Nor-Carotenoids as the Major Volatile Excretion Products of Cyanidium

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Z. Naturforsch. 34 c, 186—191 (1979); received December 5, 1978

Cyanidium, Nor-Carotenoids, Geranylacetone, β-Ionone, Methylheptonone, Dihydrotrimethylnaphthalene, Butenylidenetrimethylcyclohexene, Algal Bioassay

Cyanidium caldarium excretes a series of nor-carotenoids. A 110 litre culture was used to isolate and determine the structure of 5 compounds: methylheptone, geranylacetone, β-ionone, dihydrotrimethylnaphthalene, butenylidenetrimethylcyclohexene. An algal bioassay was used to determine their effectiveness on the growth of photoautotrophic organisms. The growth of Anabaena, Synedochococcus, Nannochoeris and Cyanidium was found to be inhibited at concentrations between 10 to 50 ppm.

During the growth of algal blooms, natural fresh waters frequently acquire odours characteristic of the dominant algal species. The odours have been described as being fishy, musty and septic or sweet and of violets and geraniums [1, 2]. The chemical nature of a very few of these odours has been determined probably due to their very low concentrations in natural waters and the resulting analytical problems concerned. One of the first examinations of an algal odour was performed with the subaerial Trentepohlia tolisthas which produced in the natural habitat the so-called “Veilchenstein”. The typically violet odour was found to be produced by β-ionone [3]. Geosmin has been shown for many years to be the causative agent for musty-earthly aromas found in association with Cyanobacteria and Actinomycetes [4, 5] and its structure has been determined as a trans-1,10-dimethyl-trans-9-decalol [6]. The studies of Tabachek and Yurkowski [7] have demonstrated its wide distribution in Cyanobacteria and in a few cases 2-methylisoborneol has also been found. An additional group of compounds is represented by aldehydes, extensively studied in Synura petersenii and Cryptomonas ovata by Collins and Kalnins [8, 9]. This group of compounds was found after steam distillation of algal cultures. Steam distillates of Chlorella species have however yielded limonene, myrcene and eucalyptol [10]. Wether these compounds can be liberated under natural conditions from these species is not known. Amines were found to be responsible for the fishy odour of the cultures of a variety of algal species [11]. Dur-

ing investigations on a bloom of Microcystis wesenbergii in the Federsee we isolated the tobacco like odorous compound β-cyclocitral [12]. Now we would like to report on the aromatic odour of Cyanidium caldarium which is produced by a series of nor-carotenoids previously unknown in microalgae.

Materials and Methods

Precultivation of the algae

Cyanidium caldarium strain 1355/1 Allen was obtained from the Culture Centre of Algae and Protozoa, Cambridge, Anabaena variabilis from Dr. N. G. Carr, Department of Biochemistry, University of Liverpool, Synedochococcus 6911 from Prof. R. Y. Stanier, Institut Pasteur, Paris, Nannochoeris cocoideis No. 251-1 from the Sammlung von Algenculturen des Pflanzenphysiologischen Instituts, Göttingen. The bioassay test organisms Anabaena, Synedochococcus and Nannochoeris were grown in a medium of Jüttner [12] at 27 °C in 300 ml cultivation tubes (see Kuhl and Lorenzen [13]) with 0.2% CO2 air aeration. Cyanidium was cultivated at 30 °C, sparged with 1.2% CO2 in air using a medium of Allen [14] with minor modifications. Full details of which are as follows: In 1 litre of distilled water, the following salts were added: 1.3 g (NH4)2SO4, 0.25 g MgSO4·7H2O, 0.26 g KH2PO4, 74 mg CaCl2·2H2O, 3.1 mg H3BO3, 2 mg MnCl2·4H2O, 290 μg ZnSO4·7H2O, 480 μg Na2MoO4·2H2O, 50 μg CuSO4·5H2O, 50 μg CoCl2·6H2O and 3.7 mg Na-FeIn-ethylene diamine tetraacetate (Fluka). The pH of the medium was adjusted to 3 with ammonia before autoclaving.

