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Phytotoxic Eremophilanes from *Ligularia macrophylla*

CHARLES L. CANTRELL,*,† STEPHEN O. DUKE,† FRANK R. FRONCZEK,‡ WESTE L. A. OSBRINK,§ LEONID K. MAMONOV,# JURIY I. VASSILYEV,# DAVID E. WEDGE,† AND FRANCK E. DAYAN†

USDA-ARS, Natural Products Utilization Research Unit, University, Mississippi 38677, Department of Chemistry, Louisiana State University, Baton Rouge, Louisiana 70803, USDA-ARS, Formosan Subterranean Termite Research Unit, New Orleans, Louisiana 70124, and Institute of Plant Biology and Biotechnology, Timiriazeva, 45, Almaty, 05040, Republic of Kazakhstan

Systematic bioassay-guided fractionation of the methylene chloride extract of the roots from *Ligularia macrophylla* was performed to identify both phytotoxic and antifungal compounds. Four phytotoxic eremophilanes (furanoeremophilan-14β,6α-olide, 6β-angeloyloxy-10β-hydroxyfuranoeremophilane, eremophil-7(11)-ene-12,8α;14β,6α-diolide, and 3α-angeloyloxybakkenolide A) and two antifungal fatty acids (linoleic acid and α-linolenic acid) were isolated. The X-ray crystal structure determination of 6β-angeloyloxy-10β-hydroxyfuranoeremophilane is reported here for the first time. All four eremophilanes substantially inhibited growth of the monocot *Agrostis stolonifera* (bentgrass) while demonstrating little activity against the dicot *Lactuca sativa* (lettuce) at 1000 μM. In a dose–response screening of all compounds for growth inhibitory activity against *Lemna paucicostata*, 6β-angeloyloxy-10β-hydroxyfuranoeremophilane was the most active with an IC_{50} of 2.94 $\pm$ 0.16 μM. This compound also caused the greatest reduction of photosynthetic electron flow; however, its mode of action remains to be determined. Evaluation of isolated compounds for activity against the Formosan subterranean termite, *Coptotermes formosanus*, is also reported. At a concentration of 0.5% (wt/wt), 6β-angeloyloxy-10β-hydroxyfuranoeremophilane significantly reduced the consumption of filter paper by *C. formosanus*.

KEYWORDS: Eremophilane; terpenoid; sesquiterpene; fatty acid; phytotoxicity; antifungal; herbicidal; *Ligularia macrophylla*; Formosan subterranean termite

INTRODUCTION

Natural products are a source of compounds that might be used directly as pesticides or as a template starting point for new synthetic pesticides (*1*). Examples of recent successful commercial pesticides discovered from natural products include the spinosad and avermectin insecticides, the strobilurin fungicides, and the triketone herbicides. Much of the natural product literature describes the isolation and identification of novel secondary products, with less research effort given to determine the biological activities of these compounds. Advances in chemical ecology have made it clear that most secondary compounds are the result of coevolution of the producing organism and those organisms with which it must coexist. In many cases, their purpose in nature is a defensive one, especially in plants which have few options other than chemical ones for avoiding and combating pests. Thus, evaluation of plants for pesticidal compounds is a highly viable strategy for pesticide discovery.

In a research program aimed at identifying natural insecticides, fungicides, and herbicides, over 340 different crude plant extracts representing 57 species of plants endemic to the Republic of Kazakhstan have been evaluated. Based on these preliminary screening results, extracts from a high altitude species, *Ligularia macrophylla* (Ledeb.) DC (Asteraceae Dumont.), were chosen for further investigation. In this paper we systematically examine *L. macrophylla* for insecticidal, fungicidal, and herbicidal activity. Many studies have been done on this species and other species of this genus from a phytochemical standpoint; however, evaluation of the biological activities of compounds from this species is sparse. We are aware of few studies for pesticide discovery from this genus except for two studies that examined *Ligularia* species for insecticidal activity (*2*, *3*) and one patent on herbicidal properties of *L. stenocephala* compounds (*4*). Of particular interest from the present study was the herbicidal activity of the eremophilanes.

* To whom correspondence should be addressed. Phone: +1-662-915-5898. Fax: +1-662-915-1035. E-mail: clcantr1@olemiss.edu.
† Natural Products Utilization Research Unit.
‡ Louisiana State University.
§ Formosan Subterranean Termite Research Unit.
# Institute of Plant Biology and Biotechnology.

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MATERIALS AND METHODS

Instrumentation. H- and ^{13}C NMR spectra were recorded in $CDCl_3$or C_6D_6 on a Varian ANOVA 400 MHz spectrometer (Palo Alto, CA). 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, Virginia) 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 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.

High-Resolution LC-MS Analysis. All isolated compounds were prepared in MeOH and injected directly into a 0.3 mL/min stream of MeOH. Twenty μL of sample (approximately 0.1 mg/mL) was injected manually at 0.5 min while mass drift compensation standards (*L*-tryptophan (negative ion), PEG (positive ion)) were injected at 1.5 min over the course of a 2 min run.

Raw Material. *Ligularia macrophylla* (Ledeb.) DC (Asteraceae Dumont.) plant raw material was collected on June 29, 2005 in Kaskelen canyon (Republic of Kazakhstan). Accompanying species were *Origanum vulgare* L., *Hypericum perforatum* L., *Dactylis glomerata* L., *Aconitum leucostomum* Worosch., *Artemisia scoparia* Waldst. et Kit., and *Trifolium repens* L. A voucher specimen number for 9412/25–1964 for *L. macrophylla* (Ledeb.) DC (Asteraceae Dumort.) has been deposited in the Institute of Botany and Phytointroduction Herbarium, Almaty, Republic of Kazakhstan.

