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ARTICLE

New Class of Algicidal Compounds and Fungicidal Activities Derived from a Chromene Amide of *Amyris texana*

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A chromene amide, *N*-[2-(2,2-dimethyl-2*H*-1-benzopyran-6-yl)ethyl]-*N*,3-dimethylbutanamide, was isolated from the EtOAc extract of the leaves of *Amyris texana* and found to have moderate antifungal activity against *Colletotrichum* spp. and selective algicidal activity against *Planktothrix perornata*, a cyanobacterium (blue-green alga) that causes musty off-flavor in farm-raised channel catfish (*Ictalurus punctatus*). To improve the selective algicidal activity and provide water solubility, a series of chromene analogues were synthesized and evaluated for algicidal activity using a 96-well microplate rapid bioassay. In addition, the chromene analogues were evaluated for antifungal and phytotoxic activities. Hydrochloride salts of a chromene amine analogue showed improved water solubility and selectivity toward *P. perornata* with activity comparable to that of the naturally occurring chromene amide.

KEYWORDS: Chromene amide; chromenes; algicide; fungicide

INTRODUCTION

Off-flavor problems cause significant economic losses to catfish farmers in the United States. *Planktothrix perornata* f. *attenuata* [Skuja], a cyanobacterium (blue-green alga) common in catfish production ponds in the southeastern United States, produces the monoterpene 2-methylisoborneol (MIB), which is absorbed into catfish flesh and imparts a musty off-flavor, rendering them unpalatable and unmarketable. Algicides such as copper sulfate and diuron [*N*′-(3,4-dichlorophenyl)-*N*,*N*-dimethylurea] that are currently used to control *P. perornata* have broad-spectrum toxicity toward other beneficial phytoplankton, such as the green alga *Selenastrum capricornutum*, as well as low biodegradability. Therefore, there is an urgent need to find algicides with high selectivity, minimum toxicities toward nontarget organisms, and biodegradability to control *P. perornata* in catfish production ponds.

Amyris texana P. Wilson (Rutaceae), also known as "Texas torchwood", is native to Texas and Mexico, distributed mainly in the arid regions in the southwestern United States. As part of our continuing efforts to search for natural product-based agrochemicals, the leaves of *A. texana* were extracted and investigated for algicidal, antifungal, and phytotoxic constituents.

MATERIALS AND METHODS

General Experimental Procedures. Extracts were analyzed on silica gel TLC plates GF with fluorescent indicator (250 μm, Analtech, Newark, DE). Iodine vapor, UV light (at 254 and 365 nm), and Dragendorff and anisaldehyde spray reagents were used for the detection of compounds. Column chromatography was carried out with kieselgel 60 (particle size = 0.063−0.2 mm, Merck) with mixtures of hexane and acetone in various amounts. All solvents were of reagent grade and used without further purification. ^{1}H and ^{13}C NMR spectra were recorded either on a Bruker AMX NMR spectrometer (Palo Alto, CA) operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR or on a Varian Mercury AS400 spectrometer operating at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. The HR-ESIMS was measured by using either a Bruker QTOF micromass spectrometer or a JEOL AccuTOF (JMS-T100LC) (Peabody, MA) mass spectrometer. GC-MS analysis was carried out on an HP5790 MSD spectrometer (Hewlett-Packard) equipped with a GC 5890 using a DB-1 column (20 m × 0.2 mm, 0.18 μm film thickness). The oven was temperature programmed from 60 °C (5 min) to 280 °C (20 min) at 5 °C/min with helium as the carrier gas.

Plant Material. Leaves of *A. texana* were collected in Cameron County in southern Texas, in June 2002, by Dr. Charles Burandt at the University of Mississippi. A voucher specimen (BUR 190204 a) is deposited at the University of Mississippi herbarium. The leaves were air-dried, ground, and stored at room temperature until they were extracted.

Extraction and Isolation of the Bioactive Constituents. Air-dried and ground *A. texana* leaves (500 g) were extracted repeatedly at room temperature (25−28 °C) with ethyl acetate (2 L × 3) by stirring with a magnetic stirrer. The resulting extracts were filtered through filter paper (Whatman no. 1) and combined, and the solvent was evaporated at 40 °C under reduced pressure to afford a dark green residue (21.6 g). This residue (20.0 g) was subjected to column chromatography on a silica gel column (3.8 cm × 81 cm) eluting with hexane and then with increasing amounts of acetone (up to 100%). Fractions of 400 mL were collected, and the elution profile of the column was monitored by TLC plates sprayed with anisaldehyde and Dragendroff spray reagents and by I_2 vapor. The fractions with similar TLC profiles were combined to yield 34 fractions. Each fraction was tested for the presence of antifungal constituents by TLC bioautography by spraying with *Colletotrichum fragariae* spores (*1*)

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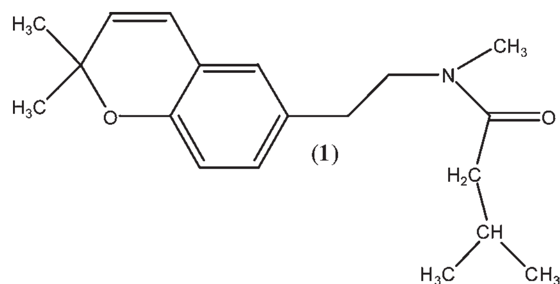

Figure 1. Structure of the natural chromene amide *N*-[2-(2,2-dimethyl-2*H*-chromen-6-yl)ethyl]-3,*N*-dimethylbutyramide.

and algicidal constituents using a rapid bioassay according to the published methods (*2*).

Chromene Amide *N*-[2-(2,2-Dimethyl-2*H*-chromen-6-yl)ethyl]-3,*N*-dimethylbutyramide (1). The pale yellow oily mass in fraction 23 was further purified by silica column chromatography with 10% EtOAc in hexane to yield an oil. The structure was confirmed as **1** (**Figure 1**), a chromene amide by comparison of ^{1}H and ^{13}C spectroscopic data with those reported in the literature (*3*). The chromene amide was also tested for algicidal and phytotoxic activity in addition to fungicidal activity.

Syntheses of Analogues. *[4-(1,1-Dimethyl-prop-2-ynyloxy)phenyl]-acetic Acid Methyl Ester* (**2**). Methyl-4-hydroxyphenylacetate (16.6 g, 100 mmol) was heated under reflux with KI (24.4 g, 140 mmol), anhydrous K_2CO_3 (24.4 g, 170 mmol), and 3-chloro-3-methyl-1-butyne (25 g, 240 mmol) in dry acetone (122 mL) under N_2 for 48 h. The mixture was allowed to cool to room temperature and filtered, and the residue was washed with acetone. The combined acetone solution was evaporated to afford a gum, which was dissolved in diethyl ether (200 mL) and partitioned between 1 M aqueous NaOH (300 mL × 2). The ether layer was dried over anhydrous $MgSO_4$ and evaporated to afford a pale yellow oil (yield, 11.5 g, 49 mmol, 49.5%): ^{1}H NMR ($CDCl_3$) δ 1.65 (6H, s), 2.58 (1H, s), 3.58 (2H, s), 3.69 (3 H, s), 7.17 (2H, d, J = 8.4 Hz), 7.20 (2H, d, J = 8.4 Hz); ^{13}C NMR ($CDCl_3$) δ 29.6, 40.4, 51.9, 72.3, 72.9, 86.1, 121.4, 128.3, 129.7, 154.7, 172.1; HRMS(ESI-TOF) m/z 233.11889 $[M + H]^+$ (calcd for $C_{14}H_{17}O_3$, 233.11777).

