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A Multiproduct Terpene Synthase from *Medicago truncatula* Generates Cadalane Sesquiterpenes via Two Different Mechanisms

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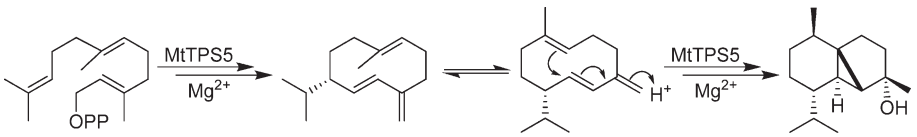

Terpene synthases are responsible for a large diversity of terpene carbon skeletons found in nature. The multiproduct sesquiterpene synthase MtTPS5 isolated from *Medicago truncatula* produces 27 products from farnesyl diphosphate (**1**, FDP). In this paper, we report the reaction steps involved in the formation of these products using incubation experiments with deuterium-containing substrates; we determined the absolute configuration of individual products to establish the stereochemical course of the reaction cascade and the initial conformation of the cycling substrate. Additional labeling experiments conducted with deuterium oxide showed that cadalane sesquiterpenes are mainly produced via the protonation of the neutral intermediate germacrene D (**5**). These findings provide an alternative route to the general accepted pathway via nerolidyl diphosphate (**2**, NDP) en route to sesquiterpenes with a cadalane skeleton. Mutational analysis of the enzyme demonstrated that a tyrosine residue is important for the protonation process.

Introduction

Sesquiterpenes are one of the structurally most diverse class of natural products isolated from plants, fungi, bacteria, and marine invertebrates.[1,2] They are known to play crucial roles when plants defend themselves against enemies, attract pollinators, and signal within and to each other. All the tens of thousands of sesquiterpenoids known to date are derived from 300 basic hydrocarbon skeletons generated by sesquiterpene cyclases (synthases) using farnesyl diphosphate (**1**, FDP) as substrate.[3] The variety of products generated by these enzymes can differ dramatically. In addition to high-fidelity enzymes, for example, the δ-cadinene synthase from *Gossypium arboreum*[4], which releases a single product, there are multiproduct enzymes, which are known to synthesize up to 52 different products.[5,6] The structural basis of varying selectivity is believed to be a consequence of the three-dimensional contour of the active site that allows or refuses different conformations of the reactive cationic intermediates.[7,8] Nevertheless, the structure and stereochemistry of the products are suggested to be mainly determined by the correct folding of the substrate and the electrostatic interactions of the cationic intermediates with amino acids of the active site.[9] The reaction cascade is initiated by a metal-ion-mediated cleavage of the diphosphate group from the substrate, resulting in a highly reactive carbocation. This reactive intermediate can undergo different cyclizations and rearrangements, such as hydride and methyl shifts. The products are released by elimination of a proton or after reaction with water, affording sesquiterpene alcohols.[10] Terpene synthases are also able

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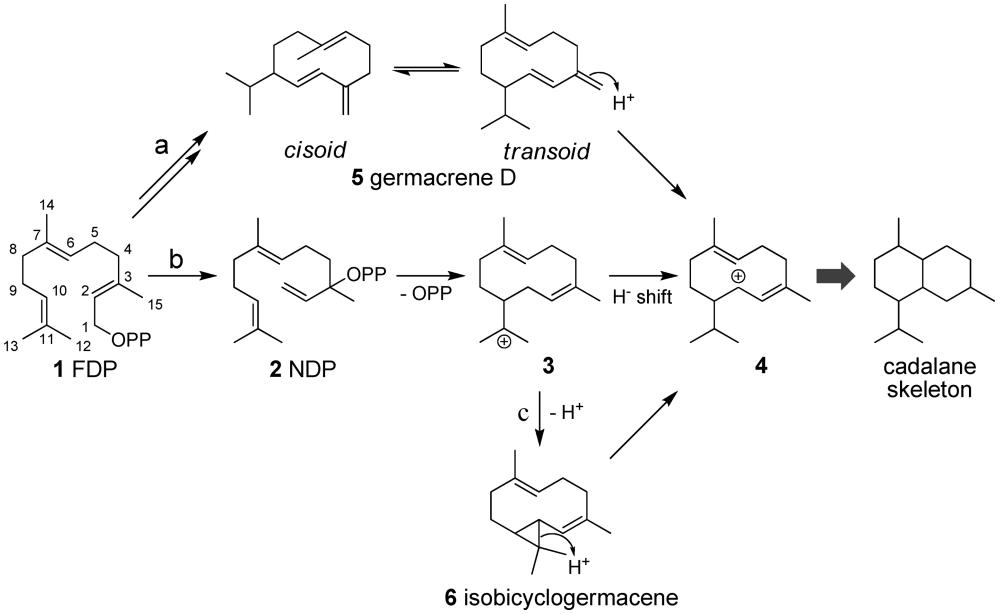
SCHEME 1. Proposed Pathways to Cadalane-Type Sesquiterpenes



to produce enzyme-bound neutral intermediates that are further cyclized after reprotonation. Germacrene A, for example, formed by a sequence consisting of the ionization of FDP (**1**), C1−C10 cyclyzation, and deprotonation at C12, is an intermediate in the reaction catalyzed by the 5-*epi*-aristolochene synthase (TEAS) from tobacco.[11] Reprotonation of the neutral intermediate initiates the reaction sequence starting with a C1−C7 cyclization, followed by a Wagner−Meerwein rearrangement of a methyl group and the loss of a proton that affords the main product 5-*epi*-aristolochene. Analysis of the crystal structure of TEAS cocrystallized with the artificial substrate farnesyl hydroxyphosphonate suggests that a catalytic triad consisting of a tyrosine residue (Tyr520) and two aspartate residues (Asp444 and Asp525) are responsible for the reprotonation of germacrene A.[12] The important role of Tyr520 in the proton-transfer reaction has been demonstrated by the exchange of the tyrosine residue with phenylalanine; this results in a protein that released germacrene A as its sole product.[13] Other germacrene-type sesquiterpenes have also been proposed as biogenic intermediates for certain groups of sesquiterpenes.[14,15] Arigoni suggested a pathway for the biosynthesis of cadalane skeletons involving germacrene D (**5**) as a neutral intermediate (pathway a, Scheme 1); such a pathway would overcome the geometric restriction hindering the direct cyclization of FDP (**1**) to 10-membered ring systems with *cis*-configured C2−C3-double bonds.[16] The key step in the reaction sequence is the conformational change of the neutral intermediate germacrene D (**5**) from the *cisoid* to the *transoid* structure, affording a *cis*-configured C2−C3-double bond after reprotonation (cation **4**, Scheme 1). In fact, the existence of this pathway has never been experimentally demonstrated. However, the enzymatic cyclization of other cadalane-type sesquiterpenes such as cubenene,[17] 1-*epi*-cubenol,[17,18] and δ-cadinene[19] has been shown to proceed via a nerolidyl diphosphate (**2**, NDP) intermediate (pathway b, Scheme 1), which allows rotation about the 2,3-single bond and ring closure to give the helmintogermacrenyl cation (**3**). In these cases, the cationic intermediate **4** originated from **3** by a hydride shift[17−19] or by protonation of the cyclopropane moiety of previously formed isobicyclogermacrene (**6**)[16] intermediate (pathway c, Scheme 1).

We recently reported a multiproduct terpene synthase from *Medicago truncatula* that catalyzes the formation of different hydrocarbon skeletons such as cadinane, copaane, cubebane, and germacrane.[20] Using an approach that combines classical labeling experiments, the determination of the absolute configuration and the enantiomeric excess of individual products by chiral gas chromatography and site-directed mutagenesis, we established the reaction cascade and the mode by which the products are cyclized. In addition, we report an alternative pathway for the formation of cadinane, cubebane, muurolane, and copaane skeletons, which proceeds via the protonation of (−)-germacrene D (**5**), proving a prior hypothesis of Arigoni.[16] Knowledge of the exact configuration of products and their optical purity allows us to elucidate the extent of enzymatic control over certain reaction steps and the initial folding of the cycling substrate.

