ChemPubSoc Europe

DOI: 10.1002/cbic.201402443

CHEMBIOCHEM Full Papers

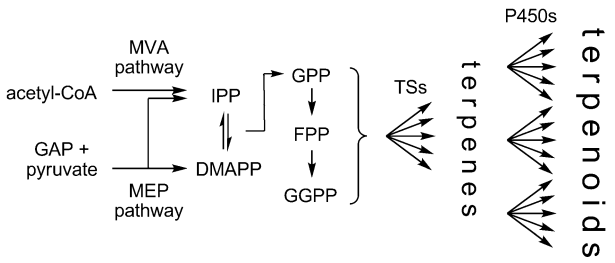
Characterization of the Gene Cluster CYP264B1-*geo*A from *Sorangium cellulosum* So ce56: Biosynthesis of (+)-Eremophilene and Its Hydroxylation

Alexander Schifrin,[a] Thuy T. B. Ly,[a, b] Nils Günnewich,[a] Josef Zapp,[c] Verena Thiel,[d] Stefan Schulz,[d] Frank Hannemann,[a] Yogan Khatri,[a] and Rita Bernhardt*[a]

Terpenoids can be found in almost all forms of life; however, the biosynthesis of bacterial terpenoids has not been intensively studied. This study reports the identification and functional characterization of the gene cluster CYP264B1–*geo*A from *Sorangium cellulosum* So ce56. Expression of the enzymes and synthesis of their products for NMR analysis and X-ray diffraction were carried out by employing an *Escherichia coli* whole-cell conversion system that provides the geoA substrate farnesyl pyrophosphate through simultaneous overexpression of the mevalonate pathway genes. The geoA product was identified as a novel sesquiterpene, and assigned NMR signals unambiguously proved that geoA is an (+)-eremophilene synthase. The very tight binding of (+)-eremophilene (~0.40 μM), which is also available in *S. cellulosum* So ce56, and its oxidation by CYP264B1 suggest that the CYP264B1–*geo*A gene cluster is required for the biosynthesis of (+)-eremophilene derivatives.

Introduction

Terpenoids, the largest group of natural products, comprise more than 55000 different molecules. They are classified according to the number of isoprene units in their structures.[1] All terpenoids are biosynthesized by the mevalonate (MVA) pathway in archaebacteria and most eukaryotes, and by a mevalonate-independent pathway (methyl erythritol pathway, MEP) in most eubacteria. Both pathways lead to the universal linear precursors geranylpyrophosphate (GPP), farnesylpyrophosphate (FPP) and geranylgeranylpyrophosphate (GGPP).[2,3] The astonishing structural diversity of the terpenoids arises in consecutive biosynthetic steps: terpene synthases (TSs; or terpene cyclases) convert the pyrophosphates into linear, mono- or multicyclic terpenes (or terpene alcohols);[1–5] then, the terpenes are derivatized by terpene-modifying enzymes, such as cytochromes P450 (Scheme 1).[6–8]



Scheme 1. Biosynthesis pathways of terpenoids. GAP: glyceraldehyde-3-phosphate; IPP: isopentenylpyrophosphate; DMAPP: dimethylallylpyrophosphate; GPP: geranylpyrophosphate; FPP: farnesylpyrophosphate; GGPP: geranylgeranylpyrophosphate; TSs: terpene synthases; P450s: cytochrome P450s.

TSs share a common alpha-helical fold and two highly conserved motifs (the aspartate-rich motif DDXXD/E and the NSE/DTE triad) in the active site; these bind three essential divalent cations (Mg^{2+} in most cases).[1–3] Sequence similarity between TSs is rather low. This explains the structural variety within the active site and the broad spectrum of terpene skeletons.

Cytochromes P450s (heme monooxygenase enzymes) are ubiquitously distributed in all forms of life. They serve as the most important metabolizers of xenobiotics, and they are involved in the biosynthesis of natural products.[9] The most common reaction catalyzed by cytochromes P450 is the introduction of an oxygen atom into nonactivated carbon–hydrogen bonds, thereby resulting in hydroxylation of the substrate.[6] The substrates, with various structures from simple alkanes to highly substituted steroids and polyketides, are converted regio- and stereo-specifically by cytochromes P450.[6,8,10]

[a] *A. Schifrin, Dr. T. T. B. Ly, Dr. N. Günnewich, Dr. F. Hannemann, Dr. Y. Khatri, Prof. Dr. R. Bernhardt*
Universität des Saarlandes, Biochemie
Campus B2.2, 66123 Saarbrücken (Germany)
E-mail: ritabern@mx.uni-saarland.de

[b] *Dr. T. T. B. Ly*
Institute of Biotechnology
Vietnam Academy of Science and Technology (VAST)
18-Hoang Quoc Viet, Hanoi (Vietnam)

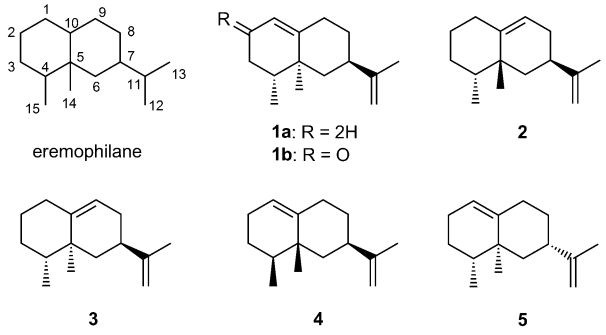
[c] *Dr. J. Zapp*
Universität des Saarlandes, Pharmazeutische Biologie
Campus C2.2, 66123 Saarbrücken (Germany)

[d] *Dr. V. Thiel, Prof. Dr. S. Schulz*
Technische Universität Braunschweig
Institut für Organische Chemie
Hagenring 30,38106 Braunschweig (Germany)

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.201402443.

Biosynthetic pathways involving both TSs and cytochromes P450 are known in plants,[11–14] fungi,[15] and more recently bacteria.[16–20] Volatile mono- (two isoprene subunits) and sesquiterpenoids (three isoprene subunits) are postulated to serve as means of chemical communication in animals, plants, insects, and microorganisms.[21–23]

For industrial production, plant terpenoids are of particularly high value,[24] because of their wide application as flavors and fragrances such as the grapefruit odorant (+)-nootkatone (Scheme 2, **1 b**),[25–30] as highly potent pharmaceuticals like the



Scheme 2. Eremophilane type sesquiterpenes: **1 a** (+)-valencene; **1 b** (+)-nootkatone; **2** 5-epiaristolochene; **3** (−)-aristolochene; **4** (−)-eremophilene; **5** (+)-eremophilene.

taxanes in tumor therapy,[31–32] and the anti-malarial drug artemisinin.[33] Cytochrome P450s are able to perform terpenoid hydroxylations[29,30] in a way that is superior to synthetic chemistry concerning selectivity and efficiency.[34–36] *Escherichia coli* and *Bacillus megaterium* have proven to be valuable hosts for in vivo cytochrome P450 bioconversions of terpenoids.[37–40] Heterologous combinations of TSs and cytochromes P450 as whole-cell biocatalysts show a high biosynthetic potential.[41]

The increasing number of sequenced bacterial genomes[42] has revealed a large number of putative TS[4–5] and cytochrome P450 genes.[43] The most widespread bacterial TS genes are 2-methylisoborneol (2-MIB) synthase,[44,45] germacradienol/geosmin synthase,[46,47] and epi-isozizaene (EIZ) synthase.[16,18,48] However, the number of functionally characterized TSs has increased only very recently.[49,50] Because of the low sequence similarities within the TS and cytochrome P450 families, it remains impossible to determine their physiological roles only from the available amino acid sequences. Functional characterization of the enzymes and structural analyses of their products are the only methods to elucidate their natural roles. The most common approach for structural analyses of the volatile terpenoids is GC/MS in EI mode combined with MS databases,[23] like NIST[51] or Massfinder,[52] and GC retention indices. However, this technique is not applicable for the identification of new structures.

