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Development of a coupled spectrophotometric assay for GlfT2, a bifunctional mycobacterial galactofuranosyltransferase

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Abstract—As a key constituent of their protective cell wall all mycobacteria produce a large structural component, the mycolyl-arabinogalactan (mAG) complex, which has at its core a galactan moiety of alternating β-(1→5) and β-(1→6) galactofuranosyl residues. Galactan biosynthesis is essential for mycobacterial viability and thus inhibitors of the enzymes involved in its assembly are potential drugs for the treatment of mycobacterial diseases, including tuberculosis. Only two galactofuranosyltransferases, GlfT1 and GlfT2, are responsible for the biosynthesis of the entire galactan domain of the mAG and we report here the first high-throughput assay for GlfT2. Successful implementation of the assay required the synthesis of multi-milligram amounts of the donor for the enzyme, UDP-Gal*f*, **1**, which was achieved using a chemoenzymatic approach. We also describe an improved expression system for GlfT2, which provides a larger amount of active protein for the assay. Kinetic analysis of **1** and a known trisaccharide acceptor for the enzyme, **2**, have been carried out and the apparent K_m and k_{cat} values obtained for the latter are in agreement with those obtained using a previously reported radiochemical assay. The assay has been implemented in 384-well microtiter plates, which will facilitate the screening of large numbers of potential GlfT2 inhibitors, with possible utility as novel anti-TB drugs.

Keywords: Assay; Spectrophotometric; Mycobacteria; UDP-Gal*f*; Galactofuranose; Glycosyltransferase

1. Introduction

Diseases caused by mycobacterial infections continue to constitute major health threats worldwide.[1] Most well known among these diseases are tuberculosis (TB), caused by *Mycobacterium tuberculosis*, and leprosy, which results from infection by *Mycobacterium leprae*. Related diseases, while less known, are also serious health issues; for example, *Mycobacterium avium* infections are common in HIV+ individuals.[2] Concerns about drug-resistant[3] strains of *M. tuberculosis* have received increased media coverage over the past year[4] and were widely publicized in the early summer of 2007 when an individual thought to have extensively drug-resistant TB (XDR-TB) potentially infected large numbers of people while traveling by air between North America and Europe.[5] Although this individual was subsequently shown[5b] not to have this most serious form of the disease, but instead multi-drug-resistant TB (MDR-TB), the incident brought attention to the need for new antibiotics to treat this disease.

TB is difficult to cure and successfully doing so requires long treatment periods with multiple antibiotics.[6] Such treatments are made necessary by the unusual

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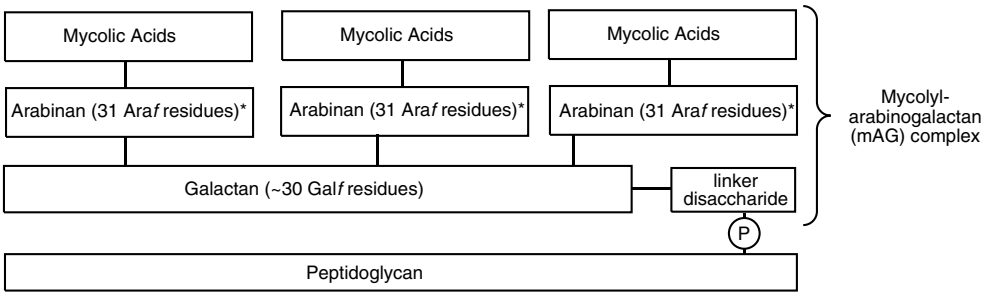
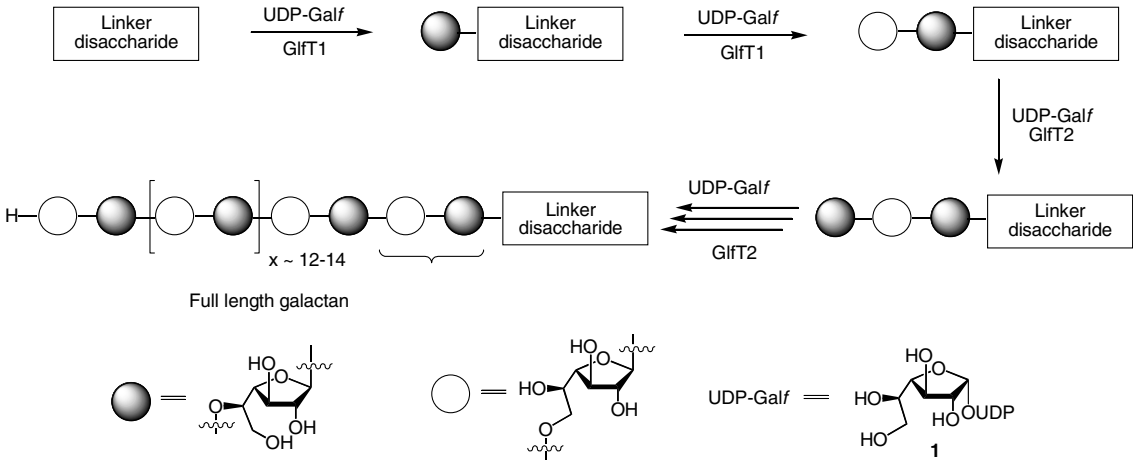


Figure 1. Schematic description of the mycolyl-arabinogalactan (mAG) complex. *It has been proposed that the arabinan domains are attached to the eighth, tenth, and twelfth Gal*f* residues of the galactan core.[12]



Scheme 1. Proposed pathway for the assembly of the galactan domain of mycobacterial mAG.[14]

structure of the mycobacterial cell wall, which serves as a significant permeability barrier to the passage of antibiotics into the organism.[7] Not surprisingly, therefore, the cell wall has been a focus of attention in novel drug development efforts.[8] These efforts have been further bolstered by the success of two inhibitors of mycobacterial cell wall biosynthesis, ethambutol and isoniazid, which are clinically used as anti-TB drugs.[6] The former inhibits one or more of the arabinosyltransferases[9] involved in the biosynthesis of the mycolyl-arabinogalactan (mAG) complex, the major structural element of the cell wall, whereas the latter blocks the assembly of branched chain lipids called mycolic acids,[10] which are key components of the mAG.

