Supporting Information for

Clickable Galactose Analogs for Imaging Glycans in Developing Zebrafish

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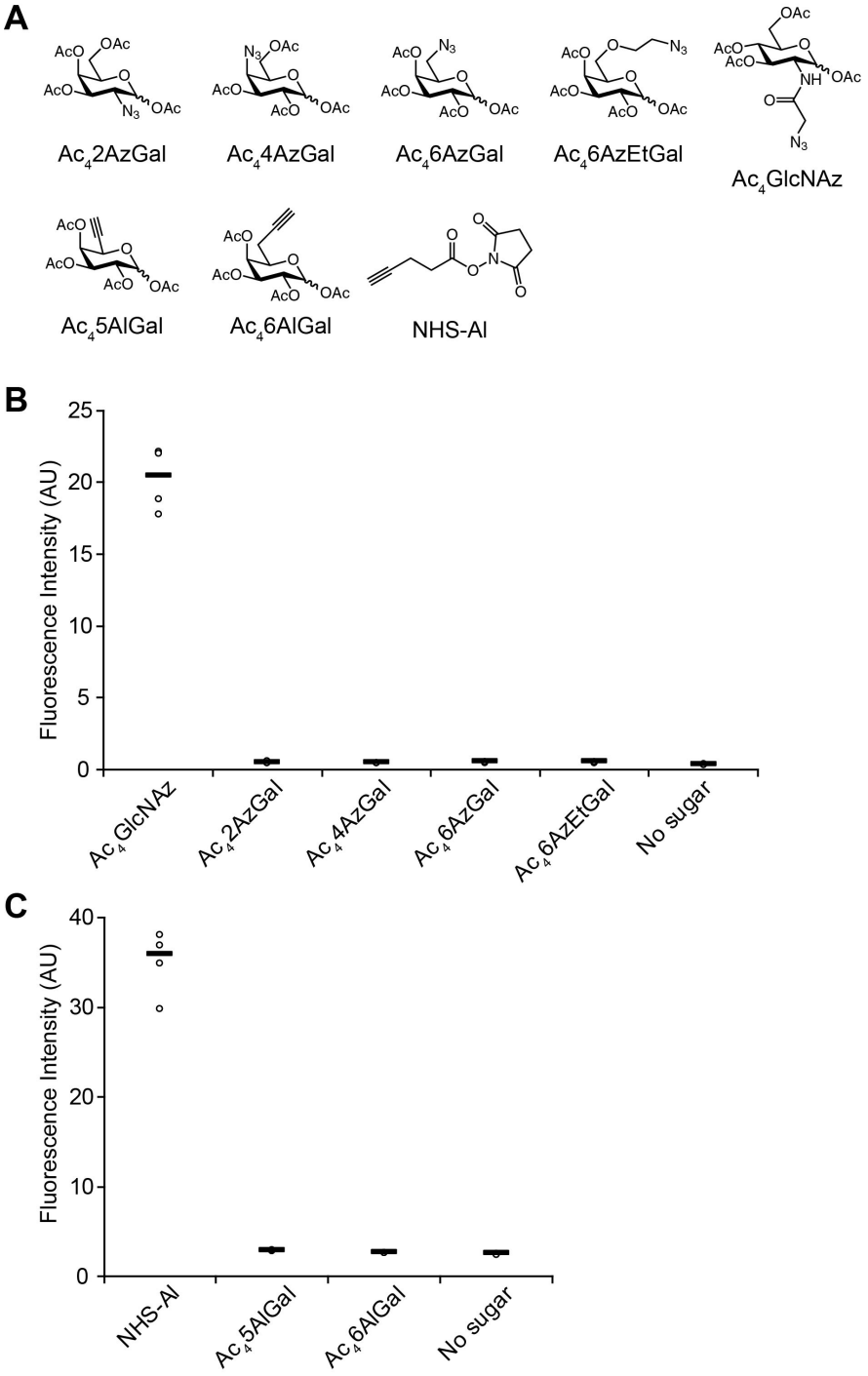


Figure S1. Unnatural Gal analogs do not metabolically label glycans in cell culture. (A) Structures of peracetylated Gal analogs containing azido (top row) or alkynyl (bottom row) groups. Indicated at the right of each group is the positive control used for labeling of cells with azides (top row) or alkynes (bottom row). (B–C) Flow cytometry analysis of HEK 293TN cells treated with the indicated compound (100 μM for 2 d for $Ac_4$4AzGal, $Ac_4$6AzGal, $Ac_4$6AzEtGal, $Ac_4$6AlGal; 50 μM for 2 d for Ac_4GlcNAz, $Ac_4$2AzGal; 250 μM for 2 d for $Ac_4$5AlGal; 100 μM for 30 min for NHS-Al) followed by CuAAC tagging

with alkyne-biotin (B) or azido-biotin (C) and staining with streptavidin-Alexa Fluor 488. Data in (B) and (C) are shown from one representative biological replicate with analysis, with the mean (black bar) indicated of three technical replicates (open circles).

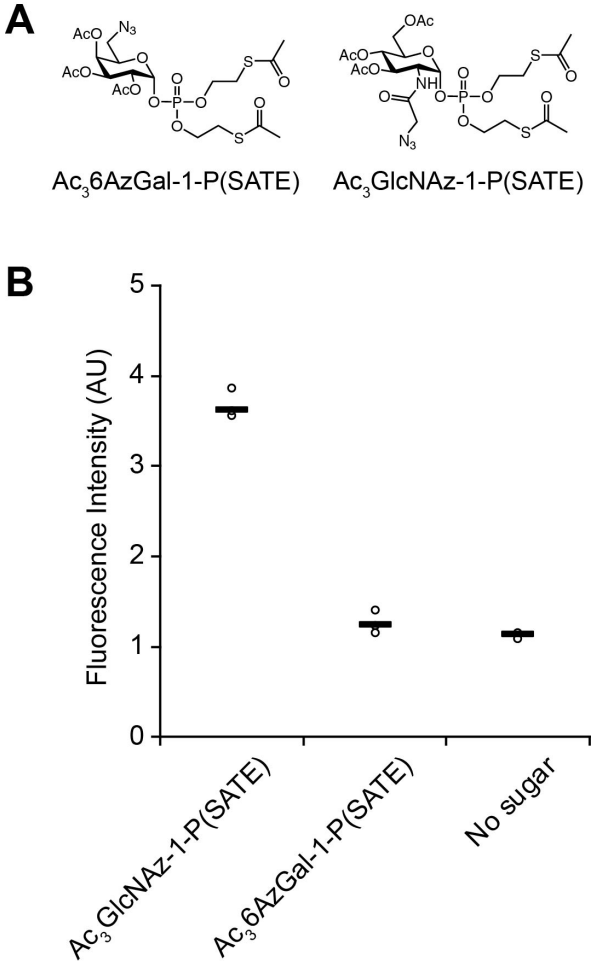


Figure S2. Unnatural Gal-1-phosphate analogs do not metabolically label glycans in cell culture. (A) Structures of peracetylated and SATE-protected analogs of Gal or *N*-azidoacetylglucosamine (GlcNAz). (B) Flow cytometry analysis of CGT-CHO cells treated with the indicated compound (200 μM for 3 d for Ac_3GlcNAz-1-P(SATE); 50 μM for 3 d for $Ac_3$6AzGal-1-P(SATE)) followed by SPAAC tagging with Cy3-DBCO. Data shown in (B) are from one representative biological replicate, with the mean (black bar) indicated of three technical replicates (open circles).

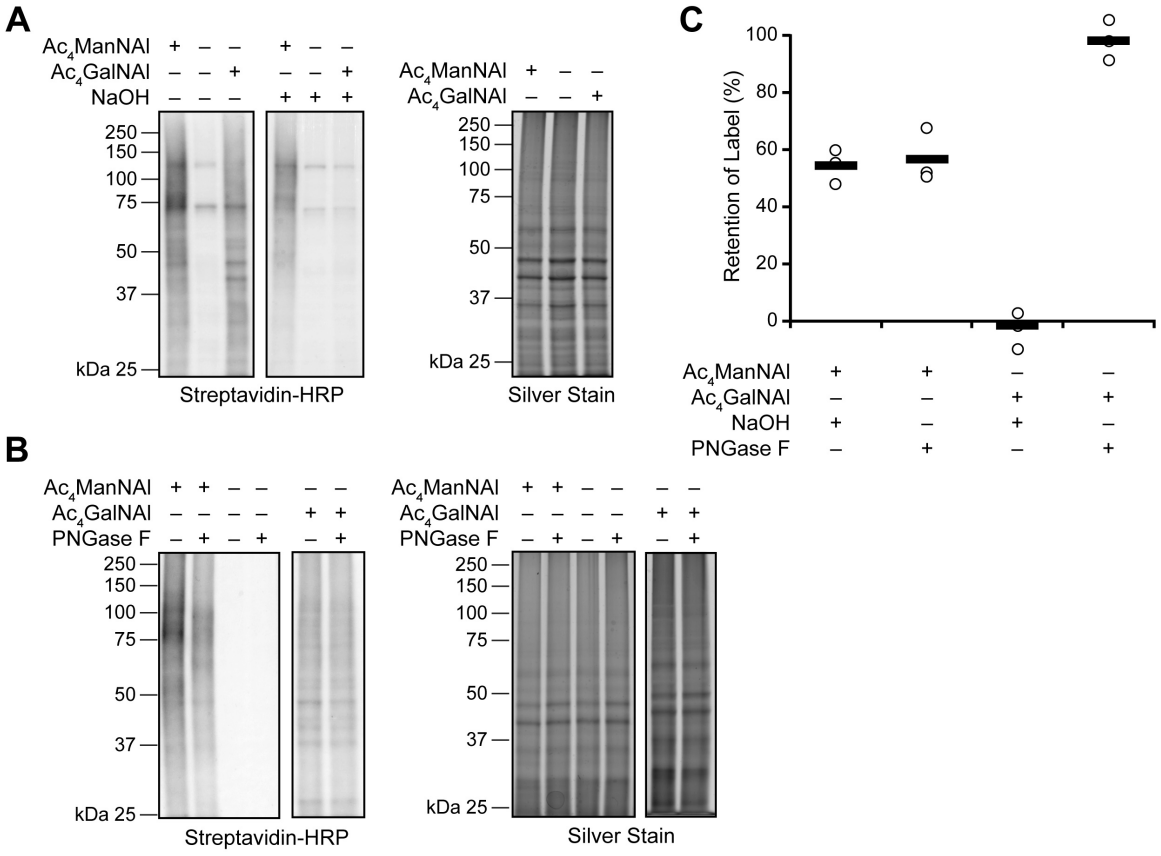
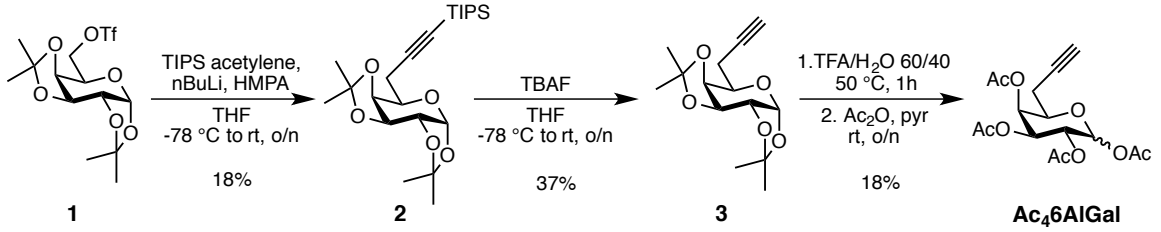
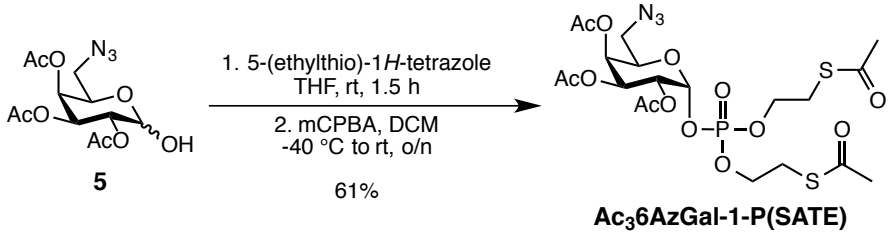


Figure S3. Control experiments for chemical and enzymatic deglycosylation studies. HeLa cells were metabolically labeled with peracetylated *N*-(4-pentynoyl)mannosamine (Ac_4ManNAl) or *N*-(4-pentynoyl)galactosamine (Ac_4GalNAl) (25 μM for 3 d), and lysates were generated. (A) Lysates were reacted with azido-biotin by CuAAC and analyzed by SDS-PAGE and transfer to a PVDF membrane. At this point, membranes were incubated with or without NaOH (55 mM, 40 °C for 22 h) to remove O-glycans, followed by Western blot with streptavidin-HRP or silver stain. (B) Lysates were incubated with PNGase F to remove N-glycans, followed by CuAAC tagging with azido-biotin and Western blot analysis with streptavidin-HRP or silver stain. (C) Quantification of Western blot data from (A) and (B). Each experiment was performed two times (biological replicates) with three technical replicates for each experiment. Quantification data shown in (C) is from one representative biological replicate, with the mean (black bar) indicated of three technical replicates (open circles).