Here we report the functional analysis of a gene cluster consisting of CYP264B1 (*sce*8551) and the terpene synthase *geo*A (*sce*8552) from the myxobacterium *Sorangium cellulosum* So ce56. CYP264B1 is one of 21 cytochrome P450s in *S. cellulosum* So ce56,[53,54] and has previously been shown to catalyze selective hydroxylation of (+)-nootkatone (**1 b**) and the norisoprenoids α- and β-ionone; however, smaller monoterpenes and bigger diterpenes and steroids were not able to bind.[55] *geo*A is one of three putative TS genes in *S. cellulosum* So ce56.[5] The in vitro enzymatic activity of geoA was examined, and *E. coli* whole-cell systems for the production of higher yields of the *geo*A and CYP264B1 products were developed. In addition, the molecular structure of geoA was elucidated by ^{1}H NMR, ^{13}C NMR, 2D-NMR, GC/MS, X-ray diffraction, and polarimetry; the in vivo CYP264B1 products were analyzed by GC/MS.

Results and Discussion

Bioinformatic analysis of the gene sequences, and vector construction

In the genome of *S. cellulosum* So ce56, a TS gene (*sce*8552) is located 63 bp downstream of CYP264B1 (*sce*8551), thus suggesting a putative gene cluster for TS and cytochrome P450. ORF *sce*8552 (annotated *geo*A in the NCBI database) is predicted to be a germacradienol/geosmin synthase.[5,46,47] Both the aspartate-rich motif (81-**DD**AYD-85) and the NSE triad (231-**N**DYF**S**LGK**E**-239) are present within its amino acid sequence.

For overexpression of *geo*A and CYP264B1, their nucleic acid sequences were optimized by employing the JAVA Codon Adaptation Tool (http://www.jcat.de)[56] and the Rare Codon Caltor (http://people.mbi.ucla.edu/sumchan/caltor.html). All *E. coli* rare codons were replaced.

In addition to their expression vectors (pET_geoA and pET22b_CYP264B1), vectors pAC-MvGo (Figure 1 A) and pET-C4oFA (Figure 1 B) were constructed for *E. coli* whole-cell conversion systems. For in vivo geoA synthesis, MVA pathway genes from *Streptomyces* sp. strain CL190 were co-expressed. For in vivo sesquiterpene oxidation by CYP264B1, the heterologous electron-transfer system consisting of Fpr and Adx[57] was used for the co-expression.

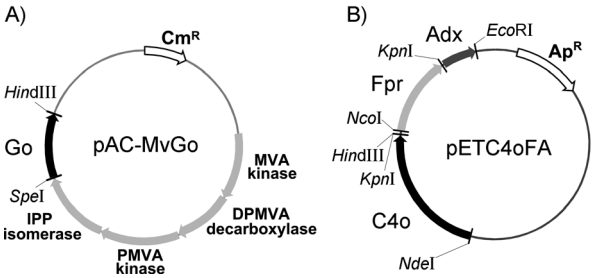


Figure 1. A) pAC-MvGo for *geo*A product synthesis, and B) pETC4oFA for synthesis of the oxidized *geo*A product in *E. coli* whole-cell systems; Gene abbreviations: Go: *geo*A; C4o: CYP264B1; Fpr: *E. coli* ferredoxin reductase; Adx: *Bos taurus* adrenodoxin.

Protein expression and purification

The purification of both TS and CYP264B1 was performed by affinity chromatography. In SDS-PAGE the masses of purified TS

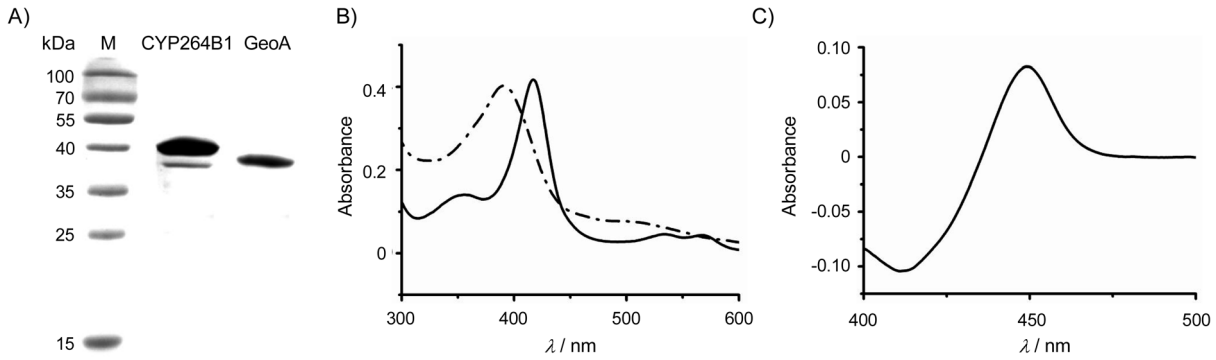


Figure 2. Analysis of purified proteins: A) SDS-PAGE of purified *geo*A gene product (GeoA) and purified CYP264B1; M: protein marker. B) UV-visible absorbance spectrum of oxidized CYP264B1 (4 μM) (——) and (+)-eremophilene (125 μM) bound CYP264B1 (-----). C) CO-difference spectrum of CYP264B1.

(~40 kDa) and CYP264B1 (~45 kDa) were in a good agreement with their predicted molar masses (38 and 46 kDa, respectively; Figure 2A). The UV–visible spectrum of CYP264B1 showed a characteristic Soret peak at 417 nm (Figure 2B). The observed CO-difference spectrum was stable for 1–2 min and did not show any appearance of a 420 nm peak (Figure 2C), whereas the 450 nm peak increased with time.[55] The yield of CYP264B1 after the purification, as determined by the CO-difference spectrum method, was 864 nmol L^{-1}.

Product analysis

A functional analysis of the gene cluster CYP264B1–*geo*A was conducted by structural characterization of the enzymatic products. In the first step, in vitro conversion by purified TS was performed to determine the molecular masses by GC/MS. Then, *E. coli* whole-cell conversion systems were used for the production of milligram quantities of the GeoA and CYP264B1 products for NMR and X-ray diffraction analyses.

In vitro conversion of FPP by geoA

In vitro conversion of FPP by the *geo*A TS resulted in a single 204 Da product (Figure 3A), which corresponds to a sesquiterpene with the chemical formula $C_{15}H_{24}$ (Figure 3B). A comparison of the EI-MS data of the sesquiterpene with the NIST Database (NIST/EPA/NIH Mass Spectral Library, Data Version NIST 05) gave eremophilene and valencene as the first two preliminary hits; both compounds have an eremophilane skeleton (Scheme 2). The GC/MS run of (+)-valencene (**1a**), as an internal standard, showed that the geoA sesquiterpene product and **1a** are different compounds (Figure 3C). Therefore, **1a** was excluded.

