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Selective Growth Inhibition of the Musty-Odor Producing Cyanobacterium *Oscillatoria* cf. *chalybea* by Natural Compounds

K. K. Schrader,[1] M. Q. de Regt,[1] P. R. Tidwell,[1] C. S. Tucker,[1] S. O. Duke[2,*]

[1]Delta Research and Extension Center, Mississippi State University, Stoneville, MS 38776, USA
[2]United States Department of Agriculture, Agricultural Research Service, Southern Weed Science Research Unit, Stoneville, MS 38776, USA

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The most common off-flavor problem in drinking water and fresh water-raised fish is earthy-musty odor. Actinomycetes and cyanobacteria are the microorganisms most frequently associated with producing geosmin and 2-methylisobomeol (MIB) which are responsible for causing earthy-musty odors (Tabachek and Yurkowski 1976; Gerber 1979). The cyanobacterium Oscillatoria cf. chalybea produces MIB (Martin et al. 1991), and is the major cause of musty off-flavor in channel catfish in west-central Mississippi (van der Ploeg et al. 1995). Channel catfish exposed to MIB will absorb the compound within hours while removal of MIB from the fish flesh may take days or weeks (Johnsen and Lloyd 1992). Channel catfish determined to be off-flavor must be held in ponds by the producer for days or weeks until they are deemed to be on-flavor and marketable. Such management dilemmas can increase production costs by an amount equivalent to 12% of the annual revenues received by catfish producers (Kinnucan et al. 1988).

Prevention of musty off-flavors in drinking water and fish could be accomplished by selectively eliminating or inhibiting the growth of undesirable cyanobacteria such as O. cf. chalybea. Copper sulfate is currently the only USEPA approved algicide for use in municipal water reservoirs and food-fish ponds; however, copper sulfate is limited in its usefulness in selectively controlling cyanobacteria in these water ecosystems due to its relatively broad spectrum toxicity towards phytoplankton. A recent approach using decomposing barley straw provided selective growth inhibition of the cyanobacterium Microcystis aeruginosa in ponds, lakes, and water reservoirs (Newman and Barrett 1993). Additional studies (Barrett et al. 1996; Everall and Lees 1996) indicate that the microbial decomposition of barley straw placed on the surface of water reservoirs releases a compound or compounds that inhibit the growth of algae and cyanobacteria. Presently, the group of compounds produced or released during barley straw decomposition is incomplete. Everall and Lees (1997) have identified several phenolic compounds produced during barley straw decomposition which may be toxic towards phytoplankton. Decomposition products of barley straw cell-wall components and compounds from the incomplete decomposition of lignin may

*Present address: USDA, ARS, NPURU, National Center for the Development of Natural Products, School of Pharmacy, University of Mississippi, University, MS 38677-8048, USA

Correspondence to: K. K. Schrader, USDA, ARS, NPURU, Post Office Box 8048, University, MS 38677-8048, USA

play roles in the algistatic effect (Newman and Barrett 1993). In situ antibiotic production by microorganisms in the decomposing barley straw may also contribute to the growth inhibition of cyanobacteria.

The objective of this study was to screen a wide range of commercially available natural compounds for selective cyanobacterial algicides. Some of the natural compounds screened in this study may be produced or released during lignin decomposition [lignin can solubilize from barley straw (Kivaisi et al. 1990)]. Other natural compounds selected for screening in this study possess similar mechanisms of toxicity as those of several synthetic compounds that were previously found to be selectively toxic towards O. cf. chalybea (*Schrader et al. in press*). The discovery of environmentally safe compounds which selectively inhibit the growth of off-flavor compound-producing cyanobacteria would benefit commercial catfish producers as well as municipal drinking water suppliers and consumers.

MATERIALS AND METHODS

Algal cultures used for preliminary screening included O. cf. chalybea, isolated from a west-central Mississippi cattish production pond (van der Ploeg et al. 1995) and Selenastrum capricornutum, a representative species for green algae (provided by Dr. J. C. Greene, USEPA, Corvallis, OR). The geosmin-producing cyanobacterium Anabaena sp. LP 691 (obtained from George Izaguirre, The Metropolitan Water District of Southern California, La Verne, CA) and the green alga Pediastrum simplex UTEX #LB 1601 (Culture Collection of Algae, University of Texas, Austin, TX) were used for additional screening of selected compounds, some of which were selectively toxic towards O. cf. chalybea.

