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Note

Synthesis of a tetrasaccharide related to the O-antigen from *Azospirillum lipoferum* SR65

Prashant Ranjan Verma, Balaram Mukhopadhyay *

Indian Institute of Science Education and Research-Kolkata (IISER-K), Mohanpur Campus, PO BCKV Campus Main Office, Mohanpur, Nadia, West Bengal, India

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ABSTRACT

Concise synthesis of a tetrasaccharide repeating unit of the LPS isolated from *Azospirillum lipoferum* SR65 has been accomplished through suitable protecting group manipulations and stereoselective glycosylation starting from commercially available L-rhamnose and D-glucose. The target oligosaccharide in the form of its *p*-methoxyphenyl glycoside is suitable for further glycoconjugate formation via selective cleavage of the OMP glycoside. Plant growth-promoting bacteria (PGPB) of genus *Azospirillum* plays important roles in the growth and development of plants. The interaction between the roots of the plants and the microbes is governed by the cell surface carbohydrate polymers (CPS, LPS, etc.). The present synthetic-based study elucidates aspects of plant-microbe interaction and future biofertiliser design.

The Gram-negative nitrogen-fixing soil bacteria of genus *Azospirillum* are known as plant growth-promoting bacteria (PGPB) as they secrete many active substances that play vital roles in plant growth and development.[1] It is clear that these bacteria have a high adaptation potential and therefore are promising for application as biofertilizers. Cell surface carbohydrate polymers such as EPS, CPS and LPS play important roles for the survival of the bacteria in adverse environmental conditions as well as they regulate the interaction with the roots of plants.[1] Literature reports indicate that the LPS of the *Azospirillum* outer membrane are involved in the formation of bacterial association with the roots of cereals; for example, mutants defective in LPS synthesis are worse adsorbers to wheat roots[2] and worse colonizers to maize roots[3] compared to their non-defective counterparts. Although the bacteria of the genus *Azospirillum* have been used as model to study associative plant-microbe relationship, only a few strains are studied so far. To get a better understanding of these plant-microbe interactions, synthetic studies on the LPSs will be useful. Recently, Fedonenko et al.[4] reported the structure of the LPS isolated from *Azospirillum lipoferum* SR65. Herein we report the total synthesis of the tetrasaccharide repeating unit (Fig. 1, **1**) of the LPS in the form of its *p*-methoxyphenyl glycoside. The choice of *p*-methoxyphenyl glycoside will enable us to conjugate the synthetic oligosaccharide with suitable aglycon, when needed.

Synthesis of the tetrasaccharide (**1**) was started with the synthesis of suitably protected L-rhamnose and D-glucose synthons followed by step-wise glycosylation and de-protection. Therefore, known *p*-methoxyphenyl 2,3-*O*-isopropylidene-α-L-rhamnopyranoside (**2**)[5] was benzylated using BnBr in the presence of NaH[6] to afford the corresponding benzylated derivative **3** in 87% yield. Hydrolysis of the isopropylidene acetal using 80% AcOH at 80 °C[7] followed by selective benzylation of the 2-OH position[8] using phase transfer catalyst furnished the required acceptor, *p*-methoxyphenyl 2,4-di-*O*-benzyl-α-L-rhamnopyranoside (**5**) in

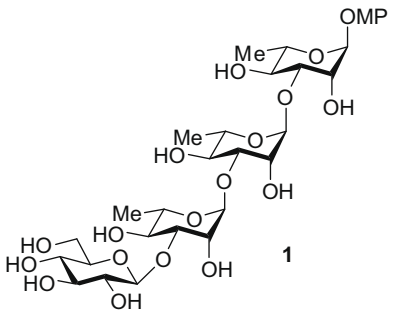

Figure 1. Structure of the target tetrasaccharide.

* Corresponding author. Tel.: +91 9748 261742; fax: +91 33 2587 3031.
E-mail address: sugarnet73@hotmail.com (B. Mukhopadhyay).