Plant Extraction. Fresh roots (1.6 kg), aerial parts (3.8 kg), and inflorescences (3.2 kg) were air-dried providing 520, 880, and 910 g of material, respectively. After grinding in a Willey-Mill plant grinder, 300 g of each plant part was soaked in their respective solvents providing root CH_2Cl_2 (24.8 g), 95% ethanol (10.3 g), and H_2O (80.8 g) extractables, aerial parts CH_2Cl_2 (10.4 g), 95% ethanol (14.0 g), and H_2O (83.3 g) extractables, and inflorescence CH_2Cl_2 (8.44 g), 95% ethanol (11.5 g), and H_2O (73.4 g) extractables.

Phytotoxicity-Guided Fractionation. Initially, 4.5 g of CH_2Cl_2 extractables from the roots were separated on a Biotage 40+M column (40–63 μm, 60 Å, 40 × 150 mm) running at 32 mL/min using a hexane:EtOAc step gradient beginning with 100:0 to 90:10 over 800 mL followed by 90:10 to 75:25 over 800 mL and finishing with 75:25 to 0:100 over 800 mL. Twenty-four mL fractions were collected and recombined based on TLC similarities into eight distinct fractions. Fraction 4 crystallized while drying providing 337 mg of pure compound **2**, whereas fraction 6 provided 584 mg of pure compound **1**. Fraction 7 (993 mg) was further purified using a Biotage 40+M column (40–63 μm, 60 Å, 40 × 150 mm) running at 32 mL/min using a hexane:EtOAc step gradient beginning with 85:15 to 75:25 over 800 mL followed by 75:25 to 50:50 over 2000 mL and finishing with 50:50 to 0:100 over 800 mL and a 400 mL MeOH wash. Twenty-four mL portions were collected and recombined based on TLC similarities into nine subfractions. Subfraction 7–8 (132 mg) was further subjected to reversed phase C-18 HPLC (Zorbax, 9.4 × 250 mm, 5 μm) running a linear gradient from 70:30 (H_2O:acetonitrile) to 0/100 (H_2O:acetonitrile) over 30 min while monitoring at 210 nm. This resulted in the isolation of 12 mg of compound **3**.

Three and a half g of CH_2Cl_2 extractables from the aerial parts were separated on a Biotage 40+M column (40–63 μm, 60 Å, 40 × 150 mm) as described above for the aerial parts. Twenty-four mL fractions were collected and recombined based on TLC similarities into 10 distinct fractions. Fraction 5 (186 mg) was subjected to reversed phase C-18 HPLC (Zorbax-SB, 21.2 × 250 mm, 5 μm) running a linear gradient from 50:50 (H_2O:acetonitrile) to 0/100 (H_2O:acetonitrile) over 15 min while monitoring at 210 nm. This resulted in the isolation of 29 mg of compound **4**.

Antifungal Bioautography-Guided Fractionation. Two and one-half g of CH_2Cl_2 extractables from the inflorescences were separated on a Biotage 40+M column (40–63 μm, 60 Å, 40 × 150 mm) as described above for the aerial parts. Twenty-four mL fractions were collected and recombined based on TLC similarities into 11 distinct fractions. Fraction 10 (469 mg) was subjected to reversed phase C-18 HPLC (Zorbax-SB, 9.4 × 250 mm, 5 μm) running an isocratic system of 15:85 (H_2O:acetonitrile) over 30 min while monitoring at 210 nm. This resulted in the isolation of 8 mg of compound **6** and 14 mg of **5**.

*Furanoeremophilan-14β,6α-olide (**1**).* High resolution ESI-MS *m/z* 269.1137 $[M + Na]^+$, calculated for $C_{15}H_{18}NaO_3$, 269.1154; *m/z* 515.2341 $[2\ M + Na]^+$, calculated for $C_{30}H_{36}NaO_6$, 515.2410; *m/z* 761.3632 $[3\ M + Na]^+$, calculated for $C_{45}H_{54}NaO_9$, 761.3666; ^1H NMR (400 MHz in $CDCl_3$) in complete agreement with published values (*5*); ^{13}C NMR (100 MHz in $CDCl_3$) δ 177.0 s, 151.1 s, 138.8 d, 120.3 s, 114.9 s, 81.9 d, 41.7 s, 41.6 d, 37.2 d, 25.5 t, 23.4 t, 20.7 t, 20.3 q, 19.0 t, 8.5 q.

*6β-Angeloyloxy-10β-Hydroxyfuranoeremophilane (**2**).* High resolution ESI-MS *m/z* 355.1952 $[M + Na]^+$, calculated for $C_{20}H_{28}NaO_4$, 355.1885; *m/z* 687.4068 $[2\ M + Na]^+$, calculated for $C_{40}H_{56}NaO_8$, 687.3873. ^1H NMR (400 MHz in $CDCl_3$), δ 7.08 (s, 1H), 6.26 (s, 1H), 6.01 (m, 1H), 3.28 (br s, 1H), 3.18 (d, 1H, $J = 17.6$), 2.63 (d, 1H, $J = 17.6$), 1.92 (d, 3H, $J = 7.0$), 1.90 (s, 3H), 1.81 (s, 3H), 1.03 (s, 3H), 0.87 (d, 3H, $J = 7.0$). ^{13}C NMR (100 MHz in $CDCl_3$) δ 167.02 s, 151.5 s, 139.0 d, 138.7 d, 127.6 s, 119.5 s, 115.4 s, 75.2 s, 70.2 d, 45.6 s, 34.1 d, 33.8 t, 33.2 t, 29.3 t, 22.5 t, 20.7 q, 16.2 q, 15.8 q, 10.2 q, 8.4 q.

