JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Isolation and Identification of Antifungal and Antialgal Alkaloids from *Haplophyllum sieversii*

C. L. CANTRELL,*,† K. K. SCHRADER,† L. K. MAMONOV,‡ G. T. SITPAEVA,§ T. S. KUSTOVA,‡ C. DUNBAR,|| AND D. E. WEDGE†

USDA-ARS, Natural Products Utilization Research Unit, University, Mississippi 38677, Institute of Plant Physiology, Genetics, and Bioengineering, Timiriazeva, 45, Almaty, 05040, Republic of Kazakhstan, Institute of Botany and Phytointroduction, Timiriazeva 36d, Almaty, 05040, Republic of Kazakhstan, The University of Mississippi, Thad Cochran Research Center, University, Mississippi 38677

Bioassay-guided fractionation of the hexane/ethyl acetate/water (H/EtOAc/H_2O) crude extract of the aerial parts of *Haplophyllum sieversii* was performed because of preliminary screening data that indicated the presence of growth inhibitory components against *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, and *Colletotrichum acutatum*. Fractionation was directed using bioautographical methods resulting in the isolation of the bioactive alkaloids flindersine, anhydroevoxine, haplamine, and a lignan eudesmin. These four compounds were evaluated for activity against *C. fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporum*, and *Phomopsis obscurans* in a dose−response growth-inhibitory bioassay at 50.0, 100.0, and 150.0 μM. Of the four compounds tested, flindersine demonstrated the highest level of antifungal activity. Additionally, flindersine, eudesmin, and haplamine were screened against the freshwater phytoplanktons *Oscillatoria perornata*, *Oscillatoria agardhii*, *Selenastrum capricornutum*, and *Pseudanabaena* sp. (strain LW397). Haplamine demonstrated selective inhibition against the odor-producing cyanobacterium *O. perornata* compared to the activity against the green alga *S. capricornutum*, with lowest observed effect concentration values of 1.0 and 10.0 μM, respectively.

KEYWORDS: ***Haplophyllum sieversii*; *Botrytis cinerea*; *Fusarium oxysporum*; *Phomopsis obscurans*; *Colletotrichum fragariae*; *Colletotrichum gloeosporioides*; *Colletotrichum acutatum*; *Oscillatoria perornata*; *Oscillatoria agardhii*; *Selenastrum capricornutum*; *Pseudanabaena*; flindersine; eudesmin; anhydroevoxine; haplamine**

INTRODUCTION

Increased microbial resistance to commercially available agrochemicals has led to a need for new pesticides. Furthermore, the desire for safe and more effective agrochemicals with reduced environmental and/or mammalian toxicity remains important. Essential to these efforts is the identification of new promising compounds possessing high levels of desirable biological activities, reduced toxicities toward nontarget species, new chemical structures, and perhaps, different modes of action to help provide protection from cross-resistance to currently used agrochemicals (*1*). Natural product-based pesticides offer advantages in that some can be specific toward a target species, and they may also have unique modes of action with little mammalian toxicity. An additional benefit is their rapid decomposition, thereby reducing harm to the environment. Our current research efforts are to identify natural product-based fungicides and algicides.

Since the early 1970s, agricultural communities have struggled with the evolution of resistance to antimicrobial agents by pathogens. This situation has resulted in an increased need for repeated fungicide applications that has overshadowed the use of fungicides for plant disease management. To address problems of resistance to pesticides, three strategies have been used as follows: (1) optimization of disease resistance control strategies, (2) discovery and development of antimicrobial agents with new modes of action, and (3) the development of new disease-resistant cultivars. One aspect of our research focuses on novel plant-derived fungicides for control of important minor crop pathogens and pests in agriculture. Pathogens of small fruits and ornamentals such as *Colletotrichum*, *Botrytis*, and *Fusarium* continue to hamper the growth and profitability of these areas of agriculture.

Another area of our research deals with the discovery of natural and natural-based algicides for use in catfish aquaculture.

* To whom correspondence should be addressed. Telephone: +1-662-915-5898. Fax: +1-662-915-1035. E-mail: ccantrell@msa-oxford.ars.usda.gov.
† Natural Products Utilization Research Unit.
‡ Institute of Plant Physiology, Genetics, and Bioengineering.
§ Institute of Botany and Phytointroduction.
|| Thad Cochran Research Center.

10.1021/jf051478v CCC: $30.25
Published on Web 09/13/2005

"Off-flavor" has been estimated to cost the channel catfish (*Ictalurus punctatus*) industry in the United States as much as $60 million annually (*2*). The most common types of off-flavors are due to the absorption of the "earthy" and "musty" compounds geosmin and 2-methylisobornoel (MIB), respectively, into the flesh of the catfish. Certain types of cyanobacteria (blue-green algae) that grow in the catfish ponds produce geosmin and MIB. Catfish producers must hold market-size catfish that are tainted by these compounds until depuration of the off-flavor occurs. Such delays in harvest increase production costs because of additional feed costs, forfeiture of income from foregone sales, and lost income from delayed harvest. In addition, catfish held in inventory can be lost from disease, water quality deterioration, and bird depredation.

The most common management approach used by catfish farmers to control earthy/musty off-flavors is the application of algicides to kill the noxious species of cyanobacteria. In west Mississippi (Delta), the leading region of catfish production in the United States, MIB-related off-flavor has been attributed to the presence of the cyanobacterium *Oscillatoria* (*Planktothrix*) *perornata* in the catfish ponds (*3*, *4*). In addition, *O. perornata* has recently been found to be present in catfish ponds in east Mississippi and west Alabama (*5*). The two types of algicides used by catfish farmers are copper-based compounds (e.g., copper sulfate and chelated-copper products) and the herbicide diuron [*N*′-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea]. Unfortunately, these chemicals have several negative attributes, including broad-spectrum toxicity toward nontarget organisms, lengthy environmental persistence, and the public's negative perception to the use of these synthetic compounds in food-fish production ponds. Because of the lack of selective toxicity of copper-based compounds and diuron, the entire phytoplankton community can be poisoned and/or killed, including beneficial types of phytoplankton (e.g., green algae). Such massive phytoplankton die-offs in catfish ponds can result in the deterioration of the water quality that can subsequently stress and/or kill the catfish.

