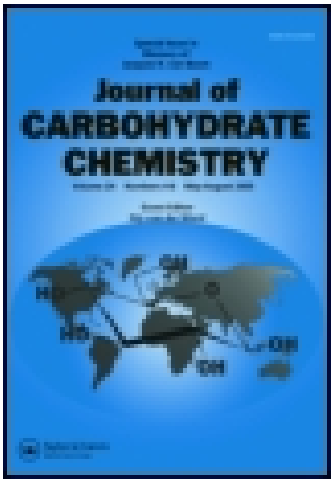

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Synthesis of a Pentasaccharide Repeating Unit of the Extracellular Polysaccharide Produced by Lactobacillus Delbrueckii Subsp. Bulgaricus 291

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Synthesis of a Pentasaccharide Repeating Unit of the Extracellular Polysaccharide Produced by *Lactobacillus Delbrueckii* Subsp. *Bulgaricus* 291

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A pentasaccharide methyl glycoside has been synthesized efficiently using a modified glycosylation strategy. This pentasaccharide is a repeating unit of the exopolysaccharides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 291.

Keywords Pentasaccharide, Glycosylation, Exopolysaccharide, *Lactobacillus delbrueckii* subsp. *bulgaricus* 291

INTRODUCTION

Lactic acid bacteria (LAB) are the organisms that beneficially affect the host animal by improving the intestinal microbial balance[1] by producing an abundant variety of exopolysaccharides (EPSs), which provide an important contribution to human health by acting as prebiotic substrates. EPSs produced by LAB have several medicinal values, possessing antitumor,[2] antimutagenic,[3] antiulcer,[4] and antibacterial activities.[5] In addition, LAB have been shown to be effective as immunostimulators[6] and blood cholesterol-lowering

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agents.[7] It has been found that the above-mentioned medicinal events arise from the whole microorganisms or cell wall components or extracellular polysaccharides.[8] Exopolysaccharides produced by lactic acid bacteria are also used to improve the body and texture of dairy products.[9] The positive role of dairy lactobacilli in human health was suggested nearly a century ego. Various brands of yogurt are consumed as prophylactics or for treatment of common intestinal infections, such as diarrhea.[10] Among several LAB, a combination of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which grow synergistically, are widely used as starter cultures for the industrial production of yogurt.[11] In another aspect, exopolysaccharides secreted by LAB play a significant role in the protection of microbial cells against phagocytosis, phage attacks, antibiotics, toxic compounds (e.g., toxic metal ions, sulfur dioxide, andethanol), osmotic stress, and bacteriocins such as nisin.[12]

Recently, the structure of the extracellular polysaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 291 has been reported by Faber et al.,[13] which is a pentasaccharide composed of D-galactose and D-glucose. In view of the importance of the exopolysaccharides for their biological use and limited natural availability, it is essential to synthesize them chemically in a concise manner. As a part of our ongoing program toward the synthesis of bacterial oligosaccharides, we report herein a chemical synthesis of the extracellular pentasaccharide as its methyl glycoside produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 291 using a robust glycosylation protocol (Figure 1).

RESULTS AND DISCUSSIONS

The synthesis of pentasaccharide **7** is shown in Scheme 1 and began with methyl α-D-glucopyranoside **(1)**. Methyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside (**2**) was prepared from methyl α-D-glucopyranoside **(1)** following a two-step reaction sequence involving the treatment with benzaldehyde dimethylacetal in the presence of *p*-toluene sulfonic acid followed by benzylation using benzyl bromide and sodium hydroxide in 85% overall yield. Treatment of compound **2** with $HClO_4$-SiO_2[14] in acetonitrile at rt furnished methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside **(3)** in 95% yield. *N*-Iodosuccinimide (NIS)-$HClO_4$-SiO_2 promoted[15] condensation of compound **3** with ethyl 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-1-thio-β-D-lactopyranoside **(5)**[16] prepared

