Available online at www.sciencedirect.com
SCIENCE DIRECT®
Bioorganic & Medicinal Chemistry Letters 15 (2005) 1205–1211
Bioorganic & Medicinal Chemistry Letters

Identification of a UDP-Gal: GlcNAc-R galactosyltransferase activity in *Escherichia coli* VW187

Pedro J. Montoya-Peleaz,[a] John G. Riley,[a,b] Walter A. Szarek,[a] Miguel A. Valvano,[c] John S. Schutzbach[b] and Inka Brockhausen[b,*]

[a]*Department of Chemistry, Queen's University, Kingston, Ontario, Canada K7L 3N6*
[b]*Department of Medicine, Department of Biochemistry, The Arthritis Centre and Human Mobility Research Centre, Queen's University, Kingston General Hospital, Kingston, Ontario, Canada K7L 2V7*
[c]*Department of Microbiology and Immunology, University of Western Ontario, London, Ontario, Canada N6A 5C1*

Received 18 September 2004; revised 29 November 2004; accepted 30 November 2004
Available online 24 December 2004

Abstract—A novel acceptor substrate for galactosyltransferase was synthesized containing GlcNAcα-pyrophosphate, covalently bound to a hydrophobic phenoxyundecyl moiety (GlcNAc α-O-PO_3-PO_3-$(CH_2)_{11}$-O-Phenyl). The new substrate was used to develop an assay for a galactosyltransferase activity from *Escherichia coli* strain VW187 that is involved in lipopolysaccharide synthesis and has not been studied by others. We showed that Gal was transferred from UDP-Gal to the novel acceptor substrate. This was a significant improvement over our previous preliminary assays of the enzyme using endogenous substrate, and showed that these synthetic substrates are useful for assaying enzymes that utilize lipid-bound substrates in O-chain synthesis in Gram-negative bacteria.

1. Introduction

The O-specific polysaccharide (O antigen) is the outermost component of the lipopolysaccharide (LPS), a major constituent of the outer membrane in Gram-negative bacteria. The outer O-chain of the LPS of *Escherichia coli* strain VW187 (O7:K1) consists of repeats of 3-VioNAc β1–2 [Rha α1–3]Man α1–4Gal β1–3 GlcNAc α1, where VioNAc is 4-acetamido-4,6-dideoxy-D-glucose.[1] The assembly of the repeat unit is thought to occur at the cytosolic face of the plasma membrane and involves a lipid-linked intermediate containing GlcNAcα1-pyrophosphoryl-undecaprenol (GlcNAc-PP-Und).[2] Additional sugars are added sequentially to the GlcNAc-PP-Und intermediate to complete the formation of the O7 subunit. Subsequently, the subunits are polymerized and then linked to lipopolysaccharide A to form LPS. These reactions are catalyzed by specific glycosyltransferases, which are either soluble enzymes or associated with the plasma membrane.

In the past, it has been very difficult to assay and characterize these enzymes because of the lack of adequate amounts of endogenous sugar-PP-Und substrate and unavailability of defined and stable synthetic substrates.[3] Our preliminary assays using endogenous acceptor substrate for galactosyltransferase from VW187 called for improved and defined assay conditions. The enzyme has not been studied by others.

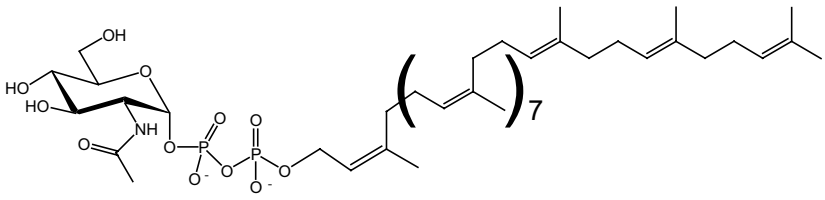
In this work, we have attempted to overcome some of these problems by chemically synthesizing an analog of the sugar-PP-Und acceptor. GlcNAc was the preferred sugar for this molecule given that it is widely used by many bacteria for the initiation reactions required for the synthesis of the O antigen repeating units.[4,5] Therefore, in this work, we report the chemical synthesis of GlcNAcα-pyrophosphate bound to a phenoxyundecyl moiety (Fig. 1) in [GlcNAc α1-O-PO_2-O-PO_2-O-$(CH_2)_{11}$-O-Phenyl]$^{2-}$ (GlcNAc-PP-PhU). This synthetic substrate was then utilized in the development of an efficient in vitro assay for a galactosyltransferase activity present in *E. coli* VW187.

2. Materials

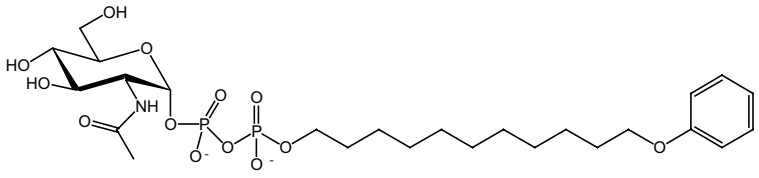
All reagents were of the highest grade available and were from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated. Protein concentrations were determined

Keywords: O-Antigen synthesis; Galactosyltransferase; Enzyme assay.
* Corresponding author. Tel.: +1 613 549 6666x6355; fax: +1 613 549 2529; e-mail: brockhau@post.queensu.ca

0960-894X/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.bmcl.2004.11.077



GlcNAc-PP-Und



GlcNAc-PP-PhU

Figure 1. Chemical structures of the GlcNAcα-pyrophosphoryl-undecaprenol (GlcNAc-PP-Und) and the novel synthetic GlcNAcα-pyrophosphate-phenoxyundecyl (GlcNAc-PP-PhU) acceptor substrates.

