Antifeedant and Mosquitocidal Compounds from *Delphinium* × cultorum Cv. Magic Fountains Flowers

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Six volatile compounds, ethylmethylbenzene (**1**), 1-isopentyl-2,4,5-trimethylbenzene (**2**), 2-(hex-3-ene-2-one)phenylmethyl ketone (**3**), E and Z isomers of 3-butylidene-3*H*-isobenzofuran-1-one (**4** and **5**), and 2-penten-1-ylbenzoic acid (**6**), were isolated from the mosquitocidal hexane extract of *Delphinium* × *cultorum* cv. Magic Fountains flowers. In addition, the ethyl acetate extract, which displayed corn earworm antifeedant activity, yielded 4-hydroxybenzoic acid (**7**) and bis(4-hydroxyphenyl)methanol (**8**). However, compounds **7** and **8** were not biologically active.

INTRODUCTION

Traditionally, plants from the genus *Delphinium* have been used as poisons and insecticides and for medicinal purposes. These activities are linked to the alkaloids present in these plants. Diterpenoid alkaloids isolated from *Delphinium* spp. demonstrated acute mammalian toxicity (Aiyar et al., 1979) in addition to insecticidal (Jennings et al., 1986), antibiotic (Atta-ur-Rahman et al., 1997), antifungal (Atta-ur-Rahman et al., 1997), and allelopathic (Waller and Burstrom, 1969) activities.

Research on *Delphinium* has focused primarily on the isolation of diterpenoid alkaloids. The few reports that have mentioned nonalkaloids from *Delphinium* included fatty acids from *Delphinium iliense* and *Delphinium ajacis* L. (Dong et al., 1991; Hasan and Osman, 1993). The seed oil of *D. ajacis* contained a high percentage of linoleic acid (Hasan and Osman, 1993). *Delphinium peregrinium* and *Delphinium carolinianum* were specifically investigated for nonalkaloid compounds, and several flavonoids were isolated (Mericli et al., 1991). Phenolic compounds such as 2,5,6-trihydroxypiperonylic acid (Mericli et al., 1991), a new benzoxepine derivative, oxformasine (Mericli et al., 1996), and 3-hydroxy-2-methyl-4*H*-pyran-4-one (Atta-ur-Rahman et al., 1997) were isolated from *Delphinium venulosum*, *Delphinium formosum*, and *Delphinium denudatum*, respectively. No biological activity has been reported for any of these nonalkaloids. In this paper, we report the isolation of several nonalkaloid compounds from the biologically active extract of *Delphinium* × *cultorum* cv. Magic Fountains.

MATERIALS AND METHODS

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were recorded on a Varian INOVA 300 MHz spectrometer at ambient temperature. GC−EIMS was performed at the Michigan State University Mass Spectrometry Facility on a JEOL-JMS-AX505H mass spectrometer configured with a 30 m DB5 column (0.32 mm × 0.25 mm). The carrier gas was He, and the solvent was acetone. The column temperature was set at 50 °C and programmed to increase to 190 °C at 10 °C/min. The ionization energy was 70 eV. All solvents were ACS reagent grade and were purchased from Aldrich Chemical Co., Inc. Statistical analyses were performed on the larval antifeedant data using the F test and least significant difference (LSD).

Plant Material. *Delphinium* × *cultorum* cv. Magic Fountains dark blue/white bee seeds were donated by Bodger Seed, Ltd., South El Monte, CA. The seeds were germinated in high porosity, peat-based growing mix in Styrofoam trays (4 in. × 6 in. × 2 in.) and grown until the first leaf appeared. The seedlings were then transplanted into pots (4 in. × 4 in. × 6 in.) and then into large clay pots (10 in. × 6 in. × 10 in.) and were grown until maturity. The plants were raised in the Bioactive Natural Products Laboratory Greenhouses, Michigan State University. The plants were subjected to a 12 h photoperiod, watered once daily, and maintained at 75 °F. Plant parts were harvested when 75−80% of the florets were fully expanded on each raceme and stored at −20 °C.