(2,2-Dimethyl-2H-chromen-6-yl)acetic Acid Methyl Ester (**3**). **2** (11 g, 47 mmol) and *N,N*-diethylaniline (40 mL) were refluxed under N_2 with stirring (210−220 °C) for 75 min. The reaction mixture was allowed to cool to room temperature and diluted with diethyl ether (300 mL). The ether solution was washed with 6 M aqueous HCl (300 mL × 2), followed by saturated aqueous NaCl, and dried over anhydrous Na_2SO_4. Ether was removed under reduced pressure to afford a pale yellow oil, which was purified by silica gel column chromatography to afford **3** (yield, 10.5 g, 45 mmol, 95.7%): ^{1}H NMR ($CDCl_3$) δ 1.41 (6H, s), 3.50 (2 H, s), 3.68 (3H, s), 5.59 (1H, d, J = 10.0 Hz), 6.28 (1H, d, J = 9.6 Hz), 6.73 (1H, d, J = 8.4 Hz), 6.88 (1H), 6.99 (1H, dd, J = 8.0, 1.6 Hz); HRMS(ESI-TOF) m/z 233.1175 $[M + H]^+$ (calcd for $C_{14}H_{17}O_3$, 233.1178).

2-(2,2-Dimethyl-2H-chromen-6-yl)ethanol (**4**). **3** (10 g, 43 mmol) and $LiAlH_4$ (3.8 g, 100 mmol) in dry diethyl ether (100 mL) were refluxed under N_2 for 30 min. Excess $LiAlH_4$ was destroyed by adding ethyl acetate (20 mL). The reaction mixture was cooled in ice, and 20% NaOH (5 mL) was added with stirring. The ether layer was decanted, the residue was washed with diethyl ether (2 × 100 mL), and the combined ether layer was dried over anhydrous $MgSO_4$ and then evaporated under reduced pressure to afford a colorless oil (yield, 8.5 g, 0.04 mol, 93%). The identity of the compound was established by comparison of ^{1}H and ^{13}C NMR with those reported in the literature (*4*).

6-(2-Bromoethyl)-2,2-dimethyl-2H-chromene (**5**). To a magnetically stirred solution of **4** (2.04 g, 10 mmol) and CBr_4 (4.15 g, 10 mmol) in CH_2Cl_2 (15 mL) at 0 °C was added Ph_3P (3.93 g, 15 mmol), and the mixture was stirred for 30 min. Diethyl ether (25 mL) was added to the mixture, and the resulting precipitate was removed by filtration. The filtrate was evaporated, and the product, **5**, was purified by silica gel chromatography eluting with 8% ethyl acetate in hexane (yield, 2.1 g, 7 mmol, 78%): ^{1}H NMR ($CDCl_3$) δ 1.42 (6H, s), 3.05 (2 H, t, J = 7.6 Hz), 3.55 (2H, t, J = 7.6 Hz), 5.61 (1H, d, J = 9.6 Hz), 6.28 (1H, d, J = 10 Hz), 6.72 (1H, d, J = 8.0 Hz), 6.81 (1H, s), 6.96(1H, d, J = 8.0 Hz); HRMS(ESI-TOF) m/z 267.03848 $[M + H]^+$ (calcd for $C_{13}H_{16}$ BrO, 267.038451).

[2-(2,2-Dimethyl-2H-chromen-6-yl)ethyl]isopropylamine Hydrochloride (**6**). Isopropylamine (5 g, 80 mmol) and **5** (1 g, 30 mmol) were reacted in a closed tube at 80 °C for 1 h. The reaction mixture was cooled, and excess amine was removed by flushing with N_2 gas. The solid obtained was partitioned between ethyl acetate (50 mL) and 5% aqueous NaOH (50 mL). The organic layer was washed with water and saturated NaCl and dried over anhydrous Na_2SO_4 to afford a gum. This gum was dissolved in ethanol (10 mL), treated with concentrated HCl (1 mL), diluted with diethyl ether (20 mL), and then left to stand for 12 h at 4 °C. The white crystals obtained were filtered to obtain **6** (yield, 700 mg, 20 mmol, 66%): ^{1}H NMR ($CDCl_3$) δ 1.34 (6H, s), 1.44 (3H, d, J = 6.4 Hz), 3.04 (2 H, m), 3.17 (2H, m), 3.31 (1H, septet, J = 6.4 Hz) 5.52 (1H, d, J = 10.0 Hz), 6.16 (1H, d, J = 10.0 Hz), 6.61 (1H, d, J = 8.0 Hz), 6.8 (1H, d, J = 2.0 Hz), 6.91 (1H, dd, J = 6.4, 2.0 Hz); ^{13}C NMR ($CDCl_3$) δ 19.1, 27.9, 31.6, 46.4, 50,5, 76.1, 116.4, 121.4, 121.9, 126.5, 128.8, 129.1, 131.0, 151.9; HRMS(ESI-TOF) m/z 246.1847 $[M + H]^+$ (calcd for $C_{16}H_{24}ON$, 246.1857).

[2-(2,2-Dimethyl-2H-chromen-6-yl)ethyl]methylamine Hydrochloride (**7**). In a closed tube, **5** (600 mg, 2.2 mmol) was reacted with a 2 M solution of methylamine in THF (5 mL) in a procedure similar to that for **6** to afford the amine as an oil after the workup of the reaction. The oil was dissolved in methanol (7 mL) and treated with concentrated HCl (0.7 mL) and then allowed to stand overnight at 4 °C to afford white crystals of the hydrochloride salt **7** (yield, 420 mg, 1.9 mmol, 87%): ^{1}H NMR ($CDCl_3$) δ 1.24 (6H, s), 2.58 (3H, s), 2.99 (4H, br m), 5.43 (1H, d, J = 9.6 Hz), 6.09 (1 H, d, J = 10.0 Hz), 6.53 (1H, d, J = 8.0 Hz), 6.72 (1H, br s), 6.82 (1H, br d, J = 6.8 Hz), 9.35 (1H, br s); ^{13}C NMR ($CDCl_3$) δ 27.8, 31.4, 33.0, 50.7, 76.1, 116.5, 121.4, 121.9, 126.4, 128.2, 129.0, 131.0, 151.8; HRMS(ESI-TOF) m/z 218.154372 $[M + H]^+$ (calcd for $C_{14}H_{20}ON$, 218.154489).

