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Spata-13,17-diene Synthase, an Enzyme with Sesqui-, Di- and Sesterterpene Synthase Activity from *Streptomyces xinghaiensis*

Jan Rinkel,[a] Lukas Lauterbach[a] and Jeroen S. Dickschat*[a]

This work is dedicated to Prof. Dr. Stefan Schulz on the occasion of his 60th birthday.

Abstract: A terpene synthase from the marine bacterium *Streptomyces xinghaiensis* was characterised, including a full structure elucidation of its products from various substrates and an in-depth investigation of the enzyme mechanism by isotopic labelling experiments, metal cofactor variations, and mutation experiments. The results revealed an interesting dependency of Mn^{2+} catalysis on the presence of Asp-217, a residue that is occupied by a highly conserved Glu in most other bacterial terpene synthases.

In terms of their structural variability, complexity, and distribution terpenes constitute one of the most successful classes of natural products. They are synthesised from geranyl (GPP), farnesyl (FPP), geranylgeranyl (GGPP), geranylfarnesyl diphosphate (GFPP), and even larger isoprenoid oligomers by terpene synthases (TS) that generate a usually polycyclic carbon framework with multiple stereogenic centres in just one enzymatic step. Although multi-product TSs such as MtTPS5 from *Medicago truncatula* are known,[1] many enzymes selectively produce a single compound with astonishing accuracy. This precision is particularly remarkable, because the action of a TS on a substrate seems to be limited to its ionisation, either by abstraction of diphosphate (type I enzymes) or by protonation (type II), and to provide a shaped and essentially water-free cavity to arrange the substrate in a reactive conformation. The reaction cascade via cationic intermediates makes then use of the inherent substrate reactivity.[2] While several type I mono- and sesquiterpene synthases (STS) have been reported from bacteria,[3] the only characterised diterpene synthases (DTSs) of this class for which the intriguing product structures and enzyme mechanisms have been thoroughly studied are the enzymes for cyclooctat-9-en-7-ol, spiroviolene, tsukubadiene, and 18-hydroxydolabella-3,7-diene, and the multi-product DTS for hydropyrene.[4] Here we present the characterisation of a TS from *Streptomyces xinghaiensis*, a marine actinomycete that has been isolated from sediments near Dalian, China,[5] that shows a broad substrate spectrum and interesting metal cofactor dependency.

The gene for an unknown TS from *S. xinghaiensis* S187 (accession no. WP_095757924) was cloned into the expression vector pYE-Express[6] by homologous recombination in yeast and expressed in *Escherichia coli*. The predicted gene product exhibited all highly conserved motifs for a functional type I TS,[7] including the aspartate-rich motif (^{74}DDQLD), the pyrophosphate (PP) sensor Arg-173, the NSE triad (^{119}NDWYSLGKE), and the ^{307}RY dimer (Figure S1). The closest characterised homolog of this enzyme is the (+)-*epi*-cubenol synthase from *Streptomyces griseus* NBRC 13350 with 51% identical amino acid residues.[8] The protein was purified (Figure S2) and incubated with GPP, FPP, GGPP and GFPP. Among these substrates GPP was not accepted, while GGPP and GFPP were converted efficiently into a di- or sesterterpene hydrocarbon, respectively, accompanied by a few side products (Figure 1). The diterpene hydrocarbons **1** and **2** were also detected in headspace extracts from *S. xinghaiensis* (Figure S3).

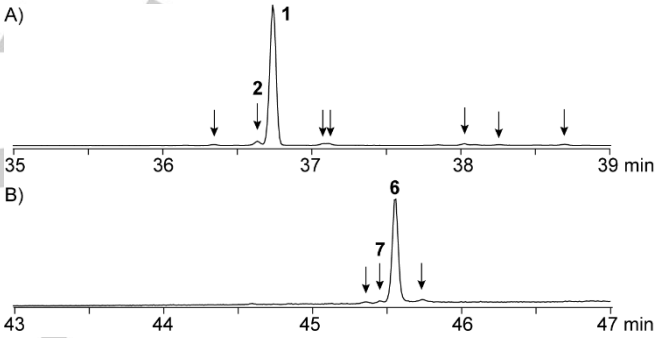

Figure 1. Total ion chromatograms of products obtained from an incubation of A) GGPP and B) GFPP with SpS. Arrows point to minor enzyme products, numbers at peaks refer to compound numbers in Scheme 1.

