

Supporting Information

Synthesis and characterization of non-hydrolysable diphosphoinositol polyphosphate second messengers

Mingxuan Wu, Barbara E. Dul, Alexandra J. Trevisan, Dorothea Fiedler*

Department of Chemistry, Princeton University, Princeton, NJ, 08544, USA

dfiedler@princeton.edu

Table of Contents

I. General Information.....	S-2
II. Chemical Synthesis	S-3
III. NMR Titration.....	S-10
IV. Inhibition of Akt T308 Phosphorylation	S-10
V. Inhibition of Ddp1 Activity.....	S-10
VI. Supporting Scheme and Figures.....	S-11
VII. NMR spectra.....	S-14
VIII. References.....	S-36

I. General Information

Commercial chemicals were purchased from Sigma–Aldrich or Alfa Aesar and used without further purification. Dichloromethane, tetrahydrofuran (THF) and toluene were dried by passing through activated alumina columns; acetonitrile was dried by passing through a column of activated molecular sieves¹. Thin layer chromatography (TLC) was performed on EMD Silica Gel 60 F₂₅₄ plates and visualized by fluorescence quenching or KMnO₄ staining.

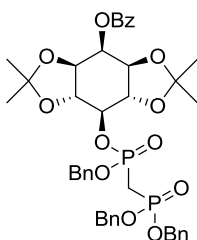
Automated column chromatography was performed using SiliCycle SiliaFlash F60 (40–53 μm) in RediSep[®] Rf cartridges and normal–phase silica flash columns on a CombiFlash[®] Rf from Teledyne Isco. High–performance liquid chromatography (HPLC) was performed on a Varian instrument with SD–1 analytical to prep solvent delivery modules, a ProStar 325 UV–Vis detector and a 440–LC fraction collector, using Water XBridge[™] 5 μm C18 columns. Protected inositol intermediates were purified on a preparative column (19 × 150 mm) using method A (50% CH₃CN in H₂O for 2 min and a linear gradient from 50% CH₃CN to 90% CH₃CN over 15 min, flow rate: 20 mL/min) or method B (40% CH₃CN in H₂O for 2 min and a linear gradient from 40% CH₃CN to 90% CH₃CN over 15 min, flow rate: 20 mL/min) at an absorbance of 220 nm. Ap₅A hydrolysis by Ddp1 was monitored on an analytical column (3.0 × 150 mm) using method C (0.1 M KH₂PO₄/K₂HPO₄ (pH = 5.5) in H₂O over 6 min, flow rate: 1 mL/min) at an absorbance of 259 nm.

¹H, ¹³C and ³¹P NMR spectra were recorded on a Bruker 500 AVANCE spectrometer (500, 125 and 202 MHz respectively). NMR data are reported as follows: chemical shift (δ ppm), multiplicity (s = singlet, d = doublet, t = triplet, q = quartet, app. t = apparent triplet, dd = doublet of doublet, td = triplet of doublet, tq = triplet of quartet, m = multiplet), coupling constant (Hz) and integration. Protons at the 2-position of the inositol ring in compounds **1**, **2**, **3** and **4** (pD < 8.8) were obscured by the water peak and could not be reported. Deuterium chloride or sodium deuterioxide was added to adjust the pD for NMR spectra of compound **1**, **2** and **4**. High resolution mass spectra (HRMS) were obtained on an Agilent 6220 using electrospray ionization time-of-flight (ESI-TOF). 3% H₂O in CH₃CN with 0.1% formic acid was used for positive ion detection mode and 3% CH₃CN in H₂O with 0.1% formic acid was used for negative ions.

NMR samples of compound **2** at pD = 2.0, pD = 6.9 and pD = 13.0 were kept at room temperature and continuously monitored for decomposition products over a period of 40 days.

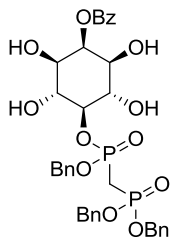
PDK1 and inactive Akt1 were purchased from Merck Millipore. Antibodies for Akt and p-Akt (T308) were purchased from Cell Signaling Technology.

II. Chemical Synthesis



2-*O*-Benzoyl-5-*O*-[[bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-1,6:3,4-di-*O*-propylidene-*myo*-inositol (**6a**).

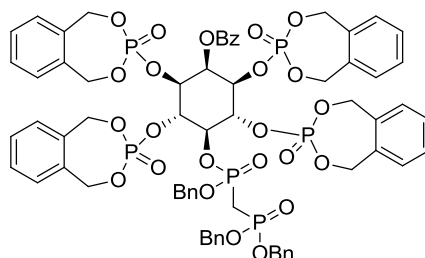
A solution of [bis(benzyloxy)phosphorylmethyl]phosphonic acid monobenzyl ester² (446 mg, 0.999 mmol) in toluene (8 mL) was treated with oxalyl chloride (172 μ L, 254 mg, 2.00 mmol) and a catalytic amount of anhydrous DMF at 0 °C for 1h. The crude phosphorochloridate was obtained after the mixture was filtered and dried under vacuum. Potassium bis(trimethylsilyl)amide (2.0 mL, 0.5 M in toluene, 1.0 mmol) was added over 4 min to another flask containing the protected inositol **5**³ (240 mg, 0.659 mmol) in THF (20 mL) at -78 °C. After 30 min, the crude phosphorochloridate in THF (5 mL) was added over 10 min, and the reaction mixture was stirred overnight, allowing it to warm to room temperature. After quenching with sat. aq. NH₄Cl (5 mL) and water (10 mL), the mixture was extracted by EtOAc (25 mL \times 3) and dried over Na₂SO₄. The organic phase was removed under vacuum and the residue was purified by flash chromatography (0% to 3% MeOH in CH₂Cl₂) to give the title compound (368 mg, 70%) as a white solid. ¹H NMR (500 MHz, CDCl₃): δ 8.02 (d, J = 7.2 Hz, 2H), 7.60 (t, J = 7.4 Hz, 1H), 7.47 (t, J = 7.8 Hz, 2H), 7.42–7.40 (m, 2H), 7.32–7.23 (m, 13H), 6.00 (t, J = 2.0 Hz, 1H), 5.25 (dd, J = 11.6, 7.3 Hz, 1H), 5.17 (dd, J = 11.6, 6.8 Hz, 1H), 5.05–4.97 (m, 4H), 4.90 (q, J = 9.3 Hz, 1H), 4.18 (td, J = 9.5, 5.5 Hz, 2H), 3.78 (td, J = 8.8, 2.0 Hz, 2H), 2.81 (tq, J = 21.5, 15.6 Hz, 2H), 1.42 (s, 3H), 1.40 (s, 3H), 1.32 (s, 3H), 1.30 (s, 3H); ¹³C NMR (125 MHz, CDCl₃): δ 165.3, 136.2 (d, J = 6.6 Hz), 136.1 (d, J = 6.6 Hz), 136.0 (d, J = 7.9 Hz), 133.5, 129.8, 129.6, 128.69, 128.65, 128.61, 128.56, 128.52, 128.51, 128.46, 128.37, 128.02, 127.99, 113.44, 113.38, 77.1 (d, J = 2.8 Hz), 77.0 (d, J = 3.6 Hz), 76.74, 76.71, 73.6 (d, J = 5.6 Hz), 68.2 (d, J = 6.2 Hz), 67.9 (d, J = 6.1 Hz), 67.7 (d, J = 6.3 Hz), 64.2, 26.90, 26.89, 26.43, 26.41, 26.2 (app. t, J = 136.6 Hz); ³¹P NMR (202 MHz, CDCl₃): δ 20.05 (d, J = 9.6 Hz, 1P), 19.70 (d, J = 9.6 Hz, 1P); HRMS [M+H]⁺ calcd for C₄₁H₄₇O₁₂P₂⁺ 793.2538, found 793.2535.



2-*O*-Benzoyl-5-*O*-[[bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-*myo*-inositol (**7a**).

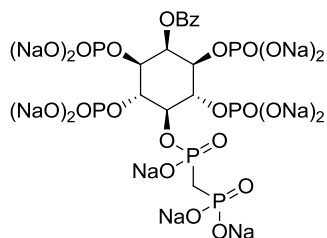
Water (68 μ L, 68 mg, 3.8 mmol) and *p*-toluenesulfonic acid monohydrate (14 mg, 0.074 mmol) were added to a solution of the acetonide **6a** (300 mg, 0.378 mmol) in acetone (15 mL). After stirring overnight, the mixture was dried under vacuum and purified by flash chromatography (2% to 10% MeOH in CH₂Cl₂) to give the title compound (178 mg, 66%) as a colorless oil. ¹H NMR (500 MHz,

CDCl₃): δ 8.01 (d, *J* = 8.0 Hz, 2H), 7.51 (t, *J* = 7.4 Hz, 1H), 7.37 (t, *J* = 7.7 Hz, 2H), 7.31–7.28 (m, 13H), 7.20–7.18 (m, 2H), 5.81 (t, *J* = 2.5 Hz, 1H), 5.73 (d, *J* = 3.4 Hz, 1H), 5.17 (d, *J* = 8.1 Hz, 2H), 5.03 (dd, *J* = 11.6, 8.4 Hz, 1H), 4.96 (dd, *J* = 11.6, 8.4 Hz, 1H), 4.87 (d, *J* = 8.6 Hz, 2H), 4.51 (d, *J* = 4.6 Hz, 1H), 4.34 (q, *J* = 9.1 Hz, 2H), 4.05 (td, *J* = 9.6, 3.4 Hz, 1H), 3.98 (td, *J* = 9.6, 4.6 Hz, 1H), 3.81 (t, *J* = 10.7 Hz, 2H), 3.70 (s, 1H), 3.45 (s, 1H), 2.58, (tq, *J* = 21.1, 15.4 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃): δ 166.6, 135.6 (d, *J* = 7.0 Hz), 135.5 (d, *J* = 6.0 Hz), 135.4 (d, *J* = 6.4 Hz), 133.3, 130.02, 129.96, 128.87, 128.81, 128.75, 128.5, 128.4, 128.3, 128.2, 82.7 (d, *J* = 8.0 Hz), 70.4, 72.5 (d, *J* = 2.3 Hz), 72.3 (d, *J* = 3.6 Hz), 70.7, 70.2, 68.9 (d, *J* = 6.4 Hz), 68.7 (d, *J* = 6.5 Hz), 68.5 (d, *J* = 6.6 Hz), 25.9 (dd, *J* = 141.2, 136.8 Hz); ³¹P NMR (202 MHz, CDCl₃): δ 21.72 (d, *J* = 7.1 Hz, 1P), 20.22 (d, *J* = 7.1 Hz, 1P); HRMS [M+H]⁺ calcd for C₃₅H₃₉O₁₂P₂⁺ 713.1911, found 713.1913.



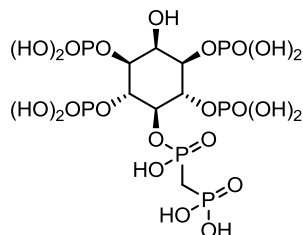
2-O-Benzoyl-5-O-[[bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-1,3,4,6-tetra-O-(o-xylylene)phosphate-myoinositol (8a).

N,N-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine (212 mg, 0.886 mmol) in CH₃CN and 1*H*-tetrazole (2.5 mL, 0.45 M in CH₃CN, 1.1 mmol) were added to a solution of the alcohol **7a** (79 mg, 0.11 mmol) in CH₃CN (10 mL) at 0 °C. The mixture was stirred for 2 h at 0 °C followed by 36 h at room temperature. After the mixture was cooled in a salted ice bath, a solution of mCPBA (298 mg, 1.33 mmol) in CH₃CN (3 mL) was added over 6 min. The mixture was stirred for 1 h at 0 °C followed by 2 h at room temperature. 10% Na₂S₂O₃ (25 mL) was added to quench the reaction and the organic CH₃CN was removed under vacuum. EtOAc (50 mL) was added to the mixture and the organic layer was washed by sat. aq. NaHCO₃ (25 mL) and sat. aq. NaCl (25 mL). The organic layer was dried over Na₂SO₄, concentrated under vacuum and purified by flash chromatography (0% to 5% MeOH in CH₂Cl₂) and preparative HPLC (Method B, *t*_R = 13.5 min) to give the title compound (46 mg, 32%) as a white solid. ¹H NMR (500 MHz, CDCl₃): δ 8.06 (d, *J* = 7.3 Hz, 2H), 7.59 (t, *J* = 7.4 Hz, 1H), 7.46 (t, *J* = 7.6 Hz, 2H), 7.34–7.19 (m, 29H), 6.95 (d, *J* = 7.0 Hz, 1H), 6.84 (d, *J* = 7.4 Hz, 1H), 6.34 (t, *J* = 2.4 Hz, 1H), 5.46 (dd, *J* = 13.9, 10.2 Hz, 1H), 5.38–4.90 (m, 25H), 4.77 (q, *J* = 9.8 Hz, 1H), 3.05 (tq, *J* = 21.8, 15.6 Hz, 2H); ¹³C NMR (125 MHz, CDCl₃): δ 165.1, 136.2 (d, *J* = 8.6 Hz), 136.1 (d, *J* = 6.9 Hz), 136.0 (d, *J* = 6.4 Hz), 135.17, 135.11, 135.06, 135.03, 134.94, 134.85, 134.2, 128.0, 129.34, 129.30, 129.2, 129.02, 128.97, 128.85, 128.78, 128.64, 128.59, 128.51, 128.49, 128.28, 128.27, 128.19, 128.11, 128.07, 76.6, 74.44, 74.40, 74.01, 73.97, 73.93, 73.89, 70.3, 69.5, 69.42, 69.40, 69.35, 69.25 (d, *J* = 7.4 Hz), 69.01, 68.99, 68.95, 68.8 (d, *J* = 6.5 Hz), 68.3 (d, *J* = 6.1 Hz), 67.8 (d, *J* = 6.2 Hz), 26.1 (app. t, *J* = 147.4 Hz); ³¹P NMR (202 MHz, CDCl₃): δ 20.92 (s, 1P), 20.39 (s, 1P), -1.09 (s, 1P), -1.98 (s, 1P), -3.25 (s, 1P), -3.56 (s, 1P); HRMS [M+H]⁺ calcd for C₆₇H₆₇O₂₄P₆⁺ 1441.2442, found 1441.2443.



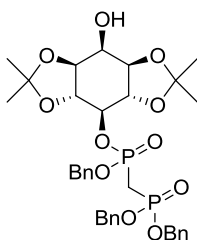
2-O-Benzoyl-myoinositol 1,3,4,6-tetra-phosphate-5-methylenediphosphate undecabasic sodium salt (4).

NaHCO₃ (14 mg, 0.16 mmol) and palladium black (34 mg, 0.32 mmol) were added to a solution of **8a** (21 mg, 0.015 mmol) in *t*-BuOH/H₂O (40:7, 3 mL) under N₂ before purging with H₂ gas. The mixture was stirred overnight and filtered through a pad of Celite. The residue on Celite was washed with ether followed by a water wash. The water filtrates were filtered through a 0.2 μm nylon syringe filter and lyophilized to yield the title product (15 mg, 99%) as a white solid. ¹H NMR (500 MHz, D₂O, pD = 2.5): δ 7.99 (d, *J* = 7.8 Hz, 2H), 7.52 (t, *J* = 7.4 Hz, 1H), 7.40 (t, *J* = 7.6 Hz, 2H), 5.79 (s, 1H), 4.43 (q, *J* = 9.4 Hz, 2H), 4.25 (t, *J* = 9.6 Hz, 1H), 4.18 (t, *J* = 9.9 Hz, 2H), 2.24 (t, *J* = 20.0 Hz, 2H); ¹³C NMR (125 MHz, D₂O, pD = 2.5): δ 167.0, 134.0, 129.8, 128.7, 128.5, 76.3, 75.0, 72.5 (d, *J* = 3.7 Hz), 71.6, 27.2 (dd, *J* = 135.5, 126.7 Hz); ³¹P NMR (202 MHz, D₂O, pD = 2.5): δ 20.71 (s, 1P), 14.49 (s, 1P), 2.59 (s, 2P), 0.57 (s, 2P); HRMS [M-H]⁻ calcd for C₁₄H₂₃O₂₄P₆⁻ 760.9010, found 760.8990.



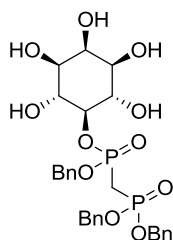
Myoinositol 1,3,4,6-tetra-phosphate-5-methylenediphosphate (3).

The benzoyl inositol polyphosphate **4** (13 mg, 0.013 mmol) was stirred with concentrated aqueous ammonia solution (5 mL) at room temperature for 4 days. After the solvent was removed under vacuum, the residue was dissolved in water (5 mL) and washed with CH₂Cl₂ (5 mL × 5). After concentrating under vacuum, the residue was purified by ion exchange chromatography (Dowex[®] 50WX8 hydrogen form) to yield the acid form of the title compound (4.4 mg, 52%) as a white solid. ¹H NMR (500 MHz, D₂O): δ 4.43 (q, *J* = 9.5 Hz, 2H), 4.33–4.28 (m, 2H), 4.17 (td, *J* = 9.6, 2.6 Hz, 2H), 2.46 (t, *J* = 21.1 Hz, 2H); ¹³C NMR (125 MHz, D₂O): δ 76.0, 75.2, 74.1 (d, *J* = 5.3 Hz), 69.7, 27.0 (dd, *J* = 136.2, 126.9 Hz); ³¹P NMR (202 MHz, D₂O): δ 18.71 (s, 2P), 0.02 (s, 2P), -0.51 (d, *J* = 9.3 Hz, 2P); HRMS [M-H]⁻ calcd for C₇H₁₉O₂₃P₆⁻ 656.8748, found 656.8741.



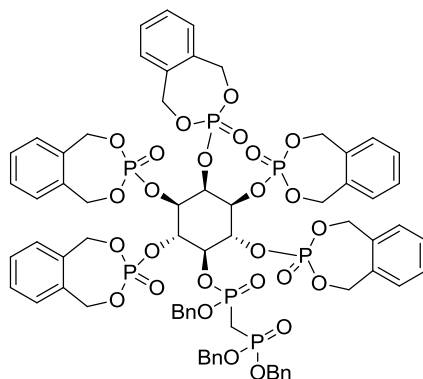
5-O-[[Bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-1,6:3,4-di-O-isopropylidene-myoinositol (6b).

To a solution of the benzoyl ester **6a** (338 mg, 0.426 mmol) in methanol (30 mL), NaOMe (1.7 mL, 0.5 M in methanol, 0.85 mmol) was added over 3 min and the mixture was stirred overnight at room temperature. Sat. aq. NH₄Cl (10 mL) and water (30 mL) were added to quench the reaction followed by extraction with EtOAc (40 mL × 3). The combined organic layers were dried over Na₂SO₄. The organic phase was removed under vacuum and the residue was purified by flash chromatography (0% to 5% MeOH in CH₂Cl₂) to give the title compound (217 mg, 74%) as a white solid. ¹H NMR (500 MHz, CDCl₃): δ 7.41–7.40 (m, 2H), 7.31–7.23 (m, 13H), 5.23 (dd, *J* = 11.7, 7.8 Hz, 1H), 5.12 (dd, *J* = 11.7, 7.1 Hz, 1H), 5.03–4.95 (m, 4H), 4.80 (q, *J* = 9.3 Hz, 1H), 4.54 (dd, *J* = 1.9, 1.7 Hz, 1H), 4.06 (q, *J* = 9.3 Hz, 2H), 3.56 (td, *J* = 9.2, 1.9 Hz, 2H), 2.78, (tq, *J* = 21.6, 15.6 Hz, 2H), 2.19 (d, *J* = 1.7 Hz, 1H), 1.42 (s, 3H), 1.42 (s, 3H), 1.40 (s, 3H), 1.38 (s, 3H); ¹³C NMR (125 MHz, CDCl₃): δ 136.17, 136.11, 136.09, 136.05, 128.63, 128.60, 128.51, 128.49, 128.44, 128.37, 128.05, 128.00, 113.01, 112.93, 78.25, 78.20, 76.1 (d, *J* = 2.7 Hz), 75.9 (d, *J* = 4.0 Hz), 73.9 (d, *J* = 5.6 Hz), 68.1 (d, *J* = 6.2 Hz), 67.9 (d, *J* = 6.1 Hz), 67.6 (d, *J* = 6.4 Hz), 63.2, 27.0, 26.6, 26.5, 26.0 (app. t, *J* = 136.9 Hz); ³¹P NMR (202 MHz, CDCl₃): δ 20.10 (d, *J* = 10.8 Hz, 1P), 19.72 (d, *J* = 10.8 Hz, 1P); HRMS [M+H]⁺ calcd for C₃₄H₄₃O₁₁P₂⁺ 689.2276, found 689.2275.



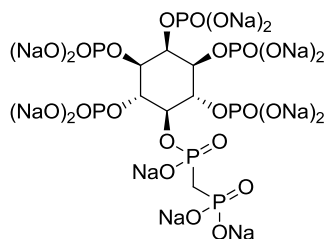
5-O-[[Bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-myoinositol (7b).