Results

MtTPS5 Is a Multiproduct Enzyme Generating 27 Different Products. Recombinant MtTPS5 incubated with FDP (**1**)

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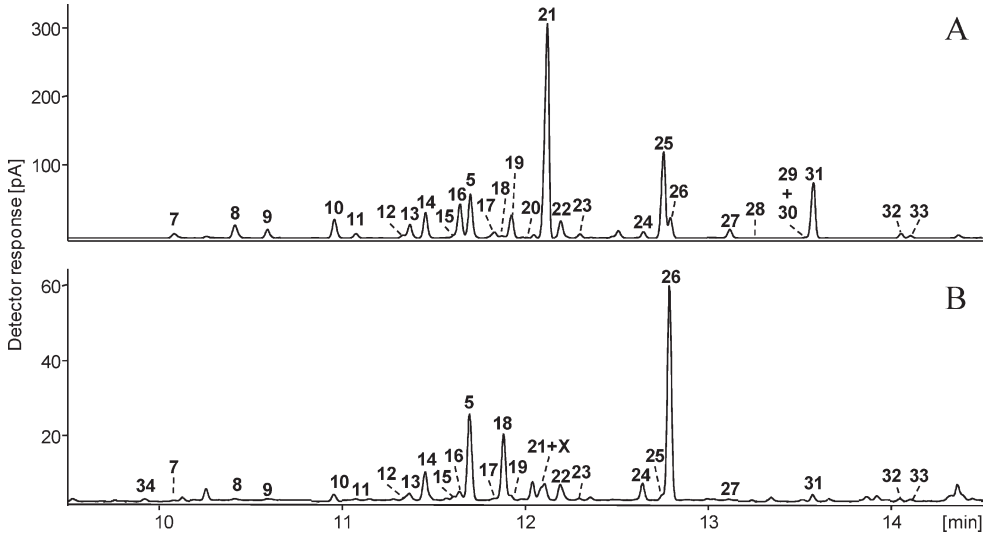

FIGURE 1. GC−FID chromatograms of enzymatic products of recombinant MtTPS5 wild type (A) and Y526F mutant (B) from incubation with (2*E*,6*E*)-FDP (**1**). The products were identified by Kováts indices and mass spectra in comparison to authentic samples: **7**, α-cubebene; **8**, α-copaene; **9**, *β*-cubebene; **10**, (*E*)-*β*-caryophyllene; **11**, *β*-copaene; **12**, cadina-3,5-diene; **13**, α-humulene; **14**, *allo*-aromadendrene; **15**, *trans*-cadina-1(6),4-diene; **16**, *γ*-muurolene; **5**, germacrene D; **17**, bicyclosesquiphellandrene; **18**, bicyclogermacrene; **19**, α-muurolene; **20**, *δ*-amorphene; **21**, cubebol; **22**, *δ*-cadinene; **23**, cadina-1,4-diene; **24**, nerolidol;[a] **25**, copan-3-ol; **26**, 4α-hydroxygermacra-1(10),5-diene; **27**, copaborneol;[b] **28**, 1-*epi*-cubenol; **29**, T-cadinol; **30**, cubenol;[b] **31**, torreyol; **32**, kunzeaol;[b] **33**, $C_{15}H_{26}O$; **34**, bicycloelemene.[c] [a]Nonenzymatic hydrolysis product of the substrate (**1**). [b]No reference compound available. [c]Thermal rearrangement product of bicyclogermacrene (**18**), X BHT-like antioxidant; peaks not indicated were contaminants from the GC column or the solvent used.

has been reported to result in 15 sesquiterpenoid products.[20] A thorough reevaluation of the GC−MS data revealed that at least 27 sesquiterpenes are formed in the presence of Mg^{2+} ions, 25 of which could unambiguously be identified by their Kováts indices and mass fragmentation in comparison with authentic references (Figure 1, Table SI-1 (Supporting Information)). In addition to the sesquiterpene hydrocarbons (31.7%), the recombinant protein generates predominantly sesquiterpene alcohols (68.3%) with cubebol (**21**) (38.6%) as the major product. The second most abundant product, **25** (14.9%), showed a mass spectrum very similar to that of **21** with a molecular ion at *m*/*z* 222 and a base peak at *m*/*z* 161, typical for the loss of an isopropyl radical. The attempted catalytic hydrogenation (5% rhodium on alumina, 1 h) of **25** showed no hydrogen uptake, proving a tricyclic backbone for **25**. Moreover, dehydratization of **20** with trifluoroacetic anhydride yielded predominantly α-copaene (**8**). A compound matching all experimental observations is copan-3-ol, previously reported from *Ocimum americanum*.[21] Like the two major products, a number of substances are formed containing a 3,7-dimethyl-10-isopropyloctaline skeleton (so-called cadalane skeleton), such as torreyol (**31**) (9.3%), *γ*-muurolene (**16**) (4.9%), α-muurolene (**19**) (3.1%), *δ*-cadinene (**22**) (2.5%), α-copaene (**8**) (2.1%), copaborneol (**27**) (1.4%), *β*-cubebene (**9**) (1.3%), bicyclosesquiphellandrene (**17**) (1.1%), α-cubebene (**7**) (0.8%), *β*-copaene (**11**) (0.7%), cadina-1,4-diene (**23**) (0.5%), and six other compounds (each of which $>0.5\%$). A second group comprises the germacranes, namely germacrene D (**5**) (5.8%), 4α-hydoxygermacra-1(10),5-diene (**26**) (2.8%), kunzeaol (**32**) (0.7%), and bicyclogermacrene (**18**) (0.3%). Additionally, α-humulene (**13**) (2.0%), (*E*)-*β*-caryophyllene (**10**) (2.6%), and *allo*-aromadendrene (**14**) (3.5%) along with an unidentified sesquiterpene alcohol (**33**, $C_{15}H_{26}O > 0.1\%$) were generated. The acyclic nerolidol (**24**) resulted from nonenzymatic hydrolysis of the substrate as demonstrated by control experiments with the heat-inactivated enzyme (data not shown).

Stereochemical Analysis of the Enzyme Products. To examine the stereochemical course of product formation, the absolute configuration of a subset of enzyme products was determined by GC−MS using chiral stationary phases. The stereochemistry of the two major alcohols, cubebol (**21**) and copan-3-ol (**25**), was inferred from the acid rearrangement products *δ*-cadinene (**22**) and α-muurolene (**19**) (Figure SI-1, Supporting Information), respectively. No information about the absolute configuration at the carbon C3 of **25** from literature was available.[21] However, the enantiomeric analysis of 17 products of the MtTPS5 (Table 1, Figure 2) revealed that germacrene D (**5**), *allo*-aromadendrene (**14**), bicyclogermacrene (**18**), and all analyzed cadalanes exhibited the (*S*)-configuration at C10. Additionally, the relative configuration of all other cadalane stereocenters turned out to be the same. These results enabled us to deduce the absolute configurations of all other products. The evaluation of the enantiomeric composition revealed that all substances were optically pure (≥98% ee). The enantiomeric excess of (−)-**16** could not be determined since the (+)-enantiomer elutes with the same retention time as (−)-**14** generated by the enzyme. In addition, the identical enantiomers of products are formed by the Y526F mutant discussed below (Table 1, Figure 2).

The identical configuration in compounds sharing the same skeleton is in agreement with a common cationic precursor and a largely predetermined conformation of the acyclic substrate in the active site. Only a limited number of conformations of (2*E*,6*E*)-FDP (**1**) allow the formation of 10- or 11-membered ring systems.[22] In fact, four (Scheme 2)

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TABLE 1. Absolute Configuration of Enzyme Products

enzyme product
(−)-α-cubebene (**7**)
(−)-α-copaene (**8**)[a]
(−)-β-cubebene (**9**)
(−)-(*E*)-β-caryophyllene (**10**)
(−)-β-copaene (**11**)
(−)-cadina-3,5-diene (**12**)
(−)-allo-aromadendrene (**14**)
(−)-γ-muurolene (**16**)
(−)-germacrene D (**5**)
(−)-bicyclosesquiphellandrene (**17**)[b]
(−)-bicyclogermacrene (**18**)
(−)-α-muurolene (**19**)
(−)-cubebol (**21**)[a]
(+)-δ-cadinene (**22**)
(−)-cadina-1,4-diene (**23**)
(+)-copan-3-ol (**25**)[a]
(−)-torreyol (**31**)[a]

[a]Not determined for Y526F mutant. [b]Not detected in Y526F.

