Published in final edited form as:

Biochemistry. 2017 October 31; 56(43): 5798–5811. doi:10.1021/acs.biochem.7b00895.

Substitution of Aromatic Residues with Polar Residues in the Active Site Pocket of *epi*-Isozizaene Synthase Leads to the Generation of New Cyclic Sesquiterpenes

Patrick N. Blank[†], Golda H. Barrow[†], Wayne K. W. Chou[‡], Lian Duan[‡], David E. Cane^{‡,*}, and David W. Christianson^{†,*}

[†]Roy and Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104-6323

[‡]Department of Chemistry, Box H, Brown University, Providence, RI 02912-9108

Abstract

The sesquiterpene cyclase *epi*-isozizaene synthase (EIZS) catalyzes the cyclization of farnesyl diphosphate to form the tricyclic hydrocarbon precursor of the antibiotic albaflavenone. The hydrophobic active site pocket of EIZS serves as a template as it binds and chaperones the flexible substrate and carbocation intermediates through the conformations required for a multistep reaction sequence. We previously demonstrated that the substitution of hydrophobic residues with other hydrophobic residues remodels the template and expands product chemodiversity [Li, R., Chou, W. K. W., Himmelberger, J. A., Litwin, K. M., Harris, G. G., Cane, D. E., and Christianson, D. W. (2014) *Biochemistry* 53, 1155–1168]. Here, we show that the substitution of hydrophobic residues – specifically, Y69, F95, F96, and W203 – with *polar* side chains also yields functional enzyme catalysts that expand product chemodiversity. Fourteen new EIZS mutants are reported that generate product arrays in which 8 new sesquiterpene products have been identified. Of note, some mutants generate acyclic and cyclic hydroxylated products, suggesting that the introduction of polarity in the hydrophobic pocket facilitates the binding of water capable of quenching carbocation intermediates. Furthermore, the substitution of polar residues for F96 yields high-fidelity sesquisabinene synthases. Crystal structures of selected mutants reveal that residues defining the three-dimensional contour of the hydrophobic pocket can be substituted without triggering significant structural changes elsewhere in the active site. Thus, more radical nonpolar-polar amino acid substitutions should be considered when terpenoid cyclase active sites are remodeled by mutagenesis with the goal of exploring and expanding product chemodiversity.

*To whom correspondence should be addressed. D.W.C.: telephone: (215) 898-5714; chris@sas.upenn.edu. D.E.C.: telephone: (401) 863-3588; david_cane@brown.edu.

ORCID

David E. Cane: 0000-0002-1492-9529

David W. Christianson: 0000-0002-0194-5212

Accession Codes

The atomic coordinates and crystallographic structure factors of EIZS mutants F95Y, W203Y, F95N, F95C, and F96S have been deposited in the Protein Data Bank (www.rcsb.org) with accession codes 6AXM, 6AXU, 6AX9, 6AXN, and 6AXO, respectively.

Notes

The authors declare no competing financial interest.

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Graphical Abstract

Introduction

The terpenome comprises an expansive library of natural products, currently numbering over 81,000 compounds produced in all domains of life (<http://dnp.chemnetbase.com>). These compounds serve myriad biological and ecological functions, e.g., in communication and defense.^{1–3} For example, oleoresin from grand fir (*Abies grandis*) is a complex mixture of terpenoids that deters insects and protects wound sites.⁴ The sesquiterpenes 7-*epi*-zingiberene and (*R*)-curcumen are insect repellents emitted by wild tomato (*Solanum habrochaites*) to protect against whiteflies (*Bemisia tabaci*).⁵ Many plants also utilize terpenoids such as (*E*)- β -ocimene and myrcene as attractants for pollinators.⁶ In another example, maize plants (*Zea mays*) emit a terpenoid mixture containing the sesquiterpene (*E*)- α -bergamotene for protection against attack by Lepidopteran species (moths and butterflies); the emitted terpenoids attract the parasitic wasp *Cotesia marginiventris*, which lays eggs in Lepidopteran larvae and thereby blocks development into adult insects.⁷

Many terpenoids are commercially important due to their physical or pharmaceutical properties. For example, the cyclic monoterpene menthol is a flavor additive in many consumer products due to its minty aroma, and it is well known for its analgesic properties; menthol is also used in topical gels as a transdermal drug delivery agent.^{8,9} Another monoterpene, D-limonene, is naturally present in lemon and orange rinds and is well known for its citrus aroma. D-Limonene is used as a flavor additive, as a fragrance in household products, and as a potential biofuel.^{10,11} Sesquiterpenes also serve as advanced biofuels: (*Z*)- α -bisabolene is a precursor of the D2 diesel fuel substitute bisabolane, while (*E*)- β -farnesene is a precursor to the bio-jet fuel farnesane.^{12–15}

Terpenoid cyclases are the enzymes responsible for the generation of the hydrocarbon skeletons of these natural products as they catalyze the cyclization of linear isoprenoid substrates, such as farnesyl diphosphate (FPP), through a well-defined reaction sequence typically characterized by multiple carbocation intermediates. There are two classes of terpenoid cyclases, denoted I and II, that are distinguished by conserved metal-binding DDXD/E and general acid DXDD amino acid sequence motifs, respectively.^{16–21} Class I cyclases additionally have a second metal-binding motif, conserved as (N, D)DXX(S, T)XX(R, K)E. Class I cyclases initiate the cyclization reaction via metal-triggered ionization of the substrate diphosphate group, whereas class II cyclases initiate catalysis through protonation of a carbon-carbon double bond or epoxide moiety. For both classes of cyclases, the hydrophobic active site cavity has a well-defined contour that serves as a template for the cyclization reaction.

The sesquiterpene cyclase *epi*-isozizaene synthase from *Streptomyces coelicolor* A3(2) (EIZS) is an excellent paradigm for the study of cyclization fidelity.^{22,23} The active site of this class I cyclase is nested within a characteristic helix bundle (designated the α fold²⁴), in which the cyclization of farnesyl diphosphate (FPP) is initiated by metal-triggered ionization of the diphosphate group. *epi*-Isozizaene (Figure 1) is the sole cyclization product at 4 °C (99%) and the major cyclization product at 30 °C (79%).^{25,26} The crystal structure of wild-type EIZS has been determined at 1.6 Å resolution in complex with 3 Mg²⁺ ions, inorganic pyrophosphate (PP_i), and the benzyltriethylammonium cation (BTAC), a lipophilic cation that partially mimics the bisabolyl carbocation intermediate.²⁵ Analysis of this structure reveals that aromatic residues define a significant portion of the active site contour. Specifically, the ring faces of F95, F96, and F198 largely define the walls of the active site and engage in cation- π interactions with BTAC.²⁵ Other aromatic residues contribute to the active site contour but are not ideally oriented to engage in cation- π interactions with bound ligands. For example, Y69 is in the “second shell” of residues defining the active site and buttresses F96, whereas the edge of the indole ring of W203 partially defines the floor of the active site (Figure 2).

Our initial studies of a library of 26 site-specific mutants of EIZS, which included 13 mutants in which active site aromatic residues were substituted mainly with nonpolar amino acids, revealed that some mutants generated an alternative predominant product (Figure 1).²⁶ The formation of alternative products is a consequence of remodeling the hydrophobic active site contour, which then redirects the cyclization cascade. Curiously, the F96Y and F198Y substitutions did not deactivate the enzyme – a newly introduced tyrosine hydroxyl group could be susceptible to alkylation by a carbocation intermediate in catalysis, but the hydrophobic active site nevertheless tolerated this substitution to yield a functional catalyst. Contrary to conventional wisdom, we hypothesized that other polar amino acids might be introduced into the EIZS active site in place of key aromatic residues without risk of incapacitating the enzyme catalyst. Substitution of aromatic residues could thus influence the template function of the active site as well as the stabilization of carbocation intermediates through substitution of cation- π interactions with cation-dipole interactions.

