Chemical Probes for Tagging Mycobacterial Lipids

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Abstract

Mycobacteria, which cause tuberculosis and related diseases, possess a diverse set of complex envelope lipids that provide remarkable tolerance to antibiotics and are major virulence factors that drive pathogenesis. Recently, metabolic labeling and bioorthogonal chemistry have been harnessed to develop chemical probes for tagging specific lipids in live mycobacteria, enabling a range of new basic and translational research avenues. A toolbox of probes ~~have~~ has been developed for labeling mycolic acids and their derivatives, including trehalose-, arabinogalactan-, and protein-linked mycolates, as well as newer probes for labeling phthiocerol dimycocerosates and potentially other envelope lipids. These lipid-centric tools have yielded fresh insights into mycobacterial growth and host interactions, provided new avenues for drug target discovery and characterization, and inspired innovative diagnostic and therapeutic strategies.

Keywords: Mycobacteria, tuberculosis, cell envelope, lipids, glycolipids, probes, click chemistry, bioorthogonal chemistry, metabolic labeling, imaging, proteomics, mycolic acids, trehalose

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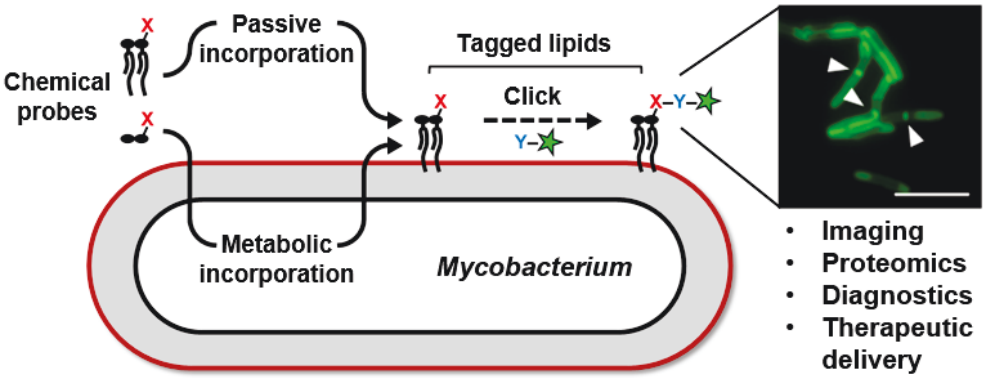
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1.1. Introduction

In the bacterial domain, mycobacteria stand out due to their remarkably complex, lipid-rich diderm cell envelope. Mycobacterial envelope lipids are major virulence factors for tuberculosis (TB)-causing *Mycobacterium tuberculosis* (*Mtb*), which is responsible for approximately 1.5 million deaths per year, as well as for related pathogens [1, 2]. The mycobacterial cell envelope comprises layers of plasma membrane, peptidoglycan, arabinogalactan, outer "mycomembrane," and capsule (Figure 1A) [3-5]. Mycobacterial lipids and glycolipids are dominant both in terms of their high content in the envelope and in their contributions to physiology and pathogenesis. Prominent mycobacteria-specific lipids include long-chain branched mycolic acids and their derivatives, trehalose-containing glycolipids, phthiocerol dimycocerosates (PDIMs) and structurally related phenolic glycolipids (PGLs), phosphatidylinositol mannosides (PIMs) and PIM-anchored lipomannan (LM) and lipoarabinomannan (LAM), and other species (Figures 1B and 1C) [5, 6]. For decades, it has been appreciated that the unique chemical structures, abundance, and spatial arrangement of these envelope lipids engender mycobacteria with their renowned cellular integrity and tolerance to external stresses (e.g., antibiotic treatment, host immune response) and, particularly for lipids exposed at the cell surface, drive key aspects of the host–pathogen interaction [6, 7]. As a result, mycobacterial lipids have long served as a source of inspiration for antibiotic development, which is underscored by the fact that multiple frontline anti-TB drugs act on lipid biosynthesis. The sequencing of the *Mtb* genome in 1998 prompted an era of rapidly accelerating research on mycobacterial lipid biosynthesis, which in turn has led to promising new TB drug targets and lead compounds that will be critical for addressing drug resistance [6-9].