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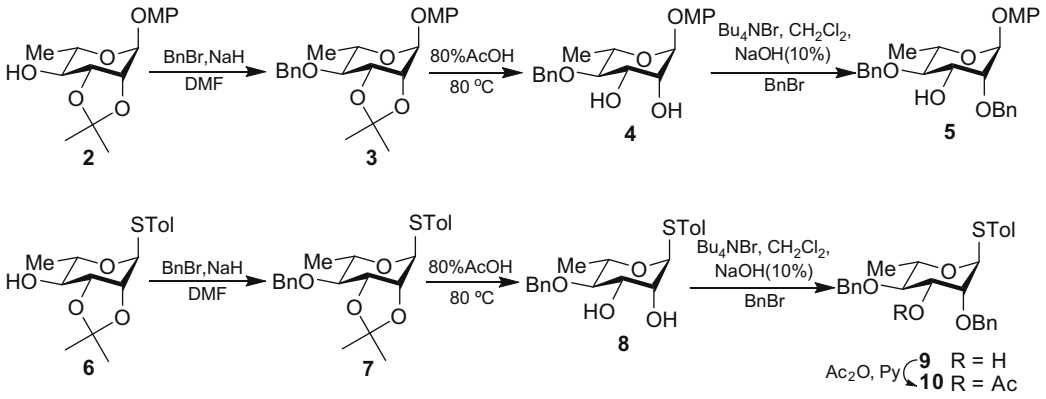
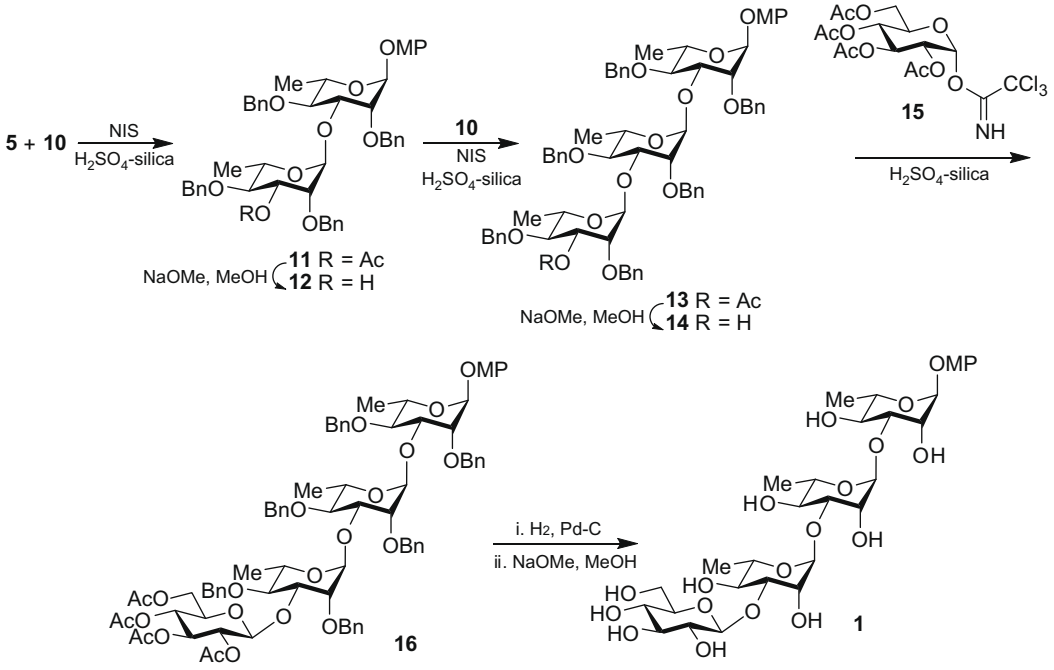


Scheme 1. Synthesis of the monosaccharide acceptor **5** and donor **10**.

78% yield over two steps. Subjected to the same benzylation-hydrolysis of the isopropylidene acetal and selective benzylation using phase transfer catalyst reaction sequence, known *p*-tolyl 2,3-*O*-isopropylidene-1-thio-α-L-rhamnopyranoside (**6**) afforded the protected derivative **9**. Compound **9** upon acetylation using Ac_2O in pyridine[9] furnished the required donor, *p*-tolyl 3-*O*-acetyl-2,4-di-*O*-benzyl-1-thio-α-L-rhamnopyranoside (**10**) in 94% yield (Scheme 1).

Glycosylation between rhamnosyl acceptor **5** and donor **10** was accomplished by using *N*-iodosuccinimide in conjunction with H_2SO_4-silica[10] to afford the disaccharide **11** in 87% yield. Use of H_2SO_4-silica as the activator of NIS was found to be beneficial over the use of traditional TfOH[11] or TMSOTf[12] as promoters. Glycosylation of acceptor **5** and donor **10** using TMSOTf and TfOH in conjunction with NIS resulted in 79% and 76% yield, respectively. The disaccharide **11** was reacted with NaOMe in MeOH to afford the disaccharide acceptor **12** in 89% yield. It was further glycosylated with rhamnosyl donor **10** following the same glycosylation strategy as mentioned above to furnish the trisaccharide **13** in 86% yield. NaOMe-catalyzed de-O-acetylation afforded the trisaccharide acceptor **14** in 94% yield. Up to this stage, activation of thioglycosides for the glycosylation reactions was satisfactory. But when we tried to couple the known *p*-tolyl 2,3,4,6-tetra-*O*-acetyl-1-thio-β-D-glucopyranoside with the trisaccharide acceptor **14**, it failed to afford the desired tetrasaccharide. Only the corresponding hemiacetal of the donor and the unreacted trisaccharide acceptor were recovered. Even the use of TMSOTf instead of H_2SO_4-silica failed to produce the desired result. Anticipating the lesser reactivity of thioglycoside donor, we used the known 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl trichloroacetimidate[13] and it gave the desired tetrasaccharide **16** in 78% yield upon activation of trichloroacetimidate by H_2SO_4-silica.[14] The protected tetrasaccharide subjected to catalytic hydrogenation using Pd–C followed by treatment with NaOMe in MeOH afforded the target tetrasaccharide **1** (Scheme 2).

