Expression and initial characterization of WbbI, a putative D-Gal*f*:α-D-Glc β-1,6-galactofuranosyltransferase from *Escherichia coli* K-12

Corin Wing,[a] James C. Errey,[b] Balaram Mukhopadhyay,[a] John S. Blanchard[b] and Robert A. Field*[a]

Received 3rd July 2006, Accepted 6th September 2006
First published as an Advance Article on the web 21st September 2006
DOI: 10.1039/b609455d

Cloning of *E. coli* K-12 *orf*8 (*wbbI*) and over-expression of the corresponding enzyme as a maltose-binding fusion protein provided recombinant WbbI β-1,6-galactofuranosyltransferase activity. Challenged with synthetic acceptor analogues in the presence of UDP-galactofuranose as a donor, WbbI showed a modest preference for pyranoside acceptor substrates of the α-D-*gluco*-configuration but it also possessed the ability to turn-over acceptor analogues.

Introduction

A wide range of pathogenic microorganisms produce macromolecules containing galactofuranose (Gal*f*): bacterial O antigens,[1,2] fungal exopolysaccharides,[3] glycolipids and cell walls,[2,4] protozoal glycoproteins and lipophosphoglycans.[5–7] Galactofuranose has been recognized to play an important structural role in *Mycobacterium tuberculosis*, where galactofuranosyltransferase (Gal*f*T) activities are essential for cell viability.[8] Sequence analysis of parasitic protozoa shows that the *Leishmania* genome encodes at least 6 candidate UDP-Gal*f* transferases and that there are more than 20 related genes present in *Trypanosoma cruzi*.[9,10] Beverley *et al.* note[1] that no candidate UDP-Gal*f* transferases have so far been reported in fungi, although they must exist given the number of Gal*f*-containing glycoconjugates known in these organisms.[11] The absence of galactofuranose in mammals[9] suggests that Gal*f* biosynthesis and metabolism has potential as a target for new anti-microbial drugs, and/or that Gal*f*-containing structures might be suitable as anti-microbial vaccines.[2]

It was originally shown in a *Salmonella* cell-free system that 5-membered ring galactofuranose-containing structures are biosynthesized from 6-membered ring UDP-galactopyranose.[12,13] Genetic and biochemical studies[14,15] showed that Gal*f* arises through the action of a novel UDP-galactopyranose mutase[16,17] that catalyzes the reversible ring-contraction of UDP-Gal*p* to UDP-Gal*f*, the putative donor substrate of galactofuranosyltransferases. The gene encoding UDP-galactopyranose mutase, GLF, was first identified by Reeves *et al.* in connection with studies on the genetics and structure of the *E. coli* K-12 O antigen, the repeat unit of which contains β-D-Gal*f* (Fig. 1).[1]

The *E. coli* K-12 *rfb* cluster,[1,18] which codes for O antigen biosynthesis, contains 11 open reading frames. Functions for most of the corresponding proteins have been identified either experimentally, or by homology to known enzymes from other species. Nassau *et al.* identified *orf*8 (otherwise known as *wbbI*, *b2034* or *yefG*)[18] of the *rfb* cluster as a putative β-1,6-galactofuranosyltransferase.[14] Assuming that this is correct,

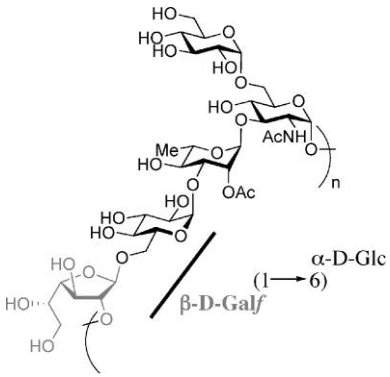


Fig. 1 Structure of the oligosaccharide repeat unit of *E. coli* K-12 O antigen,[1] with the key galactofuranose residue in grey.

the corresponding enzyme would be involved in the transfer of Gal*f* onto an α-D-*gluco*-configured acceptor substrate to form a β-1,6-linkage. To date, only the galactofuranosyltransferase that catalyses the formation of alternating β-1,5- and β-1,6-linkages in the biosynthesis of the mycobacterial cell wall has been subject to detailed analysis.[19,20] However, BLAST sequence homology analysis of WbbI and the *M. tuberculosis* transferase shows there to be no significant homology between them. Protein sequence identity between the putative β-1,6-Gal*f* transferase from *Streptococcus gordonii*, WefE, and *E. coli* WbbI is 32%.[21] It should be noted, however, that the assignment of WefE as a β-1,6-Gal*f* transferase is solely based on its sequence homology to WbbI and *S. thermophilus* Eps6N[21] (62% identity to WefE), neither of which have been experimentally characterized. Therefore, in order to confirm its assignment as a β-1,6-galactofuranosyltransferase, and to assess its suitability as a generic tool for enzymatic synthesis, we have cloned *E. coli wbbI*, over-expressed the corresponding WbbI protein in *E. coli* as a maltose binding protein (MBP) fusion protein (WbbI-MBP) and conducted a preliminary investigation of is synthetic capability.

