

Supporting information for

The role of aristolochene synthase in diphosphate activation

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Site directed mutagenesis of recombinant PR-AS cDNA

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'-GGGCATTATCTCGAGTACAAAGAGAAGGATGTAGGCAAG-3' and
5'-CTTGCCTACATCCTTCTCTTTGTACTCGAGATAATGCCC-3' for R200K;
5'-GGCATTATCTCGAGTACGAAGAGAAGGATGTAGGCAAG-3' and
5'-CTTGCCTACATCCTTCTCTTCGTA ACTCGAGATAATGCC-3' for R200E;
5'-GCATTATCTCGAGTACCAGGAGAAGGATGTAGGCAAG-3' and
5'-CTTGCCTACATCCTTCTCCTGGTACTCGAGATAATGC-3' for R200Q;
5'-TCGAGTACCGTGAGAAGCTGGTAGGCAAGGCGTTGC-3' and
5'-GCAACGCCTTGCCTACCAGCTTCTCACGGTACTCGA-3' for D203L;
5'-CATATACAGTTATGACCAGGAAGAGGAAGCGTC-3' and
5'-GACGCTTCCTCTTCCTGGTCATAACTGTATATG-3' for K251Q;
5'-GACATATACAGTTATGACCGTGAAGAGGAAGCGTCTCG-3' and
5'-CGAGACGCTTCCTCTTCACGGTCATAACTGTATATGTC-3' for K251R
5'-GGAGCAAGACCACGCGTAAATACA ACTAAGGATCCTC-3' and
5'-GAGGATCCTTAGTTGTATTTACGCGTGGTCTTGCTCC-3' for R340K;
5'-GAGCAAGACCACGCGTATGTACA ACTAAGGATCCTC-3' and
5'-GAGGATCCTTAGTTGTACATACGCGTGGTCTTGCTC-3' for R340M;
5'-CAAGACCACGCGTCGCTTTA ACTAAGGATCCTCTAG-3' and
5'-CTAGAGGATCCTTAGTTAAAGCGACGCGTGGTCTTG-3' for Y341F;

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 Eurofins MWG.

Expression of wild type PR-AS and mutants

PR-AS and mutants were overproduced in *E. coli* BL21(DE3) cells and purified as previously described.¹

GC/MS analysis

Proteins (50 μ M) were incubated with FPP (1 mM) in incubation buffer (25 mM Tris, pH 7.5, 5 mM beta-mercaptoethanol, 15% glycerol and 5 mM MgCl₂) in a total volume of 250 μ L. The reaction was overlaid with pentane and left overnight. Products were extracted with 2 portions of 500 μ L pentane and the combined organic layers passed through a short column of aluminium oxide. GC-MS analysis of incubation products was performed using a HP 6890 GC-MS system fitted with J & W Scientific HP-5MS column (30 m length and internal diameter of 0.25 mm) and a Micromass GCT Premiere mass spectrometer for detection. The program uses an initial oven temperature of 50 °C with a gradient of 4 °C min⁻¹ over 25 min followed by a gradient of 20 °C min⁻¹ over 5 min to a final temperature of 250 °C.

Steady-state kinetic assays

Kinetic assays were carried out as previously described.²⁻⁴ Steady-state parameters were obtained by direct fitting of the data to the Michaelis-Menten equation by nonlinear least squares regression using the graphical procedures developed by Lineweaver-Burk⁵ within the SigmaPlot package (Systat Software).

Table 1: Kinetic parameters and product distributions for PR-AS and mutants^a

	4	2	5	K_M	k_{cat} x10⁻³	k_{cat}/K_M
	(%)	(%)	(%)	(μM)	(s ⁻¹)	(M ⁻¹ s ⁻¹)
WT	91.5	7.5	1	0.53	84	158490
R200K	19.2	78.9	1.8	1.04	0.6	620
R200Q				Inactive		
R200E				Inactive		
D203L	17.3	79.7	3	0.74	0.75	1013
K251R	90.2	6	3.8	0.14	3.7	26428
K251Q	84.6	9.8	5.6	0.24	2	8500
R340K	54.4	42.8	2.8	1.59	0.8	510
R340M				Inactive		
Y341F	93.3	3.8	2.9	0.18	2.5	14000

^aReported values are the average of 3 measurements; all values were within 5% of the average.