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0341-0382 / 79 / 0300-0186 $ 01.00/0

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Mass production of *Cyanidium caldarium*

The mass cultivation of *Cyanidium* was performed in a 110 litre algal pilot plant [15] illuminated with 54 fluorescent tubes (Osram Interna 39/40 w) which produced a light intensity of 6000 lx (3 \times 10^4 erg cm^{-2} s^{-1}) at the surface of the culture apparatus. The algal cell suspension was circulated at a rate of 25 m/min through the 4 cm i.d. glass tubes. The temperature was held at 30–31 °C and the gas flow rate at 360 l/h. A semicontinuous culturing procedure was performed. 95 litre of the algal suspension were harvested at weekly intervals. Details of sterilization, inoculation and harvesting were similar to those previously outlined [16]. The average weekly yield of *Cyanidium* was 750 g wet weight.

Production and concentration of the odorous compounds

The stripping method was used to separate the odorous compounds from the algal suspension. The gases from the algal cultivation plant were precooled to +1 °C by passing a thermostatisized condenser which removed most of its water. The precooled gases were trapped by succession in a 4 cm i.d. U-tube to remove residual water and finally three 55 cm high 3 mm i.d. loops half dipped in a dry ice-acetone bath. This arrangement markedly reduced the formation of aerosols. The condensate of the two major traps was removed daily. The loops were washed with a small amount of diethyl ether every 3 days. The ether washings and the condensed water were united and stored. 125 g of water condensate collected within 7 days were extracted 3 times with a total of 70 ml diethyl ether and the ether extract dried over Na_2SO_4. The diethyl ether used was previously distilled over Na and paraffin wax. The residue of 10 ml of which upon evaporation exhibited no background in the gas chromatographic separation at the sensitivity usually used for analysis of the odorous compounds.

Gas chromatography and mass spectrometry

The etheric extract was gently concentrated in a rotary evaporator to 400 μl. 2 μl were injected into a gas chromatograph (model Carlo Erba 180) with an integrator (Supergrator-2/Columbia Scientific Industries) equipped with a 15 m OV-101 WCOT glass capillary column. H_2 was used as the carrier gas (100 ml/min with a split ratio of 1 : 30). The flame ionisation detector and the injection port were maintained at 250 °C. A temperature program from 40 °C to 180 °C with 5 °C/min was chosen. The mass spectra were obtained by a LKB 9000 at a ionizing energy of 70 eV, the same column but helium as carrier gas were used for separation.

Preparative separation and hydrogenation

Preparative separation of the etheric extract into 7 fractions was achieved with a Hewlett Packard 5750 G gas chromatograph with a miniprep. A packed 1.8 m long and 3 mm i.d. glass column (3% OV-17 on Chromosorb W AW DMCS 80 – 100 mesh) was used. The eluted fractions were trapped in glass vials cooled in a dry ice-acetone bath. For hydrogenation the fractions were solved in 20 μl diethyl ether, supplied with a trace of PtO_2 and hydrogenated for 2 h.

Reference substances

β-Ionone was purchased from Fluka/Neu-Ulm/Germany; methylheptenone was a kind gift of the Teranol AG, Lalden/Switzerland and a mixture of cis- and trans-geranylacetone was obtained from BASF/Ludwigshafen/Germany. 1,2-Dihydro-1,1,6-trimethylnaphthalene was prepared after the method of Karrer and Ochsner [17] from β-ionone. A pure sample was obtained by distillation and preparative gas chromatography. The 6-(but-2-enylidene)-1,5,5-trimethylcyclohex-1-enes were obtained after treatment of β-ionol with POCl_3 in pyridine. The reaction mixture of which was separated by preparative gas chromatography [18] on a 9 mm i.d. and 1.8 m glass column filled with 3% OV-17 on Chromosorb W AW DMCS 80 – 100 mesh, using a carrier gas flow of 120 ml/min and a split ratio of 1 : 15. The UV spectra were recorded in diethyl ether on a Cary spectrophotometer. The saturated compounds were synthesized by hydrogenation with H_2/PtO_2 from the unsaturated ones.

