ChemComm

COMMUNICATION

View Article Online
View Journal | View Issue

Cite this: *Chem. Commun.*, 2014, **50**, 4228

Received 8th January 2014,
Accepted 26th February 2014

DOI: 10.1039/c4cc00177j

www.rsc.org/chemcomm

Identification of isoafricanol and its terpene cyclase in *Streptomyces violaceusniger* using CLSA-NMR†

Ramona Riclea, Christian A. Citron, Jan Rinkel and Jeroen S. Dickschat*

The recently developed CLSA-NMR technique that is based on feeding experiments with ^{13}C-labelled precursors was applied in the identification of isoafricanol as the main volatile terpene emitted by *Streptomyces violaceusniger*. The isoafricanol synthase of this organism is presented, together with a recent phylogenetic analysis of bacterial terpene cyclases.

The closed-loop stripping apparatus (CLSA) was originally developed by Grob and Zürcher in 1973 for trace analysis of volatile organic compounds (VOCs) in water.[1,2] The method makes use of a circulating air stream in a closed system that is bubbled through an aqueous sample. The VOCs are stripped off and trapped in a charcoal filter that can be extracted with an organic solvent for GC/MS analysis. Instead of directing the air stream through a water sample it is also possible to interconnect a chamber that contains biological material for study of volatile natural products such as insect or spider pheromones.[3,4] The method was first applied in investigations conducted on bacterial volatiles in a series of studies with myxobacteria.[5–7] During the last few years we have systematically investigated the volatiles released by various actinomycetes using the CLSA technique.[8–13] Together with analytical data from other groups[14,15] our results demonstrated that terpenes are the major constituents of the bouquets from most actinomycetes. The unambiguous identification of terpenes in headspace extracts is based on (1) comparison of the mass spectrum to a database spectrum and of the retention index to tabulated data from the literature, or (2) direct comparison to an authentic standard. Since authentic standards of terpenes are rarely commercially available, terpene identification usually relies on the first method. Positive identification requires a high mass spectral match factor (>900; 1000 means absolutely identical mass spectra) and a good match of retention indices (difference between measured retention index and literature data <10). Compound identification is problematic, if no mass spectrum and/or no retention index of an analyte is included in the databases. Delineation of a structural proposal from the GC/MS data and synthesis of a reference compound offers an alternative, but this strategy is in case of sesqui- and diterpenes not suitable due to their structural complexity. Another option is compound isolation from liquid culture extracts and structure elucidation *via* NMR spectroscopic methods, but the required compound purification is usually very laborious and in the case of volatile terpenes the necessary concentration steps endanger a significant loss of material, and thus an unsuccessful purification process.

CLSA headspace extracts from agar plate cultures usually contain sub-microgram amounts of volatiles in complex mixtures. Therefore, direct NMR analysis of such headspace extracts does not allow for conclusive insights. To overcome this problem we have recently developed a new analytical method (CLSA-NMR) that makes use of feeding experiments with ^{13}C-labelled precursors.[16] We assumed, that if a CLSA extract is not too complex and incorporation of the ^{13}C-labelling proceeds at high rates, the volatiles trapped on the charcoal filters could be eluted with a deuterated solvent ($CDCl_3$) for direct ^{13}C-NMR analysis. Indeed, this method was successfully applied in the elucidation of the stereochemical course of the terpene cyclisation of 2-methylisoborneol in actinomycetes[16] and of α-acorenol and koraiol in *Fusarium fujikuroi*,[17] and in the structure elucidation of eudesma-11-en-4α-ol, a side product of the aristolochene synthase, in *Penicillium roqueforti*.[18] Here we present a synthetic route to obtain ^{13}C-labelled isotopomers of deoxyxylulose and their application in feeding experiments with *S. violaceusniger* for the structure elucidation of isoafricanol by CLSA-NMR.

