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Mechanistic Characterisation of Two Sesquiterpene Cyclases from the Plant Pathogenic Fungus *Fusarium fujikuroi*

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Abstract: *Two sesquiterpene cyclases from Fusarium fujikuroi were expressed in Escherichia coli and purified. The first enzyme was inactive because of a critical mutation, but activity was restored by sequence correction through site-directed mutagenesis. The mutated enzyme and two naturally functional homologues from other fusaria converted farnesyl diphosphate into guaia-6,10(14)-diene. The second enzyme produced eremophilene. The absolute configuration of guaia-6,10(14)-diene was elucidated by enantioselective synthesis, while that of eremophilene was evident from the sign of its optical rotation and is opposite to that in plants but the same as in Sorangium cellulosum. The mechanisms of both terpene cyclases were studied with various ^{13}C- and ^{2}H-labelled FPP isotopomers.*

The rice pathogenic fungus *Fusarium fujikuroi* causes severe crop losses in agriculture through the production of mycotoxins, including the fumonisins, fusarins, fusaric acid, and moniliformin.[1] Furthermore, large amounts of gibberellins, an important class of plant hormones that derive from the diterpene *ent*-kaurene, are produced by the fungus.[2] In plants, gibberellins regulate processes such as stem and leaf growth, flowering, fruiting, and seed germination. Their overproduction by fusaria causes out-of-control growth and development in infected plants that is known as the "foolish seedling" or "bakanae" disease. The distantly related *F. sporotrichioides* makes a family of mycotoxins derived from trichodiene, the trichothecenes.[3] These compounds are absent in *F. fujikuroi* and related species because the biosynthetic genes, including a trichodiene synthase gene, are missing in their genomes. Instead, the *F. fujikuroi* genome houses a bifunctional *ent*-copalyl diphosphate synthase/*ent*-kaurene synthase (CPS/KS) for gibberellin biosynthesis,[4] a lanosterol synthase, a phytoene synthase for neurosporaxanthin biosynthesis,[5] and nine sesquiterpene cyclases (STC1-9).[6] Only two of these have been characterized as (+)-koraiol (STC4) and (−)-α-acorenol synthases (STC6),[7] but knowledge about the products of the other STCs is important because it may lead to the identification of undiscovered mycotoxins that are potentially involved in plant pathogenicity. Herein, we describe the identification of the products from STC5 (FFUJ11739) and STC3 (FFUJ04067) and their mechanistic characterization by isotopic labelling techniques.[8]

Two uncharacterized STCs from *F. fujikuroi* (STC5 and STC3) were investigated through the construction of overexpression mutants. The mutants were analyzed for the production of terpenes by capturing their volatiles on charcoal filters followed by GC/MS analysis.[9] Although quantitative reverse transcription (RT)-PCR revealed higher expressions of the terpene cyclase genes, none of the mutants showed production of a new terpene compared to the wildtype, possibly because of oxidative modifications by other enzymes with genes that are clustered with the terpene cyclase genes (Figure S1 in the Supporting Information). Reverse transcription of the corresponding mRNA and amplification of the cDNA by PCR allowed gene cloning into the yeast-to-*E.-coli* shuttle vector pYE-Express through homologous recombination.[10] The proteins were expressed, purified, and incubated with geranyl (GPP), farnesyl (FPP), and geranylgeranyl (GGPP) diphosphate, followed by GC/MS analysis of the formed products.

STC5 yielded no product from any of the tested substrates, presumably because of a critically mutated gene that leads to an asparagine to lysine exchange in the NSE triad that usually displays a ND(L,I,V)XSXXXE sequence in functional terpene cyclases (Figure S2).[11] Two STC5 homologues from *F. mangiferae* and *F. proliferatum* with intact NSE triads both yielded a sesquiterpene from FPP (Figures S3 and S4 A), while no products were observed for GPP or GGPP. Sequence correction of *F. fujikuroi* STC5 through K288N mutation restored activity and gave the same product. A preparative-scale incubation of 60 mg FPP with *F. mangiferae* STC5 allowed the isolation of 1 mg (4%) of the pure compound. Its structure was established by one- and two-dimensional NMR techniques (Table S1 in the Supporting Information). The ^{13}C-NMR spectra revealed fifteen signals (three methyl groups, four sp^3 methylene and four sp^3 methine carbons, two olefinic quarternary carbons, one olefinic

