Supporting Information for:

Methylerythritol Cyclodiphosphate (MEcPP) in Deoxyxylulose Phosphate Pathway: Synthesis from an Epoxide and Mechanisms

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1. General information, Materials, and Methods

For enzymatic reactions, NMR spectra were recorded on either 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. High-resolution mass spectrometry data were obtained at the Boston University Chemical Instrumentation Center using a Waters Q-TOF mass spectrometer. All other reagents and solvents were used as supplied by Sigma-Aldrich and Pharmco without further purification. For the 2D-NMR characterization of MEcPP generated from Epoxy-HMBPP, the data were acquired at 30 ^{0}C on a 500 MHz (^{1}H) NMR spectrometer, on an "inverse detection" penta-probe (^{1}H,^{31}P,^{15}N,^{31}P,^{2}H) that is equipped with actively-shielded *z*-gradient coils. The data were processed using nmrPipe software and analyzed using nmrDraw software. Spectra were referenced in the ^{31}P dimension with respect to triphenylphosphine oxide (TPPO) as an external standard.

Epoxy-HMBPP was synthesized as previously reported (*J. Am. Chem. Soc*. **2009**, *131*, 17734-17735). Note: extensive water washing during the preparation of cellulose for purification is essential to prevent contamination of the epoxide diphosphate with inorganic salts. Alternatively, cellulose equilibrated only with elution buffer (NH_4HCO_3) provides the diphosphate free of contaminating inorganic salts.

Over-expression and purification of IspG were briefly reported on *Biochem.* **2009**, *48*, 10483-10485. The detailed procedure was described as belowing.

IpsG protein over-expression. BL21(DE3) competent cells were transformed with both IspG/pASK-IBA3+ and pDB1281 plasmids. The plasmid pDB1281 contains the *isc*-operon involved in iron-sulfur cluster maturation. From a single colony, an overnight culture of *E. coli* BL21(DE3)-IspG/pASK-IBA3+-pDB1281 was grown at 37 °C in LB medium supplemented with kanamycin (50 μg/mL), ampcilin (100 μg/mL). In the next day, the overnight culture was diluted 200-fold into fresh LB medium supplemented

with kanamycin (50 μg/mL), ampcilin (100 μg/mL) and 0.1 mM $Fe(NH_4)_2(SO_4)_2$. When the OD_{600} reached 0.1, L-arabinose was added to 0.2 g/L to induce isc-operon expression. When the OD_{600} reached 0.6, the incubation temperature was lowered to 25 °C and anhydrotetracycline was added to a final concentration of 200 ng/mL to induce IspG expression. After incubation for an additional 15 h at 25 °C, cells were harvested by centrifugation (8000*g*, 5 min) at 4 °C, washed with Tris-HCl buffer (20 mM, pH 7.5), then collected and stored at −80 °C for future use.

IpsG protein purification. Protein purification was carried out anaerobically at coy-chamber. Frozen cells (10 g) of BL21(DE3) *E. coli* harboring IspG/pASK-IBA3+ and pDB1281 constructs was thawed in 50 ml of anaerobic buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5) in an anaerobic coy chamber. Lysozyme (1.0 mg/mL) and DNase I (100 U/g cell) were then added to the cell suspension, and the mixture was incubated on ice for 40 min with gentle agitation. The cells were disrupted by sonication (10 cycles of 30 s bursts). The supernatant and the cell debris were separated anaerobically by centrifugation (20,000 g) at 4 °C for 20 min. To the supernatant, streptomycin sulfate was added to a final concentration of 1% (w/v%), and the mixture was mixed on ice for 30 min with gentle agitation. The white DNA precipitate was then separated by centrifugation (20,000 g) for 40 min. The resulting supernatant was mixed with the Strep-Tactin resin (20 mL) and incubated on ice for 30 minutes. After the cell lysate was removed by gravity, the column was washed with washing buffer (100 mM Tris-HCl, 150 mM NaCl, pH 7.5). The recombinant IspG protein was eluted with the elution buffer (2.5 mM desthiobiotin in 50 mM Tris-HCl buffer, pH 7.5). The purified IspG was stored in liquid nitrogen until use.

