

Supplementary Information

One-Step Synthesis of Novel Glycosyltransferase Inhibitors

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(1) General experimental conditions

All reagents and solvents were obtained from commercial suppliers and used without further purification unless otherwise stated. HPLC was performed on a Perkin Elmer system (Series 200 Pump; Series 200 Column Oven; Series 200 EP Diode Array Detector; Series 225 Autosampler) using a Grace reverse phase column (3 μ m C18, 150 mm x 4.6 mm). The following buffers were used: Buffer A: 100 mM potassium phosphate pH 6.5, 8 mM tetrabutylammonium bisulfate. Buffer B: 30% MeOH, 70% buffer A. The gradient for elution was as follows (sample injection volume: 50 μ L): 100% buffer A for 5 min; linear gradient to 100% buffer B over 15 min; 100% buffer B for 5 min; linear gradient to 100% buffer A over 5 min; 100% buffer A for 5 min. NMR spectra were obtained on a Bruker Avance DRX 400 spectrometer operating at 400 MHz (^1H), 100 MHz (^{13}C), 162 MHz (^{31}P) and 377 MHz (^{19}F). The spectra were obtained at 298K and samples run as dilute solutions in D_2O . All coupling constants are reported in Hertz (Hz), and multiplicities are labelled s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet) or combinations thereof. IR spectra were obtained from neat samples on a Perkin Elmer FTIR spectrometer. Low and high resolution mass spectra were recorded at the EPSRC National Mass Spectrometry Service Centre, Swansea using negative nanospray ionisation (NSI).

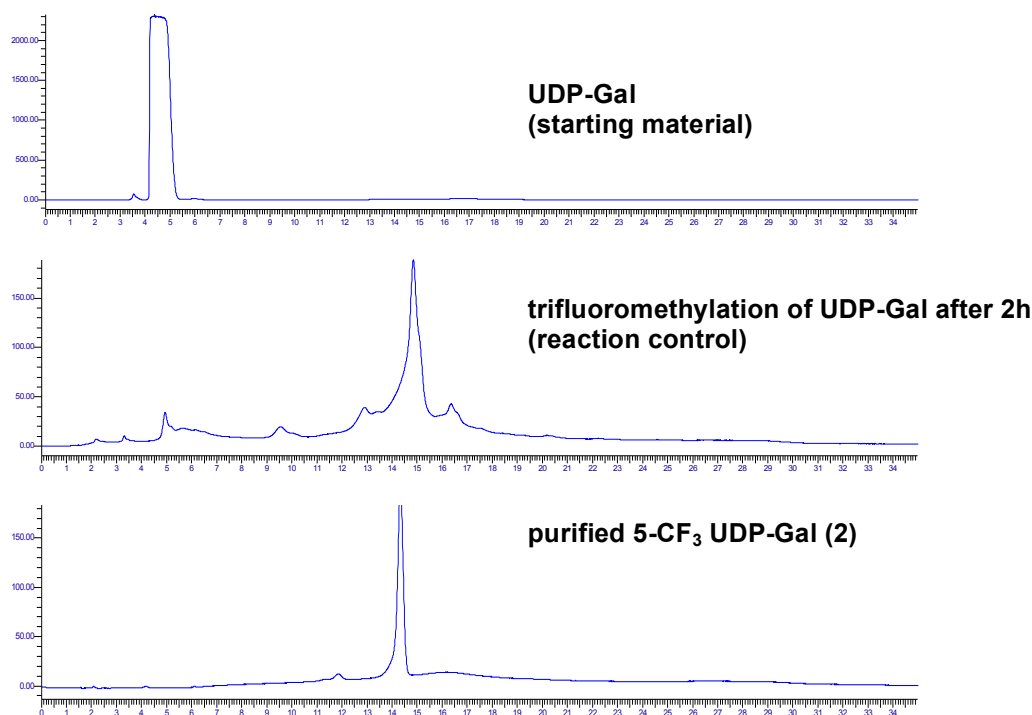
(2) Synthesis of 5- CF_3 UDP-glucose (3) and 5- CF_3 UDP (4)

5-trifluoromethyl UDP-glucose (3). The title compound was prepared from UDP-glucose x 2Na^+ (100 mg, 0.164 mmol) as described in the synthesis of **2**. After purification by ion-exchange chromatography, **3** was obtained as the triethylammonium salt in 16% yield (23 mg, 0.027 mmol, 2.3 equiv. of TEA). ^1H -NMR (400 MHz; in D_2O) δ = 8.24 (1H, s, uracil 6-H), 5.82 (1H, d, J = 4.5 Hz, ribose 1'-H), 5.49 (1H, dd, J = 7.3 Hz and 3.4 Hz, glucose 1-H), 4.32 (1H, t, J = 5.0 Hz), 4.27 (1H, t, J = 5.0 Hz), 4.22-4.07 (4H, m), 3.81-3.62 (4H, m), 3.44-3.30 (2H, m); ^{13}C -NMR (100MHz; in D_2O) δ = 162.4, 151.8, 143.2, 123.1, 120.4, 105.7, 95.5, 89.6, 83.4, 74.0, 72.8, 71.6, 69.2, 64.8, 60.3; ^{31}P -NMR (162 MHz; in D_2O) δ = -11.6 (d, J = 19.8), -13.0 (d, J = 19.8); ^{19}F -NMR (377 MHz; in D_2O) δ = -63.2; IR ν (neat)/ cm^{-1} 3248 (br), 2987, 2941, 2818, 2700, 2505, 1699, 1466, 1227; NSI-MS m/z 633 ($[\text{M}-\text{H}]^-$ 100%), 565 (29%), 316 (42%), 165 (90%); NSI-HRMS calcd. for $\text{C}_{16}\text{H}_{22}\text{F}_3\text{N}_2\text{O}_{17}\text{P}_2$ $[\text{M}-\text{H}]^-$ 633.0335, found 633.0347.

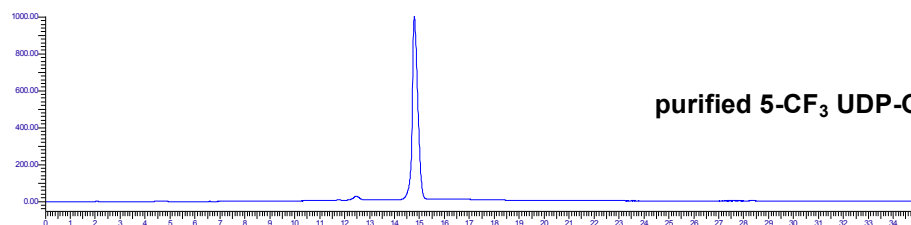
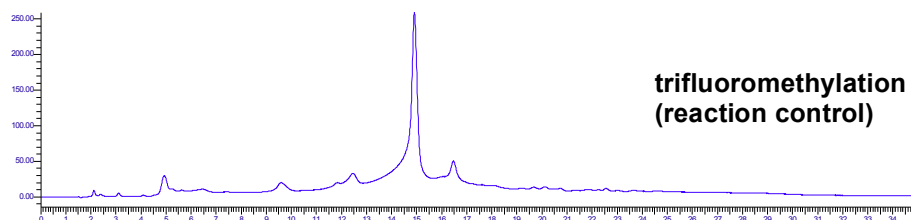
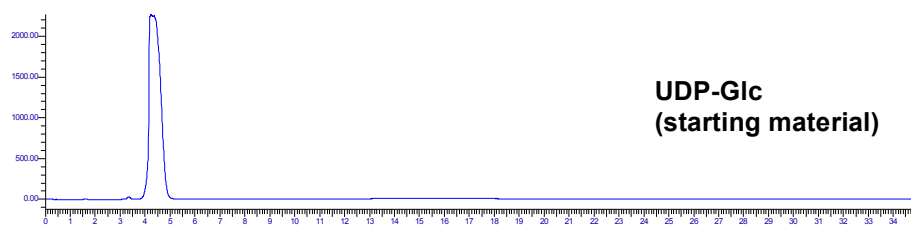
5-CF₃ UDP (4). UDP x 2Na⁺ (25 mg, 0.056 mmol) and NaSO₂CF₃ (35 mg, 0.223 mmol) were dissolved in H₂O (360 µl) in a small test tube equipped with a magnetic stirrer under rapid stirring. The reaction mixture was cooled in an ice bath and ^tBuOOH (20% in H₂O; 43 µL, 0.335 mmol) was added dropwise over 30 min. The reaction was stirred for a further 30 min, at which point HPLC indicated almost complete consumption of starting material (see chromatogram). The reaction was allowed to warm to room temperature and purified immediately by anion exchange chromatography on Macro-Prep 25Q resin, gradient 0-100 TEAB (1M, pH 7.3) against H₂O over 400 mL, flow rate 2 mL/min. Product-containing fractions were combined and co-evaporated repeatedly with MeOH to remove residual triethylammonium bicarbonate. This resulting glassy solid was freeze-dried to give the title compound in 42% isolated yield (17.5 mg, 0.023 mmol, 2.7 equiv. of TEA). ¹H-NMR (400 MHz; in D₂O) δ = 8.36 (1H, s, 6-H), 5.89 (1H, d, *J* = 4.4 Hz, 1'-H), 4.32 (1H, t, *J* = 5.1 Hz, 2'-H), 4.28 (1H, t, *J* = 4.4 Hz, 3'-H), 4.21-4.18 (1H, m, 4'-H), 4.13-4.11 (2H, m, 5'-H₂); ¹³C-NMR (100 MHz; in D₂O) 161.8 (C=O), 151.0 (C=O), 142.6 (C=CH), 123.2 (CF₃), 104.6 (C=CH), 89.7 (CH), 83.3 (CH), 74.0 (CH), 68.8 (CH), 58.9 (CH₂); ³¹P-NMR (162 MHz; in D₂O) δ = -6.61 (d, *J* = 23.2), -11.2, (d, *J* = 23.2); ¹⁹F-NMR (377 MHz; in D₂O) δ = -63.2.

