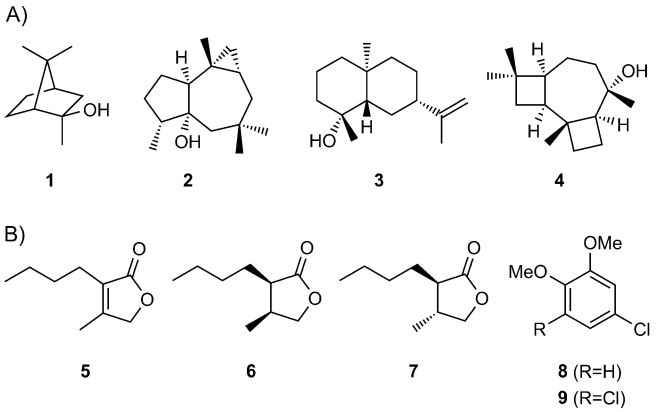
Erschienen in: ChemBioChem ; 15 (2014), 16. - S. 2379-2383
https://doi.org/10.1002/cbic.201402298

Pogostol Biosynthesis by the Endophytic Fungus *Geniculosporium*

Lena Barra,[a] Barbara Schulz,[b] and Jeroen S. Dickschat*[a]

Six ^{13}C-labelled isotopomers of mevalonolactone were synthesised and used in feeding experiments with the endophytic fungus *Geniculosporium*. The high incorporation rates of ^{13}C-label into a sesquiterpene that was found in headspace extracts of the fungus enabled unambiguous identification of this volatile as pogostol without the need for compound purification, simply by collecting the volatile fraction with a closed-loop stripping apparatus followed by direct ^{13}C NMR analysis (CLSA-NMR). The feeding experiments also gave insights into the biosynthesis of pogostol, including stereochemical aspects of the terpene cyclisation reaction. The possible biological function of pogostol is discussed.

Volatiles that are emitted by living organisms can be efficiently captured on charcoal filters by the use of a closed-loop stripping apparatus (CLSA). The experimental setup consists of a chamber containing a biological sample, and an air stream maintained by a pump and directed over the sample and then through the charcoal filter. The filter can then simply be extracted with an organic solvent, and the extract can be analysed by GC/MS.[1] This technique was originally developed in 1973 by Grob and Zürcher for trace analysis of volatile contaminants in water samples,[2,3] and has since been successfully applied in studies on volatile natural products, for example, those from insects,[4] bacteria[5,6] and fungi.[7] Unambiguous compound identification by GC/MS requires knowledge of the analyte's mass spectrum. For automated database searches, the mass spectra of thousands of compounds are collected in large electronic libraries.[8,9] In cases where several constitutional or stereoisomers of a compound have very similar mass spectra, the analyte can only be identified by additional comparison of its retention index to published retention indices. Ideally, positive compound identification in such cases requires knowledge of the retention indices of all eligible isomers. If this is not the case, synthesis of all isomers for direct comparison is a solution,[10,11] but this is highly laborious and impractical, especially with highly complex volatile natural products, such as most sesquiterpenes. An alternative approach is to purify the compound from culture extracts for structure elucidation by NMR spectroscopy. However, with volatiles significant losses in concentration steps can render the purification process unsuccessful. The major advantage of the CLSA technique is non-invasive detection of trace components in complex mixtures that cannot be analysed by NMR. Thus, to obtain NMR data from compounds in CLSA headspace extracts, we recently reported a new method; this combines feeding with ^{13}C-labelled precursors and CLSA headspace extraction for ^{13}C NMR analysis (CLSA-NMR).[12–15] The fed precursor must be one that is only incorporated into volatiles from a certain compound class (e.g., mevalonolactone or deoxyxylulose, only into terpenes). If incorporation rates are high, ^{13}C NMR analysis of the CLSA headspace extract results in a set of strongly enhanced ^{13}C NMR signals, and only of compounds from a particular class. By using this method, the volatiles 2-methylisoborneol (**1**),[12] isoafricanol (**2**),[13] eudesma-11-en-4α-ol (**3**)[14] and koraiol (**4**)[15] were identified in headspace extracts of different actinomycetes and ascomycete fungi (Scheme 1 A), and stereochemical aspects of their biosynthesis were elucidated. Here, we report the synthesis of ^{13}C-labelled mevalonolactone isotopomers, their use in feeding experiments with the endophytic fungus *Geniculosporium* sp., the identification of pogostol in headspace extracts and investigation of its biosynthesis by CLSA-NMR.



