Methyl 4-Hydroxy-3-(3′-methyl-2′-butenyl)benzoate, Major Insecticidal Principle from *Piper guanacastensis*

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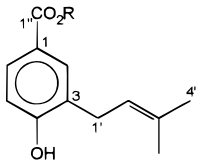
The $CHCl_3$-soluble extract of *Piper guanacastensis* (Piperaceae) was found to have noteworthy insecticidal activity to *Aedes atropalpus* mosquito larvae (LC_{50} 80.5 μg/mL). Bioassay-guided fractionation afforded methyl 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoate (**1**) as the major bioactive constituent (LC_{50} 20.5 μg/mL). The separation of compound **1** from its transesterification artifact (**2**), ethyl 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoate, was achieved by recycling reversed-phase HPLC. The flavonoids acacetin, chrysin, and pinostrobin were also isolated from the active fraction but did not display insecticidal properties.

The Piperaceae or pepper family is widely used in tropical regions throughout the world as medicines, condiments in regional cuisine, and pest-control agents.[1] As a part of a program aimed at the development of potentially useful phytochemicals as insect control agents ("green insecticides") and because of our interest in the chemical ecology of the American neotropical Piperaceae, we now report the isolation and identification of a novel prenylated phenolic from *Piper guanacastensis* C. DC., an endemic species of Costa Rica in Central America. The insecticidal and growth-reducing properties of this plant material, as well as the activity of 13 additional species in the Piperaceae, were established previously using as model insect the European corn borer *Ostrinia nubilalis* (Lepidoptera: Pyralidae).[2] This investigation confirmed that tropical American *Piper* species, including *P. guanacastensis*, have insecticidal activities comparable to previously studied African and Asian species. Bioactivity-guided fractionation of selected active crude extracts indicated the importance of phenyl propanoids (*Piper aduncum*), amides (*Piper tuberculatum*), and lignans *(Piper decurrens*) as the chemical defenses of the Piperaceae.[2,3]

In the present study, the EtOH extract of *P. guanacastensis* aerial parts was found to be toxic to mosquito larvae, *Aedes atropalpus* L. (Diptera: Culicidae), the model insect used during bioactivity-guided fractionation of the crude total extract (LC_{50} = 127.5 μg/mL) for isolation of the active principle. The insecticidal activity was traced to the $CHCl_3$-soluble fraction. Further fractionation on a silica gel column yielded a fraction rich in a mixture of prenylated benzoic acids derivatives, which had most of the insecticidal activity. HPLC separation furnished esters **1** (LC_{50} = 20.5 μg/mL) and **2** (LC_{50} = 25.7 μg/mL) in pure form as the major active components. The structure of compound **1** was elucidated as methyl 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoate on the basis of spectral analysis. Derivative **2** represents the ethyl ester analog of natural product **1** and, therefore, an artifact of extraction formed by prolonged storage of the plant material in EtOH. The flavonoids acacetin, chrysin, and pinostrobin were also isolated from the $CHCl_3$-soluble fraction but did not have pronounced insecticidal activity. These compounds have been found in other medicinally important *Piper* species, especially in those native to the Middle and Far East.[4]



1 R = CH_3
2 R = CH_2CH_3
3 R = H

Compound **1** exhibited a molecular formula of $C_{13}H_{16}O_3$, on the basis of its HREIMS data. The ^1H NMR spectrum (Table 1) contained a set of three coupled aromatic resonances at δ 6.80 (d, J = 8.0 Hz), 7.80 (d, J = 2 Hz), and 7.79 (dd, J = 8, 2 Hz). The chemical shifts and coupling constants for these signals are consistent with a 1,3,4-trisubstituted benzene ring in which the 1- and 4-substituents are strongly electron-withdrawing (−CO_2CH_3: ^1H δ 3.85; ^{13}C δ 51.82) and electron-donating (−OH: IR 3400 cm^{-1}) groups, respectively. The ^{13}C-NMR spectrum (Table 1), assisted by DEPT and $^1H-^{13}C$-HETCOR (HMQC) techniques, was in full agreement with the presence of a 3-methyl-2-butenyl moiety at C-3. With the basic fragments of compound **1** established, the connectivities between them were solved by the use of long-range heteronuclear

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Table 1. ^{1}H, HMQC, and HMBC NMR Data of Compound **1**[a]

		correlated carbon	
position	δ^1H	δ^{13}C HMQC	HMBC
1		122.52 s	
2	7.80 d (2)	131.85 d	C-4, C-6, C-1′, C-1″
3		126.83 s	
4		158.67 s	
5	6.80 d (8)	115.48 d	C-1, C-3
6	7.79 dd (8, 2)	129.66 d	C-2, C-4, C-1″
1′	3.36 br d (7.2)	29.55 t	C-2, C-3, C-4, C-2′, C-3′
2′	5.29 t (7.2)	121.11 d	C-1′, C-4′, C-5′
3′		135.38 s	
4′	1.76 s	25.77 q	C-2′, C-3′, C-5′
5′	1.76 s	17.89 q	C-2′, C-3′, C-4′
1″		167.11 s	
2″	3.85 s	51.82 q	C-1″