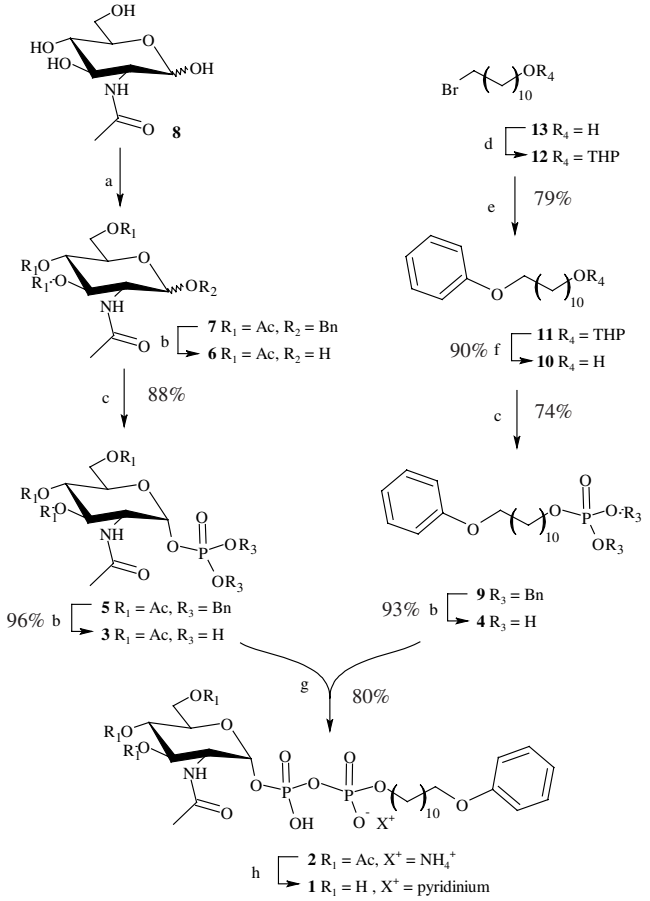
with the BioRad (Bradford) protein assay using bovine serum albumin as a standard. *Escherichia coli* strain VW187 (*E. coli* O7:K1) has been previously described.[4,6]

3. Preparation of enzyme source

Bacteria were grown at 37 °C in LB medium containing 100 μg/mL ampicillin, inoculated with 10 mL of an overnight culture of VW187. Cultures were incubated at 37 °C on a shaker at 250 rpm. After 2.5 h (A_{600} = 0.8) the bacteria were harvested by centrifugation at 5000 rpm for 10 min (IEC MultiRF Rotor). Cells were resuspended in 12 mL of PBS, pH 7.2/glycerol (v/v) 9:1, and were stored at −20 °C. All additional procedures were carried out at 4 °C. The bacterial cells were sedimented by centrifugation, washed with 3 mL of PBS, pH 7.2, and then resuspended in 0.5 mL of 10 mM Tris/acetate, pH 8.5 containing 50 mM sucrose and 1.2 mM EDTA. The cells were sonically ruptured using a Sonic Dismembrator Model 100 (Fisher Scientific) for 2 pulses of 15 s with 2 min intervals to allow for cooling on ice. Protein concentrations ranged between 2 and 12 mg/mL.

4. Synthesis of acceptor substrate

The synthesis of the GlcNAc-PP-PhU acceptor is shown in Scheme 1. All nonhydrolytic reactions were performed in oven-dried glassware under a dry nitrogen atmosphere. Dichloromethane and tetrahydrofuran (THF) were dried over CaH_2 and sodium, respectively, and then distilled. The ^1H, ^{13}C, and ^{31}P NMR spectra were recorded using Bruker Avance 300-, 400-, 500-, and 600-MHz spectrometers. Chemical shifts (δ) are reported in parts per million (ppm), and signals are described as s (singlet), d (doublet), dd (doublet of doublets), t (triplet), q (quartet), quin (quintet), and m (multiplet). Proton chemical shifts are given relative to those of internal standards $CHCl_3$, CH_3OD, or DMSO: δ = 7.26, 3.30, or 2.25 ppm, respectively. Carbon



Scheme 1. Synthesis of the GlcNAcα-*P*-*P*-phenoxyundecyl acceptor substrate. Reagents and conditions: (a) 1. NaH, DMF, benzyl bromide; 2. Pyridine, acetic anhydride, 0 °C; (b) Pd/C, CH_3OH, H_2; (c) *i*-$Pr_2NP(OBn)_2$, ^1H-tetrazole, CH_2Cl_2, −30 → 0 °C, 0.5 h, then *m*-CPBA, −40 → 25 °C, overnight; (d) Dihydropyran, CH_2Cl_2, TsOH, 0 °C → rt, 16 h; (e) Phenol, KOH, Aliquat 336, 85 °C, overnight; (f) CH_3OH, TsOH, 1.5 h, rt; (g) 1. **4**, 1,1′-carbonyldiimidazole, THF, 1.5 h, rt then CH_3OH; 2. **3**, THF, 48 h, rt; (h) $NaOCH_3$, CH_3OH, 20 min, rt, quenched with pyridinium resin. The % yield for each step is shown.

chemical shifts are given relative to those of $CDCl_3$, CD_3OD, or DMSO: $\delta = 77$, 49, or 39.5 ppm, respectively. Phosphorus chemical shifts are given relative to that of 85% phosphoric acid: $\delta = 0$ ppm. Mass spectra were obtained using an Applied Biosystems/MDS Sciex QStar XL spectrometer. TLC was performed using glass- or aluminum-backed Silicyle Silica Gel 60 F_{254}. Reactions were monitored by charring of the plates after spraying with either 5% H_2SO_4 in ethanol, or phosphomolybdic acid (PMA) in ethanol.

