ELECTRONIC SUPPORTING INFORMATION

Enzyme-catalyzed synthesis of isosteric phosphono-analogues of sugar nucleotides

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General procedures and instrumentations:

A) Synthesis and Characterizations

NMR spectra were recorded on a Bruker AV 400 (^{1}H at 400, ^{13}C at 100 and ^{31}P at 162 MHz) or a Bruker AV 500 (^{1}H at 500, ^{13}C at 125 MHz) instrument. Chemical shifts (δ in ppm) are given relative to internal standard (Tetramethylsilane (TMS) in $CDCl_3$) or residual solvent peak (chloroform at 7.21 ppm in $CDCl_3$ and water at 4.70 ppm in D_2O). *J* values are in Hz. Phosphorus-31 NMR spectra were recorded with proton decoupling and externally referenced with 85% phosphoric acid, 0.00 ppm. Assignments were made by comparison of chemical shifts, peak multiplicities, *J* values, ^{1}H-^{1}H COSY and n.O.e spectra. The abbreviation "app." (apparent) in ^{1}H-NMR assignments refers to the appearance of the multiplet observed. Hydrogen and carbons were numbered for NMR report as standard numbering of carbohydrates. The sub-assignment of "a" and "b" in ^{1}H-NMR was based on the appearance of corresponding signal at higher and lower field, respectively. Carbon spectra were recorded with proton broadband decoupling. Assignments were verified using DEPT and ^{1}H-^{13}C HMQC experiments. The central peak of chloroform at 77.1 ppm was used as the internal reference for spectra run in $CDCl_3$. Acetone in D_2O (30.63 ppm, Me) was used as external reference for spectra run in D_2O. Mass spectra were determined using ESI (Micromass LCT, ref. Erythromycin). Elemental analyses were conducted on an Exeter analytical, Inc. CE-440 Elemental Analyser. Infrared (IR) spectra were recorded in the range of 4000-500 cm^{-1} using a Perkin Elmer 1600 series FT-IR spectrometer. Optical rotation was measured with a digital Jasco polarimeter DIP-370 in a 0.5 dm cell at ambient temperature (23±1 °C) and $[\alpha]_D$ values are given in units of 10^{-1} deg cm^2 g^{-1}. High quality deionised water (>17 MΩ) was used in all purification steps. It was prepared using an Elgastat UHQII, then degassed for 30 min with a Decon F5100b ultrasonic instrument. Ion exchange resins were pre-washed several times with deionised water before use. Chromatography is medium pressure flash chromatography and was performed according to the method of Still *et al.* using Fluorochem silica gel (35-70 μm) with the eluent specified.[1] Thin layer chromatography was performed on precoated aluminium–backed silica gel plates

supplied by E. Merck, A. G. Darmstad, Germany (silica gel 60 F254, thickness 0.2 mm, Art. 5554). Chromatograms were initially examined under UV light and then visualised with aqueous potassium permanganate (dip) or phenol solution (10 g phenol, 5 mL H_2SO_4, 95 mL ethanol), followed by warming of the TLC plate with a heat gun. Thin layer cellulose chromatography was run on the Eastman chromatogram 6065 with fluorescence indicator. Spots were visualised by the specified dips. All anhydrous reactions were carried out in oven-dried glassware (>180 °C), which was cooled in desiccator and was subjected to vacuum-N_2 flashing before use. Molecular sieves were activated before applications by storing in an oven (>180 °C) for 12 h. Where necessary THF were refluxed and distilled from sodium-benzophenone ketyl, and DCM from calcium hydride, immediately before use. Anhydrous DMF was prepared through overnight stirring with calcium hydride, followed by distillation under reduced pressure. It was then collected over 3 Å molecular sieves. Triethylamine dried with CaH_2, then distilled and collected over 4 Å molecular sieves. Chloroform was distilled from P_2O_5 (3% w/v) and distillates were collected over 4 Å molecular sieves. Light petroleum refers to the fraction boiling in the range 40-60 °C that was redistilled before use. Organic extracts were dried over $MgSO_4$ before evaporation, unless otherwise specified. Evaporations were achieved using a Büchi rotary evaporator followed by drying at <1 mmHg using an Edward rotary vacuum pump. An Edward Freeze-dryer was used for lyophilization of samples. Except where specified all reagents were purchased from commercial sources and were used without further purification. Yields are for isolated, chromatographically homogenous and analytically pure products.

B) Enzymatic assays:

Enzymatic reactions were analyzed using HPLC performed on a Hewlett Packard Series 1050 instrument using an Agilent Zorbax 5 μm Rx-C18 column (150 cm x 4.6 mm). Compounds bearing a nucleotide base chromophore were monitored at an absorbance of 254 nm. Reactions were monitored by HPLC using a linear gradient from 90:10 A:B to 40:60 A:B over 8.0 min followed by a plateau at 40:60 A:B from 8.0 to 10.0 min at 1.0 mL/min where A is an aqueous buffer containing 12 mM *n*-Bu_4NBr, 10 mM KH_2PO_4, and 5% HPLC grade CH_3CN (pH 4.0) and B is 100% HPLC grade CH_3CN. Low resolution mass spectra were obtained using an Applied Biosystems hybrid triple

quadrupole linear ion trap (*Qtrap 2000*) mass spectrometer equipped with an electrospray ionization (ESI) source, used in negative ion mode. The capillary voltage was set to –4500 kV with a declustering potential of –60 V and the curtain gas was set to 10 (arbitrary units). During sample analysis, the solvent (50:50 methanol:water) was constantly infused into the ion source at 10 μL/min by the built-in syringe pump and the samples were directly injected into the mass spectrometer after dilution (to 1 μM) with the above solvent. An enhanced mass spectrum (EMS) was obtained by scanning from *m/z* 100 to 650. Product ions were then fragmented to confirm their identities with an enhanced product ion (EPI) scan using collision energy of -60 V.

Enzymatic reactions containing 1.0 mM NTP, 2.0 mM sugar-1- phosphonate, 2.2 mM MgCl2, and 0.5 EU inorganic pyrophosphatase were initiated by the addition of 2 EU nucleotidylyltransferase in Tris-HCl buffer (20 mM final buffer concentration, 50 μL reaction volume). The reactions were incubated for 30 min or 24 h at 37 °C, quenched with methanol (50 μL), and centrifuged (5 min at 12,000 x *g*) to precipitate the denatured enzymes prior to HPLC analysis. In the absence of nucleotidylyltransferase, NTP, sugar-1-phosphonate, or $MgCl_2$, no product formation was observed. Cps2L nucleotidylyltransferase was purified to an activity of 1EU/μL by methods described before.[2]

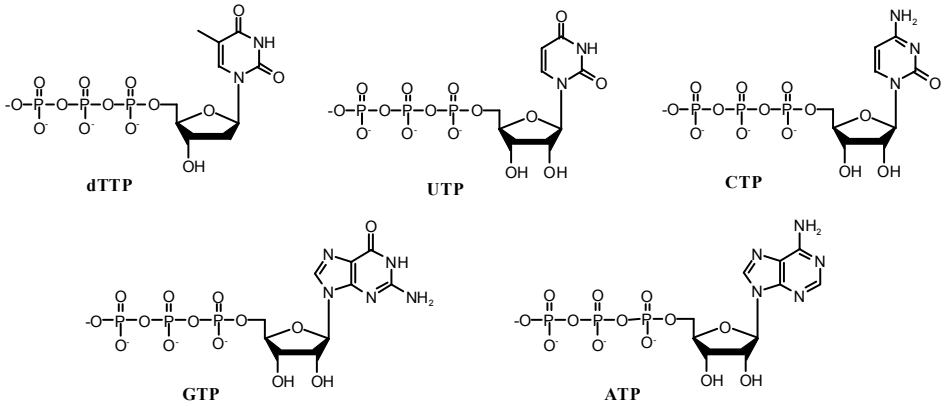


Figure S1: Nucleoside triphosphate structures

C) Kinetics Assay Conditions

Enzymatic assays were performed using same method from above. Enzymatic reactions containing 1.0 mM dTTP, 2.2 mM $MgCl_2$, 2 EU inorganic pyrophosphatase, 100 μM thymidine (internal standard) and 50 μM, 75 μM, 100 μM, 200 μM or 300 μM sugar-1-phosphate were initiated by the addition of Cps2L (0.002 EU), Cps2L (32 EU) and (1.6

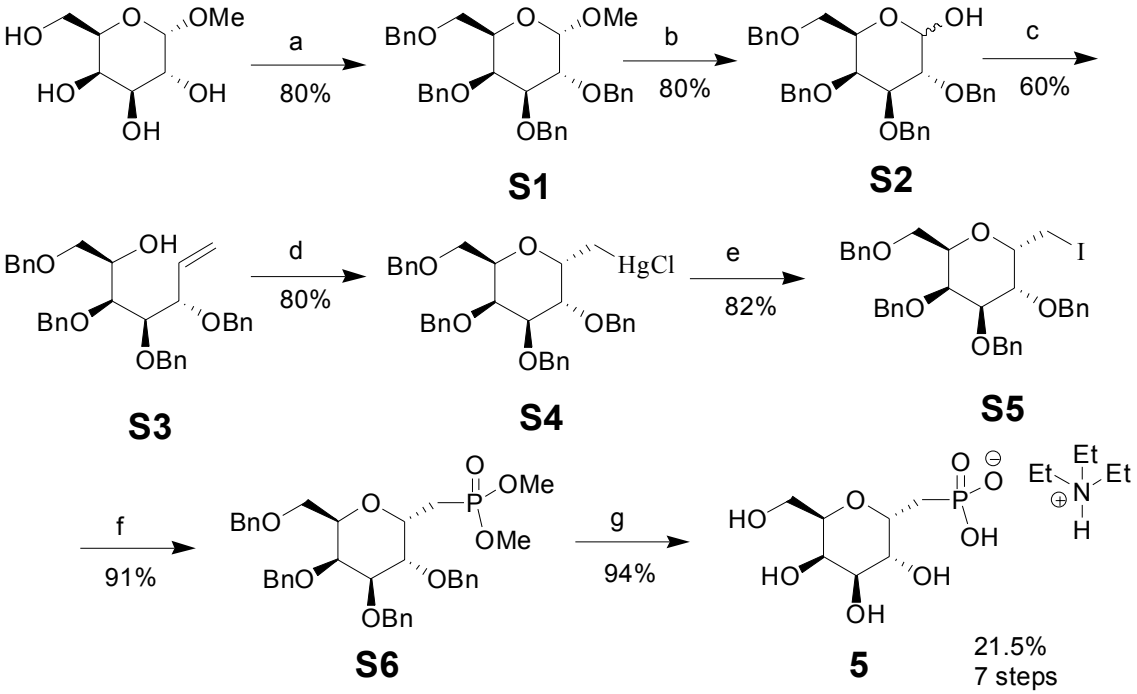
EU) for α-D-glucose-1-phosphonate, α-D-galactose-1-phosphonate and α-D-galactose-1-phosphate respectively (200 μL reaction volume). The enzymatic reactions were conducted at 37 °C and monitored at 1, 2, 4, 6, and 10 minutes (α-D-glc-1-phosphonate); 2, 4, 6, 10, and 15 minutes (α-D-gal-1-phosphonate); 10, 11, 13, 15, and 19 minutes (α-D-gal-1-phosphate). Enzymatic reaction aliquots (40 μL) were quenched with HPLC grade MeOH (40 μL) and centrifuged (5 min at 12,000 x *g*) to precipitate denatured enzymes prior to HPLC analysis. The HPLC method used to determine conversions was as described above. Final concentrations of the sugar nucleotides were determined by comparing the peak area of the product to that of the thymidine internal standard. Initial rates were determined by plotting concentration of sugar nucleotide products versus time. Michaelis-Menten and Lineweaver-Burk plots were fitted using GraFit 5.0 software.

D) pKa determination

pKa values were measured in the following way: Using an IQ Scientific Instruments IQ150 fitted with an ISFET probe, a 0.01 M solution of the diammonium salt was adjusted to pH 10 with 0.2 M NaOH. Titration was done with 5 uL aliquots of 0.2 M HCl until pH 2, and the pKa values were determined by plotting in GraFit 5.0.4 (Erithacus Software Limited).

Experimental:

A-1) Synthesis of *C*-(1-Deoxy-α-D-galactopyranosyl) methane phosphonate (5)[3]



Scheme 1. a) NaH, BnBr, (n-Bu)$_4$NI, DMF, rt, 18 h; **b)** CH_3COOH/H_2SO_4, 95 °C, 3 h; **c)** n-BuLi/Hexanes, CH_2=P(Ph)$_3$, THF, rt, 12 h; **d)** i) $Hg(CH_3COO)_2$, THF, rt, 18 h ii) KCl, rt, 10 min; **e)** I_2, DCM, rt, 16 h; **f)** $P(OCH_3)_3$, reflux, 16 h, **g)** i) TMSI, $CHCl_3$, 0 °C, 1.5 h, ii) Purification then TEA, rt, 16 h.

Methyl-2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranoside (S1)[4]

Methyl α-D-galactopyranoside (3 g, 15.4 mmol) was dissolved in anhydrous DMF (30 ml). The resulting solution was cooled and sodium hydride (60% in oil, 2.7 g, 68 mmol) was added slowly. Then, benzyl bromide (8.3 ml, 68 mmol) and tetrabutylammonium iodide (0.26 g, 0.7 mmol) were added. The resulting suspension gradually turned yellow. The solution was stirred for 18 h at room temperature under N_2. The excess NaH was destroyed by slow addition of methanol (40 ml), and the solvent was removed *in vacuo*.

DCM (50 ml) and water (50 ml) were added to residue, and the organic phase was separated, washed with water (30 ml) and brine (15 ml), dried ($MgSO_4$), filtered and concentrated *in vacuo* to give the crude product which was purified by column chromatography (light petroleum-EtOAc, 4:1) to afford compound **S1** (6.8 g, 80%) as a colourless syrup; R_f 0.33 (light petroleum-EtOAc, 4:1); δ_H (400 MHz; $CDCl_3$) 3.36 (3H, s, -OC*H*$_3$), 3.5 (2H, app. d, *J* 6.4, 6a-H and 6b-H), 3.81 (1H, app. dt, $J_{5,6a} \sim J_{5,6b} \sim 6.4$ and $J_{5,4} \sim 1$, 5-H), 3.84_6 (1H, obscured dd, $J_{3,2}$ 10.8 and $J_{3,4} \sim 3$, 3-H), 3.84_8 (1H, obscured dd, $J_{4,3} \sim 3$ and $J_{4,5} \sim 1$, 4-H), 4.04 (1H, dd, $J_{2,3}$ 10.8 and $J_{2,1}$ 3.6, 2-H), 4.39 and 4.47 (AB, each 1H, d, $^2J_{A,B}$ 11.8, CH_AH_BPh), 4.69 (1H, d, $J_{1,2}$ 3.6, 1-H), 4.68 and 4.82 (AB, each 1H, d, $^2J_{A,B}$ 12, CH_AH_BPh), 4.73 and 4.84 (AB, each 1H, d, $^2J_{A,B}$ 11.8, CH_AH_BPh), 4.57 and 4.94 (AB, each 1H, d, $^2J_{A,B}$ 11.5, CH_AH_BPh), 7.25-7.35 (20H, m, Ph); δ_C (100 MHz, $CDCl_3$) 55.4 (CH_3, -O*C*H$_3$), 69.1 (CH_2, C-6), 69.3 (CH, C-5), 73.3, 73.5, 73.6, 74.8 (all CH_2, 4x*C*H$_2$Ph), 75.2 (CH, C-4), 76.5 (CH, C-2), 79.1 (CH, C-3), 98.8 (CH, C-1), 127.5, 127.6, 127.7, 127.8, 128.1, 128.2, 128.3, 128.3_6, 128.4 (all CH, 4xPh), 138.0, 138.6, 138.7, 138.9 (all C, 4xPh); HRMS (ESI): found $(M+Na)^+$ 577.2540. $C_{35}H_{38}O_6$ requires $(M+Na)^+$, 577.2569.