Scheme 1. A) Volatiles identified from actinomycetes and ascomycete fungi by CLSA-NMR. B) Previously identified volatiles from *Geniculosporium* sp.

In previous investigations, we identified a series of structurally related lactones (**5**–**7**; Scheme 1 B) and two chlorinated veratrole derivatives (**8** and **9**) by GC/MS in headspace extracts of *Geniculosporium* sp., an endophytic fungus that had been

[a] *L. Barra, Prof. Dr. J. S. Dickschat*
Kekulé-Institut für Organische Chemie und Biochemie
Rheinische Friedrich-Wilhelms-Universität Bonn
Gerhard-Domagk-Strasse 1, 53121 Bonn (Germany)
E-mail: dickschat@uni-bonn.de

[b] *Dr. B. Schulz*
Institut für Mikrobiologie
Technische Universität Braunschweig
Spielmannstrasse 7, 38106 Braunschweig (Germany)

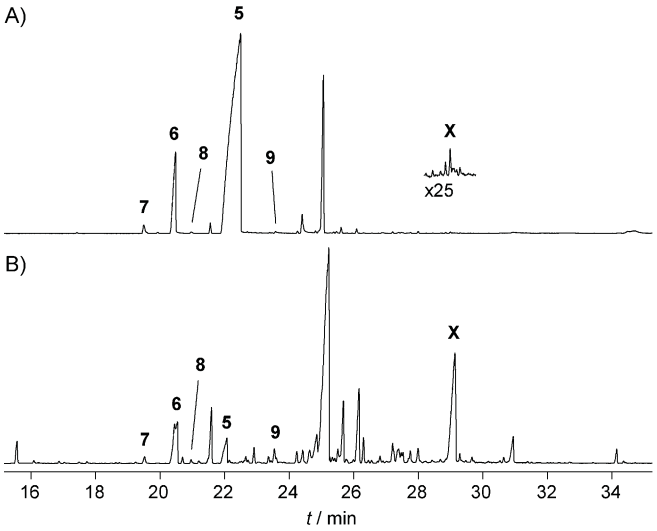
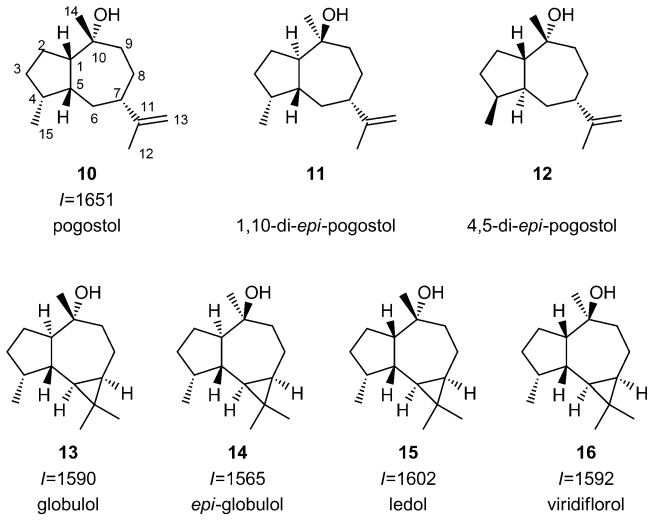


Figure 1. Total ion chromatograms of headspace extracts from *Geniculosporium* sp. cultured on potato-carrot agar medium at 20 °C, A) after three weeks of incubation, and B) after five weeks of incubation with feeding of mevalonolactone (5 mM) after four weeks. Peak numbers refer to compounds in Scheme 2. The intensity scales of chromatograms A and B are directly comparable.

