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Nostochopcerol, a new antibacterial monoacylglycerol from the edible cyanobacterium *Nostochopsis lobatus*

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**Abstract**

A new antibacterial 3-monoacyl-sn-glycerol, nostochopcerol (1), was isolated from a cultured algal mass of edible cyanobacterium *Nostochopsis lobatus* MAC0804NAN. Structure of 1 was established by the analysis of NMR and MS data while its chirality was established by comparison of optical rotation with synthetically prepared authentics. Compound 1 inhibited the growth of *Bacillus subtilis* and *Staphylococcus aureus* at MIC 50 μg/mL and 100 μg/mL, respectively.

**Keywords**

antibacterial; cyanobacterium; edible; monoacylglycerol; *Nostochopsis lobatus*
Findings

Cyanobacteria is widely accepted as a prolific source of unique bioactive metabolites [1]. Some cyanobacterial species are consumed as food, nutritional supplements, or folk medicines in many parts of the world [2], and have offered attractive opportunities for drug discovery. Results from the limited number of attempts include an antifungal lipopeptide nostofungicidine [3] and an antioxidant nostocionone [4] from *Nostoc commune*, an unusual antibacterial n-1 fatty acid from *N. verrucosum* [2], and the sacrolides, antimicrobial oxylipin macrolactones from *Aphanothece sacrum* [5, 6].

*Nostochopsis lobatus* is a freshwater species distributed in every climate zone but polar regions [7]. It grows on the riverbed rocks or cobbles in shallow streams and forms spherical to irregularly lobed, hollow, gelatinous colonies, with the size reaching up to 5.5 cm in diameter [8]. Although cosmopolitan, its occurrence is dominated in tropical regions, thus food consumption of this alga is only reported from India [9] and Thailand [10]. In India, local tribes utilize it as a dietary supplement [9]. In northern Thailand, this alga occurs in dry season from November to April and is called *Lon, Kai Hin* (stone egg), or *Dok Hin* (stone flower) [10]. It is consumed as an ingredient of salad and as a folk medicine to treat pain from stomach ulcers or fever [8]. In fact, an ethanolic extract of the air-dried alga was found to inhibit the development of gastric ulcers, suppress ethyl phenylpropiolate-induced edema on ear, and decrease writhing response induced by intraperitoneal injection of acetic acid in rodent models [10], thus supporting the ethnopharmacological testimonies. Moreover, radical scavenging activity [10, 11], hyaluronidase inhibitory activity [12], and tyrosinase inhibitory activity [13] was detected by *in vitro* testings, which further raised an expectation on its richness as the source of bioactive metabolites. However, at present, only a single drug discovery attempt has been made on this alga [12], which prompted further chemical study.
We evaluated the antimicrobial activity of the ethanolic extract of this alga and found that a mid-polar fraction inhibited the growth of two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*. Activity-guided fractionation led to the discovery of a new monoacylglycerol, nostochopcerol (1).

Figure 1: Structure of nostochopcerol (1)

**Results and Discussion**

A water-thawed algal mass of strain MAC0804NAN (374.6 g) was repeatedly extracted with EtOH. The combined extract was partitioned between 60% aqueous MeOH and CH₂Cl₂, and the latter lipophilic layer was further partitioned between 90% aqueous MeOH and *n*-hexane. The resulting three layers were tested against four Gram-positive bacteria, five Gram-negative bacteria, six fungi, and two yeasts, which detected antibacterial activity against two Gram-positive bacteria, *Bacillus subtilis* and *Staphylococcus aureus*, in the 90% aqueous MeOH layer. The responsible constituent, though prone to diffuse during chromatography, was purified with the guidance of antibacterial activity on ODS and Sephadex LH-20 and by reversed phase HPLC on ODS and styrene-divinylbenzene copolymer to yield 0.7 mg of 1 from 113.3 mg of the solvent partition fraction. The reason for the low yield of 1 was eventually understood after 1 was determined to be a monoacylglycerol, which has a surface-active property and should have deteriorated the separation capacity of the chromatographic resins.