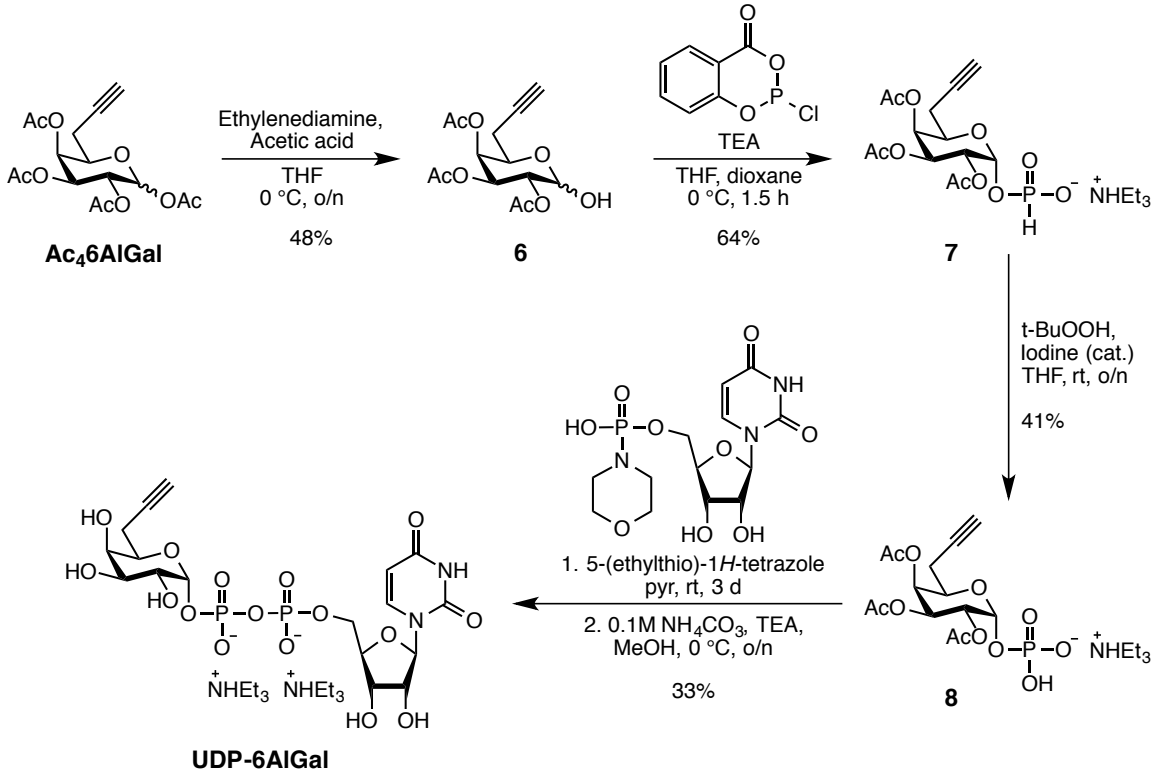
Scheme S1. Synthesis of $Ac_4$6AlGal.



Scheme S2. Synthesis of $Ac_3$6AzGal-1-P(SATE).



Scheme S3. Synthesis of UDP-6AlGal.



Materials and Methods

General materials and methods

All reagents used were analytical grade and obtained from commercial suppliers without further purification. When indicated, solvents were degassed by sparging with argon for 10 min. *N*-(4-pentynoyl)-galactosamine tetracceylated (Ac_4GalNAl), *N*-(4-pentynoyl)-mannosamine tetraacylated (Ac_4ManNAl), tris-hydroxypropyltriazolylmethlamine (THPTA), Biotin-PEG3-Azide (azido-biotin), Biotin-PEG4-Alkyne (alkyne-biotin), and Cy3-DBCO were purchased from Click Chemistry Tools. Rhodamine B isothiocyanate-Dextran (rhodamine-dextran) and cOmplete EDTA-free protease inhibitor cocktail tablets were purchased from Sigma-Aldrich; Streptavidin-HRP was purchased from GeneTex; Clarity enhanced chemiluminescence reagent was

purchased from Bio-Rad; PNGase F was purchased from NEB; N-heptadecanoyl-D-erythro-sphingosine (C17 ceramide, d18:1/17:0) was purchased from Avanti Polar Lipids; bathocuproindisulfonic disodium salt hydrate (BCS) was purchased from Beantown Chemical; Agarose was purchased from IBI Scientific; Pronase E and bovine servum albumin (BSA) was purchased from VWR. Dulbecco's modified Eagle medium (DMEM), fetal bovine serum (FBS), pencillin/streptomycin (P/S), sodium pyruvate, phosphate-buffered saline (PBS), and 0.05% trypsin-EDTA (trypsin) were purchased from Corning. Lipofectamine 2000, streptavidin-Alexa Fluor 488, and BCA assay were purchased from Thermo Fisher. HPLC purification was performed on a Shimadzu LC-20AR HPLC equipped with an SPD-20AV UV/Vis detector and a ES Industries Epic Polar C18 reverse phase column (25 cm x 10 mm, 5 μm). Flow cytometry was performed on a BD Accuri C6 flow cytometer and analyzed using the BD Accuri C6 analysis software. LCMS analysis was performed on an Agilent 6230 electrospray ionization–time-of-flight (ESI–TOF) MS coupled to an Agilent 1260 HPLC equipped with a Zorbax Rx-Sil normal phase silica column (2.1 x 50 mm, 1.8 μm). NMR spectra were recorded on a Bruker 500 MHz spectrometer with a BBO H&F cryoprobe at ambient temperature. DART MS was performed on an Exactive Plus Orbitrap Mass Spectrometer with a DART SVP ion source from Ion Sense. Confocal imaging was performed on a Zeiss LSM 800 confocal laser scanning microscope equipped with C-Apochromat 10X 0.45 NA water immersion and Plan Apochromat 40X 1.4 NA oil immersion objectives, 405, 488, 561, and 640 nm solid-state lasers, and two GaAsP PMT detectors. Images were acquired using Zeiss Zen Blue 2.3 and analyzed using FIJI.

Cell culture

CHO, HeLa, and HEK 293TN cells were cultured in a 5% CO_2, water-saturated atmosphere at 37 °C in DMEM supplemented with 10% FBS, 1% penicillin and streptomycin. HEK 293TN cells were additionally supplemented with 1% sodium pyruvate. Cells were maintained between 10^5 and $1.6x10^6$ cells/mL.

Zebrafish husbandry

Casper zebrafish embryos 0–5 d post-fertilization were kept in embryo medium (13.7 mM NaCl, 0.54 mM KCl, 0.025 mM Na_2HPO_4, 0.044 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, pH 7.2) at 28.5 °C.

Microinjection of zebrafish embryos

Casper zebrafish embryos at the one-cell stage were microinjected with 5 nL of 10 mM UDP-galactose analog or no sugar with 5% w/v rhodamine-dextran in 0.5X PBS pH 7.4. Embryos were allowed to develop by incubation at 28.5 °C. Embryos were either used for imaging or de-yolked using shear force through a trimmed P200 tip and then flash-frozen using liquid nitrogen. Frozen embryos were stored at –80 °C.

Metabolic labeling of cultured cells with peracetylated sugars

HEK 293TN cells (1.6×10^6) were seeded on 10-cm dishes and transfected using Lipofectamine 2000 with a plasmid containing CGT (pIRES-UGT8, a gift from Robyn Meech, Flinders University). Eight h post-transfection, the media was exchanged for fresh media with sugar (100 μM for $Ac_4$4AzGal, $Ac_4$6AzGal, $Ac_4$6AzEtGal, $Ac_4$6AlGal;

50 μM for Ac_4GlcNAz, $Ac_4$2AzGal; 250 μM for $Ac_4$5AlGal) or no sugar. Cells were incubated at 37 °C for 2 d. Cells were lifted using trypsin for 5 min, pelleted, resuspended in FACS buffer (1X PBS pH 7.4 + 0.5% FBS), and counted, and 200,000 cells were added to each well of a 96-well plate, in triplicate. Cells were pelleted at 500 x g at 4 °C for 3 min, resuspended in 43.3 μL of FACS buffer and 1.75 μL of master mix (50 μM $CuSO_4$, 300 μM THPTA, 100 μM of either azido-biotin or alkyne-biotin) was added followed by 5 μL of 25 mM sodium ascorbate. Cells were mixed and incubated at room temperature for 3 min. Cells were pelleted at 500 x g at 4 °C for 3 min and rinsed three times with 150 μL of FACS buffer. 50 μL of 1 μg/mL streptavidin-Alexa Fluor 488 (1 mg/mL stock) in cold FACS buffer was added to each well and reacted for 30 min at 4 °C in the dark. Cells were pelleted and rinsed three times with FACS buffer. Cell was resuspended in 170 μL of FACS buffer and analyzed by flow cytometry.

Metabolic labeling of cultured cells with protected thioester sugars

CGT-CHO cells (200,000), a CHO cell line stably expressing CGT (a gift from Tsung-Ping Su, NIH), were seeded on 10-cm dishes in media containing one of the following: 200 μM Ac_3GlcNAz-1-P(SATE), 50 μM $Ac_3$6AzGal-1-P(SATE) or no sugar. Cells were incubated at 37 °C for 3 d. Cells were lifted using trypsin for 5 min, pelleted, resuspended in FACS buffer (1X PBS pH 7.4 + 0.5% FBS), and counted. 200,000 cells, in 150 μL of FACS buffer, were added to 96-well plates (in triplicate). Cells were pelleted at 500 x g at 4 °C for 3 min. Cells were resuspended in 50 μL of 5 μM Cy3-DBCO in FACS buffer and incubated at 37 °C for 2 h. Cells were pelleted at 500 x g at 4 °C for 3 min and rinsed three times with 150 μL of FACS buffer. Cells were resuspended in 120 μL of FACS buffer and analyzed by flow cytometry.

In vitro reaction with UDP-6AzGal

HEK 293TN cells (5 x 10^6) were seeded in 10-cm dishes and allowed to grow overnight. The cells were transfected with pIRES-hUGT8 using Lipofectamine 2000 as per the manufacturer's instructions. After 24 h, cells were lysed in lysis buffer (10 mM Tris pH 7.5 and 1 mM EDTA supplemented with a protease inhibitor tablet) and sonicated for 2 pulses at 20% power. Samples were normalized to 300 μg/46 μL with lysis buffer. 12.5 μL of 4 mM C17:0 ceramide in chloroform was added to 1.5 mL Eppendorf tube. The chloroform was removed using a stream of nitrogen gas. Ceramide was then dissolved in 25 μL of sodium phosphate buffer (pH 6.8) and 4 μL of 100 mM $MgCl_2$. 25 μL of 4 mM UDP-6AzGal was added followed by 46 μL of lysate (300 μg). Solution was pipet mixed and incubated overnight at 37 °C. Reactions were then diluted with 125 μL of 20 mM acetic acid, 250 μL of methanol, and 250 μL chloroform then vortexed for 1 min and centrifuged for 1 min at 16,000 x g. The organic layer was removed and saved. 250 μL of chloroform was added and samples were vortexed and centrifuged as before. Organic layers were combined and dried under nitrogen.

Samples to be analyzed by LCMS were subjected to CuAAC labeling by a modified version of the method previously described by Thiele et al[1]. 37 μL of a reaction master mix (using degassed solvents) was added to the dried lipids. The reaction mixture consisted of 24 μL of ethanol with 0.2 mg of alkyne quaternary ammonium (Alk-QA), 7 μL of chloroform, and 6 μL of 20 mM [acetonitrile]$_4$$CuBF_4$ in acetonitrile. Tubes were flushed with argon and incubated in a 42 °C water bath (only submerged halfway)

overnight. Reactions were diluted with 113 µL of chloroform:methanol (95:5) mixture and filtered (0.45 µm) for analysis. Samples were analyzed using a gradient of 0-100% going from chloroform:methanol:ammonium hydroxide (95:7:0.5) to chloroform:methanol:water:ammonium hydroxide (60:34:5:0.5) as the carrier solvent. For detection, the MS was equipped with a dual ESI source operating in positive mode, acquiring in extended dynamic range from m/z 100-1000 at one spectrum per second; gas temperature: 325 °C, drying gas 10 L/min; nebulizer: 20 psig; fragmentor 125 V.

β-elimination of metabolically labeled zebrafish O-glycans

Casper zebrafish embryos were microinjected into the yolk at the 1-cell stage with 5 nL of 10 mM UDP-6AlGal with 5% w/v rhodamine-dextran in 0.5X PBS pH 7.4 or left uninjected. Embryos were allowed to develop by incubation at 28.5 °C. After 24 h, embryos were dechorionated using 1 mg/mL Pronase E in embryo medium for 8 min. Embryos were then pipetted onto a 1% agarose coated plates with embryo medium. Embryos were then rinsed twice with embryo medium by transferring to new dishes of media. Embryos were counted, aliquoted, and de-yolked by shear force through a trimmed P200 tip with ice cold PBS. De-yolked embryos were pelleted and rinsed three times with PBS to remove any residual yolk proteins then flash frozen using liquid nitrogen. Frozen embryos were stored at -80 °C. Flash frozen embryos were lysed in lysis buffer (4% SDS and 100 mM sodium phosphate pH 7.4 supplemented with a protease inhibitor tablet).

For positive and negative control samples using HeLa cells, lysates were generated and further processed as described below, identical to the processing for zebrafish lysates by a modified version of the method previously described by Darabedian et al[2]. A portion of the lysate was used to quantify protein amount by the BCA assay. 0.5-1.25 mg of protein was tagged via CuAAC with 1 mM $CuSO_4$ (in water, made fresh), 600 µM THPTA (in water), 100 µM azido-biotin (in water), and 50 mM sodium ascorbate (in water, made fresh) for 1 h at room temperature. Proteins were precipitated to remove excess CuAAC reagents. Water was added to a total volume of 300 µL followed by the addition of 400 µL of methanol and 150 µL of chloroform. Samples were vortexed and centrifuged at 16,000 x g at 4 °C for 5 min. The aqueous layer was removed, and 1 mL of ice-cold methanol was added. Samples were centrifuged at 16,000 x g at 4 °C for 3 min, and the supernatant was decanted. 200 µL of ice-cold methanol was added and centrifuged at 16,000 x g at 4 °C for 3 min. Methanol was decanted and protein pellet was dried at 37 °C for 5 min. Protein precipitation was performed three times.

