Supporting Information

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SI Text

Materials. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) and were used as provided. The plasmid encoding *Escherichia coli* GcpE was kindly provided by Professor Pinghua Liu. The plasmid encoding *Thermus thermophilus* GcpE was kindly provided by Drs. H. Jomaa and J. Wiesner. XL2-Blue ultracompetent cells and BL-21(DE3) competent cells were purchased from Stratagene (La Jolla, CA).

***E. coli* GcpE Protein Production and Purification.** BL21 DE3 cells harboring the *E. coli* GcpE construct were grown in Luria-Bertani media at 37 °C until the OD_{600} reached ~0.4–0.6. Induction was performed with 200 μg/L anhydrotetracycline at 20 °C for 15 hours. Cells were harvested by centrifugation at 9000 rpm for 8 mins, then stored at −80 °C. Cell pellets were resuspended and lysed in B-PER (Thermo Scientific, Rockford, IL) protein extraction reagent for about two hours at 4 °C, then the lysate was centrifuged at 250,000 rpm, for 20 mins. The supernatant was collected and loaded onto a IBA Strep-tag column equilibrated with buffer W (100 mM Tris•HCl,150 mM NaCl, pH 8.0). After washing with buffer W, protein was eluted using buffer E (buffer W containing 2.5 mM desthiobiotin). Fractions were collected and dialyzed in pH 8.0 buffer containing 50 mM Tris•HCl, 150 mM NaCl, 5% glycerol, and 1 mM DTT, three times. The purified protein was flash-frozen in liquid nitrogen, then stored at −80 °C until use.

***T. thermophilus* GcpE Protein Production and Purification.** TOP10-F'(Invitrogen) cells harboring the *T. thermophilus* GcpE construct were grown in Luria-Bertani media containing 0.2% glucose, 0.5 mM $CaCl_2$,1 mM $MgCl_2$ and 100 mg/L ampicillin at 37 °C until the OD_{600} reached ~0.6–0.8. Induction was performed with 1 mM IPTG, followed by incubation at 32 °C, overnight. Cells were harvested by centrifugation at 9000 rpm for 8 mins, then stored at −80 °C. Cell pellets were resuspended and lysed in B-PER (Thermo Scientific, Rockford, IL) protein extraction reagent for about two hours at 4 °C, then the lysate was centrifuged at 250,000 rpm, for 20 mins. The supernatant was collected and loaded onto a Ni-NTA column equilibrated with 10 mM imidazole in pH 8.0 buffer containing 50 mM Tris•HCl and 150 mM NaCl. After washing with 40 mM imidazole, protein was eluted using an imidazole gradient (40–500 mM) in pH 8.0 buffer containing 50 mM Tris•HCl and 150 mM NaCl. Fractions were collected and dialyzed in pH 8.0 buffer containing 50 mM Tris•HCl, 150 mM NaCl, 5% glycerol, and 1 mM DTT, three times. The purified protein was flash-frozen in liquid nitrogen, then stored at −80 °C until use.

Protein Reconstitution. Either *E. coli* or *T. thermophilus* GcpE proteins were transferred into a Coy Vinyl Anaerobic Chamber after being degassed on a Schlenk line. All the following steps were performed inside the anaerobic chamber with an oxygen level <2 ppm. In a typical reconstitution experiment, 10 mM DTT and ~0.5 mg of elemental sulfur were added to 3 mL 0.6 mM protein solution in a pH 8.0 buffer containing 50 mM Tris•HCl, 150 mM NaCl and 5% glycerol. After stirring for 1.5 hours, $FeCl_3$ was then added from a 30 mM stock solution, slowly, to 6 equivalents. After 3 hours, an aliquot of the solution was centrifuged and a UV-VIS spectrum recorded. The A_{410}/A_{280} ratio was typically ~0.4. The protein was then desalted by passing through a PD10 column. The reconstituted protein was concentrated by ultrafiltration, and the protein concentration determined by using a Bio-Rad (Hercules, CA) Protein Assay kit.

