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Germacrene A Is a Product of the Aristolochene Synthase-Mediated Conversion of Farnesylpyrophosphate to Aristolochene

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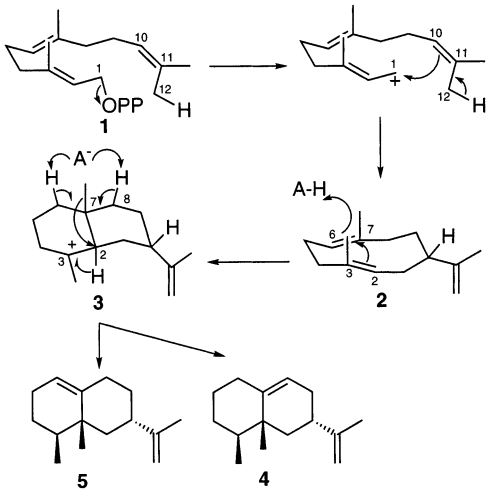
Abstract: The biosynthesis of several sesquiterpenes has been proposed to proceed via germacrene A. However, to date, the production of germacrene A has not been proven directly for any of the sesquiterpene synthases for which it was postulated as an intermediate. We demonstrate here for the first time that significant amounts of germacrene A (7.5% of the total amount of products) are indeed released from wild-type aristolochene synthase (AS) from *Penicillium roqueforti*. Germacrene A was identified through direct GC-MS comparison to an authentic sample and through production of β-elemene in a thermal Cope rearrangement. AS also produced a small amount of valencene through deprotonation of C6 rather than C8 in the final step of the reaction. On the basis of the X-ray structure of AS, Tyr 92 was postulated to be the active-site acid responsible for protonation of germacrene A (Caruthers, J. M.; Kang, I.; Rynkiewicz, M. J.; Cane, D. E.; Christianson, D. W. *J. Biol. Chem.* **2000**, *275*, 25533−25539). The CD spectra of a mutant protein, ASY92F, in which Tyr 92 was replaced by Phe, and of AS were very similar. ASY92F was approximately 0.1% as active as nonmutated recombinant AS. The steady-state kinetic parameters were measured as 0.138 min^{-1} and 0.189 mM for k_{cat} and K_M, respectively. Similar to a mutant protein of 5-epi-aristolochene (Rising, K. A.; Starks, C. M.; Noel, J. P.; Chappell, J. *J. Am. Chem. Soc.* **2000**, *122*, 1861−1866), the mutant released significant amounts of germacrene A (~29%). ASY92F also produced various amounts of a further five hydrocarbons of molecular weight 204, valencene, β-(*E*)-farnesene, α- and β-selinene, and selina-4,11-diene.

Introduction

Aristolochene synthase (AS) from *Penicillium roqueforti* is a member of the family of sesquiterpene synthases, which catalyze the cyclization of the universal acyclic precursor farnesyl pyrophosphate (FPP, **1**) (Scheme 1) in marine and terrestrial plants, fungi, bacteria, and insects, to generate more than 300 distinct sesquiterpene products, many of which have important antibiotic, antifungal, or neurotoxic activities.[1,2] Ionization of the substrate through loss of pyrophosphate triggers an exquisitely regio- and stereospecific sequence of intramolecular addition reactions and rearrangements. The enzymes control solvent access to prevent premature quenching of the extremely reactive cationic reaction intermediates by water. The starting conformation of **1** in the enzyme's active site is known to be a critical determinant of product diversity.[2] Sesquiterpene synthases are an extreme example of product maximization from a minimal substrate pool. Many mechanistic studies of FPP cyclizations have been reported.[3−6] However, little is known about the molecular details of catalysis and stereochemical control by sesquiterpene synthases, mainly because their genes have only recently been isolated.[4,7−11]

Scheme 1



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AS has previously been cloned and overproduced in *E. coli*.[7,11] The momomeric enzyme of 39 kD catalyses the bivalent metal-dependent cyclization of FPP to (+)-aristolochene (**4**), the precursor of several fungal toxins including the potentially lethal PR toxin.[7] Studies with labeled FPP,[12,13] the mechanism-based inhibitor 12-methylidenefarnesyl pyrophosphate,[14] and with (7*R*)-6,7-dihydrofarnesyl pyrophosphate[15] led to the proposal that the cyclization of FPP to aristolochene proceeds through at least two discrete intermediates, *S*-germacrene A (**2**) and eudesmane cation (**3**) (Scheme 1). However, so far germacrene A could not be detected directly in AS-catalyzed conversions of FPP to aristolochene.[15]

