

TITLE: Synthesis of substrates for the galactofuranosyl transferase GlfT2

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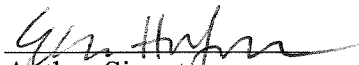
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ABSTRACT

Synthesis of substrates for the galactofuranosyl transferase GlfT2

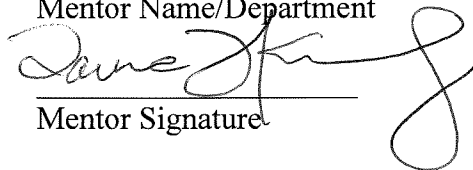
The galactan, a polymer of galactofuranose (Galf), is a key component of the hydrophobic cell wall of *Mycobacterium tuberculosis*, the bacterial species that is responsible for tuberculosis. Much remains unknown about the biosynthesis of the galactan, and there is a need for synthetic substrates that will allow study of the essential enzymes involved in galactan biosynthesis. In particular, the galactofuranosyl transferase GlfT2 exhibits interesting enzymatic activities including the ability to generate regioisomeric linkages and polymers of approximately 30 Galf residues. Recent developments in strategies for the chemical synthesis of oligosaccharides have been used to synthesize Galf-containing acceptor substrates. These substrates mimic key features of the structure of the putative endogenous acceptor for GlfT2. The availability of such substrates has been invaluable for the study of this important enzyme, and this work may assist in creating future drugs to treat bacterial diseases such as tuberculosis.

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Synthesis of substrates for the galactofuranosyl transferase GlfT2

Abstract

The galactan, a polymer of galactofuranose (Galf), is a key component of the hydrophobic cell wall of *Mycobacterium tuberculosis*, the bacterial species that is responsible for tuberculosis. Much remains unknown about the biosynthesis of the galactan, and there is a need for synthetic substrates that will allow study of the essential enzymes involved in galactan biosynthesis. In particular, the galactofuranosyl transferase GlfT2 exhibits interesting enzymatic activities including the ability to generate regioisomeric linkages and polymers of approximately 30 Galf residues. Recent developments in strategies for the chemical synthesis of oligosaccharides have been used to synthesize Galf-containing acceptor substrates. These substrates mimic key features of the structure of the putative endogenous acceptor for GlfT2. The availability of such substrates has been invaluable for the study of this important enzyme, and this work may assist in creating future drugs to treat bacterial diseases such as tuberculosis.

Background and Significance

Polysaccharides are polymers of sugars that are widespread in nature and are the most abundant organic substances on earth. Sugar monomers come in various forms including hexopyranosides and their five-membered ring counterparts, hexofuranosides. Hexofuranosides are of particular interest to researchers because of their unique distribution in nature. For example, the hexofuranoside galactofuranose (Galf, **Figure 1**) is found in many prokaryotes; however it is rare in most eukaryotes, and is completely absent in mammals.¹ Even in prokaryotes, hexofuranosides (like Galf) are rare, making the study of the biological roles of furanose sugars especially challenging. However, an understanding of furanose glycoconjugate biosynthesis is necessary since furanose polymers are major components in the cell walls of many bacteria, protozoa, and fungi.² For example, polysaccharides of Galf are found in the cell walls of many pathogenic microorganisms, including *Mycobacterium tuberculosis*, the bacterium that causes tuberculosis.² The presence of these sugars in pathogens, and their absence in humans, makes them a prime target for drug development.³

Tuberculosis is the world's most lethal bacterial disease, killing nearly 2 million people worldwide each year.⁴ Tuberculosis is extremely difficult to treat due to its essentially impenetrable hydrophobic cell wall composed of tightly packed mycolic acids, which form a thick, waxy layer

surrounding the cell (**Figure 1**). This protective layer is attached to the cell membrane by a linear polymer, known as the galactan, which is comprised of 20–40 Galf residues that are connected by alternating β -(1,5) and β -(1,6) glycosidic bonds. The enzyme GlfT2 is a critical glycosyltransferase in mycobacterial galactan assembly and is hypothesized to polymerize most of the galactan chain.^{3, 5-7}