Results and discussion

To obtain protein for analysis, the *wbbI* gene was cloned into the pMAL-c2x vector and WbbI was expressed in *E. coli* BL21 (DE3) pLysS cells as a maltose-binding protein fusion. The fusion

[a]*Centre for Carbohydrate Chemistry, School of Chemical Sciences and Pharmacy, University of East Anglia, Norwich, NR4 7TJ, U.K. E-mail: r.a.field@uea.ac.uk*
[b]*Department of Biochemistry, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, New York, 10461, USA*

protein was purified by affinity chromatography on an amylose column, typically eluting with 3 mM maltose. The resulting protein displayed an apparent molecular weight consistent with that predicted for the MalE-WbbI fusion protein (*ca.* 80 KDa) and was >90% pure, as judged by SDS-PAGE (Fig. 2). Material of this quality, of which 15 mg was obtained per litre of cell culture, was sufficiently clean for direct use in biotransformation studies.†

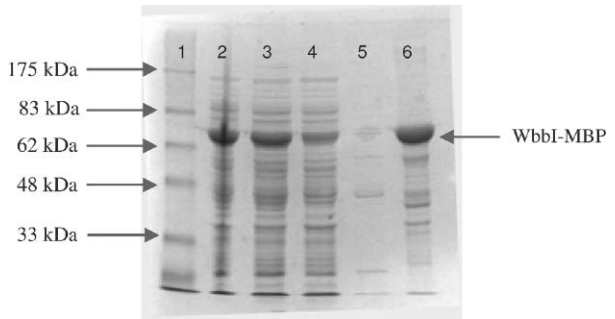
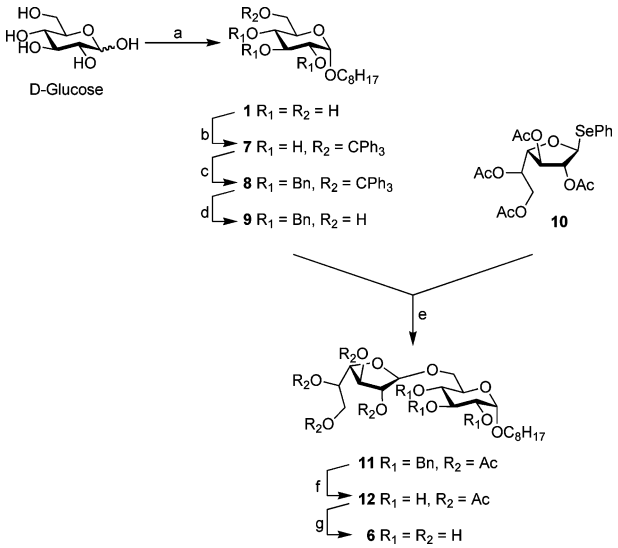


Fig. 2 Expression and purification of WbbI-MBP fusion protein. From left to right: (1) molecular weight markers; (2) cell pellet; (3) cell lysate; (4) column flow through; (5) column wash; (6) eluted WbbI-MBP fusion protein. (10% SDS-PAGE gel.)

WbbI is thought to be involved in the transfer of Gal*f* onto an α-D-glucose-based acceptor substrate to form a β-1,6-linkage. We have recently reported a convenient chemoenzymatic synthesis of UDP-Gal*f*;[22] for convenience, it can also be made *in situ* by the action of UDP-galactose mutase on commercial UDP-Gal*p*.[15] The simplest possible saccharide acceptor substrate for this enzyme is therefore an α-D-glucopyranoside. For convenient assessment of WbbI action, TLC analysis in conjunction with octyl glycoside acceptor substrates was employed.[23] Hence, octyl α-D-glucopyranoside **1** was selected as a model acceptor. In addition, substrate specificity was evaluated with a number of stereoisomers of octyl α-D-glucopyranoside (Glc*p*-α-octyl, **1**), namely octyl β-D-glucopyranoside (Glc*p*-β-octyl, **2**), octyl α-D-mannopyranoside (Man*p*-α-octyl, **3**), octyl α-D-galactopyranoside (Gal*p*-α-octyl, **4**) and the structurally similar octyl 2-acetamido-2-deoxy-α-D-glucopyranoside (GlcNAc*p*-α-octyl, **5**). These compounds were either commercially available (**1** and **2**) or were prepared essentially as described in the literature (**3**,[24] **4**,[24] **5**[25]). In addition, the putative product of WbbI action on Glc*p*-α-octyl **1**, namely octyl β-1,6-D-galactofuranosyl-α-D-glucopyranoside [Gal*f*-β-(1→6)-Glc*p*-α-octyl, **6**], was also chemically synthesized in order to validate analytical protocols. Briefly, the synthesis of authentic disaccharide product, **6**, was achieved as follows (Scheme 1). Octyl α-D-glucopyranoside **1** was prepared from D-glucose by $BF_3{\cdot}Et_2O$-promoted Fischer-type glycosylation in neat *n*-octanol; excess of the latter was subsequently removed on a silica gel column using gradient elution with CH_2Cl_2–MeOH. Selective protection of the primary alcohol of compound **1** gave trityl ether **7**, which was tri-*O*-benzylated, giving **8**, and subsequently treated with acid to give the primary alcohol **9**. Glycosylation of **9** with galactofuranosyl phenylselenide donor **10**[26] using *N*-iodosuccinimide and triflic acid afforded protected disaccharide **11**, which was hydrogenated,



Scheme 1 Synthesis of authentic disaccharide product Gal*f*-β-(1→6)-Glc*p*-α-octyl **6**. *Reagents and conditions*: (a) *n*-octanol, $BF_3{\cdot}Et_2O$, 47%; (b) trityl chloride, Py, 87%; (c) BnBr, NaH, DMF, 89%; (d) 90% aq. TFA, CH_2Cl_2, 82%; (e) NIS, TfOH, CH_2Cl_2, 78%; (f) H_2, Pd/C, EtOH, 85%; (g) NaOMe, MeOH, quant.

giving **12**, and deacetylated to afford the required octyl disaccharide **6**.