The structure of the mAG was established in a series of seminal investigations by Brennan and co-workers in the early 1990s further refined since that time (Fig. 1).[7,11,12] These studies demonstrated that the glycoconjugate is linked to cell wall peptidoglycan by way of an α-L-rhamnopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucosamine-phosphate disaccharide, which is further elaborated by a galactan moiety that forms the core of the molecule. Approximately 30 alternating β-(1→5) and β-(1→6) linked-galactofuransoyl (Gal*f*) residues comprise this galactan to which are attached three branched arabinan domains each containing, on average, 31 arabinofuranosyl (Ara*f*) residues linked α-(1→5), α-(1→3), and β-(1→2). The final structural elements of the mAG are the mycolic acids, which are esterified to the terminal ends of the arabinan domain.

Although the structure of the mAG has been known for many years, its biosynthesis is still not fully elucidated; however, significant progress in this area has been made in the past 5–10 years.[11a,13] The portion of the molecule whose biosynthesis is perhaps the best understood is the galactan. On the basis of currently available data, it is believed[14] that the galactan domain is assembled by the combined action of only two bifunctional galactofuranosyltransferases, GlfT1 and GlfT2,[‡] both of which use the sugar nucleotide UDP-Gal*f* (**1**) as the donor species (Scheme 1). Mycobacteria must produce an intact galactan to be viable,[15] and inhibitors of these galactofuranosyltransferases therefore have potential as novel anti-TB agents. Furthermore, because none of the drugs currently used to treat TB are known to inhibit these enzymes, GlfT1 and GlfT2 represent novel targets

[‡]The nomenclature used for these enzymes, GlfT1 and GlfT2, was recently proposed[14] and differs slightly from that used previously.

for the development of therapeutics. Their attractiveness in drug development is further enhanced by the absence of Gal*f*-containing glycoconjugates in humans.

In an earlier study,[16] we reported the first high-level expression of GlfT2, the enzyme that adds the third and subsequent Gal*f* residues to the growing galactan chain (Scheme 1). This enzyme, which is encoded in *M. tuberculosis* by the Rv3808c gene,[17] possesses dual β-(1→5) and β-(1→6)-galactofuranosyltransferase activity[18] and through the analysis of a panel of di- and trisaccharide substrates using a radiochemical assay developed initially by Besra and co-workers,[18,19] it was shown that trisaccharide **2** (Chart 1) is the best substrate for the enzyme, compared to a related trisaccharide, **3**, and smaller fragments.

Whereas the radiochemical assay we used in our previous study[16] is effective for evaluating relatively small numbers of compounds, it is not amenable to high-throughput screening of a large number of potential substrates and inhibitors, for example, libraries of small molecules. This radiochemical assay is also not a valid means of obtaining detailed kinetic and mechanistic information as it relies on the conversion of radiolabeled UDP-Gal*p* to UDP-Gal*f* (**1**) by the enzyme, UDP-Gal*p* mutase. This reaction heavily favors the pyranose form of the molecule, with ~7% of UDP-Gal*f* present at equilibrium,[20] making it difficult to ensure that the concentration of the donor substrate is saturated. Related to this limitation is that measuring kinetic parameters for **1** using this assay requires that the donor be available in radiolabeled form. Although ^{3}H-labeled **1** has been prepared chemically and been shown to be incorporated into an acceptor molecule,[21] its preparation necessitates access to facilities for carrying out preparative-scale radiolabeled syntheses, which are not widely available.

To address these problems, we report here the application of a known coupled spectrophotometric glycosyltransferase assay[22] to quantify GlfT2 activity. We also describe an optimized procedure for preparing **1** by a reported chemoenzymatic approach[23] and an improved method for producing recombinant GlfT2, which provides significantly larger amounts of active enzyme than previously reported.[16] Finally, we have used this spectrophotometric assay to determine kinetic parameters for **1** and the best acceptor substrate reported to date for the enzyme, trisaccharide **2** (Chart 1).

2. Experimental

2.1. Preparation of UDP-Gal*f* (1) and trisaccharide 2

2.1.1. Materials. Uridine 5′-triphosphate trisodium salt (UTP), 90% purity, uridine 5′-diphosphate sodium salt (UDP), uridine 5′-monophosphate disodium salt (UMP), uridine 5′-diphosphoglucose disodium salt (UDP-Glc*p*), and inorganic pyrophosphatase (EC 3.6.1.1) were obtained from Sigma. Tryptone and yeast extract were purchased from Becton Dickinson (Sparks, MD). All other reagents were of chemical grade. Gal*f*-1-phosphate (**4**) was synthesized as previously reported.[24] Clones of glucose-1-phosphate uridyltransferase (GalU, EC 2.7.7.9) and galactose-1-phosphate uridyltransferase (GalPUT, EC 2.7.7.12) were generous gifts from Professor Robert A. Field, John Innes Centre, Norwich, UK.

