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Article

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Alternate Cyclization Cascade Initiated by Substrate Isomer in Multiproduct Terpene Synthase from *Medicago truncatula*

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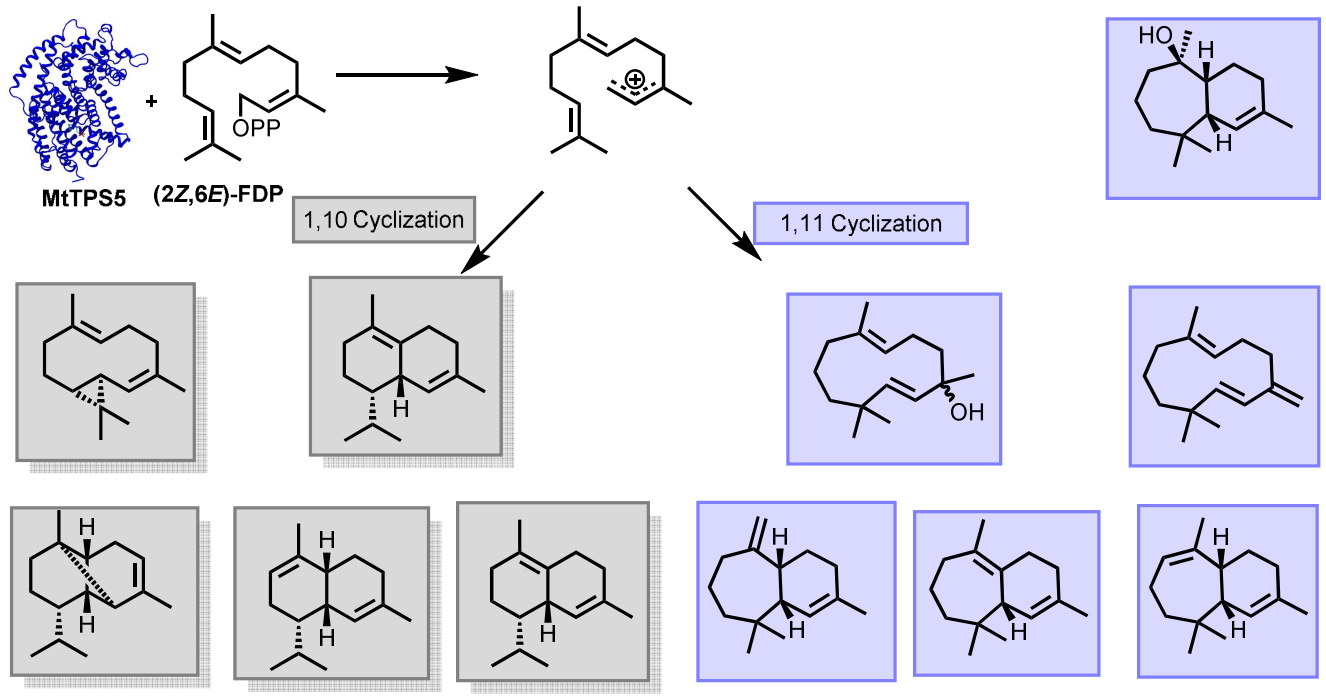

Abstract:

Promiscuity of terpene synthases results in the enormous diversity of terpenes found in nature. Multiproduct sesquiterpene synthase MtTPS5 isolated from *Medicago truncatula* generates 27 optically pure products from its natural substrate (2*E*,6*E*)-farnesyl diphosphate (FDP). In order to study the promiscuity of MtTPS5, (2*Z*,6*E*)-FDP an analogue of presumptive reaction intermediates from natural reaction cascade was utilized as a substrate. This stereoisomer induced a novel cyclization pathway leading to sesquiterpenes based on humulane, amorphene and himachalane skeletons. Interestingly, none of these products matched with those observed on incubation of MtTPS5 with natural (2*E*,6*E*)-FDP. Further determination of absolute configuration of each product helped rebuild the stereochemical route of the reaction cascade. Interestingly, the presence of only one enantiomer of each product was observed indicating the highly stereospecific nature of the enzymatic reaction. Substrate promiscuity of terpene synthases provides organism access to novel chemical bouquets of high optical purity by utilizing existing enzymes. Presence of this mechanism was indicated by the presence of these alternate products in natural herbivore-induced volatiles of *Medicago truncatula*.

Introduction:

Terpenes with more than 55,000 known members are found in almost all forms of life and constitute the most diverse class of natural products.[1-3] They serve many biological functions, such as hormones (abscisic acid, gibberellins, steroids);[4] structural components of membranes (phytosterols); pollinator attractants;[5] toxins;[6] deterrents for feeding; or oviposition to a variety of insects.[7] Terpenes are not only significant in chemical ecology but are also commercially significant as medicines, materials, fuels, chemicals, and especially as flavors and fragrances.[8-10] Volatile terpenoids which constitutes a substantial class of induced volatiles are generated by enzymes known as terpene synthases. This class of enzymes has been extensively investigated in recent times, including cloning and characterization of a number of cDNAs encoding terpenoid synthases involved in primary and secondary metabolism in plants.[11,12]

Enzymes with promiscuous functions have long been believed to evolve and acquire increased specificity and activity, and this plasticity has been achieved with minor substitutions in amino acids.[13] This promiscuity gives us the opportunity to study the evolution of enzymes and design terpene synthases as better catalysts. Catalytic promiscuity has long been known to be one of the key features of terpene synthases, especially sesquiterpene synthases.[9,10] All known sesquiterpenes are based on 300 basic hydrocarbon skeletons formed by sesquiterpene synthases from the universal precursor (2*E*,6*E*) farnesyl diphosphate (FDP).[14] This structural diversity is the result of an enzymatic environment that holds the pliable isoprenoid substrate in a desirable conformation to enforce specific C-C bond formation trajectory. Besides terpene synthases generating single products, multiproduct terpene synthases are also known for producing a collection of cyclic and acyclic products from a single substrate.[11] Currently, δ-selinene synthase and γ-humulene synthase from *Abies grandis* hold the record for generating 52 and 34 different sesquiterpenes from FDP.[15] Such biosynthetic promiscuity is assumed to be possible as a result

of active site permitting alternative conformations of the both substrate and reaction intermediates.

