Recent Progress Towards the Identification of Inhibitors of Mycobacterial Cell Wall Polysaccharide Biosynthesis

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Abstract: Mycobacterial infections have recently attracted significant attention from international health agencies due to the resurgence of these diseases worldwide. This review summarizes the recent work directed towards the identification of new anti-tuberculosis agents that act by inhibiting mycobacterial cell wall polysaccharide biosynthesis.

Keywords: Tuberculsosis, mycobacteria, polysaccharides, furanosides, bacterial cell wall, inhibitors.

1. INTRODUCTION

More than fifty different mycobacterial species are found in nature, and while most are not human pathogens, some represent serious human health threats [1]. *Mycobacterium tuberculosis* and *Mycobacterium leprae* are the most well-known pathogenic mycobacterial species. Infection by these organisms causes tuberculosis and leprosy, respectively. For individuals with weakened immune systems, infections by "atypical" mycobacteria (*e.g., M. avium*) are also a threat [2]. A third of the world's population is believed to be infected with *M. tuberculosis*, and the disease causes nearly three million deaths annually, making it the single most lethal bacterial disease [3]. Additionally, leprosy claims one million lives each year [4] and *M. avium* infections are a common cause of death in AIDS patients [5]. The reemergence of mycobacterial disease as a human health threat worldwide has spurred increasing interest in the development of new antibiotics for treating these infections, and drug resistance issues have heightened the urgency of this task [6- 8].

Successful treatment of mycobacterial disease requires strict adherence to a regimen of antibiotics that must be taken over many months [9]. The front-line antibiotics used to treat tuberculosis are shown in Fig. (**1**). The standard "short course" regimen, which is usually successful for treating non-drug-resistant mycobacterial infections provided the patient follows the full course of treatment, consists of isoniazid, rifampicin, and pyrazinamide for two months, followed by the continuation of isoniazid and rifampicin for six months [10]. This treatment is generally supplemented with ethambutol or streptomycin. With drug-resistant strains, this regimen must be altered, and second-line antibiotics are often employed [11].

One factor contributing to the difficulty in treating mycobacterial disease is the unusual structure of the organism's cell wall [12-14]. The mycobacterial cell wall serves as a formidable barrier to the passage of antibiotics into the organism, and the regimen outlined above employs drugs that interrupt cell wall biosynthesis (*i.e.,* ethambutol or isoniazid), in combination with others that have intracellular targets (*i.e.,* rifampicin or streptomycin). The goal of this treatment is to destroy the integrity of the cell wall such that other antibiotics can enter the organism more easily.

Treating individuals co-infected with HIV and *M. tuberculosis* is particularly problematic [15,16]. First, *M. tuberculosis* stimulates replication of HIV thus increasing viral load [17-19]. Second, treatment is complicated due to substantial interactions between protease inhibitors, which are part of highly active antiretroviral therapy, and rifampicin, one of the front-line drugs used for the treatment of TB. Although rifabutin can be used in place of rifampicin, the use of this drug for treating TB in HIV-infected patients has not been extensively investigated [20]. When one considers that an estimated 6 million of the 14-15 million people infected worldwide with HIV are also infected with *M. tuberculosis,* it is clear that there is not only a general need for new anti-TB drugs but also a specific need for novel antibiotics for use in treating TB in individuals co-infected with HIV.

Mycobacterial viability is dependent upon the ability of the organism to produce an intact cell wall. Therefore, compounds that interfere with the biosynthesis of the cell wall complex have the potential to be new drugs for the treatment of mycobacterial infections. The purpose of this review is to familiarize the reader with recent work directed towards identifying inhibitors of the biosynthesis of two mycobacterial cell wall polysaccharides, arabinogalactan (AG) and lipoarabinomannan (LAM). These two glycans, which are comprised largely of glycosyl residues in the furanose ring form, are the major structural components of the cell wall complex. Recent work on the characterization of the enzymes involved in AG and LAM biosynthesis will also be described, as will the assays that have been developed to measure their activity. The focus here will be on the enzymes involved in the assembly of the arabinofuranose and galactofuranose-containing portions of these polysaccharides. Given the absence of furanose oligo- and polysaccharides in mammalian biology, inhibitors of the enzymes involved in the assembly of these glycans are particularly attractive targets for drug action. A detailed discussion of the roles of these polysaccharides in the progression of mycobacterial disease will not be presented

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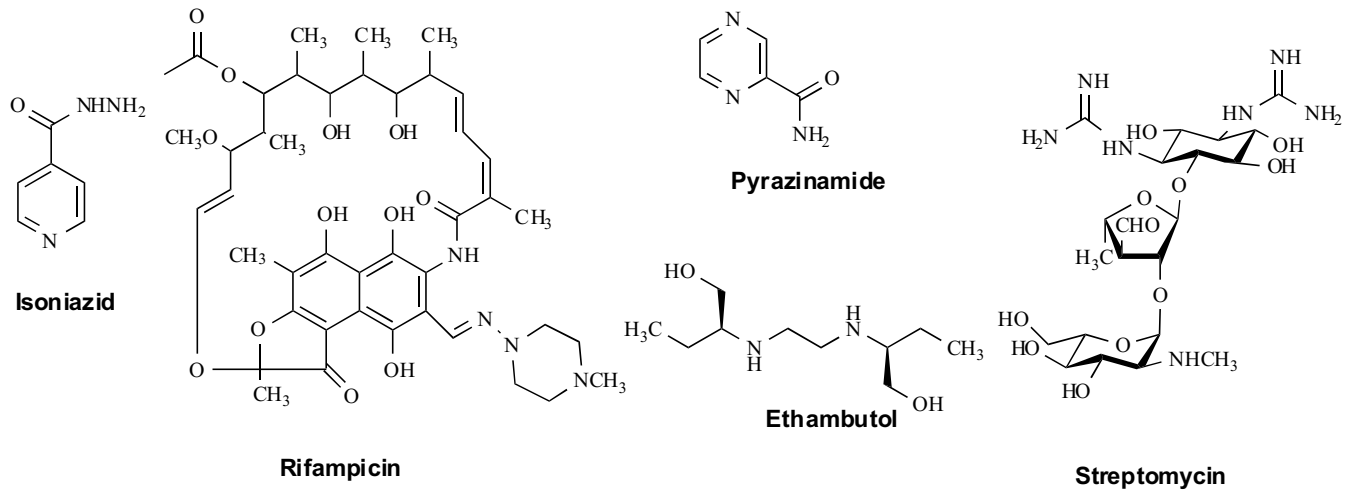


Fig. (1). Front-line antibiotics used in the treatment of tuberculosis.

here, as this topic has been the subject of recent reviews [12-14, 21, 22].

2. STRUCTURAL FEATURES OF THE MYCOBACTERIAL CELL WALL

The mycobacterial cell wall is a complex assembly comprised of polysaccharides, proteins, lipids, and glycolipids. A schematic illustration of the general structural motifs of the cell wall is shown in Fig. (**2**) [12-14]. There are four major components: 1.) Peptidoglycan, a polymer of *N*-acetylglucosamine, *N*-glycolylmuramic acid, and short cross-linked peptides; 2.) The mycolyl-AG complex, a structure consisting of a polymer of galactofuranosyl and arabinofuranosyl residues covalently linked to the peptidoglycan at the reducing end and then esterified at the non-reducing end to mycolic acids, branched-chain lipids found in mycobacteria and other actinomycetes. 3.) Glycolipids bound through noncovalent interactions with mycolate esters; and 4.) LAM, a polymer of mannopyranosyl and arabinofuranosyl residues, which is noncovalently attached to the plasma membrane through a phosphatidylinositol anchor. Although the identities of the glycolipids and mycolic acids are species specific [23-25], the structures of the polysaccharide components are virtually identical in all mycobacteria [12-14].

