

# Supporting Information

## Pentalenolactone Biosynthesis. Molecular Cloning and Assignment of Biochemical Function to PtlI, a Cytochrome P450 of *Streptomyces avermitilis*

Richard Quaderer,<sup>a</sup> Satoshi Omura,<sup>b</sup> Haruo Ikeda,<sup>c</sup> and David E. Cane<sup>a\*</sup>

<sup>a</sup>Department of Chemistry, Brown University, Box H, Providence, RI 02912-9108, USA,

<sup>b</sup>The Kitasato Institute, 9-1, Shirokane 5-chome, Minato-ku, Tokyo 108-8642, Japan

<sup>c</sup>Kitasato Institute for Life Sciences, Kitasato University, 1-15-1, Kitasato, Kanagawa 228-8555, Japan

**e-mail:** David\_Cane@brown.edu

**Materials and Methods.** General materials and methods were as described.<sup>1</sup>

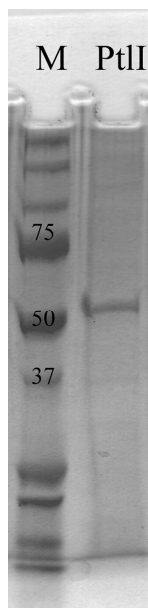
**Expression and purification of *S. avermitilis* PtlI (SAV2999).** The *ptlI* gene was amplified by PCR from template DNA from *S. avermitilis* cosmid CL\_216\_D07 using the forward (5'-CCGCGCGCCATATGTCCCAGCACACCTTCGTTC-3') and reverse (5'-GGCCGGAAGCTTACTAGTCAATTGCTACTGACCCGGCGTACGG-3') primers to introduce *Nde*I (forward primer) and *Hind*III, *Mfe*I, *Spe*I (reverse primer) restriction sites (**bold**), respectively. The reaction mixture contained cosmid DNA (1  $\mu$ L), 10x reaction buffer (5  $\mu$ L), *pfu ultra* polymerase (1  $\mu$ L), dNTP (1  $\mu$ L, 0.2 mM), forward primer (1  $\mu$ L, 0.4  $\mu$ M), reverse primer (1  $\mu$ L, 0.3  $\mu$ M), DMSO (2.5  $\mu$ L) and H<sub>2</sub>O (37.5  $\mu$ L). After purification, the size of the PCR product was confirmed by 1% agarose gel electrophoresis. The PCR product and plasmid pET28e, a variant of pET28a in which the *Xba*I site has

been replaced by an *EcoRI* site, were digested separately with *NdeI* and *HindIII* and purified by PCR purification kit before ligation with T4 DNA ligase at 4 °C overnight and transformation into *E. coli* XL-1 Blue. The DNA sequence of the insert was verified by dideoxy sequencing (DNA Sequencing Facility, UC Davis) and expression vector pET28e-*ptII* was obtained.

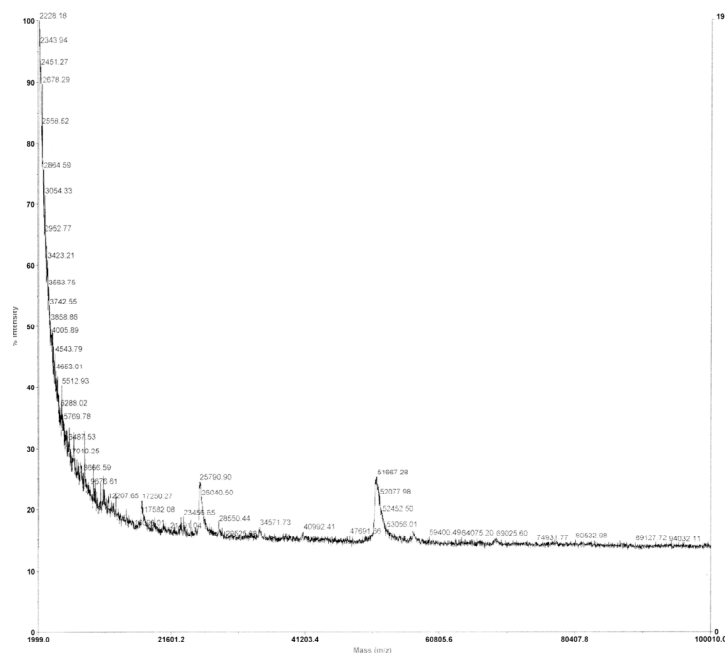
A C-terminal His-tag was introduced into the *ptII* gene by PCR from template DNA from plasmid pET28e-*ptII* using the forward (5'-GCGCCCGTCATATG**T**CCCAGCACACCTTCGTCGCC-3') and reverse (5'-TCACTCGAG**T**TAAATGATGATGATGATGATGGCTGCCCTGACCCGGCGTACGGGC-CGT-3') primers containing *NdeI* and *XhoI* restriction sites (**bold**), respectively. The reaction mixture contained plasmid pET28e-*ptII* DNA (1 µL), 10x reaction buffer (5 µL), *pfu ultra* polymerase (1 µL), dNTP (1 µL, 0.5 mM), forward primer (1 µL, 0.3 µM), reverse primer (1 µL, 0.3 µM), DMSO (3.5 µL) and H<sub>2</sub>O (36.5 µL). The PCR product and plasmid pET31b were digested separately with *NdeI* and *XhoI* followed by 1% agarose gel electrophoresis. The resultant bands at ca. 1.4 kb (*ptII*) and at 5.4 kb (pET31b), respectively, were isolated using a gel extraction kit (Qiagen). Ligation was carried out with Quick T4 DNA Ligase (New England Biolabs) at room temperature for 7 min followed by treatment with a PCR cleanup kit and transformation into *E. coli* XL-1 Blue. The DNA sequence of the insert was verified by dideoxy sequencing (DNA Sequencing Facility, UC Davis) and the resultant pET31b-*ptII* expression vector was subcloned into *E. coli* BL21(DE3).