In a research program aimed at identifying natural fungicides and algicides, over 230 different crude plant extracts representing 38 species of plants endemic to the Republic of Kazakhstan have been evaluated. On the basis of these preliminary screening results, extracts of the aerial parts from *Haplophyllum sieversii* Fisch. Et Mey (Rutaceae) were chosen for further investigation. The genus *Haplophyllum* has been extensively investigated for the presence of phytochemicals resulting in the isolation of coumarins (*6*−*8*), lignins (*9*−*11*), and alkaloids (*12*−*15*).

METHODS AND MATERIALS

Instrumentation. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ on a Bruker Avance 400 MHz spectrometer (Billerica, MA). All ^{13}C multiplicities were deduced from 90° and 135° DEPT experiments. High-resolution mass spectra were obtained using an Agilent 1100 HPLC coupled to a JEOL AccuTOF (JMS-T100LC) (Peabody, MA). Column chromatography was performed using a Biotage, Inc., Horizon Pump (Charlottesville, VA) equipped with a Horizon Flash Collector and fixed wavelength (254 nm) detector. HPLC method development work was performed using an Agilent 1100 system equipped with a quartenary pump, autosampler, diode-array detector, and vacuum degasser. Semipreparative HPLC purifications were performed using a Waters Delta-Prep system (Milford, MA) equipped with a diode-array detector and a binary pump.

High-Resolution LC−MS Analysis. All isolated compounds were prepared in methylene chloride and injected directly into a 0.4 mL/min stream of a 20% H_2O/80% MeOH solution containing 1 μg/mL *L*-tryptophan. Mass drift compensations were performed relative to *L*-tryptophan $[M + H]^+$ and/or $[2M + H]^+$ ions.

Plant Material. The aerial parts of *H. sieversii* plants in the flowering stage were collected on June 13, 2003, in Kazakhstan in the western part of the Malay-Sary mountain pass and southwest spurs of Jungar Alatauh, where it grows among accompanying Ferula-Artemisia species. Perennial green or dovish-colored object by glandular hair plants are 35−70 cm tall. The plant stem is glabrous, solitary, erect, and branched. Leaves are broad and oblong to lanceolate (2−5 cm long and 0.5−2.0 cm broad), glabrous, and acute or bluntish. Flowers are numerous, small, and combined on the top in corymbous blossom clusters. Petals are oblong-lanceolate, 3.5−6 mm long, and yellow. Capsula is unopening and glabrous with deciduous niduses. A voucher specimen number 4012a/25-1975 has been deposited in the Institute of Botany and Phytointroduction Herbarium, Almaty, Republic of Kazakhstan.

Plant Extraction. Aerial parts (1 kg) were air-dried followed by grinding in a Willey−Mill plant grinder. Ground plant material was extracted at room temperature using 6.1 L of petroleum ether, providing 19.2 g of extractables after evaporation of the solvent. Dried marc was subsequently extracted using 5.9 L of hexane/ethyl acetate/H_2O (54:44:2), providing 20 g of extractables following evaporation of the solvents. This process was again repeated using ethanol (95%) as the extraction solvent, providing 94 g of extractables. Last, extraction with H_2O provided 109 g of extractables after lyophilization to remove H_2O.

Bioassay-Guided Fractionation. Initially, 3.0 g of the H/EtOAc/H_2O (54:44:2) extract was adsorbed to silica gel and applied to a silica gel chromatography column (40−63 μm, 40 × 150 mm, 60 Å). Elution of the column was performed using increasing polarity mixtures of hexane/EtOAc in a series of three linear steps as follows: (step 1) 100:0 to 50:50 using 1200 mL, (step 2) 50:50 to 30:70 using 1200 mL, and (step 3) 30:70 to 0:100 using 402 mL. Column eluate was collected in 24-mL portions and, on the basis of TLC similarities, recombined into 11 fractions (A, 6−13, 428 mg; B, 14−23, 4 mg; C, 24−26, 486 mg; D, 27−30, 76 mg; E, 31−33, 150 mg; F, 34−45, 335 mg; G, 46−55, 131 mg; H, 56−63, 105 mg; I, 64−76, 372 mg; and J, 77−96, 129 mg). Fractions I and J were selected for further investigation based on activities in the bioautographic activity against *C. acutatum* and *C. gloeosporioides*.

Fraction I (372 mg) was adsorbed onto silica gel and applied as a dried powder to a 25 × 150 mm silica gel column (40−63 μm, 60 Å) and eluted using increasing polarity mixtures of hexane/EtOAc in a series of three linear steps (step 1, 100:0 to 75:25 using 1599 mL; step 2, 75:25 to 25:75 using 600 mL; and step 3, 25:75 to 0:100 using 600 mL). Column eluate was collected in 24-mL portions and, on the basis of TLC similarities, recombined into 4 fractions (I1, 1−23, 4 mg; I2, 24−28, 30 mg; I3, 29−38, 257 mg; and I4, 39−72, 10 mg). Fraction I2 was determined to be pure flindersine. Fraction I3 was further purified using a 25 × 150 mm silica gel column (40−63 μm, 60 Å) and eluted using increasing polarity mixtures of hexane/Et_2O in a series of three linear steps (step 1, 100:0 to 75:25 using 2304 mL; step 2, 75:25 to 0:100 using 300 mL; and step 3, 0:100 using 300 mL). Column eluate was collected in 24-mL portions and, on the basis of TLC similarities, recombined into 4 fractions (I3-1, 1−21, 32 mg; I3-2, 21−25, 163 mg; I3-3, 25−38, 5 mg; and I3-4, 39−63, 52 mg). Fraction I3-2

produced crystals after standing at room-temperature overnight. Crystals were later filtered and washed to provide pure (+)-eudesmin.

Fraction J (129 mg) was further purified using a reversed-phase C-18 HPLC column (Zorbax, 9.4 × 250 mm, 5 μm) and running isocratic conditions (60:40 H_2O/acetonitrile), while monitoring at 210 nm. Two compounds were collected, providing 56 mg of haplamine and 6 mg of anhydroevoxine.