Enzyme Inhibition Assays. All assays were performed anaerobically, at room temperature, according to Altincicek et al. (1), with minor modifications. To a pH 8.0 buffer solution containing 50 mM Tris-HCl, 150 mM NaCl, and 5% glycerol, sodium dithionite was added to 0.4 mM, methyl viologen was added to 2 mM, and *E. coli* GcpE was added to 72 nM. For enzyme assays, various amounts of MEcPP were added and the reactions monitored at 732 nm. The initial velocities were fit by using the Michaelis-Menten equation using OriginPro 8 (OriginLab Corporation, Northampton, MA). For inhibition assays, various concentrations of inhibitor were added and incubated for 10 min, prior to addition of 140 μM MEcPP. Initial velocities at different inhibitor concentrations were then plotted as dose-response curves, fitted to the following equation:

$$y = \frac{1}{1 + (\frac{x}{\mathrm{IC}_{50}})^{\mathrm{slope}}}$$

where y is the fraction inhibition and x is the inhibitor concentration, from which the IC_{50} value was determined. K_i was then estimated from the IC_{50} value by using the Cheng-Prusoff equation (2):

$$K_i = \frac{\mathrm{IC}_{50}}{1 + \frac{[S]}{K_M}}$$

where [S] is the HMBPP concentration, and K_M is the Michaelis constant.

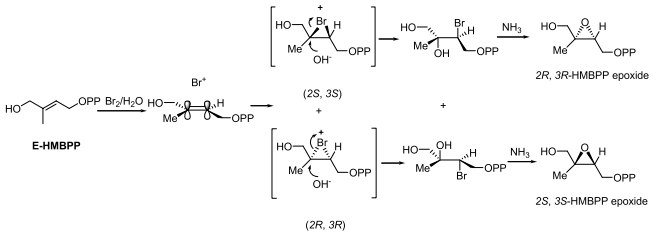
Continuous-Wave EPR Spectroscopy. All EPR samples were prepared inside a Coy Vinyl Anaerobic Chamber with an oxygen level <2 ppm. Samples for EPR spectroscopy were typically 0.2–0.4 mM in GcpE, and glycerol was added as a glassing agent to 20% (v/v). 20 equivalents of sodium dithionite were added as a reducing agent, and ligands (HMBPP, MEcPP, IPP, HMBPP epoxide, propargyl diphosphate) were added to 10 equivalents. To trap the reaction intermediate "X" from MEcPP, samples were frozen in liquid nitrogen after 1 min incubation with MEcPP at room temperature. To trap the reaction intermediate "X" from HMBPP epoxide, *E. coli* GcpE was incubated with HMBPP epoxide at room temperature for 2 min before being frozen in liquid nitrogen, while *T. thermophilus* GcpE (at 55 °C) was frozen in liquid nitrogen ~10 sec after HMBPP epoxide injection. All samples were stored in liquid nitrogen prior to spectroscopic investigation. EPR spectra were collected at X-band using a Varian E-122 spectrometer together with an Air Products (Allentown, PA) helium cryostat. Data acquisition parameters were typically: field center = 3250 G; field sweep = 800 G; modulation = 100 kHz; modulation amplitude = 5 G; time constant = 32 ms; 60 sec per scan; and 6 sec between each scan.

Samples for ENDOR and HYSCORE Spectroscopy. Samples for ENDOR and HYSCORE spectroscopy were prepared in the same way as those for EPR spectroscopy but were typically 1.5 ~ 2 mM in *T. thermophilus* GcpE protein.

Synthetic Aspects. *General methods.* All reagents used were purchased from Aldrich (Milwaukee). The purities of all compounds

investigated were confirmed by using ^{1}H and ^{31}P NMR spectroscopy at 400 MHz using a Varian (Palo Alto, CA) Unity spectrometer. HMBPP was synthesized according to a literature method (3).