(3) HPLC chromatograms

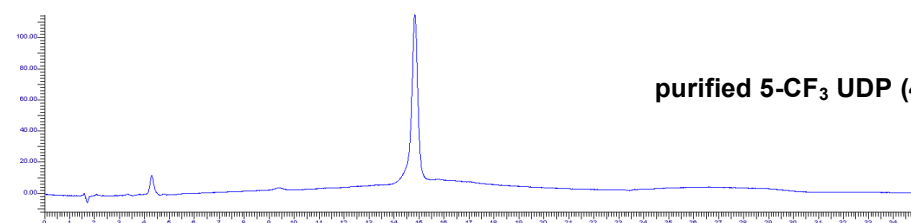
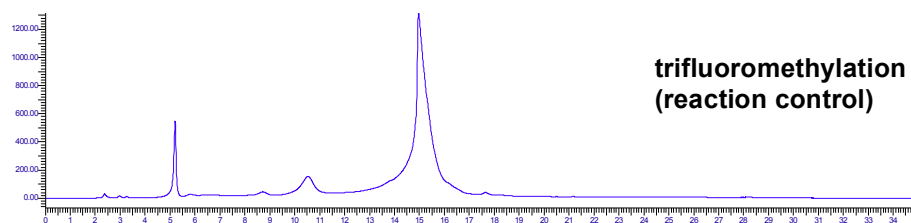
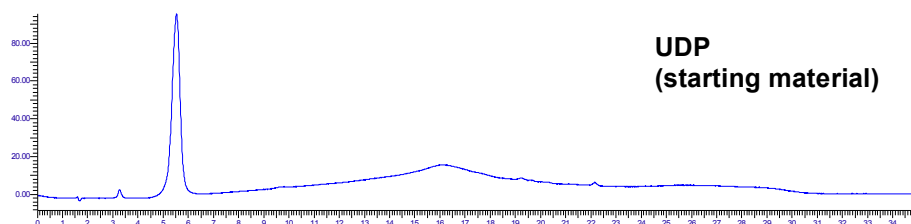
Synthesis of 5-CF₃ UDP-Gal (2)



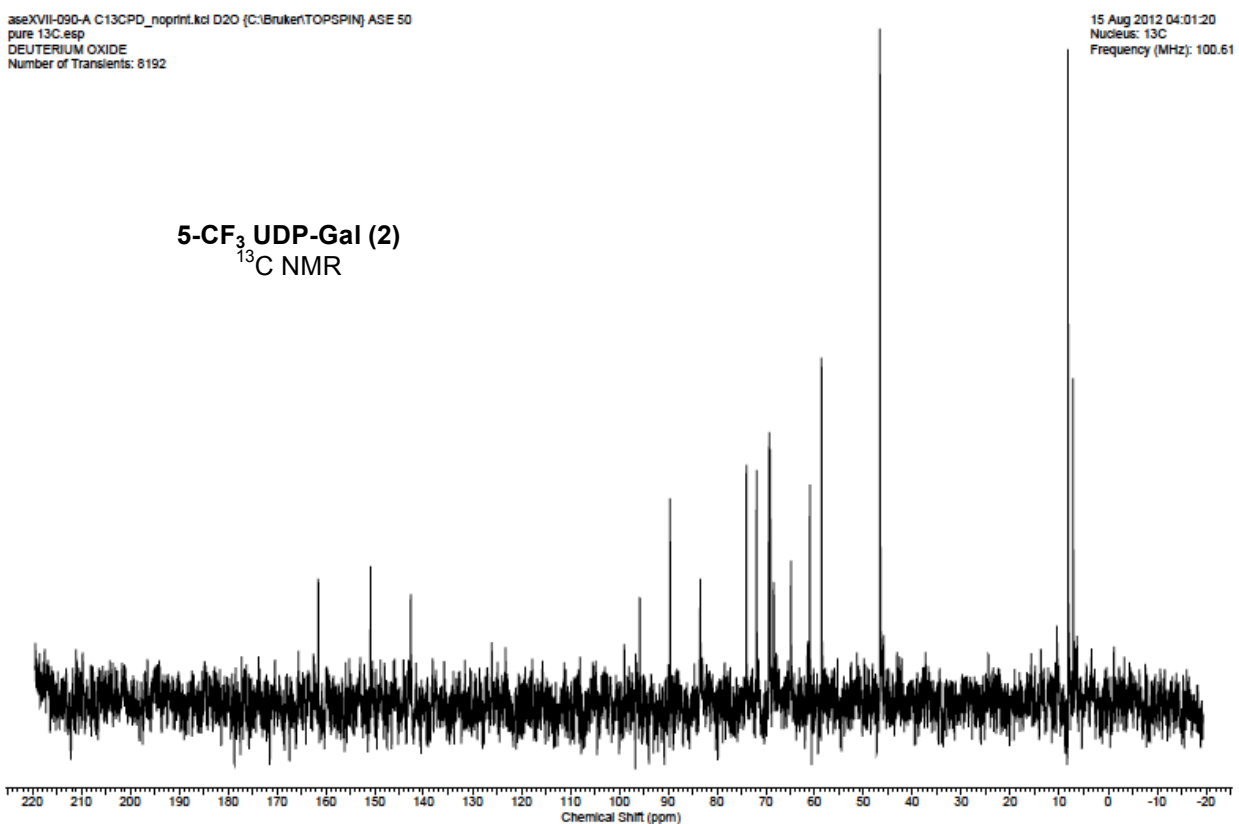
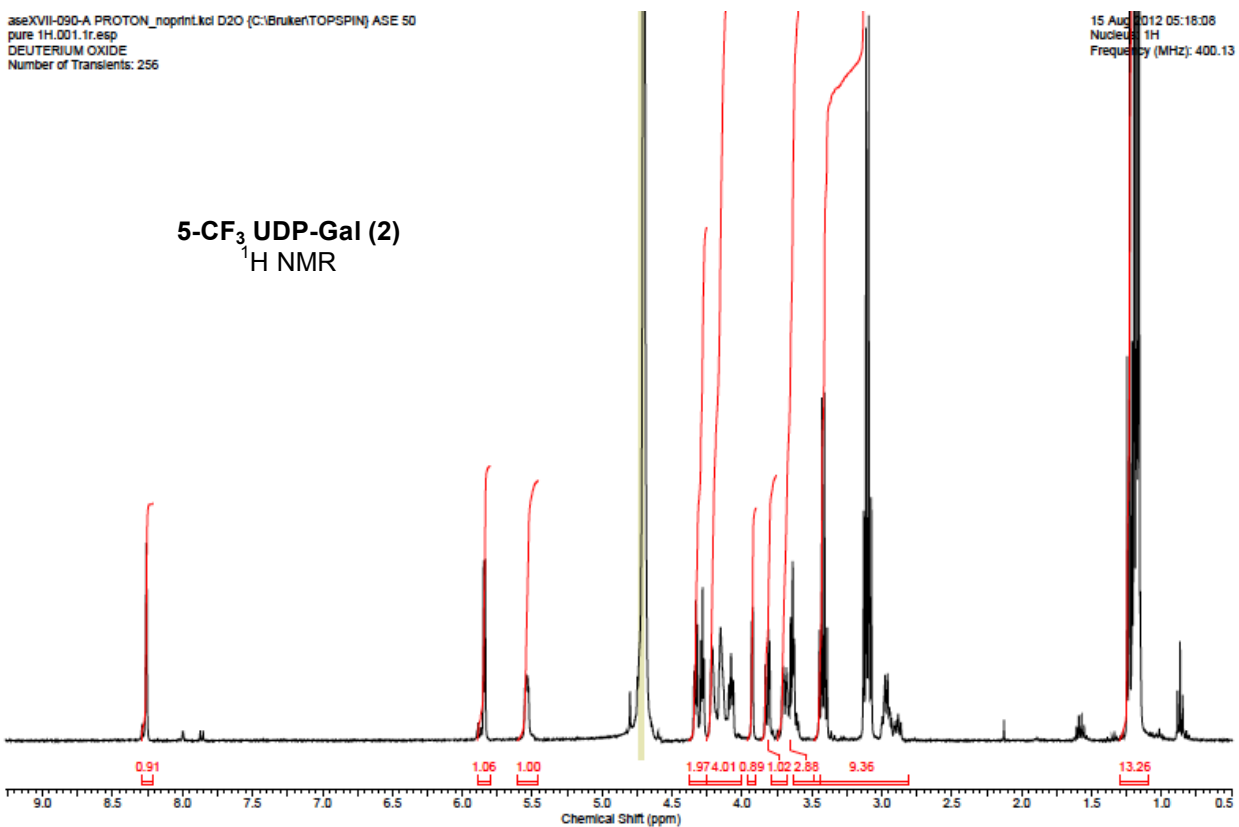
Synthesis of 5-CF₃ UDP-Glc (3)



