A Spirobicyclo[3.1.0] Terpene from the Investigation of Sesquiterpene Synthases from *Lactarius deliciosus*

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Supporting Information Placeholder

ABSTRACT: Milk cap mushrooms in the genus *Lactarius* are known to produce a wide variety of terpene natural products. However, their repertoire of terpene biosynthetic enzymes has not been fully explored. In this study, several candidate sesquiterpene synthases were identified from the genome of the saffron milk cap mushroom *L. deliciosus*, and expressed in a sesquiterpene-overproducing *Escherichia coli* strain. In addition to enzymes that produce several known terpenes, we identified an enzyme belonging to a previously unknown clade of sesquiterpene synthases that produces a terpene with a unique spiro-tricyclic scaffold. These findings further add to the rich diversity of terpene scaffolds and mushroom terpene synthases and are valuable for biotechnological applications in producing these terpenoids.

The fruiting bodies of milk cap mushrooms (genus *Lactarius*) produce a milky latex, which is exuded upon tissue injury, containing sesquiterpene compounds that are responsible for their characteristic color or flavor. Examples are the blue azulene pigment in *L. indigo*[1], and orange dihydroazulene pigment in *L. deliciosus*[2]. In many cases, the terpene compounds are fatty acyl esters, and tissue damage leads to the cleavage of the fatty acyl group and conversion of the terpene moiety into a variety of products. In *L. deliciosus*, this results in the production of a variety of substituted azulene derivatives, causing the latex to take on its characteristic hue. In other milk cap mushrooms (e.g. *L. fuliginosus*[3] and *L. chrysorrheus*[4]), this leads to the development of a compounds imparting a bitter or acrid flavor.

Despite the large number of terpenoids that have been isolated and identified in *Lactarius* mushrooms (including caryophyllene, drimane and isolactarorufin[5]), their biosynthetic pathways are not yet understood. The first step in the biosynthetic pathway of sesquiterpene natural products is generation of the terpene scaffold, catalyzed by sesquiterpene synthases (STS). Fungal STS, particularly those in mushrooms (basidiomycetes), are much less well studied compared to plant STS, and the limited homology between fungal and plant STS makes it difficult to predict their functions based on studies of the plant enzymes[6].

Several systematic investigations of mushroom STS have been reported in recent years[6-9], and several mushroom STS have been identified, though their full catalytic repertoire remains unexplored. A more complete understanding of mushroom STS would enable the identification of new terpene-derived natural products and provide new terpene scaffolds for biotechnological applications.

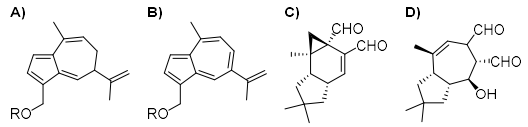

Figure 1. Examples of terpene-derived compounds in *Lactarius* mushrooms. A) The orange dihydroazulene pigment of *L. deliciosus*, and B) blue azulene pigment of *L. indigo* ($R=CH_3(CH_2)_{16}CO_2$). C) Isovelleral and D) piperdial, volatile sesquiterpene-derived dialdehydes released upon injury of the fruiting bodies of *L. rufus* and *L. piperatus* respectively, which contribute to their pungent flavour and provide chemical defense against microbes and parasites.

Results and discussion

Identification and analysis of *L. deliciosus* STS sequences

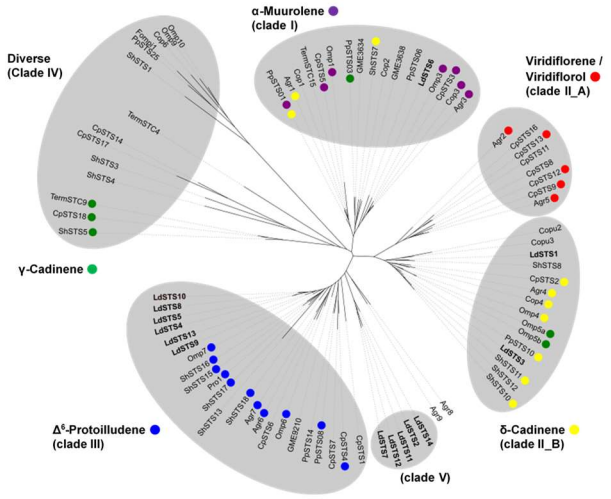

Figure 2. Maximum likelihood phylogenetic tree of previously characterized basidiomycete STS, together with predicted *L. deliciosus* STS