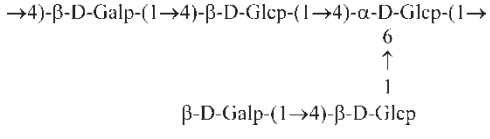
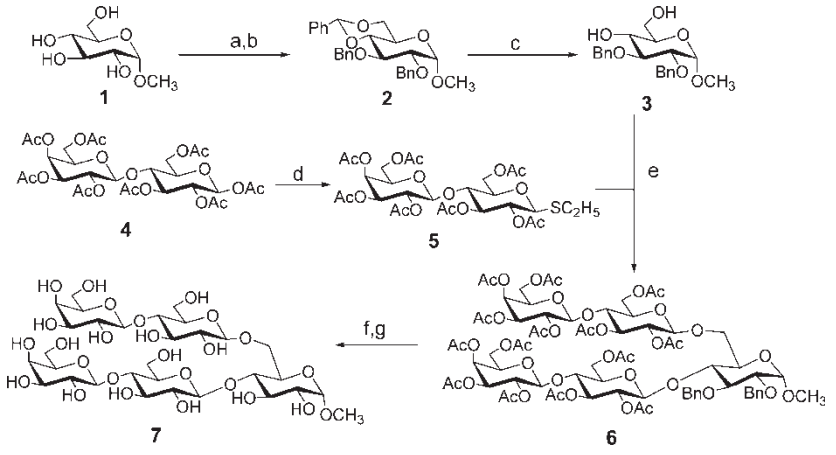


Figure 1: Structure of the extracellular pentasaccharide produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 291.



Scheme 1: Reagents: (a) $PhCH(OCH_3)_2$, *p*-TsOH, CH_3CN, rt, 5 h; (b) BnBr, 50% aq. NaOH, *n*-Bu_4NBr, CH_2Cl_2, rt, 5 h, 85% in two steps; (c) $HClO_4$-SiO_2, CH_3CN, rt, 20 min, 95%; (d) C_2H_5SH, $BF_3{\cdot}OEt_2$, CH_2Cl_2, 0°C, 12 h, 80%; (e) NIS, $HClO_4$-SiO_2, CH_2Cl_2, 0°C, 2 h, 78%; (f) CH_3ONa, CH_3OH, rt, 5 h; (g) H_2, 20% $Pd(OH)_2$-C, CH_3OH, rt, 12 h, 72% in two steps.

from D-lactoseoctaacetate and gave methyl [2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1 → 6)]-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyrano-syl-(1 → 4)-2,3-di-*O*-benzyl-α-D-glucopyranoside **(6)** in 78% yield. This glycosylation methodology has been applied in a 10 millimolar scale to achieve large quantities of pentasaccharide **(6)**. Furthermore, this protocol is equally effective, as NIS-trifluoromethanesulfonic acid (TfOH) promoted glycosylation, which has been extensively used for the activation of thioglycosides. The advantages of using $HClO_4$-SiO_2 in place of TfOH include no requirement of controlled low temperature, in some cases the catalyst system can be recovered and reused, the low cost of the catalyst, and stability toward the moisture. Zemple′n transesterification of pentasaccharide derivative **6** with sodium methoxide followed by hydrogenolysis with $H_2/Pd(OH)_2$-C furnished target pentasaccharide methyl [β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl)-(1 → 6)]-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-α-D-glucopyranoside (**7**) in 72% yield (Sch. 1). The structure of the pentasaccharide **(7)** was confirmed from its NMR and mass spectra. The presence of five anomeric signals in the ^{1}H NMR spectrum [δ 4.75 (d, J = 3.6 Hz, 1H, H-1), 6.52 (d, J = 7.8 Hz, 1H, H-1′), 4.47 (d, J = 8.1 Hz, 1H, H-1‴), 4.39 (d, J = 7.8 Hz, 1H, H-1″), 4.34 (d, J = 7.8 Hz, 1H, H-1⁗)] and ^{13}C NMR spectrum [δ 103.0 (C-1″), 102.9 (C-1⁗), 102.1 (C-1‴), 101.4 (C-1′), 99.2 (C-1)] confirmed the formation of the required pentasaccharide **(7)** (Figs. 2 and 3).

