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Sesquiterpene Synthases Cop4 and Cop6 from *Coprinus cinereus*: Catalytic Promiscuity and Cyclization of Farnesyl Pyrophosphate Geometric Isomers

Fernando Lopez-Gallego,[a] Sean A. Agger,[a] Daniel Abate-Pella,[a] Mark D. Distefano,[b] and Claudia Schmidt-Dannert*[a]

Sesquiterpene synthases catalyze with different catalytic fidelity the cyclization of farnesyl pyrophosphate (FPP) into hundreds of known compounds with diverse structures and stereochemistries. Two sesquiterpene synthases, Cop4 and Cop6, were previously isolated from *Coprinus cinereus* as part of a fungal genome survey. This study investigates the reaction mechanism and catalytic fidelity of the two enzymes. Cyclization of all-*trans*-FPP ((*E*,*E*)-FPP) was compared to the cyclization of the *cis*–*trans* isomer of FPP ((*Z*,*E*)-FPP) as a surrogate for the secondary *cisoid* neryl cation intermediate generated by sesquiterpene synthases, which are capable of isomerizing the C2–C3 π bond of all-*trans*-FPP. Cop6 is a "high-fidelity" α-cuprenene synthase that retains its fidelity under various conditions tested. Cop4 is a catalytically promiscuous enzyme that cyclizes (*E*,*E*)-FPP into multiple products, including (−)-germacrene D and cubebol. Changing the pH of the reaction drastically alters the fidelity of Cop4 and makes it a highly selective enzyme. Cyclization of (*Z*,*E*)-FPP by Cop4 and Cop6 yields products that are very different from those obtained with (*E*,*E*)-FPP. Conversion of (*E*,*E*)-FPP proceeds via a (6*R*)-β-bisabolyl carbocation in the case of Cop6 and an (*E*,*E*)-germacradienyl carbocation in the case of Cop4. However, (*Z*,*E*)-FPP is cyclized via a (6*S*)-β-bisabolene carbocation by both enzymes. Structural modeling suggests that differences in the active site and the loop that covers the active site of the two enzymes might explain their different catalytic fidelities.

Introduction

Promiscuity of function is increasingly recognized as a property of many enzymes and is important for understanding enzyme evolution and function and for the engineering of better enzymatic catalysts for a variety of applications.[1,2] A group of enzymes especially known for their catalytic promiscuity are the terpene synthases and in particular, the sesquiterpene synthases. This group of terpene synthases catalyzes the cyclization of the linear 15-carbon isoprene pyrophosphate substrate all-*trans*-farnesyl diphosphate ((*E*,*E*)-FPP) into more than 300 known mono-, bi-, or tricyclic hydrocarbon or alcohol compounds with diverse stereochemistries.[3] The resulting terpenoid hydrocarbons are intermediates in the biosynthesis of biologically active compounds that are produced by plants, bacteria, and fungi as antibiotics, toxins, and pheromones.[4,5] Sesquiterpene synthases catalyze some of the most complex carbon–carbon bond forming reactions found in chemistry and biology.[6] Several mutagenesis and protein-engineering studies aimed at understanding terpene cyclization[3] and modified product profiles of terpene synthases[7–9] have been published.

Cyclization of (*E*,*E*)-FPP is initiated by pyrophosphate cleavage, generating an initial substrate carbocation, which is rearranged and eventually quenched by a water molecule or by proton abstraction from the substrate. The active site of the enzyme functions as a chaperone that folds the isoprenoid chain, shields the carbocation from premature nucleophilic attack, and guides carbocation rearrangement until its final quenching.[10] The conformation of the bound isoprenyl chain and the residues lining the active-site binding pocket determine carbocation reactions and hence, the final product profile of a particular sesquiterpene synthase.

Two consensus sequences—an aspartate-rich DDXXD/E and a NSE/DTE motif—located at the entrance of the active site coordinate a trinuclear Mg^{2+} cluster that binds the diphosphate moiety of the isoprenoid substrate, positions the isoprenyl chain in the binding pocket, and triggers closure of the active site along with diphosphate cleavage to generate an initial *transoid*, allylic carbocation.[11–13] Enzymes that catalyze the *trans*-pathway of catalysis rearrange and quench this initial *transoid* cation. Other enzymes that catalyze the *cis*–*trans* pathway of catalysis recapture the diphosphate leaving group at carbon C3, thereby allowing rotation around the generated C2–C3 single bond to yield the nerolidyl diphosphate (NPP) intermediate. Depending on the binding of FPP in the active

[a] *Dr. F. Lopez-Gallego, Dr. S. A. Agger, D. Abate-Pella, Prof. Dr. C. Schmidt-Dannert*
Department of Biochemistry, Molecular Biology and Biophysics
University of Minnesota
1479 Gortner Avenue, St. Paul, MN 55108 (USA)
Fax: (+1) 612-625-5780
E-mail: schmi232@umn.edu

[b] *Prof. Dr. M. D. Distefano*
Department of Chemistry, University of Minnesota
207 Pleasant Street SE, Minneapolis, MN 55455 (USA)

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site, the configuration at C3 of the NPP intermediate can be *R* or *S*. This intermediate yields upon secondary diphosphate cleavage a *cisoid*, allylic cation that is subsequently rearranged and eventually quenched.[14,15]

Sesquiterpene synthases are known that are very specific for either one or the other pathway of catalysis, and yet other enzymes use both pathways. Examples of *trans*-pathway-specific enzymes with solved crystal structures include pentalenene synthase form *Streptomyces* UC5319,[16] 5-epi-aristolochene synthase from *Nicotiana tobaccum*,[17,18] and aristolochene synthases from *Aspergillus terreus* and *Penicillium roqueforti*.[19,20] Trichodiene synthase from *F. sporotrichoides*,[21,22] and very recently, δ-cadinene synthase from *Gossypium arboreum* (cotton)[23] are the only *cis–trans*-pathway-specific sesquiterpene synthases with solved crystal structures. Examples for catalytically very promiscuous sesquiterpene synthases are γ-humulene synthase and δ-selinene synthase from *grand fir*. Each enzyme produces more than 30 sesquiterpene structures (*cis–trans* and *trans*-pathway products) in addition to their major products γ-humulene (*cis–trans*-pathway product) and δ-selinene (*trans*-pathway product).[15]

Previously, we have isolated two new *cis–trans*-pathway-specific sesquiterpene synthases, Cop4 and Cop6, from the homobasidiomycete *Coprinus cinereus* as part of a survey of sesquiterpene synthase homologues from fungal genomes.[24,25] Although both enzymes catalyze the isomerization of the C2–C3 bond of the farnesyl substrate, subsequent cyclization reactions differ greatly between the two enzymes as do their catalytic promiscuities. Cop4 produced several volatile sesquiterpene products, including δ-cadinene as the major product, when expressed in *E. coli*. In contrast, Cop6 expressed in *E. coli* produced selectively α-cuprenene, which is the precursor of the lagopodin antibiotics reported to be produced by *Coprinus* species.[26]

In this work we performed kinetic and mechanistic studies with purified enzymes to investigate the different promiscuous behaviors of the two enzymes. We demonstrate that although Cop4, unlike Cop6, exhibits a very broad product profile with (*E*,*E*)-FPP under typical reaction conditions, the catalytic fidelity of Cop4 can be drastically altered by varying the reactions conditions. Structural modeling of Cop4 and Cop6 suggests reasons for the differences in catalytic fidelity observed for the two enzymes.

In addition to analyzing the cyclization routes of (*E*,*E*)-FPP catalyzed by the two Cop enzymes, we use the *cis–trans* isomer of FPP ((*Z*,*E*)-PP) as a surrogate for the secondary *cisoid* neryl cation intermediate generated by sesquiterpene synthases that can isomerize the C2–C3 π bond of all-*trans*-FPP. Here, we ask the question whether the cyclization of the *cis* isomer of FPP ((*Z*,*E*)-FPP) in which the C2–C3 π bond is already in the *cis* configuration would yield with Cop4 and Cop6 the same product profiles and promiscuous behavior as observed with their normal substrate (*E*,*E*)-FPP. For comparison, we include in our investigation a previously identified strictly *trans*-pathway-specific germacrene A synthase NS1 from *Nostoc* sp. PCC 7120, which should not accept (*Z*,*E*)-FPP as a substrate.[27]

FPP isomers and analogues have been used in studies with several sesquiterpene synthases to determine the mechanism of carbocation quenching[28–31] and the initial ionization and isomerization of *all-trans*-FPP for *cis–trans*-pathway-specific enzymes.[32–35] However, most studies compared the kinetic properties of different FPP substrate geometric isomers. Cyclization products of (*Z*,*E*)-FPP have only recently been investigated for several sesquiterpene synthase homologues from maize.[36–38] Here, we describe for the first time how the substrate's geometric conformation determines the first cyclization event. Surprisingly, and in contrast to previous studies with the maize enzymes, both Cop4 and Cop6 cyclize (*Z*,*E*)-FPP into products that are very different from those obtained with (*E*,*E*)-FPP. Most notably, cyclization of (*E*,*E*)-FPP and (*Z*,*E*)-FPP by Cop6 proceeds through opposite enantiomers of a β-bisabolyl cation intermediate.

Results

Kinetic parameters

Kinetic properties of the two fungal *cis–trans*-pathway-specific enzymes Cop4 and Cop6 and for comparison, the strictly *trans*-pathway-specific germacrene A synthase from *Nostoc* sp. PCC 7120 (NS1),[27] were analyzed by using purified recombinant proteins with (*E*,*E*)-FPP, (*Z*,*E*)-FPP and the ten-carbon (*E*)-GPP (*trans*-geranyl diphosphate) as substrates.

Kinetic parameters were first determined with (*E*,*E*)-FPP (Table 1). Cop4 and Cop6 bind (*E*,*E*)-FPP with comparable affinity (K_m), but the catalytic turnover (k_{cat}) of Cop4 with this substrate is 70-fold lower than of Cop6, resulting in a catalytic efficiency (k_{cat}/K_m) for Cop4 that is almost two orders of magnitude lower than for Cop6. NS1 has a higher affinity (K_m) for (*E*,*E*)-FPP than the two fungal enzymes, but its catalytic turnover (k_{cat}) is only slightly higher than that of Cop4.

