

Exploring biosynthetic diversity with trichodiene synthase ☆,☆☆

L. Sangeetha Vedula ^a, Yuxin Zhao ^{b,1}, Robert M. Coates ^b,
Tanetoshi Koyama ^c, David E. Cane ^d, David W. Christianson ^{a,*}^a Roy and Diana Vagelos Laboratories, Department of Chemistry, University of Pennsylvania, Philadelphia, PA 19104-6323, USA^b Department of Chemistry, University of Illinois, Urbana, IL 61801, USA^c Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, Aoba-ku, Sendai 980-8577, Japan^d Department of Chemistry, Brown University, Providence, RI 02912-9108, USA

Received 25 May 2007, and in revised form 14 June 2007

Available online 28 June 2007

Abstract

Trichodiene synthase is a terpenoid cyclase that catalyzes the cyclization of farnesyl diphosphate (FPP) to form the bicyclic sesquiterpene hydrocarbon trichodiene (89%), at least five sesquiterpene side products (11%), and inorganic pyrophosphate (PP_i). Incubation of trichodiene synthase with 2-fluorofarnesyl diphosphate or 4-methylfarnesyl diphosphate similarly yields sesquiterpene mixtures despite the electronic effects or steric bulk introduced by substrate derivatization. The versatility of the enzyme is also demonstrated in the 2.85 Å resolution X-ray crystal structure of the complex with Mg²⁺₃-PP_i and the benzyl triethylammonium cation, which is a bulkier mimic of the bisabolyl carbocation intermediate in catalysis. Taken together, these findings show that the active site of trichodiene synthase is sufficiently flexible to accommodate bulkier and electronically-diverse substrates and intermediates, which could indicate additional potential for the biosynthetic utility of this terpenoid cyclase.

© 2007 Elsevier Inc. All rights reserved.

Keywords: Terpenoid synthase; Farnesyl diphosphate; Sesquiterpene; Protein crystallography

Sesquiterpene synthases catalyze the cyclization of the universal acyclic precursor, farnesyl diphosphate (FPP),² to form one or more of over 300 known monocyclic, bicyclic, and tricyclic sesquiterpenes with a wide variety of structures and stereochemistry [1–5]. All these reactions

are known to involve a cascade of carbocationic intermediates, and the controlled manipulation of these highly reactive intermediates gives rise to impressive product diversity. Typically, the active site of a terpene cyclase serves as a template that chaperones the conformations and reactions of carbocation intermediates to yield a single or dominant cyclization product. However, some cyclases are more permissive chaperones and can generate a wide array of cyclization products. For example, δ-selinene synthase and γ-humulene synthase from Grand fir (*Abies grandis*) generate 34 and 52 distinct cyclization products, respectively [6].

Trichodiene synthase from *Fusarium sporotrichioides* is an 89 kDa homodimer that catalyzes the cyclization of farnesyl diphosphate to form the bicyclic hydrocarbon trichodiene and the coproduct inorganic pyrophosphate (PP_i) [7,8]. Three Mg²⁺ ions are required to activate the PP_i leaving group and generate a reactive allylic cation that undergoes rapid isomerization to (3*R*)-nerolidyl diphosphate, which subsequently re-ionizes to initiate a complex

[☆] This work was supported by NIH Grants GM56838 (D.W.C.), GM30301 (D.E.C.), and GM13956 (R.M.C.).

^{☆☆} Atomic coordinates of wild-type trichodiene synthase complexed with BTAC and with inorganic pyrophosphate, resulting from the reaction with 2-fluorofarnesyl diphosphate, have been deposited in the Research Collaboratory for Structural Bioinformatics (<http://www.rcsb.org/pdb>) with the following accession codes: 2Q9Y and 2Q9Z, respectively.

* Corresponding author. Fax: +1 215 573 2201.

E-mail address: chris@sas.upenn.edu (D.W. Christianson).

¹ Present address: Takeda San Diego, Inc., 10410 Science Center Drive, San Diego, CA 92121, USA.

² Abbreviations used: FPP, farnesyl diphosphate; PP_i, inorganic pyrophosphate; 2F-FPP, 2-fluorofarnesyl diphosphate; 4M-FPP, 4-methylfarnesyl diphosphate; BTAC, benzyl triethylammonium cation; GC, gas chromatography; MS, mass spectroscopy; r.m.s., root mean square.

cyclization cascade [7,9]. The first cyclic carbocation intermediate in this cascade is the bisabolyl carbocation (Figs. 1 and 2a) [8]. The PP_i coproduct inhibits trichodiene synthase with $K_i = 0.49 \mu\text{M}$ [10], and analogues of the bisabolyl cation such as *R*-azabisabolene (Fig. 2b) require PP_i for measurable inhibition with $K_i = 0.51 \mu\text{M}$ and an induced inhibition constant of $2.6 \mu\text{M}$, with the magnitude of inhibition proportional to the PP_i concentration [10]. X-ray crystallographic analyses of azabisabolene complexes with trichodiene synthase mutants show that the binding of these positively charged inhibitors in multiple conformations is stabilized by $\text{Mg}^{2+}_3\text{-PP}_i$ complexation [11,12]. A similar $\text{Mg}^{2+}_3\text{-PP}_i$ cluster also stabilizes the binding of certain aza analogues to the monoterpene synthase, bornyl diphosphate synthase [13].