Compounds **8**, **9**, and **10** were synthesized according to the published methods and characterized by spectroscopic data (*5*).

6-Bromomethyl-2,2-dimethyl-2H-chromene (***11***). **10** (1.89 g, 9.9 mmol) was reacted in a similar procedure as for the synthesis of **5** with CBr_4 (12.1 g, 36 mmol) and Ph_3P (3.8 g, 14 mmol) in CH_2Cl_2 (15 mL). Ether (30 mL) was added to the mixture and filtered. The filtrate was evaporated, and the product was purified by silica gel column chromatography eluting with 8% EtOAc in hexane to afford **11** (1.1 g, 4.3 mmol, 44%) as a pale yellow oil, which decomposed readily upon exposure to air. ^{1}H NMR ($CDCl_3$) δ 1.39 (6H, s), 3.74 (2H, s), 5.55 (1H, d, J = 10.0 Hz), 6.23 (1H, d, J = 10.0 Hz), 6.66 (1H, d, J = 8.4 Hz), 6.74 (1H, br s), 6.88 (1H, d, J = 8.4 Hz); HRMS(ESI-TOF) m/z 252.01489 $[M + H]^+$ (calcd for $C_{12}H_{14}OBr$, 252.014976).

(2,2-Dimethyl-2H-chromen-6-ylmethyl)isopropylamine (**12**). **11** (600 mg, 2.3 mmol) was treated with isopropylamine (5 mL) in a closed container and heated at 80 °C for 1 h. Excess amine was evaporated with N_2, and the solid was partitioned between aqueous 5% NaOH and EtOAc. The EtOAc layer was washed with H_2O and saturated NaCl, dried over anhydrous Na_2SO_4, and evaporated to obtain a gum as the free amine: ^{1}H NMR ($CDCl_3$) δ 1.08 (6H, d, J = 6.0 Hz), 1.40 (6H, s), 2.84 (1H, septet, J = 6.4 Hz), 3.65 (2H, s), 5.58 (1H, d, J = 10.4 Hz), 6.29 (1 H, d, J = 10.0 Hz), 6.70 (1H, d, J = 8.0 Hz), 6.92 (1H, d, J = 1.2 Hz), 7.02 (1H, br d, J = 8.0 Hz).

The free amine was treated with concentrated HCl (1 mL), diluted with ether (15 mL), and left for 12 h at 4 °C. The solid was filtered and dried to yield **12** (420 mg, 1.5 mmol, 68%) as white needle-like crystals: HRMS-(ESI-TOF) m/z 232.1702 $[M + H]^+$ (calcd for $C_{15}H_{22}ON$, 232.1701).

Compounds **13** and **14** were synthesized according to the published methods and characterized by spectroscopic data (*5*).

3-(1,1-Dimethylprop-2-ynyloxy)benzoic Acid Methyl Ester (**15**). Methyl 3-hydroxybenzoate (3.5 g, 25 mmol) was heated under reflux with KI (6.0 g), anhydrous K_2CO_3 (6.0 g), and 3-chloro-3-methyl-1-butyne (6.8 mL, 60.5 mmol) in dry acetone (300 mL) under N_2 for 48 h. The mixture was allowed to cool to room temperature and filtered, the residue was washed with acetone, and the solvent was evaporated to yield a semisolid. This was partitioned between 5% NaOH (100 mL) and ether (100 mL × 2). The ether layer was dried with anhydrous Na_2SO_4 and evaporated to dryness to yield **15** as pale yellow oil, which crystallized to yield needle-like crystals (3.8 g, 14 mmol, 40%): ^{1}H NMR ($CDCl_3$) δ 1.64 (6H, s), 2.59 (1H, s), 3.88 (3H, s), 7.30−7.41 (2H, m), 7.71 (1H, d, J = 7.6 Hz), 7.86 (1H, s); ^{13}C NMR ($CDCl_3$) δ 29.7, 52.3, 73.0, 74.7, 122.6, 124.2, 129.1, 131.3, 155.8, 167.0; HRMS(ESI-TOF) m/z 219.10223 $[M+H]^+$ (calcd for $C_{13}H_{15}O_3$, 219.10212).

2,2-Dimethyl-2H-chromene-5-carboxylic Acid Methyl Ester (***16***) *and 2,2-Dimethyl-2H-chromene-7-carboxylic Acid Methyl Ester* (***17***). **15** (3.2 g, 14.6 mmol) was refluxed with *N*,*N*-diethylaniline (15 mL). After 1 h, the reaction was allowed to cool to room temperature, diluted with ether (75 mL), washed with 6 M HCl (4 × 30 mL) and saturated NaCl, and dried over anhydrous Na_2SO_4. The solvent was removed, and the products were separated by silica gel column chromatography using 90% hexane in toluene to yield **16** as the major product (1.2 g, 5.5 mmol, 37%) and elution with 80% hexane in toluene to yield **17** as the minor product (800 mg, 3.6 mmol, 24%). **16**: ^{1}H NMR ($CDCl_3$) δ 1.41 (6H, s), 3.86 (3 H, s), 5.73 (1H, d, J = 10.0 Hz), 6.94 (1H, d, J = 8.0 Hz), 6.10 (1H, t, J = 8.0 Hz), 7.24 (1H, d, J = 10.0 Hz), 7.44 (1H, d, J = 7.6 Hz); HRMS(ESI-TOF) m/z 219.1037 [M + H]$^+$ (calcd for $C_{13}H_{15}O_3$, 219.10213).

17: ^{1}H NMR ($CDCl_3$) δ 1.41 (6H, s), 3.85 (3 H, s), 5.70 (1H, d, J = 10.0 Hz), 6.32 (1H, d, J = 10.0 Hz), 6.99 (1H, t, J = 7.6 Hz), 7.42 (1H, s), 7.50 (1H, d, J = 7.6 Hz); HRMS(ESI-TOF) m/z 219.1032 [M + H]$^+$ (calcd for $C_{13}H_{15}O_3$, 219.10213).

(2,2-Dimethyl-2H-chromen-5-yl)methanol (***18***). **16** (300 mg, 1.3 mmol) was dissolved in dry ether (50 mL) and refluxed with $LiAlH_4$ (80 mg, 2.1 mmol) for 1 h. Excess $LiAH_4$ was destroyed with EtOAc, treated with NaOH (20%, 1 mL), and stirred, and the organic layer was decanted. The residue was washed with ether (50 mL), and the combined ether layer was dried over anhydrous Na_2SO_4 and evaporated. The product was purified by silica gel column chromatography eluting with 10% EtOAc in hexane to yield **18** (220 mg, 1.1 mmol) as a colorless, viscous oil: ^{1}H NMR ($CDCl_3$) δ 1.43 (6H, s), 3.20 (1H, br s), 4.58 (2H, s), 5.66 (1H, d, J = 10.0 Hz), 6.56 (1H, d, J = 10 Hz), 6.75 (1H, d, J = 8.0 Hz), 6.83 (1H, d, J = 7.2 Hz), 7.06 (1H, t, J = 8.0 Hz); ^{13}C NMR ($CDCl_3$) δ 27.8, 62.3, 75.5, 116.4, 118.75, 119.3, 120.7, 128.7, 131.2, 136.1, 153.0.

