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Synthesis of the tetrasaccharide repeating unit of the *O*-glycan from the polar flagellum flagellin of *Azospirillum brasilense* Sp7

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Abstract: Chemical synthesis of the tetrasaccharide repeating unit of the *O*-glycan from the polar flagellum flagellin of *Azospirillum brasiliens* Sp7 in the form of its *p*-methoxyphenyl glycoside is reported. The required glycosidic linkages have been accomplished by activation of thioglycosides with *N*-iodosuccinimide in the presence of H_2SO_4-silica. H_2SO_4-silica was found to be an effective alternative to the classical acid promoters like TfOH or TMSOTf and it can lead to the formation of both 1,2-*cis* and 1,2-*trans* glycosidic linkage formation depending on the protecting group manipulation and control of the reaction condition.

Keywords: Flagellin *O*-glycan, total synthesis, H_2SO_4-silica

1. Introduction

Bacterial flagellum, consisting of the protein flagellin, is important for the movement of the bacteria in space and adapting to the environmental conditions in water, soil, plant and animal tissues. The C- and N-terminus of flagellin is conserved but the middle portion varies and it is responsible for the adhesive functions and antigenic properties of the bacteria concerned.[1,2] It is evident that the presence of carbohydrates in the middle region of flagellin affects the antigenic property of the bacteria.[3] The flagellin glycosylation occurs either independently[4] or in association with the LPS biosynthesis.[5] It is observed that

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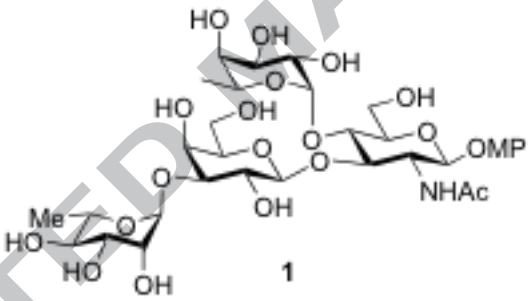
the bacterial species having glycosylated flagellin actively interact with other microorganisms.[6] The bacteria of the genus *Azospirillum* is well known for the associative plant-microbe interactions and it has a polar flagellum with glycosylated flagellin.[7] Recently Belyakov *et. al.*[8] have reported the structure of the *O*-linked repeatitive glycan chain of the polar flagellum flagellin isolated from *Azospirillum brasiliense* Sp7. Herein, we report the chemical synthesis of the tetrasaccharide repeating unit in the form of its *p*-methoxyphenyl glycoside (**1**, Figure 1). Chemical synthesis of the repeating unit will provide the route to have the target tetrasaccharide in pure form and reasonable quantity to enable better insight of the biosynthesis and antigenic behaviour of this important post translational modifications occurred in bacterial flagellum.



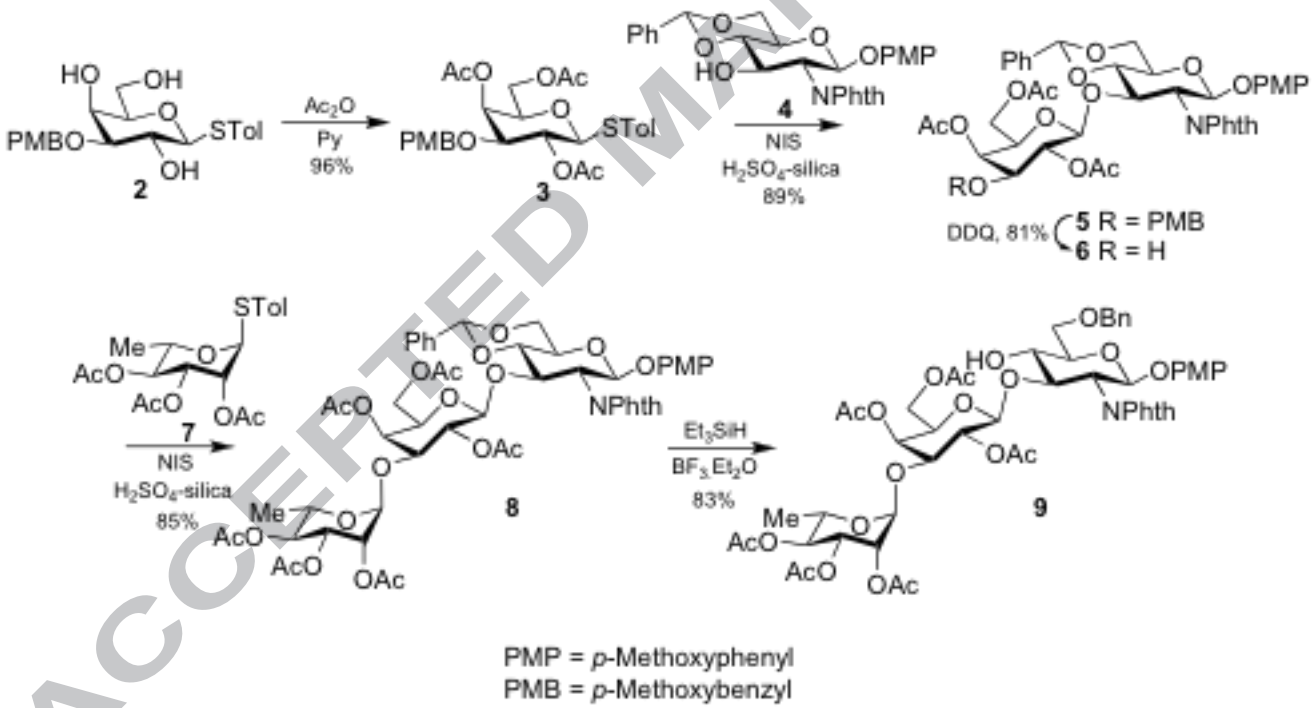
1

Figure 1. Structure of the target tetrasaccharide related to the *O*-linked glycan from the polar flagellum flagellin of *Azospirillum brasilense* Sp7

