Bacterial Terpenes

International Edition: DOI: 10.1002/anie.201507615
German Edition: DOI: 10.1002/ange.201507615

Conformational Analysis, Thermal Rearrangement, and EI-MS Fragmentation Mechanism of (1(10)*E*,4*E*,6*S*,7*R*)-Germacradien-6-ol by ^{13}C-Labeling Experiments

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Abstract: *An uncharacterized terpene cyclase from Streptomyces pratensis was identified as (+)-(1(10)E,4E,6S,7R)-germacradien-6-ol synthase. The enzyme product exists as two interconvertible conformers, resulting in complex NMR spectra. For the complete assignment of NMR data, all fifteen ($^{13}C_1$)FPP isotopomers (FPP = farnesyl diphosphate) and ($^{13}C_{15}$)FPP were synthesized and enzymatically converted. The products were analyzed using various NMR techniques, including ^{13}C,^{13}C COSY experiments. The (^{13}C)FPP isotopomers were also used to investigate the thermal rearrangement and EI fragmentation of the enzyme product.*

Terpenoids are structurally and functionally fascinating natural products. The first identified compounds from bacteria, the earthy and musty odorants geosmin and 2-methylisoborneol,[1] were isolated from streptomycetes in the 1960s, while recent research has shown that terpenes are particularly widespread in this taxon.[2] The biosynthesis of terpenes starts from a linear oligoprenyl diphosphate that is converted by a terpene cyclase in a reaction cascade via cationic intermediates into a (poly)cyclic hydrocarbon or alcohol, which usually has several contiguous stereocenters. Crystal structures of terpene cyclases[3] revealed that specific residues in the active site bind a trinuclear $(Mg^{2+})_3$ cluster that binds in turn to the substrate's diphosphate for ionization to a highly reactive cation. Hydrophobic residues shape a contour to force the substrate into a conformation for directed product formation and exclude water from the cavity to prevent quenching of immature intermediates. The products of several bacterial terpene cyclases have been characterized.[3g,4] Additionally, our structure-based mechanistic understanding of bacterial terpene cyclases has been substantially refined by quantum chemical calculations,[4s,5] site-specific mutations,[3g,h,4c,6] and isotope-labeling studies.[4b,g,q,7] Herein, we present a conformational analysis, the thermal rearrangement, and EI-MS fragmentation (EI-MS = electron impact mass spectrometry) of a sesquiterpene alcohol from *Streptomyces pratensis* by use of ^{13}C-labeling techniques.

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201507615.

The genome of *S. pratensis* ATCC 33331 encodes five terpene cyclases, four of which show close homology to the synthases for geosmin,[4c] 2-methylisoborneol,[4f,g] 7-*epi*-α-eudesmol,[4n] and *epi*-cubenol,[4l] in agreement with the production of these terpenes by the bacterium.[2a] The gene of the fifth uncharacterized terpene cyclase (accession number ADW03055; exhibiting the aspartate-rich motif ^{86}DDEYCD and the NSE triad ^{227}NDLVSYHKE) was cloned into the expression vector pYE-Express by homologous recombination in yeast.[4o] The purified protein converted farnesyl diphosphate (FPP) into (1(10)*E*,4*E*)-germacradien-6-ol (**1**), identified by GC–MS (Figure 1 A), while geranyl and geranylgeranyl diphosphate were not accepted. Two of the Cope rearrangement products of **1** (**2a** and **2b**) were also observed as a result of the thermal impact of the GC analysis (the mass spectra of **1**, **2a**, and **2b** are shown in Figure S1 in the Supporting Information). The ^{1}H and ^{13}C NMR spectra of **1** recorded in $CDCl_3$ at room temperature showed broad and poorly resolved signals (Figure S2). In contrast, the NMR spectra at −50 °C and at 0 °C showed two sets of sharp signals for the known conformers **1a** and **1b** (as shown in Figure 1 B by the "up/down" nomenclature for the methyl group pointing upwards (U) or downwards (D)).[8] The NMR data corresponded to reported, but incomplete, data for (−)-**1** from *Santolina rosmarinifolia*.[8a] The optical rotary power of $[\alpha]_D^{24} = +21.4$ for the compound pointed