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methylene, and one olefinic CH), suggesting a bicyclic system. The ^{1}H-NMR signals for the directly coupled hydrogens were assigned from the HMQC spectrum and ^{1}H,^{1}H-COSY showed three contiguous spin systems (C1-2-3-4(-15)-5-6, C11(-13)-12 and C8-9, Figure 1A). HMBC correlations from H11, H12, and H13 to C7 indicated a C7–C11 bond, while

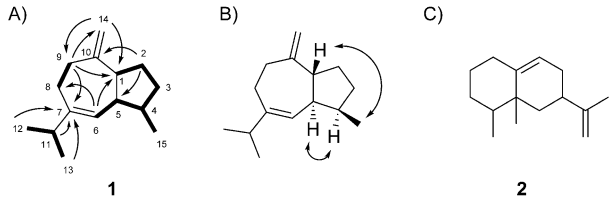
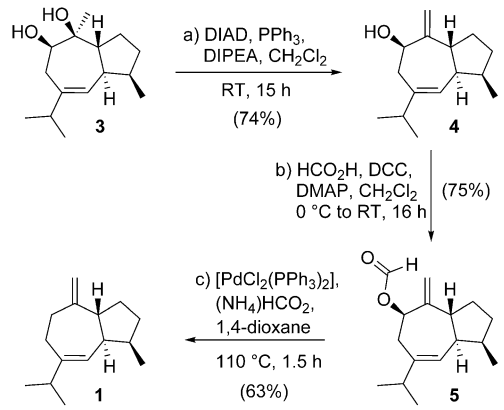


Figure 1. Structures of the enzyme products. A) Contiguous spin systems of **1** determined by ^{1}H,^{1}H-COSY (bold) and key HMBC correlations (arrows); B) key NOESY correlations for **1** (double headed arrows); C) structure of **2**.

cross-peaks from H8 and H9 to C7 and H6 to C8 revealed a C6-7-8 linkage. Correlations from H14 to C1 and C9, from H9 to C1 and C14, and from H2 to C10 placed C10 between C1 and C9. The ring closure from C1 to C5 was evident from cross-peaks between H2 and C5 and between H6 and C1, resulting in the planar structure of guaia-6,10(14)-diene (**1**). Key NOESY correlations between H1–H15 and H4–H5 suggested a *trans*-guaiane (Figure 1B).

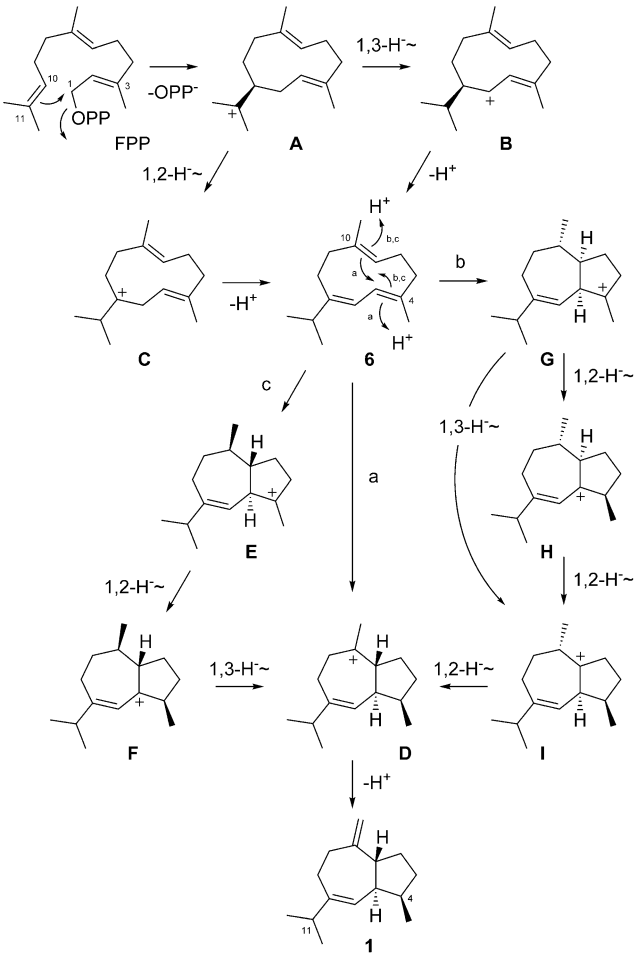
The absolute configuration of **1** was elucidated through enantioselective synthesis starting with diol **3**, a well-characterized intermediate from our englerin synthesis (Scheme 1).[12] Using a Mitsunobu protocol,[13] selective



