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Mechanisms of the Diterpene Cyclases β-Pinacene Synthase from *Dictyostelium discoideum* and Hydropyrene Synthase from *Streptomyces clavuligerus*

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Abstract: Two diterpene cyclases, one from the social amoeba *Dictyostelium discoideum* and the other from the bacterium *Streptomyces clavuligerus*, with products containing a *Z*-configured double bond between the original C2 and C3 of geranylgeranyl diphosphate, were extensively investigated for their mechanisms through isotopic labelling experiments. The participation of geranyllinalyl diphosphate, in analogy to the role of linalyl and nerolidyl diphosphate for mono- and sesquiterpene biosynthesis, as an intermediate towards diterpenes with a *Z*-configured C2=C3 double bond is discussed.

During the past decades, soil bacteria have been recognised as producers of a large variety of terpenes.[1] Starting from the identification of the pentalenene synthase,[2] this work was extended by the characterisation of several bacterial terpene cyclases (TCs).[3] Recently, also social amoebae were shown to contribute to the terpene production in soil and first TCs from these organisms have been reported.[4] The type I of these remarkable enzymes contains an $(Mg^{2+})_3$ cluster for binding of the oligoprenyl diphosphate (OPP) substrate that is itself bound to the highly conserved aspartate-rich motif DDXX(X)(D,E), the NSE triad NDXXSXX(R,K)(E,D) and the RY dimer.[5] OPP binding causes closure of the active site under movement of a catalytically active arginine,[6] followed by substrate ionisation via diphosphate (PP) abstraction to initiate the cyclisation via cationic intermediates. The dynamics of the cyclisation may be followed by modelling the reactions in the active centre of a TC[7] or by isotopic labelling experiments.[8] While the reactions of monoterpene cyclases always proceed via isomerisation of geranyl PP (GPP) to linalyl PP (LPP), for sesquiterpene cyclases the corresponding isomerisation of farnesyl PP (FPP) to nerolidyl PP (NPP) is only strictly required for products with a *Z*-configured double bond between the original C2 and C3 of FPP.[9] Here, we present the elucidation of the cyclisation mechanisms of two diterpene cyclases, one new enzyme from the social amoeba *Dictyostelium discoideum* that was demonstrated to be a β-pinacene synthase in this study, and the hydropyrene synthase from *Streptomyces clavuligerus*,[10] by isotopic labelling strategies. Both enzymes form products with *Z*-configured double bonds.

One of the TCs from *D. discoideum*, DdTPS5, exhibits all highly conserved motifs of a TC (Figure S1 in the Supporting Information), and was recently shown to have diterpene synthase activity, but the chemical identity of the diterpene product was not known.[4a] For a detailed investigation, DdTPS5 was expressed in *Escherichia coli* with an N-terminal His-tag. The purified enzyme did not convert GPP and FPP, but yielded a diterpene hydrocarbon from GGPP (geranylgeranyl pyrophosphate, Figure S3), which was isolated and identified by one- and two-dimensional NMR spectroscopy (Table S3 and Figures S4–S10 in the Supporting Information) as β-pinacene (**1**), a monocyclic diterpene with 1(14)*E*,2*Z*,6*E*,10*E* configuration and a known constituent of the essential oils from *Pinus koraiensis* and *Pinus sibirica*.[11] These experiments established the enzyme as β-pinacene synthase (PcS). The reaction mechanism from GGPP to **1** requires the isomerisation of GGPP to geranyllinalyl PP (GLPP) that can cyclise to cation **A** with a 2*Z* double bond, followed by a 1,2-hydride shift to **B** and deprotonation to **1** (Scheme 1). An alternative from **A** to **1** is a 1,3-hydride migration of $H1_R$ or $H1_S$ and deprotonation from C14 (not shown). The isomerisation of GGPP may proceed via either (*S*)- or (*R*)-GLPP, and—in analogy to LPP (linalyl PP) and NPP (nerolidyl PP) cyclisations—through *syn*-allylic transposition of PP, with opposite consequences on the fate of $H1_R$ and $H1_S$ of GGPP. For the ring closure, an *anti*-S_N2' attack of C14 at C1 can be assumed, resulting in **A** with the same absolute orientation for $H1_R$ and $H1_S$ via both enantiomers of GLPP.

