JOC The Journal of Organic Chemistry

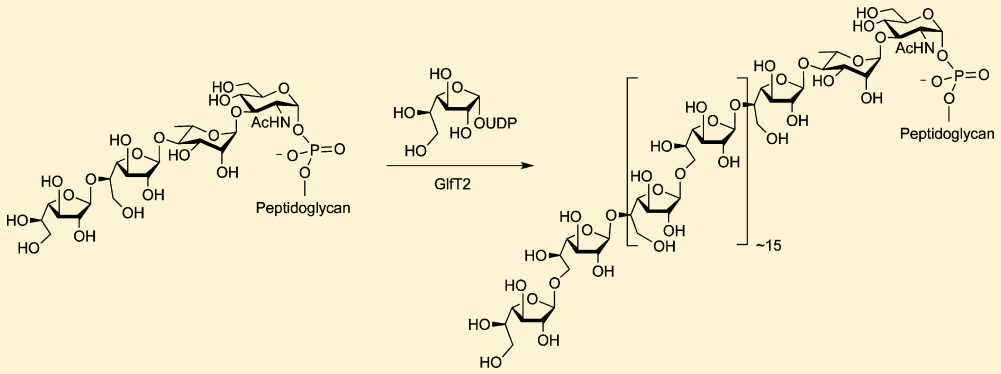
JOCSynopsis

pubs.acs.org/joc

Chemical Insight into the Mechanism and Specificity of GlfT2, a Bifunctional Galactofuranosyltransferase from Mycobacteria

Myles B. Poulin and Todd L. Lowary*

Alberta Glycomics Centre and Department of Chemistry, University of Alberta, 11227 Saskatchewan Drive, Edmonton, Alberta T6G 2G2, Canada



ABSTRACT: Mycobacteria, including the human pathogen *Mycobacterium tuberculosis*, produce a complex cell wall structure that is essential to survival. A key component of this structure is a glycoconjugate, the mycolyl−arabinogalactan−peptidoglycan complex, which has at its core a galactan domain composed of galactofuranose (Gal*f*) residues linked to peptidoglycan. Because galactan biosynthesis is essential for mycobacterial viability, compounds that interfere with this process are potential therapeutic agents for treating mycobacterial diseases, including tuberculosis. Galactan biosynthesis in mycobacteria involves two glycosyltransferases, GlfT1 and GlfT2, which have been the subject of increasing interest in recent years. This Synopsis summarizes efforts to characterize the mechanism and specificity of GlfT2, which is responsible for introducing the majority of the Gal*f* residues into mycobacterial galactan.

D-Galactofuranose (Gal*f*), the five-membered ring form of D-galactose, is found in glycoconjugates of pathogenic and nonpathogenic micro-organisms but not in mammalian glycans.[1−5] In the organisms in which they are found, the ability to make Gal*f*-containing glycoconjugates is often associated with virulence[6,7] or viability.[8,9] Consequently, the biosynthesis of Gal*f*-containing glycans has attracted attention as a target for antimicrobial development.[1,5,10]

Although a number of micro-organisms incorporate Gal*f* into their glycoconjugates, particularly notable examples are mycobacteria, including the human pathogens *Mycobacterium tuberculosis* and *M. leprae*, the causative agents of tuberculosis and leprosy, respectively. These organisms produce an essential Gal*f*-containing glycoconjugate, the mycolyl−arabinogalactan−peptidoglycan (mAGP) complex, as the main structural component of their cell wall.[11,12] The galactan domain of the mAGP is a homopolymer of 30−40 Gal*f* residues connected through alternating β-(1→5) and β-(1→6) glycosidic linkages (Figure 1). This structure is attached through an α-L-rhamnopyranosyl-(1→3)-α-D-*N*-acetylglucosamine-1-phosphate disaccharide to the peptidoglycan. Mycolated arabinan domains, consisting of ∼30 D-arabinofuranose residues each, are attached to the 8th, 10th, and 12th Gal*f* residues of the galactan.

The biosynthesis of the mAGP (Figure 2) involves formation of an α-Rha*p*-(1→4)-β-Glc*p*NAc-polyprenyl-P-P-disaccharide[13] via the sequential action of the GlcNAc-1-phosphate transferase WecA[14] and the rhamnosyltransferase WbbL.[15] Two bifunctional galactofuranosyltransferases, GlfT1 and GlfT2, then add the Gal*f* residues, using UDP-Gal*f* as the donor.[16−18] The UDP-Gal*f* is produced from UDP-Gal*p* by the action of UDP-galactopyranose mutase (UGM).[1] In the latter stages of the biosynthesis, the lipid-bound galactan is further functionalized with Ara*f* residues before its ligation to peptidoglycan and the addition of mycolic acids.[19]

Incorporation of Gal*f* into the mAGP is essential for mycobacterial viability.[8,9] There is consequently interest in developing chemical tools to explore the mechanism and specificity of the enzymes involved in galactan assembly as a prerequisite to inhibitor design. A number of approaches that make use of synthetic substrate analogues, together with biophysical and analytical methods (e.g., saturation transfer difference NMR spectroscopy, X-ray crystallography, and mass spectrometry), have been reported. This Synopsis will highlight these approaches as applied to GlfT2 (EC 2.4.1.288) with a focus on determining the substrate selectivity, mechanism,

