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# Isolation and Identification of Antifungal Fatty Acids from the Basidiomycete *Gomphus floccosus*

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Bioautography of extracts of the fruiting bodies of the basidiomycete *Gomphus floccosus* (Schw.) Singer indicated the presence of fungitoxic compounds in the ethyl acetate fraction against the plant pathogens *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, and *Colletotrichum acutatum*. Bioassay-guided fractionation of this fraction resulted in the isolation of the bioactive fatty acids (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid (**1**), (9*E*,11*Z*)-13-oxo-9,11-octadecadienoic acid (**2**), and (10*E*,12*E*)-9-oxo-10,12-octadecadienoic acid (**3**). These three oxylipins were further evaluated for activity against a greater range of fungal plant pathogens (*C. fragariae*, *C. gloeosporioides*, *C. acutatum*, *Botrytis cinerea*, *Fusarium oxysporum*, *Phomopsis obscurans*, and *Phomopsis viticola*) in in vitro dose−response studies. *Phomopis* species were the most sensitive fungi to these compounds. At 120 h of treatment, the $IC_{50}$ values for compounds **1**, **2**, and **3** for *P. obscurans* were 1.0, 4.5, and 23 $\mu$M, respectively, as compared to 1.1 $\mu$M for the captan positive control.

**KEYWORDS:** ***Gomphus floccosus*; *Botrytis cinerea*; *Fusarium oxysporum*; *Phomopsis obscurans*; *Colletotrichum fragariae*; *Colletotrichum gloeosporioides*; *Colletotrichum acutatum*; fatty acid; mushroom**

## INTRODUCTION

Evolved resistance to commercially available fungicides and the desire for environmentally and toxicologically safer agrochemicals are providing an impetus for fungicide discovery (*1*). Natural product-based pesticides offer several advantages, in that some are specific toward target species, and they often have unique modes of action with little mammalian toxicity. An additional benefit is that they generally have a relatively short environmental half-life as compared to synthetic pesticides.

Fungi are a source of novel compounds (*1, 2*). Most discovery efforts have relied on culturing the fungus to supply natural products; however, less than 1% of the estimated 1.6 billion species of fungi have been cultured. Most culture collections and screening practices have been carried out on easily cultured zygomycetes and primitive ascomycete species (*3*). Hence, discovery efforts that limit themselves to those species that can be cultured are sampling an extremely narrow part of the total fungal spectrum.

Mushrooms represent a fungal group that are composed of diploid mycelial filaments that unite with other individuals of the same species to form a highly specialized reproductive organ (the fruiting body or sporocarp) that is dedicated to above-ground spore production and dispersal. They are represented by almost all of the known 20000 species of basidiomycetes. Furthermore, the majority of the 50000 species of ascomycetes are either lichen-associated fungi or mushrooms. Thus, mushrooms represent two distinct branches of the fungal kingdom and are virtually untapped sources of novel, small molecules. Mushrooms, including shelf fungi and other fungi that produce visible fruiting bodies, have been largely uninvestigated by those searching for natural products for pest management.

Mushrooms have extremely complex nutritional requirements as compared with the more easily cultured fungi. Many of them form mycorrhizal associations with plants and grow poorly without their host, while others are obligate mycorrhizal species that perish without their micorrhizal associations (*3*). In their natural habitats, mushrooms are challenged by a wide variety of pests, such as bacteria, nematodes, insects, browsing animals, and other fungi. As a result, these fungi produce a wide and virtually unexplored variety of defensive chemicals. Investigating field-collected fungal fruiting bodies has the potential to yield new secondary metabolites, which may be used as novel chemical plant protectants against pathogens or other pests.

We initially evaluated approximately 6000 sporocarp extracts for their antifungal activity against the plant pathogens *Colletotrichum acutatum*, *Colletotrichum fragariae*, and *Colletotrichum gloeosporioides*. Among the lead candidates was the basidiomycete woolly chanterelle, *Gomphus floccosus* (Schw.)

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**Table 1.** Fungal Growth Inhibition for *G. floccosus* Crude Extract and Isolated Oxylipins Using Direct Bioautography

| test material | concentration ($\mu$g) | diameter of zone of inhibition (mm)[a] | | |
|---|---|---|---|---|
| | | *C. acutatum* | *C. fragariae* | *C. gloeosporioides* |
| crude extract | 80 | diffuse zone | 7.7 ± 0.6 | diffuse zone |
| | 10 | diffuse zone | 4.3 ± 0.6 | n/a |
| compound **1** | 80 | 19 ± 0.6 | 10.0 ± 0.6 | 7.0 ± 0.6 |
| | 20 | 5.0 ± 0.6 | 6.0 ± 0.6 | 3.0 ± 0.6 |
| compound **2** | 80 | 19 ± 0.6 | 8.0 ± 0.6 | 7.0 ± 0.6 |
| | 20 | 4.0 ± 0.6 | 6.0 ± 0.6 | 6.0 ± 0.6 |
| compound **3** | 80 | 17.0 ± 0.6 | 6.0 ± 0.6 | 7.0 ± 0.6 |
| | 20 | 8.0 ± 0.6 | 4.0 ± 0.6 | 6.0 ± 0.6 |

[a] Mean dimensions of zones (mm) of fungal inhibition ± SD. Benomyl, captan, cyprodinil, and azoxystrobin served as positive controls; data not shown.

