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Two pockets in the active site of maize sesquiterpene synthase TPS4 carry out sequential parts of the reaction scheme resulting in multiple products ☆

Tobias G. Köllner [a], Paul E. O'Maille [b], Nathalie Gatto [a], Wilhelm Boland [a], Jonathan Gershenzon [a], Jörg Degenhardt [a,*]

[a] *Max Planck Institute for Chemical Ecology, Hans-Knöll Strasse 8, D-07745 Jena, Germany*
[b] *The Salk Institute for Biological Studies, La Jolla, CA 92037, USA*

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Abstract

One of the most interesting features of terpene synthases is their ability to form multiple products with different carbon skeletons from a single prenyl diphosphate substrate. The maize sesquiterpene synthase TPS4, for example, produces a mixture of 14 different olefinic sesquiterpenes. To understand the complex TPS4 reaction mechanism, we modeled the active site cavity and conducted docking simulations with the substrate farnesyl diphosphate, several predicted carbocation intermediates, and the final reaction products. The model suggests that discrete steps of the reaction sequence are controlled by two different active site pockets, with the conformational change of the bisabolyl cation intermediate causing a shift from one pocket to the other. Site-directed mutagenesis and measurements of mutant activity in the presence of (*E*,*E*)- and (*Z*,*E*)-farnesyl diphosphate as substrates were employed to test this model. Amino acid alterations in pocket I indicated that early steps of the catalytic process up to the formation of the monocyclic bisabolyl cation are probably localized in this compartment. Mutations in pocket II primarily inhibited the formation of bicylic compounds, suggesting that secondary cyclizations of the bisabolyl cation are catalyzed in pocket II.

Keywords: Terpene synthase; Sesquiterpene biosynthesis; Multiproduct enzyme; Terpene synthase reaction mechanism

Plants produce an enormous variety of terpenes that have a multitude of functions in growth, development, reproduction, and defense [1,2]. The great structural diversity of terpenes arises mainly from the action of terpene synthases, an enzyme class that converts linear prenyl diphosphates to a large variety of carbon skeletons. One of the most striking features of terpene synthases is their ability to produce multiple terpene products from a single substrate by a carbocationic reaction mechanism that is almost unique to this class of enzymes. Our current understanding of this mechanism is based on numerous investigations reported over the last few decades, some of the most important of which have been carried out in the laboratory of Rod Croteau [3,5]. After substrate binding, the diphosphate residue is displaced and a highly reactive carbocation intermediate formed which can undergo successive ring formations, hydride shifts, and other rearrangements until the reaction is terminated by deprotonation or water capture [3,4].

In recent years, protein structural studies have begun to make important contributions to the study of the reaction mechanism of terpene synthases. For example, the elucidation of the three-dimensional structures of 5-*epi*-aristolochene synthase from *Nicotiana tabaccum* and (+)-bornyl diphosphate synthase from *Salvia officinalis* revealed that these enzymes each consist of two distinct domains [6,7]. The active sites of both proteins were determined to be

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* Corresponding author. Fax: +49 3641 1302.
E-mail address: degenhardt@ice.mpg.de (J. Degenhardt).

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hydrophobic pockets in the C-terminal domain by the location of bound substrate analogs [6,7]. Domain swapping experiments demonstrated that fragments of 60–200 amino acids from the C-terminal domain can determine product specificity [8–11].

Despite their large overall sequence diversity, terpene synthases possess several highly conserved amino acid residues [12]. An aspartate-rich DDxxD motif located at the entrance of the active site was shown to be involved in the binding of the metal ion-complexed diphosphate ester substrate [13,14,6]. Thirty five amino acids upstream of the DDxxD motif, an RxR motif is present which is thought to direct the diphosphate anion away from the reactive carbocation after ionization [6]. In the N-terminal part of monoterpene synthases, two arginine residues are present that are believed to influence the isomerization of geranyl diphosphate to linalyl diphosphate [15].

The effects of single amino acid replacements by site-directed mutagenesis have been analyzed for a few terpene synthases. For instance, in the active site cavity of 5-*epi*-aristolochene synthase from *N. tabaccum*, the reprotonation of a germacrene intermediate was demonstrated to be dependent on a tyrosine residue [16] which is assumed to work in concert with two aspartate residues forming a catalytic triad [6]. Site-directed mutagenesis of δ-selinene synthase and γ-humulene synthase from *Abies grandis* indicated that only one of these two aspartate residues is part of the reprotonation mechanism of γ-humulene synthase [17]. Additional analysis suggested that the unusually high number of different sesquiterpene products for δ-selinene synthase and γ-humulene synthase, 34 and 52, respectively, might be due to the presence of two DDxxD motifs, each of which can bind the substrate [17].

As part of our ongoing effort to investigate the regulation and function of sesquiterpene biosynthesis in maize, we have isolated and characterized the terpene synthase TPS4 [18]. This enzyme produces 14 mono- and bicyclic sesquiterpene products, mostly of the bisabolane, sesquithujane, and bergamotane types (Fig. 1). Comparative mutational