2,3,4,6-Tetra-*O*-benzyl-D-galactopyranose (S2)[4]

A solution of **S1** (670 mg, 1.2 mmol) in glacial acetic acid (15 ml) and sulphuric acid (6 ml, 1 M) was heated at 95 °C under N_2 for 3 h. The solution was then adjusted to pH = 7 with sodium hydrogen carbonate (8 g) and the solution was extracted with DCM (100 ml). The organic extract was washed with water (50 ml) and saturated aqueous $NaHCO_3$ (25 ml), dried ($MgSO_4$), filtered and concentrated. The residue was then purified by column chromatography (light petroleum-EtOAc, 4:1) to give product **S2** (433 mg, 0.96 mmol, 80%) as a colourless syrup which was a mixture of α and β anomers produced in a ratio of 1.7:1 as determined by ^{1}H-NMR; R_f 0.2 br. spot (light petroleum-EtOAc, 4:1); υ_{max} ($CHCl_3$)/cm^{-1} 3750, 3645w (free OH), 3155, 2901 (C-H, aliphatic), 1816, 1794m (C=O, aldehyde), 1643, 1462 (Ar), 1381s, 1095vs (C-O-C, ether); δ_H (500 MHz; $CDCl_3$) 2.9 (1H, br. s, α-O*H*), 3.9 (1H, br. s, β-O*H*), 3.4-3.48 (2x2H, m, α and β), 3.53 (2H, m, β), 3.69 (1H, dd, *J* 7.5 and *J* 9.6, β-2-H), 3.82 (1H, dd, *J* 2.9 and *J* ~ 4.7, α-3-H), 3.85 (1H, app. d, *J* 2.8, β-4-H), 3.9 (1H, br. s, α), 3.96 (1H, dd, *J* 3.6 and *J* ~ 9.9, α-2-H), 4.09 (1H,

app. t, α), 4.33 and 4.4 (AB, each 1H, d, $^2J_{A,B}$ 11.8, α-CH_AH_BPh), 4.34 and 4.4 (AB, each 1H, d, $^2J_{A,B}$ 11.9, β-CH_AH_BPh), 4.51 and 4.86 (AB, each 1H, d, $^2J_{A,B}$ 11.5, α-CH_AH_BPh), 4.53 and 4.87 (AB, each 1H, d, $^2J_{A,B}$ 11.6, β-CH_AH_BPh), 4.59 (1H, d, *J* 7.1, β-1-H), 4.64 and 4.76 (AB, each 1H, d, $^2J_{A,B}$ 11.7, α-CH_AH_BPh), 4.65 and 4.68 (AB, each 1H, d, $^2J_{A,B}$ 11.8, β-CH_AH_BPh), 4.67 and 4.72 (AB, each 1H, d, $^2J_{A,B}$ 11.8, α-CH_AH_BPh), 4.75 and 4.84 (AB, each 1H, d, $^2J_{A,B}$ 11.1, β-CH_AH_BPh), 5.2 (1H, d, *J* 3.4, α-1-H), 7.19-7.31 (40H, m, α/β-Ph); δ_C (125 MHz, $CDCl_3$) 68.9_8 (CH_2, β-C-6), 69.0_3 (CH_2, α-C-6), 69.6, 74.8, 76.7, 78.8 (all CH, α-C-2, C-3, C-4 and C-5) 7.7_3, 76.6_8, 80.8, 82.3 (all CH, β-C-2, C-3, C-4 and C-5) 72.9_7, 73.0_4, 73.6, 74.7 (all CH_2, 4xα-*C*H_2Ph), 73.5_6, 73.6_6, 74.7, 75.2 (all CH_2, 4xβ-*C*H_2Ph), 92.0 (CH, α-C-1), 97.9 (CH, β-C-1), 127.5_9, 127.6_6, $127._7$, 127.8, 127.9, 127.9, 128.0_8, 128.0_9, 128.2_7, 128.3_0, 128.3_2, 128.4, 128.5 (all CH, 4xα/β-Ph), 137.8, 138.0, 138.2, 138.6 (x2), 138.6, 138.7 (all C, 4xα/β-Ph); HRMS (ESI): found $(M+Na)^+$ 563.2441. $C_{34}H_{36}O_6$ requires $(M+Na)^+$, 563.2409.

3,4,5,7-Tetra-*O*-benzyl-1,2-dideoxy-D-galactoheptenitol (S3)[5]

To a cooled (-78 °C) solution of reducing sugar **S2** (1.68 g, 3.1 mmol) in anhydrous THF (15 ml) under N_2, was added a solution of *n*-butyl lithium in hexane (2.9 ml, 1.6 M) (Solution A). In a separate flask, methyltriphenylphosphonium bromide (2.3 g, 6.2 mmol) in anhydrous THF (10 ml) was cooled (–78 °C) under N_2. To this, butyl lithium (2.1 ml of 1.6 M solution in hexane) was then added to generate methylenetriphenylphosphorane *in situ* (Solution B). Solution B was then added to solution A at 0 °C, and the resulting suspension was stirred for 12 h under N_2 at room temperature. The solution was then cooled to 0 °C and NH_4Cl (20 ml, aq. sat. soln.) was added. The biphasic mixture was then allowed to warm to room temperature and diluted with DCM (200 ml). After extraction, the organic phase was separated, washed with water (100 ml) and brine (50 ml), dried ($MgSO_4$) and filtered. The filtrate was concentrated *in vacuo* and the residue was purified by column chromatography (light petroleum-EtOAc, 4:1) to give **S3** (1 g, 60%) as a brown syrup; R_f 0.45 (light petroleum-EtOAc, 4:1); υ_{max} (solution/$CHCl_3$)/cm^{-1} 3497br,w (OH, hydrogen bonded), 3155, 2901, 2869 (C-H, aliphatic), 1642, 1461 (Ar), 1095s (C-O-C, ether), 989w (C=C monosubstituted, bending); δ_H (500 MHz; $CDCl_3$) 2.97 (1H, d, $J_{OH,6}$ 5.3, 6-O*H*), 3.43 (1H, dd, $^2J_{7a,7b}$ 9.4 and $J_{7a,6}$ 6.5, 7a-H), 3.47 (1H, dd,

$^2J_{7b,7a}$ 9.4 and $J_{7b,6}$ 6.2, 7b-H), 3.75 (1H, app. obscured d, $J_{4,5}$ 5.9 and $J_{4,3}$ 4.2, 4-H), 3.75 (1H, obscured dd, $J_{5,6}$ ~ 12 and $J_{5,4}$ 5.9, 5-H), 4.03 (1H, dd, $J_{3,2}$ 7.8 and $J_{3,4}$ 4.2, 3-H), 4.06 (1H, app. br. dd, $J_{6,5}$ ~ 12, $J_{6,7a}$ ~ $J_{6,7a}$ ~ 6.5 and $J_{6,OH}$ 5.3, 6-H), 4.30 and 4.62 (AB, each 1H, d, $^2J_{A,B}$ 11.8, CH_AH_BPh), 4.31 and 4.35 (AB, each 1H, d, $^2J_{A,B}$ 11.4, CH_AH_BPh), 4.38 and 4.44 (AB, each 1H, d, $^2J_{A,B}$ 12, CH_AH_BPh), 4.7 (2H, br. s, CH_2Ph), 5.25 (A part of an ABM system, 1H, obscured dd, $J_{1a,2}$ 10.3 and $^2J_{1a,1b}$ < 0.5, 1a-H), 5.29 (B part of an ABM system, 1H, dd, $J_{1b,2}$ 17.5 and $^2J_{1b,1a}$ < 0.5, 1b-H), 5.82 (M part of an ABM system, 1H, ddd, $J_{2,1b}$ 17.5, $J_{2,1a}$ 10.3 and $J_{2,3}$ 7.8, 2-H), 7.12-7.32 (20H, m, 4xPh); δ_C (125 MHz, $CDCl_3$) 69.8 (CH, C-6), 70.4 (CH_2, *C*H_2Ph), 71.3 (CH_2, C-7), 73.1, 73.2$_4$, 75.3 (all CH_2, 4x*C*H_2Ph), 76.7 (CH, C-4), 80.8 (CH, C-3), 82.2 (CH, C-5), 119.3 (CH_2, C-1), 127.6$_6$, 127.7$_5$, 127.8, 128.0$_8$, 128.1$_8$, 128.4, 128.7$_9$ (all CH, 4xPh), 135.8$_2$ (CH, C-2), 138.1, 138.2$_5$, 138.2$_8$, 138.3 (all C, 4xPh); HRMS (ESI): found $(M+Na)^+$ 561.2631. $C_{35}H_{38}O_5$ requires $(M+Na)^+$, 561.2617.

C-(1-Deoxy 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl)-methyl mercury chloride (S4)[3, 6]

Compound **S3** (350 mg, 0.65 mmol) and mercuric acetate (207 mg, 0.65 mmol) were dissolved in anhydrous THF (12 ml) and stirred for 18 h at room temperature under N_2. To the resulting solution KCl (75 mg, dissolved in the minimum amount of water) was then added and the solution stirred for 30 min. It was then diluted with ethyl acetate (20 ml), and washed with water (2x10 ml). The organic layer was separated, dried ($MgSO_4$), filtered and concentrated *in vacuo*. The residue was then purified by column chromatography to afford **S4** as a pale yellow syrup (400 mg, 517 mmol, 80%); R_f 0.26 (light petroleum-EtOAc, 4:1); $[\alpha]_D^{25}$ +28.3 (c 0.6 in $CHCl_3$), (Found: C, 54.33; H, 4.86, Cl 4.93%; $(M+Na)^+$ 797.2322. $C_{35}H_{37}O_5ClHg$ requires C, 54.25; H, 4.82; Cl 4.58%; $(M+Na)^+$, 797.1933); δ_H (500 MHz; $CDCl_3$) 1.43 (1H, dd, $^2J_{1'a,1'b}$ 12 and $J_{1'a,1}$ 3.4, 1'a-H), 1.9 (1H, dd, $^2J_{1'a,1'b}$ 12 and $J_{1'b,1}$ 7.5, 1'b-H), 3.45 (1H, m, $J_{2,3}$ 5.7 and $J_{2,1}$ ~ 3.5, 2-H), 3.56 (1H, dd, $^2J_{6a,6b}$ 10.9 and $J_{6a,5}$ 3.6, 6a-H), 3.65 (1H, dd, $J_{3,2}$ 5.7 and $J_{3,4}$ 2.7, 3-H), 3.85 (1H, obscured br. dd, $^2J_{6a,6b}$ 10.9, 6b-H), 3.88 (1H, dd, $J_{4,5}$ 4.8 and $J_{4,3}$ 2.7, 4-H), 4.07 (1H, m 5-H), 4.24 (1H, app. p, $J_{1,1'b}$ 7.5, $J_{1,2}$ 3.5 and $J_{1,1'a}$ 3.4, 1-H), 4.38 and 4.54 (AB, each 1H, d, $^2J_{A,B}$ 11.6, CH_AH_BPh), 4.44 (2H, br. s, CH_2Ph), 4.45 (obscured) and

4.63 (AB, each 1H, d, $^2J_{A,B}$ ~ 11.8, CH_AH_BPh), 4.46 (obscured) and 4.52 (AB, each 1H, d, $^2J_{A,B}$ ~ 11.8, CH_AH_BPh), 7.17-7.28 (20H, m, 4xPh); δ_C (125 MHz, $CDCl_3$) 28.9 (CH_2, CH_2HgCl), 66.7 (CH2, C-6), 68.2 (CH, C-1), 72.7, 73.2, 73.5, 73.7 (all CH_2, 4xCH_2Ph), 73.2 (CH, C-5), 73.9 (CH, C-4), 75.3 (CH, C-3), 76.3 (CH, C-2), 127.6$_5$, 127.6$_8$, 127.8, 127.9, 128.3, 128.4, 128.5, 128.8, 128.9, 129.0 (all CH, 4xPh), 137.1, 138.3 (x2), 138.4 (all C, 4xPh).

C-(1-Deoxy 2,3,4,6-tetra-O-benzyl-α-D-galactopyranosyl) iodomethane (S5)[3]

To a solution of **S4** (383 mg, 0.5 mmol) in DCM (5ml) was added a solution of I_2 (250 mg, 1 mmol) in 5 ml DCM. The resulting solution was then stirred at room temperature for 16 h. The reaction was quenched by addition of $Na_2S_2O_3$ (250 mg in *ca.* 3 ml water) and the mixture stirred until the solution became colourless (15 min). Water (5 ml) was then added, the organic phase was separated, washed with water (5 ml) and brine (5 ml), dried ($MgSO_4$) and filtered. The filtrate was then evaporated *in vacuo* and purified by column chromatography (light petroleum-EtOAc, 9:1) to give **S5** (272 mg, 0.41 mmol, 82%) as a pale yellow syrup; R_f 0.2 (light petroleum-EtOAc, 9:1); $[\alpha]_D^{25}$ +33.3 (c 1.41 in $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 3.21 (1H, dd, $^2J_{1'a,1'b}$ 10.4 and $J_{1'a,1}$ 8.5, 1'a-H), 3.3 (1H, dd, $^2J_{1'a,1'b}$ 10.4 and $J_{1'b,1}$ 6.2, 1'b-H), 3.61 (1H, dd, $J_{3,2}$ 6.6 and $J_{3,4}$ 2.8, 3-H), 3.64 (1H, dd, $^2J_{6a,6b}$ 10.7 and $J_{6a,5}$ 4.4, 6a-H), 3.82 (1H, dd, $^2J_{6a,6b}$ 10.7 and $J_{6b,5}$ 7.2, 6b-H), 3.86 (1H, dd, $J_{2,3}$ 6.6 and $J_{2,1}$ 3.6, 2-H), 3.93 (1H, dd, $J_{4,5}$ 4.1 and $J_{4,3}$ 2.8, 4-H), 3.96 (1H, app. dd, $J_{5,6b}$ 7.2, $J_{5,6a}$ 4.4 and $J_{5,4}$ 4.1, 5-H), 4.01 (1H, ddd, $J_{1,1'a}$ 8.5, $J_{1,1'b}$ 6.2 and $J_{1,2}$ 3.6, 1-H), 4.44 and 4.6 (AB, each 1H, d, $^2J_{A,B}$ 12, CH_AH_BPh), 4.46 and 4.51 (AB, each 1H, d, $^2J_{A,B}$ 11.5, CH_AH_BPh), 4.48 and 4.53 (AB, each 1H, d, $^2J_{A,B}$ 11.3, CH_AH_BPh), 4.49 and 4.59 (AB, each 1H, d, $^2J_{A,B}$ 11.7, CH_AH_BPh), 7.17-7.29 (20H, m, 4xPh); δ_C (100 MHz, $CDCl_3$) 2.8 (CH_2, CH_2I), 67 (CH_2, C-6), 71.8 (CH, C-1), 73.1 (2xCH_2, 2xCH_2Ph), 73.3, (CH, 5-C), 73.4 and 73.6 (each CH_2, 2xCH_2Ph), 73.8 (CH, C-4), 75.9 (CH, C-2), 76 (CH, C-3), 127.6, 127.7, 127.8, 127.9, 128.0, 128.1, 128.4$_1$, 128.4$_5$, 128.4$_8$, 128.5$_5$ (all CH, 4xPh), 138.0, 138.3, 138.4, 138.4$_4$ (all C, 4xPh); HRMS (ESI): found $(M+Na)^+$ 687.1570. $C_{35}H_{37}O_5I$ requires $(M+Na)^+$, 687.1583.