1-Hydroxy-2-methyl-2, 3-epoxybutanyl-4-diphosphate (HMBPP epoxide). 1-Hydroxy-2-methyl-2, 3-epoxybutanyl-4-diphosphate (HMBPP epoxide) was synthesized following the scheme shown below:



1, 2-Dihydroxy-3-bromo-2-methylbutanyl-4-diphosphate (HMBPP bromohydrin). To a solution of 5.0 mg HMBPP in 0.50 mL water was added 3–5 drops bromine water. The mixture was left to react at room temperature for 10–20 minutes, then was lyophilized to afford a white solid. ^{1}H NMR (400 MHz, D_2O): δ 1.14 (*s*, 3H), 3.43 (*d*, 1H, J = 12.0 Hz), 3.58 (*d*, 1H, J = 12.0 Hz), 4.04–4.16 (*m*, 2H), 4.26–4.31 (*m*, 1H); ^{31}P NMR (162 MHz, D_2O): δ −10.62 (*d*, J = 20 Hz), −9.91 (*d*, J = 20 Hz).

1-Hydroxy-2-methyl-2, 3-epoxybutanyl-4-diphosphate (HMBPP epoxide). To a solution of 1, 2-dihydroxy-3-bromo-2-methylbutanyl-4-diphosphate (5.0 mg) in 0.5 mL water was added 2–3 drops ammonium hydroxide. The mixture was allowed to react at room temperature for 10–20 minutes, then the solution was lyophilized to afford a white solid. ^{1}H NMR (400 MHz, D_2O): δ 1.20 (*s*, 3H), 3.25–3.30 (*m*, 1H), 3.41 (*d*, 1H, J = 12.4 Hz), 3.53 (*d*, 1H, J = 12.8 Hz), 3.77–3.85 (*m*, 1H), 4.08–4.15 (*m*, 1H); ^{31}P NMR (162 MHz, D_2O): δ −9.46 (*d*, J = 22 Hz), −5.36 (*d*, J = 22 Hz).

1-Hydroxy-2-methyl-2, 3[^{17}O]-epoxybutanyl-4-diphosphate (HMBPP epoxide). [^{17}O]-HMBPP epoxide was made in basically the same way as the unlabeled material described above except that 60% ^{17}O-labeled water was used in place of unlabeled water. The mass spectrum (Fig. S5) of the product indicated the following isotopomeric composition: ^{16}O, 24.7%; ^{17}O, 47.7% and ^{18}O, 27.6% (commercial $H_2{}^{17}O$ contains a substantial amount of ^{18}O).

Prop-2-ynyl diphosphate (10) and [$^{13}C_3$]-prop-2-ynyl diphosphate. The syntheses of these two compounds were reported previously (4).

Biosynthesis of 2-C-methyl-D-erythritol-2,4-cyclo-diphosphate (MEcPP). MEcPP was synthesized basically according to the published protocol of Santos et al. (5). *Corynebacterium ammoniagenes* (ATCC 6872) was cultured in a peptone (10 g/L)—yeast extract (3 g/L)—NaCl (5 g/L) medium. Cells were incubated aerobically at 30 °C, with shaking at 225 rpm. When the cells reached their late logarithmic phase of growth (OD_{600} ~ 1.4), the medium was supplemented with benzyl viologen to a final concentration of 50 mg/L, and glucose to 2.0 g/L. Cell growth was allowed to continue for 18 hours, then the cells were harvested by centrifugation. The cell pellets were extracted with 7:3 (v/v) ethanol:water three times, with 40 mL of solvent each time. The supernatants were then subjected to rotary evaporation, and rediluted in deionized water. The crude extract was loaded onto a QAE-Sephadex column preequilibrated with 0.05 M NH_4OAc (pH = 7), washed with ~100 mL of the same buffer, then eluted with a linear gradient of 0.3–1.5 M NH_4OAc. The desired product eluted at approximately 0.8 M NH_4OAc. The fractions were pooled and lyophilized, and MEcPP detected by using a GcpE activity assay (6) and by ^{31}P-NMR spectroscopy (7). The MEcPP-containing fractions were then further purified using cellulose column chromatography, with acetonitrile:isopropyl alcohol:1% NH_4HCO_3 4:2:1 and 1:2:1 as eluants. The 1:2:1 solvent fractions were pooled and lyophilized.