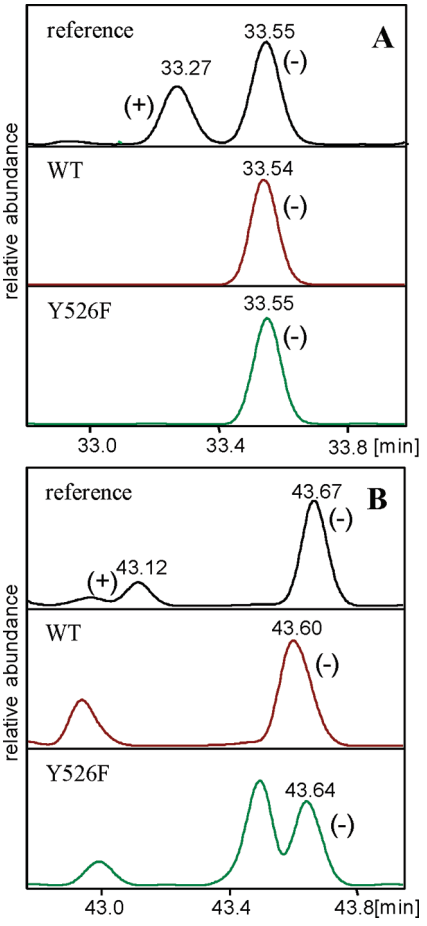

FIGURE 2. Identification of the absolute configuration of α-cubebene (**7**) (A) and germacrene D (**5**) (B) produced by the wild type MtTPS5 and the mutant Y526F enzyme.

of the eight possible diastereomeric conformations of **1** result in a (*R*)-configuration at C10 after ring closure between C1 and C10 of the substrate. Following the concept of the least motion during the course of cyclization, conformation **1a** (Scheme 2) can be inferred as the starting conformation for the majority of the enzyme's products. This conclusion is supported by relative and absolute configurations of the bridgehead atoms of (*E*)-β-caryophyllene (**10**) (2*S*,10*R*), *allo*-aromadendrene (**14**) (2*R*,6*S*), and the cadalanes (*cis*-fused decalin system).

Hydride Shifts and 1,3-Deprotonation Steps in the Formation of MtTPS5 Products. Incubation of the MtTPS5 with selectively labeled FDPs, such as (1*S*)-[1-^{2}H]-FDP, (1*R*)-[1-^{2}H]-FDP, and [1,1-$^2H_2$]-FDP, generated products with deuterium atoms in positions that allowed the reconstruction of mechanistic details of the reaction cascade. Most important are the molecular ion ($[M]^+$, number of deuterium atoms) and the cation (fragment a) resulting from the loss of the isopropyl side chain (Table 2, Figure 3, Table SI-2 (Supporting Information)).[17,23,24]

Incubating the enzyme with [1,1-$^2H_2$]-FDP resulted in a molecular ion (*m*/*z* 206 and *m*/*z* 224, respectively) for both germacrene D (**5**) and cubebol (**21**), confirming that both deuterium atoms remained in the molecules. Additional fragments at *m*/*z* 162 indicated the loss of a labeled isopropyl group, supporting that a single deuterium atom migrated from position C1 to C11. The stereochemical course of the 1,3-hydride shift was elucidated by the use of (1*R*)- and (1*S*)-[1-^{2}H]-FDP as substrates. Germacrene D (**5**) and cubebol (**21**) showed ions at *m*/*z* 205 and 161 ($[M - C_3H_6D]^+$) after incubation with (1*R*)-[1-^{2}H]-FDP, while incubation with (1*S*)-[1-^{2}H]-FDP the *m*/*z* of the corresponding ions were shifted to *m*/*z* 205 and 162, confirming the loss of an unlabeled isopropyl group. The results are consistent with a mechanism by which the C1-H_{Re} of the substrate is transferred via a suprafacial 1,3-hydride shift to the tertiary carbon atom of the isopropyl group (Scheme 3). The same result was observed for all other compounds with a germacrane or cadalane skeleton (Table SI-2, Supporting Information). The mass spectra of bicyclogermacrene (**18**) and *allo*-aromadendrene (**14**) obtained by the same series of incubations are consistent with a stereospecific 1,3-deprotonation of the (2*E*,6*E*)-germacren-11-yl cation (**35**) (Scheme 3) to give **18**. With (1*R*)-[1-^{2}H]-FDP as substrate, the C1-$^2H_{Re}$ is lost (*m*/*z* 204), while in the case of (1*S*)-[1-^{2}H]-FDP as substrate the deuterium atom remained in the product (*m*/*z* 205).

The mass spectra of *trans*-cadina-1(6),4-diene (**15**) and cadina-3,5-diene (**12**) also provided information on later hydride shifts in the reaction cascade. For example, the cadinanyl cation (**37**) (Schemes 4 and Scheme 6) suffers two consecutive 1,2-hydrogen shifts to the cations **39** and **38**, which serve as precursors for **12**, **15**, and **17**. An alternative 1,3-hydrogen shift from cation **37** to the cation **38** could be excluded since incubation of (1*S*)-[1-^{2}H]-FDP gave **15** with complete loss of deuterium. In case of a direct 1,3-shift, the deuterium atom of **37** would migrate to C7 and remain in the molecule (Scheme 4).

Formation of Cadalanes and *allo*-Aromadendrene Proceed via Protonation of Neutral Intermediates. To confirm the existence of protonation steps in the reaction cascade, we analyzed the enzyme activity in the presence of D_2O. Mass spectrometric analysis of the compounds revealed different incorporation rates of deuterium in the enzyme products (Table 3 and Table SI-3 (Supporting Information)). The germacranes (**5**, **26**, and **32**), α-humulene (**13**), (*E*)-β-caryophyllene

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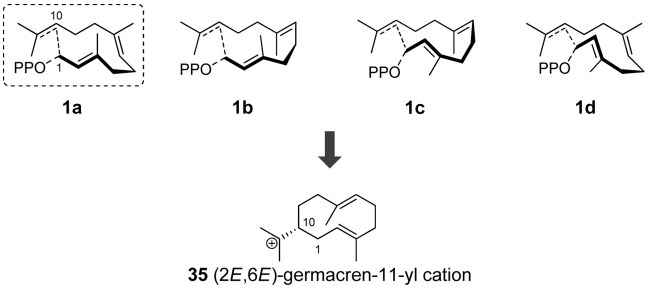
TABLE 2. Mass Spectrometric Parent and Fragment Ions from Some Nonlabeled and Deuterated Products of Recombinant MtTPS5

	ions[a] (m/z)							
	nonlabeled FDP		(1*S*)-[1-^{2}H]-FDP		(1*R*)-[1-^{2}H]-FDP		[1,1-$^2H_2$]-FDP	
	M^+	*a*	M^+	*a*	M^+	*a*	M^+	*a*
germacrene D (**5**)	204	161	205	162	205	161	206	162
cubebol (**21**)	204[b]	161	205[b]	162	205[b]	161	206[b]	162
trans-cadina-1(6),4-diene (**15**)	204	161	204	161	205	161	205	161
allo-aromadendrene (**14**)	204	161	205	162	204	161	205	162
bicyclogermacrene (**18**)	204	161	205	162	204	161	205	162

[a]Formation of ions shown in Figure 3. [b]$[M - H_2O]^+$.