Extraction. The frozen flower material was weighed and spread evenly into three stainless steel lyophilizer trays (29 cm × 31.5 cm × 3.4 cm) and lyophilized using a bulk tray lyophilizer (model TD-3B, FTS Systems, Inc., Stone Ridge, NY) at 5 °C for 48 h. The dried flower material was macerated into a fine powder in a commercial Waring blender. The water content of the fresh *D.* × *cultorum* flowers was approximately 80%. Sequential extraction of the lyophilized flower parts (409 g) with hexane and ethyl acetate (3.5 and 2.0 L × 3, respectively) yielded 8.6 and 3.6 g, respectively.

GC−MS Characterization of Compounds 1−6 in the Mosquitocidal Band II. The hexane extract (4 g) was subjected to liquid−liquid extraction with hexane (200 mL × 3) and aqueous MeOH (90%, 300 mL). This yielded hexane soluble and aqueous MeOH soluble fractions weighing 3.7 g and 283 mg, respectively. The mosquitocidal components from the aqueous MeOH soluble fraction (283 mg) from the hexane extract were isolated using preparative TLC on silica gel GF_{254} glass plates (20 cm × 20 cm; 500 mm thickness; Analtech Inc., Newark, DE) developed with $CHCl_3$. The plates were viewed under UV light (254 and 366 nm) and eluted with MeOH, yielding bands I−V with weights of 3.4, 4.6, 9.9, 3.4, and 177.4 mg, respectively. Compounds **1**−**6** were identified using GC−EIMS from the mosquitocidal band II.

Isolation of Compounds 7 and 8. The ethyl acetate extract (1.2 g) was subjected to liquid−liquid extraction with hexane (200 mL × 3) and aqueous MeOH (90%, 300 mL). This yielded hexane soluble and aqueous MeOH soluble fractions weighing 395 mg and 703 mg, respectively. The aqueous MeOH soluble fraction (2 g) accumulated from several liquid−liquid extractions of the ethyl acetate extract was fractionated

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using a Waters Sep-Pak C_{18} cartridge (1.0 mL capacity; Waters Chromatography Division, Millipore Corp., Milford, MA) eluted with aqueous MeOH (90%, 1.25 mL × 4), MeOH (5 mL), and $CHCl_3$ (3 mL). This yielded fractions I–VI with weights of 1012, 174, 79, 48, 399, and 156 mg, respectively. The corn earworm growth-inhibitory fractions I–IV were combined (1.2 g) and further separated using preparative HPLC on two Jaigel GS310-F columns in tandem (i.d. 20 mm × 300 mm), eluted with aqueous MeOH (90%) at a flow rate of 5 mL/min and detected using UV 210 nm. This yielded fractions I–VI weighing 22, 324, 621, 10,0.6, and 123 mg, respectively. The antifeedant fraction, VI, was further separated by preparative HPLC on two Jaigel S-343-15 ODS columns in tandem (i.d. 20 mm × 250 mm), eluted with aqueous MeOH (70%) at a flow rate of 5 mL/min and detected using UV 210 nm. This yielded fractions I–VI weighing 5, 24, 10, 50, 4, and 2 mg, respectively. Fractions II and IV were purified under the same conditions with the exception of the eluting solvent, which was aqueous MeOH (60%), and a flow rate of 3 mL/ min. Compounds **7** (14 mg) and **8** (26 mg) were purified from fractions II and IV, respectively, and were identified by using ^{1}H and ^{13}C NMR spectra.

Compound 1: *ethylmethylbenzene*. GC–EIMS 70 eV, *m*/*z* values (rel intensity): 120 [M$^+$] (28.75), 105 [M − 15] (100), 91 [M − 29] (12.5).

Compound 2: *1-isopentyl-2,4,5-trimethylbenzene*. GC–EIMS 70 eV, *m*/*z* values (rel intensity): 190 [M$^+$] (10.63), 133 [M − 57] (100).

Compound 3: *2-(hex-3-ene-2-one)phenylmethyl ketone*. GC–EIMS 70 eV, *m*/*z* values (rel intensity): 216 [M$^+$] (47.5), 201 (18.75), 173 (2.5), 145 (2.5), 132 (21.25), 119 (67.5), 105 (8.75), 97 (7.5), 91 (12.5), 83 (100), 77 (3.75), 65 (2.5), 55 (16.25).

