CHEMBIOCHEM

Supporting Information

IspG-Catalyzed Positional Isotopic Exchange in Methylerythritol Cyclodiphosphate of the Deoxyxylulose Phosphate Pathway: Mechanistic Implications

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1. General information

NMR spectra were recorded on a Varian 500 or 400 spectrometer. D_2O was used as the NMR solvent and its signal at 4.65 ppm in the ^{1}H NMR spectra was used to calibrate the chemical shifts of other signals; for ^{13}C NMR, DSS (3-(trimethylsilyl)-1-propanesulfonic acid sodium salt) was used as the standard and its chemical shift was set as 0.00 ppm to calibrate the chemical shifts of other signals in IspG assays. High-resolution mass spectra were obtained in the Boston University Chemical Instrumentation Center using a Waters Q-TOF spectrometer. [^{18}O]water was purchased from Cambridge Isotope Laboratories. Anion exchange resin (chloride form, AG 1-X2, Cat # 1401251, 200-400 mesh) was purchased from Bio-Rad. Fibrous cellulose powder was purchased from Whatman (CF11, Cat # 4021050). All other enzymes, reagents and solvents were used as supplied by Sigma-Aldrich and Pharma without further purification. IspC, IspD, IspE, and IspF expression constructs were kindly provided by Professor Caren Meyers at Johns Hopkins University. Those proteins were expressed and purified using Ni-NTA affinity chroma-tography. IspG purification was purified as reported by us recently.[1]

2. Mass spectrometry of the 1.0 : 1.1 mixture of 7b (1.0 mM) and 7d (1.1 mM) before and after 10 μM IspG was introduced.

The signals at 323.9919 and 325.9892 are those of **7d** and **7b**, respectively. Clearly, the signal changes in in Figure 1D are not due to MEcPP decomposition.

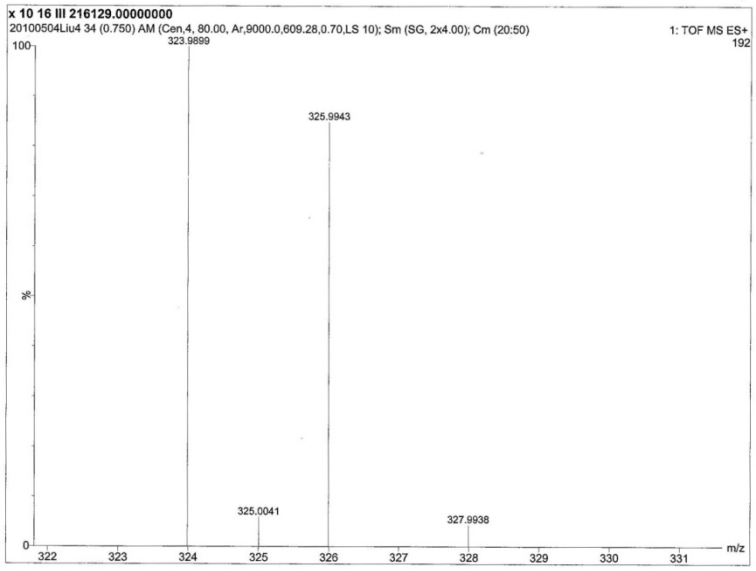


Figure 1S-1. Before IspG was introduced (Spectrum C in Figure 1).

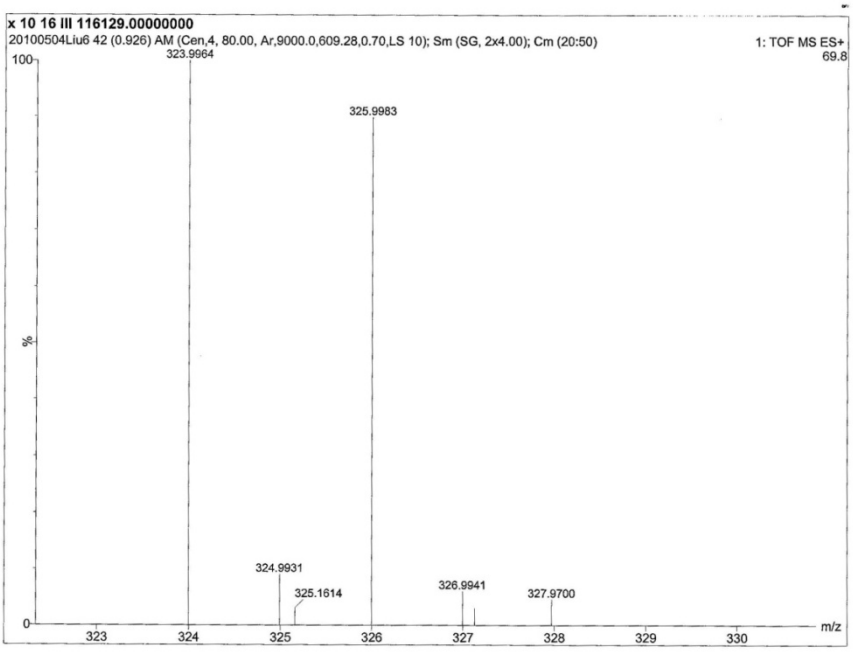


Figure 2S-2. After IspG was introduced (Spectrum D in Figure 1).

