Supporting Information for:

Magnesium-specific ring expansion/contraction catalysed by the class II diterpene cyclase from pleuromutilin biosynthesis

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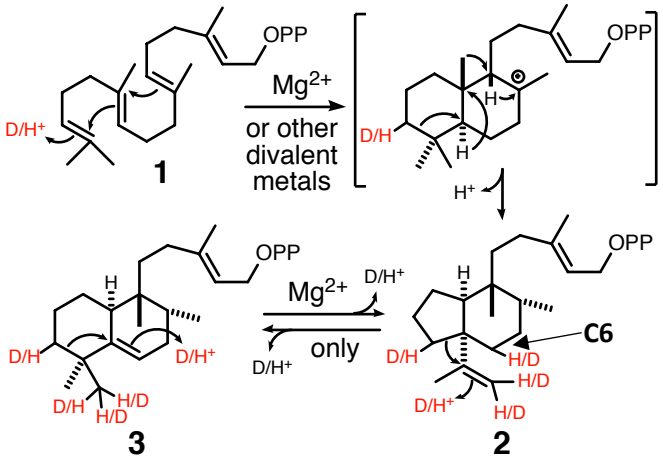
Materials and Methods

Unless otherwise stated all reagents used were purchased from Fischer Scientific. The *Cp*PS mutant constructs used in this study have been previously described (ref. 4).

To obtain protein for *in vitro* assays the *Cp*PS:D649L expression construct (pDEST14 with an incorporated histidine tag and TEV site) was transformed into the C41 strain of *E. coli* (Lucigen) and grown up in 1 L cultures of LB (in 2.8 L baffled fernbach flasks) at 37 °C with shaking at 200 RPM to an O.D. 600 value of 0.3-0.6. These were then transferred to a shaker set at 16 °C and let shake until an O.D. 600 value of 0.6-0.8 before induction with 0.1 mM IPTG. These induced cultures were let shake for 18 hours, then the cells harvested by centrifugation for 45 min. at 5000 *g*. The resulting cell pellets were resuspended in lysis buffer (50 mM Sodium Phosphate (pH 7.4), 500 mM NaCl, 10mM imidazole, 0.1% (v/v) Triton X-100, and 10% (v/v) glycerol). The cells were lysed via sonication and the cell debris pelleted out by centrifugation for 30 min. at 16,000 *g*. The lysate was then run over 1 mL of Ni-NTA agarose resin (Quiagen) equilibrated in lysis buffer using a gravity flow column. Bound protein was washed with wash buffer 1 (50 mM Sodium Phosphate (pH 6.4), 500 mM NaCl, 20mM imidazole, 0.1% (v/v) Triton X-100, and 10% (v/v) glycerol) followed by wash buffer 2 (50 mM Sodium Phosphate (pH 7.4), 150 mM NaCl, 30mM imidazole, and 10% (v/v) glycerol) then eluted with elution buffer (50 mM Sodium Phosphate (pH 7.4), 150 mM NaCl, 250mM imidazole, and 10% (v/v) glycerol). To remove the histidine tag and any co-purifying metals, *Cp*PS:D649L preparations were dialyzed overnight in dialysis buffer 1 (50 mM Sodium Phosphate (pH 7.4), 1 mM DTT, 0.5 mM EDTA, and 10% (v/v) glycerol) with TEV protease. The following morning the protein was let dialyze in dialysis buffer 2 (50mM Sodium Phosphate (pH 7.4), 500mM NaCl, 20mM Imidazole, 10% glycerol) for 3 hours prior to running on a fresh Ni-NTA agarose resin gravity flow column and collecting the protein in the flow through. The protein was then further purified using size exclusion chromatography prior to use in assays.

