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Mechanistic characterisation of a sesquiterpene synthase for asterisca-1,6-diene from the liverwort *Radula lindenbergiana* and implications for pentalenene biosynthesis†

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A sesquiterpene synthase from the liverwort *Radula lindenbergiana* was characterised and shown to produce the new sesquiterpene hydrocarbon (3*R*,9*R*)-asterisca-1,6-diene, besides small amounts of pentalenene. The biosynthesis of asterisca-1,6-diene was studied through isotopic labelling experiments, giving additional insights into the long discussed biosynthesis of pentalenene.

Terpene synthases are fascinating biocatalysts that can convert acyclic and achiral oligoprenyl diphosphates such as geranyl diphosphate (GPP), farnesyl diphosphate (FPP), geranylgeranyl diphosphate (GGPP), geranylfarnesyl diphosphate (GFPP) and even farnesylfarnesyl diphosphate (FFPP) into structurally complex terpene hydrocarbons or alcohols.[1–5] The products of these enzymatic reactions are usually chiral and formed with a high enantioselectivity, may contain several stereogenic centres, and are often polycyclic. During the terpene synthase reaction the majority of the carbons of the substrate may encounter a change in the bonding situation which makes terpene cyclisations one of the most complex transformations in nature. The dramatic structural changes in a single enzymatic step are achieved through cationic cascade reactions with multiple elementary steps. Terpene synthases have thus two major functions: first, they ionise the substrate either through the abstraction of diphosphate (type I terpene synthases) or protonation (type II enzymes), and second, they provide a confined hydrophobic space that enforces a reactive substrate conformation to make use of the inherent substrate reactivity[6] and allows the cationic cascade reaction to take place in an aqueous environment. Typical elementary steps of terpene synthase catalysis include cyclisation reactions through attack of a remote double bond to a cationic centre, Wagner–Meerwein rearrangements, hydride and proton shifts. While the multistep transformations inside the hydrophobic pocket of terpene synthases cannot be observed directly, *e.g.* through spectroscopic methods, isotopic labelling strategies have been developed to gain conclusive insights.[7–9] Alternatively, computational methods including DFT calculations[10–13] and QM/MM simulations have been widely applied.[14–16]

Bacterial and fungal type I terpene synthases are generally composed of a single domain[17] that closely resembles the α-helical fold first observed for avian farnesyl diphosphate synthase[18] and is thus called the α-domain. In contrast, plant terpene synthases exhibit either two (αβ)[19] or even three domains (αβγ),[20] but enzyme catalysis of type I plant terpene synthases seems to depend (mainly) on the α-domain, while that of type II plant terpene synthases depends (mainly) on the βγ bidomains.[21,22] In 2012 it was discovered that microbial terpene synthase like enzymes (MTPSLs) with a single α-domain occur in the lycophyte *Selaginella moellendorffii*,[23] and shortly later it was demonstrated that this type of terpene synthases is widespread in nonseed land plants,[24] including the liverwort *Marchantia polymorpha*.[25,26] Despite the fact that liverworts are a rich and extensively studied source of terpenes,[27] only a few terpene synthases have been functionally characterised from liverworts to date. A recent study from our laboratories reported on the presence of several functional MTPSLs from the liverwort *Radula lindenbergiana* whose products were characterised by GC/MS analysis. For one of these enzymes (RlMTPSL3) the production of a new sesquiterpene was observed, but the product remained uncharacterised.[28] Here we report on the isolation and structure elucidation of this sesquiterpene and an enzyme mechanistic study on RlMTPSL3 through isotopic labelling experiments. The results of these experiments are also relevant for the biosynthesis of pentalenene that has long been discussed in the literature.

The sesquiterpene synthase RlMTPSL3 from *R. lindenbergiana*[28] shows all highly conserved motifs required