Water (57 μL, 57 mg, 3.2 mmol) and *p*-toluenesulfonic acid monohydrate (12 mg, 0.063 mmol) were added to a solution of the acetonide **6b** (217 mg, 0.315 mmol) in acetone (10 mL). After stirring overnight, the mixture was dried under vacuum and purified by flash chromatography (5% to 15% MeOH in CH₂Cl₂) to give the title compound (153 mg, 80%) as a colorless oil. ¹H NMR (500 MHz, CD₃OD): δ 7.41–7.27 (m, 15H), 5.27–5.19 (m, 2H), 5.01–4.96 (m, 4H), 4.18 (q, *J* = 9.4 Hz, 1H), 3.98 (s, 1H), 3.84 (td, *J* = 9.5 Hz, *J* = 6.8 Hz, 2H), 3.41 (d, *J* = 9.8 Hz, 2H), 2.98 (qt, *J* = 21.6 Hz, 15.6 Hz, 2H); ¹³C NMR (125 MHz, CD₃OD): δ 137.7 (d, *J* = 7.9 Hz), 137.32 (d, *J* = 6.2 Hz), 137.28 (d, *J* = 6.4 Hz), 129.63, 129.62, 129.57, 129.55, 129.53, 129.37, 129.28, 129.18, 129.15, 83.9 (d, *J* = 8.1 Hz), 73.8, 73.3, 73.01 (d, *J* = 2.5 Hz), 72.94, 72.8 (d, *J* = 3.0 Hz), 69.5 (d, *J* = 6.4 Hz), 68.9 (d, *J* = 6.6 Hz), 26.3 (dd, *J* = 141.6 Hz, 136.5 Hz); ³¹P NMR (202 MHz, CD₃OD): δ 21.88 (d, *J* = 8.4 Hz, 1P), 20.27 (d, *J* = 8.4 Hz, 1P); HRMS [M+H]⁺ calcd for C₂₈H₃₅O₁₁P₂⁺ 609.1650, found 609.1649.



5-O-[[Bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-1,2,3,4,6-penta-O-(o-xilylene)-phosphate-myoinositol (8b).

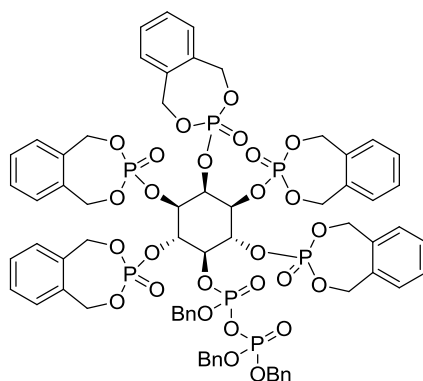
N,N-diethyl-1,5-dihydro-2,4,3-benzodioxaphosphepin-3-amine (212 mg, 0.886 mmol) in CH₃CN (2 mL) and 1*H*-tetrazole (3.0 mL, 0.45 M in CH₃CN, 1.3 mmol) were added to a solution of the alcohol **7b** (54 mg, 0.089 mmol) in CH₃CN (3 mL) at 0 °C. The mixture was stirred for 2 h at 0 °C followed by 36 h at room temperature. A solution of mCPBA (398 mg, 1.77 mmol) in CH₃CN (3 mL) was added over 6 min after the mixture was cooled in an ice bath. The mixture was stirred for 1 h at 0 °C followed by 2 h at room temperature. 10% Na₂S₂O₃ (25 mL) was added to quench the reaction and CH₃CN was removed under vacuum. EtOAc (50 mL) was added to the mixture and the organic layer was washed with sat. aq. NaHCO₃ (20 mL) and sat. aq. NaCl (20 mL). The organic layer was dried over Na₂SO₄, concentrated under vacuum and purified by flash chromatography (0% to 5% MeOH in CH₂Cl₂) and preparative HPLC (Method A, *t_R* = 10.4 min) to give the title compound (45 mg, 33%) as a white solid. ¹H NMR (500 MHz, CDCl₃): δ 7.41–7.21 (m, 33H), 6.92 (d, *J* = 7.2 Hz, 1H), 6.84 (d, *J* = 7.3 Hz, 1H), 5.77 (dd, *J* = 12.7 Hz, 10.0 Hz, 2H), 5.67 (m, 2H), 5.55–4.82 (m, 27H), 4.67 (q, *J* = 9.7 Hz, 1H), 3.04 (td, *J* = 21.8 Hz, 15.6 Hz, 1H), 2.89 (td, *J* = 21.8 Hz, 15.6 Hz, 1H); ¹³C NMR (125 MHz, CDCl₃): δ 136.2 (d, *J* = 8.2 Hz), 136.1 (d, *J* = 6.8 Hz), 136.0 (d, *J* = 6.3 Hz), 135.67, 135.65, 135.53, 135.49, 135.24, 135.07, 135.01, 134.82, 134.74, 129.6, 129.5, 129.4, 129.2, 129.06, 129.03, 128.93, 128.88, 128.77, 128.69, 128.59, 128.57, 128.55, 128.37, 128.23, 128.15, 128.12, 77.1, 76.5, 76.3, 74.4, 73.7, 69.56, 69.51, 69.47, 69.3 (d, *J* = 7.2 Hz), 68.9 (d, *J* = 6.3 Hz), 68.4 (d, *J* = 6.1 Hz), 67.9 (d, *J* = 6.2 Hz), 26.2 (app. t, *J* = 137.9 Hz); ³¹P NMR (202 MHz, CDCl₃): δ 20.82 (s, 1P), 20.34 (s, 1P), –0.60 (s, 1P), –1.73 (s, 1P), –3.67 (s, 1P), –3.73 (s, 1P), –3.83 (s, 1P); HRMS [M+H]⁺ calcd for C₆₈H₇₀O₂₆P₇⁺ 1519.2314, found 1519.2318.



Myo-inositol 1,2,3,4,6-penta-phosphate-5-methylenediphosphonate triskaidecabasic sodium salt (2).

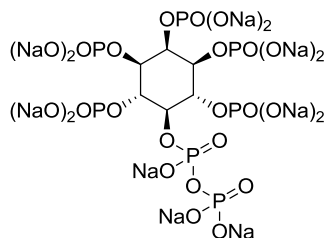
NaHCO₃ (28 mg, 0.33 mmol) and palladium black (71 mg, 0.67 mmol) were added to a solution of **8b** (39 mg, 0.026 mmol) in *t*-BuOH/H₂O (40:7, 3.5 mL) under N₂ before purging with H₂ gas. The

mixture was stirred overnight at room temperature and filtered through a pad of Celite. The residue on Celite was washed with ether followed by a water wash. The water filtrates were filtered through a 0.2 μm nylon syringe filter and lyophilized to yield the title product (23 mg, 86%) as a white solid. ^1H NMR (500 MHz, D_2O , pD = 2.1): δ 4.39 (q, J = 9.5 Hz, 2H), 4.27 (q, J = 9.5 Hz, 1H), 4.16 (t, J = 9.8, 2H), 2.46 (t, J = 20.7 Hz, 2H); ^1H NMR (500 MHz, D_2O , pD = 12.5): δ 4.45 (d, J = 10.9 Hz, 2H), 4.34 (d, J = 12.3 Hz, 1H), 4.30 (d, J = 11.1 Hz, 3H), 2.04 (t, J = 19.2 Hz, 2H); ^{13}C NMR (125 MHz, D_2O , pD = 2.1): δ 75.7, 75.6, 74.9, 72.8, 27.0 (dd, J = 136.6, 127.2 Hz); ^{31}P NMR (202 MHz, D_2O , pD = 2.1): δ 19.26 (s, 1P), 16.77 (s, 1P), 0.24 (s, 2P), 0.04 (s, 2P), -0.88 (s, 1P); ^{31}P NMR (202 MHz, D_2O , pD = 12.5): δ 23.41 (d, J = 7.5 Hz, 1P), 13.27 (d, J = 7.5 Hz, 1P), 5.15 (s, 1P), 3.89 (s, 2P), 3.87 (s, 2P); HRMS $[\text{M}-\text{H}]^-$ calcd for $\text{C}_7\text{H}_{20}\text{O}_{26}\text{P}_7^-$ 736.8411, found 736.8398.



5-O-[[Bis(benzyloxy)phosphoryloxy]benzyloxyphosphoryl]-1,2,3,4,6-penta-O-(*o*-xylylene)-phosphate-*myo*-inositol (10).

The cyanoethyl phosphate **9**⁴ (47 mg, 0.036 mmol) in CH_3CN (3 mL) was treated with triethylamine (75 μL , 0.54 mmol) and *N,O*-bis(trimethylsilyl)trifluoroacetamide (71 μL , 0.27 mmol), and the mixture was stirred for 12 h at room temperature. After drying under vacuum, the residue was dissolved in CH_2Cl_2 (1 mL) at 0 $^\circ\text{C}$. After the addition of triethylamine (19 μL , 0.11 mmol), dibenzyl phosphorochloridate (28 mg, 0.093 mmol) in CH_2Cl_2 (0.4 mL) was added over 2 min. After stirring for 30 min at 0 $^\circ\text{C}$ followed by 2 h at room temperature, the mixture was concentrated under vacuum and purified by preparative HPLC (Method A, t_{R} = 11.2 min) to yield the title compound (20 mg, 38%) as a white solid. ^1H NMR (500 MHz, CDCl_3): δ 7.49–7.21 (m, 33H), 7.01 (d, J = 6.9 Hz, 1H), 6.87 (d, J = 7.3 Hz, 1H), 5.77 (dt, J = 13.5 Hz, 9.4 Hz, 2H), 5.67 (dd, J = 13.3 Hz, 8.6 Hz, 2H), 5.59–4.81 (m, 28H); ^{13}C NMR (125 MHz, CDCl_3): δ 135.7, 135.6, 135.5, 135.43, 135.38, 135.15, 135.11, 135.04, 134.65, 134.62, 129.70, 129.65, 129.59, 129.45, 129.41, 129.27, 129.22, 129.16, 129.13, 128.94, 128.89, 128.84, 128.80, 128.72, 128.69, 128.62, 128.60, 128.37, 128.25, 76.4, 76.1, 73.7, 71.6 (d, J = 6.6 Hz), 70.8 (d, J = 5.4 Hz), 70.6 (d, J = 5.5 Hz), 69.60, 69.54, 69.46, 69.38, 69.2 (d, J = 6.7 Hz), 68.8 (d, J = 6.4 Hz); ^{31}P NMR (202 MHz, CDCl_3): δ -0.63 (s, 1P), -2.68 (s, 1P), -3.42 (s, 1P), -3.79 (s, 1P), -4.00 (s, 1P), -12.83 (d, J = 11.4 Hz, 1P), -14.06 (d, J = 11.4 Hz, 1P); HRMS $[\text{M}+\text{H}]^+$ calcd for $\text{C}_{67}\text{H}_{68}\text{O}_{27}\text{P}_7^+$ 1521.2106, found 1521.2104.



5-O-[(phosphoryloxy)phosphoryl]-1,2,3,4,6-penta-O-phosphate-*myo*-inositol triskaidecasic sodium salt (1).

NaHCO₃ (8.5 mg, 0.10 mmol) and palladium black (22 mg, 0.21 mmol) were added to a solution of **10** (12 mg, 0.0079 mmol) in *t*-BuOH/H₂O (40:7, 2 mL) under N₂ before purging with H₂ gas. The mixture was stirred overnight and filtered through a pad of Celite. The residue on Celite was washed with ether followed by a water wash. The water filtrates were filtered through a 0.2 μm nylon syringe filter and lyophilized to yield the title product (7.9 mg, 99%) as a white solid. ¹H NMR (500 MHz, D₂O, pD = 6.8): δ 4.34 (q, *J* = 9.5 Hz, 2H), 4.13 (q, *J* = 9.5 Hz, 1H), 4.02 (t, *J* = 9.4 Hz, 2H); ¹H NMR (500 MHz, D₂O, pD = 11.8): δ 4.53 (d, *J* = 10.5 Hz, 2H), 4.40 (d, *J* = 12.9 Hz, 1H), 4.33 (d, *J* = 8.6 Hz, 3H); ¹³C NMR (125 MHz, D₂O, pD = 6.8): δ 77.5, 75.5, 75.3, 73.2; ³¹P NMR (202 MHz, D₂O, pD = 6.8): δ 0.99 (s, 2P), 0.56 (s, 2P), 0.03 (s, 1P), -8.81 (s, 1P), -10.45 (d, *J* = 19.1 Hz, 1P); ³¹P NMR (202 MHz, D₂O, pD = 11.8): δ 5.04 (s, 1P), 3.98 (s, 2P), 3.69 (s, 2P), -4.09 (d, *J* = 14.2 Hz, 1P), -8.67 (d, *J* = 14.9 Hz, 1P); HRMS [M-H]⁻ calcd for C₆H₁₈O₂₇P₇⁻ 738.8204, found 738.8188.

III. NMR Titration

NMR samples containing 1 mM 5PP-IP₅ (**1**) or 5PCP-IP₅ (**2**), 140 mM KCl, 10 mM NaCl and 1mM MgCl₂ in D₂O (0.5 mL) were prepared at varying pD from 2.8 to 12.4. ¹H NMR and ³¹P NMR spectra were recorded at room temperature. Capillaries in the NMR tubes, which contained 10 mM DSS (4,4-dimethyl-4-silapentane-1-sulfonic acid) and 5 mM tetramethylphosphonium bromide in D₂O, were used to calibrated chemical shifts. Proton chemical shifts were assigned by ¹H-¹H and ¹H-³¹P correlation experiments. pH was converted to pD by the following equation⁵: pD = 0.929 × pH + 0.42. A representative example of the ¹H NMR spectra obtained for 5PCP-IP₅ is shown in Figure S1 and all data are shown in Figure S2.

IV. Inhibition of Akt T308 Phosphorylation

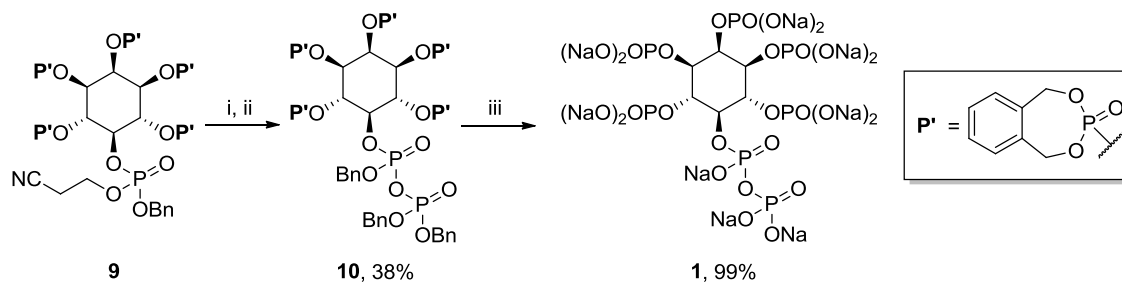
Inactive human Akt1 at a final concentration of 17 nM was treated with varying concentrations inhibitors (from 2.4 nM to 10 μM) in a buffer containing 50 mM Tris (pH = 7.5), 100 μM Mg-ATP, 1 mM Mg(OAc)₂ and 1 mM DTT in total volume of 20 μL at 30 °C for 20 min. Next, 5 μL PDK1 at a final concentration of 17 nM in the same buffer was added. After 30 min at 30 °C, SDS sample buffer was added to quench the reaction. The samples were boiled for 5 min at 95 °C and resolved by SDS-PAGE. After transferring to nitrocellulose, Akt and phosphorylated Akt were detected by western blot with Akt or Phospho-Akt (Thr 308) antibodies respectively. Bands were quantified using ImageJ software and data were analyzed by non-linear curve fitting using GraphPad Prism 5 software.

V. Inhibition of Ddp1 Activity

The vectors for expression of *S.cerevisiae* His-Ddp1 and His-Ddp1 EE/AQ were a kind gift from Adolfo Saiardi. (DDP1 was cloned in the Sall/NotI restriction sites of the pTrcHis plasmid.) His-Ddp1 and His-Ddp1 EE/AQ catalytically inactive mutant were expressed and purified as described previously⁶. The inhibition of hydrolysis of Ap₅A by Ddp1 was assayed in reactions containing 4 μg/mL Ddp1, 50 μM Ap₅A and inhibitors (from 24 nM to 100 μM) in 25 mM Hepes (pH = 6.8), 50 mM NaCl, 10 mM MgCl₂ and 1 mM DTT. As a control, we confirmed that Ap₅A showed no signs of hydrolysis when incubated with His-Ddp1 EE/AQ (Figure S3). The reactions were performed at 37 °C for 4 h before quenching by filtration through 0.2 μm syringe filter. The hydrolysis of Ap₅A was analyzed by HPLC (method C, t_R = 5.3 min). Data were analyzed by non-linear curve fitting using GraphPad Prism 5 software.

VI. Supporting Scheme and Figures

Scheme S1. Synthesis of Diphosphoinositol Polyphosphate **1**^a.



^aReagents and conditions: (i) BSTFA, TEA, CH₃CN, rt, overnight; (ii) dibenzyl phosphorochloridate, TEA, CH₂Cl₂, 0 °C to rt, 2h; (iii) H₂, Pd black, NaHCO₃, *t*-BuOH/H₂O, rt, overnight.

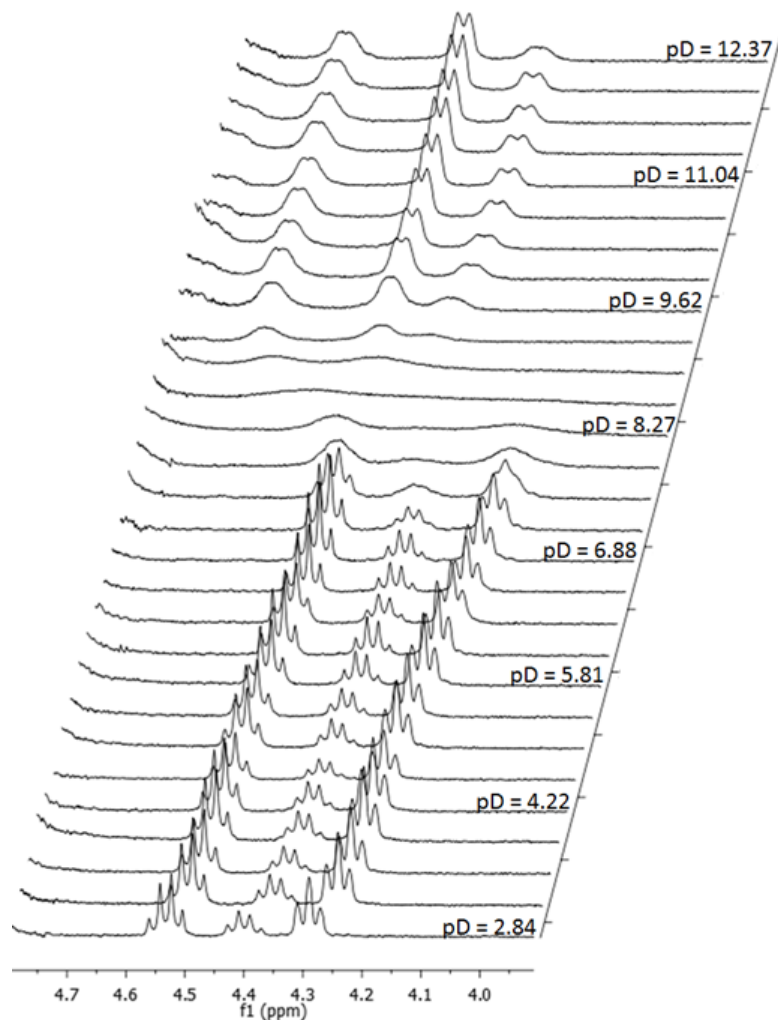


Figure S1. ¹H NMR spectra of 5PCP-IP₅ (**2**) at different pD.

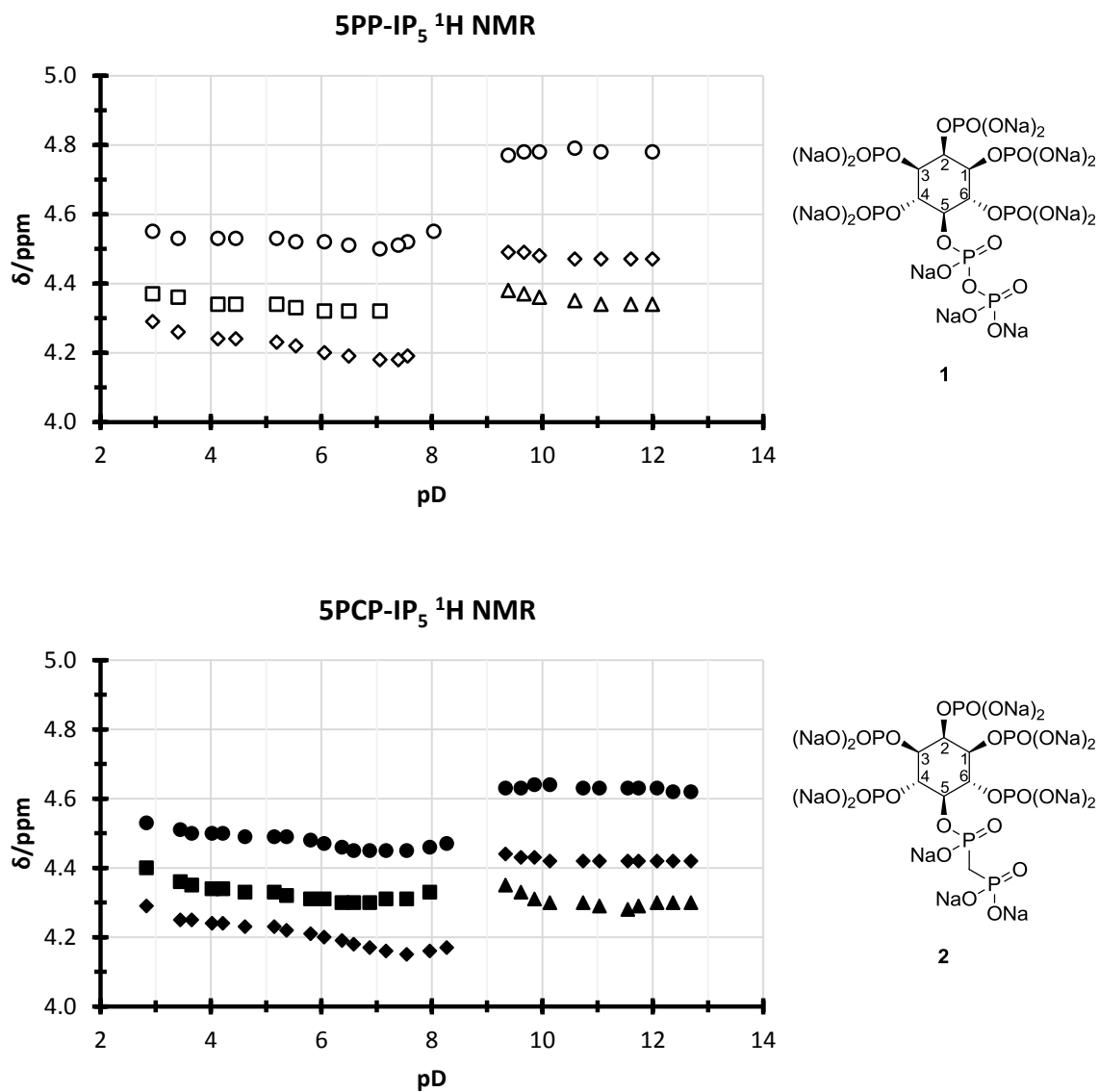


Figure S2. ¹H NMR titration data of 5PP-IP₅ (**1**) and 5PCP-IP₅ (**2**) illustrating the conformational change. Circle (○ and ●): H_{4/6}, Square (□ and ■): H₅, Diamond (◇ and ◆): H_{1/3} and Triangle (△ and ▲): H₂.