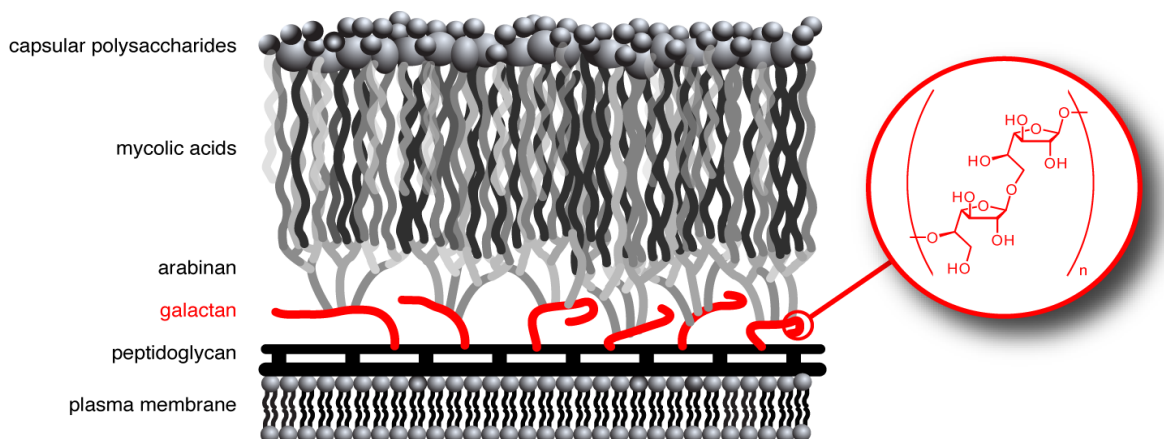


Figure 1. Schematic of the *M. tuberculosis* cell wall. Galf residues are shown in red.

Current research in the Kiessling group is aimed at synthesizing probes to provide information on the mechanisms that underlie the biosynthesis of the galactan and will eventually explore ways to inhibit galactan production. These probes have been particularly useful in studies that seek to determine the mechanism that determines the number of Galf residues added. Recombinant GlfT2 is a soluble galactofuranosyl transferase that was first shown to add up to four new Galf residues to acceptors displaying galactofuranose-terminated oligosaccharides.⁸ Polymers of lengths comparable to those observed endogenously were absent. It was postulated that the ability to vary acceptor structure could illuminate the mechanisms involved in determining polymer length. A synthetic route was developed to generate acceptors that could be converted into polymers of Galf residues.⁹ The Kiessling group recently demonstrated that recombinant His₆-GlfT2 is capable of producing galactan polymers of endogenous

length from synthetic lipid-bearing acceptors.^{9,10} Fundamental to this study was the availability of synthetic acceptors. Thus, a divergent synthetic route capable of assembling a wide range of acceptors could allow researchers to dissect the molecular features relevant for elongation and template-independent length control by GlfT2.

Results and Discussion

The first step in studying the production of galactan polysaccharides in the cell wall was to produce acceptor substrates capable of being elongated by GlfT2 into a full length polysaccharide of 20-40 Galf residues. Previous work in the Kiessling group has demonstrated that β -(1,6) diGalf acceptors were superior substrates to β -(1,5)-linked acceptors. Thus, we initially focused on the generation of β -(1,6) diGalf acceptors. The synthesis of a diGalf acceptor substrate requires three building blocks: a Galf monosaccharide donor, a Galf monosaccharide capable of acting as both a donor and an acceptor at different points along the route, and a lipid moiety.

The Kiessling group synthesized acceptor **1** (Figure 2), which was elongated by GlfT2 to produce endogenous length galactan.¹⁰ Further biochemical studies require derivatives of **1**. The development of a highly efficient route will facilitate the synthesis of a wide variety of sugar derivatives. In particular, access to longer oligosaccharides such as tri- and tetra-Galf acceptor substrates is desirable. A synthetic route toward oligomeric variants would allow us to construct acceptors with alternating β -(1,5) and β -(1,6) linkages like those seen in endogenous galactan, or to produce acceptors with non-natural, non-alternating regiochemistry.