Here, we report the preparation and analysis of 16 new EIZS mutants in which four active site aromatic residues are substituted with various amino acids: Y69, F95, F96, and W203

(Figure 2). Most mutants were designed specifically to introduce new polar functionality in the active site. Notably, through the F96S, F96M, and F96Q substitutions, we have converted EIZS into a high-fidelity sesquisabinene synthase (these mutants generate 78%, 91%, and 97% sesquisabinene A, respectively). We have also discovered the generation of 8 additional sesquiterpene hydrocarbon and alcohol products by various mutant enzymes that were not observed in our previous study.²⁶ X-ray crystal structures of selected mutants provide a framework for understanding structure-function relationships in mutant cyclases.

MATERIALS AND METHODS

Mutagenesis

A total of 16 new EIZS single-point mutants were prepared via polymerase chain reaction (PCR). Forward and reverse primers (Table S1) were used to introduce mutations into the wild-type EIZS plasmid. PCR amplification reagents were as follows: deionized H₂O (33.5 μ L), 10x Pfu Ultra HF Buffer (5 μ L), dimethyl sulfoxide (DMSO) (2.5 μ L), 10 mM dNTPs (4 μ L), 150 ng/ μ L forward primer (1 μ L), 150 ng/ μ L reverse primer (1 μ L), 50 ng/ μ L wild-type EIZS template plasmid (2 μ L), and 2.5 U/ μ L Pfu Ultra HF enzyme (1 μ L) for a solution totaling 50 μ L.

PCR reactions were run as follows: initial denaturation of the mixture at 90 °C for 1 min, followed by 30 cycles of denaturation at 90 °C for 30 s, annealing at 5–10 °C below the T_m of primers (typically 55 °C) for 1 min, and extension at 72 °C for 7 min. A final extension after 30 cycles was conducted at 72 °C for 10 min followed by a final hold at 4 °C. The PCR mixture was incubated at 37 °C for 2 h with DpnI (1 μ L), deionized H₂O (3 μ L), and 10x CutSmart Buffer (6 μ L) yielding 60 μ L of DpnI digestion products. These digested PCR products were then purified by adding 300 μ L Qiagen Buffer PB and mixing with 20 μ L of 3M sodium acetate (pH 5.0). The mixture was placed in a QIAquick PCR Purification Spin Column and centrifuged at 12,045 rcf for 1 min. A total of 750 μ L Qiagen Buffer PE (with EtOH) was added and centrifuged for 1 min at 12,045 rcf. The column was placed in a 1.5 mL tube before applying 50 μ L Qiagen Buffer EB. After incubation at room temperature for 1 min, DNA was eluted by centrifugation for 1 min at 12,045 rcf. Purified DNA was concentrated by vacuum to a volume of approximately 12 μ L, 2 μ L of which was transformed into DH5 α cells. Plasmid DNA was extracted from single colonies with a QIAprep Spin Miniprep Kit. The target mutation was verified by sequencing at the DNA Sequencing Facility of the University of Pennsylvania Perelman School of Medicine.

Expression

Mutant proteins were expressed and purified using procedures identical to those previously described.²⁶ Briefly, all EIZS mutants were expressed in BL21(DE3) *E. coli* and purified by Co²⁺ immobilized metal affinity chromatography and size exclusion chromatography. F95E and F95D EIZS formed inclusion bodies, so these mutants were not studied further.

Analysis of Sesquiterpene Product Arrays

Product array analyses were conducted using gas chromatography-mass spectrometry (GC-MS) to determine the terpenoid natural products produced by mutant proteins using

procedures identical to those previously reported.²⁶ Briefly, the extracts of organic products resulting from incubation of mutant enzymes with FPP were analyzed using an Agilent 5977A Series GC/MSD instrument. Compounds were identified by comparison of their individual mass spectra and specific retention indices with those of authentic compounds in the MassFinder 4.0 Database. Product arrays for F96M EIZS and F96Q EIZS were analyzed in duplicate to confirm high levels of sesquisabinene synthase activity.

Catalytic activity measurements

For the measurement of specific activities, a substrate stock solution containing 500 μ M FPP [97% FPP (Tris-potassium salt, Echelon Biosciences) and 3% 3 H-FPP (Tris-ammonium salt, American Radiolabeled Chemicals)] was prepared. Stock solutions of wild-type and mutant EIZS enzymes were also prepared in buffer A [50 nM enzyme, 20 mM Tris (pH 7.5), 300 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, 2 mM TCEP]; 20- μ L aliquots of each were added to 470 μ L of buffer A. After the addition of 10 μ L substrate stock solution to initiate the reaction, the final concentration of enzyme was 2 nM in a total volume of 500 μ L. After 15 min, reactions were quenched by the addition of 38 μ L of buffer B [0.5 M EDTA (pH 8.0)]. Products in the resulting organic layer, upon clear phase separation, were extracted by overlaying the reaction mixture with 2×700 μ L of hexane, vortexing for 10 s, and then removing 2×600 μ L of hexane. Great care was taken not to touch the aqueous layer containing unreacted 3 H-FPP, which would give false counts. The organic extracts were transferred to silica gel columns, and the resulting eluate was collected in scintillation vials containing 5 mL of scintillation fluid (Ecoscint). A total of 1 mL of diethyl ether was applied after both extractions had eluted and collected in the scintillation vial and mixed by vortexing. The final mixture in the scintillation vial was subject to radioactive counting using a Beckman scintillation counter. Blanks were run in the absence of radiolabeled FPP, counted, averaged, and subtracted from averaged readings measured for each mutant enzyme. Measurements were made in quadruplicate.

X-ray crystal structure determinations

Heterogeneous microseeding was used to crystallize EIZS mutants. Crystals of wild-type EIZS in complex with Mg^{2+}_3 , benzyl triethylammonium cation (BTAC), and PP_i were grown as previously described,²⁵ then manually crushed in the 8 μ L crystallization drop. A 10 μ L drop of precipitant solution [0.1 M bis-Tris (pH 5.5), 0.2 M $(NH_4)_2SO_4$, 25% (w/v) polyethylene glycol (PEG) 3350] was added to the drop containing crushed crystals, and the resulting 18 μ L drop was transferred to 162 μ L of precipitant solution to create a 10x dilution seed stock. This tube was vortexed briefly to further break up the crystals. Finally, this solution was diluted with precipitant solution (1:9) to generate a 100x dilution seed stock, which was vortexed thoroughly to produce individual crystal nuclei and stored at 4 $^{\circ}C$.

F95Y EIZS was crystallized by the sitting drop vapor diffusion method. A 0.5 μ L drop of protein solution [5 mg/mL protein, 300 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 2 mM *tris*(2-carboxyethyl)phosphine (TCEP), 10% glycerol, 2 mM sodium pyrophosphate, 2 mM BTAC] was added to 0.6 μ L of precipitant solution [0.17 M sodium acetate trihydrate, 85 mM sodium cacodylate trihydrate (pH 6.5), 25.5% PEG 8000, 15%

glycerol]. This was followed by addition of 0.1 μL of 100x dilution wild-type EIZS crystallization seed stock. The drop was equilibrated against a 100 μL reservoir of precipitant solution at room temperature. Crystals appeared after 6 days and diffracted X-rays to a resolution of 1.80 $\text{\AA}$ on NE-CAT beamline 24-ID-C at the Advanced Photon Source (APS; Chicago, Illinois). Crystals belonged to space group $P2_1$ with unit cell parameters $a = 52.1 \text{ \AA}$, $b = 46.8 \text{ \AA}$, $c = 75.7 \text{ \AA}$; $\beta = 96.9^\circ$ (one molecule in the asymmetric unit).

F95N EIZS was crystallized by the sitting drop vapor diffusion method. A 0.5 μL drop of protein solution [4 mg/mL protein, 20 mM Tris (pH 7.5), 300 mM NaCl, 10 mM MgCl_2 , 10% (v/v) glycerol, 2 mM TCEP, 2 mM sodium pyrophosphate, 2 mM BTAC] was added to 0.6 μL of precipitant solution [0.2 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.5), 25% (w/v) PEG 3350], followed by addition of 0.1 μL of 100x dilution wild-type EIZS crystallization seed stock. The drop was equilibrated against a 100 μL reservoir of precipitant solution at room temperature. Crystals appeared within 1 month and diffracted X-rays to 2.40 $\text{\AA}$ resolution at the Stanford Synchrotron Radiation Lightsource (SSRL), beamline 14-1, SLAC National Accelerator Laboratory (Menlo Park, CA). Crystals belonged to space group $P2_1$ with unit cell parameters: $a = 52.9 \text{ \AA}$, $b = 47.2 \text{ \AA}$, $c = 75.5 \text{ \AA}$; $\beta = 96.0^\circ$ (one molecule in the asymmetric unit).

