

Supporting Information

Harnessing ^{13}C -labeled *myo*-inositol to interrogate inositol phosphate messengers by NMR

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Abbreviations

1,5(PP)₂-InsP₄	1,5-bisdiphosphoinositol tetrakisphosphate
[¹³C₆]5PP-InsP₅	[¹³ C ₆]5-diphosphoinositol pentakisphosphate
[¹³C₆]InsP₆	[¹³ C ₆]inositol hexakisphosphate
5PP-InsP₅	5-diphosphoinositol pentakisphosphate
ADP	adenosine diphosphate
ATP	adenosine triphosphate
BIRD	bilinear rotation decoupling
BIRD-HMQC	HMQC experiment with BIRD-pulse
CSA	Camphorsulfonic acid
CV	column volume
DAPI	4',6-diamidin-2-phenylindol
DCM	dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMF	<i>N,N</i> -dimethylformamide
DMSO	dimethylsulfoxide
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
FID	free induction decay
FPLC	fast protein liquid chromatography
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
HMQC	Heteronuclear Multiple-Quantum Correlation
Ins(1,4,5)P₃	<i>myo</i> -inositol 1,4,5-trisphosphate
InsP	inositol polyphosphate
InsP₆	inositol hexakisphosphate
IP6K1	(human) inositol hexakisphosphate kinase 1 (Genbank: AC099668)
IP6KA	inositol hexakisphosphate kinase A from <i>Entamoeba histolytica</i> (Genbank: XP_648490.2)
IPS	inositol-3-phosphate synthase (WP_010879290.1)
<i>k</i>_{cat}	turnover number
<i>K</i>_M	Michaelis-Menten constant
<i>m</i>CPBA	<i>meta</i> -chloroperoxybenzoic acid

MES	2-(<i>N</i> -morpholino)ethanesulfonic acid
MOPS	3-morpholinopropane-1-sulfonic acid
NADH	nicotinamide adenine dinucleotide (reduced form)
NAD⁺	nicotinamide adenine dinucleotide
MeCN	acetonitrile
NMR	nuclear magnetic resonance (spectroscopy)
NTA	nitrioloacetic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
pH*	the measured pH of a deuterated solution
PPIP5K	diphosphoinositol pentakisphosphate kinase
rt	room temperature
TEA	triethylamine or triethylammonium
TEAB	triethylammonium hydrogen carbonate
TFA	trifluoroacetic acid
THF	tetrahydrofuran
TRIS	tris(hydroxymethyl)-aminomethan
V_0	initial specific velocity
V_{max}	maximum specific velocity
NAD⁺	nicotinamide adenine dinucleotide

Supporting Figures and Table

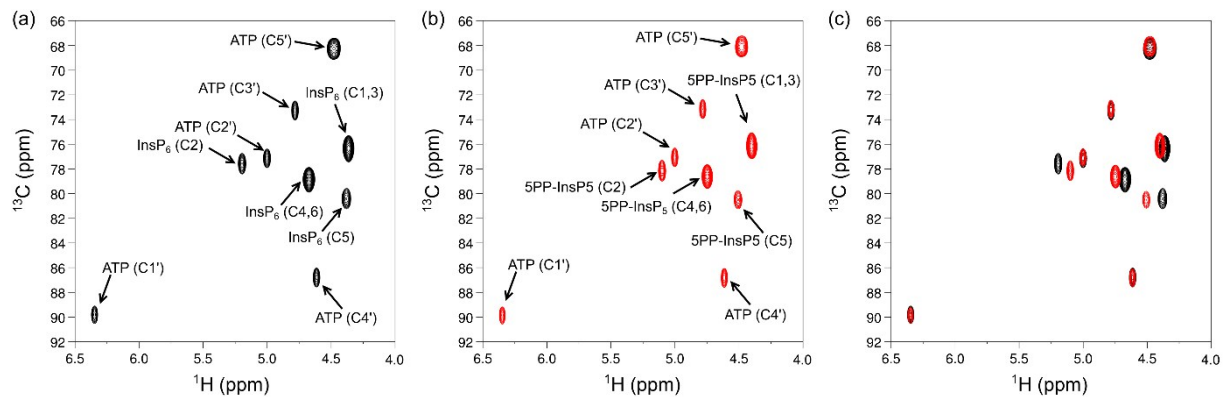


Figure S1. HMQC-Spectra to determine conversion to $^{13}\text{C}_6$ 5PP-InsP₅. (a) Control reaction without IP6KA shows no conversion of $^{13}\text{C}_6$ InsP₆. (b) Full conversion of $^{13}\text{C}_6$ InsP₆ to $^{13}\text{C}_6$ 5PP-InsP₅. (c) Overlay of a and b to illustrate that the C2 peaks are well resolved and can be used to monitor the reaction progress.

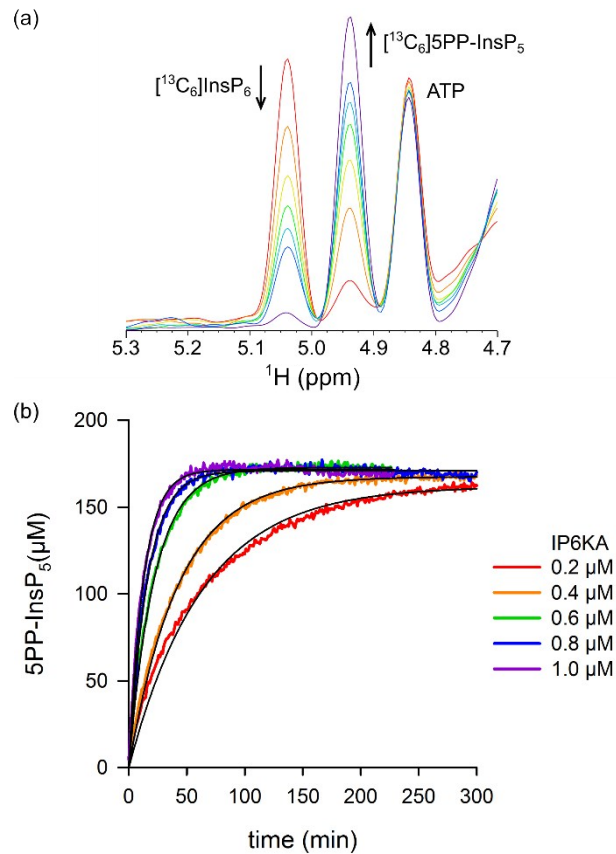


Figure S2: NMR-based measurements of IP6KA activity. (a) Superimposed pseudo-2D spin-echo difference spectra at different time points (6 min, 28 min, 50 min, 73 min, 95 min, 118 min, 455 min). The InsP_6 and 5PP-InsP_5 peaks are labeled and the change over time is indicated by the arrows. The intensity of the ATP-peak hardly changed due to the ATP regenerating system. (b) Progress curves of $[\text{C}_6]5\text{PP-InsP}_5$ synthesis at different IP6KA concentrations. An individual NMR-spectrum was recorded every 75 seconds. The $[\text{C}_6]5\text{PP-InsP}_5$ peak height was normalized against the HEPES signal and the data was fitted with a first order decay model.

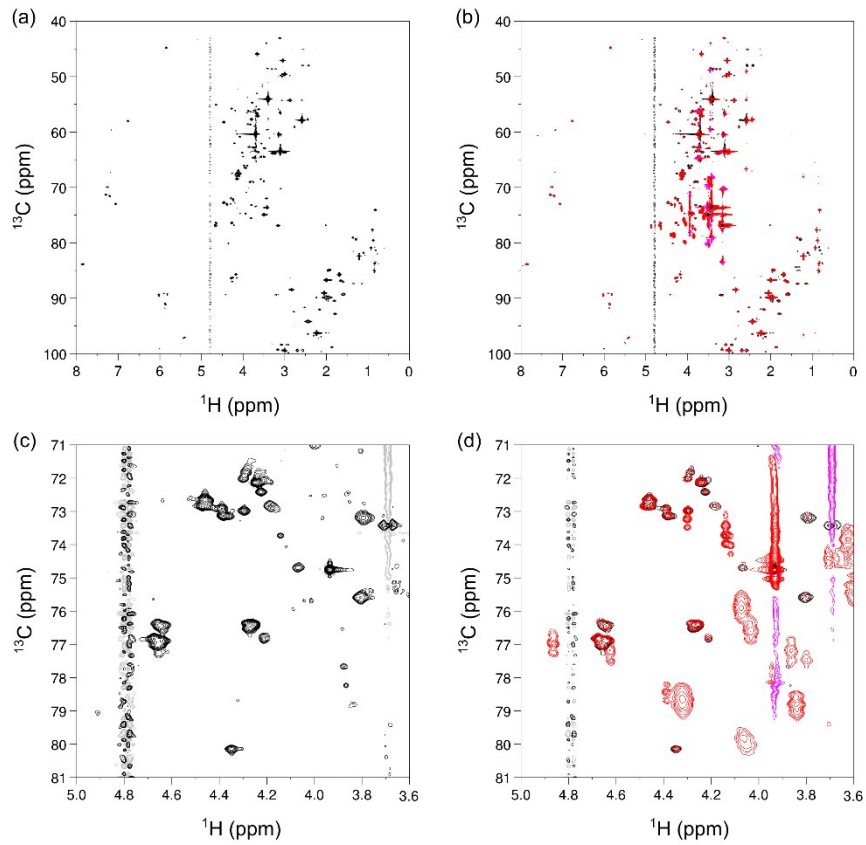
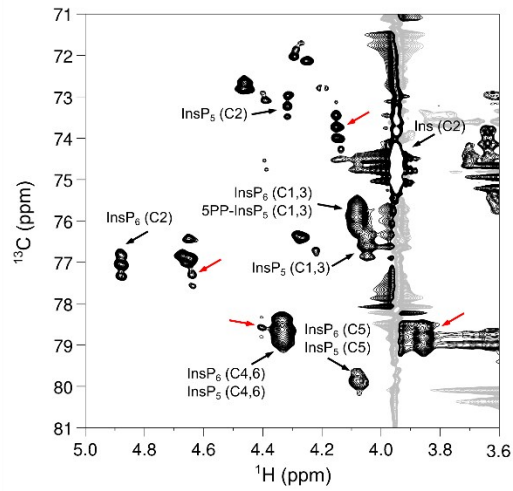


Figure S3: [$^{12}\text{C}_6$]myo-inositol labeled HCT116 wt cell extract. (a) The cells were grown in DMEM supplemented with 100 μM [$^{12}\text{C}_6$]myo-inositol and the InsPs were extracted. (b) Overlay of [$^{13}\text{C}_6$]myo-inositol-labeled (red) and unlabeled (black) HCT116 extracts. (c) Inositol phosphate region of the unlabeled HCT116 wt extract. (d) Inositol phosphate regions of overlay between [$^{13}\text{C}_6$]myo-inositol-labeled (red) and unlabeled (black) HCT116 wt extracts.



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figure S4: HEK293T cell extract. HEK293T cells were extracted with 1 M HClO_4 and the soluble fraction was measured by NMR-spectroscopy. The relevant InsP peaks are labeled and additional peaks displaying the triplet pattern are highlighted by red arrows.

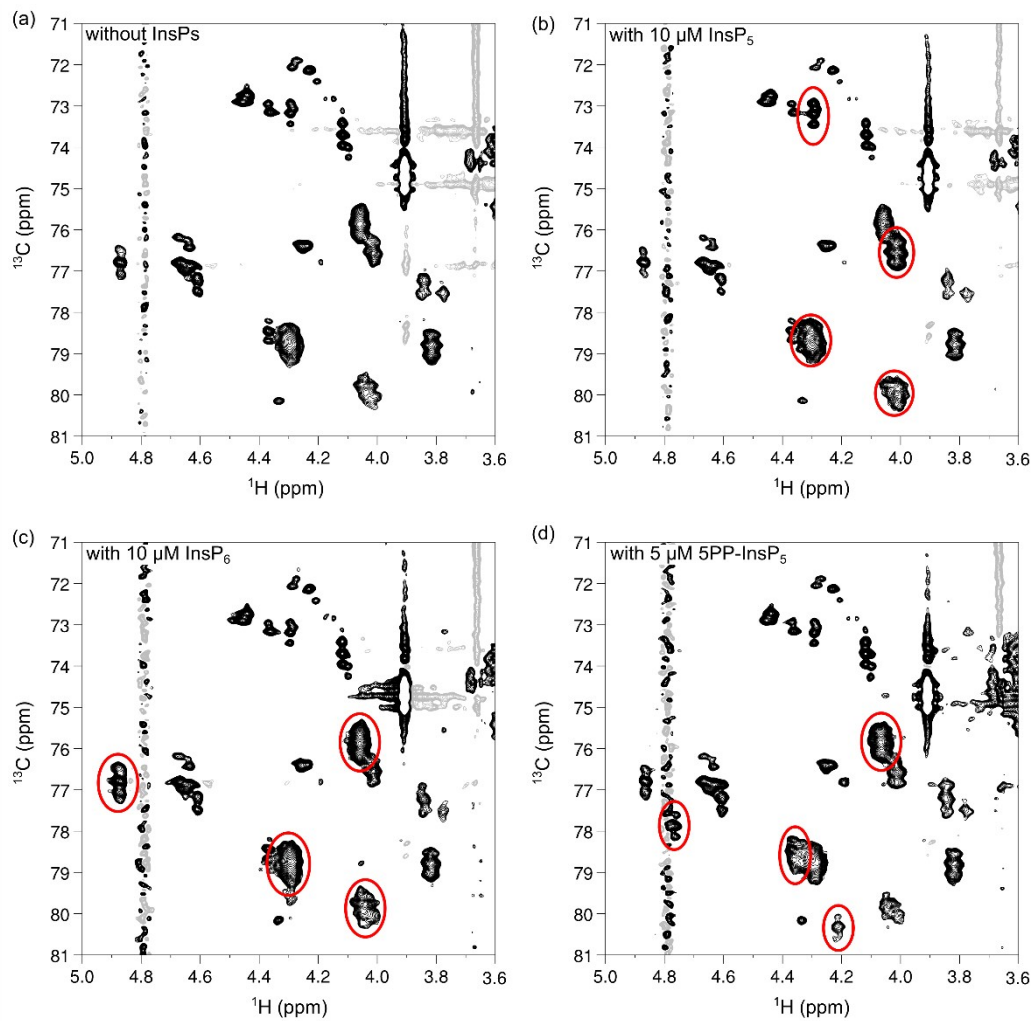


Figure S5: Spike in experiments to confirm the identity of InsP₅, InsP₆, and 5PP-InsP₅ in HCT116 extracts. (a) HCT116 extract. (b) Extract + InsP₅. (10 μM final concentration) (c) Extract + InsP₆. (10 μM final concentration) (d) Extract + 5-PP-InsP₅ (5 μM final concentration).

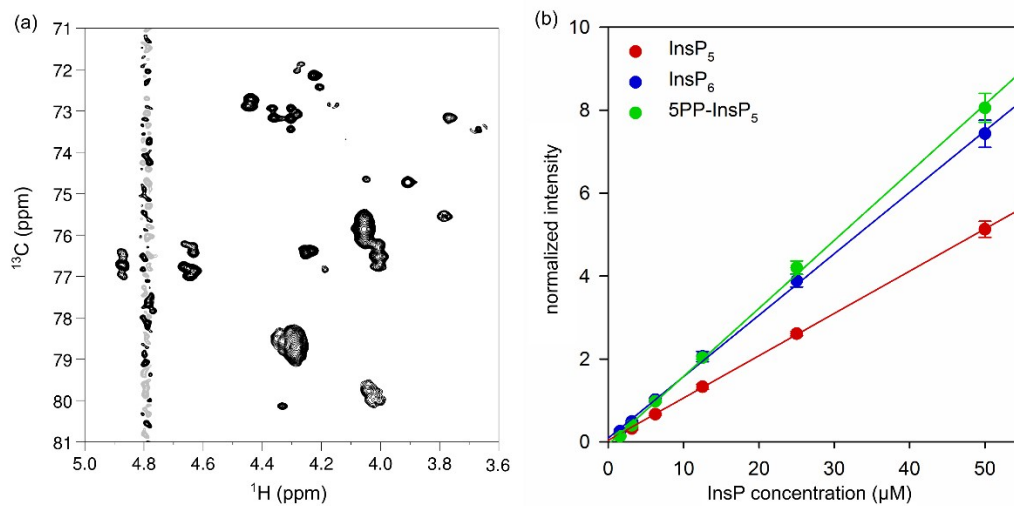
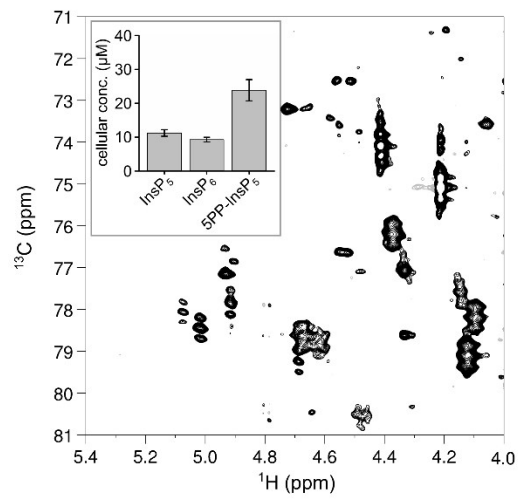


Figure S6. Absolute quantification of InsPs from HCT116 extracts. (a) 8 μM InsP₅, 10 μM InsP₆, 3 μM 5PP-InsP₅ spiked into a ^{12}C -labeled HCT116 extract. (b) Calibration curves for InsP C2 peak intensities normalized to the reference standard PME₄Br.

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figure S7. *PIP5K*^{-/-} cells were treated with 10 mM NaF for 1h prior to extraction. The insert displays triplicates of absolute cellular concentration of InsP_5 , InsP_6 and 5PP-InsP_5 based on packed cell volume.

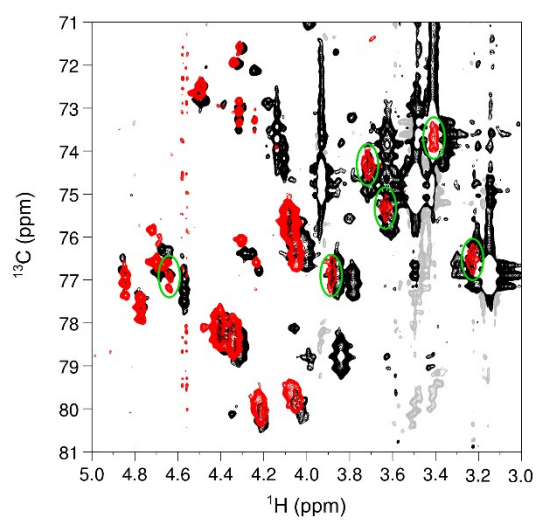


Figure S8: Overlay of TiO₂ enrichment of NaF treated, ¹³C-labeled HCT116 wt cell extract (red) and the original NaF treated, ¹³C-labeled HCT116 wt cell extract (black). One set of 6 peaks (green circles) was retained that corresponds to a putative unsymmetrical InsP₁ or InsP₂ species. All other labeled unassigned peaks were removed.

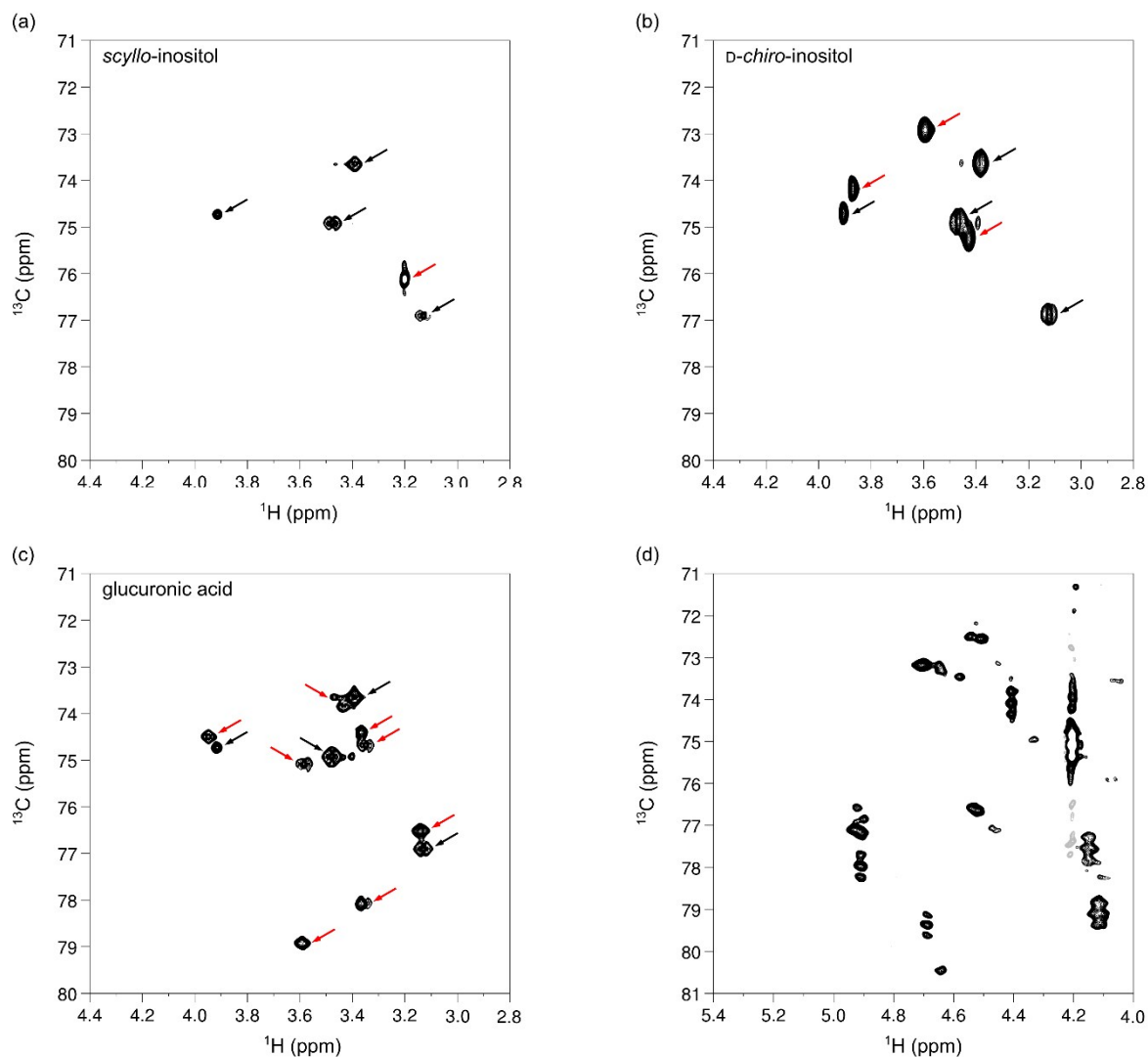


Figure S9: Preliminary analysis of unannotated NMR signals. HMQC spectrum of 1 mM (a) *scyllo*-inositol, (b) *D-chiro*-inositol, and (c) glucuronic acid (highlighted with red arrows) in KClO_4 saturated D_2O . 1 mM *myo*-inositol was included as an internal reference (black arrows). (d) Lipid extraction of HCT116 wt retained all unassigned peaks except for the putative unsymmetrical InsP_1 or InsP_2 species.