Compound 4: (*E*)-*3-butylidene-3H-isobenzofuran-1-one*. GC–EIMS 70 eV, *m*/*z* values (rel intensity): 188 [M$^+$] (28.75), 159 [M − 29] (100), 146 [M − 42] (30).

Compound 5: (*Z*)-*3-butylidene-3H-isobenzofuran-1-one*. GC–EIMS 70 eV, *m*/*z* values (rel intensity): 188 [M$^+$] (28.75), 159 [M − 29] (100), 146 [M − 42] (30).

Compound 6: *2-penten-1-ylbenzoic acid*. GC–EIMS 70 eV, *m*/*z* values (rel intensity): 190 [M$^+$](72.5), 161 [M − 29] (100), 148 [M − 42] (78.75).

Compound 8: *bis(4-hydroxyphenyl)methanol*. ^{1}H NMR (300 MHz, d_6-DMSO): δ 3.72 (1H, s, H-1), 6.67 (4H, d, J = 8.4, H-2, H-2′, H-6, H-6′), 7.74 (4H, d, J = 8.4, H-3, H-3′, H-5, H-5′). ^{13}C NMR (75 MHz, d_6-DMSO): δ 55.6 (*s*, C-7), 114.2 (s, C-2, C-2′, C-3, C-3′, C-5, C-5′, C-6, C-6′), 131.3 (*s*, C-1, C-1′), 159 (*s*, C-4, C-4′).

Methylation of Compound 8. Diazomethane was prepared by dissolving 0.5 of *N*-nitroso-*N*-methylurea in 10% KOH solution (50 mL) with ether (50 mL) over an ice bath. The mixture was stirred gently for a few minutes and poured into a 250 mL separatory funnel. The KOH solution layer was removed, and the diazomethane solution was then washed with 25 mL of ice-cold water. The diazomethane solution was poured into a brown bottle containing KOH pellets and stored at −20 °C. The diazomethane solution and a small amount of MeOH was added to a sample of compound **8** (8 mg). The sample was capped and allowed to sit in the fume hood for 2 h. The ether was evaporated with N_2 gas, and the MeOH removed in vacuo at 30 °C to yield compound **9** (8.9 mg): ^{1}H NMR (300 MHz, d_6-DMSO) δ 3.72 (1H, s, H-1), 3.84 (6H, s), 6.67 (4H, d, J = 8.4, H-2, H-2′, H-6, H-6′), 7.74 (4H, d, J = 8.4, H-3, H-3′, H-5, H-5′).

Acetylation of Compound 9. To compound **9** (8.9 mg) was added a solution of pyridine (2 mL) and acetic anhydride (1 μL). The clear solution was allowed to sit for 2 days in the dark with constant stirring. The solvent was removed in vacuo at 50 °C to yield compound **10** (10.2 mg): ^{1}H NMR (300 MHz, d_6-DMSO) d 2.35 (3H, s), 3.72 (1H, s, H-1), 3.84 (6H, s), 6.67 (4H, d, J = 8.4, H-2, H-2′, H-6, H-6′), 7.74 (4H, d, J = 8.4, H-3, H-3′, H-5, H-5′).

Mosquitocidal Assay. First instar mosquito larvae, *Aedes aegyptii* (Dipera:Culicidae), were provided by Drs. Alexander Raikel and Alan Hays, Department of Entomology, Michigan State University. The larvae were raised in reverse osmosis (RO) water in an incubator at 26 °C for 3 days. Next, 10–12 fourth instar larvae were placed in 980 mL of RO water in test tubes. Twenty microliters of test material dissolved in DMSO, to give a concentration of 250 mg 20 mL^{-1}, was added to each tube. A 20 mL aliquot of DMSO was used as a control. Treatments and controls were run in triplicate and were covered and left at room temperature. Larval mortality was recorded at 2 h intervals, up to and including 24 h (Nair et al., 1989; Roth et al., 1998).