The molecular formula of 1 was established to be C₁₉H₃₄O₄ based on a sodium adduct pseudomolecular ion at m/z 349.2348 [M + Na]⁺ observed by high resolution ESITOFMS (calcd. for C₁₉H₃₄NaO₄, 349.2355). Three degrees of unsaturation, calculated from the
molecular formula, were accounted for by a carboxyl group (δC 175.3) and two double bonds (δC 130.9, 130.6, 129.1, and 128.9) observed in the 13C NMR spectrum (Table 1), revealing that I has a linear structure. The 1H NMR spectrum contained resonances typical of an unsaturated fatty acid, such as non-conjugated olefins with four-proton integration (δH 5.34-5.32, 4H), a bisallylic methylene (δH 2.77, brt, J = 6.5 Hz, H9), a methylene adjacent to a carboxyl group (δH 2.34, brt, J = 7.5 Hz, H2), two allylic methylenes (δH 2.07, H26 and 2.05, H212), and an aliphatic methyl group (δH 0.89, t, J = 6.9 Hz, H316). Along with these resonances, several oxygenated (δH 4.13-3.54) and aliphatic signals (δH 1.62 and 1.39-1.30) were observed, implying that I is a derivative of fatty acid. Indeed, all oxygenated protons constituted a spin system (CH21’-CH2’-CH33’) in the COSY spectrum (Figure 2), and considering the lack of any terminal group besides CH316, monoacylglycerol was the only possible structure for I. This assignment was eventually proven after interpretation of the whole set of 1D and 2D NMR data. A carboxy carbon, four sp2 methines, one oxymethine, two oxymethylenes, ten aliphatic methylenes, and a methyl group were collected from the analysis of 13C NMR and HSQC spectra and these structural pieces were assembled into four spin systems by the COSY correlations: an ethyl group (C16-C15), a C8 internal hydrocarbon chain with two degrees of unsaturation (C12-C11=C10-C9-C8=C7-C6-C5), three consecutive methylenes (C4-C3-C2) with a carboxy-termination, and a glyceryl moieties (Figure 2). The Z-geometry was deduced for both double bonds (Δ7 and Δ10) from shielded chemical shift values of the allylic carbons (C6: 27.9 ppm and C12: 28.0 ppm) [14]. The first two COSY fragments were connected via the intervention of two methylene groups (CH213 and CH214) by five HMBC correlations H14/C12, H14/C13, H15/C13, H15/C14, and H16/C14, while the second and third fragments were directly connected by a correlation from H4 to C5. The (7Z, 10Z)-hexadecadienoyl unit thus constructed settled C16H27O2 of the molecular formula, leaving C3H7O2 for the glyceryl group. Finally, interconnection of the acyl and glyceryl units
via an ester linkage was verified by three HMBC correlations from the terminal protons (H1, H2, and H1') of both units to the carboxy carbon (C1), leaving two protons to occupy C2 and C3 diol. Thus, 1 was determined to be a new monoacylglycerol and named nostochopcerol after the source organism.

Figure 2: Selected COSY (bold lines) and HMBC (arrows) correlations for nostochopcerol (1)

Table 1: $^1$H (500 MHz) and $^{13}$C (125 MHz) NMR data for nostochopcerol (1) in CD$_3$OD ($\delta$ in ppm)

<table>
<thead>
<tr>
<th>Position</th>
<th>$\delta$C</th>
<th>$\delta$H, mult. ($J$ in Hz), integr.</th>
<th>HMBC ($^1$H to $^{13}$C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>175.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>34.8</td>
<td>2.34, t (7.5), 2H</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>25.8</td>
<td>1.62, qui (7.4), 2H</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>29.7</td>
<td>1.36, ovl, 2H</td>
<td>2, 3, 5, 6</td>
</tr>
<tr>
<td>5</td>
<td>30.29</td>
<td>1.39, ovl, 2H</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>27.9</td>
<td>2.07, m, 2H</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>129.1</td>
<td>5.338, m, 1H</td>
<td>9</td>
</tr>
<tr>
<td>8</td>
<td>130.6</td>
<td>5.335, m, 1H</td>
<td>6, 9</td>
</tr>
<tr>
<td>9</td>
<td>26.4</td>
<td>2.77, brt (6.5), 1H</td>
<td>8, 10</td>
</tr>
<tr>
<td>10</td>
<td>128.9</td>
<td>5.32, m, 1H</td>
<td>9, 12</td>
</tr>
<tr>
<td>11</td>
<td>130.9</td>
<td>5.34, m, 1H</td>
<td>9, 12</td>
</tr>
<tr>
<td>12</td>
<td>28.0</td>
<td>2.05, m 2H</td>
<td>11</td>
</tr>
<tr>
<td>13</td>
<td>30.34</td>
<td>1.303, ovl, 2H</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>32.5</td>
<td>1.296, ovl, 2H</td>
<td>12, 13, 16</td>
</tr>
<tr>
<td>15</td>
<td>23.5</td>
<td>1.310, ovl, 2H</td>
<td>14, 16</td>
</tr>
<tr>
<td>16</td>
<td>14.3</td>
<td>0.89, t (6.9), 3H</td>
<td>15</td>
</tr>
<tr>
<td>$1'$</td>
<td>66.4</td>
<td>4.05, dd (6.3, 11.3), 1H</td>
<td>1, 2', 3'</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4.13, dd (4.4, 11.3), 1H</td>
<td>1, 2', 3'</td>
</tr>
<tr>
<td>$2'$</td>
<td>71.2</td>
<td>3.80, m, 2H</td>
<td></td>
</tr>
<tr>
<td>$3'$</td>
<td>64.1</td>
<td>3.54, br, 1H</td>
<td></td>
</tr>
</tbody>
</table>

