Sesquiterpenes of the Geosmin-Producing Cyanobacterium *Calothrix* PCC 7507 and their Toxicity to Invertebrates

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The occurrence of sesquiterpenes was investigated with the geosmin-producing cyanobacterium *Calothrix* PCC 7507. The essential oil obtained by vacuum destillation was studied in more detail by GC-MS methods and superposition with authentic compounds. Geosmin was the dominating compound while the other sesquiterpenes were minor components. Sesquiterpenes that have not been described before in cyanobacteria were isodihydroagarofuran, eremophilone and 6,11-epoxyisodaucane. Closed-loop stripping analysis revealed that most of the sesquiterpenes were found in the biomass of *Calothrix*, while eremophilone was mainly observed in the medium of the axenic culture. Eremophilone showed acute toxicity (LC_{50}) against *Chironomus riparius* (insecta) at 29 μM and against *Thamnocephalus platyurus* (crustacea) at 22 μM. The compound was not toxic for *Plectus cirratus* (nematoda). 6,11-Epoxyisodaucane and isodihydroagarofuran exhibited no toxicity to invertebrates when applied in concentrations up to 100 μM.

Key words: Sesquiterpenes, Isodihydroagarofuran, Cyanobacterium, Insecticide

Introduction

Geosmin, a sesquiterpene tertiary alcohol that has lost an isopropyl group, is a major threat of water industry worldwide. Its extremely low threshold odour concentration of 15 ng/l (= 82 pM) (Persson, 1980) and the ease to recognize the muddy earthy odour are the primary causes of consumer complaints against drinking water (Bruchet, 1999). Producers of geosmin in the source water are planktonic and benthic cyanobacteria and in the distribution pipes microorganisms of unknown taxonomic position. In all so far investigated geosmin-producing cyanobacteria, geosmin was the major sesquiterpene accompanied by an array of sesquiterpene hydrocarbons and alcohols. But very few of these sesquiterpenes which may also contribute to the musty odour bouquet of cyanobacteria have been identified. Germacrene D and γ-cadinene were found as minor components among nine unidentified sesquiterpenes in *Oscillatoria splendida* (Tsuchiya *et al.*, 1981). In addition, several mass spectra were published, but could not be attributed to any of the known sesquiterpenes (Tsuchiya and Matsumoto, 1988). The structure determination from mass spectrometric signals is unreliable because the epimers described for higher plants must not also be typical products of lower plants. In general epimers show only minor differences in their EI mass spectra, and reference compounds that would allow identification by retention time analysis are not available. This is the reason why the structure elucidation of sesquiterpenes is severely hampered. The isolation of cyanobacterial biomass to obtain sufficient material for NMR studies is rather time-consuming, and essential oils in measurable quantities have not yet been obtained for any of these geosmin-producing cyanobacteria. Here we describe sesquiterpenes of *Calothrix* which have not yet been described for cyanobacteria. Since the biogenesis of sesquiterpenes may be similar in actinomycetes, these data will also be a contribution to the knowledge of sesquiterpenes in this group of microorganisms.

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Material and Methods

Origin and cultivation of cyanobacteria

The axenic strain *Calothrix* PCC 7507 was obtained from the Pasteur Culture Collection, Paris, France. It was grown under continuous light in 300-ml Erlenmeyer flasks without shaking as described previously (Höckelmann and Jüttner, 2004).

Closed-loop stripping of the medium

Eremophilone was isolated by closed-loop stripping from spent growth medium of *Calothrix*. For better recovery of the volatiles, the medium was supplemented with 20% NaCl before stripping. Closed-loop stripping and sorption on Tenax TA (Supelco), thermodesorption and GC-MS analysis (Fison Instruments, GC 8000 Top, MD 800) were performed as described previously (Jüttner, 1988). A chemically bound fused capillary column (DB-1301, 30 m length, 0.32 mm i.d., 0.25 μm film thickness) was used for separation. The head pressure of the He carrier gas was 50 kPa. The temperature program applied was: 4 min at 50 °C, then 5 °C/min up to 220 °C. Electron impact (EI) mass spectra were recorded in the range *m/z* 29–*m/z* 550. In the case of eremophilone, the growth medium was spiked with reference compounds to obtain superposition of the signals.