GC/MS Spectra

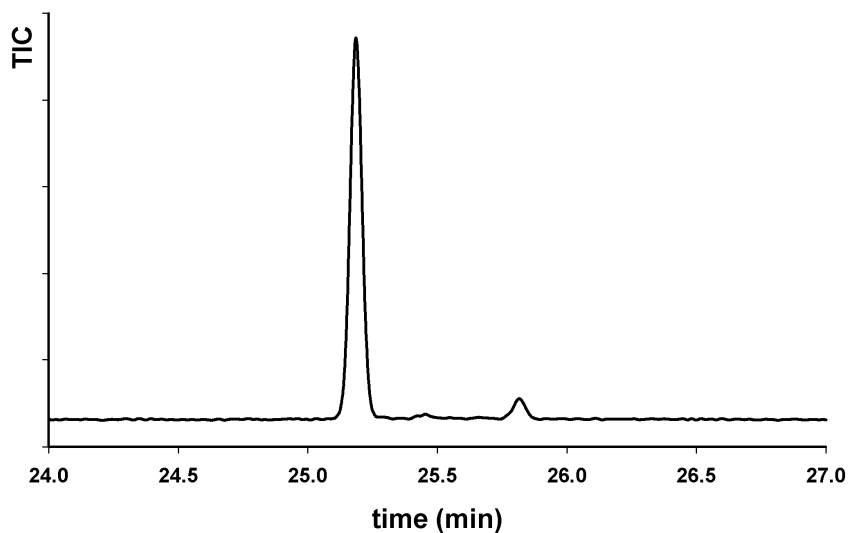


Figure S2. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS: aristolochene (retention time, 24.60 min), valencene (24.93 min) and germacrene A (25.22 min).

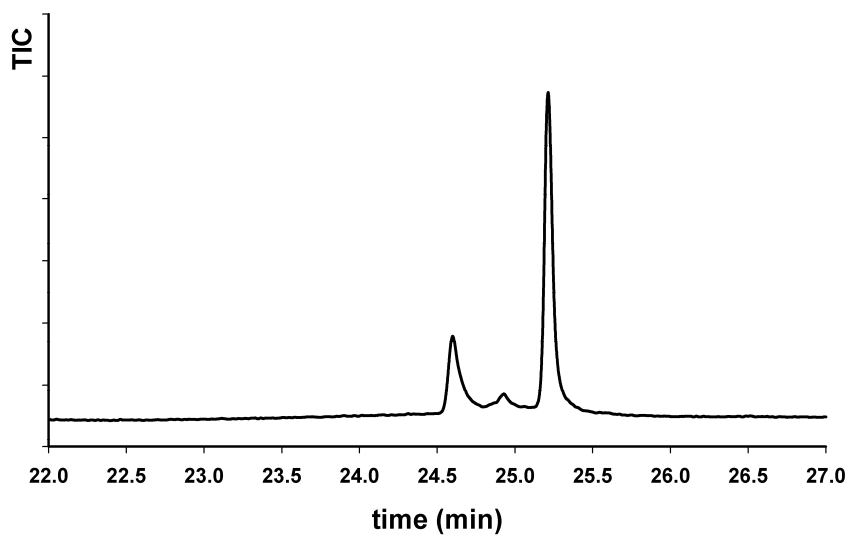


Figure S3. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-R200K: aristolochene (retention time, 24.60 min), valencene (24.93 min) and germacrene A (25.22 min).