3. Stability of MEcPP

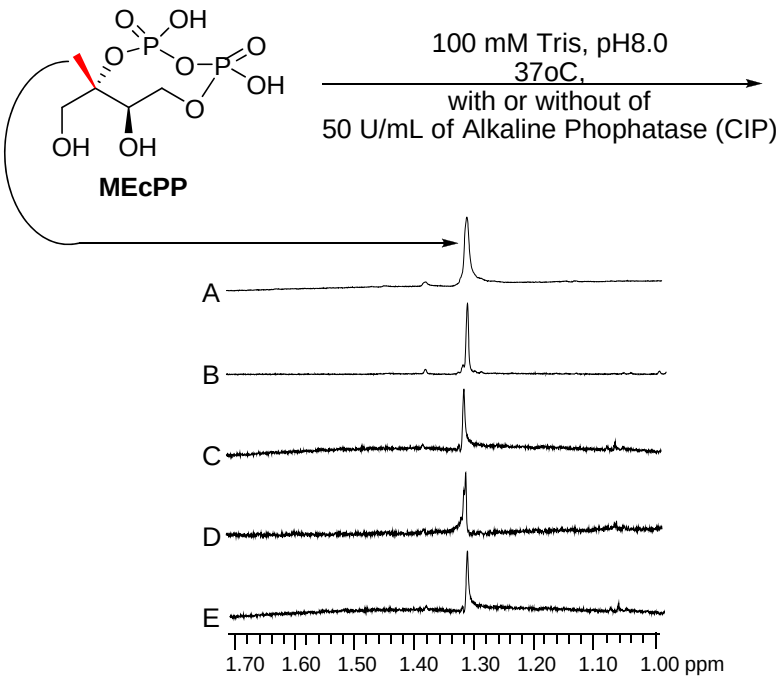


Figure 2S. The ^{1}H-NMR signals of the MEcPP C_4 methyl group with or without phosphatase. 1.0 mM MEcPP in 100 mM Tris, pH 8.0 was incubated at 37 ^{o}C for (A) 5 min., (B) 1 day; with extra 50 U/mL of alkaline phosphatase (CIP) for (C) 5 min., (D) 30 min., (E) 1 day.

4. Kinetics of positional isotopic exchange measured by ^{13}C-NMR using [2-^{13}C, ^{18}O]-MEcPP (7b) as the substrate.

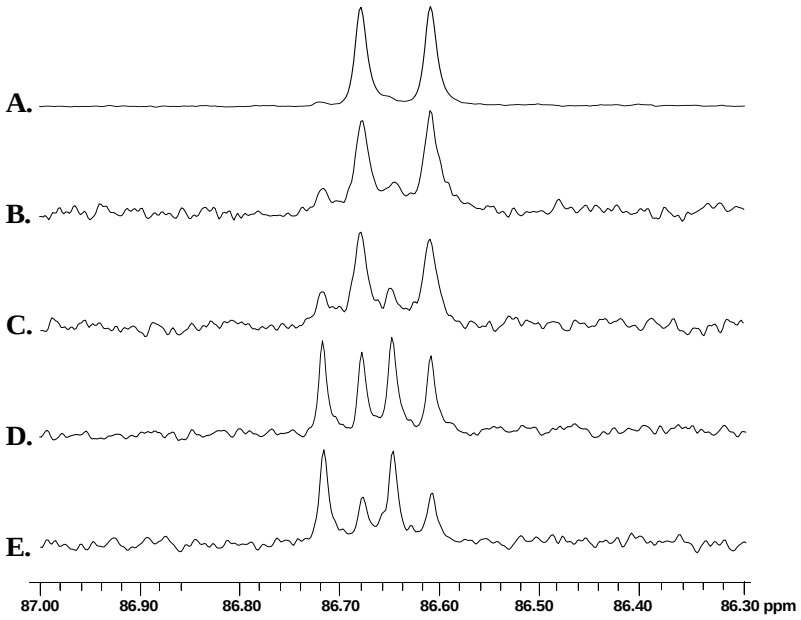
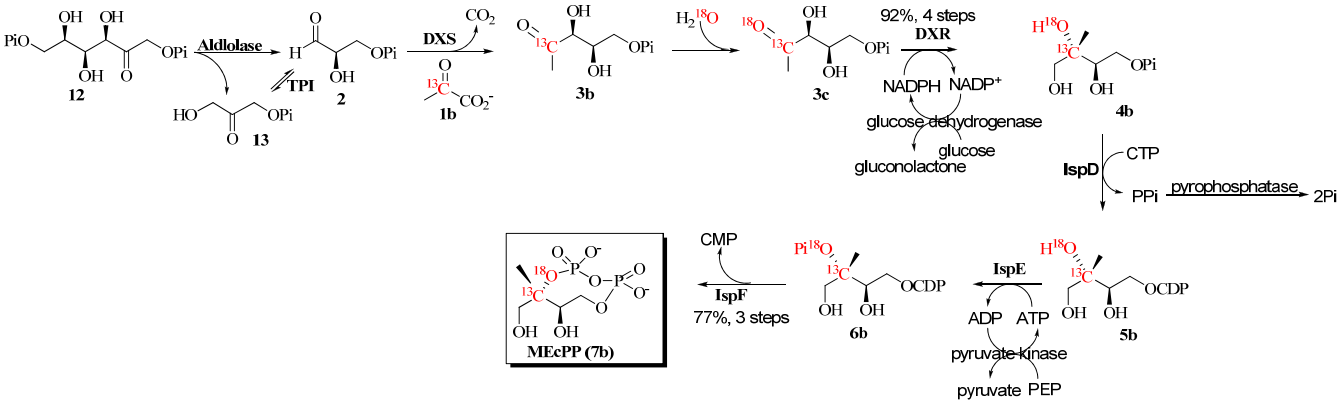


