

Enzyme Mechanisms

International Edition: DOI: 10.1002/anie.201711142
German Edition: DOI: 10.1002/ange.201711142Spata-13,17-diene Synthase—An Enzyme with Sesqui-, Di-, and Sesterterpene Synthase Activity from *Streptomyces xinghaiensis*

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Dedicated to Professor Stefan Schulz on the occasion of his 60th birthday

Abstract: A terpene synthase from the marine bacterium *Streptomyces xinghaiensis* has been characterised, including a full structure elucidation of its products from various substrates and an in-depth investigation of the enzyme mechanism by isotope 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), and geranyl-farnesyl diphosphate (GFPP), as well as even larger isoprenoid oligomers by terpene synthases (TSs) that usually generate a polycyclic carbon framework with multiple stereogenic centres in just one enzymatic step. Although multiproduct 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). Moreover, the TS provides a shaped and essentially water-free cavity to arrange the substrate in a reactive conformation. The reaction cascade that proceeds via cationic intermediates thus makes use of the inherent substrate reactivity.^[2] Although several type I mono- and sesquiterpene synthases (STSs) 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, as well as the multiproduct 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] This TS shows

a broad substrate spectrum and an 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 the highly conserved motifs for a functional type I TS,^[7] including the aspartate-rich motif (⁷⁴DDQLD), the pyrophosphate (PP) sensor Arg-173, the NSE triad (¹¹⁹NDWYSLGKE), and the ³⁰⁷RY dimer (Figure S1 in the Supporting Information). The closest characterised homologue of this enzyme is the (+)-*epi*-cubenol synthase from *Streptomyces griseus* NBRC 13350 which has 51 % identical amino acid residues.^[8] The protein was purified (Figure S2) and incubated with GPP, FPP, GGPP, and GFPP. Of these substrates, GPP was not accepted, whereas 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).

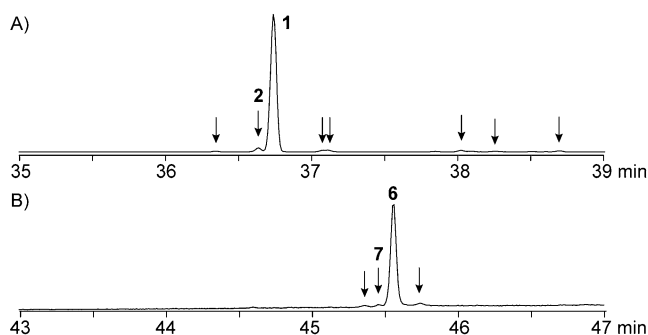
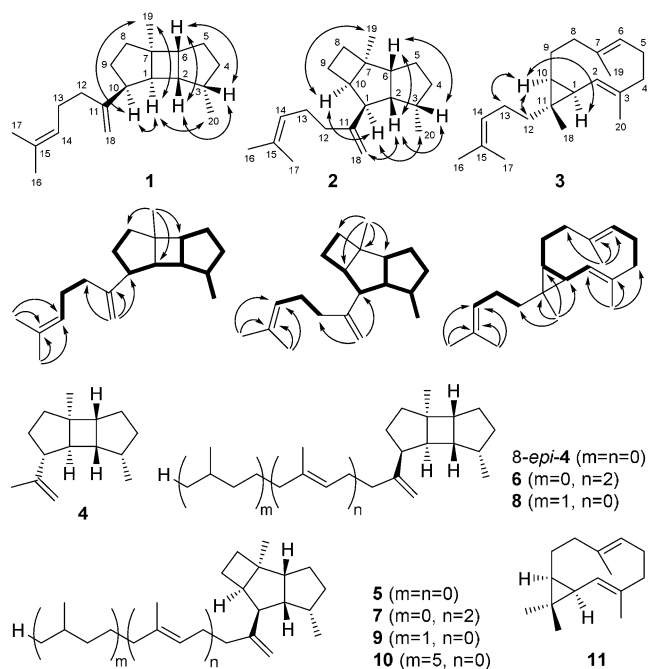


Figure 1. Total ion chromatograms of products obtained from an incubation of A) GGPP and B) GFPP with SpS. Arrows point to minor enzyme products, the numbers at peaks refer to the compound numbers in Scheme 1.