Algal bioassay

300 ml Erlenmeyer flasks filled with 100 ml algal suspension were incubated in a controlled environment incubator (model G 27, New Brunswick Sci.
with shaking at 150 rev/min. During the growth experiments on the Cyanobacteria, an incident light intensity on the cultures of 1400 lx was used, and the incubator was aerated with 0.045% CO₂ in air at a flow rate of 500 ml/min. For that of Cyanidium and Nannochloris a light intensity of 4500 lx and an aeration rate of 500 ml/min (1.5% CO₂ in air) was used. The CO₂-air mixture was controlled by an infra red gas analyser (model 20, Grubb Parsons/ Newcastle upon Tyne, UK). The culture density was determined colorimetrically (model E 1009, Metrohm) twice a day at 550 nm. The nor-carotenoids were added in the bioassays when the cultures had reached E₅₅₀cm = 0.1. Quantities of less than 1 µl were diluted with ethanol 1:10 (v/v) before addition.

Results

The aroma of *Cyanidium caldarium* can easily be recognized in small scale cultures as well as in mass culture. It resembles the odour, Cyanobacteria and Chlorophyceae often impart to the culture media. With culture sparging the bulk of these odorous compounds were stripped from the pilot plant. Extraction of the culture medium with diethyl ether or sublimation of the algae themselves (400 g wet weight) lead only to minor amounts of these compounds produced. Though a 110 litre mass culture of *Cyanidium* was used to produce the odorous compounds, µg amounts of a single compound were never surpassed, e.g. not more than 0.7 µg/d of β-ionone were obtained. Therefore, the identification of these compounds could only be performed using gas chromatography, mass and UV spectrometry, combined with microchemical reactions.

The odour of *Cyanidium* is represented by a mixture of several compounds which can be separated on a WCOT glass capillary column. Fig. 1 shows such a separation of odorous compounds found in the ether extract of the trapped condensate. The mass spectra of peak 1 (m/e 43(100%), 41(49%), 69(38%), 55(34%), 108(29%), 31(21%), 58(18%), 111(16%), 71(16%), 39(16%), 68(15%)) and 13 (m/e 177(100%), 43(61%), 44(26%), 41(22%), 159(20%), 93(18%), 91(18%), 149(16%), 135(16%), 178(14%), 192(13%)) were very similar to those reported for 6-methylhept-5-en-2-one (IV) and β-ionone (I) [19]. Hydrogenation of the ether extract lead to the formation of compounds which allowed the mass spectra of the hydrogenated derivatives to be determined. The original and hydrogenated compounds were spiked with reference substances and found to be identical on a capillary column. These compounds were always major constituents of the odorous extracts. The amount of an additional compound which can not be unequivocally correlated to a peak on the chromatogram chart presented changed markedly during the course of culture. This substance exhibited retention times and a mass spectrum (m/e 43(100%), 69(49%), 41(33%), 151(10%), 136(10%), 44(10%), 149(8%), 125(8%), 107(8%), 93(8%), 83(8%), 93(8%), 67(8%), 55(8%), 53(8%), 40(8%), 39(8%), 29(8%)) similar to those of an authentic sample of geranylacetone (V). In the literature a spectrum was reported by Buttery and Seifert [20]. Geranylacetone could be superimposed with the algal product on a capillary column. For the identification of the compounds 8 and 11 on the gas chromatogram, these were separated by preparative gas chromato-
Table I. Effect of nor-carotenoids on the growth of algae for 24 hours after the addition of various amounts (ppm) of the stated compounds, given as doubling time (h). oo = lethal for algal cells.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Added amounts [ppm]</th>
<th>β-ionone</th>
<th>Methylheptenone</th>
<th>Geranylacetone</th>
<th>Dihydrotrimethyl-naphthalene</th>
</tr>
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<tbody>
<tr>
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<td>0</td>
<td>0</td>
<td>16</td>
<td>20</td>
<td>18</td>
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<td>Anabaena</td>
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<td>Nannochloris</td>
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<td>Cyanidium</td>
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* Alga treated thus exhibited unchanged growth rate for 24 h, however after this time further growth ceased.

Table II. Effect of nor-carotenoids on the growth of algae. (−) no effect on growth, (+) growth is inhibited, (∞) lethal for algal cells.

<table>
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<th>Compound</th>
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<td>Cyanidium</td>
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inhibited only 24 h after addition of these compounds. A compilation of the effects of the tested substances on growth can be seen in Table II.