The GlcNAc sugar head group and the phospholipid tail were assembled separately and then coupled using well-established methodology. Thus, GlcNAc **8** (Scheme 1) was selectively protected at the anomeric position with a benzyl group,[7] and the remaining hydroxyl groups were acetylated. The anomeric position was then deprotected by hydrogenolysis, and the product was treated with *i*-$Pr_2NP(OBn)_2$ (dibenzyl diisopropylphosphoramidite)[8] to afford the glycosyl phosphite, which was oxidized to produce dibenzyl phosphate **5**.[9,10] Synthesis of the tail fragment required initial protection of the hydroxyl group of **13** with a THP group,[11] attachment of the phenoxy group,[12] followed by the removal of the THP group. The dibenzyl phosphate **9** was synthesized using the same conditions as employed in the case of compound **5**. Both **5** and **9** were then debenzylated by hydrogenolysis. Compound **4** was then activated by forming the phosphorimidazolidate, and compound **3** was then added.[10,13] Finally, the acetyl groups were removed using $NaOCH_3$ in methanol.[14]

5. Galactosyltransferase assay using synthetic acceptor substrate

Standard assays for galactosyl transfer to exogenously added substrate were carried out in reaction mixtures of 40 μL total volume, containing 0.5 mM GlcNAc-PP-PhU (pyridinium salt), 5 mM $MnCl_2$, 75 mM MES buffer pH 7, 0.5 mM UDP-[^{3}H]Gal (800–4400 cpm/nmol), and 20 μL of enzyme homogenate (3–12 μg protein). The reaction was stopped by the addition of 0.7 mL of ice cold water and the mixtures were applied to a 1 mL C18 Sep-Pak column that had been prewashed with 4 mL methanol followed by 6 mL water. Columns were then washed with 5 mL water and the hydrophobic product was eluted with 5 mL methanol. Fractions of 1 mL were collected and 0.5 mL samples of each fraction were counted in 5 mL Ready Safe scintillation fluid (Beckman). All assays were carried out in duplicate. For further analysis of the enzyme product, the first fraction eluted with methanol from the Sep-Pak columns was concentrated and injected into HPLC, using a C18 column and acetonitrile/water (15/85) at 1 mL/min flow rate.[15] The absorbance at 195 nm of the eluant was measured. Fractions of 2 mL were collected and the radioactivity measured by scintillation counting. The molecular weight of the enzyme product isolated from HPLC was determined by Matrix-assisted laser desorption ionization (MALDI) mass spectrometry in the negative reflectron mode (Applied Biosystems Voyager DE-STR MALDI-TOF) by Dr. B. Keller at the Department of Chemistry, Queen's University, Kingston ON. The matrix substance was 2,5-dihydroxybenzoic acid.

6. The synthetic GlcNAc-PP-PhU is an excellent acceptor substrate

The novel acceptor substrate GlcNAc-PP-PhU has amphipathic properties similar to those of the endogenous substrate GlcNAc-PP-Und, is soluble in methanol/water mixtures, and can be added directly to the reaction mixtures. The chemical and spectrometric analyses of synthetic GlcNAc α-PP-PhU showed that nearly 100% of the α-anomer was synthesized. The compound was stable at −20 °C in methanol for at least 6 months. The pyridinium salt of GlcNAc α-PP-PhU bound to C18 Sep-Pak cartridges and could be eluted with methanol and subsequently analyzed by HPLC (Fig. 2).

Our results show that GlcNAc α-PP-PhU salts are excellent substrates for galactosyltransferase from VW187 homogenates. These amphipathic compounds can easily be synthesized. Other advantages are that the substrate and the enzymatic reaction products are readily separated by HPLC using a C18 column and acetonitrile/water mixtures as the mobile phase, and that compounds containing the phenyl group can be readily detected with UV absorbance.

The Gal-transferase activity of strain VW187 catalyzed galactosyl transfer from UDP-Gal to the GlcNAc α-PP-PhU acceptor in standard assays. More than 95% of the enzyme product was eluted in the first methanol fraction from C18 Sep-Pak columns. HPLC separation revealed the presence of a new enzyme product that eluted approximately 5 min before the unmodified acceptor substrate (Fig. 2). Gal-transferase activity was linear with time for more than 10 min (Fig. 3A) and was proportional to enzyme concentration up to at least 5 μg protein per assay (Fig. 3B). Ion exchange chromatography of the fractions that did not bind to C18 Sep-Pak showed that more than 80% of the total amount of UDP-Gal added was still present after the 10 min incubation.

Enzyme product from assays using VW187 homogenates containing UDP-[^{3}H]Gal as the donor substrate and GlcNAc α-PP-PhU as the acceptor substrate was also isolated by HPLC for structural analysis. The MALDI mass spectrum showed a major $[M-H]^-$ peak at 788.6 *m/z*, and a minor peak at 626.5 *m/z* (Fig. 4). The major peak was consistent with the molecular weight of the Gal-transferase product [^{3}H]Gal-GlcNAc-O-PO_3-PO_3-$(CH_2)_{11}$-O-Phenyl (789 Da).