Biosynthesis of [u-^{13}C]-methyl-D-erythritol-2,4-cyclo-diphosphate. [u-^{13}C]-MEcPP was produced biosynthetically by employing a slightly modified version of the protocol used for unlabeled MEcPP. *C. ammoniagenes* was incubated aerobically in peptone (10 g/L)—yeast extract (5 g/L)—NaCl (5 g/L) medium at 30 °C with vigorous shaking. When the OD_{600} of the medium reached ~1.4, benzyl viologen and [u-^{13}C]-D-glucose (Cambridge Isotopes, Cambridge, MA) was added, to final concentrations of 50 mg/L and 1 g/L, respectively. Incubation was then continued for 18 hours. The labeled MEcPP was extracted and purified by using same method as that described for unlabeled MEcPP. Isotope incorporation was close to 100%, as determined by ^{13}C NMR. ^{13}C-NMR (126 MHz, D_2O) δ: 16.63, 16.30 (d); 65.72, 65.77, 66.06, 66.11 (doublet of doublets); 66.98, 67.14, 67.31 (t); 68.25, 68.44, 68.60, 68.76, 68.95 (q); 83.55, 83.87, 84.22, 84.55 (q). ^{31}P-NMR (202 MHz, D_2O) δ: −13.89, −14.00 (d); −9.95, −9.85 (d).

Biosynthesis of [2,3-^{13}C]-2-C-methyl-D-erythritol-2, 4-cyclo-diphosphate. MEcPP with ~30% overall ^{13}C labeling at C2 and/or C3 was prepared using the same protocol as used for making [u-^{13}C]-MEcPP, with minor modification. *C. ammoniagenes* was cultured in peptone—yeast extract—NaCl medium. When the OD reached ~1.4, 50 mg/L benzyl viologen and 0.5 g/L [2-^{13}C]-D-glucose were added. After another 18 hours of incubation, MEcPP was extracted and purified as described above. The overall ^{13}C-enrichment was ~30% ($^{13}C_0$, 65.4%; $^{13}C_1$, 29.4%; $^{13}C_2$, 5.2%), as determined by mass spectrometry. The sample is thus essentially a mixture of [2-^{13}C] and [3-^{13}C]-MEcPP, but for simplicity is referred to in the Text as [2,3-^{13}C]-MEcPP.

Biosynthesis of [u-^{2}H]-2-C-methyl-D-erythritol-2, 4-cyclo-diphosphate. For the biosynthesis of [u-^{2}H]-MEcPP, *C. ammoniagenes* was first "acclimatized" to growth in D_2O. 5 mL aliquots of peptone—yeast extract—NaCl media were made, with varying H_2O/D_2O contents. Cells were first cultured in 100% H_2O media, then when the OD_{600} reached ~0.8, 50 μL of the suspension was transferred to a medium with 25%:75% D_2O:H_2O. Cells were then cultured sequentially in 50%, 75% and 100% D_2O-containing media. For [u-^{2}H]-MEcPP production, we used 1L of medium with 100% D_2O, cells were grown to an OD_{600} ~ 1.4, 50 mg/L benzyl viologen and 1 mg/L [$^{2}H_7$]-D-glucose were added, and after another 18 hours incubation, cells were harvested and MEcPP isolated as described above.