Dimethyl-*C*-(1-Deoxy 2,3,5,6-tetra-*O*-benzyl-α-D-galactopyranosyl) methanephosphonate (S6)[3]

Trimethyl phosphite (7 ml) was added to iodide **S5** (257 mg, 0.38 mmole) and the solution was refluxed for 16 h. The resulting solution was concentrated at 35 °C under high vacuum and the residue purified by column chromatography (hexane-EtOAc, 5:4) to give phosphonate **S6** (223 mg, 34 mmol, 91%) as a pale yellow syrup; R_f 0.07 (hexane-EtOAc 5:4); $[\alpha]_D^{25}$ +34.8 (c 0.345 in $CHCl_3$); δ_H (400 MHz; $CDCl_3$) 2.08 (1H, ddd, $^2J_{1'a,P}$ 19.4, $^2J_{1'a,1'b}$ 15.7 and $J_{1'a,1}$ 4.5, 1'a-H), 2.17 (1H, ddd, $^2J_{1'b,P}$ 17, $^2J_{1'a,1'b}$, 15.7 and $J_{1'b,1}$ 9.4, 1'b-H), 3.64 (1H, dd, $J_{3,2}$ 7 and $J_{3,4}$ 2.7, 3-H), 3.68 (3H, d, $J_{CH3,P}$ 11, -P(O)OCH_3), 3.7 (3H, d, $J_{CH3,P}$ 11, -P(O)OCH_3), 3.73 (1H, dd, $^2J_{6a,6b}$ 10.3 and $J_{6a,5}$ 5.1, 6a-H), 3.84 (2H, m, 6b-H and 2-H), 4 (1H, dd, $J_{4,5}$ ~ 3.6 and $J_{4,3}$ 2.7, 4-H), 4.05 (1H, m, 5-H), 4.46 (1H, m, 1-H), 4.47 and 4.67 (AB, each 1H, d, $^2J_{A,B}$ 11.8, CH_AH_BPh), 4.56 and 4.69 (AB, each 1H, d, $^2J_{A,B}$ 11.7, CH_AH_BPh), 4.54 and 4.60 (AB, each 2H, obscured d, $^2J_{A,B}$ ~ 10.4, CH_AH_BPh), 7.24-7.35 (20H, m, 4xPh); δ_C (125 MHz, $CDCl_3$) 24.3 (CH_2, d, $^1J_{C1',P}$ 139.6, C-1'), 52.4 (CH_3, d, $^2J_{CH3,P}$ 5.3, -P(O)O*C*H_3), 52.6 (CH_3, d, $^2J_{CH3,P}$ 6.1, -P(O)O*C*H_3), 67.0 (CH, d, $^2J_{C1,P}$ 5, C-1), 67.3 (CH_2, C-6), 73 (CH, obscured d, $^3J_{C2,P}$ 12.5, C-2), 73.3 (CH, C-4 or C-5), 73.4 (CH, C-5 or C-4), 73.78 (CH, C-3), 72.9, 73.2, 76.3, 76.4, (all CH_2, 4x*C*H_2Ph), 127.6, 127.7, 127.9 (x2), 128.1, 128.3$_7$, 128.4, 128.5 (all CH, 4xPh), 138.1, 138.4 (x2), 138.5 (all C, 4xPh); δ_P (162 MHz, $CDCl_3$) 32.43; HRMS (ESI): found $(M+Na)^+$ 669.2585. $C_{37}H_{43}O_8P$ requires $(M+Na)^+$, 669.2593.

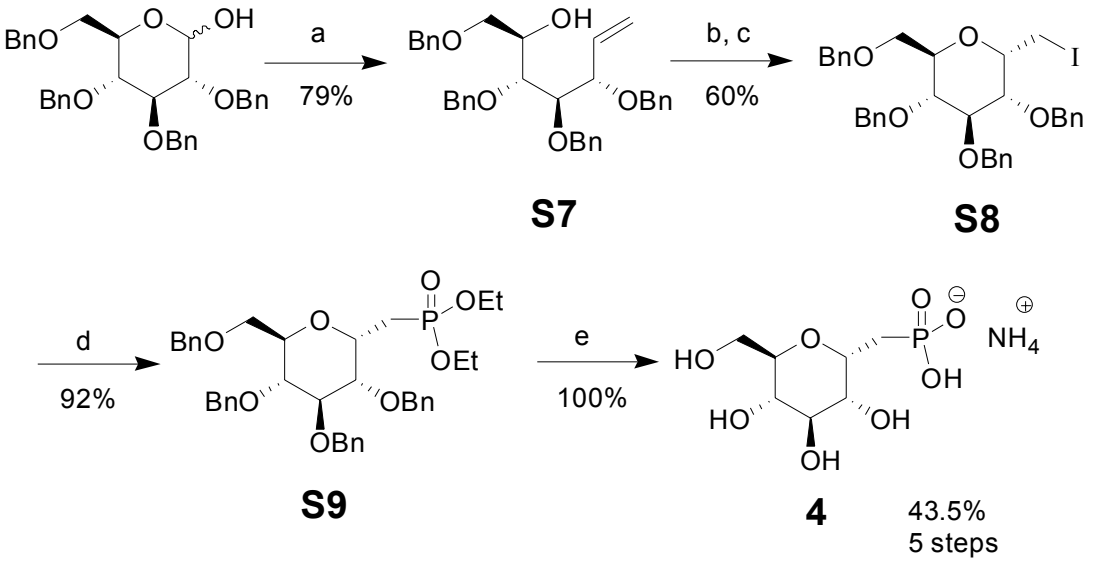
Triethylammonium *C*-(1-Deoxy-α-D-galactopyranosyl) methane phosphonate (5)[3]

Dimethyl phosphonate **S6** (200mg, 0.31 mmol) was dissolved in anhydrous $CHCl_3$ (5 ml) at 0 °C under N_2. To this solution was added trimethylsilyl iodide (6.82 mmol, 1 ml) and the solution was stirred for 45 min at 0 °C and 45 min at room temperature under N_2. Then, 5 ml methanol was added and the solvent evaporated *in vacuo*. The residue was washed several times with ether until the etheric phase remained colourless. The residue was purified by cellulose column chromatography (R_f 0.1, *n*-propanol-NH_3-water, 6:3:1) to give the deprotected-phosphonate as a white solid (79.6 mg, 0.29 mmol, 94%). To this

solid was then added water (5 ml) and triethylamine (5 ml); the resulting solution was stirred for 16 h, and was then filtered. The filtrate was concentrated under reduced pressure to afford quantitatively **4** as colourless oil. ^{1}H-NMR spectrum indicated the presence of 1 eq of triethylammonium; δ_H (400 MHz; D_2O) 1.16 **(**9H, t, *J* 7.3, (*CH*$_3$CH$_2$)$_3$NH$^+$**)**, 1.83 (1H, ddd, $^2J_{1'a,P}$ 19.6, $^2J_{1'a,1'b}$ 15.6 and $J_{1'a,1}$ 4.5, 1'a-H), 1.93 (1H, ddd, $^2J_{1'b,P}$ ~15.8, $^2J_{1'a,1'b}$, 15.6 and $J_{1'b,1}$ 10.2, 1'b-H), 3.08 **(**6H, q, *J* 7.3, (CH$_3$*CH*$_2$)$_3$NH$^+$**)**, 3.57 (1H, dd, $^2J_{6a,6b}$ 10.3 and $J_{6a,5}$ 5.1, 6a-H), 3.67 (1H, dd, $^2J_{6b,6a}$11.4 and $J_{6b,5}$ 8.1, 6b-H), 3.66 (1H, dd, $J_{3,2}$ 9.6 and $J_{3,4}$ 3.2, 3-H), 3.78 (1H, ddd, $J_{5,6b}$ 8.1, $J_{5,6a}$ 5.1 and $J_{5,4}$ 1.6, 5-H), 3.86 (1H, dd, $J_{4,3} \sim J_{4,5}$ 3.2, 4-H), 3.87 (1H, ddd, $J_{2,3}$ ~ 10 and $J_{2,1} \sim {}^4J_{2,P}$ ~ 5, 2-H), 4.3 (1H, dddd, $J_{1,P} \sim J_{1,1'b}$ 10.2 and $J_{1,2} \sim J_{1,1'a}$ 4.5, 1-H); δ_C (100 MHz, D_2O) 9.1 **(**3xCH$_3$, (*CH*$_3$CH$_2$)$_3$NH$^+$**)**, 25.1 (CH$_2$, d, $^1J_{C1',P}$ 133.7, *C*-1'), 47.4 **(**3xCH$_2$, (CH$_3$*CH*$_2$)$_3$NH$^+$**)**, 61.6 (CH$_2$, C-6), 69.1 (CH, d, $^3J_{C2,P}$ 9.6, C-2), 69.6 (CH, C-3), 70.5 (CH, C-4), 72.6 (CH, br. s, C-1), 72.7 (CH, C-5); δ_P (162 MHz, $CDCl_3$) 21.77; HRMS (ESI, negative mode): found (M+Maltose-1)$^-$, 599.1583. $C_{13}H_{30}O_8NP$ requires (M+Maltose-1)$^-$, 599.1588.

Synthesis of **5** was accomplished prior to an independent report outlining a similar synthetic strategy.[3, 7]

A-2) Synthesis of *C*-(1-Deoxy-α-D-glucopyranosyl) methane phosphonate (4)[8, 9]



Scheme 2. a) *n*-BuLi/Hexanes, CH_2=P(Ph)$_3$, DME, rt, 2 h; **b)** i) Hg(CF$_3$COO)$_2$, THF, rt, 18 h ii) KCl, rt, 5 h; **c)** I_2, DCM, rt, 2 h; **d)** P(OC$_2$H$_5$)$_3$, reflux, 16 h, **e)** i) TMSI, DCM, 0 °C, 2 h, ii) passed through Amberlite IR-120(H^+) then adjusted to pH 8 with NH_3 (aq.).

Diethyl-*C*-(1-Deoxy 2,3,5,6-tetra-*O*-benzyl-α-D-glucopyranosyl) methanephosphonate (S9)[8-10]

To a stirred suspension of methyltriphenylphosphonium bromide (23.0 g, 64.4 mmol) in anhydrous 1,2-dimethoxyethane (230 mL) at –78 ºC under nitrogen atmosphere *n*-BuLi (25.7 mL of 2.5M in hexanes, 64.4 mmol) was added dropwise. The resulting orange reaction mixture was allowed to warm to room temperature over 30 min. In a separate flask under a nitrogen atmosphere and at –78 ºC, *n*-BuLi (8.90 mL of 2.5M in hexanes, 22.2 mmol) was added to a stirred solution of 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose (12.0 g, 22.2 mmol) in anhydrous 1,2-dimethoxyethane (300 mL). This mixture was allowed to warm to room temperature over 20 min and then added to the orange ylide solution prepared above. The resultant dark orange reaction mixture turned red after being stirred at 45 ºC for 2 hr, then acetone (132 mL) was added to the reaction which

formed an immediate precipitate. The resulting solid gradually dissolved, yielding an orange solution that was stirred for an additional 2 hr. After evaporation, the residue was suspended in brine (400 mL) and extracted with diethyl ether (400 mL x 2). The combined organic extracts were dried ($MgSO_4$), filtered and concentrated. Purification by flash chromatography (hexanes-EtOAc, 85:15) afforded compound **S7** (9.47 g, 79%) as a colorless liquid; R_f 0.41 (hexanes-EtOAc, 4:1); LRMS (ESI): found $(M+Na)^+$ 561.4. $C_{35}H_{38}O_5$ requires $(M+Na)^+$ 561.3.

Under a nitrogen atmosphere, a solution of **S7** (217 mg, 0.403 mmol) and mercuric trifluoroacetate (172 mg, 0.403 mmol) in anhydrous THF (4 mL) was stirred at rt for 18 h. The reaction mixture was then charged with KCl 0.8M (4 mL) and the solution was stirred for a further 5 h. THF was removed *in vacuo* and the remaining aqueous solution was extracted with DCM (15 mL x 2). The combined organic extracts were washed with brine (20 mL) and dried (Na_2SO_4), filtered and concentrated. This residue (a single spot, R_f 0.27 (hexanes-EtOAc, 4:1) and iodine (161 mg, 0.634 mmol) were immediately dissolved in anhydrous DCM (6 mL) under a nitrogen atmosphere and stirred for 2 h. The reaction flask was then charged with 10% $Na_2S_2O_3$ (3 mL) and stirred for 10 min. The organic layer was removed and washed with 5% KI (5 mL), brine (5 mL), dried (Na_2SO_4) and concentrated. Silica column chromatography (hexanes-EtOAc, 9:1) gave a mixture of two compounds (R_f 0.60 and R_f 0.66, hexanes-EtOAc, 4:1) which were separated by a further column chromatography (hexanes-EtOAc, 92:8) to afford **S8** (161 mg, 60%, 2 steps) as a colorless solid; R_f 0.66 (hexanes-EtOAc, 4:1).

Iodo moiety **S8** (198 mg, 0.298 mmol) was refluxed in triethyl phosphite (15 mL) for 16 h, evaporated and purified over silica column (hexanes-EtOAc, 3:2) to afford compound **S9** (185 mg, 92%); R_f 0.24 (hexanes-EtOAc, 1:1); δ_H (500 MHz; $CDCl_3$) 1.27, 1.28 (6H, t, *J* 7.0, 2xOCH$_2$C*H$_3$*), 2.20 (2H, m, 1'a-H and 1'b-H), 3.79-3.61 (6H, m, 2-H, 3-H, 4-H, 5-H, 6a-H and 6b-H), 4.09, 4.07 (each 2H, q, *J* 7.0, 2xOC*H$_2$*CH$_3$), 4.54 (1H, m, $^1J_{C,H}$ 150, 1-H), 4.47, 4.50, 4.61, 4.64, 4.67, 4.77, 4.80, 4.88 (each 1H, d, *J* ~12, 4xPhC*H$_2$*), 7.10-7.35 (20H, m, 4xPh); δ_C (125 MHz, $CDCl_3$) 16.6(x2) (CH_3, d, $^3J_{CH3,P}$ 6.1, OCH$_2$*C*H$_3$), 22.6 (CH_2, d, $^1J_{C1',P}$ 145, C-1'), 61.6, 61.9 (each CH_2, d, $^2J_{CH2,P}$ 6.3, O*C*H$_2$CH$_3$), 68.8

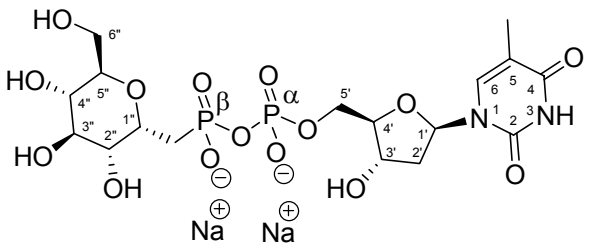
(CH_2, C-6), 69.9 (CH, d, $^2J_{C1,P}$ 5.3, C-1), 72.1 (CH, C-3 or C-4 or C-5), 73.3, 73.7, 75.1, 75.5 (all CH_2, 4xPh*C*H_2), 77.8 (CH, C-3 or C-4 or C-5), 79.3 (CH, d, $^3J_{C2,P}$ 13.0, C-2), 82.0 (CH, C-3 or C-4 or C-5), 127.8-138.7 (4C and 20CH, 4xPh); δ_P (202.5 MHz, $CDCl_3$) 29.18 (s); LRMS (ESI): found $(M+Na)^+$ 697.3. $C_{39}H_{47}O_8P$ requires $(M+Na)^+$, 697.3.