Table S1. Absolute quantification of InsP₅, InsP₆, and 5PP-InsP₅ in different cell lines.

Cell line	Condition	cellular concentration (μM) ^(a)		
		InsP ₅	InsP ₆	5PP-InsP ₅
HCT116 wt	no treatment	26.7 ± 2.3	29.4 ± 7.6	1.9 ± 0.5
	10 mM NaF ^(b)	18.6 ± 0.7	11.0 ± 0.9	14.7 ± 2.0
<i>PP1P5K</i> ^{-/-}	no treatment	19.4 ± 6.1	33.7 ± 7.8	5.1 ± 2.3
	10 mM NaF ^(b)	11.2 ± 1.0	9.3 ± 0.7	23.8 ± 3.1

(a) The C2 peak was used for quantification. The concentration in the NMR sample was calculated based on PMe₄Br as an internal standard and the cellular concentration was calculated based on the packed cell volume.

(b) Cells were treated with 10 mM NaF 1 hour before extraction. Packed cell volume was based on untreated sample.

General Information

All chemicals were purchased from Sigma Aldrich, VWR, Carl Roth, Thermo Fisher Scientific, Alfa Aesar, TCI and used without further purification unless stated otherwise. All dry solvents were purified using a solvent purification system MBRAUN MB-SPS-5 by passing through activated alumina columns. Deuterated solvents were purchased from Euriso-Top. The C18 Sep-Pak columns were purchased from Waters. Telos® was ordered from Kinesis. Normal phase flash chromatography was performed using analytical grade solvents and silica gel from VWR (40-63 μm) as stationary phase. Automated flash chromatography was performed using gradient grade solvents on a CombiFlash® Rf from Teledyne Isco using prepacked CombiFlash® columns (40-63 μm). Reversed phase flash chromatography was performed on C18 reversed phase silica gel from Teledyne Isco (40-63 μm) as stationary phase. LC-MS analysis was carried out with an Agilent 1260 Infinity Binary LC system connected to an Agilent 6130 Quadrupole LC/MS system with a ZORBAX Rapid Resolution HT Narrow Bore SB-C18 1.8 μm column (2.1 x 50mm) at 30 °C using API-ESI (atmospheric pressure ionization-electrospray) in positive ion mode. The eluent consisted of 10% ACN in water with 0.1% formic acid at 0.7 mL/min flow rate.

NMR spectra were recorded on Bruker spectrometers operating at 300 or 600 MHz for proton nuclei, 75 or 151 MHz for carbon nuclei or 122 and 244 MHz for phosphorous nuclei. NMR data are given as follows: chemical shift δ in ppm (multiplicity, coupling constant(s) J Hz, relative integral) where multiplicity is defined as: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, br = broad or combinations of the above. Measurement of the metabolic extracts was performed on Bruker AV-III spectrometers (Bruker Biospin, Rheinstetten, Germany) at 310 K using cryogenically cooled 5 mm TCI-triple resonance probe equipped with one-axis self-shielded gradients. The software used to control the spectrometer was topspin 3.5 pl6. Temperature had been calibrated using d_4 -methanol and the formula of Findeisen et al.¹

High-resolution mass spectrometer was performed by direct inject on an Orbitrap™ Q-Exactive mass spectrometer (Thermo Fisher Scientific).

For the purification *via* FPLC an NGC Quest™ 10 Chromatography System from Bio-Rad was used with an integrated NGC™ Sample Pump Module and a BioFrac™ Fraction Collector. For the spin filtration Amicon Ultra 0.5 mL centrifugal filters with a cut off of 10 kDa or 3 kDa from Merck Millipore were used.

Recombinant Protein Expression

Inositol phosphate Synthase (IPS)

The IPS-gene from *A. fulgidus* cloned into a pET23a vector (Prof. Helena Santos, Universidade Nova de Lisboa)² was transformed into *E. coli* BL21(DE3). Two 0.8 liter overnight cultures (37 °C) in Terrific Broth (TB) supplemented with Ampicillin were each diluted with 400 mL TB, prewarmed to 37 °C. After 30 min the expression was induced with 0.25 mM IPTG. After 4 hours the cells were harvested by centrifugation (3,000 g for 10 min at 4 °C) and washed with ice cold water. The pellet was stored at -80 °C until further use.

The frozen cells were resuspended in lysis buffer (50 mM Tris HCl pH 8, 250 mM NaCl). For 1 g cell pellet wet weight, 5 mL lysis buffer was used. The cell suspension was supplemented with lysozyme and DNase I, and incubated for 15 min on ice. The cells were lysed with a Microfluidizer™ LM10 at 15,000 psi with five iterations. The lysate was clarified by centrifugation (30,000 g for 30 min at 4 °C). The supernatant was incubated for 30 min at 80 °C and the precipitate was removed by centrifugation (30,000 g for 30 min at 4 °C). The volume of the heat-treated supernatant was reduced to 22 mL with spin filtration (Amicon® Ultra 15 mL 10K) and the solution was dialyzed against lysis buffer overnight. The protein solution was adjusted to 33 % (v/v) glycerol, aliquoted, frozen in liquid nitrogen, and stored at -80 °C.

Inositol hexakisphosphate kinase 1 (IP6K1)

The codon optimized IP6K1-gene from human cloned into a pTrcHis vector (a kind gift from Adam Resnick) was transformed into *E. coli* BL21 (DE3). A 1 L overnight culture (37 °C) in TB supplemented with Ampicillin was diluted with 500 mL TB, prewarmed to 37 °C. After 30 min the expression was induced with 1 mM IPTG. After 4 hours the cells were harvested by centrifugation (3,000 g for 10 min at 4 °C) and washed with ice cold water. The pellet was stored at -80 °C until further use.

The frozen cells were resuspended in lysis buffer (20 mM Tris HCl pH 7.4, 150 mM NaCl). For 1 g wet weight 10 mL lysis buffer was used. The cell suspension was supplemented with lysozyme, DNase I and 1 tablet cComplete™ protease inhibitor

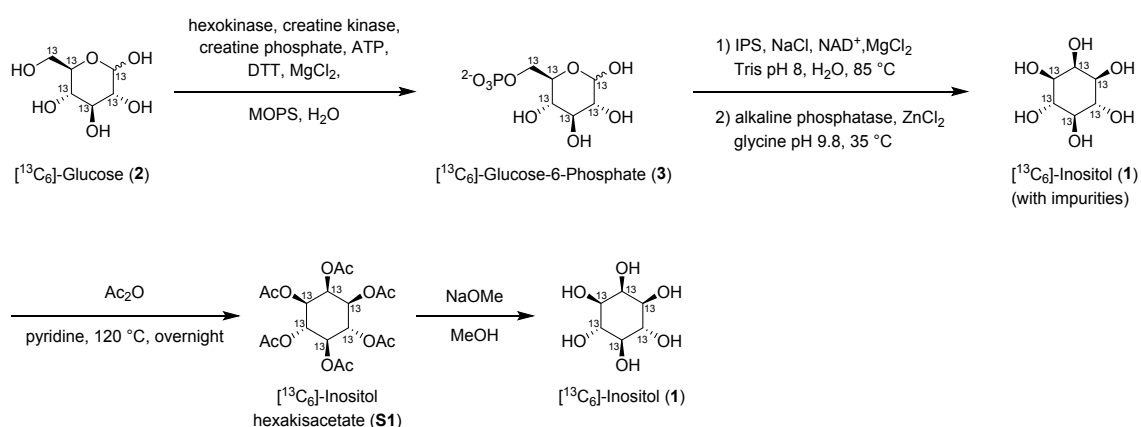
(Roche), and incubated for 15 min on ice. The cells were lysed with a microfluidizer™ LM10 at 15,000 psi with five iterations. The lysate was clarified by centrifugation (30,000 g for 30 min at 4 °C). The supernatant was adjusted to 0.1 % (v/v) Triton X-100, filtered (VWR® vacuum filter, PES 0.45 µm), and loaded onto a Co-NTA column (GE, 1 mL, HiTrap IMAC HP) that was equilibrated with lysis buffer with a flowrate of 1 mL/min. The column was washed with wash buffer (20 mM Tris HCl pH 7.4, 500 mM NaCl, 50 mM imidazole, 0.1 % (v/v) Triton X-100) until the absorption was constant. IP6K1 was eluted with a gradient of elution buffer (20 mM Tris HCl pH 7.4, 500 mM NaCl, 500 mM imidazole, 0.1 % (v/v) Triton X-100) in wash buffer from 0-100 % over 10 CV. 1 mL fractions were collected. The fractions containing IP6K1 (not more than three fractions were used) were concentrated to 0.5 mL by spin filtration (Amicon® Ultra 0.5 mL 10K) and dialyzed overnight against dialysis buffer (20 mM Tris HCl pH 7.4, 500 mM NaCl, 1 mM DTT). The protein solution was used immediately and not frozen.

Inositol hexakisphosphate kinase A (IP6KA)

The codon optimized IP6KA-gene from *E. histolytica* was subcloned from a pDest-566 vector (a kind gift from Stephen B. Shears)³ into a pET15b plasmid using NdeI and BamHI restriction sites that were introduced by PCR (forward primer (NdeI): GGCAGCCATATGAACACGAAAATCAAACGCG, reverse primer (BamHI): GCAGCCGGATCCTTACAGTGACTTAAATTCGTTTTTCG). The resulting pET15b-IP6KA plasmid was transformed into *E. coli* BL21 Arctic Express (DE3) and an overnight culture was diluted into 1 L of TB medium supplemented with Ampicillin to a final density of OD₆₀₀ 0.1. The cells were grown for 6 h at 37 °C. The culture was then switched to 18 °C for 30 min before induction with 0.1 mM IPTG for 18 hours. The cells were harvested by centrifugation (3,000 g for 10 min at 4 °C) and washed with ice cold water. The cell pellet was resuspended in lysis buffer (25 mM Tris HCl pH 7.4, 500 mM NaCl, 50 mM imidazole). For 1 g wet weight 10 mL lysis buffer was used. The cell suspension was supplemented with lysozyme, DNase I, and 1 tablet cOmplete™ protease inhibitor (Roche), and incubated for 15 min on ice. The cells were lysed with a microfluidizer™ LM10 at 15,000 psi with five iterations. The lysate was clarified by centrifugation (30,000 g for 30 min at 4 °C). The supernatant was filtered (VWR® vacuum filter, PES 0.45 µm), and loaded onto a Ni-NTA column (GE, 5 mL, HiTrap

IMAC HP) equilibrated with lysis buffer with a flowrate of 2.5 mL/min. The column was washed with lysis buffer until the absorption was constant. IP6KA was eluted with a gradient of elution buffer (25 mM Tris HCl pH 7.4, 200 mM NaCl, 500 mM imidazole) in lysis buffer from 0-100 % over 10 CV. 1.5 mL fractions were collected. The fractions containing IP6KA were concentrated by spin filtration (Amicon® Ultra 0.5 mL 10K) and dialyzed overnight against dialysis buffer (20 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM DTT). The following day the protein was adjusted to 25 % glycerol, frozen in liquid nitrogen and stored at -80 °C.

Synthesis of [¹³C₆]myo-inositol



The following stock solutions were prepared:

Hexokinase stock solution: 1000 U/mL in 50 mM citrate pH 7, 10 mM MgCl₂, 1 mg/mL BSA.

Creatine kinase stock solution: 350 U/mL in 200 mM MOPS pH 6.5, 20 mM MgCl₂, 20 mM DTT.

Inositol phosphate synthase stock solution: 4 mg/mL in 20 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM DTT.

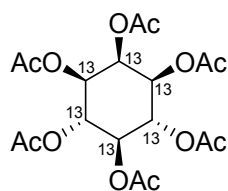
Alkaline phosphatase stock solution: 100 U/mL, 10 mM Tris pH 8.5, 5 mM MgCl₂, 0.2 mM ZnCl₂, 50 % (v/v) glycerol.

A solution containing D-[¹³C₆]glucose (2) (83 mM, 1000 mg, 5.4 mmol), MOPS (100 mM, pH 6.5), creatine phosphate (87 mM), ATP (1 mM), DTT (20 mM) and MgCl₂ (20

mM) in MilliQ® water (65 mL total reaction volume) was prepared and evenly split (32.5 mL each) into two 50 mL conical tubes. Hexokinase (1 U/mL) and creatine kinase (1.75 U/mL) were added and the reaction incubated at 30 °C overnight. The resulting solution was monitored by TLC (MeOH:H₂O:NH₄OH:AcOH, 50:30:15:5; stained by KMnO₄ or excessive heating) and upon completion diluted 5-fold with 320 mL of water. An anion-exchange column (DOWEX® 1X8) was equilibrated with 1 M (NH₄)HCO₃ and washed with water. The reaction mixture was loaded onto the column followed by washing with water to remove unreacted **2**. [¹³C₆]glucose-6-phosphate (**3**) was eluted by 0.1 M (NH₄)₂CO₃ and lyophilized to obtain the product **3** in combination with high amount of salts (4 g) as a white solid.

The product/salt mixture was redissolved in 40 mL MilliQ® water and added to a solution of Tris (50 mM, pH 8.0), NAD⁺ (0.5 mM), NaCl (50 mM) and MgCl₂ (2 mM) in MilliQ® (196 mL total reaction volume). The pH was adjusted to 8.0, if necessary, and the mixture was evenly split (49 mL each) into four 50 mL conical tubes. Recombinantly expressed inositol-3-phosphate synthase (IPS) (500 µL of 4 mg/mL stock) was added to each tube and the reaction mixture was incubated at 85 °C monitoring conversion by NMR. After 4 h, NAD⁺ (10 mg, 0.8 mM total) and IPS (500 µL, 4 mg/mL) were added to each tube and the mixture was incubated for additional 4 h at 85 °C. After full conversion of **3** into [¹³C₆]myo-inositol-3-phosphate (**4**) was observed by ¹³C NMR the reaction mixture was directly used for the subsequent dephosphorylation reaction.

For the dephosphorylation reaction, the 200 mL reaction mixture was split evenly across 5 conical tubes (40 mL each). For a total reaction volume of 50 mL, glycine (50 mM, pH 9.8) was added and the pH adjusted to 9.8. ZnCl₂ (0.25 mM) and alkaline phosphatase (0.5 U/mL) were added and the solution was filled up to 50 mL with MilliQ® water. After incubation at 35 °C overnight, all tubes were combined (250 mL total) and diluted with 300 mL MilliQ® water and the reaction mixture was applied to an ion exchange column (DOWEX® 1X8 in HCO₃⁻ form). The flow-through was collected and lyophilized to afford [¹³C₆]myo-inositol (**1**) with salts (7.5 g) as a brown solid which was directly used for the acetylation reaction.



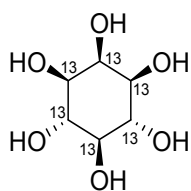
[¹³C₆]Inositol hexakisacetate (**S1**)

The crude material of **1** (7.5 g) was suspended in pyridine (161 mL) and treated with acetic anhydride (68.5 mL) at 120 °C overnight. The black solution was concentrated to minimize the amount of pyridine, re-dissolved in DCM (500 mL), and 1 M HCl was added. The resulting suspension was filtered and the filter washed with DCM and water. All filtrates were combined and organic and aqueous phase were separated. (Note: Phase separation is hard to see because of the black solution so use a flash light to find the separation layer.) The aqueous layer was extracted twice with DCM and the combined organic layers were washed with 1 M NaHCO₃. The aqueous layer was washed twice with DCM and the combined organic layers were washed with brine, dried with Na₂SO₄ and concentrated under reduced pressure. The residue was immobilized on Telos® and purified by column chromatography on silica gel changing the eluent step wise (hexane:EtOAc, 10:1 → 5:1 → 1:1 → 1:2) assisted by analysis of the fractions by LCMS to afford compound **S1** (1200 mg, 2.7 mmol) as a white solid in 50% overall yield from **2**.

¹H NMR (600 MHz, CDCl₃) δ 5.79 – 5.54 (m, 1.5H), 5.53 – 5.32 (m, 1.5H), 5.32 – 5.12 (m, 1.5H), 5.11 – 4.82 (m, 1.5H), 2.20 (s, 3H), 2.01 (s, 3H), 2.01 (s, 6H), 2.00 (s, 6H).

¹³C NMR (151 MHz, CDCl₃) δ 169.94, 169.81, 169.56, 71.68 – 70.65 (m), 70.11 – 69.15 (m), 69.11 – 67.90 (m), 20.89, 20.69, 20.60.

HRMS (ESI/Orbitrap) m/z: [M + K]⁺ calcd. for C₁₂¹³C₆H₂₄KO₁₂ 477.1101; Found 477.1091.



[¹³C₆]myo-Inositol (**1**)

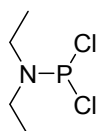
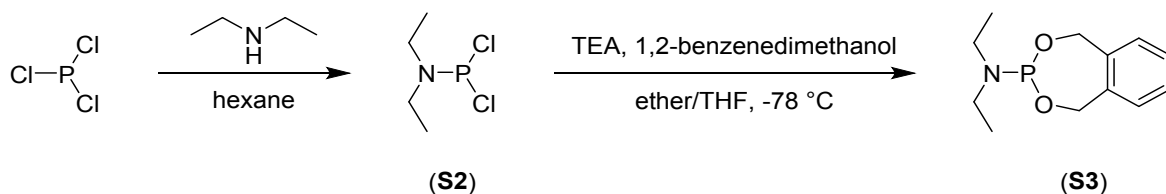
To a solution of **S1** (1200 mg, 2.7 mmol) in methanol (249 mL), 5.4 M NaOMe in MeOH (3.35 mL, 18.1 mmol) was added and the reaction was left to stir for 2 hours. The resulting suspension was neutralized by the addition of DOWEX 50W x 8 (H⁺ form) followed by filtration. The residue was washed with methanol (100 mL) and water (100 mL) to dissolve all precipitated [¹³C₆]myo-inositol and the filtrate was evaporated. The resulting solids were redissolved in H₂O and precipitated by the addition of MeCN. Subsequently, the solids were redissolved in H₂O and lyophilized to afford **1** (505 mg, 2.7 mmol) as white solid in 99% yield.

¹H NMR (600 MHz, D₂O, pD 7.0) δ 4.29 – 4.14 (m, 0.5H), 4.03 – 3.90 (m, 0.5H), 3.82 – 3.60 (m, 2H), 3.59 – 3.34 (m, 2.5H), 3.25 – 3.12 (m, 0.5H).

¹³C NMR (151 MHz, D₂O, pD 7.0) δ 74.96 – 73.80 (m), 72.86 – 71.71 (m), 71.60 – 70.50 (m).

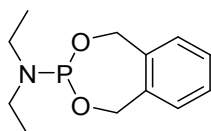
HRMS (ESI/Orbitrap) m/z: [M – H]⁻ Calcd for ¹³C₆H₁₁O₆ 185.0762; Found 185.0814.

Synthesis of xylyl phosphoamidite



N,N-diethylaminodichlorophosphine (S2)

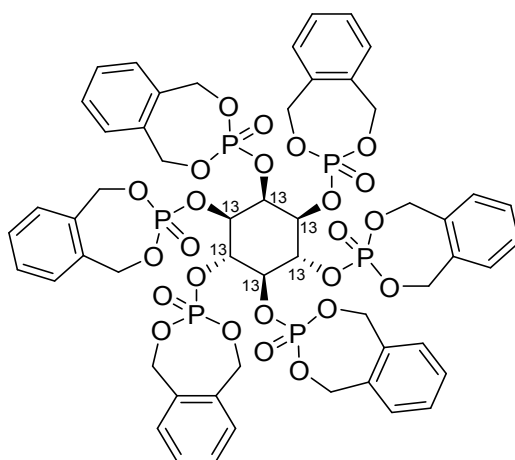
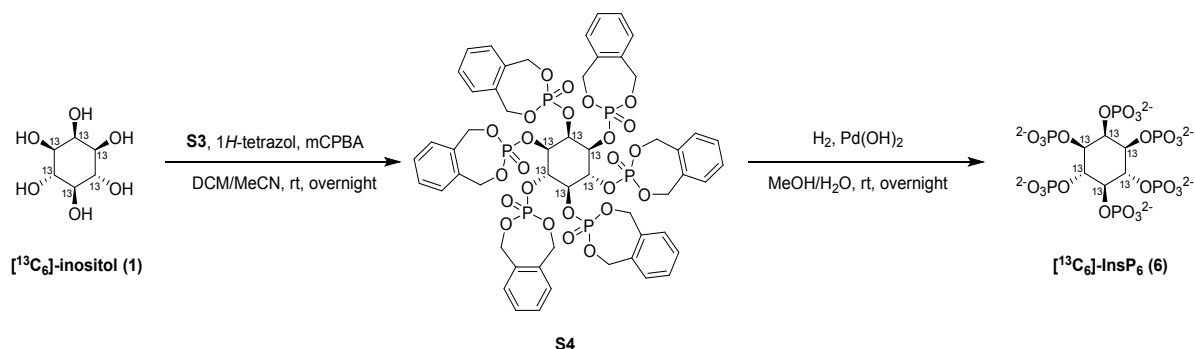
N,N-diethylaminodichlorophosphine was synthesized according to a procedure of Drent and coworkers.⁴ **S2** was obtained in 68% yield (12.9 g, 74.37 mmol) in good purity. ¹H-, ¹³C- and ³¹P NMR are in agreement with the literature procedure.



