

Supplemental data

Characterization of two UDP-Gal: GalNAc-diphosphate-lipid β 1,3-Galactosyltransferases WbwC from *Escherichia coli* Serotypes O104 and O5^{*S}

Shuo Wang¹, Diana Czuchry¹, Bin Liu², Anna N. Vinnikova¹, Yin Gao¹, Jason Z. Vlahakis³, Walter A. Szarek³, Lei Wang², Lu Feng², Inka Brockhausen¹

Synthesis of acceptor substrates 8 and 14 as outlined in Scheme 1.

General—Flash column chromatography was performed on Silicycle silica gel (230–400 mesh, 60 Å). Analytical thin-layer chromatography was performed on glass-backed pre-coated silica gel 60 F254 plates (Silicycle), and the compounds were visualized either by UV illumination (254 nm), or by heating after spraying with phosphomolybdic acid in ethanol. Melting points were measured on a Mel-Temp II apparatus and are uncorrected. ¹H, ¹³C, and ³¹P NMR spectra were recorded on a Bruker Avance 400 spectrometer in D₂O, CDCl₃, CD₃OD, or DMSO-*d*₆. The ¹³C NMR spectra were obtained using either the regular pulse program (delay time 0.2 s) or using the J-modulated spin echo pulse program (delay time 4 s). Additional spectra such as ¹H-¹H COSY and ¹H-¹³C HMQC/HSC were acquired for further structural confirmation (not shown). The chemical shifts are reported in δ (ppm), the residual proton signals from the solvent were used as an internal reference relative to the chemical shifts of tetramethylsilane. The compounds synthesized were deemed >95% pure by ¹H NMR analysis. High-resolution ESI mass spectra were recorded on an Applied Biosystems/MDS Sciex QSTAR XL mass spectrometer with an Agilent HP1100 Cap-LC system. Samples were run in 50% aqueous MeOH at a flow rate of 6 μ l/min. High-resolution EI mass spectra were recorded on a Waters/Micromass GC-TOF instrument.

Materials—Methanol was distilled from magnesium/iodine. Tetrahydrofuran was distilled from sodium/benzophenone. 1,2-Dichloroethane was distilled from calcium sulfate. D-Galactosamine hydrochloride was obtained from V-Labs, Inc. D-Glucosamine hydrochloride and all other chemicals were obtained from Sigma–Aldrich. All reactions were performed under an atmosphere of nitrogen, unless otherwise stated.

Synthetic Procedures

11-Phenoxy-1-undecanol (1).

A mixture of 11-bromo-undecanol (2.50 g, 9.95 mmol, 1 equiv), phenol (1.87 g, 19.87 mmol, 2 equiv), potassium carbonate (772 mg, 5.59 mmol, 1.2 equiv) and potassium iodide (30 mg, 0.18 mmol, 1.8 mol%) in acetone (40 ml) was heated at reflux temperature with stirring for 4 days. The reaction mixture was concentrated, and a solution of potassium carbonate (3 g) in water (120 ml) was added. The resulting white precipitate was collected by filtration and washed with a solution potassium carbonate (3 g) in water (120 ml), and then with water (120 ml). The solid (1.55 g) was dissolved in boiling pentane (80 ml) and allowed to cool in the freezer overnight. The resulting white solid was collected by filtration, washed with cold pentane (10 ml), and then air dried. High-vacuum drying gave the product (1.32 g, 4.99 mmol, 50%) as a white solid; mp 54–55 °C; *R*_f = 0.80 (EtOAc); ¹H NMR (400 MHz, CDCl₃): δ 1.20–1.40 (m, 12H), 1.40–1.52 (m, 2H), 1.52–1.64 (m, 2H), 1.72–1.84 (m, 2H), 3.60–3.70 (m, 2H), 3.95 (t, *J* = 6.4 Hz, 2H), 6.85–6.97 (m, 3H), 7.28 (app t, *J* = 7.0 Hz, 2H); ¹³C NMR (100 MHz, CDCl₃): δ 25.9, 26.2, 29.4, 29.52, 29.55, 29.65, 29.69, 29.7, 33.0, 63.2, 68.0, 114.6, 120.6, 129.5, 159.3; HRMS (EI) [M]⁺ Calcd for C₁₇H₂₈O₂: 264.2089. Found: 264.2082.

11-Phenoxyundecyl dihydrogen phosphate (2).

A flask containing hexanes (31.5 ml) was cooled to 0 °C and to this was added phosphorous oxychloride (8.36 ml, 13.75 g, 89.70 mmol, 30 equiv). Separately, the alcohol **1** (790 mg, 2.99 mmol, 1 equiv) was dissolved in triethylamine (12.50 ml, 9.08 g, 89.70 mmol, 30 equiv) at room temperature, then cooled to 0 °C. The alcohol solution was added dropwise to the POCl₃ solution at 0 °C with stirring over a period of 10 min. The mixture was stirred at 0 °C for 1 h, then at room temperature for 1 h. The mixture was cooled again to 0 °C, and carefully quenched by the addition of a solution of acetone (126 ml) / water (31.5 ml) / triethylamine (37.5 ml, 27.23 g, 269.10 mmol, 90 equiv). Stirring was continued at 0 °C for 0.5 h, then at room temperature for 2h. The mixture was concentrated, then concentrated with toluene (4×100 ml) at 60 °C and dried under high vacuum leaving a beige solid (45.80 g). The solid was ground finely under acetone (400 ml) and the solids removed by filtration. This grinding of the solid and filtration was repeated three more times (each with 400 ml of fresh acetone), and the collected pooled filtrate (1600 ml) concentrated to a golden oil, then dried under high vacuum to afford a beige oily solid residue (13.30 g). To the residue was added water (110 ml) and the mixture heated briefly to 100 °C, then cooled to 0 °C for 0.5 h. The resulting solid was collected by filtration, washed with water (4×30 ml), and air dried. High-vacuum drying gave the product (529 mg, 1.54 mmol, 52%) as a beige solid in the dihydrogen form; mp 41–42 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.13–1.21 (m, 2H), 1.23–1.35 (m, 10H), 1.35–1.45 (m, 2H), 1.48–1.57 (m, 2H), 1.64–1.74 (m, 2H), 3.09 (br s, ~2H), 3.73–3.81 (m, 2H), 3.93 (t, *J* = 6.4 Hz, 2H), 6.86–6.96 (m, 3H), 7.26 (app t, *J* = 7.8 Hz, 2H); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 25.1, 25.5, 28.7 (2C), 28.8, 28.9, 29.0 (2C), 29.9 (d, *J*_{C-P} = 7.6 Hz), 64.9 (d, *J*_{C-P} = 5.4 Hz), 67.2, 114.4, 120.3, 129.4, 158.6; ³¹P NMR (162 MHz, DMSO-*d*₆): δ 0.3; HRMS (ESI) [M-H]⁻ Calcd for C₁₇H₂₈O₅P: 343.1674. Found: 343.1661.

2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-galactopyranose (**3**).

