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Large-scale chemical and chemo-enzymatic synthesis of a spacer-containing Pk-trisaccharide ☆

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Abstract—The Pk-trisaccharide, linked to a solid carrier, is a potential agent for neutralization of shiga-like toxin in the gastrointestinal tract. Two approaches to the multigram-scale synthesis of a linkable Pk-trisaccharide derivative were therefore investigated. A four-step chemical synthesis yielded 8-methoxycarbonyloctyl β-lactoside in 75% yield from lactose. Further conversion of this derivative through either multistep organic synthesis or one-step enzymatic galactosylation with UDP-galactose and recombinant α-1,4-galactosyltransferase gave the Pk-trisaccharide derivative 8-methoxycarbonyloctyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside in 25% and 68% overall yields from commercial lactose, respectively.

Keywords: Pk-trisaccharide; Chemoenzymatic

1. Introduction

Cell-surface carbohydrates mediate cell–cell interactions through specific binding to mammalian lectins present on cell surfaces.[1–3] Whether this recognition involves migration of metastatic cells,[4] bacterial adherence,[5] or adhesion of neutrophils to the endothelium during inflammatory responses,[6] competitive carbohydrate ligands have therapeutic potential. However, there are barriers that remain in the practical synthesis[7] and use of oligosaccharides for these purposes. Cost and management of multistep synthesis is cumbersome and is often seen as a limitation for entrance of this class of molecule into the therapeutic arena. These challenges can often be met by the use of chemical synthesis in combination with the use of such enzymes as glycosyltransferases and glycosidases[8] to produce the interglycosidic linkages. In this communication, we address the preparative problems raised by the recent increased interest in the development of carbohydrate drugs for gastrointestinal diseases such as verotoxigenic *E. coli* (VTEC) infections caused by food-borne pathogens. These pathogens release a toxin whose receptor on mammalian cell surfaces has been identified[9] as the carbohydrate corresponding to the Pk-antigen, which has the α-D-Gal-(1→4)-β-D-Gal(1→4)-β-D-Glc structure present in the glycoside **1** (Scheme 1). The complications arising from *E. coli* infections, such as acute kidney failure, have been correlated with the expression of Pk-antigen in the renal glomerulus.[10,11] Currently, there is no viable anti-toxin therapy available. The Pk-trisaccharide, when covalently linked to silica gel particles, has been shown to adsorb and neutralize the toxin from the gut. Several chemical or enzymatic syntheses of Pk-trisaccharide as a reducing sugar and with a variety of aglycones have been published in the literature.[12–16] In the chemical approaches, most of the intermediates involved were purified after each step by column

