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Pristinol, a Sesquiterpene Alcohol with an Unusual Skeleton from *Streptomyces pristinaespiralis*

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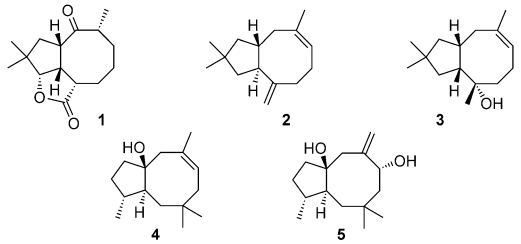
Abstract: *A terpene cyclase from Streptomyces pristinaespiralis was characterized as the synthase for (+)-(2S,3S,9R)-pristinol. The structure of this sesquiterpene alcohol, which has a new carbon skeleton, was established by NMR spectroscopy and single-wavelength anomalous-dispersion X-ray crystallography. Extensive isotopic labelling experiments were performed to distinguish between various possible cyclization mechanisms of the terpene cyclase and to decipher the EI-MS fragmentation mechanism for pristinol.*



Scheme 1. Sesquiterpenes with a 5–8 bicyclic carbon skeleton.

The thousands of naturally occurring terpenes are all derived from only a few linear and achiral precursors that are converted by terpene cyclases into (poly)cyclic homochiral compounds that usually have several stereogenic centres.[1] At least 121 different carbon skeletons are accessible from the sesquiterpene precursor farnesyl diphosphate (FPP) through the action of sesquiterpene cyclases (see Figure S1 in the Supporting Information). Most of these structures (75%) contain at least one six-membered ring (Table S1 in the Supporting Information). Five-membered rings are also particularly widespread (69%), while three- (21%) and seven-membered rings (24%) occur less often. Cyclobutane (10%) and cyclooctane (7%) systems are rare, and even larger rings are only present in single cases. Cyclooctane rings are easily recognized in the 5–8 bicyclic asteriscane sesquiterpenes asteriscanolide (**1**), which was the first isolated compound from this family,[2] asteriscadiene (**2**),[3] and 3α-hydroxyasterisc-6-ene (**3**),[4] as well as the dactylane sesquiterpenes dactylol (**4**) and poitediol (**5**), which are classified as "nonisoprenoid" because of their rearranged skeletons (Scheme 1).[5] In a few other sesquiterpenes, including clovanes, isopatchoulanes, longibornanes, longicyclanes, longifolanes, prenopsanes, and seychellanes, the presence of an 8-membered ring is less obvious (in bold in Figure S1). While the cyclooctane system is a rare structural element of sesquiterpenes, this motif is frequently seen in a number of important di- and sesterterpenes, such as fusicoccadiene,[6] the ophiobolins,[7] sesterfisherol,[8] taxa-4,11-diene,[9] cyclooctat-9-en-7-ol,[10] and odyverdiene A.[11] Herein, we report on the characterization of a bacterial terpene cyclase from *Streptomyces pristinaespiralis* ATCC 25486 that produces a 5–8 bicyclic sesquiterpene with a new skeleton.

S. pristinaespiralis encodes three type I terpene cyclases in its genome, one of which (WP_005317515) was recently characterized as selina-4(15),7(11)-diene synthase.[12] For another cyclase (WP_005321403), the function of a geosmin synthase can be assigned by homology to the characterized enzyme from *Streptomyces coelicolor*[13] and based on the reported geosmin production.[14]