SCHEME 2. Possible Starting Conformations of FDP (1) Leading to (*R*)-Configuration at C10 after C1 to C10 Cyclization



(**10**), and bicyclogermacrene (**18**) did not incorporate deuterium, which is in line with mechanistic pathways reported in the literature.[2,25] In contrast, the mass of the molecular ion of *allo*-aromadendrene (**14**) was increased by 1 amu to m/z 205, indicating that a single deuterium atom was most likely transferred to bicyclogermacrene (**18**) at C3 (Scheme 3). These results validate the suggestion that the formation of **14** is achieved by the loss of the hydrogen at C1 with subsequent closure to the three-membered ring system, as was originally proposed for the formation of kelsoene and prespatane proceeding via an *allo*-aromadendranyl cation.[26] However, because of hydrogen scrambling during the fragmentation process (cf. Schmidt et al.[27]), the exact deuterium position in **14** cannot be determined. Interestingly, all cadalanes except δ-amorphene (**20**) and T-cadinol (**29**) also incorporated deuterium, with labeling degrees up to 92% d_1 (Table 3 and Table SI-3 (Supporting Information)). These results confirm a previous hypothesis of Arigoni, that the formation of cadalane-type sesquiterpenes could be initiated by a proton transfer to the exocyclic double bond of *transoid* germacrene D (**5**) (Scheme 1, pathway a).[16] Indirect evidence for the site of protonation is provided by the lower degree of deuterium labeling of β-copaene (**11**) (60% d_1), β-cubebene (**9**) (74% d_1), and bicyclosesquiphellandrene (**17**) (73% d_1) that is consistent with the partial loss of deuterium during the final deprotonation step to the exocyclic double bond of **9**, **11**, and **17** (Scheme 5).

Identification of Tyrosine 526 as a Key Amino Acid in Proton-Transfer Reactions. The crystal structure of the 5-*epi*-aristolochene synthase from tobacco (TEAS) contains a catalytic triad of aspartate 444, tyrosine 520, and aspartate 525 which delivers a proton to the neutral intermediate germacrene A.[12] The crucial role of the phenolic hydroxyl group of Tyr520 in the proton transfer reaction was confirmed by site-directed mutagenesis.[13] A comparison of the amino acid sequence of the MtTPS5 and TEAS revealed in MtTPS5 a tyrosine in an equivalent position. The exchange of this tyrosine 526 by phenylalanine (Y526F mutant) resulted in a recombinant protein showing only 10% of the activity of the wild type (WT) and a dramatic change in the product profile (Figure 1). The Y526F mutant generated almost exclusively germacranes, namely germacrene D (**5**), 4α-hydroxygermacra-1(10),5-diene (**26**), and bicyclogermacrene (**18**), accounting for 75% of product profile (Figure 1). In contrast, the cadalane-type sesquiterpenes are reduced from 82% (WT) to 15% (Y526F mutant) supporting a crucial role of the OH-group from Tyr526 for the protonation of the germacrene D precursor. To understand the origin of the remaining cadalanes, we performed the same incubation experiments with the Y526F mutant in D_2O as described above. In these experiments the cadalanes displayed a low degree of deuteration (0% to 30% d_1) which suggests that an additional and independent reaction channel for their formation should exist. The most plausible alternative appears to be the intermediacy of NDP (**2**) and the nerolidyl cation which delivers the correct orientation for the C1 to C10 ring closure to the cation **3** (Scheme 6). This bypasses the protonation by Tyr526 en route to the cation **4a** (Scheme 6) and explains the formation of cadalanes with low deuterium labeling.

Discussion

Proposed Mechanism of the MtTPS5 Enzyme Product Formation. A general scheme integrating the results of different incubation experiments with H_2O and D_2O obtained from unlabeled FDP and chirally labeled substrates is given in Scheme 6. The mechanistic concept also takes into account the identical absolute configuration of products sharing the same skeleton and the existence of parallel and independent pathways initiated by protonation of neutral intermediates. The cyclization cascade starts by ionization of FDP prefolded according to conformation **1a** (Scheme 2) yielding the highly reactive *transoid* farnesyl cation (**42**). The C1 to C11 ring closure of **42** affords the humulyl cation (**43**), which can either lose a proton from C9 to form the achiral α-humulene (**13**) or undergo a second C2 to C10 closure by electrophilic attack on the *re*-face of the C2−C3 double bond. Final loss of a proton generates (−)-(*E*)-β-caryophyllene (**10**). The majority of the products (95%) requires the initial C1 to C10 closure and is achieved by the electrophilic attack on the *si*-face of the distal

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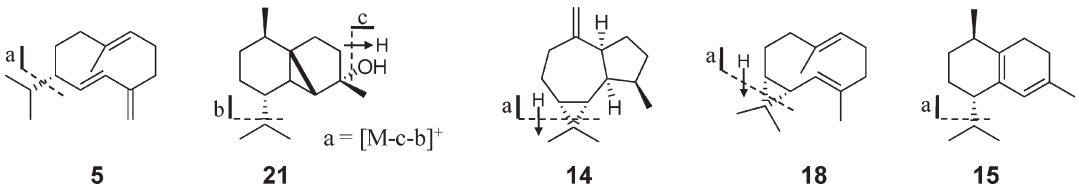
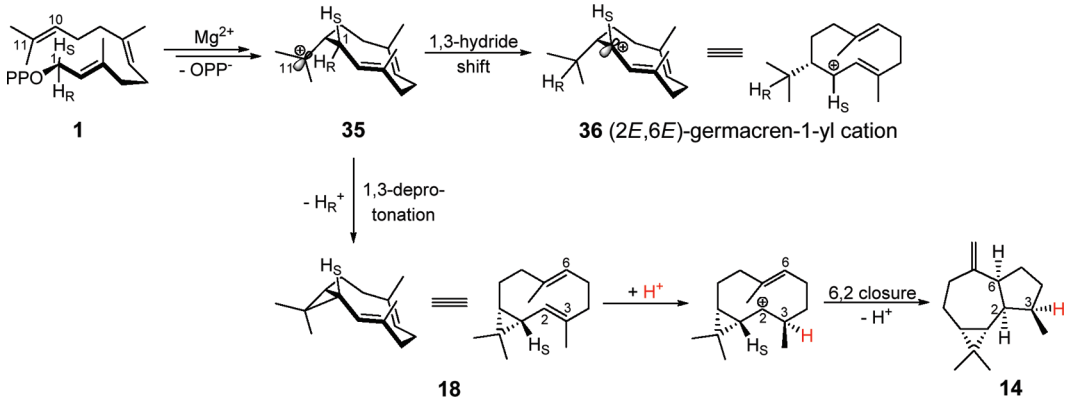


FIGURE 3. Formation of fragment ions of some products of MtTPS5.

SCHEME 3. Stereochemical Course of the 1,3-Hydride Shift and the 1,3-Deprotonation Leading to the (2*E*,6*E*)-Germacren-1-yl Cation (36), 14, and 18, Respectively



SCHEME 4. Possible Labeling Pattern of *trans*-Cadina-1(6),4-diene (15) Obtained by Incubation with (1*S*)-[1-^{2}H]-FDP along Different Types of Hydride Shifts

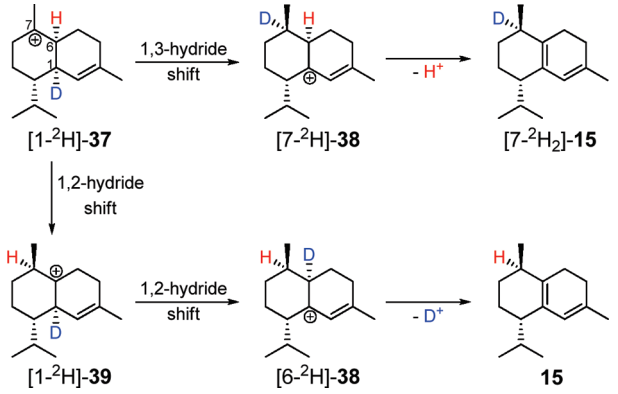


TABLE 3. Deuterium Content of a Subset of Enzyme Products from Incubating Recombinant MtTPS5 and Y526F Mutant in Highly Enriched D_2O Medium (Mean Values and Standard Deviation of Four Independent Experiments)

	deuterium content d_1 (%)	
	wild type	mutant Y526F
α-cubebene (**7**)	92 ± 1	29 ± 4
β-cubebene (**9**)	74 ± 1	24 ± 3
bicyclosesquiphellandrene (**17**)	73 ± 5	n.d.
α-copaene (**8**)	92 ± 1	30 ± 6
β-copaene (**11**)	60 ± 3	11 ± 3
α-muurolene (**19**)	91 ± 1	2 ± 5
δ-amorphene (**20**)[a]	−4 ± 0	−1 ± 1
allo-aromadendrene (**14**)	98 ± 0	64 ± 4
bicyclogermacrene (**18**)[a]	11 ± 10	0 ± 1