Synthesis of 5-CF₃ UDP (4)



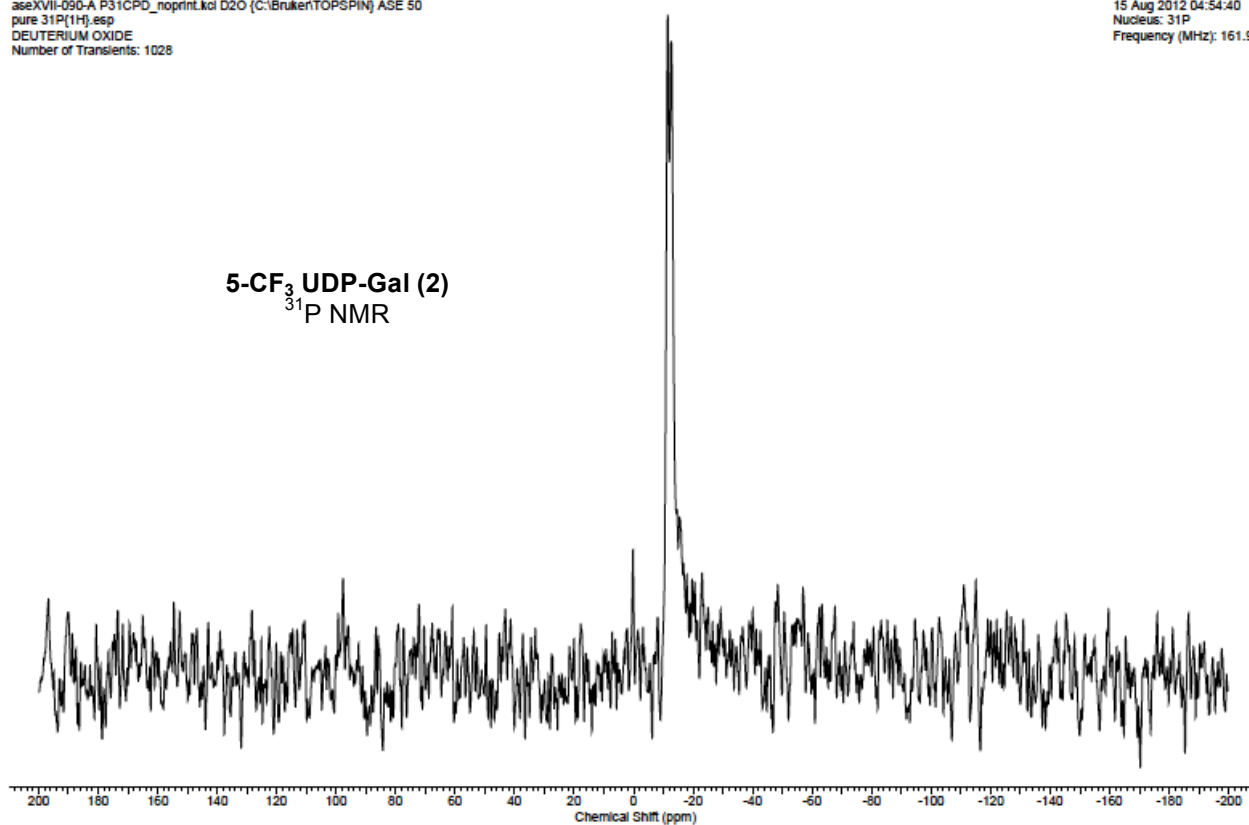
(4) NMR spectra of compounds 2, 3 and 4



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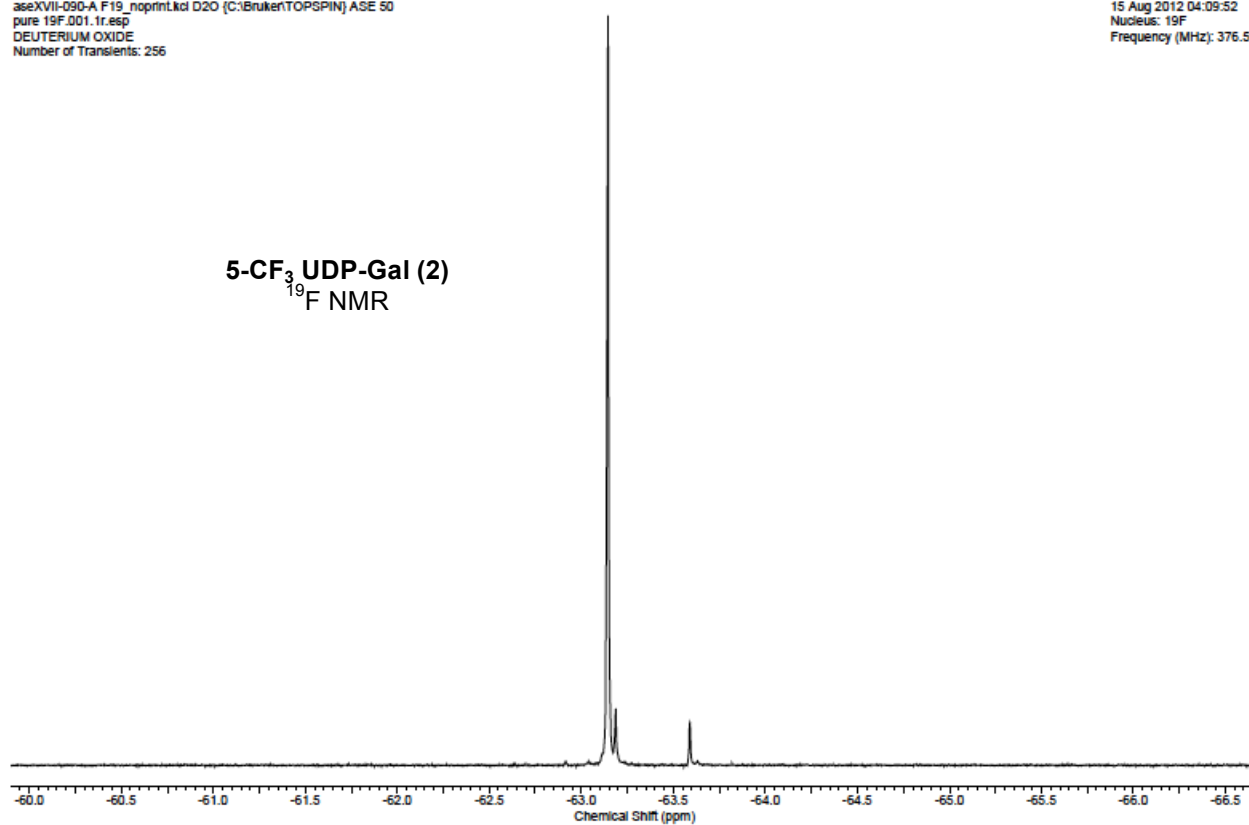
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³¹P NMR