In the proposed reaction sequence, the active site of aristolochene synthase orientates the C10−C11 bond of FPP for attack at C-1 after (or concurrent with) metal triggered diphosphate departure (Scheme 1). Deprotonation of H-12 results in the formation of the *cis*-fused Decalin *S*-germacrene A (**2**). Protonation of germacrene A at C-6 and cyclization through electron flow from the double bond at C2−C3 yield the bicyclic eudesmane cation (**3**) with an internal bond between C2 and C7. A hydride shift from C2 followed by a methyl shift from C7 to C2 and deprotonation at C8 results in the formation of aristolochene.[16]

Proof that germacrene A was an intermediate in the reaction has been precluded by the inability to demonstrate its release from any of the sesquiterpene synthases for which it was postulated as an intermediate.[17] Investigations into the mechanism of 5-epi-aristolochene synthase (EAS) from *Nicotiana tabacum*, which shows only 16% sequence identity with AS, have provided evidence for a mechanism similar to that proposed for AS.[17,18] The two synthases provide different templates for binding FPP and the reaction intermediates to produce the correct stereoisomer. Analysis of the X-ray structure of EAS and of the catalytic properties of a mutant EAS showed that Tyr 520 is the active-site acid which protonates germacrene A.[19] Mutation of Tyr 520 to phenylalanine led to the accumulation of germacrene A as the sole reaction product.[17]

On the basis of the X-ray structure of AS which has been solved at 2.5 Å in the absence of either substrate or a substrate analogue,[16] and on molecular modeling studies, it was suggested that the hydroxyl group of Tyr 92, which does not align with Tyr 520 of EAS, could act as the proton donor to C-6 of germacrene A in AS.[16] Here we report results from studies addressing the intermediacy of germacrene A in AS catalysis and the specific role of Tyr 92 during the conversion of FPP to aristolochene.

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Materials and Methods

Materials. Oligonucleotide primers were purchased from Alta Bioscience, University of Birmingham. The pZW04 plasmid encoding wild-type AS from *P. roqueforti* was kindly donated by Prof. David Cane, Brown University. Germacrene A from soldier cephalic secretion of a subterranean termite species and α-and *β*-selinene from *Albies magnifica* oleoresin were gifts from Larry Cool (Forest Products Laboratory, University of California, Berkeley). Valencene was a gift from De Monchy Aromatics Ltd. *β*-(*E*)-Farnesene was a gift from John A. Pickett, FRS, and Lynda Ireland (BBSRC − Institute for Arable Crops, Rothamsted). Aristolochene was enzymatically generated from FPP using AS. The Quick-change mutagenesis kit was obtained from Stratagene, and miniprep kits and Big Dye sequencing reagents were obtained form Qiagen and Applied Biosystems, respectively. [1-^{3}H]-FPP (21.5 mCi/mmol) was purchased from Sigma and was diluted with cold FPP to a working specific activity of 50 μCi/μmol. Q-Sepharose was obtained from Amersham Pharmacia Biotech. Ultracell Amicon YM3 membranes were purchased from Millipore, and BCA protein acid reagent was obtained from the Pierce Chemical Co. 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 (Stratagene) was used to introduce the Y92F mutation (TAC → TTC) into the AS encoding plasmid, pZW04, according to the manufacturer's instructions. The mutagenic primers were as follows: 5′-CAGAGGTTACTTGTCTTTT**T**CTTC-CCTCTTGCACTGG-3′ and 5′-CCAGTGCAAGAGGGAAG**A**AAAG ACAAGTAACCTCTG-3′ (altered bases shown in 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 in *E. coli* and Purification of Wild-type and Mutant Aristolochene Synthases. Wild-type AS and ASY92F constructs were transformed into and expressed 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 0.8−1 and were then induced with 0.5 mM isopropyl-*β*-D-1-thiogalactopyranoside and incubated for a further 2 h. Cells were harvested by centrifugation at 5000*g* for 10 min and resuspended in 20 mM Tris, pH 8, 5 mM EDTA, 5 mM *β*-mercaptoethanol. AS and ASY92F were extracted from the insoluble inclusion bodies using the base extraction procedure previously described[16] and purified on a Q-Sepharose Fast Flow column (2.5 × 20 cm), equilibrated with 20 mM Tris, pH 8, 5 mM EDTA, 5 mM 2-mercaptoethanol. Enzyme was eluted using a linear 0.1−0.6 M NaCl gradient, and the fractions containing protein were identified by absorption at 280 nm and SDS−polyacrylamide gel electrophoresis. Fractions were assayed for enzyme activity, and pooled active enzyme was dialyzed against 10 mM Tris, pH 7, 5 mM *β*-mercaptoethanol (3 × 3 L). AS and ASY92F were concentrated using an Amicon 8050 with an Ultracell amicon YM3 membrane and were judged to be >98% pure by SDS−PAGE. Protein concentrations were determined by the BCA (bicinchoninic acid) method calibrated with bovine serum albumin per the manufacturer's instructions (Pierce Chemical Co.).