Figure 3S. ^{13}C-NMR of (A) **7b**; (B) – (E) are 2.0 mM of **7b** after 10 μM IspG was introduced for 1.27, 2.0, 24, and 48 hours, respectively.

5. Synthesis of [2-^{13}C, ^{18}O]-MEcPP (7b)

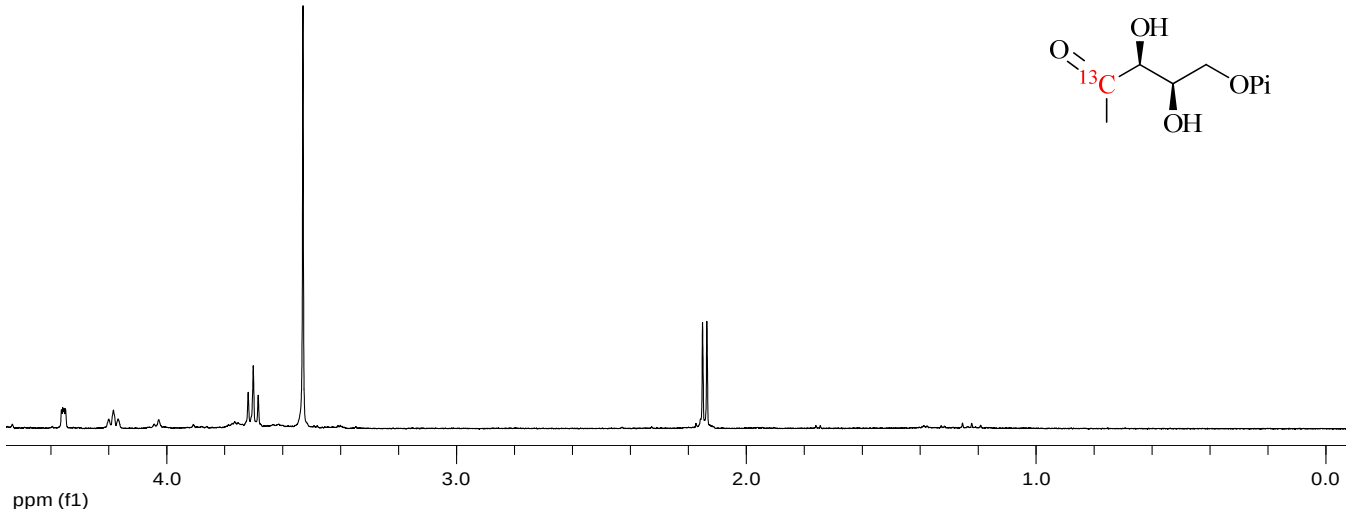
Scheme 1S



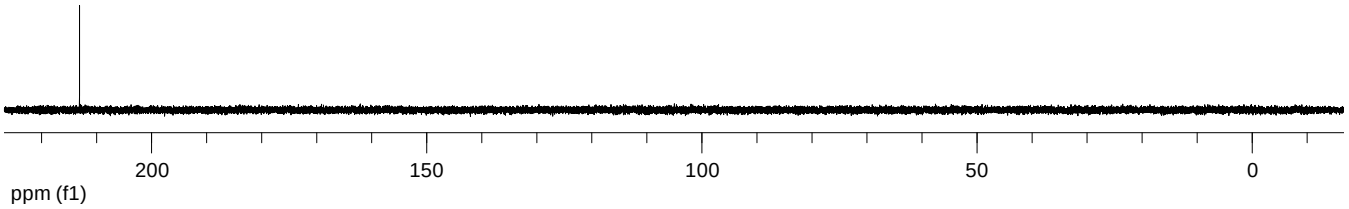
5-1. Synthesis of [3-^{13}C, ^{18}O]-MEP (**4b**)