Xylyl phosphoramidite (S3)

Xylyl phosphoamidite was synthesized according to a modified procedure of Gregory and coworkers⁵. **S2** (11.1 g, 63.79 mmol) was dissolved in dry THF (425 mL) and cooled to -78 °C. A solution of triethylamine (12.9 g, 17.8 mL, 127.59 mmol) and phenyldimethanol (8.8 g, 63.79 mmol) in dry THF (425 mL) was prepared and added to the stirring reaction mixture with a dropping funnel over 30 min. The cooling was removed and the reaction left to stir overnight at room temperature. The resulting precipitates were removed by filtration and washed with dry THF (2 × 100 mL) and the filtrate was concentrated to 500 mL. TEA (5 mL) was added to the solution and it was passed through a silica plug (equilibrated with 1% TEA in dry THF). Subsequently, 1% TEA in THF (2.0 L) was passed through the silica plug and the combined organic fractions were concentrated to afford the product (14.9 g, 62.09 mmol) as colorless oil in 97 % yield. The product can be stored at -20 °C under N₂ for several months. ¹H-, ¹³C- and ³¹P NMR are in agreement with the literature procedure.

Synthesis of [¹³C₆]inositol hexakisphosphate (**6**)



Xylyl protected [¹³C₆]inositol hexakisphosphate (**S4**)

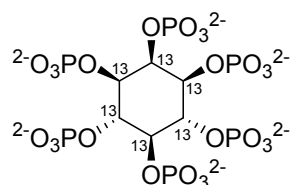
Xylyl protected [¹³C₆]inositol hexakisphosphate (**S4**) was synthesized according to a modified procedure of Podeschwa and coworkers⁶. Under nitrogen atmosphere a suspension of **1** (100 mg, 0.54 mmol) and **S3** (900 mg, 3.76 mmol) in dry DCM (38.65 mL) was prepared and sonicated for 1 min. 1*H*-tetrazole in anhydrous MeCN (14.33 mL, 6.45 mmol, 0.45 M) was added and the solution was stirred at rt overnight. For workup the solution was cooled to -40 °C and an anhydrous solution of *m*CPBA in DCM (30 mL dried with Na₂SO₄) was added. The solution was allowed to warm to rt, and the stirring was continued for another hour. The reaction mixture was diluted with DCM (300 mL) and washed consecutively with aqueous sodium bisulfite (20%, 2 × 50 mL), saturated NaHCO₃ (3 × 100 mL), and then with brine. After evaporation of the solvents, the crude was immobilized on Telos® and purified by CombiFlash® chromatography on silica gel (12 g column, gradient: 0% → 2% → 4% → 10% MeOH in DCM) and afforded the product (221 mg, 0.173 mmol) as a white solid in 32% yield.

¹H NMR (600 MHz, Chloroform-*d*) δ 7.60 – 7.49 (m, 18H), 7.44 (d, *J* = 7.7 Hz, 6H), 5.93 (dd, *J* = 13.8, 9.5 Hz, 2.5H), 5.84 (dd, *J* = 13.7, 9.1 Hz, 2H), 5.79 – 5.70 (m, 6H), 5.66 (s, 0.5H), 5.55 (dd, *J* = 13.9, 12.4 Hz, 3H), 5.50 – 5.16 (m, 15H), 5.12 (s, 1H).

¹³C NMR (151 MHz, Chloroform-*d*) δ 135.94, 135.91, 135.73, 135.69, 135.52, 134.62, 129.72, 129.60, 129.47, 129.45, 129.42, 129.40, 129.39, 129.36, 129.19, 129.13, 78.48 – 76.20 (m), 73.92 (dd, *J* = 43.9, 34.0 Hz), 69.84, 69.79, 69.68, 69.62, 69.57, 69.51, 69.45.

³¹P NMR (122 MHz, Chloroform-*d*) δ -2.80, -3.57, -4.48, -4.71.

HRMS (ESI/Orbitrap) *m/z*: [M + H]⁺ Calcd for C₄₈¹³C₆H₅₄O₂₄P₆ 1279.1705; Found 1279.1676.



[¹³C₆]inositol hexakisphosphate (**6**)

[¹³C₆]inositol hexakisphosphate (**6**) was synthesized according to a modified procedure of Godage and coworkers⁷. Compound **S4** (148 mg 0.12 mmol) was dissolved in methanol (10.53 mL) and water (2.63 mL) and 20% Pd(OH)₂/C (50% wetted with water) (68 mg, 0.49 mmol) was added. The resulting suspension was stirred at room temperature overnight under hydrogen atmosphere. The reaction mixture was passed through a PTFE syringe filter and the filtrate was evaporated under reduced pressure. The free acid was treated with NaOH (1 M) to afford the dodecasodium salt of **6** as a white solid (136 mg, 0.10 mmol, 66 w/w%) in 83% yield.

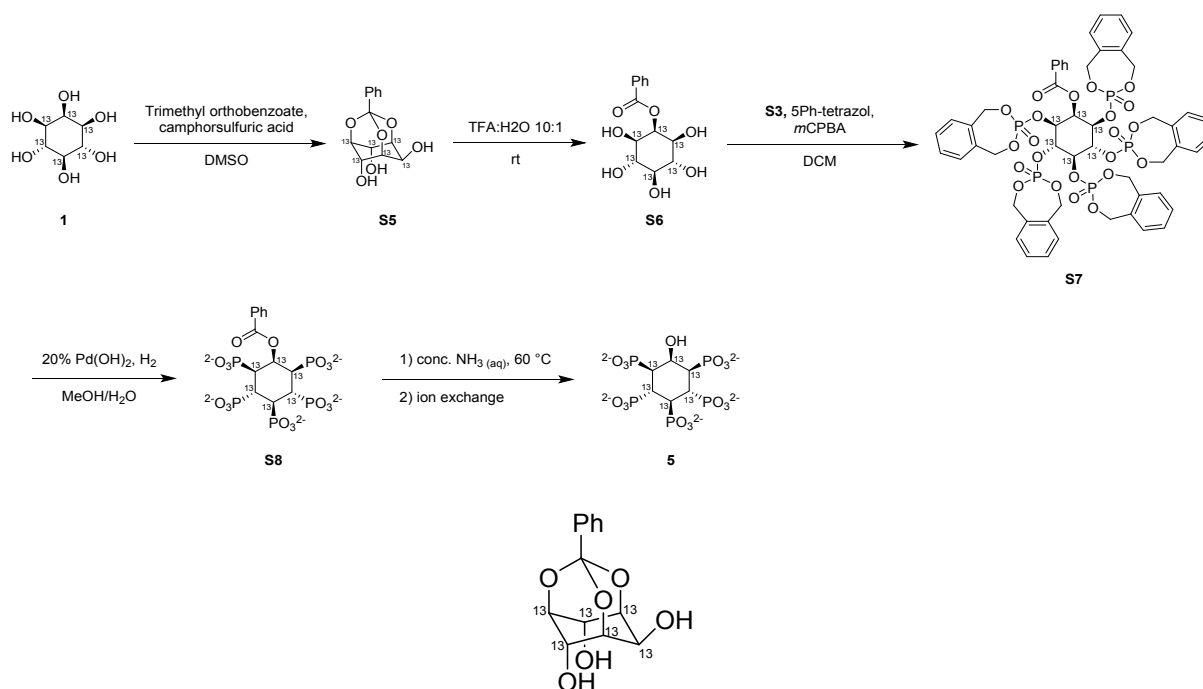
¹H NMR (600 MHz, Deuterium Oxide, pD 7.0) δ 5.01 – 4.90 (m, 0.5H), 4.71 – 4.63 (m, 0.5H), 4.48 – 4.34 (m, 1H), 4.22 – 4.04 (m, 2.5H), 3.95 – 3.78 (m, 1.5H).

¹³C NMR (151 MHz, Deuterium Oxide, pD 7.0) δ 79.99 (d, *J* = 35.7 Hz), 78.52 (t, *J* = 40.0 Hz), 77.60 – 74.58 (m).

³¹P NMR (122 MHz, Deuterium Oxide, pD 7.0) δ 1.99, 1.04, 0.77.

HRMS no ion detected.

Synthesis of and [¹³C₆]inositol pentakisphosphate



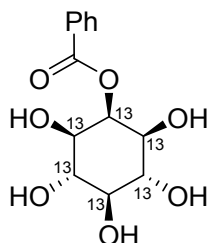
[¹³C₆]inositol-(1,3,5)-orthoester (**S5**)

[¹³C₆]inositol-(1,3,5)-orthoester (**S5**) ester was synthesized according to a modified procedure of Godage and coworkers⁷. A suspension of **1** (1.045 g, 5.61 mmol) and CSA (26.08 mg, 0.11 mmol) in DMSO (3.74 mL) was heated to 80 °C under vacuum (Rotovap, 30-40 mbar) to remove residual water. Upon the addition of trimethyl orthobenzoate (1.125 g, 6.17 mmol, 1.061 mL), the mixture was left at 80 °C under vacuum (Rotovap, 30-40 mbar) until the suspension became clear. The resulting solution was quenched with TEA (62.5 mg, 0.62 mmol, 86 μL). (Note: Quenching of acid is highly important to assure the stability of the formed orthoester during the workup.) The product was slowly precipitated through the addition of water (16 mL) at 4 °C and the solids were filtered and washed with ice cold water 3 times. The mother lye was combined with the washings and applied to reversed phase chromatography changing the eluent step wise (5 % → 25 % → 50 % MeCN in H₂O) to separate residual product from DMSO. The product containing fractions were lyophilized and the precipitates were dissolved in 50 % MeCN in H₂O and lyophilized to afford the product **S5** (1.300 g, 4.78 mmol) as white solid in 85% yield.

¹H NMR (600 MHz, Methanol-*d*₄) δ 7.71 – 7.64 (m, 2H), 7.42 – 7.30 (m, 3H), 4.70 (s, 1H), 4.45 (s, 2.5H), 4.37 (s, 0.5H), 4.18 (s, 1.5H), 4.13 (s, 0.5H).

¹³C NMR (151 MHz, Methanol-*d*₄) δ 130.12, 128.58, 126.74, 77.50 (t, *J* = 37.2 Hz), 71.63 (t, *J* = 37.7 Hz), 68.98 (t, *J* = 37.7 Hz), 60.20 (t, *J* = 36.8 Hz).

HRMS no ions detected.



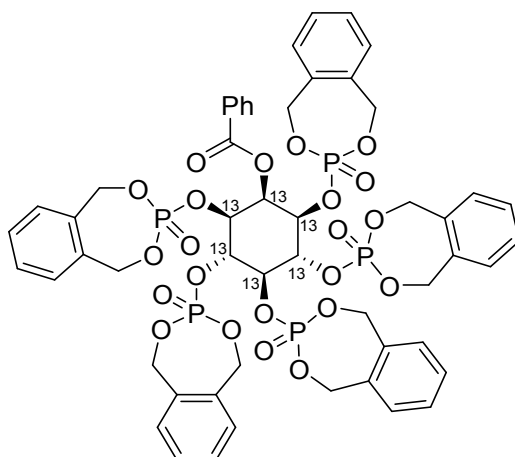
2-Benzoyl [¹³C₆]inositol (**S6**)

[¹³C₆]benzoyl inositol (**S6**) was synthesized according to a procedure of Godage and coworkers⁷. A mixture of TFA (1.8 mL) and water (180 μL) was added to **S5** (300 mg, 1.1 mmol) and the solution was stirred for 1 h and the conversion was followed by TLC (100 % EtOAc; starting material *R*_f 0.6; product *R*_f 0.0). The reaction mixture was then co-evaporated with water *in vacuo*, redissolved in water and lyophilized to obtain the product **S6** (314 mg, 1.08 mmol) as a white solid in quantitative yield.

¹H NMR (600 MHz, Deuterium Oxide, pD 7.0) δ 8.10 (d, *J* = 7.8 Hz, 2H), 7.74 (d, *J* = 7.5 Hz, 1H), 7.59 (t, *J* = 7.7 Hz, 2H), 5.86 (s, 0.5H), 5.60 (s, 0.5H), 4.02 – 3.89 (m, 2H), 3.78 – 3.66 (m, 2H), 3.58 – 3.51 (m, 0.5H), 3.36 – 3.28 (m, 0.5H).

¹³C NMR (151 MHz, Deuterium Oxide, pD 7.0) δ 170.73, 136.84, 132.46, 131.52, 77.85 (t, *J* = 38.6 Hz), 77.09 (t, *J* = 38.6 Hz), 75.62 (t, *J* = 38.8 Hz), 72.63 (td, *J* = 38.8, 6.6 Hz).

HRMS (ESI/Orbitrap) *m/z*: [M + H]⁺ calcd. for C₇¹³C₆H₁₇O₇ 291.1170; Found 291.1166.



Xylyl protected [$^{13}\text{C}_6$]benzoyl inositol (**S7**)

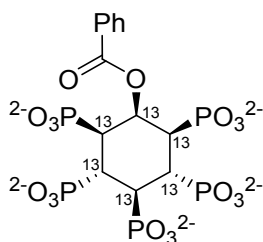
Xylyl protected [$^{13}\text{C}_6$]benzoyl (**S7**) inositol was synthesized according to a procedure of Godage and coworkers⁷. To a solution of **S6** (200 mg, 0.7 mmol) and 5-phenyltetrazole (1.01 g, 6.89 mmol) in dry DCM (4.9 mL) under nitrogen atmosphere was added **S3** (1.24 g, 5.17 mmol). The suspension was sonicated (1 min) and further stirred overnight at rt. The reaction mixture was cooled to $-40\text{ }^\circ\text{C}$, and *m*CPBA (1.70 g, 6.89 mmol) was added portion-wise while stirring. The cooling bath was removed, and the mixture was allowed to reach rt and diluted with DCM (50 mL), washed with 10% sodium sulfite solution ($2 \times 100\text{ mL}$), dried and solvent evaporated *in vacuo*. The crude was immobilized on Telos® and purified by CombiFlash® chromatography on silica gel (120 g column, gradient: 1% \rightarrow 4% \rightarrow 10% MeOH in DCM) to afford the product **S7** (651 mg, 0.54 mmol) as white solid in 79% yield

$^1\text{H NMR}$ (600 MHz, Chloroform-*d*) δ 8.06 (d, $J = 7.1\text{ Hz}$, 2H), 7.61 – 7.53 (m, 1H), 7.49 – 7.45 (m, 2H), 7.43 – 7.35 (m, 10H), 7.35 – 7.30 (m, 6H), 7.29 – 7.27 (m, 1H), 7.26 – 7.23 (m, 2H), 6.53 (s, 0.5H), 6.27 (s, 0.5H), 5.64 (ddd, $J = 17.9, 13.9, 8.1\text{ Hz}$, 4H), 5.58 – 5.47 (m, 3H), 5.41 (dd, $J = 13.7, 9.4\text{ Hz}$, 2H), 5.27 (s, 1H), 5.21 (dd, $J = 13.5, 10.9\text{ Hz}$, 3.5H), 5.16 (d, $J = 9.8\text{ Hz}$, 1H), 5.12 (d, $J = 8.7\text{ Hz}$, 1H), 5.05 (ddd, $J = 22.1, 13.8, 5.8\text{ Hz}$, 8H), 4.96 (s, 1.5H).

$^{13}\text{C NMR}$ (151 MHz, Chloroform-*d*) δ 78.00 – 76.46 (m), 74.25 (td, $J = 38.4, 36.8, 6.5\text{ Hz}$), 70.59 (t, $J = 38.5\text{ Hz}$).

$^{31}\text{P NMR}$ (122 MHz, CDCl_3) δ -3.32, -4.25, -4.90.

HRMS (ESI/Orbitrap) m/z : $[\text{M} + \text{Na}]^+$ Calcd for $\text{C}_{47}^{13}\text{C}_6\text{H}_{51}\text{NaO}_{22}\text{P}_5$ 1223.1613; Found 1223.1613.



Benzoyl [¹³C₆]inositol pentakisphosphate (**S8**)

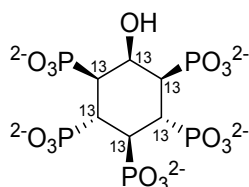
[¹³C₆]benzoyl inositol pentakisphosphate (**S8**) inositol was synthesized according to a procedure of Godage and coworkers⁷. **S7** (50 mg, 0.04 mmol) was dissolved in methanol (3.8 mL), and water (0.95 mL) and 20% Pd(OH)₂/C (50% wetted with water) (25 mg, 0.18 mmol) were added. The resulting suspension was stirred at rt overnight under hydrogen atmosphere. The catalyst was filtered through a PTFE syringe filter, and the filtrate neutralized by the addition of 1 M Et₃NHCO₃ until the pH 7.5 was reached. Lyophilization afforded the product **S8** (50 mg, 0.037 mmol, 8 × EtN₃ salt) as white solid in 88% yield. The amount of the TEA-counter ion was determined by NMR spectroscopy.

¹H NMR (600 MHz, Deuterium Oxide, pD 7.0) δ 8.19 (dd, *J* = 8.2, 1.9 Hz, 2H), 7.74 (t, *J* = 7.2 Hz, 1H), 7.61 (dd, *J* = 9.0, 6.6 Hz, 2H), 6.10 (s, 0.5H), 5.84 (s, 0.5H), 4.58 – 4.45 (m, 2H), 4.39 (s, 0.5H), 4.27 (s, 1H), 4.16 (d, *J* = 11.1 Hz, 0.5H), 3.21 (qd, *J* = 7.3, 2.5 Hz, EtN₃), 1.30 (td, *J* = 7.3, 2.6 Hz, EtN₃).

¹³C NMR (151 MHz, Deuterium Oxide, pD 7.0) δ 77.65 (t, *J* = 39.7 Hz), 76.46 (t, *J* = 39.7 Hz), 73.93 (t, *J* = 38.6 Hz), 72.53 (t, *J* = 39.9 Hz), 46.60, 8.23.

³¹P NMR (122 MHz, Deuterium Oxide, pD 7.0) δ 0.33, -0.00, -0.80.

HRMS (ESI/Orbitrap) *m/z*: [M – 2H]²⁻ calcd. for C₇¹³C₆H₁₉O₂₂P₅ 343.9634; Found 343.9954.



[¹³C₆]inositol pentakisphosphate (**5**)

[¹³C₆]inositol pentakisphosphate (**5**) was synthesized according to a procedure of Godage and coworkers⁷. Compound **S8** (50 mg, 0.037 mmol) was dissolved in concentrated aqueous ammonia solution (2.0 mL) and heated at 60 °C overnight in a Pyrex pressure tube. After evaporation of the solution under vacuum, the residue was dissolved in water and the benzamide byproduct was removed by washing with DCM. The ammonium salt of the product was obtained by evaporation of the ammonia and converted into the free acid by quick filtration (Note: Prolonged exposure causes phosphoryl group migration.) through DOWEX 50W x 8 (H⁺ form) (10-fold molar excess, previously washed with MilliQ® water) and then to its hexasodium salt by titration to pH 7.40 with 0.1 M sodium hydroxide solution. Lyophilization afforded the product **5** (17 mg, 0.024 mmol) as white solid in 65% yield.

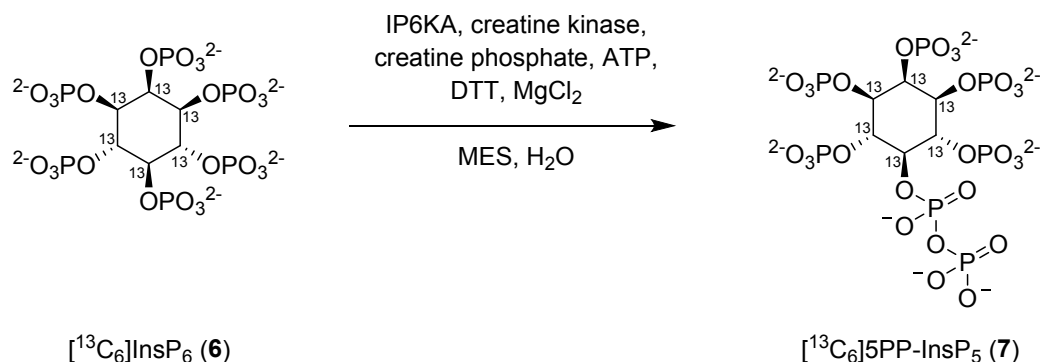
¹H NMR (300 MHz, Deuterium Oxide, pD 7.5) δ 4.72 – 4.53 (m, 1.5H), 4.47 – 4.25 (m, 1.5H), 4.24 – 4.02 (m, 1.5H), 3.96 – 3.74 (m, 1.5H).

¹³C NMR (75 MHz, Deuterium Oxide, pD 7.5) δ 78.28 – 74.98 (m), 73.92 (t, *J* = 37.7 Hz), 70.68 (t, *J* = 37.6 Hz).

³¹P NMR (122 MHz, D₂O, pD 7.5) δ 1.49, 1.23, 0.86.

HRMS no ions detected.

Synthesis of [¹³C₆]5-diphosphoinositol pentakisphosphate (7)



The following stock solutions were prepared:

Creatine kinase stock solution: 350 U/mL creatine kinase in 200 mM MOPS pH 6.5, 20 mM MgCl₂, 20 mM DTT

IP6KA stock solution: 10 mg/mL IP6KA in 20 mM Tris HCl pH 7.4, 200 mM NaCl, 1 mM DTT

ATP stock solution: 50 mM in MilliQ® water pH 6.4; (Note: Concentration was determined via UV-Vis analysis at 259 nm; $\epsilon_{259} = 15.4 \text{ E/mmol/cm}$).

