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RESEARCH ARTICLE

Functional and Mechanistic Characterization of the 4,5-*diepi*-Isoishwarane Synthase from the Liverwort *Radula lindenbergiana*

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Abstract: The microbial type sesquiterpene synthase RlMTPSL4 from the liverwort *Radula lindenbergiana* was investigated for its products, showing the formation of several sesquiterpene hydrocarbons. The main product was structurally characterized as the new compound 4,5-*diepi*-isoishwarane, while the side products included the known hydrocarbons germacrene A, α-selinene, eremophilene and 4,5-*diepi*-aristolochene. The cyclization mechanism towards 4,5-*diepi*-isoishwarane catalyzed by RlMTPSL4 was investigated through isotopic labeling experiments, revealing the stereochemical course for the deprotonation step to the neutral intermediate germacrene A, a reprotonation for its further cyclization, and a 1,2-hydride shift along the cascade. The absolute configuration of 4,5-*diepi*-isoishwarane was determined using a stereoselective deuteration approach, revealing an absolute configuration typically observed for a microbial type sesquiterpene.

Introduction

Terpene synthases are remarkable enzymes that catalyze some of the most complex transformations in natural product biosynthesis.[1-3] The type I of these enzymes[4] acts on an acyclic oligoprenyl diphosphate precursor such as geranyl diphosphate (GPP, towards monoterpenes),[5] farnesyl diphosphate (FPP, sesquiterpenes),[6] geranylgeranyl diphosphate (GGPP, diterpenes),[7] geranylfarnesyl diphosphate (GFPP, sesterterpenes),[8] or even farnesylfarnesyl diphosphate (FFPP, non-squalene derived triterpenes),[9] and converts it by the abstraction of diphosphate and through a cationic cascade reaction into a structurally complex, often polycyclic terpene hydrocarbon or alcohol. These compounds are formed in termination steps involving either a deprotonation or quenching of a cationic intermediate with water. For some terpene synthase catalyzed reactions, a neutral intermediate is passed through, which is reactivated by reprotonation to start a second cyclization cascade. Notably, such a deprotonation-reprotonation sequence with involvement of a main chain carbonyl oxygen near the helix G kink of microbial type I terpene synthases as the catalytic base/acid has been demonstrated for selina-4(15),7(11)-diene synthase through isotopic labeling experiments in conjunction with QM/MM computations.[10] In this case germacrene B serves as the neutral intermediate, while generally germacrene-type intermediates can explain the biosynthesis of many eudesmane and guaiane sesquiterpenes.[11] However, the question whether the observed compound is a neutral intermediate or just a shunt product is not instantaneously clear and requires investigation, because the hypothetical neutral intermediate may be circumvented by a direct intramolecular proton transfer.[12]

Microbial type I terpene synthases are composed of a single α-domain[13] showing the same α-helical fold as first described for avian FPP synthase.[14] Microbial terpene synthase like enzymes (MTPSLs) also occur in plants. They were first described from the lycophyte *Selaginella moellendorffii*[15] and are today known to be widespread in nonseed land plants.[16] We have recently studied the diversity and functions of MTPSLs in the liverwort *Radula lindenbergiana*,[17] including a deep mechanistic investigation of the asterisca-1,6-diene synthase (RlMTPSL3)[18] with implications for pentalenene biosynthesis.[19] Here we report on the functional and mechanistic characterization of another microbial type terpene synthase RlMTPSL4 from *R. lindenbergiana* for which sesquiterpene synthase activity has been demonstrated previously, but the main product remained unidentified.[17]

Results and Discussion

The amino acid sequence of RlMTPSL4 shows the presence of all highly conserved motifs required for enzyme function,[20] including the aspartate-rich motif (^{131}DDLVD), the NSE triad (^{316}NDVWSFKKE), the RY pair (^{405}RY), and the pyrophosphate sensor (Arg270), suggesting that RlMTPSL4 is a functional enzyme (Figure S1). Recombinant RlMTPSL4 was expressed in *Escherichia coli* and purified as reported previously.[17] Incubation experiments with oligoprenyl diphosphates demonstrated the