A 2.0 ml enzymatic reaction mixture containing 50 mM [2-^{13}C]-pyruvate (**1b**), 30 mM Fructose-1,6-diphosphate (F-1,6-BP, **12**), 1 mM DTT, 0.5 mM thiamin pyrophosphate (ThDP), 5 mM $MgCl_2$, 5.0 units of aldolase from rabbit muscle, 10 units of triosephosphate isomerase (TPI), and 0.2 mg of DXP synthase (DXS) in 50 mM Tris-Cl, pH 7.5 buffer, was incubated at 25 ^{o}C with gentle agitation overnight. Once the reaction had achieved nearly complete conversion based on ^{1}H-NMR, the reaction solution was filtered through a membrane with molecular weight cut-off of 10 kDa to remove the proteins. The filtrate was collected and lyophilized. The resulting solid power contains the desired [4-^{13}C]-DXP (**3b**). The mixture can be used for the next step without further purification. ^{1}H-NMR (D_2O, 400 MHz): δ 4.36 (q, *J* = 1.6 Hz, 1 H), 4.18 (t, *J* = 6.4 Hz, 1 H), 3.70 (t, *J* = 7.2 Hz, 2 H), 2.14 (d, *J* = 6.0 Hz, 3 H), (the giant signal at 3.54 ppm was from Tris-Cl); ^{13}C-NMR (D_2O, 100 MHz): δ 213.12.[2]

^{1}H-NMR



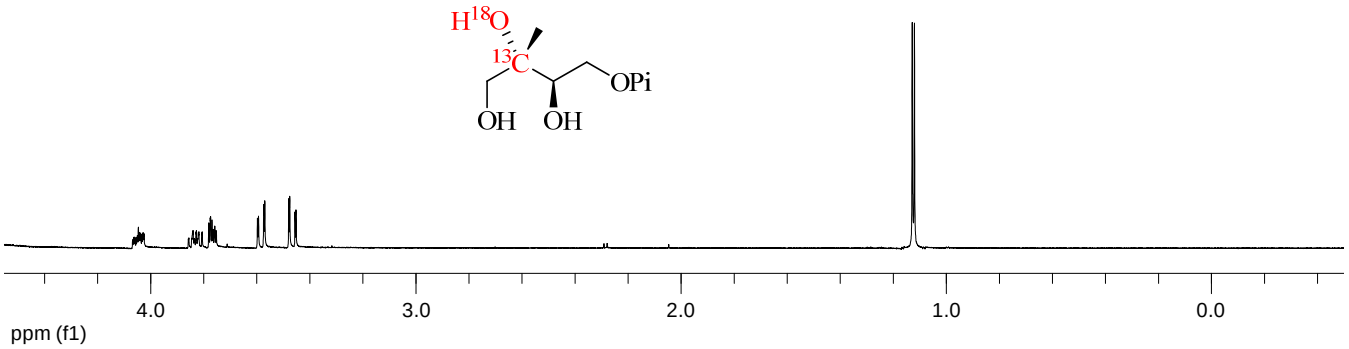
^{13}C-NMR



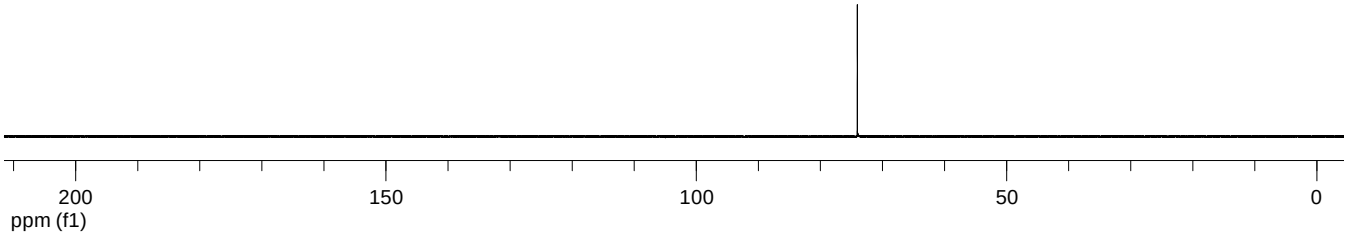
The above [4-^{13}C]-DXP containing powder was dissolved in 1 mL of [^{18}O]-water (>97% ^{18}O enrichment). An overnight incubation at room temperature led to near complete exchange to [4-^{13}C, ^{18}O]-DXP.[3]