We now report the results of incubation of trichodiene synthase with two analogues of the natural FPP substrate, 4-methylfarnesyl diphosphate (4M-FPP) and the less reactive substrate analogue 2-fluorofarnesyl diphosphate (2F-FPP) (Fig. 1). While cyclization of FPP was found to give predominantly trichodiene, accompanied by trace amounts of several aberrant sesquiterpenes previously observed only with trichodiene synthase mutants, cyclization of both FPP analogues gave rise to complex mixtures of sesquiterpene analogues. When crystals of trichodiene synthase were soaked in a buffer solution containing 2F-FPP, the resulting crystals were shown by X-ray crystallography to contain bound $\text{Mg}^{2+}_3\text{-PP}_i$. We also report the crystal structure of the complex of trichodiene with $\text{Mg}^{2+}_3\text{-PP}_i$

and the benzyl triethylammonium cation (BTAC), the chloride salt of which is used as a phase transfer catalyst for synthetic organic chemistry; BTAC partially mimics the bisabolyl carbocation or other carbocation intermediates (Fig. 2a). Taken together, these findings suggest that trichodiene synthase, and perhaps many terpene cyclases, may be capable of substantial versatility in binding and catalysis with unnatural substrates.

Materials and methods

Expression, purification, and crystallization of trichodiene synthase

The plasmid containing the gene encoding for wild-type trichodiene synthase was transformed into *Escherichia coli* BL21 (DE3); the enzyme was overexpressed, purified, and crystallized by the hanging drop vapor diffusion method as described [9]. The complex between trichodiene synthase and BTAC was prepared by soaking crystals in a buffer solution containing 1 mM MgCl_2 , 4 mM FPP, and 10 mM BTAC. The substrate turned over in the enzyme crystals, resulting in the formation of a complex between the enzyme, $\text{Mg}^{2+}_3\text{-PP}_i$, and a large molecule in the enzyme active site. Initially, we interpreted the electron density in the active site as a trichodiene molecule, but further analysis revealed that this density was better fit by a molecule of BTAC. Enzyme crystals were also soaked in a buffer solution containing 1 mM MgCl_2 and 4 mM 2F-FPP to yield the structure of the complex with $\text{Mg}^{2+}_3\text{-PP}_i$. Crystals were prepared for data collection by cryoprotection with 25% ethylene glycol and flash cooling in liquid nitrogen.

Diffraction data for crystals of both complexes were collected at the Cornell High Energy Synchrotron Source (CHESS, beamlines F1 and A1). Data were indexed and merged using the program HKL2000 [14]. Crystals were essentially isomorphous with crystals of the wild-type enzyme (space

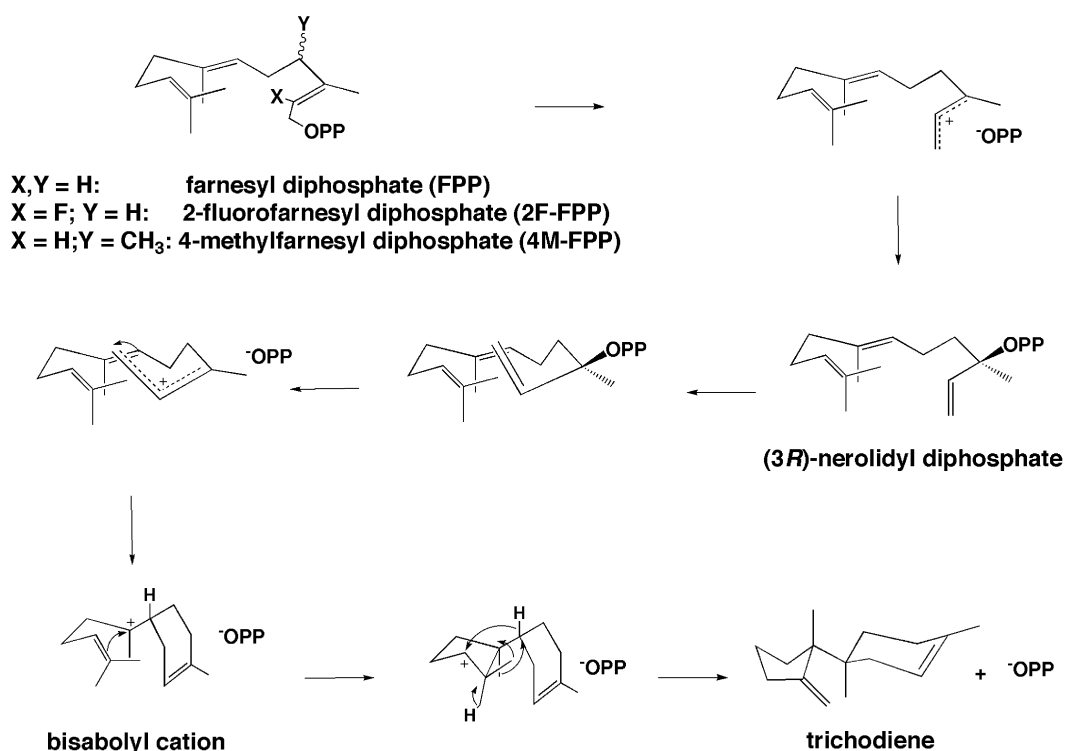


Fig. 1. Postulated mechanism for the cyclization of farnesyl diphosphate to trichodiene by trichodiene synthase (OPP, diphosphate; OPP^- , inorganic pyrophosphate anion) [8].

