Supporting Information for

# Templating Effects in Aristolochene Synthase Catalysis: Elimination versus Cyclisation

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## Site directed mutagenesis of recombinant PR-AS

The Quickchange site-directed mutagenesis kit (Stratagene) was used to introduce the desired mutations according to the manufacturer's instructions. The primers used for mutagenesis were as follows:

5'-GCCAAGTTCTCAGAGGCGACTTGTCTTTACTTCC-3' and 5'-GGAAGTAAAGACAAGTCGCCTCTGAGAACTTGGC-3' for PR-AS-V88A;

5'-GATGCCAAGTTCTCAGAGTTTACTTGTCTTTACTTCC-3' and 5'-GGAAGTAAAGACAAGTAAACTCTGAGAACTTGGCATC-3' for PR-AS-V88F;

5'-CCAAGTTCTCAGAGGTTGCGTGTCTTTACTTCCCTC-3' and 5'-GAGGGAAGTAAAGACACGCAACCTCTGAGAACTTGG-3' for PR-AS-T89A;

5'-GCCAAGTTCTCAGAGGTTTTTTGTCTTTACTTCCCTC-3' and 5'-GAGGGAAGTAAAGACAAAAAACCTCTGAGAACTTGGC-3' for PR-AS-T89F;

5'-CACTTTGCCTGCCGACTGGCGACCGTTCTCTTCCTTAT-3' and 5'-GATAAGGAAGAGAACGGTCGCCAGTCGGCAGGCAAAGTG-3' for PR-AS-L108A;

5'- CACTTTGCCTGCCGACTGAGCACCGTTCTCTTCCTTATC-3' and 5'-GATAAGGAAGAGAACGGTGCTCAGTCGGCAGGCAAAGTG-3' for PR-AS-L108S;

5' CTTTGCCTGCCGACTGGTGACCGTTCTCTTCC-3' and 5'-GGAAGAGAACGGTCACCAGTCGGCAGGCAAAG-3' for PR-AS-L108V;

5'-CACTTTGCCTGCCGACTGTTTACCGTTCTCTTCCTTATC-3' and 5'-GATAAGGAAGAGAACGGTAAACAGTCGGCAGGCAAAGTG-3' for PR-AS-L108F;

5'-CAGAGGTTACTTGTCTTCATTTCCCTCTTGCACTGGAC-3' and 5'-GTCCAGTGCAAGAGGGAAATGAAGACAAGTAACCTCTG-3' for PR-AS-Y92H

5'-GAGGTTACTTGTCTTTGGTTCCCTCTTGCACTGG-3' and 5'-CCAGTGCAAGAGGGAACCAAAGACAAGTAACCTC-3' for PR-AS-Y92W.

Plasmids were purified from overnight cultures (10 mL LB medium containing ampicillin 50 μM/mL) using the QIAGEN miniprep kit as described by the manufacturer. Mutations were confirmed by DNA sequence analysis using the internal Walesbiogrid facilities (School of Bioscience, Cardiff University, UK).

## Production of wild type PR-AS and mutants

*E. coli* BL21(DE3) cells were transformed with plasmid. One colony was added to 100 mL of LB medium containing ampillicin (50 μg/mL), and the culture was grown at 37 °C with shaking (150 rpm) overnight. 5 mL of this culture were transferred to 6 x 500 mL of LB medium containing 50 μg/mL ampillicin. Cells were incubated at 37 °C with shaking until $O.D._{600}$ reached 0.6-0.8. IPTG was added to 0.5 mM and the cultures incubated for 4 hours at 37 °C. Cells were harvested by centrifugation (4500 rpm, 10 minutes), the supernatant was discarded and the pellets were frozen at -20 °C.

Pellets were thawed and resuspended in 25 mL cell lysis buffer (20 mM Tris (pH 8.0), 5 mM EDTA, 5 mM β-mercaptoethanol (BME)) by sonication. The lysed cells were centrifuged at 16,000 rpm for 30 minutes and the supernatant was discarded (the proteins remained solely in the insoluble portion). The pellets were resuspended in 50 mL cell lysis buffer by stirring at 4 °C for 30 min; the pH of the solution was raised to 12 with 0.1 M NaOH and the solution stirred for 30 min at 4 °C. The pH was lowered to 7.5 with 0.1 M HCl and BME added to a final concentration of 5 mM. The solution was stirred for 30 min at 4 °C and centrifuged at 17,000 rpm for another 30 min. Proteins remained in the supernatant.

For further purification, a 65 mL Q Sepharose Fast Flow column (GE Healthcare, 12.5 x 2.5 cm dimensions) working at a flow rate of 10 mL/min was used. The column was washed with 5 column volumes of lysis buffer. Then the protein was loaded onto the column and washed with another 5 column volumes of lysis buffer. A linear gradient from 0 – 500 mM NaCl was applied in 6 column volumes and the desired proteins eluted at ~250 mM NaCl. Fractions containing protein were analyzed by 10% SDS-PAGE, pooled and dialysed overnight (MWCO, 30,000) against storage buffer (20 mM Tris (pH 8.0) and 5 mM BME). The volume of protein was reduced to ~ 5 mL (AMICON, YM 30). The concentration of protein was determined using the Bradford assay.[1]

## GC/MS analysis

Proteins (50 μM) were incubated with FDP (1 mM) in incubation buffer (25 mM Tris (pH 7.5), 5 mM BME, 5mM $MgCl_2$, 10% v/v glycerol) in a total volume of 250, overlaid with pentane (0.5 mL) and left overnight. Products were extracted with additional pentane (2 x 0.75 mL). The organic solutions were passed through a short (1.5 cm) column containing aluminum oxide. GC-MS analysis of the pentane extracts was performed using a HP 6890 GC-MS system fitted with J & W scientific HP-5MS column (30 m with an internal diameter of 0.25 mm) and a Micromass GCT Premiere mass spectrometer. The program uses an initial oven temperature of 50 °C with a ramp of 4 °C $min^{-1}$ (25 min) to 150 °C and a second ramp of 20 °C $min^{-1}$ (5 min) to 250 °C. The calculated errors for the product distributions were less than 2% in all cases.