2. Results and discussion

The synthesis of the desired tetrasaccharide was planned in the form of it *p*-methoxyphenyl glycoside as this can be cleaved selectively from the per-*O*-acetylated derivative and allows further glycoconjugate formation through trichloroacetimidate chemistry. Therefore, known *p*-tolyl 3-*O*-(4-methoxybenzyl)-1-thio-β-D-galactopyranoside (**2**)[9] was acetylated using Ac_2O in pyridine to afford the corresponding tri-*O*-acetyl derivative **3** in 96% yield. Glycosylation of the donor **3** with known acceptor,

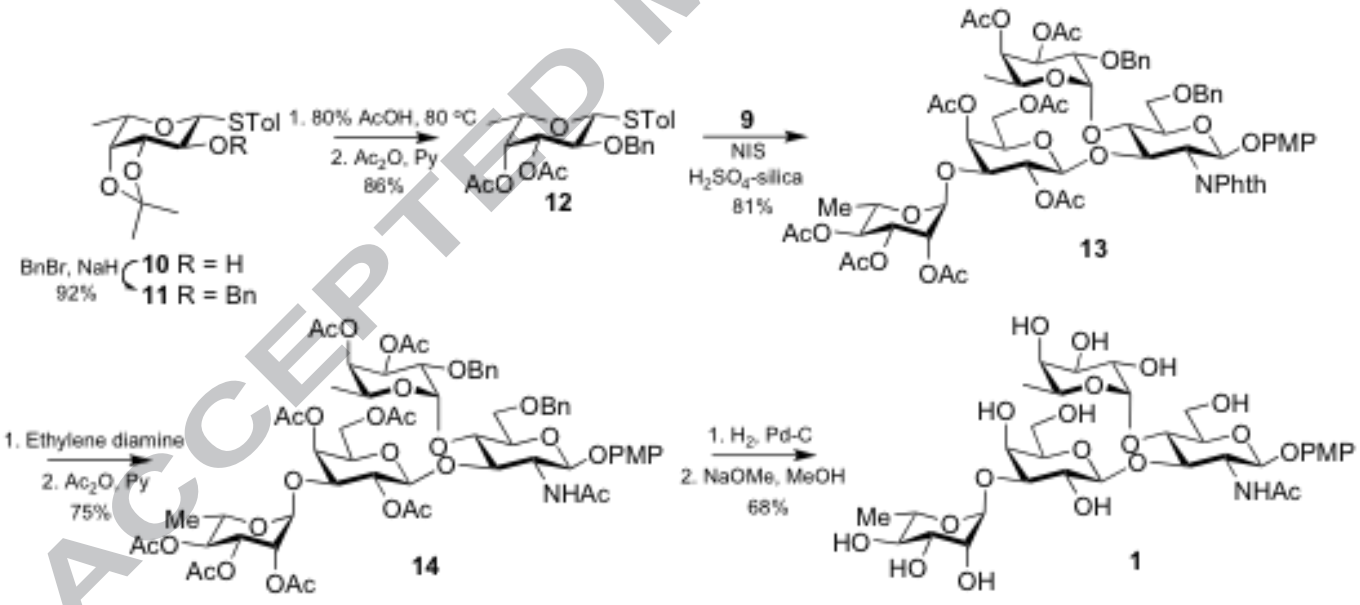
p-methoxyphenyl 4,6-*O*-benzylidene-2-deoxy-2-phthalimido-β-D-glucopyranoside (**4**)[10] using *N*-iodosuccinimide (NIS) in the presence of H_2SO_4-silica[11,12,13] afforded the disaccharide **5** in 89% yield. Use of *N*-phthalimido instead of the desired *N*-acetamido group was required as the acceptor containing NHAc becomes too unreactive to react in the glycosylation reaction.[14] The *p*-methoxybenzyl group was selectively removed by the oxidative cleavage using DDQ[15] to furnish the disaccharide acceptor **6** in 81% yield. This disaccharide acceptor was subsequently glycosylated with known donor, *p*-tolyl 2,3,4-tri-*O*-acetyl-1-thio-α-L-rhamnopyranoside (**7**).[16] NIS promoted activation of the thioglycoside gave the trisaccharide **8** in 85% yield. Finally, the reductive opening of the benzylidene acetal using triethylsilane in the presence of BF_3*Et_2O[17] afforded the trisaccharide acceptor **9** in 83% yield (**Scheme 1**).



Scheme 1:Synthesis of the trisaccharide acceptor **9**

In a separate experiment, known *p*-tolyl 3,4-*O*-isopropylidene-1-thio-α-L-fucopyranoside (**10**)[14] was benzylated using BnBr in the presence of NaH[18] to produce the fully protected derivative **11** in 92% yield. The isopropylidene group was hydrolyzed by 80% AcOH at 80 °C[19] and the free hydroxyl groups were subsequently acetylated to afford the compound **12** in 86% yield over two steps. Further, donor **12**

was coupled with the trisaccharide acceptor **9** using NIS in the presence of H_2SO_4-silica to give the protected tetrasaccharide **13** in 81% yield. It is important to note that the fucosyl donor **11** is also suitable for the required *cis*-glycosylation. However, it is found to be too reactive and failed to produce the desired tetrasaccharide in good yield. Introduction of two acetate groups instead of the isopropylidene moiety gave the donor optimum reactivity and the yield of the final glycosylation was acceptable. Once the protected tetrasaccharide is in hand, the NPhth group was cleaved using ethylene diamine[20] and converted to the corresponding acetamido group by subsequent acetylation to furnish the tetrasaccharide **14** in 75% yield. The benzyl groups were removed by catalytic hydrogenolysis using 10% Pd-C on a Thales Nano H-cube flow hydrogenation assembly with constant flow of hydrogen. Finally, the acetate groups were cleaved by transesterification using NaOMe in MeOH[21] to afford the target tetrasaccharide **1** in 68% over two steps (**Scheme 2**).