When (*Z*,*E*)-FPP was used as the substrate, only the *cis–trans*-pathway-specific enzymes Cop4 and Cop6 showed measurable

Table 1. Kinetic constants of Cop4 and Cop6 with (*E*,*E*)-, (*Z*,*E*)-FPP and (*E*)-GPP

	K_m [μM]			k_{cat} [s^{-1}]			k_{cat}/K_m ($\times 10^3$) [s^{-1} M^{-1}]		
	(*E*,*E*)-FPP	(*Z*,*E*)-FPP	(*E*)-GPP	(*E*,*E*)-FPP	(*Z*,*E*)-FPP	(*E*)-GPP	(*E*,*E*)-FPP	(*Z*,*E*)-FPP	(*E*)-GPP
NS1	3.8 ± 0.5	n.a.[a]	1.4 ± 0.5	(5.3±0.1) × 10^{-2}	n.a.[a]	(20±1) × 10^{-4}	14	n.a.[a]	1.35
Cop6	7.6 ± 2.4	n.d.[b]	1.5 ± 0.6	(67±0.7) × 10^{-2}	n.d.[b]	(3.5±0.3) × 10^{-2}	88	n.d.[b]	24
Cop4	11 ± 3	20 ± 2	24 ± 8	(1.2±0.1) × 10^{-2}	(0.25±0.04) × 10^{-2}	(1.6±0.3) × 10^{-4}	1	0.22	0.007

Kinetic constants are compared to those obtained with the bacterial germacrene A synthase NS1 that uses the all-trans pathway of catalysis. [a] n.a.: No activity detected. [b] n.d.: Activity too low for kinetic measurements.

activity, whereas expectedly no diphosphate release was detected in in vitro assays with the *trans*-pathway-specific enzyme NS1 (Table 1). However, the activity of Cop6 with (*Z*,*E*)-FPP is too low for kinetic measurements. Cop4 on the other hand converts (*Z*,*E*)-FPP with a catalytic efficiency that is about sevenfold lower than with its natural substrate (*E*,*E*)-FPP as the result of both a reduced substrate binding (40% higher K_m) and catalytic turnover (fourfold lower k_{cat}).

(*E*)-GPP is known to be converted by sesquiterpene synthases into monoterpenes.[15,30,39–41] Expectedly, all three enzymes accept (*E*)-GPP as a substrate, but the catalytic efficiency (k_{cat}/K_m) with the shorter prenyl-diphosphate substrate is lower than their longer FPP substrate (Table 1). (*E*)-GPP is a fairly good substrate for Cop6, and shows only a moderate reduction in K_m and k_{cat} compared to (*E*,*E*)-FPP. In contrast, the catalytic turnover (k_{cat}) of Cop4 and NS1 with (*E*)-GPP is more than two orders of magnitude less than that of (*E*,*E*)-FPP.

Cyclization of (*E*,*E*)-FPP

To investigate further the differences in catalytic promiscuity previously observed in *E. coli* strains expressing Cop4 or Cop6, purified Cop enzymes (and purified NS1 for comparison) were incubated for 18 h with (*E*,*E*)-FPP, and the resulting cyclization products were analyzed (Table 2, see Figures S1 and S2 in the Supporting Information for GC chromatograms and mass spectra of product peaks, respectively).

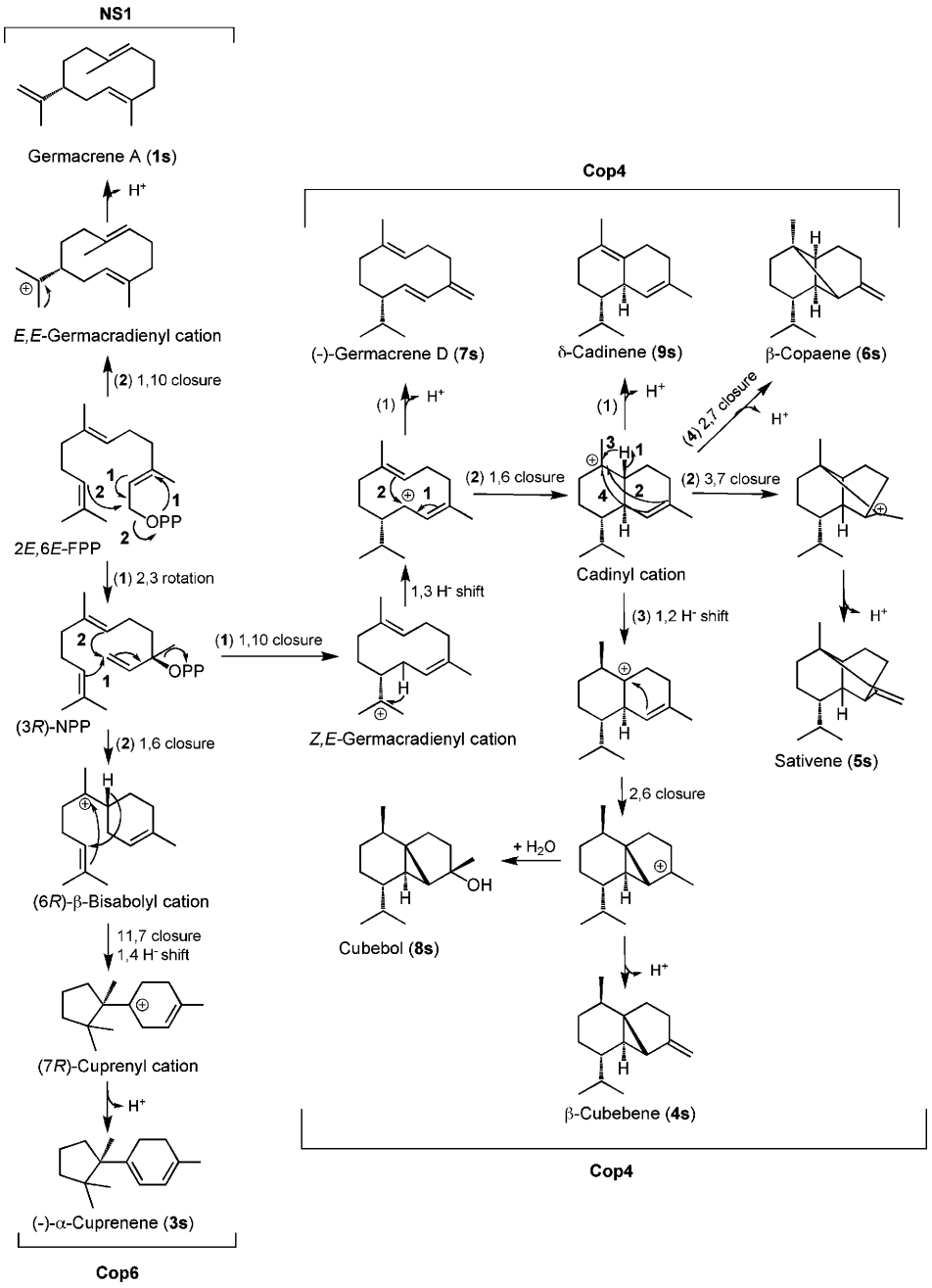
Cop6 converts (*E*,*E*)-FPP with high selectivity into (−)-α-cuprenene (**3 s**), which makes up 98% of the total sesquiterpenoids synthesized (see Figure S3 for absolute configuration determination). In contrast, Cop4 cyclizes (*E*,*E*)-FPP into six different identified sesquiterpenes in addition to a number of unidentified sesquiterpenoid compounds. (−)-Germacrene D (**7 s**; see Figure S4 for absolute configuration determination) and cubebol (**8 s**) are the major products of Cop4, each making up about 30% of the total sesquiterpenoid compounds detected (see Scheme 1 for structures and corresponding compound numbers).

All sesquiterpenoids synthesized by both Cop4 and Cop6 from (*E*,*E*)-FPP involve rotation of the C2–C3 π bond of (*E*,*E*)-FPP to yield a *cisoid* neryl cation intermediate that is rearranged and finally quenched along different routes (Scheme 1). Previous studies by Cane's group with trichodiene synthase[42] and more recently with *epi*-isozizane synthase[43] have established that ionization and isomerization of (*E*,*E*)-FPP generates the (3*R*)-NPP intermediate. Subsequent ionization and 1,6-cyclization then yields a (6*R*)-bisabolyl cation, which in the case of trichodiene synthase is further rearranged into a (7*R*)-cuprenyl cation to yield trichodiene after a final methyl shift.[44] The finding that Cop6 cyclizes (*E*,*E*)-FPP to (−)-α-cuprenene (**3 s**) and also converts NPP into α-cuprenene, albeit with noticeably reduced selectivity (Figure S5 A), suggests that Cop6 follows a cyclization pathway similar to that of trichodiene synthase until the final methyl shift. Hence, the reaction pathway shown in Scheme 1 assumes that the absolute configuration of NPP is 3*R*. Cop4 is also able to cyclize NPP and yields the same products that were obtained with (*E*,*E*)-FPP, except for one new

Table 2. Terpenoid product profiles of purified Cop4 and Cop6 with (*E*,*E*)-, (*Z*,*E*)-FPP and *E*-GPP.

	Product	**1 s**[b]	**2 s**[b]	**3 s**	**4 s**	**5 s**	**6 s**	**7 s**	**8 s**	**9 s**	**10 s**	**11 s**	**12 s**	**13 s**	**14 s**	**15 s**	**16 s**	**17 s**	n.d.[c]
	[%][a]	**1 m**	**2 m**	**3 m**	**4 m**	**5 m**	**6 m**												
	(*E*,*E*)-FPP	93.5	6.5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
NS1	(*Z*,*E*)-FPP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
	(*E*)-GPP	**25**	**9**	**66**	–	–	–												–
	(*E*,*E*)-FPP	–	–	98.2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	1.8
Cop6	(*Z*,*E*)-FPP	–	–	–	–	–	–	–	–	–	45	5.2	25.6	16.3	-	-	-	-	7.9
	(*E*)-GPP	**9.2**	**7.11**	**34.6**	**45**	**4**	–												–
	(*E*,*E*)-FPP	–	–	–	5.7	2.8	7.4	29.3	28.2	10.4	–	–	–	–	–	–	–	–	16.1
Cop4	(*Z*,*E*)-FPP	–	–	–	–	–	–	–	–	–	–	27.7	10	–	13.3	6.4	4.2	15	23.7
	(*E*)-GPP	**61**	**9.1**	–	**23.7**	**2.5**	**3.6**												9.3

Product profiles are compared to those obtained with the bacterial germacrene A synthase NS1 that uses the *all-trans* pathway of catalysis. Sesquiterpene products obtained with (*E*,*E*) or (*Z*,*E*)-FPP have compound numbers ending with an "s", whereas monoterpene products obtained with (*E*)-GPP (bold) have compound numbers ending with an "m". Structures corresponding to shown compound numbers are found in Schemes 1, 2 (sesquiterpenes), and 3 (monoterpenes). Underlined compounds represent major sesquiterpene products that could not be identified.[a] Percentages are averages calculated from product profiles of three independent in vitro reactions. The error was less than 5% in all cases. See Figures S1 and S2 (sesquiterpenes) and Figures S7 and S8 (monoterpenes) for representative GC–MS chromatograms of product profiles.[b] Compounds **1 s** and **2 s** are the heat-induced Cope rearrangement products of germacrene A: β-elemene (**1 s**) and *cis*-β-elemene (**2 s**).[76c] n.d. Sum of minor products that could not be identified.