Scheme 1. Enantioselective synthesis of (1*R*,4*R*,5*S*)-**1**.

elimination of the tertiary alcohol afforded exocyclic alkene **4** in 74% yield (notably, a natural product with identical spectroscopic data yet a different structure was published).[14] The secondary alcohol was converted into the corresponding formate **5** through Steglich esterification.[15] Pd-catalyzed hydrogenolysis[16] of the allylic formate afforded (1*R*,4*R*,5*S*)-**1** in 63% yield with an optical rotation of $[\alpha]_D^{22.8} = -22.2$ (*c* 0.15, CH_2Cl_2), while the optical rotation of the enzyme product was $[\alpha]_D^{22.0} = -19.3$ (*c* 0.15, CH_2Cl_2), thus confirming their identity. A compound with identical spectroscopic data but incompletely assigned configuration was reported from the soft coral *Nephthea chabrolii*.[17]

Several mechanisms for FPP cyclization to **1** can be proposed (Scheme 2). The initial 1,10-cyclization of FPP to the (*E*,*E*)-germacradienyl cation (**A**) may be followed by a 1,3-hydride shift to **B** or a 1,2-hydride migration to **C** and



Scheme 2. Proposed cyclization mechanisms for the biosynthesis of **1**.

deprotonation to germacrene C (**6**). Reprotonation at C4 of **6** and cyclization (path a) can give rise to **D** that yields **1** upon deprotonation. Alternatively, **6** may be cyclized by C10 reprotonation to **G**, followed by three sequential 1,2-hydride shifts via **H** and **I** to **D** (path b). Cation **I** is also accessible from **G** by 1,3-hydride migration. Finally, C10 reprotonation of **6** may initiate a cyclization to **E**, a stereoisomer of **G**, which produces **D** by a sequence of a 1,2- and a 1,3-hydride transfer (path c). The proposed mechanisms were experimentally tested through the enzymatic conversion of labelled FPP isotopomers.[18] (1,1–^{2}H$_2$,11–^{13}C)FPP resulted in labelled **1** that exhibited a singlet at $\delta = 37.8$ ppm for C11 in the ^{13}C-NMR, thus indicating that no deuterium was attached to this carbon atom (Figure 2A). In conclusion, the conversion of **A** into **6** does not proceed by a 1,3-hydride shift via **B**. GC/MS analysis of the product revealed the loss of one deuterium by

