

Characteristic oxidation behavior of β -cyclocitral from the cyanobacterium *Microcystis*

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Received: 15 September 2015 / Accepted: 29 February 2016 / Published online: 10 March 2016
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Abstract The cyanobacterium *Microcystis* produces volatile organic compounds such as β -cyclocitral and 3-methyl-1-butanol. The lysis of cyanobacteria involving the blue color formation has been occasionally observed in a natural environment. In this study, we focused on the oxidation behavior of β -cyclocitral that contributed to the blue color formation in a natural environment and compared β -cyclocitral with a structurally related compound concerning its oxidation, acidification, and lytic behavior. The oxidation products of β -cyclocitral were identified by the addition of β -cyclocitral in water, in which 2,2,6-trimethylcyclohex-1-ene-1-yl formate and 2,2,6-trimethylcyclohexanone were structurally characterized. That is, β -cyclocitral was easily oxidized to produce the corresponding carboxylic acid and the enol ester in water without an oxidizing reagent, suggesting that this

oxidation proceeded according to the Baeyer-Villiger oxidation. The oxidation behavior of β -cyclocitral in a laboratory was different from that in the natural environment, in which 2,2,6-trimethylcyclohexanone was detected at the highest amount in the natural environment, whereas the highest amount in the laboratory was β -cyclocitric acid. A comparison of β -cyclocitral with structurally similar aldehydes concerning the lytic behavior of a *Microcystis* strain and the acidification process indicated that only β -cyclocitral was easily oxidized. Furthermore, it was found that a blue color formation occurred between pH 5.5 and 6.5, suggesting that chlorophyll *a* and β -carotene are unstable and decomposed, whereas phycocyanin was stable to some extent in this range. The obtained results of the characteristic oxidation behavior of β -cyclocitral would contribute to a better understanding of the cyanobacterial life cycle.

Responsible editor: Philippe Garrigues

Electronic supplementary material The online version of this article (doi:10.1007/s11356-016-6369-y) contains supplementary material, which is available to authorized users.

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Keywords β -cyclocitral · Oxidation · Acidification · Cyanobacteria · Blue color formation · Lysis

Introduction

Cyanobacteria have occurred worldwide as water blooms in eutrophic lakes and drinking water reservoirs in recent years. This has resulted in deterioration of the landscape, problems with filtration in the water purification process, and the formation of musty odor substances, and one of the typical cyanobacteria, *Microcystis*, produces potent hepatotoxic microcystins (Watanabe et al. 1996; Chorus and Bartram 1999). Therefore, it is important to regulate the occurrence and outbreak of cyanobacteria. Although biomanipulation and algicides have been used, no permanent solution has been found (Chorus and Bartram 1999; Wu et al. 2006). Sigeo et al.

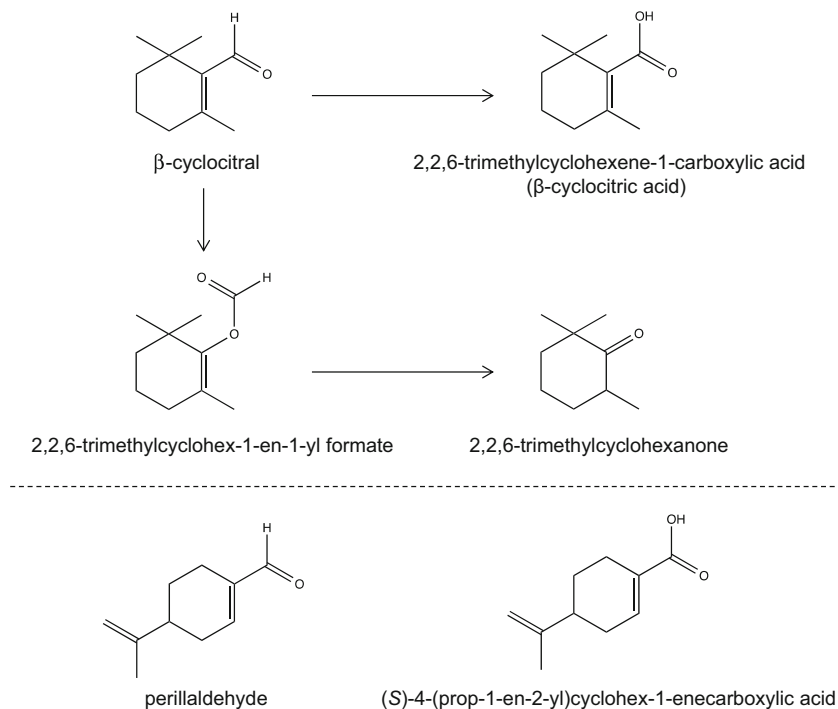
(1999) reported the biological control of cyanobacteria, i.e., principles and possibilities (Sigeo 2005). We have been attempting to establish a biological control system using microorganisms that can lyse cyanobacteria and degrade the microcystin (Hashimoto et al. 2009). Consequently, we collected a microorganism from Lake Sagami, Japan, and characterized it as *Brevibacillus* sp. (termed B-1). Because B-1 has a lytic activity against cyanobacteria, we tried to isolate the active compounds from the cultured broth of this strain (Harada et al. 2007). Although no active compounds were found from B-1, two volatile organic compounds (VOCs), β -cyclocitral (Fig. 1) and 3-methyl-1-butanol, were instead detected from the cultured broth of *Microcystis* with B-1. These VOCs, particularly β -cyclocitral, possessed a lytic activity and were produced from the cyanobacteria (Ozaki et al. 2008).

