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COMMUNICATION

A Branched Diterpene Cascade: The Mechanism of Spinodiene Synthase from *Saccharopolyspora spinosa*

Jan Rinkel, Lukas Lauterbach, and Jeroen S. Dickschat*

Abstract: A diterpene synthase from *Saccharopolyspora spinosa* was found to convert geranylgeranyl diphosphate into the new natural products spinodiene A and B, accompanied by 2,7,18-dolabellatriene. The structures and the formation mechanism of the enzyme products were investigated by extensive isotopic labelling experiments, revealing an unusual branched isomerisation mechanism towards the neutral intermediate 2,7,18-dolabellatriene. A Diels-Alder approach was used to convert the main diterpene product with its rare conjugated diene moiety into formal sesterterpene alcohols.

Among nature's diverse ways to construct small molecules, arguably terpene biosynthesis provides the largest structural variety of natural products that are all made through astonishingly simple enzymatic concepts. Thus, the most important enzymes from this pathway, terpene synthases (TSs), can be seen as evolution's combinatorial playground in generating small carbon skeletons for bioactive beneficial compounds. Type-I TSs convert a small variety of linear oligoprenyl diphosphates (OPPs) including geranyl- (GPP, C_{10}), farnesyl- (FPP, C_{15}), geranylgeranyl- (GGPP, C_{20}) and geranylfarnesyl diphosphate (GFPP, C_{25}) into mostly polycyclic hydrocarbons or alcohols with multiple stereocentres.[1] These enzymes act on the substrate by diphosphate abstraction and provide a specific active site architecture for cation stabilisation, conformational discrimination and selective termination of the reaction cascade.[2] The reaction mechanisms feature all kinds of cation chemistry such as hydride migrations, proton shifts and Wagner-Meerwein rearrangements to yield structurally complex products in only one enzymatic step. Since a time resolved experimental investigation of these cascades within the enzyme is not possible, structural studies,[1b] site-directed mutagenesis,[3] quantum chemical calculations[4] and isotopic labelling studies[5] led to a deeper understanding of the synthetic capabilities of TSs. Especially labelling experiments in combination with in vitro techniques and modern NMR spectroscopy proved to be an indispensable tool for experimental testing of proposed TS mechanisms. While the incorporation of isotopes usually results in clearly substituted product atoms and can be explained via one straight forwardly operating linear cascade, in the present study we addressed a challenging bacterial TS deviating from this rule.

The soil actinomycete *Saccharopolyspora spinosa* NRRL 18395 is known for its production of spinosyns, a class of polyketide natural products that are used as potent insecticides.[6] Its genome harbours a TS, whose sequence features previously identified conserved motifs of TSs for diphosphate binding[7] and activation[8] together with structurally important residues (Figure S1).[5g,9] This TS was characterised as a hedycaryol synthase in our previous work, but the reaction with FPP proved to be sluggish and diterpene synthase (DTS) activity was not tested.[10] Based on a phylogenetic analysis the closest characterised relative was found to be CotB2 for the diterpene cyclooctat-9-en-7-ol (Figure S2).[11] A reinvestigation of the purified recombinant enzyme (Figure S3) now demonstrated that GGPP is efficiently converted into a mixture of diterpenes (Figures 1 and S4). The compounds **1**, **2** and **3** were isolated and their structures were assigned based on one- and two-dimensional NMR experiments.

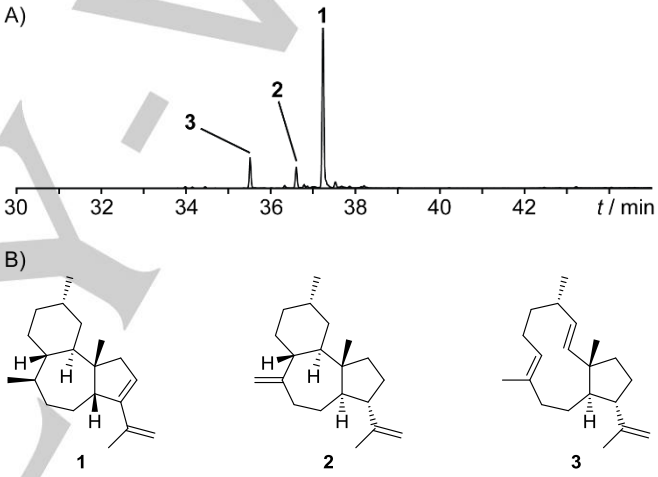

Figure 1. A) Total ion chromatogram of the products obtained from GGPP with SoS. B) Structures of the SoS main products spinodiene A (**1**), spinodiene B (**2**) and 2,7,18-dolabellatriene (**3**).

