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Chemoenzymatic Synthesis of Trehalosamine, an Aminoglycoside Antibiotic and Precursor to Mycobacterial Imaging Probes

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Abstract

Trehalosamine (2-amino-2-deoxy- α,α -D-trehalose) is an aminoglycoside with antimicrobial activity against *Mycobacterium tuberculosis*, and it is also a versatile synthetic intermediate used to access imaging probes for mycobacteria. To overcome inefficient chemical synthesis approaches, we report a 2-step chemoenzymatic synthesis of trehalosamine that features trehalose synthase (TreT)-catalyzed glycosylation as the key transformation. Soluble and recyclable immobilized forms of TreT were successfully employed. We demonstrate that chemoenzymatically synthesized trehalosamine can be elaborated to two complementary imaging probes, which label mycobacteria via distinct pathways.

Graphical Abstract

Mycobacterium tuberculosis and related bacteria possess essential metabolic pathways involving the non-mammalian disaccharide trehalose (**1**).^{1–3} Mycobacterial trehalose metabolism is an important target for the development of anti-tuberculosis drugs and diagnostics, which are urgently needed.⁴ Some trehalose analogues possess antimycobacterial activity and, when modified with detectable tags, have shown promise as mycobacteria-specific imaging probes.⁵ In particular, analogues bearing a nitrogen atom at the 2-position have proven to be valuable (Figure 1). Trehalosamine (2-amino-2-deoxy- α,α -D-trehalose, **2**), which was first isolated from *Streptomyces* in 1957, is a potent growth

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Supporting Information

Supplementary figures and characterization data.

The Supporting Information is available free of charge on the ACS Publications website.

inhibitor of *M. tuberculosis* and *M. smegmatis*.^{6–8} Our group recently found that 2-TreAz (2-azido-2-deoxy- α,α -D-trehalose, **5**) is also a growth inhibitor of *M. smegmatis*.⁹ In addition, fluorophore- and azide-tagged trehalosamine analogues (**3–5**) have been used to metabolically label and image envelope glycolipids in live mycobacterial cells either directly or through click chemistry.^{10–12}

Although trehalosamine and its derivatives are important molecules for tuberculosis research, they remain difficult to access. Isolation of trehalosamine from natural sources is arduous and may not provide material in sufficient quantity and purity.^{6,7} Chemical synthesis offers flexibility, but to date the reported routes to trehalosamine and its analogues are relatively lengthy and low-yielding.^{8,10–14} One approach to trehalose analogue synthesis is to perform a chemical glycosylation reaction between protected acceptor and donor monosaccharides. However, this approach requires custom building block preparation, and, moreover, the glycosylation reaction typically generates 4 difficult-to-separate stereoisomers (α,α ; α,β ; β,α ; and β,β) with low selectivity.¹⁵ Even when a specialized glycosylation method featuring excellent α,α -stereoselectivity was elegantly used to synthesize FITC-Tre (**4**), building block preparation and protecting group manipulations made the longest linear sequence of this convergent synthesis 8 steps with an overall yield of $< 10\%$, and the product **4** contained a non-native anomeric methyl group.¹⁰ An alternative approach that sidesteps the challenging 1,1- α,α -glycosylation is to modify native trehalose through regioselective manipulations.¹⁶ However, using this approach, 2-TreAz (**5**), trehalosamine (**2**), and 2-FITre (**3**) were generated in 9, 10, and 11 steps, respectively, and the overall yields for each were $< 5\%$.^{11,12} In general, the practical and efficient synthesis of trehalosamine and related compounds remains a difficult problem.

To develop a more efficient and convenient route to trehalosamine and its analogues, we turned to chemoenzymatic synthesis, which is now established as a powerful approach to constructing carbohydrates. In 2014, our group reported a one-step chemoenzymatic synthesis of trehalose analogues that employed trehalose synthase (TreT) from *Thermoproteus tenax*, which is a thermostable enzyme that generates trehalose from the acceptor glucose (Glc) and the nucleotide sugar donor uridine 5'-diphospho- α -D-glucose (UDP-Glc).^{17,18} We found that TreT could rapidly and efficiently convert a broad variety of Glc analogues into the corresponding trehalose analogues with complete 1,1- α,α -stereoselectivity. Therefore, we reasoned that TreT catalysis might provide an expedient route to trehalosamine. Unfortunately, the only Glc analogue containing a 2-position nitrogen atom in our initial substrate panel, 2-azido-2-deoxy- α -D-glucose (2-GlcAz), was not accepted as a substrate by TreT.¹⁷