Scheme 2: Synthesis of the target tetrasaccharide **1**

3. Conclusion

In conclusion, the total synthesis of the tetrasaccharide repeating unit of the *O*-glycan from the polar flagellum flagellin of *Azospirillum brasiliens* Sp7 is achieved in the form of its *p*-methoxyphenyl glycoside. All required glycosidic linkages have been formed by the activation of thioglycosides using NIS in the presence of H_2SO_4-silica. All glycosylation reactions were resulted in formation of single desired isomer in good to excellent yield. The *p*-methoxyphenyl group at the reducing end of the target tetrasachharide is of particular interest as it can be cleaved selectively from the per-*O*-acetylated derivative and opens up the scope for further glycoconjugate formation using trichloroacetimidate chemistry. It is worth mentioning that during the course of our synthetic experiments we have seen a report by Mandal et al[22] on the synthesis of the same repaeting unit structure but using a completely different synthetic strategy.

4. Experimental

4.1. General: All reagents and solvents were dried prior to use according to standard methods.[23] 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 carefully prepared by the addition of concentrated sulfuric acid (20 mL) to an ice-cold solution of 3,5-dihydroxytoluene (360 mg) in EtOH (150 mL) and H_2O (10 mL) was used to detect deprotected oligosaccharides by charring. Flash chromatography was performed with Silica Gel 230-400 mesh (Merck, 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 Bruker Avance spectrometer at 500 and 125 MHz respectively, using Me_4Si or CH_3OH as internal standards, as appropriate.

4.2. *p*-Tolyl 2,4,6-tri-*O*-acetyl-3-*O*-(4-methoxybenzyl)-1-thio-β-D-galactopyranoside (3): To a solution of compound **2** (2.5 g, 6.2 mmol) in dry pyridine (20 mL), Ac_2O (5 mL) was added and the solution was stirred at room temperature for 2 hours when TLC (*n*-hexane–EtOAc, 3:1) showed complete conversion of the starting material to a faster moving spot. The solvents were evaporated *in vacuo* and co-evaporated with toluene to remove residual pyridine. The syrupy residue thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (3:1) as the eluent to afford pure compound **3** (3.1 g, 96%) as a yellowish gel. $[\alpha]_D^{25}$ +108 (*c* 1.1, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.17-6.84 (4d, 8H, Ar*H*), 5.49 (dd, 1H, $J_{3,4}$ 3.5 Hz, $J_{4,5}$ 1.0 Hz, H-4), 5.10 (t, 1H, $J_{1,2}$, $J_{2,3}$ 9.5 Hz, H-2), 4.59, 4.32 (ABq, 2H, J_{AB} 11.5 Hz, $OCH_2C_6H_4OCH_3$), 4.54 (d, 1H, $J_{1,2}$ 9.5 Hz, H-1), 4.15 (m, 2H, H-6_a, H-6_b), 3.80 (m, 1H, H-5), 3.79 (s, 3H, $OCH_2C_6H_4OCH_3$), 3.52 (dd, 1H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.5 Hz, H-3), 2.32 (s, 3H, S-C_6H_4-CH_3), 2.11, 2.06, 2.05 (3s, 9H, 3×$COCH_3$). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.5, 170.4, 170.3 (3×$COCH_3$), 159.4, 138.1, 132.9(2), 129.5(4), 129.4, 129.2, 113.8(2) (Ar*C*), 86.9 (C-1), 77.1, 74.6, 70.9, 68.9, 66.1, 62.3, 55.2 ($C_6H_4OCH_3$), 21.1 (S-C_6H_4-CH_3). HRMS calcd. for $C_{27}H_{32}O_9SNa$ $(M+Na)^+$: 555.1665, found: 555.1668.

4.3. *p*-Methoxyphenyl 2,4,6-tri-*O*-acetyl-3-*O*-(4-methoxybenzyl)-β-D-galactopyranosyl-(1→3)-4,6-*O*-benzylidene-2-deoxy-2-*N*-phthalimido-β-D-glucopyranoside (5): A mixture of compound **3** (2.1 g, 3.9 mmol), compound **4** (1.5 g, 3.0 mmol) and MS 4Å (2.0 g) in dry CH_2Cl_2 (25 mL) was stirred under nitrogen for 45 min. NIS (1.1 g, 5.0 mmol) was added and the mixture was cooled to 0 °C followed by the addition of H_2SO_4-silica (100 mg) and the mixture was allowed to stir at the same temperature for 15 minutes when all acceptor monosaccharide **4** was consumed as evident by TLC (*n*-hexane-EtOAc; 3:2). The mixture was immediately filtered through a pad of Celite® and the filtrate was washed successively with aq. $Na_2S_2O_3$ (2×30 mL), saturated $NaHCO_3$ (2×30 mL) H_2O (30 mL). The organic layer was separated, dried (Na_2SO_4), filtered and concentrated *in vacuo*. The crude product thus obtained was

purified by flash chromatography using *n*-hexane–EtOAc (1:1) to give the pure disaccharide **5** (1.6 g, 89%) as white foam. $[\alpha]_D^{25}$ +78 (*c* 1.0, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.86-6.69 (m, 17H, Ar*H*), 5.65 (d, 1H, $J_{1,2}$ 8.5 Hz, H-1), 5.59 (s, 1H, C*H*Ph), 5.32 (bd, 1H, $J_{3',4'}$ 3.0 Hz, H-4′), 4.89 (dd, 1H, $J_{1',2'}$ 8.0 Hz, $J_{2',3'}$ 10.0 Hz, H-2′), 4.77 (dd, 1H, $J_{2,3}$ 10.5 Hz, $J_{3,4}$ 9.0 Hz, H-3), 4.55 (dd, 1H, $J_{1,2}$ 8.5 Hz, $J_{2,3}$ 10.5 Hz, H-2), 4.49 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 4.48, 4.15 (ABq, 2H, J_{AB} 12.0 Hz, OCH_2C$_6$H$_4$OCH$_3$), 4.37 (dd, 1H, $J_{5,6a}$ 5.0 Hz, $J_{6a,6b}$ 10.5 Hz, H-6$_a$), 4.07 (dd, 1H, $J_{5',6a'}$ 8.0 Hz, $J_{6a',6b'}$ 11.0 Hz, H-6$_a$′), 3.91-3.76 (m, 4H, H-4, H-5, H-6$_b$, H-6$_b$′), 3.75 (s, 3H, OCH$_2$C$_6$H$_4$OCH_3), 3.69 (s, 3H, OC$_6$H$_4$OCH_3), 3.41 (m, 1H, H-5′), 3.28 (dd, 1H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 3.0 Hz, H-3′), 2.08, 1.95, 1.51 (3s, 9H, 3×COCH_3). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.3, 170.1, 168.7 (3×*C*OCH$_3$), 167.7, 167.4 (2×Phthalimido *C*=O), 159.1, 155.5, 150.4, 137.0, 134.2, 131.5, 129.4, 129.1, 129.0(3), 128.2(2), 126.0(2), 123.5(2), 118.4(3), 114.4(2), 113.5(2) (Ar*C*), 101.4 (*C*HPh), 100.5 (C-1′), 97.9 (C-1), 80.8, 76.4, 75.5, 70.8, 70.7, 70.5, 68.6, 66.4, 65.4, 61.3, 55.5 (OCH$_2$C$_6$H$_4$O*C*H$_3$), 55.3 (C-2, OC$_6$H$_4$O*C*H$_3$), 20.7, 20.5, 20.2 (3×CO*C*H$_3$). HRMS calcd. for $C_{48}H_{49}NO_{17}Na$ $(M+Na)^+$: 934.2898, found: 934.2901.