D-Galactosamine hydrochloride (320 mg, 1.48 mmol, 1 equiv) was dissolved in pyridine (9 ml, 8.80 g, 111.25 mmol, 75 equiv) using mild heating, then the solution was cooled to 0 °C. To this was added acetic anhydride (6 ml, 6.48 g, 63.47 mmol, 43 equiv), and the reaction mixture stirred at room temperature for 18 h. The reaction mixture was co-evaporated with toluene (3×100 ml) at 80 °C until a white solid remained, then dried under high vacuum. The solid was dissolved in boiling methanol (24 ml), then cooled in the freezer overnight. The white crystals that formed were collected by filtration and washed with methanol (3 ml). High-vacuum drying gave the product (483 mg, 1.24 mmol, 84%) as a white solid; mp 233–234 °C; *R*_f = 0.26 (EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.78 (s, 3H), 1.91 (s, 3H), 1.99 (s, 3H), 2.04 (s, 3H), 2.12 (s, 3H), 3.99 (dd, *J* = 11.0, 6.6 Hz, 1H, H-6b), 4.05 (dd, *J* = 11.4, 5.4 Hz, 1H, H-6a), 4.09 (dd, *J* = 20.6, 9.4 Hz, 1H, H-2), 4.22 (t, *J* = 6.2 Hz, 1H, H-5), 5.07 (dd, *J* = 11.2, 3.6 Hz, 1H, H-3), 5.27 (d, *J* = 3.2 Hz, 1H, H-4), 5.65 (d, *J* = 8.8 Hz, 1H, H-1), 7.87 (d, *J* = 8.8 Hz, 1H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 20.3 (2C), 20.4, 20.5, 22.7, 48.2, 61.4, 66.4, 70.0, 70.8, 92.4, 168.9, 169.4, 169.5, 169.8, 169.9; HRMS (ESI) [M+Na]⁺ Calcd for C₁₆H₂₃NO₁₀Na: 412.1214. Found: 412.1219.

2-Methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-galactopyrano)[1,2-*d*]-1,3-oxazoline (**4**).

The tetraacetate **3** (1.00 g, 2.57 mmol, 1 equiv) was dissolved in 1,2-dichloroethane (25 ml) at room temperature. To this was added trimethylsilyl trifluoromethanesulfonate (0.51 ml, 626 mg, 2.82 mmol, 1.1 equiv) and the mixture heated at 50–60 °C for 10.5 h. The mixture was cooled to 0 °C and added to an ice-cold solution of sodium hydrogencarbonate (1 g) in water (67 ml). The mixture was extracted with dichloromethane (4×40 ml), the combined organic extracts washed successively with a saturated aqueous solution of sodium hydrogen carbonate and brine, then dried (sodium sulfate) and concentrated. High-vacuum drying left a brown oil that was purified using flash column chromatography on silica gel (ethyl acetate) to give the fairly clean product (772 mg, 2.34 mmol, 91%) as a golden oil; *R*_f = 0.47 (EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.95 (s, 3H), 2.00 (s, 3H), 2.01 (s, 3H), 2.07 (s, 3H), 3.95 (td, *J* = 6.8, 1.2 Hz, 1H), 4.02–4.08 (m, 1H), 4.12 (dd, *J* = 11.4, 7.4 Hz, 1H), 4.22–4.28 (m, 1H), 4.88 (dd, *J* = 6.8, 4.0 Hz, 1H), 5.24 (t, *J* = 3.4 Hz, 1H), 6.05 (d, *J* = 7.2 Hz, 1H, H-1); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 14.0, 20.4, 20.5 (2C), 61.8, 62.5, 65.0, 68.2, 70.8, 100.9, 165.2, 169.6, 169.8, 170.0; HRMS (ESI) [M+H]⁺ Calcd for C₁₄H₂₀NO₈: 330.1188. Found: 330.1179.

2-Acetamido-3,4,6-tri-*O*-acetyl-1-*O*-[bis(benzyloxy)phosphoryl]-2-deoxy-α-D-galactopyranose (**5**).

The oxazoline **4** (1.69 g, 5.13 mmol, 1 equiv) was dissolved in distilled 1,2-dichloroethane (37.5 ml). To this was added dibenzyl phosphate (1.71 g, 6.16 mmol, 1.2 equiv) and the mixture stirred at room temperature for 19 h. The reaction mixture was concentrated and dried under high vacuum. The remaining white solid was washed with water (3×5 ml) then with a solution of 1% sodium hydrogencarbonate in water (2×5 ml). The solid was dissolved in warm methanol (3 ml), cooled to 0 °C, then precipitated by the addition of a solution of 1% sodium hydrogencarbonate in water. The white solid was collected by filtration and washed with water (3×5 ml). High-vacuum drying gave the product (264 mg, 0.43 mmol, 8%) as a white solid; mp 42–46 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.76 (s, 3H), 1.84 (s, 3H), 1.94 (s, 3H), 2.12 (s, 3H), 3.96 (dd, *J* = 11.6, 7.2 Hz, 1H, H-6b), 4.03 (dd, *J* = 11.2, 5.2 Hz, 1H, H-6a), 4.22–4.32 (m, 1H, H-2), 4.38 (t, *J* = 6.2 Hz, 1H, H-5), 5.06–5.15 (m, 1H, H-3), 5.11 (d, *J* = 6.8 Hz, 4H, CH₂Ph), 5.41 (d, *J* = 2.4 Hz, 1H, H-4), 5.75 (dd, *J* = 6.8, 3.2 Hz, 1H, H-1), 7.35–7.44 (m, 10H, C₆H₅), 8.34 (d, *J* = 7.2 Hz, 1H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 20.2, 20.3, 20.5, 22.2, 47.3 (d, ³*J*_{C-P} = 7.8 Hz, C-2), 61.6, 66.4, 66.6, 68.1, 68.7 (d, ²*J*_{C-P} = 4.0 Hz, CH₂Ph), 95.5 (d, ²*J*_{C-P} = 6.1 Hz, C-1), 127.7 (d, ⁴*J*_{C-P} = 5.2 Hz, *ortho* C), 128.4, 128.5, 135.9 (d, ³*J*_{C-P} = 4.9 Hz, *quarternary* C), 169.6, 169.8, 170.0, 170.1; ³¹P NMR (162 MHz, DMSO-*d*₆): δ -1.20; HRMS (ESI) [M+H]⁺ Calcd for C₂₈H₃₅NO₁₂P: 608.1896. Found: 608.1892.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-galactopyranosyl dihydrogen phosphate (6).

