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Bioorthogonal Chemical Reporters for Selective In Situ Probing of Mycomembrane Components in Mycobacteria

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Abstract: *The global pathogen Mycobacterium tuberculosis and other species in the suborder Corynebacterineae possess a distinctive outer membrane called the mycomembrane (MM). The MM is composed of mycolic acids, which are either covalently linked to an underlying arabinogalactan layer or incorporated into trehalose glycolipids that associate with the MM non-covalently. These structures are generated through a process called mycolylation, which is central to mycobacterial physiology and pathogenesis and is an important target for tuberculosis drug development. Current approaches to investigating mycolylation rely on arduous analytical methods that occur outside the context of a whole cell. Herein, we describe mycobacteria-specific chemical reporters that can selectively probe either covalent arabinogalactan mycolates or non-covalent trehalose mycolates in live mycobacteria. These probes, in conjunction with bioorthogonal chemistry, enable selective in situ detection of the major MM components.*

The *Corynebacterineae* suborder consists of several genera, including *Mycobacterium*, *Corynebacterium*, *Nocardia*, and *Rhodococcus*, which contain some of the most devastating bacterial pathogens worldwide. Most notably, tuberculosis (TB) is caused by *M. tuberculosis* (*Mtb*), which infects 2 billion people, and every year causes 10 million active cases of TB and kills 1.5 million people.[1] Multi- and extensively-drug-resistant strains of TB (MDR- and XDR-TB), which have case fatality rates nearing 50%, have severely exacerbated the global health burden of TB.[2] Other important human diseases are also caused by species from the *Corynebacterineae* suborder, including leprosy, Buruli ulcer, and diphtheria.

Mycobacteria and other members of the *Corynebacterineae* have a unique cell envelope that is central to pathogenesis and provides intrinsic drug resistance (Figure 1 A and B).[3] It has plasma membrane (PM) and peptidoglycan (PG) layers in common with typical Gram-negative and -positive bacteria, but it deviates entirely at this point. PG is covalently attached to the arabinogalactan (AG) layer, which in turn is covalently modified with long-chain (C60-C90) fatty acids called mycolic acids. These AG-linked mycolates (AGM) are the foundation of the outer membrane, or mycomembrane (MM).[4] In addition to AGM, the MM hosts other non-covalently associated lipids and glycolipids, most prominently virulence-associated trehalose dimycolate (TDM).[5] Biosynthesis of the MM is mediated by the disaccharide trehalose (Figure 1 A). In the cytoplasm, Pks13 links trehalose to mycolic acid to generate trehalose monomycolate (TMM),[6] which, after translocation across the PM by MmpL3, acts as the mycolyl donor required for construction of the MM.[7] The antigen 85 complex (Ag85), consisting of several mycolyltransferases, is responsible for transferring mycolyl groups from the mycolyl donor TMM to either i) AG, leading to the formation of AGM, or ii) another molecule of TMM, generating TDM.[8] During these processes, trehalose is released and recycled by the transporter SugABC-LpqY.[9]

The MM is essential to bacterial viability and many of its constituents are required for virulence in *Mtb*, so it is viewed as an excellent target for TB drug development. Isoniazid and ethambutol, which have been used to treat TB for decades, act on pathways associated with MM biosynthesis.[10] Recent efforts to identify and develop antimycobacterial compounds continue to focus on MM pathways.[7a,11] However, progress in this area is limited by current methods for analysis of MM components, which largely rely on radiolabeling and cellular fractionation, and have drawbacks relating to convenience, specificity, and analysis in native contexts.[12]

Bioorthogonal chemical reporters[13] and related probes have recently emerged as valuable tools for rapidly detecting bacterial cell surface components in situ.[14] With respect to the MM, trehalose-based reporters for TMM and TDM have recently been described (Figure 1 C). Barry and Davis developed the first such probe, FITC-Tre, a fluorescein-modified keto trehalose analogue that allowed one-step labeling of trehalose glycolipids via a perplasmic Ag85-mediated route.[14e] The Bertozzi group created a series of azide-modified trehalose (TreAz) analogues (6-TreAz is shown in Figure 1 C), which primarily labeled trehalose glycolipids via an intracellular route and enabled their detection by bioorthogonal chemistry.[14h] We recently reported a chemoenzymatic method for rapidly synthesizing and administering trehalose-based reporters to mycobacteria.[14j] These approaches, which all involve tagging of the trehalose sugar, provide a valuable set of chemical reporters for probing trehalose glycolipids.