Scheme 1. Proposed reaction mechanism accounting for the identified products generated from (*E*,*E*)-FPP by Cop4, Cop6 and NS1. Numbered reaction arrows indicate different branch points in the cyclization reaction. Relative amounts of products formed by each enzyme are shown in Table 2.

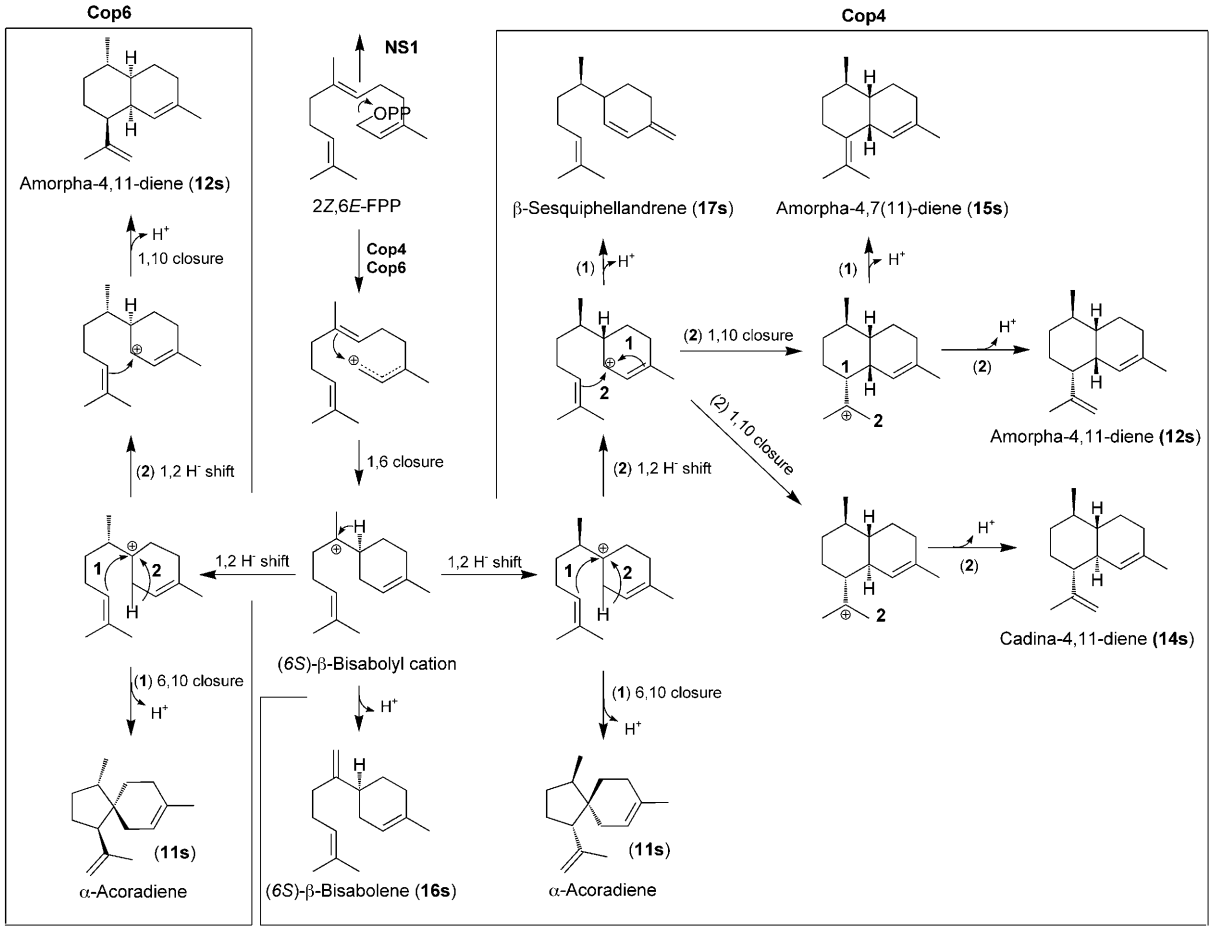
major compound (**18s**) that could not be identified (Figures S5B and S2 for mass spectrum). In the case of Cop4, ionization and 1,10-cyclization of the (3*R*)-NPP intermediate gives a (*Z*,*E*)-germacradienyl cation that upon 1,3-hydride shift and deprotonation yields (−)-germacrene D. Additional hydride shifts and ring closures produce the other cyclization products detected in the Cop4 reaction. Assuming that the configuration of the isopropyl group in (−)-germacrene D is maintained; we can postulate the reaction mechanism shown in Scheme 1.

The *trans*-pathway-specific enzyme NS1 also generates a germacradienyl cation, but with the C2–C3 π bond remaining in a *trans* configuration. This enzyme is as selective as Cop6 in the cyclization of (*E*,*E*)-FPP and synthesizes mostly germacrene A (**1s**; 93.5%, detected as its heat-induced Cope rearrangement product β-elemene;[27] Table 2, Scheme 1). The 4*Z* isomer of germacrene A, helminthogermacrene A (6.5%, detected as its Cope rearrangement product *cis*-β-elemene[45]) is produced by NS1 as a minor cyclization product.

Cyclization of (*Z*,*E*)-FPP

(*Z*,*E*)-FPP yields a *cisoid* farnesyl cation intermediate directly after diphosphate cleavage. Hence, no isomerization step precedes initiation of the subsequent carbocation rearrangement reactions. Although (*Z*,*E*)-FPP has been used as a surrogate substrate for *cis–trans*-pathway-specific enzymes for kinetic measurements,[33,36–38,46] only a few recent examples analyzed the cyclization products of (*Z*,*E*)-FPP[36–38] and found them to be comparable to those obtained with the *all-trans*-FPP isomer. We sought to determine whether cyclization of the *cis*-FPP geometric isomer by Cop4 and Cop6 would similarly yield the same product profiles and selectivities observed with their normal substrate (*E*,*E*)-FPP.

In contrast to the previous studies,[36–38] Cop4 and Cop6 generated very different cyclization products with the *cis*-FPP isomer compared to the compounds made with the all-*trans* isomer (*E*,*E*)-FPP. Moreover, the high product selectivity observed for Cop6 with all-*trans*-FPP is not obtained with the *cis*-FPP isomer (Table 2, see Figures S1 and S2 for GC chromatograms and mass spectra of product peaks, respectively). Cop4 catalyzes the cyclization of (*Z*,*E*)-FPP into α-acoradiene (**11s**) as the major product and four other significant sesquiterpenoid products (**14s**–**17s**, see Scheme 2 for structures and compound names). By determining the absolute configuration of cadina-4,11-diene (**14s**) produced by Cop4 with a cadina-4,11-diene standard with a known configuration (in *Amyris balsamifera* essential oil,[47] Figure S6), the absolute configurations of



Scheme 2. Proposed reaction mechanism accounting for the identified products generated from (*Z*,*E*)-FPP by Cop4, Cop6 and NS1. Numbered reaction arrows indicate different branch points in the cyclization reaction. Relative amounts of products formed by each enzyme are shown in Table 2. (6*S*)-β-Bisabolene is also formed as a product (18%) by the mutant form of Cop 6 (N224D); this provides strong evidence for the common 6*S* stereochemistry of the β-bisabolyl cation intermediate generated by Cop6.

the other Cop4 products shown in Scheme 2 can be postulated by assuming that stereocenters introduced in the cyclization reaction are maintained until product is released by the enzyme. Cop4 also makes a number of unidentified minor products that account for 23.7% of all sesquiterpenoid products detected.

Cop6 converts (*Z*,*E*)-FPP into a smaller and different set (except for α-acoradiene (**11 s**) and amorpha-4,11-diene (**12 s**)) of cyclization products compared to Cop4 (Table 2). The major Cop6 product (**10 s**), representing 45% of the total terpenoid products, could not be identified. The mass spectrum of this unidentified compound does not yield a complete match with published reference spectra, although its fragmentation pattern suggests it to be structurally related to α-acoradiene (**11 s**; Figure S2). Differences in retention times after chiral GC–MS separation of α-acoradiene (**11 s**) and amorpha-4,11-diene (**12 s**) produced by Cop6 and by Cop4 (Figure S6) show that conversion of (*Z*,*E*)-FPP by the two enzymes yields cyclization products with opposite absolute configuration. Knowing the absolute configuration of cadina-4,11-diene (**14 s**) produced by Cop4, we can postulate the Cop6 reaction mechanism shown in Scheme 2.

Cyclization of (*Z*,*E*)-FPP by Cop4 and Cop6 can be rationalized to proceed through a β-bisabolyl cation, which is supported by the detection of β-bisabolene (**16 s**) as a cyclization product of (*Z*,*E*)-FPP by Cop4. The detection of α-acoradiene (**11 s**) and amorpha-4,11-diene (**12 s**) as cyclization products of (*Z*,*E*)-FPP (Table 2) suggests that the cyclization of this FPP isomer by Cop4 and Cop6 proceeds through a (6*S*)-β-bisabolyl cation intermediate[41,48] rather than through a (6*R*)-β-bisabolyl cation as in the cyclization of (*E*,*E*)-FPP by Cop6. The two enzymes then catalyze a 1,2-hydride shift yielding a second cation with opposite configuration at C7 (7*S* cation in the case of Cop6 and a 7*R* cation in the case of Cop4), which give rise to cyclization products with different absolute configurations. Deprotonation yields α-acoradiene (**11 s**) whereas an additional 1,2-hydride shift leads to different isomeric carbocation intermediates en route to the products **17 s**, **15 s**, and **14 s**[29] (Scheme 2).

