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FISCHERELLIN, A NEW ALLELOCHEMICAL FROM THE FRESHWATER CYANOBACTERIUM *FISCHERELLA MUSCICOLA*[1]

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ABSTRACT

The benthic cyanobacterium Fischerella muscicola *(Thur.) Gom. UTEX 1829 produces a secondary metabolite, fischerellin, that strongly inhibits other cyanobacteria and to a lesser extent members of the Chlorophyceae. Eubacteria are not affected. The major active compound is lipophilic and exhibits a molecular ion at* m/z *408. It is heat- and acid-stable but decomposes in 1 M sodium hydroxide (80° C, 1 h). Fischerellin inhibits the photosynthetic but not the respiratory electron transport of cyanobacteria and chlorophytes. Its site of action is located in PS II. Two other species of* Fischerella *also produce fischerellin, indicating that the synthesis of such allelochemicals might be characteristic of the genus.*

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In late summer, cyanobacterial blooms of a single species often dominate in eutrophic lakes, and cyanobacterial mats consisting of only one species are frequently reported in benthic habitats (Cohen and Rosenberg 1989). Several factors lead to the mass development of cyanobacteria (Paerl 1988), including (in different species) high growth rates, tolerance to desiccation, and the production of allelochemicals.

Since Molisch (1937) coined the term allelopathy, several investigations have been made upon the role of allelochemicals and allelopathic interactions of cyanobacteria and algae (Pratt 1942, Lefèvre 1964, Keating 1977, 1978, Patterson et al. 1979, Rice 1984). In none of these were the substance or substances responsible for the inhibitory or stimulatory activity isolated and characterized. In recent years, algicidal compounds from benthic and edaphic cyanobacteria have been elucidated. Cyanobacterin, a secondary metabolite of *Scytonema hofmanni* UTEX 2349 that inhibits PS II has been extensively studied (Mason et al. 1982, Pignatello et al. 1983, Gleason and Paulson 1984, Gleason and Baxa 1986, Gleason and Case 1986, Gleason 1990). Hapalindole A and related algicidal compounds from *Hapalosiphon fontinalis* (Ag.) Bornet, strain V-3-1 were elucidated by Moore and coworkers (1984, 1987a, b, 1989). The hapalindolinones of *Fischerella* ATCC 53558 inhibit arginine vasopressin binding (Schwartz et al. 1987). Pharmacological screening programs may, though not primarily so intended, identify secondary metabolites that play a role in chemical interactions in ecosystems.

Flores and Wolk (1986) screened 65 filamentous, nitrogen-fixing cyanobacteria for the production of bacteriocins and other antibiotics that kill related strains. Of the seven strains that were found to produce effective antibiotics only *Fischerella muscicola* UTEX 1829 was shown to synthesize a substance (or substances) that kills all indicator strains tested. The substance was protease insensitive and dialyzable. We have now isolated and partially characterized the active compound, which we call fischerellin. Additional indicator strains were tested, and the mode of action of fischerellin was determined.

MATERIALS AND METHODS

Fischerella muscicola (Thur.) Gom. UTEX 1829 (Flores and Wolk 1986) and *Fischerella ambigua* (Näg.) Gom. UTEX 1903 were obtained from the Culture Collection of Algae at the University of Texas at Austin, Department of Botany, Austin, Texas. *Fischerella tisserantii* Frémy Kom. 1964/47 was obtained from Dr. Kováčik, Czechoslovak Academy of Sciences, Institute of Botany, Třeboň, Czechoslovakia. Other cyanobacteria, algae, and eubacteria were obtained from sources shown in Table 1. All cultures except *F. tisserantii* Frémy and *Scenedesmus obliquus* (Turp.) Kütz were axenic.