isolated from *Cistus monspeliensis* and was grown on potato–carrot medium for three weeks at 20 °C (Figure 1 A).[7,10] A few other unidentified compounds and traces of a sesquiterpene alcohol "**X**" were also detected. The mass spectrum of the alcohol was most similar to that of pogostol (**10**) in the database, but as the quality of the mass spectrum of the trace compound **X** was poor, the next best matches, globulol (**13**) and *epi*-globulol (**14**), could not be excluded (Scheme 2 and Figure S1 in the Supporting Information). The structures of their stereoisomers, ledol (**15**) and viridiflorol (**16**), were excluded

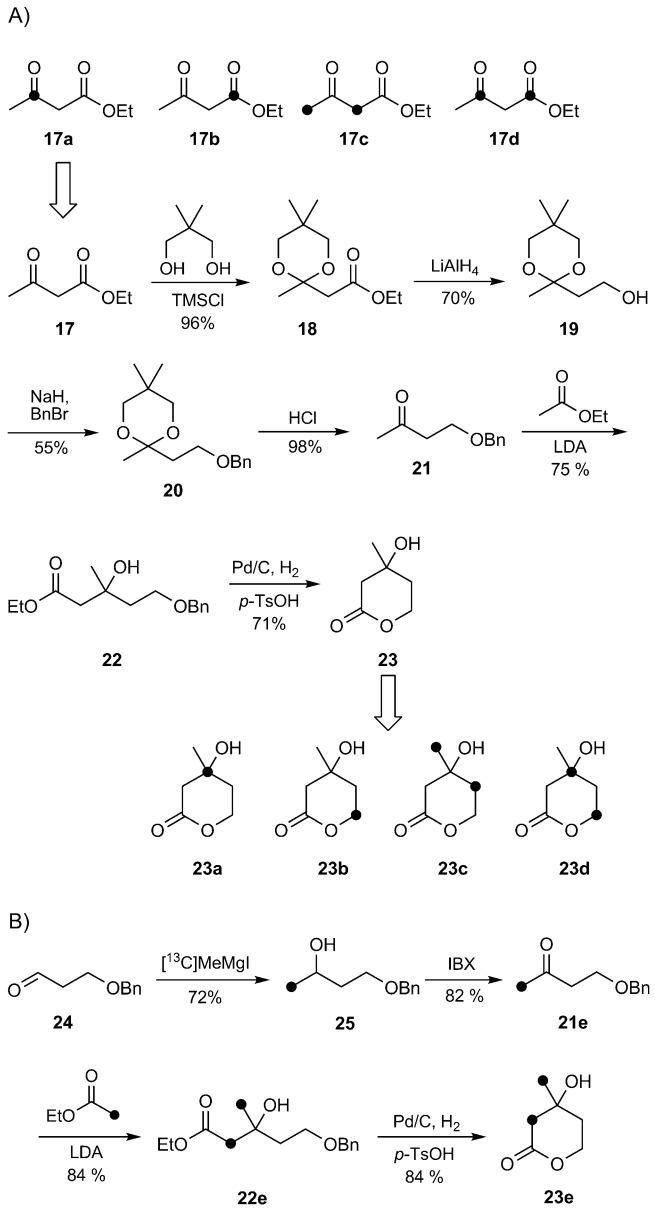


Scheme 2. Pogostol (**10**) and structurally related isomers. The retention index *I* for commercially available **14** (Sigma–Aldrich; analysed by GC-MS under the same conditions as the headspace extracts, vide infra) was determined on a HP5-MS fused silica capillary column. Retention indices of the other compounds are for a BP5 column and were taken from ref. [9].