To verify the proposed reaction mechanism via a (6*S*)-β-bisabolyl cation intermediate, we first determined by chiral GS–MS with authentic reference compounds the stereochemistry of β-bisabolene (**16 s**) produced by Cop4 as a minor compound after premature deprotonation of the corresponding cation intermediate. Figure 1 A shows that Cop4 makes (6*S*)-β-bisabo-

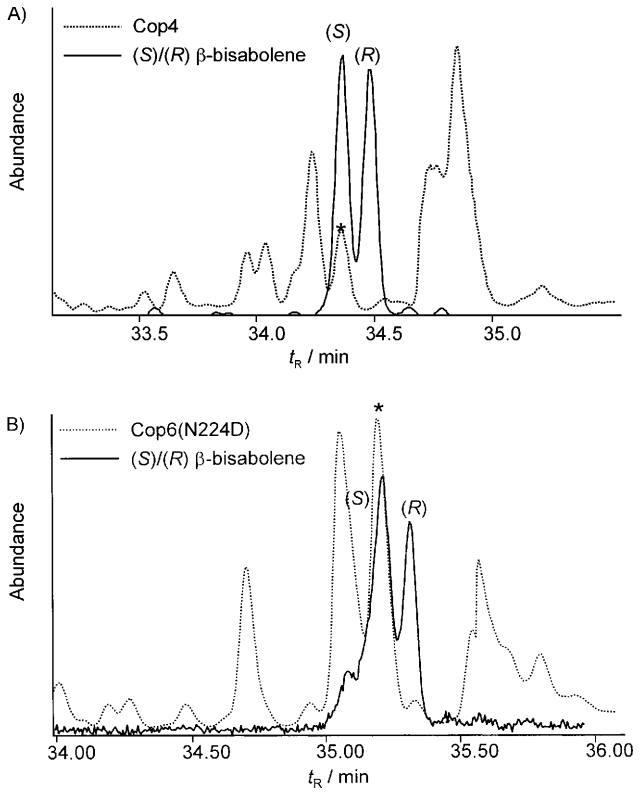


Figure 1. Stereochemical analysis of β-bisabolene products synthesized from (*Z*,*E*)-FPP. Reaction products of A) purified Cop4 and B) mutant Cop6 N224D with (*Z*,*E*)-FPP were separated by chiral GC–MS (····) and identified by using authentic β-bisabolene standards (——). Peaks labeled with an asterisk correspond to the Cop4 and Cop6 β-bisabolene reaction products.

lene (**16s**), confirming the 6*S* stereocenter of the β-bisabolyl cation.

Because Cop6 does not catalyze the premature deprotonation of the β-bisabolyl cation with either FPP isomer under the reaction conditions used (18 h incubation), a mutation, N224D, was introduced into the active site of Cop6. An analogous mutation is known to increase the premature deprotonation of the β-bisabolyl cation in trichodiene synthase.[48] Mutant Cop6N224D now makes a small amount of an additional compound that was identified by chiral GS–MS as (6*S*)-β-bisabolene (**16s**; Figure 1B). Interestingly, the active site mutation N224D does not affect the product profile of Cop6 with its normal substrate (*E*,*E*)-FPP.

Cyclization of (*E*)-GPP

Results from kinetic studies with Cop6, Cop4, and NS1 show that (*E*)-GPP is converted by all three enzymes but with very different catalytic efficiencies (Table 1). The shorter isoprene chain of (*E*)-GPP is expected to exhibit a higher degree of conformational freedom in the active site of sesquiterpene synthases compared to (*E*,*E*)-FPP. Consequently, Cop6 is expected to produce multiple products with (*E*)-GPP instead of one with (*E*,*E*)-FPP. Because cyclic monoterpenes can only be produced by terpene synthases that can isomerize the C2–C3 π bond of (*E*)-GPP,[49,50] only Cop4 and Cop6 but not NS1 are expected to cyclize (*E*)-GPP.

To confirm our expectations, purified enzymes were incubated for 18 h with (*E*)-GPP, and the products were analyzed (Table 2, see Figures S7 and S8 for GC chromatograms and mass spectra of product peaks, respectively). Cop4 and Cop6 convert (*E*)-GPP into both acyclic (**1m, 2m, 3m**) and cyclic (**4m, 5m, 6m**) monoterpenes (Table 2, Scheme 3 and Figure S7). However, cyclization of (*E*)-GPP is more efficiently catalyzed by Cop6 than by Cop4. Cop6 accumulates limonene (**4m**; 45% of total terpene products) as the major product whereas Cop4 makes mostly the acyclic terpene (*E*)-β-ocimene (**1m**; 57.6% of total terpene products; Table 2, Scheme 3). The strictly *trans*-pathway-specific sesquiterpene synthase NS1 converts (*E*)-GPP as expected exclusively into acyclic monoterpene olefins, with linalool as the major product (**3m**).

Influence of reaction conditions on product profiles

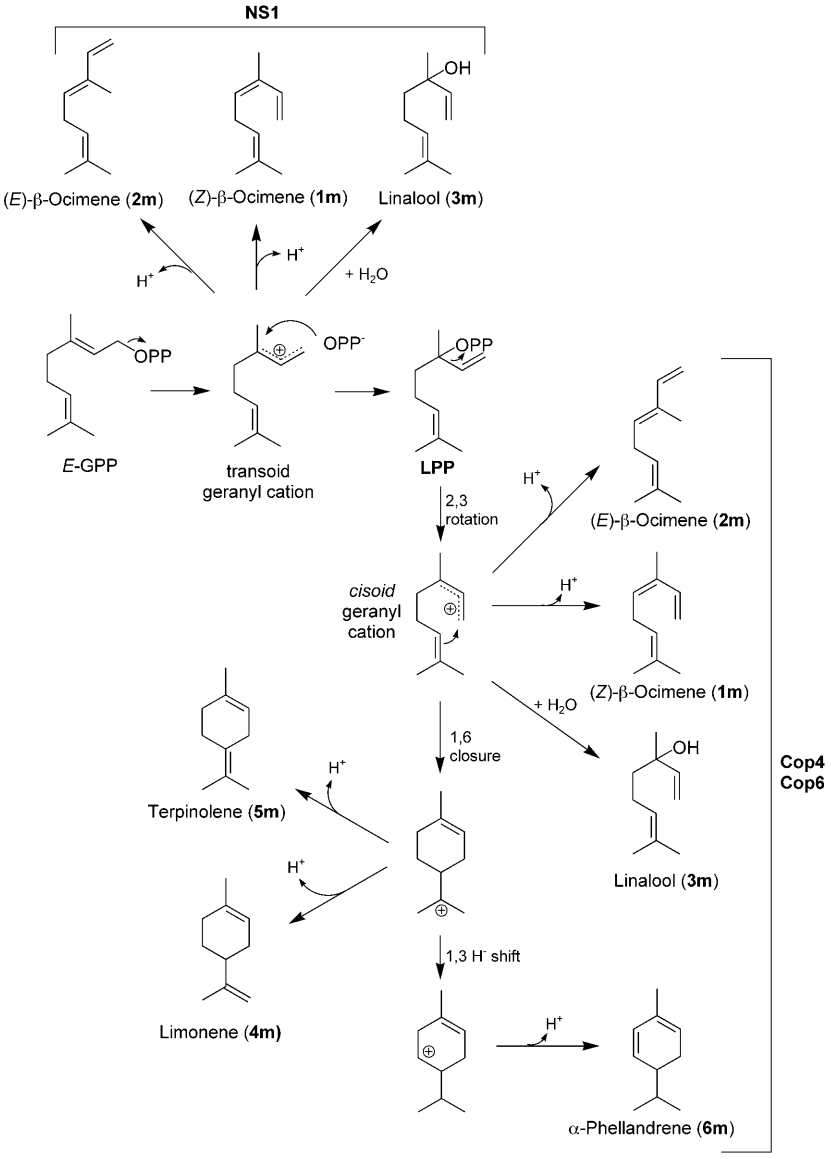
We noted that the product profile of Cop4, unlike Cop6, measured in the headspace of recombinant *E. coli* cultures (major product δ-cadinene (**9s**) is different from the product profile obtained in vitro in this study (Table 2, (−)-germacrene D (**7s**) and cubebol (**8s**) major products).[24,25] This might suggest that the cyclization reaction catalyzed by Cop4 is susceptible to changes in the reaction environment. To test this, conversion of (*E*,*E*)-FPP by Cop4 and Cop6 was analyzed under different reaction conditions.

Changes in reaction conditions were found to influence the product profiles of Cop4 and Cop6 strikingly differently. Remarkably, Cop6 does not change its product profile under any of the condition tested and always converts (*E*,*E*)-FPP highly selectively into greater than 98% of α-cuprenene as shown in Table 2. The product profile of Cop4 on the other hand is dependent on the reaction condition used, and certain conditions dramatically change its catalytic fidelity (Table 3).

Changing the ionic strengths of the reaction by adding 1 M NaCl does not affect the product specificity of Cop4 significantly (Table 3), although generation of (−)-germacrene D (**7s**) decreases somewhat in favor of cubebol (**8s**), which is located further downstream on the cyclization path (Scheme 1). In addition, the fraction of sesquiterpene olefins that could not be structurally identified increases with increasing ionic strength.

Lowering the reaction temperature from 25 to 4 °C increased the selectivity of Cop4 for (−)-germacrene D (**7s**) and decreased the fraction of structurally unidentified sesquiterpene olefins by half (Table 3). Increasing the reaction temperature to 37 °C, however, had the opposite effect and decreased the fidelity of Cop4. At this temperature Cop4 generated a relatively larger fraction of products (β-cubebene (**4s**), sativene (**5s**) δ-cadinene (**9s**) and β-copaene (**5s**)) that are derived from a cadinyl cation intermediate.

The product specificity of terpene synthases is known to be influenced by the type of metal cofactor bound by the enzyme.[38,40,51] The first notable feature of the Cop4 product profiles obtained in reactions where Mg^{2+} is replaced with either Mn^{2+} or K^+ is the disappearance of β-copaene (Table 3).



Scheme 3. Postulated mechanism of monoterpene formation from (*E*)-GPP. Acylic monoterpenes produced by NS1 are proposed to be derived from a *transoid* geranyl cation, whereas the acyclic and cyclic monoterpene products of Cop4 and Cop6 are postulated to involve the generation of a *cisoid* geranyl cation (neryl cation). Relative amounts of products formed by each enzyme are shown in Table 2.

In the presence of the divalent cation Mn^{2+}, Cop4 favors a reaction path that ends after one cyclization in (−)-germacrene D (**7s**). A larger fraction of sesquiterpene olefins that could not be structurally identified is also produced by Cop4 in the presence of Mn^{2+}. The monovalent K^+ decreases the (−)-germacrene D (**7s**) yield and increases the overall yield of tricyclic sesquiterpene olefins (**4s, 5s**, and **6s**).