*Eremophil-7(11)-ene-12,8α; 14β,6α-Diolide (**3**).* High resolution ESI-MS *m/z* 285.1114 $[M + Na]^+$, calculated for $C_{15}H_{18}NaO_4$, 285.1103; *m/z* 547.2306 $[2\ M + Na]^+$, calculated for $C_{30}H_{36}NaO_8$, 547.2307. ^1H NMR (400 MHz in $CDCl_3$) data in complete agreement with published values (*6*); ^{13}C NMR (100 MHz in $CDCl_3$) data in complete agreement with published values (*7*).

*3α-Angeloyloxybakkenolide A (**4**).* High resolution ESI-MS *m/z* 333.20922 $[M + H]^+$, calculated for $C_{20}H_{29}O_4$, 333.2066; *m/z* 355.1914 $[M + Na]^+$, calculated for $C_{20}H_{28}NaO_4$, 355.1885; *m/z* 687.3872 $[2\ M + Na]^+$, calculated for $C_{40}H_{56}NaO_8$,687.3873. ^1H NMR (400 MHz in $CDCl_3$) and ^{13}C NMR data in complete agreement with published values (*8*).

*Linoleic Acid (**5**).* High resolution ESI-MS *m/z* 279.2354 $[M - H]^-$, calculated for $C_{18}H_{31}O_2$, 279.2324; *m/z* 559.4700 $[2\ M - H]^-$, calculated for $C_{36}H_{63}O_4$, 559.4726. ^{13}C NMR data in complete agreement with published values (*9*).

α-*Linolenic Acid (**6**).* High resolution ESI-MS *m/z* 277.2167 $[M - H]^-$, calculated for $C_{18}H_{29}O_2$, *m/z* 277.2168; *m/z* 555.4371 $[2\ M - H]^-$, calculated for $C_{36}H_{59}O_4$, 555.4413. ^{13}C NMR data in complete agreement with published values (*10*).

X-ray Experimental for Compound 2. The crystal structure of **2** was determined using data collected at $T = 115K$ with Mo Kα radiation on a Nonius KappaCCD diffractometer. Crystal data: $C_{20}H_{28}O_4$, triclinic space group P1, $a = 7.5304(15)$, $b = 7.793(2)$, $c = 8.782(2)$Å, $\alpha = 65.936(11)$, $\beta = 67.821(14)$, $\gamma = 77.035(15)°$, $V = 434.24(17)$ Å^3, $Z = 1$, $R = 0.040$ (2296 data with $F^2 > 2\sigma$), $R_w = 0.103$ (all F^2) for 2467 unique data having $\theta < 30.8°$ and 226 refined parameters. Crystallographic data have been deposited with the Cambridge Crystallographic Data Centre and allocated the deposition number CCDC 657198. Copies of the data can be obtained, free of charge, on application to the Director, CCDC, 12 Union Road, Cambridge CB21EZ, UK (fax: +44-(0)1223–336033 or e-mail: deposit@ccdc.cam.ac.uk).

Bioassays Against *L. sativa* and *A. stolonifera*. Silica gel column chromatographic fractionation of the CH_2Cl_2 extract of the leaves and roots guided by *Lactuca sativa* and *Agrostis stolonifera* bioassays according to Dayan et al. (*11*) were used to identify and isolate the phytotoxic fractions. A filter paper (Whatman no. 1) and 5 mg of *L. sativa* seeds or 10 mg of *A. stolonifera* seeds were placed in each well of a 24-well multiwell plates (Corning Inc., Corning, NY). Test fractions were dissolved in acetone and mixed with distilled–deionized (DDI) H_2O such that the final concentration of acetone was 3%. To each test well, 250 μL of the DDI H_2O mixture was added. Only acetone and DDI H_2O were added to each control well. Plates were covered, sealed with Parafilm, and incubated at 26 °C in a Conviron growth chamber at 173 μmol/m^2/sec continuous light intensity. Phytotoxicity was qualitatively evaluated by visually comparing the amount of germination

of the seeds in each well with the untreated controls after 7 days for *L. sativa* and after 12 days for *A. stolonifera*. The qualitative estimate of phytotoxicity was evaluated by using a rating scale of 0–5, where 0 = no effect and 5 = no growth or no germination of the seeds. Each experiment was repeated in triplicate.

Bioassays Against *Lemna paucicostata*. *L. paucicostata* stocks were grown from a single colony consisting of a mother and two daughter fronds in a beaker on modified Hoagland media containing 1515 mg/L KNO_3, 680 mg/L KH_2PO_4, 492 mg/L $MgSO_4 \cdot 7H_2O$, 20 mg/L Na_2CO_3, 1180 mg/L $Ca(NO_3)_2 \cdot 4H_2O$, 0.5 mg/L $MnCl_2$, 0.025 mg/L $CoCl_2$, 0.025 mg/L $CuSO_4 \cdot 5H_2O$, and 18.355 mg/L Fe-EDTA. The media was adjusted to pH 5.5 with 1 M NaOH and filtered through a 0.2 μm filter. Each well of nonpyrogenic polystyrene sterile 6-well plates (CoStar 3506, Corning Inc., Corning, NY) was filled with 4950 μL of the Hoagland media mixed with 50 μL of DDI water, 50 μL of acetone with the appropriate concentration of test compound or 50 μL of EtOH with the appropriate concentration of test compound. The final concentration of acetone or EtOH was 1%. Two three-frond colonies from 4- to 5-dayold stock cultures were placed in each well. Total frond area per well was recorded by the image analysis system Scanalyzer (LemnaTec, Würselen, Germany) from days 0−4 (*12*). Percent increase at days 1−4 was determined relative to baseline area at day 0.