We therefore demonstrated that the newly assayed Gal-transferase activity in sonicated whole bacteria transfers Gal from UDP-Gal to GlcNAc-PP-PhU. GlcNAc-PP-PhU is an excellent substrate, and appears to be highly accessible to the enzyme, which is thought to be associated with the inner surface of the plasma membrane.

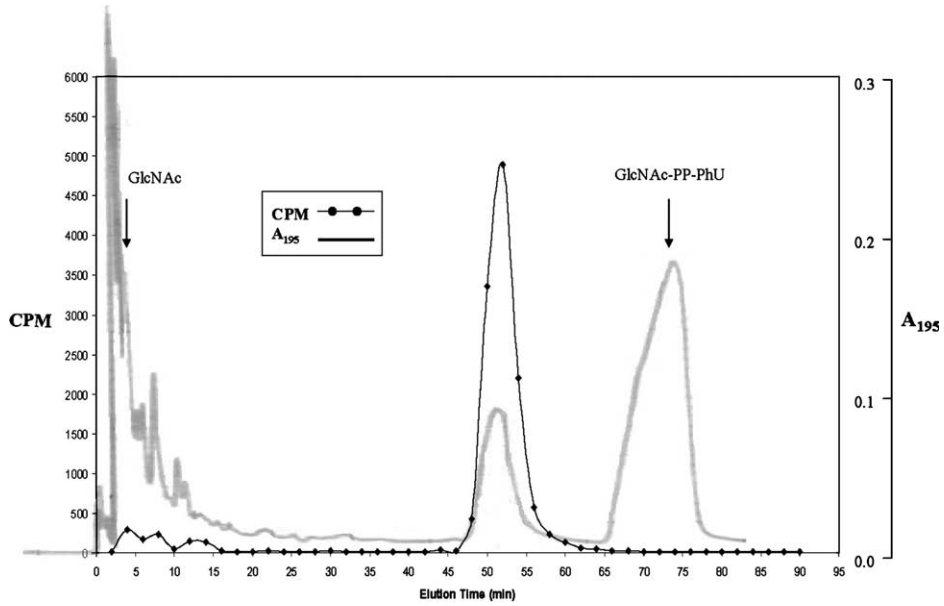


Figure 2. HPLC analysis of Gal-transferase reaction product using GlcNAc-PP-PhU as an acceptor substrate. 0.5 mM GlcNAc-PP-PhU was incubated for 10 min with homogenates from VW187 as described in Experimental Procedures. The reaction product was isolated on a C18 Sep-Pak column, by sequential fractionation with water eluting unreacted UDP-Gal and free Gal, and methanol to elute the enzyme product. The methanol fractions were concentrated, and aliquots analyzed by HPLC using a C18 column and acetonitrile/water (15/85) as the mobile phase at a flow rate of 1 mL/min. The absorbance at 195 nm was measured and the radioactivity in eluting fractions was counted. Standard compounds were GlcNAc, and substrate GlcNAc-PP-PhU.

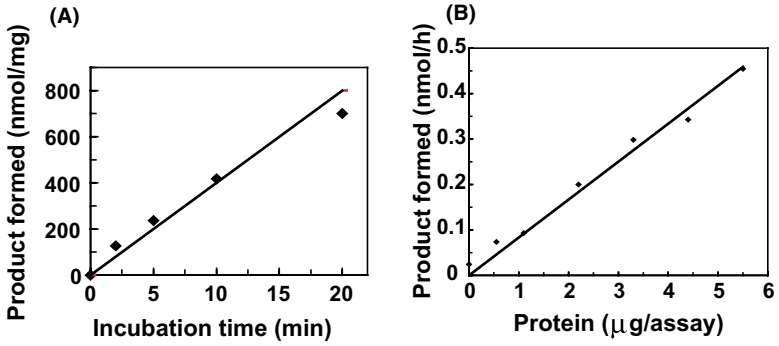


Figure 3. Initial rate of galactosyltransferase activity. Galactosyltransferase activity was assayed using UDP-[^{3}H]Gal as the donor substrate and GlcNAc-PP-UPh as the acceptor substrate at conditions described in Section 5. Enzyme product was isolated by C18 Sep-Pak columns. (A) Dependence of activity on incubation time. (B) Dependence of activity on protein concentration.

However, the membrane association and localization of the enzyme remains to be determined. Chen et al. synthesized a series of lipid-linked oligosaccharide pyrophosphate substrates to measure the activity of a GlcNAc-transferase involved in peptidoglycan synthesis from *E. coli* by a coupled fluorescence assay.[10] Compounds with short isoprene chains as well as a reduced aliphatic chain were superior to the natural substrate, with the exception of a compound containing a *trans*-configuration after the first isoprene unit. Moraprenol-pyrophosphoryl-Glc has also been shown to be an acceptor substrate for *E. coli* mannosyltransferase.[16] Thus the topology and membrane association not only of the enzymes but also of substrates are important factors for the sugar transfer reaction in bacteria. Pyrophosphate-containing lipids with a phenoxyundecyl group may also be acceptor substrates for other glycosyltransferases that catalyze the subsequent steps of O7 chain synthesis, and for glycosyltransferases of other strains of Gram-negative bacteria.