The most dramatic effect on the product spectrum of Cop4 was obtained by altering the pH of the reaction (Table 3 and Figure 2). Under both alkaline and acidic conditions Cop4 becomes a very selective enzyme with only one major product (**7s**) compared to the three major compounds (**7s**, **8s**, **9s**) produced under neutral reaction conditions. At pH 10, the cyclization reaction ends with the hydride shift and deprotonation of the germacradienyl cation to yield (−)-germacrene D (**7s**; 91% of total sesquiterpene products), whereas at pH 5.0 cyclization can proceed via a 1,6-ring closure to also yield a small amount of δ-cadinene (**9s**; 12% of total sesquiterpene products) in addition to (−)-germacrene D (**7s**).

Structural modeling

Structural models for Cop4 and Cop6 were built to understand

Table 3. Sesquiterpene product profile of purified Cop4 with (*E*,*E*)-FPP under different reaction conditions.

Conditions				Products [%][a]						
pH	NaCl [M]	*T* [°C]	Cation (10 mM)	**4s**	**5s**	**6s**	**7s**	**8s**	**9s**	n.d.[b]
10.0	0	25	Mg^{2+}	–	–	–	91.1	–	–	8.9
5.0	0	25	Mg^{2+}	–	–	–	84.3	–	12.32	3.3
8.0	0	25	Mg^{2+}	5.7	2.8	7.4	29.3	28.2	10.4	16.1
8.0	0	4	Mg^{2+}	5.9	2.3	6.2	37.8	27.2	11.7	8.9
8.0	0	37	Mg^{2+}	7.1	5.3	12.3	22.3	22.3	13.3	17.3
8.0	1	25	Mg^{2+}	4.8	1.5	4.2	17.7	33.5	10.2	28.0
8.0	0	25	Mn^{2+}	5.5	–	2.3	48.7	20.2	7.1	16.2
8.0	0	25	K^+	10.7	3.7	10.3	19.4	34.2	15	11.3

Structures corresponding to compound numbers shown are found in Scheme 1. [a] Percentages are averages calculated from product profiles of three independent in vitro reactions. The error was less than 5% in all cases. [b] Sum of minor products that could not be identified.

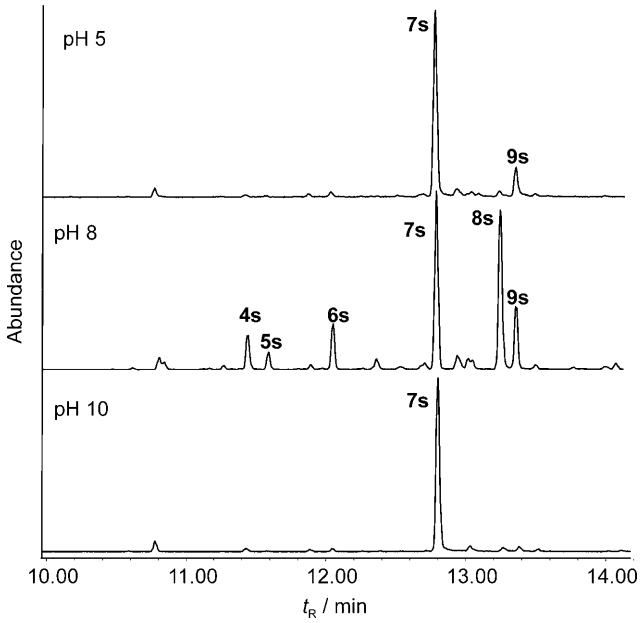


Figure 2. Effect of pH on the product selectivity of Cop4 with (*E*,*E*)-FPP. Reactions of purified Cop4 with (*E*,*E*)-FPP were carried under different pH conditions. Reaction products were separated by GC–MS, and the compound peaks were identified by comparison of mass spectra and RI values with those in reference libraries and with authentic standards.

the differences observed in their catalytic fidelities. Models in the open (no substrate and Mg^{2+} bound) and closed (Mg^{2+} and pyrophosphate (PPi) bound in the active site) conformation were built by using the structure of trichodiene synthase from *F. sporotrichoides*[48,52] for Cop6 and of aristolochene synthase from *A. terreus*[20] for Cop4. The generated models for Cop4 and Cop6 are in very good agreement with their respective templates, and conformational differences observed between the open and closed template crystal structures are also reflected in the two models.

Hydrogen-bond interactions and metal ion coordination in the PPi-bound closed conformation of the Cop models were compared to their template structures (Figure 3A and B). Residues of the two conserved motifs (DDXXD, NSE/DTE) and basic motif (RY) participating in the coordination of the $Mg^{2+}{}_3$–PPi complex in trichodiene synthase and aristolochene synthase are in perfect alignment with corresponding residues in the two Cop models; although M228 in Cop4 cannot make an ionic interaction with PPi as does the equivalent residue K226 in aristolochene synthase. The three Mg^{2+} ions assume similar positions in the two Cop models, but the PPi is rotated by 180° in the Cop4 binding pocket. Residue R304 in trichodiene synthase and residue R302 in the Cop6 model form a charge–charge interaction with the second aspartate residue in the conserved DDXXD motif (D103 and D101 in Cop6 and trichodiene synthase respectively). The corresponding residues in Cop4 (E88) and aristolochene synthase (E94), however, are not positioned to form a similar interaction.

Comparison of the active-site cavities of the Cop4 and Cop6 models in the open conformation shows that Cop4 has a much larger binding pocket compared to the narrow active-site cleft seen in the Cop6 model (Figure 3C and D). The binding pocket of the Cop4 model is much larger with more space at the cavity bottom compared to its template structure, aristolochene synthase. The active-site clefts of the Cop6 model and trichodiene synthase, however, are comparable. Volume calculations by using CASTp indicate an active-site cavity volume for Cop4 of 3376 Å^3 that is twice that measured for Cop6 (1695 Å^3), trichodiene synthase (1740 Å^3), and aristolochene synthase (1742 Å^3). The trinuclear Mg^{2+} cluster and the ligated PPi of the substrate form a plane at the entrance of the binding pocket, whereas the isoprenoid chain extends into the binding cavity. The much larger active-site cavity of Cop4 suggests that its binding pocket can accommodate multiple isoprenoid chain conformation, thus explaining the larger number of cyclization products obtained with this enzyme.

Superimposition of the Cop models with their respective fungal template structures (Figure 3E) shows that all four terpene synthases share the same α-helical fold with six helices surrounding the active-site cavity. A notable difference between the four structures is the length of the loop (H-α-1 loop in trichodiene and aristolochene synthase[20,53]) that caps the active site in the ligated, closed enzyme conformation. In the trichodiene synthase structure and Cop6 model, this loop is shorter than the other structures. Aristolochene synthase has a particularly long and flexible loop that is disordered in the open conformation. A comparison of the primary sequence of the loops shows that five out of nine residues in the Cop4 loop are basic; this results in a localized positive charge at the entrance of the binding pocket under neutral pH conditions (Figure 3E). The loop of Cop6 on the other hand is composed of mostly acidic and hydrophobic residues.

Discussion

Cop4 and Cop6 have divergent product selectivities and cyclize (*E*,*E*)-FPP via a (6*R*)-bisabolyl cation to different products

The analysis of (*E*,*E*)-FPP conversion performed in this study with purified enzymes confirms the broad product selectivity of Cop4 and the high selectivity of Cop6 previously observed in recombinant *E. coli* cultures (Table 2). However, whereas δ-cadinene (**9s**) is the major product of Cop4 in *E. coli*, (−)-germacrene D (**7s**) and cubebol (**8s**) are the major compounds made by Cop4 in vitro.

The proposed reaction mechanism for (*E*,*E*)-FPP cyclization by Cop4 involves the 1,10-cyclization of a *cisoid* neryl cation to form a (*Z*,*E*)-germacradienyl cation, which undergoes a 1,3-hydride shift to form an allylic carbocation that is either deprotonated to yield (−)-germacrene D (**7s**), the major product of Cop4, or 1,6-cyclized to the bicyclic cadinyl cation (Scheme 1). Deprotonation of this cation would produce δ-cadinene (**9s**), whereas 1,2-hydride shift followed by 2,6-ring closure and quenching of the ensuing carbocation with H_2O yields the tricyclic cubebol (**8s**) as the second major product of Cop4. Labeling studies with deuterated (*E*,*E*)-FPP and reactions with (3*R*)-NPP by using a recombinant sesquiterpene synthase from cotton have confirmed that δ-cadinene biosynthesis requires