This mechanism can be tested by enzymatic conversion of (14-^{2}H)GGPP to validate the 1,2-hydride shift from **A** to **B**. However, the labelled monomer (2-^{2}H)dimethylallyl PP (DMAPP) is easier accessible by synthesis (Scheme S1 in the Supporting Information) and was used for the elongation to (14-^{2}H)GGPP

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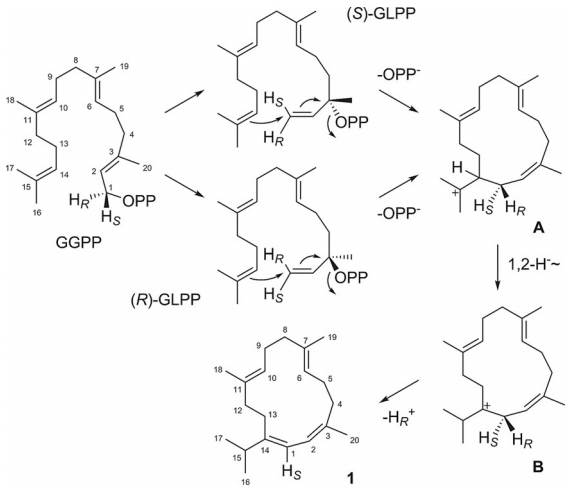
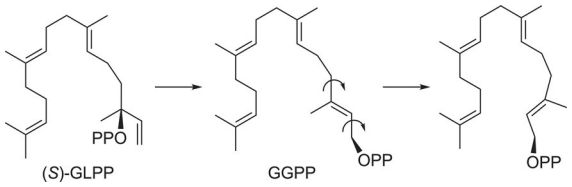


Scheme 1. Cyclisation mechanism from GGPP (geranylgeranyl pyrophosphate) to β-pinacene (**1**).

with isopentenyl PP (IPP) through the FPP synthase (FPPS) from *Streptomyces coelicolor*[4b] and the GGPP synthase (GGPPS) from *S. cyaneofuscatus* (note that the combination of FPPS and GGPPS is more effective than GGPPS alone).[12] The subsequent cyclisation by PcS yielded a product with retained deuterium that could be located in the *i*Pr group by inspection of the EI-MS (electron impact) fragmentation pattern (Figure S11), in agreement with the 1,2-hydride shift from **A** to **B**.[13] The conversion of both (*R*)- and (*S*)-(1–^{2}H)GGPP revealed the specific loss of $H1_R$ and retainment of $H1_S$ in the final deprotonation step (Figure S11). This result may point to (*R*)-GLPP as intermediate, because PP will leave towards the front side of the projection plane of Scheme 1 in the cyclisation to **A** and is thus perfectly located for the terminating abstraction of $H1_R$, similar to the observations made with spiroviolene synthase and tsukubadiene synthase, in which PP likely acts as base.[12] On the other hand, for tobacco *epi*-aristolochene synthase, a specific amino acid residue (Tyr520) and not the PP anion was suggested to participate in the deprotonation step,[7c] which challenges the argumentation above. The conversion of the pure GLPP enantiomers (Scheme S2 and Figure S12 in the Supporting Information) by PcS yielded small amounts of **1** in both cases (Figure S13; the lower efficiency than for the conversion of GGPP is at least in part due to rapid decomposition of GLPP, as revealed by controls without enzyme). This finding is explainable by uptake of both GLPP enantiomers into the active site. Coordination of the PP moiety of the wrong enantiomer to the $(Mg^{2+})_3$ cluster results in an exchanged positioning of the Me group C20 and the C1–C2 vinyl group, preventing the 1,14-cyclisation, but the docked non-native GLPP enantiomer may react to GGPP by allylic transposition of PP, followed by a conformational change that opens the way to a cyclisation via the native GLPP stereoisomer (Scheme 2).

