SUPPORTING INFORMATION

Dissecting the activation of insulin degrading enzyme by inositol pyrophosphates and their bisphosphonate analogs

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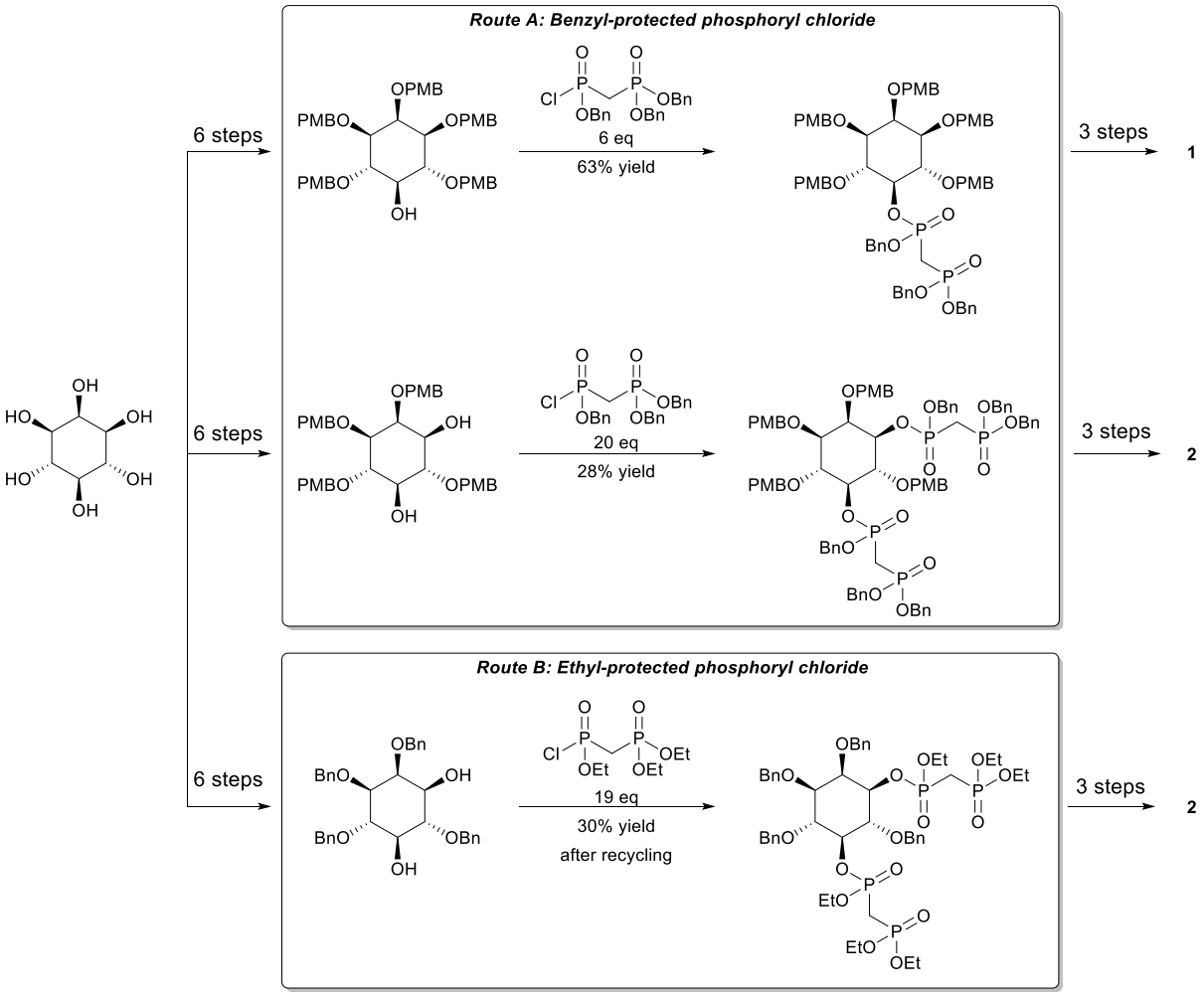
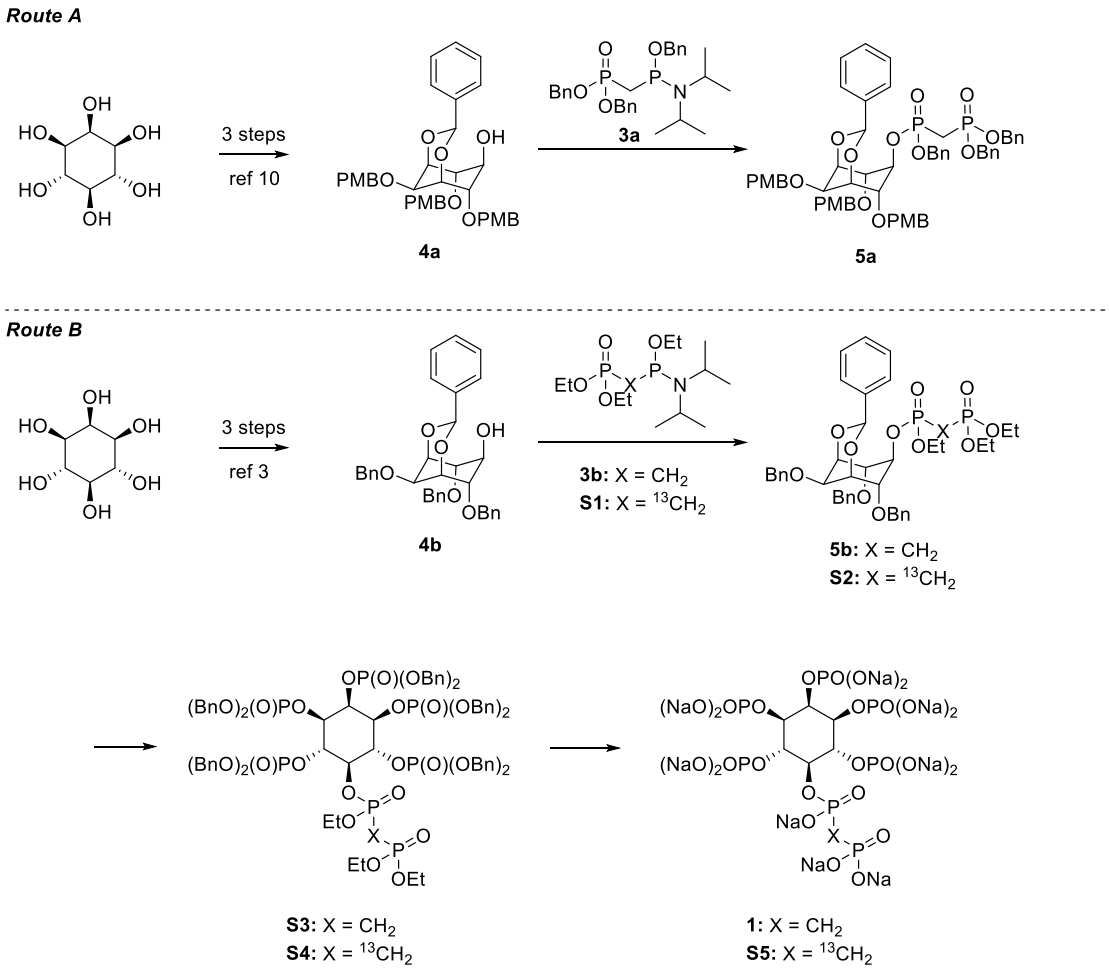
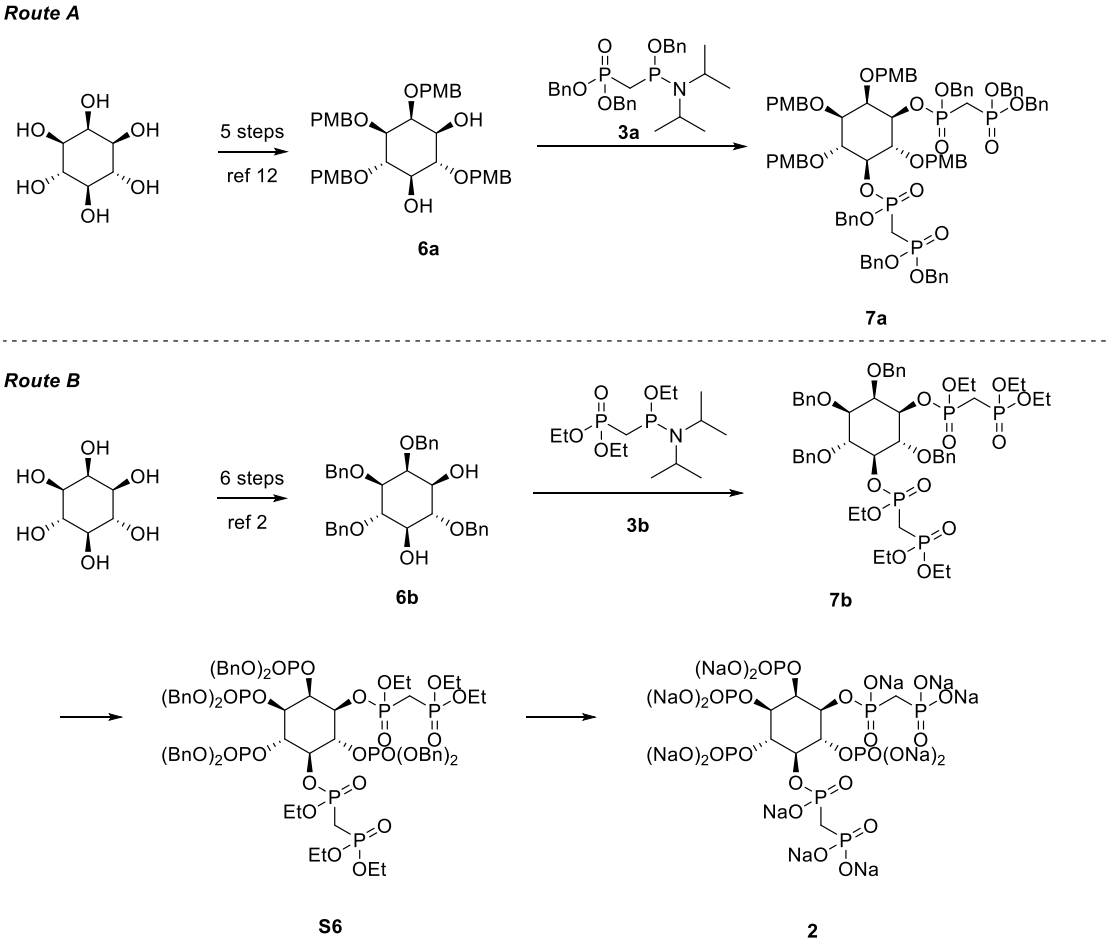
1 Supporting Figures



Scheme S1. Reported strategies for the synthesis of methylene bisphosphonate analogs of PP-InsPs



Scheme S2. Synthesis of 5PCP-InsP$_5$



Scheme S3. Synthesis of 1,5-$(PCP)_2$-$InsP_4$

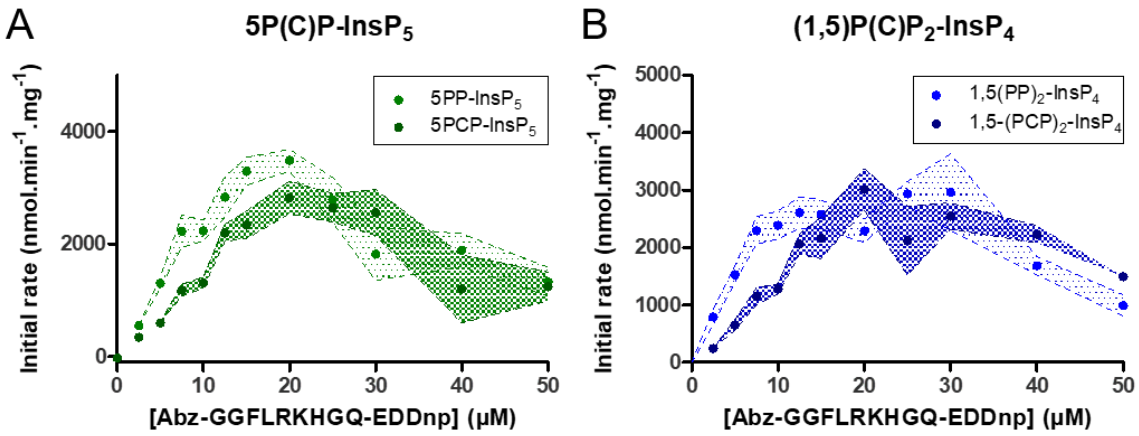
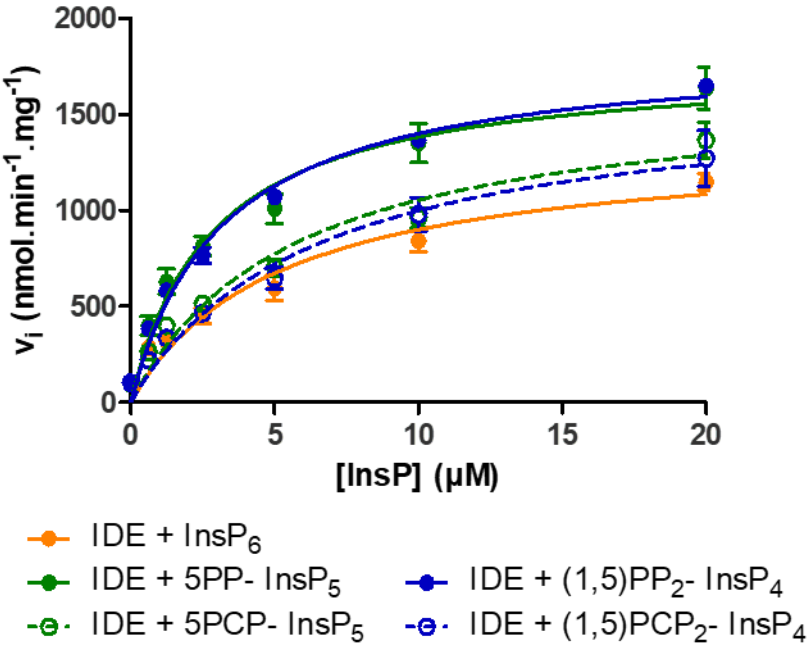


Figure S1. Activity of IDE on a fluorogenic peptide in presence of PP-InsPs and their non-hydrolyzable analogs (5 µM). All experiments were performed at least in triplicates, and colored areas represent SEM interval.



	V_{max} (nmol.min^{-1}.mg^{-1})	K_A (µM)
InsP$_6$	1361 (± 114)	5.1 (± 1.1)
5PP-InsP$_5$	1775 (± 103)	2.8 (± 0.5)
5PCP-InsP$_5$	1651 (± 132)	5.7 (± 1.1)
(1,5)PP$_2$-InsP$_4$	1847 (± 65)	3.2 (± 0.3)
(1,5)PCP$_2$-InsP$_4$	1634 (± 162)	6.4 (± 1.5)

Figure S2. Activation of IDE by InsPs and P(C)P-InsPs. Cleavage of a fluorogenic peptide (10 µM) by IDE was followed by fluorescence spectroscopy for various concentrations of InsP$_6$, PP-InsPs or PCP-InsP (0-20 µM). Initial rates were calculated for each concentration, and the resulting curve fitted using Michaelis-Menten model to obtain activation constants.

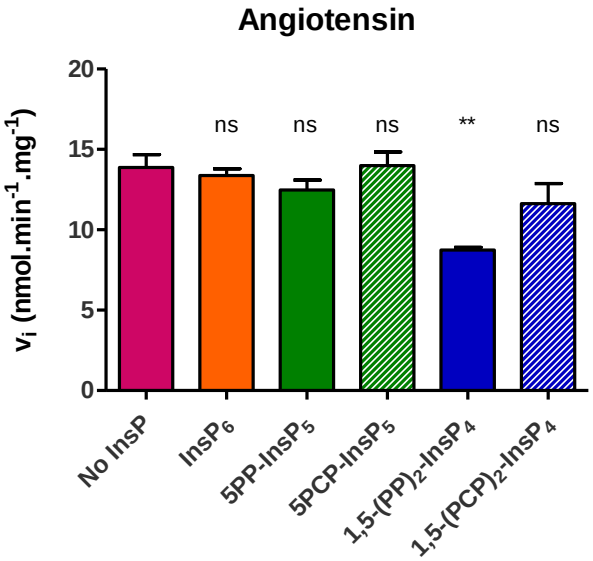


Figure S3. Angiotensin cleavage by IDE in presence of $InsP_6$ and P(C)P-InsPs. Zoom of Figure 2F.

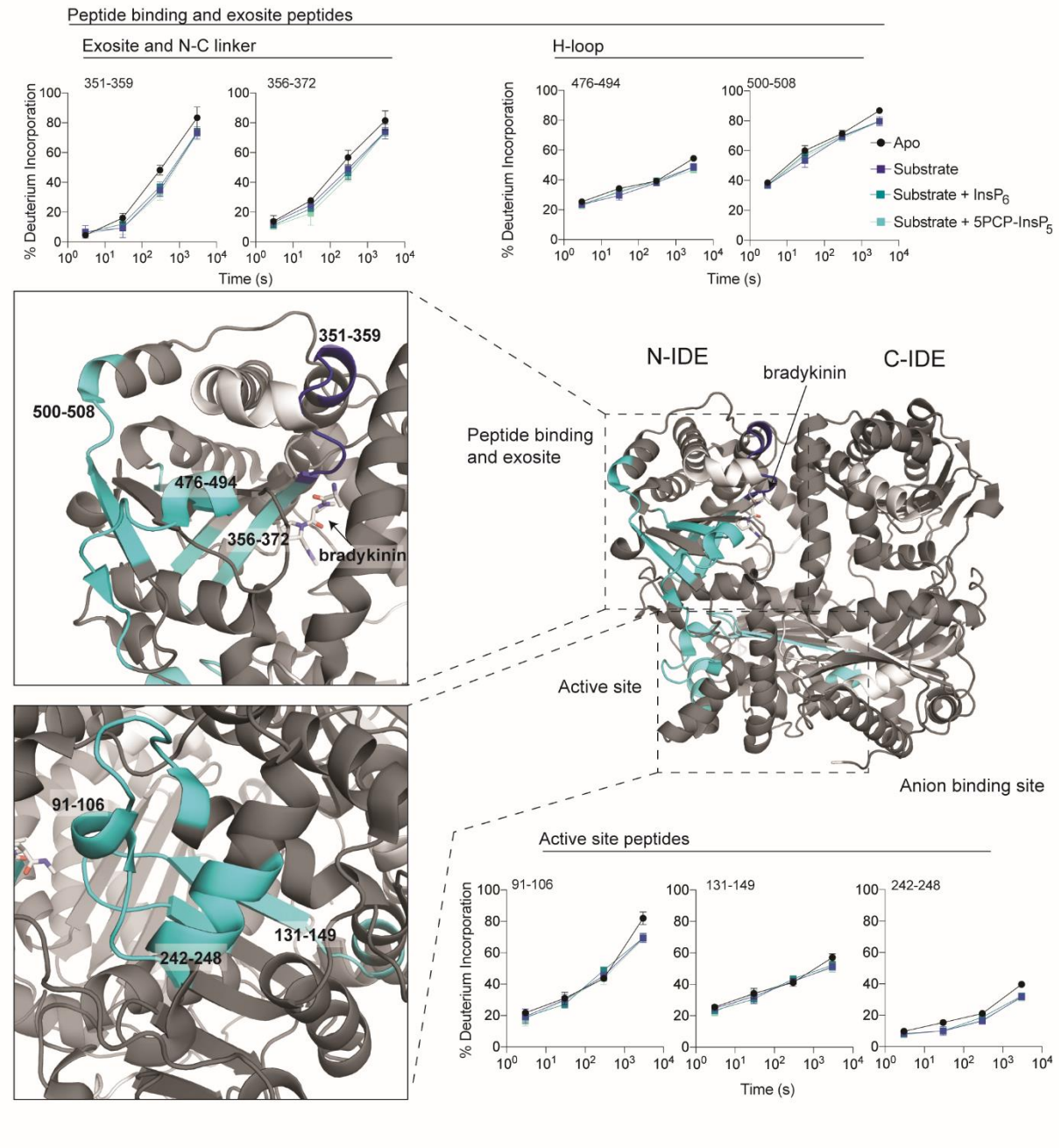


Figure S4. Bradykinin and probe induced changes in HDX of IDE. Major changes in HDX of IDE in the peptide binding and exosite region, and the active site region. Decreases in exchange of >6% are mapped in cyan, decreases in exchange >15% mapped in blue. All samples were performed in triplicate, with the exception of several samples indicated in Table S1. Model produced using PDB:3CWW (PMID: 18986166).

We compared the apo IDE to IDE combined with either ligands only ($InsP_6$ or 5PCP-$InsP_5$), substrate (bradykinin) only, or substrate and ligand. In the presence of substrate, or substrate and ligand, IDE underwent decreases in exchange in two key regions: the active site and the peptide binding site. Similar to results obtained for IDE in the presence of insulin or amyloid-beta, IDE (in the presence of InsP ligand and bradykinin) showed decreases in deuterium exchange in the exo-site, N-C linker, and the H-loop (Figure S3).[1] In the active site region, there were also decreases in exchange adjacent to the 'door' of IDE-N. Previously published HDX-MS results of IDE in complex with insulin or amyloid-beta found decreases in exchange at the 'door'. Whether this is the result of different experimental setup, or due to

the differences in substrate, is unknown. There were no differences in deuterium exchange identified within the samples containing IDE with either of the two ligands alone. Interestingly, the samples containing both bradykinin and ligands had slightly greater decreases in deuterium exchange, compared to the apo form, than the IDE and bradykinin only sample. This was more pronounced for 5PCP-$InsP_5$, for example in the peptide binding region close to the N-C linker (peptide 351-359), where the decrease in deuterium incorporation was >15% in the 5PCP-$InsP_5$+bradykinin sample only. This observation could indicate that the mechanism of increased catalytic activity towards bradykinin could be due to the ligands promoting the binding interaction with bradykinin. Whether the changes seen only in this study with bradykinin (active site peptides) are due to ligand binding or bradykinin catalysis remains unclear. No increases in exchange were seen in any of the samples.