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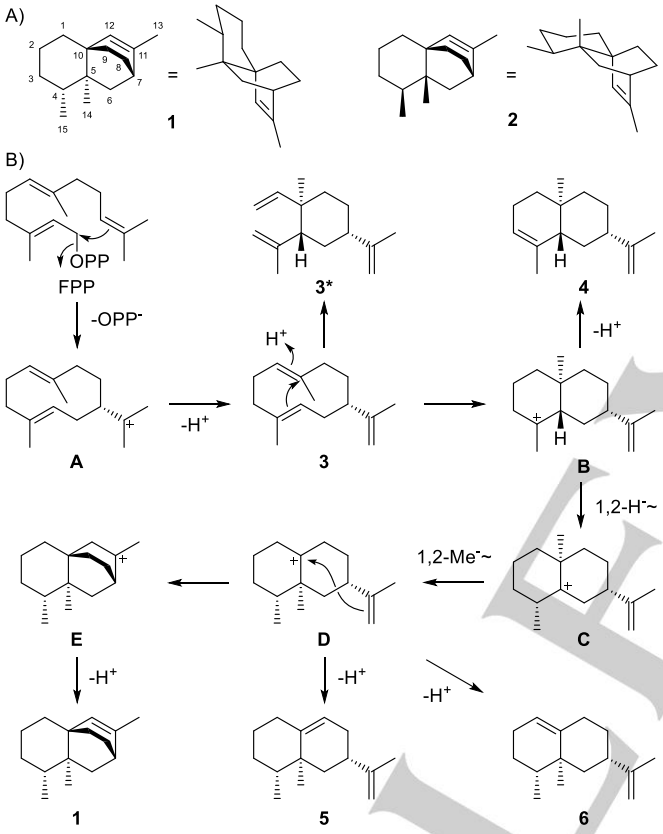
highly efficient formation of several sesquiterpene hydrocarbons from FPP, while GPP, GGPP and GFPP were not accepted as substrates (Figure S2, Table S1). The main product from FPP was isolated and structurally characterized by NMR spectroscopy (Table S2, Figures S3 – S10) as the new sesquiterpene 4,5-*diepi*-isoishwarane (**1**) (Scheme 1A). Notably, **1** is a stereoisomer of isoishwarane (**2**), the product of a recently described terpene synthase from *Streptomyces lincolnensis*,[21] and is structurally related to ishwarone from *Aristolochia indica*.[22] In addition to the main product **1** and in agreement with our previous findings,[17] the side products germacrene A (**3**) and its Cope rearrangement product β-elemene (**3***),[23] α-selinene (**4**),[24] 4,5-*diepi*-aristolochene (**5**),[25] and eremophilene (**6**)[26] were identified by GC/MS (Table S2).



Scheme 1. Biosynthesis of sesquiterpenes by RlMTPSL4. A) Structure of the main product **1** and of the related sesquiterpene isoishwarane (**2**), B) cyclization mechanism from FPP to **1**.

The hypothetical biosynthesis of **1** and its side products is shown in Scheme 1. First FPP undergoes a 1,10-cyclization to the (*E*,*E*)-germacradienyl cation (**A**) and deprotonation to germacrene A (**3**). Its reprotonation at the original C6 of FPP can induce a second cyclization to **B** that is the direct precursor of **4** by deprotonation. A subsequent 1,2-hydride shift to **C** and a 1,2-methyl group migration lead to **D** from which two alternative deprotonations give rise to **5** and **6**. Along the main path, **D** is further cyclized to **E** that yields the main product **1** upon deprotonation.

This biosynthetic hypothesis was investigated in detail through a series of isotopic labeling experiments (Table S3). The stereochemical course for the deprotonation to **3** was investigated using (12-^{13}C)FPP[27] and (13-^{13}C)FPP, in the latter case enzymatically synthesized from (9-^{13}C)GPP[28] and isopentenyl diphosphate (IPP) using *Streptomyces coelicolor* FPP synthase (FPPS) (Figure 1).[29] The conversion of (12-^{13}C)FPP with RlMTPSL4 gave mainly incorporation of labeling into the olefinic CH group of **1** (C12), but also a minor incorporation into the adjacent methyl group (C13) was observed, and the opposite outcome was obtained with (13-^{13}C)FPP. Both experiments together demonstrate that the deprotonation step towards **3** proceeds with a major deprotonation from C12 and a minor deprotonation from C13.