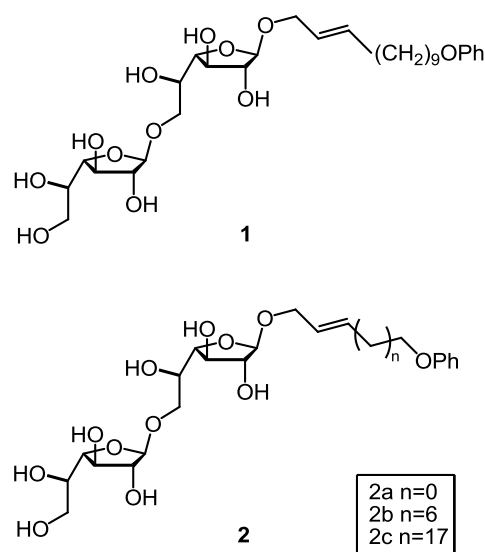
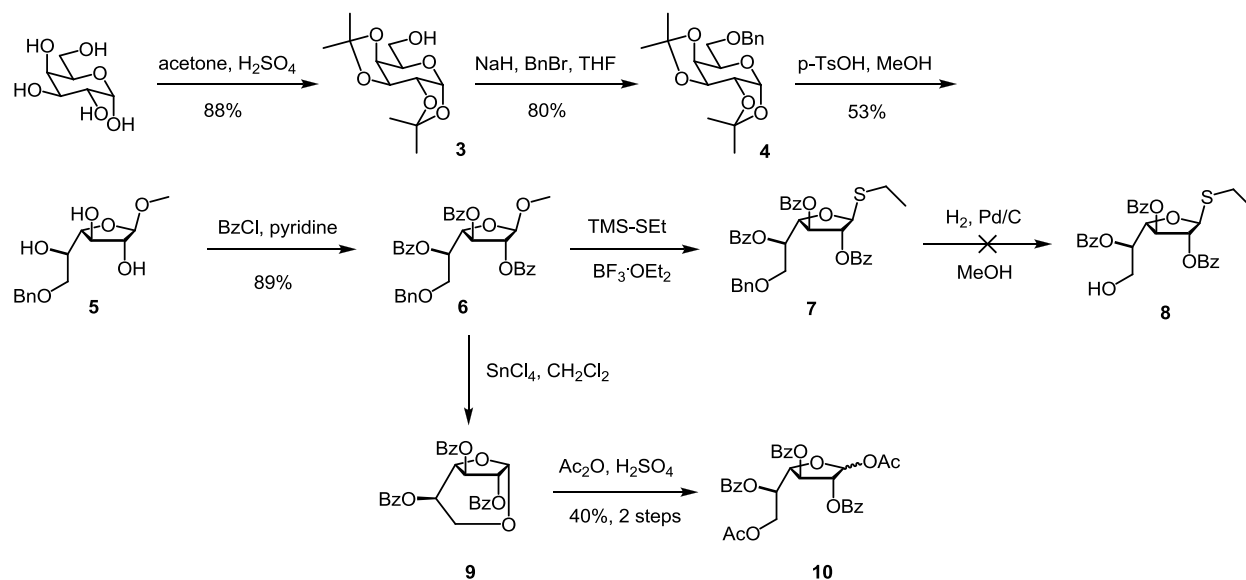


Figure 2. GlfT2 diGalf acceptor substrates.

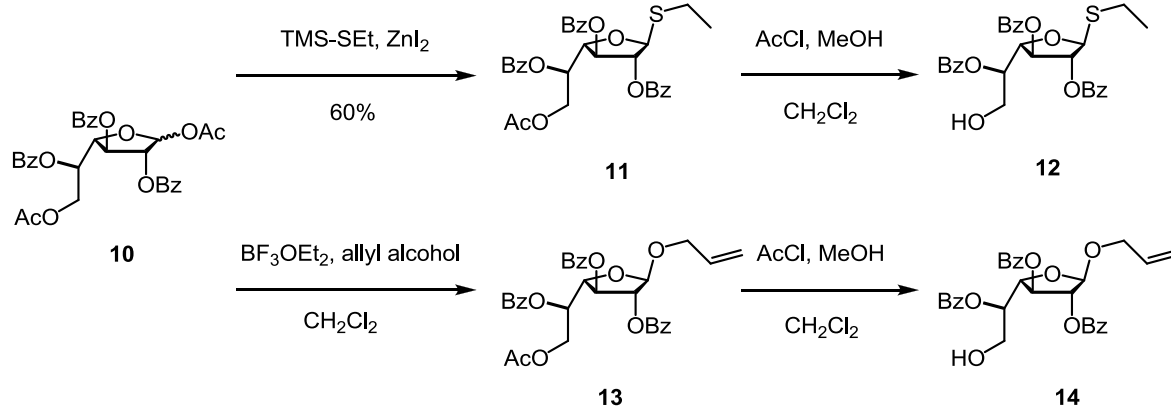
The previous route to disaccharide compounds such as **1** does not provide intermediates that can be elaborated into tri- and tetrasaccharides. Therefore, we designed thioglycoside monomers that can be orthogonally activated. For instance, ethyl thioglycosides have been shown to have orthogonal reactivity to thiazolyl (STaz) and benzoxazolyl (SBox) thioglycosides.^{11,12} Successful completion of a synthetic route incorporating these features should allow access to analogues of acceptor **1**, such as **2 a**, **b**, and **c**, as well as longer oligosaccharides.