The dibenzylated phosphate **5** (404 mg, 0.66 mmol, 1 equiv) was dissolved in deoxygenated methanol (25 ml) and 10% palladium/carbon (450 mg) was added under an atmosphere of nitrogen. The solution was then stirred at room temperature under an atmosphere of hydrogen for 23 h. Monitoring by TLC (ethyl acetate) showed only *R*_f = 0 indicating reaction was complete. The atmosphere was purged with nitrogen, and the Pd/C removed by filtration through Celite. The Celite was washed with methanol (4×5 ml) and the collected filtrate concentrated at 60 °C. The residue was concentrated with hexanes, then with diethyl ether, and dried under high vacuum leaving a white solid (270 mg). The solid was dissolved in boiling ethyl acetate (6 ml), cooled to 0 °C, and diethyl ether was slowly added. The resulting precipitate was collected by filtration and washed with diethyl ether (4×) and air dried. High-vacuum drying gave the product (173 mg, 0.40 mmol, 61%) as a white solid in the dihydrogen form; mp 120–124 °C; *R*_f = 0 (EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.80 (s, 3H), 1.90 (s, 3H), 1.98 (s, 3H), 2.11 (s, 3H), 3.30–3.50 (br s, ~2H, P(OH)₂), 3.98 (dd, *J* = 11.2, 6.4 Hz, 1H), 4.07 (dd, *J* = 11.2, 6.4 Hz, 1H), 4.16–4.25 (m, 1H), 4.32 (t, *J* = 6.2 Hz, 1H), 5.05 (dd, *J* = 12.0, 3.2 Hz, 1H), 5.36 (d, *J* = 2.0 Hz, 1H), 5.50 (dd, *J* = 6.6, 3.0 Hz, 1H, H-1), 8.13 (d, *J* = 7.6, 1H, NH); ¹³C NMR (100 MHz, CD₃OD): δ 20.4, 20.5, 20.6, 22.5, 49.1, 62.4, 68.3, 69.0, 69.1, 96.4 (d, ²*J*_{C-P} = 5.8 Hz, C-1), 171.8, 172.0, 172.1, 173.9; ³¹P NMR (162 MHz, DMSO-*d*₆): δ -1.24; HRMS (ESI) [M-H]⁻ Calcd for C₁₄H₂₁NO₁₂P: 426.0801. Found: 426.0805.

***P*¹-2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-galactopyranosyl *P*²-11-phenoxyundecyl dihydrogen diphosphate (7).**

The lipid-phosphate **2** (79 mg, 0.23 mmol, 1.2 equiv) was co-evaporated with toluene (3 ml) and diisopropylamine (3 drops) three times, then dried under high vacuum for 1.5 h. The resulting lipid-phosphate diisopropylammonium salt was dissolved in distilled tetrahydrofuran (5.4 ml), 1,1'-carbonyldiimidazole (133 mg, 0.82 mmol, 4.3 equiv) was added, and the mixture stirred at room temperature for 1.5 h. To destroy excess CDI, freshly distilled methanol (68 μ l, 0.0680 ml, 0.0538 g, 1.68 mmol, 8.8 equiv) was added and the mixture stirred at room temperature for 1 h, then concentrated at 30 °C and dried under high vacuum for 1 h. The sugar-phosphate **6** (83 mg, 0.19 mmol, 1 equiv) was also co-evaporated with toluene (3 ml) and diisopropylamine (3 drops) three times, then dried under high vacuum for 2 h. The resulting sugar-phosphate diisopropylammonium salt was dissolved in distilled tetrahydrofuran (5.4 ml) and quickly added to the flask containing the lipid-phosphoimidazolidate. The mixture was stirred at room temperature for 9 days (monitor reaction using ¹H NMR in CD₃OD), then concentrated at 30 °C and dried under high vacuum leaving a pale yellow oil. The oil was dissolved in methanol (0.3 ml), and loaded onto a preparatory-scale thin-layer chromatography plate (20 cm x 10 cm x 2 mm thick). The plate was eluted sequentially with EtOAc, 10% MeOH in EtOAc, 20% MeOH in EtOAc, 30% MeOH in EtOAc, and finally with 35% MeOH in EtOAc. The section corresponding to *R*_f range 0.29–0.53 was excised from the plate, and eluted with 80% MeOH in EtOAc, and filtered. The filtrate was treated with Amberlite IR-120 ion exchange resin in the H⁺ form (1 g), stirred at room temperature for 10 min, filtered to remove the resin, and

the filtrate concentrated. High-vacuum drying gave the product (60 mg, 0.08 mmol, 35%) as a white solid in the dihydrogen form; mp 88–92 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.16–1.35 (m, 12H), 1.36–1.44 (m, 2H), 1.45–1.55 (m, 2H), 1.65–1.74 (m, 2H), 1.83 (s, 3H), 1.88 (s, 3H), 1.99 (s, 3H), 2.10 (s, 3H), 3.70–3.83 (m, 2H), 3.93 (t, *J* = 6.4 Hz, 2H), 3.95–4.01 (m, 1H), 4.02–4.10 (m, 1H), 4.28–4.40 (m, 2H), 5.02 (dd, *J* = 11.6, 2.8 Hz, 1H, H-1), 5.27–5.34 (m, 1H), 5.37–5.45 (m, 1H), 6.85–6.95 (m, 3H), 7.22 (br s, 1H), 7.26 (app t, *J* = 7.8 Hz, 2H), 8.20 (br s, 1H); ¹³C NMR (100 MHz, CD₃OD): δ 19.3, 19.4, 20.5, 20.6, 22.8, 26.9, 27.1 (2C), 30.4, 30.5 (2C), 30.64, 30.68, 30.7, 32.0, 62.2, 68.4, 68.7, 68.8 (2C), 69.9, 115.5, 120.4, 121.4, 130.4, 160.5, 171.8, 172.0, 172.1; ³¹P NMR (162 MHz, CD₃OD): δ -9.6 (br m), -12.3 (br m); HRMS (ESI) [M-H]⁻ Calcd for C₃₁H₄₈NO₁₆P₂: 752.2448. Found: 752.2450.