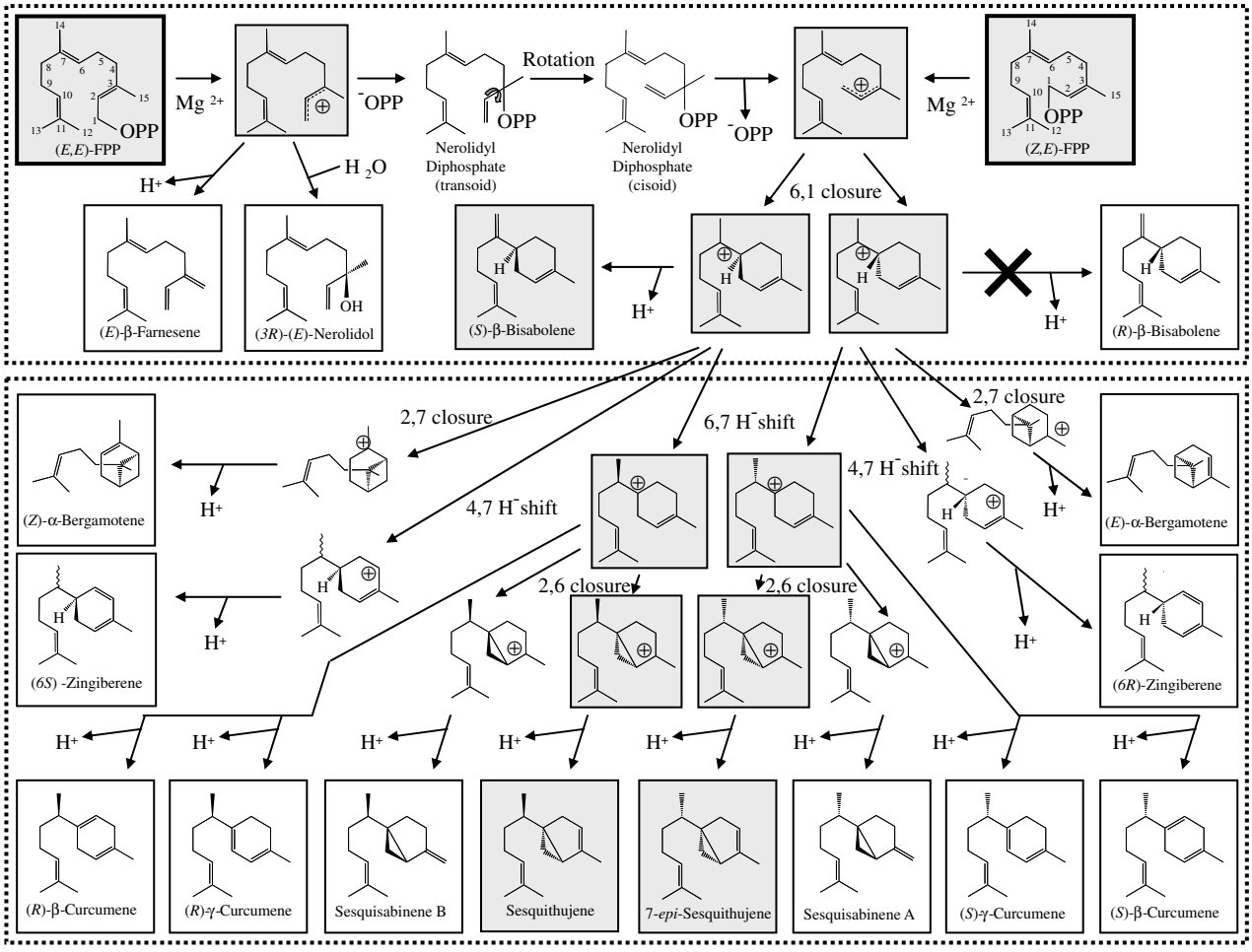

Fig. 1. Proposed reaction mechanism for the formation of sesquiterpene products by TPS4. The scheme is based on previous studies of TPS4 and TPS5 [18]. The intermediates central to the reaction mechanism are the (*S*)- and (*R*)-bisabolyl carbocations which are formed from the *cisoid* conformer of nerolidyl diphosphate and subsequently converted to a large variety of mono- and bicyclic products. Highlighted with grey shading are the substrate, major intermediates, and main products that were used in in silico docking. Hatched lines are used to indicate when more than one route for end product formation is possible. The upper and lower areas separated by dotted lines indicate the catalytic steps assumed to be located in pocket I and II, respectively. OPP indicates a diphosphate group.

analysis with the closely related maize terpene synthase TPS5 revealed a four amino acid motif that altered the stereoselectivity of the TPS4 product blend. To understand the multi-product reaction mechanism of TPS4 in more detail, we have now undertaken computational analysis via modeling and docking simulations to guide additional site-directed mutagenesis in conjunction with the feeding of (*Z*,*E*)-farnesyl diphosphate, an analog substrate with similarity to presumptive reaction intermediates. The mutational analysis indicates the presence of two pockets with different functionalities within the active site of TPS4. One pocket appears to be the site of the initial steps of the reaction sequence common to all of the major products (as well as the final steps leading to one of the major products). A conformational change of a specific intermediate, the bisabolyl carbocation, shifts it to the second pocket where it is then converted to a separate group of products. The existence of two pockets thus contributes to the multi-product character of this terpene synthase.

Materials and methods

cDNA clone and (Z,E)-FPP substrate

The cDNA clone *tps4-B73* isolated from the *Zea mays* inbred cultivar B73 was characterized in [18] and is deposited in GenBank with the Accession No.: AY518310. The (*Z*,*E*)-FPP[1] substrate was synthesized according to the method of Cane et al. [19].

Computational methods

A model of the three-dimensional structure of TPS4 was generated using the Swiss-Model Server (http://www.expasy.org [20]). For modeling, the *tps4-B73* cDNA sequence was fitted to the template structure of tobacco 5-*epi*-aristolochene synthase [6]. Hydrogen atoms were added to the structure using the program Vega ZZ [21]. Energy-minimized ligand structures were generated with the software ChemDraw and Chem3D (CambridgeSoft, Cambridge, USA). Docking of the ligands into the model of the TPS4 active site cavity was performed with the flexible ligand docking software GOLD v2.1 (Cambridge Crystallographic Data Centre, Cambridge, UK). The resulting models were visualized with the programs UCSF-Chimera [22] and Swiss-PdbViewer3.7 [23]. The software Swiss-PdbViewer3.7 was used for in silico mutagenesis.

Site-directed mutagenesis

For site-directed mutagenesis, the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, USA) was used according to the manufacturer's instructions. The PCR-based mutagenesis protocol was performed with the *tps4-B73* cDNA cloned into the expression vector pASK-IBA7 [18] using primers containing the desired mutations (Table 1). The mutagenized constructs were fully sequenced before expression.