The defining feature of the MM is covalent AGM, but to date no methods have been developed for probing AG

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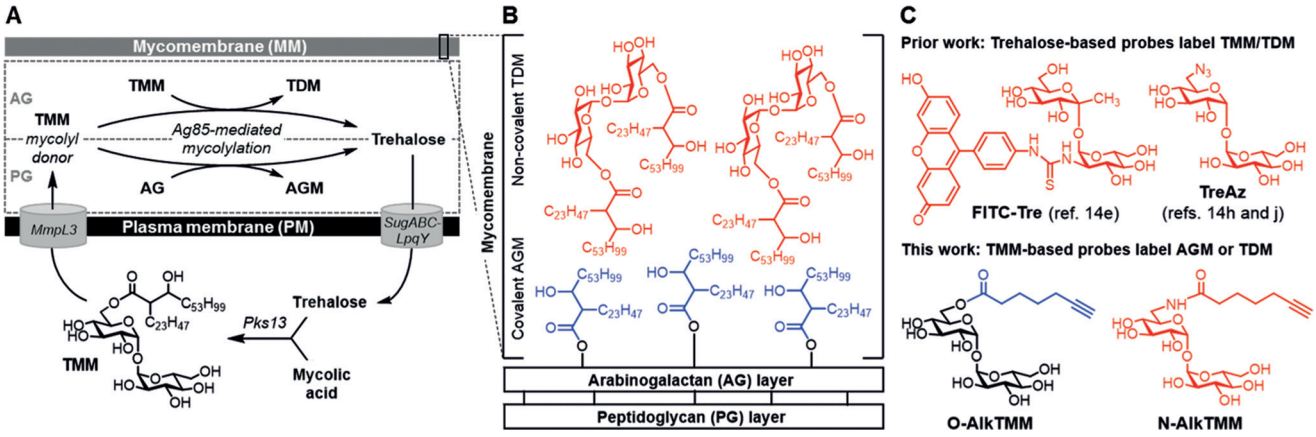
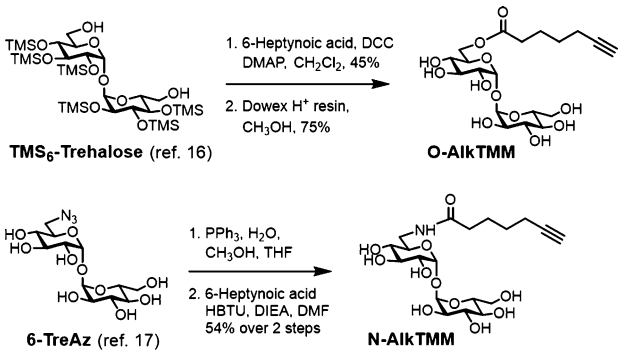


Figure 1. Mycomembrane (MM) biosynthesis, simplified structure, and chemical reporters. A) Pathways involved in MM biosynthesis. B) Structures of the major constituents of the MM: Covalent arabinogalactan-mycolate (AGM) and trehalose dimycolate (TDM). C) Reporters for selective probing of MM components: Previously described FITC-Tre and TreAz label the trehalose glycolipids TMM/TDM; O-AlkTMM and N-AlkTMM described herein selectively label either AGM or TDM, respectively.

