

Synthetic UDP-Furanoses as Potent Inhibitors of Mycobacterial Galactan Biogenesis

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SUMMARY

UDP-galactofuranose (UDP-Galf) is a substrate for two types of enzymes, UDP-galactopyranose mutase and galactofuranosyltransferases, which are present in many pathogenic organisms but absent from mammals. In particular, these enzymes are involved in the biosynthesis of cell wall galactan, a polymer essential for the survival of the causative agent of tuberculosis, *Mycobacterium tuberculosis*. We describe here the synthesis of derivatives of UDP-Galf modified at C-5 and C-6 using a chemoenzymatic route. In cell-free assays, these compounds prevented the formation of mycobacterial galactan, via the production of short “dead-end” intermediates resulting from their incorporation into the growing oligosaccharide chain. Modified UDP-furanoses thus constitute novel probes for the study of the two classes of enzymes involved in mycobacterial galactan assembly, and studies with these compounds may ultimately facilitate the future development of new therapeutic agents against tuberculosis.

INTRODUCTION

The development of new drugs against tuberculosis (TB) still presents a major challenge (Balganesh et al., 2008) because of the widespread distribution of the disease, the occurrence of multidrug and extensively drug-resistant strains of *Mycobacterium tuberculosis* (Chan and Iseman, 2008), and the deadly combination of HIV and TB infections (Corbett et al., 2003). In this area, a major target for drug development is the mycobacterial cell wall, and its biosynthesis has received increasing study in the past several years (Barry et al., 2007). At the core of this intricate structure is the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex, which is composed of covalently linked peptides, heteropolymeric carbohydrates (peptidoglycan and arabi-

nogalactan), and highly hydrophobic mycolic acids. The mAGP is a well validated target for drug development, and some of the most effective antituberculars prevent the formation of this component of the *M. tuberculosis* cell wall (Barry et al., 2007). The extremely impermeable cell wall efficiently protects pathogenic mycobacteria against multiple stress factors faced during the course of infection; at the same time, it constitutes a rather vulnerable structure, a true Achilles heel of the bacterium.

Among the four first-line anti-TB drugs, two impinge on the assembly of distinct structures of the mAGP. Isoniazid inhibits the production of mycolic acids (Takayama et al., 1972; Winder et al., 1971), and ethambutol blocks the generation of a complete arabinan domain (Mikušová et al., 1995; Takayama and Kilburn, 1989). The galactan portion of the mAGP represents yet another, and so far underexplored, potential target for anti-TB drug development because of its essential role for the viability of mycobacteria (Pan et al., 2001) and xenobiotic status of its building block, galactofuranose (Galf), in the human host (Pedersen and Turco, 2003).

In a series of investigations performed by Brennan, McNeil, and colleagues, the structure of the mAGP galactan has been established as a linear chain of about 30 alternating (1 → 5)-linked and (1 → 6)-linked β-D-Galf residues (Besra et al., 1995; Daffe et al., 1993; McNeil et al., 1987). The galactan region is linked to C-6 of N-glycolyl/N-acetyl muramic acid residues (Mahapatra et al., 2005a; Mahapatra et al., 2005b) of peptidoglycan via a linker disaccharide unit, α-L-Rhap-(1 → 3)-D-GlcNAc-1-P (Daffe et al., 1990; McNeil et al., 1990). The arabinan portions of the mAGP are, in turn, attached to O-5 of the (1 → 6)-linked Galf residues close to the reducing end of the galactofuran (Alderwick et al., 2005) in the form of three highly branched chains, each consisting of 31 Araf residues (Bhamidi et al., 2008).

The biosynthesis of the galactan component of the mAGP has, to a large extent, been elucidated (Belanova et al., 2008). As illustrated in Figure 1, its assembly begins on decaprenyl-P-P-GlcNAc-Rhap (GL-2), which is formed by the sequential action of two enzymes, a GlcNAc-1-phosphate-transferase (Rv1302) and a rhamnosyltransferase (Rv3265c) (Mikušová et al., 1996; Mills et al., 2004). We have shown that only two galactofuranosyltransferases, GlfT1 (Rv3782) and GlfT2 (Rv3808c),