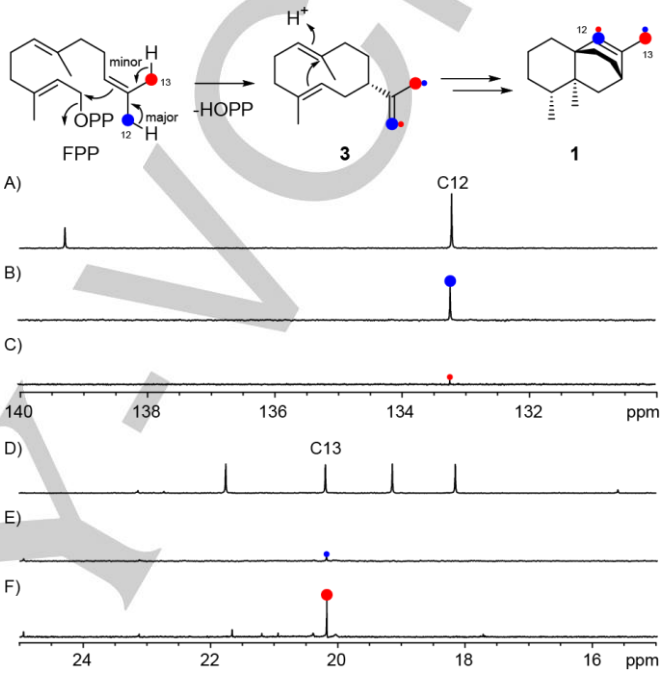


Figure 1. The stereochemical course of the deprotonation step to **3**. Partial ^{13}C-NMR spectra showing the region for C12 of A) unlabeled **1**, B) labeled **1** obtained from (12-^{13}C)FPP, and C) labeled **1** obtained from (13-^{13}C)FPP. Partial ^{13}C-NMR spectra showing the region for C13 of D) unlabeled **1**, E) labeled **1** obtained from (12-^{13}C)FPP, and F) labeled **1** obtained from (13-^{13}C)FPP.

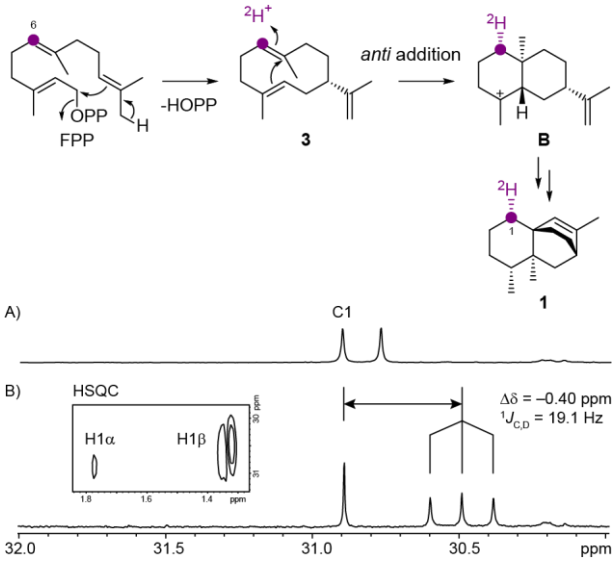


Figure 2. The reprotonation of **3** for the downstream cyclization to **1**. Partial ^{13}C-NMR spectra showing the region for C1 of A) unlabeled **1**, B) labeled **1** obtained from (6-^{13}C)FPP in deuterium oxide buffer. Section B) also shows the HSQC analysis, revealing incorporation of deuterium into the position of H1α.

The reprotonation step for the reactivation of **3** for another round of cyclization to **1** was investigated through the incubation of (6-^{13}C)FPP[27] with RlMTPSL4 in a deuterium oxide buffer. The ^{13}C-NMR analysis of the obtained products showed an upfield shifted triplet for the carbon originating from C6 of FPP (equal to C1 of **1**, $\Delta\delta$ = –0.40 ppm, $^{1}J_{C,D}$ = 19.1 Hz), as a result of the C-D spin coupling and the isotope effect on the chemical shift (Figure 2). The ring closure from **3** to **B** proceeds with an *anti* addition to the original C6=C7 double bond (FPP numbering), resulting in the incorporation of deuterium in the 1-*pro*-*S* position (H1α) of **1** (HSQC analysis in Figure 2B; because the D_2O buffer is not 100% deuterated, there is also a minor incorporation of protium as indicated by the residual signal for H1α in the HSQC spectrum and the residual singlet signal at the original shift of C1 in the ^{13}C-NMR spectrum). The 1,2-hydride migration from **B** to **C** was studied using the substrate (3-^{13}C,2-^{2}H)FPP.[30] Its conversion with RlMTPSL4 gave labeled **1** showing an upfield shifted triplet for C4 (equal to C3 of FPP, $\Delta\delta$ = –0.52 ppm, $^{1}J_{C,D}$ = 19.1 Hz), revealing that deuterium had migrated towards the labeled carbon (Figure 3).

