Supplementary Information

Gram-Scale Chemical Synthesis of Galactosyllactoses and Their Impact on Infant Gut Microbiota *In Vitro*

Evy Meeusen [a], Linqiu Cao [b], Dianne J. Delsing [b], Andre Groeneveld[b], Margreet Heerikhuisen[c], Frank Schuren[c] and Thomas J. Boltje*[a]

a. Synthetic Organic Chemistry, Institute for Molecules and Materials, Radboud University, Nijmegen, The Netherlands
b. FrieslandCampina N.V., Amersfoort, The Netherlands
c. Netherlands Organization for Applied Scientific Research (TNO), Leiden, The Netherlands
* Corresponding author, thomas.boltje@ru.nl

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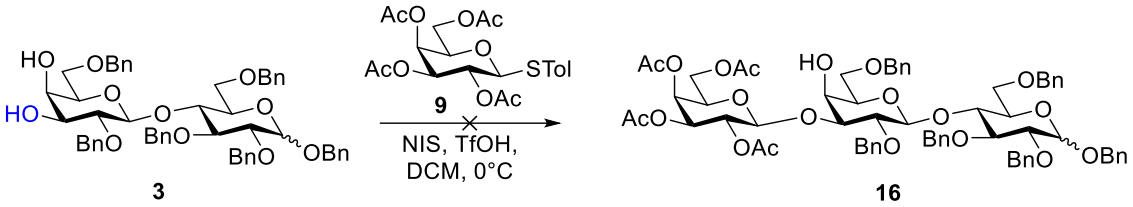
Supplementary figures and schemes



Scheme S1: Glycosylation of *cis*-diol **3** for the synthesis of protected 3'-GL **16.**

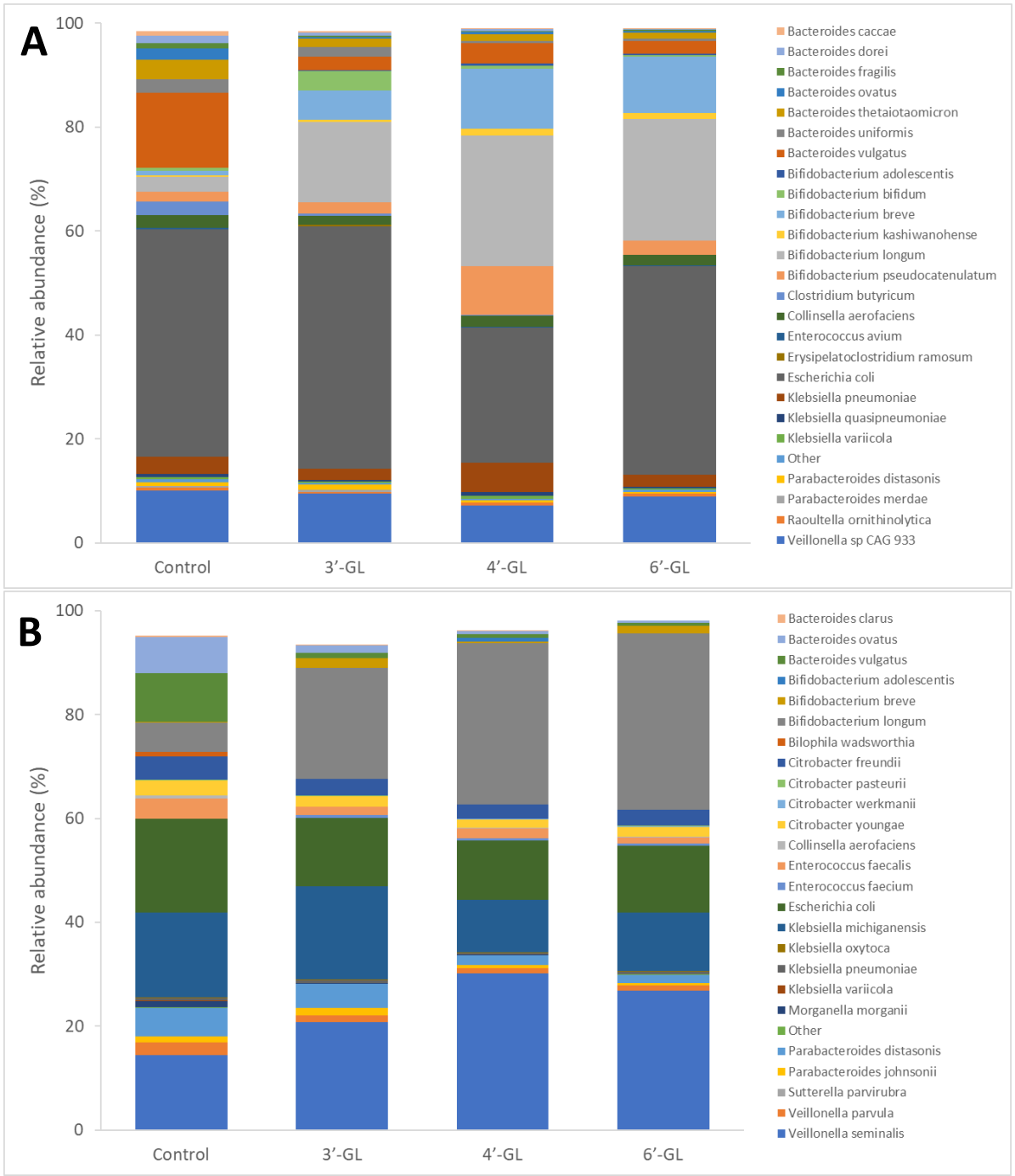


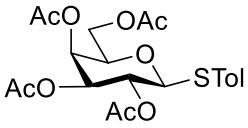
Figure S1. Bar graphs showing the relative abundances of the 25 most abundant bacterial species in infant microbiota (Panel A: formula fed infants, Panel B: breastfed infants) after exposure to 3'-GL, 4'-GL or 6'-GL.

General synthetic methods

^{1}H and ^{13}C NMR APT spectra were recorded on a Bruker 400 MHz or 500 MHz spectrometer. Chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS), or residual solvents as the internal standard. NMR data is presented as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, dd = doublet of doublets, m = multiplet and/or multiple resonances), coupling constant in hertz (Hz), integration. All NMR signals were assigned on the basis of ^{1}H NMR, ^{13}C NMR APT, COSY, HSQC, and TOCSY experiments. Mass spectra were recorded on a JEOL AccuTOF CS JMST100CS mass spectrometer. Automatic flash column chromatography was performed using Biotage Isolera Spektra One, using SNAP cartridges (Biotage, 30-100 µm, 60 Å), 4-120 g. TLC-analysis was conducted on Silicagel F254 (Merck KGaA) with detection by UV-absorption (254nm) where applicable, and by spraying with 10% sulphuric acid in methanol followed by charring at ≈300°C. DCM was freshly distilled. Molecular sieves (4Å) were flame activated under vacuum prior to use. All inert reactions were carried out under an argon atmosphere using flame-dried flasks

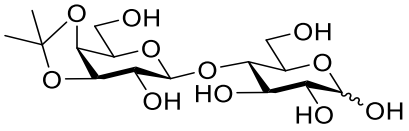
Experimental procedures

4-methylphenyl 2,3,4,6-tetra-*O*-acetyl-1-thio- β-D-galactopyranoside (9)



To a solution of penta-*O*-acetyl-β-D-galactopyranose (100 g, 256 mmol) in DCM (280 mL), were added successively, under nitrogen at 0 °C *p*-thiocresol (47.7 g, 384 mmol) and boron trifluoride diethyl etherate (51.4 mL, 410 mmol). The mixture was allowed to slowly warm up. The yellow solution was stirred for 24 hours, during which it turned purple. The mixture was washed with sat. $NaHCO_3$ (3x 200 mL), 5% aqueous sodium hydroxide solution (5x 200 mL), and brine (2x 200 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was crystallized using ethanol to yield **9** as a white solid (95 g, 210 mmol, 82%). R_f = 0.52 (50% EtOAc: *n*-heptane, v/v%). mp = 111.2 °C. **^{1}H NMR** (500 MHz, $CDCl_3$) δ 7.41 – 7.35 (m, 2H, C*H* Ar), 7.12 – 7.07 (m, 2H, C*H* Ar), 5.37 (dd, *J* = 3.4, 1.2 Hz, 1H, H-4), 5.18 (t, *J* = 10.0 Hz, 1H, H-2), 5.01 (dd, *J* = 10.0, 3.4 Hz, 1H, H-3), 4.62 (d, *J* = 10.0 Hz, 1H, H-1), 4.19 – 4.05 (m, 2H, H-6a and H-6b), 3.92 – 3.85 (m, 1H, H-5), 2.31 (s, 3H, C*H*$_3$ STol), 2.08 (s, 3H, C*H*$_3$ acetyl), 2.06 (s, 3H, C*H*$_3$ acetyl), 2.01 (s, 3H, C*H*$_3$ acetyl), 1.93 (s, 3H, C*H*$_3$ acetyl). **^{13}C NMR** (126 MHz, CDCl3) δ 170.51, 170.33, 170.20, 169.56 (4x *C*(O)OCH$_3$), 138.61, 133.30, 129.78, 128.77, 87.11 (C-1), 74.51 (C-5), 72.18 (C-3), 67.45 (C-2/C-4), 67.37(C-2/C-4), 61.72 (C-6), 21.30 (*C*H$_3$ STol), 21.00, 20.81, 20.76, 20.72 (4x *C*H$_3$ acetyl).

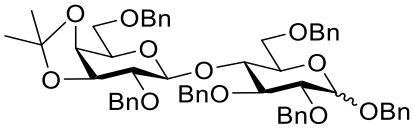
3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1→4)-D-glucopyranose (1)



Anhydrous lactose (10.0 g, 29.1 mmol) was dissolved in DMF (100 mL) and heated to 85 °C. Next, *p*-toluenesulfonic acid monohydrate (100 mg, 526 µmol) was added followed by 2,2-dimethoxypropane (8.5 mL, 69 mmol) in three portions at 5-minute intervals. After 15 minutes the lactose gradually dissolved and the reaction mixture was kept at 85 °C for an additional 30 minutes. The reaction mixture was cooled down and triethylamine was added. The reaction mixture was concentrated *in*

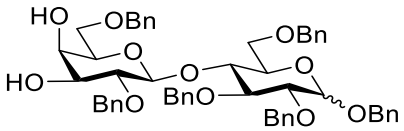
vacuo and once co-evaporated with toluene. The crude product was purified using silica column chromatography (0-20% MeOH in DCM) affording crude **1** (4.47 g, 11.7 mmol, 40%). The product was used in the next step without further purification. R_f = 0.2 (20% MeOH in DCM). **HRMS (m/z)**: [M+Na]+ calcd for $C_{15}H_{26}O_{11}$, 405.13728; found 405.13858. The spectroscopic data were identical to those reported in the literature [5].

Benzyl 2,6-di-*O*-benzyl-3,4-*O*-isopropylidene-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-D-glucopyranose (2)



To a solution of **1** (4.5 g, 11.7 mmol) in DMF (117 mL) cooled to 0°C and benzyl bromide (13.9 mL, 117 mmol) was added followed by 60wt% NaH (4.7 g, 117 mmol). The reaction was slowly allowed to warm to room temperature and was stirred for 23 hours. The reaction mixture was quenched with MeOH and concentrated *in vacuo*. The resulting residue was diluted with EtOAc (200 mL), washed with H_2O (2x 200 mL) and brine (1x 200 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude residue was purified using silica column chromatography (5-25% EtOAc in n-heptane, v/v%) yielding **2** as a pale oil (7.3 g, 7.9 mmol, 68%). Alpha/beta mixture was obtained, mainly beta. Rf = 0.56 (40% EtOAc in *n*-heptane, v/v%).**^{1}H NMR** [beta anomer] (500 MHz, $CDCl_3$) δ 7.40 – 7.12 (m, 30H, C*H* Ar), 4.94 – 4.83 (m, 3H, 3x CH*H*Ph), 4.78 – 4.59 (m, 5H, 5x CH*H*Ph), 4.56 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 4.49 – 4.45 (m, 2H, CH*H*Ph, H-1 Glc), 4.42 – 4.37 (m, 2H, H-1 Gal, CH*H*Ph), 4.28 (d, *J* = 12.0 Hz, 1H, CH*H*Ph), 4.07 (dd, *J* = 5.6, 1.8 Hz, 1H, H-4 Gal), 4.00 (dd, *J* = 6.8, 5.6 Hz, 1H, H-3 Gal), 3.94 (dd, *J* = 9.8, 8.9 Hz, 1H, H-4 Glc), 3.79 (dd, *J* = 11.0, 4.2 Hz, 1H, H-6a Glc), 3.72 (dd, *J* = 11.0, 1.9 Hz, 1H, H-6b Glc), 3.67 – 3.62 (m, 2H, H-5 Gal, H-6a Gal), 3.55 – 3.47 (m, 2H, H-3 Glc, H-6b Gal), 3.43 (dd, *J* = 9.2, 7.8 Hz, 1H, H-2 Glc), 3.36 (ddd, *J* = 9.9, 4.3, 1.9 Hz, 1H, H-5 Glc), 3.31 (dd, *J* = 8.0, 6.8 Hz, 1H, H-2 Gal), 1.36 (s, 3H, C*H*$_3$ acetonide)), 1.31 (s, 3H, C*H*$_3$ acetonide). **^{13}C NMR** [beta anomer] (126 MHz, $CDCl_3$) δ 139.13, 138.72, 138.67, 138.55, 138.43, 137.70, 128.51, 128.48, 128.47, 128.41, 128.39, 128.31, 128.17, 128.02, 128.01, 109.89 (*C* acetonide), 102.70 (C-1 Glc), 101.99 (C-1 Gal), 83.14 (C-3 Glc), 82.01 (C-2 Glc), 80.76 (C-2 Gal), 79.50 (C-3 Gal), 76.49 (C-4 Glc), 75.58 (*C*H_2Ph), 75.26 (C-5 Glc), 75.18 (*C*H_2Ph), 73.75 (C-4 Gal), 73.52 (*C*H_2Ph), 73.39 (*C*H_2Ph), 73.34 (*C*H_2Ph), 72.12 (C-5 Gal), 71.11 (*C*H_2Ph), 69.06 (C-6 Gal), 68.39 (C-6 Glc), 28.10 (*C*H_3 acetonide), 26.55 (*C*H_3 acetonide). **HRMS (m/z)**: [M+Na]+ calcd for $C_{61}H_{62}O_{11}$, 945.41898; found 945.41634

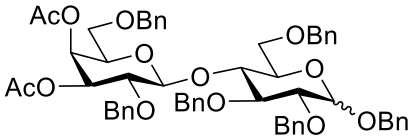
Benzyl 2,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-D-glucopyranose (3)



A solution of **2** (7.28 g, 7.89 mmol) in 80% aqueous acetic acid (80 mL) was heated to 75°C. The reaction was stirred for 5 hours. The reaction mixture was freeze-dried and the remaining solid was purified via silica column chromatography (10-40% EtOAc in n-heptane, v/v%) to yield diol **3** (5.41mg, 6.13 mmol, 78%) as a dense white solid. R_f = 0.27 (40% EtOAc in n-heptane, v/v%). Alpha/beta mixture was obtained, mainly beta. **^{1}H NMR** [beta anomer] (400 MHz, $CDCl_3$) δ 7.43 – 7.18 (m, 30H, C*H* Ar),

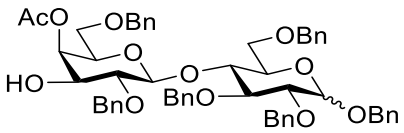
5.02 – 4.87 (m, 3H, 3x CH*H*Ph), 4.82 – 4.57 (m, 6H, 6x CH*H*Ph), 4.51 – 4.34 (m, 5H, H-1 Glc, H-1 Gal, 3x CH*H*Ph), 4.01 (t, *J* = 9.3 Hz, 1H, H-4 Glc), 3.95 – 3.89 (m, 1H, H-4 Gal), 3.82 (dd, *J* = 11.0, 4.1 Hz, 1H,, H-6a Glc), 3.75 (dd, *J* = 11.0, 1.9 Hz, 1H, H-6b Glc), 3.63 – 3.54 (m, 2H, H-6a Gal, H-3 Glc), 3.51 – 3.45 (m, 2H, H-2 Glc, H-6b Gal), 3.44 – 3.29 (m, 4H, H-2 Gal, H-3 Gal, H-5 Gal, H-5 Glc), 2.48 (d, *J* = 3.6 Hz, 1H, 4-O*H*), 2.42 – 2.37 (m, 1H, 3-O*H).* **^{13}C NMR** [beta anomer] (101 MHz, $CDCl_3$) δ 139.27, 138.69, 138.50, 138.38, 138.12, 137.65, 128.66, 128.61, 128.52, 128.47, 128.44, 128.41, 128.24, 128.20, 128.17, 128.14, 128.11, 128.05, 128.04, 127.99, 127.87, 127.84, 127.75, 127.70, 127.40, 102.72 (C-1 Gal), 102.64 (C-1 Glc), 82.99 (C-3 Glc), 81.96 (C-2 Glc), 80.17 (C-2 Gal), 76.72 (C-4 Glc), 75.41 (*C*H_2Ph), 75.29 (C-5 Glc), 75.13 (*C*H_2Ph), 75.03 (*C*H_2Ph), 73.66 (C-3 Gal), 73.62 (*C*H_2Ph), 73.37 (*C*H_2Ph), 73.02 (C-5 Gal), 71.11 (*C*H_2Ph), 68.94 (C-4 Gal), 68,82 (C-6 Gal), 68.42 (C-6 Glc). **HRMS** (m/z): [M+Na]+ calcd for $C_{54}H_{58}O_{11}$, 905. 38713; found, 905.38768

Benzyl 3,4-di-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-D-glucopyranose (17)



3 (20 mg, 23 µmol) was dissolved in pyridine (1 mL) and acetic anhydride (0.5 mL, 5 mmol) was slowly added. This reaction mixture was stirred for 6 hours and then diluted with EtOAc, washed with 1M HCl (3x 10 mL), sat. $NaHCO_3$ (3x 10 mL), and brine (1x 10 mL). The organic layer was dried using $MgSO_4$, filtered, and evaporated *in vacuo* affording **17** as a colorless oil (19 mg, 20 µmol, 87%). R_f = 0.49 (40% EtOAc in *n*-heptane). Spectral data showed a downfield shift for the H'-3 and H'-4 (**Figure S11-13)**.

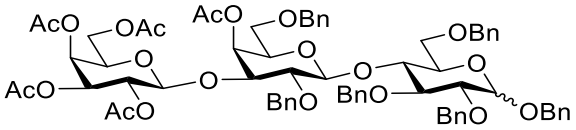
Benzyl 4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-D-glucopyranose (4)



To a solution of **3** (3.93 g, 4.45 mmol) in ACN (45 mL) was added trimethylorthoacetate (1.69 mL, 13.4 mmol) and *p*-TsOH (85 mg, 445 µmol). The reaction was stirred for 25 minutes. Next, 90% aqueous TFA (1.56 mL, 18.2 mmol) was added and this was stirred for another 25 minutes. The reaction was diluted with water and extracted with EtOAc. The combined organic layers were washed with sat. $NaHCO_3$, brine, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The residue was purified via silica column chromatography (25% EtOAc in n-heptane, v/v%) yielding **4** (3.53 g, 3.82 mmol, 86%) as a white foam. Alpha/beta mixture was obtained, mainly beta. R_f (step 1) = 0.57, R_f (step 2) = 0.42 (40% EtOAc in *n*-heptane, v/v%). **^{1}H NMR** [beta anomer] (400 MHz, $CDCl_3$) δ 7.46 – 7.15 (m, 30H, C*H* Ar), 5.35 (dd, *J* = 3.6, 1.1 Hz, 1H, H-4 Gal), 5.00 – 4.89 (m, 3H, 3x CH*H*Ph), 4.84 – 4.59 (m, 6H, 6x CH*H*Ph), 4.52 – 4.41 (m, 4H, 2x CH*H*Ph, H-1 Gal, H-1 Glc), 4.24 (dd, *J* = 12.0, 6.8 Hz, 1H, CH*H*Ph), 4.03 (dd, *J* = 9.9, 8.7 Hz, 1H, H-4 Glc), 3.82 (dd, *J* = 11.0, 4.1 Hz, 1H, H-6a Glc), 3.76 (dd, *J* = 10.9, 2.0 Hz, 1H, H-6b Glc), 3.64 (dd, *J* = 9.6, 3.5 Hz, 1H, H-3 Gal), 3.60 – 3.44 (m, 3H, H-2 Glc, H-3 Glc, H-5 Gal), 3.44 – 3.30 (m, 4H, H-2 Gal, H-5 Glc, H-6a Gal, H-6b Gal), 2.25 (s, 1H, 3-O*H*), 2.03 (s, 3H, C*H$_3$* acetyl).**^{13}C NMR** [beta anomer] (126 MHz, $CDCl_3$) δ 171.10 (C(O)CH_3), 139.18, 138.71, 138.41, 138.30, 138.08, 137.62, 128.60, 128.51, 128.49, 128.41, 128.21, 128.11, 128.03, 127.96, 127.86, 127.79, 127.77, 127.75, 127.70,

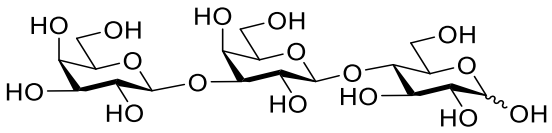
127.46, 102.63 (C-1 Glc), 102.45 (C-1 Gal), 82.87 (C-3 Glc), 81.88 (C-2 Glc), 80.25 (C-2 Gal), 76.50 (C-4 Glc), 75.40 (*C*H_2Ph), 75.19 (C-5 Glc), 75.19 (*C*H_2Ph), 75.16 (*C*H_2Ph), 73.53 (*C*H_2Ph), 73.37 (*C*H_2Ph), 72.58 (C-3 Gal), 72.12 (C-5 Gal), 71.09, (*C*H_2Ph), 69.75 (C-4 Gal), 68.30, (C-6 Glc) 67.38 (C-6 Gal), 20.91 (C(O)$\underline{CH_3}$). **HRMS** (m/z): $[M+Na]^+$ calcd for $C_{56}H_{60}O_{12}$, 947.39842; found, 947.40117

Benzyl 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl-(1→3)-4-*O*-acetyl-2,6-di-*O*-benzyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-D-glucopyranose (10)



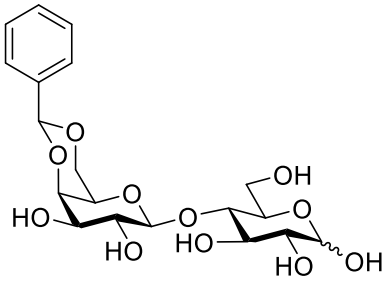
To a solution of the acceptor **4** (3.50 g, 3.78 mmol) and the thioglycosyl donor **9** (1.81 g, 3.97 mmol) in DCM (38 mL), molecular sieves (4Å) were added under an argon atmosphere. The reaction stirred for 15 minutes and was cooled to 0 °C. The thioglycoside was activated by the addition of NIS (1.12 g, 4.92 mmol) and triflic acid (67 µL, 757 µmol) The reaction was stirred for 30 minutes and quenched by the addition of triethylamine. The mixture was diluted by the addition of DCM, filtered, and washed with 10% aqueous thiosulfate solution, 1M HCl, sat. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and purified using silica column chromatography (35% EtOAc in *n*-heptane, v/v%) affording **10** (3.95 g, 3.15 mmol, 83%) as a white foam. Alpha/beta mixture was obtained, mainly beta. R_f = 0.20 (40% EtOAc in *n*-heptane, v/v%). **^{1}H NMR** [beta anomer] (400 MHz, $CDCl_3$) δ 7.42 – 7.09 (m, 30H, C*H* Ar), 5.37 (d, *J* = 3.6 Hz, 1H, H-4 Gal'), 5.31 (dd, *J* = 3.5, 1.3 Hz, 1H, H-4 Gal''), 5.12 (dd, *J* = 10.5, 7.9 Hz, 1H, H-2 Gal''), 4.98 – 4.83 (m, 4H, H-3 Gal'', 3x CH*H*Ph), 4.77 (d, *J* = 7.9 Hz, 1H, H-1 Gal''), 4.70 (dd, *J* = 10.7, 5.1 Hz, 3H, 3x CH*H*Ph), 4.65 – 4.53 (m, 3H, 3x CH*H*Ph), 4.46 – 4.36 (m, 4H, H-1 Glc, H-1 Gal', 2x CH*H*Ph), 4.26 (d, *J* = 11.8 Hz, 1H, CH*H*Ph), 4.14 – 4.10 (m, 2H, H-6 Gal''), 3.99 (dd, *J* = 9.9, 8.7 Hz, 1H, H-4 Glc), 3.80 (td, *J* = 6.7, 1.3 Hz, 1H, H-5 Gal''), 3.73 (dd, *J* = 11.1, 4.1 Hz, 1H, H-6a Glc), 3.69 – 3.62 (m, 2H, H-6b Glc, H-3 Gal'), 3.54 – 3.41 (m, 4H, H-2 Glc, H-3 Glc, H-2 Gal', H-5 Gal'), 3.35 – 3.24 (m, 3H, H-5 Glc, H-6 Gal'), 2.14 (s, 3H, C*H*$_3$ acetyl), 2.04 (s, 3H, C*H*$_3$ acetyl), 1.97 (s, 3H. C*H*$_3$ acetyl), 1.94 (s, 3H, C*H*$_3$ acetyl), 1.83 (s, 3H, C*H*$_3$ acetyl). **^{13}C NMR** [beta anomer] (101 MHz, $CDCl_3$) δ 170.54, 170.52, 170.26, 169.88, 169.16, 139.21, 138.70, 138.37, 138.24, 138.21, 137.65, 128.61, 128.54, 128.52, 128.49, 128.48, 128.46, 128.44, 128.41, 128.28, 128.24, 128.22, 128.09, 128.05, 127.99, 127.97, 127.86, 127.84, 127.81, 127.78, 127.74, 127.70, 127.68, 127.47, 102.64 (C-1 Glc), 102.16 (C-1 Gal'), 100.96 (C-1 Gal''), 82.78 (C-3 Glc), 81.87 (C-2 Glc), 80.36 (C-2 Gal'), 77.75 (C-3 Gal'), 76.16 (C-4 Glc), 75.43 (*C*H_2Ph), 75.32 (*C*H_2Ph), 75.24 (C-5 Glc), 75.18 (*C*H_2Ph), 73.74 (*C*H_2Ph), 73.43 (*C*H_2Ph), 72.77 (C-5 Gal'), 71.09 (*C*H_2Ph), 70.96 (C-3 Gal''), 70.77 (C-5 Gal''), 69.63 (C-4 Gal'), 69.38 (C-2 Gal''), 68.21 (C-6 Glc, C-6 Gal'), 67.05 (C-4 Gal''), 61.25 (C-6 Gal''), 20.84, 20.84, 20.80, 20.77, 20.71. **HRMS** (m/z): $[M+Na]^+$ calcd for $C_{70}H_{78}O_{21}$, 1277.49333; found, 1277.49402

β-D-galactopyranosyl-(1→3)-β-D-galactopyranosyl-(1→4)-D-glucopyranose (13)



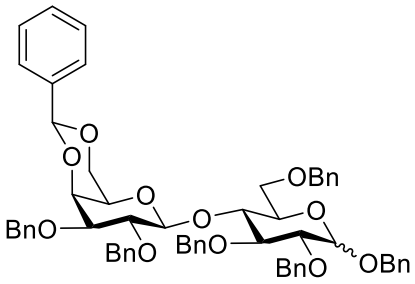
To a solution of **10** (4.67 g, 3.72 mmol) in methanol (38 mL), K_2CO_3 (154 mg, 1.12 mmol) was added. This reaction mixture was stirred for 24 hours and neutralized using Dowex H+. The reaction mixture was filtered, concentrated *in vacuo* and purified using column chromatography (6% MeOH in DCM, v/v%) yielding a white foam (3.01 g, 2.88 mmol, 77%). The resulting product (3.00 g, 2.87 mmol) was hydrogenated was hydrogenated with H_2 over Pd/C in MeOH (60 mL) for 2 days. The mixture was filtered over Celite and rinsed with MeOH and water. Next, the methanol was evaporated *in vacuo* and the resulting solution was freeze-dried to obtain 3'-galactosyllactose (**13**) (1.42 g, 2.82 mmol, 98%) as a white solid. Alpha/beta mixture was obtained; ratio beta:alpha = 0.65:0.35. R_f = 0.1 (20% H_2O in ACN). R_f = 0.19 (30% H_2O in ACN, v/v%). **^{1}H NMR** (500 MHz, D_2O) δ 5.25 (d, *J* = 3.8 Hz, 0.35H, H-1 Glc alpha), 4.69 (d, *J* = 8.0 Hz, 0.65H, H-1 Glc beta), 4.64 (d, *J* = 7.6 Hz, 1H, H-1 Gal''), 4.53 (d, *J* = 7.8 Hz, 1H, H-1 Gal'), 4.22 (d, *J* = 3.3 Hz, 1H, H-4 Gal'), 4.01 – 3.57 (m, 16H), 3.31 (t, *J* = 8.5 Hz, 0.54H, H-1 Glc beta). **^{13}C NMR** (126 MHz, D_2O) δ 104.93 (C-1 Gal''), 103.15 (C-1 Gal' beta), 103.13 (C-1 Gal' alpha), 96.38 (C-1 Glc beta), 92.44 (C-1 Glc alpha), 82.49 (C-3 Gal'), 78.88 (C-4 Glc alpha), 78.76 (C-4 Glc beta), 75.67 (C-5 Gal''), 75.59 (C-5 Gal'), 75.39 (C-5 Glc beta), 74.96 (C-3 Glc beta), 74.43 (C-2 Glc beta), 73.11 (C-3 Gal'), 72.01 (C-2 Glc alpha), 71.77 (C-3 Glc alpha), 71.63 (C-2 Gal''), 70.80 (C-2 Gal'), 70.70 (C-5 Glc alpha), 69.18 (C-5 Gal''), 69.03 (C-4 Gal'), 61.60 (C-6 Gal'), 61.56 (C-6 Gal''), 60.70 (C-6 Glc beta), 60.57 (C-6 Glc alpha). **HRMS** (m/z): [M+Na]+ calcd for $C_{18}H_{32}O_{16}$, 527.15880; found, 52715931

4,6-*O*-benzylidene-D-lactose (5)



Lactose (2.0 g, 5.8 mmol, 1 eq.) and camphorsulfonic acid (276 mg, 1.19 mmol) were added to a mixture of DMF (8 mL) and benzaldehyde dimethyl acetal (2.1 mL, 14 mmol) at room temperature. The reaction mixture was vigorously stirred at 70 °C under nitrogen for 6 hours. After cooling, triethylamine was added and concentrated in vacuo. The product was purified using silica column chromatography (10% H_2O in acetonitrile, v/v) yielding **5** as a light-yellow powder (1.86 g, 4.33 mmol, 74%). R_f = 0.28 (20% MeOH in DCM, v/v%). alpha/beta mixture was obtained; ratio = 0.4:0.6. Continued to the next step without further analysis. **HRMS** (m/z): [M+Na]+ calcd for $C_{19}H_{26}O_{11}$, 453.13728; found, 453.13736. The spectroscopic data were identical to those reported in the literature [6].

Benzyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidine- β-D-galactopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-D-glucopyranose (6)



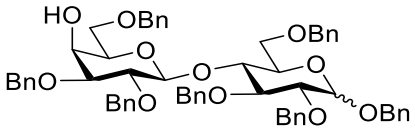
5 (10.4 g, 24.1 mmol) was dissolved in anhydrous DMF (240 mL) and cooled at 0 °C. To this mixture, benzyl bromide (28.7 mL, 241 mmol) was added, followed by slow addition of 60% w/w NaH (9.7 g, 241 mmol). The mixture was stirred for 2 hours slowly allowing it to heat up to room temperature. The suspension was quenched by the addition of methanol (40 mL) and the mixture was concentrated *in vacuo*. Next, the resulting suspension was diluted with DCM (500 mL) and washed with H_2O (2x 500 mL) and brine (500 mL), and the organic layer was dried over $MgSO_4$, filtered, and concentrated *in vacuo*. Silica column chromatography (0-40% EtOAc in n-heptane, v/v%) afforded **6** (17.8 g, 18.3 mmol, 76%) as a white solid. Alpha/beta mixture was obtained, mainly beta. R_f [beta anomer] = 0.43 (40% EtOAc in *n*-heptane, v/v%). **^{1}H NMR** [beta anomer] (500 MHz, $CDCl_3$) δ 7.53 – 7.49 (m, 2H, C*H* Ar), 7.47 – 7.43 (m, 2H, C*H* Ar), 7.39 – 7.36 (m, 4H, C*H* Ar), 7.34 – 7.15 (m, 27H, C*H* Ar), 5.46 (s, 1H, C*H* acetal benzylidene), 5.17 (d, *J* = 10.6 Hz, 1H, CH*H*Ph), 4.94 (dd, *J* = 14.2, 11.4 Hz, 2H, CH*H*Ph), 4.84 (d, *J* = 11.2 Hz, 1H, CH*H*Ph), 4.78 – 4.70 (m, 5H, 5x CH*H*Ph), 4.66 (d, *J* = 12.0 Hz, 1H, CH*H*Ph), 4.57 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 4.50 (d, *J* = 7.8 Hz, 1H, H-1 Glc), 4.46 (d, *J* = 7.8 Hz, 1H, H-1 Gal), 4.35 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 4.21 (dd, *J* = 12.4, 1.5 Hz, 1H, H-6a Gal), 4.03 (dd, *J* = 3.7, 1.1 Hz, 1H, H-4 Gal), 3.99 (t, *J* = 9.8, 8.8 Hz, 1H, H-4 Glc), 3.89 (dd, *J* = 11.0, 4.1 Hz, 1H, H-6a Glc), 3.84 (dd, *J* = 12.4, 1.9 Hz, 1H, H-6b Gal), 3.78 – 3.71 (m, 2H, H-6b Glc, H-2 Gal), 3.62 (t, *J* = 9.0 Hz, 1H, H-3 Glc), 3.51 (dd, *J* = 9.2, 7.8 Hz, 1H, H-2 Glc), 3.39 (dd, *J* = 9.6, 3.7 Hz, 1H, H-3 Gal), 3.35 (ddd, *J* = 9.9, 4.2, 1.8 Hz, 1H, H-5 Glc), 2.95 (s, *J* = 1.5 Hz, 1H, H-5 Gal).**^{13}C NMR** [beta anomer] (126 MHz, $CDCl_3$) δ 138.96, 138.88, 138.64, 138.57, 138.44, 138.11, 137.55, 128.85, 128.61, 128.37, 128.26, 128.22, 128.12, 128.09, 127.91, 127.78, 127.71, 127.60, 127.51, 127.44, 127.39, 127.27, 126.56, 102.86 (H-1 Gal), 102.55 (H-1 Glc), 101.37 (*C*H acetal benzylidene), 83.07 (C-3 Glc), 81.88 (C-2 Glc), 79.69 (C-3 Gal), 78.86 (C-2 Gal), 77.62 (C-4 Glc), 75.80 (*C*H_2Ph), 75.31 (*C*H_2Ph), 75.17 (C-5 Glc), 75.05 (*C*H_2Ph), 73.70 (C-4 Gal), 73.01 (*C*H_2Ph), 71.65 (*C*H_2Ph), 71.02 (*C*H_2Ph), 68.97 (C-6 Gal), 68.29 (C-6 Glc), 66.36 (C-5 Gal).

R_f [alpha anomer] = 0.37 (40% EtOAc in *n*-heptane, v/v%). **^{1}H NMR** [alpha anomer] (500 MHz, $CDCl_3$) δ 7.57 – 7.49 (m, 4H, C*H* Ar), 7.44 – 7.40 (m, 2H, C*H* Ar), 7.40 – 7.37 (m, 2H, C*H* Ar), 7.36 – 7.17 (m, 27H, C*H* Ar), 5.47 (s, 1H, C*H* acetal benzylidene), 5.21 (d, *J* = 10.3 Hz, 1H, CH*H*Ph), 4.83 (d, *J* = 11.2 Hz, 1H, CH*H*Ph), 4.81 (d, *J* = 3.8 Hz, 1H, H-1 Glc), 4.79 – 4.67 (m, 6H, 6x CH*H*Ph), 4.60 – 4.51 (m, 3H, 3x CH*H*Ph), 4.31 (d, *J* = 7.8 Hz, 1H, H-1 Gal), 4.28 – 4.19 (m, 2H, H-6a Gal, CH*H*Ph), 4.01 (dd, *J* = 3.7, 1.1 Hz, 1H, H-4 Gal), 3.99 – 3.93 (m, 2H, H-3, H-4 Glc), 3.90 (dd, *J* = 10.9, 3.1 Hz, 1H, H-6a Glc), 3.85 (dd, *J* = 12.4, 1.9 Hz, 1H, H-6b Gal), 3.75 (dd, *J* = 9.7, 7.9 Hz, 1H, H-2 Gal), 3.69 (dt, *J* = 9.4, 2.7 Hz, 1H, H-5 Glc), 3.55 – 3.50 (m, 1H, H-2 Glc), 3.40 (dd, *J* = 10.8, 1.9 Hz, 1H, H-6b Glc), 3.30 (dd, *J* = 9.6, 3.7 Hz, 1H, H-3 Gal), 2.89 (s, 1H, H-5 Gal). **^{13}C NMR** [alpha anomer] (126 MHz, $CDCl_3$) δ 139.36, 139.08, 138.64, 138.51, 138.28, 137.45, 129.00, 128.96, 128.55, 128.51, 128.43, 128.36, 128.30, 128.25, 128.09, 127.99, 127.93, 127.83, 127.81, 127.76, 127.57, 127.55, 127.39, 126.72, 103.11 (C-1 Gal), 101.54 (*C*H acetal benzylidene), 95.95 (C-1 Glc), 80.59 (C-3 Glc), 79.68 (C-3 Gal), 79.35 (C-2 Glc), 78.98 (C-2 Gal), 77.85

(C-4 Glc), 76.13 (*C*H_2Ph), 75.44 (*C*H_2Ph), 73.87 (H-4 Gal), 73.50 (*C*H_2Ph), 73.11 (*C*H_2Ph), 71.73 (*C*H_2Ph), 70.53 (C-5 Glc), 69.38 (*C*H_2Ph), 69.08 (C-6 Gal), 68.12 (C-6 Glc), 66.46 (C-5 Gal).

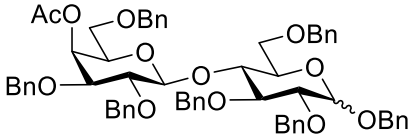
HRMS (m/z): [M+Na]+ calcd for $C_{61}H_{62}O_{11}$, 993.41898; found, 995.41486

Benzyl 2,3,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-D-glucopyranose (7)



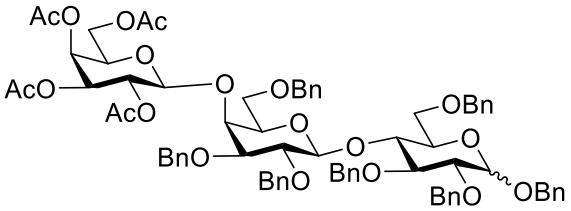
6 (10.92 g, 11.24 mmol) was dissolved in anhydrous DCM (112 mL), and molecular sieves were added. The reaction was cooled to −78 °C, and Et_3SiH (12.5 mL, 78.3 mmol) was added followed by TfOH (2.3 mL, 25.8 mmol). The reaction was stirred at −78 °C for 7 hours and then was quenched with methanol and Et_3N. The reaction was filtered, diluted with DCM, washed with 1M HCL, and sat. $NaHCO_3$, dried over $MgSO_4$ and filtered. This was concentrated *in vacuo* and purified using column chromatography (20% EtOAc in n-heptane, v/v%) yielding **7** (6.88 g, 7.1 mmol, 63%) as a white solid. Alpha/beta mixture was obtained, mainly beta. Rf = 0.53 (40% EtOAc in n-heptane, v/v%).**^{1}H NMR** [beta anomer] (400 MHz, $CDCl_3$) δ 7.42 – 7.22 (m, 35H, C*H* Ar), 5.06 – 4.88 (m, 3H, 3x CH*H*Ph), 4.83 – 4.65 (m, 7H, 7x CH*H*Ph), 4.59 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 4.51 (d, *J* = 7.6 Hz, 1H, H-1 Glc), 4.49 – 4.38 (m, 4H, H-1 Gal, 3x CH*H*Ph), 4.06 – 3.97 (m, 2H, H-4 Gal, H-4 Glc,), 3.84 (dd, *J* = 10.9, 4.3 Hz, 1H, H-6a Glc), 3.75 (dd, *J* = 10.9, 1.8 Hz, 1H, H-6b Glc), 3.68 (dd, *J* = 9.7, 7.2 Hz, 1H, H-6a Gal), 3.61 (m, 2H, H-2 Gal, H-3 Glc), 3.54 – 3.46 (m, 2H, H-2 Glc, H-6b Gal), 3.42 – 3.27 (m, 3H, H-3 Gal, H-5 Gal, H-5 Glc), 2.42 – 2.40 (m, 1H, O*H*). **^{13}C NMR** [beta anomer] (101 MHz, $CDCl_3$) δ 139.27, 138.79, 138.75, 138.50, 138.35, 138.09, 137.71, 128.61, 128.52, 128.43, 128.41, 128.34, 128.30, 128.24, 128.23, 128.20, 128.17, 128.03, 128.00, 127.98, 127.96, 127.91, 127.88, 127.84, 127.81, 127.76, 127.68, 127.62, 127.38, 102.68 (C-1 Glc, C-1 Gal), 83.08 (C-3 Glc), 81.98 (C-2 Glc), 81.28 (C-3 Gal), 79.57 (C-2 Gal), 76.75 (C-4 Glc), 75.51 (*C*H_2Ph), 75.41 (*C*H_2Ph), 75.32 (C-5 Glc), 75.15 (*C*H_2Ph), 73.66 (*C*H_2Ph), 73.30 (*C*H_2Ph), 72.92 (C-5 Gal), 72.18 (*C*H_2Ph), 71.12 (*C*H_2Ph), 68.57 (C-6 Gal), 68.41 (C-6 Glc), 66.29 (C-4 Gal). **HRMS** (m/z): $[M+Na]^+$ calcd for $C_{61}H_{64}O_{11}$, 995.43463; found, 995.43766

Benzyl 4-*O*-acetyl-2,3,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-D-glucopyranose (18)



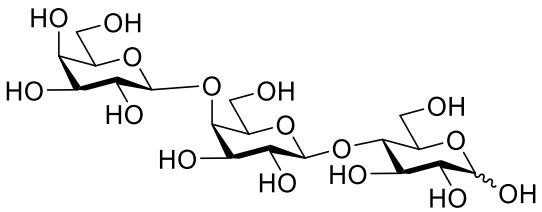
7 (29 mg, 30 µmol) was dissolved in pyridine (1 mL) and acetic anhydride (0.5 mL, 5mmol) was slowly added and this was stirred overnight. The reaction mixture was diluted with 1M HCl, sat. $NaHCO_3$, brine, dried over $MgSO_4$, filtered, and dried *in vacuo* affording **18** (24 mg, 24 µmol, 79%) as a colourless oil. R_f = 0.59 (40% EtOAc in *n*-heptane). Spectral data showed a downfield shift for the H'-4 (**Figure S31-33**).

Benzyl 2,3,4,6-tetra-*O*-acetyl- β-D-galactopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-D-glucopyranose (11)



To a solution of the acceptor **7** (6.16 g, 6.33 mmol) and the thioglycosyl donor **9** (2.90 g, 6.33 mmol) in DCM (63mL), molecular sieves (4Å) were added under an argon atmosphere. The reaction stirred for 15 minutes and was cooled to 0 °C. The thioglycoside was activated by the addition of NIS (1.85 g, 8.23 mmol) and triflic acid (112 µL, 1.27 mmol) The reaction was stirred for 1 hour and quenched by the addition of triethylamine. The mixture was diluted by the addition of DCM, filtered, and washed with 10% aqueous thiosulfate solution, 1M HCL, sat. $NaHCO_3$ and brine. The organic layer was dried over $MgSO_4$, filtered, and purified by silica column chromatography (30% ethyl acetate in n-heptane, v/v%) affording **11** (6.13 g, 4.7 mmol, 74%) as a light-yellow foam. Alpha/beta mixture was obtained, mainly beta. R_f = 0.37 (40% EtOAc in *n*-heptane, v/v%). **^{1}H NMR** [beta anomer] (500 MHz, $CDCl_3$) δ 7.61 – 7.17 (m, 35H, C*H* Ar), 5.41 (dd, *J* = 3.5, 1.2 Hz, 1H, H-4 Gal''), 5.25 (dd, *J* = 10.5, 7.8 Hz, 1H, H-2 Gal''), 5.05 – 5.01 (m, 2H, H-3 Gal'', CH*H*Ph), 4.98 – 4.88 (m, 2H, 2x CH*H*Ph), 4.83 – 4.62 (m, 8H, H-1 Gal'', 7x CH*H*Ph), 4.55 (d, *J* = 12.0 Hz, 1H, CH*H*Ph), 4.49 (d, *J* = 7.8 Hz, 1H, H-1 Glc), 4.41 – 4.37 (m, 2H, H-1 Gal', CH*H*Ph), 4.33 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 4.23 – 4.16 (m, 2H, H-6a Gal'', CH*H*Ph), 4.09 – 4.04 (m, 1H, H-6b Gal''), 4.00 (d, *J* = 2.9 Hz, 1H, H-4 Gal'), 3.93 (dd, *J* = 9.9, 8.9 Hz, 1H, H-4 Glc), 3.82 (ddd, *J* = 7.5, 5.9, 1.3 Hz, 1H, H-5 Gal''), 3.79 – 3.69 (m, 2H, H-6a and H-6b Glc), 3.66 (dd, *J* = 9.8, 6.4 Hz, 1H, H-6a Gal'), 3.59 – 3.51 (m, 2H, H-3 Glc, H-2 Gal'), 3.43 (dd, *J* = 9.3, 7.8 Hz, 1H, H-2 Glc), 3.41 – 3.26 (m, 4H, H-3 Gal', H-5 Glc, H-5 Gal', H-6b Gal'), 2.17 (s, 3H, C*H$_3$* acetyl), 2.00 (s, 3H, C*H$_3$* acetyl), 1.96 (s, 3H, C*H$_3$* acetyl), 1.88 (s, 3H, C*H$_3$* acetyl).**^{13}C NMR** [beta anomer] (126 MHz, $CDCl_3$) δ 170.47, 170.40, 170.33, 169.41 (4x *C*(O)OCH_3), 139.20, 138.93, 138.81, 138.75, 138.53, 138.28, 137.77, 129.62, 128.64, 128.49, 128.43, 128.16, 128.11, 128.05, 127.98, 127.81, 127.79, 127.75, 127.72, 127.71, 127.65, 127.56, 127.53, 127.32, 102.74 (C-1 Glc/ C-1 Gal'), 102.71 (C-1 Glc/ C-1 Gal'), 102.37 (C-1 Gal''), 82.95 (C-3 Glc), 82.02 (C-3 Gal'), 81.88 (C-2 Glc), 80.32 (C-2 Gal'), 76.85 (C-4 Glc), 76.12 (*C*H_2Ph), 75.54 (*C*H_2Ph), 75.40 (C-5 Glc), 75.27 (*C*H_2Ph), 74.76 (C-4 Gal'), 73.66 (C-5 Gal'), 73.44 (2x *C*H_2Ph), 73.28 (*C*H_2Ph), 70.99 (C-3 Gal''), 70.46 (C-5 Gal''), 69.23 (C-2 Gal''), 69.01 (C-6 Gal'), 68.33 (C-6 Glc), 67.06 (C-4 Gal''), 61.20 (C-6 Gal''), 21.01, 20.90, 20.77, 20.74 (4x *C*H_3 acetyl). **HRMS** (m/z): $[M+Na]^+$ calcd for $C_{75}H_{82}O_{20}$, 1325.52971; found, 1325.52872

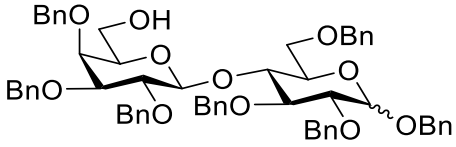
β-D-galactopyranosyl-(1→4)-β-D-galactopyranosyl-(1→4)-D-glucopyranose (14)



To a solution of **11** (6.9 g, 5.3 mmol) in methanol (53 mL), K_2CO_3 (73 mg, 0.53 mmol) was added. This reaction mixture was stirred for 16 hours and neutralized using Dowex H^+. The reaction mixture was filtered, concentrated *in vacuo*, and purified using column chromatography (70% EtOAc in *n*-heptane,

v/v%) %) yielding (5.4 g, 4.7 mmol, 90%) a white foam. The resulting white foam (5.31 g, 4.68 mmol) was hydrogenated with H_2 over Pd/C in MeOH (47 mL) for 5 days. The mixture was filtered over Celite and rinsed with MeOH and water. Next, activated charcoal was added to the mixture and filtered over Celite. The methanol was evaporated *in vacuo* and the resulting solution was freeze-dried to obtain 4'galactosyllactose (**14**) (2.18 g, 4.32 mmol, 92%) as a white solid. Alpha/beta mixture was obtained, alpha:beta ratio (4:6). R_f = 0.19 (30% H_2O in ACN, v/v%). **^{1}H NMR** (500 MHz, D_2O) δ 5.24 (d, *J* = 3.8 Hz, 0.4H, H-1 Glc alpha), 4.68 (d, *J* = 8.0 Hz, 0.6H, H-1 Glc beta), 4.62 (d, *J* = 7.8 Hz, 1H, H-1 Gal''), 4.50 (d, *J* = 7.8 Hz, 1H, H-1 Gal'), 4.21 (d, *J* = 3.3 Hz, 1H, H-4 Gal'), 4.00 – 3.57 (m, 17H), 3.30 (t, *J* = 8.5 Hz, 0.6H, H-2 Glc beta). **^{13}C NMR** (126 MHz, D_2O) δ 104.58 (C-1 Gal''), 103.28 (C-1 Gal'' beta), 103.25 (C-1 Gal' alpha), 96.13 (C-1 Glc beta), 92.19 (C-1 Glc alpha), 78.86 (C-4 Glc alpha), 78.74 (C-4 Glc beta), 77.53 (C-4 Gal'), 75.51 (C-5 Gal''), 75.18 (C-5 Glc beta), 74.88 (C-5 Gal'), 74.74 (C-3 Glc beta), 74.17 (C-2 Glc beta), 73.34 (C-3 Gal'), 73.19 (C-3 Gal''), 71.82 (C-2 Gal''), 71.79 (C-2 Gal'), 71.75 (C-3 Glc alpha), 71.50 (C-2 Glc alpha), 70.49 (C-5 alpha Glc), 68.99 (C-4 Gal''), 61.37 (C-6 Gal''), 61.12 (C-6 Gal'), 60.43 (C-1 Glc alpha), 60.30 (C-1 Glc beta). **HRMS** (m/z): $[M+Na]^+$ calcd for $C_{18}H_{32}O_{16}$, 527.15880; found, 527.15950

Benzyl 2,3,4-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-D-glucopyranose (8)



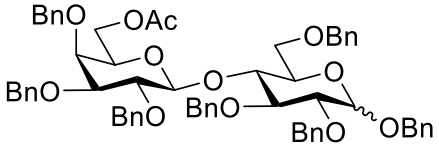
A solution of **6** (15.9 g, 16.4 mmol) in anhydrous DCM (590 mL) was added to a flame-dried round-bottom flask containing activated 4Å molecular sieves. The solution was stirred at rt under an atmosphere of argon for 30 min and was then cooled to -78 °C. Triethylsilane (3.66 mL, 22.9 mmol) and dichlorophenyl borane (3.0 mL, 22.9 mmol) were added and the reaction mixture was stirred at -78 °C. After 4 hours, TLC indicated the formation of the product and consumption of starting material. Triethylamine (23 mL) and methanol (7 mL) were added, and the reaction mixture was diluted with DCM, and washed with sat. $NaHCO_3$, dried over $MgSO_4$, filtered, and concentrated *in vacuo*. The crude product was purified by silica column chromatography (0-40% EtOAc in n-heptane, v/v%) to afford alcohol **8** (10.93 g, 11.2 mmol, 69 %) as a white foam. Alpha/beta mixture was obtained, mainly beta.

R_f [beta anomer] = 0.46 (40% EtOAc in *n*-heptane, v/v%). **^{1}H NMR** [beta anomer] (500 MHz, $CDCl_3$) δ 7.35 – 7.10 (m, 35H, C*H* Ar), 4.97 (d, *J* = 10.7 Hz, 1H, CH*H*Ph), 4.92 – 4.85 (m, 3H, 3x CH*H*Ph), 4.77 – 4.66 (m, 6H, 6x CH*H*Ph), 4.62 (d, *J* = 12.0 Hz, 1H, CH*H*Ph), 4.52 (dd, *J* = 11.8, 1.6 Hz, 2H, 2x CH*H*Ph), 4.45 (d, *J* = 7.7 Hz, 1H, H-1 Glc), 4.42 – 4.32 (m, 2H, H-1 Gal, CH*H*Ph), 3.87 (dd, *J* = 9.9, 8.8 Hz, 1H, H-4 Glc), 3.78 – 3.68 (m, 3H, H-6 Glc, H-2 Gal), 3.65 (dd, *J* = 2.9, 1.0 Hz, 1H, H-4 Gal), 3.54 – 3.48 (m, 2H, H-2 Glc, H-6a Gal), 3.43 (dd, *J* = 9.2, 7.7 Hz, 1H, H-2 Glc), 3.37 – 3.31 (m, 2H, H-5 Glc, H-3 Gal), 3.27 (dd, *J* = 11.5, 4.5 Hz, 1H, H-6b Gal), 3.17 – 3.11 (m, 1H, H-5 Gal).**^{13}C NMR** [beta anomer] (126 MHz, $CDCl_3$) δ 139.04, 138.88, 138.72, 138.68, 138.56, 138.49, 137.70, 128.57, 128.52, 128.49, 128.45, 128.42, 128.38, 128.30, 128.23, 128.20, 128.17, 128.03, 127.96, 127.87, 127.84, 127.81, 127.70, 127.65, 127.61, 127.48, 103.05 (C-1 Gal), 102.64 (C-1 Glc), 82.95 (C-3 Glc), 82.85 (C-3 Gal), 81.81 (C-2 Glc), 80.09 (C-2 Gal), 77.16 (C-4 Glc), 75.72 (*C*H_2Ph), 75.39 (*C*H_2Ph), 75.30 (C-5 Glc), 75.13 (C-5 Gal), 75.11 (*C*H_2Ph), 74.59 (*C*H_2Ph), 73.95 (C-4 Gal), 73.32 (*C*H_2Ph), 73.18 (*C*H_2Ph), 71.10 (*C*H_2Ph), 68.38 (C-6 Glc), 62.11 (C-6 Gal).

R_f [alpha anomer] = 0.41 (40% EtOAc in *n*-heptane, v/v%). **^{1}H NMR** [alpha anomer] (500 MHz, $CDCl_3$) δ 7.43 – 7.19 (m, 35H, C*H* Ar), 5.04 (d, *J* = 10.5 Hz, 1H, CH*H*Ph), 4.96 (d, *J* = 11.6 Hz, 1H, CH*H*Ph), 4.82 (d, *J* = 3.7 Hz, 1H, H-1 Glc), 4.80 – 4.67 (m, 7H, 7x CH*H*Ph), 4.59 – 4.52 (m, 4H, 4x CH*H*Ph), 4.33 (d, *J* = 12.1 Hz, 1H, CH*H*Ph), 4.27 (d, *J* = 7.8 Hz, 1H, H-1 Gal), 3.94 – 3.84 (m, 2H, H-3 Glc, H-4 Glc), 3.82 – 3.74 (m, 2H, H-6a Glc, H-2 Gal), 3.71 (ddd, *J* = 9.8, 3.2, 1.7 Hz, 1H, H-5 Glc), 3.67 (dd, *J* = 3.1, 1.0 Hz, 1H, H-4 Gal), 3.55 (dd, *J* = 11.4, 8.1 Hz, 1H, H-6a Gal), 3.48 (dd, *J* = 9.2, 3.7 Hz, 1H, H-2 Glc), 3.44 (dd, *J* = 10.7, 1.9 Hz, 1H, H-6b Glc), 3.33 – 3.27 (m, 2H, H-3 Gal, H-6b Gal), 3.17 – 3.11 (m, 1H, H-5 Gal).**^{13}C NMR** (126 MHz, $CDCl_3$) δ 139.33, 138.96, 138.74, 138.61, 138.52, 138.21, 137.45, 128.60, 128.56, 128.52, 128.51, 128.49, 128.47, 128.45, 128.31, 128.26, 128.22, 128.18, 128.10, 128.09, 127.98, 127.95, 127.86, 127.80, 127.78, 127.69, 127.61, 127.55, 127.44, 103.08 (C-1 Gal), 95.95 (C-1 Glc), 82.78 (C-3 Gal), 80.31 (C-3 Glc), 80.09 (C-2 Glc), 79.11 (C-2 Gal), 77.16 (C-4 Glc), 75.78 (*C*H_2Ph), 75.32 (*C*H_2Ph), 75.11 (C-5 Gal), 74.59 (*C*H_2Ph), 74.05 (C-4 Gal), 73.43 (*C*H_2Ph), 73.32 (*C*H_2Ph), 73.15 (*C*H_2Ph), 70.58 (C-5 Glc), 69.33 (*C*H_2Ph), 67.98 (C-6 Glc), 62.14 (C-6 Gal).

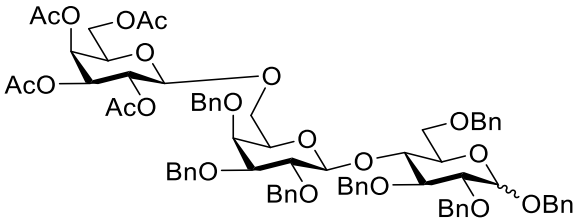
HRMS (m/z): [M+Na]+ calcd for $C_{61}H_{64}O_{11}$, 995.43408; found, 995.43463

Benzyl 4-*O*-acetyl-2,3,6-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-D-glucopyranose (19)



8 (20 mg, 21 µmol) was dissolved in pyridine (1 mL) and acetic anhydride (0.5 mL, 5 mmol) was slowly added and stirred for 3.5 hours. The reaction mixture was diluted with EtOAc (5 mL), washed with 1M HCl solution (3x 5 mL), $NaHCO_3$ (3x 5 mL), brine (1x 5 mL), dried over $MgSO_4$ and filtered. The resulting mixture was concentrated *in vacuo* yielding a colourless oil (18 mg, 18 µmol, 86%). TLC: R_f: 0.51. Spectral data showed a downfield shift for the H'-6a and H'-6b (**Figure S45-47**).