Assay for Enzymatic Activity. AS and ASY92F were assayed in a total volume of 250 μL containing 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 15% glycerol (v/v) and 0.25−15 μM or 2.5−100 μM of [1-^{3}H]-FPP for AS or ASY92F assays, respectively. Reaction mixtures were prewarmed to 30 °C and initiated by addition of 50 μL of enzyme, to give a final concentration of 0.4 μM AS or 4 μM ASY92F. Reactions were terminated by the addition of 100 μL of 100 mM EDTA, pH 7.25, and overlaid with 0.5 mL of *n*-hexane. The samples were vortex mixed for 15 s, and the hexane layer was then vortexed with 0.5 g of silica in 1 mL of *n*-hexane. The samples were extracted with an additional 2 × 1 mL of hexane, and the combined

Table 1. Kinetic Parameters for AS and ASY92F

	kinetic parameters		
enzyme E	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1}\,s^{-1}$)
AS	2.3 ± 0.5	0.03 ± 0.01	$13\,858 \pm 2989$
ASY92F	188.7 ± 52.8	0.0023 ± 0.0005	12.36 ± 0.8

hexane extracts were added to a scintillation vial containing 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 of [1-^{3}H]-FPP. Background levels of radioactivity were observed in control samples without the enzyme.

CD Spectroscopy of AS and ASY92F. Circular dichroism experiments were run for AS and ASY92F at protein concentrations of 6 μM in 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.2 mM dithiothreitol in the wavelength interval 190−300 nm using a Jasco J-810 spectropolarimeter. The CD spectra were measured at 20 °C using 1 mm quartz cuvettes.

Characterization of Products from Aristolochene Synthase Mutant by GC-MS. Purified ASY92F (50 μM) was incubated with 230 μM FPP in 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol, and 15% glycerol in a final volume of 500 μL at 30 °C for 15 min, 1 h, and 12 h. Reactions were terminated by the addition of 200 μL of 100 mM EDTA, pH 7.25, and the products were extracted by vortexing against hexane (3 × 3 mL) and purified using a silica column (containing 1.5 g of silica). Pooled hexane extracts were concentrated by rotary evaporation on ice. Identical incubations were performed with wild-type AS.

The hexane extractable products were analyzed by GC-MS using a Carlo Erba CE8000 gas chromatograph (equipped with a BPX5 30 m column) and a Prospec Mass spectrometer. High-resolution GC-MS data were obtained on a Micromass GCT with an integrated GC (HP6890) (equipped with a 30 m DB5 column). Splitless injections (5 μL) were generally performed at the relatively low injection port temperature of 100 °C to prevent the Cope rearrangement of germacrene A to β-elemene, which occurs at higher injection port temperatures. The column temperature was maintained at 60 °C for 3 min and was then increased to 150 °C at 4 °C min^{-1}.

Results and Discussion

Because several lines of indirect evidence (vide supra) had suggested that germacrene A was formed from FPP (Scheme 1), but had never been observed during AS catalysis, we have investigated the kinetics and the product distribution of AS with the use of modern mass spectrometer detectors with increased sensitivity.

AS was produced to high levels in *E. coli* and purified to apparent homogeneity. The steady-state kinetic parameters of purified recombinant AS were measured by incubation with [1-^{3}H]-FPP and monitoring the formation of tritiated, hexane extractable products.[11] The K_M and k_{cat} values for AS were determined as 2.3 ± 0.5 μM and 0.03 ± 0.01 s^{-1}, respectively (Table 1), which was in good agreement with those reported for AS isolated from *P. roqueforti* ($K_M = 0.55$ μM and $k_{cat} = 0.04$ s^{-1})[20] and suggested that the recombinant protein was properly folded. The CD spectrum of AS showed that its secondary structure was mainly α-helical as was expected from the X-ray structure (Figure 1).