In vivo conversion of mevalonolactone by *geo*A

For a detailed structural analysis of the TS sesquiterpene product by NMR and X-ray diffraction, milligram quantities were produced in vivo with an *E. coli* whole-cell system and mevalonolactone as the substrate, rather than FPP (expensive with and tedious and time consuming purification of the sensitive terpene synthase). Thus, *E. coli* was transformed with vector pAC-MvGo, which harbors MVA pathway genes from *Streptomyces* sp. strain CL190 as well *geo*A. The purified TS and the strain produce the same single

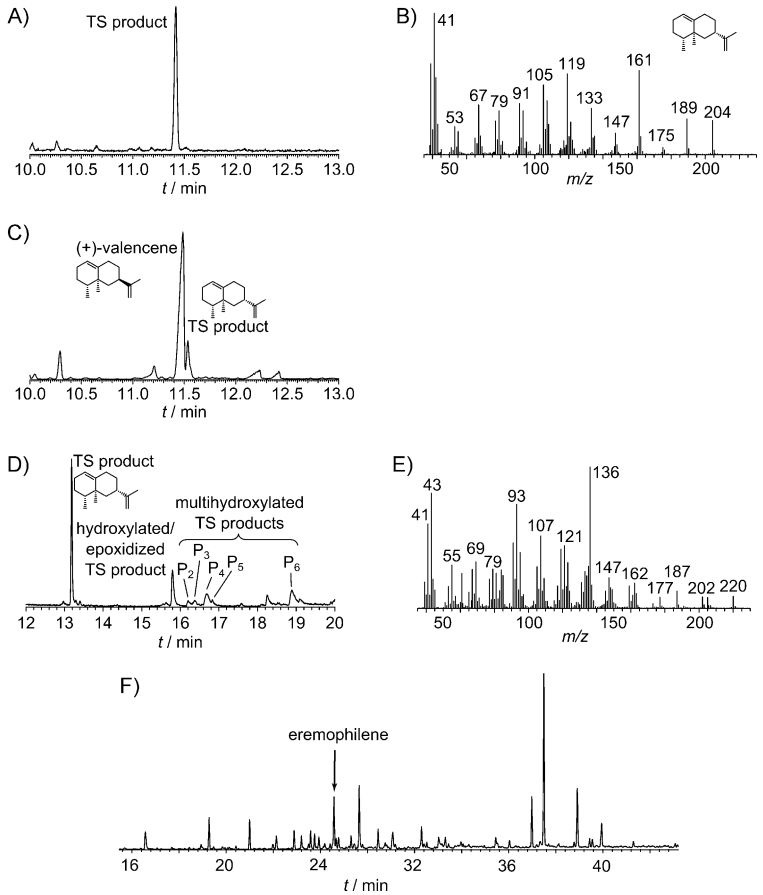


Figure 3. Total ion chromatograms (TICs) of terpenoids obtained by GC/MS. A) TIC of the in vivo conversion of mevalonolactone with *E. coli* harboring pAC-MvGo. B) Mass spectrum of the TS sesquiterpene product. C) TIC of the in vivo conversion of mevalonolactone with *E. coli* harboring pAC-MvGo with (+)-valencene (**1a**) as internal standard. D) TIC of the in vivo conversion of the TS sesquiterpene product with *E. coli* harboring pETC4oFA. E) Mass spectrum of the monohydroxylated/epoxidized TS product. F) TIC of the *S. cellulosum* So ce56 extract.

sesquiterpene product with a mass of 204 Da. The yield of the purified (+)-eremophilene was ~19 mg L^{-1} per day in *E. coli* cell culture.

Structural analysis of the TS sesquiterpene product

^{13}C,^{1}H NMR, 2D NMR and X-ray diffraction analyses of the purified sesquiterpene confirmed its relative stereochemistry in accordance with the structure of eremophilene (enantiomer **4** or **5**).

The specific rotation of the purified product was determined as $[\alpha]_D^{25} = +131.7$ ($c = 1.0$, $CHCl_3$). The absolute value is thus in agreement with previously reported data for enantiomer (−)-eremophilene (**4**) ($[\alpha]_D^{24} = -142.5$).[58] Therefore, the purified sesquiterpene was unambiguously identified as (+)-eremophilene (**5**; Figure 4; Figures S1–S7 in the Supporting Information).

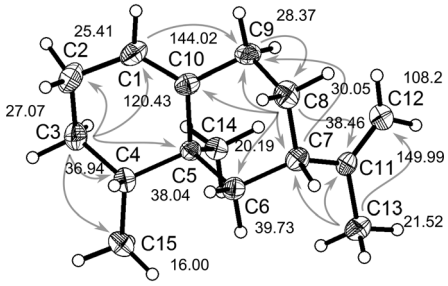


Figure 4. Crystal structure of (+)-eremophilene (**5**) at the 50 % probability level; gray arrows indicate key HMBC coupling interactions (H→C); the complete set of HMBC interactions are given in Scheme S1 and Figure S6.

Therefore, the TS is considered a (+)-eremophilene synthase.

A multitude of eremophilane-type sesquiterpenes have been isolated from different plant species and structurally elucidated by NMR, including the (+)-nootkatone (**1b**) precursor (+)-valencene (**1a**),[59] 5-epiaristolochene (**2**),[60] the precursor of the phytoalexin capsidiol,[61] (−)-aristolochene (**3**),[62] and (−)-eremophilene (**4**).[63] The structure of **4** has been a topic of discussion in a multitude of publications,[58,63–67] because the NMR signals from the nonfunctionalized hydrocarbons have similar chemical shifts and coupling constants. None of these studies gave a complete assignment of the corresponding atoms in the ^{1}H NMR spectrum.

When Hochmannová et al. first described the structural analysis of eremophilene isolated from *Petasites officinalis* and *Petasites albus*, the NMR data were sufficient to show the existence of a trisubstituted double bond and an *exo*-isopropylidene group.[63] Piers and Keziere obtained **4** by chemical synthesis and explained properly its configuration. ^{1}H NMR signals could clearly be assigned for H-1, H-12, H-13, H-14, and H-15,[64,65] as confirmed by Krepinsky et al.,[58] and Joulain and König.[67] However, because of multiple coupling and superposition of the ^{1}H NMR signals, the assignment for the secondary and tertiary carbons was hindered. Although Näf and co-workers synthesized various eremophilane derivatives (including **4**) and compared them by assignment of their ^{13}C NMR spectra, there is ambiguity in the assignment of the signals of C-8 and C-9.[66]

The present work included HSQC (Figure S5), and HMBC (Figure S6) data for **5** (Figure 4), the enantiomer of **4**, thus completing Näf's work for the assignment of the signals for tertiary carbon atoms C-4 and C-7, and secondary carbons C-8 and C-9. In the HMBC spectrum, the H-13 signal ($\delta = 1.71$ ppm, br s, 3H) shows ^{2}J coupling with C-11 ($\delta = 149.99$ ppm) and ^{3}J coupling with C-12 ($\delta = 108.20$ ppm), as well as the signal at $\delta = 38.46$ ppm, which can only belong to C-7. The signal from the two H-3 couples were via ^{3}J with C-1 ($\delta = 120.43$ ppm), C-5 ($\delta = 38.04$ ppm), and C-15 ($\delta = 20.19$ ppm), and via ^{2}J with C-2 ($\delta = 25.41$ ppm) and C-4 ($\delta = 36.94$ ppm). Thus, the ^{13}C signal at 36.94 ppm is exhibited by C-4. The adjacent carbon atoms (8 and 9) couple with each other's H atoms via ^{2}J.