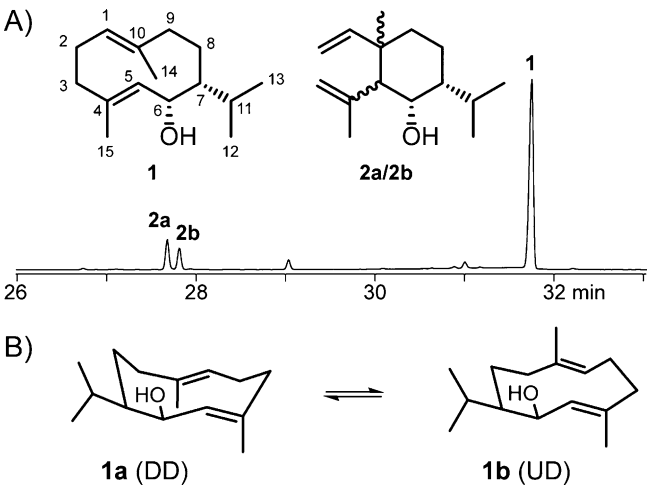

Figure 1. A) Total ion chromatogram of the sesquiterpene products from the *S. pratensis* (1(10)*E*,4*E*,6*S*,7*R*)-germacradien-6-ol (**1**) synthase. B) Structures of conformers **1a** and **1b**. U indicates that the methyl group is pointing up, D that the methyl group points downwards.

to the opposite enantiomer as in the plant ($[\alpha]_D^{20} = -14.8$), establishing the terpene from *Streptomyces pratensis* as (+)-(1(10)*E*,4*E*,6*S*,7*R*)-germacradien-6-ol. The absolute configuration of the plant metabolite **1** was recently confirmed by total synthesis.[9] Unlike the other terpene synthase products, **1** is not found in *S. pratensis* laboratory cultures (not shown).

The complex NMR spectra prevented a full assignment of the ^{1}H and ^{13}C NMR signals to **1a** and **1b**, as in a previous report.[8a] To overcome this problem, all fifteen ($^{13}C_1$)FPP isotopomers were synthesized (Figures S3–6)[10] and converted with germacradienol synthase. Each product was extracted with ($^2H_8$)toluene and analyzed by ^{13}C NMR spectroscopy, resulting in two strong signals for the labeled carbons of conformers **1a** and **1b** (Figure S7). These data unambiguously established which ^{13}C NMR signals of **1** belonged to which carbon center, but it was not possible to assign which of the two ^{13}C NMR signals observed in each single experiment belonged to which conformer. Therefore, completely labeled ($^{13}C_{15}$)FPP was synthesized and incubated with germacradienol synthase and the product was analyzed by ^{13}C,^{13}C COSY NMR experiments.[11] This experiment revealed two distinct sets of cross-peaks (Figure 2; for an enlarged version see Figure S8) that allowed for an unambiguous assignment of all 30 carbon signals to each of the 15 carbon atoms of **1a** and **1b** (Table 1). The assignment of most ^{1}H NMR resonance signals was possible from ^{1}H,^{1}H COSY, HSQC, and HMBC correlations (Figure 3) of the unlabeled compound. For a few cases, the HSQC spectra of the relevant ($^{13}C_1$)-**1** isotopomers were very useful (Figure S9).