[a]Negative values or large standard deviations were the consequence of the low concentration of products (< 0.5%), resulting in an increased influence of the background on the calculated values.

double bond of **42**, which establishes the (*R*)-configuration at C10 of the (2*E*,6*E*)-germacren-11-yl cation (**35**). This configuration brings the C1-H$_{Re}$ in a favorable position to the unoccupied *p*-orbital of the isopropyl side chain and initiates the 1,3-hydride shift to the (2*E*,6*E*)-germacren-1-yl cation (**36**). The stereoelectronics of this hydride migration are in line with a 1,3-hydride shift previously reported in the biosynthesis of (−)-germacrene D in *Solidago canadensis*.[23] However, a different geometry of the (2*E*,6*E*)-germacren-1-yl cation (**36**) favors the transfer of the C1-H$_{Si}$ rather than the C1-H$_{Re}$ in the formation of (−)-germacrene D mediated by *Streptomyces coelicolor* A3(2).[28] Alternatively, the cation **35** may suffer a 1,3-depotonoation (loss of the C1-H$_{Re}$) to (+)-bicyclogermacrene (**18**) as a minor product. The majority of **18** remains enzyme-bound and is further cyclized to (−)-*allo*-aromadendrene (**14**) by reprotonation at C3 on the *si*-face followed by concomitant C6 to C2 closure and the elimination of a proton at C14. These results give the first evidence of the important role of **18** as biogenetic precursor for sesquiterpenes with a dimethylcyclopropane ring.[14,25,29] Approximately 91% of the overall carbon flux is shuttled through the 10-membered monocyclic cation **36** that is quenched either by deprotonation to (−)-germacrene D (**5**) or by reaction with a water molecule to the germacrenyl alcohols 4α-hydroxygermacra-1(10),5-diene (**26**) and kunzeol (**32**). As evident from labeling experiments conducted in D_2O, a large fraction of (−)-**5** remains enzyme-bound and after a conformational change from the *cisoid* to the *transoid* structure its reprotonation at C15 affords the (2*Z*,6*E*)-germacren-1-yl cation **4a** along with the cadinan-7-yl cation **37a**. These results support a hypothesis of Arigoni[16] suggested for the biosynthesis of cadalane skeletons and provide an alternative to the generally accepted and well-studied pathway proceeding via

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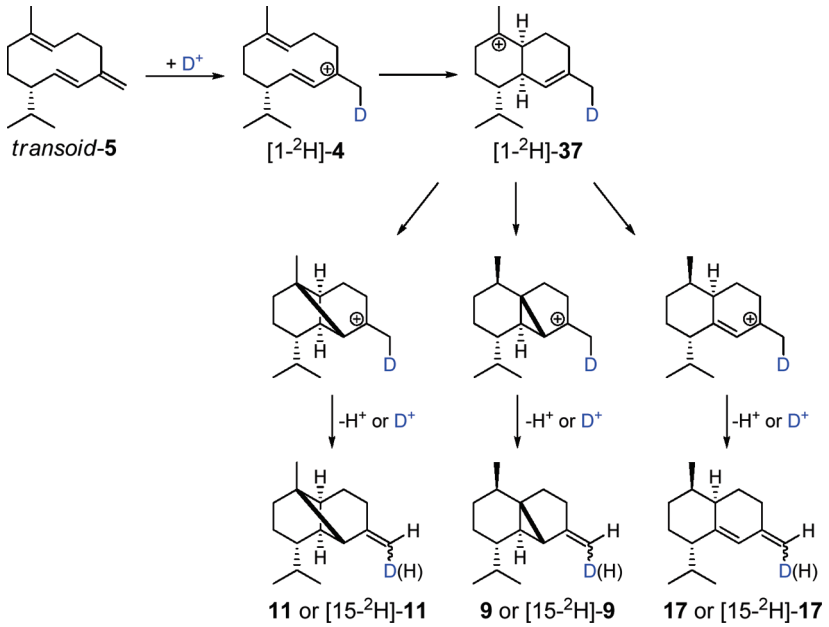
(29) Adio, A. M. *Tetrahedron* **2009**, *65*, 1533.

SCHEME 5. Proposed Labeling Pattern of Products with an Exocyclic Double Bond Obtained by Incubation of Recombinant MtTPS5 in Highly Enriched D_2O Medium