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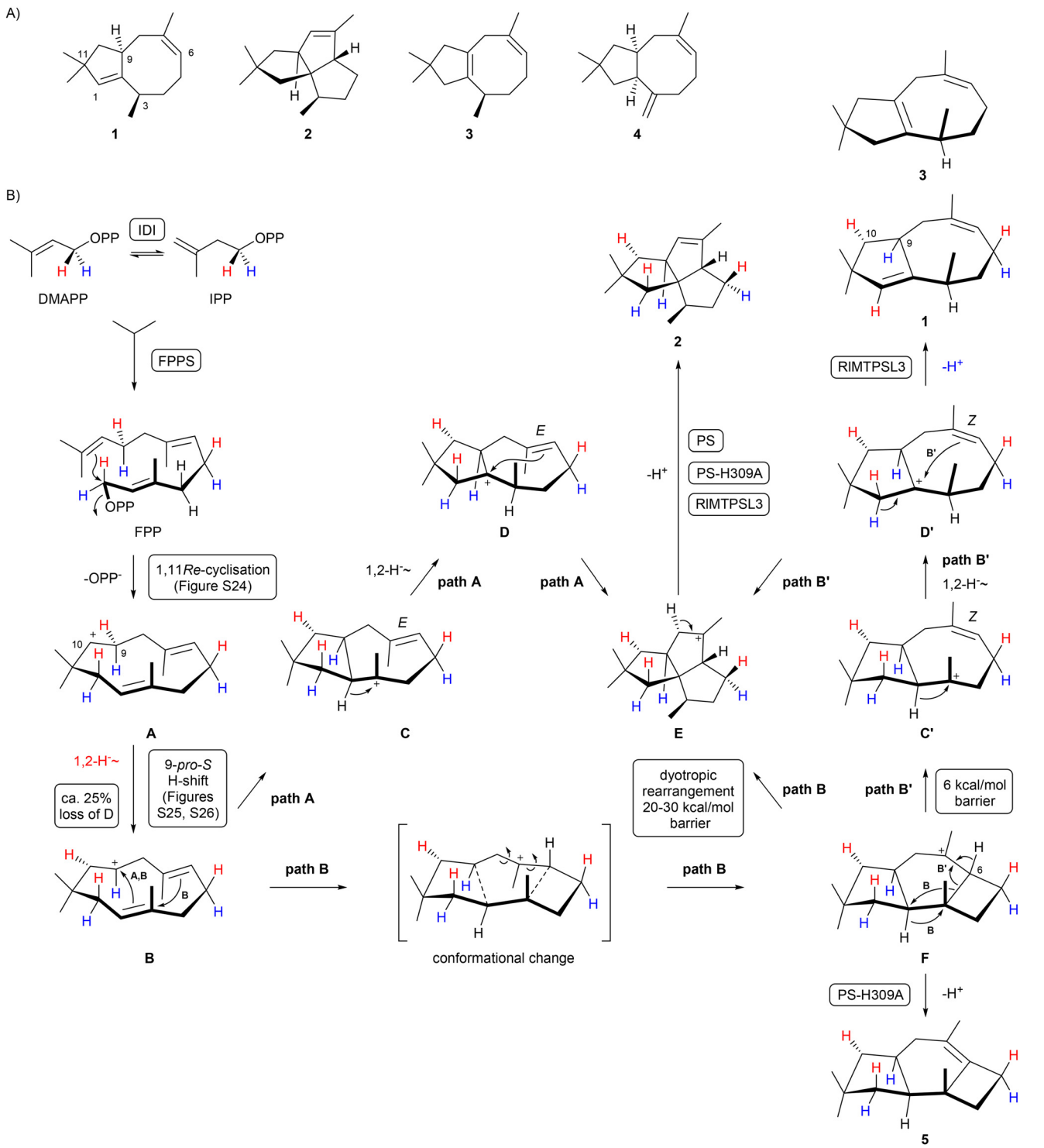
for substrate recognition and catalytic activity, including the aspartate-rich motif (DDXXD),[29] the NSE triad (ND(I,L,V)XSXXXE),[30] the RY pair,[31] and the pyrophosphate sensor, a highly conserved Arg residue within the effector triad (Fig. S1†).[32] The incubation of RlMTPSL3 with FPP resulted in the formation of one major sesquiterpene hydrocarbon (**1**) besides pentalenene (**2**), asterisca-2(9),6-diene (**3**), (*E*)-β-caryophyllene, and α-humulene that were identified by GC/MS through a comparison of mass spectra to database spectra and of retention indices to literature data[33,34] (Scheme 1A,



Scheme 1 Characterisation of RlMTPSL3. (A) Structures of the products obtained from FPP with RlMTPSL3 (**1** and **2**) and structures of related compounds. (B) Cyclisation mechanism for the conversion of FPP into **1** and **2** by RlMTPSL3.

