Demonstration of Germacrene A as an Intermediate in 5-Epi-aristolochene Synthase Catalysis

Kathleen A. Rising,[†] Courtney M. Starks,[‡] Joseph P. Noel,*[,‡] and Joseph Chappell*[,†]

Contribution from the Plant Physiology, Biochemistry, and Molecular Biology Program, University of Kentucky, Lexington, Kentucky 40546, Structural Biology Laboratory, The Salk Institute for Biological Studies, 10010 North Torrey Pines Road, La Jolla, California 92037, and Department of Chemistry and Biochemistry, University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093

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Abstract: Sesquiterpene synthases are a family of enzymes that catalyze farnesyl pyrophosphate (FPP) cyclization via alternative pathways to produce a variety of cyclic sesquiterpene products. Catalysis by several of these enzymes, including tobacco 5-epi-aristolochene synthase (TEAS), has been proposed to include the formation of germacrene A as a stable intermediate. Neither germacrene A nor any other intermediate is released from sesquiterpene synthase active sites during normal catalysis. Evidence to support the intermediacy of germacrene A has been derived from investigations of aristolochene synthases from *Aspergillus terreus* and *Penicillium roquefortii* (Cane, D. E. *Chem. Rev.* **1990**, *90*, 1089−1103 and references therein. Cane, D. E.; Bryant, C. *J. Am. Chem. Soc.* **1994**, *116*, 12063−12064. Cane, D. E.; Tsantrizos, Y. S. *J. Am. Chem. Soc.* **1996**, *118*, 10037−10040). However, until the present investigations of TEAS, formation of this postulated intermediate has never been directly demonstrated. TEAS catalyzes the cyclization of FPP to 5-epi-aristolochene, a precursor of a tobacco phytoalexin, capsidiol. Based upon the three-dimensional structure of TEAS, a detailed mechanism has been proposed for TEAS catalysis that includes the prediction that proton donation by Y520 is responsible for the activation of germacrene A to a eudesmane cation (Starks, C. M.; Back, K.; Chappell, J.; Noel, J. P. *Science* **1997**, *277*, 1815−1820). In the present investigation, a Y520F point mutation is introduced into TEAS (TEAS-Y520F) by site-directed mutagenesis. In the presence of ^{3}H−FPP, TEAS-Y520F produces hexanes−extractable ^{3}H with a catalytic efficiency approximately 3% that of nonmutated, recombinant TEAS. The hexanes−extractable ^{3}H is identified as germacrene A, *m*/*z* 204, through direct GC-MS comparison to an authentic sample. This observation confirms the intermediacy of germacrene A in TEAS catalysis, supports the postulated production of germacrene A by a variety of other sesquiterpene synthases, and also confirms the proposed role of Y520 in TEAS catalysis.

Introduction

Nicotiana tabacum (tobacco) 5-epi-aristolochene synthase (TEAS) is a member of a family of enzymes referred to as the sesquiterpene synthases (or cyclases). These enzymes convert the acyclic isoprenoid substrate, farnesyl pyrophosphate (FPP), into a variety of cyclic compounds called sesquiterpenes (C_{15}). Two related families of enzymes are the monoterpene (C_{10}) and diterpene (C_{20}) synthases, which cyclize geranyl pyrophosphate and geranylgeranyl pyrophosphate, respectively. Terpene cyclases are widespread in nature and, at present, more than 300 different carbon skeletons of the sesquiterpene type alone have been identified in sources including plants, fungi, bacteria, and insects.[1,2] In plants, sesquiterpenes have an important role in mediating plant−environment interactions. Among Solanaceous plants, including tobacco, sesquiterpene synthases catalyze chemical conversions required for the synthesis of antimicrobial compounds.[3] TEAS, for example, catalyzes the first committed step in the formation of capsidiol, an anti-fungal phytoalexin derived through hydroxylations of the sesquiterpene product of TEAS catalysis, 5-epi-aristolochene.[4,5] Overall, cyclic terpenoids constitute an important class of potential antibiotic, antitumor, and antifungal agents.[6] From this perspective, a detailed understanding of terpene cyclase catalysis may allow for the enzymatic production of novel, potentially useful, cyclic terpenoids.