4.4. *p*-Methoxyphenyl 2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-4,6-*O*-benzylidene-2-deoxy-2-*N*-phthalimido-β-D-glucopyranoside (6): To a solution of compound **5** (1.6 g, 1.8 mmol) in CH_2Cl_2–H_2O (4:1, 25 mL), DDQ (450 mg, 2.0 mmol) was added and the mixture was allowed to stir at room temperature for 2 hours when TLC (*n*-hexane-EtOAc, 1:1) showed complete conversion of the starting material to a slower moving spot. The mixture was diluted with CH_2Cl_2 (20 mL) and washed successively with H_2O (3×30 mL). The organic layer was collected, dried (Na_2SO_4), filtered and evaporated *in vacuo*. The crude residue was purified by flash chromatography using *n*-hexane–EtOAc (1:1) as the eluent to afford the pure disaccharide acceptor **6** (1.5 g, 81%) as colourless foam. $[\alpha]_D^{25}$ +103 (*c* 1.1, $CHCl_3$). ^{1}H NMR ($CDCl_3$, 500 MHz) δ: 7.92-6.74 (m, 13H, Ar*H*), 5.69 (d, 1H, $J_{1,2}$ 8.5 Hz, H-1), 5.64 (s, 1H, C*H*Ph), 5.18 (dd, 1H, $J_{3',4'}$ 3.5 Hz, $J_{4',5'}$ 1.0 Hz, H-4′), 4.86 (dd, 1H, $J_{1',2'}$ 8.0 Hz, $J_{2',3'}$ 10.0 Hz, H-2′), 4.84 (dd, 1H,

$J_{2,3}$ 10.5 Hz, $J_{3,4}$ 9.0 Hz, H-3), 4.59 (dd, 1H, $J_{1,2}$ 8.5 Hz, $J_{2,3}$ 10.5 Hz, H-2), 4.49 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1′), 4.41 (dd, 1H, $J_{5,6a}$ 5.0 Hz, $J_{6a,6b}$ 10.5 Hz, H-6_a), 4.09 (dd, 1H, $J_{5',6a'}$ 7.5 Hz, $J_{6a',6b'}$ 11.0 Hz, H-6_a′), 3.92 (m, 2H, H-4, H-6_b), 3.83 (dd, 1H, $J_{5',6b'}$ 5.5 Hz, $J_{6a',6b'}$ 11.0 Hz, H-6_b′), 3.76 (dd, 1H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 3.5 Hz, H-3′), 3.74 (s, 3H, $OC_6H_4OC\mathit{H}_3$), 3.59 (m, 1H, H-5), 3.53 (m, 1H, H-5′), 2.14, 1.98, 1.68 (3s, 9H, 3×$COC\mathit{H}_3$). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.9, 170.7, 170.2 (3×$\mathit{C}OCH_3$), 168.7, 168.4 (2×Phthalimido C=O), 155.7, 150.5, 137.0, 134.4, 131.5, 129.2, 128.3(3), 126.1(3), 123.7(2), 118.5(2), 114.5(2) (ArC), 101.4 (CHPh), 100.3 (C-1′), 98.1 (C-1), 80.6, 75.5, 72.8, 71.5, 70.6, 69.4, 68.6, 66.5, 61.3, 60.4, 55.6 ($OC_6H_4O\mathit{C}H_3$), 55.4 (C-2), 20.7, 20.6, 20.3 (3×$CO\mathit{C}H_3$). HRMS calcd. for $C_{40}H_{41}NO_{16}Na$ $(M+Na)^+$: 814.2323, found: 814.2328.

4.5. *p*-Methoxyphenyl-2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-4,6-*O*-benzylidene-2-deoxy-2-*N*-phthalimido-β-D-glucopyranoside (8): A mixture of acceptor **6** (1.5 g, 1.9 mmol), donor **7** (1.0 g, 2.6 mmol) and MS 4Å (2.0 g) in dry CH_2Cl_2 (20 mL) was stirred under nitrogen atmosphere for 10 min at 0 °C. NIS (720 mg, 3.2 mmol) was added followed by H_2SO_4–silica (100 mg) and the mixture was stirred at 0 °C for another 30 min when TLC (*n*-hexane-EtOAc, 3:2) showed complete consumption of the acceptor. The mixture was filtered through a pad of Celite® and the filtrate was washed successively with aq. $Na_2S_2O_3$ (2×30 mL), saturated $NaHCO_3$ (2×30 mL) and H_2O (30 mL). The organic layer was collected, dried (Na_2SO_4), filtered and evaporated *in vacuo* and the residue was purified by flash chromatography using *n*-hexane-EtOAc (1:1) as eluent to afford pure trisaccharide **8** (1.8 g, 89%) as white amorphous mass. $[\alpha]_D^{25}$ +73 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.75-7.35 (m, 9H, ArH), 6.76, 6.69 (2d, 4H, *J* 9.5 Hz, $OC_6\mathit{H}_4OCH_3$), 5.63 (d, 1H, $J_{1,2}$ 8.5 Hz, *H*-1), 5.59 (s, 1H, C*H*Ph), 5.17 (bd, 1H, $J_{3',4'}$ 3.0 Hz, H-4′), 5.00 (dd, 1H, $J_{1',2'}$ 8.0 Hz, $J_{2',3'}$ 10.0 Hz, H-2′), 4.98 (m, 1H, H-3″), 4.97 (t, 1H, $J_{3'',4''}$, $J_{4'',5''}$ 8.5 Hz, H-4″), 4.79 (m, 1H, H-2″), 4.77 (dd, 1H, $J_{2,3}$ 10.0 Hz, $J_{3,4}$ 8.5 Hz, H-3), 4.68 (d, 1H, $J_{1'',2''}$ 1.5 Hz, H-1″), 4.54 (dd, 1H, $J_{1,2}$ 8.5 Hz, $J_{2,3}$ 10.0 Hz,