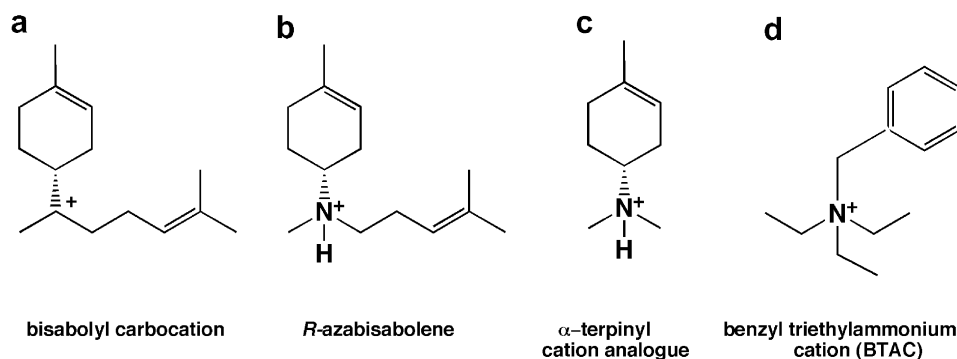


Fig. 2. (a) Bisabolyl carbocation intermediate in trichodiene synthase catalysis. (b) *R*-Azabisabolene, a cationic analogue of the bisabolyl cation in the trichodiene synthase mechanism, is a strong competitive inhibitor in the presence of inorganic diphosphate (PP_i) with K_i for PP_i = 0.51 μ M and an induced inhibition constant of 2.6 μ M [10]. (c) The aza analogue of the α -terpinyl cation is a competitive inhibitor in the presence of inorganic pyrophosphate (PP_i) with K_i for PP_i = 0.48 μ M and an induced inhibition constant of 10.9 μ M [10]. (d) The benzyl triethylammonium cation (BTAC) is a phase transfer catalyst for synthetic organic chemistry that partially mimics the bisabolyl carbocation intermediate (a).

group P3₁21, $a = b = 122.2$ Å, $c = 151.2$ Å [9] and each structure was solved by the difference Fourier technique. The programs CNS [15] and O [16] were used for refinement and model building, respectively. Noncrystallographic symmetry constraints were used in the initial stages of refinement and subsequently relaxed into appropriately weighted restraints as judged by R_{free} as refinement progressed. Molecular models shown in the figures were prepared with Raster3D V2.7d [17]. Data collection and refinement statistics are reported in Table 1.

Incubation of trichodiene synthase with farnesyl diphosphate

Analysis of cyclization products generated from FPP was performed using gas chromatography and mass spectroscopy (GC/MS) as previously described [18,19]. All glassware was washed and oven-baked prior to use. The FPP substrate (50–500 μ M) was incubated with or without purified wild-type or D100E trichodiene synthase (1 mg) in 4 mL buffer (10 mM Tris (pH 7.8), 5 mM MgCl₂, 15% glycerol, and 5 mM β -mercaptoethanol) and overlaid with HPLC-grade *n*-pentane (Fisher P399-1) in a glass test

Table 1
Data collection and refinement statistics for trichodiene synthase-ligand complexes

Ligands	Mg ²⁺ ₃ -PP _i -BTAC
Resolution range (Å)	50–2.85
Reflections (measured/unique)	127,952/27,585
Completeness (%) (overall/outer shell)	94.4/96.7
R_{merge}^a (overall/outer shell)	0.088/0.616
$\langle I/\sigma \rangle$ (overall/outer shell)	15.2/2.6
Protein atoms (no.) ^b	5717
Solvent atoms (no.) ^b	36
Metal ions (no.) ^b	3
Ligand atoms (no.) ^b	23
Reflections used in refinement (work/free)	25,786/1306
R/R_{free}^c	0.216/0.265
r.m.s. deviations	
Bonds (Å)	0.008
Angles (deg.)	1.2
Dihedral angles (deg.)	19.1
Improper dihedral angles (deg.)	0.8

^a $R_{\text{merge}} = \sum |I_j - \langle I_j \rangle| / \sum I_j$, where I_j is the observed intensity for reflection j and $\langle I_j \rangle$ is the average intensity calculated for reflection j from replicate data.

^b Per asymmetric unit.

^c $R = \sum ||F_o| - |F_c|| / \sum |F_o|$, where R and R_{free} are calculated by using the working and test reflection sets, respectively.

tube at 30 °C for 3–20 h. Reaction products were extracted with HPLC-grade *n*-pentane and purified on a 3 cm 230–400 mesh silica gel column. Purified extracts were concentrated on an ice-water mixture under reduced pressure until volume decreased to 50 μ L or less, and each resulting concentrate was analyzed by GC/MS. Reaction products were analyzed using a Hewlett–Packard 6890 gas chromatograph (GC) coupled to a 5973 mass selective detector and equipped with an HP-5MS capillary column (0.25 mm i.d. $\times$ 30 m with 0.25 μ m film) (Agilent Technologies). A total of 2 μ L of concentrate was injected in splitless mode into the gas chromatograph–mass spectrometer. The oven temperature was maintained at 35 °C for 1 min, increased at the rate of 5 °C/min to 230 °C, followed by a 20 °C/min increase to 280 °C. The temperature was held at 280 °C for 20 min.