TEAS-catalyzed cyclization of FPP is thought to proceed according to the general mechanism used to describe catalysis by all sesquiterpene synthases.[2] In this mechanism, ionization

* Correspondence may be addressed to either J. Chappell at the University of Kentucky (chappell@pop.uky.edu) or J. Noel at the Salk Institute (noel@sbl.salk.edu).

[†] University of Kentucky.

[‡] The Salk Institute for Biological Studies and University of California.

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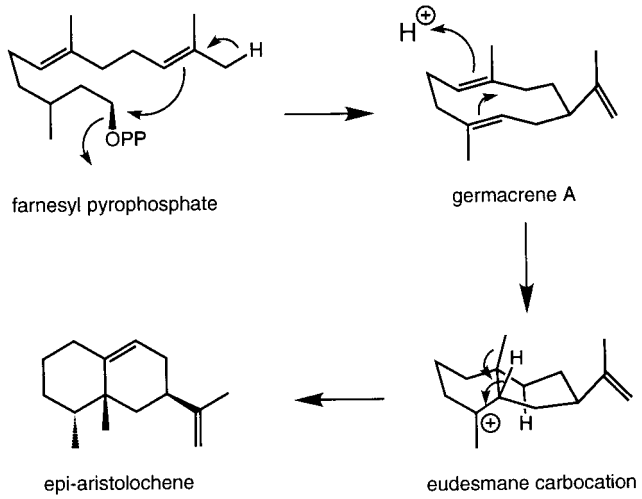
Scheme 1. Proposed Catalytic Mechanism for the Cyclization of Farnesyl Pyrophosphate to 5-Epi-aristolochene by Tobacco 5-Epi-aristolochene Synthase, TEAS [Adapted from Refs 2 and 8][a]



[a] Ionization of the pyrophosphate moiety is followed by electron flow from the C10−C11 double bond to the newly created carbocation at C1 and subsequent proton abstraction at C12, resulting in the formation of germacrene A, a cis-fused decalin. Dr. David Cane (Brown University) recently brought to our attention that our earlier depiction[22] of this partial reaction incorrectly showed the trans-fused decalin with the isopropenyl group below the structural plane of this proposed intermediate. This correction of the stereochemistry is important for accurate portrayal of the catalytic process and predicts the involvement of a general base other than D525 in the abstraction of a proton from C12 as originally proposed. Proton addition to C6 of the germacrene A intermediate, proposed to be mediated by the hydroxyl group of Y520 in TEAS, is followed by electron flow from the double bond at C2−C3, resulting in the formation of an internal bond between C7and C2 and generation of a new carbocation centered at C3. A hydride shift from C2 to this new carbocation is followed by a methyl migration from C7 and final proton abstraction from C8 resulting in the double bond between C7 and C8 and formation of epi-aristolochene.

of FPP generates an allylic carbocation that electrophilically attacks a substrate double bond, thus accomplishing the first ring closure. Catalysis continues through a series of carbocationic intermediates, resulting from transformations that may include proton abstractions, hydride and alkyl migrations, as well as additional electrophilic cyclizations. Catalysis terminates when the positive charge is quenched, either by deprotonation, or by attack of an external nucleophile. The variety of sesquiterpene products that arise in nature, from cyclization of the common FPP substrate, may result from subtle differences in active site architecture across the sesquiterpene synthase family, allowing for alternative regiochemical and stereochemical outcomes of catalysis.

Chemical rationalization of this mechanism with regard to the structure of the eremophilane product of TEAS catalysis, 5-epi-aristolochene, led to the proposal that germacrene A is formed as a catalytic intermediate (Scheme 1).[4] Germacrene A, a known sesquiterpene hydrocarbon,[7] is also proposed as a mechanistically reasonable intermediate in FPP cyclization by a variety of other sesquiterpene synthases, including vetispiradiene synthase from *Hyoscyamus muticus* (HVS),[8,9] aristolochene synthases from *Aspergillus terreus* and *Penicillium roquefortii*,[2,10−12] patchoulol synthase from *Pogostemon cablin*,[15] and *δ*-selinene synthase from *Abies grandis*.[16] Extensive investigations of the mechanism for FPP cyclization by aristolochene synthases from *A. terreus* and *P. roquefortii* have provided substantial evidence for the intermediacy of germacrene A in catalysis by these enzymes.[2,11−14] Labeling studies to elucidate details of the stereochemical course of *A. terreus* aristolochene synthase catalysis were entirely consistent with the proposed formation of germacrene A.[13,14] In addition, *A. terreus* aristolochene synthase cyclized 6,7-dihydro-FPP to dihydrogermacrene, an abortive cyclization product that is released from the active site.[12] However, direct proof for germacrene A formation has been precluded by the inability to demonstrate release of this compound from the active site of any of the sesquiterpene synthases for which it is proposed as an intermediate. This is despite recent observations on *Cichorium intybus* L. germacrene A synthase, an enzyme that catalyzes formation and release of germacrene A as its final product.[17]