Received: June 22, 2016
Published: August 24, 2016

DOI: 10.1021/acs.joc.6b01501
J. Org. Chem. 2016, 81, 8123−8130

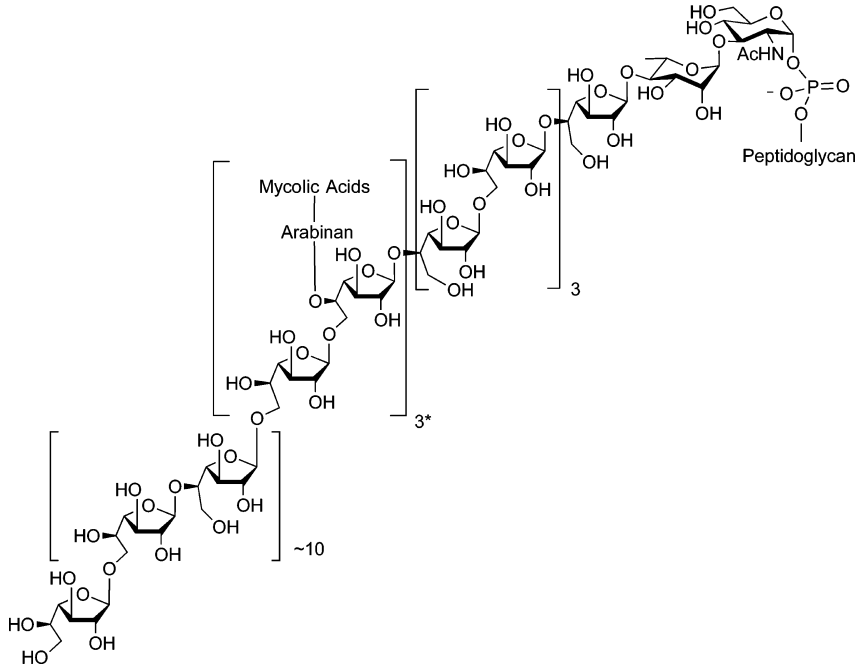


Figure 1. Structure of the mycolyl−arabinogalactan complex, highlighting the galactan domain. Only two of the three of the arabinan termini are capped with mycolic acid residues.

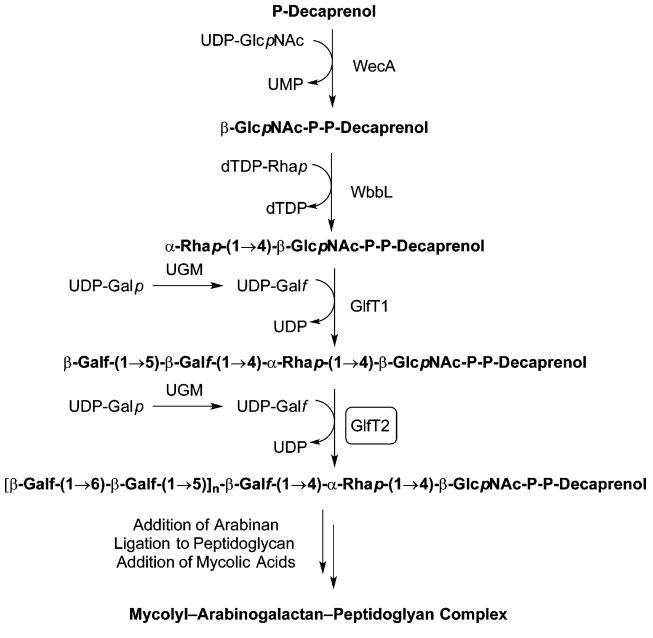


Figure 2. Proposed biosynthesis of the mAGP complex. GlfT2, is highlighted in the box; n = 30−40.

substrate-binding interactions, and regiospecificity of the enzyme.

Galf-Oligosaccharides as Probes of GlfT2 Substrate Selectivity. Galactofuranosyltransferase (GlfT) activity[20] was first observed in *M. smegmatis* by Brennan and co-workers in 2000.[21] UDP-[U−^{14}C]-Gal*p* was used to observe the incorporation of galactose into endogenous lipid-linked galactan precursors by cell membrane and cell wall preparations. [^{14}C]Gal was only incorporated when membrane fractions were supplemented with exogenous UDP-galactopyranose mutase, which produces UDP-Gal*f* from UDP-Gal*p* (Figure 2). These studies demonstrated that UDP-Gal*f* is the precursor of the Gal*f* residues in the galactan. Analysis of the *M. tuberculosis* H37Rv genome led to the identification of the first putative GlfT gene, *Rv3880c*, which was found immediately downstream from the gene encoding for UGM. The protein encoded by *Rv3880c* was later named GlfT2.[17] In the initial study, GlfT2 was overexpressed in *M. smegmatis* and *Escherichia coli* to produce membrane preparations able to incorporate [^{14}C]Gal*f* into endogenous galactan precursors.[21] Subsequent investigations using an in vitro assay with cell membrane preparations showed that GlfT2 recognized disaccharide substrates.[18] The assay was also used to screen inhibitors and acceptors for the enzyme,[22,23] and one of the outcomes of these studies was the demonstration that GlfT2 catalyzes the formation of both β-(1→5) and β-(1→6) glycosidic bonds. The enzyme is hence a bifunctional glycosyltransferase.

In 2006, we reported[24] the first expression and purification of soluble recombinant GlfT2 in *E. coli*, which facilitated exploring the substrate specificity of the enzyme using synthetic galactan fragments as acceptors (Figure 3).[25] Disaccharides **1** and **2** were found to be the minimum structural motifs required for recognition by GlfT2, but higher activity was seen for trisaccharides **3** and **4**. Acceptors in which the nonreducing terminal Gal*f* residues of **1** and **2** were replaced by L-Ara*f* (**5** and **6**) showed little to no detectible activity, indicating that the exocyclic diol is an important recognition element for the enzyme, which was later confirmed by other investigations (see below). In addition, substrates containing only the linker disaccharide substrate (i.e., **7**−**9**) were not GlfT2 substrates. However, **8** and **9** were later shown to be recognized by the other galactosyltransferase involved in galactan biosynthesis, GlfT1.[26] These results were confirmed by experiments using lipid-linked glycans isolated from mycobacteria.[17]

Taken together, these results suggested the biosynthetic model shown in Figure 2: GlfT1 adds two Gal*f* residues to the linker disaccharide, producing the minimum structural motif required by GlfT2, which adds the third and subsequent residues. When we isolated the products from reactions of

DOI: 10.1021/acs.joc.6b01501
J. Org. Chem. 2016, 81, 8123−8130

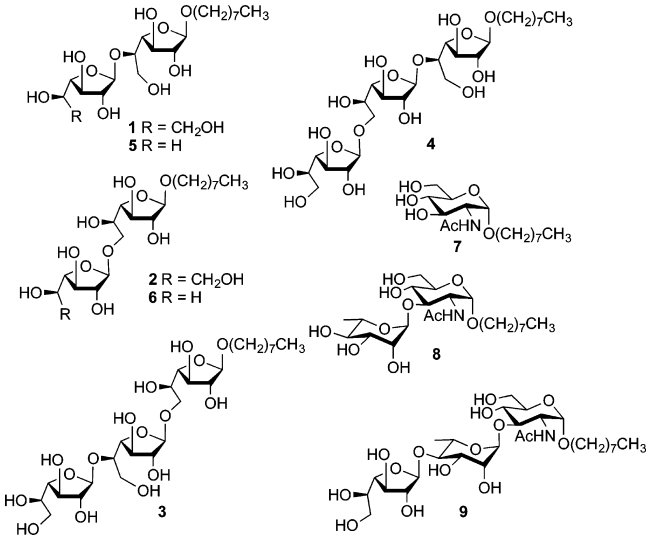


Figure 3. Mycobacterial galactan analogues tested as acceptors of GlfT2.