A solution of **6** (dodecasodium salt, 70 mg, 250 μM , 66 w/w%), MES (20 mM, pH 6.4), NaCl (50 mM), ATP (disodium salt, 2 mM), creatine phosphate (5 mM), MgCl₂ (7 mM), DTT (1 mM) in 199 mL MilliQ® water was prepared. The pH was adjusted to 6.4 and the mixture was split into four 50 mL conical tubes. The individual tubes were then equilibrated to 37 °C within 30 min without shaking. IP6KA (1 μM) and creatine kinase (1 U/mL) were added, the tube was gently inverted several times to homogenize and left to react for exactly 45 min without shaking. (Note: The correct temperature was essential to assure full conversion of the starting material within 45 min. Prolonged reaction times above 1 h led to side reactions. However, the speed of the reaction will depend on the batch and quality of the recombinantly expressed IP6KA.)

Purification: The reaction was stopped by cooling the reaction mixture down to 4 °C within 5 min with the help of a dry ice isopropanol bath. Four short C18 columns (SepPak V C18 500 mg) were each washed and equilibrated with 9 mL MeCN, then 9 mL H₂O. To remove protein, the reaction mixture was filtered through the SepPak columns (1 column per 50 mL reaction) and each column was washed with 20 mL

water. The flow through was combined (~280 mL) and the pH adjusted to 9.0-9.2 by dropwise addition of a 10 M NaOH-solution (roughly 180 μ L). 2.3 mL of a 1 M MgCl_2 solution was added which led to precipitation of InsPs as magnesium complex. The suspension was left shaking at room temperature overnight to facilitate complete precipitation. The suspension was centrifuged (5 min at 3000 g), the supernatant removed and the precipitate washed 3 times with 15 mL MgCl_2 solution (8 mM, pH 9 adjusted with NaOH)

The precipitate was resuspended in 10 mL NH_4HCO_3 buffer (10 mM, pH 8) and mixed with Amberlite IRC-748 (10 mL wetted bed volume, pre-equilibrated with NH_4HCO_3 , pH 8) until the precipitate dissolved. (Note: The Amberlite resin should be washed with 500 mL of MeOH and 500 mL of H_2O before use.) The buffer/resin suspension was added to a short Amberlite IRC-748 column (5 mL bed volume, pre-equilibrated with NH_4HCO_3 , pH 8) to remove excess Mg^{2+} . The product was flushed through the column with 50 mL water and all eluents were collected and lyophilized to afford the ammonium salt of the product (100 mg, 0.14 mmol, 35.5 w/w%) as white solid in quantitative yield.

^1H NMR (600 MHz, Deuterium Oxide, pD 7.5) δ 5.05 – 4.95 (m, 0.5H), 4.69 – 4.58 (m, 1H), 4.49 – 4.36 (m, 1.5H), 4.35 – 4.26 (m, 1H), 4.25 – 4.15 (m, 0.5H), 4.12 – 4.00 (m, 1H).

^{13}C NMR (151 MHz, Deuterium Oxide, pD 7.5) δ 80.74 – 79.46 (m), 79.32 – 77.17 (m), 76.03 (t, $J = 38.7$ Hz).

^{31}P NMR (243 MHz, D_2O) δ -1.45, -2.00, -2.45, -10.32, -13.41.

HRMS no ions detected.

NMR experiments

Progress curve measurement with IP6KA

Progress curves were recorded for different IP6KA concentrations ranging from 0.2 – 1 μM . The reactions contained 20 mM HEPES NaOD pH* 7.0, 50 mM NaCl, 1 mM DTT, 10 mM ATP, 11 mM MgCl_2 , 176 μM $[\text{}^{13}\text{C}_6]\text{InsP}_6\text{Na}_{12}$ (**6**), 5 mM creatine phosphate, 2 mM Me_4PBr , and 1 U/mL creatine kinase in D_2O . All stock solutions were prepared in D_2O . The reactions were run in a total volume of 650 μl and equilibrated at 37 $^\circ\text{C}$. The reactions were transferred into a 5 mm NMR tube and the reaction was started by the addition of the appropriate amount of IP6KA (in D_2O buffer). The NMR tubes were inserted into the NMR instrument, locked, tuned and matched, and shimmed. A spin echo difference pulse was used to measure consecutively 75 sec spectra until the reaction was finished.

Kinetic characterization of IP6K1

The $K_{\text{M,ATP}}$ and V_{max} were determined for IP6K1. The buffer contained 20 mM HEPES NaOD pH* 7.0, 50 mM NaCl, 1 mM DTT, 1 mg/mL BSA, 1 μM IP6K1, 5 mM creatine phosphate, and 1 U/mL creatine kinase in D_2O . The ATP concentration ranged from 8 mM to 62.5 μM (two-fold dilution series) and the MgCl_2 concentration was adjusted to be 5 mM plus the ATP concentration. The reaction volume was 500 μL and the reaction was started after 10 min equilibration at 37 $^\circ\text{C}$ by the addition of 175 μM $[\text{}^{13}\text{C}_6]\text{InsP}_6$. The reaction was quenched with 38 μL 0.7 M EDTA (in D_2O , pH* 8.0) after approximately 20 % conversion (5 min for IP6K1). Before the NMR measurements the pH* was adjusted to 8, if needed. The conversion of $[\text{}^{13}\text{C}_6]\text{InsP}_6$ at different ATP concentration was determined by NMR spectroscopy. The data was fitted against a

$$v = \frac{V_{\text{max}}}{1 + \frac{K_m}{S} + \frac{S}{K_i}}$$

kinetic model for substrate inhibition: using SigmaPlot 12.5.

Determination of IC_{50} values for IP6K1 inhibitors

For the IC_{50} value determination a two-fold dilution series of the appropriate inhibitor in DMSO-d_6 was used. The reactions were run in a total volume of 500 μL and contained

20 mM HEPES NaOD pH* 7.0, 50 mM NaCl, 1 mM DTT, 2.5 mM ATP, 7.5 mM MgCl₂, 0.2 μM IP6K1, 1 mg/mL BSA, 5 mM creatine phosphate, and 1 U/mL creatine kinase in D₂O. The inhibitor concentration ranged from 50 μM to 195 nM (200 x stock solutions were used). The reactions were equilibrated to 37 °C for 10 min and initiated by the addition of 175 μM [¹³C₆]InsP₆. The reactions were quenched after 3 hours by the addition of 38 μL of 0.7 M EDTA (in D₂O, pH* 8.0). Before the NMR measurements the pH* was adjusted to 8, if needed. The conversion of [¹³C₆]InsP₆ at different inhibitor concentrations was determined by NMR spectroscopy. The data was fitted against a

$$v = \frac{bottom + (top - bottom)}{1 + \frac{x^{Hillslope}}{IC_{50}}}$$

kinetic model for dose response inhibition:

using

SigmaPlot 12.5.

Metabolic labeling of mammalian cell lines

HCT116 wt, HCT116 PPIP5K^{-/-} (a kind gift from Stephen Shears)⁸ and HEK293T cells were grown in DMEM lacking *myo*-inositol and supplemented with 10 % dialyzed fetal bovine serum, 100 μM either [¹²C₆]- or [¹³C₆]*myo*-inositol, and Penicillin/Streptomycin at 37 °C and 5 % CO₂. 1 liter DMEM w/o inositol was prepared from pre-mixed medium components (8.1 g, Dulbecco), NaHCO₃ (3.7 g), HEPES (10 mL of a 1 M stock, pH 7.4), L-glutamine (584 mg), L-serine (42 mg), D-glucose (4.5 g), and NaH₂PO₄ (125 mg). The cells were grown in 15 cm dishes (for one experiment we used five to ten plates) until they reached 80-90 % confluency. For harvesting, cells were washed with PBS and 0.9 % NaCl solution, and trypsinized. The trypsin was quenched with regular DMEM. The cells were collected and washed two more times with PBS before lysis and extraction. The packed cell volume and the cell number of the preparations were determined. If needed, the cells were incubated with 10 mM NaF for one hour before harvest.

Inositol phosphate extraction

All steps were performed at 4 °C. The protocol was adapted from Azevedo et al.⁹ For HCT116 wt we used ten 15 cm dishes and for HCT116 wt with NaF treatment, HCT116

PPIP5K^{-/-} and HCT116 PPIP5K^{-/-} with NaF treatment we used three 15 cm dishes. Cell pellets were lysed with 5 mL of 1 M HClO₄, containing 3 mM EDTA, by vortexing. The lysate was incubated on ice for 30 min before the precipitate was removed by centrifugation. The supernatant was neutralized with 2 M KOH, containing 3 mM EDTA, and the pH was adjusted to 5.8-6. The resulting KClO₄ precipitate was removed by centrifugation and the supernatant was lyophilized. After lyophilization the supernatant was redissolved in 1 mL D₂O and the resulting precipitate was removed by centrifugation. The supernatant was lyophilized again and the residue was redissolved in 600 μL D₂O containing the 50–150 μM Me₄PBr standard for absolute quantification.

TiO₂ enrichment

All steps were performed at 4 °C. The protocol was adapted from Wilson *et al.*¹⁰ TiO₂ beads 4-5 mg were washed with 500 μL of water and 500 μL of 1 M HClO₄. Cell extract was adjusted to pH 1 with 1 M HClO₄ and added to the beads. The beads were rotated for 5 min and after centrifugation, the supernatant was discarded. The beads were washed twice with 500 μL of 1 M HClO₄ and the InsPs were eluted by incubating the beads twice with 200 μL 2.8 % NH₄OH solution. The eluate was lyophilized and the dry residue was dissolved in 600 μL of 1 mM MES (pH* 6.0) in D₂O.

Lipid extraction

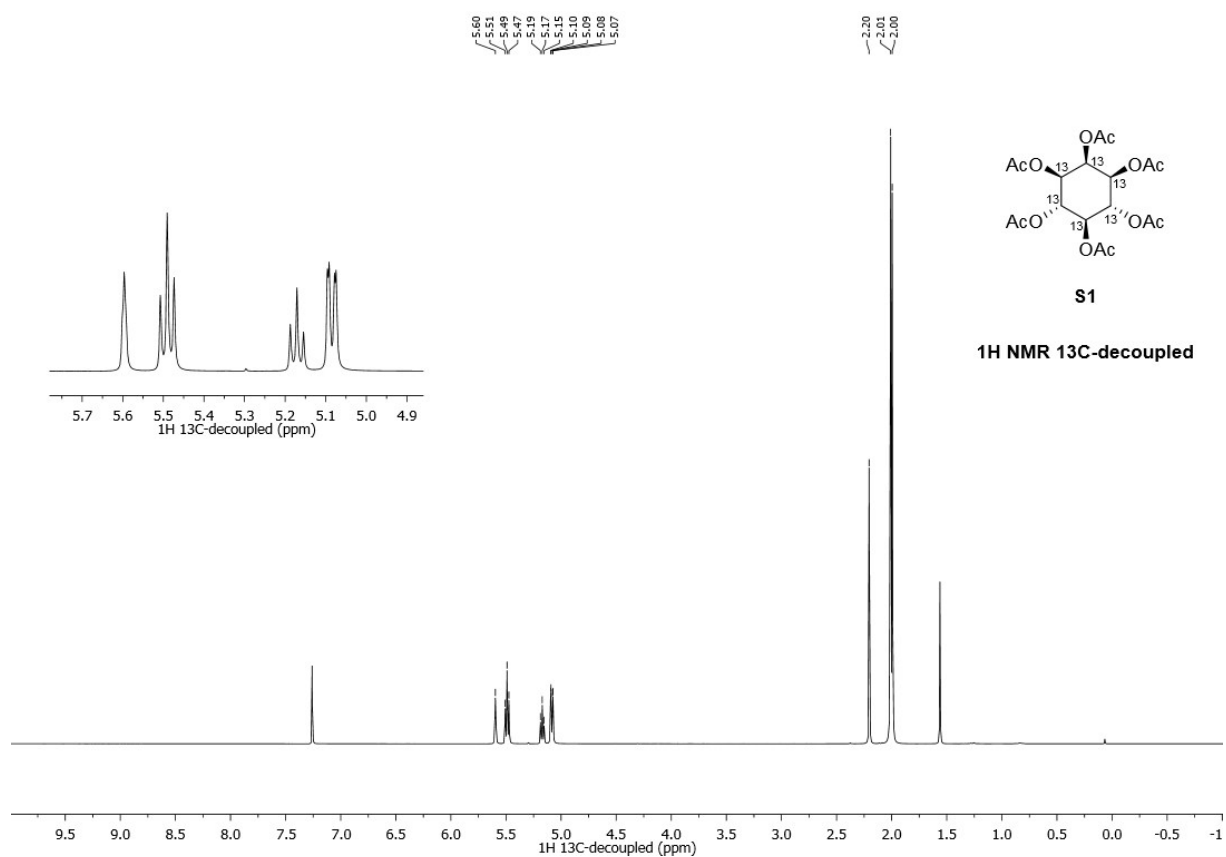
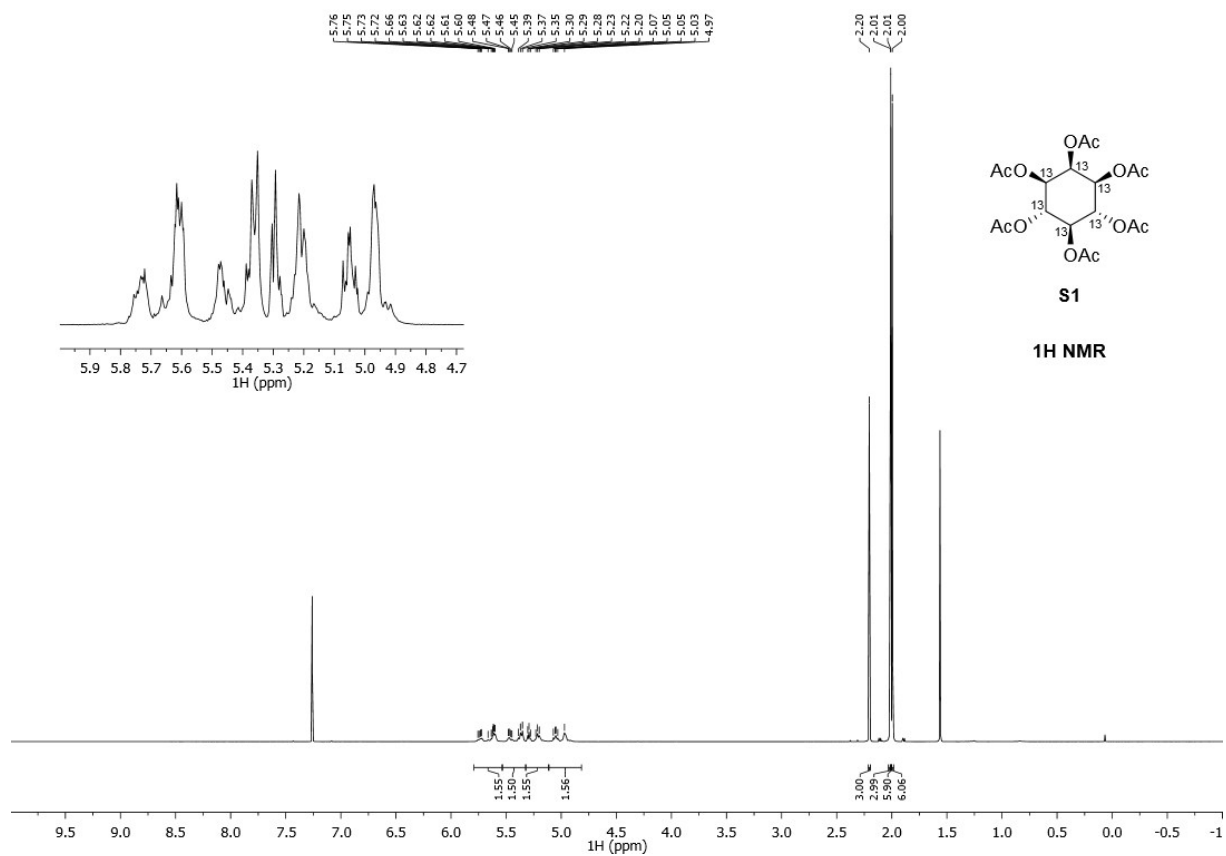
The lipid extraction was adopted from Clark *et al.*¹¹ In summary: The cells from 3 15 cm dishes were trypsinized and washed twice with PBS. The cells were resuspended in 1.7 mL Milli-Q water and 7.5 ml quench mix (48.4 mL MeOH, 24.2 mL CHCl₃, 2.4 mL 1 M HClO₄) was added. The cells were lysed by vortexing for 30 sec. 1.7 mL 1 M HClO₄ and 7.25 mL CHCl₃ were added to induce phase separation and the mixture was vortexed for 30 sec. The sample was centrifuged at 3000 g for 5 min to facilitate phase separation. The top aqueous layer was collected and washed with 7 mL CHCl₃. The aqueous layer was collected and neutralized with 2 M KOH containing 3 mM EDTA. The KClO₄ salt was removed by centrifugation and the sample was lyophilized. After lyophilization the supernatant was redissolved in 1 mL D₂O and the resulting precipitate

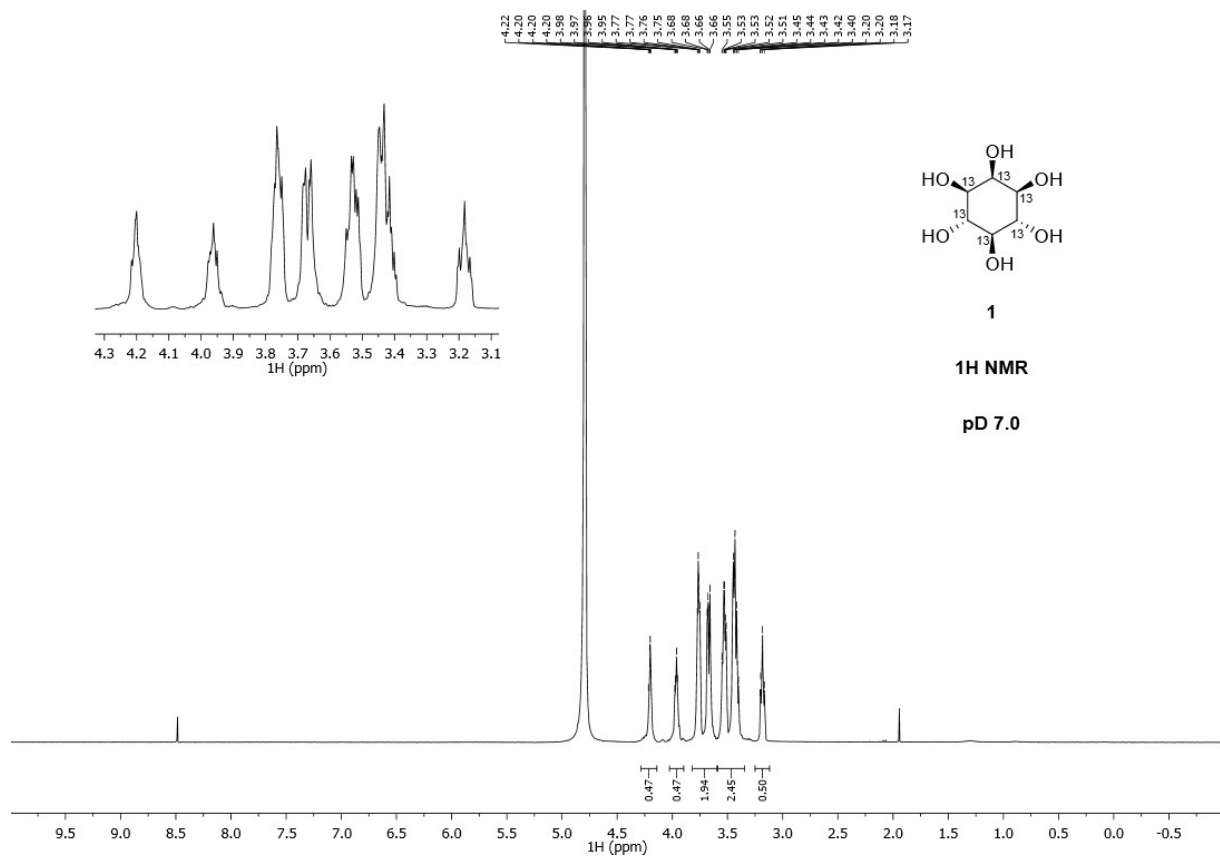
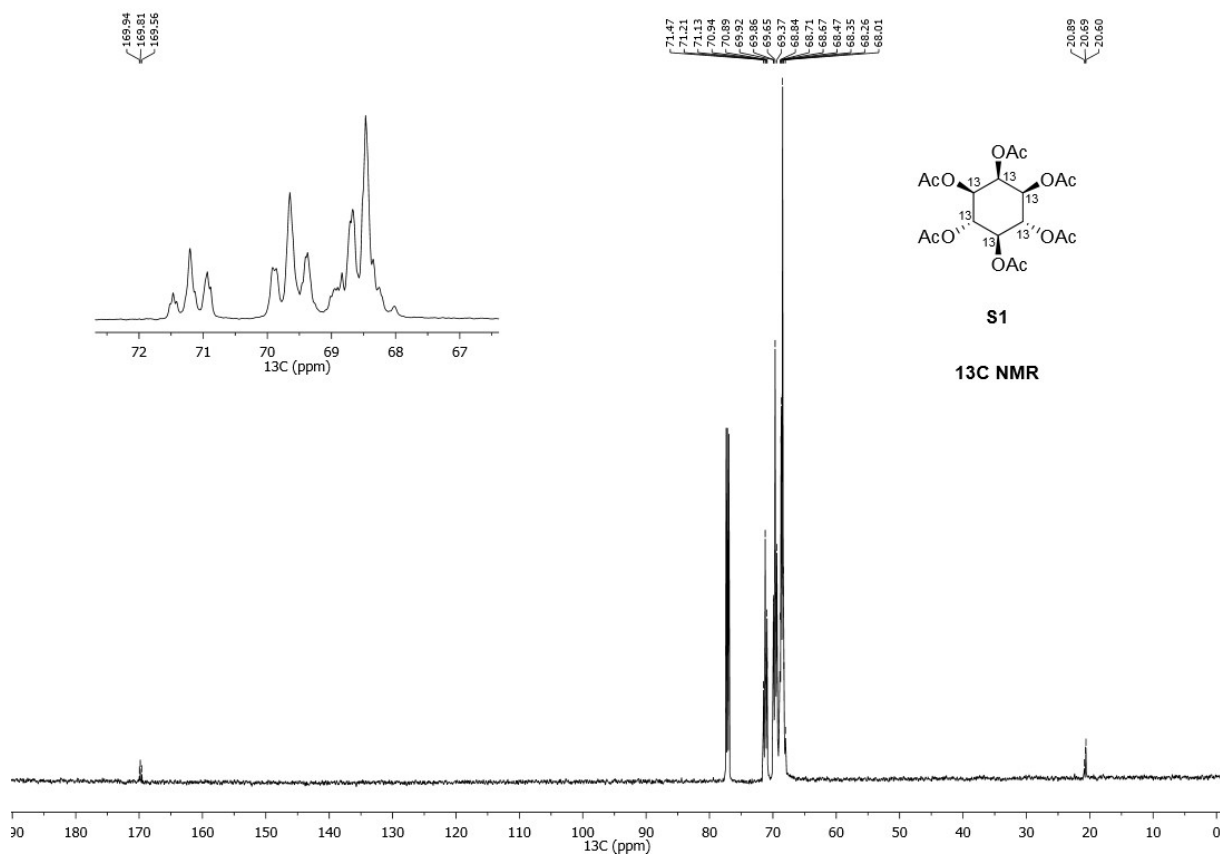
was removed by centrifugation. The supernatant was lyophilized again and the residue was redissolved in 600 μL D_2O .