In previous studies, we developed an analysis method for the VOCs from the cyanobacterium *Microcystis* using solid phase microextraction (SPME) (Fujise et al. 2010). The VOCs are classified into two types, i.e., β -ionone and β -cyclocitral from β -carotene, and 2-phenylethanol and 3-methyl-1-butanol from amino acids. Using this method, it was found that the former was detected in the intracellular fraction and the latter was in the extracellular fraction. In addition, we found that β -cyclocitral causes an interesting phenomenon, a blue color formation, during the cyanobacteria lysis process in the laboratory experiment. Such a blue color was derived from phycocyanin after the disappearance of the remaining photosynthetic pigments, when β -cyclocitral was oxidized to 2,2,6-trimethylcyclohexene-1-carboxylic acid (abbreviated as β -cyclocitric acid, Fig. 1) (Harada et al. 2009).

The lysis of cyanobacteria involving the blue color formation was observed at Lake Sagami in the north of Kanagawa Prefecture, Japan, on September 6, 2013. We collected lake water containing the cyanobacteria and investigated the VOCs, such as β -cyclocitral, β -ionone, and the oxidation products of β -cyclocitral, as well as the number of cyanobacterial cells, their damage, and pH changes. As a result of a comparison of the samples with and without the blue color formation, the following characteristic results were obtained: a higher concentration of several VOCs including β -ionone and the oxidation products of β -cyclocitral shown in Fig. 1; a lower pH; and a higher number of *Microcystis* cells and characteristic morphological change in the damaged cells (Arii et al. 2015). Particularly, they detected 2,2,6-trimethylcyclohex-1-ene-1-yl formate and 2,2,6-trimethylcyclohexanone as the major products together with β -cyclocitric acid, suggesting that the oxidation of β -cyclocitral proceeded according to the Baeyer-Villiger oxidation.

In this study, we focused on the oxidation behavior of β -cyclocitral that contributed to the blue color formation in the natural environment and compared β -cyclocitral with a structurally related compound concerning the oxidation, acidification, and lytic behavior. After identification of the oxidation products by the addition of β -cyclocitral to water was carried out, 2,2,6-trimethylcyclohex-1-ene-1-yl formate and 2,2,6-trimethylcyclohexanone (Fig. 1) were structurally characterized. To investigate the oxidation process of β -cyclocitral, an additional analysis method for β -cyclocitral and its oxidation products was optimized. Finally, to clarify the oxidation mechanism of β -cyclocitral, we used the two aldehydes and

Fig. 1 Structures of β -cyclocitral and its oxidation products, and perillaldehyde and (*S*)-4-(prop-1-en-2-yl)cyclohex-1-enecarboxylic acid



their corresponding acids and observed the lytic behavior of a *Microcystis* strain and the acidification process by addition of the four compounds.

Materials and methods

Chemicals

For the VOCs, β -cyclocitral and 2,2,6-trimethylcyclohexanone were purchased from Alfa Aesar (Ward Hill, MA, USA). Perillaldehyde and geosmin- d_3 were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemical Industries (Kyoto, Japan), respectively. Methanol, diethyl ether, methyl *tert*-butyl ether (MTBE), and other all reagents were purchased from Nacalai Tesque (Kyoto, Japan).

General experimental procedures

The $^1\text{H-NMR}$ spectrum was recorded by an ECA500 (JEOL, Tokyo, Japan) 500 MHz spectrometer using CDCl_3 as the referenced solvent at 24 °C. The $^1\text{H-NMR}$ spectrum was referenced to the $\delta_{\text{H}}7.26$ ppm solvent peak for CDCl_3 . High-resolution mass spectral data were obtained as follows: the analysis was performed using an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) equipped with an electrospray ionization probe. A sample was dissolved in water/acetonitrile/formic acid (5/95/0.1, v/v/v). The dissolved compound was directly loaded into the mass spectrometer (MS) by an infusion pump. The MS analysis was performed in the negative FTMS mode at a resolution of 7500 with the following source parameters: sheath gas flow rate was 15, tube lens voltage was -150 V, capillary voltage was -35 V, and ion spray voltage was -5.0 kV. The HPLC was performed using a pair of LC-10AD VP pumps, a DGU-12A degasser, a CTO-10A VP column oven, an SPD-M10A VP detector, and a CLASS-VP system controller (Shimadzu, Tokyo, Japan). The detection wavelength was 254 nm. The column was a TSK-gel Super-ODS column (100 \times 2 mm ID, particle size 2 μm , TOSOH, Tokyo, Japan). The mobile phase was methanol/water containing 0.1 % formic acid. The methanol concentration was increased from 40 to 80 % for 10 min in the linear gradient mode. The column temperature was 40 °C, and the flow rate was 0.2 mL min^{-1} . The gas chromatography–mass spectrometry (GC/MS) was performed using a gas chromatograph (Agilent 6890, Agilent Technologies, Palo Alto, CA, USA) connected to an Agilent Technologies VF-1ms column (60 m \times 0.25 mm ID \times 1.0 μm film) and to a mass selective detector (Agilent 5973). The injector temperature was 250 °C, and the column temperature program started at

50 °C (5 min), then to 250 °C at 20 °C min^{-1} with a 5-min hold at 250 °C. Helium was used as the carrier gas (1 mL min^{-1}). The detector temperature was 250 °C.