Ammonium *C*-(1-Deoxy-α-D-glucopyranosyl)methane phosphonate (4)[8]

Under a nitrogen atmosphere and at 0 ºC, a stirring solution of **S9** (43 mg, 0.063 mmol) in anhydrous DCM (0.5 mL) was treated with iodotrimethylsilane (500 μL, 3.51 mmol) and allowed to warm to rt over 2 hr. The reaction was quenched by methanol. The mixture was concentrated, dissolved in H_2O (10 mL) and washed with diethyl ether (10 mL x 8). The aqueous layer was passed through Amberlite IR-120 (H^+) ion exchange resin and the resulting acidic aqueous fraction was immediately adjusted to pH 8 with NH_4OH 0.2 M, concentrated to 5 mL, and lyophilized to afford the target phosphonate **5** as a colorless foam (18 mg, 100%); δ_H (500 MHz; D_2O) 1.78 (2H, m,1'a-H and 1'b-H), 3.17 (1H, dd, $J_{3,4} \sim J_{4,5} \sim 9$, 4-H), 3.43-3.55 (4H, m, 2-H, 3-H, 5-H and 6a-H), 3.65 (1H, d, $^2J_{6b,6a}$ 11.8, 6b-H), 4.20 (1H, m, 1-H); δ_C (125 MHz; D_2O) 25.9 (CH_2, d, $^1J_{C1',P}$ 129, C-1'), 61.0 (CH_2, C-6), 70.4 (CH, C-4), 71.6 (CH, d, $^3J_{C2,P}$ 8.0, C-2), 72.5 (CH, d, $^2J_{C1,P}$ 2.1, C-1), 72.9 (CH, C-3 or C-5), 73.5 (CH, C-5 or C-3); $\delta_{C\text{-H coupled}}$ 72.5 (CH, d, $^1J_{C1,H}$ 150, C-1); δ_P (202.5 MHz; D_2O) 19.90 (s); HRMS (ESI, negative mode): found $(M-H)^-$, 257.0433. $C_7H_{14}O_8P$ requires $(M-H)^-$, 257.0432.

Data is consistent with that which is presented in the literature,[8] with a correction to the value of the ^{31}P-NMR chemical shift value.

Purification of phosphono analogue of dTDP-α-D-glucopyranose (dTDP-1CP-Glcp), sodium salt (7)



A modification of the assay method described above was used to scale up enzymatic coupling in order to purify and characterize **7**. A reaction was set up containing **4** (10.0 mg, 34.2μmol), dTTP (27.0 mg, 47.8 μmol), $MgCl_2$ (4.9 mg, 51.3 μmol), and 5 EU inorganic pyrophosphatase, which was initiated by the addition of nucleotidylyltransferase (336 EU, split into two equal portions) in Tris-HCl buffer (20 mM final buffer concentration, 2 mL reaction volume). The enzymatic reaction was monitored by HPLC and was stopped following 6 h (ca. 20 % conversion based on dTTP). This was done due to concern of possible product breakdown after prolonged incubation periods, in addition to a limited quantity of fresh enzyme. Following incubation for 6 h at 37 °C, alkaline phosphatase (80 EU) was added to the mixture, and allowed to incubate overnight at 25 °C. After the set time, the protein was precipitated with 2 mL methanol and the precipitate was washed with another 2 mL of methanol. The sugar nucleotide product was purified by ion pair reversed-phase chromatography of the supernatants, and fractions containing sugar nucleotides, as judged by HPLC, were passed through a cation-exchange column as previously described.[13] Further desalting of the product mixture was performed by using a Sephadex G10 column (1.5 cm x 100 cm) with water as eluant to afford compound **7,** with an isolated yield of 6.8 mg (0.012 mmol, yield = 35% by mass, 34% by UV absorbance [$\varepsilon = 9.7 \times 10^4$ $M^{-1}cm^{-1}$ at a $\lambda_{max} = 267nm$][14]).

δ_H (500 MHz; D_2O) 1.85 (3H, br s, 5CH_3-H), 2.06 (1H, ddd, $^2J_{1"CH2a,P}$ 17, $^2J_{1"CH2a,b}$ 16 and $J_{1"CH2a,1"}$ 4, 1"CH2a-H), 2.15 (1H, ddd, $^2J_{1"CH2b,P}$ 17, $^2J_{1"CH2b,a}$ 16 and $J_{1"CH2b,1"}$ 11, 1"CH2b-H), 2.25-2.34 (2H, m, 2'a-H and 2'b-H), 3.31 (1H, dd, $J_{4",3"} \sim J_{4",5"} \sim 9.4$, 4"-H), 3.53 (1H, dd, $J_{3",2"} \sim J_{3",4"} \sim 9.4$, 3"-H), 3.58 (1H, ddd, $J_{5",4"}$ 9.4, $J_{5",6"b}$ 5 and $J_{5",6"a}$ 2.2, 5"-H), 3.61-3.64 (1H, obscured m, 2"-H), 3.64 (1H, obscured dd, $^2J_{6"a,6"b}$ 12.2 and $J_{6"a,5"}$ 5, 6"a-H), 3.76 (1H, dd, $^2J_{6"b,6"a}$12.2 and $J_{6"b,5"}$ 2.2, 6"b-H), 4.08-4.11 (3H, m, 4'-H, 5'a-H and 5'b-H), 4.35 (1H, dddd, $J_{1",P} \sim J_{1",1"CH2b} \sim 10$ and $J_{1",2"} \sim J_{1",1"CH2a} \sim 4$, 1"-H), 4.55 (1H, ddd, $J_{3',2'} \sim 6$, and $J_{3',2'a} \sim J_{3',4'} \sim 3$, 3'-H), 6.27 (1H, dd, $J_{1',2'a} \sim J_{1',2'b} \sim 7$, 1'-H), 7.67 (1H, s, 6-H); δ_C (125 MHz, D_2O) 11.7 (CH_3, 5-CH_3), 24.3 (CH_2, d, $^1J_{1"CH2,P}$ 139.7, *C*-1"CH_2), 38.6 (CH2, C-2'), 60.8 (CH_2, C-6"), 65.2 (CH_2, d, $^2J_{C5',P}$ 5.4, C-5'), 70.1 (CH, C-4"), 70.9 (CH, C-3'), 71.1 (CH, d, $^3J_{C2",P}$ 12.4, C-2"), 72.0 (CH, d, $^2J_{C1",P}$ 4.5, C-1"), 72.6 (CH, C-5"), 73.2 (CH, C-3"), 85.0 (CH, C-1'), 85.4 (CH, d, $^3J_{C2",P}$ 8.8, C-4'), 111.8 (C, C-5), 137.4 (CH, C-6), 151.9 (C, C-2), 160.3 (C, C-4); δ_P (202.5 MHz, D_2O) –11.5 (1P, d, $J_{P\alpha,P\beta}$ 27, P-α), 14.6 (1P, d, $J_{P\beta,P\alpha}$ 27, P-β). HRMS (ESI^-): found $[M-H]^-$ 561.0877. $C_{17}H_{27}N_2O_{15}P_2$ requires $[M-H]^-$, 561.0892.

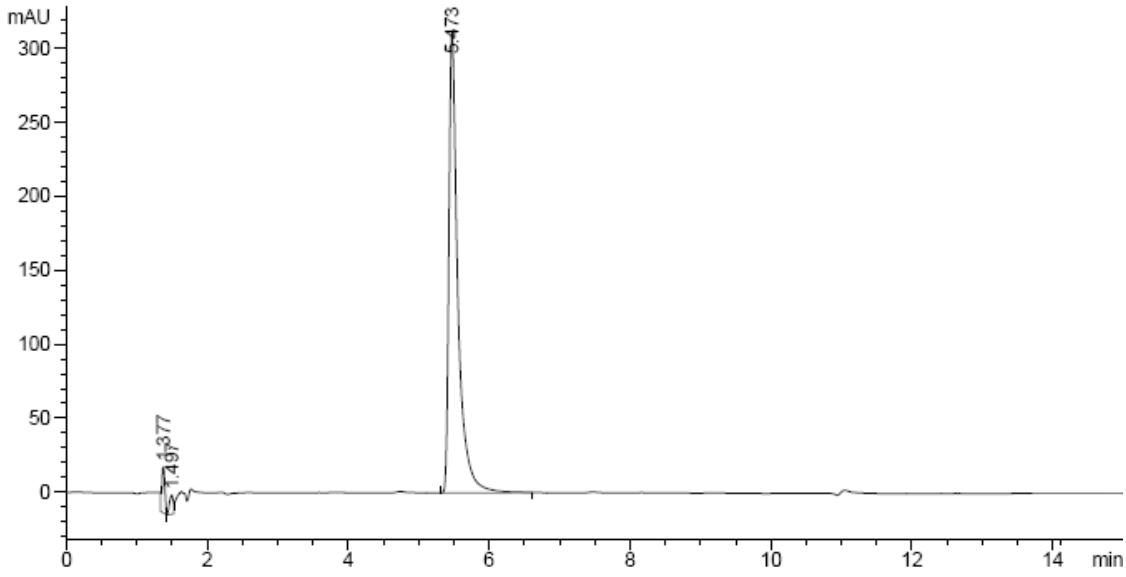
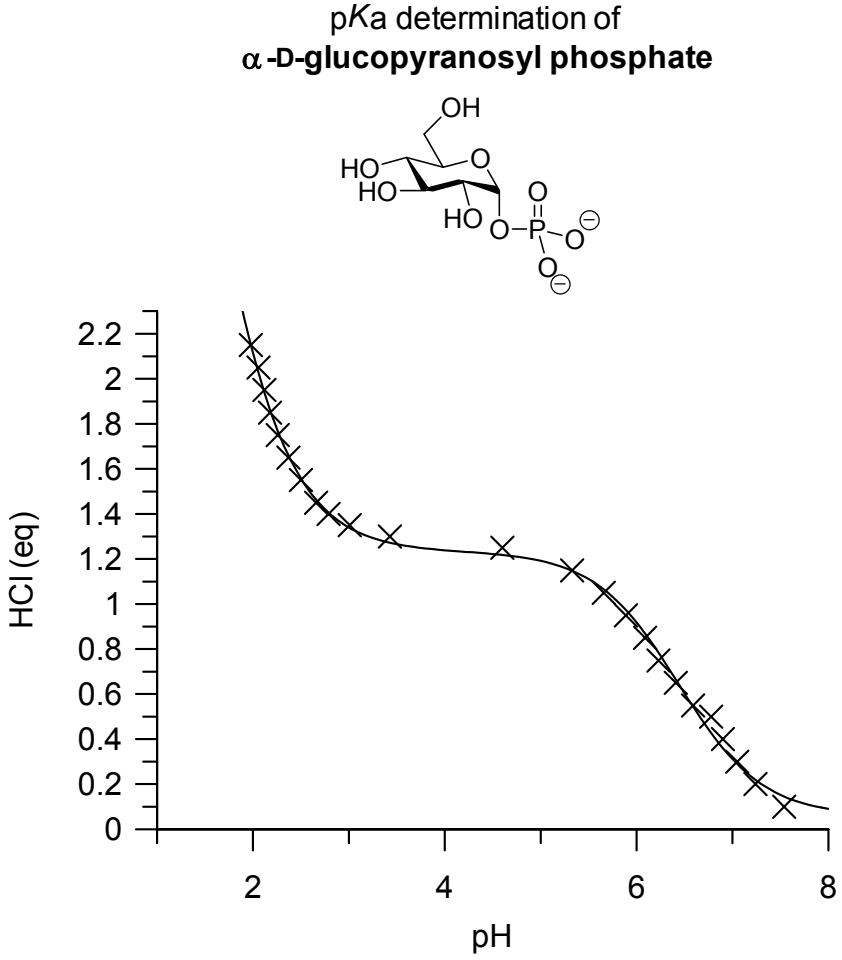
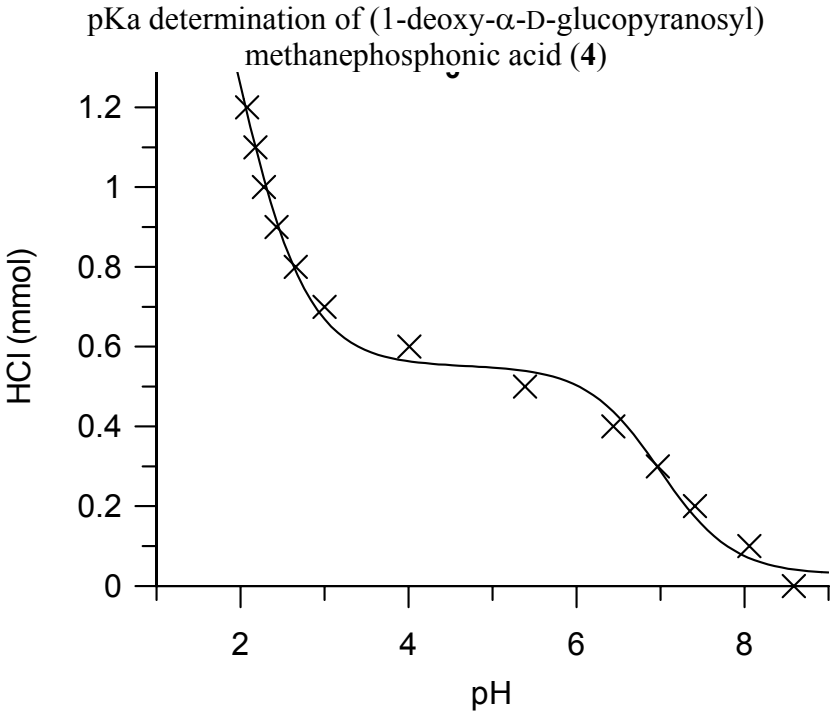


Figure S19: HPLC trace of purified **7**.

Determination of pKa 2 for α-D-glucopyranosyl phosphate (1) and (1-deoxy-α-D-glucopyranosyl)methanephosphonic acid (4)





The p*K* for the second acidic proton (p*K*a2) of α-D-glucopyranosyl phosphate (**1**) and (1-deoxy-α-D-glucopyranosyl) methanephosphonic acid (**4**) was determined to be 6.4 and 7, respectively which was in good agreement with previous reports.[11, 12]

Effect of pH on Percent Conversions

The enzyme remained active over a pH of 6-10 for α-D-Glc-1-phosphate (**1**) and α-D-Glc-1-phosphonate(**4**) with dTTP.

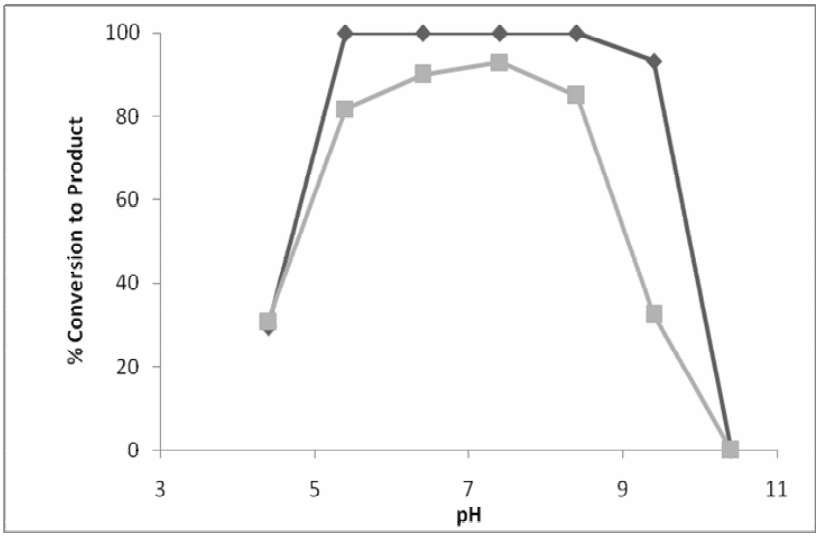


Figure S2: pH Range when Enzyme is Active for α-D-Glc-1-phosphate (♦) and α-D-Glc-1-phosphonate (■) incubations.