Unless specified otherwise, all reactions were carried out in assay buffer (50 mM Tris (8.0), 100 mM KCl, 10% (v/v) glycerol) with 50 µM GGPP and various divalent metal ions in the indicated concentrations. These assays were set up by mixing all components in a Pyrex culture tube and equilibrating for 20 min. in a 30 °C water bath. The reaction was then initiated by the addition of *Cp*PS:D649L to a final concentration of 0.1 µM, for a total volume of 1 mL, with mixing by gentle vortexing, and incubation in the 30 °C water bath for 4 hours unless indicated otherwise. DTC activity was then inactivated by 5 min. incubation in a 80 °C water bath. Dephosphorylation was carried out using calf intestinal alkaline phosphatase (CIAP, Promega), with the addition of 150 µL of the manufacturers buffer, followed by 15 µL of CIAP, with shaking at 37 °C overnight. Diterpenoids were then extracted thrice with 1 mL of hexanes, the pooled extract completely dried under N_2, the residue resuspended in 0.1

mL of hexanes, and transferred to inserts for GC-MS analysis, carried out as previously described (ref. 4). As mentioned in the text, various salt concentrations were tested in the context of this reaction with no discernable effects. The following salt conditions were tested: 50mM KCl, 100mM KCl, 250mM KCl, 50mM NaCl, 100mM NaCl, 250mM NaCl, and no added salt.



Scheme S1. Proposed positions in which deuterium can be integrated into the products during reversible reaction in the presence of a deuterated buffer.

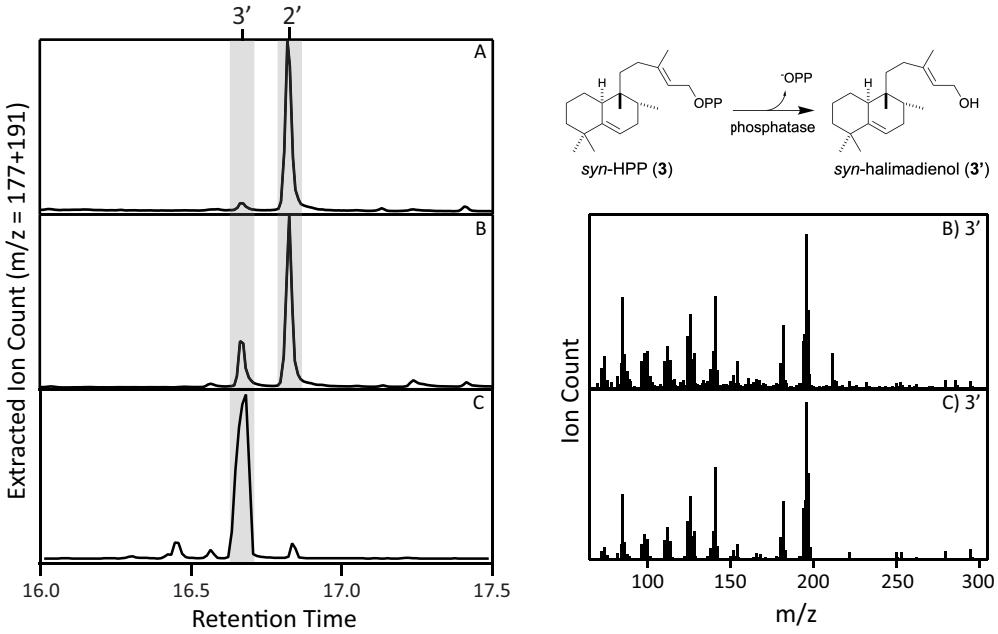


Figure S1. Production of **3** by *Cp*PS:D649L. GC-MS extracted ion count chromatograms of extracts from cultures of *E. coli* engineered to produce **1** and expressing *Cp*PS:D649L either A) 1 or B) 2 days after induction (prime notation indicates the dephosphorylated derivative of the numbered product, as described in the text). C) Authentic standard for **3**. Mass spectra comparison of **3'** observed in B) to that in C).