Cyanobacteria cultures

The axenic strain NIES-102 belonged to *Microcystis*, and it was obtained from the National Institute for Environmental Studies (NIES), Tsukuba, Japan. This strain was cultured in 1 L Erlenmeyer flasks each containing a modified MA medium (300 mL) at 25 °C for 8 days under 28 $\mu\text{E m}^{-2} \text{s}^{-1}$ continuous illumination. The MA medium consisted of a mixture of bicine (500 mg); $\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$ (50 mg); KNO_3 (100 mg); NaNO_3 (50 mg); Na_2SO_4 (40 mg); $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (50 mg); β - Na_2 glycerophosphate (100 mg); a metal mixed solution (1 mL; composed of 1 mg of Na_2EDTA , 0.1 mg of $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 1 mg of $\text{MnCl}_3 \cdot 4\text{H}_2\text{O}$, 0.1 mg of ZnCl_2 , 1 mg of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.16 mg of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 4 mg of H_3BO_3 in 200 mL of distilled water); and the resulting solution was adjusted to pH 8.6 (Ozaki et al. 2008).

Preparation and isolation of acidic compounds

Silver oxide (Ag_2O , 1.90 g) was added to water (10 mL) then NaOH (320 mg) was added to the Ag_2O solution. β -Cyclocitral (2.92 g) or perillaldehyde (2.40 g) was added to this solution with shaking at room temperature for 20 h. The reaction solution was extracted with diethyl ether to remove the starting material, and 0.5 % HCl was added to the lower layer for acidification. The resulting acidic solution was extracted with diethyl ether then the solvent was evaporated. The residue was recrystallized using benzene and cyclohexane to give colorless prism crystals with the following melting points: β -cyclocitric acid; 92–94 °C and (*S*)-4-(prop-1-en-2-yl)cyclohex-1-enecarboxylic acid; 127–128 °C determined by an MP-S3 micromelting point apparatus (Yanagimoto, Kyoto, Japan).

Preparation of 2,2,6-trimethylcyclohex-1-en-1-yl formate

An aqueous solution (20 mL) of β -cyclocitral (2.9 g) was stirred for 40 h at room temperature. The solution had a pH of 3 and was extracted with diethyl ether. The extract was evaporated to dryness to provide an oily substance (2.18 g) which was subjected to silica gel column chromatography (100 g) with benzene/*n*-hexane (1:1) as the mobile phase. 2,2,6-Trimethylcyclohex-1-en-1-yl formate (0.165 g) was obtained as an oily compound. The obtained signals in the $^1\text{H-NMR}$ spectrum are as follows: $\delta^1\text{H}$ (ppm), 1.02 (3H, s, H-7); 1.02 (3H, s, H-8); 1.49 (3H, s, H-9); 1.58 (2H, m, H-3); 1.62 (2H, m, H-4); 2.07 (2H, t, H-5); and 8.06 (1H, s, $\underline{\text{CHO}}$). The chemical shifts of 2,2,6-trimethylcyclohex-1-en-1-yl formate were assigned

based on a comparison to the proton chemical shifts of β -cyclocitral. The high-resolution mass spectral data of the deprotonated molecule at m/z 167.10848 $[\text{M-H}]^-$ indicated the exact composition for $\text{C}_{10}\text{H}_{16}\text{O}_2$ (167.10720).

Oxidation behavior of β -cyclocitral in water

The oxidation behavior of β -cyclocitral was observed by GC/MS. The final concentration (6.5 mmol L^{-1}) of β -cyclocitral was added to 100 mL of water then shook for 1 and 3 days. Two-milliliter aliquots of the samples were collected at 0, 1, 3, 5, 10, 24, and 72 h. Two milliliters of diethyl ether was added to these collected samples for extraction. The diethyl ether layer was applied to the GC/MS analysis mentioned above. The VOC peaks were obtained from the total ion chromatogram (TIC).

Quantification of β -cyclocitral and its oxidation products

Two milliliters of the samples was acidified by adding 20 μL of 1 mol L^{-1} HCl, and immediately extracted with 2 mL of MTBE containing 200 ng of geosmin- d_3 as the internal standard. The upper layer was dried over sodium sulfate for a few minutes then placed in an amber vial sealed with a Teflon-lined cap. The GC/MS was performed using a gas chromatograph (HP 6890, Agilent Technologies) connected to an Agilent Technologies DB-FFAP column ($30 \text{ m} \times 0.25 \text{ mm ID} \times 0.25 \mu\text{m}$ film) and to a mass selective detector (HP 5973). The injector temperature was $250 \text{ }^\circ\text{C}$ and the column temperature program started at $40 \text{ }^\circ\text{C}$ (4 min) then from 40 to $240 \text{ }^\circ\text{C}$ at $20 \text{ }^\circ\text{C min}^{-1}$ with a 10-min hold at $240 \text{ }^\circ\text{C}$. One microliter of the sample was injected into the GC injection port in the splitless mode using an injector (HP 6890 series). The injection port was equipped with a 2.0-mm ID single taper deactivated liner (Restek, Bellefonte, PA, USA). Helium was used as the carrier gas (1 mL min^{-1}). The MSD interface temperature was $250 \text{ }^\circ\text{C}$, and the detector temperature was $230 \text{ }^\circ\text{C}$. Electron ionization (EI) was used for the ionization at the electron energy of 70 eV and for the selected ion monitoring (SIM) mode: m/z 137 for β -cyclocitral; m/z 153 for β -cyclocitric acid; m/z 82 for 2,2,6-trimethylcyclohexanone; m/z 125 for 2,2,6-trimethylcyclohex-1-en-1-yl formate; and m/z 115 for geosmin- d_3 were monitored.