Singer (Cantharellaceae) (syn. *Cantharellus floccosus*). In previous phytochemical investigations of *G. floccosus*, the constituents responsible for the delayed gastrointestinal disturbances in individuals consuming the fruiting bodies were isolated (*4, 5*). Norcaperatic acid was isolated and found to be the cause of this effect. An additional paper reported the presence of norcaperatic acid and mannitol from *G. floccosus* (*6*). Mannitol was also reported in this species by Dominguez et al. (*7*). Min et al. (*8*) reported that an ethanol extract of *G. floccosus* had antifungal activity against *Microsporum gypseum*, with an MIC of 1 mg/mL; however, the active constituents were not identified. In this paper, we report the identity and antifungal activity of several fatty acids isolated by bioassay-guided fractionation of *G. floccosus*.

## MATERIALS AND METHODS

**Instrumentation.** $^1$H and $^{13}$C NMR spectra were recorded in $CDCl_3$ and/or $C_6D_6$ on a Varian 400 MHz spectrometer model number EUR0020 (Palo Alto, CA). All $^{13}$C multiplicities were deduced from 90 and 135° DEPT experiments. Gas chromatography−mass spectrometry (GC-MS) analysis was performed on a Varian CP-3800 GC coupled to a Varian Saturn 2000 MS/MS. High-resolution mass spectra were obtained using an Agilent 1100 HPLC coupled to a JEOL AccuTOF (JMS-T100LC) (Peabody, MA). High-performance liquid chromatography (HPLC) method development work was performed using an Agilent 1100 system equipped with a quaternary 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 while monitoring at 254 nm.

**GC-MS Analysis.** Reaction products were analyzed by GC-MS using a DB-5 column (30 m × 0.25 mm fused silica capillary column, film thickness of 0.25 $\mu$m) operated using the following conditions: injector temperature, 240 °C; column temperature, 60−240 °C at 3 °C/min then held at 240 °C for 5 min; carrier gas, He; and injection volume, 5 $\mu$L (splitless).

**High-Resolution LC-MS Analysis.** All isolated compounds were dissolved in MeOH and injected directly into a 0.3 mL/min stream of a MeOH. Twenty microliters of sample (approximately 0.1 mg/mL) was injected manually at 0.5 min, while mass drift compensation standards (L-tryptophan) were injected at 1.5 min over the course of a 2 min run. Mass drift compensations were performed relative to L-tryptophan $[M - H]^-$, $[2M - H]^-$, and $[3M - H]^-$ ions for negative ion analysis.

**Raw Material and Extract Preperation.** Fruiting bodies of *G. floccosus* were collected at Sleeping Giant State Park in Hamden, Connecticut. Their identity was verified by Ed Bosman, mycology advisor and founder of the Connecticut Valley Mycology Society. (A voucher specimen was deposited at the New York Botanical Garden Herbarium). The collection (LifePharms assession #3641) totaled 555 g wet weight. The sample was lyophilized (62 g), pulverized, and extracted with ethyl acetate (20 wt %/vol). The ethyl acetate was evaporated to give a residue (1.4 g).

**Bioassay-Guided Fractionation.** Initially, 1.4 g of crude ethyl acetate extract was dissolved in 100 mL of $CH_3OH/H_2O$ (90/10) and placed into a 500 mL separatory funnel. The phase was extracted thrice using 100 mL portions of hexane, dried over $MgSO_4$, and evaporated to dryness providing 872 mg of material. A final percent composition of 70/30 ($CH_3OH/H_2O$) was obtained by adding 28.5 mL of $H_2O$ to the aqueous phase. This aqueous phase was again extracted thrice using 100 mL portions of $CHCl_3$, dried over $MgSO_4$, and evaporated to dryness providing 371 mg of material. $CH_3OH$ was removed from the aqueous phase by rotary evaportation, returned to the separatory funnel, and extracted thrice using 50 mL portions of ethyl acetate. Drying with $MgSO_4$ and evaporation of the ethyl acetate provided 7 mg of material. $H_2O$ was removed from the aqueous phase by freeze drying providing 41 mg.

A portion of the $CHCl_3$ phase from above was further purified using a reversed-phase C-18 HPLC column (Zorbax, 9.4 mm × 250 mm, 5 $\mu$m) running a linear gradient from 40/60 ($H_2O$ with 0.1% trifloroacetic acid/acetonitrile) to 0/100 ($H_2O$ with 0.1% trifloroacetic acid/acetonitrile). Three compounds were collected providing 14 mg of **1**, 13 mg of **2**, and 8 mg of **3**.