Table 1
Primer sequences for site-directed mutagenesis

Mutation	Sequence (5′/3′)[a]	Direction[b]
E277Q	GATAGAATTGTAGAG**C**AGTATTT CTGGATGAATG	fwd
E277Q	CATTCATCCAGAAATACT**G**CTCTAC AATTCTATC	rev
T301W	ACTGACAAAGATC**TGG**GGACTTAT TACAATAATCGATG	fwd
T301W	CATCGATTATTGTAATAAGTCC**CCA** GATCTTTGTCAGT	rev
T305F	CGGGGCTTATT**TTT**ATAATCGATG ATATGTTTGATAC	fwd
T305F	GTATCAAACATATCATCGATTAT**AA** **A**AATAAGCCCCG	rev
Y382F	CGTTTAGTTGAGCTAT**T**CTCCAAG GAAATAAA	fwd
Y382F	TTTATTTCCTTGGAG**A**ATAGCTCAA CTAAACG	rev
Y382L	CGTTTAGTTGAGCTA**CT**CTCCAAG GAAATAAA	fwd
Y382L	TTTATTTCCTTGGAG**AG**TAGCTCAA CTAAACG	rev
Y382S	CGTTTAGTTGAGCTAT**C**CTCCAAG GAAATAAA	fwd
Y382S	TTTATTTCCTTGGAG**G**ATAGCTCAA CTAAACG	rev
L413A	CGCAACCATTGCT**GC**AACATGTTC TGCATATGC	fwd
L413A	GCATATGCAGAACATGTT**GC**AGCA ATGGTTGCG	rev
N452D	TTGTACGGCTCTCC**G**ACGATGTCG TATCGACC	fwd
N452D	GGTCGATACGACATCGT**C**GGAGA GCCGTACAA	rev
Y528F	CCACGGATAATATGT**T**CAGGGATC GTGACGCA	fwd
Y528F	TGCGTCACGATCCCTG**A**ACATATT ATCCGTGG	rev
Y528L	CCACGGATAATATG**CT**CAGGGATC GTGACGCA	fwd
Y528L	TGCGTCACGATCCCTG**AG**CATATT ATCCGTGG	rev
Y528S	CCACGGATAATATGT**C**CAGGGATC GTGACGCA	fwd
Y528S	TGCGTCACGATCCCTG**G**ACATATT ATCCGTGG	rev
D532N	ATGTACAGGGATCGT**A**ACGCATTG ACCTCTTC	fwd
D532N	GAAGAGGTCAATGCGT**T**ACGATCC CTGTACAT	rev

[a] The base changes are shown in boldface and underlined.
[b] fwd, forward; rev, reverse.

Protein overexpression and enzyme assay

Liquid cultures of the bacteria harboring the expression constructs were grown at 37 °C to an OD_{600} of 0.6.

[1] *Abbreviations used:* FPP, farnesyl diphosphate; SPME, solid phase microextraction; TPS4, terpene synthase 4; TEAS, 5-*epi*-aristolochene synthase.

Expression was induced by addition of anhydrotetracycline (IBA GmbH, Göttingen, Germany) to a final concentration of 200 μg/L. After 20 h incubation at 18 °C, the cells were collected by centrifugation and disrupted by a 4×30 s treatment with a sonicator (Bandelin UW2070, Berlin, Germany) in chilled extraction buffer (50 mM Mopso, pH 7.0, with 5 mM $MgCl_2$, 5 mM sodium ascorbate, 0.5 mM PMSF, 5 mM dithiothreitol, and 10% (v/v) glycerol). The cell fragments were removed by centrifugation at 14,000*g* and the supernatant was desalted into assay buffer (10 mM Mopso, pH 7.0, 1 mM dithiothreitol, and 10% (v/v) glycerol) by passage through a Econopac 10DG column (Bio-Rad, Hercules, CA, USA).

To determine the catalytic activity of the different terpene synthase mutants, enzyme assays containing 50 μl of the bacterial extract and 50 μl assay buffer with 10 μM (*E*,*E*)-FPP or (*Z*,*E*)-FPP, 10 mM $MgCl_2$, 0.05 mM $MnCl_2$, 0.2 mM $NaWO_4$, and 0.1 mM NaF in a Teflon-sealed, screw-capped 1 ml GC glass vial were performed. The substrates (*E*,*E*)-FPP and (*Z*,*E*)-FPP were compared using aliquots of the same enzyme extract. A solid phase microextraction (SPME) fiber consisting of 100 μm polydimethylsiloxane (SUPELCO, Belafonte, PA, USA) was placed into the headspace of the vial for 1 h incubation at 30 °C. For analysis of the adsorbed reaction products, the SPME fiber was directly inserted into the injector of the gas chromatograph. A comparison of the head space sampling by solid phase extraction with a direct pentane extraction of the enzyme assay showed the same characteristic blend of TPS4 products with nearly identical proportions of sesquiterpene hydrocarbons [18].

Gas chromatography

A Hewlett–Packard model 6890 gas chromatograph was employed with the carrier gas He at 1 ml min^{-1}, splitless injection (injector temperature 220 °C, injection volume 1 μl), a Chrompack CP-SIL-5 CB-MS column ((5%-phenyl)-methylpolysiloxane, 25 m × 0.25 mm i.d. × 0.25 μm film thickness, Varian, USA) and a temperature program from 40 °C (3-min hold) at 5 °C min^{-1} to 240 °C (3-min hold). The coupled mass spectrometer was a Hewlett–Packard model 5973 with a quadrupole mass selective detector, transfer line temperature 230 °C, source temperature 230 °C, quadrupole temperature 150 °C, ionization potential 70 eV, and a scan range of 40–350 amu. Compounds produced by the wild-type TPS4 and mutant enzymes with the two substrates were identified by comparison of retention times and mass spectra to those of authentic standards obtained from Fluka (Seelze, Germany), Roth (Karlsruhe, Germany), Sigma (St. Louis, MO, USA) or Bedoukian (Danbury, CT, USA), or by reference spectra in the Wiley and National Institute of Standards and Technology libraries and in the literature [24]. Many standards not commercially available were either obtained as described [18] or kindly supplied by Wilfried A. König, Hamburg. All analyses were performed at least twice.

