Inhibition of glycoprotein biosynthesis in the pathogenic bacterium *Helicobacter pylori* by masked carbohydrate phosphonates

Ebrahim Soleimani,[a,b] Aniqa Chowdhury,[b,c] Jian-She Zhu,[a] Elisa Ospanow,[a] Karen D. Moulton,[c] Danielle H. Dube[c*] and David L. Jakeman[a,d*]

[a.] *Department of Chemistry, Dalhousie University, Halifax, Nova Scotia, B3H 4J3, Canada.*
[b.] *These authors contributed equally to the research*
[c.] *Department of Chemistry and Biochemistry, Bowdoin College, Maine, USA.*
[d.] *College of Pharmacy, Dalhousie University, Halifax, Nova Scotia B3H 4R2, Canada.*

AUTHOR INFORMATION

Corresponding Authors

* Tel: +1 902 494 7159; david.jakeman@dal.ca; Tel: +1 207 798 4326; ddube@bowdoin.edu

Electronic Supporting Information Table of Contents

General biological experimental details

Organic chemicals and anti-FLAG antibodies were purchased from MilliporeSigma. *H. pylori* strain G27[1] was a gift of Manuel Amieva (Stanford University). $Ac_4GlcNAc$, $Ac_4GlcNAz$, and Phos-FLAG were synthesized following established protocols.[2] All bacterial experiments were completed using standard aseptic techniques.

Metabolic labeling to assess glycoprotein biosynthesis

H. pylori cells were streaked from frozen stock onto horse blood agar (HBA) plates (4% Columbia agar base, 5% horse blood, 10 μg/mL of vancomycin, 5 μg mL^{-1} cefsulodin, 0.3 μg/mL polymyxin B, 5 μg/mL trimethoprim, and 8 μg/mL amphotericin B) and grown for 3-4 days under microaerophilic conditions (14% CO_2, 37°C) until confluent growth was observed. Bacteria were inoculated in Brucella broth supplemented with 10% fetal bovine serum and 10 μg/mL vancomycin at OD_{600} = 0.4–0.5. Cultures were supplemented with 0.5 mM $Ac_4GlcNAz$,[3] with 0.5 mM $Ac_4GlcNAz$ alongside 0.1 – 2.0 mM **1**, **2**, **3,** or with 0.5 mM of the azide-free control $Ac_4GlcNAc$ for 3-4 days under the same conditions. Cells were then harvested, rinsed with phosphate-buffered saline (PBS), and prepared for Western blot as described below.

The horse blood agar plates were prepared in house using Oxoid Columbia agar base supplemented with 5% laked horse blood (made by LAMPIRE Biological Laboratories and sold from Fisher Scientific Laboratories).

Coomassie/western analysis

Following metabolic labeling, cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 1% Igepal, 150 mM NaCl, 1 mM EDTA, protease inhibitor cocktail from MilliporeSigma) and resultant protein lysates were standardized (BioRad's DC protein concentration assay) to a protein concentration of ~2.5 mg mL^{-1} prior to a 1:1 reaction with 500 μM Phos-FLAG overnight at room temperature. Reacted lysates were loaded onto a 12% Tris–HCl SDS-PAGE gel and separated by electrophoresis. Gels were either transferred to nitrocellulose for FLAG detection using anti-FLAG-HRP and chemiluminescence or stained with Coomassie blue. Coomassie-stained gels were destained until bands were visible and photographed to assess protein loading.

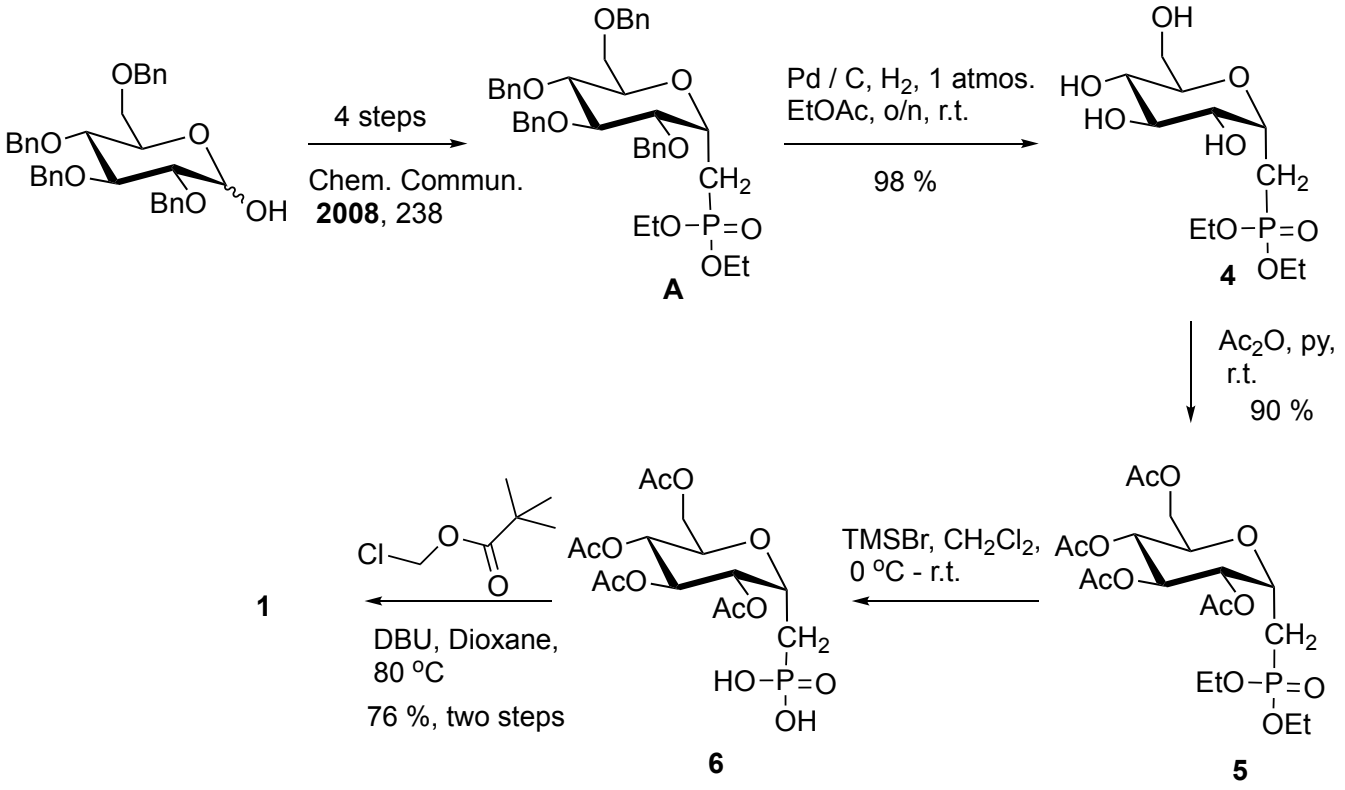
Growth curve assay

H. pylori was cultured on horse blood agar plates (4% Columbia agar, 5% horse blood, 10 mg/ml vancomycin, 5 μg/ml cefsulodin, 0.3 μg/ml polymyxin B, 5 μg/ml trimethoprim, and 8 μg/ml amphotericin B). Plates were incubated for 3-4 days under microaerophilic conditions (14% CO_2) at 37°C. Cells were then inoculated into Brucella broth at an initial optical density (OD_{600}) of ~0.1. Cultures were incubated with or without carbohydrate phosphonates inhibitors under microaerophilic conditions with gentle agitation. OD_{600} measurements were taken daily over a period of 8 days using a SPECTROStar Nano 96-well plate reader (Thermo Fisher Scientific).