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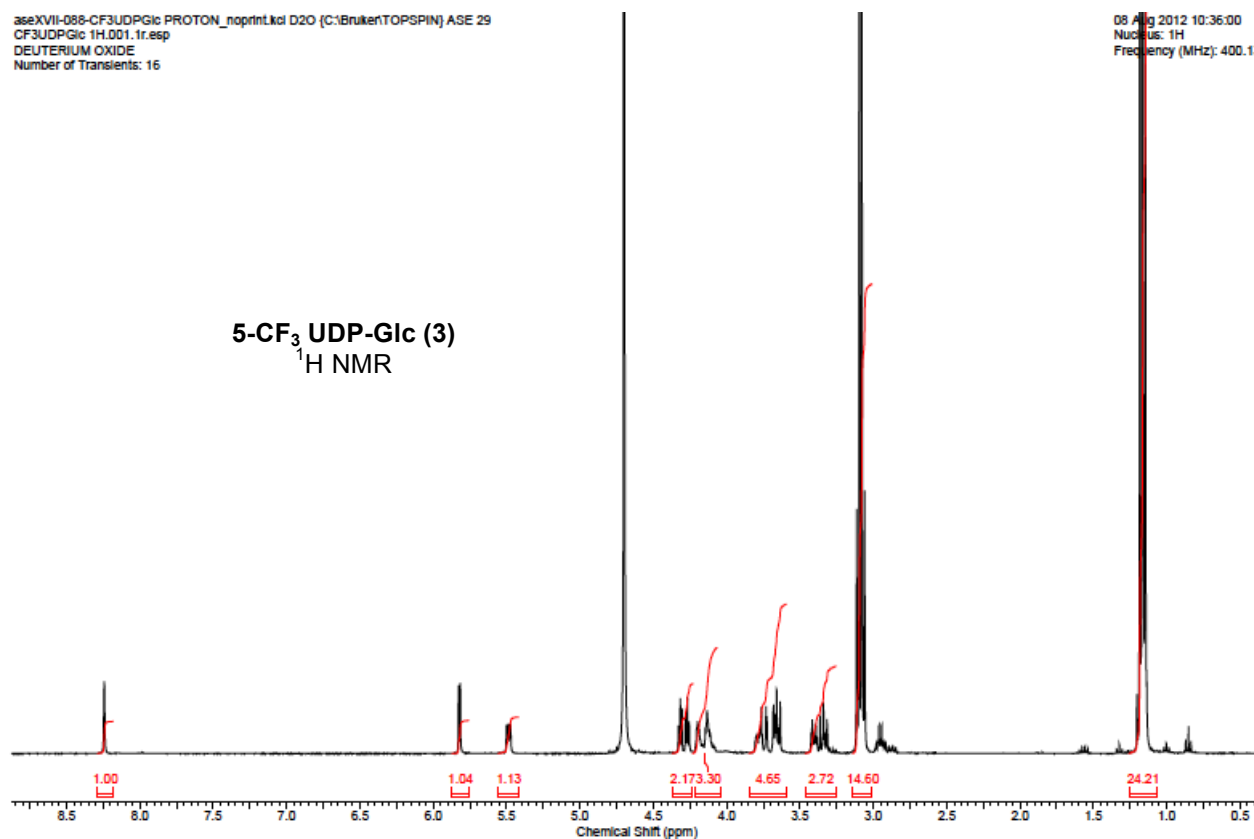
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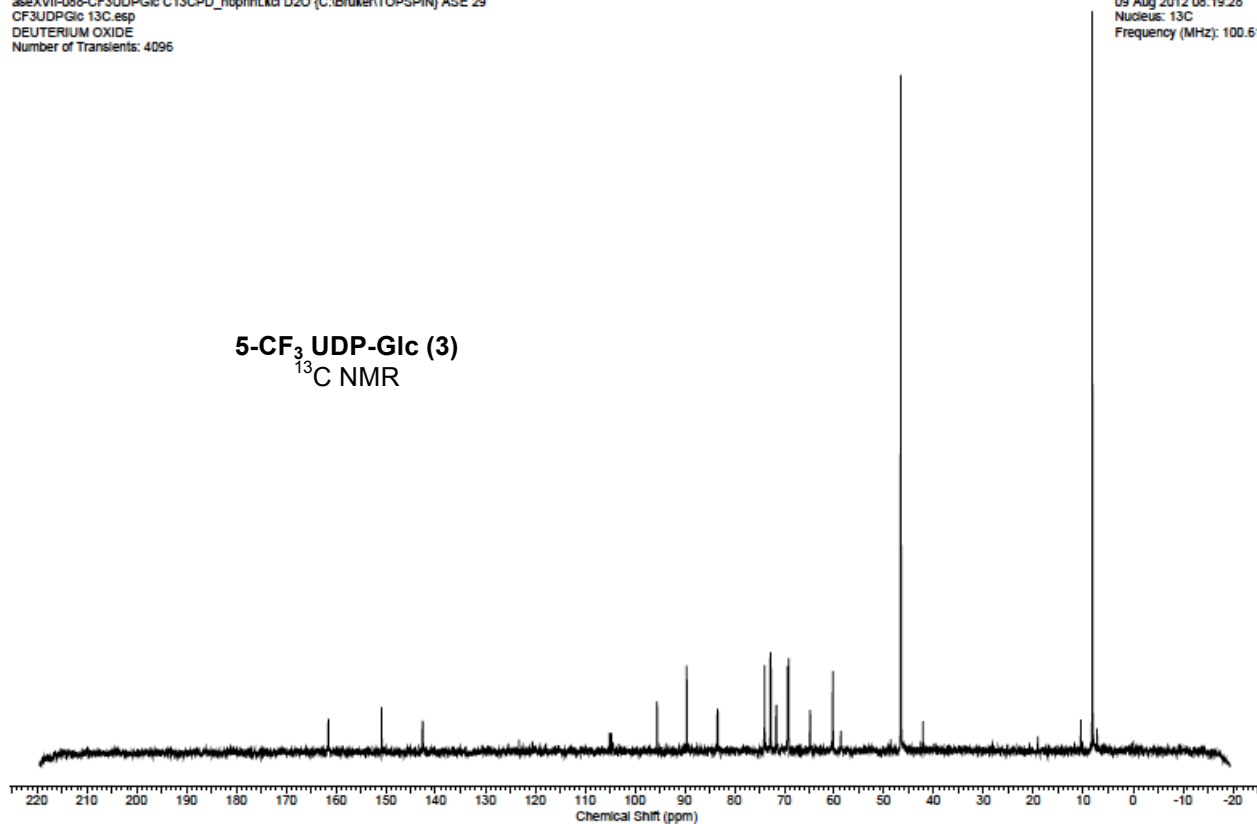
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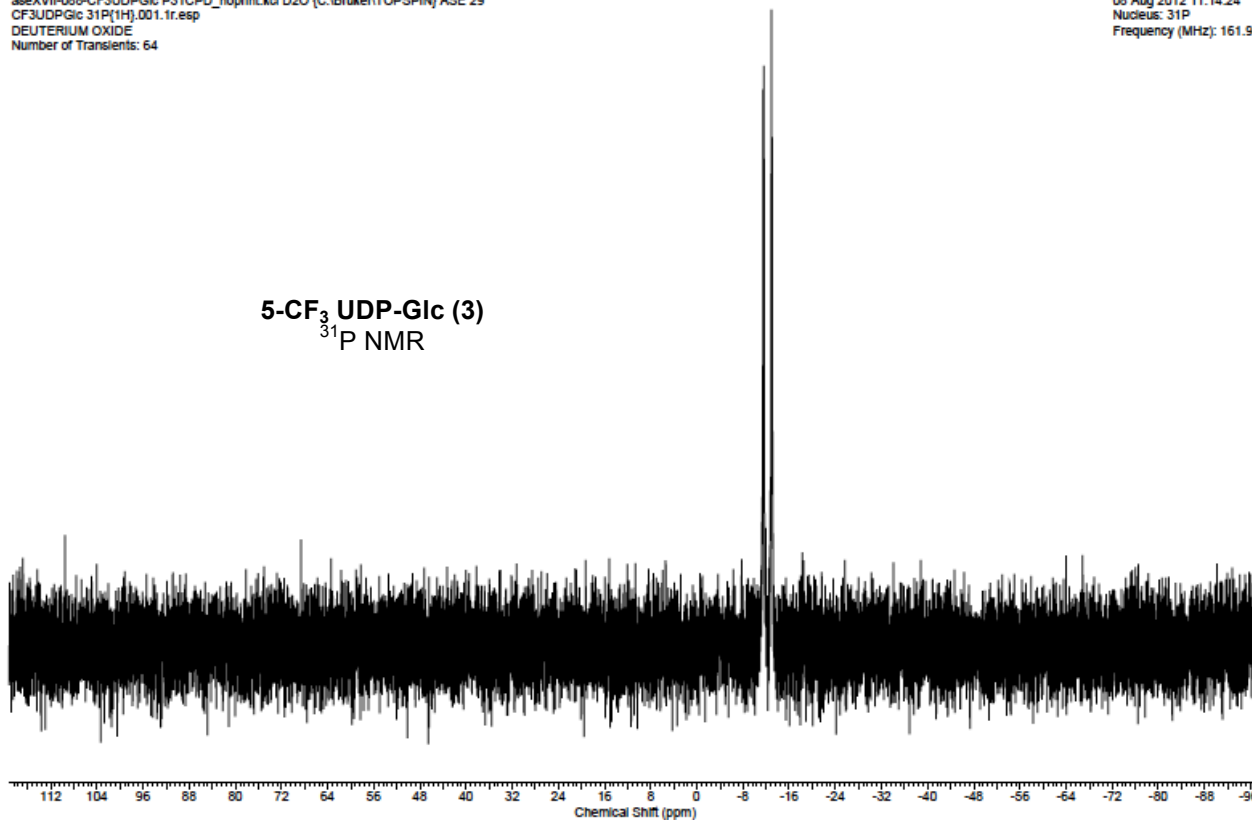
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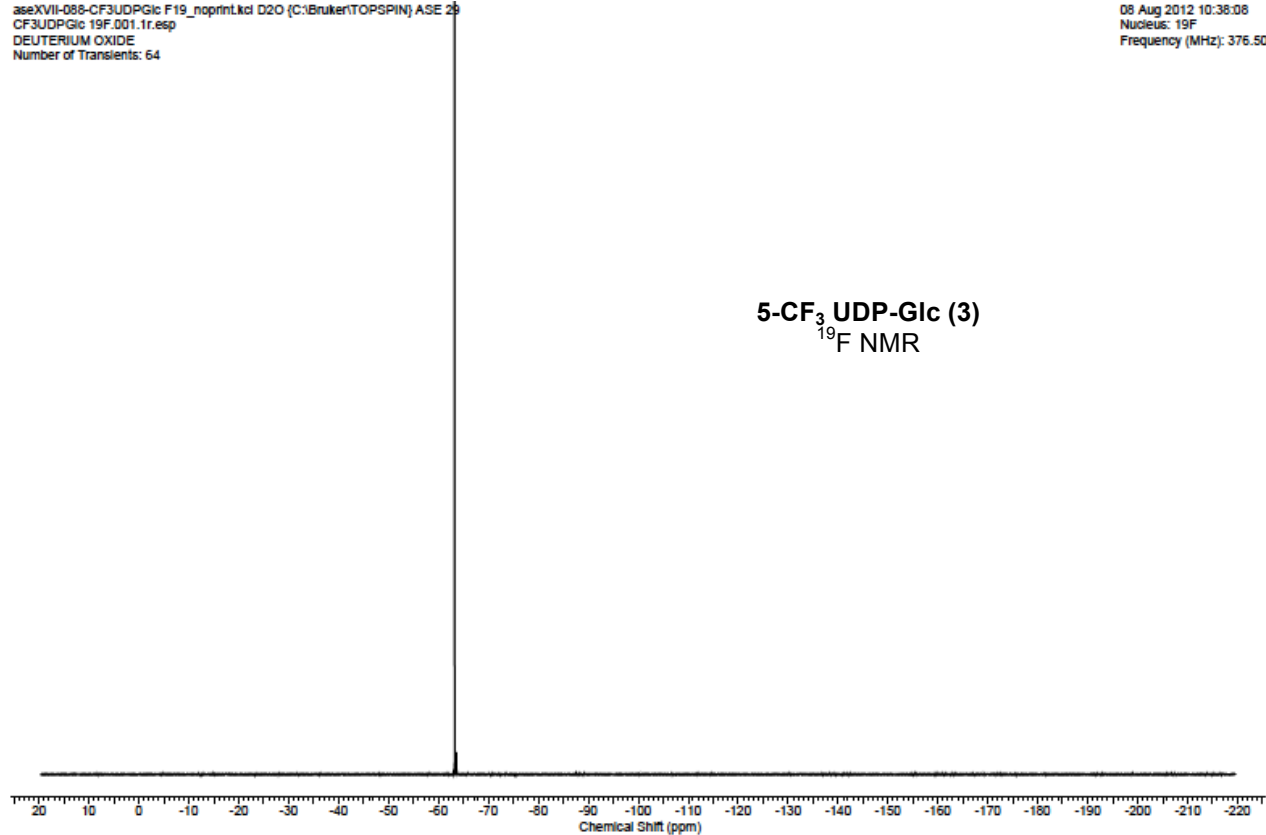
5-CF₃ UDP-Glc (3)
³¹P NMR