Results and discussion

Computational analysis of the active site cavity of TPS4 suggests the existence of two pockets with distinctive catalytic roles

The maize terpene synthase 4 (TPS4) is a multi-product enzyme that produces β-bisabolene, 7-*epi*-sesquithujene, and 12 additional sesquiterpene hydrocarbons [18]. A reaction mechanism has been proposed in which a complex pattern of cyclizations, hydride shifts, and substitutions generates the full product spectrum (Fig. 1). To explore the structural basis for the proposed reaction mechanism of this enzyme, we modeled the protein structure using the data available for 5-*epi*-aristolochene synthase (TEAS), the only plant sesquiterpene synthase for which a three-dimensional crystal structure is available [6]. TPS4 and TEAS share 53% similarity and the models of TPS4 obtained were very uniform and structurally similar to TEAS, displaying the same α-helical pattern forming the active site in the C-terminal domain of the enzyme. The active site cavity of TPS4 is ≈$15 \times 12 \times 10$ Å and is divided into two pockets by the G2 helix which reaches slightly into the cavity (Fig. 2G). Interestingly, four of the amino acids that make up the G2 helix have been previously shown to have a dramatic impact on the product specificity of TPS4 [18].

To determine the positions of substrate, reaction intermediates, and products in the catalytic site, we employed docking with flexible ligand conformations. Docking of the (*E*,*E*)-FPP substrate showed that the diphosphate moiety of the substrate is situated at the entrance of the active site. This coincides with earlier observations on other terpene synthases indicating that (*E*)-FPP interacts with the highly conserved aspartate residues 308, 309, and 312 via complexed magnesium ions [6,25]. The olefin moiety of the FPP substrate is located predominantly in one of the two pockets (pocket I, Fig. 2A), with the terminal isopentenyl unit anchored in a narrow hydrophobic cleft in the back of the catalytic site ("back cavity," Fig. 2A). The catalytic reaction is initiated by dephosphorylation and formation of an (*E*,*E*)-farnesyl carbocation. After isomerization to the (*Z*,*E*)-farnesyl carbocation which also docked in pocket I (Fig. 2B), C1 is in close proximity to C6. An electrophilic attack of C1 on the C6–C7 double bond leads to the formation of the bisabolyl cation. Docking of the (*S*)-bisabolyl cation intermediate showed surprisingly that it can be situated in two positions, in pocket I if it adopts conformation A (Fig. 2E) or in pocket II if it adopts conformation B (Fig. 2C). The (*R*)-bisabolyl cation intermediate can adopt the two positions in pocket I and pocket II as well (data not shown). While (*S*)-β-bisabolene docked in pocket I (Fig. 2F), the additional intermediates leading to the formation of the bicyclic 7-*epi*-sesquithujene and the 7-*epi*-sesquithujene products docked in pocket II (Fig. 2D). These results suggest a model in which the early steps of the catalytic sequence including dephosphorylation, isomerization, and cyclization, up to the formation of the bisabolyl carbocation, all take place in pocket I.