## Steady-state kinetic assay

Kinetic assays were carried out according to the standard, linear range, micro-assay procedure developed for limonene and bornyl diphosphate synthases[2] with modifications.[3] This protocol involves the incubation of varying amounts of [1-$^3$H]-FDP (specific activity 75 mCi/mmol) at pH 7.5 with purified PR-AS or its mutants (100 nM) in 20 mM Tris buffer containing 5 mM $MgCl_2$, 5 mM β-mercaptoethanol and 15% glycerol at pH 7.5. The reaction mixtures containing buffer, FDP and protein were prepared on ice in a total volume of 250 μL and were overlaid with 1 mL of HPLC-grade hexane prior to incubation. The assay mixtures were incubated at 30 °C for 10 or 15 min. The reactions were immediately ice-cooled and quenched by addition of 200 μL of 100 mM EDTA (pH 8.5) and brief vortexing. The hexane overlay and two additional 1 mL hexane extractions were passed through a short (1.5 cm) pipette column containing aluminium oxide. The column was washed with hexane (1 mL) and the combined eluates were analyzed by liquid scintillation counting using 15 mL of Ecoscint O (National Diagnostics) as scintillation cocktail on a Packard Tri-Carb LS analyzer model 2700TR. Steady-state kinetic parameters for wild-type PR-AS and mutants were obtained by the

graphical procedures developed by Lineweaver-Burk[4] using the commercial SigmaPlot package (Systat Software).

## Reproduction of the GC/MS spectra

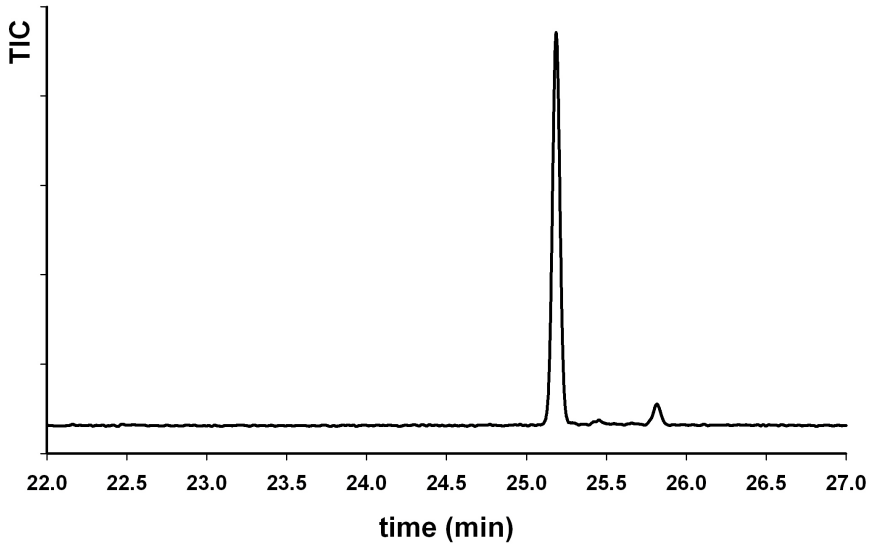


**Figure S1.** Total ion chromatogram of the pentane extractable product formed from incubation of FDP with PR-AS: aristolochene (retention time, 25.22 min), valencene (25.66 min) and germacrene A (25.82 min).

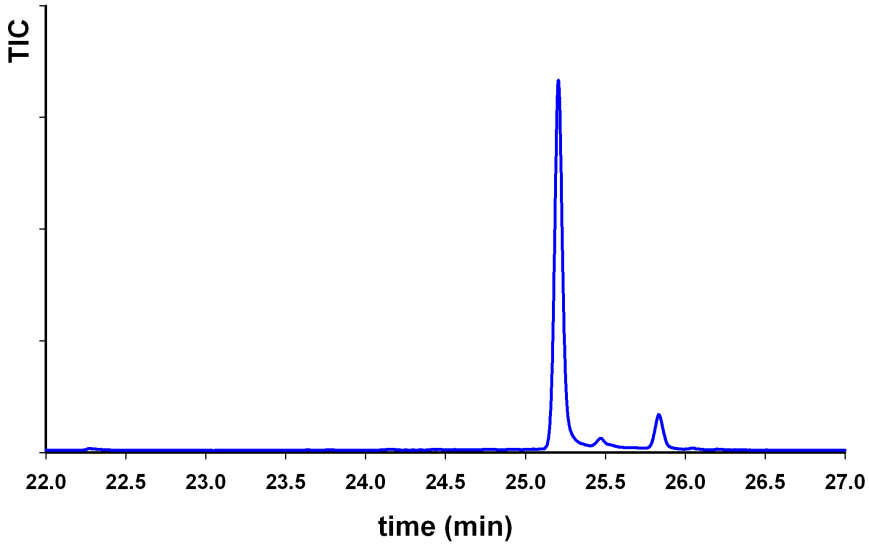


**Figure S2.** Total ion chromatogram of the pentane extractable products formed from incubation of FDP with PR-AS-V88A: aristolochene (retention time, 25.22 min), valencene (25.66 min) and germacrene A (25.82 min).