Biosynthesis of [u-^{13}C] E-1-hydroxy-2-methyl-but-2-enyl-4-diphosphate. [u-^{13}C]-HMBPP was prepared from [u-^{13}C]-MEcPP under anaerobic conditions. [u-^{13}C]-MEcPP was transferred into a Coy Vinyl Anaerobic Chamber, then placed into a Wilmad LabGlass (Vineland, NJ) screw-cap NMR tube. *E. coli* GcpE and sodium dithionite were then added, and the reaction monitored by ^{13}C-NMR spectroscopy. When the reaction was complete, the GcpE enzyme was removed by ultrafiltration using a Microcon (Billerica, MA).

1. . Altincicek B et al. (2002) LytB protein catalyzes the terminal step of the 2-*C*-methyl-D-erythritol-4-phosphate pathway of isoprenoid biosynthesis. *FEBS Lett* 532: 437–440.
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4. . Wang W, Wang K, Liu Y-L, No JH, Nilges MJ, Oldfield E (2010) Bioorganometallic mechanism of action, and inhibition, of IspH. *Proc Natl Acad Sci USA* 107:4522–4527.
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6. . Kollas AK et al. (2002) Functional characterization of GcpE, an essential enzyme of the nonmevalonate pathway of isoprenoid biosynthesis. *FEBS Lett* 532:432–436.
7. . Turner DL et al. (1992) Structure determination of a novel cyclic phosphocompound isolated from *Desulfovibrio desulfuricans*. *Biochem J* 285:387–390.