The products obtained from incubation of **1** with AS were analyzed by GC-MS. Gas chromatography of aristolochene

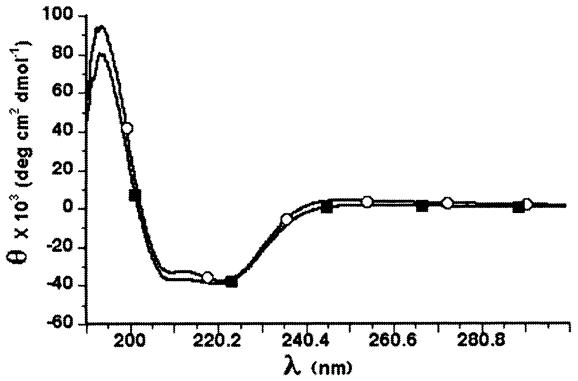

Figure 1. CD spectra of 6 μM AS (○) and 6 μM ASY92F (■) in 10 mM Tris, pH 7.5, 5 mM $MgCl_2$, 0.2 mM dithiothreitol at 20 °C.

(produced by incubation of wild-type AS with FPP) and germacrene A standards yielded distinct peaks with retention times of 20.68 and 21.37 min, respectively. The corresponding mass spectra exhibited molecular ions at 204 mass units as well as characteristic fragmentation patterns (Figure 2).[17]

The GC analysis of the products formed by AS revealed the formation of two further hydrocarbons in addition to aristolochene, which according to the total ion chromatogram represented approximately 92% of the hexane extractable material, and traces of selina-4,11-diene (**8**) and α-selinene (**9**) (vide infra) (Table 2). One of them made up approximately 7.5% of the products and showed the same retention time and fragmentation pattern as germacrene A (**4**) (Figure 2). Co-injection of a germacrene A standard and the products produced by AS led to an increase in the intensity of the peak corresponding to **4**.

Germacrene A is known to undergo a thermal Cope rearrangement to β-elemene.[21] When the hexane extractable products formed by AS were analyzed by GC-MS with an injector temperature of 200 °C, the peak corresponding to aristolochene remained unaffected, while the retention time of the major byproduct was significantly shortened (Figure 3). The new peak was identified through its mass spectrum and comparison with the known spectrum of β-elemene.[22]

The amount of germacene A formed by AS relative to aristolochene was not dependent on the extent of turnover, indicating that a small amount of germacrene A was indeed released from the active site of the wild-type enzyme. Once released, the enzyme appeared not to rebind the intermediate in good agreement with the observation that germacrene A is not a substrate for AS. Alternatively, germacrene A could be a side product of the AS-catalyzed conversion of FPP rather than an intermediate. While our results do not allow us to distinguish between these two possible mechanisms, they nevertheless represent the first demonstration that germacrene A is indeed produced at significant levels by AS and released from the enzyme for which it was postulated as an intermediate.

Interestingly, an additional hydrocarbon of molecular mass 204 was observed in the gas chromatogram (retention time 20.92 min) (Figure 2). It made up only 0.4% of the total amount of hexane extractable material (Table 2). Its mass spectrum closely matched that of valencene (**5**) in the Wiley and NIST98 libraries