In conclusion, synthesis of the tetrasaccharide repeating unit of the LPS isolated from *A. lipoferum* SR65 has been accomplished. Since the protecting group manipulation strategies and glycosylation steps were selective and high-yielding, the present synthetic strategy is capable of reasonably large-scale preparation. The syn-



Scheme 2. Synthesis of the tetrasaccharide **1**.

thetic tetrasaccharide in the form of its *p*-methoxyphenyl glycoside will be utilized for further biological experiments in due course.

1. Experimental

1.1. General methods

All reagents and solvents were dried prior to use according to standard methods.[15] Commercial reagents were used without further purification unless otherwise stated. Analytical TLC was performed on Silica Gel 60-F_{254} (Merck or Whatman) with detection by fluorescence and/or by charring following immersion in a 10% ethanolic solution of sulfuric acid. An orcinol dip, prepared by the careful addition of concentrated sulfuric acid (20 cm^3) to an ice-cold solution of 3,5-dihydroxytoluene (360 mg) in EtOH (150 cm^3) and water (10 cm^3), was used to detect deprotected compounds by charring. Flash chromatography was performed with silica gel 230–400 mesh (Qualigens, India). Optical rotations were measured at the sodium D-line at ambient temperature with a Perkin Elmer 141 polarimeter. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance spectrometer at 500 and 125 MHz, respectively, using Me_4Si or CH_3OH as internal standards, as appropriate.

1.2. *p*-Methoxyphenyl 2,4-di-*O*-benzyl-α-L-rhamnopyranoside (5)

A solution of *p*-methoxyphenyl 2,3-*O*-isopropylidene-α-L-rhamnopyranoside (**2**) (3.0 g, 9.7 mmol) in dry DMF (25 mL) was cooled to 0 °C, NaH in 50% mineral oil (930 mg, 19.4 mmol) was added followed by BnBr (1.5 mL, 12.6 mmol) and the mixture was allowed to stir at room temperature for 2 h when TLC (*n*-hexane–EtOAc; 3:1) showed complete conversion of the starting material to a faster moving spot. Excess NaH was neutralized by the careful addition of MeOH and the mixture was diluted with H_2O (30 mL). Stirring was continued for another 30 min. Then it was extracted with Et_2O (2 × 25 mL). The combined Et_2O layer was washed with water (2 × 25 mL), organic layer was separated, dried (Na_2SO_4) and evaporated in vacuo. The crude mixture thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (5:1) to afford pure *p*-methoxyphenyl 4-*O*-benzyl-2,3-*O*-isopropylidene-α-L-rhamnopyranoside (**3**) (3.4 g, 87%) as a colourless syrup. Compound **3** (3.0 g, 7.5 mmol) was then dissolved in 80% aq AcOH (25 mL) and the solution was stirred at 80 °C for 2 h. The solvents were evaporated in vacuo to afford the diol **4** which was dissolved in CH_2Cl_2 (30 mL). To it Bu_4NBr (2.4 g, 7.5 mmol), 10% aq NaOH (10 mL) and BnBr (1.2 mL, 9.8 mmol) were added and the mixture was stirred at room temperature for 36 h when TLC showed complete conversion of the starting diol to a faster moving spot. The mixture was diluted with CH_2Cl_2 (10 mL) and washed with H_2O (3 × 30 mL). Organic layer was separated, dried and evaporated in vacuo. The crude material thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (3:1) to afford *p*-methoxyphenyl 2,4-di-*O*-benzyl-α-L-rhamnopyranoside (**5**) (2.6 g, 78% over two steps) as a colourless oil. $[\alpha]_D^{25}$ +112 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.38–7.25 (m, 10H, ArH), 6.92, 6.81 (2d, 4H, O–C_6H_4–OCH_3), 5.41 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 4.90, 4.79, 4.68, 4.65 (4d, AB system, 4H, *J* 11.0 Hz, 2 × CH_2Ph), 4.13 (dt, 1H, $J_{2,3}$ 4.0 Hz, $J_{3,4}$ 9.0 Hz, $J_{3,OH}$ 9.0 Hz, H-3), 3.91 (dd, 1H, $J_{1,2}$ 1.5 Hz, $J_{2,3}$ 4.0 Hz, H-2), 3.80 (m, 1H, H-5), 3.77 (s, 3H, $C_6H_4OCH_3$), 3.40 (t, 1H, $J_{3,4}$, $J_{4,5}$ 9.0 Hz, H-4), 2.34 (d, 1H, $J_{3,OH}$ 9.0 Hz, O*H*), 1.29 (d, 3H, $J_{5,6}$ 6.0 Hz, C–CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 154.9, 150.4, 138.5, 137.6, 128.7(2), 128.4(2), 128.2, 128.0(2), 127.9(2), 127.8, 117.6(2), 114.6(2) (ArC), 96.1 (C-1), 82.2, 78.5, 75.1, 73.2, 71.5, 67.9, 55.7 ($OC_6H_4OCH_3$), 18.1 (C–CH_3). HRMS calcd for $C_{27}H_{30}O_6Na$ $(M+Na)^+$: 473.1940, found: 473.1936.