Cyanobacteria were maintained on cyanobacterial medium (Jüttner et al. 1983), and members of the Chlorophyceae on chlorophycean medium (Kuhl 1962). Both were grown at 25° C and 20 $\mu E \cdot s^{-1} \cdot m^{-2}$. Members of the eubacteria were grown either in NB-medium (8 g nutrient broth [Difco] in 1 L of distilled water) or in CPS-medium (Collins 1963). Indicator strains were grown at 28° C in 300-mL Erlenmeyer flasks in a controlled environment incubator shaker (100 mL of suspension, 120 rpm, 50 $\mu E \cdot s^{-1} \cdot m^{-2}$). *Fischerella* spp. was pregrown in 300-mL tubes (60 $\mu E \cdot s^{-1} \cdot m^{-2}$) and aerated with 0.3 $L \cdot min^{-1}$ compressed air enriched to 0.2% CO_2 (Jüttner 1982) and mass cultured in 5-L tubes (100 $\mu E \cdot s^{-1} \cdot m^{-2}$, 0.5 $L \cdot min^{-1}$ compressed air, enriched to 0.2% CO_2), both at 28° C.

Fischerella was harvested by filtration through a 100-μm net and freeze-dried. Average yields were 4 g fresh weight (0.4 g dry weight) per liter of culture suspension. One gram of lyophilized cells was extracted three times with 50 mL of methanol for 15 min in an ultrasonic bath. An equal amount of 10 mM citric acid–citrate buffer (10 mM HCl adjusted with 10 mM sodium citrate solution [2.1 g of citric acid hydrate dissolved in 20 mL of 1 M sodium hydroxide and made up to 1 L with distilled water] to pH 2.2) was added to the combined extracts. After filtration, the extract was partitioned twice with half the amount of *tert*-butylmethylether; the ether phases were combined, dried over anhydrous sodium sulfate, and evaporated to dryness. The residue was dissolved in 1.5 mL of methanol and subjected to reversed phase HPLC separation on a 25 × 0.46-cm LC18-column (No. 5-8298, Supelco, Bad Homburg, F.R.G.). Aliquots of 50–100 μL were separated by isocratic chromatography with methanol/water (99:1; v/v) using a flow rate of 1 $mL \cdot min^{-1}$. Absorptions at 267 and 415 nm were monitored to detect the active substances and contaminating pigments, respectively. One-milliliter fractions of the eluate were bioassayed by the agar diffusion test (see below). Further purification of the active compound was achieved by separation on a 10 × 0.3-cm LC8-column (Chrompack, Frankfurt/M., F.R.G.) by gradient elution (20–99% methanol in water [v/v] at a flow rate of 1 $mL \cdot min^{-1}$).

The amount of fischerellin in the culture medium was determined by passing 250 mL of medium through a C18-cartridge (500 mg of sorbens, Analytichem International, Frankfurt/M., F.R.G) and subsequently eluting this cartridge first with 5 mL of methanol and afterward with 5 mL of *tert*-butylmethylether. The eluate was evaporated under vacuum, redissolved in methanol, and subjected to the HPLC separation and agar diffusion assay.

Because the amount of purified fischerellin was not sufficient to determine the specific or molar extinction coefficients, fischerellin was quantified by its absorption maximum at 267 nm in methanol. Quantitative data refer to the absorption of the methanolic solution measured in a 1-cm cuvette.

It was possible to separate and analyze fischerellin by gas chromatography combined with mass spectrometry (5790 A MSD, Hewlett Packard, Böblingen, F.R.G.) using a fused silica capillary column (SIM-DIST-CB, 10 m length, 0.32 mm inner diameter, Chrompack, Frankfurt/M., F.R.G.). One microliter of a solution of fischerellin in *tert*-butylmethylether was injected and separated by the following temperature program: 150–285° C, 10° $C \cdot min^{-1}$, 10 min isotherm at 285° C, injection port and interface temperature at 285° C. Helium was used as the carrier gas (90 kPa head pressure). To detect H-reactive substituents, 2 μL of concentrated fischerellin solution in *tert*-butylmethylether were evaporated to dryness in a stream of nitrogen and redissolved in 2 μL each of N,O-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA) and N,N-dimethylformamide (DMFA). After 15 min a GC/MS analysis was performed.