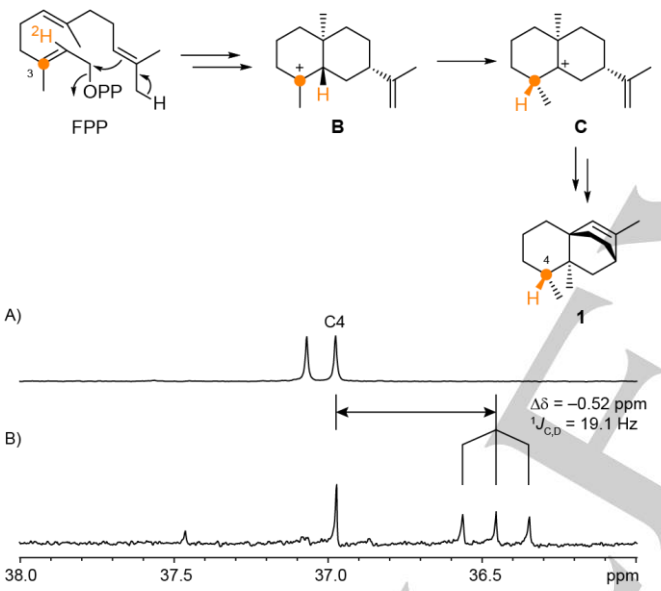


Figure 3. The 1,2-hydride shift from **B** to **C** in the biosynthesis of **1**. Partial ^{13}C-NMR spectra showing the region for C4 of A) unlabeled **1**, B) labeled **1** obtained from (3-^{13}C,2-^{2}H)FPP (the residual peak at the original shift of C4 is observed because of incomplete deuteration of the substrate).

The absolute configurations of terpenes can be investigated using stereoselectively deuterated substrates with a stereogenic center at the deuterated carbon of known absolute configuration. If this stereogenic center is unchanged during the terpene cyclization, the relative orientation between this and the naturally present stereogenic centers that can be solved by NOESY allows to conclude on the absolute configuration of the terpene under investigation. This method was applied to resolve the absolute configuration of **1** using the substrates DMAPP and (*E*)- or (*Z*)-(4-^{13}C,4-^{2}H)IPP[31] that were coupled with FPPS with a known stereochemical course.[32] The resulting stereoselectively deuterated FPP isotopologue was then converted into labeled **1**, followed by HSQC analysis of the product. The additional ^{13}C-labelings at the deuterated carbons serve to enhance the signals for the remaining protons, allowing for the sensitive detection of their incorporation pattern (Figure 4). The results revealed the absolute configuration of (4*R*,5*S*,7*S*,10*R*)-4,5-*diepi*-isoishwarane (**1**). Analogously, the absolute configurations of terpenes can be determined using the stereoselectively deuterated compounds (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)IPP.[23c] However, their conversion into labeled **1** for absolute configuration determination using *E. coli* isopentenyl diphosphate isomerase (IDI),[33] FPPS and RlMTPSL4 gave inconclusive results, and therefore, the incorporation patterns observed with these compounds were used to assign the diastereotopic hydrogen atoms at the labeled carbons C2, C6 and C8 with certainty (Figure S3, Table S3).

