Supporting Information

Synthesis and characterization of non-hydrolysable diphosphoinositol polyphosphate second messengers

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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 sieves[1]. Thin layer chromatography (TLC) was performed on EMD Silica Gel 60 F_{254} plates and visualized by fluorescence quenching or $KMnO_4$ staining.

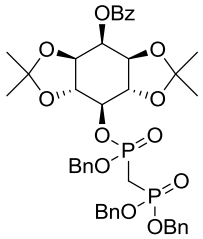
Automated column chromatography was performed using SiliCycle SiliaFlash F60 (40–53 μm) in RediSep® Rf cartridges and normal–phase silica flash columns on a CombiFlash® Rf from Teledyne Isco. High–performance liquid chromatography (HPLC) was performed on a Varian instrument with SD–1 analytical to prep 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% CH_3CN in H_2O for 2 min and a linear gradient from 50% CH_3CN to 90% CH_3CN over 15 min, flow rate: 20 mL/min) or method B (40% CH_3CN in H_2O for 2 min and a linear gradient from 40% CH_3CN to 90% CH_3CN over 15 min, flow rate: 20 mL/min) at an absorbance of 220 nm. Ap_5A hydrolysis by Ddp1 was monitored on an analytical column (3.0 × 150 mm) using method C (0.1 M KH_2PO_4/K_2HPO_4 (pH = 5.5) in H_2O over 6 min, flow rate: 1 mL/min) at an absorbance of 259 nm.

^1H, ^{13}C and ^{31}P 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 deuteroxide 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% H_2O in CH_3CN with 0.1% formic acid was used for positive ion detection mode and 3% CH_3CN in H_2O 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.

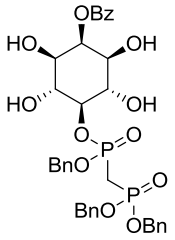
PDK1 and inactive Akt1 were purchased from Merck Millipore. Antibodies for Akt and p-Akt (T308) were purchased from Cell Signaling Technology.

II. Chemical Synthesis



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

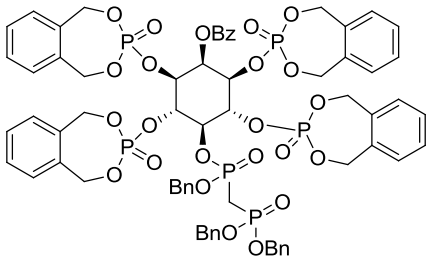
A solution of [bis(benzyloxy)phosphorylmethyl]phosphonic acid monobenzyl ester[2] (446 mg, 0.999 mmol) in toluene (8 mL) was treated with oxalyl chloride (172 μL, 254 mg, 2.00 mmol) and a catalytic amount of anhydrous DMF at 0 ℃ for 1h. The crude phosphorochloridate was obtained after the mixture was 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 ^{o}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. NH_4Cl (5 mL) and water (10 mL), the mixture was extracted by EtOAc (25 mL × 3) and dried over Na_2SO_4. The organic phase was removed under vacuum and the residue was purified by flash chromatography (0% to 3% MeOH in CH_2Cl_2) to give the title compound (368 mg, 70%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): 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); ^{13}C NMR (125 MHz, $CDCl_3$): 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); ^{31}P NMR (202 MHz, $CDCl_3$): δ 20.05 (d, *J* = 9.6 Hz, 1P), 19.70 (d, *J* = 9.6 Hz, 1P); HRMS $[M+H]^+$ calcd for $C_{41}H_{47}O_{12}P_2^+$ 793.2538, found 793.2535.



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

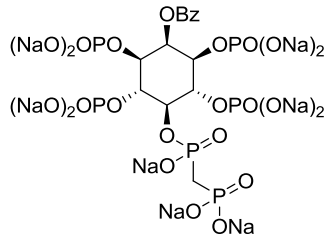
Water (68 μL, 68 mg, 3.8 mmol) and *p*–toluenesulfonic acid monohydrate (14 mg, 0.074 mmol) were added to a solution of the acetonide **6a** (300 mg, 0.378 mmol) in acetone (15 mL). After stirring overnight, the mixture was dried under vacuum and purified by flash chromatography (2% to 10% MeOH in CH_2Cl_2) to give the title compound (178 mg, 66%) as a colorless oil. ^{1}H NMR (500 MHz,

$CDCl_3$): 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); ^{13}C NMR (125 MHz, $CDCl_3$): 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); ^{31}P NMR (202 MHz, $CDCl_3$): δ 21.72 (d, *J* = 7.1 Hz, 1P), 20.22 (d, *J* = 7.1 Hz, 1P); HRMS $[M+H]^+$ calcd for $C_{35}H_{39}O_{12}P_2^+$ 713.1911, found 713.1913.



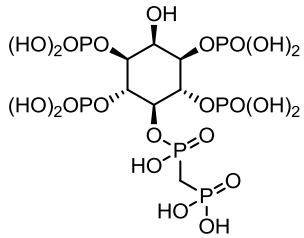
2-*O*-Benzoyl-5-*O*-[[bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-1,3,4,6-tetra-*O*-(*o*-xylylene)phosphate-*myo*-inositol (8a).

N,*N*–diethyl–1,5–dihydro–2,4,3–benzodioxaphosphepin–3–amine (212 mg, 0.886 mmol) in CH_3CN and 1*H*–tetrazole (2.5 mL, 0.45 M in CH_3CN, 1.1 mmol) were added to a solution of the alcohol **7a** (79 mg, 0.11 mmol) in CH_3CN (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 CH_3CN (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% $Na_2S_2O_3$ (25 mL) was added to quench the reaction and the organic CH_3CN was removed under vacuum. EtOAc (50 mL) was added to the mixture and the organic layer was washed by sat. aq. $NaHCO_3$ (25 mL) and sat. aq. NaCl (25 mL). The organic layer was dried over Na_2SO_4, concentrated under vacuum and purified by flash chromatography (0% to 5% MeOH in CH_2Cl_2) and preparative HPLC (Method B, t_R = 13.5 min) to give the title compound (46 mg, 32%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): 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); ^{13}C NMR (125 MHz, $CDCl_3$): 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); ^{31}P NMR (202 MHz, $CDCl_3$): 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 $C_{67}H_{67}O_{24}P_6^+$ 1441.2442, found 1441.2443.



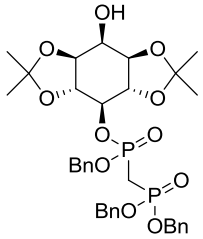
2-*O*-Benzoyl-*myo*-inositol 1,3,4,6-tetra-phosphate-5-methylenediphosphonate undecabasic sodium salt (4).

$NaHCO_3$ (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/H_2O (40:7, 3 mL) under N_2 before purging with H_2 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. ^{1}H NMR (500 MHz, D_2O, 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); ^{13}C NMR (125 MHz, D_2O, 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); ^{31}P NMR (202 MHz, D_2O, 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 $C_{14}H_{23}O_{24}P_6^-$ 760.9010, found 760.8990.



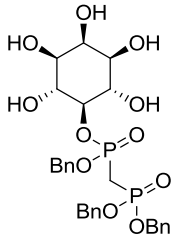
***Myo*-inositol 1,3,4,6-tetra-phosphate-5-methylenediphosphonate (3).**

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 CH_2Cl_2 (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. ^{1}H NMR (500 MHz, D_2O): 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); ^{13}C NMR (125 MHz, D_2O): δ 76.0, 75.2, 74.1 (d, *J* = 5.3 Hz), 69.7, 27.0 (dd, *J* = 136.2, 126.9 Hz); ^{31}P NMR (202 MHz, D_2O): δ 18.71 (s, 2P), 0.02 (s, 2P), –0.51 (d, *J* = 9.3 Hz, 2P); HRMS $[M–H]^-$ calcd for $C_7H_{19}O_{23}P_6^-$ 656.8748, found 656.8741.



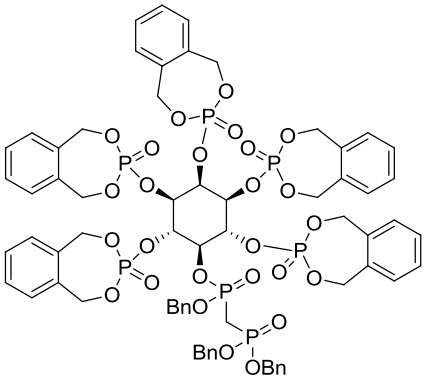
5-*O*-[[Bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-1,6:3,4-di-*O*-isopropylidene-*myo*-inositol (6b).

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. NH_4Cl (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 Na_2SO_4. The organic phase was removed under vacuum and the residue was purified by flash chromatography (0% to 5% MeOH in CH_2Cl_2) to give the title compound (217 mg, 74%) as a white solid. ^{1}H NMR (500 MHz, $CDCl_3$): 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); ^{13}C NMR (125 MHz, $CDCl_3$): 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); ^{31}P NMR (202 MHz, $CDCl_3$): δ 20.10 (d, *J* = 10.8 Hz, 1P), 19.72 (d, *J* = 10.8 Hz, 1P); HRMS $[M+H]^+$ calcd for $C_{34}H_{43}O_{11}P_2^+$ 689.2276, found 689.2275.



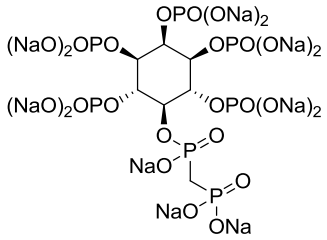
5-*O*-[[Bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-*myo*-inositol (7b).

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 CH_2Cl_2) to give the title compound (153 mg, 80%) as a colorless oil. ^{1}H NMR (500 MHz, CD_3OD): 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); ^{13}C NMR (125 MHz, CD_3OD): 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); ^{31}P NMR (202 MHz, CD_3OD): δ 21.88 (d, *J* = 8.4 Hz, 1P), 20.27 (d, *J* = 8.4 Hz, 1P); HRMS $[M+H]^+$ calcd for $C_{28}H_{35}O_{11}P_2^+$ 609.1650, found 609.1649.



5-*O*-[[Bis(benzyloxy)phosphorylmethyl]benzyloxyphosphoryl]-1,2,3,4,6-penta-*O*-(*o*-xylylene)-phosphate-*myo*-inositol (8b).

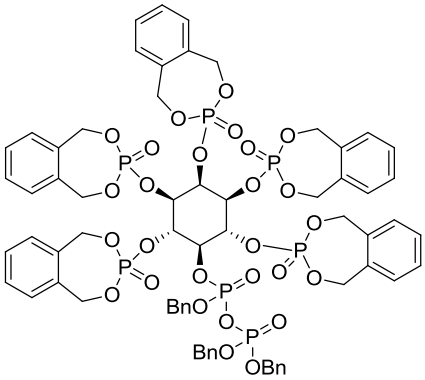
N,*N*–diethyl–1,5–dihydro–2,4,3–benzodioxaphosphepin–3–amine (212 mg, 0.886 mmol) in CH_3CN (2 mL) and 1*H*–tetrazole (3.0 mL, 0.45 M in CH_3CN, 1.3 mmol) were added to a solution of the alcohol **7b** (54 mg, 0.089 mmol) in CH_3CN (3 mL) at 0 ^oC. The mixture was stirred for 2 h at 0 ^oC followed by 36 h at room temperature. A solution of mCPBA (398 mg, 1.77 mmol) in CH_3CN (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 ^oC followed by 2 h at room temperature. 10% $Na_2S_2O_3$ (25 mL) was added to quench the reaction and CH_3CN was removed under vacuum. EtOAc (50 mL) was added to the mixture and the organic layer was washed with sat. aq. $NaHCO_3$ (20 mL) and sat. aq. NaCl (20 mL). The organic layer was dried over Na_2SO_4, concentrated under vacuum and purified by flash chromatography (0% to 5% MeOH in CH_2Cl_2) 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, $CDCl_3$): 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); ^{13}C NMR (125 MHz, $CDCl_3$): 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); ^{31}P NMR (202 MHz, $CDCl_3$): 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 $C_{68}H_{70}O_{26}P_7^+$ 1519.2314, found 1519.2318.



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

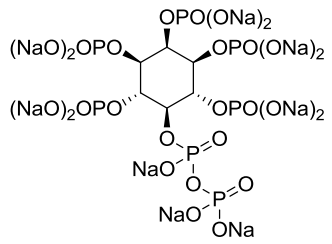
$NaHCO_3$ (28 mg, 0.33 mmol) and palladium black (71 mg, 0.67 mmol) were added to a solution of **8b** (39 mg, 0.026 mmol) in *t*–BuOH/H_2O (40:7, 3.5 mL) under N_2 before purging with H_2 gas. The

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. ^{1}H 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); ^{1}H 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 $[M–H]^-$ calcd for $C_7H_{20}O_{26}P_7^-$ 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 °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 °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. ^{1}H 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 $[M+H]^+$ calcd for $C_{67}H_{68}O_{27}P_7^+$ 1521.2106, found 1521.2104.



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

$NaHCO_3$ (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/H_2O (40:7, 2 mL) under N_2 before purging with H_2 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. ^{1}H NMR (500 MHz, D_2O, 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); ^{1}H NMR (500 MHz, D_2O, 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); ^{13}C NMR (125 MHz, D_2O, pD = 6.8): δ 77.5, 75.5, 75.3, 73.2; ^{31}P NMR (202 MHz, D_2O, 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); ^{31}P NMR (202 MHz, D_2O, 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 $C_6H_{18}O_{27}P_7^-$ 738.8204, found 738.8188.

III. NMR Titration

NMR samples containing 1 mM 5PP-IP_5 (**1**) or 5PCP-IP_5 (**2**), 140 mM KCl, 10 mM NaCl and 1mM $MgCl_2$ in D_2O (0.5 mL) were prepared at varying pD from 2.8 to 12.4. ^1H NMR and ^{31}P 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 D_2O, were used to calibrated chemical shifts. Proton chemical shifts were assigned by ^1H–^1H and ^1H–^{31}P correlation experiments. pH was converted to pD by the following equation[5]: pD = 0.929 × pH + 0.42. A representative example of the ^1H NMR spectra obtained for 5PCP-IP_5 is shown in Figure S1 and all data are shown in Figure S2.

IV. Inhibition of Akt T308 Phosphorylation

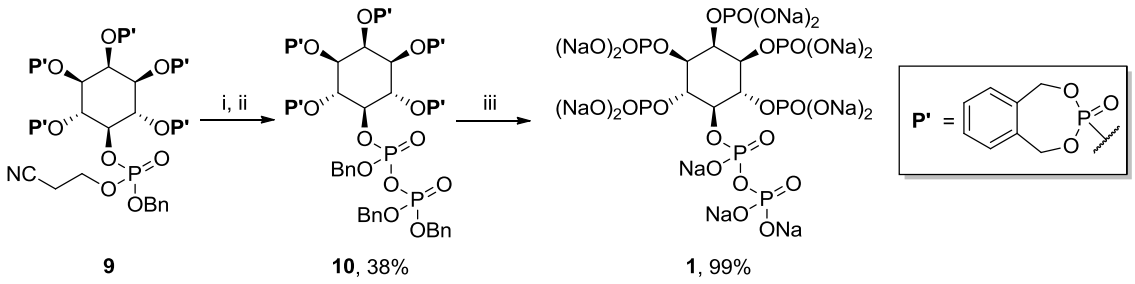
Inactive human Akt1 at a final concentration of 17 nM was treated with varying concentrations inhibitors (from 2.4 nM to 10 μM) in a buffer containing 50 mM Tris (pH = 7.5), 100 μM Mg–ATP, 1 mM $Mg(OAc)_2$ and 1 mM DTT in total volume of 20 μL at 30 ^oC for 20 min. Next, 5 μL PDK1 at a final concentration of 17 nM in the same buffer was added. After 30 min at 30 ^oC, SDS sample buffer was added to quench the reaction. The samples were boiled for 5 min at 95 ^oC 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 repectively. 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 SalI/NotI restriction sites of the pTrcHis plasmid.) His-Ddp1 and His-Ddp1 EE/AQ catalytically inactive mutant were expressed and purified as described previously[6]. The inhibition of hydrolysis of Ap_5A by Ddp1 was assayed in reactions containing 4 μg/mL Ddp1, 50 μM Ap_5A and inhibitors (from 24 nM to 100 μM) in 25 mM Hepes (pH = 6.8), 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT. As a control, we confirmed that Ap_5A showed no signs of hydrolysis when incubated with His-Ddp1 EE/AQ (Figure S3). The reactions were performed at 37 ^oC for 4 h before quenching by filtration through 0.2 μm synringe filter. The hydrolysis of Ap_5A 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].



[a]Reagents and conditions: (i) BSTFA, TEA, CH_3CN, rt, overnight; (ii) dibenzyl phosphorochloridate, TEA, CH_2Cl_2, 0 ^oC to rt, 2h; (iii) H_2, Pd black, $NaHCO_3$, *t*–BuOH/H_2O, rt, overnight.

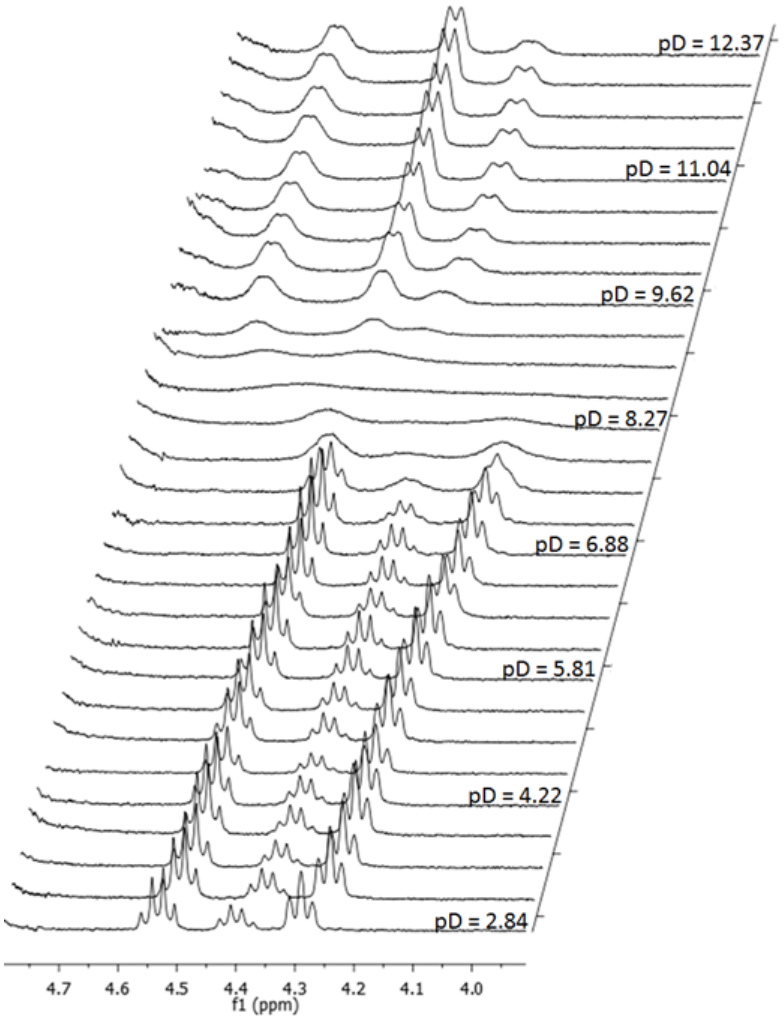


Figure S1. ^1H NMR spectra of 5PCP-IP_5 (**2**) at different pD.