2.1.2. HPLC. HPLC analysis was carried out using a Waters 600E HPLC equipped with a photodiode array (PDA) detector, with monitoring at 262 nm; the system was controlled via Empower chromatography software. Monitoring the conversion of **4** into **1** was done by analytical HPLC on a Phenomenex C18 column (4.6 × 250 mm), protected with a C18 guard column cartridge. A gradient elution of two buffers: buffer A (200 mM Et_3N–HOAc, pH 6.6) and buffer B (200 mM Et_3N–HOAc 6.6 containing 5% CH_3CN) was used. The gradient conditions employed were 96% buffer A and 4% buffer B for 10 min (isocratic), 4→100% buffer B over 15 min, 100% buffer B for 10 min. The column was re-equilibrated with a gradient of 100→4% buffer B for 1 min, followed by 96% buffer A and 4% buffer B for 10 min. Under these conditions, the retention times of UMP, UDP-Gal*f*, UDP, and UTP are 9.9, 15.8, 17.9, and 26.5 min, respectively. The flow-rate was maintained at 0.8 mL/min. UDP-Gal*f*, **1**, was purified by preparative HPLC on a Phenomenex C18 column (21.2 × 250 mm), protected with a C18 guard column cartridge, using a gradient elution of buffer C

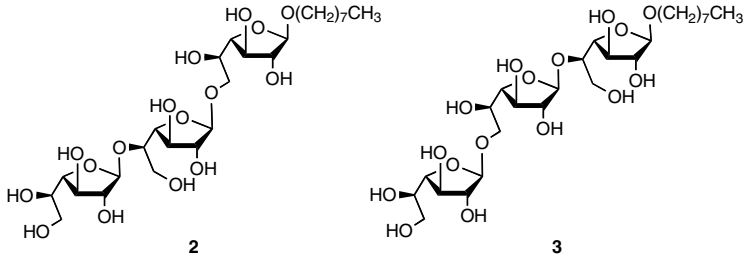


Chart 1. Reported trisaccharide substrates for GlfT2.[16]

(5 mM sodium phosphate, pH 6.8) and buffer D (MilliQ water). The gradient conditions were 0→100% buffer D for 12 min, 100% buffer D for 8 min followed by an equilibration phase of 100% buffer C for 5 min. The flow-rate was set at 6 mL/min. With this eluant system, the retention times of UMP, UDP, UTP, and UDP-Gal*f* are 10.9, 12.5, 14.2, and 17.6 min, respectively.

2.1.3. Production and immobilization of galactose-1-phosphate uridyltransferase (GalPUT). *Escherichia coli* Origami BL-21 containing GalPUT plasmid[25] was cultured in LB medium (10 g tryptone/L, 5 g yeast extract/L, and 10 g NaCl/L) supplemented with 100 μg/mL ampicillin. The protein was produced by inducing the *E. coli* culture with 1 mM IPTG (isopropyl 1-thio-β-D-galactopyranoside, Invitrogen, Burlington, ON) at $OD_{600nm} = 0.6–0.8$ and incubating at 30 °C for 5 h with shaking at 200 rpm. The cells were harvested by centrifugation at 11,300g_{max} for 20 min. Pellets were stored at −20 °C for no longer than six months. The cell pellet was resuspended at 50 mL of resuspension buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 mM imidazole, supplemented with protease inhibitor cocktail) per L cell culture. The resuspension was passed through a Thermo Spectronic French® Pressure Cell Press at 20,000 psi. The cell lysate was obtained by centrifugation at 105,000*g* for 60 min. The enzyme was purified directly from the lysate using a Ni^{2+}–NTA affinity column. After loading the cell lysate, the Ni^{2+} column was washed with six column volumes of wash buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 20 mM imidazole), followed by six volumes of GalPUT reaction buffer (50 mM HEPES, pH 8.0, containing 5 mM KCl and 10 mM $MgCl_2$). The resulting immobilized GalPUT was used in the enzymatic synthesis.

2.1.4. Production of glucose-1-phosphate uridyltransferase (GalU). *E. coli* strains Origami BL-21 containing the GalU plasmid[25] were cultured in LB medium (10 g/L tryptone, 5 g/L yeast extract, and 10 g/L NaCl) supplemented with 100 μg/mL ampicillin. The protein was produced by inducing the *E. coli* culture with 1 mM IPTG (isopropyl 1-thio-β-D-galactopyranoside, Invitrogen, Burlington, ON) at $OD_{600nm} = 0.6–0.8$ and incubating at 30 °C for 5 h with shaking at 200 rpm. The cells were harvested by centrifugation at 11,300g_{max} for 20 min. The cell pellet was resuspended at 50 mL of resuspension buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 10 mM imidazole supplemented with protease inhibitor cocktail) per L cell culture. The resuspension was passed through a Thermo Spectronic French® Pressure Cell Press at 20,000 psi. The cell lysate was obtained by centrifugation at 105,000*g* for 60 min. The enzyme was purified directly from the lysate using a Ni^{2+}–NTA affinity column. After loading the cell lysate, the Ni^{2+} column was washed with six column volumes of wash buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 20 mM Imidazole), followed by four volumes of elution buffer (50 mM sodium phosphate buffer, pH 8.0, containing 300 mM NaCl and 250 mM Imidazole). The protein was dialyzed against 50 mM HEPES buffer, pH 8.0, and was stored at −80 °C for further use.

