

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 CT CGAGATAATGCC-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'-CAAGACCACGCGTCGCTTTAACTAAGGATCCTCTAG-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.

| | | |
|----------|-------------------------------------------------------------------------|-----|
| AT-AS | -----MKKPNGTNG-----ASSSLEPPPSTFQPLCHPLVEEVSKE | 35 |
| PR-AS | MATSTETISSLAQPFVHLENPINSPLVKETIRPRNDTTITPPPTQWSYLCHPRVKEVQDE | 60 |
| SC-EI ZS | -----MHAFPHGTTATPTAIAVP-----PSLRLPVIEAAFPRQLHPYWPKLQET | 44 |
| SP-PS | -----MPQDVDFHIP-----LPGRQSPDHARAEAE | 25 |
| FS-TS | -----MENFPTEYFLNTTVRLLLEYIR-----YRDSNYTREERIEIENLHYAYNKAHHF | 47 |
| | | |
| AT-AS | VDGYFLQHWNFNPEKARKKFVAAGFSRVTCLYFPKALDDRIHFACRLLTVLFLI DDLLEY | 95 |
| PR-AS | VDGYFLENWKFPSFKAVRTFLDAKFSEVTCLYFPLALDDRIHFACRLLTVLFLI DDVLEH | 120 |
| SC-EI ZS | TRTWLLEKRLMPADKVEEYADGLCYTDLMAGYYLGAPDEVLQAIADYSAWFFVW DDRHDR | 104 |
| SP-PS | QLAWPRSLGLIRSDAAAERHLRGGYADLASRFYPHATGADLDLGVDLMSWFFLF DDLFDG | 85 |
| FS-TS | AQ--PRQQQLLKVDPKRLQASLQTI VGMVVYSWAKVSKECMADLSIHYTYTLVL DDSKD - | 104 |
| | . : : : . : : : : ** : | |
| | | |
| AT-AS | MSFEEGSAYNEKLIPISRGDVLV--DRSIPVEYIIYDL-----WESMR-----AHDR | 140 |
| PR-AS | MSFADGEAYNNRLIPISRGDVLV--DRTKPEEFILYDL-----WESMR-----AHDA | 165 |
| SC-EI ZS | DIVHGRAGAWRRLRGLLHTALDSPGDHLHHEDTLVAGF-----ADSVRRLYAFLPATWNA | 159 |
| SP-PS | PRGEN-PEDTKQLTDQVAAALDG--PLPDTAPPIAHGF-----ADIWRRTCEGMTPAWCA | 137 |
| FS-TS | DPYPTMVNYFDDLQAGREQAHPWWALVNEHFPNVLRRHFGPFCSLNLIRSTLDFEFGCWI E | 164 |
| | * : : : * | |
| | | |
| AT-AS | EMADEILEPVFLFMRAQTDRTR-ARPMGLGGY--LEY RED VDVGKELLAALMRFS--MGLK | 195 |
| PR-AS | ELANEVLEPTFVFMRAQTDRAR-LSIHELGHY--LEY REK DVGKALLSALMRFS--MGLR | 220 |
| SC-EI ZS | RFARHFHTVIEAYDREFHNRTR-GIVPGVEEY--LEL RL TFAHWIWTDLLEPS--SGCE | 214 |
| SP-PS | RSARHWRNYFDGYVDEAESRFWNAPCDSAAQY--LAM RR RHTIGVQPTVDLAERA--GRFE | 193 |
| FS-TS | QYNFGGFPGSHDYPPQFLR RM NGLGHCVGASLWPKEQFNERSLFLEITSAIAQMENWMVWV | 224 |
| | . : : . . : . | |
| | | |
| AT-AS | LSPSELQR--VREIDANCSKHLSSVNDIYSY KE LYTSKTAHSEGGILCTSVQILAQEA- | 252 |
| PR-AS | LSADELQD--MKALEANCAKQLSVVNDIYSY KE EEASRTGHKEGAFLC SAVKVLAEES- | 277 |
| SC-EI ZS | LPDAVRKHPAYRRAALLSQEFAAWYNDLCSLP KE EIAGDEVHNLGISLITHHSLTLEEAI G | 274 |
| SP-PS | VPHRVFDSAVMSAMLQIAVDVNLNLLNDIASLE KE EEARGEQNNMVMILRREHGWSKRSVS | 253 |
| FS-TS | NDLMSFY KE FDDERDQISLVKNYVVSDEISLNEALEKLTQDTLHSSKQMVAVFSDKDPQV | 284 |
| | . * * : | |
| | | |
| AT-AS | --DVTAEAAKRVLFVCREWELRHQQLLVAR-LSAEGLETPGLAAYVEGLEQMSGNELWS | 309 |
| PR-AS | --KLGIPATKRVLWSMTREWETVHDEIVA EKIASPDGCSEAAKAYMKGLEQMSGNEQWS | 335 |
| SC-EI ZS | EVRRRVEECITEFLAVERDALRFADELADGTVRGKE-LSGAVRANVGNMRNWFSSVYWFH | 333 |
| SP-PS | HMQNEVRARLEQYLLLESCLPKVGEIYQLDTAEREA----LERYRTDAVRTVIRGSYDWH | 309 |
| FS-TS | MDTIECFMHGYVTWHLCDR RY RLSEIYEKVKKEKTEDAQKFKFYEQANVGA VSPSEWA | 344 |
| | : : . . : | |
| | | |
| AT-AS | QTTL RY SVVVD----- | 320 |
| PR-AS | K T TR RY N----- | 342 |
| SC-EI ZS | HESG RY MVDSWDDRSTPPYVNNEAAGEK-- | 361 |
| SP-PS | RSSG RY DAEFALAAGAQQYLEELGSSAH-- | 337 |
| FS-TS | YPPVAQLANVRSKDVKEVQKPFLSSIELVE | 374 |