Fig. S2, and Table S1†). GPP yielded only geraniol and linalool, and GGPP and GFPP were not converted. Compound **1** was isolated and structurally characterised by NMR spectroscopy as asterisca-1,6-diene (Table S2, and Fig. S3–S10†). While **1** is a new sesquiterpene, **2** was first isolated from *Streptomyces griseochromogenes*[35] and is known as the product of the bacterial pentalenene synthases (PS) from *Streptomyces exfoliatus* and *Streptomyces bungoensis*.[36,37] Compound **3** is the product of a characterised sesquiterpene synthase from the amoeba *Dictyostelium discoideum*[34] and has been reported from the nudibranch *Phyllodesmium magnum*.[38] Furthermore, asterisca-3(15),6-diene (**4**) has been isolated from the medicinal plant *Lippia integrifolia*.[39]

The absolute configuration of **1** was determined through stereoselective deuteration experiments using DMAPP and (*E*)- or (*Z*)-(4-^{13}C,4-^{2}H)isopentenyl diphosphate (IPP) in conjunction with FPP synthase (FPPS) from *Streptomyces coelicolor*[34] and RlMTPSL3 (Fig. S11,† for a summary of labelling experiments *cf*. Table S3†).[40] Analogous experiments were conducted with (*R*)- or (*S*)-(1-^{13}C,1-^{2}H)IPP,[41] *Escherichia coli* isopentenyl diphosphate isomerase (IDI),[42] FPPS and RlMTPSL3 (Fig. S12†). Using these substrates stereogenic anchors of known absolute configuration will be introduced into **1**, which is based on the findings by Cornforth and coworkers for the stereochemical course of oligoprenyl diphosphate biosynthesis.[43] Solving the relative configuration of the naturally present stereogenic centers of **1** with respect to these anchors then allows for conclusions on its absolute configuration (for an explanation of the experimental data in the present case *cf*. the legends of Fig. S11 and S12†). Taken together the results pointed to the structure of (3*R*,9*R*)-**1**. Unfortunately, the production of **2** was too low for its isolation and determination of the absolute configuration through a comparison of its optical rotation to that of the bacterial compound.[35] For application of the stereoselective deuteration approach, NMR data with a complete assignment for all diastereotopic hydrogens are required, but such data are not available for **2** from the literature. Therefore, **2** was produced enzymatically from FPP with the PS from *S. bungoensis* for a complete assignment of its NMR data (Table S4, and Fig. S13–S20†). A reinvestigation of the data obtained from the above mentioned stereoselective deuteration experiments showed the presence of minor signals for **2**, allowing to conclude on the absolute configuration of (2*R*,3*R*,6*S*,9*S*)-**2** (Fig. S21†). This is the same absolute configuration as for the bacterial compound[44] and is with the same configurations at C3 and C9 consistent with the absolute configuration of **1**, in agreement with the common biosynthesis of **1** and **2** by RlMTPSL3. The absolute configuration of **3** was tentatively assigned based on the common biosynthesis of **1–3** by RlMTPSL3 and is opposite to that of **3** from *Dictyostelium discoideum*.[34]

As is suggestive from the common production of both compounds by one enzyme, the biosynthesis of **1** is closely linked to the biosynthesis of **2** that has received a lot of attention during the past decades. The initially suggested pathway towards **2** proceeds through 1,11-cyclisation of FPP to the (*E*,*E*)-humulyl cation (**A**), followed by a 1,2-hydride shift to **B**, cyclisation to **C**, another 1,2-hydride shift to **D**, cyclisation to **E** and deprotonation (path A in Scheme 1B).[45] Later on, based on DFT calculations, Gutta and Tantillo proposed an alternative pathway (path B) along which **B** is directly cyclised to the protoilludyl cation **F**, followed by a dyotropic rearrangement to the same intermediate **E**.[46] This rearrangement is associated with a high barrier of 20–30 kcal mol^{-1} depending on the conformation of **F**,[47] but intermediate **F** can explain the formation of protoillud-6-ene (**5**) by several enzyme variants of PS including PS-H309A generated through site-directed mutagenesis of His309.[48] Further refinement of the DFT calculations revealed another pathway (path B′) that avoids the high reaction barrier of the **F**-to-**E** conversion, but instead proceeds with ring opening of **F** to **C**′ and a 1,2-hydride shift to **D**′ with an overall barrier of only ~6 kcal mol^{-1}.[47,49] The intermediates **C**′ and **D**′ are *E*/*Z* diastereomers of **C** and **D**; they can be formed as a consequence of the conformational change associated with the cyclisation from **B** to **F**. But is path B/B′ indeed relevant for the biosynthesis of **2**? Alternatively, compounds **2** and **5** could be formed independently by PS-H309A with formation of **2** through path A and of **5** through path B/B′, respectively. The simultaneous formation of both compounds by PS-H309A allowed for an elegant labelling experiment performed by Tantillo, Peters, and Cane. The hypothesis was, if **F** is on the path towards **2**, a deuterium substitution at C6 should influence the product distribution between **2** and **5**, disfavouring **5** because of the stronger C–D bond in comparison to a C–H bond, whereas no significant influence on the product distribution would be expected, if the biosynthesis would operate independently *via* two pathways branching out from **B**. The experiment indeed showed a reduced production of **5** from (6-^{2}H)FPP, giving evidence for **F** as a true intermediate towards **2**.[49]