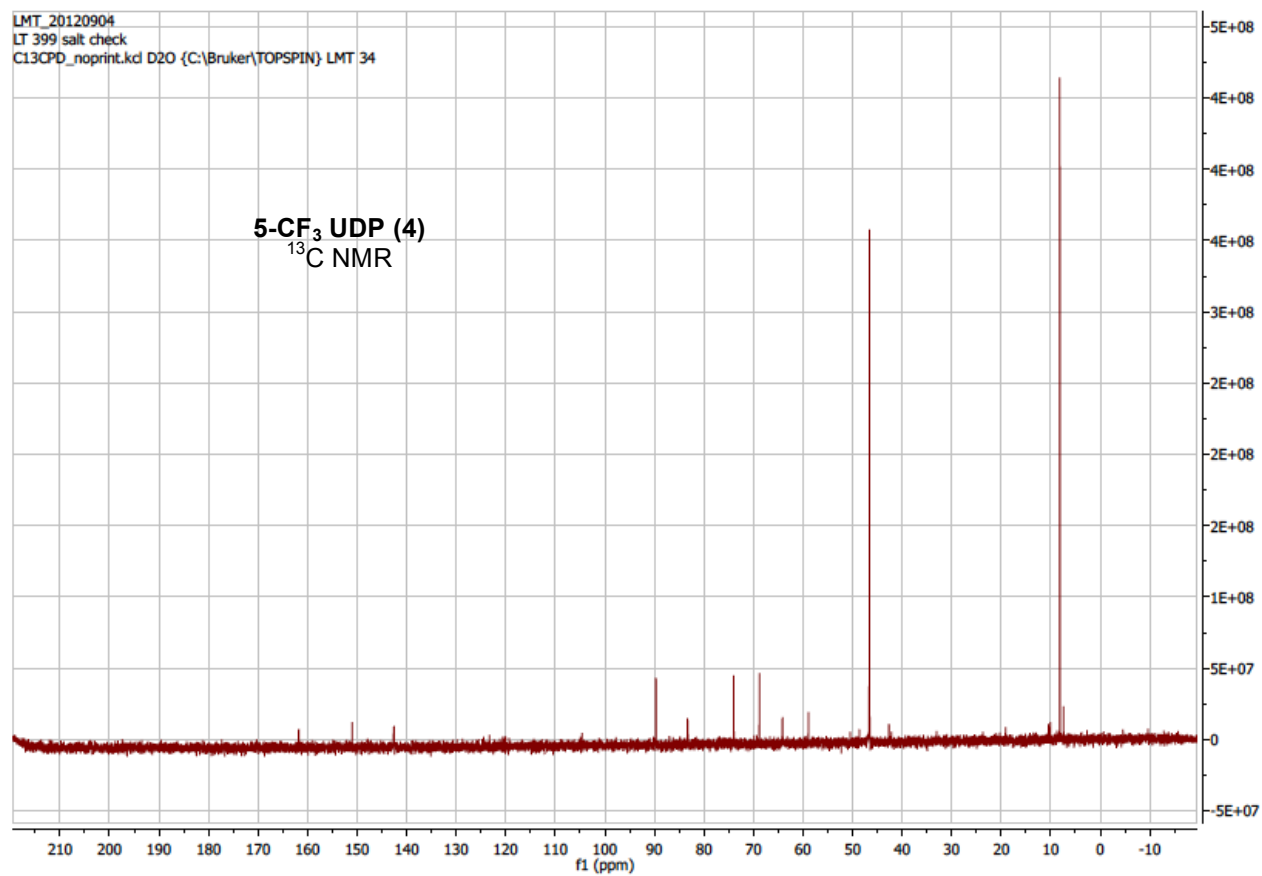
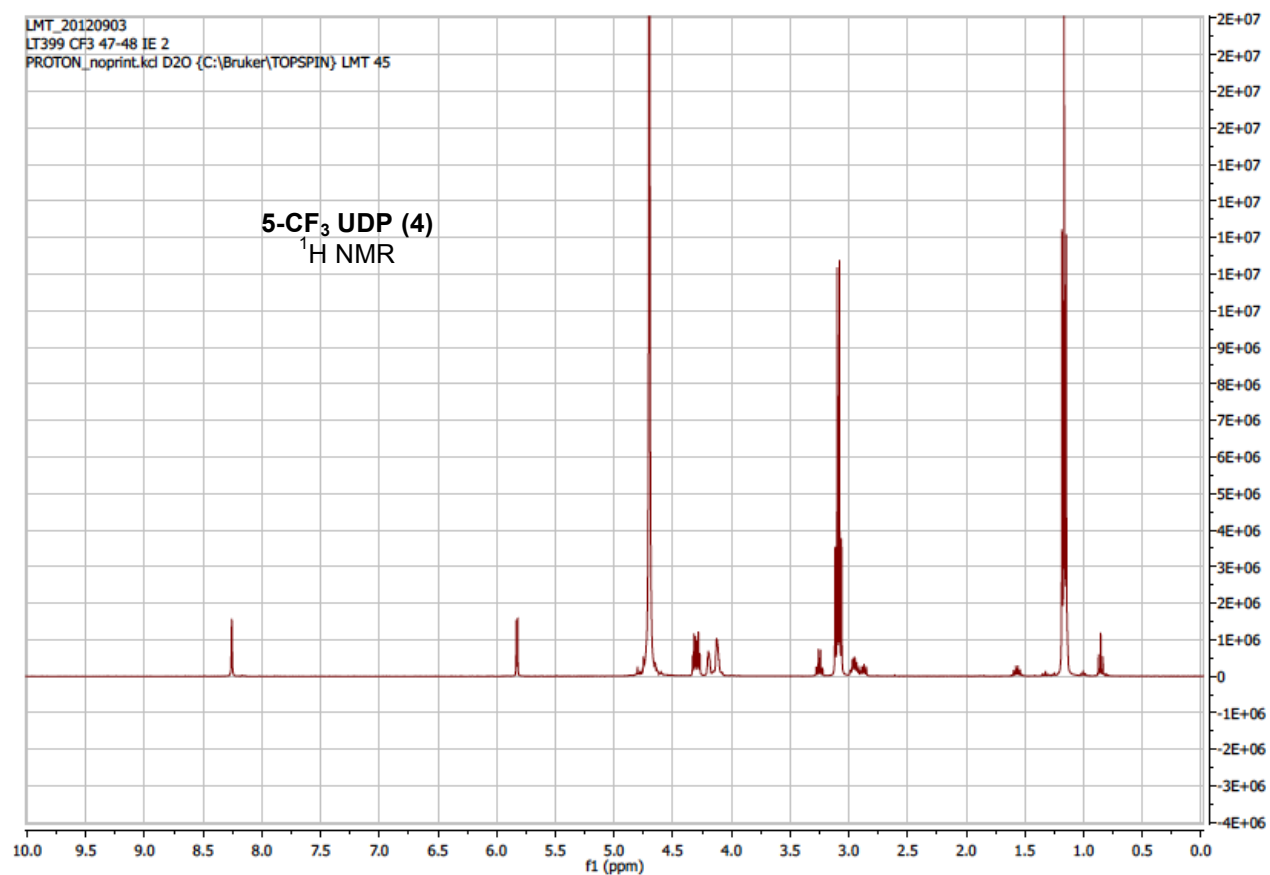