*(9S,10E,12Z)-9-Hydroxy-10,12-octadecadienoic Acid (***1***).* High-resolution ESI-MS *m/z* 295.2296 $[M - H]^-$, calculated for $C_{18}H_{31}O_3$, 295.2273; *m/z* 591.4624; $[2M - H]^-$, calculated for $C_{36}H_{63}O_6$, 591.4625. $^1$H NMR (400 MHz in $CDCl_3$): $\delta$ 6.44 (dd, 1H, $J$ = 11.2, 15.2 Hz, H-11), 5.93 (t, 1H, $J$ = 10.8 Hz, H-12), 5.62 (dd, 1H, $J$ = 6.8, 15.2 Hz, H-10), 5.42 (dt, 1H, $J$ = 7.6, 10.8 Hz, H-13), 4.11 (m, 1H, H-9), 2.30 (t, 2H, $J$ = 7.2 Hz), 2.15 (m, 2H), 2.05 (m, 2H, H-8), 1.6 (m, 2H, H-3), 1.2−1.4 (overlapping), 0.80 (t, 3H, $J$ = 6.8 Hz, H-18) (*9*). $^{13}$C NMR (100 MHz in $CDCl_3$): $\delta$ 179.6 (s, C-1), 135.8 (d, C-10), 133.2 (d, C-13), 127.9 (d, C-12), 126.1 (d, C-11), 73.1 (d, C-9), 37.5 (t), 34.2 (t, C-2), 31.7 (t, C-17 or 16), 24.5 (t), 29.3 (t), 29.2 (t), 29.1 (t), 27.9 (t, C-14?), 25.5 (t), 24.9 (t, C-3), 22.7 (t, C-17 or 16), 14.2 (q, C-18).

*(9E,11Z)-13-Oxo-9,11-octadecadienoic Acid (***2***).* High-resolution ESI-MS *m/z* 293.2128 $[M - H]^-$, calculated for $C_{18}H_{29}O_3$, 293.2117; 587.4323 $[2M - H]^-$, calculated for $C_{36}H_{59}O_6$, 587.4312. $^1$H NMR (400 MHz in $CDCl_3$): 7.49 (dd, 1H, $J$ = 11.4, 15.4 Hz, H-11), 6.16 (d, 1H, $J$ = 15.2, H-12), 6.11 (d, 1H, $J$ = 11.2, H-10), 5.90 (dt, 1H, $J$ = 7.6, 10.8 Hz, H-9), 2.54 (t, 2H, $J$ = 7.4), 2.32 (m, 2H), 1.62 (m, 2H, $J$ = 10.4), 1.31 (d, 2H, $J$= 4.4), 0.88 (t, 3H, $J$ = 6.6, H-18) (*10*). $^{13}$C NMR (100 MHz in $CDCl_3$): 201.3 (s, C-13), 179.2 (s, C-1), 142.9 (d, C-9), 137.3 (d, C-11), 129.6 (d, C-12), 127.1 (d, C-10), 41.2 (t, C-14), 34.1 (t, C-2), 31.6 (t), 29.3 (t), 29.4 (t), 29.3 (t), 29.1 (t), 28.6 (t, C-8), 24.8 (t, C-3), 24.5 (t, C-15), 22.7 (t, C-17), 14.2 (q, C-18).

*(10E,12E)-9-Oxo-10,12-octadecadienoic Acid (***3***).* High-resolution ESI-MS *m/z* 293.2130 $[M - H]^-$, calculated for $C_{18}H_{30}O_3$, 293.2117. $^1$H NMR (400 MHz in $C_6D_6$): 7.20 (dd, $J$ = 10.5, 15.2, H-11), 6.04 (d, $J$ = 16, H-10), 5.96 (dd, $J$ = 10.4, H-12), 5.8 (dt, $J$ = 6.8, 15.0, H-13), 2.73 (t, $J$ = 6.8, H-2), 2.08 (d, $J$ = 3.6, H-14), 1.88 (d, $J$ = 6.4, H-8), 1.59 (t, $J$ = 6.8, H-15), 1.48 (s, H-3), 1.27−1.10 (4−7, 16, 17), 0.92 (t, $J$ = 7.2, H-18). $^{13}$C NMR (100 MHz in $C_6D_6$): 199.6 (s, C-9), 180.1 (s, C-1), 145.2 (d, C-13), 142.7 (d, C-11), 129.7 (d, C-12), 128.8 (d, C-10), 41.2 (t, C-8), 33.6 (t, C-2), 32.0 (t, C-14), 30.9 (t), 30.0 (t), 29.7 (t), 29.5 (t), 29.0 (d,C-4), 25.3 (t, C-5), 23.2 (t, C-17), 14.6 (q, C-18).

**Diazomethane Generation.** An Aldrich (St. Louis, MO) Mini Diazald Apparatus was used for the production of diazomethane in ether. Briefly, 2.5 g of KOH was dissolved in 4 mL of deionized water and placed in the reaction vessel followed by the addition of 5 mL of ethanol. A separatory funnel containing 2.5 g of diazald dissolved in 22.5 mL of ether was placed above the reaction vessel. The reaction vessel was warmed to 65 °C using a water bath followed by the dropwise addition of the diazald solution over a period of 50 min. The receiving flask and condenser coldfinger were cooled using a dry ice/acetone bath. The codistilled diazomethane in ether solution was stored in sealed vials at −20 °C until needed.