HPLC data of Sugar Nucleotides

Table S1. Comparison of percentage conversions to sugar nucleotides after 30 min and 24 h Incubations

Enzyme	Sugar-1-Phosphonate	NTP	% Conversion [a] after 30 min	% Conversion [a] after 24 h	NDP-Sugar Retention Time (min)
Cps2L	α-D-Glucose-1-phosphonate	ATP	6	19	5.36
Cps2L	α-D-Glucose-1-phosphonate	CTP	10	16	5.79
Cps2L	α-D-Glucose-1-phosphonate	GTP	1	16	5.13
Cps2L	α-D-Glucose-1-phosphonate	dTTP	95	100	5.46
Cps2L	α-D-Glucose-1-phosphonate	UTP	55	61	5.27
Cps2L	α-D-Galactose-1-phosphonate	dTTP	42	--	5.52*
Cps2L	α-D-Galactose-1-phosphonate	UTP	--	1	5.33

[a] Percentage conversion = [NDP-sugar / (NDP-sugar + NTP)] x 100 where NDP-sugar is equal to the product peak integration and NTP is equal to the NTP peak integration.
* Product of α-D-Gal-1-phosphonate with dTTP broke down after 24 h incubation.

Table S2. Retention times of NDP, NTP and Thymidine standards

Standard	Retention Time
ATP	7.29
CTP	7.11
GTP	7.16
dTTP	7.36
UTP	7.29
ADP	5.78
CDP	5.08
GDP	5.87
dTDP	5.84
UDP	5.69
Thymidine	1.96

Sugar nucleotide ESI-MS/MS characterization data

ADP-α-D-glucose-1-phosphonate (**4** + ATP): HRMS (ESI$^-$) Accurate Mass for $C_{17}H_{26}N_5O_{14}P_2$ [M-H]$^-$ (*m/*z calcd 586.0957). Found: 586.0951; LRMS (ESI$^-$)=*m/z* 586.0. EPI fragments = *m/z* 550.1, 346.1, 239.1, 211.0

CDP-α-D-glucose-1-phosphonate (**4** + CTP): HRMS (ESI$^-$) Accurate Mass for $C_{17}H_{26}N_3O_{15}P_2$ [M-H]$^-$ (*m/*z calcd 562.0845). Found: 562.0807; LRMS (ESI$^-$)=*m/z* 562.1, EPI fragments = *m/z* 525.8, 322.1, 255.4, 239.0, 211.02

GDP-α-D-glucose-1-phosphonate (**4** + GTP): HRMS (ESI$^-$) Accurate Mass for $C_{17}H_{26}N_5O_{15}P_2$ [M-H]$^-$ (*m/*z calcd 602.0906). Found: 602.0878; LRMS (ESI$^-$)=*m/z* 602.0, EPI fragments = *m/z* 565.9, 547.6, 362.0, 300.9, 257.0, 211.1

dTDP-α-D-glucose-1-phosphonate (**4** + dTTP): HRMS (ESI$^-$) Accurate Mass for $C_{17}H_{27}N_2O_{15}P_2$ [M-H]$^-$ (*m/*z calcd 561.0892). Found: 561.0877; LRMS (ESI$^-$)=*m/*z 561.1 EPI fragments = *m/z* 321.1, 195.1, 176.9, 125.0 78.8.

UDP-α-D-glucose-1-phosphonate (**4** + UTP): HRMS (ESI$^-$) Accurate Mass for $C_{16}H_{25}N_2O_{16}P_2$ [M-H]$^-$ (*m/*z calcd 563.0685). Found: 563.0682; LRMS (ESI$^-$)= *m/*z 563.0 EPI fragments = *m/z* 323.0, 280.0, 239.2, 211.0, 193.1

dTDP-α-D-galactose-1-phosphonate (**5** + dTTP): $C_{17}H_{27}N_2O_{15}P_2$ [M-H]$^-$ LRMS (ESI$^-$)=*m/*z 561.1 EPI fragments = *m/z* 524.7, 321.0, 300.9, 257.0, 253.3, 234.9

HPLC Traces for Representative Enzymatic Reaction Mixtures:

