Supporting information for:

Isoprenoid Phosphonophosphates as Glycosyltransferase Acceptor Substrates

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I. General Procedures and Materials

All compounds were purchased from Sigma Aldrich (Milwaukee, WI), Fisher Scientific (Pittsburgh, PA), or Toronto Research Chemicals (Toronto, Canada). Solvents were purified according to the guidelines in *Purification of Laboratory Chemicals*.[1] All reactions were run under argon atmosphere unless otherwise stated. Analytical thin layer chromatography (TLC) was carried out on E. Merck (Darmstadt) TLC plates pre-coated with silica gel 60 F254 (250 μm layer thickness). Analyte visualization was accomplished using a UV lamp and by charring with *p*-anisaldehyde solution. Flash column chromatography was performed with Silicycle Flash Silica Gel (40–63 μm, 60 Å pore size) using reagent grade hexanes and ACS grade ethyl acetate (EtOAc) or acetone, or methanol (MeOH) and CH_2Cl_2. High-performance liquid chromatography was performed on a Beckman-Coulter instrument with a Vydac Protein and Peptide C18 (22 mm x 250 mm) column, using a gradient of acetonitrile in 25 mM ammonium bicarbonate. ^1H, ^{13}C, and ^{31}P nuclear magnetic resonance (NMR) spectra were recorded on a 300 MHz spectrometer (acquired at 300 MHz for ^1H NMR and 75 MHz for ^{13}C NMR), a 400 MHz spectrometer (acquired at 400 MHz for ^1H NMR, 101 MHz for ^{13}C NMR, and 162 MHz for ^{31}P NMR), a 500 MHz spectrometer (acquired at 500 MHz for ^1H NMR and 126 MHz for ^{13}C NMR), or a 600 MHz spectrometer (acquired at 600 MHz for ^1H NMR and 243 MHz for ^{13}C NMR). Chemical shifts are reported relative to tetramethylsilane or residual solvent peaks in parts per million ($CHCl_3$: ^1H: 7.26, ^{13}C: 77.16; MeOH: ^1H: 3.31, ^{13}C: 49.00; HDO: 1H: 4.79, ^{13}C referenced to ^1H; ^{31}P referenced to ^1H). Peak multiplicity is reported as singlet (s), doublet (d), doublet of doublets (dd), doublets of doublets of doublets (ddd), triplet (t), doublet of triplets (dt), etc. High resolution electrospray ionization-time of flight mass spectra (HRESI-TOF MS) were obtained on a Micromass LCT.

Assays with GlfT1 were carried out in 35 μL final volume containing 2 μM GlfT1-His_6, 100 μM acceptor substrate and 300 μM UDP-Gal*f* in 50 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.9, 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol. Reactions were incubated at 37 °C for 2 h, quenched with 35 μL of a 1:1 v/v mixture of chloroform:methanol, and evaporated to dryness on a SpeedVac SC100 (Varian). The dried mixtures were re-suspended in 35 μL of water, desalted with a ZipTip C18 pipette tip (Millipore), and spotted onto a stainless steel plate for MALDI-TOF MS analysis as a 1:1 v/v mixture with 2-(4-hydroxyphenylazo)benzoic acid matrix. MALDI spectra were recorded in negative reflectron mode on a Bruker Ultraflex III instrument. For product isolation, several reactions with a total volume of 100 μL, containing 0.5 μM GlfT1-His_6, 2 mM acceptor substrate and 1.6 mM UDP-Gal*f* in 50 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.9, 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol, were incubated at 37 °C. Following 2 h, the reactions were filtered through a 10,000 molecular weight cutoff centrifugal filter and separated by HPLC. The identity of the product-containing fraction was analyzed by MALDI-TOF mass spectrometry and NMR spectroscopy.

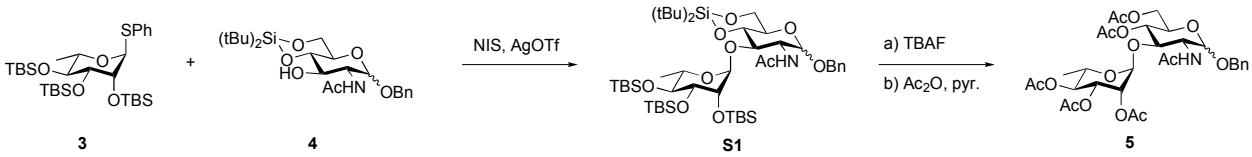
For experiments assessing glycosyltransferase activity by monitoring UDP production, three replicates of a GlfT1 reaction mixture were incubated at 37 °C for 2 h, then aliquoted in triplicate into white microwell plates and mixed 1:1 with a reagent coupling UDP production to the luciferase/luciferin reaction. Luminescence was measured on a Tecan Infinite M1000 after incubating for 1 h at ambient temperature. The luminescence readout was fitted to a standard curve made from a dilution series of known UDP concentrations measured in the same microwell plate. In kinetics experiments, three replicates of reaction mixtures containing 0.2 μM GlfT1-His_6, 0.2–200 μM acceptor substrate and 150 μM UDP-Gal*f* in 50 mM 4-morpholinepropanesulfonic acid (MOPS), pH 7.9, 10 mM $MgCl_2$ and 5 mM β-mercaptoethanol were quenched after 5 min by treatment with the reagent coupling UDP production to the luciferase/luciferin reaction and analyzed as described above. In all experiments, reaction

mixtures without acceptor substrate were utilized as negative controls. The results were fitted to the Michaelis-Menten equation using GraphPad (La Jolla, California) Prism 6.

The gene *glfT1* was amplified from purified *M. smegmatis* mc^2155 genomic DNA using the forward primer 5'-TTATTACATATGACGCACACTGAGGTCGTCTG-3' and the reverse primer 5'-TTATTAGCTAGCTCAGTGGTGGTGGTGGTGGTGCTCGAGTCGCTGGAACCT TTCGCGT-3'. Primers were employed to generate a product encoding a C-terminal hexahistidine fusion linked to the *glfT1* gene product. PCR product was digested with NdeI and NheI (New England Biolabs) and ligated into the mycobacterial expression vector pLAM12 (Addgene, plasmid 26908).[2] Positive clones were identified by Sanger sequencing and electroporated into competent *M. smegmatis* mc^2155. Transformed cells were recovered by shaking at 37 °C for 4 h in 7H9 Middlebrook media (Thermo Fisher Scientific) supplemented with ADC (bovine serum albumin, dextrose, and catalase) enrichment and 0.05% Tween 80. Cells were then plated on Luria–Bertani solid media containing 20 μg/mL kanamycin and grown for 3 d at 37 °C. One milliliter of liquid culture media was inoculated from a single colony and grown to saturation over 3 d at 37 °C with shaking. Six hundred milliliters of 7H9 Middlebrook media supplemented with ADC enrichment, 20 μg/mL kanamycin, 50 μg/mL carbenicillin, and 0.05% Tween 80 was inoculated with 0.5 mL of the saturated starter culture and grown to mid-log overnight at 37 °C. Expression of MsGlfT1-His_6 was induced by addition of acetamide to a final concentration of 0.02% w/v. The induced culture was grown overnight at 37 °C with shaking. Cells were harvested by centrifugation at 5,000 x *g* for 15 min. Liquid media was decanted and the cell pellet was stored at –80 °C until further use. The cell pellet was thawed on ice and resuspended in 20 mL of Buffer A (50 mM sodium phosphate, pH 7.6, 400 mM sodium chloride (NaCl), 15% glycerol, 20 mM imidazole) containing 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1% Triton X-100, and 96 mg of lysozyme. Once homogenous, the resuspended pellet was incubated at 37 °C for 2 h with gentle shaking (100 rpm).[3] Cells were disrupted via sonication (Branson 450 sonifier). Lysates were cleared by centrifugation at 22,000 x *g* and the soluble fraction was passed through a 0.22 μm syringe filter unit. Protein from the cleared lysate was bound to a HP HisTrap nickel column (GE Healthcare) using an ÄKTA FPLC system (Amersham Biosciences). The column was washed with 20 mL of Buffer A. A linear gradient from 0% to 100% of Buffer B (50 mM sodium phosphate, pH 7.6, 400 mM NaCl, 15% glycerol, 400 mM imidazole) was applied to the column over 20 min to elute bound protein. Fractions containing MsGlfT1-His_6 were identified by SDS/PAGE and concentrated in a 3,000 molecular weight cutoff centrifugal filter. Concentrated MsGlfT1-His_6 was then dialyzed twice into 1 L of 50 mM sodium phosphate, pH 7.6, 400 mM NaCl, 15% glycerol. MsGlfT1-His_6 aliquots were vitrified in liquid nitrogen and stored at –80 °C.

For immunoblotting, SDS/PAGE sample buffer was added to purified MsGlfT1-His_6 and the sample was loaded onto a 4–15% Tris-HCl gel. The sample was then transferred onto 0.45 μm PVDF membrane (Millipore) and blocked for 2 h at ambient temperature with a solution of Tris-buffered saline and 0.1% Tween 20 (TBS-T) containing 5% nonfat milk. The membrane was exposed to monoclonal anti-His_6 primary antibody (70796, Novagen) at 1:2000 dilution in TBS-T solution with 5% milk overnight at 4 °C. After rinsing three times in TBS-T for 5 min, the blot was treated at ambient temperature with a 1:5000 dilution of HRP-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch) in TBS-T solution containing 5% milk for 1 h at ambient temperature. The membrane was washed with TBS-T and visualized with chemiluminescent substrate (ECL, Pierce) on an ImageQuant LAS 4000 (GE Healthcare).

II. Experimental Procedures



Benzyl 2-acetamido-2-deoxy-4,6-di-*O*-acetyl-3-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-D-glucopyranoside **5**:

Compounds **3** (108 mg, 0.18 mmol, 1.5 eq) and **4** (54 mg, 0.12 mmol) were combined and coevaporated with toluene (3x). A stir bar and activated powdered 4 Å molecular sieves were added under Ar atmosphere, followed by CH_2Cl_2 (4.8 mL). The suspension was stirred at ambient temperature for 45 min, then cooled to –25 °C for 15 min. *N*-iodosuccinimide (49 mg, 0.22 mmol, 1.8 eq) and silver trifluoromethanesulfonate (6 mg, 0.02 mmol, 0.2 eq) were added and the suspension stirred at –25 °C for 2 h. The reaction was warmed to –5 °C over 20 min, then filtered through Celite. The filtrate was washed with 10% v/v sodium thiosulfate, saturated sodium bicarbonate, water, and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash chromatography (4:1 hexanes:ethyl acetate) afforded **S1** (91 mg, 80%) as a white, crystalline solid.

^{1}H (300 MHz, $CDCl_3$): δ 7.38 – 7.24 (m, 5H), 5.72 (d, J = 10.0 Hz, 1H), 4.90 (d, J = 1.9 Hz, 1H), 4.69 (d, J = 3.7 Hz, 1H), 4.66 (d, J = 11.9 Hz, 1H), 4.46 (d, J = 11.9 Hz, 1H), 4.27 (td, J = 10.0, 3.7 Hz, 1H), 4.15 – 3.69 (m, 12H), 1.87 (s, 3H), 1.19 (d, J = 6.2 Hz, 3H), 1.06 (s, 9H), 0.97 (s, 9H), 0.93 (s, 9H), 0.89 (s, 9H), 0.85 (s, 9H), 0.10 (s, 3H), 0.09 (s, 3H), 0.08 (s, 3H), 0.04 (s, 3H), 0.03 (s, 3H).

^{13}C (75 MHz, $CDCl_3$): δ 169.5, 137.1, 128.7, 128.3, 128.3, 99.4, 97.5, 76.5, 74.5, 73.3, 73.0, 72.7, 70.8, 70.1, 67.1, 66.9, 53.7, 27.8, 27.2, 26.9, 26.3, 25.8, 23.6, 22.8, 20.0, 19.3, 18.9, 18.2, 18.1, 0.1, –2.3, –3.3, –4.0, –4.0, –4.1, –4.1, –4.2.

HRMS (ESI-TOF$^+$) for $C_{47}H_{89}NNaO_{10}Si_4$ ($M+Na^+$) calcd 962.5456, found 962.5453.

Compound **S1** (348 mg, 0.37 mmol) was treated with TBAF (1.0 M in THF, 5.6 mL, 5.6 mmol, 15.0 eq) under Ar atmosphere at ambient temperature for 3 h, and then concentrated under reduced pressure. The residue was taken up in pyridine (1.5 mL) under Ar atmosphere, then cooled to 0 °C for 15 min. Acetic anhydride (3.5 mL) was added dropwise, then the solution was allowed to warm to ambient temperature. After 36 h, the reaction was concentrated under reduced pressure and coevaporated with toluene (3x) to remove residual pyridine. Purification by flash chromatography (3:2 hexanes:acetone) afforded **5** (91 mg, 80%) as a clear glass.

^{1}H (300 MHz, $CDCl_3$): δ 7.43 – 7.27 (m, 5H), 5.75 (d, J = 9.6 Hz, 1H), 5.17 – 5.05 (m, 2H), 5.00 (t, J = 10.3 Hz, 1H), 4.91 (d, J = 3.4 Hz, 2H), 4.80 (s, 1H), 4.69 (d, J = 11.8 Hz, 1H), 4.50 (d, J = 11.8 Hz, 1H), 4.42 (dt, J = 10.0, 5.2 Hz, 1H), 4.17 (dd, J = 12.3, 4.3 Hz, 1H), 4.01 (d, J = 12.3 Hz, 1H), 3.94 – 3.76 (m, 4H), 2.12 (s, 3H), 2.10 (s, 3H), 2.07 (s, 3H), 2.04 (s, 3H), 2.02 (s, 3H), 1.94 (s, 3H), 1.13 (d, J = 6.1 Hz, 3H).

^{13}C (75 MHz, $CDCl_3$): δ 170.8, 170.6, 170.5, 170.1, 169.6, 169.5, 136.6, 128.8, 128.5, 128.4, 99.7, 97.0, 80.3, 70.7, 70.2, 70.2, 70.1, 69.0, 68.4, 67.5, 62.1, 60.5, 53.9, 51.9, 51.6, 31.8, 29.8, 29.4, 25.7, 23.3, 21.2, 21.0, 20.9, 20.7, 20.4, 17.3, 14.3, 13.8.

HRMS (ESI-TOF$^+$) for $C_{31}H_{42}NO_{15}$ ($M+H^+$) calcd 668.2549, found 668.2855.