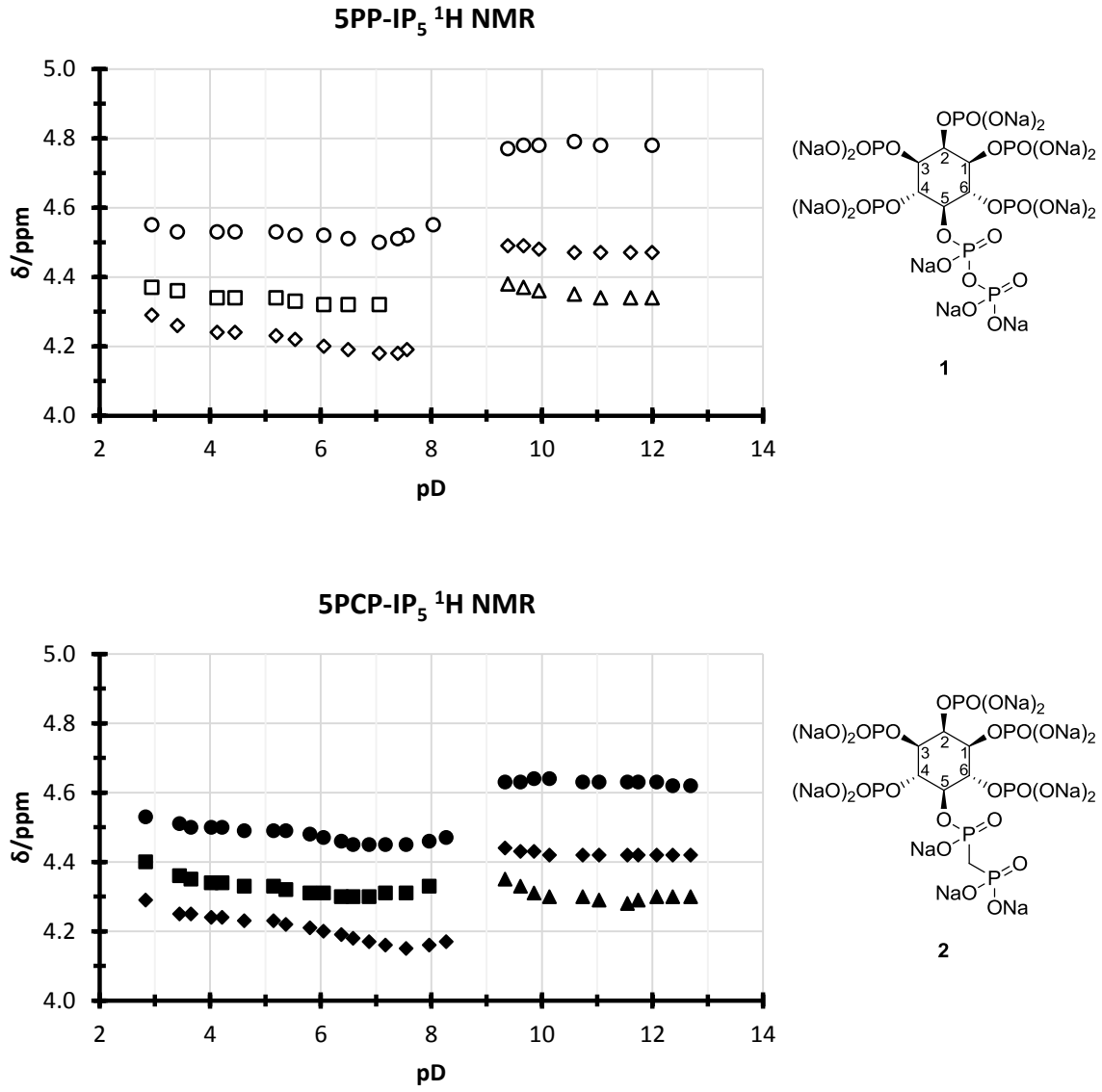


Figure S2. ^{1}H NMR titration data of 5PP-IP$_5$ (**1**) and 5PCP-IP$_5$ (**2**) illustrating the conformational change. Circle (○ and ●): $H_{4/6}$, Square (□ and ■): H_5, Diamond (◇ and ◆): $H_{1/3}$ and Triangle (△ and ▲): H_2.

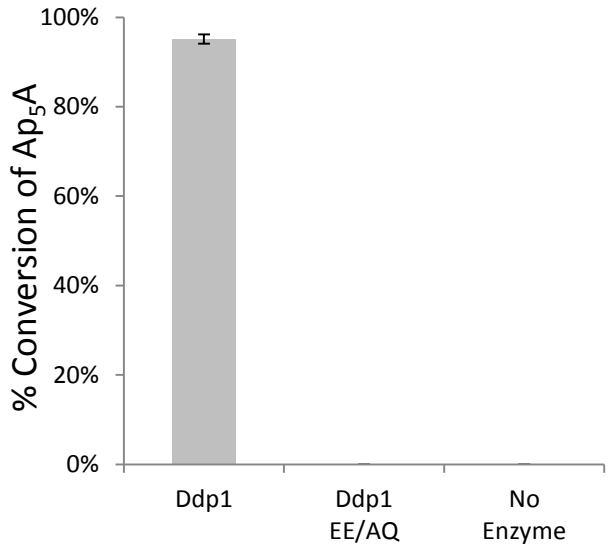


Figure S3. Conversion of 50 µM Ap_5A at 37°C for 4 h with Ddp1 (4 µg/mL), Ddp1 EE/AQ catalytically inactive mutant (16 µg/mL) and no enzyme.

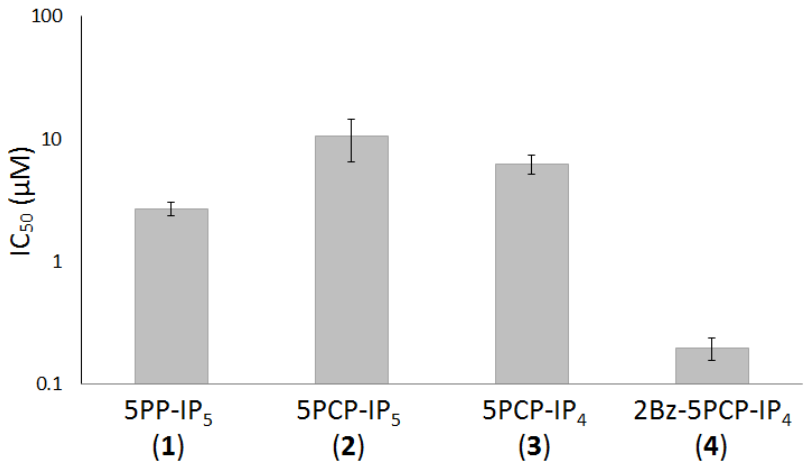
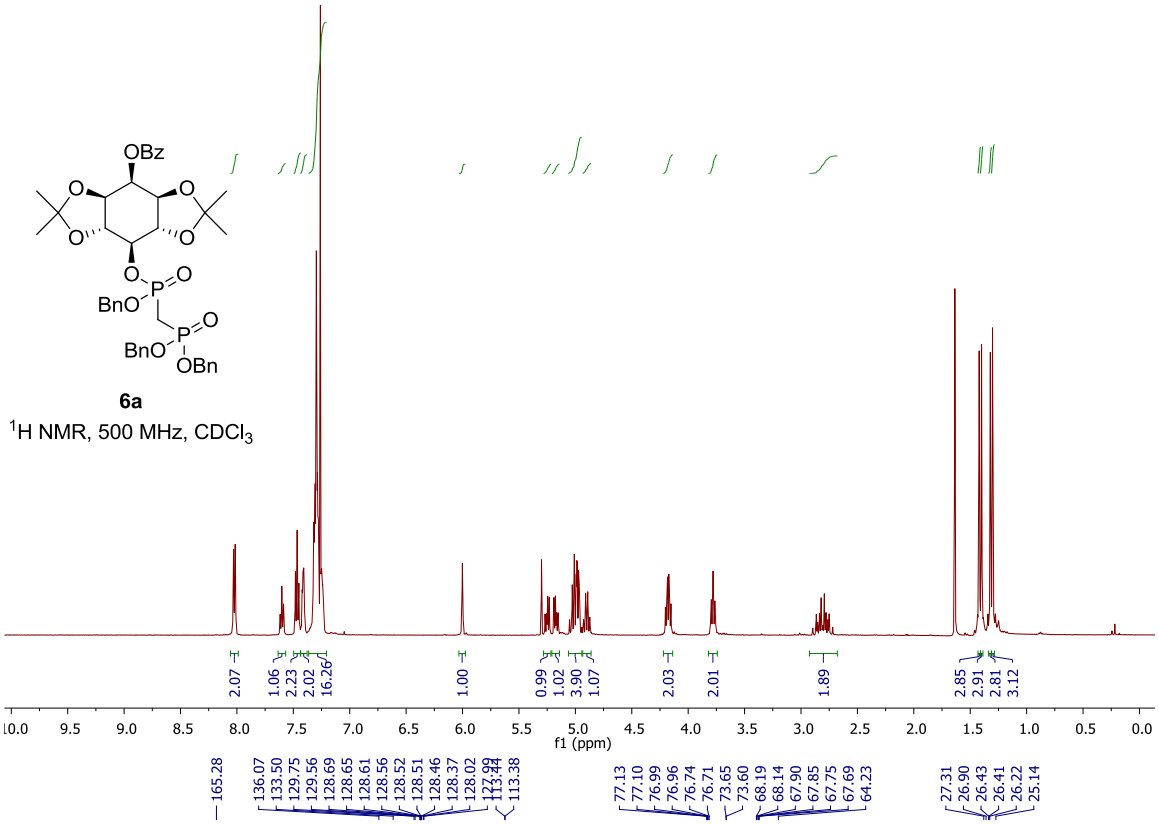
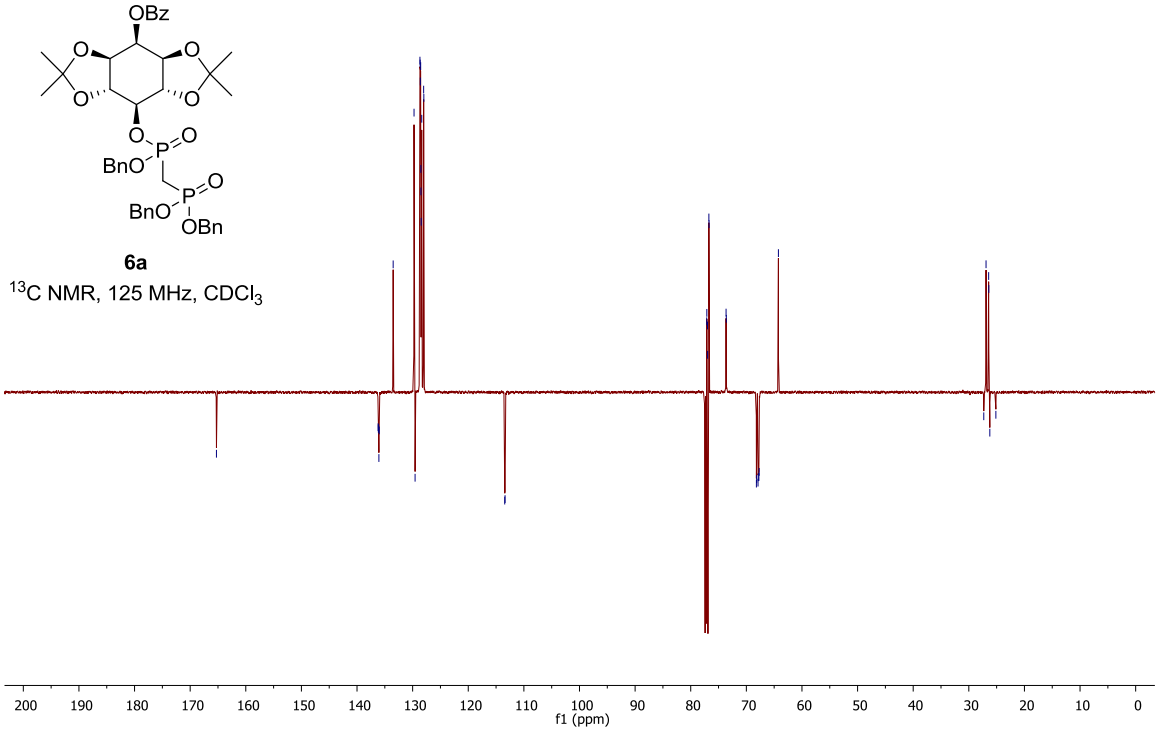
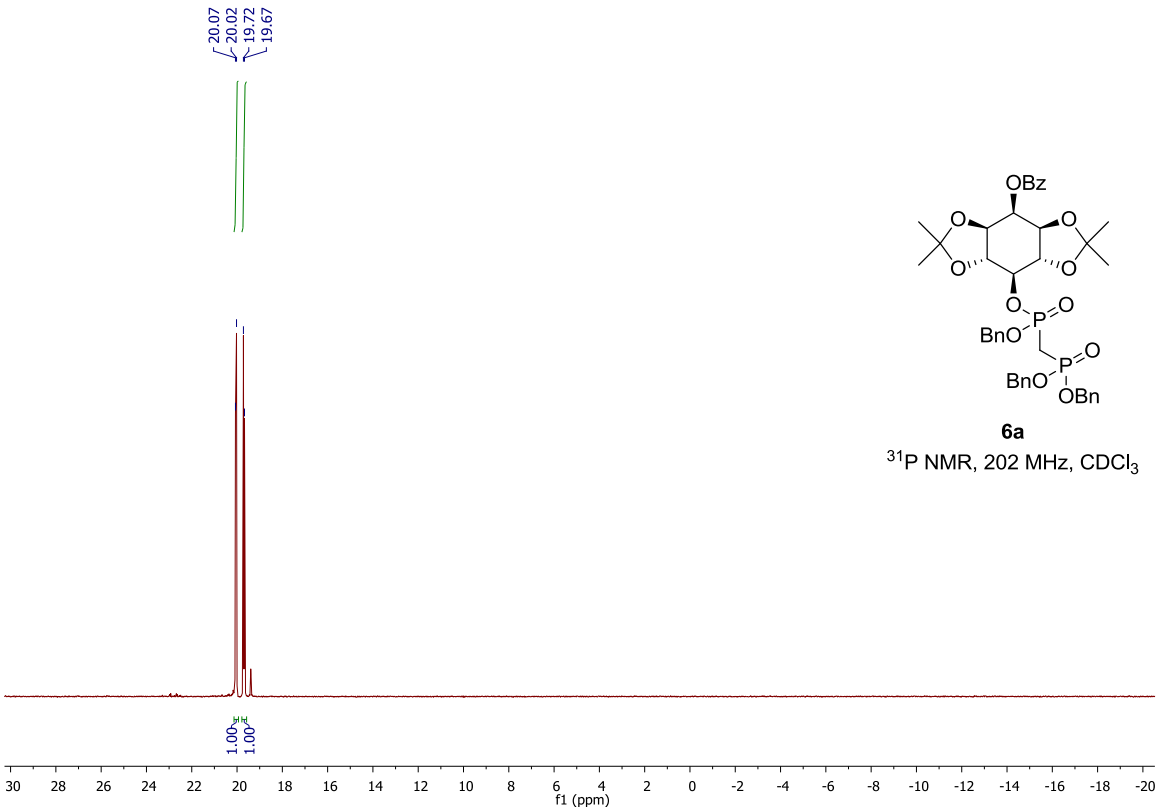


Figure S4. IC_{50} values for 5PP-IP_5 and the analogues, as measured by inhibition of hydrolysis of Ap_5A by Ddp1.

