Evidence for Differential Folding of Farnesyl Pyrophosphate in the Active Site of Aristolochene Synthase: A Single-Point Mutation Converts Aristolochene Synthase into an (*E*)-β-Farnesene Synthase[†]

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ABSTRACT: Sesquiterpene cyclases, many of which share significant structural similarity, catalyze the cyclization reactions of the universal alicyclic precursor farnesyl pyrophosphate to produce more than 300 different hydrocarbon skeletons with high regio- and stereospecificity. The molecular basis of this exquisite specificity is not well-understood, but the conformation adopted by FPP in the active site of a sesquiterpene cyclase is thought to be an important determinant of the reaction pathway. Aristolochene synthase (AS) from *Penicillium roqueforti* catalyzes the cyclization of farnesyl pyrophosphate to the bicyclic sesquiterpene aristolochene. The X-ray structure of AS suggested that the steric bulk of residue 92 was central in binding of FPP to the active site of AS in a quasi-cyclic conformation, thereby facilitating attack of C1 by the C10−C11 double bond to produce the *cis*-fused Decalin *S*-germacrene A. We demonstrate here that reduction of the size of the side chain of residue 92 leads to the production of the alicyclic sesquiterpenes (*E*)-β- and (*E*,*E*)-α-farnesene. The relative amounts of linear products formed depended linearly on the size of the residues at position 92. ASY92A, in which Tyr92 had been replaced with Ala, produced almost 80% of alicyclic sesquiterpenes, suggesting an energetic separation of less than 0.8 kcal/mol between the cyclic and noncyclic reaction pathways. A mechanism by which FPP binds to the mutant enzymes in an extended conformation is proposed to explain the altered selectivity. The mutants also produced small amounts of additional hydrocarbons with a molecular weight of 204, namely, α-selinene, β-selinene, selina-4,11-diene, (*E*,*Z*)-α-farnesene, and β-bisabolene. The production of (*E*)-β-farnesene and β-bisabolene suggested that the initial cyclization of FPP to germacrene A in AS proceeded in a stepwise fashion through farnesyl cation.

Sesquiterpenes are a large family of C15-isoprenoid natural products found in fungi, insects, bacteria, and marine and terrestrial plants. They display a wide range of biological activities. In plants, they are involved in interactions with insects and microbes and they act as phytoalexins, attractants, and deterrents. All tens of thousands of sesquiterpenes known to date are derived from just more than 300 distinct hydrocarbon skeletons with different structures and stereochemistries (*1*−*5*). These skeletons are biosynthesized from the universal alicyclic sesquiterpene precursor farnesyl pyrophosphate (FPP,[1] **1**). Sesquiterpene cyclases, each of which typically catalyzes a single cyclization reaction of FPP with often high regio- and stereospecificity, are the enzymes responsible for the generation of this product diversity. Their catalytic efficiency depends on the presence of a divalent cation, often Mg^{2+}, which mediates expulsion of pyrophosphate from FPP to generate a highly reactive allylic carbocation. Sesquiterpene cyclases chaperone the reaction intermediates along only one of many different reaction pathways involving cyclizations of exquisite specificity, hydride and methyl transfers, and deprotonation reactions while at the same time excluding solvent from the active site to prevent premature quenching by water of the extremely reactive cationic reaction intermediates.

The X-ray structures of the four sesquiterpene cyclases which have been determined to date, namely, pentalenene synthase from *Streptomyces* UC5319 (*6*), 5-epiaristolochene synthase from *Nicotiana tabacum* (*7*), aristolochene synthase from *Penicillium roqueforti* (*8*), and trichodiene synthase from *Fusarium sporotrichioides* (*9*), revealed that despite a lack of significant sequence homology they all shared the terpenoid synthase fold, which is also adopted by avian farnesylpyrophosphate synthase (*10*) and human squalene synthase (*11*). A surprising degree of sequence similarity was observed for the hundreds of sequiterpene cyclases from plants, suggesting that they all rely on the terpenoid synthase fold for catalytic activity (*5*). The starting conformation of **1** within the enzyme's active site has been postulated to be a critical determinant of product diversity (*2*, *12*). The high

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[1] Abbreviations: AS, aristolochene synthase; Y92A, tyrosine 92 → alanine mutation; Y92C, tyrosine 92 → cysteine mutation; Y92F, tyrosine 92 → phenylalanine mutation; Y92V, tyrosine 92 → valine mutation; FPP, farnesyl pyrophosphate; GC−MS, gas chromatography−mass spectroscopy; CD, circular dichroism.