The absolute configuration of the sole chiral center at C2' in the glyceryl group was addressed by comparing the optical rotation of 1 with those of synthetically prepared authentic chiral monoacylglycerols. Because (7Z, 10Z)-hexadecadienoic acid was not commercially available, methyl linoleate, having the same degree of unsaturation with a longer chain length by two carbons, was used as a source of the acyl chain. Linoleic acid, obtained by saponification of
methyl linoleate, was condensed either with (R)- or (S)-solketal (isopropylidene glycerol) by Steglich esterification. The resulting ester 2a or 2b was purified by reversed phase HPLC and deprotected by a short treatment with 80% aqueous acetic acid at 58–59°C to give 1-linoleoyl-sn-glycerol (3a) or 3-linoleoyl-sn-glycerol (3b), respectively (Scheme 1). Similarly to our experience during the isolation of 1, swapping the order of purification and deprotection severely decreased the yields (data not shown). The sn-1-acyl isomer (3a) exhibited a positive rotation ([α]22.3_D +5.5, c 0.30 in MeOH) while the sn-3-acyl isomer (3b) gave a negative rotation ([α]22.5_D −5.5, c 0.30 in MeOH), suggesting that 1 is acylated at sn-3 as judged from its negative value ([α]22.4_D −5.9, c 0.01 in MeOH).

Scheme 1: Synthesis of 1-linoleoyl-sn-glycerol (2a) and 3-linoleoyl-sn-glycerol (2b)

Compound 1 is the first as a non-glycosylated glycerolipid from cyanobacteria [15–19]. Natural 3-acylated-sn-glycerols were also reported from a fungus Sclerotinia fructicola [20] and a brown alga Ishige sinicola [21]. The 7Z, 10Z-hexadecadienoyl group have been found in galactoglycerolipids from Chlorella [22–25], kale (Brassica oleracea) [26], Daphnia [27], and meadow buttercup (Ranunculus acris, family Ranunculaceae) [28], and as a sucrose ester from rough horsetail (Equisetum hiemale, phylum Pteridophyta) [29].
Monoacylglycerols are non-ionic surfactants derivable by hydrolysis of fat, and exhibit antibacterial [30], antifungal [30], antiviral [31], and antiprotozoal [32] activities. Due to these useful properties, a wide range of industrial applications have been found as emulsifiers, antifoamers, preservatives, antistatic agents, polymer lubricants, and mold releasing agents for the production of foods, cosmetics, ointments, paints, and plastics [33]. The antimicrobial activity of 1, evaluated by a microculture method, was MIC 50 µg/mL against *B. subtilis* ATCC6633 and 100 µg/mL and *S. aureus* FDA209P JC-1 (Table 2).

Curiously, *sn*-3 linoleate 3b was more potent than its antipode 3a.

**Table 2: Antimicrobial activity of nostochopcerol (1) and synthetic analogs**

<table>
<thead>
<tr>
<th>Compound</th>
<th><em>Bacillus subtilis</em></th>
<th><em>Staphylococcus aureus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>100&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3a</td>
<td>&gt;200</td>
<td>200</td>
</tr>
<tr>
<td>3b</td>
<td>200</td>
<td>100</td>
</tr>
<tr>
<td>kanamycin sulfate&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5</td>
<td>0.63</td>
</tr>
</tbody>
</table>
<br>
<br>**a**Minimum inhibitory concentration (µg/mL). **b**Positive control.

**Experimental**

**General methods**

Cosmosil 75C18-PREP (Nacalai Tesque Inc., 75 µm) was used for ODS flash chromatography. Recycle HPLC was performed using a Shimadzu LC-6AD HPLC pump coupled with an LC-8A column holder equipped with a Rheodyne injector and a recycle valve. NMR spectra were obtained on a Bruker AVANCE 500 spectrometer using residual solvent peaks at δH/δC 3.30/49.0 ppm in CD<sub>3</sub>OH/CD<sub>3</sub>OD and 7.27/77.0 ppm in CDCl<sub>3</sub> as chemical shift reference signals. HR-ESITOFMS analysis was conducted on a Bruker microTOF mass spectrometer. Optical rotation and UV spectra were recorded on a JASCO P-1030 polarimeter and a Shimadzu UV-1800 spectrophotometer, respectively.
Biological material

*N. lobatus* MAC0804NAN was cultured as described in reference [12].