F95C EIZS was crystallized by the sitting drop vapor diffusion method. A 0.5 μL drop of protein solution [4 mg/mL protein, 20 mM Tris (pH 7.5), 300 mM NaCl, 10 mM MgCl_2 , 10% (v/v) glycerol, 2 mM TCEP, 2 mM sodium pyrophosphate, 2 mM BTAC] was added to 0.6 μL of precipitant solution [0.2 M $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.1 M bis-Tris (pH 6.5), 25% (w/v) PEG 3350], followed by addition of 0.1 μL of 100x dilution wild-type EIZS crystallization seed stock. The drop was equilibrated against a 100 μL reservoir of precipitant solution at room temperature. Crystals appeared within 1 week and diffracted X-rays to 1.90 $\text{\AA}$ resolution at SSRL, beamline 14-1. Crystals belonged to space group $P2_1$ with unit cell parameters: $a = 51.6 \text{ \AA}$, $b = 46.8 \text{ \AA}$, $c = 75.4 \text{ \AA}$; $\beta = 98.2^\circ$ (one molecule in the asymmetric unit).

W203Y EIZS was crystallized by the sitting drop vapor diffusion method. A 0.5 μL drop of protein solution [4.1 mg/mL protein, 300 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 2 mM TCEP, 10% glycerol, 2 mM sodium pyrophosphate, 2 mM BTAC] was added to 0.6 μL of precipitant solution [0.2 M $(\text{NH}_4)_2\text{SO}_4$, 0.1 M 2-(N-morpholino)ethanesulfonic acid monohydrate pH 6.5, 30% PEG monomethyl ether 5000]. This was followed by addition of 0.1 μL of 100x dilution wild-type EIZS crystallization seed stock. The drop was equilibrated against a 100 μL reservoir of precipitant solution at room temperature. Crystals appeared after 2 days and diffracted X-rays to 1.8 $\text{\AA}$ at SSRL, beamline 9-2. Crystals belonged to space group $P2_12_12_1$ with unit cell parameters $a = 46.5 \text{ \AA}$, $b = 75.0 \text{ \AA}$, $c = 107.6 \text{ \AA}$ (one molecule in the asymmetric unit).

F96S EIZS was crystallized by the sitting drop vapor diffusion method. A 0.5 μL drop of protein solution [5 mg/mL protein, 300 mM NaCl, 20 mM Tris-HCl (pH 7.5), 10 mM MgCl_2 , 2 mM TCEP, 10% glycerol, 2 mM sodium pyrophosphate, 2 mM BTAC] was added to 0.6 μL of precipitant solution [1.0 M ammonium citrate tribasic (pH 7.0), 0.1 M bis-Tris propane (pH 7.0)]. This was followed by addition of 0.1 μL of 100x dilution wild-type EIZS

crystallization seed stock. The drop was equilibrated against a 100 μ L reservoir of precipitant solution at room temperature. Crystals appeared after 2 days and diffracted X-rays to 2.10 Å resolution at APS, NE-CAT beamline 24-ID-C. Crystals belonged to space group $P2_12_12_1$ with unit cell parameters $a = 46.9$ Å, $b = 76.7$ Å, $c = 108.8$ Å (one molecule in the asymmetric unit).

X-ray diffraction data sets for F95N and F95C EIZS were indexed, integrated, and scaled using HKL2000.²⁷ Data sets for F95Y, F96S, F96H, and W203Y EIZS were indexed, integrated, and scaled using iMOSFLM.²⁸ The crystal structures of these mutants were determined by molecular replacement with PHASER²⁹ using the crystal structure of the wild-type EIZS-Mg²⁺₃-PP₁-BTAC complex (PDB 3KB9) as a search probe for rotation and translation function calculations. Manual model building and refinement were performed with COOT and PHENIX, respectively.^{30,31} Structure validation of each final model was performed using MolProbity.³² All molecular superpositions were calculated via the SSM superpose function in Coot and/or the align tool in Pymol.^{33,34} Data collection and refinement statistics for EIZS mutants F95Y, F95N, F95C, F96S, and W203Y are recorded in Table 1.

RESULTS

Sesquiterpene Product Generation

Product arrays generated by 14 EIZS mutants were analyzed using GC-MS (Table 2). All products detected and identified in the current study and in our previous study of active site mutants²⁶ represent derailment of key carbocation intermediates in the reaction sequence leading to *epi*-isozizaene (Figure 3). In general, substitution of polar amino acids for F95, F96, and W203 attenuates the production of *epi*-isozizaene by remolding the active site contour, thereby enabling the generation of alternative products. In contrast, Y69 mutations largely maintain biosynthetic fidelity for the generation of *epi*-isozizaene, with Y69F and Y69A EIZS generating 84% and 66% *epi*-isozizaene, respectively. Y69F EIZS also generates 6% zizaene and 4% β -cedrene, while Y69A EIZS also generates 7% zizaene and 5% prezizaene. Both mutants generate a small proportion of unidentified products (<8%). Thus, perturbation of Y69, which buttresses F96, has a minor impact on the template function of the active site pocket.

Polar mutations at the F95 position seem to perturb the active site contour to a lesser degree than mutations at F96, since *epi*-isozizaene is generated by all four F95 mutants. F95Y EIZS maintains the highest level of cyclization fidelity, generating 67% *epi*-isozizaene. However, acyclic side products generated by F95Y EIZS, (*E*)-nerolidol (27%) and (*E*)- β -farnesene (5%), reflect early derailment of the FPP cyclization cascade. Other F95 mutants that generate notable products include F95N EIZS, which generates 28% β -curcumene and 18% α -bisabolol, and F95Q EIZS, which is a curcumene synthase that generates 29% β -curcumene and 25% γ -curcumene. F95C EIZS also makes 22% γ -curcumene in addition to 23% α -neocallitropsene. The generation of curcumene isomers reflects successful isomerization and cyclization of FPP, as well as the 1,2-hydride transfer leading to the homobisabolyl cation. The substitution of smaller and polar residues in place of F95

uniformly compromises the cyclization of the homobisabolyl cation that would generate the acorenyl cation.

Two polar amino acid substitutions were made in place of W203. Both W203H and W203Y EIZS generate (*Z*)- γ -bisabolene (30% and 42%, respectively); W203H EIZS additionally generates 19% α -bisabolol and 7% sesquisabinene A. W203Y EIZS also generates 18% sesquisabinene A.

Polar and nonpolar amino acid substitutions in place of F96 generate significant quantities of sesquisabinene A. In particular, F96Q, F96M, and F96S EIZS generate sesquisabinene A with high fidelity (97%, 91%, and 78% respectively). Interestingly, F96Q and F96S EIZS generate minor products that reflect derailment of the cyclization cascade following the initial C1–C6 cyclization reaction yielding the bisabolyl cation, 3% β -bisabolene and 8% (*Z*)- γ -bisabolene, respectively. In contrast, F96M EIZS generates 9% 4-*epi*- β -patchoulene, which represents an alternative quenching pathway for the final carbocation intermediate.

Three more polar amino acid substitutions in place of F96 yield product arrays reflecting early quenching of reaction intermediates just before or after formation of the bisabolyl cation intermediate. F96T EIZS generates 35% (*Z*)- γ -bisabolene and 30% (*E*)- β -farnesene. F96H EIZS generates (*E*)-nerolidol with high fidelity at 73%, in addition to 18% (*E*)- β -farnesene. F96N EIZS generates 46% farnesol in addition to 18% sesquisabinene A. It is interesting to note that none of the F96 mutants generate *epi*-isozizaene except for F96S EIZS (1%), so F96 is clearly a critical residue for cyclization fidelity in the final steps of *epi*-isozizaene formation.