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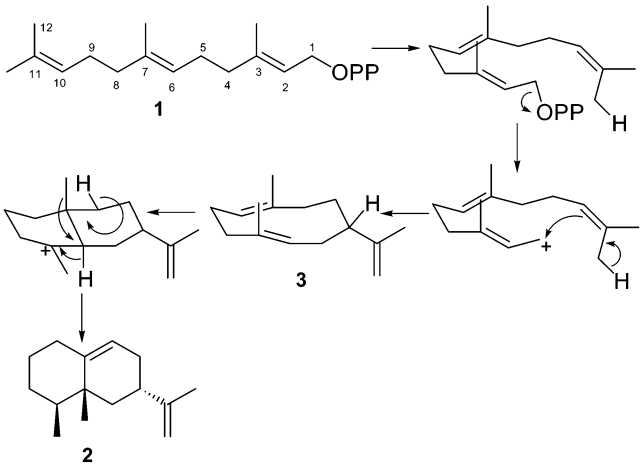
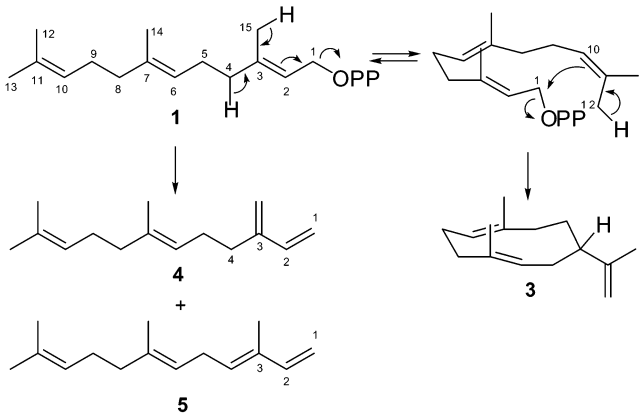
Scheme 1



Scheme 2



degree of structural similarity of sesquiterpene cyclases therefore suggests that relatively few changes in the composition of the active site residues could lead to significant alterations in the reaction pathway through differential folding of the substrate.

Aristolochene synthase (AS) from *P. roqueforti* is a monomeric enzyme with a molecular mass of 39 kDa which catalyzes the cyclization of **1** to the sesquiterpene (+)-aristolochene (**2**), the biosynthetic precursor of several toxins, including the PR toxin (*13*). A recent study has lent strong support to the proposal that AS catalyzes the conversion of FPP to aristolochene via the *cis*-fused Decalin *S*-germacrene A (**3**) (*12*). AS is thought to bind the substrate in a conformation which favors attack of C1 by the C10−C11 double bond after (or concurrent with) metal-triggered diphosphate departure (Scheme 1). Protonation of germacrene A followed by cyclization through electron flow from the C2−C3 double bond, hydride and methyl shifts, and deprotonation from C8 results in the formation of aristolochene.

The correct folding of the substrate in the active site of AS appears to be critical for prevention of the formation of alternative reaction products. We now report how alterations in the active site of AS can, most likely through alternative folding of FPP in the active site of the mutant enzymes, lead to the formation of a new major reaction product. A single-point mutation of an active site residue of AS sufficed to create an enzyme which produced 73% (*E*)-β-farnesene (**4**) (Scheme 2), an alicyclic sesquiterpene which is important for communication in plants and insects (*14*).

MATERIALS AND METHODS

Materials. Oligonucleotide primers were purchased from Alta Bioscience, University of Birmingham. α- and β-selinene from *Albies magnifica* oloresin were gifts from L. Cool (Forest Products Laboratories, University of California, Berkeley, CA). (*E*)-β-Farnesene was a gift from J. A. Pickett and L. Ireland (BBSRC-Institute for Arable Crops, Rothamsted Research), and (*E*,*E*)- and (*E*,*Z*)-α-farnesene were obtained from S. Dorn (Institute of Plant Sciences, ETH-Zurich, Zurich, Switzerland). β-Bisabolene was from Fluorochem. Aristolochene was the product of FPP incubation with AS. [1-^{3}H]FPP (16.1 Ci/mmol) was purchased from Sigma. Q-Sepharose was obtained from Amersham Pharmacia Biotech. Ultracell Amicon YM3 membranes were purchased from Millipore. EcoScint scintillation fluid was from National Diagnostics. All other chemicals were from Fluka or Sigma.

Site-Directed Mutagenesis of Recombinant AS cDNA. The Quick-Change site-directed mutagenesis kit (Strategene) was used to introduce the Y92C and Y92A mutations into the AS encoding plasmid according to the manufacturer's instructions. The mutagenic primers were as follows: 5′-CAGAGGTTACTTGTCTT**TGC**TTCCCTCTTGCACTGG and 5′-CCAGTGCAAGAGGGAA**GCA**AAGACAAGTAACCTCTG for ASY92C and 5′-CAGAGGTTACTTGTCTT**GCG**TTCCCTCTTGCACTGG and 5′-CCAGTGCAAGAGGGAA**CGC**AAGACAAGTAACCTCTG for ASY92A (altered bases are bold). Plasmids were purified from overnight LB/amp cultures (5 mL) using the Qiagen miniprep kit as described by the manufacturer. Mutations were confirmed by DNA sequence analysis, using an Applied Biosystems 3700 automated DNA sequencer (Functional Genomics Laboratory, University of Birmingham).