The main diterpene **1** was isolated by column chromatography, yielding 6 mg (14%) of pure **1**, and its structure was elucidated by NMR spectroscopy as spata-13,17-diene (Table S2, Figures S4–S10, most relevant 2D-NMR correlations are highlighted in Scheme 1), establishing the TS from *S. xinghaiensis* as spata-13,17-diene synthase (SpS). Two minor products could also be isolated and were identified by NMR as prenylkelsoene (**2**, 0.6 mg, 1%) and the known compound cneorubin Y[9] (**3**, 0.8 mg, 2%) (Tables S3 and S4, Figures S11–S23). The latter is not detectable by GC/MS, likely because it contains a Cope system resulting in thermal instability as described for germacrenes.[10] The diterpenes **1** and **2** are new natural products.

GFPP was also accepted by SpS, resulting in the formation of a main and a few side products. The main product (0.9 mg, 1%) was identified by NMR (Table S5, Figures S25–S31) and GC/MS (Figure S32) as the higher homolog of **1**, prenylspata-13,17-diene (**6**). The homolog of **2**, geranylkelsoene (**7**), was also tentatively identified by GC/MS based on the characteristic loss of ethylene from the cyclobutane portion (Figure S33). The sesterterpenes **6** and **7** are new natural products, and SpS is the first bacterial TS reported to have sesterterpene synthase activity.

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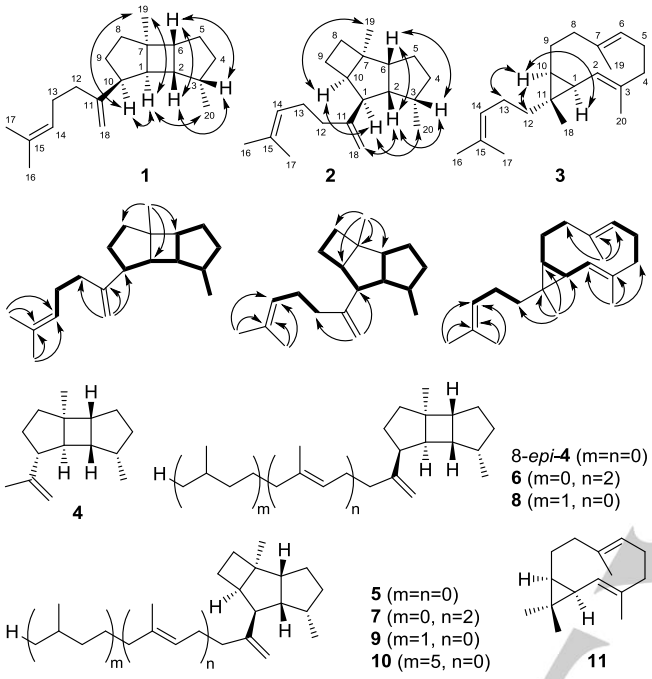
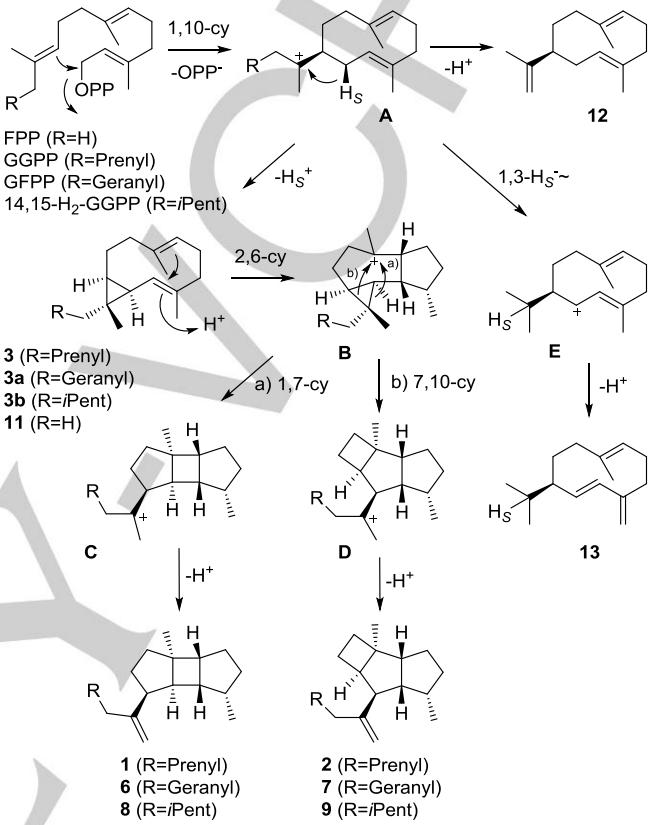
The corresponding sesquiterpene to **3** is the widespread compound bicyclogermacrene (**11**). Notably, FPP was also accepted by SpS and converted into a mixture of **11**, germacrene A (**12**) and germacrene D (**13**) that were partially detected as their Cope rearrangement products by GC/MS (Figure S34, Table S6). Compound **12** was also isolated and characterised by NMR, showing identical data to those previously reported.[11]



