Recent Advances Toward the Inhibition of mAG and LAM Synthesis in Mycobacterium tuberculosis

***Francis E. Umesiri,*[1] *Aditya K. Sanki,*[2] *Julie Boucau,*[3] *Donald R. Ronning,*[1] *and Steven J. Sucheck*[1]

[1]Department of Chemistry, University of Toledo, Toledo, Ohio 34606
[2]Syngene International Ltd, Plot No. 2 & 3, Bommasandra IV Phase, Jigani Link Road, Bangalore, India 560 099
[3]Ragon Institute of Massachusetts General Hospital, Massachusetts Institute of Technology, and Harvard, Massachusetts General Hospital and Harvard Medical School, Boston

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Abstract: Drug-resistant forms of *Mycobacterium tuberculosis* (*M. tuberculosis*) are increasing worldwide, underscoring the need to develop new drugs to treat the disease. One of the factors that make tuberculosis difficult to treat is the unique architecture of the mycobacterial cell wall. In this review, we catalogue the enzymes involved in the synthesis of the mycolylarabinogalactan (mAG), a key structural component of the mycobacterial cell wall. In addition, we review the enzymes required for the synthesis of the related lipoarabinomannan (LAM), a structure that possesses immunomodulatory properties. The integrity of the mAG and LAM is critical to the viability of mycobacteria, and many of the established antimycobacterial agents target enzymes critical to the synthesis of the mAG and LAM. Recently, new enzymes catalyzing synthetic steps in the synthesis of the mAG and LAM have been characterized and their substrate specificity determined. In this report, we review recent efforts to characterize the enzymes involved in mAG and LAM synthesis and describe the compounds used to inhibit the enzymes or characterize their catalytic activity. Med Res Rev, 30, No. 2, 290–326, 2010

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1. INTRODUCTION

Tuberculosis (TB), once thought to be under control, is one of the leading causes of death among infectious diseases.[1] On an annual basis, active cases of TB account for 1.7 million deaths around the world. There are about two billion individuals worldwide who are currently infected with *Mycobacterium tuberculosis* (*M. tuberculosis*), the causative agent for TB,

Correspondence to: Steven J. Sucheck, Department of Chemistry, University of Toledo, Toledo, OH 43606,
E-mail: steve.sucheck@uToledo.edu

but most never develop the active form of the disease. Infection rates continue to increase, further highlighting the fact that TB remains a major health concern.[2] In many countries, this increase is exacerbated by both poor public health infrastructure and TB/HIV coinfection. Combined, these factors contribute to the difficulty of TB control.

Also contributing to the difficulty in the control of TB is the rise of drug-resistant strains of *M. tuberculosis*. The first antibiotic proven effective for the treatment of TB was streptomycin, which was quickly followed by the characterization of streptomycin-resistant strains of *M. tuberculosis*.[3] Similarly, all single-drug TB treatments developed since the identification of streptomycin-resistant *M. tuberculosis* strains have promoted the emergence of drug-resistant strains. A response by physicians to single-drug-resistant strains is to employ another first-line drug that inevitably selects for multidrug resistance (MDR) strains in the case of dual resistance to isoniazid and rifampin. Failure of this strategy has led to the development and implementation of complex drug combination therapies even for those infected with strains susceptible to all first-line drugs. The complexity and length of these protocols often lead to patient non-compliance, again contributing to the emergence of those strains that exhibit resistance. Treatment of MDR-TB necessitates the use of less effective (and more expensive) second-line drugs, which can lead to further drug resistance. The resulting extensively drug-resistant strains (XDR) initially seemed unmanageable.[4] The first account of XDR-TB described an outbreak in South Africa, in 2006, in which 52 out of 53 infected patients died within a median time of 16 days from diagnosis.[5] However, a more recent study describing XDR-TB in Russia presented more optimistic results, with 48% of the patients reaching a favorable outcome after treatment.[6] It is still unclear which scenario best describes the future of XDR-TB treatment and outcome.

In addition to the selection process that leads to the accumulation of mutations promoting antitubercular drug resistance, the mycobacterial bacillus possesses a complex cell wall structure. Indeed, the thick, multilayered, extremely hydrophobic cell envelope, which results in very low cellular permeability, acts as a barrier against many classes of hydrophilic antibacterial drugs. The general structure of the mycobacterial cell envelope is now well understood. The basic model, proposed by Minnikin,[7] identified a thick asymmetric lipid bilayer that is located beyond the plasma membrane, the peptidoglycan, and an arabinogalactan (AG) layer esterified with mycolic acids (mAG; Fig. 1). Chemical analysis of the cell envelope composition has also revealed the presence of diverse noncovalently bound lipids, such as phosphatidyl-*myo*-inositol mannosides (PIMs), lipopolysaccharides, such as lipoarabinomannans (LAM), and mannose-capped lipoarabinomannans (ManLAM), depending on the *Mycobacterium* species. In slow-growing mycobacterial species, such as *M. tuberculosis*, proteins and polysaccharides are present in the outermost stratum known as the capsule. The exact location of LAM in the cell envelope remains elusive, although it is known that the phosphatidyl *myo*-inositol serves as a cell wall anchor in the bacterial plasma membrane.[8] However, LM and LAM have been shown to be exposed at the cell surface.[9]

LAM and mAG are the main lipopolysaccharides of the mycobacterial cell wall. These key components insulate the bacteria from its environment and are essential for mycobacterial survival. In addition, they play diverse roles in the bacteria–host interactions.[10] Structurally, the LAM and mAG share an arabinan moiety, but possess significantly different final superstructures. LAM is composed of a phosphatidylinositol (PI) group linked to a branched arabinomannan core, while the mAG is a branched AG polymer esterified with mycolic acids. Enzymes involved in the biosynthetic pathway of mycobacterial cell wall building blocks used to synthesize LAM and mAG have frequently been targets of antitubercular drugs. For example, the antitubercular drug isoniazid works by inhibiting the biosynthesis of the mycolic acid component of the mAG.[11] However, the strategy of targeting

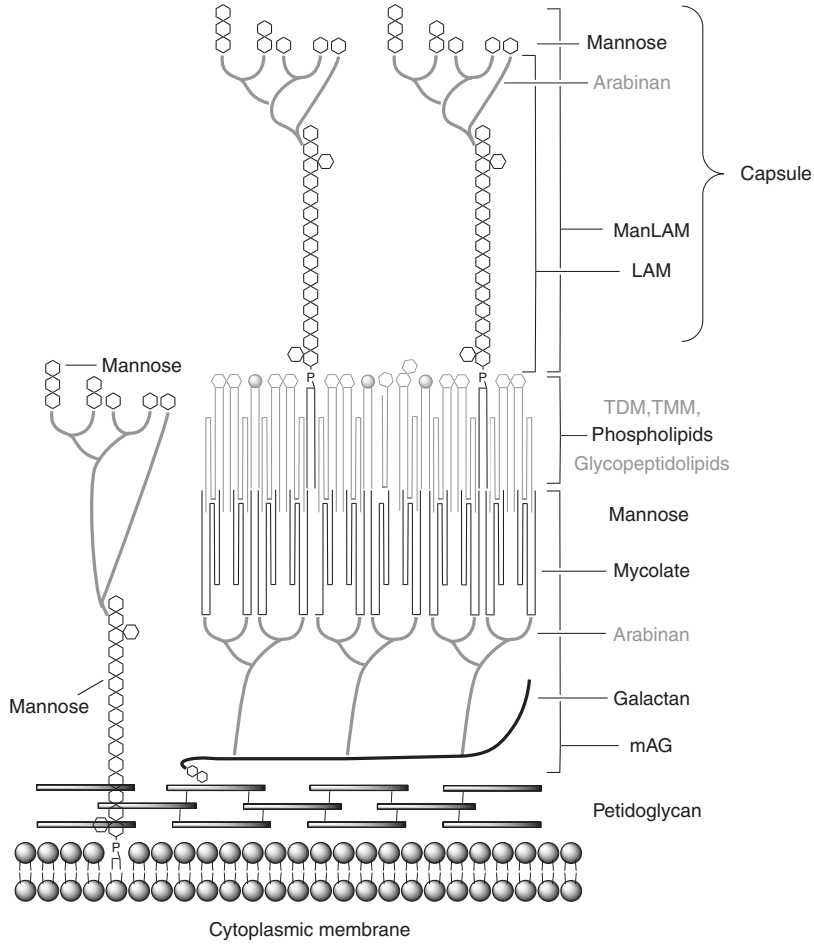


Figure 1. Schematic representation of the general structure of the mycobacterial cell wall.

a single step in a biosynthetic pathway may not be the most effective approach in the management of multidrug-resistant strains of mycobacteria.

To maximize drug effectiveness, it may be beneficial to target related enzymes with a single inhibitor. For example, the *M. smegmatis* arabinosyltransferases EmbA and EmbB are critical for formation of the hexaarabinoside motif found at the terminus of the AG.[12] Similarly, the related *M. smegmatis* arabinosyltransferase EmbC is critical for LAM synthesis.[13] Both the AG and the LAM are important for mycobacterial viability and virulence. Ethambutol (EMB), an effective antimycobacterial drug, is known to inhibit arabinosylation by acting on the arabinosyltransferases encoded by the *embCAB* gene cluster.[14,15] Thus, targeting related enzymes simultaneously may be a viable approach in developing new therapies. Another important illustration is the antigen 85 complex (Ag85), the three paralogous mycolyl transferase enzymes: Ag85A, Ag85B, and Ag85C. These enzymes catalyze the transfer of mycolyl groups to the terminal arabinosyl moieties of AG to form mAG, as well as mycolyl transfer that produces trehalose dimycolate (TDM) from two molecules of trehalose monomycolate (TMM); these two related pathways contribute to the viability of mycobacteria and may affect virulence. It is easy to see, then, how simultaneously targeting this enzymatic complex may help reduce incidence of resistance, as the possibility of all three enzymes developing mutations at the same time is miniscule. For this reason, the study of

mAG and LAM biosynthetic pathways is of interest for drug development by providing many additional targets, and new strategies aimed at inhibiting these targets will be important for the identification of new antitubercular therapies. In this report, we review the recent progress toward the inhibition of mAG and LAM biosyntheses in pathogenic mycobacteria. We will first focus on the recent advances on inhibition of the biosynthesis of LAM, and then describe the progress toward mAG biosynthesis inhibition. Because LAM and mAG bear similar structural features in the arabinan moiety, the structures share biosynthetic steps as well as common or structurally related enzymes. In the former case, their inhibition will be addressed only once. In the latter case, when homologous enzymes are used, the enzymes will be addressed separately.

2. LIPOARABINOMANNAN (LAM)

A. Structural Features and Roles of LAM and Inhibition

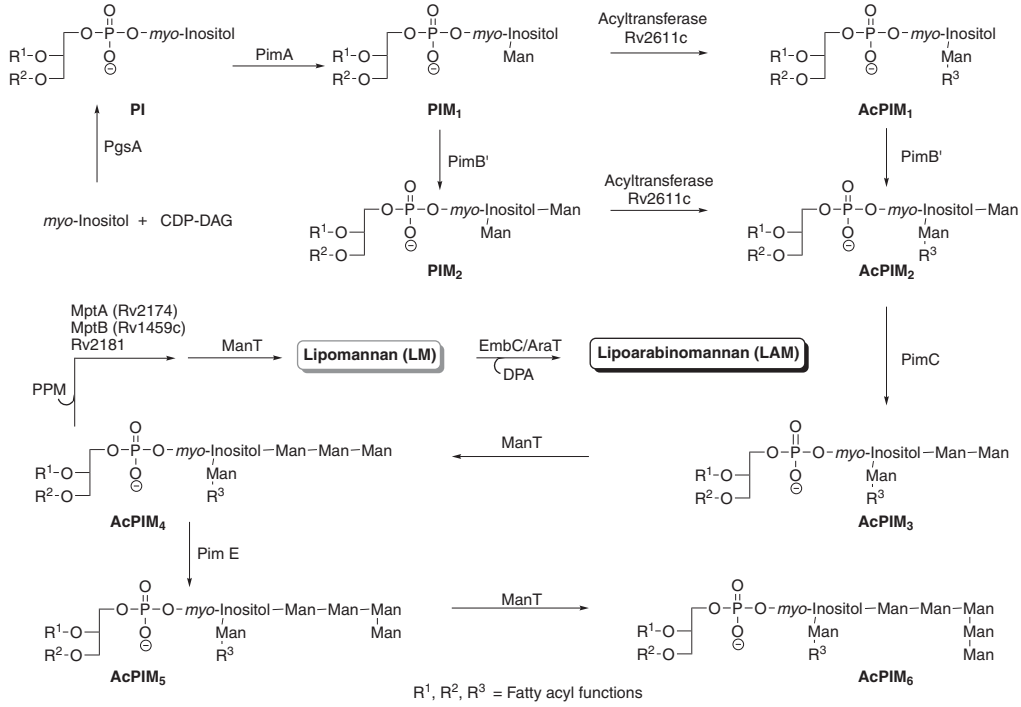
Across the *Mycobacterium* genus, LAM displays structural heterogeneity.[16] Three different structural classes of LAM have been identified: (1) a mannose-capped LAM (ManLAM) found in slow-growing mycobacteria, such as *M. tuberculosis*, *M. leprae*, *M. bovis*, *M. bovis* BCG, (2) a phospho-*myo*-inositol capped LAM (PILAM) found in fast-growing mycobacteria (*M. smegmatis* and *M. fortuitum*), and most recently (3) the LAM devoid of capping, AraLAM, found in *M. chelonae*.[10] Each LAM-type has a specific immunological activity depending on their capping motif.[16] For example, both ManLAM and PILAM play major roles in regulating host immune responses, thereby contributing to the persistence of pathogenic bacteria.[11,16] In particular, they show immunosuppressive activities that promote the survival of slow-growing bacteria in humans. PILAM has been found to activate Toll-like receptor 2 (TLR-2) promoting release of a variety of proinflammatory cytokines.[17] ManLAM was originally implicated in the inhibition of IL-12 and TNF-α production, both Th1 proinflammatory cytokines, the inhibition of *M. tuberculosis*-induced macrophage activation and apoptosis.[10] However, Pitarque et al. demonstrated that the binding to dendritic cells is complex and involved more molecules than just ManLAM.[18] Appelmelk et al. reported similar results, showing that the mannose capping motif did not govern the mycobacteria–host interactions.[19]

B. General Biosynthetic Pathway for LAM

Although a detailed description of the biosynthesis of LAM and its precursors, the lipomannan (LM) and PIMs, is beyond the scope of this review and has been extensively reviewed elsewhere,[10] a summary of the important biosynthetic steps are given below. Several of the enzymes mediating LAM biosynthesis have been identified. The current model of lipoglycan biosynthesis, which includes phosphatidyl-*myo*-inositol (PI), PIMs, and LM, follows a pathway progressing from $PI \rightarrow PIM \rightarrow LM \rightarrow LAM$,[20] although Morita et al. have shown that the biosynthesis of Ac_2PIM_2, and Ac_2PIM_6 may lie off the main pathway.[21] The current model of LAM-biosynthesis is shown in Scheme 1, as well as a structure of a ManLAM (Fig. 2).[22–24] Some of the early stages of biosynthesis for PIM homologues are not fully understood and are still under investigation.