Statistical Analysis. Data from dose–response experiments were analyzed with the add-on package for dose–response curves, drc (*13*), for *R* version 2.2.1 (*14*) using a four-parameter logistic function. Means and standard deviations were obtained using the raw data, and IC_{50} (concentration required for 50% inhibition) values were one of the parameters in the regression curves.

Effect of Compounds 1 and 2 on Onion Root Cell Division. Onion (*Allium cepa* L. cv. Evergreen bunching) seeds were germinated as previously described in the presence of 300 and 100 μM **1** and **2** at 25 °C, respectively, under a 14 h photoperiod. The test compounds were prepared in 100× stock solutions (10 mM) and acetone. Control samples received the same amount of solvent as those receiving the test compounds. The amount of acetone in the tests accounted for 1% of the total volume. Trifluralin was used as a positive control as inhibitor of mitosis. Root tips were prepared according to Armbruster et al. (*15*), and mitotic analysis was performed by counting approximately 1000 cells per slide. The data represents the average of three replications (for a total of at least 3000 cells per treatment) as described by Oliva et al. (*16*). The mitotic phases were determined according to Hess (*17*) as either in resting phase, prophase, metaphase, anaphase, or telophase. Data was analyzed with SAS version 9.1 (*18*).

Electrolyte Leakage Experiment with Compounds 1 and 2. The effect of **1** and **2** on membrane integrity was tested as described by Kenyon et al. (*19*). Twenty-five 4 mm cucumber cotyledon discs (approximately 50 mg fresh weight) were placed in a 6 cm diameter disposable Petri dish containing 5 mL of 2% sucrose and 1 mM 2-(*N*-morpholino)ethanesulfonic acid (MES, pH 6.5) with or without the test compounds. The test compounds were dissolved in acetone. Control tissues were exposed to the same amount of acetone as treated tissues but without the test compounds. The final concentration of acetone in the dishes was 1% (v/v). The cotyledon discs were incubated at 25 °C in darkness. Cellular damage was determined overtime by measuring electrolyte leakage into the bathing medium with a conductivity meter (Amber Science, model 1056, Eugene, OR) capable of assaying 1 mL of bathing medium. All measurements were done under dim-green light for the first 18 h after which the samples were placed in a high light intensity growth chamber (Percival model 30BH0, Boone, IA) to monitor light-dependent electrolyte leakage. Because of differences in background conductivity between different treatment solutions, results are expressed as a change in the conductivity. All treatments for electrolyte leakage measurements were in sextuplets.

Fluorescence Experiment with Compounds 1 and 2. Eight mm cotyledon disks were excised from 6-day-old cucumber (*Cucumis sativa* "Straight Eight"). Induced variable chlorophyll fluorescence was measured using an OptiScience pulse-modulated fluorometer (model OS5-FL, Hudson, NH) before placing the disks into a single well of a 24-well culture plate in 1 mL of 0.1% Tween 20 with or without the test compounds. Each test was triplicated. Treatments were solvent control (0.1% acetone), 100 μM compound D, 100 μM compound F, and 10 μM atrazine as positive control. The culture plate was kept at 22 °C constant with continuous light. Chlorophyll fluorescence was subsequently measured at 2, 4, 8, and 24 h posttreatment with the samples maintained under continuous light.

Bioassay against Fungal Plant Pathogenic *Colletotrichum* spp. Isolates of *Colletotrichum acutatum* Simmonds, *C. fragariae* Brooks, and *C. gloeosporioides* (Penz.) Penz. & Sacc. were obtained from Barbara J. Smith, USDA, ARS, Popularville, MS. The three *Colletotrichum* species were isolated from anthracnose lesions of strawberry (*Fragaria* × *ananassa* Duchesne). 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/sec) 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 plastic 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 for microdilution broth experiments were evaluated using a Packard model SpectraCount microplate photometer (Packard Instrument Company, Meriden, CT).

Direct Bioautography. A number of bioautography techniques were used as primary screening systems to detect antifungal activity. Matrix, one-dimensional 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 80 μg/spot. One-dimensional thin-layer chromatography (1D 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 four 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 (*24*).

Termite Bioassays Experimental. Termites from four colonies of *C. formosanus* were obtained from field sites in New Orleans, Louisiana, from bucket traps (*25*), and maintained on spruce (*Picea* spp.) slats (10 × 4 × 0.5 cm) under conditions of ca. 100% relative humidity and 26 °C. Termites were identified using keys for soldier identification from Scheffrahn and Su (*26*).

One hundred μL of an acetone solution of the compound or fraction to be tested was blotted evenly onto a 2.5 cm. diameter Whatman no. 1 filter paper. The solvent acetone was allowed to evaporate from the filter paper for several hours. Percentage (wt/wt) is defined as weight of active ingredient to weight of filter paper (substrate) or weight of extract (without solvent) to weight of filter paper (substrate), whichever is appropriate. Treated filter paper disks were placed in plastic Petri dishes (35 × 10 mm) and moistened with 100-μL water. Twenty *C. formosanus* workers (third instar or greater as determined by size) and two soldiers were placed in each treatment. Treatments were replicated four times with termites for each replicate originating from a different *C. formosanus* colony. Petri dishes were maintained at ca. 100% R.H. and 26 °C. Filter paper disks receiving water alone served as controls. It was previously determined that the filter paper treated with acetone solvent alone had no discernible effect on termite mortality or consumption compared with H_2O.