VII. NMR Spectra

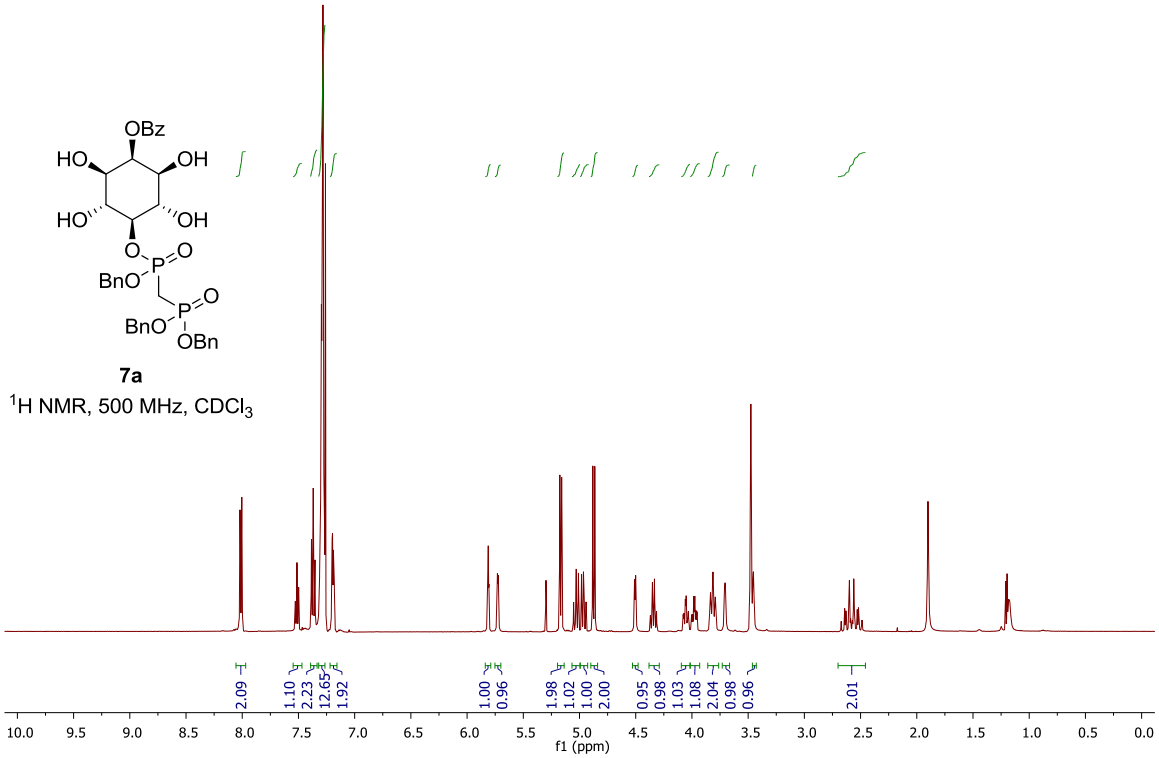






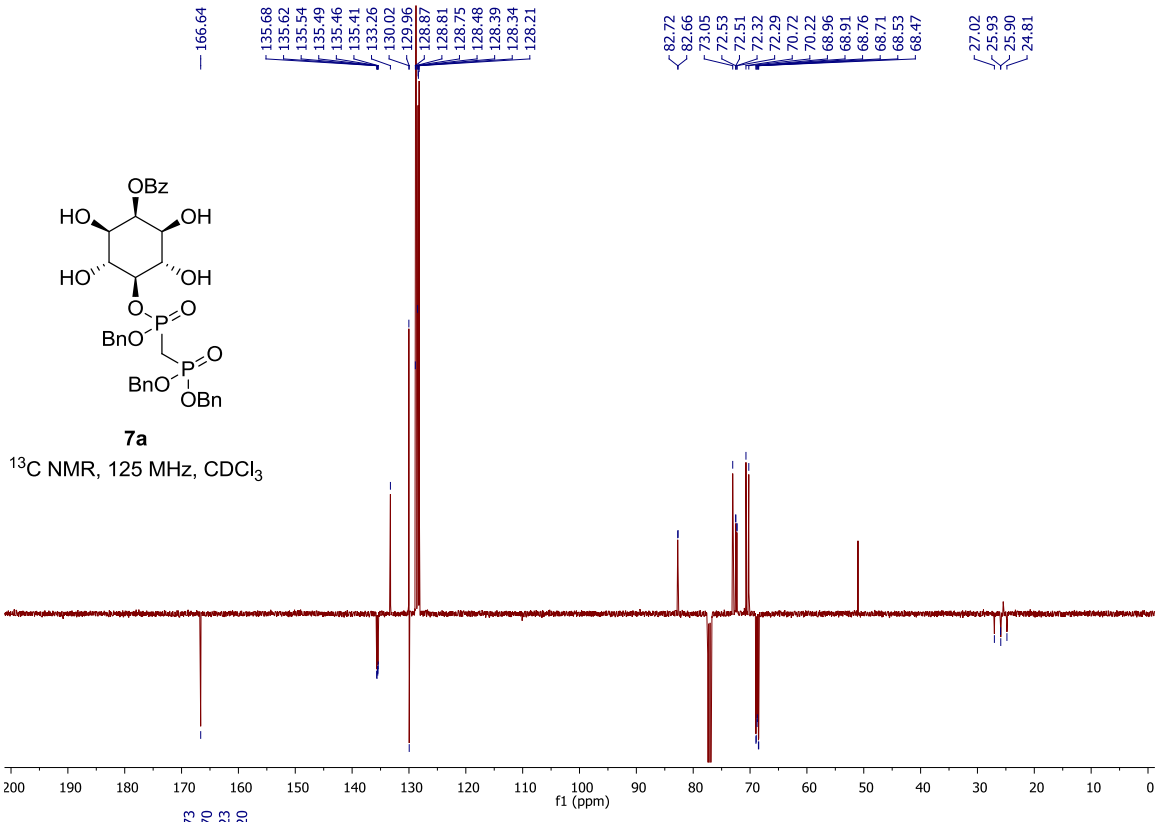
6a

^{31}P NMR, 202 MHz, $CDCl_3$



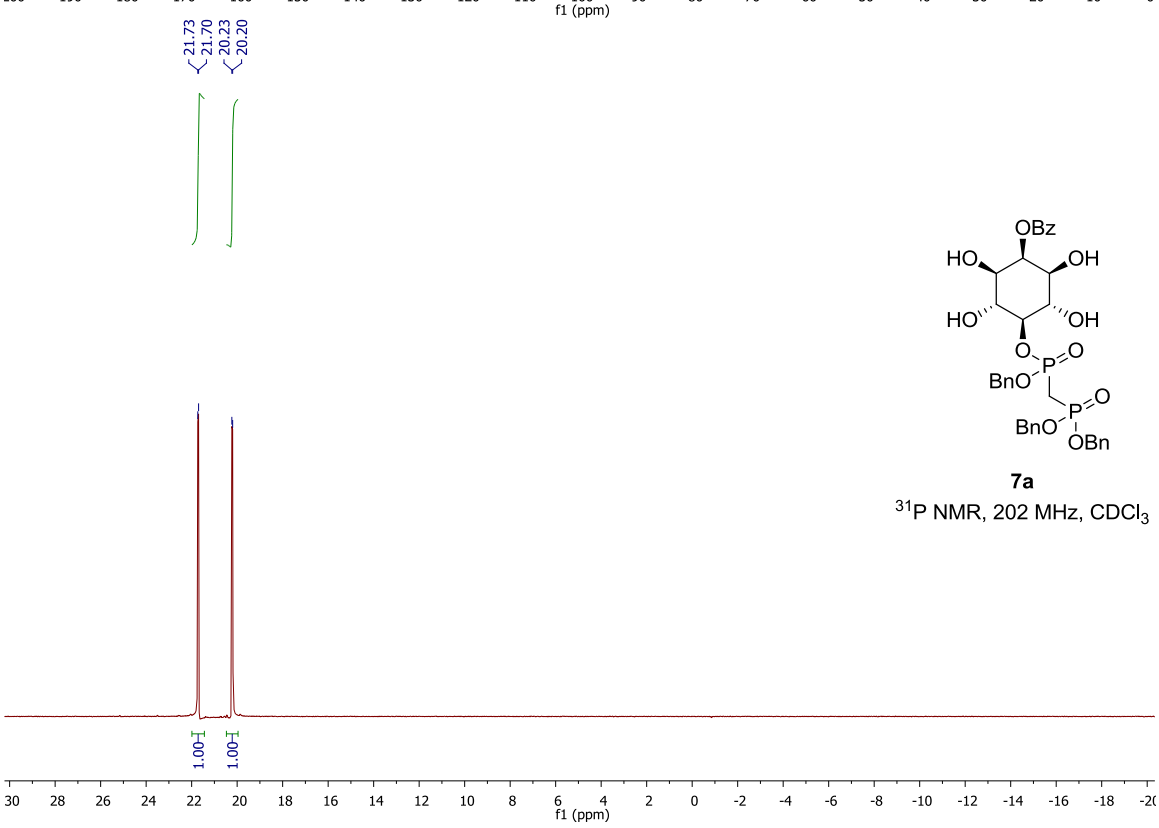
7a

^{1}H NMR, 500 MHz, $CDCl_3$



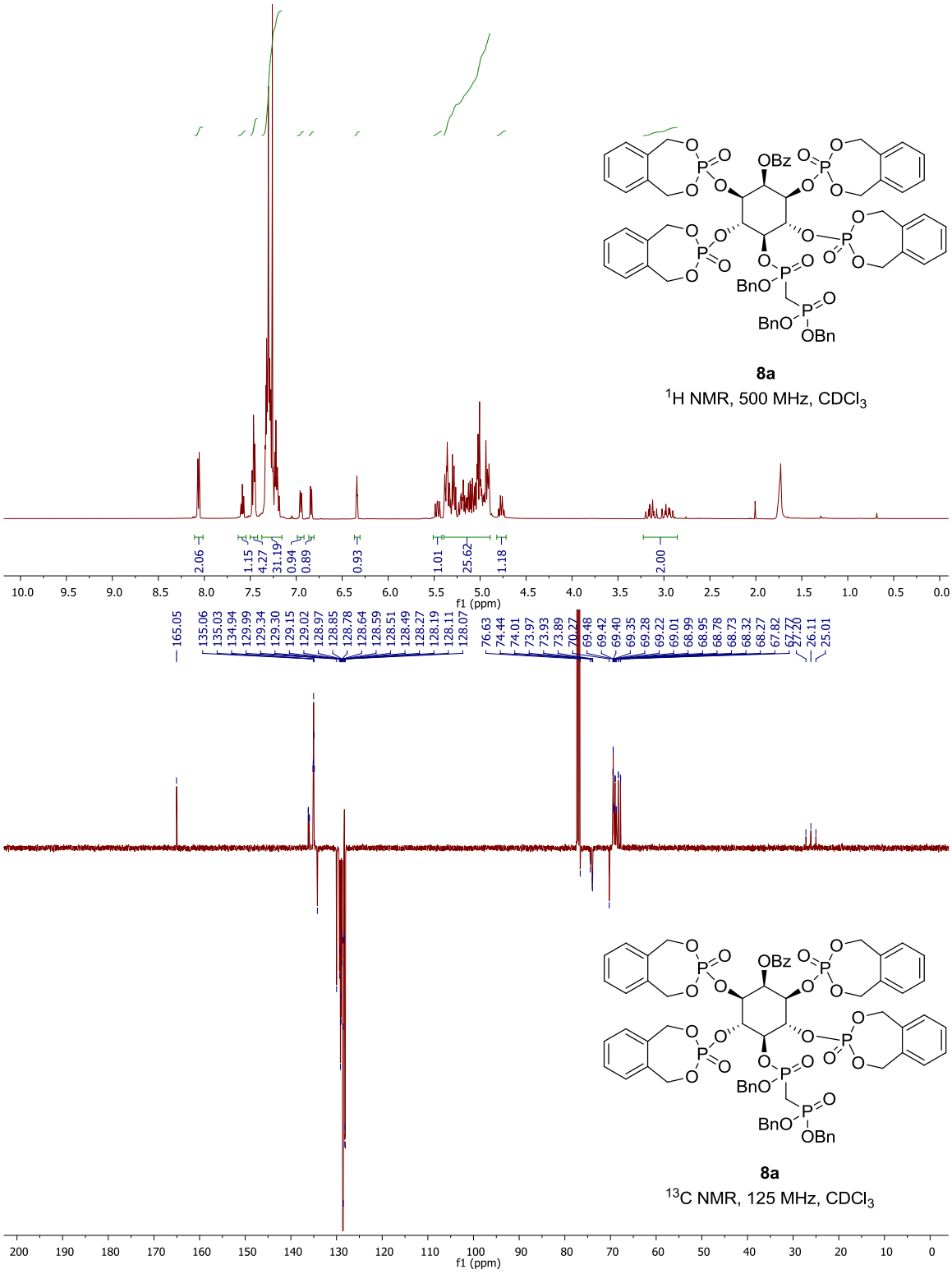
7a

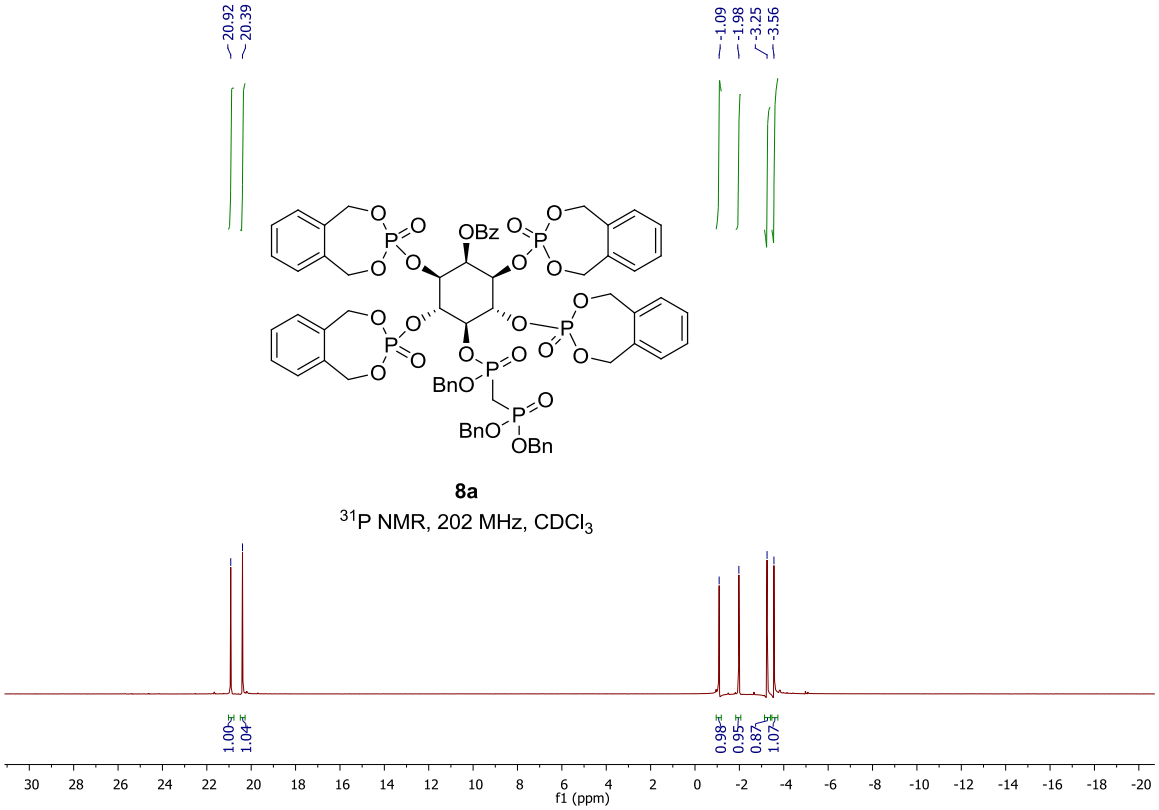
^{13}C NMR, 125 MHz, $CDCl_3$



7a

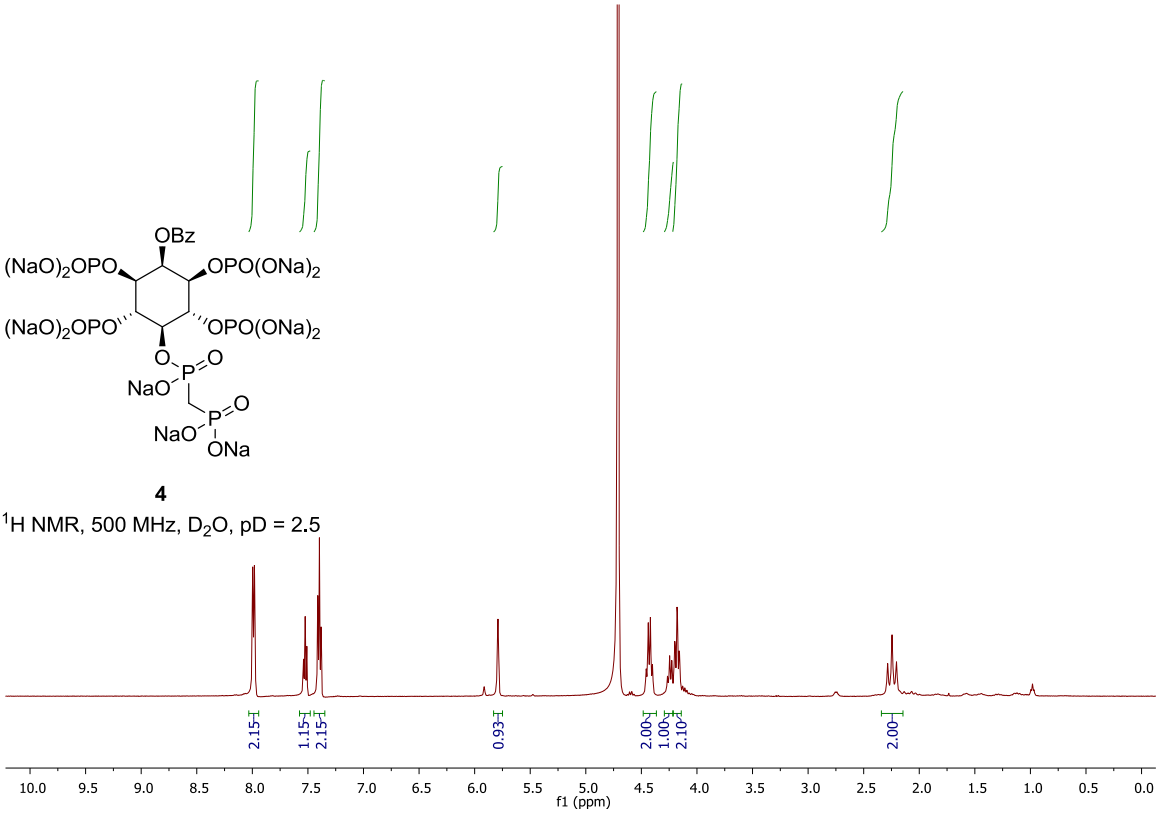
^{31}P NMR, 202 MHz, $CDCl_3$


OBz
BnO
BnO
OBn
8a
1H NMR, 500 MHz, CDCl3
2.06
1.15
4.27
31.19
0.94
0.89
0.93
1.01
25.62
1.18
2.00
f1 (ppm)
165.05
135.06
135.03
134.94
129.99
129.34
129.30
129.15
129.02
128.97
128.85
128.78
128.64
128.59
128.51
128.49
128.27
128.19
128.11
128.07
76.63
74.44
74.01
73.97
73.93
73.89
70.22
69.48
69.42
69.40
69.35
69.28
69.22
69.01
68.99
68.95
68.78
68.73
68.32
68.27
67.82
27.20
26.11
25.01
OBz
BnO
BnO
OBn
8a
13C NMR, 125 MHz, CDCl3
f1 (ppm)