7. Experimental procedures for the synthesis of GlcNAc-PP-PhU

7.1. 2[(11-Phenoxyundecyl)oxy]tetrahydro-2*H*-pyran (11)

11-Bromo-1-undecanol (**13**) (7.54 g, 30.0 mmol) and 3,4 dihydro-2*H*-pyran (5 equiv, 12.62 g, 150 mmol) were dissolved in dry CH_2Cl_2 (140 mL) and the solution was cooled to 0 °C. *p*-Toluenesulfonic acid monohydrate (0.01 equiv, 0.057 g, 0.3 mmol) was then added and the solution was stirred for 10 min; it was then stirred at rt for 16 h. The mixture was partitioned between ether and a solution made up of saturated NaCl (70 mL), saturated aqueous $NaHCO_3$ (70 mL), and

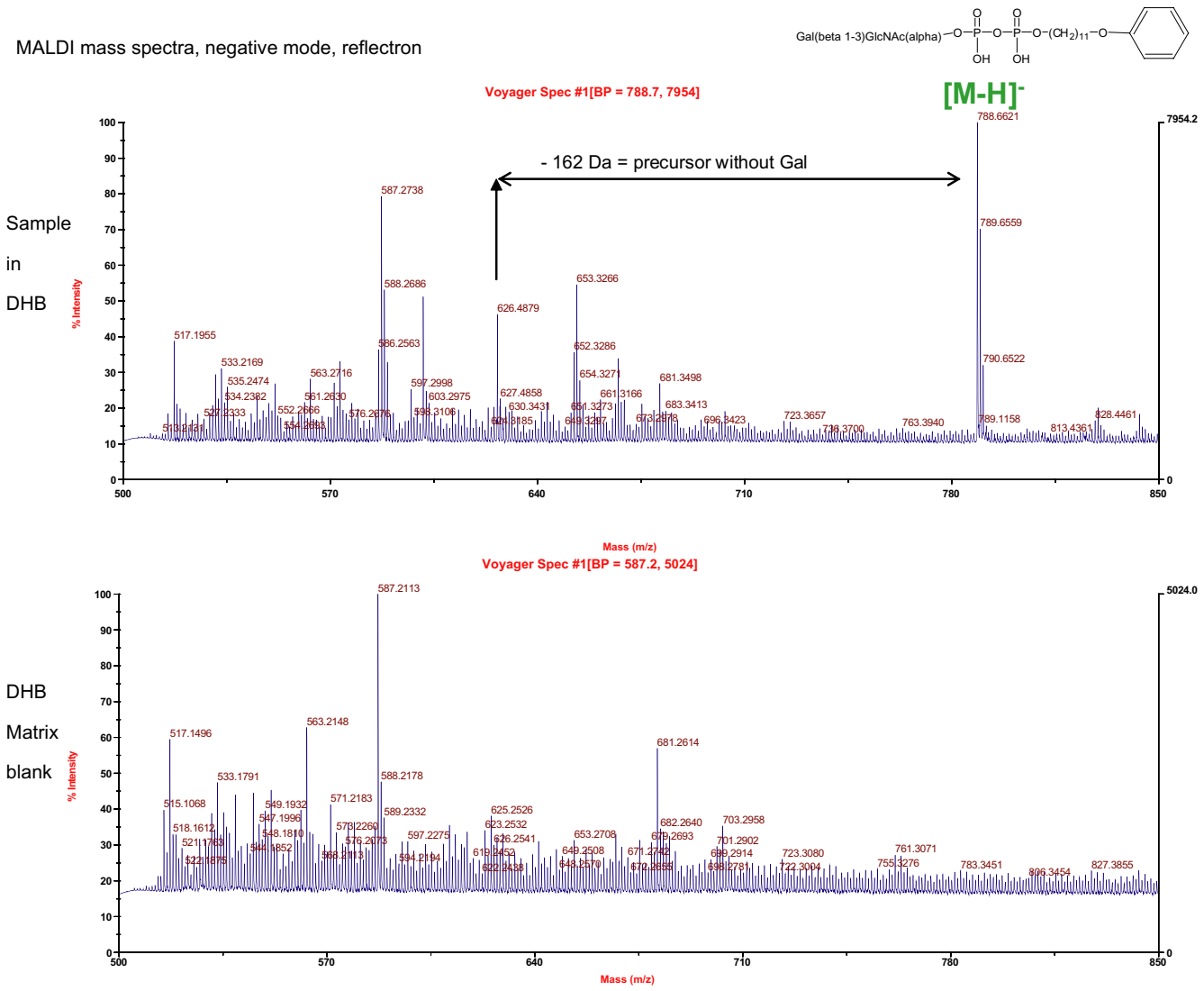


Figure 4. MALDI spectrum of purified enzyme product. Gal-transferase enzyme product from GlcNAc α1-O-PO_2-O-PO_2-O-$(CH_2)_{11}$-O-phenyl substrate was prepared using the wild type strain VW187 as the enzyme source. Enzyme product was purified on a C18 Sep-Pak column, and by HPLC using a C18 column and acetonitrile/water 6/94. Mass spectrometry was carried out using MALDI in the negative reflectron mode. DHB, 2,5-dihydroxybenzoic acid matrix substance.

water (140 mL). The organic phase was washed with saturated NaCl (50 mL × 2), dried over $MgSO_4$, and concentrated to yield a clear oil quantitatively (10.0 g). Compound **12** was used without further purification in the following step.