mycolylation in situ. Such a tool, in combination with complementary probes for trehalose glycolipids, would enable comprehensive analysis of the major MM components in their native cellular environment. We envisioned that the MM biosynthetic pathways depicted in Figure 1 A could be targeted with chemical reporters designed to selectively probe either AGM or trehalose glycolipids. First, to target AGM, we designed O-AlkTMM (Figure 1 C), which mimics the mycolyl donor TMM but contains a shortened ester-linked lipid chain with a clickable terminal alkyne in place of the native mycolate. This probe design was inspired by an early report on Ag85 substrate specificity, which showed that replacement of the native TMM mycolate chain (C60-C90) with a truncated palmitate chain (C16) did not impair processing by Ag85 in vitro.[8a] Since then, substrate analogues with even shorter alkyl chains (C4 and C6) have been used successfully in Ag85 activity assays.[11d,14e] Thus, we hypothesized that O-AlkTMM would access the cellular periplasm and serve as an unnatural substrate for Ag85, leading to transfer of the 6-heptynoyl group to terminal AG residues. Subsequently, alkyne-labeled AGM could be probed with an azido fluorophore using click chemistry. O-AlkTMM could also react with TMM to label trehalose glycolipids, which we predicted would generate comparatively little signal since AGM accounts for the majority of extracellular mycolates.[15] We also designed N-AlkTMM (Figure 1 C), an amide-linked version of the TMM-based probe that is incompetent to serve as a mycolyl donor and thus should not label AGM. Instead, similar to FITC-Tre and TreAz, we hypothesized that N-AlkTMM would serve only as a mycolylation acceptor and exclusively form a labeled version of TDM. Herein, we describe the development of O- and N-AlkTMM as reporters for selective in situ probing of AGM and trehalose glycolipids, respectively, as well as visualization of these MM components in intact mycobacteria.

The syntheses of O- and N-AlkTMM are shown in Scheme 1. For the synthesis of O-AlkTMM, we used Kulkarni's method[16] to accomplish selective 6-*O*-monoesterification of TMS_6-trehalose with 6-heptynoic acid, followed by desilylation. N-AlkTMM was accessed from 6-TreAz[17] by Staudinger reduction followed by coupling with 6-heptynoic acid. As expected, the shortened lipid chain conferred excellent water solubility to both compounds, which facilitated their storage and use in labeling experiments.



Scheme 1. Syntheses of O-AlkTMM (top) and N-AlkTMM (bottom).

O- and N-AlkTMM were first evaluated for metabolic labeling in *M. smegmatis* mc^2155 (*Msmeg*), which possesses MM biosynthetic pathways and architecture that are representative of other species in the *Corynebacterineae*. Wild-type *Msmeg* was cultured in the presence of varying concentrations (1–250 μM) of O- or N-AlkTMM until late-log phase (14 h) and then reacted with an azido fluorophore (Az488) under Cu-catalyzed azide–alkyne cycloaddition (CuAAC) conditions.[18] Flow cytometry analysis showed successful labeling for both probes, although different labeling efficiencies were observed (Figure 2 A). O-AlkTMM exhibited strong labeling even at very low concentrations; a signal-to-noise ratio (S/N) of >20 was observed at concentrations as low as 1 μM. O-AlkTMM labeling was saturable at 50 μM concentration and a S/N of >250. By comparison, N-AlkTMM labeling was less efficient, giving approximately 10-fold lower signal at the same concentrations, yet the S/N still reached >150 under the conditions tested. The high S/N at relatively low doses and the absence of growth defects during treatment (Supporting