Protein expression was carried out in TB medium (4 × 280 mL) supplemented with ampicillin (50 mg/L) and FeSO<sub>4</sub> (0.1 mM). Cells were grown at 37 °C until they reached an OD<sub>600</sub> 0.8 followed by cooling to 21 °C and addition of isopropyl-β-D-thiogalactopyranoside (IPTG, 0.1 mM), δ-aminolevulinic acid (δ-ALA, 0.48 mM), and ethanol (8.4 mL). The cells were harvested after an additional 20-22 h at 21 °C. The cells (12 g) were suspended in lysis buffer (60 mL, 50 mM Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 10% glycerol, 10 mM imidazole, 2 mM β-mercaptoethanol, 2 mg/L pepstatin, 2 mg/L leupeptin, 1 mM benzamidin, pH 7.5) plus 0.1 % Triton X-100 and disrupted by French Press (10,000 psi). The cell debris was removed by centrifugation (10000g, 1 h) and the supernatant was bound to Ni-NTA resin (nickel-nitrilotriacetic acid, 12 mL, Qiagen) by shaking at 4 °C for 40 min. The resin slurry was loaded into a column and washed with buffer without detergent containing 10 mM imidazole followed by washing with buffer containing 40 mM imidazole. The protein was eluted with 150 mM imidazole buffer (50 mM

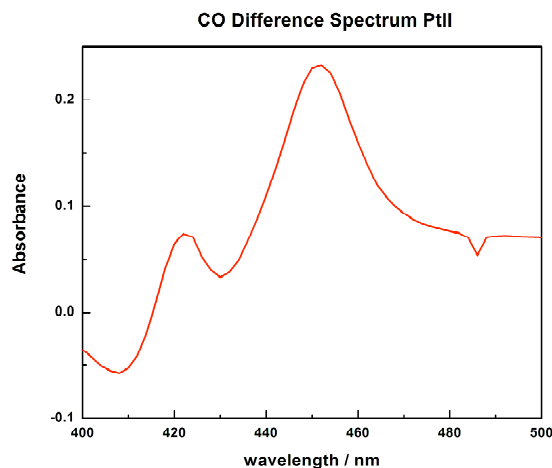
Na<sub>2</sub>HPO<sub>4</sub>, 300 mM NaCl, 10% glycerol, 150 mM imidazole, 2 mM β-mercaptoethanol, pH 7.5). The PtlI-containing protein fractions were pooled and concentrated in an Amicon Ultra (MWCO 30000) tube (Millipore) and the buffer was exchanged with a PD-10 column. Yield: 0.6 mg holo-protein (CO binding assay).<sup>2</sup> Freshly prepared recombinant PtlI was immediately used for all incubations.



