Antifungal and Larvicidal Compounds from the Root Bark of *Cordia alliodora*

Jean-Robert Ioset,† Andrew Marston,† Mahabir P. Gupta,‡ and Kurt Hostettmann*,†

Institut de Pharmacognosie et Phytochimie, Université de Lausanne, BEP, CH-1015 Lausanne, Switzerland, and Center for Pharmacognostic Research on Panamanian Flora (CIFLORPAN), Apartado 10767, College of Pharmacy, University of Panama, Panama, Republic of Panama

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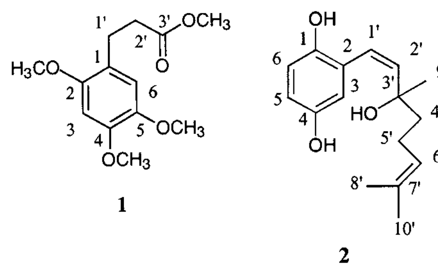
Two new natural products, a phenylpropanoid derivative characterized as 1-(3′-methoxypropanoyl)-2,4,5-trimethoxybenzene (**1**) and a prenylated hydroquinone, 2-(2*Z*)-(3-hydroxy-3,7-dimethylocta-2,6-dienyl)-1,4-benzenediol (**2**), have been isolated from the root bark of *Cordia alliodora*. Both compounds exhibited antifungal properties against the phytopathogenic mold *Cladosporium cucumerinum*. The phenylpropanoid derivative (**1**), whose structure is closely related to *β*-asarone, also demonstrated a marked activity against larvae of the yellow-fever-transmitting mosquito *Aedes aegypti*.

Cordia alliodora Oken (Boraginaceae), also known as *Cerdana alliodora* Ruiz & Pavon, is a tall tree frequently encountered in Central America, South America, and the Caribbean Islands. In Panama, *C. alliodora* is the most widespread species of the *Cordia* genus and is found all around the country up to an altitude of 1100 m.[1] A decoction of the leaves is used as a tonic for pulmonary diseases in traditional Mexican medicine and is applied on bruises and swellings in Salvador.[2] An ointment made of the plant seeds is employed in the Caribbean Islands to treat skin diseases.[2] No reports have been found about traditional use of the roots. Chemical investigations of the heartwood of *C. alliodora* have resulted in the isolation of alliodorin,[3] a prenylated hydroquinone. Six ant-repellant triterpenoids[4] have also been identified in the leaves of the same plant. In addition, other prenylated quinonoid compounds, like alliodorol and cordiachromen A, have been isolated from the plant heartwood.[5]

In our search for new antifungal and larvicidal therapeutics, we recently reported the isolation of naphthoquinones from the roots of *Cordia linnaei* Stearn[6,7] and *Cordia curassavica* Roemer & Schultes.[8] Several other *Cordia* species of Panamanian origin were then screened for biological properties. The dichloromethane extract from the root bark of *C. alliodora* exhibited activities against the phytopathogenic fungus *Cladosporium cucumerinum*,[9] the yeast *Candida albicans*,[10] and larvae of the yellow-fever-transmitting mosquito *Aedes aegypti*.[11] Based on these results, the extract was submitted to a bioactivity-guided fractionation.

The crude dichloromethane extract was first separated by column chromatography on Si gel. The antifungal and larvicidal assays performed on the 19 resulting fractions showed fractions 9 and 12 to be active against *C. cucumerinum*, while fraction 12 also demonstrated larvicidal properties against *A. aegypti*. Compounds **1** (83 mg) and **2** (12 mg) were purified from these two fractions by gel filtration on Sephadex LH-20. The structures of the pure compounds were elucidated using ^{1}H and ^{13}C NMR spectroscopy combined with EIMS and DCIMS.

The presence of ions at *m*/*z* 254 [M]$^+$ in EIMS and *m*/*z* 272 [M + NH$_4$]$^+$ in DCIMS, indicated a molecular weight of 254 amu for compound **1**. Signals for four methoxyl units