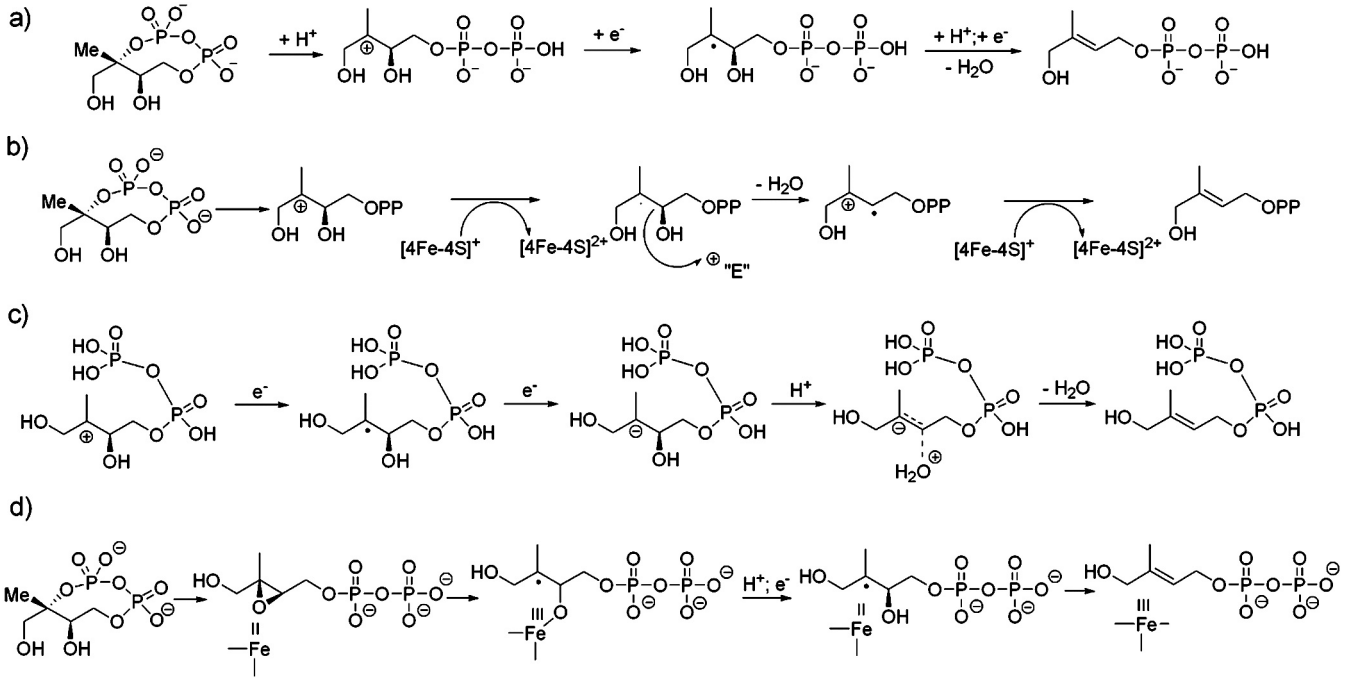

Fig. S1. Proposed GcpE catalytic mechanisms (numbers refer to references in the main text.). *A*) Cation → radical mechanism of Kollas et al. (6). *B*) Cation → radical → cation radical mechanism of Seemann et al. (7). *C*) Cation → radical → anion mechanism of Brandt et al. (8). *D*) Oxirane → radical mechanism of Rohdich et al. (9).

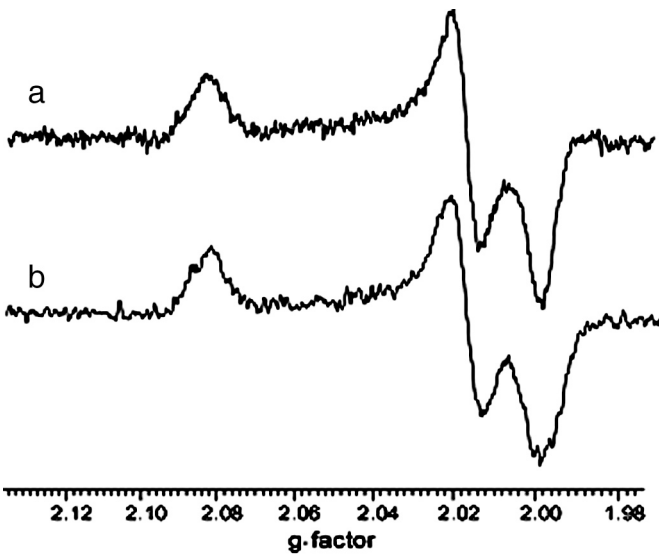

Fig. S2. 9.05 GHz EPR spectra of reaction intermediate X formed with *T. thermophilus* GcpE. (*A*) *T. thermophilus* GcpE + MEcPP, incubated for 30 sec at room temperature. (*B*) *T. thermophilus* GcpE + HMBPP epoxide, incubated for 10 sec at 55 °C. Microwave power = 0.05 mW. Temperature: *A*, 25 K; *B*, 15 K.

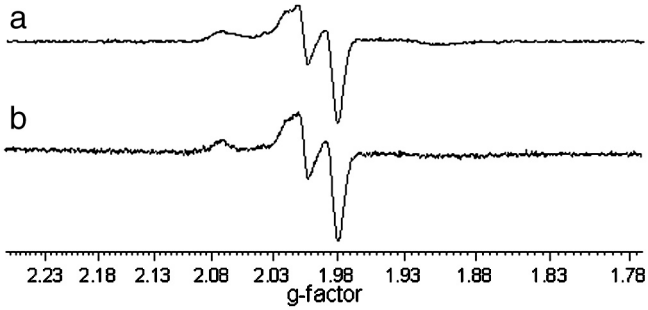

Fig. S3. 9.05 GHz EPR spectra of *T. thermophilus* GcpE + MEcPP (long incubation) or *T. thermophilus* GcpE + HMBPP. (*A*) *T. thermophilus* GcpE + MEcPP, incubated for 40 min. (*B*) *T. thermophilus* GcpE + HMBPP. Microwave power = 0.05 mW, temperature = 15 K.

