

Functional plasticity of paralogous diterpene synthases involved in conifer defense

Christopher I. Keeling, Sabrina Weisshaar, Roy P. C. Lin, and Jörg Bohlmann*

Michael Smith Laboratories, University of British Columbia, 301-2185 East Mall, Vancouver, BC, Canada V6T 1Z4

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The diversity of terpenoid compounds produced by plants plays an important role in mediating various plant–herbivore, plant–pollinator, and plant–pathogen interactions. This diversity has resulted from gene duplication and neofunctionalization of the enzymes that synthesize and subsequently modify terpenes. Two diterpene synthases in Norway spruce (*Picea abies*), isopimaradiene synthase and levopimaradiene/abietadiene synthase, provide the hydrocarbon precursors for most of the diterpene resin acids found in the defensive oleoresin of conifers. Although these paralogous enzymes are 91% identical at the amino acid level, one is a single-product enzyme, whereas the other is a multiproduct enzyme that forms completely different products. We used a rational approach of homology modeling, protein sequence comparison, domain swapping, and a series of reciprocal site-directed mutagenesis to identify the specific residues that direct the different product outcomes. A one-amino acid mutation switched the levopimaradiene/abietadiene synthase into producing isopimaradiene and sandaracopimaradiene and none of its normal products. Four mutations were sufficient to reciprocally reverse the product profiles for both of these paralogous enzymes while maintaining catalytic efficiencies similar to the wild-type enzymes. This study illustrates how neofunctionalization can result from relatively minor changes in protein sequence, increasing the diversity of secondary metabolites important for conifer defense.

biochemical evolution | natural product biosynthesis | plant defense | terpene diversity | site-directed mutagenesis

Conifers produce an oleoresin containing approximately equal amounts of monoterpenoids and diterpene acids with smaller amounts of sesquiterpenoids. The role of this secretion is thought to be largely for defense (1–3). In addition to its roles as a sticky physical barrier to invading pests and pathogens, the myriad of compounds that make up this secretion can have various behavioral and physiological roles in plant–herbivore and plant–pathogen interactions. After exposure to the atmosphere, the more volatile compounds evaporate to leave the diterpene resin acids that can harden to seal wounds (2).

Terpenoids are formed by terpene synthases (TPSs), which, particularly those of secondary metabolism in plants, comprise a large gene family believed to result from multiple gene duplications and subsequent neofunctionalization and subfunctionalization (4–6). TPSs are unique enzymes in that most form multiple products from a single substrate by providing a 3D template and stabilizing various intermediate carbocations during cyclization (7). As such, these enzymes are often suggested to have molecular promiscuity resulting from plasticity residues that slightly change the template or carbocation stabilization when the amino acid is changed and thus direct product outcome. Research in recent years has addressed how specific residues or domains in various mono-, sesqui-, and di-TPSs direct product profiles, whether it is in naturally occurring systems or through directed mutagenesis (8–15).

Diterpene resin acids are formed in two major steps. Di-TPSs use geranylgeranyl diphosphate (GGPP) to produce the diterpene olefins (5, 16), which are then oxidized to the corresponding acids by multisubstrate and multifunctional cytochromes

P450 (17, 18). To date, only a handful of di-TPSs involved in diterpene resin acid biosynthesis have been characterized, with all of them originating from gymnosperm trees (2). A series of papers from Croteau, Peters, and coworkers (14, 15, 19–23) studied an abietadiene synthase (AS) from Grand fir [*Abies grandis* (Ag)] to establish the general mechanism and specific features of this type of di-TPS. Two closely related di-TPSs identified in Norway spruce [*Picea abies* (Pa)] have 91% amino acid identity but nonoverlapping product profiles (5). One, isopimaradiene synthase (Iso), exclusively produces isopimaradiene (isopimara-7,15-diene) (Fig. 1). The other, levopimaradiene/AS (LAS), produces a mixture of four diterpenes: abietadiene (abieta-7,13-diene), levopimaradiene [abieta-8(14),12-diene], neoabietadiene [abieta-8(14),13(15)-diene], and palustradiene (abieta-8,13-diene), similar to the product profile of AgAS (20). Orthologous LAS from Loblolly pine (*Pinus taeda*) (Pt) (24) and a levopimaradiene synthase (LS) from *Ginkgo biloba* (Gb) (25) have also been characterized.