In summary, synthesis of a pentasaccharide as its methyl glycoside produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* 291 has been successfully achieved in a concise manner. Although methyl glycoside is not always

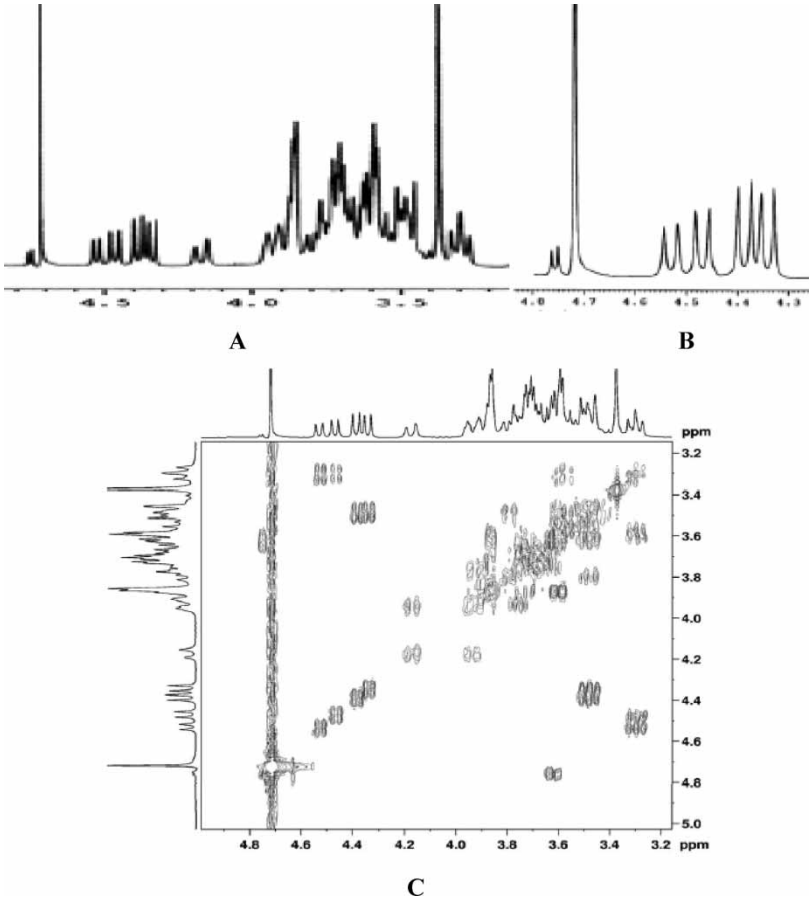


Figure 2: (A) ^{1}H NMR spectrum of compound **7**. (B) Magnified form of anomeric region of compound **7**. (C) 2D-COSY spectrum of compound **7**.

suitable for biological studies, in the synthetic scheme it can be replaced by other functional groups, such as 4-methoxyphenyl or 2-trimethylsilylethyl group, which can be removed after formation of the pentasaccharide to attach it with a spacer. The glycosylation protocol is considerably robust to be used for a scale-up reaction.

EXPERIMENTAL

General Procedure

All the reactions were monitored by thin-layer chromatography over silica gel-coated TLC plates. The spots on TLC were visualized by warming ceric sulphate (2% $Ce(SO_4)_2$ in 2 N H_2SO_4)-sprayed plates in hot plate. Silica gel