Phenol (1 equiv, 0.941 g, 10 mmol), KOH powder (1.25 equiv, 0.70 g, 12.5 mmol), and Aliquat 336 (0.02 equiv, 0.081 g, 0.2 mmol) were sonicated for 5 min at rt. The mixture was then heated to 85 °C, stirred for 5 min, and compound **12** (3.35 g, 10 mmol) was added. After 6 h at 85 °C the solution was cooled; ether (75 mL) was added and the mixture was sonicated for 5 min, passed through a Florosil plug, washed with more ether, and the solvent was removed. The crude product was purified by centrifugal chromatography {7:1 (v/v) ethyl acetate–hexane} to yield a clear liquid (2.75 g, 79%). ^{1}H NMR ($CDCl_3$) δ 1.25–1.90 (m, H), 3.40 (m, 1H), 3.52 (m, 1H), 3.76 (m, 1H), 3.90 (m, 1H), 3.97 (t, J = 6.6 Hz, 2H), 4.60 (m, 1H), 6.93 (m, 3H), 7.29 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 19.65, 25.47, 26.0, 26.2, 29.25, 29.35, 29.44, 29.49, 29.51, 29.71, 30.7, 62.3, 67.67, 67.82, 98.8, 114.4, 120.4, 129.4, 159.1.

7.2. 11-Phenoxy-1-undecanol (10)

The protected alcohol **11** (2.75 g, 7.9 mmol) was dissolved in methanol (40 mL) and *p*-toluenesulfonic acid (100 mg) and the mixture was stirred for 1.5 h. The methanol was then removed by evaporation and the remaining solid was dissolved in $CHCl_3$ (50 mL); the solution was washed with saturated aqueous $NaHCO_3$ (50 mL × 2), and dried over $MgSO_4$. Removal of the solvent afforded the pure product as a cream solid (1.88 g, 90%). ^{1}H NMR ($CDCl_3$) δ 1.28–1.38 (m, 12H), 1.47 (m, 2H), 1.59 (m, 2H), 1.80 (quin, J = 7.3 Hz), 3.66 (q, J = 6 Hz, 2H), 3.98 (t, J = 6.6 Hz, 2H), 6.94 (m, 3H), 7.29 (m, 2H). ^{13}C NMR ($CDCl_3$) δ 25.7, 26.0, 29.24,

29.35, 29.45, 29.49, 29.52, 32.6, 63.0, 67.8, 114.4, 120.4, 129.3, 159.1. MS (+TOF) *m/z* 287 $[M+Na]^+$; HRMS $[M+Na]^+$, found: 287.1986. $C_{17}H_{28}O_2Na$ requires *m/z*, 287.1981.

7.3. Dibenzyl 11-phenoxyundecyl phosphate (9)

Compound **10** (508 mg, 1.92 mmol) and tetrazole (2 equiv, 271 mg, 3.84 mmol) were dissolved in dry CH_2Cl_2 (20 mL) and the solution was cooled to −30 °C. Bis(benzyloxy)(diisopropylamino)phosphine (2 equiv, 1.219 g, 3.84 mmol) was then added drop wise over a 2 min period. The mixture was then stirred at rt for 15 h, cooled to −40 °C, and 77% *m*-CPBA (5 equiv, 2.16 g, 9.6 mmol) was added. The solution was stirred for 1 h at 0 °C, then for a further hour at rt. After addition of ethyl acetate (100 mL) the solution was washed with saturated aqueous Na_2SO_3 (50 mL × 3), water (50 mL × 2), and saturated NaCl (50 mL × 2), dried over Na_2SO_4, and concentrated to yield a yellow oil. The product was purified by column chromatography {2:3 (v/v) ethyl acetate–petroleum ether} to yield a white solid (751 mg, 74%). ^{1}H NMR ($CDCl_3$) δ 1.26–1.45 (m, 12H), 1.52 (m, 2H), 1.68 (m, 2H), 1.81 (m, 2H), 3.97–4.04 (m, 4H), 5.14 (m, 4H), 6.97 (m, 3H), 7.30–7.45 (m, 12H). ^{13}C NMR ($CDCl_3$) δ 25.2, 25.9, 29.0, 29.2, 29.26, 29.34, 29.4, 30.0, 67.8 (app t, $^3J_{CP}$ = 6.3 Hz), 68.9 (d, $^3J_{CP}$ = 5.6 Hz), 114.4, 120.3, 127.8, 128.3, 128.4, 129.3, 135.9 (d, $^4J_{CP}$ = 7.0 Hz), 159.0. ^{31}P NMR ($CDCl_3$) δ −0.45. MS (+TOF) *m/z* 547 $[M+Na]^+$; HRMS $[M+Na]^+$, found: 547.2570. $C_{31}H_{41}O_5PNa$ requires *m/z*, 547.2583.

7.4. 11-Phenoxyundecyl dihydrogen phosphate (4)

Compound **9** (517 mg, 0.986 mmol) was dissolved in deoxygenated methanol (15 mL) and 10% Pd/C (200 mg) was added. The solution was stirred overnight under a H_2 atmosphere. The Pd/C was removed by filtration through a Celite plug, and the solvent was evaporated to yield a white solid (317 mg, 93%). ^{1}H NMR (CD_3OD) δ 1.34–1.52 (m, 12H), 1.68 (app. quin, 2H), 1.78 (app. quin, 2H), 3.97 (app. q, 4H), 6.91 (m, 3H), 7.24 (m, 2H). ^{13}C NMR (CD_3OD) δ 26.7, 27.2, 30.35, 30.47, 30.53, 30.65, 30.69, 31.50, 31.58, 67.7 (d, $^3J_{CP}$ = 6.0 Hz), 68.9, 115.5, 121.5, 130.4, 160.6. ^{31}P NMR (CD_3OD) δ 0.33. MS (+TOF) *m/z* 383 $[M+K]^+$; HRMS $[M+K]^+$, found: 383.1379. $C_{17}H_{29}O_5PK$ requires *m/z*, 383.1384.