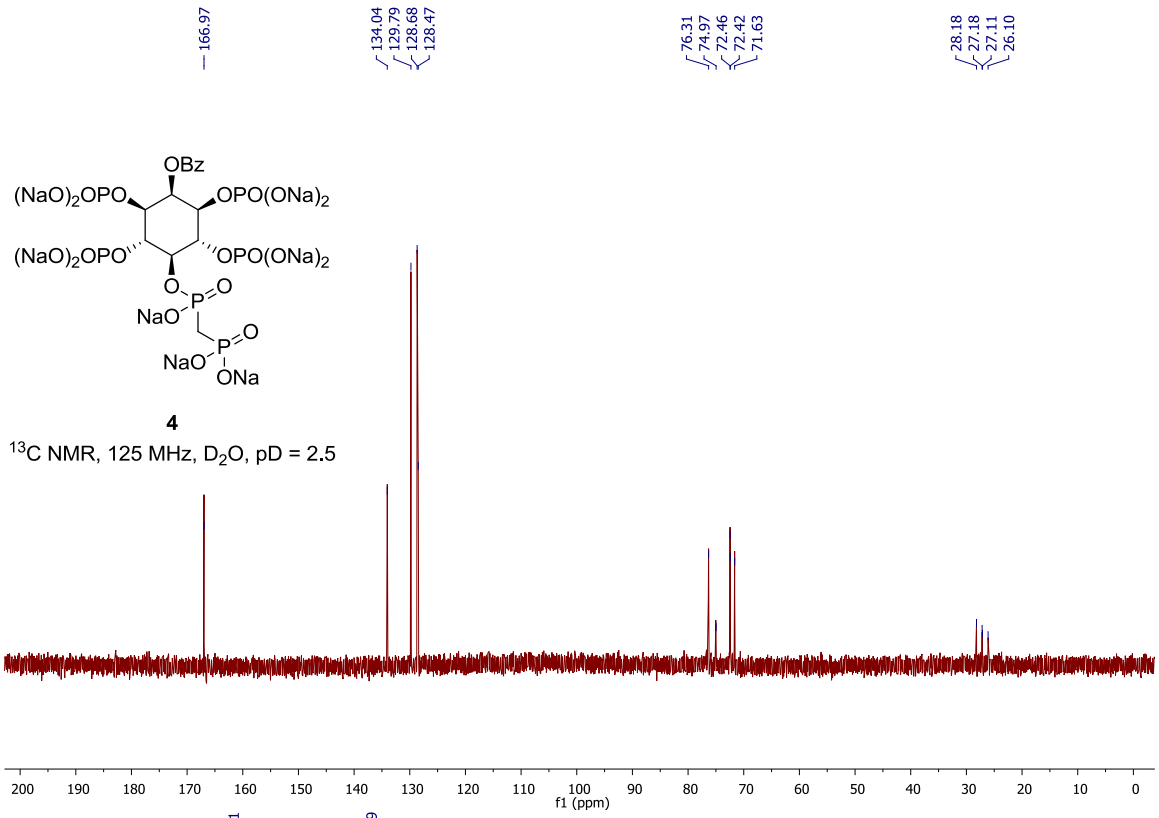
8a

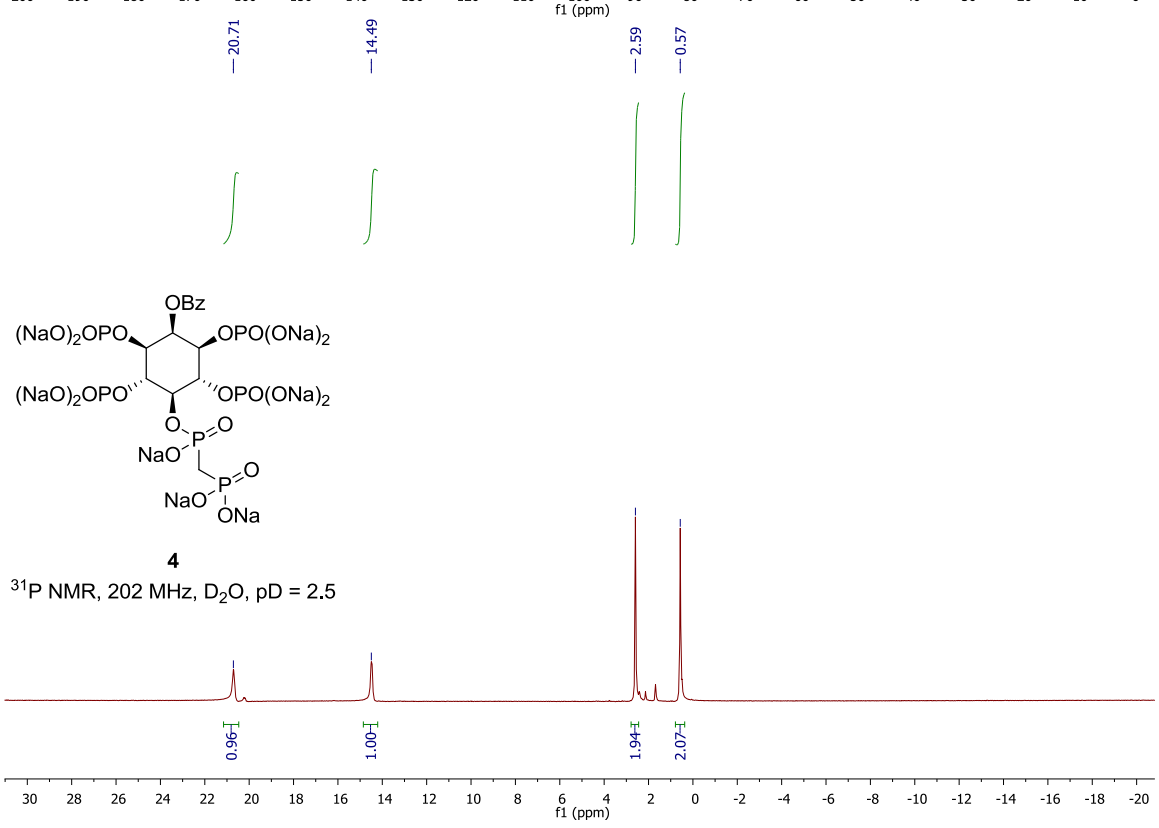
^{31}P NMR, 202 MHz, $CDCl_3$

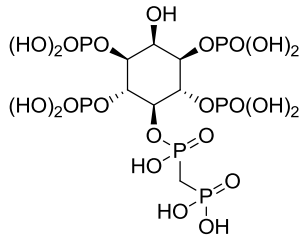


4

^{1}H NMR, 500 MHz, D_2O, pD = 2.5

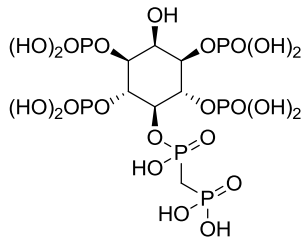

166.97
134.04
129.79
128.68
128.47
76.31
74.97
72.46
72.42
71.63
28.18
27.18
27.11
26.10
OBz
(NaO)2OPO
OPO(ONa)2
(NaO)2OPO
OPO(ONa)2
O
P
O
NaO
P
O
NaO
ONa
4
13C NMR, 125 MHz, D2O, pD = 2.5
200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
f1 (ppm)


20.71
14.49
2.59
0.57
OBz
(NaO)2OPO
OPO(ONa)2
(NaO)2OPO
OPO(ONa)2
O
P
O
NaO
P
O
NaO
ONa
4
31P NMR, 202 MHz, D2O, pD = 2.5
0.96
1.00
1.94
2.07
30 28 26 24 22 20 18 16 14 12 10 8 6 4 2 0 -2 -4 -6 -8 -10 -12 -14 -16 -18 -20
f1 (ppm)



