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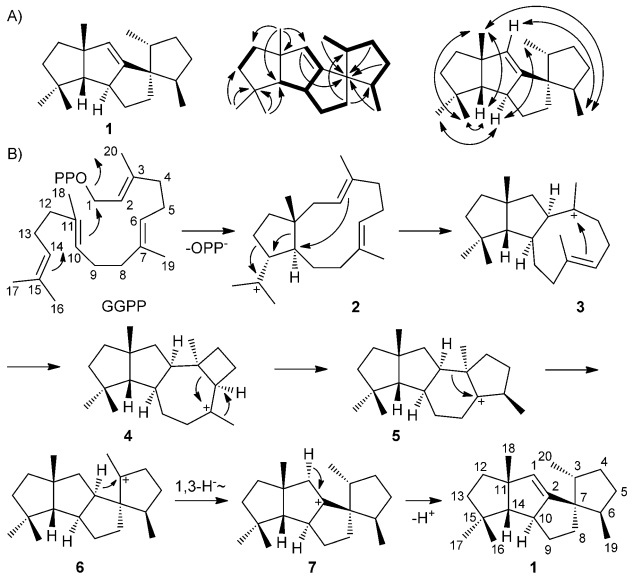
Mechanistic Investigations of Two Bacterial Diterpene Cyclases: Spiroviolene Synthase and Tsukubadiene Synthase

*Patrick Rabe, Jan Rinkel, Etilia Dolja, Thomas Schmitz, Britta Nubbemeyer, T. Hoang Luu, and Jeroen S. Dickschat**

Abstract: *The mechanisms of two diterpene cyclases from streptomycetes—one with an unknown product that was identified as the spirocyclic hydrocarbon spiroviolene and one with the known product tsukubadiene—were investigated in detail by isotope labeling experiments. Although the structures of the products were very different, the cyclization mechanisms of both enzymes proceed through the same initial cyclization reactions, before they diverge towards the individual products, which is reflected in the close phylogenetic relationship of the enzymes.*

The pioneering work by Otto Wallach in the 19th Century culminated in the identification of numerous terpenes from plants.[1] Much later it became evident that fungi,[2] bacteria,[3] and even eukaryotic microorganisms such as social amoebae[4] also produce a large variety of terpenes. The impressive conversions of linear precursors (oligoprenyl diphosphates, OPPs) into structurally complex, usually polycyclic, and chiral terpenes with several stereocenters proceed via cationic intermediates and are catalyzed by terpene cyclases (TCs). Many enzymes from bacteria have been investigated during the last two decades,[5] but the number of fully characterized diterpene cyclases (DTCs) is still limited.[6] Herein we report on the remarkable mechanisms of two bacterial DTCs that were uncovered by isotope labeling experiments.[7]

A gene for a TC homologue from *Streptomyces violens* NRRL ISP-5597, which exhibits all the highly conserved motifs of type I enzymes including the aspartate-rich motif (^{85}DDHRCD), the NSE triad (^{226}NDRHSLRKE),[8] the pyrophosphate sensor (Arg180),[9] and the ^{312}RY dimer (Figure S1), was cloned into the expression vector pYE-Express.[10] The recombinant purified protein (Figure S2) converted geranylgeranyl diphosphate (GGPP) into a diterpene hydrocarbon (Figure S3), while geranyl (GPP) and farnesyl diphosphate (FPP) were not accepted. The structure of the diterpene was elucidated by NMR spectroscopy (Table S3, Figures S4–S10), with three contiguous spin systems identified in the ^{1}H,^{1}H-COSY spectrum. Their interconnection was delineated from key HMBC correlations, while the relative configuration was established by NOESY correlations (Scheme 1 A), thereby resulting in the structure of (−)-spiroviolene (**1**) ($[\alpha]_D^{21} = -5.6$ ($c = 0.2$, C_6D_6)).