The gene of the third terpene cyclase (WP_005320742, this is a stand-alone gene that is not part of an obvious biosynthetic gene cluster) was cloned into the vector pYE-Express and expressed in *Escherichia coli*.[15] Translation of the gene sequence showed that all highly conserved motifs for enzyme functionality, including the aspartate-rich motif (^{100}DDQFD), the pyrophosphate sensor (Arg-197), the NSE triad (^{243}NDIVSLVKE), and the ^{337}RY dimer, are present.[1] The purified protein efficiently converted FPP (60 mg) into a single sesquiterpene alcohol (10 mg, 33% yield, Figure S2), while geranyl diphosphate (GPP) and geranylgeranyl diphosphate (GGPP) were not accepted. The sesquiterpene alcohol was purified and its structure was established by NMR spectroscopy (Figures S3–S9). The ^{13}C-NMR and DEPT spectra showed signals for four Me groups, four aliphatic CH_2 groups, four CH groups (including one connected to oxygen), and one aliphatic and two olefinic quarternary carbons, which suggests the structure of a bicyclic sesquiterpene alcohol (Table 1). HSQC allowed to assign the signals of directly connected hydrogens. The ^{1}H,^{1}H-COSY spectrum revealed two contiguous spin systems: C1-2-3(-15)-4-5 and C8-9-10 (Scheme 2). HMBC correlations from H12 and H13 to C1, C11, and C10 placed C11 between C1 and C10. Further HMBC correlations were observed from H4 to C6, from H2 and H5 to C6 and C7, and from H14 to C6, C7, and C8, which established the position of the C6-7-14 portion to be between C2, C5, and C8. The relative configuration was established by NOESY correlations from H2 to H12 and H15, indicating that these are on one face of the molecule, while a NOESY correlation between H9 and H13 placed them on the opposite

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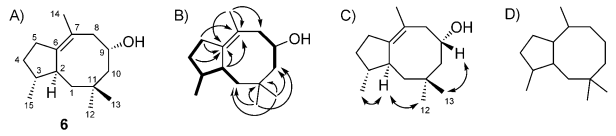
Table 1: NMR data of enzyme product **6** recorded in C_6D_6.

C[a]	^{13}C	^{1}H[b]
1	47.8 (CH_2)	1.35 (dd, ^2J = 13.9, ^3J = 11.5) 1.27 (dd, ^2J = 13.9, ^3J = 3.0)
2	48.7 (CH)	2.14 (m)
3	41.7 (CH)	1.53 (m)
4	31.9 (CH_2)	1.83 (dddd, ^2J = 12.4, ^3J = 9.1, ^3J = 6.2, ^3J = 6.2) 1.18 (dddd, ^2J = 12.4, ^3J = 8.9, ^3J = 6.2, ^3J = 6.1)
5	28.8 (CH_2)	2.25 (m) 2.09 (m)
6	141.9 (C_q)	–
7	123.0 (C_q)	–
8	42.4 (CH_2)	2.44 (dd, ^2J = 13.2, ^3J = 9.5) 2.30 (dd, ^2J = 13.3, ^3J = 4.3)
9	71.1 (CH)	3.82 (dddd, ^3J = 9.4, ^3J = 6.5, ^3J = 4.4, ^3J = 4.4)
10	46.4 (CH)	1.51 (m)
11	33.3 (C_q)	–
12	30.0 (CH_3)	1.07 (s)
13	32.8 (CH_3)	0.86 (s)
14	22.1 (CH_3)	1.64 (s)
15	20.0 (CH_3)	0.90 (d, ^3J = 6.8)

[a] Carbon numbering as shown in Scheme 2. [b] Multiplicities: s = singlet, d = doublet, t = triplet, m = multiplet; coupling constants J are given in Hz.



Scheme 2. A) Structure and carbon numbering of pristinol (**6**) (same as for FPP to allow labelling experiments to be easily followed). B) Contiguous spin systems by ^1H,^1H-COSY (bold) and key HMBC correlations (arrows). C) Key NOESY correlations (double headed arrows). D) Pristinane skeleton.