Samples were solubilized in buffer (4% SDS and 100 mM sodium phosphate pH 7.4), sonicated twice at 20% power, and denatured with 6X SDS loading buffer. CuAAC-tagged samples were run in duplicate on a 10% SDS-PAGE gel and were transferred onto PVDF membranes. One replicate membrane was placed in a sealed bag with 55 mM NaOH and the other membrane was placed in a sealed bag with water. The membranes were incubated at 40 °C for 22 h. The membranes were rinsed with water to remove any residual sodium hydroxide followed by incubation with TBS-T for 5 min. The membranes were blocked with 5% w/v BSA in TBS-T for 1 h at room temperature then incubated with 1:25,000 dilution of streptavidin-HRP in TBS-T with 5% w/v BSA for 1 h. Solution was removed, and the membranes were rinsed three times with TBS-T for 7

min followed by one rinse with TBS for 5 min. The membranes were incubated with Clarity ECL solution for 5 min, and then the chemiluminescence was imaged.

Deglycosylation of *N*-glycans with PNGase F

Casper zebrafish embryos were microinjected into the yolk at the 1-cell stage with 5 nL of 10 mM UDP-6AlGal with 5% w/v rhodamine-dextran in 0.5X PBS pH 7.4 or left uninjected. Embryos were allowed to develop by incubation at 28.5 °C. After 24 h, embryos were decorionated using 1 mg/mL Pronase E in embryo medium for 8 min. Embryos were then pipetted onto a 1% agarose coated plates with embryo medium. Embryos were then rinsed twice with embryo medium by transferring to new dishes of media. Embryos were counted, aliquoted, and de-yolked by shear force through a trimmed P200 tip with ice cold PBS. De-yolked embryos were pelleted and rinsed 3 times with PBS to remove any residual yolk proteins then flash frozen using liquid nitrogen. Frozen embryos were stored at –80 °C. Flash frozen embryos were lysed in lysis buffer (1% NP-40, 50 mM sodium phosphate buffer pH 7.5, supplemented with a protease inhibitor tablet), sonicated three times with 20% power, protein quantified by BCA assay, and normalized to 2 mg/mL with lysis buffer.

For positive and negative control samples using HeLa cells, lysates were generated and further processed as described below, identical to the processing for zebrafish lysates by a modified version of the method previously described by Darabedian et al[2]. Samples were aliquoted into 100 µg amounts. 3 µL of PNGase F or 3 µL of water was added to each sample and incubated for 6 h at 37 °C. Enzyme was then removed by protein precipitation. 250 µL of water was followed by the addition of 400 µL of methanol and 150 µL of chloroform. Samples were vortexed and centrifuged at 16,000 x g at 4 °C for 5 min. The aqueous layer was removed, and 1 mL of ice-cold methanol was added. Samples were centrifuged at 16,000 x g at 4 °C for 3 min and supernatant was decanted. 200 µL of ice-cold methanol was added and centrifuged at 16,000 x g at 4 °C for 3 min. Methanol was decanted and protein pellet was dried at 37 °C for 5 min.

Samples were solubilized in 25 µL of buffer (4% SDS, 100 µM sodium phosphate buffer pH 7.4, supplemented with a protease inhibitor tablet) and reacted by CuAAC in a total volume of 5 µL with 1 mM $CuSO_4$ (in water, made fresh), 600 µM THPTA (in water), 100 µM azido-biotin (in water), and 50 mM sodium ascorbate (in water, made fresh) for 1 h at room temperature. Samples were then precipitated as before and solubilized in 1.2% SDS in 1X PBS pH 7.4 and 6X SDS loading buffer. Samples were analyzed by SDS-PAGE and Western blot with streptavidin-HRP and detection with Clarity ECL solution as described above.

Imaging of cell-surface glycans on developing zebrafish

Casper zebrafish embryos were microinjected into the yolk at the 1-cell stage with 5 nL of 10 mM UDP-6AlGal or no sugar with 5% w/v rhodamine-dextran in 0.5X PBS pH 7.4. Embryos were allowed to develop by incubation at 28.5 °C. Injected zebrafish embryos were manually dechorionated using forceps and transferred to an agarose-coated 96-well plate (5 embryos or fewer per well) in embryo medium. Embryos were tagged via CuAAC with 45 µM $CuSO_4$, 270 µM THPTA, 50 µM azido-biotin, and 2.5 mM sodium ascorbate for 5 min (total volume 100 µL). The CuAAC reaction were stopped

with the addition of 100 µM bathocuproinedisulfonic acid (BCS) followed by 100 µL of embryo medium. Embryos were rinsed twice in embryo medium. CuAAC-tagged embryos were then incubated with 1 µg/mL streptavidin-Alexa Fluor 488 (1 mg/mL stock) for 30 min in the dark. Embryos were rinsed three times with embryo medium. Embryos were first anesthetized with 0.2% tricaine in embryo medium, mounted in 0.6% low-melt agarose, and imaged by confocal microscopy.

Synthetic Methods

$Ac_4$6AzGal[3], monodeprotected sugar **5**[3], UDP-6AzGal[3], $Ac_4$4AzGal[4], $Ac_4$6AzEtGal[5], Ac_4GlcNAz[6], $Ac_4$5AlGal[7], Ac_3GlcNAz-1-P(SATE)[8], Alk-QA[9], triflate sugar **1**[10], and bis(*S*-acetyl-2-thioethyl) *N,N*-diisopropylphosphoramidite[11] were prepared according to literature procedures.

TIPS alkyne **2**. Triisopropylsilylacetylene (700 µL, 3.12 mmol, 3.50 eq to triflate,) was added to HMPA (160 µL, 0.892 mmol, 0.26 eq to nBuLi,) and THF (3 mL). Reagents were cooled to –78 °C and nBuLi (1.4 M in hexanes, 2.47 mL, 3.43 mmol, 1.10 eq to acetylene,) was added dropwise and stirred for 30 min. Triflate sugar **1** (350 mg, 0.892 mmol, 1.00 eq) in 900 µL of THF was added dropwise to the reaction. The reaction was warmed and stirred overnight. The reaction was quenched with 20 mL of saturated ammonium chloride and extracted twice with EtOAc, washed with water and brine, dried with sodium sulfate, and filtered. The reaction was concentrated and purified by flash column chromatography (12:1, hexanes/EtOAc, v/v) to yield an oil with a small amount of residual triisopropylsilylacetylene (69.0 mg, 18%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.50 (d, *J* = 5.0 Hz, 1H), 4.60 (dd, *J* = 8.0, 2.3 Hz, 1H), 4.34 (dd, *J* = 8.0, 1.8 Hz, 1H), 4.28 (dd, *J* = 5.0, 2.3 Hz, 1H), 3.91 (ddd, *J* = 8.3, 6.1, 1.8 Hz, 1H), 2.64 (dd, *J* = 16.5, 8.7 Hz, 1H), 2.53 (dd, *J* = 16.5, 6.1 Hz, 1H), 1.52 (s, 6H), 1.44 (s, 3H), 1.32 (s, 6H), 1.05 (d, *J* = 4.6 Hz, 18H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 113.0, 109.2, 108.6, 104.6, 96.7, 82.3, 71.4, 70.9, 70.8, 67.2, 65.6, 31.7, 26.2, 26.1, 25.1, 24.3, 22.0, 18.7, 18.7, 11.4, 11.2. HRMS (DART) m/z: $[M+H]^+$ calcd. for $C_{23}H_{41}O_5Si^+$ 425.27178; found 425.27330.

Bisacetonide alkyne **3**. TIPS alkyne **2** (100 mg, 0.235 mmol, 1.00 eq) was dissolved in THF (1.2 mL) and cooled to 0 °C. TBAF (1 M in hexanes, 710 µL, 0.706 mmol, 3.00 eq) was added slowly, warmed to room temperature, and reacted overnight. The reaction was diluted with EtOAc then washed twice with water and once with brine. The crude mixture was dried with sodium sulfate, filtered, and concentrated. The brown oil was purified by flash column chromatography (15:1 hexanes/EtOAc, v/v) to yield the bisacetonide protected alkyne **3** (23.4 mg, 37%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.51 (d, *J* = 5.0 Hz, 1H), 4.63 (dd, *J* = 7.8, 2.4 Hz, 1H), 4.45 – 4.25 (m, 2H), 3.92 (ddd, *J* = 8.5, 6.4, 1.9 Hz, 1H), 2.58 (ddd, *J* = 16.5, 8.4, 2.7 Hz, 1H), 2.51 (ddd, *J* = 16.5, 6.5, 2.7 Hz, 1H), 2.01 (t, *J* = 2.7 Hz, 1H), 1.55 (s, 3H), 1.45 (s, 3H), 1.36 (s, 3H), 1.33 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 109.5, 108.8, 96.7, 80.4, 71.5, 70.9, 70.6, 70.0, 66.7, 26.2, 26.1, 25.0, 24.6, 20.5. HRMS (DART) m/z: $[M+NH_4]^+$ calcd. for $C_{14}H_{24}NO_5^+$ 286.16490; found 286.16599.

$Ac_4$6AlGal. Water (21 mL) and TFA (32 mL) was added to alkyne sugar **3** (1.44 g, 5.37 mmol, 1.00 eq) at 0 °C. The reaction was warmed to room temperature then heated to 50 °C for 1 h. The reaction was then concentrated and co-evaporated with toluene three times. Trace amounts of toluene were removed under reduced pressure, and the sugar residue was dissolved in pyridine (6.5 mL) and acetic anhydride (4 mL) and stirred overnight at room temperature. The reaction was quenched with 20 mL of cold water and extracted three times with EtOAc and washed three times with 1 M HCl and once with brine. Crude mixture was dried with sodium sulfate, filtered, and concentrated. The reaction was purified by flash column chromatography (4:1 hexanes/EtOAc, v/v) to give the desired compound as a complex mixture of isomers (338 mg, 18%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.29 (d, *J* = 3.5 Hz, 1H, α), 5.65 (d, *J* = 8.3 Hz, 1H, β), 5.57 (d, *J* = 3.0 Hz, 1H, α), 5.51 (d, *J* = 3.3 Hz, 1H, β), 5.32 (dd, *J* = 11.0, 3.2 Hz, 1H, α), 5.26 (td, *J* = 8.0, 3.9 Hz, 2H, α & β), 5.07 (dd, *J* = 10.5, 3.4 Hz, 1H, β), 4.27 – 4.19 (m, 1H, α), 3.94 (dd, *J* = 9.0, 5.9 Hz, 1H, β), 2.60-2.34 (m, 4H, α & β), 2.16-1.94 (m, 13H, *CH_3*-COO & C≡*CH*). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.2, 170.1, 170.0, 170.0, 169.9, 169.4, 169.0, 92.2, 92.1, 89.8, 89.8, 77.9, 77.7, 72.5, 71.7, 71.5, 71.0, 71.0, 69.7, 68.3, 67.8, 67.7, 67.6, 66.4, 20.9, 20.8, 20.7, 20.7, 20.7, 20.6, 20.3. HRMS (ESI) m/z: $[M+NH_4]^+$ calcd. for $C_{16}H_{24}NO_9^+$ 374.14456; found 374.14419.

$Ac_3$6AzGal-1-P(SATE). Monodeprotected sugar **5** (45.0 mg, 0.136 mmol, 1.00 eq), THF (270 µL), and bis(*S*-acetyl-2-thioethyl) *N,N*-diisopropylphosphoramidite (60.0 mg, 0.163 mmol, 1.20 eq) were added to a flask. 5-(ethylthio)-1*H*-tetrazole (53 mg, 0.408 mmol, 3.00 eq) was then added and the reaction was stirred for 1.5 h. The reaction was cooled to –40 °C and mCPBA (52% v/w, 59.0 mg, 0.177 mmol, 1.30 eq) dissolved in 340 µL of DCM was added. After addition, the reaction was then allowed to warm to room temperature and stirred overnight. The reaction was quenched with 3 mL of 10% sodium thiosulfate, extracted with DCM (3 x 3 mL), and washed with 2 mL of saturated $NaHCO_3$ followed by water (3 x 2 mL). The combined organic layer was dried with sodium sulfate, filtered, and concentrated. The crude was purified by flash column chromatography (2:1 hexanes/EtOAc, v/v) to yield an oil (51.0 mg, 61%). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.91 (dd, *J* = 6.7, 3.3 Hz, 1H), 5.50 – 5.44 (m, 1H), 5.32 (dd, *J* = 10.9, 3.0 Hz, 1H), 5.28 – 5.20 (m, 1H), 4.33 (td, *J* = 6.0, 5.2, 3.1 Hz, 1H), 4.16 (ddtd, *J* = 10.6, 8.1, 6.5, 2.9 Hz, 4H), 3.47 (dd, *J* = 12.8, 7.7 Hz, 1H), 3.19 (ddt, *J* = 17.5, 10.3, 4.5 Hz, 6H), 2.34 (d, *J* = 2.5 Hz, 6H), 2.15 (s, 3H), 2.08 (s, 3H), 1.98 (s, 3H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 194.8, 194.78, 170.1, 170.1, 169.9, 94.7 (d, *J* = 5.2 Hz), 70.1, 68.2, 67.0, 66.9, 66.5 (d, *J* = 5.7 Hz), 66.4 (d, *J* = 5.6 Hz), 50.5, 30.6, 29.1 (d, *J* = 7.3 Hz), 29.1 (d, *J* = 7.6 Hz), 20.8, 20.7. ^{31}P NMR (200 MHz, $CDCl_3$) δ -3.37. HRMS (DART) m/z: $[M+NH_4]^+$ calcd. for $C_{20}H_{34}NO_{13}PS_2^+$ 633.12959; found 633.13128.