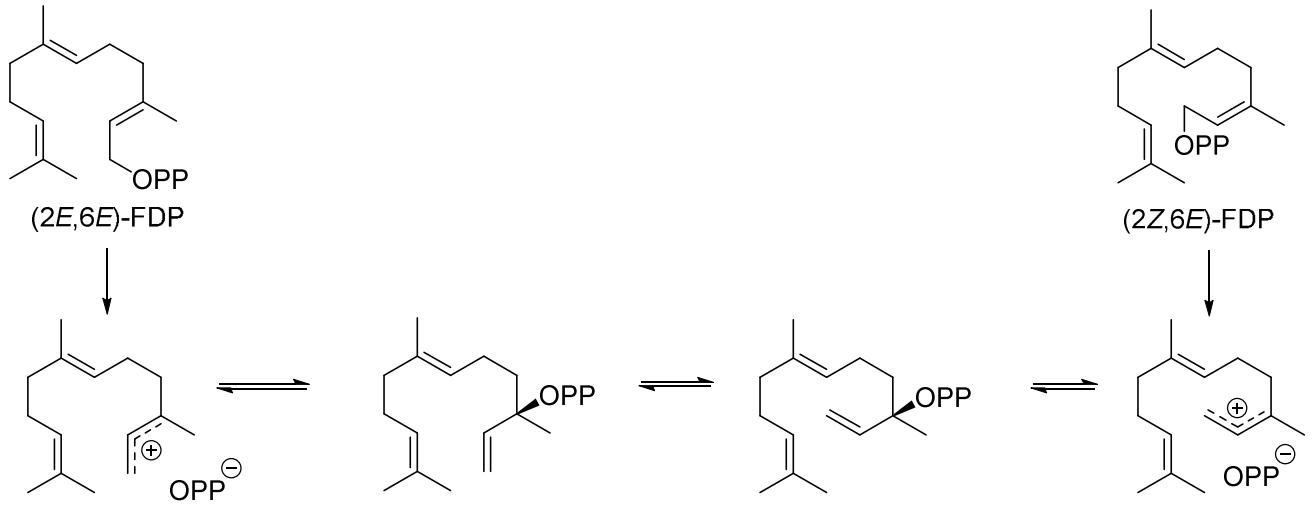

Figure 1: (2*E*,6*E*)-Farnesyl diphosphate (FDP) and (2*Z*,6*E*)- farnesyl diphosphate (FDP). The initial steps of farnesyl carbocation formation and corresponding isomerization double bond.

During terpenoid biosynthesis, the unsaturated diphosphates (FDP, etc.) dissociate into highly reactive cations and remainder diphosphate anions. These electrophilic carbocations react with nearby electron-rich double bounds, followed by intramolecular cyclizations; rearrangements, including methyl or hydride shifts prior to stabilization by either reaction with a nucleophile; or deprotonation.[16] Due to the (*E*)-configuration of the substrate, this intramolecular cyclization progresses through the remote double bonds. In contrast, the direct intramolecular cyclization of (2*E*,6*E*)-FDP with the neighboring C(5)-C(6) double bond to cyclohexenyl cations is not possible and requires a preceding isomerization. This isomerization is achieved by a suprafacial migration of the diphosphate moiety in the substrate that leads to a tertiary allylic intermediate bound to the enzyme active site (Figure 1).[17-19] It was believed that there is a high free-energy barrier for the direct rotation of the allylic cation but recently geranyl cation isomerization has shown to be feasible in a self-assembled cavity.[20,21] Presence of this intermediate prompted the

use of (2*Z*,6*E*)-FDP as a substrate mimicking the secondary cisoid nerolidyl cation intermediate to study the substrate promiscuity of terpene synthases (Figure 1). The efficient synthesis of (2*Z*,6*E*)-FDP, has been previously described,[22] and used to study the kinetics of various terpene synthases, but few studies have compared the cyclization with highly promiscuous multiproduct enzymes.[23-26] For example, Cop4 and Cop6 from *Coprinus cinereus* resulted in the opposite enantiomer (6S)-β-bisabolene from (2*Z*,6*E*)-FDP rather than (6R)-β-bisabolene and (2*E*,6*E*)-germacradienyl carbocation, respectively, from (2*E*,6*E*)-FDP.[27] In our previous work with multiproduct terpene synthases from *Zea mays*, we observed that this rotation had huge turnover benefits but generated an identical product profile.[28] Hence, we wanted to test the substrate promiscuity of MtTPS5 enzyme with (2*Z*,6*E*)-FDP through variations in catalytic mechanisms among its 27 optically pure products.