Flindersine. High-resolution ESI−MS *m/z* 228.0989 [M + H]$^+$, calculated for $C_{14}H_{14}NO_2$, 228.1024. ^{1}H NMR (400 MHz in $CDCl_3$) δ: 12.42 (br s, 1H, H-6), 7.88 (d, 1H, J = 8.4 Hz, H-10), 7.47 (t, 1H, J = 6.8 Hz, H-8), 7.44 (d, 1H, J = 8.4 Hz, H-7), 7.18 (t, 1H, J = 6.8 Hz, H-9), 6.80 (d, 1H, J = 10 Hz, H-4), 5.56 (d, 1H, J = 10 Hz, H-3), 1.54 (s, 6H, H-11 and H-12) (*16*). ^{13}C NMR (400 MHz in $CDCl_3$) δ: 163.1 (s, C-5), 157.4 (s, C-10b), 138.3 (s, C-6a), 130.9 (d, C-8), 126.3 (d, C-3), 122.6 (d, C-10), 122.2 (d, C-9), 117.4 (d, C-4), 116.4 (d, C-7), 115.4 (s, C-10a), 105.9 (s, C-4a), 79.2 (s, C-2), 28.5 (q, C-11 and C-12).

Haplamine. High-resolution ESI−MS *m/z* 258.1133 [M + H]$^+$, calculated for $C_{15}H_{16}NO_3$, 258.1130. ^{1}H NMR (400 MHz in $CDCl_3$) δ: 12.78 (br s, 1H, H-6), 7.39 (d, 1H, J = 8.8 Hz, H-7), 7.23 (d, 1H, J = 2.0 Hz, H-10), 7.11 (d, 1H, J = 8.4 Hz, H-8), 6.80 (d, 1H, J = 10 Hz, H-4), 5.54 (d, 1H, J = 10 Hz, H-3), 3.86 (s, 3H, OMe), 1.54 (s, 6H, H-11 and H-12). ^{13}C NMR (400 MHz in $CDCl_3$) δ: 162.7 (s, C-5), 156.9 (s, C-10b), 155.1 (s, C-9), 133.1 (s, C-6a), 126.3 (d, C-3), 120.7 (d, C-8), 117.9 (d, C-7), 117.5 (d, C-4), 115.8 (s, C-10a), 106.1 (s, C-4a), 103.4 (d, C-10), 79.1 (s, C-2), 55.8 (q, OMe), 28.5 (q, C-11 and C-12) (*17*).

Anhydroevoxine. High-resolution ESI−MS *m/z* 330.1359 [M + H]$^+$, calculated for $C_{18}H_{20}NO_5$, 330.1342. ^{1}H NMR (400 MHz in $CDCl_3$) δ: 7.98 (d, 1H, J = 9.2 Hz, H-5), 7.59 (d, 1H, J = 2.4 Hz, H-2), 7.25 (d, 1H, J = 9.2 Hz, H-6), 7.04 (s, 1H, J = 2.4 Hz, H-3), 4.43 (2, 3H, 4-OMe), 4.39 (dd, 1H, J = 6.0, 11.2 Hz, H-1′), 4.30 (dd, 1H, J = 6.0, 11.2 Hz, H-1′), 4.13 (s, 3H, 8-OMe), 3.25 (t, 1H, J = 4.8 Hz, H-2′), 1.39 (s, 3H, H-4′), 1.36 (s, 3H, H-5′). ^{13}C NMR (400 MHz in $CDCl_3$) δ: 164.5 (s, C-9a), 157.4 (s, C-4), 151.3 (s, C-7), 143.5 (d, C-2), 143.4 (s, C-8), 141.9 (s, C-8a), 118.3 (d, C-5), 115.8 (s, C-4a), 115.1 (d, C-6), 104.8 (d, C-3), 102.6 (s, C-3a), 69.7 (t, C-1′), 61.9 (q, 8-OMe), 61.8 (d, H-2′), 59.2 (q, 4-OMe), 58.5 (s, C-3′), 24.8 (q, C-4′), 19.2 (q, C-5′) (*18*).

(+)-Eudesmin. High-resolution ESI−MS *m/z* 387.1841 [M + H]$^+$, calculated for $C_{22}H_{27}O_6$, 387.1808. $[\alpha]^{25}_D$ = +63.1 (*c* 0.13, $CHCl_3$). ^{1}H NMR (400 MHz in $CDCl_3$) δ: 6.91 (br s, 2H, H-1′), 6.88 (d, 2H, J = 8.4 Hz, H-6′), 6.84 (d, 2H, J = 8.4 Hz, H-5′), 4.76 (br s, 2H, H-2 H-6), 4.26 (m, 2H, H-4 H-8), 3.89 (s, 6H, 4′ OCH_3), 3.87 (s, 6H, 3′ OCH_3), 3.90 (m, 2H, H-4 H-8), 3.11 (m, 2H, H-1 H-5). ^{13}C NMR (400 MHz in $CDCl_3$) δ: 149.4 (s, 3′), 148.8 (s, 4′), 133.8 (s, C-1′), 118.4 (d, C-6′), 111.3 (d, C-5′), 109.5 (d, C-2′), 85.9 (d, C-2 C-6), 71.9 (t, C-4 C-8), 56.1 (q, 3′-OCH_3), 56.1 (q, 4′-OCH_3), 54.3 (d, C-1 C-5) (*19*).

Fungal Isolates and Media. Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. and Sacc. were obtained from B. J. Smith, USDA, ARS, Poplarville, MS. Cultures of *Phomopsis viticola* and *P. obscurans* were obtained from Mike Ellis, The Ohio State University, OH, and *Botrytis cinerea* Pers. and *Fusarium oxysporum* Schlechtend were isolated in our laboratory at Oxford, MS. The three *Colletotrichum* species and *Phomopsis obscurans* were isolated from strawberry (*Fragaria x ananassa* Duchesne), while *Phomopsis viticola* and *Botrytis cinerea* were isolated from commercial grape (*Vitis vinifera* L.) and *Fusarium oxysporum* from orchid (*Cynoches* sp.). Fungi were grown on potato dextrose agar (PDA, Difco, Detroit, MI) in 9-cm Petri dishes and incubated in a growth chamber at 24 ± 2 °C under cool-white fluorescent lights (55 ± 5 μmol m^{-2} s^{-1}) with a 12-h photoperiod.

Conidia Preparation. Conidia were harvested from 7- to 10-day-old cultures by flooding plates with 5 mL of sterile distilled water and dislodging conidia by softly brushing the colonies with an L-shaped glass rod. Aqueous conidial suspensions were filtered through sterile Miracloth (Calbiochem−Novabiochem Corp., La Jolla CA) to remove mycelia. Conidia concentrations were determined photometrically (*20*, *21*) from a standard curve based on absorbance at 625 nm, and suspensions were adjusted with sterile distilled water to a concentration of 1.0 × 10^6 conidia/mL.