The genome of *L. deliciosus* was previously sequenced and assembled by Li *et al.*[10]. Protein coding genes were predicted using AUGUSTUS[11] (version 3.2.2, default parameters with *Coprinus cinereus* as training set). Candidate STS sequences were identified by a HMMER search[12], with a profile created from an alignment of 393 putative basidiomycete STS (reported by Wawrzyn *et al.*[8]). This yielded 14 complete sequences (Table 1, Data S1). The amino acid sequences revealed conservation of the metal-binding region (NSE motif and D(D/E/N)xx(D/E) motif, Figure S1).

Gene	Enzyme	Clade	Product(s)
g3408	LdSTS6	1	α-muurolene, γ-Gurjunene#, α-selinene#, etc.
g833	LdSTS1	2	1,10-di-epi-cubenol
g1979	LdSTS3	2	myrcene, trans-β-ocimene etc.
g2294	LdSTS4	3	N.I.
g2295	LdSTS5	3	N.I.
g4554	LdSTS8	3	N.D.
g4592	LdSTS9	3	N.I.
g5296	LdSTS10	3	N.D.
g7402	LdSTS13	3	N.I.
g1333	LdSTS2	5	N.D.
g4057	LdSTS7	5	new
g5987	LdSTS11	5	new
g6779	LdSTS12	5	N.D.
g9127	LdSTS14	5	N.D.

Table 1. Predicted *L. deliciosus* sesquiterpene synthases, their cyclization clades, and products when expressed in a terpene-overproducing *E. coli* strain (N.I. = not investigated; N.D. = no product detected; # indicates that the compounds are predicted with a relatively modest accuracy).

The classification of basidiomycete STS into several cyclization clades was previously reported[7, 8]. To classify the newly identified STS, a multiple sequence alignment including the new STS and previously characterized basidiomycete STS (compiled by Zhang *et. al*[6]) was performed with Clustal Omega[13], and a phylogenetic analysis was conducted with PhyML[14] (Figure 2, Table 1). Of the predicted STS, one belongs to clade I (α-muurolene, 1,10 cyclase), two belong to clade II (δ-cadinene, 1,10 cyclases), six belong to clade III (putative 1,11 cyclases), and the remaining five belong to an unknown clade V.

Determination of STS terpene products by GC-MS

Since *L. deliciosus* is known to produce guaiene-based terpenes, which are products of 1,10 cyclization, we first focused our experiments on the STS from clades I, II and subsequently on the unknown clade V. The genes for selected STS were synthesized, cloned into pET28a vectors, and transformed into an engineered *E. coli* BL21 strain overproducing the sesquiterpene precursor farnesyl pyrophosphate (FPP) used in in previous study[15].

We first investigated the *L. deliciosus* STS belonging to the δ-cadinene (LdSTS1, LdSTS3) and α-muurolene (LdSTS6) clades (Table 1), which catalyze 1,10-cyclization as expected for guaiane STS. LdSTS1 produced a single major product (1,10-di-epi-cubenol). LdSTS3 is a highly promiscuous terpene synthase with ~40 terpene products including monoterpenes (myrcene, trans-β-ocimene, etc.) and sesquiterpenes (copaene#, β-cubebene, γ-cadinene, etc.; here # indicates that the compounds were predicted based on the GC-MS fragmentation against the NIST library). LdSTS6 is also a promiscuous terpene synthase with several sesquiterpene products (α-muurolene, γ-Gurjunene#, α-selinene#, etc). Contrary to our expectation, none of the three 1,10 cyclases produced a guaiene terpene as its major product under our experimental conditions. We also investigated two of the STS belonging to the Δ6-protoilludene clade (LdSTS8 and LdSTS10), but no products were detected.