The biomasses of different strains of *Fischerella* and sterile cell extracts were tested for inhibitory activity by a slight modification of the agar diffusion assay described by Flores and Wolk (1986). Methanolic or ethanolic cell extracts (usually 5 μL) were diluted with an equal amount of sterile water and spotted on plates of

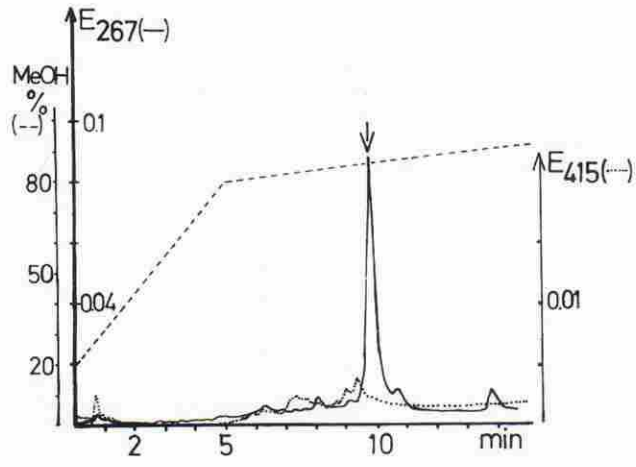

FIG. 1. C8-HPLC separation of purified fischerellin. Gradient elution from 20 to 99% methanol in water (v/v) at a flow rate of 1 mL·min^{-1} and detection at 267 and 415 nm.

medium solidified with 1% agar. The liquid was dried in a stream of sterile air and the plate was then overlaid with 10 mL of a suspension of indicator cells in agar (1%). Inhibitory activities could be seen by an area of clearing around cell inocula or spots after 3–10 days of incubation in the light. Quantification of the inhibitory effects was estimated by preparing serial dilutions and by the resulting diameter of the clearing zones. Controls were performed with 50% methanol or ethanol in sterile water.

To determine the effect of fischerellin on the growth of liquid cultures of *Synechococcus*, different concentrations of fischerellin dissolved in 150 μL of ethanol were added to 150 mL of a growing culture (5×10^4 cells·mL^{-1}) three days after inoculation. Control cultures received an equal amount of ethanol. Optical density was determined at 530 nm and cell number with the cell analyzer system, Casy 1 (Schärfe System, Reutlingen, F.R.G.). Chlorophyll *a* (chl *a*) was determined spectrophotometrically after extraction with cold methanol as described by Wu and Jüttner (1988). Protein was determined with amido black (Schaffner and Weissmann 1973).

Rates of photosynthetic evolution of O_2 by the P9 derivative of *Anabaena variabilis* ATCC 29413 and of respiratory uptake of O_2 by *Nannochloris* sp. were measured with a Clark type electrode that was mounted in a 1.2-mL cuvette. The addition of chemicals followed procedures given by Allen and Holmes (1986). Cultures were harvested during the postexponential growth phase by centrifugation, washed, and resuspended in 50 mM sodium phosphate buffer, pH 7.9 (Scherer et al. 1988). The resulting suspension was diluted in this buffer in the cuvette to yield 17 μg chl·mL^{-1}. To measure the effect of fischerellin on photosystem II (PS II), the basal rate of electron transport of PS II was determined after addition of 2,6-dichlorophenol–indophenol (167 μM DCPIP) that inhibited PS I and served as an artificial electron acceptor of PS II. Electron transport of PS II was subsequently determined in the presence of fischerellin, atrazine, or ethanol.

In the case of PS I the basal rate was determined in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (12.5 μM DCMU). Because no artificial electron donor was added, the resulting electron transport was very low. After addition of sodium ascorbate (4.2 mM)/DCPIP (167 μM) as artificial electron donor and 8.3 μM N,N-dimethyl-4-nitrosoaniline (PNDA) as artificial electron acceptor, the electron transport was measured after the addition of fischerellin, atrazine, or ethanol.