2.1. Structural Features of the Mycolyl-AG Complex [12-14]

The AG is covalently bound to the peptidoglycan via an α-L-Rha-(1$\rightarrow$3)-α-GlcNAc-OPO_3 disaccharide (**1**, Fig. (**3**)) to which is attached a chain of roughly 30 galactofuranose (Gal*f*) residues. This linear galactan is comprised of alternating β-(1$\rightarrow$5) and β-(1$\rightarrow$6) Gal*f* residues (**2**). Periodically along this galactan are branch points to which

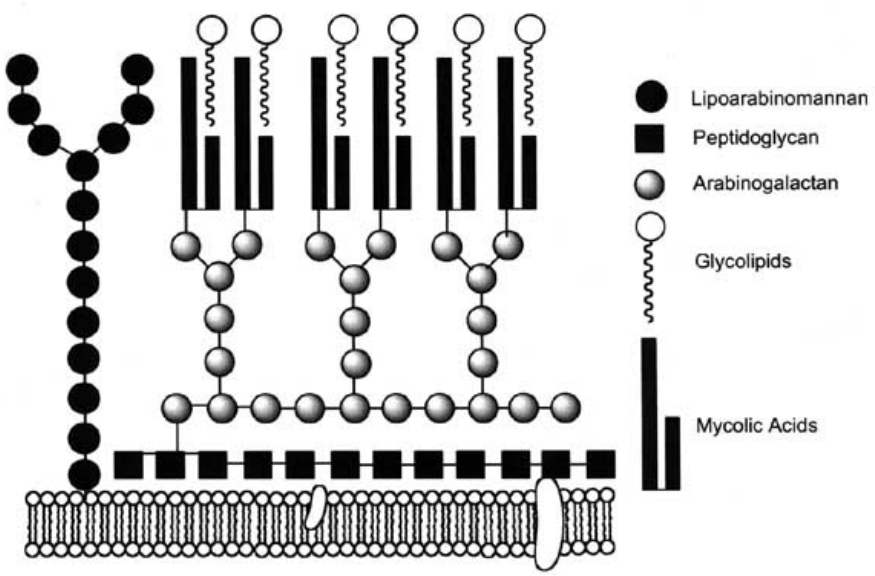
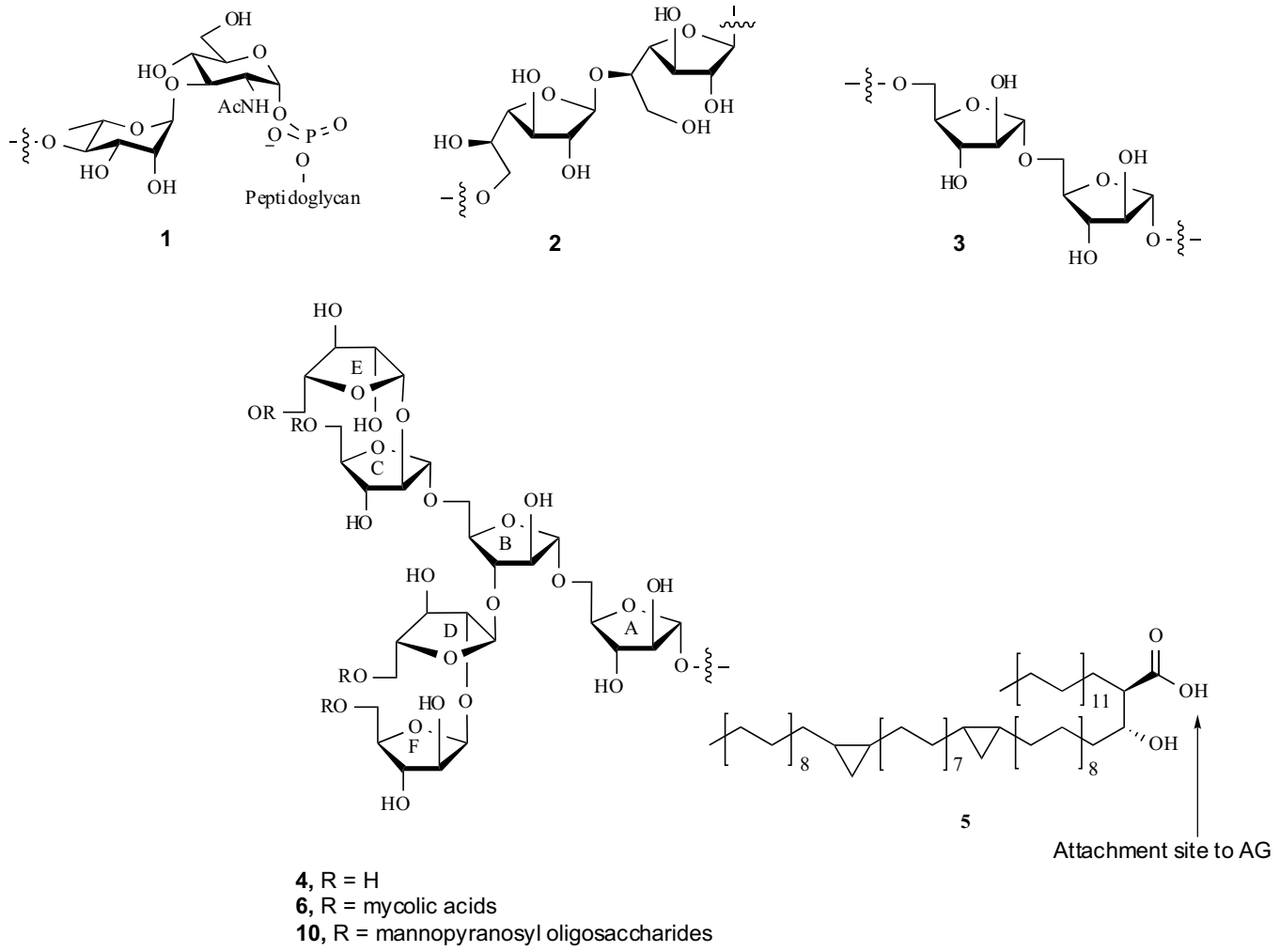


Fig. (2). Schematic drawing of the mycobacterial cell wall.



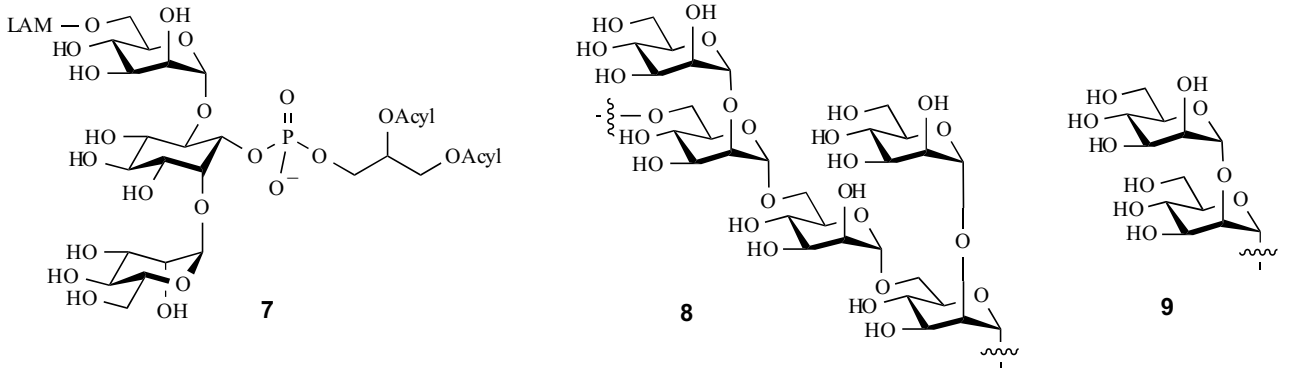


Fig. (3). Structural motifs present in mycobacterial arabinogalactan and lipoarabinomannan.

the arabinan portions of the AG are attached. Made up of approximately 70 arabinofuranose (Ara*f*) residues, this arabinan is predominantly an α-(1→5) linked linear polymer (**3**). However, there are periodic branch points to which another linear arabinan chain is attached, via the 3 position of an Ara*f* residue. The nonreducing termini of these arabinan chains is capped with a branched hexasaccharide (**4**). Approximately two-thirds of the hexasaccharide motifs of a given AG molecule have mycolic acid residues (*e.g.,* **5**) esterified to all four primary hydroxyl groups giving **6** while the remaining motifs are unsubstituted. The mycolic acid residues of the AG form a tightly packed assembly that dramatically decreases the permeability of the cell wall and protects the organism not only from passive transport of antibiotics, but also from the immune system of the host [24, 25]. The incorporation of mycolic acids into the cell wall depends upon the organism's ability to synthesize the arabinan portions of the AG. Hence, inhibitors of the enzymes involved in arabinan biosynthesis are expected to be potent anti-mycobacterial agents.