1.3. *p*-Tolyl 2,4-di-*O*-benzyl-3-*O*-acetyl-1-thio-α-L-rhamnopyranoside (10)

To a cold (0 °C) solution of *p*-tolyl 2,3-*O*-isopropylidene-1-thio-α-L-rhamnopyranoside (**6**) (3.0 g, 9.7 mmol) in dry DMF (25 mL), NaH in mineral oil (700 mg, 14.6 mmol) was added followed by BnBr (1.5 mL, 12.6 mmol). After complete addition, the mixture was allowed to stir at room temperature for 2 h when TLC (*n*-hexane–EtOAc; 3:1) showed complete conversion of the starting material to a faster moving spot. Excess NaH was neutralized by the careful addition of MeOH and the mixture was diluted with H_2O (30 mL). Stirring was continued for another 30 min. Then it was extracted with Et_2O (2 × 25 mL). The combined Et_2O layer was washed with water (2 × 25 mL), organic layer was separated, dried (Na_2SO_4) and evaporated in vacuo. The crude mixture thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (5:1) to afford pure *p*-tolyl 4-*O*-benzyl-2,3-*O*-isopropylidene-1-thio-α-L-rhamnopyranoside (**7**) (3.4 g, 89%) as a colourless gel. Compound **7** (3.0 g, 7.5 mmol) was then dissolved in 80% aq AcOH (25 mL) and the solution was stirred at 80 °C for 2 h. The solvents were evaporated in vacuo to afford the diol **8** which was dissolved in CH_2Cl_2 (30 mL). To it Bu_4NBr (2.4 g, 7.5 mmol), 10% aq NaOH (10 mL) and BnBr (1.1 mL, 9.0 mmol) were added and the mixture was stirred at room temperature for 36 h when TLC showed complete conversion of the starting diol to a faster moving spot. The mixture was diluted with CH_2Cl_2 (10 mL) and washed with H_2O (3 × 30 mL). Organic layer was separated, dried and evaporated in vacuo. The crude material thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (3:1) to afford *p*-tolyl 2,4-di-*O*-benzyl-1-thio-α-L-rhamnopyranoside (**9**) (2.6 g, 76% over two steps) as a colourless syrup. Compound **9** (2.5 g, 5.5 mmol) was dissolved in dry pyridine (15 mL) followed by Ac_2O (5 mL) and the solution was stirred at room temperature for 2 h. Solvents were evaporated and co-evaporated with toluene to remove residual pyridine. The crude material was purified by flash chromatography using *n*-hexane–EtOAc (4:1) as an eluent to give *p*-tolyl 2,4-di-*O*-benzyl-3-*O*-acetyl-1-thio-α-L-rhamnopyranoside (**10**) (2.6 g, 94%) as a colourless gel. $[\alpha]_D^{25}$ +101 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.34–7.09 (m, 14H, ArH), 5.39 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 5.18 (dd, 1H, $J_{2,3}$ 3.0 Hz, $J_{3,4}$ 8.5 Hz, H-3), 4.74–4.46 (3d, 4H, *J* 12.0 Hz, 2 × CH_2Ph), 4.22 (m, 1H, H-5), 4.09 (dd, 1H, $J_{1,2}$ 1.5 Hz, $J_{2,3}$ 3.0 Hz, H-2), 3.69 (t, 1H, $J_{3,4}$, $J_{4,5}$ 8.5 Hz, H-4), 2.31 (s, 3H, $SC_6H_4CH_3$), 1.95 (s, 3H, $COCH_3$), 1.34 (d, 3H, $J_{5,6}$ 6.0 Hz, C–CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.1 ($COCH_3$), 138.2, 137.6, 132.2(2), 130.5, 129.8(2), 128.4(5), 127.9(2), 127.8, 127.7, 127.6(2) (ArC), 85.6 (C-1), 79.2, 74.9, 73.6, 72.4, 72.1, 69.0, 21.1 (S-Ph-CH_3), 20.9 ($COCH_3$), 17.9 (C–CH_3). HRMS calcd for $C_{29}H_{32}O_5SNa$ $(M+Na)^+$: 515.1868, found: 515.1864.