Standard conidial concentrations were determined from a standard curve for each fungal species. Standard turbidity curves were periodically validated using both a Bechman/Coulter Z1 particle counter and hemocytometer counts. Conidial and mycelial growth are evaluated using a Packard model SpectraCount microplate photometer (Packard Instrument Company, Meriden, CT). Conidial growth and germ tube development were evaluated using an Olympus IX 70 inverted microscope and recorded with a DP12 digital camera as appropriate for compounds that affected spore germination and early germ tube development.

Direct Bioautography. A number of bioautography techniques were used as primary screening systems to detect antifungal activity. Matrix, one-dimensional, and two-dimensional bioautography protocols on silica gel TLC plates along with *Colletotrichum* spp. as the test organisms were used to identify the antifungal activity according to published methods (*22*, *23*). Matrix bioautography was used to screen large numbers of crude extract at 20 mg/mL. One-dimensional thin-layer chromatography (1D TLC) were subsequently used to purify and identify the number of antifungal agents in extracts. Each plate was subsequently sprayed with a spore suspension (10^5 spores/mL) of the fungus of interest and incubated in a moisture chamber for 3 days at 26 °C with a 12-h photoperiod. Clear zones of fungal growth inhibition on the TLC plate indicated the presence of antifungal constituents in each extract. Inhibition of fungal growth was evaluated 3−4 days after treatment. Antifungal metabolites were readily located on the plates by visually observing clear zones where the active compounds inhibited fungal growth (*24*).

96-Well Fungal Microbiassay. A reference method [M27-A from the National Committee for Clinical Laboratory Standards (NCCLS)] for broth-dilution antifungal susceptibility testing of yeast was adapted for evaluation of antifungal compounds against sporulating filamentous fungi (*21*). This 96-well microtiter assay is used to determine and compare the sensitivity of fungal plant pathogens to natural and synthetic compounds with known fungicidal standards (*25*).

The 96-well microtiter assay was used to determine the sensitivity *of C. acutatum*, *C. fragariae*, *C. gloeosporioides*, *F. oxysporum*, *B. cinerea*, *P. obscurans*, and *P. viticola* to the various antifungal agents in comparison with several commercial fungicides. The fungicides benomyl, azoxystrobin, and captan were used as standards in these assays. Each fungal species was challenged in a dose−response format so that the final test compound concentrations of 0.3, 3.0, and 30.0 μM were achieved (in duplicate) in the different columns of the 96-well plate.

Table 1. Fungal and Algal Inhibition Screening Results for *H. sieversii* Crude Extracts of the Aerial Parts

	LCIC (ppm)[a]		diameter of zone of inhibition (mm)[b]		
solvent[c]	*O. perornata*	*S. capricornutum*	*C. acutatum*	*C. fragariae*	*C. gloeosporioides*
PE	>100	>100	9.1 (±0.3)	9.2 (±0.3)	9.5 (±0.0)
H/EtOAc/H_2O	100	>100	10.4 (±0.3)	10.1 (±0.2)	12.8 (±0.7)
EtOH	100	>100	DZ	DZ	DZ
H_2O	>100	>100	9.2 (±0.4)	7.4 (±0.3)	7.6 (±0.3)

[a] Lowest complete inhibition concentration. [b] Mean dimensions of zones (mm) of fungal inhibition produced by sample extracts ±SD. All extracts tested at 80 μg. DZ, diffuse zone, indicating no clear zone of inhibition observed. Benomyl, captan, cyprodinil, and azoxystrobin served as positive controls. [c] PE, petroleum ether; H/EtOAc/H_2O = 54:44:2 hexane/ethyl acetate/H_2O.

Fungal growth was evaluated by measuring the absorbance of each well at 620 nm at 0, 24, 48, and 72 h, except for *P. obscurans* and *P. viticola*, where the data was also recorded at 120 h. Mean absorbance values and standard errors were graphed, and graphs were used to evaluate fungal growth. Differences in spore germination and mycelial growth in each of the wells in the 96-well plate demonstrate sensitivity to particular concentrations of pure compounds and indicated fungistatic or fungicidal effects.

Algicide Bioassay. Isolates of the cyanobacteria *O. agardhii* and *O. perornata* were obtained from water samples collected from Mississippi catfish ponds (*26*). A culture of the cyanobacterium *Pseudanabaena* sp. (strain LW397) was obtained from George Izaguirre, Metropolitan Water District of Southern California, La Verne, CA. An isolate of the green alga *S. capricornutum* was obtained from Dr. J. C. Greene, United States Environmental Protection Agency, Corvallis, OR, and *S. capricornutum* was used as a representative of green algae in the bioassay to determine selective toxicity of the extract and pure compounds. Each culture was maintained separately in continuous, steady-state growth using the conditions outlined in Schrader et al. (*27*) to provide a source of cells growing at a fairly constant rate.

The rapid bioassay of Schrader et al. (*27*) was used to evaluate the crude extracts from *H. sieversii* and the pure compounds flindersine, eudesmin, and haplamine. Stock solutions of the crude extracts and each pure compound were made in 100% dichloromethane (technical-grade quality) at 20.0, 200.0, and 2000.0 ppm for the crude extract and 2.0, 20.0, 200.0, and 2000.0 μM for each pure compound. Stock solutions of each test material were micropipeted to the empty wells (10 μL/well) of a reusable 96-well quartz microplate (Hellma Cells, Inc., Forest Hills, NY), and the dichloromethane was allowed to evaporate completely before adding continuous culture sample material (200 μL/well) from each of the continuous culture systems (only one test organism per well). Controls did not include any test material, only culture material (200 μL/well). Final treatment concentrations for the crude extracts were 1.0, 10.0, and 100.0 ppm, and final concentrations for each pure compound were 0.1, 1.0, 10.0, and 100.0 μM. Three replications were used for each concentration of crude extract, pure compound, and the control. Experiments were repeated. Microplates were placed in a growth chamber held at 29 ± 1°C and were illuminated continuously by fluorescent lights (40 W, cool white) at a photon flux density of 21−27 μE m^{-2} s^{-1}. Absorbance measurements of each well were measured at 650 nm at 24-h intervals for 4 days using a Packard model SpectraCount microplate photometer. Mean values and standard deviations of absorbance measurements were calculated and graphed to determine the lowest observed effect concentration (LOEC) and lowest complete inhibition concentration (LCIC). The 96-h 50% inhibition concentration (96-h IC_{50}) was determined for flindersine and haplamine for *O. perornata*, *Pseudanabaena* sp. (LW397), and *S. capricornutum* using the method outlined in Schrader et al. (*28*).