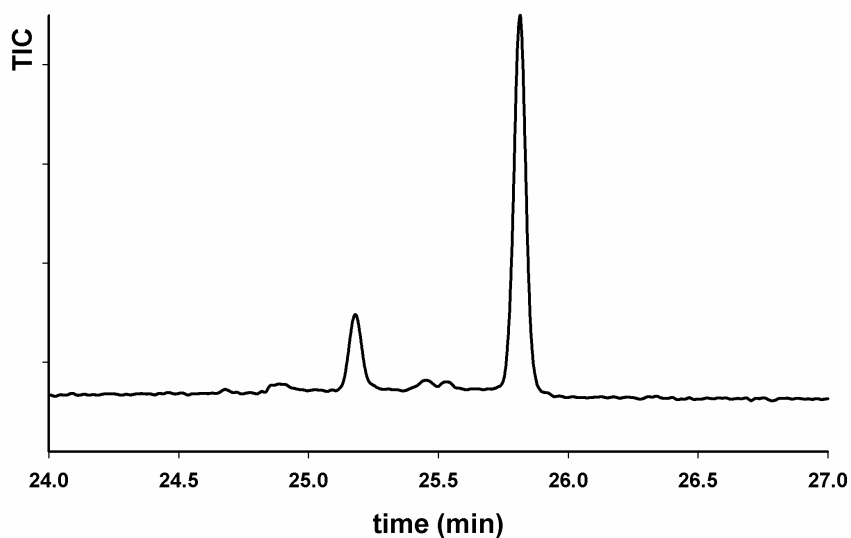


Figure S4. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-D203L: aristolochene (retention time, 25.18 min), valencene (25.48 min) and germacrene A (25.82 min).

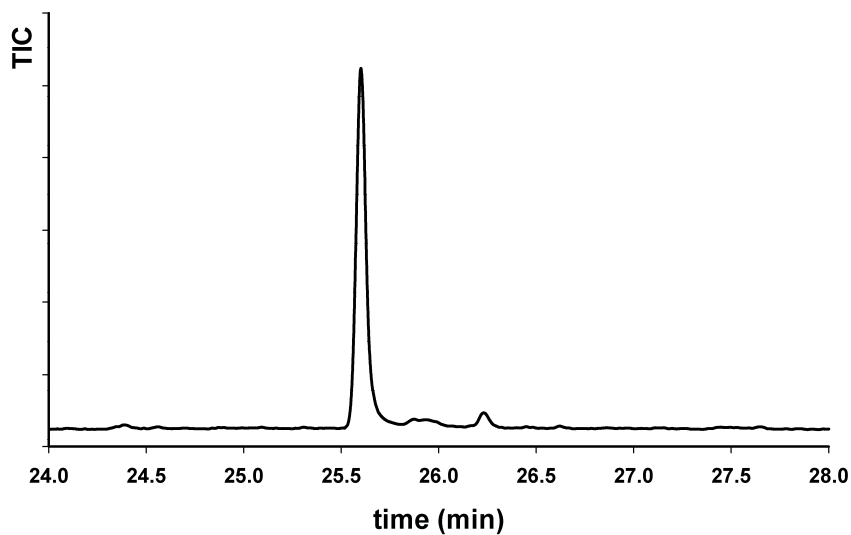


Figure S5. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-K251Q: aristolochene (retention time, 25.60 min), valencene (25.88 min) and germacrene A (26.23 min).