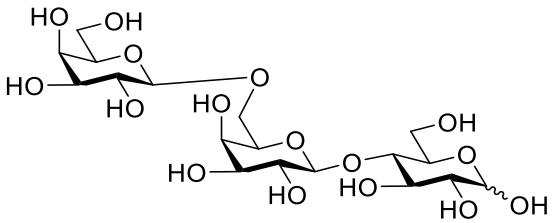
Benzyl 2,3,4,6-tetra-*O*-acetyl- β-D-galactopyranosyl-(1→6)-2,3,4-tri-*O*-benzyl-β-D-galactopyranosyl-(1→4)- 2,3,6-tri-*O*-benzyl-D-glucopyranose (12)



To a solution of the acceptor **8** (10.92 g, 11.22 mmol) and the thioglycosyl donor **9** (5.61 g, 12.35 mmol) in DCM (112 mL), molecular sieves (4Å) were added under a nitrogen atmosphere. The reaction stirred for 15 minutes and was cooled to 0°C. The thioglycoside was activated by the addition of NIS (3.28 g, 14.59 mmol) and triflic acid (199 µL) The reaction was stirred for 15 minutes and quenched by the addition of triethylamine. The mixture was diluted by the addition of DCM, filtered, and washed with 10% aqueous thiosulfate solution, sat. $NaHCO_3$ and brine. The organic layer was dried ($MgSO_4$), filtered, and purified by silica column chromatography (0-30% EtOAc in *n*-heptane, v/v%) affording **12** (7.65 g, 5.87 mmol, 52 %). Alpha/beta mixture was obtained, mainly beta. R_f = 0.43 (40% EtOAc in *n*-heptane, v/v%)**. ^{1}H NMR** [beta anomer] (500 MHz, $CDCl_3$) δ 7.43 – 7.02 (m, 35H, C*H* Ar), 5.17 (dd, *J* =

3.4, 1.2 Hz, 1H, H-4 Gal''), 5.11 (dd, *J* = 10.5, 8.0 Hz, 1H, H-2 Gal''), 5.03 (m, 2H, 2x CH*H*Ph), 4.95 (m, 2H, 2x CH*H*Ph), 4.89 – 4.81 (m, 4H, H-3 Gal'', 3x CH*H*Ph), 4.77 – 4.73 (m, 2H, 2x CH*H*Ph), 4.71 – 4.63 (m, 3H, H-1 Gal'', 2x CH*H*Ph), 4.59 (d, *J* = 11.2 Hz, 1H, CH*H*Ph), 4.55 – 4.49 (m, 2H, H-1 Glc, CH*H*Ph), 4.45 – 4.39 (m, 2H. H-1 Gal', CH*H*Ph), 4.08 (dd, *J* = 10.0, 8.8 Hz, 1H, H-4 Glc), 3.89 (dd, *J* = 10.8, 8.3 Hz, 1H, H-6a Gal''), 3.84 – 3.78 (m, 3H, H-2 Gal', H-6a Glc, H-6a Gal'), 3.74 – 3.70 (m, 2H, H-4 Gal', H-6b Glc), 3.67 – 3.61 (m, 2H, H-6b Gal', H-6b Gal''), 3.57 – 3.48 (m, 2H, H-2 Glc, H-3 Glc), 3.40 (dd, *J* = 9.7, 3.0 Hz, 1H, H-3 Gal'), 3.35 (m, 2H, H-5 Glc, H-5 Gal'), 2.77 – 2.70 (m, 1H, H-5 Gal''), 2.07 (s, 3H, C*H*$_3$ acetyl), 2.02 (s, 3H, C*H*$_3$ acetyl), 1.98 (s, 3H, C*H*$_3$ acetyl), 1.92 (s, 3H, C*H*$_3$ acetyl). **^{13}C NMR** [beta anomer] (101 MHz, CDCl3) δ 170.40, 170.31, 170.23, 169.90 (4x *C*(O)CH$_3$), 139.35, 139.17, 138.84, 138.66, 138.51, 138.21, 137.82, 128.73, 128.68, 128.57, 128.54, 128.51, 128.49, 128.48, 128.41, 128.40, 128.39, 128.33, 128.32, 128.18, 128.15, 128.10, 128.05, 128.04, 128.02, 127.96, 127.90, 127.89, 127.81, 127.80, 127.73, 127.65, 127.60, 127.57, 127.50, 103.00 (C-1 Glc), 102.85 (C-1 Gal'), 100.66 (C-1 Gal''), 84.23 (C-3 Glc), 82.65 (C-3 Gal'), 82.27 (C-2 Glc), 79.88 (C-2 Gal'), 76.49 (*C*H$_2$Ph), 76.04 (C-4 Glc), 75.37 (*C*H$_2$Ph), 75.20 (*C*H$_2$Ph), 75.07 (C-5 Glc/C-5 Gal'), 74.95 (*C*H$_2$Ph), 74.74, (C-5 Glc/C-5 Gal') 74.36 (C-4 Gal'), 73.21 (*C*H$_2$Ph), 73.10 (*C*H$_2$Ph), 71.32 (C-3 Gal''), 70.95 (*C*H$_2$Ph), 69.46 (C-5 Gal''), 69.27 (C-2 Gal''), 68.20 (C-6 Glc), 67.65 (C-6 Gal'), 67.10 (C-4 Gal''), 60.81 (C-6 Gal''), 21.27, 20.82, 20.78, 20.71 (4x *C*H$_3$ acetyl). **HRMS** (m/z): [M+Na]+ calcd for $C_{75}H_{82}O_{16}$ 1325.52917; found, 1325.52971

β-D-galactopyranosyl-(1→6)- β-D-galactopyranosyl-(1→4)-D-glucopyranose (15)



To a solution of **12** (8.0 g, 6.1 mmol) in methanol (61 mL), K_2CO_3 (42 mg, 0.31 mmol) was added. This reaction mixture was stirred for 24 hours and neutralized using Dowex H^+. The reaction mixture was filtered, concentrated *in vacuo*, and purified using column chromatography (0-80% EtOAc in n-heptane, v/v%) yielding a white foam (5.5 g, 4.8 mmol, 79%). The resulting product was hydrogenated with H_2 over Pd/C in MeOH/H_2O (100 mL, 1:1, v/v) for 8 days. The mixture was filtered over Celite and rinsed with MeOH and water. The methanol was evaporated *in vacuo* and the resulting solution was freeze-dried to obtain 6'galactosyllactose (**15**) (2.25 g, 4.46 mmol, 96%) as a white solid. Alpha/beta mixture was obtained, alpha:beta ratio (4:6). R_f = 0.19 (30% H_2O in ACN, v/v%). **^{1}H NMR** (500 MHz, D_2O) δ 5.25 (d, *J* = 3.8 Hz, 0.36H, H-1 Glc alpha), 4.69 (d, *J* = 8.0 Hz, 0.63H, H-1 Glc beta), 4.52 – 4.46 (m, 2H. H-1 Gal' and Gal''), 4.10 (ddd, *J* = 9.5, 4.0, 2.3 Hz, 1H, H-6 Gal'), 4.00 – 3.54 (m, 16.45H), 3.34 – 3.29 (m, 0.59H, H-2 Glc beta). **^{13}C NMR** (126 MHz, D_2O) δ 104.05 (C-1 Gal''), 103.85 (C-1 Gal' beta), 103.82 (C-1 Gal' alpha), 96.41 (C-1 Glc beta), 92.53 (C-1 Glc alpha), 80.16 (C-4 Glc alpha), 79.90 (C-4 Glc beta), 75.82 (C-5 Gal''), 75.40 (C-5 Glc beta), 75.25 (C-3 Glc beta), 74.71 (C-5 Gal'), 74.46 (C-2 Glc beta), 73.33 (C-3 Gal' alpha/beta), 73.31 (C-3 Gal' alpha/beta), 73.15 (C-3 Gal''), 72.34 (C-3 Glc alpha), 71.78 (C-2 Glc alpha), 71.55 (C-2 Gal' alpha and beta), 71.51 (C-2 Gal''), 70.67 (C-5 Glc alpha), 69.78 (C-6 Gal' beta), 69.75 (C-6 Gal' alpha), 69.34 (C-4 Gal' alpha and beta), 69.18 (C-4 Gal''), 61.71 (C-6 Gal''), 60.88 (C-6 Glc beta), 60.73 (C-6 Glc alpha). **HRMS (m/z)**: [M+Na]+ calcd for $C_{18}H_{32}O_{16}$, 527.15826; found 527.15880

Purity checks of 3'-, 4'- and 6'-GL

The identification of 3'-, 4'- and 6'-GL was based on the standards purchased on Carbosynth. The GLs (25 µL, 5 ppm in Milli-Q) were injected into a CarboPac PA1250 x 4 mm anion-exchange column, equipped with CarboPac PA1: 50 x 4 mm pre-column. The temperature was set at 30°C and the flow rate was set at 1.0 mL/min of 150 mM NaOH in Milli-Q. The analyses were ran for 30 minutes and a PAD with an AU-electrode was used as detector. The purity of the GLs was based on relative peak percentage.

Table 1: Purity of 3'-, 4'- and 6'-GL

Compound	Purity
3'-GL	95.36%
4'-GL	94.96%
6'-GL	98.1%

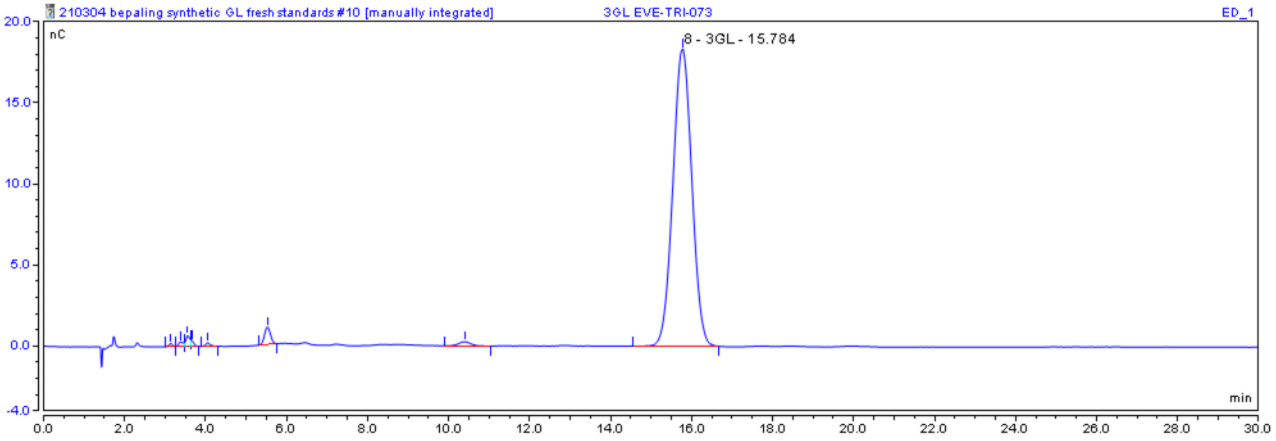


Figure S2 Purity check of 3'-GL

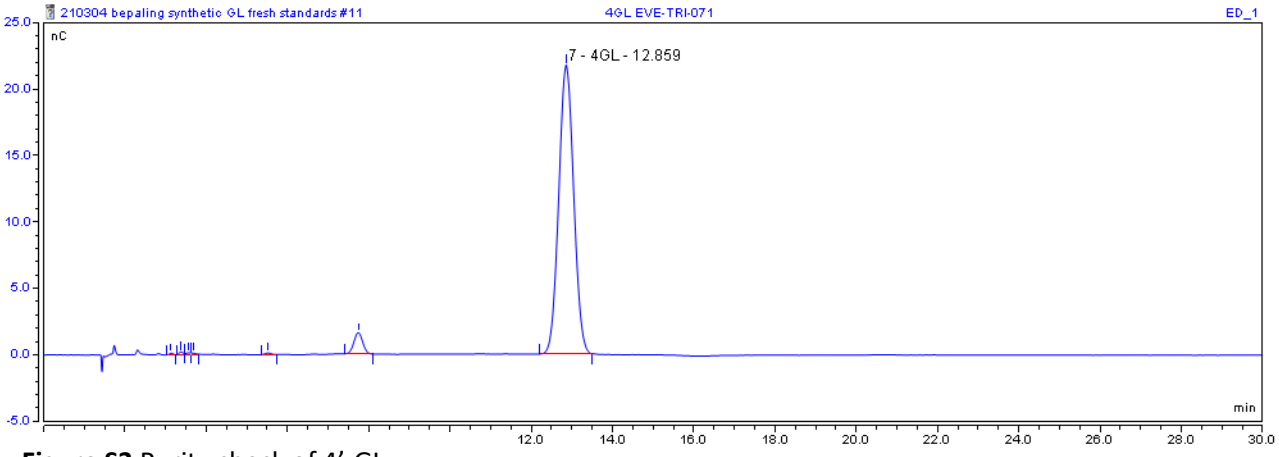


Figure S3 Purity check of 4'-GL

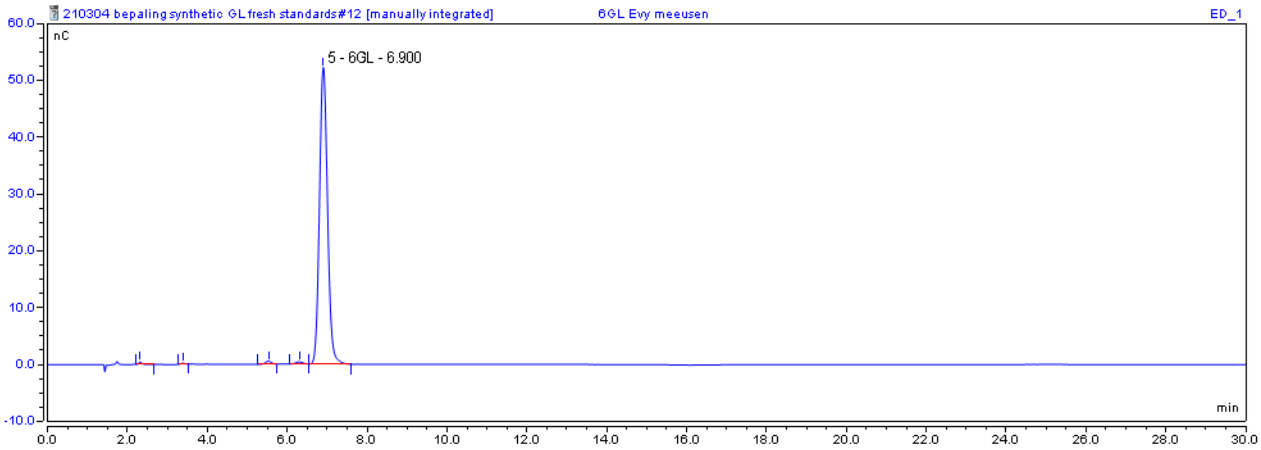


Figure S4 Purity check of 6'-GL

General methods *in vitro* studies human infant intestinal microbiota

i-screen.

To determine the effect of the galactosyllactoses on healthy human infant intestinal microbiota, the TNO i-screen model was used [1]. The i-screen is an *in vitro* system that allows the anaerobic cultivation of microorganisms obtained from faecal material, suitable for testing the effects of multiple components in small volumes. For the experiment, the i-screen system was inoculated with two different starting microbiota: A pool consisting of faecal material from 3 breastfed or from 3 formula-fed infants between 3 and 6 months of age. The samples were collected once and anonymously and are therefore considered not subject to "Medical Research Involving Human Subjects Act (WMO)" in The Netherlands. Before starting the i-screen incubations with the GLs, the two faecal microbiota pools were pre-incubated for 4 hours in standard ileal effluent medium (SIEM)[2] under anaerobic conditions, at 37°C and with shaking at 300 rpm. The microbiota was transferred to microtiter plates and the GLs were added at a concentration of 4 mg/ml. An untreated control (only microbiota) was taken along. All experimental conditions, including the controls, were run in triplicate. After 18 hours of fermentation under anaerobic conditions at 37°C, samples were collected and used for DNA isolation and SCFA analysis.

Total DNA was extracted from the samples using an Agowa/PurePrep protocol. To each 150 µl sample, 500 µl zirconium beads (0.1 mm) and 800 µl CD1 solution (DNeasy 96 Powersoil Pro QIAcube HT kit) were added. Cells were disrupted by bead beating twice for 2 min, with cooling on ice in between and afterwards. After centrifugation for 6 min at 3,000 RPM, 350 µl supernatant was mixed with 300 µl Agowa binding buffer and 10 µl Agowa magnetic beads. Samples were further purified using the PurePrep 96 system (Molgen, The Netherlands) with two wash steps and a final elution step in 65 µl.

Libraries for whole-genome sequencing were prepared using the Illumina DNA prep protocol according to the instructions of Illumina (Illumina DNA Prep Reference Guide). DNA concentrations were standardized across samples. After the tagmentation and clean-up steps, PCR-mediated standard indexed i5 and i7 adapters were added and the library was amplified. Next, the libraries were cleaned-up and pooled. Whole-genome sequencing was performed using the Illumina MiSeq sequencer applying MiSeq V3 chemistry. Sequence pre-processing (host filtering) and analysis (mapping, merging of de paired-end reads, classification and normalization) were performed using the MetaPhlAn software package release 3 (https://elifesciences.org/articles/65088) with the ChocoPhlAn database release 3 (clade-specific marker genes) for taxonomic classification[3]. The

classification was performed at the species level. Multi-Dimensional Scaling (MDS) and PERMANOVA analysis were applied to microbiome data transformed using
the Wisconsin double standardization. Differentially expressed species between treatments were determined by DESeq2 [4].

qPCR analysis

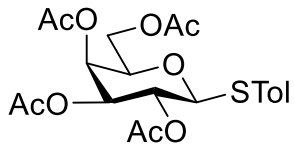
To discriminate *Bifidobacterium longum* subsp *longum* from *Bifidobacterium longum* subsp *infantis*, qPCR was performed on samples. In short, DNA was amplified in the following conditions: Maxima Master Mix, Forward primer 0.4 µM, Reverse primer 0.4 µM, Probe 0.2 µM and DNA template. Amplification was performed on QuantStudio 5 Real-Time PCR System starting with a single incubation of 2 min at 50°C, followed by a single incubation of 10 min at 95°C, followed by 40 cycles of 15 sec at 95°C and 1 min 60°C. The following primers were used for amplification:

Bifidobacterium longum subsp *longum*	forward	5'-TGG AAG ACG TCG TTG GCT TT-3'
	reverse	5'- ATC GCG CCA GGC AAA A-3'
	probe	5'- CGC ACC CAC CGC A -3'
Bifidobacterium longum subsp *infantis*	forward	5'-CGC GAG CAA AAC AAT GGT T-3'
	reverse	5'- AAC GAT CGA AAC GAA CAA TAG AGT T-3'
	probe	5'-TTC GAA ATC AAC AGC AAA A-3'

SCFA analysis
Sample derivatization: Faecal samples were diluted 20x in 75% methanol. 50 µl of internal standard was added to 50 µl of faecal material followed by 50 µl of 50 mM 3-Nitrophenylhydrazine, 50 µl of 50 mM 1-Ethyl-3-(3-dimethylamino-propyl)carbodiimide and 50 µl of pyridine (7.5% in 75% methanol). Samples were incubated for 30 min at 600 rpm at room temperature. Then 250 of 2% formic acid was added and mixed. Samples were then stored at -80°C until analysis.

LC-MS/MS analysis: The derivatized samples were analyzed by LC-MS by reversed phase chromatography using a Waters BEH C18 column (100 x 2.1 mm i.d.) (Waters, USA), a Waters Acquity H-Class UPLC system (Waters, USA), and a Thermo Q-Exactive high resolution mass spectrometer (ThermoFisher, USA). A linear regression model was constructed from the calibration standard data. SCFA concentrations in the samples were calculated by applying the regression model to the sample data. Statistical analysis was based on Nonparametric pairwise multiple comparisons in independent groups using Dunn's test