With regard to catalytic activity, all 14 EIZS mutants exhibit specific activities that range 21–44% of that measured for the wild-type enzyme (Table 3). No correlation is evident between the measured activity loss and the site of mutation or the polarity of the mutation. Although the mutation of aromatic residues in the enzyme active site reduces the rate of production of hydrocarbon products, it is notable that rate reductions are relatively minor, in that all mutants exhibit specific activities that are within an order of magnitude of that measured for wild-type EIZS.

Crystal Structures of Selected EIZS Mutants

Crystal structures of 6 representative EIZS mutants were determined at resolutions of 1.80 – 2.51 Å. We present crystal structures of three F95 mutants, two F96 mutants, and one W203 mutant. The crystal structures of mutants at F96 are the first structures solved for EIZS mutants at this position. These mutants were selected for further structural study to determine the consequences of aromatic-to-polar residue substitutions in the active site.

The 1.8 Å resolution structure of the F95Y EIZS-Mg²⁺₃-PP_i-BTAC complex is quite similar to that of the wild-type EIZS-Mg²⁺₃-PP_i-BTAC complex, with a root-mean-square deviation (rmsd) of 0.18 Å for 284 C α atoms. The aromatic rings of Y95 and F95 superimpose nearly perfectly, and the hydroxyl group of Y95 forms a new hydrogen bond with the backbone carbonyl of T197 (Figure 4). T197 is located at the break in helix G; backbone carbonyl groups at this break, as well as the helix dipole, are hypothesized to play a role in stabilizing

carbocation intermediates in catalysis.^{35,36} The position of the aromatic ring of BTAC is very similar to that observed in its complex with wild-type EIZS. There are no significant changes in PP_i and Mg²⁺ binding to F95Y EIZS compared to wild-type EIZS.

The 1.82 Å resolution structure of the W203Y EIZS-Mg²⁺₃-PP_i-BTAC complex is very similar to that of the wild-type EIZS-Mg²⁺-PP_i-BTAC complex, with an rmsd of 0.19 Å for 316 Cα atoms. The W203Y substitution deepens the active site, and the hydroxyl group of Y203 forms a new hydrogen bond with S92 (Figure 5). The increased active site volume resulting from the W203Y substitution enables the binding of two new ordered water molecules at the base of the active site. One of these water molecules, #102, forms hydrogen bonds with Q233 and the backbone carbonyl of F198. The second water molecule, #31, is hydrogen bonded to water #102 and also makes a 4.4 Å contact with the BTAC cation. The BTAC molecule is characterized by clear electron density and the orientation of its phenyl ring is similar to that observed in its complex with the wild-type enzyme; some conformational differences are observed for BTAC ethyl groups. There are no significant changes in PP_i and Mg²⁺ binding to W203Y EIZS compared to wild-type EIZS.

The 2.40 Å resolution structure of the F95N EIZS-Mg²⁺₃-PP_i-BTAC complex is very similar to that of the wild-type EIZS-Mg²⁺-PP_i-BTAC complex, with an rmsd of 0.18 Å for 304 Cα atoms. Although electron density is weak for the Nδ atom of the side chain of N95, the Nδ and Oδ atoms of the substituted side chain nearly perfectly superimpose on the Cδ1 and Cδ2 atoms of F95 in the wild-type enzyme (Figure 6). Additionally, the Nδ atom of N95 forms a new hydrogen bond with Y91, and the Oδ atom is 3.1 Å from the phenyl ring of BTAC.

The increased active site volume resulting from the F95N substitution appears to cause BTAC to flip relative to its position in the wild-type enzyme. However electron density for BTAC is very noisy, which may be a consequence of the moderate resolution of the structure determination, molecular disorder, or both. Disordered BTAC binding in the EIZS active site enlarged by the F95N substitution could indicate that binding of substrate and carbocation intermediate is also disordered. Such disordered binding may be responsible for the generation of quenching products derived from carbocations generated early in the cyclization cascade, such as 28% β-curcumen and 18% α-bisabolol. This is consistent with previous studies in which changes in BTAC conformation signaled derailed cyclization cascades for EIZS mutants.^{25,26} The appearance of product α-bisabolol is particularly notable, since such hydroxylated products were not detected in product arrays resulting from nonpolar amino acid substitutions in the active site.²⁶ The additional active site volume and polarity resulting from the F95N substitution presumably allow a water molecule to become trapped in the active site, which can then quench the bisabolyl cation intermediate.

The 1.90 Å resolution structure of the F95C EIZS-Mg²⁺₃-PP_i-BTAC complex is very similar to that of the wild-type EIZS-Mg²⁺₃-PP_i-BTAC complex, with an rmsd of 0.17 Å for 265 Cα atoms. Electron density for C95 indicates two conformations; the major conformation (75% occupancy) places the Sγ atom 3.6 Å away from hydroxyl group of Y91, and the minor conformation (25% occupancy) places the Sγ atom 4.5 Å away from the hydroxyl group of Y172 (Figure 7). Side chain flexibility for C95 may contribute to the emergence of a previously unobserved product, α-neocallitropsene (23%), which is generated at only

minor levels by F95N EIZS (1%) and F95Q EIZS (<1%). The binding mode of BTAC is essentially unchanged from that observed in the wild-type enzyme, although noisy electron density may reflect some degree of disorder.

The 2.1 Å resolution structure of unliganded F96S EIZS is similar to that of unliganded wild-type EIZS, with an rmsd of 0.24 Å for 246 Ca atoms. Significant disorder is observed in the A57–L67 and G337–N355 segments located in helices adjacent to the active site. In previously solved structures of unliganded wild-type EIZS and unliganded D99N EIZS, the A57–L67 segment is ordered but the S336–N355 segment is disordered.^{25,26} Additionally, the I249–E253 segment is disordered in unliganded wild-type EIZS, but this segment is ordered in F96S EIZS. No electron density is observed for BTAC, Mg²⁺ ions, or PP_i in the crystal structure of F96S EIZS even though these ligands were included in the crystallization drop.

Two conformers of S96 are observed: The first (64% occupancy) places the hydroxyl group 2.5 Å from the backbone carbonyl oxygen of A93 and 2.9 Å from the backbone NH group of V97; the second (36% occupancy) places the hydroxyl group 3.1 Å from the S96 backbone carbonyl oxygen (Figure 8).

DISCUSSION

For the past 20 years,^{37–39} X-ray crystal structures of class I and class II terpenoid cyclases have revealed hydrophobic active site cavities that serve as templates to chaperone the folding of flexible lipophilic substrates, thereby specific binding conformations leading to the formation of specific cyclization products. The most critical function of the template is to stabilize the flexible substrate in the correct binding conformation before the initial carbocation is formed. Class I cyclases such as *epi*-isozizaene synthase generate an initial allylic carbocation through the metal ion-triggered ionization of the substrate diphosphate group,²⁵ whereas class II cyclases such as *ent*-copalyl diphosphate synthase generate an initial tertiary carbocation by protonation of an isoprenoid carbon-carbon double bond (or epoxide) with an aspartic acid serving as a general acid.^{40,41} Such carbocation intermediates are highly reactive and susceptible to rapid quenching by water in the aqueous milieu of the cell. The terpenoid cyclase active site has evolved with protective measures, however, enclosing carbocation intermediates in a hydrophobic pocket that is usually inaccessible to bulk solvent. Typically, chemically-inert aliphatic and aromatic amino acid residues define the contour of the active site pocket – i.e., the cyclization template – with the added benefit of carbocation stabilization by aromatic residues through cation- π interactions.

Conventional wisdom regarding the active site pocket of a terpenoid cyclase is occasionally subject to curious exceptions – some active site pockets contain polar and potentially reactive elements. For example, the crystal structures of bornyl diphosphate synthase and aristolochene synthase complexed with analogues of substrates and carbocation intermediates reveal the binding of trapped water molecules in the enclosed active site, even though these cyclases do not utilize water in their reaction mechanisms.^{42,43} Additionally, the crystal structure of methylisoborneol synthase reveals a negatively charged glutamate at the base of the active site pocket, yet this negatively charged residue does not influence the

cyclization cascade.⁴⁴ Nature thus defies conventional wisdom, in that polar residues, charged residues, and even solvent molecules are rendered inert when they are incorporated in the active site pocket of a terpenoid cyclase.