1.4. *p*-Methoxyphenyl 3-*O*-acetyl-2,4-di-*O*-benzyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranoside (11)

To a solution of acceptor **5** (1.2 g, 2.7 mmol) and donor **10** (1.7 g, 3.5 mmol) in dry CH_2Cl_2 (25 mL) was added MS 4 Å (2.0 g) and the mixture was stirred under nitrogen for 30 min. Then NIS (1.0 g, 4.6 mmol) was added followed by H_2SO_4-silica (50 mg) and the mixture was allowed to stir at room temperature for 45 min when TLC (*n*-hexane–EtOAc; 3:1) showed complete consumption of the acceptor. The mixture was filtered through a pad of Celite, washed with CH_2Cl_2 (10 mL) and the combined filtrate was washed successively with $Na_2S_2O_3$ (2 × 30 mL), saturated $NaHCO_3$ (2 × 30 mL) and brine (30 mL). The organic layer was separated, dried (Na_2SO_4) and evaporated in vacuo. The crude material thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (4:1) as an eluent to afford pure disaccharide **11** (1.9 g, 87%) as white foam. $[\alpha]_D^{25}$ +128 (*c* 1.1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.43–7.14 (m,

20H, ArH), 6.93, 6.80 (2d, 4H, $C_6H_4OCH_3$), 5.37 (s, 1H, H-1), 5.33 (dd, 1H, $J_{2',3'}$ 2.8 Hz, $J_{3',4'}$ 9.6 Hz, H-3′), 5.20 (s, 1H, H-1′), 4.85–4.53 (m, 6H, 3 × CH_2Ph), 4.32, 4.19 (2d, J 12.4 Hz, 2H, CH_2Ph), 4.29 (dd, 1H, $J_{2,3}$ 2.8 Hz, $J_{3,4}$ 9.6 Hz, H-3), 3.90 (bd, 2H, J 2.8 Hz, H-2, H-2′), 3.83 (m, 2H, H-5, H-5′), 3.76 (s, 3H, $C_6H_4OCH_3$), 3.70 (t, 1H, $J_{3,4}$, $J_{4,5}$ 9.6 Hz, H-4), 3.62 (t, 1H, $J_{3',4'}$, $J_{4',5'}$ 9.6 Hz, H-4′), 1.95 (s, 3H, $COCH_3$), 1.29, 1.27 (2d, 3H, J 6.0 Hz, 2 × C–CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.2 ($COCH_3$), 154.8, 150.3, 138.5, 138.4, 137.9, 137.8, 128.5(2), 128.4(2), 128.3(2), 128.2(2), 127.8(2), 127.7(2), 127.6(2), 127.5(2), 127.4(2), 127.0(2), 117.5(2), 114.5(2) (ArC), 99.6 (C-1′), 96.7 (C-1), 80.7, 79.0, 77.9, 77.6, 74.7, 74.6, 73.5, 72.9, 72.6, 68.9, 68.4, 55.6 ($C_6H_4OCH_3$), 21.1 ($COCH_3$), 18.1, 17.9 (2 × C–CH_3). HRMS calcd for $C_{49}H_{54}O_{11}Na$ $(M+Na)^+$: 841.3564, found: 841.3562.

1.5. *p*-Methoxyphenyl 2,4-di-*O*-benzyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranoside (12)