Figure S1. Amino acid sequence alignment of fungal/bacterial sesquiterpene synthases
ClustalW2.⁶ AT-AS and PR-AS, *A. terreus* and *P. roqueforti* aristolochene synthases; EIZS, *S. coelicolor* epi-isozizane synthase; PS, *Streptomyces sp.* pentalenene synthase; TS, *Fusarium sporotrichioides* trichodiene synthase.

GC/MS Spectra

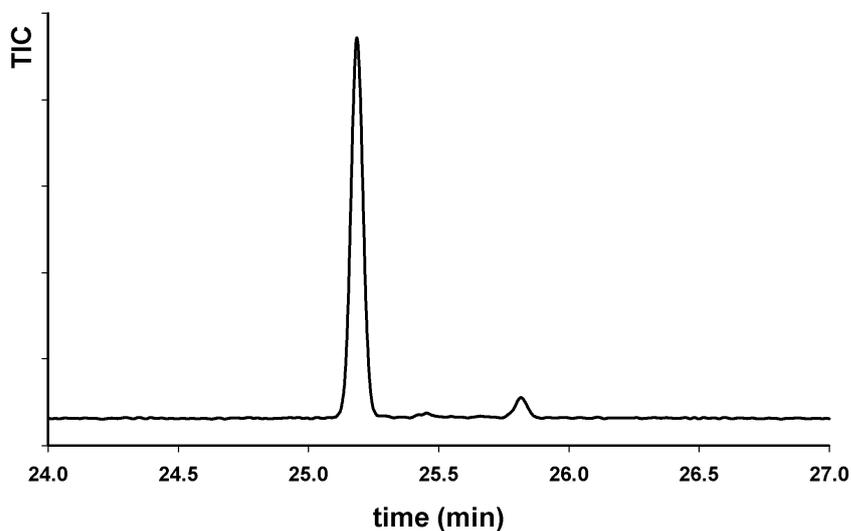


Figure S2. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS: aristolochene (retention time, 24.60 min), valencene (24.93 min) and germacrene A (25.22 min).