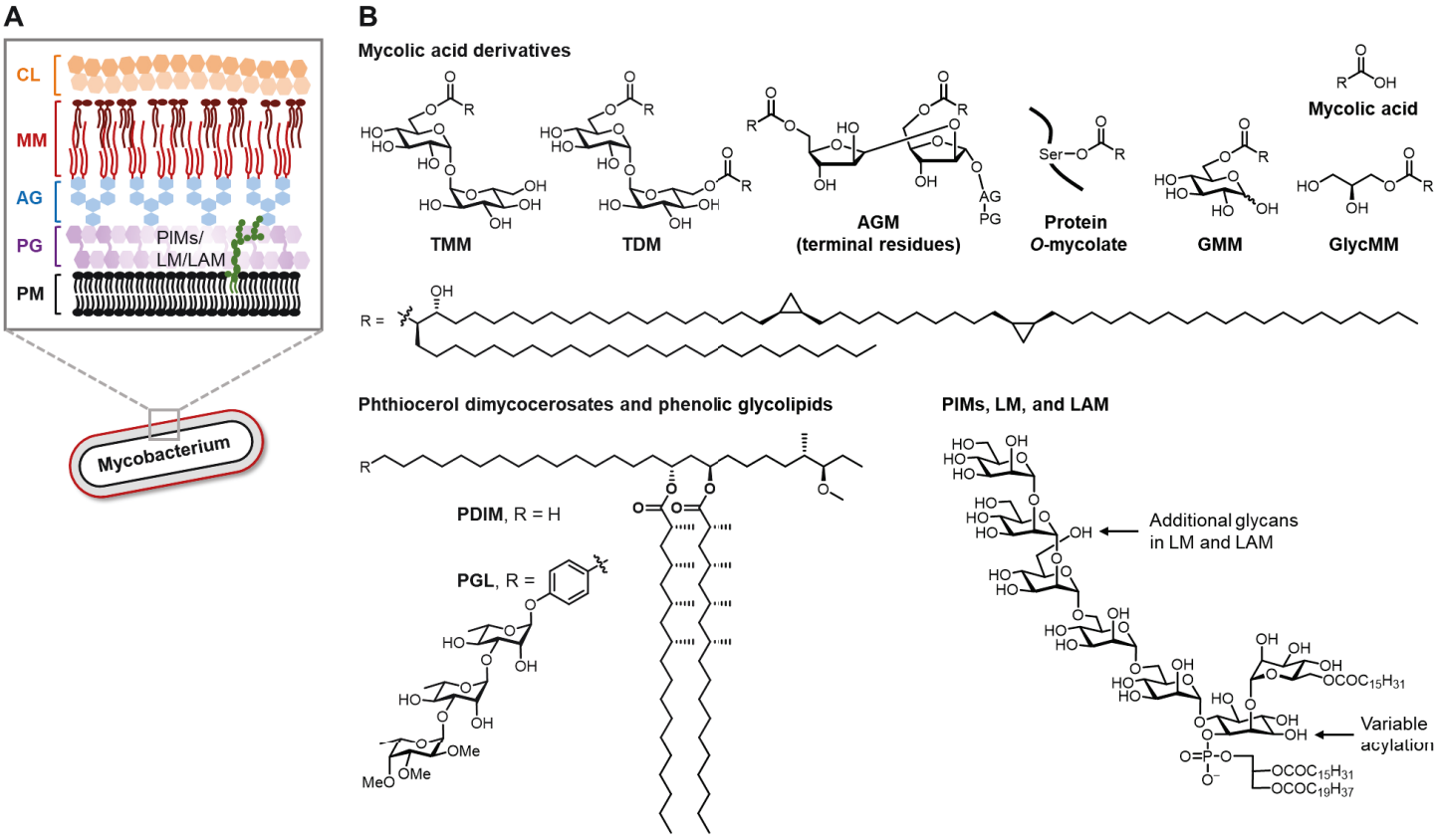

Figure 1. (A) Schematic representation of the mycobacterial cell envelope. AG, arabinogalactan; CL, capsular layer; MM, mycomembrane; PG, peptidoglycan; PM, plasma membrane. (B) Mycobacteria-specific lipids for which chemical probes have been developed. Among other lipids not represented here are additional trehalose glycolipids (e.g., diacyltrehalose, polyacyltrehalose, sulfolipid-1, trehalose polyphleates), glycopeptidolipids (GPLs), lipooligosaccharides (LOSs), and plasma membrane phospholipids. AGM, arabinogalactan-linked mycolate; GMM, glucose monomycolate; GlycMM, glycerol monomycolate; LAM, lipoarabinomannan; LM, lipomannan; PDIM, phthiocerol dimycocerosate; PIM, phosphatidylinositolmannoside; PGL, phenolic glycolipids; TDM, trehalose dimycolate; TMM, trehalose monomycolate.