Synthesis of 2-fluorofarnesyl diphosphate

The sample of *trans*, *trans*-2-fluorofarnesyl diphosphate (2F-FPP) was obtained by methods similar to those reported for the preparation of 2-fluorogeranyl diphosphate [20] i.e., conversion of *trans*, *trans*-2-fluorofarnesol [21] to *trans*, *trans*-2-fluorofarnesyl chloride [22] and displacement with tetrabutylammonium pyrophosphate [23]. Procedures and characterization data for *trans*, *trans*-2-fluorofarnesyl chloride and 2F-FPP will be published separately.

Incubation of trichodiene synthase with 2-fluorofarnesyl diphosphate

The fluorinated substrate 2F-FPP (50–500 μ M) was incubated with or without purified protein (1 mg) in 4 mL buffer (10 mM Tris (pH 7.8), 5 mM MgCl₂, 15% glycerol, and 5 mM β -mercaptoethanol) and overlaid with HPLC-grade *n*-pentane in a glass test tube at 30 °C. In anticipation of a slower reaction rate with a less reactive substrate, 1 mg of enzyme was added to the reaction mixture at 10 and 20 h reaction time points in order to maximize turnover and product detection. Reaction products were extracted, concentrated, and subject to GC/MS analysis as described above.

Incubation of trichodiene synthase with racemic 4-methylfarnesyl diphosphate

The methylated substrate 4M-FPP [24] (1–2 mg) was incubated with or without purified protein (1 mg) in 4 mL buffer (10 mM Tris (pH 7.8), 5 mM MgCl₂, 15% glycerol, and 5 mM β -mercaptoethanol) and overlaid with HPLC-grade *n*-pentane in a glass test tube at 30 °C. Reaction products were extracted after $\sim$ 18 h, concentrated, and subject to GC/MS analysis as described above.

Inhibition of trichodiene synthase by BTAC

For inhibition assays, 1 mL of reaction buffer (40 ng trichodiene synthase, 10 mM Tris (pH 7.8), 5 mM MgCl₂, 15% glycerol, and 5 mM β-mercaptoethanol) was overlaid with 0.75–1 mL of HPLC-grade *n*-pentane and incubated at 30 °C for 7 min. Inhibition of trichodiene synthase with BTAC alone was studied using ³H-FPP (Sigma), diluted with cold FPP (Echelon Biosciences), and pre-washed with *n*-pentane. Concentrations of 50–1200 nM FPP (~80 Ci/mol) and 50 μM BTAC were used. The reactions were carried out both with and without BTAC for each substrate concentration. Inhibition of trichodiene synthase in the presence of BTAC and PP_i was studied using 1 μM ³H-FPP and a range of BTAC and PP_i concentrations. Each reaction was quenched using 75 μL of 0.5 M EDTA (pH 8.0) and vortexed for 25 s. Products were extracted using an additional 3 × 0.75 mL *n*-pentane. Extracts were passed over a silica gel column in a Pasteur pipette. The column was washed with an additional 0.75 mL of *n*-pentane directly into 5 mL scintillation cocktail. Five different concentrations of BTAC ranging from 0 to 30 μM were used in combination with four different PP_i concentrations ranging from 5 to 20 μM. All reactions were run 3–5 times. Inhibition constants for PP_i and BTAC were calculated by fitting all data simultaneously to the general kinetic expression for cooperative competitive inhibition in Eq. (1) [10,25]

$$\frac{V_{\max}}{v} = \frac{K_M}{[FPP]} \left(1 + \frac{[PP_i]}{K_I} + \frac{[BTAC]}{K_J} + \frac{[PP_i][BTAC]}{\alpha K_I K_J} \right) + 1 \quad (1)$$

where K_I and K_J are the inherent inhibition constants of PP_i and BTAC, respectively, α is a cooperativity factor, and $[BTAC]/K_J \approx 0$ since BTAC is ineffective as an inhibitor in the absence of PP_i.

Results

Incubation of trichodiene synthase with unnatural FPP derivatives

Overnight incubation of 2F-FPP in reaction buffer containing 0.25 mg/mL trichodiene synthase results in the generation of multiple pentane-extractable products (Fig. 3a), the concentrations of which increase considerably by lengthening the incubation time to 36 h and by adding enzyme to the reaction mixture at 0, 10, and 20 h time points. Control incubations carried out in the absence of enzyme confirm that there is no background formation of these products. The GC/MS data indicate that eight products exhibit molecular ion peaks of m/z 221 (consistent with the M-1 ions of fluorinated sesquiterpenes), while two addi-

tional products exhibit molecular ion peaks of m/z 203 (consistent with the loss of fluorine during the reaction catalyzed by trichodiene synthase or during the GC/MS analysis). Notably, when crystals of wild-type trichodiene synthase are soaked in buffer solutions containing 4 mM 2F-FPP, only the Mg²⁺₃-PP_i cluster is observed to bind to monomer B of the enzyme (data not shown). The structure of this Mg²⁺₃-PP_i complex is identical to that determined previously [9] and is consistent with generation of a fluorinated sesquiterpene product that is released from the enzyme active site while the PP_i coproduct remains bound.