All of these enzymes are bifunctional with two catalytically independent active sites. The first active site (class II) in the N-terminal domain contains a DXDD motif that catalyzes the protonation-initiated cyclization of GGPP to (+)-copalyl diphosphate [(+)-CPP; Fig. 1]. The (+)-CPP then freely diffuses to a second active site (class I) in the C-terminal domain, containing the Mg²⁺-binding DDXXD motif that catalyzes the diphosphate ionization-initiated cyclization of (+)-CPP to produce the C8-sandaracopimarenyl cation intermediate (21–23). Deprotonation of this carbocation at alternate sites results in isopimaradiene or sandaracopimaradiene [sandaracopimara-8(14),15-diene] (Fig. 1, routes a and c). Alternatively, intramolecular proton transfer and a 1,2-methyl migration result in the abietenyl cation intermediate (Fig. 1, route b) that is subsequently deprotonated at one of four sites to produce abietadiene, levopimaradiene, neoabietadiene, or palustradiene.

The few amino acid differences (Fig. 2) in the second active site between the paralogous PaIso and PaLAS enzymes are sufficient to direct PaIso to deprotonate the C8-sandaracopimarenyl cation while PaLAS proceeds further through an abietenyl cation intermediate, resulting in alternate products (Fig. 1). These paralogous enzymes provide a unique opportunity to examine the domains and residues that determine product diversity of di-TPSs involved in conifer secondary metabolism. The objective of the present work was to identify those residues that are different between PaLAS and PaIso and result in the observed product profile differences. We used reciprocal domain-swapping and mutagenesis strategies directed by homol-

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*To whom correspondence should be addressed. E-mail: bohlmann@msl.ubc.ca.

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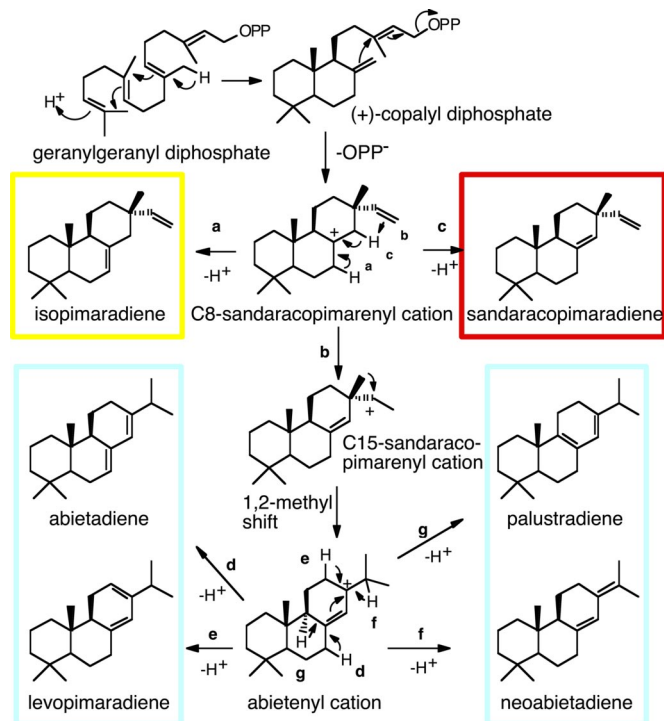


Fig. 1. Proposed reaction mechanisms for the di-TPSs PaIso and PaLAS. Routes a and b are the pathways for PaIso and PaLAS, respectively. Route c occurs with some of the mutants. Deprotonations leading to the multiple products of PaLAS are indicated by routes d–g.

ogy modeling and sequence comparisons to delineate and identify the specific amino acids that determine the product profiles of these naturally occurring paralogues.

Results

We developed a homology model of the second active site for both PaIso and PaLAS by using Deep View/Swiss-PDBViewer

and SWISS-MODEL (26) based on the structure of 5-*epi*-aristolochene synthase from *Nicotiana tabacum* (Protein Data Bank ID code 5EAT) (27). Although 5-*epi*-aristolochene synthase is an angiosperm sesqui-TPS that shares only 18% identity to gymnosperm PaIso and PaLAS, the tertiary structures of TPSs are well conserved (7, 27), allowing us to model these enzymes when no comparable di-TPS structure has been described.