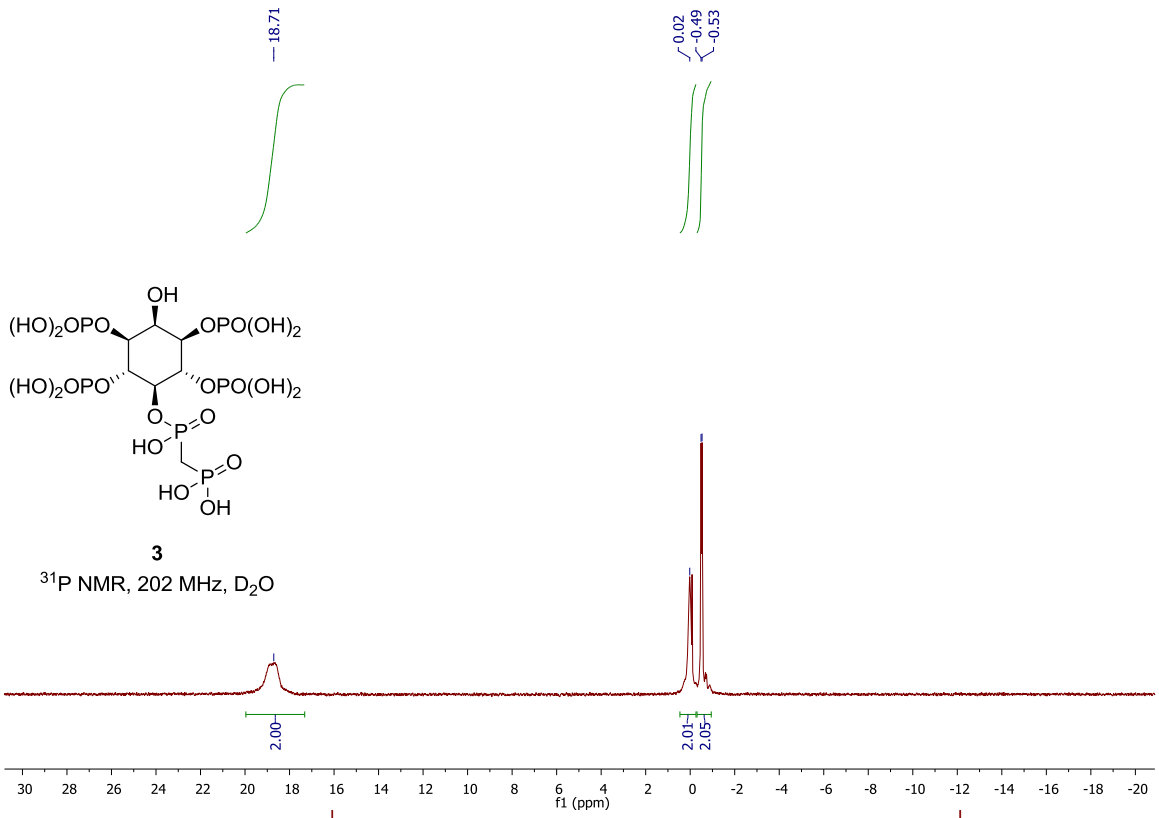
3

^{1}H NMR, 500 MHz, D_2O



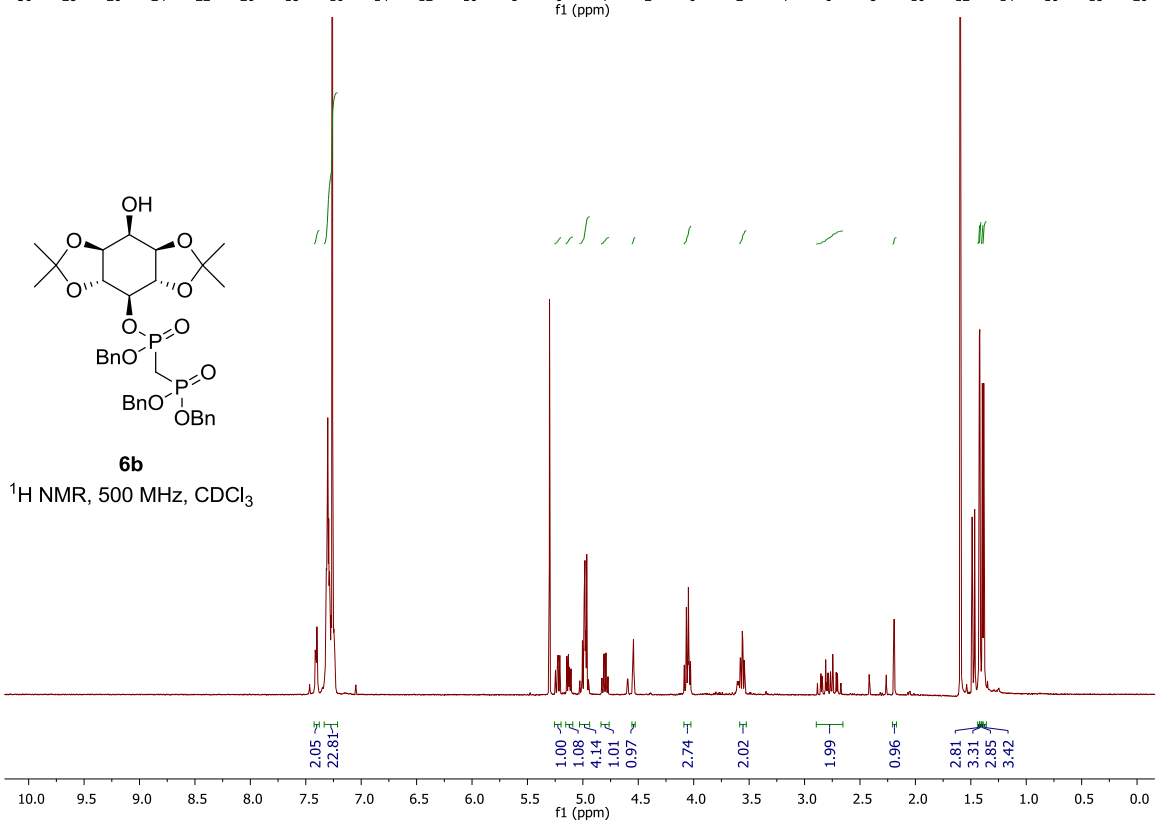
3

^{13}C NMR, 125 MHz, D_2O



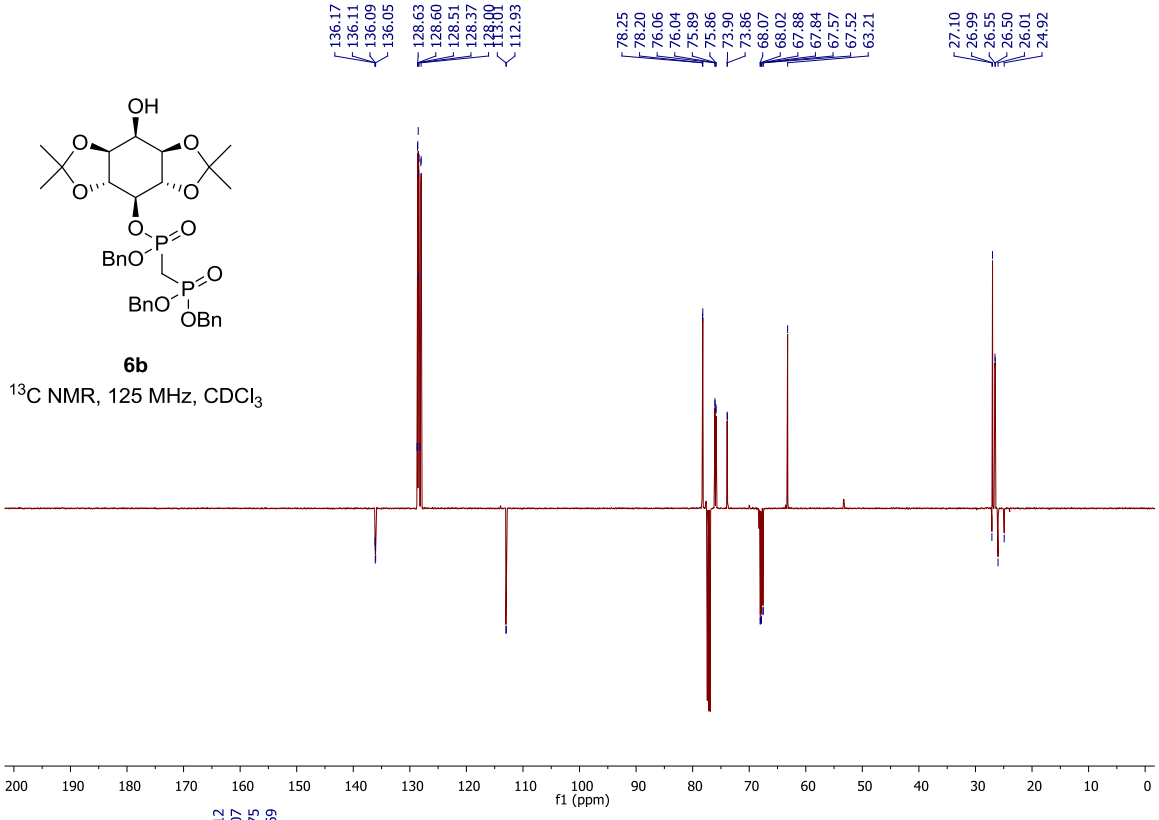
3

^{31}P NMR, 202 MHz, D_2O



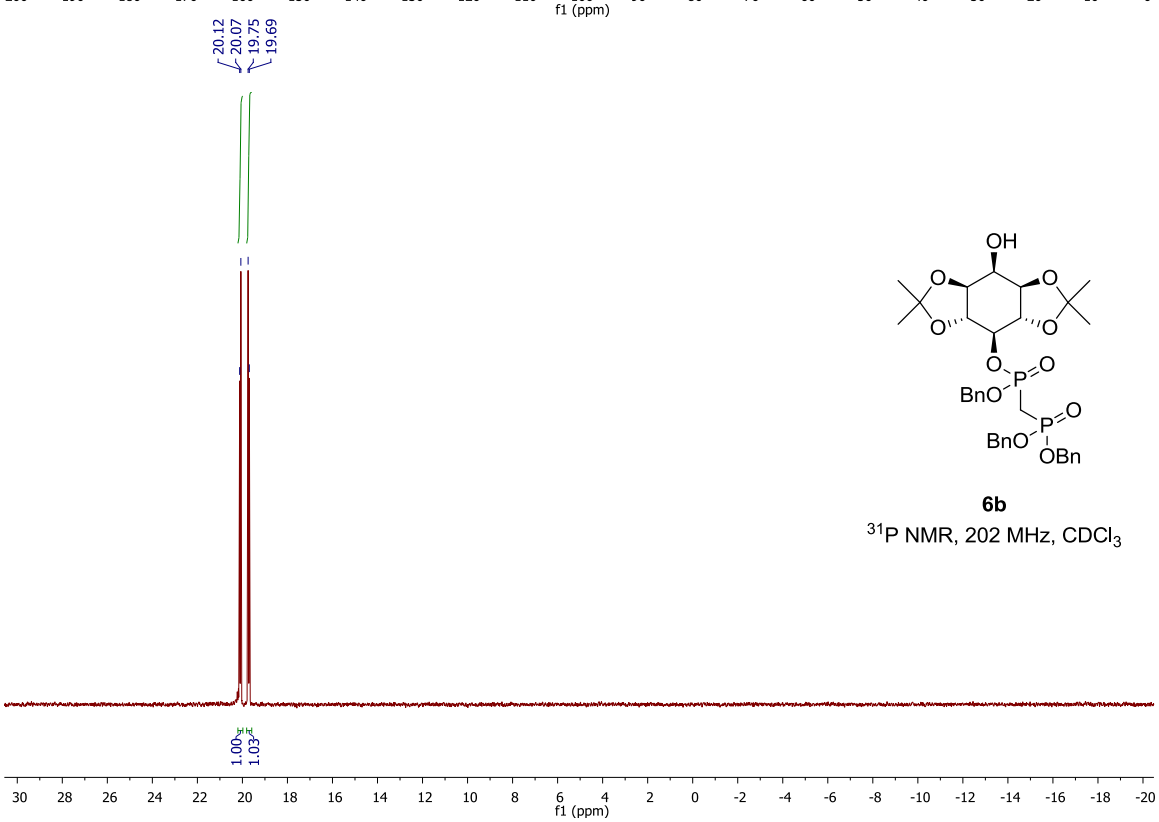
6b

^{1}H NMR, 500 MHz, $CDCl_3$



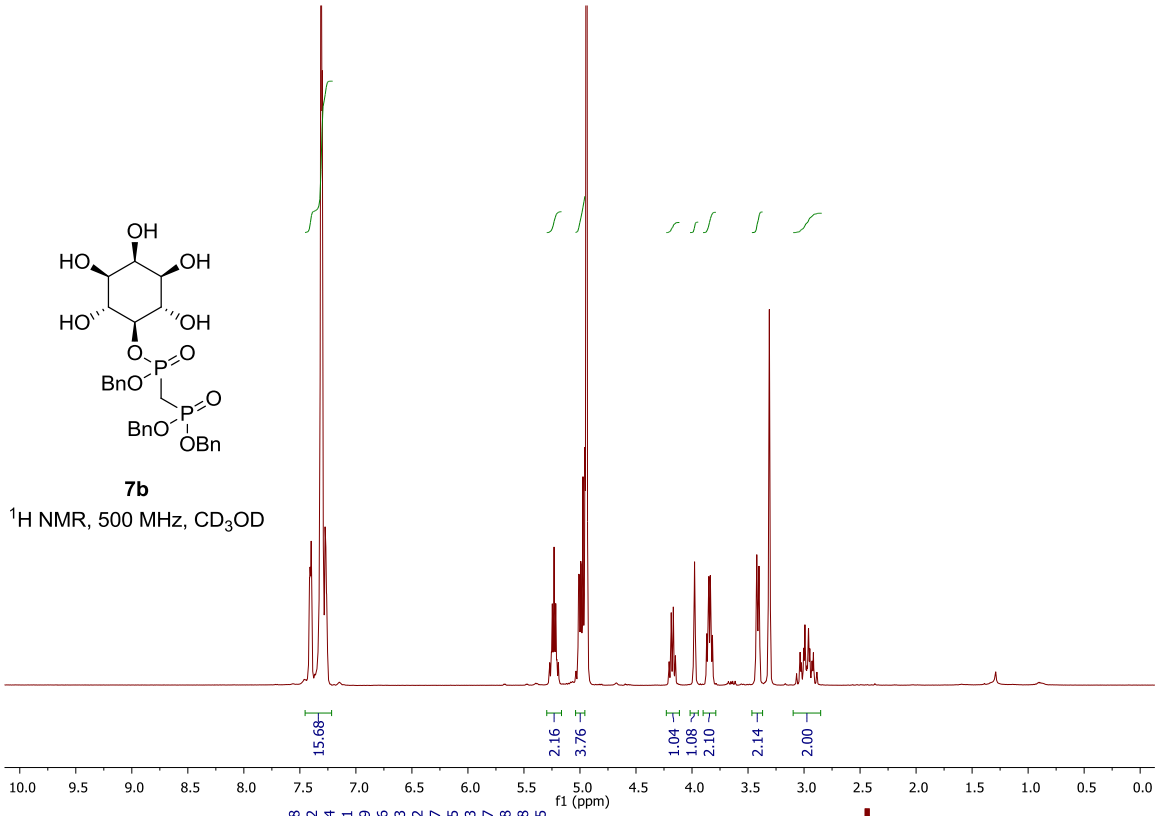
6b

^{13}C NMR, 125 MHz, $CDCl_3$



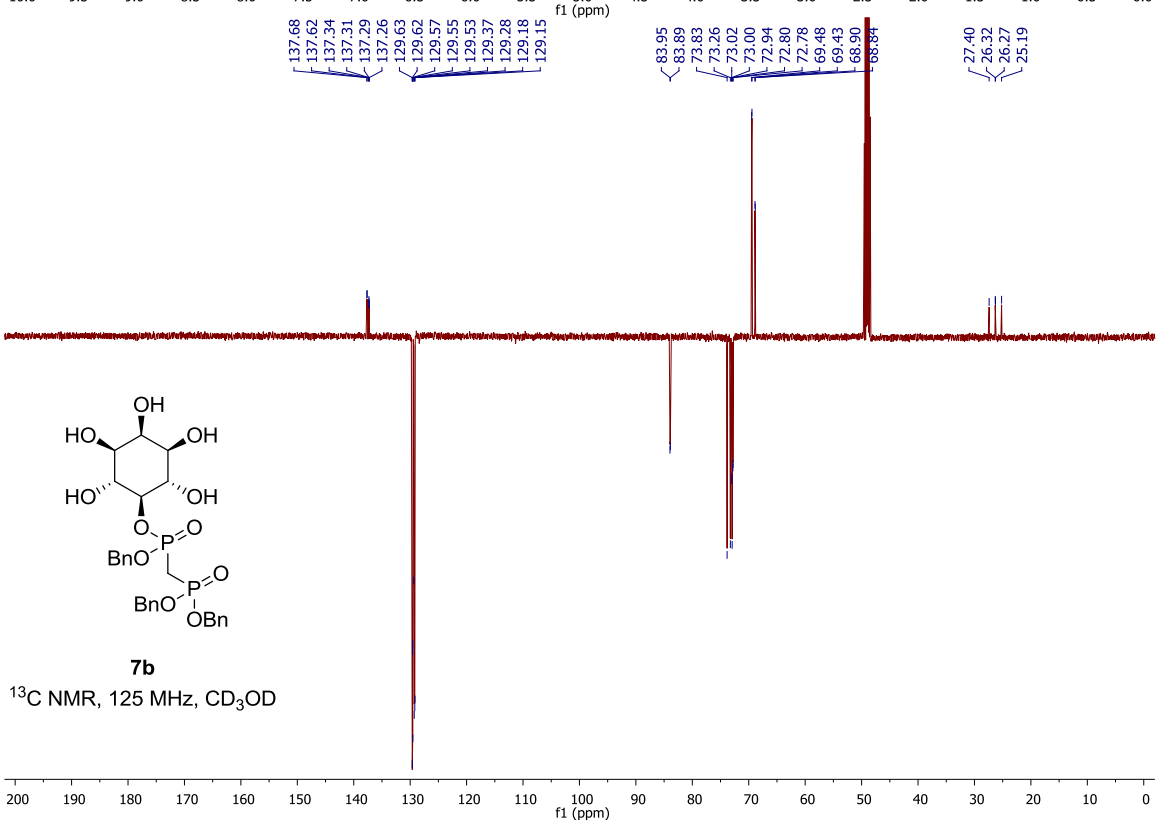
6b

^{31}P NMR, 202 MHz, $CDCl_3$



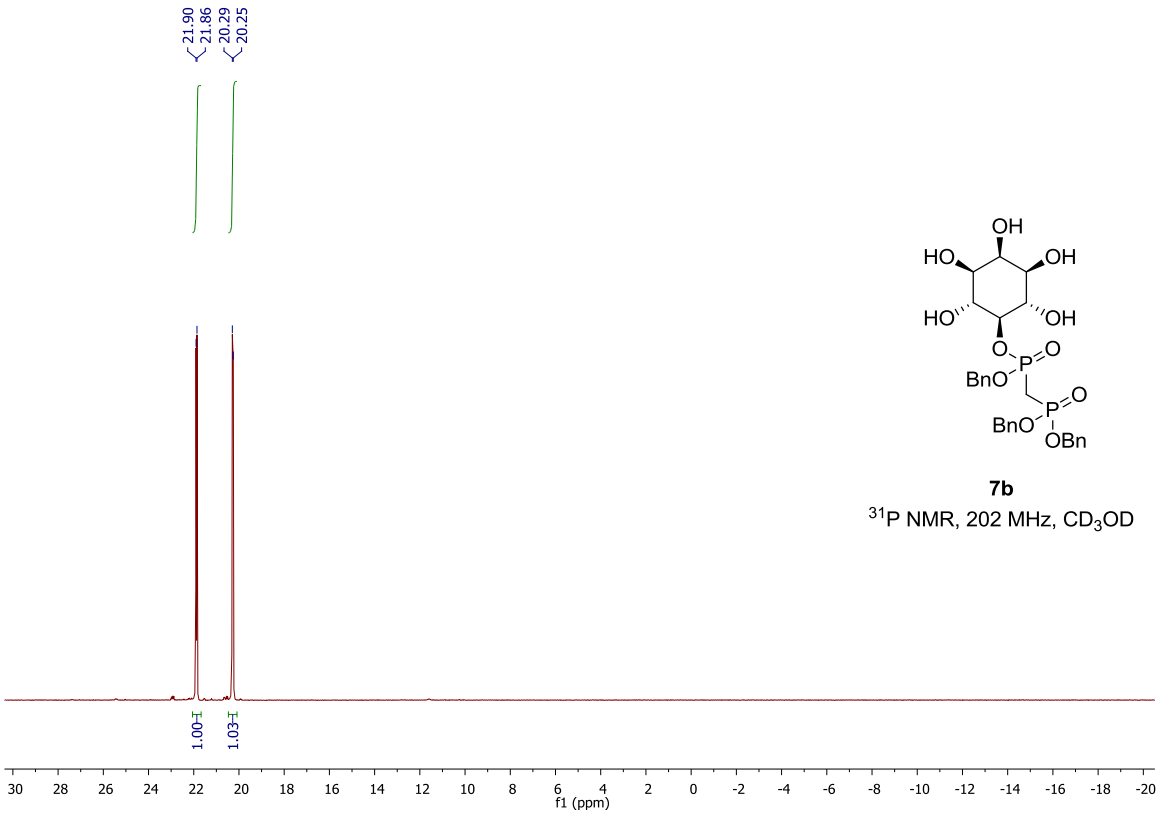
7b

^{1}H NMR, 500 MHz, CD_3OD



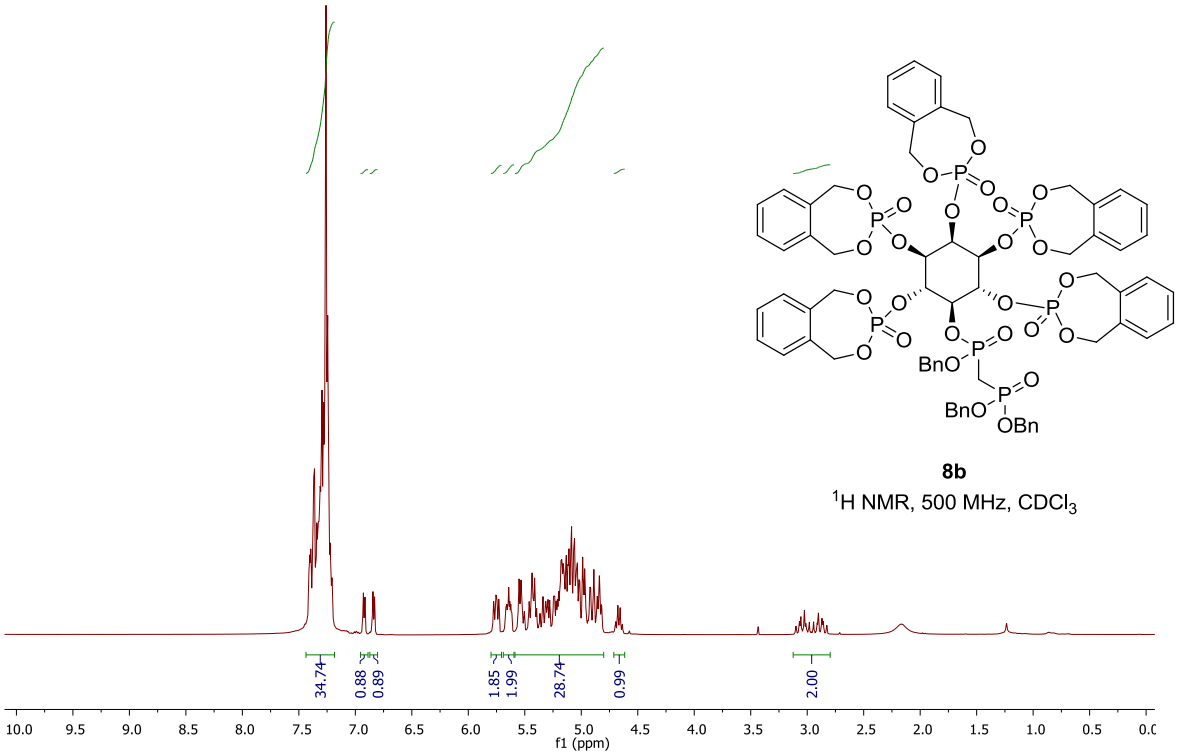
7b

^{13}C NMR, 125 MHz, CD_3OD



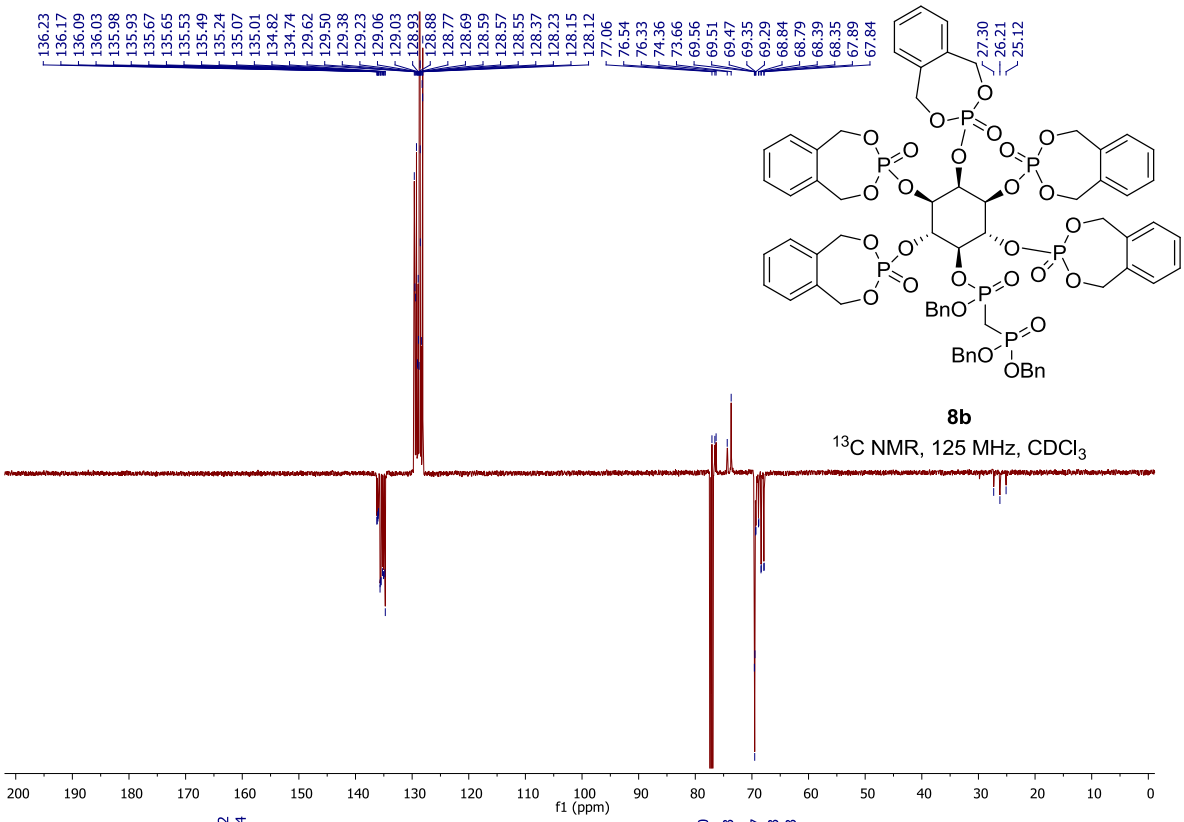
7b

^{31}P NMR, 202 MHz, CD_3OD