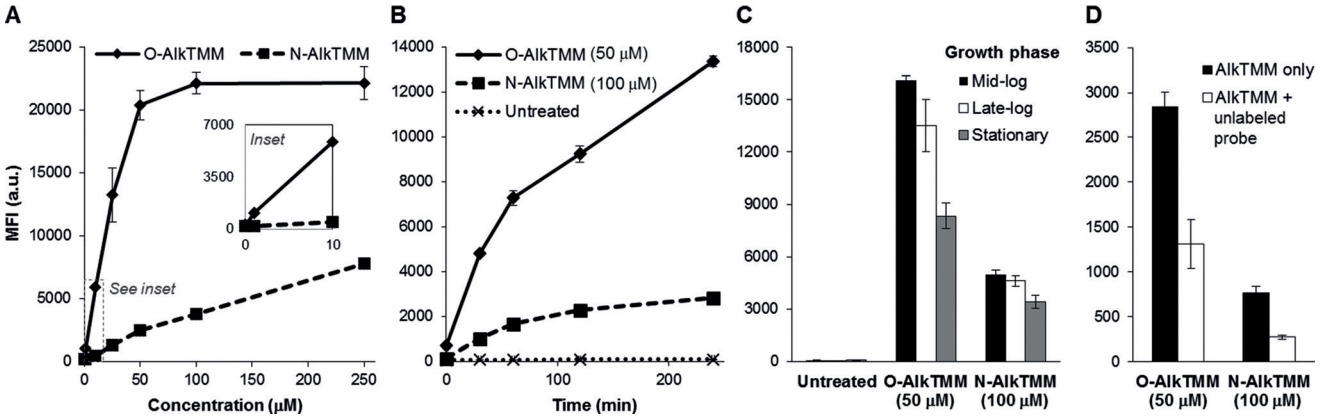


Figure 2. O- and N-AlkTMM undergo metabolic incorporation into live *Msmeg* cells. *Msmeg* was incubated with AlkTMM (or left untreated), reacted with Az488 by CuAAC, and analyzed by flow cytometry to assess A) concentration-, B) time-, and C) growth phase-dependence of labeling. D) *Msmeg* was incubated with AlkTMM alone or with AlkTMM plus 5 mM of competing unlabeled versions of the probes lacking alkynes. Cells were reacted with Az488 by CuAAC and analyzed by flow cytometry. Error bars denote the standard deviation of three replicate experiments. MFI, mean fluorescence intensity; a.u., arbitrary units.

Information, Figure S1) make both O- and N-AlkTMM appealing as chemical reporters.

The observed dose-dependent labeling suggested that active metabolic incorporation of the AlkTMM probes was occurring, which was further supported by experiments evaluating the time- and growth phase-dependence of labeling, as well as competition experiments (Figure 2 B–D). Both O- and N-AlkTMM exhibited time-dependent increases in labeling until signal saturation occurred between 2–4 h, which is the approximate doubling time for *Msmeg*. Of note, significant labeling could be detected for O- and N-AlkTMM after only a few seconds of culture time, suggesting that they will be useful for probing mycolylation processes that occur on extremely short timescales. Growth phase-dependence experiments showed a significant decrease in O- and N-AlkTMM labeling between log- and stationary-phase *Msmeg*. Labeling was also reduced by competition with unlabeled versions of the probes (synthesized by Pd-catalyzed alkyne reduction of O- and N-AlkTMM), further confirming metabolic incorporation of the AlkTMM probes. Because low millimolar concentrations of the competitors were required to halve the signal, the possibility of some non-specific probe uptake cannot be completely ruled out. However, similar competition profiles have been observed for a number of other metabolic reporters, including FITC-Tre and 3-TreAz reporters for trehalose glycolipids[14e,h] and unnatural D-alanine reporters for peptidoglycan.[14i] Additionally, our specificity data, presented below, strongly support the proposed labeling routes and molecular targets for O- and N-AlkTMM.

To establish that O- and N-AlkTMM were incorporated into MM components through Ag85, we performed labeling experiments in the presence of ebselen, which was recently identified as a covalent inhibitor of all Ag85 isoforms in *M. tuberculosis*.[11d] Upon treatment of *Msmeg* with ebselen, O- and N-AlkTMM labeling decreased by approximately 70% and 85%, respectively, confirming their Ag85 specificity (Figure 3 A). To rule out the possibility that the observed signal loss was due to a general reduction in cellular