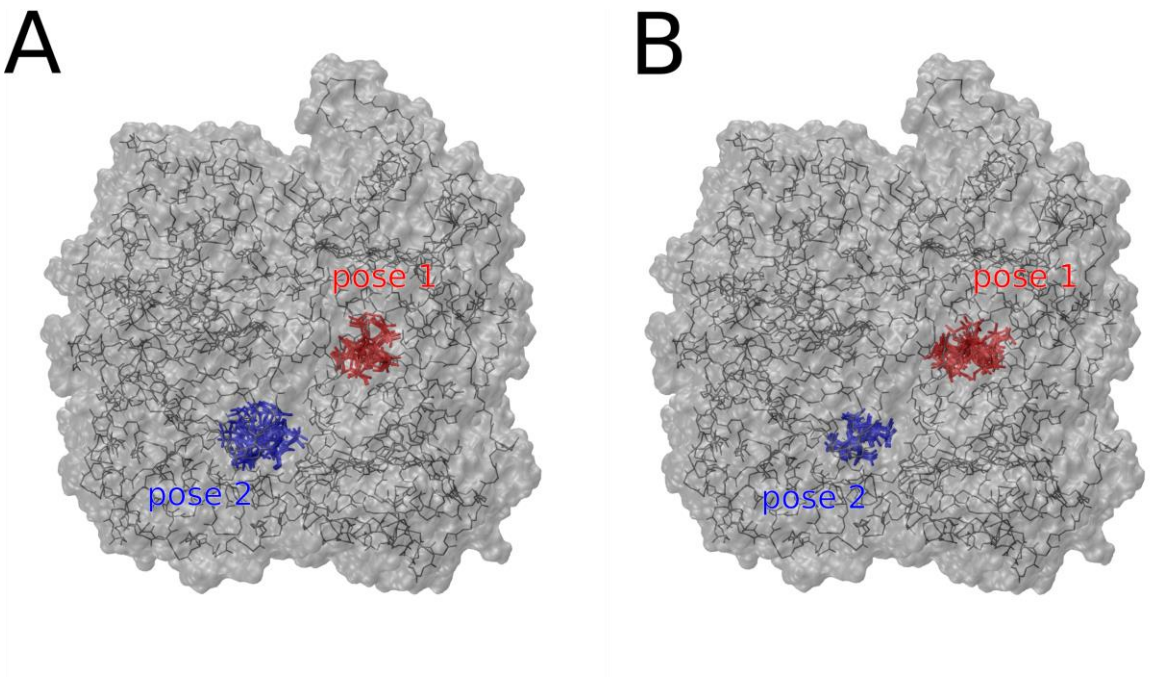


Figure S5. Heterogeneity of the binding poses observed for $InsP_6$ (A) and 5PCP-$InsP_5$ (B) derived from molecular docking. Poses 1 and 2 are coloured in red and blue, respectively. The protein matrix is highlighted as grey sphere and the backbone as grey trance.

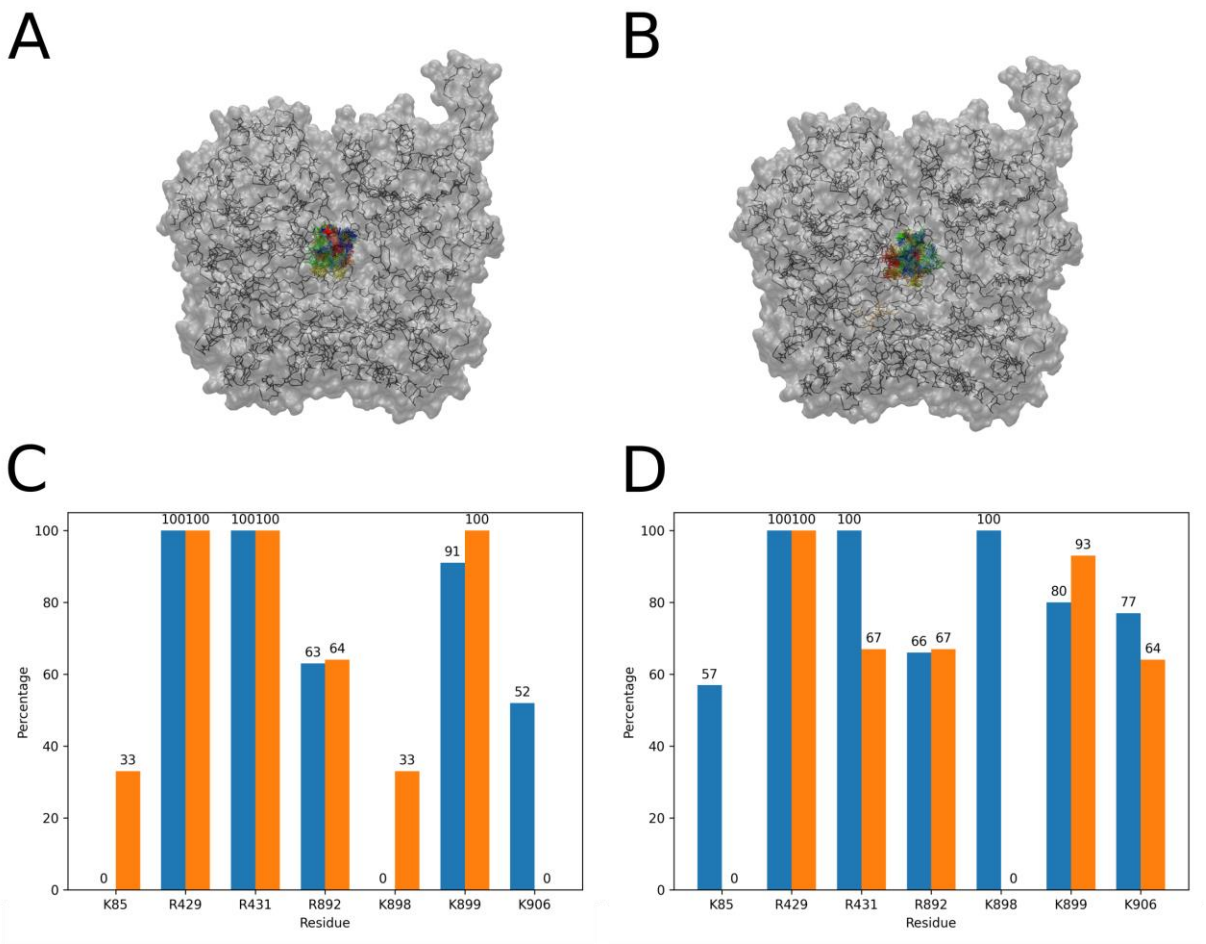


Figure S6. MD Simulations (**A+B**) Heterogeneity of the binding modes shown for $InsP_6$ and 5PCP-$InsP_5$ during the last 20 ns of all individual MD trajectories starting from pose 1 (blue, cyan, green) and pose 2 (red, orange, yellow). The protein and its backbone are shown as grey sphere and trace, respectively. (**C**+**D**) Interaction probability of selected amino acids with $InsP_6$ and 5PCP-$InsP_5$. Simulations starting from pose 1 and pose 2 are highlighted as blue and orange bars, respectively. The interactions are calculated with a cut-off of 3 Å and are averaged over the last 20 ns of three independent replicas per model.

Table S1. Full statistics on all hydrogen deuterium exchange mass spectrometry (HDX-MS) experiments according to guidelines from the International Conference on HDX-MS (PMID: 31249422) (refers to Figure 3).

Data Set	IDE + Ligand + Substrate
HDX reaction details	%D_2O=84.9% $pH_{(read)}$= 7.2 Temp= 23°C
HDX time course	3s, 30s, 300s, 3000s at 23°C
HDX controls	N/A
Back-exchange	Corrected using a fully deuterated (FD) sample
Number of peptides	123
Sequence coverage	81.8%
Average peptide length/ redundancy	Length = 12.4 Redundancy = 3.9
Replicates	3 (2 for substrate 30s and for substrate + IP6 3s)
Repeatability	Average StDev = 1.897%
Significant differences in HDX	>6% and >0.4 Da and unpaired t-test <0.05

Table S2. Ligands used for molecular docking

Ligand **L**	charge	protons	K^+ ions	
$InsP_6$	-8	4	-	$H_4\mathbf{L}^{8-}$
$InsP_6$ ionized	-5	4	3	$H_4\mathbf{L}^{8-} + 3K^+$
5PCP-$InsP_5$	-9	4	-	$H_4\mathbf{L}^{9-}$
5PCP-$InsP_5$ ionized	-6	4	3	$H_4\mathbf{L}^{9-} + 3K^+$

2 Chemical Synthesis

2.1 General Information

Abbreviations

MeCN	acetonitrile
EA	ethyl acetate
RBF	Round-bottomed flask
THF	tetrahydrofuran
TEA	triethylamine
RM	Reaction mixture

Solvents and chemicals

Commercially available chemicals were purchased from Sigma-Aldrich, Acros-Organics, Alfa-Aesar, and used as received unless otherwise stated. Dry THF and dry hexane were purchased from Acros-Organics and used as received. Dry MeCN and DCM was obtained using a solvent-purification system.

Silica gel column chromatography

Automated column chromatography was carried out on normal–phase silica columns on a CombiFlash® Rf from Teledyne Isco.

For the purification of mixed P^{III}-P^{V} species, high-purity grade silica (Sigma) was used, and column chromatography was performed by hand under N_2 atmosphere using solvents containing 1% TEA.

Reactions and chromatography fractions were monitored by thin-layer chromatography using SiO_2 on aluminium plates and visualized using a 254 nm UV lamp and/or by treatment with a suitable staining solution (potassium permanganate or phosphomolybdic acid (PMA) or cerium sulfate), followed by heating.

NMR

NMR measurements of isolated compounds and quantification were performed on a Bruker AV 300, or a AV600 spectrometer. ^{31}P spectra were referenced to a solution of tetramethylphosphonium bromide(PMe_4Br) standard (22.77 ppm) in a D_2O capillary insert.

HPLC

Preparative high-performance liquid chromatography (HPLC) was performed on a Varian system with SD-1 prep solvent delivery system, a ProStar 325 UV-Vis detector and a 440-LC fraction collector, using a Waters XBridge™ 5 µm C18 column (19 × 150 mm). All compounds were purified using the following method: 70% MeCN in H_2O for 2 min, 70 to 95 or 100% MeCN in H_2O in 20 min, 100% MeCN for 2 min, monitoring at 214 nm.

2.2 General procedures

General procedure A: Synthesis of PCP-amidites

Anhydrous THF (4 mL) was introduced in an oven-dried 25-mL RBF under a dry N_2 atmosphere, and diisopropylamine (295 µL, 2.1 mmol, 2.1 eq) was added. The solution was cooled in an acetone-$CO_{2(s)}$ bath and briefly degased (~ 5 min) under high vacuum. The temperature of the reaction mixture was kept at -78°C until otherwise stated. A BuLi solution (2.5 M in hexanes, 0.85 mL, 2.1 mmol, 2.1 eq.) was added dropwise with vigorous stirring. The mixture was stirred for 30 minutes, then a solution of methyl phosphonate (1.0 mmol, 1 eq) in anhydrous THF (1 mL) was added. The reaction mixture was stirred for a further 45-50 minutes. During this time, a ~ 1 M solution of N,N-diisopropylchlorophosphoramidite in dry hexane was prepared and filtered through a syringe equipped with a PTFE filter to remove diisopropylamine hydrochloride salt. 1.2 mL of this filtered solution were added dropwise to the solution. The reaction mixture was stirred for two further hours, then EtOH (85 µL) was added to quench the reaction. The cooling bath was removed, and the mixture was poured in a bilayer system of EA (15 mL) and saturated aqueous $NaHCO_3$ (15 mL). The mixture was quickly shaken and decanted. The organic layer was dried over Na_2SO_4, filtered and concentrated to an oil. The crude mixture was purified by silica gel column chromatography.

General procedure B: Coupling of PCP-amidite to inositol derivatives

Inositol derivative (0.1 mmol, 1 eq) and PCP-amidite (0.2 mmol, 2 eq) were evaporated with dry acetonitrile (2 x 4 mL) and dried under high vacuum for 15-30 minutes, then put under a dry N_2 atmosphere. They were then dissolved in dry acetonitrile (4 mL) and cooled in an ice bath. DCI (0.4 mmol, 4 eq) was added, and the reaction mixture was left to stir overnight, during which time it reached room temperature. The reaction mixture was then cooled in an acetone-CO_2 bath, and mCPBA was added in one portion. The cooling bath was removed after 5-10 minutes, and RM was allowed to stir for one hour. A saturated aqueous NH_4Cl solution (20 mL) was then added to the mixture, and extracted twice with ethyl acetate (2 x 20 mL). The organic layers were pooled and washed with saturated aqueous $NaHCO_3$ (1 x 30 mL) and brine (1 x 30 mL). The organic layer was dried over Na_2SO_4, filtered, concentrated under reduced pressure, and purified by silica gel flash column chromatography.

General procedure C

The synthesis was performed by adapting published procedures.[2,3] Inositol derivative (0.080 mmol, 1 eq.) was dissolved in a mixture of MeOH (2.2 mL), THF (2.2 mL) and water (0.55 mL) under N_2. AcOH (10 drops) was added, followed by $Pd(OH)_2/C$ (20% wt loading, 50% water, 58.0 mg). The 50-mL RBF was purged with H_2 and stirred for 50 h at RT. The reaction mixture was then filtered over a 0.45 µm GHP syringe filter. The filter was washed with methanol (2 ×5 mL), and solvent was removed from the pooled filtered solutions by rotary evaporation. The residue was evaporated once with dry acetonitrile (2 mL) and dried under high vacuum for 30 min. It was then suspended in dry DCM (2.5 mL) under a dry N_2 atmosphere. The solution was cooled in an ice bath, and DCI (192.7 mg, 1.63 mmol, 20 eq.) was added, followed by dibenzyl diisopropylphosphoramidite (0.27 mL, 0.819 mmol, 10 eq.). The ice bath was removed, and the reaction mixture was stirred for 3 h at room temperature. The fine suspension was then cooled in an ice-brine bath, and mCPBA (268.4 mg, 1.20 mmol, 15 eq.) was added portionwise. After 1 h, the cooling bath was removed and the mixture was stirred for a further 1 h. The reaction mixture was then diluted with EA (20 mL) and washed with 10% aq. $Na_2S_2O_3$ (20 mL), sat. aq. $NaHCO_3$ (20 mL) and brine (20 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to an oil. The resulting crude was purified by flash column chromatography (0-10% MeOH in DCM). The resulting oil was further purified by preparative reversed phase HPLC (70-95% MeCN in water), yielding the desired compound as an oil.

General procedure D

The procedure was adapted from published procedures.[2,4]

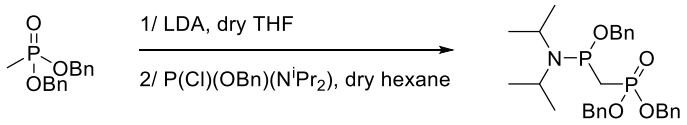
Briefly, the Ins derivative (25 µmol, 1 eq) was dissolved in dry DCM (1.1 mL) under a dry N_2 atmosphere, and cooled in an ice bath. Trimethylsilylbromide TMSBr (0.55 mL) was added dropwise, and the mixture was allowed to stir for 3 days. Solvents were then removed under reduced pressure, and 5.5 mL MeOH were added. The resulting solution was stirred for 1 h at RT, and solvent was removed under reduced pressure. The resulting crude mixture was taken up in water (5.5 mL) and extracted with 3 x 5.5 mL Et_2O. The aqueous layer was diluted with 5.5 mL of a "2X precipitation buffer" (2X: 14 mM $MgCl_2$, 20 mM HEPES pH 6.8, 50 mM NaCl). The pH was adjusted to 9 with 10 M NaOH, and the resulting suspension was left to precipitate overnight. The suspension was then centrifuged 15 min at 3,000g and the supernatant was discarded. The resulting white pellet was resuspended in 7 mL and centrifuged 15 min at 3,000 g. The supernatant was discarded, and the operation was repeated once more. The solid was gently shaken with 9 mL Amberlite IRC748 resin for 2h. The solution was collected, and the resin was washed with 10 volumes of water. Water was removed by rotary evaporation followed by lyophilization.

2.3 Synthesis of PCP-amidites

Mixed P^{III}-P^{V} species (PCP-amidites) were synthesized by adapting literature procedures.[5]

Diethyl ^{13}C-methyl phosphonate,[6] dibenzyl methyl phosphonate[7] and *N, N* - diisopropyl benzyl chlorophosphoramidite[8,9] were synthesized following literature procedures.

*PCP(OBn) (**3a**)*



3a

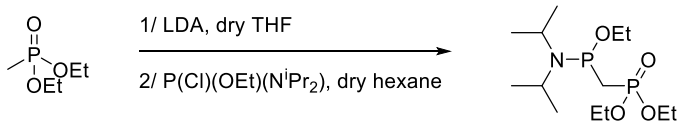
Compound **3a** was synthesized according to general procedure A from dibenzyl methyl phosphonate (800 mg, 2.9 mmol, 1 eq) and benzyl N,N-diisopropylchlorophosphoramidite (3.5 mL of a ~ 1 M solution in dry hexane, 3.5 mmol, 1.2 eq). The crude mixture was purified by silica gel column chromatography (33% Ethyl Acetate in Hexane, 1%TEA), yielding 893 mg (1.74 mmol, 60 %) of the desired compound as a colorless to slightly yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32 – 7.20 (m, 15H, C_5H_6), 5.09 – 4.89 (m, 4H, CH_2-C_5H_6(P^{V})), 4.64 (qd, J = 12.1, 8.1 Hz, 2H, CH_2-C_5H_6 (P^{III})), 3.62 – 3.39 (m, 2H, N(C*H*$(CH_3)_2)_2$)), 2.53 (ddd, J = 19.8, 14.6, 3.9 Hz, 1H, P^{V}CH*H*P^{III}), 2.01 (ddd, J = 20.6, 14.6, 4.2 Hz, 1H, P^{V}C*H*HP^{III}), 1.18 (d, J = 6.6 Hz, 6H, N(CH(C$H_3)_2)_2$)), 1.10 (d, J = 6.8 Hz, 6H, N(CH(C$H_3)_2)_2$)).

^{13}C NMR (75 MHz, $CDCl_3$) δ 139.29 72 (d, *J* = 9.5 Hz, 1C), 136.63 (d, *J* = 6.4 Hz, 1C), 136.59 (d, *J* = 6.5 Hz, 1C), 128.44, 128.43, 128.23, 128.14, 128.11, 127.91, 127.89, 127.35, 127.29, 68.94 (d, *J* = 15.1 Hz, 1C), 67.15(d, *J* = 15.1 Hz, 1C), 44.77 (d, *J* = 11.1 Hz, 2C, N(*C*H$(CH_3)_2)_2$), 31.18 (dd, *J* = 132.5, 32.4 Hz, P^{III}*C*H_2P^{V}), 24.20 ((m, 2C, N(CH(*C*$H_3)_2)_2$)).

^{31}P NMR (122 MHz, $CDCl_3$) δ 117.1 (d, J = 54.4 Hz, P^{III}), 21.8 (d, J = 54.4 Hz, P^{V}).

*PCP(OEt) (**3b**)*



3b

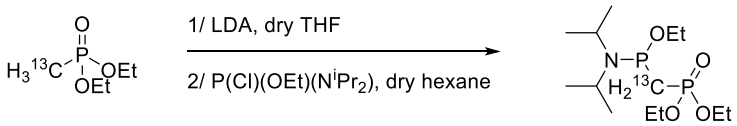
Compound **3b** was synthesized according to general procedure A from diethyl methyl phosphonate (146 µL, 1.0 mmol, 1 eq) and ethyl N,N-diisopropylchlorophosphoramidite (1.2 mL of a ~ 1 M solution in dry hexane, 1.2 mmol, 1.2 eq). The crude mixture was purified by silica gel column chromatography (15% Ethyl Acetate in Hexane, 1%TEA), yielding 238 mg (0.66 mmol, 66 %) of the desired compound as a colorless to slightly yellow oil.

^{1}H NMR (300 MHz, $CDCl_3$) δ 4.17 – 3.97 (m, 4H, $P^V(O)(OCH_2CH_3)_2$), 3.62 (dqd, *J* = 9.2, 7.0, 4.2 Hz, 2H, $P^{III}(OCH_2CH_3)$), 3.43 (dp, *J* = 10.1, 6.7 Hz, 2H, $N(CH(CH_3)_2)_2$), 2.31 (ddd, *J* = 19.5, 14.6, 3.5 Hz, 1H, P^VCHHP^{III}), 1.87 (ddd, *J* = 20.4, 14.6, 3.4 Hz, 1H, P^VCHHP^{III}), 1.28 (t, *J* = 7.1 Hz, 6H, $P^V(O)(OCH_2CH_3)_2$), 1.17 (t, *J* = 7.2 Hz, 3H, $P^{III}(OCH_2CH_3)$), 1.13 (d, *J* = 6.5 Hz, 6H, $N(CH(CH_3)_2)_2$), 1.07 (d, *J* = 6.8 Hz, 6H, $N(CH(CH_3)_2)_2$).