Results and Discussion:

We had previously elucidated the complexities involved in the mechanistic pathway of MtTPS5 reaction cascade with (2*E*,6*E*)-FDP using a combination of techniques, including labelling experiments.[29] In brief, the disassociation of diphosphate moiety initiates the reaction cascade leading to the formation of a highly reactive farnesyl carbocation. The ring closure involving C1 to C11 carbons of the farnesyl carbocation generates the humulyl cation, which leads to compounds such as α-humulene and β-caryophyllene. Most of the other products follow the initial C1 to C10 closure to generate the germacren-11-yl cation; further cyclization of this cation leads to products like germacrene D and germacrenyl-based alcohols. (2*Z*,6*E*)-germacren-1-yl cation and cadinan-7-yl cation are the other key cationic intermediates leading to about 80% products which are generated by the isomerization of FDP to the nerolidyl diphosphate (NDP). Substitution of even single amino acid in MtTPS5 has a dramatic effect on the product profile; the substitution of tyrosine with phenylalanine stops the formation of a key intermediate via the

protonation of germacrene D as a neutral intermediate. The 27 optically pure products are the result of a series of cyclization steps and hydride shifts under the tight control of the enzyme. Hence, the enzyme response after incubation with (2*Z*,6*E*)-FDP would allow us to study the effects of the initial isomerization step on the reaction cascade. These alterations in product profile via different reaction channels depend on the nature of the initial carbocationic intermediates; ease of deprotonation and their stability.

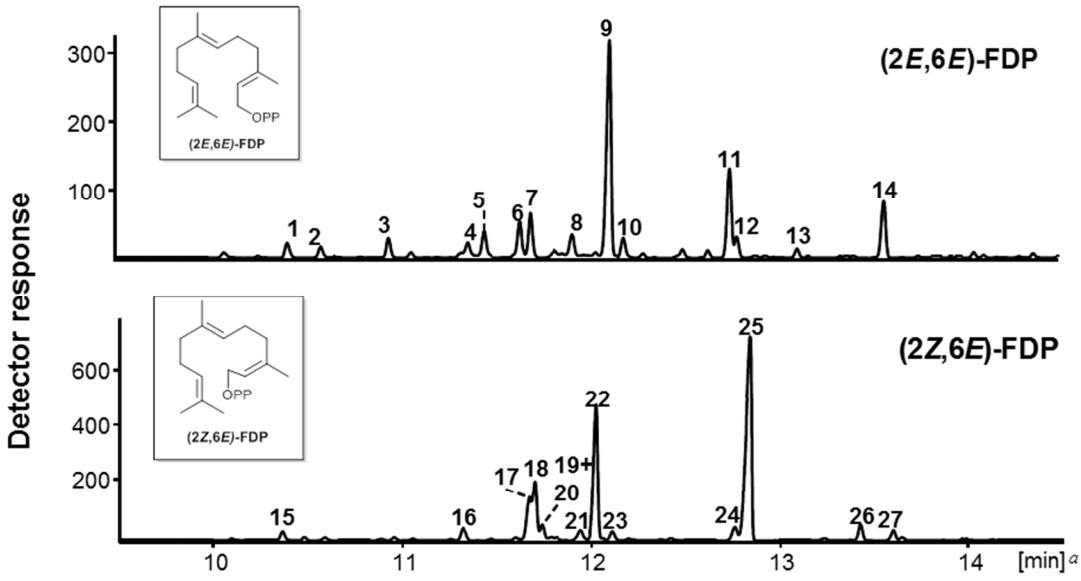

Figure 2: GC-FID Chromatogram of major sesquiterpenes after the incubation of recombinant MtTPS5 with (2*E*,6*E*)-FDP and (2*Z*,6*E*)-FDP. The compounds were identified by their Kovats indices and mass spectra, and compared to authentic references. Only major sesquiterpenes are labelled.

(2*E*,6*E*)-FDP: (**1**) α-copaene, (**2**) β-cubebene, (**3**) (*E*)- β-caryophyllene, (**4**) cadina-3,5-diene, (**5**) *allo*-aromadendrene, (**6**) γ-muurolene, (**7**) germacrene D, (**8**) α-muurolene, (**9**) cubebol, (**10**) δ-cadinene, (**11**) copan-3-ol,(**12**) 4α-hydroxygermacra-1(10),5-diene, (**13**) copaborneol, (**14**) torreyol.

(2*Z*,6*E*)-FDP: (**15**) α-ylangen, (**16**) α-himachalene, (**17**) isobicyclogermacrene, (**18**) α-amorphene, (**19**) γ-humulene, (**20**) γ-himachalene, (**21**) β-himachalene, (**22**) δ-amorphene, (**25**) humula-4,9-dien-8-ol and (**27**) 2-himachalen-7-ol. Sesquiterpene alcohols (**23**), (**24**) and (**26**) ($C_{15}H_{26}O$ II-IV) could not identified.

Interestingly, unlike the incubation of (2*Z*,6*E*)-FDP with maize sesquiterpene synthases, which showed only quantitative differences within the natural product profile, MtTPS5 gave a completely distinct 23 product profile with no similarity to the (2*E*,6*E*)-FDP product profile (Figure. 2). Out of these 23 compounds in GC-FID chromatogram, 13 sesquiterpenes were isolated and identity of eight compounds were confirmed based on a comparison of their mass spectra and retention indices with authentic references and two terpenoids were identified by other means. The most interesting aspect of these results was the absence of the dominant terpenoid skeletons that were observed with (2*E*,6*E*)-FDP. The product profile of (2*Z*,6*E*)-FDP consisted predominantly of mono- and bicyclic sesquiterpenes comprising humulane, amorphene and himachalane skeletons, none of which were observed in the reaction with the natural substrate. These results suggested that product formation with (2*Z*,6*E*)-FDP by MtTPS5 followed a completely novel pathway as compared to natural mechanistic pathway.