GC/MS analysis of CLSA headspace extracts from *S. violaceusniger* Tü4113 showed the presence of one major sesquiterpene alcohol (**X**) besides 2-methylisoborneol (**1**), geosmin (**2**), and african-1-ene (**3**) (Fig. 1). The best matching mass spectrum in our mass spectral libraries in comparison to the mass spectrum of **X** was that of maaliol, but the mass spectral match factor was fairly poor

Institut für Organische Chemie, Hagenring 30, 38106 Braunschweig, Germany. E-mail: j.dickschat@tu-braunschweig.de; Fax: +49 531 3915272; Tel: +49 531 3915264

† Electronic supplementary information (ESI) available: Mass spectra of **16a** and maaliol, phylogenetic tree of bacterial terpene cyclases, NMR spectra obtained in feeding experiments and tabulated NMR data of related sesquiterpene alcohols, experimental procedures, and NMR spectra of synthetic compounds. See DOI: 10.1039/c4cc00177j

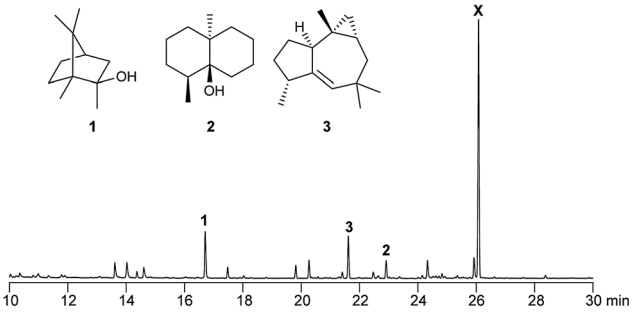
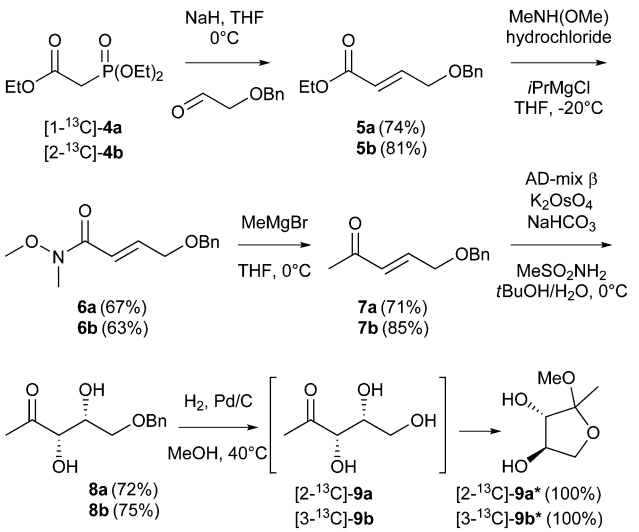


Fig. 1 Total ion chromatogram of a CLSA headspace extract from *S. violaceusniger*.

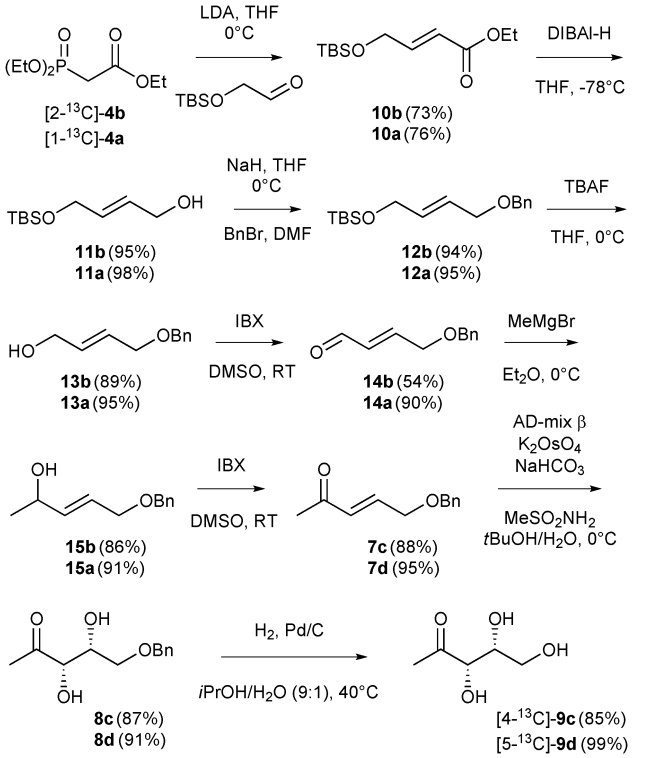
(Fig. S1 of ESI†). We decided to elucidate the structure of **X** *via* the CLSA-NMR technique, avoiding the need for compound purification from liquid culture extracts. For this purpose the synthesis of four isotopomers of [$^{13}C_1$]deoxyxylulose with labellings in different positions (**9a–9d**) was performed, while the fifth isotopomer [1-^{13}C]deoxyxylulose (**9e**) was already available in our laboratories.[16]