2.1.5. Synthesis and purification of 1. The preparative synthesis of UDP-Gal*f* (**1**) was carried out in HEPES buffer (50 mM, pH 8.0) containing $MgCl_2$ (10 mM), KCl (5 mM), UTP (24.5 mg, 40 μmol), Gal*f*-1-phosphate (**4**,[24] 19.4 mg, 42 μmol), GalU (20 U), inorganic pyrophosphatase (5 U), and immobilized GalPUT (1 mL of Ni^{2+}–NTA-agarose resins containing 20 U of enzyme) in a total reaction volume of 1.5 mL. The reaction was initiated by the addition of UDP-Glc*p* (0.1 mg, 0.15 μmol). The mixture was incubated at rt overnight with shaking and the reaction was stopped when HPLC analysis of the spin-filtered (Amicon YM10) solution indicated that the reaction was complete (See Section 2.1.2). At the end of the reaction, the immobilized enzymes were removed by passing the incubation mixture through an empty 10 mL BioRad cartridge, equipped with a filter. The filtrate was then collected by passage through Amicon® Ultra-15 centrifuge tubes with a molecular weight cut off of 10,000 Daltons; this filtrate was centrifuged to remove inorganic pyrophosphatase. Following centrifugation, the filtrate was collected and was applied to a Sephadex G-15 gel filtration column to remove salts. Purification of **1** was achieved by concentrating target fractions of a Sephadex G-15 gel filtration column and purification of the mixture using preparative HPLC (Section 2.1.2). The fractions containing UDP-Gal*f* were concentrated under reduced pressure below 25 °C to less than 10 mL and then salts were removed on a Sephadex G-15 column. The purity and identity of UDP-Gal*f* was confirmed by ^1H NMR spectroscopy.[26]

2.1.6. Preparation of 2. The preparation of trisaccharide **2** is described elsewhere.[27]

2.2. Expression and purification of GlfT2

Newly transformed *E. coli* Rosetta™ (DE3) Quarters™ competent cells (EMD Biosciences, La Jolla, CA) carrying recombinant plasmids, pET-15b/Rv3808c,[16] were cultured while shaking at 200 rpm overnight at 30 °C in 100 mL of TB broth supplemented with 1× TB salts, 1% glucose, and 100 μg/mL ampicillin. Cultures were expanded to a volume of 1 L and the OD_{600nm} regularly monitored. Overexpression of the target protein was achieved by inducing the *E. coli* culture with 1.0 mM

IPTG (isopropyl 1-thio-β-D-galactopyranoside, Invitrogen, Burlington, ON) at OD_{600nm} of 0.6–0.8 and incubating for a further 20 h at 20 °C. Cells were harvested at 4 °C by centrifugation at 11,300g_{max} and the pellets were stored at −20 °C until further use. Upon use, the cell pellets were thawed on ice and resuspended at a ratio of 1:10 in ice-cold 50 mM sodium phosphate buffer, pH 7.6, containing 5 mM β-mercaptoethanol, 10 mM imidazole, 300 mM NaCl, and 0.1% Triton X-100 supplemented with complete protease inhibitor cocktail (Roche, Indianapolis, IN). All subsequent steps were performed at 4 °C.

Lysates were prepared by a single passage of the resuspension mix through a pre-chilled bench top cell disrupter (Constant Systems Incorporated, Sanford, NC) set to 20,000 psi. The cell lysate was centrifuged at 105,000*g* for 60 min to remove cellular debris and other insoluble proteins. The supernatant fraction was applied directly to a pre-equilibrated Ni^{2+}–NTA-agarose affinity column using a flow-rate of 0.5 mL/min. After loading the cell lysate, the column was washed with six column volumes of binding buffer (5 mM β-mercaptoethanol, 20 mM imidazole, 0.3 M NaCl, 50 mM sodium phosphate buffer, pH 7.6), followed by six volumes of washing buffer (5 mM β-mercaptoethanol, 50 mM imidazole, 0.3 M NaCl, 50 mM sodium phosphate buffer, pH 7.6). The over-expressed protein was eluted with a linear gradient containing 50–200 mM imidazole. The absorbance at 280 nm was monitored with a UV detector, and fractions containing the purified enzyme were pooled and concentrated to ~0.5 mL using an Amicon® Ultra-15 centrifuge tube with a molecular weight cut off of 30,000 Daltons (Millipore, Billerica, MA). The concentrated enzyme fraction was applied to a Sephacryl S-100 HR (Amersham Biosciences, Piscataway, NJ) column pre-equilibrated with 50 mM MOPS, pH 7.6, containing 1 mM DTT, 100 mM NaCl, and 10 mM $MgCl_2$, then eluted with the same buffer. Evaluation by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS–PAGE) indicated that the final preparation was more than 95% pure (see Section 3.2 below).

2.3. GlfT2 assay development

2.3.1. Materials. Potassium chloride (KCl), β-nicotinamide adenine dinucleotide (NADH) as the reduced disodium salt, phosphoenolpyruvic acid monocyclohexylammonium salt (PEP), pyruvate kinase (PK, type III, lyophilized powder, rabbit muscle), and lactate dehydrogenase, (LDH, type XI, salt free, rabbit muscle) were from Sigma–Aldrich (St. Louis, MO). 3-(*N*-Morpholino)propanesulfonic acid (MOPS), BioUltraPure grade, was from BioShop (Burlington, ON), and magnesium chloride hexahydrate was obtained from EMD Biosciences (La Jolla, CA). Proteins were quantified with the Pierce Biotechnology BCA method (Rockford, IL) using bovine serum albumin as a standard. Flat-bottomed 384-well polystyrene, non-binding surface assay plates were obtained from Corning (Corning, NY).