Expression of Mutants in Escherichia coli and Purification of ASY92C and ASY92A. ASY92C and ASY92A were produced in *E. coli* BL21(DE3) cells. The cells were grown at 37 °C in LB medium with 0.3 mM ampicillin until they reached an A_{600} of 0.5. They were induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside and incubated for a further 3 h. Cells were harvested by centrifugation at 8000*g* for 10 min and resuspended in 20 mM Tris (pH 8), 5 mM EDTA, and 5 mM 2-mercaptoethanol. ASY92C and ASY92A were purified essentially as described previously (*12*). Purified ASY92C and ASY92A were concentrated using an Amicon 8050 cell and an Ultracell Amicon YM3 membrane. They were pure as judged by SDS gel electrophoresis. Protein concentrations were determined by amino acid analysis (Alta Bioscience, University of Birmingham) or from their extinction coefficient at 280 nm.

Assay for Enzymatic Activity. ASY92C and ASY92A were assayed in a total volume of 250 μL containing 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 15% glycerol, and [1-^{3}H]FPP (0.8−160 μM) (*12*). After the reactions had been terminated by addition of 200 μL of 100 mM EDTA (pH 7.25), the samples were extracted several times with *n*-hexane. The combined hexane extracts were mixed with 4 mL of Ecoscint and analyzed for radioactivity. The percentage conversion of [1-^{3}H]FPP to hexane extractable products was determined by comparing the observed radioactivity to that observed with a known concentration

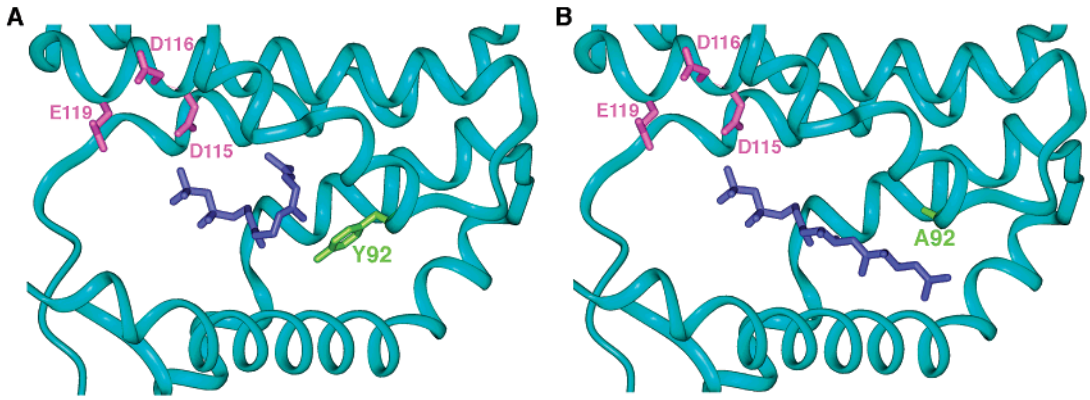


FIGURE 1: (A) Representation of the active site of aristolochene synthase from *P. roqueforti* (*8*) illustrating the pyrophosphate binding site (D115, D116, and E119), the position of Y92, and a proposed reactive conformation of FPP favoring cyclization to geramacrene A. (B) Hypothetical active site structure of ASY92A generated from the X-ray structure of the wild-type enzyme.

of [1-^{3}H]FPP. Background levels of radioactivity were observed in control samples without the enzyme. Energetic separations of the cyclic and linear kinetic pathways were derived from the Arrhenius equation.

CD Spectroscopy. Circular dichroism experiments were carried out with ASY92C and ASY92A at a protein concentration of 6 μM in 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, and 0.2 mM dithiothreitol in the wavelength interval of 190−300 nm using a Jasco J-810 spectropolarimeter. The CD spectra were recorded at 20 °C using 1 mm quartz cuvettes.

Characterization of Products from ASY92C and ASY92A by GC−MS. ASY92C (25 μM) and ASY92A (25 μM) were incubated with FPP (100 μL, 10 mM) in a volume of 500 μL containing 10 mM Tris (pH 7.5), 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 15% glycerol for 60 h. The reactions were stopped by the addition of 100 μL of 100 mM EDTA (pH 7.25), extracted with *n*-hexane (3 × 3 mL), and vortexed with silica (1.5 g). The solvent was removed *in vacuo*, and the concentrated samples were analyzed by GC−MS using a ThermoQuest Finnigan GC 8000 gas chromatograph (equipped with a 30 m ZB5 column) and a MD 1000 mass spectrometer. Splitless injections (2 μL) were performed at 110 °C. The column temperature was increased from 50 to 150 °C at a rate of 4 °C/min and was maintained for 15 min at 150 °C.