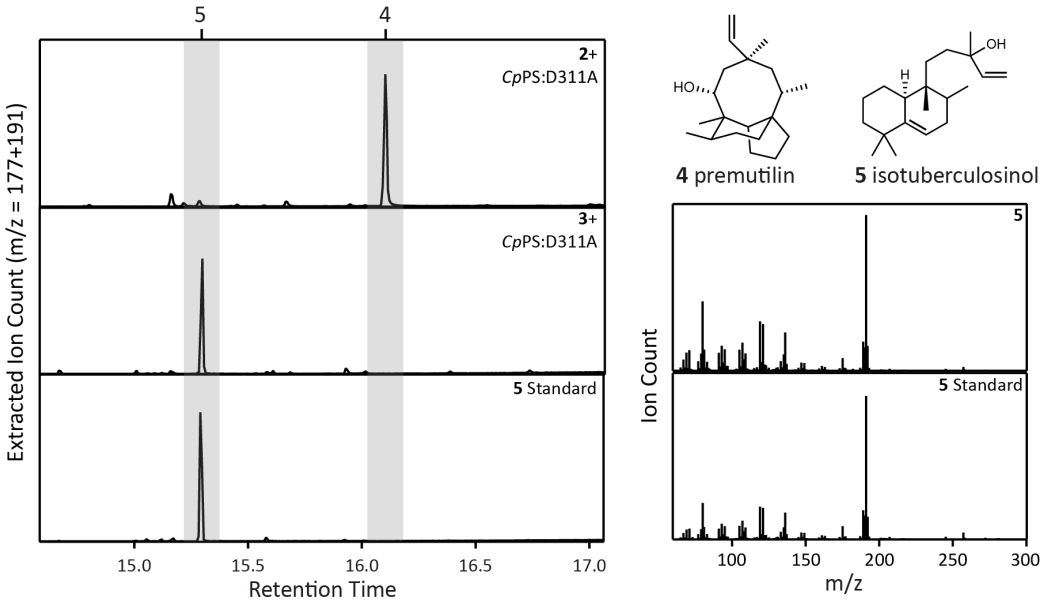


Figure S2. Only **2** is converted to premutilin (**4**) by the class I diterpene synthase activity of *Cp*PS (DTC activity is blocked by the D311A mutation; ref. 4). GC-MS chromatograms from cultures co-expressing *Cp*PS:D311A in *E. coli* also producing **2** (using *Cp*PS:D649L) or **3** (using *Os*CPS4:H501D; ref. 6) only leads to production of **4** from **2**, while the tertiary alcohol derivative of **3** (isotuberculosinol/nosyberkol, **5**) is observed with **3** instead.

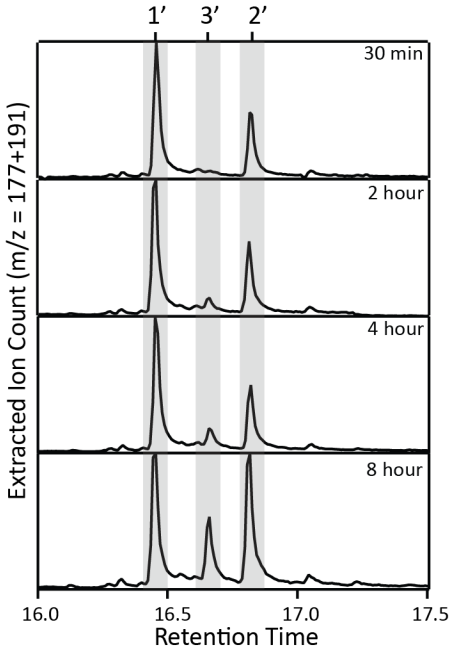


Figure S3. **3** is only observed after accumulation of **2**. GC-MS chromatograms from time course of *Cp*PS DTC activity *in vitro* (using D649L mutant), with subsequent dephosphorylation to the corresponding alcohol (as indicated by prime notation) to enable extraction and GC based analysis.