Our first objective in this work was to identify a nitrogenous substrate that was tolerated by TreT. To assess TreT activity on unnatural substrates relative to the native substrates Glc and UDP-Glc, we used a commercially available luminescence-based glycosyltransferase assay that detects UDP released in the reaction.¹⁹ First, we evaluated acceptor monosaccharides with 2-N modifications, including D-glucosamine (GlcN), *N*-acetyl-D-glucosamine (GlcNAc), and 2-GlcAz (Figure 2). Despite our earlier work showing that TreT has good tolerance for various 2-position modifications (e.g., deoxy, fluoro, and stereochemical modifications),¹⁷ all three of these compounds were poor substrates and thus not suitable for

trehalosamine synthesis. This failure led us to investigate whether exploiting the opposite mode of TreT-catalyzed analogue synthesis—reacting unnatural nucleotide sugar donors with unmodified Glc—would reveal substrate tolerances that were useful for trehalosamine synthesis. We screened two commercially available 2-N-containing nucleotide sugar donors: UDP-N-acetyl- α -D-glucosamine (UDP-GlcNAc) and UDP-N-acetyl- α -D-galactosamine (UDP-GalNAc). We were surprised to observe that, while UDP-GalNAc showed no activity, UDP-GlcNAc reacted with Glc just as efficiently as UDP-Glc did (Figure 2). In the absence of a crystal structure for TreT, it is challenging to speculate as to the reason for this substrate preference. Regardless, with the product of this reaction being *N*-acetyl-2-amino-2-deoxy- α , α -D-trehalose (TreNAc, **6**), we expected that *N*-deacetylation could readily deliver the target molecule trehalosamine.

With UDP-GlcNAc identified as a suitable substrate, we proceeded to the synthesis of trehalosamine. First, the enzymatic reaction conditions were optimized. Typically, the reaction is carried out using 10 μ M TreT enzyme, 20 mM Glc (or Glc analogue), 40 mM UDP-sugar, 20 mM MgCl₂, and 300 mM NaCl in 50 mM Tris-HCl buffer (pH 8.0) at 70 °C for 60 min. Using microscale reactions (50 μ L) and TLC analysis, we optimized the reaction stoichiometry to minimize the use of UDP-GlcNAc while maintaining complete conversion of Glc to the TreNAc product. It was found that 1.5 equivs. of UDP-GlcNAc could be used, giving final substrate concentrations of 20 mM Glc and 30 mM UDP-GlcNAc (Figure S1, Supporting Information). These conditions were applied to a larger scale reaction (6 mL). After 60 min reaction time, the enzyme was removed by spin dialysis, then the reaction mixture was treated with mixed-bed ion exchange resin, which removed all ionic species and left behind the neutral TreNAc product in aqueous solution. After removal of water, pure TreNAc was obtained in 78% yield. Five independent enzymatic reaction trials gave yields of approximately 70–80%, demonstrating the reproducibility of the method. NMR and MS analyses confirmed the structure and purity of the product (see Supporting Information). Key ¹H NMR absorptions included the anomeric positions, whose coupling constants (J = 3.0 and 3.5 Hz) established the 1,1- α , α -stereochemistry of the glycosidic bond, as well as the *N*-acetyl methyl group absorption at 2.0 ppm.

In order to improve the efficiency of the TreT synthesis process, we sought to reduce waste of valuable enzyme. Semi-preparative and preparative scale reactions quickly deplete TreT, which is produced from *E. coli* in a moderate yield of 1–4 mg/L. To address this issue, we developed recyclable bead-immobilized TreT. Recombinant TreT was coupled to either *N*-hydroxysuccinimide (NHS)-activated magnetic or agarose beads, both of which are commercially available. TreT retained its activity when immobilized to either bead type and allowed quantitative conversion of Glc and UDP-Glc to trehalose in microscale reactions (50 μ L) over 10 rounds of use, as shown by TLC (Figure S2, Supporting Information). To demonstrate the benefit of these systems, we used agarose-immobilized TreT to carry out multiple rounds of TreNAc synthesis on a semi-preparative scale. However, it is important to note that isolated TreNAc yields were significantly lower (45–60%) when using immobilized TreT, likely due to non-specific binding of substrate(s) and/or product to the beads during the reaction, so there appears to be a tradeoff between enzyme preservation and product yield. In general, the agarose beads were preferred due to their lower cost and more

efficient recovery as compared to magnetic beads, which were more expensive and gradually lost during recovery using a magnetic tube rack.