Incubation of WbbI-MBP fusion protein with *n*-octyl α-D-glucopyranoside **1** as a putative acceptor and authentic UDP-Gal*f* as a donor substrate gave a new product as a function of time, as judged by TLC (Fig. 3). Similar results were obtained regardless of whether authentic UDP-Gal*f* was used, or it was generated *in situ* from UDP-Gal*p* by the action of UDP-galactose mutase. Reactions with UDP-Gal*p* in the absence of UDP-galactose mutase gave no product.‡

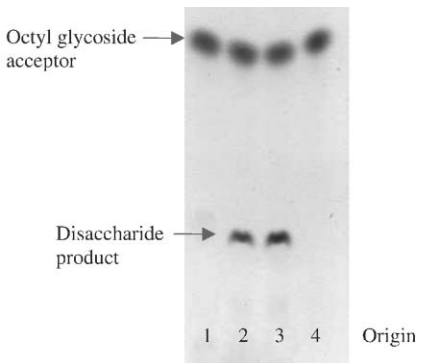


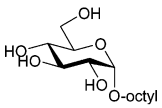
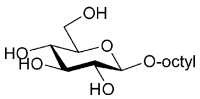
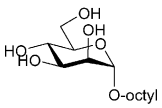
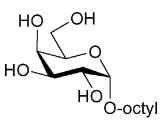
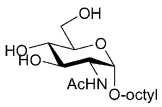
Fig. 3 TLC analysis of a time course of the *E. coli* WbbI-MBP Gal*f* transfer reaction using synthetic UDP-Gal*f* as donor and *n*-octyl α-D-glucopyranoside **1** as acceptor. (1) time = 0; (2) time = 1 h; (3) time = 3 h; (4) *n*-octyl α-D-glucopyranoside standard.

These results suggest that WbbI is a galactofuranosyltransferase which can use *n*-octyl α-D-glucopyranoside as an acceptor substrate for the addition of galactofuranose from the donor

† Cleavage of the fusion protein could be effected with factor Xa, giving WbbI as essentially a single species of molecular mass 38 236 Da (predicted 38 234 Da), as judged by electrospray ionization mass spectrometry.

‡ In enzyme activity analyses the intact WbbI-MBP fusion protein and the cleaved re-purified WbbI behaved similarly.

Table 1 Transformations and product characterization for the action of WbbI on prospective octyl glycoside acceptor substrates

Substrate	Product	Turnover	^{1}H NMR data	Mass spectrometry data
Glc*p*-α-octyl **1** 	Gal*f*-β-(1→6)-Glc*p*-α-octyl **6**	100%	H1: 4.75 ppm (3.6 Hz) H1′: 4.88 ppm (1.2 Hz)	Calcd for $C_{20}H_{38}O_{11}$ 454 Found [M + Na^+] 477.4
Glc*p*-β-octyl **2** 	Gal*f*-β-(1→6)-Glc*p*-β-octyl	<5%	—	—
Man*p*-α-octyl **3** 	Gal*f*-β-(1→6)-Man*p*-α-octyl	50%	H1: 4.69 ppm (1.2 Hz) H1′: 4.85 ppm (1.2 Hz)	Calcd for $C_{20}H_{38}O_{11}$ 454 Found [M + Na^+] 477.2
Gal*p*-α-octyl **4** 	Gal*f*-β-(1→6)-Gal*p*-α-octyl	25%	H1: 4.73 ppm (3.2 Hz) H1′: 4.78 ppm (1.2 Hz)	Calcd for $C_{20}H_{38}O_{11}$ 454 Found [M + Na^+] 477.2
GlcNAc*p*-α-octyl **5** 	Gal*f*-β-(1→6)-GlcNAc*p*-α-octyl	75%	H1: 4.76 ppm (3.3 Hz) H1′: 4.82 ppm (0.9 Hz)	Calcd for $C_{22}H_{40}NO_{11}$ 495 Found [M + Na^+] 518.2

substrate UDP-galactofuranose. In order to confirm the structure of the product formed, reactions were scaled up accordingly and octyl disaccharide product was purified by C18 reverse phase HPLC. ^{1}H NMR spectroscopy and mass spectrometry characterization showed the enzymatic product to be identical to authentic octyl β-1,6-D-galactofuranosyl-α-D-glucopyranoside [Gal*f*-β-(1→6)-Glc*p*-α-octyl, **6**] produced by chemical synthesis (see Scheme 1 and Table 1).