C. Biosynthesis of LM

In the early stages of linear LM biosynthesis (Scheme 1), *myo*-inositol is first phosphorylated by a PI synthase, PgsA (Rv2612c), which uses CDP-diacylglycerol (CDP-DAG) as a



Scheme 1. Schematic representation of LM and LAM biosynthetic pathway in *M. tuberculosis*. First mannosylation occurs at C-2 position of *myo*-Inositol to form PIM_1 and second mannosylation at C-6 position of *myo*-Inositol in PIM_1 or $AcPIM_1$. First acylation occurs at C-6 position of Man in PIM_1. CDP-DAG, CDP-diacylglycerol; ManT, mannopyranosyltransferase; AraT, arabinofuranosyltransferase; PPM, polyprenol monophosphomannose; DPA, decaprenyl-1-monophosphoryl-arabinose.

diacylglycerol phosphate (DAG) donor to form PI.[25] PI is glycosylated by PimA (Rv2610c) using an α-mannopyranosyl (Man*p*) residue from GDP-mannose (GDP-Man) at the C-2 position of PI to form PIM_1.[26,27] Biochemical experiments along with the crystal structure of *M. smegmatis* PimA offer a clear understanding of the enzyme mechanism and provide helpful information for future inhibitor design.[28–30] PIM_1 is further glycosylated at the C-6 position of the inositol moiety of PIM_1, producing PIM_2.[31] This second glycosylation may occur before or after the acylation of PIM_1 by an acyltransferase (Rv2611c)[32] to give $AcPIM_2$. Recent work shows that this second α(1→6) mannosylation is catalyzed by an enzyme now called PimB′ (Rv2188c),[33] whereas the enzyme formerly called PimB (Rv0557) has been renamed MgtA.[34] MgtA has in fact been shown to be involved in the synthesis of a novel mannosylated glycolipid, 1,2-di-*O*-C_{16}/$C_{18:1}$-(α-D-mannopyranosyl-(1→4)-(α-D-glucopyranosyluronicacid)-(1→3)-glycerol ($Man_1GlcAGroAc_2$).[23,34]

$AcPIM_2$ is elaborated by α(1→6) mannosylation to form the LM core. This core is found branched at approximately half of the mannose residues by α(1→2) mannosylation. Further mannosylation of $AcPIM_2$ to $AcPIM_3$ was assigned to PimC (RvD2-ORF1);[35] however, this was questioned by the lack of change in PIM composition upon the disruption of PimC in *M. bovis*.[21] The enzyme catalyzing the following step in the pathway, marking the beginning of the LM synthesis, through biosynthesis of $AcPIM_4$ from $AcPIM_3$, has yet to be identified. PimE (Rv1159) is associated with the late stage biosynthesis of PIMs and is involved in the biosynthesis of $AcPIM_5$ and, potentially, $AcPIM_6$.[36] PimF (Rv1500) was thought to be involved in the synthesis of $AcPIM_6$,[37] but was identified as LosA, a glycosyltransferase not involved in this pathway.[38] Kovacevic and coworkers identified a lipoprotein, LpqW, involved in the conversion of PIM to LAM. A mutant lacking LpqW was

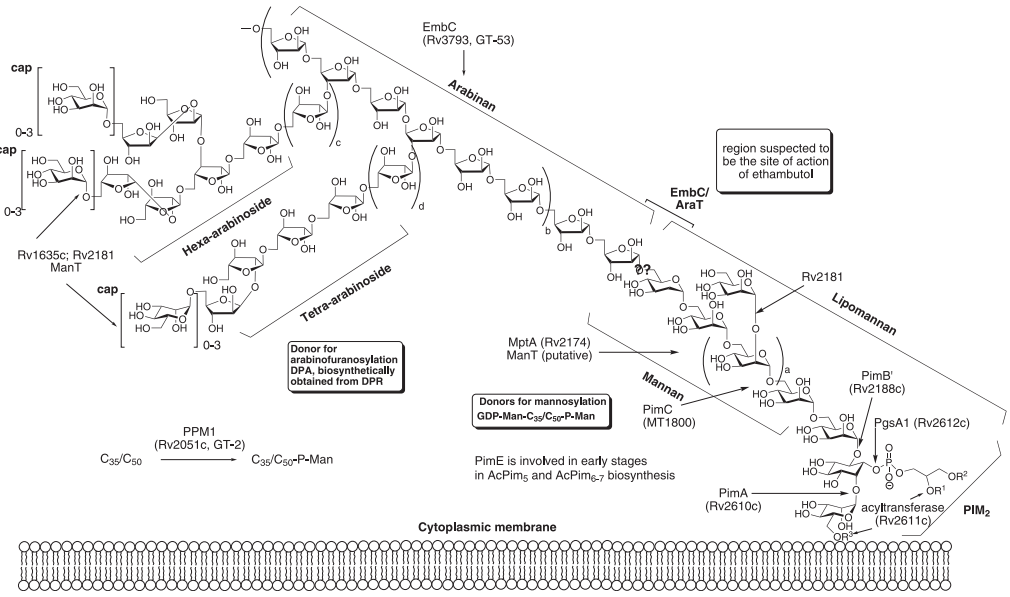


Figure 2. Current model of *M. tuberculosis* ManLAM with structurally related components PIMs, LM, and LAM. The exact nature of the LM-arabinan linkage is yet unknown, and the question mark reflects this uncertainty. PIM_2 is a precursor both for LM and LAM. In both LM and LAM, the mannan domain contains a α(1→6)-linked Man*p* backbone substituted at C-2 by a single Man*p* unit. The arabinan domain is a linear α(1→5)-linked arabinfuranosyl polymer with two types of oligosaccharide branch points; (a) branched hexa-arabinfuanosides: [β-D-Ara*f*(1→2)-α-D-Ara*f*(1→]$_2$(3,5)-α-D-Ara*f*(1→5)-α-D-Ara*f* and (b) linear tetra-arabinofuranosides: β-D-Ara*f*(1→2)-α-D-Ara*f*(1→5)-α-D-Ara*f*(1→5)-α-D-Ara*f*. The mannose cap terminating the arabinan domain has either a mono Man*p* residue, a dimannoside α-D-Man*p*(1→2)-α-D-Man*p*, or trimannoside α-D-Man*p*(1→2)-α-D-Man*p*(1→2)-α-D-Man*p* found at C-5 of terminal Ara*f*. R^1, R^2, and R^3 are fatty acyl-chains. C_{35}/C_{50}-P-Man*p* indicates a polyprenylmonophosphomannose. The a, b, c, and d values are species dependent. Enzymes indicated by arrows are involved in the biosynthesis of these lipoglycans. PimC was found in *M. tuberculosis* CDC1551 but absent in *M. tuberculosis* H37Rv. Classification of the glycosyltransferase by their CAZy family is indicated in brackets (CAZy stands for carbohydrate-active enzymes; www.cazy.org).

unable to form LAM; however, another mutation in PimE partially restored the LAM biosynthesis.[39–41] The exact function of LpqW is under investigation.

The use of a polyprenylphosphate sugar donor (i.e. polyprenyl monophosphomannose, PPM) synthesized by polyprenol monophosphomannose synthase (Ppm1) takes place after the formation of $AcPIM_4$ in *M. tuberculosis*.[23,34,42,43] LM can be viewed as PIMs extended with additional Man*p* residues supplied by C_{35}/C_{50} PPM (C_{35}/C_{50}-P-Man*p*), forming "linear" LM that contain α(1→6) Man*p* residues.[20,22] Ppm1 (Rv2051c), encoded by the *ppm1* gene, catalyzes the synthesis of C_{35}/C_{50}-P-Man*p* from GDP-Man*p* and polyprenols. Recently, a novel enzyme α-mannopyranosyltransferase (ManT), MptA (Rv2174) involved in the synthesis of core mannan backbone, was reported.[44–46] MptB was also characterized as a PPM-dependent α(1→6) mannosyltranferase required in the early stages of mannan biosynthesis for proximal α(1→6) core Cg-LM-A and Cg-LM-B in *C. glutamicum*.[23] The core mannan backbone is branched via glycosylation catalyzed by Rv2181, resulting in the characteristic α(1→2)-linked branches found in LM and LAM.

D. Biosynthesis of LAM

Glycosylation of the "mature" LM with arabinan results in formation of LAM. The elaboration of the mannan core with arabinosyl residues is accomplished by a group of arabinosyltransferases (EmbC and other AraTs) where the arabinofuranosyl moieties in the arabinan core is α(1→5)-linked. Work by Berg et al. extended the model of arabinan

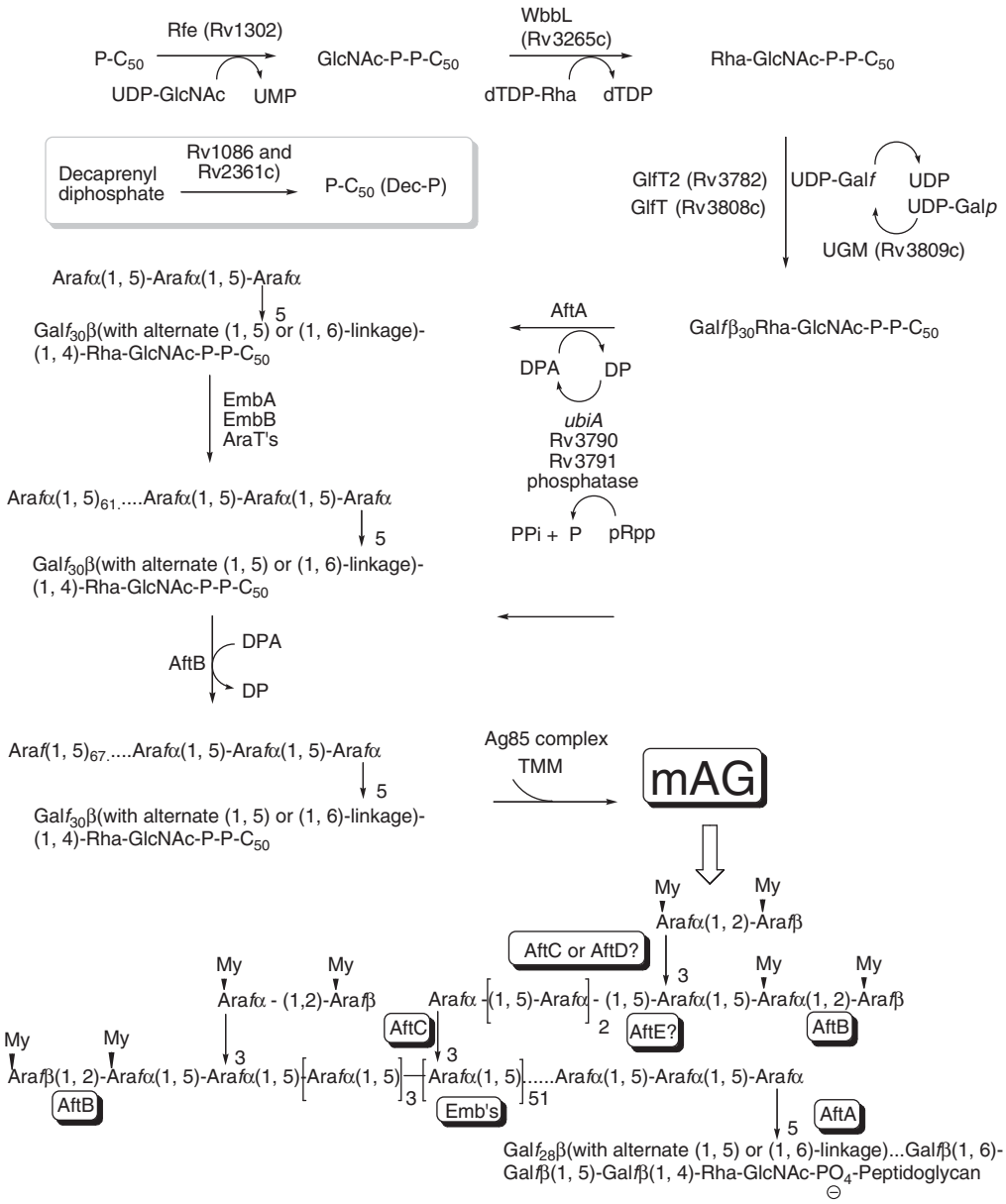
biosynthesis by showing that EmbC plays a major role in the LAM-arabinan biosynthesis.[47] The EmbC protein appears to possess two domains. The N-terminal domain has 13 predicted transmembrane spanning helices. The C-terminal hydrophilic extracytoplasmic domain promotes chain extension in LAM, as established by using an *embC* knock-out mutant of *M. smegmatis* complemented with plasmids expressing truncated *embC* genes, and an EmbC-specific peptide antibody.[48] The length of the arabinan core is species-dependent,[49] but in all species the arabinan backbone is punctuated with a branched hexaarabinofuranoside motif and a straight-chained tetra-arabinofuranoside motif. The penultimate arabinose residue of each branching chain is glycosylated via $\beta(1\rightarrow2)$-linkage to the terminal arabinofuranosides, which often terminates with $\alpha(1\rightarrow5)$-linked mannose residues. The composition of this mannose cap is also species-dependent. Some species lack a capping structure, whereas others possess up to a trimannoside of $\alpha(1\rightarrow6)$-Man*p* linked sugars. Multiple ManTs are involved in the sequential addition of mannopyranosyl units donated by decaprenyl-P-Man*p* on the periplasmic side of the plasma membrane. The distinct role of ManT (Rv2181), involved in the formation of di- and tri-Man*p* motifs of ManLAM, is a subject of interest in the light of evidence that Rv2181 plays a dual role of Man capping and mannan-core branching.[50] The investigation provides a tool for further study of ManLAM in the pathogenesis of TB.[51] Recent findings have identified another novel enzyme, Rv1635c (and MT1671), responsible for the addition of terminal Man*p* residues to the mature LAM in *M. tuberculosis*.[52]