With TreNAc successfully obtained using either solution or immobilized TreT, we proceeded to the *N*-deacetylation step using hydrazinolysis (Scheme 1). Although *N*-deacetylation of TreNAc in 85% aqueous hydrazine was slow at 80 °C, requiring 5 days, nearly complete conversion was observed. After evaporation of water and excess hydrazine, the crude product was subjected to HPLC purification on a semi-preparative aminopropyl column to separate trehalosamine from the acetylhydrazide byproduct, while unreacted TreNAc could be removed using ion-exchange chromatography. Following the *N*-deacetylation step, trehalosamine was obtained in 50% isolated yield or 57% based on recovered starting material (BRSM), thus the two-step yields were 39% (isolated) or 45% (BRSM).

Trehalosamine (**2**) can be readily elaborated to established mycobacterial imaging probes (Scheme 1). We converted **2** into the known fluorophore-tagged trehalose analogue 2-FITre (**3**) in 82% yield by treatment with fluorescein isothiocyanate (FITC) in bicarbonate buffer (pH 9.0) as reported by Rodriguez-Rivera et al.¹² To access clickable trehalose analogue 2-TreAz (**5**) we performed a diazo transfer reaction using triflic azide in the presence of a Cu(II) catalyst,²⁰ obtaining the product in 75% yield. With the two probes in hand, we evaluated their mechanisms of metabolic incorporation in *M. smegmatis*. Published data suggest that 2-TreAz labels cell envelope glycolipids in mycobacteria via an “inside-out” mechanism that requires initial uptake of the probe into the cytoplasm via the trehalose-specific transporter LpqY-SugABC.^{11,21} On the other hand, while it is established that the larger fluorescein-tagged trehalose analogues (e.g., **3** and **4**) efficiently label mycobacterial cells,^{10,12} it is not clear whether or not their incorporation is also LpqY-SugABC-dependent. To address this question, we performed metabolic labeling experiments with **3** and **5** in three *M. smegmatis* strains,¹⁷ including wild type, the *sugC* mutant (LpqY-SugABC deleted), and the *sugC::sugC* complement (LpqY-SugABC restored). Flow cytometry analysis revealed that while 2-TreAz labeling was completely dependent on LpqY-SugABC, 2-FITre labeling was not affected by deletion of the transporter (Figure 3). Rather, it is likely that 2-FITre is anchored to the cell envelope through lipidation by extracellular antigen 85 (Ag85) mycoloyltransferases, as previously suggested.^{10,12} These data suggest that the size of reporter group has a significant impact on the mechanism of incorporation, and, furthermore, that **3** and **5** label mycobacteria via distinct metabolic pathways. These experiments demonstrate how improved access to compounds such as **3** and **5**, enabled by TreT catalysis, can facilitate progress in the development and characterization of imaging probes for mycobacteria.

In summary, we described herein a highly efficient synthesis of trehalosamine. Instead of traditional chemical synthesis approaches, we opted to investigate chemoenzymatic synthesis employing *T. tenax* TreT, which is a trehalose-producing glycosyltransferase with exceptional properties for synthetic applications. Indeed, TreT possessed the requisite substrate tolerance and robustness to generate the key intermediate TreNAc (**6**) in one step and in high yield using either soluble or bead-immobilized recyclable enzyme. Simple deacetylation delivered the target molecule trehalosamine (**2**), which has been known as an

aminoglycoside antibiotic against mycobacteria for decades. This fact, coupled with the antimicrobial and anti-biofilm activities observed for other types of trehalose analogues in numerous previous studies,^{9,10,22–27} motivates the development of novel trehalosamine-based antibiotics. The reported method is expected to enable ready access to trehalosamine derivatives for such future investigations. More recently, trehalose-based mycobacteria-specific imaging probes have capitalized on trehalosamine as a useful synthetic intermediate. In this context, the reported method will facilitate access to known imaging probes, such as **3–5**, as well as streamline the development of novel entries in this emerging class of compounds. The finding in this work that TreT can tolerate modifications to the UDP-sugar donor provides a new route to access novel trehalose analogues. However, a current limitation is that few UDP-sugar derivatives are commercially available at a reasonable cost. Thus, it is of interest to couple existing chemoenzymatic methods for the production of UDP-sugars²⁸ with TreT catalysis to further improve the scope and efficiency of this synthetic approach to trehalose analogues.