(*δ* 3.81, s, 6H; *δ* 3.73, s, 3H, and *δ* 3.61, s, 3H), two aromatic protons (*δ* 6.80, s, 1H; *δ* 6.66, s, 1H), and four aliphatic protons (*δ* 2.81, t, $J = 7.7$ Hz, 2H; *δ* 2.52, t, $J = 7.7$ Hz, 2H) were detected in the ^{1}H NMR spectrum. The 13 resonances observed in the ^{13}C NMR experiment were established to be four CH$_3$, two CH$_2$, two CH, and five quaternary carbons through a DEPT experiment. These NMR data, combined with the mass spectroscopy measurements, indicated a molecular formula of $C_{13}H_{18}O_5$ for compound **1**. Three of the methoxyl groups were assigned to the aromatic ring of compound **1** after observation of HMBC correlations between ^{1}H NMR signals at *δ* 3.81 ppm (2-OCH$_3$ and 4-OCH$_3$) and ^{13}C resonances at *δ* 99.3 (C-3), 152.6 (C-2), and 149.8 (C-4) and correlations between *δ* 3.73 ppm (5-OCH$_3$) and *δ* 144.0 (C-5). The fourth methoxyl group was found to be part of a CH$_2$−CH$_2$−COO−CH$_3$ subunit by observation of a HMBC correlation between the ^{13}C signal belonging to the ester function at *δ* 173.7 (C-3′) and the ^{1}H NMR resonance at *δ* 3.61 (3′-OCH$_3$). This unit was linked to C-1 by HMBC correlations between C-1 and protons at *δ* 2.81 (H-1′) and *δ* 2.52 (H-2′). The ^{1}H and ^{13}C NMR data obtained for **1** were closely related to those of *β*-asarone[12] and isohexyl dihydrocaffeate.[13] To our knowledge, compound **1** is a new natural phenylpropanoid derivative.

Observation of ions at *m*/*z* 244 [M − H$_2$O]$^+$ in the EIMS and at *m*/*z* 280 [M + NH$_4$]$^+$ and *m*/*z* 262 [M]$^+$ in the DCIMS suggested a molecular weight of 262 amu for compound **2**. An AMX system was identified in the ^{1}H NMR spectrum by the presence of aromatic protons at *δ* 6.64 (d, $J = 8.7$ Hz), 6.57 (dd, $J = 8.7$, 3.1 Hz), and 6.48 ppm (d, $J = 3.1$ Hz). HSQC correlations proved these signals to be part of a 2-substituted hydroquinone moiety characterized by resonances at *δ* 147.0 (C-1), 122.0 (C-2), 112.8 (C-3), 149.2 (C-4), 115.4 (C-5), and 116.7 (C-6) in the ^{13}C NMR spectrum. Another 10 ^{13}C NMR signals were observed and assigned to the terpenoid substituent of the hydroquinone.

* To whom correspondence should be addressed. Tel.: 41 21 692 4561. Fax: 41 21 6924565. E-mail: kurt.hostettmann@ipp.unil.ch.
† Université de Lausanne.
‡ University of Panama.

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Table 1. Antifungal and Larvicidal Activities for Compounds **1** and **2**

compound	*C. cucumerinum*[a]	*C. cucumerinum*[b]	*C. albicans*[a]	*C. albicans*[b]	*A. aegypti*[c]
1	5	15	>50	n.d.[d]	12.5
2	5	15	>50	n.d.	>50
nystatin	0.2	1	0.1	1	
plumbagin					3
β-asarone					25

[a] Minimal amount (μg) of compound to inhibit growth on a Si gel TLC plate. [b] Minimal inhibition concentration MIC (μg/mL) of compound in an agar-dilution assay. [c] Minimal concentration (μg/mL) of compound required to kill all the larvae after 24 h. [d] n.d. = not determined.

Six of them were included in a $CH_2-CH_2-CH{=}C(CH_3)_2$ sequence (δ 40.9, 22.7, 124.1, 131.7, 25.6, and 17.6; C-4′, C-5′, C-6′, C-7′, C-8′, and C-10′) according to HSQC and DFQ−COSY data. The presence of a *cis*-CH=CH moiety was shown by ^{1}H NMR signals at δ 6.27 (d, J = 9.8 Hz, H-1′) and 5.59 (d, J = 9.8 Hz, H-2′), connected to ^{13}C resonances at δ 122.6 and 131.0 (C-1′ and C-2′, respectively). Its position on the aromatic ring was established by HMBC correlations observed between H-1′ and the ^{13}C signals at δ 147.0 (C-1), 122.0 (C-2), and 112.8 (C-3). The HMBC cross-peaks seen between the quaternary carbon at δ 78.1 (C-3′) and the protons centered at δ 6.27 (H-1′), 5.59 (H-2′), 1.71(H-4′), and 1.36 ppm (CH_3-9′) positioned this carbon atom between the double bond and the $CH_2-CH_2-CH{=}C(CH_3)_2$ chain, with the methyl group linked to it. The chemical shift of C-3′ suggested an attached hydroxy group, consistent with the molecular weight for compound **2**. Finally, the established structure was confirmed by comparison of spectral data with those of 2-(2*E*)−(3-hydroxy-3,7-dimethylocta-2,6-dienyl)-1,4-benzenediol, the trans isomer of compound **2**, isolated from the tunicate *Amaroucium multiplicatum* Sluiter (Polyclinidae).[14]