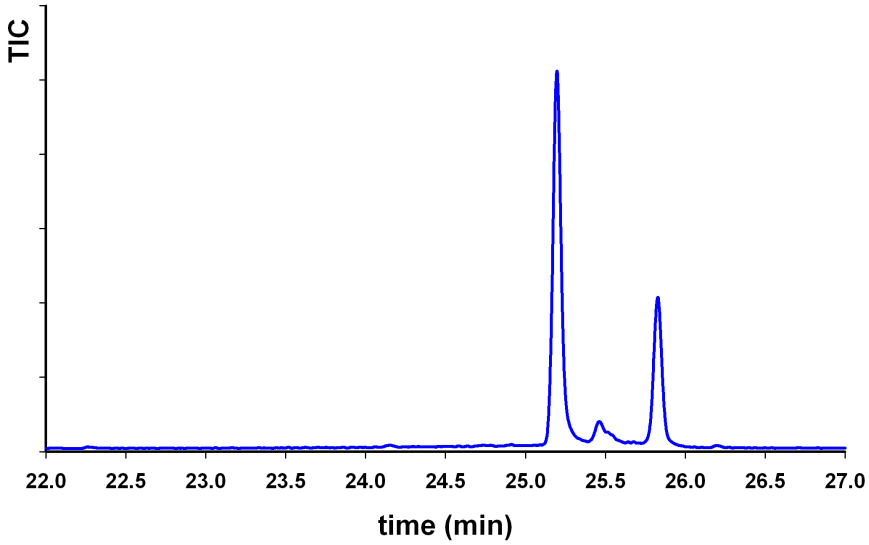


**Figure S3.** Total ion chromatogram of the pentane extractable products formed from incubation of FDP with PR-AS-V88F: aristolochene (retention time, 25.22 min), valencene (25.50 min) and germacrene A (25.82 min).

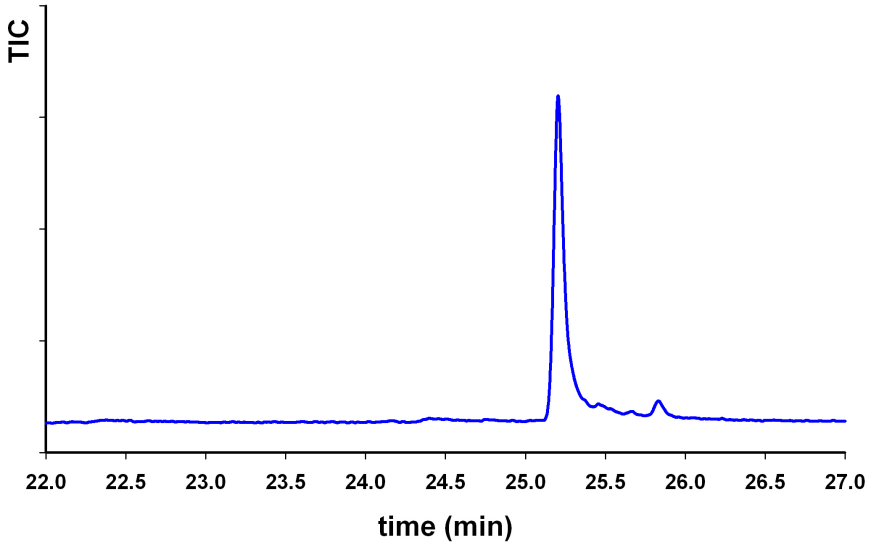


**Figure S4.** Total ion chromatogram of the pentane extractable products formed from incubation of FDP with PR-AS-T89A: aristolochene (retention time, 25.22 min), valencene (25.50 min) and germacrene A (25.82 min).

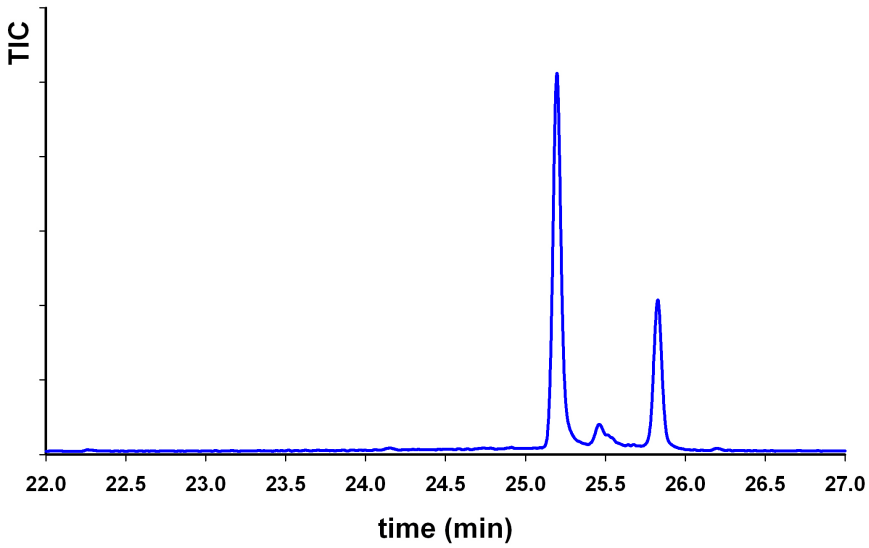


**Figure S5.** Total ion chromatogram of the pentane extractable products formed from incubation of FDP with PR-AS-T89F: aristolochene (retention time, 25.22 min), valencene (25.50 min) and germacrene (25.82 min).