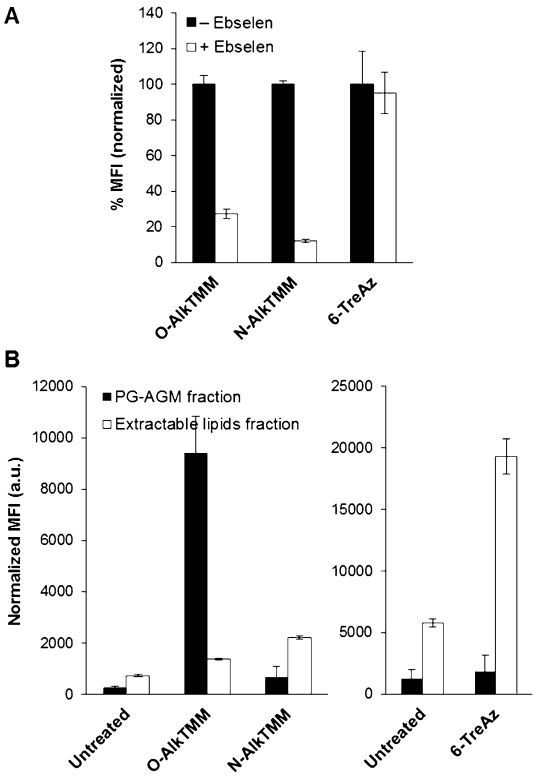


Figure 3. A) O- and N-AlkTMM labeling is Ag85-dependent. *Msmeg* was incubated in the presence or absence of the Ag85 inhibitor ebselen (50 $\mu g\,mL^{-1}$), then labeled with the indicated chemical reporter, reacted with the appropriate fluorophore by CuAAC, and analyzed by flow cytometry. MFI was normalized to show percent of maximum fluorescence for each reporter. B) O- and N-AlkTMM selectively label covalent AGM and extractable trehalose glycolipid fractions, respectively. *Msmeg* was treated with the indicated chemical reporter (or left untreated), reacted with an appropriate fluorophore by CuAAC, fractionated into PG-AGM material and extractable lipids, and analyzed using a fluorescence plate reader. MFI was normalized to cell density of samples. Flow cytometry data for intact cells prior to fractionation is shown in Figure S4. Error bars denote the standard deviation of three replicate experiments.

metabolism, we confirmed that ebselen treatment did not reduce *Msmeg* labeling using 6-TreAz, which is known to be incorporated into cell wall TMM in an Ag85-independent manner; rather, it traverses an intracellular route consisting of SugABC-LpqY/Pks13/MmpL3.[14h] It was also verified that, in contrast to 6-TreAz, incorporation of O- and N-AlkTMM was not dependent on the trehalose transporter SugABC-LpqY (Figure S2). Together, these data support the proposed periplasmic route of AlkTMM incorporation by Ag85.

As noted above, we hypothesized that O- and N-AlkTMM would have distinct targets in the MM, with O-AlkTMM selectively reporting on AGM and N-AlkTMM reporting on trehalose glycolipids. To differentiate between these possibilities, we treated *Msmeg* with O- or N-AlkTMM, conjugated Az488 by CuAAC, and then used an established procedure[12] to fractionate *Msmeg* cells into i) insoluble cell wall material, including the PG-AGM complex, and ii) soluble extractable lipids, including the trehalose glycolipids. TLC analysis confirmed separation of trehalose glycolipids from the PG-AGM material (Figure S3). Analysis of solubilized samples revealed that nearly all of the fluorescence from O-AlkTMM-treated *Msmeg* was present in the PG-AGM fraction, while N-AlkTMM-treated *Msmeg* showed signal only in the extractable lipids fraction, supporting the anticipated probe selectivity (Figure 3B). As a control, we performed the same experiment with 6-TreAz, which is known to label trehalose glycolipids with high efficiency but not AGM.[14h] As expected, the 6-TreAz-treated sample showed signal exclusively in the extractable lipids fraction, matching the results from N-AlkTMM. The nominal fluorescence observed in the extractable lipids fraction of the O-AlkTMM-treated sample likely corresponds to a small fraction of this probe reacting with TMM to form alkyne-labeled TDM (estimated at $\leq 10\,\%$ based on Figure 3B).

Next, we assessed the specificity of O- and N-AlkTMM for species in the *Corynebacterineae* suborder. While these species possess the conserved MM biosynthetic machinery (Figure 1A), canonical Gram-negative and -positive bacteria do not. Thus, we evaluated O- and N-AlkTMM labeling in *Msmeg* and *C. glutamicum*, both of which are members of the *Corynebacterineae*, as well as in *Escherichia coli* and *Bacillus subtilis*, which are representative Gram-negative and -positive species, respectively. As expected, both *Msmeg* and *C. glutamicum* showed strong labeling of the cell surface by O- and N-AlkTMM, while *E. coli* and *B. subtilis* showed no labeling above background (Figure 4A and B).

To demonstrate how chemical reporters can provide insight into MM dynamics, we used O- and N-AlkTMM to image AGM and TDM biosynthesis in *Msmeg* using time-course fluorescence microscopy (Figures 4C and S5). O-and N-AlkTMM exhibited similar labeling features, characterized by intensely fluorescent septa in dividing cells, highly polar labeling for short pulses, and diffuse surface labeling for longer pulses. We also showed that AGM and trehalose glycolipids can be simultaneously imaged by co-administering O-AlkTMM and 6-TreAz, which bear orthogonal reactive tags, followed by delivery of green and red fluorophores using appropriate bioorthogonal reactions (Figure S6). See the Supporting Information for additional imaging data and discussion. These experiments represent the first direct visualization of the AGM layer in mycobacteria, and they underscore how chemical probes can advance our understanding of mycobacterial growth and division processes, which remain poorly characterized in comparison to other types of bacteria.[15]