Triethylammonium 2-acetamido-2-deoxy-4,6-di-*O*-acetyl-3-*O*-(2,3,4-tri-*O*-acetyl-α-L-rhamnopyranosyl)-α-D-glucopyranosyl phosphate **6**:

Compound **5** (30 mg, 45 μmol) was dissolved in ethanol (4.5 mL) under Ar atmosphere. Palladium on carbon (15 mg, 10%) was added, then the reaction was placed under H_2 atmosphere by evacuating the flask and backfilling with H_2 via balloon (4x). The suspension stirred vigorously at ambient temperature for 45 h. The reaction was filtered through a cotton plug and a 0.22 μm filter, then concentrated under reduced pressure to afford the lactol as a clear oil that was carried onto the next step without further purification.

The lactol (26 mg, 45 μmol) was coevaporated with toluene (3x), then dissolved in CH_2Cl_2 (2.3 mL). This solution was added via cannula to a solution of 1*H*-tetrazole (15 mg, 0.21 mmol) and dibenzyl *N*,*N*-diisopropylphosphoramidite (46 μL, 0.14 mmol) in CH_2Cl_2 (2.3 mL) at ambient temperature. The reaction stirred at ambient temperature for 2 h, then it was cooled to –40 °C for 15 min. *meta*-Chloroperoxybenzoic acid (77% max, 43 mg, 0.25 mmol) was added in one portion, and the cloudy solution stirred at –40 °C for 10 min. The reaction was then allowed to warm to ambient temperature. After stirring for 1.5 h at ambient temperature, the reaction was diluted with CH_2Cl_2 (15 mL), washed with 10% v/v sodium thiosulfate (2 x 20 mL), saturated sodium bicarbonate (2 x 20 mL), and water (2 x 20 mL). The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash chromatography (3:2 acetone:hexanes) afforded the phosphotriester as a light yellow oil.

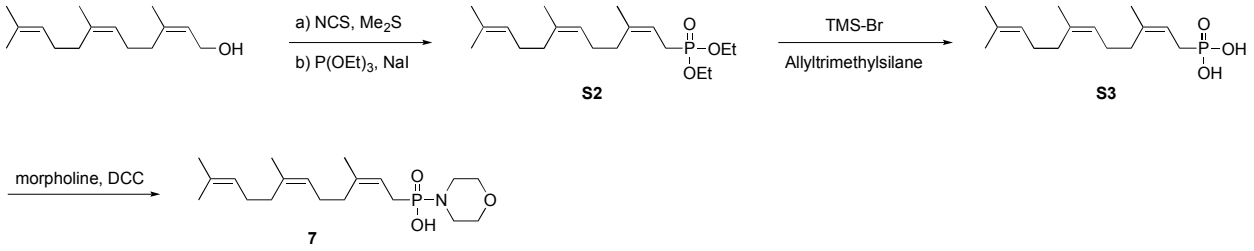
Triethylamine (0.60 mL, 20% v/v w.r.t. ethanol) was added to a solution of the phosphotriester in ethanol (3.0 mL) under Ar atmosphere. Palladium on carbon (3.0 mg, 10%) was added, then the reaction was placed under H_2 atmosphere by evacuating the flask and backfilling with H_2 via balloon (4x). The suspension stirred vigorously at ambient temperature for 17 h. The reaction was filtered through a cotton plug and a 0.22 μm filter, then concentrated under reduced pressure to afford **6** (28 mg, 69% over 4 steps) as a white solid.

^{1}H (300 MHz, $CDCl_3$): δ 5.43 (dd, J = 6.9, 3.2 Hz, 1H), 5.17 (dd, J = 3.3, 2.0 Hz, 1H), 5.14 – 5.06 (m, 2H), 5.01 – 4.92 (m, 3H), 4.26 – 4.16 (m, 3H), 4.13 – 4.00 (m, 2H), 3.92 – 3.80 (m, 1H), 3.16 (q, J = 7.3 Hz, 15H), 2.12 (s, 3H), 2.08 (s, 3H), 2.06 (s, 3H), 2.04 (s, 3H), 2.03 (s, 3H), 1.93 (s, 3H), 1.30 (t, J = 7.3 Hz, 22H), 1.14 (d, J = 6.2 Hz, 3H).

^{13}C (126 MHz, CD_3OD): δ 178.3, 172.6, 171.2, 170.3, 170.1, 170.1, 169.7, 99.3, 93.8 (d), 78.5, 70.7, 69.9, 69.7, 69.0, 68.5, 67.0, 61.8, 53.2 (d), 46.1, 22.4, 21.6, 20.0, 19.4, 19.3, 19.2, 16.4, 7.8.

^{31}P (162 MHz, CD_3OD): δ –0.65.

HRMS (ESI-TOF$^-$) for $C_{24}H_{35}NO_{18}P$ (M^-) calcd 656.1597, found 656.1610.



Morpholino((2*Z*,6*Z*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)phosphinic acid, N,N'-dicyclohexyl-4-morpholinecarboxamidine salt **7**:

A solution of *N*-chlorosuccinimide (155 mg, 1.16 mmol, 1.11 eq) in CH_2Cl_2 (2.10 mL) under Ar atmosphere was cooled to –30 °C for 15 min. Methyl sulfide (91 µL, 1.23 mmol, 1.18 eq) was added and the solution was warmed to 0 °C for 20 min. The solution was cooled to –30 °C for 10 min, then a solution of (2*Z*,6*Z*)-farnesol[4,5] (233 mg, 1.05 mmol) in CH_2Cl_2 (2.1 mL) was added dropwise via cannula. The solution was warmed to 0 °C and stirred for 1.5 h before warming to ambient temperature. After stirring at ambient temperature for 1 h, the reaction was diluted with hexanes, filtered, and washed with brine (2x). The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure to afford crude (2*Z*,6*Z*)-farnesyl chloride (203 mg). Sodium iodide (25 mg, 0.17 mmol) and triethyl phosphite (320 µL, 1.86 mmol) were added under Ar atmosphere, and the suspension stirred vigorously at 110 °C for 19 h. The reaction was cooled to ambient temperature, diluted with Et_2O, and washed with saturated sodium thiosulfate (2x) and brine. The organic layer was dried over $MgSO_4$, filtered, and concentrated under reduced pressure. Purification by flash chromatography (3:2 ethyl acetate:hexanes) afforded **S2** (203 mg, 70%) as a clear oil.

^{1}H (400 MHz, $CDCl_3$): δ 5.20 (q, *J* = 7.1 Hz, 1H), 5.12 (m, 2H), 4.16 – 4.01 (m, 4H), 2.56 (dd, *J* = 21.8, 7.3 Hz, 2H), 2.05 (d, *J* = 9.5 Hz, 8H), 1.75 (d, *J* = 5.0 Hz, 3H), 1.69 (s, 6H), 1.61 (s, 3H), 1.31 (t, *J* = 7.1 Hz, 6H).

^{13}C (101 MHz, $CDCl_3$): δ 140.1 (d), 135.5, 131.4, 124.5, 124.2, 113.1 (d), 61.7, 61.6, 32.2, 32.2, 31.87, 26.6, 26.2 (d), 26.0 (d), 25.6, 23.5, 23.4, 23.3, 17.6, 16.4, 16.4.

^{31}P (162 MHz, $CDCl_3$): δ 28.7.

HRMS (ESI-TOF$^+$) for $C_{19}H_{36}O_3P$ ($M+H^+$) calcd 343.2397, found 343.2409.

Compound **S2** (34 mg, 0.10 mmol) was coevaporated with toluene (3 x 1.5 mL), then dissolved in CH_2Cl_2 (1.4 mL) under Ar atmosphere. Allyltrimethylsilane (23 µL, 0.15 mmol, 1.5 eq) and bromotrimethylsilane (42 µL, 0.32 mmol, 3.2 eq) were added dropwise at ambient temperature and the reaction stirred for 24 h. Additional bromotrimethylsilane (0.13 mL, 9.6 eq) was added to ensure complete deprotection. The reaction stirred for an additional 4 h. The reaction was concentrated under reduced pressure, then diluted with ammonium bicarbonate (1 M, 0.9 mL), flash-frozen and lyophilized to afford **S3** (31 mg, 97%) as an off-white fluffy solid.

^{1}H (400 MHz, CD_3OD): δ 5.34 (q, *J* = 7.0 Hz, 1H), 5.21 – 5.10 (m, 2H), 2.37 (dd, *J* = 20.8, 7.5 Hz, 2H), 2.12 – 2.03 (m, 8H), 1.72 (m, 3H), 1.68 (s, 6H), 1.62 (s, 3H).

^{13}C (126 MHz, CD_3OD): δ 137.5 (d), 136.0, 132.3, 126.3, 125.4, 119.4 (d), 33.2 (d), 32.9, 30.3 (d), 27.7, 27.3 (d), 25.9, 23.9 (d), 23.7, 17.7.

^{31}P (162 MHz, CD_3OD): δ 21.4.

HRMS (ESI-TOF$^-$) for $C_{15}H_{27}O_3P$ (M^-) calcd 285.1625, found 285.1636.

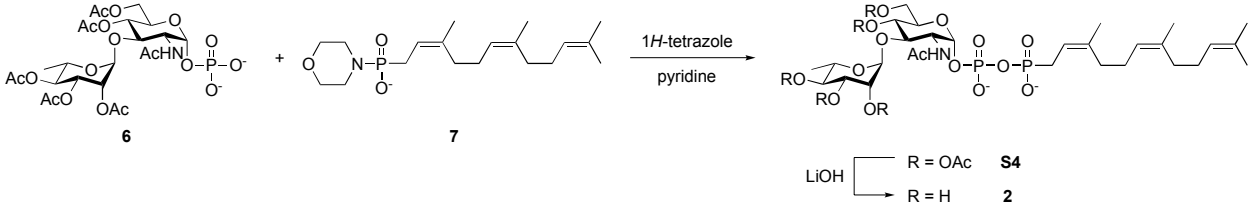
Morpholine (73 µL, 0.84 mmol, 8 eq) was added to a solution of **S3** (31 mg, 0.11 mmol) in 1:1 *tert*-butanol:water (10.5 mL) under Ar atmosphere at ambient temperature. The solution stirred at ambient temperature for 10 min, then it was heated to 100 °C. After refluxing for 15 min, a solution of dicyclohexylcarbodiimide (433 mg, 2.1 mmol, 20 eq) in *tert*-butanol (3.5 mL) was added over 2.5 h. The reaction was refluxed for an additional 3 h before being concentrated under reduced pressure. The residue was taken up in water (40 mL) and washed with diisopropyl ether (3x). The aqueous layer was flash-frozen and lyophilized. The residue was taken up in methanol (1.5 mL) and loaded onto two Waters Oasis Plus MAX cartridges connected in series. Product eluted with 50 mM ammonium bicarbonate, pH 8.25. Further purification by flash chromatography (80:20:1 $CHCl_3$:MeOH:Et_3N) afforded **7** (27.6 mg, 43%) as a white solid.

^{1}H (400 MHz, CD_3OD): δ 8.52 (s, 1H), 5.29 (q, *J* = 7.3 Hz, 1H), 5.17 – 5.04 (m, 2H), 3.72 – 3.68 (m, 10H), 3.63 – 3.57 (m, 1H), 3.53 (t, *J* = 4.6 Hz, 4H), 3.38 (t, *J* = 4.8 Hz, 9H), 3.30 – 3.24 (m, 5H), 3.13 (s, 1H), 3.01 (q, *J* = 4.5 Hz, 4H), 2.30 (dd, *J* = 19.2, 7.7 Hz, 2H), 2.12 – 1.99 (m, 7H), 1.95 – 1.83 (m, 12H), 1.83 – 1.75 (m, 9H), 1.75 – 1.60 (m, 16H), 1.58 (s, 1H), 1.56 – 1.49 (m, 1H), 1.46 – 1.24 (m, 25H), 1.24 – 1.08 (m, 7H).

^{13}C (126 MHz, CD_3OD): δ 157.9, 134.9, 134.7, 130.9, 124.9, 124.8 (d), 124.0, 118.1 (d), 117.3, 67.5 (d), 65.9, 63.7, 54.6, 48.4, 45.1, 43.0, 33.1, 31.9, 31.5, 31.5 (d), 29.4, 27.8, 26.3, 25.9, 24.9, 24.7, 24.6 (d), 22.5, 22.4, 22.3, 16.4.

^{31}P (162 MHz, CD_3OD): δ 21.4.

HRMS (ESI-TOF$^-$) for $C_{19}H_{33}NO_3P$ (M^-) calcd 354.2203, found 354.2210.



(2-acetamido-2-deoxy-3-*O*-(α-L-rhamnopyranosyl)-α-D-glucopyranosyl phosphoryl) ((2*Z*,6*Z*)-3,7,11-trimethyldodeca-2,6,10-trien-1-yl)phosphonate **2**:

Compounds **6** (11 mg, 12 µmol) and **7** (16 mg, 25 µmol, 2.0 eq) were coevaporated with toluene (2 x 1.5 mL) and pyridine (2 x 1.5 mL) then placed on high vacuum overnight. A stir bar and

1*H*-tetrazole (2.8 mg, 40 μmol, 3.2 eq) were added under Ar atmosphere, followed by pyridine (0.25 mL). The reaction stirred at ambient temperature for 5 d. It was diluted with methanol and concentrated under reduced pressure. The crude was purified by semi-preparative HPLC on a C18 column (25% B for 5 min, 25% B to 70% B over 45 min, A = 50 mM ammonium bicarbonate and B = acetonitrile). Fractions containing compound **S4** were pooled and concentrated under reduced pressure to a white solid.

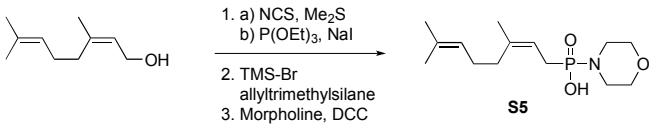
Fractions containing compound **S4** were treated with 0.5 M LiOH (1.0 mL) at ambient temperature for 4 h. The reaction mixture was neutralized, and purified immediately by semi-preparative HPLC on a C18 column (25% B for 5 min, 25% B to 70% B over 45 min, A = 50 mM ammonium bicarbonate and B = acetonitrile). Compound **2** eluted at 29 min (1.8 mg, 20% over 2 steps) as a white solid.

^{1}H (500 MHz, D_2O):δ 5.45 (dd, *J* = 7.3, 3.1 Hz, 1H), 5.29 – 5.17 (m, 3H), 4.85 (s, 1H), 4.08 (dt, *J* = 10.3, 3.0 Hz, 1H), 3.99 (dq, *J* = 10.1, 6.3 Hz, 1H), 3.94 (ddd, *J* = 10.6, 4.8, 2.2 Hz, 1H), 3.90-3.68 (m, 5H), 3.57 (t, *J* = 9.6 Hz, 1H), 3.41 (t, *J* = 9.7 Hz, 1H), 2.56 (dd, *J* = 21.5, 7.6 Hz, 2H), 2.14 – 2.04 (m, 12H), 1.73 (d, *J* = 4.8 Hz, 3H), 1.68 (s, 6H), 1.62 (s, 3H), 1.22 (t, *J* = 6.2 Hz, 3H).