GlfT2 with trisaccharide acceptors, we found the products exclusively consisted of alternating β-(1→5)- and β-(1→6)-linked residues.[24] Subsequent work by Kiessling and co-workers demonstrated that the identity of the aglycone influenced substrate activity and that disaccharides possessing longer, more lipophilic groups were better substrates than **1** and **2**.[27]

Initial studies[21,24] on GlfT2 employed UDP-Gal*f* generated in situ from UDP-Gal*p* by UGM, which highly favors (~9:1) the pyranose ring form. In situ generation of the donor complicated carrying out kinetic measurements of GlfT2. Thus, we sought to use UDP-Gal*f* directly as the donor species. UDP-Gal*f* has been chemically synthesized using a number of methods.[28−31] However, we have found it more practical to prepare the substantial quantities of UDP-Gal*f* needed for carrying out large numbers of kinetic assays using the chemoenzymatic method developed by Field and co-workers.[32] This approach uses a promiscuous galactose-1-phosphate uridyltransferase (GalPUT)[33,34] from *E. coli* to couple Gal*f*-1-phosphate and UMP derived from UDP-glucose.[35] Using this method, we have prepared UDP-Gal*f* on a 100 mg scale, thus facilitating the development of a spectrophotometeric assay to measure GlfT2 activity,[36] which can be run in 384-well plate format.

One Active Site or Two? GlfT2 is one of a growing number of glycosyltransferase (GT) enzymes that have been shown to catalyze the construction of more than one type of glycosidic linkage. In the case of GlfT2, the enzyme catalyzes the synthesis of both β-Gal*f*-(1→5)-β-Gal*f* and β-Gal*f*-(1→6)-β-Gal*f* linkages. There are two scenarios that could account for the formation of two different glycosidic linkages by a single GT. In one, GlfT2 could possess two active sites, each catalyzing a single reaction, with the growing chain moving between active sites following Gal*f* addition. This is the case for a number of bifunctional GTs including hyaluronan synthases.[37] Alternatively, the enzyme could possess a single active site that catalyzes both reactions, which has been observed in a bacterial polymerizing sialyltransferase.[38]

To differentiate these possibilities, we, in collaboration with Pinto and co-workers, used saturation transfer difference (STD) NMR spectroscopy to probe the ability of GlfT2 to bind to trisaccharides **3** and **4**, which mimic substrates for the β-(1→6)-Gal*f* and β-(1→5)-Gal*f* transferase activities, respectively.[24] Competitive titration experiments showed that **3** and **4** compete for binding to the same site in GlfT2, consistent with a single bifunctional active site model.[39] Further work showed that the enzyme binds to UDP-Gal*f* in a manner consistent with the recognition of GTs with sugar nucleotides; that is, the nucleotide portion is most strongly bound.[40]

Site-directed mutagenesis and structural studies by Kiessling subsequently offered further support for a single active site. The DXD amino acid motif is a conserved feature of nearly all members of the GT-A superfamily of GTs.[41,42] The primary sequence of GlfT2 contains multiple putative DXD motifs, but only one of these is flanked by four N-terminal hydrophobic amino acids,[18] a structural feature crucial for substrate recognition and turnover in other GTs.[41−43] Molecular modeling of the putative catalytic GT-2 domain of GlfT2 also revealed that only two of these motifs (a DDA motif consisting of D_{371}, D_{372}, and A_{373} and a DDD motif consisting of $D_{256–258}$) were conserved in related enzymes.[44] Subsequent crystallo-

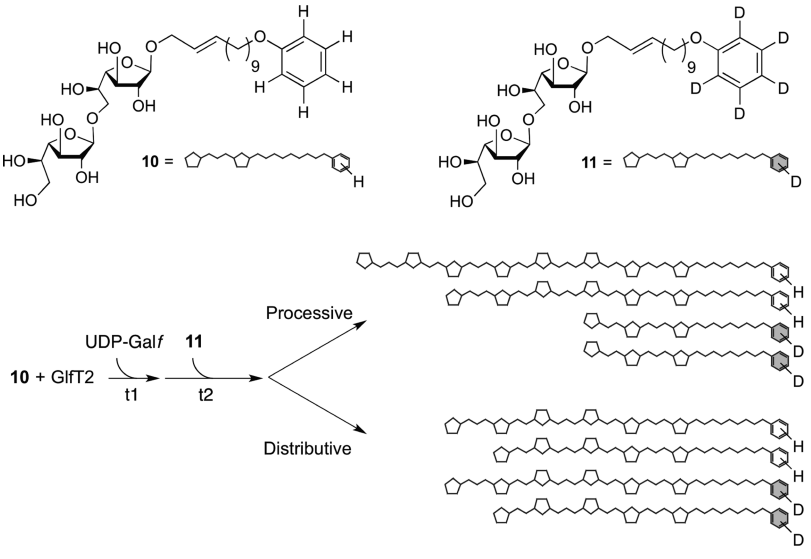


Figure 4. Use of isotopically labeled substrates **10** and **11** to probe the processive or distributive nature of GlfT2-catalyzed polymerization.