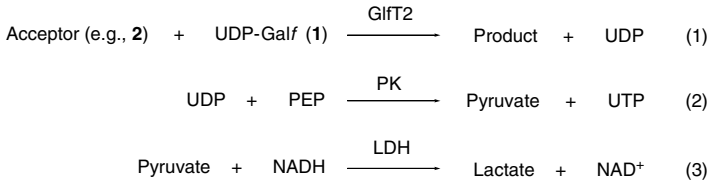
2.3.2. Assay conditions

2.3.2.1. Spectrophotometric assays. Spectrophotometric assays were performed in 384-array microtiter plate wells containing 50 mM MOPS, pH 7.9, 50 mM KCl, 20 mM $MgCl_2$, 0.825 or 1.1 mM NADH (see Section 3.3), 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). MOPS, pH 7.9, was added as a 20-fold stock solution. KCl, $MgCl_2$, and the acceptor and donor substrates were dissolved in de-ionized distilled water. All other assay components were prepared in 50 mM MOPS, pH 7.9, with the exception of PEP, which, due to its acidity, was buffered in 250 mM MOPS, pH 7.9. Stock solutions of NADH, PEP, PK, LDH, and donor were made fresh on the day of use. A standard assay reaction contained UDP-Gal*f* (**1**) at a final concentration of 3 mM and acceptor trisaccharide **2** at 2 mM. Each reaction was initiated by the addition of 0.75 μg GlfT2 to the assay mixture. The final assay volume was 40 μL.

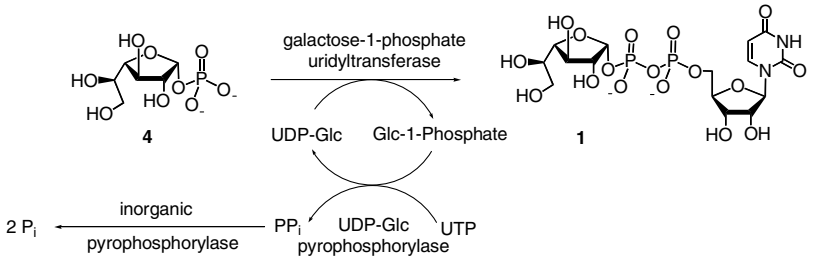
Reactions were continuously monitored at 37 °C using a Spectra Max 340PC microplate reader controlled with SOFTmax® PRO software (Molecular Devices, Sunnyvale, CA) in the kinetics read mode. The wavelength was set at 340 nm and each reaction was monitored at 12–15 s intervals for up to 8 min. The rate of NADH oxidation was converted to pmoles by using a pathlength of 0.37 cm and an extinction co-efficient of 6300 $(M\ cm)^{-1}$. Data were graphed and analyzed using GraphPad Prism® Version 4. For Michelis–Menten plots, a non-linear regression fit was employed. Data were transformed as a Lineweaver–Burk (double reciprocal) plot. We assumed that all of the GlfT2 had full catalytic activity, and so we report V_{max} values in units of pmol/min and k_{cat} values in min^{-1}.

Acceptor kinetics were performed with concentrations of trisaccharide **2** at 0.0156–1.5 mM and donor **1** at 4.0 mM. For donor kinetics, trisaccharide **2** was used at 3.0 mM with donor **1** at 0.125–4.0 mM. When donor kinetics were performed, reaction velocities were linear for up to 1.5 min at the lowest donor concentration, and for up to 5 min at the highest donor levels. Trisaccharide **2** kinetics were linear for at least a 3 min span. Control assays lacking the donor substrate for donor kinetics and the acceptor substrate for acceptor kinetics were performed to correct for the presence of endogenous activity.

2.3.2.2. Radiochemical assays. Radiochemical assays were carried out as described previously.[16]



Scheme 2. Coupled spectrophotometric assay for measuring GlfT2 activity.



Scheme 3. Chemoenzymatic synthesis of UDP-Gal*f*, **1**, reported by Field and co-workers.[23]

Donor**1** was generated in situ from 5.0 mM UDP-Gal*p* with 55 μg of UDP-Gal*p* mutase.[28] Trisaccharide **2** was used at concentrations ranging from 0.015625 to 1.5 mM and 0.02 μg of GlfT2 was used.

3. Results and discussion

The coupled spectrophotometric assay[22] that we used to measure GlfT2 activity relies on detection of UDP liberated from **1** upon transfer of Gal*f* to an acceptor substrate (e.g., **2**). The UDP produced in the reaction is coupled to the oxidation of NADH by way of two enzymes, pyruvate kinase (PK), and lactate dehydrogenase (LDH), as outlined in Scheme 2. The decrease in absorption at 340 nm, resulting from NADH oxidation, is proportional to GlfT2 activity.

One of the challenges in assaying galactofuranosyltransferases is that the donor substrate for the reaction, UDP-Gal*f* (**1**), is not commercially available. As mentioned above, in the radiochemical assay employed earlier by us,[16] and others,[18,19] this problem was overcome by in situ generation of **1** from commercially-available UDP-Gal*p*, through the action of UDP-Gal*p* mutase,[28,29] which was added to the incubation mixture,[16] or was present in the membrane fraction used as the GlfT2 source.[18,19] Using the same approach with this spectrophotometric assay would be difficult as the mutase must be in a reducing environment to be active[28] and this is most conveniently achieved by adding 10 mM NADH to the storage and assay mixtures. As the spectrophotometric assay measures NADH oxidation, we envisioned that the addition of an excess of this reducing agent would complicate monitoring. Therefore, the first task was to develop a robust method for producing multi-milligram quantities of **1**.

3.1. Preparation of UDP-Gal*f*, 1

In developing a method for the synthesis of UDP-Gal*f*, we first considered one of the chemical approaches that has been used to prepare this sugar nucleotide, and analogs, in both radiolabeled[21] and non-radiolabeled form.[30] In all cases, however, the overall product yields are generally poor to modest, and we felt that accessing 50–100 mg quantities of this material by a purely chemical strategy would be difficult. An enzymatic synthesis of **1** from UDP-Gal*p* using UDP-Gal*p* mutase has also been reported,[20] but this approach is limited by the equilibrium position of the reaction, which favors UDP-Gal*p* over UDP-Gal*f* by a 93:7 ratio, thereby limiting the practicality of preparing large amounts of **1** by this method. We therefore focused our attention on a chemoenzymatic synthesis of **1**, which has been previously reported by Field and co-workers.[23] In this approach, galactofuranosyl 1-phosphate (**4**, Scheme 3), a compound that can be straightforwardly produced in gram quantities,[24] is converted to **1** by the action of a galactose-1-phosphate uridyltransferase with a broad substrate specificity.