Overnight incubation of 4M-FPP in reaction buffer containing 0.25 mg/mL enzyme and GC-MS analysis of the *n*-pentane elutable extract reveal the generation of one major product and 16 minor products (Fig. 3b), each with molecular ion peaks of m/z 217, consistent with the M-1 ions of methylated sesquiterpenes. Unexpectedly, control incubations of wild-type trichodiene synthase with FPP indicate that although trichodiene is the major product (89%), the formation of trichodiene was also accompanied by the generation of 1–2% of each of five additional sesquiterpenes identical by GC/MS with the more abundant aberrant products (total 37%, β-farnesene, α- and β-bisabolene, cuprenene, and isochamigrene) that are known to be produced [18] by mutants of trichodiene synthase (Fig. 3c).

Inhibition of trichodiene synthase by BTAC and PP_i

Although 50 μM BTAC alone does not inhibit the cyclization of FPP by trichodiene synthase, 5–30 μM BTAC acts as a competitive inhibitor of trichodiene synthase reaction in the presence of 5–20 μM PP_i (PP_i itself is a strong competitive inhibitor with $K_I = 0.49$ μM [10]). The apparent inhibition constant of PP_i in the presence of BTAC is determined to be 0.7 μM while the induced inhibition constant for BTAC in the presence of PP_i is 36 μM (Fig. 4). The dependence of BTAC inhibition on the presence of PP_i is similar to that previously observed for ammonium analogues of the bisaboly carbocation intermediate, such

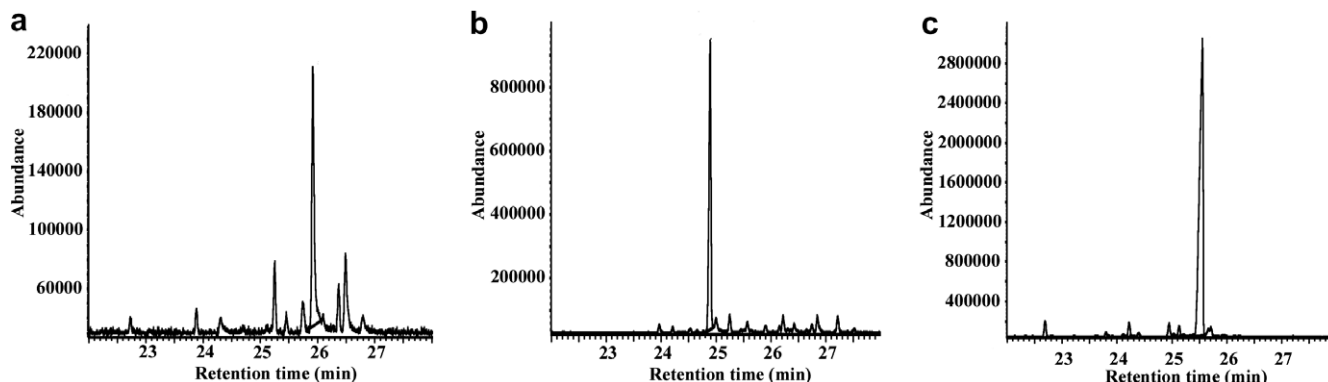


Fig. 3. Gas chromatograms of (a) pentane extract resulting from the incubation of trichodiene synthase with 2F-FPP, (b) pentane extract resulting from the incubation of trichodiene synthase with 4M-FPP, and (c) pentane extract resulting from the incubation of trichodiene synthase with FPP.

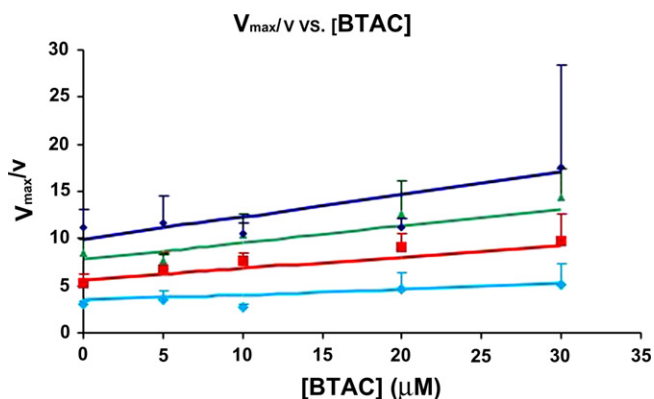


Fig. 4. Plot of $V_{\max}/v$ as a function of [BTAC] at variable [PP_i]: (♦) = 5 μM; (■) = 10 μM; (▲) = 15 μM; (●) = 20 μM [PP_i]. Positive standard deviations for data are represented by vertical lines of the color corresponding to each [PP_i]. The data were fit to Eq. (1), as described. The calculated inhibition constant K_i for inorganic pyrophosphate is 0.7 μM; the induced inhibition constant for BTAC in the presence of inorganic pyrophosphate is 36 μM.

as *R*-azabisabolene, the aza-analogue of the α -terpinyl cation (Fig. 2), and trimethylamine [10] (Table 2).

Structure of the trichodiene synthase-Mg²⁺₃-PP_i-BTAC complex

The Mg²⁺₃-PP_i cluster and BTAC bind to the active site of monomer B of the dimer (Fig. 5). The enzyme undergoes ligand-induced conformational changes similar to those observed in the wild-type enzyme complex with Mg²⁺₃-PP_i [9]. No ligand binding is observed in monomer A because the ligand-induced conformational changes are hindered by interlattice crystal contacts. The r.m.s. deviation between liganded and unliganded trichodiene synthase structures is 1.3 Å for 350 C α atoms. The overall conformational changes observed in the BTAC-Mg²⁺₃-PP_i complex are similar to those observed in the Mg²⁺₃-PP_i complex, with an r.m.s. deviation of 0.338 Å for 352 C α atoms between monomers B of the two complexes.