To delineate the larger regions of PaIso and PaLAS that determine the different product outcomes, we prepared a series of chimeric constructs dividing the second active site into two sections that were reciprocally swapped, alone or in combination, between PaLAS and PaIso (Fig. 2). Most of the residues within the modeled second active site occurred in an area of increased sequence divergence between PaIso and PaLAS (residues 571–730 of PaIso; Fig. 2). We named the portion of these enzymes corresponding to residues 568–640 of PaIso the AB domain and the portion corresponding to residues 651–733 of PaIso the BC domain, as shown in Fig. 2.

By swapping the full AC domain between PaIso and PaLAS, we completely reversed the product profiles of these two enzymes (Fig. 3A). Thus, the AC domain fully encompassed those residues that determine product profile differences between the two wild-type enzymes. We then characterized chimeras for which the AB and BC domains were individually swapped. PaIso containing the BC domain of PaLAS (PaIso~LAS BC) and PaLAS~Iso AB chimeras formed products similar to each other and wild-type PaLAS (Fig. 3) except for additionally producing 6% isopimaradiene (the product of route a in Fig. 1). Expression of both the PaIso~LAS AB and PaLAS~Iso BC chimeras failed to produce any His-tagged enzyme after Ni-affinity purification. Analysis of the active chimeras indicated that the BC domain determined almost all of the functional differences between PaIso and PaLAS but that the AB domain also had a minor influence on product profile. Precise quantitative data for the results shown in Fig. 3 appear in [supporting information \(SI\) Table 2](#).

We then examined residues of PaIso and PaLAS in an increasing sphere corresponding to that around the phosphorus atom of the hydroxyphosphonate group of the substrate ana-