IspG NMR activity assay. The IspG NMR assay was set up in a Coy-Chamber and monitored by ^{1}H-NMR under anaerobic conditions. A routine NMR steady-state IspG activity assay mixture typically contained various amounts of IspG depending on its activity, 1.0 mM MEcPP (or 1.0 mM of Epoxy-HMBPP), in 100 mM Tris-HCl, pH 8.0 with a total volume of 400 μL with 50% D_2O.

IspG reaction product purification. The IspG reaction mixture was filtered through a membrane with 10 kDa molecular weight cut-off and then purified by HPLC. MEcPP has a retention time of ~ 5 min. HPLC purification was carried out on SHIMADZU CBM-20A system using a Waters Atlantis® Prep T3 column (10 x 250 mm) with the following method (100% A, 1-15 min; 100% A-5% B, 15-20 min; 5% B-20% B, 20-30 min; 20% B, 30-35 min; 20% B-100% A, 35-40 min; 100% A, 40-60 min with a flow rate of 3 mL/min. Solvent A: 50 mM ammonium bicarbonate; Solvent B: acetonitrile).

2. Figure 1S. ^{1}H-NMR and High-resolution mass spectrometry of MEcPP purified from the reaction mixture shown in Figure 2B.

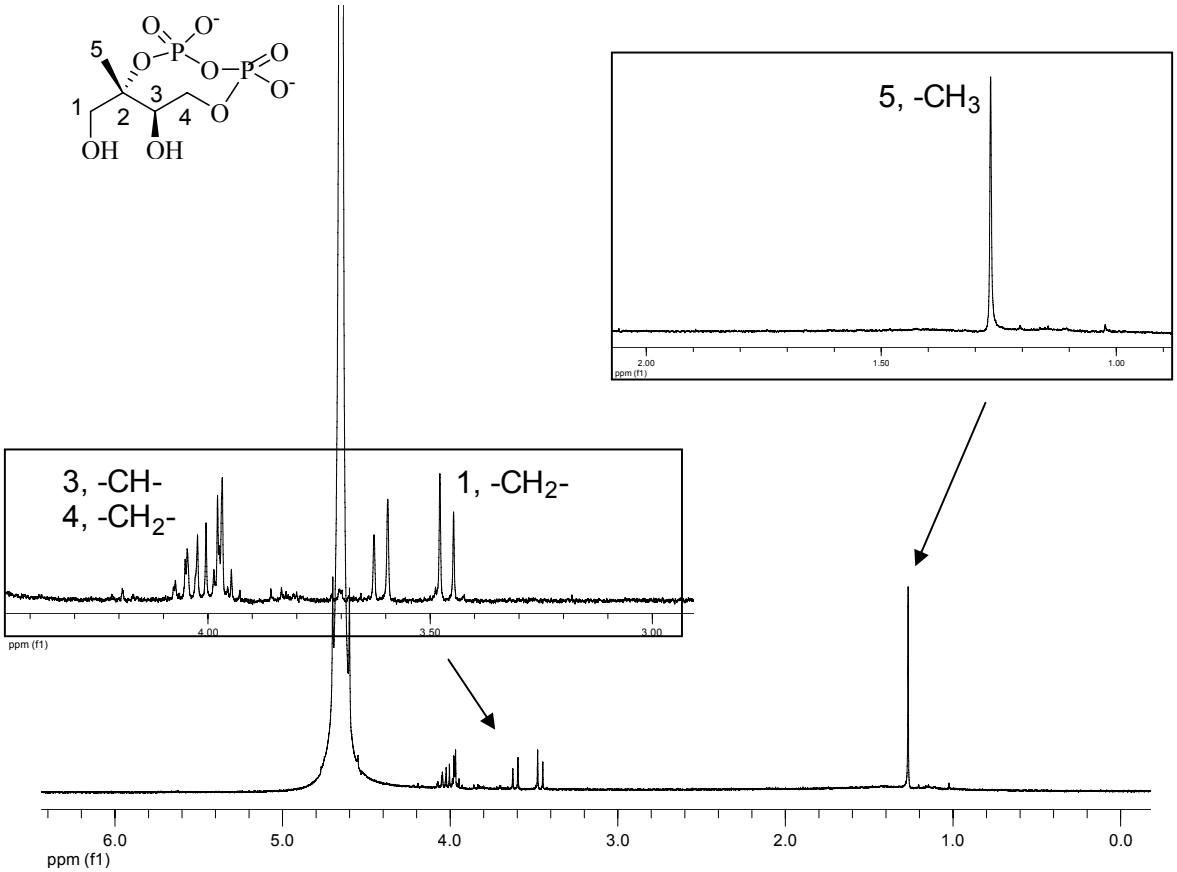

Figure 1S-1. The ^{1}H-NMR spectrum of MEcPP purified from the reaction mixture shown Figure 2B. ^{1}H NMR (400 MHz, D_2O): δ 3.96-4.05 (m, 3 H), 3.60 (d, J = 12.4 Hz, 1 H), 3.46 (d, J = 12.4 Hz, 1 H), 1.26 (s, 3 H).