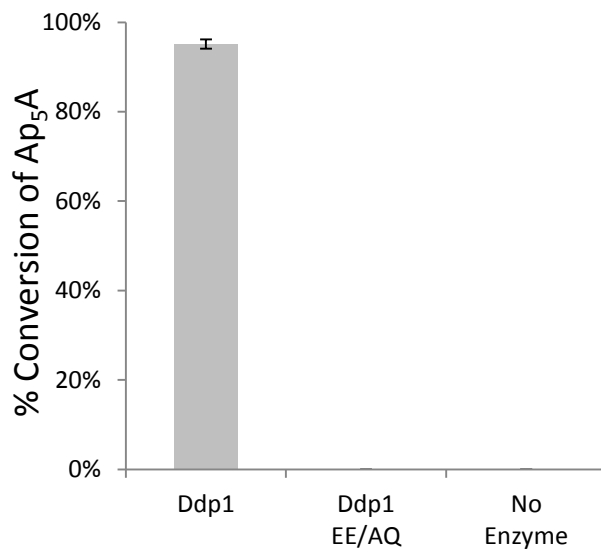


Figure S3. Conversion of 50 μM Ap₅A at 37°C for 4 h with Ddp1 (4 $\mu\text{g}/\text{mL}$), Ddp1 EE/AQ catalytically inactive mutant (16 $\mu\text{g}/\text{mL}$) and no enzyme.

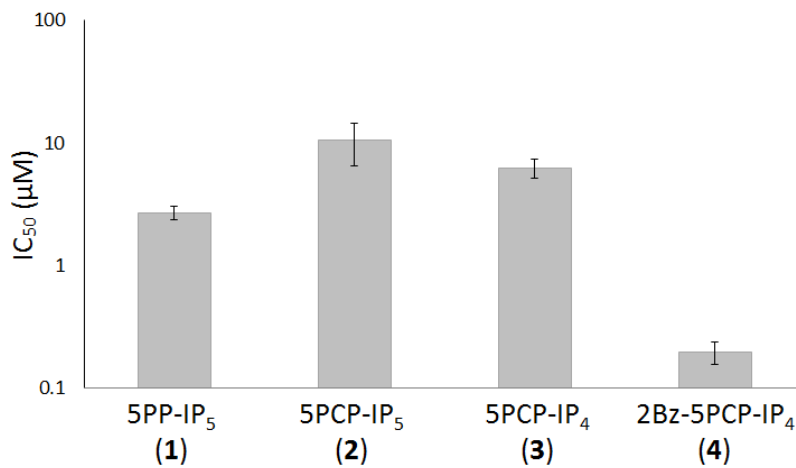
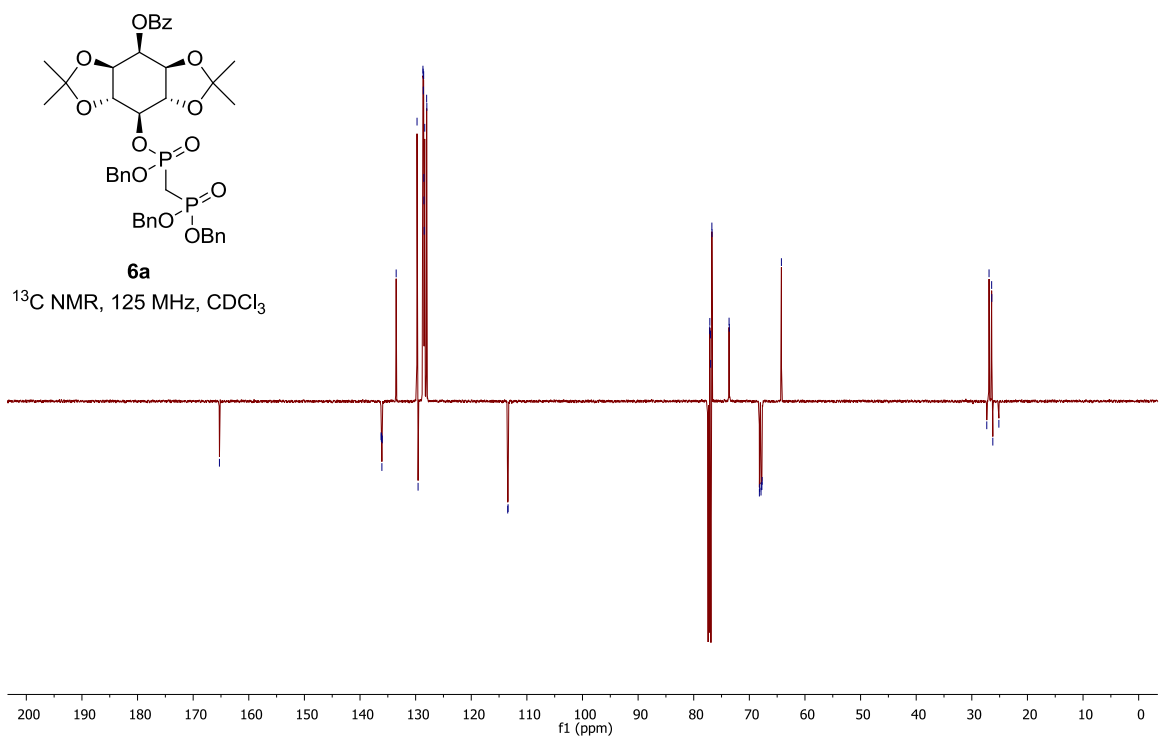
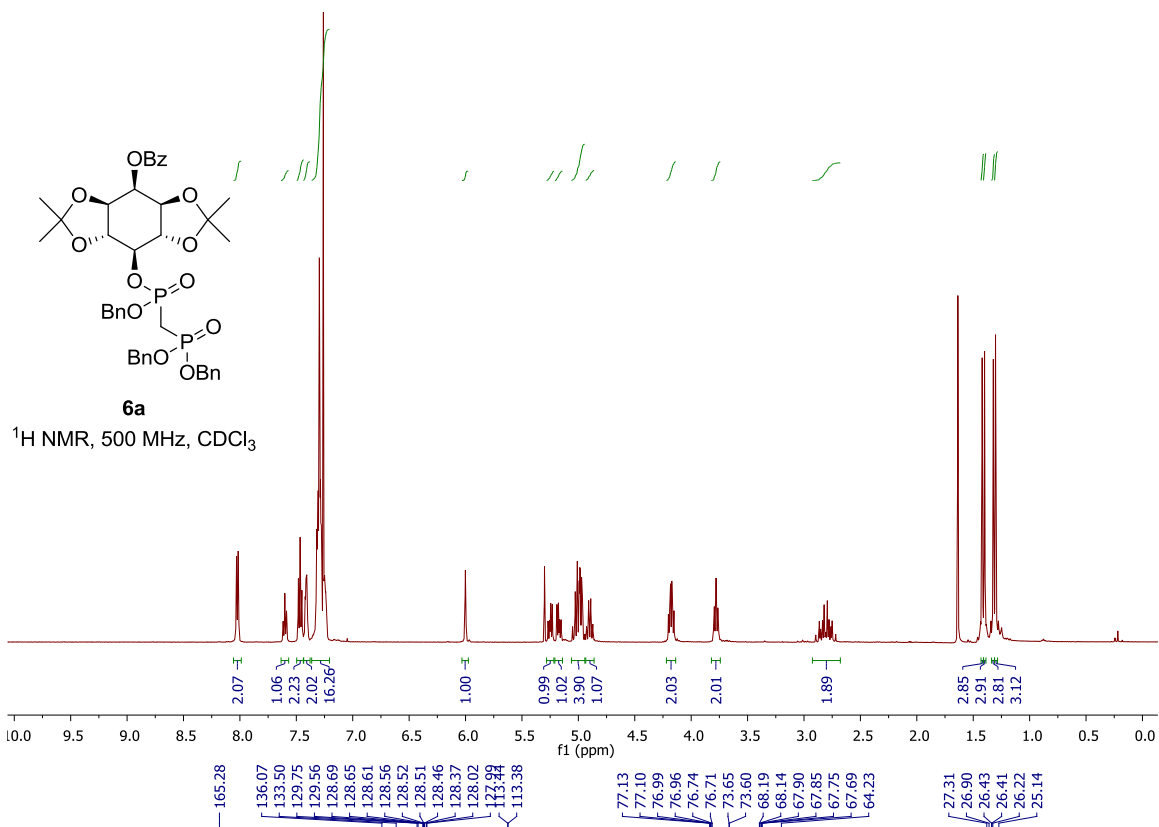
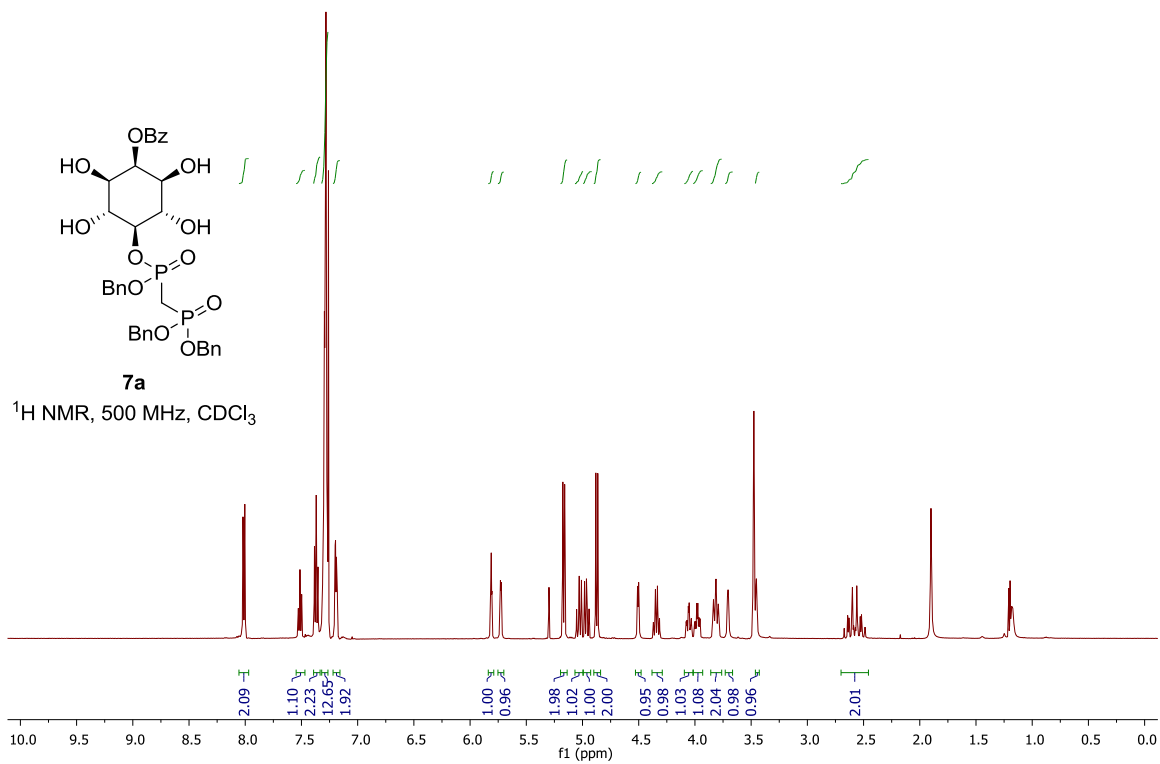
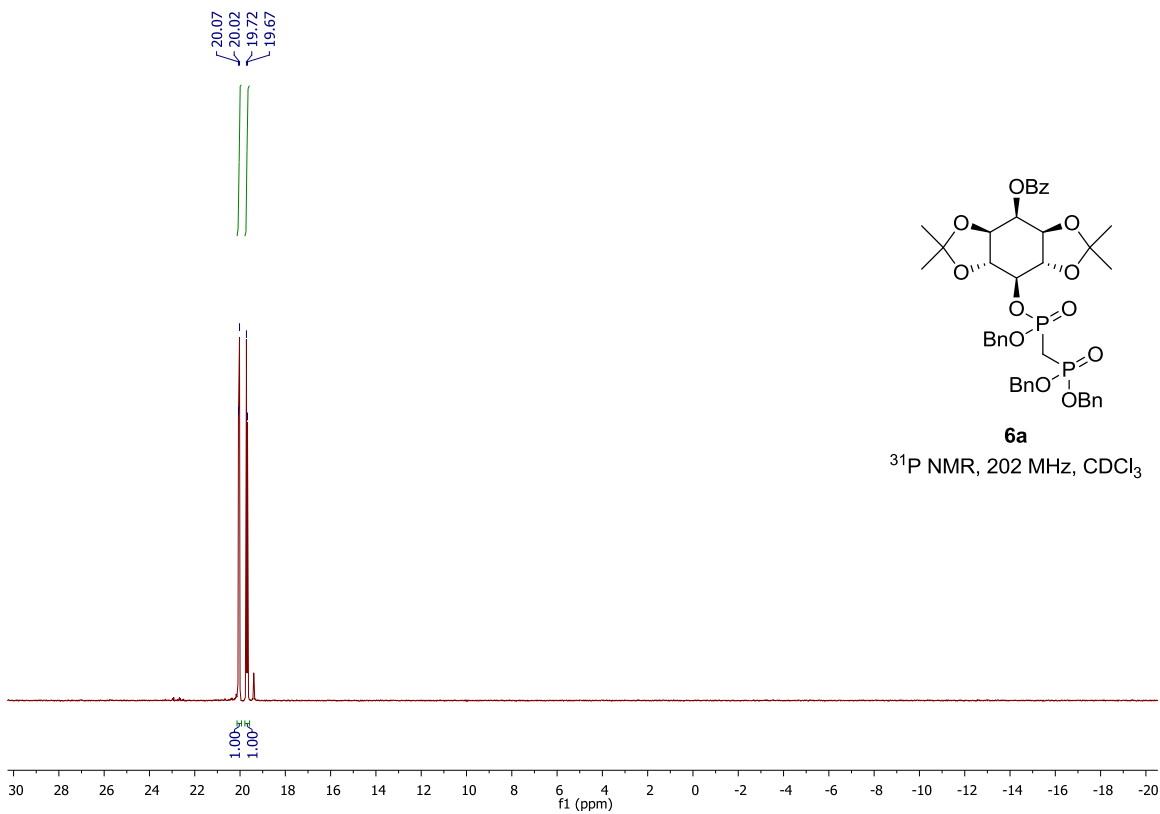
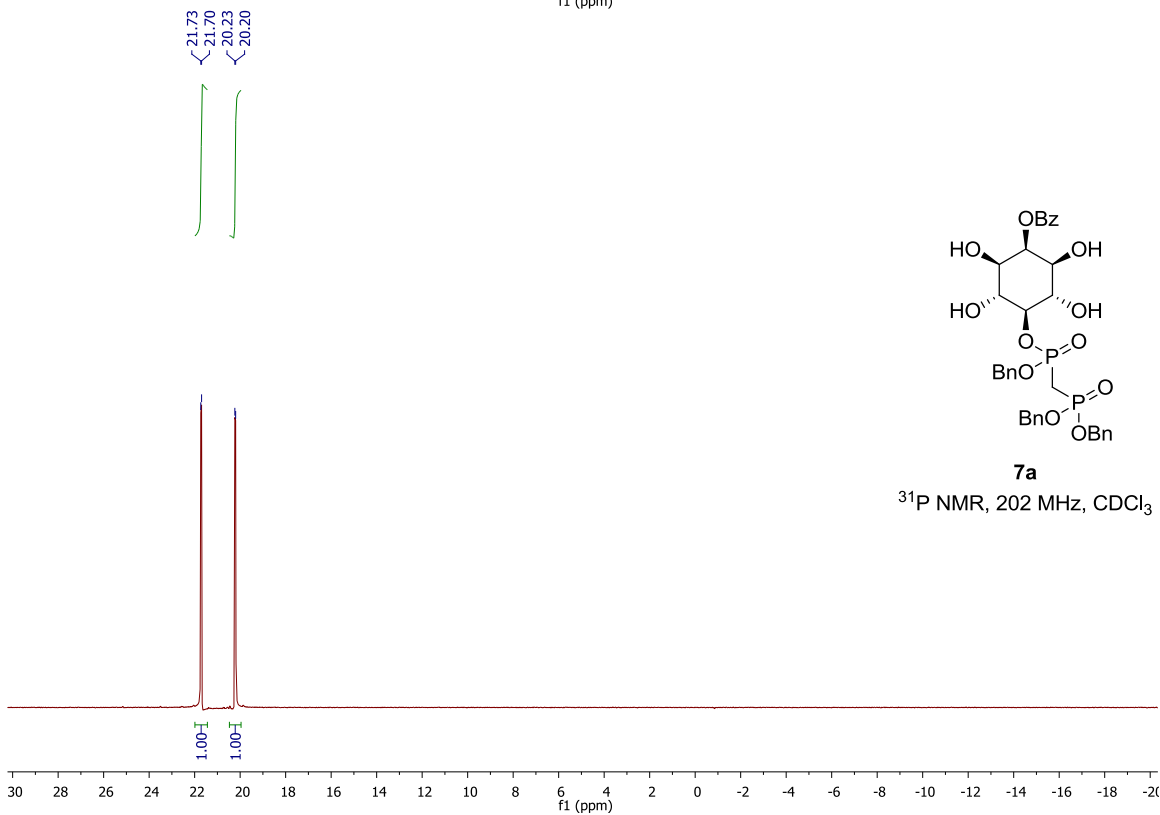
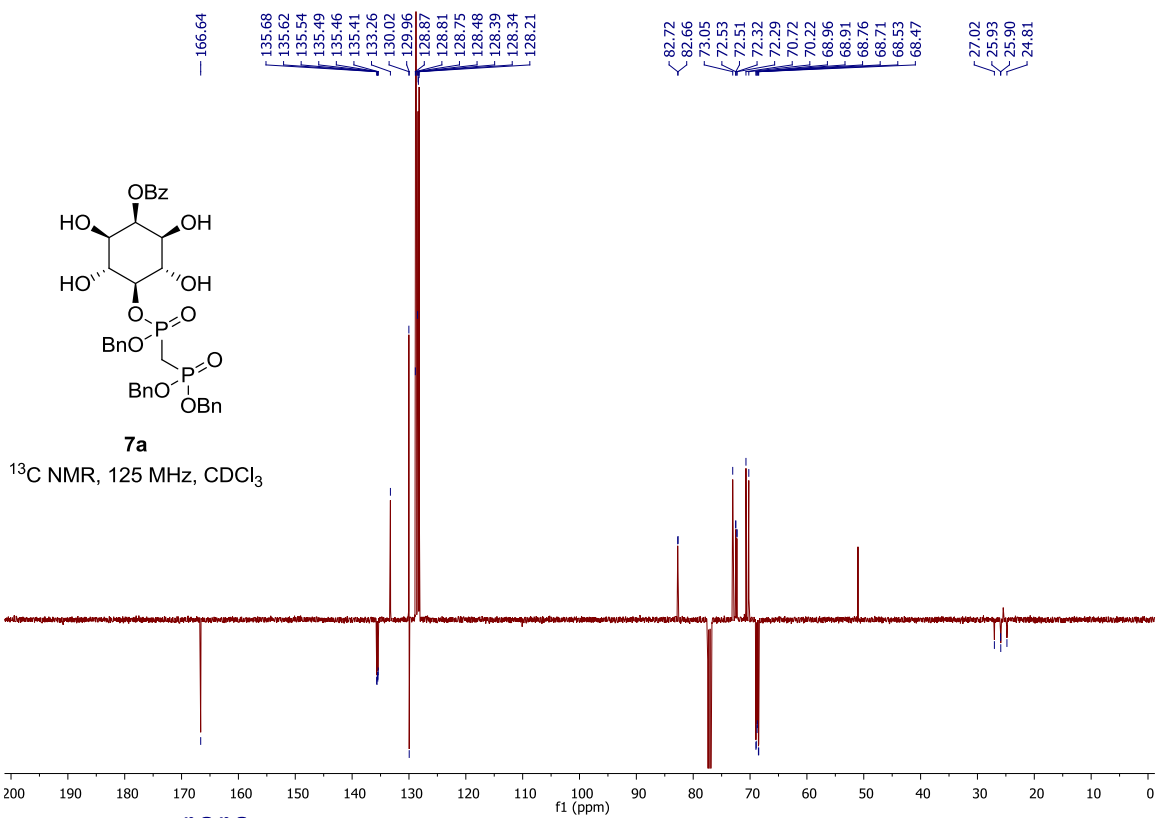