***P*¹-2-Acetamido-2-deoxy- α -D-galactopyranosyl *P*²-11-phenoxyundecyl disodium diphosphate (8).**

A sample of sodium (33 mg, 1.43 mmol, 3.5 equiv) which had been washed with hexanes and dried under high vacuum was added to methanol (50 ml) under an atmosphere of nitrogen. The mixture was stirred at room temperature until the reaction was completed, then this solution of sodium methoxide (3.5 equiv) in methanol was added to the triacetate **7** (353 mg, 0.41 mmol, 1 equiv). The mixture was stirred at room temperature for 20 min (pH=9), then quenched by the addition of Amberlite IR-120 ion exchange resin in the H⁺ form (1 g), stirred at room temperature for 20 min, and filtered to remove the resin. The filtrate was treated with Amberlite IR-120 ion exchange resin in the Na⁺ form (3 g), stirred at room temperature for 20 min, and filtered to remove the resin. The filtrate was again treated with Amberlite IR-120 ion exchange resin in the Na⁺ form (3 g), stirred at room temperature for 20 min, and filtered to remove the resin. The filtrate was concentrated at 50 °C, concentrated with diethyl ether, then dried under high vacuum. The resulting white solid was washed with ethyl acetate (3 ml). High-vacuum drying gave the product (273 mg, 0.41 mmol, 100%) as a white solid in the disodium salt form; mp 190–195 °C (dec); ¹H NMR (400 MHz, D₂O): δ 1.22–1.41 (m, 12H), 1.41–1.50 (m, 2H), 1.59–1.69 (m, 2H), 1.73–1.83 (m, 2H), 2.09 (s, 3H), 3.52 (br s, ~1H), 3.70–3.84 (m, 2H), 3.90–4.02 (m, 3H), 4.05 (br s, 1H), 4.11 (t, *J* = 6.4 Hz, 2H), 4.21 (t, *J* = 5.8 Hz, 1H), 4.27 (app d, *J* = 10.8 Hz, 1H), 5.53 (dd, *J* = 7.0, 3.4 Hz, 1H, H-1), 7.00–7.10 (m, 3H), 7.39 (app t, *J* = 7.6 Hz, 2H); ¹³C NMR (100 MHz, D₂O): δ 22.3, 25.4, 25.8, 29.0, 29.3 (2C), 29.5 (2C), 30.3, 40.0, 58.9, 61.2, 66.9, 67.5, 67.8, 68.5, 72.1, 94.7 (~m, C-1), 114.4, 120.6, 129.4, 158.6, 174.7; ³¹P NMR (162 MHz, D₂O): δ -9.41 (d, ²*J*_{P-P} = 21.4 Hz), -11.88 (d, ²*J*_{P-P} = 20.9 Hz); HRMS (ESI) [M-Na]⁻ Calcd for C₂₅H₄₁NO₁₃P₂Na: 648.1950. Found: 648.1942.

2-Acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy- α -D-glucopyranose (9).

D-Glucosamine hydrochloride (10 g, 46.38 mmol, 1 equiv) was dissolved in pyridine (281 ml, 274.82 g, 3.47 mol, 75 equiv) using mild heating, then the solution was cooled to 0 °C. To this was added acetic anhydride (188 ml, 203.04 g, 1.99 mol, 43 equiv), and the reaction mixture stirred at room temperature for 21 h. The reaction mixture was co-evaporated with toluene (9×200 ml) at 60 °C until a beige oily solid remained, then dried under high vacuum. The oily solid was dissolved in boiling methanol (~200 ml), cooled to 0 °C, then diethyl ether (5 ml) was added. The solution was cooled in the freezer overnight and the white solid that formed was removed by filtration. The filtrate was concentrated, the residue again recrystallized from methanol/diethyl ether, and the solid removed by filtration. The filtrate was concentrated with diethyl ether and then with hexanes, and dried under high vacuum. The resulting beige solid (15.18 g) was diluted with a saturated aqueous solution of sodium hydrogen carbonate (100 ml) and extracted with dichloromethane (2×100 ml). The extract was washed with brine (50 ml), dried (sodium sulphate), then concentrated to a brown oil. High-vacuum drying gave the product (11.71 mg, 30.08 mmol, 65%) as a beige solid; mp 58–60 °C; *R*_f = 0 (EtOAc); ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.80 (s, 3H), 1.95 (s, 3H), 1.98 (s, 3H), 2.01 (s, 3H), 2.18 (s, 3H), 3.99 (dd, *J* = 12.0, 1.6 Hz, 1H), 4.11 (app d, *J* = 10.0 Hz, 1H), 4.16 (app d, *J* = 4.0 Hz, 1H), 4.19 (app d, *J* = 4.0 Hz, 1H), 4.21–4.30 (m, 1H), 4.99 (t, *J* = 9.8 Hz, 1H), 5.17 (t, *J* = 10.2 Hz, 1H), 5.93 (d, *J* = 3.6 Hz, 1H, H-1), 8.05 (d, *J* = 8.8 Hz, 1H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 20.40, 20.43, 20.5, 20.9, 22.2, 49.9, 61.4, 68.1, 69.1, 69.9, 89.7, 169.1, 169.2, 169.9, 170.0, 170.1; HRMS (ESI) [M+Na]⁺ Calcd for C₁₆H₂₃NO₁₀Na: 412.1219. Found: 412.1221.

2-Methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy- α -D-glucopyrano)[1,2-*d*]-1,3-oxazoline (10).

The tetraacetate **9** (200 mg, 0.51 mmol, 1 equiv) was dissolved in 1,2-dichloroethane (5 ml) at room temperature. To this was added trimethylsilyl trifluoromethanesulfonate (0.10 ml, 124 mg, 0.56 mmol, 1.1 equiv) and the mixture heated at 50–60 °C for 10 h. The mixture was cooled to 0 °C and added to an ice-cold solution of 1% sodium hydrogen carbonate in water (20 ml). The mixture was extracted with dichloromethane (2×), the combined organic extracts washed with a 1% solution of sodium hydrogen carbonate in water, dried (sodium sulfate) and concentrated. High-vacuum drying gave the product (161 mg, 0.49 mmol, 96%) as a golden oil; $R_f = 0.48$ (EtOAc); $^1\text{H NMR}$ (400 MHz, DMSO- d_6): δ 2.00 (s, 3H), 2.02 (s, 3H), 2.03 (s, 3H), 2.06 (s, 3H), 3.54–3.62 (m, 1H), 4.03–4.10 (m, 2H), 4.10–4.16 (m, 1H), 4.79 (d, $J = 9.6$ Hz, 1H), 5.05 (t, $J = 2.0$ Hz, 1H), 6.03 (d, $J = 7.6$ Hz, 1H, H-1); $^{13}\text{C NMR}$ (100 MHz, DMSO- d_6): δ 13.5, 20.5, 20.6, 20.7, 62.9, 64.2, 66.7, 68.2, 69.9, 98.6, 165.5, 169.0, 169.3, 170.1; HRMS (EI) $[\text{M}]^+$ Calcd for $\text{C}_{14}\text{H}_{19}\text{NO}_8$: 329.1111. Found: 329.1117.

2-Acetamido-3,4,6-tri-O-acetyl-1-O-[bis(benzyloxy)phosphoryl]-2-deoxy- α -D-glucopyranose (11).

The oxazoline **10** (450 mg, 1.37 mmol, 1 equiv) was dissolved in distilled 1,2-dichloroethane (8 ml). To this was added dibenzyl phosphate (581 mg, 2.09 mmol, 1.5 equiv) and the mixture stirred at room temperature for 6 days. The reaction mixture was concentrated at 60 °C, then concentrated with hexanes. The resulting brown oil was washed with boiling hexanes (3×4 ml) and dried under high vacuum. The oil was dissolved in warm ethyl acetate (2 ml) and purified using flash column chromatography on silica gel (elute first with 1:1 EtOAc–hexanes, then with EtOAc). High-vacuum drying gave the product (276 mg, 0.45 mmol, 33%) as a brown oil (still containing some dibenzylphosphate); $R_f = 0.5$ (EtOAc); $^1\text{H NMR}$ (400 MHz, CDCl_3): δ 1.71 (s, 3H), 2.01 (br s, 6H), 2.03 (s, 3H), 3.91 (dd, $J = 12.4, 1.6$ Hz, 1H, H-6b), 3.96–4.03 (m, 1H, H-5), 4.13 (dd, $J = 12.6, 3.8$ Hz, 1H, H-6a), 4.32–4.42 (m, 1H, H-2), 5.03–5.11 (m, 4H, CH_2Ph), 5.11–5.19 (m, 2H, H-3 and H-4), 5.64–5.72 (m, 2H, H-1 and NH), 7.33–7.43 (m, 10H, aro); $^{31}\text{P NMR}$ (162 MHz, CDCl_3): δ -1.27; HRMS (ESI) $[\text{M}+\text{Na}]^+$ Calcd for $\text{C}_{28}\text{H}_{34}\text{NO}_{12}\text{PNa}$: 630.1716. Found: 630.1711.