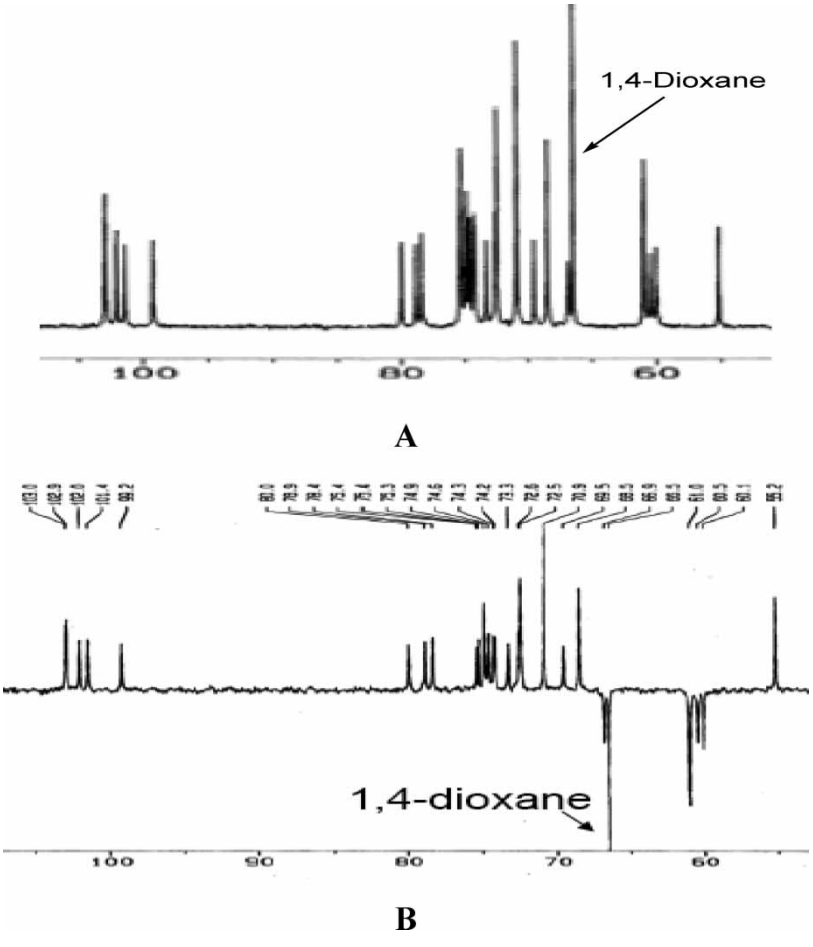


Figure 3: (A) ^{13}C NMR spectrum of compound **7**. (B) DEPT 135 spectra of compound **7**.

230–400 mesh was used for column chromatography. ^{1}H and ^{13}C NMR was recorded on a Brucker Advance DPX 300 MHz using $CDCl_3$ and D_2O as solvents and TMS as internal reference and 1,4-dioxane as external reference. Chemical shift value is expressed in (ppm. ESI-MS were recorded on a MICRO-MASS QUTTRO II triple quadrupole mass spectrometer. Elementary analysis was carried out on Carlo ERBA-1108 analyzer. Optical rotations were measured at 25°C on a Rudolf Autopol III polarimeter. Commercially available grades of organic solvents of adequate purity are used in many reactions.

Preparation of $HClO_4$-SiO_2

$HClO_4$ (1.8 g, 12.5 mmol, as a 70% aq solution) was added to a suspension of SiO_2 (230–400 mesh, 23.7 g) in Et_2O (70.0 mL). The mixture was concentrated

and the residue was heated at 100°C for 72 h under vacuum to furnish $HClO_4$-SiO_2 (0.5 mmol/g) as a free flowing powder.

Methyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-α-D-glucopyranoside (2)