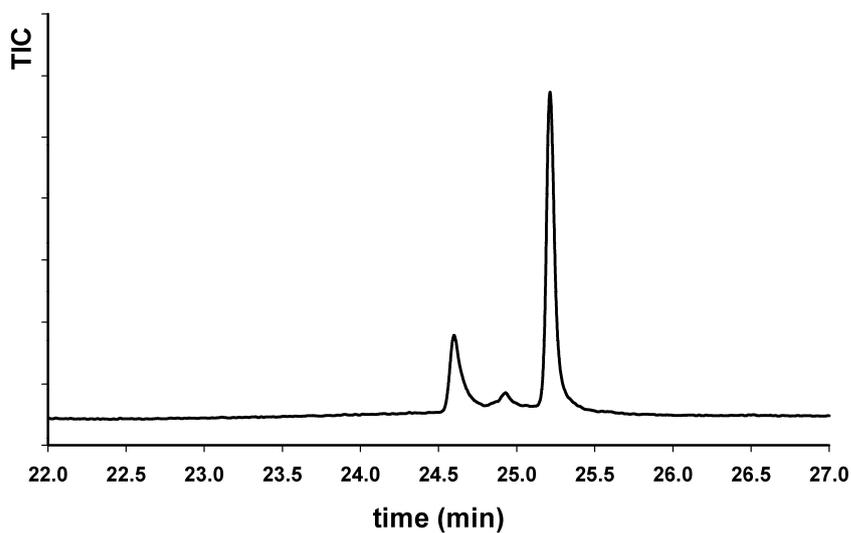


Figure S3. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-R200K: aristolochene (retention time, 24.60 min), valencene (24.93 min) and germacrene A (25.22 min).

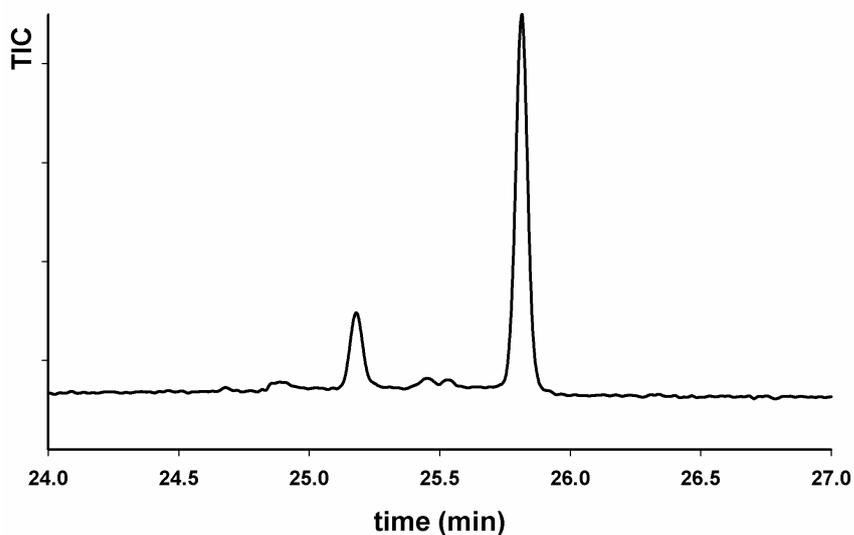


Figure S4. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-D203L: aristolochene (retention time, 25.18 min), valencene (25.48 min) and germacrene A (25.82 min).

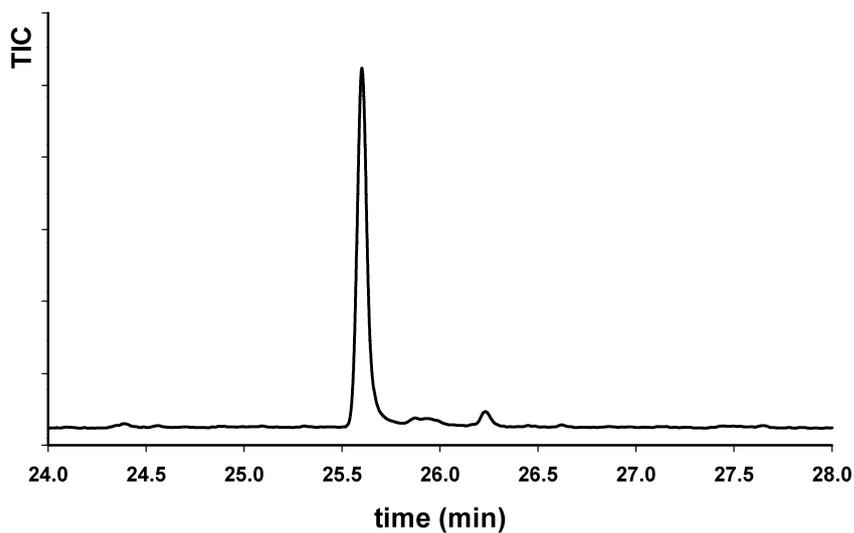


Figure S5. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-K251Q: aristolochene (retention time, 25.60 min), valencene (25.88 min) and germacrene A (26.23 min).