The absolute configuration of α- and δ-amorphene (**18**, **22**), α - and γ- himachalane (**16**, **20**), as well as of α-ylangen (**15**), was determined by GC-MS with a chiral phase column (Figure 3, supporting information). The stereochemical analysis of the enzymatic products with (2*Z*,6*E*)-FDP revealed the retention of configuration among products with the same hydrocarbon skeleton also between related structures. One common factor observed with both geometric isomers is that the enantiomeric composition of all substances had a high degree of optical purity. The absolute configuration and identity of individual compounds was confirmed by comparison with authentic standards obtained from various sources (supporting information). Accordingly, the formation of only one enantiomer of the products shows that MtTPS5 exerts strong control on the initial conformation of the farnesyl cation. In contrast, the trichodiene synthase from *Fusarium sporotrichioides* and the multiproduct terpene synthase TPS4 and TPS5 from *Zea mays* generate a mixture of diastereomeric and racemic products.[30,31] For α-amorphene (**18**), the determination of the enantiomeric excess was not possible because the (+)-

enantiomer co-eluted with that of (+)-δ-amorphene **22** (supporting information). All compounds generated from MtTPS5 with (2*E*,6*E*)-FDP as a substrate shared (*S*)-configured stereochemistry at C10. In contrast, the bridgehead hydrogen atoms (C1-H, C6-H) of α-ylangen (**15**), as well as α- and δ-amorphene (**18**, **22**) of (2*Z*,6*E*)-FDP had an opposite orientation to those from volatiles generated by (2*E*,6*E*)-FDP (**1**, **8** and **10**) (Figure. 3).

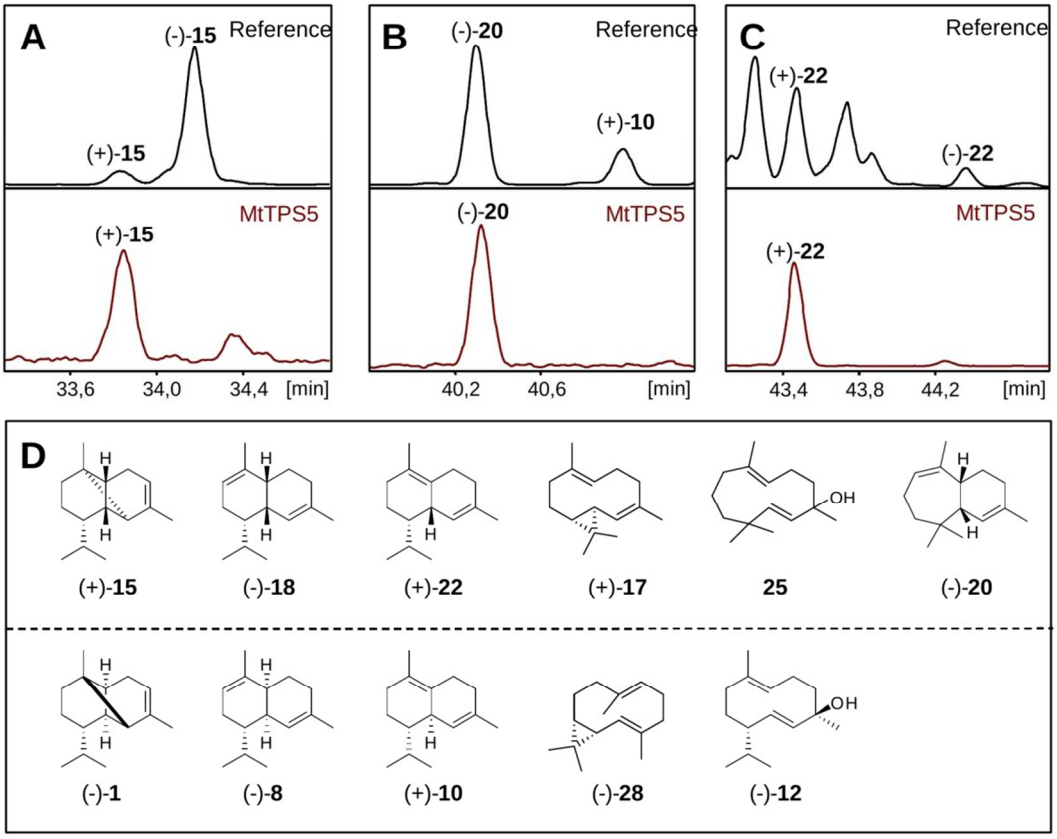

Figure 3: Determination of the absolute configurations of some products obtained after the incubation of MtTPS5 with (2*Z*,6*E*) -FDP. The separation of the enantiomers was carried out by GC-MS on a chiral phase (shown for **15** (A), **20** (B) and **22** (C)). D. Juxtaposition of isomeric compounds starting from (2*Z*,6*E*)-FDP (top row: isobicyclogermacrene (**17**)) and (2*E*,6*E*)-FDP (bottom row: bicyclogermacrene (**28**)).