^{13}C NMR (75 MHz, $CDCl_3$) δ 62.72 (d, *J* = 21.1 Hz, 1C, $P^{III}(OCH_2CH_3)$), 61.55 (ddd, *J* = 10.1, 6.3, 1.1 Hz, 2C, $P^V(OCH_2CH_3)_2$), 44.62 (d, *J* = 11.0 Hz, 2C, $N(CH(CH_3)_2)_2$), 31.18 (dd, *J* = 132.8, 32.0 Hz, $P^{III}CH_2P^V$), 24.43 (2C, $N(CH(CH_3)_2)_2$), 24.04 (d, *J* = 7.6 Hz, 2C, $N(CH(CH_3)_2)_2$)), 17.08 (d, *J* = 8.0 Hz, 1C, $P^{III}(OCH_2CH_3)$), 16.46 (dd, *J* = 6.3, 2.1 Hz, 2C, $P^V(OCH_2CH_3)_2$).

^{31}P NMR (122 MHz, $CDCl_3$) δ 114.04 (d, *J* = 55.3 Hz, P^{III}), 28.18 (d, *J* = 55.3 Hz, P^V).

*P^{13}CPOEt (**S1**)*



S1

Compound **S1** was synthesized according to general procedure A from diethyl ^{13}C-methyl phosphonate (163.2 mg, 1.1 mmol, 1.1 eq) and ethyl N,N-diisopropylchlorophosphoramidite (1.1 mL of a ~ 0.9 M solution in dry hexane, 1.0 mmol, 1.0 eq). The crude mixture was purified by silica gel column chromatography (15% Ethyl Acetate in Hexane, 1%TEA), yielding 227.7 mg (0.69 mmol, 69 %) of the desired compound as a colorless to slightly yellow oil.

^{1}H NMR (300 MHz, CDCl$_3$) δ 4.17 – 3.99 (m, 4H, P^{V}(O)(OC*H$_2$*CH$_3$)$_2$), 3.64 (dqd, *J* = 9.2, 7.0, 4.2 Hz, 2H, P^{III}(OC*H$_2$*CH$_3$)), 3.45 (dp, *J* = 10.1, 6.7 Hz, 2H, N(C*H*(CH$_3$)$_2$)$_2$), 2.32 (dddd, *J* = 125.7, 19.5, 14.6, 3.5 Hz, 1H, P^{V}C*H*HP^{III}), 1.89 (dddd, *J* = 124.7, 20.4, 14.6, 3.4 Hz, 1H, P^{V}CH*H*P^{III}), 1.29 (t, *J* = 7.1 Hz, 6H, P^{V}(O)(OCH$_2$C*H$_3$*)$_2$), 1.18 (t, *J* = 6.9 Hz, 3H, P^{III}(OCH$_2$C*H$_3$*)), 1.15 (d, *J* = 6.7 Hz, 6H, N(CH(C*H$_3$*)$_2$)$_2$), 1.08 (d, *J* = 6.8 Hz, 6H, N(CH(C*H$_3$*)$_2$)$_2$).

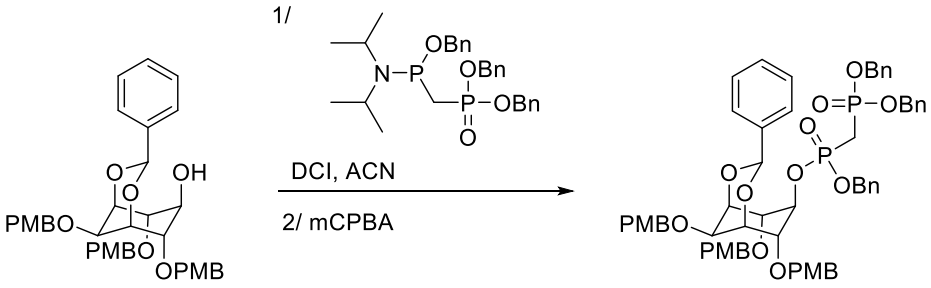
^{13}C NMR (75 MHz, CDCl$_3$) δ 62.74 (dd, *J* = 21.1, 4.3 Hz, P^{III}(O*C*H$_2$CH$_3$), 61.56 (ddd, *J* = 10.2, 6.3, 1.1 Hz, 2C, P^{V}(O*C*H$_2$CH$_3$)$_2$), 45.11 – 44.18 (m, 2C, N(*C*H(CH$_3$)$_2$)$_2$), 31.22 (dd, *J* = 132.8, 32.0 Hz, P^{III}[^{13}CH$_2$]P^{V}), 24.67 – 24.28 (m, 2C, N(CH(*C*H$_3$)$_2$)$_2$), 24.07 (d, *J* = 7.6 Hz, , 2C, N(CH(*C*H$_3$)$_2$)$_2$)), 17.12 (d, *J* = 8.0 Hz, P^{III}(OCH$_2$*C*H$_3$)), 16.50 (dd, *J* = 6.3, 2.1 Hz, 2C, P^{V}(OCH$_2$*C*H$_3$)$_2$).

^{31}P NMR (122 MHz, CDCl$_3$) δ 114.03 (dd, *J* = 55.3, 31.9 Hz, 1P, P^{III}), 28.16 (dd, *J* = 132.8, 55.3 Hz, 1P, P^{V}).

2.4 Synthesis of 5PCP-InsP$_5$ derivatives

Compounds **4a**[10] and **4b**[3] were synthesized according to reported procedures.

*Synthesis of **5a***



Method 1: Synthesis with *in situ* generation of PCP-amidite **3b**

Dibenzyl methyl phosphonate (426 mg, 1.54 mmol, 1.9 eq) was dissolved in anhydrous, degassed THF (6.1 mL) under a dry N_2 atmosphere. The solution was cooled in a $CO_{2(s)}$-acetone bath, and a 2.5 M BuLi solution (0.6 mL, 1.5 mmol, 1.9 eq) was added dropwise. The resulting clear yellow solution was stirred for 30 min at -78°C. A solution of benzyl diisopropylamine chlorophosphoramidite (422 mg, 1.54 mmol, 1.9 eq) in dry THF (3 mL) was then added dropwise, and the solution was stirred for a further 30-45 min at -78°C. The cooling bath was then replaced by an ice-brine bath, and a solution of compound **4a** (510 mg, 0.81 mmol, 1 eq) in dryf THF (3 mL) was added, followed by DCI (364 mg, 3.1 mmol, 3.8 eq). Reaction mixture was left to reach RT overnight. The suspension was then cooled in an ice-brine bath. mCPBA (77 %, 697 mg, 3.1 mmol, 3.8 eq) was carefully added portionwise. The resulting yellow solution was stirred 5 min, then the cooling bath was removed and RM was stirred for a further 15 min. A saturated aqueous NH_4Cl solution (30 mL) and water (30 mL) were added at 0°C, and the resulting solution was extracted with EA (2 x 90 mL). Combined organic layers were washed once with brine (75 mL), dried over Na_2SO_4, filtered and concentrated. Purification by silica gel flash chromatography (40 to 100% EA in hexane) yielded compound **5a** (554 mg, 0.524 mmol, 34%).

Method 2: Synthesis using isolated PCP-amidite **3b**

Compound **5a** was synthesized following General procedure B from inositol derivative **4a** (33 mg, 0.052 mmol, 1 eq.) and PCP-amidite **3b** (42 mg, 0.083 mmol, 1.6 eq) in presence of DCI (20 mg, 0.165 mmol) in dry CAN (5 mL), followed by oxidation with mCPBA (77%, 18 mg, 0.081 mmol, 1.6 eq). The compound was purified by silica gel flash chromatography (50 to 100% ethyl acetate in hexane) to yield compound **5a** (8 mg, 0.008 mmol, 14%) of the desired compound as an oil.

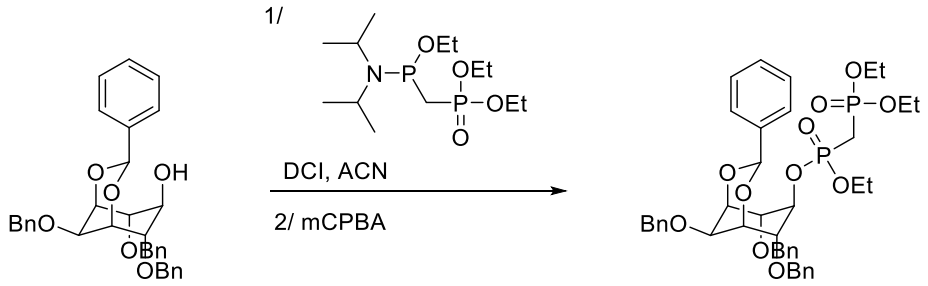
An improved synthesis of this compound is reported elsewhere.[11] NMR characterization was consistent with this previous report.

^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 – 7.99 (m, 0.5H), 7.60 – 7.51 (m, 2H), 7.47 – 7.30 (m, 6H), 7.30 – 7.18 (m, 15.5H), 7.18 – 7.10 (m, 2H), 6.94 – 6.85 (m, 2H), 6.80 – 6.67 (m, 4H), 5.75 (s, 1H), 5.11 – 4.82 (m, 6H), 4.68 – 4.59 (m, 5H), 4.52 – 4.39 (m, 4H), 4.16 (t, *J* = 8.7 Hz, 2H), 3.79 (s, 3H), 3.71 (s, 3H), 3.70 (s, 3H), 3.63 (t, *J* = 2.4 Hz, 1H), 2.59 (t, *J* = 21.5 Hz, 2H).

^{13}C NMR (75 MHz, $CDCl_3$) δ 159.38, 159.36, 159.30, 137.84, 136.01, 135.93, 135.88, 135.83, 135.79, 129.75, 129.59, 129.49, 129.45, 129.05, 128.91, 128.57, 128.55, 128.50, 128.45, 128.43, 128.25, 128.03, 127.85, 126.59, 113.96, 113.91, 113.81, 92.82, 80.08, 79.64, 79.59, 78.37, 78.27, 77.30, 73.18, 73.04, 70.74, 70.66, 70.60, 68.26, 68.17, 67.77, 67.69, 67.65, 67.57, 55.28, 55.22, 55.20, 26.22 (t, J = 137.4 Hz).

^{31}P NMR (122 MHz, $CDCl_3$) δ = 20.24 (d, *J*=9.1 Hz, 1P), 19.63 (d, *J*=9.1 Hz, 1P).

*Synthesis of **5b***



4b **5b**

Compound **5b** was synthesized according to General procedure B from inositol derivative **4b** (1.30 g, 2.41 mmol, 1 eq.) and PCP-amidite **3b** (1.58 g, 4.83 mmol, 2 eq). The compound was purified by silica gel flash chromatography (0 to 100% ethyl acetate in hexane) to yield compound **5b** (1.83 g, 2.34 mmol, 97%) of the desired compound as an oil.

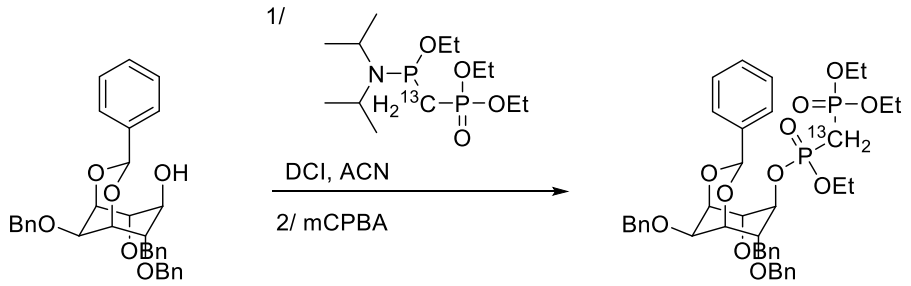
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.61 – 7.54 (m, 2H), 7.47 – 7.24 (m, 18H), 5.83 (s, 1H), 4.83 – 4.56 (m, 7H), 4.44 (d, *J* = 2.3 Hz, 2H), 4.24 (dd, *J* = 14.8, 8.2 Hz, 2H), 4.18 – 4.05 (m, 3H), 4.05 – 3.92 (m, 3H), 3.68 (t, *J* = 2.4 Hz, 1H), 2.43 (t, *J* = 21.3 Hz, 2H), 1.30 (t, *J* = 7.2 Hz, 4H), 1.25 (t, *J* = 7.0 Hz, 3H), 1.17 (t, *J* = 7.1 Hz, 3H).

^{31}P NMR (122 MHz, $CDCl_3$) δ 19.27 (d, *J* = 8.7 Hz), 19.02 (d, *J* = 8.6 Hz).

^{13}C NMR (75 MHz, $CDCl_3$) δ 137.88, 137.76 (1 × C), 137.16, 137.14 (1 × C), 129.57 (1 × C), 128.60, 128.56, 128.52, 128.50 (9 × C), 128.11, 127.96, 127.94, 127.86, 127.80, 127.73, 127.70 (10 × C), 126.66 (2 × C), 93.08 (1 × C), 80.77, 80.73, 80.61, 80.56 (2 × C), 78.12, 78.03 (1 × C), 73.21 (1 × C), 73.00 (1 × C), 71.26 (1 × C), 71.06 (2 × C), 67.97 (1 × C), 62.81, 62.74, 62.73, 62.66 (2 × C), 62.26, 62.18 (1 × C), 27.61, 25.81, 25.77, 23.97 (1 × C, (dd, *J* = 138.8, 136.0 Hz), 16.48, 16.42, 16.40, 16.33 (2 × C), 16.21, 16.11 (1 × C).

HRMS (ESI): calculated for $C_{41}H_{50}O_{11}P_2Na^+$: 803.2721 $[M+Na]^+$, found 803.2758.

*Synthesis of **S2***



4b **S2**

Compound **S2** was synthesized according to General procedure B from inositol derivative **4b** (54.3 mg, 0.101 mmol, 1 eq.) and PCP-amidite **S1** (66.3 mg, 0.202 mmol, 2 eq). The compound was purified by silica gel flash chromatography (0 to 100% ethyl acetate in hexane) to yield 40.9 mg (0.077 mmol, 76%) of the desired compound as an oil.

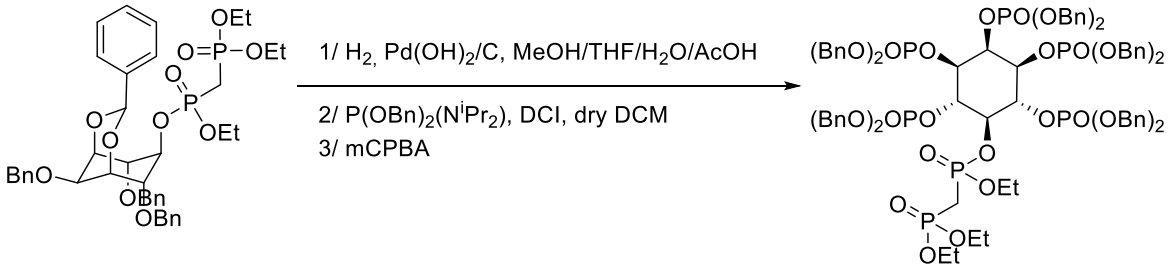
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.64 – 7.52 (m, 2H), 7.48 – 7.23 (m, 18H), 5.83 (s, 1H), 4.86 – 4.53 (m, 7H), 4.44 (d, *J* = 2.3 Hz, 2H), 4.24 (dd, *J* = 14.8, 8.2 Hz, 2H), 4.11 (m, *J* = 7.2, 3.2 Hz, 3H), 4.05 – 3.92 (m, 3H), 3.68 (t, *J* = 2.4 Hz, 1H), 2.43 (dt, *J* = 124.9, 21.3 Hz, 2H), 1.30 (t, *J* = 7.9 Hz, 3H), 1.25 (t, *J* = 7.8 Hz, 3H), 1.17 (t, *J* = 7.1 Hz, 3H).

^{31}P NMR (122 MHz, $CDCl_3$) δ 19.27 (dd, *J* = 138.8, 8.5 Hz), 19.01 (dd, *J* = 136.0, 8.6 Hz).

^{13}C NMR (75 MHz, $CDCl_3$) δ 137.88, 137.76 (1 × C), 137.17, 137.14(1 × C), 129.56 (1 × C), 128.60, 128.56, 128.51, 128.50 (9 × C), 127.96, 127.94, 127.91, 127.86, 127.80, 127.70 (10 × C), 126.66 (2 × C), 93.08 (1 × C), 80.77, 80.73, 80.61, 80.56 (2 × C), 78.12, 78.02 (1 × C), 73.20 (1 × C), 73.00 (1 × C), 71.26 (1 × C), 71.05 (2 × C), 67.97 (1 × C), 62.80, 62.74, 62.72, 62.65 (2 × C), 62.25, 62.17 (1 × C), 61.32, 61.23 (imp), 59.05, 58.96 (imp), 25.79 (^{13}C, dd, J = 138.8, 135.9 Hz), 16.48, 16.42, 16.40, 16.33 (2 × C), 16.21, 16.11 (1 × C).

HRMS (ESI): calculated for $C_{40}\{^{13}C\}H_{50}O_{11}P_2Na^+$ 804.2754 $[M+Na]^+$, found 804.2796.

*Synthesis of **S3***



5b **S3**

Compound **S3** was obtained following general procedure C from inositol derivative **5b** (1.83 g, 2.34 mmol, 1 eq), yielding the desired compound as an oil (2.14 g, 1.24 mmol, 58% over three steps and after isolation).

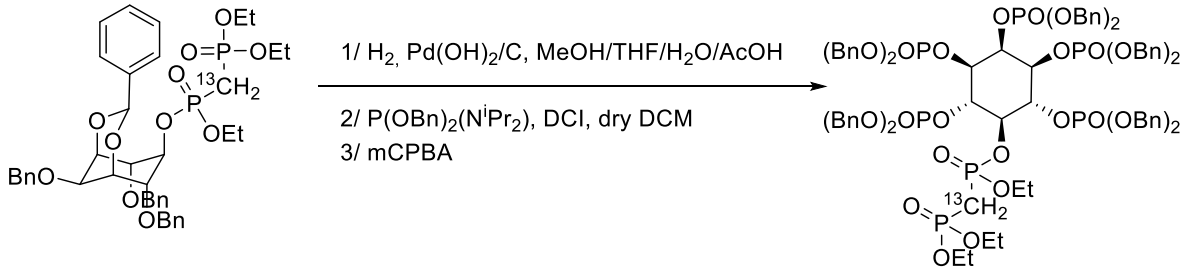
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.40 – 6.99 (m, 50H, P(O)(OCH_2-C_6H_5)$_2$), 5.64 (dt, *J* = 9.3, 2.4 Hz, 1H, 2-*CH-Ins*), 5.37 – 4.72 (m, 22H, OCH_2-C_6H_5 + 2 C*H*-Ins), 4.52 (dd, *J* = 20.5, 9.5 Hz, 1H, 5-C*H*-Ins), 4.43 – 4.16 (m, 4H, OCH_2CH_3), 4.15 – 3.95 (m, 4H, OCH_2CH_3 + 2 C*H*-Ins), 2.79 (dddd, *J* = 116.3, 22.8, 20.8, 15.5 Hz, 2H, PCH_2P), 1.44 – 1.01 (m, 9H, OCH_2CH_3).

^{31}P NMR (122 MHz, $CDCl_3$) δ 21.64, 19.67, -0.63, -0.81, -1.51, -1.63, -2.45.

^{13}C NMR (75 MHz, $CDCl_3$) δ 135.87, 135.78, 135.70, 135.66, 135.61, 128.52, 128.51, 128.47, 128.46, 128.43, 128.41, 128.39, 128.34, 128.25, 128.21, 128.19, 128.16, 128.12, 128.10, 128.07, 127.90, 127.84, 70.08, 69.86, 69.79, 69.71, 64.29, 62.61, 62.53, 62.18, 62.09, 16.34, 16.25.

HRMS (ESI): calculated for $C_{83}H_{93}O_{26}P_7Na_2^{2+}$ 884.1951 $[M+2Na]^{2+}$, found 884.2012.

*Synthesis of **S4***



S2 **S4**

Compound **S4** was obtained following general procedure C from inositol derivative **S3** (55.8 mg, 0.071 mmol, 1 eq.). The crude was purified by flash column chromatography (0 to 100% EA in hexane, then isocratic 100 % EA). The resulting oil was further purified by reversed phase HPLC (70 to 100% MeCN in H_2O), yielding the desired compound as an oil (63.1 mg, 0.037 mmol, 51% over three steps and after isolation).

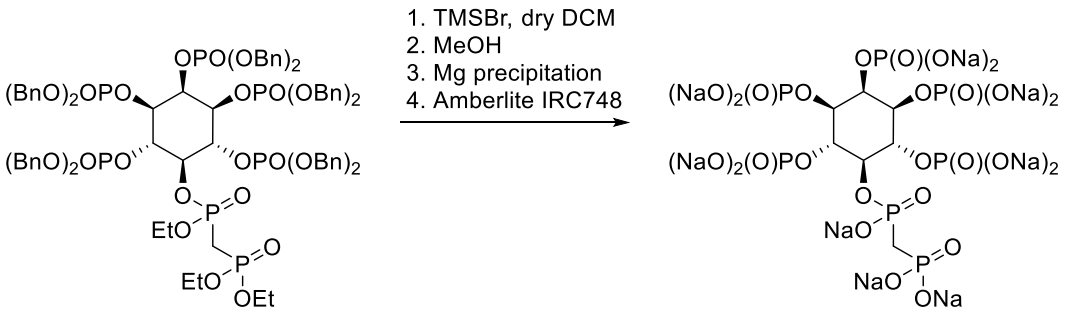
^{1}H NMR (300 MHz, $CDCl_3$) δ 7.66 – 6.81 (m, 50H), 5.64 (dt, J = 9.2, 2.4 Hz, 1H), 5.44 – 4.69 (m, 22H), 4.59 – 4.46 (m, 1H), 4.43 – 3.94 (m, 8H), 3.34 – 2.25 (m, 2H), 1.40 – 1.05 (m, 9H).