TEAS, a 64 kDa monomer, has been cloned and expressed in bacteria,[18−20] and is among the first sesquiterpene synthases for which the three-dimensional structure has been solved.[21,22] The three-dimensional structure of the TEAS active site, solved in the presence of substrate analogues and subsequently modeled in the presence of FPP,[22] was used in conjunction with biochemical data to propose a detailed sequence of chemical transformations describing the specific mechanism of FPP cyclization within the TEAS active site.[22] Consistent with the general catalytic mechanism proposed for sesquiterpene synthases, this reaction scheme incorporates the formation of a stable, uncharged germacrene A intermediate during the conversion of FPP to a eudesmane cation. According to the proposed mechanism, germacrene A arises as the product of proton abstraction from the carbocation generated in the initial cyclization. Activation to a eudesmane cation is then initiated by donation of the Y520 hydroxyl proton to a double bond of the germacrene A intermediate. In an effort to test the validity of both the proposed intermediacy of germacrene A in TEAS catalysis and the specific role assigned to Y520 in the activation of this intermediate, active site-directed mutagenesis was undertaken to alter Y520 to Phe. Functional characterization of this TEAS point mutant is described in this paper.

Materials and Methods

Materials. [1-^{3}H]FPP (20.5−21.5 Ci/mmol) was purchased from DuPont-NEN. Germacrene A was a gift from Larry Cool (Forest

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Products Laboratory, University of California, Berkeley) and was used as a GC-MS standard. The source of germacrene A was the soldier cephalic secretion of a subterranean termite species. 5-Epi-aristolochene was a gift from Professor Robert Coates (Department of Chemistry, University of Illinois, Urbana-Champaign) and was used as a GC-MS standard. Using TEAS prepared as described below, Coates and coworkers enzymatically generated the 5-epi-aristolochene from FPP, isolated the compound to approximately 90% purity according to GC analysis, and confirmed the 5-epi-aristolochene structure by NOE NMR. Unless indicated otherwise, all other chemicals were purchased from Sigma or Fisher.

Site-Directed Mutagenesis of TEAS cDNA. The Y520F mutation (TAT → TTC) was introduced by QuikChange Site-Directed Mutagenesis (Stratagene kit) using standard kit protocols, with the exception that PCR annealing and extension temperatures were 45 and 62 °C, respectively. The template was a pET28b (Novagen) TEAS expression vector containing sequence encoding a hexahistidine tag at the amino-terminus of the TEAS cDNA.[20] The mutagenic primers were as follows (altered bases in bold): 5′ GCTCGTATTGTTGAGGTTACAT**TC**ATACACAATCTAGATGG 3′ and 5′ CCATCTAGATTGTGTAT**GA**ATGTAACCTCAACAATACGAGC 3′. Large-scale plasmid preparations were done using the Qiagen Midi plasmid purification kit. Incorporation of the Y520F mutation was verified by sequencing over a span of nucleotides encompassing codon 485 through the stop codon, using the following sense primer: 5′ GCAACTGGAATTGAGTGCTGC 3′. Sequence data indicated an additional mutation, E532K, that was attributable to an error in the reported sequence.[9,22,23]