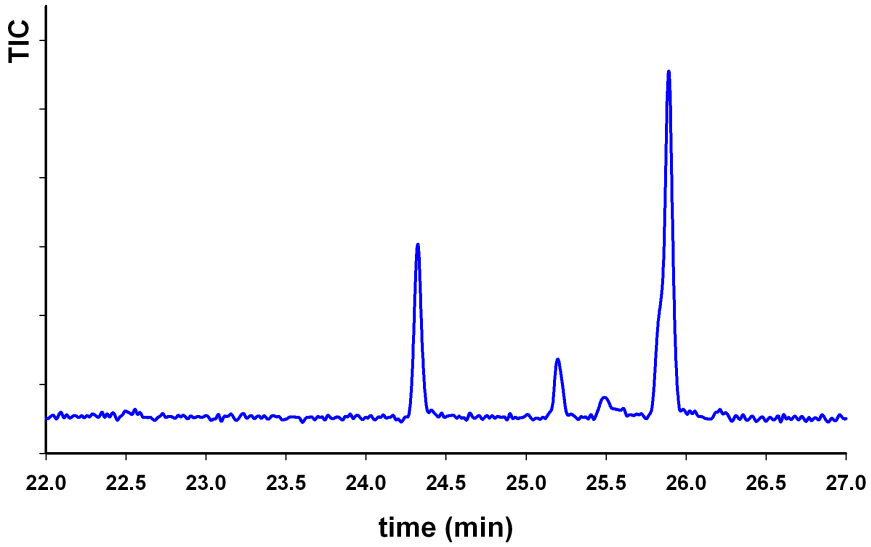


**Figure S6.** Total ion chromatogram of the pentane extractable products formed from incubation of FDP with PR-AS-L108A: (*E*)-β-farnesene (retention time, 24.31 min), aristolochene (25.22 min), (*Z, E*)-α-farnesene (25.48 min), valencene (25.50 min), germacrene A (25.82 min) and (*E, E*)-α-farnesene (25.88 min).

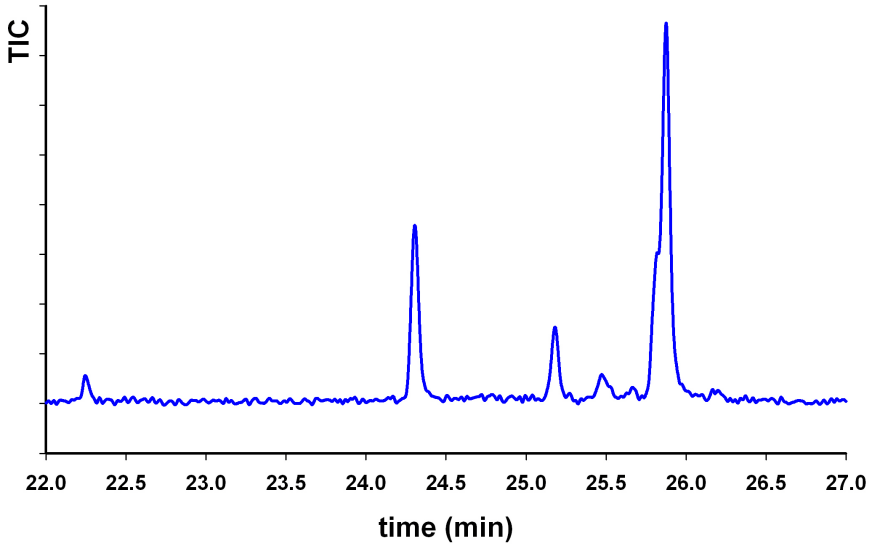


**Figure S7.** Total ion chromatogram of the pentane extractable products formed from incubation of FDP with PR-AS-L108S: (*E*)-β-farnesene (retention time, 24.31 min), aristolochene (25.22 min), (*Z, E*)-α-farnesene (25.48 min), valencene (25.50 min), germacrene A (25.82 min) and (*E, E*)-α-farnesene (25.88 min).

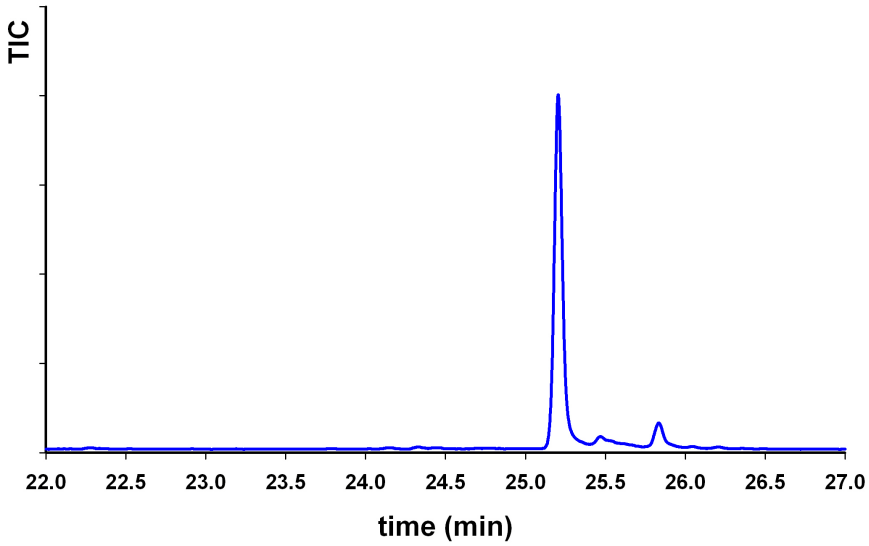


**Figure S8.** Total ion chromatogram of the pentane extractable products formed from incubation of FPP with PR-AS-L108V: aristolochene (retention time, 25.22 min), valencene (25.50 min) and germacrene A (25.82 min).

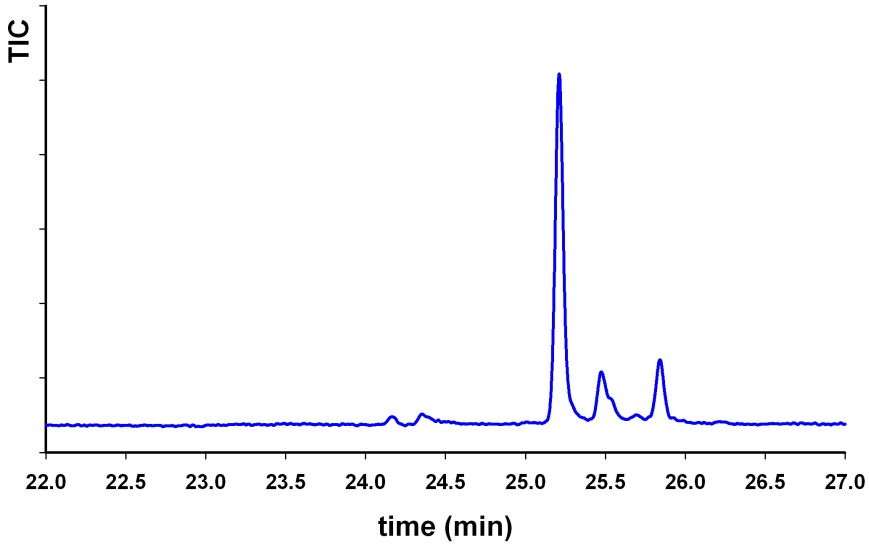


**Figure S9.** Total ion chromatogram of the pentane extractable products formed from incubation of FDP with PR-AS-L108F: aristolochene (retention time, 25.22 min), valencene (25.50 min) and germacrene A (25.82 min).