face. Monocrystals obtained by crystallization from pentane allowed us to determine the absolute configuration by single-wavelength anomalous dispersion (SAD) X-ray crystallography (Figure 1, Table S2), which resulted in the structure of (2*S*,3*S*,9*R*)-**6**. We propose the trivial name pristinol for **6**, which represents a new 5–8 bicyclic (pristinane) skeleton that was confirmed by ^{13}C,^{13}C-COSY NMR of completely labelled ($^{13}C_{15}$)-**6** obtained enzymatically from synthetic ($^{13}C_{15}$)FPP[16] (Figure S10). The method of ^{13}C,^{13}C-COSY NMR has previously been used in the structure elucidation of a number of other natural products that were completely labelled for this purpose.[17] Pristinol has an optical rotation of $[\alpha]_D^{21} = +12.9$ (*c* 0.23, CH_2Cl_2). Closely related enzymes that may also produce **6** are found in *Streptomyces* spp. NRRL F-5123 (70% identical sites) and URHA0041 (72% identity; Figure S11).

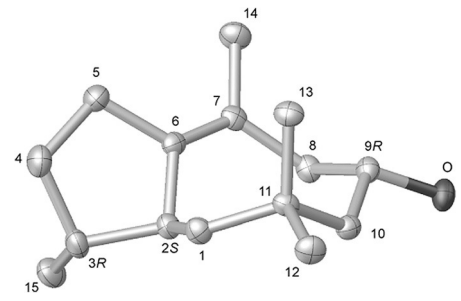
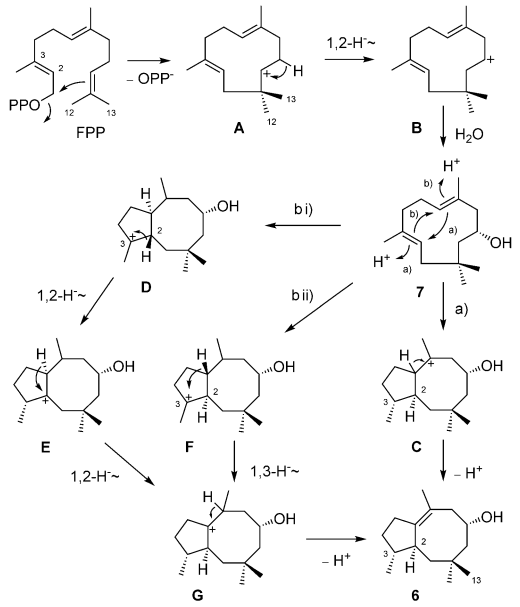


Figure 1. ORTEP of (2*S*,3*S*,9*R*)-**6** (Flack parameter: −0.02(6), further crystallographic parameters in Table S2).

Three cyclization mechanisms for the enzymatic conversion of FPP into **6** by the pristinol synthase are possible (Scheme 3). All three mechanisms start with the cyclization of FPP to the humulyl cation (**A**), a 1,2-hydride shift to **B**, and capture with water to (*R*)-hyemalol (**7**), a sesquiterpene alcohol that was first isolated from *Zanthoxylum hyemale* (Rutaceae)[18] and is also produced by a terpene cyclase from *Vitis vinifera*,[19] with unknown absolute configuration in the latter case. The most straightforward mechanism to **6** is a protonation of **7** at C3 followed by cyclization to **C** and deprotonation (pathway a). Alternatives are a protonation at C7, cyclization to **D**, and two sequential 1,2-hydride shifts via **E** to **G** that form **6** upon deprotonation (pathway b i). Furthermore, a protonation at C7 may induce a cyclization to **F**, a stereoisomer of **D** that can react to **G** through 1,3-hydride transfer (pathway b ii).



Scheme 3. Possible mechanisms for the enzymatic cyclization of FPP to **6**.