DOI: 10.1021/acs.joc.6b01501
J. Org. Chem. 2016, 81, 8123−8130

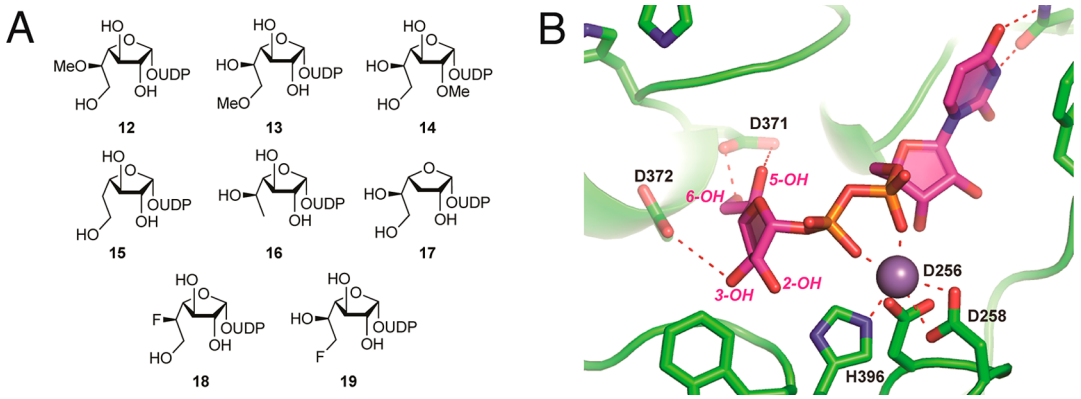


Figure 5. (A) Synthetic UDP-Gal*f* mimics evaluated as substrates for GlfT2. (B) Model of the donor-binding site of GlfT2 based upon an X-ray structure of the enzyme in complex with UDP. The UDP-Gal*f* has been modeled into the active site using the UDP as an anchor.

graphic studies of GlfT2 (see below) confirmed that a single GT-2 domain exists.[45] From the structure of the GT-2 domain, D372 serves as the predicted general base that deprotonates the acceptor nucleophile in the GT mechanism. A D372A mutation resulted in a loss of both β-(1→5) and β-(1→6) transferase activities.[44,45] Thus, all evidence indicates that GlfT2 catalyzes the formation of two distinct glycosidic linkages via a single bifunctional active site.

Isotopically Labeled Galactan Analogues as Probes of Polymerase Activity. GlfT2 adds multiple β-Gal*f* residues to the growing galactan through alternating β-(1→5) and β-(1→6) glycosidic linkages. Polymerization could arise through either a distributive mechanism, where the galactan acceptor is released between subsequent Gal*f* transfers, or a processive mechanism, where the galactan remains bound through multiple Gal*f* additions. To gain insight into this process for GlfT2, Kiessling and co-workers carried out a series of pulse–chase experiments, making use of two isotopically labeled synthetic disaccharides (Figure 4).[46] The light, hydrogen-containing isotopologue (**10**) was incubated with GlfT2 and UDP-Gal*f* followed by introduction of the heavy, deuterium-containing isotopologue (**11**), after an incubation interval t_1. Following an additional incubation period (t_2), the products were observed by MALDI-TOF MS, which allowed direct quantification of the isotope distribution in products of differing length. The product distribution would be alike for both substrates for a distributive mechanism or favor the light isotope in longer products for a processive mechanism. In these experiments, an enrichment of the light isotopologue in longer polymerized products was observed. From this observation, it was proposed that the enzyme proceeds by a processive mechanism where the initial isotopologue remains bound to the enzyme.

This experiment is an elegant use of isotope labeling to probe an enzymatic mechanism. However, a concern is that the time interval between the pulse and chase (30 s) and the enzyme concentration (0.25 μM) provide conditions that could allow the heavy disaccharide (**11**) to be converted to longer products. It has been previously noted that rate of GlfT2-catalyzed glycosylation is at least 2–3-fold faster with trisaccharide acceptor substrates compared to disaccharides.[24] If similar increases in rate are observed with even longer oligosaccharides, the observed isotope distribution could also arise from a distributive mechanism. In this case, as the product becomes longer, it would be preferentially used by the enzyme. A corollary of this is that shorter pulse times would lead to smaller differences in rates. In fact, when a shorter pulse length was used, no difference in the isotopologue distribution was observed.[46] Thus, a more detailed analysis of the GlfT2 reaction with acceptor substrates of different lengths should be carried out to more strongly establish the processivity of the enzyme.

UDP-Gal*f* Analogues as Probes of GlfT2 Substrate Selectivity. Carbohydrate derivatives where individual hydroxyl groups have been replaced by a hydrogen (deoxy), fluorine (fluoro), or a methoxy (OMe) group have been widely used to probe hydrogen-bonding contacts and steric constraints in carbohydrate–protein recognition.[47−50] Using a panel of singly modified deoxy and OMe UDP-Gal*f* derivatives (Figure 5A), we surveyed GlfT2–donor substrate binding.[51] The relative enzymatic activity with deoxy analogues report the importance of hydrogen-bonding interactions between the substrate and the protein, whereas the OMe analogues introduce additional bulk and thus provide insight into steric constraints in the enzyme active site.

When tested, none of the OMe UDP-Gal*f* analogues (**12**–**14**) functioned as effective donor substrates for GlfT2, pointing to a sterically constrained donor-binding site.[51] More differences in activity were seen with the deoxygenated analogues. Both the C-5′ and C-6′ deoxy analogues (**15** and **16**) served as moderate substrates for GlfT2, indicating that these hydroxyl groups are not involved in hydrogen-bonding interactions essential to catalysis. On the other hand, the 3′-deoxy UDP-Gal*f* analogue **17** showed very low levels of turnover, suggesting that the C-3′ hydroxyl group is essential for catalysis. Compound **17** did, however, function as a moderate GlfT2 inhibitor with an affinity comparable to that of UDP-Gal*f* (K_i for **17** = 120 ± 20 μM and K_M for UDP-Gal*f* = 250 ± 40 μM). These observations are consistent with STD-NMR studies looking at both acceptor and donor substrate binding.[39,40] In a related investigation, Kiessling and co-workers investigated GlfT2 activity with 5′- and 6′-fluoro UDP-Gal*f* derivatives (**18** and **19**).[52] As was the case for the deoxy analogues **15** and **16**, both of these fluorinated analogues were readily transferred onto a synthetic galactan acceptor. The investigations with **15**, **16**, **18**, and **19** with the purified enzyme and synthetic substrates mirrored earlier work using a cell-free assay system.[53] In that work, the modified Gal*f* residue present in those sugar nucleotides was shown to be effectively incorporated into endogenous acceptors, leading to "dead-end" products. The result was inhibition of galactan polymerization.