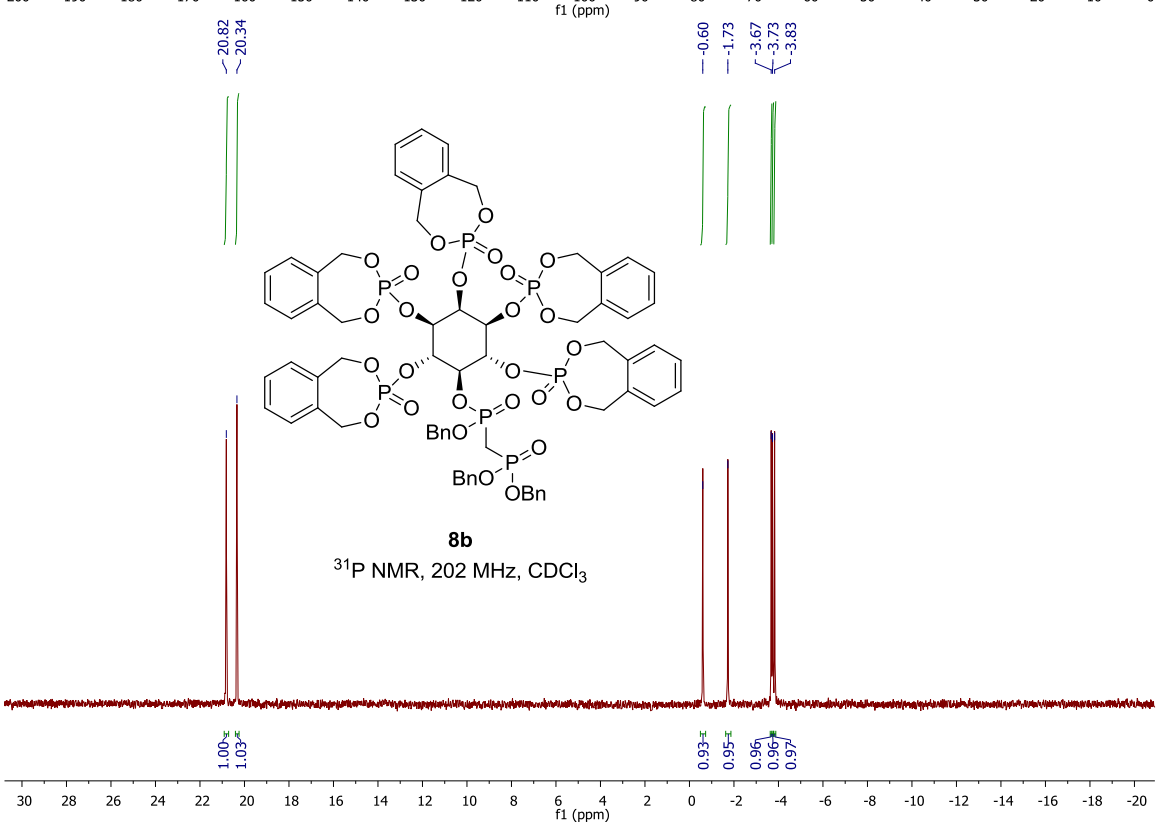
8b

^{1}H NMR, 500 MHz, $CDCl_3$



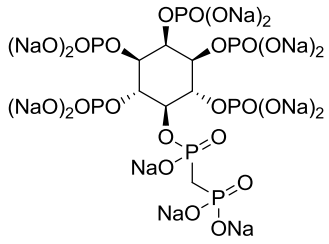
8b

^{13}C NMR, 125 MHz, $CDCl_3$



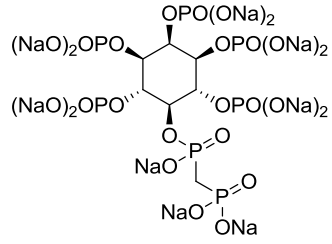
8b

^{31}P NMR, 202 MHz, $CDCl_3$



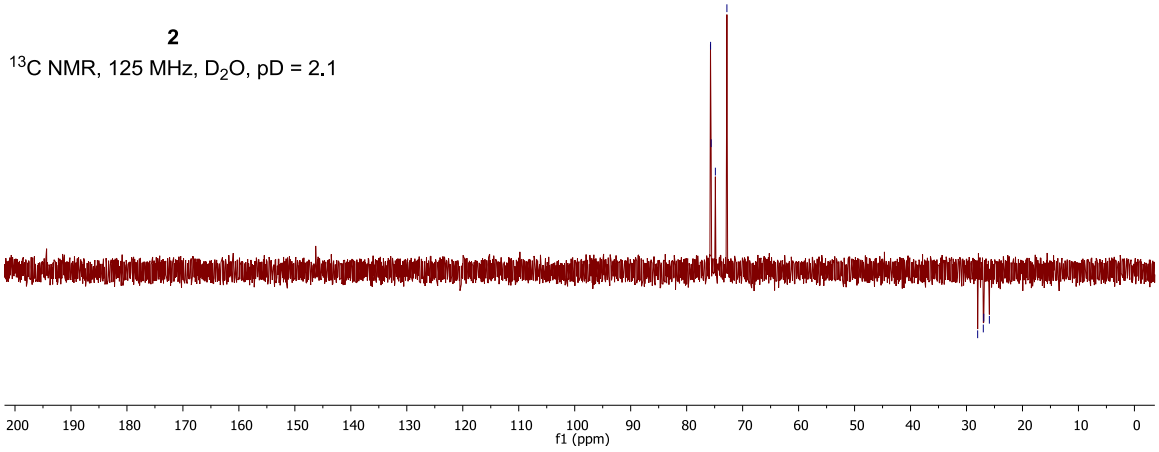
2

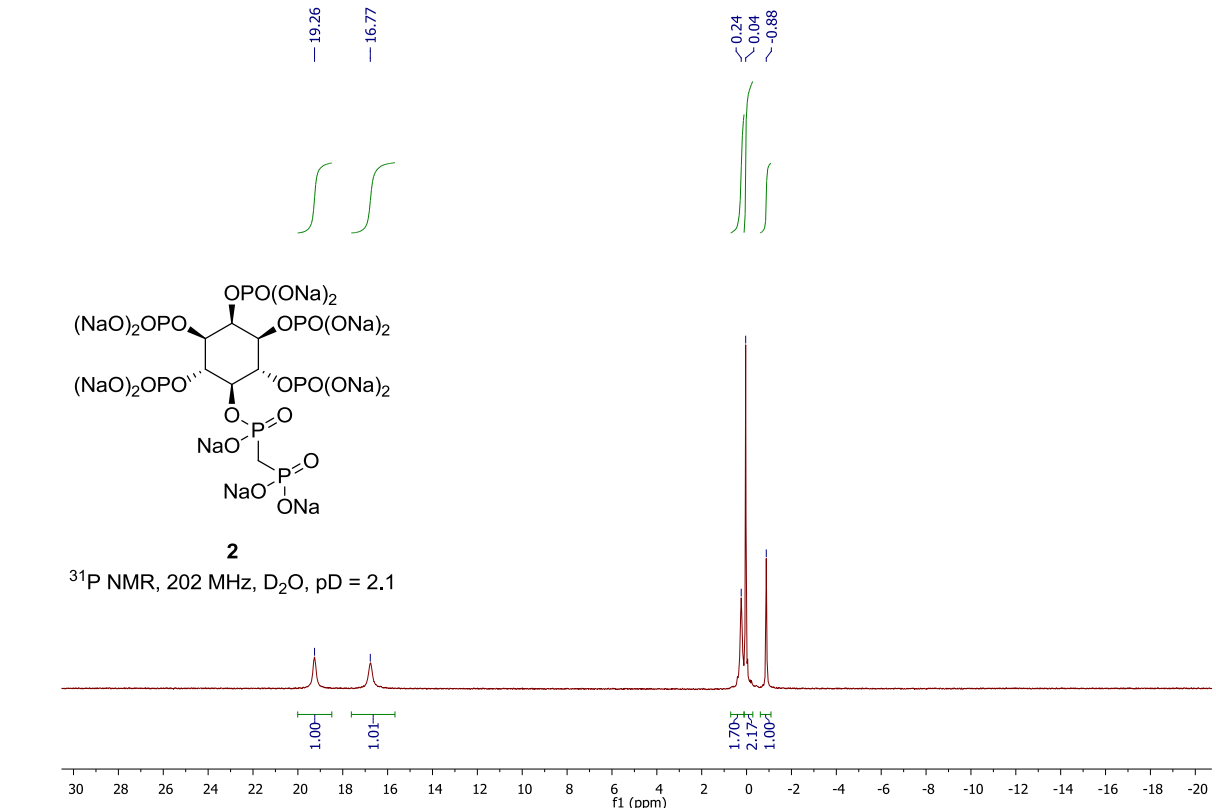
^{1}H NMR, 500 MHz, D_2O, pD = 2.1

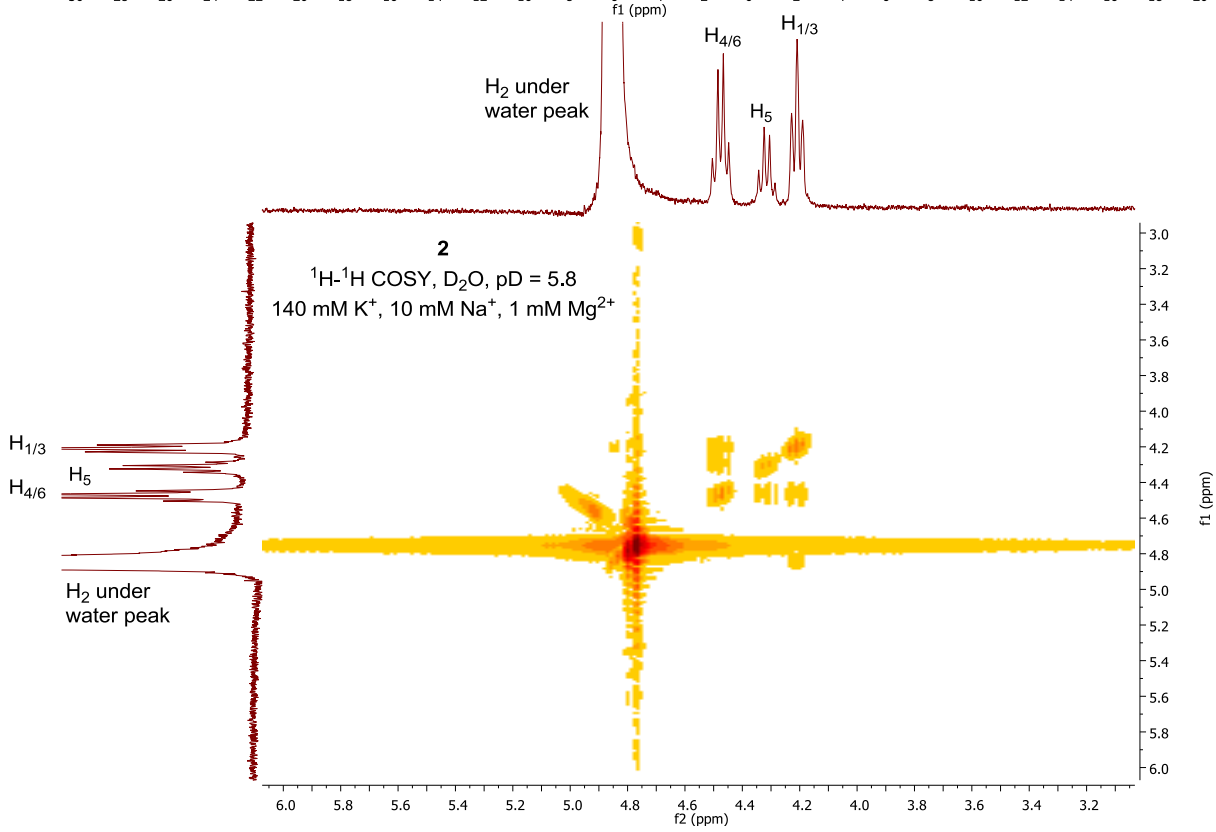


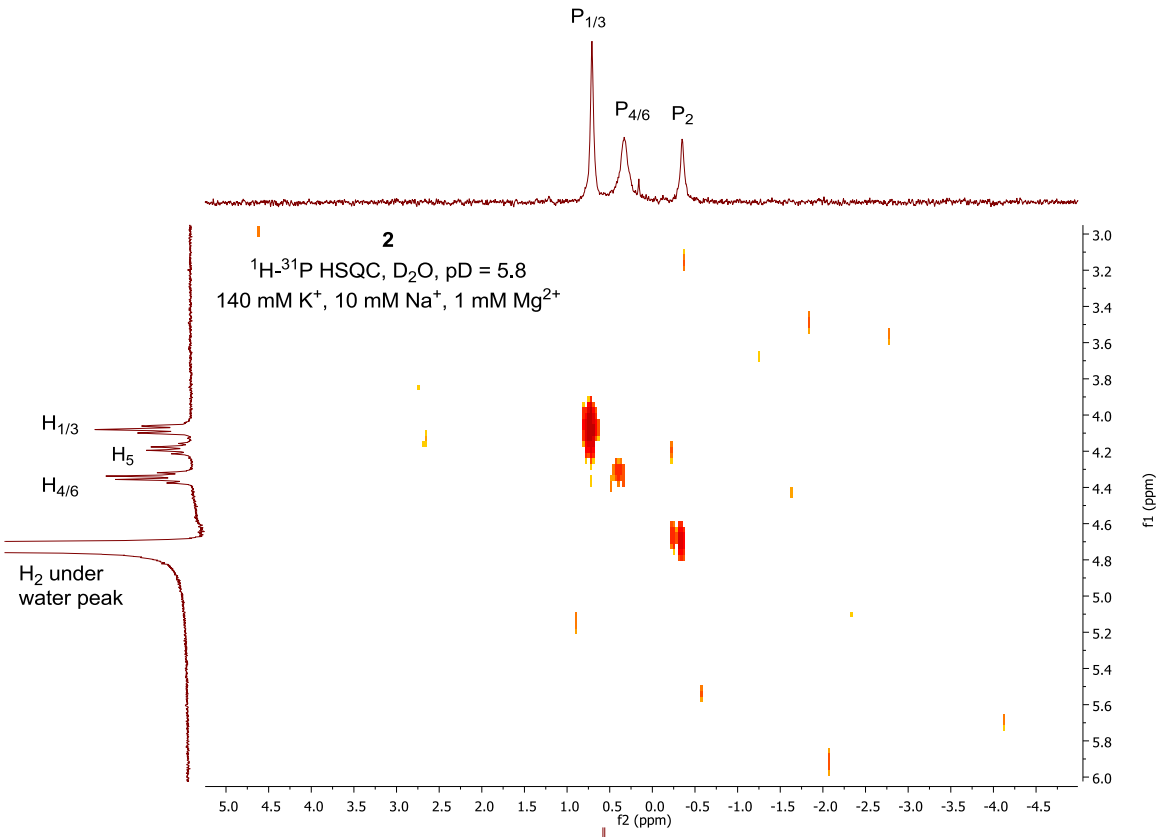
2

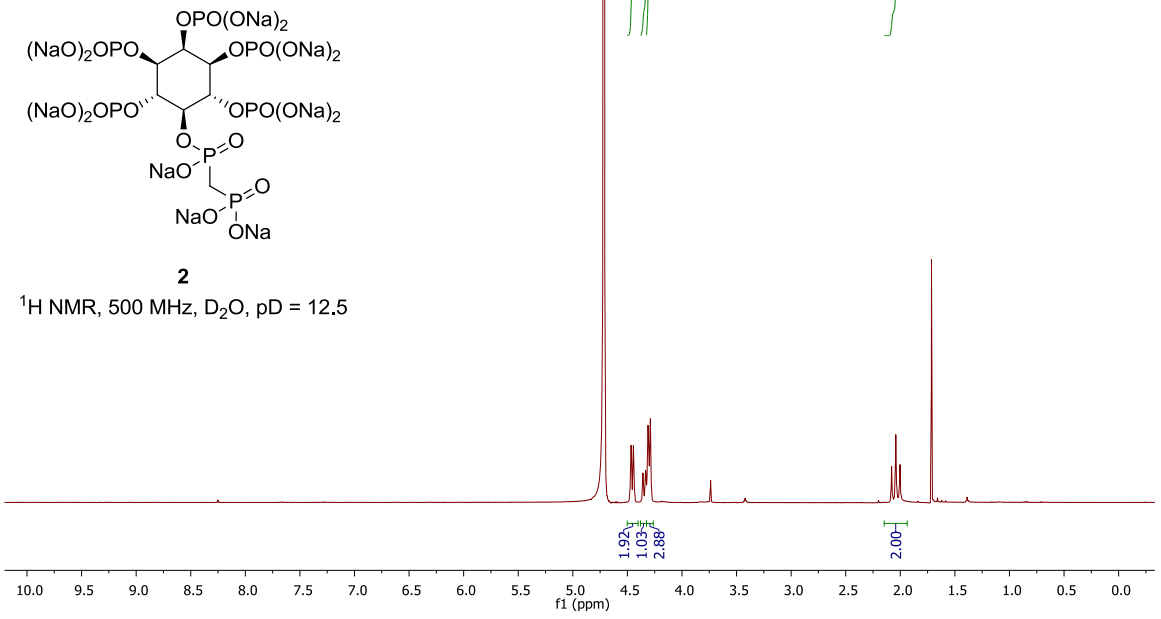
^{13}C NMR, 125 MHz, D_2O, pD = 2.1




19.26
16.77
0.24
0.04
-0.88
OPO(ONa)2
(NaO)2OPO
OPO(ONa)2
(NaO)2OPO
OPO(ONa)2
NaO
NaO
ONa
2
31P NMR, 202 MHz, D2O, pD = 2.1
1.00
1.01
1.70
2.17
1.00
f1 (ppm)