Monodeprotected **6**. 95 µL of ethylenediamine (1.42 mmol, 1.10 eq) was added to 8 mL of THF at 0 °C then 96 µL of acetic acid (1.68 mmol, 1.30 eq) was added slowly at 0 °C and stirred for 10 min. **$Ac_4$6AlGal** (460 mg, 1.29 mmol, 1.00 eq) in 8 mL of THF was added slowly and stirred overnight at 0 °C. The reaction was concentrated, dissolved in 30 mL of DCM, and washed twice with 20 mL of 1 M HCl, once with 30 mL of saturated sodium bicarbonate, and once with 40 mL of brine. Crude mixture was dried with sodium sulfate, filtered, and concentrated. The reaction was purified by flash column

chromatography (6:1 hexanes/EtOAc, v/v) to yield **6** as a complex mixture of isomers (101 mg, 48% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 5.53 (d, *J* = 3.3 Hz, 1H, α), 5.50 (d, *J* = 2.9 Hz, 1H, β), 5.48 (d, *J* = 3.1 Hz, 2H, α & β), 5.41 (dd, *J* = 10.9, 3.4 Hz, 1H, α), 5.12 (dd, *J* = 10.9, 3.6 Hz, 1H, α), 5.09 – 5.00 (m, 2H, β), 4.68 (t, *J* = 7.8 Hz, 1H, β), 4.40 (t, *J* = 7.4 Hz, 1H, α), 3.86 (dd, *J* = 8.7, 6.3 Hz, 1H, β), 3.49 (d, *J* = 3.4 Hz, 1H, α), 2.59 (ddd, *J* = 14.6, 6.7, 2.6 Hz, 1H, β), 2.54 (dd, *J* = 6.2, 2.7 Hz, 1H, β), 2.43 (qtd, *J* = 16.7, 9.2, 7.8, 2.7 Hz, 2H, α), 2.16 (s, 1H), 2.15 (s, 3H), 2.09 (s, 3H), 1.98 (s, *3*H). ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6, 170.4, 170.3, 170.3, 170.2, 96.0, 90.8, 78.9, 78.3, 71.9, 71.5, 71.1, 71.1, 70.7, 69.3, 68.4, 68.2, 67.6, 67.2, 21.0, 20.8, 20.8, 20.8, 20.7, 20.6. HRMS (ESI) m/z: $[M+NH_4]^+$ calcd. for $C_{14}H_{22}NO_8^+$ 332.13399; found 332.13367.

Phosphite **7**. Monodeprotected sugar **6** (167 mg, 0.531 mmol, 1.00 eq) was dissolved in 700 µL of THF and 1.4 mL of dioxane and cooled to 0 °C. TEA (150 µL, 1.063 mmol) was then added and stirred for 30 min. 2-Chloro-1,3,2-benzodioxaphosphorin-4-one was dissolved in 700 µL of THF and added to reaction at 0 °C and stirred for 1.5 h. The reaction was stopped with the addition of 2 mL of water and concentrated, followed by co-evaporation three times with 5 mL of THF. The crude product was dissolved in 3 mL of THF, filtered, and purified by flash column chromatography (7:1 to 3:1 chloroform/methanol + 1% acetic acid, v/v) to yield **7** as a sub-stoichiometric TEA salt (162.0 mg, 64%). ^{1}H NMR (500 MHz, MeOD) δ 6.88 (d, $J_{P\text{-}H}$ = 641.9 Hz, 1H), 5.75 (dd, *J* = 8.3, 3.4 Hz, 1H), 5.57 (d, *J* = 3.1 Hz, 1H), 5.37 (dd, *J* = 10.8, 3.2 Hz, 1H), 5.20 – 5.07 (m, 1H), 4.41 (t, *J* = 7.4 Hz, 1H), 3.21 (q, *J* = 7.2 Hz, 4H, NCH_2CH_3), 2.46 (td, *J* = 11.7, 10.9, 6.3 Hz, 2H), 2.34 (q, *J* = 3.5, 2.5 Hz, 1H), 2.15 (s, 3H), 2.07 (s, 3H), 1.96 (s, 3H), 1.32 (t, *J* = 7.2 Hz, 6H, NCH_2CH_3). ^{13}C NMR (125 MHz, MeOD) δ 171.9, 171.8, 171.6, 93.3, 79.5, 72.2, 70.2, 69.8, 69.2, 68.9, 68.8, 47.8, 20.9, 20.7, 20.6, 20.5, 9.2. ^{31}P NMR (200 MHz, MeOD) δ -0.12. HRMS (ESI) m/z: $[M]^-$ calcd. for $C_{14}H_{18}O_{10}P^-$ 377.06431; found 377.06326.

Phosphate **8**. Phosphite sugar **7** (138 mg, 0.366 mmol, 1.00 eq) was dissolved in 6 mL of THF and then Dowex 50W H+ resin was added to the flask. The resin was removed by filtration, and the solution was concentrated. The residue was then dissolved in 5.5 mL of THF and 133 µL of t-BuOOH (0.732 mmol, 2.00 eq) was added, followed by 11 mg of iodine (0.11 mmol, 0.30 eq) in 500 µL of THF. The reaction was stirred overnight at room temperature. TEA (100 µL, 2.00 eq) was added and concentrated. The crude was purified by flash column chromatography (8:1 to 3:2 chloroform/methanol, v/v) to yield **8** as a sub-stoichiometric TEA salt (74.0 mg, 41%). ^{1}H NMR (500 MHz, MeOD) δ 5.70 (dd, *J* = 7.6, 3.4 Hz, 1H), 5.67 – 5.59 (m, 1H), 5.41 (td, *J* = 10.5, 3.4 Hz, 1H), 5.15 – 5.04 (m, 1H), 4.50 (ddd, *J* = 27.7, 10.3, 5.2 Hz, 1H), 3.15 (q, *J* = 7.3 Hz, 4H), 2.69 – 2.40 (m, 2H), 2.31 (t, *J* = 2.7 Hz, 1H), 2.15 (s, 3H), 2.07 (s, 3H), 1.96 (s, 3H), 1.30 (t, *J* = 7.2 Hz, 6H). ^{13}C NMR (125 MHz, MeOD) δ 172.2, 172.0, 171.6, 93.5, 93.4, 79.5, 79.4, 72.2, 70.1, 69.4, 69.0, 47.6, 20.6, 20.5, 20.5, 9.3. ^{31}P NMR (200 MHz, MeOD) δ -1.03. HRMS (ESI) m/z: $[M]^-$ calcd. for $C_{14}H_{18}O_{11}P^-$ 393.05812; found 393.05786.

UDP-6AlGal. Phosphate sugar **8** (63.0 mg, 0.128 mmol, 1.00 eq) was dissolved in 7:3 chloroform:methanol, and ion exchange was performed as above using Dowex 50W H+.

The solution was concentrated and co-evaporated with pyridine (3 x 5 mL) on a rotary evaporator that had been previously dried by evaporation of toluene from a separate flask. Uridine 5′-monophosphomorpholidate 4-morpholine-N,N′-dicyclohexylcarboxamidine salt (175 mg, 0.255 mmol, 2.00 eq) and 5-(ethylthio)-*1H*-tetrazole (33.0 mg, 0.255 mmol, 2.00 eq) were added to the flask and co-evaporated with pyridine (3 x 5 mL). The reagents were then dried under reduced pressure to remove any moisture. 13 mL of pyridine was added to the reaction and stirred for 3 d under nitrogen and in the dark. The reaction was concentrated, co-evaporated 3 times with toluene, and dissolved in 10 mL of methanol. 13 mL of 0.1 M NH_4CO_3 was added at 0 °C followed by TEA (508 µL, 3.64 mmol, 28.6 eq) and stirred overnight. The pH was adjusted to 7–7.5 using Dowex 50WX8, followed by filtration to remove the resin. The crude mixture was concentrated and dissolved at 20 mg/mL in 10% acetonitrile in water. The product was isolated by semi-preparative C18 HPLC purification using a 0–20% gradient of 0.1 M TEAA (triethylammonium acetate) buffer in water (solvent A) to acetonitrile (solvent B) (33.0 mg, 33%). Excess TEAA salt was present after purification. ^{1}H NMR (500 MHz, D_2O) δ 7.95 (d, *J* = 8.1 Hz, 1H), 5.94 (d, *J* = 8.1 Hz, 1H), 5.92 (d, *J* = 4.1 Hz, 1H), 5.54 (dd, *J* = 7.1, 3.6 Hz, 1H), 4.35 – 4.29 (m, 2H), 4.26 – 4.19 (m, 2H), 4.19 – 4.12 (m, 1H), 4.06 (d, *J* = 3.2 Hz, 1H), 3.87 (dd, *J* = 10.3, 3.3 Hz, 1H), 3.73 (dt, *J* = 10.4, 3.1 Hz, 1H), 3.05 – 2.95 (m, 1H), 2.50 (dd, *J* = 7.6, 2.7 Hz, 2H), 2.36 (t, *J* = 2.7 Hz, 1H). ^{13}C NMR (125 MHz, D_2O) δ 179.1, 166.1, 151.7, 141.7, 102.6, 95.9, 95.8, 88.6, 83.2, 83.1, 80.3, 73.8, 71.3, 70.0, 69.5, 69.4, 69.2, 68.3, 68.3, 64.8, 64.8, 46.6, 22.2, 19.7, 8.2. ^{31}P NMR (200 MHz, D_2O) δ -11.18, -12.85. HRMS (ESI–TOF) m/z: $[M]^-$ calcd. for $C_{17}H_{23}N_2O_{16}P_2^-$ 573.0528; found 573.0541.

Supporting Information References

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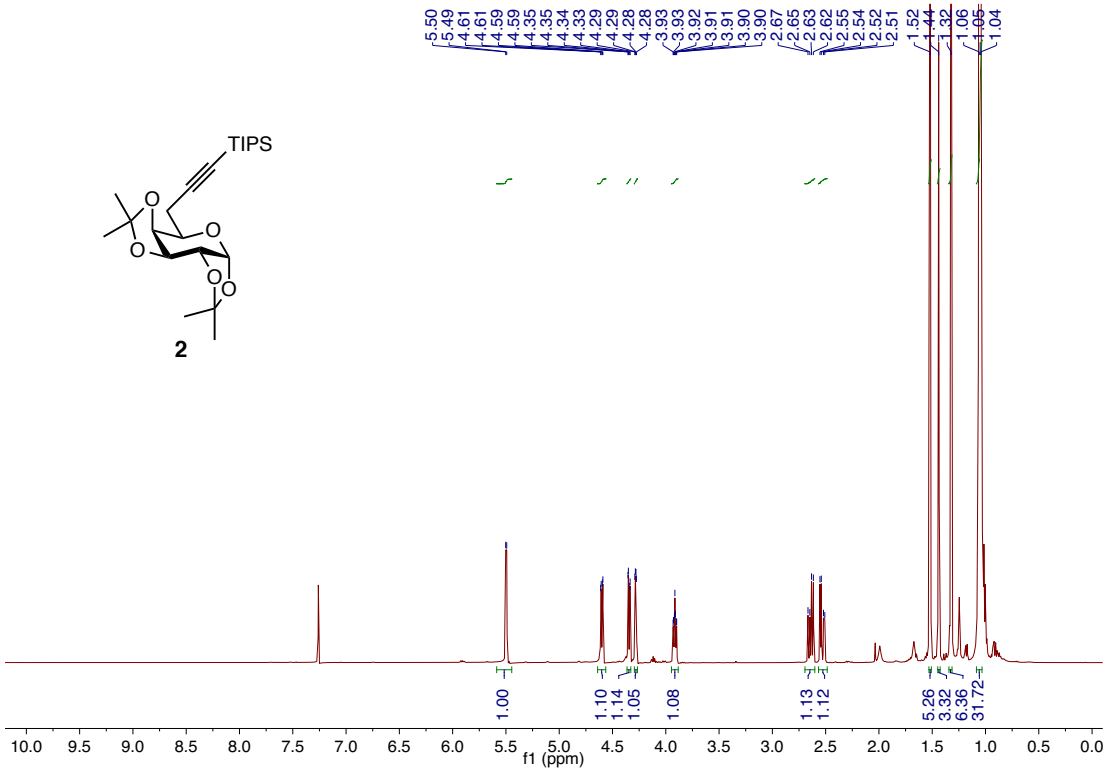
^{1}H, ^{13}C, and ^{31}P NMR Spectra