Unialgal cultures were grown and maintained in continuous, steady-states at 29°C under continuous light by the method of van der Ploeg et al. (1995) except that light intensity was 18-29 $\mu E/m^2/sec$ and air flow rate was 16-33 L/hr. Cell density in the continuous cultures was maintained at 0.18-0.27 A (absorbance) for O. cf. chalybea, 0.19-0.26 A for S. capricornutum, 0.28-0.33 A for Anabaena sp. LP 691, and 0.20-0.24 A for P. simplex. Cell density was measured at 750 nm using a Gilford model Response UV-VIS spectrophotometer (Gilford Instrument Laboratories, Inc., Oberlin, OH).

A rapid microtiter plate-based assay developed previously for screening compounds for selectivity as cyanobacterial algicides was used (Schrader et al. 1997). Technical grade compounds were used in this study (Table 1). Compound purity was compensated for when preparing stock solutions. Test solutions were prepared in tenfold concentration increments for each compound, and four replications were used for each concentration and control.

Microplates were placed in a growth chamber at 25-27°C and illuminated by three

overhead fluorescent lamps (40 W, cool white) at a light intensity of 20-28 $\mu E/m^2/sec$. Optical densities of each well were measured daily for 5 d at 650 nm using a Packard model Spectracount microplate photometer (Packard Instrument Company, Meriden, CT). Mean values of the optical density measurements for each concentration and controls were graphed (data not shown). Graphs were used to determine compound LCIC (Lowest-Complete-Inhibition Concentration) and LOEC (Lowest-Observed-Effect Concentration) which were used to calculate differential sensitivity values for the cultures used in primary screening. Several compounds that exhibited toxic selectivity towards O. cf. chalybea were screened further using continuous cultures of Anabaena sp. LP 691 and P. simplex.

Table 1. Natural compounds screened.

Compound	purity (%)	Solvent	Manufacturer
trans-Cinnamic acid	99.0	Ethanol	Fisher Scientific Co.
p-Coumaric acid	99.0	Ethanol	Sigma Chemical Co.
Caffeic acid	99.0	Ethanol	Sigma Chemical Co.
Chlorogenic acid	99.0	Ethanol	Sigma Chemical Co.
trans-Ferulic acid	Unknown[a]	Ethanol	Sigma Chemical Co.
Sinapic acid	99.0	Ethanol	Fluka Chemical Co.
Tannic acid	Unknown[a]	H_2O	Chem Service, Inc.
Anthraquinone	97.0	Ethanol	Aldrich Chemical Co.
2,3-Dichloronaphthoquinone	98.0	Ethanol	Aldrich Chemical Co.
2-Methylanthraquinone	95.0	Ethanol	Aldrich Chemical Co.
1,4-Naphthoquinone	97.0	Ethanol	Aldrich Chemical Co.
9,10-Phenanthraquinone	99.0	Ethanol	Aldrich Chemical Co.
Ubiquinone- 10	99.0	Ethanol	Aldrich Chemical Co.
Vitamin K_1	100.0	Ethanol	Sigma Chemical Co.
Vanillin	Unknown[a]	Ethanol	Sigma Chemical Co.
Vanillic acid	Unknown[a]	Ethanol	Sigma Chemical Co.
Syringic acid	99.0	Methanol	Sigma Chemical Co.
Syringaldehyde	99.0	Ethanol	Sigma Chemical Co.
p-Hydroxybenzaldehyde	Unknown[a]	Ethanol	Eastman Kodak Co.
Artemisinin	98.0	Methanol	Aldrich Chemical Co.
1,8-Cineole	Unknown[a]	Ethanol	Sigma Chemical Co.
Eugenol	Unknown[a]	Ethanol	Eastman Kodak Co.
Hypericin	85.0	Ethanol	Sigma Chemical Co.
Juglone	97.0	DMSO/H_2O[b]	Fluka Chemical Corp.
Phosphinothricin	98.0	H_2O	Sigma Chemical Co.
Sorgoleone	Unknown[a]	Ethanol	CI Nimbal[c]

[a]For unknowns, no correction for purity was applied when making stock solutions.
[b]Dimethyl sulfoxide (DMSO) was the initial solvent used before further dilution with H_2O; highest DMSO concentration used in the screening was no greater than 0.01%.
[c]Provided by CI Nimbal, Dept. of Horticulture and Landscape Architecture, Univ. of Kentucky, Lexington, KY.

RESULTS AND DISCUSSION

Decomposition of straw cell walls can release phenolic compounds such as p-coumaric acid and ferulic acid. These compounds may undergo oxidation under alkaline, well-oxygenated conditions to form oxidized phenolics (Everall and Lees 1997) such as quinones which have been shown to be highly toxic towards Microcystis aeruginosa (Pillinger et al. 1994). In addition, phototransformation of phenolics can form phytotoxic hydrogen peroxide and superoxide radicals (Appel 1993).