Scheme 1. A) Structure of spiroviolene (**1**), continuous spin systems in ^{1}H,^{1}H-COSY (bold), key HMBC and NOESY correlations (single- and double-headed arrows, respectively). B) Cyclization of GGPP to form **1**. The numbering of the carbon atoms of **1** indicates the origin of each carbon atom from GGPP.

The proposed biosynthesis of **1** by the spiroviolene synthase (SvS) starts with the cyclization of GGPP to form the bicyclic cation **2**, followed by a double Wagner–Meerwein rearrangement (WMR) and cyclization to form **3**. If the transformation of GGPP to **2** and of **2** to **3** are formulated as concerted processes, with the electron density migrating along the C14−C15 and C10−C14 bonds, secondary cation intermediates can be avoided and retention of configuration at C10 and C14 can be assumed. A third cyclization to generate **4** and dyotropic rearrangement[11] yield **5**, which can undergo a ring contraction to produce the spirocyclic cation **6**. A 1,3-hydride migration to form **7** followed by deprotonation result in **1**. This hypothetical pathway was tested by conversion of all 20 isotopomers of ($^{13}C_1$)GGPP with SvS. Although a route for their chemical synthesis was established,[12] an easier approach to the labeled probes is by enzymatic reactions of the 15 isotopomers of ($^{13}C_1$)FPP that we have previously synthesized[13] and isopentenyl diphosphate (IPP), or of FPP and synthetic (1-^{13}C)IPP, (3-^{13}C)IPP, or (4-^{13}C)IPP

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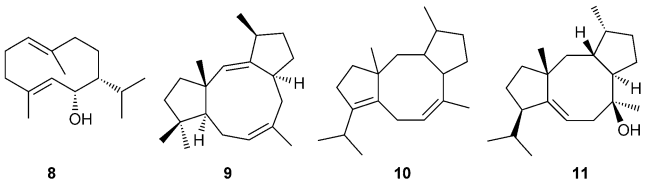
(Scheme S1). The elongations were performed using the GGPP synthase (GGPPS) from *Streptomyces cyaneofuscatus*. Only (2-^{13}C)GGPP and (20-^{13}C)GGPP were directly synthesized (Scheme S2). Small-scale reactions (1 mg) of the 20 isotopomers of ($^{13}C_1$)GGPP with SvS followed by extraction with C_6D_6 and direct ^{13}C NMR analysis without product purification showed the ^{13}C label in the expected positions in all cases (Figure S11). For the geminal methyl groups C16 and C17 of GGPP SvS showed a strictly controlled stereochemical course (Figures S11 R + S), as observed for most terpene cyclases.[5d,e,14] One of the few exceptions is (−)-guaia-6,10-(14)-diene synthase from *Fusarium fujikuroi*, which reveals a distribution of the label between C12 and C13 of FPP.[15]

Stereoselectively deuterated OPPs can be used for the determination of the absolute configurations of terpenes and of the intermediate α-terpinyl cation en route to the achiral monoterpene 1,8-cineol.[5f,13d,16,17] The enzymatic conversion of these probes results in products with the deuterium label in diastereotopic positions, which can be distinguished by NMR spectroscopy. To increase the sensitivity of the probes in HSQC spectroscopy both enantiomers of (1-^{13}C,1-^{2}H)GPP and (1-^{13}C,1-^{2}H)FPP were synthesized with an additional ^{13}C label (Scheme S3) and with high enantiomeric purity (Figure S12). Incubation of SvS and GGPPS with (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)FPP and IPP followed by product analysis by HSQC and comparison to the unlabeled compound allowed assignment of one of the two stereochemically different hydrogen atoms at the ^{13}C-labeled carbon atom to the site of deuterium incorporation and the other one to the site of protium incorporation, with a known absolute configuration of the stereocenter at this carbon atom (Figure S13). The absolute configuration of the other stereocenters in **1** was deduced by assignment of their relative configuration with respect to the stereocenter at the labeled carbon atom, thus resulting in the structure of (3*R*,6*R*,10*S*,11*R*,14*R*)-**1**. Consistent findings were obtained using the same strategy for the conversion of (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)GPP (Figure S14). These experiments make use of the known inversion of configuration in chain elongations of OPPs with IPP by OPP synthases.[18]