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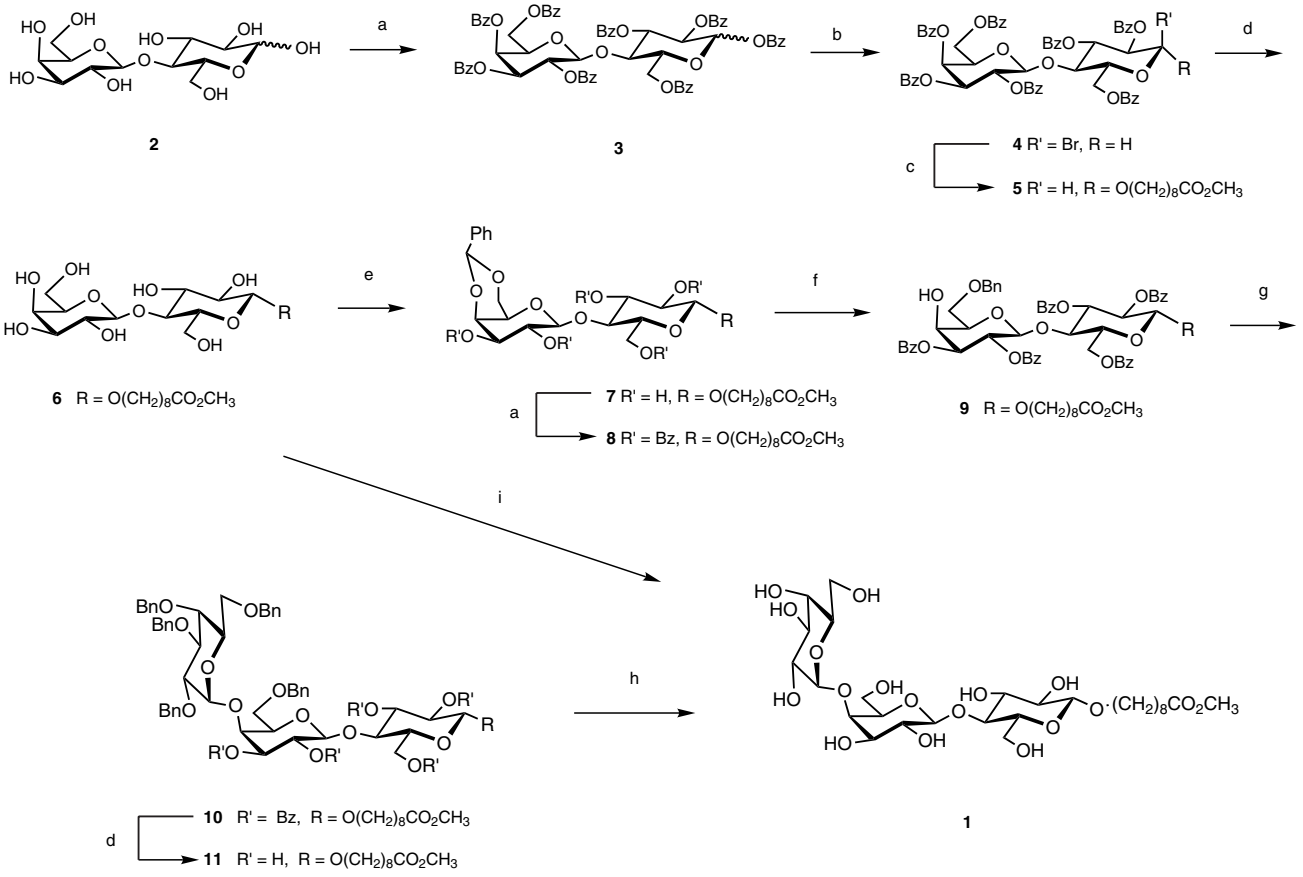

Scheme 1. Reagents and conditions: (a) benzoyl chloride, pyridine; (b) HBr in acetic acid; (c) AgOTf, $HO(CH_2)_8CO_2CH_3$, tetramethylurea; (d) $NaOCH_3$; (e) $PhCH(OCH_3)_2$, TsOH; (f) $AlCl_3$, $BH_3{\cdot}Et_3N$, trifluoroacetic acid; (g) 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl chloride, AgOTf, tetramethylurea; (h) H_2, Pd–C; (i) UDP-Galactose, α-1,4-Gal-T.

chromatography. In this paper, we report a practical, large-scale chemical synthesis of the Pk-trisaccharide glycoside **1** in 10 steps starting from lactose. The synthesis involves only one column chromatographic purification while all other intermediates were purified by either crystallization or trituration, or were taken forward without any purification. For comparison, a large-scale enzymatic synthesis of **1** was also carried out, starting from the synthesized lactose derivative **6**.