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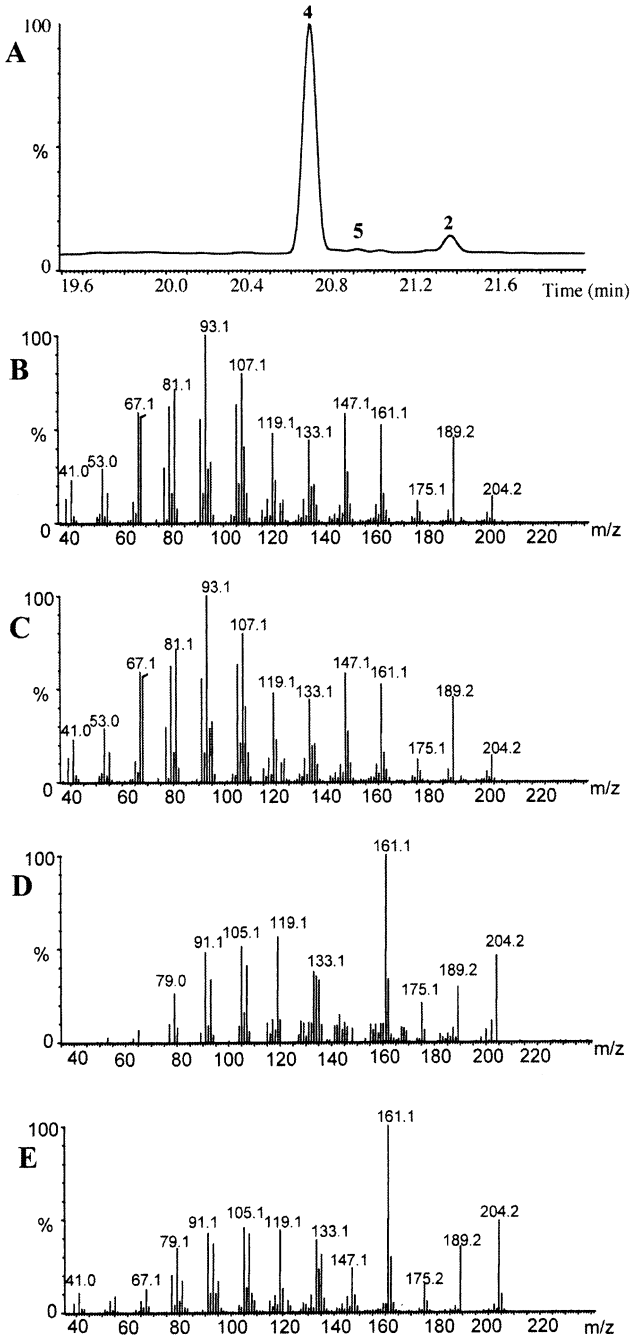

Figure 2. GC-MS analysis of hexane extractable products of FPP utilization by AS. (a) Total ion chromatogram of the hydrocarbons produced by AS from FPP; (b) mass spectrum of product **2** of AS catalysis; (c) mass spectrum of GC fraction from authentic germacrene A (**2**); (d) mass spectrum of product **5** of AS catalysis; (e) mass spectrum of GC fraction from authentic valencene.

of mass spectra (Figure 2). Co-injection with a reference sample of valencene confirmed the identity of this hydrocarbon that is most likely produced by the abstraction of a proton from C6 rather than from C8 as is observed in the production of aristolochene (Scheme 1). Inspection of the crystal structure of AS indicated that the hydroxyl of Tyr 92 was approximately equidistant to C6 and C8 of eudesmane cation (**3**), suggesting that the phenolate anion could act as the base abstracting either of these protons. The yield of valencene produced was, however, very low (Table 2), and a residue other than Tyr 92 might have acted as the base (vide infra).

