# Auto-Growth Inhibitory Substance from the Fresh-Water Cyanobacterium *Phormidium tenue*[1)]

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**An extract of the cyanobacterium *P. tenue* showed a significant inhibitory effect on its own growth. Bioassay-directed fractionation has led to the identification of the auto-growth inhibitory substance as a mixture of fatty acids. Unsaturated fatty acids such as linoleic and linolenic acids appear to be predominantly responsible for the auto-growth inhibitory effect.**

**Keywords** cyanobacterium; *Phormidium tenue*; auto-growth inhibitory substance; unsaturated fatty acid; linoleic acid; linolenic acid

Recent advances in plant biology have shown that many terrestrial plants excrete allelopathic substances which inhibit the growth of other species of plants.[3)] However, little is known about allelopathic substances of aquatic plants.[4)] It has often been suggested that algal extracellular metabolites play an important role in controlling succession in algal populations. The presence of anti-algal substances in culture filtrates of various algae has been demonstrated by several workers.[5)] Thus, it seems that allelopathic effects are a significant factor in bloom sequence determination, but virtually nothing is known about natural anti-algal substances and the manner in which these substances influence algal growth. The lack of chemical identification of algal-inhibitory substances has been an obstacle to investigation of the ecological significance and the mechanisms of algal antibiosis.

The fresh-water cyanobacterium *P. tenue* is distributed widely in Japan and is one of the typical cyanobacteria that induce water blooms, producing an earthy, musty odor in drinking water. In the course of preparing an axenic clone of *P. tenue*, one of us noticed that the culture filtrate of the alga inhibited its own growth.[6)] Here, we present a full account of the isolation and identification of the auto-growth inhibitory substance of the cyanobacterium *P. tenue*.

## Results and Discussion

Lyophilized *P. tenue* was successively extracted with acetone and 80% MeOH at room temperature. The acetone extract, showing the growth inhibitory activity, was further purified, with monitoring of the activity, to give two active fractions, fr. 3 and fr. 5. Fraction 3 was rechromatographed using octadecylsilanized silica gel to furnish a growth-inhibitory substance. The $^1$H-NMR spectrum of this substance showed several signals due to primary methyl groups and two broad signals assignable to a mass of methylene protons and olefinic protons, whereas the IR exhibited absorption bands due to carboxylic groups in the range of 3400 to 2600 cm$^{-1}$ and 1700 cm$^{-1}$. In the electron impact-MS (EI-MS), the active substance showed no molecular ion peak because of its poor volatility, while the corresponding methyl ester exhibited molecular ion peaks at 242 ($C_{14:0}$), 268 ($C_{16:1}$), 270 ($C_{16:0}$), 292 ($C_{18:3}$), 294 ($C_{18:2}$), and 296 ($C_{18:1}$). Thus, the auto-growth inhibitory substance was proved to be a mixture of fatty acids. The composition of fatty acids was determined by gas-liquid chromatography–mass spectroscopy (GC-MS) to be a mixture of myristic, palmitic, palmitoleic, *cis*-vaccenic, linoleic, and linolenic acids in a ratio of 5 : 4 : 5 : 4 : 1 : 47 : 31. Fraction 5 consisted mostly of monogalactosyl diacylglycerols that also exhibit the activity.[7)]

We next examined the growth inhibition by commercially available fatty acids. Algal culture for bioassay was grown to the late logarithmic growth phase (10 d). After injection with test samples at the concentrations of 0.5, 1.0, 2.5, 5.0, 10, 25, 50, and 100 ppm, individual cultures were allowed to grow for 3 d. The algal growth ratio was determined by measuring chlorophyll a as described by Parsons and Strickland.[8)] The lowest concentrations resulting in less than 50% viability of the alga were expressed as the minimum growth inhibitory concentration. The values of minimum growth inhibitory concentration for *P. tenue* of the purified commercial fatty acids and the fatty acid mixture isolated from the alga are summarized in Table I. Among the tested fatty acids, unsaturated fatty acids such as linoleic and linolenic acids were potent growth inhibitors, while saturated fatty acids were inactive even at 100 ppm. The fatty acid mixture from *P. tenue* was as potent a growth inhibitor as linoleic or linolenic acid. This finding is consistent with the high proportion of these acids in the fatty acid mixture from *P. tenue*. We next examined the growth-inhibitory effects of the sodium salts and methyl esters of the previously tested fatty acids. Table I includes their activities. The sodium salts and methyl esters are apparently less effective that the corresponding free fatty acids. However, less unsaturated compounds tend to have

TABLE I. Minimum Growth Inhibitory Concentrations of Fatty Acids, Sodium Salts, and Methyl Esters (ppm)

| | Free fatty acid | Na salt | Methyl ester |
|---|---|---|---|
| Myristic acid ($C_{14:0}$) | >100 | >100 | >100 |
| Palmitic acid ($C_{16:0}$) | >100 | >100 | >100 |
| Palmitoleic acid ($C_{16:1}$) | 2.5 | >100 | >100 |
| Oleic acid ($C_{18:1}$) | 1.0 | >100 | 50 |
| *cis*-Vaccenic acid ($C_{18:1}$) | 5.0 | >100 | 50 |
| Linoleic acid ($C_{18:2}$) | 0.5 | 5.0 | 5.0 |
| Linolenic acid ($C_{18:3}$) | 0.5 | 1.0 | 1.0 |
| Fatty acid from *P. tenue* | 0.5 | — | — |