DOI: 10.1021/acs.joc.6b01501
J. Org. Chem. 2016, 81, 8123–8130

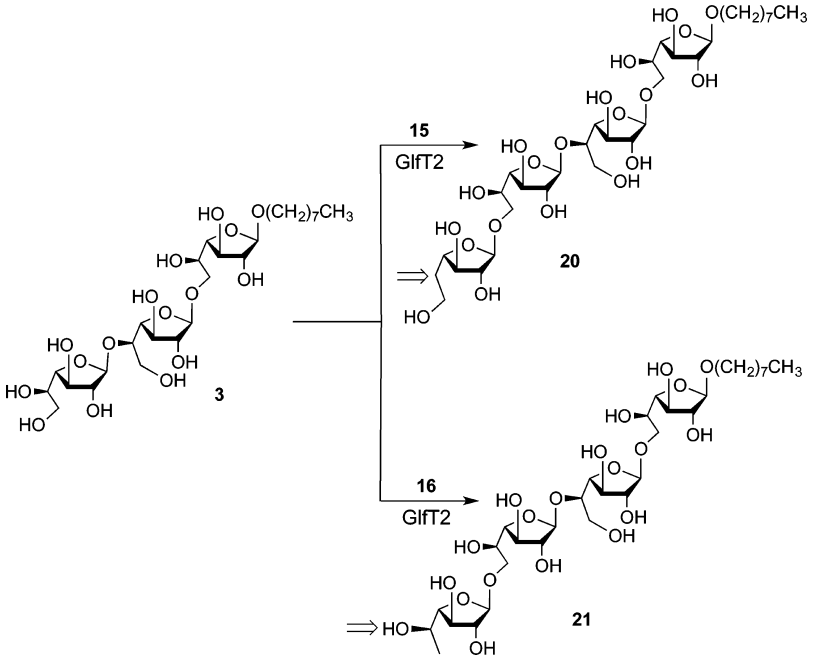


Figure 6. Tetrasaccharide products produced from the GlfT2-catalyzed transfer of the carbohydrate residue of **15** or **16** onto trisaccharide **3**. The site of the next Gal*f* addition (in a molecule possessing a full complement of hydroxyl groups) is indicated by the double-barreled arrow.

Further insight into the donor-binding pocket was provided by the X-ray crystal structure of the enzyme. Although it has not been possible to obtain a structure of GlfT2 cocrystallized with either a donor or acceptor substrate, the structure with UDP has been solved.[45] Using this structure, it was possible to model in the Gal*f* portion of the donor (Figure 5B). This model was validated through the generation of site-directed mutants, which supported the results obtained with the UDP-Gal*f* analogues.[51]

Probing the Alternating Specificity of GlfT2 Using UDP-Gal*f* Analogues. UDP-Gal*f* analogues have also been employed to probe the mechanism that controls the regioselectivity of GlfT2.[51,52] Native galactan consists of alternating β-Gal*f*-(1→5)-β-Gal*f*-(1→6) residues; however, it is not clear what mediates this alternating specificity. To gain insight into this process, we explored the effect of deoxy UDP-Gal*f* analogues **15** and **16** on polymerization. Such analogues are incorporated into the growing galactan, leading to products having terminal Gal*f* residues lacking a C-5 or C-6 hydroxyl group, respectively (**20** and **21**, Figure 6).[51] Deoxygenation at the site of the subsequent glycosylation reaction could give two outcomes, depending on the fidelity of the enzyme. If GlfT2 has high fidelity for alternating linkages, further chain elongation would be prevented. On the other hand, if the enzyme has low fidelity, it is possible that additional residues could be added to the adjacent hydroxyl group (e.g., the C-6 hydroxyl group in a substrate lacking a C-5 hydroxyl group).

Using trisaccharide **3** as the acceptor and **15** as the donor, only a tetrasaccharide product was observed, resulting from the addition of a single β-(1→6)-linked 5-deoxy residue (**20**). Assuming alternating specificity, the next glycosylation would not be possible as **20** lacks the appropriate hydroxyl group. Indeed, no products resulting from the introduction of subsequent "incorrect" β-(1→6)-linkage were observed. An identical result was observed when **16** was used as a donor substrate—only tetrasaccharide **21** was produced. However, in this case, the product still had an available hydroxyl group at C-5, which could undergo glycosylation. Yet, no further polymerization was observed. Identical results were also obtained when trisaccharide **4** was used as the acceptor substrate: only tetrasaccharide products were formed. Thus, it appears that both hydroxyl groups in the exocyclic diol of the terminal Gal*f* residue in the acceptor are required for subsequent GlfT2 activity, regardless the glycosidic linkage to be formed. Modeling of both **3** and **4** in the crystal structure of GlfT2 shows the nonreacting hydroxyl group of this exocyclic diol moiety interacting with H_{296}, E_{300}, and Y_{344} residues of GlfT2 (Figure 7).[45] These interactions could serve to anchor the reacting hydroxyl group in the correct orientation to react with UDP-Gal*f*. Products lacking this interaction would not adopt the correct geometry and, thus, not serve as substrates.

Similar studies have been carried out using fluorinated UDP-Gal*f* analogues **18** and **19** with comparable although slightly different results.[52] When **18** or **19** is used as the donor substrate, up to two additional fluorinated residues were incorporated, as long as the hydroxyl group required for the alternating reaction was present. This is likely due to the electronegative fluorine atom mimicking the hydrogen bond acceptor properties of the hydroxyl group.

Thus, the studies with the deoxy analogues **15** and **16**, as well as the fluoro analogues **18** and **19**, show that GlfT2 has high fidelity, introducing Gal*f* residues only with alternating regioselectivity. Moreover, it appears that anchoring of the nonreacting terminal hydroxyl group serves to orient the substrate.[51,52] However, it is not clear if disrupting these interactions (by mutating H_{296}, E_{300}, or Y_{344}) would result in a loss of alternating regioselectivity as these mutants have, to date, not been reported. It should be noted that a very recent report by Kiessling and co-workers on the GlfT2-catalyzed extension of unnatural trisaccharides (i.e., β-Gal*f*-(1→6)-β-Gal*f*-(1→6)-β-Gal*f* and β-Gal*f*-(1→5)-β-Gal*f*-(1→5)-β-Gal*f*) is consistent with the role of the terminal exocyclic diol in controlling regioselectivity.[20]

DOI: 10.1021/acs.joc.6b01501
J. Org. Chem. 2016, 81, 8123−8130

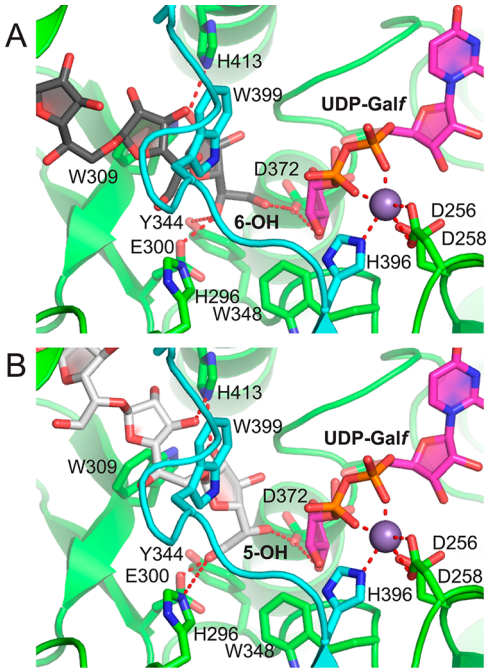


Figure 7. Model of the GlfT2 active site with UDP-Gal*f* and acceptor trisaccharide **3** (A) and trisaccharide **4** (B). The key interactions between the protein and the nonreacting hydroxyl group in the terminal exoxylic diol are highlighted.