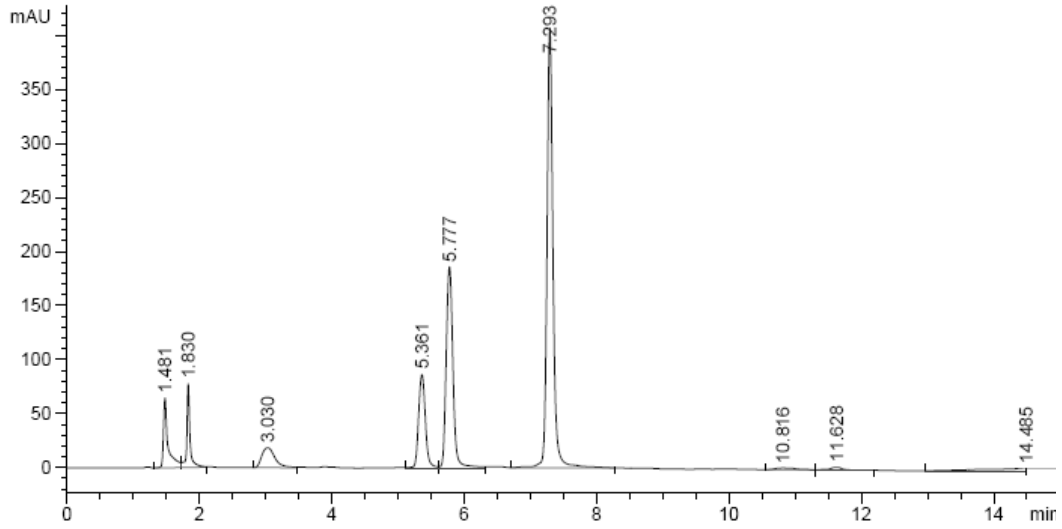


Figure S3: Cps2L + α-D-Glc-1-phosphonate (**4**) + ATP after 24 h

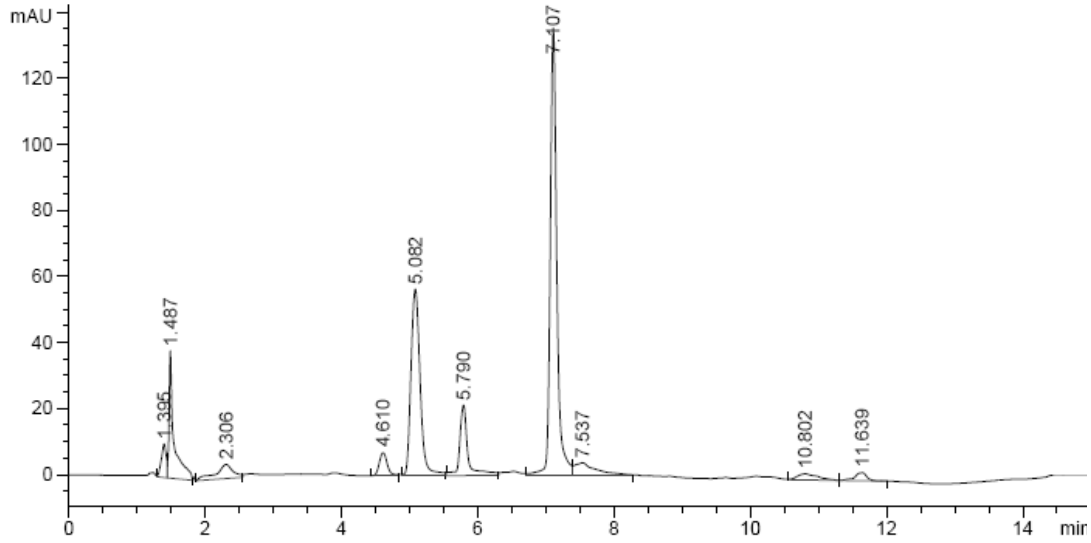


Figure S4: Cps2L + α -D-Glc-1-phosphonate (**4**) + CTP after 24 h

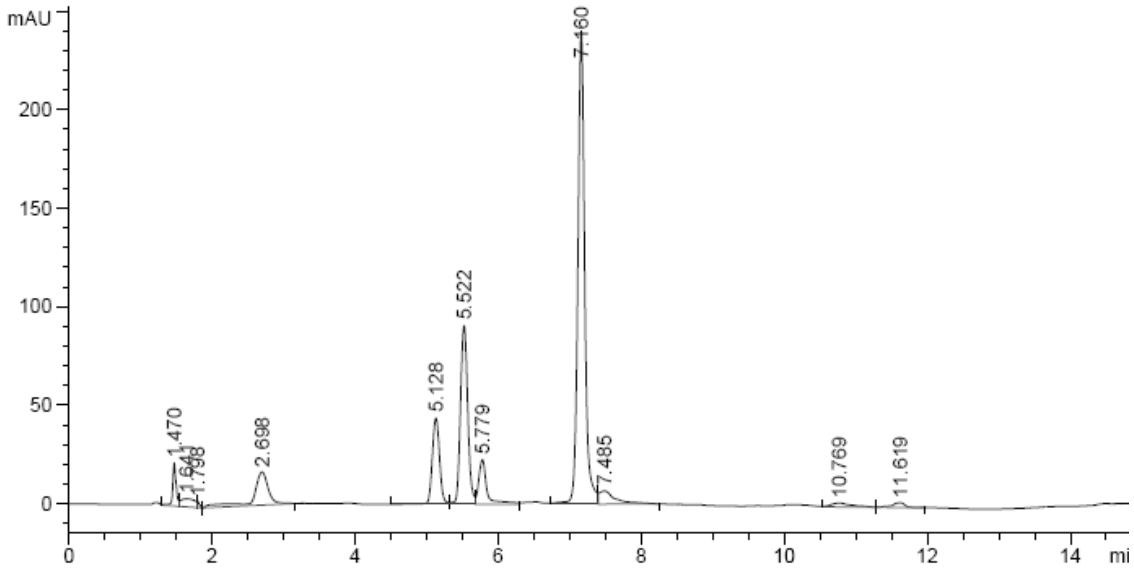


Figure S5: Cps2L + α -D-Glc-1-phosphonate (**4**) + GTP after 24 h

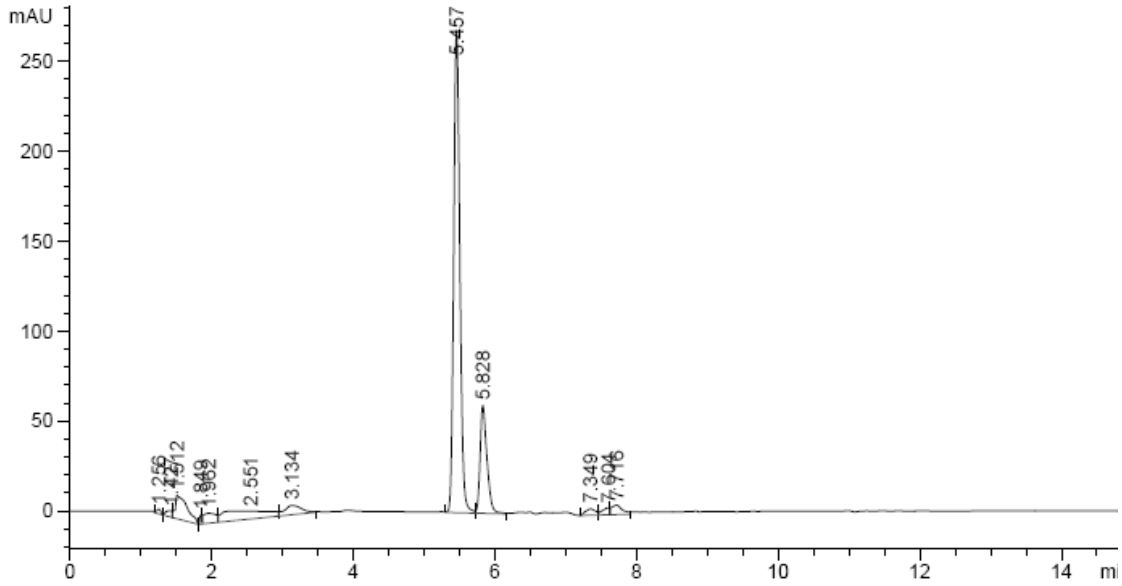


Figure S6: Cps2L + α -D-Glc-1-phosphonate (**4**) + dTTP after 24 h

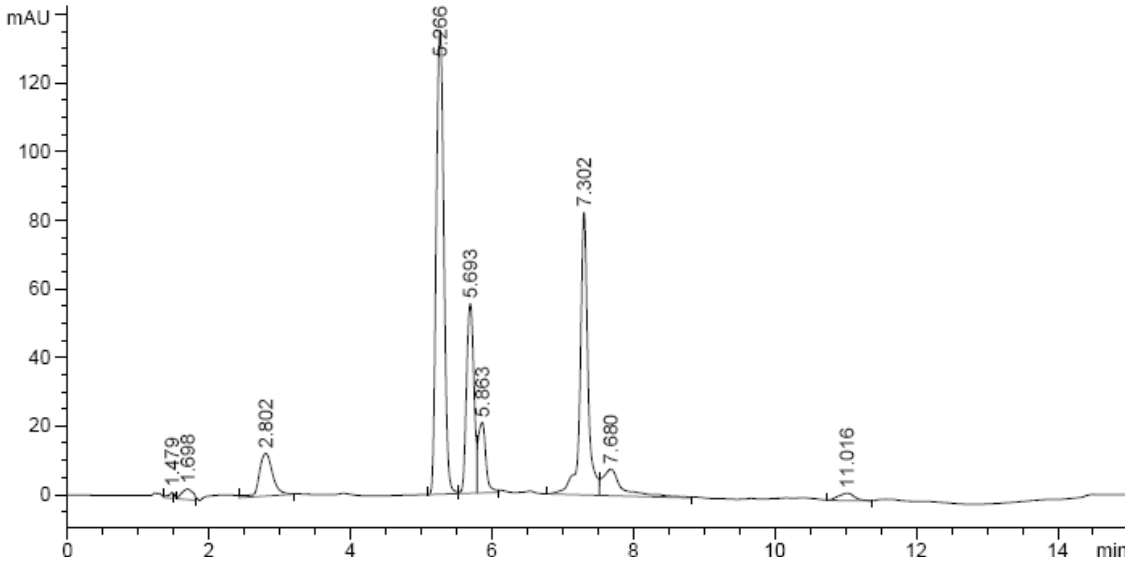


Figure S7: Cps2L + α -D-Glc-1-phosphonate (**4**) + UTP after 30min

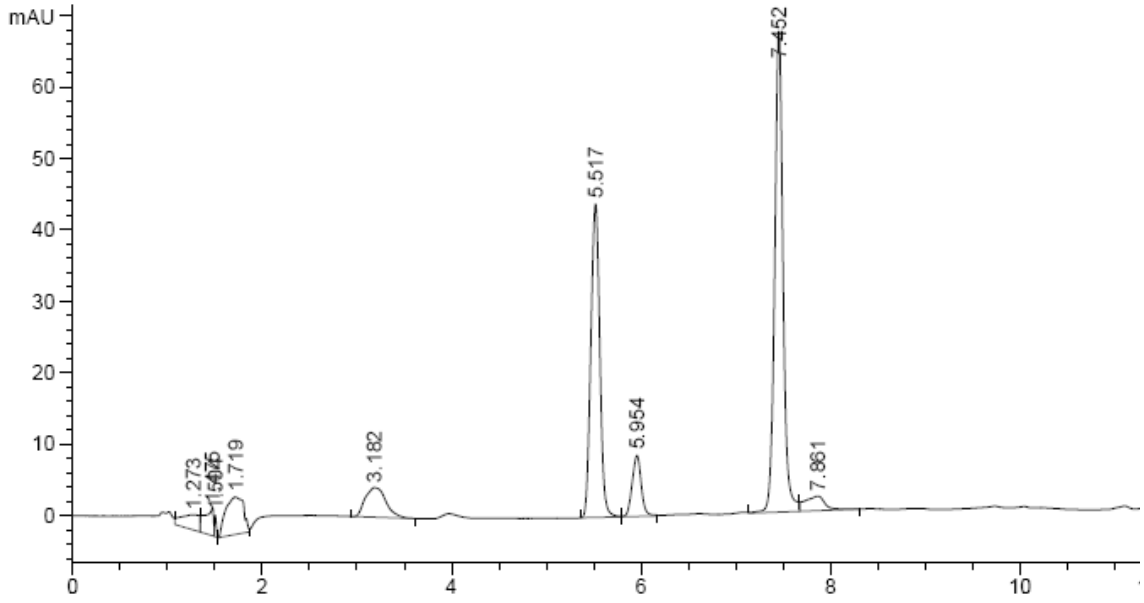


Figure S8: Cps2L + α -D-Gal-1-phosphonate (**5**) + dTTP after 30min

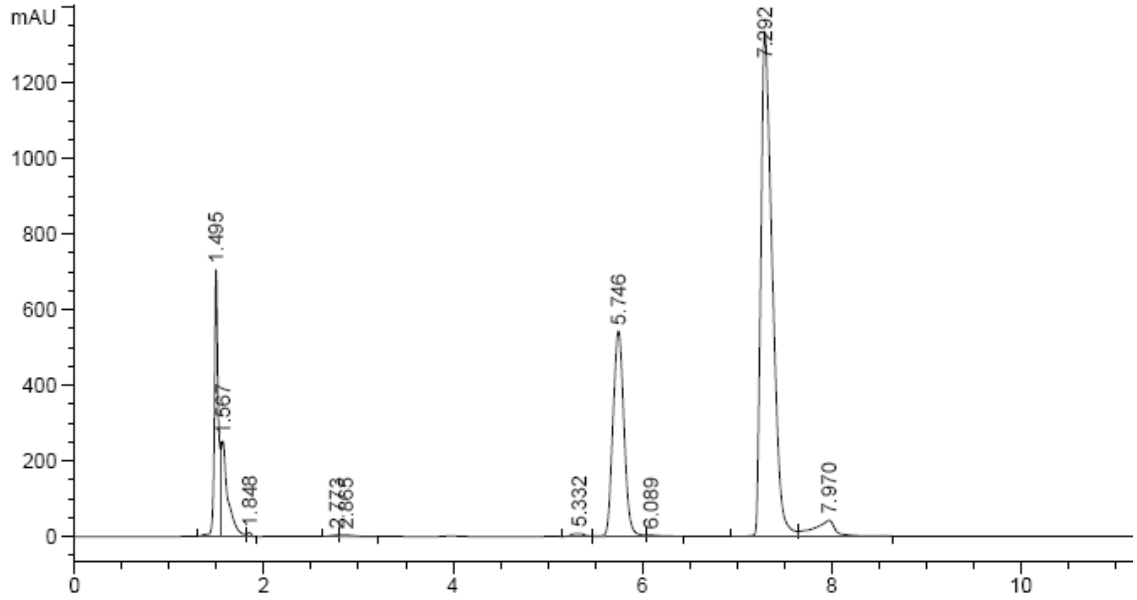


Figure S9: Cps2L + α -D-Gal-1-phosphonate (**5**) + UTP after 24 h

ESI-MS/MS EPI Scan Data of Enzyme-Catalyzed Production of Sugar Nucleotides

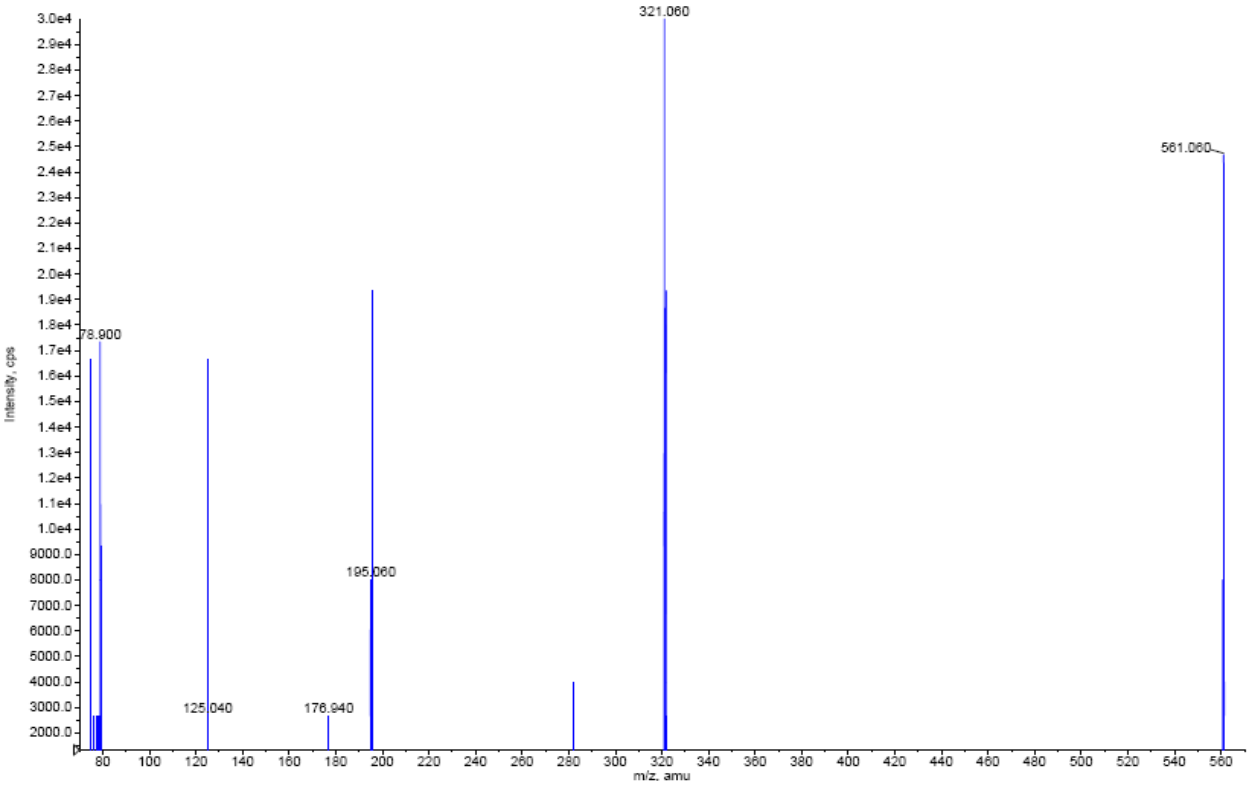


Figure S10: Cps2L + α -D-Glc-1-phosphonate (**4**) + dTTP

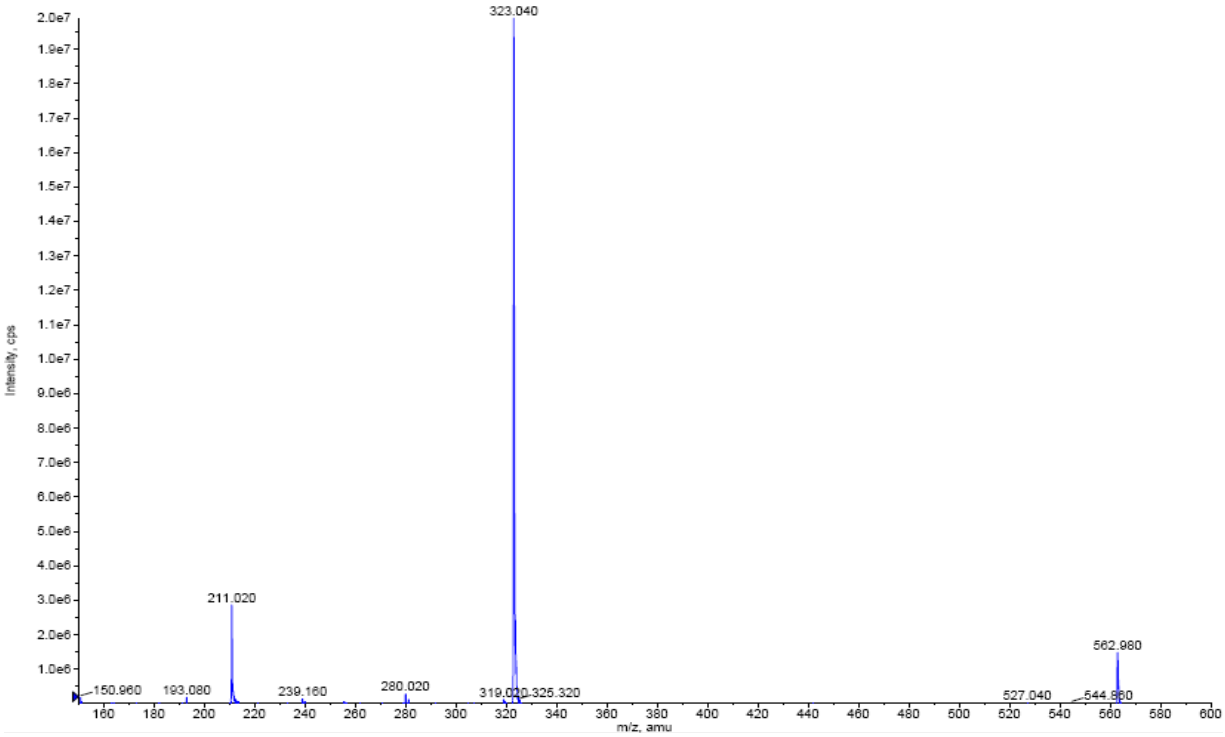


Figure S11: Cps2L + α -D-Glc-1-phosphonate (**4**) + UTP

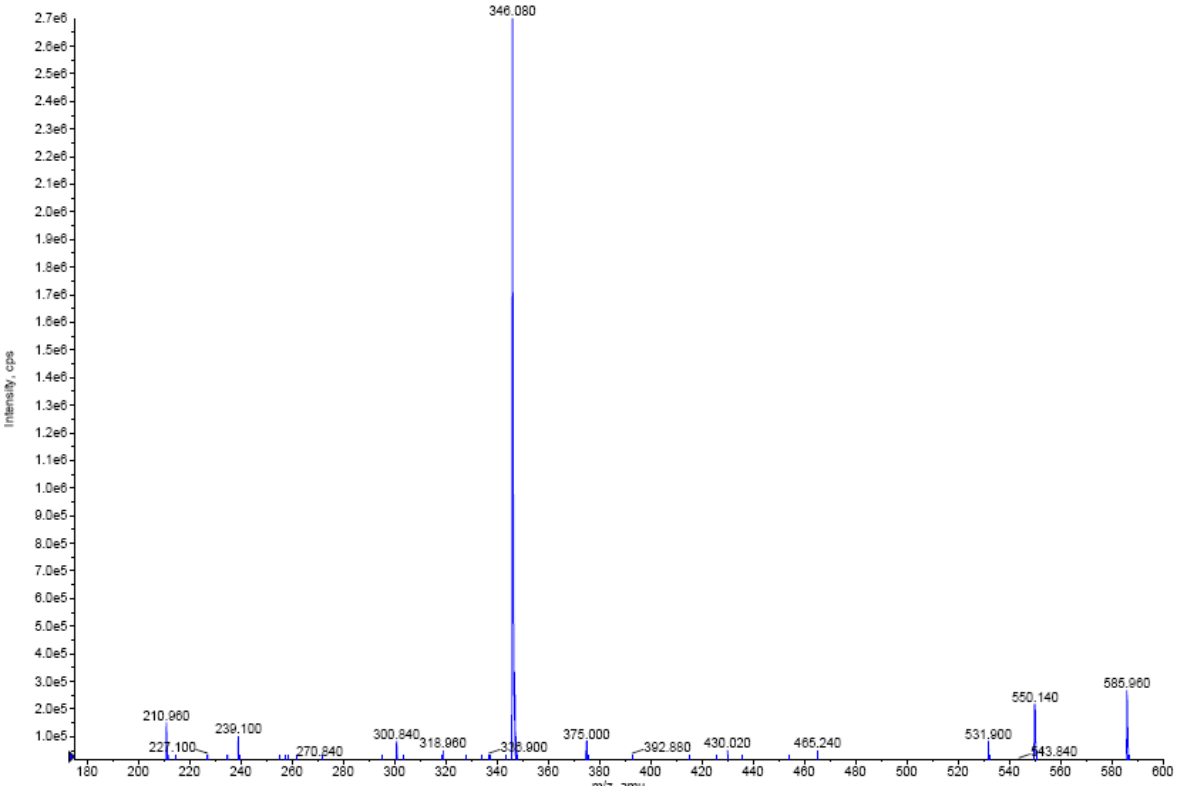


Figure S12: Cps2L + α -D-Glc-1-phosphonate (**4**) + ATP

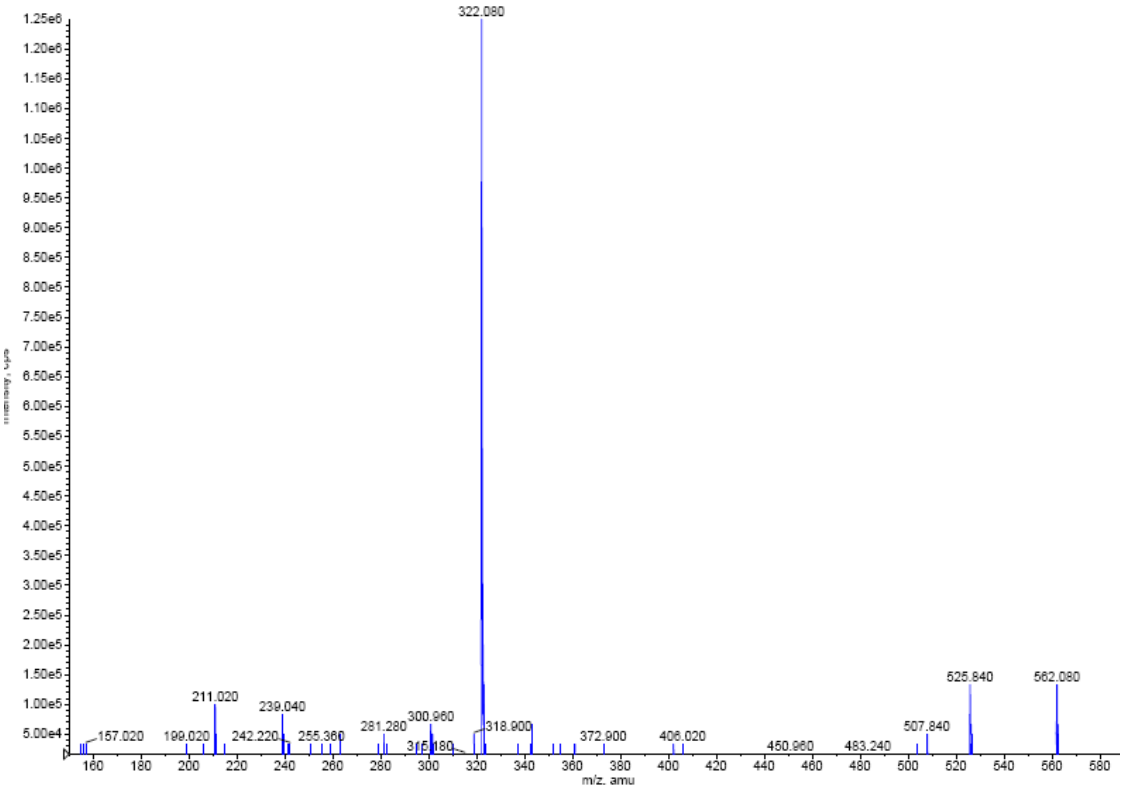


Figure S13: Cps2L + α -D-Glc-1-phosphonate (**4**) + CTP