based on their mass spectra (data not shown). Also, the retention index of **X** ($I = 1659$) matched best that of **10** ($I = 1651$), whereas the retention indices of **13**–**16** deviated significantly.[9] Unfortunately, published mass spectra and retention indices for all other stereoisomers of pogostol and of globulol were not available, so these compounds were also candidate structures for **X**. The ^{13}C NMR data for all five compounds[16–20] and the two pogostol stereoisomers (**11** and **12**)[21] have been reported, and therefore we strived to confirm the identities of **X** and **10** by obtaining ^{13}C NMR data for **X** by CLSA-NMR (note: the originally published structure of **10**[22] was later found to be incorrect[21] and then corrected;[23] in ref. [17] the wrong structure is shown for **13**). Therefore, we had to find culture conditions under which the production of **X** was significantly enhanced. The production of **X** was much higher after five weeks of incubation with feeding of mevalonolactone (5 mM) after four weeks (Figure 1 B), thus suggesting that the availability of terpenoid monomers was limiting.

For further investigations on the structure of **X** by CLSA-NMR, a series of ^{13}C-labelled isotopomers of mevalonolactone was synthesised from commercially available ^{13}C-labelled ethyl acetoacetate isotopomers by a known route (Zamir and Nguyen;[24] Scheme 3 A). The route was first tested with unlabelled material, thus revealing that the reported yields were accessible. Ethyl acetoacetate (**17**) was converted into ketal **18** by reaction with neopentyl glycol and trimethylchlorosilane. Subsequent reduction with $LiAlH_4$ to alcohol **19** and protection of the hydroxy function gave the benzyl ether **20**, which was transformed into ketone **21** by acid deprotection. Aldol reaction with the ester enolate of ethyl acetate yielded ester **22**, which upon catalytic hydrogenation and treatment with *p*-toluenesulfonic acid provided mevalonolactone in 19% yield via six steps. This reliable protocol allowed us to convert commercially available isotopomers of ethyl acetoacetate **17a**–**d** into the corresponding mevalonolactones **23a**–**d**. For the synthesis of **23e** we used a route that we previously developed for the synthesis of deuterated isotopomers of mevalonolactone (Scheme 3 B).[25] Starting from the aldehyde **24**, reaction with [^{13}C]methylmagnesium iodide yielded the alcohol **25**, which was oxidised with IBX to ketone **21e**. This was transformed into mevalonolactone **23e** (as above). Finally, [4-^{13}C]mevalonolactone (**23f**) was synthesised by a reported procedure (Cane and Levin).[26]

All six mevalonolactone isotopomers (**23a**–**f**) were fed to agar plate cultures of *Geniculosporium* sp., and the volatiles emitted by these agar plate cultures were trapped on charcoal by CLSA. The charcoal filter was extracted with $CDCl_3$ daily for one week. The extracts were collected in an NMR tube and subjected to ^{13}C NMR analysis (^{13}C NMR and DEPT spectra; results in Figure 2). Each feeding experiment resulted in the incorporation of labelling at three or six carbons of **X**, depending on whether a singly or doubly labelled mevalonolactone isotopomer was fed. This resulted in a set of strongly enhanced ^{13}C NMR signals. These signals perfectly matched the chemical shifts of **10**, whereas the chemical shifts of its known stereoisomers revealed significant differences (Table S1). The ^{13}C NMR shifts of **13** and its known stereoisomers also did not match. In



Scheme 3. Synthesis of ^{13}C-labelled isotopomers of mevalonolactone. A) Synthesis of isotopomers **23 a**–**d** starting from ethyl acetoacetate according to Zamir and Nguyen.[24] The yields are those obtained in the synthesis of unlabelled **23**. (For yields with labelled compounds see the Supporting Information.) B) Synthesis of **23 e** by a route that was previously developed in our laboratories.[25] Black dots indicate ^{13}C-labelled carbons.