To a solution of compound **11** (1.8 g, 2.2 mmol) in dry MeOH (10 mL) was added NaOMe in MeOH (0.5 M, 1 mL) and the solution was allowed to stir at room temperature for 2 h. Then the solution was neutralized with DOWEX 50W H^+ resin and filtered. The solvents were evaporated in vacuo and the residue was purified by flash chromatography using *n*-hexane–EtOAc (3:1) to afford pure compound **12** (1.5 g, 89%) as white foam. $[\alpha]_D^{25}$ +112 (*c* 1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.40–7.15 (m, 20H, ArH), 6.94, 6.81 (2d, 4H, $C_6H_4OCH_3$), 5.37 (s, 1H, H-1), 5.26 (s, 1H, H-1′), 4.93 (d, 1H, J 11.2 Hz, CH_2Ph), 4.83–4.73 (m, 4H, 2 × CH_2Ph), 4.65 (d, 1H, J 11.2 Hz, CH_2Ph), 4.35, 4.13 (2d, J 11.6 Hz, 2H, CH_2Ph), 4.31 (dd, 1H, $J_{2,3}$ 2.5 Hz, $J_{3,4}$ 9.6 Hz, H-3), 4.02 (m, 1H, H-3′), 3.89 (br s, 1H, H-2), 3.86 (m, 2H, H-5, H-5′), 3.77 (s, 3H, $C_6H_4OCH_3$), 3.75 (br s, 1H, H-2′), 3.69 (t, 1H, $J_{3,4}$, $J_{4,5}$ 9.6 Hz, H-4), 3.62 (t, 1H, $J_{3',4'}$, $J_{4',5'}$ 9.3 Hz, H-4′), 2.30 (d, 1H, J 9.2 Hz, OH), 1.30, 1.27 (2d, 3H, J 6.0 Hz, 2 × C–CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 154.8, 150.3, 138.7, 138.4, 137.8, 137.7, 128.5(2), 128.4(2), 128.3(2), 128.2(2), 127.7(8), 127.6(2), 126.8(2), 117.6(2), 114.6(2) (ArC), 98.8 (C-1′), 96.7 (C-1), 82.2, 80.9, 79.1, 77.7, 74.8, 74.7, 72.9, 72.5, 71.6, 69.0, 67.9, 55.6 ($C_6H_4OCH_3$), 18.1, 18.0 (2 × C–CH_3). HRMS calcd for $C_{47}H_{52}O_{10}Na$ $(M+Na)^+$: 799.3458, found: 799.3455.

1.6. *p*-Methoxyphenyl 3-*O*-acetyl-2,4-di-*O*-benzyl- α-L-rhamnopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranoside (13)

Glycosylation between disaccharide acceptor **12** (1.4 g, 1.8 mmol) and donor **10** (1.1 g, 2.3 mmol) was accomplished by following the same reaction protocol as described above for the preparation of disaccharide **11**. After purification, the trisaccharide **13** (1.8 g, 86%) was obtained as a colourless gel. $[\alpha]_D^{25}$ +83 (*c* 1.0, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.44–7.11 (m, 30H, ArH), 6.93, 6.80 (2d, 4H, $C_6H_4OCH_3$), 5.38 (s, 1H, H-1), 5.28 (m, 1H, H-3″), 5.22 (s, 1H, H-1′), 5.14 (s, 1H, H-1″), 4.80–4.57 (m, 8H, 4 × CH_2Ph), 4.44 (s, 2H, CH_2Ph), 4.31, 4.15 (2d, J 12.4 Hz, 2H, CH_2Ph), 4.27 (dd, 1H, $J_{2,3}$ 2.4 Hz, $J_{3,4}$ 9.6 Hz, H-3), 4.21 (dd, 1H, $J_{2',3'}$ 2.0 Hz, $J_{3',4'}$ 9.6 Hz, H-3′), 3.92 (br s, 2H, H-2, H-2″), 3.83–3.78 (m, 4H, H-2′, H-5, H-5′, H-5″), 3.76 (s, 3H, $C_6H_4OCH_3$), 3.65 (t, 1H, $J_{3,4}$, $J_{4,5}$ 9.6 Hz, H-4), 3.62 (t, 1H, $J_{3',4'}$, $J_{4',5'}$ 9.2 Hz, H-4′), 3.60 (m, 1H, H-4″), 2.01 (s, 3H, $COCH_3$), 1.26, 1.21, 1.17 (3d, 9H, J 6.0 Hz, 3 × C–CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.1 ($COCH_3$), 154.8, 150.3, 138.8, 138.5, 138.2, 138.0, 137.9, 137.8, 128.5(3), 128.4(3), 128.3(4), 128.2, 127.7(3), 127.6(2), 127.5(2), 127.4(3), 127.3(3), 127.1(3), 126.8(3), 117.5(2), 114.5(2) (ArC), 99.6(2) (C-1′, C-1″), 96.5 (C-1), 80.6, 80.5, 79.0, 78.5, 78.3, 78.0, 74.9, 74.6, 73.5, 72.8, 72.6, 72.4, 68.8, 68.4, 55.6 ($C_6H_4OCH_3$), 21.0 ($COCH_3$), 18.1, 17.9, 17.8 (3 × C–CH_3). HRMS calcd for $C_{69}H_{76}O_{15}Na$ $(M+Na)^+$: 1167.5082, found: 1167.5080.