Scheme 1. Products of SpS and structures of related natural products. The carbon numberings for **1** – **3** follow the GGPP numbering to indicate the biosynthetic origin of each carbon. This numbering is different to previously introduced numbering systems (cf. comment on page 1 of SI).

Poduran (**10**) is a tetraterpenoid with the same tricyclic core as in **2** and a saturated side chain that was reported from the springtail *Podura aquatica*.[12] The broad substrate specificity of SpS suggested that a similar enzyme may be responsible for the biosynthesis of **10**. This prompted us to investigate whether 14,15-dihydro-GGPP can also be converted. For this purpose, 10,11-dihydro-FPP was synthesised (Scheme S1), elongated with IPP by the GGPP synthase (GGPPS) from *Streptomyces cyaneofuscatus*,[4b] and converted by SpS, yielding 1.8 mg (5%) of spat-13-ene (**8**) (Table S7, Figures S32 and S35–S41). A minor product was tentatively identified from its mass spectrum as isopentylkelsoene (**9**) that showed a loss of 28 Da from the molecular ion (Figure S33), as reported for **10**.[12]

The biosynthesis of **1** and **2** can be rationalised by 1,10-cyclisation to **A**, followed by deprotonation with formation of a cyclopropane ring to **3**. This neutral intermediate can be reprotonated at C-3 for a second cyclisation to **B**. This cation can react by two alternative ring openings of the cyclopropane, either via pathway a) to **C** that is the precursor of **1**, or via pathway b) to **D**, the direct precursor of **2**. Starting from GFPP or 14,15-dihydro-GGPP, essentially the same reactions can explain the formation of **6**/**7** and **8**/**9**, respectively, via the hypothetical intermediates **3a** and **3b**. In contrast, the enzymatic conversion of FPP stopped at **11** and did not proceed to tricyclic analogs of **1** and **2**, but produced major amounts of **12** by alternative deprotonation of **A**, and **13** by 1,3-hydride migration and deprotonation.



Scheme 2. Biosynthetic mechanism for SpS.

The proposed biosynthetic pathway of Scheme 2 was investigated by incubation of all twenty isotopologs of (^{13}C)GGPP, obtained by chemical synthesis or enzymatically from the corresponding labelled FPP or IPP isotopologs using *S. cyaneofuscatus* GGPPS,[4b,13] resulting in the incorporation of labelling into the expected positions of **1**–**3** in all cases (Figures S42–S44). In particular, these results demonstrate the formation of **1** by cyclopropane ring closure to **3** and reverse ring opening with carbon backbone rearrangement.

The sesquiterpene prespatane (bourbon-11-ene, **4**) has been reported from the sponge *Cymbastella hooperi*[14] and from several liverworts[15] and actinobacteria.[16] In all these organisms **4** co-occurs with kelsoene (tritomarene, **5**), but the different relative orientation of the isopropenyl group seems to exclude a simple biosynthetic mechanism via a common intermediate **11** as we have found for the pairs **1**+**2** and **6**+**7**. A comparison of the ^{13}C-NMR data of **1** and **2** to those reported for **4** and **5**[14] suggests that the structure of **4** needs correction to 8-*epi*-**4** that also better fits into the biosynthesis scheme (Figure S45). The structural revision for prespatane was recently also independently described by Weng and coworkers.[17] In this report a biosynthesis

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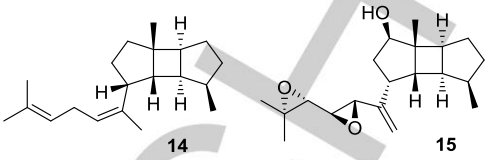
of 8-*epi*-**4** via germacrene A, B or C was suggested, but the larger homolog **3** of bicyclogermacrene (**11**) as intermediate can better explain the common formation of the two products **1** and **2**.