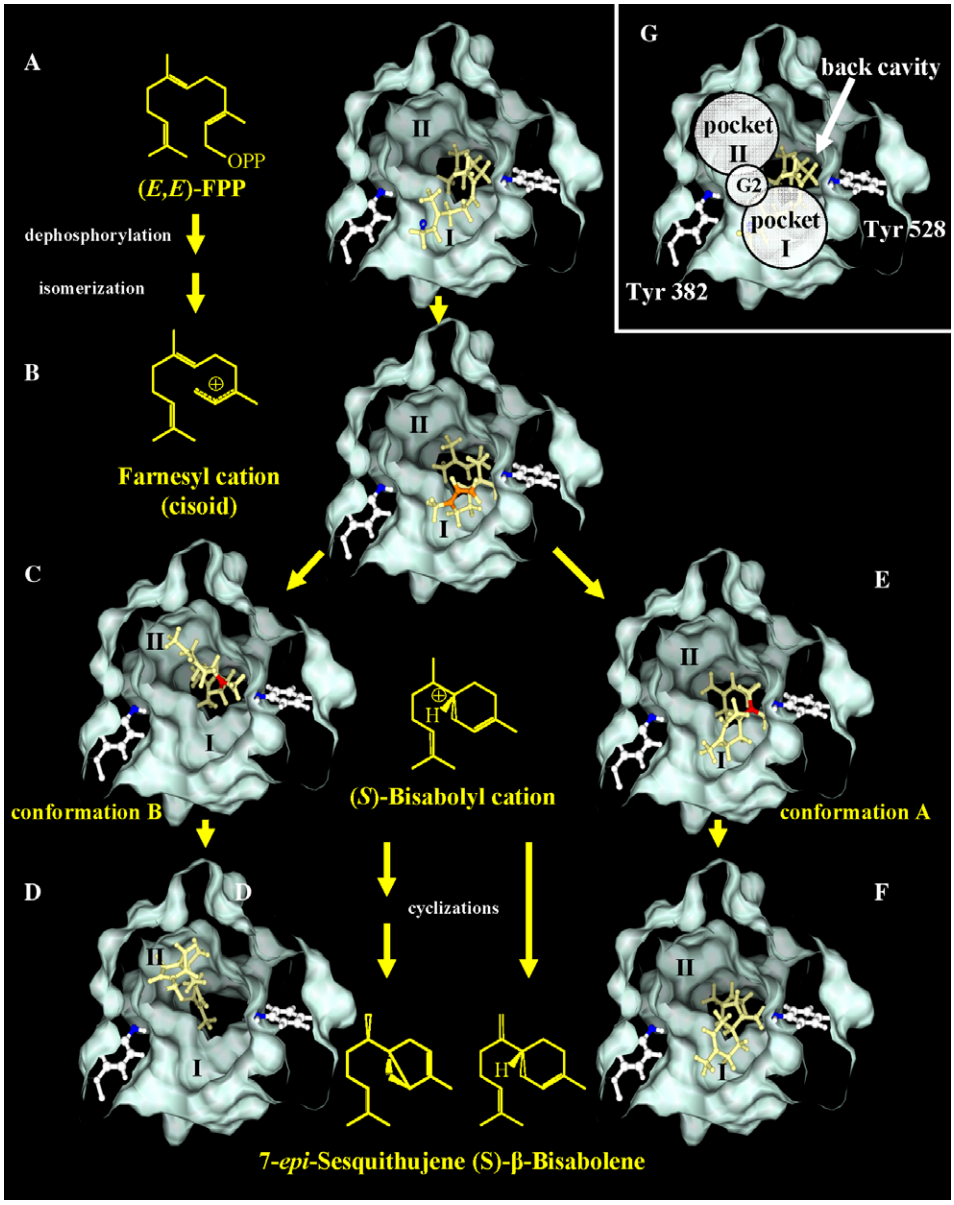

Fig. 2. Model of the TPS4 active site based on the structure of TEAS [6] with the proposed positions of the (*E*,*E*)-FPP substrate, carbocationic reaction intermediates, and sesquiterpene products. The model offers views of the surface of the active site with the docking of the (*E*,*E*)-FPP substrate (A), the farnesyl carbocation (B), the bisabolyl carbocation in conformation A (E) and B (C), and the products (*S*)-β-bisabolene (F) and 7-*epi*-sesquithujene (D). The diphosphate group of (*E*,*E*)-FPP which binds to the aspartate residues complexed with magnesium ions at the entrance of the site is cut off and the protein structures covering the active site are not shown. The reaction pockets I and II are labeled with roman numerals and the side chains of Y528 and Y328 are shown (G). G2 denotes the position of helix G2.

When the bisabolyl cation adopts conformation A, proton loss occurs and (*S*)-β-bisabolene is formed, also in pocket I. When the bisabolyl carbocation adopts conformation B, it shifts to pocket II. Then, a variety of additional cyclizations, hydride shifts, and deprotonations occur in pocket II leading to the formation of bicyclic products like 7-*epi*-sesquithujene.

Pocket I is the site of (E,E)-FPP substrate binding and isomerization

To verify the essential aspects of this two pocket model, site-directed mutagenesis was performed on various amino acid residues in the catalytic center of TPS4. Fig. 3 shows the most prominent residues that form the wall of the active site cavity and their location relative to the farnesyl carbocation and 7-*epi*-sesquithujene product. In pocket I, Y528 is a prominent amino acid residue with its functional group located close to the early intermediates of the reaction (Fig. 3). This tyrosine corresponds to Y520 in TEAS which was implicated in the protonation of a reaction intermediate [16]. We tested the function of Y528 in TPS4, an enzyme that does not catalyze the type of protonation that TEAS does. Removal of the tyrosine hydroxyl group in the Y528F mutant reduced TPS4 activity to only trace levels (Fig. 4A).

Loss of activity was also observed with the mutations Y528L and Y528S. The activity of these mutants was also tested with the (*Z*,*E*) isomer of the natural (*E*,*E*)-FPP substrate which was previously used to establish the initial (*E*,*E*)- to (*Z*,*E*)-isomerization of FPP in the reaction mechanisms of many sesquiterpene synthases [26]. Surprisingly, the three mutants of Y528 were active with the (*Z*,*E*)-FPP substrate, suggesting that Y528 is crucial for the isomerization of FPP and/or for earlier catalytic steps such as binding of the (*E*,*E*)-FPP substrate or its dephosphorylation (Fig. 4B). The similarity of the terpene products of Y528F, Y528L, and Y528S indicates that both the hydroxyl group and the aromatic ring of this tyrosine residue are important for enzyme activity.

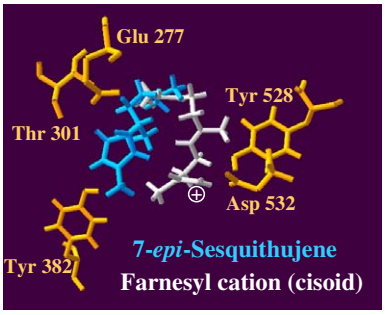

Fig. 3. Model of the reaction intermediates in pockets I and II of the TPS4 active site and some amino acid residues lining the pockets. The spatial relationships between the *cisoid* farnesyl cation in pocket I (white), the 7-*epi*-sesquithujene in pocket II (blue), and selected amino acids of the active cavity are shown. The amino acid residue L413 is part of the pocket II surface but is above the plane of the model and not included.

Starks et al. [6] postulated that the corresponding Y520 residue in TEAS is closely associated with two aspartate residues that initiate protonation of the germacrene A intermediate through polarization of the hydroxyl group of tyrosine. Sequence comparisons with TPS4 show that one of these two aspartate residues is conserved in position D532 while the position of the second aspartate is filled by an asparagine, N452 (both in pocket I). Site-directed mutagenesis was used to determine whether these residues interact with Y528 in TPS4. Alteration of D532 to N abolished activity with the (*E*,*E*)-FPP substrate but (*Z*,*E*)-FPP was still converted to the wild-type product blend (Fig. 4). This mutant behavior is similar to that of Y528F, Y528L, and Y528S, and suggests that both Y528 and D532 are involved in the same catalytic process(es). The mutation of N452 to