The ^{13}C signal at 28.37 ppm couples with H-1 ($\delta = 5.32$ ppm), H-7 ($\delta = 2.01$ ppm) and the ^{1}H signals at 1.67 and 1.65 ppm, which are bound to the carbon atom at 30.05 ppm. These ^{1}H signals (besides the ^{13}C signal at 28.37 ppm) couple with C-6 ($\delta = 39.73$ ppm), C-7 ($\delta = 38.46$ ppm), C-10 ($\delta = 144.02$ ppm), and C-11 ($\delta = 149.99$ ppm). The peak at 28.37 ppm belongs to C-9, and the peak at 30.05 ppm belongs to C-8 (Figure 4). Thus, we unambiguously determined all ^{1}H and ^{13}C NMR signals for **5** (Table 1). The product was obtained as single crystals, which were subjected to X-ray diffraction. The result confirmed the NMR data.

Table 1. NMR data: Chemical shifts and multiplicities of (+)-eremophilene in $CDCl_3$, ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz) (Figure S1–S4).

C atom	^{13}C NMR [δ in ppm]	^{1}H NMR [δ in ppm, m]
1	120.43	5.32, m
2	25.41	2.02, m; 1.93, m
3	27.07	1.43, m, 2H
4	36.94	1.50, m
5	38.04	–
6	39.73	1.51, m, 2H
7	38.46	2.01, m
8	30.05	1.67, m; 1.65, m
9	28.37	2.37, m; 1.94, m
10	144.02	–
11	149.99	–
12	108.20	4.70, m; 4.68, m
13	21.52	1.71, br s, 3H
14	20.19	0.90, s, 3H
15	16.00	0.85, d, $J = 6.45$ Hz, 3H

Most importantly, (+)-eremophilene (**5**) was also detected as one of the most abundant among a multitude of sesquiterpenoids in the cell extract of *S. cellulosum* So ce56 (Figure 3F). This confirms the importance of this new compound in myxobacterial metabolism.

Binding and conversion of (+)-eremophilene (5) by CYP264B1

For the characterization of CYP264B1, purified **5** ((+)-eremophilene, its natural substrate) was employed for binding and conversion studies. The binding of **5** with CYP264B1 showed a high spin shift (~99%; Figure 2B), and very tight binding was

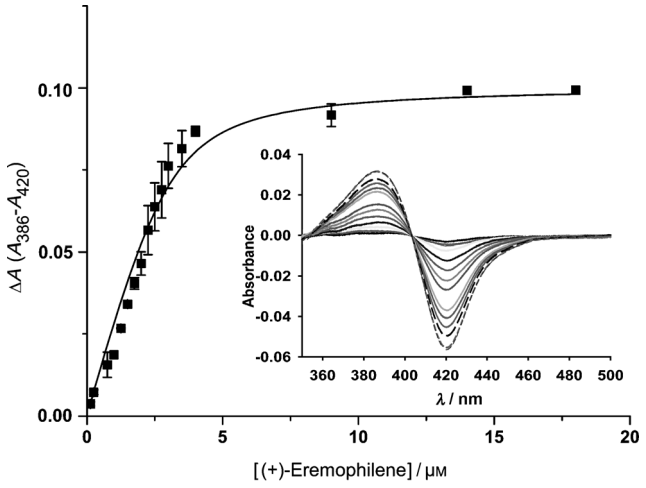


Figure 5. Spectral change of CYP264B1 with various concentrations of (+)-eremophilene; inset: spectral shifts induced by (+)-eremophilene binding to CYP264B1.

observed (K_d 0.40 μM; Figure 5). Both findings suggest the physiological relevance of **5** as a geoA product. During in vivo conversion, at least six products were observed in the extraction of **5** (Figure 3D). The most abundant one (t_R = 15.8 min, Figure 3D) had a monoisotopic mass of 220 Da, which corresponds to a monohydroxylated or epoxidized sesquiterpene with the formula $C_{15}H_{24}O$ (Figure 3E). The same product was also observed in an extract of a whole-cell system containing two strains, one harboring the plasmid pAC-MvGo and the other with pETC4oFA (data not shown). We also observed multihydroxylated products P_2–P_6 (Figure 3D) during the in vivo conversion of **5**. However, because of the low abundance of the products, the precise masses could not be accurately measured. (Figure S8–S12). Although we attempted to purify the monohydroxylated (+)-eremophilene product, we could not obtain it in a sufficient amount because of degradation or derivatization during the purification. Nevertheless, none of the CYP264B1 products were detected in a cell extract of *S. cellulosum* So ce56 (Figure 3F), thus suggesting further conversion or derivatization of the products by this bacterium.

Conclusion

We have proven the functional role of the gene cluster consisting of *geo*A and CYP264B1 from the myxobacterium *S. cellulosum* So ce56 by structural analysis of its sesquiterpenoid products. The geoA TS synthesizes the previously undescribed sesquiterpene (+)-eremophilene (**5**); the sesquiterpene hydroxylase CYP264B1 converts this compound into various products.

To the best of our knowledge, this is the first description of an (+)-eremophilene synthase and its product (**5**), which is the natural substrate of CYP264B1. Although Ghalib et al. reported the presence of **5** in the $CHCl_3$ extract of *Cinnamomum iners*, only GC/MS-TOF was employed for the analytical approach and product characterization. As this technique is not suitable for the determination of absolute stereochemistry, an unambiguous identification of **5** was not performed.[68] The detection of **5** as one of the most important sesquiterpenoids in the volatile fraction of the *S. cellulosum* So ce56 extracts proves its importance in myxobacterial metabolism (Figure 3F).

CYP264B1 oxidizes **5** (its physiological substrate) to various products (Figure 3D). The most abundant product has a mass of 220 Da, thus suggesting a monohydroxylated or epoxidized (+)-eremophilene derivative (Figure 3E). It is likely that this molecule is an intermediate in the biosynthesis of multioxidized eremophilanes or other (+)-eremophilene derivatives.

An eudesmane-type sesquiterpene adenoside with antibacterial properties has been isolated from *S. cellulosum* KM1003.[69] The fungal multioxidized eremophilene derivative sporogen-AO 1 has been found to have cytotoxic, phytotoxic, and antiviral properties.[70,71] Oxidized plant eremophilanes including petasins have been reported to have anti-inflammatory[72] and cytotoxic effects.[73]

Myxobacteria are known producers of terpenoids.[74–75] In the genome of *S. cellulosum* So ce56, a terpene pathway gene cluster is also evident,[53] thus probably highlighting a need for the TS product. As myxobacteria are known to form fruiting bodies upon starvation,[76] single cells need to communicate in order to facilitate the complex lifestyle,[77] and the emission and sensing of such volatile terpenes could be a mechanism of chemical communication. Trace amounts of eremophilene have also been detected in the volatiles extracted from another myxobacterial species, *Chondromyces crocatus*, though its stereochemistry has not been elucidated.[78] (−)-Eremophilene (**4**) has been isolated from a multitude of plants.[63,79–81] It was also found in extracts of the coral *Plexaurella fusifera*,[82] and has been synthesized by different chemical methods.[64–66] This work proves the existence of pairs of enantiomers of natural products: the (−)-enantiomer in plants and the (+)-enantiomer one in bacterium. This provides indirect proof of parallel evolution of enantiocomplementary TS enzymes in different species.

Experimental Section

Bioinformatic analysis of the gene sequences: The coding sequence for CYP264B1 (sce8551) was identified previously.[54,55] The sequences of the genes in immediate proximity of CYP264B1 were determined by using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The open reading frame *sce*8552 (*geo*A) showed sequence similarities with terpene synthases and was further investigated for the conserved aspartate rich DDXX(D/E) motif and NSE/DTE triad, (N/D)DXX(S/T)XX(K/R)(D/E).