Insights into Length Control by GlfT2. An important issue to understand for all carbohydrate polymerases is the mechanism by which chain length is controlled. Polysaccharide biosynthesis, unlike protein synthesis, is template-independent and thus control of chain length must be achieved in ways other than templated assembly.[54] Mycobacterial galactan biosynthesis, like that of many polysaccharides, is believed to occur at the plasma membrane, on polyprenol-bound substrates embedded in the lipid bilayer.[11,55] Direct evidence for the interaction of the GlfT2 with the membrane has not been obtained; however, early work[18] on galactan biosynthesis used membrane preparations as the enzyme source supporting the notion that GlfT2 is membrane associated in vivo. Any model for galactan chain length control should take into consideration the cellular location of this process.

In 2009, Kiessling and co-workers showed that GlfT2 can produce full-length galactan polymers, containing 30−35 Gal*f* residues, from a series of Gal*f*-(1→6)-Gal*f* oligosaccharide acceptors (**22**−**26**, Figure 8).[27] It was observed that the identity of the aglycone influenced the size of galactan produced, which led to the conclusion that there was a lipid-binding site on the enzyme, distal to the active site, that dictates chain length. It was proposed that when the chain reaches an appropriate length, the substrate dissociates from the enzyme, hence preventing further addition of carbohydrate residues. This is an intriguing hypothesis; however, it requires that the enzyme can initially extract the polyprenol-linked substrate from the lipid bilayer and then release it following chain extension. Although the enzyme may be able to use both soluble and membrane-associated substrates, the latter is more likely to be the biologically relevant context.

The crystal structure of the GlfT2, reported in 2012, reveals no lipid-binding site distal to the active site.[45] The structure does, however, provide additional clues as to how the protein might control chain length. GlfT2 exists as a homotetramer in

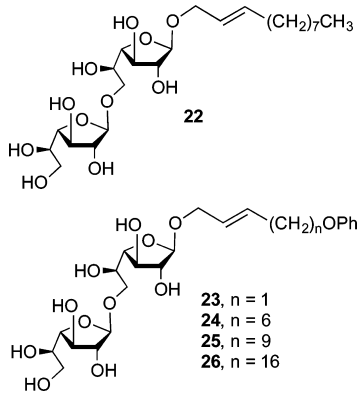


Figure 8. Disaccharides **22**−**26** evaluated as acceptor substrates for GlfT2 by Kiessling and co-workers.[27]

both the X-ray structure[45] and in solution,[24] with the active site of each monomer facing inward toward a central cavity with a ∼60 000 Å^3 volume. The C-terminal face of the tetramer consists almost entirely of positively charged and hydrophobic amino acids (Figure 9A), suggesting that this surface associates

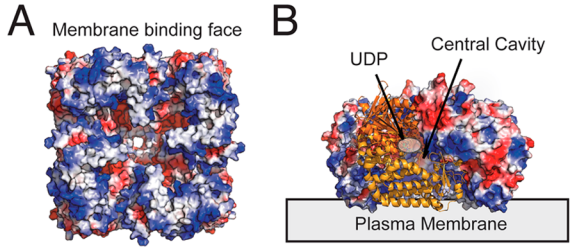


Figure 9. (A) C-terminal face of the GlfT2 tetramer showing the largely hydrophobic and positively charged surface and the central cavity where polymerization occurs. (B) Model of GlfT2 interacting with the plasma membrane; the UDP-binding site and central cavity are depicted.

with the plasma membrane. Based on the GlfT2 tetramer architecture, a membrane-embedded polyprenol-linked acceptor could be contained within the central cavity of the enzyme, with the chain elongation also occurring within that space. Given these structural features, another plausible hypothesis is that galactan length could be controlled, at least in part, by the volume of this cavity. That is, as the chain is elongated, the cavity fills and at some point steric congestion would prevent further polymerization. The size of the cavity appears to be large enough to harbor 120−150 Gal*f* residues, which is sufficient for four fully elongated galactan chains, if one assumes that all four tetramer active sites produce product at similar rates.[45]

Both hypotheses proposed above result largely from the observation that GlfT1 and GlfT2 working in consort with UGM can produce full-length galactan polymers in vitro. However, in vivo, there are likely other proteins required. For example, the addition of the arabinan to the galactan and the transfer of the resulting arabinogalactan to peptidoglycan are believed to occur in the periplasm.[11,55] Thus, the galactan must be transported through the cell membrane following its synthesis. An ABC transporter thought to function by translocating the galactan to the periplasm has been reported.[19] Two protein components of the transporter, encoded by the conserved *MSMEG_6366* and *MSMEG_6369* genes of *M.*

DOI: 10.1021/acs.joc.6b01501
J. Org. Chem. 2016, 81, 8123−8130

smegmatis mc^{2}155, respectively, are co-transcribed with the gene encoding for GlfT1, suggestive of a role in galactan biogenesis. As an alternative to the two hypotheses presented above, another possibility is that this transporter binds to full-length galactan as it is produced by GlfT2, in turn transferring it to the periplasm and effectively terminating chain extension. Further studies are required to establish a direct role for this transporter in galactan assembly and determine its function, if any, in galactan length control in vivo. More generally, additional work is required before the mechanism of chain-length control by GlfT2 can be unequivocally understood.