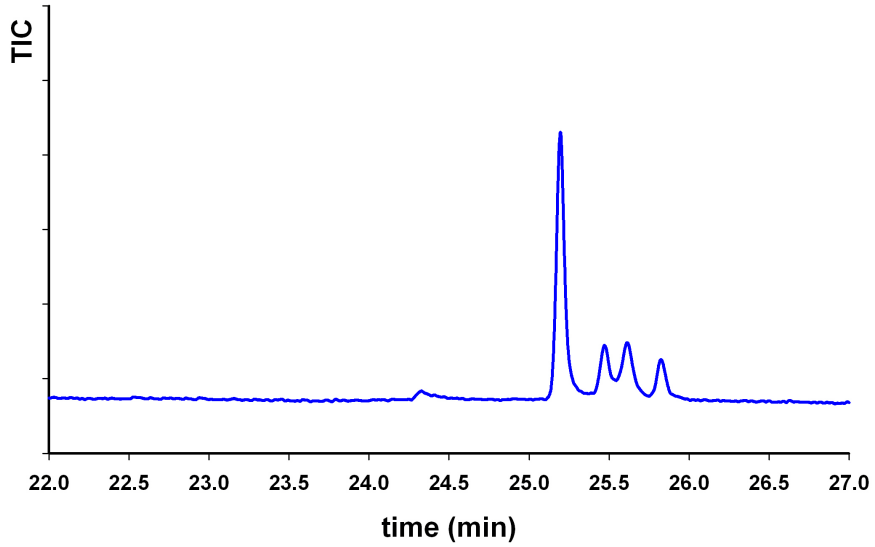


**Figure S10.** Total ion chromatogram of the pentane extractable products formed from incubation of FDP with PR-AS-Y92H: aristolochene (retention time, 25.22 min), valencene (25.50 min) and germacrene A (25.82 min).

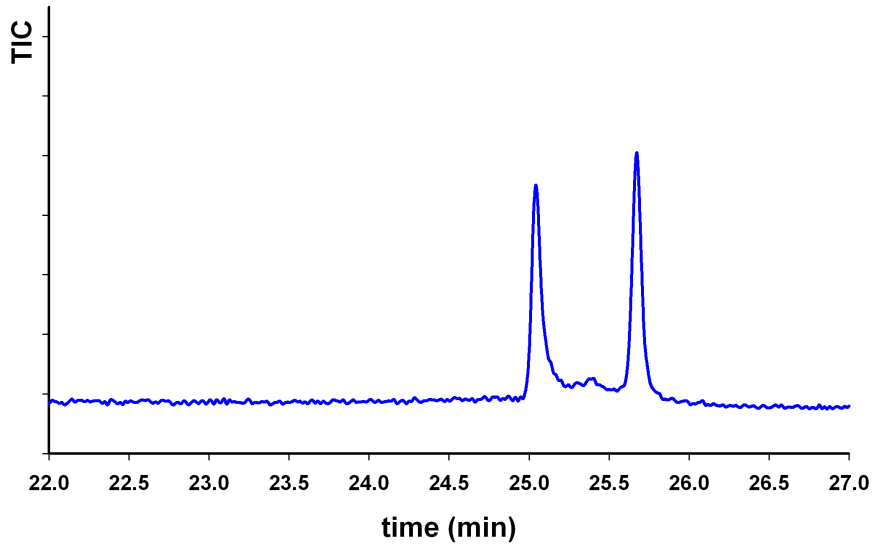


**Figure S11.** Total ion chromatogram of the pentane extractable product formed from incubation of FDP with PR-AS-Y92W: aristolochene (retention time, 25.22 min), valencene (25.50 min) and germacrene A (25.82 min).

## Synthesis of (*E*)-β-farnesene, (*Z, E*)-α-farnesene and (*E, E*)-α-farnesene

Following the same procedure previously described for geranyl acetate,[5] to a stirred solution of farnesyl acetate (1 mmol) in dry THF (3 mL) was added 5 % mol $Pd(PPh_3)_4$ (60 mg) at room temperature and under argon. After overnight incubation, the solvent was removed under reduced pressure and the residue purified by silica gel chromatography using hexane to give farnesenes (130 mg, 64 %). GC-MS analysis (see below) revealed a composition of 62% (*E*)-β-farnesene (24.31 min), 24% (*Z, E*)-α-farnesene (25.48 min) and 14% (*E, E*)-α-farnesene (25.88 min).

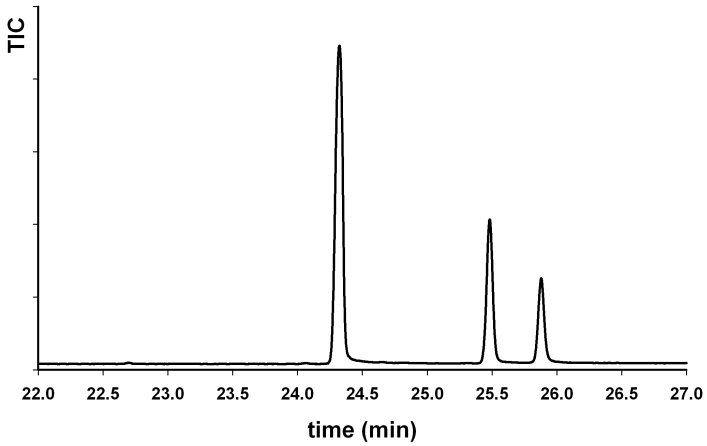


**Figure S12.** (*E*)-β-farnesene (retention time, 24.31 min), (*Z, E*)-α-farnesene (25.48 min), and (*E, E*)-α-farnesene (25.88 min).