Progress in mycobacterial lipid research has been supported by various experimental techniques. In addition to genetic techniques, most routinely used are conventional lipid isolation and analysis methods, such as radiolabeling, solvent extraction, TLC, and MS, including MS-based lipidomics [10, 11]. In addition, purified natural or synthetic mycobacterial lipids have been used to investigate lipids' effects on host cell membrane structure and immunological response [12]. Imaging techniques, particularly electron microscopy, have provided important insights into mycobacterial cell envelope architecture and membrane morphology [13, 14]. Various lipophilic dyes that associate non-covalently with envelope lipids (e.g., carbol fuchsin, auramine-rhodamine, Congo red) have been used with light and fluorescence microscopy to assess gross cell envelope characteristics and, in fact, are the basis for TB diagnosis via sputum smear microscopy [15, 16]. Collectively, these techniques have been indispensable for the study of mycobacterial lipids and membranes. However, the inability to directly

tag, visualize, and interrogate the interactions of specific lipid classes in live mycobacteria has been a major deficiency, ultimately limiting our understanding of lipid biosynthesis, transport, remodeling, and interactions with both intrinsic (bacterial) and extrinsic (host) factors. To address these technical and knowledge gaps, chemists have begun to apply emerging chemical biology approaches—namely metabolic labeling and bioorthogonal click chemistry—toward the creation of tools for probing mycobacteria-specific envelope components in their native environment [12, 17, 18]. Here, we describe probes that have been developed to chemically tag mycobacterial lipids in live cells, and we highlight how these tools have been applied to drive forward the investigation and targeting of mycobacterial lipids.

1.2. Tagging the tail: approaches to lipid probe development

Two complementary approaches have been harnessed to install functional tags into lipids in live mycobacteria: metabolic incorporation and passive incorporation (Figure 2A). In metabolic incorporation, the probe is a synthetic, chemically tagged mimic of a natural biosynthetic precursor that, upon administration to live cells, is processed through endogenous biosynthetic pathway(s) to attach the tag to the targeted lipid. In passive incorporation, the probe is a synthetic or semi-synthetic tagged version of a mature lipid structure that, upon administration to cells, is directly intercalated into the membrane in a metabolism-independent manner. Metabolic probes are generally smaller and easier to synthesize, and, assuming successful processing through endogenous biosynthetic machinery, end up in the lipid's native location in the cell. Importantly, in addition to tagging the lipid to enable its direct analysis, metabolic probes report on the activity of lipid biosynthesis, transport, and remodeling pathways. Passively incorporated probes are structurally more complex, which in principle may necessitate more challenging synthetic chemistry. Their incorporation does not rely on cellular biosynthetic machinery, which bypasses potential problems with respect to substrate intolerance, but on the other hand prevents read-outs of metabolic pathway activity and also poses the risk that the tagged lipid will not reach the appropriate cellular location. The nature and location of the tag on the probe depends on the objective of the study. Most commonly, chemists design probes that contain tags with detection, isolation, and/or photo-cross-linking capabilities, while also aiming to achieve synthetic accessibility and minimize the tag size, which reduces potential for substrate tolerance problems and/or biological perturbation. While the progenitor tag type is a fluorescent dye used in one-step incorporation approaches, the rise of bioorthogonal click chemistry has

provided an exceptionally powerful and versatile two-step approach to tagging lipids and other biomolecules in living systems (Figure 2B) [19, 20]. Finally, a lipid-specific probe design consideration is whether the tag is incorporated into the lipid tail or the polar head group. In the following sections, we will see a diverse range of probe types that have been designed and deployed in the service of advancing mycobacterial lipidology.

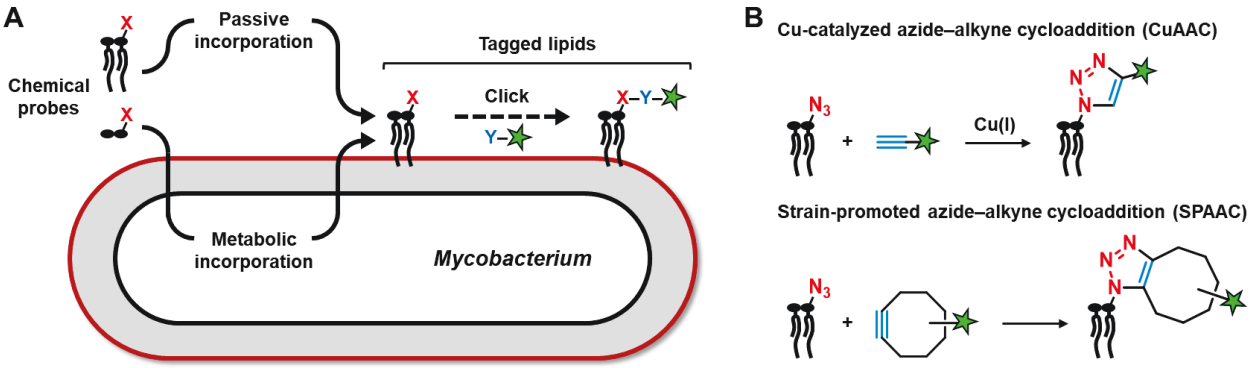

Figure 2. (A) Metabolic and passive incorporation approaches to tagging mycobacterial lipids with chemical probes. X = fluorescent dye or other tag (e.g., affinity, photo-cross-linking) for one-step incorporation or bioorthogonal click functionality for two-step incorporation; Y = complementary bioorthogonal click functionality for two-step incorporation; star = fluorescent dye or other tag; dashed line indicates usage of bioorthogonal click chemistry in the two-step incorporation approach. (B) Commonly used bioorthogonal click reactions used for two-step incorporation approaches.