2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranosyl dihydrogen phosphate (12).

The dibenzylated phosphate **11** (813 mg, 1.34 mmol, 1 equiv) was dissolved in deoxygenated methanol (51 ml) and 10% palladium/carbon (914 mg) was added under an atmosphere of nitrogen. The solution was then stirred at room temperature under an atmosphere of hydrogen for 23 h. Monitoring by TLC (ethyl acetate) showed only $R_f = 0$ indicating reaction was complete. The atmosphere was purged with nitrogen, and the Pd/C removed by filtration through Celite. The Celite was washed with methanol (~100 ml) and the collected filtrate concentrated at 50 °C. The residue was concentrated with hexanes, then with diethyl ether. High-vacuum drying gave the product (560 mg, 1.31 mmol, 98%) as a white solid in the dihydrogen form; mp 113–115 °C; $R_f = 0$ (EtOAc); $^1\text{H NMR}$ (400 MHz, DMSO- d_6): δ 1.82 (s, 3H), 1.94 (s, 3H), 1.98 (s, 3H), 2.02 (s, 3H), 4.05 (dd, $J = 12.0, 2.0$ Hz, 1H), 4.08–4.17 (m, 2H), 4.19 (app d, $J = 4.0$ Hz, 1H), 4.95 (t, $J = 9.6$ Hz, 1H), 5.16 (dd, $J = 10.8, 9.6$ Hz, 1H), 5.41 (dd, $J = 6.8, 3.2$ Hz, 1H, H-1), 7.98 (d, $J = 8.4$ Hz, 1H, NH); $^{31}\text{P NMR}$ (162 MHz, DMSO- d_6): δ -1.47; HRMS (ESI) $[\text{M}-\text{H}]^-$ Calcd for $\text{C}_{14}\text{H}_{21}\text{NO}_{12}\text{P}$: 426.0801. Found: 426.0809.

P^1 -2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy- α -D-glucopyranosyl P^2 -11-phenoxyundecyl hydrogen diisopropylammonium diphosphate (13).

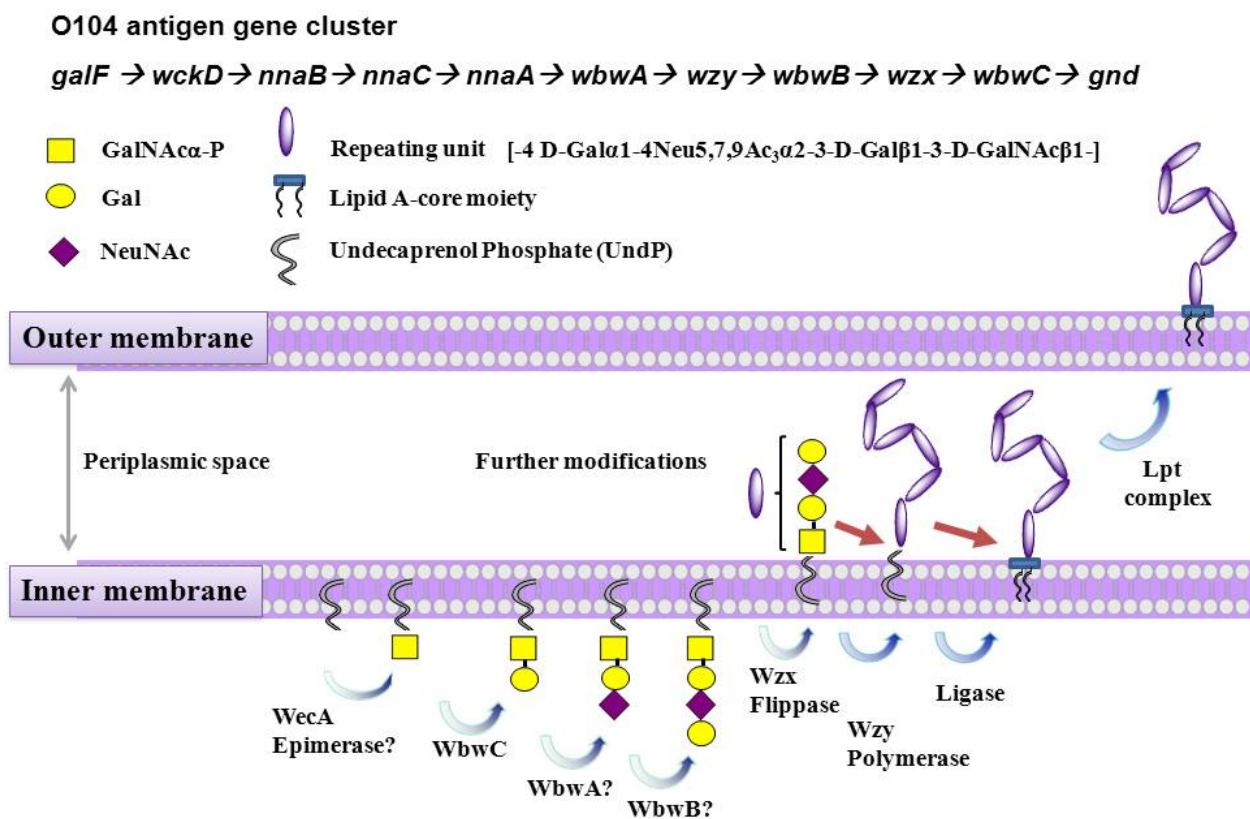
The lipid-phosphate **2** (272 mg, 0.79 mmol, 1.2 equiv) was co-evaporated with toluene (10 ml) and diisopropylamine (10 drops) two times, then dried under high vacuum for 1.5 h. The resulting lipid-phosphate diisopropylammonium salt was dissolved in distilled tetrahydrofuran (18.5 ml), 1,1'-carbonyldiimidazole (461 mg, 2.84 mmol, 4.3 equiv) was added, and the mixture stirred at room temperature for 1.5 h. To destroy excess CDI, freshly distilled methanol (0.234 ml, 0.185 g, 5.77 mmol, 8.7 equiv) was added and the mixture stirred at room temperature for 1 h, then concentrated at 30 °C and dried under high vacuum for 1 h. The sugar-phosphate **12** (280 mg, 0.66 mmol, 1 equiv) was also co-evaporated with toluene (10 ml) and diisopropylamine (10 drops) two times, then dried under high vacuum for 1.5 h. The resulting sugar-phosphate diisopropylammonium salt was dissolved in distilled tetrahydrofuran (18.5 ml) and quickly added to the flask containing the lipid-phosphoimidazolidate. The mixture was stirred at room temperature for 9 days (monitor reaction using $^1\text{H NMR}$ in CD_3OD), then concentrated at 30 °C and dried under high vacuum leaving a golden oil. The oil was purified using flash column chromatography on silica gel; the