Corn Earworm Antifeedant Assay. Corn earworm eggs, *Helicoverpa zea* Boddie (Lepidoptera:Noctuidae) and dry corn earworm diet was purchased from North Carolina State Insectory, Department of Entomology, North Carolina State University, Raleigh, NC. The eggs were hatched in an incubator at 27 °C. The dry diet was dispensed into scintillation vials (940 mg) for each treatment. Test material was dissolved in DMSO to give a concentration of 1250 mg 25 mL^{-1}, unless otherwise stated. Next, 25 mL of the test solution was mixed thoroughly with the portions of dry diet. An aliquot of 25 mL of DMSO was used as a control. Agar solution (1.4%) was mixed and autoclaved for 5 min at 15 psi and 125 °C to melt the agar. This solution was held in a water bath at 50 °C, and added to the dry diet until the total diet weighed 5 g. The final concentration of test extracts and compounds was 250 mg/mL. The wet diet was mixed thoroughly, and 3–4 drops of diet were dispensed into 3.5 mL polystyrene vials. The freshly poured portions of diet were allowed to cool and dry for at least 1 h. After drying, one neonate larva was placed in each vial, and the vials were capped. The treatment and control vials were held in a growth chamber at a photoperiod of 16 h day and 8 h night with day temperature at 28 °C and night temperature at 24 °C. Each treatment had 15 replicates. The treatments were arranged in a completely randomized design. Larvae were weighed (mg) after 6 days (Zhang et al., 1997).

Gypsy Moth Caterpillar Antifeedant Assay. Gypsy moth eggs, *Lymantria dispar* L. (Lepidoptera:Lymantriidae) were obtained from the Insect Production Unit of the Canadian Forest Service, Sault Ste. Marie, Canada. The dry diet was mixed in the Bioactive Natural Products Laboratory, Department of Horticulture, Michigan State University, and consisted of wheat germ (36 g), casein (7.5 g), Wesson's salt mix (2.4 g), sorbic acid (0.6 g), methylparaben (*p*-hydroxybenzoic acid methyl ester) (0.3 g), and Hoffman-La Roche 26862 vitamin mixture (Hoffman-La Roche, Inc., Nutley, NJ; 3.0 g). The eggs were hatched in an incubator at 27 °C. The dry diet was dispensed into scintillation vials (845 mg) for each treatment. The rest of this assay was identical to that of the corn earworm assay (Zhang et al., 1997).

RESULTS AND DISCUSSION

The flowers of *D.* × *cultorum* were separated from the stems, lyophilized, and macerated into a fine powder prior to the sequential extraction with hexane and ethyl acetate. These crude extracts were tested at 250 mg/mL concentrations for antifungal, antibacterial, topoisomerase inhibitory, mosquitocidal, and larval antifeedant activities. The hexane extract showed 93% and 59% growth inhibition against *H. zea* and *L. dispar*, respectively. In addition, it produced 100% mortality at 10 mg/mL concentration in *A. aegyptii* larvae at 2 h. The ethyl acetate extract showed 88% growth inhibition against *H. zea*. Both extracts were subjected to liquid–liquid extraction with hexane and aqueous MeOH as a preliminary means of separation.

Bioassay-directed fractionation of the hexane extract was conducted using fourth instar *A. aegyptii* and *H. zea* neonates. Both the hexane soluble and aqueous MeOH soluble fractions exhibited LD_{100} in 2 h at 100 mg/mL concentration when tested against *A. aegyptii*. Similarly, these fractions showed significant antifeedant activity against *H. zea* and *L. dispar*; however, the