PaIso	1	MALLSSLSQIPTGSH ^{EL} TH ⁻⁻⁻ ---TQCIPHF ST TINAC ^{IS} AGK ^{PR} S ^F YL ^R WGKGSNK ^I IACVGE ^{CT} TSLPYQSAEKT ^{DS} LSAPTLVKREFPPGFWKDH
PaLAS	1	MALLSSLSQIPTGAH ^{EL} TLNAYANTQCIPHF ST TINAC ^{IS} AGK ^{RS} SL ^F YL ^R WGKGSNK ^I IACVGE ⁻⁻⁻⁻⁻ -----DLSAPTLVKREFPPGFWKDH
PaIso	96	VIDSLTSSHKVSA ^{AE} EKR ^{ME} TLISEIKN ^I FRSMGYG ^{ET} NP ^S AYDTAWVARIPAVDGE ^{PE} FPETLEWILQNLKDGSGWEGFYFLAYDRILATLACIIT
PaLAS	88	VIDSLTSSHKVAAS ^{DE} KRI ^{ET} LISEIKN ^I FRSMGYG ^{DT} NP ^S AYDTAWVARIPAVDGE ^{PE} FPETLEWILQNLKDGSGWEGFYFLAYDRILATLACIIT
PaIso	196	LTLWR ^{TGE} ^Q IRK ^{GI} E ^{FF} KTQAGKIEDEADSHRPSGFEIVFPAMLKEAKVGLGLDLPYELPF ^{IK} Q ^{II} E ^K REAKLERLP ^{TN} ILYALPTLLYSLEGLQEI ^{VD}
PaLAS	188	LTLWR ^{TGE} ^Q Q ^{VG} K ^{GI} E ^{FF} KTQAGKIEDEADSHRPSGFEIVFPAMLKEAKVGLGLDLPYELPF ^{IK} Q ^{II} E ^K REAKLERLP ^{TN} ILYALPTLLYSLEGLQEI ^{VD}
PaIso	296	W ^K IK ^{IK} LQSKDGSFL ^T SPASTAAVFMRTGNKKCLEFLN ^F V ^L KKFGNHVPC ^H YPLDLFERLWAVDT ^{VER} L ^G ID ^R H ^F K ^{EE} IKD ^{AL} DYVYSHWDERGIGWARE
PaLAS	288	W ^K IK ^{IK} LQSKDGSFL ^T SPASTAAVFMRTGNKKCLEFLN ^F V ^L KKFGNHVPC ^H YPLDLFERLWAVDT ^{TER} L ^G ID ^R H ^F K ^{EE} IKD ^{AL} DYVYSHWDERGIGWARE
PaIso	396	NP ^I PD ^{IDD} TAMGLRILRLHGYNVSSDVLK ^T FRDENG ^{EFF} CFLGQ ^T Q ^R GVTDMLNVNRC ^{SH} VAF ^P GETIM ^Q EAK ^I CTERYLRNALEDVGA ^F DKWALKKNIR
PaLAS	388	NP ^V PD ^{IDD} TAMGLRILRLHGYNVSSDVLK ^T FRDENG ^{EFF} CFLGQ ^T Q ^R GVTDMLNVNRC ^{SH} VAF ^P GETIM ^Q EAK ^I CTERYLRNALEDVGA ^F DKWALKKNIR
PaIso	496	GEVEYALKYPWH ^{RS} MP ^R LEARSYIEHYGPN ^D V ^L GK ^T MYM ^{MP} YISN ^K YLELAKLDFNHVQSLHQKELRDLRR ^W W ^K SSGL ^S SELK ^F TRER ^V TEIYFS ^{AS}
PaLAS	488	GEVEYALKYPWH ^{RS} MP ^R LEARSYIEHYGPN ^D V ^L GK ^T MYM ^{MP} YISN ^K YLELAKLDFNHVQSLHQKELRDLRR ^W W ^K SSG ^F TELK ^F TRER ^V TEIYFS ^{AS}
PaIso	596	IFEP ^E FAT ^{CR} D ^V YTK ^{IS} SN ^F T ^V ILDDLYDAHG ^T LD ^N L ^F SE ^{CV} K ^R W ^D LSLVDRMPQDMKIC ^F T ^V LYNT ^V NEIA ^{VE} BGRK ^R QGRDVLGYIRNV ^{LE} IL ^{LA} HT
PaLAS	588	MFEP ^E FAT ^{CR} D ^V YTK ^{IS} SN ^F T ^V ILDDLYDAHG ^T LD ^N L ^F FS ^D SV ^K K ^W DLSLVDRMPQDMKIC ^F M ^G FYNT ^V NEIA ^{VE} BGRK ^R QGRDVLGYIRNV ^{LE} IL ^{LA} HT
PaIso	696	KEA ^E WSAARYVPSFDEYIENASV ^{IS} LG ^T LV ^L IS ^V LV ^L FTG ^E IL ^T DDVLSKIGR ^S R ^F LQ ^L MGLT ^G R ^L VND ^T KTYE ^A ERGQGEV ^{AS} AVQ ^C YMK ^H PEISEE
PaLAS	688	KEA ^E WSAARYVPSFDEYIDNASV ^{IA} LG ^T V ^L IS ^A LV ^L FTG ^E IL ^T DDVLSKIGR ^S R ^F LQ ^L MGLT ^G R ^L VND ^T KTYE ^A ERGQGEV ^{AS} AVQ ^C YMK ^H PEISEE
PaIso	796	ALKHVY ^T VMENALDELNREFVNNR ^D VPD ^S CR ^{RL} VF ^{ET} ARIMQ ^L FY ^{ME} GDGL ^T LSH ^E MEI ^{KE} HVK ^N CLFQ ^P VA
PaLAS	788	ALKHVY ^T VMENALDELNREFVNNR ^D VPD ^S CR ^{RL} VF ^{ET} ARIMQ ^L FY ^{MD} GDGL ^T LSH ^E MEI ^{KE} HVK ^N CLFQ ^P VA

Fig. 2. Protein alignment of PaIso and PaLAS. Amino acids with white, gray, and black backgrounds indicate identical, similar, and nonsimilar residues, respectively. Asterisks indicate residues mutated in this study. Circles indicate the four residues found in this study that determined product profile differences. Red letters and colored backgrounds indicate AB and BC domain boundaries. A triangle indicates the start of the pm constructs. The conserved DXDD and DDXXD motifs for the first and second active sites, respectively, and the KRX₆W motif are underlined.