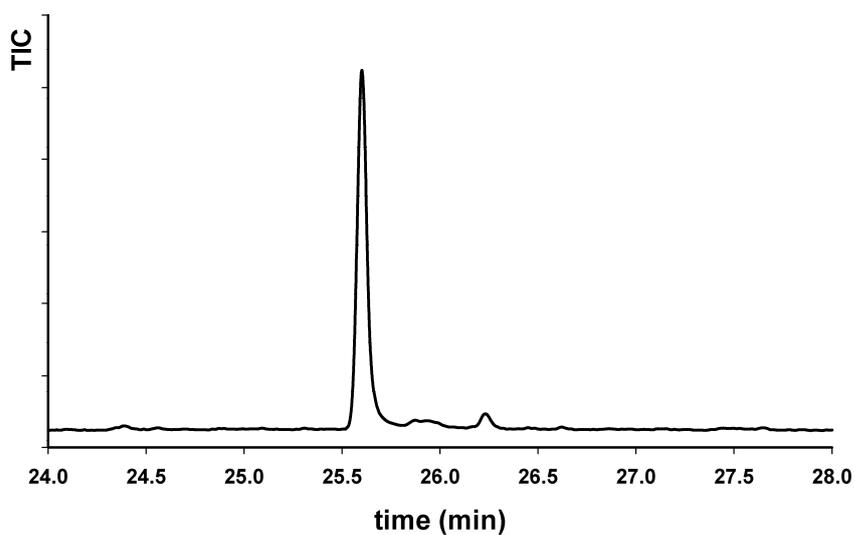


Figure S6. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-K251R: aristolochene (retention time, 25.60 min), valencene (25.88 min) and germacrene A (26.23 min).

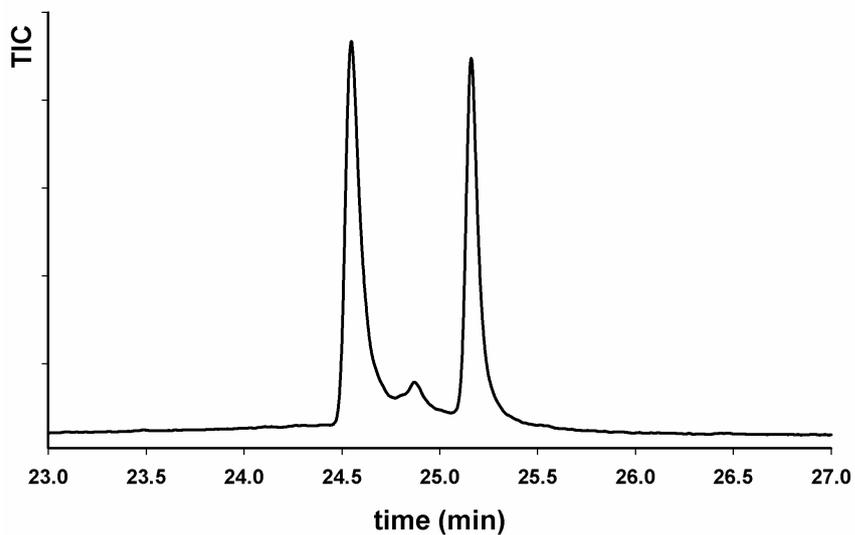


Figure S7. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-R340K: aristolochene (retention time, 24.55 min), valencene (24.93 min) and germacrene A (25.22 min).