For structure elucidation of the two Cope rearrangement products observed during GC–MS analysis, **1** was subjected to a microwave reaction in toluene at 225 °C. The products were separable by column chromatography and proved to be identical to **2a** and **2b** in terms of their mass spectra and GC retention times. Their structures were determined by one- and two-dimensional NMR spectroscopy (Table 1), resulting in their identification as shyobunol (**2a**) and 5,10-di-*epi*-shyobunol (**2b**).[12] The relative configurations were determined from key NOESY correlations (Figure 4 A). The assignment of NMR data was confirmed by Cope rearrangement of ($^{13}C_{15}$)-**1**, obtained by enzymatic conversion of ($^{13}C_{15}$)FPP, and subsequent analysis of the product by ^{13}C,^{13}C COSY NMR experiments (Figure S10). From these experiments, two distinct sets of cross-peaks were detected for **2a** and **2b** that gave direct insights into the carbon–carbon connectivities. The absolute configurations of **2a** and **2b** can be deduced from the stereocenters at C-6 and C-7 of **1** that are not affected by the Cope rearrangement, as is known for various

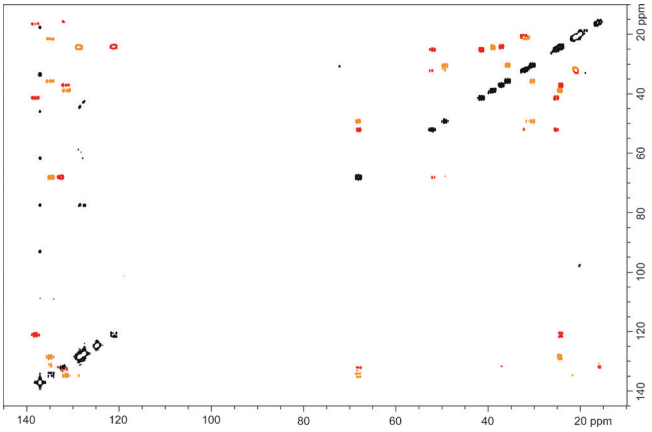

Figure 2. ^{13}C,^{13}C COSY spectrum of ($^{13}C_{15}$)-**1** obtained by enzymatic conversion of ($^{13}C_{15}$)FPP. The two sets of cross-peaks for the conformers are shown in yellow (for **1a**) and red (for **1b**).

Table 1: NMR data of the conformers **1a** and **1b** of (1(10)*E*,4*E*,6*S*,7*R*)-germacradien-6-ol in ($^2H_8$)toluene recorded at −50 °C, and of **2a**/**2b** in ($^2H_6$)benzene at 25 °C.[a]