**Methylation of Linoleic Acids.** A 1.0 mg solution of fatty acid **1**, **2**, or **3** in 2.0 mL of MeOH was treated at room temperature with a solution of $CH_2N_2$ in diethyl ether (2 mL). The solution was placed in a laboratory fume hood overnight to complete the reaction and

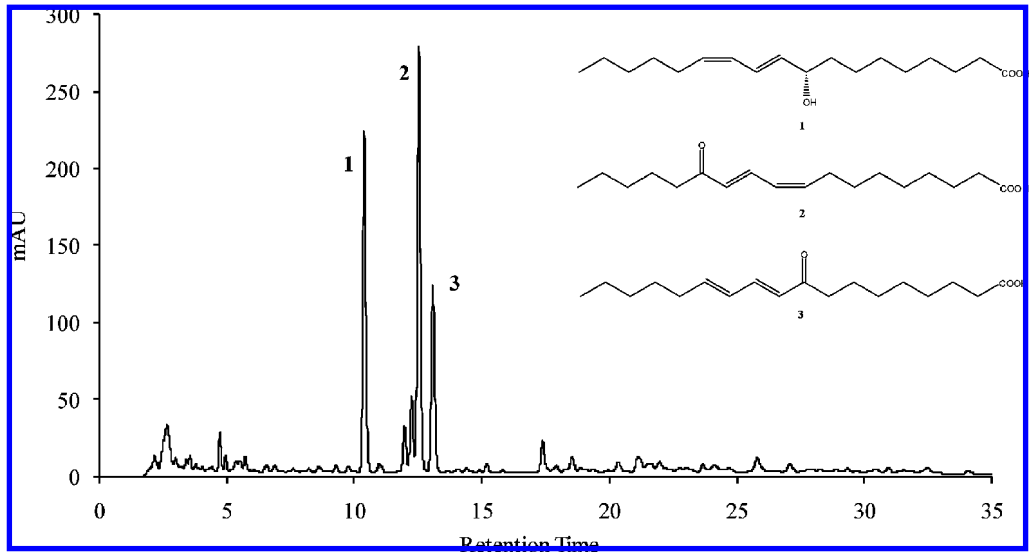


**Figure 1.** Representative HPLC chromatogram (254 nm) used during the purification of the chloroform partition. (9*S*,10*E*,12*Z*)-9-Hydroxy-10,12-octadecadienoic acid (**1**), (9*E*,11*Z*)-13-oxo-9,11-octadecadienoic acid (**2**), and (10*E*,12*E*)-9-oxo-10,12-octadecadienoic acid (**3**) identified bioactive compounds were purified.