Can we follow Nature's lead and similarly defy conventional wisdom by introducing polar residues in the active site pocket of a terpenoid cyclase? EIZS serves as an excellent paradigm system to explore this question. We now show that polar residues can be substituted for aromatic residues to yield functional EIZS catalysts that generate new product arrays with appreciable catalytic activity. There can be limitations, however, particularly with the most radical amino acid substitutions. For example, F95D EIZS and F95E EIZS expressed poorly, and although expression was confirmed the mutant proteins were generally insoluble. As with any mutagenesis experiment, certain amino acid substitutions can compromise protein folding and stability, and the substitution of aromatic residues in the hydrophobic protein core or a hydrophobic active site may be particularly susceptible to such deleterious effects. Even so, for those mutants that did express well as folded proteins and yielded X-ray crystal structures, the amino acid substitutions did not trigger significant compensatory structural changes in enzyme active sites. Thus, to a close approximation, the structural consequences of remolding a terpenoid cyclase active site by substitution of surface residues (Figures 4–7) ought to be readily predictable by molecular modeling.

Even so, this expectation is not universal. For example, the F96S substitution exposes the formerly-buttressing residue Y69, which undergoes compensatory structural changes as a new surface residue additionally poised to engage in cation- π interactions, taking the place of F96 in this role (Figure 8). Moreover, F96 undergoes induced-fit conformational changes upon the binding of $\text{Mg}^{2+}_3\text{-PP}_i$ in the EIZS active site,^{26,27} so this residue exemplifies the challenges in molecular modeling approaches: the three-dimensional contour of the active site in the fully closed conformation can differ from that in the open, unliganded conformation.

In the current work, we successfully prepared and analyzed 14 new EIZS mutants in which active site aromatic residues were substituted mainly with neutral polar residues (serine, threonine, asparagine, glutamine, histidine, and tyrosine) or weakly polar residues (cysteine and methionine) as summarized in Table 2. Importantly, these mutants generate 9 new products that have not been observed in the product arrays of any previously reported EIZS mutants, with three mutants generating sesquisabinene A as the predominant product (Figure 3). New products include those resulting from proton elimination from a carbocation intermediate, including γ -curcumene, β -bisabolene, and 4-*epi*- β -patchoulene; additionally, *cis*- α -bergamotene results from a 7,2 ring closure reaction with the bisaboly l cation. Other products result from water capture of a carbocation intermediate, including farnesol, nerolidol, acorenol, and α -bisabolol. Thus, the introduction of polarity into a terpenoid cyclase active site can enable the generation of hydroxylated terpenoid products.

Strikingly, the F96S, F96M, and F96Q substitutions convert EIZS into a high-fidelity sesquisabinene synthase, with these mutants generating 78%, 91%, and 97% sesquisabinene A in their respective product arrays. The conversion of EIZS into a high-fidelity

sesquisabinene synthase has not been previously observed. While D99E EIZS generates 24% sesquisabinene A,²⁵ and other aromatic mutants generate 1–19% sesquisabinene A (Table 2), the substitution of smaller polar residues for F96 seems to be of chief importance in redirecting the FPP cyclization cascade toward sesquisabinene formation through conformational control of the preceding homobisabolyl carbocation intermediate. Molecular features that contribute to this new enzyme activity include the introduction of additional volume in the position of F96 in the closed conformation, and the retention of potential cation- π interactions through compensatory structural changes of Y69 – the ring face of this formerly-butressing residue forms a new wall of the active site, as observed in F96S EIZS (Figure 8).

In essence, F96 appears to be a hot spot for sesquisabinene formation when substituted with smaller, polar residues. It is intriguing, however, that although the F96S, F96M, and F96Q substitutions convert EIZS into a sesquisabinene synthase, the F96T and F96N substitutions do not. F96T EIZS generates (*Z*)- γ -bisabolene and (*E*)- β -farnesene as its predominant products, and F96N EIZS generates a mixture of sesquiterpene products in which farnesol predominates. At present, we do not have a plausible explanation for this observation. However, it is interesting to note that (*Z*)- γ -bisabolene can also derive from the homobisabolyl carbocation intermediate, as does sesquisabinene, so perhaps the unique steric and polar environment resulting from the F96T substitution is simply less conducive for the transannular ring closure leading to sesquisabinene formation; a simple proton elimination to yield (*Z*)- γ -bisabolene is instead favored.

Interestingly, two naturally occurring sesquisabinene synthases, SASQS1 and SASQS2, have recently been isolated from *Santalum album* L. (Indian sandalwood), which is well known for its fragrant heartwood oil.⁴⁵ This essential oil comprises a blend of sesquiterpene hydrocarbons and alcohols and exhibits useful antimicrobial, antiinflammatory, and antitumor properties.^{46–48} SASQS1 and SASQS2 each contain 566 residues and are related by 80% amino acid sequence identity; based on amino acid sequence analysis, each enzyme adopts the characteristic $\alpha\beta$ domain architecture^{49,50} of a plant sesquiterpene cyclase. As class I terpenoid cyclases, the locus of catalytic activity resides exclusively in the α domain of each enzyme.

Despite the fact that crystal structures of SASQS1 and/or SASQS2 have not been reported to date, it is reasonable to assume that the three-dimensional contours of their active sites are similar to those of F96 EIZS mutants that generate sesquisabinene with high fidelity. Although a detailed comparison between the naturally occurring enzymes and EIZS mutants must await experimental crystal structure determinations of SASQS1 and/or SASQS2, it is interesting to note that both sesquisabinene synthases have a polar amino acid side chain in a position that aligns with F96 of EIZS based on amino acid sequence analysis. On helix D of EIZS, the amino acid sequence is F⁹⁵FVWDDRHD, whereas the corresponding sequence of both sesquisabinene synthases is I³¹²TTIDDVYD. Thus, F96 of EIZS aligns with T313 of sesquisabinene synthase. Perhaps the polar amino acid side chain on the active site face of helix D of sesquisabinene synthase plays a role in facilitating sesquisabinene formation. Future structural and functional studies of the sesquisabinene synthases may clarify this speculation.

Finally, it is interesting to compare the results acquired with EIZS with those reported for mutants of 5-*epi*-aristolochene synthase in which nucleophilic cysteine and glutamate residues were similarly introduced in the hydrophobic active site pocket.⁵¹ Alkylation and inactivation of certain mutants was observed; however, the substituted nucleophilic side chain was not the site of covalent modification. Instead, conserved active site residues were alkylated in response to perturbation of the active site structure. Thus, for a nucleophilic side chain to react with a carbocation in a terpenoid cyclase active site, it must be precisely positioned for reaction. If not, then the nucleophile is essentially inert.

CONCLUDING REMARKS

Including the 14 new mutants described in the current work, our EIZS library now contains 40 different single-site mutants in which the hydrophobic active site pocket is modified. Each mutant exhibits a distinct product profile – remolding the contour of the active site pocket, even by just a single residue, can unmask new reaction pathways. Notably, each new pathway represents a derailment of the natural cyclization cascade, in that each new product identified derives from a key carbocation intermediate encountered in the cyclization pathway leading to *epi*-isozizaene. Overall, we have quintupled the number of characterized products generated by EIZS. Moreover, we have converted EIZS into 9 different sesquiterpene synthases, as summarized in Figure 1.