1+**2** C[a]		**1a** (DD) ^{1}H	^{13}C	**1b** (UD) ^{1}H	^{13}C	**2a** ^{1}H	^{13}C	**2b** ^{1}H	^{13}C
1	CH	4.80 (d, *J* = 11.6, 1 H)	129.0	4.87 (t, *J* = 7.5, 1 H)	121.5	5.78 (dd, *J* = 17.5, 10.8, 1 H)	150.3	5.85 (dd, *J* = 17.6, 10.8, 1 H)	150.2
2	CH_2	2.17 (m, 1 H)	24.8	2.24 (m, 1 H)	24.6	4.98 (dd, *J* = 17.5, 1.2, 1 H, *E*)	110.1	4.90 (dd, *J* = 17.6, 0.8, 1 H, *E*)	110.4
		1.95 (m, 1 H)		1.89 (m, 1 H)		4.94 (dd, *J* = 10.8, 1.2, 1 H, *Z*)		4.86 (dd, *J* = 10.8, 0.8, 1 H, *Z*)	
3	CH_2	2.04 (m, 1 H)	39.2	2.09 (m, 1 H)	37.5	5.02 (br s, 1 H, *Z*)	113.3	4.90 (br s, 1 H)	113.9
		2.00 (m, 1 H)		1.94 (m, 1 H)		4.91 (br s, 1 H, *E*)		4.70 (br s, 1 H)	
4	C_q	–	131.8	–	132.0	–	146.8	–	145.5
5	CH	5.06 (d, *J* = 7.0, 1 H)	135.1	5.04 (d, *J* = 8.5, 1 H)	133.0	1.69 (d, *J* = 1.7, 1 H)	56.6	2.32 (d, *J* = 6.8, 1 H)	57.4
6	CH	4.54 (d, *J* = 6.0, 1 H)	68.5	4.55 (d, *J* = 6.0, 1 H)	68.4	3.81 (br s, 1 H)	70.2	3.94 (m, 1 H)	71.9
7	CH	0.75 (d, *J* = 9.0, 1 H)	49.7	0.66 (d, *J* = 9.5, 1 H)	52.5	0.74 (m, 1 H)	49.8	1.54 (m, 1 H)	44.6
8	CH_2	1.95 (m, 2 H)	30.7	1.80 (m, 1 H)	25.6	1.63 (m, 1 H)	21.1	1.56 (m, 2 H)	22.3
		1.39 (d, *J* = 13.8, 1 H)		1.30 (m, 1 H)		1.49 (m, 1 H)			
9	CH_2	2.44 (d, *J* = 13.1, 1 H)	36.1	2.14 (m, 1 H)	41.8	1.48 (m, 1 H)	41.1	1.53 (m, 1 H)	33.7
		1.62 (t, *J* = 13.5, 1 H)		1.79 (m, 1 H)		1.29 (m, 1 H)		1.25 (m, 1 H)	
10	C_q	–	135.3	–	138.5	–	40.2	–	30.3
11	CH	1.78 (m, 1 H)	32.0	1.73 (m, 1 H)	32.6	1.68 (m, 1 H)	29.4	1.88 (oct, *J* = 6.8, 1 H)	27.4
12	CH_3	1.00 (d, *J* = 6.0, 3 H)	21.4	1.09 (d, *J* = 6.3, 3 H)	21.2	0.92 (d, *J* = 6.8, 3 H)	20.8	0.94 (d, *J* = 6.8, 3 H)	21.9
13	CH_3	1.05 (d, *J* = 6.5, 3 H)	21.6	1.00 (d, *J* = 6.0, 3 H)	21.3	0.94 (d, *J* = 6.9, 3 H)	21.3	1.20 (d, *J* = 6.6, 3 H)	23.1
14	CH_3	1.55 (s, 3 H)	22.0	1.48 (s, 3 H)	16.9	1.46 (s, 3 H)	20.3	0.93 (s, 3 H)	23.1
15	CH_3	1.32 (s, 3 H)	16.3	1.30 (s, 3 H)	16.2	1.72 (s, 3 H)	27.8	1.68 (s, 3 H)	26.8

[a] Carbon numbering as shown in Figure 1. Chemical shifts δ in ppm, multiplicity m (s = singlet, d = doublet, t = triplet, oct = octet, m = multiplet, br = broad), coupling constants *J* are given in Hertz. Carbon assignments for **1** were deduced from incubation experiments with ^{13}C-labeled FPP isotopomers (see the main text).

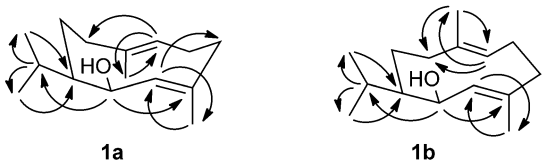

Figure 3. Key HMBC correlations that enabled the assignment of ^{1}H NMR signals of unlabeled **1**.

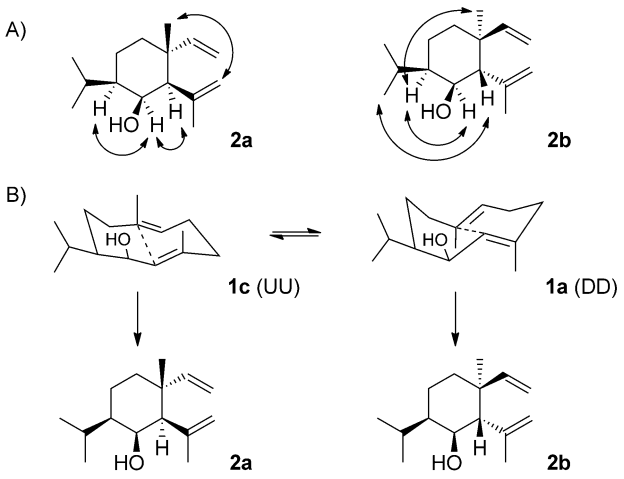

Figure 4. Cope rearrangement of **1**. A) Key NOESY correlations for determination of the relative configurations of **2a** and **2b**. B) Conformations of **1** explaining the formation of **2a** and **2b**.