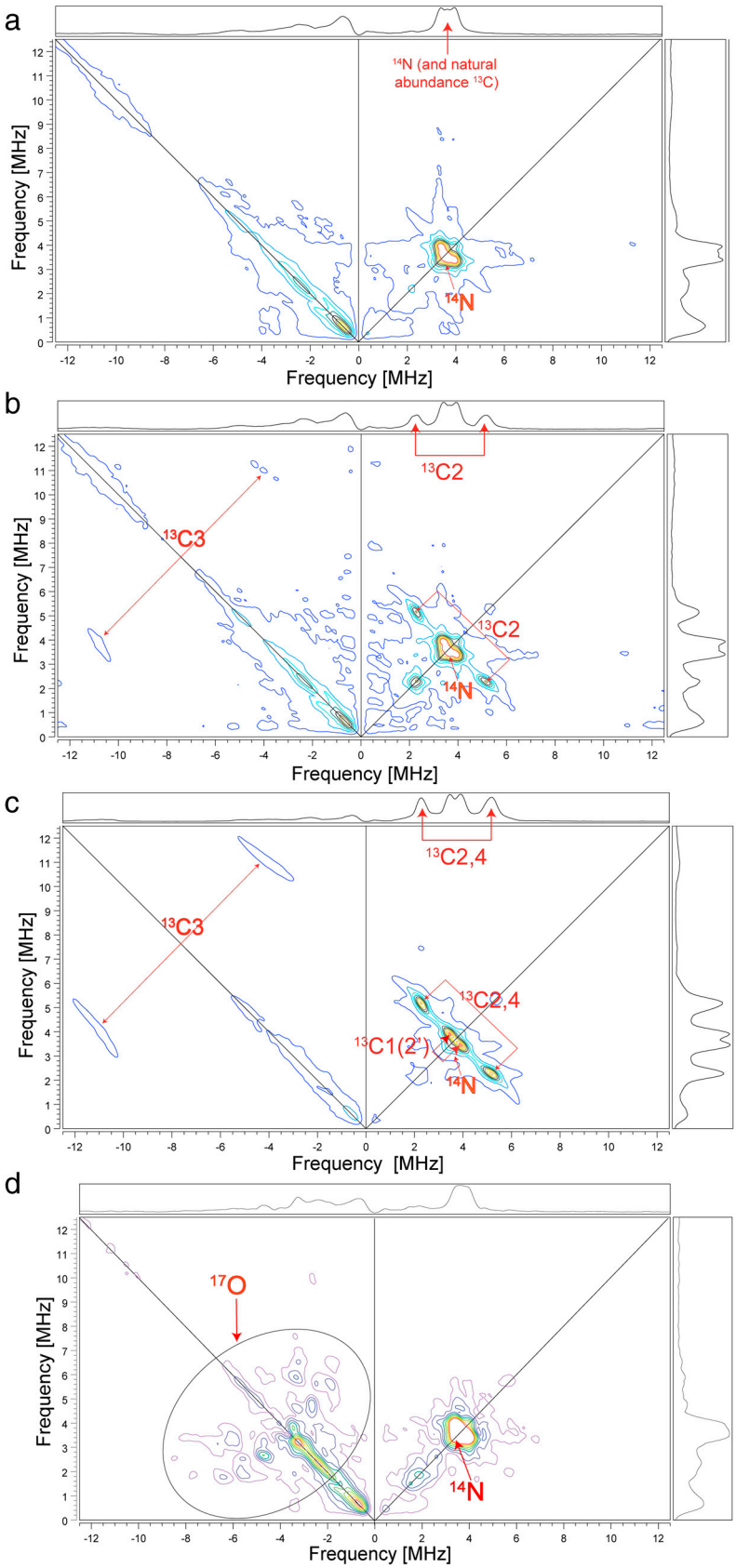

Fig. S4. HYSCORE spectra of *T. thermophilus* GcpE + MEcPP. (*A*) *T. thermophilus* GcpE with unlabeled MEcPP; (*B*) with [2,3-^{13}C]- labeled MEcPP; (*C*) with [u-^{13}C]-labeled MEcPP; (*D*) with [2,3-^{17}O]-HMBPP-epoxide.