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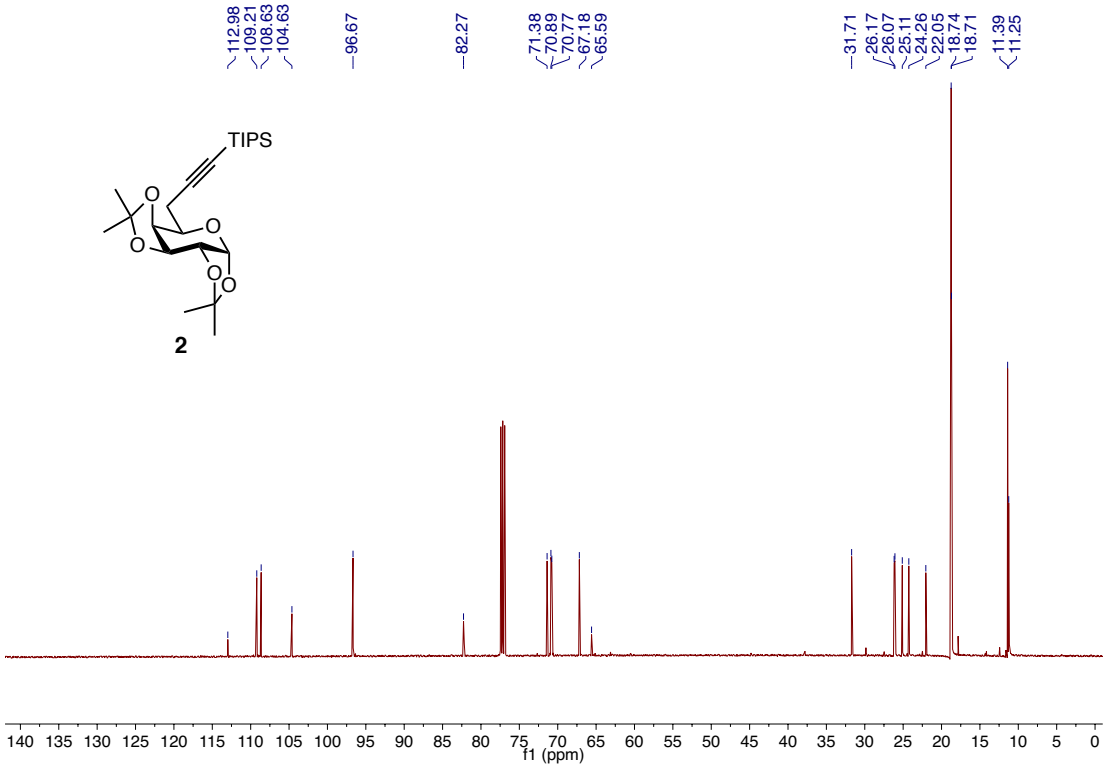

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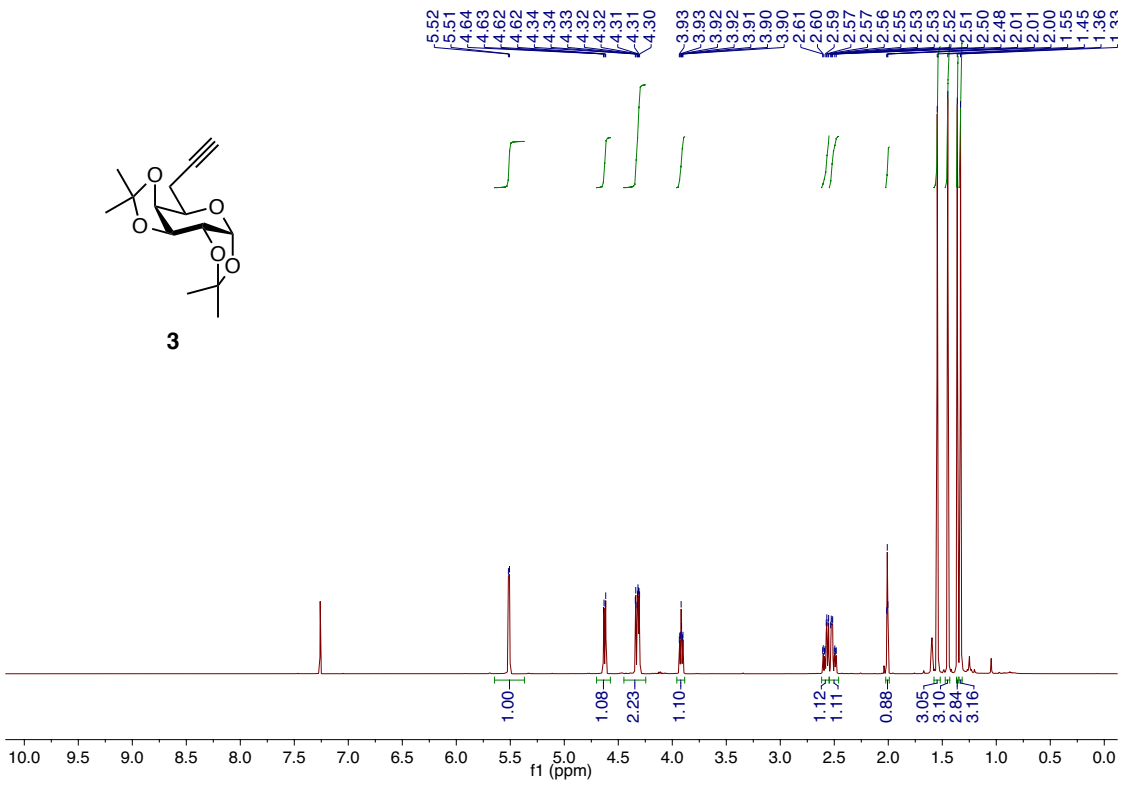

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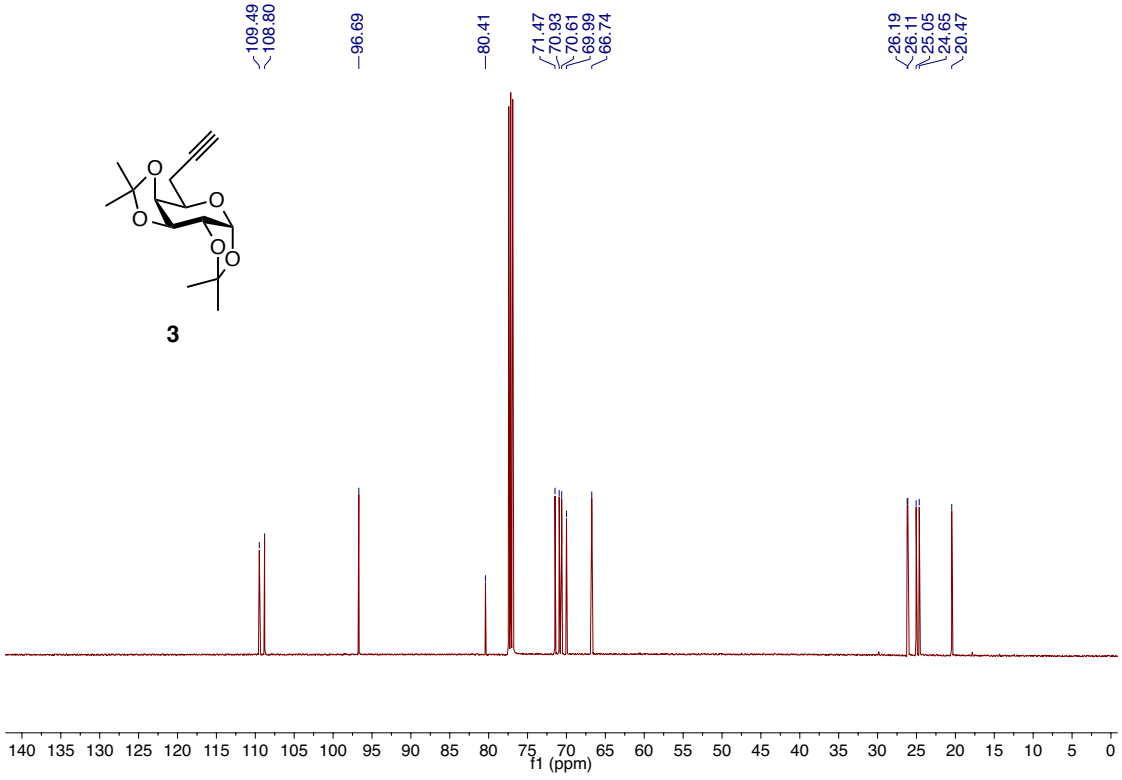

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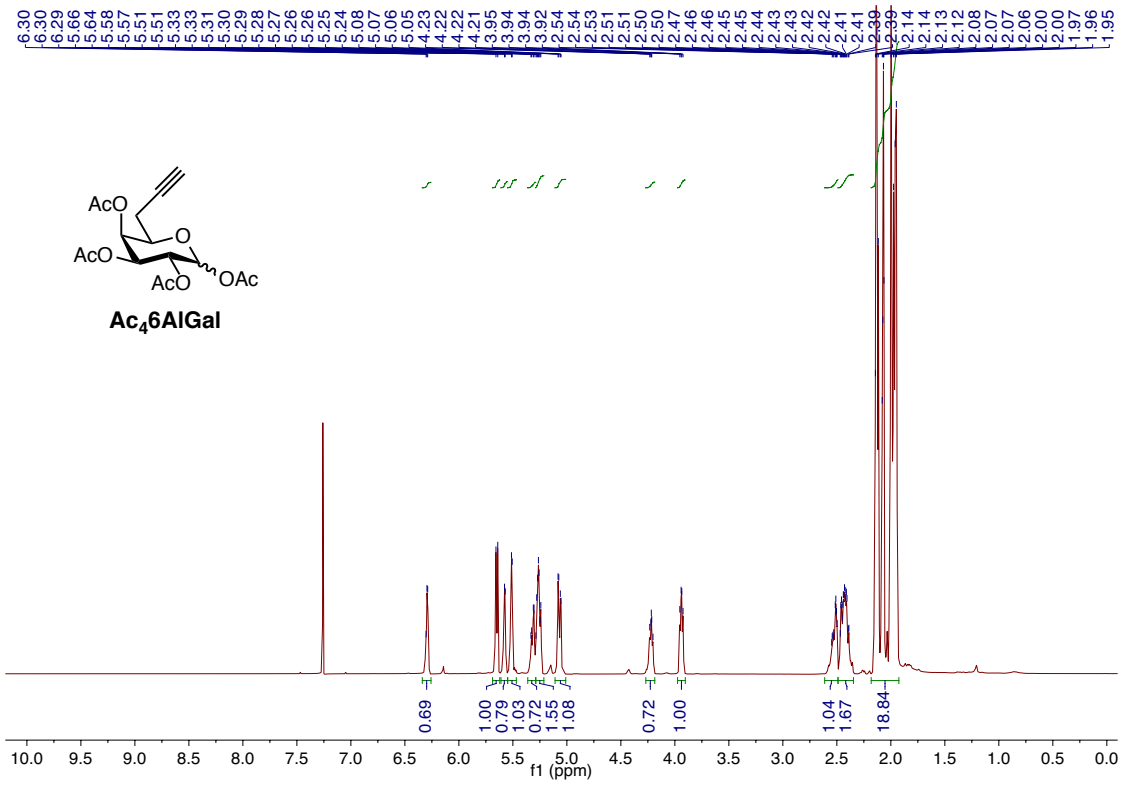

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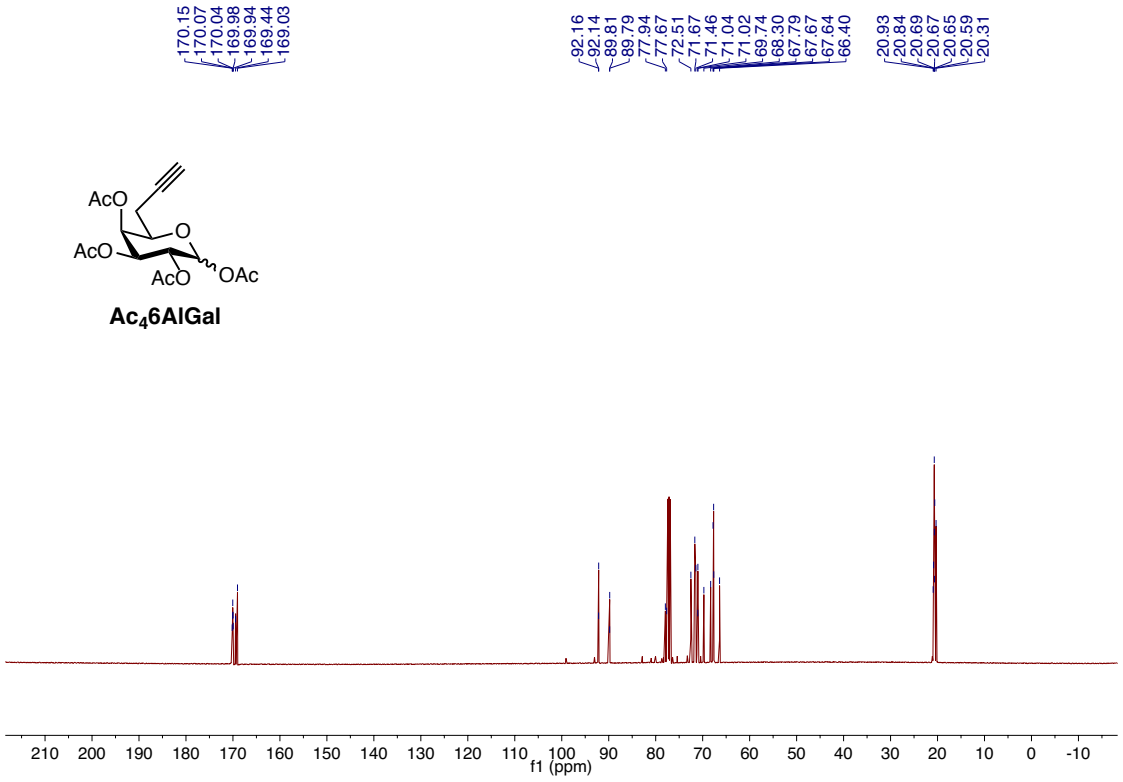