1.7. *p*-Methoxyphenyl 2,4-di-*O*-benzyl- α-L-rhamnopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranoside (14)

Compound **13** (1.5 g, 1.3 mmol) was de-O-acetylated by following the same procedure as described above for the preparation of compound **12** to afford the trisaccharide acceptor **14** (1.4 g, 94%) as white foam. $[\alpha]_D^{25}$ +78 (*c* 1.1, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.37–7.04 (m, 30H, ArH), 6.86, 6.71 (2d, 4H, $C_6H_4OCH_3$), 5.32 (s, 1H, H-1), 5.14 (s, 1H, H-1′), 5.10 (s, 1H, H-1″), 4.79–4.51 (m, 8H, 4 × CH_2Ph), 4.34 (s, 2H, CH_2Ph), 4.22, 4.13 (2d, J 12.4 Hz, 2H, CH_2Ph), 4.21 (dd, 1H, $J_{2,3}$ 2.4 Hz, $J_{3,4}$ 9.6 Hz, H-3), 4.01 (dd, 1H, $J_{2',3'}$ 2.0 Hz, $J_{3',4'}$ 9.6 Hz, H-3′), 3.89 (m, 3H, H-2, H-2″, H-3″), 3.80–3.72 (m, 4H, H-2′, H-5, H-5′, H-5″), 3.66 (s, 3H, $C_6H_4OCH_3$), 3.59 (m, 2H, H-4, H-4′), 3.22 (t, 1H, $J_{3'',4''}$, $J_{4'',5''}$ 9.0 Hz, H-4″), 2.32 (br s, 1H, OH), 1.18, 1.16, 1.11 (3d, 9H, J 6.0 Hz, 3 × C–CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 154.9, 150.4, 138.8, 138.7, 138.3, 138.1, 138.0, 137.8, 128.5(3), 128.4(3), 128.3(3), 128.3(3), 128.2(3), 127.8(3), 127.6(4), 127.5(4), 127.4, 127.2(2), 126.7, 117.6(2), 114.6(2) (ArC), 99.8 (C-1′), 99.0 (C-1″), 96.6 (C-1), 82.2, 81.0, 80.6, 79.2, 78.7, 78.2, 74.9, 74.7, 74.6, 72.9, 72.5, 72.4, 71.6, 68.1(2), 67.8, 55.7 ($C_6H_4OCH_3$), 18.2, 18.1, 18.0 (3 × C–CH_3). HRMS calcd for $C_{67}H_{74}O_{14}Na$ $(M+Na)^+$: 1125.4976, found: 1125.4973.

1.8. *p*-Methoxyphenyl 2,3,4,6-tetra-*O*-acetyl-β-D-glucopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranosyl-(1→3)-2,4-di-*O*-benzyl-α-L-rhamnopyranoside (16)

A mixture of compound **14** (1.0 g, 0.9 mmol), compound **15** (890 mg, 1.8 mmol) and MS 4 Å (1.0 g) in dry CH_2Cl_2 (15 mL) was stirred under nitrogen atmosphere for 30 min. H_2SO_4-silica (20 mg) was added and the mixture was allowed to stir at room temperature for 45 min when the TLC (*n*-hexane–EtOAc; 3:1) showed complete conversion of the starting material. The mixture was filtered through a pad of Celite and washed with CH_2Cl_2 (10 mL). The combined filtrate was washed successively with aq satd $NaHCO_3$ (2 × 20 mL) and brine (20 mL). The organic layer was separated, dried (Na_2SO_4), filtered through cotton plug and evaporated in vacuo. The crude mixture thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (3:1) as an eluent to afford pure tetrasaccharide **16** (1.0 g, 78%). $[\alpha]_D^{25}$ +118 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.40–7.21 (m, 30H, ArH), 6.93, 6.80 (2d, 4H, $C_6H_4OCH_3$), 5.37 (d, 1H, $J_{1,2}$ 1.5 Hz, H-1), 5.22 (br s, 1H, H-1′), 5.14 (t, 1H, $J_{3''',4'''}$, $J_{4''',5'''}$ 9.5 Hz, H-4‴), 5.10 (dd, 1H, $J_{1''',2'''}$ 7.5 Hz, $J_{2''',3'''}$ 9.5 Hz, H-2‴), 5.07 (br s, 1H, H-1″), 5.03 (t, 1H, $J_{2''',3'''}$, $J_{3''',4'''}$ 9.5 Hz, H-3‴), 4.83 (d, 1H, $J_{1''',2'''}$ 7.5 Hz, H-1‴), 4.84–4.70 (m, 6H, 3×CH_2Ph), 4.65, 4.57 (2d, 2H, J 11.5 Hz, CH_2Ph), 4.52 (m, 2H, CH_2Ph), 4.39–4. 31 (m, 3H, H-6a‴, CH_2Ph), 4.26 (dd, 1H, $J_{2,3}$ 3.5 Hz, $J_{3,4}$ 10.0 Hz, H-3), 4.15 (dd, 1H, $J_{2',3'}$ 3.0 Hz, $J_{3',4'}$ 9.0 Hz, H-3′), 4.11–4.07 (m, 2H, H-3″, H-6b‴), 3.90–3.78 (m, 6H, H-2, H-2′, H-2″, H-5, H-5′, H-5″), 3.76 (s, 3H, $C_6H_4OCH_3$), 3.66 (t, 1H, $J_{3,4}$, $J_{4,5}$ 9.5 Hz, H-4), 3.55 (t, 2H, J 9.5 Hz, H-4′, H-4″), 3.47 (m, 1H, H-5‴), 2.01, 1.99, 1.83, 1.81 (4s, 12H, 4 × $COCH_3$), 1.26, 1.23, 1.17 (3d, 9H, J 6.0 Hz, 3 × C–CH_3). ^{13}C NMR (125 MHz, $CDCl_3$) δ: 170.4, 170.3, 169.4, 169.3 (4 × $COCH_3$), 154.9, 150.3, 138.6, 138.5, 138.4, 138.3, 138.1, 137.9, 128.6(2), 128.5(5), 128.4(3), 128.3, 128.2(3), 127.9(3), 127.8(3), 127.7(3), 127.6, 127.5(2), 127.4(2), 127.1, 126.9, 117.6(2), 114.6(2) (ArC), 101.4 (C-1‴), 100.4 (C-1′), 99.4 (C-1″), 96.6 (C-1), 80.7, 80.6, 80.2, 80.0, 79.0, 78.7, 78.2, 78.0, 77.6, 77.3, 74.9, 74.8, 73.4, 73.1, 72.9, 72.2, 71.8(2), 69.2, 68.9, 68.7, 68.5, 61.7, 55.7 ($C_6H_4OCH_3$), 20.6(2), 20.5, 20.4 (4 × $COCH_3$), 18.1, 18.0, 17.9 (3 × C–CH_3). HRMS calcd for $C_{81}H_{92}O_{23}Na$ $(M+Na)^+$: 1455.5927, found: 1455.5923.