The most essential Galf building block requires the presence of a nucleophilic alcohol and an anomeric leaving group orthogonal to the anomeric leaving group on the donor monosaccharide, such as compound **8**. To generate monosaccharide **8**, known intermediate **6** can be assembled in four steps from D-galactose.^{13,14} The C1, C2, C3, and C4 oxygens of D-galactose were protected as acetonides via treatment with acetone and sulfuric acid to produce compound **3**. The primary hydroxyl group at C6 was benzylated to afford fully protected galactopyranose **4**. Deprotection using p-toluenesulfonic acid (TsOH) and methanol was accompanied by attendant isomerization to furanose **5**. Benzoyl esters were generated from the free hydroxyl groups to give compound **6**. Importantly, compound **6** has a differentially protected hydroxyl at C6. Following conversion of the anomeric methoxy ether of **6** to a thioethyl moiety (compound **7**), we planned to remove the benzyl ether by standard hydrogenation conditions to produce the thioglycoside **8** (**Scheme 1**).¹⁵ However, the sulfur in the thioethyl was found to poison the palladium catalyst.



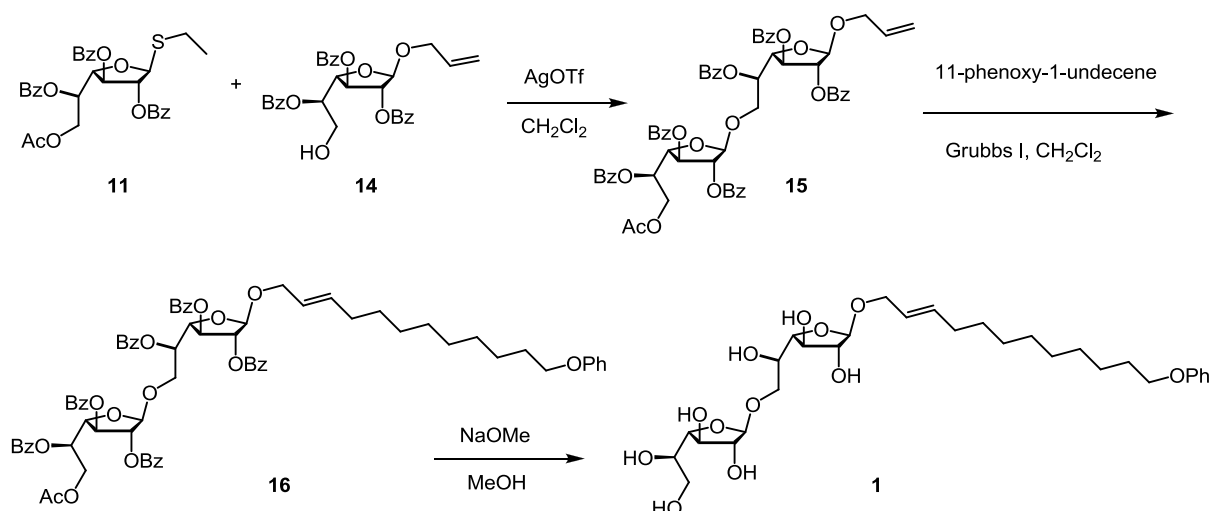
Scheme 1. Synthesis of key Galf intermediates **8** and **10**.

Our alternative approach took advantage of previously synthesized compound **6**. From **6**, a second ring contraction was performed with tin(IV) chloride in dichloromethane to produce bicyclic compound **9**. Intermediate **10** was formed via acetolysis of **9** with acetic anhydride and sulfuric acid (**Scheme 1**). Compound **10** can yield several different acceptor compounds with a differentially protected hydroxyl at C6, such as thioglycoside **11**, which is produced by reacting **10** in the presence of (ethylthio)trimethylsilane (TMS-SEt) and zinc iodide, or compound **13** which is formed through an allylation of the anomeric position (**Scheme 2**).