Apart from a conformational change in the side chain of D100 that appears to weaken coordination interactions

with Mg²⁺_A and Mg²⁺_C, the metal ion coordination and hydrogen bond networks formed in the Mg²⁺₃-PP_i-BTAC complex are similar to those observed in the Mg²⁺₃-PP_i complex [9]. The positively charged BTAC molecule is presumably stabilized by favorable long-range electrostatic interactions with the PP_i anion: the closest PP_i oxygen atom is 4.7 Å from the quaternary ammonium nitrogen atom of BTAC and 3.8 Å from the closest *N*-ethyl carbon atom of BTAC, which is notable insofar that the positive charge of the cation is inductively shared by its neighboring atoms. The phenyl ring of BTAC resides deep within the hydrophobic active site cleft, where it makes van der Waals contacts with the hydrophobic side chains of I70, L97, and F157.

Discussion

The crystal structure of trichodiene synthase complexed with BTAC and Mg²⁺₃-PP_i provides the first definitive proof that a terpenoid cyclase active site is capable of accommodating a positively charged ligand. Although the structures of trichodiene synthase mutants complexed with the presumably positively charged ligands *R*-azabisabolene or *S*-azabisabolene plus Mg²⁺₃-PP_i have previously been reported [11,12], the protonation states of their tertiary amino nitrogen atoms cannot be determined in the 2.5–2.95 resolution electron density maps of proteins. In contrast, the four hydrocarbon substituents of the quaternary ammonium group of BTAC are readily visible in the electron density map, thereby conclusively confirming that the ligand bound in the active site of trichodiene synthase is positively charged. Therefore, just as the trichodiene synthase active site can accommodate and stabilize a positively charged quaternary ammonium group, so too can it accommodate and stabilize a positively charged carbocation intermediate in catalysis.

In the presence of PP_i, trichodiene synthase is inhibited by positively-charged aza compounds that partially mimic the shape and charge of a critical carbocation intermediate in the FPP cyclization reaction (Fig. 2); in the absence of PP_i, these compounds are ineffective as inhibitors [10]. These aza compounds exhibit a 100-fold range of binding affinities in the presence of PP_i (Table 2). Structural studies show that both the *R*- and the *S*-azabisabolene analogues are bound in a variety of orientations in the active sites of Y305F and R304K trichodiene synthases [11,12]. Interestingly, while BTAC binds ~15-fold less tightly than either *R*- or *S*-azabisabolene (Table 2 and Fig. 4), it is bound in a single, well-defined orientation (Fig. 5), possibly due to the fact that it has three identical alkyl groups.

Structural comparisons of the trichodiene synthase-Mg²⁺₃-PP_i complex and the trichodiene synthase-Mg²⁺₃-PP_i complexes with bound ammonium ion analogues *R*-azabisabolene, *S*-azabisabolene, or BTAC, reveal that the average PP_i-metal ion coordination distances increase from 2.3 to 2.5 when positively charged ammonium ligands bind in the enzyme active site. Thus, it appears that

Table 2
Cooperative competitive inhibition of trichodiene synthase by inorganic pyrophosphate (PP_i) and quaternary ammonium analogues of the bisabolyl carbocation intermediate

Inhibitor	PP _i K_i (μM)	Inhibitor αK_i^a (μM)
PP _i ^b	0.49	—
Trimethylamine ^b	0.68	172.0
BTAC	0.7	36
α -Terpinyl cation analogue ^b	0.48	10.9
<i>R</i> -Azabisabolene ^b	0.51	2.6
<i>S</i> -Azabisabolene ^b	0.47	2.9

^a Calculated induced inhibition constant of the ammonium analogues (Fig. 2) in the presence of PP_i, using Eq. (1) for cooperative competitive inhibition [10,25].

^b From Ref. [10].

