Terpene Biosynthesis

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Structures and Biosynthesis of Corvol Ethers—Sesquiterpenes from the Actinomycete *Kitasatospora setae***

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Abstract: *Here we present the functional characterization of a sesquiterpene cyclase from Kitasatospora setae. The enzyme converts the sesquiterpene precursor farnesyl diphosphate (FPP) into two previously unknown and unstable sesquiterpene ethers for which we propose the trivial names corvol ethers A and B. Both compounds were purified and their structures were determined by one- and two-dimensional NMR spectroscopy. A biosynthetic mechanism for the FPP cyclization by the corvol ether synthase was proposed. The results from the incubation experiments of the corvol ether synthase with isotopically labeled precursors were in line with this mechanism, while alternative mechanisms could clearly be ruled out.*

Terpenoids represent the largest class of secondary metabolites with more than 50 000 known compounds from all forms of life. Their biosynthesis starts from the linear precursors geranyl diphosphate (GPP, precursor of monoterpenes), farnesyl diphosphate (FPP, sesquiterpenes), or geranylgeranyl diphosphate (GGPP, diterpenes), which are converted by terpene cyclases into terpene hydrocarbons, alcohols, or ethers. This conversion proceeds through substrate ionization by abstraction of diphosphate (type I terpene cyclases) or by protonation (type II enzymes) to yield a highly reactive cationic intermediate that subsequently undergoes a domino reaction with usually several elementary steps. These steps can include cyclization reactions by intramolecular attack of an olefinic double bond to the cationic center, hydride and proton shifts, Wagner–Meerwein rearrangements, and a terminating deprotonation or attack of water.[1,2] Since many variations of these reactions are possible in terms of their sequential order and stereochemical courses, a large variety of different carbon skeletons is accessible. Post-cyclization modifications by oxidations, O acylations, and methylations further increase the exceptionally large structural diversity of natural terpenoids.

The crystal structures of terpene cyclases, especially of those in complex with nonreactive substrate surrogates, gave important insights into the conformational folds of the enzyme's substrates in the active sites, thereby allowing conclusions to be made on the mechanisms of this fascinating class of enzymes.[3–10] Such a cocrystal can visualize the starting point of a terpene cyclase reaction, but cationic intermediates and elementary steps along the cyclization cascade are difficult to observe. To date, several bacterial terpene cyclases have been characterized for their products by in vitro incubation of the substrate with the purified enzyme or by heterologous expression.[9–27] Here we present the cloning and expression of a terpene cyclase gene from *Kitasatospora setae*, purification of the enzyme, and structure elucidation of its two sesquiterpene products. Finally, enzyme mechanistic investigations by isotopic labeling experiments are presented.

The genome of *K. setae* KM-6054 encodes, besides the characterized (2*Z*,6*E*)-hedycaryol synthase[9] and two terpene synthases with predicted functions as geosmin synthase and 2-methylisoborneol synthase,[28] two additional putative sesquiterpene synthases with unknown products. One of these terpene cyclase genes (accession number YP_004903082) was cloned into pYE-Express, a pET28-derived plasmid that can be used for efficient cloning by homologous recombination in yeast and for heterologous expression of His-tagged recombinant enzymes in *Escherichia coli*.[25]

Although GGPP was not accepted as a substrate, incubation of the purified enzyme with GPP yielded small amounts of several acyclic and cyclic monoterpenes, with linalool as the main product (see Figure S1 in the Supporting Information). The enzyme's native substrate FPP was efficiently converted into two main products (Figure 1 A). Their EI mass spectra (Figure S2) were not included in our mass spectral libraries and did not exhibit clear molecular ions at $m/z = 204$ or $m/z = 222$, as normally observed for sesquiterpenes ($C_{15}H_{24}$ or $C_{15}H_{26}O$, respectively). Detectable fragment ions at $m/z = 179$ pointed to a molecular ion at $m/z = 222$ (the mass difference of 43 Da is, for example, explainable by loss of an isopropyl group C_3H_7, whereas the mass difference of 25 Da would be difficult to explain). Accordingly, the CI-HRMS spectrum of the main product **2** showed ions at $m/z = 223.20864$ (calcd for $C_{15}H_{27}O^+$, $[M+H]^+$: 223.20564) and $m/z = 240.23495$ (calcd for $C_{15}H_{30}NO^+$, $[M+NH_4]^+$: 240.23219). Traces of both volatiles were detected by GC/MS[29] in the headspace of *K. setae* in a similar ratio (3:1) as produced by the recombinant enzyme (Figure S3).