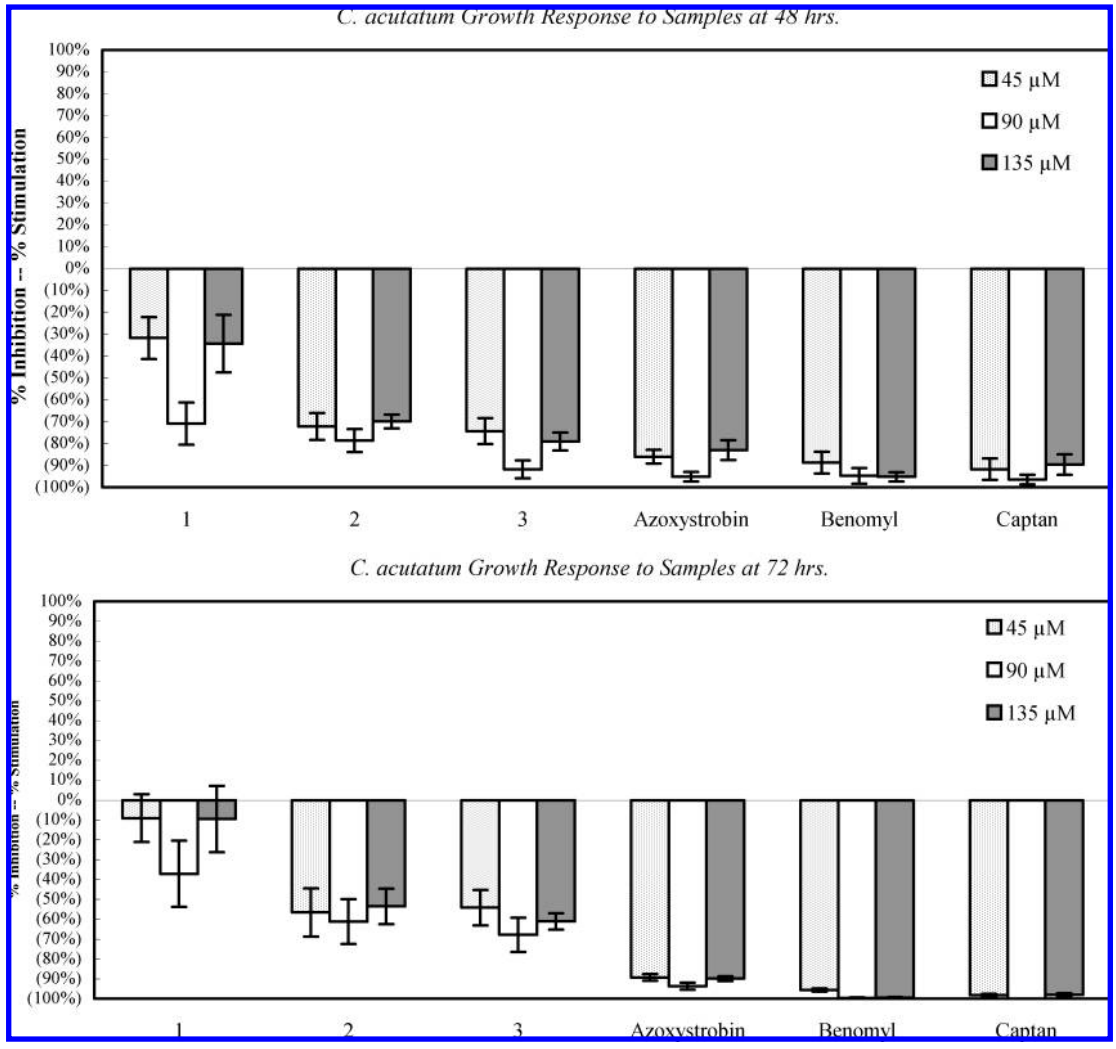


**Figure 2.** Growth inhibition/stimulation of *C. acutatum* after 48 and 72 h using 96 well microtiter format in a dose response to **1**−**3** and the commercial fungicide standards azoxystrobin, benomyl, and captan. Means from percent growth inhibition/stimulation were pooled from two experiments replicated in time.

allow for evaporation of solvent and $CH_2N_2$. Methyl ester products were dissolved in $CH_2Cl_2$ and analyzed by GC-MS and $^1H$ NMR.

**Preparation of the Trimethylsilylated Linoleic Acid Methyl Esters.** Following methylation of compounds **1**, **2**, or **3**, solvent was evaporated, 0.3 mL of BSTFA containing 1% trimethylchlorosilane

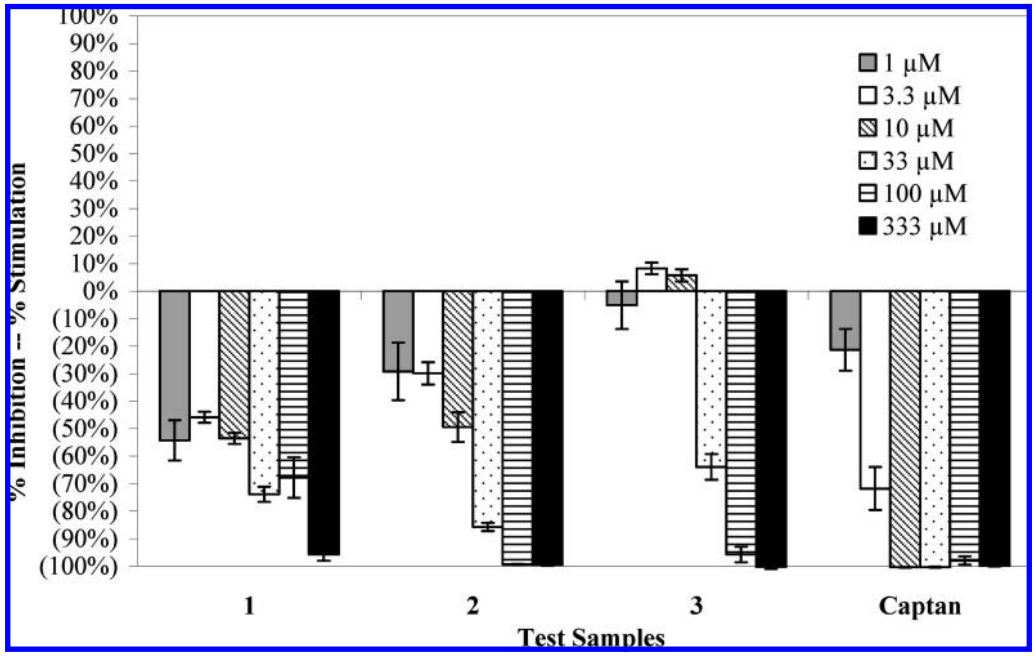


**Figure 3.** Growth inhibition of *P. obscurans* after 144 h using 96 well microdilution broth in a dose response to **1**−**3** and the commercial fungicide standard captan. Percent growth inhibition means were pooled from two experiments replicated twice in time.