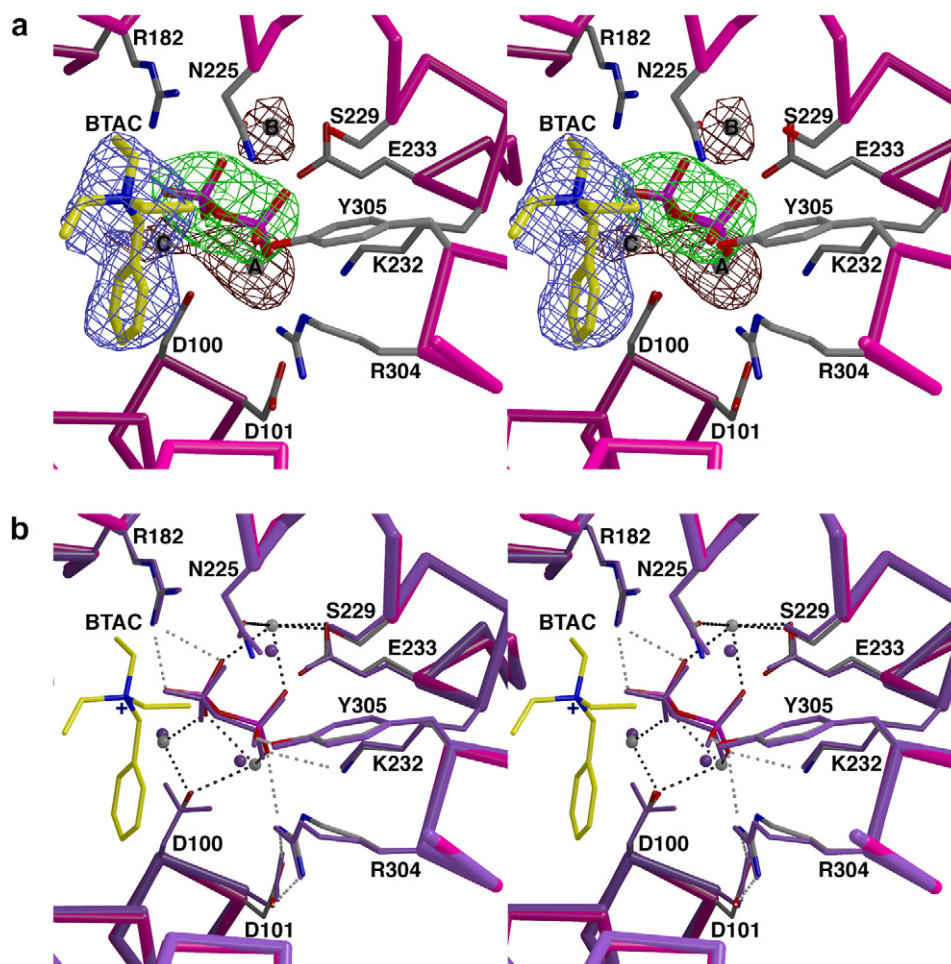


Fig. 5. Active site of trichodiene synthase. (a) Simulated annealing omit maps of BTAC (blue, 4.5σ), PP_i (green, 7.6σ), and magnesium ions A, B, and C (maroon, 4.1σ). Atoms are color-coded as follows: carbon (protein), gray; carbon (BTAC), yellow; oxygen, red; nitrogen, blue; phosphorus, magenta; magnesium, gray. (b) Superposition with the trichodiene synthase-Mg²⁺₃-PP_i complex (purple). Hydrogen bond and metal coordination interactions are represented by white and black broken lines, respectively.

electrostatic interactions in the Mg²⁺₃-PP_i cluster are weaker when a positive charge is bound or generated in catalysis. The weakening of electrostatic interactions in the Mg²⁺₃-PP_i cluster, which “locks” the active site template in the closed conformation, could contribute to the anomalous binding modes observed for *R*- and *S*-azabisabolens in complexes with Y305F and R304K trichodiene synthases [11,12]. Moreover, it is possible that metal coordination interactions are weakened upon initial carbocation formation in the cyclization cascade. This could “loosen” the active site template and thereby account for the formation of aberrant cyclization products by wild-type and mutant trichodiene synthases. Additionally, this could facilitate the potential function of PP_i as a base in the final step of catalysis and also facilitate product release.

The finding that wild-type trichodiene synthase generates 89% trichodiene and 11% side products (13 total) contrasts with our earlier observation, obtained with less sensitive instrumentation, indicating that cyclization of FPP generates trichodiene as the exclusive product [18,19]. This low but real rate of formation of aberrant

products that is enhanced in active site mutants is reminiscent of the properties of other wild-type sesquiterpene cyclases that have been found to generate multiple products. For example, aristolochene synthase from *Penicillium roqueforti* generates (*S*)-germacrene-A (4%) and (–)-valencene (2%) in addition to aristolochene, although aristolochene synthase from *Aspergillus terreus* generates 100% aristolochene [26]. The promiscuity of product formation exhibited by a terpene synthase can therefore vary even from one species to another. Strikingly, γ -humulene synthase from *A. grandis* generates 52 products from FPP [6]. However, a relatively small number of mutations (5 or less) of the residues that define the active site contour of γ -humulene synthase can substantially enhance the proportion of a single cyclization product while decreasing the relative abundance of the remaining sesquiterpene coproducts, without significant decrease in the overall rate of product formation [27]. The occurrence of minor side products in the reaction catalyzed by a terpenoid cyclase therefore indicates the evolutionary potential of the cyclase to generate a significantly different product or product array.

As noted by Petsko and colleagues [28], low levels of alternative catalytic activity provide a “toehold” for the evolution and optimization of enzyme function toward such alternative activity.

The cyclization of farnesyl diphosphate by trichodiene synthase is initiated by the departure of the diphosphate group from C1, resulting in the generation of an allylic carbocation intermediate with the positive charge delocalized over C1–C3 (Fig. 1). The electron-withdrawing effect of the C2 fluorine atom in 2F-FPP is expected to destabilize the allylic cation and therefore retard if not abolish the cyclization reaction. This would be the case regardless of whether the enzyme utilizes the mechanism in Fig. 1 or the mechanism recently proposed by Hong and Tantillo [29] for FPP cyclization. Nonetheless, 2F-FPP is converted to a mixture containing predominantly fluorinated sesquiterpene derivatives. The fact that 4M-FPP can also serve as a surrogate substrate further illustrates the ability of the trichodiene synthase active site to accommodate derivatives of the natural FPP substrate. Future work will focus on determining the structures of the sesquiterpene analogues that are generated from such alternative substrates and the kinetics of their formation. Coupled with binding studies of unnatural carbocation analogues such as BTAC, the current work sets a foundation for studying diversity in ligand binding and catalysis by trichodiene synthase.