^{31}P NMR (122 MHz, $CDCl_3$) δ 21.63 (d, J 139.2), 19.69 (d, J 136.2), -0.64, -0.81, -1.47, -1.58, -2.46.

^{13}C NMR (75 MHz, $CDCl_3$) δ 135.87, 135.79, 135.71, 135.67, 135.62, 128.52, 128.51, 128.47, 128.46, 128.43, 128.41, 128.39, 128.37, 128.33, 128.24, 128.21, 128.19, 128.16, 128.12, 128.11, 128.07, 127.90, 127.84, 70.08, 69.87, 69.79, 69.71, 62.61, 62.53, 62.17, 62.09, 25.51 (dd, *J* = 139.1, 136.2 Hz, ^{13}C), 16.35, 16.28, 16.23, 16.19.

HRMS (ESI): calculated for $C_{82}\{^{13}C\}H_{93}O_{26}P_7Na_2^{2+}$ 884.6968 $[M+2Na]^{2+}$, found 884.7035.

*Synthesis of **1***

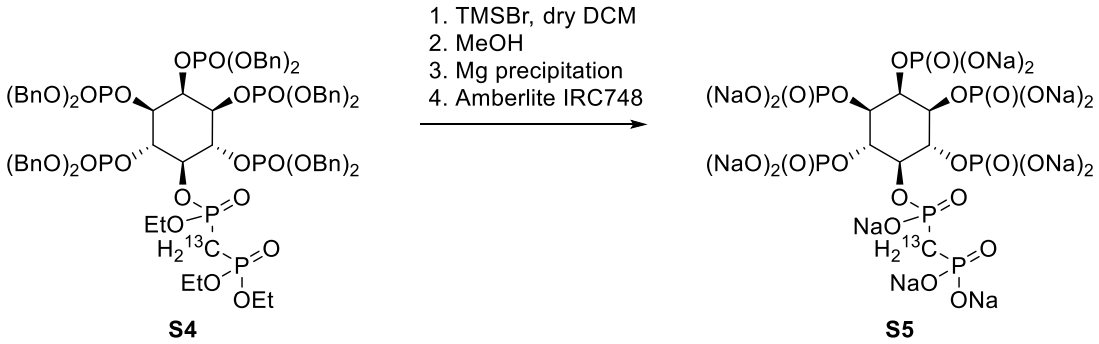


S3 **1**

Compound **1** was obtained by submitting compound **S3** (300 mg, 0.174 mmol, 1 eq) to TMSBr (4 mL) and following general procedure D, affording the desired Na-salt of **1** as a white solid (136 mg, 0.133 mmol, 76%).

NMR characterization was found consistent with previously reported data.[12]

*Synthesis of **S5***



S4 **S5**

Compound **S5** was obtained from **S4** (37 mg, 22 µmol, 1 eq) following general procedure D. It was obtained as a white solid (15 mg, 14 µmol, 67%).

^{1}H NMR (300 MHz, D_2O) δ 4.51 (d, *J* = 11.4 Hz, 1H), 4.38 (d, *J* = 10.2 Hz, 2H), 2.11 (dt, *J* = 121.2, 19.3 Hz, 2H).

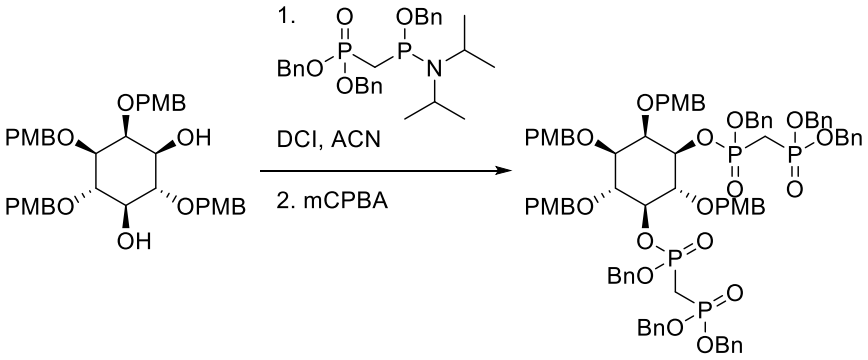
^{31}P NMR (122 MHz, D_2O) δ 22.99 (d, 1P, *J* = 121.5 Hz), 13.10 (d, 1P, *J* = 115.5 Hz), 5.19 (1P), 3.95 (4P).

^{13}C NMR (75 MHz, D_2O) δ 27.22 (t, *J* = 119.4 Hz).

2.5 Synthesis of 1,5-$(PCP)_2$-$InsP_4$

The racemic diol **7a**[12] enantiopure diol **7b**[2] were synthesized according to published procedures.

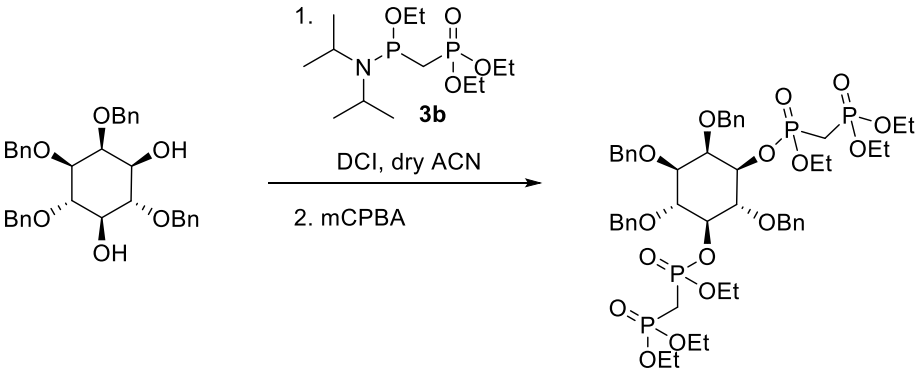
*Synthesis of compound **7a***



7a

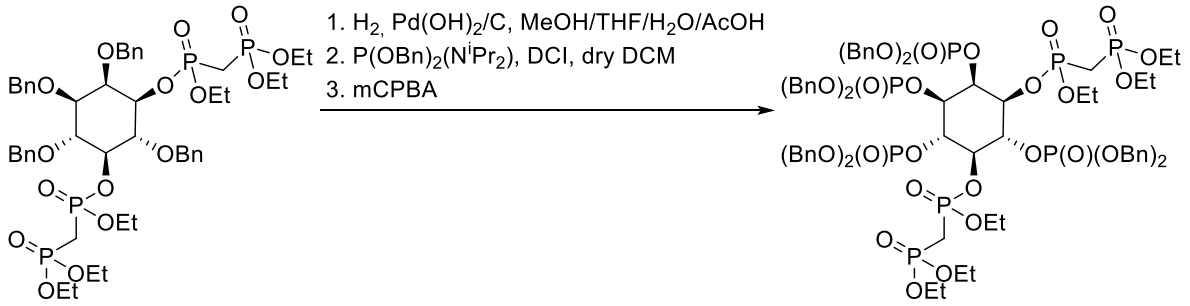
The racemic diol (52 mg, 80 µmol, 1 eq) was reacted with PCP-amidite **3a** (162 mg, 0.32 mmol, 4 eq) following General procedure B. The crude mixture contained a mixture of starting inositol derivative, mono-functionalized Ins derivative and a minority of desired product, as seen by mass spectrometry. The crude was thus not further purified.

*Synthesis of compound **7b***



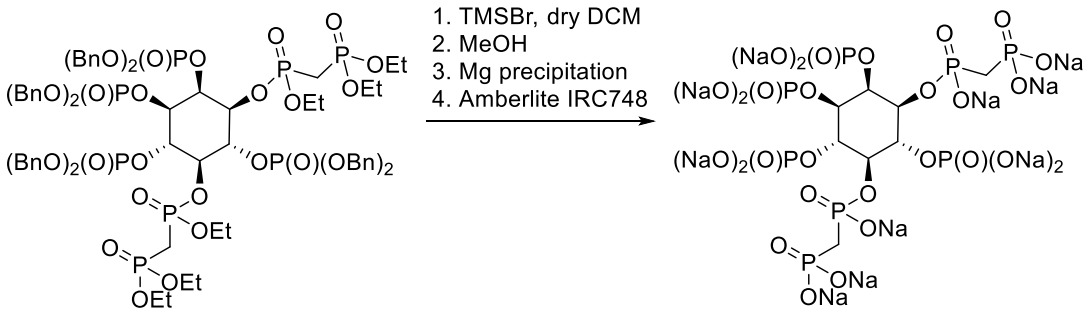
The enantiopure diol[2] (1.21 g, 2.24 mmol, 1 eq.) was evaporated with 2 mL dry acetonitrile and dried under high vacuum for 30 min. It was then dissolved in dry acetonitrile (80 mL) under a dry N_2 atmosphere, and cooled in an ice-brine bath. A solution of phosphoramidite **3b** (2.49 g, 7.61 mmol, 3.4 eq.) evaporated with dry acetonitrile and dried under high vacuum beforehand) in dry acetonitrile (30 mL) was then added. DCI (1.8 g, 15.2 mmol, 6.8 eq.) was finally added, and the mixture was stirred overnight, during which time it reached RT. It was then cooled in an ice-brine bath, and mCPBA (77%, 2.0 g, 8.96 mmol, 4 eq.) was **carefully** added portionwise. After 15 min, the cooling bath was removed an RM was stirred for a further 30 min at RT. 10% aq. $Na_2S_2O_3$ (110 mL) were added to RM, and the mixture was extracted with EA (3x 110 mL). Combined organic layers were then washed with sat. aq. $NaHCO_3$ (110 mL) and brine (110 mL), then dried over Na_2SO_4, filtered and concentrated by rotary evaporation. This crude material was purified by flash column chromatography (0 to 10 % MeOH in EA), followed by a second purification by silica gel flash chromatography (0-10% MeOH in DCM) to yield compound **7b** (2.17 g, 2.11 mmol, 95%). CHaracerization by NMR was consistent with reported values.[2]

*Synthesis of compound **S6***



Compound **S6** was obtained by adapting slightly a published procedure.[2] Compound **7b** (1.73 g, 1.65 mmol, 1 eq.) was dissolved in a mixture of MeOH (65 mL), THF (65 mL) and water (1 mL) under N_2. AcOH (1.2 mL) was added, followed by $Pd(OH)_2/C$ (20% wt loading, 50% water, 2.3 g). The RBF was purged with H_2 and stirred for 4 days at RT. The reaction mixture was then filtered over a celite plug. Filtrate was collected and solvents were removed by rotary evaporation. The residue was evaporated once with dry acetonitrile (10 mL) and dried under high vacuum for 30 min. It was then suspended in dry DCM (70 mL) under a dry N_2 atmosphere. The solution was cooled in an ice bath, and DCI (3.33 g, 28.2 mmol, 17 eq.) was added, followed by dibenzyl diisopropylphosphoramidite (4.6 mL, 13.8 mmol, 8.3 eq.). The ice bath was removed, and the reaction mixture was stirred overnight at room temperature. The fine suspension was then cooled in an ice-brine bath, and mCPBA (77%, 4.87 g, 18.2 mmol, 17 eq.) was added **portionwise** over 30 min. After 1 h, the cooling bath was removed and the mixture was stirred for a further 1h30. The reaction mixture was then diluted with EA (200 mL) and washed with 10% aq. $Na_2S_2O_3$ (100 mL), sat. aq. $NaHCO_3$ (100 mL) and brine (100 mL). The organic phase was dried over Na_2SO_4, filtered and concentrated to an oil. The resulting crude was purified by flash column chromatography (0 to 10% MeOH in DCM). The resulting oil was further purified by reverse-phase preparative HPLC (70-95% MeCN in H_2O), yielding the desired pure compound as an oil (1.03 g, 0.6 mmol, 36% over three steps and after isolation).

*Synthesis of **2***



Compound **2** was obtained by submitting compound **S6** (500 mg, 0.293 mmol, 1 eq.) to TMSBr (7.3 mL) and following general procedure D, affording the desired Na-salt of **2** as a white solid (294 mg, 0.261 mmol, 89%).

NMR characterization was found consistent with previously reported data.[12]

3 Biochemical assays

Protein purification

Wild-type IDE was expressed in *E. coli* from pProEx-IDE-wt plasmid and purified according to a reported procedure.[13]

The Dnp-labeled amino acid and fluorogenic peptide were synthesized according to published procedures.[14]

Data processing

Kinetic data were processed and fitted using GraphPad Prism software, which was also used for statistical analysis.

Activity assay using a fluorogenic peptide[14,15]

Fluorogenic assays were performed at 37°C in 50 mM Tris buffer pH 7.5 and 50 mM NaCl (to maintain ionic strength), containing 0.5 % DMSO. Divalent cations were not included in the reaction buffer, since they were reported to affect activation of IDE by anionic ligands.[16] 2X solutions of IDE and InsP on the one hand, and peptide on the other hand, were incubated at 37°C 15 min prior to experiment start. Reaction was started by adding 50 µL of 2X IDE+InsP solution to 50 µL of peptide solution. Cleavage of the fluorogenic peptide was followed by recording fluorescence signal (ex: 320 nm; em: 420 nm) in a 96-well low-binding plates using a Tecan Safire plate reader. Final concentrations: 0.05 µg/mL IDE (0.005µg/well), 5 µM InsP if present, 0-20 µM peptide. Calibration curves for cleaved peptide were obtained by measuring fluorescence signal of reaction mixtures at various peptide concentrations after complete cleavage.

HPLC assay

The cleavage of angiotensin and bradykinin substrates was followed by measuring the disappearance of the parent peptide by HPLC, by adapting a reported procedure. [15] Reaction conditions: 5 µg/mL IDE, 5 µM InsP if present, 10 µM peptide, 50 mM NaCl, 50 mM Tris buffer pH 7.5, 37°C. Reaction was started by mixing equal volumes of a 2X IDE+InsP solution and a 2X peptide solution, equilibrated at 37°C for 15 min prior to mixing. 300 µL aliquots were incubated at 37°C with gentle shaking, and quenched by addition of 30 µL 5% aq TFA at given timepoints. 75 µL were injected in triplicate onto a Vydac C18 column. Peptides were eluted with a linear gradient of 5–50% acetonitrile, and quantified by measuring peak areas. All experiments were performed at least in triplicates.

Activation constants

Activation constants were measured following the procedure of fluorogenic activity assay.

Conditions: 0.002 mg/mL IDE (0.2 µg/well), 0-20 µM InsP, 50 mM Tris buffer pH 7.5, 50 mM NaCl, 0.5%DMSO, 10 µM fluorogenic peptide, final volume: 100 µL.

Data were fitted using Michaleis-Menten model in GraphPad Prism software.

4 Hydrogen-deuterium Exchange (HDX) experiments

Materials and methods

HDX reactions were conducted on apo IDE, IDE in the presence of the IP6 or PCP7 probe, IDE in the presence of bradykinin substrate, or IDE in the presence of bradykinin and either probe. There were a total of 6 sample types. Protein, substrate, and probe (or buffer blanks) were mixed together for a starting concentration of 10 uM IDE, 200 uM bradykinin and 100 uM probe. Of this, 2 μL was incubated at RT for 20 minutes before initiation of deuterium exchange reactions with 18 μL of 97% deuterium buffer (50 mM Tris 7.5, 50 mM NaCl). Final concentrations in the deuterium reactions was 1 uM of IDE, 10 uM of bradykinin, 20 uM of probe, and 84.9% deuterium. The different sample conditions were completed for 4 time points (3 s, 30 s, 300 s, 3000 s) in duplicate, at 23C. Reactions were quenched with the addition of 50 μL quench buffer (final concentration 0.6 M guanidine-HCl, 0.86% formic acid). Samples were snap frozen in liquid nitrogen and stored at -80 until mass analysis.

Measurement of Deuterium Incorporation

Samples were rapidly thawed and injected onto an ultra-performance liquid chromatography (UPLC) at 4 C. The protein was run over an immobilized pepsin column (Trajan, ProDx Pepsin Column), and the peptides were collected onto a precolumn trap (Trajan, ProDx Trap column). The trap was eluted in line with a Hypersil GOLD analytical HPLC column (Thermo Scientific, 25002-051030, 50 X 1 mm C18, Particle size 1.9 uM) with a gradient of 5-36% buffer B over 16 mins (buffer A 0.1% formic acid, buffer B 90% acetonitrile). Mass spectrometry experiments were performed on an Orbitrap Elite (Thermo Scientific) acquiring over a mass range from 150-2000 m/z using an electrospray ionization source operated at a temperature of 220C and a spray voltage of 3.8 kV. Peptides from IDE were identified using data-dependent acquisition methods following tandem MS/MS experiments. MS/MS datasets were analysed using MaxQuant against a database of purified proteins and known contaminants.

Mass analysis of peptide centroids and measurement of deuterium incorporation

Deuterium incorporation of peptides was automatically calculated using HD-Examiner Software (Sierra Analytics). Peptides were manually inspected for correct parameters (charge state, retention time, isotopic distribution etc). Results are presented with a back exchange control of a fully deuterated IDE sample. Changes in any peptide at any time point greater than specified cut-offs (>6% deuterium incorporation and 0.4 Da between apo and different conditions with an unpaired student t-test of $p<0.05$) were considered significant. The raw data is presented in a separate file in Supporting Information with analysis statistics shown above in Table S1.

5 Molecular docking

Docking of the DFT-optimized ionized and non-ionized forms of $InsP_6$ and 5PCP-$InsP_5$ to the ATP binding site of IDE was performed with Glide implemented in Schrödinger 2019.3[17,18]. The structure of the protein containing the ATP ligand was extracted from the protein database (pdb:3tuv).[19] Missing loops and atoms were added with the Prime routine.[20,21] The centroid of ATP served as centre for defining the docking grid. The docking was performed using a box size of 33×33×33 $Å^3$ (inner box: 15×15×15 $Å^3$). All docking poses were calculated in the XP mode and scored and ranked according to the Glide score. During the docking the protein was kept rigid, while the ligands were flexible. Table S1 summarizes the ligands used for the docking approach.

6 Molecular dynamics simulations

Classical all-atom molecular dynamics (MD) simulations were performed for non-ionized $InsP_6$ and 5PCP-$InsP_5$ starting from the two energetically most favourable docking poses, *pose1* and *pose2.* For this, the protein was treated with the CHARMM36 force field.[22] Missing parameters for $InsP_6$ and 5PCP-$InsP_5$ were derived with the SwissParam server[23] and water was simulated with the TIP3P model.[24]

The simulations were run with the CPU version of Gromacs 2018.3.[25]. Preceding to the 100 ns long NPT (constant number of particles, pressure, and temperature) production runs, the models were energy minimized and thermally equilibrated in an NVT ensemble (constant number of particles, volume, and temperature). The production simulations were performed at 300 K and under a pressure of 1 bar. The isotropic and periodic cell was realized by a Parrinello-Rahman barostat.[26] Van der Waals and short-ranged electrostatic interactions were truncated at 1.2 nm. Long-ranged electrostatics were calculated with the Particle-Mesh Ewald summation.[27] The time step of 2 fs was enabled by constraining all bonds to hydrogen atoms with the Lincs algorithm.[28]

All simulations were repeated in triplicates using different Boltzmann distributed initial velocities.