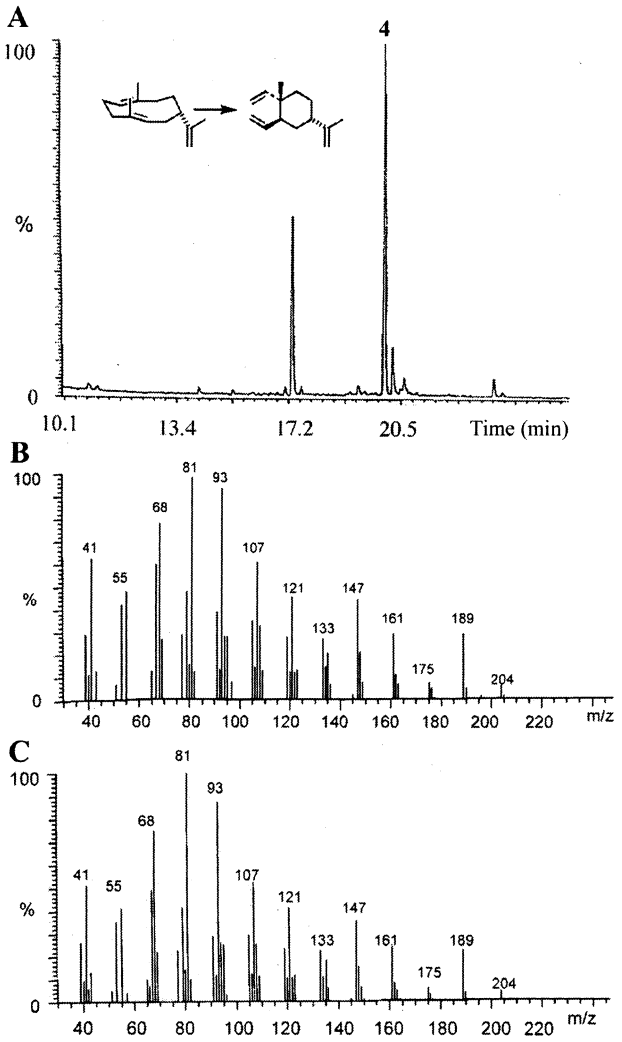

Figure 3. Analysis of the temperature-induced reaction of the hexane extractable products of FPP utilization by AS. (a) GC analysis of the hydrocarbons produced by AS from FPP at an injection port temperature of 200 °C. Note that the material corresponding to **4** is not affected by the increased temperature, while the retention time of the byproduct is significantly shortened; (b) mass spectrum of the material eluting at 17.3 min in (a); (c) mass spectrum of GC fraction from authentic β-elemene.

X-ray crystallography[19] and mutational studies[17] have suggested that a tyrosine residue of EAS served as the active-site acid required for the protonation of germacrene A. In the final step of 5-epi-aristolochene production, the conjugate base abstracts a proton from C8. While Tyr 92 of AS is not in a homologous position to Tyr 520 of EAS, inspection of the crystal structure of AS[16] suggested that it could nevertheless fulfill a similar function during AS catalysis. Similarly, a phenolate anion could abstract a proton from C8 to produce aristolochene. Occasionally, a proton appeared to be abstracted from C6 to produce **5** (Scheme 1). To investigate the role of Tyr 92 within the active site of AS, we have replaced Tyr 92 by phenylalanine to create the mutant ASY92F.

ASY92F was produced in *E. coli* and purified to apparent homogeneity. The steady-state kinetic parameters were determined as described for AS. Relative to the wild-type enzyme, ASY92F displayed an 80-fold increased value for K_M, while the turnover number k_{cat} was reduced to 0.0023 s^{-1}, resulting in an overall decrease of the catalytic efficiency of more than 3 orders of magnitude (Table 1). While the replacement of Tyr

Table 2. Relative Amounts of Hexane Extractable Hydrocarbon Products from Incubations with FPP Determined by GC Analysis

	product						
	2 germacrene A	**4** aristolochene	**5** valencene	**6** β-farnesene	**7** β-selinene	**8** selina-4,11-diene	**9** α-selinene
retention time (min)	21.37	20.68	20.92	19.78	20.81	20.37	21.03
WTAS	7.5	91.5	0.4	0	0	0	0
Y92F	28.6	56.2	5.9	1.8	1.8	2.1	3.6

92 with Phe reduced the catalytic power of the enzyme significantly, it is important to keep in mind that the catalytic efficiencies of wild-type AS and other terpene cyclases are intrinsically rather low. These enzymes have evolved to produce cyclic hydrocarbons with often exquisite specificity rather than with high speed. The significant decrease in the catalytic power of the mutant was not a consequence of a grossly altered structure of the enzyme. The CD spectra of AS and ASY92F were very similar, indicating that they had similar secondary structure (Figure 1). Given the similar hydrophobicities of germacrene A and aristolochene, the decreased k_{cat} observed for ASY92F was most likely due to a decrease of the rate of the chemical steps rather than of the rate of product release which has been shown to be rate limiting in several WT-sesquiterpene cyclases.[23,24]

The hexane extractable materials produced from **1** by ASY92F were analyzed by GC-MS (Figure 4). While the mutant enzyme produced a significantly increased amount of germacrene A (28.6%) when compared to WT-AS (Table 2), it still produced aristolochene. However, substantial amounts of further five hydrocarbon products, valencene (**5**), β-(*E*)-farnesene (**6**), β-selinene (**7**), selina-4,11-diene (**8**), and α-selinene (**9**) (Scheme 2), were also observed in the GC trace (Figure 4). The major one of these (5.9%) was valencene (**5**) (Table 2), which had already been identified as a minor product of the reaction of **1** with AS. Three further bicyclic hydrocarbons were generated through proton loss from C2, C4, and the methyl group on C2 of eudesmane cation (**3**) to produce selina-4,11-diene (**8**), α-selinene (**9**), and β-selinene (**7**) (Scheme 2). The relative amounts for **8**, **9**, and **7** were 2.1, 3.6, and 1.8% of the total hexane extractable products (Table 2). Germacrene A is known to undergo a slow and spontaneous acid-catalyzed rearrangement to form selinenes in chloroform. However, no evidence for such a rearrangement was obtained in hexane, which was the solvent in our studies. Hydrocarbons **7**, **8**, and **9** appeared therefore to be true enzymatic products of ASY92F.