## Lineweaver-Burk plots

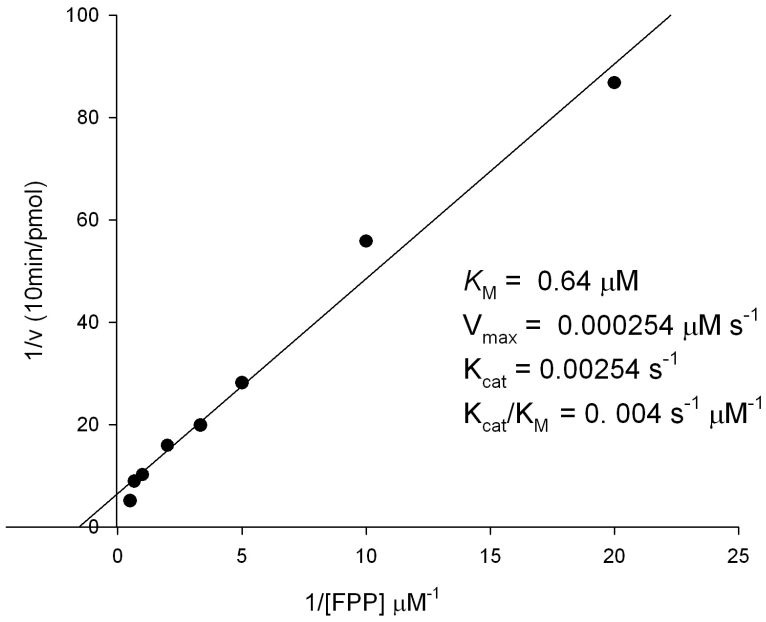


**Figure S13.** Lineweaver-Burk plot for incubations of FDP with PR-AS-V88A.

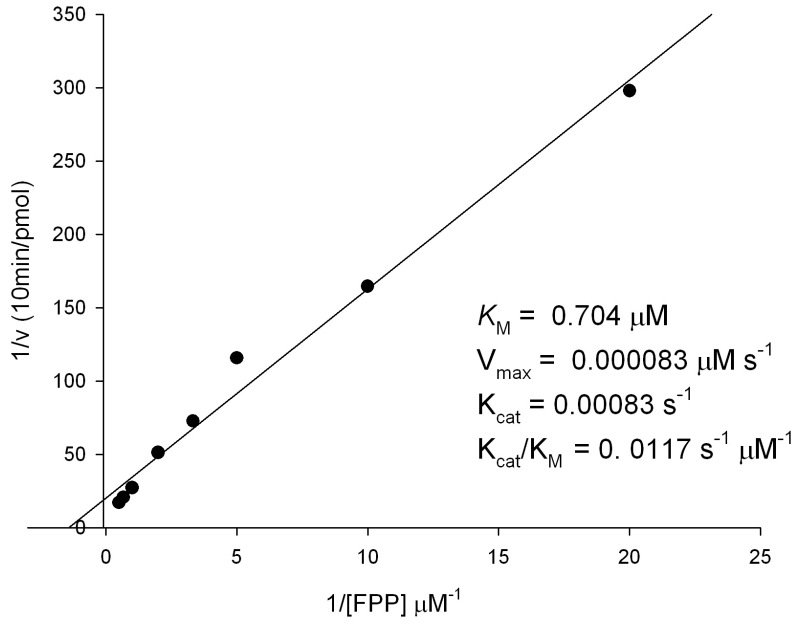


**Figure S14.** Lineweaver-Burk plot for incubations of FDP with PR-AS-V88F.

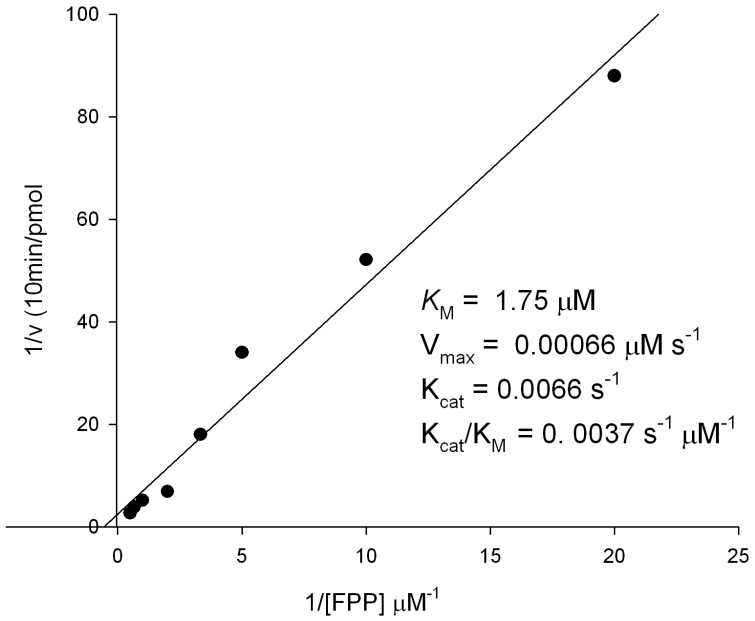


**Figure S15.** Lineweaver-Burk plot for incubations of FDP with PR-AS-T89A.

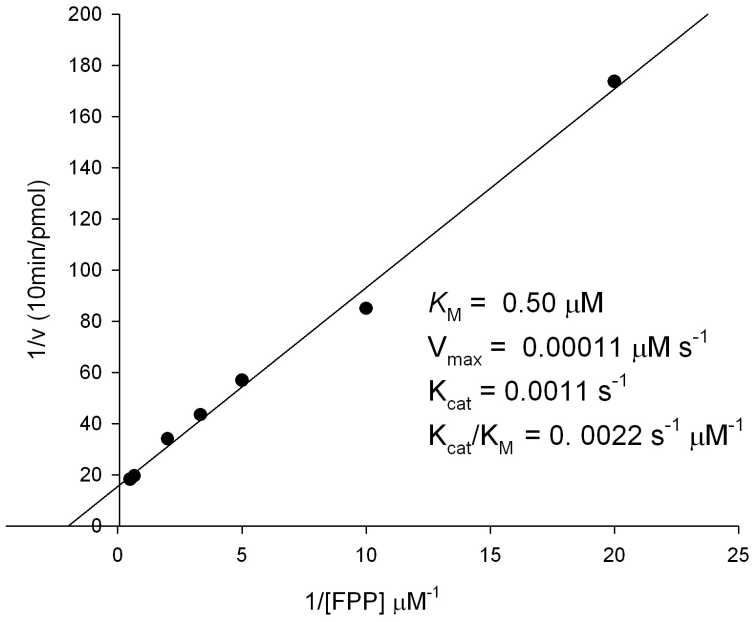


**Figure S16.** Lineweaver-Burk plot for incubations of FDP with PR-AS-T89F.

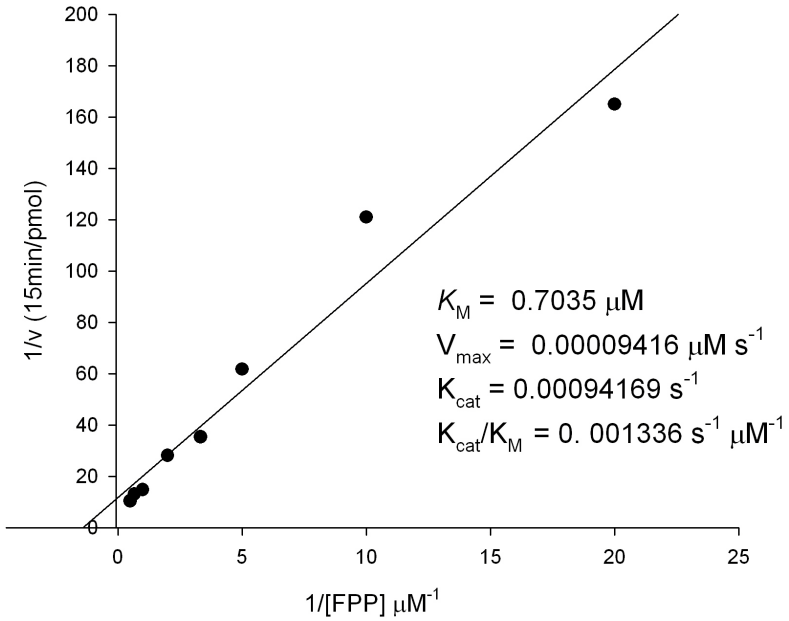