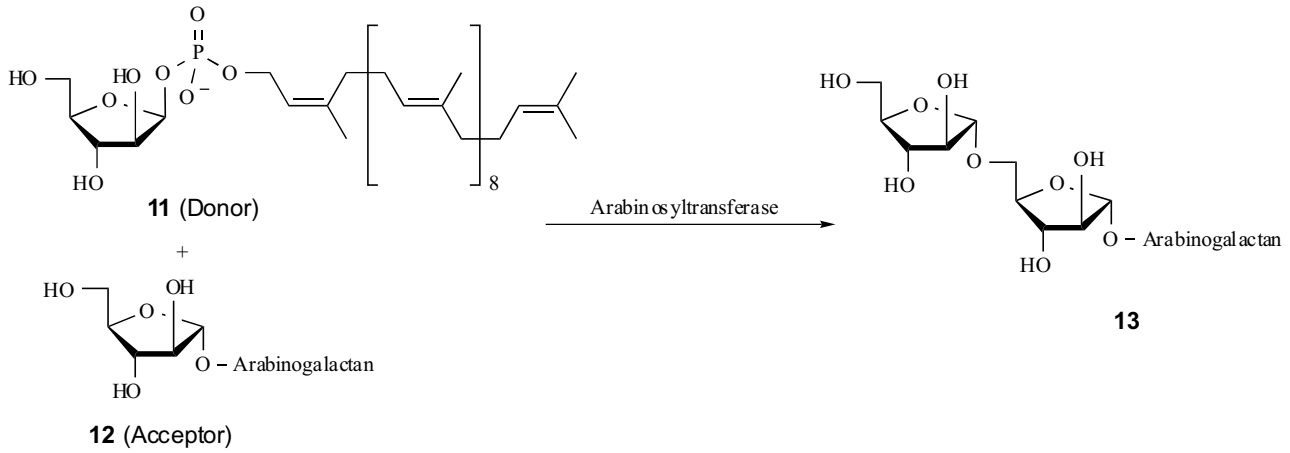


Fig. (4). Representative reaction catalyzed by a mycobacterial arabinosyltransferase.

2.2. Structural Features of LAM [12-14]

The LAM is noncovalently bound to the cytoplasmic membrane of the organism via the diacylglycerol moiety of a phosphadtidylinositol moiety (**7**) that is found at the reducing end of the polysaccharide. Extending from the inositol residue is a mannan comprised of α-(1→6)-linked mannopyranosyl residues. This polymer is further modified by the presence of α-(1→2) mannopyranosyl residues on approximately half of the sugar residues of the backbone glycan (**8**). At the non-reducing end of this mannan is attached an arabinan that is structurally similar to the one found in the AG: a linear α-(1→5) linked chain of Ara*f* residues possessing occasional α-(1→3) branch points. As in the AG, the chains of the arabinan in the LAM are capped at the nonreducing end with hexasaccharide **4**. However, in contrast to the AG, this hexasaccharide moiety is not esterified to mycolic acids. Instead, this motif is either unsubstituted or further glycosylated with α-(1→2) linked mannopyranosyl oligosaccharides (**9**) to give ManLAM (**10**). The terminal mannose residues in ManLAM have been postulated to be involved in the infection process through their binding to human mannose-binding proteins [26, 27]. In addition, it was recently demonstrated that these terminal mannose residues bind to human dendritic cells; this interaction may be important in the progression of the disease [28-31]. Other LAM modifications are known [32-34].

3. BIOSYNTHESIS OF THE ARABINAN AND GALACTAN PORTIONS OF AG AND LAM

Work over the past 5-10 years has led to a general understanding of the pathways by which the arabinan and galactan portions of the mycobacterial cell wall are assembled. A description of these processes is outlined below and the reader is referred to earlier reviews covering some aspects of this topic [12-14, 35, 36]. While great progress has been made in recent years, there is still much to be learned concerning the fine details of these processes. Additionally, although assays for many of these biosynthetic

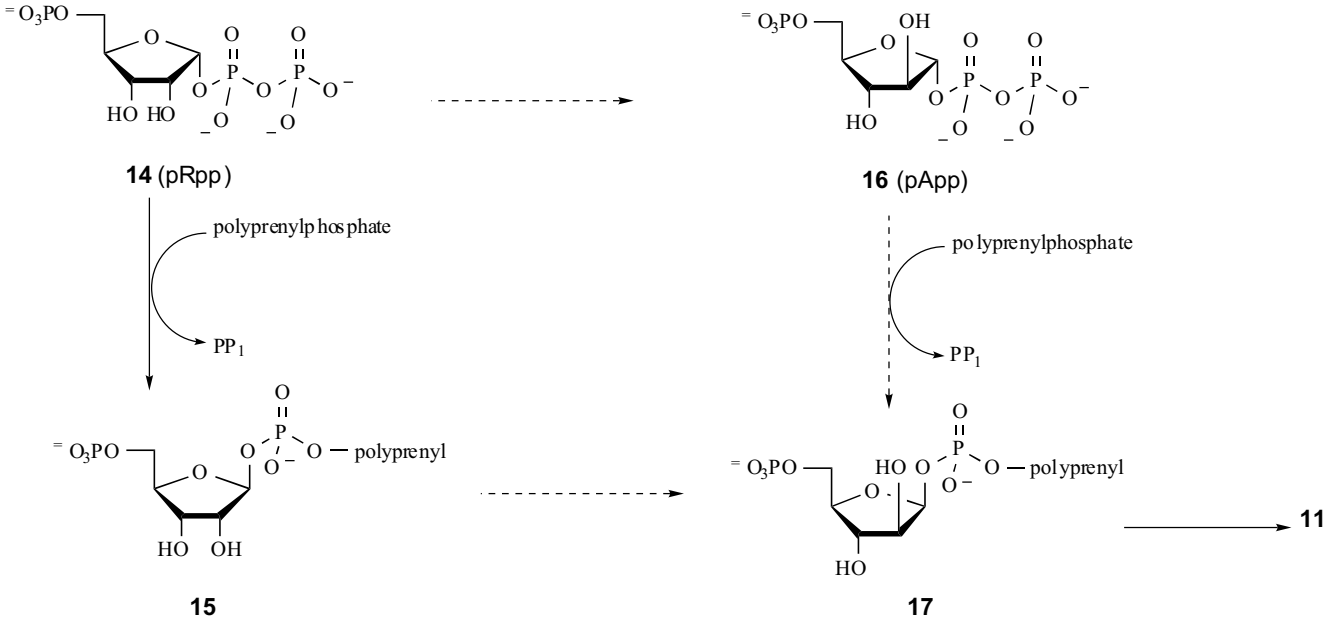


Fig. (5). Postulated biosynthesis of decaprenolphosphoarabinose (DPA, **11**). Established transformations are indicated by solid arrows, proposed transformations are indicated by dashed arrows.

enzymes are now available, most are not high-throughput and thus are not readily amenable to the screening of large numbers of potential inhibitors.

3.1. Biosynthesis of the Arabinan [35]

A family of arabinosyltransferases (AraT's) are involved in the assembly of the arabinan portions of the AG and LAM. A representative example of the reaction catalyzed by these enzymes is shown in Fig. (**4**). The donor substrate used by some, if not all, of these enzymes is the glycophospholipid decaprenolphosphoarabinose (DPA, **11**) [37]. The acceptor substrate for the AraT's is the growing arabinogalactan polysaccharide (*e.g.,* **12**). Although the natural acceptor substrates for these enzymes are lipid-bound intermediates, these AraT's also recognize small oligosaccharides (disaccharides or larger) [38, 39]. The proposed biosynthesis of DPA is given in Fig. (**5**) [40, 41]. It is known that 5-phospho-D-ribosyl pyrophosphate (pRpp, **14**) is the precursor to **11**, but the exact intermediates in the pathway have not been unequivocally proven.

To date, efforts to isolate these AraT's have not been successful. However, three genes (*embA*, *embB,* and *embC*) believed to encode for one or more of these AraT's in *M. smegmatis* and *M. tuberculosis* have been cloned, sequenced, and characterized [42, 43]. Recent work [44] has demonstrated that *M. smegmatis* mutants in which the *embA* and *embB* genes have been inactivated produce AG with reduced arabinose content. In particular, the DF disaccharide moiety of hexasaccharide **4** (Fig. (**3**)) is largely missing, thus suggesting that these genes encode for an α-(1$\rightarrow$3) AraT. The *embC*$^{-}$ mutant appeared to produce normal AG, but the arabinosylation of LAM was abolished. Based on amino acid sequences, the Emb proteins are proposed to be membrane-bound and possess 11-13 transmembrane domains, with a *C*-terminal globular domain located in the periplasm. Although the enzymes have not been purified, an assay for AraT activity that uses a mycobacterial membrane preparation as the enzyme source has been developed [39, 45]. The assay measures the incorporation of radiolabeled arabinose into arabinan from **11**, and it has been used to screen potential substrates and inhibitors.