Acknowledgments

We thank the Cornell High Energy Synchrotron Source and Elettra Synchrotron Light Source for access to X-ray crystallographic data collection facilities, we thank Dr. Barry Cooperman for the use of the scintillation counter, and we thank Kevin Jude, Tatiana Zakharian, and Xin Lin for helpful scientific discussions.

References

- [1] D.E. Cane, *Acc. Chem. Res.* 18 (1985) 220–226.
- [2] R. Croteau, D.E. Cane *Methods in Enzymology Steroids and Isoprenoids (Part A)*, vol. 110, Academic Press, New York, 1985, pp. 383–405.
- [3] D.E. Cane, *Chem. Rev.* 90 (1990) 1089–1103.
- [4] K.U. Wendt, G.E. Schulz, *Structure* 6 (1998) 127–133.
- [5] D.W. Christianson, *Chem. Rev.* 106 (2006) 3412–3442.
- [6] C.L. Steele, J. Crock, J. Bohlmann, R. Croteau, *J. Biol. Chem.* 273 (1998) 2078–2089.
- [7] T.M. Hohn, F. VanMiddlesworth, *Arch. Biochem. Biophys.* 251 (1986) 756–761.
- [8] D.E. Cane, S. Swanson, P.P.N. Murthy, *J. Am. Chem. Soc.* 103 (1981) 2136–2138.
- [9] M.J. Rynkiewicz, D.E. Cane, D.W. Christianson, *Proc. Natl. Acad. Sci. USA* 98 (2001) 13543–13548.
- [10] D.E. Cane, G. Yang, R.M. Coates, H.-J. Pyun, T.M. Hohn, *J. Org. Chem.* 57 (1992) 3454–3462.
- [11] L.S. Vedula, M.J. Rynkiewicz, H.-J. Pyun, R.M. Coates, D.E. Cane, D.W. Christianson, *Biochemistry* 44 (2005) 6153–6163.
- [12] L.S. Vedula, D.E. Cane, D.W. Christianson, *Biochemistry* 44 (2005) 12719–12727.
- [13] D.A. Whittington, M.L. Wise, M. Urbansky, R.M. Coates, R.B. Croteau, D.W. Christianson, *Proc. Natl. Acad. Sci. USA* 99 (2002) 15375–15380.
- [14] Z. Otwinowski, W. Minor *Methods in Enzymology. Macromolecular Crystallography (Part A)*, vol. 276, Academic Press, San Diego, 1997, pp. 307–326.
- [15] A.T. Brünger, P.D. Adams, G.M. Clore, W.L. DeLano, P. Gros, R.W. Grosse-Kunstleve, J.-S. Jiang, J. Kuszewski, M. Nilges, N.S. Pannu, R.J. Read, L.M. Rice, T. Simonson, G.L. Warren, *Acta Crystallogr. D* 54 (1998) 905–921.
- [16] T.A. Jones, J.Y. Zou, S.W. Cowan, M. Kjeldgaard, *Acta Crystallogr. A* 47 (1991) 110–119.
- [17] E.A. Merritt, D. Bacon *Methods in Enzymology. Macromolecular Crystallography (Part B)*, vol. 277, Academic Press, San Diego, 1997, pp. 505–524.
- [18] D.E. Cane, Q. Xue, B.C. Fitzsimons, *Biochemistry* 35 (1996) 12369–12376.
- [19] D.E. Cane, J.H. Shim, Q. Xue, B.C. Fitzsimons, T.M. Hohn, *Biochemistry* 34 (1995) 2480–2488.
- [20] D.C. Hyatt, B. Youn, Y. Zhao, B. Santhamma, R.M. Coates, R.B. Croteau, C.H. Kang, *Proc. Natl. Acad. Sci. USA* 104 (2007) 5360–5365.
- [21] Y. Jin, D.C. Williams, R. Croteau, R.M. Coates, *J. Am. Chem. Soc.* 127 (2005) 7834–7842.
- [22] A.I. Meyers, E.W. Collington, *J. Org. Chem.* 36 (1971) 3044–3045.
- [23] A.B. Woodside, Z. Huang, C.D. Poulter, *Org. Synth. CV* 8 (1993) 616–620.
- [24] S. Ohnuma, M. Ito, T. Koyama, K. Ogura, *Tetrahedron* 45 (1989) 6145–6160.
- [25] W.W. Cleland, in: P.D. Boyer (Ed.), *Enzymes*, vol. 2, Academic Press, New York, 1970, pp. 1–65.
- [26] B. Felicetti, D.E. Cane, *J. Am. Chem. Soc.* 126 (2004) 7212–7221.
- [27] Y. Yoshikuni, T.E. Ferrin, J.D. Keasling, *Nature* 440 (2006) 1078–1082.
- [28] G.A. Petsko, G.L. Kenyon, J.A. Gerlt, D. Ringe, J.W. Kozarich, *Trends Biochem. Sci.* 18 (1993) 372–376.
- [29] Y.J. Hong, D.J. Tantillo, *Org. Lett.* 8 (2006) 4601–4604.