H-2), 4.50 (d, 1H, $J_{1',2'}$ 8.0 Hz, H-1′), 4.35 (dd, 1H, $J_{5,6a}$ 5.0 Hz, $J_{6a,6b}$ 10.5 Hz, H-6_a), 4.00 (dd, 1H, $J_{5',6a'}$ 7.5 Hz, $J_{6a',6b'}$ 11.0 Hz, H-6_a′), 3.90 (m, 3H, H-4, H-5″, H-6_b), 3.78 (dd, 1H, $J_{5',6b'}$ 5.5 Hz, $J_{6a',6b'}$ 11.0 Hz, H-6_b′), 3.70 (m,1H, H-5), 3.68 (s, 3H, $OC_6H_4OCH_3$), 3.60 (dd, 1H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 3.0 Hz, H-3′), 3.40 (m, 1H, H-5′), 2.14, 2.04, 2.02, 2.00, 1.91, 1.57 (6s, 18H, 6×$COCH_3$), 1.11 (d, 3H, $J_{5'',6''}$ 6.0 Hz, C-CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.4, 170.1(2), 169.9, 169.4, 168.8 (6×$COCH_3$), 167.9, 167.7 (2×phthalimido *C*O), 155.5, 150.4, 137.0, 134.1(2), 131.7(2), 129.2, 128.3(2), 126.0(2), 123.6, 123.3, 118.4(2), 114.4(2) (Ar*C*), 101.4 (*C*OPh), 100.2 (C-1′), 98.2 (C-1″), 97.9 (C-1), 80.8, 76.0, 75.3, 71.1, 70.5, 70.4, 70.0, 68.6, 68.0, 67.9, 67.2, 66.3, 61.3, 55.4 ($OC_6H_4OCH_3$), 55.2, 20.7(2), 20.6, 20.5, 20.4, 19.9 (6×$COCH_3$), 17.2 (C-CH_3). HRMS calcd. for $C_{52}H_{57}O_{23}NNa$ $(M+Na)^+$: 1086.3219, found: 1086.3223.

4.6. *p*-Methoxyphenyl-2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-6-*O*-benzyl-2-deoxy-2-*N*-phthalimido-β-D-glucopyranoside (9): To a stirred solution of compound **8** (1.8 g, 1.7 mmol) and Et_3SiH (3.2 mL, 20 mmol) in dry CH_2Cl_2 (20 mL), BF_3*Et_2O (0.4 mL, 3.4 mmol) was added at 0 °C and the mixture was allowed to stir for 2 min when TLC (*n*-hexane–EtOAc; 1:1) showed complete conversion of the starting material to a slower moving spot. The mixture was washed successively with H_2O (2×30 mL), saturated $NaHCO_3$ (2×30 mL) and brine (30 mL). The organic layer was collected, dried (Na_2SO_4), filtered and evaporated *in vacuo*. The residue was purified by flash chromatography using *n*-hexane–EtOAc (3:4) as eluent to afford pure trisaccharide acceptor **9** (1.5 g, 83%) as colourless foam. $[\alpha]_D^{25}$ +111 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.77-7.26 (m, 9H, Ar*H*), 6.80, 6.65 (2d, 4H, *J* 9.5 Hz, $OC_6H_4OCH_3$), 5.47 (d, 1H, $J_{1,2}$ 8.0 Hz, *H*-1), 5.26 (bd, 1H, $J_{3',4'}$ 3.5 Hz, H-4′), 5.18 (dd, 1H, $J_{1',2'}$ 8.0 Hz, $J_{2',3'}$ 10.0 Hz, H-2′), 5.02 (dd, 1H, $J_{2'',3''}$ 3.0 Hz, $J_{3'',4''}$ 10.0 Hz, H-3″), 4.98 (t, 1H, $J_{3'',4''}$, $J_{4'',5''}$ 10.0 Hz, H-4″), 4.85 (m, 1H, H-2″), 4.67 (d, 1H, $J_{1'',2''}$ 1.5 Hz, H-1″), 4.61, 4.58 (ABq, 2H, J_{AB} 12.0 Hz, CH_2Ph), 4.54 (m, 2H, H-2, H-3), 4.35 (d, 1H, $J_{1',2'}$

8.0 Hz, H-1′), 4.18 (dd, 1H, $J_{5,6a}$ 3.5 Hz, $J_{6a,6b}$ 12.0 Hz, H-6_a′), 3.98 (m, 2H, H-6_a, H-6_b′), 3.89 (dd, 1H, $J_{2',3'}$ 10.0 Hz, $J_{3',4'}$ 3.5 Hz, H-3′), 3.84 (m,1H, H-5′′), 3.71 (m, 4H, H-4, H-5, H-5′, H-6_b), 3.67 (s, 3H, $OC_6H_4OCH_3$), 2.19, 2.06, 2.05, 2.02, 1.93, 1.52 (6s, 18H, 6×$COCH_3$), 1.13 (d, 3H, $J_{5'',6''}$ 6.5 Hz, C-CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.4, 170.3, 170.0, 169.9, 169.4, 168.8 (6×*C*OCH$_3$), 168.0, 167.8 (2×phthalimido *C*O), 155.3, 150.7, 138.3, 134.4(2), 131.4(2), 128.2(2), 127.5(2), 127.4, 123.7, 123.5, 118.3(2), 114.3(2) (Ar*C*), 101.2 (C-1′), 98.4 (C-1′′), 97.4 (C-1), 82.4, 75.9, 75.8, 73.4, 71.6, 70.4, 70.2, 69.9, 69.8, 69.5, 68.2, 67.9, 67.5, 62.0, 55.5 ($OC_6H_4OCH_3$), 54.6, 20.7, 20.5(2), 20.4(2), 19.7 (6×$COCH_3$), 17.3 (C-CH_3). HRMS calcd. for $C_{52}H_{59}NO_{23}Na$ $(M+Na)^+$: 1088.3376, found: 1088.3380.