The synthesis of all isotopomers of **9** was performed starting from commercially available [1-^{13}C]- and [2-^{13}C]triethyl phosphonoacetate (**4a** and **4b**). A Horner–Wadsworth–Emmons (HWE) olefination with benzyloxyacetaldehyde (Scheme 1) yielded the esters **5** that were converted into the Weinreb amides **6.** Treatment with methylmagnesium bromide resulted in the methyl ketones **7** that upon Sharpless dihydroxylation with AD-mix β to **8** and cleavage of the benzyl protecting groups by catalytic hydrogenation in MeOH afforded [2-^{13}C]-**9a** and [3-^{13}C]-**9b** that further reacted to afford the corresponding methyl acetals **9a*** and **9b*** (Scheme 1).

Synthesis of the remaining two isotopomers of **9** was performed *via* HWE olefination of *tert*-butyldimethylsilyloxybenzaldehyde with **4a** and **4b** to the esters **10**, followed by DIBAl-H reduction to the allyl alcohols **11** (Scheme 2). Treatment with sodium hydride and benzyl bromide and cleavage of the TBS protecting groups with TBAF gave the allyl alcohols **13** *via* **12**. IBX oxidation to **14** and



Scheme 1 Synthesis of deoxyxylulose isotopomers **9a** and **9b**.

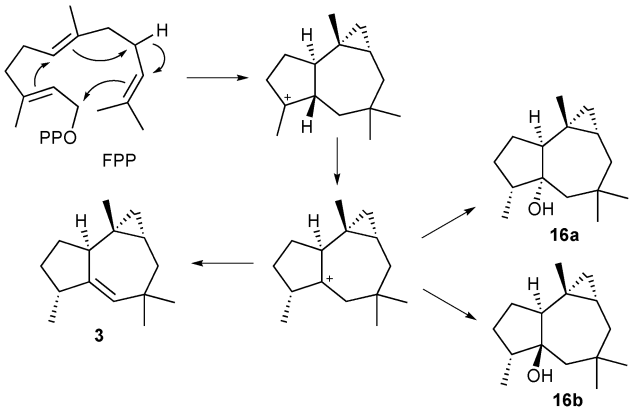


Scheme 2 Synthesis of deoxyxylulose isotopomers **9c** and **9d**.

reaction with methylmagnesium bromide resulted in the methyl carbinols **15** that upon oxidation to the ketones **7**, Sharpless dihydroxylation to **8** and deprotection resulted in [4-^{13}C]-**9c** and [5-^{13}C]-**9d**. To avoid the formation of the acetals as in the synthesis of **9a** and **9b** different solvents were tested in the deprotection step. The mixture of iPrOH/H_2O (9 : 1) proved to be optimal,[19] affording **9c** and **9d** in the pure form.

The unidentified sesquiterpene alcohol **X** was assumed to be structurally related to **3**, because only one uncharacterised terpene cyclase is encoded in the genome of *S. violaceusniger* (accession number ZP_07605120, recently updated by record YP_004815539, Fig. S2 of ESI†),[20] suggesting that **3** and **X** should both be formed by this enzyme. This was further supported by the similarities between the mass spectra of **X** and other sesquiterpene alcohols with a 5-7-3 tricyclic backbone (palustrol and ledol). Biosynthetic considerations suggested the capture of a cationic intermediate en route to **3** with water (Scheme 3), pointing to the structures of *e.g.* isoafricanol (**16a**) or 8-*epi*-isoafricanol (**16b**), but other constitutional or stereoisomers also seemed possible (Fig. S3 of ESI†).