(2,2-Dimethyl-2H-chromen-7-yl)methanol (***19***). **17** (250 mg, 1.1 mmol) was reacted with $LiAlH_4$ (80 mg, 2 mmol) in a similar manner as above to yield **19** (180 mg, 0.94 mmol) as a colorless, viscous oil: ^{1}H NMR ($CDCl_3$) δ 1.41 (6H, s), 3.0 (1H, br s), 4.50 (2H, s), 5.67 (1H, d, J = 10.0 Hz), 6.29 (1H, d, J = 10 Hz), 6.75 (1H, s), 6.78 (1H, d, J = 8.4 Hz), 6.91 (1H, t, J = 7.6 Hz); ^{13}C NMR ($CDCl_3$) δ 27.9, 64.7, 76.2, 114.8, 119.2, 120.4, 120.0, 126.3, 130.5, 142.3, 152.9.

3-(4-Hydroxyphenyl)propionic Acid Methyl Ester (***20***). A solution of 4-hydroxybenzenepropanoic acid (5 g, 30 mmol) in dry MeOH (25 mL) was treated with concentrated H_2SO_4 (0.25 mL) and refluxed for 24 h. The solvent was removed under vacuum, and the oil was partitioned with EtOAc and aqueous $NaHCO_3$. The organic layer was washed with water and saturated NaCl, dried over $MgSO_4$, and evaporated to yield **20** (4.2 g, 23 mmol, 92%) as a white solid: ^{1}H NMR ($CDCl_3$) δ 2.61 (2H, t, J=7.6 Hz), 2.87 (2H, t, J = 8 Hz), 3.67 (3H, s), 6.71 (1H, s), 6.76 (2H, d, J=7.6 Hz), 7.02 (2H, d, J = 8 Hz).

3-[4-(1,1-Dimethylprop-2-ynyloxy)phenyl]propionic Acid Methyl Ester (***21***). **20** (4.1 g, 22.8 mmol) was heated under reflux with KI (6.0 g), anhydrous K_2CO_3 (6.0 g), and 3-chloro-3-methyl-1-butyne (6.8 mL, 60.5 mmol) in dry acetone (30 mL) under N_2 for 48 h. The reaction mixture was filtered, and the solvent was evaporated to yield a gum. The gum was dissolved in ether (100 mL) and washed with 1 M NaOH (30 mL). The ether layer was dried over anhydrous $MgSO_4$ and evaporated to yield **21** (4.2 g, 17 mmol) as a pale yellow oil, which soon crystallized upon cooling to needle-like crystals: ^{1}H NMR ($CDCl_3$) δ 1.59 (6H, s), 2.54 (1H, s), 2.58 (2H, t, J = 7.6 Hz), 2.87 (2H, t, J = 7.6 Hz), 3.61 (3H, s), 7.05−7.11 (4H, m); ^{13}C NMR ($CDCl_3$) δ 29.8, 30.4, 36.0, 51.7, 72.5, 74.12, 86.4, 121.8, 128.9, 135.2, 154.1, 173.5; HRMS(ESI-TOF) m/z 247.133531, [M + H]$^+$ (calcd for $C_{15}H_{19}O_3$, 247.13342).

3-(2,2-Dimethyl-2H-chromen-6-yl)propionic Acid Methyl Ester (***22***). **21** (4.0 g, 16.3 mmol) was refluxed with *N*,*N*-diethylaniline (20 mL) under N_2 for 90 min. The reaction mixture was cooled, acidified with 5% HCl, and extracted with EtOAc. The organic layer was washed with water, dried over anhydrous $MgSO_4$, and evaporated to yield a pale yellow oil. The product was purified by silica gel column chromatography using 5% EtOAc in hexane to afford (**22**) (3.3 g, 13.4 mmol): ^{1}H NMR ($CDCl_3$) δ 1.40 (6H, s), 2.57 (2H, t, J = 7.6 Hz), 2.83 (2H, t, J = 7.6 Hz), 3.65 (3H, s), 5.58 (1H, d, J = 9.6 Hz), 6.27 (1H, d, J = 9.6 Hz), 6.68 (1H, d, J = 8.0 Hz), 6.79 (1H, d, J = 1.6 Hz), 6.91 (1H dd, J = 6.9, 1.6 Hz); ^{13}C NMR ($CDCl_3$) δ 28.1, 30.3, 36.1, 51.7, 76.2, 116.4, 121.4, 122.5, 126.3, 129.0, 131.0, 132.8, 151.6, 173.4; HRMS(ESI-TOF) m/z 269.11113 [M + Na]$^+$ (calcd for $C_{15}H_{18}NaO_3$, 269.11536).

3-(2,2-Dimethyl-2H-chromen-6-yl)propan-1-ol (***23***). **22** (2.8 g, 11 mmol) was refluxed with $LiAlH_4$ (600 mg, 16 mmol) in dry diethyl ether (100 mL) for 3 h. Excess $LiAlH_4$ was destroyed with EtOAc, and the reaction mixture was treated with concentrated NaOH (5 mL) and stirred for 30 min. The ether layer was decanted, and the residue was washed with ether. The combined ether layer was dried over $MgSO_4$ and evaporated to yield **23** (2.3 g, 10 mmol, 90%) as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.41 (6H, s), 1.84 (2H, multiplet) 2.59 (2H, t, J=8 Hz), 3.64 (2H, t, J= 6 Hz), 5.58 (1H, d, J=9.6 Hz), 6.27 (1H, d, J=9.6 Hz), 6.70 (1H, d, J= 8.0 Hz), 6.80 (1H, d, J=1.6 Hz), 6.93 (1H dd, J=6.9, 1.6 Hz).