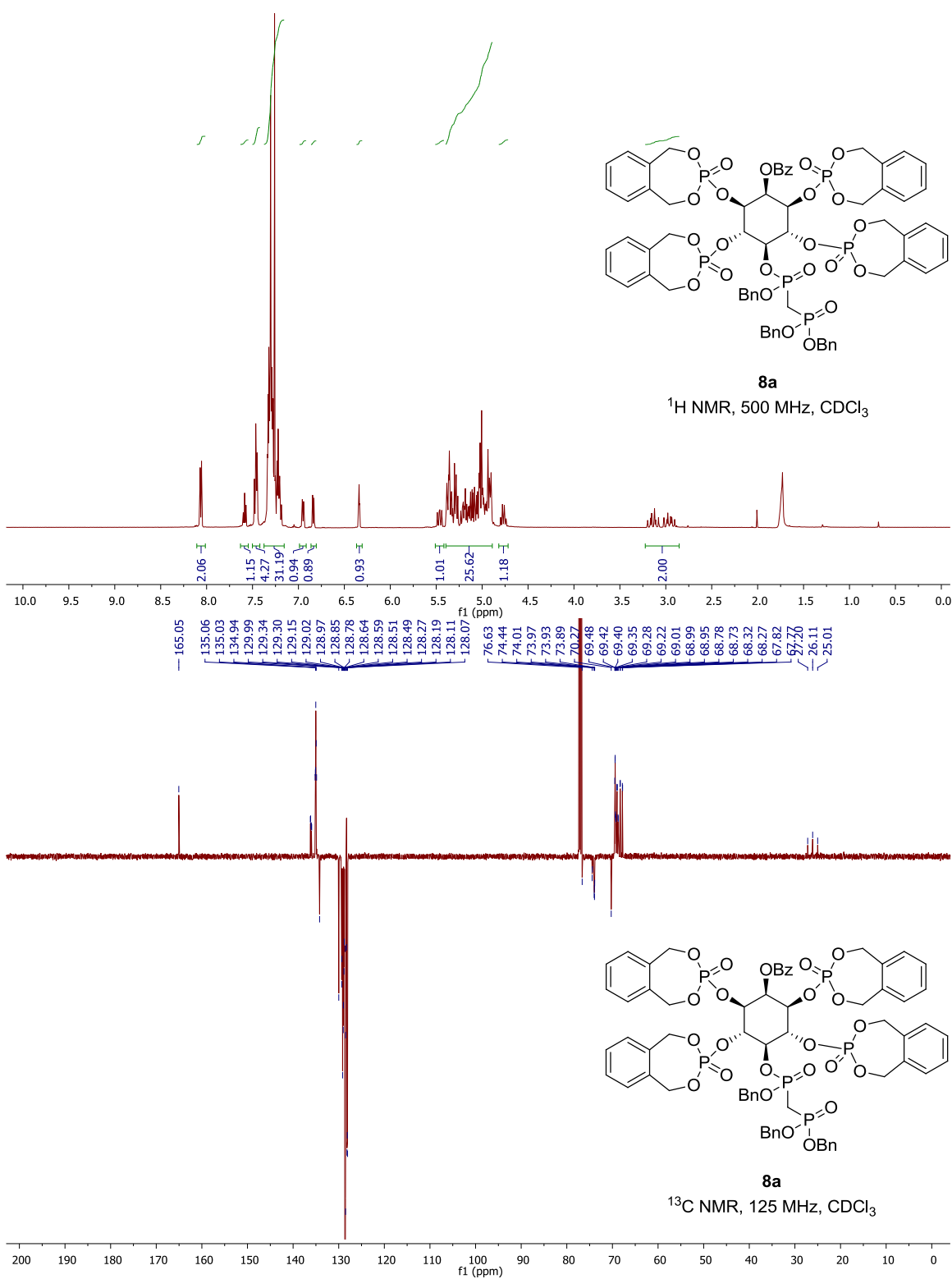
Figure S4. IC₅₀ values for 5PP-IP₅ and the analogues, as measured by inhibition of hydrolysis of Ap₅A by Ddp1.

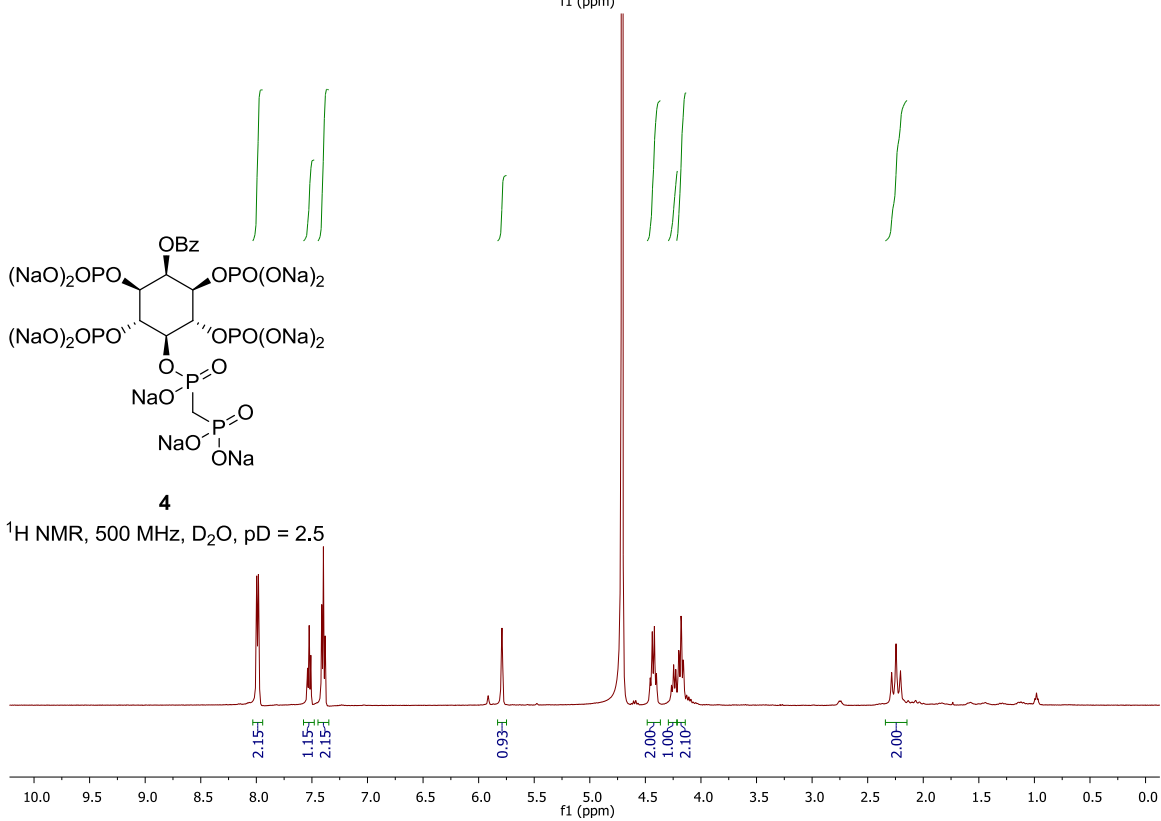
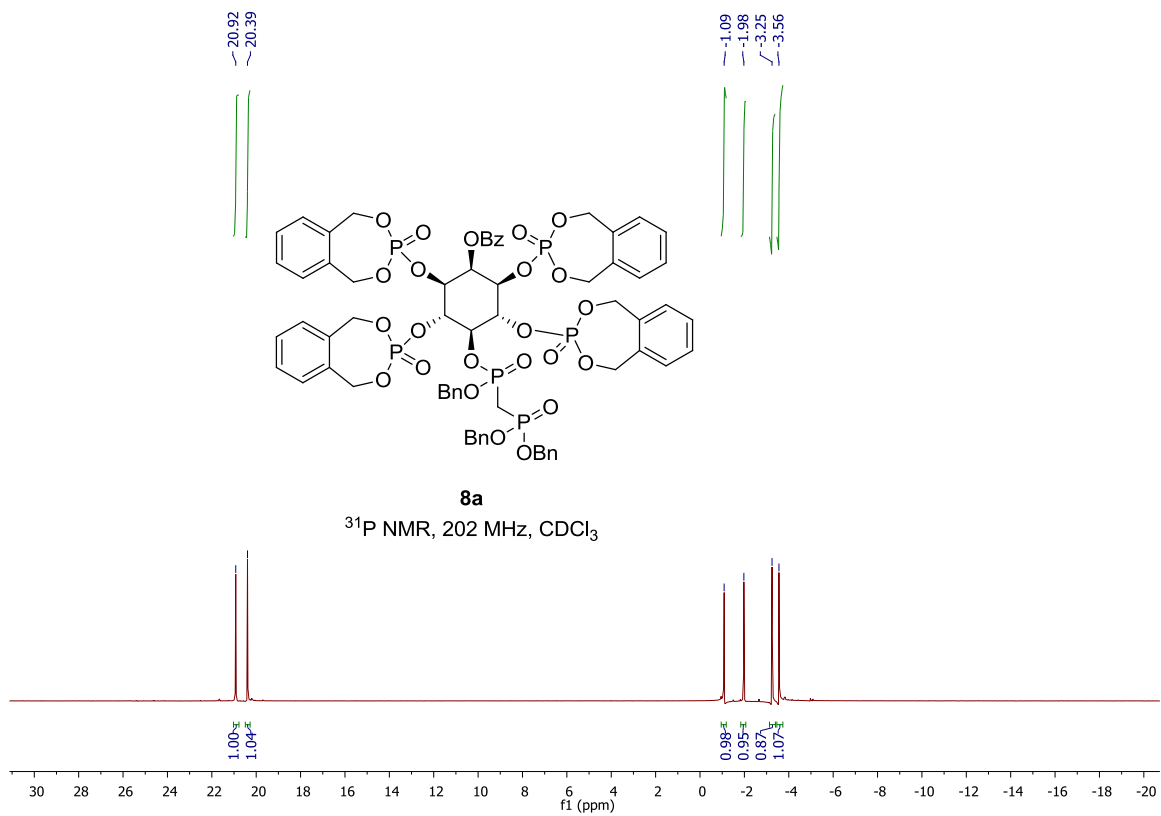
VII. NMR Spectra

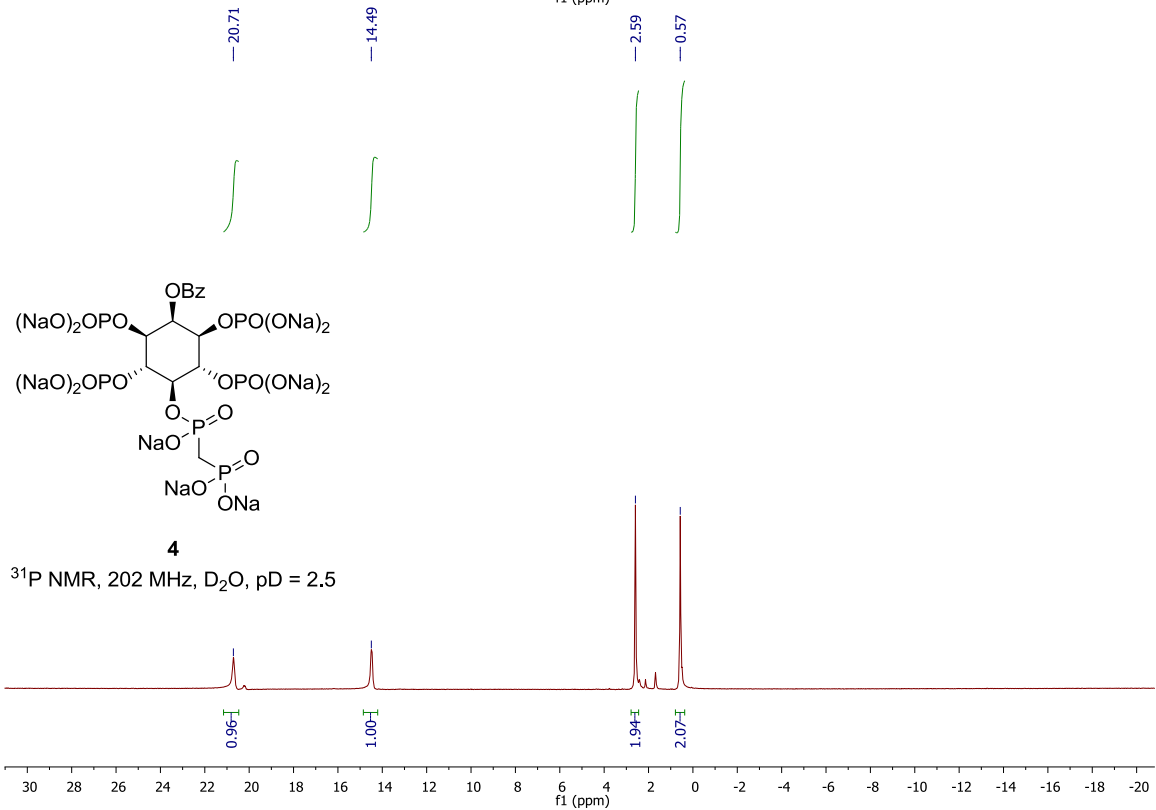
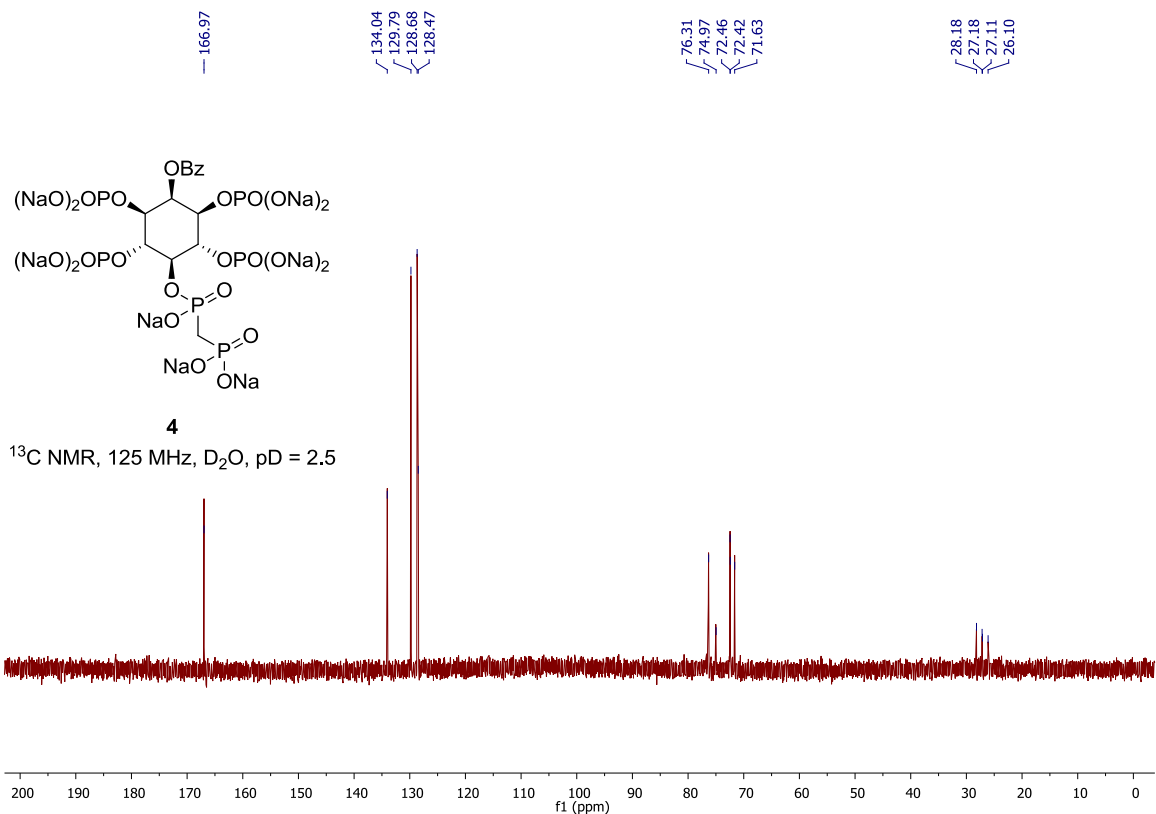


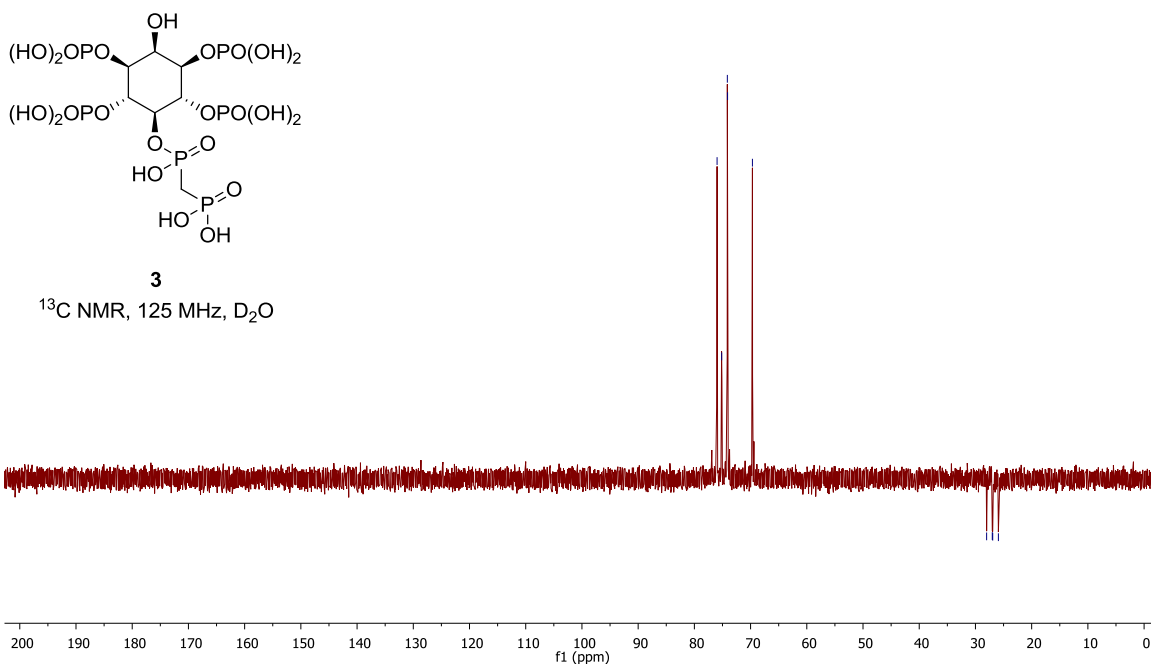
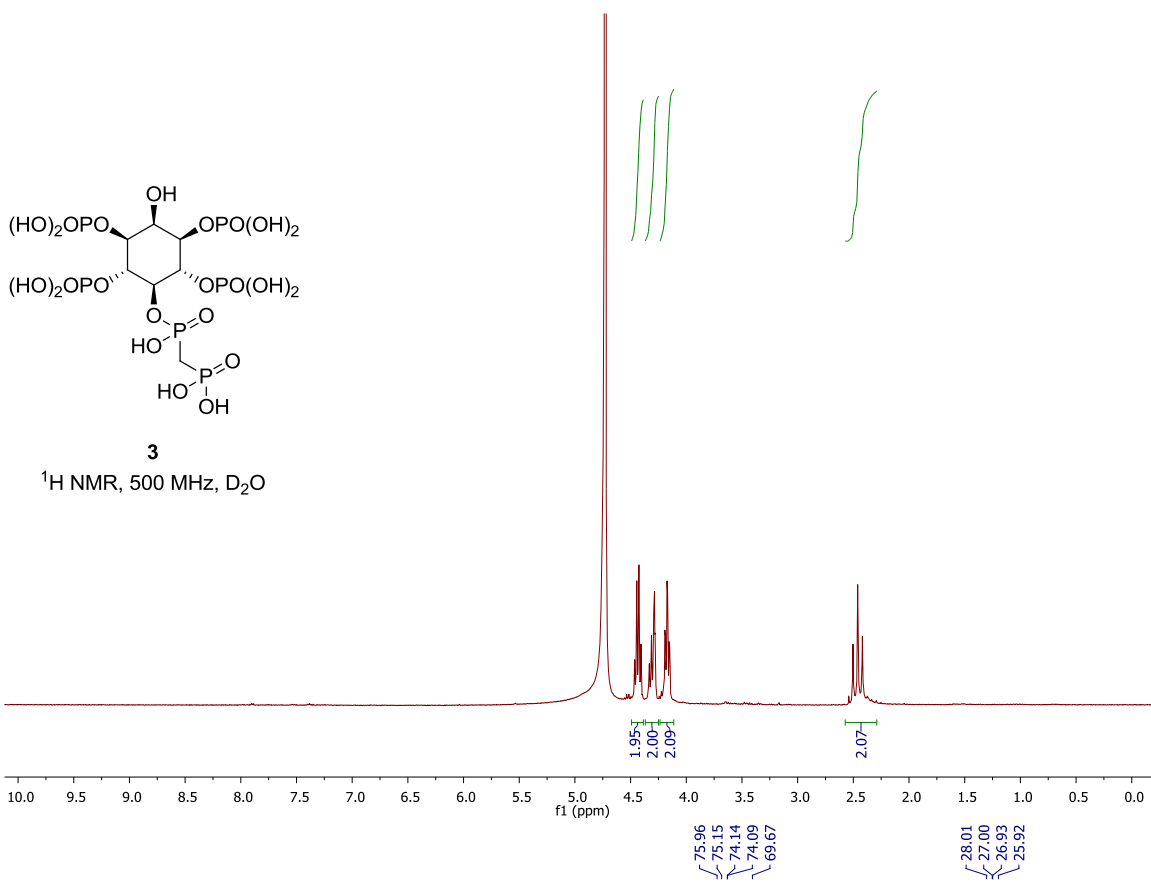