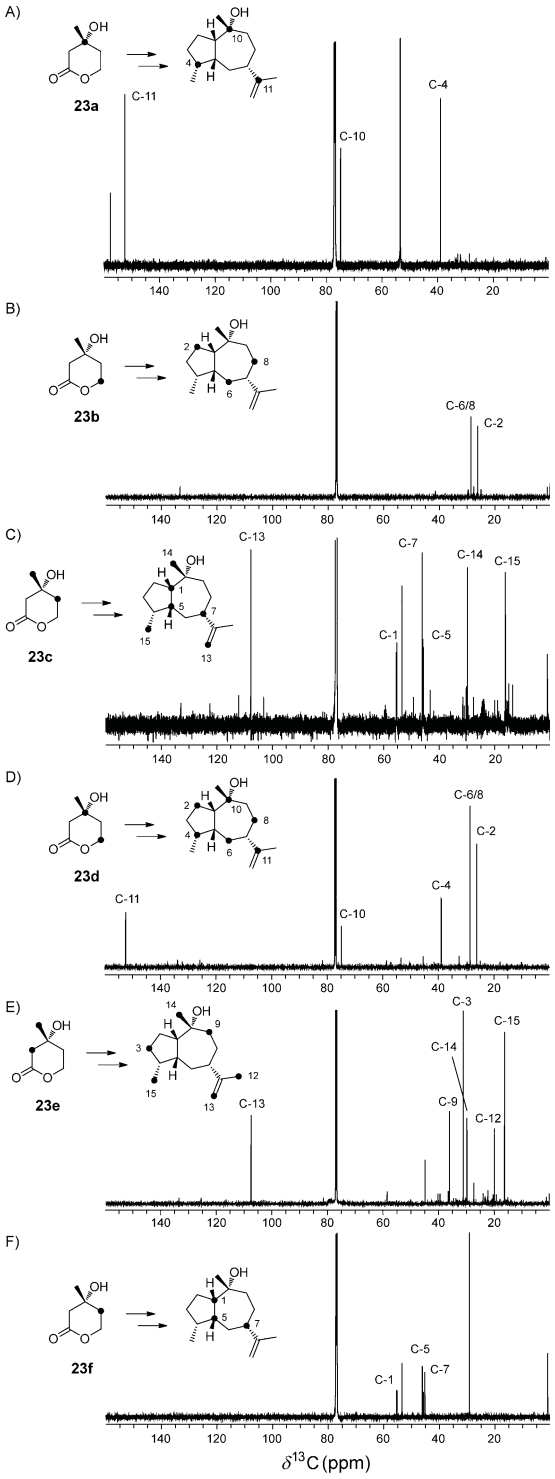
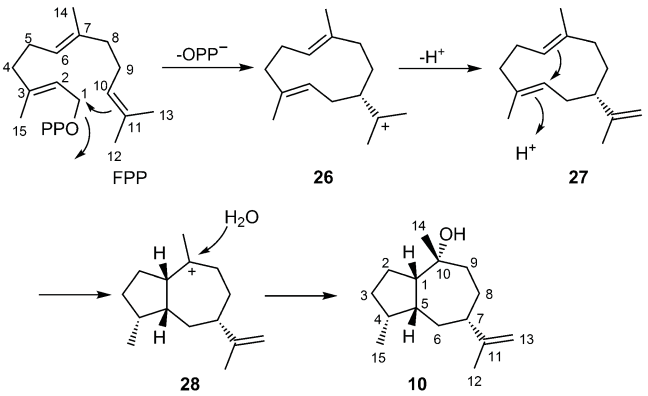


Figure 2. Results of feeding experiments with synthetic mevalonolactone isotopomers **23 a**–**f**. The ^{13}C NMR spectra were obtained in CLSA-NMR experiments from crude extracts. Chemical shifts of ^{13}C signals, multiplicities and coupling constants for ^{n}J(C,C) couplings are summarised in Table S1.

particular, the presence of signals for olefinic carbons in the experiments with **23 a** and **23 c**–**e** clearly ruled out **13**–**16** and their unknown stereoisomers. Based on these data, the identity of **X** as pogostol was unequivocally established. The full biosynthetic pathway for the incorporation of label from **23** into **10** is presented in Scheme S1.