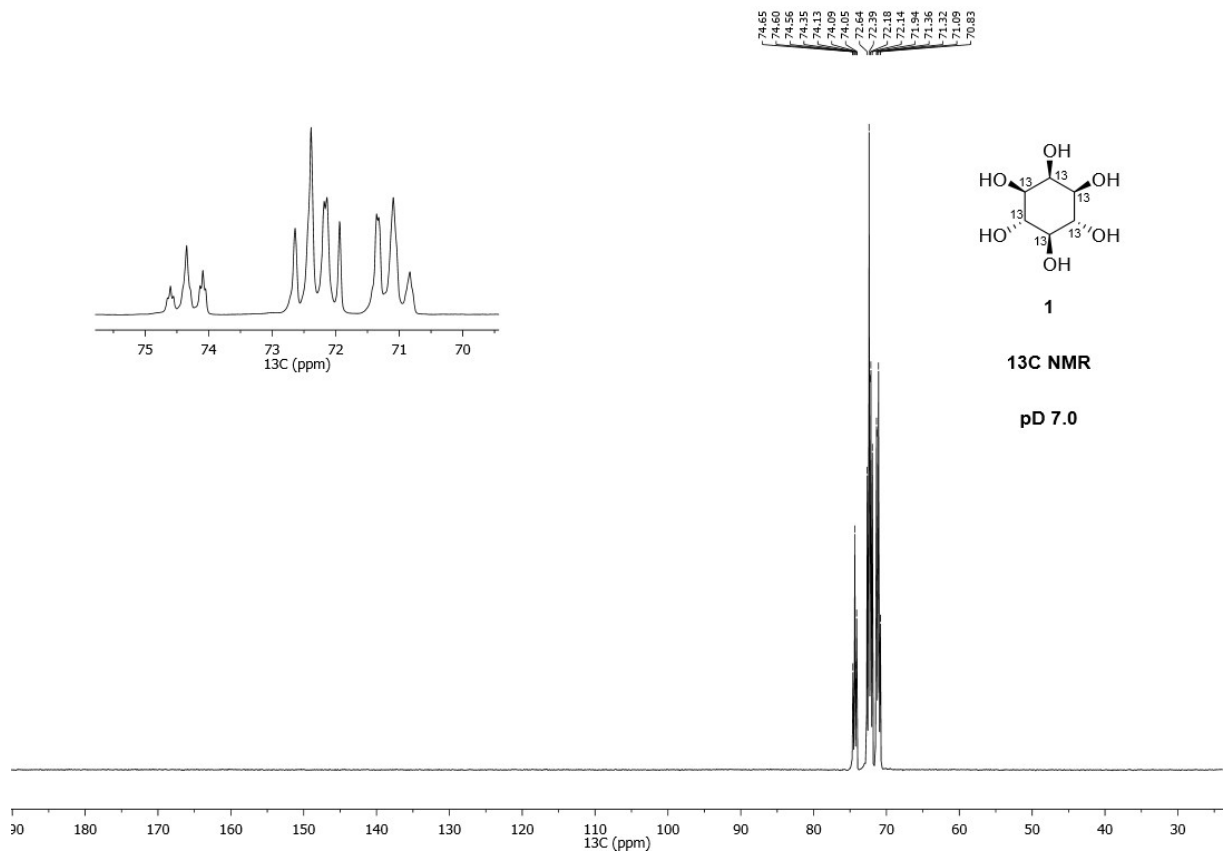
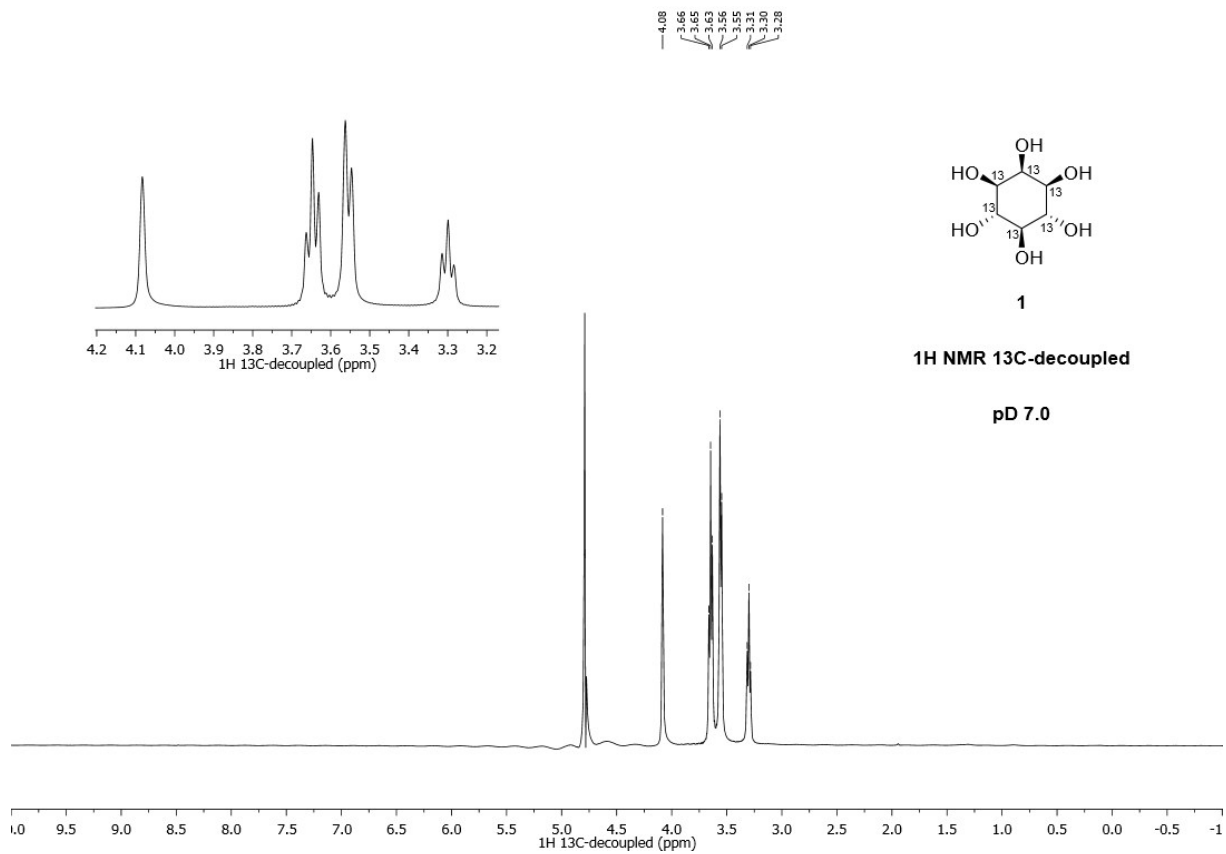
References

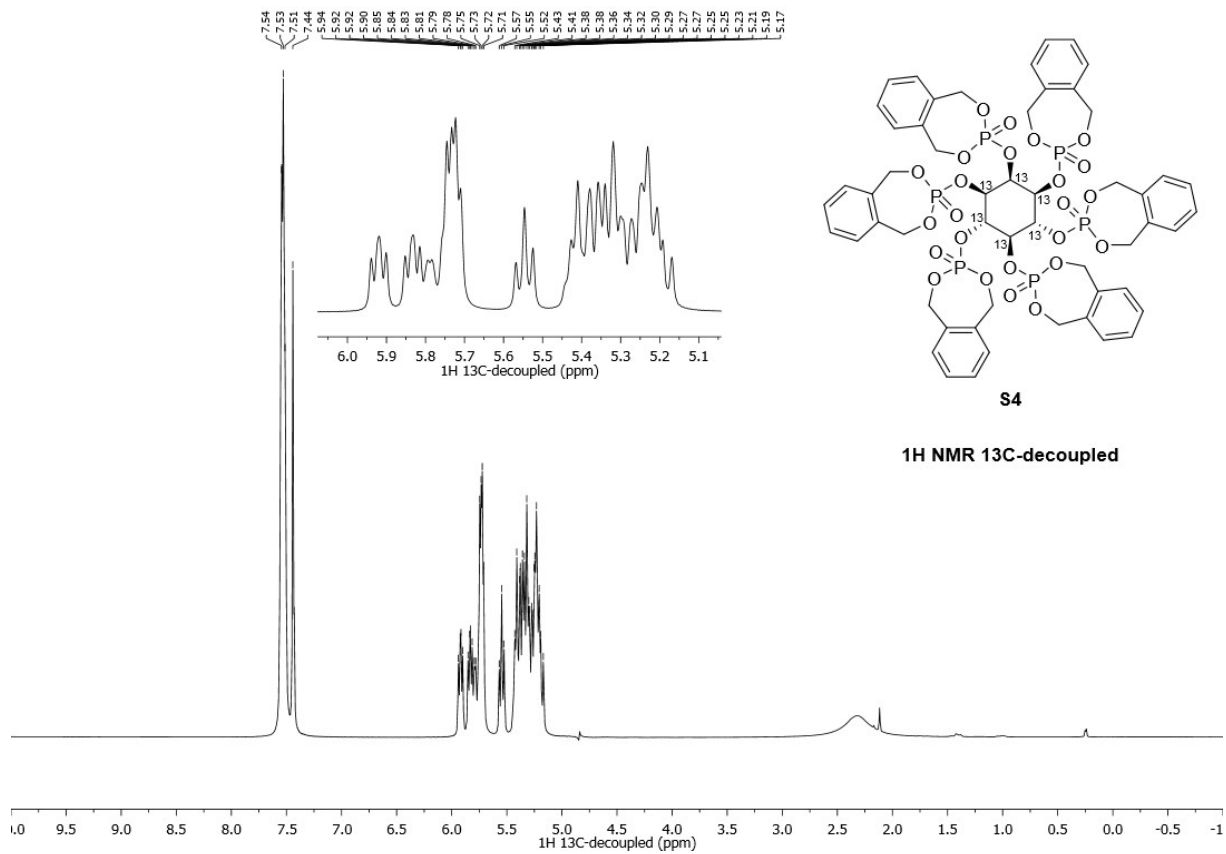
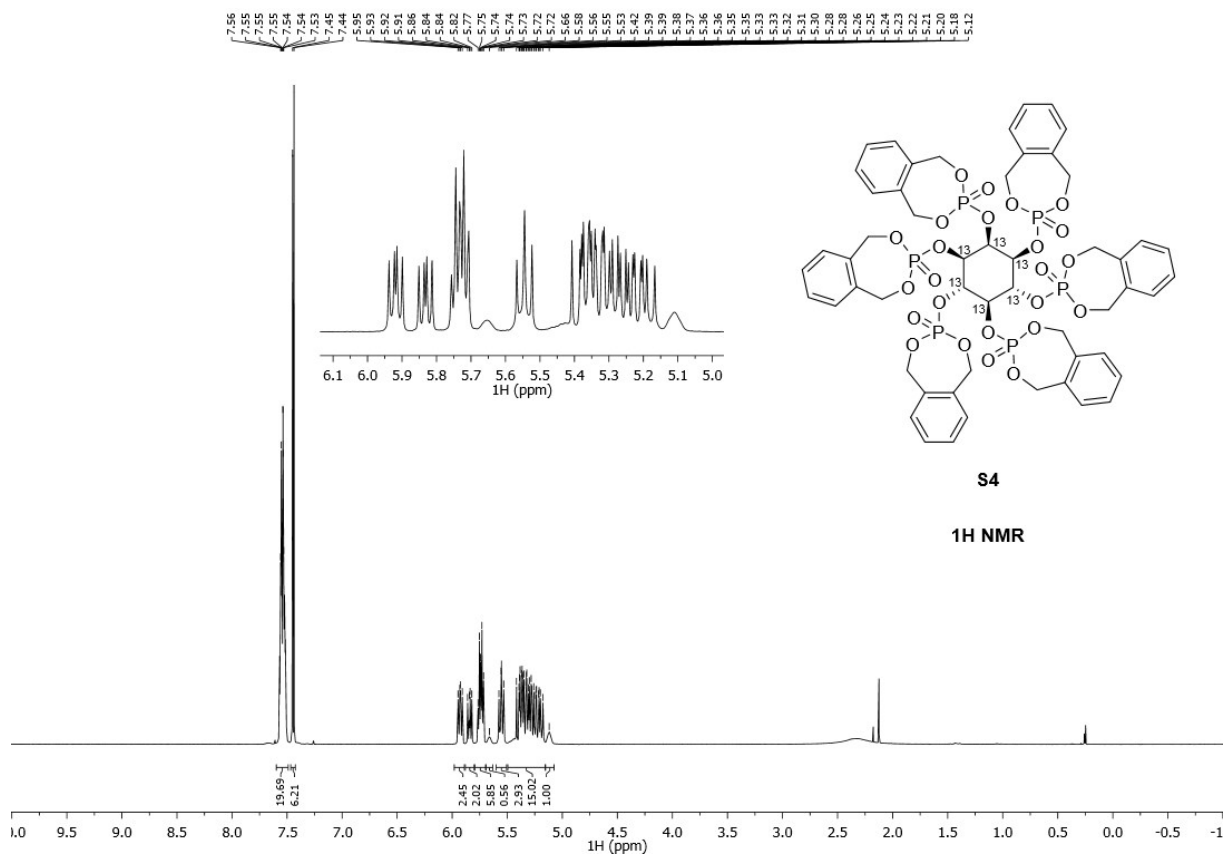
- 1 M. Findeisen, T. Brand and S. Berger, *Magn. Reson. Chem.*, 2007, **45**, 175–178.
- 2 M. V. Rodrigues, N. Borges, M. Henriques, P. Lamosa, R. Ventura, C. Fernandes, N. Empadinhas, C. Maycock, M. S. Da Costa and H. Santos, *J. Bacteriol.*, 2007, **189**, 5405–5412.
- 3 H. Wang, E. F. Derose, R. E. London and S. B. Shears, *Nat. Commun.*, 2014, **5**, 4178.
- 4 E. Drent, R. van Dijk, R. van Ginkel, B. van Oort and R. I. Pugh, *Chem. Commun.*, 2002, **2**, 964–965.
- 5 M. Gregory, B. Catimel, M. X. Yin, M. Condrón, A. W. Burgess and A. B. Holmes, *Synlett*, 2016, **27**, 121–125.
- 6 M. Podeschwa, O. Plettenburg, J. Vom Brocke, O. Block, S. Adelt and H. J. Altenbach, *European J. Org. Chem.*, 2003, **2003**, 1958–1972.
- 7 H. Y. Godage, A. M. Riley, T. J. Woodman, M. P. Thomas, M. F. Mahon and B. V. L. Potter, *J. Org. Chem.*, 2013, **78**, 2275–2288.
- 8 C. Gu, H.-N. Nguyen, D. Ganini, Z. Chen, H. J. Jessen, Z. Gu, H. Wang and S. B. Shears, *Proc. Natl. Acad. Sci. U. S. A.*, 2017, **114**, 11968–11973.
- 9 C. Azevedo and A. Saiardi, *Nat. Protoc.*, 2006, **1**, 2416–2422.
- 10 M. S. C. Wilson, S. J. Bulley, F. Pisani, R. F. Irvine and A. Saiardi, *Open Biol.*, 2015, **5**, 150014.
- 11 J. Clark, K. E. Anderson, V. Juvin, T. S. Smith, F. Karpe, M. J. O. Wakelam, L. R. Stephens and P. T. Hawkins, *Nat. Methods*, 2011, **8**, 267–272.

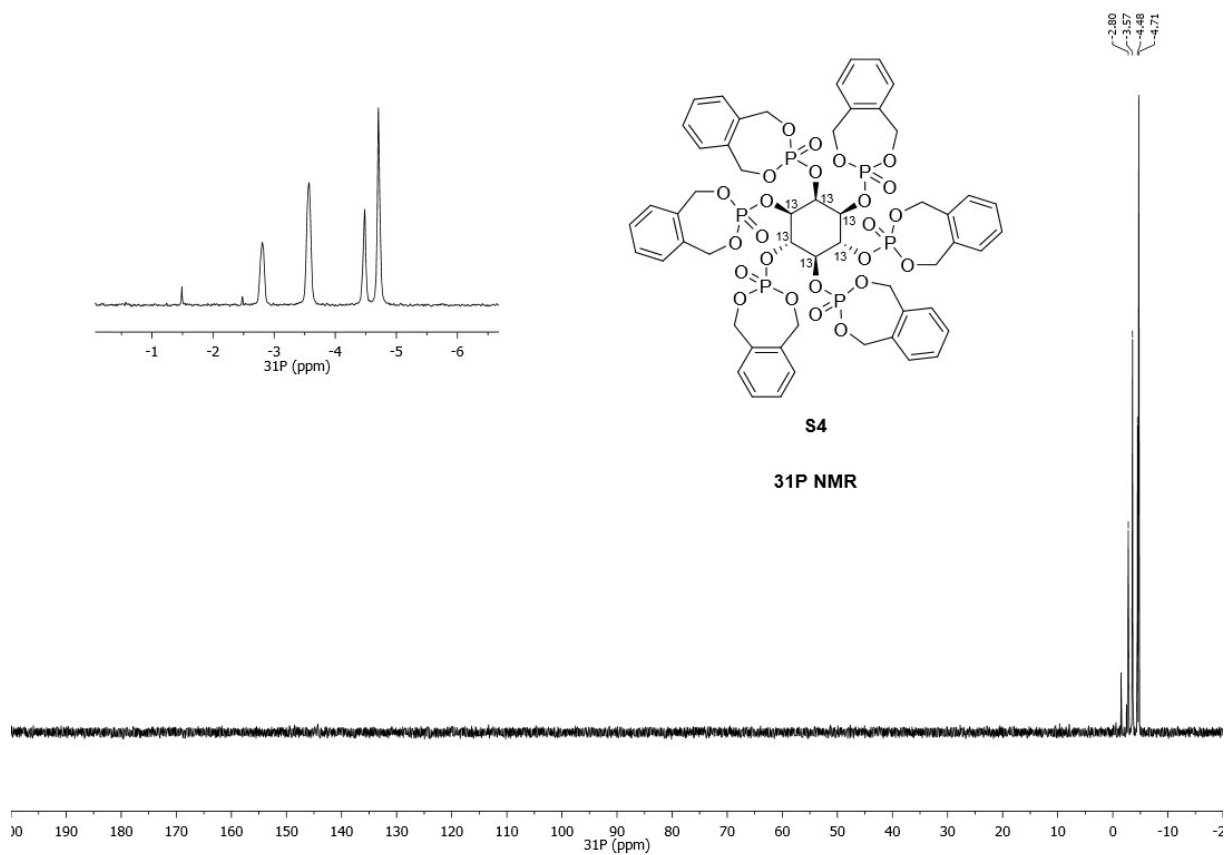
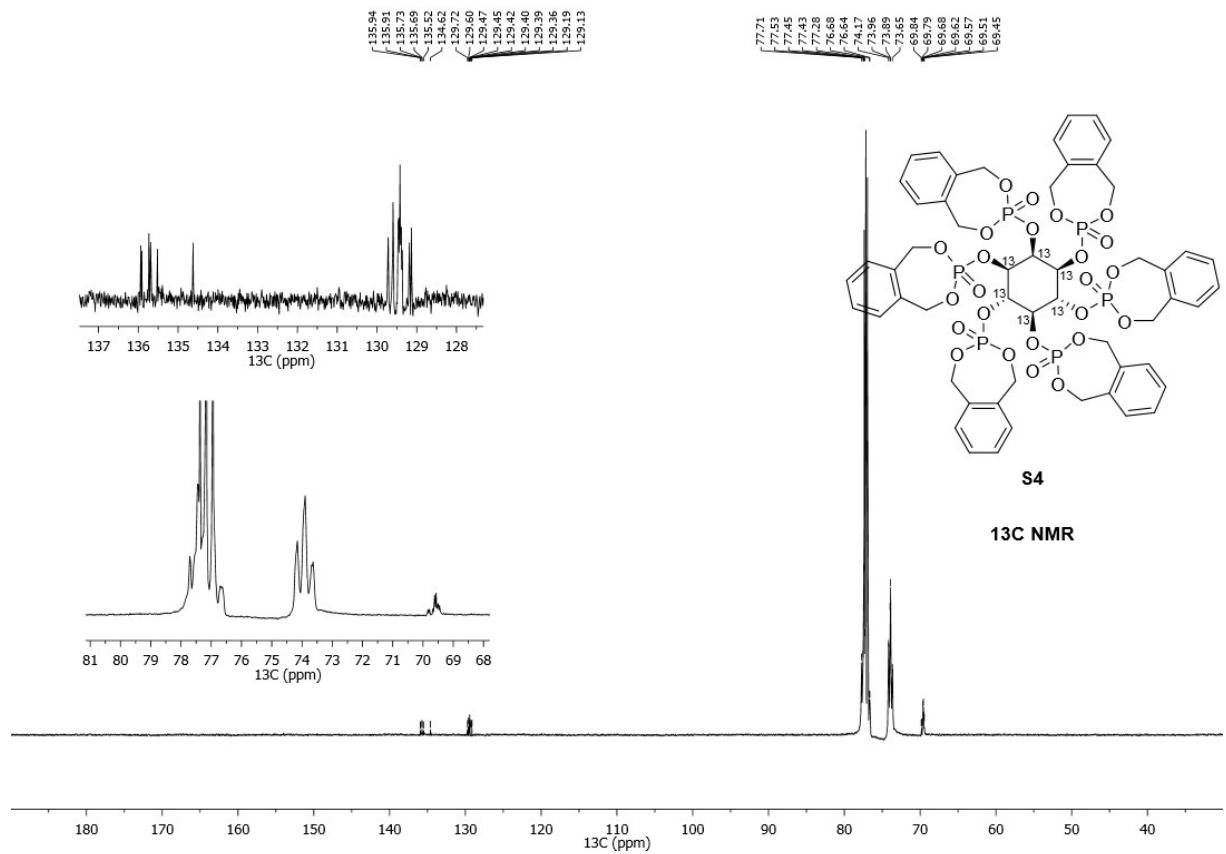
NMR Spectra