Closed-loop stripping analysis of cyanobacterial biomass

To analyze the sesquiterpenes in fresh biomass of *Calothrix,* an extraction procedure had to precede the stripping procedure. *Calothrix* biomass was collected on a glass-fibre filter. The filter was transferred into the stripping vessel and extracted with 2 ml of methanol. Then 50 ml of water and 20% NaCl (as a solid) were added, and the suspension was stripped for 45 min. Without the preceding extraction procedure, the detection of sesquiterpenes in fresh biomass of *Calothrix* by closed-loop stripping was not successful.

Fractionation of sesquiterpenes

The wet biomass of *Calothrix* (about 200 g) was first extracted with methanol, then with hexane. The extracts were combined and the solvents removed. The residue was subjected to high-vacuum distillation to separate the volatiles from the non-volatiles. The essential oil obtained was separated into several sesquiterpene fractions by HPLC (Jasco RI-2031 Plus, Omnilab, Mettmenstetten, Switzerland; column: RT 250–10, Li Chrosphere Si 60, 250 mm length, 10 mm i.d., 7 μm particle size, Merck, Darmstadt, Germany) under isocratic conditions. The solvent was hexane/*tert*-butylmethyl ether (95:5, v/v) and the flow rate 4 ml/min.

Preparative gas chromatography

A preparative gas chromatograph (Thermo, Finnigan, Waltham, MA, USA) equipped with a capillary column (SUPELCOWAX 10, 30 m length, 0.75 mm i.d., 1 μm film thickness) was used to enrich and purify epoxyisodaucane and dihydroagarofuran of the sesquiterpene fractions. The head pressure of the carrier gas (N_2) was 50 kPa. The temperature program was as follows: 3 min at 100 °C, then 2 °C/min up to 160 °C. The retention times of 6,11-epoxyisodaucane and dihydroagarofuran were 14.9 min and 17.9 min, respectively. The eluted sesquiterpenes were adsorbed on Tenax TA and desorbed with deuterobenzene.

Superposition with reference compounds

The sesquiterpene fractions obtained by preparative GC (isodihydroagarofuran, 6,11-epoxyisodaucane) were used for superposition with authentic compounds to establish the identity of the compounds. Without the purification step due to the large number of similar sesquiterpenes an unequivocal superposition was not possible. Superpositions with isodihydroagarofuran and 6,11-epoxyisodaucane were performed on a magnetic GC-MS instrument (MAT 95, Finnigan) equipped with a HP-INNOWax capillary column (60 m length, 0.25 mm i.d., 0.25 μm film thickness). The temperature program was as follows: 4 min at 30 °C, then 10 °C/min up to 70 °C, 1.5 °C/min up to 140 °C, and 8 °C/min up to 240 °C. EI mass spectra were recorded in the range *m/z* 24–*m/z* 350.

^{13}C NMR spectroscopy

NMR spectra were recorded on a Bruker Avance 500 instrument with a TCI cryoprobehead in C_6D_6 and/or $CDCl_3$ using TMS as the internal standard.

Reference compounds

Reference compounds of isodihydroagarofuran were from Firmenich SA (Geneva, Switzerland),

of *trans*-dihydroagarofuran and eremophilone from Givaudan AG (Dübendorf, Switzerland), and of 6,11-epoxyisodaucane (99% purity), an isolate from the liverwort *Tritomaria polita* (Adio *et al.*, 2003), from the Institute of Organic Chemistry, University of Hamburg, Germany.