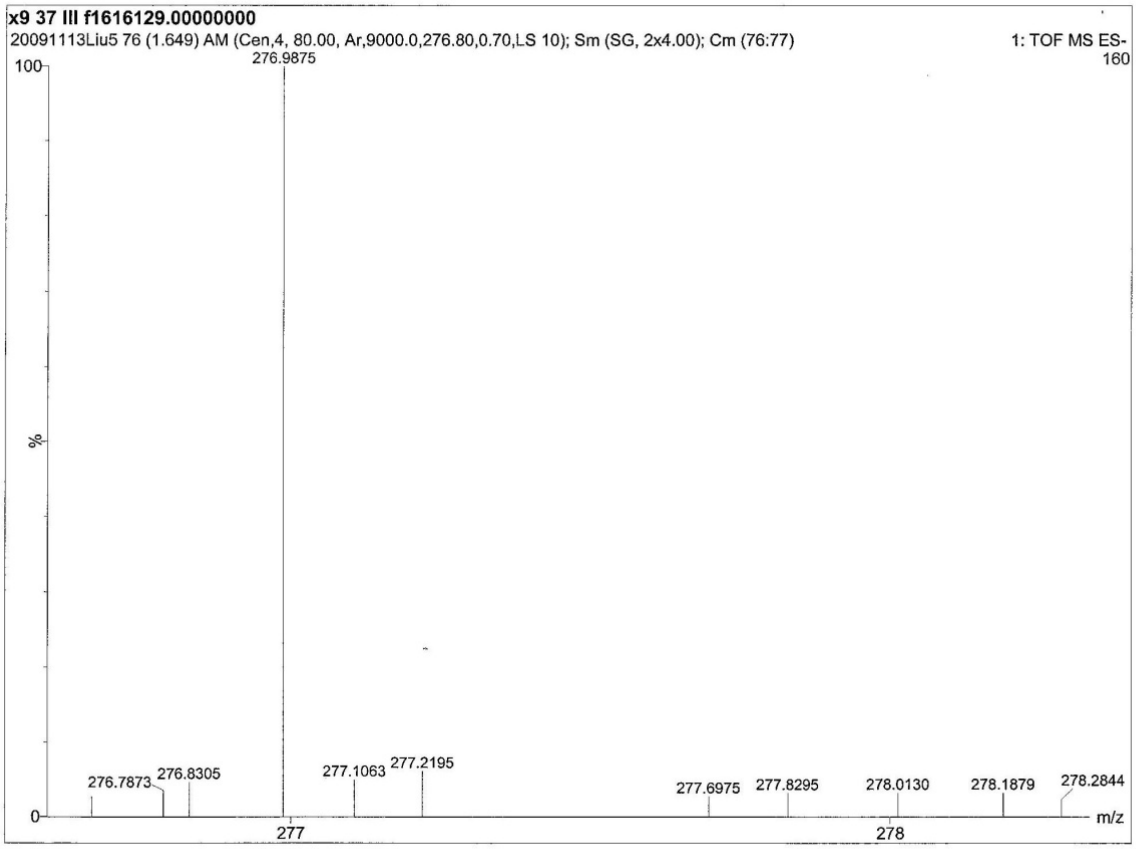

Figure 1S-2. High resolution ESI-MS of MEcPP purified from the reaction mixture shown in Figure 2B reaction mixture (calculated molecular weight in the $[M-H]^-$ form for MEcPP was 276.9878, and found at 276.9875).

3. Figure 2S. 2D-NMR characterization of MEcPP (1) produced from Epoxy-HMBPP (2).

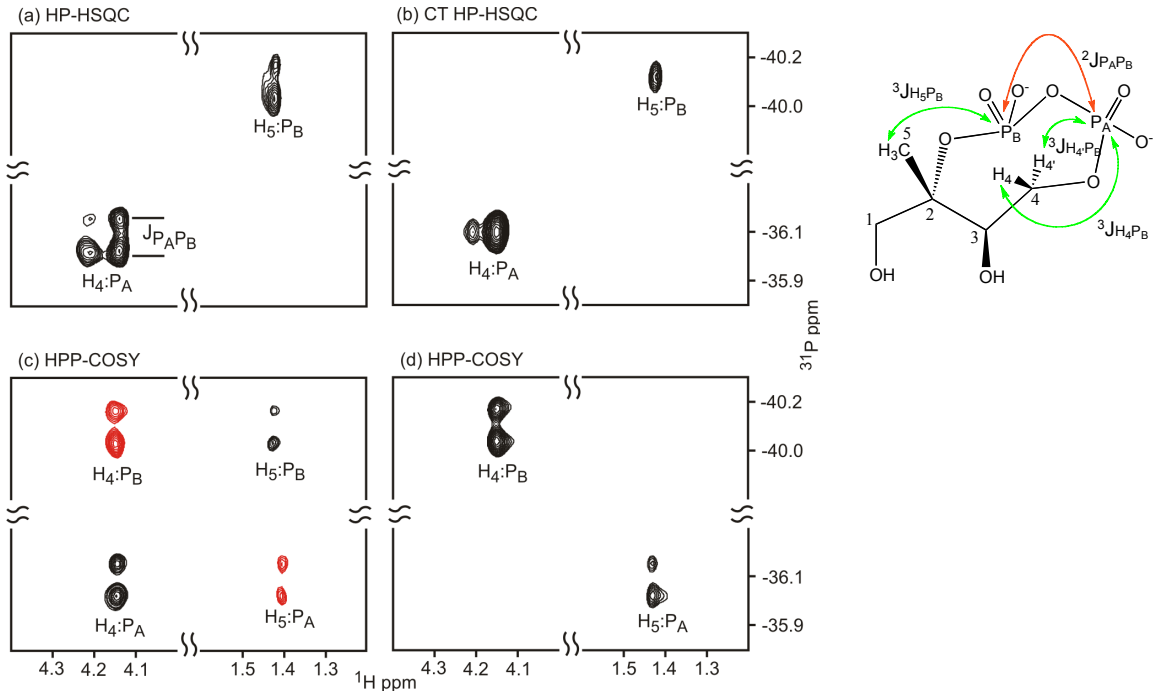

Figure 2S. 2D H,P-NMR of the sample reported in Figure 2B. 2D-NMR spectra of MEcPP (**1**) produced from Epoxy-HMBPP (**2**) by $[4Fe\text{-}4S]^{2+}$- containing IspG in the absence of extra reductants

a) ^{1}H-^{31}P-HSQC spectrum of MEcPP. J_{PP} coupling is observed in the ^{31}P dimension indicating the presence of a diphosphate linkage.