Table 1 summarizes a preliminary assessment of the acceptor substrate specificity of WbbI-MBP, based on challenging the enzyme with a series of octyl monosaccharide acceptor analogues (*vide supra*). Whilst the enzyme exhibits a strong preference for α-configured acceptor sugars (**1** > **5** > **3** > **4** ≫ **2**), it is less fussy about the relative configuration elsewhere on the acceptor sugar ring (**1** > **3** > **4**) and it will also tolerate replacement of the C-2 hydroxyl groups by an acetamido group (**1** *vs.* **5**). This suggests that WbbI-MBP is a potentially versatile biocatalyst for *in vitro* synthesis of Gal*f*-containing saccharides. For instance, the Gal*f*-β-(1→6)-Man*p*-α-octyl disaccharide arising from WbbI action on Man*p*-α-octyl **3** represents a fragment analogue of the phosphoglyceroglycolipid antigen of *Paracoccidioides brasiliensis*, the causative agent of a systemic mycosis, which contains Gal*f*-β-1,6-Man-β-1,3-Man-β-1,2-*myo*-inositol.[27]

Conclusions

In summary, we have cloned *E. coli* K-12 *wbbI* and over-expressed the corresponding protein as a maltose-binding fusion protein. The fusion protein shows activity as a UDP-Gal*f*-dependent β-1,6-galactofuranosyltransferase capable of glycosylating octyl α-D-glucopyranoside but not its β-anomer, in keeping with the α-configuration of the natural oligosaccharide acceptor for WbbI. The relaxed acceptor substrate specificity displayed by WbbI *in vitro* identifies this enzyme as a potentially useful biocatalyst for formation of Gal*f*-β-1,6-Gly*p* units. However, given that WbbI does not appear to require more than a single sugar residue for acceptor recognition, this raises questions about its *in vivo* specificity. Further kinetic analysis is ongoing to investigate this point.

Experimental

All chemicals were purchased from Sigma-Aldrich. *Escherichia coli* strain BL21 (DE3) pLysS cells and factor Xa were purchased from Novagen. All restriction enzymes, T4 DNA ligase, amylose resin and pMAL-c2X vector were obtained from New England Biolabs. PCR primers and pCR-Blunt plasmid kit were obtained

from Invitrogen. *Pfu* DNA polymerase was purchased from Stratagene. The *Klebsiella pneumoniae* UDP-galactopyranose mutase clone was kindly provided by Prof. Chris Whitfield, University of Guelph, Canada.[15]

General methods

Solution pH values were measured at 25 °C with an Accumet model 20 pH meter and Accumet combination electrode standardized at pH 7.0 and 4.0 or 10.0. Protein purification was performed at 4 °C using an FPLC system (Amersham-Pharmacia Biotech). Protein concentration was determined using the bicinchoninic acid (BCA) protein assay (Pierce) with bovine serum albumin as a standard. Spectrophotometric assays were performed using a UVIKON XL double beam UV-vis spectrophotometer (BIOTEK Instruments). ^{1}H NMR spectra were recorded on a Varian Gemini spectrometer at 300 MHz and are referenced to residual HOD at δ_H 4.75 ppm in D_2O. ESI mass spectra were recorded on an ABI QSTAR Pulsar QTOF spectrometer.

Cloning of *wbbI*, over-expression and purification of WbbI-MBP

The *E. coli* K-12 *wbbI* gene was cloned as described previously[14] but due to problems in obtaining soluble protein the gene was further sub-cloned. The gene was amplified by PCR using the primers: 5-GATATAGAATTCATGTATTTTTTGAATCAT-3 and 5-GAATTCGGATCCTTAGAGAGTTTTAAG-3, which were designed to create *Eco*RI and *Bam*HI restriction endonuclease sites (underlined) using the initial construct as a template. The PCR product was digested with *Eco*RI and *Bam*HI and was subjected to agarose (0.8%) gel electrophoresis. The amplified DNA product was ligated into the pCR-Blunt plasmid and transformed into One Shot TOP10 cells. Plasmid DNA isolated from these cells was then digested with *Eco*RI and *Bam*HI and the purified insert was ligated into purified plasmid pMAL-c2x previously linearized with the same restriction enzymes, yielding an expression plasmid for *E. coli* WbbI with an *N*-terminal maltose binding protein fusion tag. The plasmid (pMAL-c2x:*wbbI*) was then isolated, sequenced, and used to transform *E. coli* BL21 (DE3) pLysS cells. Transformed cells were grown overnight in 50 mL of LB broth containing 100 μg mL^{-1} ampicillin and 34 μg mL^{-1} of chloramphenicol. 1 L cultures were then inoculated to an A_{600} of ~0.05. The cells were grown at 37 °C to an A_{600} of 0.8. Cells were cooled for 1 h at 4 °C and protein expression was subsequently induced by the addition of 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) and left to grow for a further 16 h at 20 °C. Expression of the protein was confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

All protein purification steps were carried out at 4 °C. The cell pellet (30 g) was resuspended in 50 mL of 20 mM HEPES, 200 mM NaCl, pH 8, containing two tablets of complete protease inhibitor cocktail (Roche). Cells were disrupted on ice by sonication using a Branson Sonifier 450. The suspension was then centrifuged (10 000 *g* for 30 min) to remove cell debris. The supernatant was then filtered using a 0.2 μm syringe filter. The filtered supernatant was applied to a pre-equilibrated (Buffer A; 20 mM HEPES, 200 mM NaCl, pH 8) amylose column (3 × 15 cm). The column was washed at 1 mL min^{-1} with 8 column volumes of the same buffer and then eluted with a linear gradient of maltose (0–5 mM over 10 column volumes) in Buffer A. Protein was detected with an on-line detector monitoring A_{280} and column fractions were collected and analyzed by SDS-PAGE. Fractions containing the *ca.* 80 kDa protein were pooled and dialyzed twice against 4 L of 20 mM HEPES (pH 7.5), 200 mM NaCl, 1 mM EDTA, pH 8 and concentrated using a YM10 Amicon ultrafiltration membrane to a final concentration of 20 mg mL^{-1} and stored in 50% glycerol at −20 °C.