Oxidation behavior of β -cyclocitral in water, medium solution, and cultured broth

The final concentration (6.5 mmol L^{-1}) of β -cyclocitral was added to 100 mL of water, MA medium, and the *Microcystis aeruginosa* NIES-102 cultured broth. These solutions were shaken for 1 and 3 days. Two-milliliter aliquots of the samples

were collected at 0, 1, 3, 5, 8, 22, 48, and 72 h. These samples were treated as described above, and β -cyclocitral and its oxidation products were quantified by GC/MS.

Lytic study with β -cyclocitral and related compounds

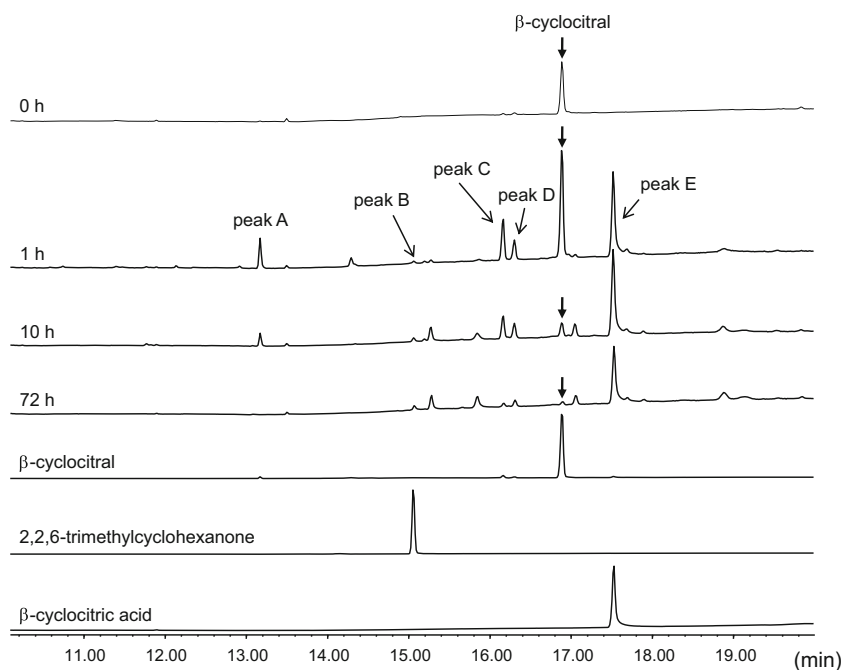
The anticyanobacterial activity was determined by measuring the absorbance of chlorophyll *a* (Uchida et al. 1998). Briefly, the final concentrations (6.5 mmol L^{-1}) of β -cyclocitral and related compounds (β -cyclocitric acid, perillaldehyde, (S)-4-(prop-1-en-2-yl)cyclohex-1-enecarboxylic acid) were added to 100 mL of the cultured cyanobacteria (8 days), and incubated at $25 \text{ }^\circ\text{C}$ with shaking for 1 and 7 days under continuous illumination ($8 \mu\text{E m}^{-2} \text{ s}^{-1}$). The culture medium was used as the negative control, which did not lyse cyanobacteria. Five-milliliter aliquots of the culture medium were withdrawn according to the color change during the first day and then once per day until the seventh day. Simultaneously, the culture broth was photographed. The pH of the withdrawn samples was measured using a Navi F-52 pH meter (Horiba, Ltd., Kyoto, Japan). After the filtration using a GF/A filter (Whatman International, Ltd., Maidstone, UK), the cyanobacteria on the filters were soaked in 2.5 mL of methanol and the resulting mixture stored at $4 \text{ }^\circ\text{C}$ in the dark overnight. Aliquots of 200 μL of the supernatant were placed on a 96-well microplate (Kartell, Milan, Italy), and the optical density was measured at 665 nm using an MPR-A4iII microplate reader (TOSOH, Tokyo, Japan). The anticyanobacterial activity was determined by the decrease in the absorbance. Five microliters of the sample was filtered using an Ultrafree-MC membrane centrifuge filtration unit (hydrophilic PTFE, 0.2 μm , Millipore, Bedford, MA, USA). The filtrates were analyzed by HPLC mentioned above.

Results

Addition of β -cyclocitral to water and confirmation of the oxidation products

To identify the oxidation products, β -cyclocitral was added to water and the reaction mixture was allowed to stand for 3 days. After 0, 1, 3, 5, 10, 24, and 72 h, the reaction products were extracted with diethyl ether and analyzed by GC/MS. Figure 2 shows the typical chromatograms of the reaction products in water after 0, 1, 10, and 72 h. There are several peaks in the total ion chromatogram. We focused on peaks C and E because they were observed with a higher intensity than others in the chromatogram after 1 h. The peak of β -cyclocitral had almost disappeared after 72 h. Peak C was identified as the enol ester, 2,2,6-trimethylcyclohex-1-ene-1-yl formate. The structure determination of this compound will

Fig. 2 Time course of the resulting β -cyclocitral, β -cyclocitric acid (*peak E*), 2,2,6-trimethylcyclohex-1-ene-1-yl formate (*peak C*), and 2,2,6-trimethylcyclohexene (*peak B*) by addition of β -cyclocitral to water



be described in detail in the next section. Peak E with a slight tailing was identified as β -cyclocitric acid by comparison with an authentic sample (Fig. S1). Peak B was identified as 2,2,6-trimethylcyclohexanone by comparison with standard material (Fig. S1). Peak A with m/z 124 (M^+) disappeared after 72 h, and peak D with m/z 140 (M^+) continued to appear for 72 h. However, these two peaks may be impurities of β -cyclocitral because they were already present in the standard reagent (Fig. S2).