To the above mixture, the following components were added as solids to make a reaction mixture containing: 200 mM glucose, 100 U/mL glucose dehydrogenase, 50 µM DXR, 400 µM $NADP^+$, and 5 mM $MgCl_2$. The mixture was incubated at 37 ^oC for half hour and the reaction was monitored by ^{1}H and ^{13}C-NMR. The solution was filtered through a membrane with molecular weight cut-off of 10 kDa. The filtrate was purified by anion-exchange chromatography (Bio-Rad, AG 1-X2, Cl^- type, 1.5 x 10 cm) using 30 mL × 30 mL a linear gradient of 0 - 200mM of NaCl in H_2O. The column was further eluted with 20 mL of 200 mM NaCl solution. Fractions containing pure [3-^{13}C, ^{18}O]-MEP were combined and lyophilized to give 92 µmol of [3-^{13}C, ^{18}O]-MEP (92% yield, 4 steps)[4]. ^{1}H-NMR (D_2O, 500 MHz): δ 4.02-4.07 (m, 1 H), 3.81-3.86 (m, 1 H), 3.75-3.78 (m, 1 H), 3.58 (dd, *J* = 11.5, 2.0 Hz, 1 H), 3.46 (dd, *J* = 11.5, 2.0 Hz, 1 H), 1.12 (d, *J* = 4.0 Hz, 3 H); ^{13}C-NMR (D_2O, 126 MHz): δ 74.05 (3-^{13}C); HR-ESI MS: calculated for $C_4{}^{13}CH_{12}O_6{}^{18}OPNa_2$ (disodium salt) m/z 264.0192 [$M+H^+$]; found 264.0339.

^{1}H-NMR



^{13}C-NMR



5-2. Synthesis of [2-^{13}C, ^{18}O]-MEcPP (**7b**)

The synthesis of [2-^{13}C, ^{18}O]-MEcPP was performed in one pot by tandem incubations with IspD, IspE, and IspF enzymes.[5]

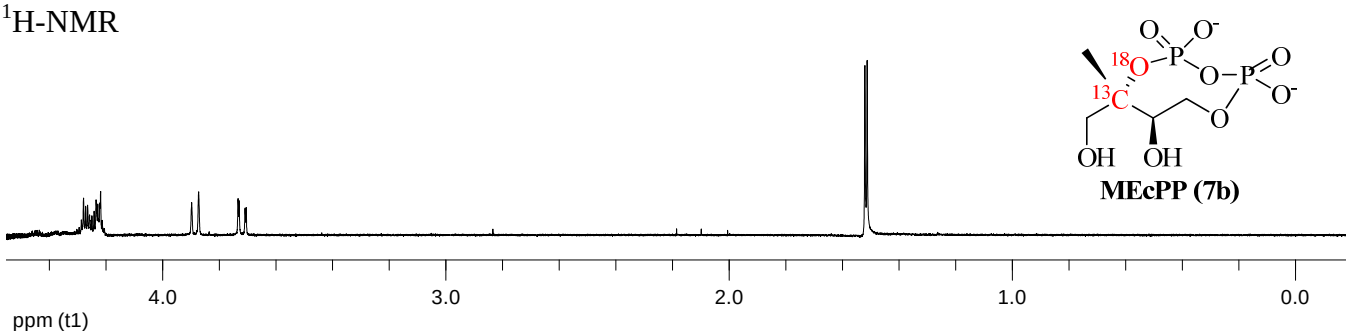
A 5 mL IspD reaction mixture contained: 10 mM [3-^{13}C, ^{18}O]-MEP, 12.0 mM CTP, 1.0 mM DTT, 1 U/mL of inorganic pyrophosphate, 5.0 mM $MgCl_2$, and 20 µM IspD in 100 mM Tri-Cl, pH 8.0 buffer. The mixture was incubated at 37 ^{o}C for 1 hour with over 95% conversion as indicated by ^{1}H-NMR.

The IspD product was carried on without further purification. To the IspD reaction mixture, 5 mM DTT, 15 mM phosphoenpyruvate (PEP), 20 U/mL pyruvate kinase (PK), 2 mM ATP and 20 µM IspE were added. The reaction mixture was incubated at 37 °C for 1 hour and over 95% conversion was achieved as judged by ^{1}H-NMR.

IspF (100 µM) was added to the crude IspE enzymatic reaction mixture. Over 95% conversion was achieved after incubation at 37 ^{o}C for 1.5 hour based on ^{1}H-NMR.