Factor Xa cleavage of WbbI-MBP

Cleavage of the fusion protein was achieved by the addition of 400 U of Factor Xa to 50 mg of fusion protein during dialysis against 4 L of 20 mM HEPES, 200 mM NaCl, 5 mM $CaCl_2$ for 36 h at 4 °C, with the subsequent re-application of the protein to the amylose column to effect removal of cleaved maltose binding protein.

Donor and acceptor substrates

UDP-Gal*f* was prepared as described previously.[23] Octyl α-D-glucopyranoside **1** and octyl β-D-glucopyranoside **2** were purchased from Sigma-Aldrich. Octyl α-D-mannopyranoside **3**,[24] octyl α-D-galactopyranoside **4**[24] and octyl 2-acetamido-2-deoxy-α-D-glucopyranoside **5**[25] were prepared essentially according to literature procedures. Authentic octyl β-D-galactofuranosyl-(1→6)-α-D-glucopyranoside **6** was prepared as described below.

Octyl 6-*O*-trityl-α-D-glucopyranoside 7. A solution of octyl α-glucoside **1** (4.0 g, 14 mmol) and trityl chloride (5.9 g, 21 mmol) in pyridine (30 mL) was stirred at room temperature for 12 h. Solvent was then removed *in vacuo* and the resulting residue was dissolved in CH_2Cl_2 (50 mL) and washed successively with 1 M HCl (2 × 50 mL), $NaHCO_3$ solution (2 × 50 mL) and H_2O (50 mL). The organic extract was dried (Na_2SO_4) and concentrated to a syrup. Purification of the crude product on silica gel column using EtOAc afforded trityl ether **7** (6.4 g, 87%) as a light yellow gum. $[\alpha]_D^{23}$ +131 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 7.46–7.21 (15H, ArH), 4.85 (d, 1H, $J_{1,2}$ = 4.0 Hz, H-1), 3.77–3.67 (m, 2H, H-3, O–CH_2), 3.53–3.42 (m, 3H, H-2, H-4, H-6a), 3.42–3.32 (m, 2H, H-6b, O–CH_2), 2.98 (m, 1H, H-5), 2.69 (bs, 1H, OH), 2.22 (bd, 2H, 2 OH), 1.63 (m, 2H, O–CH$_2$–CH_2), 1.34 (m, 10H, octyl-CH_2), 0.93 (t, 3H, octyl-CH_3). ^{13}C NMR ($CDCl_3$) δ: 143.8, 128.7, 128.0, 127.2 (ArC), 97.9 (*C*-1), 87.0, 74.9, 72.3, 71.7, 70.0, 68.3, 63.9 (C-6), 31.7, 29.4, 29.3, 29.1, 26.1, 22.5, 14.0. HRMS [M + NH_4]$^+$ calcd. for $C_{33}H_{46}O_6N$ 552.3325, found 552.3321.

Octyl 2,3,4-tri-*O*-benzyl-α-D-glucopyranoside 9. To a solution of trityl ether **7** (4.0 g, 7.5 mmol) in DMF (30 mL), was added NaH (810 mg, 50% suspension in mineral oil) followed by BnBr (3.2 mL, 27 mmol) and the mixture was stirred at room temperature for 8 h. Excess NaH was destroyed by careful addition of MeOH and solvent was removed *in vacuo*. The resulting residue was dissolved in CH_2Cl_2 (70 mL) and washed with H_2O (3 × 70 mL); the organic extract was dried (Na_2SO_4) and concentrated to a syrup. The crude product was purified on a silica gel column using EtOAc–*n*-hexane (1 : 5) to give octyl 2,3,4-tri-*O*-benzyl-6-*O*-trityl-α-D-glucopyranoside **8** (5.4 g, 89%) as light yellow oil. An aqueous solution of TFA (90% v/v, 10 mL) was added to a solution of compound **8** (5.0 g, 6.2 mmol) in CH_2Cl_2 (30 mL) and the mixture was stirred at room temperature for 30 min. The reaction mixture

was diluted with CH_2Cl_2 (20 mL) and washed successively with H_2O (50 mL), aqueous $NaHCO_3$ solution (2 × 50 mL) and brine (50 mL). The organic extract was dried (Na_2SO_4) and concentrated to a syrup. The crude product was purified on a silica gel column using EtOAc–*n*-hexane (1 : 3) to give compound **9** (2.9 g, 82%) as a foam. $[\alpha]_D^{23}$ +103 (*c* 1.2, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 7.39–7.26 (m, 15H, ArH), 5.04–4.65 (6d, 6H, J_{AB} = 10.8 Hz, 3CH_2Ph), 4.71 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.04 (t, 1H, $J_{2,3}$ = $J_{3,4}$ = 9.3 Hz, H-3), 3.80–3.66 (m, 4H, H-5, H-6a, H-6b, OCH_2), 3.64 (t, 1H, $J_{3,4}$, $J_{4,5}$ = 10.2 Hz, H-4), 3.51 (dd, 1H, $J_{1,2}$, $J_{2,3}$, H-2), 3.42 (m, 1H, OCH_2), 2.28 (bs, 1H, OH), 1.61 (m, 2H, O–CH_2–CH_2), 1.33 (m, 10H, octyl-CH_2), 0.92 (t, 3H, octyl-CH_3). ^{13}C NMR ($CDCl_3$) δ: 138.9, 138.4, 138.2, 128.6, 128.5, 128.4, 128.2, 128.0, 127.9, 127.8, 127.6 (ArC), 96.8 (*C*-1), 81.9, 80.2, 75.6, 75.0, 73.1, 70.6, 68.2, 61.9 (*C*-6), 31.7, 29.3, 29.1, 26.1, 22.5 (2), 14.0. HRMS [M + NH_4]$^+$ calcd. for $C_{35}H_{50}O_6N$ 580.3638, found 580.3635.