RESULTS AND DISCUSSSION

Site-directed mutagenesis experiments had indicated that Tyr92 of AS was the active site acid responsible for the protonation of germacrene A during the conversion of FPP to aristolochene. Phenoxide anion was most likely the base in the final deprotonation from C8 to yield aristolochene (*12*). These studies and inspection of the X-ray structure of AS (*8*) together with molecular modeling studies suggested that Tyr92 not only could act as a general acid and base during AS catalysis but also might have a function in directing the proper folding of FPP. A mutant in which Tyr92 was replaced with the slightly smaller phenylalanine produced a small amount of the alicyclic (*E*)-β-farnesene (**4**) (*12*). When Tyr92 was replaced with Val, more than 40% of the noncyclic product **4** was formed by the corresponding mutant (*15*). In the X-ray structure of AS, the active site was in an open and water accessible conformation, most likely due to the absence of either the substrate or a substrate analogue in the crystals (*8*). The side chains of the active site residues were relatively mobile, and neither FPP nor any of the proposed intermediates could be reliably modeled into the active site. Nevertheless, the X-ray structures of AS (*8*) and of other sesquiterpene cyclases (*7*, *9*) suggested that FPP was anchored to the active site through the pyrophosphate binding site DDVIE, while the side chain of Tyr92 blocked a hydrophobic cavity at the bottom of the active site of AS, thereby forcing FPP to adopt a cyclic conformation (Figure 1A). In this conformation, cyclization of **1** to germacrene A was the preferred reaction. The presence of the bulky side chain of Tyr92 may be necessary to prevent **1** from binding in an extended conformation, which would favor the production of linear sesquiterpenes such as (*E*)-β-farnesene (**4**) and (*E*,*E*)-α-farnesene (**5**) (Scheme 2 and Figure 1B). Reduction of the bulkiness of residue 92 should in turn lead to a decrease in the proportion of FPP molecules bound in the cyclic conformation and increased generation of elimination products such as **4** and **5**. To test this hypothesis, we have generated ASY92C and ASY92A, in which Tyr92 was replaced with the relatively hydrophobic amino acids cysteine and alanine, respectively.

Table 1: Kinetic Constants for AS and Mutants ASY92C and ASY92A[a]

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M (M^{-1} s^{-1})
AS[b]	2.3 ± 0.50	0.03 ± 0.01	13043.0 ± 2989.0
ASY92C	50.27 ± 9.11	(4.91 ± 1.3) × 10^{-4}	10.2 ± 4.3
ASY92A	83.40 ± 10.53	(1.37 ± 0.14) × 10^{-3}	16.5 ± 2.4

[a] Standard deviations were determined from a minimum of three identical measurements. [b] From refs *12* and *15*.

ASY92C and ASY92A were produced at high levels in *E. coli* and purified to apparent homogeneity. The CD spectra of the mutants indicated that they adopted mainly α-helical secondary structure similar to that of wild-type AS (data not shown). The steady state kinetic parameters of ASY92C and ASY92A were determined by incubating them with [1-^{3}H]FPP and monitoring the formation of tritiated, hexane extractable products. Both enzymes exhibited catalytic activities similar to those previously reported for ASY92F and ASY92V (Table 1) (*12*, *15*). The K_M values were determined to be 50.27 and 83.4 μM for ASY92C and ASY92A, respectively. The turnover numbers k_{cat} were 0.03 and 0.08 min^{-1}, respectively, which were approximately 2 orders of magnitude lower than the value that had been observed for the wild-type enzyme (*12*). Though the overall catalytic

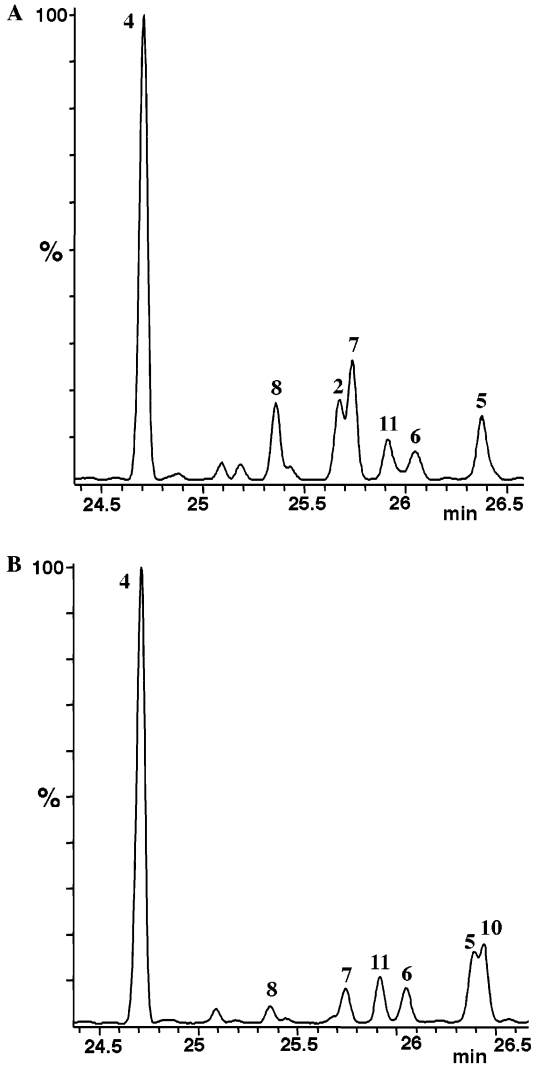


FIGURE 2: Total ion chromatograms from GC−MS analysis of the hexane extractable products of FPP utilization by ASY92C (A) and ASY92A (B).

efficiency of the mutant enzymes was reduced by nearly 3 orders of magnitude relative to that of the wild-type enzyme, it is important to keep in mind that the catalytic efficiencies of most terpene cyclases are intrinsically rather low. These enzymes have evolved to produce cyclic hydrocarbons with often exquisite specificity rather than with high speed.