[4-(1,1-Dimethylprop-2-ynyloxy)-3-methoxyphenyl]acetic Acid Ethyl Ester (***24***). Ethyl 4-hydroxy-3-methoxyphenylacetate (2 g, 9.52 mmol) was heated under reflux with KI (2.4 g), anhydrous K_2CO_3 (2.4 g), and 3-chloro-3-methyl-1-butyne (2.6 mL, 24 mmol) in dry acetone (12 mL) under N_2 for 48 h. The mixture was allowed to cool to room temperature and filtered, and the residue was washed with acetone. The combined acetone solution was evaporated to afford a gum, which was dissolved in diethyl ether (100 mL) and partitioned between 1 M aqueous NaOH (100 mL × 2). The ether layer was dried over anhydrous $MgSO_4$ and evaporated to afford **24** as a pale yellow solid (1.15 g, 4.1 mmol, 43%): ^{1}H NMR ($CDCl_3$) δ 1.21 (3H, t, J = 7.2 Hz) 1.60 (6H, s), 2.49 (1H, s), 3.51 (2H, s), 3.75 (3H, s), 4.10 (2H, q, J = 7.2 Hz), 6.74 (1H, d, J = 8.8 Hz), 6.80 (1H, s), 7.30 (1H, d, J = 8.8 Hz); ^{13}C NMR ($CDCl_3$) δ 14.4, 29.5, 41.3, 55.9, 61.0, 73.6, 74.1, 86.6, 113.4, 121.3, 130.1, 143.9, 153.0, 171.8; HRMS(ESI-TOF) m/z 277.14184, [M + H]$^+$ (calcd for $C_{16}H_{21}O_4$, 277.14398).

(8-Methoxy-2,2-dimethyl-2H-chromen-6-yl)acetic Acid Ethyl Ester (***25***). **24** (1 g, 3.6 mmol) was refluxed with *N*,*N*-diethylaniline (5 mL) under N_2 for 90 min. The reaction was cooled to room temperature, acidified with aqueous HCl, and extracted with EtOAc (100 mL × 2). The EtOAc layer was washed with water, dried over anhydrous $MgSO_4$, and evaporated. The product was purified by silica gel column chromatography using 5% EtOAc in hexane to yield **25** (730 mg, 2.6 mmol, 73%): ^{1}H NMR ($CDCl_3$) δ 1.25 (3H, t, J = 7.2 Hz), 1.45 (6H, s), 3.48 (2H, s), 3.84 (3H, s), 4.14 (2H, q, J = 7.2 Hz), 5.58 (1H, d, J = 9.6 Hz), 6.26 (1H, d, J = 9.6 Hz), 6.33 (1H, s), 6.69 (1H, s); HRMS(ESI-TOF) m/z 277.14384, [M + H]$^+$ (calcd for $C_{16}H_{21}O_4$, 277.14398).

2-(8-Methoxy-2,2-dimethyl-2H-chromen-6-yl)ethanol (***26***). **25** (700 mg, 2.7 mmol) was refluxed with $LiAlH_4$ (200 mg, 5 mmol) in dry ether (10 mL) for 1 h. Excess $LiAlH_4$ was destroyed with EtOAc, and the reaction mixture was treated with concentrated NaOH (2 mL) and stirred for 30 min. The ether layer was decanted, and the residue was washed with ether. The combined ether layer was dried over $MgSO_4$ and evaporated to yield **26** (590 mg, 2.5 mmol, 93%) as a pale yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.32 (6H, s), 2.62 (2H, t, J = 6.4 Hz), 3.64 (2H, t, J = 6.4 Hz), 3.70 (3H, s), 5.48 (1H, d, J = 8.4 Hz), 6.14 (1H, d, J = 8.4 Hz), 6.37 (1H, s), 6.52 (1H, s); ^{13}C NMR ($CDCl_3$) δ 27.7, 38.7, 56.3, 63.4, 76.5, 113.4, 119.3, 122.0, 122.4, 130.9, 131.2, 140.4, 148.0.

6-(2-Bromoethyl)-8-methoxy-2,2-dimethyl-2H-chromene (***27***). **26** (480 mg, 2.1 mM) was treated with CBr_4 (850 mg, 2.56 mM) and Ph_3P (870 mg, 3.1 mM) in dry CH_2Cl_2 (8 mL) at 0 °C under N_2. After 30 min, solvent was evaporated, and the residue was applied to a silica gel column and eluted with 2.5% EtOAc in hexane to yield **27** (480 mg, 1.6 mmol, 77%) as a yellow oil: ^{1}H NMR ($CDCl_3$) δ 1.48 (6H, s), 3.07 (2H, t, J = 7.6 Hz), 3.54 (2 H, t, J = 7.6 Hz), 3.80 (3H, s), 5.63 (1H, d, J = 10.0 Hz), 6.29 (1H, d, J=9.6 Hz), 6.49 (1H, d, J=1.6 Hz), 6.63 (1H, d, J=1.6 Hz); HRMS(ESI-TOF) m/z 297.04516, [M + H]$^+$ (calcd for $C_{14}H_{18}BrO_2$, 297.04902).

Isopropyl[2-(8-methoxy-2,2-dimethyl-2H-chromen-6-yl)ethyl]amine Hydrochloride (***28***). **27** (308 mg, 1.04 mmol) was refluxed with isopropylamine (2 mL) in CH_3CN (8 mL) for 3 h. The solvent was evaporated, and the crystalline solid was partitioned between dilute HCl and ether. The acidic layer was basified with NaOH and extracted with ether (50 mL × 2). The organic layer was washed with water, dried over $NaSO_4$, and evaporated to yield the free amine (250 mg) as a gum. This gum was dissolved in ethanol (10 mL) and treated with concentrated HCl (0.5 mL). The solvent was evaporated under vacuum, and the crystalline product was recrystallized from ethanol and ether to give **28** as a white crystalline solid (220 mg, 70 mmol, 69%): ^{1}H NMR ($CDCl_3$) δ 1.44 (6H, s), 1.50 (6H, d, J = 6.4 Hz), 3.10 (2H, br m), 3.26 (2H, br m), 3.49 (1H, septet, J=5.2 Hz), 5.60 (1H, d, J=10.0 Hz),

6.21 (1H, d, $J = 9.6$ Hz), 6.51 (1H, s), 6.64 (1H, s), 9.67 (1H br s); HRMS-(ESI-TOF) *m*/*z* 276.20056, $[M + H]^+$ (calcd for $C_{17}H_{26}O_2N$, 276.19635).

6-(3-Bromopropyl)-2,2-dimethyl-2H-chromene (***29***). Compound **23** (1.2 g, 5.5 mmol) was treated with CBr_4 (2.28 g, 6.88 mM) and PPh_3 (2.17 g, 8.26 mM) in dry CH_2Cl_2 (10 mL) at 0 °C under N_2. After 30 min, solvent was evaporated, and the residue was applied to a silica gel column and eluted with 2.5% EtOAc in hexane to yield **29** as a yellow oil (1.1 g, 3.9 mmol, 70%): ^1H NMR ($CDCl_3$) δ 1.43 (6H, s), 2.14 (2H, quintet, $J = 6.8$ Hz), 2.69 (2H, t, $J = 7.2$ Hz), 3.40 (2H, t, $J = 6.4$ Hz), 5.63 (1H, d, $J = 9.6$ Hz), 6.31 (1H, d, $J = 10$ Hz), 6.72 (1H, d, $J = 8$ Hz) 6.82 (1H, d, $J = 1.6$ Hz), 6.95 (1H, dd, $J = 6.4$, 1.6 Hz); ^{13}C NMR ($CDCl_3$) δ 28.0, 33.1, 33.2, 34.3, 76.1, 116.2, 121.2, 122.3, 126.3, 129.0, 131.0, 132.6, 151.3; HRMS-(ESI-TOF) *m*/*z* 281.05452 $[M + H]^+$ (calcd for $C_{14}H_{18}OBr$, 281.05410).