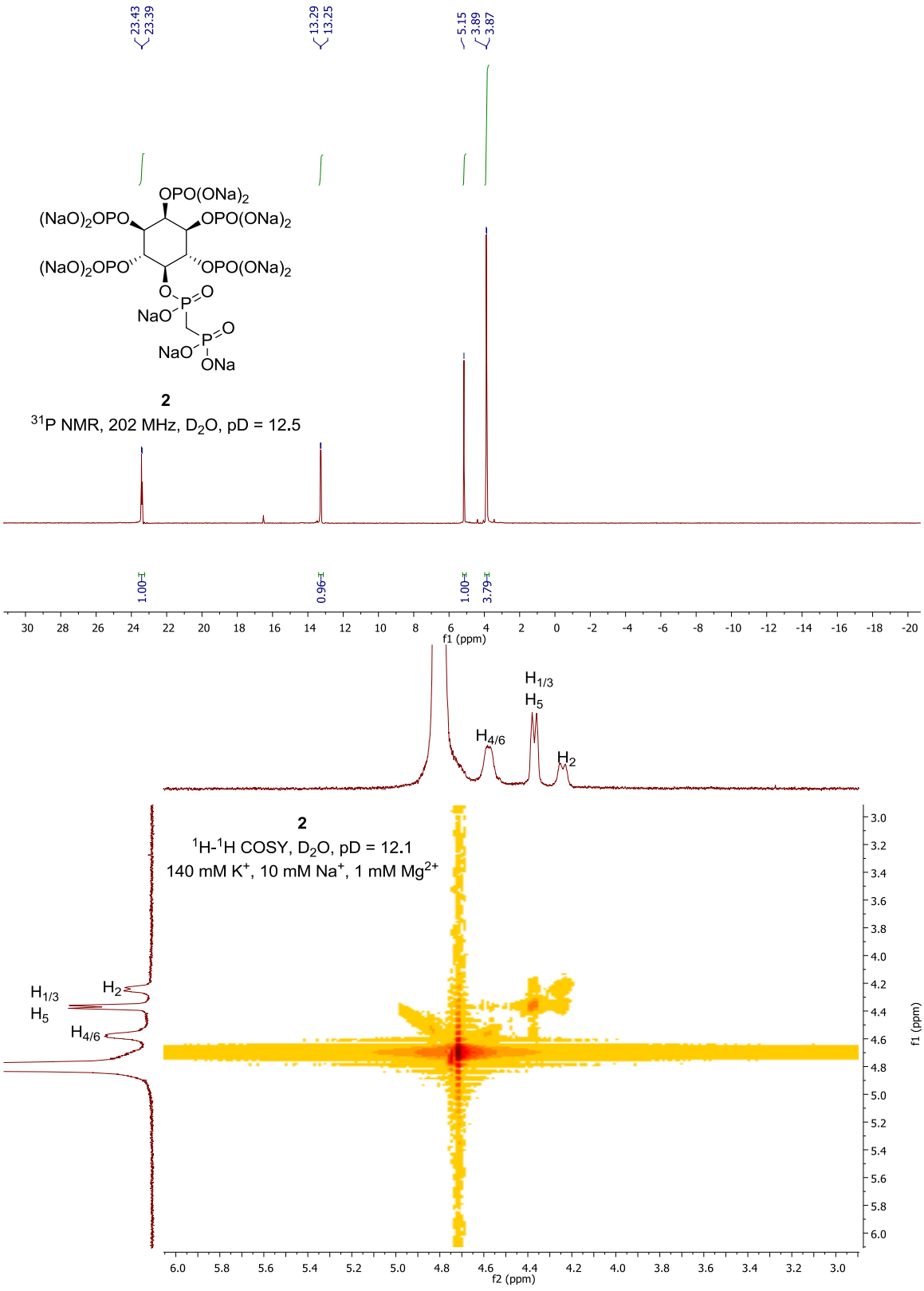

H2 under water peak
H4/6
H5
H1/3
2
1H-1H COSY, D2O, pD = 5.8
140 mM K+, 10 mM Na+, 1 mM Mg2+
H1/3
H5
H4/6
H2 under water peak
f2 (ppm)
f1 (ppm)

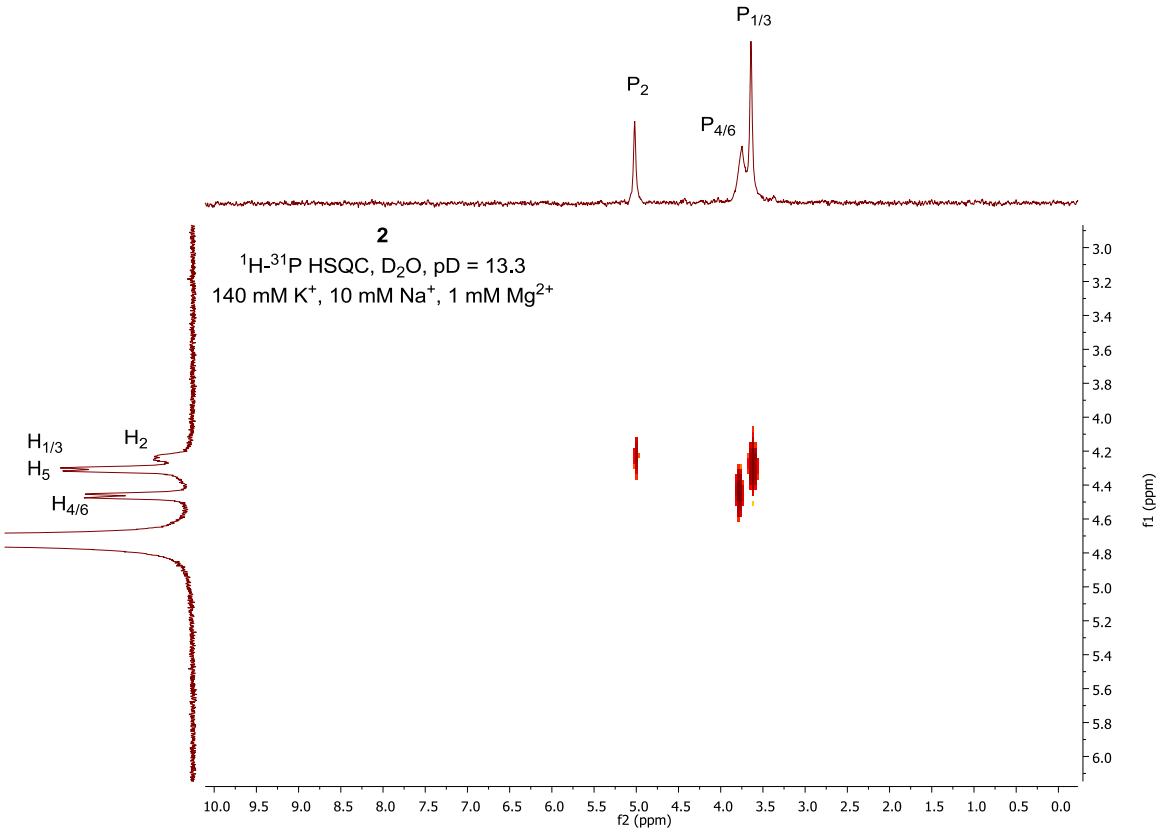


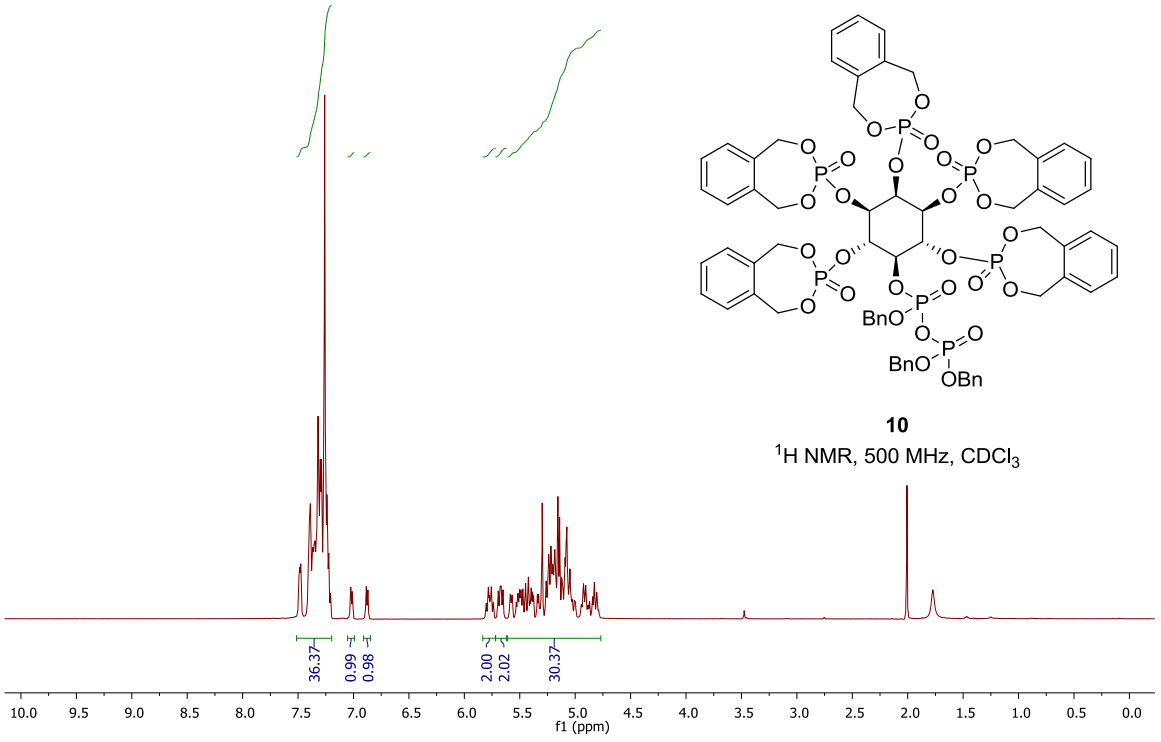


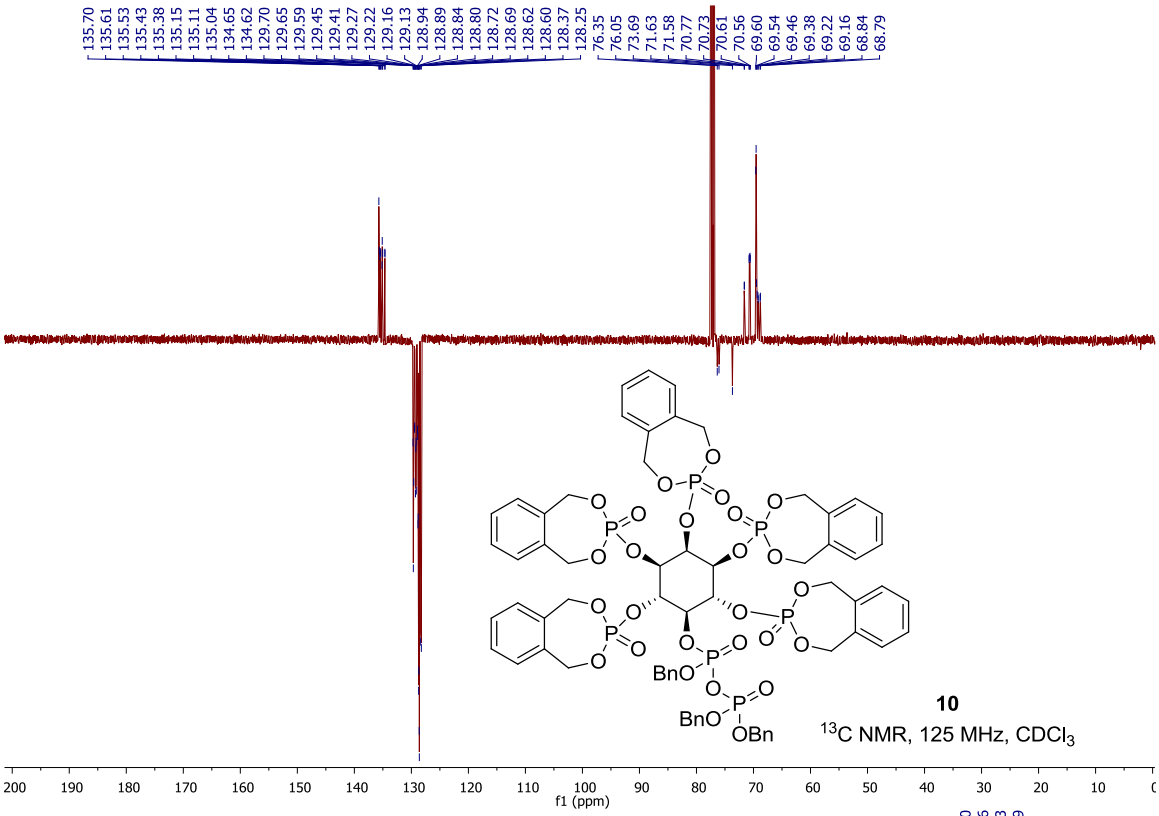
2

^{1}H NMR, 500 MHz, D_2O, pD = 12.5


23.43
23.39
13.29
13.25
5.15
3.89
3.87
OPO(ONa)2
(NaO)2OPO
OPO(ONa)2
(NaO)2OPO
OPO(ONa)2
O
P
O
NaO
P
O
NaO
ONa
2
31P NMR, 202 MHz, D2O, pD = 12.5
1.00
0.96
1.00
3.79
30 28 26 24 22 20 18 16 14 12 10 8 6 4 2 0 -2 -4 -6 -8 -10 -12 -14 -16 -18 -20
f1 (ppm)
H1/3
H5
H4/6
H2
2
1H-1H COSY, D2O, pD = 12.1
140 mM K+, 10 mM Na+, 1 mM Mg2+
H1/3
H5
H2
H4/6
3.0 3.2 3.4 3.6 3.8 4.0 4.2 4.4 4.6 4.8 5.0 5.2 5.4 5.6 5.8 6.0
f1 (ppm)
6.0 5.8 5.6 5.4 5.2 5.0 4.8 4.6 4.4 4.2 4.0 3.8 3.6 3.4 3.2 3.0
f2 (ppm)

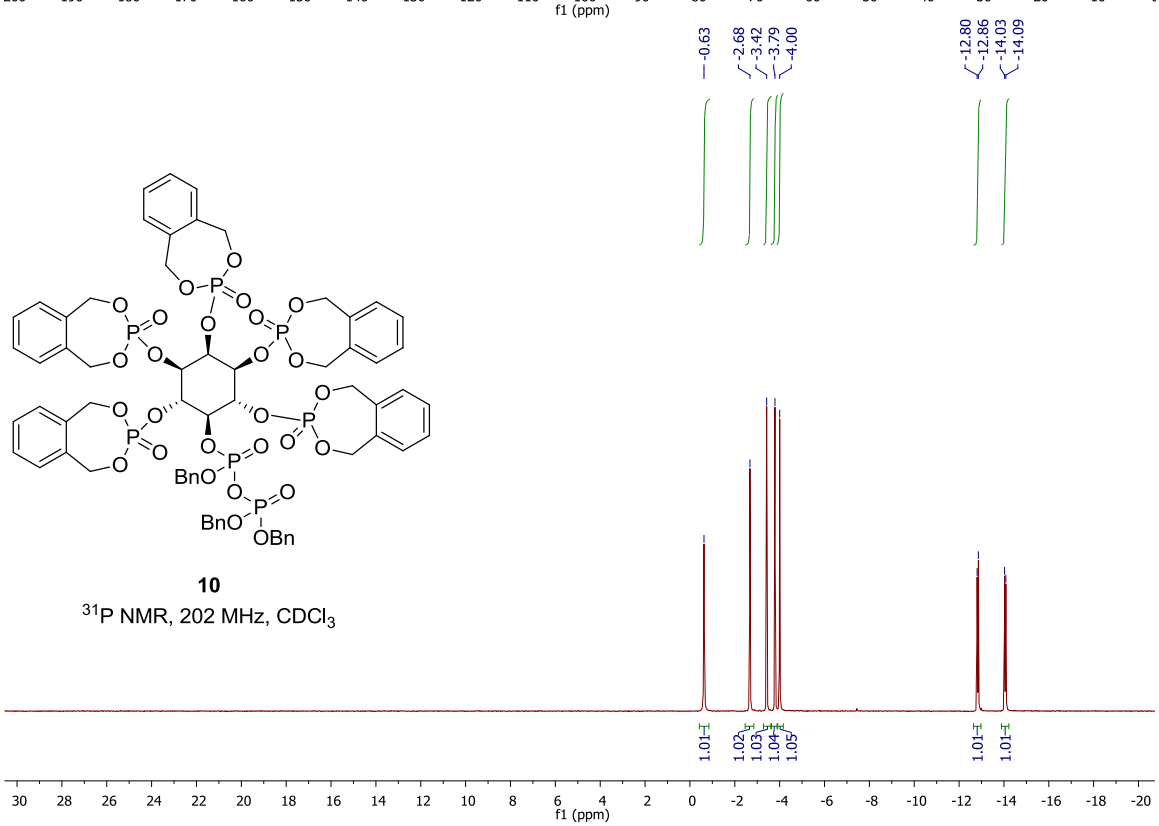

P1/3
P2
P4/6
2
1H-31P HSQC, D2O, pD = 13.3
140 mM K+, 10 mM Na+, 1 mM Mg2+
H1/3
H2
H5
H4/6
f2 (ppm)
f1 (ppm)


BnO
BnO
OBn
10
1H NMR, 500 MHz, CDCl3
36.37
0.99
0.98
2.00
2.02
30.37
f1 (ppm)