Using this assay, it was shown [39] that incubation of arabinofuranosyl di- and trisaccharides together with synthetic radiolabeled **11** and a membrane preparation from *M. smegmatis* produced tri- and tetrasaccharide products containing additional β-(1$\rightarrow$2) or α-(1$\rightarrow$5) linked arabinofuranose residues. This work therefore demonstrated that **11** is the precursor to the β-(1$\rightarrow$2) or α-(1$\rightarrow$5) linked arabinofuranose residues in AG and LAM. In contrast, no products with α-(1$\rightarrow$3) linkages could be detected. This could be attributed to the absence of the α-(1$\rightarrow$3) transferase in the membrane preparation or the instability of the enzyme outside of the organism. It is also possible that the oligosaccharide acceptor substrates used were too small to be recognized by the enzymes, or that another donor serves as the precursor to the α-(1$\rightarrow$3) linked residues. With regard to the last possibility, a single report has described the presence of an arabinofuranosyl-containing sugar nucleotide, UDP-Ara*f,* in mycobacteria [46], but its incorporation into mycobacterial arabinan has not been demonstrated. In further support of the position that **11** is the only source of arabinofuranose residues in mycobacterial cell wall polysaccharides, it was shown [47] that the arabinan synthesized from radiolabeled **11** and a membrane preparation (using endogenous acceptors) is virtually identical to the natural polymer. Furthermore, the radiolabel was distributed equally throughout the polymer. Taken together these results suggest that **11** is the major (or possibly only) source of Ara*f* residues in mycobacterial AG.

The AraT's involved in arabinan biosynthesis have been validated as suitable targets for drug action as ethambutol (Fig. (**1**)), one of the drugs currently used to treat TB, is an inhibitor of one or more of these enzymes [48-50]. It has been suggested that the primary site of ethambutol action is an α-(1$\rightarrow$3) AraT involved in AG biosynthesis [39]. A mutation in the genes that are believed to encode for these enzymes gives rise to ethambutol-resistant strains of mycobacteria [42, 51, 52].

3.2 Biosynthesis of the Galactan

It could be expected that three different galactosyltransferases (GalT's) are necessary to assemble the galactan portions of the AG; one for the assembly of the β-(1$\rightarrow$5) linkages, another for the β-(1$\rightarrow$6) linkages, and a third for the attachment of the first Gal*f* residue to the rhamnose at the reducing end of the polysaccharide. It has

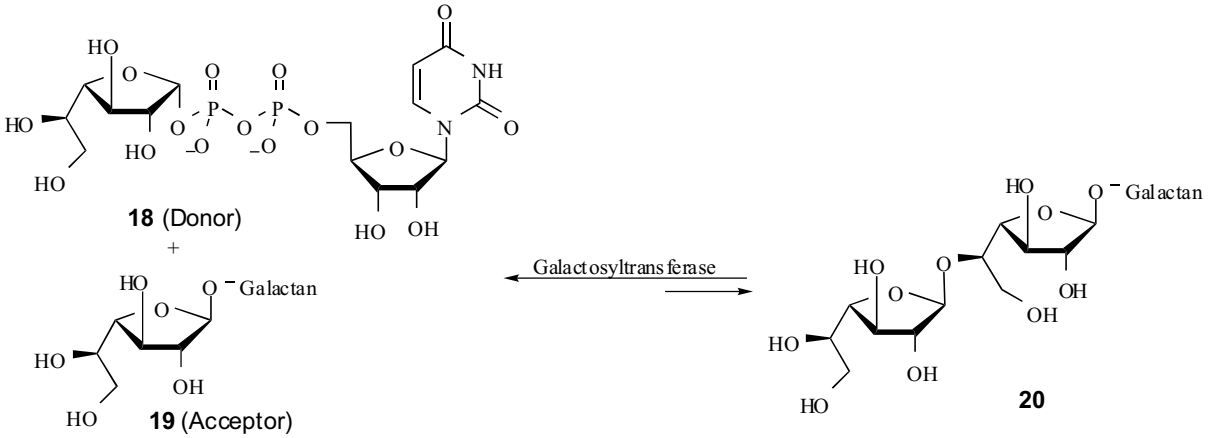


Fig. (6). Representative reaction catalyzed by a mycobacterial galactosyltransferase.

been established, however, that a single enzyme, *glfT*, is responsible for the synthesis of all three of the galactofuranosyl linkages in the mycobacterial cell wall [53, 54]. The broad substrate specificity of *glfT* is an unexpected discovery although other bifunctional glycosyltransferases have been reported [55, 56].

This enzyme transfers galactofuranose from the donor, UDP-galactofuranose (UDP-Gal*f,* **18**), to galactofuranose-containing oligo- or polysaccharides (**19**, Fig. (**6**)). The identification of *glfT* has allowed for the development of an assay that can be used for the screening of potential substrates and inhibitors of the enzyme [53]. Like the previously mentioned AraT assay, this GalT assay involves the use of synthetic oligosaccharide substrates and a membrane preparation containing the enzyme. The progress of the enzymatic reaction is monitored by the transfer of radioactivity from the labeled donor substrate, UDP-Gal*f*, which is usually produced *in situ*.

The UDP-Gal*f* required by this GalT is synthesized in a single step from UDP-Gal*p* (**21**) via the enzyme, UDP-Gal*p* mutase (Fig. (**7**)). At equilibrium, this enzyme produces a 93:7 UDP-Gal*p*:UDP-Gal*f* ratio and thus it is likely that this reaction is somehow coupled to the GalT involved in galactan biosynthesis. The mechanism of the unique transformation catalyzed by this enzyme has been the subject of an increasing number of investigations, but is still not understood [57-60]. The crystal structure of UDP-Gal*p* mutase from *E. coli* has been solved [61] and there is great similarity between this enzyme and the mycobacterial mutase. A particularly curious feature of the enzyme is that it requires a FAD cofactor. The role that this cofactor plays in this transformation, which appears not to be a redox process, has been the subject of some debate [36], and a recent paper has proposed that the reaction proceeds via a single-electron process involving both galactopranosyl and galactofuranosyl radicals [62]. Two assays are now available for the measurement of UDP-Gal*f* mutase activity. The first involves quanititation of the products using HPLC [63]. More recently, a high throughput assay has been developed (see Section 4.3) [64].

Through the use of gene knockouts it has been demonstrated that mycobacterial viability is dependent upon the ability of the organism to biosynthesize the galactan portion of the AG [65, 66]. Inhibitors of either the bifunctional GalT, *gtfT* or UDP-Gal*p* mutase are therefore potential antimycobacterial agents. To date, however, none of the drugs currently used to treat mycobacterial disease have been shown to inhibit cell wall galactan formation and therefore these biochemical pathways appear to be an untapped source for drug development.

3.3. Attachment of the Arabinan and Galactan to other Components of the Cell Wall

It has been proposed that in AG biosynthesis, the entire polysaccharide is assembled as a polyprenol diphosphate intermediate, which is transferred to the peptidoglycan prior to the addition of the mycolate esters [25]. In LAM biosynthesis, the arabinan portion is transferred to the mannan portion of the LAM from the same polyprenol diphosphate intermediate [45, 67].

4. SYNTHESIS OF POTENTIAL INHIBITORS OF MYCOBACTERIAL ARABINAN AND GALACTAN BIOSYNTHESIS

As outlined above, the pathways by which mycobacterial AG and LAM are assembled are now generally understood, and assays are now available for measuring the activities of the biosynthetic enzymes. The development of these assays now makes it possible to screen potential inhibitors against these enzymes in order to identify lead compounds for new anti-mycobacterial drugs. Progress in this area has been further enhanced by an increased number of methods for determining the susceptibility of mycobacteria to potentially lethal compounds [68-76]. Notable among these is a fluorescence-based high-throughput system, the "Alamar Blue" assay [77], which is increasingly being used to screen large numbers of potential anti-mycobacterial agents in a microtiter plate format. The identification of new drugs for the treatment of mycobacterial disease has been further facilitated by the establishment of a National Institutes of Health facility, the Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF), whose mission is to "encourage and support both academic researchers and pharmaceutical companies to reenter the area of TB drug development" [78]. Compounds sent to this facility are screened free of charge to determine their anti-mycobacterial potential [79].