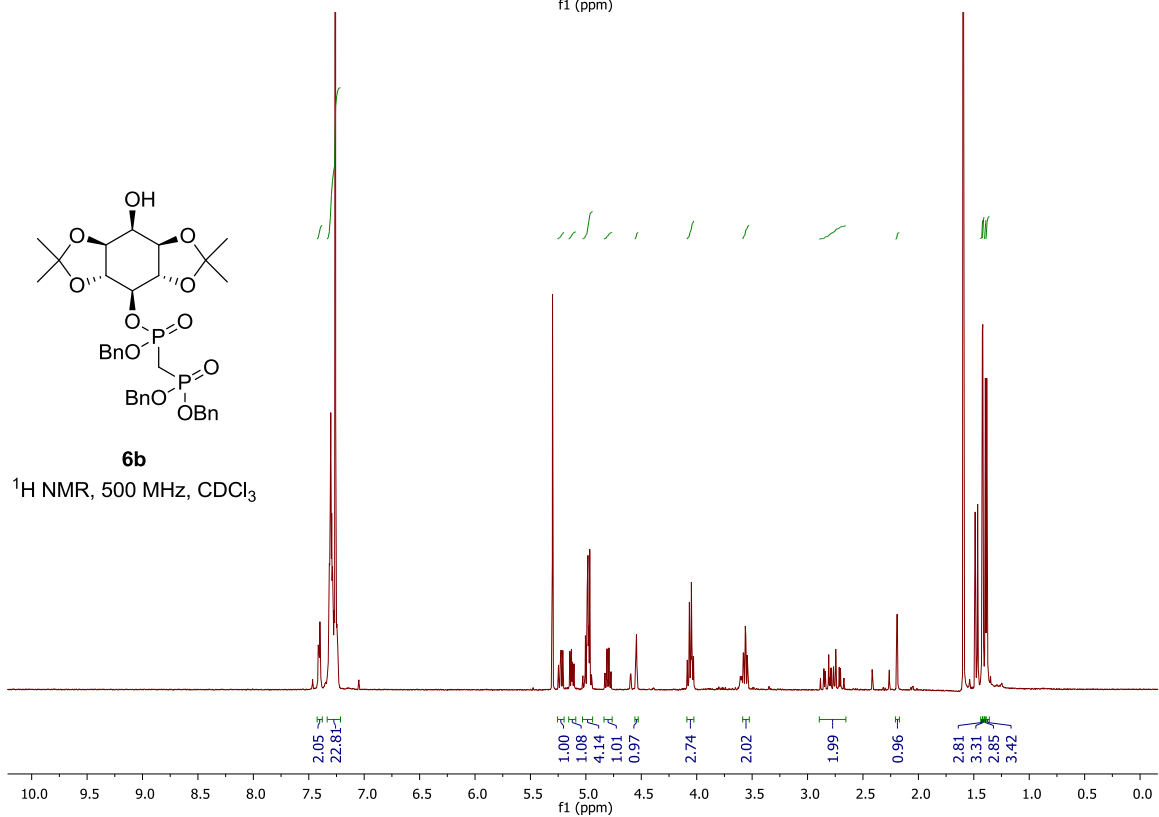
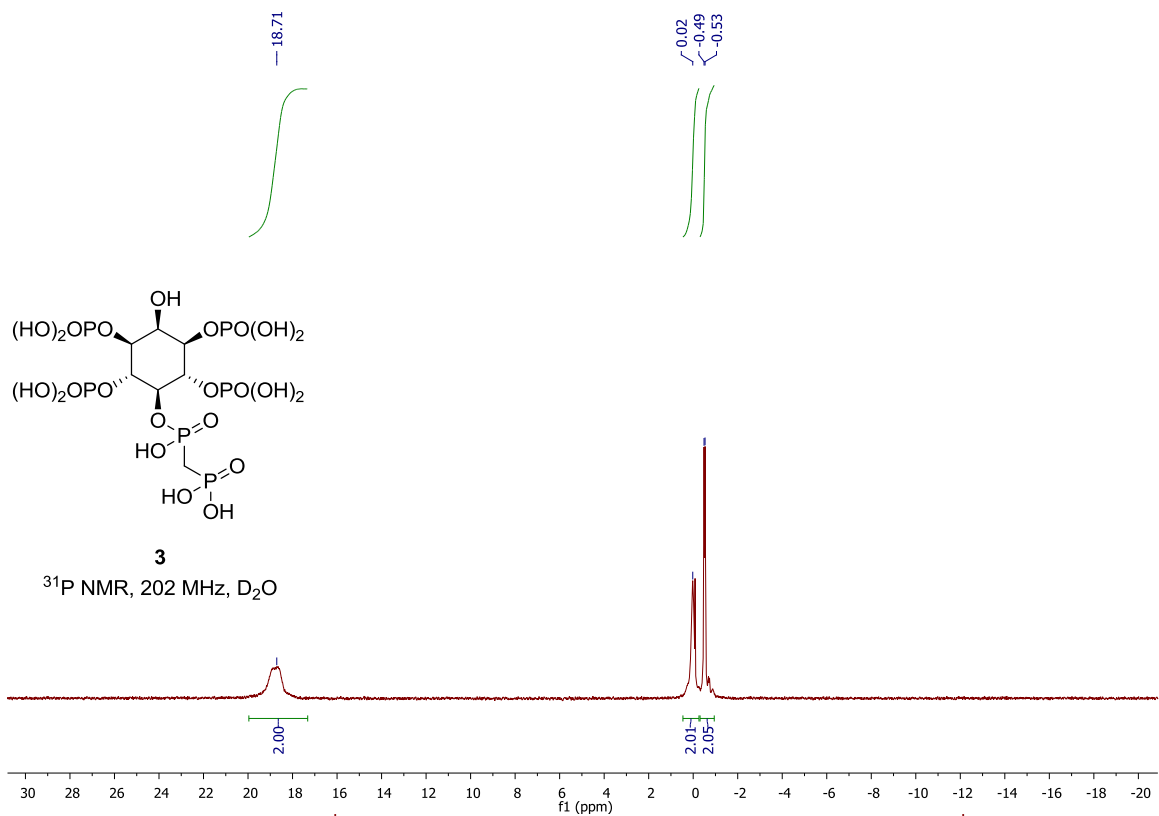


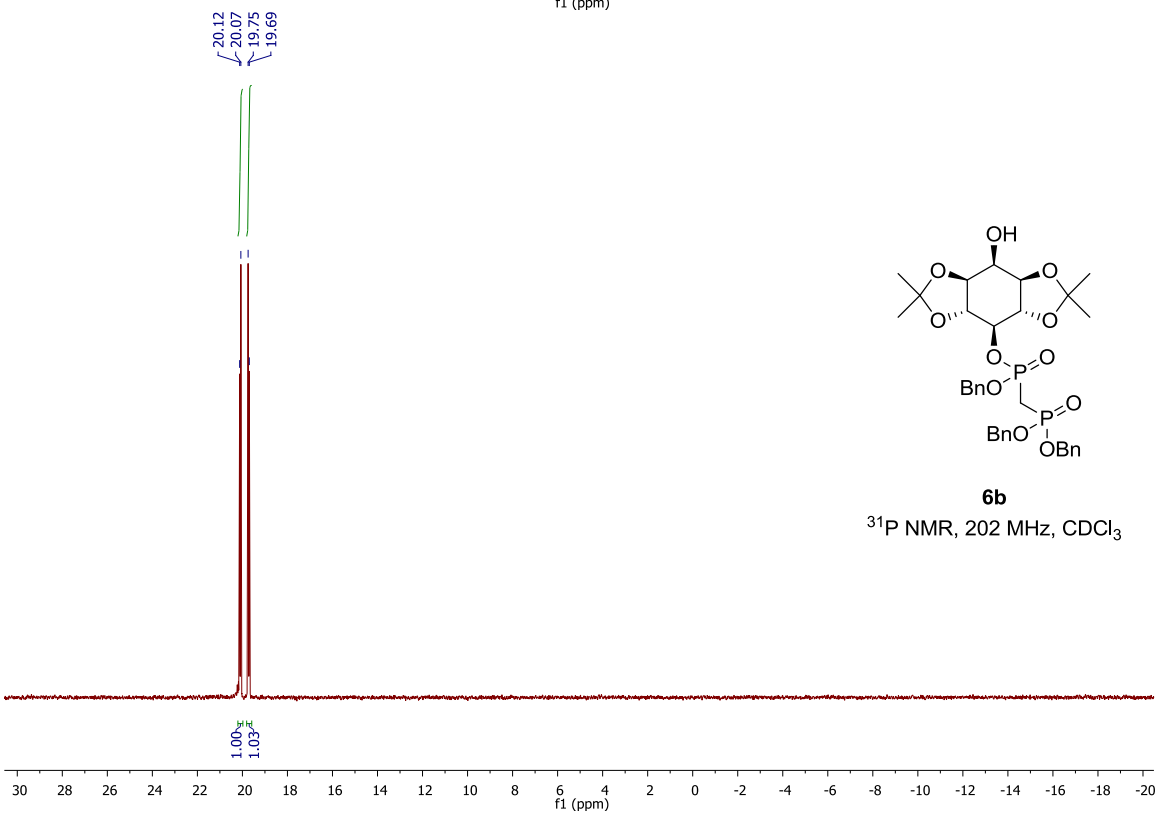
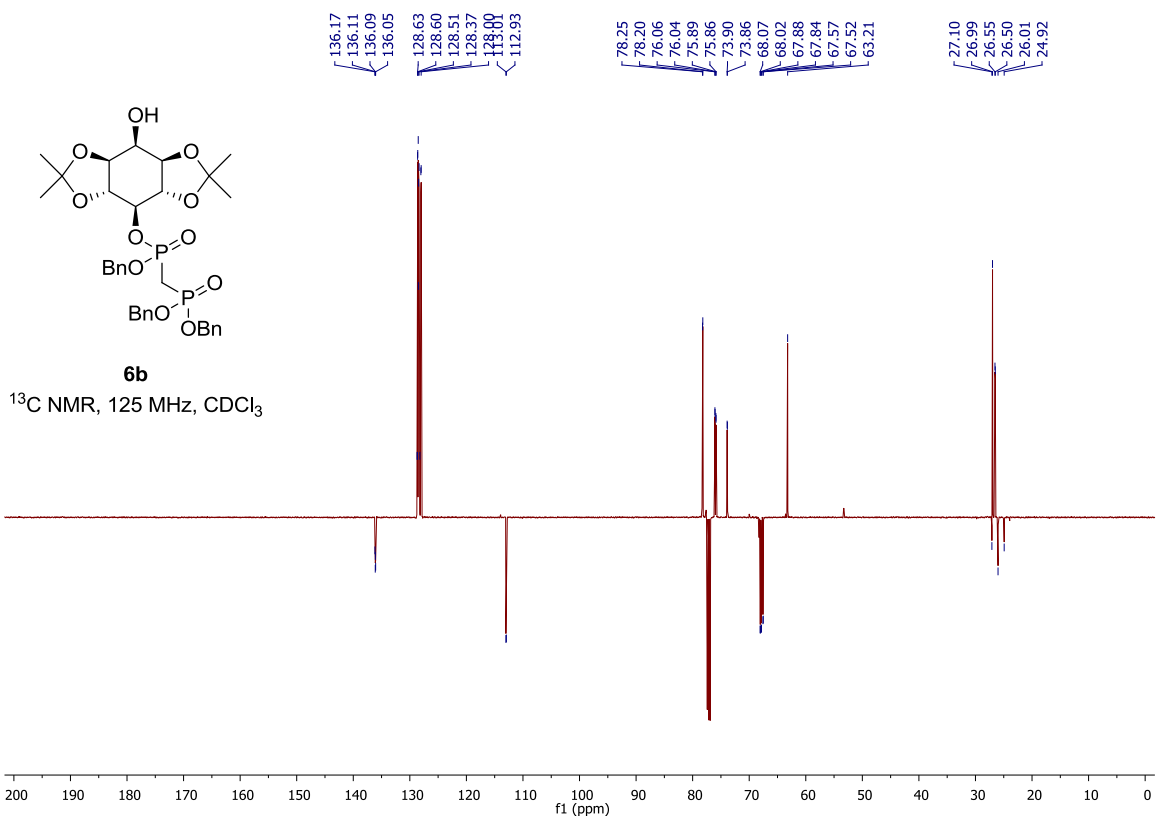


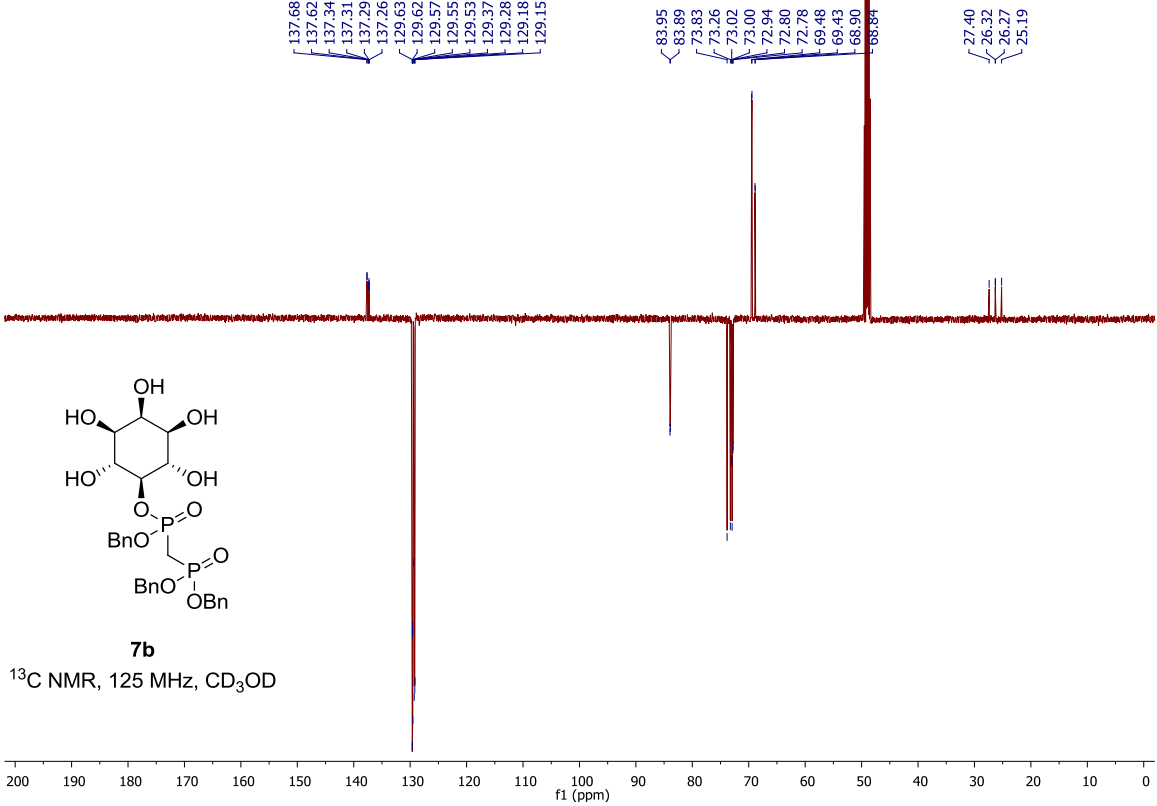
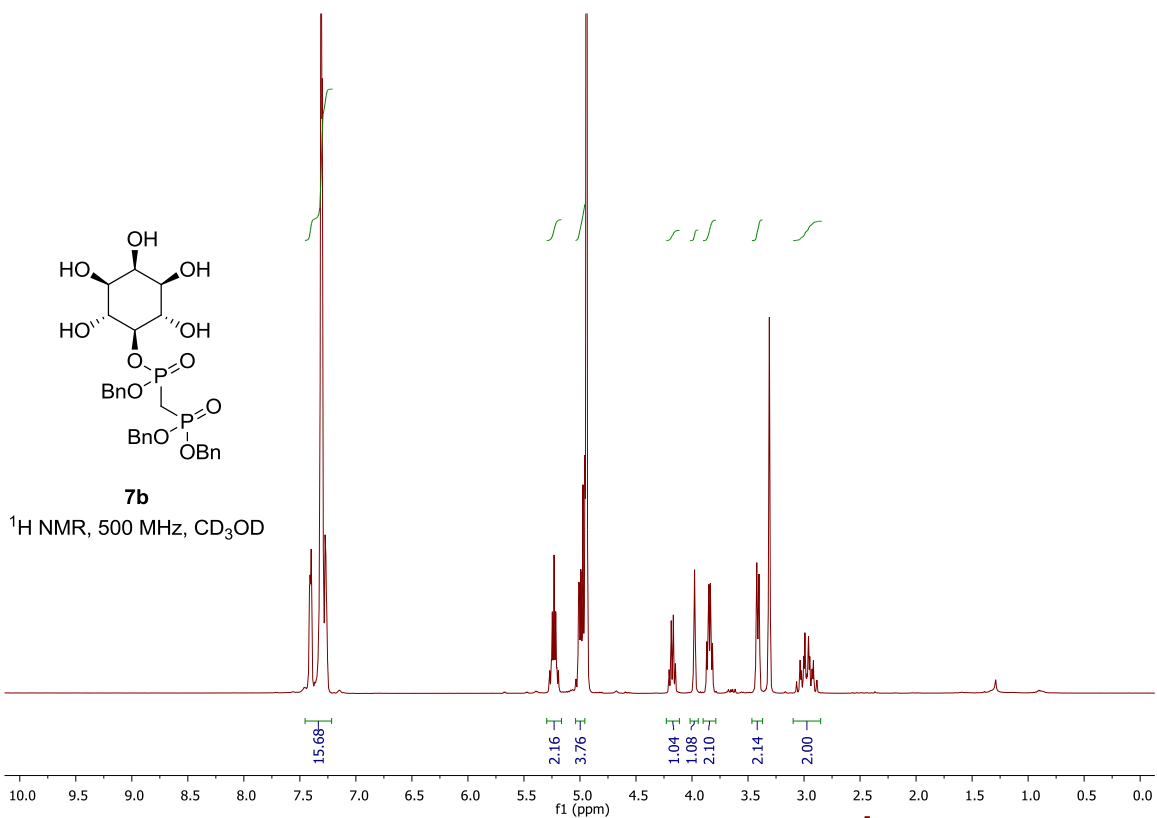


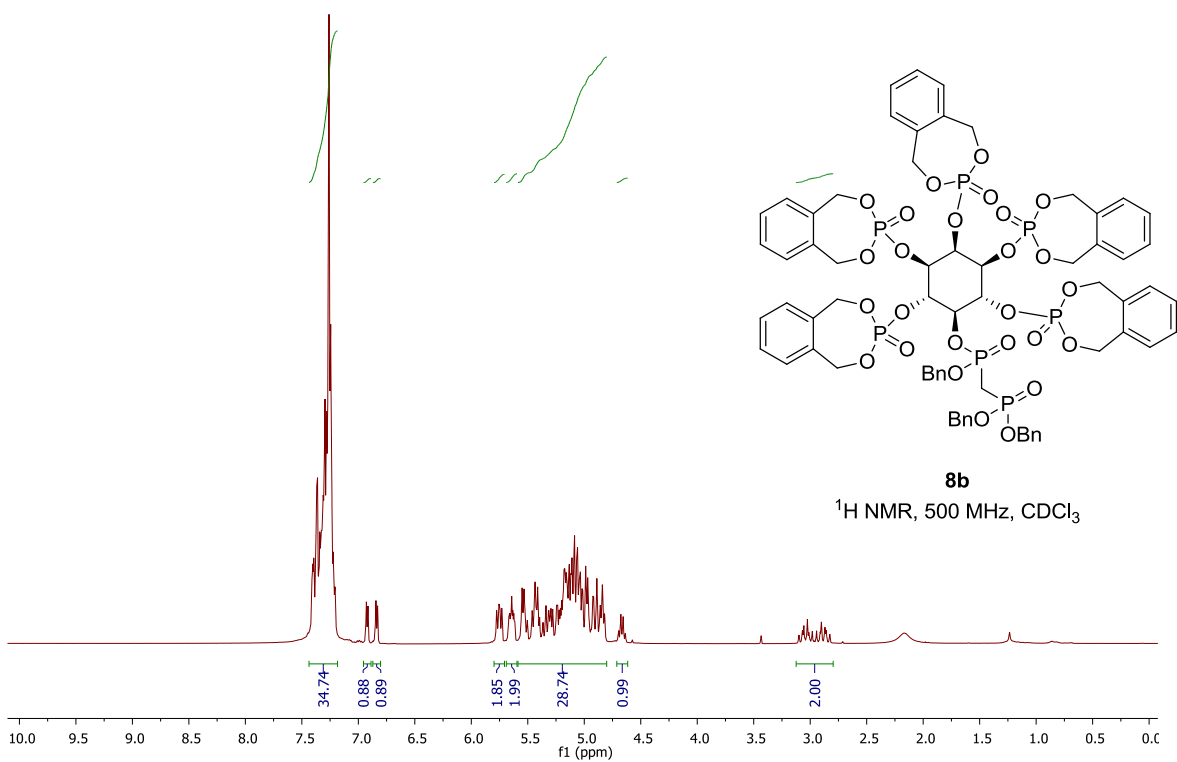
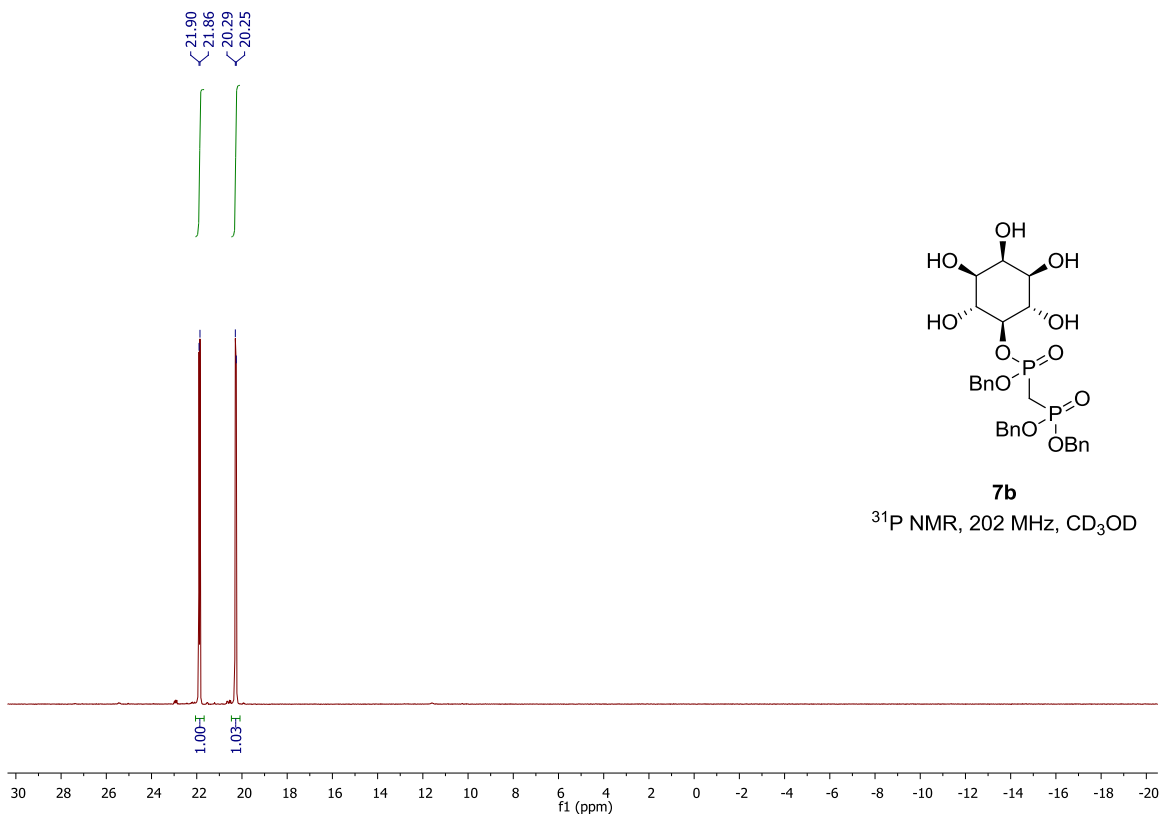