^{13}C (126 MHz, D_2O): δ 163.8, 139.5 (d), 137.1, 133.7, 125.3, 124.4, 115.5 (d), 101.5, 94.5 (d), 79.5, 73.3, 71.9, 70.8, 70.2, 68.9, 68.2, 60.4, 53.3, 31.5 (d), 31.2, 28.5 (d), 25.9, 25.6 (d), 25.0, 22.8 (d), 22.5, 22.2, 17.0, 16.5.

^{31}P (162 MHz, D_2O): δ 16.63 (d, *J* = 28.4 Hz), –13.09 (d, *J* = 28.4 Hz).

HRMS (ESI-TOF$^+$) for $C_{29}H_{55}N_2O_{15}P_2$ ($M+NH_4^+$) calcd 733.3073, found 733.3052.



(*Z*)-(3,7-dimethylocta-2,6-dien-1-yl)(morpholino)phosphinic acid **S5**:

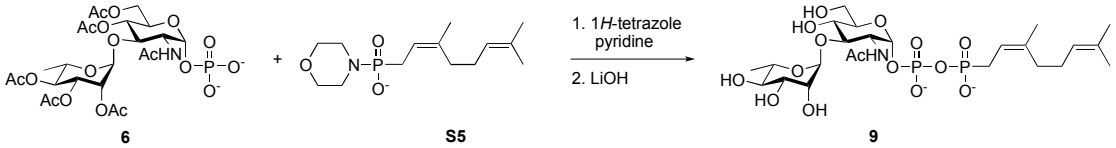
Prepared from nerol (60% over 4 steps) as in **7** as a clear oil.

^{1}H (500 MHz, CD_3OD): δ 8.52 (br s, 1H), 5.32 (q, *J* = 7.1 Hz, 1H), 5.17-5.11 (m, 1H), 3.76-3.72 (m, 6H), 3.59-3.54 (m, 4H), 3.44-3.39 (m, 6H), 3.37-3.32 (m, 2H), 3.05 (q, *J* = 4.2 Hz, 4H), 2.35 (dd, *J* = 19.3, 7.5 Hz, 2H), 2.11-2.07 (m, 4H), 1.99 - 1.87 (m, 6H), 1.87-1.75 (m, 6H), 1.74-1.64 (m, 9H), 1.62 (s, 3H), 1.48-1.32 (m, 9H).

^{13}C (126 MHz, CD_3OD): δ 159.3, 137.0 (d), 132.4, 125.4, 119.6 (d), 68.9, 68.9, 67.3, 56.0, 49.8, 46.4, 34.4, 33.2, 30.0, 29.0, 27.5, 27.5, 26.3, 26.2, 26.0, 23.8 (d), 17.8.

^{31}P (162 MHz, CD_3OD): δ 21.0.

HRMS (ESI-TOF$^-$) for $C_{14}H_{25}NO_3P$ (M^-) calcd 286.157, found 286.1593.



(2-acetamido-2-deoxy-3-*O*-(α-L-rhamnopyranosyl)-α-D-glucopyranosyl phosphoryl) ((*Z*)-(3,7-dimethylocta-2,6-dien-1-yl)phosphonate **9**:

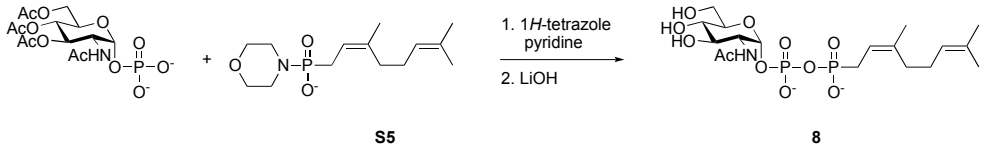
Prepared from disaccharide **6** (triethylammonium salt, 39 mg, 45 μmol) and neryl phosphonomorpholidate **S5** (66 mg, 0.11 mmol) as in **2** as a white solid (7.2 mg, 24% over 2 steps).

^{1}H (500 MHz, D_2O): δ 5.44 (dd, *J* = 7.1, 3.1 Hz, 1H), 5.28 – 5.17 (m, 2H), 4.84 (s, 1H), 4.08 (dt, *J* = 10.3, 2.9 Hz, 1H), 4.02 – 3.95 (m, 1H), 3.94 – 3.90 (m, 1H), 3.89 – 3.66 (m, 5H), 3.57 (t, *J* = 9.6 Hz, 1H), 3.40 (t, *J* = 9.6 Hz, 1H), 2.55 (dd, *J* = 21.5, 7.6 Hz, 2H), 2.15 – 2.08 (m, 4H), 2.07 (s, 3H), 1.72 (d, *J* = 4.7 Hz, 3H), 1.67 (s, 3H), 1.61 (s, 3H), 1.21 (d, *J* = 6.3 Hz, 3H).

^{13}C (126 MHz, D_2O): δ 166.4, 139.6 (d), 133.7, 124.3, 115.4 (d), 101.4, 94.5 (d), 79.5, 73.3, 71.9, 70.7, 70.2, 68.9, 68.2, 60.4, 53.3 (d), 31.2, 28.5 (d), 25.8, 24.9, 22.7 (d), 22.2, 17.0, 16.5.

^{31}P (162 MHz, D_2O): δ 17.13 (d, *J* = 28.1 Hz), –13.01 (d, *J* = 28.1 Hz).

HRMS (ESI-TOF$^+$) for $C_{24}H_{47}N_2O_{15}P_2$ ($M+NH_4^+$) calcd 665.2447, found 665.2438.



(2-acetamido-2-deoxy-α-D-glucopyranosyl phosphoryl) ((*Z*)-(3,7-dimethylocta-2,6-dien-1-yl)phosphonate **8**:

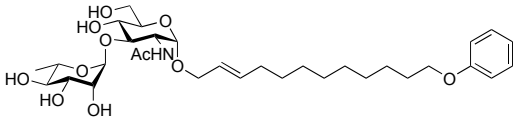
Prepared from 2-acetamido-2-deoxy-3,4,6-tri-O-acetyl-α-D-glucopyranosyl phosphate[6] (diisopropylethylammonium salt, 40 mg, 54 μmol) and neryl phosphonomorpholidate **S5** (63 mg, 0.11 mmol) as in **2** as a white solid (9.6 mg, 33% over 2 steps).

^{1}H (600 MHz, D_2O): δ 5.49 (dd, *J* = 7.2, 3.3 Hz, 1H), 5.32 – 5.23 (m, 2H), 4.00 (dt, *J* = 10.2, 3.0 Hz, 1H), 3.96 (ddd, *J* = 10.2, 4.5, 2.3 Hz, 1H), 3.90 (dd, *J* = 12.4, 2.3 Hz, 1H), 3.87 – 3.79 (m, 2H), 3.60 – 3.53 (m, 1H), 2.59 (dd, *J* = 21.5, 7.7 Hz, 2H), 2.20 – 2.12 (m, 4H), 2.09 (s, 3H), 1.76 (d, *J* = 4.8 Hz, 3H), 1.71 (s, 3H), 1.66 (s, 3H).

^{13}C (126 MHz, D_2O): δ 174.8, 139.6 (d), 133.7, 124.4, 115.5 (d), 94.4 (d), 73.0, 71.1, 69.6, 60.4, 53.7, 31.2, 28.5 (d), 25.8 (d), 24.9, 22.7 (d), 22.2, 17.0.

^{31}P (243 MHz, D_2O): δ 16.77 (d, *J* = 28.1 Hz), –12.98 (d, *J* = 28.1 Hz).

HRMS (ESI-TOF$^-$) for $C_{18}H_{32}NO_{11}P_2$ (M^-) calcd 500.1456, found 500.1461.



S6

12-Phenoxydodec-2-enyl 2-acetamido-2-deoxy-3-*O*-(α-L-rhamnopyranosyl)-D-glucopyranoside **S6**:

^{1}H (500 MHz, CD_3OD): δ **7**.27 – 7.21 (m, 2H), 6.91 – 6.86 (m, 3H), 5.81 – 5.71 (m, 1H), 5.66 – 5.53 (m, 1H), 4.84 (d, *J* = 1.6 Hz, 1H), 4.74 (m, 1H), 4.18 – 4.08 (m, 1H), 4.08 – 4.03 (m, 1H), 3.99 – 3.90 (m, 4H), 3.85 – 3.80 (m, 1H), 3.76 – 3.68 (m, 3H), 3.66 – 3.60 (m, 2H), 3.47 – 3.34 (m, 2H), 2.13 – 2.02 (m, 2H), 1.98 (s, 3H), 1.80 – 1.72 (m, 2H), 1.52 – 1.44 (m, 2H), 1.43 – 1.27 (m, 18H), 1.24 (d, *J* = 6.2 Hz, 3H).

^{13}C (126 MHz, CD_3OD): δ 173.3, 160.6, 136.5, 130.4, 126.8, 121.5, 115.5, 103.1, 97.5, 81.5, 74.0, 73.8, 72.7, 72.2, 70.8, 70.4, 68.9, 62.6, 54.5, 33.4, 30.6, 30.5, 30.5, 30.5, 30.3, 28.5, 27.2, 22.6, 17.9.

HRMS (ESI-TOF$^+$) for $C_{32}H_{51}NaNO_{11}$ ($M+Na^+$) calcd 648.3355, found 648.3358.

References

1. Perrin, D. D.; Armarego, W. L. F. *Purification of Laboratory Chemicals*, 3rd ed.; Pergamon Press, Oxford, 1988.
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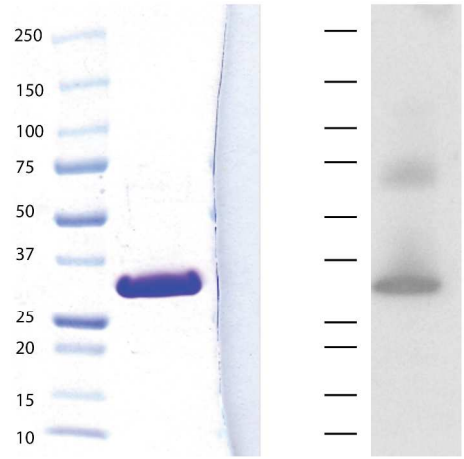
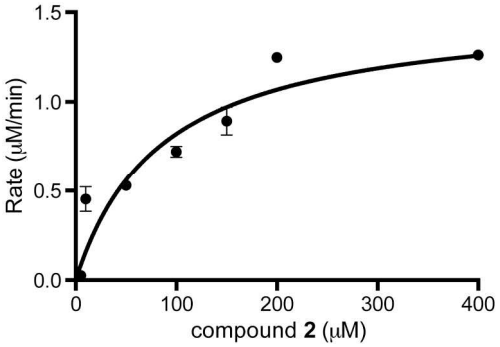
6. Sim, M. M.; Kondo, H.; Wong, C.-H. *J. Am. Chem. Soc.* **1993**, *115*, 2260.

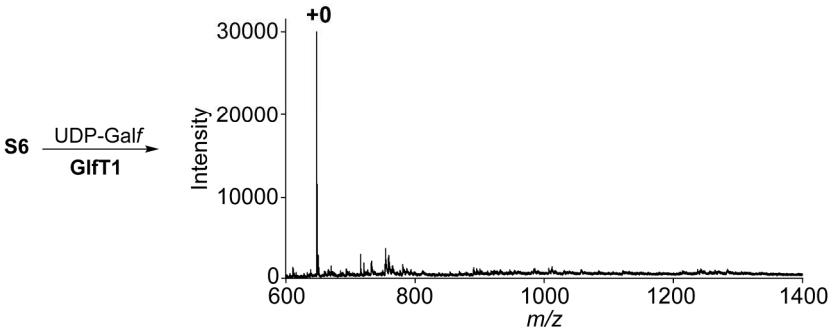
III. Supporting Figures



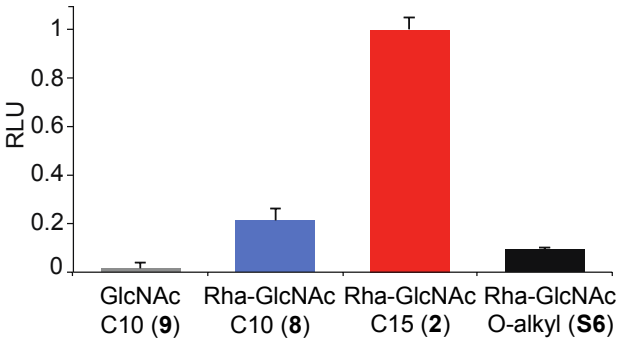
Supporting Figure 1. Visualization of *M. smegmatis* GlfT1-His_6 on SDS-PAGE gel via Coomassie Brilliant Blue stain (left) and Western blot with anti-His antibody (right).



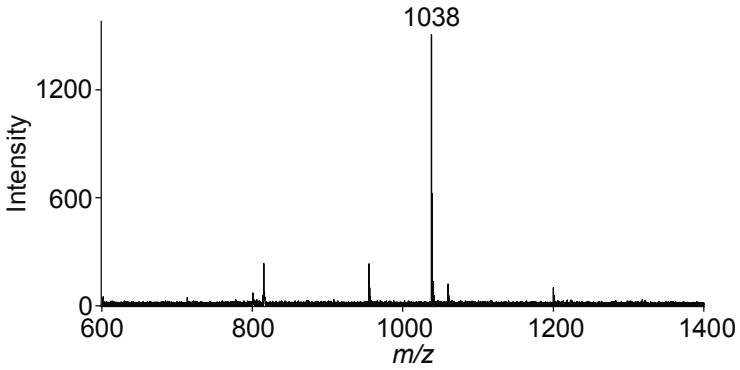
Supporting Figure 2. GlfT1 kinetics analysis with acceptor **2**. Values are plotted as the average of three replicates ± SD and fitted to the Michaelis-Menten equation.