7 Bibliography

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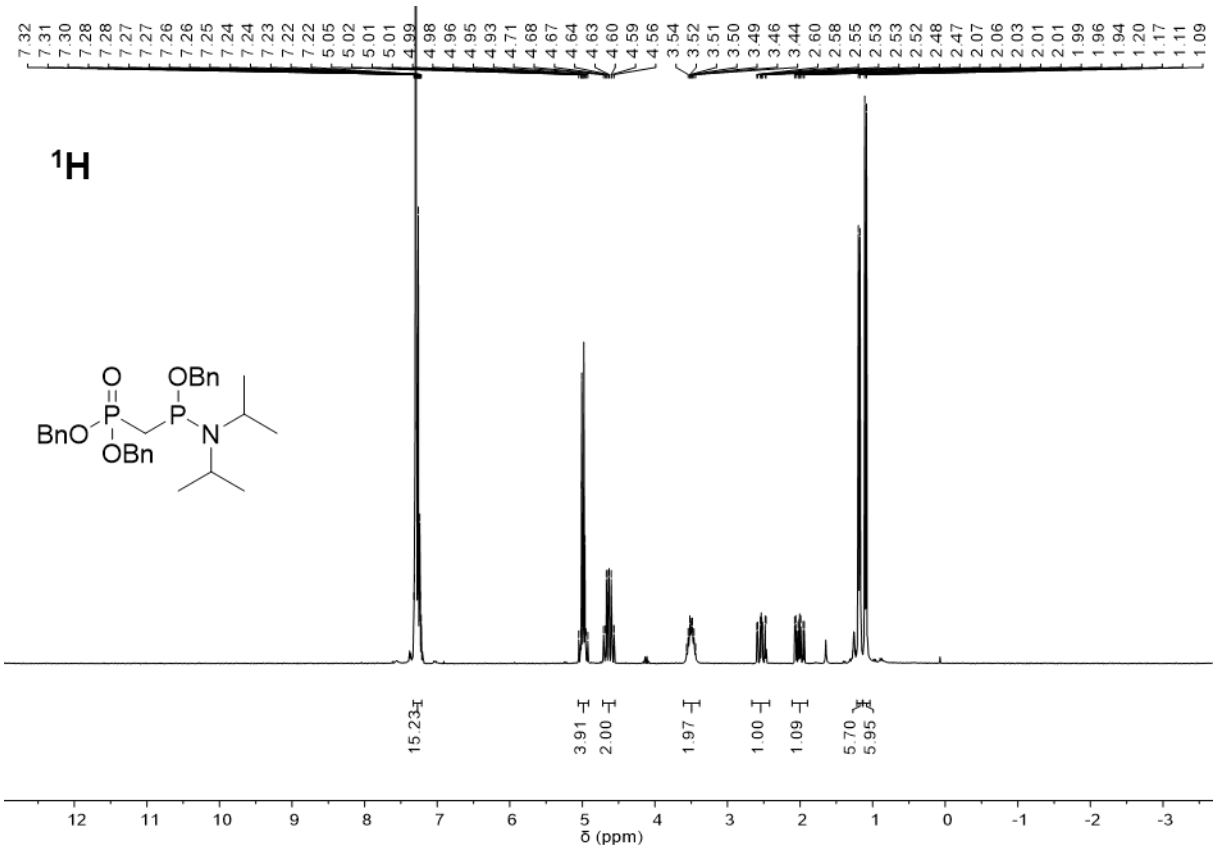
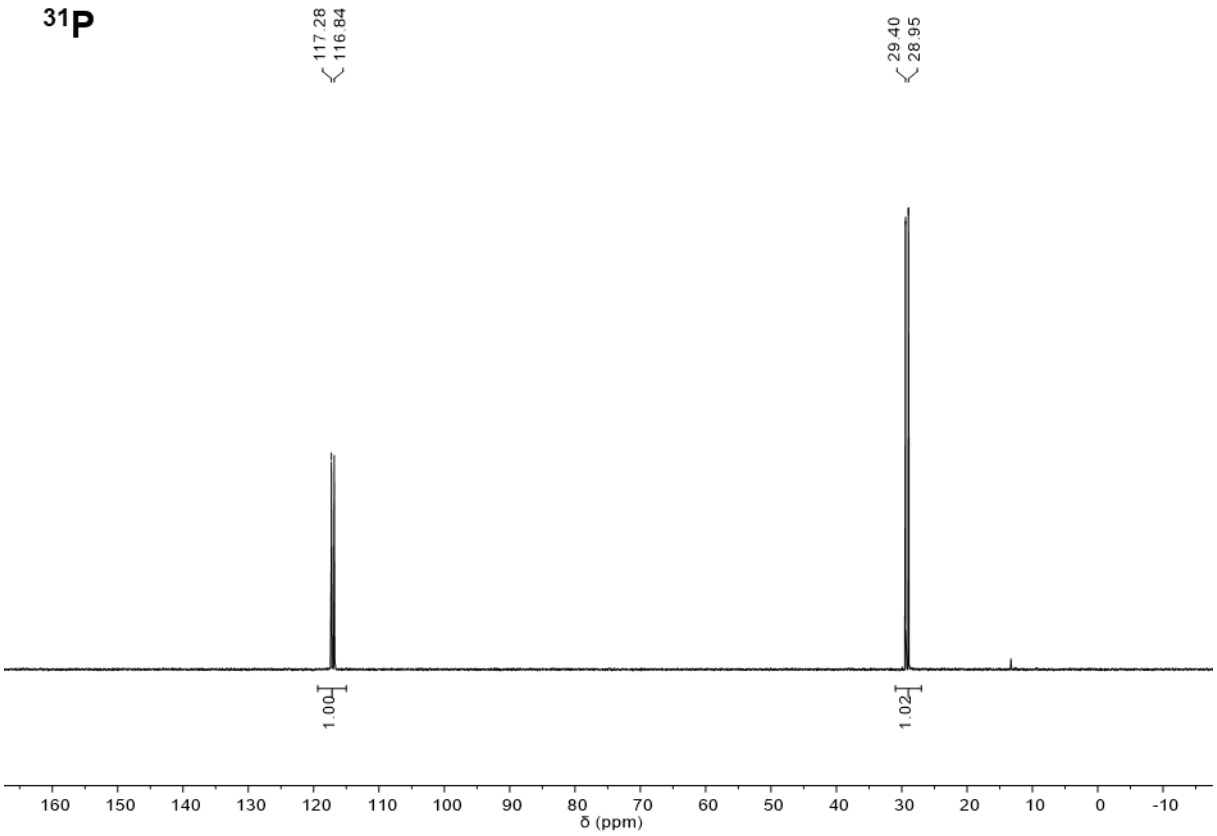
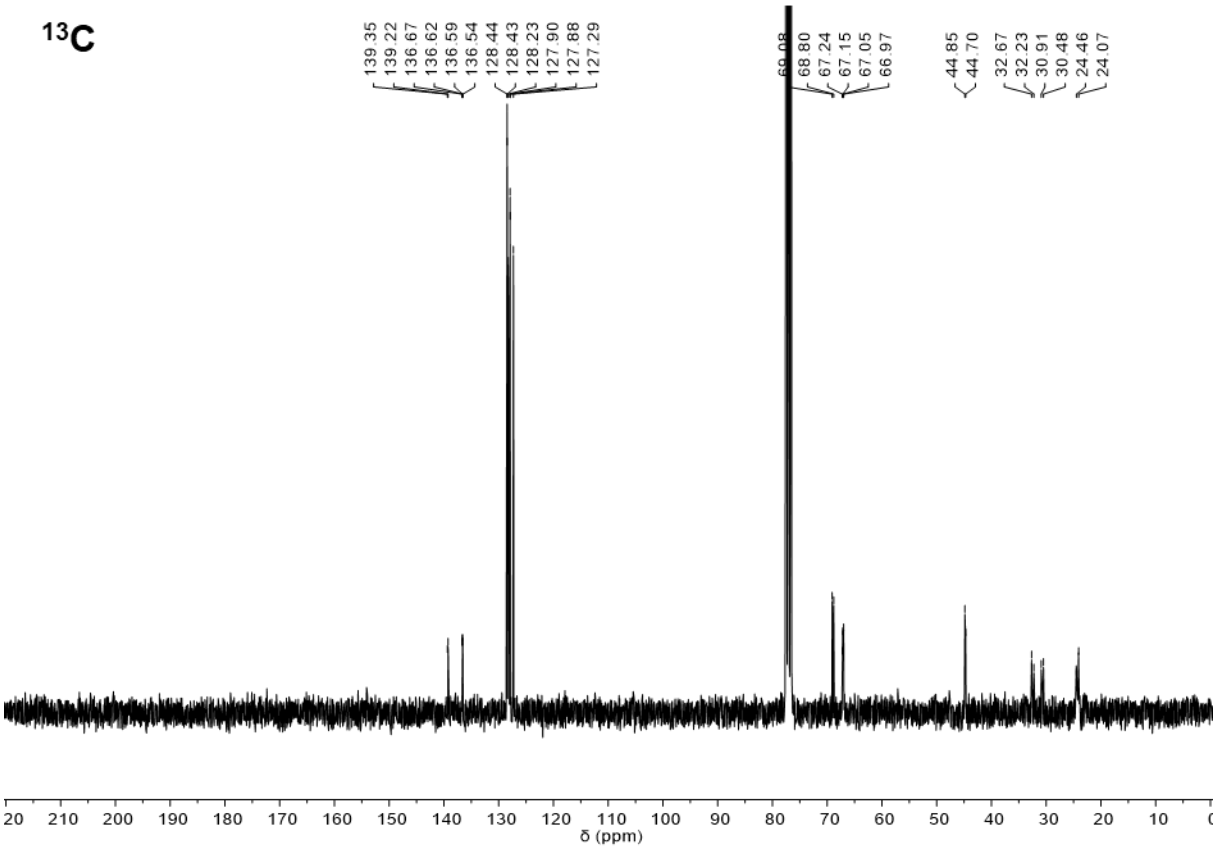
8 Appendix: NMR and HRMS Spectra

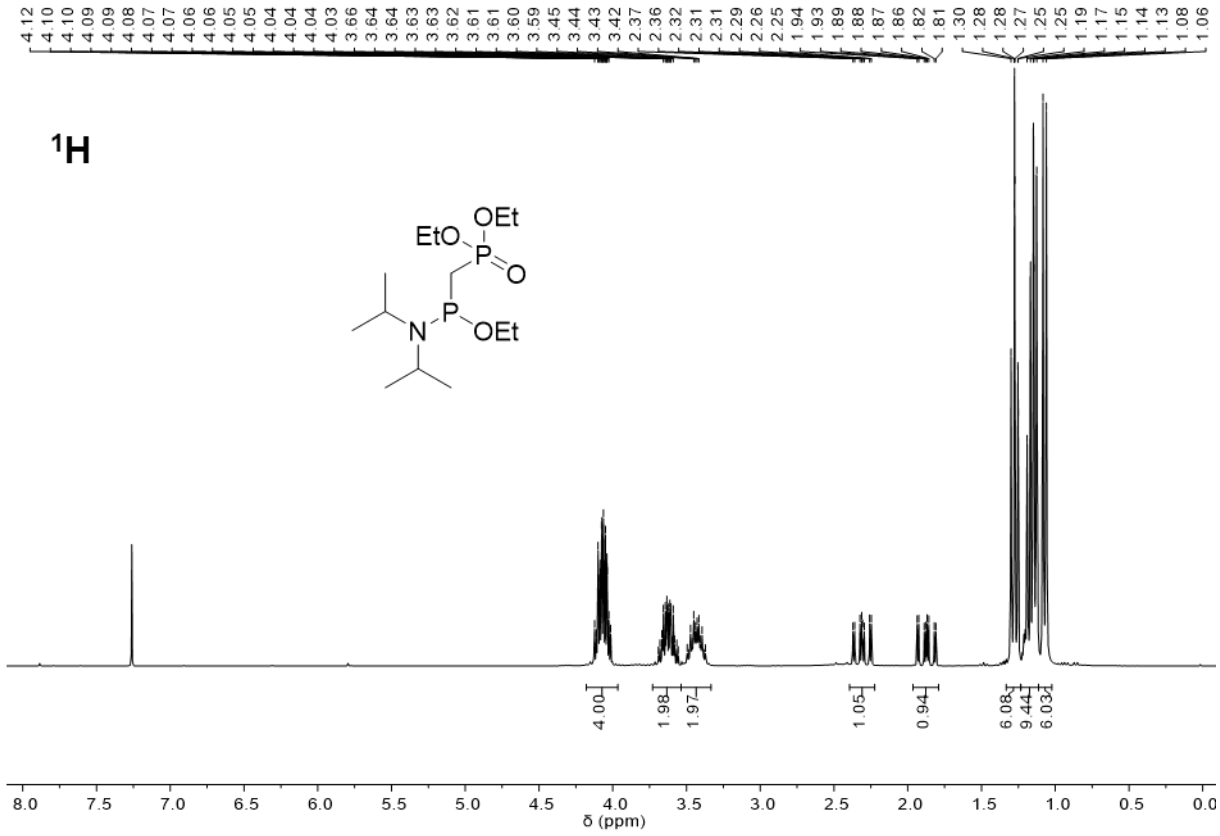
Compound 3a

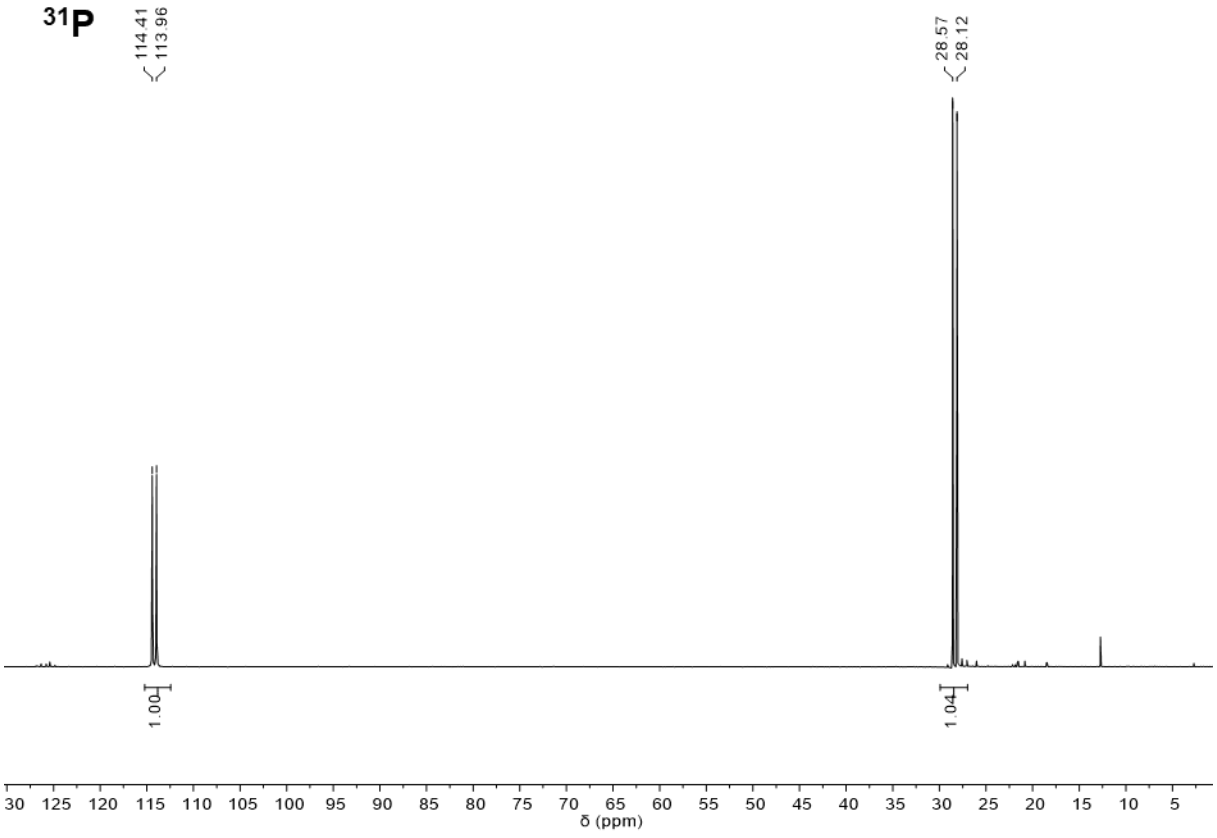


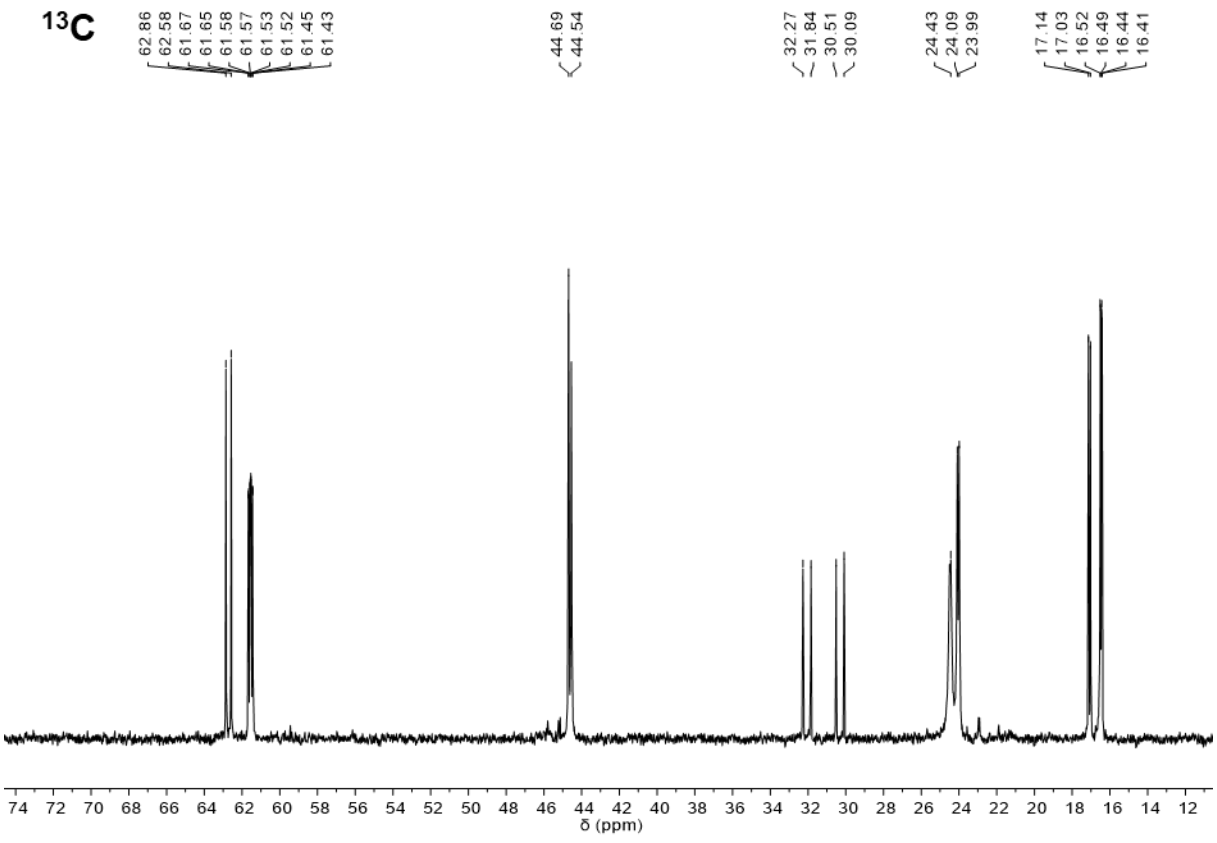



13C
139.35
139.22
136.67
136.62
136.59
136.54
128.44
128.43
128.23
127.90
127.88
127.29
69.08
68.80
67.24
67.15
67.05
66.97
44.85
44.70
32.67
32.23
30.91
30.48
24.46
24.07
δ (ppm)

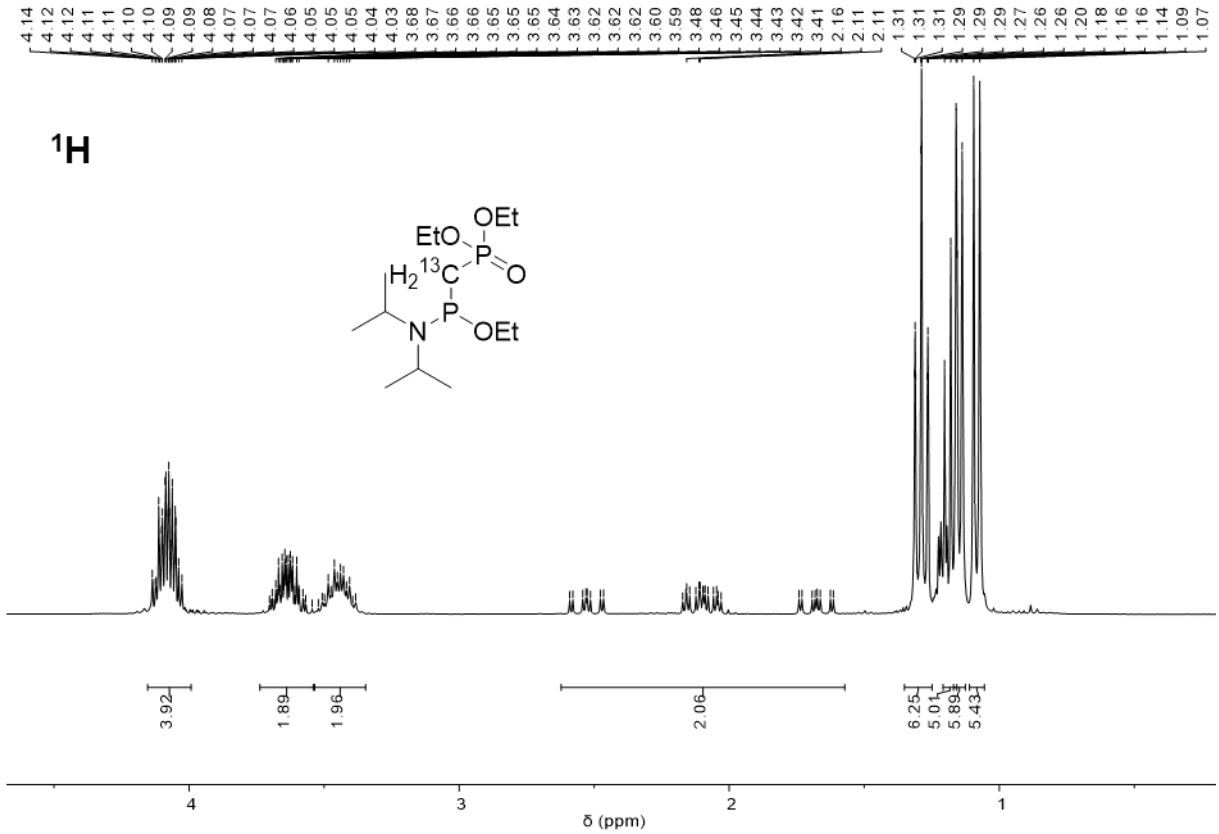
Compound 3b

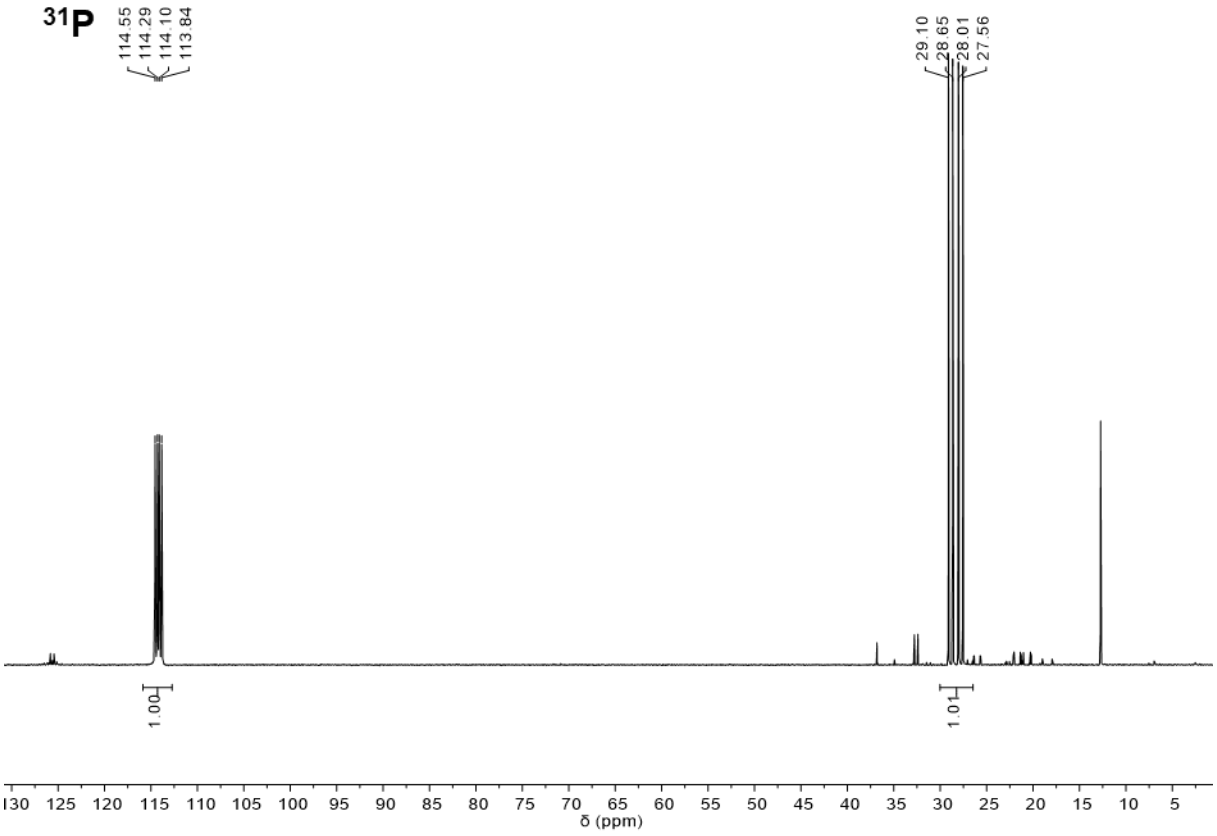

4.12 4.10 4.10 4.09 4.09 4.08 4.07 4.07 4.06 4.06 4.05 4.05 4.04 4.04 4.04 4.03 3.66 3.64 3.64 3.63 3.63 3.62 3.61 3.61 3.60 3.59 3.45 3.44 3.43 3.42 2.37 2.36 2.32 2.31 2.31 2.29 2.26 2.25 1.94 1.93 1.89 1.88 1.87 1.86 1.82 1.81 1.30 1.28 1.28 1.27 1.25 1.25 1.19 1.17 1.15 1.14 1.13 1.08 1.06
1H
EtO
OEt
P
O
N
P
OEt
4.00
1.98
1.97
1.05
0.94
6.08
9.44
6.03
8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
δ (ppm)