4.7. *p*-tolyl 2-benzyl-3,4-*O*-isopropylidene-1-thio-β-D-fucopyranoside (11): To a solution of known compound **10** (2.0 g, 6.4 mmol) in dry DMF (20 mL) was added NaH (620 mg, 25.8 mmol, 50% in mineral wax) followed by benzyl bromide (1.0 mL, 8.4 mmol) and the mixture was stirred at room temperature for 2 hours. Excess NaH was neutralized by careful addition of MeOH (2.0 mL) and the mixture was evaporated *in vacuo*. The residue was dissolved in diethyl ether (30 mL) and washed successively with H_2O (30 mL) and brine (30 mL). The diethyl ether layer was collected, dried (Na_2SO_4), filtered and evaporated. The residue was purified by flash chromatography using *n*-hexane–EtOAc, 6:1 to afford pure compound **11** (2.4 g, 92%) as a pale yellow solid. $[\alpha]_D^{25}$ -34 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.45-7.08 (m, 9H, Ar*H*), 4.82, 4.67 (ABq, 2H, J_{AB} 11.5 Hz, CH_2Ph), 4.52 (d, 1H, $J_{1,2}$ 9.5 Hz, *H*-1), 4.21 (t, 1H, $J_{2,3}$,$J_{3,4}$ 6.0 Hz, *H*-3), 4.04 (dd, 1H, $J_{3,4}$ 6.0 Hz, $J_{4,5}$ 2.0 Hz, *H*-4), 3.79 (qd, $J_{4,5}$ 2.0 Hz, $J_{5,6}$ 6.5 Hz, 1H, *H*-5), 3.48 (dd, 1H, $J_{1,2}$ 9.5 Hz, $J_{2,3}$ 6.0 Hz, *H*-2), 2.33 (s, 3H, $SC_6H_4CH_3$), 1.41, 1.36 (2s, 6H, 2×isopropylidene CH_3), 1.39 (d, 3H, $J_{5,6}$ 6.5 Hz, C-CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 138, 137.5, 132.7 (2), 129.8, 129.5 (2), 128.5, 128.3, 128.2 (2), 127.7 (Ar*C*), 109.6 [*C*$(CH_3)_2$], 86.5 (*C*-1), 79.9, 78.2, 73.5, 72.4, 27.9, 26.4 (2×isopropylidene *C*H_3), 21.1 ($SC_6H_4CH_3$), 16.9 (C-*C*H_3). HRMS calcd. for $C_{23}H_{28}O_4SNa$ $(M+Na)^+$: 423.1606, found: 423.1611.

4.8. *p*-tolyl 2-benzyl-3,4-di-*O*-acetyl-1-thio-β-D-fucopyranoside (12): A suspension of compound **11** (2.4 g, 5.9 mmol) in 80% AcOH (20 mL) was stirred at 80 °C for 2 hours when TLC (*n*-hexane–EtOAc, 2:1) showed complete convertion of the starting material to a slower moving spot. The solvents were evaporated *in vacuo* and co-evaporated with toluene to remove residual AcOH. The syrupy residue thus obtained was dissolved in 15 mL pyridine and 10 mL of Ac_2O as added. The solution was stirred at room temperature for 2 hours when TLC (*n*-hexane–EtOAc, 4:1) showed complete conversion of the starting material to a faster moving spot. The solvents were evaporated *in vacuo* and co-evaporated with toluene to remove residual pyridine. The syrupy residue thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (4:1) as the eluent to afford pure compound **12** (2.3 g, 86%) as a white amorphous mass. $[\alpha]_D^{25}$ -48 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.32-7.12 (m, 9H, Ar*H*), 5.24 (bd, 1H, $J_{3,4}$ 3.0 Hz, H-4), 5.02 (dd, 1H, $J_{2,3}$ 9.5 Hz, $J_{3,4}$ 3.0 Hz, H-3), 4.85, 4.58 (ABq, 2H, J_{AB} 11.0 Hz, CH_2Ph), 4.65 (d, 1H, $J_{1,2}$ 9.5 Hz, *H*-1), 3.72 (t, 1H, $J_{1,2}$, $J_{2,3}$ 9.5 Hz, H-2), 3.71 (m, 1H, *H*-5), 2.34 (s, 3H, $SC_6H_4CH_3$), 2.14, 1.93 (2s, 6H, 2×COCH_3), 1.20 (d, 3H, $J_{5,6}$ 6.5 Hz, C-CH_3). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.2, 169.7 (2×C*O*CH_3), 137.8, 137.5, 132.4(2), 129.7(2), 128.1(2), 127.9(2), 127.7, 127.6 (Ar*C*), 87.5 (*C*-1), 75.1, 74.9, 74.4, 72.4, 70.6, 20.9, 20.5 (2×CO*C*H_3), 20.4 ($SC_6H_4CH_3$), 16.3 (C-*C*H_3). HRMS calcd. for $C_{24}H_{28}O_6SNa$ $(M+Na)^+$: 467.1504, found: 467.1509.