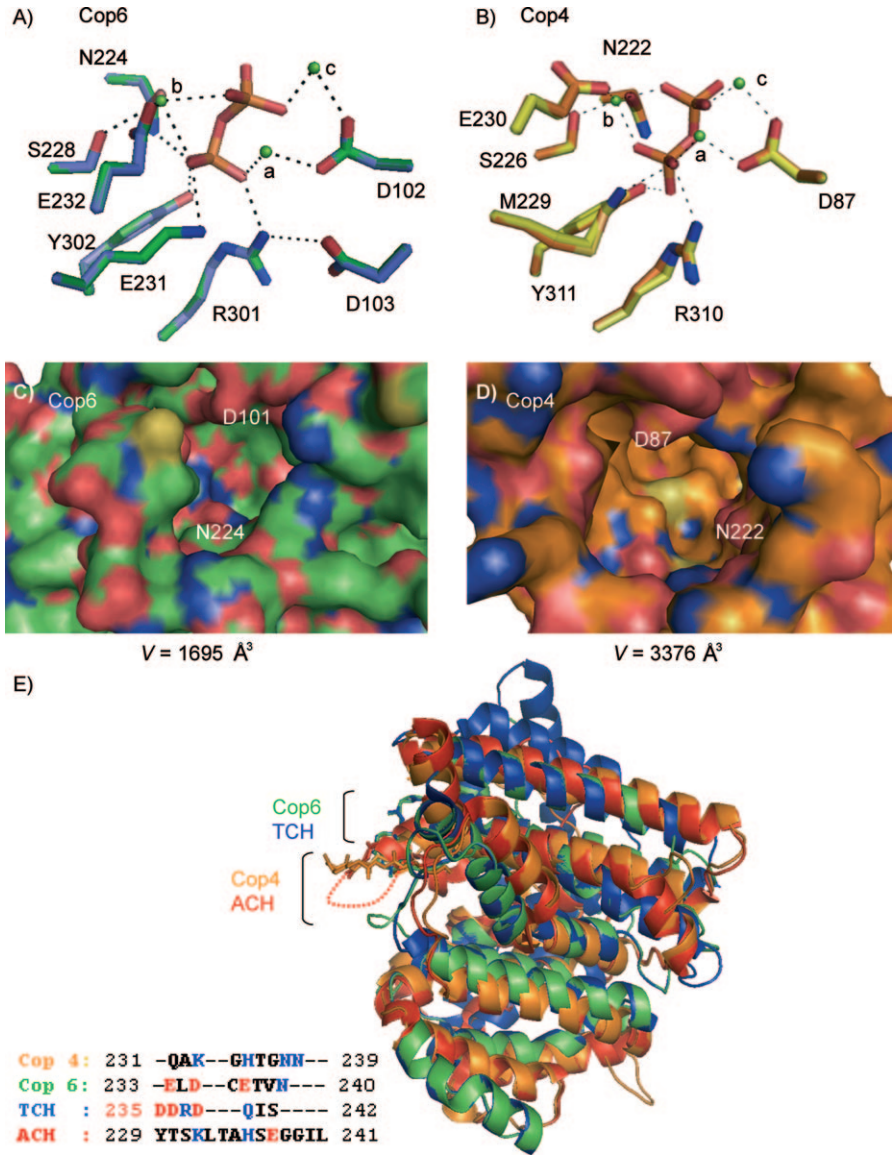


Figure 3. Structural modeling of Cop4 and Cop6 based on the structures of aristolochene synthase (ACH)[20] and trichodiene synthase (TCH),[48] respectively. Top panels: Hydrogen bond and metal coordination interactions in the enzyme–$Mg2+$–PPi complex for the A) Cop6 (green side chains) and B) Cop4 (orange side chains) models. Depicted side chains of the conserved DDXXD/E, NSE/DTE and basic motif (XRY) are superimposed with corresponding side chains from their respective template structures (TCH, purple; ACH, yellow). Mg^{2+} ions are shown in green and complexed PPi is located in the center of the networks. Center panels: View into the active-site cavities of unligated C) Cop6 (green alpha carbons) and D) Cop4 (orange alpha carbons). Positively charged and negatively charged amino acid residues are shown in blue and red respectively. Bottom panel: Superimposition of the unligated Cop4 and Cop6 models with the unligated structures of THC and ACH (E). Loops covering the active sites of the enzymes are labeled and colors correspond to those of their α-carbon backbones in the superimposition. Inset shows the amino acid residues of the different loops. Basic (blue) and acidic (red) amino acid residues are highlighted.

the isomerization (*E*,*E*)-FPP.[28,32] A similar mechanism via (3*R*)-NPP has also been described for the synthesis of (−)-germacrene D by germacradienol/germacrene D synthase from *Streptomyces coelicolor*.[54] It should be noted that (−)-germacrene D can also be derived from the (*E*,*E*)-germacradienyl cation,[55,56] but cyclization products derived from a cadinyl cation bearing a C2–C3 π bond in a *cis* configuration require the isomerization of (*E*,*E*)-FPP.

Unlike Cop4, Cop6 makes almost exclusively (−)-α-cuprenene (**3s**; 98%). Comparable selectivities for their major terpene products have been reported for trichodiene synthase (89%),[48] aristolochene synthases from *A. terreus* (>99%), and *P. roqueforti* (94%)[57] and δ-cadinene synthase from cotton (98%).[32] The proposed reaction mechanism for the cyclization of (*E*,*E*)-FPP to (−)-α-cuprenene by Cop6 (Scheme 1) follows the same cyclization route described for trichodiene synthase until the formation of the (7*R*)-cuprenyl cation,[13,33,42] at which point wild-type trichodiene synthase continues with a carbocation rearrangement whereas Cop6 and some trichodiene synthase mutants[35,44,48,58] quench the carbocation to form (−)-α-cuprenene. Specifically, Cop6 and trichodiene synthase first generate a (6*R*)-β-bisabolyl carbocation from a *cisoid* neryl cation following 1,6-ring closure. A second 11,7-ring closure and 1,4-hydride shift then yields the (7*R*)-cuprenyl cation, which is deprotonated by Cop6, whereas trichodiene synthase catalyzes two additional methyl shifts prior to deprotonation.

The remarkable differences in product selectivity observed with Cop4 and Cop6 correlate with the different sizes of their active sites. Whereas, Cop6 has a relatively narrow active-site cleft that likely binds (*E*,*E*)-FPP with high accuracy in one cyclization-competent conformation, the large active-site cavity of Cop4 allows a much higher degree of conformational freedom for cyclization intermediates, giving rise to multiple alternative cyclization pathways. Interestingly, the larger binding pocket of Cop4 does not support the 1,6-cyclization of (3*R*)-NPP to the β-bisabolyl cation, although this monocyclic cyclization product with its flexible isoprenoid tail is accommodated in the much smaller active-site cleft of Cop6. Instead, Cop4 exclusively catalyzes the 1,10-cyclization of the proposed (3*R*)-NPP intermediate and multiple reaction pathways become possible only after this initial cyclization step.

Similar product diversification after initial farnesyl cation cyclization is also observed for other terpene synthases,[14,59,60] whereas for instance, γ-humulene synthase catalyzes different ring closures of a *cisoid* neryl cation.[15]

Both the high-fidelity enzyme Cop6 and the promiscuous Cop4 bind (*E*,*E*)-FPP with comparable affinity (Table 1), but the catalytic turnover of Cop4 is about 70-fold lower than the k_{cat} of Cop6. The steady-state kinetic properties of Cop4 suggest that it binds (*E*,*E*)-FPP with high affinity in one selective conformation that yields the (*Z*,*E*)-germacradienyl cation. Once this carbocation is formed, multiple conformations, including unproductive cyclization conformations might become possible, thus reducing the overall catalytic efficiency of this enzyme.

(*Z*,*E*)-FPP is cyclized by Cop4 and Cop6 via a (6*S*)-β-bisabolyl cation intermediate

(*Z*,*E*)-FPP has been used as a surrogate substrate for sesquiterpene synthases that isomerize the C2–C3 π bond of (*E*,*E*)-FPP to form a *cisoid* neryl cation.[33,36–38,46] Cop4 catalyzes the cyclization of the *cis*-FPP isomer with only a modestly reduced binding affinity (K_m) and catalytic turnover (k_{cat}; Table 1); this suggests that its large active-site cavity can readily accept the sterically more demanding *cis*-FPP isomer. Cop6 on the other hand shows measurable activity with (*Z*,*E*)-FPP only after prolonged overnight incubation; this suggests that the *cis*-FPP isomer cannot easily be accommodated by its narrow active-site cleft. Nevertheless, Cop6 can catalyze diphosphate cleavage of (*Z*,*E*)-FPP to initiate subsequent cyclization reactions. The strictly *trans*-pathway-specific enzyme NS1 expectedly does not convert (*Z*,*E*)-FPP because it either is unable to bind (*Z*,*E*)-FPP or catalyze diphosphate cleavage.

Very few studies have compared the cyclization products obtained with the two FPP geometric isomers, although pioneering work by Croteau's group on the cyclization mechanism of monoterpene synthases investigates the cyclization products obtained with (*E*)-GPP and (*Z*)-GPP.[49,61,62] Recent studies with sesquiterpene synthases TPS4, TPS6, and TPS11 from maize report comparable product profiles with both FPP isomers.[36–38] An enzyme preparation from the liverwort *Heteroscyphus planus*[46] converted (*E*,*E*)-FPP and (*Z*,*E*)-FPP to different isomeric cadinanes, but the cyclization paths of the two FPP substrates diverge only after 1,10-cyclization of the *cisoid* farnesyl cation intermediate. The maize enzymes and the liverwort enzyme preparation therefore appear not to discriminate between (*E*,*E*)- or (*Z*,*E*)-FPP when it comes to generation and cyclization of the first *cisoid* allylic carbocation intermediate. Cop4 and Cop6, however, yield very different products with (*E*,*E*)- and (*Z*,*E*)-FPP. As discussed below, the two Cop enzymes must bind the two geometric isomers as different conformers, resulting in the generation of opposite enantiomers of the first cyclic cation intermediate. This has not been observed before, and it remains to be seen whether other *cis*–*trans*-pathway-specific sesquiterpene synthase show the same discrimination between the two geometric FPP isomers like Cop4 and Cop6 or generate the same products with both isomers like the maize enzymes.[36–38]

In detail, Cop4 catalyzes a 1,10-ring closure of a *cisoid* neryl cation derived from its normal substrate (*E*,*E*)-FPP. Despite its large active-site cavity, Cop4 is unable to catalyze the same cyclization reaction with a *cisoid* carbocation derived from (*Z*,*E*)-FPP. Instead Cop4 now catalyzes a 1,6-ring closure to generate a β-bisabolyl cation intermediate from (*Z*,*E*)-FPP (Table 2, Scheme 2).

Remarkably, Cop6 cyclizes (*Z*,*E*)-FPP through a (6*S*)-β-bisabolyl cation intermediate rather than the (6*R*)-β-bisabolyl cation postulated for Cop6 with (*E*,*E*)-FPP. Cop4 also cyclizes (*Z*,*E*)-FPP through a (6*S*)-β-bisabolyl cation intermediate. This means that the *cis*-FPP isomer must bind as the right-handed helical conformer in the active sites of the Cop enzymes, whereas the normal *trans*-FPP substrate must bind as the left-handed helical conformer in the two active sites to explain the different stereochemistries seen in the cyclization pathways of the two FPP substrates.[50]

FPP adopts an extended conformation in solution,[63] but the active site of a terpene synthase must bind FPP in a helical conformation to facilitate cyclization. It is obvious that the conformation of the substrate in the active site controls the stereochemical course of the cyclization reaction. Crystallographic studies of aristolochene synthase and fluorinated FPP analogues suggest that binding of the substrate diphosphate moiety to the trinuclear magnesium cluster triggers active-site closure and controls the conformation of the FPP substrate in the active site.[64] The diphosphate moiety of different substrates/analogues will likely be bound in the same orientation independently of its relative position in the isoprenoid chain of different substrates. Hence, the relative position of the diphosphate group in the substrate will determine the positioning of the isoprenoid moiety in the active site of each terpene synthase. Because the diphosphate group is positioned differently in (*Z*,*E*)-FPP compared to (*E*,*E*)-FPP, it is safe to assume that the isoprenoid chain of the two FPP geometric isomers adopt different binding conformations in the active sites of Cop4 and Cop6. Alternatively or in addition, isomerization of (*E*,*E*)-FPP might control the binding conformation of the isoprenoid chain as has been demonstrated for limonene synthase by using different substrate isomers.[65] In contrast, maize terpene synthase TPS4 not only cyclizes (*E*,*E*)-FPP and (*Z*,*E*)-FPP to similar products, but appears to bind each geometric isomer both in the right and left-handed conformations, resulting in cyclization products that are derived from both the (6*S*)- and (6*R*)-β-bisabolyl cation intermediate.[37] A structural model of this sesquiterpene synthase suggests that its active-site cavity has two binding pockets that can accommodate the two different β-bisabolyl cation enantiomers.[37] The structural models of Cop4 and Cop6 do not indicate the existence of two active-site binding pockets.