Termite Bioassay Data Analysis. Daily termite mortality was evaluated for 3 weeks. Consumption was determined by subtracting dried posttreatment from pretreatment filter paper weights. Cumulative

Table 1. Phytotoxicity and Fungal Screening Results for *Ligularia macrophyll*[a] Crude Extracts

		phytotoxicity screening[b]		antifungal screening[c] diameter of zone of inhibition (mm)		
plant part	solvent[a]	lettuce	agrostis	*C. acutatum*	*C. fragariae*	*C. gloeosporioides*
inflorescence	CH_2Cl_2	0	0	NA	5.0 ± 0	NA
	EtOH	0	0	NA	NA	NA
	H_2O	0	0	NA	NA	NA
leaves	CH_2Cl_2	1	4	NA	4.5 ± 0	NA
	EtOH	0	0	NA	NA	NA
	EtOH ppt	0	0	NA	NA	NA
	H_2O	0	0	NA	NA	NA
roots	CH_2Cl_2	2	5	5.5 ± 0.7	7.0 ± 0	4.8 ± 0.4
	EtOH	0	0	NA	NA	NA
	H_2O	0	0	NA	NA	NA

[a] CH_2Cl_2 = methylene chloride. [b] All extracts tested at 1 mg/mL. [c] Mean dimensions of zones (mm) of fungal inhibition produced by sample extracts ± SD. All extracts tested at 80 μg. NA = Inactive.

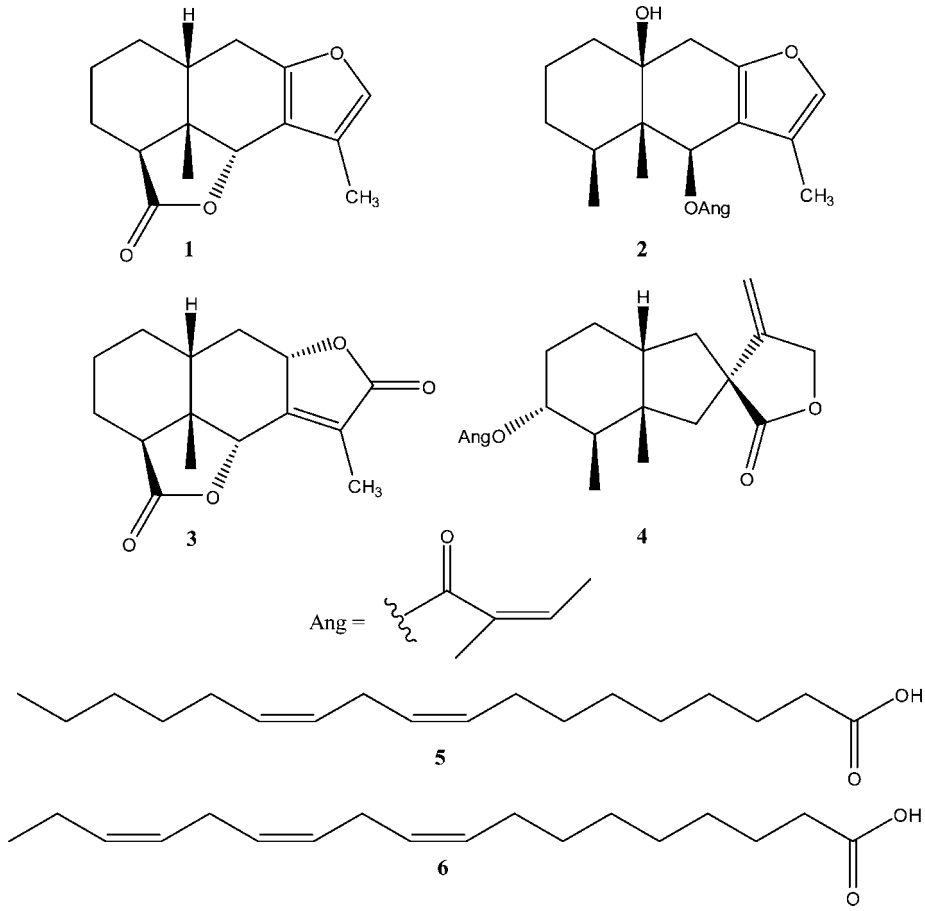

Figure 1. Bioactive compounds isolated from *Ligularia macrophylla*.

daily mortality and consumption (mean and standard deviation) was calculated from the four replicates (n = 20) of each treatment. Treatments are compared using ANOVA and means separated using a protected Fisher least-significant difference (LSD) test ($P < 0.05$; PROC GLM, SAS Institute 1990). LSD means separations test followed transformation to arcsine square root percent mortality (*27*). Actual percent mortality is reported in the tables.

RESULTS AND DISCUSSION

Roots, leaves, and inflorescences of *L. macrophylla* (Ledeb.) DC. (Asteraceae) were sequentially extracted using CH_2Cl_2, 95% ethanol, and water providing nine unique extracts which were subjected to both antifungal and phytotoxicity screens (**Table 1**). Evaluation against both the monocot *Agrostis stolonifera* (bentgrass) and the dicot *Lactuca sativa* (lettuce) at 1 mg/mL indicated the CH_2Cl_2 extract of the leaves and roots caused phytotoxicity rankings of 4 and 5, respectively, against bentgrass and 1 and 2, respectively, against lettuce where 0 = no effect and 5 = no growth or no germination of the seeds. All remaining extracts were inactive against both bentgrass and lettuce at 1 mg/mL.