2. Results and discussion

Both the chemical and enzymatic synthesis of **1** utilized the 8-methoxycarbonyloctyl β-lactoside **6**, which was synthesized in four steps from commercially available lactose (Scheme 1). The strategy involved converting lactose into its octabenzoate **3**, using benzoyl chloride in pyridine. Treatment of the crude octabenzoate with hydrogen bromide in acetic acid gave the lactosyl bromide **4**, which was in turn used in a silver triflate-promoted glycosylation of 8-methoxycarbonyloctanol to give **5**. Debenzoylation of **5** with sodium methoxide and precipitation gave **6** in a 75% yield from lactose, which is a significant improvement from earlier reported syntheses in literature.[13] For the chemical synthesis of **1**, a benzylidene group was introduced at the 4′,6′ position of compound **6**, using benzaldehyde dimethyl acetal and a catalytic amount of camphorsulfonic acid. The resulting product **7** was directly benzoylated with benzoyl chloride in pyridine to give **8**, which could be crystallized from methanol in 85% yield. Subsequent reductive opening of the benzylidene ring using anhydrous aluminum chloride, borane–triethylamine complex, and trifluoroacetic acid gave the desired glycosyl acceptor **9**,[17] which was crystallized readily from methanol in 86% yield. The structure of **9** was confirmed by ^1H NMR spectroscopy using in situ derivatization with trichloroacetyl isocyanate, which resulted in a downfield shift of the H-4″ proton signal to 6.65 ppm.

Several conditions were attempted for the glycosylation of **9**. The best conditions found were as follows: Compound **9** was reacted with 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl chloride (obtained by treating commercially available ethyl 2,3,4,6-tetra-*O*-benzyl-1-thio-β-D-galactopyranoside with *N*-chlorosuccinimide and tetraethylammonium chloride) using silver triflate promotion at ambient temperature[18] and tetramethyl-

urea as acid scavenger. The crude reaction mixture, containing **10**, was directly debenzoylated (sodium methoxide), and the resulting mixture was then subjected to column chromatography using a dichloromethane–methanol gradient as eluant. The desired fractions were pooled and evaporated to give pure **11** in 75% yield from the alcohol **9**. Finally, the benzyl groups were removed by catalytic hydrogenation using 10% Pd/C. The desired Pk-trisaccharide glycoside **1** was obtained in a crystalline form in 80% yield (from **11**). The structure of **1** was verified by high field ^{1}H and ^{13}C NMR spectroscopy. The data agreed closely with those previously published[14] for the Pk-trisaccharide bromoethyl glycoside.

For comparison, we also carried out enzymatic galactosylation of **6** on a relatively large scale (Scheme 1), utilizing UDP-galactose and recombinant α-1,4-galactosyltransferase from *N. meningitidis*. This gave 27 g of compound **1** in 90% yield, similar to what has been reported before [16a] for small-scale (210 mg) synthesis of the corresponding methyl glycoside. The largest scale reported for Pk-trisaccharide is 188 g/L of the reducing sugar using metabolically engineered bacteria.[15a]

In summary, the enzymatic route to **1** gave a 68% overall yield from lactose, whereas the corresponding yield of **1** by the chemical route was 25%. Both routes were shown to be suitable for pilot-plant size batches of material.

3. Experimental

3.1. General methods

All solvents were of commercial grade and used without purification or drying. Melting points were measured with a Fisher–Johns instrument and are uncorrected. NMR spectra were recorded on a Bruker AMX-300 instrument using the signals from either internal Me_4Si (δ 0.00, $CDCl_3$) or DOH (δ 4.80, D_2O) as references. FTIR spectra were recorded on a Perkin–Elmer Spectrum-1000 instrument. Mass spectra were recorded on a Micromass ZabSpec Hybrid Sector-TOF instrument in positive electrospray ionization mode using a 1% solution of AcOH in 1:1 methanol–water as the liquid carrier. Optical rotation was measured with a Perkin–Elmer 241 polarimeter. Abbreviations used: CH_2Cl_2; EtOAc, ethyl acetate; MeCN, acetonitrile; MeOH, methanol; Et_3N, triethylamine; TFA, trifluoroacetic acid; EDTA, ethylenediaminetetraacetic acid.