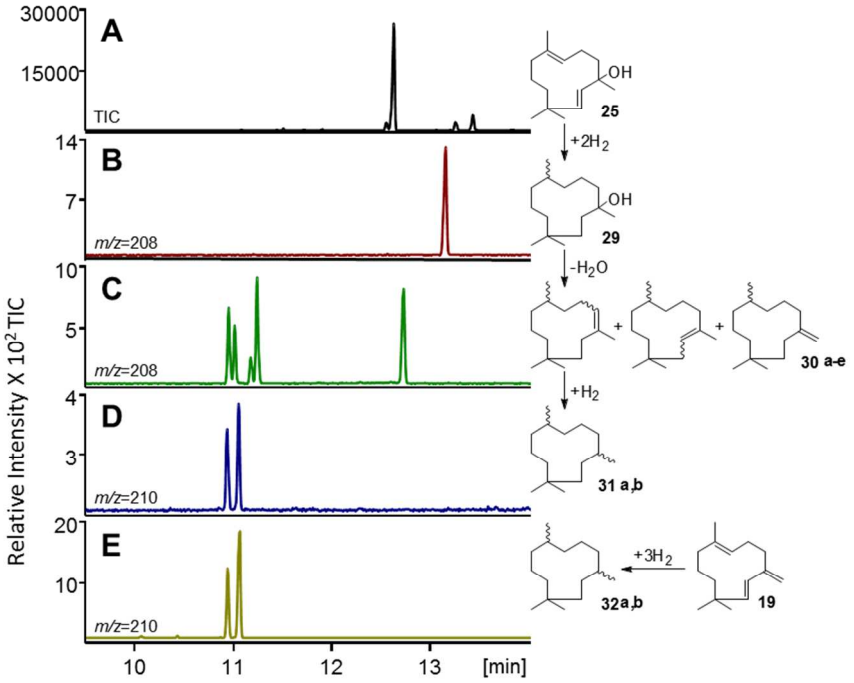

Figure 4: Structure elucidation of humula-4,9-dien-8-ol (25). GC-MS chromatograms, which were obtained by reactions of a column chromatography on silica gel, purified a fraction of **25** and its secondary products. (A): an **25**-enriched fraction, (B) products by catalytic hydrogenation of **25**, (C) products after acid catalyzed dehydration of **29**, (D) products by catalytic hydrogenation of **30a-e**, (E) reference, obtained from **19** by hydrogenation **32a, b**.

The available mass spectra libraries and Kovats indices were unsuccessful in identifying the major product **25**. The structure of compound **25** was elucidated by classical step-by-step derivatization (Figure. 4). The mass spectrum of **25** (supporting information) showed a weak molecular peak at *m*/*z*=222 and fragment ions at *m*/*z* =207 $[M\text{-}Me]^+$ and *m*/*z* =204 ($[M\text{-}H_2O]^+$), indicating the structure of a sesquiterpene. The catalytic hydrogenation of a highly enriched fraction **25** yielded compound **29** (Figure. 4B) with an increased mass (4 amu) of the fragment ion $[M\text{-}H_2O]^+$ *m*/*z* = 208 (Figure 4). This increase in mass indicated that compound **25** is monocyclic in nature wherein two double bonds were reduced. After acid-catalyzed dehydration of **29** with trifluoroacetic anhydride, five compounds (**30a-e**) were separated by gas chromatography (Figure. 4C), which had the expected molecular peak at *m/z* = 208. The detection of five stereoisomeric products helped us conclude that the hydroxy group could

only be located on a tertiary carbon atom, as a secondary alcohol, would have provided only four products. Further proof that **25** is as an 11- membered ring system was observed after the hydrogenation of γ-humulene (**19**) (Figure. 4E). The products diastereomeric humulane (**32a,b**) matched exactly with the hydrogenation products of the mixture of substances (**30a-e**) (Figure. 4D,E). Connecting all the observed results, the product can be identified as humula-4,9-dien-8-ol (**25**), which was isolated by Pentegova et al., from the resin of silver fir (*Abies alba*).[32]