3. MYCOLYLARABINOGALACTAN (mAG): STRUCTURE AND BIOSYNTHESIS

A comprehensive review of the structure and biosynthesis of the mycolylarabinogalactan (mAG) component of mycobacterial cell wall has been adequately covered elsewhere.[49] Nevertheless, a summary of the biosynthetic pathway is included as a guide to understanding the design, synthesis and evaluation of current inhibitors of the mAG pathway. The mAG is a major structural component of the *M. tuberculosis* cell wall. The polysaccharide component of the mAG is the AG. The linear galactan portion consists of alternating $\beta(1\rightarrow5)$ and $\beta(1\rightarrow6)$ galactofuran residues. The arabinan portion is attached to the galactan through the C-5 position of the $\beta(1\rightarrow6)$-linked galactofuranosides and consists of $\alpha(1\rightarrow5)$-linked arabinofuranosides with some branching introduced by 3,5-α-D-arabinofuranosides. Using an endogenous arabinase to solubilize the arabinan region of the cell wall, Bhamidi et al. have also demonstrated that succinyl esters are present on O-2 of the inner-branched 1,3,5-α-D-arabinofuranosyl residues.[45] The nonreducing termini of the arabinan are esterified by mycolic acids. This massive structure contains a Rha-$\alpha(1\rightarrow3)$-GlcNAc disaccharide at the reducing end of the AG, which serves as a linker. The GlcNAc is $\beta(1\rightarrow4)$ linked through a phosphodiester bond to the peptidoglycan. Depending on the species, the amount of mycolated arabinan termini varies.[49] A schematic representation of the mAG moiety and the main enzymes involved in the biosynthetic pathway are presented in Scheme 2.

The biosynthetic pathway of mAG starts with the synthesis of polyprenyl phosphate molecules. Mycobacteria use polyprenyl phosphate molecules as lipid carriers of activated sugars for the polysaccharide synthesis. *M. tuberculosis* uses a decaprenyl phosphate (Dec-P) carrier obtained from decaprenyl diphosphate, itself synthesized by prenyl diphosphate synthases (Rv1086 and Rv2361c; Scheme 2).[49] Rv1086 encodes *E,Z*-farnesyl diphosphate synthase and Rv2361c encodes decaprenyl synthase.[53–57]

Once the Dec-P is available, the true AG synthesis begins with the synthesis of the linker, followed by parallel synthesis of the arabinan and the galactan. It is believed that a uridine diphosphate-*N*-acetylglucosamine (UDP-GlcNAc) transferase, Rfe (Rv1302, ortholog to



Scheme 2. Roles of glycosyltransferases in the mycobacterial mycolated arabinogalactan biosynthesis. AftD and AftE are predicted enzymes but are yet to be confirmed. Pds, prenyl diphosphate synthases; My, mycolate; P, phosphate. TMM, trehalose monomycolate.

E. coli WecA) loads GlcNAc-P onto Dec-P to start the synthesis of the linker.[49] The second saccharide is then added by the rhamnosyltransferase WbbL (Rv3265c).[58] Inactivation of a temperature-sensitive mutant of *M. smegmatis* mc^2155 showed that WbbL is essential for the formation of the disaccharide linker connecting AG and peptidoglycan; therefore, inhibition of WbbL is expected to shut down the mycobacterial cell wall formation. Once the linker is formed, the galactan is then elaborated onto the linker by galactofuranosyltransferases (GlfT; Scheme 2). To date, only two GlfT enzymes have been identified. These enzymes

use UDP-Gal*f* as the activated galactofuranosyl donor, which is synthesized by UDP-Gal*p* mutase (UGM, Rv3809c) through the conversion of uridine diphosphate (UDP)-galacto*pyranose* (Gal*p*) to the corresponding *furanose* (Gal*f*), which is obtained by the conversion of UDP-glucose to UDP-Gal*p* by an epimerase, predicted to be Rv3634.[59] The transfer of the first Gal*f* residue onto the Rha-α(1→3)-GlcNAc linker is performed by a galactosyltransferase, GlfT2 (Rv3782), to yield decaprenyl-P-P-GlcNAc-Rha-Gal*f*-Gal*f*.[61] After the priming of the pathway by Rv3782, the rest of the Gal*f* moieties are transferred onto decaprenyl-P-P-GlcNAc-Rha-Gal*f*-Gal*f* by another GlfT (Rv3808c). Rose et al. showed that Rv3808c is bifunctional and is able to synthesize β(1→5) and β(1→6) linkages; this result might explain why only two GlfT enzyme have been identified.[62]

The arabinan synthesis is believed to be simultaneous with galactan chain elongation.[44,45] Immediately following addition of the first β(1→6)Gal*f*, it can be decorated at the C-5 position by Ara*f* residues. The enzymes responsible for the addition of Ara*f* are arabinofuranosyltransferases (AraT, Scheme 2). In contrast with the simplicity of the GlfTs with only two members, the AraTs have at least two characterized classes of enzymes, Emb and Aft. AraTs use glycophospholipid, decaprenolphosphoarabinose (DPA) as an Ara*f* donor during glycosylation. Biosynthesis of DPA from 5-phosphoribopyrophosphate (pRpp) is performed by three enzymes: Rv3806c, Rv3790, Rv3791.[63,64] First, the decaprenylphosphoryl-5-phosphoribose (DPPR) synthase Rv3806c, identified as UbiA in *C. glutamicum*,[65] transfers pRpp to decaprenyl phosphate to form DPPR,[66] then the 5′-phosphate is removed to give decaprenylphosphoryl ribose (DPR) by a phosphatase, putatively Rv3807c,[67] and DPR is then epimerized to DPA. The epimerization was initially proposed to occur via an oxidation–reduction process involving two enzymes (Rv3790 and Rv3791);[68] Rv3790 (or $DprE_1$) functions as decaprenylphosphoryl-β-D-ribose oxidase and Rv3791 (or $DprE_2$) as decaprenylphosphoryl-D-2-keto erythropentose reductase.[69] Interestingly, a recent report by Meniche et al. suggests that a third enzyme, Rv2073c, might also be involved in the epimerization reaction.[67]

Alderwick et al. identified and characterized a new enzyme, arabinofuranosyltransferase A (AftA) (Rv3792), from the *emb* locus.[70] The enzyme catalyzes the transfer of the first arabinofuranosyl moiety to the galactan for further elaboration by the Emb proteins toward formation of the AG heteropolysaccharide core, thereby playing a key role in "priming" the AG biosynthesis. Although both protein classes are arabinofuranosyltransferases, they cannot functionally replace each other. Additionally, AftA is not inhibited by EMB. Therefore, AftA represents a distinct drug target for TB. Most recently, Seidel et al. identified and characterized another arabinofuranosyltransferase B (AftB) (Rv3805c), which plays a pivotal role in the formation of β(1→2)-linkages in the terminal step of cell wall arabinan biosynthesis in *Corynebacterianeae* species such as *C. glutamicum* and *M. tuberculosis*.[71] Like AftA, AftB activity is not inhibited by EMB, thereby representing a new drug target for TB. In addition to the previously characterized arabinosyltransferases (AftA and AftB), Birch et al. have identified another enzyme, arabinofuranosyltransferase C (AftC) (Rv2673), responsible for the formation of α(1→3)-glycosidic linkages in the mycobacterial arabinan biosynthesis.[72] On the basis of the analysis of the *M. smegmatis* AftC mutant and the Ara:Gal ratio in AG, it is predicted that there are two more arabinofuranosyltranferases, AftD and AftE (Scheme 2), required for α(1→3)-glycosidic linkages and linear α(1→5)-glycosidic linkages, respectively.

The specific roles of EmbA and EmbB proteins in the arabinan biosynthesis were well established in their initial reports and were identified as the target of the antitubercular drug EMB.[12–15] Khasnobis et al. characterized the combined activity of these enzymes as being responsible for the transfer of two Ara*f* residues, and completion of β(1→2) disaccharide formation to make the terminal hexaarabinofuranosyl motif, Ara_6[73] Knockout stains of

M. smegmatis lacking a functional gene for either EmbA or EmbB proteins did not produce any detectable β(1→2) linked products. Also, Zhang et al. recently identified a new arabinosyltransferase activity independent from the Aft or Emb enzymes, thereby indicating the presence of additional arbinosyltransferase targets.[74]

The final step in the biosynthesis of mAG is the esterification of the terminal Ara*f* residues with mycolic acids. The three members of the Ag85 complex (Ag85A, Ag85B, and Ag85C) catalyze this step (Scheme 2).[75] General details about Ag85 and how it catalyzes mycolation of terminal ends of the AG are provided in the discussion involving current synthetic targets toward the inhibition of biosynthesis of mAG pathway.

4. INHIBITION OF LM/LAM BIOSYNTHETIC PATHWAY

Table I summarizes some of the main enzymes involved in the LM/LAM biosynthetic pathway and refers to a number of synthetic compounds related to their inhibition. Using the substrate requirements of PimA and PimB′, Dinev et al. developed galactose-derived phosphonate analogs **1** and **2** of *myo*-inositol-1-phosphate (IP) and PI, respectively, as possible inhibitors of those enzymes (Fig. 3).[76] In a cell-free radiolabeled mannosyltransferase assay, the IP analog was an effective inhibitor of PimA at 0.01 mM, while PI displayed a weak inhibition of PimB′ at concentrations up to 1 mM.

In order to probe the biosynthesis of the mannan portion of LM/LAM, a series of prenyl-based photoactivable probes with a benzophenone moiety and flexible linkers were prepared by Guy et al. through the utilization of Ppm synthase and a PPM-dependent α(1→6)mannosyltransferase, and tested for their ability as substrates for recombinant Ppm synthase (Mt-Ppm1/D2).[77] These probes were also tested for inhibitory activity, upon photoactivation, against recombinant Mt-Ppm1/D2, and *M. smegmatis* α(1→6)mannosyltransferases. All the synthesized probes functioned as very good substrates for Mt-Ppm1 in the Ppm synthase assay, suggesting that changes (such as saturation, unsaturation, chain length, etc.) at the lipid portion of polyprenyl phosphates may be well tolerated. Upon photoactivation, several of these compounds showed inhibitory activity against Mt-Ppm1 obtained from cell extracts. The probes were also screened for activity in a radiolabeled mannosyltransferase assay. Probe **3** was the best among them and showed 71.5 and 83.9% inhibition in mannosyltransferase activities against *M. smegmatis* α(1→6)-mannosyltransferase and Mt-Ppm1/D2, respectively (Fig. 4).[77]

The understanding of the substrate scope of PPM-dependent α(1→6)-mannosyltransferase and substrate–enzyme interactions is essential for the design and synthesis of potential inhibitors. Brown et al. designed and studied a panel of octyl α(1→6)-polysaccharides (α-D-Man*p*(1→6)-α-D-Man*p*-*O*-octyl) as substrate analogs.[78] The synthetic mannosides (**4–7**; Fig. 4) were mannosylated in a cell-free assay. In a similar approach, Subramanian et al. first used monofunctionalized disaccharides with modification at the 2′-position (**8–11**; Fig. 4) to probe the substrate scope of the PPM-dependent α(1→6)-mannosyltransferase.[79] The synthetic analogs with small substituents (**9–11**) acted as substrates for the enzyme with the exception of compound **8** bearing a methoxy substituent at the 2′-position. In a cell-free assay for PPM-dependent mannosyltransferase activity, none of the synthetic analogs showed inhibitory activity against the enzyme. Furthermore, the 2′-deoxygenated analog **9** was recognized by the enzyme as a substrate, implying that hydrogen bonding interactions between the protein and the 2′-OH are not critical for catalytic activity. Given the outcome of the substrate scope and inhibitory potency of the monofunctionalized analogs presented above, a series of di-functionalized disaccharides **12–17** (Fig. 4) having a modification at the 2-OH and 6-OH positions on the nonreducing end and