**Figure S1.** SDS PAGE of recombinant PtlI. **M**, MW markers (band sizes in kD). **PtlI**, PtlI-His<sub>6</sub>.



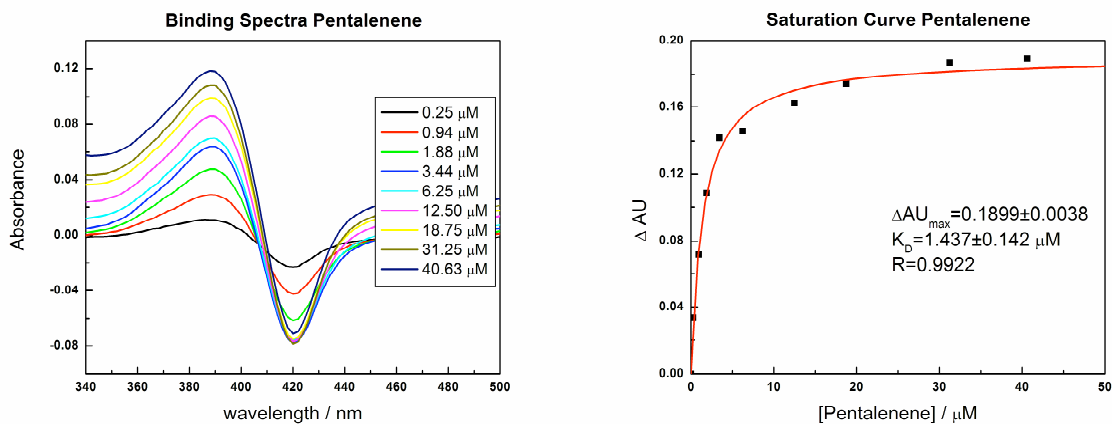
**Figure S2.** MALDI TOF MS of PtlI-His<sub>6</sub>



**Figure S3.** CO difference spectrum<sup>2</sup> of PtII-His<sub>6</sub>. Buffer: 50 mM phosphate, 300 mM NaCl, 10% glycerol (v/v), pH 7.3.

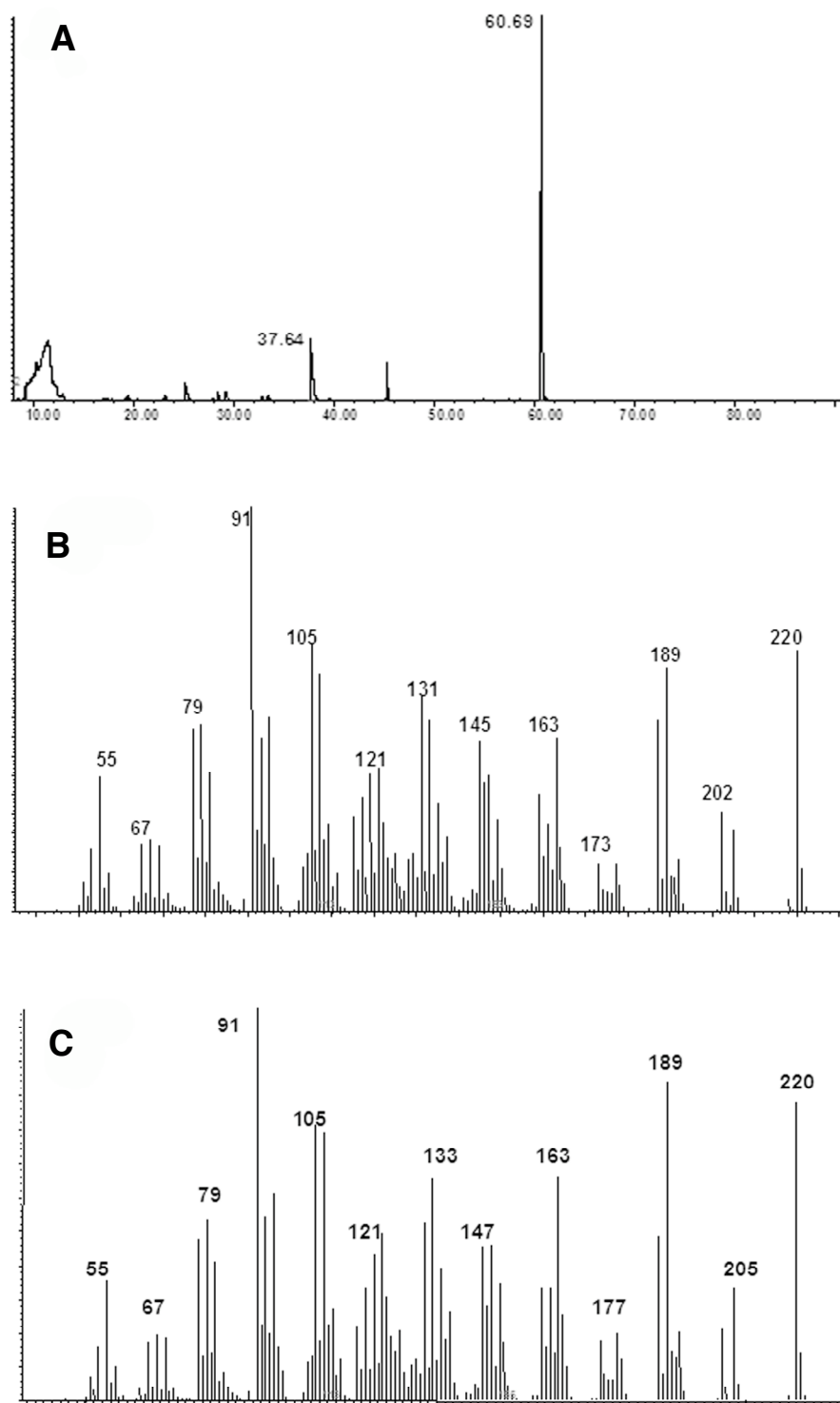
**Dissociation Constant  $K_D$  of Pentalenene and PtII.** Pentalenene (**3**) dissolved in DMSO was added at increasing concentrations to a stock solution of PtII (50 mM Tris-HCl, 10% glycerol, pH 8; [P450]=0.98  $\mu$ M) and the concentration of DMSO was adjusted to 1.75% (v/v). UV difference spectra were recorded ([pentalenene]=0.25 – 40.6  $\mu$ M; all concentrations are given for a single enantiomer, although racemic ( $\pm$ )-pentalenene was used). A blank spectrum with an equal amount of DMSO (1.75% v/v) added to the P450 solution was also recorded. The resulting difference spectra are shown below (Figure S4, left). A saturation curve of the measured delta value ( $\Delta AU = A_{390 \text{ nm}} - A_{420 \text{ nm}}$ ) versus substrate concentration is shown in Figure S4 (right). The data were fit by nonlinear least squares regression to Eqn (1) to give  $\Delta AU_{\text{max}} = 0.190 \pm 0.004$  and a  $K_D$ -value of  $1.44 \pm 0.14 \mu\text{M}$ .