In summary, the described chemical reporters enable sensitive, selective, and simultaneous detection of AGM and trehalose glycolipids in situ, providing a platform to study the MM in its native setting. O-AlkTMM is of particular interest

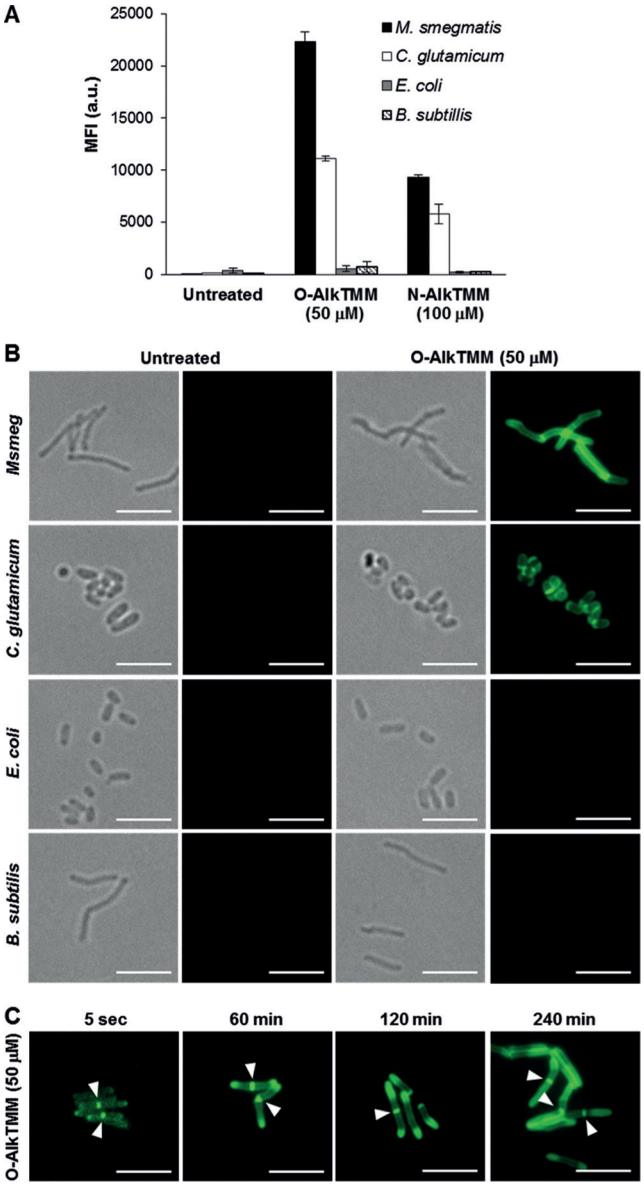


Figure 4. A) and B) O- and N-AlkTMM are specific for MM-containing species of the *Corynebacterineae* suborder. Different species were cultured in O- or N-AlkTMM, reacted with Az488 by CuAAC, and analyzed by A) flow cytometry and (B) fluorescence microscopy (shown for O-AlkTMM). C) Time-course fluorescence microscopy of O-AlkTMM labeling in *Msmeg* (see Figure S5 for images of N-AlkTMM-treated and control samples). Error bars denote the standard deviation of three replicate experiments. Scale bars, 5 μm. White triangles in C mark the septa of dividing cells.

because it allows rapid detection of AG mycolylation in whole cells for the first time. Like the previously reported FITC-Tre and TreAz series, the TMM-based reporters described here are mycobacteria-specific, connoting potential for detection of bacteria in complex settings, for example, in sputum samples or during infection. Importantly, our work demonstrates that Ag85 exhibits remarkable promiscuity in the cellular environment, not only for unnatural mycolyl acceptors like FITC-Tre and TreAz, as previously shown,[14e,h] but also for TMM-mimicking mycolyl donors that have ester-linked unnatural lipids. The ability of Ag85 to catalyze the transfer of unnatural lipids from TMM mimics to AG represents a strategy for cell surface modification of live mycobacteria. Coupled with the ease of synthesizing TMM mimics, this strategy will facilitate chemical remodeling of the MM for various applications.

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