The 1,3-hydride shift from **6** to **7** was investigated by conversion of (3-^{13}C,2-^{2}H)GGPP, which was prepared by using a reported method for deuterium introduction at C2 (Scheme S4).[19] With this probe, the hydride migration resulted in a direct bond between deuterium and the ^{13}C-labeled carbon atom in **1**, as indicated by a triplet in the ^{13}C NMR spectrum of the obtained product as a result of ^{13}C-^{2}H spin coupling (Figure S15). The stereochemical course of the final deprotonation step was addressed using (*R*)- and (*S*)-(1-^{2}H)GGPP (Scheme S3). GC/MS analysis of the enzyme products revealed the specific loss of deuterium from (*S*)-(1-^{2}H)GGPP but not from the *R* enantiomer (Figure S16). These experiments demonstrate that the proton *syn* to the migrating hydride in the reaction from **6** to **7** is abstracted, if inversion of configuration at C1 is assumed for the intitial 1,11-cyclization, as described for several terpene cyclases.[13b,20] A candidate base for the deprotonation of **7** is the diphosphate anion that leaves C1 of GGPP towards the backside of the projection plane (Scheme 1) and is thus perfectly oriented to take up the proton in the final step.

A BLAST search using the SvS sequence as a probe was performed to identify closely related enzymes in other bacteria. A phylogenetic tree constructed from SvS and the 65 closest hits is shown in Figure S17. Organisms that were available from the DSMZ strain collection and that encode the most closely related enzymes to the SvS from *S. violens* were investigated for their production of **1**, which could be shown for the streptomycetes *S. ochraceiscleroticus*, *S. sclerotialus*, and *S. ambofaciens* as well as the actinomycete *Allokutzneria albata* (Figure S18). Closely related enzymes include the cyclases for (+)-(1(10)*E*,4*E*,6*S*,7*R*)-germacradien-6-ol (**8**, Scheme 2),[13] tsukubadiene (**9**), and cyclooctat-



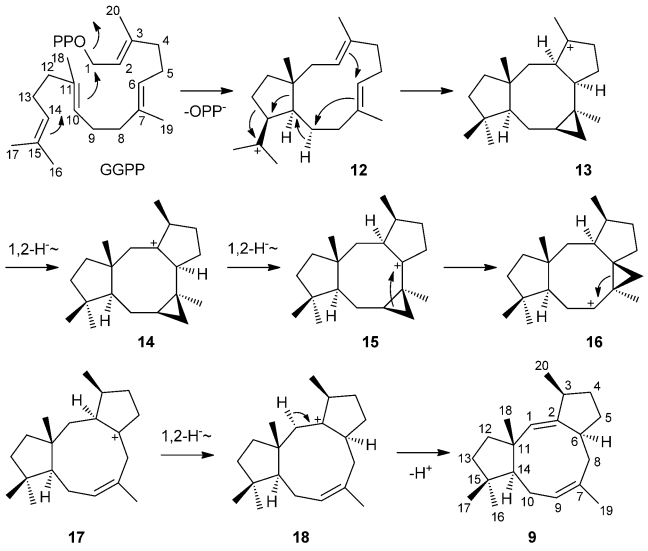
8 9 10 11

Scheme 2. Terpenes produced by enzymes with a close relationship to SvS (**8**–**10**) and structure of cyclooctat-9-en-7-ol (**11**).

7(8),10(14)-diene (**10**).[6b] Although their enzymes are phylogenetically distant, the structural similarity of the products **10** and cyclooctat-9-en-7-ol (**11**) suggests that the cyclization mechanism for **10** may be similar to the mechanism for **11**, which proceeds through a surprising rearrangement of a cyclopropane intermediate.[6a] This prompted us to investigate whether **9** is also formed by a related mechanism.