Structure determination of 2,2,6-trimethylcyclohex-1-ene-1-yl formate and 2,2,6-trimethylcyclohexanone

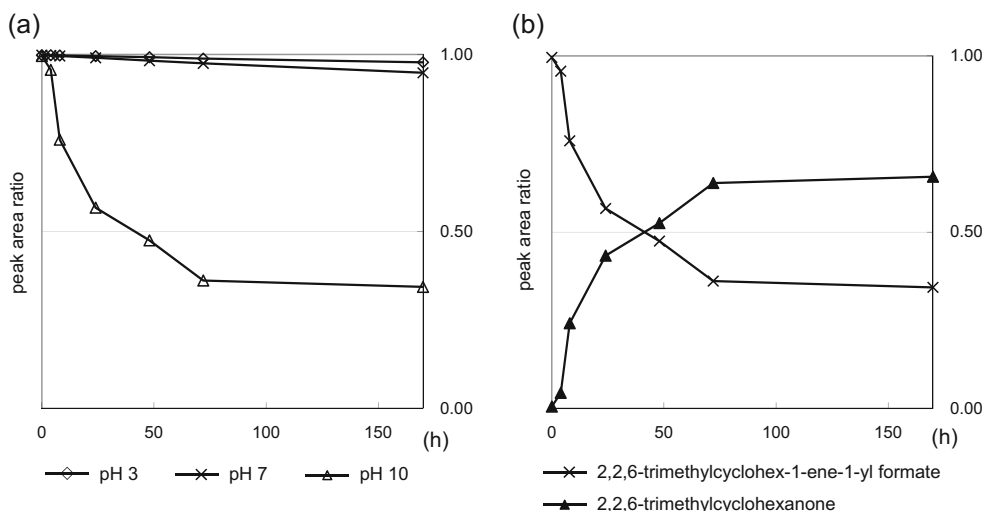
The EI mass spectrum of peak C is shown in Fig. S3, in which the ion at m/z 168 appears as the molecular ion (M^+) and the following fragment ions, m/z 153, 140, and 125, are also observed. These ions can be assigned to (M^+ -15), (M^+ -28), and (m/z 140-15), respectively, and these assignments suggest that peak C is the enol ester, 2,2,6-trimethylcyclohex-1-ene-1-yl formate (Fig. 1). A characteristic formyl proton signal at 8.06 ppm in the $^1\text{H-NMR}$ spectrum (Fig. S4) supported the above structure characterization. In the EI mass spectrum of peak B, the M^+ was observed at m/z 140. As a result of the library search of the spectrum, it was considered to be 2,2,6-trimethylcyclohexanone and a direct comparison with a commercially available reagent showed that peak B was identified as 2,2,6-trimethylcyclohexanone (Fig. S1). To further confirm this conclusion, 2,2,6-trimethylcyclohex-1-ene-1-yl formate was prepared from β -cyclocitral, and subjected to a stability test in pH 3, 7, and 10 solutions. Although 2,2,6-

trimethylcyclohex-1-ene-1-yl formate was significantly stable under the acidic and neutral conditions, it was degraded to 2,2,6-trimethylcyclohexanone under the basic condition. The half-life time was estimated to be about 40 h (Fig. 3). These results indicated that β -cyclocitral was subjected to oxidation in water and produced two products, β -cyclocitric acid and 2,2,6-trimethylcyclohex-1-ene-1-yl formate (Fig. 1).

Oxidation behavior of β -cyclocitral under the different conditions

To investigate the oxidation behavior of β -cyclocitral, we had to precisely quantify β -cyclocitral and its oxidation products. Because the conventional method had some problems concerning accuracy, a method was optimized using the following procedures: to efficiently extract β -cyclocitric acid, hydrochloric acid was used for the acidification; methyl *tert*-butyl ether (MTBE) was used as the extraction solvent instead of diethyl ether; the addition of geosmin- d_3 as an internal standard (IS) to the extract made possible the accurate quantification; and the GC/MS column was changed to a DB-FFAP column to obtain a good separation of the peak of β -cyclocitric acid without tailing. A typical chromatogram by this method for the standard solution of β -cyclocitral and related compounds is shown in Fig. S5. The quantification of each compound was carried out in the selected ion monitoring (SIM) mode using the base peak of each compound as the target ion. The quantification limit of each compound was obtained as $10 \mu\text{g L}^{-1}$, and the calibration curve of all compounds showed a good linearity in the concentration range from 10 to $1000 \mu\text{g L}^{-1}$ (Fig. S6).