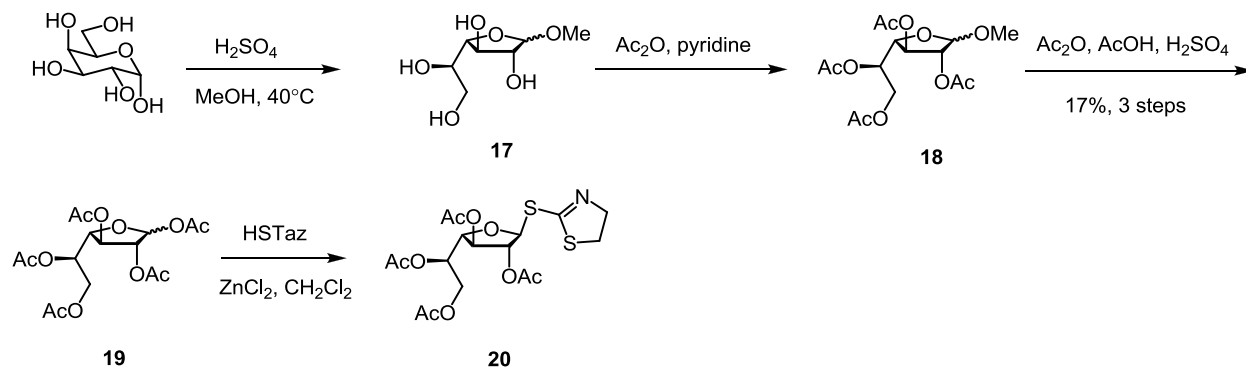
Scheme 2. Synthesis of orthogonally protected Galf monosaccharides. Thioglycoside **11** is capable of acting as either a donor or acceptor.

Monosaccharides **12** and **14** are capable of acting as glycosylation acceptors, and thus they can be used to assemble Galf oligosaccharides. To accomplish this, the primary acetate protecting group of compound **11** and **13** can be selectively removed to afford compounds **12** and **14**, respectively, without removal of the benzoyl protection or the thiol anomeric groups. To assemble β -(1,6)-linked diGalf acceptors, monosaccharide acceptor **14** can be activated in the presence of thioglycoside **11** to assemble disaccharide **15** (**Scheme 3**). Several acidic thiophilic reagents have been shown to be capable of thioglycoside activation.¹⁶ For ethyl thioglycosides, we chose to use *N*-iodosuccinimide and silver triflate. Olefin cross-metathesis of lipids and disaccharide **15**, followed by removal of the ester protecting groups with sodium methoxide can provide a collection of acceptor substrates for study of GlfT2. For instance, disaccharide **15** reacted with 11-phenoxy-1-undecene in the presence of Grubbs I, followed by deprotection to yield **1** (**Scheme 3**).^{9,10} Analogues **2 a-c** can be produced by varying the alkenyl lipid used in the olefin cross-metathesis step.



Scheme 3. Synthesis of diGalf acceptor **1** via ruthenium-catalyzed olefin cross-metathesis.