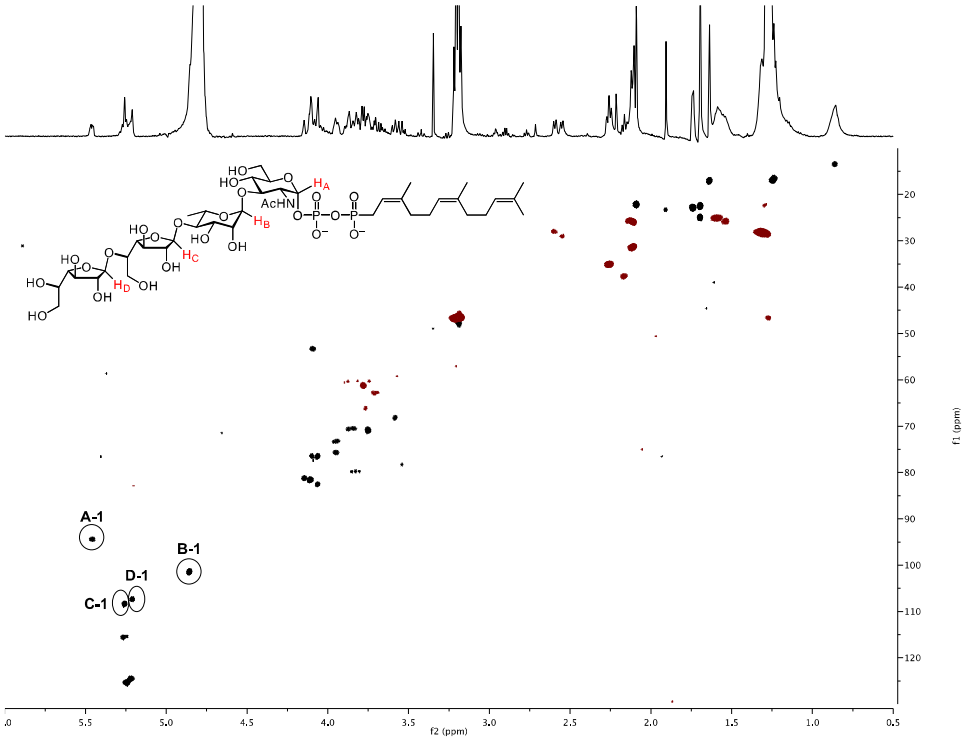
Supporting Figure 3. *O*-alkyl disaccharide **S6** is not elongated by purified GlfT1 in the absence of a lipid environment.



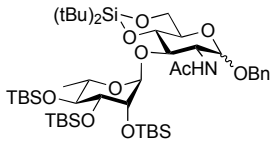
Supporting Figure 4. Relative output of UDP-Gal*f* turnover by GlfT1 with acceptors **2**, **8**, **9**, and **S6**.



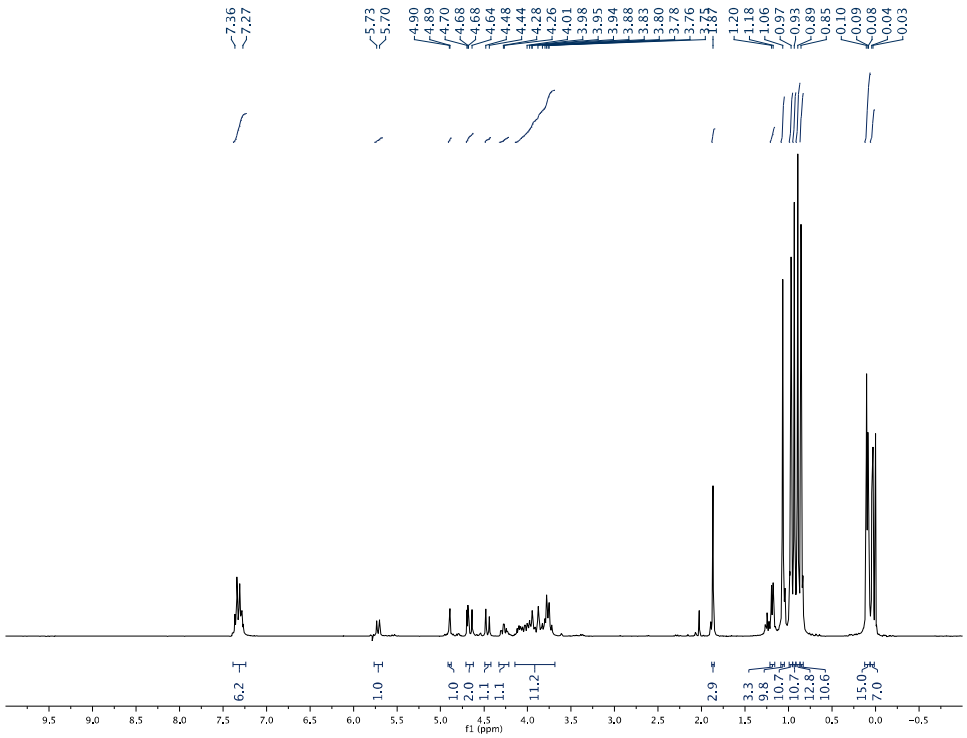
Supporting Figure 5. MALDI-TOF MS spectrum of isolated tetrasaccharide product obtained following extension of acceptor **2** with GlfT1. LRMS: calcd $C_{41}H_{70}NO_{25}P_2$ $[M\text{-}H]^-$ 1038.9, observed 1038.5.



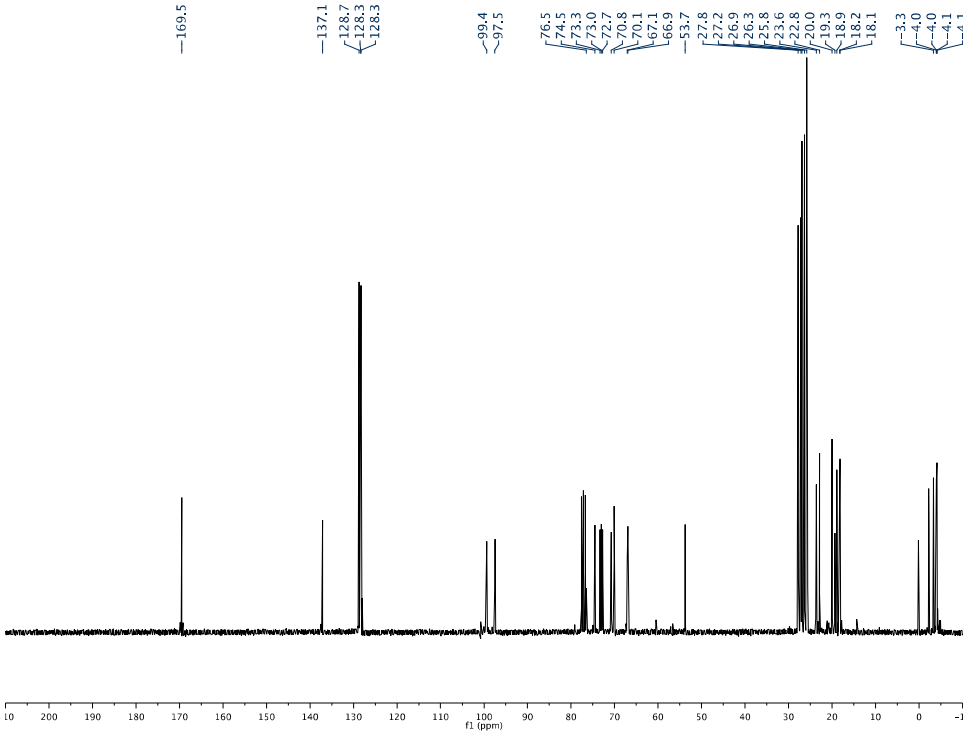
Supporting Figure 6. $^{1}H–^{13}C$ HSQC spectrum of isolated tetrasaccharide product obtained following extension of acceptor **2** with GlfT1. Labels correspond to sugar resonances in the inset structure.

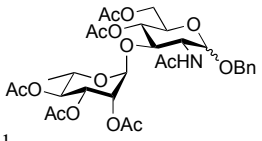


^{1}H (300 MHz, $CDCl_3$):

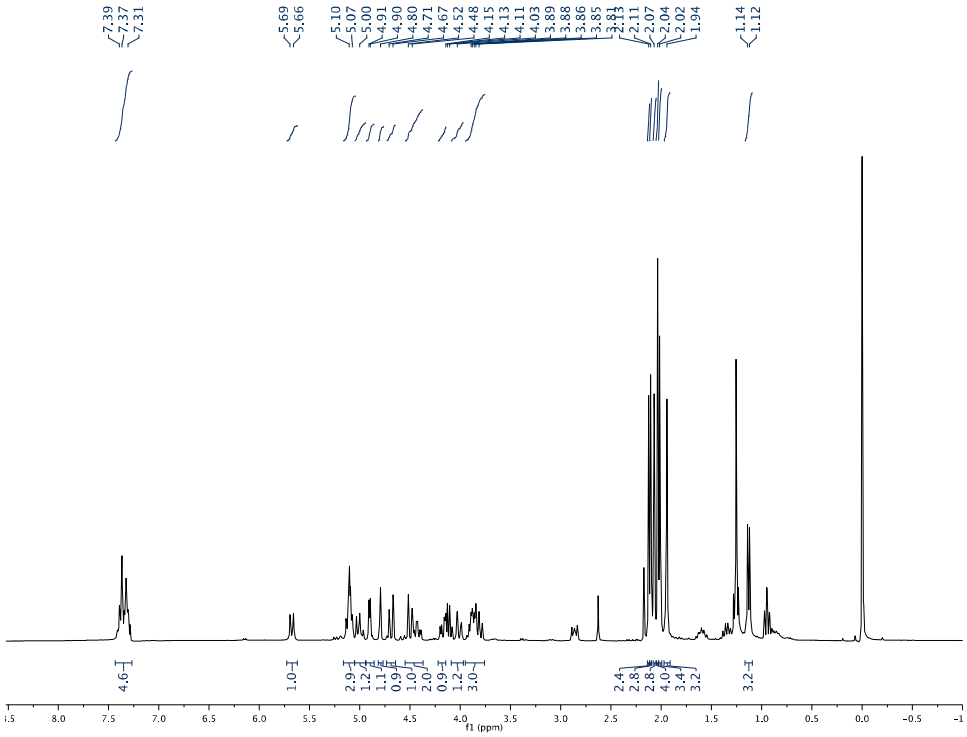


^{13}C (75 MHz, $CDCl_3$):

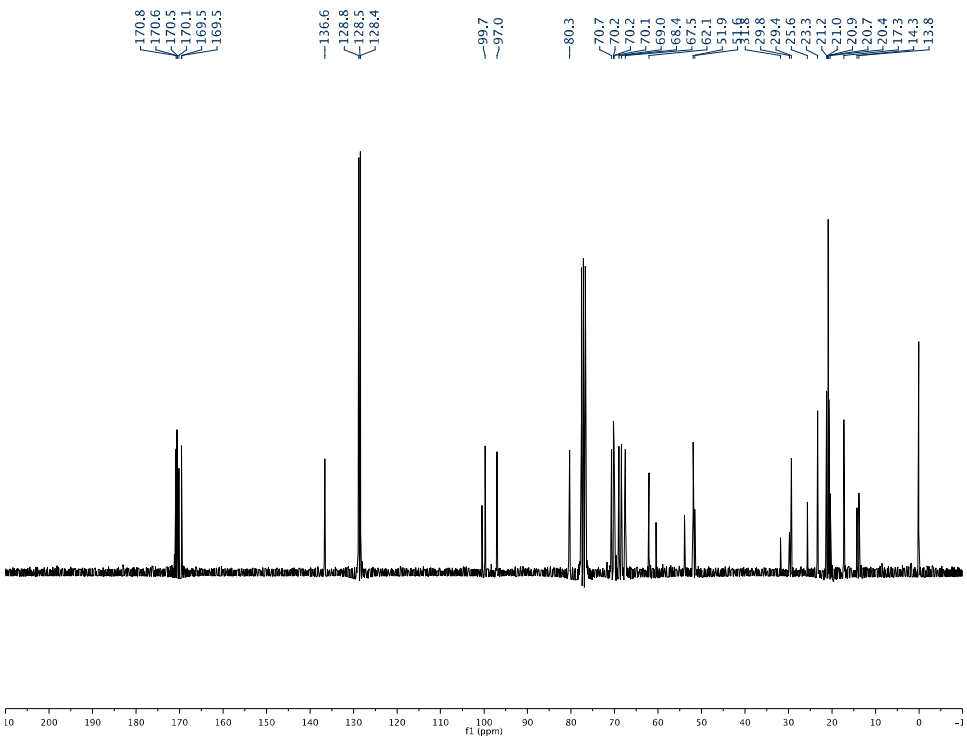


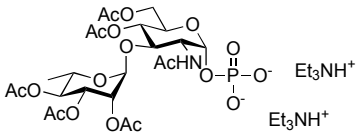


^{1}H (300 MHz, $CDCl_3$):

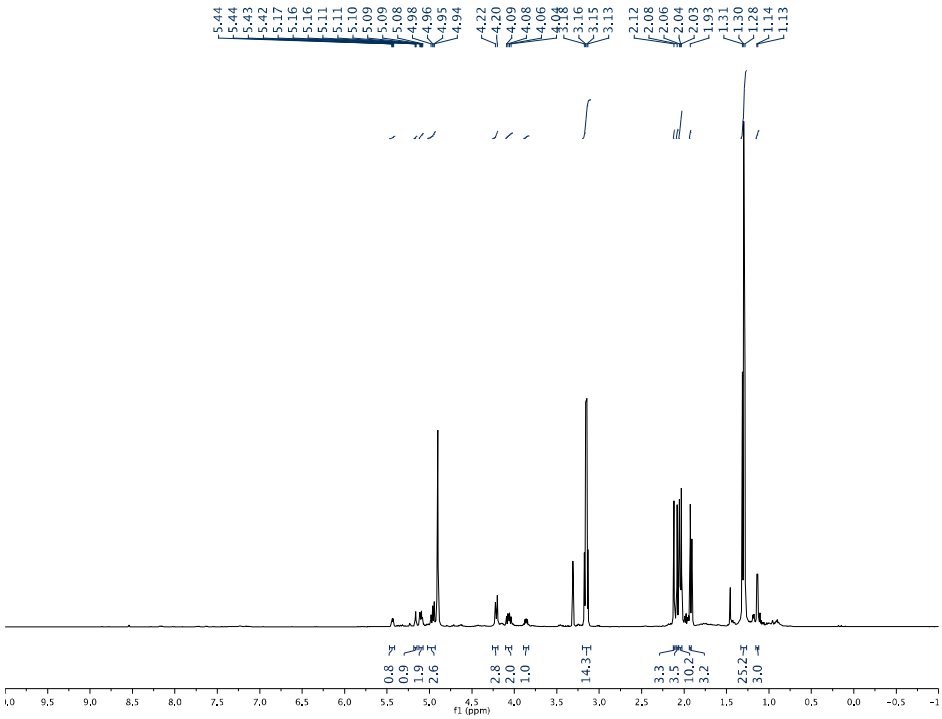


^{13}C (75 MHz, $CDCl_3$):