3.2. Synthesis

3.2.1. 2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-1,2,3,6-tetra-*O*-benzoyl-α,β-D-glucopyranose (lactose octabenzoate, 3). Commercially available lactose (**2**, 400 g, 1.10 mol) was stirred in pyridine (4.5 kg) at ambient temperature. Benzoyl chloride (1.7 kg, 12.3 mol) was added to the reaction vessel and the mixture was stirred at 65 °C. The progress of the reaction was monitored by TLC (85:15, toluene–EtOAc; product α anomer R_f 0.58, β anomer R_f 0.51). Excess benzoyl chloride was decomposed by the addition of MeOH (250 g) and the mixture was concentrated to a syrup. The crude syrup was redissolved in CH_2Cl_2 and washed with water, 5% aq HCl, and 6% aq $NaHCO_3$. The organic layer was dried (Na_2SO_4) and this solution was carried forward to the next step.

3.2.2. 2,3,4,6-Tetra-*O*-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzoyl-α-D-glucopyranosyl bromide (benzobromolactose, 4). To the solution of **3** in CH_2Cl_2 was added 30% w/w HBr–acetic acid (1110 g, 4.12 mol) and the mixture was stirred at ambient temperature. Progress of the reaction was monitored by TLC (85:15, toluene–EtOAc; product R_f 0.53). The organic layer was washed with water and 6% aq $NaHCO_3$, then evaporated to a syrup. The syrup was triturated with hexane and the supernatant liquid was decanted. The resulting solid was dried and taken forward to the next step without any further purification.

3.2.3. 8-Methoxycarbonyloctyl β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (8-methoxycarbonyloctyl β-lactoside, 6). Compound **4** (1300 g, 1.17 mol) dissolved in CH_2Cl_2 (2 kg) was added to a reaction vessel containing CH_2Cl_2 (10 kg), 4 Å molecular sieves (1.5 kg), 8-methoxycarbonyloctanol (263 g, 1.40 mol), tetramethylurea (147 g, 1.26 mol) and silver triflate (389 g, 1.51 mol). The mixture was stirred at ambient temperature until TLC (85:15 (v/v), toluene–EtOAc, product R_f 0.48) showed complete consumption of the starting material. The mixture was neutralized with Et_3N and then filtered through Celite. The filtrate was washed with 5% aq NH_4OH and then neutralized with AcOH. The organic layer was evaporated to a syrup, which was triturated with hexane. The supernatant was decanted and the resulting solid dried, and then dissolved in MeOH (5.5 kg), followed by the addition of 1 M anhydrous NaOMe (300 g, to pH > 11). The mixture was kept at 45 °C until TLC (65:35:8, $CHCl_3$–MeOH–H_2O, product R_f 0.48) showed complete conversion. The mixture was neutralized by the addition of AcOH to pH 5–7. The solvent was evaporated and the crude product triturated with hexane to give a crude solid. The compound was finally crystallized from MeOH–EtOAc to furnish the desired product **6** (75% yield, 400 g). Mp 158–160 °C; $[\alpha]_D$ −1.41° (*c* 0.5, H_2O); ^{1}H NMR (D_2O/CD_3OD) δ 4.51 (d, 1H, *J* 8 Hz, H-1′), 4.47 (d, 1H, *J* 8 Hz, H-1), 3.71 (s, 3H, $-OCH_3$), 2.40 (t, 2H, $-CH_2CO$), 1.20–1.78 (m, 12H, $-CH_2-$); ^{13}C NMR (D_2O/CD_3OD) δ 177.7, 103.8, 103.0, 79.3, 76.1, 75.5, 75.3, 73.7, 73.4,

71.7, 71.2, 69.4, 61.7, 60.9, 52.5, 34.4, 29.6, 29.2, 29.1, 29.0, 25.8, 25.1; IR (KBr) 3395, 2920, 2851, 1729, 1438, 1086, 1064, 1037 cm^{-1}; HRMS calcd for $C_{22}H_{40}O_{13}[M+H]^+$ 513.2547, found 513.2536.