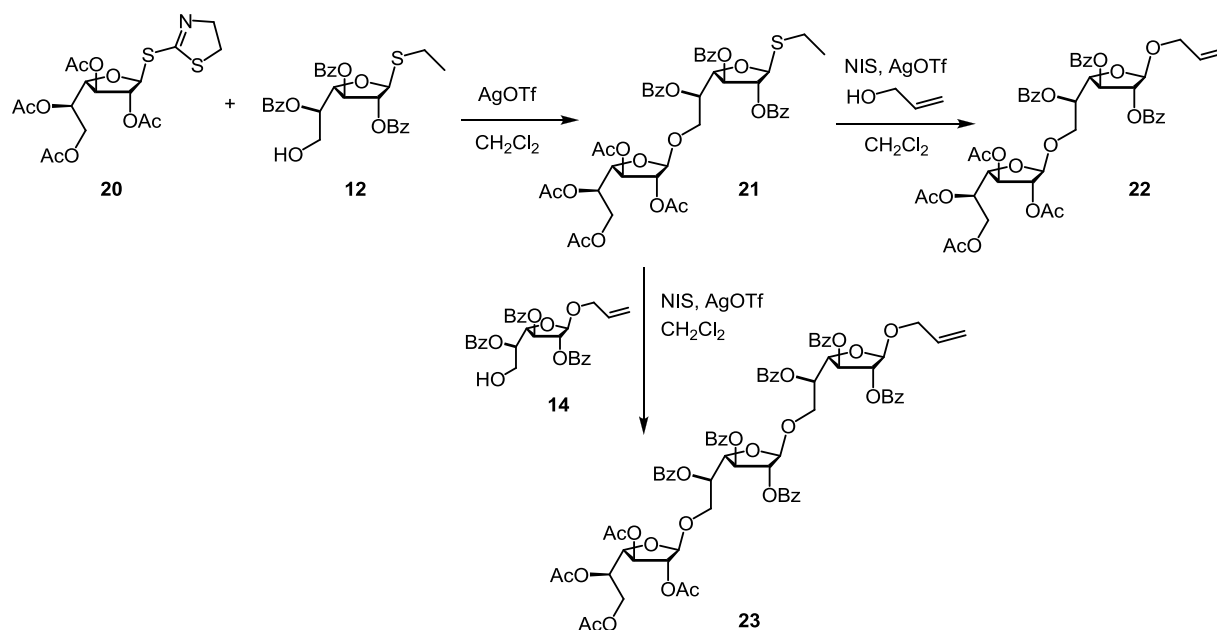
A monosaccharide Galf donor, compound **20**, was also prepared from D-galactose. D-Galactose was converted to per-acetylated furanose **19** over a series of three reactions spanning five days.¹⁷ Compound **19** could be converted into thioglycoside **20** with 2-thiazoline-2-thiol and zinc chloride (**Scheme 4**).¹¹



Scheme 4. Synthesis of Galf donor **20**.

Future Directions

Thioglycoside **11** is an attractive intermediate because it has the unique ability to act as both donor and acceptor according to synthetic requirements. Specifically, as mentioned above, prior to acetate deprotection, thioglycoside **11** can be activated with *N*-iodosuccinimide and silver triflate in the presence of an appropriate acceptor (e.g., allyl glycoside **14**) to generate a new glycosidic linkage (i.e. diGal β **15**). Alternatively, the primary acetate can be removed from **11** to produce **12**. Peracylated monosaccharide **20** can be activated with stoichiometric silver triflate in the presence of thioglycoside acceptor **12**. Under these conditions, the thioethyl functionality of compound **12** remains unactivated, allowing efficient glycosylation to form diGal β **21** (**Scheme 5**). This disaccharide can act as a donor in glycosylation reactions with lipid alcohols (e.g., allyl alcohol to yield compound **22**) or compounds such as allyl monosaccharide **14** (**Scheme 5**). Ruthenium-catalyzed olefin cross metathesis of trisaccharide **23** will generate trisaccharide glycolipids with non-natural non-alternating β -(1,6) glycosidic linkages. This trisaccharide could in turn be used to study the effects of regiochemistry on the activity of the enzyme GlfT2. Similar chemistry could be used to create non-alternating β -(1,5) glycosidic linkages (data not shown) or a tetrasaccharide. My work provides efficient access to a variety of Gal β oligomers including di-, tri-, and even tetrasaccharides.



Scheme 5. Synthesis of diGalf acceptor **22** and proposed synthesis of the β -(1,6) linked trisaccharide **23**.

Conclusion

This work has provided key intermediates (i.e. **11**, **12**, **14**, and **20**) that are important for the synthesis of Galf disaccharide glycolipids, which are invaluable in studying the mechanism behind galactan synthesis. Such compounds are also intermediates that can be used in the creation of other oligosaccharides which can also be used as probes for studying galactan formation, such as tri- and tetrasaccharides. The future synthesis of tetrasaccharide probes will be particularly useful for studying regiospecificity in the biosynthesis of the galactan. Increased knowledge of how the galactan is synthesized in the cell wall of *Mycobacterium tuberculosis*, specifically looking at the role that the enzyme GlfT2 plays in the process may lead to many potential drug targets.

Materials and Methods

For the thesis work discussed, I have synthesized compounds **3-7**, **9-11**, and **17-19**. These compounds were previously synthesized in the Kiessling group. Synthetic procedures and

characterization can be found in reference 10 (compounds **1**, **2a**, **2b**, **2c**, **13**, **14**, **22**), reference 11 (compounds **3-6**) and reference 12 (compounds **9**, **10**). In addition to my efforts, compounds **7**, **11**, **12**, **15**, **16**, **20**, and **21** were synthesized by Rebecca A. Splain of the Kiessling research group (unpublished results, manuscript pending).