The reprotonation of **3** at C-3 for the second cyclisation to **B** (Scheme 2) was investigated by incubation of (3-^{13}C)IPP[4b] and FPP with GGPPS and SpS in D_2O. The obtained product (3-^{13}C,3-^{2}H)-**1** showed a triplet in the ^{13}C-NMR in agreement with reprotonation at C-3 (Figure S46). The stereochemical course of the deprotonation from **A** to **3** was investigated by enzymatic conversion of (*S*)- and (*R*)-(1-^{13}C,1-^{2}H)GGPP[18] with SpS. Labelled **1** obtained from (*S*)-(1-^{13}C,1-^{2}H)GGPP showed a singlet in ^{13}C-NMR, while (*R*)-(1-^{13}C,1-^{2}H)GGPP gave a product exhibiting a triplet due to ^{13}C-^{2}H spin coupling, establishing the loss of the *pro-S* and retainment of the *pro-R* hydrogen from C-1 in the cyclisation to **3** which was also supported by GC/MS analysis (Figure S47). Analogous results were obtained for **6** with (*S*)- and (*R*)-(1-^{13}C,1-^{2}H)GFPP[13b] (Figure S48) and for **11** with (*S*)- and (*R*)-(1-^{2}H)FPP (Figure S49). For **13** a stereospecific shift of the *pro-S* hydrogen into the isopropyl group was observed by GC/MS (Figure S50). Assuming inversion of configuration at C-1 for the initial 1,10-cyclisation step to **A** as reported for other terpene cyclisations,[19] these data are in favour of the absolute configurations of the products of SpS as shown in Scheme 2. In particular, the stereochemical course of the 1,3-hydride shift from C-1 of FPP into the isopropyl group as for **13** was shown to be indicative for the absolute configuration of sesquiterpenes.[20]

The absolute configuration of **12** was independently established by compound isolation from its optical rotation ($[\alpha]_D^{20}$ = –7.2, *c* 0.5, CCl_4) and comparison to different literature data for **12** ($[\alpha]_D^{25}$ = –3.2, *c* 14.4, CCl_4; $[\alpha]_D^{25}$ = –26.8, *c* 1.0, CCl_4) and its enantiomer ($[\alpha]_D^{25}$ = +42.1, *c* 1.0, CCl_4).[21] Compound **12** was also converted into its Cope rearrangement product β-elemene in refluxing toluene to yield a material of $[\alpha]_D^{20}$ = +17.9 (*c* 0.06, $CHCl_3$), consistent with the earlier reported conversion of (+)-**12** into (–)-β-elemene ($[\alpha]_D^{25}$ = –15.8 (*c* 0.50, $CHCl_3$)).[20c] The absolute configurations of **1** – **3** were investigated by conversion of stereoselectively deuterated (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)GPP and (*R*)- and (*S*)-(1-^{13}C,1-^{2}H)FPP[4b] that were elongated to the corresponding GGPPs using *S. cyaneofuscatus* GGPPS (Figures S51–S56). This reaction is known to proceed with inversion of configuration at C-1 of GPP and FPP.[22] The obtained stereoselectively deuterated GGPP isotopologs were converted into **1** – **3** by SpS. The installed stereochemical anchors with known absolute configurations allowed to deduce the absolute configurations at the other stereocentres simply by solving the relative configurations of the obtained stereoselectively deuterated products. The additional ^{13}C label was introduced for an efficient and sensitive product analysis by HSQC. The deduced absolute configurations for **1** – **3** are in line with their biosynthetic relationship. The absolute configuration of **1** corresponds to that of prespatane (8-*epi*-**4**) from the alga *Laurencia pacifica*.[17] The same absolute configuration as found here for **3** can be assigned to **3** from *Cneorum tricoccon* based on the same sign for optical rotations (found here: $[\alpha]_D^{20}$ = –31, *c* 0.05, acetone, reported: $[\alpha]_D^{20}$ = –49.1, 0.3%, acetone).[23] The absolute configuration of **2** ($[\alpha]_D^{20}$ = +20 (*c* 0.05, C_6D_6)) is the same as reported for natural (+)-kelsoene that was established by synthesis of its enantiomer.[24] The main product **1** of SpS is structurally related to a series of spatanes that were reported from different brown algae, including (13*Z*)-spata-13(15),17-diene (**14**) and spatol (**15**, Scheme 3).[25] These compounds exhibit the opposite absolute configuration as determined for **1**.



Scheme 3. Known spatanes from brown algae.