3.2.4. 8-Methoxycarbonyloctyl 2,3-di-*O*-benzoyl-4,6-*O*-benzylidene-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (8). Compound **6** (340 g, 0.66 mol) and benzaldehyde dimethyl acetal (322 g, 2.10 mol) was stirred in MeCN (5.7 kg). The pH of the suspension was adjusted to 3 by addition of camphorsulfonic acid (14 g, 60 mmol). The mixture was kept at 40 °C for 16–24 h until the reaction was complete as indicated by TLC (65:35:8, $CHCl_3$–MeOH–H_2O; product R_f 0.77). The mixture was then neutralized by addition of Et_3N, and the solvents were evaporated to give crude **7**, which was directly taken up in pyridine (4.7 kg) containing 4-dimethylaminopyridine (84 g, 0.68 mol). Benzoyl chloride (742 g, 5.28 mol) was added dropwise at room temperature and the reaction was then stirred overnight. The progress of the reaction was monitored by TLC (85:15, toluene–EtOAc; product R_f 0.35). Excess benzoyl chloride was decomposed by addition of MeOH (250 g). The solvent was evaporated and the residue was dissolved in CH_2Cl_2 and washed with water, 5% aq HCl, 6% aq $NaHCO_3$, and water. The solvents were removed by evaporation and the residue was crystallized from MeOH to give pure **8** (85% yield, 632 g), mp 164–166 °C; $[\alpha]_D$ +98.18° (*c* 1.7, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.10–8.07 (m, 30H, C_6H_5), 5.29 (s, 1H, benzylidene), 4.85 (d, 1H, *J* 8 Hz, H-1′), 4.66 (d, 1H, *J* 8 Hz, H-1), 3.65 (s, 3H, O═C─O─CH_3), 2.21 (m, 2H, –CH_2CO), 0.92–1.7 (m, 12H, –CH_2–); ^{13}C NMR ($CDCl_3$) δ 174.2, 166.0, 165.6, 165.3, 165.0, 164.8, 137.4, 133.1, 133.0, 132.9, 129.9, 129.8, 129.7, 129.65, 129.6, 129.5, 128.9, 128.8, 128.7, 128.4, 128.3, 128.28, 128.26, 128.2, 127.9, 126.3, 101.4, 100.7, 100.6, 76.8, 76.5, 74.0, 73.0, 72.7, 72.6, 72.3, 69.4 66.4, 51.4, 50.8, 34.0, 29.2, 29.0, 28.9, 25.6, 24.8; IR (KBr) 3433, 3064, 2936, 1724, 1602, 1452, 1274, 1070, 708 cm^{-1}; HRMS calcd for: $C_{64}H_{64}O_{18}$ $[M+Na]^+$ 1143.3990, found 1143.4004.

3.2.5. 8-Methoxycarbonyloctyl (2,3-di-*O*-benzoyl-6-*O*-benzyl-β-D-galactopyranosyl)-(1 → 4)-2,3,6-tri-*O*-benzoyl-β-D-glucopyranoside (9). Anhydrous aluminum chloride (204 g, 1.5 mol) was slowly added to THF (3.3 kg) at 0 °C. After the reaction had subsided, $BH_3{\cdot}Et_3N$ (112 g, 1.5 mol) and compound **8** (340 g, 0.3 mol) were added. Once the mixture had stabilized at 0 °C, TFA (175 g, 1.5 mol) was added dropwise. The reaction was monitored by TLC (85:15:2, toluene–EtOAc–MeOH; product R_f 0.63). Upon completion of the reaction as detected by TLC, the mixture was washed twice with 5% aq H_2SO_4, and then with 6% aq $NaHCO_3$ until the pH was above 7. The solvents were removed by evaporation to give a solid residue, which was recrystallized from MeOH to give the desired product **9** (86% yield, 292 g), $[\alpha]_D$ +55.30° (*c* 1.6, $CHCl_3$); mp 80–82 °C; ^1H NMR ($CDCl_3$) δ 7.17–8.03 (m, 30H, C_6H_5), 4.76 (d, 1H, *J* 8 Hz, H-1′), 4.65 (d, 1H, *J* 8 Hz, H-1), 3.64 (s, 3H, O═C─O─CH_3), 2.21 (m, 2H, –CH_2CO), 0.92–1.54 (m, 12H, –CH_2–); ^{13}C NMR ($CDCl_3$) δ 174.2, 165.7, 165.6, 165.3, 165.2, 165.0, 137.6, 133.2, 133.1, 129.9, 129.8, 129.77, 129.7, 129.67, 129.65, 129.4, 129.0, 128.8, 128.4, 128.38, 128.3, 128.29, 127.7, 127.4, 101.2, 100.8, 76.3, 73.5, 73.2, 72.9, 71.9, 70.0, 69.9, 67.3, 51.4, 34.0, 29.2, 28.95, 28.9, 25.6, 24.8; IR (KBr) 3448, 3066, 2930, 1729, 1602, 1452, 1274, 1107, 708 cm^{-1}; HRMS calcd for $C_{64}H_{66}O_{18}$ $[M+Na]^+$ 1145.4146, found 1145.4152.