After the clarification that path B/B′ is relevant for the biosynthesis of **2**, the remaining question is whether it is path B or path B′ that is taken towards this compound. Path B′ proceeds through **D**′ that is the direct precursor to the main product **1** (and the side product **3**) of RlMTPSL3 by deprotonation and is potentially an on-path intermediate towards **2**. Thus, the common production of **1** and **2** by RlMTPSL3 allows for a similar experiment as described above. A deuterium substitution at C1 should influence the product distribution between **1** and **2**, if **D**′ is a common intermediate towards both compounds, but it should not, if two independent pathways (B and B′) branching out from **F** are used. The conversion of (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)IPP with IDI, FPPS and RlMTPSL3 showed a selective deprotonation from C1 to **1** with loss of the 1-*pro*-*R* hydrogen (Fig. S22†), and indeed a strongly enhanced production of **2**, if this hydrogen is substituted with deuterium (Fig. S23†), demonstrating that also **D**′ is an on-path intermediate towards **2**.

Further isotopic labelling experiments on the biosynthesis of **1** were conducted using (12-^{13}C)FPP and (13-^{13}C)FPP.[7] Their conversion with RlMTPSL3 revealed an initial 1,11-cyclisation with attack at C11 from the *Re* face (Fig. S24†). The above mentioned experiments with (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)IPP furthermore demonstrated a retainment of the 9-*pro*-*R* hydrogen of

FPP at C9, and a selective 1,2-hydride shift of the 9-*pro-S* hydrogen from C9 to C10 in the step from **A** to **B**, with *ca.* 25% washout of the migrating deuterium (Fig. S12, S21 and S25†). The migrating hydrogen ultimately ends up in the 10-*pro-R* position of **1** (Fig. S26†). The observed deuterium loss points to a deprotonation of **A** by a basic residue in the enzyme to the neutral intermediate α-humulene that is subsequently reprotonated to **B**, with some D^+/H^+ exchange at the protonated active site residue. For the bacterial PS, isotopic labelling experiments revealed the same stereochemical course for this hydrogen shift.[50] Notably, enzyme structural data pointed for PS to a guidance of the migrating hydrogen in the 1,2-hydride shift through C9–H···π-interactions with an active site phenylalanine (F76).[51] This residue is located four residues upstream of the Asp-rich motif, and a Phe residue located three positions upstream of the Asp-rich motif may have a similar function in RlMTPSL3. Furthermore, the experiments with (*E*)- and (*Z*)-(4-^{13}C,4-^{2}H)IPP showed the selective loss of the 8-*pro-S* proton of FPP in the terminal deprotonation to **2** (Fig. S27†). This is again the same stereochemical course as observed for the biosynthesis of **2** by bacterial PS.[52] Finally, conversion of (2-^{2}H,3-^{13}C)FPP[53] with RlMTPSL3 and product analysis through ^{13}C-NMR spectroscopy confirmed the 1,2-hydride shift from **C′** to **D′** in the biosynthesis of **1** (Fig. S28†).

Conclusions

Taken together, a new terpene synthase was characterised from the liverwort *Radula lindenbergiana* that produces (3*R*,9*R*)-asterisca-1,6-diene (**1**) as the main product, and pentalenene (**2**), asterisca-2(9),6-diene (**3**), (*E*)-β-caryophyllene, and α-humulene as side products. Isotopic labelling experiments not only gave insights into the absolute configurations of both compounds, but also revealed that a previously proposed intermediate towards **2** is indeed on the pathway towards this compound. The in all detail coinciding stereochemical courses for the reactions catalysed by RlMTPSL3 and PS are in line with the same absolute configuration of **2** from both enzymes, and demonstrate that the overall cyclisation mechanisms of both enzymes are very similar, despite the fact that they are not evolutionary related. Thus, the experimental evidence obtained in this study for RlMTPSL3 revealing that **D′** is an intermediate on the path to **2**, is most likely transferable to the biosynthesis of this compound by bacterial PS.

Conflicts of interest

There are no conflicts to declare.

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