The conversion of (16-^{13}C)- and (17-^{13}C)GGPP with PcS, obtained from (12-^{13}C)- and (13-^{13}C)FPP and IPP through GGPPS, yielded **1** with a shared labelling in both Me groups of the *i*Pr moiety (Figure S14). The stereochemical course for the terminal geminal Me groups of OPP substrates is tightly controlled for



Scheme 2. Conversion of the non-native GLPP (geranyllinalyl PP) enantiomer (hypothetically (*S*)-GLPP) by PcS (β-pinacene synthase). Analogous reactions are possible from (*R*)-GLPP.

most investigated TCs.[4b,12,14] One of the few enzymes causing a similar distribution of labelling is (1*R*,4*R*,5*S*)-guaia-6,10(14)-diene synthase (GdS) from *Fusarium fujikuroi*.[15] Notably, the cyclisation reactions of GdS and PcS both proceed with a 1,2-hydride shift into an *i*Pr group, while for systems with 1,3-hydride shifts such as the TCs for T-muurolol, 4-*epi*-cubebol and γ-cadinene always a strict stereochemical course is found.[14e] These findings reflect the different topological constraints of the two possible reactions: For the 1,2-hydride shift, the equal distances of the moving hydride to the two planes of the cation cause a scrambling of labelling in the *i*Pr group, while the well-defined target plane for the 1,3-hydride migration does not (Figure 1). In contrast to the observations made here and with GdS, a conformational pre-organisation could enforce a defined stereochemical course for the 1,2-hydride shifts in other systems.

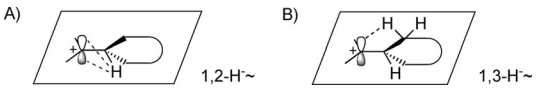
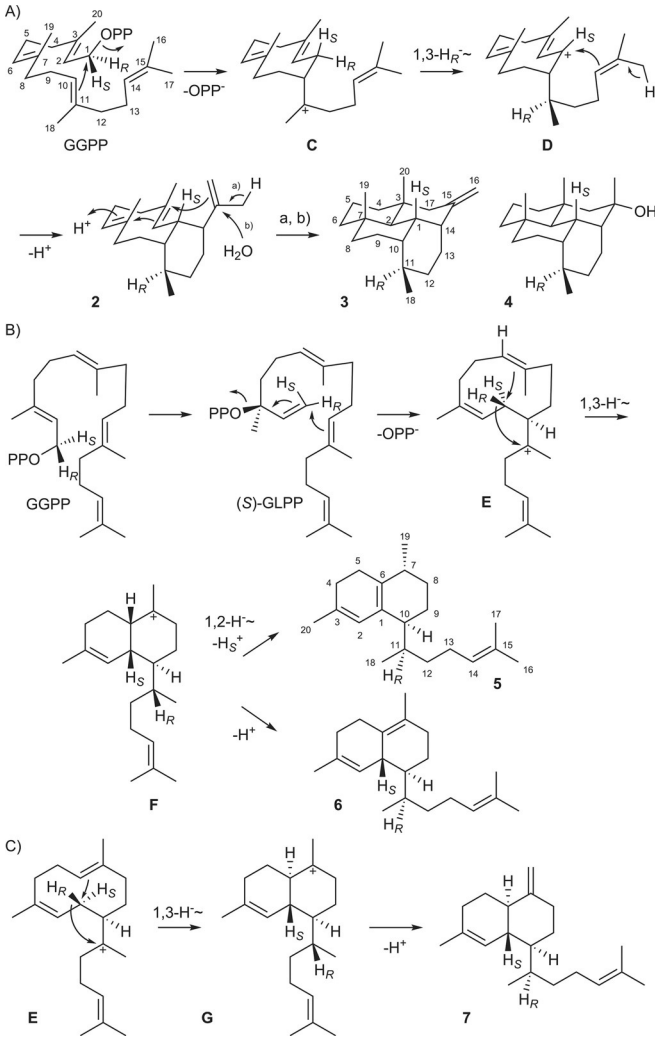


Figure 1. Topological constraints for A) 1,2- and B) 1,3-hydride transfers into *i*Pr groups during terpene cyclisations.