Molecular cloning: For the genes *geo*A and CYP264B1, *E. coli* expression vectors (pET_geoA and pET22b_CYP264B1) and vectors for *E. coli* based whole-cell conversion systems (pAC-MvGo, pETC4oFA) were constructed. *geo*A was amplified PCR from genomic DNA of *S. cellulosum* So ce56 by using forward primer 5′-cca atc *ata tg*t cat ctg atc gaa cta gcg-3′ (NdeI restriction site in italics) and reverse primer 5′-cca at*a agc t*t cag tga tgg tga tgg tga tgt cca gcg cgc acg atc a-3′ (HindIII site in italics). The purified PCR product was inserted into pET-17b (Invitrogen) at NdeI and HindIII sites to create pET_geoA. For the construction of vector pAC-MvGo, a sequence-optimized *geo*A gene flanked by restriction sites SpeI and HindII (Life Technologies) was synthesized and cloned at the same restriction sites into the pAC-Mv vector,[83,84] which was generously

provided by Prof. Dr. Norihiko Misawa (Ishikawa Prefectural University, Ishikawa, Japan). The CYP264B1 expression vector pET22b_CYP264B1 was obtained by cloning the sequence-optimized *CYP264B1* gene flanked by restriction sites NdeI and HindIII (Life Technologies) at the same restriction sites into pET-22b (Invitrogen). The vector pETC4oFA was constructed from three fragments: NdeI/HindIII-flanked CYP264B1, EcoRI/HindIII-digested pET_MR8,[57] and NdeI/EcoRI-digested pET-17b.

Expression and purification of the *geo*A and CYP264B1 gene products: A single colony of *E. coli* BL21(DE3) or C43(DE3) cells co-transformed with pET_geoA and Gro12_groES–groEL[88] or pET22b_CYP264B1 was grown overnight in Nutrient Broth I (Sifin Diagnostics) containing ampicillin (100 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) at 37 °C with shaking. Terrific Broth medium (250 mL) containing these antibiotics in a 2 L Erlenmeyer flask was inoculated with overnight culture (2.5 mL) and grown under the same conditions until the absorbance (A_{600}) reached 0.6–1.0. Expression of proteins was induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM); for the expression of CYP264B1, 5-aminolevulinic acid (0.5 mM) was added to support heme synthesis. The cultures were left for 48 h at 28 °C with shaking (100 rpm). CYP264B1 was purified as described before.[55] For the purification of geoA, cells were harvested by centrifugation (10000 *g*, 10 min, 4 °C) and then resuspended in Tris·HCl buffer (50 mM, pH 7) containing NaCl (500 mM), imidazole (2.5 mM), mercaptoethanol (10 mM), glycerol (10%, *v/v*), and Tween 20 (1%, *v/v*), then lysed on ice with lysozyme (750 μg mL^{-1}, 1 h). The cell suspension was centrifuged (12000 *g*, 20 min, 4 °C), and the supernatant (containing the TS) was loaded on TALON resin (Clontech). The TS was purified according to the manufacturer's instructions. Purity of the fractions was assessed by SDS-PAGE. The pure fractions were pooled and loaded on PD-10 columns (GE Healthcare) for buffer exchange (HEPES buffer (500 μL, 50 mM, pH 7.5) containing dithiothreitol (2.5 mM), $MgCl_2$ (10 mM), KCl (10 mM), and glycerol (10%, *v/v*)). The TS was used immediately after purification. CYP264B1 was stored at −20 °C until further use.

Determination of the cytochrome P450 concentration: The concentration of cytochrome P450 was determined at room temperature in a UV2100PC double-beam spectrophotometer (Shimadzu Kyoto, Japan), based on the reduced CO-difference spectra method.[85] The enzyme in potassium phosphate buffer (10 mM, pH 7.4) with glycerol (20%) was reduced with a few grains of sodium dithionite and divided into two cuvettes. The baseline was recorded between 400 and 500 nm. The sample cuvette was bubbled gently with carbon monoxide for 1 min, and a spectrum was recorded. The concentration of functional CYP264B1 was estimated by using $\varepsilon_{450\text{-}490} = 91\ mM^{-1}cm^{-1}$.[85]

In vitro TS activity assay: The terpene cyclase assay was conducted in HEPES buffer (500 μL, 50 mM, pH 7.5) containing dithiothreitol (2.5 mM), $MgCl_2$ (10 mM), KCl (10 mM), and glycerol (10%, *v/v*) with the purified *geo*A gene product and FPP (10–30 μM). The assay was overlaid with *n*-hexane (500 μL) to trap the volatile terpenes and incubated for 1 h at 30 °C; the enzymatic reaction was stopped on ice. After incubation, the assay mixture was mixed vigorously, centrifuged (14000 *g*, 1 min), and the hexane phase was removed. The sample was extracted a second time, and the combined hexane phases were concentrated (200 μL) under a stream of N_2, and analyzed by GC/MS.

***E. coli* whole-cell conversion of mevalonolactone to (+)-eremophilene (5):** A single colony of *E. coli* C43(DE3) cells transformed with pAC-MvGo (or pAC-Mv for the negative control) was grown overnight in Nutrient Broth I containing chloramphenicol (30 μg mL^{-1}) at 37 °C with shaking (100 rpm). Terrific Broth medium (100 mL) containing chloramphenicol (30 μg mL^{-1}) in a 1 L baffled flask was inoculated with overnight culture (1 mL), and cells were grown under the same conditions until A_{600} reached 0.6–1.0. Expression was induced with IPTG (1 mM), mevalonolactone (500 μM) was added, and the flask was closed tightly. The cultures were left for 24 h at 30 °C with shaking (100 rpm). Conversion was stopped by cooling the flask on ice, and the products were extracted twice with ethyl acetate. The combined organic phases were dried over $MgSO_4$ and evaporated to dryness. An aliquot (2 mL) of the organic phase was concentrated (200 μL) under a stream of N_2 and analyzed by GC/MS. The whole-cell conversion extracts were kept at −20 °C until purification.

Spin-state shift and (+)-eremophilene binding: The spin-state shift upon (+)-eremophilene binding was assayed at room temperature under aerobic conditions in an UV-2101PC UV-Vis scanning photometer (Shimadzu) equipped with two tandem cuvettes containing CYP264B1 (1 μM) as previously described.[86] The dissociation constant (K_d) of (+)-eremophilene for CYP264B1 was calculated with SigmaPlot 9.0 (Systat Software, San Jose, CA) by fitting the peak-to-trough difference against the substrate concentration to the non-linear tight-binding quadratic Equation (1):

$$\Delta A = (A_{max}/2[E])\,\{(K_d + [E] + [S]) - \{(K_d + [E] + [S])^2 - 4\,[E]\,[S]\}^{1/2}\}$$

where ΔA is the observed peak-to-trough absorbance difference at each substrate addition, A_{max} is the maximum absorbance difference at substrate saturation, [E] is the total concentration of CYP264B1, and [S] is the (+)-eremophilene concentration.