Thus, **4** was synthesized and subjected to the multienzyme reaction depicted in Scheme 3.[23] Monitoring of the reaction by HPLC was done by following the disappearance of UTP. Because the commercial UTP used contained small amounts of both UMP and UDP,[§] an HPLC purification method was developed to purify UDP-Gal*f* from these compounds, which could be expected to interfere with the spectrophotometric assay. We found this approach provided **1** in a higher degree of purity than using the ion-exchange method previously

[§] As determined by HPLC.

reported.[23] We also observed that the presence of unreacted starting materials and reaction byproducts, such as **4**, UTP, and UDP, resulted in a decrease in the stability of UDP-Gal*f* upon storage. Following purification of the incubation mixture by preparative reversed-phase HPLC, the product was desalted on a Sephadex G15 column. This desalting step is essential because when stored at high salt concentrations, **1** readily decomposes. Once free from other impurities and salts, **1** can be stored dessicated at −20 °C for at least four months without appreciable decomposition. By using these modifications of this earlier method,[23] a typical synthesis can be carried out in less than one week, including purification. Each synthesis provides 30 mg of pure UDP-Gal*f* in an overall yield of 80% from **4**.

3.2. Expression of GlfT2

In our previous report[16] on the characterization of GlfT2, we expressed the His-tagged recombinant enzyme in *E. coli* C41(DE3) cells and a three-step purification process (successive ion-exchange, affinity, and gel filtration chromatography) was employed to isolate the protein from the crude cell lysate. Using this method, the yield of isolated pure protein was 5–7 mg/L of cell culture. We have continued to explore alternate expression systems and have found that using *E. coli* Rosetta (DE3) cells, followed by purification of the enzyme using a Ni^{2+}–NTA-agarose affinity column and a subsequent Sephacryl S-100-HR gel filtration column, allows us to obtain significantly more pure protein (>95% purity by SDS–PAGE, Fig. 2).

Although the expression level was not significantly different, the recombinant protein from *E. coli* Rosetta (DE3) cells binds better to the Ni^{2+}–NTA affinity column than the protein expressed in *E. coli* C41(DE3) cells. The use of this alternate expression system allowed us to produce GlfT2 in quantities of ~40 mg/L of cell culture. In addition, the need for only two purification steps, as opposed to three, contributed to the increased yield of active enzyme. The enzyme can be stored at both 4 °C and −80 °C with 20% glycerol (v/v). The activity of the enzyme does not decrease significantly during four months when stored at −80 °C and is stable for two weeks at 4 °C.

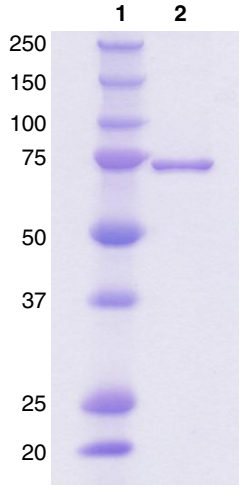


Figure 2. 10% SDS–PAGE of GlfT2. Lane 1, protein molecular weight standards; Lane 2, recombinant GlfT2 expressed in *E. coli* Rosetta (DE3) cells following purification by Ni^{+}–NTA affinity chromatography and Sephacryl™ S-100 gel filtration. The His-tagged protein has a molecular weight of ~73.5 kDa,[16–18] consistent with the GlfT2 band observed.

3.3. Assay development and application

Having established robust protocols for the synthesis of **1** and the expression and purification of GlfT2, we turned our attention to the development of the spectrophotometric assay. A key consideration was the costs of reagents, in particular **1**, and we first investigated if the assay could be carried out in 384-well microtiter plates as opposed to the 96-well plates reported previously.[22] This modification was indeed successful and sufficient signal was observed to provide reproducible results, thus allowing the potential measurement of 384 samples simultaneously. Under the standard assay conditions (see Section 2.3.2.1) this resulted in a total of 68 μg of **1** and 49 μg **2** being used per well.

We next evaluated the proportionality of both the amount of Rosetta (DE3) GlfT2 present in the assay mixture and the length of incubation time. Determining the effect of enzyme concentration on activity involved carrying out the reaction for up to 20 min with 3.0 mM **1** and 2.0 mM **2**, while varying the amount of recombinant GlfT2 from 0.125 to 2.0 μg. As shown in Figure 3, up to 2.0 μg of protein, there is a linear relationship between the rate of decrease in absorbance and transferase concentration.

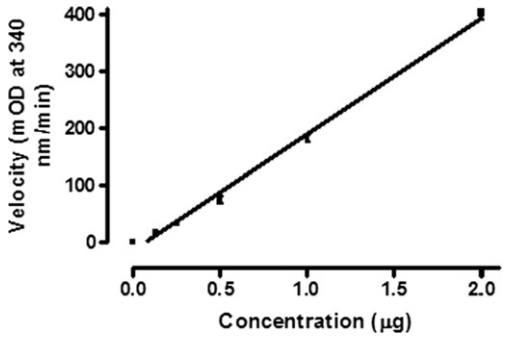


Figure 3. Proportionality of the spectrophotometric assay to the amount of Rosetta (DE3) GlfT2 present in the reaction mixture. The 40 μL reaction mixtures contained 3 mM UDP-Gal*f* (**1**) and 2 mM trisaccharide **2**, and were monitored for up to 20 min at 37 °C. The error bars indicate the standard deviation derived from duplicate experiments.

