Supporting Information for:

A single residue switch converts abietadiene synthase into a pimaradiene specific cyclase

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Material and methods

General

Unless otherwise noted all chemicals were purchased from Fisher Scientific (Loughborough, Leicestershire, UK) and molecular biology reagents from Invitrogen (Carlsbad, CA, USA).

Mutagenesis

Wild-type pseudo-mature AgAS[1] was transferred via PCR amplification and directional topoisomerase-mediated insertion into the Gateway system pENTR/SD/D-TOPO vector and verified by complete sequencing. Site directed mutagenesis was carried out via PCR amplification with overlapping mutagenic primers of the pENTR/AgAS vector. The resulting AgAS:A723S mutant was verified by complete sequencing. Both the wild-type and A723S mutant of AgAS were then transferred by directional recombination into the T7-promoter based Gateway expression vector pDEST14.

Product analysis

For product analysis, the wild-type and A723S mutant of AgAS were co-expressed in the OverExpress C41 strain of *E. coli* (Lucigen, Middleton, WI) with an (*E,E,E*)-geranylgeranyl diphosphate (GGPP) synthase, using the previously described pGG vector.[2] Gas chromatography-mass spectrometry (GC-MS) was performed on organic extracts of the resulting recombinant cultures using an HP1-MS column on an Agilent (Palo Alto, CA) 6890N GC instrument with 5973N mass selective detector in electron-ionization mode (70 eV) located in the W.M. Keck Metabolomics Research laboratory at Iowa State University, much as previously described.[3] Briefly, 5 µL of sample was injected at 40°C in splitless mode, the oven temperature held at 40°C for 3 min., then raised at 20°C/min. to 300°C, and held there for 3 min. MS data was collected from 50 to 500 *m/z* during the temperature ramp and final hold. The enzymatically produced diterpenes were identified by comparison of retention time and mass spectra to authentic samples (e.g. Supporting Figure).

Kinetic characterization

Comparison of the diterpene yield from recombinant bacterial cultures co-expressing wild type or mutant diterpene synthases with a GGPP synthase has been correlated with relative catalytic efficiency in vitro,[4] and such comparative analysis demonstrated that the AgAS:A723S mutation actually increased diterpene production ~2-fold. For more detailed in vitro analysis AgAS constructs were expressed and purified as previously described.[5] To selectively determine the effect of the A723S mutation on the relevant class I activity assays were run with [1-$^{3}H_{1}$]-CPP. Given our limited quantities of this radiolabeled substrate, only specific activity was measured. Briefly, duplicate reactions were run in assay buffer (50 mM Hepes, pH 7.2, 7.5 mM $MgCl_2$, 10% glycerol, 0.1 mg/mL α-casein, and 5 mM fresh DTT) containing 5 µM CPP and 10

nM AgAS for 1 min. at room temperature and stopped by the addition of KOH to 0.2 M and EDTA to 15 mM. The produced diterpenes were then extracted in hexanes, the pooled extract passed over a short silica gel column, and product formation assessed by scintillation count. Under these conditions AgAS:A723S exhibited specific activity of 0.3 units (nmole product/min.)/mg protein, while the wild type enzyme = 0.2 units/mg protein (both with standard error of <20%).

Literature References

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(3)Xu, M.; Hillwig, M. L.; Prisic, S.; Coates, R. M.; Peters, R. J. *Plant J.* **2004**, *39*, 309-318.

(4)Xu, M.; Wilderman, P. R.; Peters, R. J. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 7397-7401.

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Supporting Figure: Mass spectra for A) the AgAS:A723S major product (retention time = 13.33 min.) and B) authentic isopimara-7,15-diene (retention time = 13.32 min.).