**Discussion**

Preliminary experiments have demonstrated a great number of volatile odorous compounds liberated from *Cyanidium* into the medium. Some of these have also been found in Cyanobacteria and Chlorophyceae. The analysis in the latter classes is complicated by the heavy contamination with branched and unbranched hydrocarbons. *Cyanidium* however liberates only small amounts of these alkanes and thus this alga presented an ideal opportunity for a detailed study on this subject. The odorous compounds can be obtained in a virtually pure form by the stripping method used. The analysis and identification of the major products showed that all those so far determined belong to nor-carotenoid type molecules. Monoterpenes, characteristic for higher plants have not been found in this study. In this respect the Rhodophycean alga *Cyanidium* [23] resembles the Cyanobacteria, in which up to now no genuine monoterpenes have been found [12]. The fact that α-ionone has not been detected is in agreement with the experiments of Sanderson et al. [24] demonstrating that nor-carotenoids originate from carotenoids, and of Allen et al. [25] who detected only β-carotene in *Cyanidium*. The occurrence of butenylidenetrimethylcyclohexene does not provide opposing evidence for this postulate since the movement of double bonds from the β- to the α-position in the molecule is frequently encountered during the dehydration of β-ionol under a variety of experimental conditions. Dihydrotrimethylnaphthalene is formed *in vitro* when β-ionone epoxide is treated in acid [26]. In fact, the medium of *Cyanidium* has an acid reaction (pH 2–3) but no indication was found for the existence of such a precursor molecule during our investigation. Methylheptenone and geranylacetone are the most likely degradation products of open chain carotenoids. Though open chain carotenoids have not yet been reported in *Cyanidium*, their existence in this organism can be deduced from the general biosynthetic pathway of carotenoids in other organisms, the assumption of which should also be valid for *Cyanidium*. The formation of nor-carotenoids in *Cyanidium* resembles the pathways operating in flowers and fruits of higher plants. Butenylidenetrimethylcyclohexene has been found in the passion fruit [27], dihydrotrimethylnaphthalene in peach fruit [21], β-ionone, methylheptenone and geranylacetone in a great variety of fruits. Methylheptenone has also been found in fungi [28] but, nevertheless, there are no reports of these compounds in bacteria. There is some suggestive evidence that nor-carotenoids are generally distributed throughout the major algal classes, e.g. β-cycloalitrin has been found in the Cyanobacterium *Microcystis* [12] and β-ionone in the Chlorophyceae *Trentepohlia* [3].

This study demonstrated that nor-carotenoids have an effect on photoautotrophic organisms. The threshold values of their efficiency in effecting growth was similar for all algae tested despite their phylogenetic separation. With the exception of methylheptenone the compounds assayed revealed an inhibition between 10 and 50 ppm. It is well known that nor-carotenoids are extremely effective as growth modifiers. Firn et al. [29] described the inhibitory properties of a compound on the growth of higher plants which was found to be xanthoxin. Methylheptenone has a stimulatory effect on the germination of uredospores of several species of rust as has β-ionone [30]. Zajic and Kuehn [31] demonstrated a greater antimicrobial activity of β-ionone against soil bacteria than fungi. However, concentrations as high as 2000 ppm were used. In several studies β-ionone was demonstrated to effect carotene biosynthesis without being metabolized itself. Methylheptenone has been shown to inhibit, β-ionone to activate carotene biosynthesis [32, 33]. In natural waters Sakevich [34] has demonstrated the liberation of volatile inhibitory compounds from natural blooms of Cyanobacteria. Their formation was dependent on the physiological state of the algae and on the growth environment. Though their chemical nature was not determined they may have well been nor-carotenoids. An effect of nor-carotenoids under natural conditions is not ruled out, but the concentrations reached may not be high enough to cause a short term inhibition of organisms in natural waters. In these natural conditions a long term inhibition is a much more likely event.

I am greatly indebted to Mr. K. Wurster for his excellent assistance, to BASF/Ludwigshafen/Germany for a gift of geranylacetone and β-ionone and Ciba Geigy/Basel/Switzerland for methylheptenone. I am very appreciative of the financial support of the Deutsche Forschungsgemeinschaft.