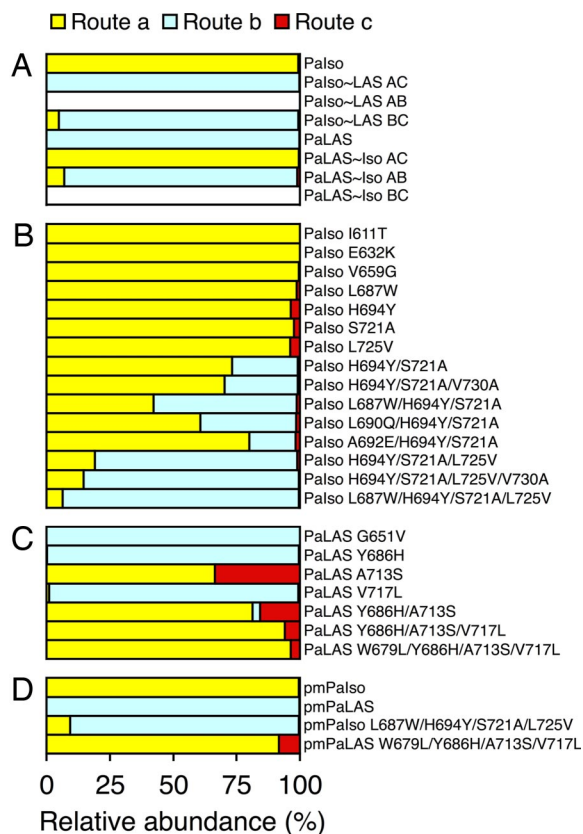


Fig. 3. Relative abundance of enzyme assay products from routes a, b, and c (see Fig. 1) for each enzyme tested. (A) Wild-type and chimeric enzymes. (B) Site-directed mutants of PaIso. (C) Site-directed mutants of PaLAS. (D) Pm wild-type and quadruple mutants.

logue in the sesqui-TPS structure superimposed on our di-TPS homology models. Table 1 lists the residues divergent between PaIso and PaLAS within a 20-Å sphere. We focused site-directed mutations to those uniquely different in PaIso compared with four other related di-TPSs (PaLAS, AgAS, PtLAS, and GbLS; Table 1 and SI Fig. 6), which all favor formation of the abietenyl cation (Fig. 1, route b). We hypothesized that those residues that were conserved between the four enzymes but divergent in PaIso were most likely to direct differences in product outcomes (Table 1, Fig. 2, and SI Fig. 6).

Within a predicted 10-Å sphere, we identified two residues, H694 and S721 in PaIso, and Y686 and A713 in PaLAS, that had previously been speculated to determine product outcome in these enzymes (5). However, reciprocal mutagenesis at these residues was not sufficient to fully reverse product profiles (Fig. 3B). Both PaIso H694Y and PaIso S721A mutations individually did not result in products from route b but did produce 2–4% sandaracopimaradiene, the product from route c. However, the double mutant PaIso H694Y/S721A produced 26% of the products from route b. The reciprocal PaLAS Y686H mutant did not change product outcome from wild-type PaLAS, but the PaLAS A713S mutant completely abolished products from route b and instead produced a 2:1 ratio of products from routes a and c. The double mutant PaLAS Y686H/A713S produced an even greater ratio of product from route a but also produced 3% products from route b. Thus, although reciprocally mutating these two residues was not sufficient to completely reverse product profiles, it had a substantial impact on redirecting catalytic routes and thus appeared necessary for determining product profiles.

We then used site-directed mutagenesis to characterize additional divergent residues within the AC domain as shown in Table 1 that may direct product profile differences. Because the initial mutations of PaIso and PaLAS described above suggested that it was harder to change the function of PaIso rather than that of PaLAS, PaIso was used as the initial template for further mutations. Although none of the individual mutations of PaIso gave products from route b, the quadruple mutant PaIso L687W/H694Y/S721A/L725V almost exclusively formed products from route b (93%; Fig. 3B). All four of these mutated residues occurred within the BC domain. A comparison of the product profile of this mutant with those of PaIso~LAS BC and PaLAS~Iso AB suggested that these four mutations account for all of the critical residues in the BC domain necessary for directing product outcome (Fig. 3A and B). Other mutations characterized within the AC domain did not produce substantial differences in product outcome.

To further validate the role of these four residues in determining product outcome, we made reciprocal mutations with PaLAS as template and determined that the PaLAS W679L/Y686H/A713S/V717L mutant no longer produced any diterpenes from route b but rather 96% from route a and 4% from route c (Fig. 3C). Although the single-residue A713S mutation of PaLAS appeared to have a major impact redirecting product profile, the additional three mutations substantially enhanced the product outcome toward that of PaIso. Thus in both cases, the quadruple reciprocal mutants were effective in nearly completely reversing the product profiles for these two paralogous diTPS enzymes, PaLAS and PaIso (Figs. 3 and 4).