In summary, investigations over the past several years have provided crucial insights into the process by which mycobacterial galactan is assembled. A major focus has been on the enzyme GlfT2, a polymerizing glycosyltransferase that installs the majority of the Gal*f* residues in this polysaccharide. Achievements have included the recombinant expression of the protein in large quantity[24] as well as the development of a robust activity assay[36] and synthetic routes[25,35] for the substrates used by the enzyme. In addition, the catalytic machinery[44] and substrate specificity[51,52] of the enzyme is now understood as is its three-dimensional structure as determined by X-ray crystallography.[45] However, a number of factors remain to be elucidated. Primary among these is understanding the mechanism by which the enzyme installs two different glycosidic linkages in alternating fashion using a single active site. Further work is also needed to determine how GlfT2 controls the length of the galactan chain. In the broader context of galactan assembly, future investigations should focus on characterizing the other glycosyltransferase involved in the process, GlfT1, as well as the putative ABC transporter that transfers the galactan across the plasma membrane. With regard to GlfT1, it should be noted that a recent investigation reports the recombinant expression of the enzyme and the development of an assay for monitoring its activity.[56] Thus, it appears that further investigations, along the lines of those already carried out for GlfT2, are now in reach for GlfT1.

AUTHOR INFORMATION

Corresponding Author

*E-mail: tlowary@ualberta.ca. Phone: 780-492-1861. Fax: 780-492-7705.

Notes

The authors declare no competing financial interest.

Biographies

Todd L. Lowary is the R.U. Lemieux Professor of Carbohydrate Chemistry at the University of Alberta and the Scientific Director of the Canadian Glycomics Network. His research interests are in the area of synthesis, biosynthesis, and immunomodulatory activity of microbial glycans.

Myles B. Poulin obtained his Ph.D. at the University of Alberta studying enzymes involved in furanose sugar biosynthesis before completing a postdoctoral position in biochemistry at the Albert Einstein College of Medicine. In 2016, he will join the Department of Chemistry and Biochemistry at the University of Maryland College Park as an Assistant Professor.

ACKNOWLEDGMENTS

Support from the Alberta Glycomics Centre and the Natural Science and Engineering Research Council (NSERC) of Canada is gratefully acknowledged. In addition, M.B.P. thanks NSERC and Alberta Innovates Technology Futures for fellowships while a graduate student. We thank Professor Robert Campbell (University of Alberta) for providing comments on the manuscript.

REFERENCES

(1) Richards, M. R.; Lowary, T. L. *ChemBioChem* **2009**, *10*, 1920−1938.

(2) Tefsen, B.; Ram, A. F. J.; van Die, I.; Routier, F. H. *Glycobiology* **2012**, *22*, 456−469.

(3) Marino, C.; Gallo-Rodriguez, C.; de Lederkremer, R. M. In *Glycans: Biochemistry, Characterization and Applications*, 1st ed.; Mora-Montes, H. M., Ed.; Nova Science Publishers, Inc., 2012; pp 207−268.

(4) Chlubnova, I.; Legentil, L.; Dureau, R.; Pennec, A.; Almendros, M.; Daniellou, R.; Nugier-Chauvin, C.; Ferrières, V. *Carbohydr. Res.* **2012**, *356*, 44−61.

(5) Eppe, G.; El Bkassiny, S.; Vincent, S. P. In *Carbohydrates in Drug Design and Discovery*; Jimenez-Barbero, J., Canada, F. J., Martın-Santamaria, S., Eds.; Royal Society of Chemistry, 2015; pp 209−241.

(6) Schmalhorst, P.; Krappmann, S.; Vervecken, W.; Rohde, M.; Muller, M.; Braus, G.; Contreras, R.; Braun, A.; Bakker, H.; Routier, F. *Eukaryotic Cell* **2008**, *7*, 1268−1277.

(7) Damveld, R. A.; Franken, A.; Arentshorst, M.; Punt, P. J.; Klis, F. M.; Van Den Hondel, C. A. M. J. J.; Ram, A. F. J. *Genetics* **2008**, *178*, 873−881.

(8) Pan, F.; Jackson, M.; Ma, Y.; McNeil, M. *J. Bacteriol.* **2001**, *183*, 3991−3998.

(9) Pan, F.; Jackson, M.; Ma, Y.; McNeil, M. *J. Bacteriol.* **2001**, *183*, 3991−3998.

(10) Pedersen, L.; Turco, S. *Cell. Mol. Life Sci.* **2003**, *60*, 259−266.

(11) Angala, S. K.; Belardinelli, J. M.; Huc-Claustre, E.; Wheat, W. H.; Jackson, M. *Crit. Rev. Biochem. Mol. Biol.* **2014**, *49*, 361−399.

(12) Jankute, M.; Cox, J. A. G.; Harrison, J.; Besra, G. S. *Annu. Rev. Microbiol.* **2015**, *69*, 405−423.

(13) Mikusová, K.; Mikus, M.; Besra, G. S.; Hancock, I.; Brennan, P. J. *J. Biol. Chem.* **1996**, *271*, 7820−7828.