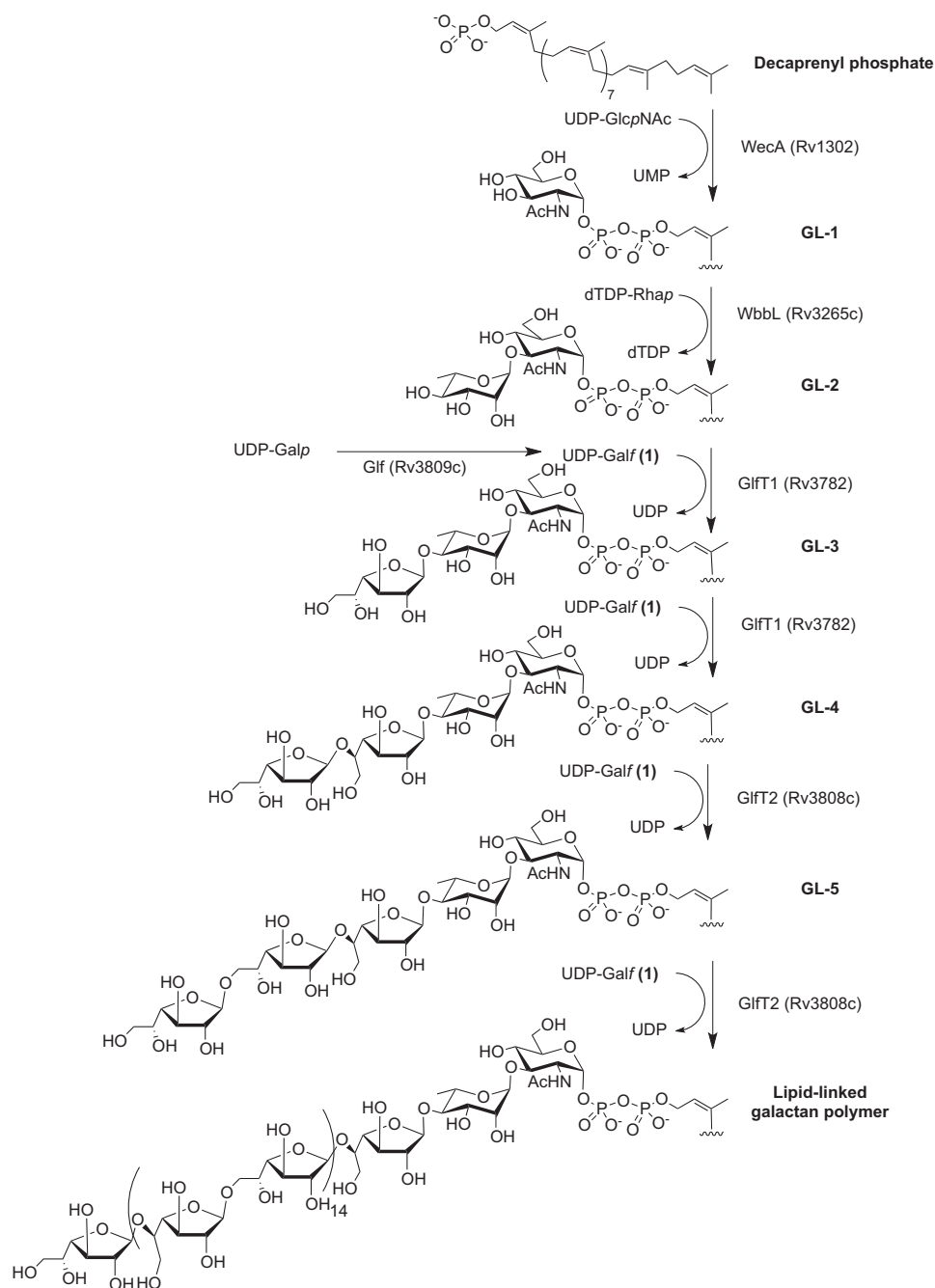


Figure 1. Metabolic Pathway for Biosynthesis of Mycobacterial Galactan

The pathway can be followed by *in vitro* reaction using mycobacterial enzyme fractions and UDP-[^{14}C] Galp as a tracer. Glycolipids GL-1 to GL-5 are extracted into $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1), and lipid-linked galactan polymer into more polar solvents.

appear to account for full galactan synthesis (Belanova et al., 2008). The bifunctional enzyme GifT1 attaches the first two Galp residues to the GL-2 intermediate forming both Galp- β -(1 \rightarrow 4)-Rhap and Galp- β -(1 \rightarrow 5)-Galp glycosidic bonds (Belanova et al., 2008; Mikusova et al., 2006). The product of this reaction, decaprenyl-P-P-GlcNAc-Rhap-Galp-Galp (GL-4), serves as the direct substrate for the full galactan polymerization catalyzed by GifT2, another dual-acting enzyme with both β -(1 \rightarrow 5)

and β -(1 \rightarrow 6) activity (Kremer et al., 2001; Mikusova et al., 2000; Rose et al., 2006). Galactofuranose residues for transferase reactions are donated by UDP- α -D-Galp (1) (Figure 2), which is produced from UDP- α -D-Galp by the action of UDP-galactopyranose mutase (Gif, Rv3809c) (Weston et al., 1997; Soltero-Higgin et al., 2004).

Despite the information presented above, questions remain about the specificity of GifT1 and GifT2, and more detailed

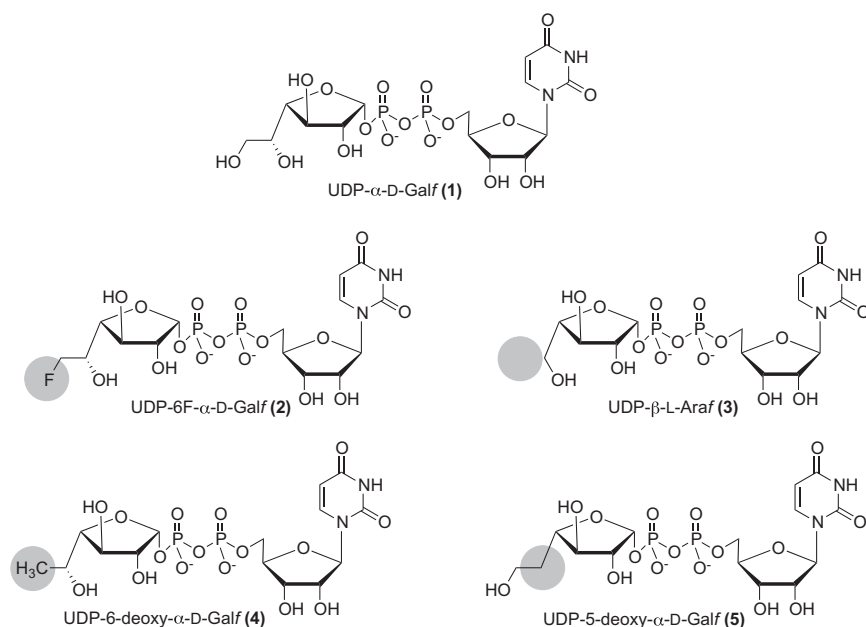


Figure 2. Structure of UDP-Galf (1) and Analogs 2–5 Modified at C-5 or C-6

we investigated the impact of UDP-Galf analogs **2–5** on the biosynthesis of mycobacterial galactan. A better understanding of this process could lead to targeting of this crucial structure of the mycobacterial cell wall for the development of new drugs against TB.

RESULTS

Chemical Synthesis of UDP-Galf Analogs Modified at C-5 or C-6 (2–5)

The 6-deoxy-6-fluoro-UDP-Galf derivative **2** and UDP-L-Araf **3** were prepared as previously described (Peltier et al., 2007; Peltier et al., 2008b).

The synthesis of **4** (Figure 3) started from the known methyl glycoside **6** (Completo and Lowary, 2008; Pathak et al.,

1999), which was converted in three steps and excellent overall yield into alcohol **7**. Next, treatment of **7** with triphenylphosphine and iodine at room temperature in the presence of imidazole (Garegg and Samuelsson, 1980) gave primary iodide **8** in a 96% yield. Subsequent cleavage of the carbon-iodine bond by catalytic hydrogenation at elevated pressure (15 psi) afforded a 90% yield of **9**. Treatment of **9** with 44% HBr in HOAc led to the corresponding glycosyl bromide, which, following its formation, was immediately reacted with dibenzyl phosphate, thus yielding **10** as the pure α -isomer in 59% yield. The modest yield of the reaction is ascribed to the hydrolysis of the less stable β -anomer upon chromatography, which has been reported in the preparation of α -galactofuranosyl phosphate (de Lederkermer et al., 1994). The two benzyl groups in **10** were cleaved by catalytic hydrogenation and the three benzoyl groups were then removed by treatment with 5:2:1 methanol-triethylamine-water to give 6-deoxy- α -D-galactofuranosyl phosphate **11** in 50% overall yield from **10**.

investigations of these enzymes has been hampered by the lack of access to their donor and acceptor substrates. However, recently we have published procedures for the preparation of a panel of synthetic analogs that correspond to GL-2 and GL-3, the natural acceptor substrates for GltT1, as well as analogs of GL-4 and GL-5, substrates for GltT2 (Completo and Lowary, 2008). In addition, recent advances in the preparation of the donor substrate, UDP- α -D-Galf (Peltier et al., 2007; Timmons et al., 2008; Rose et al., 2008), have paved the way for further examination of these galactofuranosyltransferases, which will lead to better understanding of these intriguing bifunctional enzymes.