***E. coli* whole-cell conversion of (+)-eremophilene (5) to oxidized eremophilenes:** A single colony of *E. coli* C43(DE3) cells transformed with pETC4oFA was grown overnight in Nutrient Broth I containing ampicillin (100 μg mL^{-1}) at 37 °C with shaking (100 rpm). Terrific Broth medium (100 mL) containing ampicillin (100 μg mL^{-1}) in a 1 L baffled flask was inoculated with 1 mL of the overnight culture, and cells were grown under the same conditions until A_{600} reached 0.6–1.0. Expression was induced with IPTG (1 mM), and 5-aminolevulinic acid (0.5 mM) was added. After 24 h at 30 °C with shaking (100 rpm) the cells were harvested by centrifugation (4000 *g*) and resuspended in potassium phosphate buffer (100 mL, 50 mM, pH 7.4) containing glycerol (4%, *v/v*). (+)-Eremophilene (**5**, 200 μM), IPTG (1 mM), and 5-aminolevulinic acid (0.5 mM) were added, and the conversion was carried out for another 24 h at 30 °C with shaking (100 rpm) in a sealed baffled flask. The reaction was stopped by cooling the flask on ice, and the products were extracted twice with ethyl acetate. The combined organic phases were dried over $MgSO_4$ and evaporated to dryness. An aliquot (2 mL) of the organic phase was concentrated (200 μL) under a stream of N_2 and analyzed by GC/MS. The whole-cell conversion extracts were kept at −20 °C until purification.

GC/MS analysis of the *S. cellulosum* So ce56 volatiles: *S. cellulosum* So ce56 was grown in liquid MD 1 medium as described before.[75] The cultures were connected to a closed-loop stripping apparatus, the volatiles were collected for 24 h, and then analyzed by GC/MS, according to the method of Fuhlendorff et al.[78]

GC/MS analysis of the produced terpenoids: Each sample was analyzed in a Focus GC/DSQ II GC/MS spectrometer (Thermo Scientific) equipped with an HP-5 capillary column (25 m × 0.32 mm i.d., 0.52 μm film; Agilent Technologies) with the following conditions: inlet pressure, 50 kPa; column flow, 10 mL min^{-1}; injection volume, 1 μL; transfer line, 280 °C; electron energy, 70.5 eV. The tempera-

ture gradient was 50 °C for 1 min, increasing (10 °C min^{-1}) to 310 °C, and holding at 310 °C for 5 min (operated in splitless mode). The carrier gas was He (1 mL min^{-1}). Mass spectra were recorded over *m/z* 35–350.

Product purification and characterization: Products for purification were obtained from in vivo conversions in a total reaction volume of 1.9 L. (+)-Eremophilene (**5**) was purified on silica gel with an isocratic eluent mixture of *n*-hexane and ethyl acetate (99:1). The fractions containing the product were identified by TLC in a solvent tank containing the same eluent and stained with anisaldehyde. The obtained product (12 mg) was used for NMR characterization. The NMR spectra of the product were recorded in $CDCl_3$ with a DRX 500 NMR spectrometer (Bruker). All chemical shifts (δ in ppm) are relative to $CHCl_3$ at $\delta = 7.24$ (^{1}H NMR) or $CDCl_3$ at $\delta = 77.00$ ppm (^{13}C NMR). The 2D NMR spectra were recorded as gradient-selected-(gs-) HH-COSY, gs-NOESY, gs-HSQC, and gs-HMBC. The optical rotation of **5** was analyzed on a model 241 Polarimeter (PerkinElmer) with (+)-valencene (**1a**) as the standard.

X-ray diffraction of 5: Crystals suitable for single-crystal X-ray analysis were obtained at −20 °C. The data were collected at −140 °C on an AXS X8 Apex CCD diffractometer (Bruker) with graphite-monochromatized Cu Kα radiation. Frames of 0.5° oscillation were exposed, thus deriving data in a θ range of 2–63.4° (completeness ~98%). Unit cell: orthorhombic, $P2_12_12_1$, $a = 6.0493(4)$, $b = 7.7094(4)$, $c = 27.274(2)$ Å, 1271.96(13) Å^3. Structure solution and full least-squares refinement with anisotropic thermal parameters of all non-hydrogen atoms and free refinement of the hydrogen were performed in SHELX.[87] The final refinement resulted in $R1 = 0.027$. The absolute structure could not be determined reliably. CCDC 1009046 contains the supplementary crystallographic data for this paper. These data can be obtained free of charge from The Cambridge Crystallographic Data Centre via www.ccdc.cam.ac.uk/data_request/cif

Acknowledgements

This work was supported by a grant from the Deutsche Forschungsgemeinschaft to R.B. (Be1343/23–1), and from the DAAD (Deutscher Akademischer Austauschdienst) as well as from the Vietnam National Foundation for Science and Technology Development (NAFOSTED) under grant number 106-NN.02–2013.57 to T.T.B.L. The authors are very grateful to Prof. Dr. Norihiko Misawa for providing the plasmid pAC-Mv, to Prof. Dr. Rolf Müller for providing the S. cellulosum *So ce56 extract, to Dr. Volker Huch for help with the X-ray diffraction analysis, to Reiner Wintringer for help with GC/MS measurements, to Prof. Dr. Andreas Speicher for help with the polarimetric measurements and to Dr. Michael Ringle and Dipl. Chem. Maël Charpentier for helpful advice.*

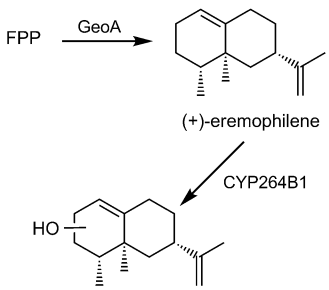
Keywords: cytochromes · eremophilenes · hydroxylation · *Sorangium cellulosum* So ce56 · terpenes