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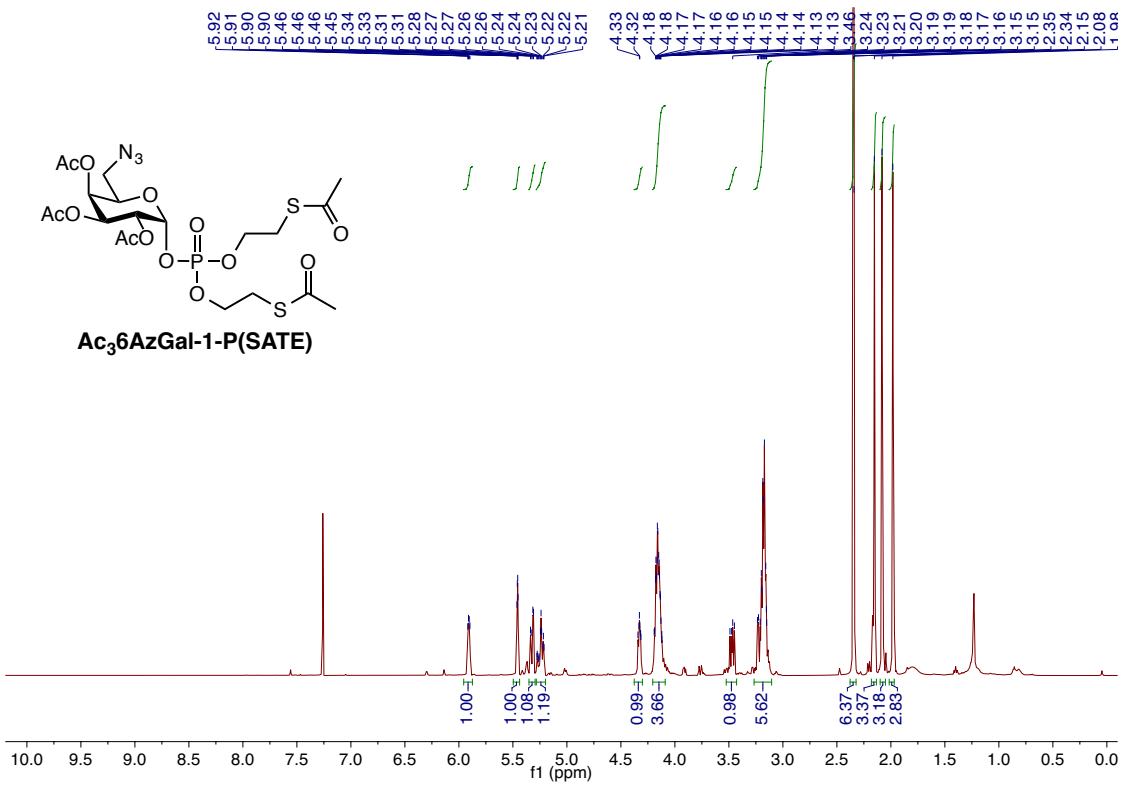

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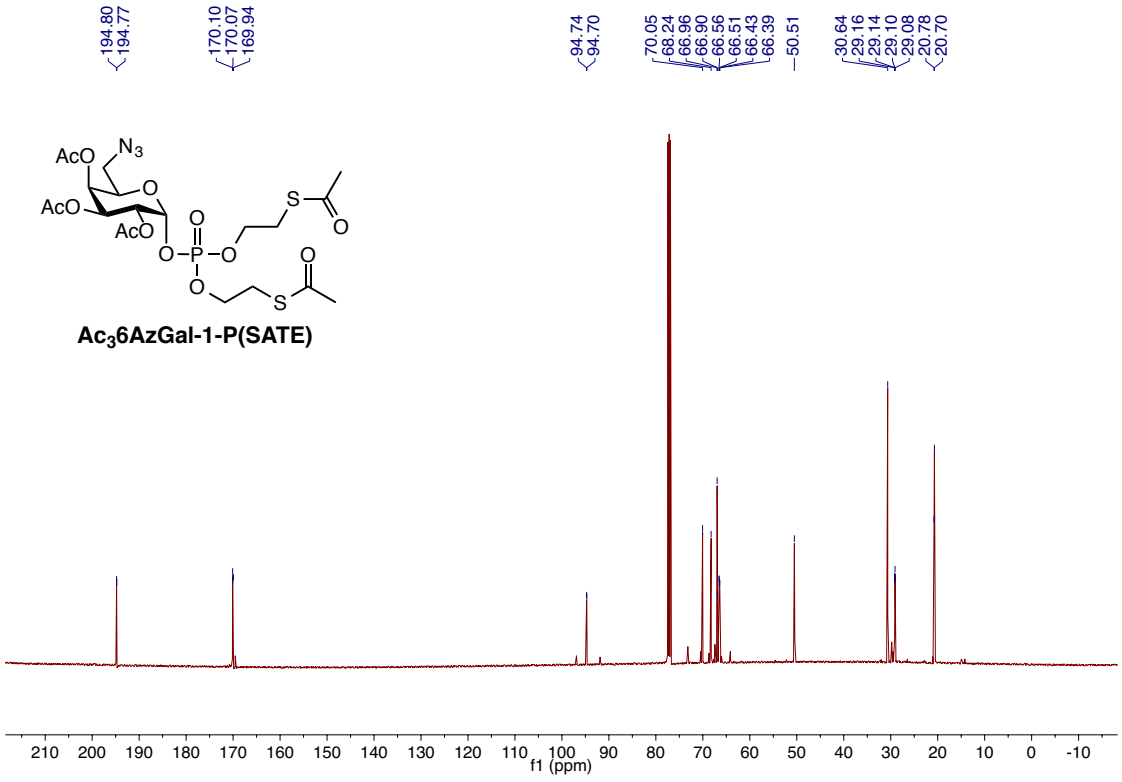

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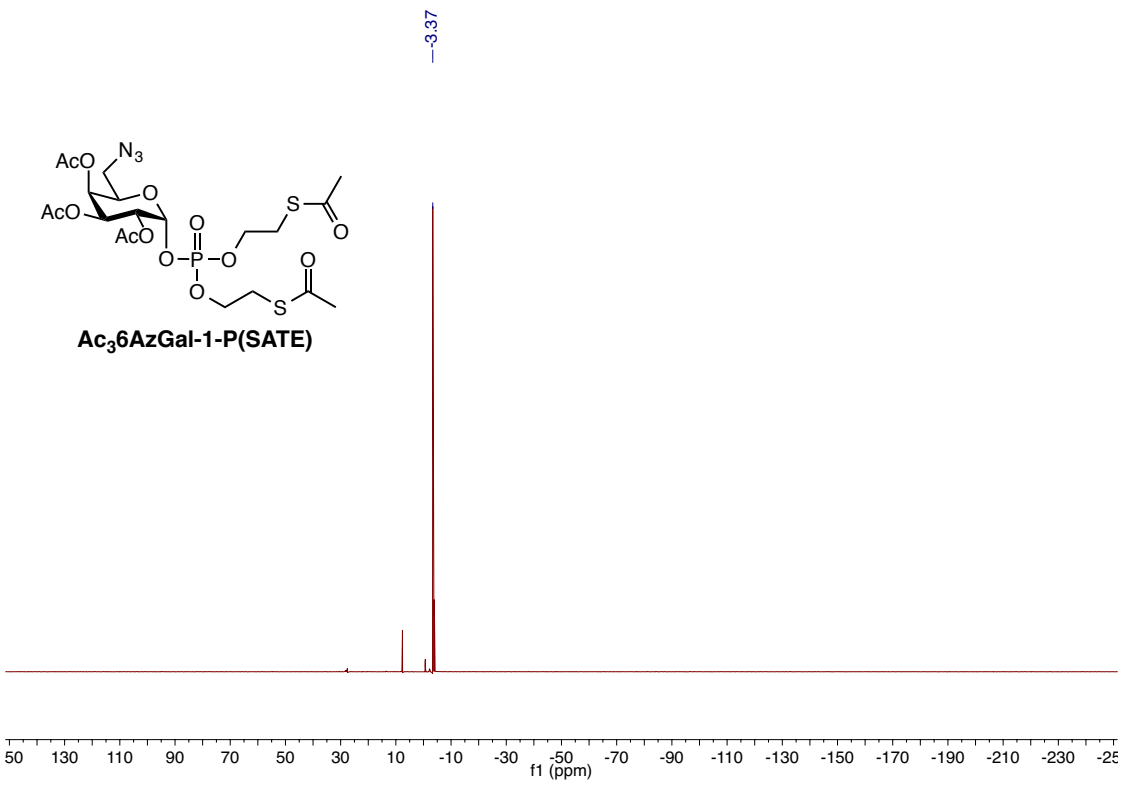
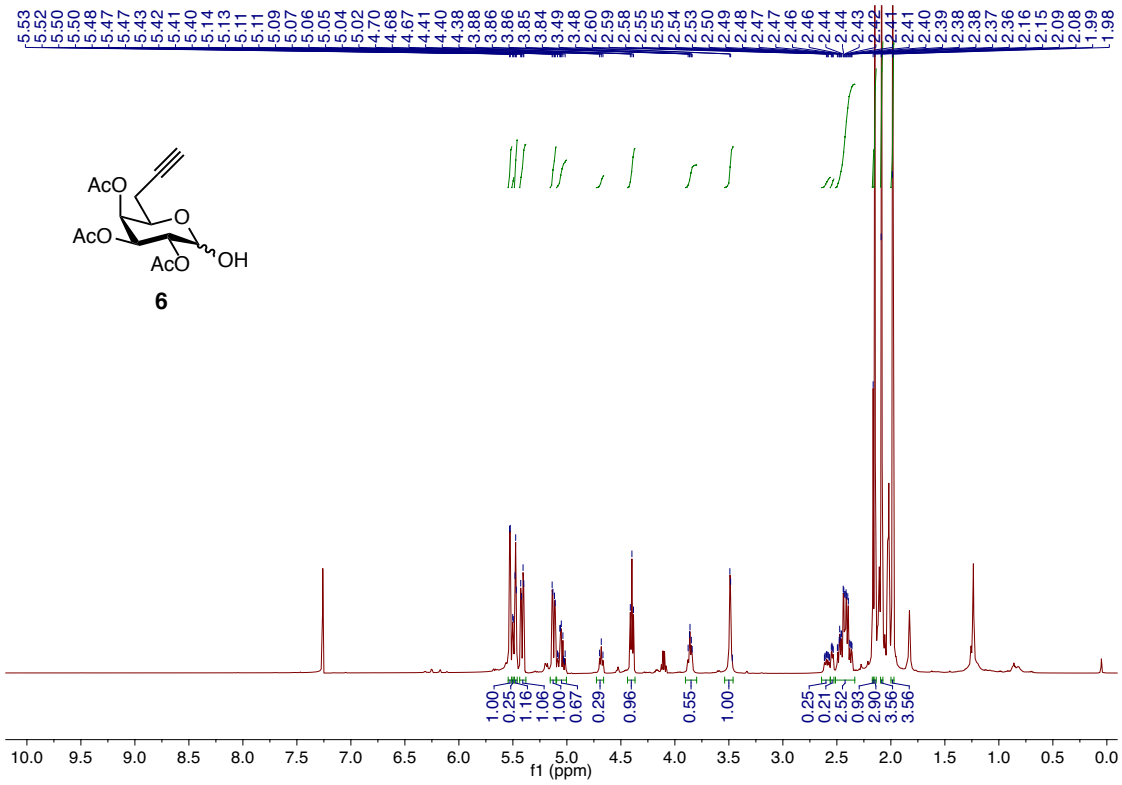
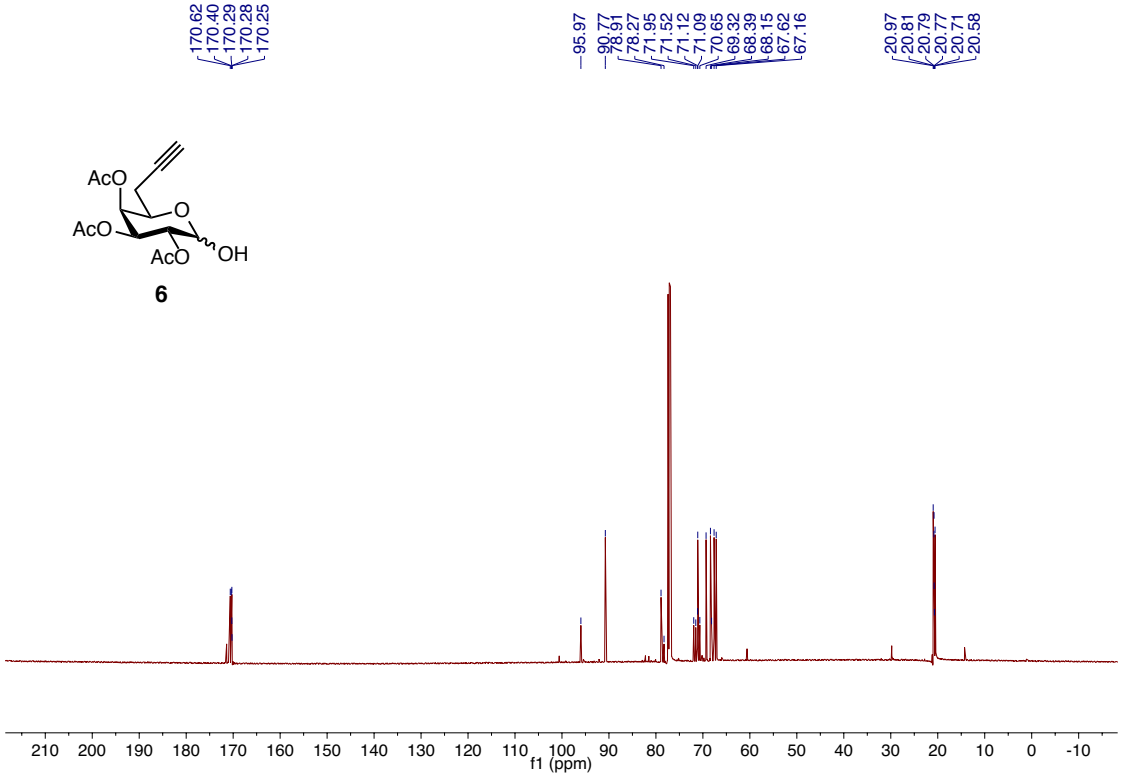