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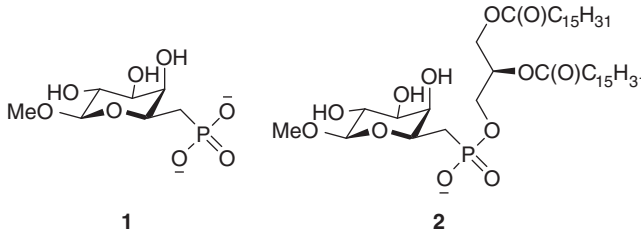
Table I. Enzymes Involved in PIM/LM/LAM Biosynthesis, Along With Current Synthetic Inhibitors

Enzyme, Rv #	Species found	Function	Essential	Synthetic Inhibitor/ Probe#	Refs.
PgsA (Rv2612c)	*M. smegmatis*	Acyltransferase, phosphorylates myo-inositol	YES		25
PimA (Rv2610c)	*M. smegmatis*	Mannosyltransferase, adds mannose to PI to give PIM_1	YES	**1** (Fig. 3)	26,29,76
Rv2611c	*M. smegmatis*	Acylation of PIM_1	YES		32
PimB′ (Rv2188c)	*M. tuberculosis* *C. glutamicum* (*NcgI2106*)	Mannosyltransferase, forms PIM_2	Unknown	**2** (Fig. 3)	33,76,148
PimC (RvD2-ORF1)	*M. tuberculosis* *CDC1551*	Mannosyltransferase, forms PIM_3	NO		35
PimE (Rv1159)	*M. smegmatis*	Mannosyltransferase, forms mannosylated $AcPIM_5$	Unknown		36,41
Ppm1 Synthase (Rv2051c)	*M. smegmatis*	Mannosyltransferase; forms LM	Unknown	**3, 12–14** (Fig. 4)	42,77,80
MptA (Rv2174)	*M. tuberculosis* *H37Rv*	Mannosyltransferase, mannan backbone of LM	Unknown		44,45
MptB (NCg12093)	C. *glutamicum*	Mannosyltransferase, mannan backbone of LM	Unknown		23
EmbC (Rv3793)	*M. smegmatis*	Arabinosylation of LM to form LAM	YES		149
Rv2181	*M. smegmatis*	Mannosyltransferase, Man capping (ManLAM) and mannan-core branching	YES		50,51
Rv1635c (MT1671)	*M. tuberculosis* *CDC1551*	Mannosyltransferase, addition of first Man*p* capping residue	Unknown		19,51,52

PIM, phosphatidyl-*myo*-inositol mannosides; LAM, lipoarabinomannan; LM, lipomannan; Ppm1, polyprenol monophosphomannose synthase; Mpt, mannopyranosyltransferase; Emb, Ethambutol.



1 2

Figure 3. Galactose-derived phosphonate analogs of IP and PI. IP, *myo*-inositol-1-phosphate; PI, phosphatidylinositol.

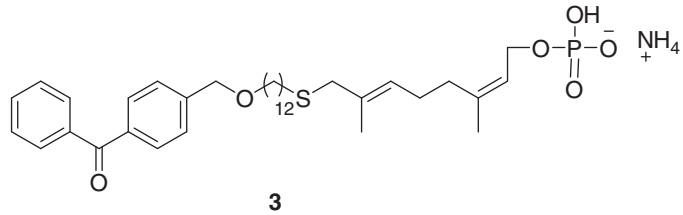
an anhydro-sugar **18** derived from D-mannose were synthesized. In the PPM-dependent ManT assay, none of the compounds proved to be promising substrates for the enzyme. These results can be explained by the absence of a hydroxyl group at C-6′ position which is required for further glycosylation by the enzyme. Using a 0.2 mM concentration of compound **20** as a substrate for mannose transfer, a 2.0 mM concentration of amino or halide substituted analogs produced weak to moderate inhibition (30–57%). Compounds **12, 13,** and **14** inhibited the reaction by 57, 33, and 30%, respectively.[80]

To further test the substrate scope of the enzyme, a homologous series of mono- through tetrasaccharides (**19,**[81] **20–22**; Fig. 4) was synthesized and screened to probe the effect of acceptor length on activity. In addition, a panel of deoxy and methoxy analogs of **20** (**23–34**; Fig. 4) were synthesized and probed using the previously reported protocol.[82] The di-saccharide motif is the most significant feature recognized by the enzyme, as increases in activity were not obtained by moving to larger oligosaccharides. Among other important findings, methylation of hydroxyl groups at C-2 of either mannopyranose residues in the disaccharide substrates resulted in complete loss of activity, suggesting that $\alpha(1\rightarrow2)$-mannopyranosyl branches in the mannan core of LM/LAM occurs after the assembly of the larger $\alpha(1\rightarrow6)$-linked mannan. By using extensively modified disaccharides (either deoxy or methoxy at each position), the authors shed light on the adaptable substrate scope of the synthetic analogs for use in inhibitor design. Deoxygenation at all positions, except C-2′/C-4 in the parent disaccharide **20,** led to loss of activity, indicating possible hydrogen bonding interactions between the enzyme active site and the acceptor analogs.[83]

Two additional structural motifs were discovered linked to mannopyranose capping residues, 5-deoxy-5-methylthio-pentose, and its sulfoxide counterpart from *M. tuberculosis* and *M. kansasii*.[84–86] Turnbull et al. established that the motif was 5-deoxy-5-methylthio-α-xylose (**35** MTX),[87] and Lowary and co-workers assigned these monosaccharides as having a D-configuration and established their linkage as $\alpha(1\rightarrow4)$ to mannopyranose residue of LAM (Fig. 5).[88] The disaccharides formed between α-methyl mannoside and either MTX **35** or MSX **36** are shown in Figure 5.

5. INHIBITION OF THE mAG BIOSYNTHETIC PATHWAY

A list of some of the enzymes involved in the mAG biosynthesis, along with current synthetic inhibitors, is shown in Table II. Because the biosynthetic pathway of mAG starts with the synthesis of polyprenyl phosphate molecules, targeting prenyl phosphate synthase (Rv1086 and Rv2361c) is a possible route to the inhibition of mAG pathway. Schulbach et al. synthesized ω,*E*-geranyl diphosphate as substrate analogs of the *Z*-farnesyl diphosphate synthase; however, only one of the compounds **(37)** showed any significant activity (Fig. 6).[55]



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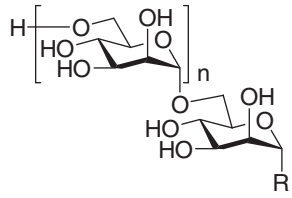
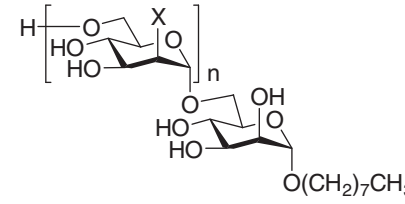
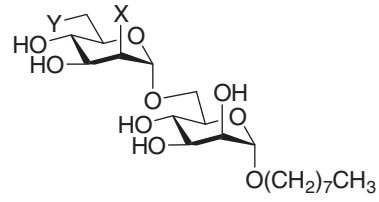


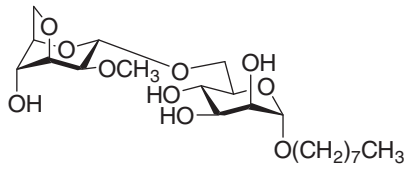
4 R = $S(CH_2)_7CH_3$; n = 1
5 R = $O(CH_2)_7CH_3$; n = 1
6 R = $O(CH_2)_8CH_3$; n = 1
7 R = $O(CH_2)_8CH_3$; n = 2



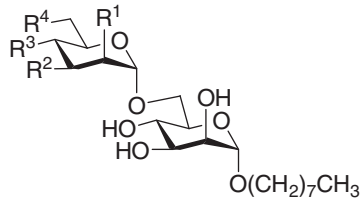
8 X = OCH_3; n = 1
9 X = H; n = 1
10 X = F; n = 1
11 X = NH_2; n = 1
19 X = OH; n = 0
20 X = OH; n = 1
21 X = OH; n = 2
22 X = OH; n = 3



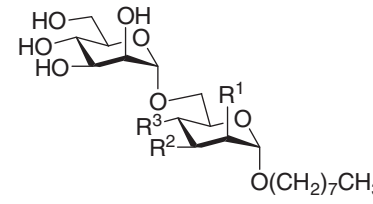
12 X = F; Y = NH_2
13 X = F; Y = F
14 X = NH_2; Y = NH_2
15 X = NH_2; Y = F
16 X = OCH_3; Y = NH_2
17 X = OCH_3; Y = F



18

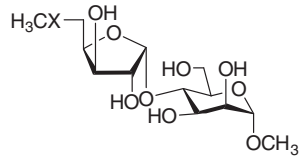


23 R^1 = OH; R^2 = OCH_3; R^3 = OH; R^4 = OH
24 R^1 = OH; R^2 = H; R^3 = OH; R^4 = OH
25 R^1 = OH; R^2 = OH; R^3 = OCH_3; R^4 = OH
26 R^1 = OH; R^2 = OH; R^3 = H; R^4 = OH
27 R^1 = OH; R^2 = OH; R^3 = OH; R^4 = OCH_3
28 R^1 = OH; R^2 = OH; R^3 = OH; R^4 = H



29 R^1 = OCH_3; R^2 = OH; R^3 = OH
30 R^1 = H; R^2 = OH; R^3 = OH
31 R^1 = OH; R^2 = OCH_3; R^3 = OH
32 R^1 = OH; R^2 = H; R^3 = OH
33 R^1 = OH; R^2 = OH; R^3 = OCH_3
34 R^1 = OH; R^2 = OH; R^3 = H

Figure 4. Prenyl-based photoreactive probe and mannose-derived substrates used in the study of ManTs.



35 X = S (MTX)
36 X = S=O (MSX)

Figure 5. Xylose residues found in mannan capping motifs.

Following the availability of Dec-P, actual AG synthesis begins with the synthesis of the diglycosylphosphoryl linker. Currently no study of the UDP-GlcNac transferase, the first enzyme of the linker synthesis, has been reported. The second saccharide is added by the rhamnosyltransferase WbbL (Rv3265c). Although no inhibitors of the rhamnosyltransferase have been reported to date, WbbL has generated some interest since Grzegorzewicz et al. developed a microtiter plate-based assay to evaluate potential inhibitors of the rhamnosyltransferase.[89]

A significant amount of research has been dedicated to the development of UDP–sugar analogs as inhibitors of the UDP-Gal*p* mutase (UGM). Tangallapally et al. developed libraries of nitrofurans as UGM inhibitors; some of the compounds **(38–77)** displaying inhibitory activity are presented in Figure 7.[90] A few of the nitrofuranylamides were further characterized by Hurdle et al.;[91] the microbiological assessment confirmed that the nitrofuranylamides are lead compounds for drug development.

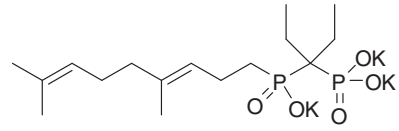
A time-dependent inactivation of UGM, using fluorinated UDP-Gal*f* derivatives **78a** and **78b,** was first shown by Liu et al.[92] Later, Caravano et al. extended this work using the nonfluorinated exo-cyclic UDP-glycal derivative **79**.[93] Pursuing their synthetic efforts, Caravano et al. synthesized two more fluorinated analogs **80a** and **80b** (Fig. 8) and subjected them to a time-dependent study in anticipation of improved inactivation.[94] Both **80a** and **80b** showed significantly slower inactivation kinetics compared with the nonfluorinated analog. Most recently, the same group reported the synthesis of three *C*-glycoside analogues of UDP-Gal*p* as conformational probes to explore the binding pocket of UGM.[95] The compounds **81** and **82** achieved lower inhibition percentages than the furanose compounds when tested against *E. coli* UGM. However, **81** and **82** did not display a time-dependent inactivation like all the other tested compounds, suggesting that these derivatives might act as competitive inhibitors.

In addition, a biochemical study of UGM revealed that the enzyme uses flavin adenine dinucleotide (FAD) as a cofactor.[96] Using this information, Pinto et al. synthesized transition-state mimics of the proposed oxocarbenium intermediate.[97,98] The synthesized sulfonium, ammonium, and selenium ions **83–88** (Fig. 9) did not display a very strong inhibitory activity against UGM at a concentration of 10 mM. Itoh et al. worked on a linear substrate analog, UDP-galactitol **89** shown in Figure 9.[99] The linear compound inhibited UGM activity by 54% at 50 μM. Another attempt by Pan et al. involved synthesis of acyclic inhibitors **90** and **91** (Fig. 9) that produced weak inhibition (0–11%) at 2 M.[100] Martin and co-workers prepared UDP-Gal*f* analogs and transition-state analogs **92–98** built around a 1,4-dideoxy-1,4-imino-D-galactitol.[101–103] The inhibitory activities of the various galactitol-based inhibitors against UGM are shown in Figure 9.