1.3. Origin and expansion of lipid-tagging trehalose probes

Trehalose, a non-mammalian disaccharide of glucose, is the key mediator of mycomembrane lipid synthesis in mycobacteria (Figure 3A) [21]. Trehalose shuttles mycolic acids from their site of synthesis in the cytoplasm to the periplasm in the form of trehalose monomycolate (TMM) [22, 23]. Then, TMM donates its mycoloyl chain to various acceptor molecules through the action of mycolic acid-exchanging antigen 85 (Ag85) enzymes, producing the major mycomembrane components arabinogalactan mycolate (AGM) and trehalose dimycolate (TDM), among other mycolate ester-containing species (Figures 1A and 3A) [24, 25]. This pathway, which is essential for mycobacterial viability and is a prime target for anti-TB drugs, served as inspiration for the development of the first lipid-tagging probes for mycobacteria. In 2011, Backus *et al.* demonstrated that a fluorescent trehalose probe (FITC-Tre, Figure 3B) could hijack the extracellular Ag85 pathway by serving as an unnatural mycoloyl acceptor substrate that, upon mycoloylation, generated fluorescently tagged trehalose mycolates that anchored into the mycomembrane [26]. In 2012, Swarts *et al.* showed that clickable azido trehalose probes (TreAz, Figure 3B) were similarly metabolically incorporated into mycomembrane-resident

trehalose mycolates, albeit via an alternative intracellular route initiated by uptake through the trehalose-specific transporter, owing to their smaller modifications [27, 28]. Following TreAz incorporation, click chemistry could be performed to ligate an alkyne-modified fluorophore (or other tag) to the azide-labeled lipid. The FITC-Tre and TreAz probes, both of which install tags on the polar head groups of trehalose mycolates in live mycobacteria, are complementary both in terms of the experimental approach (one-step vs. two-step; fluorescent vs. clickable) and the biochemical process being reported on (extracellular mycolic acid exchange vs. intracellular *de novo* synthesis of trehalose mycolates).