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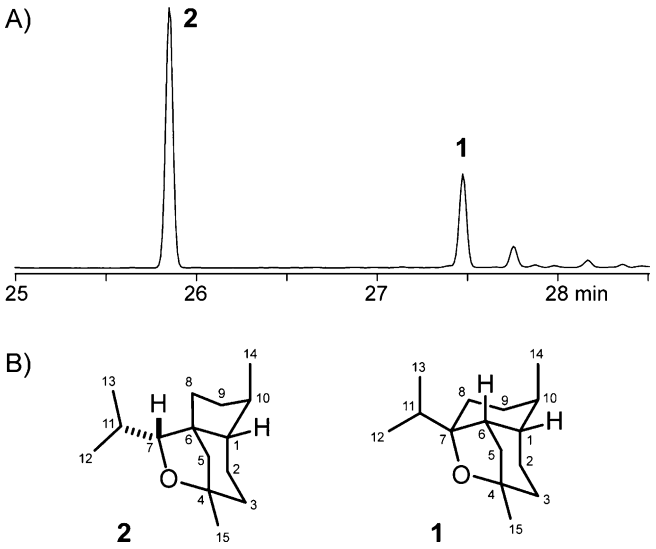


Figure 1. Products of the corvol ether synthase from *K. setae*. A) Total ion chromatogram of the extracted products from a FPP incubation with purified enzyme, B) structures of corvol ethers A (**1**) and B (**2**).

The two enzyme products **1** and **2** were difficult to separate by column chromatography on silica gel, because they exhibited very similar polarities and proved to be unstable. A mixture of 20 mg of **1** and **2**, obtained from an incubation of 50 mg FPP with purified enzyme from 6 L of *E. coli* culture, only yielded about 2 mg of **2** and less than 1 mg of **1**, while the rest of the material decomposed. The use of other standard materials for column chromatography did not give better results (basic aluminum oxide also resulted in decomposition and florisil gave no successful separation). Repeated incubation of three 50 mg FPP batches with freshly prepared enzyme and a swift separation by silica gel chromatography with cooled (4 °C) solvents (pentane and diethyl ether) finally yielded 6.4 mg of **2** and 2.5 mg of **1**.

The structures of both sesquiterpenes were elucidated by extensive one- and two-dimensional NMR spectroscopy. The ^{13}C NMR and DEPT (distorsionless enhancement by polarization transfer) spectra of **1** exhibited 15 carbon signals (4 methyl, 5 methylene, 4 methine, and 2 oxygenated quaternary carbon atoms, Table 1). None of the signals appeared in the olefinic region, thus indicating the structure of a tricyclic sesquiterpene ether. The corresponding signals of the protons bound to each of the CH_3, CH_2, and CH carbon atoms were assigned by HSQC (heteronuclear single quantum correlation) spectroscopy. The ^{1}H NMR spectrum together with ^{1}H,^{1}H correlation spectroscopy (COSY) revealed three connected spin systems (C-8-9-10-14, C-5-6-1-2-3, and C-12-11-13, Figure 2 A) and a methyl group (C-15) bound to a quaternary carbon atom that showed long-range couplings to H-5a and H-6 in the ^{1}H,^{1}H COSY spectrum. As a result of the signals of H-1 and H-10 overlapping, the connection between the corresponding carbon atoms could not be established with certainty from the ^{1}H,^{1}H COSY spectrum and was determined by HMBC (heteronuclear multiple bond correlation) spectroscopy instead (Figure 2 A). Other C-C connectivities that were inferred from the HMBC spectrum included connections from C-6, C-8, and C-11 to the quarternary C-7, and from C-3, C-5, and C-15 to quarternary C-4. The relative configuration of **1** was determined by nuclear Overhauser spectroscopy (NOESY). Key correlations between H-6 and H-1 and between H-6 and H-14 placed these on the same face of the molecule and established the *cis*-decalin system of **1** (Figure 2 B). The ether linkage between C-4 and C-7 can

