β-Cyclocitral and Alkanes in *Microcystis* (Cyanophyceae)

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A tobacco-like odorous compound isolated from a bloom of the blue-green alga *Microcystis wesenbergii* and a laboratory culture of *M. aeruginosa* was analysed by gas-liquid chromatography and mass spectrometry. Its structure was determined as β-cyclocitral. No other monoterpenes have been detected in these and in other blue-green algae. The hydrocarbon content was 0.10–0.18% of the dry weight. *n*-Hexadecane, *n*-heptadecane, 4-methylheptadecane and *n*-octadecane constituted the main components. The relation of the different alkanes to each other, especially 4-methylheptadecane and *n*-octadecane, revealed marked differences in the two organisms.

The increasing eutrophication of bodies of fresh waters has produced an increase in the occurrence of algal blooms. Correspondingly, the knowledge about odoriferous compounds released by these algae has gained in importance. Cyanophyceae are mainly responsible for bloom-formation in fresh waters. However the odorous compounds of this group have been investigated but only to a very limited extent. Some sulfurous compounds, such as mercaptans, dimethylsulfide and dimethyldisulfide associated especially with aged blue-green algae were identified by Jenkins *et al.*[1]. Saffermann *et al.*[2] identified the so-called geosmin in *Symploca muscorum* and Medsker *et al.*[3] also found it in *Oscillatoria tenuis*. Later the structure of this musty, earthy-smelling compound was determined by Gerber[4] as dimethyldecalol. Sirenko and Sakevich[5] reported the occurrence of essential oils in *Microcystis* as well as in other blue-green algae, although they were not able to identify any of the compounds. The composition of the alkanes in the Cyanophyceae, which are of special interest for sedimentology, have been studied to a greater extent[6–9].

In 1973, a bloom of *Microcystis* appeared in Lake Federsee (Southwest Germany). We took advantage of this occurrence to examine this very important bloom-forming alga for odorous compounds and compared the results with those of a form cultivated under laboratory conditions.

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Materials and Methods

Collection and culture of Algae

During the summer of 1973, a bloom of *Microcystis wesenbergii* Komárek appeared in the eutrophic Lake Federsee (Southwest Germany). The algae were concentrated in baskets lined with nylon netting (25 μm, mesh width), the dense algal suspension, having been cooled with ice, was transported within 3 hours time to the laboratory to Tübingen and was immediately separated in a continuous-flow centrifuge, type LG 209-1 (Westfalia Separator AG, Oelde/Germany). Thus, 6 kg (wet weight) of *M. wesenbergii* were attained and subsequently stored at −30 °C. A microscopic examination of the algae revealed almost no contamination by other algal species. *M. aeruginosa* Kütz., strain LB 1450-1, was obtained from the Sammlung von Algenkulturen des Pflanzenphysiologischen Institutes der Universität Göttingen (Germany) in liquid culture. Approximately 1 kg (wet weight) was grown in 30 l tower-type units[10]. The optimized medium had the following composition per litre distilled water: 88 mg $CaCl_2 \times 2\,H_2O$, 680 mg $NaNO_3$, 99 mg $MgSO_4 \times 7\,H_2O$, 91 mg $K_2HPO_4 \times 3\,H_2O$. Trace elements were added to 1 l medium in the following amounts (modified after Allen and Stanier[11]): 3.7 mg NaFeEDTA, 3.1 mg H_3BO_3, 2.0 mg $MnCl_2 \times 4\,H_2O$, 290 μg $ZnSO_4 \times 7\,H_2O$, 480 μg $Na_2MoO_4 \times 2\,H_2O$, 50 μg $CuSO_4 \times 5\,H_2O$, and 50 μg $CoCl_2 \times 6\,H_2O$. The pH value was 7.5. 200 g of *Anabaena flos-aquae* Bréb, strain A 37, a gift of Prof. R. G. Tischer (State College, Mississippi, USA), were produced in the same way. 400 g of *Synechococcus* NRC-1, labelled *Microcystis aeruginosa* NRC-1, from the American Type Culture Collection (Maryland, USA) were cultivated in a 110 litre algal plant[12, 13] in the same medium at 29 °C, bubbled with air containing 0.5% CO_2 and irradiated with 1000 lx from two sides.