Kiessling et al. proposed a slightly different approach, moving away from substrate and transition analogs by using a fluorescence polarization-binding assay to screen commercially available libraries.[104] This initial screening led to the discovery of three hits **99–101** (Fig. 10) with a common five-membered thiazolidinone core. A library of compounds with the thiazolidinone core was designed and screened for activity, revealing three more possible inhibitors **102–103** with a 5-arylidene 4-thiazolidinone structure. Unfortunately, further

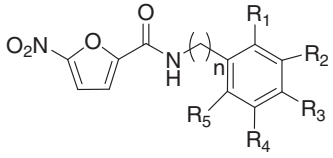
Table II. Enzymes Involved in mAG Biosynthesis, Along With Current Synthetic Inhibitors

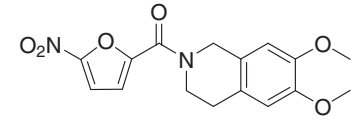
Enzyme, Rv #	Species found	Function	Essential	Synthetic inhibitor	Refs.
UbiA (Rv3806)	*M. tuberculosis*	Phosphoribosyl transferase	YES		66,150
DprE1 (Rv3790)	*M. tuberculosis*	Decaprenylphosphoryl-D-ribose oxidase	YES	**113** (Fig. 12)	67–69,150
DprE2 (Rv3791)	*M. tuberculosis*	Decaprenylphosphoryl-D-2′-keto erythro pentose reductase	YES		67–69,150
Rv1086	*M. tuberculosis*	Prenyl diphosphate synthase, for synthesis Dec-P	YES	**37** (Fig. 6)	49,53,55–57
Rv2361c	*M. tuberculosis*	Decaprenyl diphosphate synthase	YES		49
Rv1302, ortholog to *E. coli* WecA	*E. coli*	UDP-GlcNAc transferase, loads GlcNAc-P to Dec-P	YES		58,88
WbbL (Rv3265c)	*M. smegmatis MC2155*	Rhamnosyltransferase for formation of disaccharide linker	YES		58
UGM (Rv3809c)	*M. smegmatis*	UDP-Gal*p* mutase, converts UDP-Gal*p* to a furanose	YES	**38–82** (Figs. 7,8) **83–98** (Fig. 9) **99–108** (Fig. 10)	94,95,97–106
GlfT2 (Rv3782)	*M. tuberculosis*	A galactosyltransferase (GlfT); transfers first galactofuranoside	YES	**109–111** (Fig. 11)	61,107,108
GlfT (Rv3808c)	*M. tuberculosis*	Transfers Gal*f* moeities	YES	**112** (Fig. 11)	62,109
AftA (Rv3792)	*M. tuberculosis*	Transfer first arabinofuranosyl moiety to the galactan	YES		70
AftB (Rv3805c)	*C. glutamicum*	Plays role in formation of β(1→2)-linkages in arabinan biosynthesis	YES	**141–155**, **156–164** (Fig. 15), **171–179** (Fig. 16)	61,123–126 127,128
AftC (Rv2673) MSMEG2785 Ncgl1822	*M. smegmatis* *C. glutamicum*	Required for formation of α(1→3)-linkages in arabinan biosynthesis	YES	**125** (Fig. 14)	72,118
EmbA (Rv3794)	*M. tuberculosis*	Transfers two Ara*f* residues, completes Ara_6 motif	YES	**114–120** (Fig. 13)	12–14,110–113
EmbB (Rv3795)	*M. tuberculosis*	Arabinosyltransferase	Yes?		65,12–14
Antigen85	*M.tuberculosis*	Acyltransferase	Yes	**189–214** (Figs. 18,19)	132–147



37 IC_{50} = 300 μM

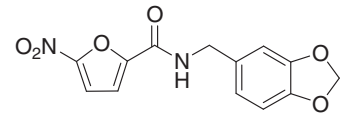
Figure 6. ω,*E*-Geranyl diphosphate inhibitor for Rv1086.



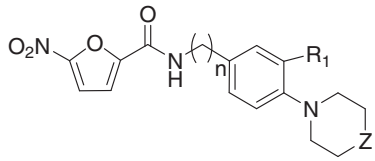


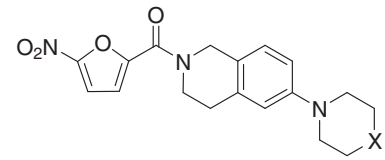
51 MIC_{90} = 0.15 μg/mL

38 n = 0, $R_1 = R_2 = R_3 = R_4 = R_5$ = H; MIC_{90} = 0.8 μg/mL
39 n = 0, $R_1 = R_2 = R_3 = R_5$ = H, R_4 = Cl; MIC_{90} = 0.8 μg/mL; IC_{50} = 15 μM
40 n = 0, R 1= $R_3 = R_4 = R_5$ = H, R_2 = F; MIC_{90} = 0.8 μg/mL; IC_{50} = 28 μM
41 n = 0, $R_1 = R_2 = R_4 = R_5$ = H, R_3 = OMe; MIC_{90} = 0.4 μg/mL
42 n = 0, $R_1 = R_2 = R_3 = R_5$ = H, R_4 = OMe; MIC_{90} = 0.8 μg/mL
43 n = 1, $R_1 = R_2 = R_3 = R_5$ = H, R_4 = OMe; MIC_{90}= 0.1 μg/mL; IC_{50} = 115 μM
44 n = 1, $R_1 = R_2 = R_5$ = H, $R_3 = R_4$ = OMe; MIC_{90} = 0.1 μg/mL
45 n = 1, $R_2 = R_4 = R_5$ = H, $R_1 = R_3$ = OMe; MIC_{90} = 0.4 μg/mL
46 n = 1, $R_1 = R_5$= H, $R_2 = R_3 = R_4$= OMe; MIC_{90} = 0.8 μg/mL
47 n = 1, $R_1 = R_4 = R_5$ = H, R_2 = OMe, R_3 = OH; MIC_{90} = 0.15 μg/mL
48 n = 1, $R_1 = R_2 = R_4 = R_5$ = H, $R_3 = NMe_2$; MIC_{90} = 0.1 μg/mL
49 n = 2, $R_1 = R_2 = R_4 = R_5$ = H, R_3 = OMe; MIC_{90} = 0.8 μg/mL
50 n = 2, $R_1 = R_2 = R_5$ = H, $R_3 = R_4$ = OMe; MIC_{90} = 0.4 μg/mL



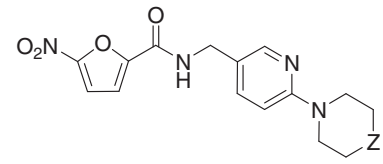
52 MIC_{90} = 0.15 μg/mL





53 X = NBn, MIC_{90} = 0.0062 μg/mL
54 X = NMe, MIC_{90} = 0.2 μg/mL
55 X = S, MIC_{90} = 0.4 μg/mL

56 n = 0, R_1 = H, Z = N-Bn; MIC_{90} = 0.8 μg/mL
57 n = 0, R_1 = H, Z = 0; MIC_{90} = 0.2 μg/mL
58 n = 0, R_1 = H, Z = N-Me; MIC_{90} = 0.2 μg/mL
59 n = 1, R_1 = H, Z = N-Bn; MIC_{90} = 0.0125 μg/mL
60 n = 1, R_1 = H, Z = CH-Bn; MIC_{90} = 0.8 μg/mL
61 n = 1, R_1 = H, Z = S; MIC_{90} = 0.1 μg/mL
62 n = 1, R_1 = F, Z = N-Bn; MIC_{90} = 0.025 μg/mL
63 n = 1, R_1 = F, Z = N-Me; MIC_{90} = 0.8 μg/mL
64 n = 1, R_1 = F, Z = S; MIC_{90} = 0.8 μg/mL
65 n = 1, R_1 = F, Z = O; MIC_{90} = 0.4 μg/mL
66 n = 1, R_1 = F, Z = CH-Bn; MIC_{90} = 0.2 μg/mL
67 n = 1, R_1 = H, Z = NCO_2Et; MIC_{90} = 0.0062 μg/mL
68 n = 1, R_1 = H, Z = NCO_2Me; MIC_{90} = 0.1 μg/mL
69 n = 1, R_1 = H, Z = $NCO_2{}^nBu$; MIC_{90} = 0.05 μg/mL
70 n = 1, R_1 = H, Z = $NCO_2{}^iPr$; MIC_{90} = 0.05 μg/mL
61 n = 1, R_1 = H, Z = NCO_2allyl; MIC_{90} = 0.5 μg/mL
72 n = 1, R_1 = H, Z = $NCONH{}^nPr$; MIC_{90} = 0.2 μg/mL
73 n = 1, R_1 = H, Z = $NCO_2{}^iPr$; MIC_{90} = 0.8 μg/mL



74 n = 1, Z = $NCO_2{}^tBu$; MIC_{90}= 0.05 μg/mL
75 n = 1, Z = N-Bn; MIC_{90} = 0.062 μg/mL
76 n = 1, Z = $NCONH{}^iPr$; MIC_{90} = 0.56 μg/mL
77 n = 1, Z = NCO_2Et; MIC_{90} = 0.05 μg/mL

Figure 7. UGM inhibitors based on nitrofuran derivatives.

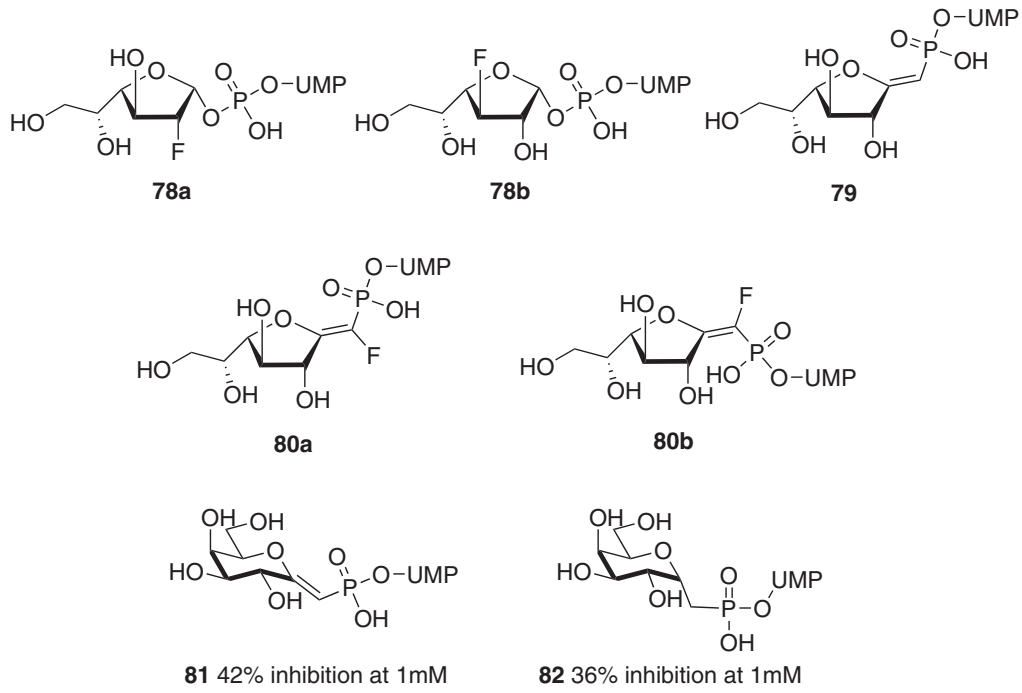


Figure 8. A panel of UDP-Gal*f* analogs for time-dependent inhibition study.

characterization of those compounds demonstrated that the synthetic compounds react with thiols under physiological conditions, compromising their future as leads in drug discovery. Further studies on this type of compounds revealed that a 2-aminothiazole scaffold could be used to replace the thiazolidinone core.[105] From this work, four compounds emerged as potential molecules of interest for drug development **104–107** (Fig. 10). More recently, Dykhuizen et al. expanded this family of inhibitors with compounds displaying higher affinity to UGM, with compound **108** as the most potent inhibitor at IC_{50} value of 3.5 μM.[106] The inhibitors are believed to bind the substrate-binding pocket and an adjacent subsite, explaining the higher binding affinity.

Wen et al. developed a series of analogs of the linker to inhibit GlfT2.[107] Three compounds **109–111** (Fig. 11) displayed interesting inhibitory activity in a cell-free GlfT2 inhibition assay. When they reported the identification of Rv3808c, Rose et al. also probed the substrate specificity of the recombinant enzyme and discovered a preference for trisaccharide substrates over mono- or disaccharides.[62] The same group later reported a coupled spectrophotometric assay allowing a high-throughput screening of potential inhibitors.[108] Before the spectrophotometric assay was reported, efforts to inhibit the GlfT had been reported. Cren et al. designed iminosugar analogs **112a** and **112b** as potential inhibitors of Rv3808c.[109] Both compounds were synthesized with a deoxygenated C-2 position to promote stability, and the racemic products were evaluated against UDP-Gal*f* transferase from *M. smegmatis* using a formaldehyde release assay. Compound **112a** showed moderate inhibitory activity (IC_{50} = 4.8 mM) compared with its diastereomer **112b** that only showed 40% inhibition at 8 mM (Fig. 11).