Experimental

General experimental for synthesis.

Materials and reagents were obtained from commercial sources without further purification unless otherwise noted. TreT was expressed and purified from *E. coli* as previously described.^{17,18} UDP-Glucose was obtained from Abcam. UDP-GlcNAc was obtained from CarboSynth. Anhydrous solvents were obtained either commercially or from an alumina column solvent purification system. Chemical synthesis reactions were carried out in oven-dried glassware under inert gas. Analytical TLC was performed on glass-backed silica 60 Å plates (thickness 250 µm) and detected by charring with 5% H₂SO₄ in ethanol. NMR spectra were obtained at room temperature on a Varian INOVA 500, with ¹H and ¹³C NMR spectra recorded at 500 MHz and 125 MHz respectively. Coupling constants (*J*) are reported in hertz (Hz). High-resolution electrospray ionization (HR ESI) mass spectra were obtained using a Waters LCT Premier XE with raffinose or reserpine as the lock mass for accurate mass determinations.

Luminescence glycosyltransferase assay.

TreT activity was measured using the UDP-Glo glycosyltransferase assay (Promega) as previously described.¹⁸ Briefly, for each experimental condition, three replicates of TreT reaction mixture containing 1 µg TreT, 200 mM NaCl, 20 mM MgCl₂, 10 mM glucose (or analogue), and 0.4 mM UDP-sugar (all UDP-sugars used in the UDP-Glo assay were obtained from Promega) in 25 µL 50 mM Tris-HCl buffer at pH 7.0 were set up in a white 96-well microplate. The reactions were incubated for 2 min at room temperature. After equilibrating to room temperature, 25 µL UDP detection reagent were added, which coupled UDP production to a luciferase reaction. After incubation at room temperature for 60 min, the luminescence signal was recorded using a microplate reader (Tecan Infinite F200 Pro). The luminescence signal was fitted to a standard curve made from a dilution series of known UDP concentrations measured in the same 96-well microplate. Relative light units (RLUs) given by the luminescence reader were converted to percent enzyme activity. In all experiments, reactions without acceptor substrate were used as negative controls.

Immobilization of TreT on NHS-activated agarose and magnetic beads.

Coupling to agarose beads: 151 mg of dry NHS-activated agarose resin (Pierce) were added to a 2 mL spin column (Pierce). After addition of 2 mL of TreT (~300 µg/mL) in coupling buffer (100 mM NaHCO₃, 300 mM NaCl) to the column, the mixture was rotated end-over-end for 1 h. The spin column was placed into a 15 mL conical tube and centrifuged at 1500 rpm for 1 min, after which the flow-through was discarded. The resin was rinsed with 2 mL of coupling buffer and centrifuged twice. To ensure maximum coupling of enzyme to resin, an additional 2 mL of TreT were added to the column, coupled, and rinsed as described above. After rinsing, 2 mL of 1 M ethanolamine were added to the column, which was then rotated end-over-end for 15 min. The spin column was placed into a 15 mL conical tube and centrifuged at 1500 rpm for 1 min. The resin was rinsed with 2 mL Tris-HCl buffer (50 mM Tris, 300 mM NaCl, pH 8.0) and centrifuged three times, then resuspended in Tris-HCl buffer and stored in a microcentrifuge tube at 4 °C.