[a] Chemical shifts in ppm relative to TMS; coupling constants J (Hz). C-multiplicities were established by DEPT. HMBC (J = 8 Hz).

chemical shift correlations (HMBC, Table 1). To the best of our knowledge, this is the first report of methyl 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoate from a natural source. However, the title compound (**1**) has been prepared during the synthesis of its benzoic acid precursor (**3**), which has proved to be an ulcer inhibitor and effective in the treatment of hepatitis in murine models.[5,6] The free acid (**3**) was previously isolated as a phytotoxic phenol from culture filtrates of *Discula* sp., the fungus responsible for dogwood anthracnose.[7]

The present investigation constitutes the first evaluation of prenylated benzoic acid derivatives as a potential "green insecticide". Compounds of this type and their chromene homologs have been previously reported as the antimicrobial and molluscicidal principles of *P.aduncum*.[8] Prenylated benzoic acid derivatives represent a biosynthetically inexpensive and effective defense against insects, together with isobutylamides and lignans, for a limited number of highly successful Piperaceae species from the neotropics.

Experimental Section

General Experimental Procedures. Melting points were taken on a Gallenkamp capillary melting point apparatus and are uncorrected. ^{1}H (500 MHz), ^{13}C (125.7 MHz), DEPT, HMQC, and HMBC NMR spectra were recorded using the Bruker AMX-500 spectrometer. $CDCl_3$ was used as solvent and internal reference: $CHCl_3$ (^{1}H, δ 7.25) or $CDCl_3$ (^{13}C, δ 77.00). Mass spectra were obtained on a VG 7070E spectrometer using electron impact ionization. The TLC R_f values were obtained by using Si gel 60 F_{254} precoated 0.25-mm aluminum sheets (Merck) in CH_2Cl_2−Me_2CO (7:3) as solvent system; after development, the samples were visualized by UV light or staining with a 5% solution of phosphomolybdic acid in EtOH, followed by heating at 110 °C. Preparative column chromatography was done on Si gel 60 Merck (70−230 mesh). The analytical HPLC t_R values were determined on a reversed-phase C_{18} column (5 μm, 4 × 250 mm) using a Beckman system (Series 332), equipped with a Model 165 variable-wavelength UV−vis detector. All compounds were eluted isocratically with 60% CH_3CN and 40% H_2O (flow rate = 1 mL/min) and monitored at 250 nm. Preparative HPLC was performed using a LC-908 recycling preparative system (Japan Analytical Industry Co., Ltd.).

Plant Material. *P. guanacastensis* C. DC. was collected in a secondary forest in Costa Rica near Mirador, Guanacaste Province, in October 1993. Identification of the material was carried out by L. Poveda and P. Sanchez, and voucher specimens were retained at the Universidad Nacional de Heredia (Costa Rica) and the University of Ottawa (Canada).

Biological Screening Procedures. Insect bioassays were determined by larval toxicity of the rock hole breeding mosquito, *Aedes atropalpus* L. (Diptera: Culicidae). A laboratory colony was maintained according to previously described procedures.[2,9] Ten second-instar larvae were transferred into glass vials containing 10 mL of water and test samples, i.e., extracts, fractions, or pure compounds, at concentrations of 200, 100, 50, 10, and 1 μg/mL. After 24 h at room temperature, the surviving larvae were counted. The same protocol was used for controls containing solvent vehicle (EtOH 5%). Bioassays were conducted in triplicate, and LC_{50} values were obtained by probit analysis.[10]

Extraction and Isolation. Fresh leaves (830 g) were immediately immersed in 95% EtOH after collection and were stored until processed for approximately 1 year. The extracts were concentrated to dryness under reduced pressure at 30 °C to a thick dark-green residue (97.67 g). The whole extract was dissolved in EtOH−H_2O (7:3) and subjected to partition with hexane, $CHCl_3$ (three times), and butanol. The insecticidal activity (LC_{50} = 80.5 μg/mL) was found in the $CHCl_3$ extract (30.92 g), which was subjected to chromatography over Si gel (150 g) in a gravity column using a gradient of Me_2CO−MeOH in CH_2Cl_2. A total of 120 fractions (50 mL each) were collected and combined into five pools (I−V) on the basis of similar TLC profiles. Insecticidal activity was found in fractions 14−28 (pool I), which were eluted with CH_2Cl_2.