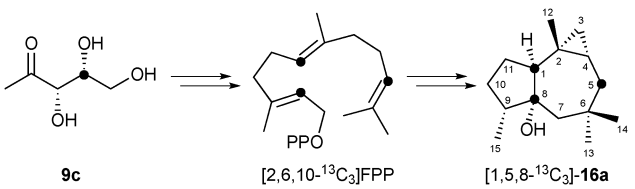
For an unambiguous structural assignment of **X** all five isotopomers of deoxyxylulose (**9a–9e**) were fed to agar plate cultures of *S. violaceusniger* that are converted along well-known pathways into farnesyl diphosphate (FPP),[21] followed by cyclisation to **X** and **3** by the bacterial sesquiterpene cyclase. In the case of **9a** and **9b** the acetals were fed, because we assumed that these would hydrolyse during culturing, and indeed both compounds were efficiently incorporated. Capture of the emitted volatiles *via* CLSA, extraction of the charcoal



Scheme 3 Biosynthesis of **3** and related sesquiterpene alcohols.

filters with $CDCl_3$, and direct analysis by ^{13}C-NMR and DEPT spectroscopy yielded for each feeding experiment three major ^{13}C-NMR signals (Fig. S4–S8 of ESI†), along with some peaks of lower intensities originating from incorporation of labelling into terpenes that are produced in small amounts, such as **1–3**. The relevant ^{13}C-NMR data for structure elucidation of **X** are summarised in Table S1 of ESI,† together with a summary of ^{13}C-NMR data from the literature of structurally related known compounds. All fifteen ^{13}C-NMR signals perfectly matched the reported chemical shifts of isoafricanol (**16a**),[22] thus confirming its identity with **X**, while all alternative structures could be ruled out. Particularly interesting was the outcome of the feeding of **9c** that resulted in incorporation into C-1 and C-8 of **16a** (Scheme 4). Each molecule of **16a** with incorporation of labelling into both carbons showed up as doublets in the ^{13}C-NMR spectrum ($^2J_{C,C}$ = 36.9 Hz), confirming the C-1/C-8 ring closure in **16a**, while incorporation into only one of these carbons gave the usual singlets (Fig. S6 of ESI†). Due to the full assignment of all chemical shifts for **16a**,[23] the feeding experiments with **9b** and **9e** also gave insights into the stereochemical course of the terpene cyclisation. The terminal (*E*)-methyl group of FPP, labelled after feeding of **9b**, is converted into C-13 of **16a** (δ = 31.1 ppm), while the terminal (*Z*)-methyl group that is labelled after feeding of **9e**, ends up as C-14 of **16a** (δ = 31.6 ppm).

Isoafricanol has previously been isolated, first from the ascomycete *Leptographium lundbergii*[23] and later from the liverwort *Nardia scalaris*[24] and the marine red alga *Laurencia mariannensis*,[25] but never from bacteria. Since the genome of *S. violaceusniger* has been sequenced and only one terpene cyclase with unassigned function is encoded (ZP_07605120, updated by record YP_004815539),[20] we can use the data presented here to assign the function of this terpene cyclase to isoafricanol synthase (Fig. S2 of ESI†).



Scheme 4 Incorporation of labelling into **16a** after feeding of **9c**. Black circles indicate ^{13}C-labelled carbons.

S. violaceusniger produces one previously unidentified major sesquiterpene alcohol **X** along with a few known minor terpenes. We have established a robust synthetic route to achieve all five singly ^{13}C-labelled isotopomers of deoxyxylulose and successfully applied these compounds in feeding experiments together with the newly developed CLSA-NMR technique. The NMR data obtained allowed for unambiguous assignment of the structure of isoafricanol to **X**. Furthermore, the function of one uncharacterised sesquiterpene cyclase in *S. violaceusniger* could be assigned as isoafricanol synthase. With the synthetic ^{13}C-labelled deoxyxylulose isotopomers in our hands application of the CLSA-NMR technique for structure elucidation of several more bacterial terpenes is now possible.