RESULTS

Isolation of fischerellin. Freshly harvested cells of *Fischerella* or lyophilized cell material were extracted with methanol as described above. To obtain a better separation of fischerellin from chlorophyll *a* and derivatives of chlorophyll, the acidic extraction procedure was established. The methanolic crude extract was treated with citric acid/citrate buffer to convert chlorophyll into phaeophytin. This procedure resulted in a crude extract that contained only low amounts of chlorophyll derivatives. The methanolic crude extract was further subjected to reversed phase HPLC separation on a C18-column. As indicated by the agar diffusion assay, the fraction of the HPLC separation responsible for most of the activity contained a sharp peak at 7.2 ± 0.1 min when the absorption at 267 nm was recorded. When this fraction was chromatographed on a C8-HPLC column in a solvent gradient (Fig. 1), only one peak appeared that was responsible for all the inhibitory activity and that coeluted with the compound absorbing strongly at 267 nm. Because the inhibitory activity and the strong absorption were found in the same fraction in all separation systems, both features were assumed to belong to the same compound. This compound, which is further characterized below, was named fischerellin. According to the agar diffusion assay, two other less inhibitory substances that have not been studied further were present in the crude extract. The inhibitory compounds were completely extracted with lipophilic solvents, and no inhibitory activity was left in the cell residue when tested with the agar diffusion assay. The yields of inhibitory activity of the medium were 50–100-fold lower than the yields of the cells that were suspended in the medium.

Characterization of fischerellin. Fischerellin was readily soluble in several organic solvents, including methanol, ethanol, acetonitrile, acetone, diethylether and *tert*-butylmethylether, but was only weakly soluble in water. Freeze dried cells, methanolic extracts, and the purified compound could be stored for months at +4° C without any loss of activity. Fischerellin proved to be very heat resistant: unchanged activities and peak heights (absorption at 267 nm) after treatment for 1 h at 100° C were obtained with the HPLC separation. No differences in the yield of fischerellin were found after extraction with 50% methanol at pH 1, pH 7, and pH 13 at room temperature. Treatment with 1 M hydrochloric acid at 80° C for 1 h showed no effect, while 1 M sodium hydroxide at 80° C for 1 h led to the decomposition of fischerellin and loss of its inhibitory activity. Fischerellin could not be retained efficiently on either anionic or cationic exchange resins. Therefore, the presence of carboxy- and amino-groups in the molecule can be ruled out.

When HPLC-purified fischerellin was subjected to capillary gas chromatography/mass spectrometry

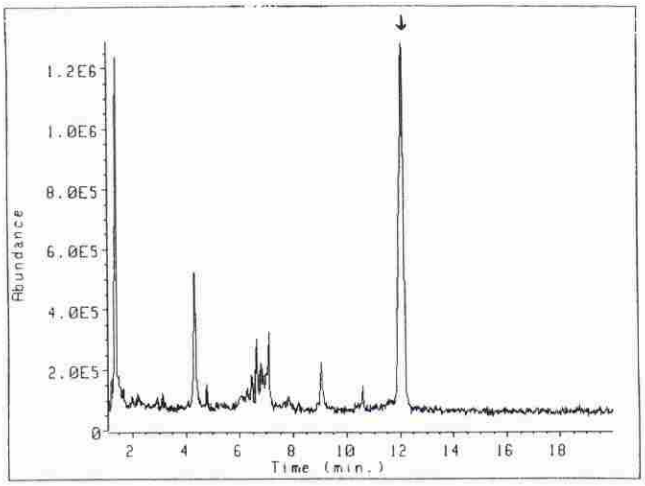

FIG. 2. GC separation of purified fischerellin (arrow).

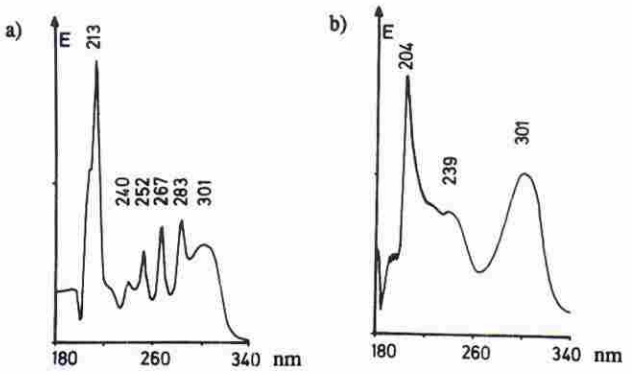

FIG. 3. UV-spectra in methanol: a) fischerellin, b) hydrogenated fischerellin.