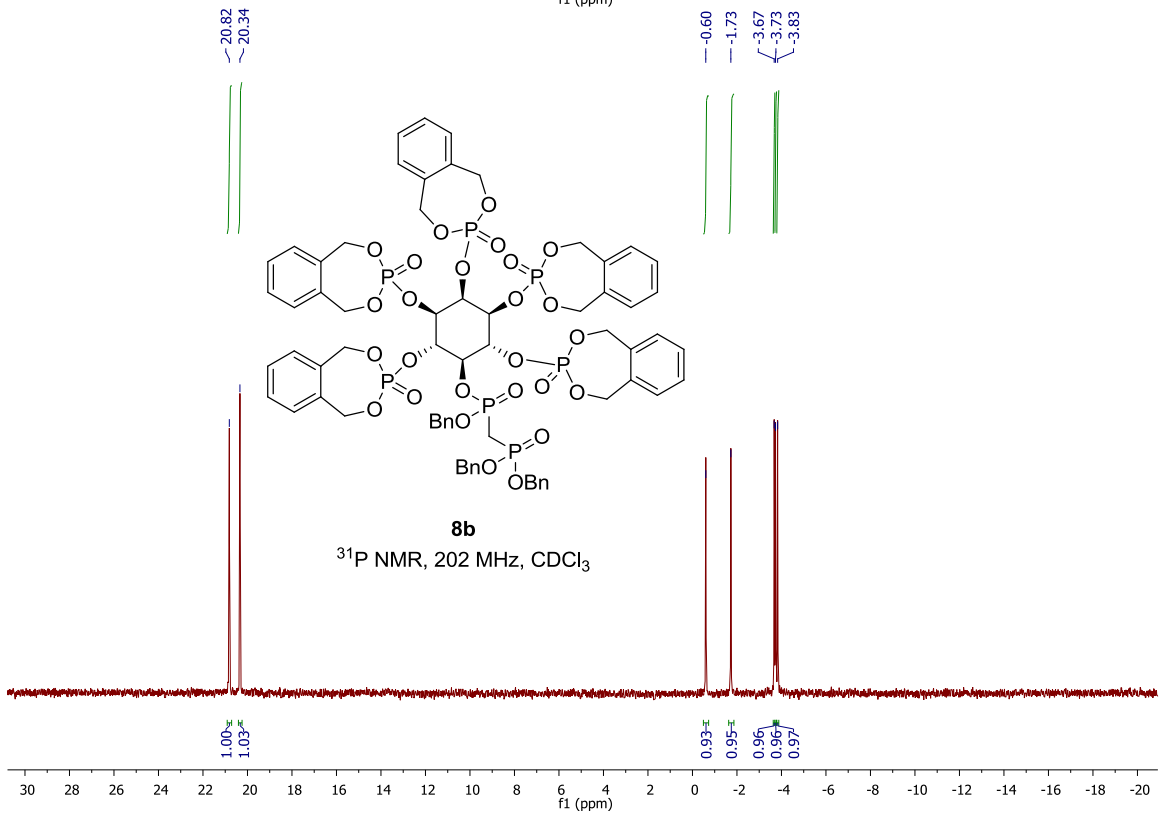
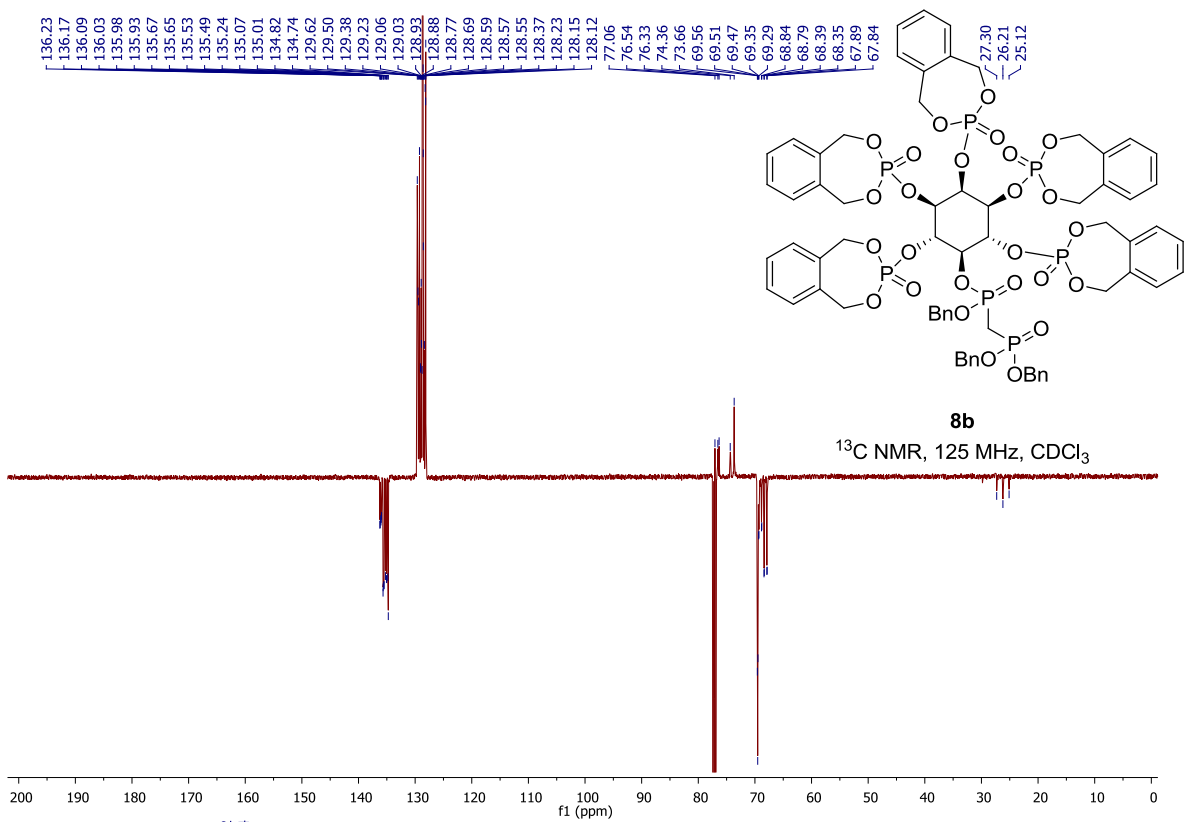


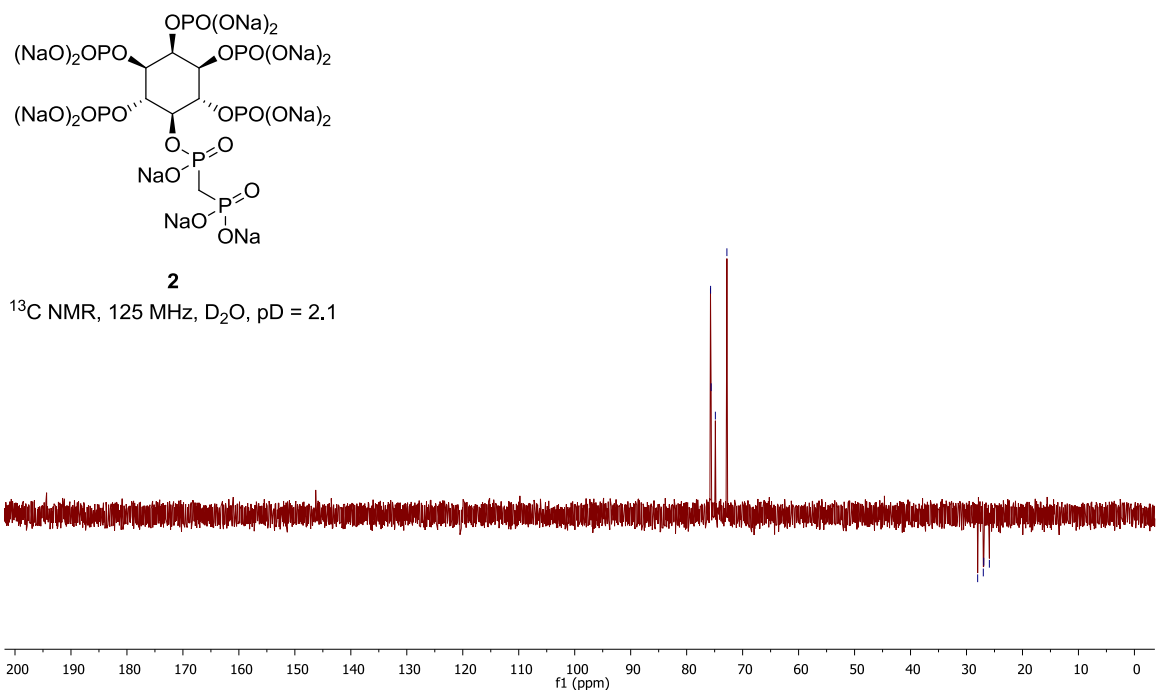
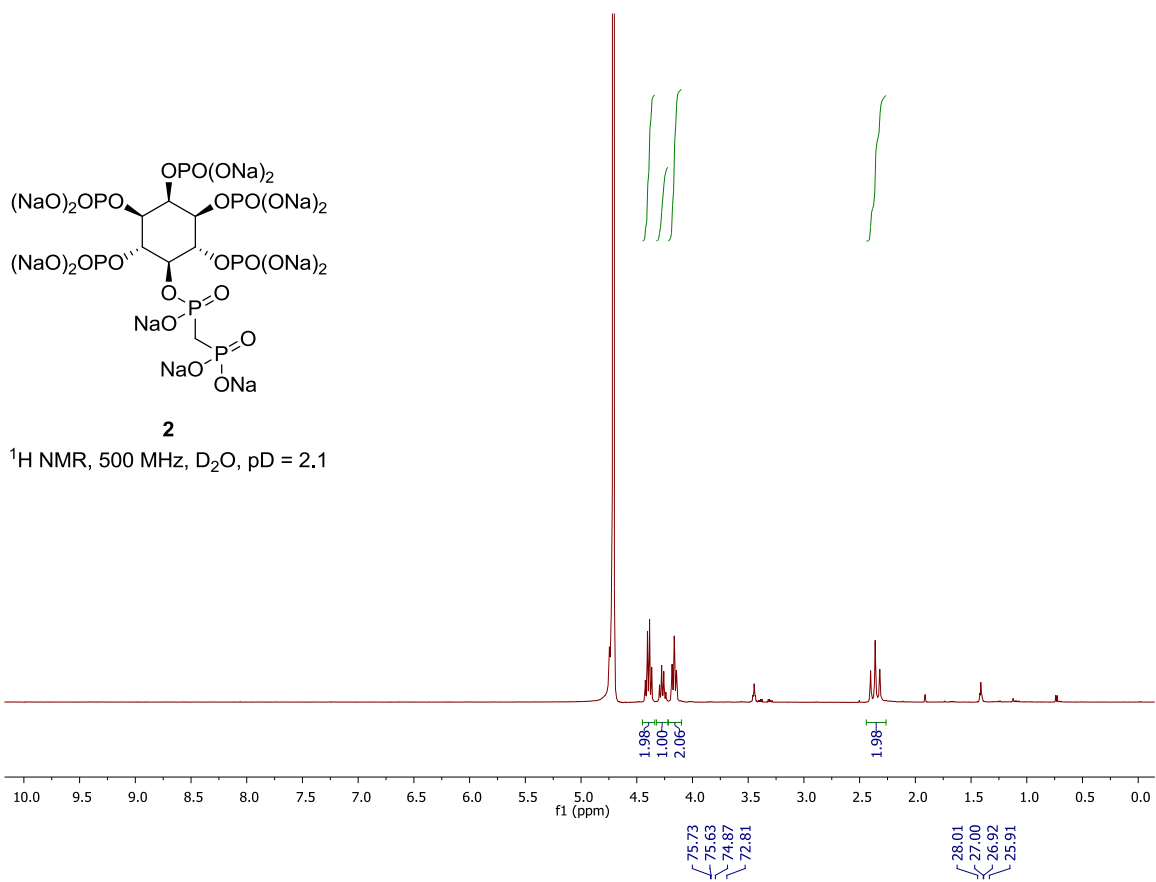


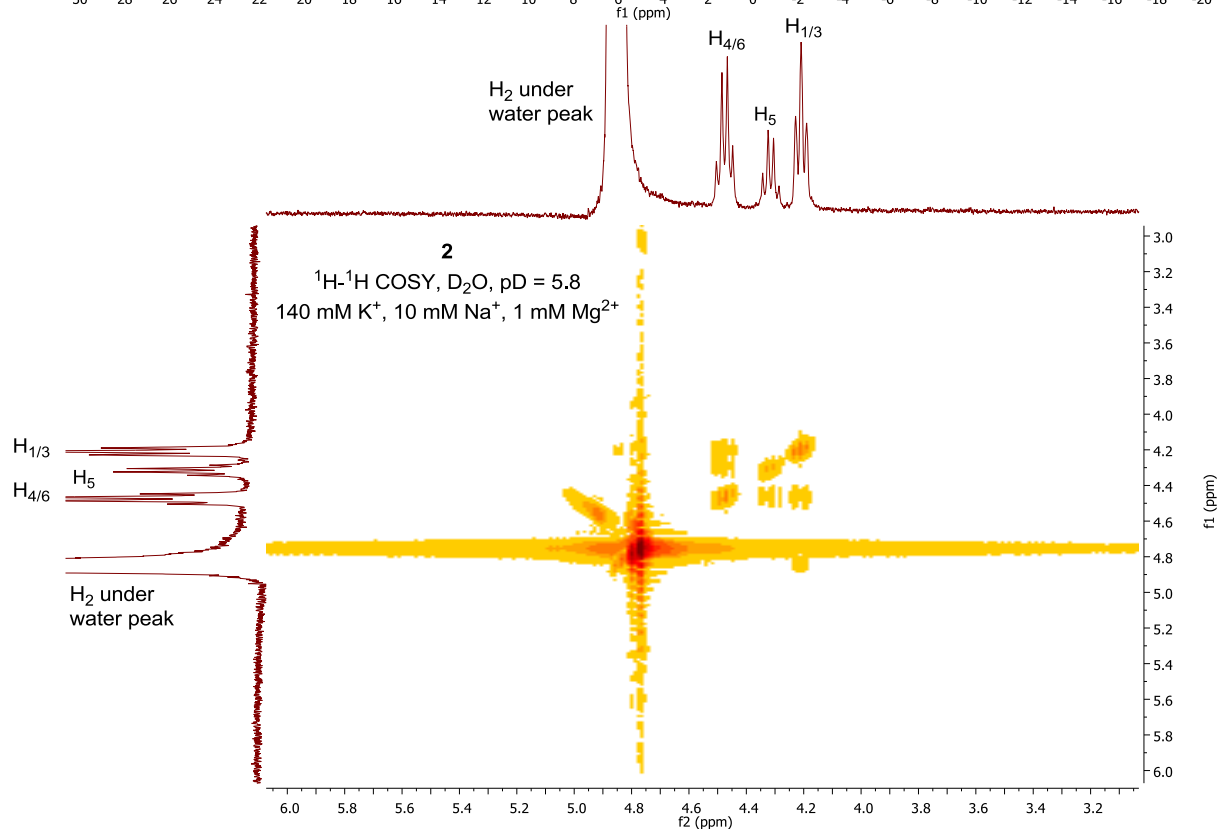
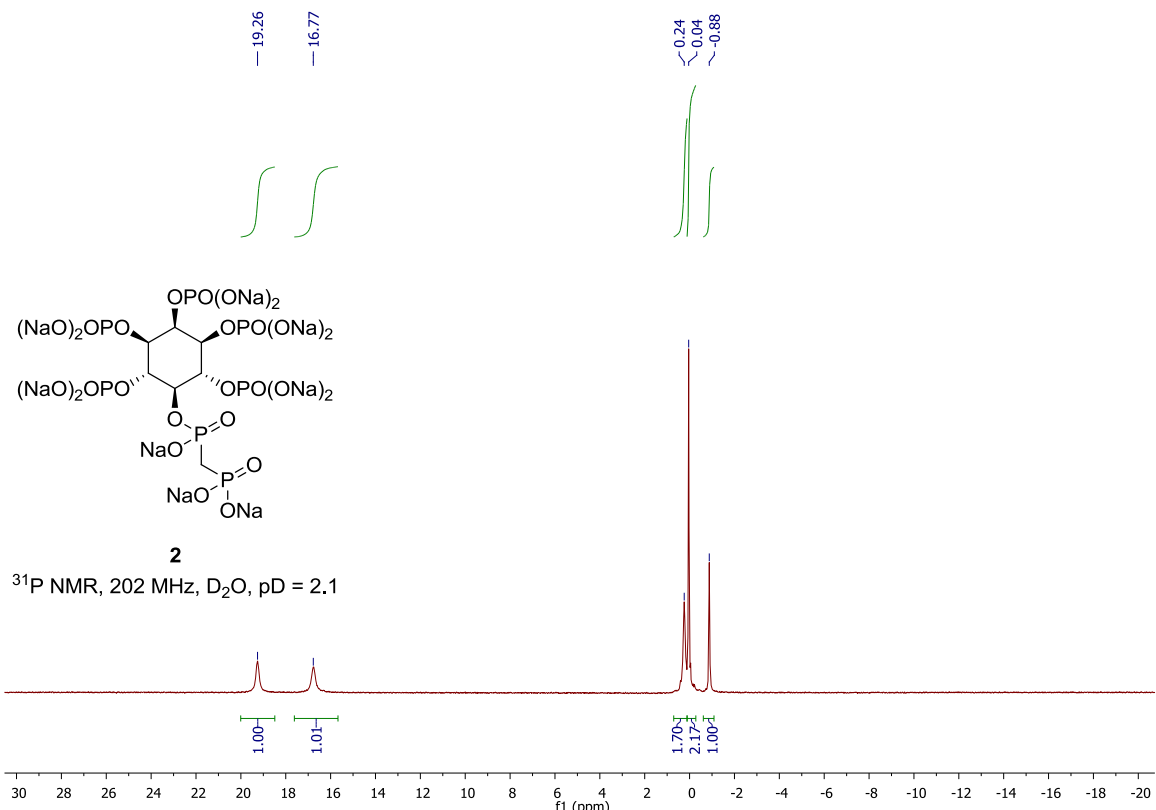