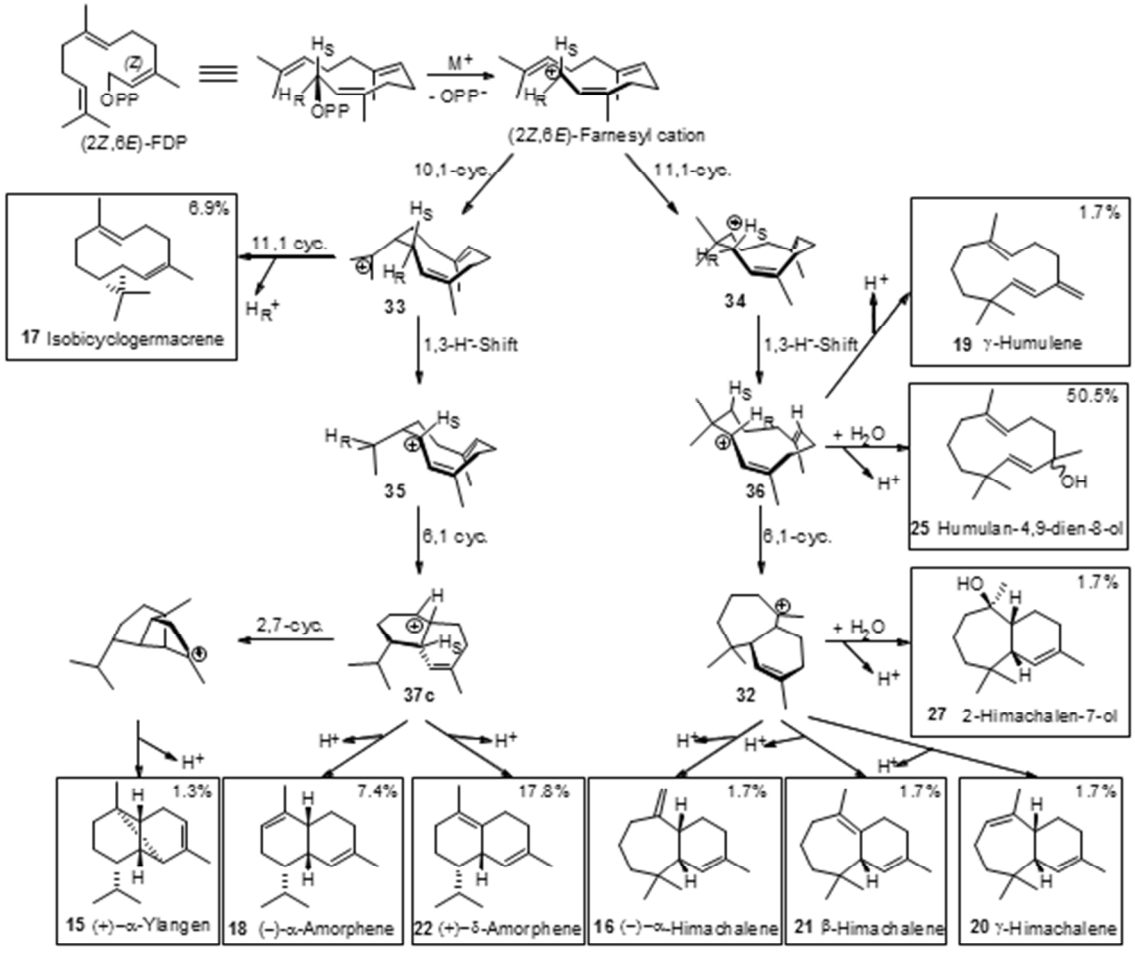

Figure 5: Proposed mechanism of formation of the products after incubation of (2*Z*,6*E*) -FDP with MtTPS5.

Previous labelling and mechanistic studies of MtTPS5 with (2*E*,6*E*)-FDP showed that the enzyme is capable of generating cadalane sesquiterpenes via two different pathways.[29] A large proportion of the released compounds (~33%) from (2*Z*,6*E*)-FDP had a cadalane backbone; however, these cadalenes possessed the opposite absolute configuration (1*S*, 6*R*) at the bridgehead carbon atoms in contrast to the natural products. The determination of the absolute configurations of some of the products helped us reconstruct the stereochemical course of the reaction cascade from the starting conformation of the (2*Z*)-isomeric substrate (Figure. 5). The highly reactive (2*Z*,6*E*)-farnesyl cation, which is formed after the cleavage of the diphosphate group of (2*Z*,6*E*)-FDP, predominantly undergoes (~57%) a C1-C11 ring closure to generate the (2*Z*,6*E*)-humul-10-yl cation. The transfer of a positive charge from C10 to C1 is possible only by a direct 1,3-hydride shift. The presence of a quaternary carbon atom between these two positions eliminates the possibility of two consecutive 1,2-hydride shifts. The deprotonation of the (2*Z*,6*E*)-humul-1-yl cation (**36**) generates γ-humulene (**19**); γ-humulene (**19**) on further reaction with water results in the main product (**25**). Furthermore, the himachalanes **16**, **21**, **20** and **27** are released from **36** through an electrophilic attack on the *si* face of the C6-C7 double bond, followed by the elimination of a proton or by reaction with a water molecule.

Another reaction channel, which starts from the (2*Z*,6*E*)-farnesyl cation, passes through the ten-membered macrocyclic (2*Z*,6*E*)-germacrene-11-yl cation (**33**) after the initial C1-C10 cyclization. In this step, the (*R*) configuration at C10 is initially established, which is conserved in all the cadalane sesquiterpenes. Analogous to the proposed reaction mechanism with (2*E*,6*E*)-FDP (a 1,3-deprotonation of C1-H_{Re} leading to bicyclogermacrene), a similar mechanism is observed with (2*Z*,6*E*)-FDP, wherein the isomer isobicyclogermacrene (**17**) is formed. In addition, the absolute configuration at C1 of isobicyclogermacrene (**17**) suggests that a shift of 1- H_{Re} from C1 to C11 generates the (2*Z*,6*E*)-germacrene-11-yl cation (**33**). 1,3 hydride shift in carbocation (**33**) yields **35** and a further C1-C6 ring-closure leads to cadinane-7-yl cation (**37c**) and the further loss of a proton in the two double bond

resulting in amorphenes **22** and **18**. The tricyclic rings are formed after a C2-C7 additional cyclization of **37c** followed by deprotonation to (-)-α-ylangen (**15**).

A close correlation exists between the two cyclization cascades, starting from both (2*E*,6*E*)- and (2*Z*,6*E*)-FDP. The essential elements, such as the 1,3-hydride shift, initial 1,10- or 1,11- cyclization and the formation of bicyclic compounds by a second-ring closure, are found in both reaction mechanisms. The huge structural difference in the products is mainly due to the different starting conformation of the two substrates, as each of the reactive moieties occupies a different spatial arrangement, which needs further examination with help of docking studies. The large influence of the initial folding of farnesyl cation in the active site, depending on the configuration of the C2-C3 double bond, has already been shown for the 5-epi-aristolochene synthase (TEAS) of tobacco.[33] The availability of crystal structures of enzyme-substrate complexes of TEAS with fluorine analogs of (2*E*,6*E*)- and (2*Z*,6*E*)-FDP helped us understand the structural basis of this conformational difference. However, in case of multiproduct terpene synthases lack of available crystal structures hinders the structural explanation for different stereo-and regiochemical reaction cascades resulting from the two starting conformations.[33,34] Work is in progress to co-crystalize the 3-bromo analogs of FDP with MtTPS5 to decipher the structural basis of this diversity and substrate promiscuity.[35]