Octyl 2,3,5,6-tetra-*O*-acetyl-β-D-galactofuranosyl-(1→6)-2,3,4-tri-*O*-benzyl-α-D-glucopyranoside 11. A mixture of primary alcohol **9** (2.0 g, 3.6 mmol), galactofuranosyl phenylselenide **10**[26] (2.3 g, 4.6 mmol) and 4 Å molecular sieves (3 g) in CH_2Cl_2 (30 mL) was stirred under nitrogen for 2 h. After cooling to 0 °C, NIS (1.4 g, 6 mmol) was added followed by TfOH (53 μL, 0.6 mmol) and the mixture was stirred at 0 °C for 45 min. After complete consumption of the starting material, the mixture was diluted with CH_2Cl_2 (20 mL), filtered through Celite and washed successively with aqueous $Na_2S_2O_3$ solution (2 × 50 mL), $NaHCO_3$ solution (2 × 50 mL) and brine (50 mL). The organic extract was dried (Na_2SO_4) and concentrated to a syrup. The crude product was purified on a silica gel column using EtOAc–*n*-hexane (1 : 3) to give pure compound **7** (2.5 g, 78%) as a foam. $[\alpha]_D^{23}$ +78 (*c* 0.9, $CHCl_3$). ^{1}H NMR ($CDCl_3$) δ: 7.39–7.25 (m, 15H, ArH), 5.39 (m, 1H, H-5′), 5.09 (d, 1H, $J_{2',3'}$ = 2.1 Hz, H-2′), 5.04 (s, 1H, H-1′), 5.01 (dd, 1H, $J_{2',3'}$, $J_{3',4'}$ = 6.0 Hz, H-3′), 5.05–4.59 (6H, J_{AB} = 11.2 Hz, 3CH_2Ph), 4.75 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.33 (dd, 1H, $J_{5',6a'}$ = 4.2 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6a′), 4.28 (m, 1H, H-5′), 4.21 (dd, 1H, $J_{5',6b'}$ = 7.5 Hz, $J_{6a',6b'}$, H-6b′), 4.02 (t, 1H, $J_{2,3}$, $J_{3,4}$ = 9.0 Hz, H-3), 3.86 (m, 1H, H-6a), 3.80 (dd, 1H, $J_{3',4'}$ = 9.9 Hz, $J_{5',6a'}$, H-4′), 3.69–3.63 (m, 2H, H-6b, OCH_2), 3.58 (t, 1H, $J_{3,4}$, H-4), 3.55–3.49 (m, 2H, H-2, H-5), 3.41 (m, 1H, OCH_2), 2.13, 2.06, 2.04, 2.03 (4s, 12H, 4 COCH_3), 1.64 (m, 2H, O–CH_2–CH_2), 1.28 (m, 10H, octyl-CH_2), 0.94 (t, 3H, octyl-CH_3). ^{13}C NMR ($CDCl_3$) δ: 170.5, 170.0, 169.9, 169.4 (4 *C*OCH$_3$), 138.8, 138.3 (2), 128.4, 128.3 (2), 127.9, 127.8, 127.7, 127.6, 127.5 (ArC), 105.7 (*C*-1′), 96.5 (*C*-1), 81.9, 81.0, 80.0, 79.5, 77.6, 76.2, 75.5, 74.9, 72.8, 69.7, 69.0, 68.0, 66.3, 62.6 (*C*-6), 31.6, 29.2, 29.1, 29.0, 26.0, 22.4, 20.5, 20.4, 20.3 (2), 13.8. HRMS [M + NH_4]$^+$ calcd. for $C_{49}H_{68}O_{15}N$ 910.4583, found 910.4586.

Octyl 2,3,5,6-tetra-*O*-acetyl-β-D-galactofuranosyl-(1→6)-α-D-glucopyranoside 12. To a solution of protected disaccharide **11** (2.0 g, 2.2 mmol) in EtOH (25 mL) was added 10% Pd/C (70 mg); the mixture was stirred at room temperature under a positive hydrogen pressure for 48 h. After filtration through Celite, the filtrate was evaporated and dried under vacuum. Column chromatography of the crude product with EtOAc as eluent afforded partially esterified disaccharide **12** (1.2 g, 85%) as a foam. $[\alpha]_D^{23}$ +48 (*c* 1.1, MeOH). ^{1}H NMR (CD_3OD) δ: 5.36 (m, 1H, H-5′), 5.14 (s, 1H, H-1′), 5.03 (d, 1H, $J_{2',3'}$ = 1.8 Hz, H-2′), 4.98 (dd, 1H, $J_{2',3'}$, $J_{3',4'}$ = 5.7 Hz, H-3′), 4.76 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 4.32 (dd, 1H, $J_{5',6a'}$ = 4.2 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6a′), 4.32 (t, 1H, $J_{3',4'}$, $J_{4',5'}$ = 5.7 Hz, H-4′), 4.19 (dd, 1H, $J_{5',6b'}$ = 7.2 Hz, $J_{6a',6b'}$ = 12.0 Hz, H-6b′), 3.93 (bd, 1H, H-6a), 3.74–3.60 (m, 2H, H-6b, OCH_2), 3.45 (t, 1H, $J_{2,3}$, $J_{3,4}$ = 5.7 Hz, H-3), 3.43 (m, 1H, OCH_2), 3.38 (dd, 1H, $J_{1,2}$, $J_{2,3}$, H-2), 3.34–3.25 (m, 2H, H-4, H-5), 2.09, 2.08, 2.06, 2.02 (4s, 12H, 4 COCH_3), 1.63 (m, 2H, O–CH_2–CH_2), 1.38 (m, 10H, octyl-CH_2), 0.96 (t, 3H, octyl-CH_3). ^{13}C NMR (CD_3OD) δ: 172.4, 172.0, 171.8, 171.4 (4 *C*OCH$_3$), 107.1 (*C*-1′), 100.0 (*C*-1), 82.5, 81.4, 78.0, 75.0, 73.4, 72.7, 71.8, 70.8, 69.2, 67.7, 63.8, 32.9, 30.5, 30.4, 30.3, 27.2, 23.6, 20.7, 20.6 (2), 20.5 (4 CO*C*H$_3$), 14.4. HRMS [M + NH_4]$^+$ calcd. for $C_{28}H_{50}O_{15}N$ 640.3175, found 640.3181.