Bioassays of acute toxicity

Three groups of freshwater invertebrates were applied as test organisms: neonate larvae from a culture of *Chironomus riparius* (diptera) were used to detect 24-h acute toxicity of insects, 24-h-old instar larvae hatched from resting eggs of *Thamnocephalus platyurus* (anostraca) (Thamnotoxkit F, G. Persoone, State University of Ghent, Belgium) were used for crustaceans (Todorova and Jüttner, 1996), and worms of *Plectus cirratus* (nematoda), an isolate from a *Calothrix/Rivularia/Tolypothrix* biofilm that covered stones of the littoral zone of Lake Zurich, Switzerland, were used for nematodes (Höckelmann *et al.*, 2004). Five concentrations of eremophilone (5, 10, 50, 100 and 125 μM) were tested (each three replicates) on 10 animals of the different invertebrate groups. The toxicity assays were performed in 24-well tissue culture plates. Eremophilone dissolved in ethanol was added in different amounts to the wells. After adding 0.5 ml standard synthetic freshwater (moderately hard; Weber, 1993), 10 animals were added to each vial and examined under a dissection microscope after 24 h. The final content of ethanol did not exceed 1% of the solution. The mortalities of the control group (no eremophilone added) were substracted from the observed mortalities. A sigmoidal four-parametric logistic curve was chosen to determine the concentration causing 50% mortality (LC_{50}).

Results

Stripping analysis combined with GC-MS was applied to study the sesquiterpene bouquet of the freshwater cyanobacterium *Calothrix* PCC 7507 that was available as an axenic culture. Around thirty sesquiterpene hydrocarbons and oxygenated sesquiterpenes were observed exhibiting molecular ions at *m/z* 204 $[M]^+$ and *m/z* 222 $[M]^+$, respectively. In some cases the intensity of the molecular ion at *m/z* 222 was very weak, but the observed fragment ions at *m/z* 204 and *m/z* 207 obtained by abstraction of a water molecule or a methyl group, and the longer retention time were indicative for oxygenated sesquiterpenes. Only the more prominent sesquiterpenes were studied in more detail. Monoterpenes with a regular carbon skeleton could not be detected.

Table I. The retention times (DB-1301; temperature program, 4 min at 50 °C, 5 °C/min up to 220 °C), relative amounts (percentage of total ions; geosmin, 100%), and state of identification (MS, identical spectrum with mass spectrum of the essential oil library and literature; MS/R, identical mass spectrum and retention time with a reference compound; MS/SUP, identical mass spectrum and successful superposition with a reference compound) of sesquiterpenes and nor-carotenoids in extracts from *Calothrix* PCC 7507 (growing culture).

Compound	R_t [min]	Rel. amount (%)	State of identification
8,10-Dimethyl-1-octalin	15.58	14.3	MS
8,10-Dimethyl-1(9)-octalin	15.75	5.1	MS
Dimethyloctalin	16.00	0.3	tentatively
Dimethyloctalin	16.01	3.0	tentatively
Dimethyloctalin	16.20	2.2	tentatively
β-Cyclocitral	16.62	0.6	MS/R
β-Ylangene	20.96	1.7	MS
Geosmin	21.32	100	MS/R
6,11-Epoxyisodaucane	22.32	8.2	MS/SUP
Germacrene D	22.80	18.3	MS/R
Isodihydroagarofuran	23.24	4.5	MS/SUP
β-Ionone	23.98	2.3	MS/R
6,11-Epoxyeudesmane epimer	24.06	0.8	MS
Rosifoliol epimer	26.11	1.6	MS
1(10),5-Germacradiene-11-ol	27.46	12.5	MS
Heptadecane	27.71	5.8	MS/R

The structures of some well-known compounds could easily be determined by MS and retention time analysis. Reference compounds allowed the identification of geosmin, germacrene D and the two nor-carotenoids *β*-cyclocitral and *β*-ionone. The mass spectra of *β*-ylangene (essential oil library, essential oil of *Marsupella emarginata*, Hepaticae) and 1(10)(*E*),5(*E*)-germacradiene-11-ol (Ganßer *et al.*, 1995) were identical with published data. The identified compounds, their retention times on a DB-1301 capillary column and their percentage in relation to geosmin are given in Table I.

To identify further sesquiterpenes, more complex work was necessary. A combination of prefractionation of the essential oil on an HPLC column and preparative GC afforded pure sesquiterpenes that allowed superposition with reference compounds. For this purpose the essential oil of about 200 g fresh biomass of *Calothrix* was separated and fractionated by HPLC. The first fraction (3.7–4.0 min) contained primarily dimethyloctalins, the second (4.0–4.7 min) dihydroagarofuran and the third (4.7–5.4 min) epoxyisodaucane. Applying preparative GC, pure fractions of dihydroagarofuran and epoxyisodaucane were obtained. Since the amounts were less than 1 μg, they were not sufficient for NMR. However, it should be mentioned that in C_6H_6 it was possible to verify in the ^{1}H NMR spectrum of epoxyisodaucane the very characteristic signal of the ether bond [δ 3.20 (*t* 1H, H-6, J = 9.8 Hz)] that was similar to the reference compound [δ 3.22 (*t,* 1H, H-6, J = 9.8 Hz)] (Adio *et al.*, 2003).