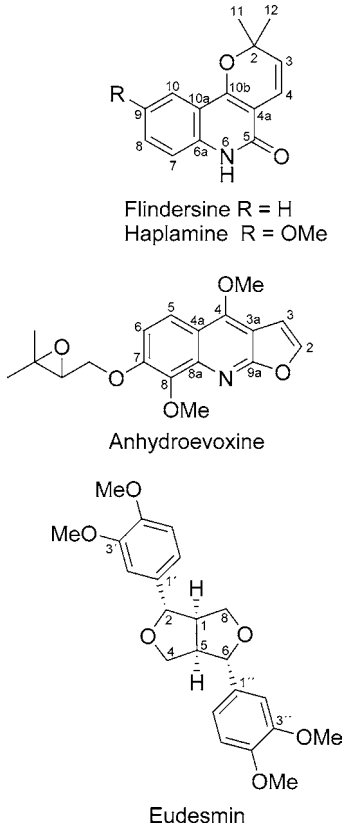

Figure 1. Compounds isolated from *H. sieversii*.

RESULTS AND DISCUSSION

Preliminary bioautographic screening of crude extracts from plants native to the Republic of Kazakhstan against *C. fragariae*, *C. gloeosporioides*, and *C. acutatum* prompted us to further investigate the hexane/EtOAc/H_2O extract from the aerial parts of *H. sieversii* (**Table 1**). This crude extract showed no selectivity between the three fungal species; however, large zones of inhibition prompted us to investigate further. Bioassay-guided fractionation of this extract using a hexane/ethyl acetate gradient suggested that fractions I and J contained the compounds responsible for the activity of the extract as a whole.

Fraction I was further purified using a hexane/ethyl acetate gradient providing a compound with a HRESIMS ion of *m*/*z* 228.0989, which suggested a possible molecular formula of $C_{14}H_{13}NO_2$ and hence 9° of unsaturation. Inspection of the ^1H

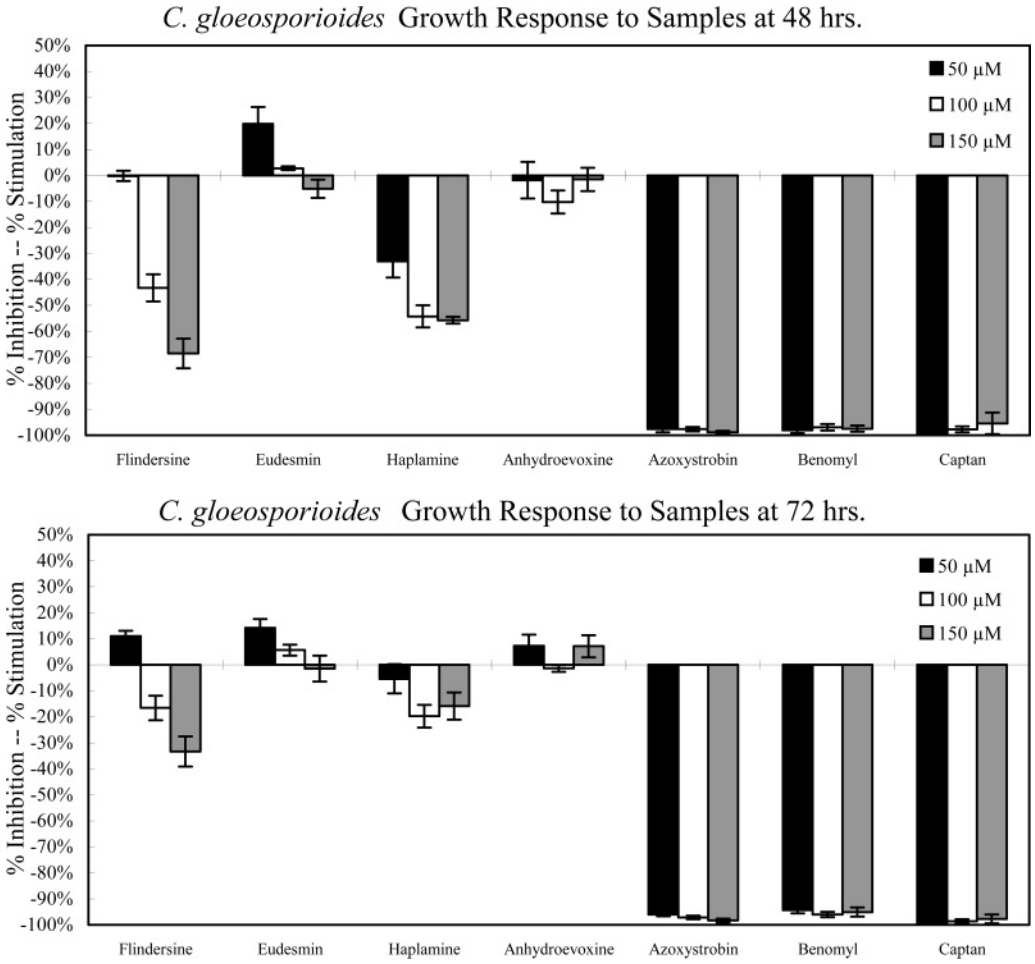

Figure 2. Growth inhibition/stimulation of *C. gloesporioides* after 48 and 72 h using a 96-well microtiter format in a dose–response to flindersine, haplamine, eudesmin, and anhydroevoxine and the commercial fungicide standards azoxystrobin, benomyl, and captan. Means from percent growth inhibition/stimulation were pooled from two experiments replicated in time.