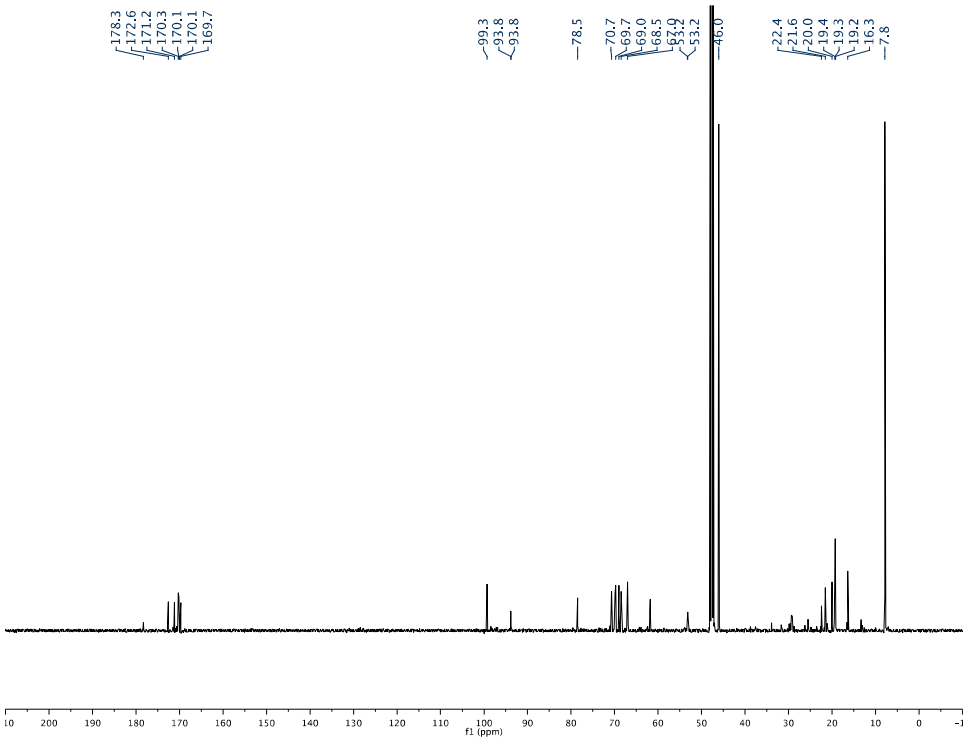




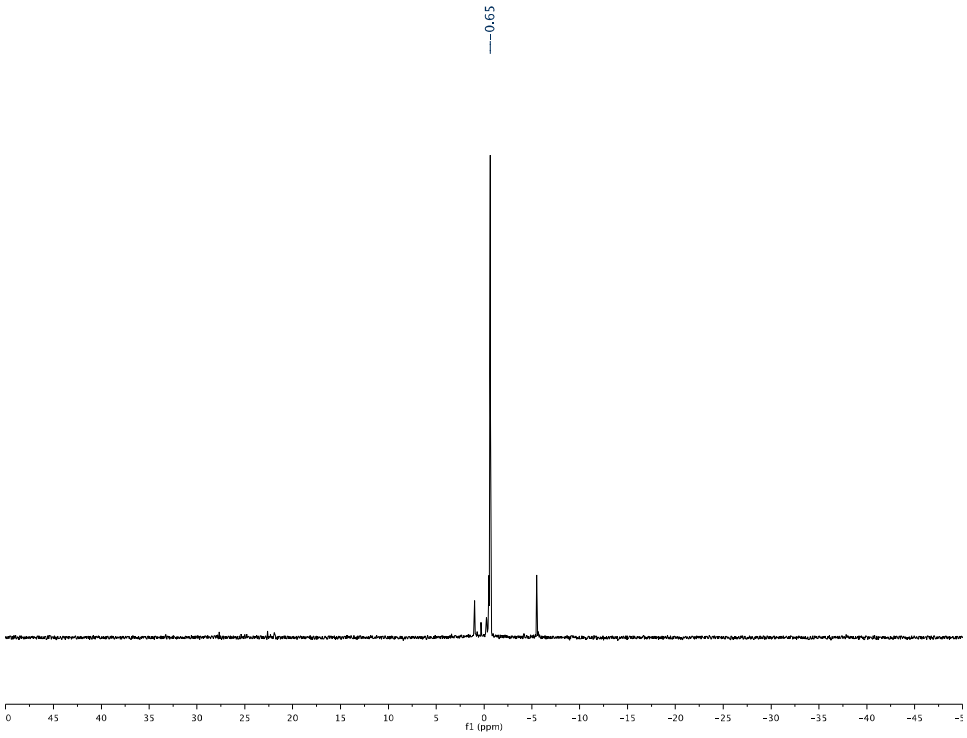
^{1}H (300 MHz, $CDCl_3$):

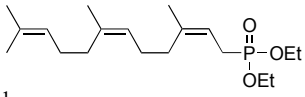


^{13}C (126 MHz, CD_3OD):

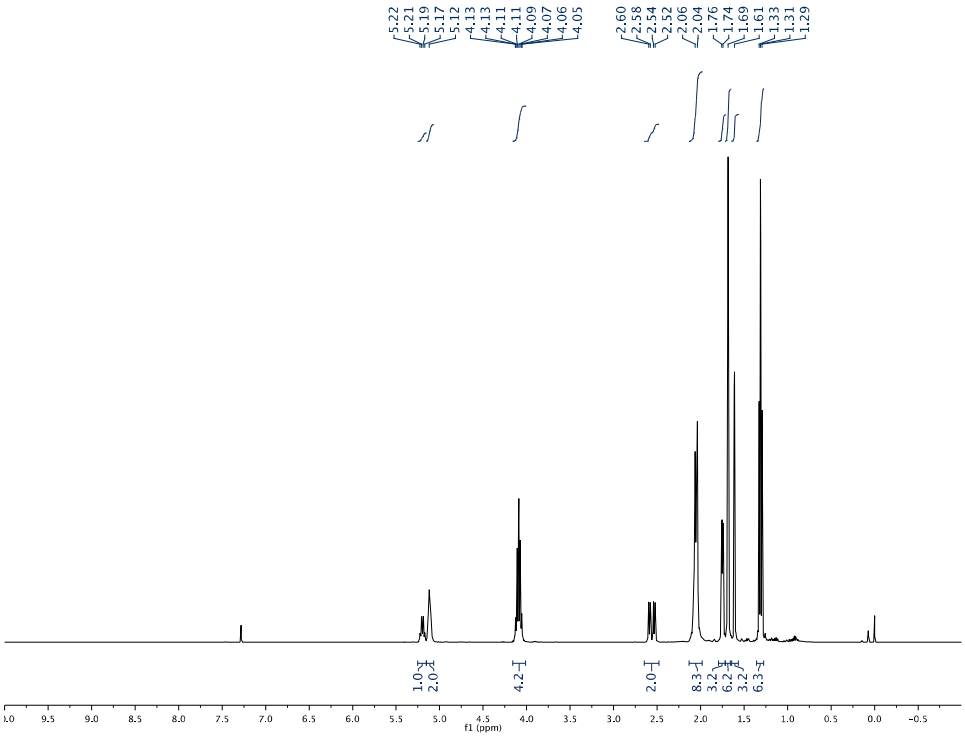


^{31}P (162 MHz, CD_3OD):

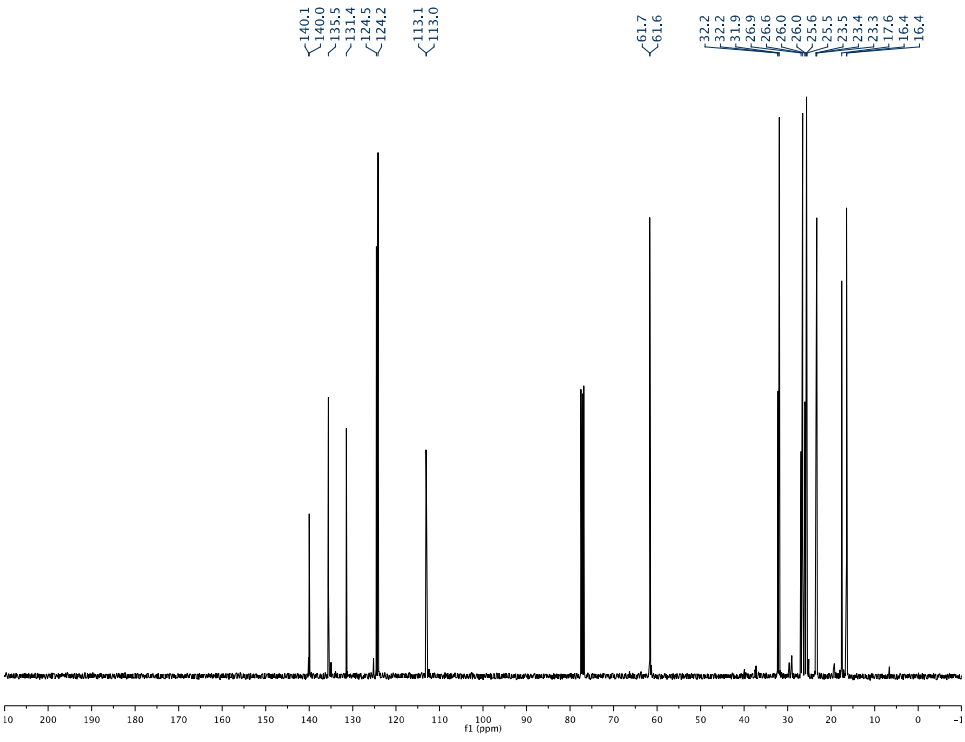




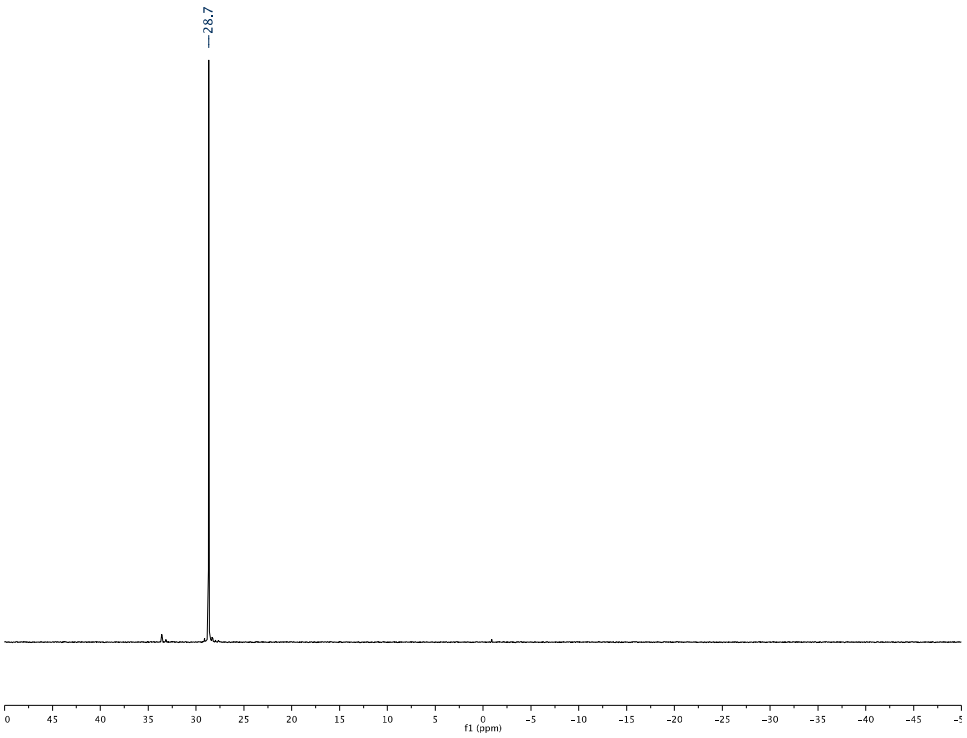
^{1}H (400 MHz, $CDCl_3$):

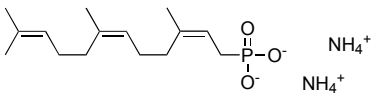


^{13}C (101 MHz, $CDCl_3$):

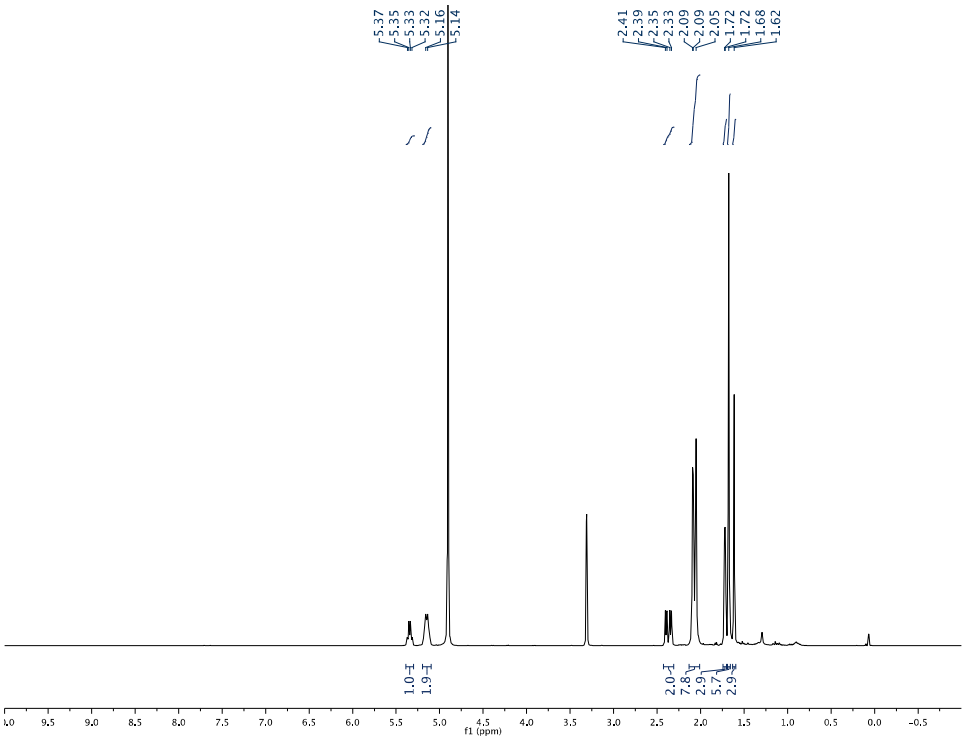


^{31}P (162 MHz, $CDCl_3$):