$$(1) \Delta AU_{\text{obsd}} = \Delta AU_{\text{max}} [S] / (K_D + [S])$$



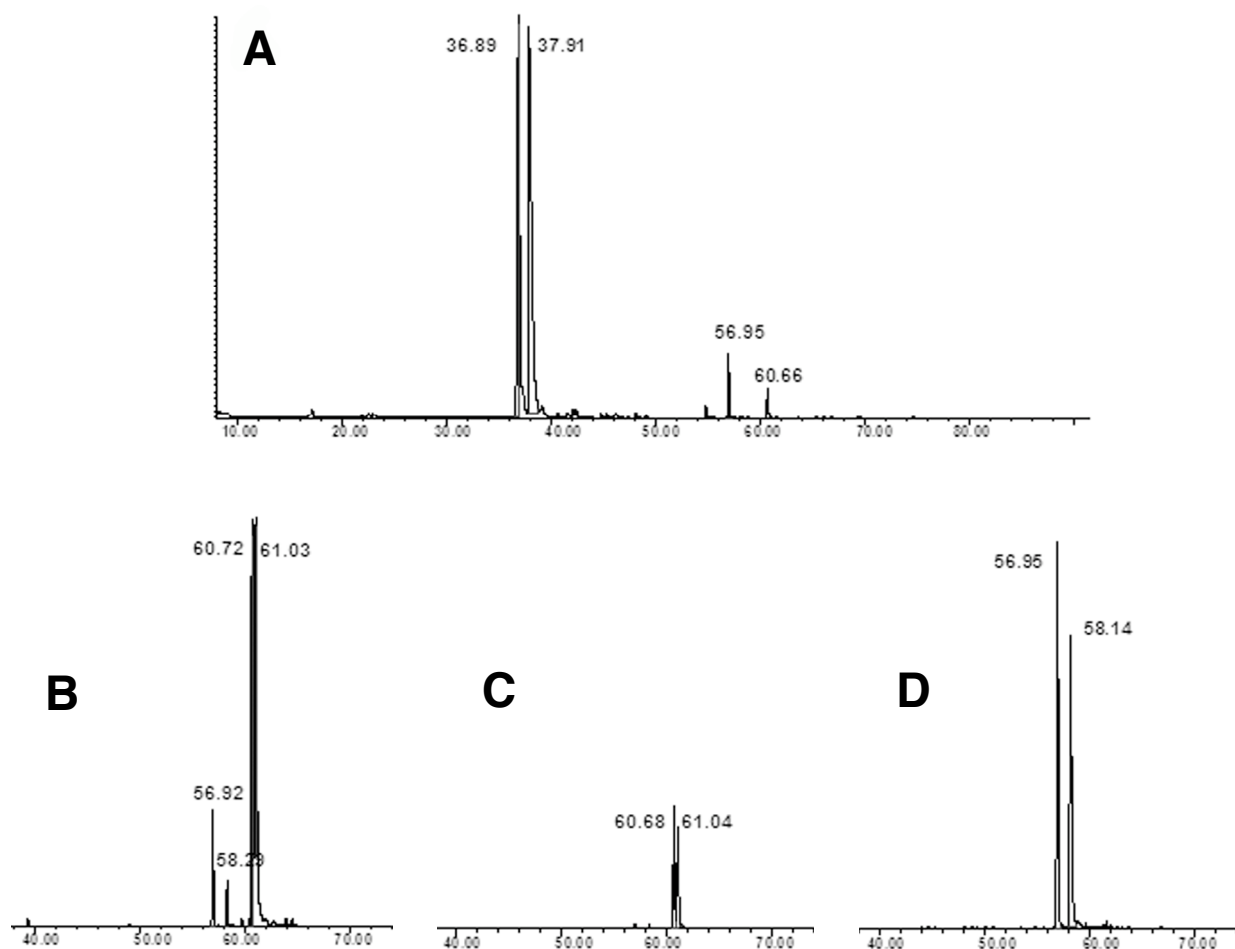
**Figure S4.** P450 substrate UV difference binding spectra (left), and saturation curve (right) for PtlI and (±)-pentalenene (**3**).