The purified compounds were used to establish their identity with reference sesquiterpenes by superposition on a 60 m capillary column (HP-INNOWax). The EI mass spectrum of a sesquiterpene was identical to 6,11-epoxyisodaucane (Adio *et al.*, 2003) and the superposition of the isolated compound with the reference compound was achieved. However, the absolute configuration has yet to be determined. Another sesquiterpene exhibited the mass fragmentation pattern of a dihydroagarofuran [*m/z* (%) = 41 (23), 55 (22), 69 (20), 81 (18), 95 (21), 109 (35), 125 (17), 137 (57), 149 (27), 151 (15), 164 (19), 179 (8), 189 (37), 207 (100), 208 (18), 222 (14)]. A reference compound that contained primarily *trans*-dihydroagarofuran (verified by NMR spectrometry) from *Galbanum* resin (Kaiser, personal communication) was applied for superposition (R_t 46.38 min) with the

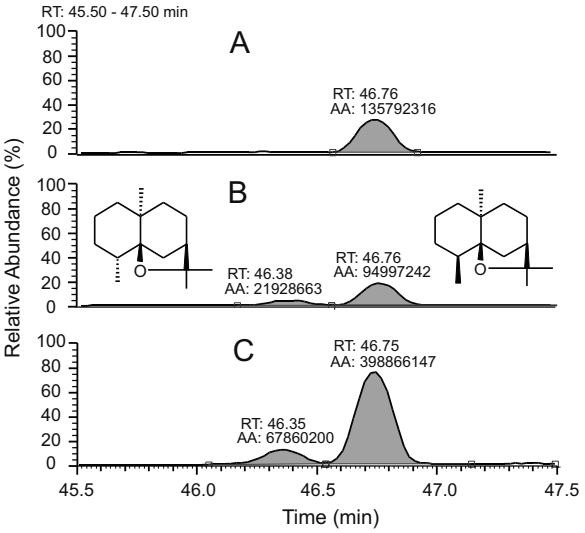

Fig. 1. Superposition of isodihydroagarofuran from *Calothrix* with a reference compound. (A) Isomer isolated from the biomass of *Calothrix* PCC 7507. (B) Reference compound containing 19% *trans*-dihydroagarofuran and 81% isodihydroagarofuran. (C) Coinjection of A and B.

compound from *Calothrix*. It could be demonstrated that both compounds were different. However, the dihydroagarofuran isolated from *Calothrix* was coeluted with a later eluting minor peak (R_t 46.76) of this reference. Another dihydroagarofuran sample obtained from Firmenich, also isolated from *Galbanum* resin (see Thomas and Ozainne, 1976), contained this second and later eluting compound as a major component. The superposition with this dihydroagarofuran epimer was successful (Fig. 1). The ^{13}C NMR spectrum of the reference [125 MHz, $CDCl_3$: δ = 38.0 (CH_2, C-1), 21.3 (CH_2, C-2), 32.1 (CH_2, C-3), 32.1 (CH, C-4), 87.3 (C, C-5), 33.4 (CH_2, C-6), 43.7 (CH, C-7), 25.0 (CH_2, C-8), 36.0 (CH_2, C-9), 38.6 (C, C-10), 81.0 (C, C-11), 30.2 (CH_3, C-12), 23.0 (CH_3, C-13), 23.5 (CH_3, C-14), 15.7 (CH_3, C-15)] was in accordance with isodihydroagarofuran. Isodihydroagarofuran was the only epimer (Cavalli *et al.*, 2004) in the essential oil of *Calothrix*.

Two other sesquiterpenes from *Calothrix* exhibited mass fragmentation patterns of rosifoliol (Southwell, 1978; Beagley *et al.*, 1982) and 6,11-epoxyeudesmane (Adio *et al.*, 2003). Although the retention times were within the expected range, a superposition was not possible

due to the lack of reference compounds. Therefore the epimers in position C-4 of both compounds, that are much more likely to occur on the base of biosynthetic arguments, are also possible structures of these two compounds.