We prepared pseudomature (pm) constructs of PaIso, PaLAS, PaIso L687W/H694Y/S721A/L725V, and PaLAS W679L/Y686H/A713S/V717L (truncated at residues 84 and 76 of PaIso and PaLAS, respectively) to allow direct comparison of the kinetic parameters with the pm version of AgAS (19) and evaluate the catalytic efficiency of the PaIso and PaLAS mutants. The products from these N-terminally His-tagged pm enzymes did not differ from their full-length counterparts (Fig. 3A and D) or C-terminally His-tagged pm constructs (data not shown). Kinetic data for the pm enzymes with GGPP as substrate are shown in Fig. 4. We observed substrate inhibition and overall kinetic properties similar to those reported for AgAS (20). PaLAS appeared a more efficient enzyme than PaIso. In addition, the efficiency of the quadruple mutants increased or decreased toward the efficiency of the reciprocal wild-type enzyme, suggesting that these reciprocal mutations did not impair catalytic function. Although the kinetics parameters were not determined for the other intermediate mutants, none appeared to be severely impaired in our assay conditions.

The four pm enzymes were also characterized at different pHs and temperatures. These enzymes were active between pH 6.0 and 9.5 but showed greatly reduced or no activity at lower pH (data not shown). Both pmPaIso and pmPaLAS W679L/Y686H/A713S/V717L did not change product profiles over this pH range, but pmPaLAS and pmPaIso L687W/H694Y/S721A/L725V produced slightly more abietadiene at lower pH. We observed no differences in product profiles over the range of 4°C to 37°C with the enzymes tested except for a slight increase in the amount of sandaracopimaradiene produced with increasing temperature for pmPaLAS W679L/Y686H/A713S/V717L (data not shown).

Discussion

Our rational approach to identifying the specific residues that determine product profile differences between PaIso and PaLAS was successful in identifying four residues that nearly completely reverse the product outcome between these two enzymes. By examining the location of these residues within the homology models, we found that the three residues (H694, S721, and L725)

Table 1. Divergent residues in Palso and PaLAS homology models within an increasing radius from the phosphorous atom of the substrate analogue in the structure of 5-*epi*-aristolochene synthase from *N. tabacum* and the corresponding residues in other related di-TPSS

Radius, Å	Residue in:					Domain	Unique	Mutated
	Palso	PaLAS	PtLAS	AgAS	GbLS			
5								
10	L690	Q682	Q	Q	L	BC		Palso
	H694	Y686	Y	Y	Y	BC	Yes	Both
	S721	A713	A	A	A	BC	Yes	Both
15	I611	T603	T	T	T	AB	Yes	Palso
	I613	N605	N	N	C	AB		
	L687	W679	W	W	W	BC	Yes	Both
	A692	E684	E	E	A	BC		Palso
	E714	D706	G	E	E	BC		
	L725	V717	V	V	V	BC	Yes	Both
20	A592	P584	P	P	V	AB		
	N630	D622	N	D	D	AB		
	E632	K624	K	K	K	AB	Yes	Palso
	V659	G651	G	G	G	BC	Yes	Both
	L660	F652	F	F	L	BC		
	E842	D834	D	Q	Y			
	M852	T844	M	M	K			
>20	V730	A722	A	A	I	BC		Palso

The domain in which the residue resides and whether the residue is uniquely divergent in Palso are indicated. The rightmost column indicates whether mutants in Palso, both Palso and PaLAS, or neither were characterized at this residue.

in Palso, and the corresponding residues in PaLAS, form a line of amino acids running the length of the predicted active-site cavity, with H694 nearest the entrance and L725 furthest into the cavity (Fig. 5). Palso L687 and PaLAS W679 do not line the active-site cavity, but because of their proximity to the cavity and their relative size differences may have a secondary effect on the size and shape of the active-site cavity that modestly influences product outcome.