Next, we investigated the *L. deliciosus* STS in the unknown clade 5 (LdSTS2, LdSTS7, LdSTS11, LdSTS12, LdSTS14, Table 1). No products were detected for LdSTS2, LdSTS12 and LdSTS14. Intriguingly, LdSTS7 produced one minor and one major product, both with identical m/z of 204.2 according to GC-MS analysis (Figure 3). Preliminary analysis suggest that the terpene product could be guaiene terpene that we were after. LdSTS11 produced a lower yield of the same compounds corresponding to the major product of LdSTS7, based on their identical retention times and mass spectra.

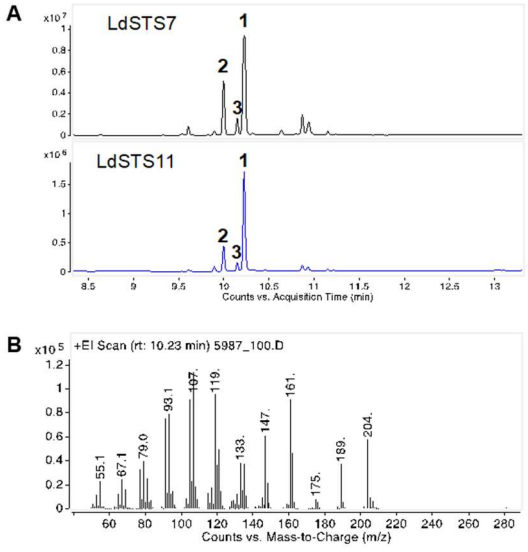

Figure 3. GC-MS detection of the terpene products of the clade 5 enzymes LdSTS7 and LdSTS11. A) Total ion chromatograms of

the fermentation products of *E. coli* overexpressing LdSTS7 and LdSTS11, showing a major product **1** and two other minor products (**2** & **3**). B) GC-MS trace of the main compound **1** produced by LdSTS7.

Purification of the unknown LdSTS7 terpene product

To enhance the yield of LdSTS7 terpene products, the vector backbone was changed from pET28a to a p15A origin of replication-based vector[16] that expressed the gene *ispA* (encoding for *E. coli* FPP synthase). This LdSTS7 plasmid was transformed into an engineered *E. coli* BL21 strain containing the SPS01 plasmid that overexpresses all the mevalonate pathway genes derived from previous study [15] for the large-scale fermentation. At the end of the production process, the extracted terpenoids were analysed using GC-MS to determine the level of the terpenoid products along with 10 mg/L of caryophyllene standard. The calculated yield of the product mixture was estimated at ~80 mg/L.

The isolated terpene sample contained primary a mixture of the major **1** and minor product **2** (*c.a.* 2:1 peak area based on GC-TIC, Figure 3) with trace amounts of product **3**. Further attempts to isolate the components by flash column chromatography or preparative thin-layer chromatography (TLC) were unsuccessful, suggesting that the products could be structural isomers with very similar physical characteristics.

The GC-MS spectra of both products had m/z = 204 (Figure S2c), which was corroborated by LC-HRMS. The IR spectrum did not show the presence of any hydroxy groups (2500–3600 cm^{-1}). Combining elemental analysis (absence of nitrogen group) with the chemical formula ($C_{15}H_{24}$), a sesquiterpene hydrocarbon with 4 degrees of unsaturation is proposed.

NMR analysis of the LdSTS7 terpene products

Initial 1D and 2D NMR experiments revealed significant peak overlaps (see SI, Figure S4c) as well as low signal intensity due to limited sample quantity. Thus, selective and semi-selective 1D and 2D experiments were employed for *de novo* structure elucidation to resolve and rigorously identify individual signals from each component in the mixture while concurrently obtaining accurate J-couplings and ^{1}H multiplicities.