Makarov et al. recently reported the design, synthesis, and in vitro testing of 1,3-benzothiazin-4-ones (BTZ) represented by compound **113a** (Fig. 12).[69] The *S* enantiomer **113b**, called BTZ043, displayed a minimum inhibitory concentration (MIC) of 1 ng/mL (2.3 nM) against *M. tuberculosis* H37Rv, and 4 ng/mL (9.3 nM) against *M. smegmatis*. The uptake, intracellular killing, and potential cytotoxicity ex vivo as well as the in vivo efficacy of BTZ

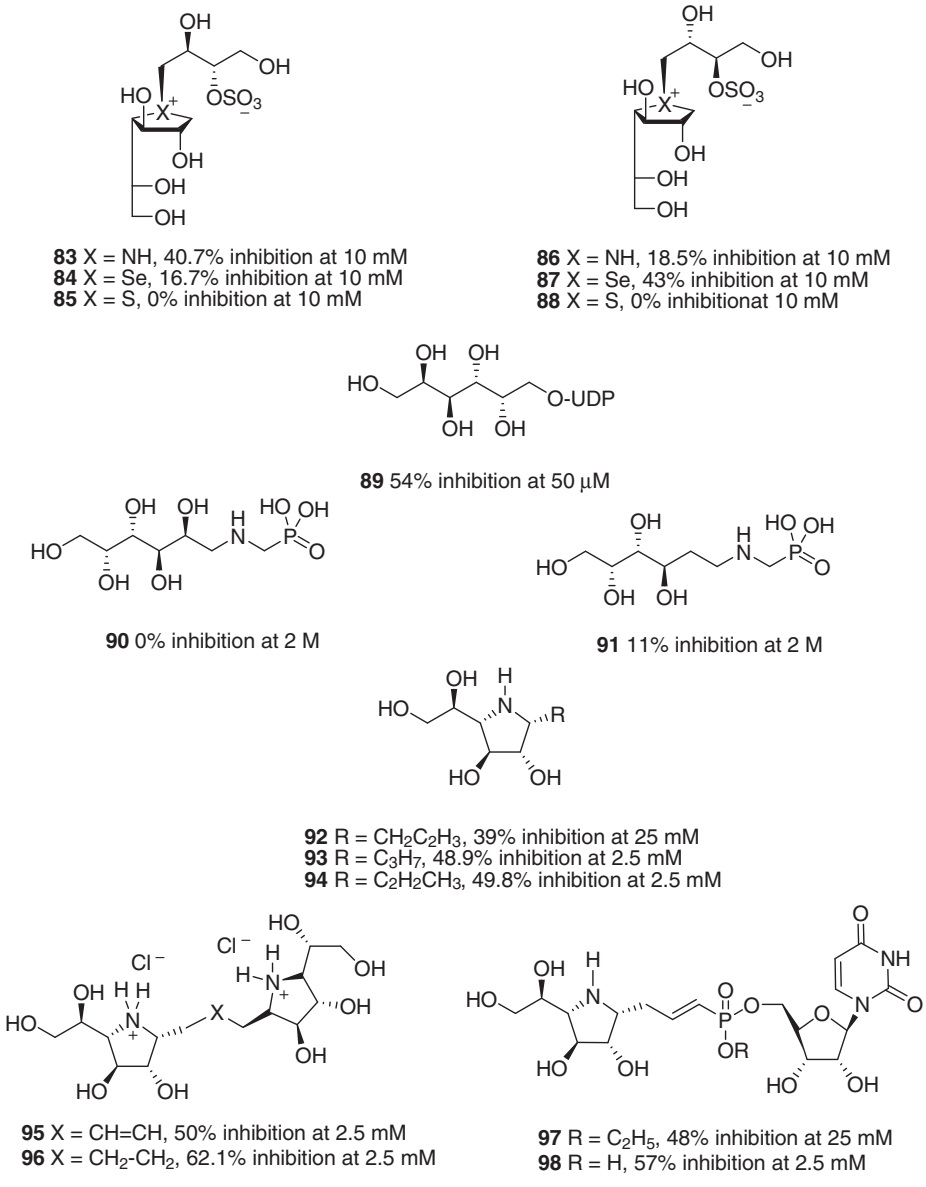


Figure 9. Transition-state inhibitors and substrate analogs for UGM based on galactitol.

were evaluated. Because the compound displayed bactericidal activities in the range of INH, its target was investigated. The BTZ family of compounds was found to target $DprE_1$ (Rv3790).

As for the AraTs, current research efforts are aimed at the development of EMB analogs. Jia et al. reported the pharmacodynamic and pharmacokinetic characterization of a promising EMB analog, **SQ 109** (**114**), shown in Figure 13.[110] Similarly, Bogatcheva et al. studied new diamine scaffolds as potential EMB analogs.[111] Compound **SQ 775** (**115**) also emerged from in vitro and in vivo testing as a lead for drug development.[111] Yendapally et al. recently published the synthesis of analogs **116** and **117** that inhibited the growth of mycobacteria at comparable concentration to EMB (1.6, 1.6, and 0.8 μg/mL, respectively).[112]

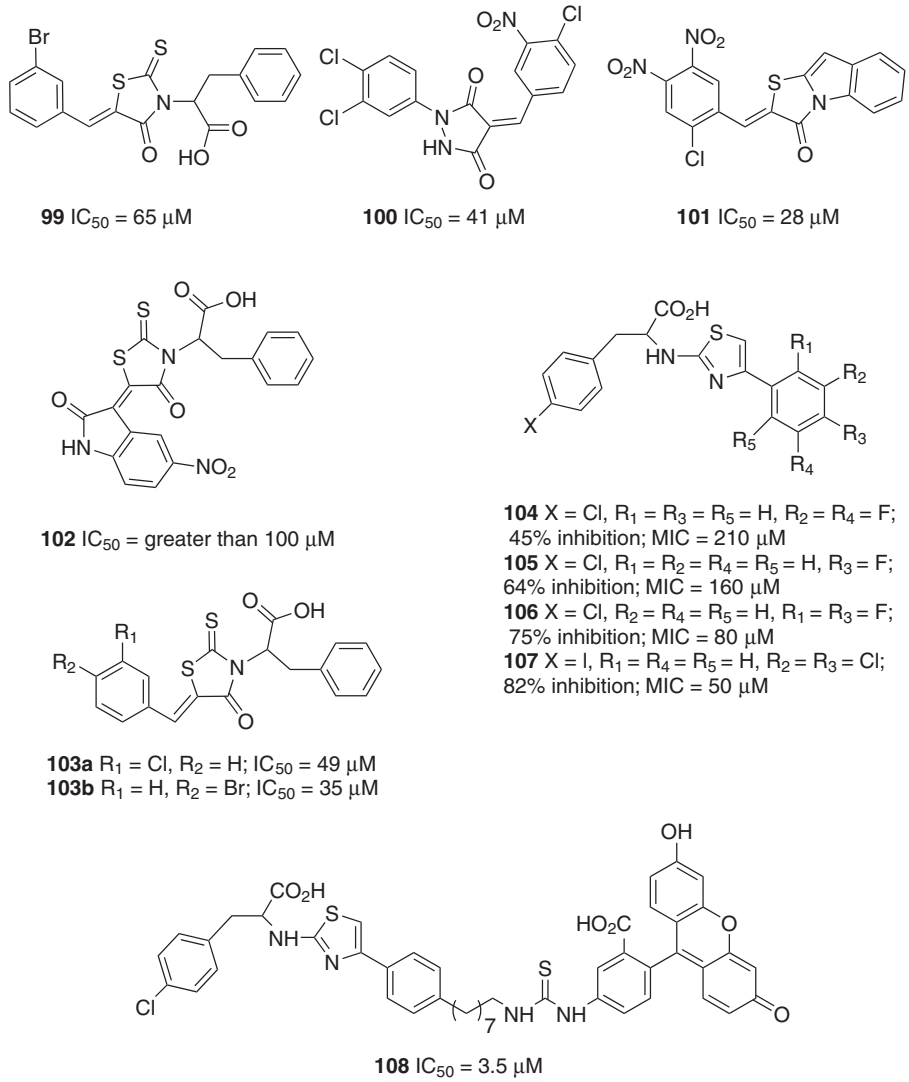


Figure 10. Thiazolidinone and 2-aminothiazole-based UGM inhibitors. For **105–108**, each compound was used at 50 μM against UGM inhibition.

Faugeroux et al. developed EMB analogs **118–120** (Fig. 13) with a more rigid scaffold; unfortunately, the presence of conformational restriction seemed to lower the activity of the compounds.[113]

Most of the research efforts for inhibition of the arabinan biosynthesis, for both LAM and mAG, have focused on the development of DPA analogs. An octyl 5-*O*-(α-D-arabinofuranosyl)-α-D-arabinofuranoside disaccharide with a free hydroxyl (the octyl chain mimicks the lipid component of the natural donor, DPA) is a recognized acceptor by arabinofuranosyltransferases involved in the biosynthesis of arabinan portions of AG and LAM in mycobacterial cell wall synthesis. Recently, partially blocked disaccharide analogs at the nonreducing end have been shown as modest inhibitors of arabinofuranosyltransferases in a cell free assay.[114–116] Pathak et al. synthesized a series of disaccharides modified at the

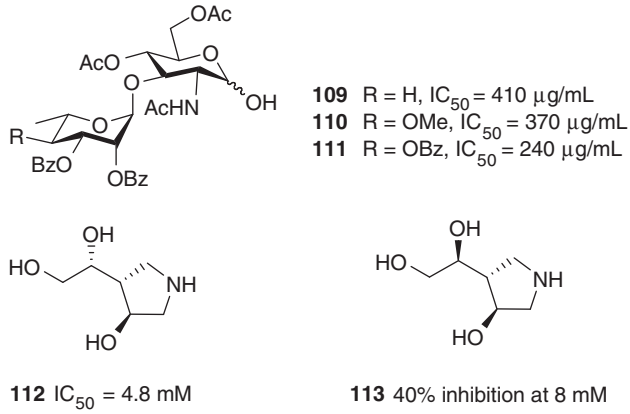


Figure 11. mAGP linker analogs and deoxygenated iminosugars as inhibitors of galactosyltransferase.

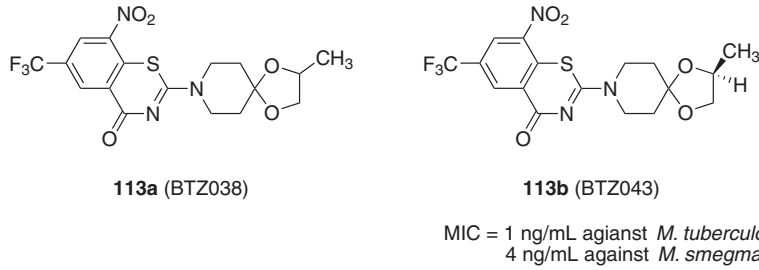


Figure 12. BTZ043, a representative member of 1,3-benzothiazin-4-ones (BTZs), a new class of antibacterial agents. BTZ043 kills *M. tuberculosis* by inhibition of decaprenylphosphoryl-β-D-ribose 2′-epimerase responsible for formation of decaprenylphosphoryl arabinose, a precursor required for the synthesis of cell-wall arabinans.

C-5′-position of the disaccharide and utilized them to probe their ability to act as acceptors as well as inhibitors for *M. tuberculosis* H37Ra (Fig. 14).[117] When probed for their ability as potential acceptors of [^{14}C]Ara*f* from DP[^{14}C]A in the transferase assay,[118] **121, 123, 125–129** did not show any detectable acceptor activity due to the blocking of C-5 hydroxyl group at the nonreducing end. Among all the synthetic analogs, compound **125**, having a sterically large dicyclohexylamino substitution, displayed promising inhibitory activity, exhibiting an $IC_{50} = 1.56$ mM as determined in a cell-free enzyme assay. It also exhibited an MIC of 8 μg/mL in a bacterial growth inhibition assay against *M. tuberculosis* H37Ra. Compound **125** also showed inhibitory activity against *M. avium* complex (MAC) NJ211 with an MIC of 16 μg/mL. Expansion of the library of C-5′-modified disaccharides led to compounds **130–132** (Fig. 14),[119] followed by the recent report of **133–135**.[120] This α(1→5)-linked arabinofuranoside disaccharide library revealed additional leads for future drug development.

From a panel of *C*-phosphonate analogs of DPA synthesized by Centrone et al., one of the *C*-phosphonate derivatives, **136,** displayed promising inhibitory potency against *M. tuberculosis* strain H37Rv with an MIC of 3.13 μg/mL.[121] Based on this success, a series of sulfone and phosphonic acid analogs of DPA were synthesized (Fig. 15; see only active compounds **137–140**) and tested for their ability to prevent growth of *M. tuberculosis* strain H37Rv using a fluorescence-based Alamar Blue microplate assay.[122] Weak to modest growth inhibitory activity for the synthetic compounds was reported, as shown in Figure 15. In the same approach, Cociorva et al. reported the synthesis of a panel of oligosaccharides containing C-5 arabinofuranosyl residues **141–155** (Fig. 15).[123] It should be noted that

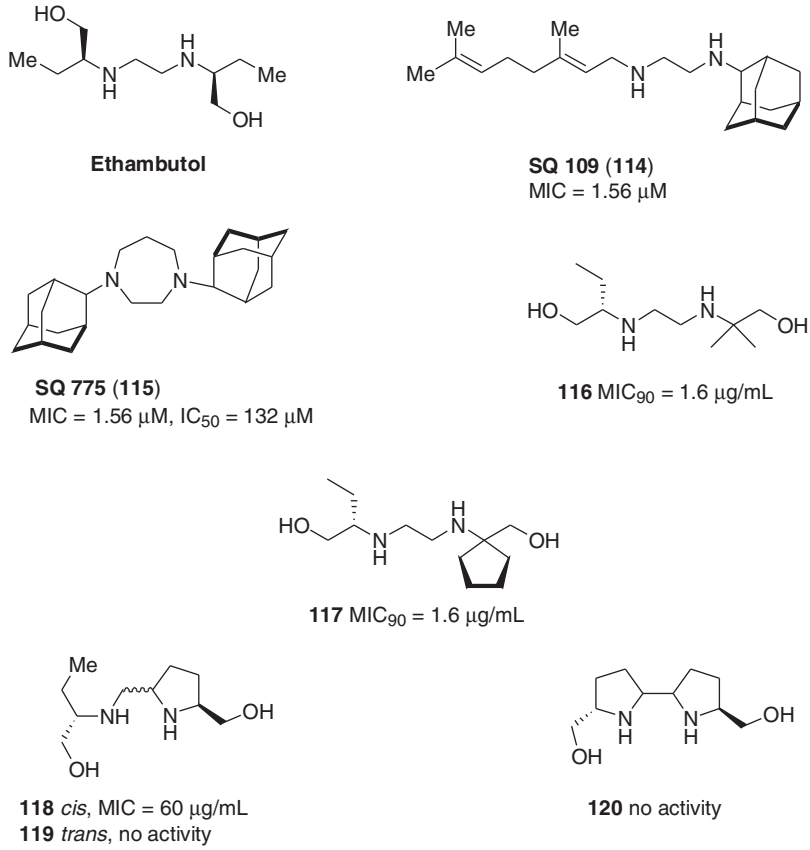
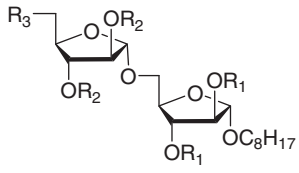


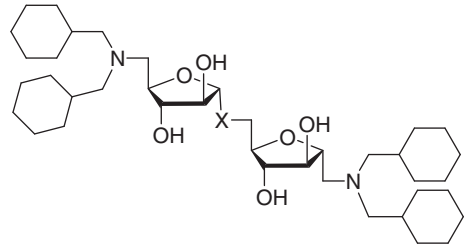
Figure 13. Ethambutol, a firstline antitubercular drug. Currently being investigated for their ability to overcome mycobacterial resistance associated with ethambutol, **114–120** are synthetic analogs of ethambutol.

compounds **141–143** represent structural motifs found within the mycobacterial arabinan. Each member of the panel contained a modification at the C-5 position at the nonreducing end, independently with amino, azido, fluoro, and methoxy functionalities. To date, the inhibitory activity for **144–155** against bacterial growth has not been reported.