Table 1: NMR data of corvol ether A (**1**) and corvol ether B (**2**) in C_6D_6.

1			**2**		
C[a]	^{1}H (δ, m, J, int)[b]	^{13}C (δ)[c]	C[a]	^{1}H (δ, m, J, int)[b]	^{13}C (δ)[c]
1	1.39 (m, 1 H)	39.9 (CH)	1	1.01 (ddt, ^4J = 1.2, ^3J = 4.9, 12.6, 1 H)	56.1 (CH)
2a	1.59 (dd, ^2J = 8.7, ^3J = 5.5, 1 H)	29.1 (CH_2)	2a	1.56 (dddd, ^2J = 13.2, ^3J = 5.2, 5.2, 0.9, 1 H)	22.7 (CH_2)
2b	1.61 (ddd, ^2J = 8.7, ^3J = 5.2, 0.8, 1 H)		2b	1.81 (dddd, ^2J = 13.0, ^3J = 12.9, 11.8, 5.8, 1 H)	
3a	1.35 (ddd, ^2J = 12.9, ^3J = 8.7, 8.3, 1 H)	39.1 (CH_2)	3a	1.24 (ddd, ^2J = 13.2, ^3J = 11.7, 5.7, 1 H)	38.5 (CH_2)
3b	1.69 (m, 1 H)		3b	1.64 (dddd, ^2J = 13.1, ^3J = 5.8, 1.3, ^4J = 2.2, 1 H)	
4		79.7 (C_q)	4		78.6 (C_q)
5a	1.15 (d, ^2J = 11.4, 1 H)	41.2 (CH_2)	5a	1.21 (d, ^2J = 10.4, 1 H)	54.5 (CH_2)
5b	1.82 (ddd, ^2J = 11.4, ^3J = 5.0, 2.4, 1 H)		5b	1.93 (dd, ^2J = 10.4, ^4J = 2.4, 1 H)	
6	1.99 (dd, ^3J = 4.7, 4.7, 1 H)	37.4 (CH)	6		53.3 (C_q)
7		86.2 (C_q)	7	3.31 (dd, ^3J = 10.5, ^4J = 1.1, 1 H)	93.7 (CH)
8a	1.72 (m, 2 H)	24.5 (CH_2)	8a	1.48 (ddd, ^2J = 14.1, ^3J = 9.4, 6.2, 1 H)	28.8 (CH_2)
8b			8b	1.52 (ddd, ^2J = 14.0, ^3J = 11.9, 4.7, 1 H)	
9a	0.83 (m, 1 H)	27.9 (CH_2)	9a	1.14 (dddd, ^2J = 13.3, ^3J = 12.0, 10.0, 6.2, 1 H)	33.9 (CH_2)
9b	1.74 (m, 1 H)		9b	1.87 (dddd, ^{2}J = 13.3, ^3J = 9.3, 8.2, 4.6, 1 H)	
10	1.42 (q, ^3J = 7.0, 1 H)	33.8 (CH)	10	1.73 (m, 1 H)	37.6 (CH)
11	1.75 (sept, ^3J = 6.8, 1 H)	36.8 (CH)	11	2.16 (dsept, ^3J = 10.5, 6.4, 1 H)	29.3 (CH)
12	1.11 (d, ^3J = 6.8, 3 H)	16.1 (CH_3)	12	0.81 (d, ^3J = 6.4, 3 H)	20.0 (CH_3)
13	0.78 (d, ^3J = 7.0, 3 H)	19.1 (CH_3)	13	1.23 (d, ^3J = 6.4, 3 H)	22.5 (CH_3)
14	0.87 (d, ^3J = 7.0, 3 H)	21.4 (CH_3)	14	0.91 (d, ^3J = 6.4, 3 H)	19.2 (CH_3)
15	1.29 (s, 3 H)	27.5 (CH_3)	15	1.31 (s, 3 H)	26.8 (CH_3)