In general, Palso and PaLAS form different products because the predicted active-site cavities of the two enzymes differ in size, shape, and polarity. Once formed, the C8-sandaracopimarenyl cation seems to be stabilized deeper into the cavity of Palso and in a slightly different orientation than in PaLAS. The polarity of S721 of Palso appears positioned to stabilize the C8-sandaracopimarenyl cation. This residue is an alanine in PaLAS and thus cannot stabilize the cation. The dramatic change in product profile from the PaLAS A713S mutant supports this residue's influence in directing product outcome. Near this residue is a conserved serine (Palso S719/PaLAS S711), which may also play a role in stabilizing both the C8-sandaracopimarenyl and abietenyl cations. In AgAS, mutation of this conserved serine to alanine greatly impairs catalysis (15). Although PaLAS has this conserved serine to stabilize the C8-sandaracopimarenyl cation, Y686 in PaLAS is better positioned to stabilize the abietenyl cation (Fig. 5). The corresponding residue in Palso is H694, which, owing to its positive charge, may destabilize the abietenyl cation. Palso S721 and H694, and the corresponding PaLAS A713 and Y686, appear to strongly influence the polarity of the active site and thus determine which of the alternate carbocation intermediates is favorably stabilized. Palso L725 and the corresponding PaLAS V717 residue influence the shape in the deeper portions of the cavity, with Palso L725 impinging into the modeled cavity further than does PaLAS V717. As this single mutation has a negligible influence on product outcome, we hypothesize that this difference changes the orientation of the intermediates and thus affects differential stabilization of the carbocations by other residues.

Interestingly, mutation of PaLAS more easily resulted in large changes in product profiles compared with the reciprocal mutations in Palso. This finding may suggest that the subsequent formation of the abietenyl cation from the C8-sandaracopimarenyl cation (Fig. 1) is particularly sensitive to single amino acid changes in the active site. The single PaLAS A713S mutation dramatically changed product profile, abolishing all products derived from the abietenyl cation and instead producing a 2:1 ratio of isopimaradiene and sandaracopimaradiene. No mutants of Palso produced as much sandaracopimaradiene as did the mutants of PaLAS.

There is conservation around the DDXD motif between these two enzymes (Fig. 2), and our focus of study was those residues that influence product profile after formation of the C8-sandaracopimarenyl cation. Thus, it would seem that the differences we observed in the kinetics between enzymes were not caused by differences in the kinetics of initial formation of the C8-sandaracopimarenyl cation but rather in final product formation and subsequent release of the products from the enzyme. Although formation of the abietenyl cation in route b requires an intramolecular proton transfer, passes through a less stable secondary carbocation intermediate, and includes a methyl migration, the kinetic parameters of enzymes that used this pathway appeared more efficient than the enzymes that simply deprotonate the C8-sandaracopimarenyl cation in route a. The difference in kinetic efficiencies between these enzymes cannot be explained with our current understanding of these enzymes.

For those enzymes expressed in both full-length and pm forms, the resulting product profiles did not differ between full-length and pm forms. Interestingly, the relative ratio of products resulting from route b (i.e., abietadiene, levopimaradiene, neoabietadiene, palustradiene, and two minor unknown products not previously identified from these enzymes) did not differ among all of the mutations, even though the relative amounts of products from routes a, b, and c varied widely. However, mutations in this study only targeted those residues that differ between PaLAS and Palso in a reciprocal fashion. Different

applied to His SpinTrap Ni-affinity columns (GE Healthcare) and eluted with 20 mM NaPO₄, 500 mM NaCl, and 500 mM imidazole, pH 7.4 at 4°C following the manufacturer's protocol. Purified enzymes were used immediately for enzyme assays as described below.

For preparative purifications, cell pellets from 1-liter cultures were resuspended in (2 ml/g pellet) ice-cold 20 mM Hepes (pH 7.5), 150 mM NaCl, 20 mM imidazole, 5 mM DTT, 5 mM PMSF, and 0.5 mg/ml lysozyme. After 30 min of gentle mixing at 4°C, the lysates were sonicated, clarified by centrifugation, and applied to a 1-ml HisTrap HP Ni-affinity column by using an ÄKTApurifier 10 fast protein liquid chromatograph (GE Healthcare). The column was washed with 60 ml of 20 mM Hepes, 150 mM NaCl, and 20 mM imidazole, pH 7.5 before eluting the His-tagged proteins with 20 mM Hepes, 150 mM NaCl, and 350 mM imidazole, pH 7.5. Buffer composition of eluate was exchanged to 20 mM Hepes and 150 NaCl, pH 7.5 with an Econo-Pac 10DG desalting column (Bio-Rad). Protein concentration was quantified (7–13 mg/ml) by direct measurement of absorbance at 280 nm with a spectrophotometer (NanoDrop Technologies) using the molar extinction coefficients calculated by Vector NTI software (Invitrogen). Fresh DTT was added to 5 mM final concentration and then the purified enzymes were aliquoted, flash-frozen in liquid N₂, and stored at –80°C where they were stable for several months.