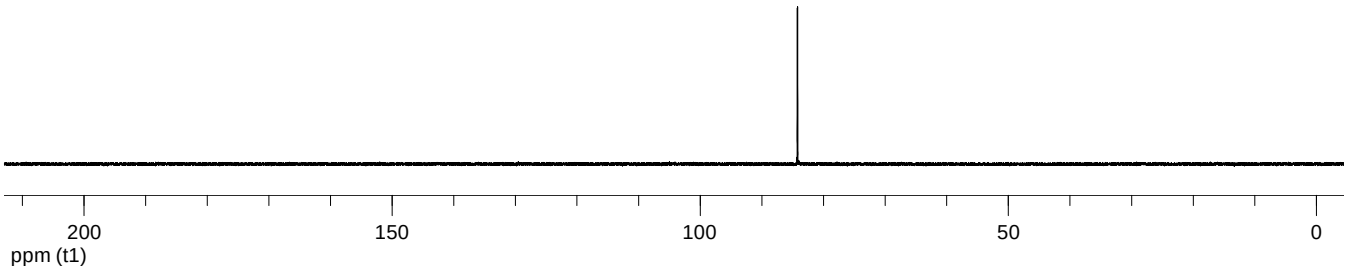
The solution was filtered through a membrane with 10 kDa molecular weight cut-off and purified by anion-exchange chromatography (Bio-Rad, AG 1-X2, Cl^- type, 1.5 x 10 cm) using 60 mL × 60 mL linear gradient of 0-500 mM of NaCl in H_2O. The column was further eluted with 20 mL of 500 mM NaCl solution. Fractions containing [2-^{13}C, ^{18}O]-MEcPP were combined and lyophilized. MEcPP was extracted from the lyophilized powder using methanol and further purified by cellulose column chromatography (1.5 x 15 cm) using a solvent system containing isopropylalcohol : acetonitrile : 100 mM NH_4HCO_3 (aq) 2.5:1.5:1 and 2:1:1. [2-^{13}C, ^{18}O]-MEcPP was obtained in 77% yield (38.5 µmol, 3 steps). ^{1}H-NMR (D_2O, 500 MHz): δ 4.22-4.30 (m, 3 H), 3.89 (d, *J* = 12.5 Hz, 1 H), 3.72 (dd, *J* = 12.0, 2.0 Hz, 1 H), 1.52 (d, *J* = 4.0 Hz, 3 H); ^{13}C-NMR (D_2O, 126 MHz): δ 86.64 (d, *J* = 8.4 Hz, ^{13}C-3); ^{31}P-NMR (D_2O, 202 MHz): δ –10.24 (d, *J* = 22.6 Hz); -14.53 (dd, *J* = 22.8, 8.5 Hz); HR-ESI MS: Calcd. for $C_4{}^{13}CH_{11}O_8{}^{18}OP_2Na_2$ (disodium salt) m/z 325.9750 [$M+H^+$]; found 325.9920.

^{1}H-NMR

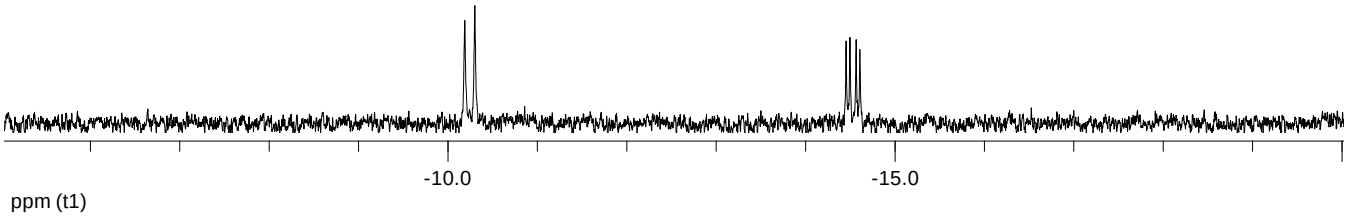


MEcPP (7b)