b) Constant time spectrum resolving peaks in the ^{31}P dimension.

c) ^{1}H-^{31}P-^{31}P COSY spectrum with 50% magnetization transfer between P_A and P_B.

d) ^{1}H-^{31}P-^{31}P COSY spectrum with 100% magnetization transfer between P_A and P_B. ^{1}H-^{31}P-^{31}P COSY analysis confirms the presence of a diphosphate linkage through P_A and P_B to unambiguously establish the H_4-P_A-P_B-H_5 network.

4. Figure 3S. Kinetic studies of [4Fe-4S]$^{2+}$ containing IspG catalyzed Epoxy-HMBPP (2) to MEcPP (1) conversion.

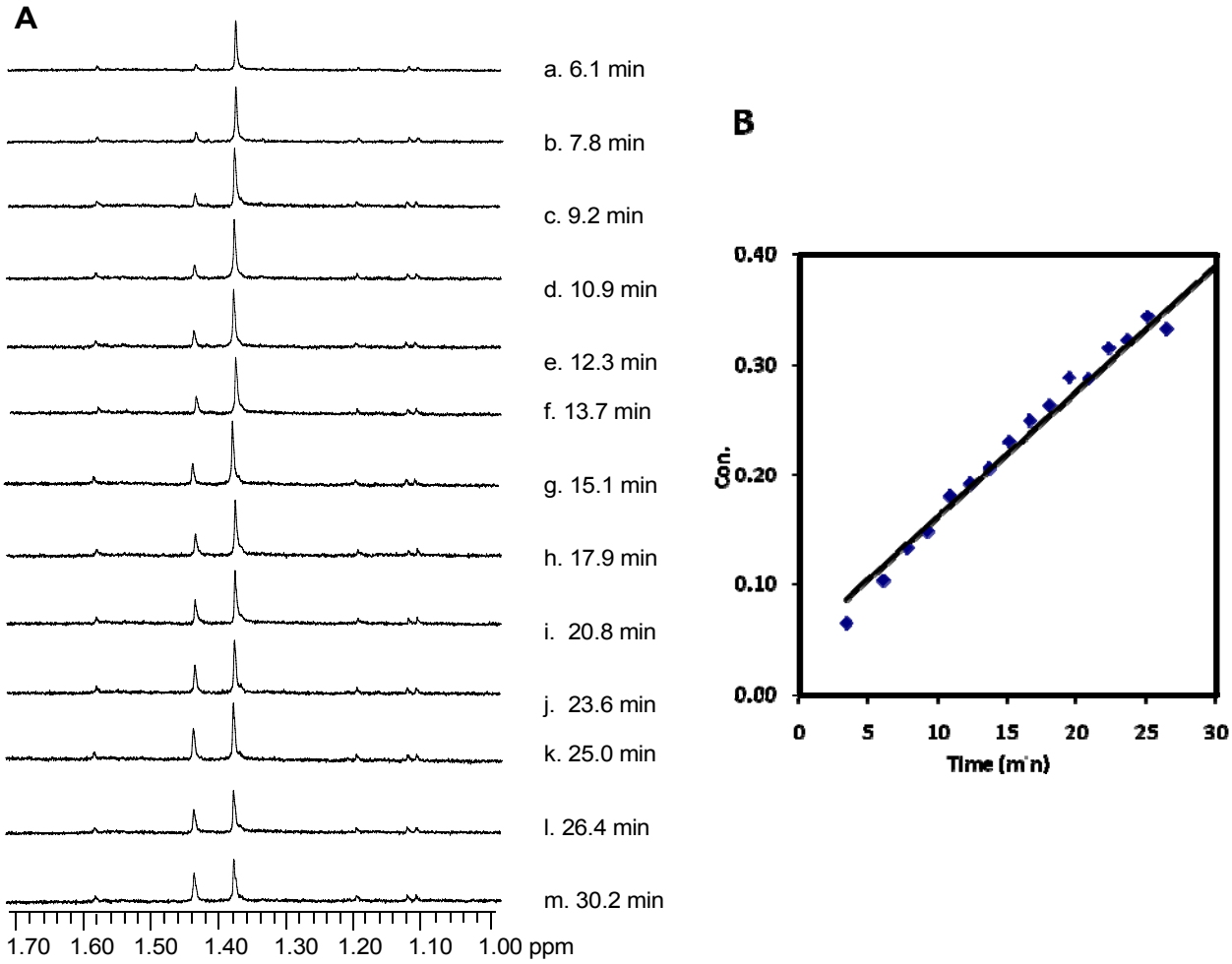

Figure 3S. Kinetic study of IspG-catalyzed Epoxy-HMBPP (**2**) to MEcPP (**1**) conversion in the absence of extra reductants. A) The time course for one of the kinetic experiments (5.0 μM of [4Fe-4S]$^{2+}$ containing IspG, 1.0 mM Epoxy-HMBPP (**7**) in 100 mM Tris, pH 8.0 at 