General synthetic experimental details

All reactions without water as a solvent were carried out under an inert atmosphere of argon in oven-dried glassware. All reagents were used directly as commercial reagent grade without further purification prior to use and purchased from Sigma-Aldrich and Astatech. Analytical thin-layer chromatography (TLC) was performed on Silicycle aluminum-backed ultrapure silica gel TLC plates, monitored by an ultraviolet lamp (UV) and/ or developed with potassium permanganate. Column chromatography with

silica gel was performed using the dry-load technique with celite absorbent from Sigma-Aldrich. Neutral alumina column chromatography was performed using neutral aluminum columns from Hawach, 25 g cartridges while adhering to recommended flow rates, 25 mL/minute. HPLC grade methanol and 1mM phosphate buffer (pH 7.4) in deionized water were used as the weak and strong solvents, respectively. NMR spectra were recorded on Bruker instruments within the NMR3 centre, Dalhousie University. Mass spectrometry data were collected on instruments within the Department of Chemistry, Dalhousie University. ^{1}H nuclear magnetic resonance (NMR) spectra were recorded at 300 or 500 MHz, ^{13}C NMR spectra were recorded at 125 MHz and ^{31}P NMR were recorded at 202 MHz. Chemical shift values are reported in parts per million, and coupling constants are reported in hertz. ^{1}H multiplicity, as observed in 1D ^{1}H NMR spectra, is reported using the abbreviations s = singlet, bs = broad singlet, d = doublet,, dd = doublet of doublets, t = triplet, q = quartet, m = multiplet. Structural and stereochemical assignments, where required, were made using correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC) and heteronuclear multiple bond correlation (HMBC) 2D NMR experiments. Electrospray ionization (ESI) mass spectra were collected in both positive and negative modes and are reported as the observed molecular ion. Mass spectra were acquired using a Bruker microTOF Focus Mass Spectrometer, using an ESI (+ or -) - ionization source.



The compound **A** was synthesized according to a literature procedure in three steps from 2,3,4,6-tetra-*O*-benzyl-D-glucopyranose[4]

Diethyl (((2S,3R,4S,5S,6R)-3,4,5-trihydroxy-6-(hydroxymethyl)tetrahydro-2H-pyran-2-yl)methyl)phosphonate (4).

To a solution of **A** (674 mg, 1 mmol) in methanol/ethyl acetate (1:1, 10 mL) palladium hydroxide (600 mg, 20% Pd on carbon) was added. The mixture was degassed three times and each time replacing the vacuum by hydrogen. The reaction mixture, connected to a balloon of hydrogen, was stirred at room temperature for overnight. The catalyst was removed by filtration through a pad of Celite, and the filtrate was concentrated to provide the crude product **4** as a white foam (307 mg, 0.977 mmol, 98%) which was used in subsequent reactions without further purification. ^{1}H NMR (300 MHz, D_2O), δ 1.28 (t, J = 7.1 Hz, 6H), 2,14-2.47 (m, 2H), 3.37-3.73 (m, 6H), 4.11 (q, J = 7.1 Hz, 4H), 4.37-4.41 (m, 1H); ^{13}C NMR (125 MHz, D_2O), δ 16.8 (d, J = 5.9 Hz), 23.1 (d, J = 143.5 Hz), 62.7, 63.3 (d, J = 6.4 Hz), 63.6 (d, J = 6.4 Hz), 71.7, 72.4 (d, J = 13.4 Hz), 72.6 (d, J = 5.2 Hz), 74.8, 75.4; ^{31}P NMR (121 MHz, D_2O), δ 32.6; HRMS-ESI calcd for $C_{11}H_{23}NaO_8P$ $[M + Na]^+$ 337.1028, found 337.1030.

(2R,3R,4S,5R,6S)-2-(Acetoxymethyl)-6-((diethoxyphosphoryl)methyl)tetrahydro-2H-pyran-3,4,5-triyl triacetate (5).

To a solution of **4** (314 mg, 1 mmol) in pyridine (5 mL), Ac_2O (5 mL) was added. The reaction mixture was stirred at room temperature for overnight. The resulting solution was concentrated at 50 °C under high vacuum and the residue purified by column chromatography (hexane-EtOAc, 1:2) to give product **5** as a colorless syrup (433 mg, 0.890 mmol, 90%). ^{1}H NMR (500 MHz, $CDCl_3$), δ 1.31 (td, J = 7.0 Hz, J = 1.6 Hz, 6H), 1.92-1.98 (m, 1H), 2.01 (s, 6H), 2.04 (s, 3H), 2.07 (s, 3H), 2.24-2.33 (m, 1H), 3.96 (dt, J = 9.0 Hz, J = 3.4 Hz, 1H), 4.08-4.15 (m, 5H), 4.29 (dd, J = 12.3 Hz, J = 4.0 Hz, 1H), 4.57-4.59 (m, 1H), 5.03 (t, J = 12.3 Hz, 1H), 5.08 (ddd, J = 9.0 Hz, J = 5.6 Hz, J = 1.9 Hz, 1H), 5.23 (t, J = 9.0, 1H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 16.6 (d, J = 4.3 Hz), 20.73, 20.75, 20.78, 20.83, 24.1 (d, J = 145.4 Hz), 61.8, 61.9 (d, J = 6.3 Hz), 62.4 (d, J = 6.3 Hz), 68.3, 68.5 (d, J = 4.0 Hz), 69.7, 69.7 (d, J = 11.8 Hz), 70.1, 169.4, 169.5, 170.1, 170.7; ^{31}P NMR (202 MHz, $CDCl_3$), δ) 26.8; HRMS-ESI calcd for $C_{19}H_{31}NaO_{12}P$ $[M + Na]^+$ 505.1445, found 505.1448.

(2R,3R,4S,5R,6S)-2-(Acetoxymethyl)-6-((bis((pivaloyloxy)methoxy)phosphoryl)methyl) tetrahydro-2H-pyran-3,4,5-triyl triacetate (6).