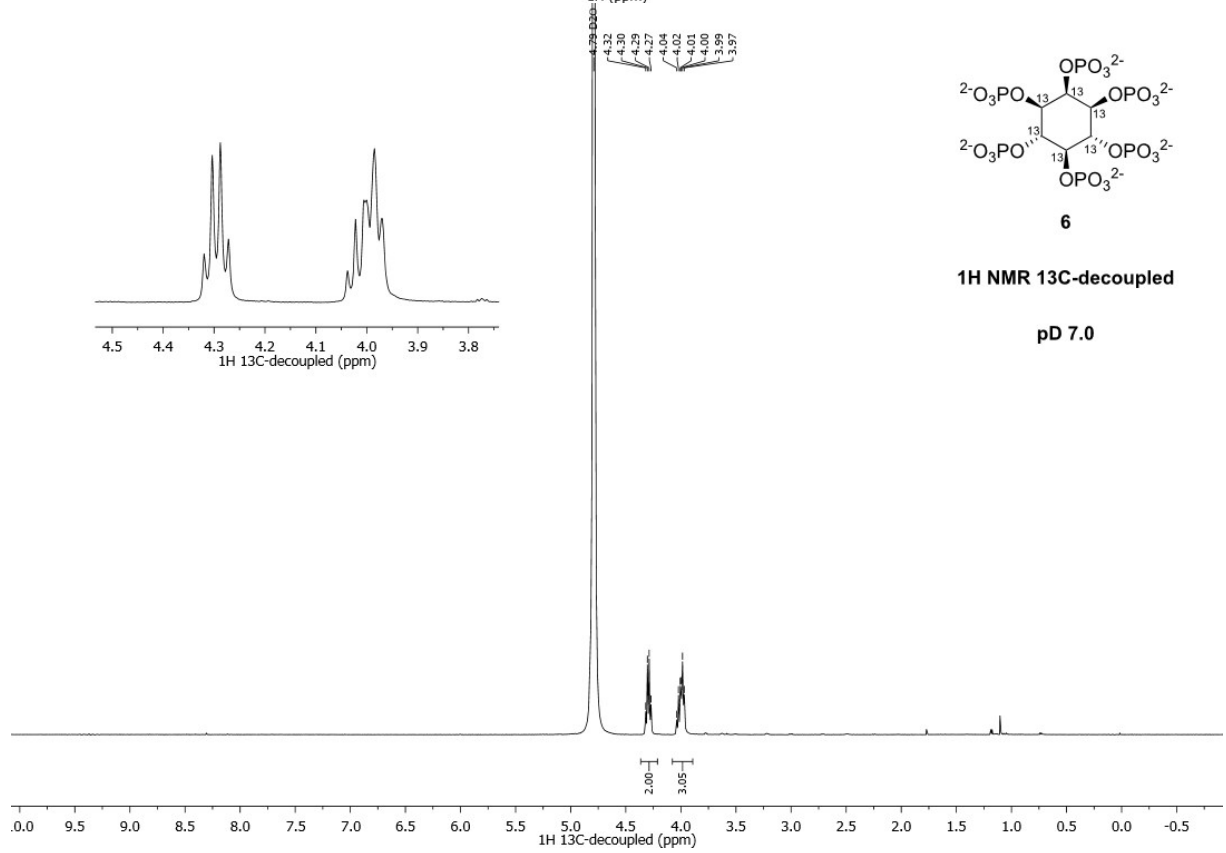
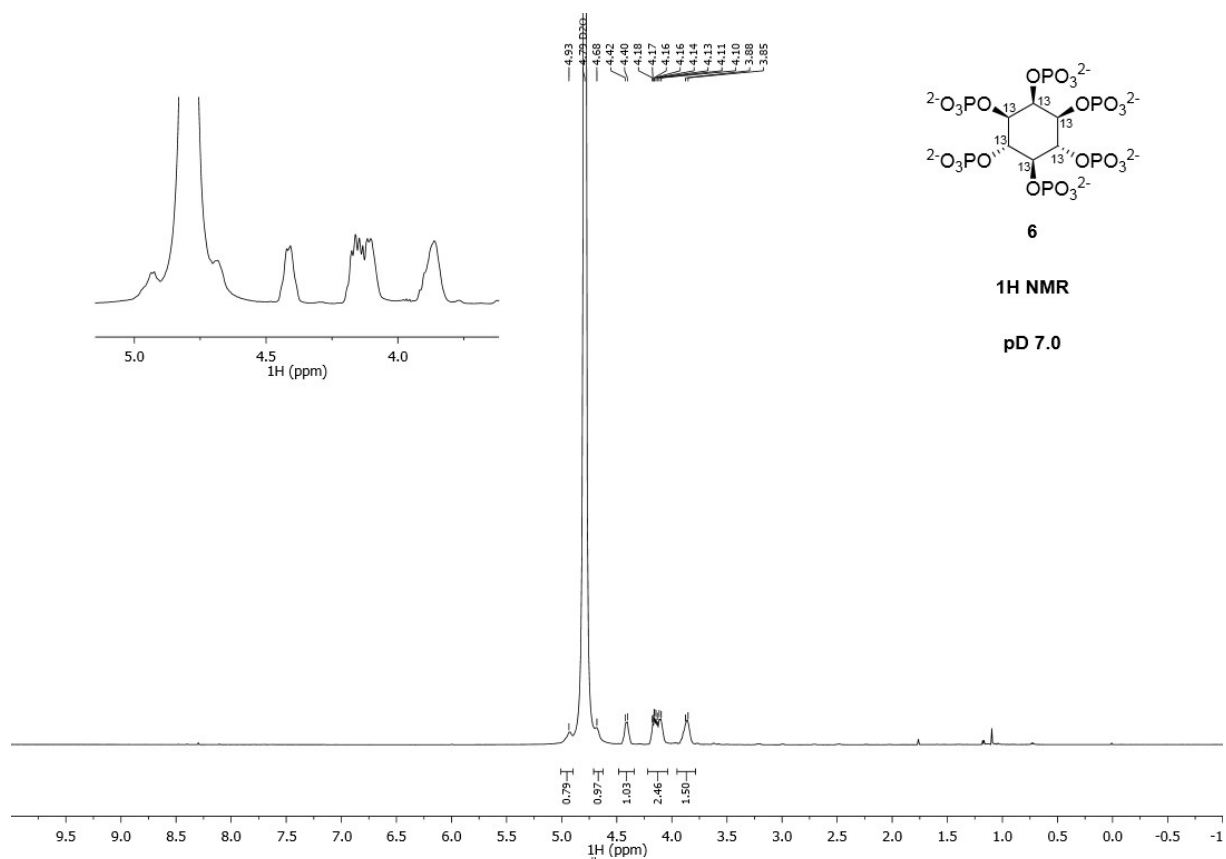


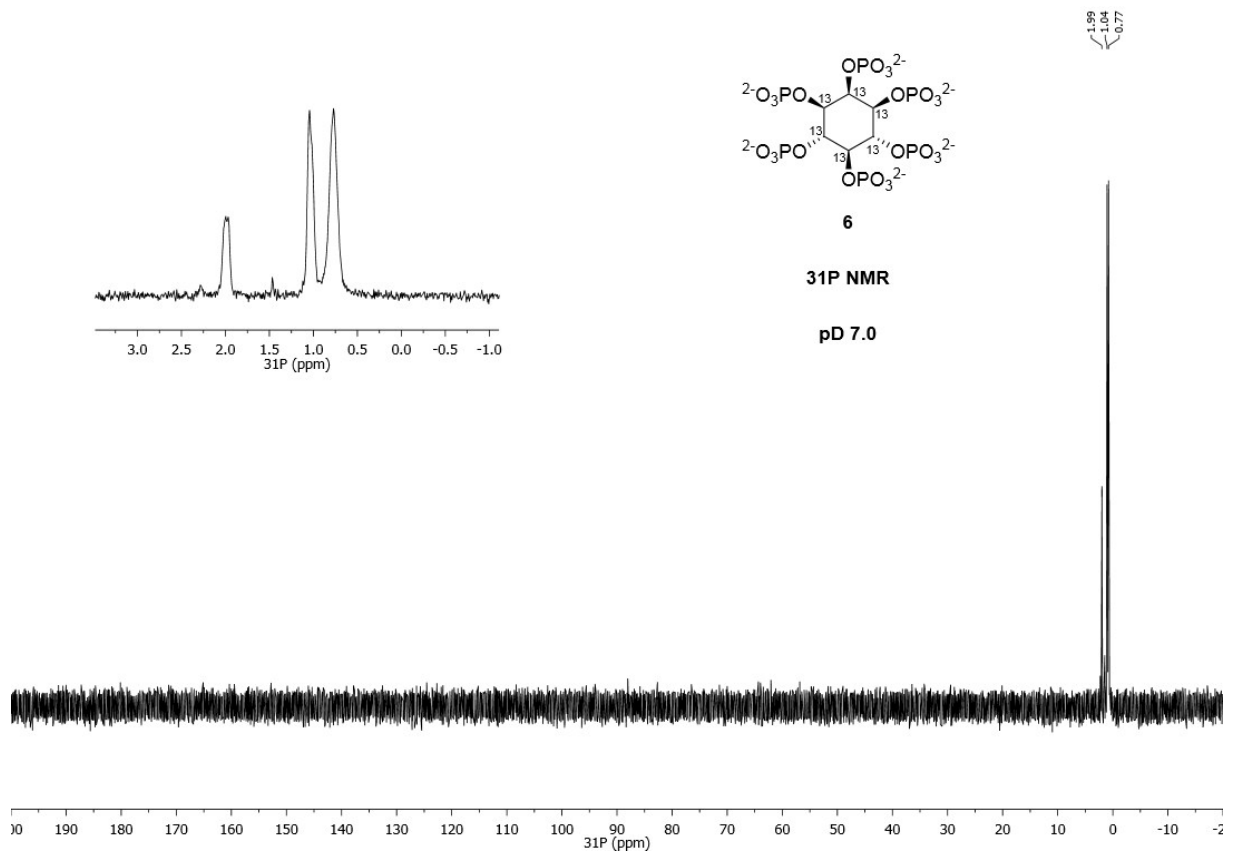
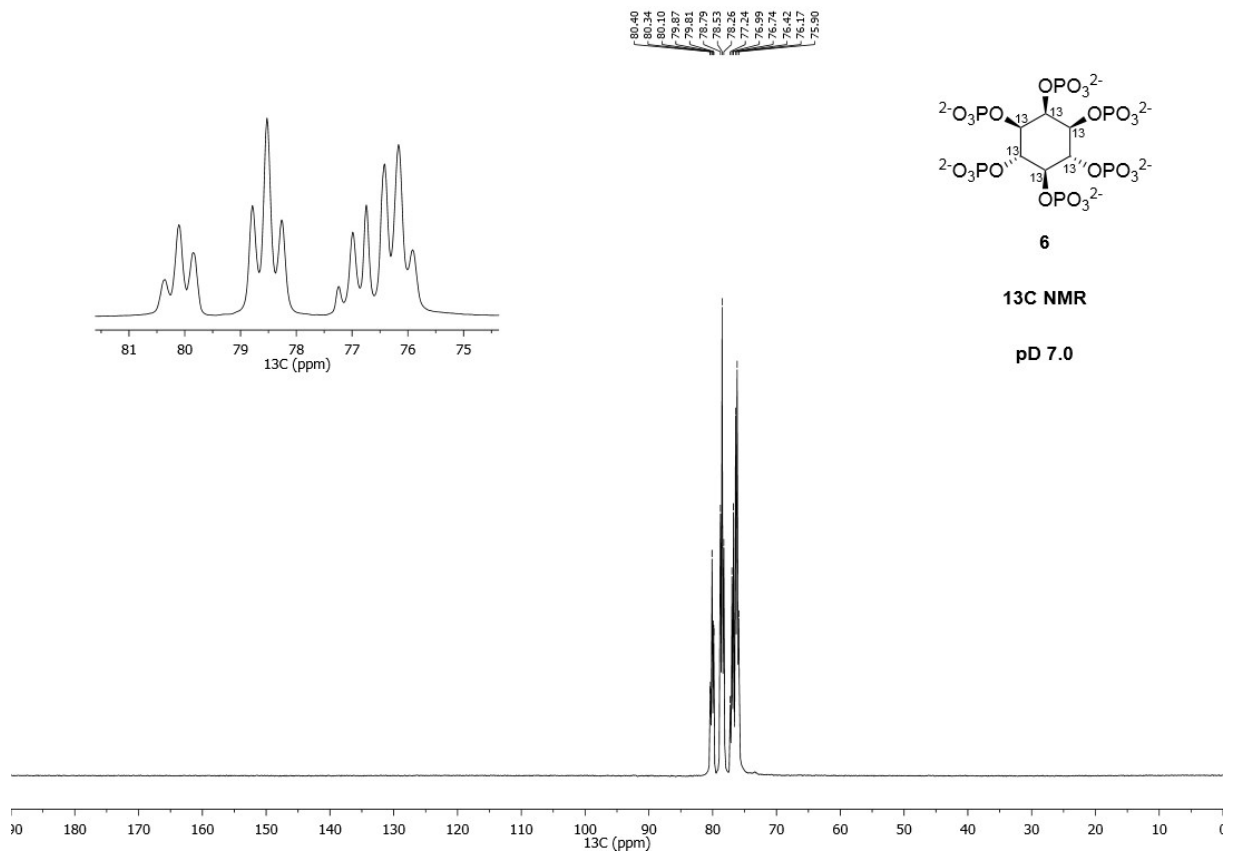