Five different isomers of dimethyloctalin could be separated. Dimethyloctalins can be formed from geosmin by treatment with hydrochloric acid (Rosen *et al.*, 1968) and possibly by pyrolysis. To test whether the large number of dimethyloctalins in *Calothrix* were degradation products of geosmin that was present in high amounts, pure geosmin was stripped and analyzed by GC-MS under the same conditions as the sample of *Calothrix*. Dimethyloctalins were not formed under these conditions. This observation rules out that the dimethyloctalins observed were analytical artefacts. The mass spectra of two major dimethyloctalins were consistent with spectra published for 8,10-dimethyl-1-octalin and 8,10-dimethyl-1(9)-octalin (Nawrath *et al.*, 2008). 8,10-Dimethyl-1-octalin was the dimethyloctalin with the highest relative abundance.

Unlike the other sesquiterpenes, eremophilone was primarily found in spent medium of *Calothrix*. It exhibited a mass spectrum identical to a reference compound and could successfully be superpositioned. Only traces of eremophilone were observed in the essential oil obtained from the biomass of *Calothrix*. Also stripping analysis of sesquiterpenes in the biomass of *Calothrix* confirmed the low concentration of this compound in the biomass.

To show any effect of the age of the culture on the intracellular pattern of the sesquiterpenes, a growing (2- to 3-month-old) and an old culture (5- to 8-month-old) were analyzed. *Calothrix* is a slowly growing cyanobacterium. There were marked differences of the percentage values of the sesquiterpenes (Table II). It is interesting to note that particularly the abundance of dimethyloctalins and germacrene D was much higher in growing rather than in old cultures. The nor-carotenoids β-cyclocitral and β-ionone exhibited much lower contents in the old cultures indicating low activity of carotene oxygenases.

Since eremophilone was available in sufficient amounts and was excreted into the medium, the acute toxicity of this compound was tested on invertebrates. *Chironomus riparius* was used as a test organism for insects, *Thamnocephalus platyurus* for crustaceans, and *Plectus cirratus* for freshwater nematodes. The concentrations applied were between 5 and 125 μM and showed acute toxicity for *Chironomus* and *Thamnocephalus*, but not for the nematode *Plectus cirratus* (Fig. 2). The LC_{50} value was 29 μM for *Chironomus* and 22 μM for *Thamnocephalus*. Though tested for a period of 48 h, a toxic response even for the highest concentration of eremophilone could not be seen for the freshwater nematode *Plectus cirratus*. Toxicity assays of 6,11-epoxyisodaucane and isodihydroagarofuran were performed with *C. riparius*. Concentrations up to 100 μM showed no mortality in the 48-h bioassay.

Table II. Relative abundance of sesquiterpenes and nor-carotenoids in the biomass of *Calothrix* PCC 7507 of different age (2- to 3- and 5- to 8-month-old standing cultures). Standard deviations are given in brackets (n = 3).

Compound	Relative abundance	
	2–3 months	5–8 months
Dimethyloctalins[a]	27.2 (2.1)	13.7 (4.7)
β-Cyclocitral	0.6 (0.5)	0.1 (0.2)
β-Ylangene	1.6 (0.1)	0.5 (0.2)
Geosmin	100	100
6,11-Epoxyisodaucane	7.6 (0.5)	4.9 (0.8)
Germacrene D	18.5 (2.0)	5.8 (3.0)
Isodihydroagarofuran	3.9 (0.5)	3.6 (1.3)
β-Ionone	2.4 (0.4)	0.5 (0.6)
6,11-Epoxyeudesmane epimer	0.8 (0.0)	0.7 (0.3)
Rosifoliol epimer	1.3 (0.3)	0.8 (0.2)
1(10),5-Germacradiene-11-ol	11.1 (1.7)	13.2 (6.9)

[a] Sum of 6 isomers.