The main diterpene **1** was isolated by column chromatography (6 mg of pure product, 14 %) and its structure was elucidated by NMR spectroscopy to be spata-13,17-diene (Table S2, Figures S4–S10; the most relevant 2D NMR correlations are highlighted in Scheme 1), thus 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 spectroscopy as prenylkelsoene (**2**; 0.6 mg, 1 %) and the known compound cneorubin Y^[9] (**3**;

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Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under:
<https://doi.org/10.1002/anie.201711142>.



Scheme 1. Products of SpS and structures of related natural products. Single-headed arrows indicate HMBC, double-headed arrows indicate NOESY correlations, and bold lines show contiguous spin systems in the $^1\text{H}, ^1\text{H}$ -COSY spectrum. The carbon atom numbering for 1–3 follows the GGPP numbering to indicate the biosynthetic origin of each carbon atom. This numbering is different to previously introduced numbering systems (see comment on page 1 of the Supporting Information).

0.8 mg, 2%; Tables S3 and S4, Figures S11–S23). The latter compound is not detectable by GC/MS, likely because it contains a Cope system that results in thermal instability, as described for germacrenes.^[10] The diterpenes **1** and **2** are new natural products.

GFPP was also accepted by SpS and resulted in the formation of a main product and a few side products. The main product (0.9 mg, 1%) was identified by NMR spectroscopy (Table S5, Figures S25–S31) and GC/MS (Figure S32) as the higher homologue of **1**, namely prenylspata-13,17-diene (**6**). The homologue of **2**, geranylkelsoene (**7**), was also tentatively identified by GC/MS on the basis of 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.

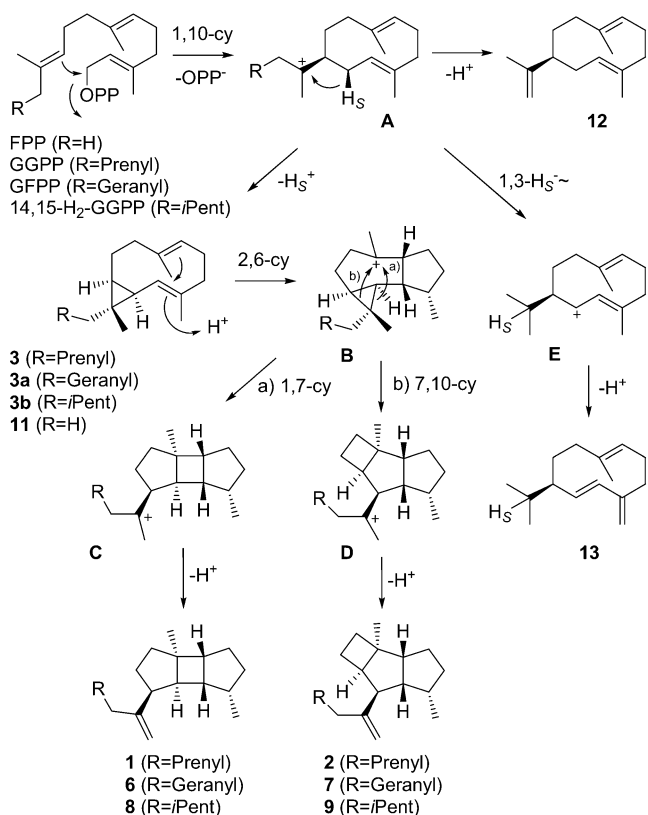
The corresponding sesquiterpene to **3** is the widespread compound bicyclogermacrene (**11**). Notably, FPP was also accepted by SpS and converted into a mixture of **11**, germacrene A (**12**), and germacrene D (**13**), which were partially detected as their Cope rearrangement products by GC/MS (Figure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