[3-(2,2-Dimethyl-2H-chromen-6-yl)propyl]isopropylamine Hydrochloride (***30***). **29** (500 mg, 1.7 mmol) was refluxed with isopropylamine (2 mL, 23 mmol) and CH_3CN (8 mL) for 3 h. Excess amine was evaporated under reduced pressure, and the residue was partitioned between aqueous 5% NaOH and EtOAc. The EtOAc layer was washed with H_2O and saturated NaCl, dried over anhydrous Na_2SO_4, and evaporated to obtain a gum as the free amine. The free amine was isolated by silica gel column chromatography using 30% EtOAc in hexane as a viscous oil (270 mg, 61%): ^1H NMR ($CDCl_3$) δ 1.04 (6H, d, $J = 6$ Hz), 1.42 (6H, s), 1.76 (2H, quintet, $J = 7.2$ Hz), 2.55 (2H, t, $J = 8.0$ Hz), 2.61 (2H, t, $J = 7.6$ Hz), 2.74−2.80 (1H, m) 5.58 (1H, d, $J = 10.0$ Hz), 6.28 (1H, d, $J = 10.0$ Hz), 6.69 (1H, d, $J = 8.4$ Hz), 6.79 (1H, d, $J = 1.6$ Hz), 6.91 (1H, dd, $J = 8.0$, 1.6 Hz); ^{13}C NMR ($CDCl_3$) δ 23.1, 27.9, 32.1, 32.9, 74.1, 48.7, 75.9, 116.0, 121.0, 122.4, 126.1, 128.8, 130.7, 134.3, 150.9; HRMS(ESI-TOF) *m*/*z* 260.201426 $[M + H]^+$ (calcd for $C_{17}H_{25}NO$, 260.20143). The free amine (250 mg) was dissolved in ethanol (2 mL), treated with concentrated HCl (1 mL), diluted with diethyl ether (5 mL), and kept for 12 h at 4 °C. The hydrochloride salt of the amine **30** was crystallized as white needle-like crystals (220 mg).

2-(2,2-Dimethyl-2H-chromen-6-yl)ethylamine (***31***). **5** (3 g, 11.24 mM) was reacted with NaN_3 (0.952 g, 14 mmol) in anhydrous DMF (40 mL) under N_2 at room temperature for 3 h. The reaction mixture was added to water (100 mL), extracted with diethyl ether (250 mL), and evaporated to dryness to obtain the azide. This azide was dissolved in absolute ethanol (20 mL) and hydrogenated with Pd/C (10%) at 10 psi for 4 h. The mixture was filtered through Celite, and the solvent was evaporated to give a viscous oil. **31** was obtained as a viscous oil after silica gel column chromatography using 30% EtOAc in hexane as the solvent (1.9 g, 83%): ^1H NMR ($CDCl_3$) δ 1.42 (6H, s), 1.78 (2H, quintet, $J = 7.6$ Hz), 2.64 (2H, t, $J = 7.6$ Hz), 2.71 (2H, t, $J = 7.6$ Hz), 5.63 (1H, d, $J = 10.0$ Hz), 6.31 (1H, d, $J = 10.0$ Hz), 6.71 (1H, d, $J = 8.4$ Hz), 6.80 (1H, d, $J = 1.6$ Hz), 6.93 (1H, dd, $J = 8.0$, 1.6 Hz).

The free amine was dissolved in ethanol (10 mL) and treated with concentrated HCl (1 mL), diluted with ether (25 mL), and left at 4 °C for 48 h to yield the hydrochloride salt as a white powder.

Bioassay for Evaluating Algicidal Activity. Algicidal activities of the compounds were tested in 96-well microplates in a dose−response format according to the previously published methods (*2*). The lowest observed effect concentration (LOEC), the lowest complete inhibition concentration (LCIC), and the 50% inhibition concentration (IC_{50}) were determined by graphing the absorbance data. Whereas previous studies have used the traditional approach designation of *Oscillatoria perornata* f. *attenuata* [Skuja], we used the modern approach designation of *P. perornata* f. *attenuata* [Skuja] in the current study.

Bioassay for Fungicidal Activity against Plant Pathogenic Fungi. Bioautography on silica gel TLC plates was used to detect the presence of antifungal constituents in the extracts, column fractions, and purified compounds and synthesized analogues according to published methods (*6*). To evaluate the quantitative fungicidal activity, the active compounds identified by bioautography were evaluated in a dose−response format in the 96-well microbioassay using modifications of published methods (*6*, *7*). Chromene derivatives were evaluated at three concentrations (1.0, 10.0, and 100.0 μM) and compared to the commercial fungicide captan for activity against *Colletotrichum fragariae*, *Colletotrichum gloeosporioides*, *Colletotrichum acutatum*, *Botrytis cinerea*, and *Fusarium oxysporium*.

Bioassay for Phytotoxic Activity. The compounds were evaluated for phytotoxicity on a monocot and a dicot using lettuce (*Lactuca sativa* cv. Iceberg) and bent grass (*Agrostis stolonifera* cv. Pencross) according to published methods (*8*). Phytotoxic activities were ranked on a scale from 1 to 5 visually, with 1 representing no inhibition and 5 representing complete inhibition of germination. The compounds were further evaluated for activity on plant cell integrity by determination of electrolyte leakage from cucumber (*Cucumis sativus*) cotyledon disks according to a published method (*9*).

RESULTS AND DISCUSSION

Preliminary bioautography of the ethyl acetate extract of the leave of *A. texana* indicated the presence of antifungal compounds against *C. fragariae*. Bioassay-guided fractionation of the extract led to the isolation of the chromene amide **1** (**Figure 1**) as the major antifungal constituent. Algicidal assay of this compound showed selective activity toward *P. perornata*, with an LCIC of 10.0 μM and an IC_{50} of 2.3 μM compared to those for *S. capricornutum* with an LCIC of 100.0 μM and an IC_{50} of 12.6 μM. However, the low water solubility and low activity of **1** preclude further research including efficacy studies as a useful agent to control *P. perornata* in catfish production ponds. To identify the structural features responsible for the algicidal activity of this type of compound and also to develop water-soluble analogues, a series of chromene derivatives based on the natural chromene amide were synthesized and evaluated (**Figure 2**).