Compound **5** (241 mg, 0.5 mmol) was dissolved in anhydrous dichloromethane (10 mL) under nitrogen. The resulting solution was cooled and bromotrimethylsilane (20 equiv., 1.3 ml, 10 mmol) was added dropwise. The solution was stirred for overnight at room temperature, and then solvent was removed *in vacuo*. THF (5 mL) and water (1 mL) were added to residue, and the solution stirred for 30 min at room temperature. THF was removed in vacuo and the remaining aqueous solution and washed with diethyl ether (10 mL). The resulting aqueous solution was concentrated to provide the crude product which was

used in subsequent reactions without further purification. Then the crude product was dissolved in dry dioxane (20 mL), and DBU (2 equiv., 304 mg, 2 mmol) was added and subsequently POM-Cl (4 equiv., 602 mg, 4 mmol) was added and the solution was refluxed for overnight. The solids were filtrated and after evaporation of solvent, the residue was purified by flash column chromatography (hexanes-EtOAc, 4:1) to afford the title product **1** as a colorless oil (249 mg, 0.380 mmol, 76%); ^{1}H NMR (500 MHz, $CDCl_3$), δ 1.35 (s, 18H), 2.04 (s, 6H), 2.07 (s, 3H), 2.10 (s, 3H), 2.15-2.20 (m, 1H), 2.38-2.44 (m, 1H), 3.97 (dt, *J* = 9.2 Hz, *J* = 3.7 Hz, 1H), 4.16 (dd, *J* = 12.4 Hz, *J* = 2.8 Hz, 1H), 4.30 (dd, *J* = 12.4 Hz, *J* = 4.1 Hz, 1H), 4.59-4.62 (m, 1H), 5.05 (t, *J* = 8.9 Hz, 1H), 5.12-5.13 (m, 1H), 5.27 (t, *J* = 8.9, 1H), 5.69-5.72 (m, 4H); ^{13}C NMR (125 MHz, $CDCl_3$), δ 20.7, 20.8, 20.9, 24.9 (d, *J* = 145.3 Hz), 27.0, 38.9, 61.8, 68.2, 68.3, 69.4 (d, *J* = 13.4 Hz), 70.0 (d, *J* = 17.8 Hz), 81.7 (d, *J* = 5.9 Hz), 81.9 (d, *J* = 5.6 Hz), 169.4, 169.5, 170.1, 170.8, 177.0, 177.1; ^{31}P NMR (202 MHz, $CDCl_3$), δ) 27.3; HRMS-ESI calcd for $C_{27}H_{43}NaO_{16}P$ $[M+Na]^+$ 677.2181, found 677.2179.

NMR data

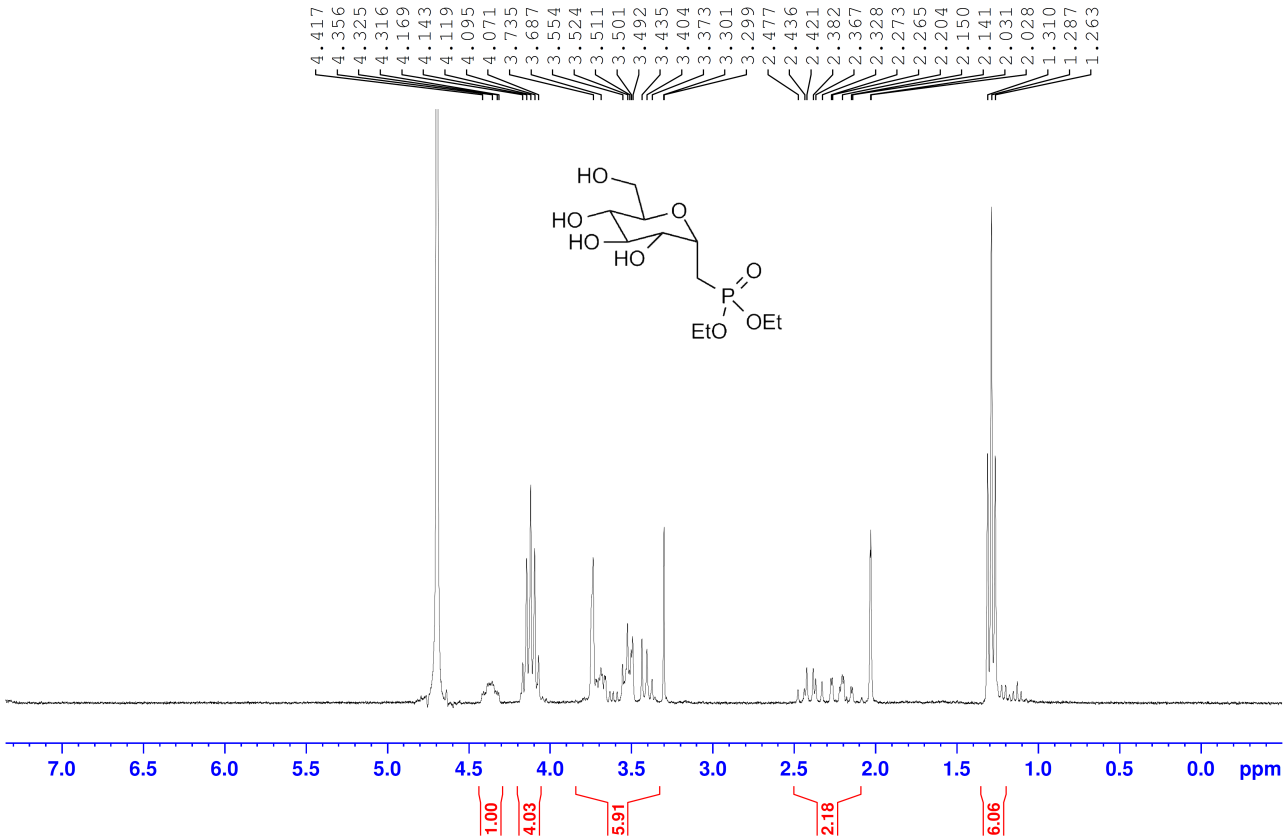


Figure S1. 1H NMR spectrum (300 MHz, D2O) of 4.