37^{o}C). The ^{1}H-NMR spectra were recorded at 37^{o}C (the C_5 methyl group chemical shifts collected at this condition for both Epoxy-HMBPP and MEcPP were shifted down-field by 0.18 ppm relative to those in Figure 2B, whose spectrum was collected at 25^{o}C). B) Calculation of the rate of conversion of Epoxy-HMBPP to MEcPP.

5. Figure 4S. IspG concentration dependence of the Epoxy-HMBPP (2) to MEcPP (1) conversion.

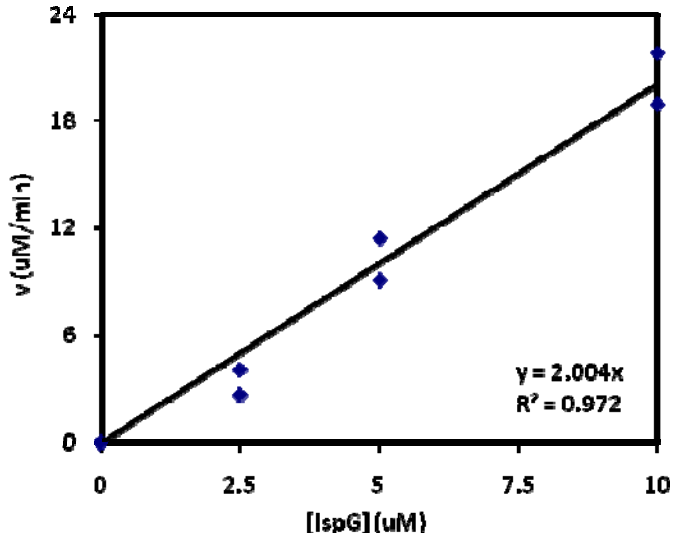

Figure 4S. IspG concentration dependence of the Epoxy-HMBPP (**2**) to MEcPP (**1**) conversion at a saturating Epoxy-HMBPP concentration (1.0 mM). All steps were carried out under anaerobic conditions in an NMR tube at 37°C. Assay mixtures contained 100 mM Tris-HCl, pH 8.0, $[4Fe\text{-}4S]^{2+}$-containing IspG at the indicated concentrations, and 1.0 mM Epoxy-HMBPP (**2**).