Our previous study²⁶ focused on the substitution of aromatic and aliphatic residues in the EIZS active site with other nonpolar residues. We had assumed that the introduction of polar residues would disfavor binding of the lipophilic substrate and risk reaction with carbocation intermediates to deactivate the enzyme. Surprisingly, however, EIZS appears to be quite tolerant of polar residues introduced in the hydrophobic active site pocket. Indeed, the substitution of polar residues for F96 led to the generation of new high-fidelity sesquisabinene synthases. Thus, more radical nonpolar-polar amino acid substitutions in the hydrophobic pockets of terpenoid cyclases should be considered in efforts to explore and expand product chemodiversity through protein engineering.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Jaclyn Robustelli and Alaina Stockhausen for technical assistance, and we thank Dr. Travis Pemberton for helpful scientific discussions. We also thank the National Institute of General Medical Sciences (NIGMS) for grants GM56838 (D.W.C.) and GM30301 (D.E.C.) in support of this research. Additionally, we are grateful for access to synchrotron data collection facilities at SSRL (beamlines 9-2 and 14-1) and the Advanced Photon Source (NE-CAT beamline 24-ID-C). The SSRL, SLAC National Accelerator Laboratory, is supported by the DOE Office of Science, Office of Basic Energy Sciences, under Contract No. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by NIGMS (including P41GM103393). This work is also based upon research conducted at the Northeastern Collaborative Access Team beamlines, which are funded by NIGMS (P41 GM103403). The Pilatus 6M detector on 24-ID-C beam line is funded by a NIH-ORIP HEI grant (S10 RR029205). This research used resources of the Advanced Photon Source, a U.S. Department of Energy (DOE) Office of Science User Facility operated for the DOE Office of Science by Argonne National Laboratory under Contract No. DE-AC02-06CH11357.

References

1. Gershenzon J, Dudareva N. The function of terpene natural products in the natural world. *Nat Chem Biol.* 2007; 3:408–414. [PubMed: 17576428]
2. Pichersky E, Noel JP, Dudareva N. Biosynthesis of plant volatiles: nature's diversity and ingenuity. *Science.* 2006; 311:808–811. [PubMed: 16469917]
3. Quin MB, Flynn CM, Schmidt-Dannert C. Traversing the fungal terpenome. *Nat Prod Rep.* 2014; 31:1449–1473. [PubMed: 25171145]
4. Little D, Croteau R. Alteration of product formation by directed mutagenesis and truncation of the multiple-product sesquiterpene synthases δ -selinene synthase and γ -humulene synthase. *Arch Biochem Biophys.* 2002; 402:120–135. [PubMed: 12051690]
5. Bleeker PM, Diergaarde PJ, Ament K, Schuetz S, John B, Dijkink J, Hiemstra H, de Gelder R, de Both MTJ, Sabelis MW, Haring MA, Schuurink RC. Tomato-produced 7-epizingiberene and *R*-curcumene act as repellents to whiteflies. *Phytochemistry.* 2011; 72:68–73. [PubMed: 21074818]
6. Tholl D. Terpene synthases and the regulation, diversity and biological roles of terpene metabolism. *Curr Opin Plant Biol.* 2006; 9:297–304. [PubMed: 16600670]
7. Schnee C, Kollner T, Held M, Turlings T, Gershenzon J, Degenhardt J. The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. *Proc Natl Acad Sci U S A.* 2006; 103:1129–1134. [PubMed: 16418295]
8. Galeotti N, Mannelli L, Mazzanti G, Bartolini A, Ghelardini C. Menthol: A natural analgesic compound. *Neurosci Lett.* 2002; 322:145–148. [PubMed: 11897159]
9. Kamatou GPP, Vermaak I, Viljoen AM, Lawrence BM. Menthol: A simple monoterpene with remarkable biological properties. *Phytochemistry.* 2013; 96:15–25. [PubMed: 24054028]
10. Sun J. D-Limonene: Safety and clinical applications. *Altern Med Rev.* 2007; 12:259–264. [PubMed: 18072821]
11. Pourbafrani M, Forgacs G, Horvath IS, Niklasson C, Taherzadeh MJ. Production of biofuels, limonene and pectin from citrus wastes. *Bioresour Technol.* 2010; 101:4246–4250. [PubMed: 20149643]
12. Peralta-Yahya PP, Ouellet M, Chan R, Mukhopadhyay A, Keasling JD, Lee TS. Identification and microbial production of a terpene-based advanced biofuel. *Nat Commun.* 2011; 2:483. [PubMed: 21952217]
13. Rude MA, Schirmer A. New microbial fuels: A biotech perspective. *Curr Opin Microbiol.* 2009; 12:274–281. [PubMed: 19447673]
14. Gowdagiri S, Cesari XM, Huang M, Oehlschlaeger MA. A diesel engine study of conventional and alternative diesel and jet fuels: Ignition and emissions characteristics. *Fuel.* 2014; 136:253–260.
15. Ryder, JA. Fuel composition, useful to power any equipment such as an emergency generator or internal combustion engine, which requires a fuel such as jet fuels or missile fuels, comprises limonene and farnesane. US097813P. US393024. Sep 17. 2008 25 Feb 2009
16. Christianson DW. Structural biology and chemistry of the terpenoid cyclases. *Chem Rev.* 2006; 106:3412–3442. [PubMed: 16895335]
17. Christianson DW. Structural and Chemical Biology of Terpenoid Cyclases. *Chem Rev.* 2017; 117:11570–11648. [PubMed: 28841019]
18. Wendt KU, Schulz GE. Isoprenoid biosynthesis: manifold chemistry catalyzed by similar enzymes. *Structure.* 1998; 6:127–133. [PubMed: 9519404]
19. Degenhardt J, Kollner TG, Gershenzon J. Monoterpene and sesquiterpene synthases and the origin of terpene skeletal diversity in plants. *Phytochemistry.* 2009; 70:1621–1637. [PubMed: 19793600]
20. Miller DJ, Allemann RK. Sesquiterpene synthases: Passive catalysts or active players? *Nat Prod Rep.* 2012; 29:60–71. [PubMed: 22068697]
21. Gao Y, Honzatko RB, Peters RJ. Terpenoid synthase structures: a so far incomplete view of complex catalysis. *Nat Prod Rep.* 2012; 29:1153–1175. [PubMed: 22907771]
22. Lin X, Hopson R, Cane DE. Genome mining in *Streptomyces coelicolor*: Molecular cloning and characterization of a new sesquiterpene synthase. *J Am Chem Soc.* 2006; 128:6022–6023. [PubMed: 16669656]