References

- (1) Pan, F. et al. Cell wall core galactofuran synthesis is essential for growth of mycobacteria. *J. Bacteriol.* **2001**, *183*, 3991-3998.
- (2) Remond, C. et al. An original chemoenzymatic route for the synthesis of β -D galactofuranosides using an α -L-arabinofuranosidase. *Carbohydr. Res.* **2005**, *340*, 637-644.
- (3) Mikusova, K. et al. Biosynthesis of the galactan component of the mycobacterial cell wall. *J. Biol. Chem.* **2000**, *275*, 33890-33897.
- (4) In *Fact Sheet No. 104 Tuberculosis*; World Health Organization: Geneva, **2007**.
- (5) Belanova, M.; Dianiskova, P.; Brennan, P. J.; Completo, G. C.; Rose, N. L.; Lowary, T. L.; Mikusova, K. Galactosyl transferases in mycobacterial cell wall synthesis. *J. Bacteriol.* **2008**, *190*, 1141-1145.
- (6) Mikusova, K.; Belanova, M.; Kordulakova, J.; Honda, K.; McNeil, M. R.; Mahapatra, S.; Crick, D. C.; Brennan, P. J. Identification of a novel galactosyl transferase involved in biosynthesis of the mycobacterial cell wall. *J. Bacteriol.* **2006**, *188*, 6592-6598.
- (7) Kremer, L.; Dover, L. G.; Morehouse, C.; Hitchin, P.; Everett, M.; Morris, H. R.; Dell, A.; Brennan, P. J.; McNeil, M. R.; Flaherty, C.; Duncan, K.; Besra, G. S. Galactan Biosynthesis in *Mycobacterium tuberculosis*: identification of a bifunctional UDP-galactofuranosyltransferase. *J. Biol. Chem.* **2001**, *276*, 26430-26440.
- (8) Rose, N. L.; Completo, G. C.; Lin, S. J.; McNeil, M.; Palcic, M. M.; Lowary, T. L. Expression, purification, and characterization of a galactofuranosyltransferase involved in *Mycobacterium tuberculosis* arabinogalactan biosynthesis *J. Am. Chem. Soc.* **2006**, *128*, 6721-6729.
- (9) Splain R. A.; Kiessling L. L. Synthesis of galactofuranose-based acceptor substrates for the study of the carbohydrate polymerase GlfT2. *Bioorg. Med Chem.* **2010**, *18*, 3753-3759.
- (10) May, J. F. et al. A tethering mechanism for length control in a processive carbohydrate polymerization. *Proc. Nat. Acad. Sci.* **2009**, *106*, 11851-11856.
- (11) Demckenko, A. V. et al. Potent, versatile, and stable: Thiazolyl thioglycosides as glycosyl donors. *Angew. Chem. Int. Ed.* **2004**, *43*, 3069-3072.
- (12) Kaeothip, S. et al. Unexpected orthogonality of S-benzoxazolyl and S-thiazolinyl glycosides: application to expeditious oligosaccharide assembly. *Org. Lett.* **2009**, *11*, 799-802.
- (13) Choudhury, A. K. and Roy, N. Synthesis of some galactofuranosyl disaccharides using a galactofuranosyl trichloroacetimidate as donor. *Carbohydr. Res.* **1998**, *308*, 207-211.
- (14) Sarkar, S. K. et al. An efficient method for the synthesis of a 1,6-anhydro-D-galactofuranose derivative and its application in the synthesis of oligosaccharides. *J. Carbohydr. Chem.* **1999**, *18*, 1121-1130.
- (15) Completo, G. C. and Lowary, T. L. Synthesis of galactofuranose-containing acceptor substrates for mycobacterial galactofuranosyltransferases. *J. Org. Chem.* **2008**, *73*, 4513-4525.
- (16) Pornsuriyasak, P. et al. S-Thiazolinyl (STaz) glycosides as versatile building blocks for convergent selective, chemoselective, and orthogonal oligosaccharide synthesis. *Chem. Eur. J.* **2006**, *12*, 6630-6646.
- (17) Chittenden, G.J.F. Synthesis of β -D-galactofuranose 1-phosphate. *Carbohydr. Res.* **1972**, *25*, 35-41.