To a solution of methyl α-D-glucopyranoside (**1**; 5 g, 25.7 mmol) in anhydrous CH_3CN (20 mL) were added benzaldehyde dimethylacetal (5.85 mL, 39.0 mmol) and *p*-toluene sulfonic acid (100 mg) and the reaction mixture was stirred at rt for 5 h. The reaction was quenched with triethyl amine and evaporated to dryness. To a solution of the crude mass in CH_2Cl_2 (100 mL) were added 50% aq. NaOH solution (50 mL) followed by benzyl bromide (9.0 mL, 75.8 mmol) and tetrabutylammonium bromide (100 mg) and the reaction mixture was stirred vigorously at rt for 5 h. The reaction mixture was diluted with water (100 mL) and extracted with CH_2Cl_2 (150 mL). The organic layer was washed with water, dried (Na_2SO_4), and concentrated under reduced pressure. The crude product was purified over SiO_2 using hexane-EtOAc (7:1) as eluant to furnish pure compound **2** (10 g, 85%) as a white solid, $[\alpha]_D$ +21.9 (*c* 1.0, $CHCl_3$); IR (KBr): 2926, 2368, 1595, 1368, 1087, 1052, 739, 693 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.44–7.23 (m, 15H, aromatic proton), 5.49 (s, 1H, PhC*H*), 4.86 (d, $J = 11.5$ Hz, 1H), 4.81–4.76 (m, 2H), 4.65 (d, $J = 12.1$ Hz, 1H), 4.53 (d, $J = 3.3$ Hz, 1H, H-1), 4.23 (dd, $J = 9.3$ and 3.8 Hz, 1H), 3.99 (t, $J = 9.1$ and 9.1 Hz, 1H), 3.84–3.69 (m, 2H), 3.64–3.46 (m, 2H), 3.37 (s, 3H, OCH_3); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 139.2, 138.7, 137.9, 129.2–126.5 (aromatic carbon), 101.7, 99.6, 82.7, 79.7, 78.9, 75.6, 74.0, 69.4, 62.8, 55.7; ESI-MS (462): *m*/*z* 485 [M + Na]; Anal. Calcd. for $C_{28}H_{30}O_6$: C, 72.71; H, 6.54; found: C, 72.55; H, 6.75.

Methyl 2,3-di-*O*-benzyl-α-D-glucopyranoside (3)

To a solution of compound **2** (1.5 g, 3.25 mmol) in CH_3CN (15 mL) was added $HClO_4$-SiO_2 (250 mg) and the reaction mixture was stirred at rt for 20 min. The reaction mixture was filtered through a celite bed and evaporated to dryness to give the crude product. Column chromatography of the crude product over a short pad of silica gel using hexane-EtOAc (1:1) furnished pure compound **3** (1.15 g, 95%); $[\alpha]_D$ +17.4 (*c* 1.0, $CHCl_3$); IR (neat): 2924, 1719, 1454, 1363, 1198, 1054, 743, 700 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.30–7.25 (m, 10H, aromatic proton), 4.95 (d, $J = 11.5$ Hz, 1H), 4.75 (d, $J = 11.5$ Hz, 1H), 4.69 (d, $J = 11.5$ Hz, 1H), 4.58 (d, $J = 11.5$ Hz, 1H), 4.54 (d, $J = 3.5$ Hz, 1H, H-1), 3.77–3.60 (m, 3H), 3.56–3.51 (m, 1H), 3.47–3.38 (m, 2H), 3.34 (s, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 139.3, 138.6, 128.7–127.9 (aromatic carbon), 98.5, 81.8, 80.2, 76.3, 73.3, 71.6, 70.3, 62.0, 55.5; ESI-MS

(374): *m/z* 397 [M + Na]; Anal. Calcd. for $C_{21}H_{26}O_6$: C, 67.36; H, 7.0; found: C, 67.10; H, 7.28.

Ethyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-1-thio-β-D-glucopyranoside (5)