Using phytotoxic activity against both bentgrass and lettuce as a guide, bioassay directed fractionation of the CH_2Cl_2 extract of the roots was performed. A single silica-gel column chromatographic purification of the extract produced crystalline compounds **1** and **2** (**Figure 1**). When analyzed using positive ion high resolution LC-MS, compound **1** gave pseudo molecular ion peaks at m/z 269.1154 and m/z 515.2341 corresponding to $[M + Na]^+$ and $[2\ M + Na]^+$, respectively, and suggesting a molecular formula of $C_{15}H_{18}O_3$ and seven sites of unsaturation. Examination of the literature for compounds from the genus

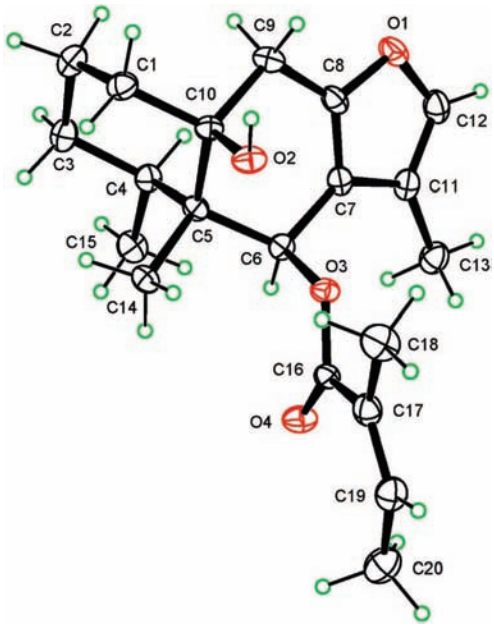

Figure 2. ORTEP drawing of compound **2**.

Ligularia revealed a complete match of the ^{1}H NMR (400 MHz) data with published values (*5*) allowing the assignment of its structure as that of furanoeremophilan-14β,6α-olide (**1**). ^{13}C NMR (100 MHz) data are reported here for the first time, and multiplicities were determined from 90° and 135° DEPT experiments.

When analyzed using positive ion high resolution LC-MS, compound **2** gave pseudo molecular ion peaks at *m*/*z* 355.1952 and *m*/*z* 687.4068 corresponding to $[M + Na]^+$ and $[2 M + Na]^+$, respectively, and suggesting a molecular formula of $C_{20}H_{28}O_4$. Ultimately, structure determination of compound **2** was completed using single crystal X-ray diffraction studies which unequivocally established the structure as that of 6β-angeloyloxy-10β-hydroxyfuranoeremophilane (**2**). The X-ray crystal structure determination of compound **2** is reported here for the first time. Although isolation and structure determination of compound **2** had been reported in the literature previously ((*5*), (*28*), (*29*)), ^{1}H and ^{13}C NMR data had not been reported and is reported here for the first time. ^{13}C NMR multiplicities were determined from 90° and 135° DEPT experiments.

The crystal structure of eremophilane **2** is illustrated in **Figure 2**. The cis-fusion of the two six-membered rings causes a marked nonplanarity in the ring system, with the best planes of these two rings forming a dihedral angle of 73.60(7)°. The saturated ring has a chair conformation, with endocyclic torsion angle magnitudes in the range 50.3(3) − 57.1(2)°. The unsaturated six-membered ring has a half-chair conformation, with C5 lying 0.605(3) Å from the best plane of the other five atoms. The furan ring is planar, with largest deviation 0.004(4) Å. The angelate substituent is slightly nonplanar, having a twist of 12.6(3)° about the C16–C17 bond. The OH group forms an intermolecular hydrogen bond with angelate carbonyl O4, leading to hydrogen-bonded chains. Crystal structures of several cis-fused eremophilanes have been previously reported, and they have similar conformations. These include β-tetradymodiol (*30*), 1-methoxy-decompositin (*31*), and 3β-(angeloyloxy)-1,10-epoxy-furanoeremophilane (*32*).

Further bioassay directed purification of fractions from the CH_2Cl_2 extract of the roots using reversed phase C-18 HPLC led to the isolation of compound **3**. When analyzed using positive ion high resolution LC-MS, compound **3** gave pseudo molecular ion peaks at *m*/*z* 285.1114 and 547.2306 corresponding to $[M + Na]^+$ and $[2 M + Na]^+$, respectively, and suggesting a molecular formula of $C_{15}H_{18}O_4$. Examination of the literature for compounds possessing the same molecular formula and similar ^{1}H and ^{13}C NMR chemical shifts allowed us to establish the structure as that of eremophil-7(11)-ene-12,8α; 14β,6α-diolide (**3**).[1] H NMR and ^{13}C NMR data were in complete agreement with published values (*6*, *7*).