**Optimization of the PtlI assay for conversion of pentalenene (**3**) to pentalen-13-ol (**6**).** The pH-dependence of the PtlI-catalyzed oxidation of pentalenene to pentalen-13-ol was determined using a series of 5 buffers: bis-tris (20 mM, 10% glycerol, pH 6), bis-tris (20 mM, 10% glycerol, pH 6.5), phosphate (50 mM, 10% glycerol, pH 7), phosphate (50 mM, 10% glycerol, pH 7.5) and Tris-HCl (50mM, 10% glycerol, pH 8). Parallel incubations were run for 2 h at 25 °C with shaking (120 rpm) using P450 (ca. 0.2 μM), *E. coli* Fld (1.4 μM), *E. coli* Fdr (2.3 μM), glucose-6-phosphate (0.43 mM), glucose-6-phosphate dehydrogenase (1 u), NADPH (0.32 mM) and (±)-pentalenene (0.14 mM) and 0.8% DMSO. Reactions were acidified to pH 2 with 1 M HCl and extracted with ether. Ether was removed *in vacuo* and the residue resuspended in 4:1 MeOH:benzene (500 μL). Trimethylsilyl-diazomethane (1 M in hexane, 20 μL) was added and after 20 min at room temperature the volume reduced to 150 μL. Organic extracts were analyzed by GC-MS. A 1 μL sample was injected and the intensity of the product peaks from each incubation was determined. Maximum pentalen-13-ol formation was observed at pH 8 (aldehyde and methyl ester formation was not observed under these conditions). In a second set of experiments, the pH range from 7.75 to 8.5 was analyzed following the procedure described above (buffer: 50mM Tris-HCl, 10% glycerol; pH values: 7.75, 8, 8.25, 8.5). Maximum pentalen-13-ol formation was observed at pH 8.