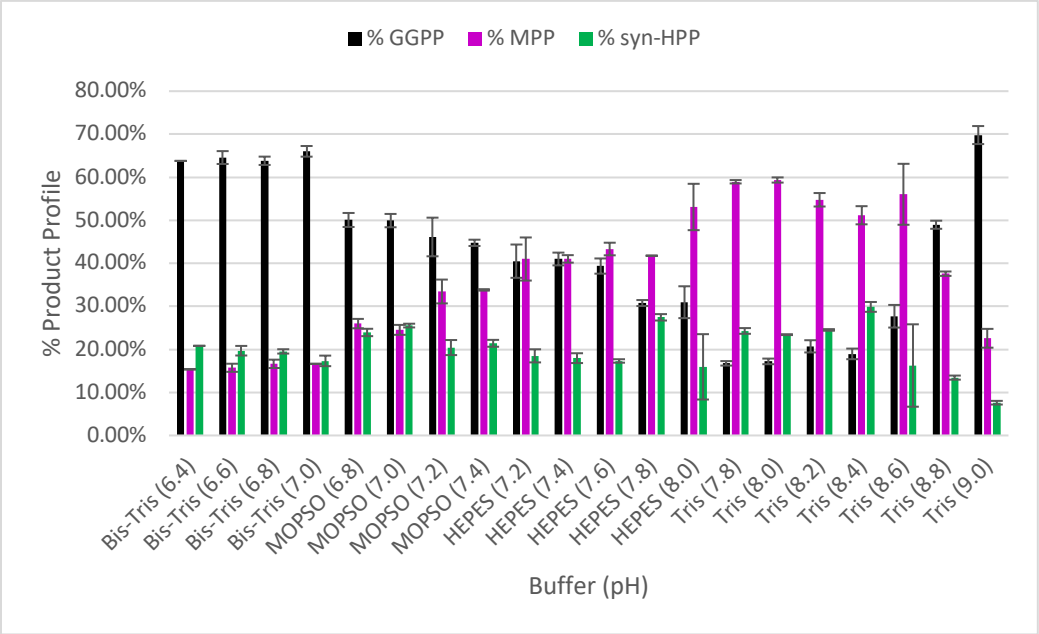


Figure S4. CpPS exhibits the most activity in a Tris buffer at a pH of 7.8 – 8.0. Production of **3** does not seem to be significantly affected by changes in pH.

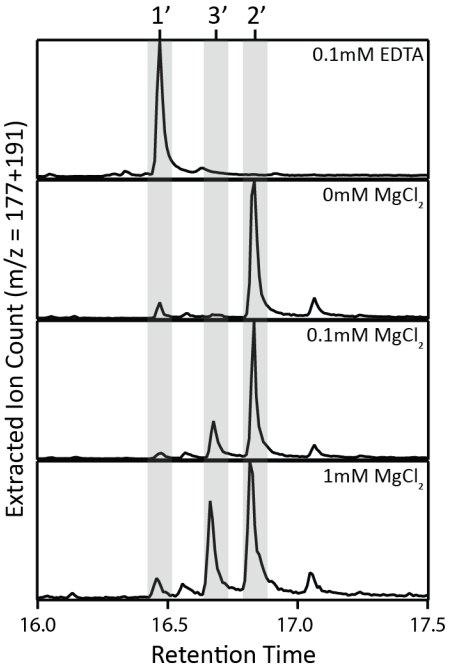


Figure S5. Effect of Mg^{2+} concentration on the rate of production of **3** by *Cp*PS DTC activity (using D649L mutant). GC-MS chromatograms of *in vitro* assays at the indicated concentration of Mg^{2+}, with subsequent dephosphorylation to the corresponding alcohol (as indicated by prime notation) to enable extraction and GC based analysis. Note that there are residual divalent metal ions present, as indicated by activity in the absence of Mg^{2+}, and loss of activity in the presence of 0.1mM EDTA.