column was loaded using EtOAc (3×1 ml), then eluted sequentially with EtOAc, 20% MeOH in EtOAc, 30% MeOH in EtOAc, 40% MeOH in EtOAc, and finally with 50% MeOH in EtOAc. High-vacuum drying gave the product (397 mg, 0.46 mmol, 70%) as a clear oil in the monodisopropylammonium salt form; ¹H NMR (400 MHz, DMSO-*d*₆): δ 1.14–1.34 (m, 24H), 1.34–1.44 (m, 2H), 1.44–1.53 (m, 2H), 1.64–1.73 (m, 2H), 1.81 (s, 3H), 1.85 (s, 3H), 1.95 (s, 3H), 2.01 (s, 3H), 3.27 (br s, 2H, DIPA), 3.67–3.77 (m, 2H, POCH₂), 3.93 (t, *J* = 6.4 Hz, 2H, PhOCH₂), 3.95–4.05 (m, ~1H, H-6b), 4.05–4.21 (m, 3H, H-6a, H-5, H-2), 4.91 (t, *J* = 9.6 Hz, 1H, H-4), 5.14 (t, *J* = 10.0 Hz, 1H, H-3), 5.33 (dd, *J* = 8.0, 3.2 Hz, 1H, H-1), 6.84–6.96 (m, 3H, aro), 7.09 (br s, 1H, possibly POH), 7.26 (app t, *J* = 8.0 Hz, 2H, aro), 8.91 (br s, 2H, possibly DIPA), 9.47 (d, *J* = 9.2 Hz, 1H, NH); ¹³C NMR (100 MHz, DMSO-*d*₆): δ 18.8 (4C, DIPA), 20.4 (2C), 20.5, 22.3, 25.5, 28.7, 28.8, 28.9, 29.00, 29.04, 29.1, 30.3, 30.4, 45.8 (2C, DIPA), 51.1 (d, ³*J*_{C-P} = 7.4 Hz, possibly C-2), 61.6 (C-6), 64.4 (d, ²*J*_{C-P} = 5.9 Hz, POCH₂), 67.2 (CHOPh), 67.6, 68.6, 71.5, 93.7 (d, ²*J*_{C-P} = 6.5 Hz, C-1), 114.4 (2C, CH aro), 120.3 (CH aro), 129.4 (2C, CH aro), 158.7 (quarternary aro), 169.3, 169.6, 170.1 (2C); ³¹P NMR (162 MHz, DMSO-*d*₆): δ -10.41 (d, ²*J*_{P-P} = 22.5 Hz), -13.77 (d, ²*J*_{P-P} = 22.5 Hz); HRMS (ESI) [M-C₆H₁₆N]⁺ Calcd for C₃₁H₄₈NO₁₆P₂: 752.2448. Found: 752.2443.

***P*¹-2-Acetamido-2-deoxy- α -D-glucopyranosyl *P*²-11-phenoxyundecyl disodium diphosphate (14).**

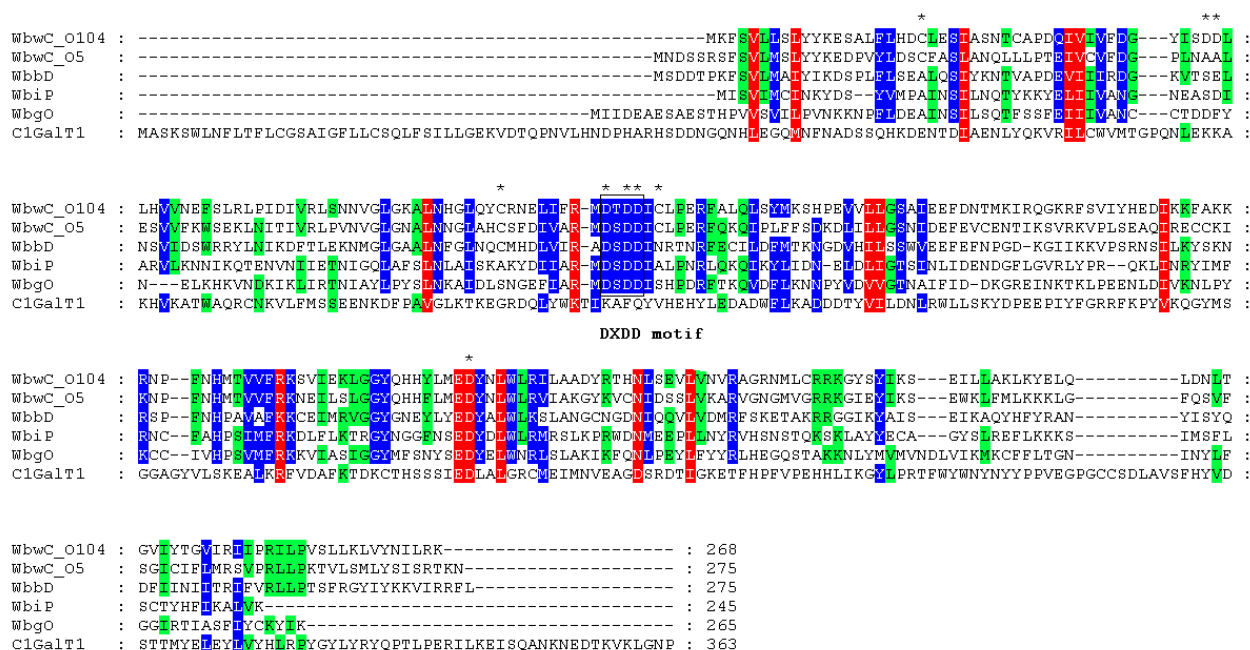
A sample of sodium (33 mg, 1.43 mmol, 3.25 equiv) which had been washed with hexanes and dried under high vacuum was added to methanol (50 ml) under an atmosphere of nitrogen. The mixture was stirred at room temperature until the reaction was completed, then this solution of sodium methoxide (3.25 equiv) in methanol was added to the triacetate **13** (380 mg, 0.44 mmol, 1 equiv). The mixture was stirred at room temperature for 20 min (pH=9), then quenched by the addition of Amberlyst 15 ion exchange resin in the H⁺ form (1 g), stirred at room temperature for 20 min (pH=6), and filtered to remove the resin. The filtrate was treated with Amberlite IR-120 ion exchange resin in the Na⁺ form (3 g), stirred at room temperature for 20 min (pH=6), and filtered to remove the resin. The filtrate was again treated with Amberlite IR-120 ion exchange resin in the Na⁺ form (3 g), stirred at room temperature for 20 min (pH=7), and filtered to remove the resin. The filtrate was concentrated at 40 °C, concentrated with diethyl ether, then dried under high vacuum. The resulting beige solid (295 mg) was dissolved in methanol (0.8 ml), and loaded onto a preparatory-scale thin-layer chromatography plate (10 cm x 10 cm x 2 mm thick). The plate was eluted sequentially with ethyl acetate, then twice with 30% methanol in ethyl acetate. The section corresponding to *R*_f range 0–0.38 was excised from the plate, and eluted with 80% methanol in ethyl acetate, and filtered. The filtrate was treated with Amberlite IR-120 ion exchange resin in the Na⁺ form (3 g), stirred at room temperature for 20 min, filtered to remove the resin, the filtrate concentrated at 40 °C, then concentrated with diethyl ether. High-vacuum drying gave the product (206 mg, 0.31 mmol, 70%) as a beige solid in the disodium salt form; mp 155–160 °C; ¹H NMR (400 MHz, exchanged with D₂O first, then acquire spectrum in DMSO-*d*₆): δ 1.11–1.35 (m, 12H), 1.35–1.45 (m, 2H), 1.45–1.58 (m, 2H), 1.63–1.75 (m, 2H), 1.85 (br s, 3H), 3.03–3.11 (m, 1H), 3.11–3.25 (m, 2H), 3.63–3.83 (m, 5H), 3.93 (t, *J* = 6.4 Hz, 2H), 5.23–5.38 (br m, 1H, H-1), 6.84–6.97 (m, 3H, aro), 7.26 (app t, *J* = 7.8 Hz, 2H, aro); ³¹P NMR (162 MHz, DMSO-*d*₆): δ -9.18 (br s), -11.44 (br s); HRMS (ESI) [M-Na]⁺ Calcd for C₂₅H₄₁NO₁₃P₂Na: 648.1950. Found: 648.1946.