Briefly, 1D selective TOCSY experiments identified two well-defined spin systems for the major compound **1**, leaving two CH_3 singlets and two quaternary carbons based on the chemical formula (Figure 4B). The identity of the spin systems were confirmed by 2D HSQC experiments (see SI, Figure S4n). The connectivities of the fragments were obtained by 2D semi-selective HMBC experiments, revealing the main spiro[4.5]dec-6-ene skeleton bearing a key spiro centre, as well as a gem-dimethylcyclopropyl motif (Figure 4C). 1D selective NOESY experiments then identified protons that were close in space (Figure 4D), thus confirming the relative stereochemistries about the 4 stereogenic centres and affording the final structure of compound **1**.

To further analyse if this scaffold has been previously reported, a search of the first spin system on Reaxys® revealed 74 structurally similar hits such as premnaspirodiene and hinesene that bear the spiro[4.5]dec-6-ene skeleton. However, none of them fulfilled the subsequent structural conditions of (a) possessing the elucidated second spin system and (b) containing no further olefinic groups.

The remaining minor compounds **2** and **3** were identified to be 9-aristolene (**2**) and 1-aristolene (**3**) based on similar *de novo* structural elucidation from (semi-selective) 1D and 2D NMR experiments, followed by subsequent verification after a structure search (Reaxys®) and comparison of ^{1}H and ^{13}C NMR chemical shifts from literature data (see SI, sections S4.3 and S4.4).

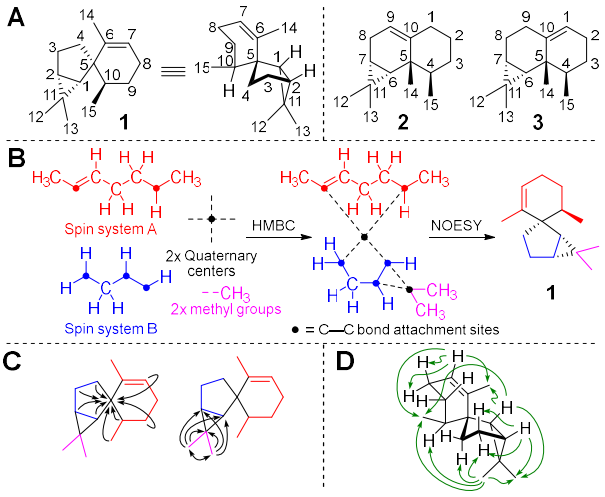

Figure 4. A) Numbering nomenclature for compound (**1**), 9-aristolene (**2**) and 1-aristolene (**3**) used in NMR analysis. B) Structure elucidation process for compound **1**. C) Observed key HMBC correlations for **1**. D) Observed key NOE correlations for **1**.

Proposed mechanism for formation of 1, 2 and 3

Our phylogenetic analysis suggests that clade V STS (which include LdSTS7) are closely related to clade III STS (which produces Δ^{6}-protoilludene, a product of 1,11 cyclization) and to the enzymes Agr8 and Agr9 (which produce several sesquiterpenes including γ-muurolene and β-cadinene, products of 1,10 cyclization)[6]. To account for the formation of major product **1** and minor products **2** and **3**, we hypothesize the mechanistic pathway shown in Figure 5, which draws on analogous proposed mechanisms for other STS. Cyclization of farnesyl diphosphate (FPP, **4**) to form the cyclopropyl moiety could proceed through either 1,10 cyclization to form a germacrenyl carbocation **7**, 1,11 cyclization to form a humulyl carbocation **5**, or direct formation of the nonclassical carbocation intermediate/transition state **6**, involving a three-center two-electron bond between C1, C10 and C11, as proposed for viridiflorol synthase[17]. Deprotonation of **5** or **7** gives bicyclogermacrene **8**.