The obtained data were also used to investigate the biosynthesis of **10** (Scheme 4). The conversion of farnesyl diphosphate (FPP) into **10** proceeds most likely first through a 1,10-cyclisation to the (*E*,*E*)-germacradienyl cation (**26**), which might proceed via nerolidyl diphosphate (not shown), and deprotonation to germacrene A (**27**). Its reprotonation initiates a second cyclisation to cation **28**, which is trapped with water to yield **10**. Feeding of **23 f** resulted in labelling of FPP at C-2, C-6 and C-10 (converted into C-5, C-1 and C-7 of **10**). A C–C single bond between C-5 and C-1 is formed by the second ring clo-



Scheme 4. Biosynthesis of pogostol (**10**).

sure, thus resulting in doublets in the respective ^{13}C NMR signals (coupling constant $^{1}J(C,C) = 33.1$ Hz), thereby confirming the biosynthesis of **10** as shown in Scheme 4. Feeding experiments with **23c** and **23e** gave insights into the stereochemical course of the terpene cyclisation. The methyl group of mevalonolactone that is labelled in these two isotopomers is converted into the terminal (*Z*)-methyl group of FPP (C-13). Both feeding experiments showed that the deprotonation step of the terpene cyclisation to the neutral intermediate **27** proceeds with stereospecific deprotonation of C-13 and not C-12 of FPP, thus ending up as the olefinic C-13 of **10**. This suggests that free rotation around the C-10/C-11 single bond in cation **26** is not possible, as similarly observed in the biosynthesis of 2-methylisoborneol.[12] A possible explanation is cation–π stabilisation[27] of cation **26** with an adjacent aromatic amino acid residue in the active centre of the terpene cyclase, similarly to other terpene cyclisation reactions.[28–31]

Stereochemical questions in terpene biosynthesis have frequently been solved by isotopic labelling.[32] Other compound classes can be investigated by related methods; for example, Bode and co-workers recently presented a combined strategy of gene knockout and cultivation in D_2O to identify D-amino acids in nonribosomally synthesised peptides.[33] Pogostol was first isolated from patchouli (*Pogostemon cablin*),[34] and was recently reported in the endophytic fungus *Biscogniauxia nummularia* from the plum yew *Cephalotaxus harringtonia*, where it co-occurs with its oxidation product xylaranone (xylaranone arises by oxidation at C-3 of **10** to the corresponding ketone).[23] Both **10** and xylaranone were shown to inhibit seed germination by *Raphanus sativus* (radish)—the particularly strong activity of xylaranone is comparable to that of glyphosate. Intrigued by this we reanalysed our *Geniculosporium* headspace extracts for the presence of oxidation products of **10**. We observed traces of candidate compounds, but the amounts were too small for detection by CLSA-NMR in the ^{13}C NMR spectra, and the mass spectra did not match database spectra in our mass spectral libraries. In an ecological context, both sesquiterpenoids **10** and xylaranone secreted by the endophytes might be directed towards the endophyte's host. In particular, endophytic fungi produce herbicidal metabolites that are assumed to play a role in maintaining a balance of antagonisms between host and endophyte.[35–37] Seed germination inhibiting metabolites such as **10** and xylaranone might also benefit the host plant by inhibiting the proliferation of other species in the area. Currently, the toxic effects of glyphosate towards humans have become a concern and have prompted discussion as to whether glyphosate should be substituted. Natural germination inhibitors such as **10** and xylaranone might be suitable alternatives and could be made available by biotechnological approaches in the near future.

Experimental Section

Culture conditions and feeding experiment: The endophytic fungus *Geniculosporium* sp. 9910, isolated from the leaves of *Cistus monspeliensis*, was cultivated for three weeks (without feeding) or four weeks (feeding experiment) on potato-carrot agar medium[38] (20 mL) in glass petri dishes at 20 °C. For the feeding experiments, cultures were supplemented with an aqueous solution of mevalonolactone isotopomer (**23a–f**, 10 mg in sterile-filtered water (200 μL)). The resulting solution was injected with a syringe (10 μL) into the agar. The cultures were incubated at 20 °C for a further day, and collection of the volatiles was by use of a closed-loop stripping apparatus as described previously.[39] For the feeding experiments, collection of volatiles was continued for the next seven days. The charcoal filter was extracted every 24 h with of $CDCl_3$ (50 μL). The extracts were combined and analysed by ^{13}C NMR and DEPT spectroscopy. Spectra were recorded on an AV II-600 spectrometer (150 MHz; Bruker) and referenced to TMS.