Fig. 3 Stability test for 2,2,6-trimethylcyclohex-1-ene-1-yl formate **a** under different conditions at pH of 3, 7, and 10, and **b** under a basic condition at pH 10

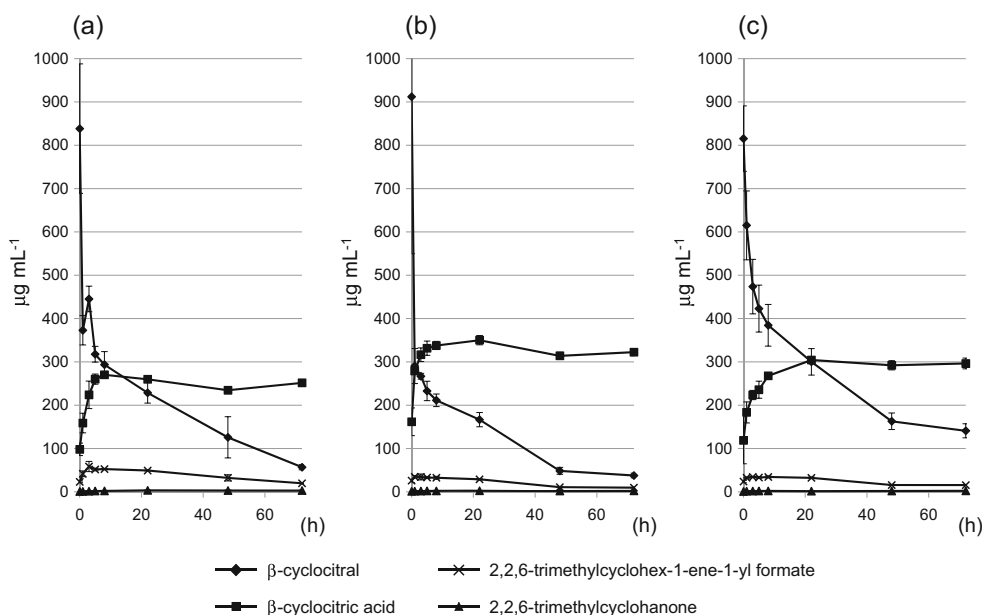


In this experiment, β -cyclocitral at a concentration of 6.5 mmol L^{-1} was separately added to water, MA medium, and the cultured broth of *M. aeruginosa* NIES-102 (abbreviated as NIES-102). Water and the MA medium provided almost the same behavior, but the NIES-102 cultured broth showed a different behavior compared to the other solutions (Fig. 4). While the half-lives of β -cyclocitral in the water and the MA medium were within 1 h, that of the cultured broth was about 3 h. After 22 h, the concentration of β -cyclocitral in the cultured broth was approximately equal to that of β -cyclocitric acid, whereas the concentration of β -cyclocitral was lower than that of the β -cyclocitric acid in the water and the MA medium. Also, after 72 h, the concentration of β -cyclocitral in the cultured broth was two times higher than the others. This suggested that β -cyclocitral was replenished from the cyanobacterium and then converted into the

corresponding acid. The concentration ratio of 2,2,6-trimethylcyclohex-1-ene-1-yl formate to β -cyclocitric acid gradually continued to decrease from about 20 % and that of 2,2,6-trimethylcyclohexanone to β -cyclocitric acid was less than 1 %. This indicated that the route to β -cyclocitric acid was the main one and that to 2,2,6-trimethylcyclohex-1-ene-1-yl formate and 2,2,6-trimethylcyclohexanone was the minor one in this laboratory experiment (Fig. 1).

The pHs of the reaction mixtures were measured after 1, 3, 5, 8, 22, 48, and 72 h. The pH of each solution decreased from the start of the addition until 8 h and thereafter constant (Fig. S7). However, the constant pHs of the water, MA medium, and NIES-102 cultured broth were 3.4, 4.7, and 6.4, respectively. These values were reflected by the presence or absence of a buffer action and photosynthesis. In the culture broth, the blue color formation was observed with

Fig. 4 Time course of the concentrations of β -cyclocitral and its oxidation products in **a** water, **b** MA medium, and **c** NIES-102 cultured broth



lysis of the cyanobacteria after 8 h. The concentration of β -cyclocitric acid increased until 8 h from the start of the experiment and thereafter remained constant. These results indicated that β -cyclocitric acid has a considerable effect on the pH of the solution.

Lytic behavior of *Microcystis* NIES-102 by the addition of β -cyclocitral, perillaldehyde, β -cyclocitric acid, and (S)-4-(prop-1-en-2-yl)cyclohex-1-enecarboxylic acid and their oxidation behavior

As already mentioned, β -cyclocitral was found to be easily oxidized. In the present experiment, we investigated the oxidation behavior of β -cyclocitral by comparison to that of perillaldehyde. The addition of perillaldehyde changed the cultured broth's green color to yellow after 5 h, then provided a colorless solution after 1 day (Fig. S8 (b)). This indicated that perillaldehyde possesses the strongest lytic activity among the tested compounds, and this behavior can be expected based on the MIC value of the perillaldehyde (Ozaki et al. 2008). However, the characteristic blue color was not observed during this process. The lytic behavior of β -cyclocitric acid was similar to that of perillaldehyde (Fig. S8 (c)). As expected, the addition of β -cyclocitral provided a slightly bluish color after 5 h and a blue-colored solution after 1 day (Fig. S8 (a)). For the (S)-4-(prop-1-en-2-yl)cyclohex-1-enecarboxylic acid, the characteristic behavior was observed in which the green color continued for 1 day, whereas the blue color appeared by day 2 and continued until day 3. This result indicated that the acid possessed a weaker lytic activity, but had the ability for the blue color formation.