NMR spectra



9

Figure S5 ^{1}H-NMR spectrum of **9**

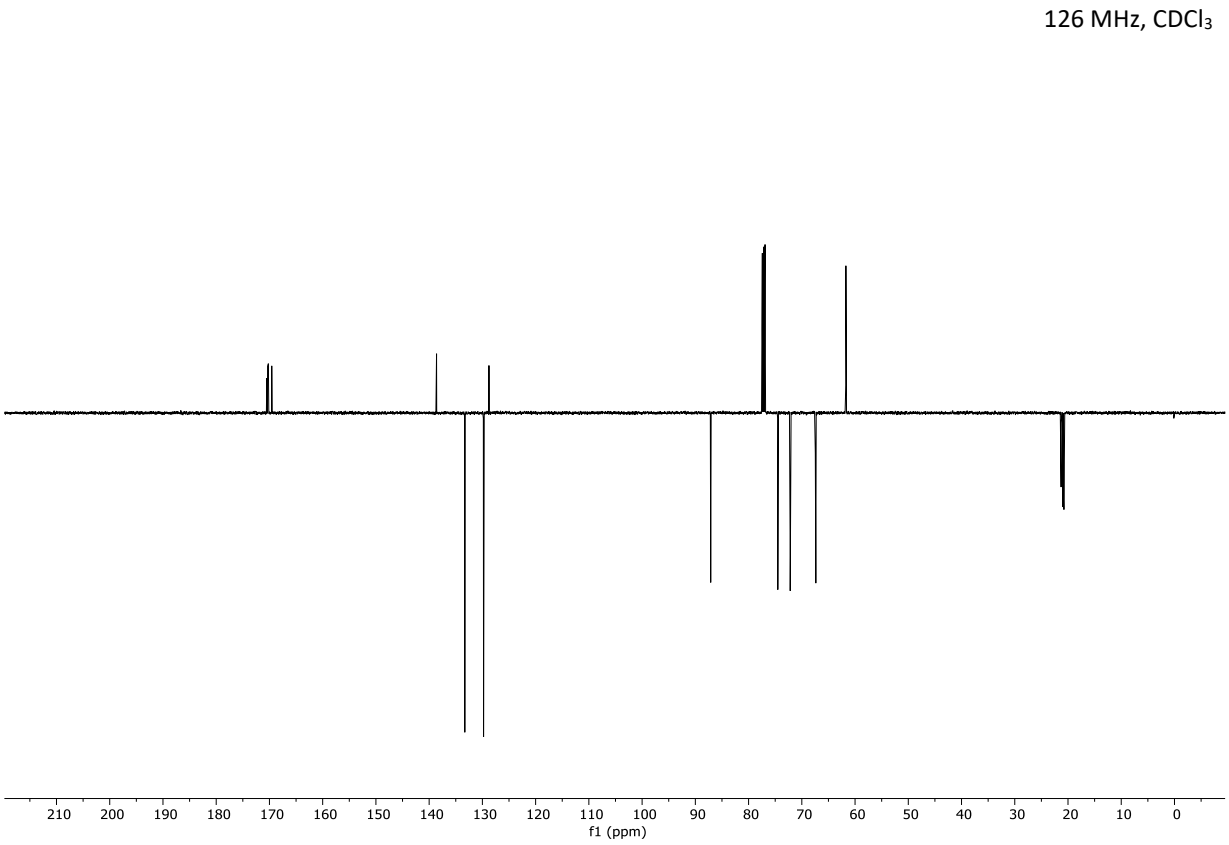


Figure S6 ^{13}C-NMR spectrum of **9**

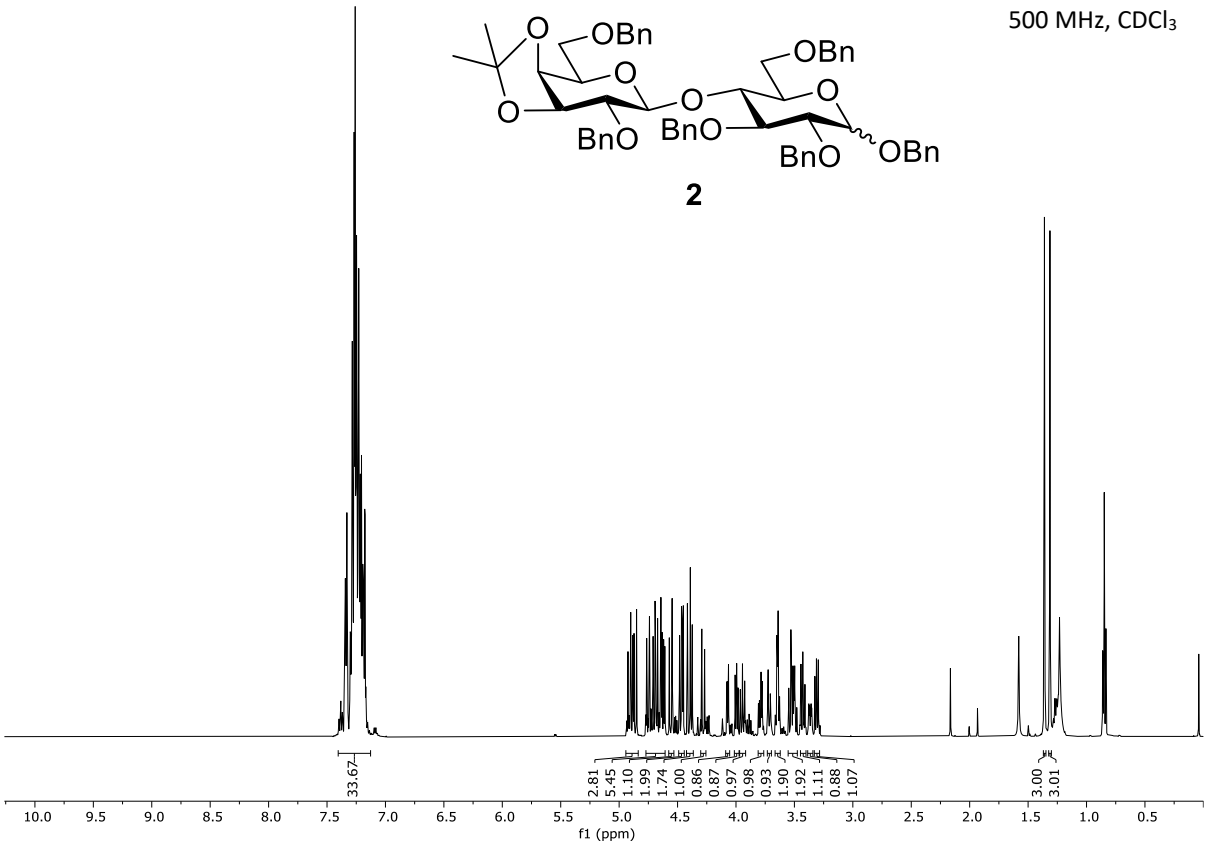


Figure S7 ^{1}H-NMR spectrum of **2**

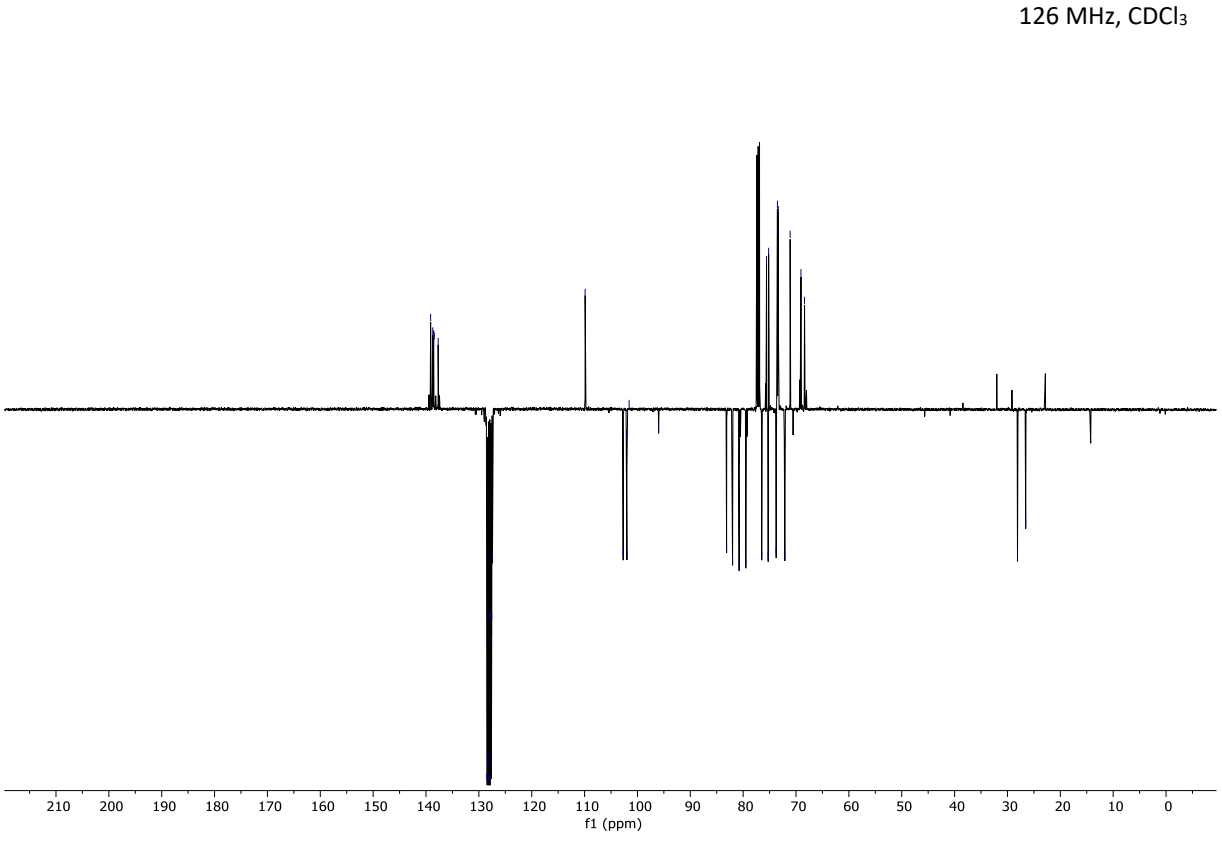


Figure S8 ^{13}C-NMR spectrum of **2**

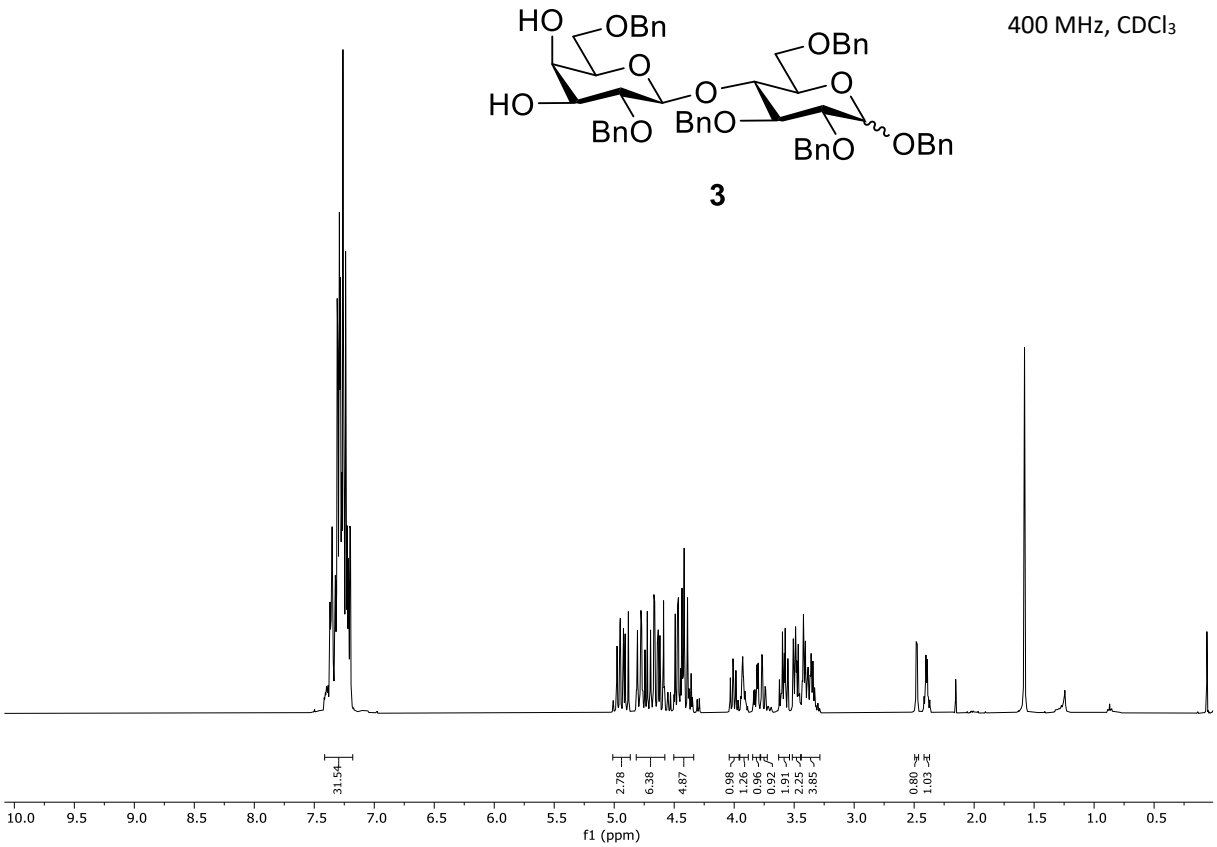


Figure S9 ^{1}H-NMR spectrum of **3**

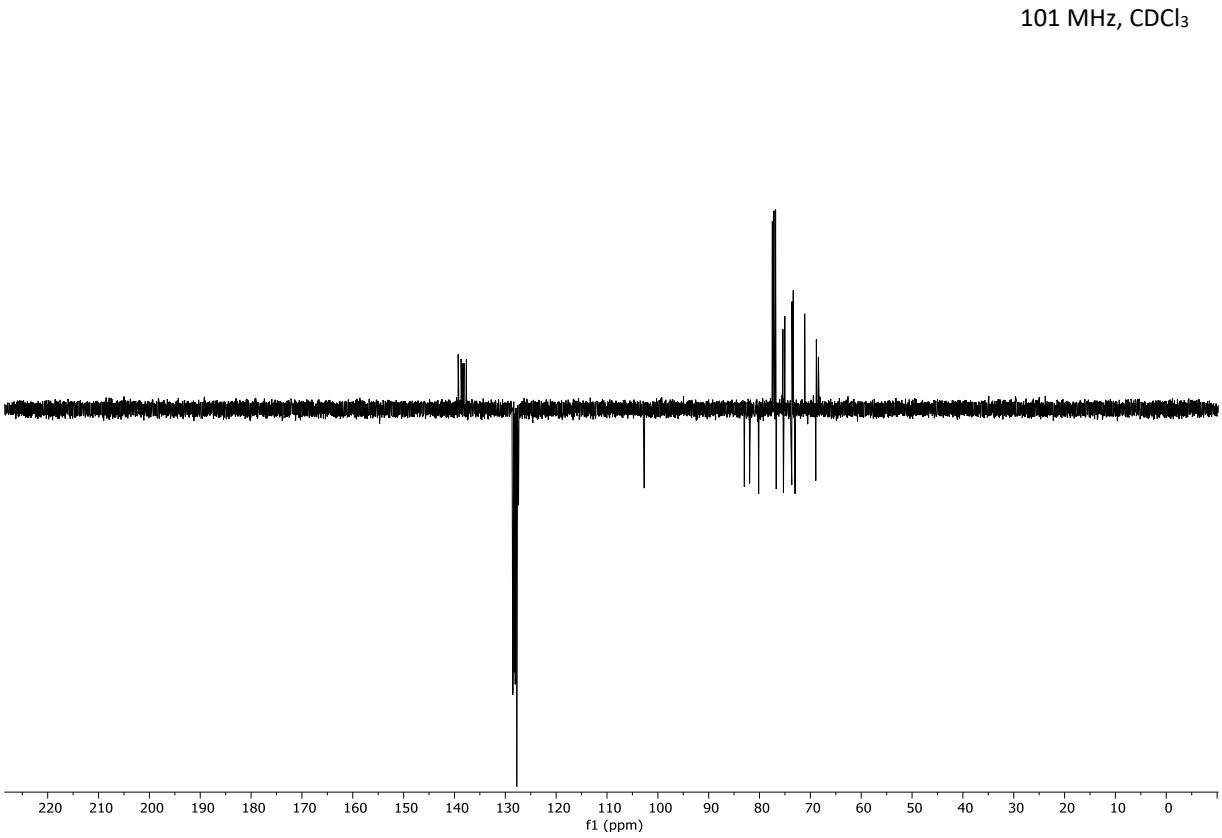


Figure S10 ^{13}C-NMR spectrum of **3**

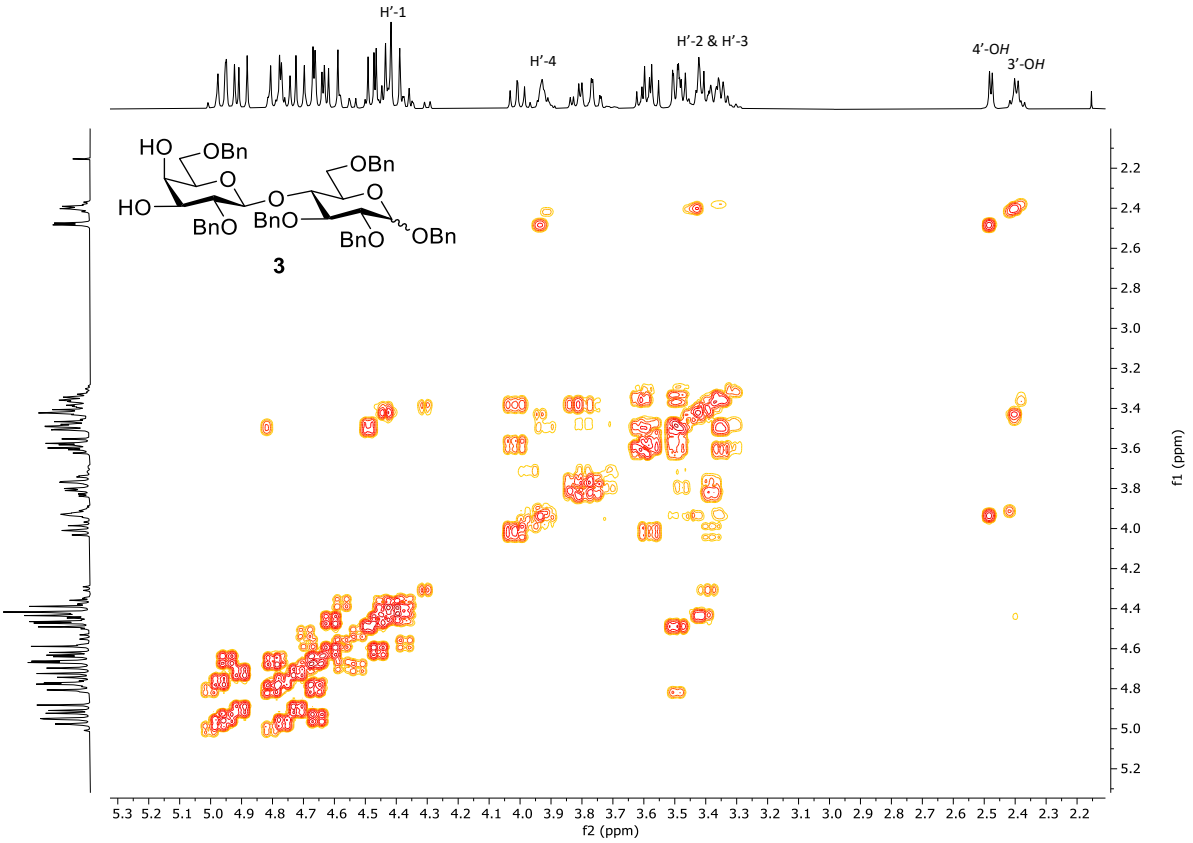


Figure S11 COSY spectrum of **3**

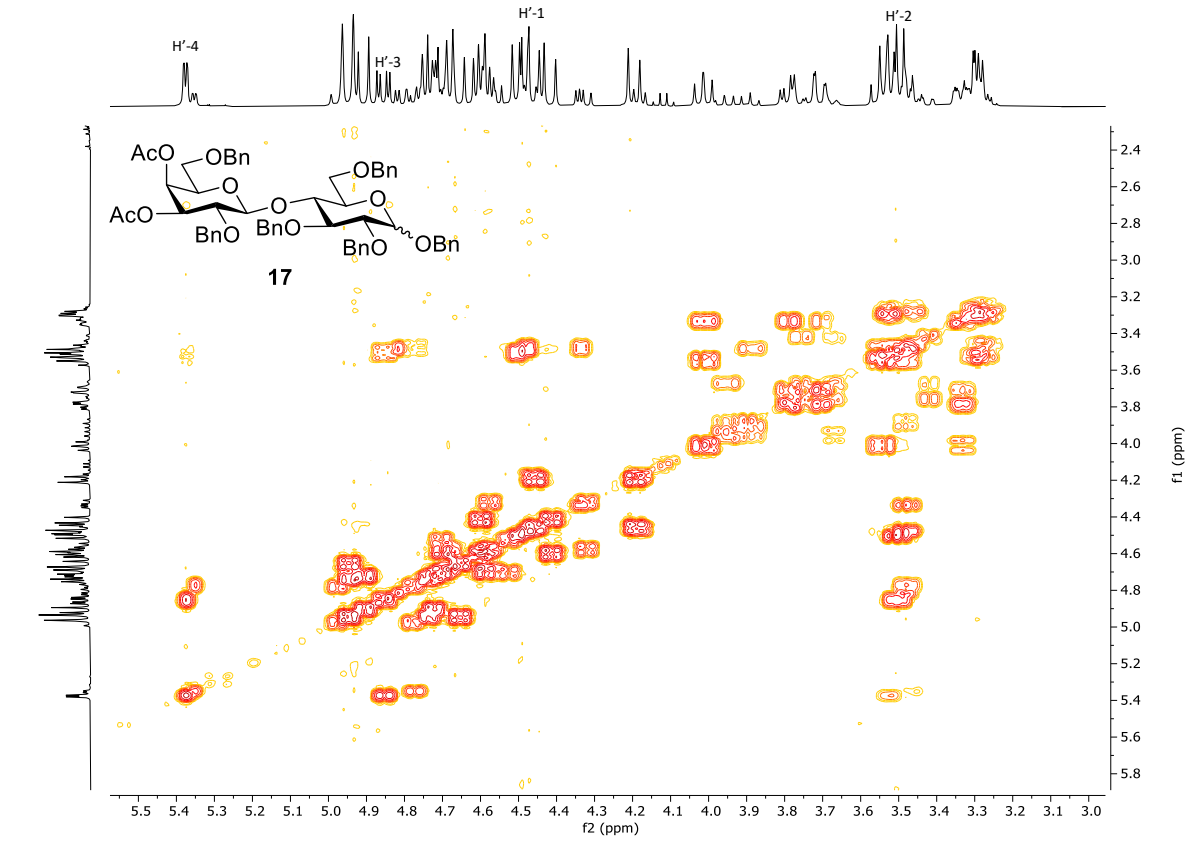


Figure S12 COSY spectrum of **17**

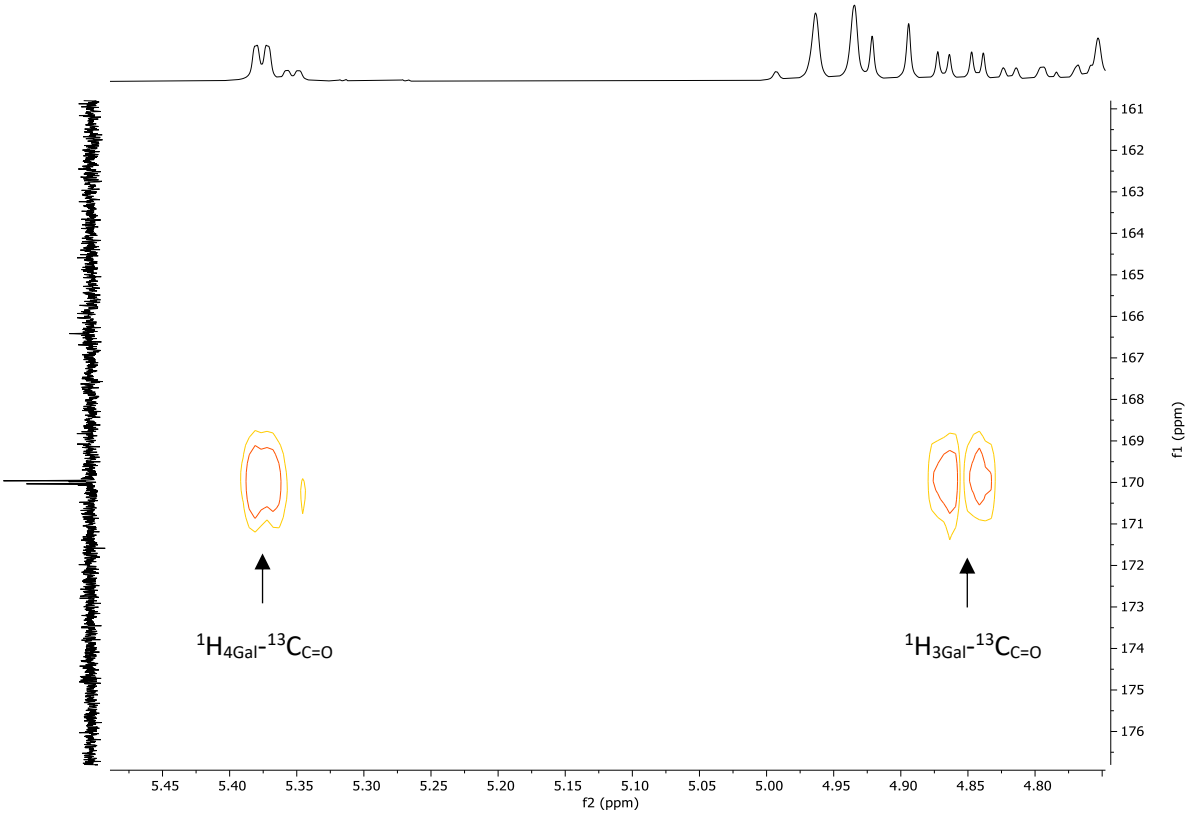


Figure S13 HMBC spectrum of **17**

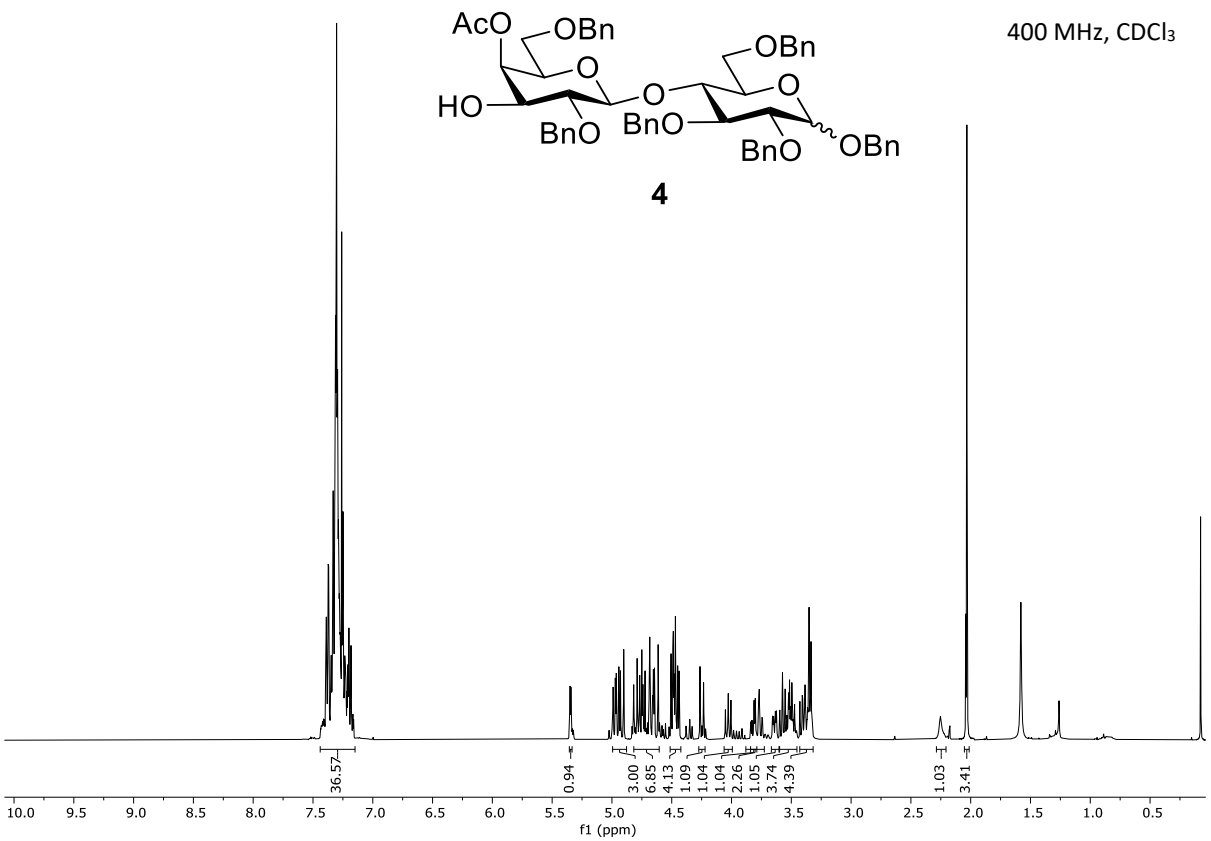


Figure S14 ^{1}H-NMR spectrum of **4**

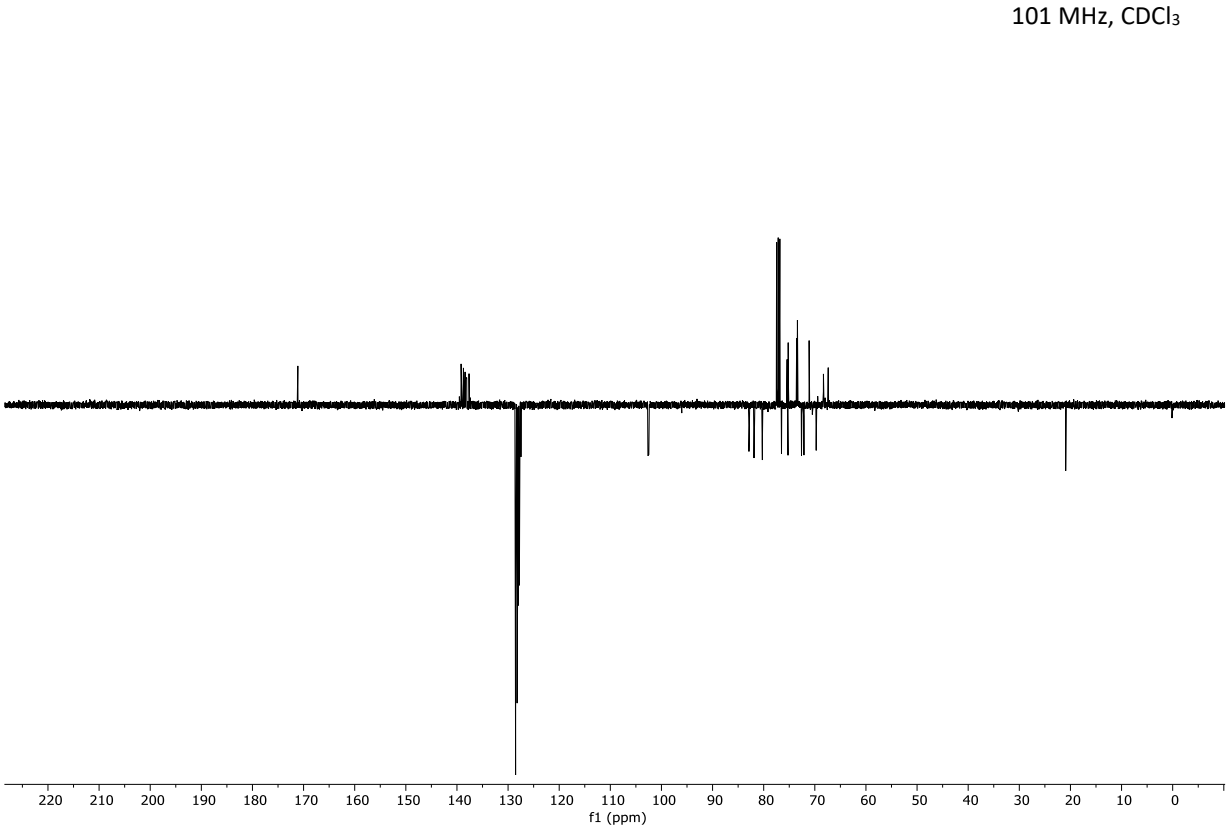


Figure S15 ^{13}C-NMR spectrum of **4**

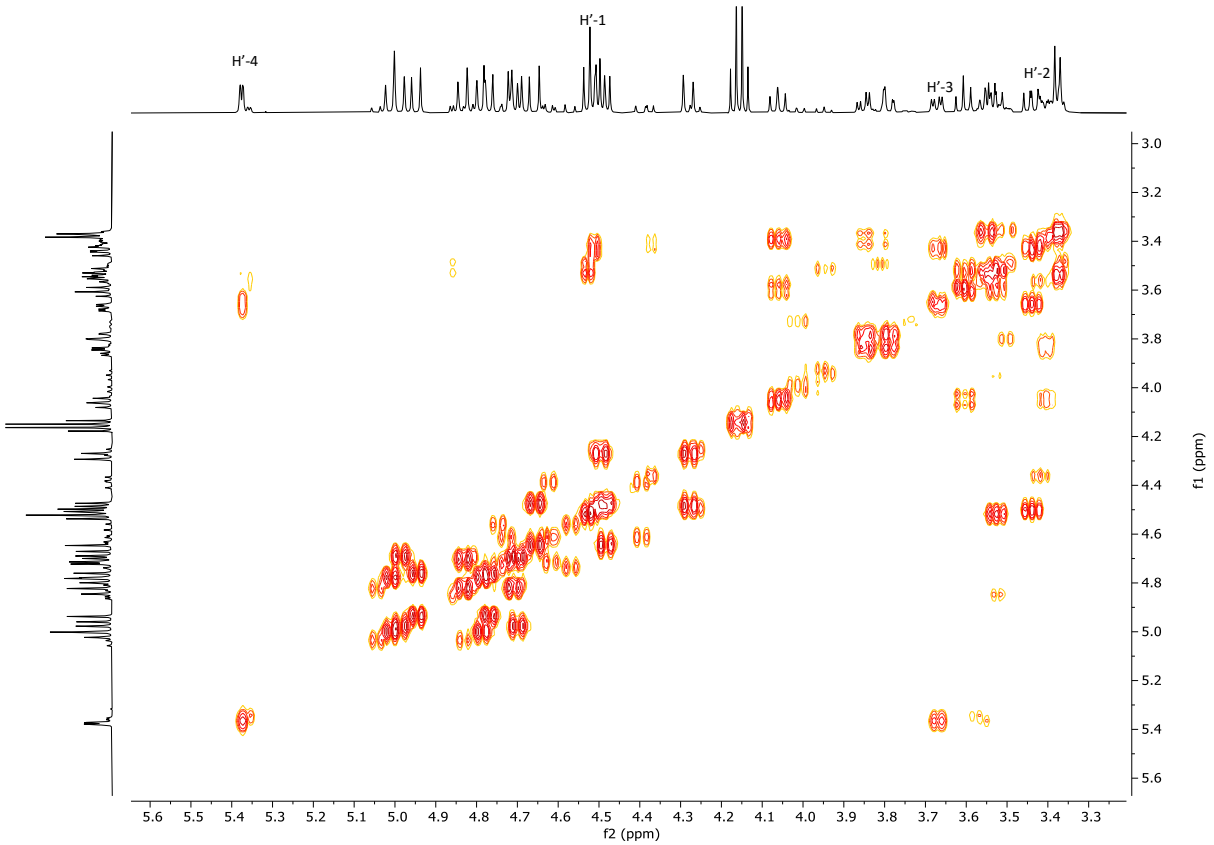