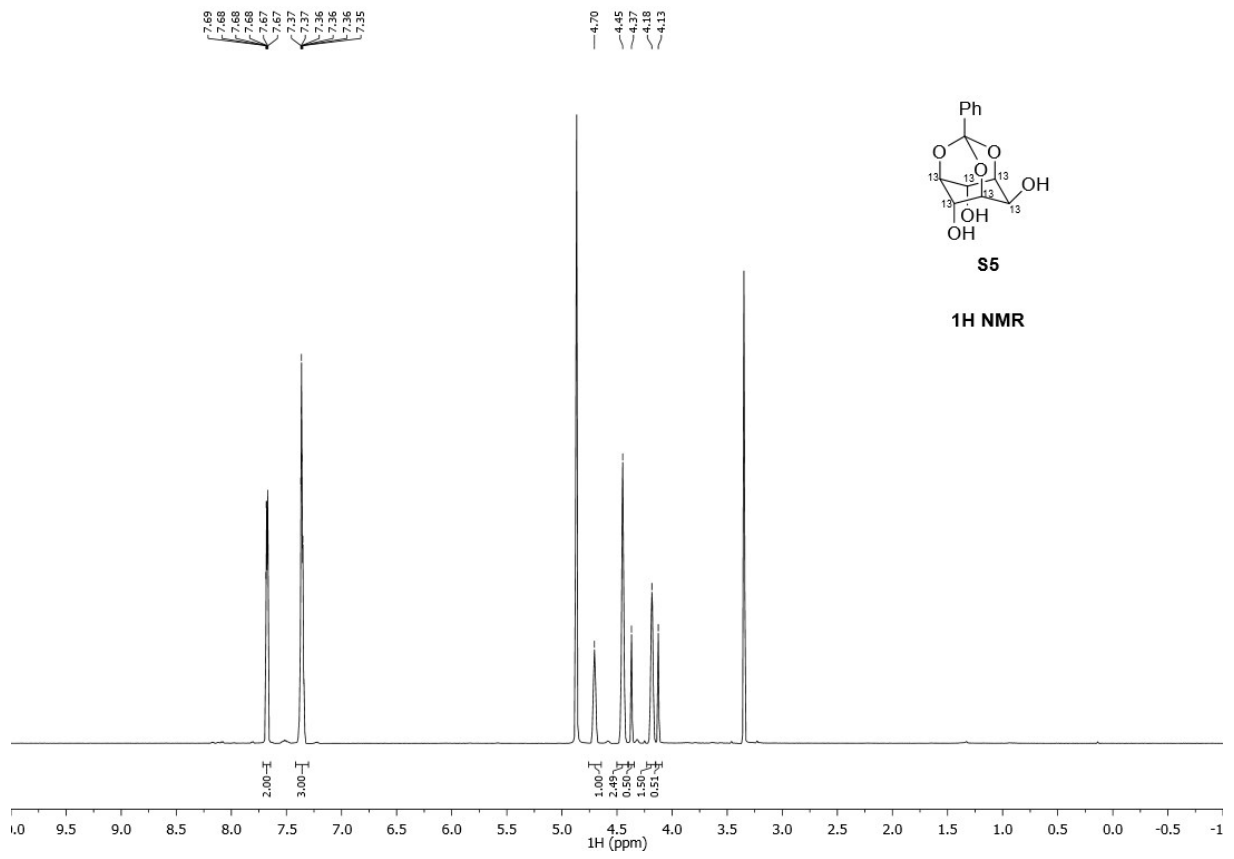


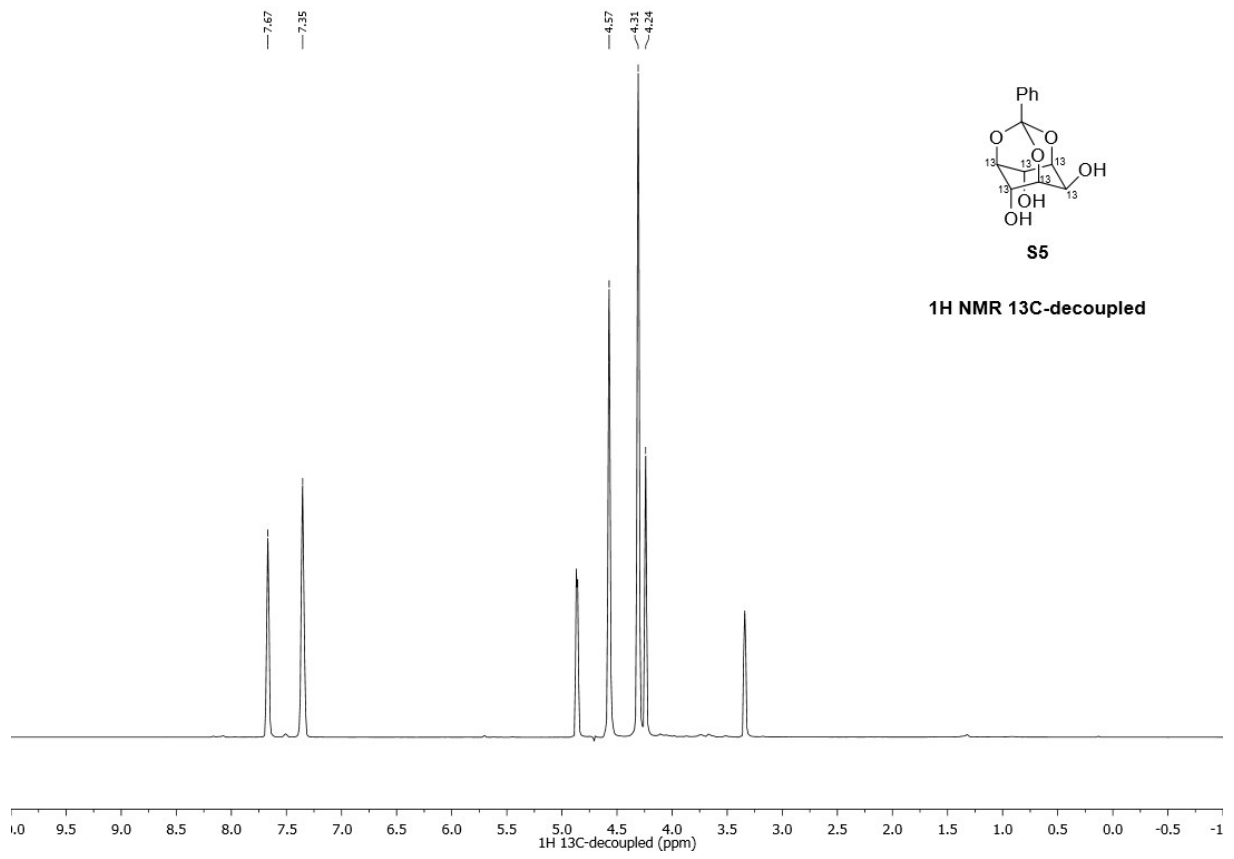


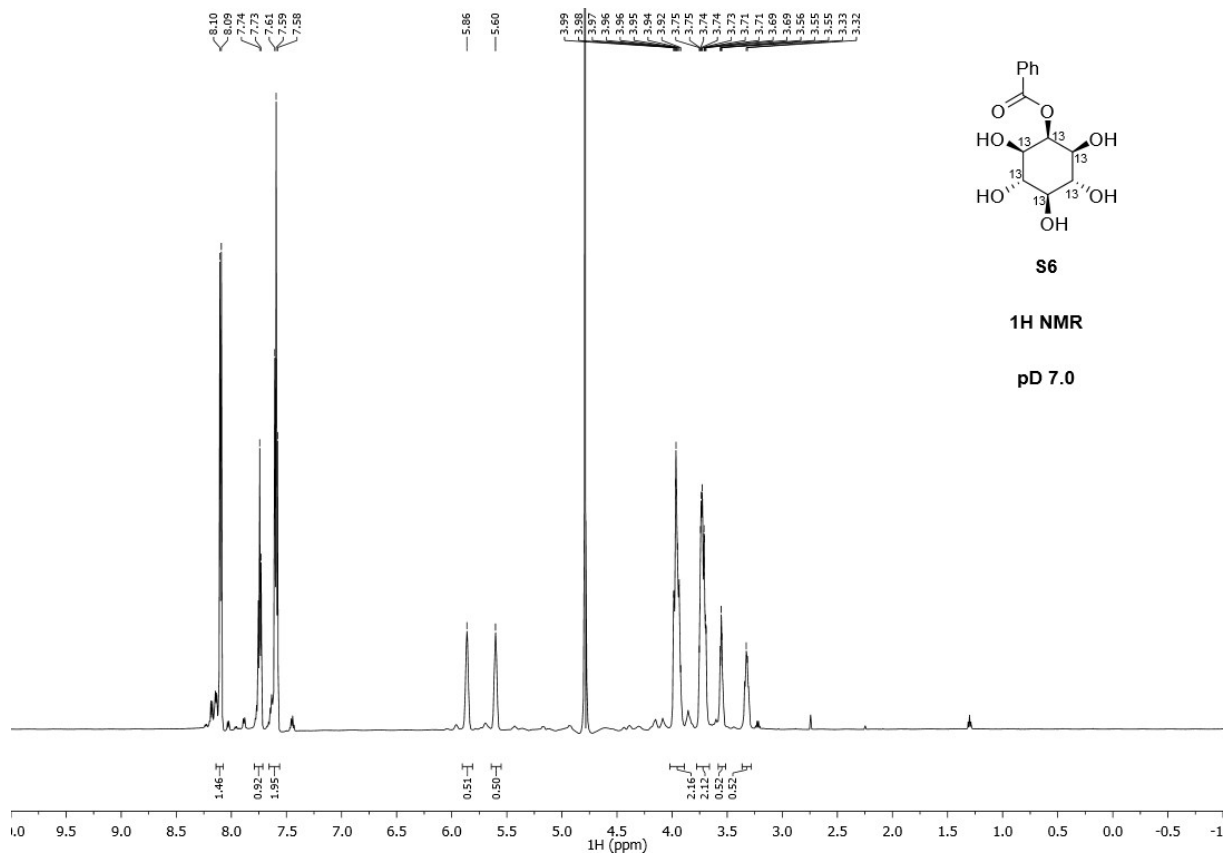
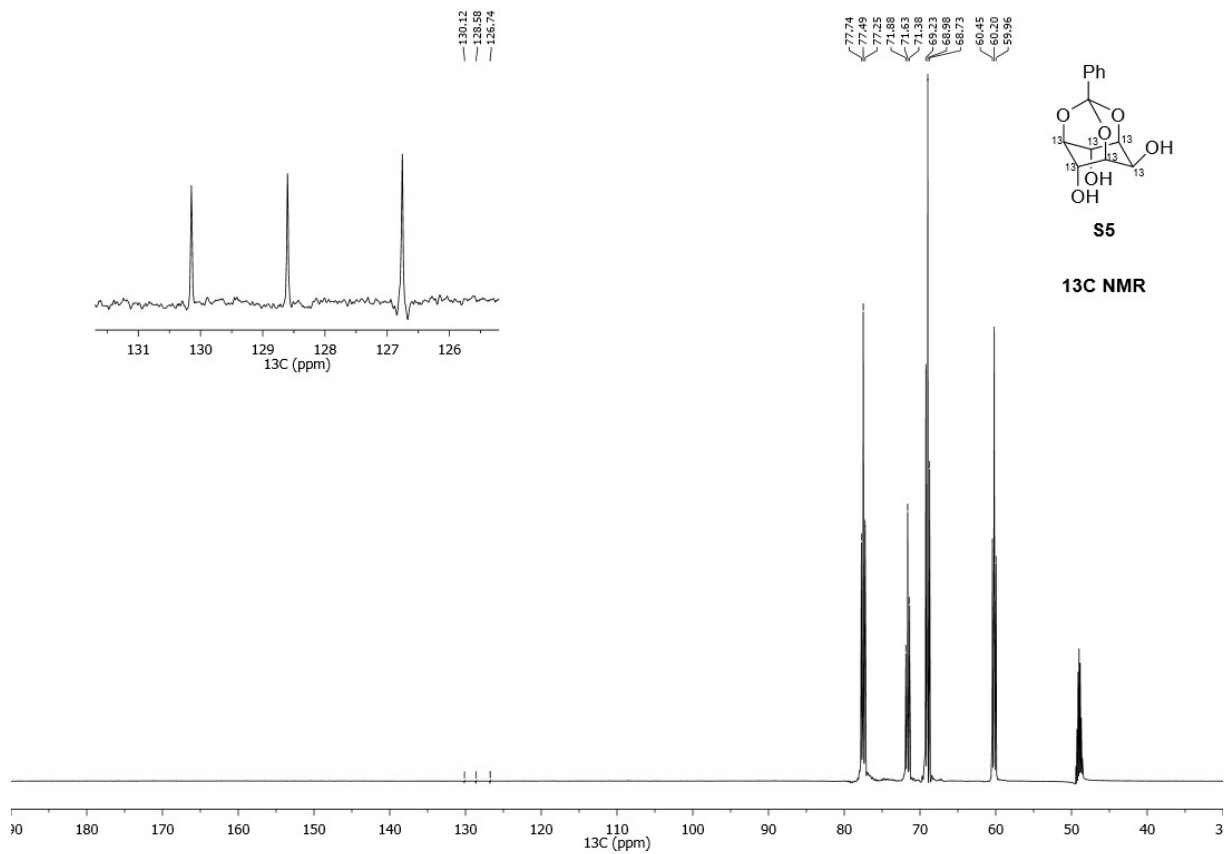


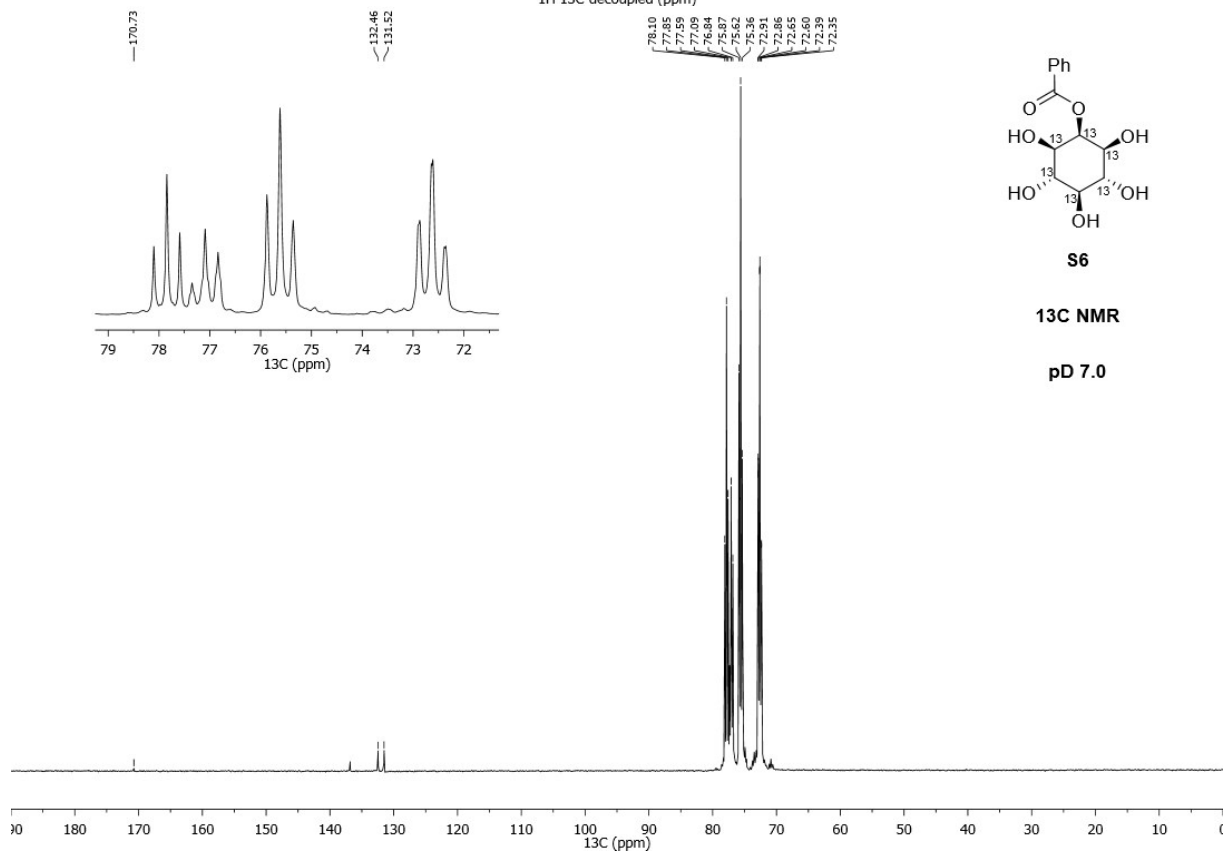
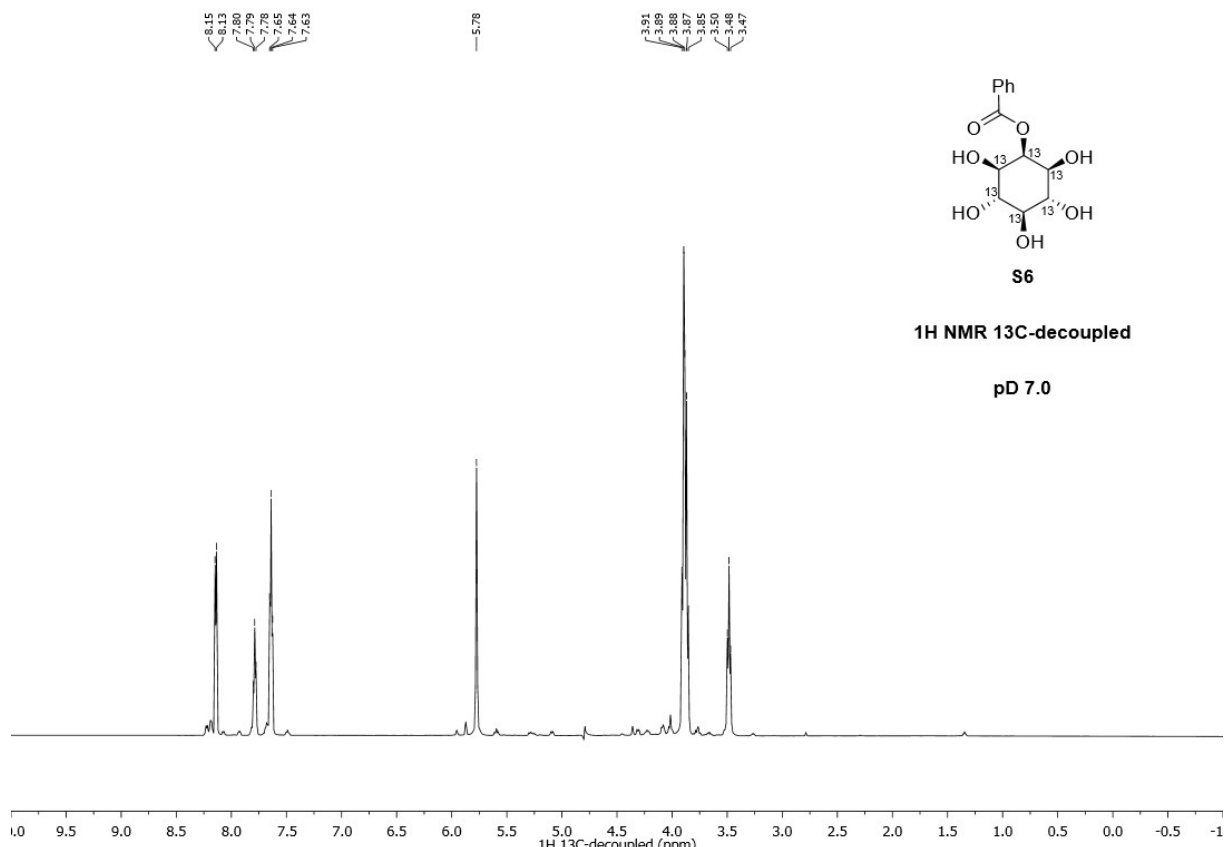


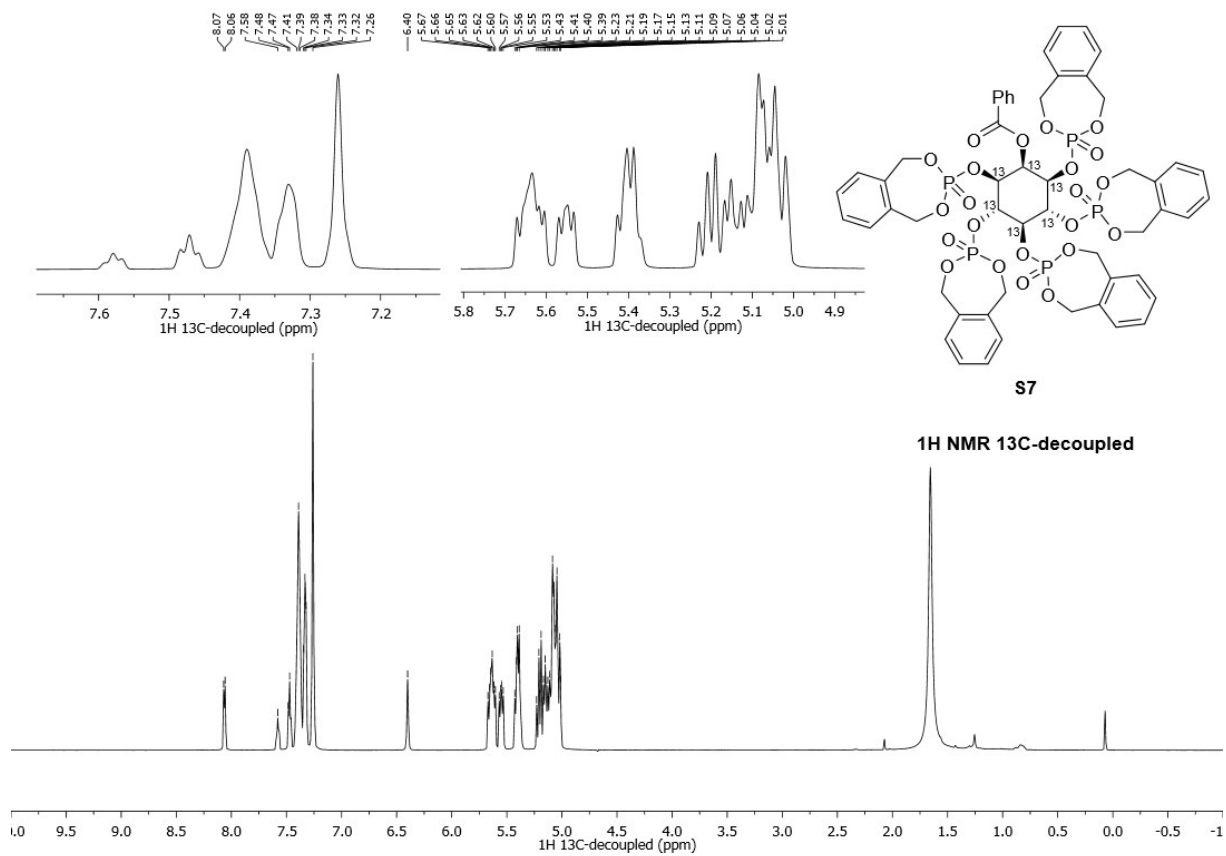
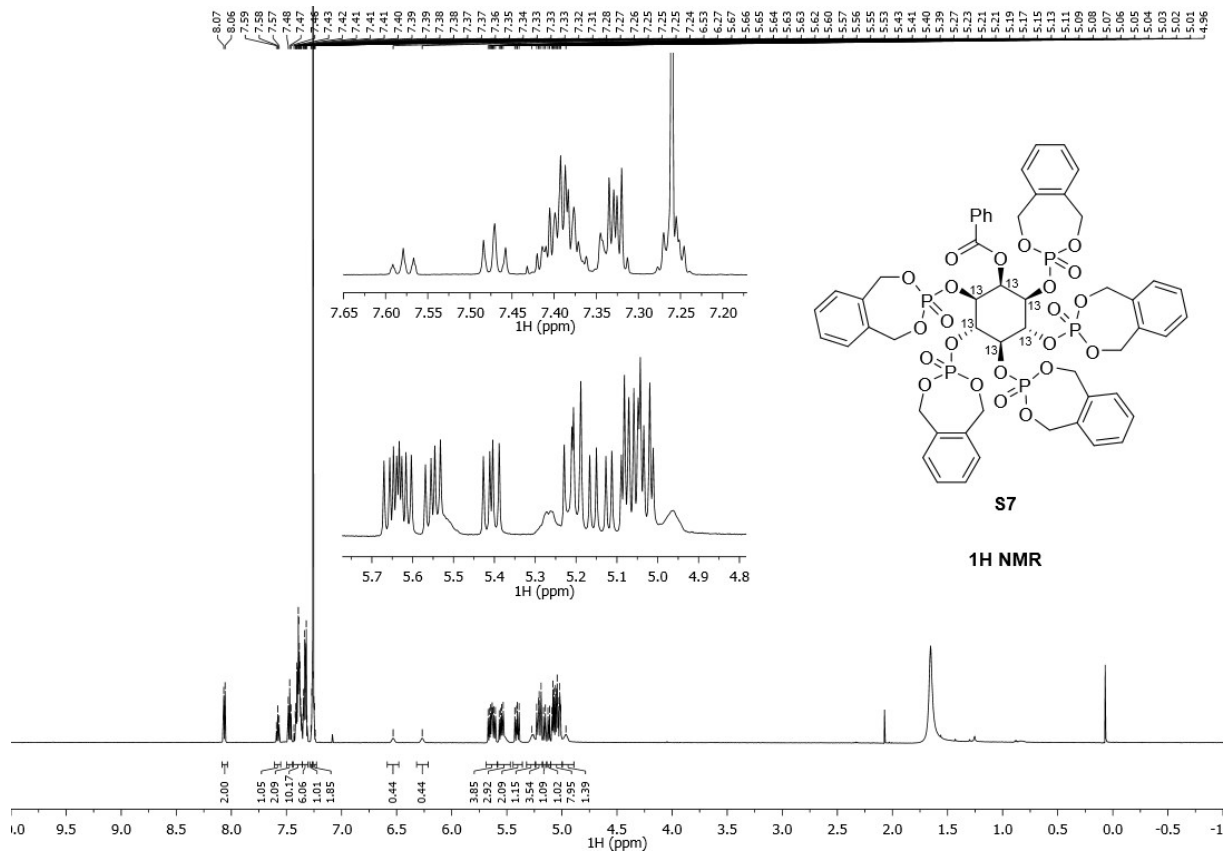