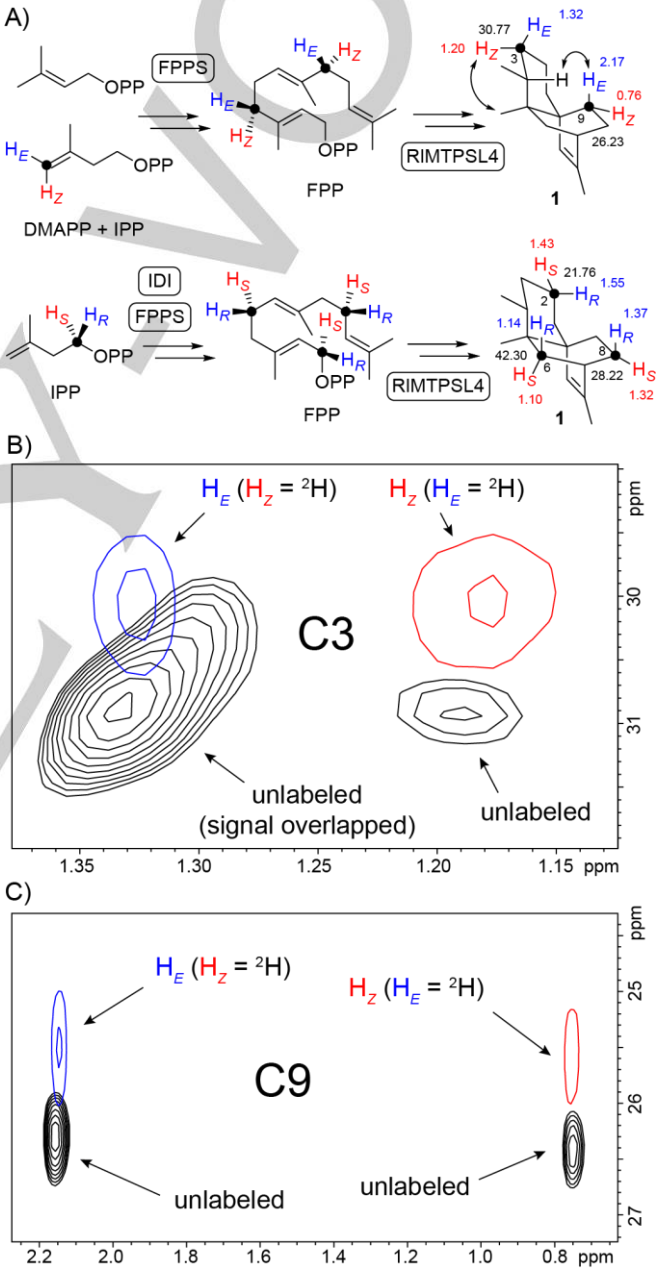


Figure 4. The absolute configuration of **1**. A) Enzymatic synthesis of labeled **1** from DMAPP and (*E*)- and (*Z*)-(4-^{13}C,4-^{2}H)IPP, and from (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)IPP. Values at labeled carbons (black dots) and at blue and red hydrogens are chemical shifts in ppm for unlabeled **1**. B) Three overlaid HSQC spectra for C3 of unlabeled **1** (black crosspeaks), of labeled **1** obtained from (*Z*)-(4-^{13}C,4-^{2}H)IPP (blue crosspeak), and of labeled **1** obtained from (*E*)-(4-^{13}C,4-^{2}H)IPP (red crosspeak). C) Three overlaid HSQC spectra for C9, analogous to B).

Conclusion

In summary, we have identified the main product of the microbial type sesquiterpene synthase RlMTPSL4 from *Radula lindenbergiana* as the new sesquiterpene hydrocarbon 4,5-*diepi*-isoishwarane. The cyclization mechanism was investigated through a series of isotopic labeling experiments. Firstly, the incubation of (12-^{13}C)FPP and (13-^{13}C)FPP revealed a defined stereochemical course, albeit with a minor distribution of labeling over both positions, for the deprotonation to germacrene A. This compound is likely a true intermediate, and not just a shunt product, as demonstrated by the incubation of (6-^{13}C)FPP in deuterium oxide buffer, leading to an uptake of deuterium from the incubation buffer. Also because of the long distance between C12 and C6 in the intermediate **A**, the alternative of a direct proton transfer would be difficult to understand, but a water-mediated proton transfer as described for the biosynthesis of myrothec-15(17)-en-7-ol cannot fully be excluded.[34] Isotopic labeling experiments also gave access to the absolute configuration of (4*R*,5*S*,7*S*,10*R*)-**1**. Herein, the 7*S* configuration is remarkable, because plant compounds formed via germacrene A usually show the opposite configuration at this stereogenic center,[11a] while the observed configuration in **1** is typical for bacterial and fungal compounds. Thus, this finding is in line with the formation of **1** by a microbial type terpene synthase.

Experimental Section

Incubation experiments with recombinant RlMTPSL4. The heterologous expression and protein purification of RlMTPSL4 in *Escherichia coli* was performed as reported previously.[17] Test incubations were carried out with GPP, FPP, GGPP and GFPP (0.5 mg each) dissolved in substrate buffer (100 μL; 25 mM NH_4HCO_3). After dilution with incubation buffer (0.5 mL; 50 mM Tris/HCl, 10 mM $MgCl_2$, 20% glycerol, pH = 8.2), an RlMTPSL4 protein preparation (0.4 mL) was added. The reaction mixtures were incubated at 30 °C with shaking overnight, followed by extraction with cyclohexane (150 μL). The organic layers were dried with $MgSO_4$ and analyzed by GC/MS.