The hexane extractable materials produced by ASY92C and ASY92A were also analyzed by GC−MS (Figure 2). As was observed in mutants where Tyr92 was replaced with Phe or Val, ASY92C still produced a small amount of aristolochene (6.8%), while no aristolochene was detected when the smaller alanine replaced Tyr92 (Table 2). Decreasing the size of the side chain of residue 92 led to a decrease in the relative amount of aristolochene produced and an increase of the amount of (*E*)-*β*-farnesene (Figure 2). Indeed, **4** was the major product generated by both ASY92C and ASY92A at 61.3 and 72.6%, respectively (Table 2). A smaller amount of (*E*,*E*)-α-farnesene (**5**) was also produced by the mutant proteins (Figure 2). The fact that ASY92C and ASY92A produced 69.4 and 78.2% of the alicyclic products, respectively, suggested energetic separations of 0.48 and 0.76 kcal/mol, respectively, between the noncyclic and cyclic pathways in the two mutants. **4** and **5** were both

Table 2: Relative Amounts (%) of Hexane Extractable Hydrocarbon Products[a] from Incubations of FPP with AS and Mutants ASY92F, ASY92V, ASY92C, and ASY92A Determined by GC Analysis

	2	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
AS[b]	91.5	7.5	0	0	0	0	0	0.4	0	0
Y92F[b]	56.2	28.6	1.8	0	3.6	1.8	2.1	5.9	0	0
Y92V[c]	28.2	11.2	42.6	0	1.0	6.9	4.0	6.1	0	0
Y92C	6.8	0	61.3	8.1	3.1	10.0	7.0	0	0	3.7
Y92A	0	0	72.6	5.6	4.2	4.1	1.8	0	6.3	5.4

[a] **2**, aristolochene; **3**, *S*-germacrene A; **4**, (*E*)-*β*-farnesene; **5**, (*E*,*E*)-α-farnesene; **6**, α-selinene; **7**, *β*-selinene; **8**, selina-4,11-diene; **9**, valencene; **10**, *β*-bisabolene; and **11**, (*E*,*Z*)-α-farnesene. [b] Data for AS and ASY92F from ref *12*. [c] From ref *15*.

identified from their mass spectra by comparison with the spectra in the Wiley library (*16*) (Figure 3). Co-injection with authentic samples of (*E*)-*β*-farnesene and (*E*)-α-farnesene confirmed their identity.

(*E*)-*β*-Farnesene and (*E*,*E*)-α-farnesene were generated through deprotonation from C4 and C15 of either FPP or the corresponding allylic cation (Scheme 2). Inspection of the X-ray structure of AS (*8*) and of 5-epiaristolochene synthase (*7*) revealed no suitable amino acid residue in the vicinity of C4 and C15 that could act as the base for this deprotonation. However, the substrate's diphosphate group is close to both C4 and C15 and might therefore serve as the proton acceptor in this process. A similar mechanism has been invoked to explain the production of small amounts of (*E*)-*β*-farnesene in a trichodiene synthase mutant (*17*).

The reduction in size of residue 92 of AS clearly had a dramatic effect on the product distribution. The relative amount of (*E*)-*β*-farnesene that was produced increased almost linearly with the decreasing van der Waals volume of the side chain. While no (*E*)-*β*- or (*E*)-α-farnesene was produced by wild-type AS and only 1.8% of (*E*)-*β*-farnesene was generated by ASY92F, almost 80% of the products generated by ASY92A were the linear sesquiterpenes **4** and **5** (Table 2). The X-ray structure of AS (*8*) suggested that the replacement of Tyr92 with Ala might prevent FPP from adopting the cyclic conformation necessary for efficient cyclization to **2**. In ASY92A, FPP might bind in a more extended conformation (Figure 1B), which would dramatically reduce the efficiency of cyclization to germacrene A. Indeed, ASY92A and ASY92C produced no germacrene A. FPP most likely prefers to bind to hydrophobic active sites in an extended conformation. Terpene cyclases such as AS must therefore have evolved active sites that direct FPP to bind in a cyclic conformation as a prerequisite for efficient product formation. In AS, this is achieved by placing the relatively bulky side chain of Tyr92 at the bottom of the active site close to the 6,7-double bond, where it prevents the isoprenoid tail from penetrating deeper into the hydrophobic cavity (Figure 1A).