Acid hydrolysis of the parent amide is expected to yield an amine, which could then be converted to an acid salt to yield a water-soluble analogue. Due to limited availability of the parent compound, a synthetic procedure was developed to synthesize these analogues. The general synthetic procedure is shown in **Figure 3**. Thus, the corresponding methyl or ethyl esters of phenolic acids were reacted with 3-chloro-3-methyl-1-butyne to obtain the propynyloxy ether, which was subsequently cyclized to obtain the corresponding chromene derivative (**Figure 3**). The reduction of ester yielded the corresponding alcohol, which was converted to its bromide. Treatment of the bromide with amines yielded chromene amines, which were crystallized as hydrochloride salts.

The treatment of bromide **5** with methylamine yielded the amine analogue **7**. The hydrochloride salt of this amine gave a crystalline water-soluble compound. This compound showed improved activity toward *P. perornata* (LOEC = 1.0 μM) but showed reduced selectivity based upon LCIC values when compared to *S. capricornutum* (**Table 1**). The chromene analogues **3** and **4** did not show toxicity toward *P. perornata*. These results indicated that the amide in the side chain is not a prerequisite for algicidal activity of the chromenes, but the amine group is important for activity. A previous study with anthraquinone analogues showed that the amine groups on the side chain can have a great influence on the selective algicidal activity of the analogues (*10*). In this study, the isopropylamine analogue yielded the optimum activity and selectivity toward *P. perornata*. Treatment of bromide **5** with isopropylamine followed by treatment with HCl yielded the corresponding amine analogue **6**. The hydrochloride salt **6** showed enhanced selectivity toward *P. perornata* compared to *S. capricornutum*, but no improvement on the activity compared to the parent amide **1** (**Table 1**). The primary amine analogue **31** of this compound was prepared by treatment of bromide **5** with sodium azide and subsequent reduction with H_2 and Pd/C. This amine showed improved toxicity toward both *P. perornata* and *S. capricornutum*, but with reduced selectivity.

To study the influence of the length of the side chain on the activity, isopropylamine analogues of chromenes with one-carbon (**12**) and three-carbon (**30**) side chains were prepared. Hydrochloride salts of compounds **30** and **12** showed equal or less activity, respectively, compared to the two-carbon chain analogue **6**, and both compounds lacked selectivity.

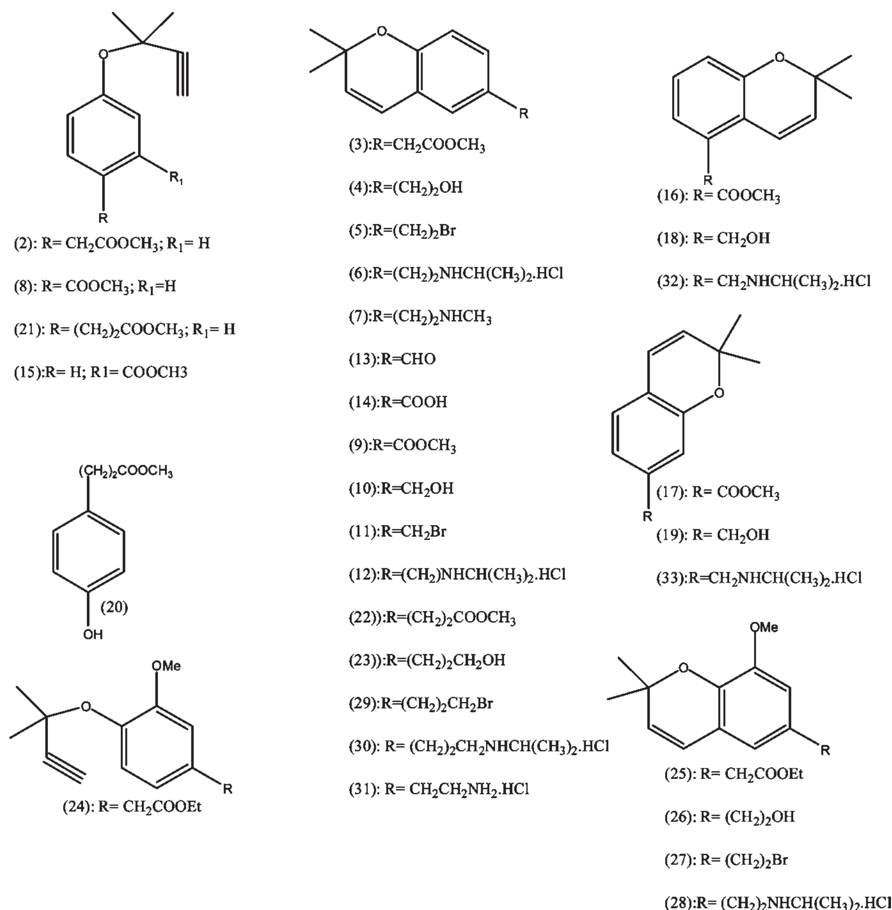

Figure 2. Structures of chromene derivatives.

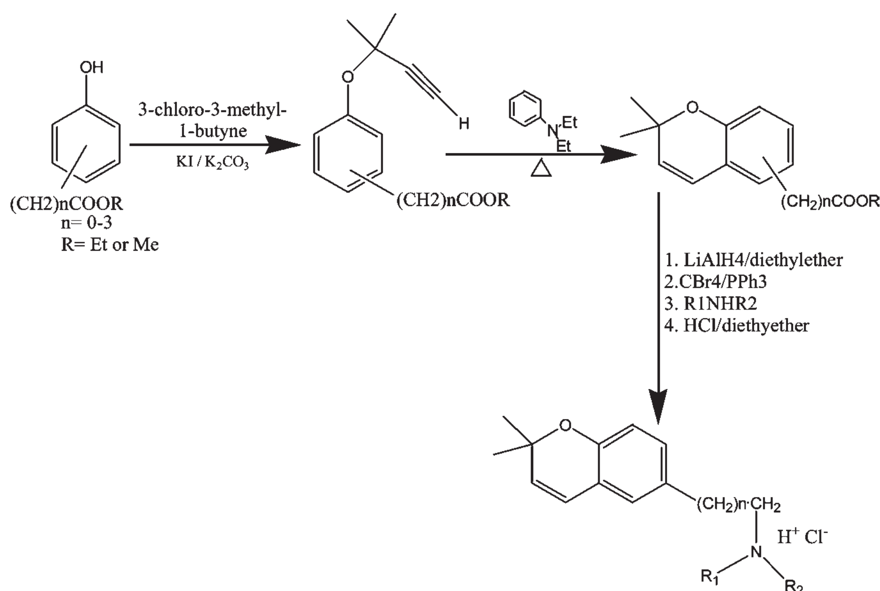

Figure 3. General synthetic procedure for water-soluble chromene derivatives.