[a] Carbon numbering as shown in Figure 1. [b] Chemical shifts δ in ppm, multiplicity m (s = singlet, d = doublet, m = multiplet), coupling constants ^nJ are over n bonds and given in Hertz, integral (number of protons). [c] Carbon assignments (CH_3, CH_2, CH, and C_q) were delineated from a DEPT spectrum. All one- and two-dimensional NMR spectra of **1** and **2** are shown in Figures S4–S17 in the Supporting Information.

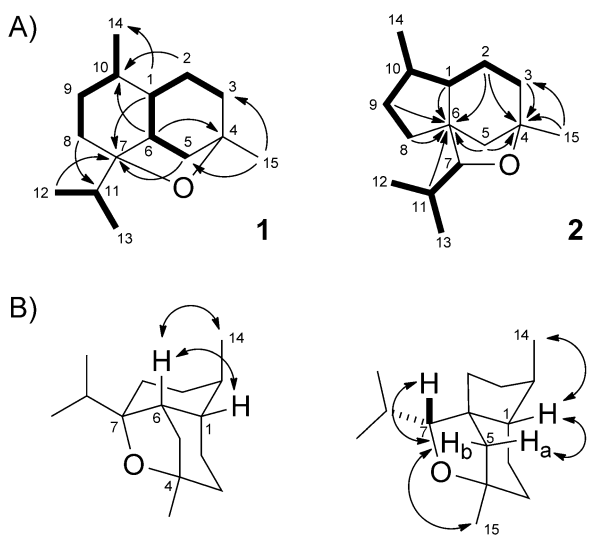
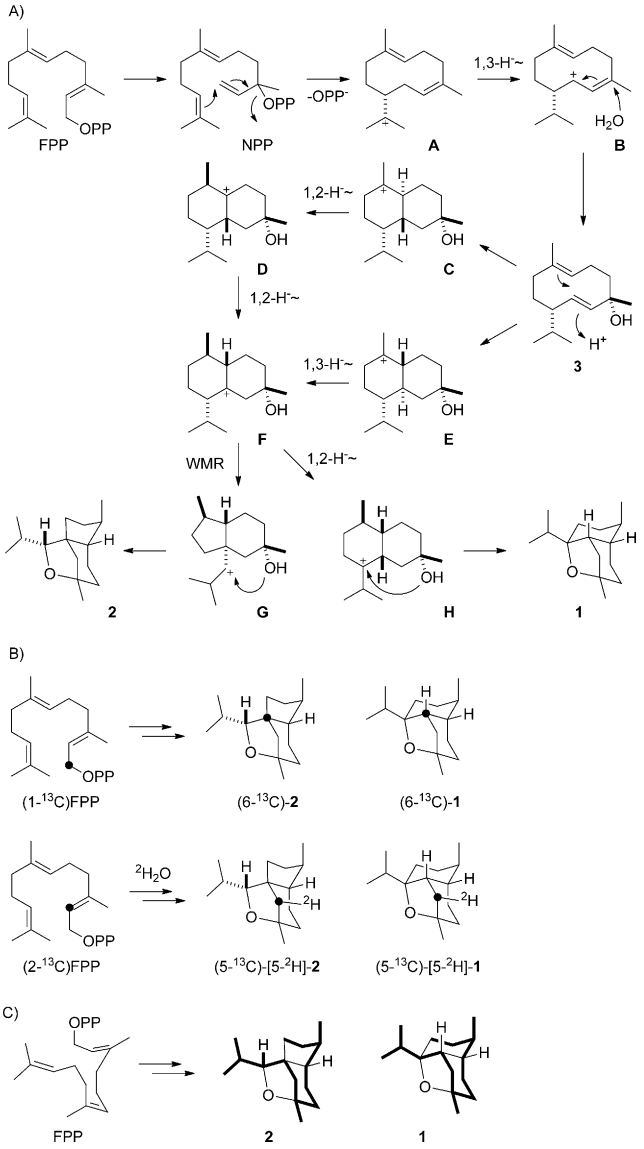