Synthetic substrate analogs, particularly deoxygenated and fluorinated compounds (Pongdee and Liu, 2004), are valuable tools for probing the mechanism of enzyme-catalyzed reactions. Many are good enzyme inhibitors, some of which have been developed into clinically useful chemotherapeutic agents (Pongdee and Liu, 2004). Recently, we have prepared a series of synthetic fluorinated and deoxygenated analogs of UDP- α -D-Galf (**1**) that are subtly modified in the two-carbon exocyclic side chain (Figure 2) (Peltier et al., 2007; Timmons et al., 2008). In place of the hydroxymethyl group at the C-5 position of (**1**), UDP-6F- α -D-Galf (**2**) bears a fluoromethyl function; the fluorine atom is nearly isosteric to a hydroxyl group but acts only as hydrogen bond acceptor, rather than as both a hydrogen bond donor and acceptor. UDP- β -L-Araf (**3**) lacks the C-5 hydroxymethyl altogether. UDP-6-deoxy- α -D-Galf (**4**) carries a hydrophobic methyl group in place of the hydroxymethyl substituent, and UDP-5-deoxy- α -D-Galf (UDP-5-deoxy- β -L-arabino-hexofuranose, **5**) lacks the polar hydroxyl group at C-5; both are expected to have altered hydrogen-bonding capabilities compared with the parent substrate. Moreover, as depicted in Figure 1, these analogs may be able to interfere with the elongation of the lipid-linked galactan polymer, through their incorporation into the growing chain by GltT1 and GltT2. In the present study,

With **11** in hand, it was converted to the corresponding sugar nucleotide **4** using an enzymatic approach, as has been previously reported for the preparation of UDP-Galf by Field and coworkers (Errey et al., 2004). Thus, incubation of **11** with UDP-glucose and UTP in the presence of three enzymes—a promiscuous galactose-1-phosphate uridylyltransferase, UDP-glucose pyrophosphorylase and inorganic pyrophosphatase—yielded **4** in 70% overall yield (Rose et al., 2008). The anomeric proton in the ^1H NMR spectrum of **4** appeared as a doublet of doublets ($^3J_{\text{H1,H2}} = 5.9$ Hz and $^3J_{\text{H1,P}\alpha} = 4.2$ Hz), and in the ^1H -decoupled ^{31}P NMR spectrum, two doublets ($J = 20.7$ Hz) were present at $\delta_{\text{P}} = -10.2$ and -10.6 ppm, arising from the two coupled phosphorous atoms of the sugar nucleotide moiety. These data are in good agreement with those reported previously for the parent sugar nucleotide, UDP-Galf (Koplin et al., 1997).

The preparation of **5** (Figure 3) was achieved starting from primary alcohol **7** by treatment with silver oxide in DMF, which

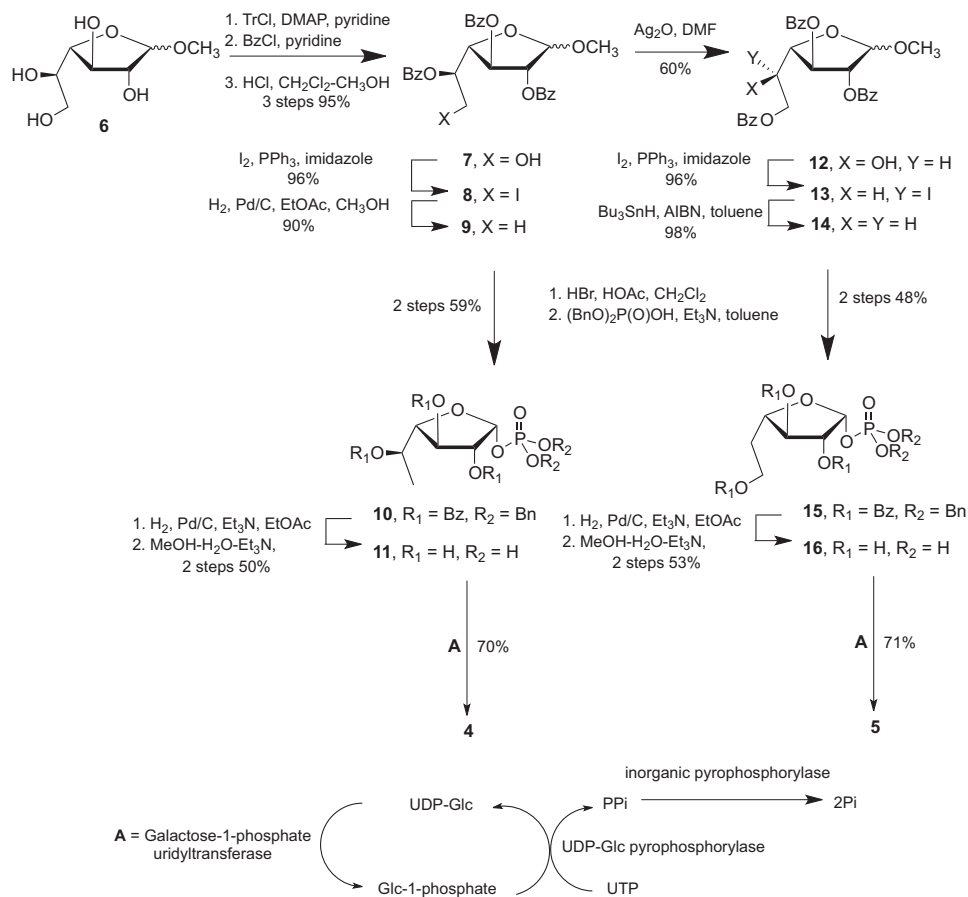


Figure 3. Synthesis of 4 and 5

induced a benzoyl migration reaction that gave secondary alcohol **12** in a 60% yield. Reaction of **12** with triphenylphosphine and iodine, as was done for the preparation of **8**, provided the secondary iodide **13** in excellent (96%) yield. Although in the formation of **8**, the reaction proceeded easily at room temperature, in the case of **13**, it was necessary to heat the reaction mixture at 90°C . To reduce **13**, catalytic hydrogenation was investigated. Using conditions that had worked well in the synthesis of the 6-deoxy derivative, none of the anticipated product was obtained. Even increasing the hydrogen pressure to 45 psi (3 atm) was unsuccessful. Radical deoxygenation was therefore employed to cleave the carbon-iodine bond (Patroni et al., 1986). Thus, **13** was heated at reflux with tin hydride and AIBN to afford **14** in 98% yield. As described for the preparation of **10**, deoxy-glycoside **14** was converted into a glycosyl bromide by reaction with 44% HBr in HOAc, and this product was treated with dibenzyl phosphate and triethylamine to afford 5-deoxy- β -L-*arabino*-hexofuranoside phosphate **15** in 48% overall yield. The product was deprotected in two steps to yield **16** in 53% yield. The same enzymatic synthesis employed in the preparation of **4** was used to convert **16** into the target compound **5** in a 71% yield. Proof of the structure was obtained from NMR spectroscopy: H-1 appears as a doublet of doublet ($^3J_{\text{H1,H2}} = 5.9$ Hz and $^3J_{\text{H1,P}\alpha} = 4.2$ Hz) in the ^1H NMR spectrum, and the proton-

decoupled ^{13}P NMR spectrum shows two doublets (-7.6 and -9.1 ppm, $^3J_{\text{P,P}} = 20.7$ Hz).