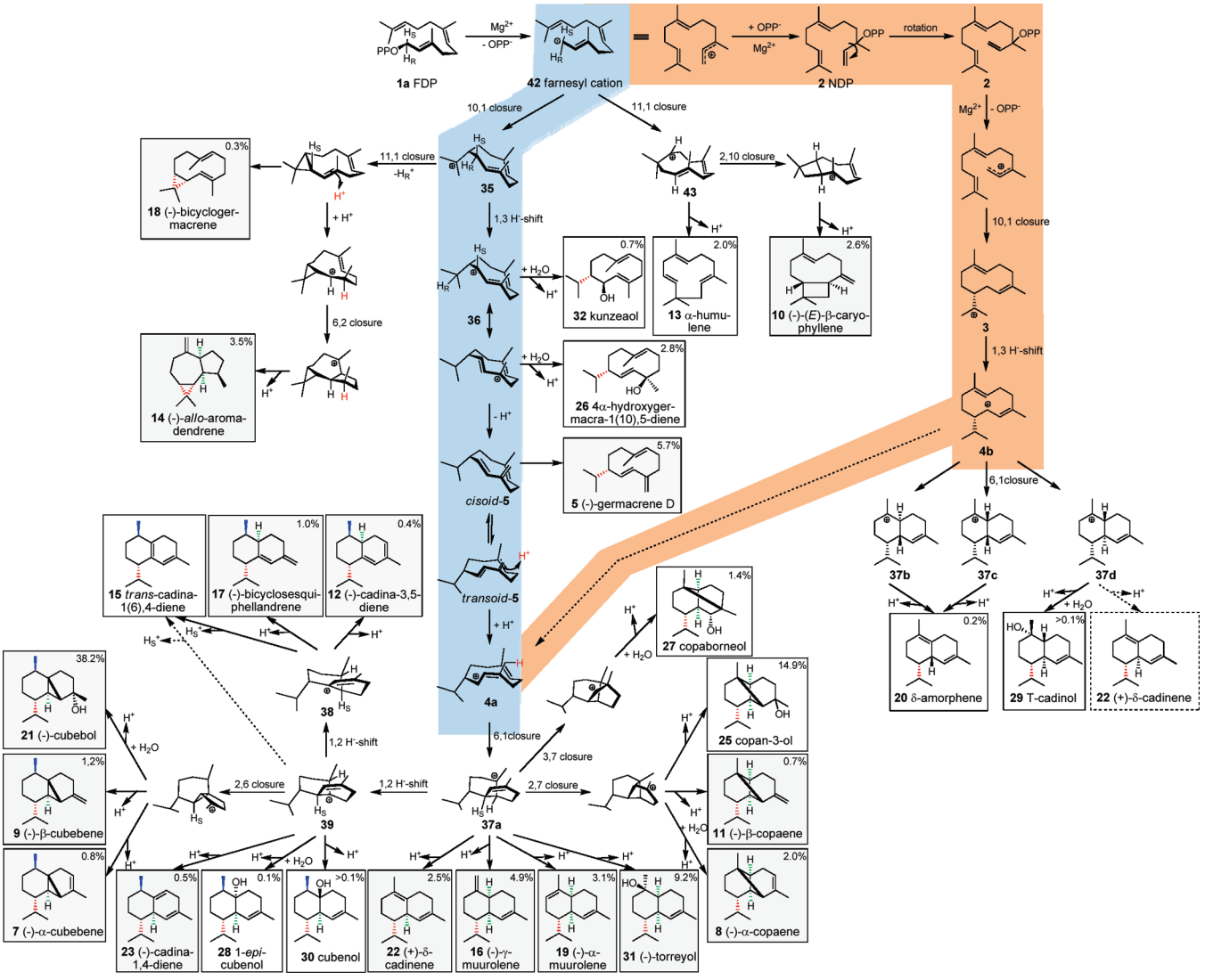


the intermediacy of NDP (**2**).[17−19,30] The cation **37a** represents the key branching point for all cadalanes with a *cis*-decalin ring system (ca. 80% of the product profile) and stabilizes itself by an unspecific loss of a vicinal proton to *δ*-cadinene (**22**), *γ*-muurolene (**16**), and α-muurolene (**19**) or by capture of a water molecule affording torreyol (**31**). After ring closure between C2 and C7 of **37a**, the new cation eliminates a proton or adds a water molecule to afford the copaane-type sesquiterpenes α-, *β*-copaene (**8**, **11**) and copan-3-ol (**25**). This occurs in concurrence with a C3 to C7 ring closure and addition of water to copaborneol (**27**). Another reaction channel via a *syn* 1,2-hydride shift transforms **37a** into the cation **39**, which is either deprotonated to (−)-cadina-1,4-diene (**23**) or reacts with water to the epimeric cubenols **28** and **30**. The same hydride shift has been previously documented for (+)-*epi*-cubenol produced by *Heteroscyphus planus* and *Streptomyces* sp LL-B7.[31] A second *syn* 1,2-shift of cation **39** produces cation **38** that leads to either (−)-bicyclosesquiphellandrene (**17**), (−)-cadina-3,5-diene (**12**), or *trans*-cadina-1(6),4-diene (**15**); the latter one could also be formed by direct deprotonation from **39**. The principal product (−)-cubebol (**21**), as well as (−)-α-cubebene (**7**) and (−)-*β*-cubebene (**9**), are formed from cation **39** after C2 to C6 ring closure followed by deprotonation or the capture of a water molecule. Cadalane structures originating from cadinanyl cations with different relative configurations of the angular hydrogen atoms and the isopropyl group (cations **37b**−**d**), such as T-cadinol (**29**) and *δ*-amorphene (**20**), seem not to be biosynthesized via protonation of the *transoid* (−)-**5** as suggested by the labeling experiments with D_2O. In these cases, the cationic key intermediate **4b** is generated by a sequence starting from farnesyl diphosphate (**1**) with subsequent isomerization to the tertiary allylic intermediate, nerolidyl diphosphate (**2**). After rotation around the C2−C3 single bond and the cleavage of the diphosphate group, the stereospecific C10 to C1 closure establishes the (*R*)-configuration at C10 of the cation **3**. Finally, a stereospecific 1,3-hydride shift of the C1-H_{Re} affords cation **4b**. The different conformations of **4b** determine the configurations of the angular hydrogens, which are realized after the electrophilic attack on the C6−C7 double bond. Both cations **37b** and **37c**, which exhibit a *trans*-configuration of the C1-H and the isopropyl group, are potential precursors of **20**, which is released after deprotonation. The formation of **29** involves the electrophilic attack on the C6−C7 double bond of **4b**, establishing the *trans*-configuration (1*R*,6*S*) (cation **37d**) of the bridgehead hydrogen atoms and quenching of the positive charge by a water molecule. Labeling experiments with the Y526F mutant conducted in D_2O revealed that all other cadalane products, such as **19** and **31**, could also arise from the nerolidyl diphosphate pathway, as indicated by the absence of deuterium in the products. This may also explain why the labeling degrees of the cadalanes obtained by D_2O experiments with the wild type do not exceed 91% d_1 (although 98% d_1 is possible, as obtained for **14**). These results suggest that about 92% of the key intermediate cation **4a** is derived by the protonation of (−)-**5** (highlighted blue in Scheme 6) and about 8% arises from the nerolidyl diphosphate pathway (highlighted orange in Scheme 6). The MtTPS5 enzyme seems to use both pathways to produce the same compounds, reflecting the high flexibility of multiproduct enzymes. In addition, 5-*epi*-aristolochene synthase from tobacco is able to cyclize both FDP (**1**) and its tertiary allylic isomer NDP (**2**), but different products were generated by both pathways.[32,33]

Enzymatic Control Exerted on Reaction Channels. The sesquiterpene synthase gene *MtTPS5*, isolated from *Medicago truncatula*, encodes an enzyme that generates from FDP (**1**) at least 27 different sesquiterpenes. The stereochemical analysis of 17 enzyme products reveals the conservation of configurations

(30) Nabeta, K.; Fujita, M.; Komuro, K.; Katayama, K.; Takasawa, T. *J. Chem. Soc., Perkin Trans. 1* **1997**, 2065.
(31) Cane, D. E.; Tandon, M. *Tetrahedron Lett.* **1994**, *35*, 5351.
(32) O'Maille, P. E.; Chappell, J.; Noel, J. P. *Arch. Biochem. Biophys.* **2006**, *448*, 73.
(33) Faraldos, J. A.; O'Maille, P. E.; Dellas, N.; Noel, J. P.; Coates, R. M. *J. Am. Chem. Soc.* **2010**, *132*, 4281.

SCHEME 6. Proposed Reaction Mechanism for the Formation of Sesquiterpene Products by MtTPS5[a]



[a]Stereocenters with the same absolute configuration are marked in green, red, and blue. Reaction channels leading to **4a**,**b** by protonation of germacrene D (**5**) (light blue) or by the intermediacy of NDP (**2**) (orange) are highlighted. Absolute configuration determined (gray highlighted boxes).

among compounds with the same hydrocarbon skeleton as well as between related structures. This allows the deduction of common cationic precursors and the initial conformation of the cyclizing substrate in the active site. In addition, compounds belonging to a common structural group are almost optically pure (≥98% ee). For example, the initial cyclization of FDP (**1**) to the 10-membered ring system of (−)-germacrene D (**5**) and the follow-up product α-cubebene (**7**) (Scheme 6) leads to enantiomerically pure products including the alcohols **26**, **32**, and **21**, originating from cationic intermediates en route to cadalane skeletons (Figure 2). In contrast, the multiproduct terpene synthase TPS4 from *Zea mays* and the trichodiene synthase from *Fusarium sporotrichioides* generate racemic and diastereomeric products that differ in configuration at C6 or C7, which is explained by invoking the intermediacy of both (*R*) and (*S*)-bisabolyl cation enantiomers.[34,35] In TPS4 this has been attributed to two different pockets at the active site of the enzyme that are populated by different conformations of the farnesyl cation, each of which produces a single enantiomer of the initially formed bisabolyl cation.[36] MtTPS5 apparently exerts a stronger control on the initial conformation of the farnesyl cation (**42**) allowing only the formation of the (*R*)-enantiomer of the (2*E*,6*E*)-germacren-11-yl cation (**35**) and its follow-up products (germacranes, cadalanes). Most importantly, the configuration of the product spectrum discussed so far is basically determined by the starting conformation of FDP (**1**) and the subsequently formed farnesyl cation (**42**). Nevertheless, the correct folding of the FDP (**1**) does not alone dictate the structure of the cyclization products. The enzyme-active site

(34) Kollner, T. G.; Schnee, C.; Gershenzon, J.; Degenhardt, J. *Plant Cell* **2004**, *16*, 1115.

(35) Vedula, L. S.; Jiang, J.; Zakharian, T.; Cane, D. E.; Christianson, D. W. *Arch. Biochem. Biophys.* **2008**, *469*, 184.

(36) Kollner, T. G.; O'Maille, P. E.; Gatto, N.; Boland, W.; Gershenzon, J.; Degenhardt, J. *Arch. Biochem. Biophys.* **2006**, *448*, 83.

seems to be spacious or flexible enough to enable conformational changes and rearrangements of the initial cation **35** to give **37a** after protonation of the neutral intermediate (−)-**5**. These results are in line with theoretical studies on cyclization mechanisms of several sesquiterpenes, which suggest that certain productive conformations of the bisabolyl cation could not be directly derived from FDP (**1**). Only after conformational changes from accessible to initially inaccessible bisabolyl cation conformers certain sesquiterpenes are formed.[37] Once the central cation **37a** is formed it stabilizes by a multitude of independent reaction channels (hydride shifts, cyclizations, deprotonations, or addition of water) to hydrocarbons or alcohols as the main products. Apparently, the enzyme exerts less control on the pathways diverting from cation **37a**, which is consistent with a lack of properly positioned functional groups that could direct the final cyclizations and deprotonations to defined cadalane end products.