**Figure S17.** Lineweaver-Burk plot for incubations of FDP with PR-AS-L108A.

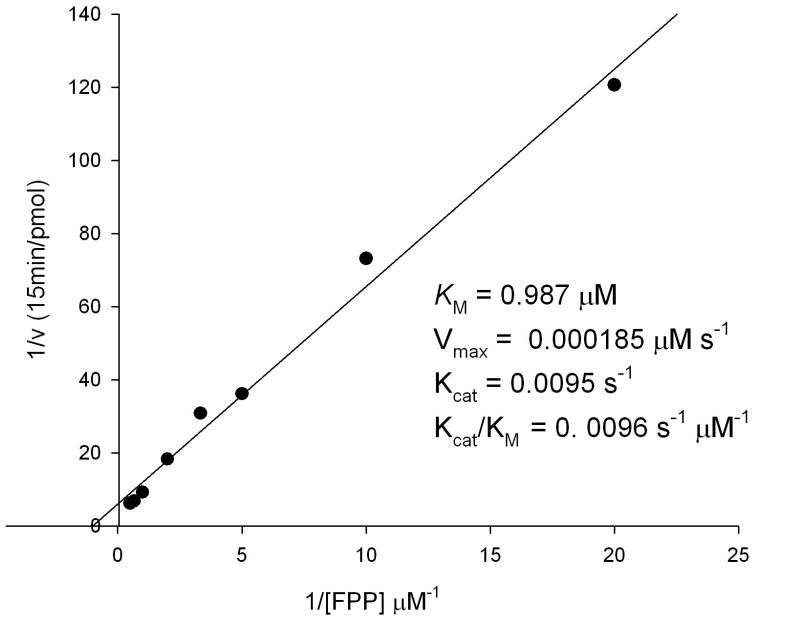


**Figure S18.** Lineweaver-Burk plot for incubations of FDP with PR-AS-L108S.

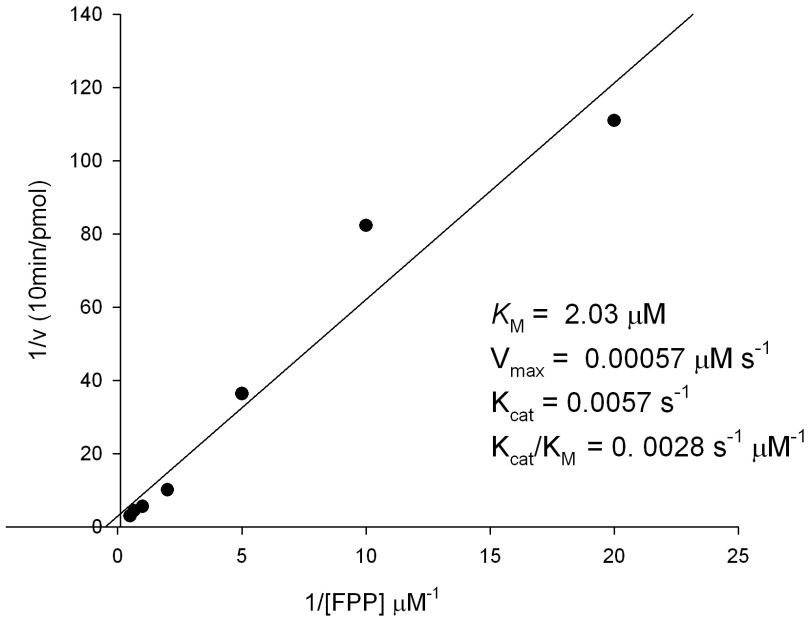


**Figure S19.** Lineweaver-Burk plot for incubations of FDP with PR-AS-L108V.

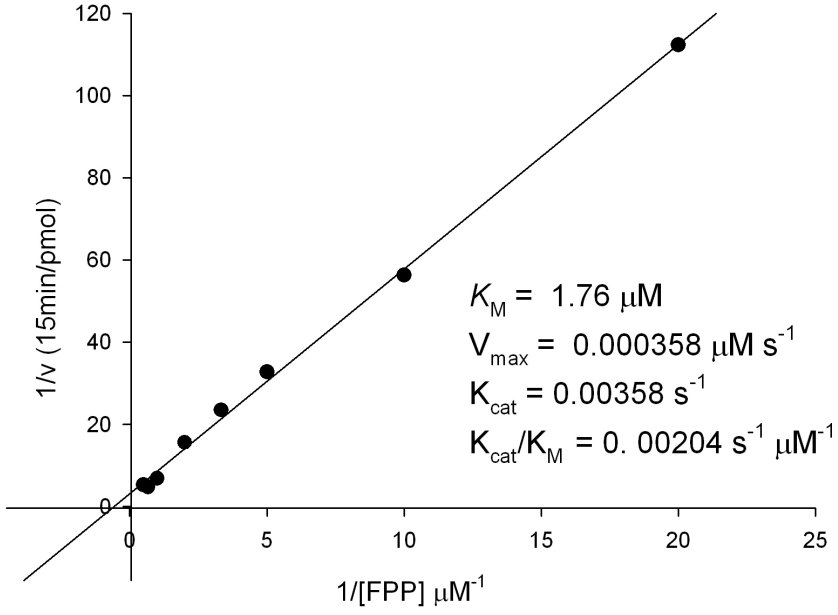


**Figure S20.** Lineweaver-Burk plot for incubations of FDP with PR-AS-L108F.

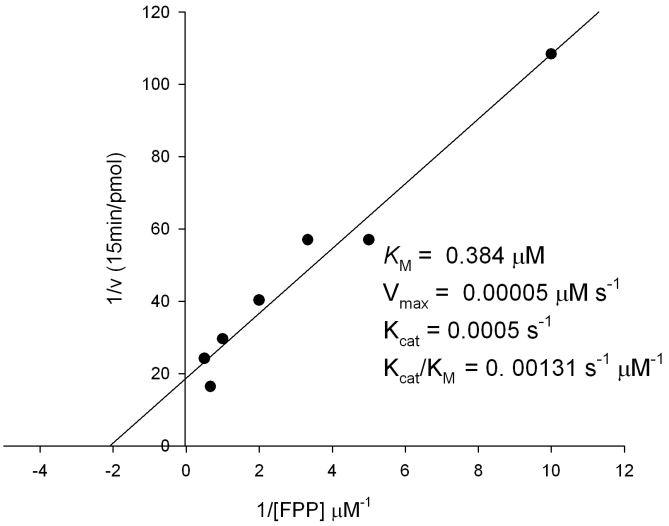