1.9. *p*-Methoxyphenyl β-D-glucopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranosyl-(1→3)-α-L-rhamnopyranoside (1)

A dilute solution of compound **16** (800 mg, 0.6 mmol) in MeOH (80 mL) was passed through the flow hydrogenation assembly fitted with a Pd–C cartridge at room temperature and 2 atm pressure of hydrogen. After completion, as evident from TLC (*n*-hexane–EtOAc; 1:1), the solvents were evaporated in vacuo and the residue was re-dissolved in dry MeOH (10 mL). NaOMe (1 mL, 0.5 M in MeOH) was added and the solution was stirred overnight at room temperature. It was neutralized with DOWEX 50W H^+ resin, filtered and evaporated in vacuo to afford the pure target tetrasaccharide **1** (330 mg, 82% over two steps) as amorphous white powder. $[\alpha]_D^{25}$ +62 (*c* 0.8, H_2O). ^1H NMR (500 MHz, D_2O) δ: 7.02, 6.90 (2d, 4H, *J* 8.5 Hz, $C_6H_4OCH_3$), 5.33 (s, 1H, H-1), 4.99 (s, 1H, H-1′), 4.98 (s, 1H, H-1″), 4.62 (d, 1H, $J_{1''',2'''}$ 8.0 Hz, H-1‴), 4.24 (br s, 1H, H-2), 4.15 (br s, 1H, H-2′), 4.09 (br s, 1H, H-2″), 3.93 (m, 2H, H-3, H-3′), 3.85–3.78 (m, 5H, H-4, H-4′, H-4″, H-5‴, H-6a‴), 3.72 (s, 3H, $C_6H_4OCH_3$), 3.64 (dd, 1H, $J_{5''',6b'''}$ 5.0 Hz, $J_{6a''',6b'''}$ 12.0 Hz, H-6b‴), 3.56–3.51 (m, 2H, H-5, H-5′), 3.48 (t, 1H, $J_{3''',4'''}$, $J_{4''',5'''}$ 9.0 Hz, H-4‴), 3.42 (t, 1H, $J_{2''',3'''}$, $J_{3''',4'''}$ 9.0 Hz, H-3‴), 3.37 (m, 1H, H-5″), 3.31 (dd, 1H, $J_{1''',2'''}$ 8.0 Hz, $J_{2''',3'''}$ 9.0 Hz, H-2‴), 1.22, 1.18, 1.15 (3d, 9H, *J* 6.0 Hz, 3×C-CH_3). ^{13}C NMR (125 MHz, D_2O) δ: 154.7, 149.3, 118.8(2), 115.1(2) (ArC), 103.7 (C-1‴), 102.3 (C-1′), 102.1 (C-1″), 99.0 (C-1), 79.9, 78.3, 77.9, 75.7, 75.5, 73.3, 71.3(2), 71.0, 69.9, 69.8, 69.7, 69.6, 69.5, 69.3, 69.0, 60.5, 55.7 ($C_6H_4OCH_3$), 16.6, 16.5, 16.4 (3 × C–CH_3). HRMS calcd for $C_{31}H_{48}O_{19}Na$ $(M+Na)^+$: 747.2688, found: 747.2686.

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