Mycobacterial Galactan Biogenesis Is Severely Inhibited by UDP-Galp Analogs 2–5 Modified at C-5 or C-6

Previously, we have shown that mycobacterial galactan build-up can be monitored in a cell-free reaction containing mycobacterial membrane and cell wall fractions, and UDP- ^{14}C Galp (Mikusova et al., 2000). The lipid carrier for galactan assembly, decaprenyl phosphate, was supplied by the crude enzymatically active membranes and cell wall. The reaction mixture was supplemented with UDP-GlcPNAc and dTDP-Rhap for in situ formation of the GifT1 substrate, decaprenyl-P-P-GlcPNAc-Rhap (GL-2), and occasionally by recombinant Gif for more efficient conversion of UDP-Galp into UDP-Galf. In this experiment, production of three distinct glycolipids, GL-3, GL-4, and GL-5, is observed, which can be extracted with CHCl_3 - CH_3OH (2:1). Further extraction of the reaction mixture with more polar solvents (Mikusova et al., 2000) results in the isolation of a heterogeneous population of metabolic intermediates, termed lipid-linked galactan polymer. We have shown that these compounds contain GlcNAc, Rha, and the expected (1 \rightarrow 5)-linked and (1 \rightarrow 6)-linked Galp residues (Mikusova et al., 2000).

We have tested the effect of analogs **2–5** (Figure 2) on galactan build-up in the above-described system, along with

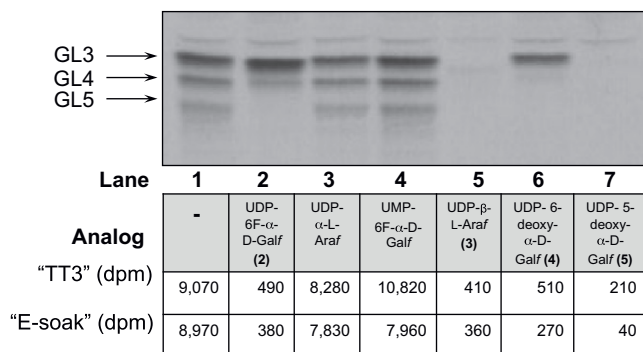


Figure 4. Effects of UDP-Galf Analogs on the Cell-Free Production of GL-3 to GL-5 and Lipid-Linked Galactan Polymer

TLC profile of CHCl_3 - CH_3OH (2:1) fraction; bands were visualized by autoradiography. Quantification of the lipid-linked galactan polymer was performed by scintillation counting.

UMP-6F- α -D-Galf and UDP- α -L-Araf (the 1,2-*trans* isomer of **3**) (Peltier et al., 2008b), which we expected would serve as negative controls (Figure 4). Each compound was added to the reaction mixtures at a concentration of 100 $\mu\text{g}/\text{ml}$ and, after incubation, the products were extracted as described in the Experimental Procedures. TLC analysis of the CHCl_3 - CH_3OH (2:1) fraction revealed that the addition of UDP- β -L-Araf (**3**) and UDP-5-deoxy- α -D-Galf (**5**) resulted in the complete inhibition of galactose-containing glycolipids, whereas significant reduction of galactolipid production, with particular absence of GL5, was observed in case of UDP-6F- α -D-Galf (**2**) and UDP-6-deoxy- α -D-Galf (**4**). The synthesis of the lipid-linked galactan polymer, obtained by extraction of the incubation mixture with CHCl_3 - CH_3OH - H_2O (10:10:3) and "E-soak," was almost completely inhibited in the presence of the tested analogs **2-5**. As expected, addition of UMP-6F- α -D-Galf and UDP- α -L-Araf did not have any effect on elongation of the galactan chain.

UDP-Galf Analogs 2-5 Serve as Substrates of Gift1

Such a severe inhibition of galactan build-up by the UDP-Galf analogs could be explained in two ways. One possibility is that

the compounds are inhibiting one or more of the three enzymes involved in galactan assembly: Gift1, Gift2, or Gif. The second possibility is that the compounds are serving as efficient substrates for these enzymes, resulting in competition between the natural substrate and the synthetic analog. Incorporation of nonradioactive substrates into the galactan products would appear as a decrease in radioactivity in the bands corresponding to the individual galactolipids.

We examined these possibilities by direct investigation of the effects of **2-5** on Gift1. These compounds were included in the assay for examination of Gift1 activity, as recently described elsewhere (Belanova et al., 2008). In our previous work, we exploited UDP-Galf and Gif as a source of UDP-Galf (**1**) for the reaction catalyzed by Gift1. However, synthetic **1** can also be used. Thus, the reaction mixture contained the crude lysate of *Escherichia coli* cells expressing recombinant Gift1 from *Mycobacterium smegmatis*, radiolabeled GL-1/2 mixture, in which GL-2 served as a natural acceptor substrate, as well as the natural donor substrate UDP-Galf (**1**) and/or its synthetic analogs. Reaction products were analyzed by TLC (Figure 5).

From these experiments it is clear that analogs **2-5** serve as substrates for Gift1, giving rise to glycolipids migrating on TLC slightly higher than GL-3 or GL-4 because of the more hydrophobic nature of the incorporated analogs (Figure 5, lanes 3-6). It appears that Gift1 incorporates two residues from UDP- β -L-Araf (**3**) (Figure 5, lane 4), and only one residue from other three tested compounds. It can be expected that, particularly in case of the 5-deoxy analog (**5**), chain termination should occur at the first Gift1 reaction. Transfer of the 5-deoxy-Galf residue to GL-2 produces a modified GL-3 analog, lacking the requisite hydroxyl group for chain extension by the second transferase activity of Gift1.