The participation of pathway a, the shortest and therefore supposedly most likely mechanism for the cyclization of **7** to **6**, was excluded by incubation with (3-^{13}C)FPP[16] with pristinol synthase in deuterium oxide. If this pathway were relevant, deuterium should be introduced at C3 of FPP through the reprotonation of **7**, resulting in (3-^{13}C,3-^{2}H)-**6** that should yield a triplet for C3 in the ^{13}C-NMR due to ^{13}C-^{2}H spin coupling, but the experiment shows a clear singlet for C3 (Figure 2 A). A similar experiment using (7-^{13}C)FPP in D_2O to investigate the alternative reprotonation of **7** at C7 as is relevant for both variants of pathway b was not performed,

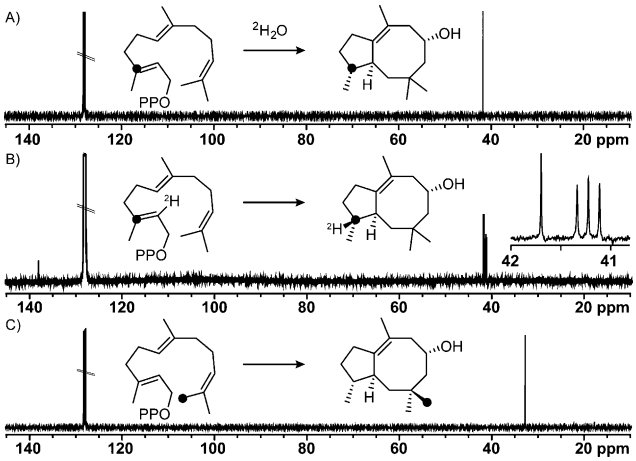
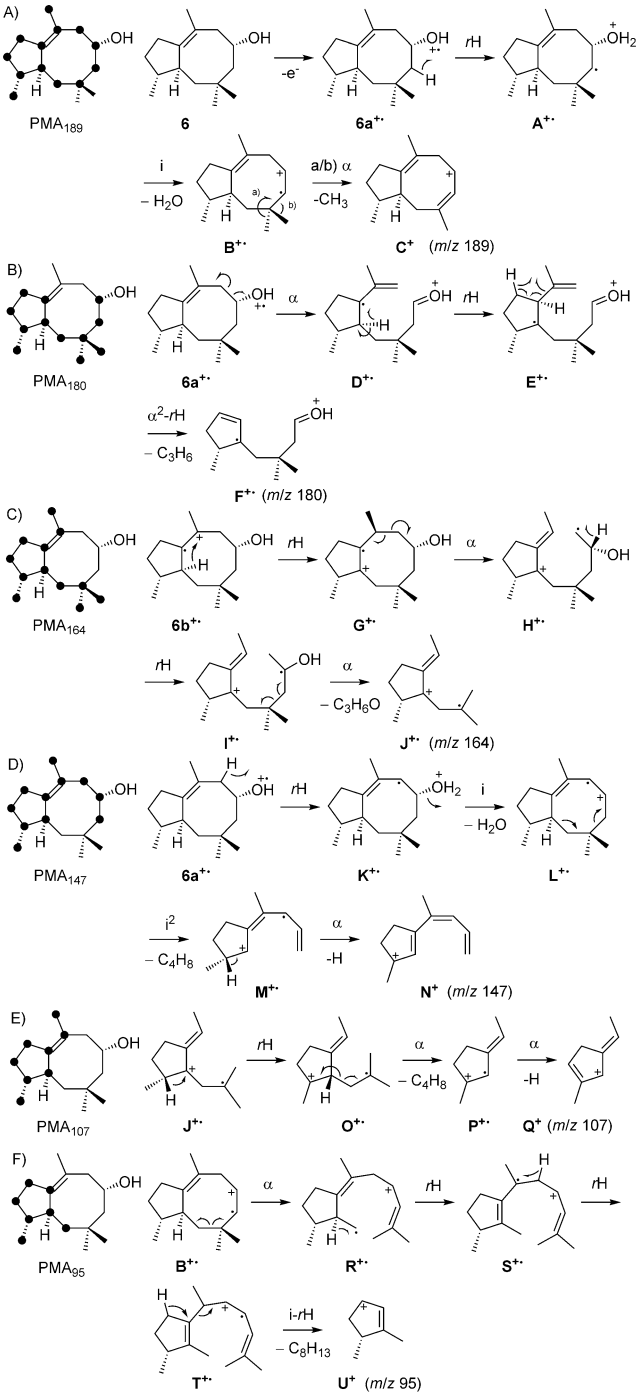