In order to access additional substrates for AraT inhibition, Cociorva et al. modified compounds **141–143** at C-3(C-3′) position(s) with amino, azido, or methoxy functionality to constitute a library of additional compounds (Fig. 15).[124] Compounds **156–160** and **162–164** having modification at C-3(C-3′) position(s) were screened for their ability to act as substrates for AraTs responsible for $\alpha(1\rightarrow5)$ and $\beta(1\rightarrow2)$-linked arabinan chain using a membrane fraction (devoid of enzyme for the installation of $\alpha(1\rightarrow3)$-linked residues in mycobacterial arabinan) from mycobacteria. All of the synthetic analogs **156–160** and **162–164** showed modest inhibition at 3.6 mM against AraTs, with those of **142** being the most potent. In a subsequent Alamar Blue mycobacterial growth assay, only low levels of activity of the synthetic compounds were reported when screened at a concentration of 3.6 μg/mL.

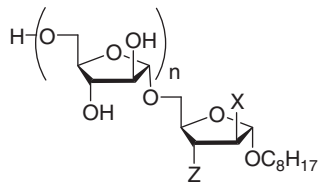
Davis et al. also synthesized galactofuranosyl alkyl thioglycosides **165–167** (Fig. 16) as possible DPA analogs.[125] The antibacterial activity was evaluated against *M. smegmatis* ATCC 14468. The compounds exhibited moderate to good activity, with **166** having the highest antibacterial activity with an MIC of 1 μg/mL. A parent family of *N*,*N*-dialkyl sulfenamides and sulfonamides **168–170** (Fig. 16) was later synthesized and displayed

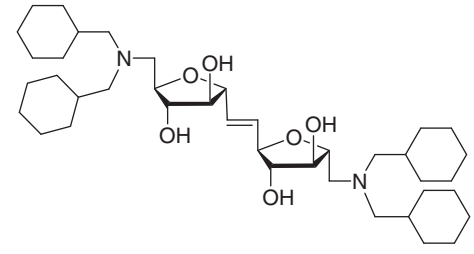




121 $R_1 = R_2 = H$, $R_3 = N_3$; $IC_{50} = 2.93$ mM
122 $R_1 = R_2 =$ TBDMS, $R_3 = N_3$
123 $R_1 = R_2 = H$, $R_3 = NH_2$
124 $R_1 =$ TBDMS, $R_2 = H$ $R_3 = N(CH_2C_6H_{11})_2$
125 $R_1 = R_2 = H$, $R_3 = N(CH_2C_6H_{11})_2$; MIC = 8 μg/mL, $IC_{50} = 1.56$ mM
126 $R_1 = R_2 = H$, $R_3 = NHCOC(CH_3)_3$; $IC_{50} = 2.49$ mM
127 $R_1 = R_2 = H$, $R_3 = NHCOC_6H_5$; $IC_{50} = 2.25$ mM
128 $R_1 = R_2 = H$, $R_3 = NHSO_2C_2H_5$; $IC_{50} = 3.55$ mM
129 $R_1 = R_2 = H$, $R_3 = NHSO_2C_6H_4$-*p*-Me; $IC_{50} = 1.82$ mM

130 X = O; $IC_{50} = 2.80$ mM
131 X= CH_2; $IC_{50} = 3.44$ mM





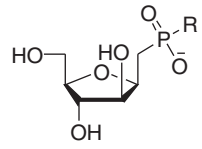
133 n = 0, X = H , Z = OH; $IC_{50} = 2.45$ mM
134 n = 1, X = OH, Z = H; $IC_{50} = 2.15$ mM
135 n = 1, X = F, Z = OH; $IC_{50} = 3.58$ mM

132 $IC_{50} = 4.15$ mM

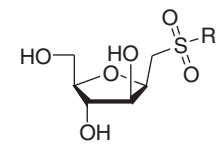
Figure 14. A series of arabinose-derived known acceptor analogs.

a similar level of activity.[126] Marotte et al. reported the synthesis of imino sugar-oligo-arabinofuranoside hybrids **171–177** (Fig. 16) as possible inhibitors of arabinofuranosyl transferase.[126] Designed as DPA analogs, the compounds were assayed using an arabino-furanosyltransferase assay employing [1-^{14}C]-β-D-arabinofuranosyl-1-decaprenyl phosphate ([1-^{14}C]-DPA) as the arabinose donor and an octyl 5-*O*-α-D-arabinofuranosyl-α-D-arabino-furanoside as the synthetic acceptor, and showed good inhibitory activity. Chaumontet et al. continued the synthetic efforts around the imino-sugar oligoarabinofuranoside hybrid skeleton and reported the synthesis of two hydrolytically stable arabinofuranoside analogs **178** and **179** (Fig. 16).[128] The authors mentioned that **178** displayed an activity similar to parent compound **172**.

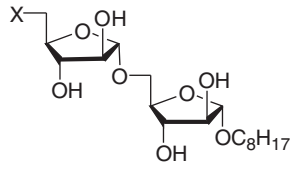
A different series of metabolically stable DPA isosteres, β-D-arabinofuranosyl triazole compounds having various hydrophobic side chains, were synthesized by Wilkinson et al. and their inhibitory potency was tested against *M. Bovis* BCG.[129] A weak to moderate activity for **180–182** was reported (Fig. 16). The inhibitory activity was shown to be dependent on the nature of hydrophobic group attached to the heterocyclic moiety, although a precise structure–activity relationship is not fully understood from their study. Bosco et al. synthesized DPA analogs **183** and **184** containing an arabinose or an aza-arabinose phosphonate carrying a polyprenyl chain (Fig. 17).[130] The compounds displayed modest inhibitory activity against *M. tuberculosis* H37Rv. In a similar fashion, Joe et al. synthesized analogs **185–188** with a 2-deoxy-2-fluoro-arabinofuranosyl moiety, a phosphate group, and a polyprenyl chain (Fig. 17).[131] Antibacterial activities of the synthetic analogs are not reported.



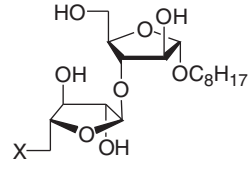
136 R= $O(CH_2)_4O(CH_2)_{15}CH_3$;
MIC = 3.13 μg/mL
92% inhibition in the fluorescence assay
137 R = $(CH_2)_{11}$ CH_3; 17% inhibition



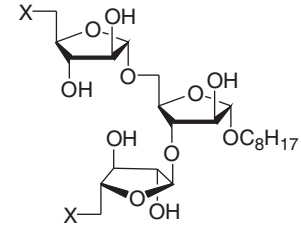
138 R = $(CH_2)_9$ CH_3; 21% inhibition
139 R = $(CH_2)_{11}$ CH_3; 45% inhibition
140 R = $(CH_2)_2CH(CH_3)(CH_2)_3CH(CH_3)_2$;
20% inhibition



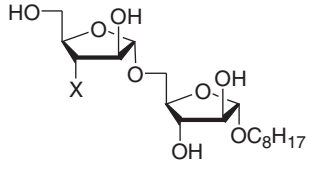
141 X = OH
144 X = F
147 X = OCH_3
150 X = N_3
153 X = NH_2



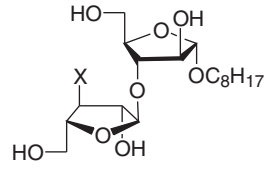
142 X = OH
145 X = F
148 X = OCH_3
151 X = N_3
154 X = NH_2



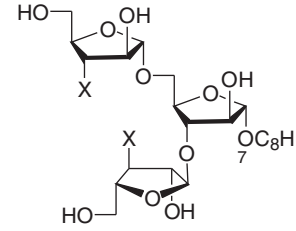
143 X = OH
146 X = F
149 X = OCH_3
152 X = N_3
155 X = NH_2



156 X = OCH_3; 34% (8%)
159 X = N_3; 36% (13%)
162 X = NH_2; 23% (4%)



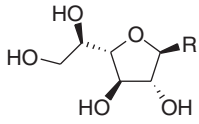
157 X = OCH_3; 59% (14%)
160 X = N_3; 75% (17%)
163 X = NH_2; 49% (10%)



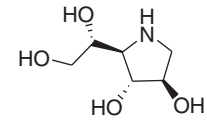
158X = OCH_3; 37% (20%)
161 X = N_3
164 X = NH_2; 25% (12%)

Figure 15. A panel of arabinfuranose-based compounds as potential inhibitors of mycobacterial arabinofuranosyltransferases. Compounds **137–140** were screened at 6.25 μg/mL concentration. For **156–160** and **162–164**, percentage in parenthesis is for mycobacterial growth inhibition and the same outside parenthesis is for AraT inhibition. AraT inhibition experiments were carried at 3.6 mM of synthetic analogs, and a concentration of 3.6 μg/mL for each compound was used in mycobacterial growth assay.

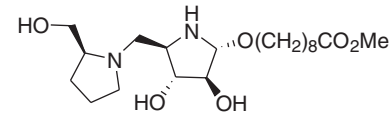
Esterification of the terminal Ara*f* residues with mAG is the final step in the biosynthesis of mAG, catalyzed by Ag85 complex (Scheme 2). These enzymes represent interesting drug targets for many reasons. First, the Ag85 enzymes are secreted to the periplasmic space via the SecA/SecYEG pathway promoted by the N-terminal signal sequence found on all three Ag85 enzymes, which should promote more efficient targeting by minimizing the effects of drug efflux pumps and drug modification systems. Second, Ag85 catalyzes a mycolyltransfer reaction, where the mycolate moiety is removed from TMM and transferred to a carbohydrate acceptor molecule. The hypothesis for all three enzymes using TMM as the mycolyl-donor is based on genetic data, biochemical data, and thorough characterization and



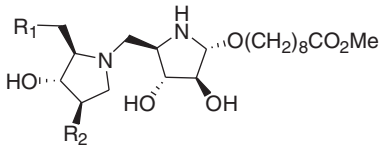
165 R = $S(CH_2)_7CH_3$; MIC > 32 μg/mL
166 R = $S[CH(CH_2)_7CH_3]_2$; MIC > 1 μg/mL
167 R = $S(O)_2[(CH_2)_7CH_3]_2$; MIC > 4 μg/mL
168 R = $SN[(CH_2)_7CH_3]_2$; MIC > 4 μg/mL
169 R = $S(O)_2N[(CH_2)_7CH_3]_2$; MIC > 2 μg/mL
170 R = $S[N(CH_2)_9CH_3]_2$; MIC > 1 μg/mL



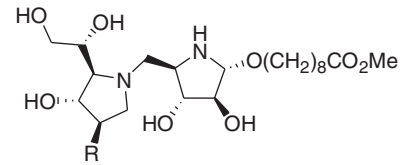
171; 46% inhibition



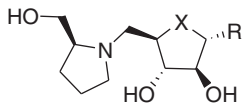
172; 64% inhibition



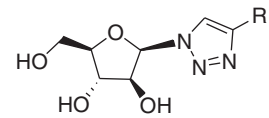
173 R_1 = Me, R_2 = OH; 80% inhibition
174 R_1 = OH, R_2 = OH; 80% inhibition
175 R_1 = OH, R_2 = F; 58% inhibition



176 R_1 = OH; 47% inhibition
177 R_1 = F; 59% inhibition



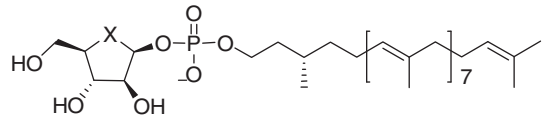
178 X = O, R = $CH_2OC_{16}H_{33}$
179 X = CH_2, R = $OC_{16}H_{33}$



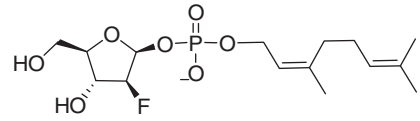
180 R = $(CH_2)_7CH_3$; MIC = 62 μg/mL
181 R = $CH_2O(CH_2)_{13}CH_3$; MIC = 31 μg/mL
182 R = $CH_2OCH_2{=}C(CH_3)(CH_2)_2CH{=}C(CH_3)(CH_2)_2$ $CH{=}C(CH_3)_2$; MIC = 62 μg/mL