^{1}H (400 MHz, CD_3OD):



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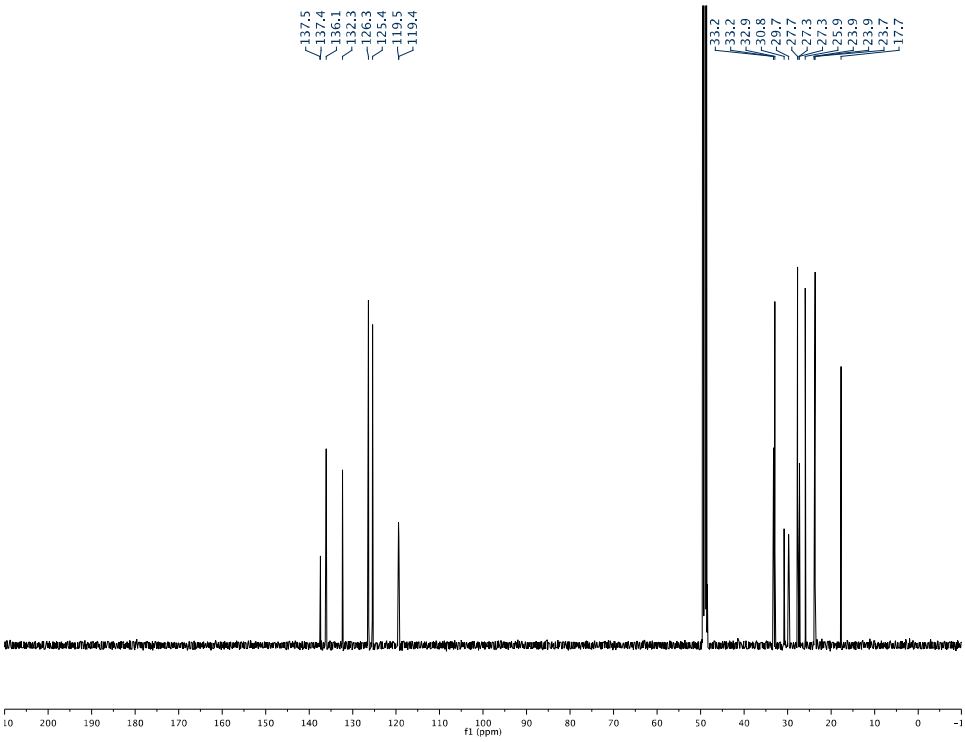

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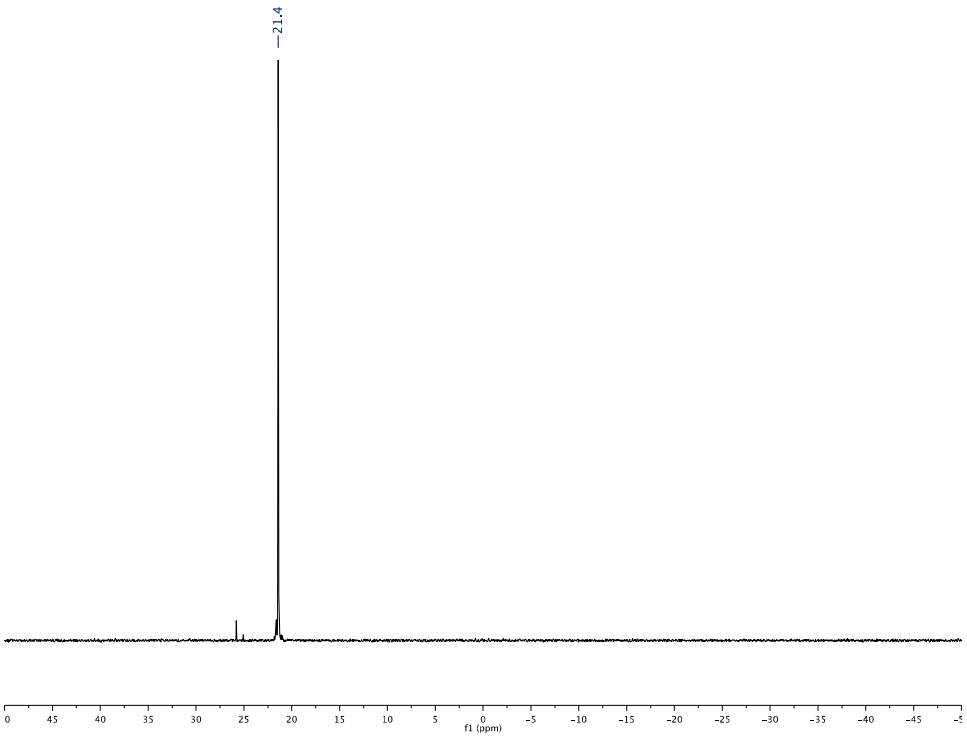

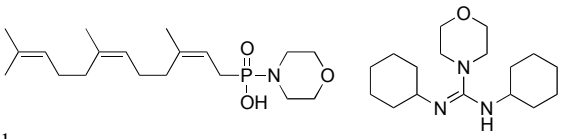

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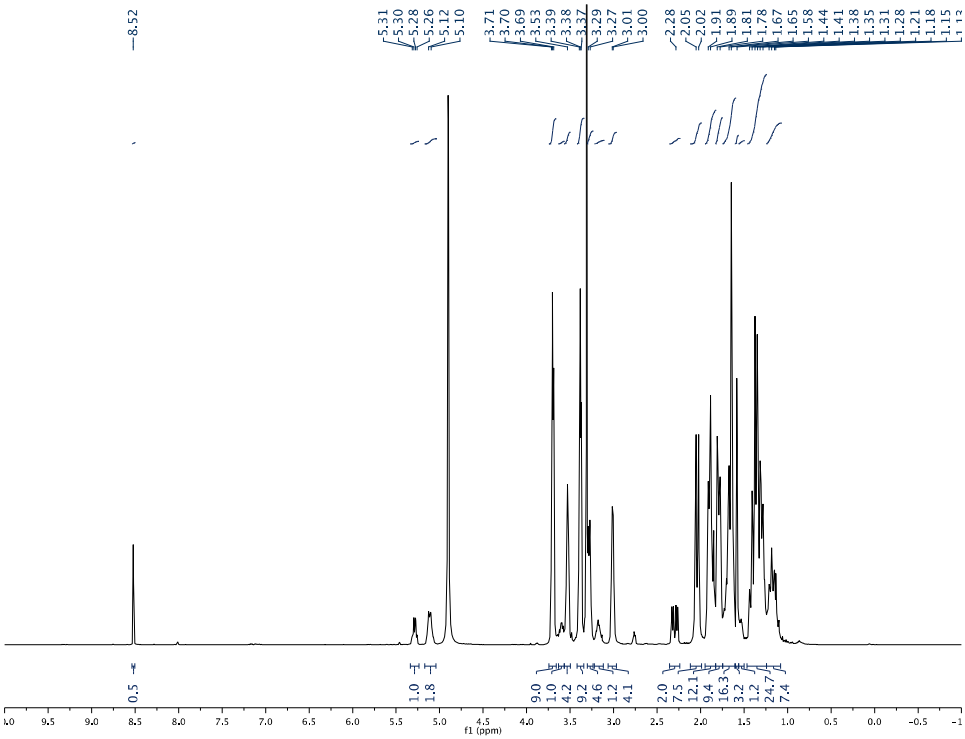

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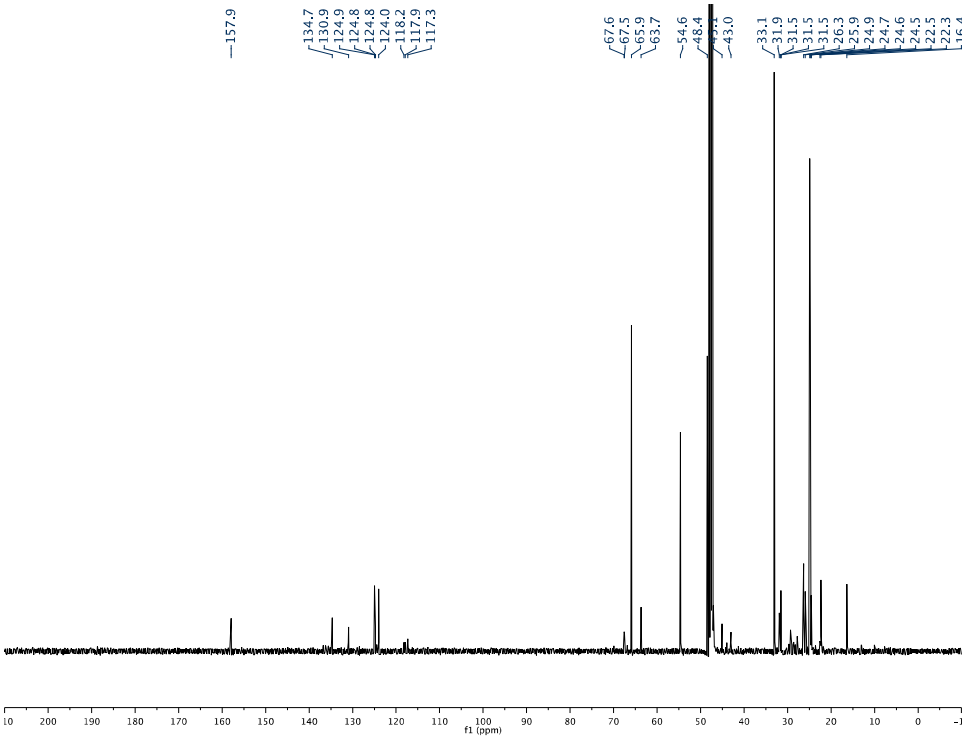

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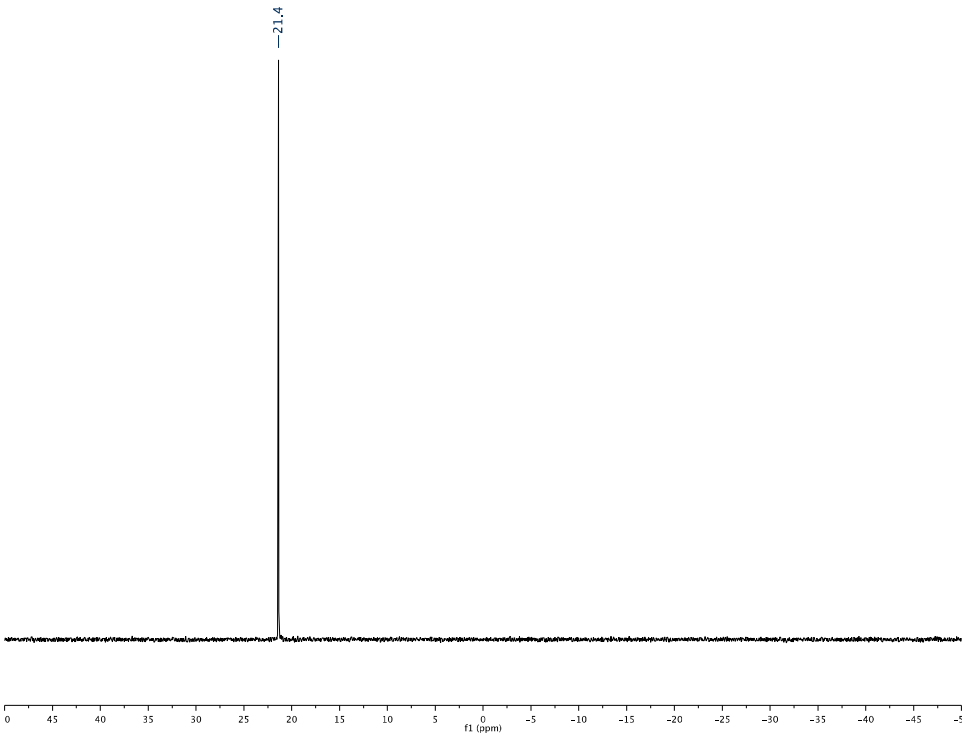
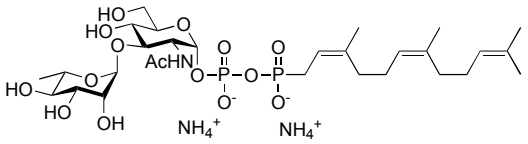
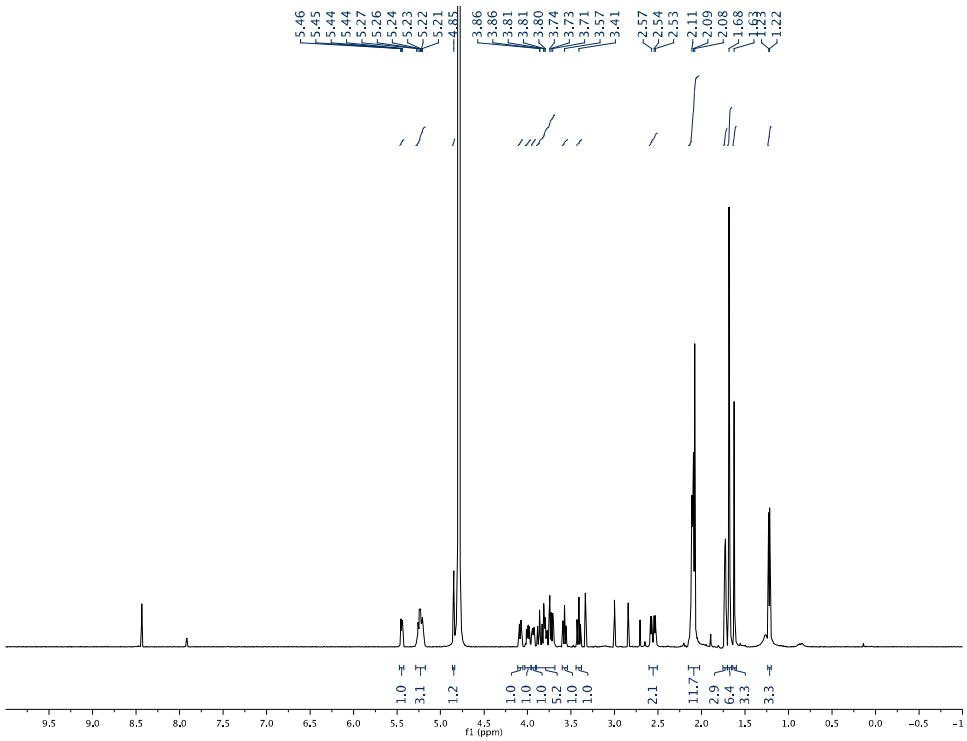