When the tested compounds were included in the reaction mixture containing an equimolar concentration of the natural donor substrate **1**, we observed synthesis of the natural product of the reaction, GL-4, as well as another compound, which we propose corresponds to modified GL-4, containing both Galf and its modified counterpart (Figure 5, lanes 7-14). These products were formed regardless of the manner in which UDP-Galf was provided to the enzyme, either directly in the form of **1**

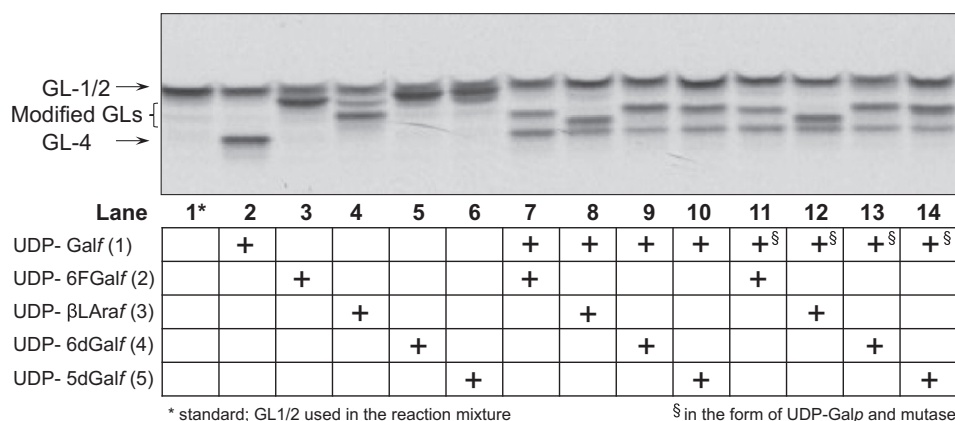


Figure 5. Effects of 2-5 on the Activity of Recombinant Gift1

TLC profile of CHCl_3 - CH_3OH (2:1) fraction; the bands were visualized by autoradiography. Natural substrate and synthetic analogs were used in the same 200 μM concentration.

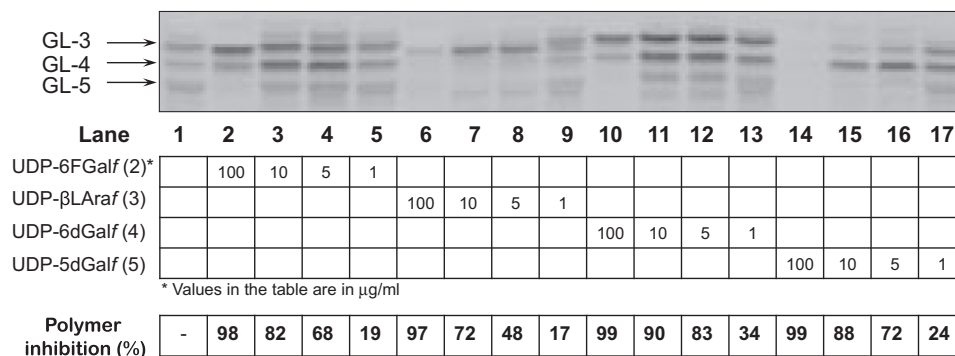


Figure 6. Dose Response for the Addition of 2–5 into the In Vitro Reactions for Mycobacterial Galactan Build-Up

TLC of the [^{14}C]Gal α f-containing glycolipids was produced in the reaction mixtures with decreasing concentration of 2–5. Inhibition of galactan polymer production was evaluated by scintillation counting.

(Figure 5, lanes 7–10), or indirectly from UDP-Galp and Gif (Figure 5, lanes 11–14). These results indicate that none of the compounds 2–5 appreciably inhibit the mutase enzyme that produces UDP-Galf.

Incorporation of UDP-Galf Analogs into GL-4 Results in the Production of “Dead-End” Intermediates

The finding that 3 and 5 are GifT1 substrates came as rather surprising, because lack of radiolabeled galactolipid products in the initial experiments gave the impression of the efficient inhibition of the metabolic utilization of the radioactive UDP-Galp by these analogs (Figure 4, lanes 5 and 7). However, as mentioned above, we could rule out inhibition of Gif, because when UDP-Galp and Gif were used as source of UDP-Galf (1), the production of natural GL-4 and modified GL-4 was observed in the presence of the analog, confirming efficient in situ production of UDP-Galf (Figure 5, lanes 11–14).

One explanation for this apparent contradiction would be if 3 and 5 had a rather high affinity for GifT1, resulting in the preferential production of modified glycolipids GL-3 and/or GL-4, containing solely the modified residues, which would not be radiolabeled in the experiment using UDP-[^{14}C]Galp. To test this hypothesis, we used the above assay for a competition study with varying concentrations of all four compounds 2–5 (Figure 6). It is clear from the TLC profile of CHCl_3 – CH_3OH (2:1) extracts that, indeed, a ten-fold lowering of the concentrations of UDP- β -L-Araf (3) or UDP-5-deoxy- α -D-Galf (5) in the reaction mixtures from the originally used 100 $\mu\text{g/ml}$ led to less efficient competition of the natural substrate UDP-Galf (1) with the analogs, resulting in the synthesis of radiolabeled compounds (Figure 6, lanes 7 and 15).

Of all the compounds, the UDP-5-deoxy- α -D-Galf (5) was the most potent competitor with 1 in the reaction catalyzed by GifT1, and UDP-6-deoxy- α -D-Galf (4) was comparable to UDP-6F- α -D-Galf (2). In the presence of about 15-fold higher amount of the latter two analogs in the reaction mixtures compared to the radioactive natural substrate, efficient production of radiolabeled modified glycolipids is still observed, confirming incorporation of much less abundant radioactive natural substrate in the reaction mixture to the products (Figure 6, lanes 2 and 10). This is in contrast with the results for 5 (Figure 6, lane 14), which appears to be used preferentially as a substrate by GifT1 under

these conditions. In the dose response experiment performed with compounds 2–5, less than equimolar concentration of the analogs (9 μM) compared to the radioactive natural substrate (11 μM) was sufficient to achieve about 50%–80% inhibition of galactan synthesis, respectively (Figure 6, lanes 4, 8, 12, and 16). We thus propose that in the presence of 2–5 modified “dead-end” glycolipid intermediates are formed (Figure 7A) leading to truncation of galactan polymerization.

To test this hypothesis, we made use of a synthetic GL-2 analog, octyl disaccharide 17 (Completo and Lowary, 2008) (Figure 7B), which had previously been shown to be a GifT1 substrate (Belanova et al., 2008). Disaccharide 17 and 5 were first incubated in the presence of GifT1 and then the reaction mixture was analyzed by LC-MS. As predicted, a product corresponding to trisaccharide 18 (Calculated $m/z = 648.3202$ [$\text{M} + \text{Na}^+$], Found $m/z = 648.3191$) was formed (data not shown). Similar reaction of 17 with the 6-deoxy analog 4 gave a trisaccharide product 19 (Calculated 648.3202 [$\text{M} + \text{Na}^+$], Found $m/z = 648.3194$).