As already mentioned, β -cyclocitral and (S)-4-(prop-1-en-2-yl)cyclohex-1-enecarboxylic acid showed the characteristic blue color, whereas perillaldehyde and β -cyclocitric acid did not, suggesting that this depended on the pH of the culture broth of the cyanobacteria. Figure 5 demonstrates the changes in pH of the broths after the addition of the four compounds or water. The broth, which water was added 0.1 mL, was used as the negative control, and the broth showed the expected pH around 10. While perillaldehyde showed a constant pH around 8 during the experiment, β -cyclocitric acid maintained a pH lower than 5 after 5 h. The HPLC chromatograms of these two compounds in the broths did not change during this experiment (Fig. S9). For β -cyclocitral, the pH was changed to around pH 6 after 5 h (Fig. 5). Although there were two peaks (the aldehyde and the corresponding acid) after 5 h, the aldehyde completely changed to the corresponding acid after 1 day. A mixture of the aldehyde and acid probably had a pH around 6, and this may be suitable for the blue color formation. Figure 5 also shows the change in the pH with (S)-4-(prop-1-en-2-yl)cyclohex-1-enecarboxylic acid in which the pH changed from 8.0 to about 5.5 to 6.0. In the chromatogram, only the peak of the acid was observed. These results indicated

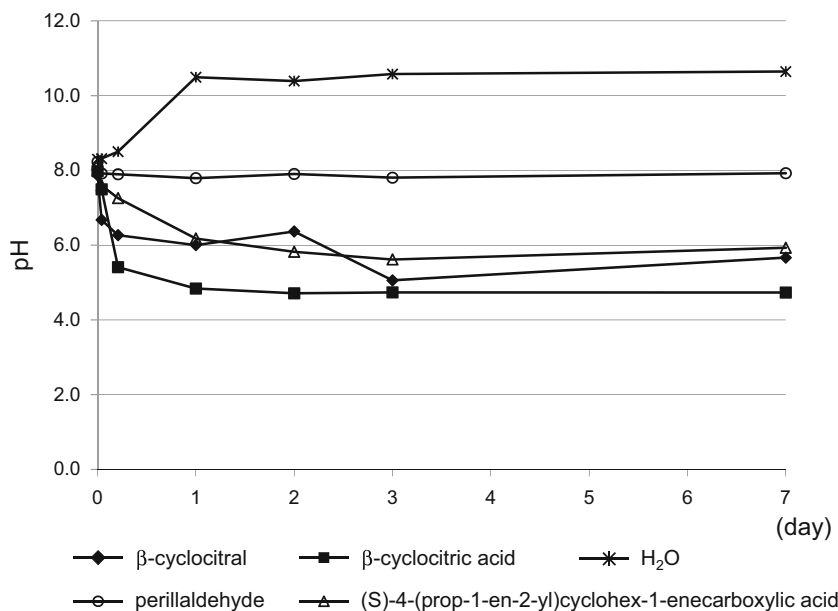
that the blue color formation occurred between pH 5.5 and 6.5, suggesting that chlorophyll *a* and β -carotene are unstable and decomposed, while phycocyanin is stable to some extent in this range.

Discussion

In a natural environment, the lytic phenomena associated with the occasional blue color are known. Fallon and Brook (1979) reported that the lysis was indicated by the presence of a blue opalescent sheen on the surface waters, resulting from the release of the phycocyanin pigments and gas vesicles from the lysing algae. For the lysis with the blue color formation, they considered lytic organisms (bacteria and protozoa) and photo-oxidation due to the effect of a high light exposure, and described that although both factors have shown to be potentially harmful to the cyanobacterial populations, the exact relationships regarding the effect under natural conditions is still poorly understood. They could neither identify the cause nor clarify the mechanism of the blue color formation (Fallon and Brook 1979). We also observed the lysis of cyanobacteria involving the blue color formation in the natural environment on August 5, 2008, and detected phycocyanin from the filtrate of the lysed cyanobacteria scum and β -cyclocitral, β -cyclocitric acid, and other VOCs (Arii et al. 2015). The pH of the lysed scum samples from Lake Tsukui indicated that the blue color formation started at 6.70, and that of blue color formation finished at 5.37. However, this behavior was not observed in the non-lysed scum samples, whose pH was about 9. These results indicated that β -cyclocitral was significantly related to the phenomenon including the blue color formation and disappearance of the cyanobacteria.