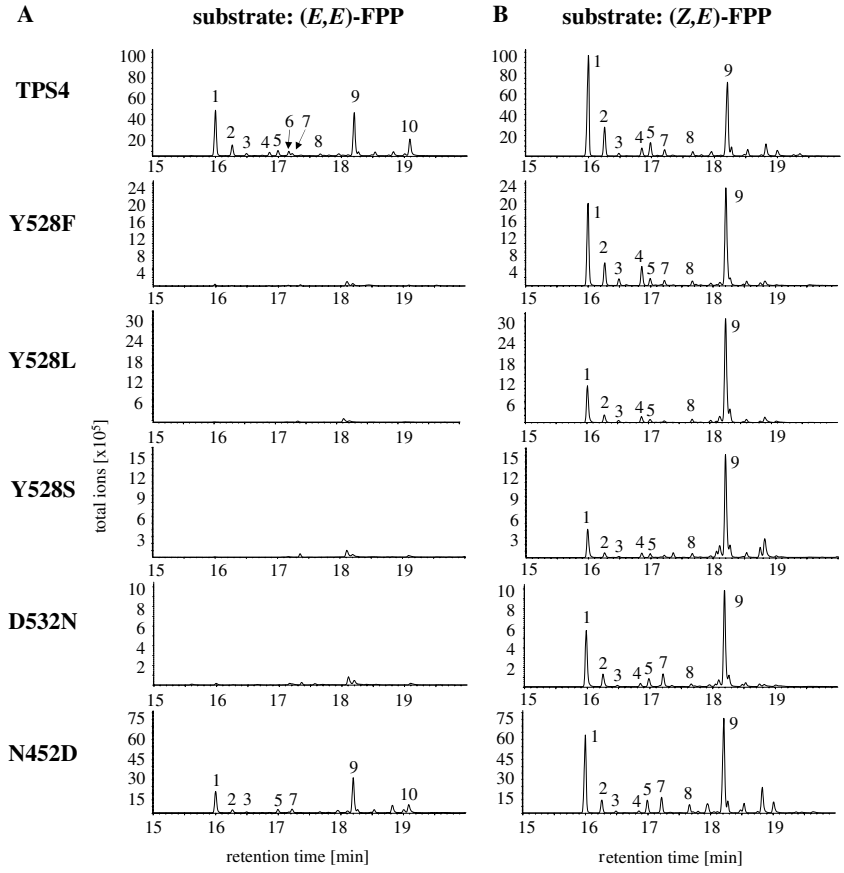

Fig. 4. Amino acid alterations in pocket I affect early substrate binding and isomerization. The amino acids Y528, D532, and N452 were changed by site-directed mutagenesis and the activities of the altered enzymes were determined with (*E*,*E*)-FPP (A) and (*Z*,*E*)-FPP (B). Total ion chromatograms of GC–MS analyses of the terpene products are shown. The sesquiterpene olefins were identified by mass spectrometry and comparison of mass spectra and retention times to those of authentic standards. 1, 7-*epi*-sesquithujene; 2, sesquithujene; 3, (*Z*)-α-bergamotene; 4, (*E*)-α-bergamotene; 5, sesquisabinene A; 6, (*E*)-β-farnesene; 7, sesquisabinene B; 8, γ-curcumene; 9, β-bisabolene; 10, (*E*)-nerolidol.

D had no significant effect on enzyme activity but the mutation of N452 to L was completely inactive with both substrates, probably because of disturbance of the active site cavity by the large, hydrophobic leucine side chain (data not shown). Mutational analysis of γ-humulene synthase and δ-selinene synthase of *A. grandis* suggested that the function of both of the corresponding aspartate residues in these enzymes are also important for the early steps of the reaction mechanism leading up to the protonation of the germacrene intermediates in these enzymes [17]. In contrast to the catalytic triad of Y520, D444, and D525 postulated for the TEAS mechanism [6], TPS4 appears to require a dyad of Y528 and D532 to form the (*Z*,*E*)-FPP intermediate. Although the precise function of these amino acids is not known, they clearly affect the early steps of the TPS4 catalytic process. Since these residues are located in or near pocket I, they support the model which predicts that the initial dephosphorylation, isomerization, and cyclization take place in this pocket.

Pocket II is the site of the second cyclization

To investigate the function of pocket II, amino acid residues forming the pocket II cavity were mutated. The functional group of tyrosine 382 is located close to the position of docked intermediates in pocket II (Figs. 2 and 3). Alteration of Y382 to F, L or S resulted in almost complete loss of the main bicyclic product, 7-*epi*-sesquithujene, and the minor bicyclic sesquiterpenes while the acyclic and monocyclic products were still produced (Fig. 5). The same pattern was observed after incubation with (*Z*,*E*)-FPP, indicating that mutation of Y328 blocks the second cyclization predicted to occur in pocket II according to the model, but has no effect on the steps leading up to the formation of the bisabolyl carbocation predicted to occur in pocket I (Fig. 3). The inactivity of Y382F and Y382L demonstrates the crucial role of the tyrosine hydroxyl group in the stabilization of the carbocation intermediate. The inactivity of Y382S may arise because the serine hydroxyl group is not properly positioned or because the aromatic ring is also essential for catalysis.

To further study the function of pocket II, we mutated the amino acid residues E277, T301, and L413 which constitute part of the pocket II surface (Fig. 3). Mutation of E277 to Q resulted in a decreased production of 7-*epi*-sesquithujene, both with (*E*,*E*)- and (*Z*,*E*)-FPP similar to the results obtained on mutation of Y382 (Fig. 5). The alteration of the neighboring T301 to W, however, resulted in complete inactivity of the enzyme (Fig. 6B). A model of pocket II including this mutation shows that the larger W residue may not only block most of pocket II but leads to steric interaction with L413 and a relative dislocation of the α-helices forming the catalytic center (Fig. 6A). To restore the positions of these α-helices and overall enzyme activity,