4.1. Inhibitors of Mycobacterial Decaprenolphosphoarabinose Biosynthesis

The donor source of the arabinose residues in the mycobacterial cell wall is decaprenolphosphoarabinose, **11**, a glycolipid that is biosynthesized from 5-phospho-D-ribosyl pyrophosphate, pRpp, **14** (Fig. (**5**)). Two possible pathways for this transformation have been postulated [40] but neither has been proven. Compounds that block any of the putative enzymes should be potential anti-mycobacterial agents as they would shut down all arabinan biosynthesis by blocking

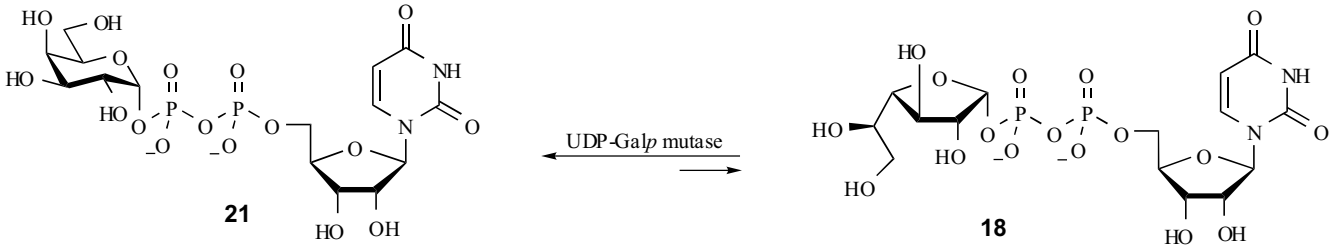


Fig. (7). The ring contraction reaction catalyzed by UDP-Galp mutase.

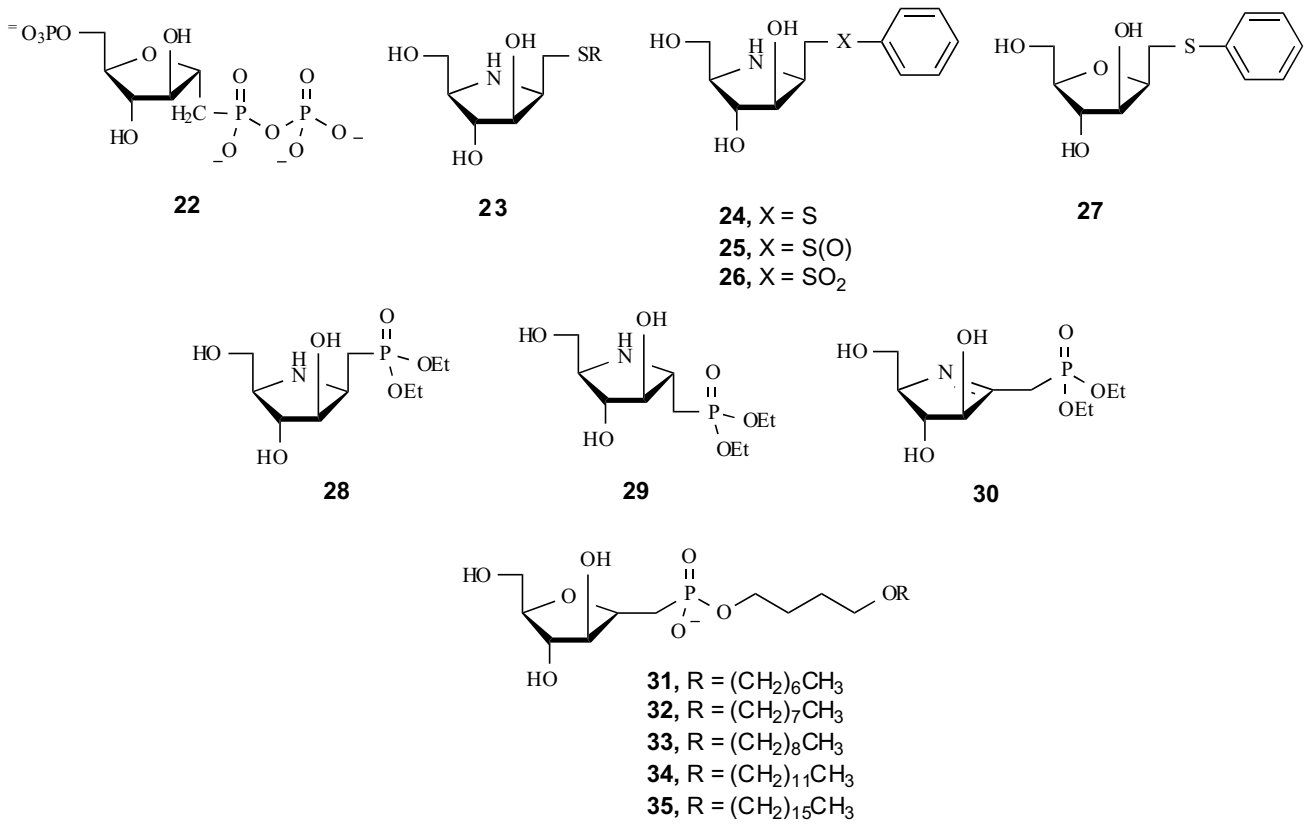


Fig. (8). *C*-phosphonate tested as an inhibitor of decaprenolphosphoarabinose (DPA) biosynthesis (**22**) and examples of DPA analogs screened as inhibitors of mycobacterial arabinosyltransferases (**23**-**35**).

the formation of **11**. With that goal in mind, Field and coworkers have reported [80] the synthesis of 5-phospo-α-D-arabinosyl *C*-phosphonophosphate, **22** (Fig. (**8**)), an analog of 5-phospho-D-α-arabinosyl pyrophosphate, **16**. *C*-phosphonate **22** was designed based on the belief that it should block the conversion of **16** into **17** (Fig. (**5**)). Once synthesized, **22** was tested for its ability to inhibit the conversion of radiolabeled pRpp (**14**) into organic-soluble material (*e.g.,* glycolipid **11**). However, at 5 mM concentration of **22**, less than 20% inhibition was observed. Based on these results, no further investigations with **22** were carried out.

4.2. Inhibitors of Mycobacterial AraT's

When considering the preparation of inhibitors of mycobacterial AraT's, analogs of either the donor or acceptor substrates are potential candidates. Both approaches have been explored and, to date, donor analogs have received more detailed attention than acceptor analogs.

4.2.1. Decaprenolphosphoarabinose (Donor) Analogs

The first paper describing the synthesis and biochemical evaluation of decaprenolphosphoarabinose analogs reported a family of substituted pyrroldines (*e.g.,* **23**, Fig. (**8**)), which differed in the identity of the exocyclic alkylthio group [81]. Evaluation of these compounds was done *in vitro* against not only *M. tuberculosis* but also five *M. avium* isolates using a microdilution assay [82]. Selected analogs were also assayed using alternate screening methods [83]. Most of the compounds synthesized showed little to marginal activity against these bacteria. However, one compound, the thiophenyl derivative **24**, was effective against three of the *M. avium* strains in infected macrophages [83] at a level that was not cytotoxic (MIC = 4 μg/mL). The activity was attributed in part to Tumor Necrosis Factor-α (TNF-α) production, which occurred when the macrophages were treated with **24**. When tested against *M. tuberculosis*-infected macrophages, **24** was inactive; however, an increase in TNF-α was observed. In further studies it was demonstrated that analogs closely related to **24**, compounds **25**–**27**, were inactive in *M. avium* infected macrophages. The site of action of these compounds was not reported; it therefore remains to be determined whether these pyrrolidines block the action of mycobacterial AraT's by preventing the binding of DPA to the enzyme.

Nearly concurrently with the previous report, Eustache and coworkers described the synthesis of the phosphonate derivatives **28**–**30 (**Fig. (**8**)), which were designed to be mimics of DPA [84, 85]. To date, however, no reports have appeared detailing the ability of these compounds to either inhibit mycobacterial AraT's or prevent mycobacterial growth.

More recently, a series of *C*-phosphonate ester analogs of DPA (**31**-**35**, (Fig. (**8**)) have been synthesized and tested as anti-mycobacterial agents [86]. These compounds differ only in the length of the alkyl chain on the phosphonate ester.