3.2.6. 8-Methoxycarbonyloctyl 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl-(1 → 4)-6-*O*-benzyl-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranoside (11). To a solution of **9** (312 g, 0.28 mol) in CH_2Cl_2 (5.9 kg) was added 4 Å molecular sieves (312 g), tetramethylurea (84 g, 0.72 mol) and silver triflate (173 g, 0.67 mol). The mixture was stirred for 1 h and then 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl chloride (470 g, 0.84 mol) was added. The reaction was allowed to proceed at room temperature for 16–24 h and the progress of the reaction was monitored by TLC (85:15, toluene–EtOAc; product R_f 0.5). The mixture was filtered and the filtrate extracted with aq EDTA and water. The organic layer was dried, filtered and the solvents evaporated. The residue, containing crude **10**, was taken directly to the next step without further purification. Thus, 1 M anhydrous NaOMe (164 g) was added to a solution of **10** (234 g) in MeOH (3.0 kg) to give a pH > 11. The mixture was stirred at 45 °C and the progress of the reaction monitored by TLC (95:5, CH_2Cl_2–MeOH; product R_f 0.25). The mixture was neutralized by the addition of AcOH to pH 5–7. The solvent was evaporated and the residue was redissolved into EtOAc and washed with water, and 6% brine. The organic layer was evaporated and the crude product purified by silica gel column chromatography using a Biotage 150 flash chromatography system. The fractions containing product were pooled together and the solvents evaporated to furnish pure **11** in 75% yield (234 g) as foam. $[\alpha]_D$ +8.75° (*c* 2.7, $CHCl_3$); ^1H NMR ($CDCl_3$) δ 7.10–8.15 (m, 25H, C_6H_5), 3.65 (s, 3H, O═C─O─CH_3), 2.29 (t, 2H, *J* 8 Hz, –CH_2CO), 1.20–1.70 (m, 12H, –CH_2–); ^{13}C NMR ($CDCl_3$) δ 174.2, 138.3, 138.0, 137.7, 137.3, 128.6, 128.5, 128.4, 128.38, 128.34, 128.3, 128.2, 127.9, 127.87, 127.84, 127.8, 127.7, 127.5, 104.2, 102.5, 100.5, 84.0, 80.9, 78.7, 76.5, 76.3, 74.9, 74.5, 74.4, 74.2, 74.1, 73.9, 73.8, 73.5, 73.3, 73.1, 71.7, 71.3, 70.1, 69.2, 63.2, 51.4, 34.0, 29.5, 29.1, 29.08, 29.0, 25.8, 24.8; IR (KBr) 3448, 3054, 2930, 2305, 1732, 1454, 1265, 1097, 896, 739 cm^{-1}; HRMS calcd for $C_{63}H_{80}O_{18}$ $[M+Na]^+$ 1147.5242, found 1147.5241.