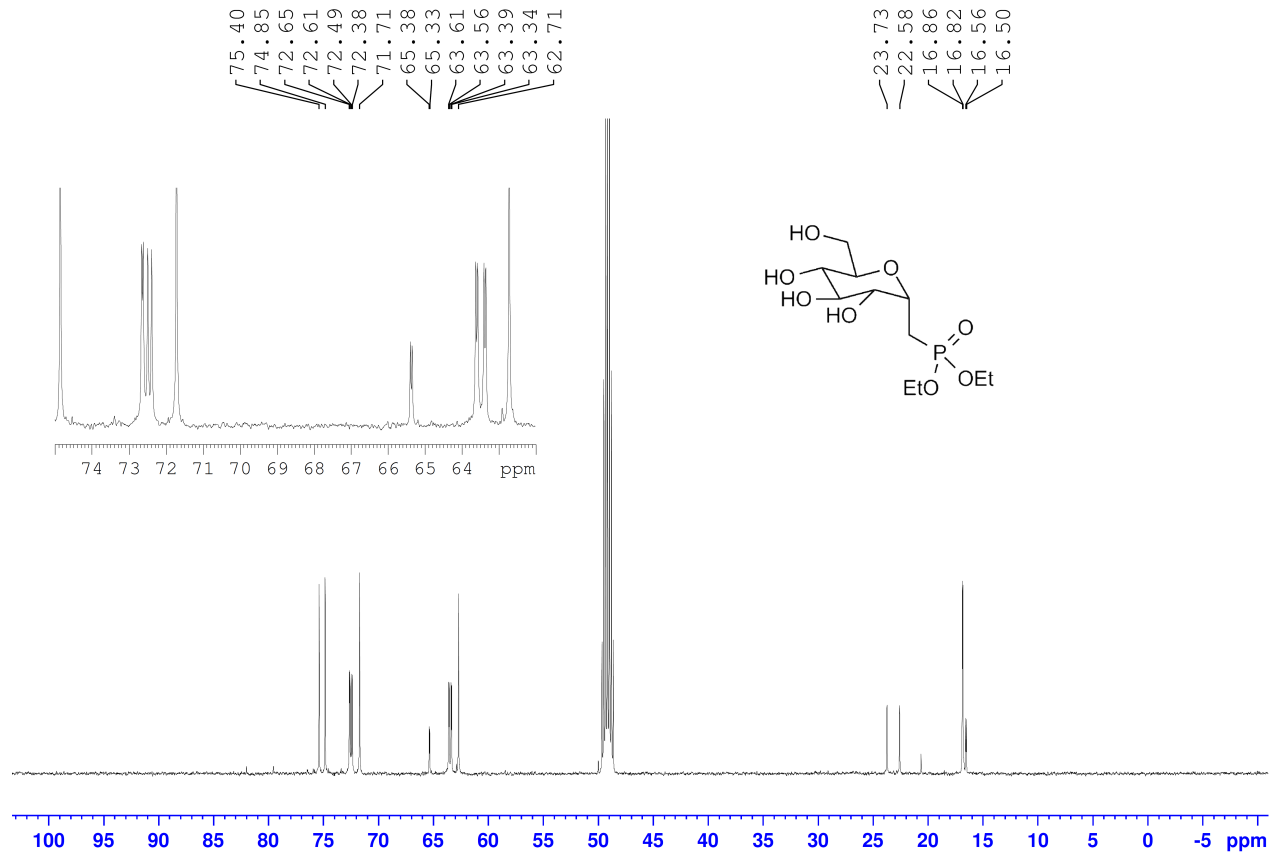


*Figure S2. ^{13}C NMR spectrum (125 MHz, D_2O) of **4**.*

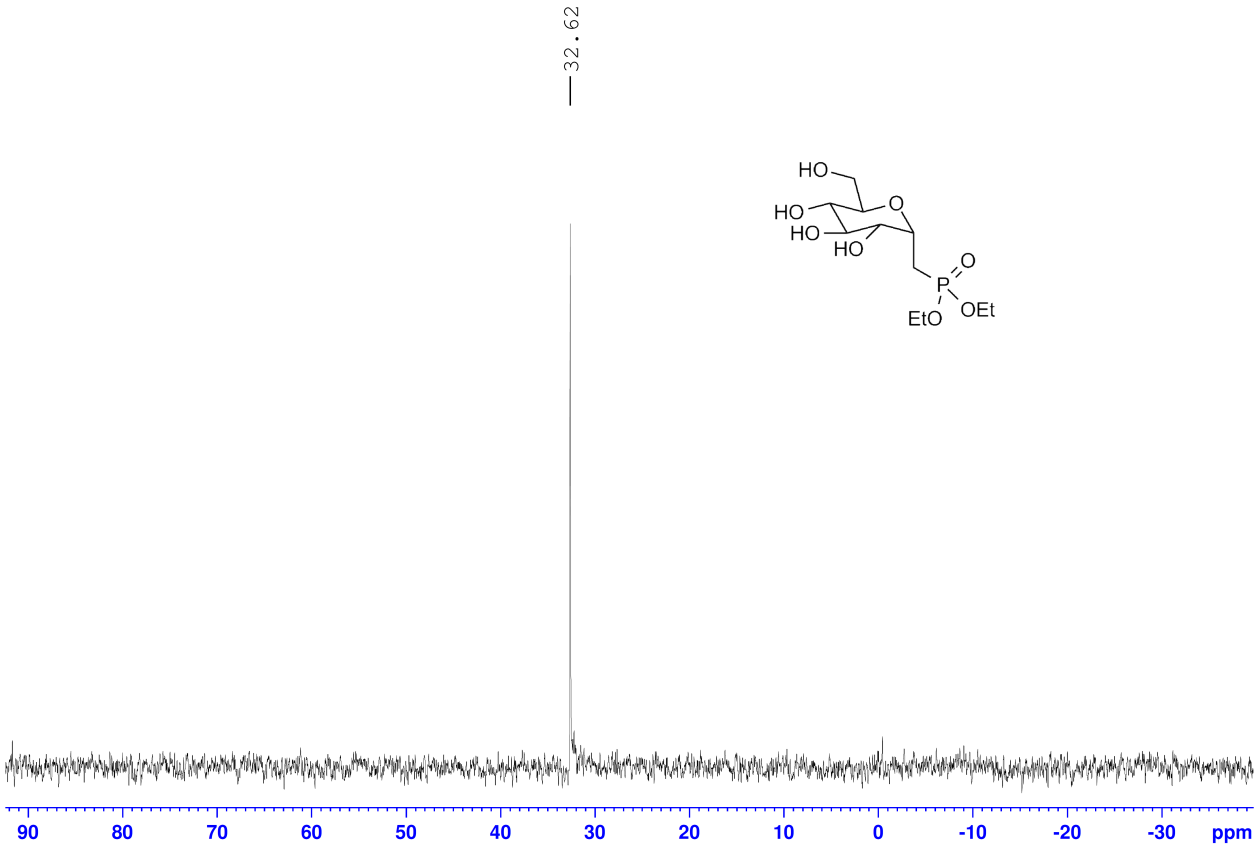


*Figure S3. ^{31}P NMR spectrum (121 MHz, D_2O) of **4**.*

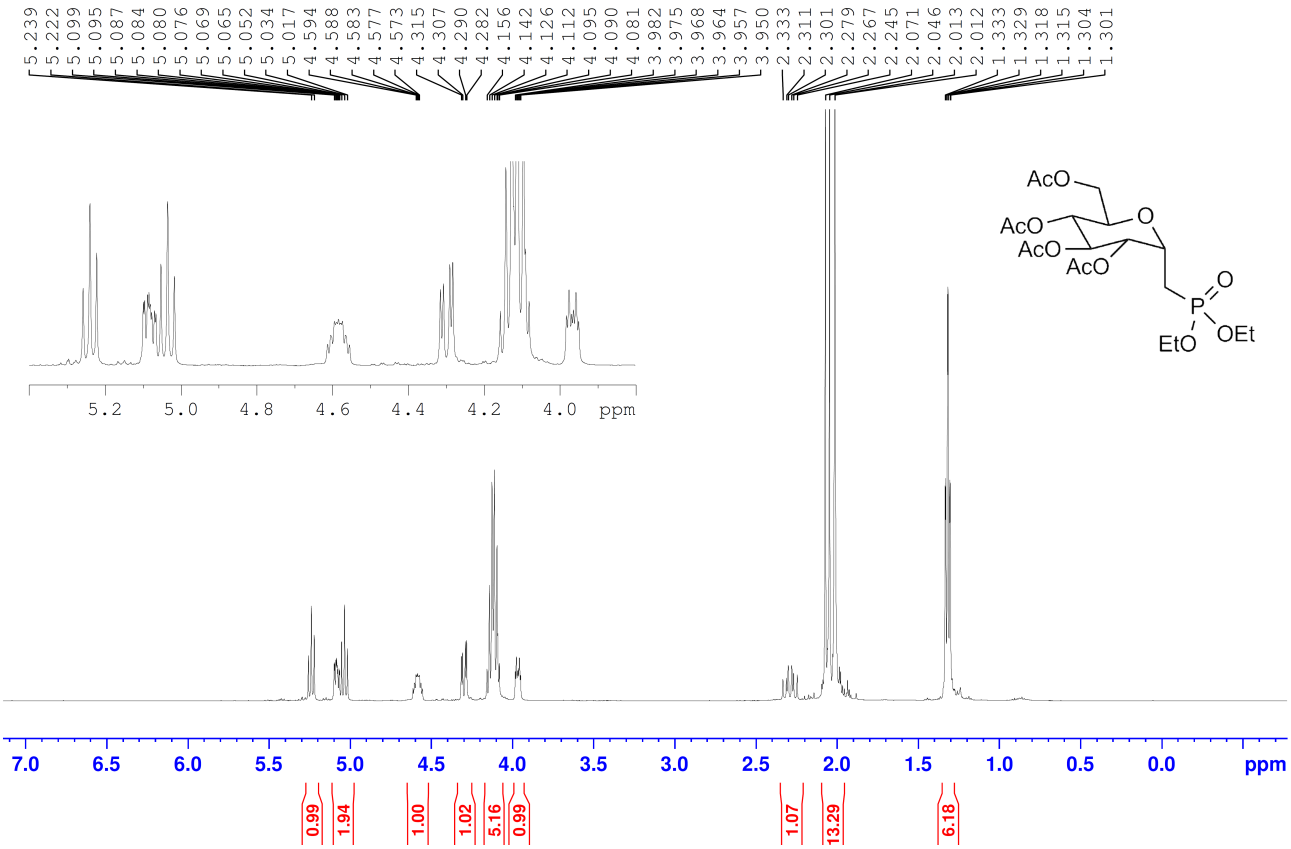


*Figure S4. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **5**.*

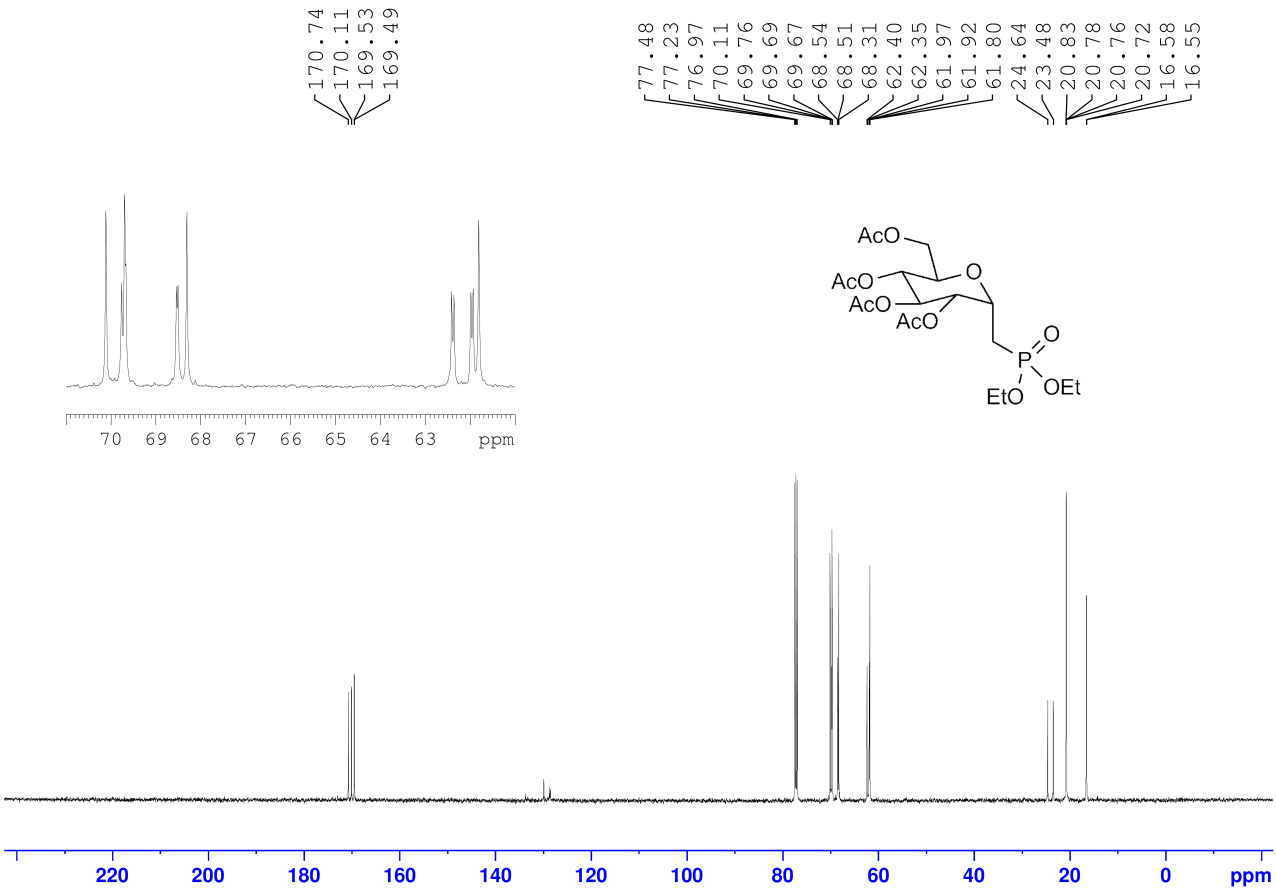


*Figure S5. ^{13}C NMR spectrum (500 MHz, $CDCl_3$) of **5**.*

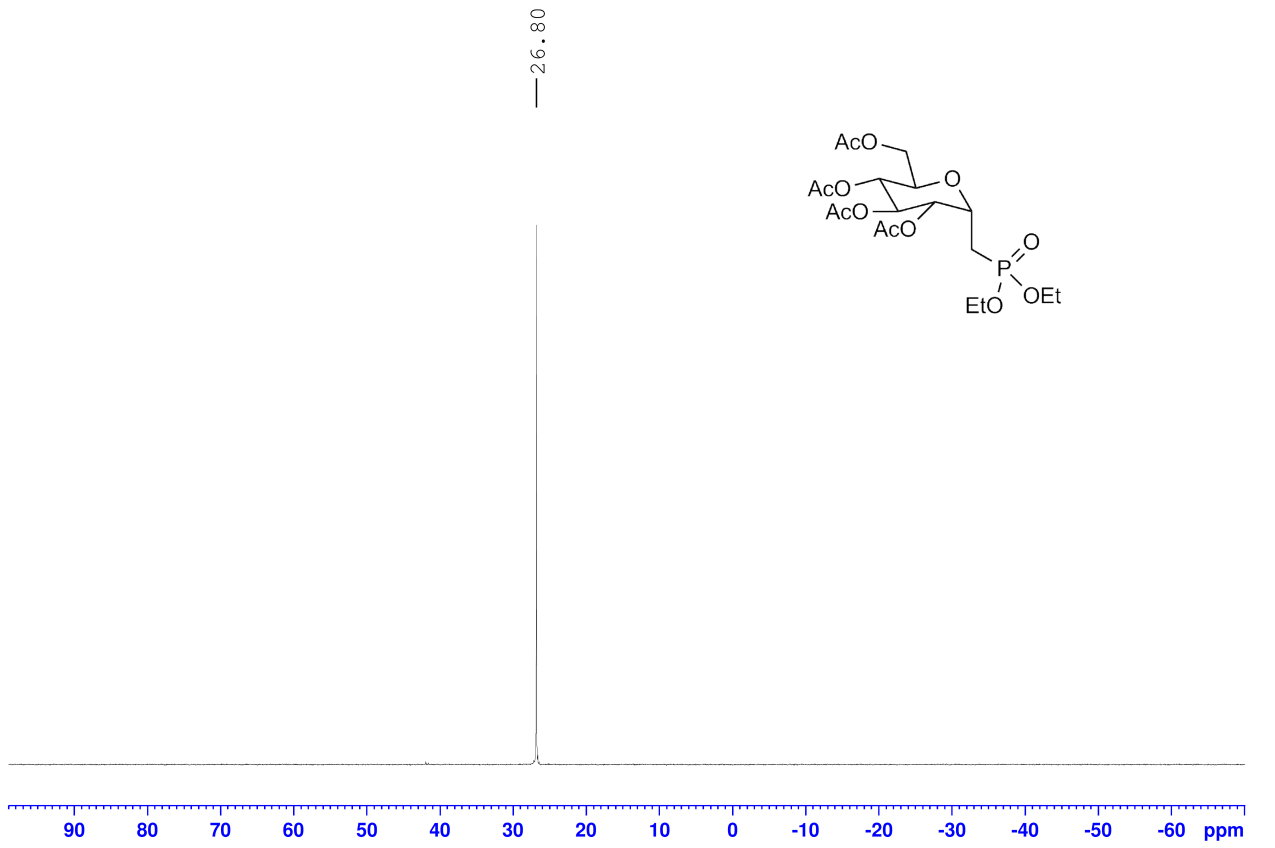


*Figure S6. ^1H NMR spectrum (500 MHz, $CDCl_3$) of **5***

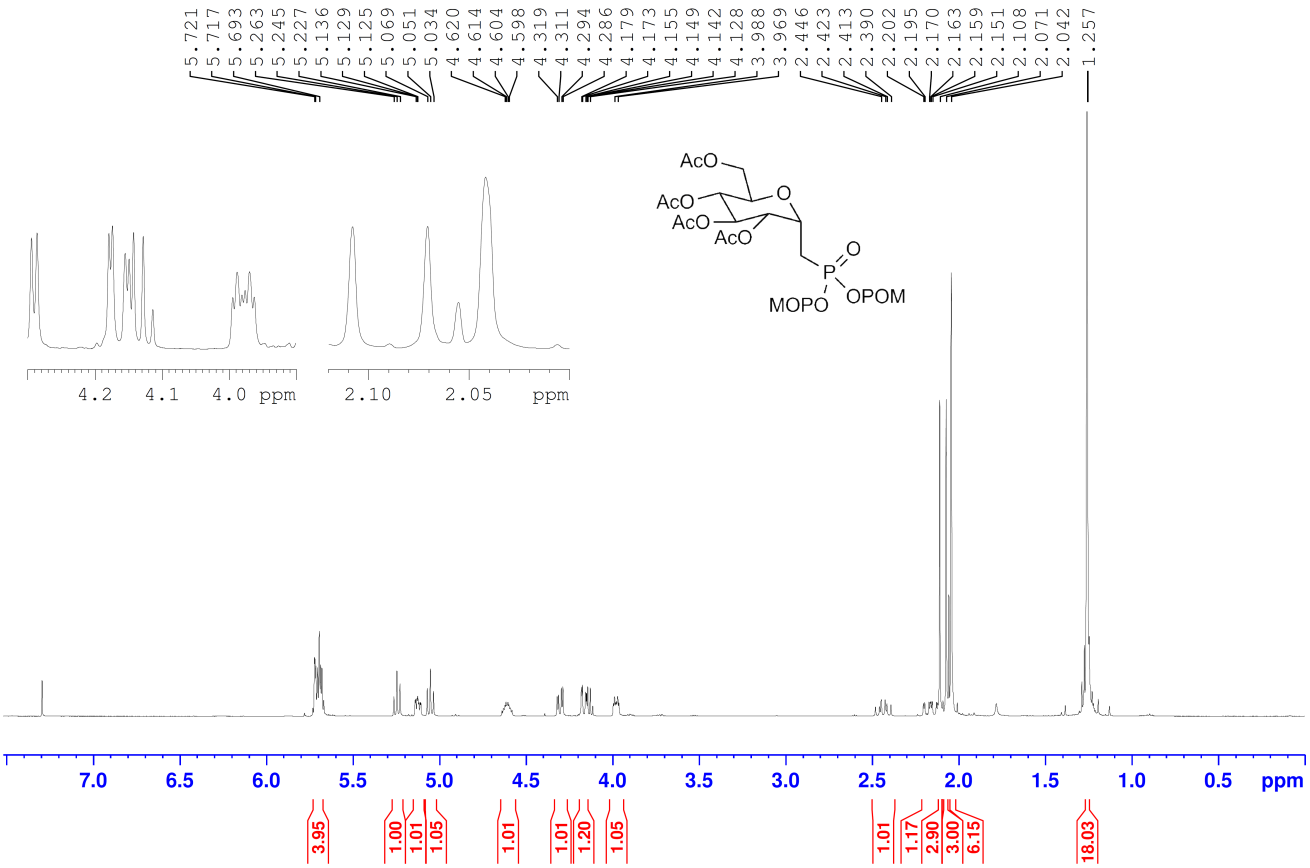


*Figure S7. ^1H NMR spectrum (500 MHz, $CDCl_3$) of **6**.*