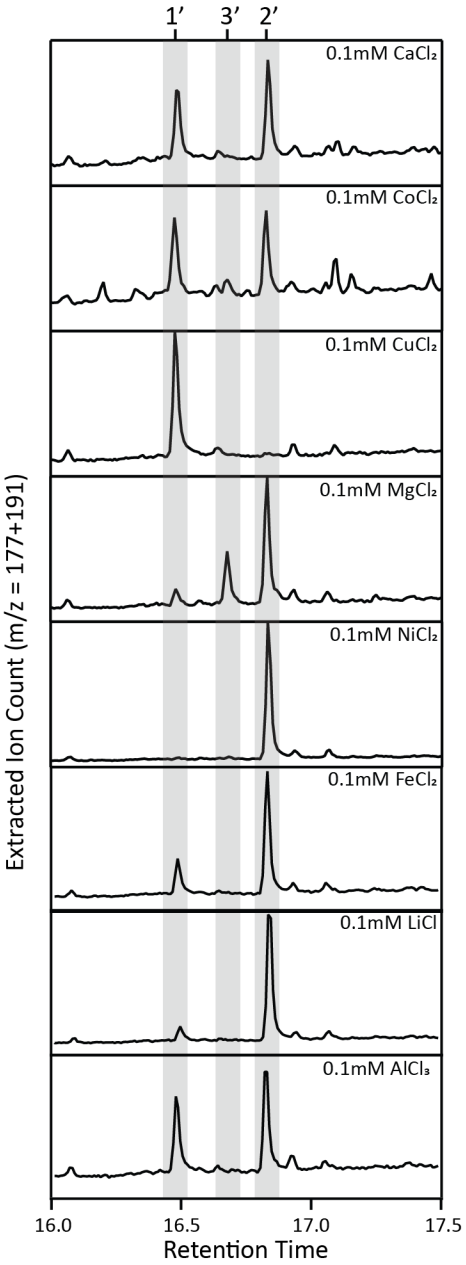


Figure S6. Effect of various metal ion on the production of **3** by *Cp*PS DTC activity (using D649L mutant). GC-MS chromatograms from *in vitro* assays with the indicated metal, with subsequent dephosphorylation to the corresponding alcohol (as indicated by prime notation) to enable extraction and GC based analysis.

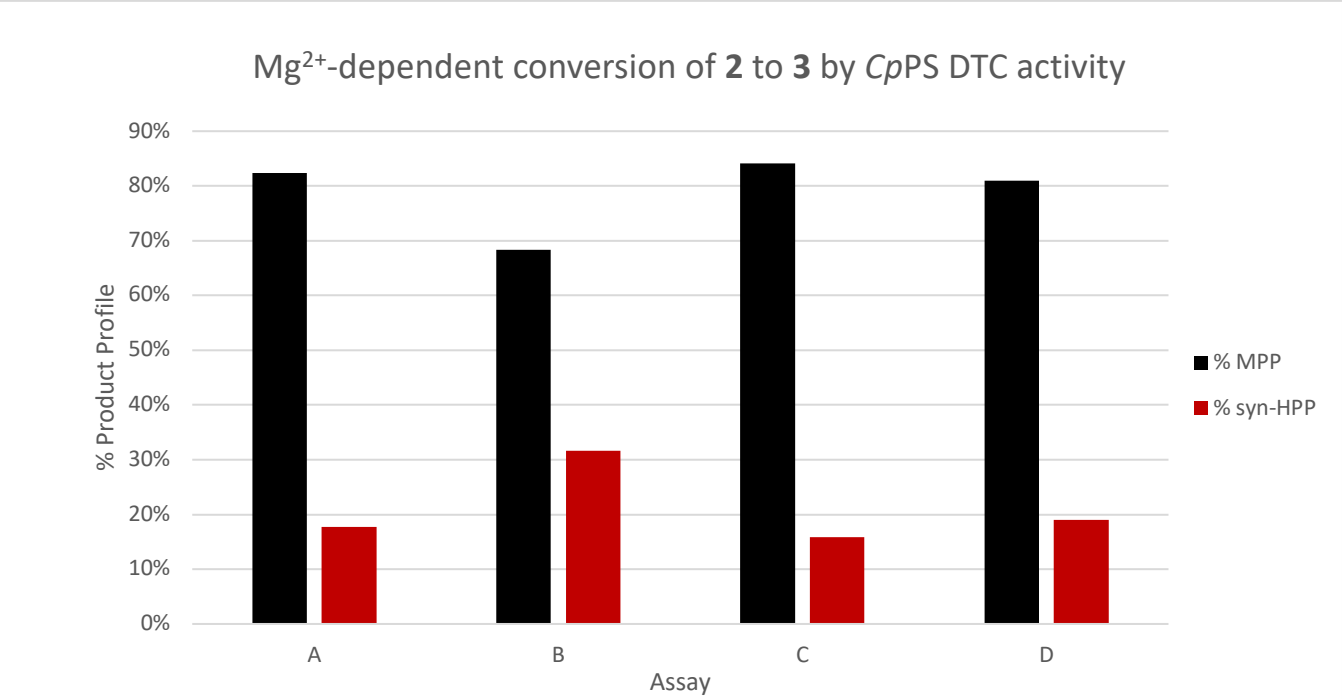


Figure S7. *In vitro* assay with *Cp*PS:D649L was performed for 4 hours before aliquoting into four experimental groups. A) No treatment. This sample was immediately dephosphorylated. B) An additional aliquot of *Cp*PS was added to the assay and $MgCl_2$ was added to 1mM and the reaction let proceed for another 4 hours. C) *Cp*PS was heat inactivated (5 min. at 80 °C) prior to addition of $MgCl_2$ to 1mM and the reaction again let proceed for 4 hours. D) An additional aliquot of *Cp*PS was added to the assay in the absence of $MgCl_2$ and the reaction again let proceed for 4 hours.