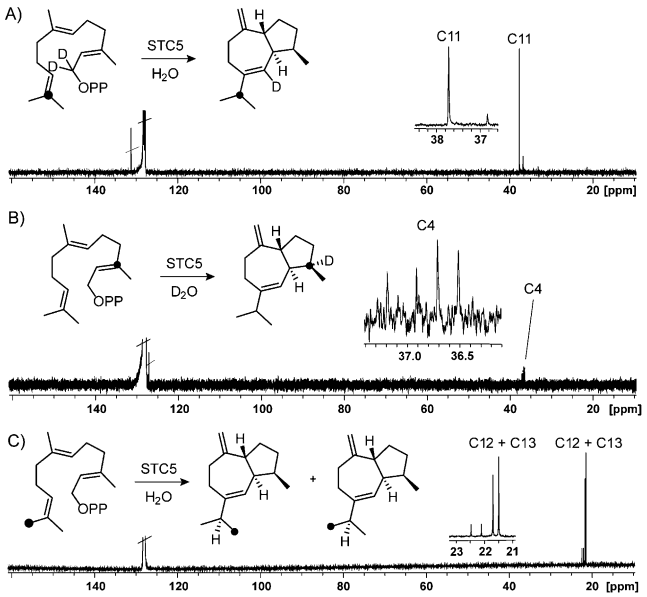
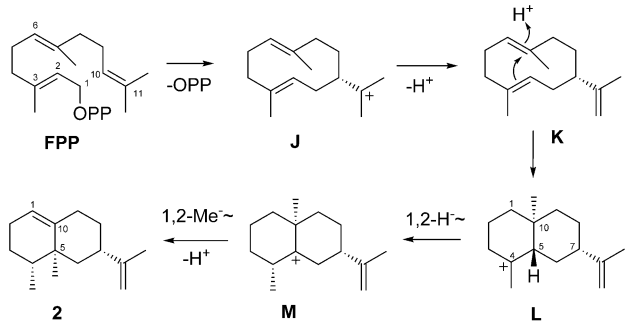


Figure 2. Enzymatic mechanism of STC5. ^{13}C-NMR spectrum of A) (6-^{2}H,11–^{13}C)-**1** obtained from (1,1-$^{2}H_2$,11-^{13}C)FPP, B) (4-^{2}H,4-^{13}C)-**1** from (3-^{13}C)FPP in D_2O, C) mixture of (12-^{13}C)-**1** and (13-^{13}C)-**1** obtained from (13-^{13}C)FPP. Black dots indicate ^{13}C-labelled carbons.



Scheme 3. Proposed cyclization mechanism for the biosynthesis of **2**.

a molecular ion at *m*/*z* 206, as is expected for the pathway via **C** (Figure S4B). Enzymatic conversion of (10-^{2}H)FPP[19] yielded a product with an increased molecular mass of *m*/*z* 205 in its mass spectrum, but still the same base peak ion at *m*/*z* 161 as unlabeled **1** (Figure S4C). Since this fragment ion arises through isopropyl cleavage, the deuterium labelling in the terpene obtained from (10-^{2}H)FPP can be localized in the isopropyl group, thus corroborating the 1,2-hydride shift from **A** to **C**. To distinguish between the options for the reprotonation of **6**, incubation experiments in D_2O were performed.[20] Incubation of (3-^{13}C)FPP in D_2O yielded a triplet for C4 of **1** at $\delta = 36.7$ ppm ($\Delta\delta = -0.5$ ppm, $^{1}J_{C,D} = 20.1$ Hz), thus indicating that deuterium was connected to C4 (Figure 2B). This finding is in agreement with the conversion of **6** into **D** and **1** via pathway a, but not via pathways b or c. The singlet at $\delta = 37.2$ ppm indicated that a portion of the labelled material was not deuterated at C4 because the incubation buffer contained residual water, as was also evident from GC/MS analysis of the sample (Figure S4D). To exclude the possibility that a minor fraction of **6** may react via pathway b or c to cation **D**, (2-^{13}C)FPP and (6-^{13}C)FPP were enzymatically converted in D_2O, yielding (^{2}H,5-^{13}C)-**1** and (^{2}H,1-^{13}C)-**1**, which only exhibited singlets for C5 and C1 (Figure S5), while deuterium uptake was evident from the mass spectra (Figures S4E and S4F). In conclusion, pathway a is the only one that is relevant for the reaction from **6** to **D**. An incubation experiment with (13-^{13}C)FPP resulted in amplified ^{13}C signals for both C12 and C13 (Figure 2C), thus indicating that the isopropyl group in cation **A** is not conformationally fixed but can freely rotate prior to the 1,2-hydride shift to **C**.