Isolation of β-cyclocitral and alkanes

The volatile compounds were sublimated at 5×10^{-2} torr in a stainless steel sublimation apparatus: A simple U-tube in a dry ice or liquid air bath was used as a trap. 150 g of algae were sublimated during each run. After thawing, the opacious water was saturated with NaCl and extracted 3 times with freshly distilled diethylether. The ether extracts were combined, dried for several days over anhydrous Na_2SO_4 and then carefully concentrated to nearly 200 µl.

Gas-liquid chromatography

5 µl of the concentrate was injected into a Hewlett Packard model 5750 gas chromatograph employing a glass column (1.8 m × 3 mm i.d.) filled with 3% OV 17 on Chromosorb W AW DMCS, a nitrogen carrier-gas flow of 30 ml/min and a temperature program from 70 – 200 °C (10 °C/min). Registration of the mass spectra was performed with the same column at 70 eV in a combined gas chromatograph mass spectrometer system, LKB 9000 *. For the selective substraction according to Bierl *et al.*[14], two different reaction loops were used: a) for the substraction of aldehydes, a 15 cm column filled with 5% *o*-dianisidine on Chromosorb W AW DMCS at 140 °C and b) for the substraction of ketones, a 15 cm column filled with 20% benzidine on Chromosorb W AW DMCS at 140 °C.

Registration of UV-spectra

The terpene was separated from the other compounds by preparative gas liquid chromatography and dissolved in freshly distilled diethylether and its spectrum was registered on a recording spectrophotometer (Zeiss M4QIII).

Reduction of monoterpenes

Reduction products of the monoterpene were produced by adding PtO_2 to the ether extract and hydrogenating for 1 h under continuous stirring. The 1,1,2,3-tetramethylcyclohexanes and 1-formyl-2,6,6-trimethylcyclohexanes were prepared in the same way from an etheric solution of β-cyclocitral **.

Preparation of 4-methylheptadecane

4-Methylheptadecane was prepared from bromotridecane and methylpropylketone according to a procedure for branched alkanes [6].

* We are reatly indebted to Prof. W. Koenig, Organisch-Chemisches Institut der Universität Hamburg, for making the MS-spectra.

** β-Cyclocitral was a kind gift of Dr. H. Raman Ansari, Bush Boake Allon, Ltd., London, England.

Quantitative determination of alkanes

10 g (wet weight) of algae were centrifuged, the supernatant discarded and the remaining water absorbed with filter paper. The sedimented algae were resuspended in 100 ml methanol and extracted for 0.5 h. After renewed centrifugation, the sediment was extracted for 30 min with 100 ml freshly distilled diethylether and twice with 100 ml *n*-hexane: the supernatants being combined and gently evaporated to dryness under reduced pressure. The lipids were redissolved in 10 ml *n*-hexane, the weight determined, and 5 µl were injected into the gas chromatograph. By comparison with the peak heights of weighed authentic samples, the yield of the identified alkanes was determined.

Quantitative determination of monoterpenes

100 g (wet weight) of *Microcystis* were sublimated, the ethereal extract, derived as above, was gently concentrated to less than 0.5 ml and adjusted to a defined volume. 5 µl were injected into the gas chromatograph and peak heights were compared with those derived from weighed samples.

Results

Odoriferous compounds of algae are characterized by the property to evoke, in very small concentrations, strong physiological effects in the human olfactory system. Therefore a quantitative and selective concentration method was essential to enrich the odorous compounds of *Microcystis*. Extraction methods are only of a limited value since they furnish an extremely high number of gas-chromatographically-detectable substances. The more selective steam distillation very often results in the decomposition of compounds. With the method we applied, vacuum sublimation, no decomposition was noticed and volatile substances could be selectively concentrated. The gas-gromatographic separation of a sublimate isolated from the naturally-occurring *M. wesenbergii* is shown in Fig. 1. Organoleptic checking at the exit port (extinguished FID) of the gas chromatograph, revealed a tobacco-like odor in the region of peak 1. Alkane contaminants, such as hexadecane, heptadecane, octadecane and 4-methylheptadecane, were shown to be the source of the other peaks. The retention time of the odorous compound was a little shorter than that of pulegone. This, together with the mass spectrum (Fig. 2), which exhibited a strong molecular ion of *m/e* 152, strongly alluded to a cyclic unsaturated monoterpene