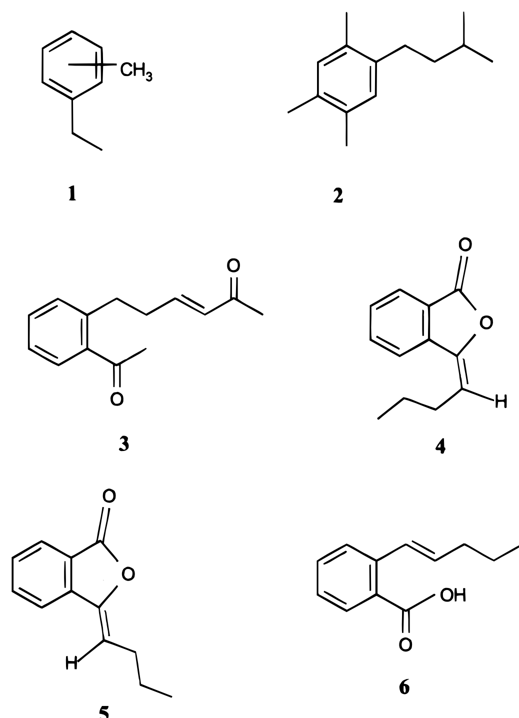


Figure 1. Compounds characterized by GC−MS analysis of the mosquitocidal fraction, band II, from the hexane extract.

aqueous MeOH soluble fraction was the most active. Separation of the aqueous MeOH soluble fraction was conducted by preparative TLC, yielding bands I−V. All five bands were assayed against *A. aegyptii*. Band II produced 100% mortality at 1 mg/mL concentration in 2 h. Because of the volatility of this band, isolation of pure compounds from it was very difficult. Therefore, this band was analyzed by GC−EIMS. The total ion current (TIC) profile indicated a total of 15 peaks, six of which were characterized and assigned structures (Figure 1) based on their fragmentation patterns with R_t values of 3.00, 6.98, 13.11, 13.22, 13.75, and 13.90 min, respectively.

Peak 1, showing M^+ at *m/z* 120 and fragment ions at *m/z* 105 and 91, indicating the loss of ethyl and methyl substituents, respectively, was assigned the structure identical to **1**, an isomer of ethylmethylbenzene (National Institute of Science and Technology [NIST], 1998). Similarly, peak 2 with M^+ at *m/z* 190 and the fragment ion at *m/z* 133 due to the loss of an isobutyl moiety, was identical to 1-isopentyl-2,4,5-trimethylbenzene (**2**) (Kingston et al., 1988). The third peak was the most abundant in the TIC. The M^+ at *m/z* 216 produced fragment ions at *m/z* 201, 173,119, and 83 due to the cleavage of a methyl, 2-propanone, hex-2-ene-1-one, and phenylmethyl ketone, respectively. Peak 3 was characterized from its EIMS fragmentation pattern as compound **3**, 2-(hex-3-ene-2-one)phenylmethyl ketone. Literature search revealed that this is the first report of compound **3**. The MS analysis revealed that TIC peaks 4 and 5 showed identical fragmentation profiles with an M^+ ion at *m/z* 188. The fragment ions at *m/z* 159 due to cleavage of an ethyl moiety and 146 suggesting a McClafferty rearrangement followed by the loss of a propyl group. Hence, these compounds were identified as isomers of each other and differed only in retention times. A survey of the NIST mass spectral database and the literature revealed that they were the E and Z isomers of 3-butylidene-3*H*-isobenzofuran-1-one, respectively (**4** and **5**)

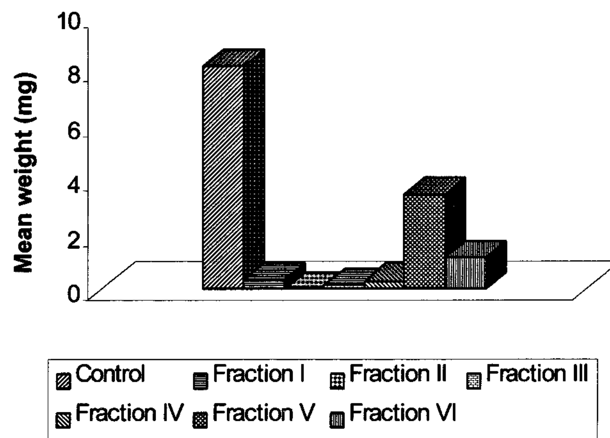
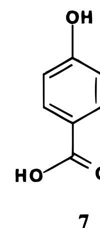


Figure 2. Antifeedant assay of fractions I−VI from C_{18} Sep-Pak separation of ethyl acetate extract from *D. X cultorum* flower parts on *H. zea* at 250 mg/mL concentration after 6 days; $p \leq 0.01$.