Effects of UDP-Galf Analogs 2–5 on GifT2

Although the work outlined above suggests that 2–5 exert their effect on galactan polymerization via GifT1, the overall inhibition of galactan synthesis in the presence of these compounds could be further strengthened by inhibition of GifT2. We have therefore examined the effect of these compounds on recombinant GifT2 using a recently described spectrophotometric assay (Rose et al., 2008). Using a trisaccharide acceptor substrate, and a 2 mM concentration of the synthetic analogs and 0.5 mM concentration of UDP-Galf, we have observed 25% inhibition of GifT2 activity for both UDP-6F- α -D-Galf (2) and UDP-5-deoxy- α -D-Galf (5), and 32% inhibition in case of UDP-6-deoxy- α -D-Galf (4). The 6-deoxy-derivative also serves as a substrate for GifT2 achieving 50% of the activity of the natural substrate at 2 mM. UDP- β -L-Araf (3) served as a very weak substrate of the enzyme ($\sim 3\%$ of the natural substrate); it did not act as an inhibitor of GifT2.

DISCUSSION

The method described here for the preparation of 4 and 5 makes use of a chemoenzymatic approach that has previously been employed for the preparation of UDP-Galf (Rose et al., 2008;

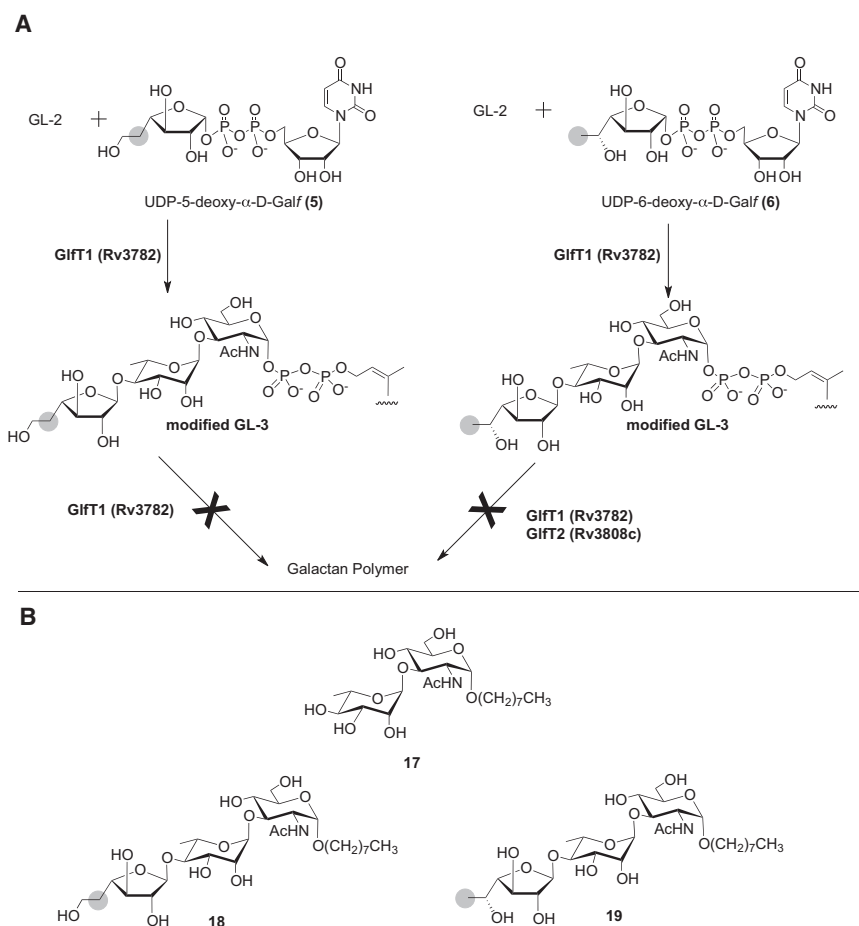


Figure 7. UDP-Furanoses Are Substrates of GIFT1

(A) “Dead-end” galactolipid intermediates inhibit further elongation of mycobacterial galactofuran. (B) Synthetic GL-2 analog 17 and the reaction products produced upon its reaction with either 4 or 5 in the presence of GIFT1.

inhibit mycobacterial growth in the same range as the clinically used antituberculosis agents ethambutol and rifampicin (Dykhuzen et al., 2008).

Over the past 10 years, several reports on the preparation of compounds that could serve as probes and potential inhibitors of GifT1 and GifT2 have been published, but the focus has been primarily on acceptor analogs. Among them, β -D-Galf-(1 \rightarrow 4)- α -L-Rhap and β -(1 \rightarrow 5)- and β -(1 \rightarrow 6)-linked octyl Galf disaccharides were shown to serve as substrates for these enzymes and showed moderate antimycobacterial effects (Pathak et al., 1999; Pathak et al., 2001). Modest inhibition of galactosyltransferase activity has also been reported for analogs of the arabinogalactan linker disaccharide α -L-Rhap-(1 \rightarrow 3)- α -D-GlcpNAc (Wen et al., 2003). More recent investigations have focused on the synthesis and use of Galf oligomers for probing the specificity of GifT1 and GifT2 in vitro (Belanova et al.,

Errey et al., 2004). The advantage of this approach over a purely chemical approach is that the formation of the sugar nucleotide from the corresponding Galf-1-phosphate derivative (i.e., **11** and **16**) proceeds more efficiently and in higher yield. An obvious limitation of this approach is that the enzyme may not recognize all modified Galf-1-phosphate analogs of interest. Nevertheless, the successful preparation of **4** and **5**, as well as the preparation of **2** and **3** reported earlier (Peltier et al., 2008b), demonstrates that the key uridyl transferase will tolerate modification of the exocyclic diol moiety, albeit with varying degree of efficiency.

The straightforward availability of UDP-Galf synthetic analogs has prompted us to test their effects on mycobacterial galactan biosynthesis. Our data confirm that **2–5** in a concentration of 100 μ g/ml almost completely abolish cell-free production of the galactan polymer. To the best of our knowledge, this is the first report of compounds that so severely inhibit galactan biosynthesis.

Three enzymes are involved in mycobacterial galactan assembly, Gif and the glycosyltransferases GifT1 and GifT2. Gif has received the most attention to date (Eppe et al., 2009; Yuan et al., 2008; Barlow and Blanchard, 2000; Zhang and Liu, 2001a; Richards and Lowary, 2009), and a number of inhibitors of this enzyme have been identified (Kovensky et al., 1999; Liautard et al., 2008; Itoh et al., 2007). Most notable are a family of derivatives, some of which have also been demonstrated to

2008; Completo and Lowary, 2008; Gandolfi-Donadio et al., 2003).

Despite the focus on acceptor derivatives, some work has addressed the preparation of donor analogs as probes of GifT1 and GifT2. For example, Fleet and coworkers studied the effects of Galf iminosugar analogs on galactan biosynthesis and found about 60% inhibition at a concentration of 200 μ g/ml, but this was attributed to inhibition of Gif, which was needed in the assay to generate the donor species (Lee et al., 1997). In other studies, Thomas and coworkers extended the investigation of iminosugars to develop inhibitors based on a proposed model of the transition state of the transferase reaction. These investigations led to the identification of the first donor-based inhibitor of galactosyltransferase activity (presumably GifT2) in *M. smegmatis* with an IC_{50} value of 4.8 mM (Cren et al., 2004). Finally, a recent investigation by Bugg and coworkers described the synthesis and evaluation of uridine-linked transition state mimics of the GifT2 transfer reaction, which inhibited the enzyme, although the level of inhibition was not quantified (Trunkfield et al., 2010).