Octyl β-D-galactofuranosyl-(1→6)-α-D-glucopyranoside 6. To a solution of partially acetylated disaccharide **8** (1.0 g, 1.6 mmol) in MeOH (20 mL) was added methanolic NaOMe (2 mL, 0.5 M); the mixture was stirred at room temperature for 2 h. After neutralization with Dowex 50 W × 8 (H^+) resin, the mixture was filtered through Celite and the filtrate was evaporated to afford octyl disaccharide **9** (730 mg, quantitative) as a white powder. $[\alpha]_D^{23}$ +43 (*c* 1.0, MeOH). ^{1}H NMR (CD_3OD) δ: 4.88 (d, 1H, $J_{1',2'}$ = 1.2 Hz, H-1′), 4.75 (d, 1H, $J_{1,2}$ = 3.6 Hz, H-1), 1.61 (m, 2H, O–CH_2–CH_2), 1.29 (m, 10H, octyl-CH_2), 0.95 (t, 3H, octyl-CH_3). ^{13}C NMR (CD_3OD) δ: 111.8 (*C*-1′), 100.5 (*C*-1), 88.0, 82.1, 80.7, 75.3, 73.6, 73.1, 72.4, 72.2, 69.5, 67.9, 65.0, 33.0, 30.6, 30.5, 30.4, 27.3, 23.7, 14.4. HRMS [M + NH_4]$^+$ calcd. for $C_{20}H_{42}O_{11}N$ 472.2752, found 472.2754.

Measurement of enzyme activity

Assay mixtures contained 50 mM MOPS, 2 mM magnesium chloride, 5 mM sodium dithionite pH 7.5, in addition to 10 mM acceptor and 10 mM UDP-galactopyranose. Reactions were initiated by the addition of 50 μM UDP-galactopyranose mutase[15] and 125 μM galactofuranosyltransferase fusion protein in a total volume of 100 μL and were maintained at 37 °C. Reactions were terminated with 50 μL ethanol, followed by centrifugation at 10 000 rpm for 5 min. Reactions were monitored by TLC using glass-backed 10 cm by 10 cm, 250 μm, 60 Å K6F silica gel TLC plates run in $CHCl_3$–CH_3OH–H_2O (65 : 25 : 4.1) using a variation of a literature method.[28] Compounds were detected with an orcinol solution (20 mg of orcinol dissolved in 10 mL of 70% sulfuric acid) by heating at 100 °C. Spots containing sugars appeared pink-violet on a white background. The detection limit was *ca.* 0.5 nmol (*ca.* 1 μg) of total sugar per spot. Octyl glycoside turnover was estimated using a densitometer (SynGene Gel Analysis and Documentation System).

Product purification and analysis

Octyl glycoside (*ca.* 8 mg, 27 μmol) and UDP galactopyranose (40 mg, 65 μmol) were dissolved in buffer (20 mL, 50 mM TRIS, 10 mM sodium dithionate and 2 mM $MgCl_2$, pH 8.0). Purified recombinant *Klebsiella pneumoniae* UDP-galactopyranose mutase (4 mg, 93 nmol)[15] and purified UDP-galactofuranosyltransferase fusion protein (10 mg, 125 nmol) was added, the solution was flushed with nitrogen and the reaction was stirred at 30 °C for 16 h. The reaction mixture was passed through an Amicon YM10 spin filter (13 000 rpm, 4 °C). A proportion of the flow through was applied to a C18 Phenomenex Luna 5 μ column (4.6 mm × 250 mm) and eluted with a gradient from 0.5% to

99.5% acetonitrile in water over 25 column volumes at a flow rate of 1 mL min^{-1}. UDP galactopyranose eluted at the beginning of the gradient, whilst octyl disaccharide typically eluted at *ca.* 50% acetonitrile. Relevant samples were combined and concentrated *in vacuo* to give the required compounds, which were subjected to NMR and mass spectrometry analysis (Table 1).