Several TSs have been investigated by site-directed mutagenesis (SDM), including the fungal trichodiene synthase and the bacterial TSs for pentalenene, *epi*-isozizaene, (2*Z*,6*E*)-hedycaryol and selina-4(15),7(11)-diene (Tables S8–S12).[7a,26] This work underpinned the importance of highly conserved motifs such as the Asp-rich motif,[7a,26c,f,g] the NSE triad,[7a] the RY dimer,[26a,b,g] and the PP sensor[26g] for catalysis. A detailed analysis of 51 characterised bacterial type I TSs,[3] their close relatives with presumably the same function from sequenced bacteria, and SpS by sequence alignment resulted in the identification of four highly conserved amino acid residues whose importance for catalysis have not been shown so far. These include P83 and L90, located 21 and 14 positions upstream of the Asp-rich motif, and E184 and E217 that are placed 19 positions upstream and 14 positions downstream of the PP sensor (Table S13, SpS numbering, the usually found E217 is altered to D217 in native SpS). P83A exchange resulted in a poor yield of soluble enzyme and a complete loss of activity (Figures S57 and S58). Also the L90A variant showed a lower expression level and significantly reduced activity (3%) compared to native SpS. The crystal structure of selinadiene synthase from *Streptomyces pristinaespiralis*[26g] shows that its corresponding residues (P61 and V68) have an important structural role at the link between two α-helices (Figure S59). Their exchange in SpS may result in an incorrect enzyme folding, causing poor activity and yield of enzyme. The efficiently expressed E184Q variant of SpS was nearly inactive (0.5%). The structure of selinadiene synthase shows that the corresponding E159 is involved in Mg^{2+} binding (Figure S60) that may be critical for activity. Exchange of D217 to the usual Glu (D217E) resulted in an increased expression and catalytic efficiency (170%). Comparison to the structure of selinadiene synthase reveals that the corresponding E192 in helix G is part of a salt bridge to R144 in helix F (Figures S60 and S61). In SpS, D217 can substitute for this function, but the D217E variation seems to fit better to the structural requirements of a type I TS.

Catalysis by type I TSs requires a trinuclear cluster of divalent cations (usually Mg^{2+} or Mn^{2+}) that binds to the Asp-rich motif, the NSE/DTE triad and the substrate's PP to initiate its ionisation.[27] Other divalent cations were sometimes reported to be ineffective for catalysis,[28] but most of the recently described enzymes have not been systematically investigated for their metal ion dependency. Incubation experiments with SpS and various cations (Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}) resulted in efficient catalysis by Mg^{2+} and, with ca. threefold higher rates, by Mn^{2+} (Figure S62), while all other cations gave no product.

Interestingly, incubation of the highly productive D217E variant with Mn^{2+} gave no diterpene product from GGPP, possibly because this mutation causes a conformational rearrangement in the helices F and G that disturbs the active site residues involved in metal cofactor binding. A BLAST search and phylogenetic analysis (Figure S63) revealed the presence of two closely related homologs of SpS in *Streptomyces albus* NRRL F-4971 (WP_030543144, 89% identity) and in *Streptomyces fradiae* ATCC 19609 (WP_050363727, 96%). Both enzymes also exhibit an aspartate residue in the position corresponding to Asp-217 of SpS. Future experiments will address whether the metal cofactor requirement also for other bacterial TSs can be tuned by mutation of this highly conserved Asp.

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Keywords: biosynthesis • enzyme mechanisms • isotopes • metal cofactors • terpenes

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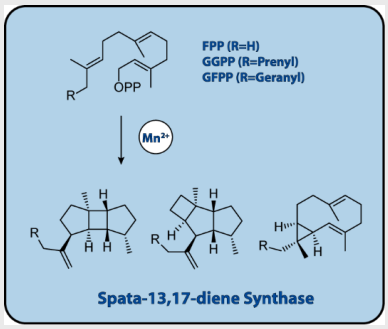
A terpene synthase from *Streptomyces xinghaiensis* with sesqui-, di, and sesterterpene synthase activity was identified. The enzyme mechanism was investigated by isotopic labelling experiments. Site-directed mutagenesis uncovered several previously unrecognised highly conserved residues that are important for catalysis. One of these residues was linked to Mg^{2+} versus Mn^{2+} cofactor requirement.



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Spata-13,17-diene Synthase, an Enzyme with Sesqui-, Di- and Sesterterpene Synthase Activity from *Streptomyces xinghaiensis*