Compounds **7** and **9** were identified from their MS spectra and comparison with reference spectra of the Wiley and the NIST 98 libraries of mass spectra. Co-injections with reference samples of α- and β-selinene confirmed their identity. The mass spectrum of selina-4,11-diene was identical to the reference spectra in the NIST 98 mass spectral library.

The final hydrocarbon which was generated in 1.8% yield by ASY92F could be identified as β-(*E*)-farnesene (**6**) (Scheme 2) by co-injection with authentic material and comparison of the mass spectra. The production of **6** by this mutant suggested strongly that the initial cyclization of FPP to germacrene A was not concerted as had been suggested previously.[16] Instead, it appeared to follow a stepwise mechanism in which the departure of the pyrophosphate group preceded deprotonation of the *cis*-methyl group (C12) (Scheme 1).

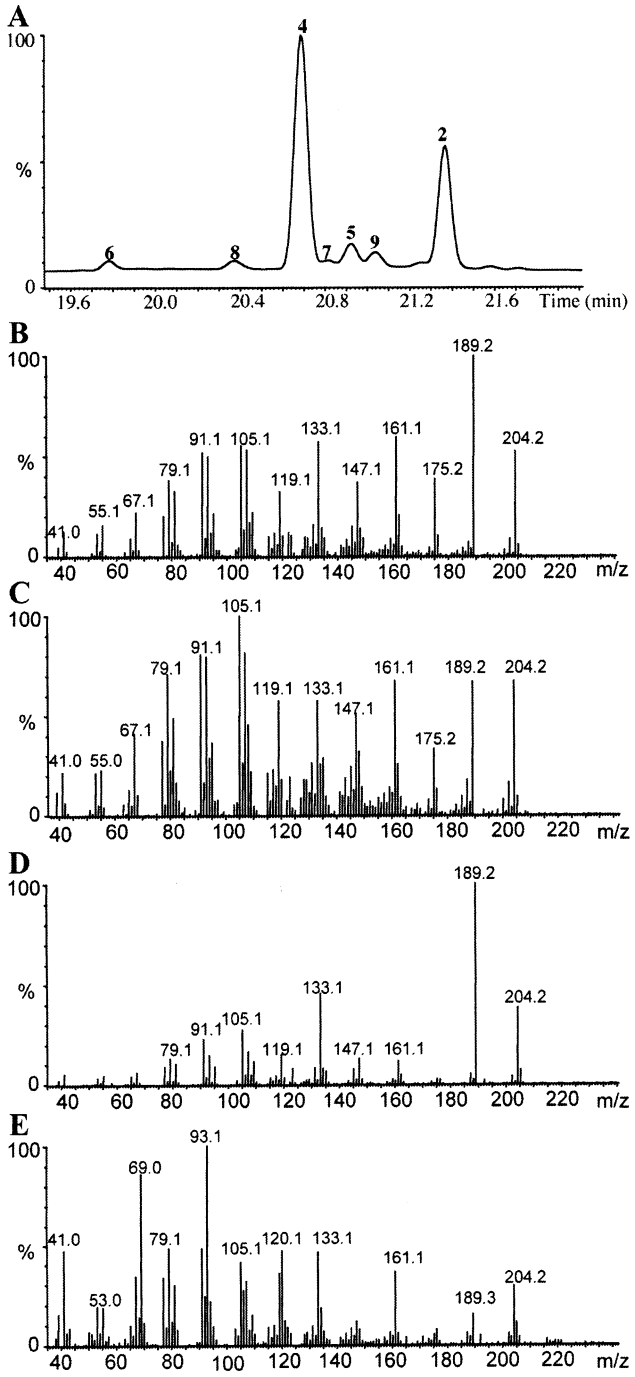

Figure 4. GC-MS analysis of hexane extractable products formed from FPP by ASY92F. (a) Gas chromatogram of the products of ASY92F catalysis; mass spectra of **9** (b), **7** (c), **8** (d), and **6** (e) produced from FPP by ASY92F.