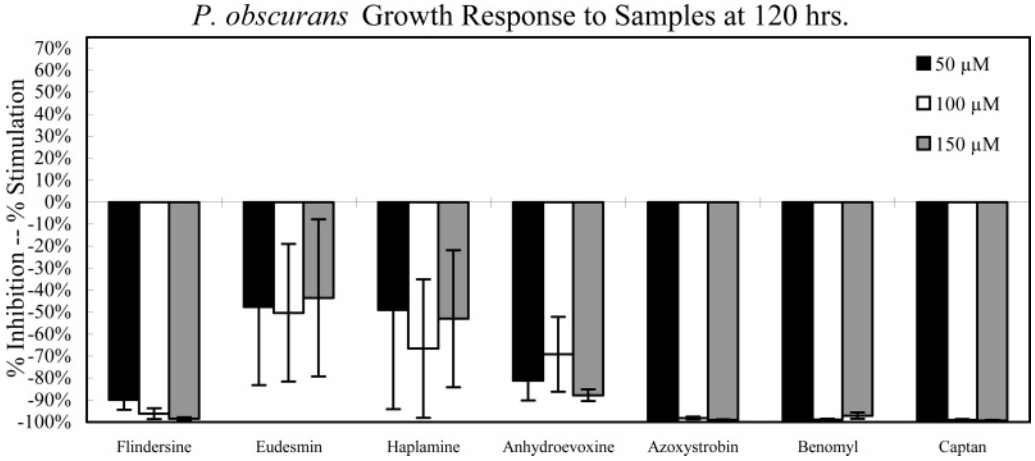

Figure 3. Growth inhibition of *P. obscurans* after 120 h using a 96-well microtiter format in a dose–response to flindersine, haplamine, eudesmin, and anhydroevoxine and the commercial fungicide standards azoxystrobin, benomyl, and captan. Means from percent growth inhibition were pooled from two experiments replicated in time.

NMR spectral data indicated the presence of four aromatic hydrogen atoms (δ 7.88 d, H-10; 7.47 t, H-8; 7.44 d, H-7; and 7.18 t, H-9), two olefinic doublets (δ 6.80, H-4 and 5.56, H-3), and a six-proton singlet (δ 1.54, H-11 and H-12). ^{13}C NMR analysis confirmed the presence of 14 carbons. One- and two-dimensional (HMBC and HSQC) NMR investigations confirmed the structure as that of flindersine, and complete NMR assignment data is reported herein. ^{1}H NMR data is in complete agreement with that reported in the literature by Ahmad et al. (*16*), providing further confirmation of its structure as that of flindersine (**Figure 1**).

Additional purification of fraction I using a hexane/ether gradient provided a crystalline compound that gave a HRESIMS molecular ion of *m*/*z* 387.1841 corresponding to $[M + H]^+$, suggesting a possible molecular formula of $C_{22}H_{27}O_6$, and implying 10° of unsaturation. ^{1}H NMR analysis revealed the presence of three aromatic protons, one singlet (δ 6.91) and two doublets (δ 6.88 and 6.84), an oxygenated methine (δ 4.76),

Table 2. Evaluation of Pure Compounds from *H. sieversii* Extracts for Antialgal Activity

	O. perornata[b]			*O. agardhii*[b]			*Pseudanabaena* sp. LW397[b]			*S. capricornutum*[b]		
compound[a]	LOEC	LCIC	IC_{50}	LOEC	LCIC	IC_{50}	LOEC	LCIC	IC_{50}	LOEC	LCIC	IC_{50}
flindersine	10	100	15.9	>100	>100	nd	>100	>100	nd	10	100	17.8
eudesmin	10	>100	nd	>100	>100	nd	100	100	nd	100	>100	nd
haplamine	1.0	10	1.8	>100	>100	nd	1.0	100	2.0	10	100	15.9

[a] Anhydroevoxine not tested because of an insufficient sample quantity available. [b] LOEC, lowest-observed-effect concentration; LCIC, lowest-complete-inhibition concentration; IC_{50} = 96-h 50% inhibition concentration; nd, not determined. All data expressed in micromolar concentrations.

an oxygenated methylene (δ 4.26 and 3.90), an aliphatic methine (δ 3.11), and two deshielded methoxyl groups (δ 3.89 and 3.87). ^{13}C NMR analysis indicated the presence of only 11 carbons, exactly half of the proposed molecular formula. The above information led us to suspect a molecule with a plane of symmetry, and the structure was assigned to (+)-eudesmin (**Figure 1**). ^{1}H and ^{13}C NMR data are in complete agreement with data reported previously (*19*), and optical rotation data is in complete agreement with data reported by Latip et al. (*29*).

Purification of the bioactive fraction J using HPLC resulted in the isolation of two pure compounds. Analysis of the major compound by HRESIMS revealed an $[M + H]^+$ at *m*/*z* 258.1133, suggesting a possible molecular formula of $C_{15}H_{15}NO_3$ and nine sites of unsaturation. Analysis of the ^{1}H NMR spectrum indicated a highly deshielded singlet at δ 12.78, three aromatic protons (δ 7.39, 7.23, and 7.11), two olefinic doublets (δ 6.80 and 5.54), one aromatic methoxyl group (δ 3.86), and two equivalent methyls (δ 1.54). ^{13}C NMR revealed 14 carbons as expected because one carbon corresponded to the two equivalent methyl groups. Further analysis by DEPT, HMQC, and HMBC unequivocally confirmed the structure as that of haplamine (**Figure 1**). ^{1}H and ^{13}C NMR data were in complete agreement with that reported by Campbell et al. (*17*).

The second compound purified from fraction J yielded a strong ion at *m*/*z* 330.1359, corresponding to the $[M + H]^+$ molecular ion when analyzed by HRESIMS. A search of possible empirical formulas suggested $C_{18}H_{20}NO_5$, implying that this compound had an empirical formula of $C_{18}H_{19}NO_5$ and 10 sites of unsaturation. ^{1}H NMR spectral analysis indicated the presence of four aromatic protons (δ 7.98, 7.59, 7.25, and 7.04), two aromatic methoxyl groups (δ 4.43 and 4.13), two deshielded methylene protons (δ 4.39 and 4.30), one methine proton (δ 3.25), and two methyl groups (δ 1.39 and 1.36). Analysis of the ^{13}C NMR, DEPT, HMQC, and HMBC spectral data confirmed the structure as that of anhydroevoxine (**Figure 1**). ^{13}C NMR data were in complete agreement with that reported in the literature (*18*).

The results of evaluation of the isolated compounds for antifungal activity are provided in **Figures 2** and **3** and in the Supporting Information. Of the four compounds tested, flindersine demonstrated the highest level of activity against all of the fungal species, except for *B. cinerea*, where haplamine was the most active (see the Supporting Information). *P. obscurans* appeared to be the most sensitive fungal species of those tested to this set of compounds (**Figure 3**). For example, more than 80% inhibition was observed for the 50.0 μM concentration at both 120 and 144 h. The large error bars observed in **Figure 3** are a result of the asynchronous spore germination that occurs for *P. obscurans* and is typical for assays of this organism. Also noteworthy was the general trend of decreased fungal inhibition over time by the test compounds against all of the fungal species used in the bioassay. For example, flindersine at 150.0 μM demonstrated greater than 65% inhibition at 48 h against *C. gloeosporioides*; however, at 72 h, less than 40% inhibition was observed (**Figure 2**). This decrease in sensitivity to chemicals over time suggests that the test organism may have inducible enzyme systems or other resistance mechanisms to bypass these natural product inhibitors (*30*). There was no loss of activity in the three commercial fungicide standards tested at these concentrations. None of the compounds tested was as active as the positive controls azoxystrobin, benomyl, or captan.