^{1}H (500 MHz, D_2O):



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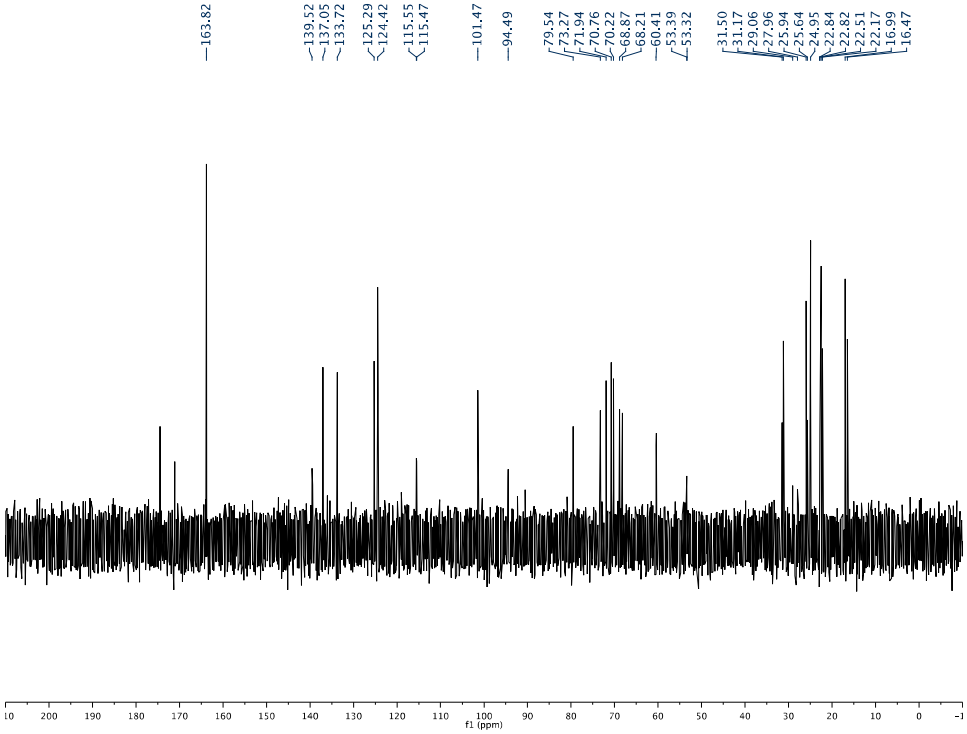

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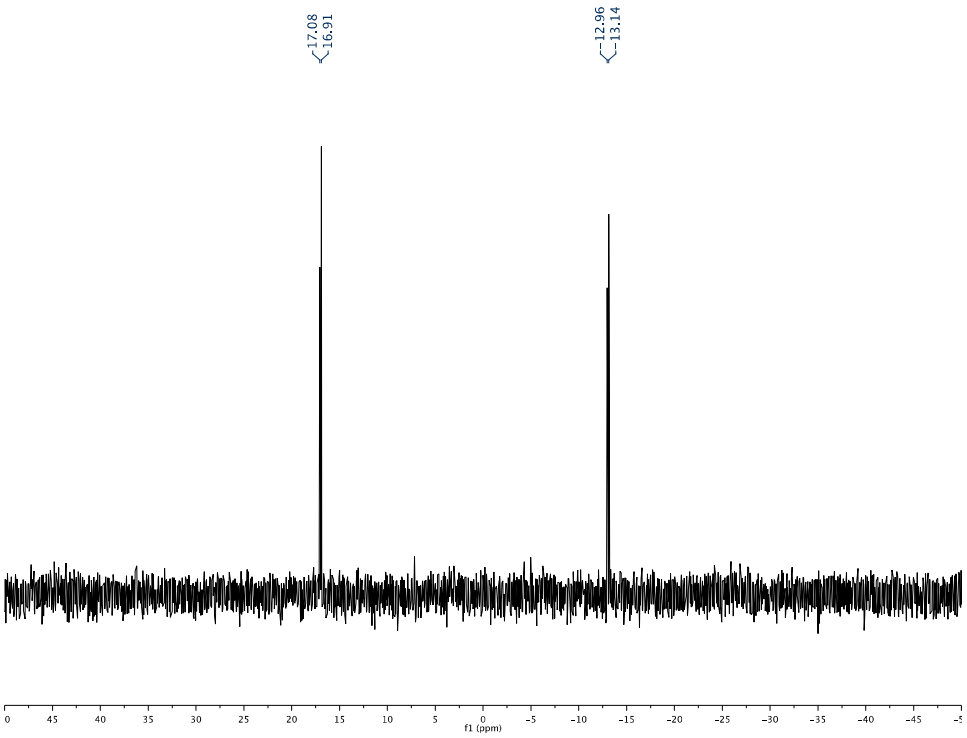

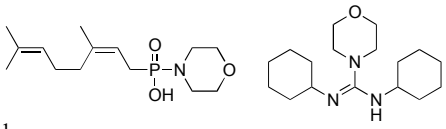

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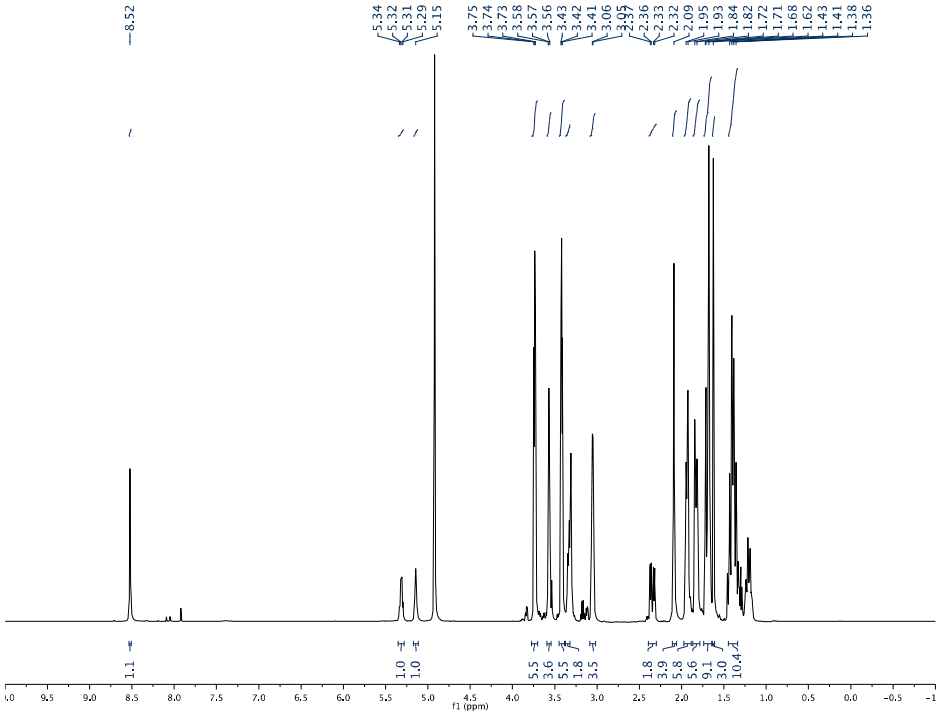
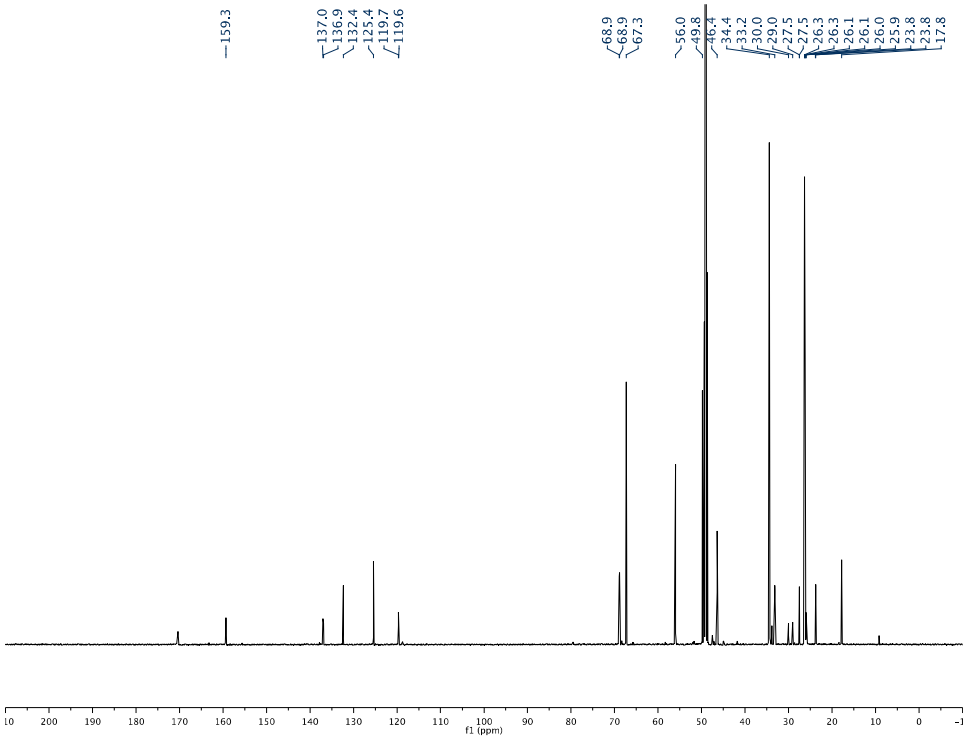
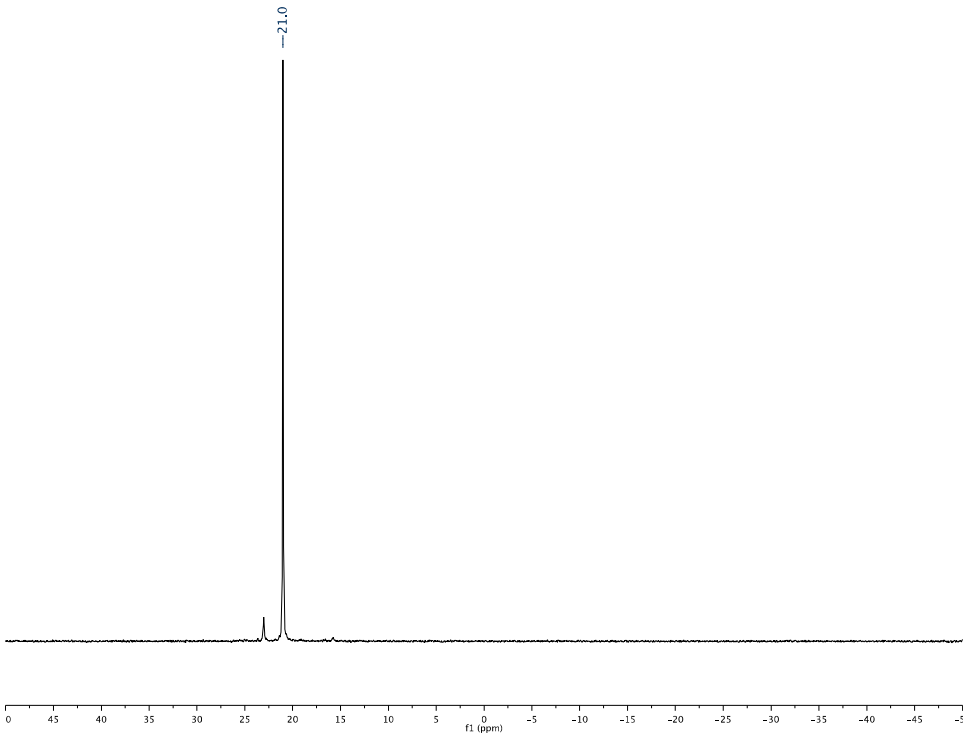
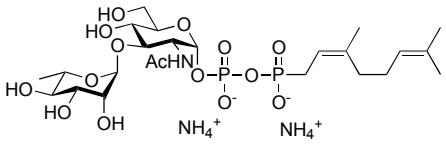


^{13}C (126 MHz, CD_3OD):

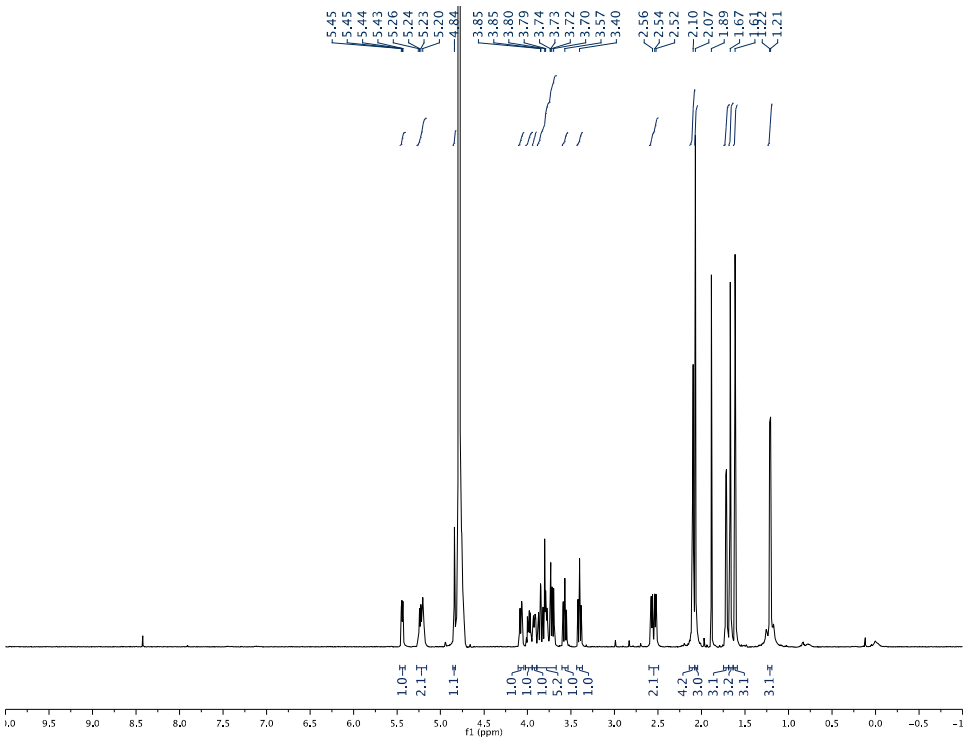


^{31}P (162 MHz, CD_3OD):