a strong peak was obtained that exhibited a molecular ion of m/z 408 at a retention time of 12.2 min (Fig. 2). The molecular ion shifted to m/z 418 when the substance was hydrogenated over platinum under hydrogen gas. Fischerellin showed a distinct ultraviolet absorption spectrum with maxima at 213, 240, 252, 267, 283, and 301 nm. Hydrogenated fischerellin lost the maxima at 252, 267, and 283 nm (Fig. 3). H-reactive substituents did not occur in the molecule as neither the treatment of fischerellin with acetic anhydride (30 min, 70° C) nor BSTFA in DMFA changed the retention time or absorption intensity upon separation by HPLC nor the retention time or mass fragmentation pattern after GC/MS.

Distribution of fischerellin in the genus Fischerella. To determine whether or not the production of fischerellin is restricted to *F. muscicola*, two other species of *Fischerella* were investigated. By HPLC separation and UV spectroscopy, fischerellin could be clearly identified in *F. ambigua*. Extracts of the nonaxenic *F. tisserantii* exhibited slight absorptions at 267 and 283 nm at the fischerellin retention time in HPLC, and the corresponding fraction was inhibitory in the agar diffusion assay. Fischerellin may, therefore, be present also in this species.

Inhibitory activity. Fischerellin purified by HPLC inhibited the growth of green algae as well as several previously untested cyanobacteria on agar (Table 1). Inhibition of cyanobacteria was about 50–1000-fold stronger than the inhibition of the chlorophyte genera *Ankistrodesmus*, *Nannochloris*, and *Scenedesmus*. However, fischerellin did not inhibit the growth of aquatic and soil eubacteria (Table 1).

The inhibitory effect of fischerellin could also be observed in liquid cultures of *Synechococcus* PCC 6911 when the inhibitor was added dissolved in ethanol (Fig. 4). Immediately upon addition to exponentially growing cultures of *Synechococcus*, fischerellin inhibited the increase of optical density at 530 nm, cell number, chlorophyll *a*, and protein concentration. When 150 μL of a relatively dilute solution of fischerellin (A_{267} = 0.1) were added, the growth of *Synechococcus* was significantly and immediately inhibited, but the cells recovered after one week. With more highly concentrated solutions of fischerellin (A_{267} = 0.5), no recovery of the cells was observed. Assuming a molar absorption coefficient of 6000 (assumed for an extended aromatic or conjugated system), the effective concentration of fischerellin appears to be in the range of a few ng·mL^{-1}. According to this assumption *Fischerella* contains 100–150 μg of fischerellin per gram dry weight. This is less than has been found for cyanobacterin in *Scytonema hofmanni* and hapalindole A in *Hapalosiphon fontinalis*. The compounds were reported to be present in the cells at a concentration of 3 and 5.8 mg·g^{-1} dry weight, respectively (Pignatello et al. 1983, Moore et al. 1984).

Mode of action of fischerellin. Because fischerellin appeared to inhibit specifically the growth of photosynthetic organisms in the light (Table 1) and no effect on respiratory electron transport could be found with *Nannochloris* (data not shown), experiments were conducted with the P9 strain of *Anabaena* (Table 2) to determine whether photosynthesis was affected. When PS II was inhibited by DCMU, the electron flow between the artificial electron donor (DCPIP reducted by sodium ascorbate) and acceptor (PNDA) of PS I showed no difference when either ethanol alone or with fischerellin were added. However, the electron flow from water to DCPIP, which accepts electrons from PS II (Trebst 1972), was effectively inhibited by fischerellin.