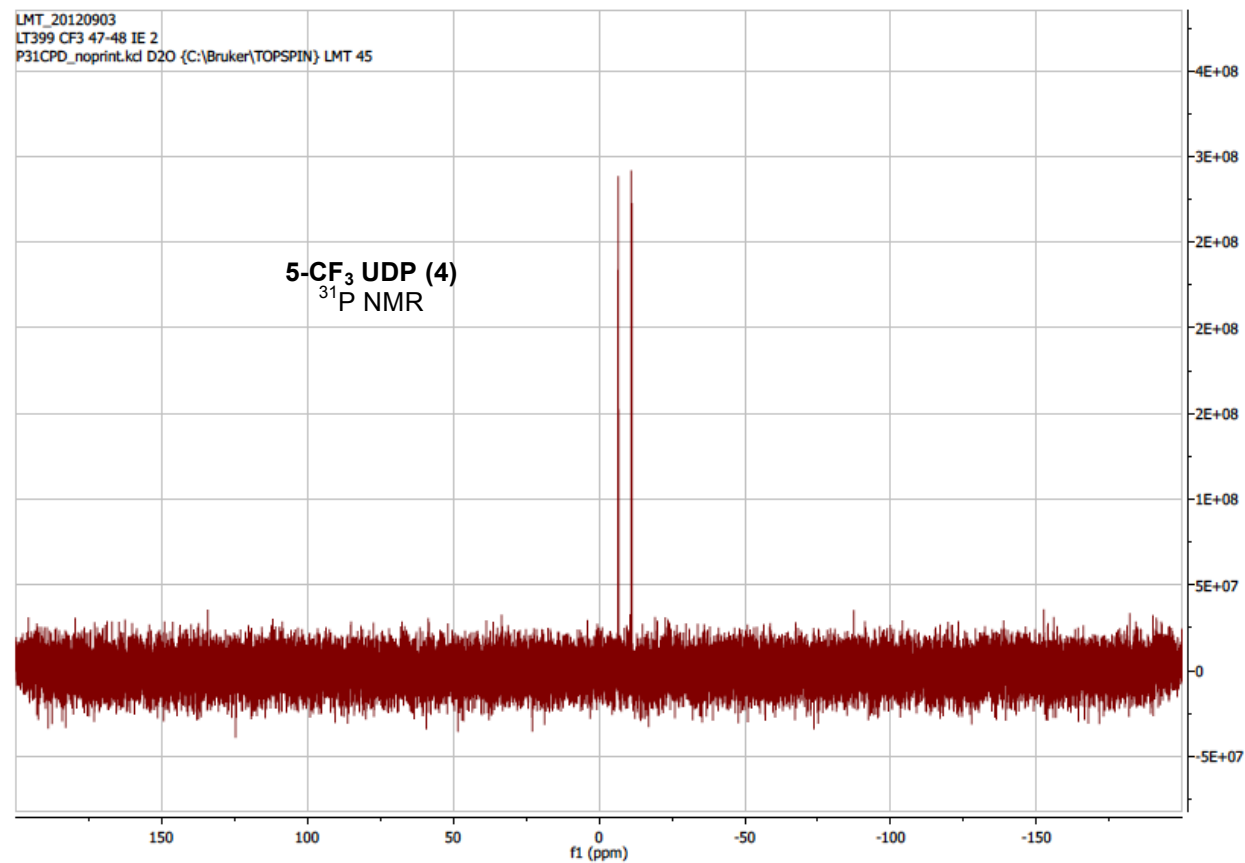
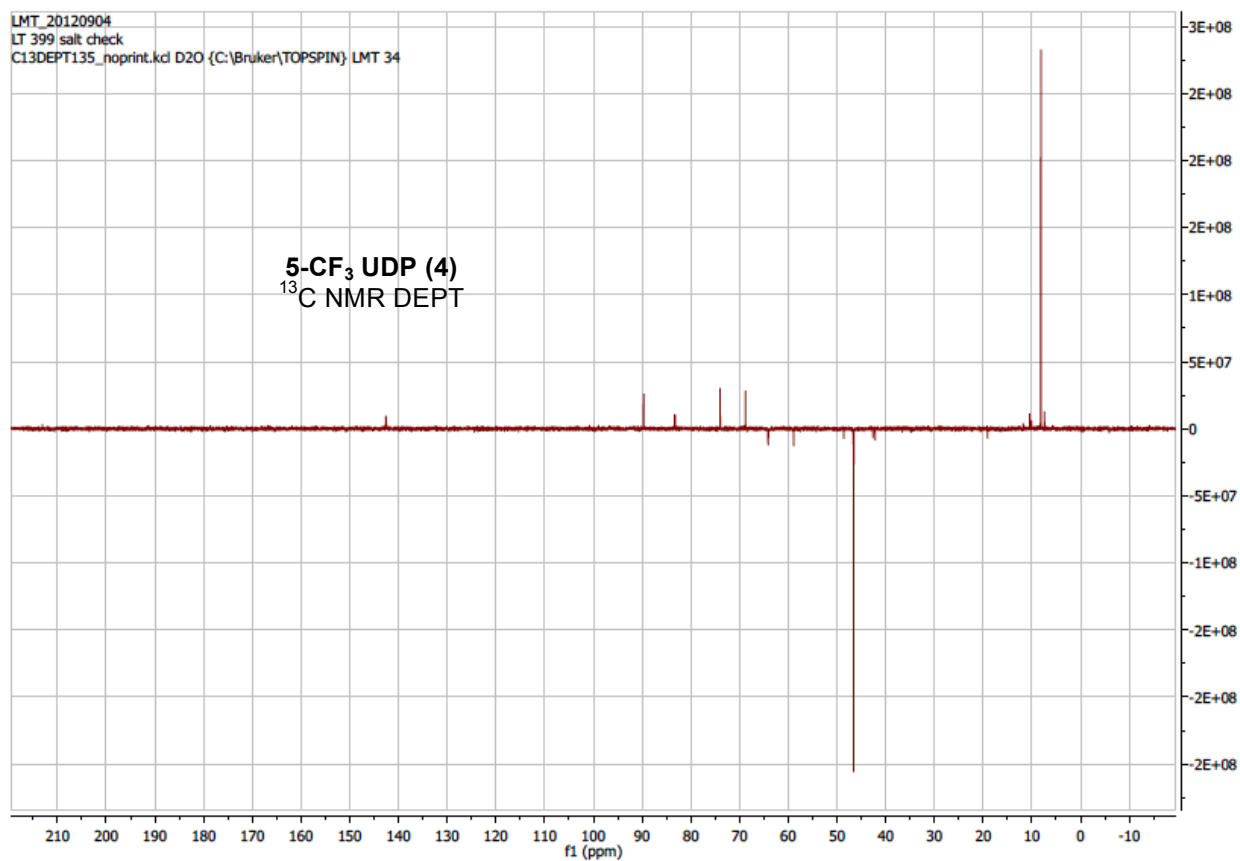
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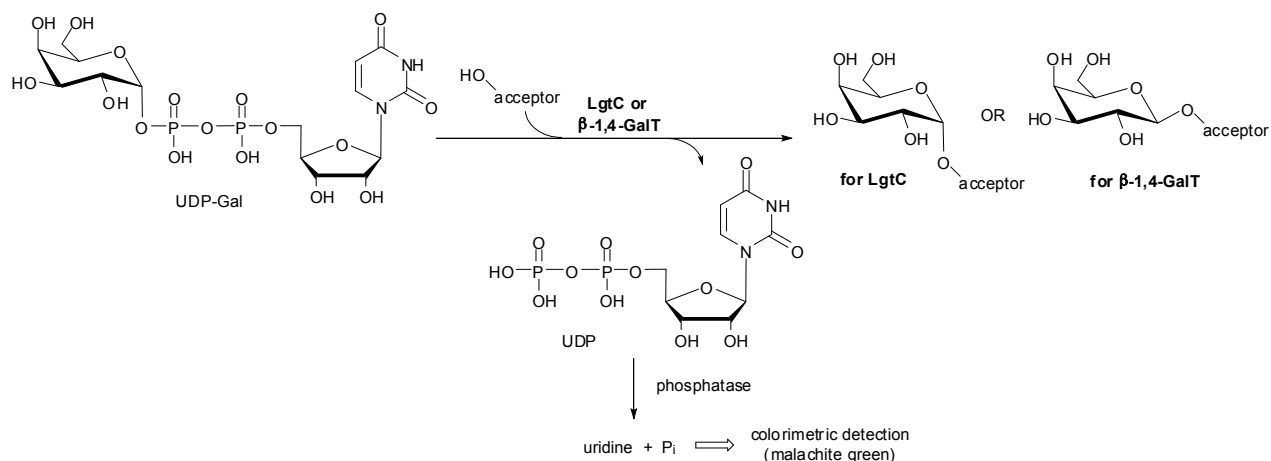
5-CF₃ UDP-Glc (3)
¹⁹F NMR