[1] Y. Gao, R. B. Honzatko, R. J. Peters, *Nat. Prod. Rep.* **2012**, *29*, 1153 – 1175.
[2] E. Oldfield, F.-Y. Lin, *Angew. Chem. Int. Ed.* **2012**, *51*, 1124 – 1137; *Angew. Chem.* **2012**, *124*, 1150 – 1163.
[3] D. J. Miller, R. K. Allemann, *Nat. Prod. Rep.* **2012**, *29*, 60 – 71.
[4] D. E. Cane, H. Ikeda, *Acc. Chem. Res.* **2012**, *45*, 463 – 472.
[5] Y. Yamada, D. E. Cane, H. Ikeda in *Methods in Enzymology, Vol. 515: Natural Product Biosynthesis by Microorganisms and Plants, Part A* (Ed.: D. Hopwood), Academic Press, San Diego, **2012**, pp. 123 – 162.
[6] R. Bernhardt, *J. Biotechnol.* **2006**, *124*, 128 – 145.
[7] J. Kirby, J. D. Keasling, *Annu. Rev. Plant Biol.* **2009**, *60*, 335 – 355.
[8] F. Hannemann, A. Bichet, K. M. Ewen, R. Bernhardt, *Biochim. Biophys. Acta* **2007**, *1770*, 330 – 344.
[9] R. Bernhardt, M. R. Waterman in *Metal Ions in Life Sciences Vol. 3: The Ubiquitous Roles of Cytochrome P450 Proteins* (Eds.: A. Sigel, H. Sigel, R. K. O. Sigel), Wiley, New York, **2007**, pp. 361 – 396.
[10] R. Bernhardt, V. Urlacher, *Appl. Microbiol. Biotechnol.* **2014**, *98*, 6185 – 6203.
[11] A. Aharoni, A. P. Giri, F. W. A. Verstappen, C. M. Bertea, R. Sevenier, Z. Sun, M. A. Jongsma, W. Schwab, H. J. Bouwmeester, *Plant Cell* **2004**, *16*, 3110 – 3131.
[12] D. P. Drew, B. Dueholm, C. Weitzel, Y. Zhang, C. W. Sensen, H. T. Simonsen, *Int. J. Mol. Sci.* **2013**, *14*, 9080 – 9098.
[13] B. Hamberger, D. Hall, M. Yuen, C. Oddy, B. Hamberger, C. Keeling, C. I. Ritland, K. Ritland, J. Bohlmann, *BMC Plant Biol.* **2009**, *9*, 106.
[14] B. Hamberger, T. Ohnishi, B. Hamberger, A. Séguin, J. Bohlmann, *Plant Physiol.* **2011**, *157*, 1677 – 1695.
[15] H.-C. Lo, R. Entwistle, C.-J. Guo, M. Ahuja, E. Szewczyk, J.-H. Hung, Y.-M. Chiang, B. R. Oakley, C. C. C. Wang, *J. Am. Chem. Soc.* **2012**, *134*, 4709 – 4720.
[16] B. Zhao, L. Lei, D. G. Vassylyev, X. Lin, D. E. Cane, S. L. Kelly, H. Yuan, D. C. Lamb, M. R. Waterman, *J. Biol. Chem.* **2009**, *284*, 36711 – 36719.
[17] B. Zhao, M. R. Waterman, *IUBMB Life* **2011**, *63*, 473 – 477.
[18] S. Takamatsu, X. Lin, A. Nara, M. Komatsu, D. E. Cane, H. Ikeda, *J. Microb. Biotechnol.* **2011**, *4*, 184 – 191.
[19] S. C. Moody, B. Zhao, L. Lei, D. R. Nelson, J. G. L. Mullins, M. R. Waterman, S. L. Kelly, D. C. Lamb, *FEBS J.* **2012**, *279*, 1640 – 1649.
[20] R. Quaderer, S. Omura, H. Ikeda, D. E. Cane, *J. Am. Chem. Soc.* **2006**, *128*, 13036 – 13037.
[21] J. Gershenzon, N. Dudareva, *Nat. Chem. Biol.* **2007**, *3*, 408 – 414.
[22] R. Kramer, W.-R. Abraham, *Phytochem. Rev.* **2012**, *11*, 15 – 37.
[23] S. Schulz, J. S. Dickschat, *Nat. Prod. Rep.* **2007**, *24*, 814 – 842.
[24] J. Zhou, G. Du, J. Chen, *Curr. Opin. Biotechnol.* **2014**, *25*, 17 – 23.
[25] M. A. Fraatz, R. G. Berger, H. Zorn, *Appl. Microbiol. Biotechnol.* **2009**, *83*, 35 – 41.
[26] H. Zorn, M. A. Fraatz, S. J. L. Riemer, M. Takenberg, U. Krings, R. G. Berger, S. Marx, K. Zelena, WO 2011147453 A1, **2011**.
[27] C. Gavira, R. Höfer, A. Lesot, F. Lambert, J. Zucca, D. Werck-Reichhart, *Metab. Eng.* **2013**, *18*, 25 – 35.
[28] D. Belsito, D. Bickers, M. Bruze, P. Calow, H. Greim, J. M. Hanifin, A. E. Rogers, J. H. Saurat, I. G. Sipes, H. Tagami, *Food Chem. Toxicol.* **2008**, *46*, S1 – S71.
[29] R. J. Sowden, S. Yasmin, N. H. Rees, S. G. Bell, L.-L. Wong, *Org. Biomol. Chem.* **2005**, *3*, 57 – 64.
[30] M. Girhard, K. Machida, M. Itoh, R. Schmid, A. Arisawa, V. Urlacher, *Microb. Cell Fact.* **2009**, *8*, 36.
[31] L. S. Bachegowda, D. F. Makower, J. A. Sparano, *Anticancer Drugs* **2014**, *19*, 19.
[32] B. Orlikova, N. Legrand, J. Panning, M. Dicato, M. Diederich in *Cancer Treatment and Research, Vol. 159: Advances in Nutrition and Cancer* (Eds.: V. Zappia, S. Panico, G. L. Russo, A. Budillon, F. Della Ragione), Springer, Berlin, **2014**, pp. 123 – 143.
[33] K. Bruxvoort, C. Goodman, S. P. Kachur, D. Schellenberg, *PLoS One* **2014**, *9*, e84555.
[34] S. Bleif, F. Hannemann, M. Lisurek, J. P. von Kries, J. Zapp, M. Dietzen, I. Antes, R. Bernhardt, *ChemBioChem* **2011**, *12*, 576 – 582.
[35] S. Bleif, F. Hannemann, J. Zapp, D. Hartmann, J. Jauch, R. Bernhardt, *Appl. Microbiol. Biotechnol.* **2012**, *93*, 1135 – 1146.
[36] S. Janocha, J. Zapp, M. Hutter, M. Kleser, J. Bohlmann, R. Bernhardt, *ChemBioChem* **2013**, *14*, 467 – 473.
[37] F. Hannemann, C. Virus, R. Bernhardt, *J. Biotechnol.* **2006**, *124*, 172 – 181.
[38] D. Zehentgruber, F. Hannemann, S. Bleif, R. Bernhardt, S. Lütz, *ChemBioChem* **2010**, *11*, 713 – 721.
[39] S. Janocha, R. Bernhardt, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 7639 – 7649.
[40] D. Schmitz, J. Zapp, R. Bernhardt, *FEBS J.* **2012**, *279*, 1663 – 1674.
[41] M. Farhi, E. Marhevka, J. Ben-Ari, A. Algamas-Dimantov, Z. Liang, V. Zeevi, O. Edelbaum, B. Spitzer-Rimon, H. Abeliovich, B. Schwartz, T. Tzfira, A. Vainstein, *Nat. Biotechnol.* **2011**, *29*, 1072 – 1074.
[42] M. J. Dark, *Infect. Drug Resist.* **2013**, *6*, 115 – 123.