Isoprenoid pyrophosphates have previously been observed to bind to enzymes in quasi-linear conformations. The cocrystal structure of protein farnesyltransferase and FPP revealed that the isoprenoid moiety of the substrate bound in an extended conformation in a funnel-like active site lined with aromatic amino acid residues, while the pyrophosphate group was located at the top of the funnel close to positively charged residues (*18*). Similarly, avian farnesylpyrophosphate synthase, which catalyzes the sequential chain elongation of

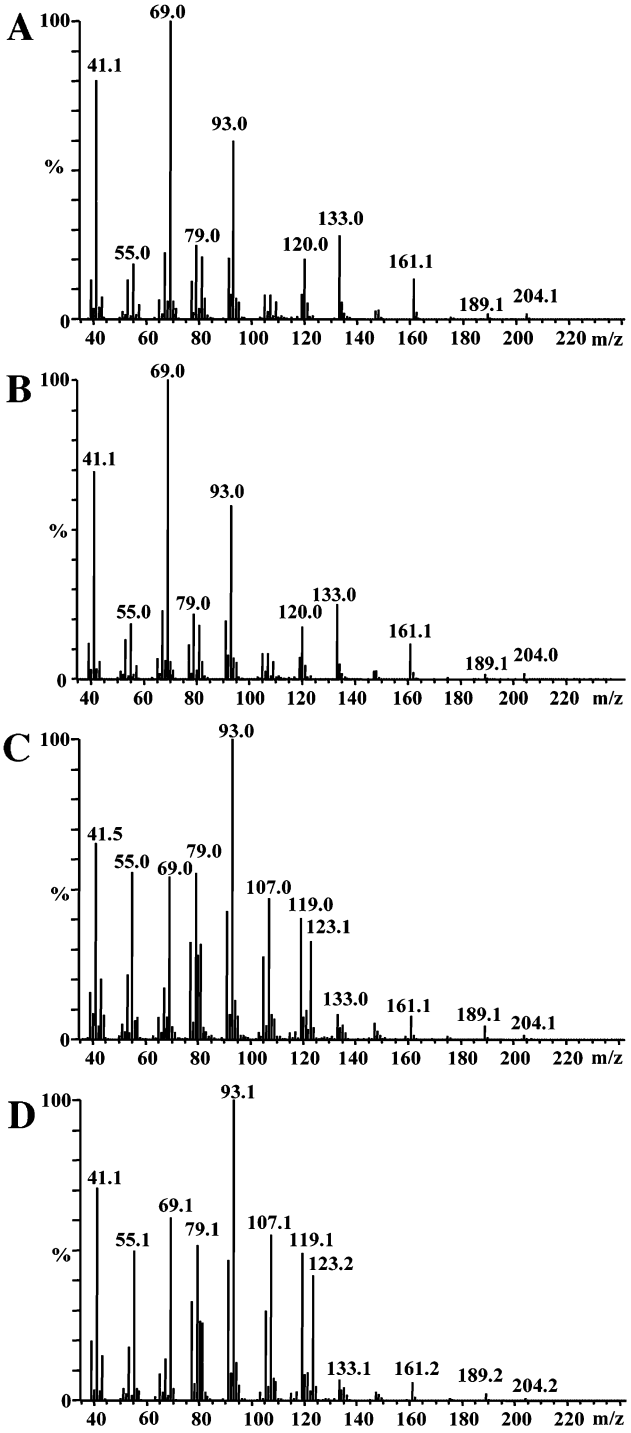
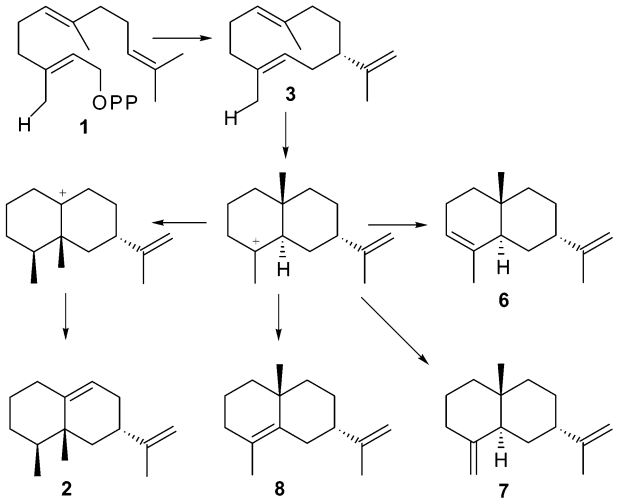


FIGURE 3: GC−MS analysis of the alicyclic sesquiterpenes produced by ASY92C on incubation with FPP: (A) mass spectrum of product **4** of ASY92C catalysis, (B) mass spectrum of the GC peak corresponding to authentic (*E*)-β-farnesene, (C) mass spectrum of product **5** of ASY92C catalysis, and (D) mass spectrum of the GC peak corresponding to authentic (*E*)-α-farnesene.

dimethyl-allyl pyrophosphate to geranyl pyrophosphate and of geranyl pyrophosphate to FPP, contains a long central cavity with two hydrophobic depressions which bind the substrates in extended conformations (*10*, *19*). Energy minimizations and molecular mechanics simulations had previously revealed a relatively solvent independent preference of an extended conformation for the isoprenoid chain of farnesol (*20*, *21*). One nanosecond molecular dynamics

Scheme 3



simulations for FPP using the AMBER force field revealed a similar preference of the isoprenoid tail for an extended conformation in chloroform and to a lesser extent in water (P. J. Shrimpton, C. J. Weston, and R. K. Allemann, unpublished observations). NMR studies of ^{13}C-labeled FPP confirmed the preference for an extended conformation in five organic solvents and in water (*21*).