Figure 2. Structure elucidation of **1** and **2**. A) Spin systems of **1** and **2** determined by ^{1}H,^{1}H COSY (bold) and selected HMBC correlations through three bonds (arrows). B) Key NOE correlations (arrows) indicative of the relative configurations of **1** and **2**.

then only be realized with the shown relative configuration. All other observed NOESY correlations were in agreement with the structure of **1**.

In agreement with its formation from FPP, the ^{13}C and DEPT spectra revealed a carbon skeleton for compound **2** composed of 15 sp^3 carbon atoms (4 methyl, 5 methylene, 4 methine, and 2 quarternary carbon atoms, Table 1). One of the methine and one of the quarternary carbon atoms were bound to oxygen, again pointing to the structure of a tricyclic sesquiterpene ether. The directly linked hydrogen atoms were identified by HSQC. The ^{1}H and ^{1}H,^{1}H COSY spectra showed the presence of two extended spin systems (C-8-9-10(-14)-1-2-3 and C-7-11(-12)-13, Figure 2 A) plus separate methylene (C-5) and methyl groups (C-15). Diagnostic HMBC correlations indicated binding of C-1, C-5, C-7, and C-8 to the quarternary carbon C-6, and of C-3, C-5 and C-15 to quarternary C-4. Key NOESY correlations placed H-7, H-5b, and H_3-15 on the same face of the tetrahydrofuran ring, while a correlation of H-5a to H-1 established the relative configuration at C-1. The relative configuration at C-10 was in turn inferred from a NOESY correlation between H-1 and H_3-14, thereby demonstrating their *syn* orientation at the cyclopentane ring. The deduced relative configuration was further supported by a cross-peak between H-1 and H-7 in the ^{1}H,^{1}H COSY spectrum arising from a W coupling.

Both sesquiterpene ethers are new natural products and we propose the trivial names corvol ether A (**1**) and B (**2**) (named upon the group leaders suggestion after their discoverer P. Rabe, lat. raven = corvus). Compound **2** represents a sesquiterpene ether with a new skeleton, while 4,7-epoxy-5-hydroxymuurolane, a formal oxidation product of **1**, has been reported from *Fabiana imbricata* (Solanaceae).[30] A plausible biosynthetic mechanism for the corvol ether synthase is shown in Scheme 1 A. The mechanism starts with the isomerization of FPP to NPP, followed by a 1,10-cyclization to the



Scheme 1. Biosynthesis of corvol ethers A and B. A) Proposed biosynthetic mechanism for the formation of **1** and **2** by the corvol ether synthase. B) Incubation experiments with isotopically labeled precursors. C) Alternative folding of FPP for the cyclization to **1** and **2**.

germacradienyl cation (**A**). A subsequent 1,3-hydride shift to **B** and attack of water yield the neutral intermediate germacrene D-4-ol (**3**). Protonation at the original C-2 of FPP initiates a second cyclization to **C**, followed by two sequential 1,2-hydride shifts via **D** to **F**. Alternatively, just one 1,3-hydride shift could substitute for these two steps, but this would require another stereochemical course in the cyclization step (to **E**) to establish the correct stereochemistry in **F** (note that all the hydride migrations must be suprafacial). The main product corvol ether B (**2**) is formed by a Wagner–Meerwein rearrangement with ring contraction to **G** and intramolecular attack of the alcohol function on the cationic center. If the reaction from **F** to **2** would be a concerted process, the intermediate formation of the explicitly shown secondary cation **G** could be avoided. This would require an inversion of configuration and result exactly in the observed

relative configuration of **2**. Alternatively, **F** may react to form **H** by another 1,2-hydride shift, followed by intramolecular attack of the hydroxy function to yield **1**. Although these steps proceed via a more-stable tertiary cation, again this final reaction could be concerted with inversion of configuration.