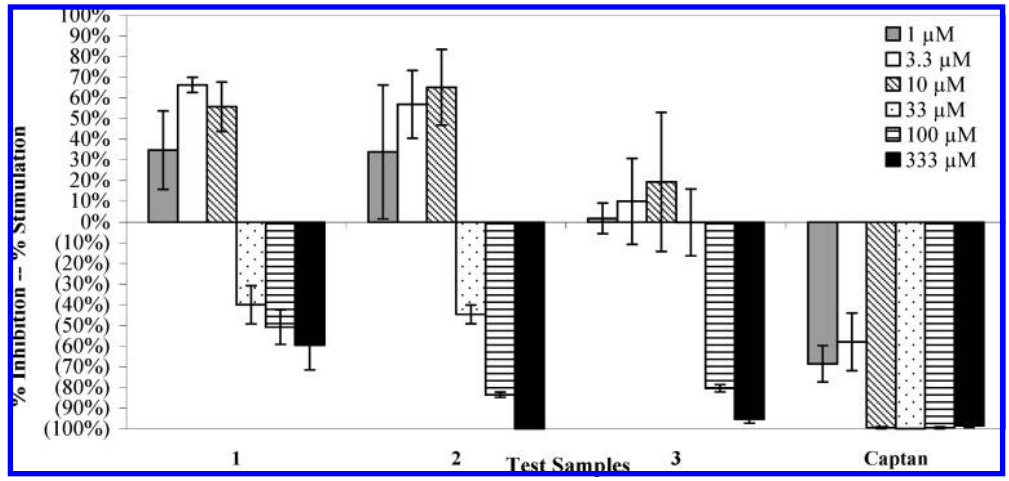


**Figure 4.** Growth inhibition of *P. viticola* after 144 h using 96 well microdilution broth assay in a dose response to **1**−**3** and the commercial fungicide standard captan. Percent growth inhibition means were pooled from two experiments replicated twice in time.

was added, and the mixture was carefully heated in an oven for 60 min at 60 °C. Secondary containment was used as a precaution. The solvents were evaporated under $N_2$, dissolved in methylene chloride, and analyzed by GC-MS.

**Fungal Isolates and Media.** Isolates of *C. acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. were obtained from Barbara J. Smith (Agricultural Research Service, U.S. Department of Agriculture, Popularville, MS). Cultures of *Phomopsis viticola* and *Phomopsis obscurans* were obtained from Mike Ellis (Ohio State University, OH), and *Botrytis cinerea* Pers. and *Fusarium oxysporum* Schlechtend were isolated in our laboratory at Oxford, Mississippi. *F. oxysporum* identification was confirmed by Wade H. Elmer (Connecticut Agricultural Experiment Station, New Haven, CT), and the identity of *Botrytis cinerea* was confirmed by Kenneth Curry (University of Southern Mississippi, Hattiesburg, MS). The three *Colletotrichum* species and *P. obscurans* were isolated from strawberry (*Fragaria* × *ananassa* Duchesne), while *P. viticola* and *Botrytis cinerea* were isolated from commercial grape (*Vitis vinifera* L.) and *F. 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 $\mu$mol/m$^2$/s) with a 12 h photoperiod.

**Table 2.** Concentrations of Test Compounds Causing 50% Growth Reduction ($IC_{50}$) Determined from Dose−Response Studies Using a 96 Well, Liquid Bioassay

| | | $IC_{50}$ ($\mu$M) | | | |
|---|---|---|---|---|---|
| organism | time point (h) | compound **1** | compound **2** | compound **3** | captan |
| *B. cinerea* | 48 | >333 | 120 | 90 | 1.4 |
| | 72 | >333 | >333 | >333 | 2.8 |
| *C. acutatum* | 48 | >333 | >333 | >333 | 2.5 |
| | 72 | >333 | >333 | >333 | 5.2 |
| *C. fragariae* | 48 | >333 | 160 | 91 | 1.6 |
| | 72 | >333 | 270 | >333 | 3.0 |
| *C. gloeosporioides* | 48 | >333 | >333 | >333 | 2.1 |
| | 72 | >333 | >333 | >333 | 4.7 |
| *F. oxysporum* | 48 | >333 | >333 | >333 | 2.8 |
| | 72 | >333 | >333 | >333 | 16 |
| *P. obscurans* | 120 | 10 | 4.5 | 23 | 1.1 |
| | 144 | 6.0 | 10 | 26 | 2.0 |
| *P. viticola* | 120 | >333 | 115 | 110 | <1.0 |
| | 144 | 100 | 38 | 65 | <1.0 |

**Conidia Preparation.** Conidia were harvested from 7−10 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 plastic rod. Aqueous conidial suspensions were filtered through sterile Miracloth (Calbiochem-Novabiochem Corp., La Jolla, CA) to remove mycelia. Conidia concentrations were determined photometrically (*11, 12*) from a standard curve based on absorbance at 625 nm, and suspensions were adjusted with sterile distilled water to a concentration of $1.0 \times 10^6$ 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 for microdilution broth experiments were evaluated using a Packard model SpectraCount microplate photometer (Packard Instrument Co., Meriden, CT).

**Direct Bioautography.** A number of bioautography techniques were used as primary screening systems to detect antifungal activity. Matrix, one-dimensional (1D) protocols on silica gel thin-layer chromatography (TLC) plates along with *Colletotrichum* spp. as the test organisms were used to identify the antifungal activity according to published methods (*13, 14*). Matrix bioautography was used to screen large numbers of crude extract at 80 $\mu$g/spot. One-dimensional TLC was 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 4 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. Fungal growth inhibition was evaluated 4−5 days after treatment by measuring zone diameters. Antifungal metabolites were readily located on the plates by visually observing clear zones where the active compounds inhibited fungal growth (*15*).

**Microdilution Broth Assay.** 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 (*12*). This 96 well microdilution assay was used to determine and compare the sensitivity of fungal plant pathogens to natural and synthetic compounds with known fungicidal standards (*16*).