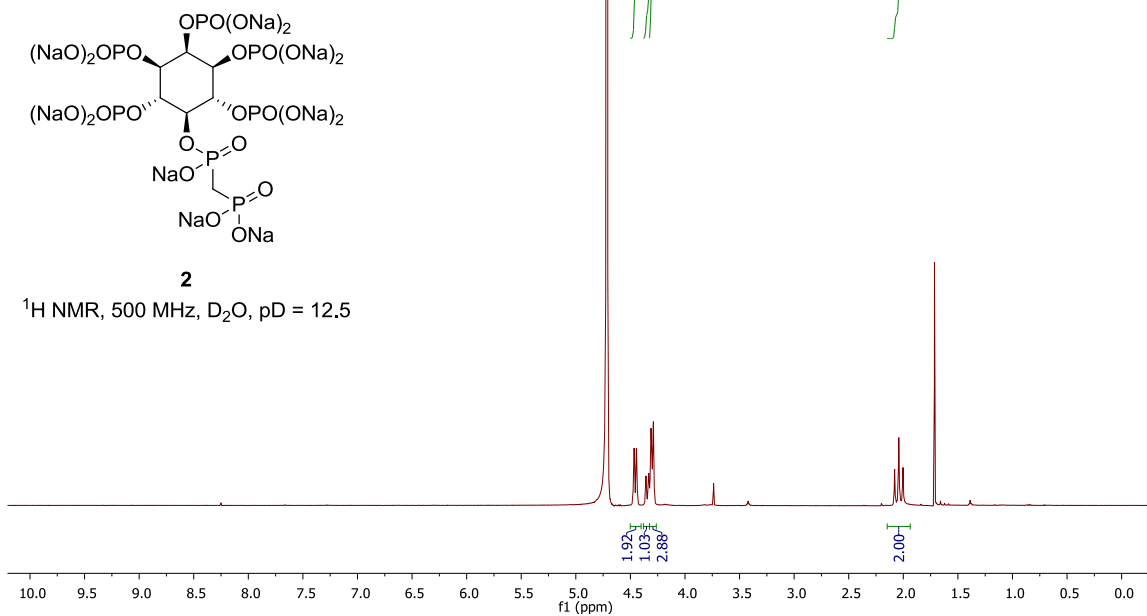
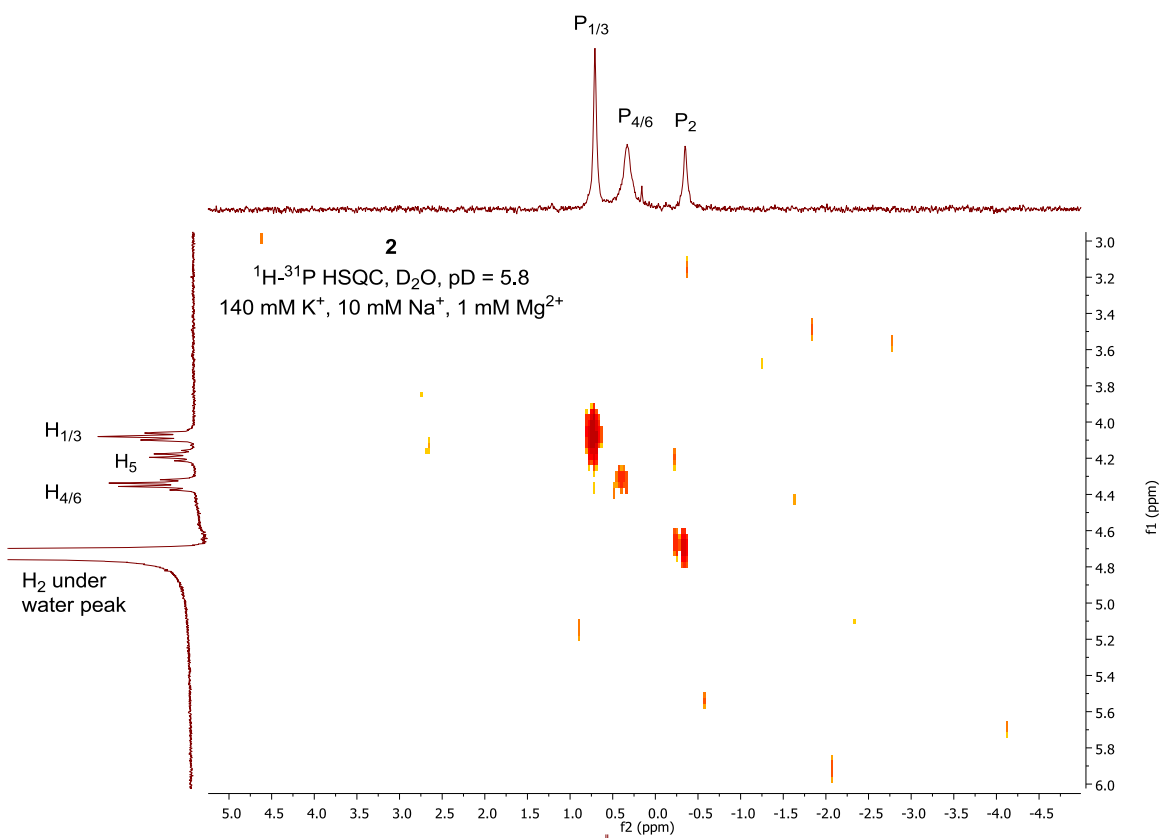


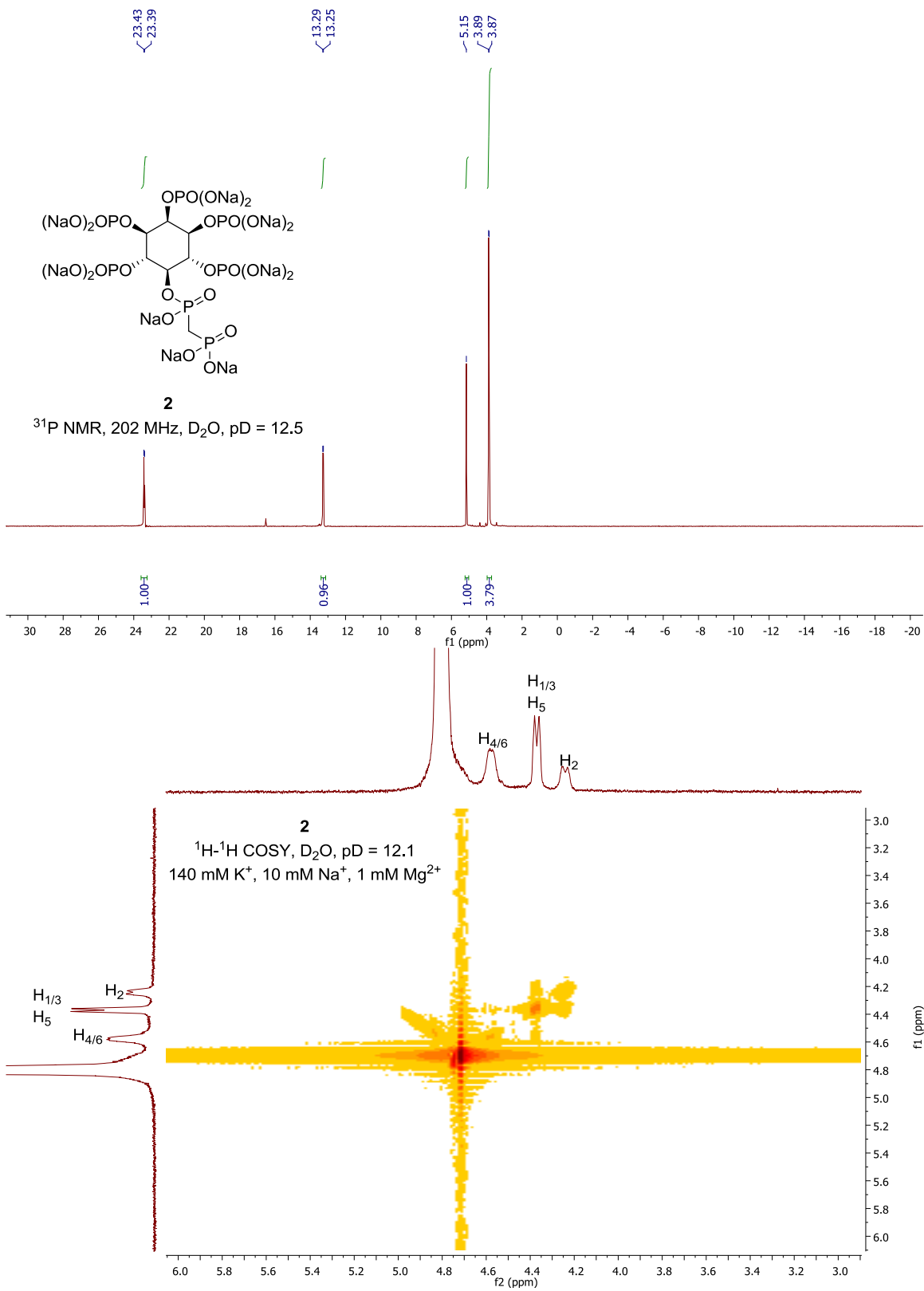


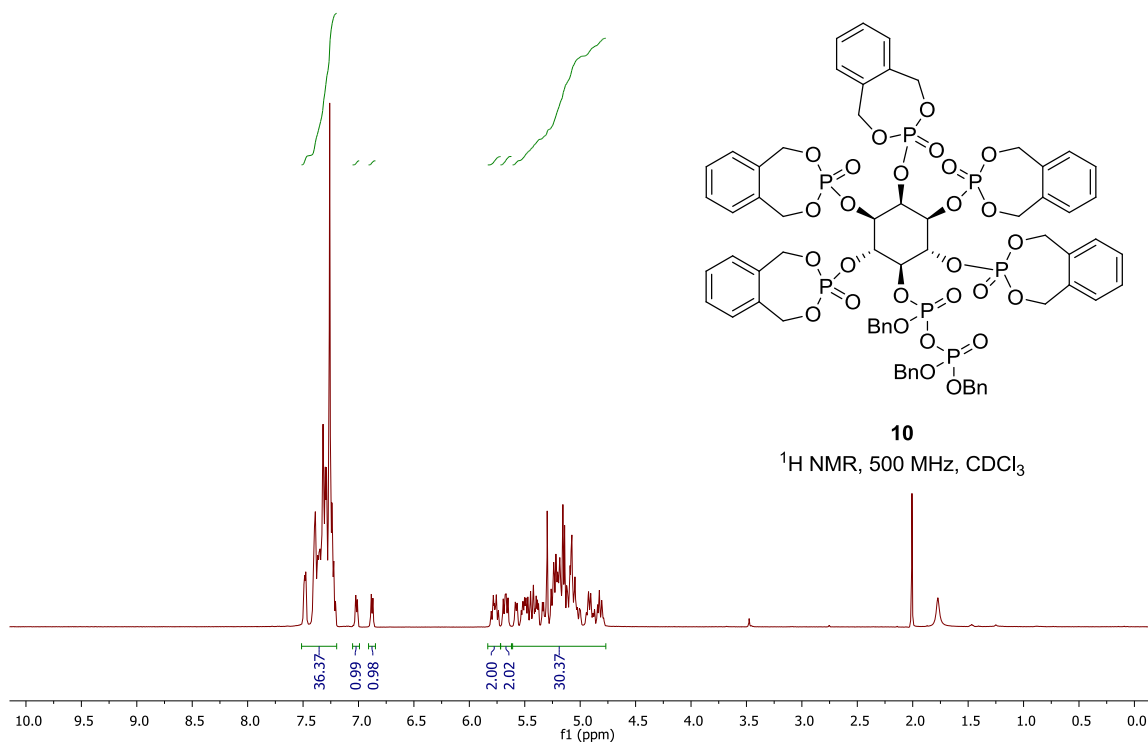
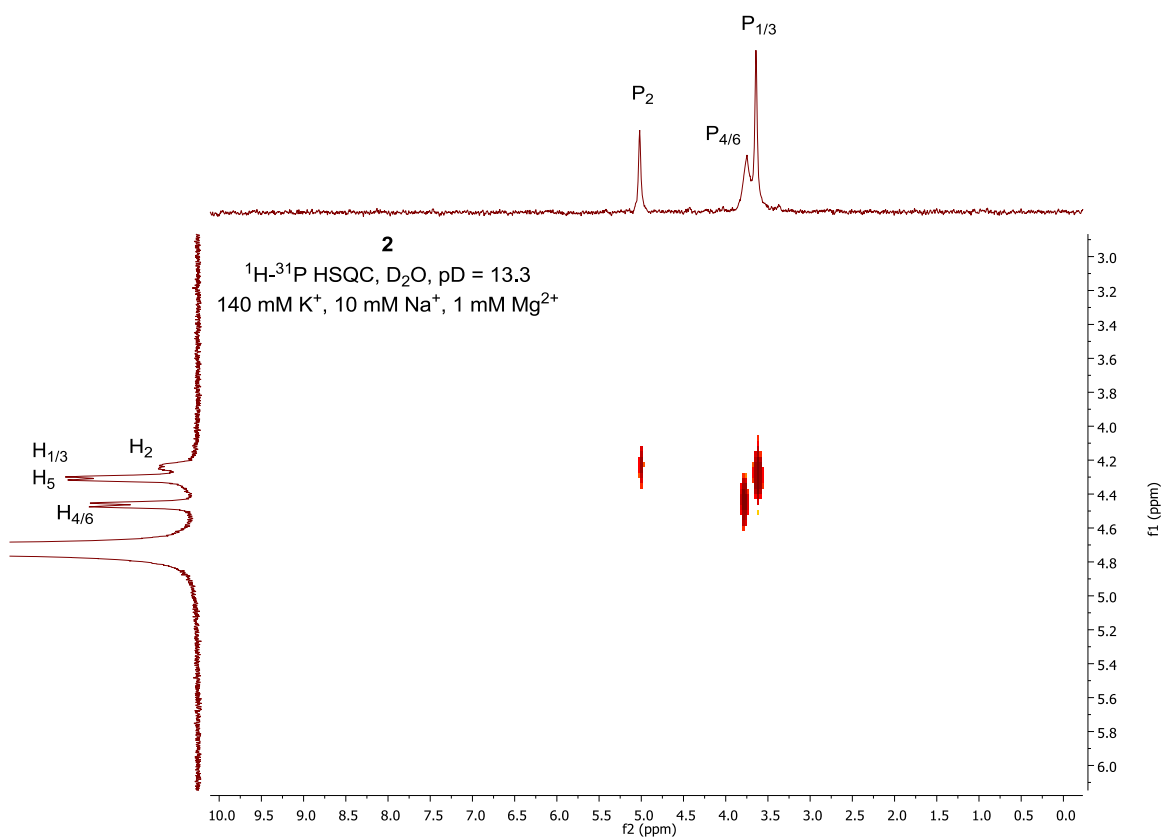


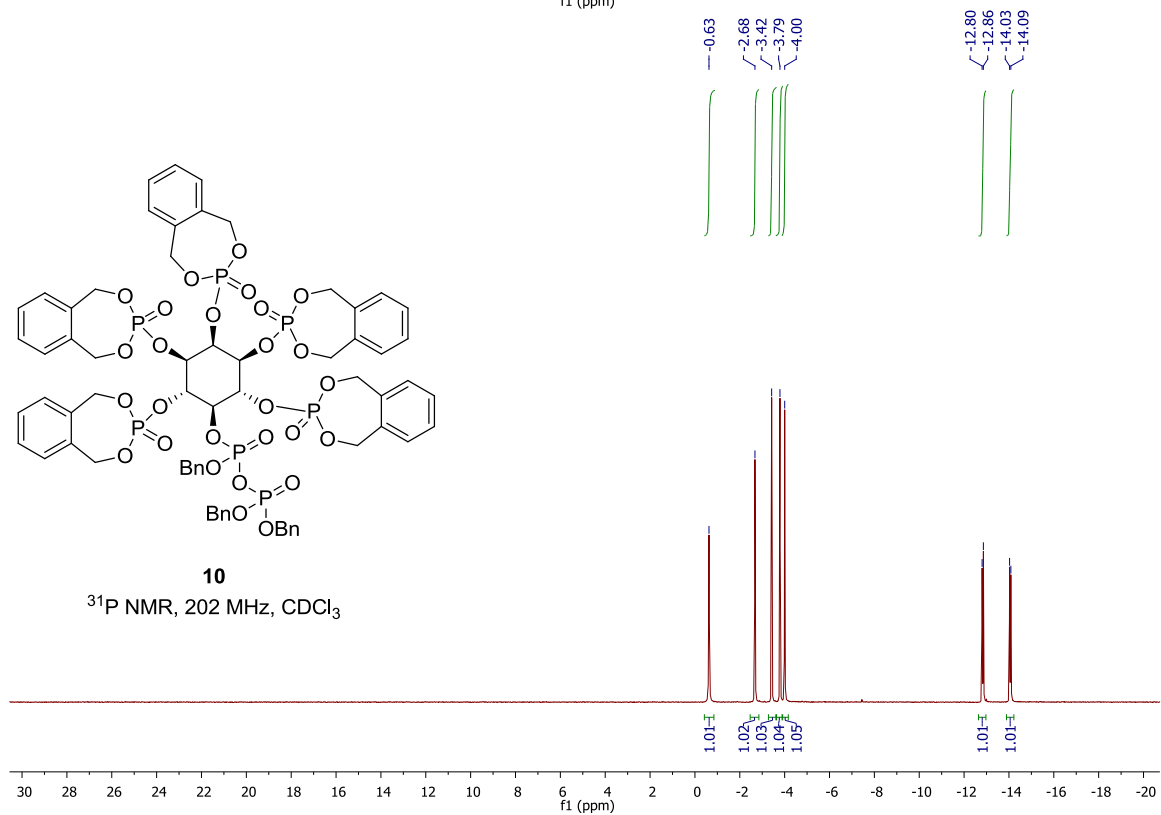
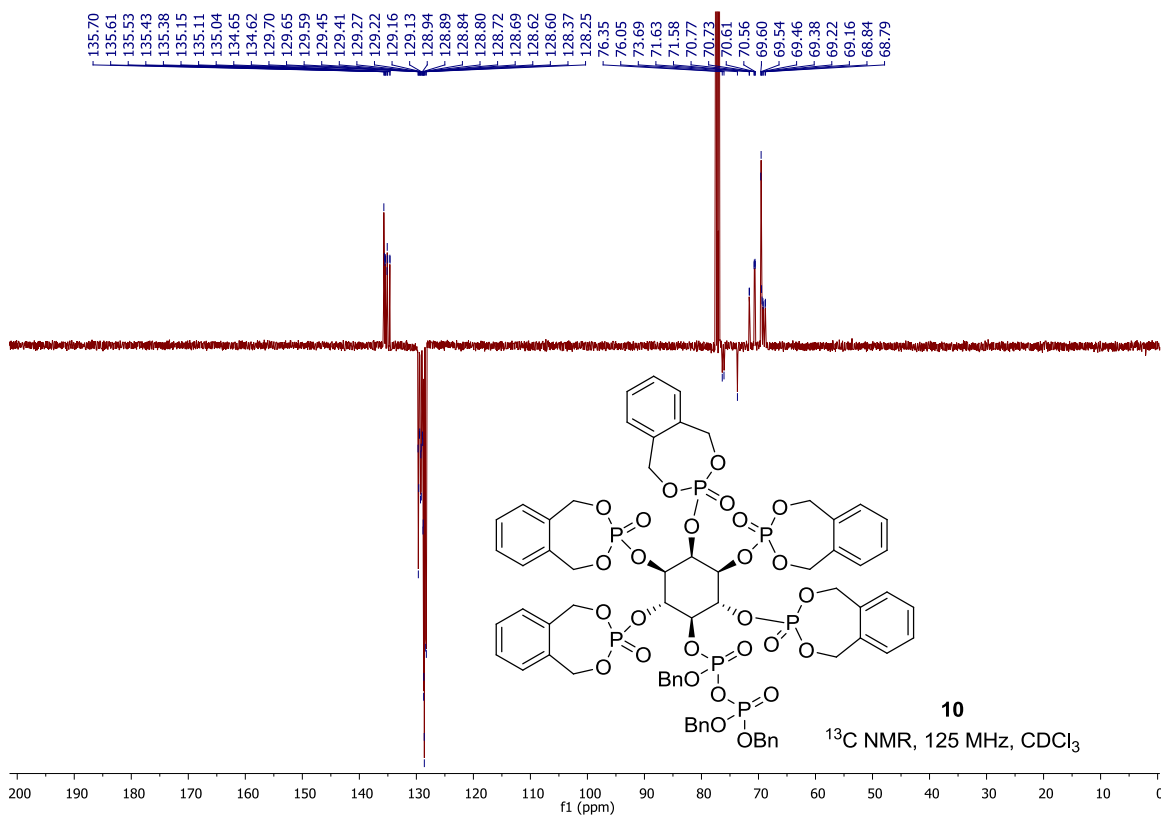