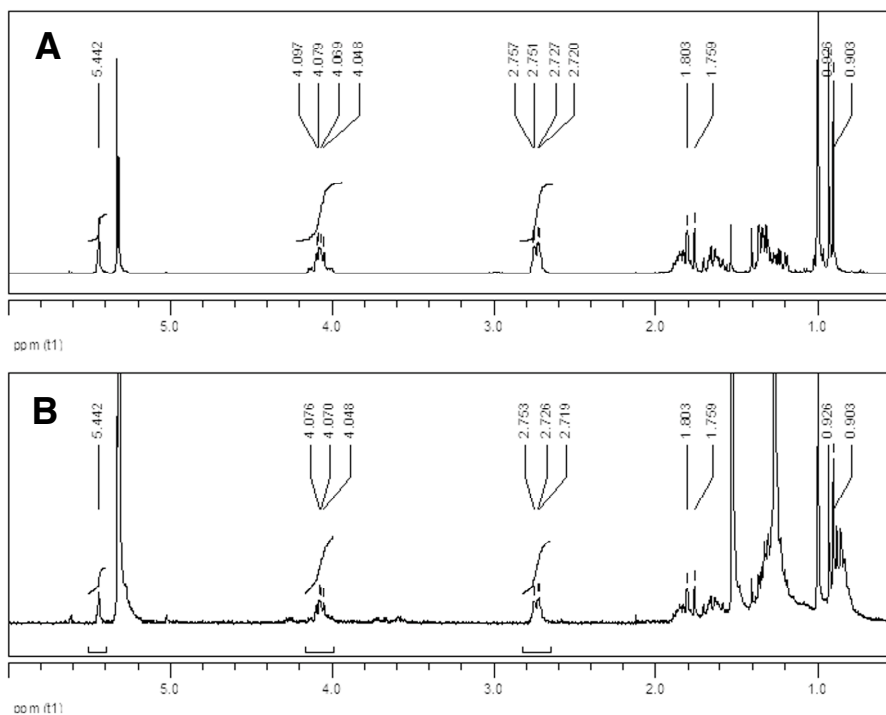


**Figure S5.** Chiral capillary GC-MS of enzymatically generated pentalen-13-ol (**6**) obtained by reduction of the total pentane extract (**6** plus **7**) with  $\text{NaBH}_4$ . Hydrodex- $\beta$ -6-TBDM column (25 m  $\times$  0.25 mm, Macherey-Nagel); temperature ramp of 45 °C to 180 °C at 2 °C/min, inlet temperature 150 °C; run time 91.5 min (Hewlett-Packard Series 2 GC-MSD, 70 eV). A. TIC. B. enzymatically generated pentalen-13-ol (**6**), ret. time 60.69 min. C. Synthetic ( $\pm$ )-pentalen-13-ol.

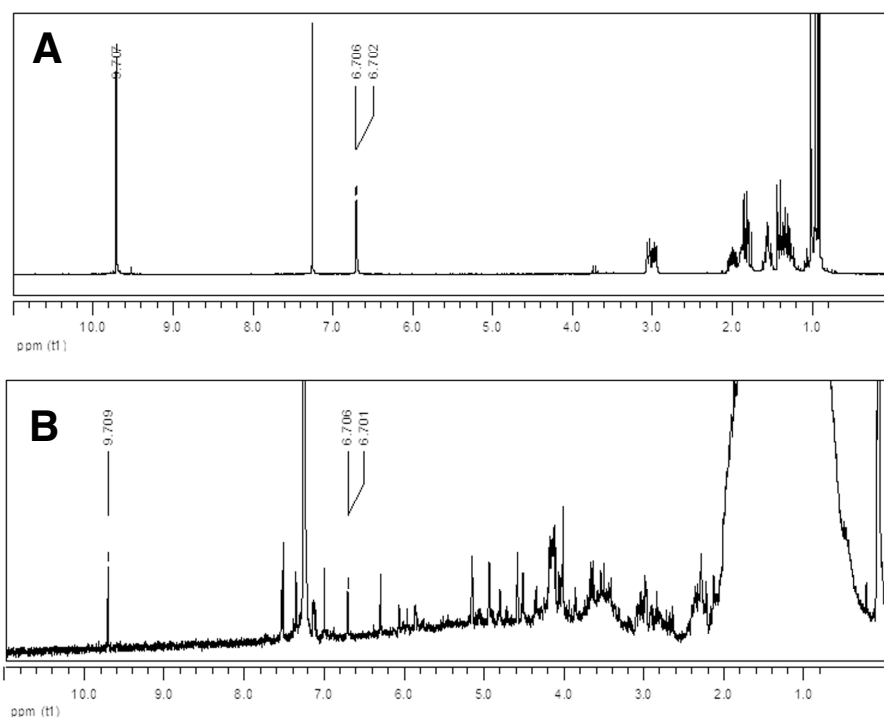
**Incubation of ( $\pm$ )-pentalenene (**3**) and of ( $\pm$ )-pentalen-13-ol (**6**) with PtII and analysis by chiral capillary GC-MS.** Incubations containing PtII (ca. 0.2  $\mu$ M), Fld (3.1  $\mu$ M), Fdr (1.6  $\mu$ M), glucose-6-phosphate (2.1 mM), glucose-6-phosphate dehydrogenase (5 u), NADPH (1 mM), ( $\pm$ )-pentalenene (0.48 mM), and 1% DMSO, final volume 4.85 mL, were run for 4 h at 25  $^{\circ}$ C with shaking at 60 rpm. The reaction mixtures were acidified to pH 2 with 1 M HCl and extracted with pentane, followed by treatment as described above for optimization of the PtII assay. The pentane extracts were analyzed by chiral GC-MS.