Coupling to magnetic beads: 300 µL of NHS-activated magnetic resin (Pierce) was separated from its storage solution by a magnet. The supernatant was discarded, and the beads were resuspended in 1 mL of ice-cold 1 mM HCl. The resuspended resin was vortexed for 15 seconds and then placed next to a magnet. The supernatant was discarded, and the beads were resuspended in 100 µg of TreT in 300 µL of coupling buffer. The mixture was rotated end-over-end for 1 h at room temperature. The beads were collected with a magnet, and the supernatant was discarded. The resin was resuspended in 1 mL of 0.1 M glycine wash buffer, then the beads were collected with a magnet and this wash step was repeated again. The resin was resuspended in 1 mL of 3 M ethanolamine to quench any remaining reactive sites, and the mixtures was rotated end-over-end for 2 hours. The beads were collected with a magnet and resuspended in dH₂O. The beads were collected with a magnet, resuspended in storage buffer (100 mM NaHCO₃, 300 mM NaCl, and 0.005% Triton X-100), and stored in a microcentrifuge tube at 4 °C.

Semi-preparative chemoenzymatic synthesis of *N*-acetyl-2-amino-2-deoxy- α , α -D-trehalose (TreNAc, compound 6) using soluble TreT.

To a 15 mL conical tube was added glucose (0.120 mmol, 21.6 mg), UDP-GlcNAc (0.180 mmol, 117.2 mg), and MgCl₂ • 6 H₂O (0.120 mmol, 24.4 mg). TreT in Tris-HCl buffer (50 mM Tris, 300 mM NaCl, pH 8.0), plus additional Tris-HCl buffer if needed, were added to achieve a final volume of 6 mL and a final protein concentration of 10 µM. The reaction was incubated at 70 °C with shaking at 300 rpm for 60 min, then the tube was cooled by placing it on ice. An Amicon Ultra-15 centrifugal filter unit (nominal molecular weight limit (NMWL) 10 kDa) was pre-rinsed with 3 mL deionized water three times by centrifugation at 3214 x g for 20 min to remove trace glycerol in the membrane. After transferring the cooled enzymatic reaction mixture to the pre-rinsed centrifugal filter unit, it was spun at 3214 x g for 20 min. The upper chamber of the centrifugal filter unit was rinsed two times with 3 mL of deionized water and centrifuged again using the same speed and time. After discarding the upper chamber of the centrifugal filter unit, mixed-bed ion-exchange resin (3 g of Bio-Rad Bio-Rex RG 501-X8) was added to the tube and stirred for 1 h at room temperature. Next, the supernatant was decanted and filtered. The remaining resin was rinsed two times with 5 mL of deionized water and the supernatant was decanted, filtered, and combined with

the rest of the product. The purified product was concentrated by rotary evaporation and dried under high vacuum to give **6** (36.4 mg, 78.3% yield) as a colorless glass. TLC (*n*-butanol/ethanol/H₂O 5:3:2): *R_f* = 0.38. ¹H NMR (500 MHz, D₂O): δ 5.16 (d, *J* = 2.5 Hz, 1 H, H1'), 5.13 (d, *J* = 4.0 Hz, 1 H, H1), 3.93 (dd, *J* = 4.0, 11 Hz, 1H, H2') 3.89–3.80 (m, 3 H, H3', H5', 1xH6'), 3.77–3.73 (m, 4 H, 3H, 1xH6', 2xH6), 3.62 (dd, *J* = 4.0, 10.5 Hz, 1 H, H2), 3.54–3.51 (m, 1 H, H5), 3.49 (t, *J* = 10 Hz, 1 H, H4'), 3.44 (t, *J* = 9.5 Hz, 1 H, H4), 2.01 (s, 3 H, CH₃). ¹³C NMR (125 MHz, D₂O): δ 174.2 (C=O), 93.4 (C1), 91.7 (C1'), 72.6 (C5), 72.5 (C3), 72.2 (C5'), 70.8 (C2), 70.00 (C3' and C4' overlapping), 69.2 (C4), 60.4 (C6'), 60.0 (C6), 53.4 (C2'), 21.6 (CH₃). Peak assignments were aided by COSY and HSQC NMR spectra. HR ESI-TOF MS negative mode: calcd. for C₁₄H₂₄NO₁₁ [M-H]⁻ *m/z*, 382.1349; found, 382.1352.

Semi-preparative chemoenzymatic synthesis of TreNAc (compound **6**) using agarose-immobilized TreT.