The analysis of the hexane extractable material revealed that in addition to aristolochene and the main product (*E*)-β-farnesene, additional sesquiterpenes had been produced by ASY92C and ASY92A (Figure 2). As had been observed with ASY92F (*12*) and ASY92V (*15*), α-selinene (**6**) and β-selinene (**7**) as well as selina-4,11-diene (**8**) were generated (Table 2) by deprotonation of eudesmane cation (Scheme 3). These products were identified from their mass spectra and by comparison of the mass spectra to the mass spectra of authentic samples as previously described (*12*). While germacrene A is known to undergo acid-induced cyclizations to form selinenes in chloroform, no evidence for such a reaction was obtained in hexane, suggesting that **6**−**8** were genuine reaction products of ASY92C and ASY92A.

Unlike the case with AS and the mutants ASY92F and ASY92V, no valencene (**9**) appeared to be produced by ASY92C and ASY92A. Valencene is generated by deprotonation from C6 rather than from C8 in the final step of aristolochene production. However, ASY92A produced two additional compounds, **10** and **11**, which were characterized by retention times of 26.44 and 25.92 min in the gas chromatogram (Figure 2). **10** made up approximately 6.3% of the total amount of hexane extractable products (Table 2). It was only observed with ASY92A. Its mass spectrum closely matched that of β-bisabolene (**10**) (Figure 4). Co-injection with an authentic sample confirmed the identity of this hydrocarbon, in which the 2,3-double bond is in the *Z*-configuration (Scheme 4). The 2,3-double bond of FPP must therefore have undergone an isomerization reaction through the formation of an allylic cation and rotation around the 2,3-bond (Scheme 4).

ASY92C and ASY92A also produced 3.7 and 5.4% of compound **11**, respectively (Table 2). **11** was identified as the alicyclic sesquiterpene (*E*,*Z*)-α-farnesene from its mass spectrum and through co-injections with authentic material (Figure 4). **11** must have been formed by deprotonation from

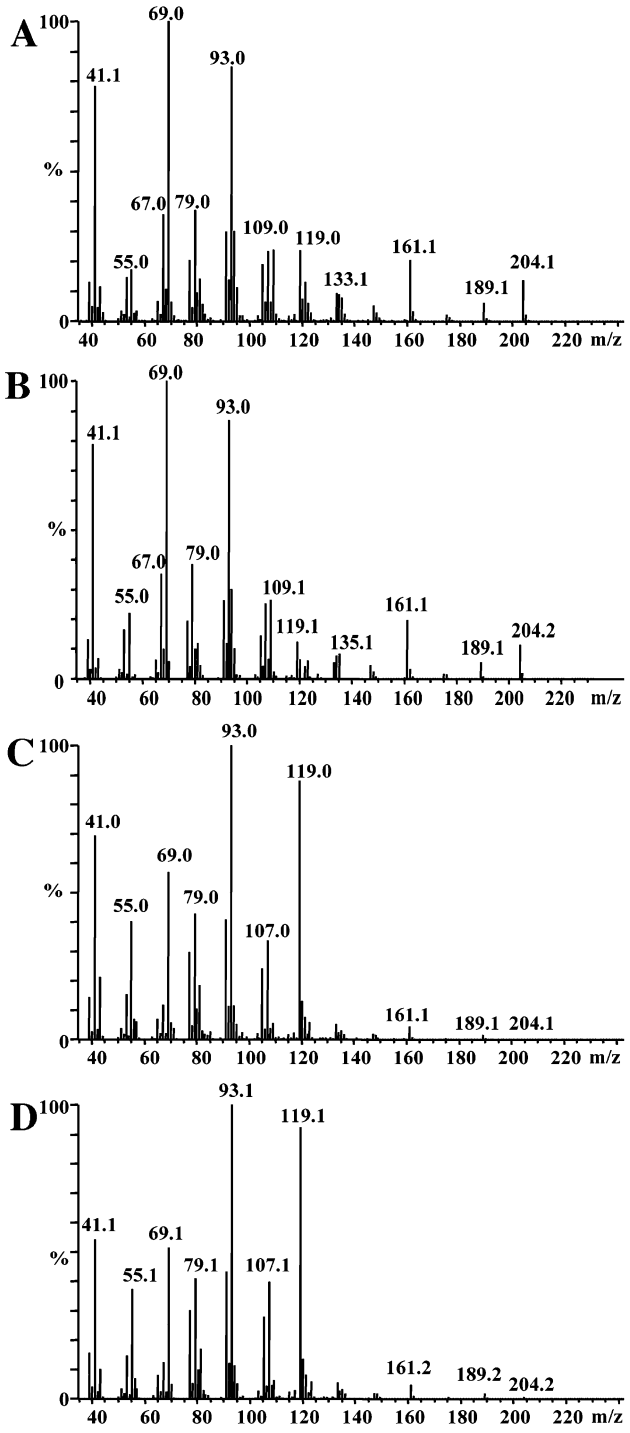
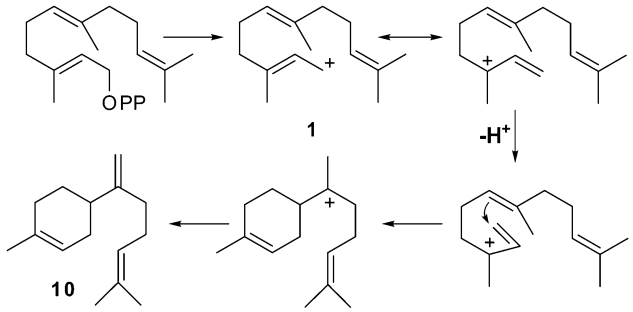