Bacterial Expression and Purification of Recombinant TEAS and the TEAS-Y520F Mutant. The expression and purification of TEAS and TEAS-Y520F were based on previously published procedures,[20] with modifications as described here. Cells collected from 300 mL of culture were resuspended in a minimal volume of Buffer A (0.5 M NaCl, 20 mM Tris-Cl, pH 7.9), frozen overnight at −80 °C, and thawed at room temperature. Additional Buffer A was added to a maximum volume of approximately 12 mL and the suspensions were supplemented with 1 mg/mL of lysozyme. Following incubation on ice for 30 min, the cells were disrupted by sonication (3 × 30 s pulses) and the lysate was clarified by centrifugation at 39000 g for 20 min. The supernatant, containing TEAS or TEAS-Y520F, was filtered (0.45 μm) and applied to a 2 mL column of His-Bind Ni^{2+}-affinity resin (Novagen), equilibrated in Buffer A at a flow rate of approximately 20 mL/h. The column was washed with 20 mL of Buffer A and the enzyme was eluted with a 20 mL linear gradient to 250 mM imidazole in Buffer A. Fractions were assayed for protein according to Bradford,[24] using the Bio-Rad reagent, and the protein peak was dialyzed against Buffer B (50 mM HEPES, pH 7.5, 5 mM $MgCl_2$) containing 1 mM DTT. At this stage of purification, TEAS-Y520F preparations were concentrated to approximately 10 mg/mL using a centrifugal filter unit (Millipore Ultrafree-4 Biomax-30; 30 kDa MWCO). Glycerol was added to 50%, and the protein was stored at −80 °C.

Recombinant TEAS was purified further by anion exchange chromatography on a MonoQ HR5/5 column (Pharmacia). The column was equilibrated in Buffer B at a flow rate of 1 mL/min. Following application of the dialyzed protein, the column was washed with 10 mL of Buffer B and eluted with a 30 mL linear gradient to 0.3 M NaCl in Buffer B. The protein peak was dialyzed, concentrated to approximately 40 mg/mL, and stored as described above.

Assay for Enzymatic Activity. The assay used to measure the activity of TEAS and TEAS-Y520F is based upon partitioning of the hydrophobic product(s) (^{3}H-5-epi-aristolochene in the case of TEAS) into an organic solvent while the hydrophilic substrate (^{3}H−FPP) remains in the aqueous phase.[19,20,25] Reactions (50 μL) contained 200 mM Tris-Cl, pH 7.5, 40 mM $MgCl_2$, 10% glycerol, 0.2 μCi ^{3}H−FPP, unlabeled FPP (0.2−50 μM), and enzyme (10 nM TEAS; 300−500 nM TEAS-Y520F). Enzyme was added at fixed intervals to reaction mixtures preequilibrated at 30 °C. Exactly 5 min after the addition of enzyme, reactions were quenched with 50 μL of 0.2 M KOH, 0.1 M EDTA and placed on ice. Reactions were extracted with 0.5 mL of hexane, 0.4 mL of which was treated with approximately 50 mg of silica powder to remove any contaminating FPP or farnesol, the latter of which may be generated by contaminating phosphatase activity, or by treatment with the quenching mixture. Reactions were extracted twice more with 0.4 mL of hexane, each time removing 0.4 mL for silica treatment. A 100 μL aliquot from the pooled, silica-scrubbed, hexane extract was mixed with 4 mL of liquid scintillation cocktail and analyzed for radioactivity (disintegrations per min). Determination of reaction rate was based upon the percent conversion of FPP to product, the value for which was obtained by comparing the radioactivity in the hexane extract to that in an untreated aliquot of the assay mixture. Using the assay conditions described, the percent conversion of FPP to product was consistently less than 10% in all reactions. Near-background levels of radioactivity were observed in hexane extracts derived from control reactions lacking enzyme. In addition, silica treatment did not significantly alter the amount of radioactivity observed in hexane extracts, indicating insignificant phosphatase contamination and nonenzymatic FPP degradation. Data were analyzed using EnzymeKinetics V1.5 (Trinity Software). Kinetic constants are reported with standard errors.

GC-MS Analysis of TEAS-Y520F Reaction Products. TEAS-Y520F reactions (100 μL each) contained 200 mM Tris-Cl, pH 7.5, 40 mM $MgCl_2$, 30 μM FPP, and 5 μM TEAS-Y520F and were incubated for 30 min at 30 °C. Reactions were pooled and extracted by vortexing against excess hexane (Fisher Optima, GC-MS grade). Pooled hexane extracts were maintained in an ice−water mixture and were dried to a minimal volume by rotary evaporation. The final concentration of hexanes−extractable products (≥30 μg/mL) was estimated using a measurement of the percent conversion of substrate to product in a portion of the reaction mixture spiked with ^{3}H−FPP.