germacranes.[13] The bacterial shyobunol stereoisomers isolated here are the enantiomers of plant compounds from *Acorus calamus*,[12] in agreement with the fact that also bacterial **1** is the optical antipode of a plant terpene. Whereas **2b** arises from the reported conformer **1a**,[8a] the formation of **2a** is possible from the up/up conformer **1c** (Figure 4B, UU). Both rearrangement products are formed via chair-like transition states, similar to the Cope rearrangements of several other germacranes.[8b,14]

The relative configurations of **2a** and **2b** gave additional support for the *syn* orientation of the hydroxy and isopropyl groups in **1**. In fact, the ^{13}C NMR chemical shifts of **1** and its *anti* stereoisomer kunzeaol are very similar (Table S11), and the *syn* or *anti* orientation of the substituents in the conformationally flexible compounds **1** and kunzeaol[15] are difficult to determine. However, the NOESY spectra of the more rigid compounds **2a** and **2b** unambiguously proved the *syn* arrangement of the hydroxy and the isopropyl groups, thereby giving indirect evidence for the correct assignment of the relative configuration of **1**.

Since isotopically labeled compounds are very useful in studying MS fragmentation mechanisms,[16] the products obtained from all fifteen isotopomers of (^{13}C)FPP with germacradienol synthase were also subjected to GC/EI-MS and GC/EI-MS-QTOF analysis. The observed fragment ion patterns for the isotopomers of (^{13}C)-**1** (Figure S11) gave direct insight into the fragmentation mechanism. Electron impact ionization of **1** proceeds preferably with the loss of one electron from an oxygen lone pair to yield the molecular ion

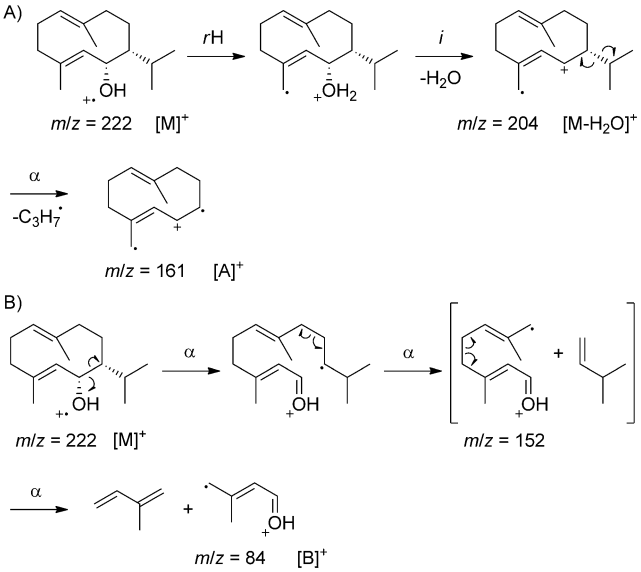

Figure 5. EI-MS fragmentation of **1**. Mechanisms for the formation of fragment ion $[A]^+$ ($m/z = 161$) and the base peak ion $[B]^+$ ($m/z = 84$).