To test the proposed biosynthetic mechanism for the FPP cyclization to **1** and **2**, incubation experiments with ^{13}C-labeled isotopologues of FPP were performed (Scheme 1 B). Incubation of (1-^{13}C)FPP (for the synthesis, see Scheme S1) with corvol ether synthase yielded a mixture of (6-^{13}C)-**2** and (6-^{13}C)-**1**, as unambigously determined by ^{13}C NMR spectroscopy (Figure 3 A). This observation is in line with the proposed terpene cyclization mechanism and rules out an alternative folding of the FPP precursor, as shown in Scheme 1 C. In a second experiment (2-^{13}C)FPP was incubated with corvol ether synthase in a 1:1 mixture of water and deuterium oxide. The second cyclization step starting from the neutral intermediate **3** is proposed to proceed with protonation at C-2 of FPP. This mechanism was evident from the observation of triplets for C-5 of both products **1** and **2** through ^{2}H-^{13}C coupling in the ^{13}C NMR spectrum (Figure 3 B).

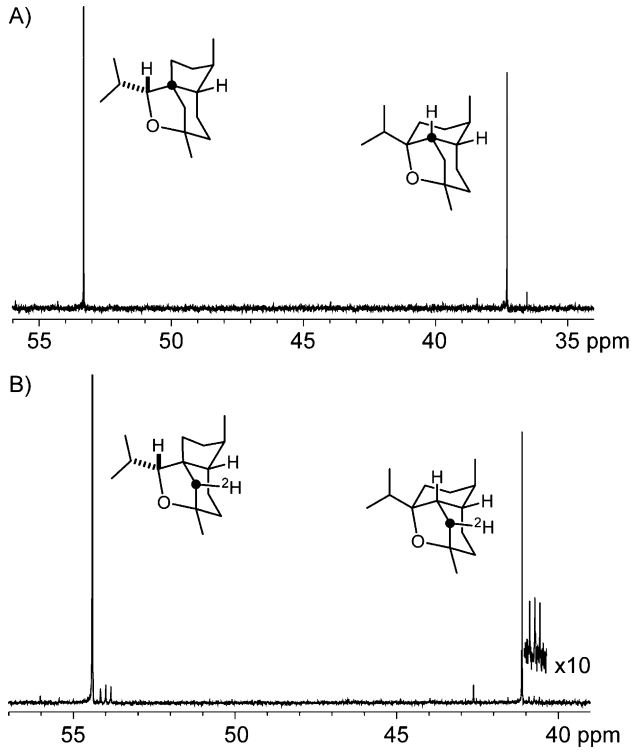


Figure 3. Analysis of isotopically labeled products **1** and **2** obtained from incubation of FPP isotopologues with corvol ether synthase.

We have characterized the products of a sesquiterpene cyclase from the actinomycete *Kitasatospora setae* as corvol ethers A and B. A proposed biosynthetic mechanism for their formation was interrogated by incubation experiments with ^{13}C-labeled precursors that allowed the highly sensitive detection of diagnostic carbon atoms by ^{13}C NMR spectroscopy. The labeling techniques presented here offer detailed insights into elementary steps of terpene cyclization mechanisms. The complementary usage of deuterium labeling in combination with mass spectrometry is an interesting alternative in mechanistic investigations, for example, to follow epimerization reactions in the biosynthesis of nonribosomal peptides.[31,32] We will soon report about further mechanistic studies on terpene cyclases using isotopic labels.

Keywords: biosynthesis · enzyme mechanisms · isotopic labeling · NMR spectroscopy · terpenoids

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