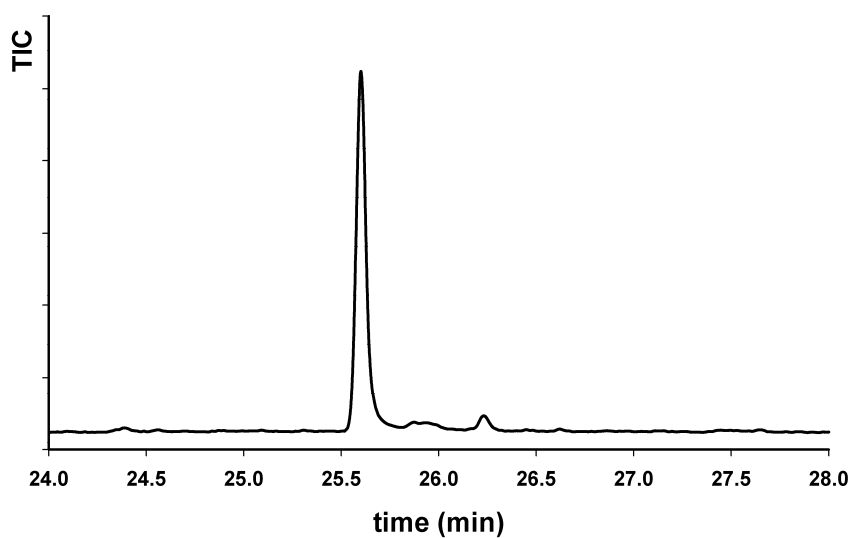


Figure S6. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-K251R: aristolochene (retention time, 25.60 min), valencene (25.88 min) and germacrene A (26.23 min).

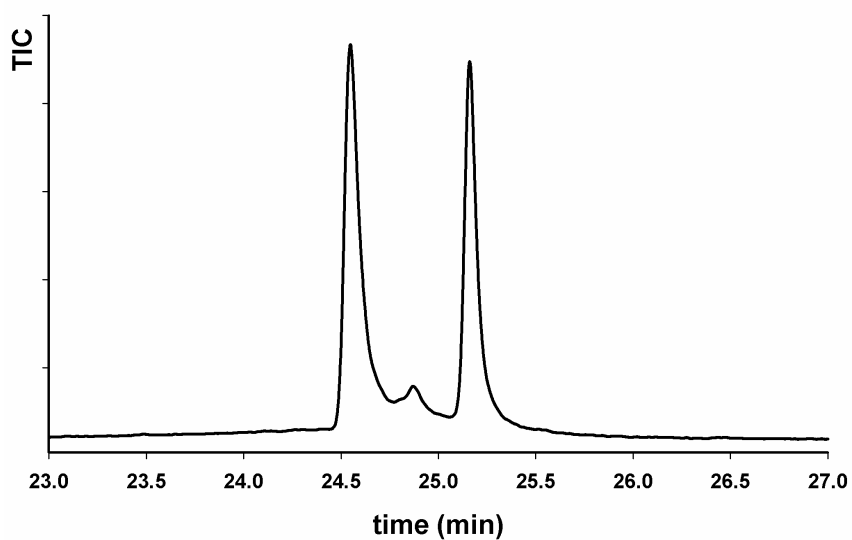


Figure S7. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-R340K: aristolochene (retention time, 24.55 min), valencene (24.93 min) and germacrene A (25.22 min).

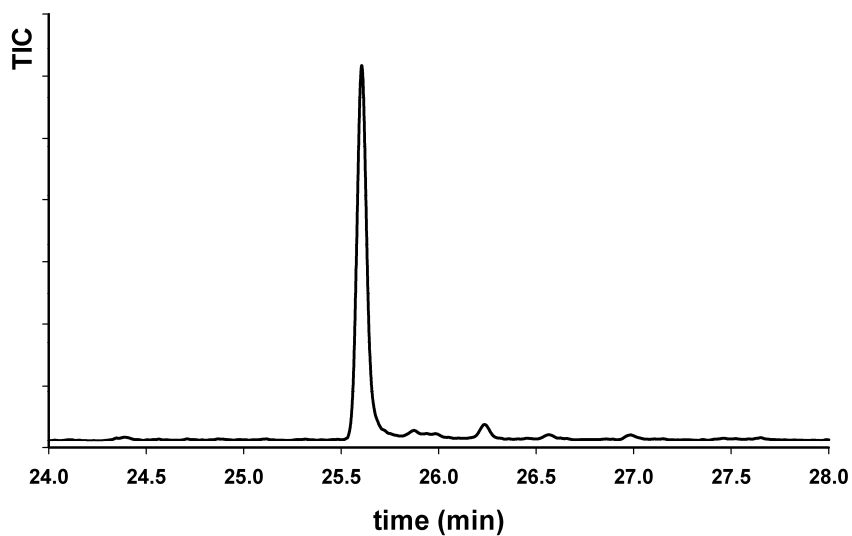


Figure S8. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-Y341F: aristolochene (retention time, 25.60 min), valencene (25.88 min) and germacrene A (26.23 min).