Discussion

The ability to synthesize geosmin is scattered over filamentous heterocystous and non-heterocystous cyanobacterial species, but is lacking in the chroococcales (Jüttner and Watson, 2007). When the structure of geosmin was established (Gerber, 1968), this odour compound was already regarded as a sesquiterpene alcohol that has lost an isopropyl group. A rich bouquet of sesquiterpene hydrocarbons and oxygenated sesquiterpenes accompanies geosmin in cyanobacteria. This has been shown for *Calothrix* and is consistent with observations concerning myxobacteria (Dickschat *et al.*, 2004), actinomycetes (Pollak and Berger, 1996) and the moss *Symphyogyna*

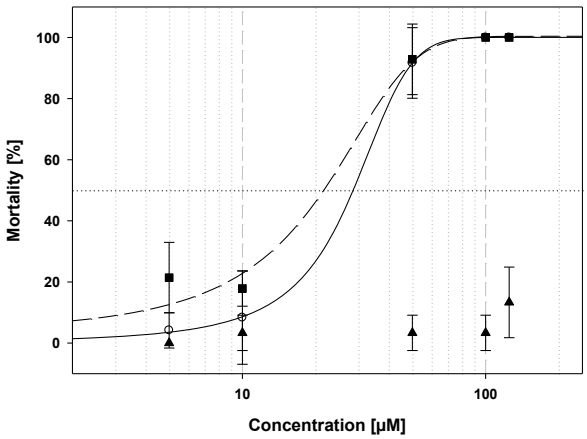

Fig. 2. Concentration response curve for eremophilone. Mortality (%) is given for *Chironomus riparius* (solid line and circles, $R^2 = 0.99$), *Thamnocephalus platyurus* (dashed line and squares, $R^2 = 0.99$) and *Plectus cirratus* (triangles).

brongniartii (Spörle *et al.*, 1991). Germacrene D and γ-cadinene have been described before for cyanobacteria. Here we describe for the first time the presence of isodihydroagarofuran, 6,11-epoxyisodaucane and 1(10),5-germacradiene-11-ol in a cyanobacterial species. In addition, several other sesquiterpenes of unknown structure were present.

Recent studies have shown that the single enzyme germacradienol/germacrene synthase is responsible for the formation of geosmin from farnesyl pyrophosphate (Jiang *et al.*, 2007; Jiang and Cane, 2008). Several intermediates such as germacradienol and dimethyloctalin are compounds of the reaction sequence. Obviously a part of them is not further metabolized to the final product geosmin. In a side reaction, as shown for recombinant germacradienol/germacrene D synthase, germacrene D is formed. Since the same intermediates as in *Streptomyces* can be found in *Calothrix* as major components, the function of such an enzyme is also most likely for cyanobacteria. Experiments with a geosmin-forming *Phormidium* have shown that sesquiterpene synthase genes homologous to that of streptomycetes can be found in cyanobacteria (Ludwig *et al.*, 2007). It is still an open question how a shift of the intracellular pattern of sesquiterpenes as observed for cultures of different age can be explained.

The mass spectra and retention times of two compounds were consistent with those of rosifoliol and 6,11-epoxyeudesmane. However, from the standpoint of biosynthesis the compounds epimeric at the C-4 atom are much more likely. Since reference compounds were not available and the amounts of the isolated compounds were too low, the determination of the configurations was not possible.

Sesquiterpenes have been shown to be potent allelochemicals against insects and to protect organisms from being eaten (Ainge *et al.*, 2001; Panella *et al.*, 2005; Kiran *et al.*, 2007). In a screening of 41 dihydro-β-agarofurans from plant species of the Celastraceae, three components showed insecticidal activity to larvae of the moth *Spodoptera littoralis*, whereas 38 showed antifeedant activity (Gonzalez *et al.*, 1997). In our experiments insecticidal activity could be demonstrated for eremophilone, but no acute toxicity was found for isodihydroagarofuran and 6,11-epoxyisodaucane. However, additional ecological properties of allelochemicals such as deterrence activity should be investigated for these compounds. The toxicity of eremophilone from *Calothrix* PCC 7507 against chironomid larvae definitely is another example for the prevalence of insecticidal compounds in biofilm-forming cyanobacteria (Becher *et al.*, 2007) that are habitat for aquatic insects and other invertebrates exerting a strong grazing pressure.

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