Acknowledgements

This work was supported by the EPSRC and the Weston Foundation. We are indebted to the EPSRC Mass Spectrometry Service Centre, Swansea, for invaluable support. We thank Dr Steve Martin (GSK, Stevenage, UK) for helpful discussions during the early phases of this project and Prof. Chris Whitfield, University of Guelph, Canada, who kindly provided the UDP-galactopyranose mutase clone.[15]

References

1 G. Stevenson, B. Neal, D. Liu, M. Hobbs, N. H. Packer, M. Batley, J. W. Redmond, L. Lindquist and P. Reeves, *J. Bacteriol.*, 1994, **176**, 4144–4156.
2 L. L. Pedersen and S. J. Turco, *Cell. Mol. Life Sci.*, 2003, **60**, 259–266.
3 W. N. Haworth, H. Raistrick and M. Stacey, *Biochem. J.*, 1937, **31**, 640–644.
4 J. P. Latgé, H. Kobayashi, J. P. Debeaupuis, M. Diaquin, J. Sarfati, J. M. Wieruszeski, E. Parra, J. P. Bouchara and B. Fournet, *Infect. Immun.*, 1994, **62**, 5424–5433.
5 D. H. Mendelzon and A. J. Parodi, *J. Biol. Chem.*, 1986, **261**, 2129–2133.
6 J. O. Previato, R. Wait, C. Jones, G. A. DosReis, A. R. Todeschini, N. Heise and L. M. Previato, *Adv. Parasitol.*, 2004, **56**, 1–41.
7 R. M. de Lederkremer and W. Colli, *Glycobiology*, 1995, **5**, 547–552.
8 F. Pan, M. Jackson, Y. Ma and M. McNeil, *J. Bacteriol.*, 2001, **183**, 3991–3998.
9 S. M. Beverley, K. L. Owens, M. Showalter, C. L. Griffith, T. L. Doering, V. C. Jones and M. R. McNeil, *Eukaryotic Cell*, 2005, **4**, 1147–1154.
10 K. Zhang, T. Barron, S. J. Turco and S. M. Beverley, *Mol. Biochem. Parasitol.*, 2004, **136**, 11–23.
11 Although no galactofuranosyltransferase genes have been identified in fungi, the UDP-galactose mutase from *Aspergillus fumigatus* has recently been cloned: H. Bakker, B. Kleczka, R. Gerardy-Schahn and F. H. Routier, *Biol. Chem.*, 2005, **386**, 657–661.
12 M. Sarvas and H. Nikaido, *J. Bacteriol.*, 1971, **105**, 1063–1072.
13 H. Nikaido and M. Sarvas, *J. Bacteriol.*, 1971, **105**, 1073–1082.
14 P. M. Nassau, S. L. Martin, R. E. Brown, A. Weston, D. Monsey, M. R. McNeil and K. Duncan, *J. Bacteriol.*, 1996, **178**, 1047–1052.
15 R. Koplin, J.-R. Brisson and C. Whitfield, *J. Biol. Chem.*, 1997, **272**, 4121–4128.
16 D. A. R. Sanders, A. G. Staines, S. A. McMahon, M. R. McNeil, C. Whitfield and J. H. Naismith, *Nat. Struct. Biol.*, 2001, **8**, 858–863.
17 M. Soltero-Higgin, E. E. Carlson, T. D. Gruber and L. L. Kiessling, *Nat. Struct. Biol.*, 2004, **11**, 539–543.
18 F. R. Blattner, G. Plunkett, C. A. Bloch, N. T. Perna, V. Burland, M. Riley, J. Collado-Vides, J. D. Glasner, C. K. Rode, G. F. Mayhew, J. Gregor, N. W. Davis, H. A. Kirkpatrick, M. A. Goeden, D. J. Rose, B. Mau and Y. Shao, *Science*, 1997, **277**, 1453–1474.
19 L. Kremer, L. G. Dover, C. Morehouse, P. Hitchini, M. Everett, H. R. Morris, A. Dell, P. J. Brennan, M. R. McNeil, C. Flaherty, K. Duncan and G. S. Besra, *J. Biol. Chem.*, 2001, **276**, 26430–26440.
20 N. L. Rose, G. C. Completo, S.-J. Lin, M. McNeil, M. M. Palcic and T. L. Lowary, *J. Am. Chem. Soc.*, 2006, **128**, 6721–6729.
21 D. Q. Xu, J. Thompson and J. O. Cisar, *J. Bacteriol.*, 2003, **185**, 5419–5430.
22 J. C. Errey, B. Mukhopadhyay, K. P. Kartha and R. A. Field, *Chem. Commun.*, 2004, 2706–2707.
23 J. R. Brown, T. K. Smith, M. A. J. Ferguson and R. A. Field, *Bioorg. Med. Chem. Lett.*, 1998, **8**, 2051–2054.
24 T. Mizutani, T. Kurahashi, T. Murakami, N. Matsumi and H. Ogoshi, *J. Am. Chem. Soc.*, 1997, **119**, 8991–9001.
25 M. Nuchter, B. Ondruschka and W. Lautenschlager, *Synth. Commun.*, 2001, **31**, 1277–1284.
26 B. D. Johnston and B. M. Pinto, *Carbohydr. Res.*, 1997, **305**, 289–292.
27 S. B. Levery, M. S. Toledo, E. Suzuki, M. E. Salyan, S. Hakomori, A. H. Straus and H. K. Takahashi, *Biochem. Biophys. Res. Commun.*, 1996, **15**, 639–45.
28 A. K. Pathak, V. Pathak, L. Seitz, J. A. Maddry, S. S. Gurch, G. S. Besra, W. J. Suling and R. C. Reynolds, *Bioorg. Med. Chem.*, 2001, **9**, 3129–3143.