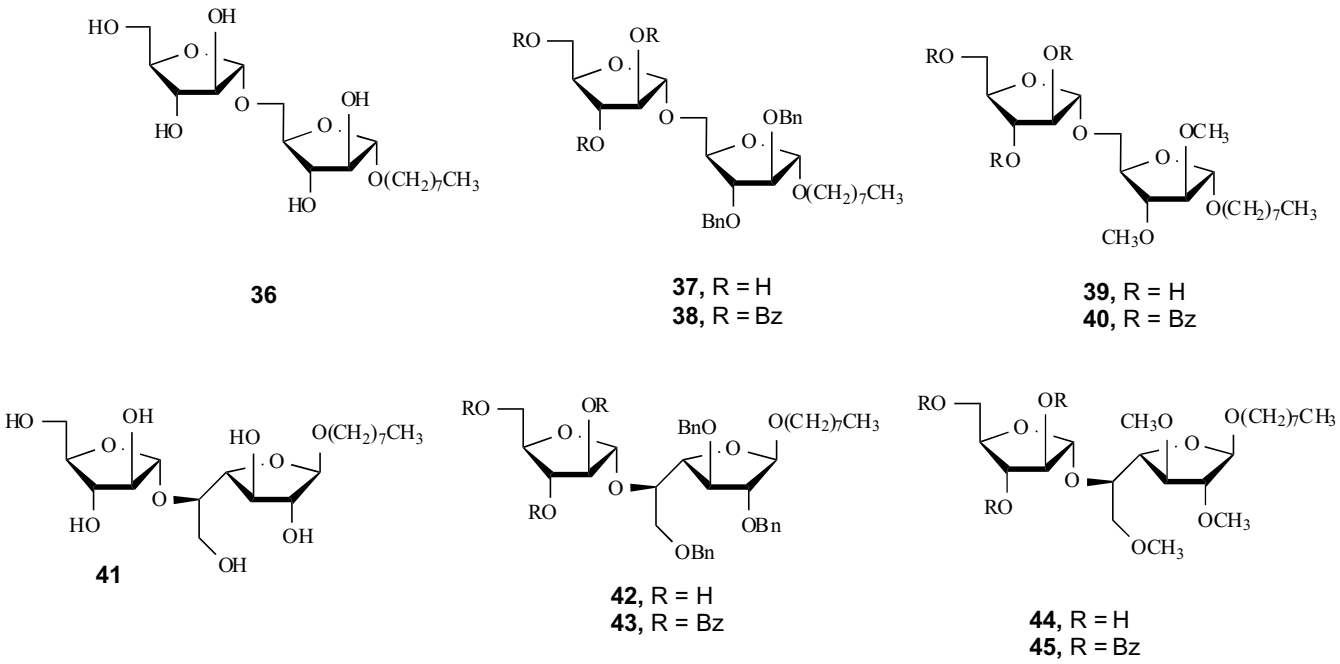


Fig. (9). Disaccharides tested as inhibitors of mycobacterial arabinosyltransferases and mycobacterial growth, Abbreviations: Bn = benzyl; Bz = benzyl.

When tested against *M. tuberculosis* $H_{37}Rv$, only the analog with the longest alkyl chain (**35**) was active. This compound had an MIC value against *M. tuberculosis* $H_{37}Rv$ of 3.13 μg/mL, and an IC_{50} value of 36.4 μg/mL against a Vero cell line. It is currently being evaluated in other assays to further explore its therapeutic potential.

4.2.2. Acceptor Analogs

In recent years, significant effort has been directed towards the synthesis of arabinofuranosyl-containing oligosaccharides that can serve as acceptor substrates for mycobacterial AraT's. Efficient methods for the synthesis of these compounds are now available [87-101]. In contrast, only a few reports have described the testing of acceptor analogs for their ability to inhibit these enzymes or to prevent mycobacterial growth.

Reynolds and coworkers have investigated the ability of disaccharides comprised of arabinofuranose and galactofuranose residues to inhibit both mycobacterial

Table 1. Activity of Disaccharides 36-45 Against Mycobacterial Strains and as Inhibitors of Mycobacterial AraT's.[a]

Compound	MIC (μg/mL)				AraT Assay	
	MTB[b] $H_{37}Ra$	MAC[c] NJ 168	MAC[c] NJ 211	MAC[c] NJ 304	K_m (mM)	IC_{50} (mM)
36	>128	ND[d]	>128	ND[d]	ND[d]	ND[d]
37	>12.8≤128	ND[d]	>12.8≤128	ND[d]	ND[d]	1.12
38	>128	ND[d]	>128	ND[d]	ND[d]	ND[d]
39	>128	ND[d]	>128	ND[d]	ND[d]	3.70
40	>128	ND[d]	>128	ND[d]	ND[d]	ND[d]
41	>128	>128	>128	>128	5.7	ND[d]
42	32	>128	>128	>128	>128	1.16
43	>128	ND[d]	>128	ND[d]	ND[d]	ND[d]
44	>128	>128	>128	>128	>128	3.20
45	>128	>128	>128	>128	ND[d]	ND[d]

a. Compiled from references 102 and 103.
b. MTB, *Mycobacterium tuberculosis*.
c. MAC, *Mycobacterium avium* Complex.
d. ND, Not determined.

AraT's and mycobacterial proliferation [102, 103]. Disaccharides **36**-**45** (Fig. (**9**)) were synthesized using established protocols, and then MIC values of these compounds were determined against a small panel of mycobacterial strains (Table 1). The majority of the compounds were weakly active against these bacteria with the most effective compounds being those (**37** and **42**) that are fully benzylated on the reducing end residue and unprotected on the non-reducing residue. Both compounds were, however, significantly poorer inhibitors of mycobacterial growth than ethambutol. In further investigations, it was demonstrated that the fully deprotected oligosaccharides, **36** and **41**, were substrates of mycobacterial AraT's with K_m values in the mM range. The partially or fully protected oligosaccharides **37**-**40** and **42**-**45** were not substrates for these AraT's. Yet some of these compounds (**37**, **39**, **42**, and **44**) competitively inhibited these enzymes when tested against the parent disaccharides (*e.g.,* **36** and **41**). The IC_{50} values for these inhibitors, which were all in the low mM range, are presented in Table 1.

4.2.3. Ethambutol Analogs

The relatively simple structure of ethambutol has prompted interest in the synthesis of analogs for use as new anti-mycobacterial drugs. Indeed, ethambutol itself was identified through the preparation of a number of ethylenediamine derivatives after *N*,*N*'-diisopropylethylenediamine was shown to be an anti-tuberculosis agent in mice [104]. Initial current day efforts in this area were reported by Reynolds and coworkers who prepared a series of carbohydrate derivatives that were conjugated to an acyclic fragment resembling a portion of ethambutol [105]. These compounds, (*e.g.,* **46**-**49**, Fig. (**10**)) were chosen based on a previous hypothesis that ethambutol inhibited mycobacterial AraT's by binding to the active site normally bound by DPA [106]. Screening of these compounds against both *M. tuberculosis* strain $H_{37}Ra$ and various *M. avium* strains revealed that all of the compounds had MIC values >128 μg/mL and were thus poor antimycobacterial agents when compared to ethambutol (MIC values 8-32 μg/mL).

More recent work has focused on the synthesis of analogs in which the groups on the diamine core have been modified. A small panel of analogs was prepared by Stütz and coworkers (*e.g.,* **50**-**52**, Fig. (**10**)), but none of these was found to be superior to ethambutol [107]. A substantially larger panel of ethylenediamine derivatives has just been reported [108]. Through the use of solid phase synthesis, a total of 63,238 ethylenediamines were synthesized as groups of 10 or 30 compounds. These pools of compounds were screened against *M. tuberculosis* strain $H_{37}Rv$ and ~2800 compound mixtures possessed anti-tuberculosis activity. These mixtures were deconvoluted and those analogs that