The obtained NMR data for the main product **1** (Table S1, Figures S5 – S12) suggested the structure of a diterpene with a 6-7-5 ring system featuring a rare conjugated diene. NOE correlations fixed the relative configuration with an unusual *cis* stereochemistry at the fused 5-membered ring. Based on ring sizes some similarity of **1** to the dolastane diterpenes[12] can be found, but the ring arrangement is different and **1** represents a new diterpene skeleton. With respect to the origin of its producing enzyme, **1** was named spinodiene A and the TS from *S. spinosa* is characterised as a spinodiene synthase (SoS). Compound **2**, spinodiene B, (Table S2, Figures S13 – S20) has a similar structure with the same skeleton as **1**, but a different stereochemistry at the 5-membered ring and an exocyclic double bond. The third product **3** (Table S3, Figures S21 – S27) was characterised as (+)-2,7,18-dolabellatriene with an optical rotation of $[\alpha]_D^{20}$ = +23.0 (*c* 0.32, $CHCl_3$), pointing to the enantiomer of *ent*-**3** ($[\alpha]_D^{20}$ = –21, *c* 0.03, $CHCl_3$) that is known from the brown alga *Dilophus spiralis*.[13]

The biosynthesis of the three SoS products was investigated using all twenty ($^{13}C_1$)GGPP isotopomers that were obtained by synthesis[14] or enzymatically prepared from other synthetic ^{13}C

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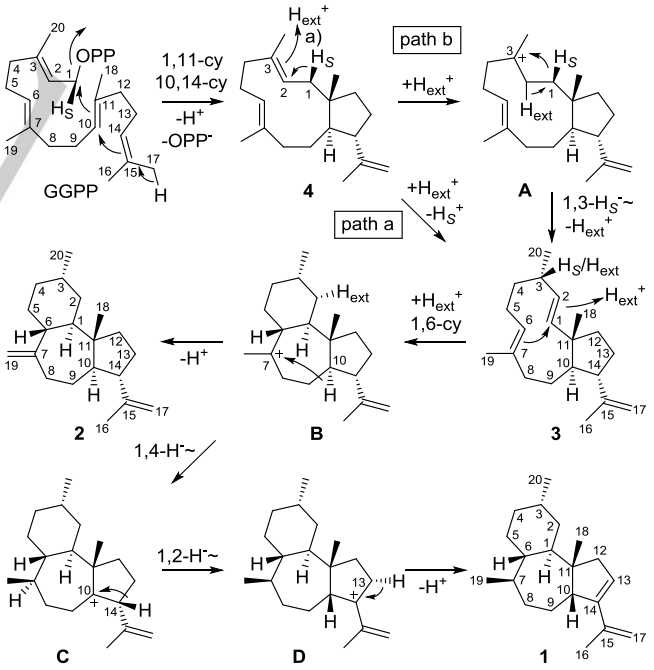
labelled precursors GPP,[12c,15] FPP[16] and isopentenyl diphosphate (IPP)[14] using a GGPP synthase (GGPPS) from *Streptomyces cyaneofuscatus*,[14] and converted by SoS to give a product mixture of singly ^{13}C labelled diterpenes **1**-**3**. ^{13}C-NMR analysis of these samples allowed for an unambiguous assignment of all carbon atoms in these products to their originating positions in GGPP (Figures S28 – S30). The absolute configurations of **1**-**3** were determined by conversion of enantioselectively deuterated and ^{13}C labelled probes. This method uses sensitive HSQC experiments to delineate the incorporation of deuterium into diastereotopic positions in the products and thus simplifies the problem of solving the absolute configuration to the determination of the relative orientation of all stereocentres with respect to the known absolute configuration of the labelled methylene groups in the target molecule. For this purpose (1*R*)- and (1*S*)-(1-^{13}C,1-^{2}H)GPP[14] were converted into (9*R*)- and (9*S*)-(9-^{13}C,9-^{2}H)GGPP through elongation with IPP using GGPPS with an established inversion of configuration at C-1 of GPP.[17] The obtained HSQC data after conversion with SoS (Figures S31 – S33) resulted in the absolute configurations as shown in Figure 1 which is in line with the literature data for *ent*-**3**.[13] The same principle was extended for other positions using (1*R*)- and (1*S*)-(1-^{13}C,1-^{2}H)FPP,[14] which was only applicable to **2** (Figure S34), because of overlaying signals at C-5 for **1** and **3**. Dimethylallyl diphosphate (DMAPP) was also elongated with (*E*)- and (*Z*)-(4-^{13}C,4-^{2}H)IPP[5f] in a known stereochemical course[18] with FPP synthase (FPPS) from *S. coelicolor*[19] and GGPPS to target three more positions (C-4, C-8 and C-12) in a single experiment (Figures S35 – S37). All experiments resulted in the same conclusion on the absolute configurations of **1**-**3**.