Incubation of STC3 with FPP yielded a sesquiterpene, while GPP and GGPP were not accepted. GC/MS analysis (Figure S6A) suggested the structure of valencene or eremophilene, but it was not possible to clearly assign one of these structures. A large-scale incubation of 50 mg FPP yielded 4.6 mg (19%) of the pure sesquiterpene with ^{1}H- and ^{13}C-NMR data matching those of eremophilene (**2**; Table S1).[21] Determination of the rotary power as $[\alpha]_D^{20.8} = +86.1$ (*c* 0.36, $CHCl_3$) (lit.: $[\alpha]_D^{25} = +131.7$ (*c* 1.0, $CHCl_3$))[21] pointed to the opposite enantiomer to in plants and the coral *Plexaurella fusifera*,[22] but the same as in the myxobacterium *Sorangium cellulosum*.[21] The proposed biosynthesis of **2** (Scheme 3) starts with a 1,10-cyclization of FPP to the (*E*,*E*)-germacradienyl cation **J**, followed by deprotonation to germacrene A (**K**). Cyclization upon reprotonation at C6 leads to the cation **L**, and subsequent 1,2-hydride and 1,2-methyl migrations and deprotonation yield **2**. The 1,2-hydride shift to **M** was shown by enzymatic conversion of (2-^{2}H,3-^{13}C)FPP, which resulted in a triplet at $\delta = 36.6$ ppm ($\Delta\delta = -0.5$ ppm, $^{1}J_{C,D} = 19.0$ Hz) in the ^{13}C-NMR spectrum of **2**, thus indicating the connection of deuterium to C4 (Figure 3A). A singlet for the non-deuterated compound is also visible at $\delta = 37.1$ ppm as a result of the deuteration grade of the substrate (86% by MS, Figure S6B). The reprotonation in **K** occurs at the same carbon atom from which a proton is lost in the final step. To test whether it is the same or another proton that is first introduced and then lost, an enzymatic conversion of FPP in D_2O was conducted. Indeed GC/MS analysis of the product gave no evidence for any deuterium uptake from D_2O (Figure S6C). Finally, the

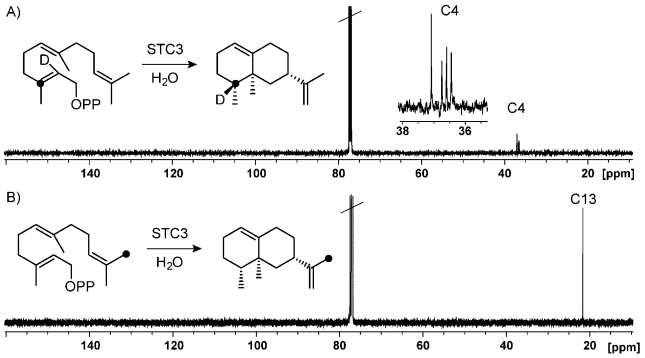


Figure 3. Enzymatic mechanism of STC3. ^{13}C-NMR spectra of A) (4-^{2}H,4–^{13}C)-**2** obtained from (2-^{2}H,3-^{13}C)FPP, B) (13-^{13}C)-**2** obtained from (13-^{13}C)FPP. Black dots indicate ^{13}C-labelled carbons.

stereochemical fate of the terminal methyl groups of FPP was followed by enzymatic conversion of (13-^{13}C)FPP, which resulted in the incorporation of labelling only into the methyl group C13 and not C12 of **2** (Figure 3B). In contrast to STC5, STC3 shows a strict stereochemical course in this aspect, as previously described for several other terpene cyclases.[18a,23]

In summary we have identified STC3 and STC5 from *F. fujikuroi* as (+)-eremophilene and (−)-guaia-6,10(14)-diene synthases. STC5 in *F. fujikuroi* was only active after a sequence correction in the mutated NSE triad, while homologues from *F. proliferatum* and *F. mangiferae* are naturally functional. The biosynthesis of both compounds was investigated in detail to show the stereospecific course of the cyclizations, reprotonations, and hydride shifts. A detailed investigation of the multilayer regulation of the STC5 gene cluster by pathway-specific and epigenetic processes in *F. fujikuroi* is currently in progress.

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Keywords: enzyme mechanisms · *Fusarium fujikuroi* · isotopic labelling · NMR spectroscopy · terpenes

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