Using phytotoxic activity against both bentgrass and lettuce as a guide, bioassay directed fractionation of the CH_2Cl_2 extract of the leaves was also performed. Column chromatographic purification followed by reversed phase C-18 purification resulted in the isolation of compound **4**. When analyzed using positive ion high resolution LC-MS, compound **4** gave pseudo molecular ion peaks at *m*/*z* 333.2092, 355.1914, and 687.3872 corresponding to $[M + H]^+$, $[M + Na]^+$, and $[2 M + Na]^+$, respectively, and suggesting a molecular formula of $C_{20}H_{28}O_4$. Examination of the literature for compounds possessing the same molecular formula and similar ^{1}H and ^{13}C NMR chemical shifts allowed us to establish the structure as that of 3α-angeloyloxy-

Table 2. Phytotoxicity (day 7) Screening Results for *L. macrophylla* Isolated Compounds Versus *L. sativa*, and *A. stolonifera*

		ranking (0–5)	
compound	concentration (μM)[a]	*L. sativa*	*A. stolonifera*
1	10	0	1
	100	0	1
	1000	0	4
2	10	0	0
	100	0	1
	1000	1	4
3	10	0	0
	100	0	0
	1000	2	5
4	10	0	0
	100	0	0
	1000	1	3
5	10	0	0
	100	0	0
	1000	0	2
6	10	0	0
	100	0	0
	1000	0	0

[a] Compounds were also evaluated at 1 μM, however all rankings were 0 against both *L. sativa* and *A. stolonifera*.

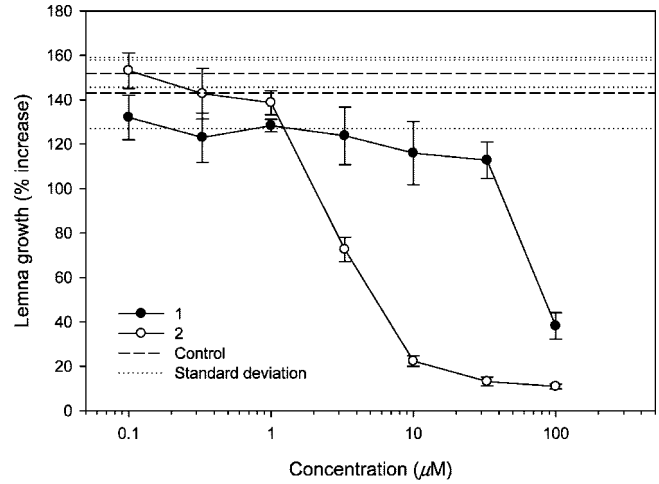

Figure 3. Effect of compounds **1** and **2** on growth of *L. paucicostata* at 4 days. Bars represent the standard error of each mean. Bold dashed lines represent the mean values of untreated plants, and dotted lines represent the standard errors of the means of these control values.

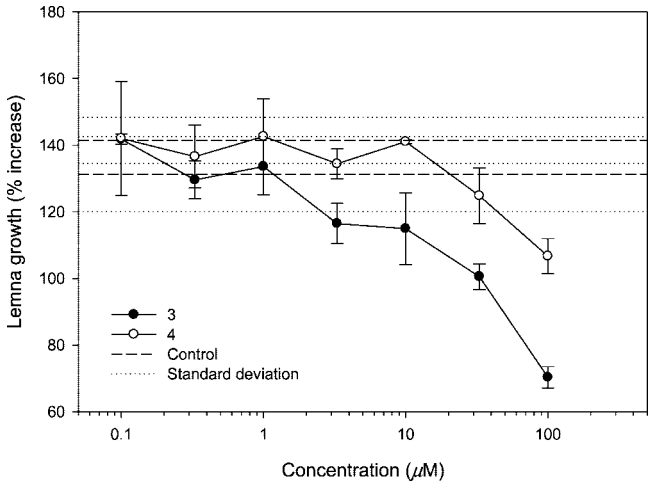

Figure 4. Effect of compounds **3** and **4** on growth of *L. paucicostata* at 4 days. Bars represent the standard error of each mean. Bold dashed lines represent the mean values of untreated plants, and dotted lines represent the standard errors of the means of these control values.

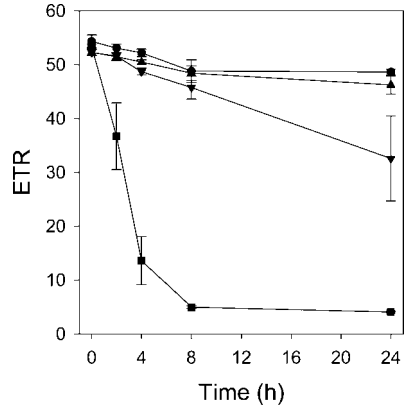

Figure 5. Photosynthetic electron transport rate (ETR) of cucumber cotyledon disks incubated in the presence of 100 μM 1 (●), 100 μM 2 (▼), and 10 μM atrazine (a photosystem II inhibitor) (■). Control samples (▲) received equivalent amount of solvent.

bakkenolide A (*4*). ^{1}H NMR and ^{13}C NMR data were in complete agreement with published values (*8*).

Compounds which had been isolated using the above bioassay-guided fractionation approach were evaluated for activity against both *L. sativa* and *A. stolonifera* using previously established methods (**Table 2**) (*11*). Compounds were evaluated from 1000 to 1 μM. As observed with crude extracts and fractions, compounds **1**–**4** were consistently more active against the monocot bentgrass than the dicot lettuce. Compounds **1**–**4** all exhibited good activity at 1000 μM; however, upon diluting the activity was quickly lost in this bioassay. Compounds **5** and **6** were also evaluated in this bioassay but were inactive at all concentrations.

Phytotoxic effects of eremophilanes **1**–**4** on *L. paucicostata* were evaluated in a dose–response manner from 100 to 0.1 μM. 6β-angeloyloxy-10β-hydroxyfuranoeremophilane (**2**) was clearly the most active compound with an IC_{50} of 2.94 ± 0.16 μM (**Figure 3**) while compounds **1**, **3**, and **4** were not active enough at 100 μM to allow determination of an IC_{50} (**Figures 3** and **4**). Due to the high level of activity observed for compounds **1** and **2** and the substantial quantities available for additional studies, both compounds will be examined in further studies in an attempt to understand the mode of action of these compounds.