Ac$_3$6AzGal-1-P(SATE)

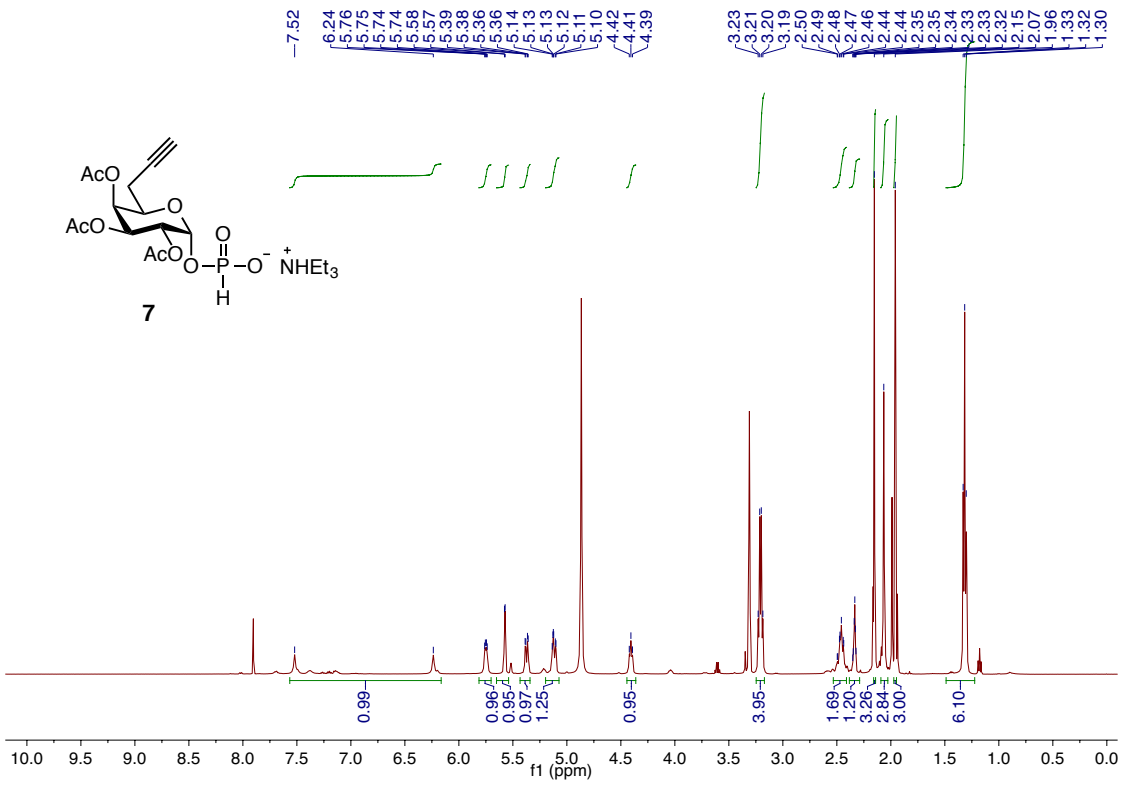
^{31}P NMR of **Ac$_3$6AzGal-1-P(SATE)** (200 MHz, $CDCl_3$)



^{1}H NMR of compound **6** (500 MHz, $CDCl_3$)



^{13}C NMR of compound **6** (125 MHz, $CDCl_3$)



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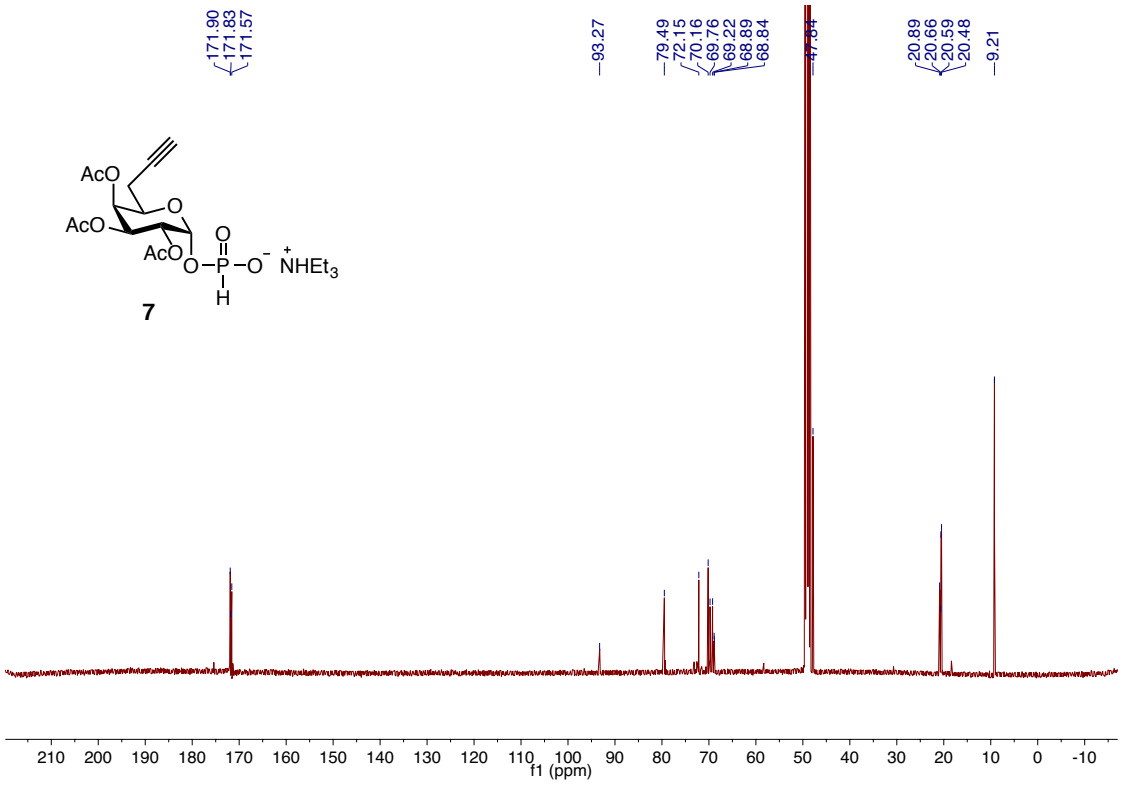
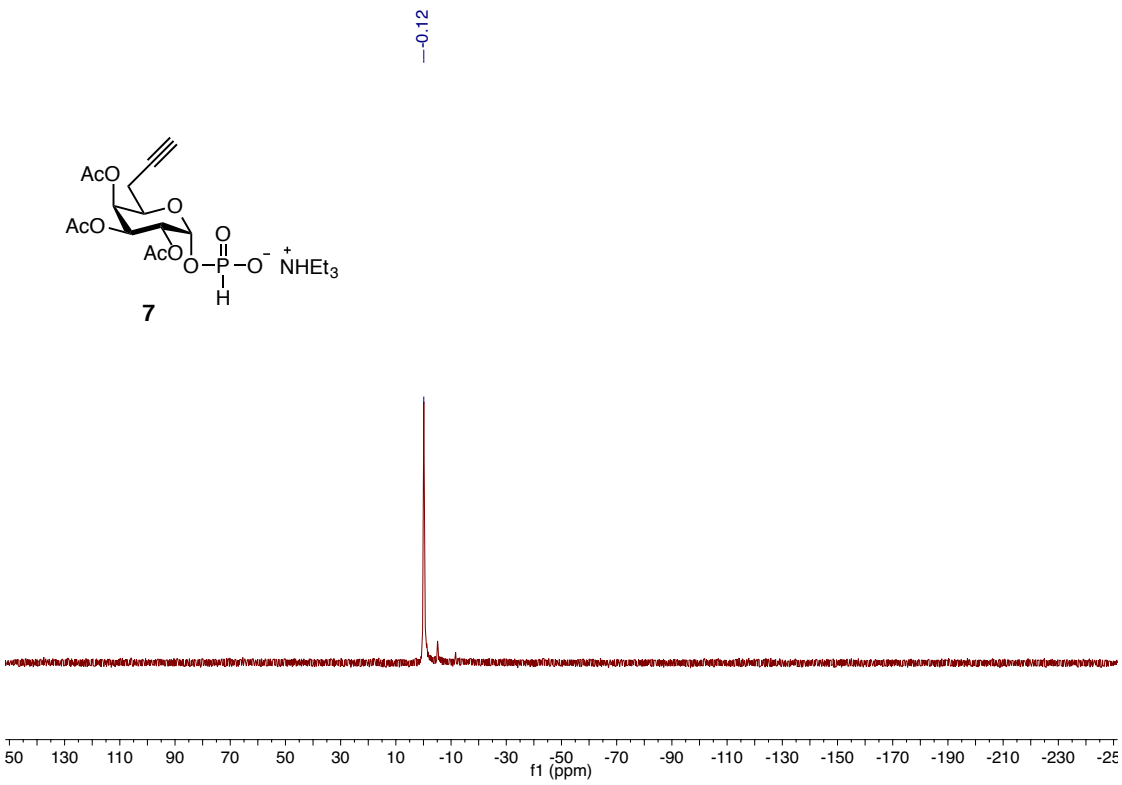
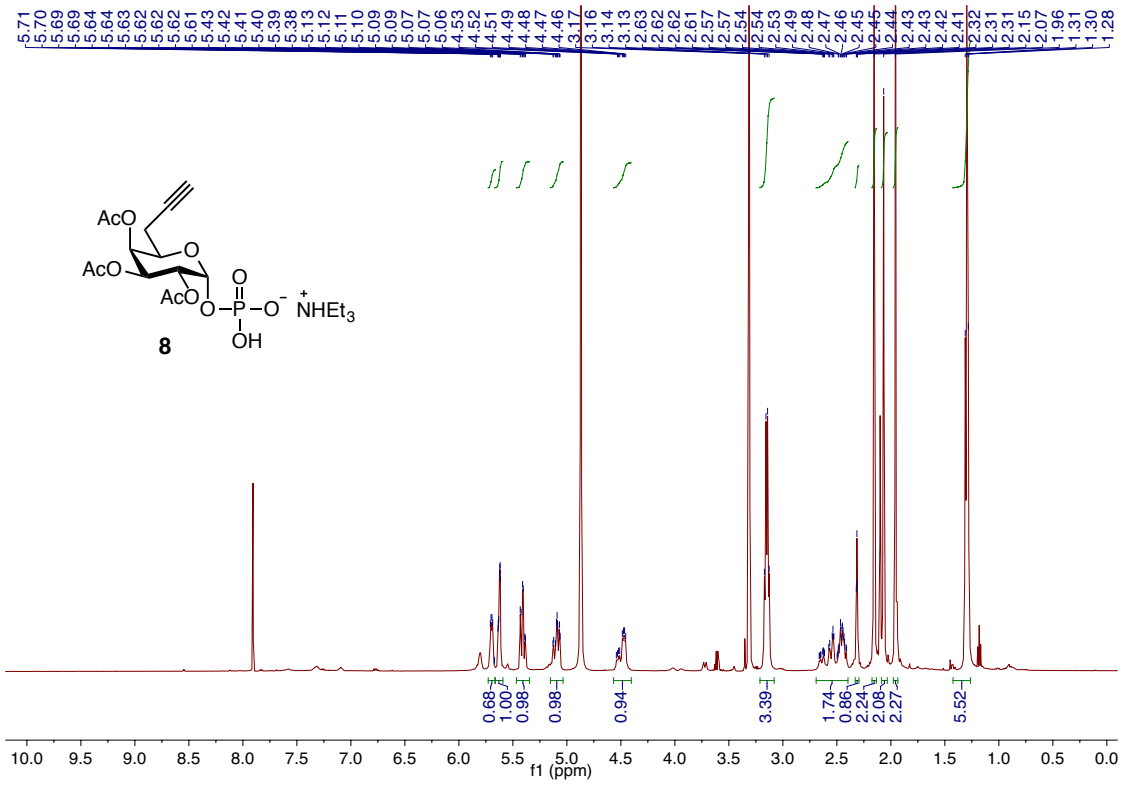