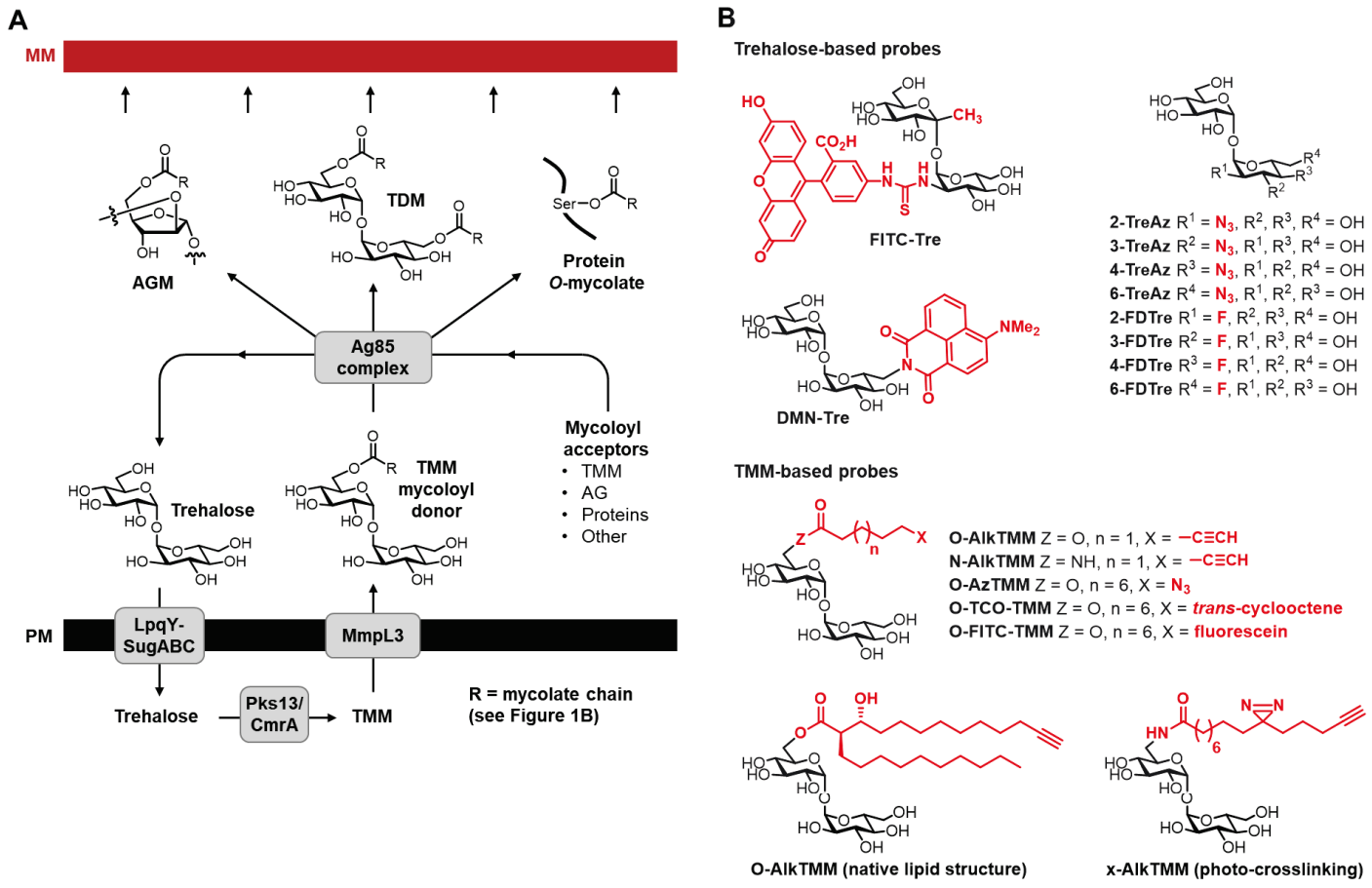

Figure 3. (A) Trehalose-mediated biosynthesis of mycolate-containing lipids present in the mycomembrane. (B) Synthetic trehalose- and TMM-based probes for metabolically tagging the polar head group and lipid tail of specific mycolate lipids, respectively.

The remarkable substrate tolerance of trehalose-processing enzymes afforded opportunities to expand the trehalose probe toolbox. In 2016, Foley *et al*. reported a novel class of probes based on TMM, which serves as both a mycoloyl donor and acceptor in Ag85-catalyzed mycolic acid exchange (Figure 3A) [29]. Placement of an alkyne tag on the ester-linked lipid tail of a synthetic TMM probe (O-AlkTMM, Figure 3B) allowed mimicry of

the TMM donor function, meaning that promiscuous Ag85 enzymes transferred the unnatural clickable lipid tail of O-AlkTMM onto mycoloyl acceptors. In this way, it was possible to tag the tail of all mycolate-containing lipids in the cell envelope, including TDM and, for the first time, AGM, which forms the foundational inner leaflet of the mycomembrane. In parallel, by designing an amide-linked TMM probe (N-AlkTMM) that was incapable of serving as a mycoloyl donor, it was possible to mimic solely the acceptor function of TMM and thus exclusively label TDM. Therefore, the lipid linkage of the TMM scaffold can be tuned to direct probe incorporation into the non-polar tail of specific mycolate lipid sub-types via distinct mechanisms. This platform was subsequently extended to create a variety of TMM-based probes bearing fluorescent, azido, *trans*-cyclooctene, and photo-cross-linking tags, as well as FRET pairs and more-native mycolate structures, thus providing broad experimental versatility (Figure 3B) [30-32]. In the following sections, we illustrate how trehalose- and TMM-based probes have provided valuable new insights into mycomembrane construction and proteomic composition, as well as opened new opportunities for targeting mycobacteria with diagnostic and therapeutic cargo.

1.4. Lipids in motion: trehalose probes reveal mycomembrane dynamics

The lipid composition and layered organization of the mycobacterial cell envelope are fairly well characterized (Figure 1). In comparison, the spatiotemporal dynamics of envelope assembly—when and where specific envelope construction events occur—have remained challenging to study, preventing a holistic molecular-level understanding of how mycobacteria and related organisms grow and divide under varied conditions [33]. The availability of lipid-tagging trehalose and TMM probes sparked a series of studies that leveraged these new tools to provide unprecedented views of mycomembrane assembly and remodeling [29, 31, 34-38]. We highlight two recent studies here. In 2019, Zhou *et al*. employed a multi-probe approach to visualize how the different layers of the *Corynebacterium glutamicum* envelope are assembled [37]. By using D-amino acid probes to tag peptidoglycan [39], TMM probes to tag mycomembrane inner-leaflet AGM, and trehalose probes to tag mycomembrane outer-leaflet trehalose mycolates, the team showed that these layers of the envelope are constructed via two different mechanisms: (i) synchronous assembly at the tips of cells during polar growth and (ii) sequential assembly at the division plane, or septum, during cytokinesis. Strikingly, it was found that sequential septal envelope assembly involved the formation of perforations in a peripheral peptidoglycan barrier, which permitted the rapid mobilization of mycomembrane lipids to complete the septal