Pool I (4.95 g; LC_{50} = 48.5 μg/mL) was dissolved in CH_2Cl_2 and treated with activated charcoal. An amorphous white solid (4.0 g) was obtained and analyzed by ^{1}H-NMR and MS. These spectra revealed the presence of a mixture of two aromatic esters of related structure with the same chromatographic behavior in TLC (R_f 0.52; $CHCl_3$−hexene−Me_2CO 7:2.5:0.5). In the mass spectrum, corresponding $[M]^+$ ions were present at m/z 220 (minor) and 234 (major). The whole residue was recrystallized from MeOH to afford 3.57 g (0.27% w/w) of the mixture of compounds **1** and **2** (LC_{50} = 22.8 μg/mL). Recycling preparative HPLC equipped with a reversed-phase C_{18} column (20 × 250 mm; 15 μm) and an UV detector set at 254 nm and running isocratically with 70:30 CH_3CN−H_2O (flow rate = 3.5 mL/min) was effective in separating 147 mg of the minor component (**1**, LC_{50} = 20.5 μg/mL with a 95% confidence interval of 36.0−10.6 μg/mL) from 500 mg of the mixture of esters **1** (t_R = 30 min) and **2** (348 mg; t_R = 37 min; LC_{50} 25.7 with a 95% confidence interval of 39.0−12.3 μg/mL).

Pool II, eluted with CH_2Cl_2−Me_2CO (9:1), afforded by fractional crystallization in $CHCl_3$−MeOH 1.28 g of acacetin (t_R = 8.0 min) and 268.3 mg of chrysin. Pool III afforded 42 mg of chrysin (t_R = 5.8 min). Pool IV, eluted with CH_2Cl_2−Me_2CO (7:3), left a residue that was recrystallized from MeOH to yield 964.2 mg of a mixture of chrysin and pinostrobin in ratio of 2:1. Finally, pool V, eluted with CH_2Cl_2−Me_2CO−MeOH (7:3:0.5), af-

forded 301.5 mg of pinostrobin (t_R = 3.8 min). These known flavonoids were identical (mp, NMR, TLC, and HPLC) to standard samples and exhibited no insecticidal activity.[11,12]

Methyl 4-hydroxy-3-(3′-methyl-2′-butenyl)benzoate (1): white crystalline solid; mp 86−87 °C; TLC R_f 0.60; HPLC t_R 8.3 min; HRMS *m/z* found M^+ 220.10887, $C_{13}H_{16},O_3$ requires M^+ 220.10994; EIMS (70 eV) *m/z* (rel int) $[M]^+$ 220 (49.1), 203 $[M - OH]^+$ (13.5), 189 $[M - OCH_3]^+$ (15.5), 173 (18.1), 165 $[M - C_4H_7]^+$ (100), 161 $[189 - CO]^+$ (19.3), 145 (25.3), 146 (12.4), 147 (9.5), 133 (14.5), 105 (12.5), 91 (11.3), 77 (12.6), 59 (16.0); ^1H and ^{13}C NMR spectral data, see Table 1.

Compound 2: white crystalline solid; mp 76−77 °C; TLC R_f 0.60; HPLC t_R 11.2 min; ^1H NMR ($CDCl_3$, 500 MHz) δ 7.80 (1 H, d, J = 2.1 Hz, H-2), 7.78 (1 H, dd, J = 2.1, 8.3 Hz, H-6), 6.81 (1 H, d, J = 8.3 Hz, H-5), 5.29 (1 H, t, J = 7.2 Hz, H-2′), 4.32 (2 H, q, J = 7.1 Hz, O*CH*$_2$-Me), 3.36 (2 H, br d, J = 7.2, H-1′), 1.74 (6 H, s, Me-4′ and Me-5′), 1.35 (3 H, t, J = 7. 1 Hz, OCH_2*Me*); ^{13}C NMR ($CDCl_3$, 125.7 MHz) δ 167.01 (*CO*OCH_2Me), 158.76 (C-4), 134.89 (C-3′), 131. 79 (C-2), 129.55 (C-6), 127.12 (C-3), 122.55 (C-1), 121.30 (C-2′), 115.34 (C-5), 60.75 (O*CH*$_2$Me), 29.32 (C-1′), 25.74 (C-4′), 17.86 (C-5′), 14.33 (OCH_2*Me*); EIMS (70 eV) m/z (rel int) $[M]^+$ 234 (60.4), 189 $[M - OC_2H_5]^+$ (30.7), 179 $[M - C_4H_7]^+$ (100), 161 $[189 - CO]^+$ (26.3), 151 (13.8), 147 (22.8), 146 (11.3), 145 (9.6), 133 (22.9), 129 (10.3), 91 (13.4), 77 (13.2); HRMS *m/z* found M^+ 234.10887, $C_{14}H_{18},O_3$ requires M^+ 234.10994.

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