For mechanistic investigations on the hydropyrene synthase (HpS, Figure S1) from *S. clavuligerus*, the corresponding gene was cloned into the *E. coli* expression vector pYE-Express.[16] The purified protein gave no products from GPP and FPP, but produced a mixture of diterpenes, including hydropyrene (**3**), hydropyrenol (**4**), isoelisabethatriene (**5**) and traces of isoelisabethatriene B (**6**) from GGPP (Figure S15), in agreement with the results from the heterologous expression in *Streptomyces avermitilis*.[10] In addition to the identification through comparison of recorded to published mass spectra,[10] the known products **3**–**5** of HpS were re-isolated and unambiguously characterised by NMR (Tables S4–S6), only the production of **6** was too low for its isolation. A further compound that was so far not reported from HpS was tentatively identified by GC-MS as biflora-4,10(19),15-triene (**7**), based on an identical mass spectrum to a library spectrum and on a retention index that matched published data (Figure S15). A possible cyclisation mechanism to **3** and **4** starts with 1,10-cyclisation of GGPP to **C**, followed by a 1,3-hydride shift to **D** and a second ring closure with deprotonation to **2** (Scheme 3). Reprotonation at C6 induces two more ring closures to set the unique carbon skeleton of **3** and **4** that arise by final deprotonation or attack of



Scheme 3. Cyclisation mechanism of HpS (hydropyrene synthase). A) Cyclisation of GGPP via **2** to **3** and **4**, B) cyclisation of GGPP to **5** and **6** and C) cyclisation of GGPP to **7**.

water. The formation of **5** and **6** with a *Z* double bond in their bicyclic system requires GGPP isomerisation to GLPP, a 1,10-cyclisation to **E** and a 1,3-hydride migration and second ring closure to **F**. Another 1,2-hydride shift and deprotonation results in **5**, while **6** requires simple deprotonation. The reactions to **7** start from **E** in a different conformation and proceed via a 1,3-hydride shift and cyclisation to **G** and deprotonation.

These mechanisms were tested by conversion of all 20 isotopomers of ($^{13}C_1$)GGPP, prepared with GGPPS from the 15 isotopomers of ($^{13}C_1$)FPP and IPP, from FPP and (1-^{13}C)IPP, (3-^{13}C)IPP and (4-^{13}C)IPP, and by direct synthesis of (2-^{13}C)GGPP and (20-^{13}C)GGPP.[12,17] All 20 GGPP isotopomers were converted by HpS in small scale reactions (1 mg), followed by extraction with C_6D_6 and direct ^{13}C-NMR analysis without product purification. In each sample, strong ^{13}C-NMR signals for the labelled carbons of **3**, **4** and **5** were observed, with the labelling in the expected positions and without a distribution of labelling from C16 and C17 (Figures S16–S18). In all 20 experiments, major signals appeared that could not be assigned to a known product, but were in line with intermediate **2** (Figure S19). The isolation of this compound turned out to be difficult, because of its instability under the conditions of column chromatography, but swift work yielded an almost pure sample of sufficient quality for unambiguous structure elucidation by NMR (Table S7 and Figures S20–S26). The neutral biosynthesis intermediate **2** was named prehydropyrene.

The mechanism of HpS was further tested using doubly labelled probes. The 1,3-hydride shift from **C** to **D** and from **E** to **F** was addressed by conversion of (*S*)- and (*R*)-(1-^{13}C,1-^{2}H)GGPP, synthesised with high enantiomeric purity (Scheme S3 and Figure S27), and product analysis by ^{13}C-NMR, which yielded enhanced triplets for C1 of **2**, **3** and **4** from (*S*)-(1-^{13}C,1-^{2}H)GGPP, but a singlet for **5** (Figure S28 A). These data indicated the stereospecific migration of H1$_R$ from C1 and are in line with the loss of H1$_S$ in the final step to **5**, as confirmed by GC-MS (Figure S29). Using the *R* enantiomer, a singlet was observed for all four compounds, again consistent with migration of H1$_R$ (Figure S28 B). The final destination of this hydrogen at C11 was proven using (7-^{13}C)FPP and (2,2-$^{2}H_2$)IPP, synthesised as shown in Scheme S4, with an enzyme mix of GGPPS and HpS, which produced triplets for C11 of **3** and **4** and pointed to attachment of deuterium to this carbon (Figure S28 C). Notably, the stereochemical course of the hydride movement is the same in both cases, regardless of the double bond configuration at C2 for both **C** and **E**. The reprotonation of **2** towards **3** and **4** was investigated by incubation of (2-^{13}C)FPP and IPP with GGPPS and HpS in a deuterium oxide buffer, resulting in upfield shifted triplets for C6 in the ^{13}C-NMR, besides singlets for the non-deuterated compounds due to residual water in the enzyme preparation (Figure S30). HSQC analysis of the products allowed to follow the stereochemical course of the reprotonation with specific incorporation of deuterium into H6$_\beta$ (Figure S31).