envelope, and, subsequently, separation of the two daughter cells. Presumably, this pre-division mycomembrane confluency event is an important feature of mycobacterial cytokinesis, since it involves release of daughter cells with fully intact envelopes—thus avoiding temporary vulnerability. This finding could have implications for drug development, as interruption of proper septal construction could conceivably lower the defenses of actively dividing bacteria and sensitize them to antibiotics. In another study, published in 2021, Pohane *et al.* used O-AlkTMM and N-AlkTMM probes to tag inner-leaflet AGM and outer-leaflet TDM, respectively, to investigate mycomembrane biosynthesis and remodeling in starved, non-replicating mycobacteria [38]. Previous studies with trehalose and TMM probes established that actively replicating mycobacteria grown under nutrient-rich conditions synthesize mycomembrane lipids from their poles [26, 27, 29, 31, 34]. However, Pohane *et al*. showed that there is a dramatic re-wiring of mycomembrane assembly in response to nutrient starvation, as outer-leaflet TDM is degraded while inner-leaflet AGM synthesis continues around the entire periphery of the cell, rather than from the poles. A fascinating consequence of this remodeling event is that TDM-depleted, AGM-reorganized mycobacteria exhibit reduced permeability, implying that, despite TDM breakdown, mycomembrane remodeling fortifies the cell envelope in response to stress. This represents another mechanism that can potentially be targeted to enhance the effectiveness of antibiotics against mycobacterial pathogens. Collectively, these studies are compelling representative examples of how lipid-tagging probes can provide novel insights into the spatiotemporal dynamics of mycobacterial envelope construction.

1.5. Uncovering the mycomembrane proteome: probes for tagging lipid-modified and -interacting proteins

In contrast to the relatively well-characterized lipid composition of the mycomembrane, wide gaps in knowledge still exist with respect to the mycomembrane proteome, including the identities of various proteins expected to be involved in mycomembrane construction, remodeling, turnover, and host interactions [40]. Indeed, while mycobacteria are predicted to have as many as 100 mycomembrane proteins [41], very few have been identified and characterized due to the complex nature of the mycobacterial envelope and the lack of suitable techniques. Motivated by the possibility that surface-accessible mycomembrane proteins could be promising targets for therapeutic development, chemical probes have recently been developed to tag and identify mycolate-modified or -interacting proteins. At least a subset of mycomembrane-containing organisms (e.g., *C.*

glutamicum) produce *O*-mycoloylated proteins, which are post-translationally modified at serine residues by mycolate lipids and are localized within the mycomembrane [42, 43]. Because TMM is the universal mycoloyl donor, TMM-based probes should, in principle, metabolically tag *O*-mycoloylated proteins to permit protein detection and identification. In 2016, Kavunja *et al*. demonstrated that O-AlkTMM specifically labeled *O*-mycoloylated proteins in *C. glutamicum*, and exploited this capability to facilitate the identification and characterization of novel mycolate modifications of several mycomembrane porins [44]. Numerous additional proteins were also labeled by O-AlkTMM and related TMM probes, indicating that this lipid modification is widespread and that the probe will be instrumental in future efforts to globally profile *O*-mycoloylated proteins. In 2017, Issa *et al*. used an alkyne fatty acid probe to tag *O*-mycoloylated proteins, although this approach lacks the specificity of TMM probes and requires overexpression of proteins of interest to achieve detection, and thus its use as a discovery tool is limited [45]. Moving from tools focused on *O*-mycoloylated proteins in *C. glutamicum* to a more broadly applicable approach, in 2020 Kavunja *et al*. reported new TMM probes outfitted with photo-cross-linking and click chemistry tags (x-AlkTMM, Figure 3B) [46]. x-AlkTMM probes exploit Ag85 activity to metabolically embed into mycolate lipids in any mycobacterial species, enabling live-cell photocapture and downstream click-mediated detection, affinity enrichment, and identification of mycolate-interacting proteins. This approach was coupled with label-free quantitative proteomics to identify approximately 100 candidate mycolate-interacting proteins in *M. smegmatis*, including mycomembrane-related enzymes and transporters (e.g., Ag85, CmrA MmpL3, Tdmh, MspA). Together, these probes enable tagging of proteins that are either covalently modified by, or non-covalently interact with, mycolate lipids, providing the first chemical toolset for characterizing the notoriously intractable mycomembrane proteome.