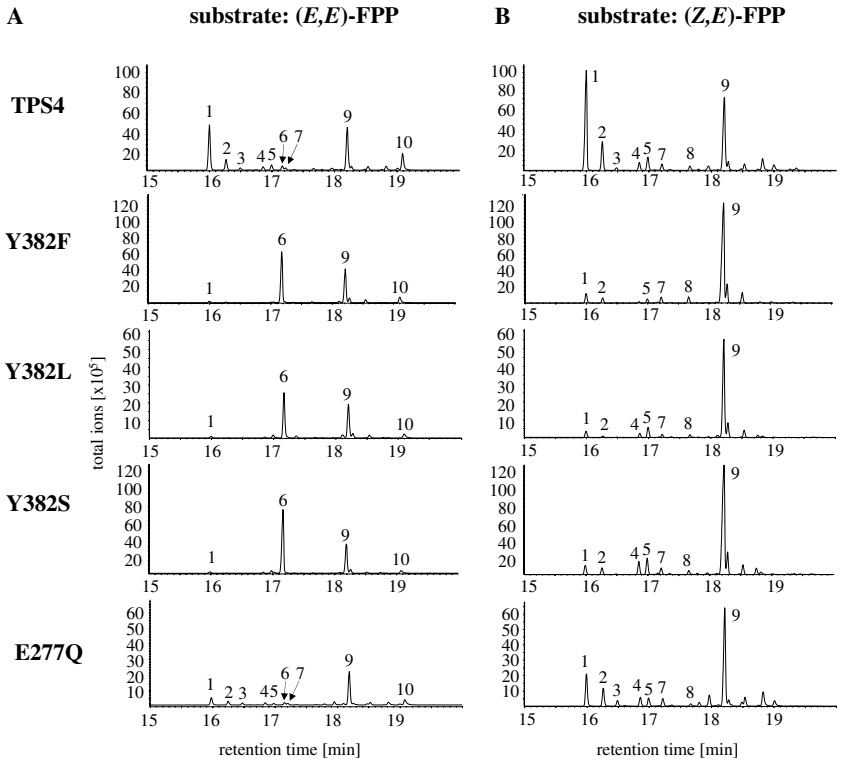

Fig. 5. Amino acid alterations in pocket II affect formation of the bicyclic sesquiterpene products. The amino acids Y382 and E277 were changed by site-directed mutagenesis and the activities of the altered enzymes were determined with (*E*,*E*)-FPP (A) and (*Z*,*E*)-FPP (B). Total ion chromatograms of GC–MS analyses of the terpene products are shown. The sesquiterpene olefins were identified by mass spectrometry and comparison of mass spectra and retention times to those of authentic standards. 1, 7-*epi*-sesquithujene; 2, sesquithujene; 3, (*Z*)-α-bergamotene; 4, (*E*)-α-bergamotene; 5, sesquisabinene A; 6, (*E*)-β-farnesene; 7, sesquisabinene B; 8, γ-curcumene; 9, β-bisabolene; 10, (*E*)-nerolidol.

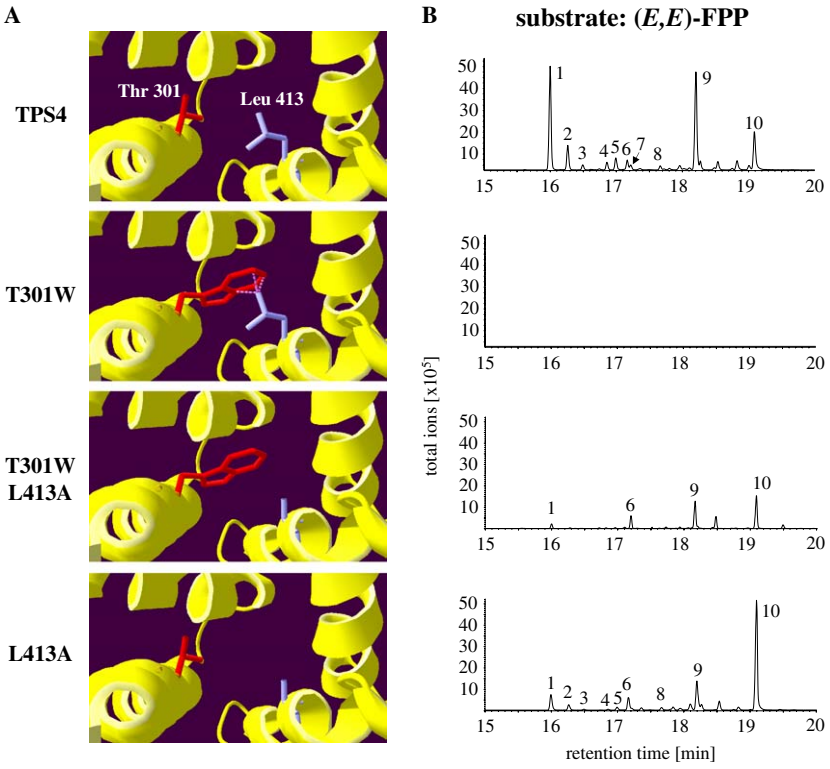

Fig. 6. Spatial constraints in pocket II affect enzyme activity. **(A)** Model of the pocket II cavity with the amino acids threonine 301, leucine 413, and their alterations by site-directed mutagenesis. **(B)** Product formation of the altered enzymes with (*E*,*E*)-FPP. Total ion chromatograms of GC–MS analyses of the terpene products are shown. 1, 7-*epi*-sesquithujene; 2, sesquithujene; 3, (*Z*)-α-bergamotene; 4, (*E*)-α-bergamotene; 5, sesquisabinene A; 6, (*E*)-β-farnesene; 7, sesquisabinene B; 8, γ-curcumene; 9, β-bisabolene; 10, (*E*)-nerolidol.

we decreased the size of the L413 side chain by mutation to alanine. The T301W L413A double mutant was active but formed only traces of bicyclic products, as might be predicted from the intrusion of the large tryptophan side chain in pocket II. The L413A mutation by itself also decreased bicyclic product formation, presumably by alteration of the pocket II cavity. In addition, this mutant produced a large amount of (*E*)-nerolidol, indicating that water molecules likely enter the enlarged cavity and react with the nerolidyl carbocation intermediate produced during the course of the reaction before it is converted to the bisabolyl cation. These mutations provide further support that pocket II is the likely site of the second cyclizations of TPS4. The overall consistency of the experimental results with the model suggests that the postulated three-dimensional protein structure based on TEAS is essentially correct.