23. Lin X, Cane DE. Biosynthesis of the sesquiterpene antibiotic albaflavenone in streptomyces coelicolor. mechanism and stereochemistry of the enzymatic formation of *epi*-isozizaene. J Am Chem Soc. 2009; 131:6332–6333. [PubMed: 19385616]
24. Oldfield E, Lin FY. Terpene biosynthesis: modularity rules. Ang Chem Int Ed. 2012; 51:1124–1137.
25. Aaron JA, Lin X, Cane DE, Christianson DW. Structure of *epi*-isozizaene synthase from streptomyces coelicolor A3(2), a platform for new terpenoid cyclization templates. Biochemistry. 2010; 49:1787–1797. [PubMed: 20131801]
26. Li R, Chou WKW, Himmelberger JA, Litwin KM, Harris GG, Cane DE, Christianson DW. Reprogramming the chemodiversity of terpenoid cyclization by remodeling the active site contour of *epi*-isozizaene synthase. Biochemistry. 2014; 53:1155–1168. [PubMed: 24517311]
27. Otwinowski Z, Minor W. Processing of X-ray diffraction data collected in oscillation mode. Methods Enzymol. 1997; 276:307–326.
28. Batty TGG, Kontogiannis L, Johnson O, Powell HR, Leslie AGW. iMOSFLM: A new graphical interface for diffraction-image processing with MOSFLM. Acta Cryst. 2011; D67:271–281.
29. McCoy AJ, Grosse-Kunstleve RW, Adams PD, Winn MD, Storoni LC, Read RJ. Phaser crystallographic software. J Appl Cryst. 2007; 40:658–674. [PubMed: 19461840]
30. Emsley P, Lohkamp B, Scott WG, Cowtan K. Features and development of Coot. Acta Cryst. 2010; D66:486–501.
31. Adams PD, Afonine PV, Bunkoczi G, Chen VB, Davis IW, Echols N, Headd JJ, Hung L, Kapral GJ, Grosse-Kunstleve RW, McCoy AJ, Moriarty NW, Oeffner R, Read RJ, Richardson DC, Richardson JS, Terwilliger TC, Zwart PH. PHENIX: A comprehensive python-based system for macromolecular structure solution. Acta Cryst. 2010; D66:213–221.
32. Chen VB, Arendall WB III, Headd JJ, Keedy DA, Immormino RM, Kapral GJ, Murray LW, Richardson JS, Richardson DC. MolProbity: All-atom structure validation for macromolecular crystallography. Acta Cryst. 2010; D66:12–21.
33. Krissinel E, Henrick K. Secondary-structure matching (SSM), a new tool for fast protein structure alignment in three dimensions. Acta Cryst. 2004; D60:2256–2268.
34. The PyMOL Molecular Graphics System, Version 18. Schrödinger, LLC;
35. Pandit J, Danley DE, Schulte GK, Mazzalupo S, Pauly TA, Hayward CM, Hamanaka ES, Thompson JF, Harwood HJ. Crystal structure of human squalene synthase. J Biol Chem. 2000; 275:30610–30617. [PubMed: 10896663]
36. Baer P, Rabe P, Citron CA, de Oliveira Mann CC, Kaufmann N, Groll M, Dickschat JS. Hedycaryl synthase in complex with nerolidol reveals terpene cyclase mechanism. ChemBioChem 2014. 2014; 15:213–216.
37. Lesburg CA, Zhai G, Cane DE, Christianson DW. Crystal structure of pentalenene synthase: mechanistic insights on terpenoid cyclization reactions in biology. Science. 1997; 277:1820–1824. [PubMed: 9295272]
38. Starks CM, Back K, Chappell J, Noel JP. Structural basis for cyclic terpene biosynthesis by tobacco 5-*epi*-aristolochene synthase. Science. 1997; 277:1815–1820. [PubMed: 9295271]
39. Wendt KU, Poralla K, Schulz GE. Structure and function of a squalene cyclase. Science. 1997; 277:1811–1815. [PubMed: 9295270]
40. Köksal M, Hu H, Coates RM, Peters RJ, Christianson DW. Structure and mechanism of the diterpene cyclase *ent*-copalyl diphosphate synthase. Nat Chem Biol. 2011; 7:431–433. [PubMed: 21602811]
41. Köksal M, Potter K, Peters RJ, Christianson DW. 1.55 Å-Resolution structure of *ent*-copalyl diphosphate synthase and exploration of general acid function by site-directed mutagenesis. Biochim Biophys Acta. 2014; 1840:184–190. [PubMed: 24036329]
42. Whittington DA, Wise ML, Urbansky M, Coats RM, Croteau RB, Christianson DW. Bornyl diphosphate synthase: structure and strategy for carbocation manipulation by a terpenoid cyclase. Proc Natl Acad Sci U S A. 2002; 99:15375–15380. [PubMed: 12432096]
43. Chen M, Al-lami N, Janvier M, D'Antonio EL, Faraldos JA, Cane DE, Allemann RK, Christianson DW. Mechanistic insights from the binding of substrate and intermediate analogues to aristolochene synthase. Biochemistry. 2013; 52:5441–5453. [PubMed: 23905850]

44. Köksal M, Chou WKW, Cane DE, Christianson DW. Structure of 2-methylisoborneol synthase from *Streptomyces coelicolor* and implications for the cyclization of a noncanonical C-methylated monoterpene substrate. *Biochemistry*. 2012; 51:3011–3020. [PubMed: 22455514]
45. Srivastava PL, Daramwar PP, Krithika R, Pandreka A, Shankar SS, Thulasiram HV. *Sci Rep*. 2015; 5:10095. [PubMed: 25976282]
46. Jirovetz L, Buchbauer G, Denkova Z, Stoyanova A, Murgov I, Gearon V, Birkbeck S, Schmidt E, Geissler M. Comparative study on the antimicrobial activities of different sandalwood essential oils of various origin. *Flavour Frag J*. 2006; 21:465–468.
47. Sharma M, Levenson C, Clements I, Castella P, Gebauer K, Cox ME. East Indian sandalwood oil (EISO) alleviates inflammatory and proliferative pathologies of psoriasis. *Front Pharmacol*. 2017; 8:125. [PubMed: 28360856]
48. Dwivedi C, Zhang Y. Sandalwood oil prevents skin tumour development in CD1 mice. *Eur J Cancer Prev*. 1999; 8:449–455. [PubMed: 10548401]
49. Oldfield E, Lin F-Y. Terpene biosynthesis: modularity rules. *Angew Chem Int Ed*. 2012; 51:1124–1137.
50. Chen M, Harris GG, Pemberton TA, Christianson DW. Multi-domain terpenoid cyclase architecture and prospects for proximity in bifunctional catalysis. *Curr Op Struct Biol*. 2016; 41:27–37.
51. Kersten RD, Diedrich JK, Yates JR III, Noel JP. *ACS Chem Biol*. 2015; 10:2501–2511. [PubMed: 26378620]

Figure 1.

Wild-type *epi*-izozizaene synthase (EIZS) generates 99% and 79% *epi*-izozizaene at 4 °C and 30 °C, respectively, from substrate farnesyl diphosphate (FPP). Substitution of active site aromatic residues leads to the formation of alternative predominant acyclic and cyclic products in multiproduct mixtures.²⁶ The current study reports a new predominant hydrocarbon product, sesquisabinene A, generated by F96 mutants with exceptionally high fidelity (97% for F96Q EIZS). Additionally, the sesquiterpene alcohol products farnesol and nerolidol are generated predominantly by the F96N and F96H mutants, respectively.

Figure 2.

Stereoview of the active site contour (grey) defined by aromatic residues in the active site of wild-type EIZS complexed with 3 Mg²⁺ ions (magenta spheres), inorganic pyrophosphate (PP_i; P = orange, O = red), and the benzyltriethylammonium cation (BTAC; C = green, N = blue). The aromatic ring faces of F95, F96, and F198 form walls of the active site and make cation- π interactions with BTAC. Aromatic residue Y69 buttresses F96, and the ring edge of W203 contributes to the floor of the active site contour; neither residue makes cation- π interactions with BTAC.

Figure 3.

Biosynthetic manifold illustrating the proposed reaction mechanisms of FPP cyclization leading to products identified by GC-MS analysis catalyzed by wild-type and mutant EIZS enzymes. The predominant product generated by the wild-type enzyme, *epi-isozizaene*, is highlighted in a cyan box. Each product that is generated predominantly by a mutant enzyme is highlighted in a blue box. Minor products are shown in yellow boxes. This manifold updates that previously reported by Li and colleagues.²⁶ New products identified in the current study are highlighted by red borders, and new EIZS mutants are italicized. With regard to product stereochemistry, the absolute configurations of (+)-*epi-isozizaene*, (+)-β-cedrene, and (–)-β-cedrene shown above have been experimentally determined.^{22,26} The absolute configurations of all remaining sesquiterpene products have not been experimentally determined and are arbitrarily illustrated. The generation of their enantiomers can be readily accommodated by simple variations of the reaction mechanisms shown.

Figure 4.

(a) Stereoview of a simulated annealing omit map of the F95Y EIZS- Mg^{2+}_3 -PP_i-BTAC complex contoured at 3.0σ . Atoms are color coded as follows: C = slate (Y95), yellow (protein), or green (BTAC); N = blue; O = red; P = orange; Mg^{2+} ions = magenta spheres. The phenolic hydroxyl group of Y95 forms a new hydrogen bond with the backbone carbonyl oxygen of T197 (dashed line). Selected active site residues are indicated. (b) Superposition of the F95Y EIZS- Mg^{2+}_3 -PP_i-BTAC complex (yellow) with the wild-type EIZS- Mg^{2+}_3 -PP_i-BTAC complex (blue; PDB 3KB9). Minimal structural differences are observed for active site aromatic residues and BTAC.

Figure 5.

(a) Stereoview of a simulated annealing omit map of the W203Y EIZS-Mg²⁺₃-PP_i-BTAC complex contoured at 2.5σ. Atoms are color coded as follows: C = slate (Y203), yellow (protein), or green (BTAC); N = blue; O = red; P = orange; Mg²⁺ ions = magenta spheres. The phenolic hydroxyl group of Y203 forms a new hydrogen bond with S92. The steric void resulting from the W203Y substitution allows two hydrogen bonded water molecules to bind, one of which forms a hydrogen bond with Q233. Selected active site residues are indicated; hydrogen bonds are shown as dashed lines. (b) Superposition of the W203Y EIZS-Mg²⁺₃-PP_i-BTAC complex (yellow) with the wild-type EIZS-Mg²⁺₃-PP_i-BTAC complex (blue; PDB 3KB9). Apart from structural differences at position 203, the mutation has minimal effect on active site aromatic residues and BTAC.