To investigate the influence of functional groups on the chromene ring on algal toxicity, the methoxy analogue **28** was prepared and tested for algicidal activity. The hydrochloride salt of this amine had lower activity compared to that of the demethoxy analogue **6** and lacked selectivity. The halogenated chromene ring analogues were not prepared as the intention of this work was to find compounds very close to natural products in nature. To investigate the influence of the position of the side chain on the chromene ring on algicidal activity, analogues **32** and **33** were prepared and assayed. Cyclization of propynyloxy ether **15** yielded a mixture of chromene analogues, which were separated by column chromatography and converted to the corresponding isopropylamine analogues **32** and **33**. Algicide bioassay results of these compounds indicate that toxicity is enhanced when the side chain is at the 7-position compared to when the side chain is at the 5-position; however, selective toxicity is reduced (**Table 1**).

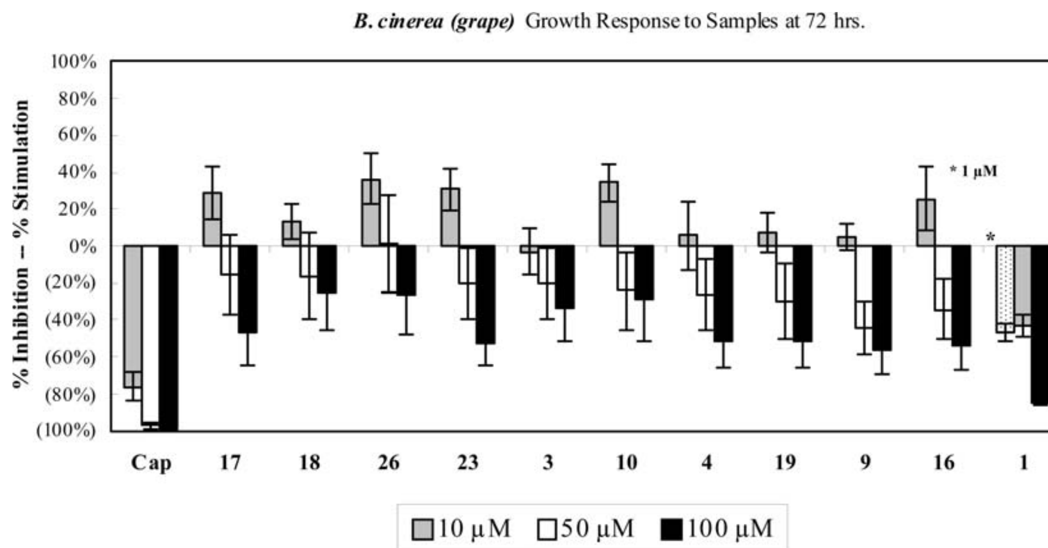

Figure 4. Fungal growth inhibition of *Botrytis cinerea* at 72 h against chromene derivatives. Captan was used as the positive control.

Table 1. Algicide Activity of Chromene Derivatives

	test organism			
	Planktothrix perornata		*Selenastrum capricornutum*	
test compound	LOEC[a] (μM)	LCIC[b] (μM)	LOEC (μM)	LCIC (μM)
chromene amide **1**	10	10	10	100
3	>100	>100	>100	>100
4	>1000	>1000	>1000	>1000
6	10	10	10	1000
7	1.0	10	10	10
12	10	100	10	100
28	10	100	10	100
30	10	10	10	10
31	10	10	10	10
32	10	100	10	10
33	1	10	1	1

[a] LOEC, lowest observed effect concentration. [b] LCIC, lowest complete inhibition concentration.

These results suggest that a two-carbon side chain is necessary for highest algicidal activity among these analogues. Extending the carbon chain of **6** by one carbon as in **30** did not improve the algicidal activity. For the amine group, the isopropylamine moiety as in **6**, **30**, and the methylamine moiety as in **7** had the same level of activity as the natural product **1**. The analogue **28** with a methoxy group in the aromatic ring was less active. Therefore, we can conclude that a two-carbon side chain with an isopropylamine moiety had the highest selective algicidal activity among the chromene derivatives that we tested against *P. perornata* when compared to results for *S. capricornutum*.

It has been reported that **4**, a compound isolated from *Eutypa lata*, which is the fungus responsible for dying-arm disease in grapevines, and the corresponding aldehyde of **4** are phytotoxic (*5*). However, in the bioassays carried out in our laboratory following published procedures (*8*), none of our chromene analogues showed any phytotoxicity at the concentrations that we tested (0.1−1000 μM). We have also performed assays on effects on cellular integrity with cucumber cotyledon disks for **4**, **6**, and **10** and found no phytotoxicity (data not shown).

TLC bioautography of the chromene analogues when tested against *C. fragariae* indicated that only the analogues that had CH_2OH and ester moieties in the side chain were significantly antifungal. Interestingly, none of the amine derivatives showed fungicidal activity on TLC bioautography. Therefore, the structural requirements for algicidal and fungicidal activities should be different. The analogues that showed significant antifungal activity were further evaluated for fungicidal activity by microbioassay in a dose−response manner according to the published methods (*6*, *7*, *11*). Captan was used as the positive standard, and the fungal strains used were *C. fragariae*, *C. acutatum*, *C. gloeosporioides*, *B. cinerea*, and *F. oxysporum*. The data for *B. cinerea* are shown in **Figure 4**. According to the microbioassay, *B. cinerea* was the most sensitive to the chromenes among the fungal strains that we tested. The natural chromene amide was the most active against *B. cinerea*, with 80% inhibition at 100.0 μM. Among the synthetic chromene analogues, **17**, **23**, **4**, **19**, **9**, and **16** at 100.0 μM showed >50% growth inhibition of *B. cinerea* at 72 h after treatment. Both **3**, an ester, and **10**, an alcohol with one CH_2 group in the side chain at the 6-position of the aromatic ring, showed about 30% growth inhibition at 72 h after treatment on the same fungal strain. Compound **4**, having a chain with two CH_2 groups at C-7, showed higher activity than **23** with three CH_2 groups at C-7. Between the isomers **18** and **19**, where the only difference is the position of the CH_2OH moiety, the chromene analogue **19** was more active. Therefore, the side chain at the 7-position of the chromene ring shows better antifungal activity. None of our chromene analogues showed better or comparable activity to that of captan, a widely used commercial fungicide. The synthetic chromenes did not show significantly higher activity than the natural product **1**. Thus, a different strategy is needed to develop more potent fungicides that can compete with the commercial fungicides that are currently available.

Chromenes occur in many plants belonging to diverse families (*12*−*17*). These compounds exhibit a diverse array of biological activities such as antifeedent, fungicidal, phytotoxic, cytotoxic, larvicidal, and phytotoxic properties. Plants in general produce secondary metabolites as part of their defense mechanism. Hence, we can postulate that the naturally occurring chromene amide **1** as a natural fungicide produced by *A. texana* acts as a defense compound. Despite the numerous biological activities reported for chromenes, algicidal activity has not previously been reported in the literature. This study is the first describing the algicidal activity of chromenes.

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