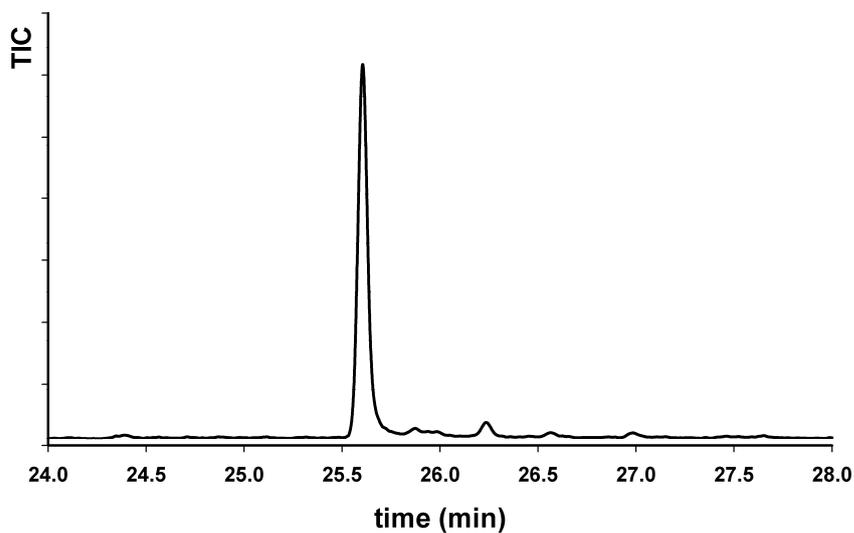


Figure S8. Representative total ion chromatogram of the pentane extractable products formed from incubation of FDP and PR-AS-Y341F: aristolochene (retention time, 25.60 min), valencene (25.88 min) and germacrene A (26.23 min).

Lineweaver-Burk plots

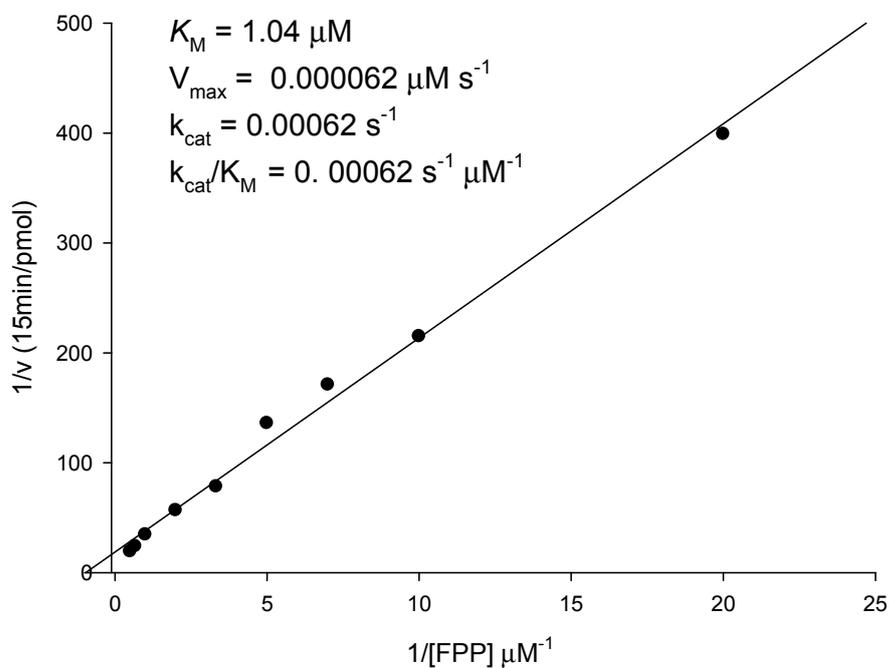


Figure S9. Representative Lineweaver-Burk plot for PR-AS-R200K.