4.9. *p*-Methoxyphenyl-4-*O*-(2,3-di-*O*-acetyl-2-*O*-benzyl-α-L-fucopyranosyl)-2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-6-*O*-benzyl-2-deoxy-2-*N*-phthalimido-β-D-glucopyranoside (13): A mixture of acceptor **9** (1.5 g, 1.4 mmol), donor **12** (810 mg, 1.8 mmol) and MS 4Å (2.0 g) in dry CH_2Cl_2 (20 mL) was stirred under nitrogen atmosphere for 10 min at 0 °C. NIS (720 mg, 3.2 mmol) was added and the solution was kept at -45 °C for 15 minutes followed by the addition of H_2SO_4–silica (100 mg) and the mixture was stirred at -45 °C for another 30 min when TLC (*n*-hexane-EtOAc; 1:1) showed complete consumption of the acceptor. The mixture was filtered through a pad of Celite® and the filtrate was washed successively with aq. $Na_2S_2O_3$ (2×30 mL), saturated

$NaHCO_3$ (2×30 mL) and H_2O (30 mL). The organic layer was collected, dried (Na_2SO_4), filtered and evaporated *in vacuo* and the residue was purified by flash chromatography using *n*-hexane-EtOAc (3:4) as eluent to afford pure tetrasaccharide **13** (1.6 g, 81%) as white amorphous mass. $[\alpha]_D^{25}$ +138 (*c* 0.9, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.89-7.26 (m, 14H, Ar*H*), 6.73, 6.64 (2d, 4H, *J* 9.0 Hz, $OC_6H_4OCH_3$), 5.41 (d, 1H, $J_{1,2}$ 8.5 Hz, H-1), 5.26 (bd, 1H, $J_{3',4'}$ 2.5 Hz, H-4′), 5.24 (m, 3H, H-3′′, H-4′′, H-1′′′), 5.06-5.00 (m, 2H, H-2′, H-5′′′), 4.97 (ABq, 2H, J_{AB} 10.0 Hz, CH_2Ph), 4.85 (m, 2H, H-2′′, $J_{3''',4'''}$, $J_{4''',5'''}$ 7.5 Hz, H-4′′′), 4.64 (ABq, 1H, J_{AB} 11.5 Hz, CH_2Ph), 4.61 (d, 1H, $J_{1'',2''}$ 1.5 Hz, H-1′′), 4.51 (m, 4H, H-2, H-3, H-3′′′, CH_2Ph), 4.35 (m, 2H, H-6_a′, H-6_b′), 4.14 (d, 1H, $J_{1',2'}$ 8.0 Hz, H-1′), 4.07 (t, 1H, $J_{3,4}$ 9.5 Hz, H-4), 3.91 (m, 3H, H-6_a, H-3′, H-5′′), 3.69 (s, 3H, $C_6H_4OCH_3$), 3.67 (m, 3H, H-5, H-5′,H-6_b), 3.38 (dd,1H, $J_{1'',2''}$ 4.0 Hz, $J_{2'',3''}$ 3.5 Hz, H-2′′), 2.18, 2.14, 2.10, 2.09, 2.07, 2.02, 1.94(2) (7s, 24H, 8×COCH_3), 1.28 (d, 3H, $J_{5''',6'''}$ 6.5 Hz, H-6′′′), 1.13 (d, 3H, $J_{5'',6''}$ 6.5 Hz, H-6′′). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.9, 170.7, 170.6, 170.2, 169.7(2), 169.5, 169.4 (8×*C*OCH_3), 168.0, 167.8 (2×phthalimido *C*O), 155.5, 150.8, 138.1, 137.9, 134.7(2), 131.3(2), 128.4(2), 128.3(2), 127.9, 127.7(2), 127.5, 123.8(2), 118.8(2), 114.4(2) (Ar*C*), 100.5 (C-1′), 98.1 (C-1′′), 97.8 (C-1), 97.2 (C-1′′′), 75.7, 75.5, 74.2, 73.8, 73.7, 73.3, 73.0, 72.2, 71.4, 70.9, 70.6, 70.2, 68.1, 67.9, 67.3, 67.1, 64.5, 61.6, 56.3, 55.6 ($OC_6H_4OCH_3$), 20.9(2), 20.8(2), 20.7, 20.6(2), 20.5 (8×CO*C*H_3), 17.4 (C-*C*H_3), 15.9 (C-CH_3). HRMS calcd. for $C_{69}H_{79}O_{29}NNa$ $(M+Na)^+$: 1408.4635, found: 1408.4639.

4.10. *p*-Methoxyphenyl-4-*O*-(2,3-di-*O*-acetyl-2-*O*-benzyl-α-L-fucopyranosyl)-2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl-(1→3)-2,4,6-tri-*O*-acetyl-β-D-galactopyranosyl-(1→3)-6-*O*-benzyl-2-acetamido-2-deoxy-β-D-glucopyranoside (14): To a solution of the compound **13** (1.6 g, 1.1 mmol) in *n*-butanol (15 mL) was added ethylene diamine (0.15 mL, 1.3 mmol) and the solution was stirred at 110 °C for 24 hours. After evaporating the solvents *in vacuo*, the residue was dissolved in pyridine (10 mL) followed by Ac_2O (10 mL) and the solution was stirred at room temperature for 4 hours when TLC (*n*-hexane–EtOAc,