To a 15 mL conical tube was added glucose (0.080 mmol, 14.5 mg), UDP-GlcNAc (0.120 mmol, 78.2 mg), and MgCl₂ (0.080 mmol, 16.3 mg). 1.8 mL of a suspension of TreT-coupled agarose beads (prepared as described above) in Tris-HCl buffer (50 mM Tris, 300 mM NaCl, pH 8.0) was added to the tube, followed by 2.2 mL additional Tris-HCl buffer to achieve a final volume of 4 mL. The reaction was incubated at 70 °C with shaking at 300 rpm for 60 min. After collection of the supernatant, the beads were rinsed with 2 mL Tris-HCl buffer twice, with the rinses being combined with the supernatant. To this solution was added mixed-bed ion-exchange resin (3 g of Bio-Rad Bio-Rex RG 501-X8), resulting in a suspension that was stirred for 1 h at room temperature. Next, the supernatant was decanted and filtered. The remaining ion-exchange resin was rinsed two times with 5 mL of deionized water, and the rinses were combined with the rest of the product. The purified product was concentrated by rotary evaporation and dried under high vacuum to give **6** (19.5 mg, 64%) as a colorless glass. The TreT-coupled agarose beads recovered from this reaction were re-used in two subsequent semi-preparative scale reactions using the same procedure, and the resulting isolated yields of **6** were 44% and 46%. In all three cases, TLC indicated quantitative conversion of Glc to TreNAc, suggesting that substrate or product may have been lost due to non-specific binding to the beads.

Hydrazinolysis of TreNAc (**6**) to give 2-amino-2-deoxy-α,α-D-trehalose (trehalosamine, compound **2**).

To a 15 mL PTFE round-bottomed flask containing **6** (35.4 mg, 0.0923 mmol) was added 0.30 mL DI H₂O and 2.56 mL anhydrous N₂H₄. The reaction was stirred under an inert atmosphere at 100 °C for 5 days at which time TLC analysis indicated no further progress. The reaction mixture was concentrated by rotary evaporation and dried under high vacuum. Crude product was dissolved in DI H₂O and purified by HPLC (Luna Amino 250 × 100 mm column, isocratic elution with 80% MeCN and 20% DI H₂O over 40 min, flow rate 4.5 mL/min). TLC analysis of HPLC fractions indicated the co-elution of **2** and trace amounts of **6**. Fractions containing co-eluted **2** and **6** were combined and loaded onto a column packed with Dowex 50WX8 400 (primed with 5 M HCl), then eluted using 3 column volumes (CV) of H₂O, 3 CV of 0.1% NH₄OH, and 3 CV of 5% NH₄OH successively. Desired product identified by TLC was pooled, concentrated by rotary evaporation, and dried under high

vacuum to give **2** (15.6 mg, 49.5% yield, 57.1% based on recovered TreNAc) as a faintly yellow glass. TLC (ethyl acetate/isopropanol/water 2:2:1): R_f = 0.036. ^1H NMR (500 MHz, D_2O): δ 5.17 (d, J = 3.0 Hz, 1 H, H1), 5.15 (d, J = 3.5 Hz, 1 H, H1'), 3.86–3.72 (m, 7 H, H3, H5, H5', 2xH6, 2xH6'), 3.67 (t, J = 10 Hz, 1 H, H3'), 3.62 (dd, J = 3.5, 10 Hz, 1 H, H2), 3.42 (t, J = 9.5 Hz, 1 H, H4), 3.41 (t, J = 9.5 Hz, 1 H, H4'), 2.81 (dd, J = 3.5, 10.0 Hz, 1 H, H2'). ^{13}C NMR (125 MHz, D_2O): δ 93.5 (C1'), 92.8 (C1), 73.7 (C3'), 72.4 (C5 or C5'), 72.4 (C5 or C5'), 72.2 (C3), 70.8 (C2), 69.6 (C4'), 69.5 (C4), 60.5 (C6 or C6'), 60.4 (C6 or C6'), 54.6 (C2'). Peak assignments were aided by COSY and HSQC NMR spectra. HR ESI-TOF MS positive mode: calcd. For $\text{C}_{12}\text{H}_{26}\text{NO}_{10}$ $[\text{M}+\text{H}]^+$ m/z , 342.1400; found, 342.1410.