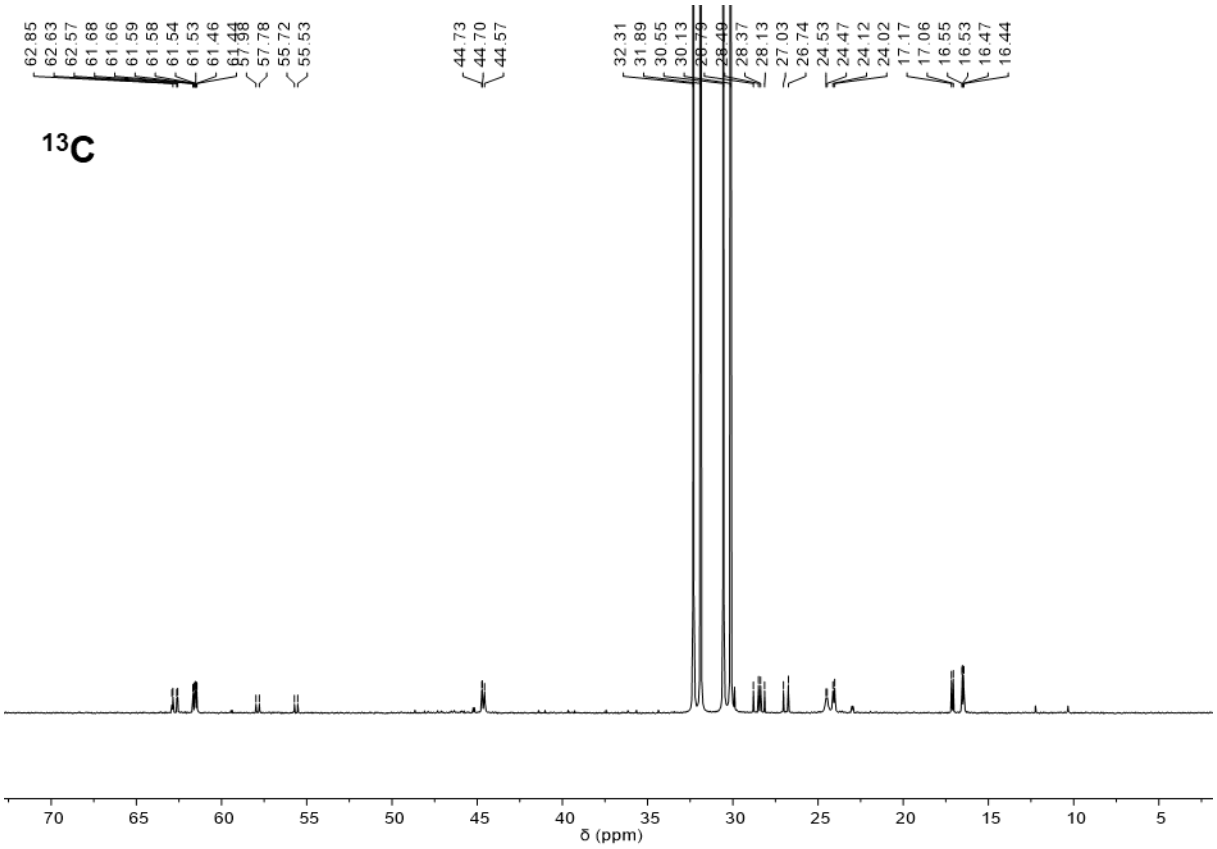

31P
114.41
113.96
28.57
28.12
1.00
1.04
30 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5
δ (ppm)


13C
62.86
62.58
61.67
61.65
61.58
61.57
61.53
61.52
61.45
61.43
44.69
44.54
32.27
31.84
30.51
30.09
24.43
24.09
23.99
17.14
17.03
16.52
16.49
16.44
16.41
74 72 70 68 66 64 62 60 58 56 54 52 50 48 46 44 42 40 38 36 34 32 30 28 26 24 22 20 18 16 14 12
δ (ppm)

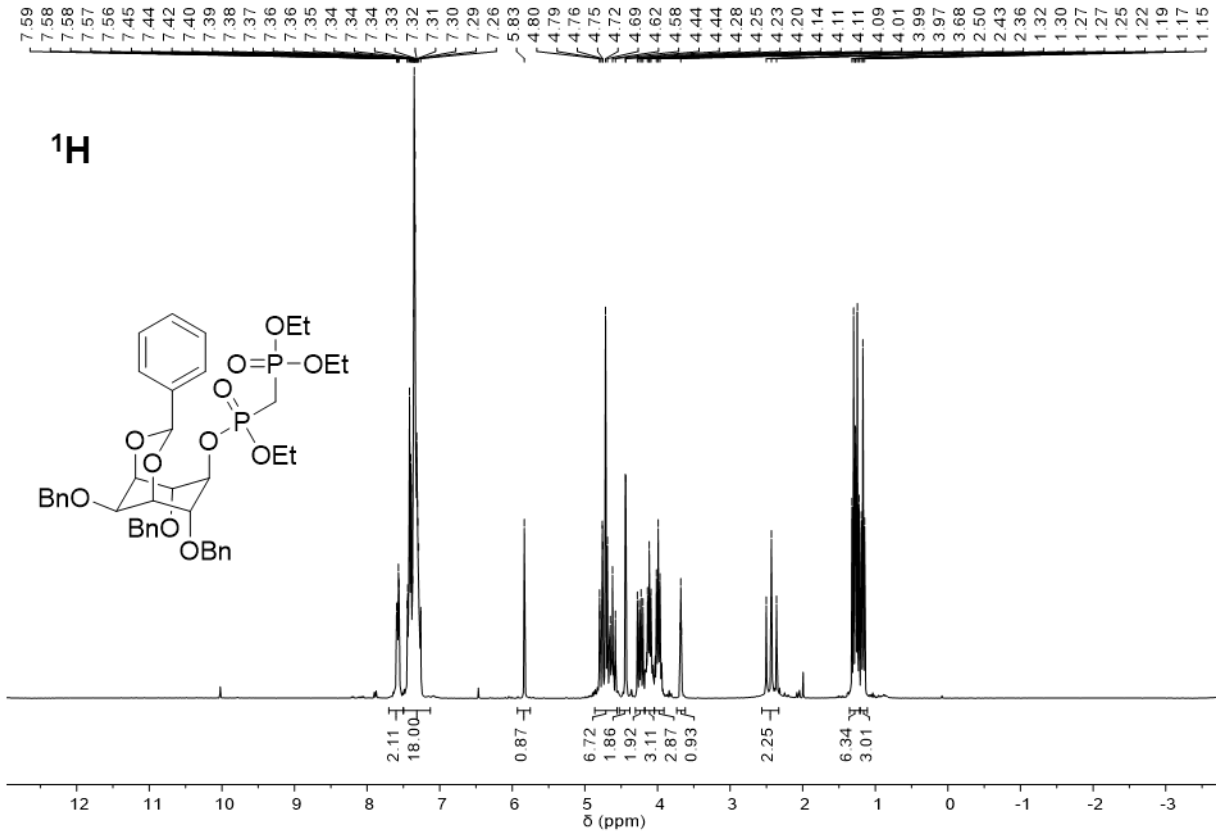
Compound S1

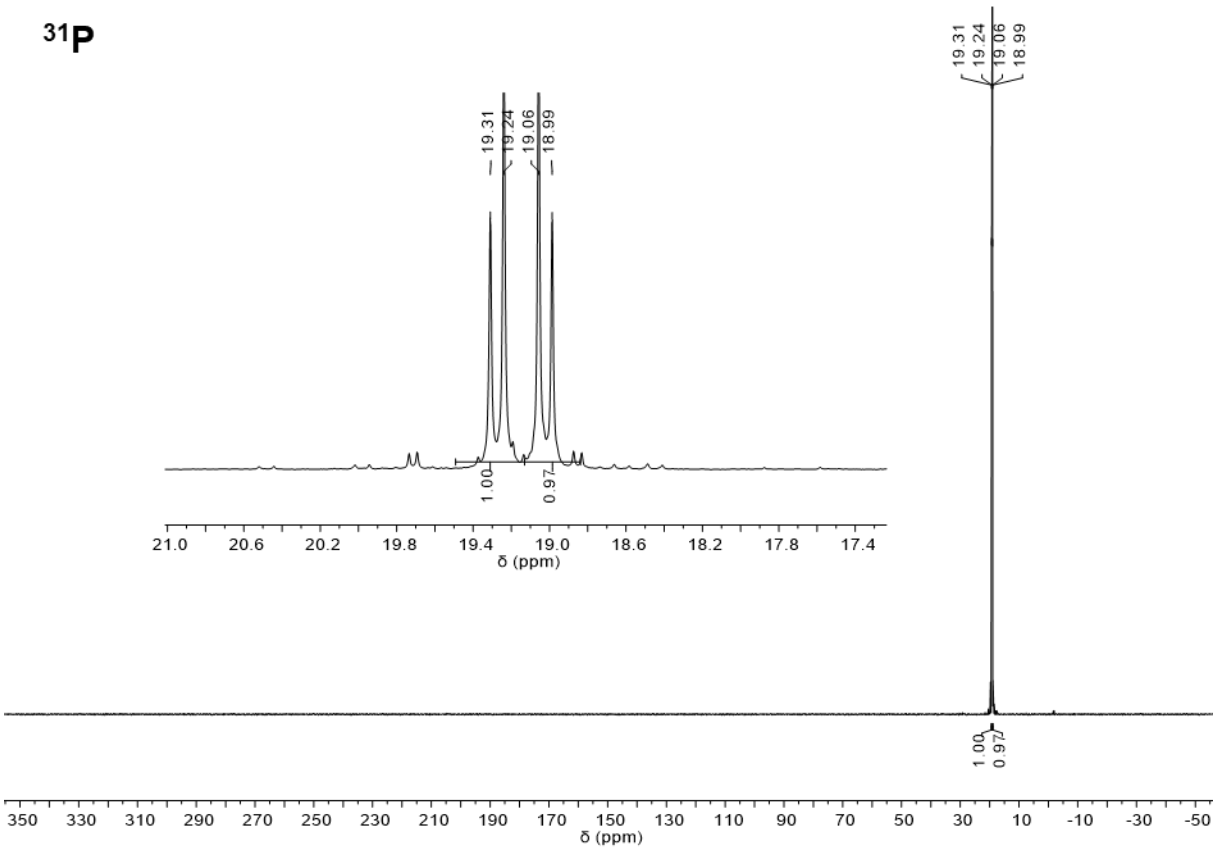

4.14 4.12 4.12 4.11 4.11 4.10 4.10 4.09 4.09 4.09 4.08 4.07 4.07 4.07 4.06 4.05 4.05 4.05 4.04 4.03 3.68 3.67 3.66 3.66 3.65 3.65 3.65 3.64 3.63 3.62 3.62 3.62 3.60 3.59 3.48 3.46 3.45 3.44 3.43 3.42 3.41 2.16 2.11 2.11 1.31 1.31 1.31 1.29 1.29 1.29 1.27 1.26 1.26 1.20 1.18 1.16 1.16 1.14 1.09 1.07
1H
EtO
OEt
P
O
H2 13C
P
N
OEt
3.92
1.89
1.96
2.06
6.25
5.01
5.89
5.43
4
3
2
1
δ (ppm)

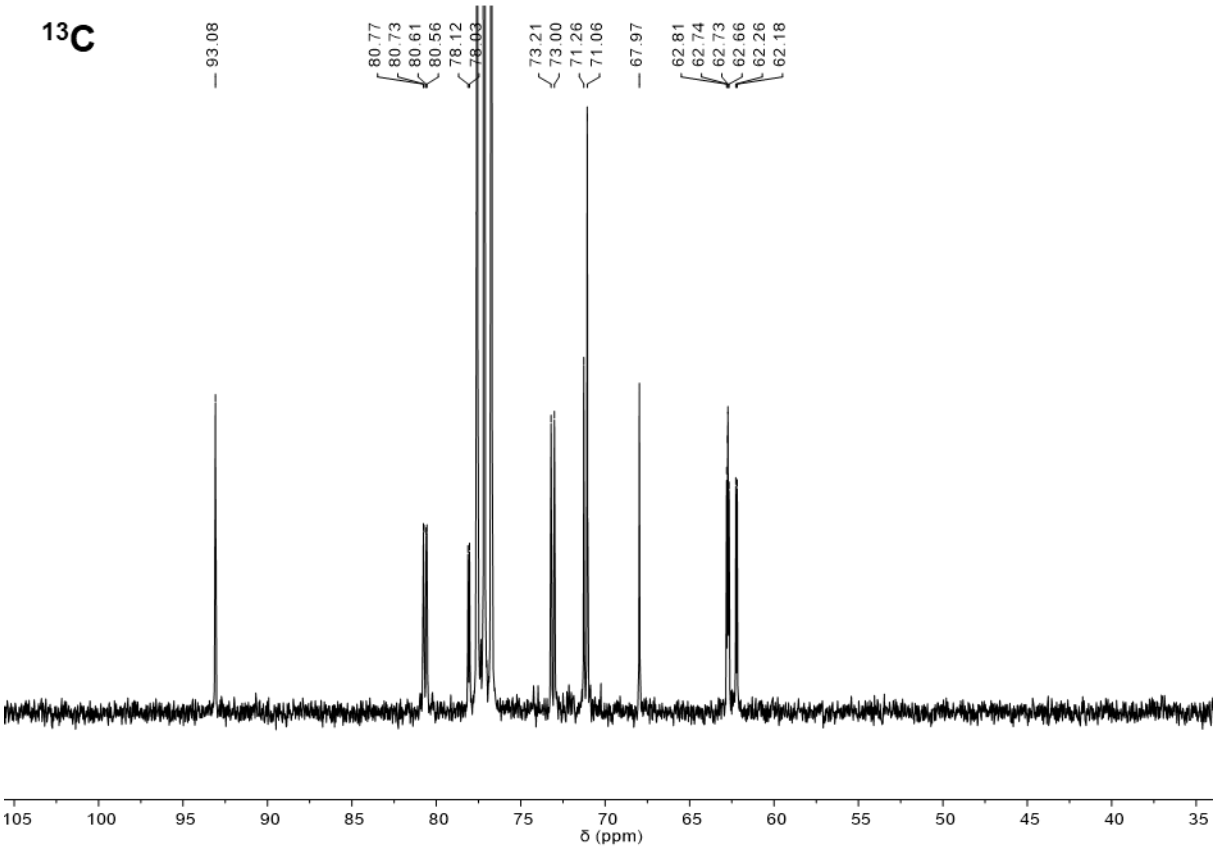

31P
114.55
114.29
114.10
113.84
29.10
28.65
28.01
27.56
1.00
1.01
130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5
δ (ppm)


62.85
62.63
62.57
61.68
61.66
61.59
61.58
61.54
61.53
61.46
57.78
55.72
55.53
44.73
44.70
44.57
32.31
31.89
30.55
30.13
28.37
28.13
27.03
26.74
24.53
24.47
24.12
24.02
17.17
17.06
16.55
16.53
16.47
16.44
13C
70
65
60
55
50
45
40
35
30
25
20
15
10
5
δ (ppm)

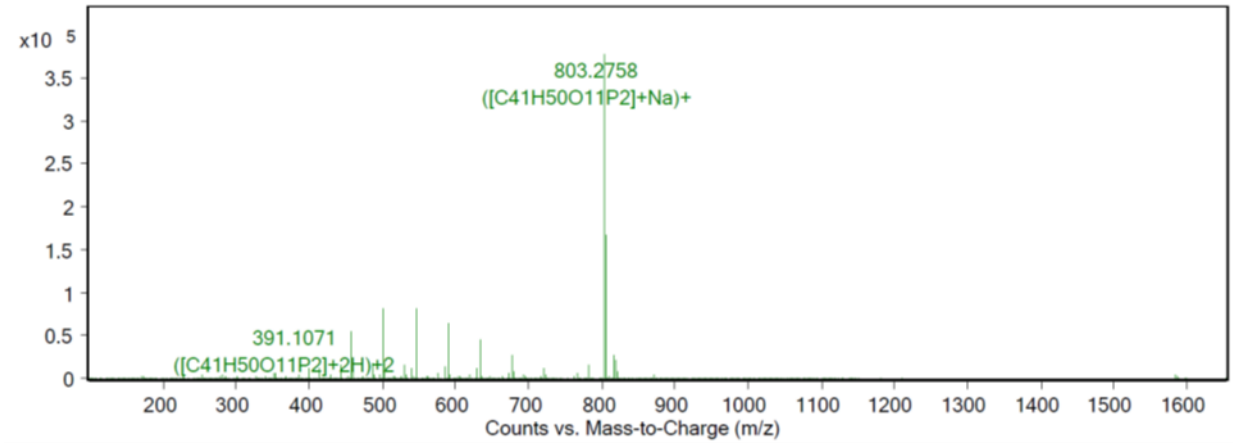
Compound 5b


1H
OEt
O=P–OEt
O
P
OEt
O
O
O
BnO
BnO
OBn
δ (ppm)