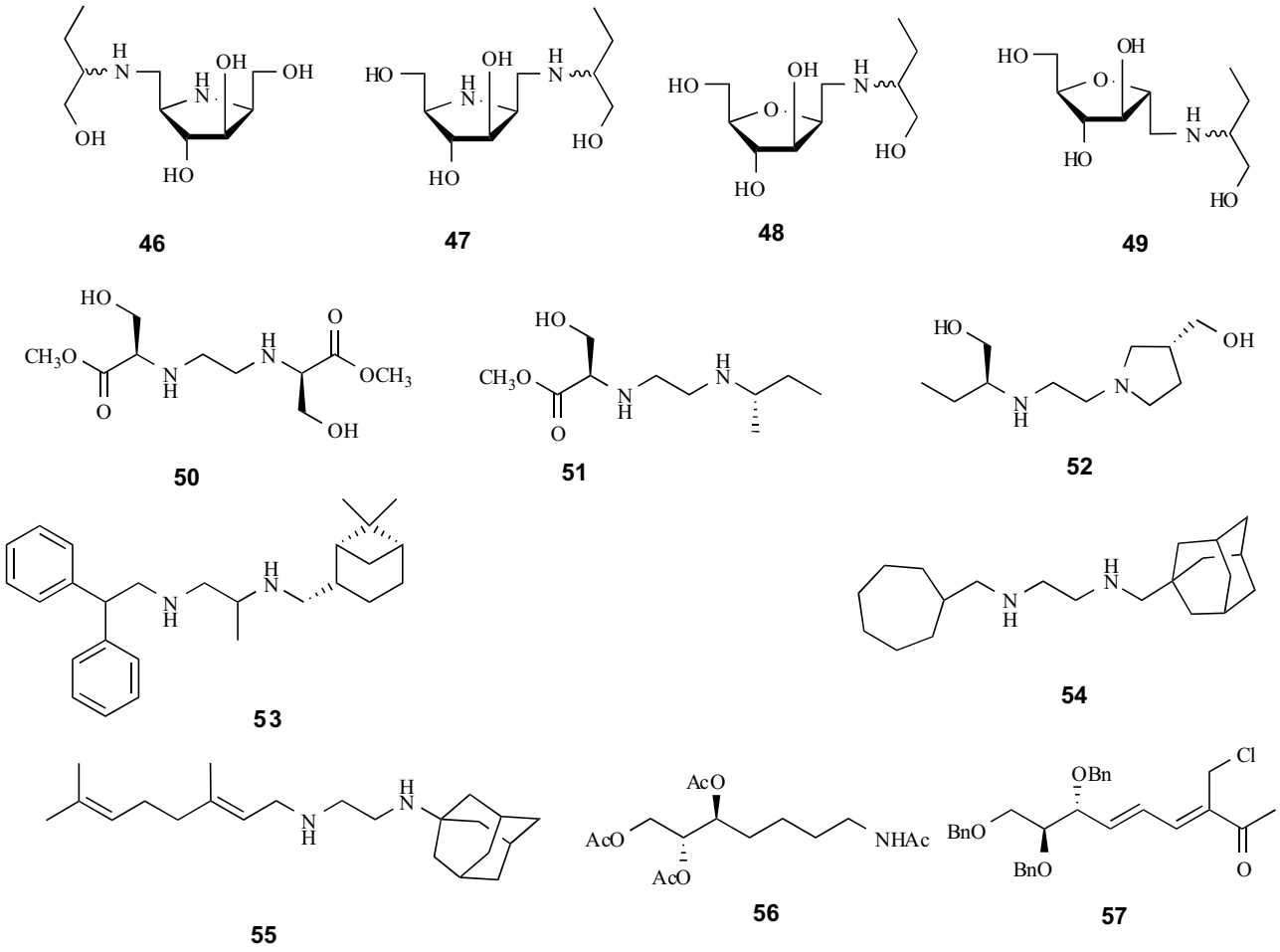


Fig. (10). Examples of novel ethambutol analogs tested as anti-mycobacterial agents. Abbreviations: Ac = acetyl; Bn = benzyl.

had MIC values <12.5 μg/mL (69 compounds) were the resynthesized in larger quantities for further screening. This process led to the identification of 26 compounds that possessed MIC values lower than ethambutol. Representative examples of these compounds, **53**-**55**, are shown in Fig. (**10**) and all are characterized by the presence of large hydrophobic groups. The most active compound, **55** (MIC = 0.2 μg/mL), contained both an adamantyl group and a geranyl group. It was suggested that the potent activity of this compound is a result of the geranyl group mimicking the polyprenol chain of decaprenolphosphoarabinose in the active site of a mycobacterial AraT's. Further screening of these compounds is in progress.

Other acyclic compounds, inspired by the structure of ethambutol, have also been reported. In 2002, a series of acyclic acetylated aminodeoxysugars were synthesized (*e.g.,* **56**) and some of these compounds possessed MIC values below 12 μg/mL when screened against *M. tuberculosis* strain $H_{37}Rv$ [109]. The same group also reported that chloroketone **57**, an intermediate in the synthesis of a family of amines (through displacement of the chlorine) was active against *M. tuberculosis* strain $H_{37}Rv$, with a MIC value below 12 μg/mL [110]. Interestingly, **57** was more active than any of the amines synthesized from it. The mechanism by which these compounds exert their anti-mycobacterial effect is currently unclear, but it was postulated that these species bind in the active site of AraT's.

4.3. Inhibitors of Mycobacterial UDP-Gal*p* Mutase

The first reported inhibitors of UDP-Gal*p* mutase were a series of pyrrolidine derivatives **58**-**60** (Fig. (**11**)) that were synthesized by Fleet and coworkers [111]. Using an *in vitro* assay, compounds **58** and **59** were demonstrated to strongly inhibit the incorporation of radiolabeled galactose into mycobacterial arabinogalactan, while compound **60** was a less potent inhibitor. In addition, both **58** and **59** (but not **60**) were also shown to inhibit the conversion of UDP-Gal*p* into UDP-Gal*f* as well as the reverse reaction (UDP-Gal*f*→UDP-Gal*p*) using an UDP-Gal*f* mutase from *Klebsiella pnuemoniae*. From these studies, it appears that in order for these pyrrolidines to inhibit the enzyme the presence of a two carbon exocyclic group attached to C-4 is necessary (see **60**, Fig. (**11**)). Subsequent work by the same group [112] reported the synthesis an analog of **58**, the amino derivatives **61**, as well as two protected derivatives, **62** and **63**. None of these second-generation compounds were inhibitors of UDP-Gal*p* mutase, thus indicating the deleterious effect of the exocyclic amino group on activity. The Fleet group has also reported [112] the synthesis of the UDP-pyrrolidine conjugates, **64** and **65** (Fig. (**11**)), which were designed to serve as a mimic for the entire UDP-Gal*f* structure. To date, the biological activity of neither **64** or **65** has been reported.

A very recent account has described the development of a high-throughput assay for UDP-Gal*f* mutase activity and its use in the screening of a library of UDP derivatives [64]. The assay relies on the periodate-induced liberation of tritiated formaldehyde from C-6 tritiated UDP-Gal*f* but not UDP-Gal*p*. Its development is an important step forward in the identification of UDP-Gal*p* mutase inhibitors, as the previously available assay [63], which involved quantitation of the reaction products by HPLC, was not high-throughput.