In the present report, we show that selected UDP-Galf analogs modified at C-5 or C-6 inhibit galactan assembly in an *M. smegmatis* cell-free assay by 50% or more at a concentration of only 9 μ M (5 μ g/ml). The incorporation of these analogs into the growing galactan chain by GifT1, leading to modified dead-end

glycolipid intermediates, appears to be the predominant mechanism by which polymer production is halted. However, at the same time, UDP-6-deoxy- α -D-Galf (**4**) showed activity as the substrate of GifT2 and thus this enzyme may also introduce 6-deoxy-Galf residues into the polymer chain leading to similar “dead-end” intermediates. In addition, compounds **2**, **4**, and **5** served also as weak inhibitors of GifT2. Earlier work has shown that UDP-6F- α -D-Galf (**2**) and UDP- β -L-Araf (**3**) serve as substrates for Gif from *E. coli* (Eppe et al., 2009; Zhang and Liu, 2001b); thus, in principle, these compounds can compete with the natural substrate, in turn inhibiting the efficiency of galactan polymerization. However, again this pathway also appears to be comparatively minor as experiments studying the effect of **3** and **5** on GL-4 formation showed that comparable results were obtained either when synthetic UDP-Galf was used in the reaction, or when this intermediate was produced in situ using UDP-Galp and Gif.

Although sugar nucleotides such as **2–5** are expected to have limited potential as lead drug candidates as a result of their poor cell penetration, these species nevertheless have the potential to be very useful tools in probing galactan assembly in mycobacteria. For example, because GifT1 catalyzes two sequential glycosyl transfer reactions, the product of the first glycosylation (GL-3) is a substrate for the second transferase activity leading to the formation of GL-4. As described above, GifT1 uses the 5-deoxy-UDP-Galf derivative **5** to generate a GL-3 derivative that is incapable of being further glycosylated. Hence, this derivative could prove useful in studies in which it is desirable to dissect the two different transferase activities of the enzyme. Analogous investigations with GifT2 can be envisioned. These compounds will also be useful in studying the formation of galactofuranose-containing glycoconjugates in other organisms (Richards and Lowary, 2009; Peltier et al., 2008a). In addition to various Gifs and galactofuranosyltransferases from a host of organisms, such investigations could be extended to a recently identified protein that transfers UDP-Galf across the Golgi membrane in *Aspergillus fumigatus* (Engel et al., 2009).

SIGNIFICANCE

In this article, we report that derivatives of the sugar nucleotide UDP-Galf prevent the formation of mycobacterial galactan, an important cell wall component in the organism that causes the disease tuberculosis, *M. tuberculosis*. Four UDP-Galf derivatives, modified at C-5 or C-6 by replacement of a single hydroxyl group with either hydrogen or fluorine or through removal of C-6 and the associated hydroxyl group, were synthesized using a chemoenzymatic route, which has significant advantages over the chemical synthesis of these compounds. When evaluated in a cell-free assay at 100 μ g/ml, all four compounds abolished the formation of the full-length galactan polymer. Instead, short “dead-end” intermediates, resulting from incorporation of the modified carbohydrate residues into the growing carbohydrate chain, were produced. Thus, these compounds have potential as chain terminators in studies of galactan biosynthesis in mycobacteria and as probes of Galf metabolism in other microorganisms.

EXPERIMENTAL PROCEDURES

Synthesis of UDP-Galf analogs modified at C-5 or C-6

See Supplemental Experimental Procedures available online.

Preparation of Enzymatically Active Membranes and Cell Envelope from *M. smegmatis*

Enzymatically active membranes and cell envelope (wall and membrane) were prepared essentially as described from *M. smegmatis* mc²155 grown in Nutrient Broth (Mikusova et al., 1996). Briefly, cells (10 g) were suspended in about 40 ml of 50 mM MOPS buffer (pH 7.9) containing 5 mM 2-mercaptoethanol and 10 mM MgCl₂ (buffer A), subjected to probe sonication, and centrifuged at 23,000 \times g for 20 min at 4°C. The pellet was resuspended in buffer A, and Percoll (Amersham Pharmacia Biotech) was added to achieve a 60% suspension, which was centrifuged at 23,000 \times g for 60 min at 4°C. The white upper band was isolated, and Percoll was removed by repeated suspension in buffer A and centrifugation. The fraction (cell envelope) was resuspended in buffer A to a protein concentration of 10–20 mg/ml for use. Membranes were obtained by centrifugation of the 23,000 \times g supernatant at 100,000 \times g for 2 hr at 4°C and suspended in buffer A to give a protein concentration of 20–30 mg/ml.

Preparation of the *E. coli* Cell Lysate Containing Recombinant GifT1 for the In Vitro Assays

One hundred milliliters of culture of the overproducing strain *E. coli* BL21(DE3)/pET28a-MSMEG_6367 (Belanova et al., 2008) was grown to OD₆₀₀ 0.6 and placed on ice for 1 hr. The culture was induced with IPTG at a final concentration of 0.2 mM, overnight at 16°C. The cells were harvested and disintegrated by probe sonication (10 \times 15-s pulses with 40-s cooling intervals between pulses) in 5-fold excess of buffer A. The cell lysate was cleared by centrifugation for 20 min at 20,000 \times g at 4°C, and the supernatant was used as a source of GifT1.

Preparation of dTDP-Rha

The synthesis of dTDP-Rha relied on the presence of the full array of the Rha synthetic enzymes and endogenous cofactors in *M. smegmatis* and was prepared from dTDP-Glc using the 100,000 \times g supernatant of disrupted *M. smegmatis* as an enzyme source, as described elsewhere (Mikusova et al., 1996).

Preparation of UDP-Galp Mutase

Histidine-tagged mutase from *Klebsiella pneumoniae* was purified essentially as described elsewhere (Beis et al., 2005). The recombinant plasmid for the preparation of Gif was kindly provided by Prof. David A. R. Sanders from the University of Saskatchewan, Saskatoon, Canada.

Reaction Mixtures, Fractionation, and Analysis of Reaction Products

For the initial establishment of the effects of the synthetic analogs of UDP-Galf on galactan build-up, we prepared reaction mixtures in which mycobacterial membrane (0.7 mg of protein) and cell envelope (1 mg of protein) fractions served as enzyme sources, and UDP-[U-¹⁴C]Galp (NEN; 278 mCi/mmol, 0.25 μ Ci) was used to monitor formation of the galactofuran biosynthetic intermediates. NADH was added to the reaction mixtures in 2.5 mM concentration to promote the activity of the endogenous mutase, and cold sugar nucleotides for in situ production of GL-2, UDP-GlcNAc, and TDP-Rha were added in 20 μ M concentration. The reactions were supplemented with the UDP-Galf analogs in concentrations between 1 and 100 μ g/ml. Volumes of the reactions were adjusted to 80 μ l with buffer A.