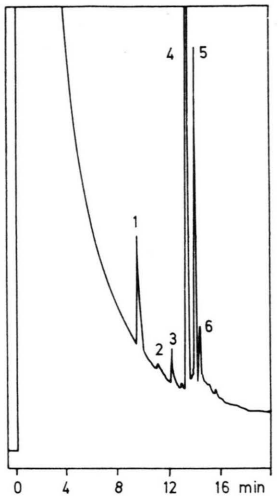

Fig. 1. Gaschromatogram of the sublimated products of *Microcystis wesenbergii*. 1 β-cyclocitral, 2 pentadecane, 3 hexadecane, 4 heptadecane, 5 4-methylheptadecane, 6 octadecane.

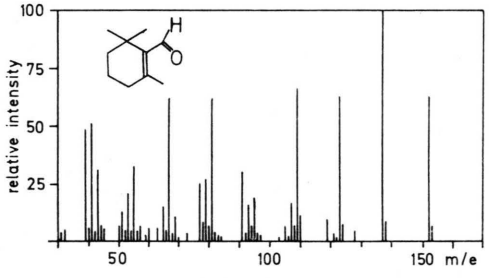

Fig. 2. Mass spectrum of β-cyclocitral isolated from *Microcystis aeruginosa*.

with an oxygen function. 110 μg of an identical compound were isolated from 1 kg of a laboratory culture of *Microcystis aeruginosa*. The ethereal extract was used for further experiments without previous separation of the odoriferous compound from the alkane contaminants. Because of the limited amount of matter available, reactive gas chromatographic methods according to Bierl *et al.*[14] were employed. An *o*-dianisidine reaction column was used for the extraction of aldehydes and a benzidine reaction column for removal of ketones: their functioning was checked with citral and cyclohexanone which were both approximately 90% eliminated. The peak of the odoriferous compound was only slightly diminished by the *o*-dianisidine loop whereas it was almost completely eliminated by the benzidine loop, which either indicated the presence of a ketone, or, more likely, a protected aldehyde function. Other monoterpene ketones such as pulegone, carvone and camphor were not affected by the benzidine reaction loop. A test for hydroxyl groups was performed by incubation of the sample with trifluoroaceticanhydride, TriSil and N-methyl-bis(trifluoroaceticanhydride): No peak shift was noticed. Reduction with Pt/H_2, however, yielded 5 compounds. The first compound eluted on the OV 17 column had a molecular weight of 126 and its mass fragmentation pattern was consistent with the pattern of 1,1,3-trimethylcyclohexane[15], the next were the two isomeres of the pure carbon skeleton (1,1,2,3-tetramethylcyclohexane) of the monoterpene with molecular ions of *m/e* 140, and finally the two isomeres of 1-formyl-2,6,6-trimethylcyclohexane with molecular ions of *m/e* 154 revealed the hydrogenation of one double bond. They were identical both in retention time and mass fragmentation pattern with compounds obtained by the reduction of an authentic sample of cyclocitral. In order to determine the position of the double bond, the odorous compound was separated by preparative gas chromatography and the UV-spectrum determined. The strong maximum appearing near 243 nm in diethylether is typical for a α-position of a double bond to an oxo group. From these results, it could be deduced that the compound was probably β-cyclocitral and subsequently the identity was confirmed by an authentic sample *. The approximate amounts of β-cyclocitral and alkanes in *Microcystis* are tabulated in Tab. I. Aside from β-cyclocitral there was no indication of the presence of monoterpenes in *Microcystis*. In other blue-green algae, no monoterpene at all could be found even when 200 g of *Anabaena Flos-aquae* or 400 g of Synechococcus were analysed.

Table I. Concentrations of β-cyclocitral and alkanes in *Microcystis aeruginosa* and *M. wesenbergii*.