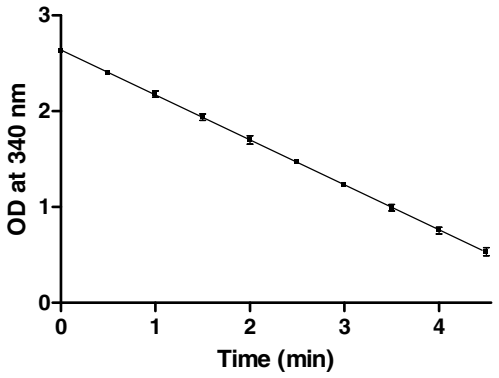
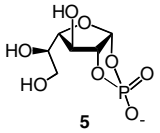


Figure 4. Linearity of the spectrophotometric assay over time. The 40 μL reaction mixtures contained 3 mM UDP-Gal*f* and 2 mM trisaccharide **2**, and 1 μg GlfT2. The error bars indicate the standard deviation derived from duplicate experiments.

Furthermore, carrying out the reaction of **1** and **2**, at 3.0 mM and 2.0 mM concentration, respectively, with 1 μg of GlfT2 and determining the reaction rate at 30 s intervals allowed us to demonstrate that the change in absorbance was linear to 4.5 min (Fig. 4). Taken together, these results show that the decrease in absorbance at 340 nm is proportional to both the amount of GlfT2 present and the length of incubation time. Based on these results, we carried out all subsequent incubations for 5 min with 0.75 μg of transferase derived from the Rosetta (DE3) system. In a separate series of proportionality experiments, which contained a twofold increase in concentration of linking enzymes, we confirmed that the reaction velocities remained unchanged and linear with respect to GlfT2 concentration.

While in general the assay was reproducible, there was occasional variability, which we could attribute to the use of preparations of **1** that were contaminated with residual UDP. On the basis of the equations presented in Scheme 2, it can be predicted that this impurity will interfere with the assay by causing NADH depletion by PK and LDH in the absence of the transferase. Similar problems have been reported when this assay was used with other particularly labile sugar nucleotides, for example, CMP-NeuNAc.[22a] Given the lability of furanosyl sugar nucleotides to hydrolysis, and the particular propensity of UDP-Gal*f* to decompose to UMP and the galactofuranose-1,2-cyclic phosphate **5**,[21,26] it could be expected that this problem would be encountered with GlfT2. Therefore, before carrying out a series of assays, we found it advantageous to check the purity of **1** by HPLC. In cases where the purity of UDP-Gal*f* was less than 85%, it was found that increasing NADH to 1.1 mM (from 0.825 mM) was sufficient to result in stable absorbance rates prior to initiating the reaction with GlfT2.



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We next compared the kinetic parameters for trisaccharide **2**, with those measured using the radiochemical assay (Table 1). Representative substrate-velocity and Lineweaver–Burk plots for both methods are shown in Figures 5 and 6. In the spectrophotometric assay, an apparent K_m value of 6×10^2 μM for **2** was obtained; the V_{max} was 4.0×10^3 pmol/min (Fig. 5 and Table 1). The use of the radiochemical assay gave an apparent K_m of 4.6×10^2 μM and a V_{max} of 1.3×10^2 pmol/min (Fig. 6 and Table 1). With both assays, similar k_{cat} values (4.8×10^2 min^{-1} vs 4.3×10^2 min^{-1}) were obtained.

Given the different conditions under which the spectrophotometric and radiochemical assays are carried out, discrepancies between the absolute values of these

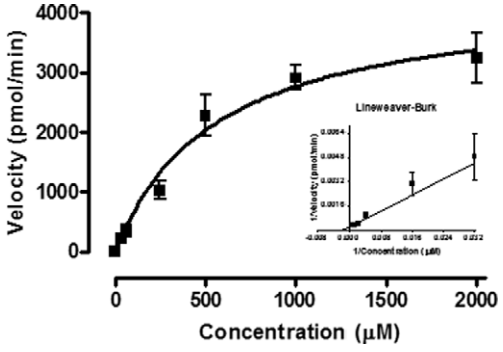

Figure 5. Acceptor kinetics for **2** in the spectrophotometric assay. The reactions were performed in duplicate and the error bars indicate the standard deviation from the mean. The inset illustrates the Lineweaver–Burk plot of the data. UDP-Gal*f* was present at 4 mM and 0.75 μg GlfT2 was used in each assay. The reactions were monitored for 5 min at 37 °C.

Table 1. Kinetic parameters for **1** and **2**

Assay method	**1**[a]			**2**[b]		
	$K_{m\,app}$ (μM)	V_{max} (pmol/min)	k_{cat} (min^{-1})	$K_{m\,app}$ (μM)	V_{max} (pmol/min)	k_{cat} (min^{-1})
Radiochemical	ND[c]	ND[c]	ND[c]	4.6 (±0.2) × 10^2	1.31 (±0.03) × 10^2	4.8 (±0.2) × 10^2
Spectrophotometric	3.8 (±0.6) × 10^2	4.4 (±0.2) × 10^3	4.3 (±0.2) × 10^2	6 (±2) × 10^2	4.3 (±0.5) × 10^3	4.3 (±0.5) × 10^2

[a] Determined using a 3.0 mM concentration of **2**.
[b] Determined using a 4.0 mM concentration of **1** in the spectrophotometric assay and 5.0 mM of Gal*p* in the radiochemical assay.
[c] Not determined.