This work was funded by the Deutsche Forschungsgemeinschaft (DFG) with an Emmy-Noether grant (DI1536/1-3), a Heisenberg grant (DI1536/4-1) and a grant ''Duftstoffe aus Actinomyceten'' (DI1536/2-1). We thank Hans-Peter Fiedler (Tübingen) for *Streptomyces violaceusniger* Tü4113.

Notes and references

1 K. Grob, *J. Chromatogr.*, 1973, **84**, 255.
2 K. Grob and F. Zürcher, *J. Chromatogr.*, 1976, **117**, 285.
3 W. Boland, P. Ney, L. Jänicke and G. Grassmann, A ''Closed-loop-stripping'' technique as a versatile tool for metabolic studies of volatiles, in *Analysis of Volatiles: Method, Applications*, ed. P. Schreier, de Gruyter, Berlin, 1984, p. 371.
4 M. D. Papke, S. E. Riechert and S. Schulz, *Anim. Behav.*, 2001, **61**, 877.
5 S. Schulz, J. Fuhlendorff and H. Reichenbach, *Tetrahedron*, 2004, **60**, 3863.
6 J. S. Dickschat, S. C. Wenzel, H. B. Bode, R. Müller and S. Schulz, *ChemBioChem*, 2004, **5**, 778.
7 J. S. Dickschat, H. B. Bode, S. C. Wenzel, R. Müller and S. Schulz, *ChemBioChem*, 2005, **6**, 2023.
8 J. S. Dickschat, T. Martens, T. Brinkhoff, M. Simon and S. Schulz, *Chem. Biodiversity*, 2005, **2**, 837.
9 C. A. Citron, J. Gleitzmann, G. Laurenzano, R. Pukall and J. S. Dickschat, *ChemBioChem*, 2012, **13**, 202.
10 R. Riclea, B. Aigle, P. Leblond, I. Schoenian, D. Spiteller and J. S. Dickschat, *ChemBioChem*, 2012, **13**, 1635.
11 C. A. Citron, P. Rabe and J. S. Dickschat, *J. Nat. Prod.*, 2012, **75**, 1765.
12 P. Rabe and J. S. Dickschat, *Angew. Chem., Int. Ed.*, 2013, **52**, 1810.
13 T. Wang, P. Rabe, C. A. Citron and J. S. Dickschat, *Beilstein J. Org. Chem.*, 2013, **9**, 2767.
14 C. E. G. Schöller, H. Gürtler, R. Pedersen, S. Molin and K. Wilkins, *J. Agric. Food Chem.*, 2002, **50**, 2615.
15 K. Wilkins and C. Schöller, *Actinomycetologica*, 2009, **23**, 27.
16 N. L. Brock, S. R. Ravella, S. Schulz and J. S. Dickschat, *Angew. Chem., Int. Ed.*, 2013, **125**, 2154.
17 C. A. Citron, N. L. Brock, B. Tudzynski and J. S. Dickschat, *Chem. Commun.*, 2014, DOI: 10.1039/C3CC45982A.
18 N. L. Brock and J. S. Dickschat, *ChemBioChem*, 2013, **14**, 1189.
19 O. Meyer, J.-F. Hoeffler, C. Grosdemange-Billiard and M. Rohmer, *Tetrahedron*, 2004, **60**, 12153.
20 X. Chen, B. Zhang, W. Zhang, X. Wu, M. Zhang, T. Chen, G. Liu and P. Dyson, *Genome Announc.*, 2013, **1**, e00494-13.
21 J. S. Dickschat, *Nat. Prod. Rep.*, 2011, **28**, 1917.
22 W. Fan and J. B. White, *J. Org. Chem.*, 1993, **58**, 3557.
23 W.-R. Abraham, L. Ernst, L. Witte, H.-P. Hanssen and E. Sprecher, *Tetrahedron*, 1986, **42**, 4475.
24 U. Langenbahn, G. Burkhardt and H. Becker, *Phytochemistry*, 1993, **33**, 1173.
25 N.-Y. Ji, X.-M. Li, K. Li, L.-P. Ding, J. B. Gloer and B.-G. Wang, *J. Nat. Prod.*, 2007, **70**, 1901.