Cop4 and Cop6 convert (*E*)-GPP into cyclic and acyclic monoterpenes

Sesquiterpene synthases are known to accept (*E*)-GPP as a substrate to produce monoterpenes.[15,30,39–41]All three enzymes tested in this study expectedly catalyze the conversion of (*E*)-

GPP into different monoterpenes (Table 1). Cop4 binds (*E*)-GPP with a much-reduced affinity compared to both FPP isomers. Its large binding cavity most likely does not provide a good fit for binding of the shorter substrate in a productive conformation. Cop6, on the other hand, can convert (*E*)-GPP with only a moderately reduced efficiency compared to (*E*,*E*)-FPP, but its fidelity suffers with this non-natural substrate, resulting in the generation of multiple products (Table 2, Scheme 3). In contrast to (*E*,*E*)-FPP, (*E*)-GPP likely adopts multiple conformations in the enzyme's binding pocket, which causes different catalytic outcomes.

Studies with monoterpene synthases have shown that only terpene synthases that can isomerize the C2–C3 π bond of *E*-GPP can make cyclic monoterpenes.[49,50] Likewise, only sesquiterpene synthases known to isomerize the C2–C3 π bond of (*E*,*E*)-FPP have been reported to synthesize cyclic products from (*E*)-GPP.[15,30,39–41] In this study, only Cop4 and Cop6 produce monocyclic monoterpene olefins (**4m**, **5m**, **6m**), whereas the strictly *trans*-pathway-specific enzyme NS1 only makes acyclic monoterpenes (**1m**, **2m**, **3m**). The absence of any detectable cyclic products with NS1 confirms its inability isomerize the C2–C3 π bond of allylic isoprenoid diphosphate substrates.

The acyclic monoterpenes (*Z*)-β-ocimene (**1m**) and linalool (**3m**) make up more than 90% of the monoterpenes produced by NS1 (Table 2). Their formation can be rationalized to originate from a *transoid*, *endo* conformation of the geranyl cation, whereas the minor NS1 cyclization product (*E*)-β-ocimene (**2m**) originates from a *transoid*, *exo* conformation of the bound prenyl chain.[49] The same monocyclic terpenes can also be obtained from a *cisoid* geranyl cation (neryl cation) in which the *exo* conformation would now yield **1m** and **3m**, whereas the *endo* conformation would give **2m**. Cop4 and Cop6 convert 30% and 50%, respectively, of (*E*)-GPP into cyclic monoterpene products (Table 2). With both enzymes, limonene (**4m**) is synthesized as the major cyclic monoterpene product. Limonene can be derived from either a *cisoid*, *exo* or *cisoid*, *endo* conformation of the initial geranyl cation.[49] The accumulation of **1m** (Cop4) and of **1m** and **3m** (Cop6) as major acyclic products by the two Cop enzymes, suggests that the *cisoid* geranyl cation is likely predominantly bound in an *exo* conformation, which yields both acyclic and cyclic products.

Reaction conditions strongly influence the product profile of Cop4 but not of Cop6

A major determinant for product selectivity is the degree of conformational flexibility that the substrate possesses in the active site of a terpene synthase. Hence, Cop6 with its narrow binding pocket is a high-fidelity enzyme, whereas the large cavity of Cop4 yields multiple cyclization products (Table 2). Consequently, by modifying the conformational flexibility and/or fit of the bound substrate in the active site, the fidelity of the cyclization reaction might be altered. This can be done by protein engineering or modifying the physical environment of an enzyme. Whereas there are many examples that use protein engineering to explore the catalytic promiscuity of terpene synthases,[7,9] only very few studies have investigated how reaction conditions affect the fidelity of terpene synthases.[9,38,40,51,66,67]

In this study we have compared the effects that different reaction conditions have on the cyclization fidelity of Cop4 and Cop6. As expected, the product profile of Cop6 never changed in response to any of the conditions tested because its active site must provide a rigid template for binding of (*E*,*E*)-FPP in an optimal conformation (Table 2). In contrast, the product profile of the low-fidelity Cop4 was easily altered by varying reaction conditions (Table 3, Figure 2).

Substitution of Mg^{2+} with Mn^{2+} as the divalent metal ion shifts the product profile of Cop4 to germacrene D, disfavoring subsequent ring closures that would produce the cadinyl cation and its tricyclic descendents (Scheme 1). The larger size of the Mn^{2+} ion has been suggested to reduce the size of the active site of terpene synthases and thus, change the conformational flexibility of the bound substrate.[68] The product selectivity of amorphadiene-4,11-diene synthase increased from 80 to 90% for amorphadiene in the presence of Mn^{2+}.[68] In other examples, replacement of Mg^{2+} with Mn^{2+} increases premature quenching of carbocation intermediates.[38,40]

Because temperature influences both protein (and hence, flexibility of the active site) as well as prenyl chain motion, product fidelity is expected to decrease with increasing temperature. A modest decrease in catalytic fidelity at higher reaction temperature has been observed with *epi*-aristolochene synthase.[9] Temperature has a much more pronounced effect on the cyclization fidelity of Cop4 (Table 3).

The most notable and dramatic effect on the catalytic fidelity of Cop4 enzyme was obtained when enzyme reactions are carried out under alkaline (pH 10) or acidic (pH 5.0) conditions. Under both conditions, Cop4 becomes a very selective germacrene D synthase (Table 3, Figure 2). At pH 10, none of the cadinyl-cation-derived products at pH 8 are present in the reaction. The strong effect of pH on product selectivity seen with Cop4 is in contrast to what has been observed with amorphadiene-4,11-diene synthase.[68] The product selectivity of this enzyme was not influenced by the surrounding pH, which was explained by supposing that the loop covering the active site in the closed conformation will completely shield the active side from outside solvent effects. The product selectivity of Cop6 also is not affected by the pH of the reaction. Apart from the size of their active sites, Cop4 and Cop6 show differences in their loops that are supposed to cover the active site upon substrate binding. In contrast to Cop6 (and also to trichodiene and aristolochene synthase), Cop4 contains a larger number of basic amino acid residues in its loop (Figure 3E). The histidine side chain in the Cop4 loop, in particular, will have a strong impact on the net charge of the loop at different pH values. Changes in its net charge will impact interactions with residues of the active-site entrance and thus might influence protein conformation and consequently, diphosphate binding, positioning, and ionization of the substrate in the binding pocket. Mutagenesis studies have so far largely been focused on residues lining the active-site entrance that are directly involved in coordinating the trinuclear Mg^{2+} cluster and binding of the substrate diphosphate.[13,34,35,48,53] The results obtained in this

work with Cop4 suggest that this lid might play an important role in catalysis. Additional mutagenesis studies in the lid regions of terpene synthases are necessary to define their functions and explore whether changes in this region impact the catalytic promiscuity of terpene synthases.

Conclusions

In this work we describe the reaction mechanism of two new fungal sesquiterpene synthases that both isomerize the C2–C3 π bond of (*E*,*E*)-FPP via an NPP intermediate prior to catalysis of subsequent cyclization reactions. We show that Cop6 is a high-fidelity enzyme whereas Cop4 is very promiscuous, generating multiple cyclization products from (*E*,*E*)-FPP. The fidelity of Cop6 with (*E*,*E*)-FPP can be attributed to the enzymes' narrow active site, which restricts binding of (*E*,*E*)-FPP to one cyclization-competent conformation. In contrast, the promiscuous Cop4 has a large active-site cavity that allows binding of (*E*,*E*)-FPP in several cyclization-competent conformations.

We demonstrate that the promiscuity of Cop4, but not of Cop6, is strongly influenced by the reaction conditions. Changing the pH of the reaction dramatically changed the product profile of Cop4, converting the enzyme from a very promiscuous sesquiterpene synthase into a high-fidelity enzyme. Such a dramatic effect of reaction condition on the product profile of a terpene synthase has not been reported previously. Inspection of the Cop4 structural model and comparison with structures of other fungal sesquiterpene synthases and the Cop6 model suggests that the amino acid residues of the loop that covers the active site of terpene synthases might be important in determining the cyclization products of sesquiterpene synthases. Hence, mutating the loop region of terpene synthase might be a different strategy for engineering of terpene synthases with desired product profiles. Studies are underway to test this approach with different terpene synthases.

Finally, by analyzing the cyclization products generated by Cop4 and Cop6 with the two geometric isomers of FPP, we show for the first time that the two FPP isomers must be bound as different helical conformers in the active sites of Cop4 and Cop6 to rationalize the different cyclization products obtained with each FPP isomer. This observation is in contrast to the results obtained with maize terpene synthases,[37] which produce the same products with either FPP isomer. However, these plant enzymes seem to have two FPP-binding pockets in their active site that can accommodate both helical FPP conformers, whereas the two Cop enzymes like many other *cis*–*trans*-pathway-specific enzymes have only one binding pocket. Studies with other *cis*–*trans*-pathway enzymes to determine the cyclization products with both geometric isomers of FPP should confirm our observations.

Experimental Section

Chemicals: (*E*,*E*)-FPP and *E*-GPP were purchased from Sigma–Aldrich. (*Z*,*E*)-FPP was synthesized from (*E*,*E*)-farnesol as described by Shao et al.[69] DNA-modifying enzymes were obtained from New England Biolabs; (6*S*)- and (6*R*)-β-bisabolene standards were gifts from Prof. Jörg Degenhardt, Max Planck Institute for Chemical Ecology, Jena, Germany. α-Cuparene (98% (+)-enantiomer) was obtained from Chromadex (Irvine, CA). Other chemicals were from suppliers as described or from Sigma–Aldrich. Nerolidol was purchased from Sigma–Aldrich and phosphorylated according to the method of Popjak et al.[70] with one modification. After all the diethylamine phosphate was added and left stirring for 2 h, the crude reaction mixture was purified by RP-HPLC (solvent A: 0.1% trifluoroacetic acid (TFA) in H_2O, solvent B: 0.1% TFA in MeCN, gradient: 0% B over 5 min, 0–60% B over 25 min, 60–100% B over 5 min). Nerolidyl diphosphate elutes at 42% B and was subsequently lyophilized to give a white powder. Such powder was dissolved in methanol/water (3:7) at 1 mM final concentration.