Hexane extracts from TEAS-Y520F reactions, as well as standards of germacrene A and 5-epi-aristolochene, were analyzed by GC-MS using a Varian 3400 gas chromatograph and a Finnigan INCOS-50 quadrupole mass selective detector. The GC was equipped with a capillary DB-5MS column (15 m × 0.25 mm, 0.25 μm phase thickness), with He as the carrier gas (10 psi). Splitless injections were done at an injection port temperature of 100 °C. The column temperature was maintained at 50 °C for 1 min and was then increased to 150 °C with a gradient of 4 °C per min. Relatively low GC temperatures were used due to the tendency of germacrene A to undergo a Cope rearrangement to β-elemene at high temperatures.[7,17,26] Following passage through the GC column, samples were introduced directly to the electron impact ionization source. Mass spectra were recorded at 70 eV, scanning from 20 to 420 atomic mass units. MS data were compared to those published for 5-epi-aristolochene[4] and germacrene A.[27]

Results and Discussion

Generation and characterization of TEAS containing a Y520F mutation were undertaken in an effort to test the validity of both specific chemical transformations proposed to occur within sesquiterpene synthase active sites and specific roles assigned to TEAS active site residues proposed to participate in these transformations. In particular, if germacrene A is an intermediate in TEAS catalysis, and if Y520 is the source of the proton required to activate germacrene A to a eudesmane cation, then removal of the Y520 hydroxyl group should cause TEAS catalysis to abort following formation of germacrene A. Despite its thermal and acid lability, germacrene A is a reasonably stable compound[7,17] that is also hydrophobic. Release of germacrene A from the TEAS-Y520F active site should be detectable using an assay identical with that described for measurement of 5-epi-aristolochene production by TEAS.

The Y520F mutation was successfully incorporated into TEAS using as template the TEAS cDNA contained within a pET28b expression construct.[20] Expression of the hexahistidine-

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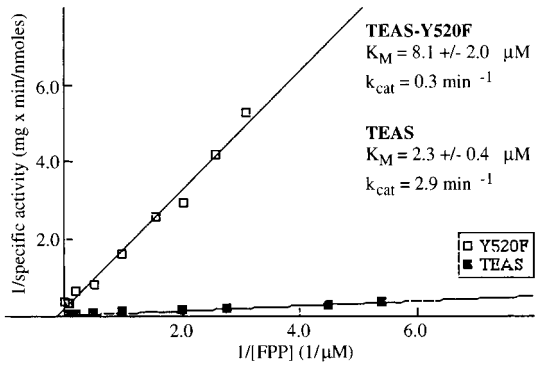

Figure 1. Lineweaver−Burk transformations of initial velocity data for FPP utilization by TEAS and TEAS-Y520F. Initial rates were measured as described, monitoring the production of hexanes−extractable ^{3}H from ^{3}H−FPP. A representative determination of the kinetic constants for each enzyme is shown. The kinetic constants were determined in duplicate for TEAS and in triplicate for TEAS-Y520F.

tagged TEAS-Y520F protein was comparable to that of nonmutated TEAS, with nickel-affinity purification of either protein yielding approximately 3.5 mg of protein/100 mL of culture. SDS-PAGE analysis indicated a TEAS-Y520F purity of >80% following nickel-affinity chromatography. Nonmutated TEAS preparations that were further fractionated through MonoQ anion exchange chromatography were >95% pure according to SDS-PAGE analysis.

The kinetics of TEAS-Y520F catalysis were characterized with respect to the observed ability of the enzyme to produce hexanes−extractable ^{3}H in the presence of ^{3}H−FPP. For comparison, a parallel characterization was completed for TEAS-catalyzed cyclization of ^{3}H−FPP to ^{3}H-5-epi-aristolochene (Figure 1). Relative to nonmutated TEAS, the Y520F mutation results in a 3.5-fold increase in the K_M for FPP, from 2.3 ± 0.4 μM for TEAS to 8.1 ± 2.0 μM for TEAS-Y520F**.** The same mutation results in an approximately 10-fold decrease in the k_{cat} for production of hexanes−extractable ^{3}H from ^{3}H−FPP, from 2.9 min^{-1} for TEAS to 0.3 min^{-1} for TEAS-Y520F. Thus, TEAS-Y520F produces hexanes−extractable ^{3}H from ^{3}H−FPP with a catalytic efficiency (k_{cat}/K_M) that is approximately 3% that of wild-type TEAS.