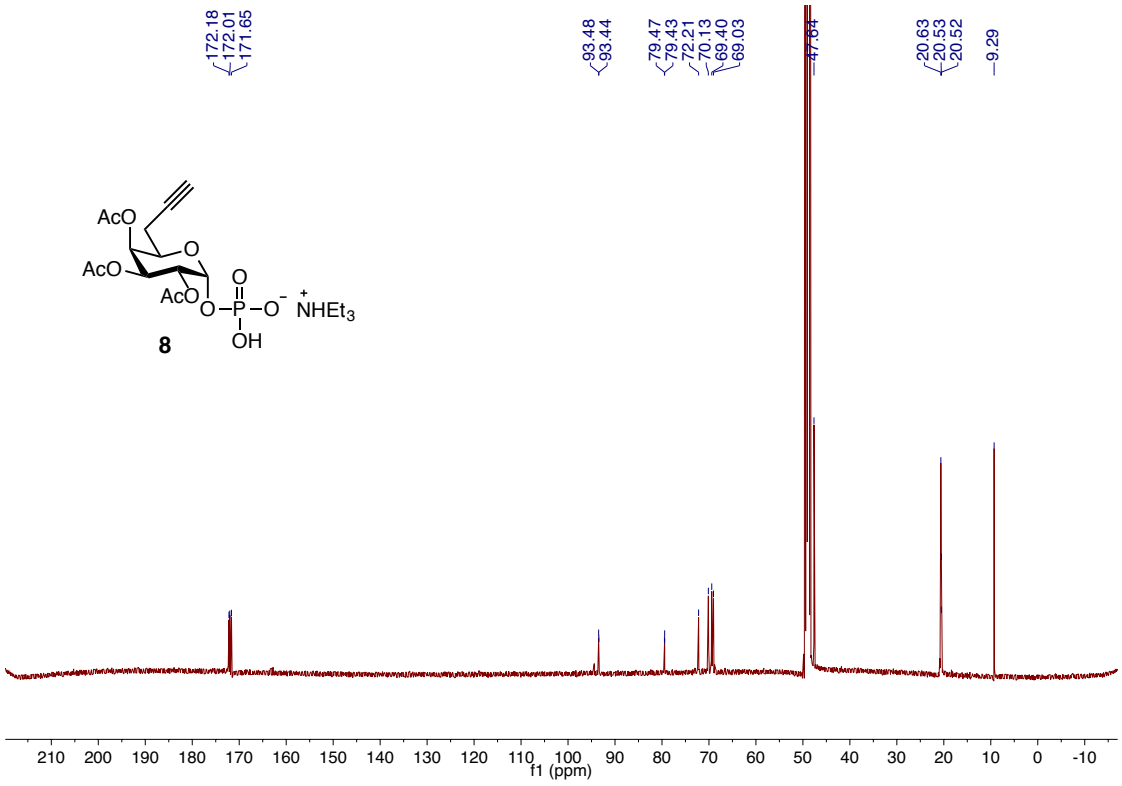
^{13}C NMR of compound **7** (125 MHz, MeOD)



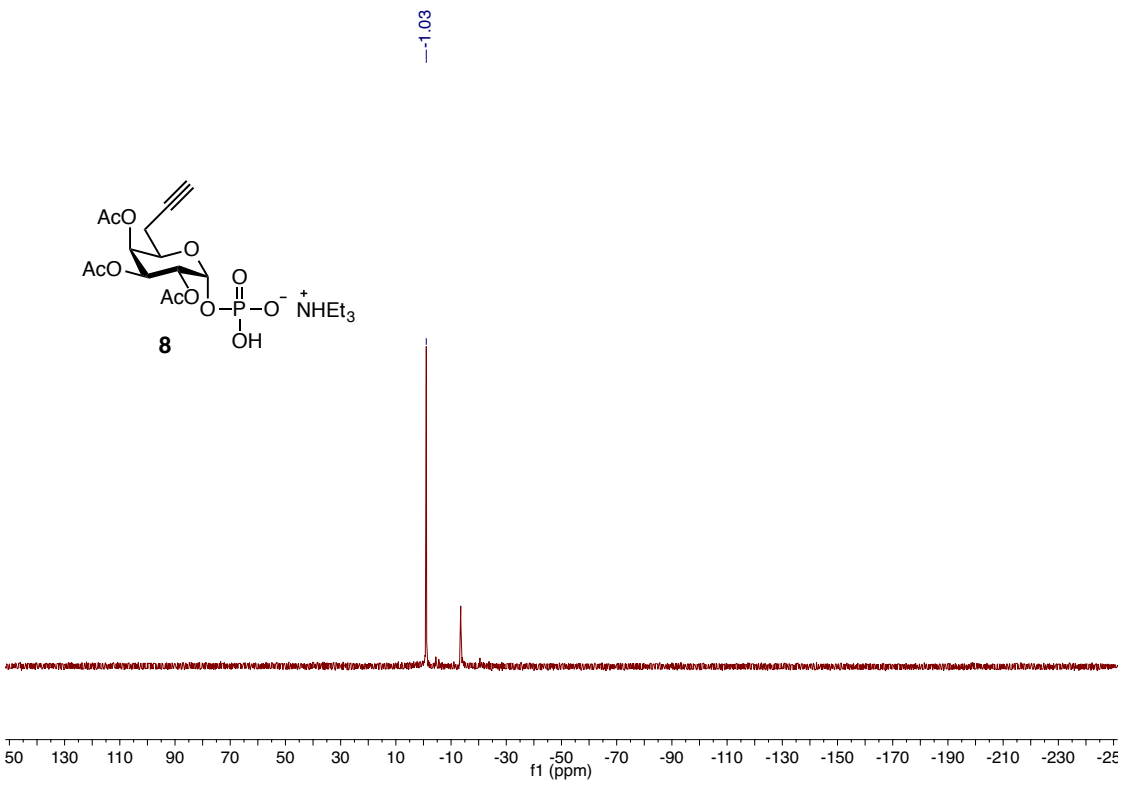
^{31}P NMR of compound **7** (200 MHz, MeOD)



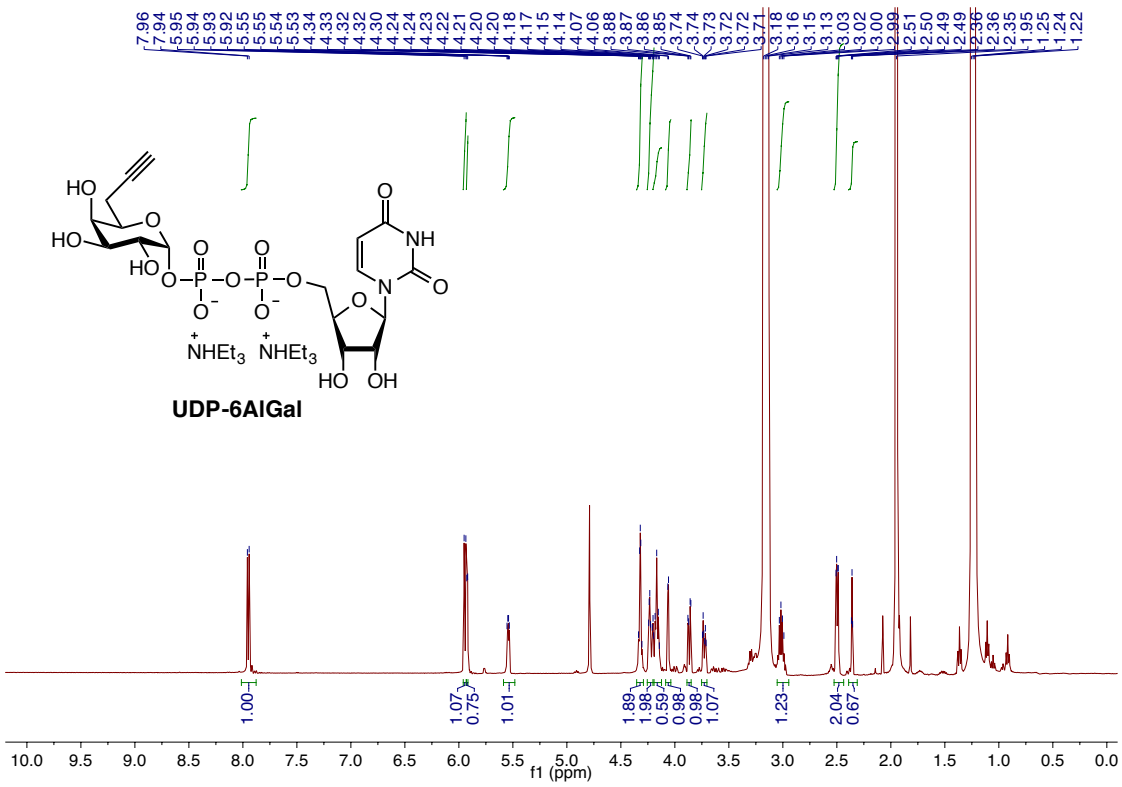
^{1}H NMR of compound **8** (500 MHz, MeOD)



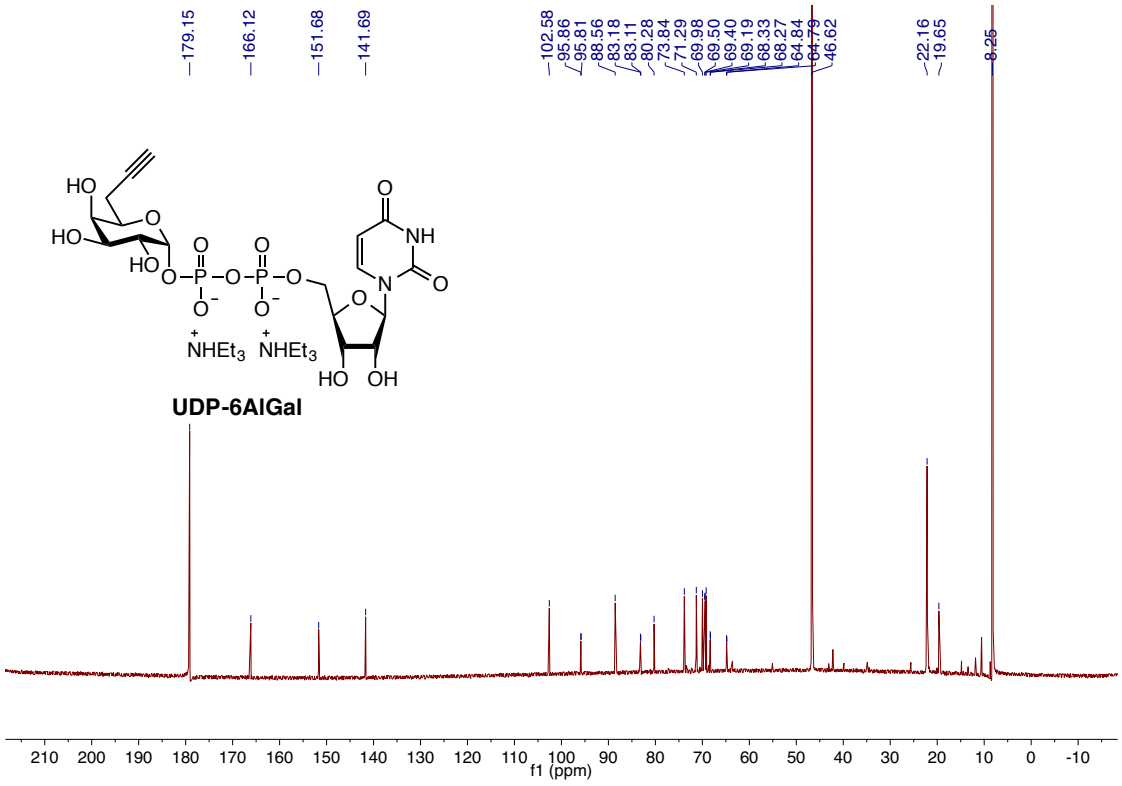
^{13}C NMR of compound **8** (125 MHz, MeOD)



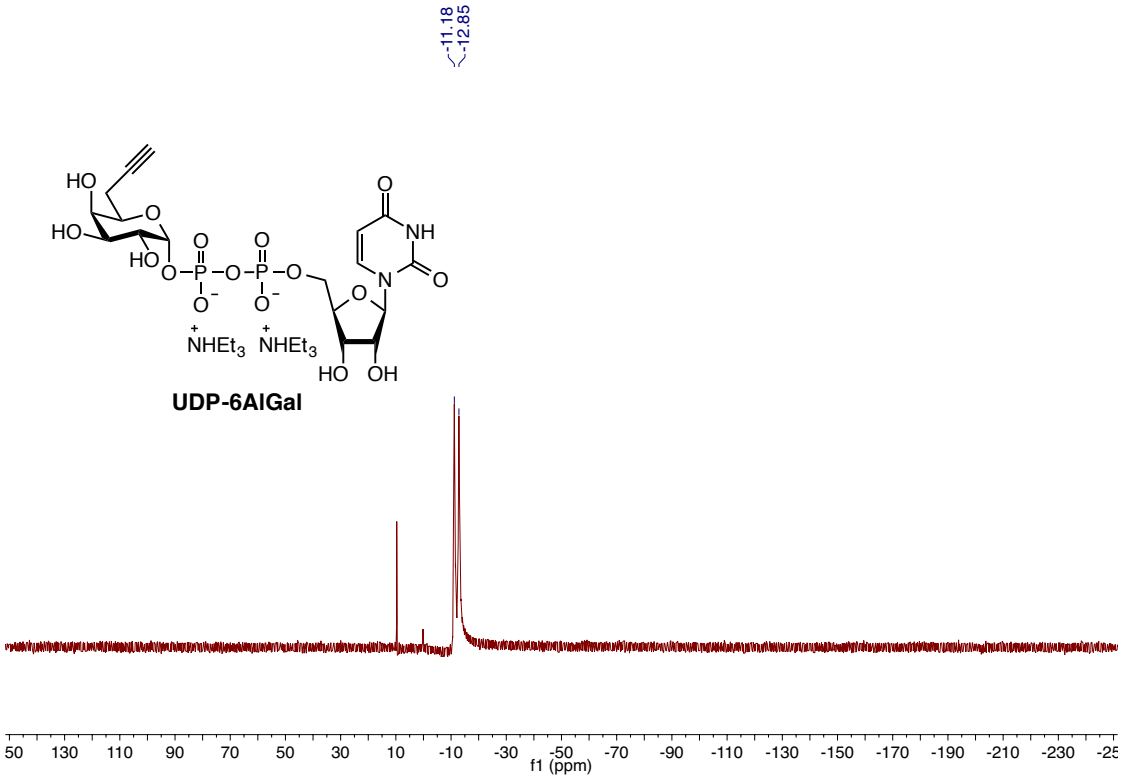
^{31}P NMR of compound **8** (200 MHz, MeOD)



^{1}H NMR of **UDP-6AlGal** (500 MHz, D_2O)



^{13}C NMR of **UDP-6AlGal** (125 MHz, D_2O)



^{31}P NMR of **UDP-6AlGal** (200 MHz, D_2O)