For this purpose, the gene for the tsukubadiene synthase (TdS) from *Streptomyces tsukubaensis* NRRL 18488 was cloned and expressed in *E. coli* (Figure S2). Consistent with the reported findings by heterologous expression in *Streptomyces avermitilis*,[6b] the purified protein converted GGPP into a single product (Figure S19), while GPP and FPP were not accepted. Since only the planar structure of **9** had been reported, the diterpene product was isolated and its structure fully elucidated by extensive NMR spectroscopy (Table S4, Figures S20–S26). The relative configuration determined by NOESY experiments, as shown in Scheme 2, was in line with the suggested structure based on a comparison of the recorded and calculated NMR data for all stereoisomers of **9**.[21]

The proposed cyclization mechanism of TdS starts with the cyclization of GGPP to **12** (Scheme 3). The further reactions, including a ring expansion and ring contraction by WMR, a 1,2-hydride migration, as well as a 7,9- and a 2,6-cyclization to form **13**, may be a concerted process with retention of configuration at C14 to avoid secondary cation intermediates. Two subsequent 1,2-hydride migrations via **14** to generate **15** may be followed by a rearrangement of the cyclopropane ring, similar to a key step in the biosynthesis of **11**.[6a] A ring opening to produce **17**, 1,2-hydride migration to form **18**, and deprotonation yield **9**. This suggested mechanism was tested by conversion of all 20 isotopomers of



Scheme 3. Cyclization of GGPP to generate **9**. The numbering of the carbon atoms of **9** indicates the origin for each carbon atom from GGPP.

($^{13}C_1$)GGPP with TdS, which resulted in the appearance of label in each of the expected positions and, thus, confirmed the model (Figures S27 and S28).

The absolute configuration of **9** was determined by the same strategy as described above for **1**. The conversion of both enantiomers of (1-^{13}C,1-^{2}H)FPP yielded products with specific incorporation of deuterium into one of two stereochemically distinct hydrogen atoms, as determined by HSQC (Figure S29). The experiments with both labeled probes consistently established the absolute configuration of (3*S*,6*S*,11*R*,14*S*)-**9**.

The stereochemical course of the 1,2-hydride shift from C9 to C10 in the reaction from **12** to **13** was investigated by enzymatic conversion of (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)GPP and IPP with GGPPS and TdS. The ^{13}C NMR spectrum of the product obtained from (*R*)-(1-^{13}C,1-^{2}H)GPP showed a strong triplet for C9, while the product formed from the *S* enantiomer exhibited an upfield-shifted singlet that indicated the presence of a deuterium at a neighboring carbon atom (Figure S30). ^{1}H NMR analysis of the product obtained from (*S*)-(1-^{2}H)GPP[17] revealed simplified multiplicities for H9 and H14, and demonstrated the specific incorporation of deuterium into H10$_\beta$, which was confirmed by NOESY experiments (Figures S30 and S31). Overall, these data established the stereospecific migration of H9$_\alpha$ with inversion of configuration at C10 in the step from **12** to **13**.

The two sequential 1,2-hydride shifts from intermediate **13** to **14** and from **14** to **15** were addressed with deuterated and ^{13}C-labeled substrates. The possibility of a single 1,3-hydride migration with direct formation of **15** from **13** was also taken into consideration. ^{13}C NMR analysis of the product obtained from (3-^{13}C)IPP and (2-^{2}H)FPP (Schemes S1 and S4) with GGPPS and TdS showed a singlet for the C3 atom of **9**, which is not consistent with a possible 1,3-hydride transfer (Figure S32 B). Comparison of the chemical shift for C3 to the ^{13}C NMR data of unlabeled **9** (Figure S32 A) indicated a slight upfield shift ($\delta\Delta = 0.04$ ppm), in agreement with deuterium being attached to a neighboring carbon atom (C6). In contrast, incubation of (3-^{13}C,2-^{2}H)GGPP (Scheme S4) with TdS gave a product showing a strong triplet in the ^{13}C NMR spectrum (Figure S32 C, $^{1}J_{C,D} = 19.6$ Hz, $\delta\Delta = 0.44$ ppm). These data established the direct ^{13}C–^{2}H connection, which is in agreement with two sequential 1,2-hydride shifts. Similar findings were reported for the cyclization mechanisms of cyclooctat-9-en-7-ol synthase[22] and corvol ether synthase.[23] The tentatively assigned relative orientation of the cyclopropane ring in the intermediates **13**–**16** cannot be deduced with certainty from the structure of the final product **9**, but the orientation as shown in Scheme 3 requires an inversion of configuration at C6 in the conversion of **14** into **16**, which seems more likely than retention of configuration.