To the best of our knowledge, no report has been published about this type of reaction in which the β -cyclocitral was very smoothly oxidized in water without an oxidizing reagent to produce the corresponding carboxylic acid and the enol ester, which may be produced according to the Baeyer-Villiger oxidation, and the former was predominant (Fig. 4). However, the production ratio of the acid and the enol ester plus 2,2,6-trimethylcyclohexanone was approximately 1:2 in a sample with the blue color formation taken from Lake Sagami in Sept. 2013 (Arii et al. 2015). Based on these results, the oxidation of β -cyclocitral in a natural environment suggested that the formation of the enol ester may be related to the Baeyer-Villiger monooxygenases. Leisch et al. (2011) reported that the Baeyer-Villiger monooxygenases catalyze the oxidation of aldehydes and produce the corresponding acid and formate. They also showed that the oxidation predominantly gave the formate over the acid. Therefore, it is suggested that β -cyclocitral was oxidized to the acid and the enol ester by the Baeyer-Villiger monooxygenases in a natural environment and 2,2,6-trimethylcyclohex-1-en-1-yl formate is further hydrolyzed to

Fig. 5 Time course of the resulting pH by the addition of β -cyclocitral, perillaldehyde, β -cyclocitric acid, and (S)-4-(prop-1-en-2-yl)cyclohex-1-enecarboxylic acid to NIES-102 cultured broth



2,2,6-trimethylcyclohexanone due to the basic conditions in the lake water. In a laboratory experiment, the half-life of β -cyclocitral in water was about 1 h (Fig. 4), and the solution gradually became acidic by the production of β -cyclocitric acid. On the other hand, 2,2,6-trimethylcyclohexanone is considered to be produced by hydrolysis of 2,2,6-trimethylcyclohex-1-en-1-yl formate under basic conditions; the half-life was about 40 h (Fig. 3). These results suggested that β -cyclocitral is immediately oxidized by the Baeyer-Villiger monooxygenases after releases from the cells. At present, it is difficult to determine the exact reason why β -cyclocitral is easily oxidized in water without an oxidizing reagent. However, it should be emphasized that this property is significantly correlated with the lysis and the blue color formation of the cyanobacteria.

In a laboratory experiment, we compared the pH-lowering effect of β -cyclocitral to those of several similar aldehydes, such as citral, cinnamaldehyde, perillaldehyde, and vanillin, from plants (Harada et al. 2009). When these compounds were added to water, the pH of the water solution of cinnamaldehyde, perillaldehyde, and β -cyclocitral decreased to about 4. However, for the MA cultivation medium, the pH quickly decreased to 4.5 when β -cyclocitral was only added (Harada et al. 2009). The reason for the decrease in pH with β -cyclocitral in the MA medium solution is due to the fact that β -cyclocitral is easily oxidized to β -cyclocitric acid, thus contributing to the pH-lowering effect (Harada et al. 2009). These results suggest that the prompt oxidation of β -cyclocitral and subsequent acidification are important in order to clarify the function of β -cyclocitral. In a separate study, we oxidized β -cyclocitral and perillaldehyde with silver oxide that is a representative oxidizing reagent of aldehydes to carboxylic acids and found that the oxidation rate of β -cyclocitral was faster

than that of perillaldehyde. Because only β -cyclocitral was completely oxidized, it is more easily oxidized compared to similar aldehyde compounds (data not shown).

All four tested compounds showed individual lytic behaviors against the *Microcystis* strain (NIES-102), and the perillaldehyde possessed the strongest lytic activity (Fig. S8). The substantial lytic activity of the VOCs may depend on their MIC (Ozaki et al. 2008) and solubility in water. Although it is difficult to estimate the solubility of hydrophobic compounds, such as the VOC, in water, we have proposed a method using log D (Nozawa et al. 2009). The log D of β -cyclocitral was calculated to be 3.26, which means that the added β -cyclocitral was dissolved at approximately one two thousandth (1/2000) in water. In the present study, we found that the blue color formation could occur under favorable pH conditions (pH 5.5–6.5), in which chlorophyll *a* and β -carotene are unstable and decompose, while the phycocyanin is stable to some extent in this range (Fig. 5).

Conclusions

In this study, we performed rudimentary experiments to clarify that VOCs, such as β -cyclocitral, are involved to the blue color formation by lowering the pH of the lake water. We confirmed that it is possible to detect the oxidation products of β -cyclocitral observed in a natural environment by the addition of β -cyclocitral to water. 2,2,6-Trimethylcyclohex-1-ene-1-yl formate and 2,2,6-trimethylcyclohexanone, the oxidation products of β -cyclocitral, were structurally characterized using GC/MS and NMR methods. These indicated that β -cyclocitral was easily oxidized to provide the corresponding carboxylic acid and the enol ester in water without an

oxidizing reagent, suggesting that this oxidation proceeded according to the Baeyer-Villiger oxidation. The oxidation behavior of β -cyclocitral in the laboratory was different from that in the natural environment, in which 2,2,6-trimethylcyclohexanone was detected at the highest amount in the natural environment, whereas the highest in the laboratory was β -cyclocitric acid. This result suggested that the Baeyer-Villiger monooxygenases may participate in the oxidation of β -cyclocitral in the natural environment. On the other hand, we also investigated the lytic behavior of a *Microcystis* strain and the acidification process of β -cyclocitral by comparison with perillaldehyde that has a structure similar to β -cyclocitral. It was found that β -cyclocitral was only easily oxidized, and a blue color formation occurred between pH 5.5 and 6.5, suggesting that chlorophyll *a* and β -carotene are unstable and decompose, while phycocyanin was more stable in this range. Consequently, β -cyclocitral and its characteristic oxidation behavior is considered to have closely contributed to the blue color formation with the lysis of cyanobacteria, and plays a key role in the life of *Microcystis*. By revealing the function and role of β -cyclocitral, it may be possible to regulate the *Microcystis* population.

Acknowledgments We acknowledge Drs. Atsushi Miyachi and Andrea Roxanne J. Anas and Mr. Kohei Kawai for measurement of the high-resolution mass spectral data, NMR measurement, and technical assistance, respectively, in this study.

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