Compounds	Organisms	
	M. aeruginosa	*M. wesenbergii*
lipids	10.5% of d.w.	9.0% of d.w.
hydrocarbons	0.18% of d.w.	0.10% of d.w.
β-cyclocitral	1.1 μg/g d.w.	n.d.
n-hexadecane	41 μg/g d.w.	106 μg/g d.w.
n-heptadecane	893 μg/g d.w.	715 μg/g d.w.
4-methylheptadecane	20 μg/g d.w.	141 μg/g d.w.
n-octadecane	845 μg/g d.w.	43 μg/g d.w.

d.w., dry weight. n.d., not determined.

* We are greatly indebted to Dr. H. Raman Ansari for making this proposal based on data in his collection of mass spectra.

An extraction method was used for the quantitative determination of alkanes in the two algae because the vacuum sublimation method yielded only a small part of the total hydrocarbons. The pattern consisted of *n*-hexadecane, *n*-heptadecane, *n*-octadecane and 4-methylheptadecane. The structure of 4-methylheptadecane could be elucidated from its mass fragmentation pattern. It was very similiar to both a spectrum published by Han *et al.*[6] for *Chlorogloea fritschii* and to a spectrum of an authentic synthesized sample. Between the two species of *Microcystis* used, marked differences in the quantitative relationships among the various alkanes were noticed. Whereas *M. aeruginosa* possessed a high percentage of octadecane and only small amounts of 4-methylheptadecane, the converse is true for *M. wesenbergii*.

Discussion

As far as this limited survey reveals, monoterpenes are not generally found in blue-green algae. The occurrence of β-cyclocitral seems to be unique and typical for *Microcystis*. The two closely-related species, *M. aeruginosa* and *M. wesenbergii*, contain appreciable amounts of this monoterpene. As far as we know, no other monoterpene has been found in the Cyanophyceae. β-Cyclocitral was found in a few of the higher plants, but only in small amounts. Karlsson *et al.*[16, 17] reported finding small amounts of β-cyclocitral in the volatile constituents of several *Carphephorus* species. The concentration of β-cyclocitral in tobacco leaves was approximately 0.1 ppm as stated by Kimland *et al.*[18]. Goryaev *et al.*[19], however, found a better yield in *Perovskia abrotanoides* (32 ppm). This monoterpene does not seem to be biosynthetically comparable to those monoterpenes generally found in eukaryotic plants. There are some arguments that β-cyclocitral is formed by a degradation process of carotenes. Indeed, β-cyclocitral is very similar to the β-ionon part of β-carotene, the only carotene isomer found in blue-green algae. It may, therefore, be formed from this compound by an oxidative degradation process similar to that proposed by Stevens[20] for the formation of geranylacetone, farnesylacetone, β-ionone, α-ionone etc. in tomatoes. Thus, a genuine monoterpene, typical for eukaryotic plants, still remains unknown in the Cyanophyceae.

The total amount of alkanes in *Microcystis* (0.10 − 0.18%) is comparable to the values found by Fehler and Light[21] for *Anabaena variabilis* (0.09%) and Winters *et al.*[7], from 0.05 to 0.12%, for various other blue-green algae. Gelpi *et al.*[8], however, reported a very low amount (0.005%) for *Anacystis nidulans*. *Microcystic aeruginosa*, defined as *Anacystis cyanea* according to Drouet's nomenclature, has already been investigated for alkanes by Gelpi *et al.*[8]. Besides the main component heptadecane, 13% of the total alkanes were reported as belonging to 7- and 8-methylheptadecane. Whereas we also found heptadecane to be the main component, large amounts of hexadecane and especially octadecane were detected. 7- and 8-methylheptadecane were lacking, instead the occurrence of another branched alkane, 4-methylheptadecane was noticed. The mass spectrum of this compound gave no indication of the occurrence of not separated 7- and 8-methylheptadecanes. The marked difference in the relative proportions of octadecane and 4-methylheptadecane between *M. aeruginosa* and *M. wesenbergii* is a physiological support for Komárek's opinion[22] to separate these two forms into different species.

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