Role of Tyrosine 526 in the Catalysis Mediated by MtTPS5. Introduction of the Y526F mutation in the active site of MtTPS5 results in almost exclusive formation of germacranes (80% of the product profile) accompanied by strong reduction of cadalane type sesquiterpenes (82% WT → 15% Y526F mutant). The enhanced accumulation of the precursor related products verifies the intermediacy of germacrene D (**5**) in MtTPS5 catalysis and strongly supports the crucial role of the hydroxyl group of Y526 in the proton transfer to the exocyclic double bond of (−)-**5**. Further evidence is given by the low incorporation rates of deuterium in cadalane products obtained by incubation experiments with D_2O (0% to 30% d_1). Nevertheless, the exchange of tyrosine by a phenylalanine does not fully disable the reprotonation of (−)-**5** as indicated by the noticeable incorporation of deuterium in cubebanes and copaanes (11−30% d_1), which in turn suggests that Tyr526 is not the direct proton donor. It is more likely that a water molecule trapped in the active site might serve as the requisite Lewis acid. A similar function for a solvent molecule has been proposed for the aristolochene synthase from *Penicillium roqueforti*.[38] Evidence for the presence of water in the active site of MtTPS5 comes from the high amounts of sesquiterpene alcohols formed by the wild type (68%) and the Y526F mutant (53%). The Lewis acid properties of water might be adjusted by hydrogen bonds to the Tyr526 and other polar amino acid side chains. The study of the crystal structure of the aristolochene synthase from *Aspergillus terreus* revealed a diphosphate moiety derived from the substrate FDP (**1**) as another possible acid driving the reprotonation of (−)-**5**.[39] The reported strong cofactor-dependence of the macrocarpene biosynthesis mediated by TPS6 and TPS11 from *Z. mays,* which includes the protonation of β-bisabolene, provides further evidence for this hypothesis.[40] Neither this hypothesis nor that which claims another amino acid is the actual proton donor could be excluded without more detailed structural information and additional experiments.

The Tyr526 is also involved in the proton transfer to (−)-bicyclogermacrene (**18**) as evident from the reduced deuterium content of (−)-*allo*-aromadendrene (**14**) released from the mutant enzyme (98% d_1 WT to 64% d_1 Y526F mutant). However, in MtTPS5 the influence of the tyrosine is in fact smaller compared to that observed for cadalane type sesquiterpenes, leading to the suggestion that other amino acids are more important for this proton transfer. Notably, the production of (−)-**18** is strongly enhanced in the mutant enzyme, accounting for 12.9% of the overall product outcome (0.3% WT). These results demonstrate that the tyrosine residue might influence the equilibrium of the competing pathways branching from (2*E*,6*E*)-germacren-11-yl cation (**35**, Scheme 6). The proportion of the 1,3-deprotonation affording (−)-**18** competing with the 1,3-hydride shift is slightly enhanced in the mutant enzyme. A possible explanation is a disturbed hydrogen bonding network, activating the active site base which facilitate the removal of the C1-H_{Re}. This remains more speculative, since no information about the structural bases favoring hydride shifts are available.

Conclusions

Our studies using the multiproduct enzyme MtTPS5 from *Medicago truncatula* demonstrate viable mechanistic pathways for the formation of a structural diverse group of sesquiterpene hydrocarbon skeletons sharing the 3,7-dimethyl-10-isopropyloctalin skeleton as their common structural feature (cadalane skeleton). We validated a proposal of Arigoni[16] that includes a pathway for the production of compounds containing a *cis*-configured C2−C3-double bond representing an alternative to the generally accepted and well-studied pathway proceeding via initial FDP−NDP isomerization. Furthermore, our results underline the importance of proton transfer reactions in sesquiterpene biosynthesis and show how these reactions expand the evolutionary flexibility of this highly versatile class of enzymes. The alteration of only a single amino acid can have a dramatic effect on the product profile; as we could show, the alteration of tyrosine to phenylalanine in MtTPS5 prevents the formation of a key intermediate via protonation of germacrene D (**5**). This gives the plant the opportunity to adapt very quickly to environmental changes. Furthermore, knowing the key amino acids that govern the carbon flux in distinct directions, we can tailor enzymes accordingly. Our analysis of the stereochemical course of the reaction cascade by incubation experiments with chirally labeled FDPs and the determination of the absolute configuration of enzyme products gave us information about the timing and the enzymatic control of the reaction steps. The cyclization steps, as well as the hydride shifts, establish new stereocenters under tight control of the enzyme, resulting in optically pure products. In contrast, the last steps, involving deprotonation and reaction with the nucleophile water, are less controlled, as evident from the formation of double bond isomers and epimeric alcohols. In summary, every cationic species led to a direct product, indicating that the enzyme was unable to prevent the premature quenching of the emerging cationic intermediates.

Experimental Section

Protein Expression. Strains of *E. coli* (BL21-CodonPlus(DE3)) harboring the recombinant vectors of MtTPS5[20] and mutant Y526F carrying an N-terminal His_8-tag were grown to A_{600} = 0.5 at 37 °C

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(38) Felicetti, B.; Cane, D. E. *J. Am. Chem. Soc.* **2004**, *126*, 7212.
(39) Shishova, E. Y.; Di Costanzo, L.; Cane, D. E.; Christianson, D. W. *Biochemistry* **2007**, *46*, 1941.
(40) Kollner, T. G.; Schnee, C.; Li, S.; Svatos, A.; Schneider, B.; Gershenzon, J.; Degenhardt, J. *J. Biol. Chem.* **2008**, *283*, 20779.

in LB-medium with kanamycin at 50 μg mL^{-1}. After induction with isopropyl β-D-1-thiogalactopyranoside (IPTG, final concentration 1 mM), cultures were shaken overnight at 16 °C and 200 rpm. Cells were harvested by centrifugation for 20 min at 4000 rpm, and the pellet was resuspended in lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and incubated with lysozyme (1 mg mL^{-1}) for 1 h at 4 °C. Disruption of the cells was achieved by sonication for 2 × 2 min. The cell debris was removed by centrifugation at 10000*g* for 30 min. The supernatant was passed over a column of Ni^{2+}-NTA-Agarose (QIAGEN, Hilden, Germany), equilibrated with eight bed volumes of lysis buffer. After being washed twice with four bed volumes of washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0), the protein was eluted with elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein was desalted into a TRIS-buffer (50 mM TRIS, pH 7.5, 10 mM NaCl, 10% glycerol) by passing through a NAP 25 column (Amersham Biosciences, Uppsala, Sweden), diluted to reach a concentration of 1 mg mL^{-1} and stored at −20 °C. Protein quantification was performed by using a method of Bradford et al.[41]

Site-Directed Mutagenesis. For site-directed mutagenesis, the QuickChange site-directed mutagenesis kit was used according to the manufacturer's instructions. The PCR-based mutagenesis protocol was performed with the cDNA of MtTPS5 cloned into the pHis8−3 expression vector[42] using primers containing the desired mutations (Y526Ffwd GTTTTATGGATGTTATTTTCAAAAACAAAGATAAC, Y526Frev GTTATCTTTGTTTTTGAAAATAACATCCATAAAAC). The mutagenized construct was fully sequenced before expression.