The proposed cyclisation mechanism of SoS (Scheme 1) starts from GGPP with a combined 1,11- and 10,14-cyclisation driven by the abstraction of OPP$^-$ and deprotonation to build up (*E*,*E*)-3,7,18-dolabellatriene (**4**),[13] which undergoes isomerisation to **3**, either by a direct reprotonation-deprotonation sequence (path a) or with a 1,3-hydride migration via cation **A** (path b). Protonation of **3** at C-2 induces further cyclisation to **B**, which provides access to **2** upon deprotonation of the Me group. A suprafacial 1,4-hydride shift in **B** yields cation **C**, which stabilises to the allylic tertiary cation **D** with another 1,2-hydride shift that proceeds with *anti*-attack and thus explains the *cis*-fused 5-membered ring of **1**. A final deprotonation at C-13 builds its conjugated diene system.

The incubations with (16-^{13}C)GGPP and (17-^{13}C)GGPP showed a flexible stereochemical course in the initial deprotonation towards **4** by a scrambled incorporation of label into the Me and the methylene groups (Figures S28 – S30), hinting to similar distances of the deprotonating base to both positions. To address the isomerisation problem, (1*R*)- and (1*S*)-(1-^{13}C,1-^{2}H)GGPP[20] were converted with SoS, resulting in a singlet in the (*S*) case and an upfield shifted triplet due to a direct ^{13}C-^{2}H bond for the (*R*) case, for C-1 of **1**-**3** in the ^{13}C-NMR spectrum (Figure S38). This experiment demonstrates that selectively H_R stays at C-1 during cyclisation, while H_S moves away. However, GC/MS analysis surprisingly demonstrated a partial loss of deuterium from the H_S position for **1**-**3**, but full retainment of labelling from H_R (Figure S39). An incubation experiment with enzymatically prepared (3-^{13}C)GGPP in D_2O buffer followed by GC/MS analysis of the products showed the uptake of one or two deuterium atoms for **1** and **2**, but no incorporation or uptake of only one deuterium for **3** (Figure S40). These findings support a full incorporation of one deuterium downstream of **3**, in agreement with its protonation induced cyclisation to **B** that is thus only observed for **1** and **2**. An additional partial deuterium incorporation is found for the reactions preceding **3**. The ^{13}C-NMR spectrum of the product from (3-^{13}C)GGPP in D_2O buffer revealed both a slightly upfield shifted singlet and a more strongly upfield shifted triplet for C-3 of **1** and **3**, demonstrating that the partial uptake of deuterium goes into position C-3, while the slight upfield shift of the singlet indicated additional deuterium incorporation at the neighbouring carbon C-2 (Figure S41). Conclusively, C-3 of the enzyme products carries either a hydrogen from bulk water (H_{ext}), or another hydrogen, for which we assumed the migrating H_S from C-1 would be a good candidate. Direct evidence for the partial H_S movement from C-1 to C-3 was obtained using the substrates (3-^{13}C,1,1-$^{2}H_2$)GGPP, and (1*R*)- and (1*S*)-(3-^{13}C,1-^{2}H)GGPP that were synthesised (Scheme S1, Figure S42). Conversion of these substrates with SoS indeed resulted in a singlet and a triplet for C-3 of **1** with (3-^{13}C,1,1-$^{2}H_2$)GGPP and also with the (1*S*)-deuterated sample, but only in a singlet with (1*R*)-(3-^{13}C,1-^{2}H)GGPP (Figure S43), which allowed for a direct tracking of the partial H_S location. Combining these results, the challenging isomerisation of **4** to **3** can be rationalised by two simultaneously operating pathways (Scheme 1). Path (a) proceeds by a protonation (C-3) and deprotonation (C-1) sequence, which leads to a partial loss of H_S, and path (b) involves a protonation (C-2), 1,3-hydride migration and deprotonation (C-2) sequence, which explains the observed partial movement of H_S to C-3.