The compounds separated from the root bark of *C. alliodora* were found to be less active than nystatin (Table 1), a commercially available fungicide, against *C. cucumerinum* in both bioautographic and agar-dilution assays.[15] The same products were inactive against *C. albicans* on TLC plates at the highest tested concentrations and were thus not tested in dilution assays. These latter tests were required to quantify the activity of **1** and **2** against *C. cucumerinum*, as bioautographic assays only gave a semiquantitative estimation of their antifungal properties. In adddition to these results, compound **1** demonstrated a larvicidal effect against *A. aegypti* weaker than plumbagin, a remarkably toxic naphthoquinone, but comparable to the structural analogue β-asarone, which is known for its larvicidal, insect repelling, and insect sterilizing properties (Table 1).[11,16]

Experimental Section

General Experimental Procedures. ^{1}H and ^{13}C NMR were obtained on a Varian Unity Inova NMR instrument. ^{1}H and ^{13}C NMR spectra were recorded in $CDCl_3$ at 500 and 125 MHz, respectively. TMS was the internal standard. UV was measured on a Varian DMS 100S UV−vis spectrophotometer; $[\alpha]_D$, on a Perkin-Elmer-241 polarimeter; TLC, on Si gel 60 F_{254} Al sheets (Merck) and diol HPTLC plates (Merck); column chromatography, with Si gel (63−200 μm; 700 × 55 mm i.d., Merck) and Sephadex LH-20 (530 × 20 mm i.d., Pharmacia). EIMS and DCIMS were obtained on a Finnigan MAT TSQ-700 triple stage quadrupole instrument. The purity control of the isolated compounds was performed by HPLC/UV/DAD with a Nova-Pak RP18 column (4 μm; 250 × 3.9 mm i.d.; Waters) using an $MeOH-H_2O$ gradient (35:65 →100:0) in 30 min, followed by an isocratic elution with MeOH in 10 min.

Plant Material. Roots of *C. alliodora* Oken were collected in March 1997, at Gamboa, in the Panama Channel Zone, Panama. A voucher is deposited at the National Herbarium of Panama (FLORPAN 2775) and at the Institut de Pharmacognosie et Phytochimie, Lausannne, Switzerland (no. 97027).

Extraction and Isolation. Air-dried, powdered root bark of *C. alliodora* (650 g) was extracted at room temperature with dichloromethane to afford 4.9 g of extract. The dichloromethane extract was separated by column chromatography on Si gel with a petroleum ether−EtOAc gradient (6:1→0:1), giving 19 fractions. Fraction 9 was purified on Sephadex LH-20 with $CHCl_3-MeOH$ (1:1) to yield 12 mg of compound **1**. Compound **2** (83 mg) was obtained from fraction 12 after gel filtration on Sephadex LH-20 using $CHCl_3-MeOH$ (1:1) as mobile phase.

Sample Preparation for Bioautographic Assays. Geometric dilutions were obtained from freshly prepared stock solutions of isolated and reference compounds at a concentration of 5 mg/mL in an appropriate solvent. Of these solutions, 10 μL were applied on the TLC plates using graduated capillaries.

Bioautographic Assays. Direct bioautography with *C. cucumerinum*: after application of the samples on a Si gel 60 F_{254} Al sheet (Merck), the TLC plate was developed in petroleum ether−EtOAc (1:1) solvent system and thoroughly dried for complete removal of solvents. The plate was then sprayed with a suspension of *C. cucumerinum* in a nutritive medium and incubated for 2−3 days in polystyrene boxes with a moist atmosphere. Clear inhibition zones appeared against a dark gray background. Nystatin (Sigma) was used as reference compound.

Agar overlay bioautography with *C. albicans*: after application of the samples on a Si gel 60 F_{254} glass plate (Merck), the samples were developed using a petroleum ether−EtOAc (1:1) solvent system and thoroughly dried for complete removal of solvents. An inoculum of yeast (approximately 10^7 cells/mL) in molten malt agar (Biokar Diagnostics) was distributed over the plates.The medium solidified as a thin layer (approximately 1 mm), and the plates were then incubated overnight at 30 °C in polystyrene boxes with a moist atmosphere. Inhibition zones were visible after spraying with an aqueous solution of methylthiazolyltetrazolium bromide (2.5 mg/mL). Active compounds appeared as clear spots against a purple background. Nystatin (Sigma) was used as reference compound.