The isolation of a (2*Z*,6*E*)-FDP synthase from *Mycobacterium tuberculosis*, which is involved in the bacterial cell wall biosynthesis, indicates the relevance of (2*Z*,6*E*)-FDP in other biological systems.[36,37] In a recent work, an novel terpene synthase gene family from evolutionary perspective has been reported in the striped flea beetle, these enzymes have evolved from trans-isoprenyl diphosphate synthases and are involved in the biosynthesis of a sesquiterpene which acts as a male-specific aggregation pheromone.[38] Interestingly, a novel sesquiterpene biosynthesis pathway from other isomer (2*Z*,6*Z*)-FDP in the wild tomato *Solanum habrochaites* has also been reported.[39] Natural presence of (2*Z*,6*E*)-FDP in other systems invoked our interest in looking for presence of these alternate volatiles from MtTPS5 in the herbivore induced terpenoid volatiles of *Medicago truncatula*. Interestingly, we

found five hydrocarbons α, β, γ himachalanes (**16**, **21**, **20**), γ-humulene (**19**) and α-ylangen (**15**) in the volatile blends upon *Spodoptera littoralis* or *Tetranychus urticae* infestation which were emitted by *Medicago truncatula*.[40] These compounds are absent in the product profile of the known terpene synthases from *Medicago truncatula* on incubation with (2*E*,6*E*)-FDP.[41] As a natural substrate (2*Z*,6*E*)-FDP may lead to volatile production in *Medicago truncatula*. Presence of these compounds in natural volatile blends also indicates the possibility of a "sleeping" cyclization pathway starting from (2*Z*,6*E*)-FDP, but none has been discovered yet.

Conclusion

In this work, we probe the reaction mechanism of multiproduct terpene synthase from *Medicago truncatula* with an analogue that mimics the isomerization via an NDP intermediate of the C2=C3 π bond of (2*E*,6*E*)-FDP. The results show that MtTPS5 is a promiscuous enzyme, generating 23 alternate cyclization products starting from (2*Z*,6*E*)-FDP. The (2*Z*,6*E*)-FDP product profile consisted predominantly of mono- and bicyclic sesquiterpenes based on humulane, amorphene and himachalane ring skeletons. We showed that MtTPS5 retains the exceptional stereochemical control over the reaction cascade through determination of the absolute configuration of the final products which indicated the presence of only one enantiomer. Additionally, the detection of some alternate products in herbivore-induced volatiles indicates the possible natural use of substrate promiscuity by terpene synthases. This promiscuity provides organisms with access to novel chemical defenses that are adaptable with available enzymes and possible evolutionary advantage. Along with help of site-directed mutagenesis, these alternative geometric analogs can be used to generate novel cyclic products with known terpene synthases

Experimental Section

General methods.

Reactions were performed under Argon gas. Solvents were dried based on standard procedures. Chemical shifts of ^{1}H, ^{13}C and ^{31}P NMR was given in ppm (δ) based on solvent peaks. (^{1}H NMR) $CDCl_3$: 7.27 and 77.4 ppm (^{13}C NMR). D_2O/ND_4OD: 4.79 (^{1}H NMR); ^{13}C NMR and ^{31}P NMR were referenced to external standard 3-(trimethylsilyl)-propionic acid-d_4 sodium salt (TSP; 3 % in D_2O) and phosphoric acid (H_3PO_4, 10 % in D_2O), respectively. GC-MS: equipped with a DB5 (15 m × 0.25 mm, 0.25 μm); helium served as carrier gas. Molecular composition of prepared compounds was determined by ESI-MS using a tandem quadrupole mass spectrometer (geometry quadrupole-hexapole-quadrupole) equipped with an electrospray (ESI) source. High-resolution ESI-MS (HR-EI-MS) were recorded at resolution ca 2500. High-resolution MS (EI) data were obtained in positive ion mode using 70 eV ionization energy. A DB-5 capillary column, 30 m × 0.25 mm, 0.25 μm film thickness using helium (1.5 mL s^{-1}) as carrier gas was used for separation.

Heterologous expression of terpene synthases.

Strains of *E. coli* (BL21-CodonPlus(DE3)) containing the recombinant vectors of MtTPS5[41] with an N-terminal His_8-tag were cultivated to optical density(at 600 nm) A_{600} = 0.5 at 37 °C in Luria-Bertani (LB) broth -medium with kanamycin (50 μg mL^{-1}) stirring at 200 rpm. The expression medium was induced with isopropyl β-d-1-thiogalactopyranoside (IPTG, final concentration 1 mM), and the cultures were shaken overnight at 16 °C at 200 rpm. Cells were centrifuged at 4000 rpm for 20 min, and the cell pellet was resuspended in lysis buffer (300 mM NaCl,10 mM imidazole, 50 mM NaH_2PO_4, pH 8.0) and incubated for 1 h at 4 °C with lysozyme (1 mg mL^{-1}). The cell disruption was performed by sonication for 2 × 2 min. The cell debris was separated by centrifugation at 10000*g* for 30 min. A column of Ni^{2+}-NTA-Agarose, was equilibrated with eight bed volumes of lysis buffer, and the supernatant after centrifugation was passed over it. It was further washed twice with four bed volumes of washing buffer (300 mM NaCl,50 mM NaH_2PO_4, 20 mM imidazole, pH 8.0), the protein was eluted with two bed

volumes of elution buffer (300 mM NaCl, 50 mM NaH_2PO_4 250 mM imidazole, pH 8.0). The purified protein was directly desalted) by passing it through a NAP 25 column into a TRIS-buffer (50 mM TRIS, pH 7.5, 10 mM NaCl, 10% glycerol, diluted to reach a concentration of 1 mg mL^{-1} and stored at −20 °C. The protein concentration was quantified by using the method explained by Bradford et al.[42]