(5) Enzyme assays



Scheme 1 The glycosyltransferase reaction, and set-up of the colorimetric assay

Protein expression and general assay conditions. LgtC from *Neisseria meningitidis* and bovine β-1,4-GalT were expressed and purified as previously reported [1-3]. The plasmid for LgtC was a generous gift from Warren Wakarchuk (Toronto). The construct for bovine β-1,4-GalT (pET29b_b4GalT1Δ129 C342T) was kindly provided by Christelle Breton (Grenoble). The protocol for renaturation of inclusion bodies and refolding of β-1,4-GalT was adapted from Ramakrishnan et al. [3]. The colorimetric glycosyltransferase assay (see Scheme 1) was adapted from the previously published protocol by Wu et al. [4]. Full details of our modifications to the published protocol will be reported elsewhere. In brief, assays were carried out in Nunc clear, flat-bottom 96-well plates. The total volume per well was 150 μl and comprised of buffer (13 mM HEPES, 50 mM KCl, pH 7.0), MnCl₂ (5 mM), chicken egg-white lysozyme (1mg/ml), calf-intestinal alkaline phosphatase (CIAP; 10 U/ml), glycosyltransferase, acceptor (LgtC = lactose at 2 mM; β-1,4-GalT = GlcNAc at 10 mM), and either inhibitor in DMSO (10% v/v) or DMSO only (10% v/v). The reaction was started by addition of donor (UDP-Gal). MnCl₂, lysozyme, CIAP, glycosyltransferase, acceptor and inhibitor were added in 15 μL aliquots of the respective stock solution (10x assay concentration). UDP-Gal was added in 30 μL aliquots of the stock solution (5x assay concentration). The concentrations of glycosyltransferase, donor and inhibitor were varied depending on the experiment (see below). The reaction was allowed to run for 20 min at 30°C, after which time the reaction was stopped by the addition of 30 μl malachite reagent A. After thorough shaking, 30 μl of malachite reagent B was added and the colour allowed to develop over 20 min. The absorbance at 620 nm was recorded and the data used to calculate initial reaction velocities for each well. Each experiment was carried out in triplicate.

Calibration curve. In all assays, a calibration curve was constructed by using, instead of UDP-Gal, UDP (25, 12.5, 6.25, 3.13, 1.56 and 0 μM) as the starter. The absorbance at 620 nm was plotted against the concentration of UDP (Figure 1, red plot). This experiment also served as a positive control for CIAP activity.

Examination of 5-CF₃ UDP (4) as a CIAP substrate. A calibration curve was constructed for 5-CF₃ UDP **4** (0-25 μM) in the same way as for UDP. The absorbance at 620 nm was plotted against the concentration of **4** (Figure 1, blue plot). Comparison with the standard UDP calibration curve shows that the two plots are practically identical. This demonstrates that in the relevant concentration range (0-25 μM), UDP derivative **4** is recognized as a substrate by CIAP to the same extent as UDP itself under the conditions of the assay (20 min incubation, 10 U/ml CIAP).