^{13}C-NMR

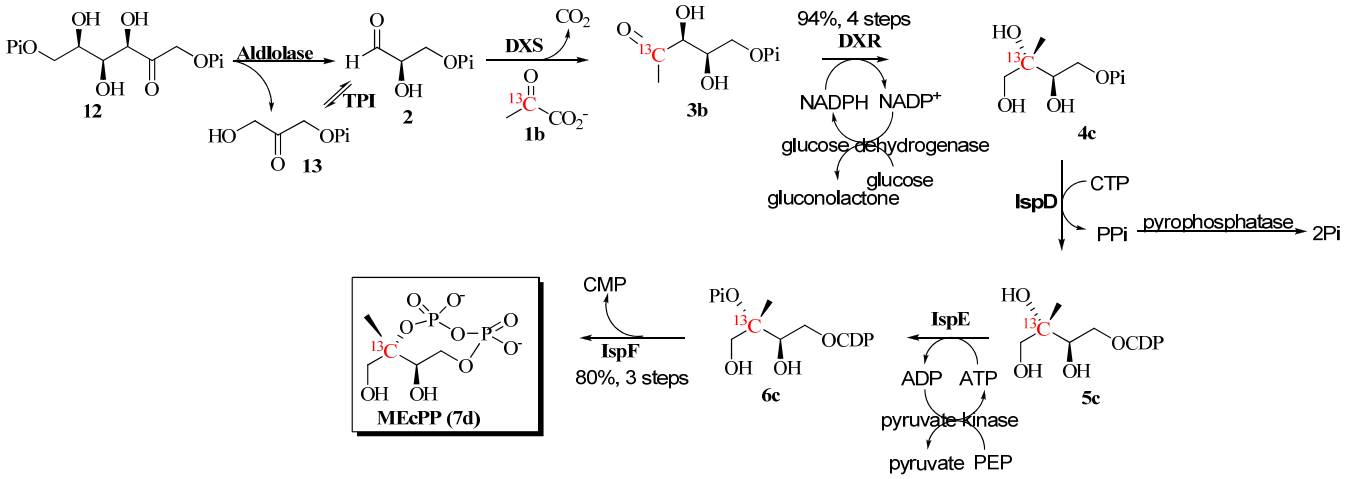


^{31}P-NMR



6. Synthesis of [2-^{13}C]-MEcPP (7d)

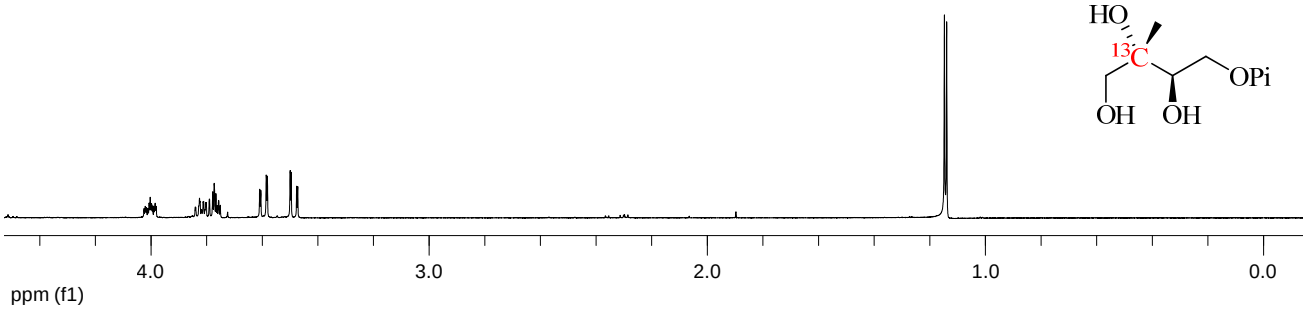
Scheme 2S



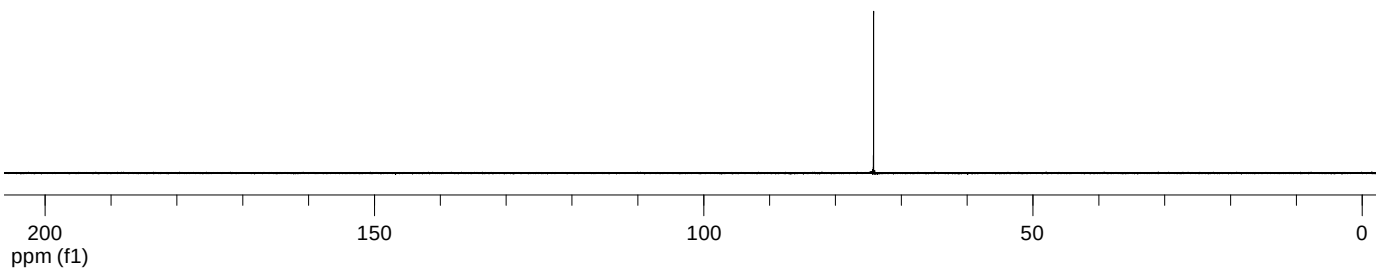
5-1. Synthesis of [3-^{13}C]-MEP (**4c**)

Following a procedure similar to that described for compound [3-^{13}C, ^{18}O]-MEP (**4b**) in section 5-1, but eliminating the [^{18}O]-water exchange step, 94 μmol of [3-^{13}C]-MEP (94% yield, 4 steps) was obtained. ^{1}H-NMR (D_2O, 500 MHz): δ 4.00-4.04 (m, 1 H), 3.77-3.86 (m, 2 H), 3.61 (dd, *J* = 11.5, 2.0 Hz, 1 H), 3.50 (dd, *J* = 12.0, 2.0 Hz, 1 H), 1.16 (d, *J* = 4.0 Hz, 3 H); ^{13}C-NMR (D_2O, 126 MHz): δ 74.20 (^{13}C-3); ^{31}P-NMR (D_2O, 202 MHz): δ 4.12; HR-ESI MS: Calcd. for $C_4{}^{13}CH_{12}O_7PNa_2$ (disodium salt) m/z 262.0150 [M+H$^+$]; found 262.0197.