Two crude extracts from the aerial portions of *H. sieversii* were found to be selectively toxic toward *O. perornata* when compared to *S. capricornutum* (**Table 1**); therefore, evaluation of the isolated compounds (eudesmin, flindersine, and haplamine) for algicidal activity was conducted. Haplamine was found to be the most toxic of the three isolated compounds toward *O. perornata* with a LCIC of 10 μM, while the LCIC of flindersine and eudesmin were 100 and >100 μM, respectively (**Table 2**). In addition, haplamine was selectively toxic toward *O. perornata* when comparing LCIC results with the other species tested. The 24-h IC_{50} results further establish the selective toxicity of haplamine toward *O. perornata* because the 24-h IC_{50} of haplamine for *O. perornata* (1.8 μM) was approximately 9× less than the 24-h IC_{50} for *S. capricornutum* (15.9) (**Table 2**). Haplamine was also selectively toxic toward *Pseudanabaena* LW397 based on 24-h IC_{50} (2.0 μM); however, haplamine showed no toxicity toward *O. agardhii*, similar to results for flindersine and eudesmin.

There are several considerations before efficacy testing of haplamine can be performed using limnocorrals (fiberglass enclosures) placed in catfish ponds (*31*). Because haplamine is insoluble in water, either formulation development of haplamine or modification of the chemistry of haplamine to instill water solubility would need to be performed to maintain the compound in an active algicidal state in the pond water column (*32*). Additional testing of haplamine is required to determine any potential mutagenic and/or antibiotic properties, unfavorable characteristics for a particular compound being considered for use as a selective algicide in food-fish production ponds. Also, the persistence of haplamine in the pond water column would need to be monitored closely because previous studies have found that some natural algicides do not persist for a long enough period to be effective (*33*).

It should be noted that investigations were also performed on bioactive petroleum ether and water extracts (**Table 1**), resulting in the isolation of the same bioactive compounds reported above. No additional compounds were found in these extracts that had not already been isolated from the hexane/EtOAc/H_2O extract.

In summary, our bioassay-guided efforts to isolate the antifungal and antialgal constituents present in extracts of *H. sieversii* have resulted in the isolation of four compounds, three alkaloids, and one lignin. One of these alkaloids, flindersine, demonstrated activity against *P. obscurans*, *C. gloeosporioides*, *C. fragariae*, *C. acutatum*, and *F. oxysporum*, with its highest level of activity observed against *P. obscurans* at 150 μM. Another alkaloid, haplamine, demonstrated selective inhibition

against the odor-producing cyanobacterium *O. perornata* compared to the activity against the green alga *S. capricornutum*, with lowest observed effect concentration values of 1.0 and 10.0 μM, respectively. Clearly, this study has progressed from a bioactive crude extract to the isolation of both antifungal and antialgal constituents with varying degrees of activity against many species of microorganisms.

ACKNOWLEDGMENT

The authors thank Solomon Green III and Amber Callahan for technical assistance.

Supporting Information Available: Results of growth inhibition/stimulation evaluation of the isolated compounds for antifungal activity (Figures 1S−5S). This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) Duke, S. O.; Baerson, S. R.; Dayan, F. E.; Rimando, A. M.; Scheffler, B. E.; Tellez, M. R.; Wedge, D. E.; Schrader, K. K.; Akey, D. H.; Arthur, F. H.; De Lucca, A. J.; Gibson, D. M.; Harrison, H. F., Jr.; Peterson, J. K.; Gealy, D. R.; Tworkoski, T.; Wilson, C. L.; Morris, J. B. United States Department of Agriculture-Agricultural Research Service research on natural products for pest management. *Pest Manage. Sci.* **2003**, *59*, 708−717.

(2) Tucker, C. S. Off-flavor problems in aquaculture. *Rev. Fish. Sci.* **2000**, *8*, 45−88.

(3) Martin, J. F.; Izaguirre, G.; Waterstrat, P. A planktonic *Oscillatoria* species from Mississippi catfish ponds that produces the off-flavor compound 2-methylisoborneol. *Water Res.* **1991**, *25*, 1447−1451.

(4) van der Ploeg, M.; Tucker, C. S.; Boyd, C. E. Geosmin and 2-methylisoborneol production by cyanobacteria in fish ponds in the southeastern United States. *Water Sci. Technol.* **1992**, *25*, 283−290.

(5) Schrader, K. K.; Dennis, M. E. Cyanobacteria and earthy/musty compounds found in commercial catfish (*Ictalurus punctatus*) ponds in the Mississippi Delta and Mississippi−Alabama Blackland Prairie. *Water Res.* **2005**, *39*, 2807−2814.

(6) Filippini, R.; Piovan, A.; Innocenti, G.; Caniato, R.; Cappelletti, E. M. Production of coumarin compounds by *Haplophyllum patavinum in vivo* and *in vitro*. *Phytochemistry* **1998**, *49*, 2337−2340.

(7) Gunes, H. S. (−)-Haplonolide, a novel terpenoid from *Haplophyllum vulcanicum* (Rutaceae). *J. Fac. Pharm. Gazi Uni.* **2001**, *18*, 35−41.

(8) Ivanova, A.; Mikhova, B.; Stambolijska, T.; Kostova, I. Lignan and coumarin glycosides from *Haplophyllum suaveolens*. *Z. Naturforsch., C: J. Biosci.* **2001**, *56*, 329−333.

(9) Innocenti, G.; Puricelli, L.; Piacente, S.; Caniato, R.; Filippini, R.; Cappelletti, E. M. Patavine, a new arylnaphthalene lignan glycoside from shoot cultures of *Haplophyllum patavinum*. *Chem. Pharm. Bull.* **2002**, *50*, 844−846.

(10) Prieto, J. M.; Giner, R. M.; Recio, M. C.; Schinella, G.; Manez, S.; Rios, J. L. Diphyllin acetylapioside, a 5-lipoxygenase inhibitor from *Haplophyllum hispanicum*. *Plant. Med.* **2002**, *68*, 359−360.