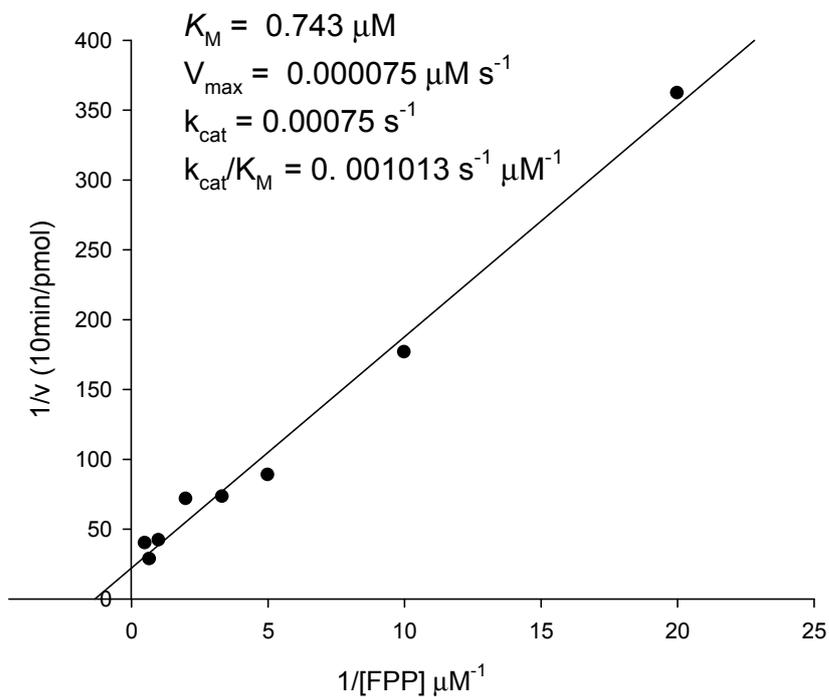


Figure S10. Representative Lineweaver-Burk plot for PR-AS-D203L.

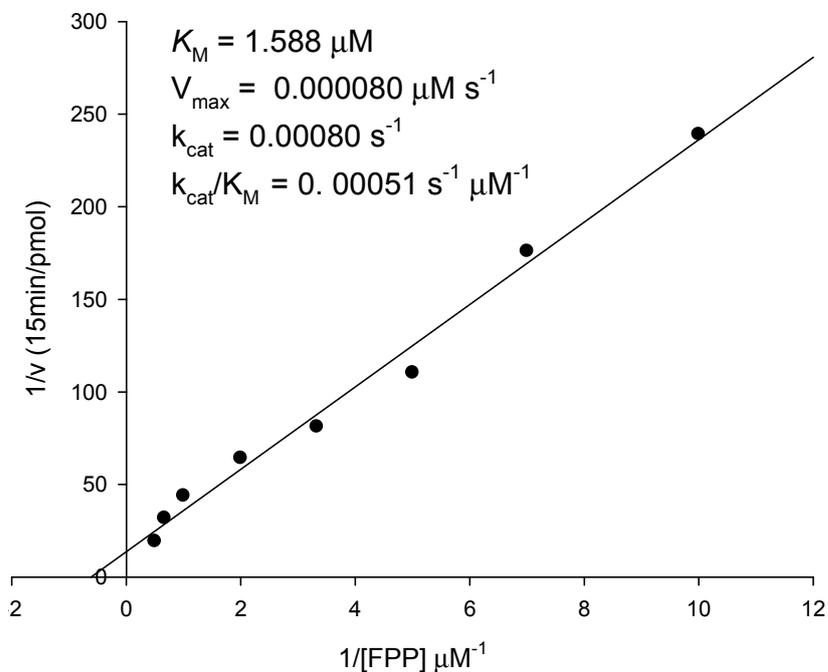


Figure S11. Representative Lineweaver-Burk plot for PR-AS-R340K.

Michaelis-Menten plots

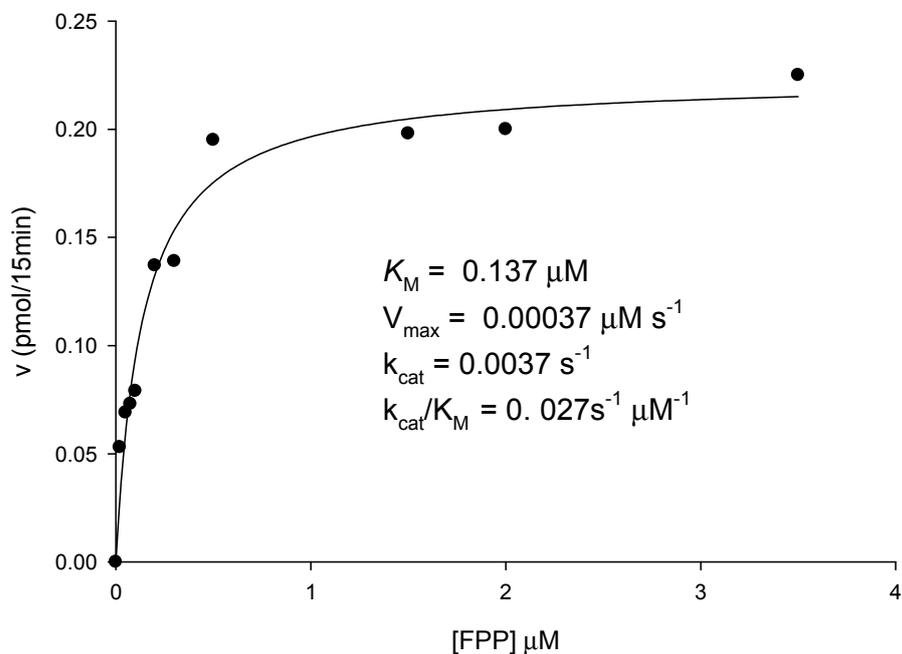


Figure S12. Representative Michaelis-Menten plot for PR-AS-K251R.