Single-Vial Enzyme Assays. Enzyme assays were completed in 2-ml amber glass GC sample vials as described (31). Buffer (500 μ l) consisted of 20 mM Hepes, 20 mM Mes, 20 mM Mops (pH 7.0), 100 mM KCl, 7.5 mM MgCl₂, 0.02 mM MnCl₂, 5 mM fresh DTT, 5% glycerol, and 40 μ M GGPP (Sigma–Aldrich). Assays were started by adding 50–100 μ g of purified enzyme, gently mixed, overlaid with 500 μ l of pentane containing 1.6 μ M 1-eicosene as an internal standard, and then placed in a 30°C water bath for 1 h. The vials were then vigorously vortexed for 30 s to stop the reaction (by denaturing the enzyme) and to extract the products from the aqueous layer. Phases were separated by centrifugation at 1,000 \times g for 30 min at 4°C.

GCMS Analysis. Assay products were analyzed on Agilent HP5ms (5% phenyl methyl siloxane, 30 m \times 250 μ m i.d., 0.25- μ m film) and DB-WAX (polyethylene glycol, 30 m \times 250 μ m i.d., 0.25- μ m film) columns at 1 ml/min He on an Agilent 6890N GC, 7683B series autosampler (vertical syringe position of 8), and a 5975 Inert XL MS Detector at 70 eV. GC temperature programs were as follows: HP5ms, 40°C for 1 min, 7.5°C/min to 250°C, hold 2 min, pulsed splitless injector held at 250°C; DB-WAX, 40°C for 3 min, 10°C/min to 240°C, hold 15 min, pulsed splitless injector held at 240°C. Compounds were identified by comparison to authentic standards, retention indices, and mass spectra from Adams (32) and

National Institute of Standards and Technology 2005 MS library searches. The relative contributions of the different pathway products of mutant and wild-type enzymes were calculated by using total ion currents.

Analysis of Enzyme Activity and Product Profiles at Various pHs and Temperatures. To assess the effect of pH on enzyme activity and product profile, single-vial assays were performed as above on preparatively Ni-affinity-purified pmPaLAS, pmPalso, pmPalso L687W/H694Y/S721A/L725V, and pmPaLAS W679L/Y686H/A713S/V717L in 20 mM Hepes, 20 mM Mes, 20 mM Mops, 100 mM KCl, 7.5 mM MgCl₂, 0.02 mM MnCl₂, 5 mM fresh DTT, 20 μ M GGPP, and 5% glycerol at 0.5 pH units between 5.0 and 9.5 at 22°C. These enzymes were also assayed at pH 7.0 at 4°C, 16°C, 22°C, and 37°C for 135, 60, 45, and 30 min, respectively, to determine whether product profiles varied with temperature.

Kinetic Analysis of pmPalso, pmPaLAS, pmPalso L687W/H694Y/S721A/L725V, and pmPaLAS W679L/Y686H/A713S/V717L. Single-vial assays were used as described above but with the addition of 0.1 mg/ml BSA to the buffer to stabilize the recombinant enzymes at the lower concentrations (\approx 10 nM). Assays were completed in triplicate with 0.5–42 μ M GGPP for 10 min at 22°C. Assays were analyzed on the GC mass spectrometer by using selected ion monitoring of *m/z* 83 (for internal standard) and both 257 and 272 for diterpene products (abietadiene and isopimaradiene were used to quantify products). The response of the GC mass spectrometer at each substrate concentration was determined relative to the internal standard by using excess enzyme in the single-vial assay and allowing the reaction to proceed to completion for 2 h. Kinetic parameters were determined by nonlinear regression using the substrate inhibition model in ANEMONA (33).

Note Added in Proof: Wilderman and Peters (34) recently described results from site-directed mutagenesis of AgAS A723S confirming our observations with PaLAS A713S.

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