Phytotoxic effects of compounds **1** and **2** on mitotic indices of *A. cepa* root tips were determined at 100 and 300 μM. No significant differences between solvent control and treatments were observed for either compound at 100 and 300 μM (Supporting Information Figure 1), suggesting that the growth inhibition is not associated with inhibition of mitotic processes. The effect of compounds **1** and **2** on membrane integrity was also evaluated as described by Kenyon et al. (*19*). Neither compound had membrane-disrupting effects (data not shown). On the other hand, a reduction of photosynthetic electron rate was observed in response to a 100 μM treatment with **2** (**Figure 5**). The effect was much less pronounced than that observed with atrazine, a potent inhibitor of photosystem II, indicating that the reduction of electron transport rate (ETR) by **2** is most likely unrelated to its actual mode of action. Indeed, photosynthetic efficiency is often reduced in plants experiencing physiological or environmental stresses. Compound **1** did not affect ETR at the concentration tested (**Figure 5**).

Evaluation of the nine extracts of *L. macrophylla* in direct bioautography assay at 80 μg/spot against *C. acutatum*, *C fragariae*, and *C. gloeosporioides* indicated the presence of antifungal metabolites. Bioassay of the CH_2Cl_2 extracts against *C. fragariae* demonstrated clear zones of fungal growth inhibition for inflorescences (5.0 mm), leaves (4.5 mm) and roots (7.0 mm) (**Table 1**). The CH_2Cl_2 extract of the roots was also active against *C. acutatum* and *C. gloeosporioides* with growth inhibition mean values 5.5 mm and 4.8 mm, respectively.

Bioassay-directed fractionation of the CH_2Cl_2 extracts of the inflorescences, leaves, and roots was performed in an effort to identify the antifungal constituents present in these extracts. Column chromatography fractionation followed by HPLC purification of the antifungal fractions resulted in the isolation of two antifungal compounds **5** and **6**. When analyzed using negative ion high resolution LC-MS, compound **5** gave pseudo molecular ion peaks at *m*/*z* 279.2354 and 559.4700 corresponding to $[M - H]^-$ and $[2\ M - H]^-$, respectively, and suggesting a molecular formula of $C_{18}H_{30}O_2$. Examination of the literature for compounds possessing the same molecular formula and similar ^{13}C NMR chemical shifts allowed us to establish the structure as that of linoleic acid (**5**). ^{13}C NMR data were in complete agreement with published values (*9*). Compound **6**

Table 3. Cumulative % Mortality of *C. formosanus* and Filter Paper Consumed

	% mortality (mean ± S.D.)[1,2] days				
treatment	1	6	13	21	monsumption (mg ± S.D.)
compound **1** (0.5%)	1.3 ± 2.5A	1.3 ± 2.5A	3.8 ± 4.8A	8.8 ± 2.5A	43.1 ± 3.0A
compound **2** (0.5%)	1.3 ± 2.5A	3.8 ± 2.5A	16.3 ± 0.2A	18.8 ± 18.9A	16.5 ± 8.4B
compound **4** (0.5%)	0A	2.5 ± 2.9A	2.5 ± 2.9A	2.5 ± 2.9A	40.0 ± 4.2A
compound **5** (0.5%)	2.5 ± 5.0A	3.8 ± 4.8A	5.0 ± 4.1A	6.3 ± 6.3A	44.6 ± 0.0A
compound **6** (0.5%)	0A	0	5.0 ± 4.1A	6.3 ± 4.8A	44.2 ± 0.8A
solvent control	0A	2.5 ± 5.0A	3.8 ± 4.8A	3.8 ± 4.8A	41.4 ± 5.6A

[1] Twenty workers (>third instar))/1 soldier per rep. Four reps. [2] Means within a column/treatment with the same letter are not significantly different, LSD: $P < 0.05$.

was analyzed using a similar approach to that used for compund **5** which established its structure as that of α-linolenic acid (**6**). ^{13}C NMR data were in complete agreement with published values (*10*). The purified compounds **5** and **6** were evaluated for activity against *C. acutatum*, *C. fragariae*, and *C. gloeosporioides* in a bioautographical screen at 10 μg/spot. Both compounds generated clear zones of inhibition of 2.9 ± 0.1 mm against *C. fragariae* and no inhibition against *C. acutatum* and *C. gloeosporioides*. Specific activity of **5** and **6** against *C. fragariae* confirms the fungal sensitivity results seen in the extract testing. Due to the lack of activity at lower concentrations in the bioautographical screens, these compounds were not pursued further in microdilution broth bioassays as is typically done (*33*).

Lastly, compounds **1–3** and **5–6** were evaluated for activity against the Formosan subterranean termite, *Coptotermes formosanus*. All compounds were evaluated at a concentration of 0.5% for 21 days. Percent mortality was determined every few days, and none of the compounds caused any significant mortality. At the conclusion of this 21 day assay, consumption of filter paper was determined for all treatments and the solvent control. Compound **2** demonstrated a significant low level of consumption relative to the control. All other treatments appeared to be equivalent to the solvent control.

In summary, we have examined *L. macrophylla* for compounds active against insects, fungal plant pathogens, and weeds. The most promising compound as a potential pesticide lead was **2.** Its activity on *L. paucicostata* was higher than that of many commercial herbicides (*12*). It deserves further study for structure optimization and to determine is mode of action.

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Supporting Information Available: More information is shown in one additional figure and seven tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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