GC-MS analysis of headspace extracts: CLSA headspace extracts were obtained from *Geniculosporium*, with or without feeding of mevalonolactone isotopomers, and analysed by GC-MS (total ion chromatograms in Figure 1). GC-MS analyses were carried out an HP7890A GC system (Agilent) connected to an HP5975C Mass Selective Detector equipped with a HP-5 MS fused silica capillary column (30 m × 0.22 mm, 0.25 μm; Agilent): inlet pressure 67 kPa, He 23.3 mL min^{-1}, injection volume 1 μL, injector 250 °C, transfer line 300 °C, electron energy 70 eV. The GC was programmed as follows: 50 °C (5 min isothermic), then increasing (5 °C min^{-1}) to 320 °C, operated in splitless mode (60 s valve time), He 1.2 mL min^{-1}.

Acknowledgements

Funding by the Deutsche Forschungsgemeinschaft with an Emmy Noether grant (DI1536/1–3) and a Heisenberg grant (DI1536/4–1) is gratefully acknowledged.

Keywords: isotopic labeling · NMR · terpenoids · trace analysis · volatiles

[1] J. S. Dickschat, *Nat. Prod. Rep.* **2014**, *31*, 838 – 861.
[2] K. Grob, *J. Chromatogr.* **1973**, *84*, 255 – 273.
[3] K. Grob, F. Zürcher, *J. Chromatogr.* **1976**, *117*, 285 – 294.
[4] W. Boland, P. Ney, L. Jänicke, G. Grassmann in *Analysis of Volatiles: Method, Applications* (Ed.: P. Schreier), Walter de Gruyter, Berlin, **1984**, pp. 371 – 380.
[5] S. Schulz, J. Fuhlendorff, H. Reichenbach, *Tetrahedron* **2004**, *60*, 3863 – 3872.
[6] C. A. Citron, J. Gleitzmann, G. Laurenzano, R. Pukall, J. S. Dickschat, *ChemBioChem* **2012**, *13*, 202 – 214.