**Figure S21.** Lineweaver-Burk plot for incubations of FDP with PR-AS-Y92H.

## Michaelis Menten Plots

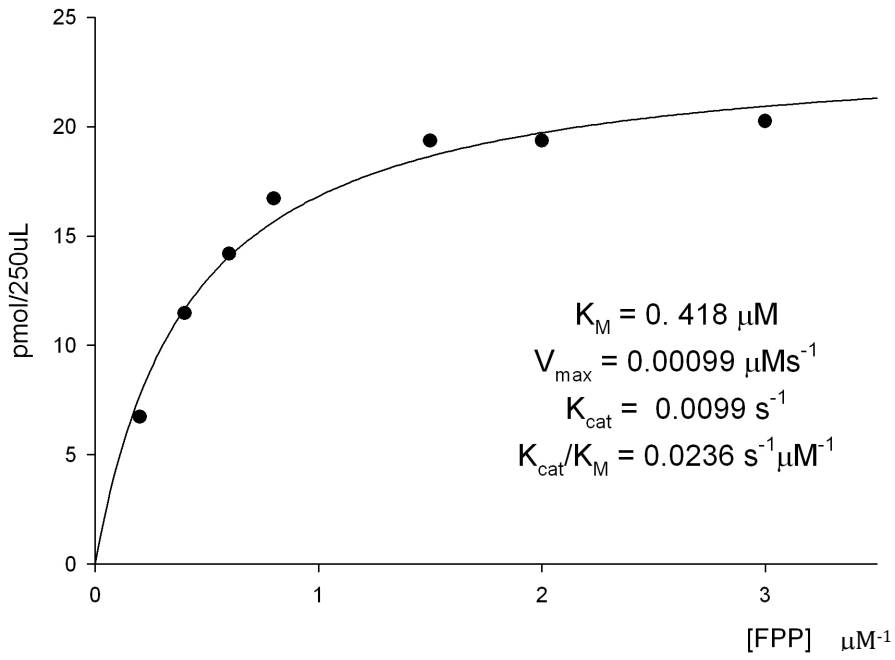


**Figure S22.** Michaelis-Menten plot for incubations of FDP with PR-AS-Y92W.

## References

1. M. Bradford, *Anal. Biochem.,* 1976, **72**, 248-254.

2. F. Karp, Z. Yuxin, B. Santhamma, B. Assink, R. M. Coates and R. B. Croteau, *Arch. Biochem. Biophys.* 2007, **468**, 140-146.

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