The phenolic compounds, ferulic acid and sinapic acid, were inhibitory to O. cf. chalybea at 1 and 10 µM, respectively, based on LOEC results (Table 2). Sinapic acid completely inhibited the growth of O. cf. chalybea at 100 µM and was selectively toxic towards O. cf. chalybea (based on LCIC). Ferulic acid had a high differential sensitivity (DS=3) based on LOEC results (Table 2) and, therefore, was the most selectively toxic phenolic compound screened. However, ferulic acid was not selectively toxic towards another cyanobacterium, Anabaena sp. LP 691 (Table 3). Therefore, ferulic acid might be of use in selectively controlling the growth of particular species of cyanobacteria such as O. cf. chalybea, but could not be used as a broad-spectrum cyanobacterial algicide.

Cinnamic acid (trans- isomer) was also found to be selectively toxic towards O. cf. chalybea (DS=2) based on LOEC results, but was not selectively toxic based on LCIC results. Cinnamic acid (3-phenyl-2-propenoic acid) was the only compound that we screened which has also been identified as a compound released from decomposing barley straw placed in a water reservoir (Everall and Lees 1997). Everall and Lees (1997) found 10- 100 µg/L of cinnamic acid in water downstream from the decomposing barley straw; however, cinnamic acid was not present when a significant decline in the numbers of Oscillatoria tenuis in the treated reservoir occurred. In our screening with O. cf. chalybea, the LCIC of trans-cinnamic acid was 100 µM (14.8 mg/L) while the LOEC was 0.1 µM (14.8 µg/L) (Table 2). Cinnamic acid by itself may not be toxic enough to completely inhibit the growth of cyanobacteria in aquatic ecosystems.

Several quinones were selectively toxic towards O. cf. chalybea (Table 2) and anthraquinone and 2,3-dichloronaphthoquinone were the most toxic with LCIC of 0.1 µM. Fitzgerald et al. (1952) reported that only 2.0 µg/L (0.01 µM) of 2,3-dichloronaphthoquinone was required to completely kill Microcystis aerurginosa. In contrast, our study revealed that Anabaena sp. LP 691 requires greater than 100 µM of 2,3-dichloronapthoquinone to completely inhibit growth, as is the case for many of the other quinones screened (Table 3). The most toxic quinone to Anabaena sp. LP 691 was 9,10-phenanthrenequinone (LCIC = 10 µM) which has been reported to be even more toxic to Microcystis aeruginosa with 50% growth inhibition at 0.1 µM (Pillinger et al. 1994). These results indicate that the toxicity of certain quinones toward different cyanobacterial species can vary greatly. The

Table 2. LCIC and LOEC of compounds screened for toxic selectivity towards Oscillatoria cf. chalybea.

Compound	LCIC (μM):	O. cf. chalybea	S. capricornutum	DS[a]	LOEC (μM):	O. cf. chalybea	S. capricornutum	DS[a]
trans-Cinnamic acid		100	100	0		0.1	10	2
p-Coumaric acid		1000	1000	0		1000	1000	0
Caffeic acid		1000	1000	0		1000	1000	0
Chlorogenic acid		>100	>100	0		>100	>100	0
trans-Ferulic acid		1000	1000	0		1	1000	3
Sinapic acid		100	1000	1		10	100	1
Tannic acid		100	100	0		100	100	0
Anthraquinone		0.1	>100	4[b]		0.1	>100	4[b]
2,3-Dichloronaphthoquinone		0.1	100	3		0.1	100	3
2-Methylanthraquinone		1	>100	3[b]		0.1	>100	4[b]
1,4-Naphthoquinone		10	100	1		0.1	10	2
9,10-Phenanthrenequinone		1	10	1		0.1	10	2
Ubiquinone-10		>100	>100	0		>100	>100	0
Vitamin K_1		100	100	0		10	100	1
Vanillin		>1000	>1000	0		100	1000	1
Vanillic acid		1000	1000	0		100	100	0
Syringic acid		1000	>1000	1[b]		100	1000	1
Syringaldehyde		1000	1000	0		100	1000	1
p-Hydroxybenzaldehyde		>1000	>1000	0		100	1000	1
Artemisinin		1	10	1		0.1	10	2
1,8-Cineole		1000	>1000	1[b]		100	>1000	2[b]
Eugenol		10	10	0		10	10	0
Hypericin		>100	>100	0		>100	>100	0
Juglone		1	>10	2[b]		0.1	1	1
Phosphinothricin		1000	1000	0		1000	1000	0
Sorgoleone		100	>1000	2[b]		100	100	0

[a]DS=Differential sensitivity; as order of magnitude; derived by LCIC (or LOEC) of S. capricornutum/LCIC (or LOEC) of O. cf. chalybea.
[b]DS could be higher since LCIC or LOEC of S. capricornutum is greater than the highest level of compound screened.