Figure S16 COSY spectrum of **4**

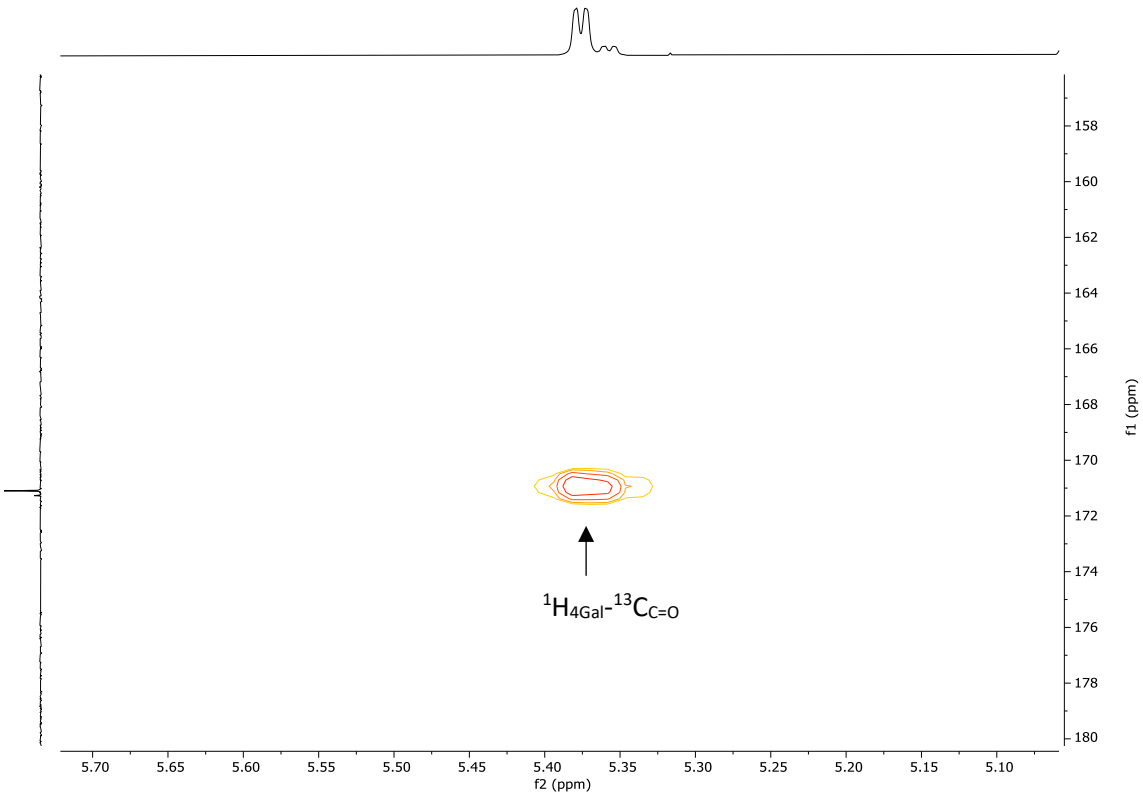


Figure S17 HMBC spectrum of **4**

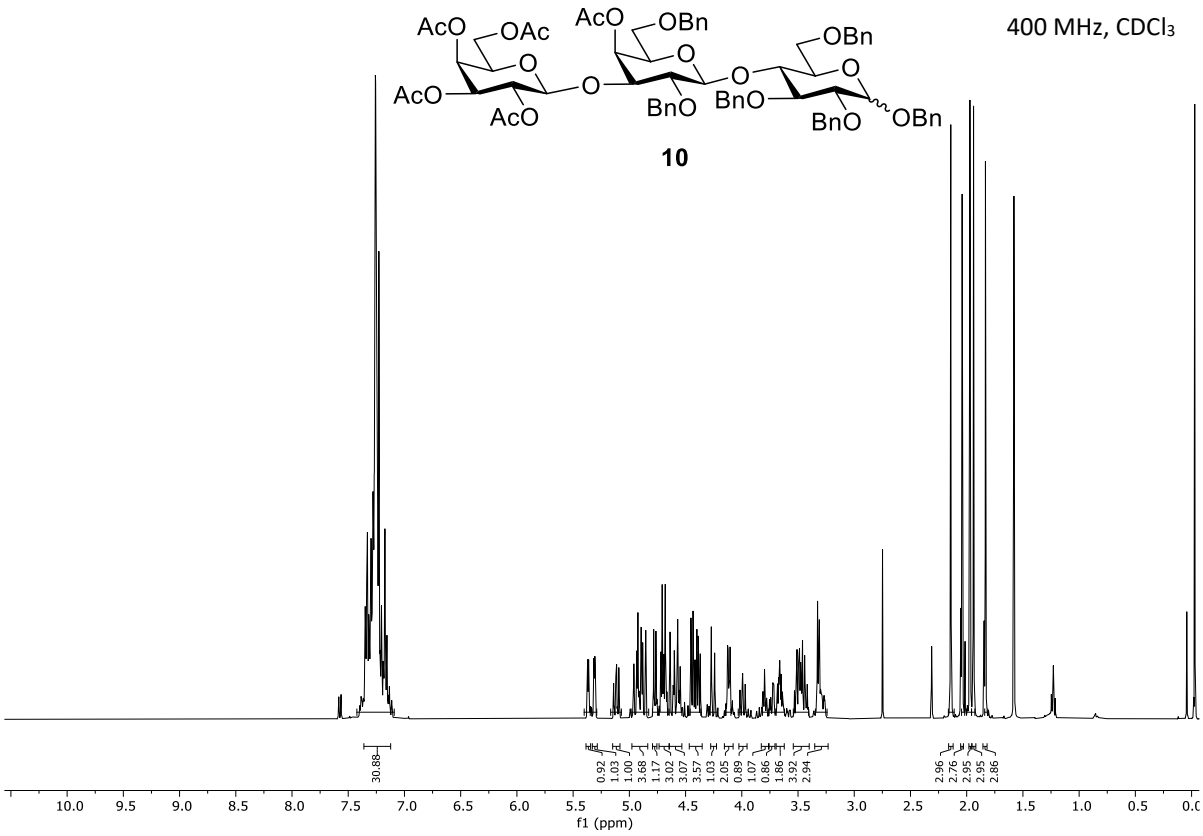


Figure S18 ^{1}H-NMR spectrum of **10**

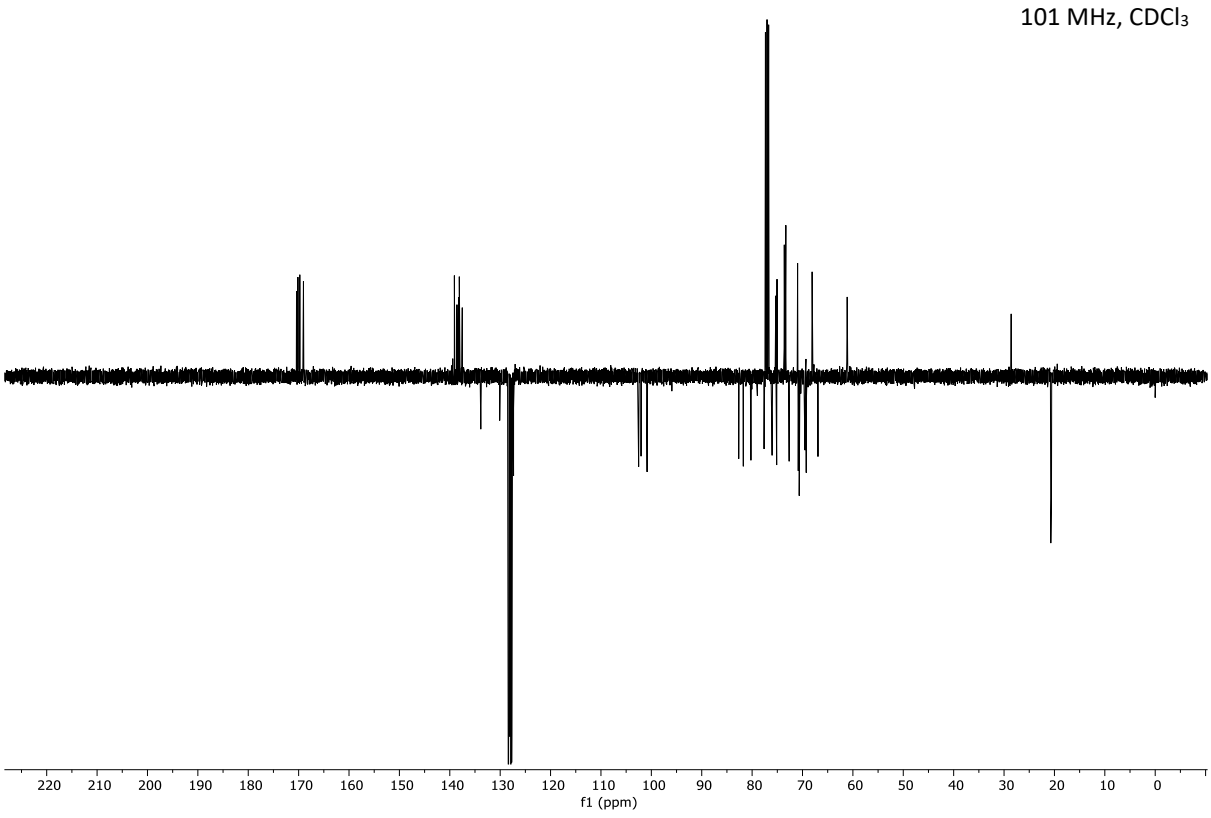


Figure S19 ^{13}C-NMR spectrum of **10**

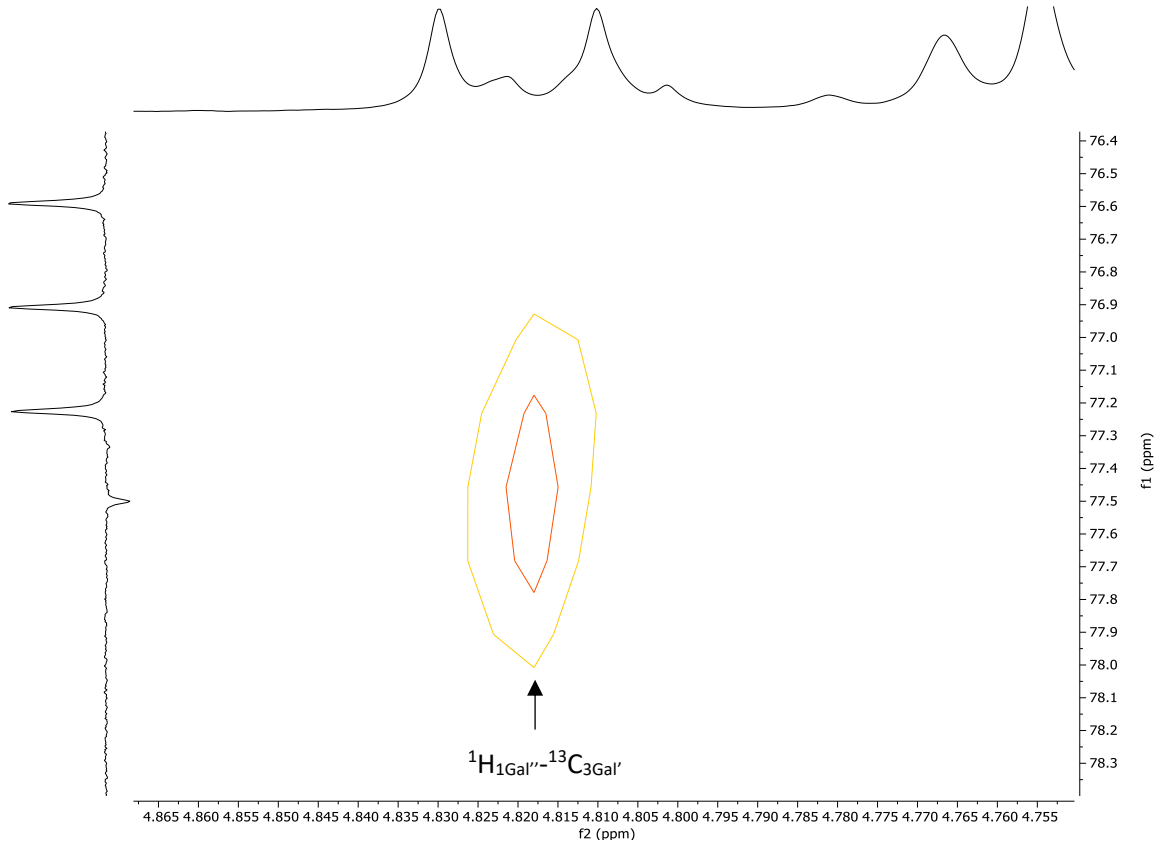


Figure S20 $^{1}H_{1Gal''}$-$^{13}C_{3Gal'}$ HMBC spectrum of **10**

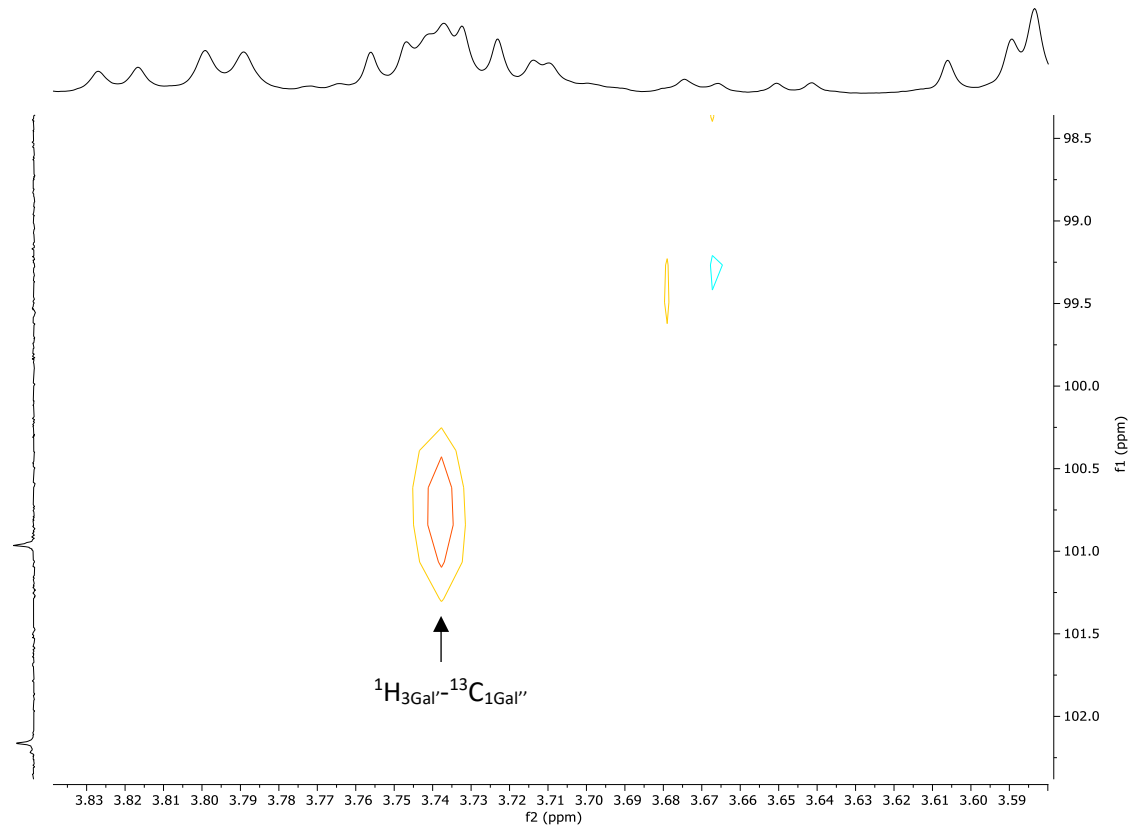


Figure S21 $^{1}H_{3Gal'}$-$^{13}C_{1Gal''}$ HMBC spectrum of **10**

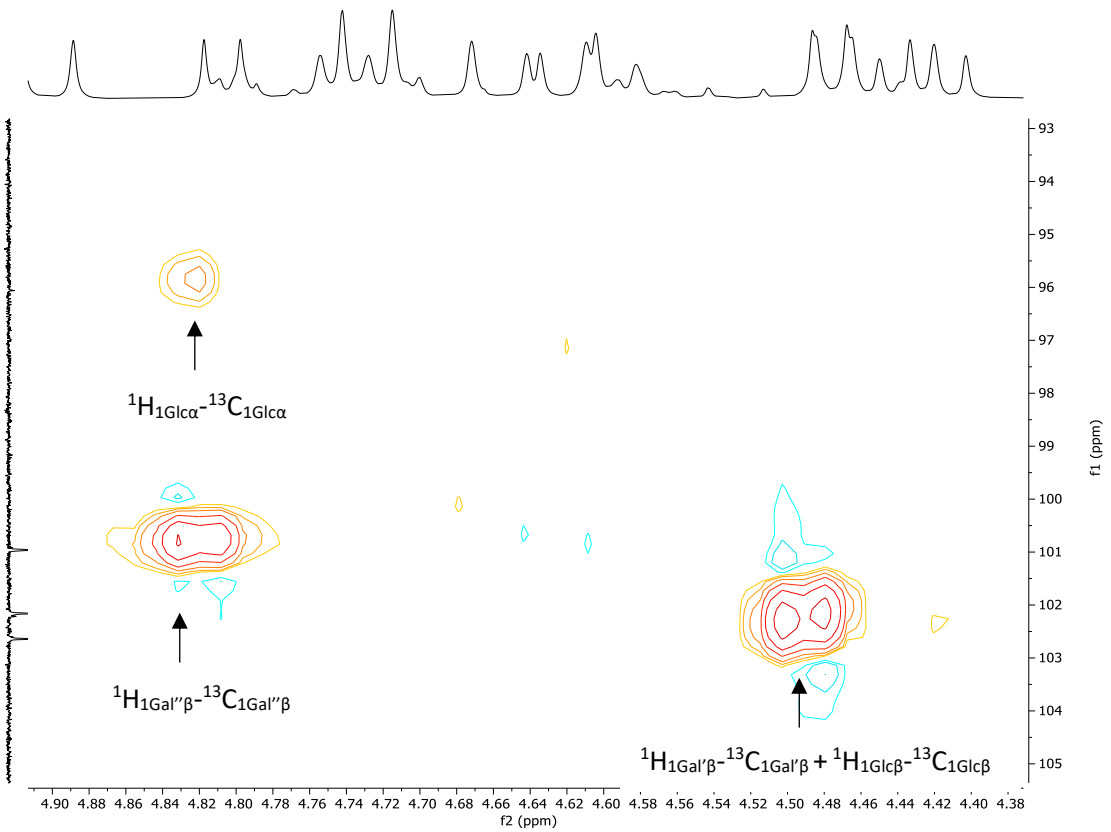


Figure S22 HSQC spectrum of **10**

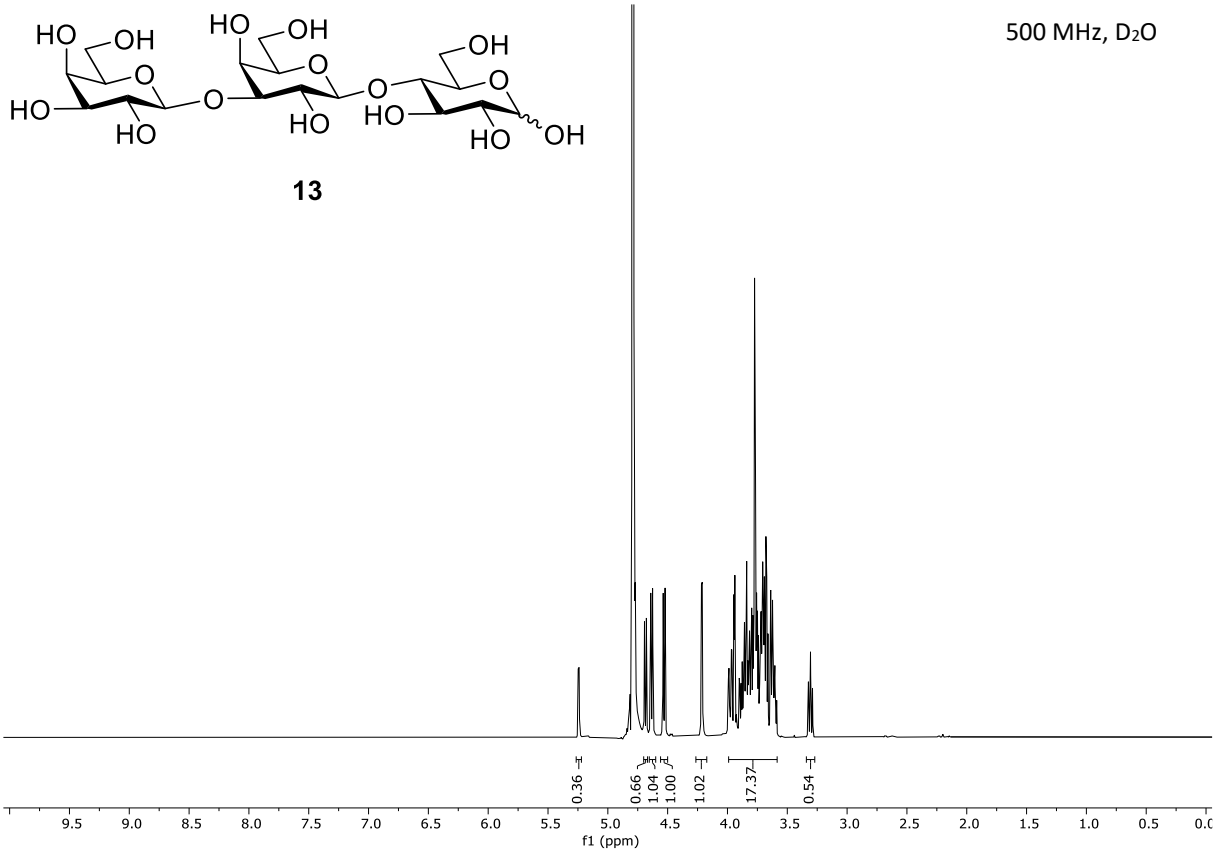


Figure S23 ^{1}H-NMR spectrum of **13**

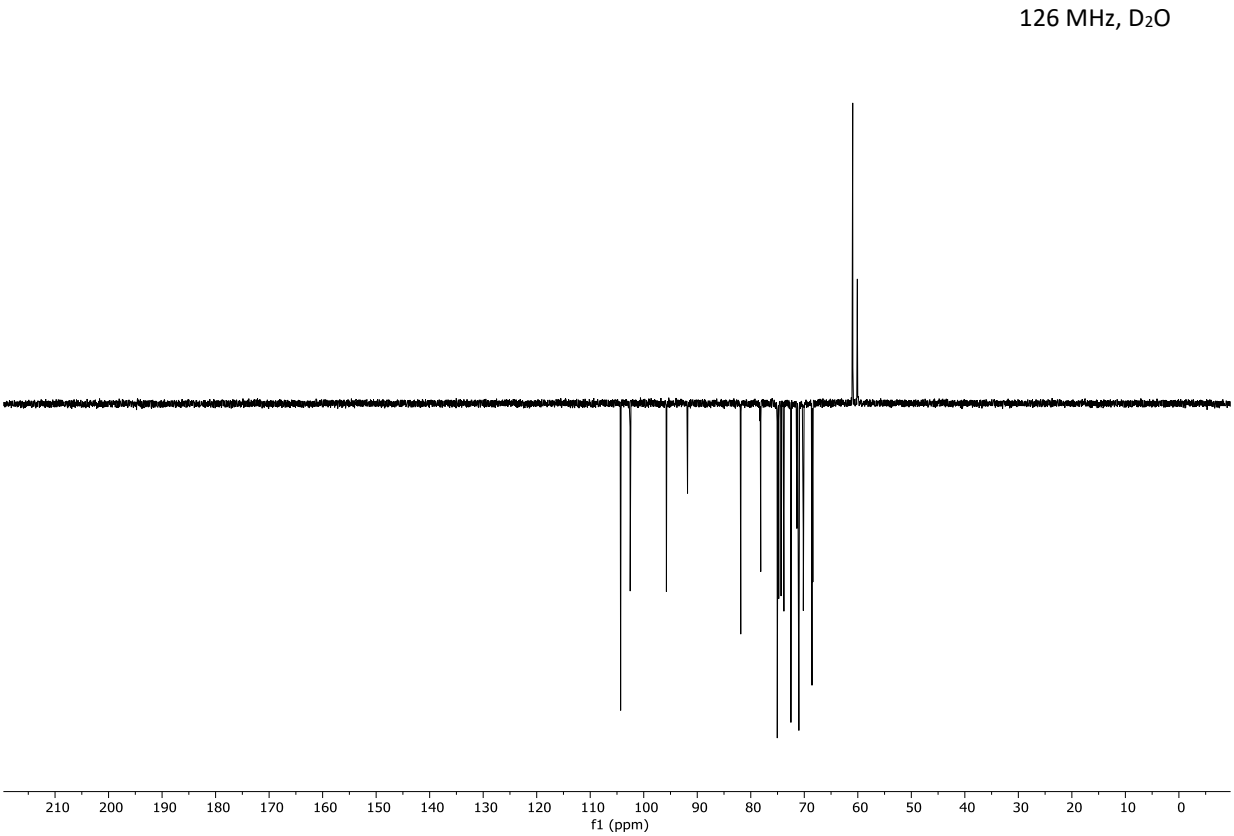


Figure S24 ^{13}C-NMR spectrum of **13**

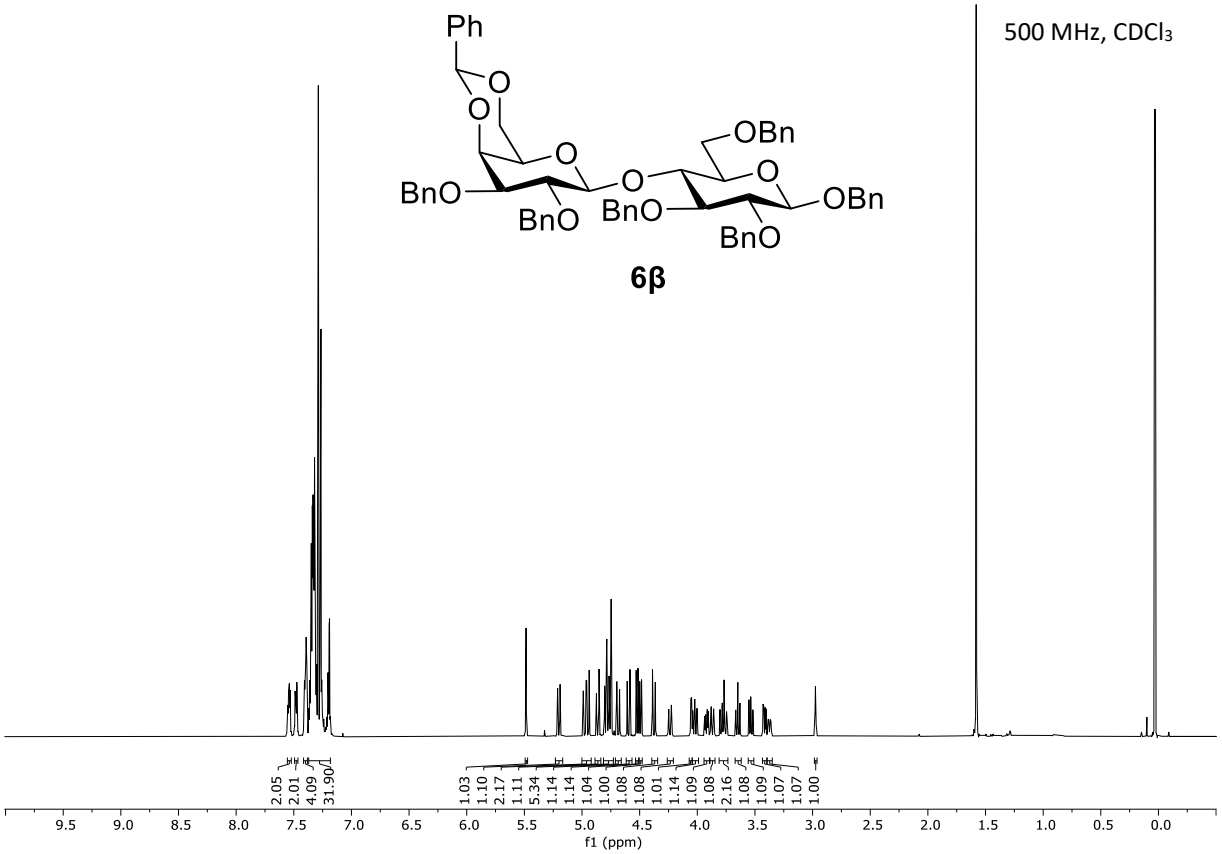


Figure S25 ^{1}H-NMR spectrum of **6β**

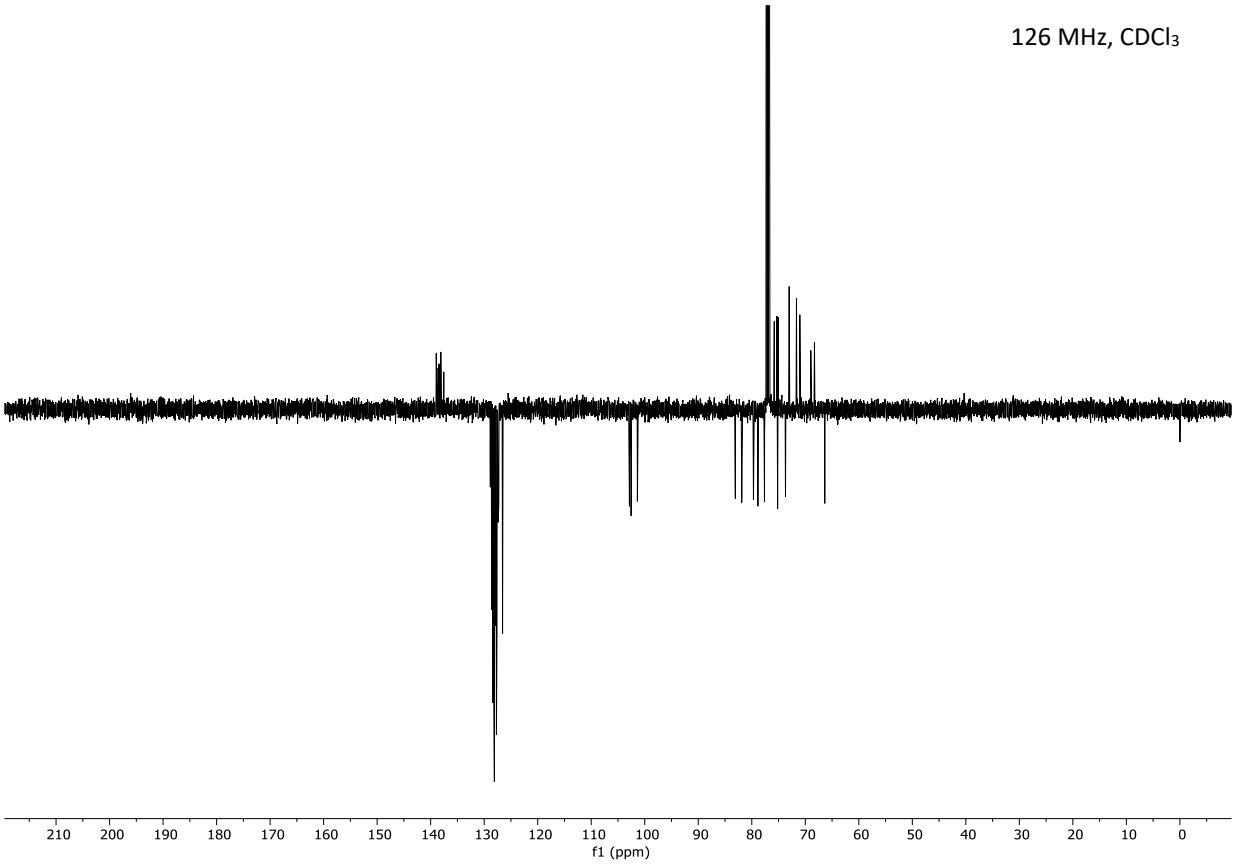


Figure S26 ^{13}C-NMR spectrum of **6β**