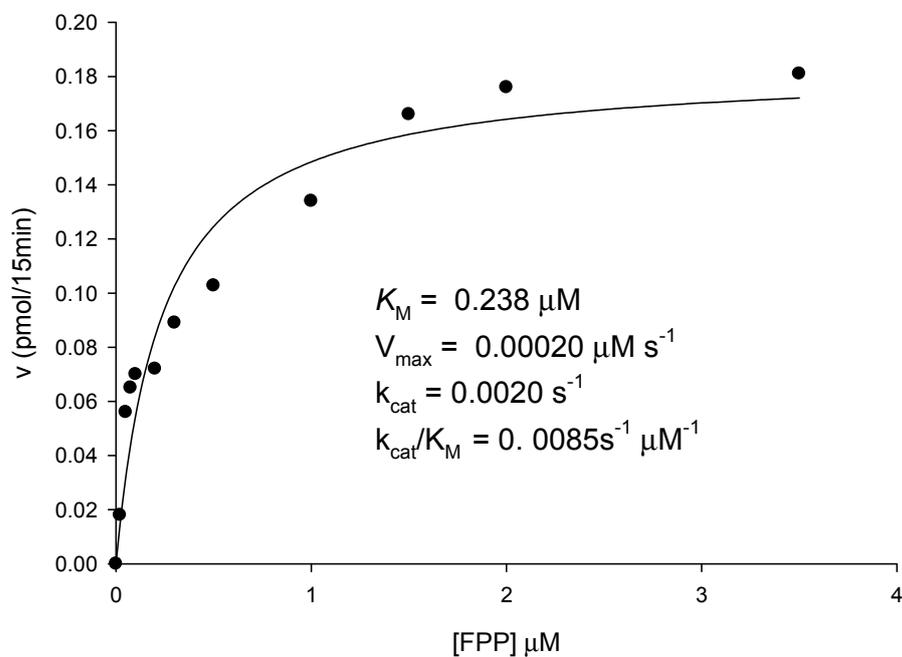


Figure S13. Representative Michaelis-Menten plot for PR-AS-K251Q.

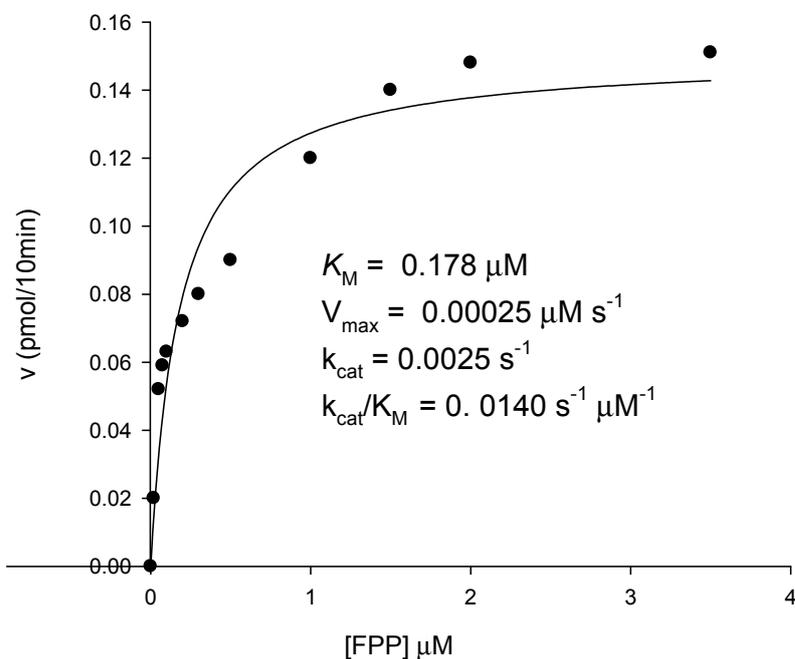


Figure S14. Representative Michaelis-Menten plot for PR-AS-Y341F.

References

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