To a solution of β-D-lactose octaacetate (5.0 g, 7.37 mmol) in dry CH_2Cl_2 (20 mL), ethanethiol (1.6 mL, 21.5 mmol) was added under inert atmosphere. The reaction mixture was cooled to 0°C and borontrifluoride diethyletherate (1.85 mL, 14.74 mmol) was added to it and the reaction mixture was stirred at 0°C for 5 h. The progress of the reaction was monitored by thin-layer chromatography over silica gel-coated plates. After completion of the reaction, the reaction mixture was diluted with CH_2Cl_2 and washed with aq. sodium bicarbonate solution and water in succession. The organic layer was dried over anhydrous Na_2SO_4 and concentrated to dryness under reduced pressure. The crude reaction mixture was purified over SiO_2 using hexane-EtOAc as eluant to furnish desired ethyl thioglycosides **5** (4.0 g, 80%); m.p.: 72°C; $[\alpha]_D$ −6 (*c* 1.0; $CHCl_3$); IR (neat): 2930, 1750, 1372, 1225, 1051, 769 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 5.23 (d, $J = 1.2$ Hz, 1H), 5.10 (t, $J = 9.0$ and 9.3 Hz, 1H), 5.09–4.94 (m, 1H), 4.89 (dd, $J = 10.5$ and 3.3 Hz, 1H), 4.82 (t, $J = 9.6$ and 9.6 Hz, 1H), 4.48 (d, $J = 7.5$ Hz, 1H), 4.43 (d, $J = 9.9$ Hz, 1H), 4.39 (dd, $J = 10.4$ and 2.0 Hz, 1H), 4.07–3.99 (m, 3H), 3.85 (t, $J = 6.6$ and 6.9 Hz, 1H), 3.71 (t, $J = 9.0$ and 9.6 Hz, 1H), 3.58–3.54 (m, 1H), 2.66–2.51 (m, 2H), 2.09, 2.04, 2.0 (3 s, 9H, 3 COCH_3), 1.99 (s, 3H, COCH_3), 1.97 (s, 6H, 2 COCH_3), 1.96, 1.89 (2 s, 6H, 2 COCH_3), 1.23–1.17 (m, 3H); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.1 (3 C), 169.8, 169.6, 169.5, 168.9, 101.3, 83.4, 77.0, 76.7, 74.2, 71.2, 70.8, 70.5, 69.4, 66.9, 62.7, 61.0, 24.3, 21.0 (2 C), 20.9 (2 C), 20.8 (2 C), 20.7, 15.2; ESI-MS (680): *m/z* 703.4 [M + Na]; Anal. Calcd. for $C_{28}H_{40}O_{17}S$: C, 49.41; H, 5.92; found: C, 49.12; H, 6.20.

Methyl (2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl)-(1 → 6))-2,3,4,6-tetra-*O*-acetyl-β-D-galacto-pyranosyl-(1 → 4)-2,3,6-tri-*O*-acetyl-β-D-glucopyranosyl-(1 → 4)-2,3-di-*O*-benzyl-α-D-glucopyranoside (6)

To a solution of compound **3** (600 mg, 1.60 mmol) and thioglycoside donor **5** (2.8 g, 4.12 mmol) in anhydrous CH_2Cl_2 (20 mL) was added powdered MS-4Å (4 g) and the reaction mixture was stirred at rt under argon for 1 h. After cooling the reaction mixture to 0°C, *N*-iodosuccinimide (1.2 g, 5.33 mmol) was added to it followed by $HClO_4$-SiO_2 (150 mg) and it was allowed to stir at 0°C

for 2 h. The reaction mixture was quenched by adding 5% aq. $Na_2S_2O_3$, diluted with CH_2Cl_2 and filtered through a celite bed. The organic layer was washed successively with aq. $NaHCO_3$ and water, dried (Na_2SO_4), and concentrated under reduced pressure. The crude product was purified over SiO_2 using hexane-EtOAc (2:1) to afford pure pentasaccharide **6** (2.0 g, 78%); $[\alpha]_D + 5.9$ (*c* 1.0, $CHCl_3$); IR (neat): 2942, 1752, 1595, 1377, 1232, 1055, 601 cm^{-1}; ^1H NMR ($CDCl_3$, 300 MHz): δ 7.35–7.34 (m, 4H, aromatic protons), 7.28–7.26 (m, 6H, aromatic protons), 5.37–5.35 (m, 2H), 5.21 (t, $J = 7.8$ Hz, 1H), 5.12–5.07 (m, 3H), 5.00–4.99 (d, $J = 3.3$ Hz, 1H), 4.94–4.92 (m, 4H), 4.89–4.87 (m, 1H), 4.70–4.67 (m, 2H), 4.60 (bs, 1H), 4.57–4.50 (m, 4H), 4.41 (d, $J = 7.8$ Hz, 1H), 4.19 (d, $J = 12$ Hz, 1H), 4.15–4.09 (m, 7H), 3.95–3.92 (m, 1H), 3.90–3.86 (m, 3H), 3.83–3.78 (m, 4H), 3.75–3.65 (m, 2H), 3.41–3.39 (m, 1H), 3.36 (s, 3H, OCH_3), 2.18, 2.17, 2.14, 2.08 (4 s, 12H, 4 $COCH_3$), 2.07 (s, 6H, 2 $COCH_3$), 2.06, 2.05, 2.04, 2.03, 2.01, 2.00, 1.99, 1.98 (8 s, 24H, 8 $COCH_3$); ^{13}C NMR ($CDCl_3$, 75 MHz): δ 170.3 (3 C), 170.2, 170.1 (2 C), 170.0 (2 C), 169.7 (2 C), 169.5 (2 C), 169.0, 168.9, 139.2, 137.8, 128.3 (2 C), 128.2 (2 C), 128.0 (2 C), 127.9, 127.2, 126.6 (2 C), 101.0 (2C), 100.8, 99.8, 97.6, 79.4, 78.9, 78.1, 76.0, 75.7, 74.3, 73.1, 72.8, 72.7, 72.6, 72.4, 72.2, 71.6, 70.9 (2C), 70.5 (2C), 69.5, 69.0, 68.9, 68.3, 66.5 (2 C), 61.9, 61.7, 60.7 (2 C), 55.1, 20.8 (3 C), 20.6 (6 C), 20.5 (5 C); ESI-MS (1610): m/z 1633.4 [M + Na]; Anal. Calcd. for $C_{73}H_{94}O_{40}$: C, 54.41; H, 5.88; found: C, 54.10; H, 6.14.