Identification and Determination of the Stereochemistry of Enzyme Products. GC−MS analysis was performed on an instrument equipped with a ZB-5 capillary column (0.25 mm i.d. × 15 m with 0.25 μm film). One microliter of the sample was injected in splitless mode at an injection port temperature of 220 °C. The oven temperature was kept at 50 °C for 2 min followed by a ramp of 10 °C min^{-1} to 240 °C followed by an additional ramp of 30 °C min^{-1} to 280 °C and finally kept for 2 min. Helium at a flow rate of 1.5 mL min^{-1} served as carrier gas. Ionization potential was set to 70 eV, and scanning was performed from 40 to 250 amu. Compounds were identified by comparing their mass spectra and Kováts indices (retention indices) with those of published reference spectra in MassFinders' (software version 3.5) and Adams'[43] terpene library and in the NIST database. In addition, retention indices (RI) of sesquiterpene peaks derived by calibrating GC runs with a C8−C20 alkane standard were compared with RI values of authentic reference compounds (Table SI-1, Supporting Information). Many of the references not commercially available were either obtained as described below or kindly provided by Stefan von Reuss, Hamburg, Germany; Charles Fehr, Fimenich, Geneva, Switzerland; Alois Fürstner, Mühlheim an der Ruhr, Germany; and Simon Egli, Birmensdorf, Switzerland. Essential oils with known composition containing relevant sesquiterpenoids were purchased from a commercial supplier or were generously provided by Stefan von Reuss, Hamburg, Germany. Additional references were generated by acid-catalyzed rearrangements of germacrene D (**5**) (W. A. König) and α-copanene (**8**) using conditions reported in the literature.[44,45] The liverwort *Preissia quadrata* was collected by H. J. Zuendorf (Herbarium Haussknecht, Jena, Germany) near Talbürgel, Germany.

For quantification of enzyme products, the compounds were first separated on a gas chromatograph (H_2 carrier gas 1.5 mL min^{-1}, injection volume 2 μL) under the conditions described above and subsequently analyzed on a flame ionization detector (FID) (250 °C). Correction of the different response factors of sesquiterpene hydrocarbons and alcohols was achieved using calibration curves obtained from samples with different concentrations of (*E*)-β-caryophyllene (**10**) and torreyol (**31**). The average and standard deviations of relative ratios were determined by at least four independent samples setting the sum of identified compounds to 100%.

The enantiomers of the enzyme products were separated and identified by GC−MS using a heptakis(2,3-di-*O*-methyl-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin column (50% in OV1701, w/w) (FS-Hydrodex β-6TBDM) (0.25 mm i.d. × 25 m × 0.25 μm film) operated with helium at 1 mL min^{-1} as carrier gas, a splitless injection of 1 μL sample at 220 °C and a temperature program starting from 60 °C kept for 5 min, followed by a ramp of 2 °C min^{-1} (for α-copaene (**8**) 1 °C min^{-1}) to 160 °C followed by an additional ramp of 30 °C min^{-1} to 220 °C with 2 min hold. The separation of torreyl enantiomers was achieved by using a heptakis-(2,3,6-tri-*O*-methyl)-β-cyclodextrin column (50% in OV1701, w/w) (FS-Hydrodex β-PM) (0.25 mm i.d. × 25 m × 0.25 μm film). Helium was used as carrier gas at a constant flow rate of 1 mL min^{-1}, and samples (1 μL) were injected at 220 °C. The GC was programmed with an initial oven temperature of 110 °C (15-min hold), which was then increased 2 °C min^{-1} up to 160 °C followed by a 30 °C min^{-1} ramp until 220 °C (2-min hold). Samples containing both enantiomeres were either gifts from von Reuss or were prepared by mixing pentane extracts of the liverwort *Preissia quadrata* (containing generally the opposite enantiomer of the relevant sesquiterpene[46]) with the corresponding reference (Table SI-4, Supporting Information) or obtained by acid-catalyzed rearrangements of racemic germacrene D (**5**) and α-copaene (**8**) (see above). No information about the absolute configuration of the torreyol (**31**) isolated from *Cortinarius odorifer* Britz (gift from Simon Egli) was available.[47] Therefore, we determined the optical rotation with a polarimeter. The value of $[\alpha]_D = +100.6$ ($c = 0.9$, $CHCl_3$) establishes the configuration of **31** isolated from the fungi to be the opposite of that reported by Borg-Karlson et al.[48]

Enzyme Assays for Product Analysis. Standard assays contained 600 nM purified protein in assay buffer (25 mM HEPES, pH 7.5, 10% glycerol, 10 mM $MgCl_2$, 1 mM DTT) with 50 μM substrate (FDP, (1*S*)-[1-^{2}H]-FDP, (1*R*)-[1-^{2}H]-FDP, or [1,1-$^2H_2$]-FDP) in a final volume of 1 mL. Deuterated FDPs were synthesized as previously described.[49,50] The reaction mixture was covered with 100 μL of pentane containing 1 ng μL^{-1} of dodecane as an internal standard to trap the reaction products. After being incubated for 90 min at 30 °C, the reaction was stopped by vortexing for 20 s. The whole mixture was frozen in liquid nitrogen, and the pentane layer was removed after thawing and analyzed by GC−MS as described above.

To analyze the protonation reaction, 50 μL of the purified enzyme (1 mg mL^{-1}) was lyophilized and redissolved in 50 μL of D_2O and incubated for 30 min on ice to ensure proper H−D exchange of the enzyme. An aliquot of 20 μL of the protein sample was analyzed in assay buffer prepared with D_2O (>99% d_1) containing 50 μM FDP. The assays were incubated

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(50) Cane, D. E.; Iyengar, R.; Shiao, M. S. *J. Am. Chem. Soc.* **1981**, *103*, 914.

for 1 h at 30 °C. The reaction products were collected by a solid-phase microextration fiber (SPME) consisting of 100 μm polydimethylsiloxane and analyzed by GC−MS. The labeling degree of each product was calculated from the intensities of the respective $[M]^+$, $[M + 1]^+$ for hydrocarbons and $[M - H_2O]^+$, $[M - H_2O+1]^+$ for alcohols after correcting for the abundance of their ^{13}C satellite peaks.

For identifying and determining the stereochemistry of copan-3-ol (**25**) and cubebol (**21**), two 10-mL assays containing 600 nM purified enzyme in assay buffer with 50 μM FDP were covered with 10 mL of pentane. After being incubated overnight at 30 °C, the mixture was extracted three times with 5 mL of pentane. The combined organic phases were passed through a Pasteur pipet containing Na_2SO_4, and the volume was reduced to ∼100 μL. Alcohols were separated on silica (1 g, Pasteur pipet) using pentane/ether (6:1, v/v) for elution. Two fractions highly enriched in the desired compounds were obtained, besides fractions containing both compounds.

Acid-Catalyzed Dehydratization and Rearrangement of Copaen-3-ol (25) and Cubebol (21). The solvent of samples containing ∼500 ng (GC) of the sesquiterpene alcohol was removed under an argon stream. The compounds were redissolved under argon atmosphere in 200 μL of dry CH_2Cl_2, and 5 μL of trifluoroacetic anhydride (derivatization grade) was added. The solution was stirred for 30 min. Subsequently, after the addition of 1 mL of pentane, the reaction was quenched with 1 mL of 10% $NaHCO_3$. The aqueous phase was extracted twice with 1 mL of pentane. The unified organic extracts were dried (Na_2SO_4) and reduced to a volume of ∼100 μL under a stream of argon. Samples were analyzed by GC−MS as described above.

Dehydratization of copan-3-ol (**25**) resulted in α-copaene (**8**) (60%) and in the three rearrangement products α-muurolene (**19**) (21%), γ-muurolene (**16**) (9%), and δ-cadinene (**22**) (9%). The rearrangement of cubebol (**21**) afforded δ-cadinene (**22**) (70%), cadina-1,4-diene (**23**) (18%), and *trans*-cadina-1(6),4-diene (**15**) (12%) (Figure SI-1, Supporting Information).

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Supporting Information Available: Complete tables of retention indices, relative ratios of products, sources of references, mass spectra of all enzyme products in comparison to authentic references, mass spectrometric data from incubation experiments with deuterium-containing substrates, deuterium content of products obtained by experiments conducted in highly enriched D_2O medium, and gas chromatographic data of the chiral separation of enzyme products. Total ion chromatograms of catalyzed dehydratization products of copan-3-ol (**25**). This material is available free of charge via the Internet at http://pubs.acs.org.