The identity of the hexanes−extractable material produced from FPP by TEAS-Y520F was elucidated through GC-MS analysis. Gas chromatography of 5-epi-aristolochene and germacrene A standards yielded distinct peaks with retention times of 16.0 and 16.7 min, respectively (Figure 2a). The associated mass spectra exhibited molecular ions at 204 daltons, as well as fragmentation patterns corresponding to data published for these compounds (Figure 2b,c).[4,27] A gas chromatograph of the hexane extract from a TEAS-Y520F reaction showed a single, distinct peak with a retention time of 16.5 min (Figure 3a). The corresponding mass spectrum was identical with that of germacrene A (Figure 3b). The slight difference in the GC retention time of the TEAS-Y520F reaction product (16.5 min), compared to that of authentic germacrene A (16.7 min), was attributed to previously observed variations between sequential GC analyses of identical samples. Co-injection of germacrene A and the TEAS-Y520F reaction extract demonstrated coelution of germacrene A with the single reaction product synthesized from FPP by the mutated enzyme (GC retention time of 16.7 min; Figure 3c). As expected, the corresponding mass spectrum was indistinguishable from that characterizing authentic germacrene A (Figure 3d). Thus, TEAS-Y520F produces germacrene A as its sole product.

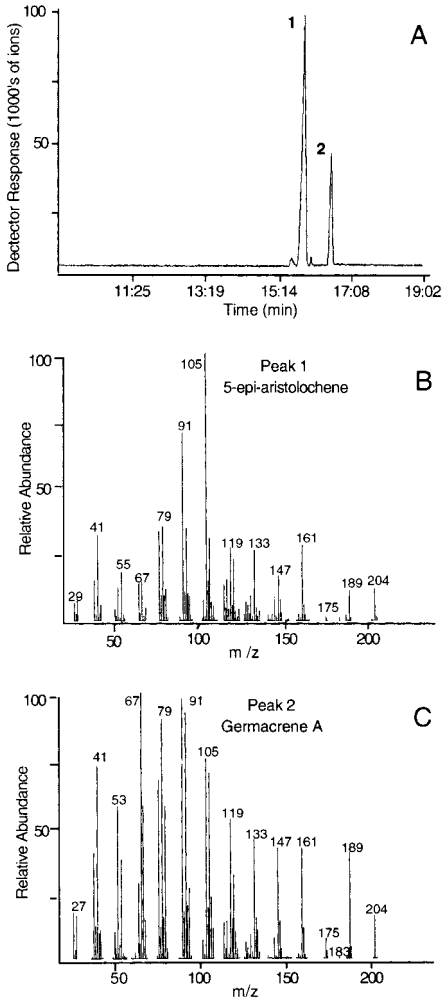

Figure 2. GC-MS analysis of authentic samples of 5-epi-aristolochene and germacrene A. (a) Total ion chromatogram of co-injected authentic samples of 5-epi-aristolochene and germacrene A; (b) mass spectrum of authentic 5-epi-aristolochene; and (c) mass spectrum of authentic germacrene A.

These observations represent the first direct demonstration of the germacrene A intermediate in sesquiterpene synthase catalysis. Coupled with the extensive evidence for formation of germacrene A during catalysis by *A. terreus* and *P. roquefortii* aristolochene synthases,[2,11−14] these data strongly support the general proposal that germacrene A is formed during catalysis by those sesquiterpene synthases for which it is a mechanistically reasonable intermediate, including TEAS, HVS, *P. roquefortii* and *A. terreus* aristolochene synthases, *A. grandis* δ-selinene synthase, and *P. cablin* patchoulol synthase. It is probable that active site architectures within this group of sesquiterpene synthases have common features required for the formation of germacrene A, as well as divergent features that dictate the specific chemical transformations required for the conversion of germacrene A to products as various as eremophilanes and vetispiranes. Active site features involved in catalysis and substrate/product selectivity are likely to include chemical features associated with active site residues, such as proton donation by Y520 of TEAS, as well as steric factors associated with the three-dimensional shape of the sesquiterpene synthase active site pocket. As additional sequences and three-dimensional structures of sesquiterpene synthases become available, sequence comparisons viewed in the context of three-dimensional space may uncover additional molecular determinants relevant to the catalytic chemistry as well as to the stereochem-