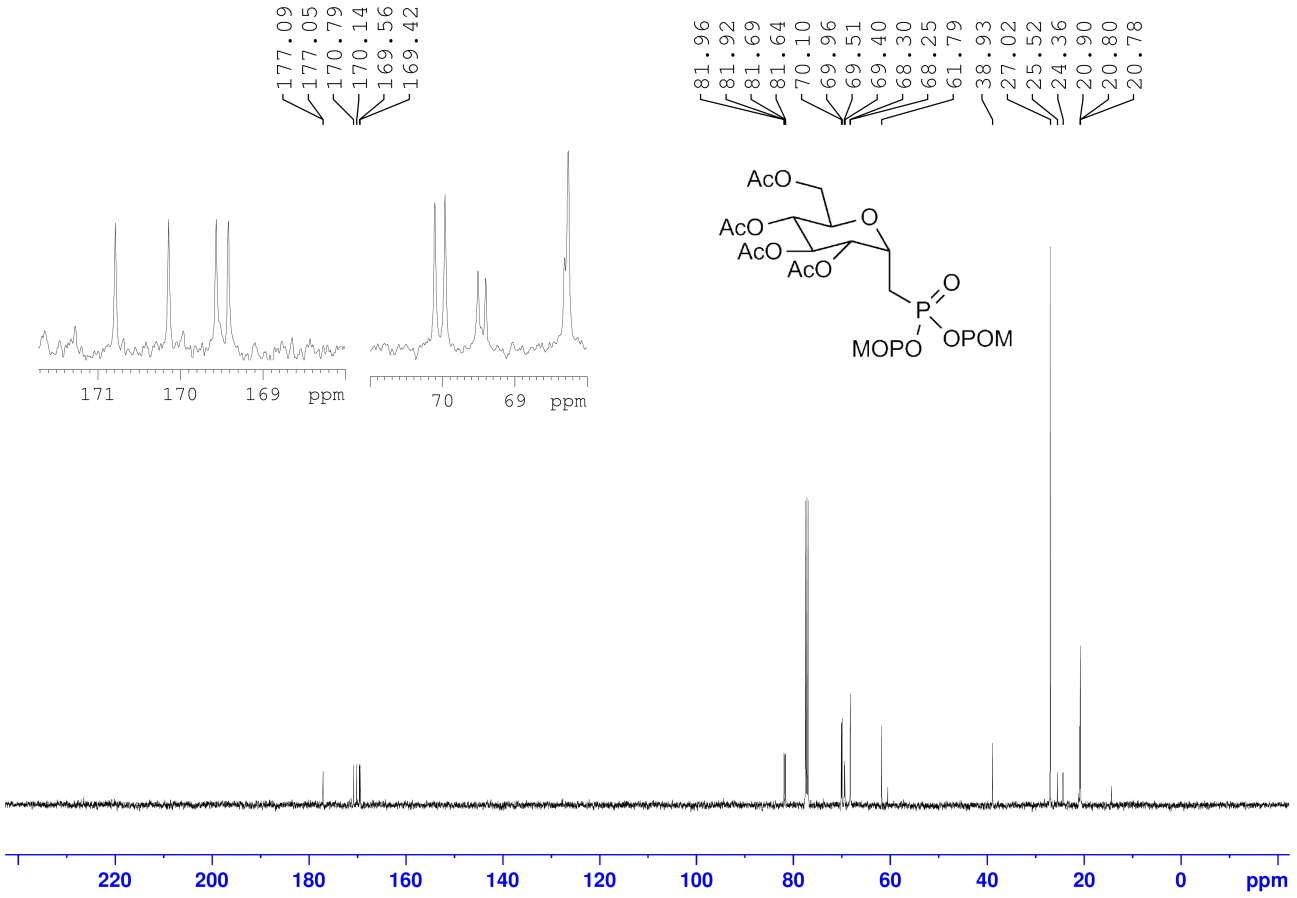


*Figure S8. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **6**.*

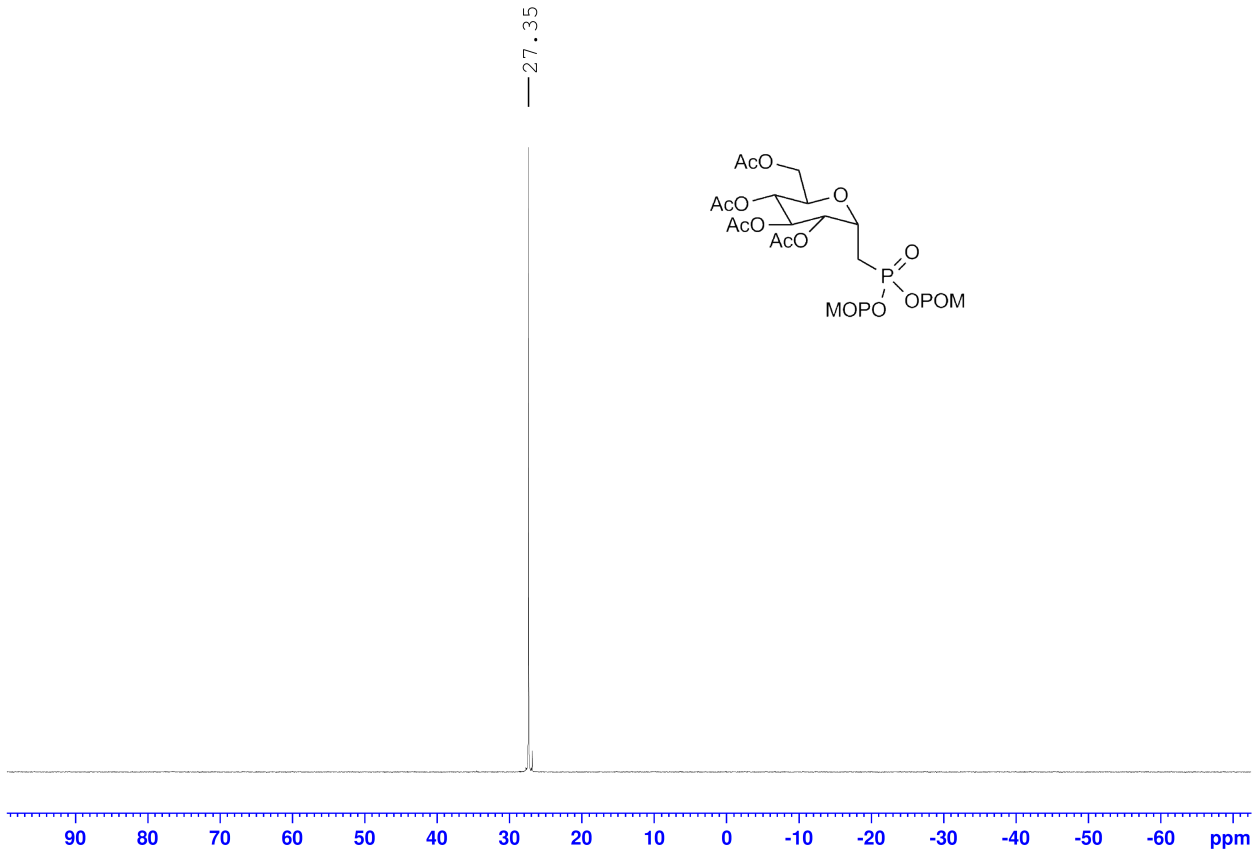


*Figure S9. ^{1}H NMR spectrum (500 MHz, $CDCl_3$) of **6**.*

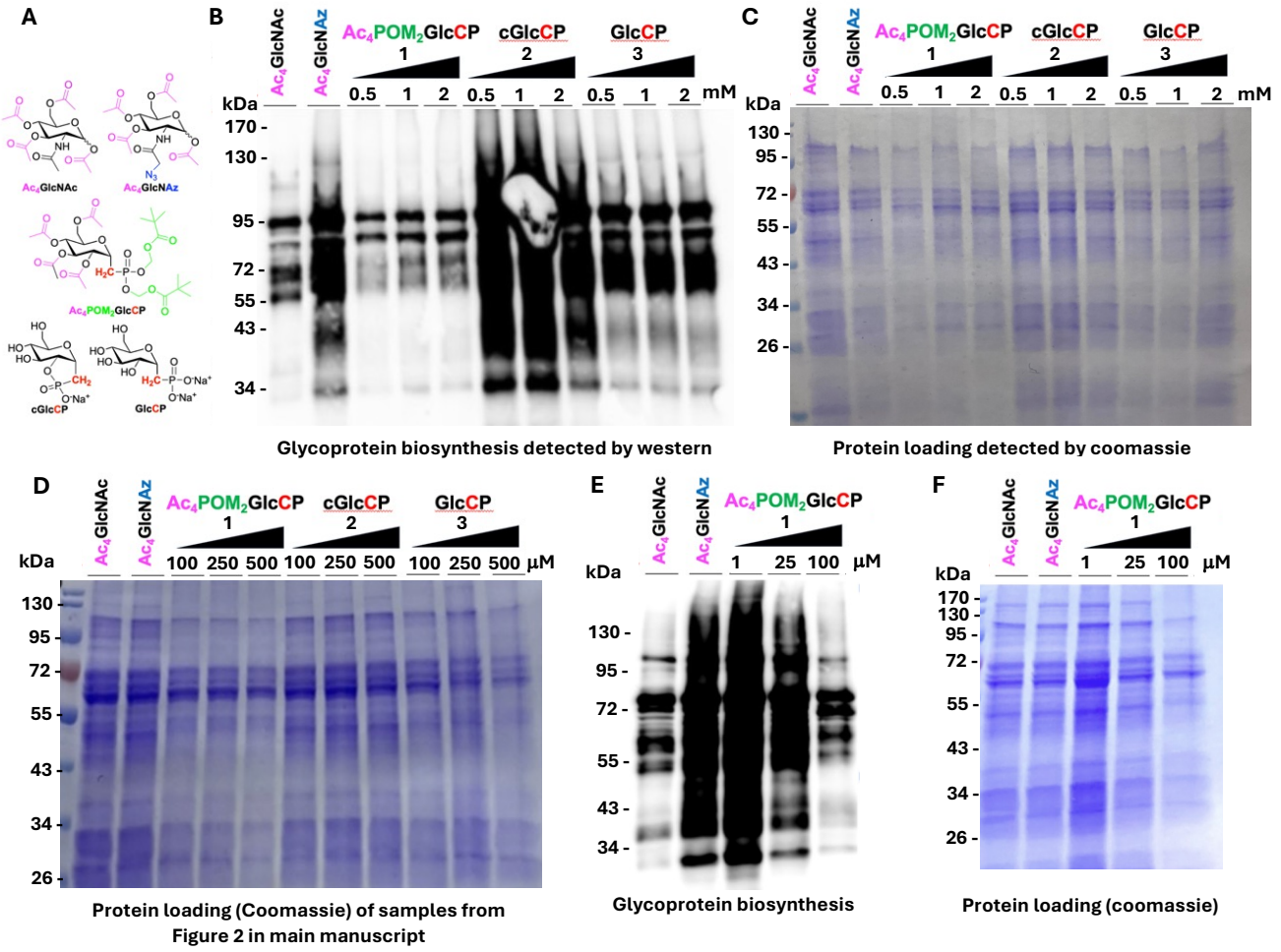


*Figure S10. Inhibition of masked phosphonate **1** and corresponding protein loading controls.*

A) Compounds used in this study. B) Western analysis revealed compound 1 exhibited H. pylori glycoprotein biosynthesis inhibition, yet 2 and 3 exhibited minimal effects even at millimolar concentrations. C) Coomassie staining for samples analyzed by western in (B) had equivalent protein concentrations. D) Protein loading controls stained by Coomassie for samples from Figure 2 in main manuscript had roughly equivalent protein levels. E) Western analysis revealed compound 1 exhibited concentration-dependent inhibition of H. pylori glycoprotein biosynthesis at micromolar levels. F) Coomassie staining for samples analyzed by western in (E) had equivalent protein concentrations. These data are representative of replicate experiments.

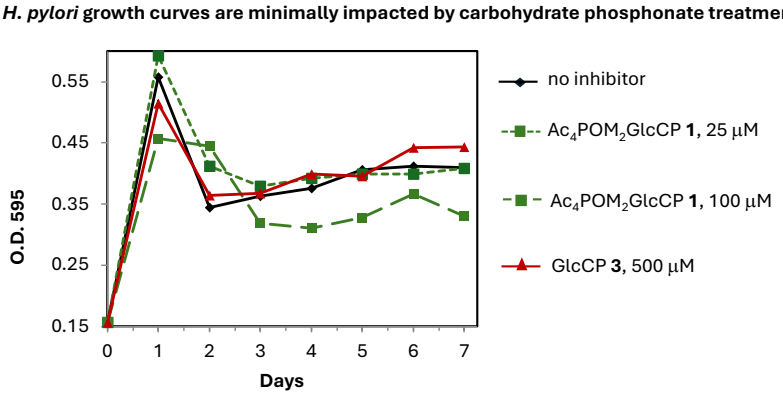


Figure S11. H. pylori growth curves are minimally impacted by carbohydrate phosphonate treatment.