Poduran (**10**) is a tetraterpenoid with the same tricyclic core as in **2** as well as a saturated side chain that was reported in 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. This yielded 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**), which 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 give **3** (Scheme 2). 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 by pathway a to give **C**, which is the precursor of **1**, or by pathway b to give **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 analogues of **1** and **2**, but produced major amounts of **12** by an alternative deprotonation of **A**, and **13** by a 1,3-hydride migration and deprotonation.

The proposed biosynthetic pathway of Scheme 2 was investigated by incubation of all 20 isotopomers of ($^{13}\text{C}_1$)GGPP, obtained by chemical synthesis or enzymatically from the corresponding labelled FPP or IPP isotopomers



Scheme 2. Biosynthetic mechanism for SpS. Cy = cyclisation.

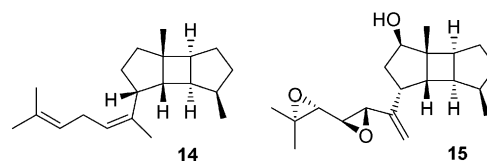
using *S. cyaneofuscatus* GGPPs,^[4b,13] which resulted in incorporation of the label at the expected positions of **1–3** in all cases (Figures S42–S44). In particular, these results demonstrate the formation of **1** by ring closure to form the cyclopropane in **3** and reverse ring opening with rearrangement of the carbon backbone.

The sesquiterpene prespatane (bourbon-11-ene, **4**) has been reported from the sponge *Cymbastella hooperi*^[14] as well as 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 the common intermediate **11**, as we found for the pairs **1** + **2** and **6** + **7**. A comparison of the ¹³C NMR data of **1** and **2** with those reported for **4** and **5**^[14] suggests that the structure of **4** needs correction to 8-*epi*-**4**, which also better fits into the biosynthesis scheme (Figure S45). The structural revision of prespatane was recently also independently described by Weng and co-workers.^[17] In this report a biosynthesis of 8-*epi*-**4** via germacrene A, B, or C was suggested, but the intermediacy of the larger homologue **3** of bicyclogermacrene (**11**) 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 (3-¹³C)IPP^[4b] and FPP with GGPPS and SpS in D₂O. The obtained product (3-¹³C,3-²H)-**1** showed a triplet in the ¹³C NMR spectrum, 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*)-(1-¹³C,1-²H)GGPP^[18] with SpS. Labelled **1** obtained from (*S*)-(1-¹³C,1-²H)GGPP showed a singlet in the ¹³C NMR spectrum, while (*R*)-(1-¹³C,1-²H)GGPP gave a product exhibiting a triplet as a consequence of ¹³C-²H spin coupling, thus establishing the loss of the *pro-S* and retention of the *pro-R* hydrogen atoms from C-1 in the cyclisation to form **3**. This was also supported by GC/MS analysis (Figure S47). Analogous results were obtained for **6** with (*S*- and (*R*)-(1-¹³C,1-²H)GFPP^[13b] (Figure S48) and for **11** with (*S*- and (*R*)-(1-²H)FPP (Figure S49). In the case of **13**, a stereospecific shift of the *pro-S* hydrogen atom into the isopropyl group was observed by GC/MS (Figure S50). Assuming inversion of the 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 of the absolute configuration of sesquiterpenes.^[20]