Lineweaver-Burk plots

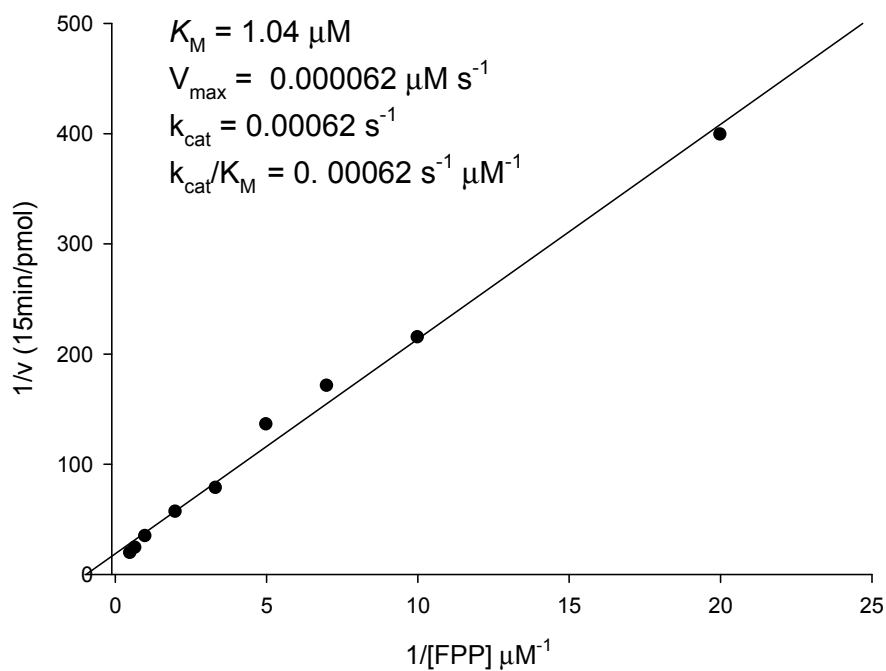


Figure S9. Representative Lineweaver-Burk plot for PR-AS-R200K.

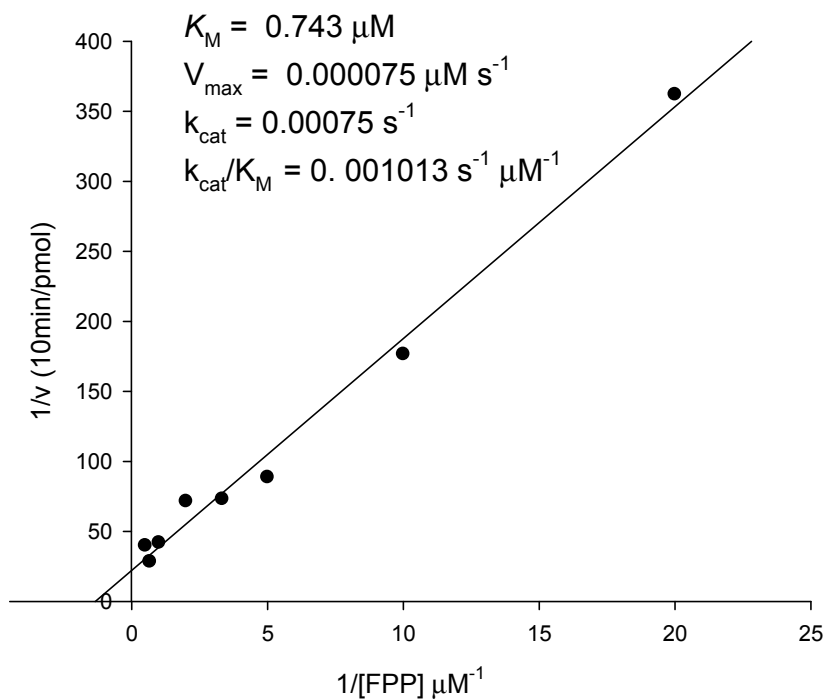


Figure S10. Representative Lineweaver-Burk plot for PR-AS-D203L.