***N*-(Fluorescein-5-yl)-thioureido-2-amino-2-deoxy- α,α -D-trehalose (2-FITre, compound 3).**

2-FITre (**3**) was prepared from trehalosamine (**2**) as described by Rodriguez-Rivera et al.¹² NMR was consistent with the reported data.¹²

2-Azido-2-deoxy- α,α -D-trehalose (2-TreAz, compound 5).

To a 15 mL round-bottomed flask containing a stirring solution of trehalosamine (8.4 mg, 0.0246 mmol) in 120 μL H_2O was added K_2CO_3 (5.7 mg, 0.0412 mmol), $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$ (0.218 mg, 0.874 μmol), and freshly prepared TfN_3 ¹⁹ (approximately 0.098 mmol), then MeOH was added until homogeneity for a final volume of 2 mL. The reaction was stirred for 18 h, then concentrated and purified by silica gel column chromatography ($\text{CHCl}_3/\text{MeOH}$ 1.5:1). Fractions containing the product (TLC: R_f = 0.41) were combined, filtered through a 0.22 μm syringe filter, and dried to give **5** (7.2 mg, 75% yield) as a white solid. NMR was consistent with the reported data.¹¹

Metabolic labeling and flow cytometry analysis.

Cultures of *M. smegmatis* wild type, *sugC* mutant, or *sugC::sugC* complement were generated by inoculating a single colony from a freshly streaked agar plate (with appropriate antibiotic, if needed) 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 log phase. Bacteria were mixed with liquid medium and probe stock solution (or vehicle solution for controls) in sterile flat-bottom 96-well plates to achieve the desired cell density and probe concentration at a final volume of 200 μL . For the 2-TreAz experiment, the final probe concentration was 25 μM , and the corresponding untreated control cells were incubated in 7H9 medium. For the 2-FITre experiment, the final probe concentration was 100 μM in 7H9 medium with 1% DMSO, and the corresponding untreated control cells were incubated in 7H9 medium with 1% DMSO. Plates were incubated at 37 °C with shaking in a Tecan plate reader (Infinite F200 PRO operated by Tecan iControl software) at 37 °C for 16 h, after which cells were pelleted (5,000 rpm for 5 min) and washed three times with phosphate-buffered saline supplemented with 0.5% BSA (PBS-B), then fixed by treatment with 4% paraformaldehyde in PBS for 15 min. After fixing, 2-TreAz-treated or -untreated cells were pelleted and washed once with PBS-B, then reacted with alkyne-modified carboxyrhodamine 110 fluorophore (Click Chemistry Tools) via Cu-catalyzed azide–alkyne cycloaddition (CuAAC) according to a reported procedure.¹⁷ Following CuAAC, cells were pelleted and washed

three times with PBS, then resuspended in PBS. Cell suspensions were then transferred to 5 mL polystyrene Falcon tubes (BD Biosciences) and analyzed by flow cytometry on a BD Biosciences FACS Aria II flow cytometer. Fluorescence data was collected for 50,000 cells at an event rate of 500–1,000 events/sec and processed using BD FACSDIVA 8.0.1. All flow cytometry experiments were performed with three replicate samples, and data shown were representative of at least two independent experiments. Scatter-gated fluorescence analysis was used to obtain mean fluorescence intensities with doublet discrimination.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Structures of trehalose (1), trehalosamine (2), and trehalosamine analogues used to image mycobacteria (3–5).

Figure 2. Relative activity of TreT on various nitrogen-containing acceptor and donor substrates, as assessed by a luminescence glycosyltransferase assay. Error bars represent the standard deviation from three replicate experiments.

Figure 3.

Metabolic labeling of *M. smegmatis* with 2-FITre (**3**) and 2-TreAz (**5**) proceeds via distinct pathways. The indicated *M. smegmatis* strains were cultured in 2-TreAz (25 μ M), 2-FITre (100 μ M), or left untreated. 2-TreAz-treated cells (and an aliquot of control cells) were subjected to Cu-catalyzed azide–alkyne cycloaddition (CuAAC) with an alkyne-modified 488 fluorophore. All probe-treated and control cells were then analyzed by flow cytometry. Error bars represent the standard deviation from three replicate experiments. A.u., arbitrary units.

Scheme 1.

Chemoenzymatic synthesis of trehalosamine and elaboration to mycobacterial imaging probes **3** and **5**.