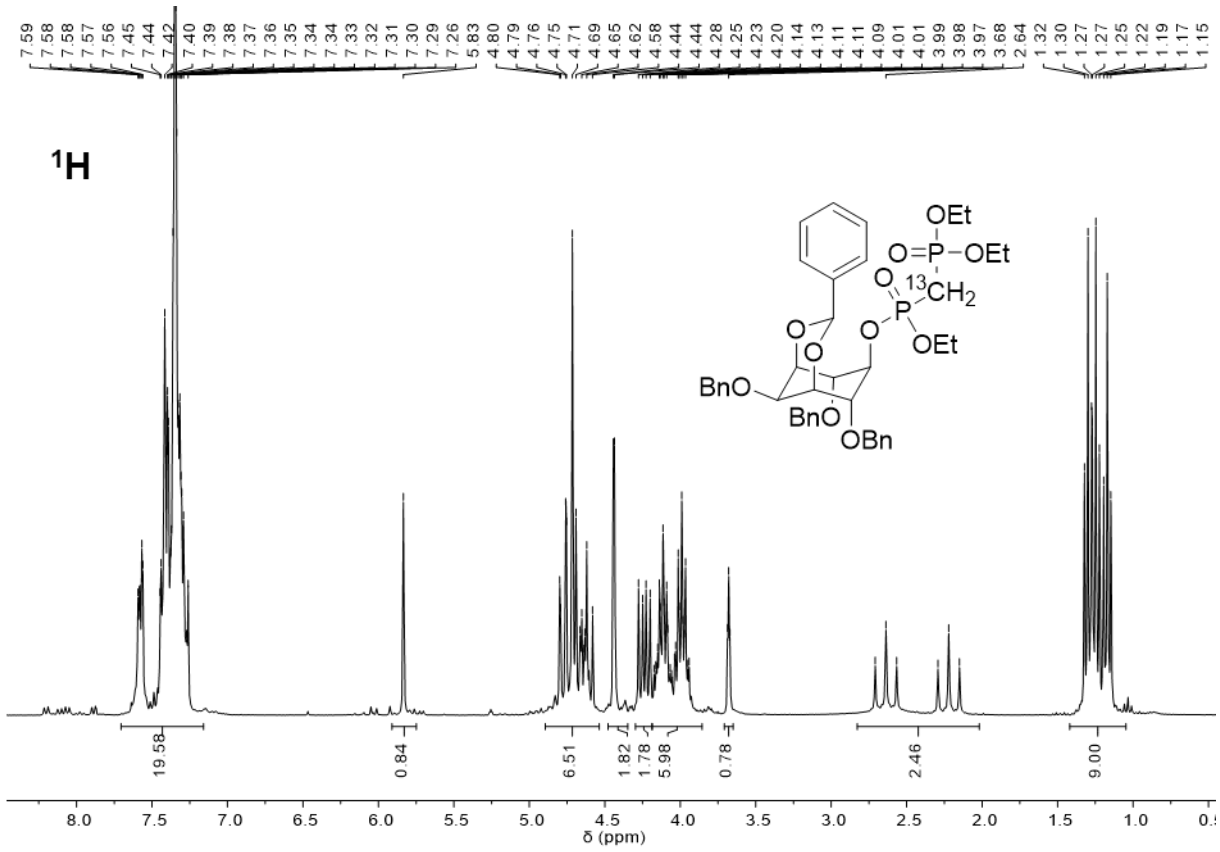

31P
19.31
19.24
19.06
18.99
1.00
0.97
δ (ppm)

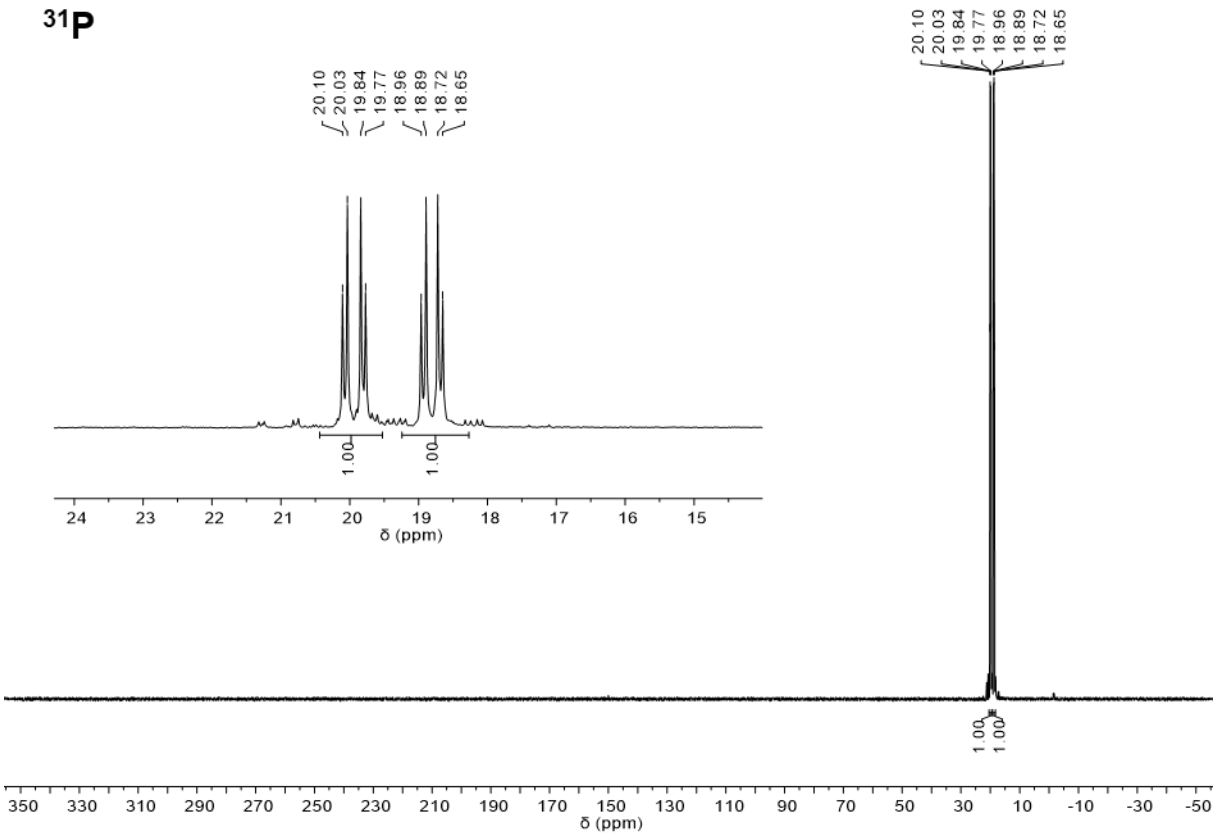

13C
93.08
80.77
80.73
80.61
80.56
78.12
78.03
73.21
73.00
71.26
71.06
67.97
62.81
62.74
62.73
62.66
62.26
62.18
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
δ (ppm)

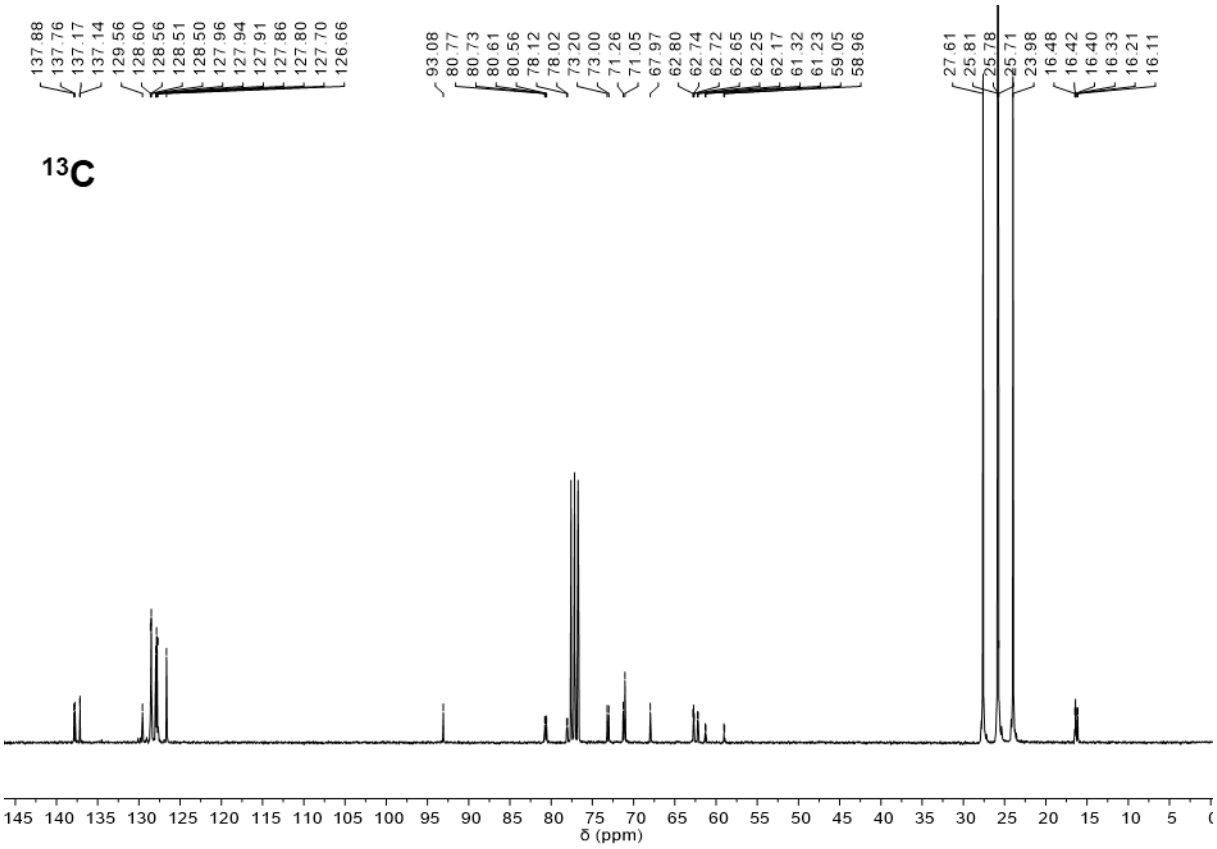
HRMS


x10 5
3.5
3
2.5
2
1.5
1
0.5
0
803.2758
([C41H50O11P2]+Na)+
391.1071
([C41H50O11P2]+2H)+2
200
300
400
500
600
700
800
900
1000
1100
1200
1300
1400
1500
1600
Counts vs. Mass-to-Charge (m/z)

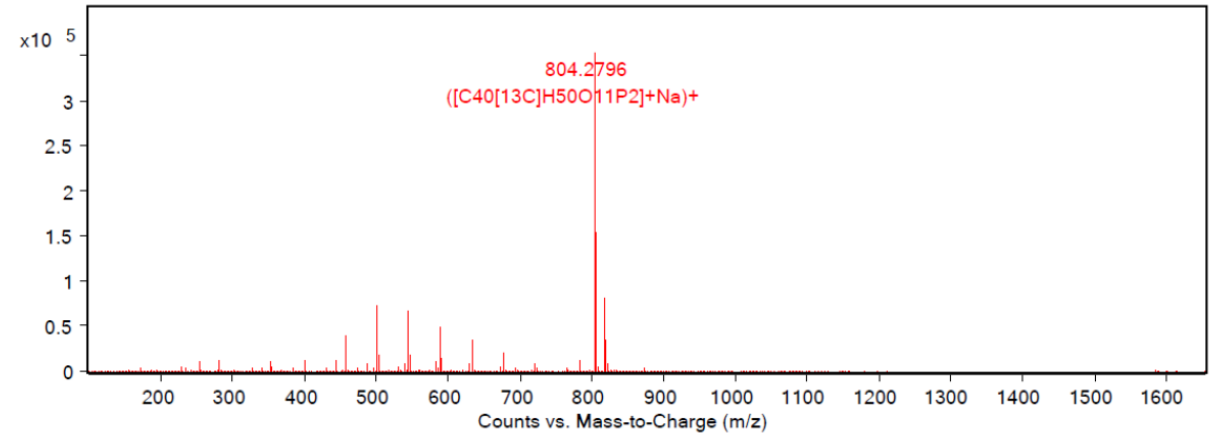
Compound S2



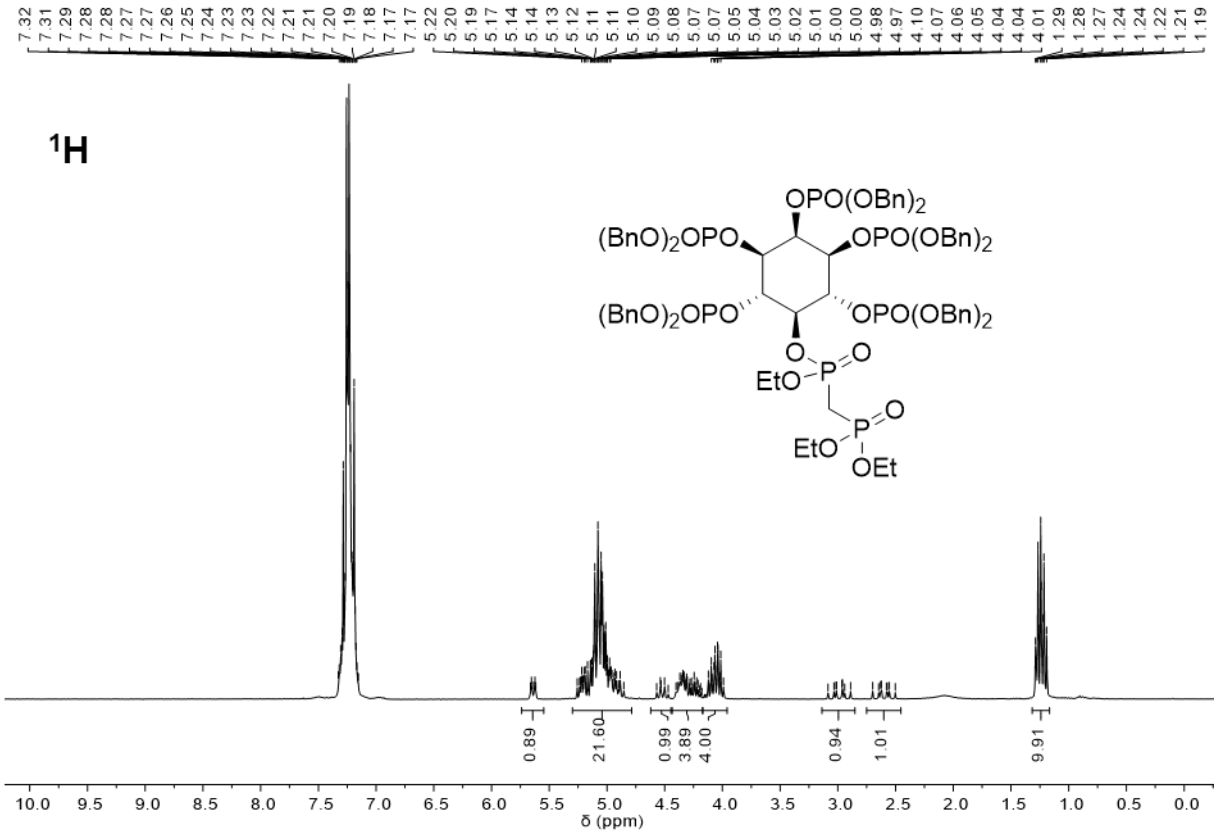


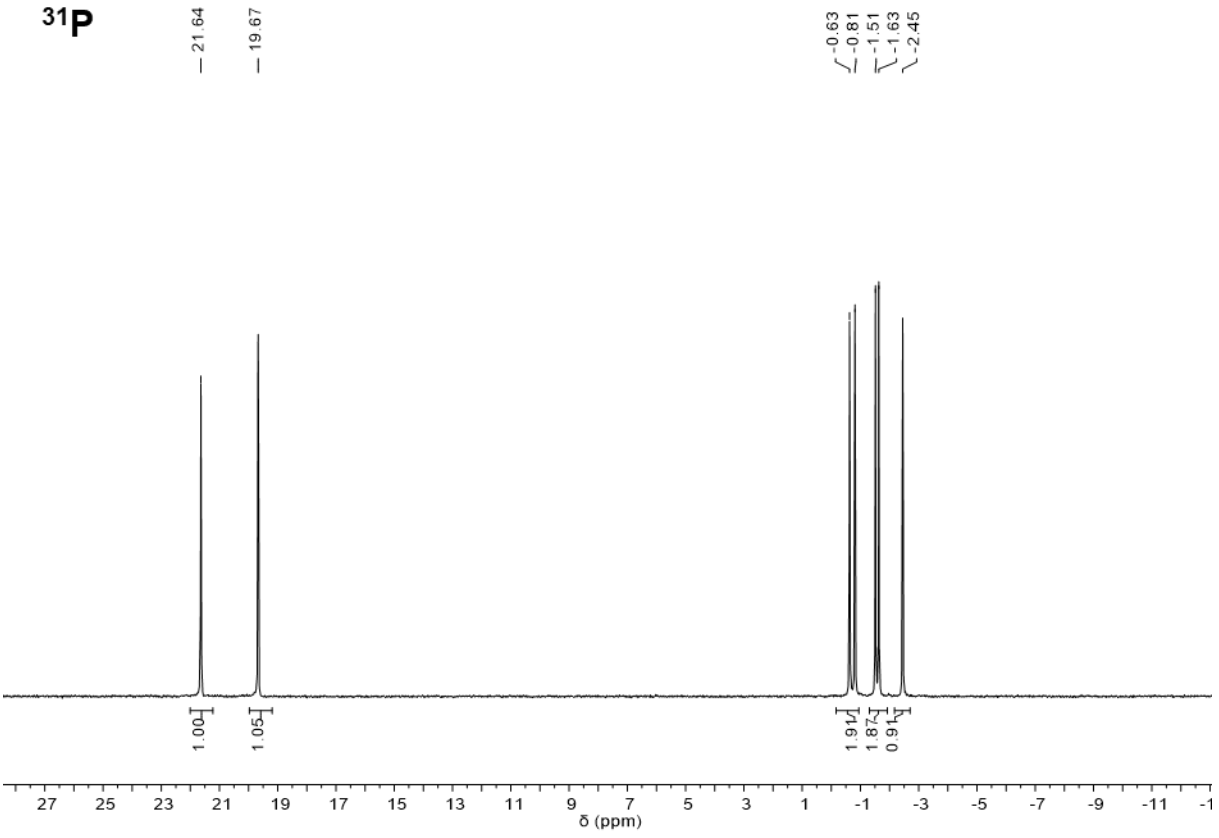

137.88
137.76
137.17
137.14
129.56
128.60
128.56
128.51
128.50
127.96
127.94
127.91
127.86
127.80
127.70
126.66
93.08
80.77
80.73
80.61
80.56
78.12
78.02
73.20
73.00
71.26
71.05
67.97
62.80
62.74
62.72
62.65
62.25
62.17
61.32
61.23
59.05
58.96
27.61
25.81
25.78
25.71
23.98
16.48
16.42
16.40
16.33
16.21
16.11
13C
145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5
δ (ppm)

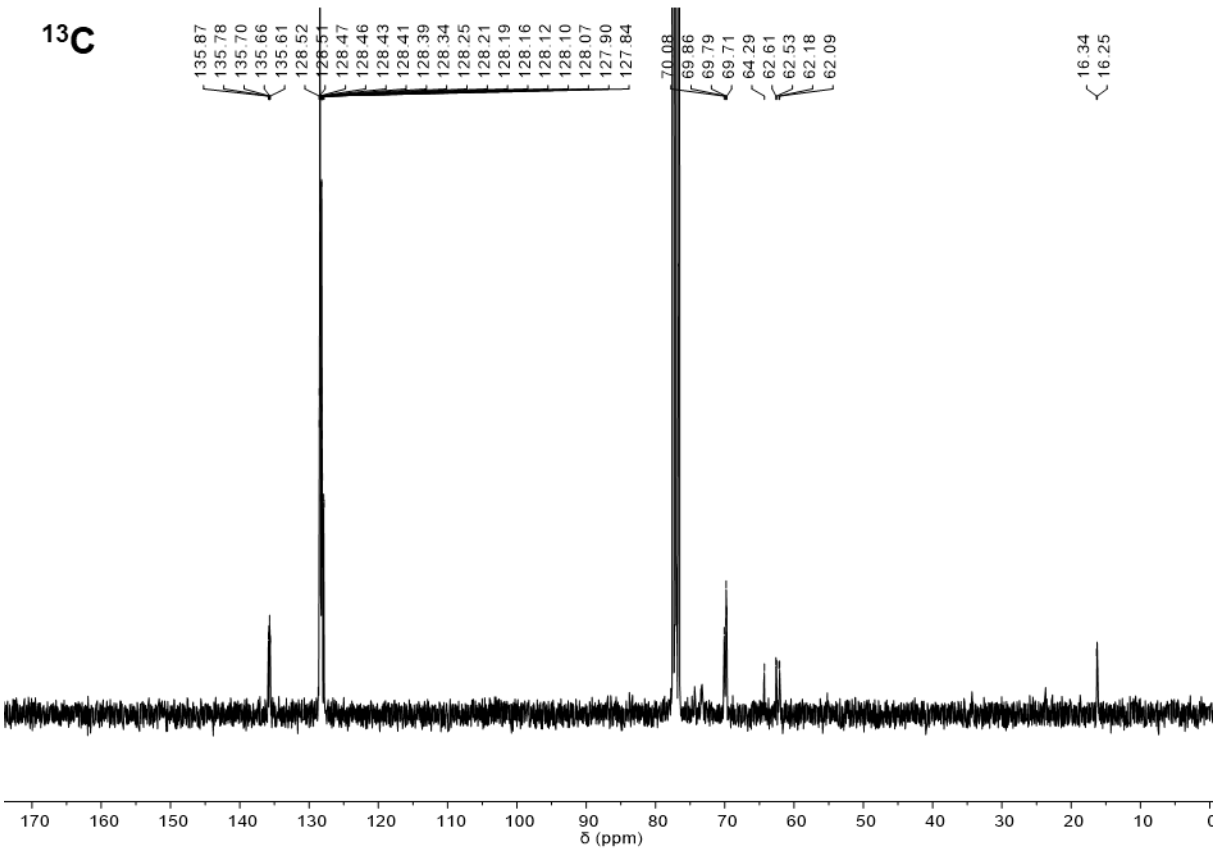
HRMS


x10 5
3
2.5
2
1.5
1
0.5
0
804.2796
([C40[13C]H50O11P2]+Na)+
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600
Counts vs. Mass-to-Charge (m/z)