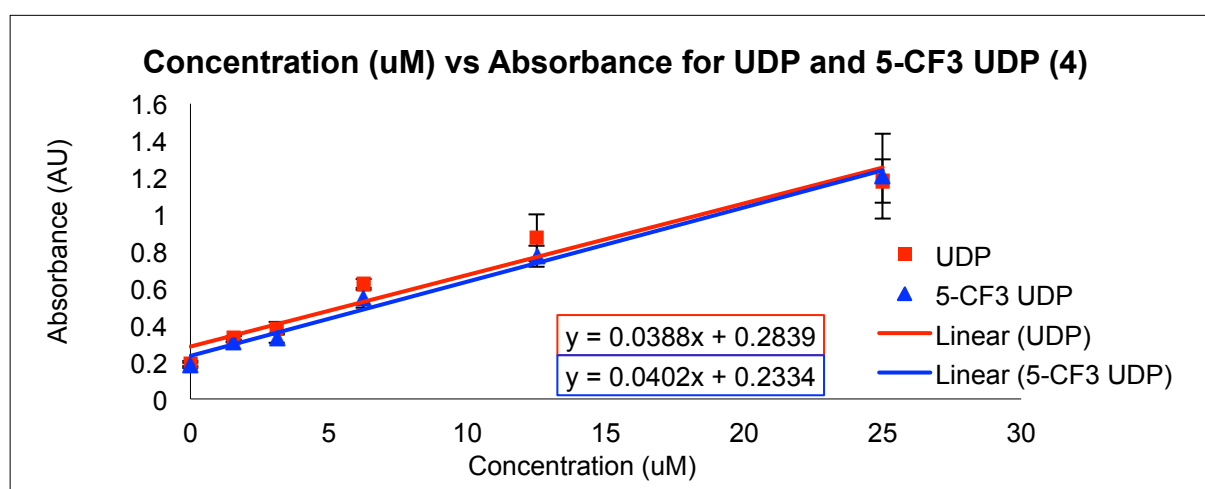


Figure 1 Calibration curves for the CIAP phosphatase reaction with UDP and 5-CF₃ UDP (**4**)

Examination of UDP-Gal analogues as glycosyltransferase substrates – determination of optimal glycosyltransferase concentration. The UDP-Gal analogue concentration was held at 50 μM and the concentration of glycosyltransferase was varied. A background was run where all components except the glycosyltransferase were present. The total turnover at 20 minutes (minus the background) was plotted against the amount of glycosyltransferase. This plot was used to determine the concentration of glycosyltransferase required to achieve 5% turnover over the 20 min period.

Examination of UDP-Gal analogues as glycosyltransferase substrates – determination of Michaelis-Menten kinetic values. The glycosyltransferase concentration was held constant at the value determined and the UDP-Gal analogue concentration was varied (100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 0 μM). A set of backgrounds at all UDP-Gal analogue concentrations were run, where all components were the same except for omitting the acceptor. After subtracting the appropriate background, the initial velocities were plotted against the UDP-Gal analogue concentration and the values for K_M and v_{max} were calculated using the Michaelis-Menten curve-fitting algorithm in Kaleidagraph 4.1.3 (Synergy Software).

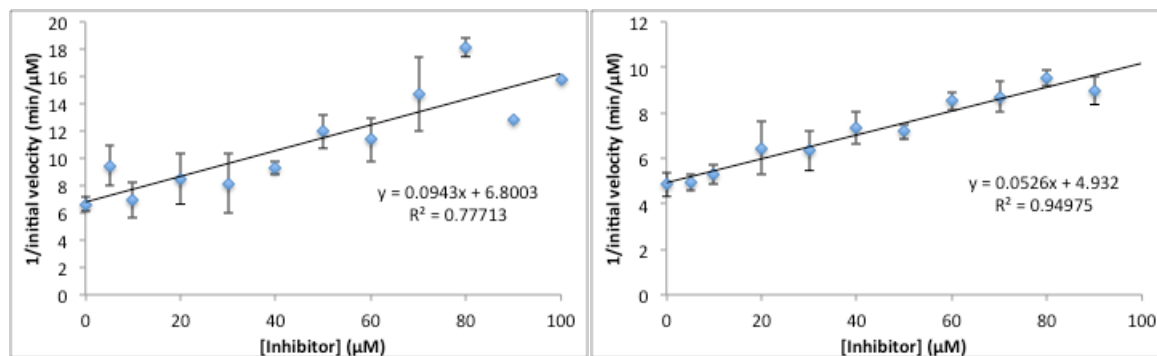
Examination of UDP-Gal analogues as inhibitors of glycosyltransferases. This experiment was performed after establishing that turnover of the requisite UDP-Gal analogue at the glycosyltransferase concentration used was negligible. Glycosyltransferase and UDP-Gal concentrations were held constant (LgtC at 1mU/ml and UDP-Gal at 26 μ M ($2\times K_M$); β -1,4-GalT at 0.4mU/ml and UDP-Gal at 18 μ M ($2\times K_M$)) and inhibitor concentration was varied (100, 90, 80, 70, 60, 50, 40, 30, 20, 10, 5, 0 μ M). A set of backgrounds at all UDP-Gal analogue concentrations were run, where all components were the same except for omitting the acceptor. After subtracting the appropriate background, the reciprocal of the initial velocity was plotted against inhibitor concentration and K_i values were determined according to the method of Dixon [5].

References

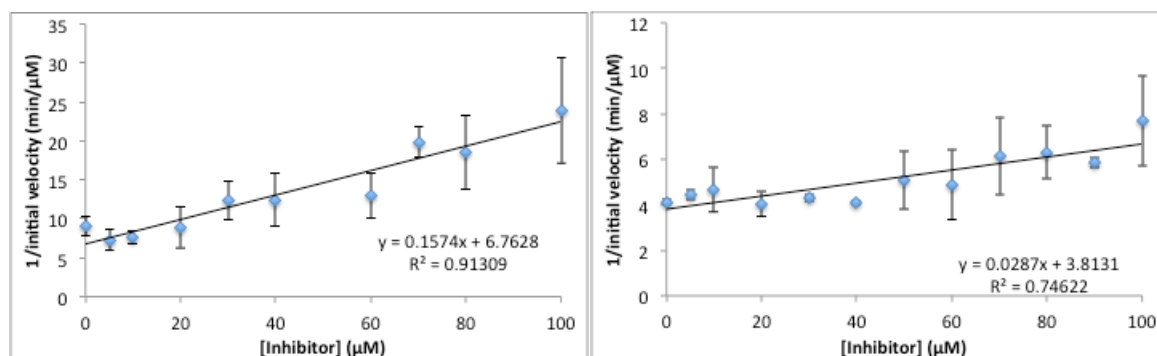
- [1] Persson, K., Ly, H.D., Dieckelmann, M., Wakarchuk, W.W., Withers, S.G. & Strynadka, N.C.J. Crystal structure of the retaining galactosyltransferase LgtC from *Neisseria meningitidis* in complex with donor and acceptor sugar analogs. *Nat. Struct. Biol.* **2001**, 8, 166-175.
- [2] Boeggeman, E. & Qasba, P.K. Studies on the metal binding sites in the catalytic domain of β 1,4-galactosyltransferase. *Glycobiology* **2002**, 12, 395-407.
- [3] Ramakrishnan, B., Shah, P.S. & Qasba, P.K. α -Lactalbumin (LA) stimulates milk β -1,4-galactosyltransferase I (β 4Gal-T1) to transfer glucose from UDP-glucose to *N*-acetylglucosamine. *J. Biol. Chem.* **2001**, 276, 37665-37671.
- [4] Wu, Z.L., Ethen, C.M., Prather, B., Machacek, M. & Jiang, W. Universal phosphatase-coupled glycosyltransferase assay. *Glycobiology* **2011**, 21, 727-733.
- [5] Irwin Segel, "Enzyme kinetics: behaviour and analysis of rapid equilibrium and steady-state enzyme systems", 1975, John Wiley and Sons

Dixon plots for inhibition experiments

5- CF_3 UDP-Gal (**2**) towards β -1,4-GalT (left) and LgtC (right)



5- CF_3 UDP-Glc (**3**) towards β -1,4-GalT (left) and LgtC (right)



UDP-Glc towards β -1,4-GalT (left) and LgtC (right)