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TABLE II. Minimum Inhibitory Concentrations of Four Representative Detergents (ppm)

| | |
|---|---|
| Tween 80 | >100 |
| Triton X-100 | >100 |
| Sodium laurylsulfate | 25 |
| Sodium laurylbenzenesulfate | 25 |

weaker activities in all three classes of compounds. Linoleic and linolenic acids have a surface-activity that may account for the growth-inhibitory effect toward the alga. Thus, the growth-inhibitory effects of four representative surface-active agents were examined, and the results are summarized in Table II. Nonionic detergents, Tween 80 and Triton X-100, showed no activity up to 100 ppm, whereas both sodium laurylsulfate and sodium laurylbenzenesulfonate (anionic detergents) exhibited the activity, but to a lesser degree. Thus, the growth-inhibitory effect of linoleic and linolenic acids toward the alga may be attributed not to surface-activity but to the ability to injure the functional integrity of the membrane owing to penetration of the fatty acids into the hydrophobic region of the membrane.[9)]

Unsaturated fatty acids are known to be oxidized readily by oxygen to give hydroperoxides. In the course of the present investigation, we detected hydroxy and hydroperoxy fatty acids in the culture medium of the alga as well as fatty acids.[10)] It took linoleic acid more than 12 h to inhibit the growth of *P. tenue* at the concentration of 100 ppm. On the other hand, Scutt reported that the hydroxyperoxides of oleic and linoleic acids were autoinhibitors produced by the green alga *Chlorella vulgaris*.[11)] The unsaturated hydroxy fatty acids were also found to exhibit several biological activities.[12)] On the basis of these findings, we examined the growth-inhibitory effects of hydroxy and hydroperoxy fatty acids generated from linoleic acid toward the alga in order to elucidate whether the unsaturated fatty acids free from oxidation are a genuine growth inhibitors. Specifically, we examined the activities of two hydroxy and two hydroperoxy fatty acids derived from linoleic acid, *viz.* 10*E*,12*Z*-9-hydroxy- and 10*E*,12*Z*-9-hydroperoxy-octadecadienoic acid, 9*Z*,11*E*-13-hydroxy- and 9*Z*,11*E*-13-hydroperoxy-octadecadienoic acid. Although each oxidized derivative of linoleic acid showed the activity at 100 ppm, we concluded that the genuine growth inhibitory substances are not the oxidized derivatives of the unsaturated fatty acids, but the native compounds.

In summary, we have isolated and identified a fatty acid mixture as the auto-growth inhibitory substance of the cyanobacterium *P. tenue*. There are a few reports of growth-inhibitory activity of fatty acids against some algae.[4a,13)] However, this is the first example, to our knowledge, of purification and physicochemical characterization of the auto-growth inhibitory substance released by a microalga.

## Experimental

IR spectra were recorded on a JASCO IRA-2 spectrometer. $^1$H NMR spectra were obtained with a JEOL EX-270 (270 MHz) spectrometer using tetramethylsilane as an internal standard. EI-MS were determined with a JEOL DX-300 spectrometer. Gas-liquid chromatography (GLC) was carried out on a Shimadzu GC-14A. The conditions for identification of methyl esters of fatty acids were as follows: column, ULBON HR-SS-10 (0.25 mm i.d. × 50 m, Shinwa Kako Co., Ltd.); column temperature, 150—220 °C, 3 °C/min; injection temperature, 250 °C; carrier gas, $N_2$, 2.2 kg/cm$^2$. For GC-MS analysis, a JEOL D-300 mass spectrometer interfaced to a Hewlett Packard 5710A gas chromatograph with a JMA 2000 data processing system was employed. The conditions for GC-MS measurement were as follows. Gas chromatography: column, Silicone OV-101 (0.25 mm i.d. × 50 m); injection temperature, 300 °C; column temperature, 250—280 °C, 3 °C/min; carrier gas, He, 1.0 ml/min. Mass spectrometry: ionizing energy, 70 eV; ion source temperature, 230 °C. Chlorophyll a was determined with a Shimadzu UV-2100 spectrophotometer. Thin layer chromatography (TLC) was performed on Merck precoated Kieselgel 60$F_{254}$, and spots were detected by illumination with an ultraviolet lamp, or by spraying 5% vanillin–70% $HClO_4$, 1% $Ce(SO_4)_2$–10% $H_2SO_4$ followed by heating. Column chromatography was performed on Silica gel BW-200 (Fuji Davison Chemicals Co., Ltd.). The fatty acids were purchased from Funakoshi Co., Ltd. The sodium salts of fatty acids, were prepared by adding equimolar of NaOH to the fatty acids in ethanol, then evaporating the solvent.