Scheme 1. Cyclisation mechanism of SoS to its products **1**-**3**. Numbering of carbons indicates their origin from GGPP by same number and deviates from the numbering used in reference [13].

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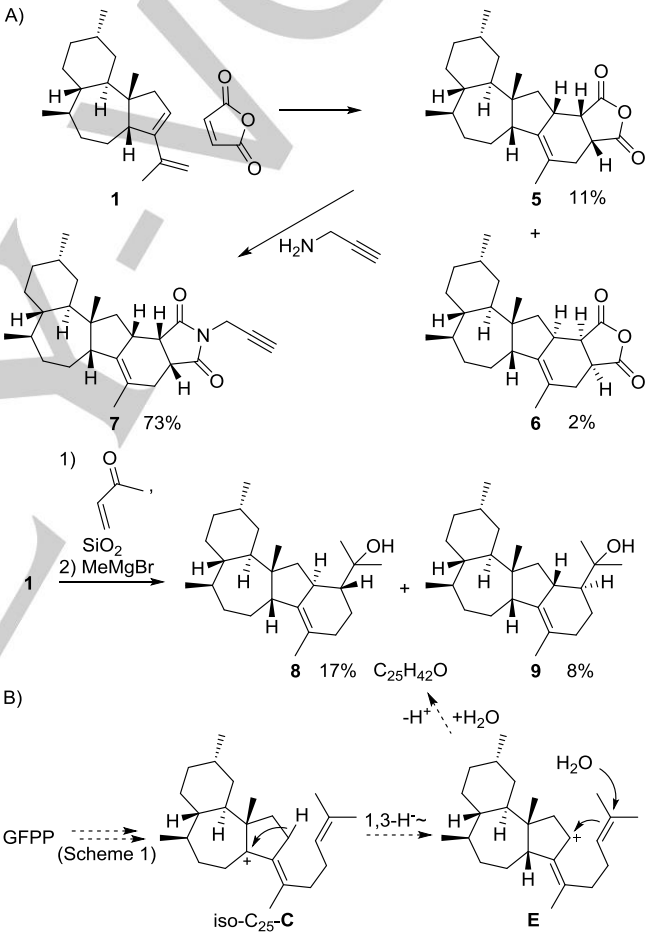
The protonation at C-2 of **3** for which already hints were obtained by the experiments described above (Figures S40 and S41) was followed by incubation of (2-^{13}C)GGPP with SoS in D_2O buffer, showing a complete incorporation of deuterium by triplets for C-2 of **1** and **2** (Figure S44). Reprotonation of **3** proceeds with a strict stereochemical course through addition to the *Si*-face as followed by HSQC. The 1,4-hydride shift from **B** to **C** was confirmed by incubation of (2-^{2}H)GPP[12c] and (3-^{13}C)IPP with SoS and GGPPS (Figure S45, triplet for C-7), and the 1,2-hydride shift to **D** was demonstrated by incubation of (2-^{2}H)DMAPP[20] and (2-^{13}C)IPP, synthesised from (2-^{13}C)acetic acid (Scheme S2), with SoS, FPPS and GGPPS (Figure S46, triplet for C-10). For the stereochemical investigation of the final deprotonation step, (1*R*)- and (1*S*)-(1-^{2}H)IPP were synthesised (Scheme S3, Figure S47) and incubated with isopentenyl diphosphate isomerase (IDI) from *Serratia plymuthica*,[5g] followed by its heat inactivation and elongation of the resulting (1*R*)- and (1*S*)-(1-^{2}H)DMAPP with excess unlabelled IPP by FPPS and GGPPS to suppress incorporation of additional labelled IPP units. This process yielded (13*S*)- and (13*R*)-(13-^{2}H)GGPP that was converted into **1** by SoS with selective deprotonation of H_S (Figure S48). The product profiles obtained from these samples indicated a significant shift in the product ratios depending on the isotopic nature of H_S, i. e. a significant drop in the yield of **1** in case of H_S = ^{2}H. Especially the usage of deuterium can sometimes lead to interesting effects, e. g. for the H309A variant of pentalenene synthase a change in the product distribution helped to distinguish between alternative cyclisation mechanisms.[21] The nature of **3** as an intermediate was challenged by a conversion of **3** to **1** by SoS. While the enzymatic conversion of **3** failed, likely because the active site architecture is in a wrong state if the reaction is not started from GGPP, it was possible to intercept the cyclisation of (20-^{13}C)GGPP by the addition of unlabelled **3** that shows a moderate exchange with active site bound intermediate ^{13}C-labelled **3** and is further converted into **1** (Figure S49).