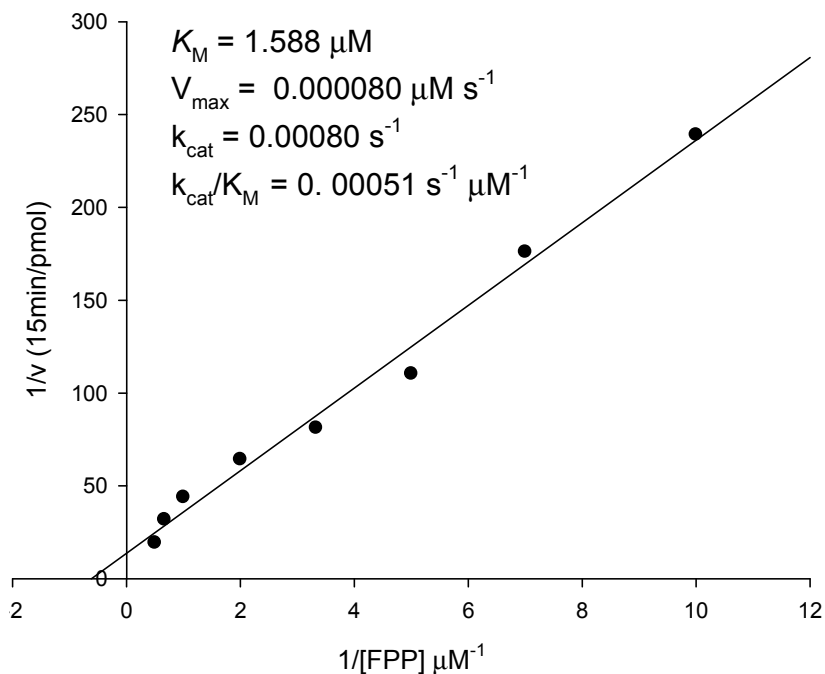


Figure S11. Representative Lineweaver-Burk plot for PR-AS-R340K.

Michaelis-Menten plots

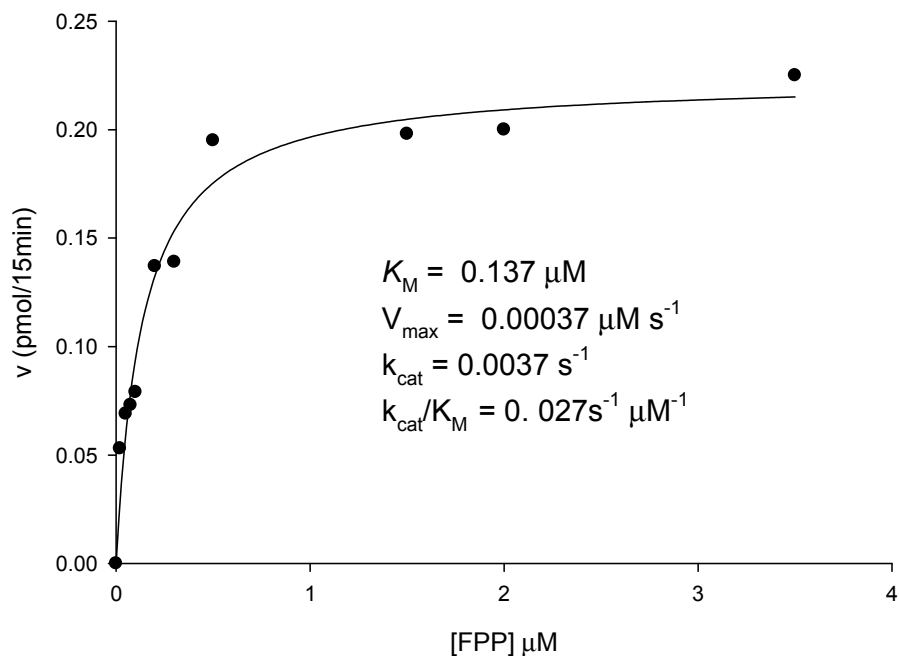


Figure S12. Representative Michaelis-Menten plot for PR-AS-K251R.