Supplemental FIGURE 1. Proposed biosynthesis of the O104 antigen-specific lipopolysaccharide. The biosynthesis of the *E.coli* O104 repeating unit is thought to be initiated at the cytoplasmic side of the inner membrane. Undecaprenol phosphate serves as an acceptor for the transfer of GlcNAc α -phosphate by sugar-phosphate transferase WecA which needs to be converted by a 4-epimerase to form the acceptor for WbwC, GalNAc-PP-Und. It may also be possible that WecA directly transfers GalNAc-P. The WbwC and two other transferases WbwA and WbwB of which genes are found in the O104 antigen gene cluster then complete the repeating unit synthesis. Flippase Wzx flips the repeating unit to the periplasm where polymerase Wzy transfers the unit to the reducing end of the growing chain. The completed O-polysaccharide is then transferred by a ligase to the outer core oligosaccharide-linked to lipid A to form the completed LPS. The Lpt complex then extrudes the large O antigen-containing LPS to the outer membrane where the O antigen is exposed to the external environment.

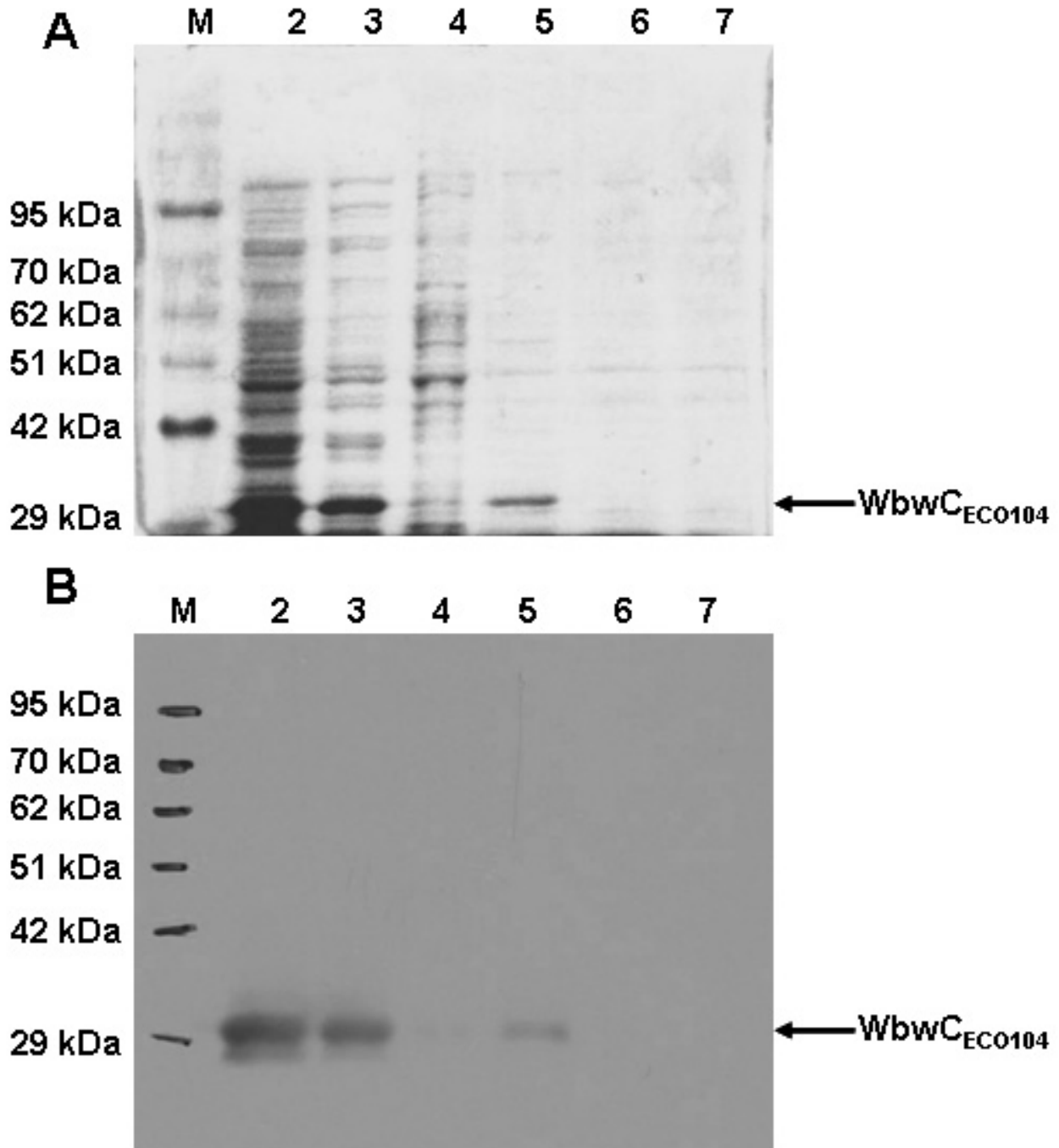


Supplemental FIGURE 2

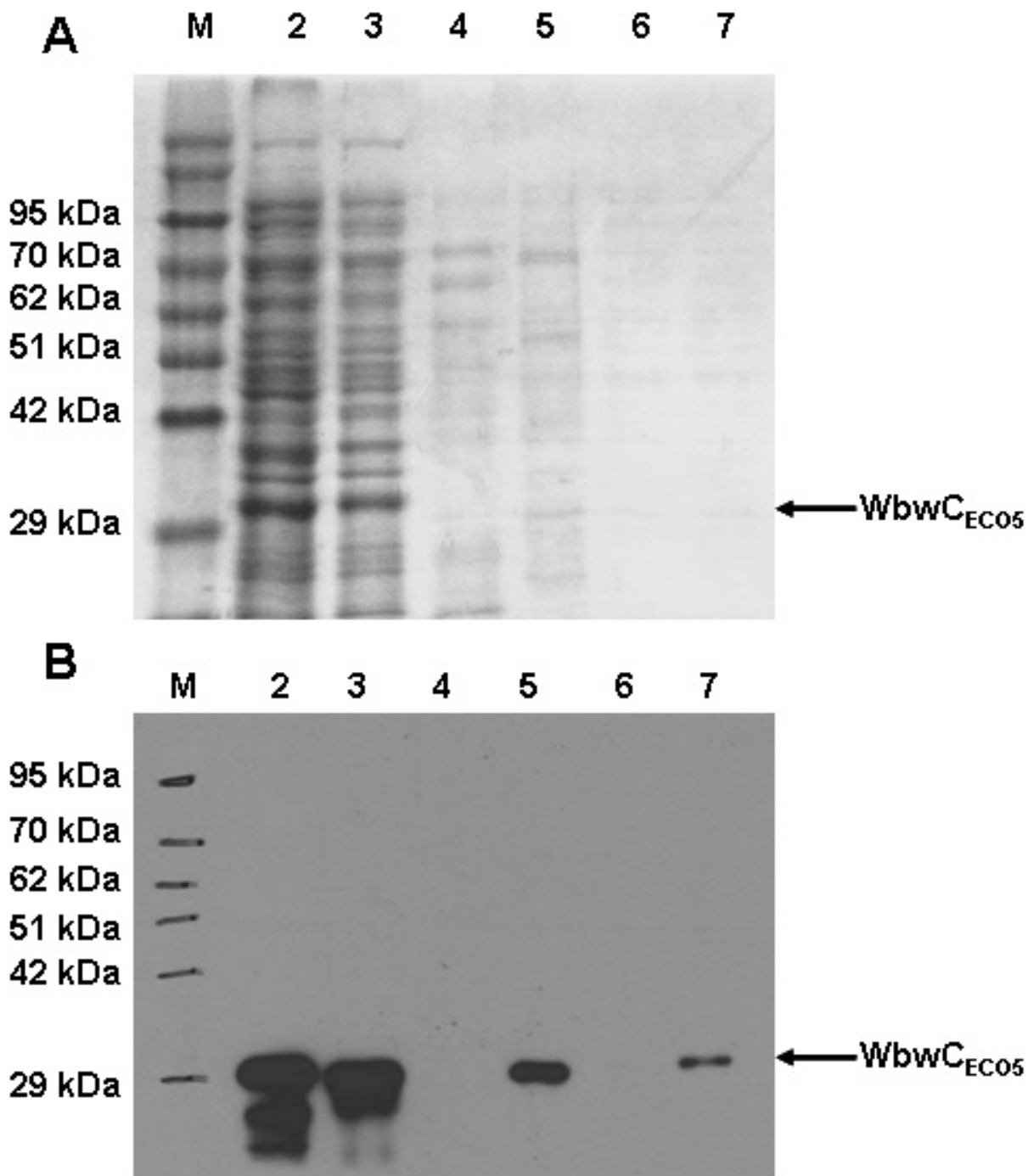
Multiple sequence alignment of WbwC from *E. coli* O104 and O5 with other bacterial Gal-transferases and human core 1 β 1,3-Gal-transferase, C1GalT1. WbbD is from *E. coli* O7: K1; WbiP is from *E. coli* O127:H6 and WbgO is from *E. coli* O55:H7. WbiP synthesizes a Gal β 1-3GalNAc linkage, whereas WbbD and WbgO synthesize a Gal β 1-3GlcNAc linkage. Using similarity groups, the red colour shows 100% conserved residues, the blue shows 80% or greater conserved residues and the green shows 60% or greater conserved residues. WbwC_{O104} mutation sites are indicated by an asterisk above the sequence and the conserved DxDD motif is shown below the alignment. This alignment was made using ClustalW and was reformatted in GeneDoc (<http://www.nrbsc.org/gfx/genedoc/index.html/>).