A 96 well microdilution 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 isolated. The fungicide captan was used as a positive standard in all assays. Azoxystrobin and benomyl were used in some in addition to captan in some studies. Each fungal species was initially challenged in microdilution broth assay using three-point dose−response format so that the final test concentrations of 45, 90, and 135 $\mu$M were achieved (in duplicate) in the different columns of the 96 well plate. Subsequently, six-point formats with the final test compound concentrations of 1, 3.3, 10, 33, 100, and 333 $\mu$M were used to establish $IC_{50}$ values. Experiments were performed twice, and the mean values were pooled for all experiments. Linear growth phases were utilized for computing activities of both the standard positive control, captan, and the test compounds; all solvents were tested for their effects on fungal growth previously when the linear phase of growth for each isolate was determined (*12*).

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 were also recorded at 120 and 144 h. Mean absorbance values and standard errors were determined and represented graphically, and the graphs were used to evaluate fungal growth inhibition. Differences in spore germination and mycelial growth in each of the wells in the 96 well plate demonstrated sensitivity to particular concentrations of pure compounds and indicated fungistatic or fungicidal effects.

## RESULTS AND DISCUSSION

An ethyl acetate extract of the fruiting bodies of *G. floccosus* contained sufficient quantities of crude extract for chemical investigations. Spots of 10 and 80 $\mu$g generated clear zones of fungal growth inhibition using bioautography against *C. fragariae* and diffuse zones against both *C. acutatum* and *C. gloeosporioides* (**Table 1**). Diffuse inhibitory zones are regions on the bioautography plate where fungal growth is visually reduced and interspersed with few mycelia and reproductive structures and are often characteristic of fungistatic compounds. These results indicated potential "selective" activity of *G. floccosus* extract to *C. fragariae*.

The ethyl acetate extract was partially dissolved in methanol/water (90/10) and extracted thrice using hexane followed by three extractions using chloroform and three with ethyl acetate. Biological evaluation of the partitions using bioautography against *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* was conducted. The chloroform extract had the highest activity against all three species. This chloroform extract was further purified using semipreparative reversed phase HPLC utilizing an acetonitrile/water gradient providing three compounds **1**, **2**, and **3** (**Figure 1**).

Negative ion high-resolution LC-MS indicated that compound **1** gave a parent molecular ion of *m/z* 295.2296 corresponding to $[M - H]^-$ and suggesting a possible molecular formula for **1** of $C_{18}H_{32}O_3$ and three sites of unsaturation. $^1$H NMR analysis indicated the presence of four olefinic protons, one oxygenated methine, a methyl triplet, and several aliphatic methylene protons. $^{13}$C and DEPT NMR analysis indicated the presence of one carbonyl, four olefinic carbons, one hydroxylated carbon, one methyl, and several methylene carbons.

The above data indicated that **1** was possibly a hydroxylated octodecadienoic acid isomer, and reaction with diazomethane and conversion to its corresponding trimethylsilyl ether provided a compound that gave a parent molecular ion of *m/z* 382 when analyzed by GC-MS. The above reactions confirmed the presence of both carboxylic acid and hydroxyl functional groups and inspection of the literature, and comparison of $^1$H NMR data with that previously reported (*9*) indicated complete agreement providing structural confirmation of **1** as (9*S*,10*E*,12*Z*)-9-hydroxy-10,12-octadecadienoic acid. Stereochemistry at C-9 was established by comparison of optical rotation data with that which had been previously reported for **1** (*23*). We report here for the first time $^{13}$C NMR data in $CDCl_3$ for **1** along with carbon assignment data established by inspection of $^1$H−$^1$H correlation spectroscopy (COSY), heteronuclear single quantum coherence (HSQC), and heteronuclear multiple-bond correlation (HMBC) spectral data.

Negative ion high-resolution LC-MS analysis of compound **2** gave a parent molecular ion of *m/z* 293.2128 corresponding to $[M - H]^-$ and suggesting a possible molecular formula for **2** of $C_{18}H_{30}O_3$ and four sites of unsaturation. $^1$H NMR analysis indicated the presence of four olefinic protons, a methyl triplet, and several aliphatic methylene protons. $^{13}$C and DEPT NMR analysis indicated the presence of two carbonyls one being a ketone, four olefinic carbons, one methyl, and several methylene carbons. The above data indicated **2** was likely an oxo-octodecadienoic acid isomer, and inspection of the literature and comparison of $^1$H and $^{13}$C NMR data with that previously reported (*11, 17*) indicated complete agreement providing structural confirmation of **2** as (9*E*,11*Z*)-13-oxo-9,11-octadecadienoic acid.

Negative ion high-resolution LC-MS of compound **3** gave a parent molecular ion of *m/z* 293.2130 corresponding to $[M - H]^-$ and suggesting a possible molecular formula for **3** of $C_{18}H_{30}O_3$ and four sites of unsaturation. $^1$H NMR analysis indicated the presence of four olefinic protons, a methyl triplet, and several aliphatic methylene protons. $^{13}$C and DEPT NMR

analysis indicated the presence of two carbonyls one being a ketone, four olefinic carbons, one methyl, and several methylene carbons. The above data indicated that **3** was also an oxo-octodecadienoic acid isomer. Inspection of the literature and comparison of mass spectrometry fragmentation data and $^{1}H$ and $^{13}C$ NMR data with that previously reported (*18*) indicated complete agreement providing structural confirmation of **3** as (10*E*,12*E*)-9-oxo-10,12-octadecadienoic acid.