After incubation of the reaction mixtures for 1 hr at 37°C, CHCl₃-CH₃OH (2:1; 1.5 ml) was added, which was left rocking at room temperature for 10 min and centrifuged (3,000 \times g). The CHCl₃-CH₃OH phase was removed from the pellet and treated as described before (Mikusova et al., 2000). To remove residual radiolabel from the pellet, 50% CH₃OH in H₂O containing 0.9% NaCl (0.5 ml) was added, and the mixture was briefly bath-sonicated and centrifuged at 3,000 \times g. The supernatant was discarded, and the pellet was further extracted with 50% CH₃OH in H₂O (0.5 ml) and 100% CH₃OH (0.5 ml), which were also discarded. The washed pellet was extracted with 0.5 ml of the solvent “TT3” (CHCl₃-CH₃OH-H₂O; 10:10:3) (Rush et al., 1993)

to remove more polar products (the lipid-linked galactofuran polymer) and finally with 0.5 ml "E-soak" (water-ethanol-diethyl ether-pyridine-concentrated ammonium hydroxide; 15:15:5:1:0.017) (Angus and Lester, 1972) to obtain [^{14}C]Gal-labeled lipid-linked products of greater polarity. To the $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1) extract, 170 μl of buffer A was added to achieve a biphasic mixture. The upper aqueous phase was removed and discarded, and the bottom phase was backwashed with $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O}$ (3:47:48) (Folch et al., 1957). The backwashed bottom phase was dried under a stream of N_2 at room temperature and redissolved in 50 μl of $\text{CHCl}_3\text{-CH}_3\text{OH-H}_2\text{O-NH}_4\text{OH}$ (65:25:3.6:0.5). Amounts of the radiolabeled products in the individual extracts were quantified by scintillation counting. Qualitative analysis of $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1) extracts was performed by TLC on Silica Gel plates (Merck) in $\text{CHCl}_3\text{-CH}_3\text{OH-NH}_4\text{OH-1 M ammonium acetate-H}_2\text{O}$ (180:140:9:9:23), and the radiolabeled lipid bands were visualized by autoradiography.

To examine direct effects of the studied UDP-Galp analogs on the activity of Gift1, we have prepared crude GL-1/2 mixture in vitro and used it in the reaction. Reaction mixture for GL-1/2 production contained 9 mg of membranes from *M. smegmatis* mc 2 155, 2 μCi of UDP-[^{14}C]GlcNAc (NEN, 288 mCi/mmol), 20 μM TDP-Rha, and buffer A in a final volume of 640 μL . After 1 hr incubation at 37°C, [^{14}C] GlcNAc-radiolabeled glycolipids were extracted with $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1) and subjected to biphasic Folch wash as described above (Folch et al., 1957). In vitro reactions using radioactive GL-1/2 as the galactose acceptors were performed as follows: 3000 dpm of glycolipid preparation were dried in the stream of N_2 . This was followed by addition of the enzyme source [crude lysate of *E. coli* BL21(DE3)/pET28a-MSMEG_6367 producing recombinant Gift1 (Belanova et al., 2008); ~ 0.3 mg of protein] and UDP-Galp and/or the studied analogs in 200 μM concentration. In cases where UDP-Galp was used as the source of UDP-Galp, the reaction mixture was supplemented with purified Gift and 2.5 mM freshly prepared NADH. Volume of the reaction was adjusted with buffer A to the final volume 80 μL . The whole mixture was subsequently briefly bath sonicated. Incubation was performed for 1 hr at 37°C. Extraction of the reaction products and their TLC analysis was performed as described above.

For LC-MS analysis of the Gift1 reaction products mixtures containing 3.2 mM GL-2 acceptor analog 17 (Completo and Lowary, 2008), 150 μM donor analogs 4 or 5, and 1 mg of crude Gift1 enzyme (as above) and buffer A in a final volume of 320 μl were incubated in triplicate for 2 hr at 37°C. The reactions were stopped by addition of 6 ml of $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1), followed by addition of 680 μl of water to achieve a Folch wash (Folch et al., 1957). The water phase was backwashed twice with 4 ml of CHCl_3 and 1 ml of CH_3OH . Organic phases from the triplicates were combined and dried under the stream of N_2 . The extract was subjected to mild acid hydrolysis in 300 μl of 1-propanol and 600 μl of 20 mM HCl at 60°C for 30 min. After cooling, the mixture was neutralized with 30 μl of 0.2 mM NaOH, dried, and subjected to *n*-butanol-water partitioning, as follows: 1.5 ml *n*-butanol saturated with water and 1.5 ml water were added to the dried samples, mixed for 20 min, centrifuged and the upper *n*-butanol phase was kept. The water phase was extracted two more times with *n*-butanol and the combined *n*-butanol fractions were dried under N_2 . This sample was further hydrolyzed in mild alkali conditions with 500 μl of $\text{CHCl}_3\text{-CH}_3\text{OH}$ (2:1) and 500 μl 0.2 M NaOH in CH_3OH at 37°C for 20 min. Following neutralization with 2 μl of glacial acetic acid, the reaction products were again extracted by *n*-butanol-water partitioning, as described above, and then dried. Samples were analyzed on Waters Q-TOF Premier LC-MS system in positive ion mode.

Spectrophotometric Assay for Monitoring Gift2 Activity

Recombinant Gift2 was prepared as described elsewhere (Rose et al., 2006). Spectrophotometric Gift2 assays were performed in 384-array microtiter plate wells, as described elsewhere (Rose et al., 2008). The reaction buffer contained 50 mM MOPS (pH 7.9), 50 mM KCl, 20 mM MgCl_2 , 1.1 mM NADH, 3.5 mM PEP, 7.5 U pyruvate kinase (PK, EC 2.7.1.40), and 16.8 U lactate dehydrogenase (LDH, EC 1.1.1.27). A standard assay reaction contained UDP-Galp (1) at a final concentration of 0.5 mM and a trisaccharide acceptor substrate, octyl β -D-galactofuranosyl-(1 \rightarrow 5)- β -D-galactofuranosyl-(1 \rightarrow 6)- β -D-galactofuranoside (Rose et al., 2008) at 2 mM. Each reaction was initiated by the addition of 0.75 μg Gift2 to the assay mixture. The final assay volume was 40 μL . Reactions were monitored at 37°C using a Spectra Max 340PC microplate reader controlled with SOFTmax $^{\text{®}}$ PRO software (Molecular Devices,

Sunnyvale, CA) in the kinetics read mode, as described elsewhere (Rose et al., 2008).

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures and can be found with this article online at doi:10.1016/j.chembiol.2010.10.014.

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