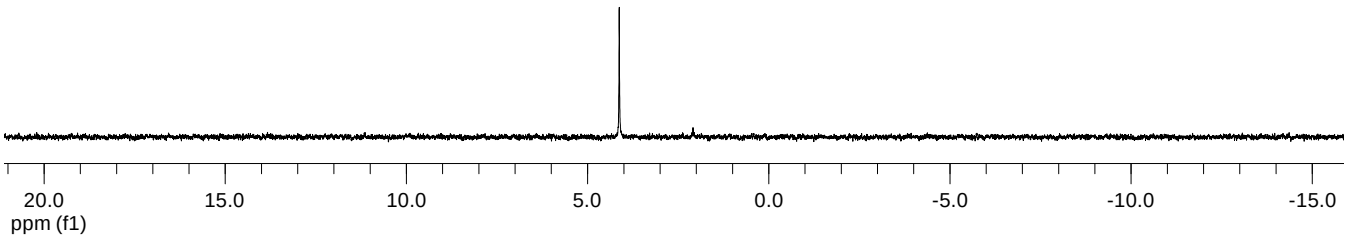
^{1}H-NMR



^{13}C-NMR



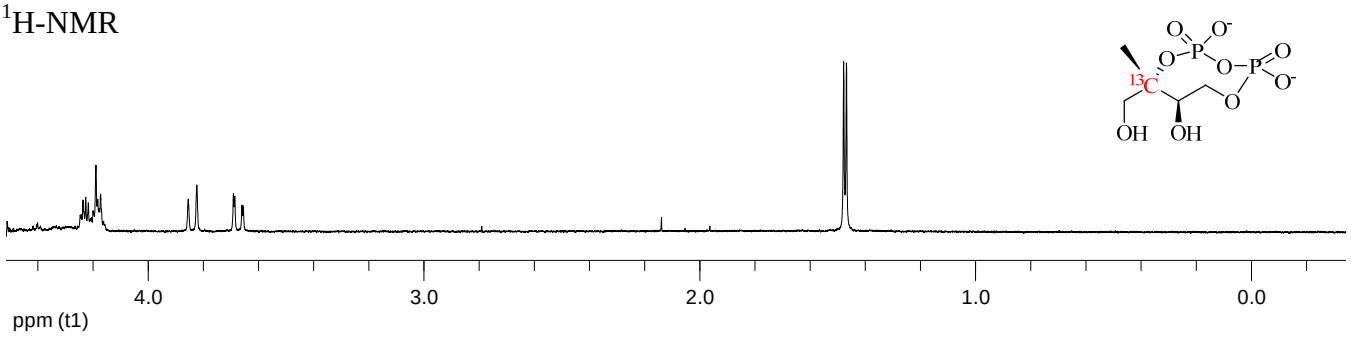
^{31}P-NMR



6-2. Synthesis of [3-^{13}C]-MEcPP (**1d**)

Following a procedure similar to that described for compound [2-^{13}C, ^{18}O]-MEcPP (**7b**) in section 5-2, 20.0 µmol of [2-^{13}C, ^{18}O]-MEcPP (80% yield, 3 steps) was obtained. ^{1}H-NMR (D_2O, 400 MHz): δ 4.17-4.25 (m, 3 H), 3.84 (d, *J* = 12.0 Hz, 1 H), 3.67 (dd, *J* = 12.0, 2.0 Hz, 1 H), 1.47 (d, *J* = 4.0 Hz, 3 H); ^{13}C-NMR (D_2O, 126 MHz): δ 86.68 (d, *J* = 8.3 Hz, 3- ^{13}C); ^{31}P-NMR (D_2O, 202 MHz): δ –10.25 (d, *J* = 22.6 Hz); -14.52 (dd, *J* = 22.5, 8.5 Hz); HR-ESI MS: calculated for $C_4{}^{13}CH_{11}O_9P_2Na_2$ (disodium salt) m/z 323.9797 [M+H$^+$]; found 323.9768.

^{1}H-NMR



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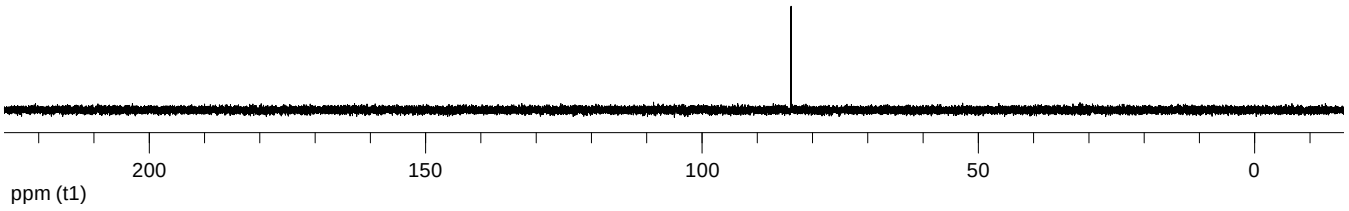

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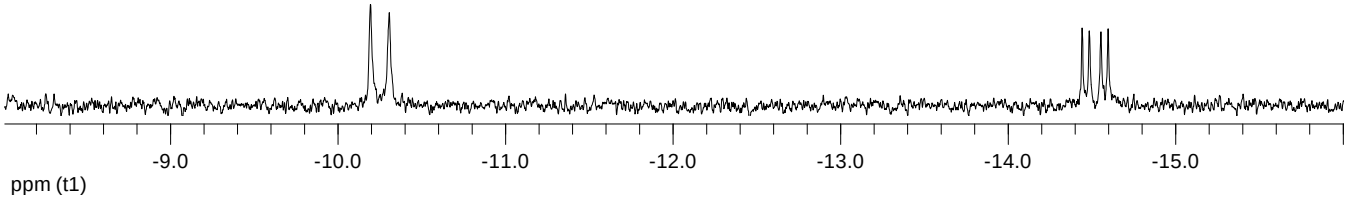

7. Reference

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