The activities of the three compounds were evaluated individually in a 96 well microdilution format against the three *Colletotrichum* species used in the bioautography trials, as well as *F. oxysporum*, *B. cinerea*, *P. obscurans*, and *P. viticola.* Bioautographical data were collected after 4 days of growth, and microtiter data were collected at 24, 48, 72, 120, and 144 h, depending on the growth curves for each organism. The most sensitive species were those in the genus *Phomopsis*, with *P. obscurans* being most susceptible.

Bioautography of compound **1** demonstrated clear zones of antifungal activity against all three *Colletotrichum* species (**Table 1**). In the microtiter plate bioassays, compound **1** inhibited growth of *C. acutatum* 79% at a dose of 90 $\mu$M after 48 h (**Figure 2**). This activity dropped to 37% after 72 h. Doses lower and higher than this had significant activity, which also decreased over time. Stimulation of growth at a subtoxic concentration (hormesis) is common with fungicides (e.g., *19*). In fact, the term hormesis was coined in a study with a natural product fungicide (*20*). Against the other two species of *Colletotrichum* in the microtiter format, compound **1** stimulated growth at doses up to 100 $\mu$M and had no effect at 333 $\mu$M (see the Supporting Information). Compound **1** also showed hormetic activity against *F. oxysporum*, although stimulation was highest at the highest dose (333 $\mu$M) tested in this case (see the Supporting Information). Concentrations higher than this were not tested, since they would be unrealistic for potential practical use. Against *P. obscurans*, compound **1** caused increasing inhibition with increasing dose at 144 h (**Figure 3**). Against *P. viticola* at 144 h of treatment, doses of 1−10 $\mu$M were hormetic, and those of 33−333 $\mu$M were increasingly inhibitory with concentation (**Figure 4**).

Compounds **2** and **3** appeared slightly more active in the microtiter environment than compound **1** (**Figures 2–4**). However, they had similar activity to **1** in the bioautography assay (**Table 1**). Lower doses of both compound **2** and **3** caused stimulation of *P. viticola* (**Figure 4**), *C. gloeosporioides*, and *F. oxysporum* growth (see the Supporting Information).

The antifungal activity of the three oxylipins described in this study is of sufficient magnitude on some of the test pathogens to warrant further study. Data obtained from six-point microdilution broth assays indicated that oxylipins are selective and more active against *Phomopis* species and less active against the other test fungi by a large margin (**Table 2**). Growth inhibitory concentrations ($IC_{50}$ values) of **2** ($IC_{50}$ = 4.5 $\mu$M) against *P. obscurans* at 120 h were lower than for compounds **1** ($IC_{50}$ = 10 $\mu$M) or **3** ($IC_{50}$ = 23 $\mu$M) in comparison to captan standard ($IC_{50}$ = 1.1 $\mu$M). $IC_{50}$ values indicated that *Phomopis viticola* was much less sensitive to the oxylipins than *P. obscurans.* $IC_{50}$ values for **2** ($IC_{50}$ = 38 $\mu$M) and **3** ($IC_{50}$ = 65 $\mu$M) indicated that compounds **2** and **3** were more against *P. viticola* at 144 h than compound **1** ($IC_{50}$ = 100 $\mu$M) in comparison to the captan standard ($IC_{50}$ = <1.0 $\mu$M).

In a study of the antimicrobial activity of oxylipins, Prost et al. (*21*) evaluated compounds **1**−**3** at 100 $\mu$M against various bacterial and fungal species. They found **1** and **2** to have little or no activity against seven bacterial plant pathogens and **3** to be mildly active to three of the test species. In their study, **1** and **3** had good activity against *Phytophthora parasitica* and *Cladosprium herbarum*, with little or no activity against *B. cinerea*, *Phytophthora infestans*, *F. oxysprorum*, *Rhizopus* spp., or *Alternaria brassicicola*. Although these workers used only one dose, making it difficult to compare results, their findings do not conflict with ours. Their conclusions were that this family of compounds can contribute to plant protection not only by the induction of defensive responses to plant pathogens but also by direct antimicrobial activity in some cases.

In the present study, we used a bioassay-guided approach to isolate active antifungal metabolites from the basidiomycete *G. floccosus*, confirming the presence of some oxylipins in the sporocarp of this basidiomycete. Oxylipins are known to be important in fungal life cycle processes, including basidiomycetes (e.g., *22*). Our study demonstrated that *Phomopsis* species are most sensitive to compounds **1**−**3**, with other fungi showing considerably less sensitivity. Our results suggest that the oxylipins might deserve further testing for activity as plant protectants to control *Phomopsis* species, which cause small fruit blight, leaf spot, and rot. Because oxylipins are quite common as phytochemicals in the food supply, naturally occurring ones might have reduced regulatory requirements for approval.

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**Supporting Information Available:** Figures of growth inhibition/stimulation and six-point inhibition curves. This material is available free of charge via the Internet at http://pubs.acs.org.

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