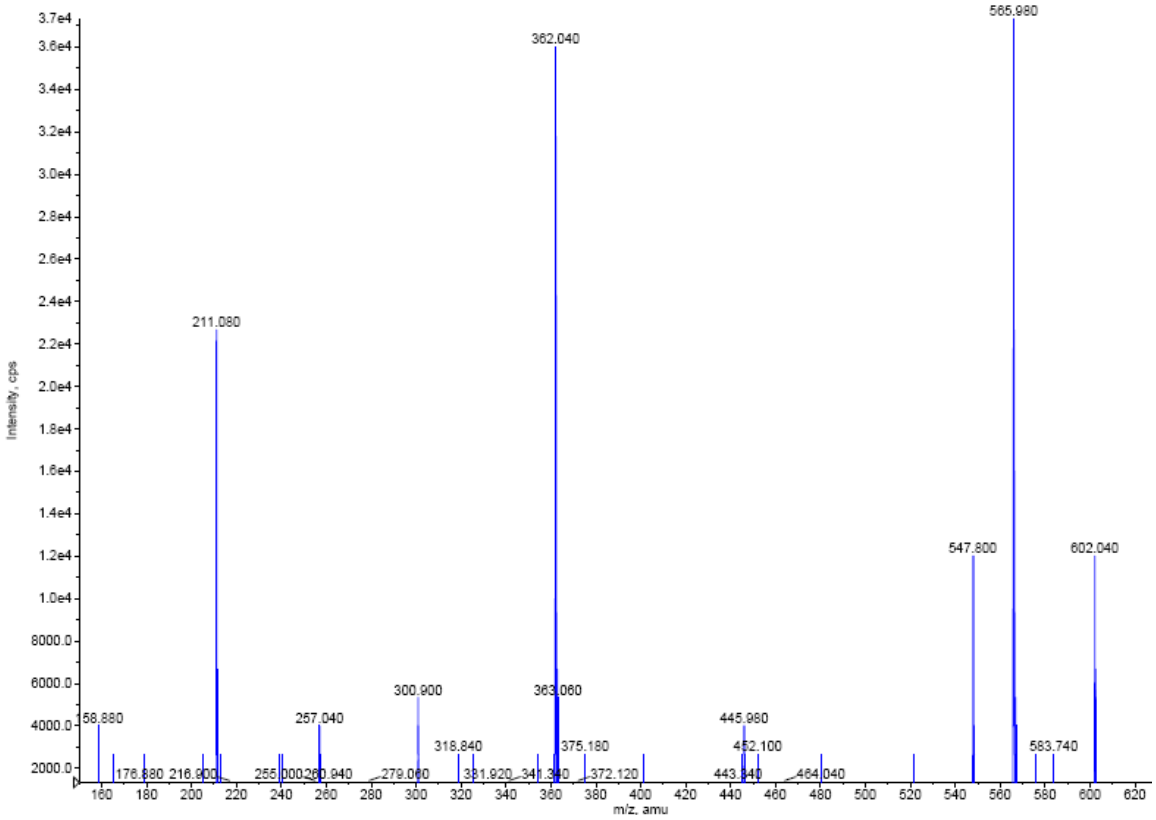


Figure S14: Cps2L + α -D-Glc-1-phosphonate (**4**) + GTP

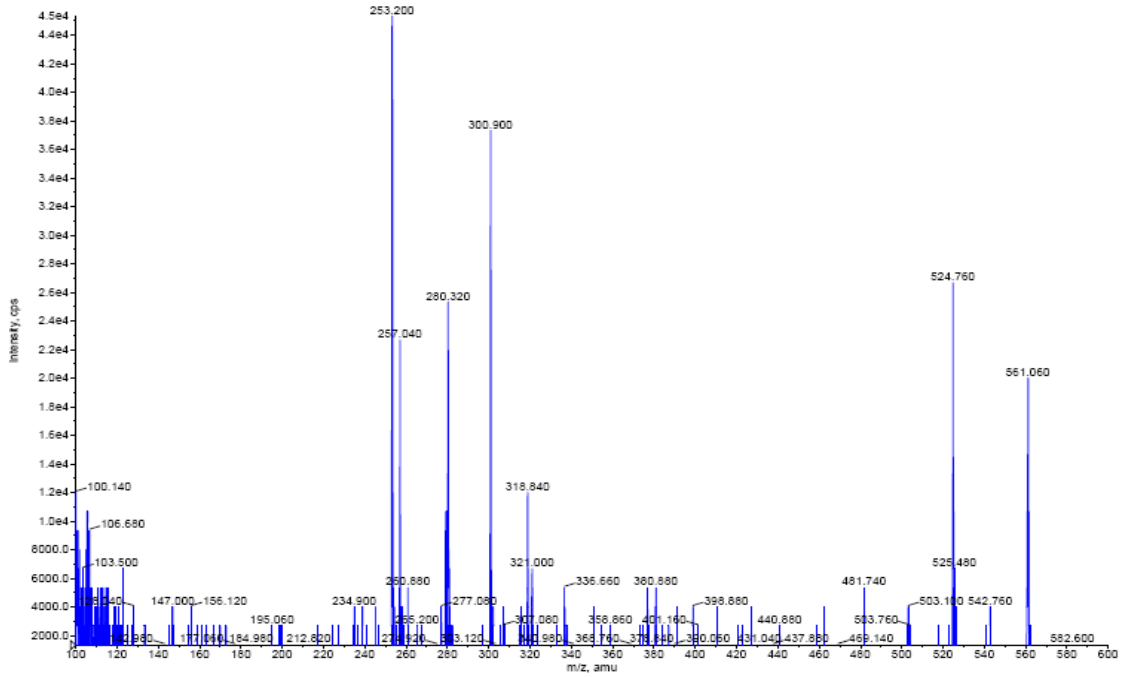


Figure S15: Cps2L + α -D-Gal-1-phosphonate (**5**) + dTTP

Kinetic Parameters with respect to α-D-glucose-1-phosphonate, α-D-galactose-1-phosphonate and α-D-galactose-1-phosphate

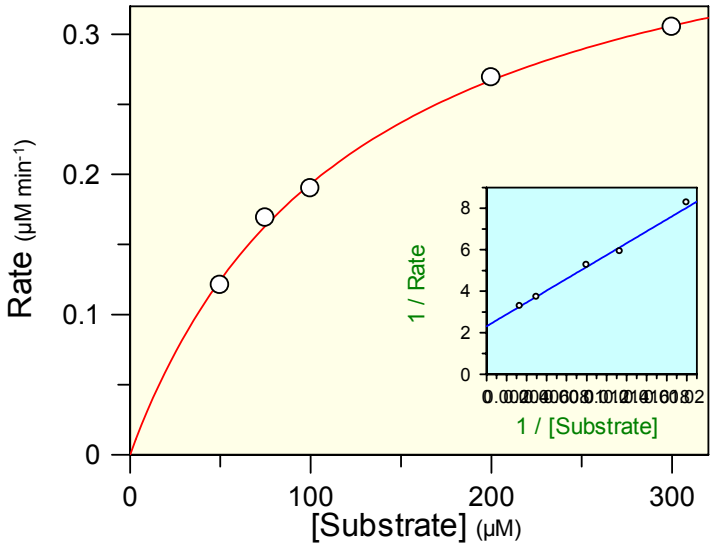


Figure S16: Michaelis-Menten and Lineweaver-Burk plots for α-D-Glc-1-phosphonate (**4**). Michaelis-Menten plot with inset Lineweaver-Burk plot demonstrating a V_{max} of 0.43 μM min^{-1} and a K_m of 123.5 μM with respect to α-D-Glc-1-phosphonate.

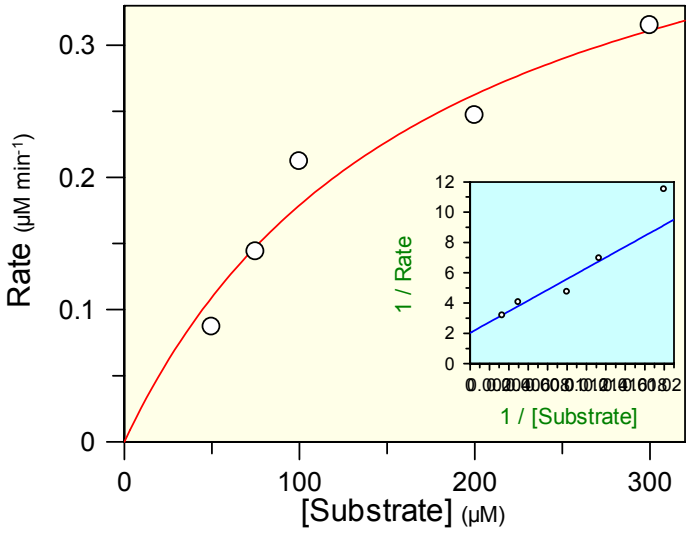


Figure S17: Michaelis-Menten and Lineweaver-Burk plots for α-D-Gal-1-phosphonate (**5**). Michaelis-Menten plot with inset Lineweaver-Burk plot demonstrating a V_{max} of 0.49 μM min^{-1} and a K_m of 175.6 μM with respect to α-D-Gal-1-phosphonate.

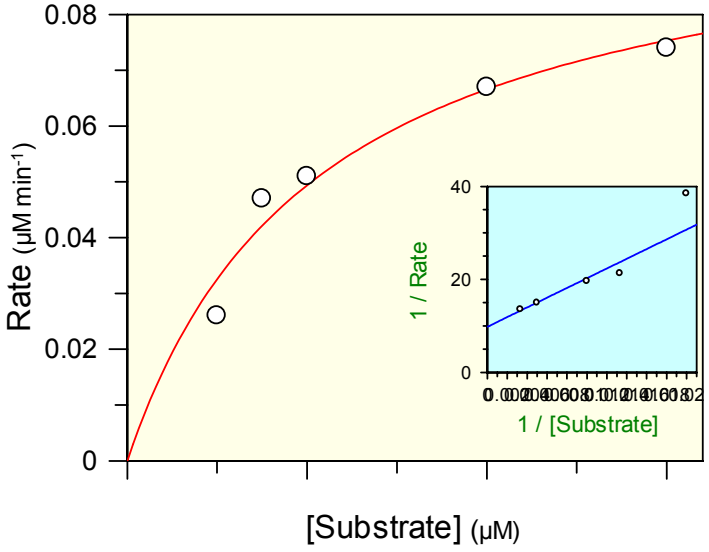
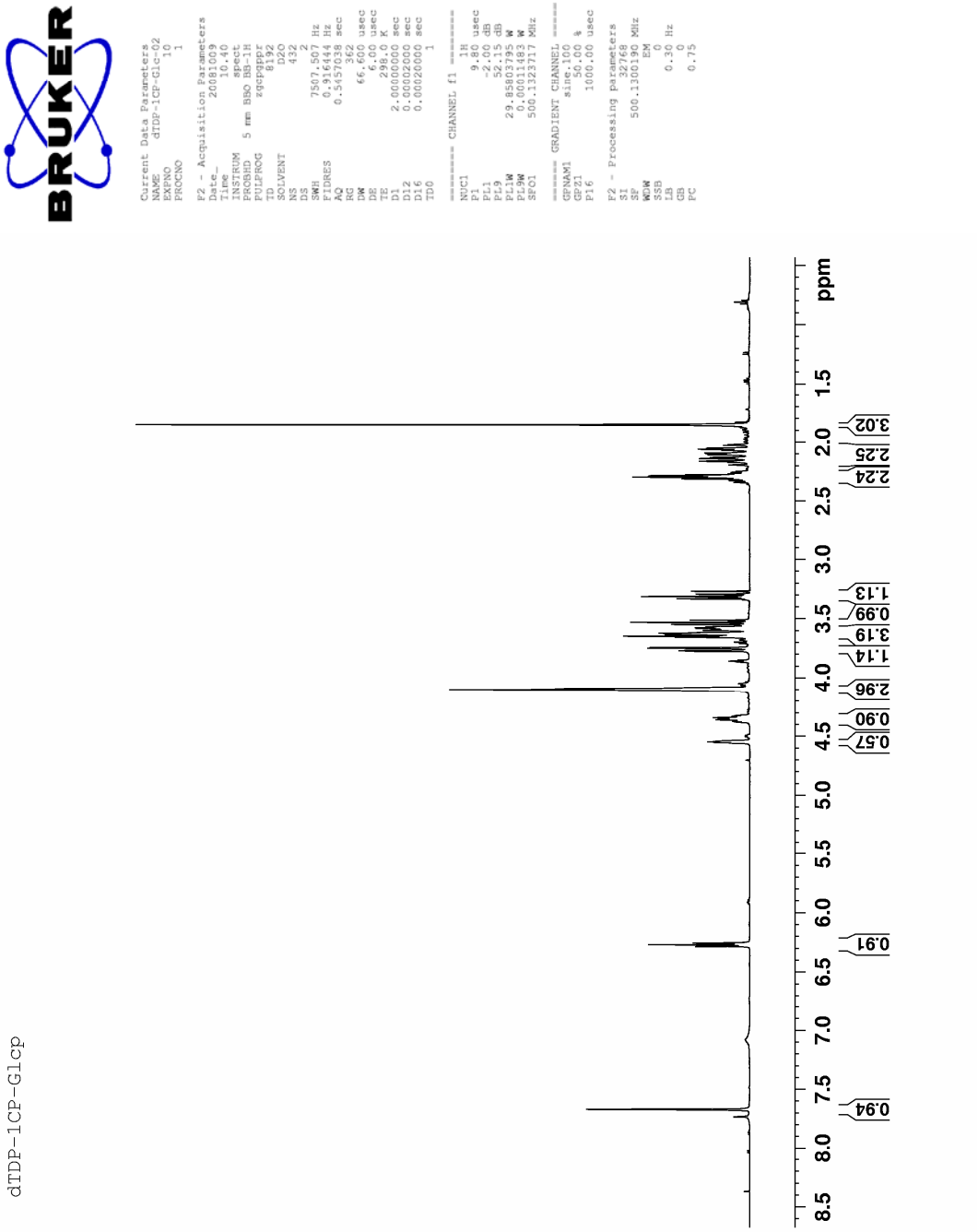


Figure S18: Michaelis-Menten and Lineweaver-Burk plots for α-D-Gal-1-phosphate (**6**). Michaelis-Menten plot with inset Lineweaver-Burk plot demonstrating a V_{max} of 0.102 μM min^{-1} and a K_m of 106.9 μM with respect to α-D-Gal-1-phosphate.