[43] Y. Khatri, F. Hannemann, O. Perlova, R. Müller, R. Bernhardt, *FEBS Lett.* **2011**, *585*, 1506 – 1513.
[44] M. Komatsu, M. Tsuda, S. Ōmura, H. Oikawa, H. Ikeda, *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 7422 – 7427.
[45] C.-M. Wang, D. E. Cane, *J. Am. Chem. Soc.* **2008**, *130*, 8908 – 8909.
[46] B. Gust, G. L. Challis, K. Fowler, T. Kieser, K. F. Chater, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1541 – 1546.
[47] D. E. Cane, R. M. Watt, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 1547 – 1551.
[48] B. Zhao, X. Lin, L. Lei, D. C. Lamb, S. L. Kelly, M. R. Waterman, D. E. Cane, *J. Biol. Chem.* **2008**, *283*, 8183 – 8189.
[49] R. Riclea, C. A. Citron, J. Rinkel, J. S. Dickschat, *Chem. Commun.* **2014**, *50*, 4228 – 4230.
[50] P. Baer, P. Rabe, K. Fischer, C. A. Citron, T. A. Klapschinski, M. Groll, J. S. Dickschat, *Angew. Chem. Int. Ed.* **2014**, *53*, 7652 – 7656; *Angew. Chem.* **2014**, *126*, 7783 – 7787.
[51] F. W. McLafferty, *Wiley Registry of Mass Spectral Data, 7th Edition*, Wiley, New York, **2005**.
[52] Terpenoids Library http://massfinder.com/wiki/Terpenoids_Library.
[53] S. Schneiker, O. Perlova, O. Kaiser, K. Gerth, A. Alici, M. O. Altmeyer, D. Bartels, T. Bekel, S. Beyer, E. Bode, H. B. Bode, C. J. Bolten, J. V. Choudhuri, S. Doss, Y. A. Elnakady, B. Frank, L. Gaigalat, A. Goesmann, C. Groeger, F. Gross, et al., *Nat. Biotechnol.* **2007**, *25*, 1281 – 1289.
[54] Y. Khatri, F. Hannemann, K. M. Ewen, D. Pistorius, O. Perlova, N. Kagawa, A. O. Brachmann, R. Müller, R. Bernhardt, *Chem. Biol.* **2010**, *17*, 1295 – 1305.
[55] T. T. B. Ly, Y. Khatri, J. Zapp, M. Hutter, R. Bernhardt, *Appl. Microbiol. Biotechnol.* **2012**, *95*, 123 – 133.
[56] A. Grote, K. Hiller, M. Scheer, R. Münch, B. Nörtemann, D. C. Hempel, D. Jahn, *Nucleic Acids Res.* **2005**, *33*, W526 – W531.
[57] M. Ringle, Y. Khatri, J. Zapp, F. Hannemann, R. Bernhardt, *Appl. Microbiol. Biotechnol.* **2013**, *97*, 7741 – 7754.
[58] J. Křepinský, O. Motl, L. Dolejš, L. Novotný, V. Herout, R. B. Bates, *Tetrahedron Lett.* **1968**, *9*, 3315 – 3318.
[59] G. L. K. Hunter, W. B. Brogden, Jr., *J. Food Sci.* **1965**, *30*, 1 – 4.
[60] I. M. Whitehead, D. R. Threlfall, D. F. Ewing, *Phytochemistry* **1989**, *28*, 775 – 779.
[61] Y. Zhao, D. J. Schenk, S. Takahashi, J. Chappell, R. M. Coates, *J. Org. Chem.* **2004**, *69*, 7428 – 7435.
[62] T. R. Govindachari, P. A. Mohamed, P. C. Parthasarathy, *Tetrahedron* **1970**, *26*, 615 – 619.
[63] J. Hochmannová, V. Herout, *Collect. Czech. Chem. Commun.* **1964**, *29*, 2369 – 2376.
[64] E. Piers, R. J. Keziere, *Tetrahedron Lett.* **1968**, *9*, 583 – 586.
[65] E. Piers, R. J. Keziere, *Canadian J. Chem.* **1969**, *47*, 137 – 144.
[66] F. Näf, R. Decorzant, W. Thommen, *Helv. Chim. Acta* **1982**, *65*, 2212 – 2223.
[67] D. Joulain, W. A. König, *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons*, E.B. Verlag, Hamburg, **1998**.
[68] R. M. Ghalib, R. Hashim, O. Sulaiman, S. H. Mehdi, Z. Anis, S. Z. Rahman, B. M. K. Ahamed, A. M. S. Abdul Majid, *Nat. Prod. Res.* **2011**, *26*, 2155 – 2158.
[69] J.-W. Ahn, K. H. Jang, S.-C. Chung, K.-B. Oh, J. Shin, *Org. Lett.* **2008**, *10*, 1167 – 1169.
[70] S. Tanaka, K. Wada, S. Marumo, H. Hattori, *Tetrahedron Lett.* **1984**, *25*, 5907 – 5910.
[71] G. Schneider, H. Anke, O. Sterner, *Nat. Prod. Lett.* **1997**, *10*, 133 – 138.
[72] B. L. Fiebich, M. Grozdeva, S. Hess, M. Hüll, U. Danesch, A. Bodensieck, R. Bauer, *Planta Med.* **2005**, *71*, 12 – 19.
[73] R. T. Gallagher, J. Wei-Dong, J. Zhi-Dong, WO 1998000121 A1, **1998**.
[74] W. Lorenzen, M. W. Ring, G. Schwär, H. B. Bode, *J. Bacteriol.* **2009**, *191*, 5849 – 5853.
[75] J. S. Dickschat, T. Nawrath, V. Thiel, B. Kunze, R. Müller, S. Schulz, *Angew. Chem. Int. Ed.* **2007**, *46*, 8287 – 8290; *Angew. Chem.* **2007**, *119*, 8436 – 8439.
[76] S. Huntley, N. Hamann, S. Wegener-Feldbrügge, A. Treuner-Lange, M. Kube, R. Reinhardt, S. Klages, R. Müller, C. M. Ronning, W. C. Nierman, L. Søgaard-Andersen, *Mol. Biol. Evol.* **2011**, *28*, 1083 – 1097.
[77] D. Kaiser, *Curr. Opin. Microbiol.* **1998**, *1*, 663 – 668.
[78] S. Schulz, J. Fuhlendorff, H. Reichenbach, *Tetrahedron* **2004**, *60*, 3863 – 3872.
[79] J. C. Chalchat, J. L. Chabard, M. S. Gorunovic, V. Djermanovic, V. Bulatovic, *J. Essent. Oil Res.* **1995**, *7*, 147 – 152.
[80] W. S. Schlotzhauer, R. J. Horvat, O. T. Chortyk, S. F. Nottingham, D. M. Jackson, *J. Essent. Oil Res.* **1995**, *7*, 265 – 269.
[81] M. Tori, A. Watanabe, S. Matsuo, Y. Okamoto, K. Tachikawa, S. Takaoka, X. Gong, C. Kuroda, R. Hanai, *Tetrahedron* **2008**, *64*, 4486 – 4495.
[82] J. Frenz-Ross, J. Enticknap, R. G. Kerr, *Mar. Biotechnol.* **2008**, *10*, 572 – 578.
[83] H. Harada, K. Shindo, K. Iki, A. Teraoka, S. Okamoto, F. Yu, J.-i. Hattan, R. Utsumi, N. Misawa, *Appl. Microbiol. Biotechnol.* **2011**, *90*, 467 – 476.
[84] H. Harada, N. Misawa, *Appl. Microbiol. Biotechnol.* **2009**, *84*, 1021 – 1031.
[85] T. Omura, R. Sato, *J. Biol. Chem.* **1964**, *239*, 2379 – 2385.
[86] Y. Khatri, F. Hannemann, M. Girhard, R. Kappl, M. Hutter, V. B. Urlacher, R. Bernhardt, *FEBS J.* **2014**; DOI: 10.1111/febs.13104.
[87] G. M. Sheldrick, *Acta Crystallogr. Sect. A Found. Crystallogr.* **2008**, *64*, 112 – 122.
[88] K. Nishihara, M. Kanemori, M. Kitagawa, H. Yanagi, T. Yura, *Appl. Environ. Microbiol.* **1998**, *64*, 1694 – 1699.

Received: August 6, 2014
Published online on ■■ ■■, 0000

Terpene synthases (TSs) and cytochromes P450 are key enzymes in biosynthesis pathways of terpenoids. We report the functional characterization of the gene cluster *geo*A-CYP264B1 from *S. cellulosum* So ce56. The TS forms the previously unknown sesquiterpene enantiomer (+)-eremophilene, the physiological substrate of the cytochrome P450. The sesquiterpene hydroxylase CYP264B1 conducts multiple hydroxylations on (+)-eremophilene.



*A. Schifrin, T. T. B. Ly, N. Günnewich, J. Zapp, V. Thiel, S. Schulz, F. Hannemann, Y. Khatri, R. Bernhardt**

■■ – ■■

Characterization of the Gene Cluster CYP264B1-*geo*A from *Sorangium cellulosum* So ce56: Biosynthesis of (+)-Eremophilene and Its Hydroxylation