Supplemental FIGURE 3. Expression and purification of WbwC_{ECO104}. WbwC_{ECO104} eluted from the Ni²⁺-NTA Sepharose Fast Flow column and fractions were run on SDS-PAGE gels. (M)-Molecular mass markers; (2)-WbwC_{ECO104} Pellet; (3)- Flow-through fraction from NTA Sepharose column; (4)-Wash with 25 mM imidazole; (5-7) Fractions eluted with 250 mM imidazole. (A) SDS-PAGE analysis of WbwC_{ECO104} proteins. (B) Western blot analysis of purified WbwC_{ECO104}. An anti-His₆- tag antibody was used to detect the WbwC_{ECO104} protein.



Supplemental FIGURE 4. Expression and purification of WbwC_{ECO5}. WbwC_{ECO5} eluted from the Ni²⁺-NTA Sepharose Fast Flow column and fractions were run on SDS-PAGE gels. (M)-Molecular mass markers; (2)-WbwC_{ECO5} Pellet; (3)-Flow-through fraction from NTA Sepharose column; (4)-Wash with 25 mM imidazole; (5-7) Fractions eluted with 250 mM imidazole. (A) SDS-PAGE analysis of WbwC_{ECO5} proteins. (B) Western blot analysis of purified WbwC_{ECO5}. An anti-His₆ tag antibody was used to detect the WbwC_{ECO5} protein.



Supplemental FIGURE 5. Test for TF antigen reactivity of WbwC enzyme products. Samples (50 nmol) were incubated with TF antigen-specific antibody as the primary antibody and horseradish peroxidase-conjugated anti-mouse IgG as the secondary antibody followed by separation on a C18 Sep-Pak column. MeOH elution fractions were concentrated, added to small spots on Nitrocellulose membrane and visualized with developer. Lanes 1,2: Samples before separation on Sep-Pak; lanes 3,4: Samples after elution from Sep-Pak. **8**, GalNAc-PP-PhU; **14**, GlcNAc-PP-PhU; WbdN product, Glc β 1-3GalNAc-PP-PhU; WbwC_{ECO104} and WbwC_{ECO5} products, Gal β 1-3GalNAc-PP-PhU. Gal β 1-3GalNAc α -Bn (TF antigen) served as the positive control.