NMR spectra of purified phosphono analogue of dTDP-α-D-glucopyranose: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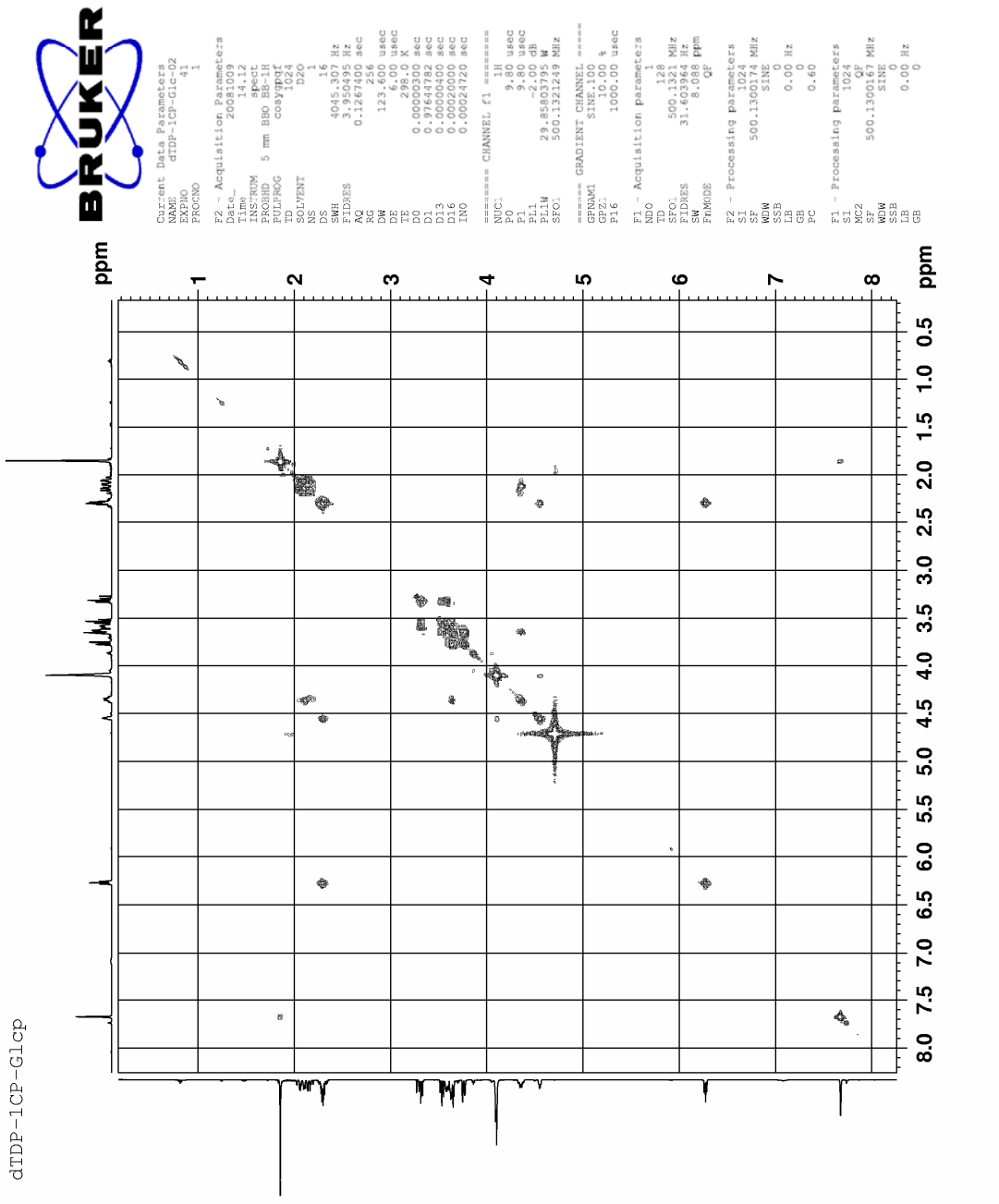
BRUKER
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PULPROG cosygpqf
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DS 1
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FIDRES 3.950499 Hz
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RG 256
DW 123.600 usec
DE 6.00 usec
TE 298.0 K
D0 0.00000300 sec
D1 0.97644782 sec
D13 0.00000400 sec
D16 0.00020000 sec
IN0 0.00024720 sec
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P0 9.80 usec
P1 9.80 usec
PL1 -2.00 dB
PLW1 29.85803795 W
SFO1 500.1321249 MHz
====== GRADIENT CHANNEL =====
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GPZ1 100.00 %
P16 1000.00 usec
F1 - Acquisition parameters
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TD 128
SFO1 500.1321 MHz
FIDRES 31.603964 Hz
SW 8.088 ppm
FnMODE QF
F2 - Processing parameters
SI 1024
SF 500.1300174 MHz
WDW SINE
SSB 0
LB 0.00 Hz
GB 0
PC 0.60
F1 - Processing parameters
SI 1024
MC2 QF
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LB 0.00 Hz
GB 0
ppm
1
2
3
4
5
6
7
8
ppm
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2.0
2.5
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8.0
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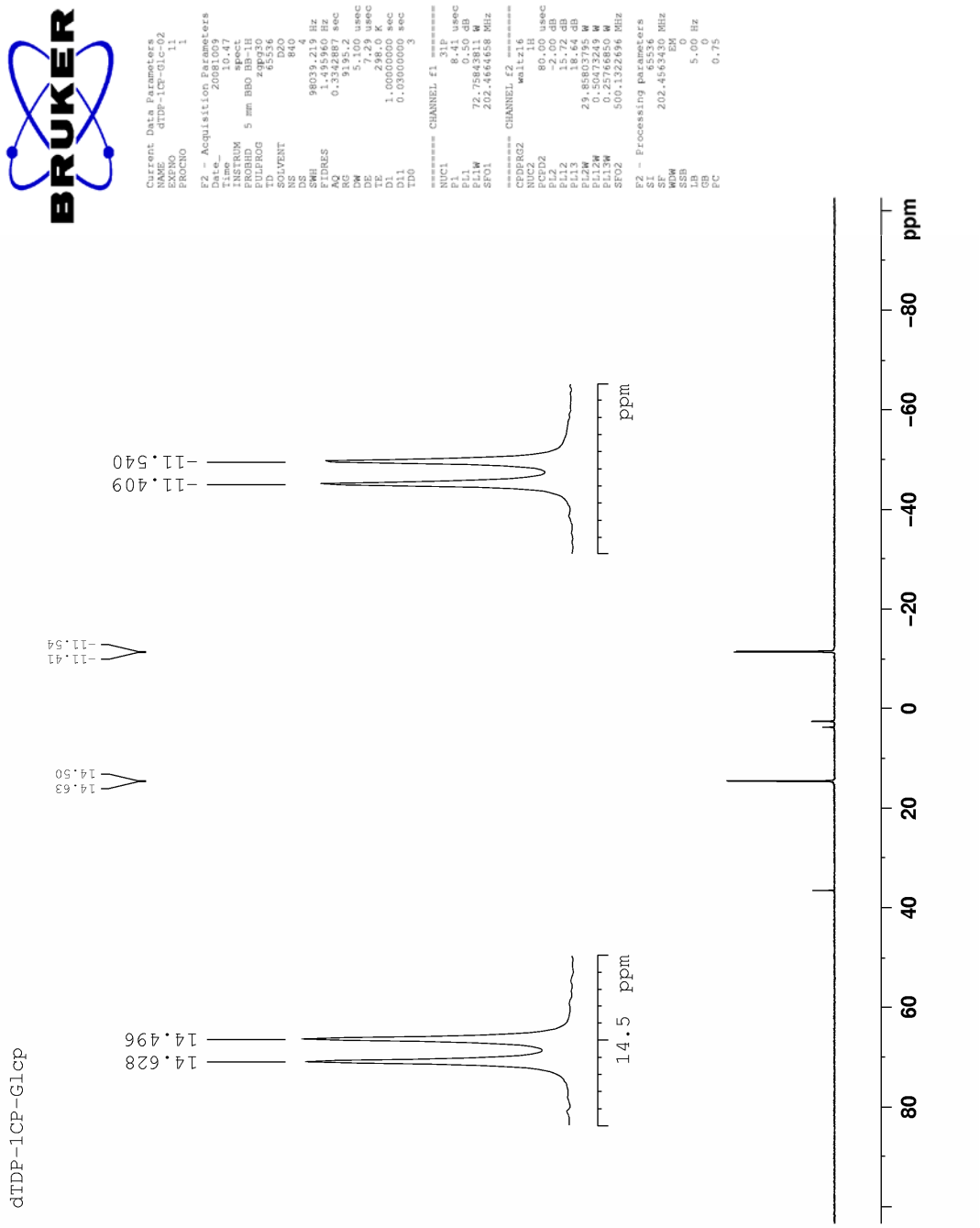
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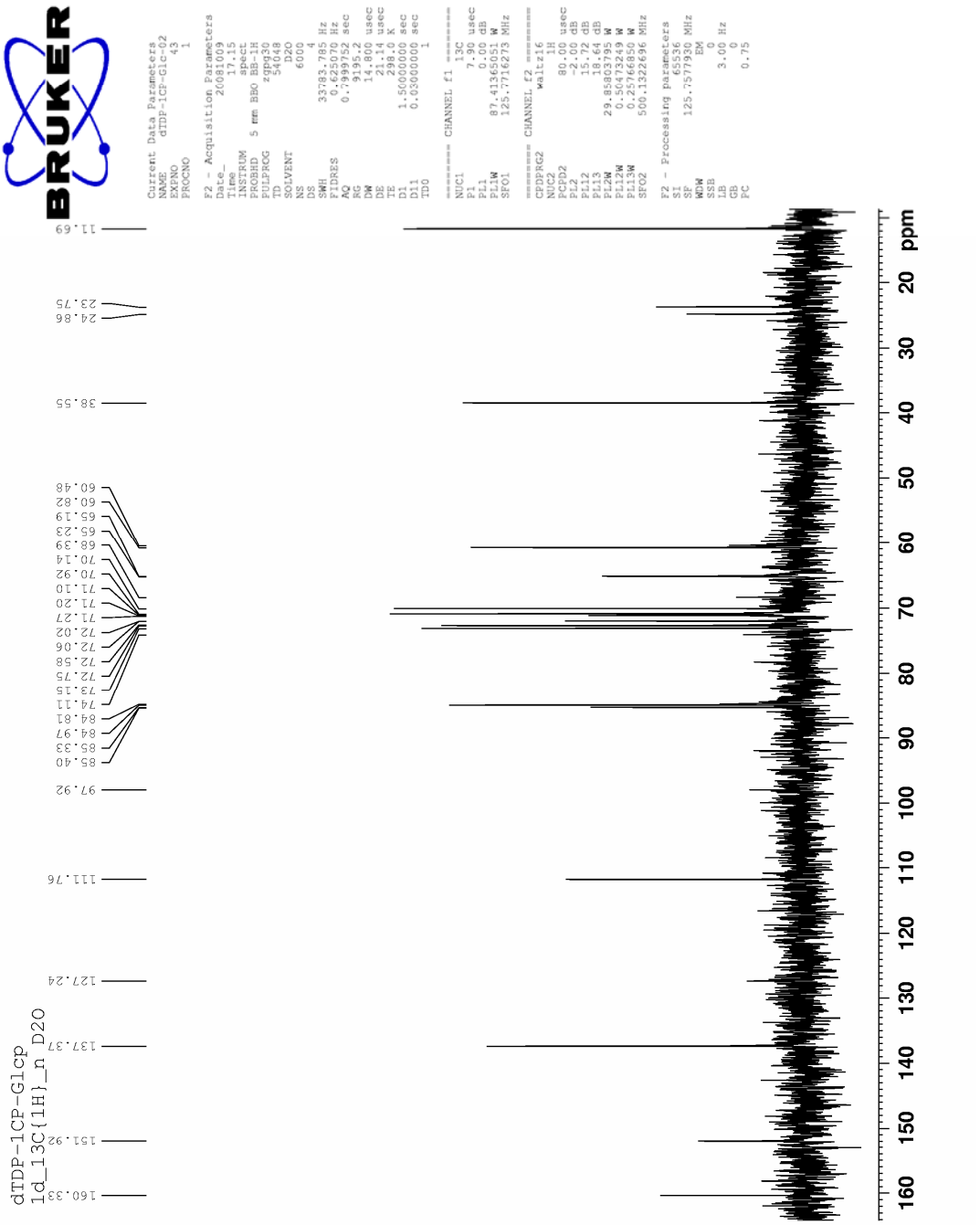
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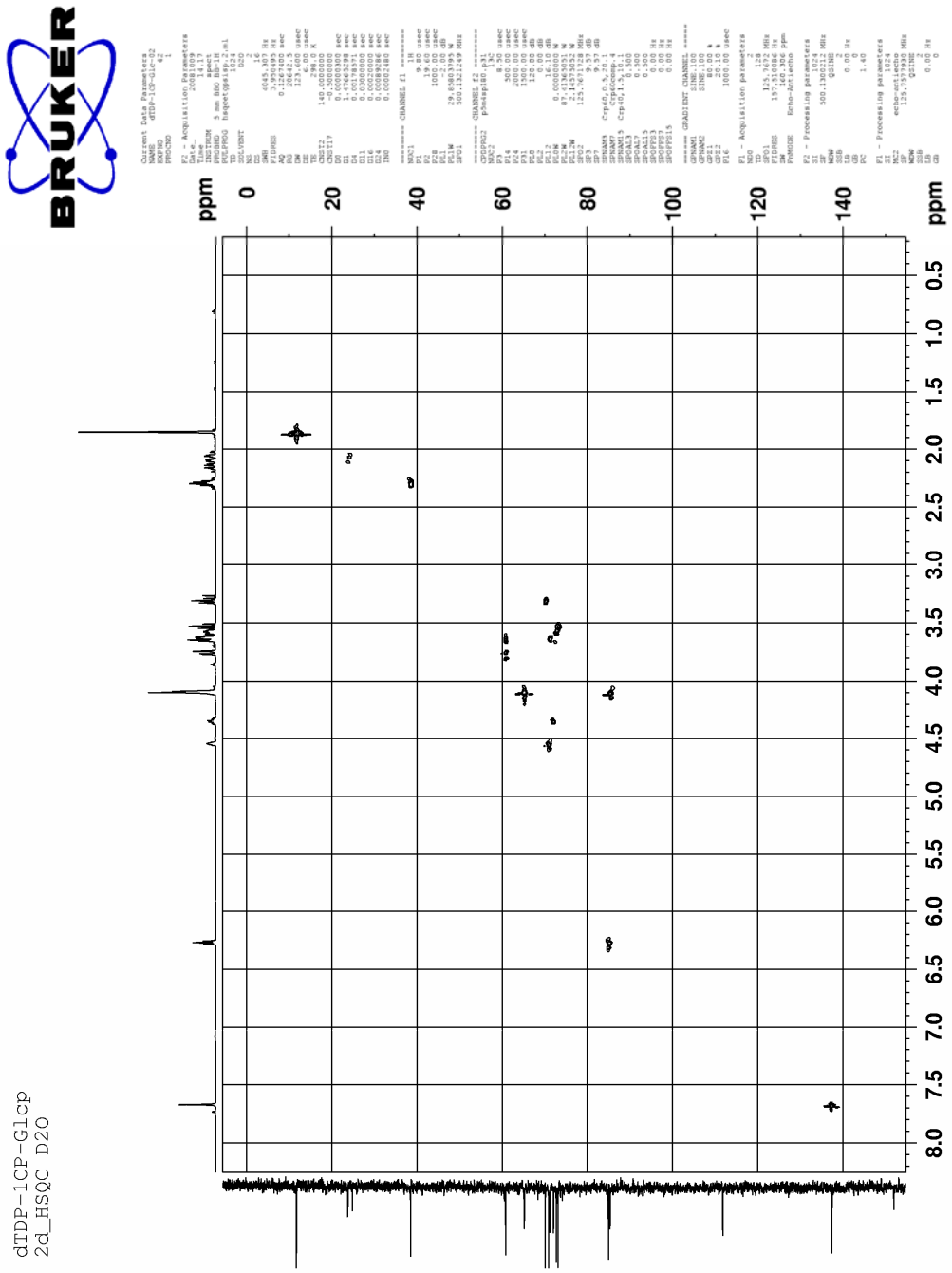
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8.0

References:

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