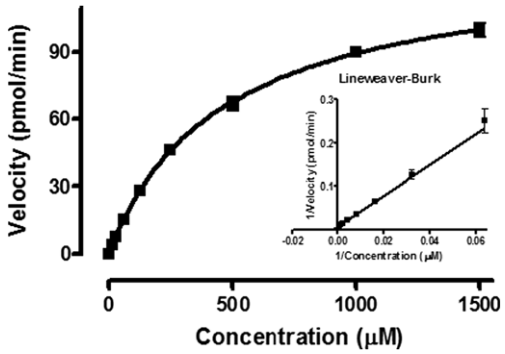

Figure 6. Acceptor kinetics for **2** in the radiochemical assay. Measurements were done in duplicate in a final volume of 40 μL and the error bars indicate the standard deviation from the mean. The inset illustrates the Lineweaver–Burk plot of the data. UDP-Gal*f* was generated in situ from 5 mM UDP-Gal*p* using 55 μg UDP-Gal*p* mutase, 0.02 μg of GlfT2 was used in each assay and reactions were stopped following a 90-min incubation at 37 °C.

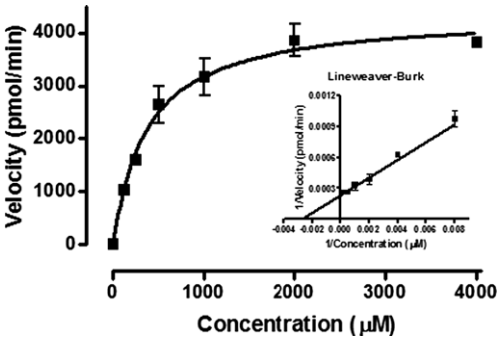

Figure 7. Donor kinetics for UDP-Gal*f* (**1**) in the spectrophotometric assay. The reactions were performed in duplicate and the error bars indicate the standard deviation from the mean. The inset illustrates the Lineweaver–Burk plot of the data. Trisaccharide **2** was present at 3 mM and 0.75 μg GlfT2 was used in each assay; reactions were monitored for 5 min at 37 °C.

parameters can be expected. In particular, when using the radiochemical assay the donor UDP-Gal*f* is generated in situ from the radiolabeled UDP-Gal*p* and quantifying its concentration is not possible. Therefore, typically an excess of UDP-Gal*p* is used and kinetic parameters are determined under the theoretical assumption that the furanosyl donor, **1**, is never depleted. Indeed, the concentration of UDP-Gal*p* used in the radiochemical assay has a significant impact on the k_{cat} of the enzyme, with a less substantial influence on the apparent K_m. When a 1 mM concentration of UDP-Gal*p* was used, corresponding to an approximate concentration of **1** of 70 μM (~0.2 × apparent K_m) assuming the mutase leads to a 93:7 pyranose–furanose ratio,[20] the k_{cat} is 2.7×10^2 min^{-1} and the apparent K_m is 4.3×10^2 μM. A much larger (20 mM) concentration of UDP-Gal*p* (leading to a 4 × apparent K_m concentration of **1**) yields a k_{cat} of 5.7×10^2 min^{-1} and an apparent K_m of 3.5×10^2 μM. The results presented in Table 1 reflect an intermediate case where a 5 mM concentration of UDP-Gal*p* (estimated concentration of **1** = 350 μM, which is approximately 1 × apparent K_m) was used. In this regard, it is important to note that the kinetics done in our earlier study were carried out at 1 mM UDP-Gal*p* hence yielding a concentration of **1** well below its apparent K_m.[16]

We also measured the kinetic parameters for **1** when using **2** as the acceptor substrate (Fig. 7 and Table 1). The apparent K_m was determined to be 3.8×10^2 μM and the V_{max} 4.4×10^3 pmol/min (Fig. 7 and Table 1). As described above, the ability to carry out kinetic studies on **1** and other donor analogs is one of the advantages of this spectrophotometric assay. The use of a radiochemical assay for GlfT2 donor kinetics would require access to pure **1**, in radiolabeled form. Although this compound has been prepared using a purely chemical approach,[21] and could also be obtained using the enzymatic route illustrated in Scheme 3, doing so would require facilities approved for preparative-scale radiochemical synthesis.

4. Conclusions

In summary, we describe the first continuous spectrophotometric assay for GlfT2, a bifunctional galactofuranosyltransferase from mycobacteria. Successful implementation of this assay required reliable access to multi-milligram amounts of UDP-Gal*f*, **1**, the donor for the enzyme, which was achieved by modification of a reported[23] chemoenzymatic synthesis of this sugar nucleotide.

Assay development was further facilitated by improvements in the expression and purification of recombinant GlfT2, which was achieved through the use of Rosetta (DE3) *E. coli*, as opposed to the C41(DE3) strain, which was used in our earlier study.[16] Although the protein concentrations of purified GlfT2 obtained from both expression systems were similar, the amount of active protein in the former is apparently higher. This may be related to the more efficient (two vs three-step) purification employed with these preparations. In addition, we occasionally observed varying results with different preparations of the enzyme produced using the C41(DE3) strain (unreported results), which we now attribute to the differing amounts of active protein in each preparation. Both the expression levels reported here, and our previous results,[16] are in contrast to a recent report by Besra and co-workers, which describes that soluble GlfT2 can only be expressed in the presence of chaperone proteins that facilitate folding.[31]

Once in place, the assay was used to determine kinetic parameters for **1** and a known trisaccharide substrate, **2**. Comparison of the apparent K_m values obtained for **2**, with those obtained using a radiochemical assay,

showed good agreement. Discrepancies in absolute values of these parameters can be attributed to differences in conditions between the two assays, most notably the in situ generation of **1** in the radiochemical method. The assay has been implemented in 384-well microtiter plates, which will facilitate the screening of large numbers of potential GlfT2 inhibitors, which have possible utility as novel anti-TB drugs.

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