The absolute configuration of a terpene can be deduced from enantioselectively deuterated precursors by incorporation of labelling into one of two diastereotopic positions, if the stereocentre at the deuterated carbon remains unchanged (or is changed with a known course) during terpene biosynthesis, because the configurations of all other stereogenic centres in the product can be inferred from their relative orientation to this stereogenic centre.[4b,12,13,18] Using this approach, the absolute configurations of **3**, **4** and **5** were delineated from incubations of (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)FPP with IPP, GGPPS and HpS (Figure S32–S34), which resulted in the absolute configurations for **2**, **3**, **4** and **5** as in Scheme 3. Consistent findings were obtained using both enantiomers of (1-^{13}C,1-^{2}H)GPP (Figure S35–S37). These experiments were built on the known inversion of configuration at C1 in the OPP elongations with IPP[19] and confirmed the tentatively assigned absolute configuration for **3** from crystallographic data of its epoxide.[10b] The HpS product (−)-**5** ($[\alpha]_D^{20} = -5.5^\circ$, *c* 0.1, C_6D_6) is likely the enantiomer of isoelisabethatriene isolated from the coral *Pseudopterogorgia elisabethae*.[20] While no optical rotary power was reported, its absolute configuration can be tentatively assigned based on the fact that **5** is biosynthetically linked to the pseudopterosins that were isolated from the same organism and for which the absolute configurations are well established.[21]

A model for the stereochemical course of the reactions towards **5** and the installation of its absolute configuration involves an *anti*-S_N2' reaction in the 1,10-cyclisation of (*S*)-GLPP to **E** that establishes a *syn* orientation of $H1_R$ and C11, as required for the next 1,3-hydride shift to **F** and the correct absolute configuration at C10 (a hypothetical pathway via (*R*)-GLPP would produce **E** with *syn*-oriented $H1_S$ and C11). The second ring closure to **F** likely proceeds with inversion at C1, which defines the absolute configuration at this carbon. The *cis*-decalin system of **F** can be inferred from the configuration at C7 of **5**, because the 1,2-hydride shift from **F** to **5** must be suprafacial. Conclusively, the configurations of all stereocentres in **F** can be deduced either from those in the product **5** or from the observed $H1_R$ shift from **E** to **F**. This information can finally be used to deduce the tentative structure of **6**, a compound that is produced in too low amounts for isolation and structure elucidation, because it likely arises directly from **F**. In this context it is interesting to note that both deprotonations from **F** to **5** and **6** happen from the same hemisphere above the projection plane of Scheme 3, which is identical to the hemisphere into which the PP anion is expelled from (*S*)-GLPP, consistent with PP acting as the terminating base. The cyclisation of **E** to the *trans*-decalin system of **G** results in the deprotonation of the Me group C19, which also points into this hemisphere, to yield **7**. However, similar to the observations made with PcS, both (*S*)- and (*R*)-GLPP were converted by HpS, albeit with lower efficiency as for GGPP, but clearly yielding **3**, **4** and **5** (Figure S38). The same mechanism as discussed above for PcS can explain the product formation from both GLPP enantiomers (Scheme 2).

In summary, we have investigated the mechanistic details of each single elementary reaction along the cyclisation cascades of two diterpene cyclases by extensive labelling experiments. PcS from *D. discoideum*, previously shown to have diterpene cyclase activity,[4a] is a newly characterised enzyme producing β-pinacene. For several products of HpS from *S. clavuligerus*, the absolute configurations were elucidated and a mechanistically predicted neutral intermediate was discovered by ^{13}C-labelling experiments that was previously overlooked, likely because of its instability. Notably, both enzymes PcS and HpS form products with a *Z*-configured double bond that are explainable by GGPP isomerisation to GLPP. Both of its enantiomers were accepted by PcS and HpS, albeit with lower efficiency than for GGPP, which reflects the adaption of the enzymes to their natural substrates. It is interesting to see that diterpene cyclases use the same strategy of substrate isomerisation by allylic PP transposition for the installation of *Z* double bonds as is well established for mono- and sesquiterpene cyclases.

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Conflict of interest

The authors declare no conflict of interest.

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