H. pylori were inoculated in rich media containing no inhibitor or phosphonates 1 or 3 at the indicated concentrations. Optical density at 595 nm was monitored daily. Growth of treated cells was similar to growth of untreated cells; 25 mM of Ac4POM2GlcCP 1 had no appreciable effect on growth and 100 mM of Ac4POM2GlcCP 1 led to a very subtle growth suppression relative to untreated controls. These data are representative of replicate experiments.

References

(1) Baltrus, D. A.; Amieva, M. R.; Covacci, A.; Lowe, T. M.; Merrell, D. S.; Ottemann, K. M.; Stein, M.; Salama, N. R.; Guillemin, K. The Complete Genome Sequence of Helicobacter Pylori Strain G27. *J. Bacteriol.* **2009**, *191* (1), 447–448.

(2) Laughlin, S. T.; Bertozzi, C. R. Metabolic Labeling of Glycans with Azido Sugars and Subsequent Glycan-Profiling and Visualization via Staudinger Ligation. *Nat. Protoc.* **2007**, *2* (11), 2930–2944.

(3) Koenigs, M. B.; Richardson, E. A.; Dube, D. H. Metabolic Profiling of *Helicobacter Pylori* Glycosylation. *Mol. BioSyst.* **2009**, *5* (9), 909.

(4) Beaton, S. A.; Huestis, M. P.; Sadeghi-Khomami, A.; Thomas, N. R.; Jakeman, D. L. Enzyme-Catalyzed Synthesis of Isosteric Phosphono-Analogues of Sugar Nucleotides. *Chem. Commun.* **2009**, 238–240.