(11) Puricelli, L.; Innocenti, G.; Piacente, S.; Caniato, R.; Filippini, R.; Cappelletti, E. M. Production of lignans by *Haplophyllum patavinum in vivo* and *in vitro*. *Heterocycles* **2002**, *56*, 607−612.

(12) Al-Rehaily, A. J.; Al-Howiriny, T. A.; Ahmad, M. S.; Al-Yahya, M. A.; El-Feraly, F. S.; Hufford, C. D.; McPhail, A. T. Alkaloids from *Haplophyllum tuberculatum*. *Phytochemistry* **2001**, *57*, 597−602.

(13) Khalifa, A. A.; Youssef, D. T. Lignans and an alkaloid from *Haplophyllum tuberculatum*. *Bull. Pharm. Sci.* **1999**, *22*, 157−166.

(14) Ulubelen, A.; Mericli, A. H.; Kaya, U. An alkaloid and lignans from *Haplophyllum telephioides*. *Phytochemistry* **1994**, *35*, 1600−1601.

(15) Ulubelen, A.; Mericli, A. H.; Mericli, F.; Sonmez, U.; Ilarslan, R. Alkaloids and coumarins from *Haplophyllum thesioides*. *Nat. Prod. Lett.* **1993**, *1*, 269−272.

(16) Ahmad, S. Flindersine from *Fagara heitzii*. *J. Nat. Prod.* **1984**, *47*, 391−392.

(17) Campbell, W. E.; Davidowitz, B.; Jackson, G. E. Quinolinone alkaloids from an *Agathosma* species. *Phytochemistry* **1990**, *29*, 1303−1306.

(18) Al-Rehaily, A. J.; Ahmad, M. S.; Muhammad, I.; Al-Thukair, A. A.; Perzanowski, H. P. Furoquinoline alkaloids from *Teclea nobilis*. *Phytochemistry* **2003**, *64*, 1405−1411.

(19) Roy, S. C.; Rana, K. K.; Guin, C. Short and stereoselective total synthesis of furano lignans (±)-dihydrosesamin, (±)-lariciresinol dimethyl ether, (±)-acuminatin methyl ether, (±)-sanshodiol methyl ether, (±)-lariciresinol, (±)-acuminatin, and (±)-lariciresinol monomethyl ether and furofuran lignans (±)-sesamin, (±)-eudesmin, (±)-piperitol methyl ether, (±)-pinoresinol, (±)-piperitol, and (±)-pinoresinol monomethyl ether by radical cyclization of epoxides using a transition-metal radical source. *J. Org. Chem.* **2002**, *67*, 3242−3248.

(20) Espinel-Ingroff, A.; Kerkering, T. M. Spectrophotometric method of inoculum preparation for the *in vitro* susceptibility testing of filamentous fungi. *J. Clin. Microbiol.* **1991**, *29*, 393−394.

(21) Wedge, D. E.; Kuhajek, J. M. A microbioassay for fungicide discovery. *SAAS Bull. Biochem. Biotechnol.* **1998**, *11*, 1−7.

(22) Homans, A. L.; Fuchs, A. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. *J. Chromatogr.* **1970**, *51*, 327−329.

(23) Wedge, D. E.; Nagle, D. G. A new 2D-TLC bioautography method for the discovery of novel antifungal agents to control plant pathogens. *J. Nat. Prod.* **2000**, *63*, 1050−1054.

(24) Tellez, M. R.; Dayan, F. E.; Schrader, K. K.; Wedge, D. E.; Duke, S. O. Composition and some biological activities of the essential oil of *Callicarpa americana* (L.). *J. Agric. Food Chem.* **2000**, *48*, 3008−3012.

(25) Wedge, D. E.; Galindo, J. C. G.; Macias, F. A. Fungicidal activity of natural and synthetic sesquiterpene lactone analogs. *Phytochemistry* **2000**, *53*, 747−757.

(26) van der Ploeg, M.; Dennis, M. E.; and de Regt, M. Q. Biology of *Oscillatoria* cf. *chalybea*, a 2-methylisoborneol producing blue-green alga of Mississippi catfish ponds. *Water Sci. Technol.* **1995**, *31*, 173−180.

(27) Schrader, K. K.; de Regt, M. Q.; Tucker, C. S.; Duke, S. O. A rapid bioassay for selective algicides. *Weed Technol.* **1997**, *11*, 767−774.

(28) Schrader, K. K.; de Regt, M. Q.; Tidwell, P. D.; Tucker, C. S.; Duke, S. O. Compounds with selective toxicity towards the off-flavor metabolite-producing cyanobacterium *Oscillatoria* cf. *chalybea*. *Aquaculture* **1998**, *163*, 85−99.

(29) Latip, J.; Hartley, T. G.; Waterman, P. G. Lignans and coumarins metabolites from *Melicope hayesii*. *Phytochemistry* **1999**, *51*, 107−110.

(30) Wedge, D. E.; Curry, K. J.; Boudreaux, J. E.; Pace, P. F.; Smith, B. J. A microtiter assay demonstrates sensitivity and resistance profiles for *Botrytis cinerea* isolates from Louisiana strawberry farms. *Adv. Strawberry Res.* **2001**, *20*, 27−33.

(31) Schrader, K. K.; Tucker, C. S.; de Regt, M. Q.; Kingsbury, S. K. Evaluation of limnocorrals for studying the effects of phytotoxic compounds on plankton and water chemistry in aquaculture ponds. *J. World Aquacult. Soc.* **2000**, *31*, 403−415.

(32) Schrader, K. K.; Nanayakkara, N. P. D.; Tucker, C. S.; Rimando, A. M.; Ganzera, M.; Schaneberg, B. T. Novel derivatives of 9,-10-anthraquinone are selective algicides against the musty-odor

cyanobacterium *Oscillatoria perornata*. *Appl. Environ. Microbiol.* **2003**, *69*, 5319−5327.

(33) Schrader, K. K.; Duke, S. O.; Kingsbury, S. K.; Tucker, C. S.; Duke, M. V.; Dionigi, C. P.; Millie, D. F.; Zimba, P. V. Evaluation of ferulic acid for controlling the musty-odor cyanobacterium, *Oscillatoria perornata*, in aquaculture ponds. *J. Appl. Aquacult.* **2000**, *10*, 1−16.

Received for review June 22, 2005. Revised manuscript received August 11, 2005. Accepted August 12, 2005. Financial support from the International Science and Technology Center (ISTC) project K790 is greatly appreciated.

JF051478V