Strains and growth conditions: *E. coli* strain JM109 was used for cloning and recombinant proteins were expressed in *E. coli* strain BL21 (DE3). *E. coli* cultures were grown in Luria–Bertani (LB) medium supplemented with appropriate antibiotics ampicillin (100 μg mL^{-1}) or kanamycin (30 μg mL^{-1}) at 30 °C, 250 rpm.

Gene cloning: Genes encoding Cop6 and Cop4 were subcloned from their respective pUCmod plasmids[24,25] into the NdeI and NotI sites of pHIS8,[71] in the case of Cop6, and into the NdeI and XhoI of pET21b (Novagen), in the case of Cop4, for overexpression under the control of the T7 promoter. Cop4 and Cop6 are expressed with a His_6 (pET21b-Cop4) or His_8 tag (pHIS8-Cop6) added to their N termini. Cloning of NS1 into the expression vector pET21b (pET-NS1) is described in ref. [27]. The Cop6 N224D mutant was obtained by overlap extension PCR by using mutagenic forward and reverse oligonucleotide primer. The PCR product was digested with NdeI and NotI for cloning into plasmid pHIS8.

Protein expression and purification: Expression vectors pHIS8-Cop6 and pET21b-Cop4 were transformed into *E. coli* BL21 (DE3). For protein overexpression, a culture (50 mL) was inoculated with an overnight culture (1 mL) and grown at 30 °C until it reached an OD_{600} of 0.6, at which point protein expression was induced by the addition of 1 mM IPTG and cultivation was continued for 18 h at 30 °C. Cells were harvested by centrifugation and stored at −20 °C until used. For protein purification, harvested cells were resuspended in terpene synthase buffer (10 mM Tris–HCl, 10 mM $MgCl_2$, and 1 mM β-mercaptoethanol at pH 8.0) and sonicated. Cell debris was cleared by centrifugation, and the cleared protein extract was purified by metal affinity chromatography. Soluble protein was loaded onto a Talon Resin (Invitrogen) equilibrated with terpene synthase buffer containing 10 mM imidazole. Following protein binding to the column, the column was washed three times with terpene synthase buffer containing 20 mM imidazole prior to elution with 300 mM imidazole. Overexpression and purification of recombinant sesquiterpene synthase NS1 followed the same procedure previously described in ref. [27]. Protein concentrations were determined by using Bradford reagent (BioRad).

Kinetic parameters: Steady-state kinetics of sesquiterpene synthases (Cop6, Cop4, and NS1) were determined with varying concentrations (1–100 μM) of prenyl diphosphate substrates ((*E*,*E*)-FPP, (*Z*,*E*)-FPP and *E*-GPP) by measuring the release of pyrophosphate (PPi) as described in ref. [27]. Briefly, PPi was detected by using a coupled enzyme system consisting of PPi-dependent fructose-6-phosphate kinase, aldolase, triosephosphate isomerase, and α-glycerophosphate dehydrogenase. The enzymes are supplied as pyrophosphate reagent by Sigma–Aldrich (product number P7275) and were reconstituted in assay buffer prior to use (16.7 mg in 1 mL terpene synthase buffer). PPi release was measured by the consumption of NADH, resulting in a decrease in absorbance at

340 nm. Microplate assays were carried out with pyrophosphate reagent (50 μL), assay buffer (90 μL), and varying concentrations of different substrates (10 μL). Blank reactions without substrate were run in parallel. Assay mixtures were allowed to equilibrate for 5 min at 30 °C prior to the addition of enzyme (5 μL; 0.2 mg mL^{-1}) to start the reaction. The activity was determined as the difference between the decrease of absorbance per minute of the sample and of the blank. By using an extinction coefficient for NADH of $\varepsilon_{340\,nm} = 6.22 \times 10^3$ M^{-1} mL^{-1}, one unit of activity was defined as the amount of enzyme needed to release 1 μmol of PPi, inducing the consumption of 2 μmol of NADPH. The K_m and V_{max} values were determined by using a nonlinear fit of *V* versus [S] plot. The analysis was carried out by running a macro in Xcel 2007.

In vitro analysis of sesquiterpene product profiles: Sesquiterpene product profiles of Cop4, Cop6 and NS1 were analyzed by incubating purified enzyme (20 μL; 0.1 mg mL^{-1} in the case of Cop6 and 0.2 mg mL^{-1} in the case of Cop4 and NS1) in terpene synthase buffer (180 μL) containing one of the four prenyl diphosphate substrates investigated ((*E*,*E*)-FPP, (*Z*,*E*)-FPP, (±)NPP, and (*E*)-GPP) to yield a final assay concentration of 100 μM. Reactions were carried out in a glass vial for 18 h at 25 °C before the headspace of the glass vial was sampled for 10 min by solid-phase microextraction (SPME) by using a 100 μm polydimethlysiloxane fiber from (Supelco/Sigma–Aldrich). After 10 min absorption, the fiber was inserted into the injection port of a GC–MS for thermal desorption.

To measure the influence of reaction conditions on the product profiles of Cop4 and Cop6 with 100 μM (*E*,*E*)-FPP as the substrate, the terpene synthase buffer was modified by the addition of NaCl or KCl (final assay concentration: 1 M) or substitution of 10 mM $MgCl_2$ with 10 mM $MnSO_4$. The pH of the reactions was changed by substituting 10 mM Tris–HCl in the terpene synthase buffer with 10 mM of sodium carbonate (pH 10.0) or 10 mM of sodium acetate (pH 5.0) buffer. Reactions were carried out for 18 h at 25, 4, and 37 °C prior to the analysis of sesquiterpene hydrocarbon products as described above.

Gas chromatography–mass spectrometry (GC–MS) analysis: GC–MS analysis was carried out on a HP GC 7890 A coupled to anion-trap mass spectrometer HP MSD triple axis detector (Agilent Technologies). Separation was carried out by using a HP-1MS capillary column (30 m × 0.25 mm, i.d.: 1.0 μm) with an injection port temperature of 250 °C and helium as a carrier gas. Mass spectra were recorded in electron-impact ionization mode. Volatile compounds adsorbed on a fiber from the enzyme reaction headspace were desorbed for 10 min in the injection port. The temperature program started at 60 °C and ramped up 8 °C min^{-1} to a final oven temperature of 250 °C. Mass spectra were scanned in the range of 5–300 atomic mass units at 1 s intervals.

For product identification, the retention index (RI) of each compound peak was determined by calibrating the GC–MS first with a C8–C40 alkane mix. Retention indices and mass spectra of compound peaks were compared to reference data in MassFinder's (software version 3) terpene library.[47] In addition, essential oils with known terpene compositions were used as authentic standards as described in Table S1.

Absolute configuration determination: To determine the absolute configuration of several sesquiterpenes described in this study, we used chiral GC–MS analysis for comparison of retention times with reference compounds. Sesquiterpenes were separated on a Quiral β-cyclodextrin column (25 m × 0.25 mm × 0.125 μm; Chirasil-Dex, Varian Inc.) by using a temperature program that started at 40 °C for 2 min followed by ramping the temperature at 3 °C min^{-1} to a final oven temperature of 200 °C. Mass spectra were scanned in the range of 5–300 atomic mass units at 1 s intervals.

For chiral GC–MS analysis of β-bisabolene, racemic β-bisabolene was kindly provided by Prof. Degenhardt and used as an authentic standard for comparison with Cop4 and Cop6 reaction products (Figure 1). Enantiomers were assigned by comparison with the β-bisabolene present in Bergamot essential oil, which contains only (6*S*)-β-bisabolene (**16s**), as described by Köllner et al.[38] The absolute configuration of germacrene D (**7s**) synthesized by Cop4 was determined by comparison with germacrene D enantiomers from the essential oil of *Solidago canadensis*. In this essential oil, the (+) enantiomer is more abundant (Figure S4).[72] *Amyris balsamifera* essential oil contains only one enantiomer of cadina-4,11-diene (**14s**) with known absolute configuration.[47] Cadina-4,11-diene (**14s**) in this essential oil was therefore used as reference compound to determine the absolute configuration of the cadina-4,11-diene (**14s**) that was synthesized by Cop4 with (*Z*,*E*)-FPP as the substrate (Figure S6). Finally, the absolute configuration of the Cop6 reaction product α-cuprenene (**3s**) was indirectly determined by comparison of its oxidation product, α-cuparene with a synthetic standard compound containing 98% (+)-α-cuparene and 2% (−)-α-cuparene. Dauben and Oberhänsli[73] reported the isolation and synthesis of cuprenenes that under retention of absolute configuration slowly convert into the corresponding, aromatic cuparenes after prolonged air exposure. In vitro reactions of Cop6 with (*E*,*E*)-FPP as a substrate were therefore left standing for up to 30 d at 30 °C with periodic analysis of products formed. Over time, the ring-oxidized α-cuparene accumulated (Figure S3).

Structural modeling of Cop4 and Cop6 sesquiterpenes synthases: Structural models in the open, unligated conformation were built by using the structure of trichodiene synthase from *F. sporotrichoides*[52] (PDB ID: 1JFA, chain A) for Cop6 (44% amino acid sequence similarity) and of aristolochene synthase from *A. terreus* (PDB ID: 2E40, chain D)[20] for Cop4 (39% amino acid sequence similarity). Crystal structures of trichodiene (PDB ID: 2Q9z, chain B)[48] and of aristolochene synthase (PDB ID: 2A6, chain D)[20] in the closed formation, ligated with Mg^{2+} and pyrophosphate (PPi), were used to build the corresponding models for Cop6 and Cop4. Models were built by using the alignment mode of the Swiss Model homology-modeling server .[74] This method assesses protein structures by using 3D profiles. Structures are validated by comparison of an atomic model with its amino acid sequence and assignment of positive (good compatibility) or negative scores for each amino acid position. Models generated in this study have very good compatibility scores. Protein models were visualized and aligned with their template structure by using PyMol 0.99 developed by DeLano Scientific LLC (San Francisco, CA). Active site volumes were calculated with CASTp[75] (by using the CASTpyMol version 2.0).

Abbreviations: FPP, farnesyl diphosphate (also-pyrophosphate); NPP, nerolidyl diphosphate; GPP, geranyl diphosphate; PPi, pyrophosphate.

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