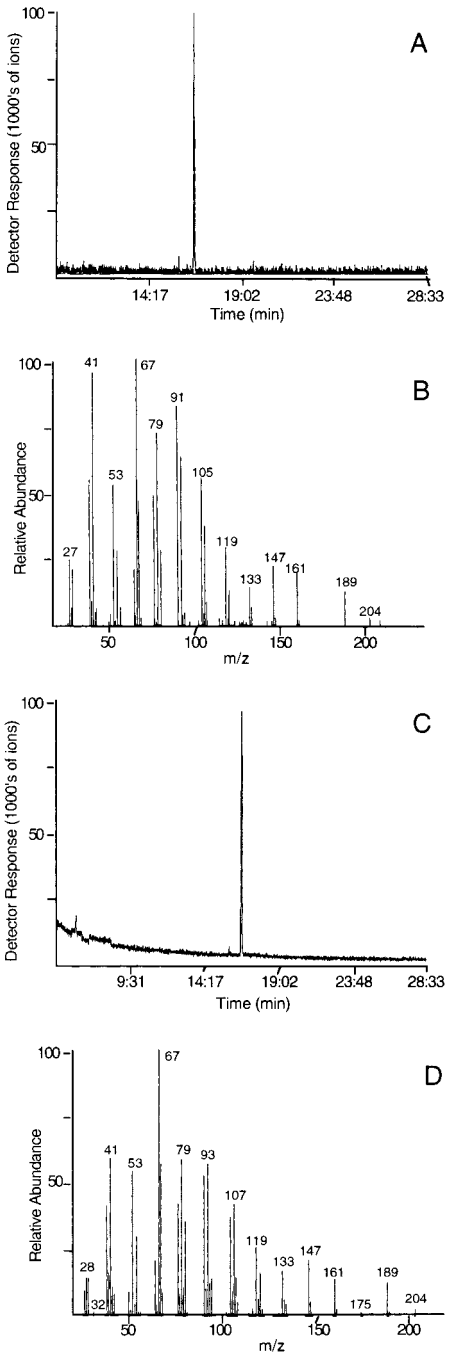

Figure 3. GC-MS analysis of the hexanes−extractable product of FPP utilization by TEAS-Y520F. (a) Total ion chromatogram of the product of FPP utilization by TEAS-Y520F; (b) mass spectrum of the product of TEAS-Y520F catalysis; (c) total ion chromatogram of co-injected authentic germacrene A and the hexane extract from a TEAS-Y520F reaction with FPP; and (d) mass spectrum derived from co-injected germacrene A and the TEAS-Y520F reaction hexane extract.

ical control accompanying product formation from highly reactive carbocationic intermediates within a hydrophobic active site pocket.

Three sesquiterpene synthases for which the nucleic acid sequences are known, and between which a relevant comparison can be made are HVS,[9] *P. roquefortii* aristolochene synthase,[28] and *Lycopersicon esculentum* cv. VFNT cherry tomato germacrene C synthase.[27] HVS and TEAS share an overall amino acid identity of 77% and a similarity of 81%.[9] All active site residues

Scheme 2. Minimal Mechanism for the Cyclization of Farnesyl Pyrophosphate to 5-Epi-aristolochene by Tobacco 5-Epi-aristolochene Synthase [Adapted from Ref 20]

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\bullet}S \xrightarrow{k_2} E{\bullet}P \xrightarrow{k_3} E + P$$

proposed to participate directly in TEAS catalysis are conserved in HVS, including Y520, suggesting an obvious target for mutagenesis. In contrast, the *P. roquefortii* aristolochene synthase shares little amino acid sequence similarity with TEAS, although these two enzymes catalyze nearly identical chemical reactions.[29] Determination of the three-dimensional structure of *P. roquefortii* aristolochene synthase may provide insight into steric factors that influence active site shape and thereby direct the formation of germacrene A. Germacrene C synthase catalyzes the formation of germacrene C, a process that requires a 1,2- or 1,3-hydride shift within the initial cyclization product, prior to deprotonation.[27] TEAS shares an overall amino acid identity of 45% and a similarity of 64% with germacrene C synthase and is no more similar in sequence to this enzyme than are other sesquiterpene synthases that produce unrelated products.[27] However, a parallel has been drawn between germacrene C synthase and TEAS with the proposal that germacrene C synthase catalysis terminates upon germacrene formation due to the absence of an Asp residue that is spatially equivalent to D444 in TEAS.[27] In TEAS, D444 is proposed to initiate germacrene A protonation through polarization of the Y520 hydroxyl group.[22] Germacrene C synthase contains residues Y521 and N445 in positions equivalent to TEAS residues Y520 and D444, respectively. A more direct comparison awaits the determination of the amino acid and gene sequences for *C. intybus* L. germacrene A synthase.