The conjugated diene moiety in **1** inspired us to explore the Diels-Alder (DA) chemistry of this new natural product. Challenged by the low availability of starting material from in vitro incubations, **1** was reacted with maleic anhydride (Scheme 2), which gave the main *endo* products **5** (Table S4, Figures S50 – S57) and **6** (Table S5, Figures S58 – S65). The main product **5** was further converted into the imido-alkyne **7** (Table S6, Figures S66 – S73). We also aimed to exploit a DA reaction for the conversion of diterpene **1** into a formal sesterterpene. The DA reaction of **1** with methyl vinyl ketone on silica[22] produced a complex mixture of inseparable regio- and stereoisomers, but from the product mixture obtained by a following Me Grignard reaction the sesterterpene alcohols **8** (Table S7, Figures S74 – S81) and **9** (Table S8, Figures S82 – S89) could be purified. These compounds **8** and **9** represent formal sesterterpene alcohols with an isoprenoid-like carbon skeleton, for which a hypothetical biosynthesis can be rationalised via similar steps as determined for the SoS mechanism proceeding to *iso*-C_{25}-**C** with a double bond at C-14 instead of C-15. A 1,3-hydride shift to **E**, cyclisations with different stereochemical courses and attack of water can give rise to **8** and **9**.

In summary, we have characterised the function of a TS from *S. spinosa* as spinodiene synthase. This fascinating enzyme converts GGPP into spinodienes A (**1**) and B (**2**) featuring a new diterpene skeleton, and 2,7,18-dolabellatriene (**3**). Extensive labelling studies were used to investigate the cyclisation mechanism of SoS, which harbours an interesting and challenging isomerisation step, for which two different coexisting mechanisms lead to the same products. In a combined synthesis-biosynthesis approach[23] enzymatically prepared **1** was tested for DA reactions leading to the synthesis of the artificial sesterterpene alcohols **8** and **9**. Their formation can be rationalised by a terpene cyclisation mechanism similar to the mechanism of SoS, but a sesterterpene synthase is unknown. The astonishing synthetic and mechanistic potential of TSs will also pave the way for future investigations in nature's enzymatic playground for small molecule biosynthesis.



Scheme 2. A) Diels-Alder reactions with **1**. B) Hypothetical terpene cyclisation mechanism from GFPP to **8** and **9**.

Acknowledgements

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Keywords: biosynthesis • enzyme mechanisms • isotopes • terpenes • NMR spectroscopy

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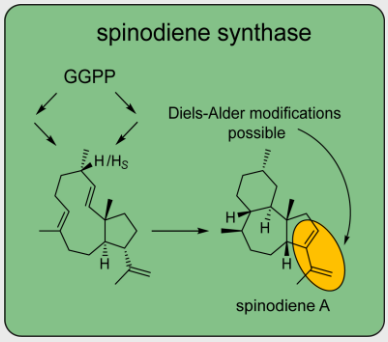
Taking the challenge: Investigations on a bacterial diterpene synthase revealed its unusual cyclisation mechanism to spinodiene A and B via neutral intermediates. Spinodiene A features a rare conjugated diene moiety providing chemical access to artificial sesterterpene alcohols by a Diels-Alder approach.



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