**Extraction and isolation**

A water-thawed specimen (374.6 g) was homogenized with an equal amount of Celite in EtOH (400 mL). The resulting slurry was paper-filtered to separate an ethanolic extract and an algal cake, and the latter was extracted three more times. The combined extract was concentrated *in vacuo* and the resulting suspension was diluted with MeOH to adjust its concentration to 60% (v/v). This was extracted with CH$_2$Cl$_2$ for three times, and the CH$_2$Cl$_2$-soluble layer was partitioned between aqueous 90% MeOH and n-hexane. The most active aqueous MeOH layer (113.3 mg) was subjected to ODS flash chromatography with a stepwise elution by MeCN-50 mM NaClO$_4$ 30/70, 45/55, 60/40, 75/25, 90/10, and chloroform-MeOH-H$_2$O 6/4/1 to give six fractions. Antibacterial activity against *S. aureus* FDA209P JC-1 and *B. subtilis* ATCC6633 was detected from the second and fourth fractions. The latter was gel-filtered on Sephadex LH-20 (MeCN-50 mM NaClO$_4$ 75/25) to see the separation of activity at the top two and slow-eluting fractions. The top fraction was purified by repeated HPLC first on an ODS column (Cosmosil AR-II φ 1 × 25 cm) and second on a styrene-divinylbenzene polymer column (Hamilton PRP-1 φ 1 × 25 cm) both eluted with MeCN-50 mM NaClO$_4$ 75/25 to yield 1 (0.7 mg).

Nostochopcerol (1): [α]$^{22.4}_{D}$ = -5.9 (c 0.01, MeOH); UV(MeOH) $\lambda$$_{\text{max}}$ 200 nm (log ε 1.7); HRESIMS: m/z 349.2348 [M + Na]$^+$ (calcd. for C$_{19}$H$_{34}$NaO$_4$, 331.2355). IR (ATR) $\nu$$_{\text{max}}$ 3350, 2921, 2852, 1601, 1457, 1195, 1103, 1015, 875, 696 cm$^{-1}$.

**Paper disk-agar diffusion method**
Antimicrobial potency of the chromatographic fractions was evaluated by a paper disk-agar diffusion method. Fractions at each purification stage were diluted to the same concentration with MeOH, and 10 µL aliquots were impregnated into 6 mm-diameter paper disks, which were left stand until completely dried. A loop of the test organism, suspended in a small amount of water, was mixed with liquefied agar medium precooled to nearly body temperature, and the inoculated medium was quickly poured into a sterile plastic dish. The composition of the medium is 0.5% yeast extract, 1.0% tryptone, 1.0% NaCl, 0.5% glucose, and 1.5% agar. After agar solidified, the drug-impregnated disks were placed on the medium, and the test cultures were incubated at 32 °C for a day or two until the diameters of inhibitory haloes turned measurable.

**Microculture antimicrobial testing**

To each well of a sterile 96-well microtiter plate was dispensed 100 µL of tryptic soy broth. Additionally, 98 µL of the same medium and 2 µL of the solutions of test compounds in MeOH or a reference antibiotic, kanamycin monosulfate, in H2O, were added to the wells at the top row. To make two-fold serial dilutions along the column, 100 µL aliquots in the wells of the top row were taken and added to the well in the second row and mixed gently with the pre-dispensed medium by pipetting. In the same manner, 100 µL aliquots were transferred from the second row to the third row. This operation was repeated until the transfer of diluted drug solutions reached the bottom row. The excess 100 µL in the bottom row was discarded to equalize the volume of the medium in the wells. The test strains, *S. aureus* FDA209P JC-1 and *B. subtilis* ATCC6633, were recovered on tryptic soy agar, and a loopful of bacterial masses was transferred to tryptic soy broth in a φ 16 mm tube. The tubes were shake-cultured for several hours at 37°C at 306 rpm until the turbidity measured by the absorbance at 600 nm (ABS600) exceeded 0.1. The liquid culture was diluted to adjust the turbidity to ABS600 0.09–
0.1 (0.5 McFarland), which corresponds to the cell density of $1.5 \times 10^8$ cfu/mL. This was further diluted by 75 times to prepare a cell suspension of $2.0 \times 10^6$ cfu/mL, of which 100 μL was dispensed to the wells to give microcultures with the final cell density of $1.0 \times 10^6$ cfu/mL. The plates were incubated at 37 °C for 48 h and the concentration at which the growth of microbes was completely inhibited was defined as the minimum inhibitory concentration (MIC).

**Supporting Information**

Supporting information features procedures for synthesis of chiral α-linoleoyl glycerols, NMR signal assignments for synthetic compounds, copies of NMR spectra for nostochopcerol (1), 3-linoleoyl-1,2-O-isopropylidene-sn-glycerol (2b), and 1-linoleoyl-sn-glycerol (3a)

Supporting Information File 1
File Name: nostochopcerol_SI
File Format: PDF
Title: Text: Supporting Information for: Nostochopcerol, a new antibacterial monoacylglycerol from the edible cyanobacterium Nostochopsis lobatus

**Acknowledgments**

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**References**