The minimal mechanism shown in Scheme 2 was developed previously to interpret kinetic constants determined in pre-steady-state studies of several plant sesquiterpene synthases, all of which are closely related to TEAS.[20] According to this mechanism, k_1 and k_{-1} represent substrate binding and dissociation steps that allow for a rapid equilibration of the enzyme with FPP to form the E·S complex ($k_{-1} \gg k_2$). K_2 and k_3 then represent the rate constants describing the conversion of FPP to the final reaction product and product release steps of catalysis. In our earlier study, the overall rate-limiting step corresponded to that described by k_3 ($k_3 \approx k_{cat}$). This step, however, may represent the slow release of the reaction product from the enzyme, or could encompass some combination of a limiting partial reaction within the catalytic mechanism along with slow product release.

Kinetic analysis of the Y520F mutant provides an opportunity to further evaluate these possibilities. If, for example, the chemical conversion of germacrene A to 5-epi-aristolochene within the TEAS enzyme were rate-limiting and a determining factor in establishing the k_3 rate constant, then the Y520F mutant would be expected to exhibit a greater catalytic efficiency than the TEAS enzyme. This is not the case. In fact, this mutation results in a 10-fold decrease in the k_{cat} and a 3.5-fold increase in the K_M. The decrease in catalytic efficiency of the Y520F mutant therefore seems more likely to represent a decrease in the rate of product release from the enzyme. This makes structural sense because the Y520F mutation, in essence the removal of a hydroxyl side chain projecting into the catalytic active site, should create a more hydrophobic pocket from which the hydrophobic reaction product must be released. Thus, the

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release of germacrene A from TEAS-Y520F is predicted to be slower than the release of 5-epi-aristolochene from TEAS. Precedent for rate-limiting product release among enzymes of isoprenoid metabolism is also found in the enzyme farnesyl diphosphate synthase.[30]

The increase in the K_M for the Y520F mutant is more difficult to rationalize in terms of a kinetic argument. Our earlier pre-steady-state kinetic studies of terpene synthases did not agree with a simplified monoreactant, rapid equilibrium Michaelis−Menton mechanism where $K_M = K_D$. Instead, the earlier results were more consistent with the complex relationship where $K_M \approx (k_{-1}/k_1)(k_3/k_2)$.[20] Because the rate constant for product release (k_3) apparently decreases in the Y520F mutant, the observed increase in K_M could arise by an increase in the substrate dissociation rate constant (k_{-1}), a decrease in the substrate association rate constant (k_1), a decrease in the rate constant defining the chemical transformation of FPP to germacrene A (k_2), or some combination of all of these. Determining these rate constants for the Y520F mutant in additional pre-steady-state kinetic studies is necessary before we can assess the contribution of any one of these parameters in establishing the K_M values for mutant and wild-type terpene synthases.

Introduction of the Y520F mutation into the TEAS active site results in formation of germacrene A as the final product of catalysis. This observation verifies the intermediacy of germacrene A in TEAS catalysis and strongly supports the proposal that the hydroxyl group of Y520 is indeed the proton donor initiating transformation of germacrene A to the eudesmane cation.[22] In addition, the observation that germacrene C synthase contains N445 in a position equivalent to TEAS D444[27] supports the proposal that protonation of the germacrene intermediate in TEAS catalysis requires the presence of D444 for polarization of the Y520 hydroxyl group.[22] Since TEAS-Y520F catalysis is characterized by a relatively slow release of its germacrene A product, crystallization of TEAS-Y520F in the presence of FPP may allow visualization of germacrene A within the enzyme active site, thus providing a snapshot along the reaction coordinate that describes TEAS-catalyzed conversion of FPP to 5-epi-aristolochene.

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