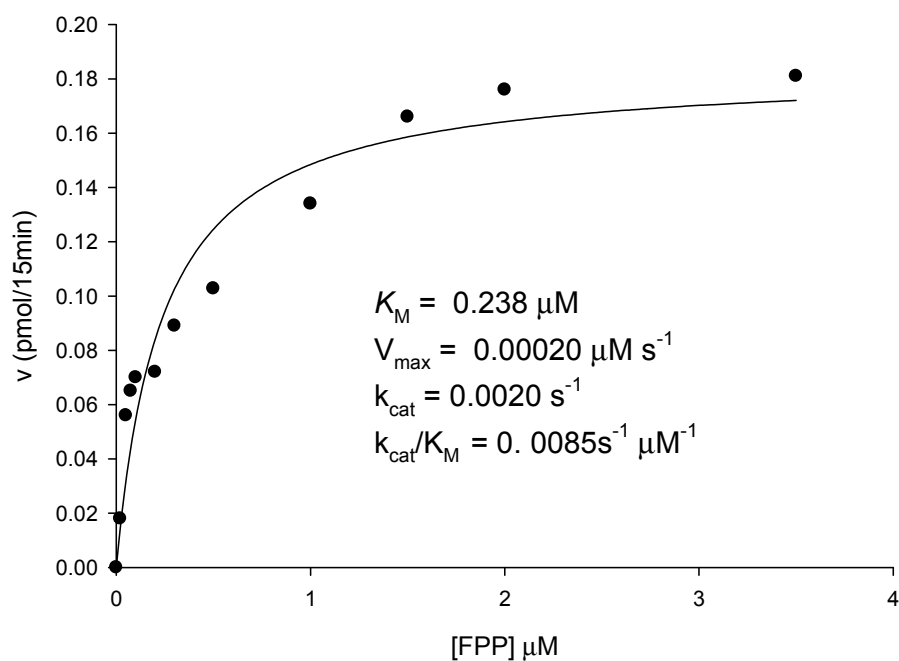


Figure S13. Representative Michaelis-Menten plot for PR-AS-K251Q.

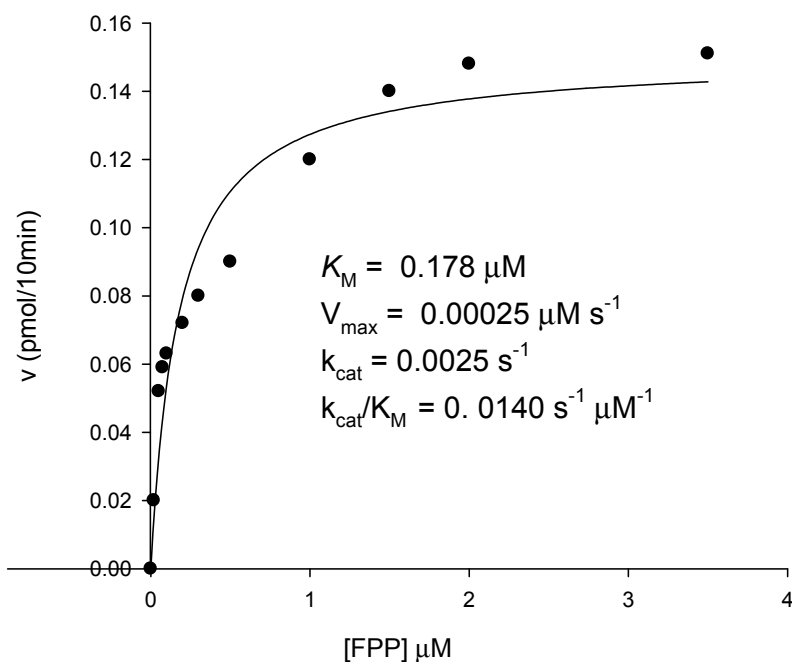


Figure S14. Representative Michaelis-Menten plot for PR-AS-Y341F.

References

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