**Figure S6.** Chiral capillary GC-MS analysis (TIC) of PtII-incubations (same conditions as for Fig. S5). A. Pentane extract obtained from incubation of PtII with ( $\pm$ )-pentalenene (**3**) showing formation of single enantiomers of both pentalen-13-al (**7**) (ret. time 56.95 min) and pentalen-13-ol (**6**) (ret. time 60.66). B. Pentane extract obtained from incubation with ( $\pm$ )-pentalen-13-ol (**6**) showing formation of pentalen-13-al (**7**) (ret. time 56.92 min) as major product and selective depletion of the natural enantiomer of **6** (ret. time 60.72 min). C. Synthetic ( $\pm$ )-pentalen-13-ol (**6**). D. Synthetic ( $\pm$ )-pentalen-13-al (**7**) (peak areas are equal).

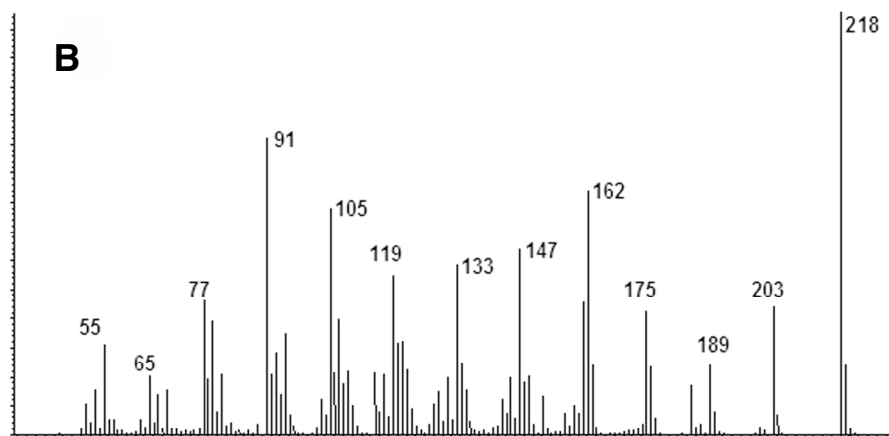
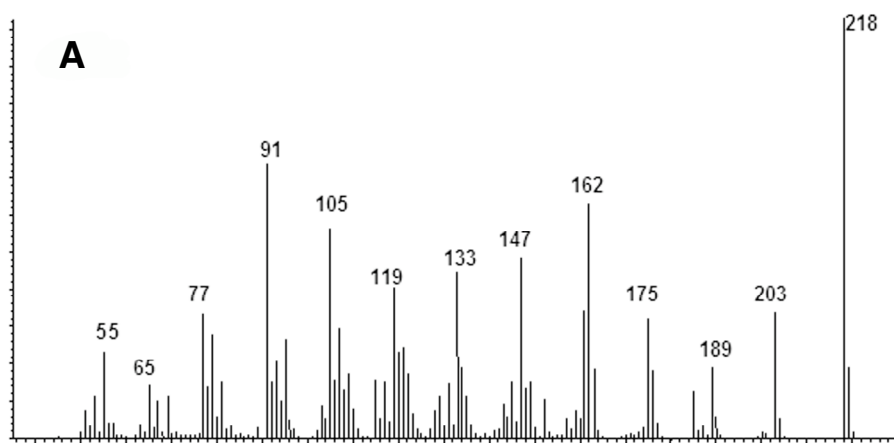


**Figure S7.** 400 MHz  $^1\text{H}$  NMR spectra ( $\text{CDCl}_3$ ) of pentalen-13-ol (**6**). A. Synthetic ( $\pm$ )-pentalen-13-ol. B. Enzymatically-generated pentalen-13-ol.

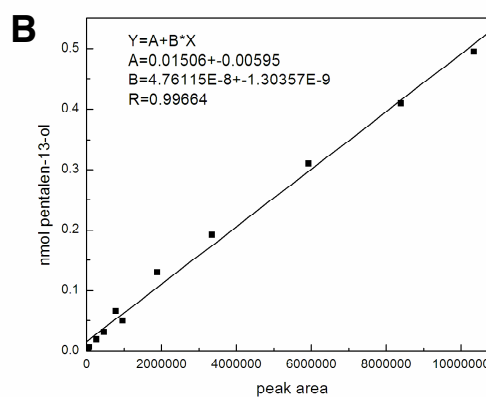
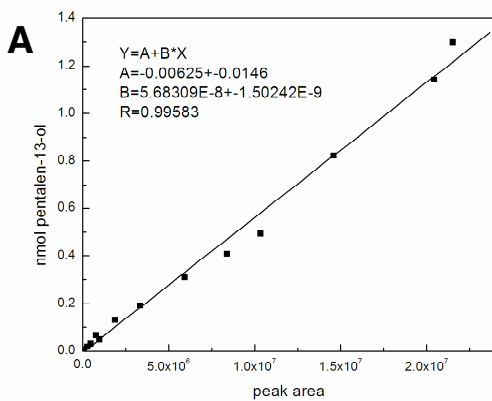