DOI: 10.1021/acs.joc.6b01501
J. Org. Chem. 2016, 81, 8123−8130

(14) Jin, Y.; Xin, Y.; Zhang, W.; Ma, Y. *FEMS Microbiol. Lett.* **2010**, *310*, 54−61.
(15) Mills, J.; Motichka, K.; Jucker, M.; Wu, H.; Uhlik, B.; Stern, R.; Scherman, M.; Vissa, V.; Pan, F.; Kundu, M.; Ma, Y.; McNeil, M. *J. Biol. Chem.* **2004**, *279*, 43540−43546.
(16) Mikusova, K.; Belanova, M.; Kordulakova, J.; Honda, K.; McNeil, M.; Mahapatra, S.; Crick, D.; Brennan, P. *J. Bacteriol.* **2006**, *188*, 6592−6598.
(17) Belánová, M.; Dianiskova, P.; Brennan, P.; Completo, G.; Rose, N.; Lowary, T.; Mikušová, K. *J. Bacteriol.* **2008**, *190*, 1141−1145.
(18) 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. *J. Biol. Chem.* **2001**, *276*, 26430−26440.
(19) Dianiskova, P.; Kordulakova, J.; Skovierova, H.; Kaur, D.; Jackson, M.; Brennan, P.; Mikusova, K. *Gen. Physiol. Biophys.* **2011**, *30*, 239−250.
(20) Yamatsugu, K.; Splain, R. A.; Kiessling, L. L. *J. Am. Chem. Soc.* **2016**, *138*, 9205−9211.
(21) Mikušová, K.; Yagi, T.; Stern, R.; McNeil, M. R.; Besra, G. S.; Crick, D. C.; Brennan, P. J. *J. Biol. Chem.* **2000**, *275*, 33890−33897.
(22) Pathak, A. K.; Pathak, V.; Seitz, L.; Maddry, J. A.; Gurcha, S.; Besra, G.; Suling, W. J.; Reynolds, R. C. *Bioorg. Med. Chem.* **2001**, *9*, 3129−3143.
(23) Cren, S.; Gurcha, S.; Blake, A. J.; Besra, G.; Thomas, N. *Org. Biomol. Chem.* **2004**, *2*, 2418−2420.
(24) Rose, N. L.; Completo, G. C.; Lin, S. J.; McNeil, M.; Palcic, M. M.; Lowary, T. L. *J. Am. Chem. Soc.* **2006**, *128*, 6721−6729.
(25) Completo, G.; Lowary, T. *J. Org. Chem.* **2008**, *73*, 4513−4525.
(26) Alderwick, L.; Dover, L.; Veerapen, N.; Gurcha, S.; Kremer, L.; Roper, D.; Pathak, A.; Reynolds, R.; Besra, G. *Protein Expression Purif.* **2008**, *58*, 332−341.
(27) May, J. F.; Splain, R. A.; Brotschi, C.; Kiessling, L. L. *Proc. Natl. Acad. Sci. U. S. A.* **2009**, *106*, 11851−11856.
(28) Zhang, Q.; Liu, H. *J. Am. Chem. Soc.* **2000**, *122*, 9065−9070.
(29) Tsvetkov, Y. E.; Nikolaev, A. V. *J. Chem. Soc., Perkin Trans. 1* **2000**, 889−891.
(30) Marlow, A.; Kiessling, L. *Org. Lett.* **2001**, *3*, 2517−2519.
(31) Peltier, P.; Daniellou, R.; Nugier-Chauvin, C.; Ferrieres, V. *Org. Lett.* **2007**, *9*, 5227−5230.
(32) Errey, J. C.; Mukhopadhyay, B.; Kartha, K. P. R.; Field, R. A. *Chem. Commun.* **2004**, 2706−2707.
(33) Ichikawa, Y.; Wang, R.; Wong, C. H. *Methods Enzymol.* **1994**, *247*, 107−127.
(34) Liu, Z.; Zhang, J.; Chen, X.; Wang, P. *ChemBioChem* **2002**, *3*, 348−355.
(35) Poulin, M. B.; Lowary, T. L. *Methods Enzymol.* **2010**, *478*, 389−411.
(36) Rose, N.; Zheng, R.; Pearcey, J.; Zhou, R.; Completo, G.; Lowary, T. *Carbohydr. Res.* **2008**, *343*, 2130−2139.
(37) Weigel, P.; DeAngelis, P. *J. Biol. Chem.* **2007**, *282*, 36777−36781.
(38) Keys, T. G.; Fuchs, H. L. S.; Galuska, S. P.; Gerardy-Schahn, R.; Freiberger, F. *Glycobiology* **2013**, *23*, 613−618.
(39) Szczepina, M. G.; Zheng, R. B.; Completo, G. C.; Lowary, T. L.; Pinto, B. M. *ChemBioChem* **2009**, *10*, 2052−2059.
(40) Szczepina, M.; Zheng, R. B.; Completo, G.; Lowary, T. L.; Pinto, B. M. *Bioorg. Med. Chem.* **2010**, *18*, 5123−5128.
(41) Wiggins, C. A. R.; Munro, S. *Proc. Natl. Acad. Sci. U. S. A.* **1998**, *95*, 7945−7950.
(42) Lairson, L. L.; Henrissat, B.; Davies, G. J.; Withers, S. G. *Annu. Rev. Biochem.* **2008**, *77*, 521−555.
(43) Shibayama, K.; Ohsuka, S.; Tanaka, T.; Arakawa, Y.; Ohta, M. *J. Bacteriol.* **1998**, *180*, 5313−5318.
(44) May, J. F.; Levengood, M. R.; Splain, R. A.; Brown, C. D.; Kiessling, L. L. *Biochemistry* **2012**, *51*, 1148−1159.
(45) Wheatley, R. W.; Zheng, R. B.; Richards, M. R.; Lowary, T. L.; Ng, K. K. S. *J. Biol. Chem.* **2012**, *287*, 28132−28143.
(46) Levengood, M. R.; Splain, R. A.; Kiessling, L. L. *J. Am. Chem. Soc.* **2011**, *133*, 12758−12766.
(47) Lowary, T. L.; Hindsgaul, O. *Carbohydr. Res.* **1994**, *251*, 33−67.
(48) Tam, P. H.; Besra, G. S.; Lowary, T. L. *ChemBioChem* **2008**, *9*, 267−278.
(49) Bundle, D. R.; Nycholat, C.; Costello, C.; Rennie, R.; Lipinski, T. *ACS Chem. Biol.* **2012**, *7*, 1754−1763.
(50) Johnson, M. A.; Cartmell, J.; Weisser, N. E.; Woods, R. J.; Bundle, D. R. *J. Biol. Chem.* **2012**, *287*, 18078−18090.
(51) Poulin, M. B.; Zhou, R.; Lowary, T. L. *Org. Biomol. Chem.* **2012**, *10*, 4074−4087.
(52) Brown, C. D.; Rusek, M. S.; Kiessling, L. L. *J. Am. Chem. Soc.* **2012**, *134*, 6552−6555.
(53) Peltier, P.; Beláňová, M.; Dianišková, P.; Zhou, R.; Zheng, R. B.; Pearcey, J. A.; Joe, M.; Brennan, P. J.; Nugier-Chauvin, C.; Ferrières, V.; Lowary, T. L.; Daniellou, R.; Mikušová, K. *Chem. Biol.* **2010**, *17*, 1356−1366.
(54) King, J. D.; Berry, S.; Clarke, B. R.; Morris, R. J.; Whitfield, C. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 6407−6412.
(55) Berg, S.; Kaur, D.; Jackson, M.; Brennan, P. *Glycobiology* **2007**, *17*, 35R−56R.
(56) Martinez Farias, M. A.; Kincaid, V. A.; Annamalai, V. R.; Kiessling, L. L. *J. Am. Chem. Soc.* **2014**, *136*, 8492−8495.

DOI: 10.1021/acs.joc.6b01501
J. Org. Chem. 2016, 81, 8123−8130