1:2) showed complete conversion of the starting material to a slower moving spot. The solvents were evaporated *in vacuo* and coevaporated with toluene to remove residual pyridine. The crude material thus obtained was purified by flash chromatography using *n*-hexane–EtOAc (1:4) to give pure compound **14** (1.1 g, 75%) as a colourless foam. $[\alpha]_D^{25}$ +122 (*c* 1.0, $CHCl_3$). ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.30-7.23 (m, 10H, Ar*H*), 6.98, 6.78 (2d, 4H, *J* 9.0 Hz, $OC_6H_4OCH_3$), 6.51 (d, 1H, $J_{2,NH}$ 9.0 Hz, N*H*Ac), 5.40 (d, 1H, $J_{3',4'}$ 3.5 Hz, H-4′), 5.25 (m, 5H, H-1, H-2′, H-1‴, H-3‴, H-4‴), 5.12 (dd, 1H, $J_{2'',3''}$ 3.0 Hz, $J_{3'',4''}$ 10.0 Hz, H-3″), 5.05 (t, 1H, $J_{3'',4''}$, $J_{4'',5''}$ 10.0 Hz, H-4″), 4.98 (dd, 1H, $J_{1'',2''}$ 2.0 Hz, $J_{2'',3''}$ 3.0 Hz, H-2″), 4.93 (d, 1H, $J_{1'',2''}$ 1.5 Hz, H-1″), 4.82(d, 1H, *J* 8.0 Hz, H-1′), 4.57 (ABq, 2H, J_{AB} 11.5 Hz, CH_2Ph), 4.46, 4.38 (ABq, 2H, J_{AB} 12.0 Hz, CH_2Ph), 4.18 (m, 4H, H-2, H-3, H-6_a′, H-6_b′), 4.03 (m, 3H, H-6_a, H-5′, H-5″), 3.90 (m, 3H, H-4, H-6_b, H-3′), 3.83 (m, 1H, H-5), 3.76 (m, 1H, H-4, H-2‴), 3.75 (s, 3H, $C_6H_4OCH_3$), 2.14, 2.13, 2.11, 2.05(3), 2.00, 1.95 (7s, 24H, 8×COCH_3), 1.75 (s, 3H, NHCOCH_3), 1.21 (d, 3H, $J_{5'',6''}$ 6.0 Hz, H-6″), 1.18 (d, 3H, $J_{5''',6'''}$ 7.0 Hz, H-6‴). ^{13}C NMR ($CDCl_3$, 125 MHz) δ: 170.5(3), 170.2, 170.1, 170.0(2), 169.6(2) (8×O*C*OCH$_3$, NH*C*OCH$_3$), 155.0, 150.9, 138.2, 137.3, 128.5(2), 128.3(2), 128.2, 128.1(2), 127.7(2), 127.5, 118.0(2), 114.5(2) (Ar*C*), 99.7 (C-1′), 98.3 (C-1, C-1″), 93.5 (C-1‴), 75.5, 75.3, 73.5, 73.4, 73.2, 72.1, 71.5, 71.4, 70.8, 70.7, 70.3, 70.2, 69.9, 69.3, 68.5, 68.1, 67.4, 65.1, 61.5, 55.6 ($OC_6H_4OCH_3$), 20.9(3), 20.8, 20.7(2), 20.6(2), 20.5 (8×OCOCH_3, NHCOCH_3), 17.4 (C-CH_3), 15.9 (C-CH_3). HRMS calcd. for $C_{63}H_{79}O_{28}NNa$ $(M+Na)^+$: 1320.4686, found: 1320.4689.

4.11. *p*-Methoxyphenyl-4-*O*-α-L-fucopyranosyl-α-L-rhamnopyranosyl-(1→3)-β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-glucopyranoside (1): A methanolic solution (200 mL) of the tetrasaccharide **14** (1.1 g, 0.8 mmol) was passed through an H-cube flow hydrogenation assembly using a 10% Pd-C cartridge with a flow rate of 1 mL/min. Complete debenzylation was achieved after three cycles, as confirmed by the mass spectrum. Solvents were evaporated *in vacuo* and the residue was purified by flash column using CH_2Cl_2–MeOH (12:1). The compound thus obtained was dissolved in

MeOH (20 mL). NaOMe (2.0 mL, 0.5 M in MeOH) was added and the solution was stirred at room temperature for 24 hours. The solution was neutralized with DOWEX 50W H^+ resin, filtered and the solvents were evaporated under reduced pressure and the residue was purified by a short flash column using CH_2Cl_2–MeOH (3:1) to afford the target tetrasaccharide **1** (450 mg, 68%). ^{1}H NMR (CD_3OD, 500 MHz, selected data) δ: 6.99, 6.85 (2d, 4H, *J* 9.0 Hz, $OC_6H_4OCH_3$), 5.12 (bs, 1H, H-1″), 5.09 (d, 1H, $J_{1''',2'''}$ 4.0 Hz, H-1‴), 4.98 (d, 1H, $J_{1,2}$ 8.0 Hz, H-1), 4.47 (d, 1H, $J_{1',2'}$ 7.5 Hz, H-1′), 3.77 (s, 3H, $OC_6H_4OCH_3$), 2.03 (s, 3H, $NHCOCH_3$), 1.28 (d, 3H, *J* 6.0 Hz, C-CH_3), 1.22 (d, 3H, *J* 6.5 Hz, C-CH_3). ^{13}C NMR (CD_3OD, 125 MHz) δ: 174.3 ($NHCOCH_3$), 156.8, 153.1, 119.2(2), 115.6(2) (Ar*C*), 105.1 (C-1′), 103.7 (C-1″), 101.7 (C-1), 99.6 (C-1‴), 81.1, 78.6, 77.6, 76.7, 74.1, 73.8, 73.4, 72.1(2), 72.0, 71.2, 70.2, 70.0, 69.6, 67.7, 62.6, 61.3, 57.4, 56.1 ($OC_6H_4OCH_3$), 23.2 ($NHCOCH_3$), 18.0 (C-CH_3), 16.6 (C-CH_3). HRMS calcd. for $C_{33}H_{51}O_{20}NNa$ $(M+Na)^+$: 804.2902, found: 804.2905.

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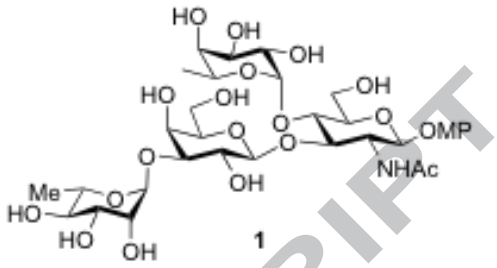
Synthesis of the tetrasaccharide repeating unit of the *O*-glycan from the polar flagellum flagellin of *Azospirillum brasilense* Sp7 Kumar Bhaskar Pal, Balaram Mukhopadhyay	 **1**

Highlights

1. Total synthesis of the tetrasaccharide repeating unit of the flagellin *O*-glycan
2. Activation of thioglycoside using NIS in the presence of H_2SO_4-silica
3. Use of OPMP glycoside for further glycoconjugate formation