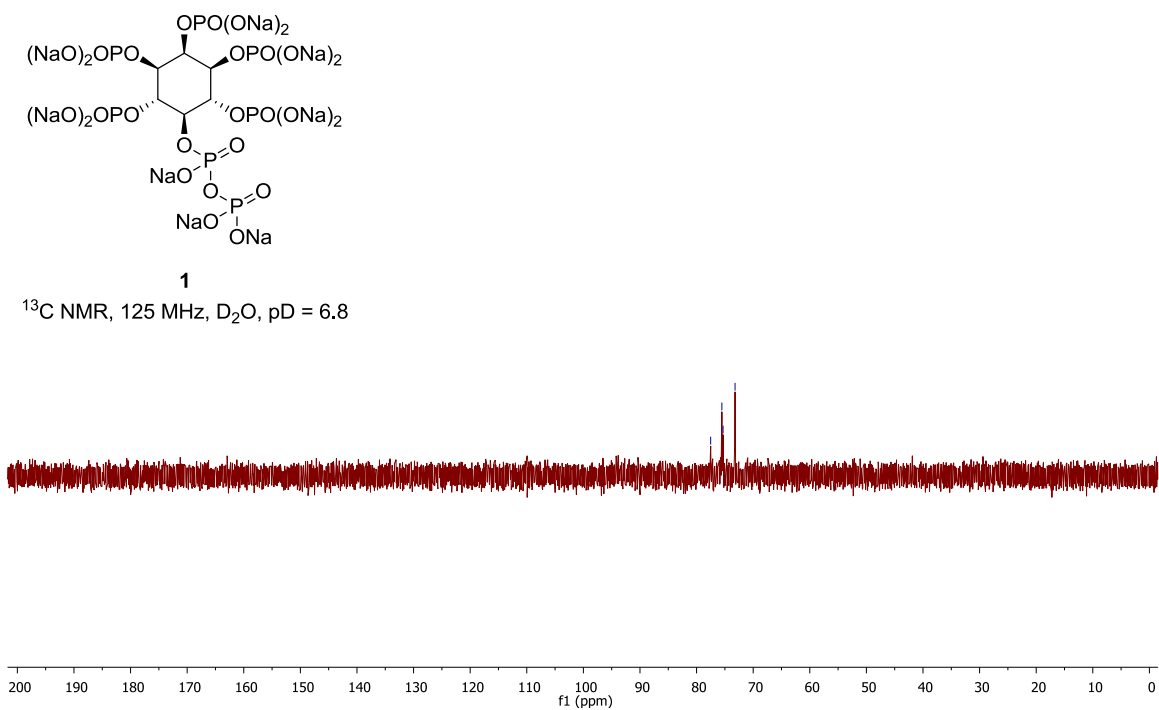
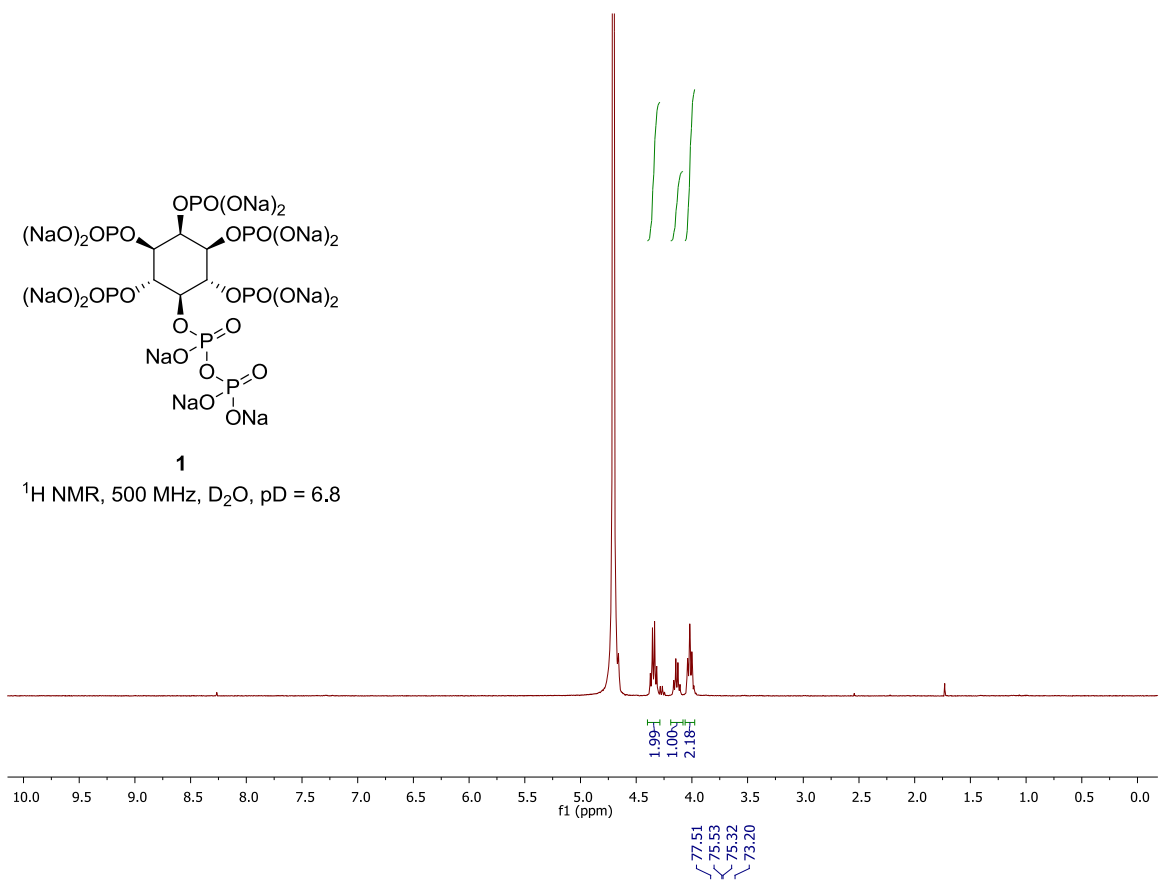


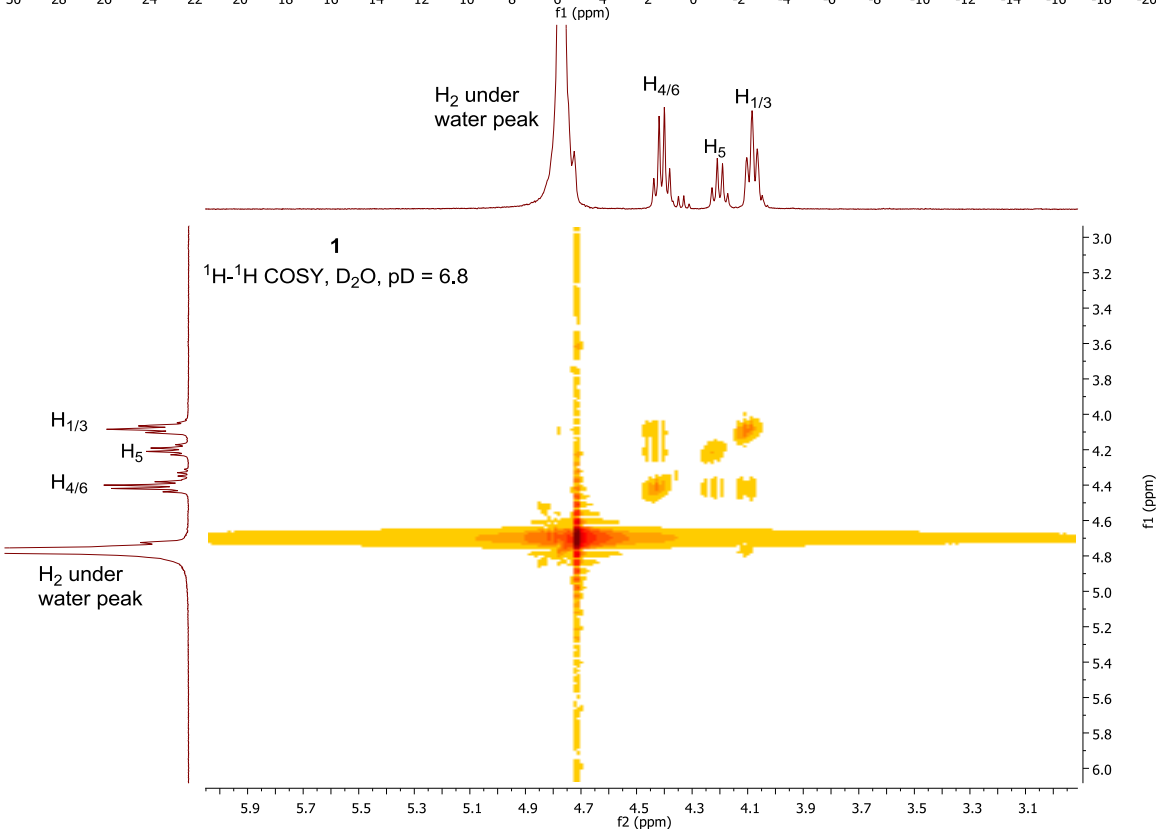
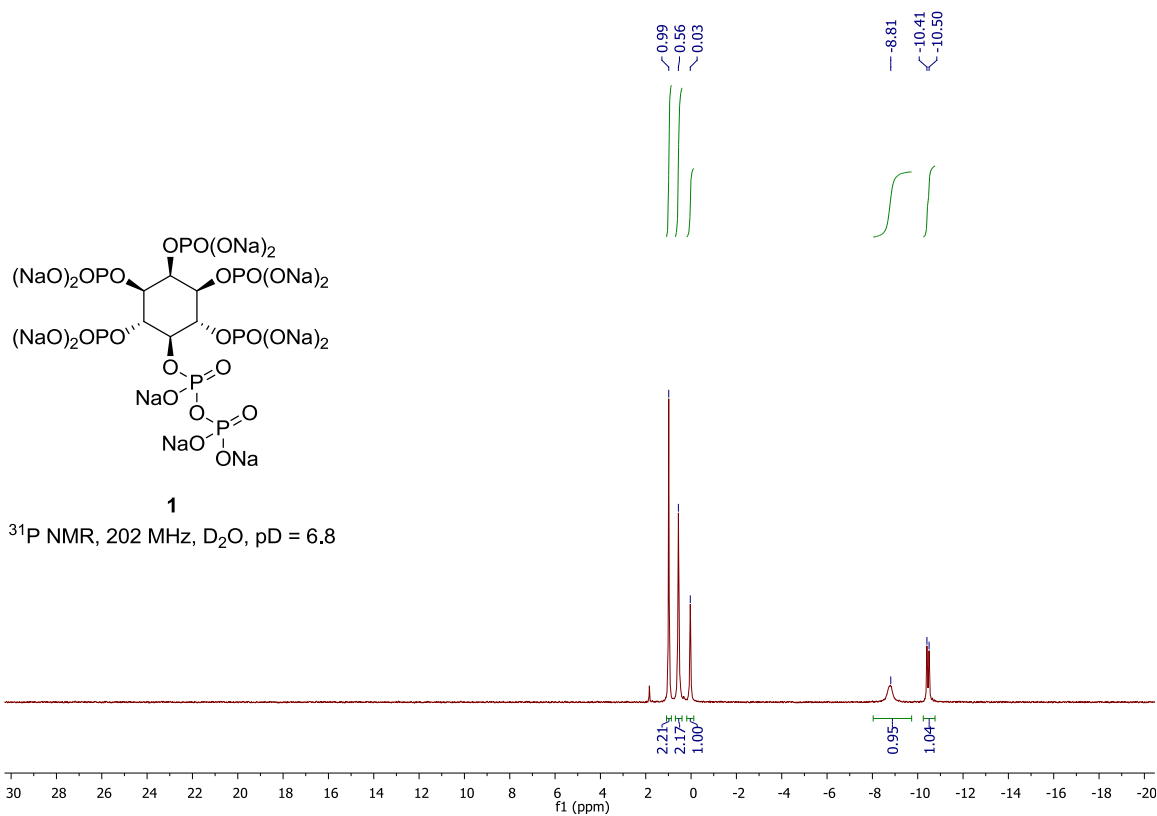


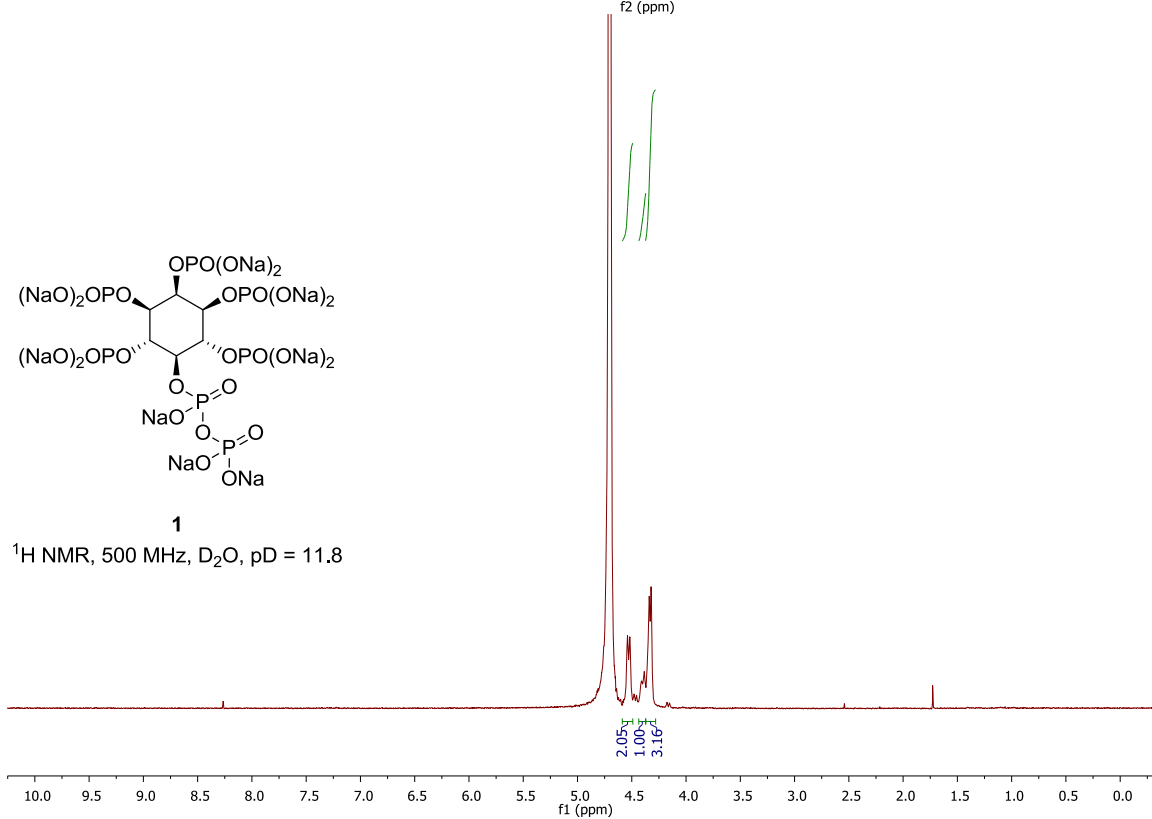
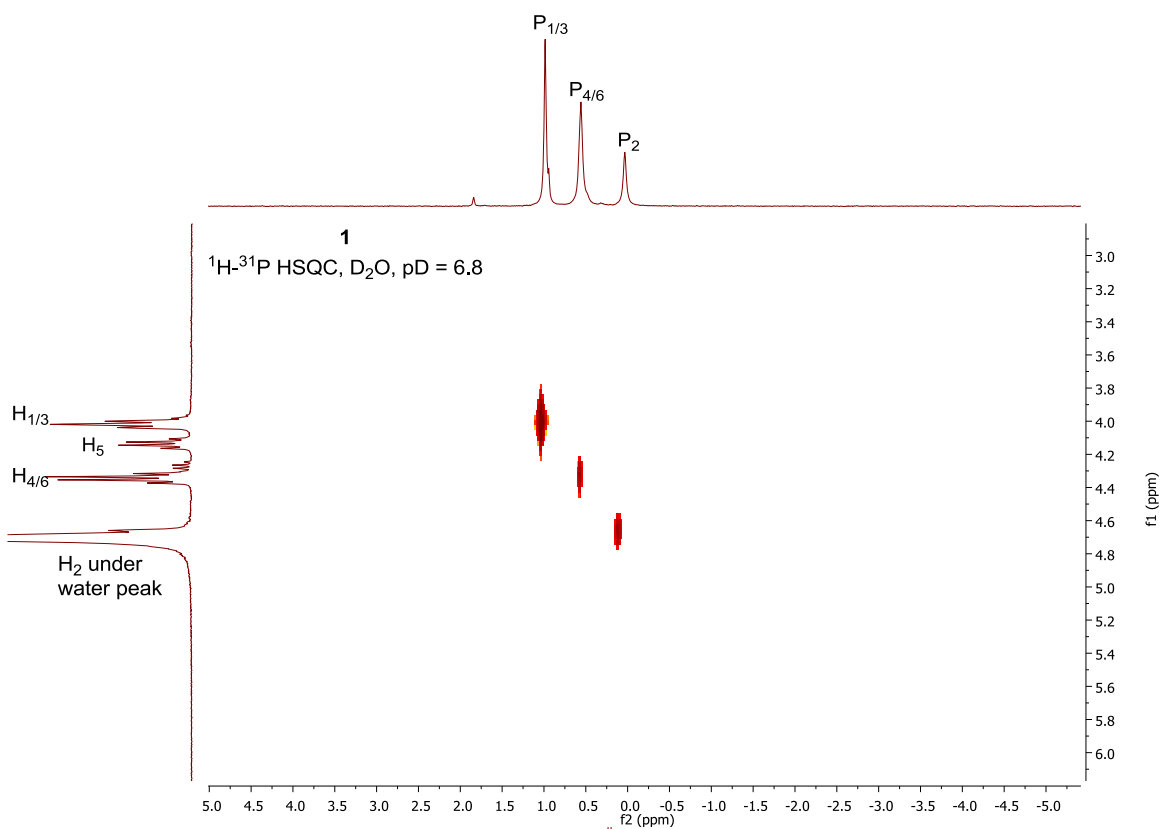


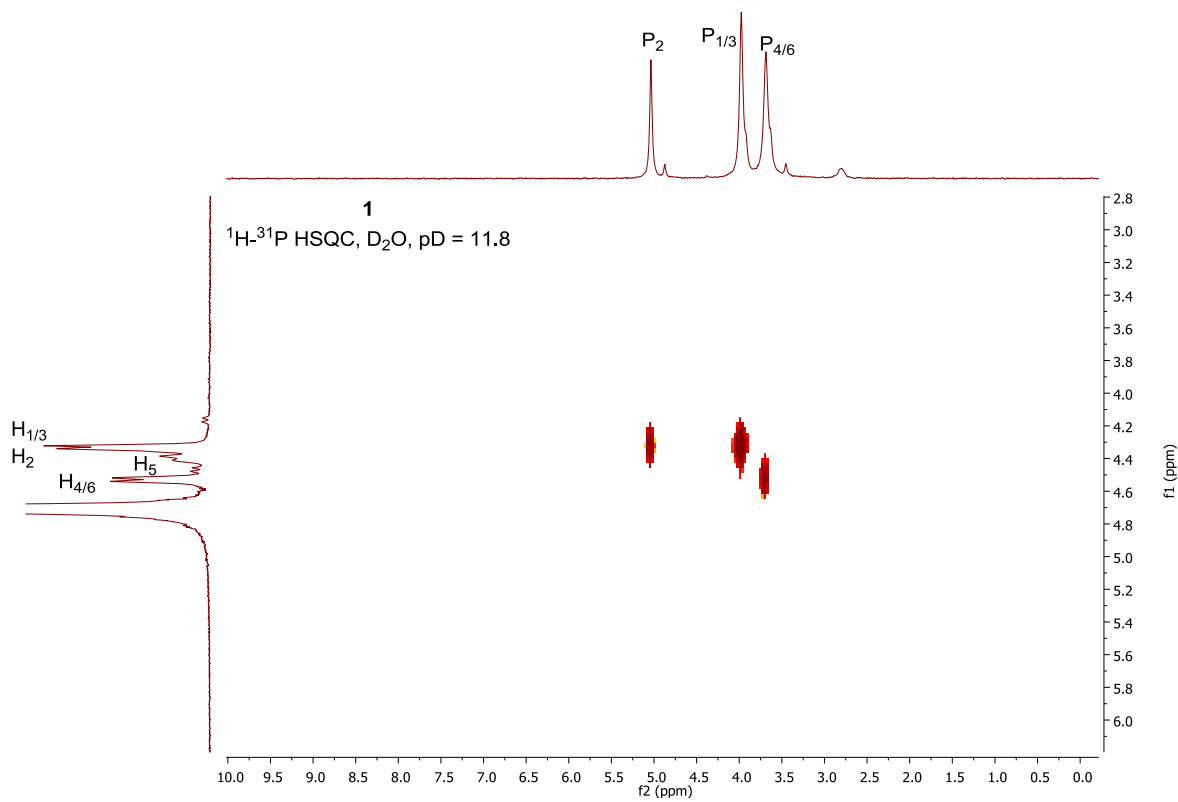
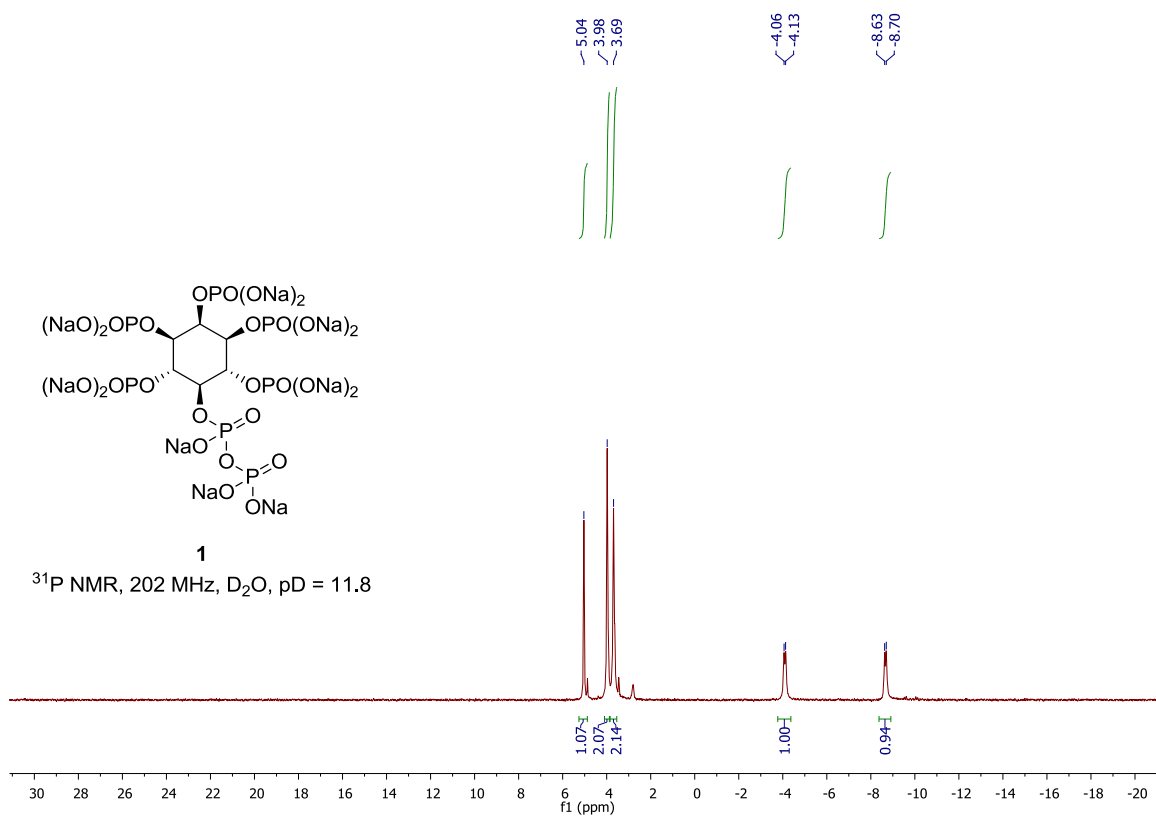












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