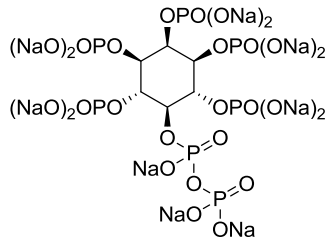
10

^{13}C NMR, 125 MHz, $CDCl_3$



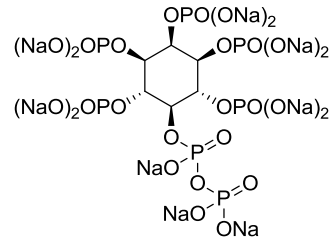
10

^{31}P NMR, 202 MHz, $CDCl_3$



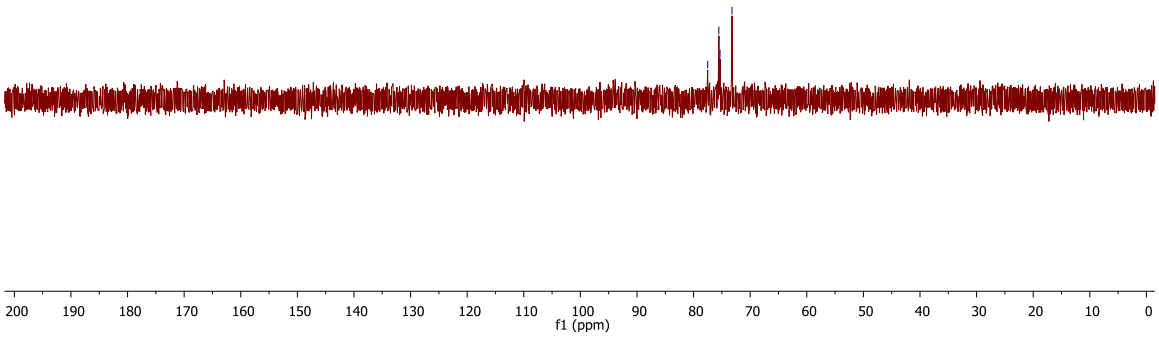
1

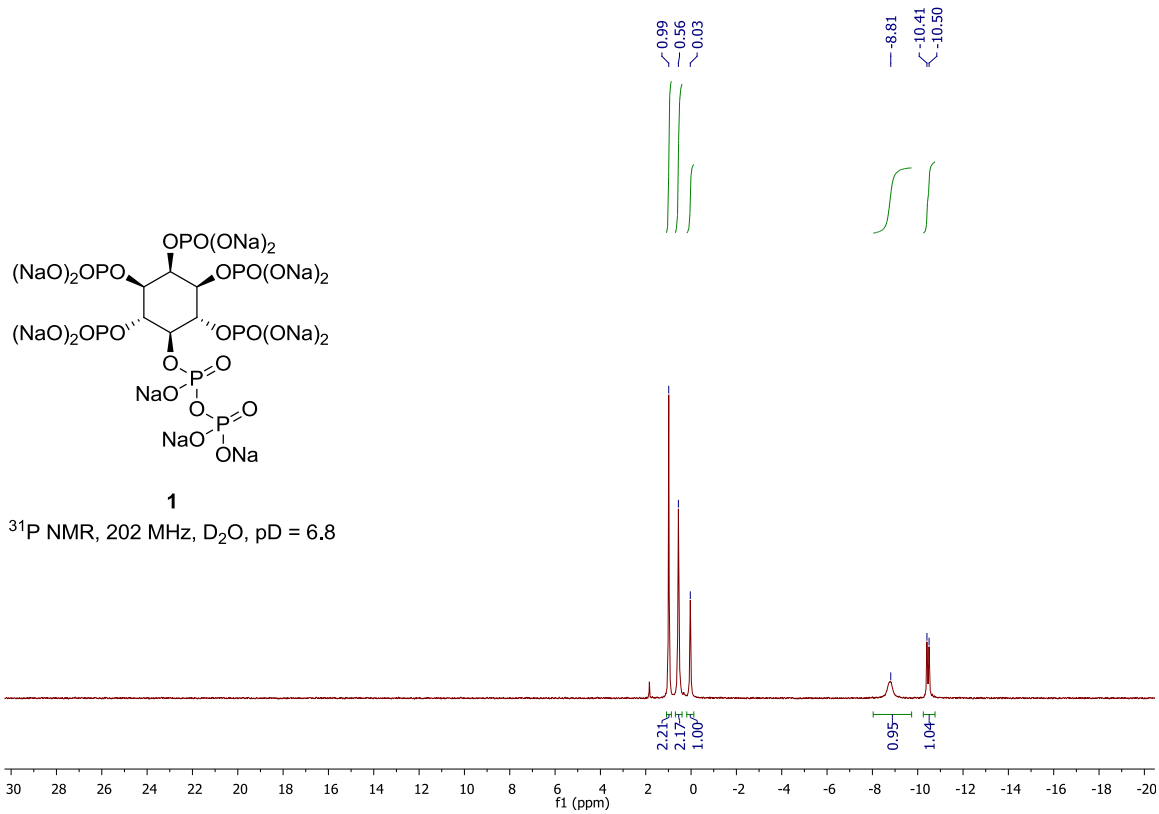
^{1}H NMR, 500 MHz, D_2O, pD = 6.8



1

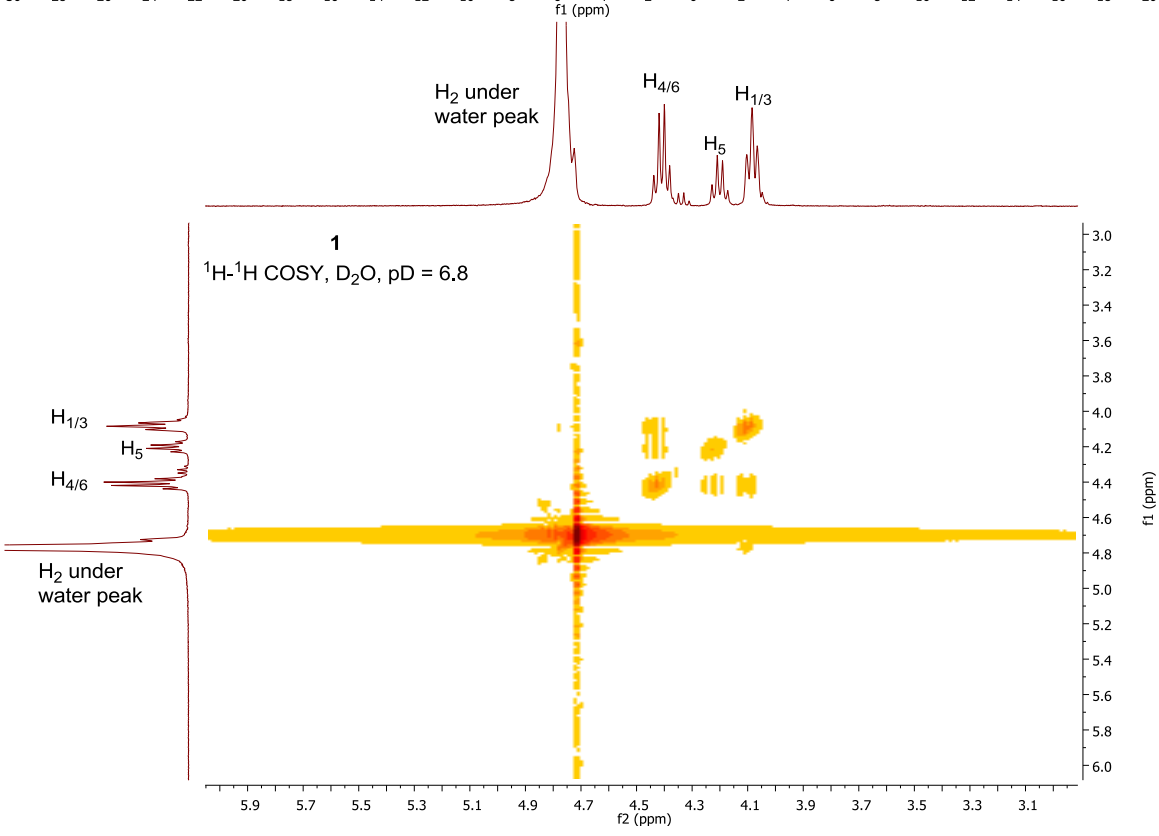
^{13}C NMR, 125 MHz, D_2O, pD = 6.8

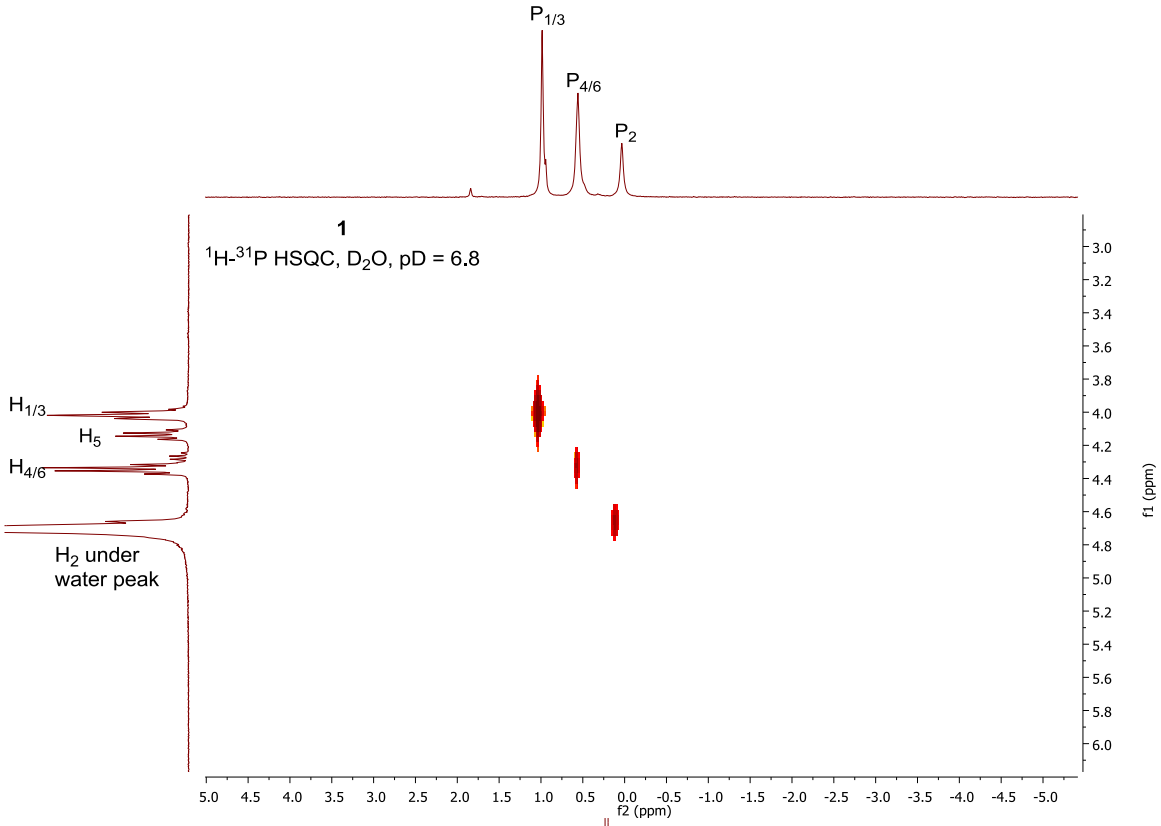


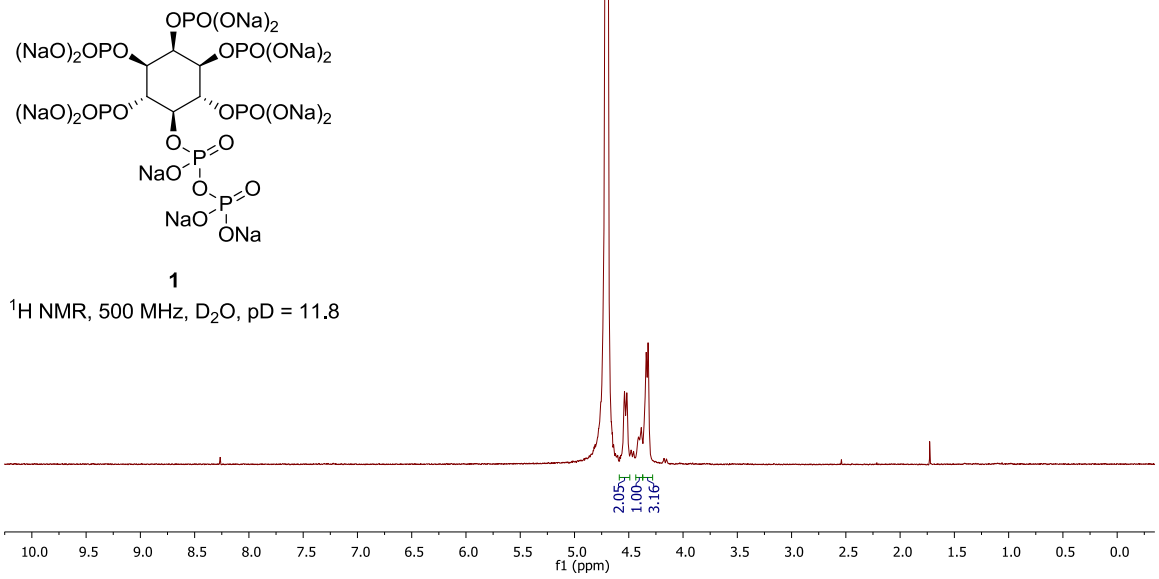


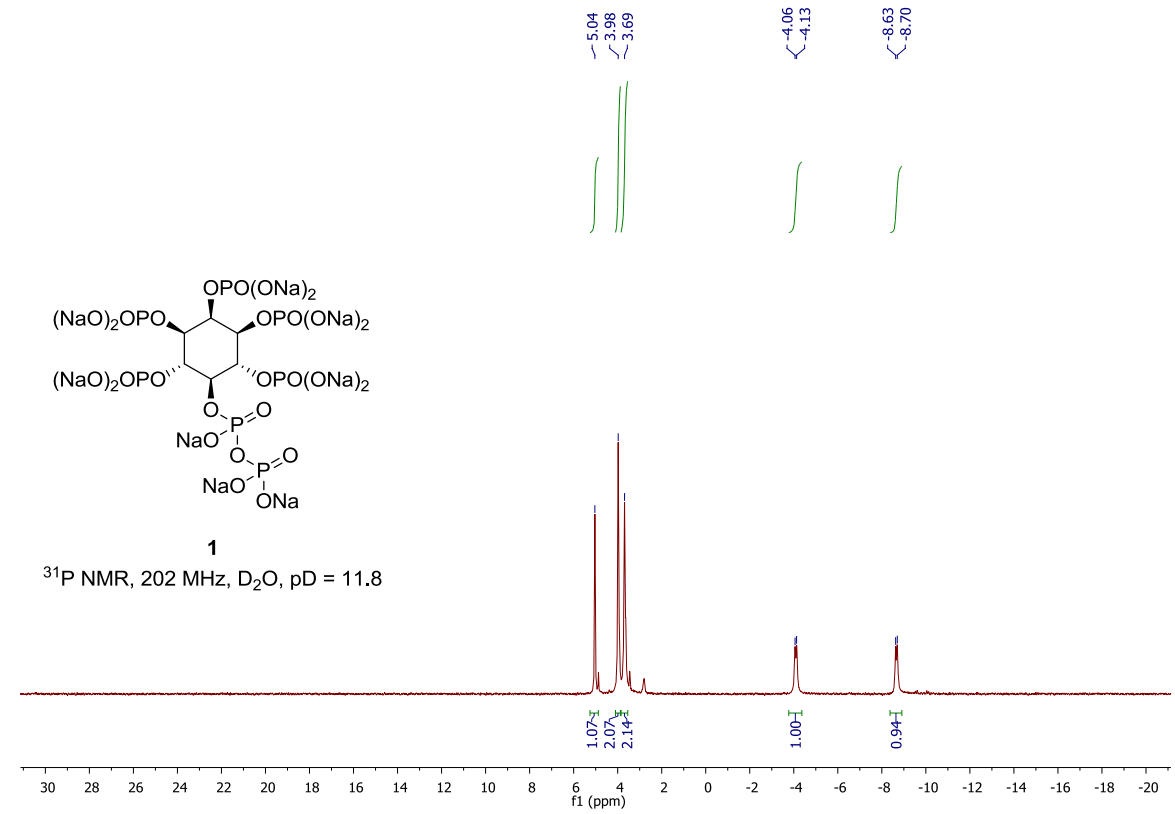
1

^{31}P NMR, 202 MHz, D_2O, pD = 6.8



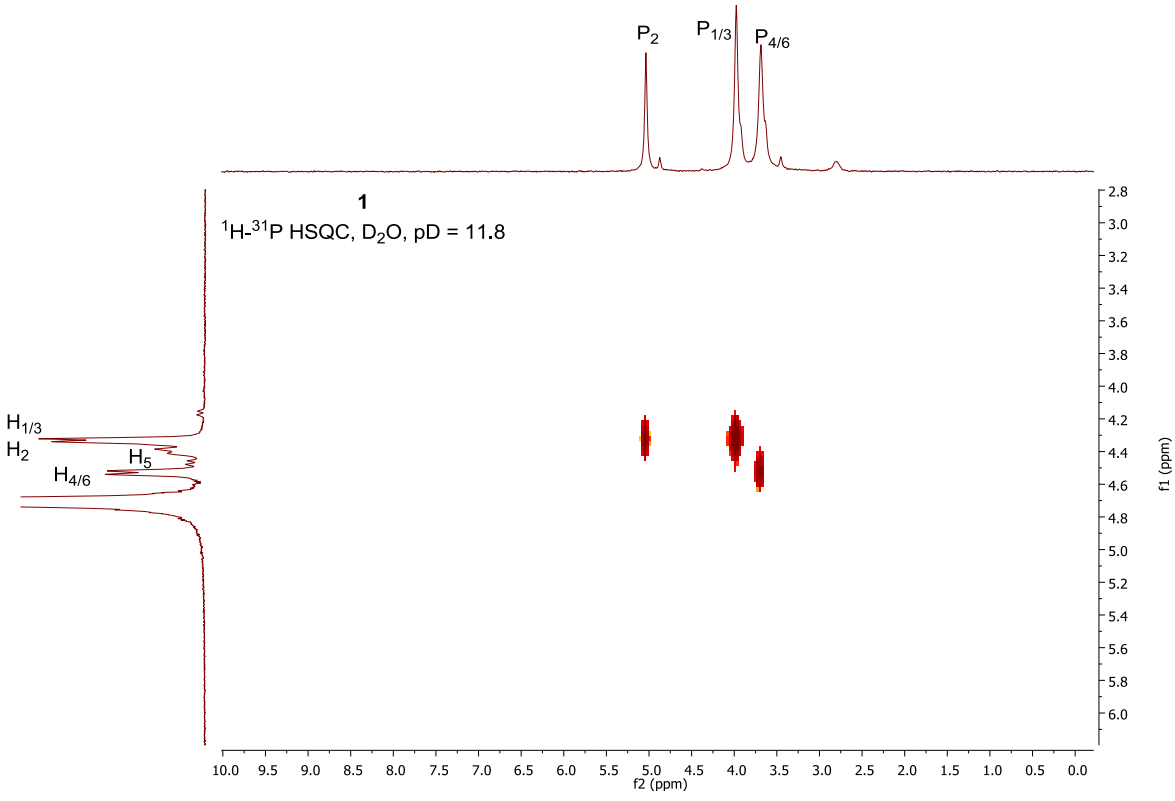

P1/3
P4/6
P2
1
1H-31P HSQC, D2O, pD = 6.8
H1/3
H5
H4/6
H2 under
water peak
f2 (ppm)
f1 (ppm)


OPO(ONa)2
(NaO)2OPO
OPO(ONa)2
(NaO)2OPO
OPO(ONa)2
NaO
NaO
ONa
1
1H NMR, 500 MHz, D2O, pD = 11.8
2.05
1.00
3.16
f1 (ppm)



1

^{31}P NMR, 202 MHz, D_2O, pD = 11.8



1

^{1}H-^{31}P HSQC, D_2O, pD = 11.8

VIII. References

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