6. Examining the presence of an IspG-mediated equilibrium between Epoxy-HMBPP and MEcPP.

To further examine the relationship between MEcPP and Epoxy-HMBPP in IspG-catalyzed reaction, we also examine the option of having MEcPP and Epoxy-HMBPP existing in an equilibrium. To examine this possibility, alkaline phosphatase was introduced to perturb the equilibrium (Figures 5S & 6S).[16,23,24] MEcPP is stable to phosphatase, and there is no sign of MEcPP degradation at 37 °C for up to 3 days (Figure 6D). However, monophosphates and linear diphosphates readily undergo rapid dephosphorylation by phosphatase (Figure 6B). We hypothesized that if IspG does facilitate an equilibrium between MEcPP and Epoxy-HMBPP, the introduction of phosphatase will shift the equilibrium to the Epoxy-HMBPP side by converting Epoxy-HMBPP to alcohol **5** or monophosphate **4** (Figure 5S). Under such a condition, MEcPP could be consumed to produce alcohol **5** through the Epoxy-HMBPP intermediate if enough IspG and alkaline phosphatase are included in the reaction mixture.

Compounds **2**, **4**, **5** are easily distinguished by their C_5 methyl group chemical shift differences in the ^{1}H-NMR spectrum (Figure 6E). After the activity from Calf Intestinal Alkaline phosphatase (CIP) was calibrated using Epoxy-HMBPP as the substrate, it was then used to perturb an equilibrium between Epoxy-HMBPP and MEcPP (Figure 5S). Briefly, Epoxy-HMBPP was incubated with $[4Fe\text{-}4S]^{2+}$-containing IspG. When the reaction reached the point of ~ 50% conversion to MEcPP (Figure 6A), alkaline phosphatase was added in 100-fold excess relative to IspG (in terms of calibrated reaction units), and the time course was monitored by ^{1}H-NMR from 5 min up to 1 day (Figure 6B-C). As shown in Figure 6B, the alkaline phosphatase efficiently dephosphorylates Epoxy-HMBPP to alcohol **13** within 5 min. However, under these conditions, the ratio of MEcPP (**1**) to **5** remains constant over a long period examined (1 day, Figure 6C). The results from the alkaline phosphatase experiments suggest that

Epoxy-HMBPP is either not produced from MEcPP or the rate of this conversion is very slow and is below our ^{1}H-NMR assay detection limit.

Figure 5S. Experimental design to examine the presence of an IspG-mediated equilibrium between MEcPP and Epoxy-HMBPP.

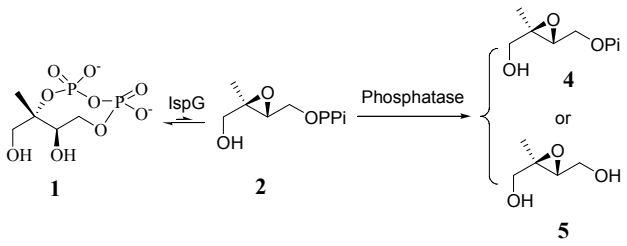

Figure 6S. Phosphatase assay for studying the presence of an IspG-mediated equilibrium between Epoxy-HMBPP and MEcPP. ^{1}H-NMR of the Epoxy-HMBPP and MEcPP C_5 methyl group region for: A) a reaction mixture containing 10 μM $[4Fe\text{-}4S]^{2+}$ containing IspG with 1.0 mM of Epoxy-HMBPP (**2**). NMR spectrum was acquired after the mixture was incubated at 37 °C for 30 min, which resulted in roughly 50% conversion of Epoxy-HMBPP (**2**) to MEcPP (**1**); B) Into sample A, 100 × of alkaline phosphatase was added and ^{1}H-NMR was acquired in less than 5 min. Under such a condition, all Epoxy-HMBPP (**2**) was dephosphorylated to the alcohol (**5**); C) Into sample A, 100 × of alkaline phosphatase was added and ^{1}H-NMR was recorded after 1 day; D) Pure 1.0 mM MEcPP in the reaction buffer with the same amount of phosphatase at 37 °C for 3 days; E) a mixture of synthetic **2**, **4**, **5** in a ration of 1:0.5:1 in the IspG reaction buffer.