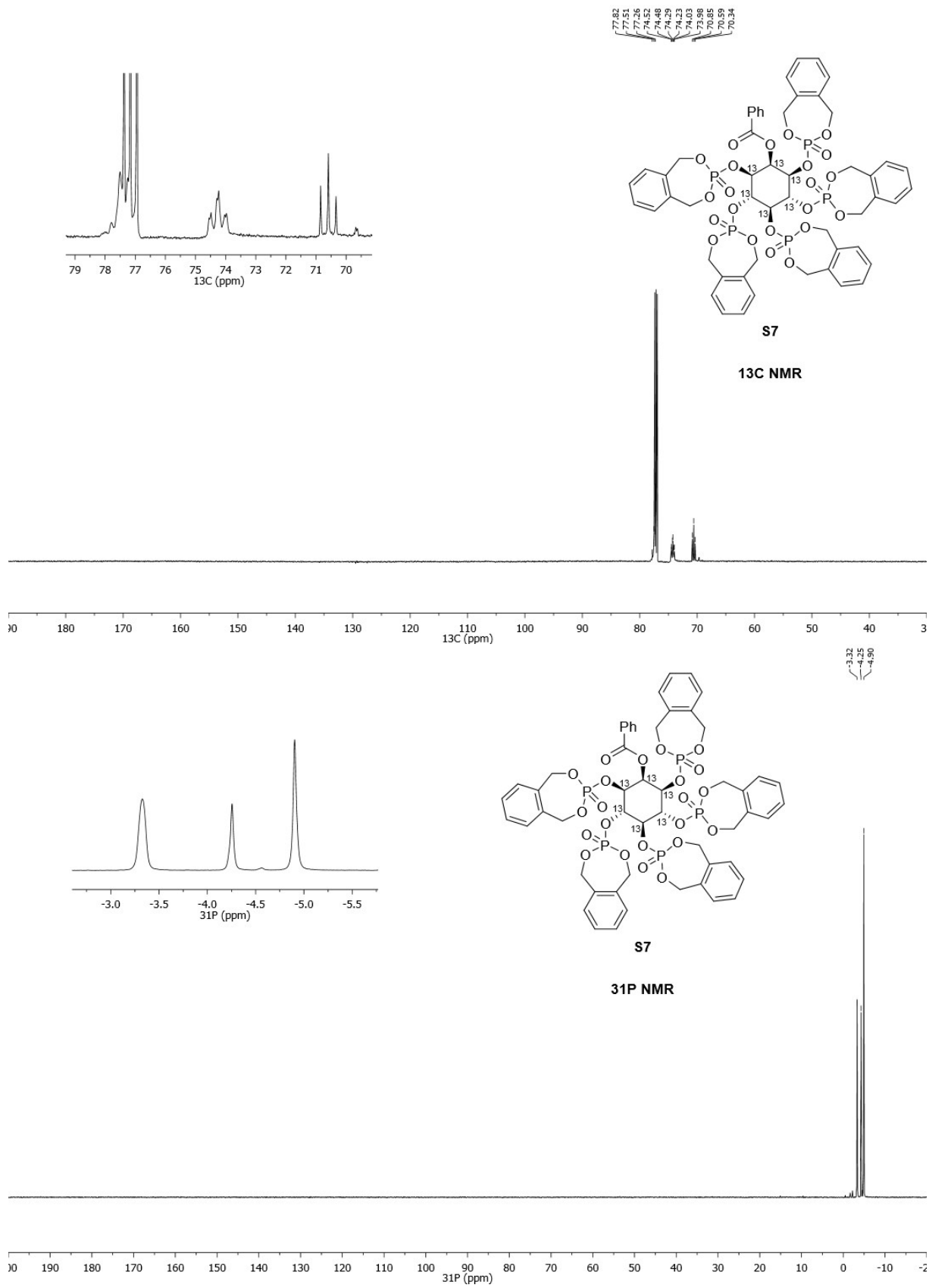


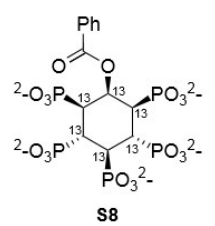
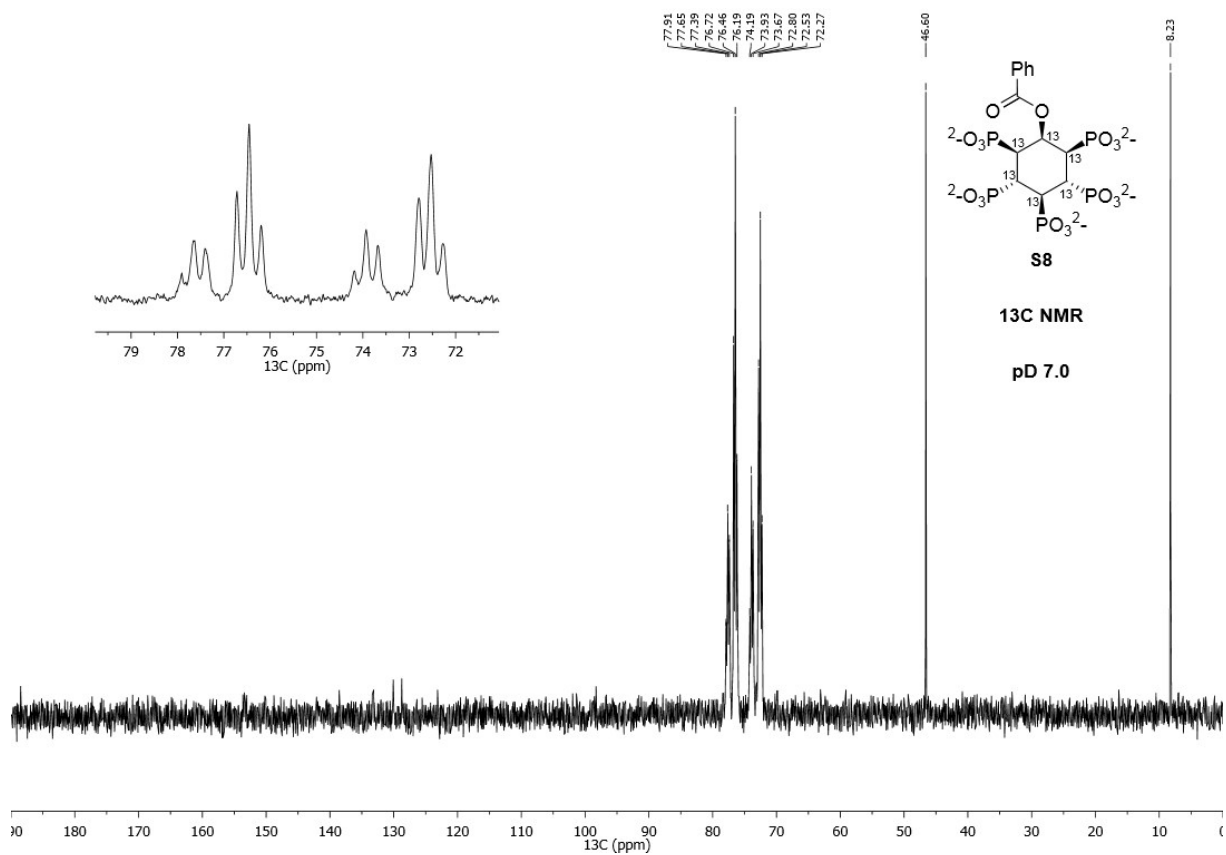
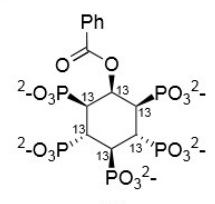
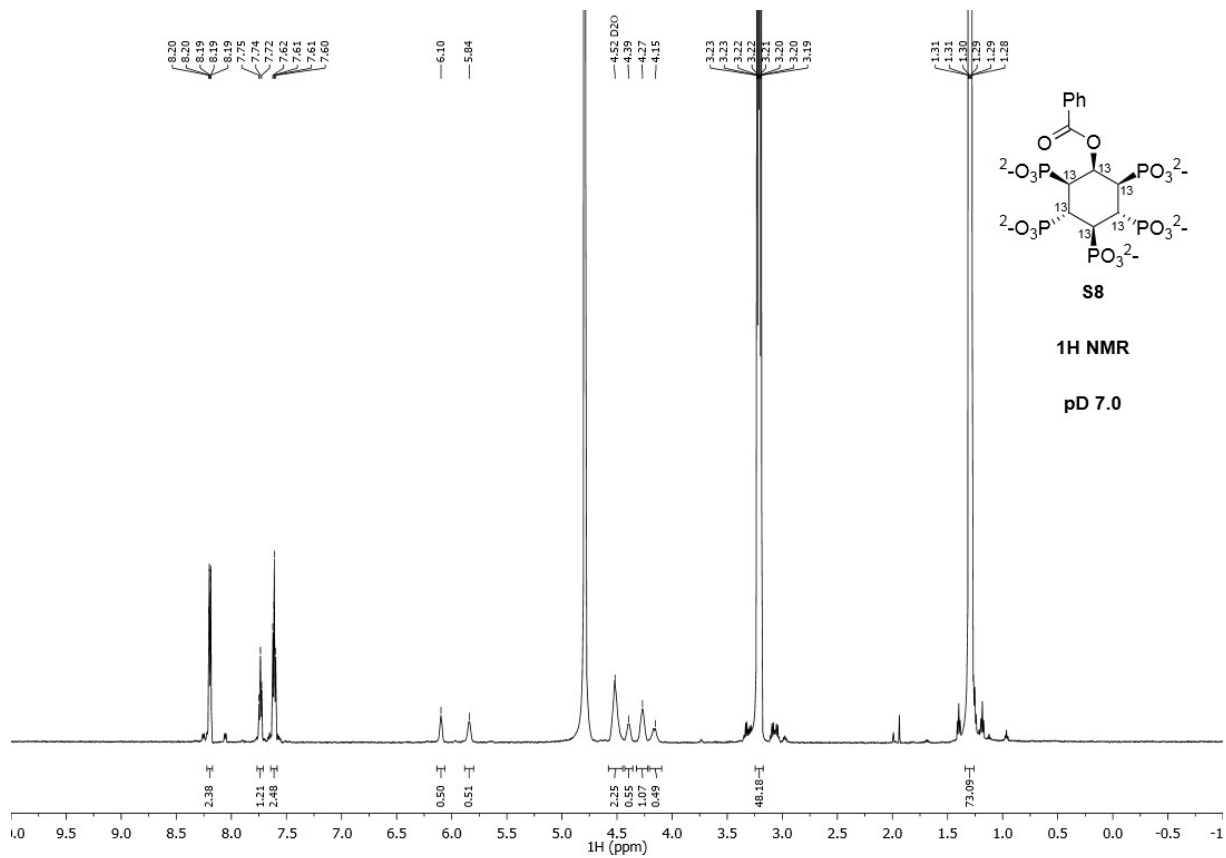


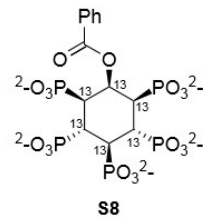
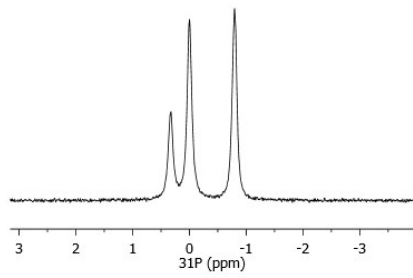




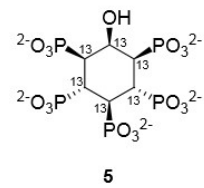
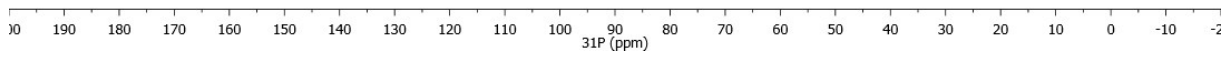




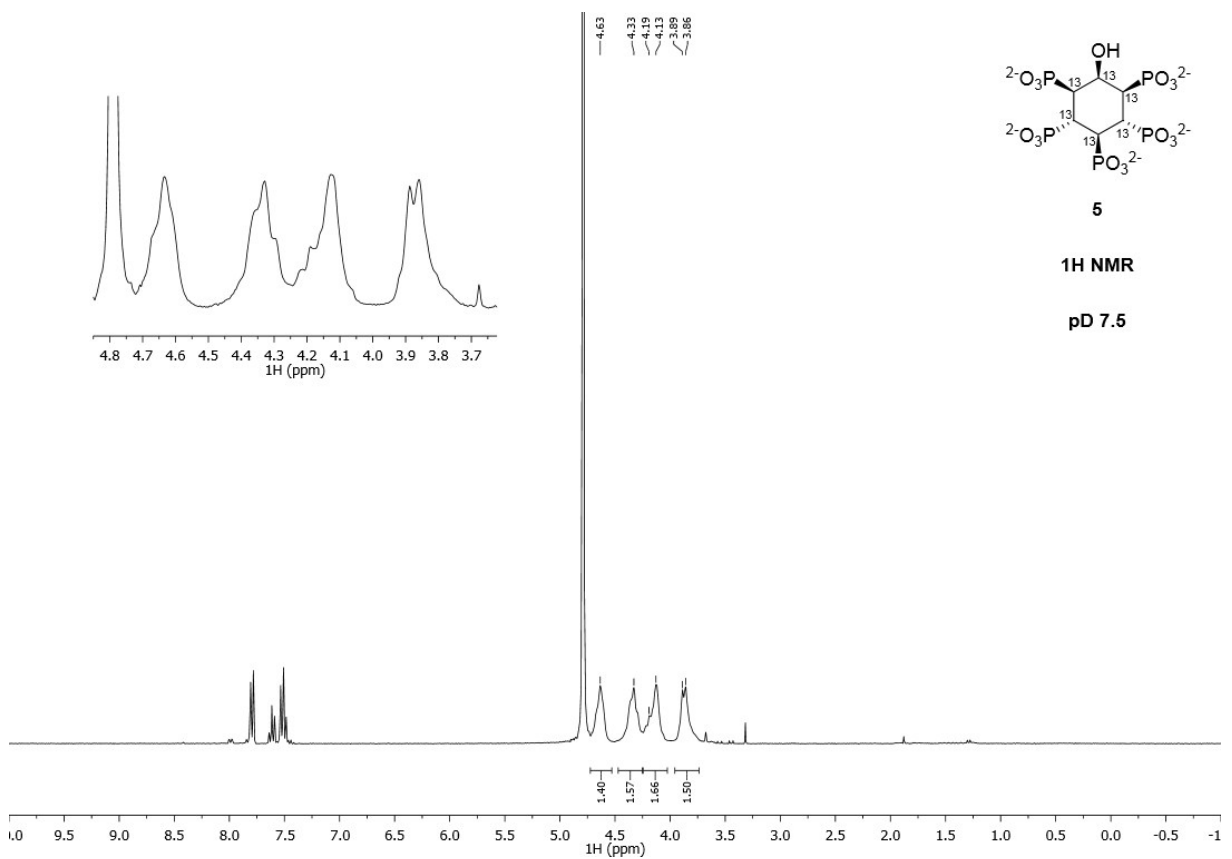
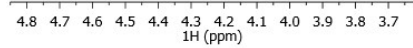


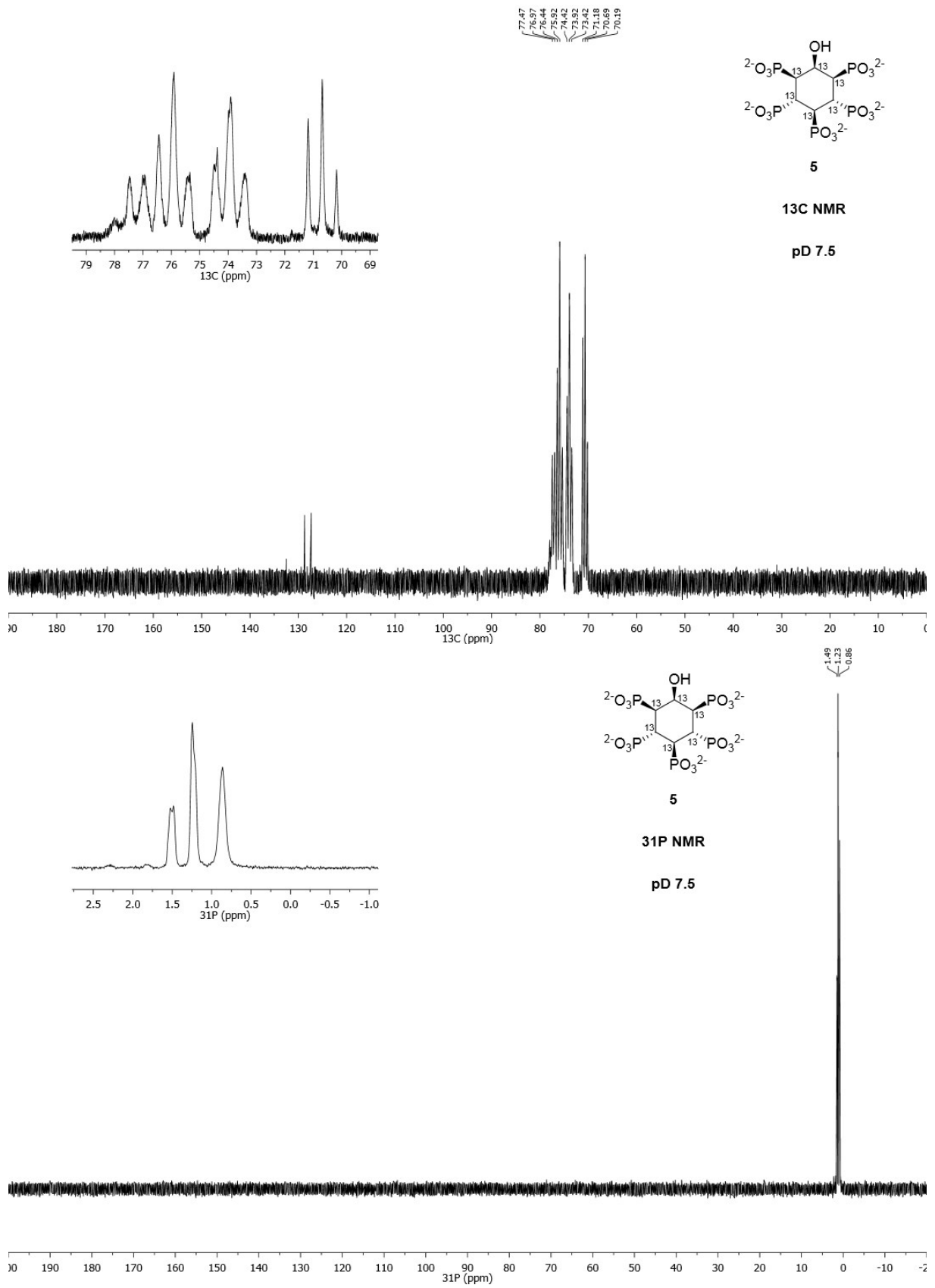


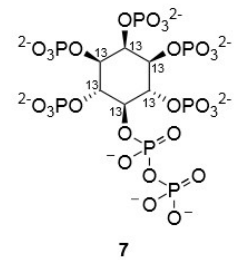
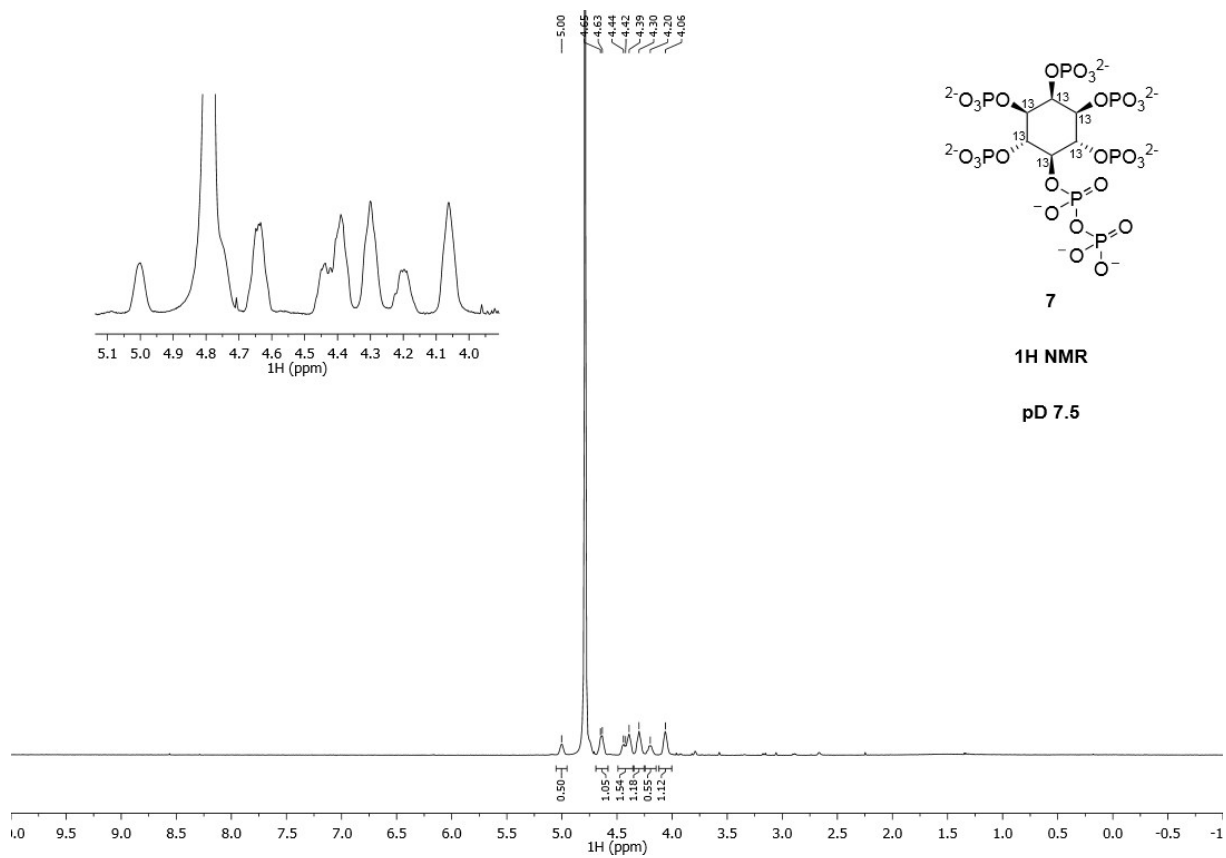
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pD 7.0



1H NMR
pD 7.5

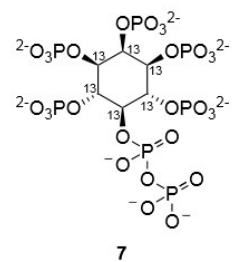
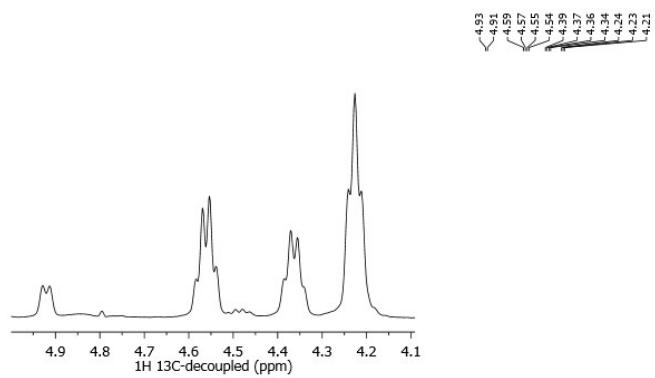






¹H NMR

pD 7.5



¹H NMR ¹³C-decoupled

(water suppression)

pD 7.5