1.6. Adapting lipid-tagging trehalose probes for diagnostic and therapeutic applications

In addition to fundamental TB research, there is increasing interest in applying lipid-tagging probe scaffolds in the clinical realm, i.e. adapting these tools to deliver diagnostic or therapeutic payload specifically to mycobacterial pathogens within infected host organisms. This idea is supported by the fact that the biosynthetic machinery responsible for modifying trehalose (or its synthetic derivatives) with lipids—and thereby anchoring it to the mycobacterial envelope—is absent from humans and other microbes. In an effort to improve the century-old sputum smear microscopy TB diagnosis technique, several fluorogenic probes have been developed that

fluoresce only upon metabolic processing by mycobacteria [31, 47-49]. Among these, a solvatochromic trehalose derivative (DMN-Tre, Figure 3B) reported by Kamariza *et al*. in 2018 has shown exceptional promise, as it successfully illuminated *M. tuberculosis* in TB-positive human sputum samples [47]. Toward non-invasive imaging of mycobacteria within human patients, in 2016 and 2019 Rundell *et al*. and Peña-Zalbidea *et al*. demonstrated that ^{18}F-modified trehalose derivatives may have potential for *in vivo* positron emission tomography (PET) imaging of mycobacterial infections (Figure 3B) [50, 51]. Trehalose-mediated delivery of therapeutic cargo has also been pursued. In 2015, Jayawardana *et al*. showed that trehalose-conjugated nanoparticles loaded with the antimycobacterial drug isoniazid targeted to and killed *M. smegmatis* more efficiently than non-functionalized nanoparticles [52]. Similarly, in 2019 Dutta *et al*. reported that trehalose-conjugated photosensitizers incorporated into and killed mycobacteria upon light exposure [53]. Other trehalose derivatives have been shown to inhibit mycobacterial growth and biofilm formation by interrupting mycomembrane lipid biosynthesis and remodeling [26, 54, 55]. While all of these efforts are still in early stages, it is exciting to witness the evolution of lipid-tagging trehalose probes into nascent strategies for addressing mycobacterial infections in the clinic.

1.7. Beyond trehalose: emerging tools to tag PDIMs and other mycobacterial lipids

As explained in the Introduction, mycobacteria are replete with virulence-associated lipids, presenting opportunities for new lipid-tagging strategies that extend the robust set of mycolate-targeting trehalose probes. For example, PDIMs are abundant mycomembrane lipids that both provide cellular defense and drive immune evasion during infection, although the mechanism of the latter function has remained unclear [56, 57]. To address this question, in 2020 Cambier *et al*. reported a method for tagging PDIMs in live mycobacteria, which enabled visualization of PDIMs during infection of a live host organism [58]. Without a clear pathway to metabolically label PDIMs, the team developed an elegant strategy for tagging PDIMs via passive incorporation. PDIMs were extracted from whole cells of *M. marinum* with petroleum ether and chemically modified with a clickable azido group to form the probe AzDIM, which was then passively incorporated back into delipidated *M. marinum* cells, enabling click-mediated fluorophore ligation and imaging (Figure 4A). Importantly, the AzDIM-modified bacteria fully copied the virulence characteristics of unmodified bacteria, indicating that the probe retained PDIM's native bioactivity. Using AzDIM in combination with a *M. marinum*–zebrafish infection model and click chemistry, it was

shown that PDIM prevents immune recognition by spreading into host epithelial membranes, a phenomenon that is (i) dependent upon PDIM's extensive mobility-promoting methyl branching and (ii) aided by PDIM-binding host cholesterol. Finally, cholesterol-lowering statins dampened PDIM spread and protected against infection in zebrafish, suggesting that these compounds may be useful in preventing mycobacterial infections, yet another example of how probe-driven mechanistic investigation has yielded novel insights for TB therapeutic development.