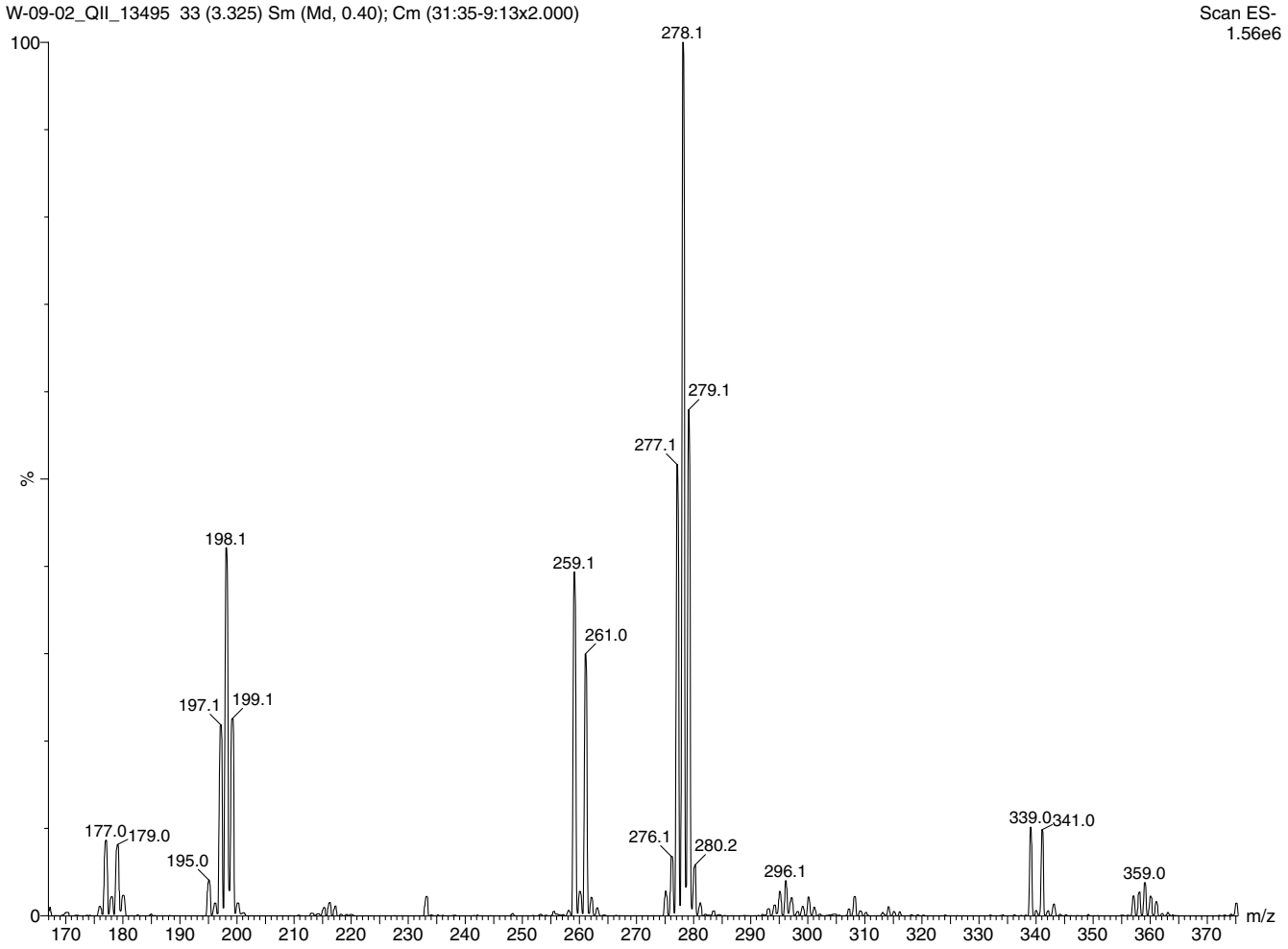

Fig. S5. ESI-MS of ^{17}O-HMBPP epoxide.