Figure 16. Galacto-based DPA analogs and imino-sugar-oligoarabinofuranoside hybrids for arabinofuranosyltransferase inhibition. The percentage in parenthesis represents amount of AraT inhibition. Each imino-sugar hybrid was assayed at 4 mM concentration using octyl 5-*O*-α-D-arabinofuranosyl-α-D-arabinofuranoside acceptor at 0.4 mM concentration.

inspection of the six available crystal structures.[132–134] The possibility of all three Ag85 enzymes using TMM as a common mycolyl-donor simplifies inhibitor development and allows simultaneous targeting of all three Ag85 enzymes with the same compound. Although studies have shown that Ag85 possess some redundant biochemical activity, no evidence exists for a viable organism where two or more Ag85 genes have been knocked out. Studies have shown that at least three different sugars act as mycolyl-acceptors producing TDM, mAG, and glucose monomycolate.[74,135,136] The basis for mycolyl-acceptor discrimination is just now being studied. Third, recent studies by Harth et al. show that inhibiting Ag85 activity while administering isoniazid exhibits a synergistic growth inhibition.[137] This indicates that successful inhibitors of the Ag85 enzymes will improve delivery of other antitubercular drugs. This was very recently shown to be true for *M. smegmatis* by Nguyen and Pieters who showed an Ag85A knockout strain that exhibited increased sensitivity to imipenum.[138] Fourth, the mechanistic details of the Ag85 enzymatic reaction are now well characterized and this information has been used to design mechanism-based inhibitors. In fact, the rationale for most of these designs is based on the mechanistic knowledge of the Ag85 enzymes. These enzymes catalyze mycolyl transfer using a double-displacement mechanism that proceeds through a



183 X = NH; MIC = 35 μM
184 X = O; MIC = 50 μM



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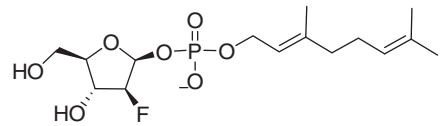

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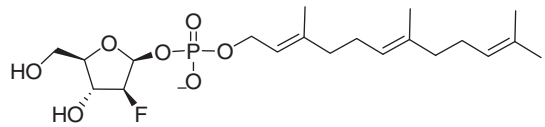

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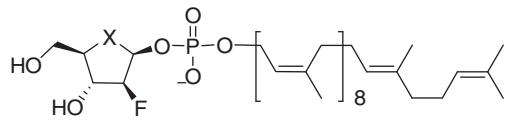
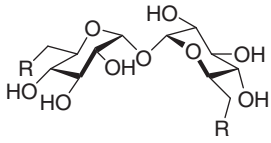


188

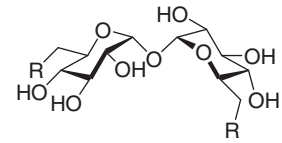
Figure 17. Additional arabinofuranosyl-based DPA analogs bearing polyprenyl chains.

covalent tetrahedral transition state, similar to the mechanism of serine proteases. After the initial report by Belisle et al. that 6-azido-6-deoxytrehalose (ADT) inhibited Ag85 activity in their assay,[75] other inhibitors were designed and synthesized. To date, various libraries have been designed based upon phosphonate compounds (**196–201**),[139,140] sulfonate compounds (active compound **202**)[141] to mimic the tetrahedral transition state, and two types of trehalose analogs **189–195** (Fig. 18) designed, two of them, **189** and **190**, before the crystal structure was reported.[142,143] Once synthesized, the compounds were assayed using the radiometric assay described by Belisle.[75] The second trehalose-based library **191–195** was derived from mechanistic studies and included substrate analogs.[143] The two libraries of compounds were assayed using a disk diffusion assay against *M. smegmatis* (ATCC 14468) and their inhibitory activities are presented in Figure 18. Only the most potent compounds are shown.

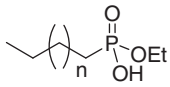
Recently, Boucau et al. reported a spectrophotometric coupled assay measuring *M. tuberculosis* Ag85C activity.[144] This assay was used in two different lines of inquiry. First, it was used on a semipreparative scale to allow characterization of the formed products.[145] Belisle et al. had previously shown the production of TDM using Ag85C.[75] Therefore, Sanki



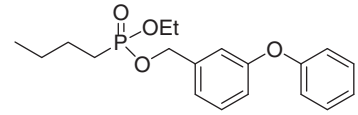
189 R = $CH_2NH(CO)CH_2NHC_8H_{17}$;
DZI (10 mg/mL) = 20mm, MIC > 256μM
190 R = $NH(CO)CH_2NHC_7H_{15}$; DZI (10 mg/mL) = 19 mm, MIC = ND (solubility issue)



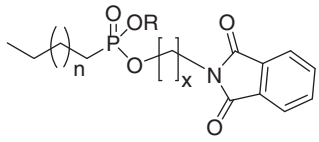
191 R = $C_8H_{17}SO_2NH$; MIC = 16-32 μg/mL
192 R = $C_8H_{17}NH$; MIC = 4 μg/mL
193 R = $C_{12}H_{25}NH$; MIC = 8 μg/mL
194 R = $C_{10}H_{21}NH$; MIC > 1.3 <13 μg/mL
195 R = $C_4H_9CH(C_2H_5)CH_2NH$; MIC = 32 μg/mL



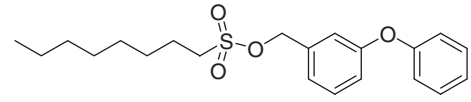
196 n = 3; IC_{50} = 3.56 μM
197 n = 4; IC_{50} = 1.06 μM



198 IC_{50} = 2.01 μM; MIC = 248.8 μg/mL



199 n = 4, x = 1, R = Et; IC_{50} = 1.31 μM, MIC inactive
200 n = 3, x = 2, R = H; IC_{50} = 4.39 μM
201 n = 4, x = 2, R = H; IC_{50} = 1.47 μM



202 IC_{50} = 4.3 μM

Figure 18. A panel of phosphonate, sulfonate, and trehalose-based inhibitors for antigen 85 complex.

et al. synthesized a number of arabinofuranosides that were tested as acyl acceptors in the Ag85C catalyzed transfer reaction.[145] Using both NMR and ESI-MS, this study clearly showed that Ag85C readily catalyzes acylation at the 5-OH position of arabinofuranose-based compounds in vitro. Although both α- and β-arabinofuranoside were modified at the two and five positions, an arabinosylarabinoside more closely mimicking the nonreducing termini of the AG was modified only at the five position, even though a 2-OH was available for acylation. Second, the assay was tested for feasibility in high-throughput screening applications using the NIH Clinical Collection (NCC). Interestingly, two known mycobacterial drugs, ebselen and clofazimine (a first line leprosy drug), showed inhibition of Ag85C activity (unpublished data), thereby indicating that the Ag85 complex may be one of the targets inhibited by these compounds. Other compounds exhibited significant inhibitory activity of both Ag85C and *M. smegmatis* growth, and so are being pursued as lead compounds for the design of Ag85 inhibitors.

The assay was then used to test the possible inhibitory activity of methyl 5-*S*-alkyl-5-thio-D-arabinofuranosides (**203–208**) synthesized by Sanki et al.[146] Although compounds containing a 5-*S*-octyl side chain showed activity in a growth inhibition assay against *M. smegmatis* ATCC 14468, the compounds were inactive in the spectrophotometric coupled assay. Most recently in another attempt, Sanki et al. synthesized a panel of D-arabinose (**209–211**) and trehalose (**212–214**) based compounds having ester, α-ketoester, and α-ketoamide functions, so as to design transition state inhibitors for Ag85C (Fig. 19).[147] In the disk diffusion assay, none of the analogs showed inhibition against the growth of *M. smegmatis* ATCC 14468. In the enzymatic assay, among all the compounds tested, only the methylester **209** was found to show activity at millimolar concentration (Fig. 19).

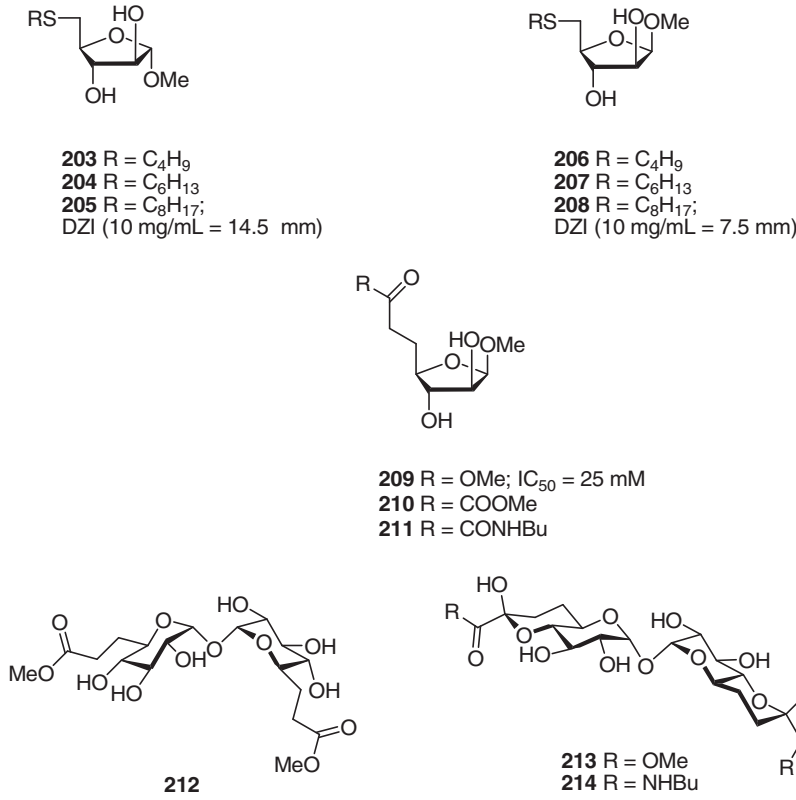


Figure 19. Methyl 5-*S*-alkyl-5-thio-D-arabinofuranosides **203–208**, arabinosides **209–211,** and trehalose derivatives **212–214** based on 1,2-dicarbonyl compounds as transition state inhibitors for Ag85C.

6. CONCLUSION

It is clear that extensive and innovative research is being conducted in broad areas of chemistry to identify which mycobacterial biosynthetic enzymes may serve as potential targets for development of new antitubercular therapeutics. This review presents the recent progress toward the inhibition of mAG and LAM synthesis with an emphasis on the discovery of new enzymes, the characterization of known drug targets, as well as the compositions of substrate analogs, transition-state analogs, and the development of high-throughput assays for the screening of existing libraries of compounds. From the studies highlighted here, several new enzymes and their specific roles have been discovered and characterized, which may be attractive targets for tubercular intervention. It appears that among several antibacterial agents developed so far, the nitrofuranylamide compounds (see Fig. 7) appear to be the most promising candidates for glycosyltranferase inhibition. However, much of the work presented herein is very recent, and it is too early to conclude which of these targets and approaches are likely to be the most effective.

What is clear is that there are an increasing number of reports of drug-resistant TB worldwide. Thus, there is a continued need to identify new drug targets and new avenues of treatment. It is anticipated that the discovery of these targets will eventually lead to the development of small molecule inhibitors with the potential to add to the arsenal of drugs available to treat drug-resistant forms of *M. tuberculosis*.

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Francis E. Umesiri, M.S. *is a doctoral candidate at the University of Toledo. His research is focused on the development of carbohydrate-based antitubercular agents. He obtained his M.S. in Chemistry in 2005 from Central Michigan University, and his B.S. in Chemical Education from the University of Lagos, Nigeria.*

Aditya K. Sanki, Ph.D. *performed his research work at the National Chemical Laboratory and received his Ph.D. in Carbohydrate Chemistry in 2003 from the University of Pune, India. He held postdoctoral positions at the University of Hawaii at Manoa, University of Texas at Austin and, most recently, at the University of Toledo. He is currently a Scientific Manager at Syngene International Limited, Bangalore, India.*

Julie Boucau, Ph.D. *obtained her Ph.D. degree in biochemistry from the University of Toledo in 2008 for her work on mycobacterial proteins important for viability and pathogenesis, including the Antigen 85 family. She is currently a postdoctoral research associate in Dr. Le Gall's laboratory at the Ragon Institute, working on HIV antigen processing.*

Donald R. Ronning, Ph.D. *obtained his B.S. degree from the University of Minnesota, 1995; and Ph.D. from Texas A&M University (2001) under James Sacchettini. In 2001, Ronning joined Fred Dyda's lab at the National Institutes of Health where he studied the catalytic and regulatory mechanisms of Tn7 and ISHp608 transposases. In 2005, he joined the faculty at the University of Toledo where he is combining his backgrounds in mycobacterial biochemistry and macromolecular complex formation to better understand the interactions between mycobacterial and human proteins, and the macromolecular complexes that promote metabolic changes in mycobacteria.*

Steven J. Sucheck, Ph.D. *obtained his B.S. from the University of Toledo, 1992; Ph.D. from the University of Virginia, 1998, under Professor S. M. Hecht; NIH postdoctoral scholar, The Scripps Research Institute under Professor C.H. Wong. In 2000, he joined Optimer Pharmaceuticals, Inc. where he worked on the discovery and development of antibiotics and tumor-associated carbohydrate antigens. In 2005, he joined the University of Toledo as an Assistant Professor where he studies the chemistry of carbohydrates.*