[7] C. A. Citron, S. M. Wickel, B. Schulz, S. Draeger, J. S. Dickschat, *Eur. J. Org. Chem.* **2012**, 6636 – 6646.
[8] D. Joulain, W. A. König, *The Atlas of Spectral Data of Sesquiterpene Hydrocarbons*, E. B. Verlag, Hamburg, **1998**.
[9] R. P. Adams, *Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry*, Allured, Carol Stream, **2009**.
[10] T. Wang, P. Rabe, C. A. Citron, J. S. Dickschat, *Beilstein J. Org. Chem.* **2013**, *9*, 2767 – 2777.
[11] C. A. Citron, P. Rabe, J. S. Dickschat, *J. Nat. Prod.* **2012**, *75*, 1765 – 1776.
[12] N. L. Brock, S. R. Ravella, S. Schulz, J. S. Dickschat, *Angew. Chem. Int. Ed.* **2013**, *52*, 2100 – 2104; *Angew. Chem.* **2013**, *125*, 2154 – 2158.
[13] R. Riclea, C. A. Citron, J. Rinkel, J. S. Dickschat, *Chem. Commun.* **2014**, *50*, 4228 – 4230.
[14] N. L. Brock, J. S. Dickschat, *ChemBioChem* **2013**, *14*, 1189 – 1193.
[15] C. A. Citron, N. L. Brock, B. Tudzynski, J. S. Dickschat, *Chem. Commun.* **2014**, *50*, 5224 – 5226.
[16] A. A. Stierle, D. B. Stierle, E. Goldstein, K. Parker, T. Bugni, C. Baarson, J. Gress, D. Blake, *J. Nat. Prod.* **2003**, *66*, 1097 – 1100.
[17] M. L. Bolte, J. Bowers, W. D. Crow, D. M. Paton, A. Sakurai, N. Takahashi, M. Ujiie, S. Yoshida, *Agric. Biol. Chem.* **1984**, *48*, 373 – 376.
[18] H. J. M. Gijsen, K. Kanai, G. A. Stork, J. B. P. A. Wijnberg, R. V. A. Orru, C. G. J. M. Seelen, S. M. van der Kerk, A. de Groot, *Tetrahedron* **1990**, *46*, 7237 – 7246.
[19] H. J. M. Gijsen, J. B. P. A. Wijnberg, G. A. Stork, A. de Groot, M. A. de Waard, J. G. M. van Nistelrooy, *Tetrahedron* **1992**, *48*, 2465 – 2476.
[20] A. San Feliciano, M. Medarde, M. Gordaliza, E. del Olmo, J. M. M. del Corral, *Phytochemistry* **1989**, *28*, 2717 – 2721.
[21] K. I. Booker-Milburn, H. Jenkins, J. P. H. Charmant, P. Mohr, *Org. Lett.* **2003**, *5*, 3309 – 3312.
[22] P. Weyerstahl, H. Marschall, U. Splittgerber, D. Wolf, *Flavour Fragrance J.* **2000**, *15*, 153 – 173.
[23] S. Amand, A. Langenfeld, A. Blond, J. Dupont, B. Nay, S. Prado, *J. Nat. Prod.* **2012**, *75*, 798 – 801.
[24] L. O. Zamir, C.-D. Nguyen, *J. Labelled Compd. Radiopharm.* **1988**, *25*, 1189 – 1196.
[25] J. S. Dickschat, C. A. Citron, N. L. Brock, R. Riclea, H. Kuhz, *Eur. J. Org. Chem.* **2011**, 3339 – 3346.
[26] D. E. Cane, R. H. Levin, *J. Am. Chem. Soc.* **1976**, *98*, 1183 – 1188.
[27] D. A. Dougherty, *Science* **1996**, *271*, 163 – 168.
[28] 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.
[29] M. J. Rynkiewicz, D. E. Cane, D. W. Christianson, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 13543 – 13548.
[30] C. A. Lesburg, G. Zhai, D. E. Cane, D. W. Christianson, *Science* **1997**, *277*, 1820 – 1824.
[31] J. M. Caruthers, I. Kang, M. J. Rynkiewicz, D. E. Cane, D. W. Christianson, *J. Biol. Chem.* **2000**, *275*, 25533 – 25539.
[32] J. S. Dickschat, *Nat. Prod. Rep.* **2011**, *28*, 1917 – 1936.
[33] C. Kegler, F. I. Nollmann, T. Ahrendt, F. Fleischhacker, E. Bode, H. B. Bode, *ChemBioChem* **2014**, *15*, 826 – 828.
[34] H. Hikino, K. Ito, T. Takemoto, *Chem. Pharm. Bull.* **1968**, *16*, 1608 – 1610.
[35] S. Peters, S. Draeger, H.-J. Aust, B. Schulz, *Mycologia* **1998**, *90*, 360 – 367.
[36] B. Schulz, C. Boyle, S. Draeger, A.-K. Römmert, K. Krohn, *Mycol. Res.* **2002**, *106*, 996 – 1004.
[37] B. Schulz, C. Boyle, *Mycol. Res.* **2005**, *109*, 661 – 686.
[38] U. Höller, A. D. Wright, G. F. Matthée, G. M. König, S. Draeger, H.-J. Aust, B. Schulz, *Mycol. Res.* **2000**, *104*, 1354 – 1365.
[39] J. S. Dickschat, S. C. Wenzel, H. B. Bode, R. Müller, S. Schulz, *ChemBioChem* **2004**, *5*, 778 – 787.