7.5. 3,4,6-Tri-O-acetyl-2-acetamido-1-O-[bis(benzyloxy)phosphoryl]-2-deoxy-α-D-glucopyranose (5)

Compound **6** (0.508 mg, 1.16 mmol) and tetrazole (10 equiv, 832 mg, 11.6 mmol) were dissolved in dry CH_2Cl_2 (20 mL) and the solution was cooled to −20 °C. Bis(benzyloxy)(diisopropylamino)phosphine (5 equiv, 1.84 g, 5.8 mmol) was then added drop wise over a 2 min period. The mixture was stirred at rt for 5 h, cooled to −40 °C, and 77% *m*-CPBA (10 equiv, 2.6 g, 11.6 mmol) was added. The solution was stirred for 30 min at 0 °C, then overnight at rt. After addition of ethyl acetate (100 mL) the solution was washed with saturated Na_2SO_4 (50 mL × 3), water (50 mL × 2), and saturated NaCl (50 mL × 2), dried over Na_2SO_4, and concentrated to yield a yellow oil. The product was purified by column chromatography {3:1 (v/v) ethyl acetate–petroleum ether, then 100% ethyl acetate} to give a clear oil (0.617 mg, 88%). ^{1}H NMR ($CDCl_3$) δ 1.70 (s, 3H), 2.02 (m, 9H), 3.93 (dd, *J* = 12.5, 2.2 Hz, 1H), 4.00 (m, 1H), 4.13 (dd, *J* = 12.5, 3.9 Hz, 1H), 4.36 (m, 1H), 5.10 (m, 6H), 5.66 (m, 2H), 7.34 (m, 10H). ^{13}C NMR ($CDCl_3$) δ 20.52, 20.58, 20.61, 22.7, 51.8 (d, $^4J_{CP}$ = 7.6 Hz), 61.2, 67.3, 69.6, 69.9 (app t, $^3J_{CP}$ = 5.5 Hz), 70.1, 96.2 (d, $^3J_{CP}$ = 6.6 Hz), 128.0, 128.1, 128.77, 128.81, 128.9, 135.2 (d, $^4J_{CP}$ = 6.5), 135.3 (d, $^4J_{CP}$ = 6.3 Hz), 169.1, 170.1, 170.5, 171.1. ^{31}P NMR ($CDCl_3$) δ −2.3. MS (+TOF) *m/z* 630 (M^++Na); HRMS $[M+Na]^+$, found: 630.1700. $C_{28}H_{34}NO_{12}NaP$ requires *m/z*, 630.1710.

7.6. 3,4,6-Tri-O-acetyl-2-acetamido-2-deoxy-α-D-glucopyranosyl dihydrogen phosphate (3)

Compound **5** (251 mg, 0.413 mmol) was dissolved in deoxygenated methanol (15 mL) and 10% Pd/C (300 mg) was added. The solution was stirred overnight under an H_2 atmosphere. The Pd/C was removed by filtration through a Celite plug, and the solvent was evaporated to yield a white solid (170 mg, 96%). ^{1}H NMR (CD_3OD) δ 1.96 (s, 3H), 2.01 (s, 3H), 2.03 (s, 3H), 2.07 (s, 3H), 4.14 (dd, *J* = 12.3, 2.1 Hz, 1H), 4.25 (m, 1H), 4.32 (m, 2H), 5.11 (t, *J* = 10 Hz, 1H), 5.31 (dd, *J* = 10.9, 9.3 Hz, 1H), 5.60 (dd, 6.2, 3.3 Hz, 1H). ^{13}C NMR (CD_3OD) δ 20.57, 20.62, 20.7, 22.4, 53.1, 62.7, 69.7, 70.3, 71.5, 96.0 (d, *J* = 6.2 Hz), 171.2, 172.0, 172.4, 173.7. ^{31}P NMR (CD_3OD) δ −2.0. MS (−TOF) *m/z* 426 $[M-H]^-$; HRMS $[M-H]^-$, found: 426.0805. $C_{14}H_{22}NO_{12}P$ requires *m/z*, 426.0806.

7.7. Ammonium P^1-3,4,6-tri-O-acetyl-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2–11-phenoxyundecyl hydrogen diphosphate (2)

Both sugar head group **3** (250 mg, 0.589 mmol) and tail **4** (1.1 equiv, 223 mg, 0.648 mmol) were dried separately by coevaporation (×3) from toluene (3 mL)–diisopropylamine (3 drops). Compound **4** was left on a vacuum line for another 30 min, then dry THF (15 mL) was added followed by 1,1′-carbonyldiimidazole (4.3 equiv, 417 mg, 2.53 mmol); the solution was stirred at rt for 1.5 h. Dry methanol (212 μL) was added, and the solution was stirred for a further hour. The solvent was removed under reduced pressure and the residue was kept on a vacuum line for 1 h (no bubbling). A solution of compound **3** in dry THF (15 mL) was added, the reaction was stirred for 48 h, and evaporated. The crude product was purified by a Sephadex G-15 size-exclusion column using methanol (0.1% NH_4HCO_3) as eluent to yield a white solid (0.365 mg, 80 %). ^{1}H NMR (CD_3OD) δ 1.26–1.45 (m, 12H), 1.50 (m, 2H), 1.69 (m, 2H), 1.79 (m, 2H), 2.06 (m, 12H), 3.43 (m, 2H), 3.96 (m, 3H), 4.21 (m, 2H), 4.56–4.69 (m, 3H), 5.12 (t, *J* = 9.7 Hz, 1H), 5.33 (t, *J* = 9.7 Hz, 1H), 5.66 (dd, *J* = 3.3, 7.3 Hz, 1H), 6.92 (m, 3H), 7.25 (t, *J* = 8.3 Hz, 2H), 8.75 (d, *J* = 8.6 Hz, 1H). ^{13}C NMR (CD_3OD) δ 19.5, 20.6,