FIGURE 4: (A) Mass spectrum of product **10** in the GC−MS analysis of the hexane extractable material generated from FPP by ASY92A. (B) Mass spectrum of the GC peak corresponding to authentic β-bisabolene. (C) Mass spectrum of **11** in the gas chromatogram of the hexane extractable material generated from FPP by ASY92A. (D) Mass spectrum of authentic (*E*,*Z*)-α-farnesene.

C4 of FPP or farnesyl cation either in its cyclic form or in an extended conformation after rotation around the C3−C4 single bond. The observation that no (*E*,*Z*)-α-farnesene was produced by AS, ASY92F, and ASY92V, all of which produce predominantly cyclic products, suggested that **11** was formed from FPP in a quasi-linear conformation.

The production of **10** by ASY92A and of (*E*)-β-farnesene by all mutants of AS with substitutions of residue 92

Scheme 4



suggested strongly that the initial cyclization of FPP to germacrene A was not a concerted reaction. Ionization of **1** to generate pyrophosphate and farnesyl cation must precede ring closure and deprotonation of C12. It had previously been shown that AS from *Aspergillus terreus* catalyzed cyclization of **1** to germacrene A with inversion of configuration at C1 of **1** (*22*). Like AS from *P. roqueforti*, this enzyme exclusively catalyzed the production of the (+)-enantiomer of **2**. Farnesyl cation must therefore be bound very tightly in the active site of AS to prevent a possible rotation around the 2,3-bond in the allylic cation. It appears that in ASY92A and ASY92C the prevention of this rotation is partly lost, thereby allowing the formation of small amounts of **10**.

In summary, Tyr92 plays a dual role during the conversion of FPP to aristolochene by AS from *P. roqueforti*. Previous work had indicated that Tyr92 acted as the critical proton donor responsible for the activation of **3** to produce eudesmane cation (*12*), which subsequently undergoes rearrangements and deprotonation to produce **2**. However, Tyr92 appeared also to play a pivotal role during cyclization of **1** to germacrene A. Its presence was required to ensure the correct regiochemical outcome of the reaction. Tyr92 was central for the binding of FPP in a conformation favoring the production of germacrene A through cyclization between C1 and C10. A reduction in the size of the side chain of residue 92 appeared to allow the mutant enzyme to bind FPP in the sterically preferred extended conformation, thereby preventing the efficient cyclization of FPP to germacrene A.

All sesquiterpene cyclases characterized so far share a common three-dimensional structure (*6*−*9*). The specific arrangements of the amino acid side chains in the active sites of individual enzymes distinguish one terpene cyclase activity from another. The enzymes act as high-fidelity templates for the binding of the substrate in the desired active conformations. They chaperone their substrate and intermediates along the reaction coordinate on an energy surface with many energetically similar minima and saddle points. The structural similarities of terpene cyclases and of their reaction products suggest that small changes in the active site geometries must be sufficient to alter the selectivity of the reaction, thereby ensuring easy product maximization from a limited substrate pool. The generation of multiple products even by individual terpene cyclases is well-documented (*23*−*27*). (*E*)-β-Farnesene synthase, for instance, has been reported to produce 5% of δ-cadenine and 8% of (*Z*)-β-farnesene in addition to (*E*)-β-farnesene (*14*). The most extreme cases reported so far are δ-selinene and γ-humulene synthases, for which 34 and 52 products were identified, respectively (*28*).

The larger active site cavities, which resulted from replacement of Tyr92 of AS with Phe, Val, Cys, or Ala, led to an increased number of aberrant reaction products most likely as a consequence of increased conformational freedom of both substrates and intermediates. A similar observation had been made for a mutant of trichodiene synthase, where replacement of Asp100 with Glu in the aspartate rich motif led to the production of five aberrant reaction products in addition to trichodiene (*17*). Interestingly, however, one of the AS mutants reported here, ASY92A, displayed a very strong preference for the production of (*E*)-*β*-farnesene. Our results therefore provide evidence that simple amino acid substitutions form the basis of the general mechanism for the generation of chemical diversity employed by terpene cyclases. This mechanism may at least in part rely on differential folding of the substrates within the enzymes' active sites.

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