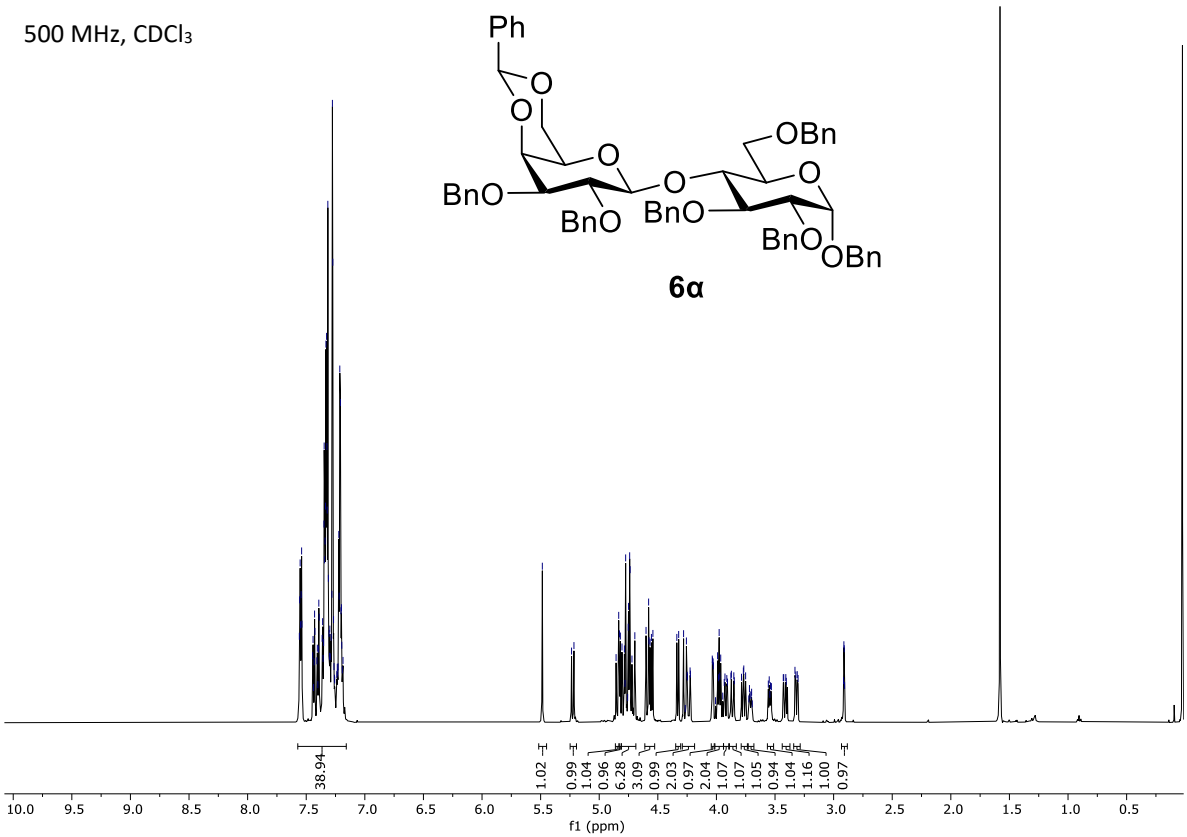


Figure S27 ^{1}H-NMR spectrum of **6α**

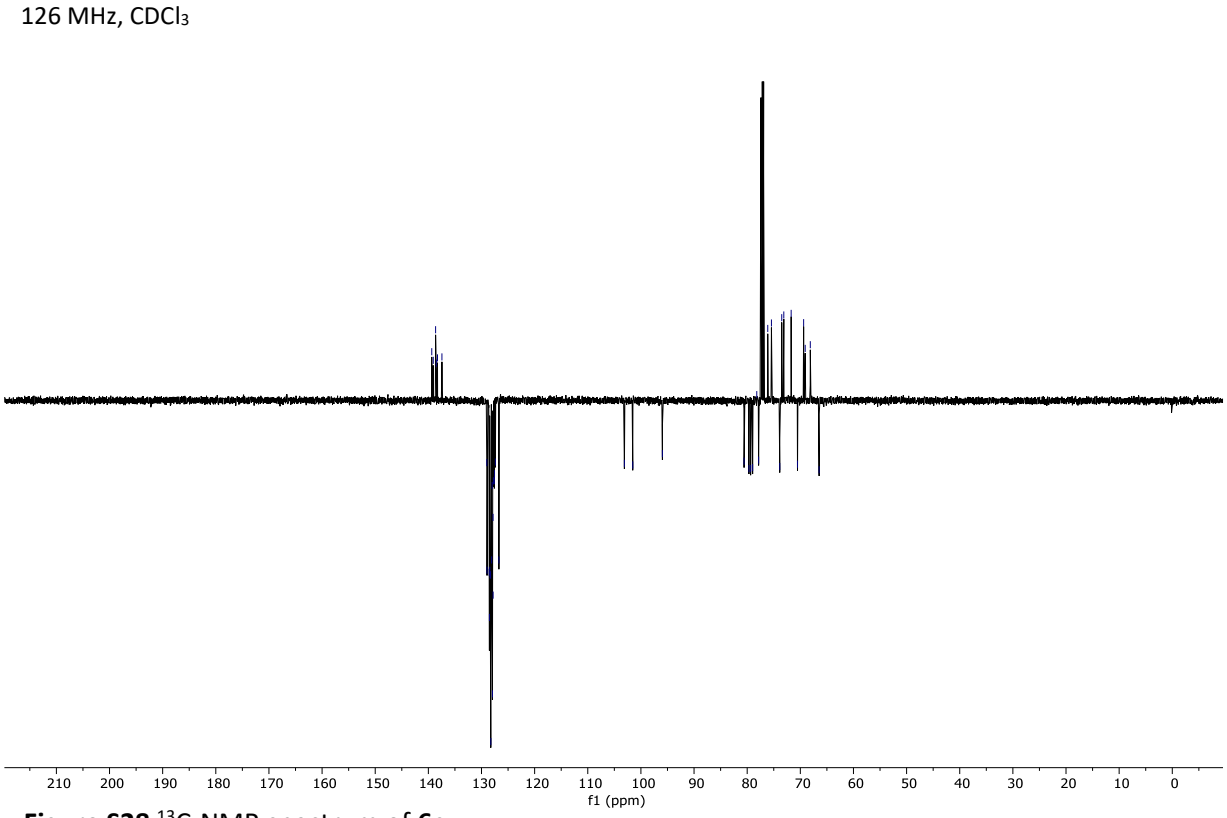


Figure S28 ^{13}C-NMR spectrum of **6α**

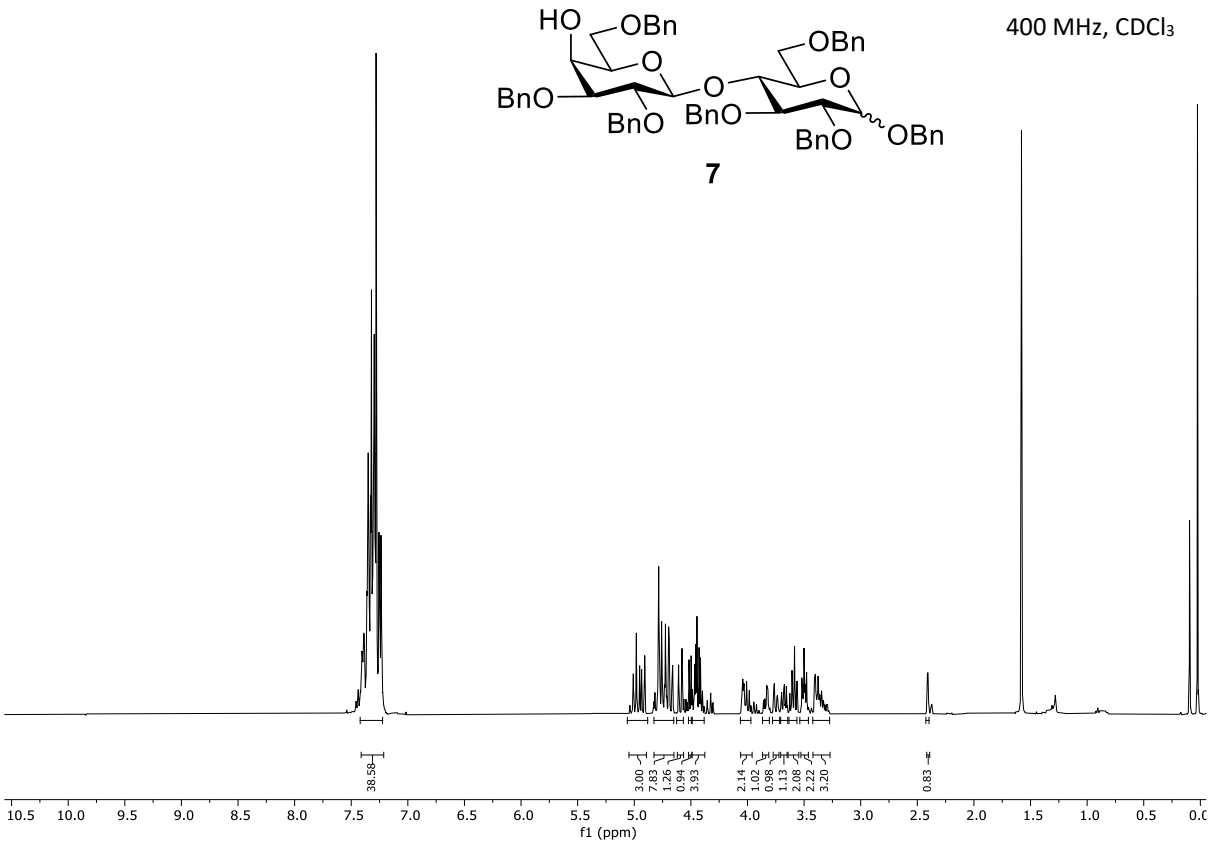


Figure S29 ^{1}H-NMR spectrum of **7**

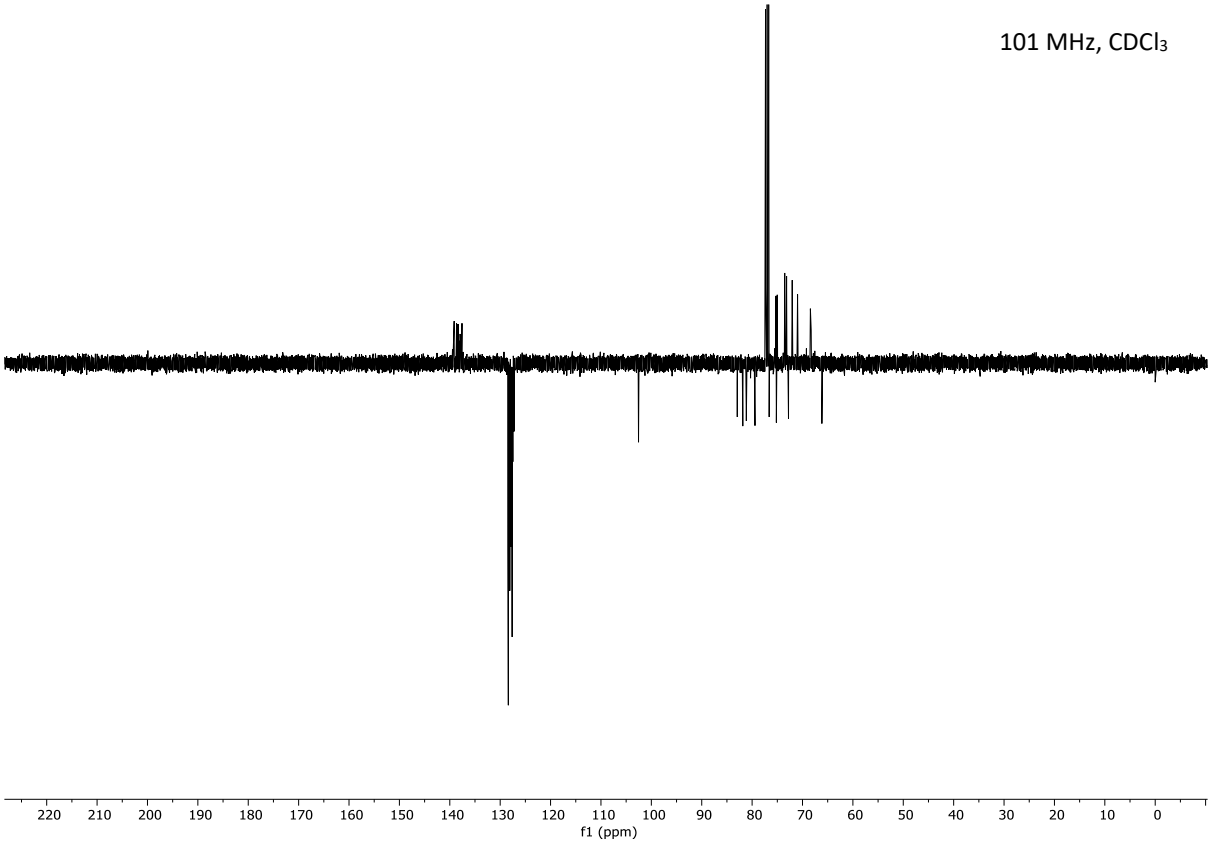


Figure S30 ^{13}C-NMR spectrum of **7**

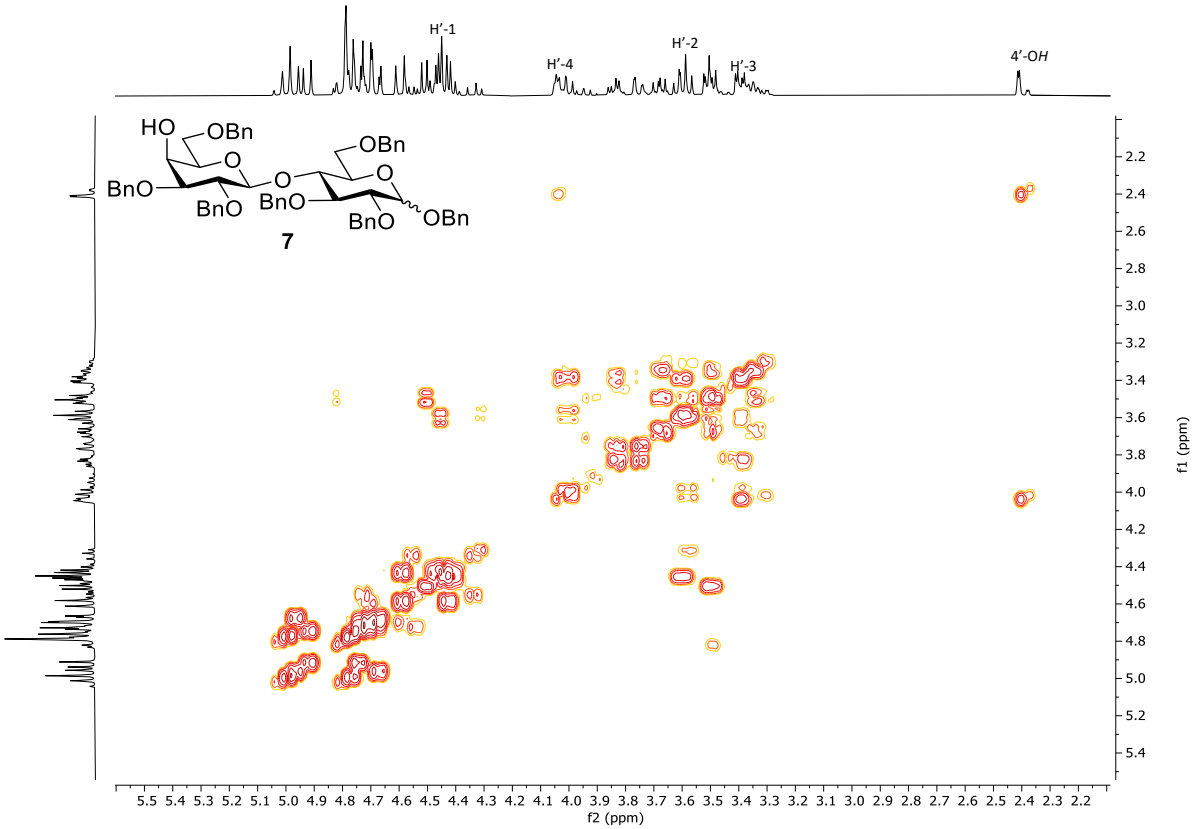


Figure S31 COSY spectrum of **7**

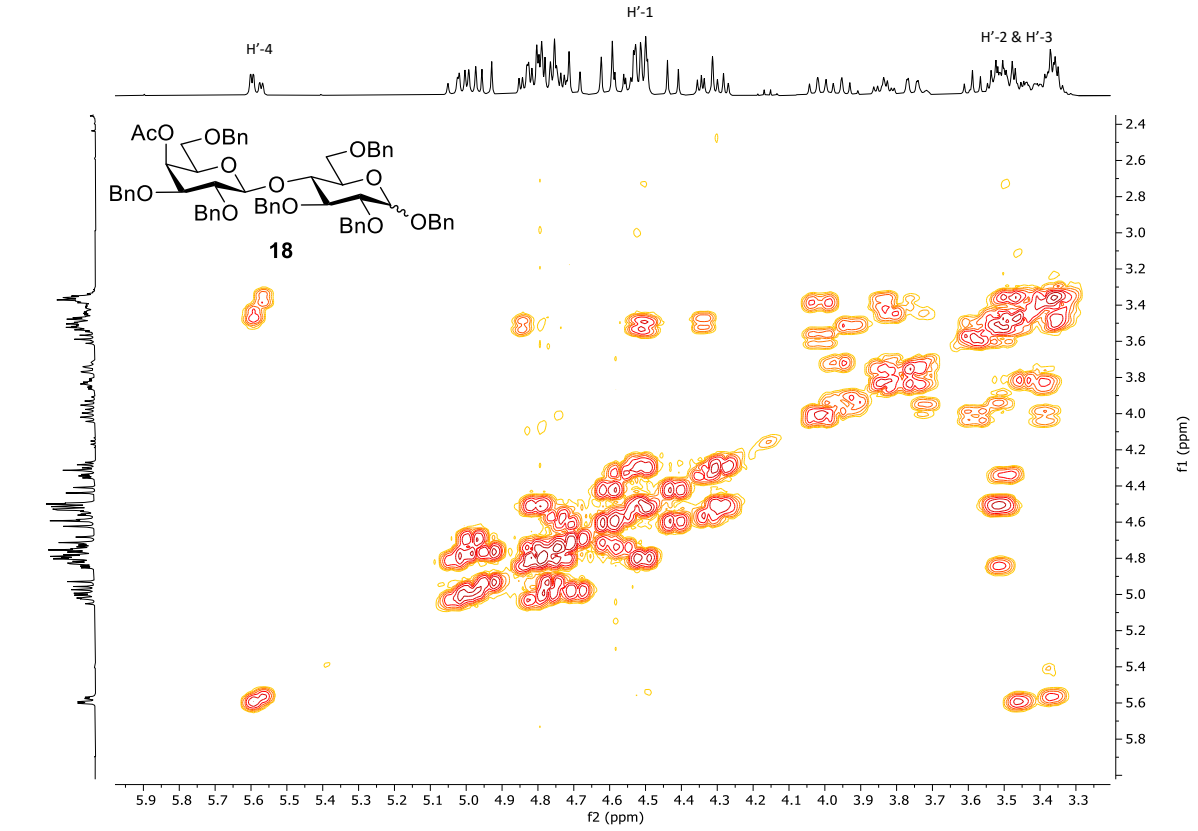


Figure S32 COSY spectrum of **18**

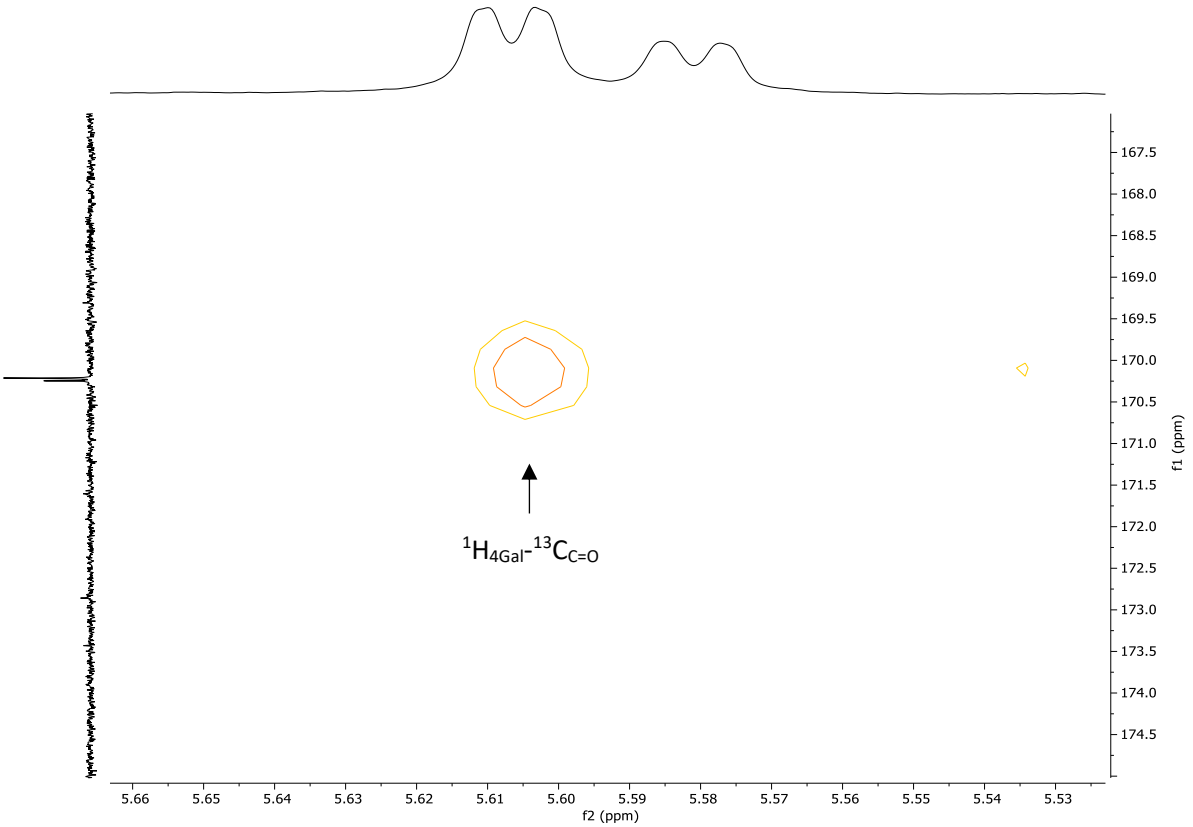


Figure S33 HMBC spectrum of **18**

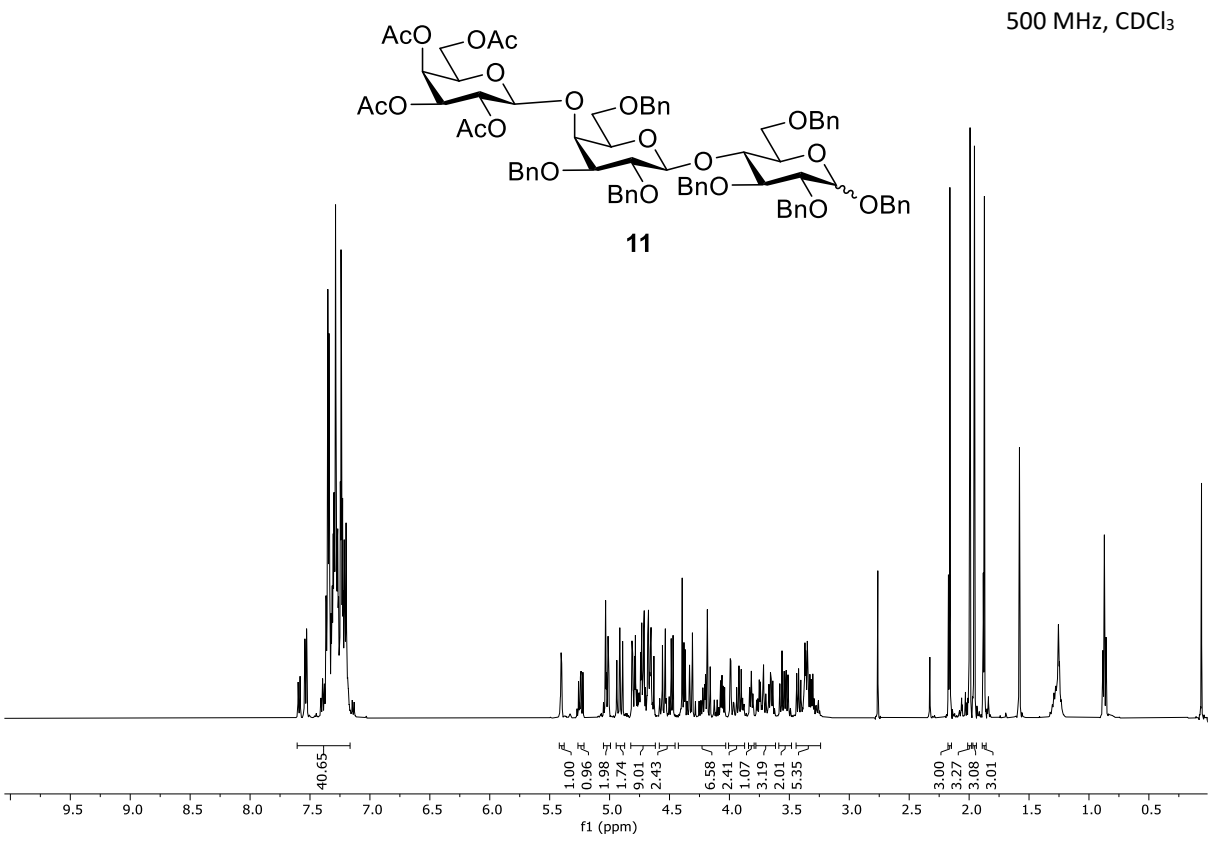


Figure S34 ^{1}H-NMR spectrum of **11**

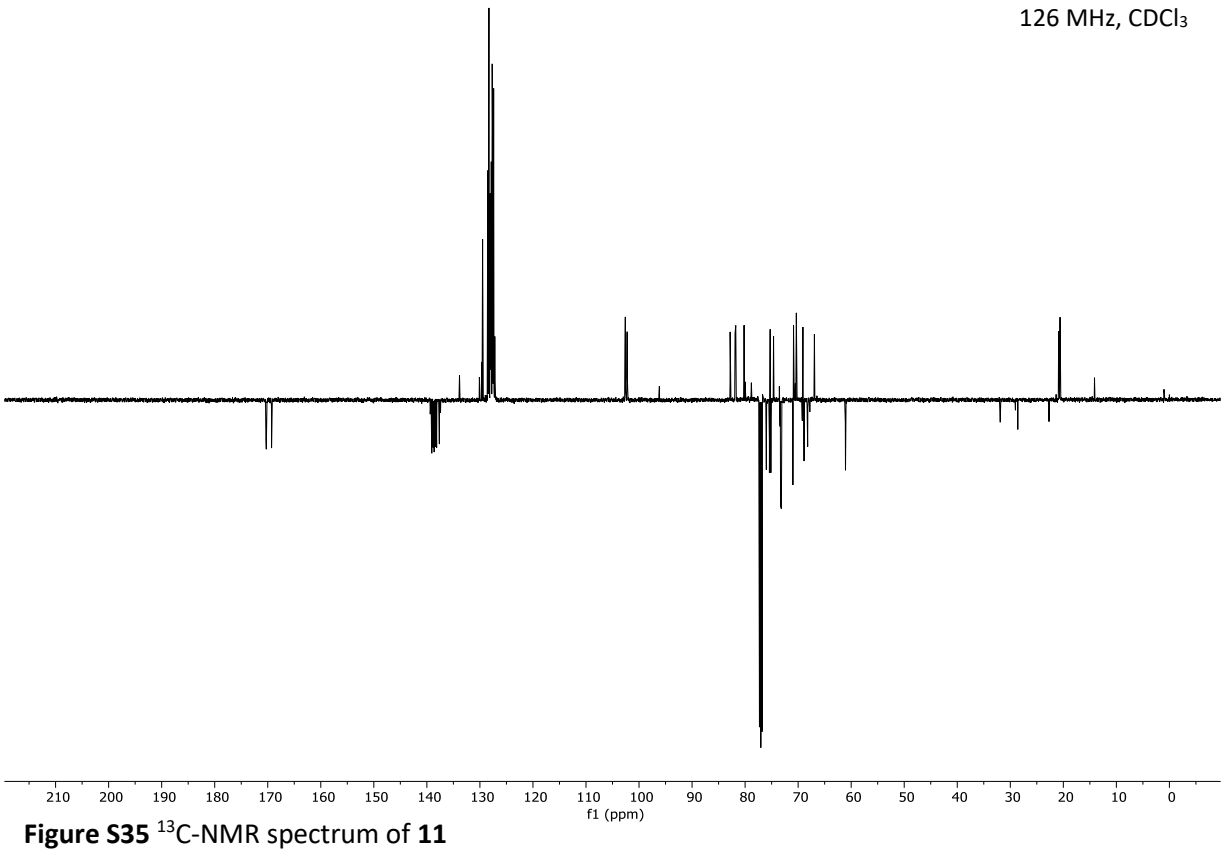


Figure S35 ^{13}C-NMR spectrum of **11**

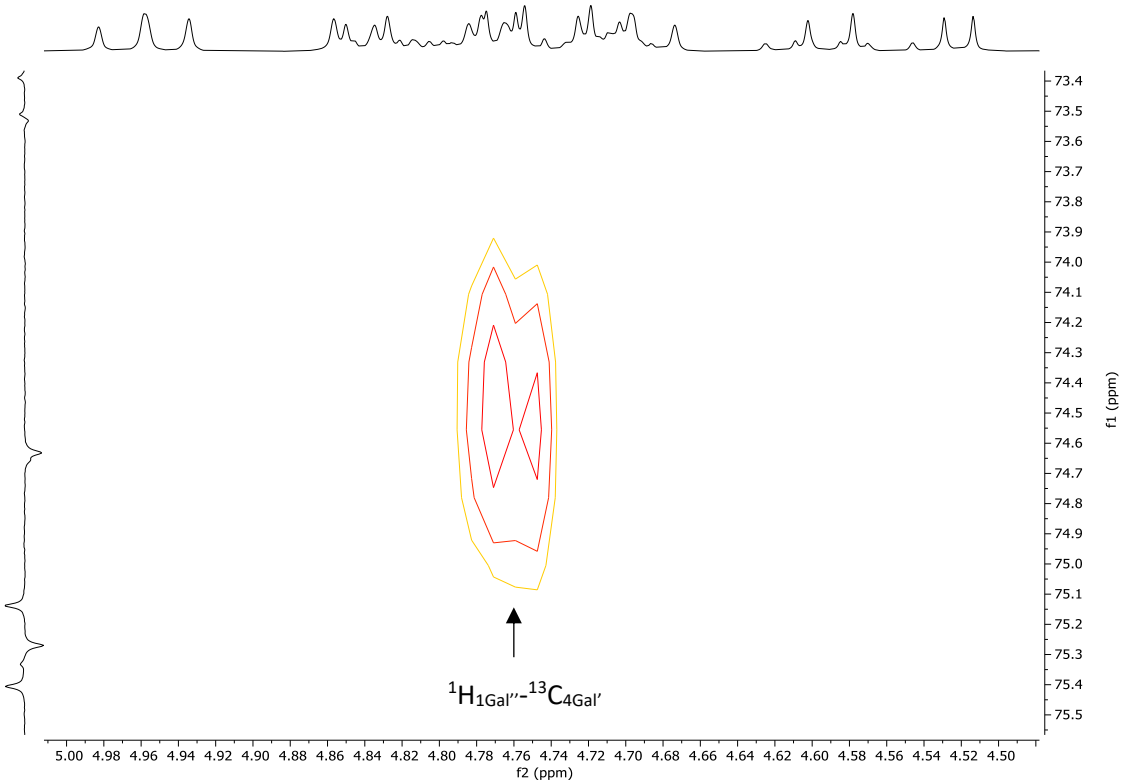


Figure S36 HMBC spectrum of **11**

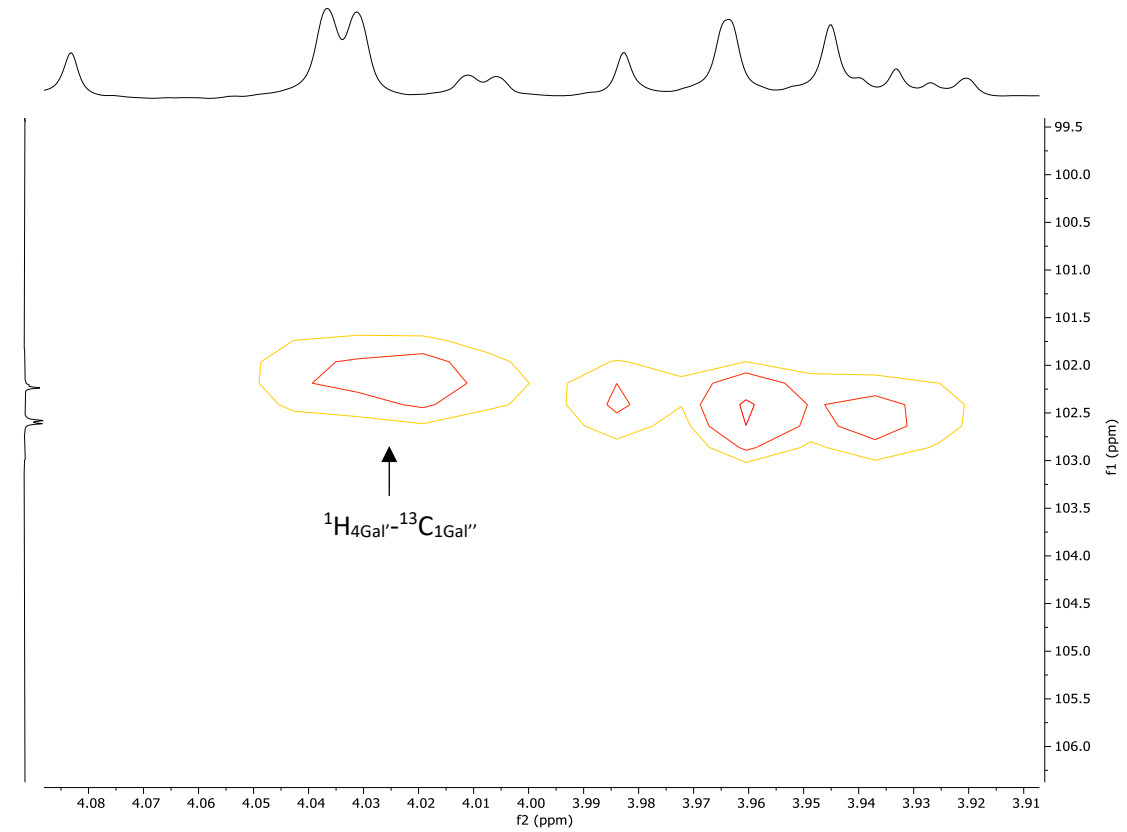


Figure S37 HMBC spectrum of **11**