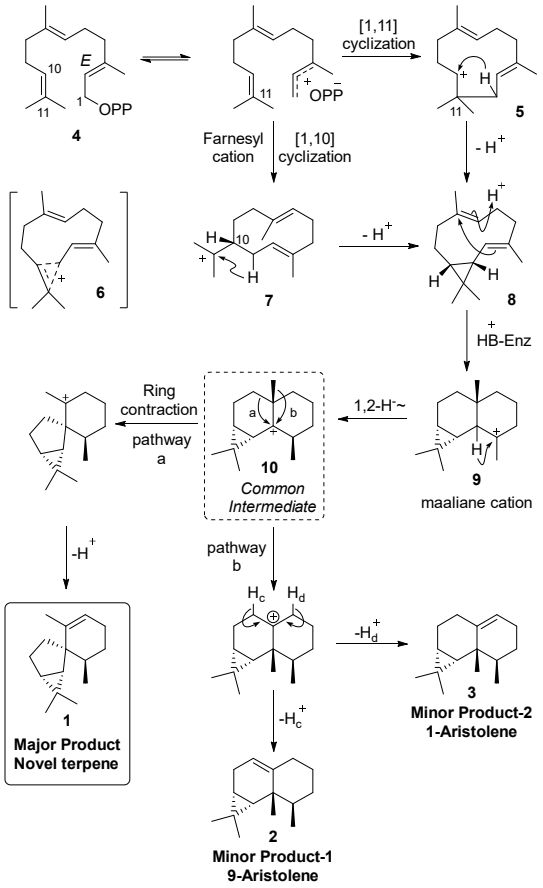

Figure 5. Proposed bifurcated mechanistic pathway for conversion of farnesyl pyrophosphate to **1**, **2** and **3**.

Subsequently, the neutral **8** is reprotonated, followed by Markovnikov-directed 1,6 cyclization to give maaliane carbocation **9**. Finally, a 1,2-hydride shift from **9** would lead to common intermediate **10**. Conversion of common intermediate **10** to the observed products then follows a bifurcated pathway: a) **10** can either undergo a Wagner–Meerwein type rearrangement with ring contraction to yield **1** (pathway a) or b) a 1,2-methyl group migration that results in **2** and **3** upon deprotonation (pathway b). These pathways are consistent with those involved in the conversion of germacrene A to hinesene and selina-4,11-diene[18]. Based on the current results, the presence of the aristolane isomers coupled with the *syn*-protons observed on the dimethylcyclopropane products (*cf.* Lepidozene[19]) suggests that they might have originated from a common biosynthetic source activated by a bicyclogermacrene synthase similar to those responsible for other structurally related sesquiterpenes[20].

Conclusions

In conclusion, our studies of STS in *L. deliciosus* has led to the discovery of a new clade of STS producing compound **1** with a structurally unique spirobicyclo[3.1.0] terpene sesquiterpene scaffold. The involvement of this STS in the biosynthesis of terpene natural products in *L. deliciosus* is to-date unknown, but its discovery highlights the latent biosynthetic capabilities and potential for discovery of new enzymatic activity in Basidiomycete fungi. The characterization of this new enzyme also adds to the repertoire of terpene synthases and scaffolds available for biotechnological application. In particular, the spirocyclic carbon skeleton and rigid dimethylcyclopropane moiety of the new terpene has attractive characteristics for medicinal chemistry[21-23]. Further studies on the elucidation of the mechanistic pathways are currently underway in our laboratories.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at

Methods and material of cell cultures, spectral characterization, additional figures, as well as GC-MS, HRMS, NMR, FTIR and elemental-analysis.

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Author Contributions

‡ C. B. C and G. P. contributed equally. Y. W. and C. Z. carried out the bioinformatics and cloning. C. Z. and R. T. carried out strain optimization, fermentation, crude extraction of the terpene products and initial GC-MS analysis. G. P. and Y. H. L. carried out purification and analytical chemistry. C. B. C and G. P. carried out NMR experiments and analysis. C. Z., Y. H. L, E. L. A. and H. Z. carried out funding acquisition and supervision.

Notes

The authors declare no competing financial interests.

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