Table 3. LCIC and LOEC of compounds screened against Anabaena sp. LP 691 and Pediastrum simplex.

Compound	LCIC (μM)		LOEC (μM)	
	Anabaena sp.	P. simplex	Anabaena sp.	P. simplex
trans-Ferulic acid	>1000	1000	1000	1000
Anthraquinone	>100	>100	>100	>100
2,3-Dichloronaphthoquinone	>100	100	>100	10
2-Methylanthraquinone	>100	100	>100	100
1,4-Naphthoquinone	>100	100	100	100
9,10-Phenanthrenequinone	10	10	10	1
Ubiquinone-10	>100	>100	>100	>100
Vitamin K_1	>100	>100	100	100
Vanillin	>1000	>1000	1000	>1000
Vanillic acid	>1000	>1000	1000	1000
Syringic acid	>1000	1000	1000	100
Syringaldehyde	>1000	1000	1000	100
p-Hydroxybenzaldehyde	>1000	>1000	1000	>1000

application of such toxins to aquaculture ponds and water reservoirs could drastically change the overall phytoplankton community structure in these aquatic environments.

Ubiquinone-10 (coenzyme Q-10), not found in cyanobacteria (Threlfall 1980), and vitamin K_1 (2-methyl-3-phytyl-1,4-naphthoquinone), found in chloroplasts (Robinson 1967) and probably present in the acceptor complex of Photosystem I of cyanobacteria (Klughammer and Pace 1997), were not highly toxic to the phytoplankton used in this study. Among the quinones screened, anthraquinone, 2,3-dichloronaphthoquinone, 2-methylanthraquinone, and juglone (5-hydroxy-1,4-naphthoquinone) had the highest differential sensitivity values based on LCIC results. However, direct application of a quinone or quinones to food-fish ponds to selectively control a particular species of cyanobacteria, such as O. cf. chalybea, may not be an alternative due to the potential direct toxicity of the quinone(s) towards fish.

Vanillin, syringaldehyde, and p-hydroxybenzaldehyde are lignin-related molecules derived from the fungal decomposition of lignin while vanillic acid and syringic acid are intermediate compounds formed by the fungal decomposition of phenolics. These five compounds were toxic only at high concentrations (LCIC equal to or greater than 1000 μM) to the phytoplankton used in this study (Tables 2 and 3) and only syringic acid was selectively toxic towards O. cf. chalybea based on LCIC results (Table 2). Among the non-quinones screened, artemisinin was the most toxic to O. cf. chalybea (LCIC = 1 μM) and was selectively toxic towards O. cf. chalybea (Table 2). Artemisia annua, commonly referred to as annual wormwood, produces artemisinin which is toxic to malarial parasites and

to some plants (Duke et al. 1987). The activity of artemisinin against cyanobacteria and algae is unreported. The antialgal use of A. annua leaves and/or flowers in ways similar to those using barley straw for controlling the growth of cyanobacteria in water reservoirs needs to be preceded by testing to determine if any objectionable tastes or odors are added to the water and to food fish by A. annua. Sorgoleone was not highly toxic to O. cf. chalybea (LCIC = 100 µM) but was selectively toxic (DS=2) based on LCIC results (Table 2). Sorgoleone is produced by grain sorghum (Sorghum bicolor) and inhibits photosynthetic oxygen evolution in some plants (Nimbal et al. 1996). 1,8-Cineole was also not highly toxic towards O. cf. chalybea (LCIC = 1000 µM) but was selectively toxic (DS=1) based on LCIC results. Caffeic acid, chlorogenic acid, p-coumaric acid, eugenol, hypericin, phosphinothricin, and tannic acid were not selectively toxic towards O. cf. chalybea.

Of the potential barley-straw decomposition products screened in this study, ferulic acid, trans-cinnamic acid, and several quinones appear to be the lead compounds for controlling the MIB-producing O. cf. chalybea in aquaculture ponds. It is possible that other barley-straw decomposition products that were not screened in this study, or that still remain unidentified, could be responsible for antialgal properties of decomposing barley straw in water reservoirs. In addition, a "cocktail" of several compounds produced or released during barley straw decomposition could be responsible for the antialgal effects.

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