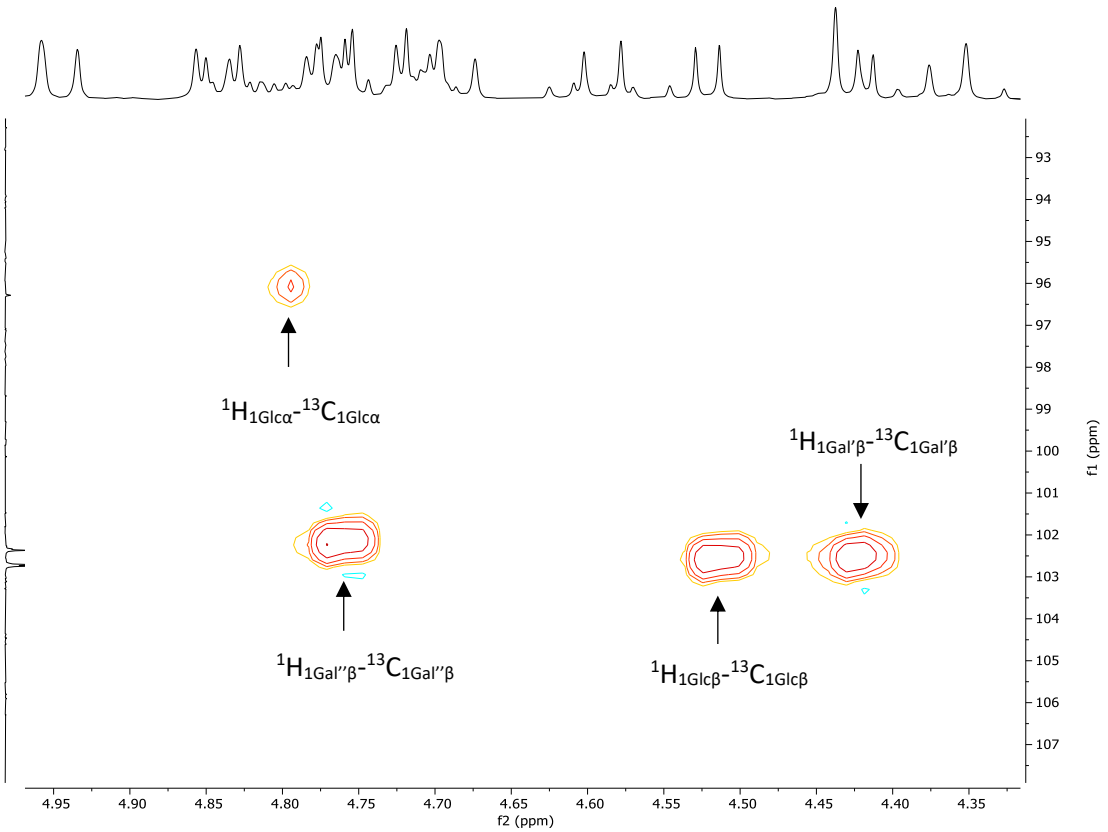


Figure S38 HSQC spectrum of **11**

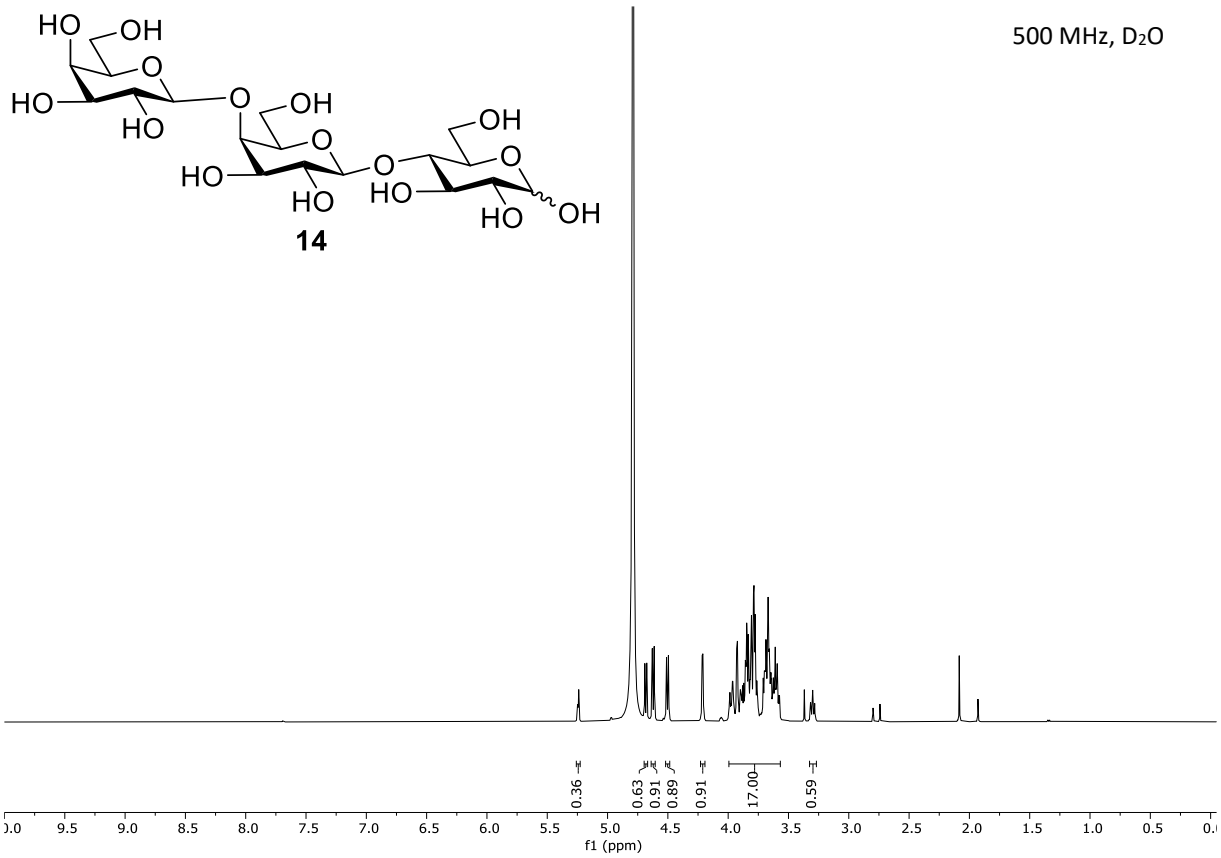


Figure S39 ^{1}H-NMR spectrum of **14**

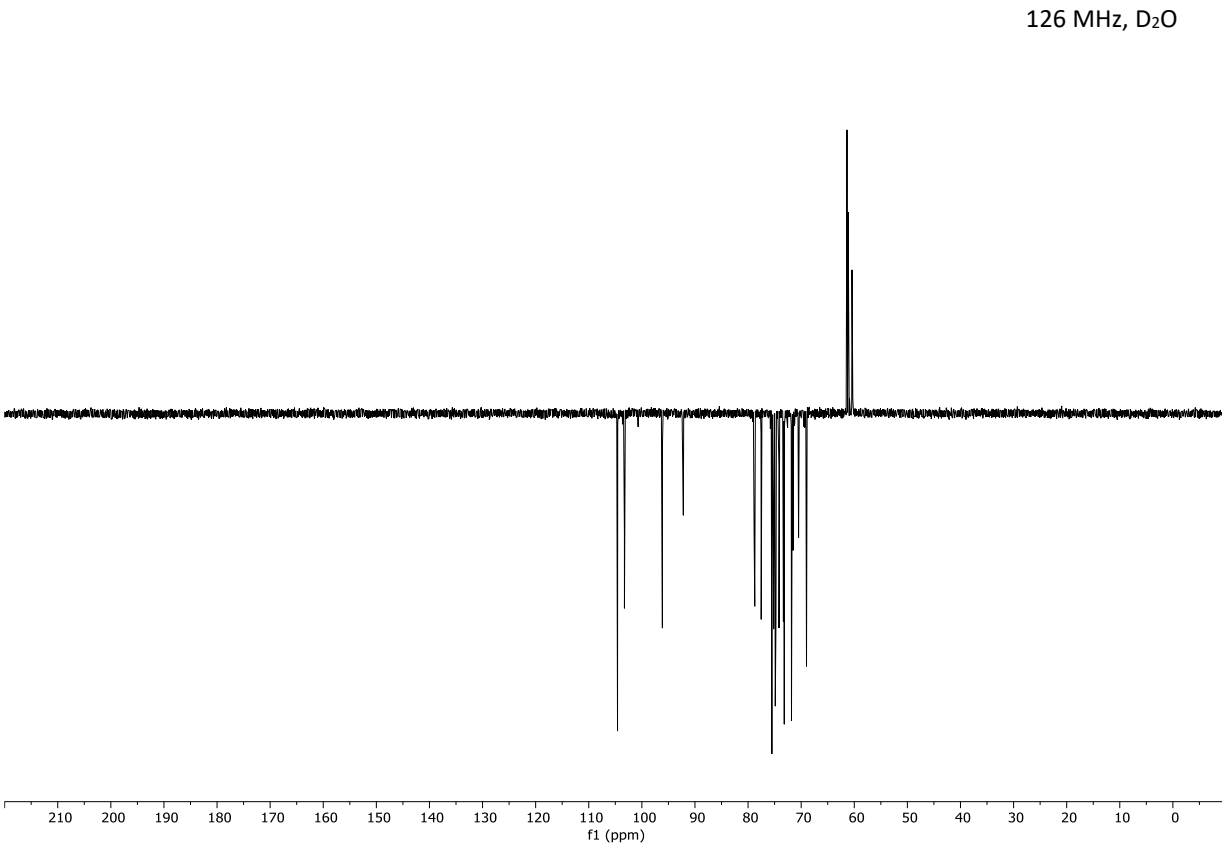


Figure S40 ^{13}C-NMR spectrum of **14**

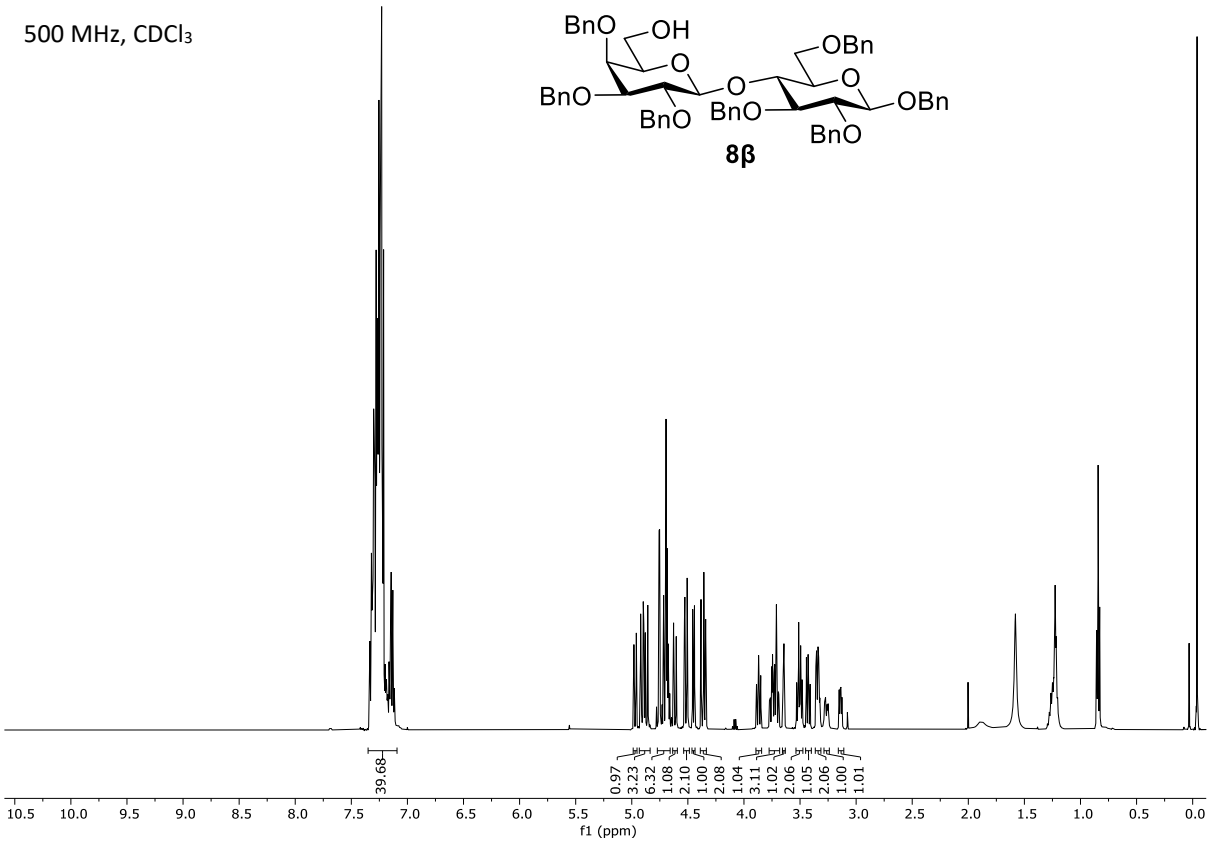


Figure S41 ^{1}H-NMR spectrum of **8β**

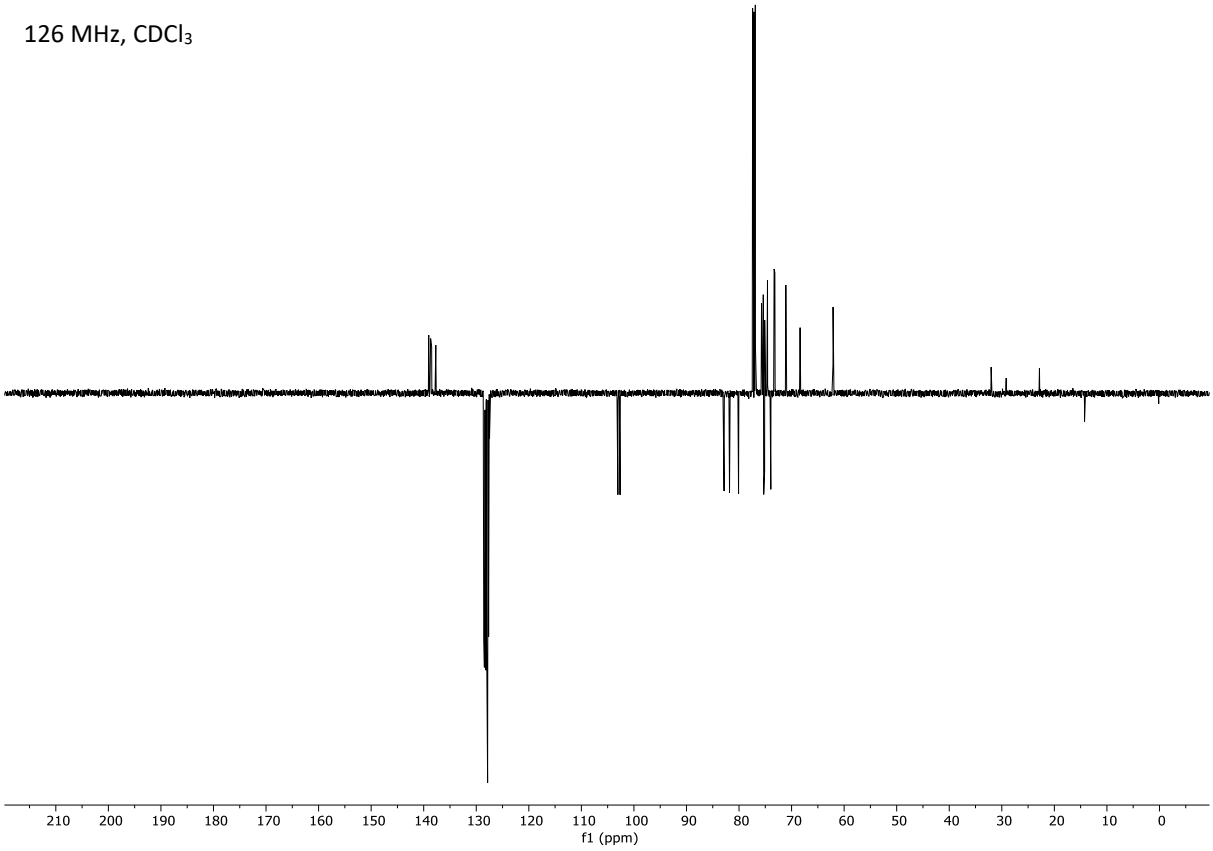


Figure S42 ^{13}C-NMR spectrum of **8β**

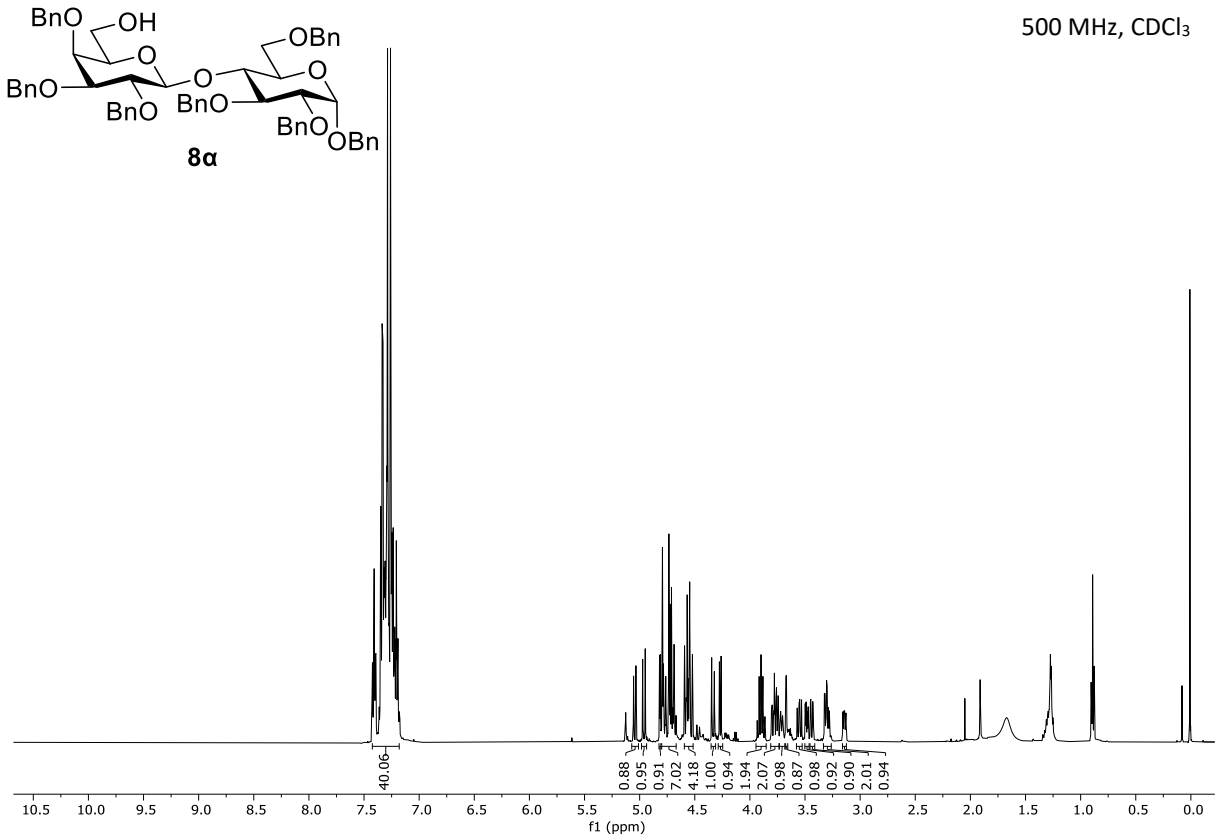


Figure S43 ^{1}H-NMR spectrum of **8α**

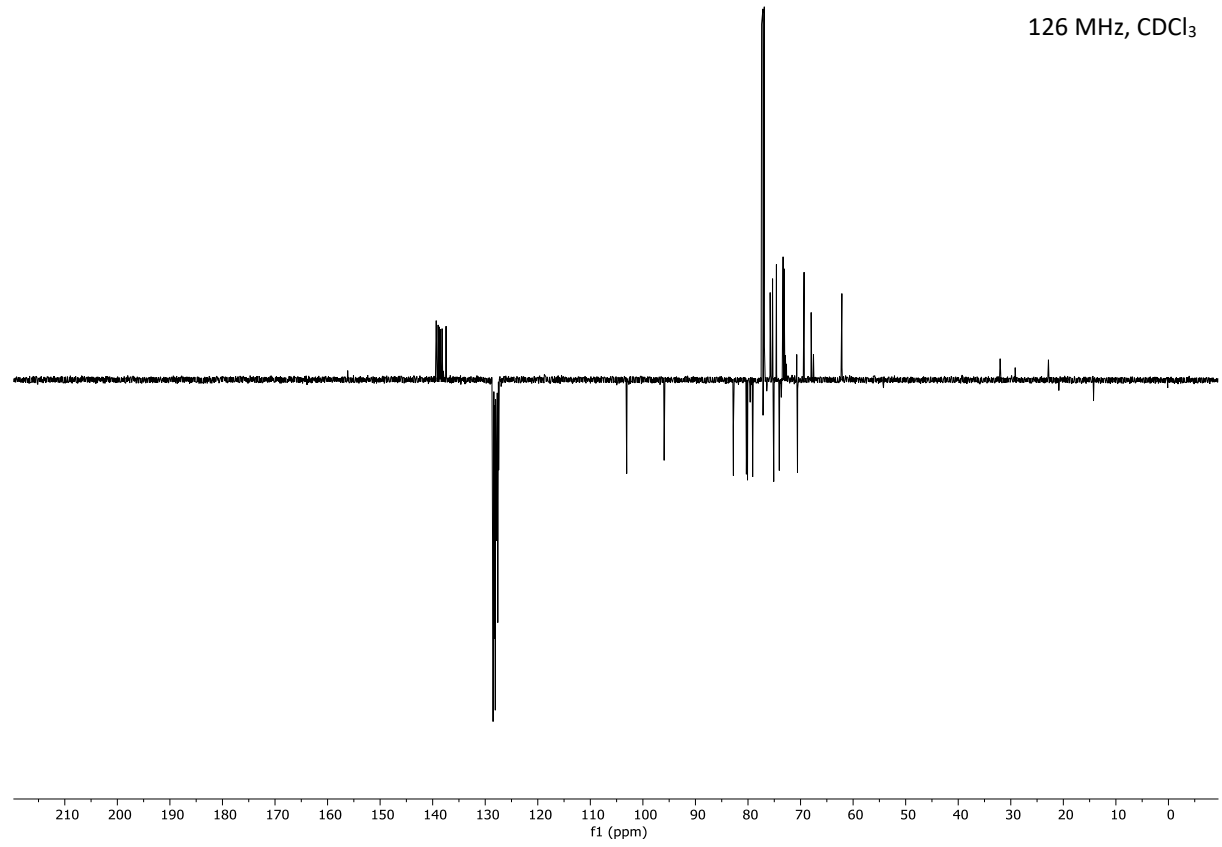


Figure S44 ^{13}C-NMR spectrum of **8α**

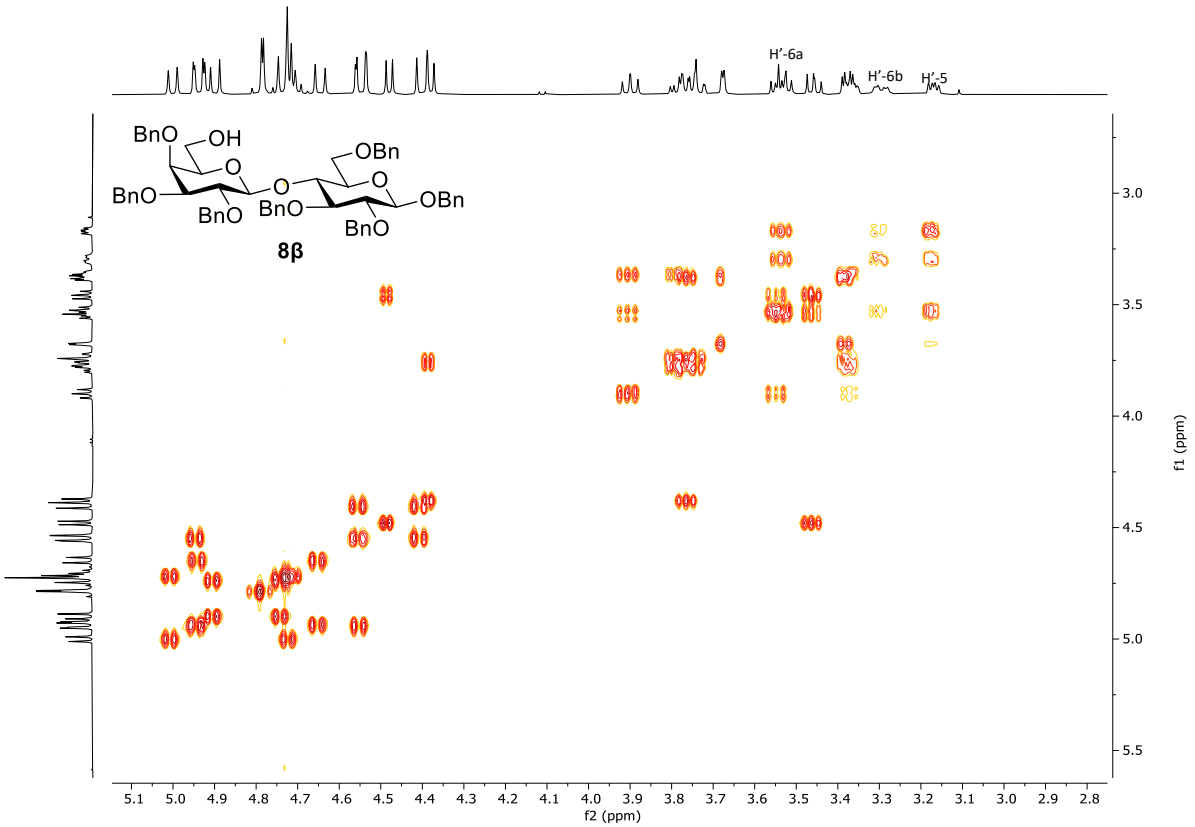


Figure S45 COSY spectrum of **8β**

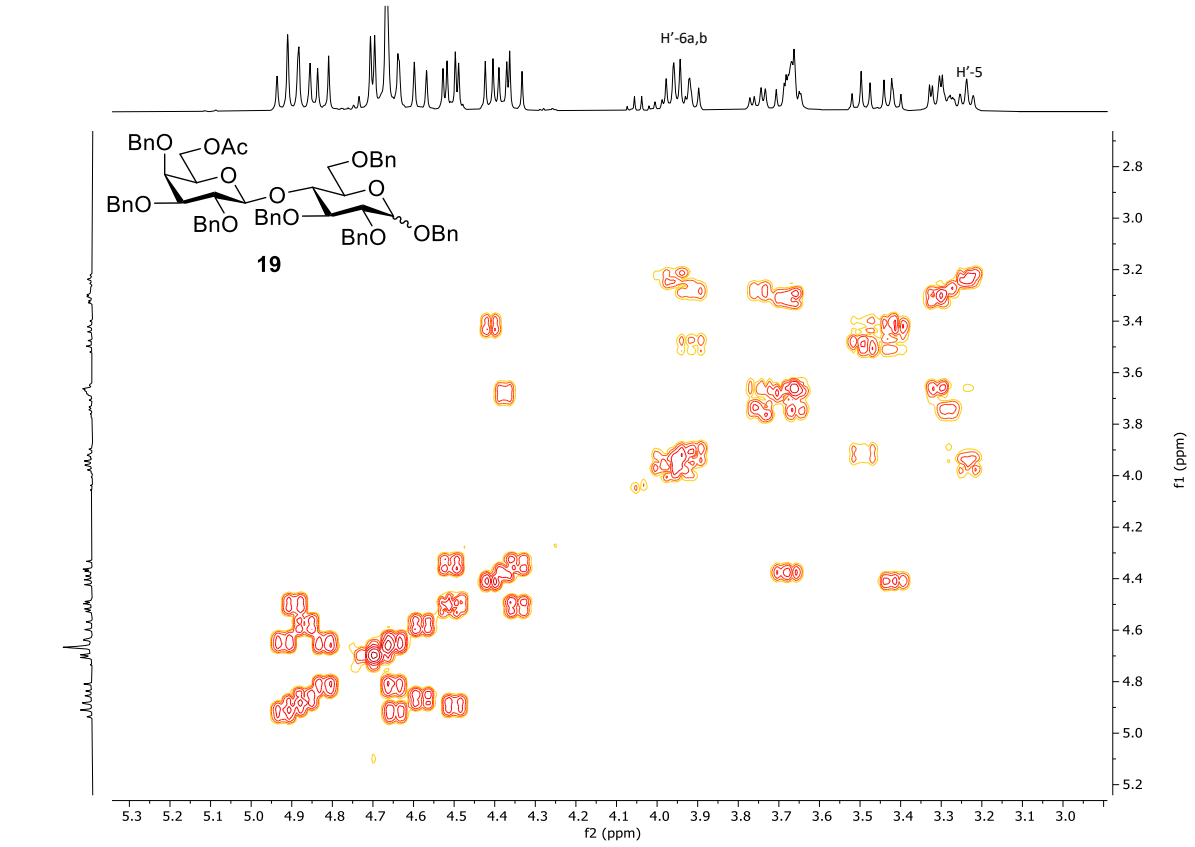


Figure S46 COSY spectrum of **19**

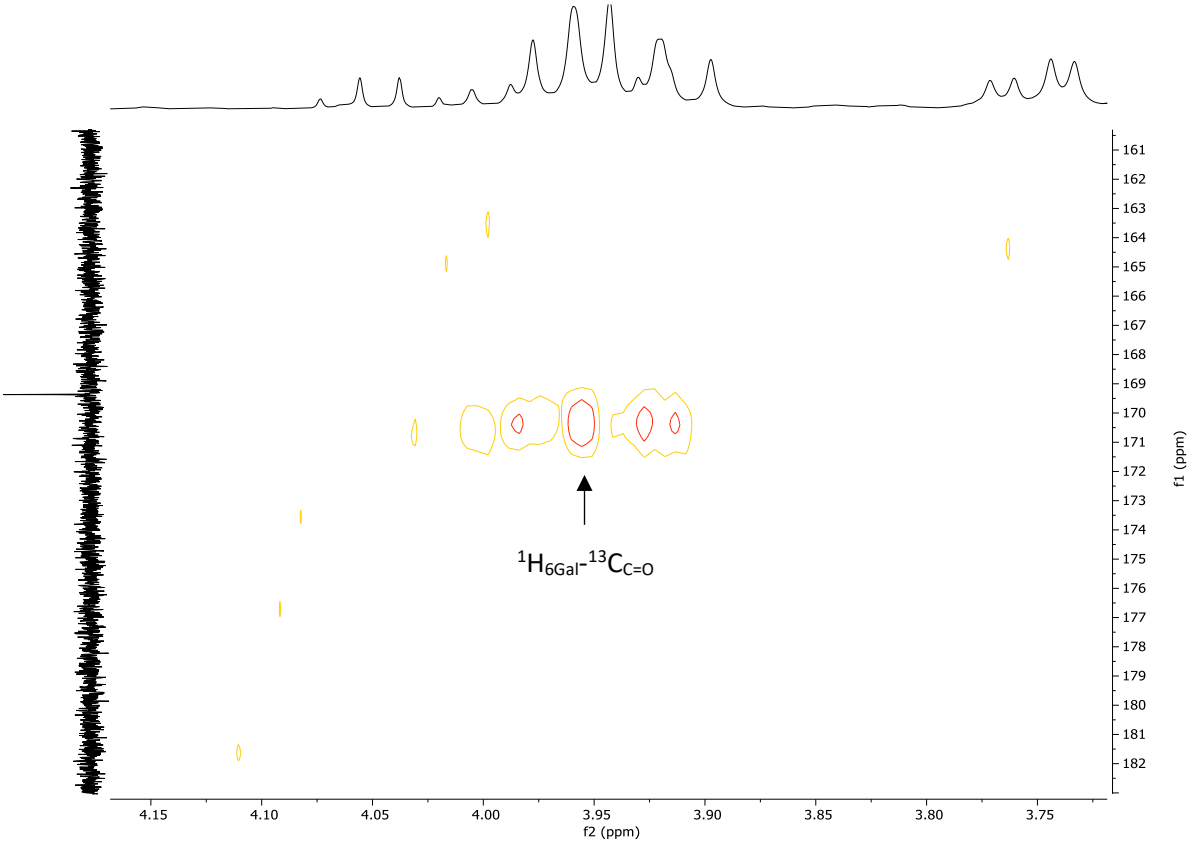


Figure S47 HMBC spectrum of **19**

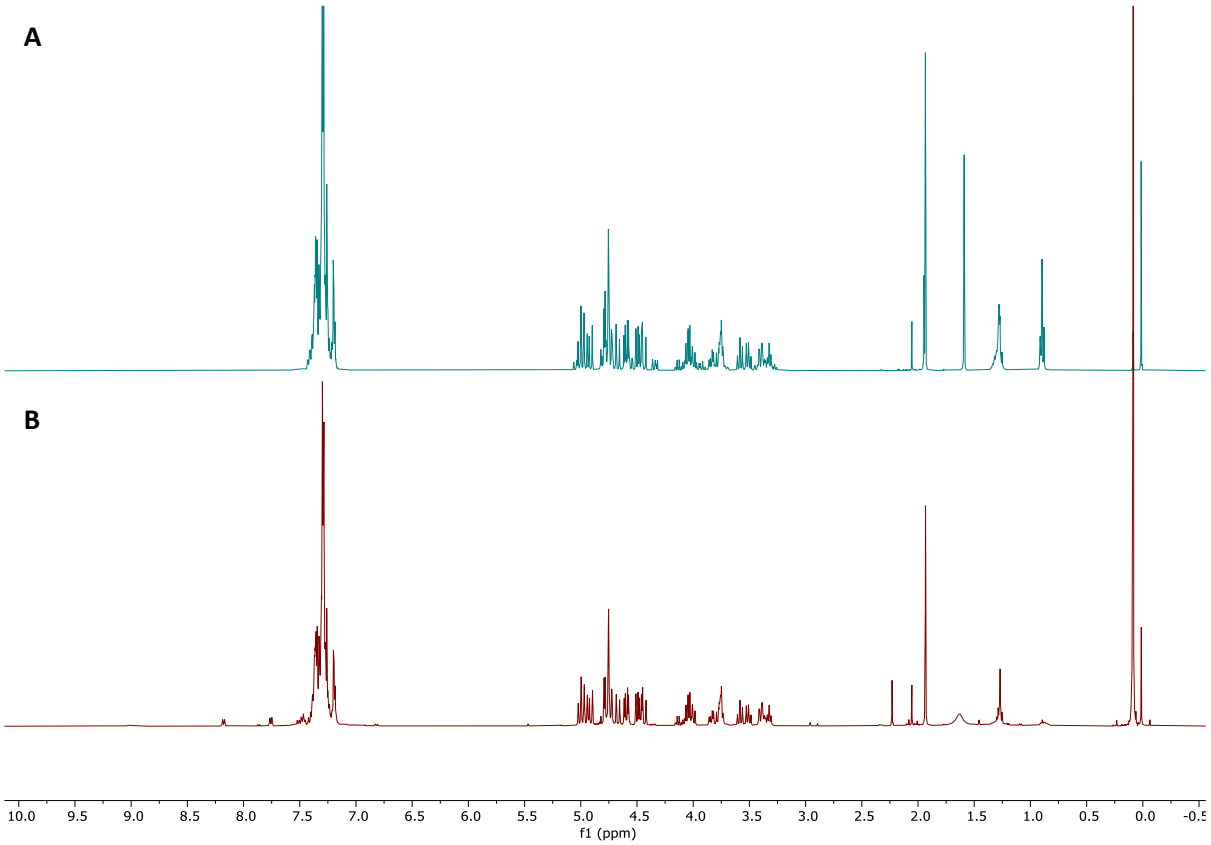