C. cucumerinum **Dilution Assays.** Geometric dilutions of the isolated and reference compounds were freshly prepared in DMSO from stock solutions at 3 mg/mL (in DMSO). Aliquots of these dilutions were added to Sabouraud agar medium (Biokar Diagnostics), which was distributed in hermetically sealed 24-well plates. The final concentration of DMSO in the assay did not exceed 2%. A suspension of *C. cucumerinum* in distilled water was spread over the agar. Incubation was performed at 30 °C for 24 h. Control experiments without test compounds were carried out for verification of fungal growth. All samples were measured in duplicate. Data given in Table 1 are averages of these measurements. Nystatin (Sigma) was used as reference compound.

Larvicidal Assays. Geometric dilutions of the isolated and reference compounds were freshly prepared from stock solutions at 5 mg/mL in DMSO. Aliquots of these dilutions were added to a graduated tube containing approximately 20 instar II larvae of *A. aegypti* in tap water, and the final volume was adjusted to 10 mL. The tubes were incubated in darkness at 26−28 °C for 24 h. Larvae lethality was observed under lab light. All samples were measured in duplicate. Data given in

Table 1 are an average of these measurements. Plumbagin (Roth) and β-asarone (Roth) were used as reference compounds.

1-(3′-Methoxypropanoyl)-2,4,5-trimethoxybenzene (**1**): yellow gum; UV (MeOH) λ_{max} (log ϵ) 231 (3.83), 290 (3.58) nm; ^{1}H NMR ($CDCl_3$) δ 6.80 (1H, s, H-6), 6.66 (1H, s, H-3), 3.81 (6H, s, 2-OCH_3 and 4-OCH_3), 3.73 (3H, s, 5-OCH_3), 3.61 (3H, s, 3′-OCH_3), 2.81 (2H, t, J = 7.7 Hz, H-1′), 2.52 (2H, t, J = 7.7 Hz, H-2′); ^{13}C NMR ($CDCl_3$) δ 173.7 (C-3′), 152.6 (C-2), 149.8 (C-4), 144.0 (C-5), 120.9 (C-1), 116.3 (C-6), 99.3 (C-3), 57.1 (5-OCH_3), 56.5[a] (2-OCH_3), 56.4[a] (4-OCH_3), 51.5 (3′-OCH_3), 34.9 (C-2′), 26.1 (C-1′); EIMS m/z 254 $[M]^{+\cdot}$ (66), 181 (100), 151 (19); DCIMS m/z 272 $[M + NH_4]^+$ (100), 255 $[M + H]^+$ (14);[a] represents interchangeable signals.

2-(2*Z*)−(3-Hydroxy-3,7-dimethylocta-2,6-dienyl)-1,4-benzenediol (**2**): yellow gum; $[\alpha]^{25}_{D}$ −8.8° (c 0.5, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 220 (4.18), 264 (sh, 3.50), 332 (3.36) nm; ^{1}H NMR ($CDCl_3$) δ 6.64 (1H, d, J = 8.7 Hz, H-6), 6.57 (1H, dd, J = 8.7, 3.1 Hz, H-5), 6.48 (1H, d, J = 3.1 Hz, H-3), 6.27 (1H, d, J = 9.8 Hz, H-1′), 5.59 (1H, d, J = 9.8 Hz, H-2′), 5.09 (1H, m, H-6′), 2.10 (2H, m, H-5′), 1.71 (2H, m, H-4′), 1.66 (3H, s, CH_3-8′), 1.57 (3H, s, CH_3-10′), 1.36 (3H, s, CH_3-9′); ^{13}C NMR ($CDCl_3$) δ 149.2 (C-4), 147.0 (C-1), 131.7 (C-7′), 131.0 (C-2′), 124.1 (C-6′), 122.6 (C-1′), 122.0 (C-2), 116.7 (C-6), 115.4 (C-5), 112.8 (C-3), 78.1 (C-3′), 40.9 (C-4′), 26.0 (CH_3-9′), 25.6 (CH_3-8′), 22.7 (C-5′), 17.6 (CH_3-10′); EIMS m/z 244 $[M - H_2O]^+$ (10), 161 (100); DCIMS m/z 280 $[M + NH_4]^+$ (6), 262 $[M]^+$ (100), 246 (100), 244 (21), 180 (18), 161 (100).

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