$[M]^+$ (Figure 5A) that is observed at $m/z = 222$ for unlabeled **1** and at $m/z = 223$ for all ($^{13}C_1$)-**1** isotopomers (Table S12). Rearrangement of one hydrogen atom (*r*H) and inductive cleavage (*i*) with the neutral loss of water yields the fragment ion $[M-H_2O]^+$ that is detected at $m/z = 204$ for natural **1** and at $m/z = 205$ for all ($^{13}C_1$)-**1** isotopomers. A subsequent α cleavage (α) with loss of the isopropyl group is the only relevant mechanism that yields fragment ion $[A]^+$. This is evident from the observation of $[A]^+$ at $m/z = 161$ in the mass spectrum for unlabeled **1** as well as for all isotopomers that contain ^{13}C labeling within the isopropyl group, that is, for the products obtained from (11-^{13}C)FPP, (12-^{13}C)FPP, and (13-^{13}C)FPP. All other ($^{13}C_1$)FPP isotopomers had a shifted signal for $[A]^+$ at $m/z = 162$ (Figure S11). This mechanism was further supported by HRMS data for $[A]^+$, which established its molecular formula as $C_{12}H_{17}^+$ for all signals at $m/z = 161$ or as $^{13}C_1{}^{12}C_{11}H_{17}^+$ for all at $m/z = 162$ (Table S12), and by MS^2 analysis showing the direct formation of $[A]^+$ from $[M-H_2O]^+$.

For the formation of the base peak $[B]^+$ at $m/z = 84$, the mechanism was shown to proceed by two α-cleavage reactions with the loss of the neutral molecule 3-methylbut-1-ene to a form a fragment ion at $m/z = 152$ and a third subsequent α cleavage with the neutral loss of isoprene (Figure 5B). The last step was confirmed by a shift of the signal for $[B]^+$ to $m/z = 85$ for all ($^{13}C_1$)-**1** isotopomers in which the isotopic labeling appears in the $[B]^+$ forming portion, that is for **1** derived from (1-^{13}C)FPP, (2-^{13}C)FPP, (3-^{13}C)FPP, (4-^{13}C)FPP, and (15-^{13}C)FPP (Figure S11, Table S12). Furthermore, HRMS data established the molecular formulae for molecular fragments with $m/z = 84$ ($C_5H_8O^+$) and $m/z = 85$ ($^{13}C_1{}^{12}C_4H_8O^+$). The formation of $[B]^+$ as a daughter ion from the fragment ion at $m/z = 152$ was investigated by MS^2 analysis, but because of the low abundance of this ion this experiment was inconclusive. The formation of $[B]^+$ is thus better described as a concerted process of three simultaneous α fragmentations.

In summary we have characterized a bacterial terpene cyclase from *S. pratensis* as (+)-(1(10)*E*,4*E*,6*S*,7*R*)-germacradien-6-ol synthase. Only one closely related homologue with 99.4% identical sites is found in *Streptomyces* sp. PAMC26508. As is typical for germacranes, **1** exists in different well-defined conformers that are observable by NMR at low temperatures. Extensive labeling experiments using synthetic ^{13}C-labeled FPP isotopomers enabled a full assignment of ^{1}H and ^{13}C NMR data of **1**, which had until this point not been possible for **1** and related germacranes as a result of their complex NMR spectra.[8] A thermal rearrangement of **1** via chair-like transition states yielded two products whose absolute configurations were deduced from the absolute configuration of **1**, while the relative configurations of the rearrangement products reconfirmed that **1** is different from its epimer kunzeaol. Using ^{13}C-labeled FPP isotopomers, we have also laid the groundwork for analyses of EI-MS fragmentation patterns of sesquiterpenes, and we present a first showcase study here. Future experiments in our laboratories will include the usage of the FPP isotopomers to address various other intricate problems of sesquiterpene chemistry.

Acknowledgements

This work was funded by the Deutsche Forschungsgemeinschaft (DI1536/7-1) and the Beilstein-Institut zur Förderung der Chemischen Wissenschaften with a Ph.D. scholarship to P.R. We thank Dr. Senada Nozinovic (Bonn) for NMR measurements.

Keywords: conformation analysis · isotopic labeling · mass spectrometry · NMR spectroscopy · terpenoids

How to cite: *Angew. Chem. Int. Ed.* **2015**, *54*, 13448–13451
Angew. Chem. **2015**, *127*, 13649–13653

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Received: August 14, 2015
Published online: September 11, 2015