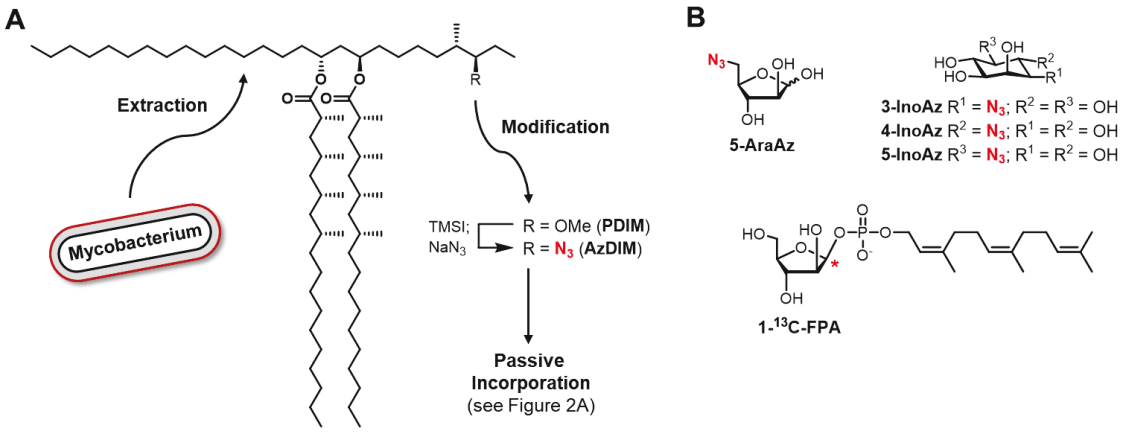

Figure 4. (A) Semi-synthesis and passive incorporation of PDIM-based probes into mycobacteria. (B) Arabinose and inositol derivatives as potential probes for mycobacterial glycolipids. Asterisk, ^{13}C label; FPA, (*Z*,*Z*)-farnesyl phosphoryl arabinofuranose; TMSI, trimethylsilyl iodide.

Among other mycobacterial lipids that have received recent attention are PIMs, LM, and LAM, which are an abundant family of lipid-anchored glycans that provide envelope structural integrity and are involved in host immunomodulation [59, 60]. The pentose sugar arabinose is a major component of LAM and AGM glycolipids, which inspired Kolbe *et al*. to study the metabolic incorporation of azido pentose derivatives into mycobacteria. Although 5-azido arabinose (5-AraAz, Figure 4B) incorporated mycobacteria, labeling of LAM and related glycolipids was not observed, so additional characterization of the tagged species is needed [61]. PIMs, LM, and LAM share a generally conserved lipid-anchoring core consisting of phosphatidylinositol, suggesting that inositol-based probes may tag these structures, as previously shown for mammalian glycosylphosphatidylinositol (GPI) anchors [62]. In 2020, Ausmus *et al*. reported the synthesis of azido inositol derivatives (InoAz, Figure 4B) as potential probes for this purpose, although evaluation of these compounds is still ongoing [63]. Since many mycobacterial glycolipids are biosynthesized from lipid-linked sugar donors, a promising alternative strategy to tag these molecules may be to employ lipid-linked sugar donor analogues. Indeed, Calabretta *et al*.

demonstrated in 2019 that a ^{13}C-labeled, lipid-linked arabinofuranose probe (1-^{13}C-FPA, Figure 4B) metabolically incorporated into AGM in *C. glutamicum* and could conceivably label LAM in mycobacteria, paving the way for the development of next-generation fluorescent or clickable chemical probes [64].

1.8. Conclusions

Mycobacterial envelope lipids—extraordinarily complex in structure and often enigmatic in biological function—have long served as a source of inspiration for chemists, providing immense challenges and opportunities in the realms of structural elucidation and chemical synthesis. In recent years, the persistent threat of TB and related diseases, the rise of drug resistance, and the emergence of powerful chemical biology techniques have coincided to usher in a new period of research focused on the creation and applications of tools that allow direct probing of specific mycobacterial lipids in native settings. Early efforts have focused on the eponymous mycolic acids and their glycan conjugates, which represent the defining chemical structures of all mycobacterial species and are high-priority targets for drug development. With trehalose being the essential mediator of mycolic acid transport and transfer, this disaccharide has served as a scaffold on which to design and build probes that install an array of tags onto the head groups or tails of mycolate glycolipids, as well as the proteins they modify or interact with. Trehalose- and TMM-based metabolic labeling probes have advanced our understanding of how mycobacteria grow and divide, provided avenues to discover and characterize potential drug targets, and inspired new strategies to detect and treat mycobacterial infections. These advances provided a foundation for more recent work aimed at bioorthogonal metabolic labeling of other mycobacterial lipid-anchored glycans (e.g., PIMs, LM, LAM), although such efforts are nascent. In addition to metabolic labeling, passive incorporation of semi-synthetic lipid probes has proven to be a successful approach in mycobacteria, specifically for PDIMs, and it could be extended to other surface-exposed lipid types. The PDIM tagging approach yielded a particularly impressive example of applying chemical probes *in vivo* to elucidate the molecular-level details of an important host–pathogen interaction. As the described chemical probes and their associated usage protocols become more robust and readily accessible to the community, we can expect to see continued innovation in their deployment to answer pressing questions in the field. On the probe development side, there remains a trove of virulence-associated lipids in the *Mycobacterium* genus that can be targeted for tool development, aided by the insights and practices gleaned from the work described herein. Chemists will continue

to play an essential role in these interdisciplinary efforts.

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