22.8, 26.9, 27.2. 30.4, 30.5, 30.71, 30.74, 53.2, 62.8, 67.2, 68.8, 69.7, 69.8, 73.0, 95.8, 115.5, 121.4, 130.4, 160.5, 171.2, 171.8, 172.4, 174.0. ^{31}P NMR (CD_3OD) −12.4 (J = 22 Hz), −9.4 (J = 22 Hz). MS (−TOF) m/z 752 $[M-H]^-$; HRMS $[M-H]^-$, found: 752.2479. $C_{31}H_{48}NO_{16}P_2$ requires m/z, 752.2453.

7.8. Pyridinium P^1-2-acetamido-2-deoxy-α-D-glucopyranosyl P^2-11-phenoxyundecyl hydrogen diphosphate (1)

A solution of compound **2** (25.1 mg, 0.0325 mmol) in 0.0325 M $NaOCH_3$ (4 mL) was stirred for 20 min at rt until reaction was complete {thin layer chromatography, 6:2.5:0.4 (v/v/v) $CHCl_3$–methanol–H_2O}; it was then quenched with an excess of IR-120 resin (Aldrich) in the pyridinium form, and the solution was stirred for another 40 min. The reaction mixture was filtered and the resin was washed with methanol. The filtrate and washings were combined and concentrated under vacuum to yield a white solid (20.1 mg, 88%). ^{1}H NMR (CD_3OD) δ 1.26–1.45 (m, 12H), 1.50 (m, 2H), 1.69 (m, 2H), 1.79 (m, 2H), 2.06 (s, 3H), 3.45 (m, 1H), 3.73 (m, 2H), 3.82 (m, 1H), 3.91–4.05 (m, 6H), 5.64 (br s, 1H), 6.92 (m, 3H), 7.25 (t, J = 8.3 Hz, 2H), 8.04 (br s, 3H), 8.56 (m, 1H), 8.96 (br s, 2H). ^{13}C NMR (methanol) δ 19.2, 22.7, 26.6, 27.0, 30.2, 30.3, 30.5, 31.4, 55.1, 62.5, 67.8, 68.7, 71.73, 72.7, 75.0, 96.5, 115.4, 121.3, 128.0, 130.4, 143.7, 146.6, 160.4, 174.0. ^{31}P NMR (methanol) δ −11.7 (J = 16.4 Hz), −9.6 (J = 16.4 Hz). MS (−TOF) m/z 626 $[M-H]^-$; HRMS $[M-H]^-$, found: 626.2126. $C_{25}H_{42}NO_{13}P_2$ requires m/z, 626.2131.

Acknowledgements

The authors thank Bernd Keller for the mass spectrometric analyses, and Eddie Khoo for galactosyltransferase assays. This work was supported by grants from the Natural Science and Engineering Research Council of Canada and The Arthritis Society of Canada.

References and notes

1. L'vov, V. L.; Shaskov, A. S.; Dmitriev, B. A.; Kochetkov, N. K. *Carbohydr. Res.* **1984**, *126*, 249–259.
2. Whitfield, C.; Valvano, M. A. *Adv. Microb. Physiol.* **1993**, *35*, 135–246.
3. Guan, S.; Clarke, A. J.; Whitfield, C. *J. Bacteriol.* **2001**, *183*, 3318–3327.
4. Alexander, D. C.; Valvano, M. A. *J. Bacteriol.* **1994**, *176*, 7079–7084.
5. Amer, A. O.; Valvano, M. A. *Microbiology* **2002**, *148*, 571–582.
6. Valvano, M. A.; Crosa, J. H. *Infect. Immun.* **1989**, *57*, 937–943.
7. Vauzeilles, B.; Dausse, B.; Palmier, S.; Beau, J.-M. *Tetrahedron Lett.* **2001**, *42*, 7567–7570.
8. Bannwarth, W.; Trzeciak, A. *Helv. Chim. Acta* **1987**, *70*, 175–186.
9. Sim, M. M.; Kondo, H.; Wong, C.-H. *J. Am. Chem. Soc.* **1993**, *115*, 2260–2267.
10. Chen, L.; Men, H.; Ha, S.; Ye, X.-Y.; Brunner, L.; Hu, Y.; Walker, S. *Biochemistry* **2002**, *41*, 6824–6833.
11. Bernady, K. F.; Floyd, M. B.; Poletto, J. F.; Weiss, M. J. *J. Org. Chem.* **1979**, *44*, 1438–1447.
12. Warren, C. D.; Jeanloz, R. W. *Biochemistry* **1972**, *11*, 2565–2572.
13. Loupy, A.; Sansoulet, J.; Vaziri-Zand, F. *Bull. Soc. Chim. Fr.* **1987**, *6*, 1027–1035.
14. Hoard, D. E.; Ott, D. G. *J. Am. Chem. Soc.* **1965**, *87*, 1785–1788.
15. Brockhausen, I.; Lehotay, M.; Yang, J.; Qin, W.; Young, D.; Lucien, J.; Coles, J.; Paulsen, H. *Glycobiology* **2002**, *12*, 33–45.
16. Jann, K.; Pillat, M.; Weisgerber, C.; Shibaev, V. N.; Torgov, V. I. *Eur. J. Biochem.* **1985**, *151*, 393–397.