Active site mutations can affect the secondary structure of TPS4

Mutations of amino acid residues may not only alter the side chains involved in catalysis but can also affect the secondary structure of the protein. These structural changes can be reflected in a reduction in activity or alterations of product specificity. The added size of the tryptophan side chain in the T301W mutation as discussed above appears to have led to alterations in secondary structure which caused a lack of activity with (*E*,*E*)-FPP (Fig. 6). Interestingly, this mutant showed 10% of wild-type activity when incubated with (*Z*,*E*)-FPP, but produced a different blend of sesquiterpenes with particular alterations in the relative proportions of bicyclic sesquiterpenes which according to the model are formed in pocket II (Fig. 7). The high concentration of sesquithujene in this blend is reminiscent of that of TPS5, a closely related maize terpene synthase which forms predominantly products of different stereospecificity to TPS4 due to a four amino acid change in the G2 helix which forms the boundary between pockets I and II. These results indicate that both the early steps leading to the isomerization of the FPP substrate and the later steps involved in the second cyclization are disturbed by the T301W mutation, suggesting alterations in both pockets. A disturbance of both reaction pockets is also observed after mutation of T305, an amino acid that is not part of the cavity surface. The mutant T305F is inactive with (*E*,*E*)-FPP but active with (*Z*,*E*)-FPP and forms a different product blend with the (*Z*,*E*)-FPP substrate than the wild-type enzyme (Fig. 7). This blend contains large amounts of sesquisabinene A, sesquisabinene B, and γ-curcumene which are usually minor products of TPS4.

The two functional pockets facilitate the formation of multiple products by TPS4

Domain swapping between two different terpene synthases has often been used as a tool to gain a more

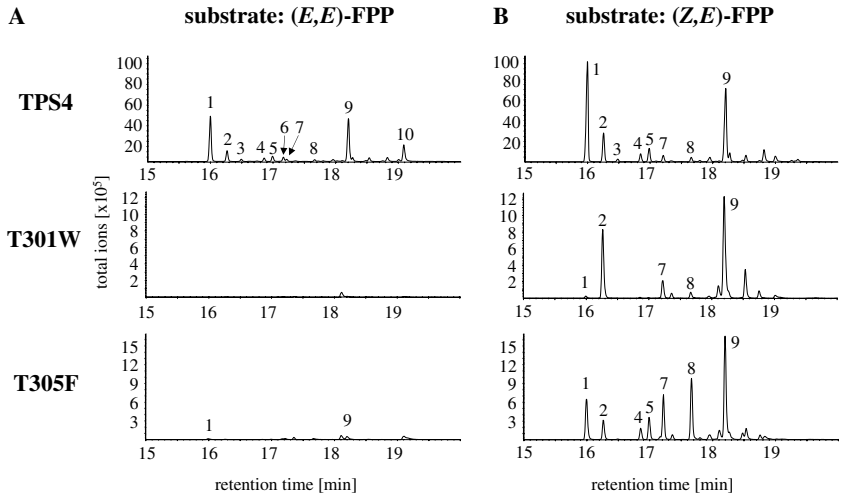

Fig. 7. Amino acid alterations can affect early substrate binding, isomerization, and formation of bicyclic sesquiterpene products. The amino acids T301 and T305 were changed by site-directed mutagenesis and the activities of the altered enzymes were determined with the (*E*,*E*)-FPP (A) and (*Z*,*E*)-FPP (B) substrates. Total ion chromatograms of GC–MS analyses of the terpene products are shown. The sesquiterpene olefins were identified by mass spectrometry and comparison of mass spectra and retention times to those of authentic standards. 1, 7-*epi*-sesquithujene; 2, sesquithujene; 3, (*Z*)-α-bergamotene; 4, (*E*)-α-bergamotene; 5, sesquisabinene A; 6, (*E*)-β-farnesene; 7, sesquisabinene B; 8, γ-curcumene; 9, β-bisabolene; 10, (*E*)-nerolidol.

complete understanding of structure–function relationships in terpene synthases. For example, the analysis of monoterpene synthases from *S. officinalis* and *A. grandis* demonstrated that product specificity is determined within the C-terminal domain which encodes the active site [10,11]. A domain swapping analysis of γ-terpinene and β-pinene synthases of *Citrus limon* revealed that the respective product specificities are determined by a segment of 200 amino acids in the first half of the C-terminal domain which includes a region that corresponds to pocket II of TPS4 [9]. Similarly, determinants of the second cyclization carried out by (+)-bornyl diphosphate synthase from *S. officinalis* are located between amino acids 430 and 499 within the C-terminal domain [10]. Exchanges close to the C-terminus in the region of pocket I decreased enzyme activity dramatically, verifying that this region is essential for catalysis [9,10].

Site-directed mutagenesis allows a more detailed analysis of protein structures. Application of this method to maize TPS4 has supported the concept of two pockets with different functionalities within the active site. Pocket I is the site of the early steps of the reaction and the formation of one of the major products, (*S*)-β-bisabolene. Conformational change of the intermediate bisabolyl carbocation moves it from the first to the second pocket and leads to the formation of a different group of products including the major and minor bicyclic sesquiterpenes. Such conformational mobility may also serve to expand the product spectrum in other terpene synthases that form multiple products from a single substrate. The energy required for such conformational changes is likely to be very small and determined both by the chemical nature of the intermediate and the surrounding amino acids. Overall, the results of the modeling and the mutagenesis experiments support the reaction mechanism originally proposed for TPS4 [18].

Previous investigations of the terpene synthase reaction mechanism have attributed the formation of multiple products to the fact that the reactive carbocationic intermediates can have many metabolic fates even while accommodated within a single active site. Our results suggest that conformational shifts of intermediates dictated by the substructure of the active site may direct their metabolic fate and thus influence the final product composition. It is conceivable that the terpene synthases which allow conformational changes of reaction intermediates between reactive pockets form a more complex blend of products than those that limit intermediates to one confirmation. However, not only the size but also the functional groups in the cavity are important for the outcome of the reaction. Mutations in the aristolochene synthase of *Penicillium roqueforti* that increased the cavity without additional stabilization of the reaction intermediates allowed the first intermediate to assume its energetically lowest conformation, the acyclic farnesyl carbocation which was subsequently deprotonated to (*E*)-β-farnesene [27]. Further research into the reaction mechanisms of other terpene synthases and their relation to protein structure is necessary to determine if the presence of functional pockets in the active site is a more general phenomenon. Unraveling the structure–function relationships of terpene synthases will not only lead to a greater understanding of their fascinating reaction mechanisms but also enhance the ability to engineer terpene synthases with a specific, commercially desired terpene spectrum [28].

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