**Figure S8.** 400 MHz  $^1\text{H}$  NMR spectra ( $\text{CD}_2\text{Cl}_2$ ) of pentalen-13-al (**7**). A. Synthetic ( $\pm$ )-pentalen-13-al. B. Crude neutral organic extract of product of incubation of pentalenene (**3**) with PtII showing key aldehydic and olefinic protons of enzymatically generated **7**. Also evident are the signals for the olefinic and allylic hydroxymethyl protons of **6** at  $\delta$  5.25 and 4.15, respectively.



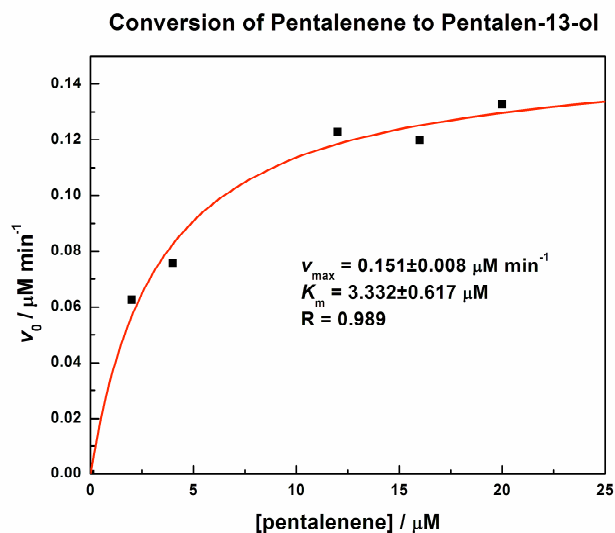


**Figure S9.** EI-MS of A. synthetic ( $\pm$ )-pentalen-13-ol (**7**); B. Enzymatically generated **7**.



**Figure S10.** Calibration of GC-MS detection of pentalen-13-ol (**6**) (30 m x 0.25 mm HP5MS capillary column and temperature program of 50-280 °C, 20 °C/min, run time 15 min). The Y-axis is the quantity of injected **6** and the X-axis is the measured peak area for each injection. A. Entire calibration range. B. Low range

**Oxidation of pentalenene (3) to pentalen-13-ol (6) – Steady State Kinetics.** Kinetic assays were carried out at 25 °C with PtII (0.3  $\mu\text{M}$ ) in 50 mM Tris-HCl, 10% glycerol, pH 8, containing *E. coli* flavodoxin (3  $\mu\text{M}$ ), *E. coli* flavodoxin reductase (1.5  $\mu\text{M}$ ), NADPH (0.56 mM) in a total vol. of 2 mL. After preincubation for 5 min, reactions were initiated by adding a solution of ( $\pm$ )-pentalenene (3) in DMSO to a concentration of 4 - 40  $\mu\text{M}$  (final DMSO conc. 0.8%). The reactions were shaken at 60 rpm and quenched with 1 M HCl after 10 min to give a final pH 2. The mixtures were extracted with ether and analyzed by calibrated GC-MS.



**Figure S11.** Michaelis-Menten plot of initial velocity of formation of pentalen-13-ol (6) as a function of pentalenene (3) concentration (based on a single enantiomer of 3).

### References – Supporting Information

(Ref 14b) (full citation): Zhao, B.; Guengerich, F. P.; Bellamine, A.; Lamb, D. C.; Izumikawa, M.; Lei, L.; Podust, L. M.; Sundaramoorthy, M.; Kalaitzis, J. A.; Reddy, L. M.; Kelly, S. L.; Moore, B. S.; Stec, D.; Voehler, M.; Falck, J. R.; Shimada, T.; Waterman, M. R. *J. Biol. Chem.* **2005**, *280*, 11599-11607.

(1) (a) Cane, D. E.; Watt, R. M. *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1547-1551. (b) Tetzlaff, C. N.; You, Z.; Cane, D. E.; Takamatsu, S.; Omura, S.; Ikeda, H. *Biochemistry* **2006**, *45*, 6179-6186.

(2) Omura, T.; Sato, R. *J. Biol. Chem.* **1964**, *239*, 2370-2378.