Identification of enzyme products

A ZB-5 capillary column equipped instrument (0.25 mm i.d. × 15 m with 0.25 μm film) was used to perform GC−MS analysis. 1μl of the sample was injected in splitless mode at 220 °C injection port temperature. The oven temperature was initially maintained at 50 °C for 2 min then followed by a ramp up of 10 °C min^{-1} until 240 °C followed by an additional ramp of 30 °C min^{-1} upto 280 °C and finally maintained for 2 min. Ionization potential was set at 70 eV, and with a scanning range from 40 to 250 amu. Carrier gas was helium at a flow rate of 1.5 mL min^{-1} .

Compound identification was performed by comparison of individual mass spectra and Kováts indices (retention indices) with those of published reference spectra in Adams' terpene library[43], MassFinders' (software version 3.5) and the NIST database. Moreover, retention indices (RI) of sesquiterpene peaks calibrated with GC runs of C8−C20 alkane standard mixture were compared with authentic reference compounds RI values (Table A-1, supporting information). Essential oils containing relevant sesquiterpenoids were generously provided by Prof. Dr. Stephan von Reuss or bought from commercial supplier.

Quantification of products

For quantification of enzymatic products, the products were first separated by gas chromatography (injection volume 2 μL, H_2 carrier gas 1.5 mL min^{-1}) under the above described conditions and further analyzed on a flame ionization detector (FID) at 250°C. Calibration curves obtained from samples with different concentrations of (*E*)-β-caryophyllene and torreyol was used for correction of the different response factors of sesquiterpene hydrocarbons and alcohols. The average and standard deviations of

relative ratios were determined by at least four independent samples setting the sum of identified compounds to 100%.

Determination of the stereochemistry of the enzyme products

The separation and identification of product enantiomers were performed 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) and operated with helium at 1 mL min^{-1} as carrier gas. A splitless injection of sample (1 μL) at 220 °C and a temperature program was started from 60 °C and kept for 5 min, ramped up 2 °C min^{-1} to 160 °C followed by an additional ramping up of 30 °C min^{-1} to 220 °C with 2 min hold. Samples containing both enantiomers were prepared by mixing pentane extracts of the liverwort *Preissia quadrata*[44] or *Scarpania undulata*[45] with the corresponding reference or were gifts from Prof. Stephan von Reuss.

Enzyme assays for product analysis

Standard assays were performed with 600 nM purified protein in assay buffer (25 mM HEPES, pH 7.5, 1 mM DTT, 10% glycerol, 10 mM $MgCl_2$) with 50 μM substrate (FDP) making final volume of 1 mL. The reaction mixture was overlayed with 100 μL of pentane containing 1 ng μL^{-1} of dodecane (internal standard) on static platform to trap the volatile products. After incubation for 90 min at 30 °C, the reaction was halted by vortexing (20 s) due to enzyme denaturation. The entire reaction mixture was frozen in liquid nitrogen; the pentane layer extracted after thawing and analyzed by GC−MS.

Mass Spectra of Compound **25:**

MS (ESI): m/z=222, 207 $[M\text{-}Me]^+$, 204 ($[M\text{-}H_2O]^+$).

Preparative assays

For the identification of the unknown compound (25), two 10-mL assays consisting 600 nM purified enzyme in assay buffer and FDP (50 μM) was overlayed with 10 mL of pentane. After overnight incubation at 30 °C, the volatiles were further extracted with 5 mL of pentane three times. The combined pentane extracts were passed through a Pasteur pipette containing Na_2SO_4, the volume was

then reduced to a final volume of ~100 µL. Alcohols were separated using pentane/ether (6:1, v/v) for elution on silica column (1 g, Pasteur pipette). Two fractions of highly enriched compound 25 were obtained, and reduced to a volume of approximately 1 ml under a light argon stream.

Catalytic hydrogenation

For the catalytic hydrogenation, the solution of the respective sesquiterpene alcohol was concentrated to dryness under argon stream. The residue was taken up in 300 µl of pentane, and a spatula tip catalyst (5% rhodium on aluminum, ~10µg) was added. The gas space of the reaction vessel was flushed with H_2 for 10s, and the reaction mixture was stirred for a further 15 minutes under a H_2 atmosphere (1 atm). The suspension was then filtered over cotton, and the solid components were rinsed with 500 µl of pentane. The volume was reduced to ~ 200 µl and the sample was analyzed by GC-MS.

Acid-catalyzed dehydration

The sample (~500ng GC) of the alcohol was re-dissolved in 200 µL of dry CH_2Cl_2, and 5 µL of trifluoroacetic anhydride (derivatization grade) was added under argon atmosphere. The reaction mixture was stirred for 30 min, and after the addition of pentane (1 ml), 10% $NaHCO_3$ (1 ml) was added to quench the reaction. The aqueous phase was extracted 2 x 1 mL of pentane. The combined organic extracts were dried (Na_2SO_4) and reduced to ~100 µL under argon. Concentrated samples were then analyzed by GC−MS.

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Supporting information

Complete tables of relative ratios of products, retention indices, source of references. Spectra of enzyme products in comparison with authentic reference standards. Mass spectrometric data from incubation experiments with substrates and synthetic procedure of (2*Z*,6*E*)-FDP.

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