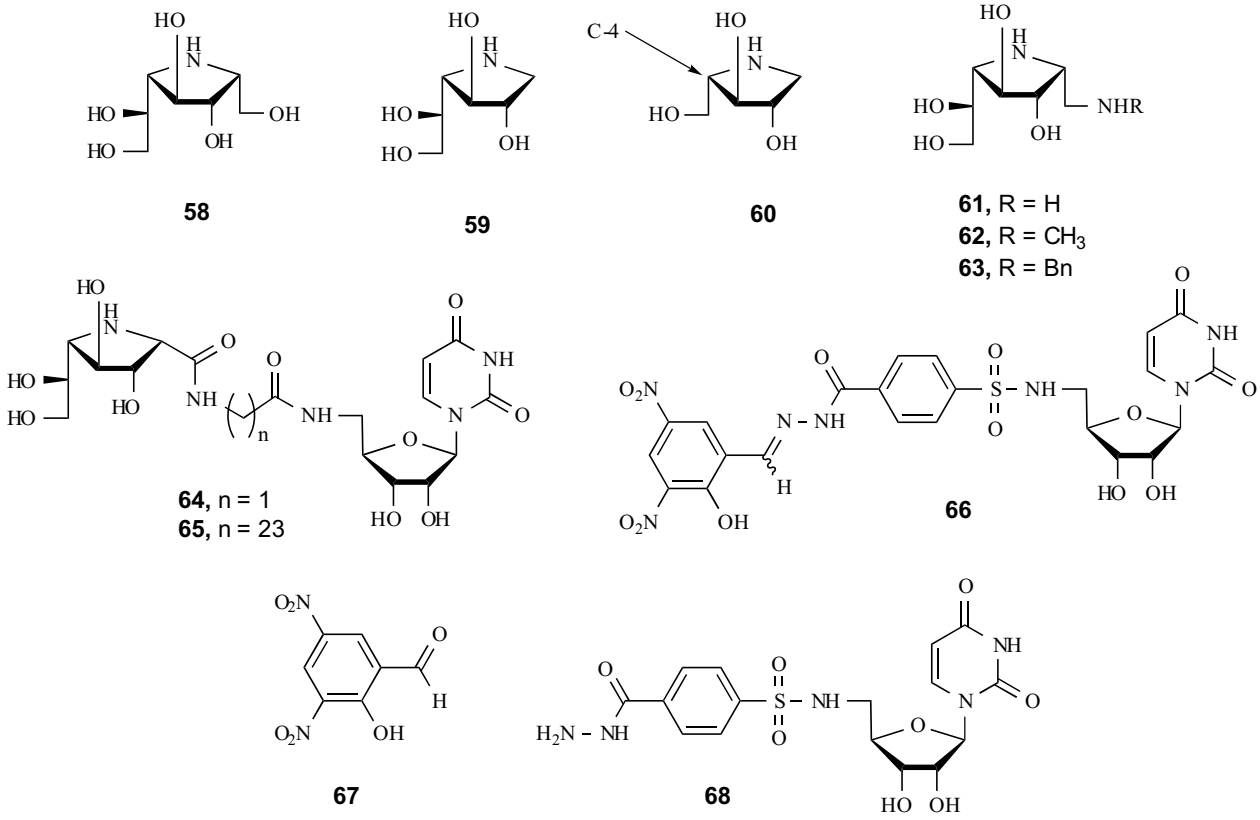


Fig. (11). Established and potential UDP-Galp mutase inhibitors reported to date.

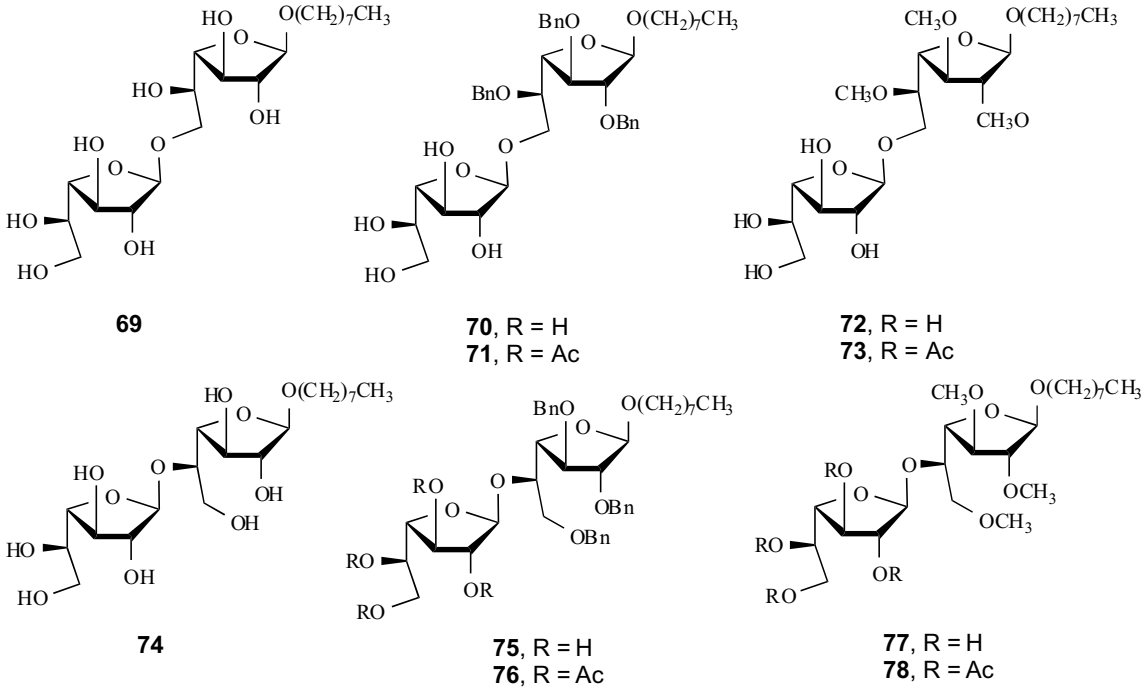


Fig. (12). Disaccharides tested as inhibitors of mycobacterial galactosyltransferases. Abbreviations: Ac = acetyl; Bz = benzoyl.

Upon screening a library of 1300 UDP derivatives, a single compound, **66** (Fig. (**11**)), was identified as an inhibitor with an IC_{50} of 6 μM. The two compounds that were used to synthesize the active compound, **67** and **68**, were also screened in the assay. Aldehyde **67** was shown to have slight activity at 100 μM but not at lower concentrations, while **68** was inactive at all concentrations. Unfortunately, when tested against *M. tuberculosis*, compound **66** showed no inhibitory effect on bacterial growth.

Finally, to probe the mechanism of the reaction catalyzed by UDP-Galp mutase, some fluorinated UDP-Gal*p* analogs have been synthesized and tested as substrates for the enzymes [58, 59, 113] However, the ability of these compounds to prevent the growth of mycobacteria has not been reported.

4.4. Inhibitors of Mycobacterial GalT's

As is true for arabinofuranosyl-containing oligosaccharides, there has been increasing interest in the synthesis of oligosaccharides comprised of galactofuranose residues for use as mycobacterial GalT substrates [114-119]. However, to date, only one paper has reported the evaluation of potential inhibitors of these enzymes [120]. In this study,

Table 2. Activity of Disaccharides 69-78 Against Mycobacterial Strains and as Inhibitors of Mycobacterial GalT's.[a]

Compound	MIC (μg/mL)				GalT Assay	
	MTB[b] $H_{37}Ra$	MAC[c] NJ 168	MAC[c] NJ 211	MAC[c] NJ 304	K_m (mM)	IC_{50} (mM)
69	>128	>128	>128	>128	2.60	ND[d]
70	>12.8≤128	ND[d]	>12.8≤128	>128	ND[d]	ND[d]
72	>128	>128	>128	>128	31.73	3.65
73	>128	>128	>128	>128	ND[d]	ND[d]
74	>128	>128	>128	>128	3.77	ND[d]
75	>12.8≤128	ND[d]	>12.8≤128	>128	ND[d]	ND[d]
76	>128	ND[d]	>128	>128	ND[d]	ND[d]
77	>12.8	>12.8	>12.8	>12.8	5.95	3.32
78	>12.8	>12.8	>12.8	>12.8	ND[d]	ND[d]

a. Compiled from reference 120.
b. MTB, *Mycobacterium tuberculosis*.
c. MAC, *Mycobacterium avium* Complex.
d. ND, Not determined.

a series of disaccharide analogs (**69**-**78**, Fig. (**12**)) were prepared and screened against *M. tuberculosis* $H_{37}Ra$ and three *M. avium* species. The MIC values for each compound are given in Table 2. Most of the compounds were inactive but some (*e.g.,* **77** and **78**) prevented the growth of these species at concentrations above 12.8 μg/mL. In addition, using the previously reported assay for the mycobacterial galactosyltransferase assay [53], disaccharides **69** and **74** were shown to be substrates for this enzyme, with K_m values of 2.60 and 3.77 mM, respectively. The methylated derivatives **72** and **77** were also substrates, although in the former case, the K_m value was significantly higher than those obtained with the fully deprotected parent structures. Both **72** and **77** were also competitive inhibitors of the enzyme, with IC_{50} values of 3.65 and 3.32 mM, respectively.

5. CONCLUSIONS

Work done over the past decade has provided us with a firm understanding of the biosynthetic pathways by which mycobacterial cell polysaccharides are assembled. Among the most important achievements has been the development of assays for many of the enzymes involved in the biosynthesis of these glycans. In addition, there is an increasing number of papers devoted to the synthesis and evaluation of compounds that may potentially inhibit these enzymes. Much work remains to be done, however, in order to identify clinically useful compounds that block mycobacterial cell wall polysaccharide assembly. Future challenges include the development of high-throughput assays for more of the biosynthetic enzymes, which will complement the rapid screening methods that are now available to measure inhibition of mycobacterial growth. Furthermore, new classes of compounds that inhibit these enzymes need to be identified. So far, the bulk of the effort has been focused on the use of carbohydrate-based compounds or on the generation of additional analogs of an existing drug (ethambutol). The identification of new small molecule anti-mycobacterial pharmacophores, in particular those that can be readily modified by combinatorial chemistry, will be an important goal in the future. As the health issues related to mycobacterial disease continue to concern global health officials it is certain that research in this area will continue to increase.

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