Methyl (β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl)-(1 → 6))-β-D-galactopyranosyl-(1 → 4)-β-D-glucopyranosyl-(1 → 4)-α-D-glucopyranoside (7)

To a solution of pentasaccharide derivative **6** (500 mg, 0.31 mmol) in CH_3OH (10 mL), solid CH_3ONa was added until the pH was ~10. The reaction mixture was allowed to stir at rt for 5 h followed by neutralization with Amberlite IR-120 (H^+) cation exchange resin. The reaction mixture was filtered and evaporated to dryness. To a solution of the crude product in CH_3OH (5 mL) was added 20% $Pd(OH)_2$-C (100 mg) and the reaction mixture was stirred at rt under a positive pressure of hydrogen for 12 h. The reaction mixture was filtered through a celite bed and concentrated to a white powder, which was further purified through a Sephadex LH-20 using CH_3OH-H_2O (4:1) as eluent to furnish pure pentasaccharide **7** as an amorphous powder (190 mg, 72%); $[\alpha]_D$ +10.5 (*c* 1.0, H_2O); ^1H NMR ($CDCl_3$, 300 MHz): δ 4.75 (d, $J = 3.6$ Hz, 1H, H-1), 4.52 (d, $J = 7.8$ Hz, 1H, H-1′), 4.47 (d, $J = 8.1$ Hz, 1H, H-1‴), 4.39 (d, $J = 7.8$ Hz, 1H, H-1″), 4.34 (d, $J = 7.8$ Hz, 1H, H-1⁗), 4.17 (d, $J = 11.1$ Hz, 1H, H-6_a), 3.96–3.95 (m, 1H), 3.93–3.90 (m, 1H), 3.87–3.84 (m, 3H), 3.81–3.66 (m, 11H), 3.64–3.55 (m, 6H), 3.51–3.45 (m, 3H), 3.37 (s, 3H, OCH_3), 3.32–3.27 (m, 2H); ^{13}C NMR ($CDCl_3$, 75 MHz, 1,4-dioxan as external standard): δ 103.0 (C-1″), 102.9 (C-1⁗⁗⁗⁗),

102.1 (C-1‴), 101.4 (C-1′), 99.2 (C-1), 79.9, 78.8, 78.4, 75.4, 75.3, 74.9 (2C), 74.6, 74.3, 74.2, 73.3, 72.6, 72.5 (2C), 70.9 (3C), 69.5, 68.5 (2C), 66.8, 61.0 (2C), 60.5, 60.0, 55.2; ESI-MS (842): m/z 865.3 [M + Na]; Anal. Calcd. for $C_{31}H_{54}O_{26}$: C, 44.18; H, 6.46; found: C, 43.90; H, 6.70.

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