(Ogawa et al., 1995; Watanabe et al., 1993). However, we could not assign which peak corresponded to which isomer; we did not have standards, and the GC−MS of these compounds are not reported. TIC peak 6 showed M^+ at *m/z* 190 and fragment ions at *m/z* 161, indicating loss of an ethyl moiety, and at *m/z* 148, suggesting a McClafferty rearrangement followed by cleavage of a propyl group. Compound **6** was identified from these diagnostic ions as 2-penten-1-ylbenzoic acid (Li et al., 1993). As a result of the volatility of the mosquitocidal band II containing compounds **1**−**6** (Figure 1), the yield was very low and did not allow further bioassays to be conducted on the pure compounds or the isolation of further biologically active components.

Bioassay-directed fractionation of the ethyl acetate extract was conducted using *H. zea* neonates. The aqueous MeOH fraction from liquid−liquid extraction of the ethyl acetate extract was further separated using a C_{18} sep-pak cartridge. The resulting fractions I−VI were tested against *H. zea* for antifeedant activity, and fractions I−IV were combined on the basis of their similar TLC profiles and similar levels of antifeedant activity as indicated by larval weight reduction by >90% (Figure 2).

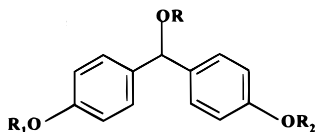
The combined fractions were purified by preparative HPLC using a series of size exclusion columns. The antifeedant compounds were eluted in the last fraction at R_t = 46−110 min and further purified by HPLC on ODS columns. The fraction eluting at R_t = 52 min was identified as 4-hydroxybenzoic acid (**7**) (Mericli et al., 1991). The fraction that eluted at R_t = 57 min was



identified as bis(4-hydroxyphenyl)methanol (**8**) (Elliger, 1985).

Compound **8** gave an AB pair of doublets at δ 6.67 and 7.74 (J = 8.4 Hz) and each signal integrated for four protons in the ^{1}H NMR spectrum. This suggested that **8** is a symmetrical molecule, containing two

benzene rings that had a 1,4 substitution pattern. A one proton singlet at δ 3.72 further supported the symmetry in **8**. The ^{13}C NMR data of **8** gave signals at δ 55.6 (C-7) and 159 (C-4, C-4′), which indicated oxygen functionalities at these positions.



8 : R = R_1 = R_2 = H
9 : R = H; R_1 = R_2 = Me
10 : R = Ac; R_1 = R_2 = Me

To confirm the oxygen functionalities, compound **8** was methylated using diazomethane to yield **9**. It was evident from the ^1H NMR of **9** that **8** contained 2-OH moieties as indicated by a singlet at δ 3.84 that integrated for six protons. Also, a singlet at δ 3.91 in compound **9** indicated the presence of a third OH functionality in **8**.

To confirm the presence of the third hydroxyl group in **9**, it was acetylated using acetic anhydride and pyridine. The ^1H NMR analysis of the product (**10**) gave a signal at δ 2.35 that integrated for three protons and confirmed the presence of a hydroxy group at C-7 in **8**. Hence, the structure of compound **8** was assigned as bis-(4-hydroxyphenyl)methanol. Compounds **7** and **8** did not show antifeedant activity on *H. zea* at 250 mg/mL concentration.

This is the first bioassay-directed phytochemical investigation of the hybrid *D.* × *cultorum* and the first analysis of volatile compounds from *Delphinum* flowers. This study yielded compounds **1**−**6** and **8** new to the genus *Delphinium*, of which **3** is novel. In addition, this is the first report of compounds **1**−**6** from *Delphinium* species. Compounds **1**, **2**, and **6** have never been reported from natural sources. E and Z isomers of 3-butylidene-3*H*-isobenzofuran-1-one (**4** and **5**) are commonly found in essential oils of *Angelica* (Dai and Qiu, 1996), *Apium* (Gijbels et al., 1985), and *Ligusticum* species (Luo et al., 1996). Compound **7** has been isolated previously from *D. venulosum* Boiss (Mericli et al., 1991).

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