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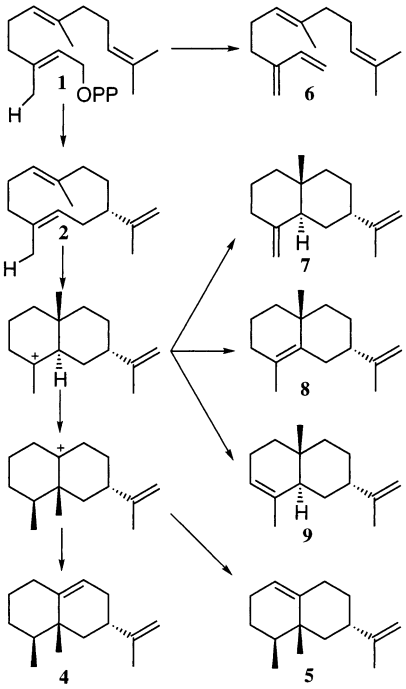
Scheme 2



The ability of terpene synthases to produce multiple products has been well documented and may be a consequence of the reaction mechanism employed by these enzymes. The most extreme cases reported so far are δ-selinene and γ-humulene synthase for which 34 and 52 discrete products were identified.[25] The recent publication of the X-ray structures of EAS from *Nicotiana tabacum*,[19] AS from *Penicillium roqueforti*,[16] pentalenene synthase from *Streptomyces* UC5319,[26] and trichodiene synthase from *Fusarium sporotrichioides*[27] revealed that the three-dimensional structures of the active-site domains of these and most likely many other terpene cyclases are very similar. Only relatively few active-site residues appear to account for the formation of different products. It is therefore not surprising that a relatively small change in the active site of AS can lead to a significant increase in product diversity. Terpene cyclases, which have evolved to form tightly fitting templates for their substrates, subtly channel conformation and stereochemistry during the cyclization reactions. Alterations of active-site residues, introduced by either nature or man, therefore often result in the formation of desired or aberrant cyclization products, thereby shedding light on the molecular mechanisms that control these exquisitely specific reactions.

The identification of a substantial amount of germacrene A during AS catalysis and the formation of large amounts of **2** by ASY92F confirmed that **2** was indeed formed during AS catalysis. The possibility that **2** was a side product in AS catalysis could not be ruled out completely, in which case changing Tyr 92 to Phe would have affected the relative rates of formation of germacrene A and aristolochene. However, our results together with previous studies using mechanism-based inhibitors and labeled FPP as well as the high structural similarity of AS with EAS are consistent with the proposal that germacrene A is indeed an intermediate during the AS-catalyzed formation of aristolochene from FPP.

The results presented above indicated that Tyr 92 might act as the critical proton donor responsible for the activation of **2** to eudesmane cation (**3**), its absence severely impairing the enzyme's catalytic ability. The production of a significant amount of **2** by ASY92F suggested that if Tyr 92 was indeed the active-site acid during AS catalysis, then some other group must have served as the surrogate acid in the mutant. Inspection of the X-ray structure of AS revealed that the side chain of Lys 206,[16] which is involved in a hydrogen-bonding network with Asp 203 and Arg 200, might act as the proton donor. It is ideally placed to serve as the active-site acid in ASY92F to protonate the 6,7-double bond of germacrene A. Subsequently, because the ϵ-amino group of Lys 206 is approximately equidistant to carbons 2, 4, 6, 8, and the carbon of the methyl group on C3 of eudesmane cation (**3**), it could also abstract a proton from **3** to produce **7**, **8**, and **9** or from C6 and C8 to produce valencene (**5**) and aristolochene (**4**), respectively (Scheme 2).

In the wild-type enzyme, Lys 206 is also hydrogen bonded to Tyr 92. This hydrogen-bonding network provides a proton shuttle from Arg 200, which lies at the top of the active-site cleft and is exposed to the solvent, to the double bond at C6−C7 of germacrene A, which is deeply buried in the active site of the enzyme. This hydrogen-bonding network might enhance the acidity of Tyr 92 sufficiently to allow protonation of the C6−C7 double bond in **2**.

Despite minimal sequence identity, *P. roqueforti* AS and *N. tabacum* EAS appear to share some common active-site features. While the amino acid residues that stabilize Tyr 520 in EAS are different from those involved in the hydrogen-bonding network observed in AS, a tyrosine residue appears to act as the general acid responsible for protonating C6 of germacrene A in both enzymes. It may be possible that such a tyrosine is the common proton donor in all sesquiterpene synthases whose mechanism proceeds via germacrene A.

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