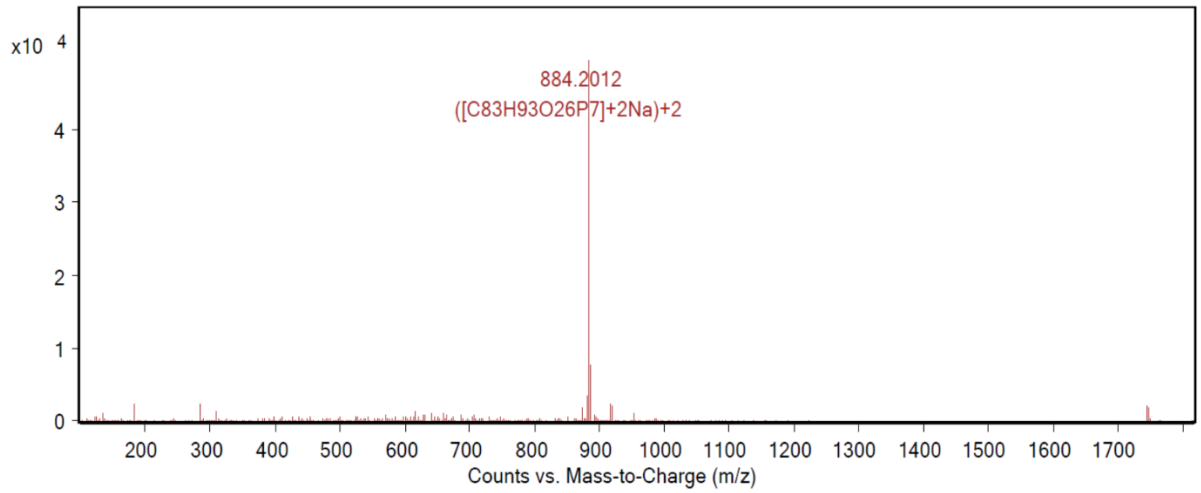
Compound S3


7.32 7.31 7.29 7.28 7.28 7.27 7.27 7.26 7.25 7.24 7.23 7.23 7.22 7.21 7.21 7.20 7.19 7.18 7.17 7.17
5.22 5.20 5.19 5.17 5.14 5.14 5.13 5.12 5.11 5.11 5.10 5.09 5.08 5.07 5.07 5.05 5.04 5.03 5.02 5.01 5.00 5.00 4.98 4.97 4.10 4.07 4.06 4.05 4.04 4.04 4.01 1.29 1.28 1.27 1.24 1.24 1.22 1.21 1.19
1H
OPO(OBn)2
(BnO)2OPO
OPO(OBn)2
(BnO)2OPO
OPO(OBn)2
EtO
P
O
P
O
EtO
OEt
0.89
21.60
0.99
3.89
4.00
0.94
1.01
9.91
10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
δ (ppm)

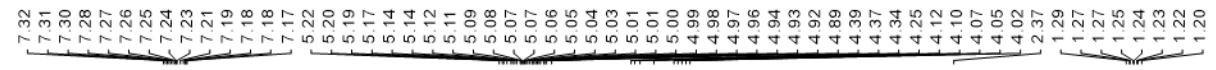

31P
21.64
19.67
-0.63
-0.81
-1.51
-1.63
-2.45
1.00
1.05
1.91
1.87
0.91
27 25 23 21 19 17 15 13 11 9 7 5 3 1 -1 -3 -5 -7 -9 -11 -1
δ (ppm)


13C
135.87
135.78
135.70
135.66
135.61
128.52
128.51
128.47
128.46
128.43
128.41
128.39
128.34
128.25
128.21
128.19
128.16
128.12
128.10
128.07
127.90
127.84
70.08
69.86
69.79
69.71
64.29
62.61
62.53
62.18
62.09
16.34
16.25
170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
δ (ppm)

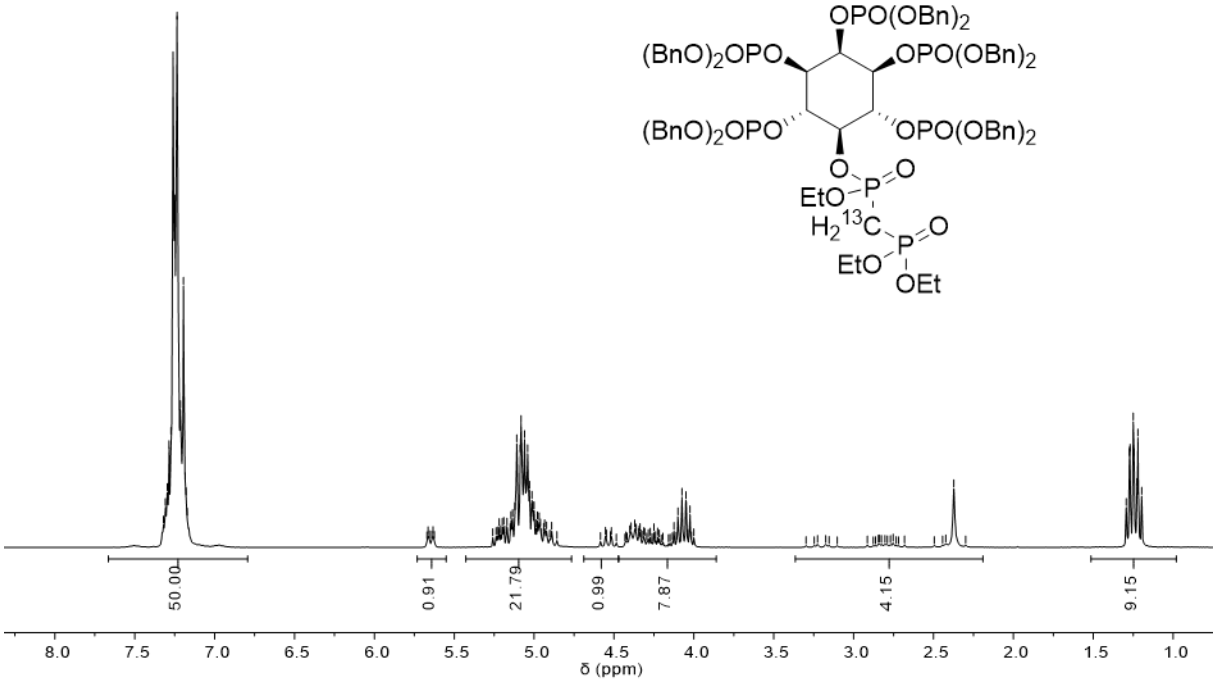
HRMS


x10 4
4
3
2
1
0
884.2012
([C83H93O26P7]+2Na)+2
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700
Counts vs. Mass-to-Charge (m/z)

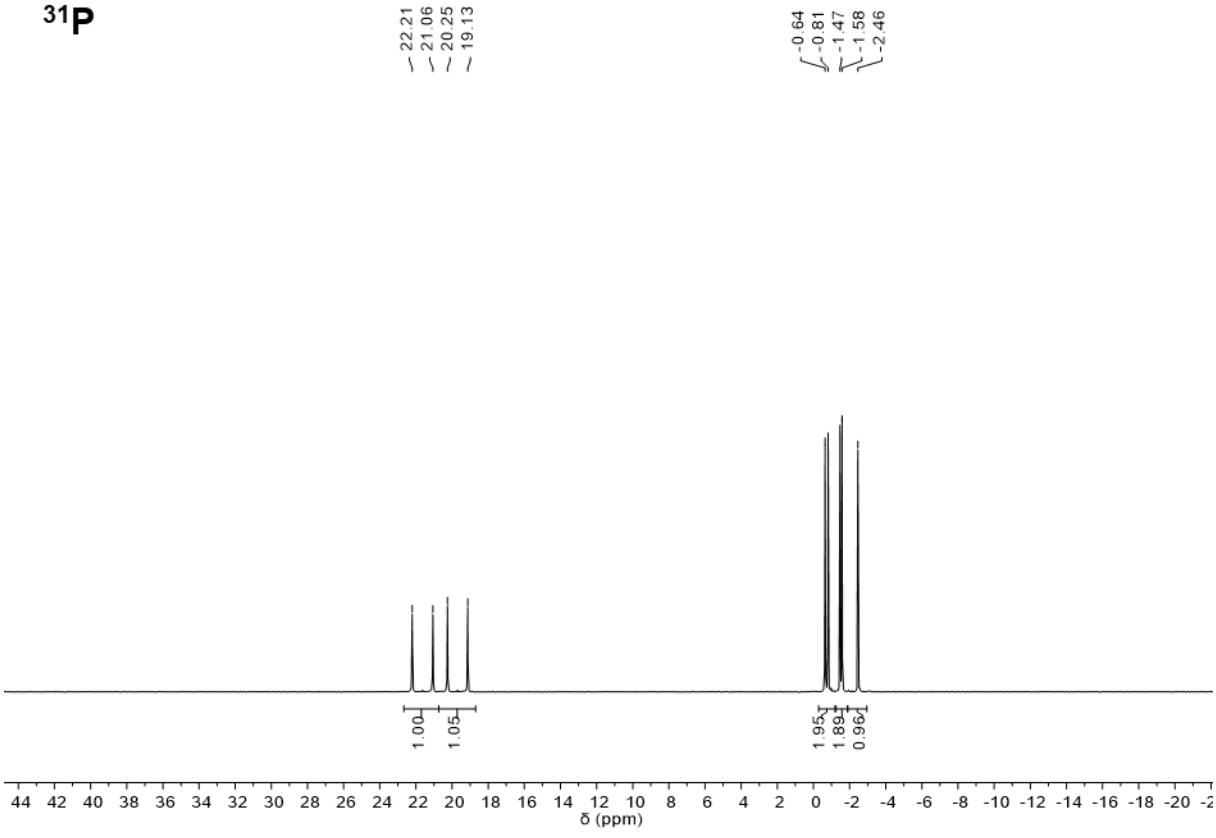
Compound S4

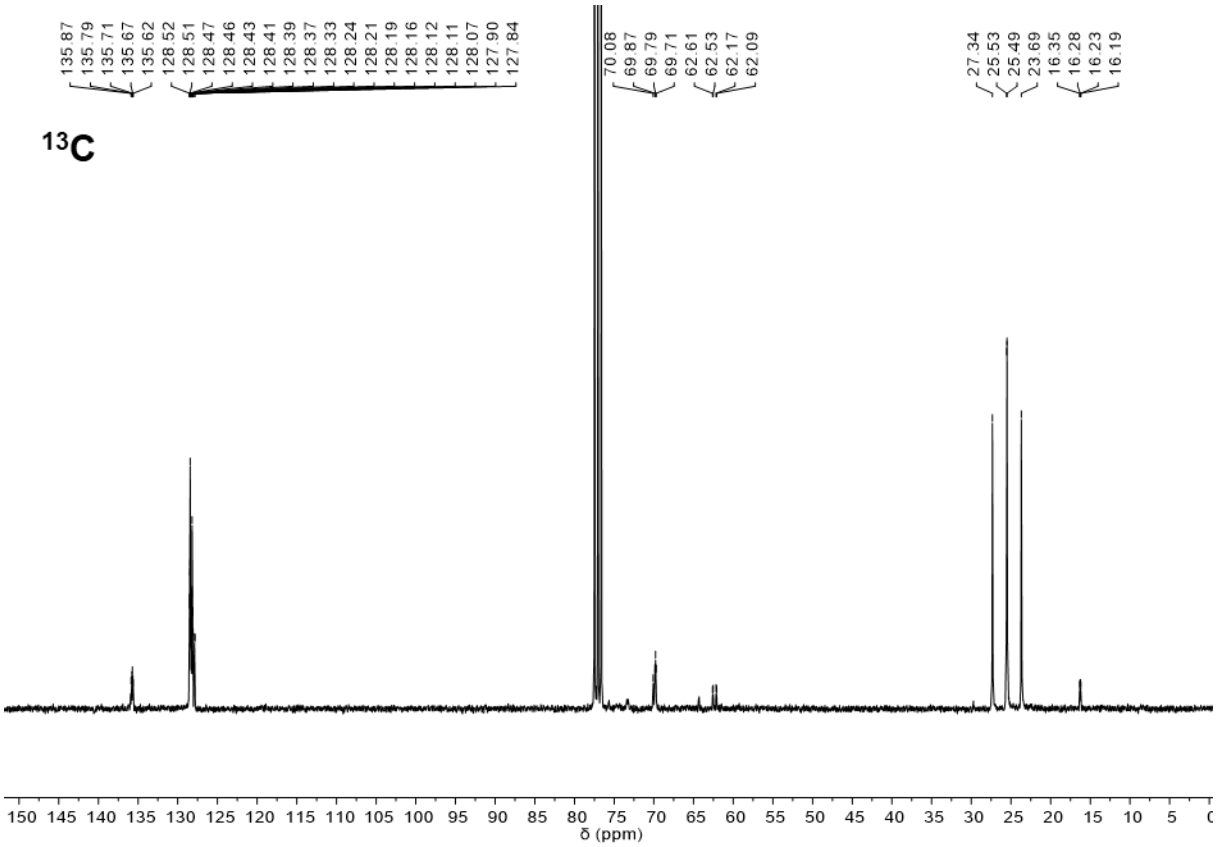


^{1}H

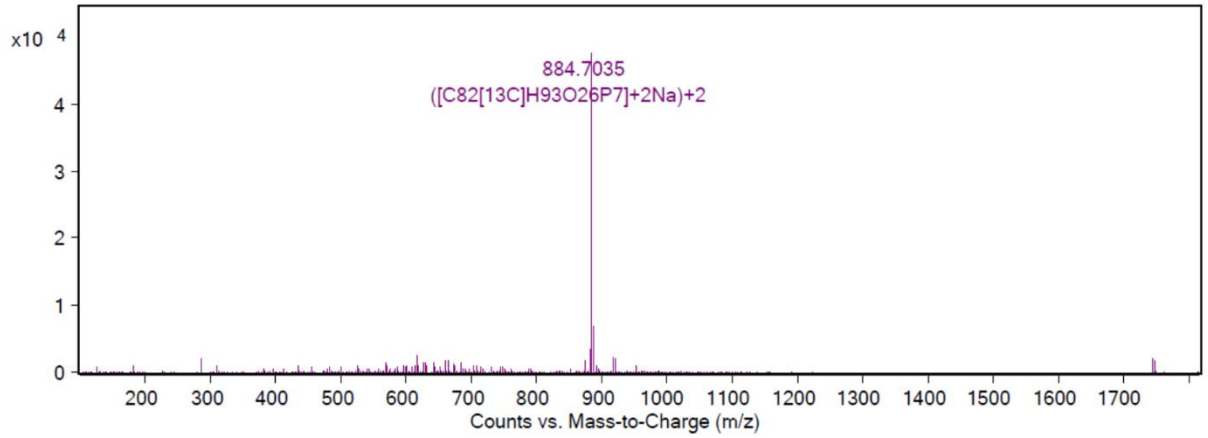


^{31}P



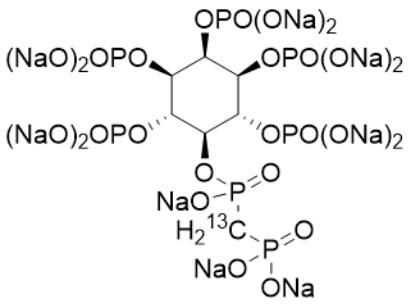

135.87
135.79
135.71
135.67
135.62
128.52
128.51
128.47
128.46
128.43
128.41
128.39
128.37
128.33
128.24
128.21
128.19
128.16
128.12
128.11
128.07
127.90
127.84
70.08
69.87
69.79
69.71
62.61
62.53
62.17
62.09
27.34
25.53
25.49
23.69
16.35
16.28
16.23
16.19
13C
150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5
δ (ppm)

HRMS

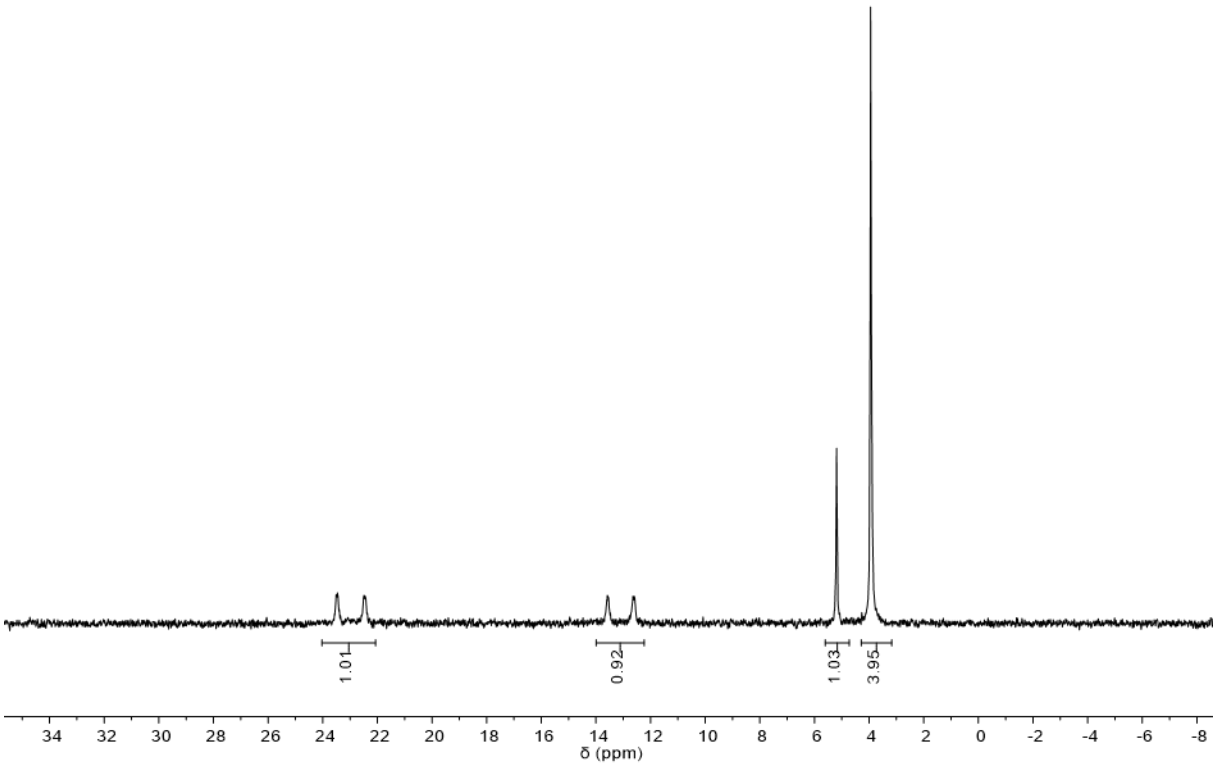

x10 4
4
3
2
1
0
884.7035
([C82[13C]H93O26P7]+2Na)+2
200 300 400 500 600 700 800 900 1000 1100 1200 1300 1400 1500 1600 1700
Counts vs. Mass-to-Charge (m/z)

Compound S5

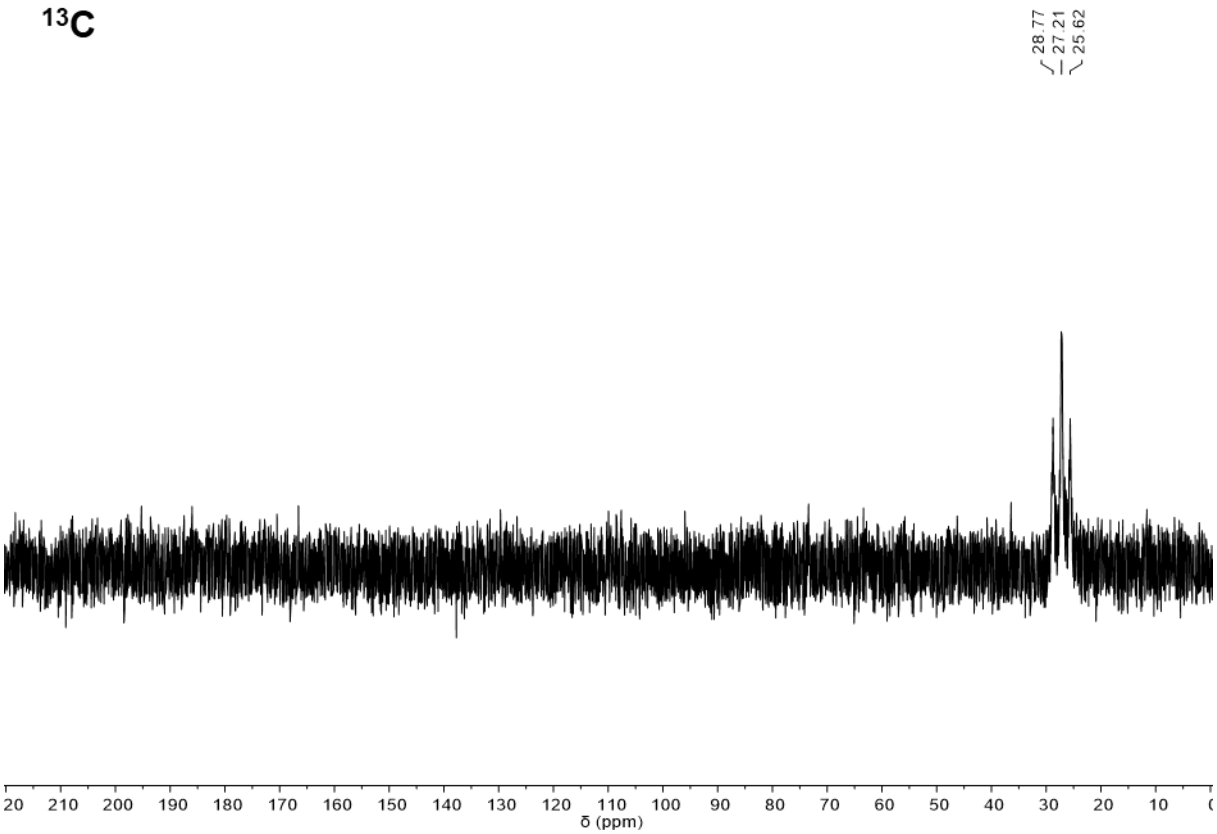
^{1}H



^{31}P



^{13}C


28.77
27.21
25.62
20 210 200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10
δ (ppm)