The absolute configuration of **12** was independently established from the optical rotation of the isolated compound ($[\alpha]_D^{20} = -7.2$, $c = 0.5$, CCl₄) through comparison with different literature data for **12** ($[\alpha]_D^{25} = -3.2$, $c = 14.4$, CCl₄; $[\alpha]_D^{25} = -26.8$, $c = 1.0$, CCl₄) and its enantiomer ($[\alpha]_D^{25} = +42.1$, $c = 1.0$, CCl₄).^[10b,21] Compound **12** was also converted into its Cope rearrangement product β -elemene in refluxing toluene to yield a material with $[\alpha]_D^{20} = +17.9$ ($c = 0.06$, CHCl₃), consistent with the earlier reported conversion of (+)-**12** into (–)- β -elemene ($[\alpha]_D^{25} = -15.8$ ($c = 0.50$, CHCl₃)).^[10b] The absolute configurations of **1–3** were investigated by elonga-

tion of the stereoselectively deuterated (*R*)- and (*S*)-(1-¹³C,1-²H)GPP and (*R*- and (*S*)-(1-¹³C,1-²H)FPP^[4b] to the corresponding GGPPs using *S. cyaneofuscatus* GGPPS (Figures S51–S56). This reaction is known to proceed with inversion of the configuration at C-1 of GPP and FPP.^[22] The obtained stereoselectively deuterated GGPP isotopologues were converted into **1–3** by SpS. The installed stereochemical anchors with known absolute configurations allowed the absolute configurations at the other stereocentres to be deduced simply by solving the relative configurations of the obtained stereoselectively deuterated products. The additional ¹³C label was introduced to allow efficient and sensitive product analysis by HSQC. The deduced absolute configurations of **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 tricocon* on the basis of the same sign of their optical rotations (found here: $[\alpha]_D^{20} = -31$, $c = 0.05$, acetone, reported: $[\alpha]_D^{20} = -49.1$, 0.3 %, acetone).^[9] The absolute configuration of **2** ($[\alpha]_D^{20} = +20$ ($c = 0.05$, C₆D₆)) is the same as that reported for natural (+)-kelsoene, which was established by synthesis of its enantiomer.^[23] The main product **1** of SpS is structurally related to a series of spatanes, including (13*Z*)-spata-13(15),17-diene (**14**) and spatol (**15**, Scheme 3), known from different brown algae.^[24] 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, 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,25] This work underpinned the importance of highly conserved motifs such as the Asp-rich motif,^[7,25c,f] the NSE triad,^[7a] the RY dimer,^[7b,25a,b] and the PP sensor^[7b] 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, respectively, of the Asp-rich motif, and E184 and E217 that are placed 19 positions upstream and 14 positions downstream, respectively, 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 a soluble enzyme and a complete loss of activity (Figures S57 and S58). The L90A variant also showed a lower expression level and significantly reduced activity (3 %) compared to native SpS. The crystal structure of

selinadiene synthase from *Streptomyces pristinaespiralis*^[7b] 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, thereby causing poor activity and a low yield of the 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), which may be critical for activity. Exchange of D217 to the usual Glu (D217E) resulted in an increased expression and catalytic efficiency (170%). A comparison with 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 PP of the substrate to initiate its ionisation.^[26] Other divalent cations have sometimes been reported to be ineffective for catalysis,^[27] 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 rates about threefold higher—by Mn^{2+} (Figure S62), while no product was obtained with all other cations. 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 helices F and G that disturbs the active-site residues involved in the binding of metal cofactors. A BLAST search and phylogenetic analysis (Figure S63) revealed the presence of two closely related homologues 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 requirement of a metal cofactor also for other bacterial TSs can be tuned by mutation of this highly conserved Asp.

Acknowledgements

This work was funded by the DFG (DI1536/7-1) and by the Fonds der Chemischen Industrie. We thank Seocho Kim for skillful assistance in the synthesis of 10,11-dihydro-FPP.

Conflict of interest

The authors declare no conflict of interest.

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

How to cite: *Angew. Chem. Int. Ed.* **2017**, *56*, 16385–16389
Angew. Chem. **2017**, *129*, 16603–16607

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Manuscript received: October 30, 2017

Accepted manuscript online: November 10, 2017

Version of record online: November 23, 2017