Figure 6.

(a) Stereoview of a simulated annealing omit map of the F95N EIZS-Mg²⁺-PP_i-BTAC complex contoured at 3.0σ. Atoms are color coded as follows: C = slate (N95), yellow (protein), or green (BTAC); N = blue; O = red; P = orange; Mg²⁺ ions = magenta spheres. The carboxamide NH₂ group of N95 forms a new hydrogen bond with Y91 (dashed line). Selected active site residues are indicated. (b) Superposition of the F95N EIZS-Mg²⁺-PP_i-BTAC complex (yellow) with the wild-type EIZS-Mg²⁺-PP_i-BTAC complex (blue; PDB 3KB9). The orientation of BTAC is flipped as one structure is compared with the other.

Figure 7.

(a) Stereoview of a simulated annealing omit map of the F95C EIZS-Mg²⁺-PP_i-BTAC complex contoured at 3.0σ. Atoms are color coded as follows: C = slate (C95), yellow (protein), or green (BTAC); N = blue; O = red; P = orange; Mg²⁺ ions = magenta spheres. Selected active site residues are indicated. (b) Superposition of the F95C EIZS-Mg²⁺-PP_i-BTAC complex (yellow) with the wild-type EIZS-Mg²⁺-PP_i-BTAC complex (blue; PDB 3KB9). The side chain of C95 is disordered between two conformations.

Figure 8.

(a) Simulated annealing omit map of unliganded F96S EIZS contoured at 3.0σ . Atoms are color coded as follows: C = slate (S96) or yellow (protein); N = blue; O = red. (b) Superposition of unliganded F96S EIZS (yellow) with unliganded wild-type EIZS (teal; PDB 4LTV) and the wild-type EIZS-Mg²⁺-PP_i-BTAC complex (blue; PDB 3KB9). The side chains of S96 and D99 are each disordered between two conformations, and the side chain of Y69 exhibits significant conformational variability in response to structural changes in F96, the residue that it buttresses.

Table 1

Data collection and refinement statistics

ElIZS Mutant (Mg ²⁺ ₃ -PP ₁ -BTAC complex)	F95Y	W203Y	F95N	F95C	F96S (unliganded)
<i>Data Collection</i>					
Beamline	APS	SSRL	SSRL	SSRL	APS
Wavelength (Å)	0.98	0.98	1.18	1.18	0.98
Resolution (Å) (outer shell)	51.74-1.80 (1.84-1.80)	37.52-1.82 (1.86-1.82)	39.95-2.40 (2.49-2.40)	32.53-1.90 (1.97-1.90)	54.39-2.10 (2.18-2.10)
Total reflections	97646 (5764)	193111 (11067)	52707 (4296)	54127 (10152)	144593 (11901)
Unique reflections ^a	32978 (1920)	33846 (1923)	14666 (1432)	28111 (2603)	23391 (1886)
Completeness ^a	97.6 (98.6)	98.0 (95.1)	99.9 (99.2)	98.8 (92.5)	99.1 (98.2)
Redundancy ^a	3.0 (3.0)	5.7 (5.8)	3.6 (3.6)	4.1 (3.9)	6.2 (6.3)
I/σ(I) ^a	7.0 (2.5)	9.1 (2.8)	5.6 (2.2)	9.9 (2.5)	6.1 (2.8)
R _{merge} ^{a,b}	0.125 (0.688)	0.116 (0.538)	0.132 (0.458)	0.143 (0.553)	0.428 (2.410)
CC _{1/2} ^{a,c}	0.986 (0.572)	0.993 (0.802)	0.988 (0.853)	0.992 (0.810)	0.970 (0.520)
<i>Refinement</i>					
R _{work} /R _{free} ^{a,d}	0.170/0.213 (0.223/0.264)	0.195/0.223 (0.245/0.271)	0.186/0.243 (0.227/0.256)	0.154/0.192 (0.188/0.224)	0.176/0.222 (0.212/0.273)
Rms deviations					
bonds (Å)	0.008	0.004	0.008	0.015	0.006
angles (deg)	0.9	0.7	1.1	1.4	0.8
Number of non-hydrogen atoms					
protein	2710	2710	2699	2719	2497
ligands	26	26	26	31	--
solvent	262	340	48	187	243
Average B-factors (Å ²)					
protein	15	16	32	24	19
ligands	18	17	31	28	
solvent	27	28	30	31	29
Ramachandran plot ^e					
favoured	99	99	97	99	99
allowed	1.2	0.86	2.96	1.48	1.31

EIZS Mutant (Mg ²⁺ , 3 ⁺ -PP ₁ -BTAC complex)	F95Y	W203Y	F95N	F95C	F96S (unliganded)
outliers	0	0	0.30	0	0
PDB accession code	6AXM	6AXU	6AX9	6AXN	6AXO

^aValues in parentheses refer to the data from the highest shell.

^b $R_{\text{merge}} = \frac{\sum |I_h - \langle I \rangle|}{\sum I_h}$, where $\langle I \rangle$ is the average intensity calculated for reflection h from replicate measurements.

^cPearson correlation coefficient between random half-datasets.

^d $R_{\text{work}} = \frac{\sum ||F_o| - |F_c||}{\sum |F_o|}$ for reflections contained in the working set; $|F_o|$ and $|F_c|$ are the observed and calculated structure factor amplitudes, respectively. R_{free} is calculated using the same expression for reflections contained in the test set held aside during refinement.

^eCalculated with MolProbity.

Table 2

Sesquiterpene products generated by EIZS mutants^a

Product	RI ^b	Wild-type ^c	F95N	F95Y	F95Q	F95C	F96S	F96T	F96H	F96N	F96M	F96Q	Y69F	Y69A	W203Y	W203H
4- <i>epi</i> - β -patchoulene	1376										9				3	6
cis- α -bergamotene	1411									4						
α -cedrene	1418	2			<1			3		5						
β -cedrene	1424			2				5					4	5		
selina-4(15),5-diene	1433					<1										
sesquisabinene A	1435	2				1	78			18	91	97	1		19	9
<i>epi</i> -isozizaene	1444	79	5	67	6	6	1						84	66	6	
(<i>E</i>)- β -farnesene	1446	5	1	5	1	1		30	18					2		
prezizaene	1452												1	5	<1	
zizaene	1456	9											6	7	9	<1
α -neocallitropsene	1475		1		<1	23		5		2				2		
γ -curcumene	1475				25	22										
(<i>E,E</i>)- α -farnesene	1498					1		5								
β -bisabolene	1503							6	<1	15		3		<1		
β -curcumene	1503		28		29	2								<1		
(<i>Z</i>)- γ -bisabolene	1505		3		5	3	8	35		5				3	42	38
(<i>E</i>)-nerolidol	1553		6	27	3	9		5	73	2				2	10	24
acoreanol	1667				2	3										
α -bisabolol	1673		18		13	4										24
farnesol	1694						5			46						
Unknown	--		39		14	25	7	5	8	2			3	5	6	
Trace products	--				<1	<2		<3		<2				<3	1	

^a Each product is reported as a percentage of total products generated.^b RI, retention index (MassFinder 4.0 Database).^c Refs. 25 and 26

Table 3

Catalytic activities of EIZS mutants

Enzyme	Specific Activity (nmol product/min)/(μM enzyme)
Wild-type	18 ± 2
F95Y	8 ± 4
W203Y	6 ± 2
F96Q	8 ± 2
F96M	7.0 ± 0.6
F96N	4 ± 1
W203H	4.2 ± 0.6
F96S	4 ± 2
F96H	5.8 ± 0.6
F96T	5.9 ± 0.4
F95Q	5.916 ± 0.002
F95C	5.5 ± 0.6
F95N	5 ± 2
Y69A	5 ± 2
Y69F	7.6 ± 0.4