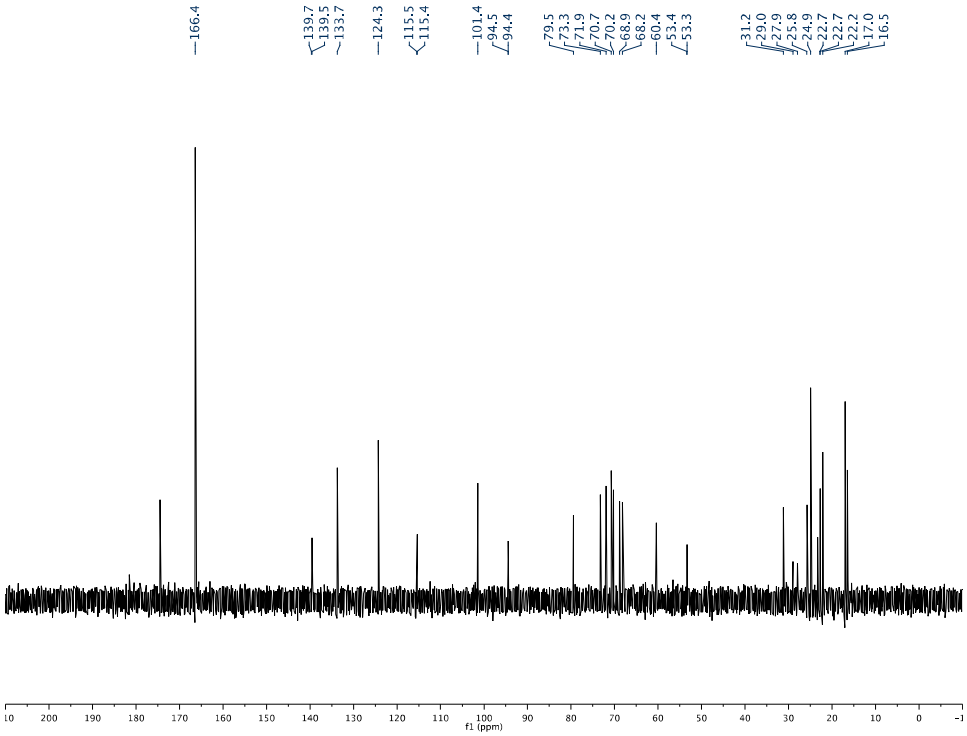




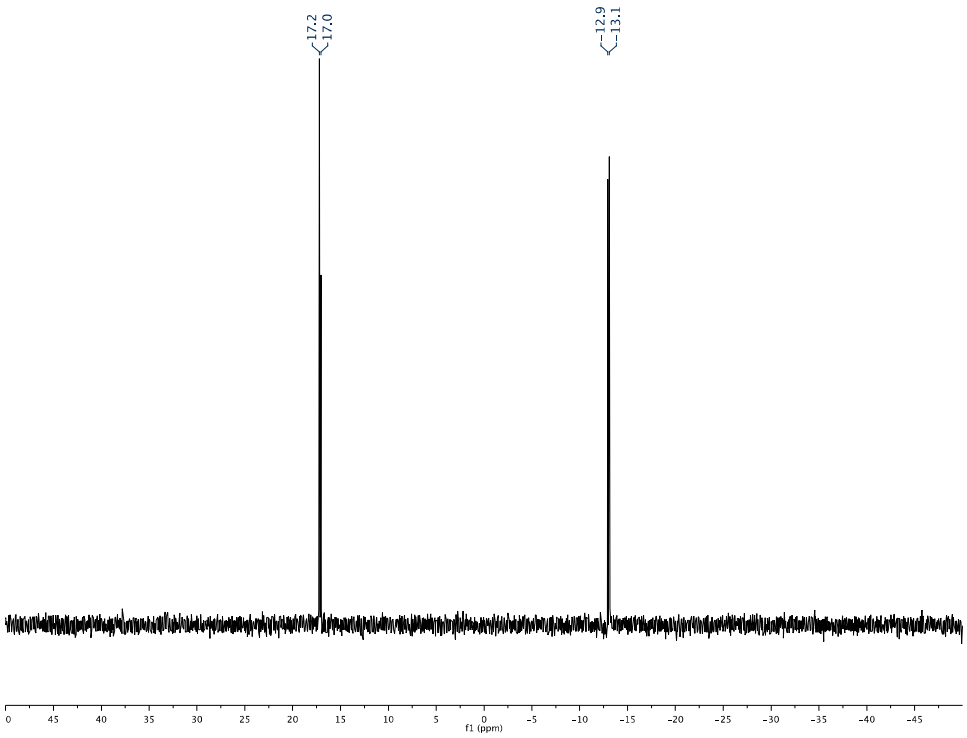
^{1}H (500 MHz, D_2O):

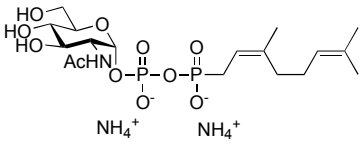


^{13}C (126 MHz, D_2O):

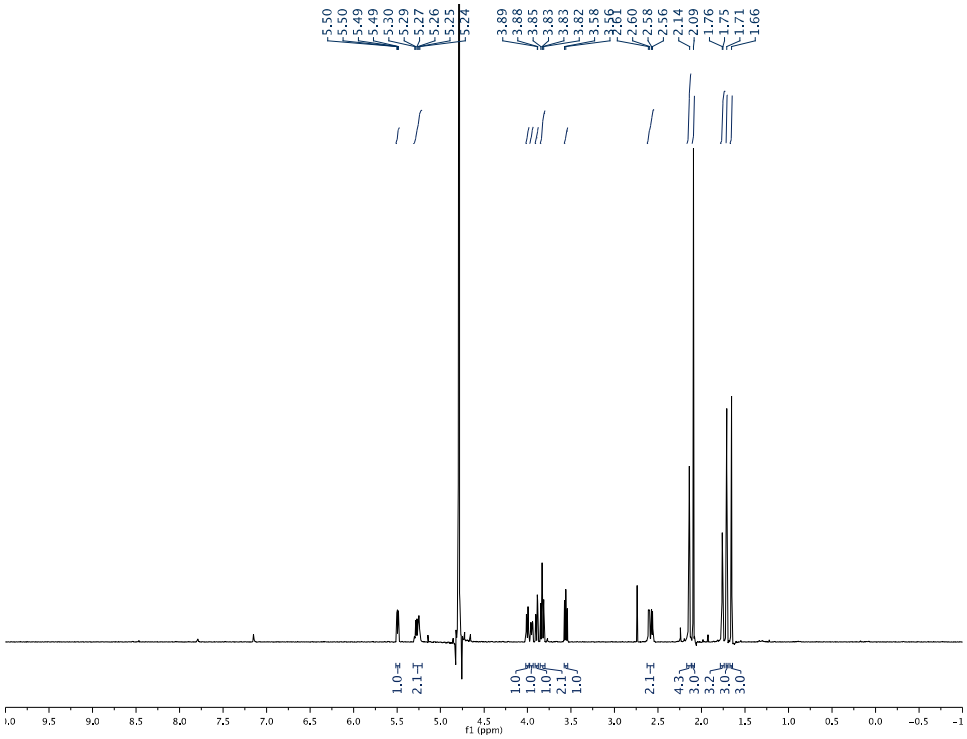


^{31}P (162 MHz, D_2O):

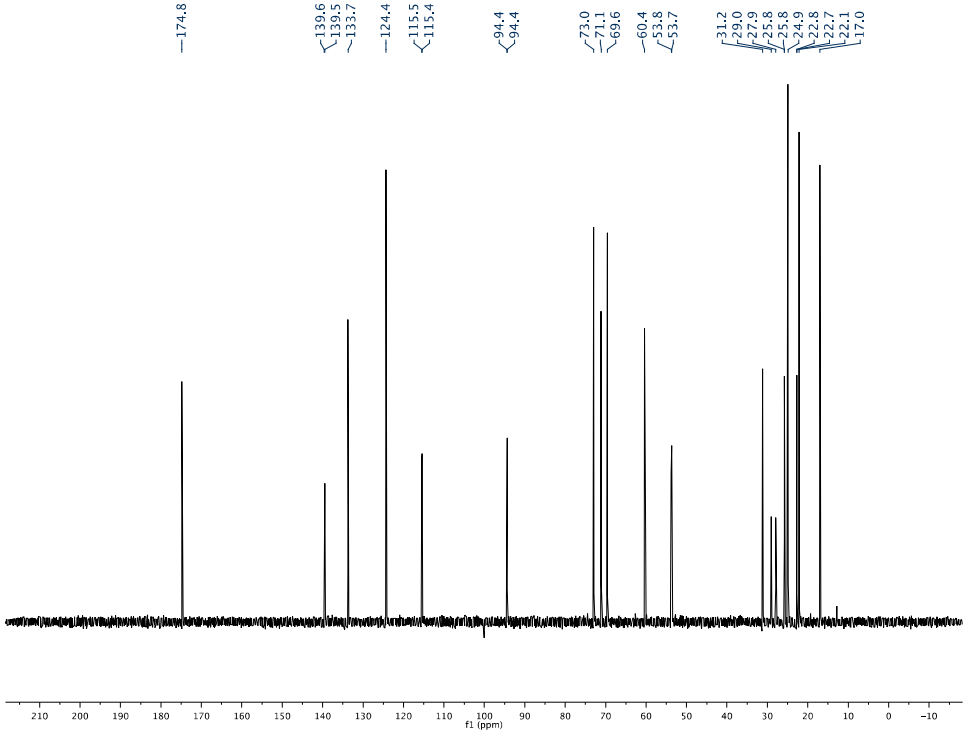




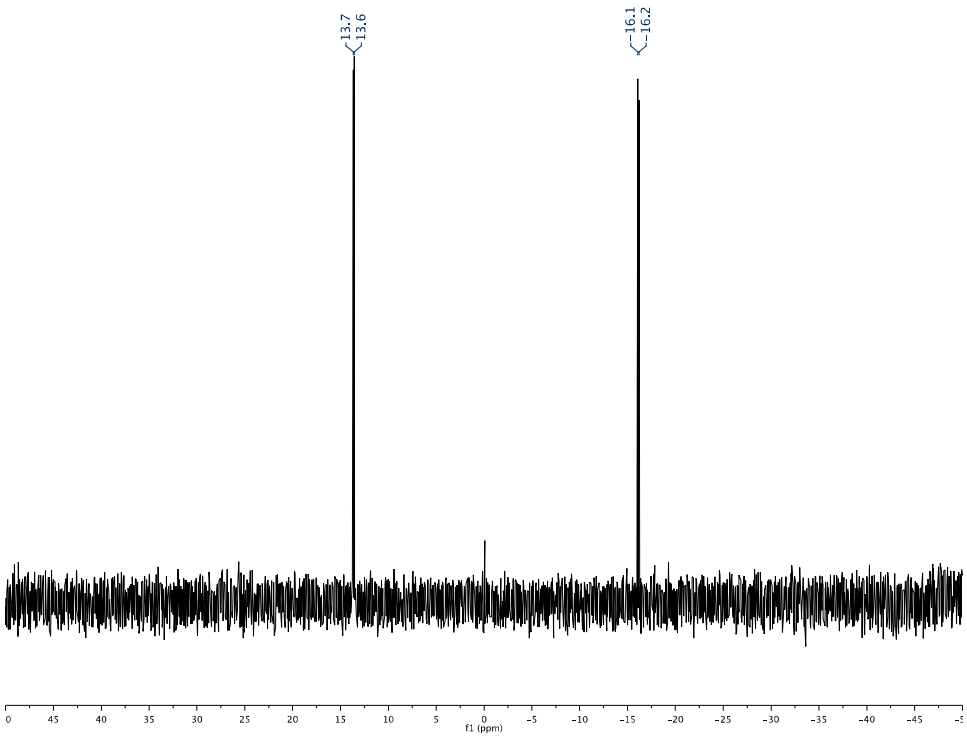
^{1}H (600 MHz, D_2O):

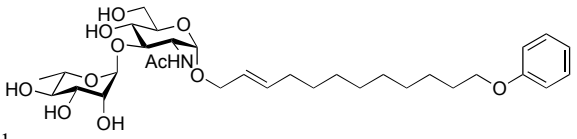


^{13}C (126 MHz, D_2O):

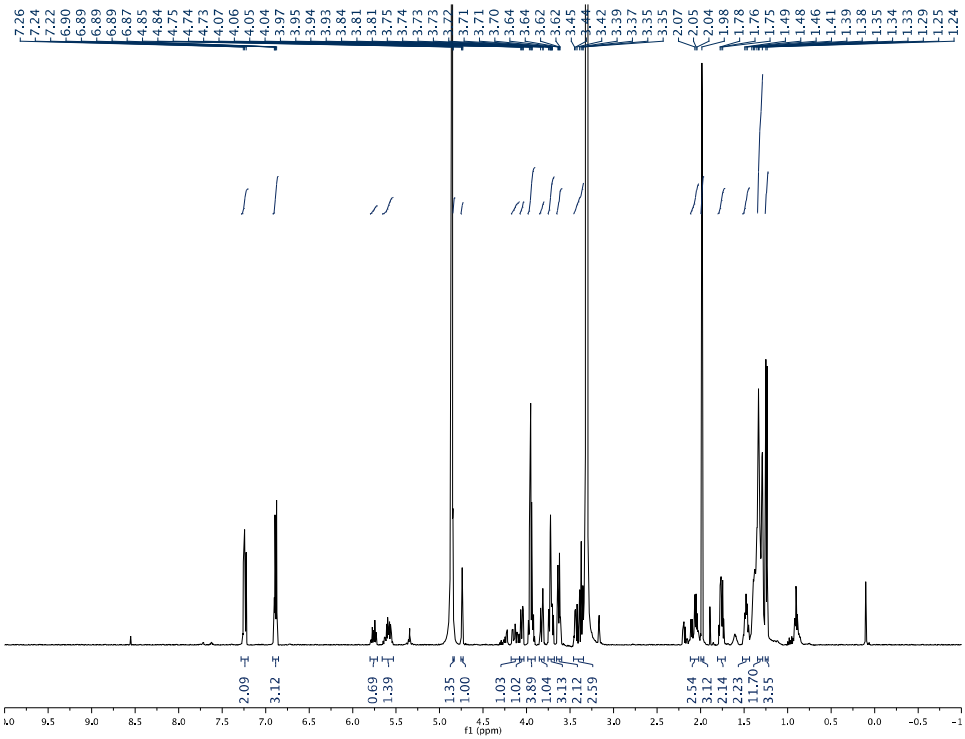


^{31}P (243 MHz, D_2O):





^{1}H (500 MHz, CD_3OD):



^{13}C (126 MHz, CD_3OD):