For a preparative scale incubation, trisammonium FPP (80 mg, 185 μmol) was dissolved in substrate buffer (50 mL), followed by addition of incubation buffer (125 mL). The reaction was started by the addition of RlMTPSL4 protein preparation (25 mL) obtained from 8 L of expression culture and incubated at 30 °C with stirring overnight. The reaction mixture was then extracted with Et_2O (3 x 100 mL). The organic layers were dried with $MgSO_4$ and concentrated under reduced pressure. Column chromatography on silica gel with n-pentane yielded **1** (0.5 mg, 2.5 μmol, 1.3%) as a colorless oil.

4,5-*diepi*-Isoishwarane (1). TLC (n-pentane): R_f = 0.84. GC (HP-5MS): *I* = 1478. IR (diamond ATR): $\tilde{\nu}$ / cm^{-1} = 2955 (s), 2923 (s), 2856 (m), 1666 (w), 1460 (m), 1378 (w), 1260 (m), 1093 (m), 1018 (m), 802 (m). HR-MS (APCI): calc. for $[C_{15}H_{25}]^+$ *m/z* = 205.1951; found: *m/z* = 205.1947. Optical rotation: $[\alpha]_D^{25}$ = –22.0 (*c* 0.05, CH_2Cl_2). NMR data are given in Table S2 and the EI mass spectrum is shown in Figure S2.

GC/MS. GC/MS analyses were carried out using a 5977A GC/MSD system (Agilent, Santa Clara, USA) with a 7890B GC and a 5977A mass selective detector. The GC was fitted with a HP5-MS fused silica capillary column (30 m, 0.25 mm i. d., 0.50 μm film). GC settings were 1) inlet pressure: 77.1 kPa, He at 23.3 mL min^{-1}, 2) injection volume: 1 μL, 3) temperature program: 5 min at 50 °C increasing at 10 °C min^{-1} to 320 °C, 4) 60 s valve time, and 5) carrier gas: He at 1.2 mL min^{-1}. MS settings were 1) source: 230 °C, 2) transfer line: 250 °C, 3) quadrupole: 150 °C and 4) electron energy: 70 eV. Retention indices (*I*) were determined from retention times in comparison to the retention times of *n*-alkanes (C_7-C_{40}).

NMR spectroscopy. NMR spectra were recorded on a Bruker (Billerica, MA, USA) Avance III HD Cryo (700 MHz) NMR spectrometer. Spectra were measured in C_6D_6 and referenced against solvent signals (^{1}H-NMR, residual proton signal: δ = 7.16; ^{13}C-NMR: δ = 128.06).[35]

Incubation experiments with isotopically labeled substrates. Isotopic labeling experiments were performed with substrates (ca. 1.0 mg each, in 1 mL 25 mM aq. NH_4HCO_3), incubation buffer (5 mL) and enzyme preparations (2 mL each). The substrates and enzyme preparations are listed in Table S3. After incubation at 30 °C with shaking overnight, the products were extracted twice with C_6D_6 (500 μL and 200 μL). The extracts were dried with $MgSO_4$ and analyzed by NMR and GC-MS.

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Keywords: biosynthesis • enzymes • isotopes • liverwort • terpenes

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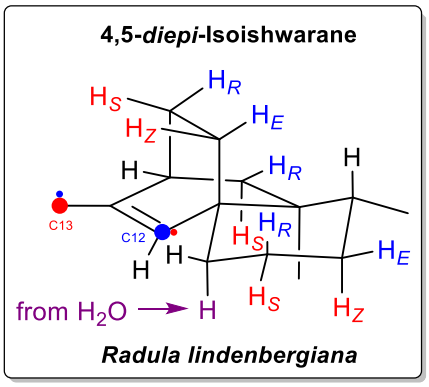

The microbial type and multiproduct sesquiterpene synthase RIMTPSL4 from the liverwort *Radula lindenbergiana* produces the new sesquiterpene hydrocarbon 4,5-*diepi*-isoishwarane as the main product, besides the known compounds germacrene A, α-selinene, eremophilene and 4,5-*diepi*-aristolochene. The enzyme mechanism for the formation of the main product and its absolute configuration were determined through isotopic labeling experiments.