3.2.7. 8-Methoxycarbonyloctyl α-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-β-D-glucopyranoside (1). To a nitrogen-purged solution of **11** (120 g, 0.1 mol) in MeOH (4.9 kg) was added 10% Pd/C (70 g). The mixture was stirred while hydrogen gas was bubbled through the solution for 5–8 h. The progress of the reaction was monitored by TLC (65:35:8, $CHCl_3$–MeOH–H_2O; product R_f 0.67). Upon completion of the reaction, the catalyst was removed by filtration and the filtrate was concentrated. The residue was crystallized from MeOH–1-propanol to give **1** in 80% yield (57 g), mp 156–158 °C; $[\alpha]_D$ +46.98° (*c* 1.9, H_2O); ^1H NMR (D_2O) δ 4.95 (d, 1H, $J_{1'',2''}$ 4.0 Hz, H-1″), 4.51 (d, 1H, $J_{1',2'}$ 7.8 Hz, H-1′), 4.48 (d, 1H, $J_{1,2}$ 8.1 Hz, H-1), 4.35 (ψt, 1H, H-5″), 4.04 (d, 1H, H-4′), 4.03 (d, 1H, H-4″), 3.99 (1H, H-6a), 3.93 (1H, H-6a′), 3.91 (1H, H-3″), 3.84 (2H, *J* 10.5 Hz, H-2″, H-6b′), 3.83 (1H, H-6b), 3.79 (1H, H-5′), 3.74 (2H, H-6a″, H-3), 3.70 (m, 1H, H-6b″), 3.69 (s, 3H, –OCH_3), 3.65 (1H, H-4), 3.64 (dd, *J* 9.8 Hz, H-3), 3.58 (2H, H-5, H-2′), 3.30 (dd, 1H, *J* 9.2 Hz, H-2), 2.39 (m, 2H, –CH_2CO), 1.30–1.80 (m, 12H, –(CH_2)–); ^{13}C NMR (D_2O) δ 178.1, 103.5 (1C, C1′), 102.3 (1C, C-1), 100.6 (1C, C-1″), 79.0, 77.7, 75.7, 75.1, 74.8, 73.2, 72.5, 71.2, 71.1, 71.0, 69.4, 69.2, 68.9, 60.8, 60.7, 60.4, 52.4, 34.0, 29.0, 28.7, 28.6, 28.4, 25.3, 24.6; IR (KBr) 3401, 2930, 1736, 1438, 1073, 810, 700, 545 cm^{-1}; HRMS calcd for $C_{28}H_{50}O_{18}$ $[M+Na]^+$ 697.2894, found 697.2903.

3.2.8. Enzymatic synthesis of 1. A 1.13 L solution of **6** (22.5 g, 43.9 mmol), UDP-Gal.2Na (35.7 g, 58.5 mmol), recombinant *N. meningitidis* α-1,4-galctosyltransferase[15,16] (283 U) in 68 mM MOPS-NaOH buffer, pH 7.5, containing 0.2 mg/mL bovine serum albumin, 2 mM $MnCl_2$ and alkaline phosphatase (3660 U), was gently stirred in a 2 L plastic beaker at room temperature for 38 h. The progress of the reaction was monitored by TLC (4:1:0.2, EtOAc–MeOH–H_2O) and by a radiochemical assay[19] until conversion of **6** to product **1** was complete (product **1** R_f 0.29, substrate **6** R_f 0.47). The mixture was filtered and the filtrate was applied to a reverse-phase C_{18} column (column size: 370 mL). The column was washed with Milli-Q water (1.5 L) and then the product was eluted with 0.5 L HPLC-grade MeOH. The solvent was evaporated to dryness to give a residue, which was re-dissolved in H_2O and lyophilized to dryness to furnish the desired product **1** as a white powder (26.9 g, yield 90%). The spectral data matched those of a chemically prepared batch of **1**. HRMS calcd for $C_{28}H_{50}O_{18}$ $[M+Na]^+$ 697.2894, found 697.2900.

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