DISCUSSION

In this paper we describe a new algicidal substance, which we term "fischerellin," from the benthic cyanobacterium *Fischerella muscicola*. Fischerellin is a low molecular weight, lipophilic, and polyunsaturated compound. Its protease insensitiv-

TABLE 1. *Inhibition of growth of different indicator strains by fischerellin. Growth inhibition of indicator strains was determined by the agar diffusion assay. The amount of fischerellin and the area of lysis were taken into consideration. +++ very strong inhibition, ++ strong inhibition, + weak inhibition, − no inhibition observed.*

Indicator strain	Source	Inhibition
Cyanobacteria		
Anabaena variabilis P9 derivative of ATCC 29413	R. D. Simon	+++
Anabaena sp. ATCC 27893 PCC 7120	R. Haselkorn	+++
Phormidium sp. UTEX 1540	UTEX	++
Synechococcus sp. PCC 6911	PCC	+++
Synechocystis spec. CB-3	T. Vaara	+++
Chlorophyceae		
Ankistrodesmus falcatus SAG 202-3	SAG	++
Nannochloris spec. SAG 55-81	SAG	++
Scenedesmus armatus SAG 276-4c	B. Meier	+
Scenedesmus communis SAG 276-46	SAG	+
Scenedesmus falcatus SAG 2.81	SAG	+
Scenedesmus obliquus	B. Hickel	++
Eubacteria		
Alcaligenes eutrophus DSM 531	M. Höfle	—
Arthrobacter globiformis DSM 820124	M. Höfle	—
Bacillus cereus DSM 626	M. Höfle	—
Bacillus subtilis DSM 347	M. Höfle	—
Cytophaga johnsonae C21	M. Höfle	—
Pseudomonas fluorescens DSM 50090	M. Höfle	—

Sources: R. D. Simon, Dept. of Biology, State University of New York Geneseo, New York; R. Haselkorn, Dept. Molecular Genetics and Cell Biology, University of Chicago, Illinois; PCC, Pasteur Culture Collection, Institut Pasteur, Paris, France; T. Vaara, Helsinki, Finland; UTEX, The Culture Collection of Algae, University of Texas at Austin Collection, Dept. of Botany, Austin, Texas; SAG, Sammlung von Algenkulturen, Pflanzenphysiologisches Institut der Universität, Göttingen, FRG; B. Meier, B. Hickel, and M. Höfle, Max-Planck-Institut für Limnologie, Plön, F.R.G.

ity and ability to diffuse through dialysis membranes (Flores and Wolk 1986) are consistent with the molecular weight of 408 deduced from the mass spectrum. The mass spectrum did not show ions (M+2), which would indicate atoms of chlorine and bromine. Because after hydrogenation the molecular ion shifted from m/z 408 to m/z 418, at least five double bonds or an equivalent amount of triple and double bonds can be assumed in the molecule. The loss of maxima at 252, 267, and 283 nm in the UV spectrum after hydrogenation further indicated the presence of conjugated double bonds.

Several benthic cyanobacteria that belong to the typological group V (Rippka et al. 1979) have recently been shown to produce physiologically active compounds. The morphological similarities of these organisms may also be accompanied by similarities in their secondary metabolism. Although it remains possible that fischerellin has a structure related to

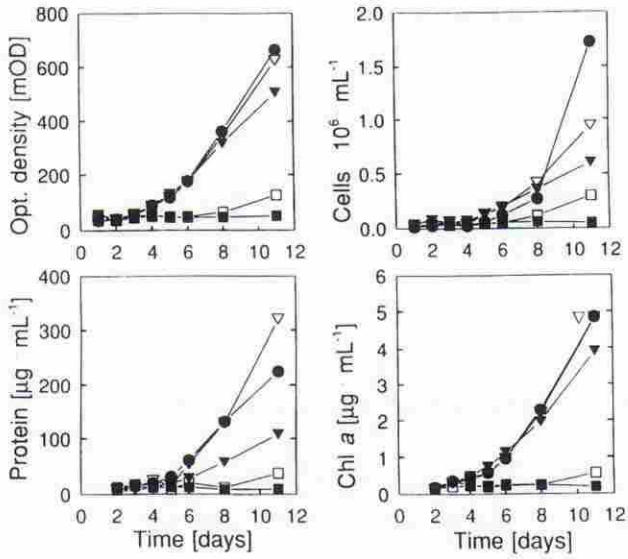

FIG. 4. Effect of fischerellin on growth parameters of *Synechococcus* PCC 6911: optical density at 530 nm, cell number, chlorophyll *a*, and protein concentration. Fischerellin dissolved in ethanol was added to the cultures during the early exponential growth phase (third day of experiment). The control cultures received no (●) or equal amounts of ethanol (1 µL·mL^{-1} culture medium; ▽). The amount of added fischerellin was determined spectrophotometrically; concentrations equivalent to A_{267} = 0.005 (▼), A_{267} = 0.1 (□), and A_{267} = 0.5 (■) were added.