Figure 2. Mechanistic investigations for the cyclization of FPP to **6**. Black circles indicate ^{13}C-labelled carbons.

because the same deuterium that may be introduced by protonation of **7** will be lost in the final deprotonation step from **G**. Accordingly, the conversion of (3-^{13}C)FPP in D_2O did not shown any deuterium uptake by GC/MS (Figure S12). Incubation with (2-^{2}H,3-^{13}C)FPP (for synthesis, see Scheme S1) resulted in (3-^{13}C,3-^{2}H)-**6**, as demonstrated by a triplet for C3 in the ^{13}C-NMR (Figure 2B, the additional singlet is observed because of the 89% deuteration grade of the substrate), giving experimental evidence for the 1,2-hydride migration from **D** to **E** and thereby confirming pathway b i, while pathway b ii is ruled out. Finally, incubation with (13-^{13}C)FPP[16] resulted in the incorporation of labelling only into C13 of **6**, thus demonstrating a strict stereochemical course for the cyclization of FPP to **A**, with attack of C11 from the *Si* face (Figure 2C). A similar tight control of the fate of the geminal methyl groups has previously been reported for other terpene cyclases.[20] In summary, these experiments clearly indicate a cyclization of FPP to **6** through pathway b i, which is surprisingly not the most direct mechanism, thus demonstrating that the mechanism of terpene cyclases can only be established with certainty by experimental work with isotopically labelled precursors, as recently performed for several other terpene cyclases.[8,10,21] There have also been other cases, like cyclooctat-9-en-7-ol, where labelling experiments have revealed surprising mechanistic details.[10]

The EI-MS fragmentation mechanism of **6** was investigated by an approach that we have recently developed for *epi*-isozizaene.[22] The method makes use of the enzymatic conversion of all 15 isotopomers of ($^{13}C_1$)FPP[16] and investigation of the obtained products by GC/EI-MS (Figure S13). A position-specific mass-shift analysis ($PMA_{m/z}$) summarizes for a certain fragment ion m/z whether the labelling of a carbon atom causes an increase of this fragment ion by +1 or not (marked in black in Scheme 4, partially increased fragment ions are shown in gray and point to multiple mechanisms yielding this fragment ion that represent different parts of the analyte). The data obtained from the labelling experiments could be used to explain the formation of six major fragment ions in the mass spectrum of **6** by the



Scheme 4. EI-MS fragmentation of **6**. α: α-cleavage, i: inductive cleavage, *r*H: hydrogen rearrangement. Black and gray circles indicate carbon atoms that contribute fully or partially to a fragment ion.

mechanisms shown in Scheme 4 through well-established elementary steps, including α- and inductive cleavages, hydrogen rearrangements, and combinations thereof. A detailed description of the mechanisms is given in the Supporting Information.

In summary, we have identified a terpene cyclase from *S. pristinaespiralis* as (+)-(2*S*,3*S*,9*R*)-pristinol synthase. Its product is a sesquiterpene alcohol with a novel skeleton containing

an 8-membered ring, which is a rare feature in sesquiterpenes. Notably, production of neither this sesquiterpene nor its biosynthetic intermediate hyemalol could be observed in *S. pristinaespiralis* laboratory cultures (Figure S2). Isotopic labelling experiments unraveled a cyclization mechanism that does not represent the most straightforward cyclization reaction, which explicitly demonstrates that mechanistic investigations for each terpene cyclase by isotopic labelling experiments must be performed to obtain clear-cut evidence. Finally, we also provided detailed insight into the EI-MS fragmentation by performing isotopic labelling experiments. We will continue to investigate other interesting terpene cyclases and their products.

Acknowledgments

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Keywords: enzyme mechanisms · isotopic labelling · mass spectrometry · NMR spectroscopy · terpenes

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