As demonstrated by enzymatic cyclization of stereospecifically deuterated (*R*)- and (*S*)-(1-^{2}H)GGPP, the final deprotonation step towards **9** proceeds with loss of deuterium from the *S* enantiomer, while the deuterium from the *R* enantiomer is retained (Figure S33). Assuming inversion of configuration in the initial 1,11-cyclization,[14b,20] these experiments reveal a deprotonation from C1 *syn* to the migrating hydride in the reaction from **17** to **18**. The diphosphate anion leaves C1 towards the backside of the projection plane (Scheme 3) and is perfectly located to act as the base in the terminal deprotonation to **9**.

Isotope labeling experiments continue to be an important method to obtain mechanistic insights into the remarkable reactions of terpene cyclases.[5b,6a,13,14d,15,24] We have shown here that not only many details of the cyclization mechanism, including stereochemical aspects, but also the absolute configurations of terpenes can be elucidated using stereospecifically deuterated precursors. The knowledge of the absolute configurations of **1** and **9** allows for a comparison of the cyclization mechanisms of SvS and TdS. The first elementary reaction for both enzymes proceeds by 1,11*Si*-cyclization of GGPP. In this step, the same absolute configuration at C11 is installed and is not changed during the further process. Simultanously, SvS catalyzes a 10*Re*,14*Si*-cyclization, while for TdS a 10*Re*,14*Re* process is observed, which requires a different relative orientation of C10 and C15 of the folded GGPP in the active site. The methyl group C16 and H14, which are *cis*-oriented in GGPP, end up in both cases on the same faces of **1** and **9**, thus showing the strict stereochemical course in the 10,14-cyclization by both enzymes. The initially formed bicyclic cations **2** and **12** are diastereomers, and the downstream reactions to the individual products deviate to yield the structurally very different products **1** and **9**. However, the final deprotonation step proceeds in both cases from the side to which the diphosphate is expelled in the ionization step, thereby suggesting that diphosphate may act as the base in the terminal step. It is interesting to note that SvS and TdS are closely related, as shown by a phylogenetic analysis of more than 1000 bacterial terpene cyclases (Figure S34), which suggests that a few critical substitutions in the amino acid sequence of a terpene cyclase can cause a minor change of the substrate's conformational fold in the active site, but with a dramatic effect on the structure of the product.

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Keywords: biosynthesis · isotopes · NMR spectroscopy · reaction mechanisms · terpenes

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Communications

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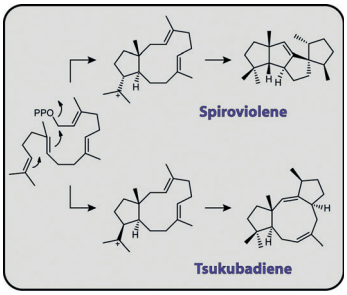
P. Rabe, J. Rinkel, E. Dolja, T. Schmitz, B. Nubbemeyer, T. H. Luu, J. S. Dickschat* ——— ■■■–■■■

Mechanistic Investigations of Two Bacterial Diterpene Cyclases: Spiroviolene Synthase and Tsukubadiene Synthase



A close relationship: The products of two phylogenetically related diterpene cyclases from streptomycetes were identified. The mechanisms of the enzymes were investigated by extensive labeling experiments. Although the structures of the diterpenes are very different, some steps of the cyclization cascade are very similar, thus suggesting that only minor conformational changes of the substrate are responsible for the formation of the unique products.