**Algal Material** *P. tenue* was isolated from a water sample collected from the moat around Nagoya Castle in 1981. The axenic cultures were prepared by the reported capillary pipette washing method and have been maintained in Clostridium–Tris (CT) medium adjusted to pH 8.0 at 25 °C under cool-white fluorescent illumination of 1000 lux.

**Cultivation of *P. tenue*** The alga was cultured in 5-l Erlenmeyer flasks containing CT medium, *viz.* in g/l, $Ca(NO_3)_2 \cdot 4H_2O$ 0.15; $KNO_3$ 0.1; $\beta$-$Na_2$ glycerophosphate 0.05; $MgSO_4 \cdot 7H_2O$ 0.04; minor element solution 0.1 ml/l; trace elements solution 0.1 ml/l. The minor elements solution was composed of, in g/l, $FeCl_3 \cdot 6H_2O$ 0.196; $MnCl_2 \cdot 4H_2O$ 0.036; $ZnSO_4 \cdot 7H_2O$ 0.022; $CoCl_2 \cdot 6H_2O$ 0.004; $Na_2MnO_4 \cdot 2H_2O$ 0.0025; $Na_2EDTA \cdot 2H_2O$ 1.0. The trace element solution consisted of, in g/l, vitamin $B_{12}$ 0.1; biotin 0.1; thiamine·HCl 10.0. The pH of the medium was adjusted to 8.0 with sodium hydroxide prior to autoclaving. Cultures were illuminated continuously at an incident intensity of 1000 lux with cool-white fluorescent lamps and vigorously aerated with sterilized air passed through a 0.2 $\mu$m membrane filter (Millipore, Mirex FG-50) at the rate of 0.5 l/min. After three weeks, the alga was harvested by centrifugation at 20000 ***g*** from the combined 40-l culture and lyophilized to give a lyophilized alga (7.85 g).

**Extraction and Isolation** The lyophilized alga (7.85 g) was homogenized in acetone in an ice-cooling bath and the mixture was allowed to stand at room temperature for 6 h. Then the mixture was filtrated through a membrane filter (0.4 $\mu$m). The residue was extracted with 80% MeOH at room temperature for 6 h. Each extraction was carried out three times. The acetone extract (235 mg) was chromatographed on silica gel (10 g) using the eluents in Fig. 1. Fraction 3 was subjected to flash chromatography using octadecylsilanized silica gel (YMC AQ-120-S50, 3 g) with the solvent mixture in Fig. 1 to afford an active substance (5.0 mg).

**Preparation of Hydroperoxy- and Hydroxy-octadecadienoic Acids from Linoleic Acid** Linoleic acid (100 mg) in a 100 ml beaker was allowed to stand at 40 °C for 36 h. The reaction mixture was treated with ethereal diazomethane to give a product, which was separated by $SiO_2$ column chromatography (*n*-hexane : EtOAc = 9 : 1) to give a mixture of methyl hydroperoxyoctadecadienoates (31.0 mg). The mixture was purified by normal-phase (Nucleosil 50-5) and reversed-phase (Develosil ODS A-5) HPLC to afford methyl 10*E*,12*Z*-9-hydroperoxy-(10.0 mg) and 9*Z*,11*E*-13-hydroperoxyoctadecadienoate (9.2 mg). The two hydroperoxy methyl esters (4.0 mg) were each treated with 5% KOH–MeOH (1.0 ml) at room temperature for 5 min. The reaction mixture was acidified with 10% HCl and extracted with EtOAc. The EtOAc layer was washed with brine and dried over $MgSO_4$. Removal of the solvent *in vacuo* gave 10*E*,12*Z*-9-hydroperoxy- and 9*Z*,11*E*-13-hydroperoxy-octadecadienoic acid (3.8 mg, quant.). The two hydroperoxy methyl esters (4.0 mg) were each treated with sodium borohydride (8.0 mg) in MeOH (1 ml) at room temperature for 10 min. The reaction mixture was acidified with 10% HCl and extracted with $Et_2O$. The $Et_2O$ layer was washed with brine and dried over $MgSO_4$. After removal of the solvent *in vacuo*, the residue was treated with 5% KOH–MeOH (1.0 ml) at room temperature for 5 min. Work-up of the reaction mixture as described above gave 10*E*,12*Z*-9-hydroxy- and 9*Z*,11*E*-13-hydroxy-octadecadienoic acids (3.4 mg, 94%). The four compounds were identical with corresponding authentic samples in terms of their physicochemical properties.[10)]

**Examination of Growth Inhibitory Activity** Algal cultures (100 ml) for bioassay were grown for 10 d, then portions (20 ml) were transferred to 50 ml Erlenmeyer flasks. Test samples were dissolved in acetone or dimethyl sulfoxide (DMSO), and these solutions were sterilized through membrane filters (Bio-Rad Co., Ltd., Micro Pre-Disc, 0.2 $\mu$m pore size). Then 0.2 ml aliquots were added to the culture medium in 50 ml flasks. The controls

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were cultured with only 0.2 ml of MeOH added. Individual cultures were incubated at 25 °C for 3 d, then the concentrations of chlorophyll a were determined with an ultraviolet-visible spectrophotometer as described by Parsons and Strickland.[8] The relative growth rate of the alga was expressed as a percentage of the control.

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