hapalindole, some features of fischerellin appear inconsistent with hapalindole A and other compounds from the edaphic cyanobacterium *Hapalosiphon fontinalis* (Ag.) Bornet, strain V-3-1 (Moore et al. 1984)

TABLE 2. *Effect of fischerellin on photosynthesis of* Anabaena *P9. Basal rate of PS I was determined after addition of DCMU, of PS II after addition of DCPIP, and of PS I + II without any addition. Electron flow rates were determined after addition of sodium ascorbate, DCPIP, and PNDA in PS I and after addition of fischerellin, atrazine, or ethanol in both PS. The difference is the electron flow rate minus basal rate. The oxygen consumption and evolution by PS I, PS II, and PS I + II were determined by a Clark type electrode. PS I + II was determined directly, PS I and PS II after decoupling using artificial electron acceptors and donors. Fischerellin was added as 10 µL of an ethanolic solution having the indicated value of A_{267}. Controls were performed with an equal volume of ethanol or without additions.*

Addition	Oxygen exchange (µmol O_2·mg Chl^{-1}·h^{-1}) Basal rate	Electron flow rate	Difference
PS I + II			
Ethanol	81	81	0
Fischerellin A_{267} = 0.1	68	42	−26
Fischerellin A_{267} = 10.0	47	0	−47
PS I			
Control	8	−205	−213
Ethanol	9	−173	−182
Fischerellin A_{267} = 1.0	8	−173	−181
PS II			
Ethanol	38	30	−8
Fischerellin A_{267} = 0.1	40	21	−19
Fischerellin A_{267} = 0.25	34	0	−34
Atrazine (17 µM)	50	5	−45

and the hapalindolinones from *Fischerella* ATCC 53558 (Schwartz et al. 1987). The mass spectrum gave no indication of the presence of chlorine atoms, and the stability of the molecule to acidic hydrolysis is inconsistent with the presence of an isonitrile (Bornemann et al. 1988) or isothiocyanate group, both being characteristic of the hapalindoles (Moore et al. 1984). The molecular weights of the hapalindoles are also much lower than that of fischerellin.

In addition to *F. muscicola, F. ambigua* and *F. tisserantii* showed inhibitory activity. Our investigations indicate that fischerellin may be present in extracts of the nonaxenic *F. tisserantii*, but the HPLC separations were not as good as with extracts of the axenic strains. However, fischerellin could be clearly identified in *F. ambigua*. The production of fischerellin may, therefore, be a characteristic feature of the genus *Fischerella*.

Because fischerellin strongly inhibits photosynthetic organisms, chlorophytes as well as cyanobacteria, it is an algicide. Cyanobacterin, an algicide from *Scytonema hofmanni*, also inhibits PS II (Mason et al. 1982, Gleason and Paulson 1984), but unlike fischerellin contains an atom of chlorine and has a higher molecular weight (Pignatello et al. 1983). Other biogenic algicides that inhibit photosynthesis have been isolated from *Pandorina morum* (Patterson et al. 1979) and *Chara* (Wium-Andersen et al. 1982). The first was of unknown structure and inhibited the electron flow of PS II, the latter were cyclic sulfur compounds and active against epiphytic *Nitzschia palea* and natural phytoplankton. Hapalindole A of *H. fontinalis* also exhibits antialgal activity (Moore et al. 1984). With the exception of the active extract of *Pandorina*, all of these inhibitory substances are lipophilic. The ease with which lipophilic compounds can pass through cell membranes and can be accumulated in the thylakoid membranes of the target organisms may be responsible for this feature. In the two-dimensional habitat of algal and cyanobacterial mats, direct contact between photosynthetic organisms is quite common, and an exchange of allelochemicals between cells that are attached or close to each other can easily be assumed. In these habitats allelochemical interactions seem to be a powerful competitive strategy. Studies of the ecological impact of fischerellin as an allelochemical are warranted.

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