Figure S48 Stacked ^{1}H-NMR spectra of **19**. **A.** ^{1}H-NMR spectra of **19** obtained as byproduct in the synthesis of protected 6'-GL **B.** ^{1}H-NMR spectra of **19** obtained after acetylation of **8**

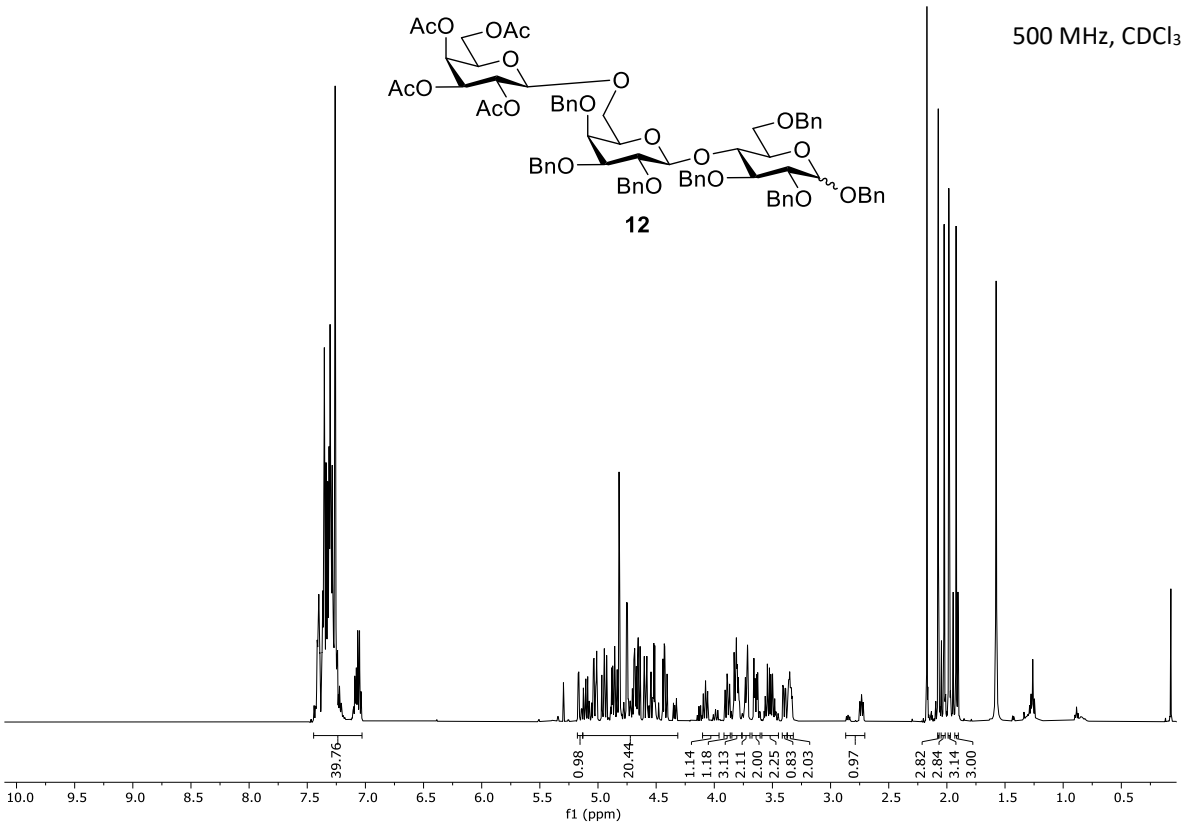


Figure S49 ^{1}H-NMR spectrum of **12**

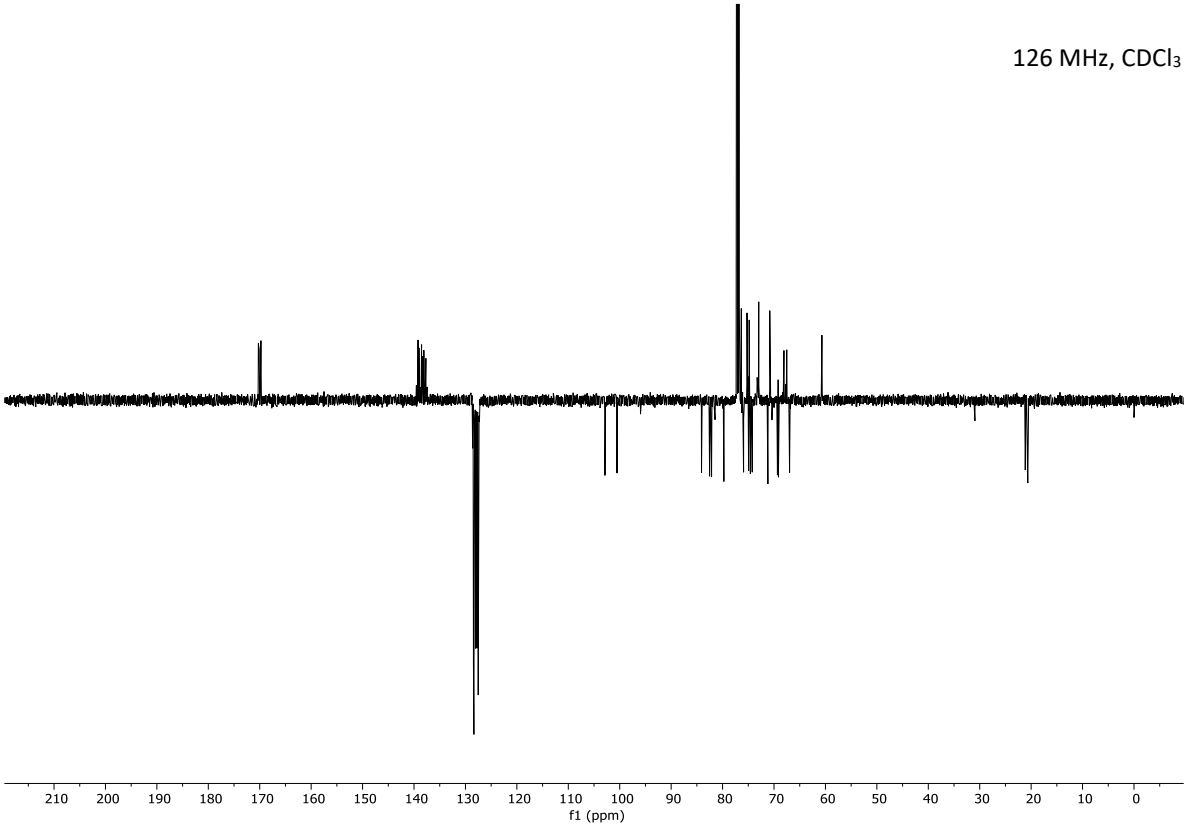


Figure S50 ^{13}C-NMR spectrum of **12**

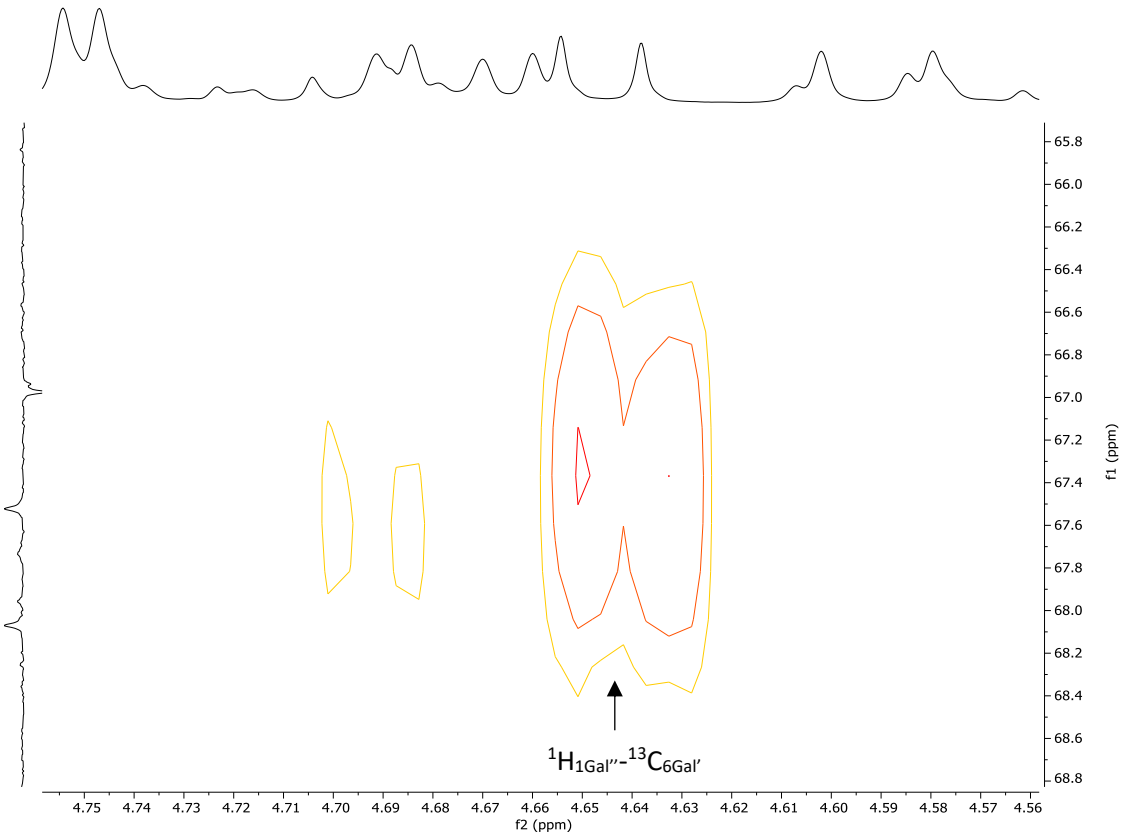


Figure S51 HMBC spectrum of **12**

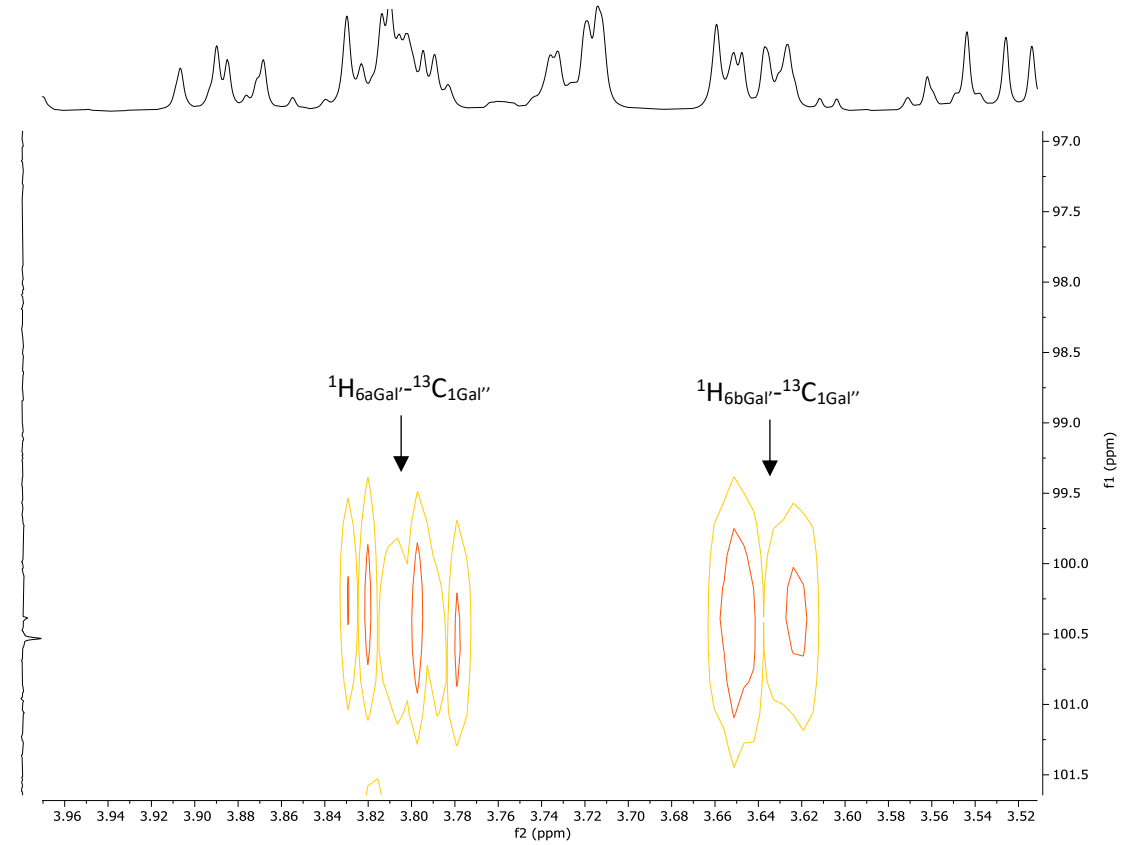


Figure S52 HMBC spectrum of **12**

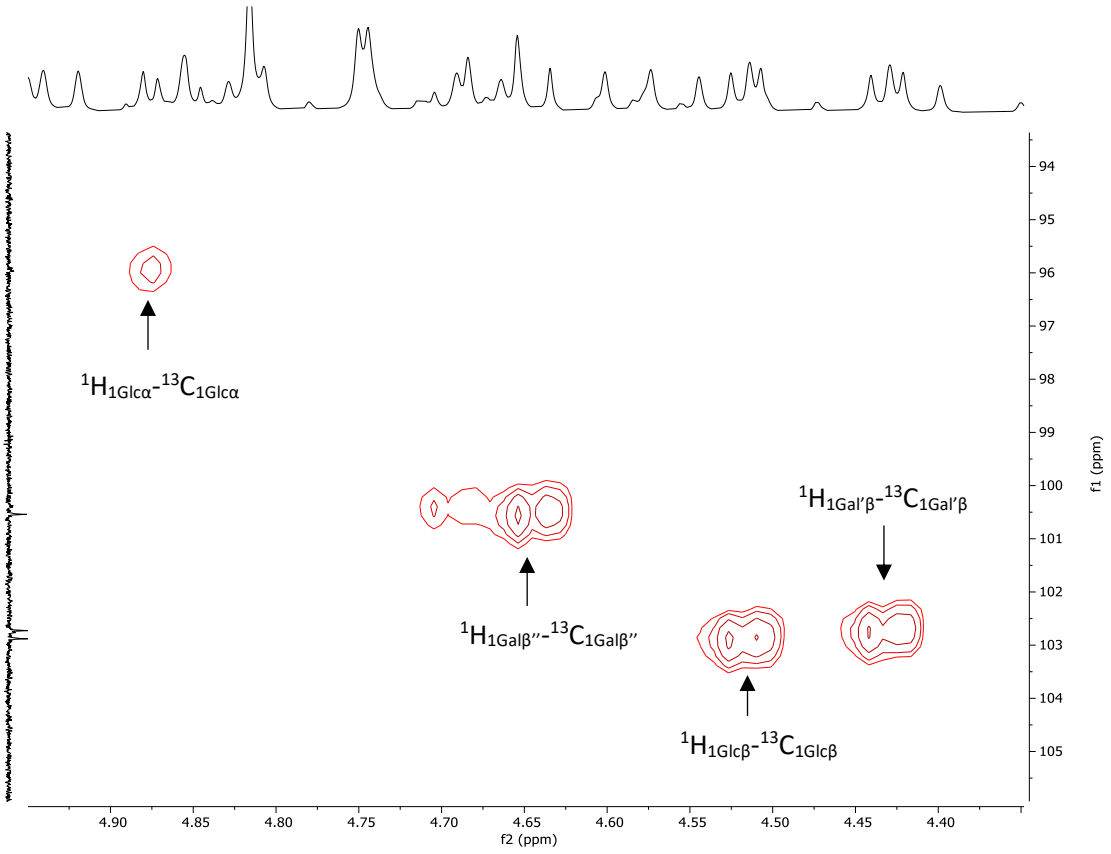


Figure S53 HSQC spectrum of **12**

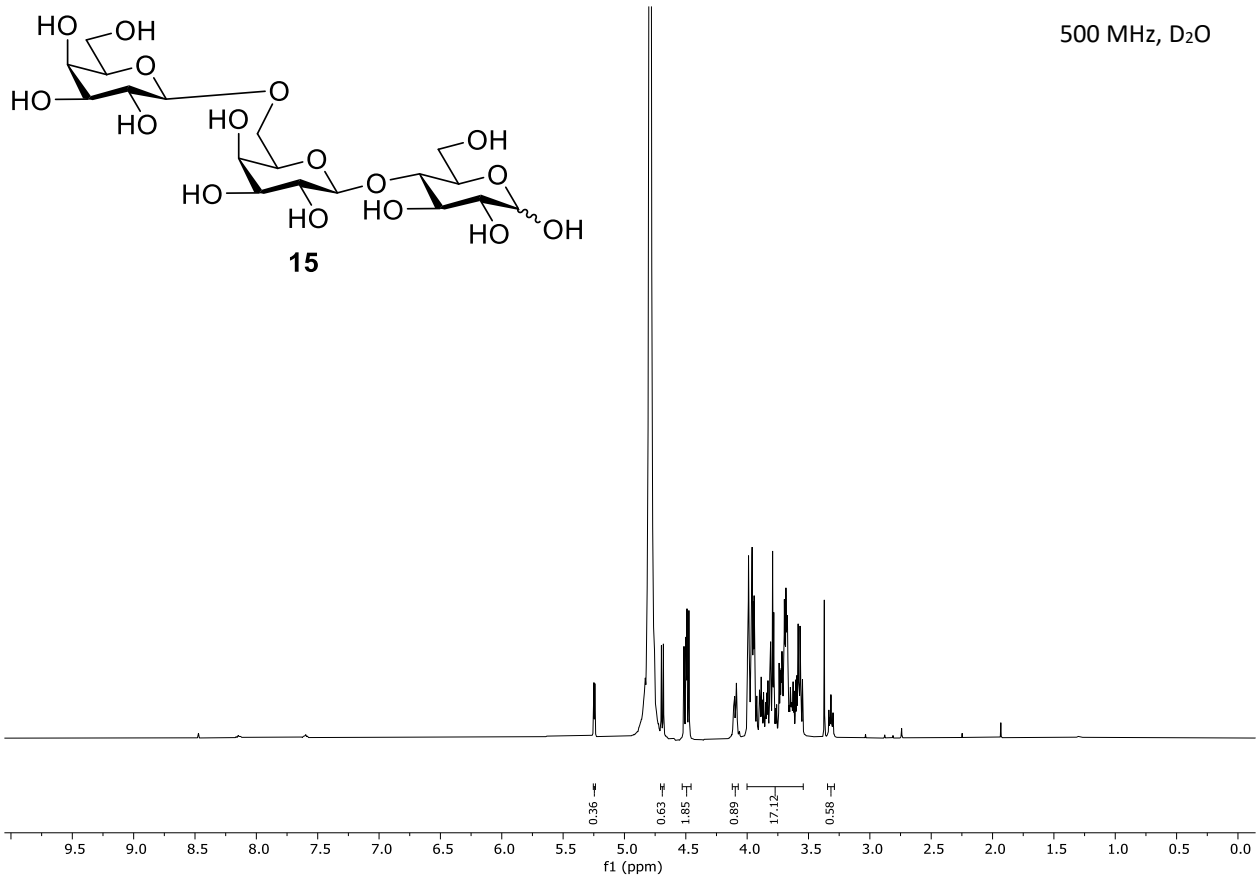


Figure S54 ^{1}H-NMR spectrum of **15**

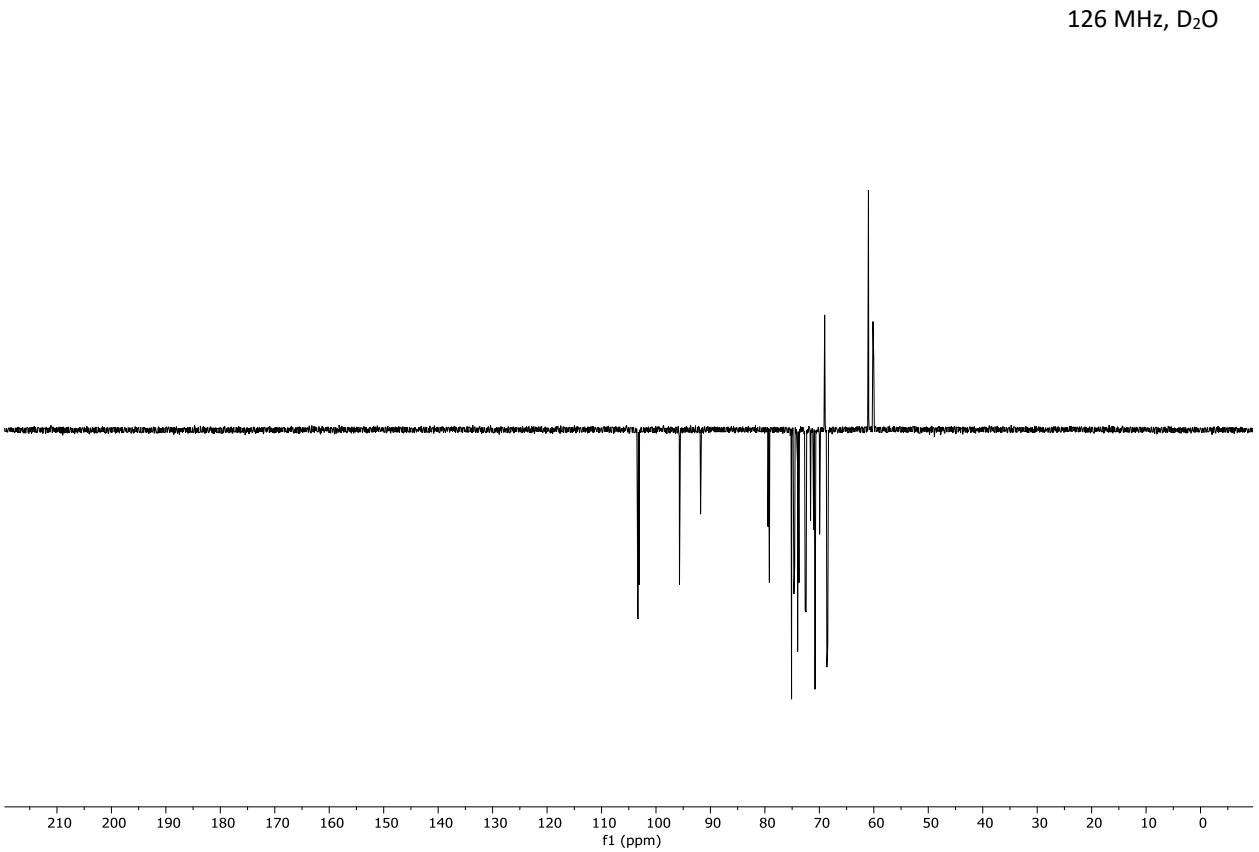


Figure S55 ^{13}C-NMR spectrum of **15**

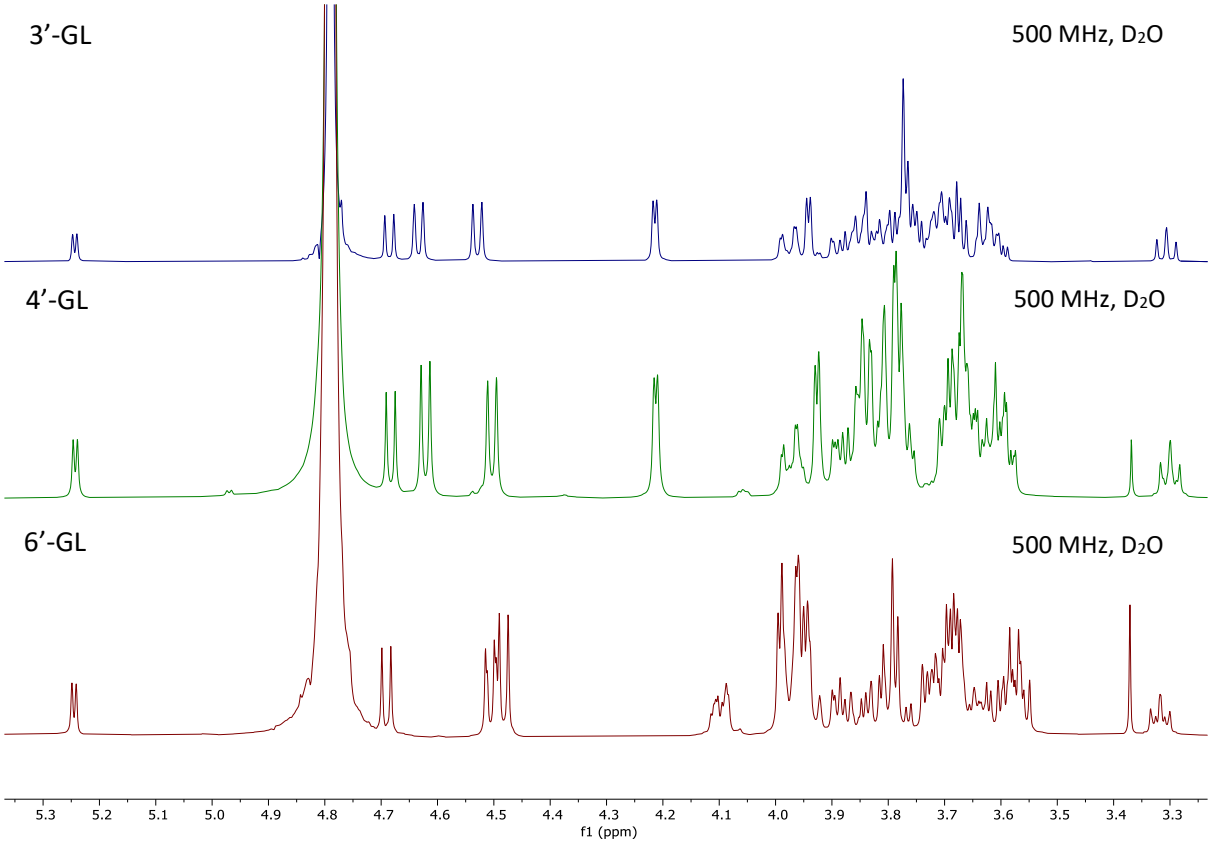


Figure S56 stacked ^{1}H-NMR spectra of 3’-GL (**13**), 4’-GL (**14**) and 6’-GL (**15**)

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