Bioactive Aromatic Compounds from Leaves and Stems of *Vanilla fragrans*

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Alcoholic extracts of leaves and stems of *Vanilla fragrans* were fractionated with ethyl acetate and aqueous butanol. All three fractions of ethyl acetate, butanol, and water were screened for toxic bioactivity against mosquito larvae. The results of these experiments showed that the fractions from the ethyl acetate and butanol phases were both active in the bioassay. Bioactivity of the ethyl acetate fraction was found to be much greater than that from the butanol fraction in mosquito larvae toxicity. The water phase appeared to contain no substances that impaired mosquito larval growth. Repeated column chromatography of the ethyl acetate fraction on silica gel led to the isolation of 4-ethoxymethylphenol (**1**), 4-butoxymethylphenol (**2**), vanillin (**3**), 4-hydroxy-2-methoxycinnamaldehyde (**4**), and 3,4-dihydroxyphenylacetic acid (**5**). Compounds **4** and **5** were isolated from *Vanilla* species for the first time and **2** has not been reported to have been found in a natural form. 4-Ethoxymethylphenol (**1**) was the predominant compound, but 4-butoxymethylphenol (**2**) showed the strongest toxicity to mosquito larvae. The structures of the compounds were determined on the basis of their mass spectra and ^{1}H or ^{13}C NMR data.

Keywords: *Vanilla fragrans; bioactivity; insecticidal properties; 4-ethoxymethylphenol; 4-butoxymethylphenol; vanillin; toxicity*

INTRODUCTION

Vanilla is a most popular flavoring material that has been used for hundreds of years. The plant is a climbing orchid and is said to attain a length of 100 m (*1*). There are about 110 wild-growing species of the genus *Vanilla* known throughout the tropics and subtropics, of which only three are cultivated for their economic importance: *V. planifolia* Andrews (*V. fragrans)*, *V. tahitensis* Moore, and *V. pompona* Schiede (*2*). Because of the specific fragrance of vanilla the plant has become the only orchid used for commercial purposes other than for their ornamental value. Vanilla is now widely used for the production of foods, and in perfumes and pharmaceutical preparations. For this reason, the chemical constituents that are responsible for the flavor, aroma, and taste of vanilla bean extract have been extensively investigated. More than 180 volatile constituents from cured vanilla beans have been identified (*3*−*7*), and among those components over one-third are aromatic volatiles. Vanillin is the most abundant ingredient, and the other major volatile constituents of vanilla aroma are *p*-hydroxybenzoic acid, *p*-hydroxybenzaldehyde, vanillic acid, *p*-hydroxybenzyl alcohol, and vanillyl alcohol. It is now well-known that these aromatic compounds contribute a great deal to the typical vanilla flavor but little is known about the biological activity of those aromatic volatiles. Although there is a large amount of information concerned with the constituents of vanilla bean extract, little work has been done to characterize the components of vegetative portions of this plant. It was observed by us that greenhouse grown *V. fragrans* did not appear to be as frequently attacked by insects as were some others, and this interesting observation led us to determine whether the extract of vegetative portions of vanilla possesses insecticidal activity. The present paper deals with the isolation, structural identification, and biological activity against mosquito larvae of several aromatic compounds from the leaves and stems of *V. fragrans*.

MATERIALS AND METHODS

Plant Material. Vegetative aerial parts of *Vanilla fragrans* were collected in June 1999 from the greenhouse of Cook College, Rutgers University, New Brunswick, New Jersey.

Chemicals. All reagents used for column chromatography and thin-layer chromatography were of HPLC grade. Ethyl alcohol (95%) was used for extraction. Pyridine and acetic anhydride were obtained from Sigma Chemical Company. All other solvents were analytical grade or better. Silica gel (Merck, 230−400 mesh) used for column chromatography was purchased from Aldrich Chemical Co., Inc.

Extraction and Isolation. *Extraction*. Before extraction, the freshly collected leaves and stems (3 kg of each) were washed with distilled water to eliminate impurities and organic fertilizers. The washed material was then chopped into fine pieces within an electric blender in the presence of ethyl alcohol. The chopped tissue was transferred to an extraction container into which a total volume of 4000 mL of ethyl alcohol (95%) was added. The container was kept at room temperature for 4 to 5 days. The alcoholic extraction solution was filtered and this treatment was repeated twice more with ethyl alcohol (2 × 3000 mL). The combined aqueous ethyl alcohol phase was evaporated in a rotary evaporator under reduced pressure until the final concentration of alcohol was approximately 20%. The concentrated solution was kept in a freezer to precipitate chlorophyll and insoluble material, which were than filtered off. Evaporation of the remaining alcohol in the filtrate left an orange water phase. This was extracted with ethyl acetate (3 × 150 mL), followed by aqueous butanol (3 × 300 mL). After

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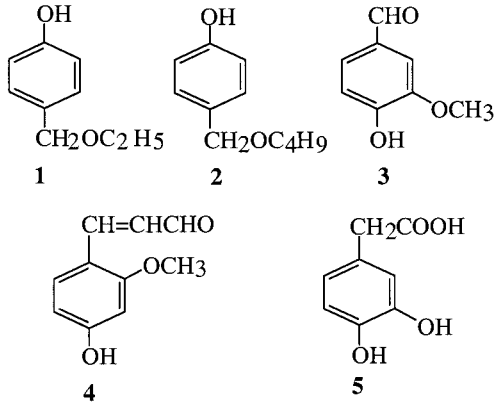

Figure 1. Structures of compounds **1**−**5**.

evaporation of solvents, 2 and 22 g of extracts were obtained from the ethyl acetate and butanol fractions, respectively. The water phase was also evaporated to dryness under reduced pressure to provide 43 g of yellowish solid substance.

Each of the above-mentioned extracts was subjected to toxicity bioassays using mosquito larvae as test organisms for initial screening. Results of these preliminary bioassays showed that both the ethyl acetate extract and the butanol extracts were biologically active, whereas the yellowish solid substance from the water extract was neither toxic nor beneficial to mosquito larvae. The lethality of the ethyl acetate fraction to mosquito larvae was very much stronger than that of the butanol fraction. Therefore, the ethyl acetate extraction (2 g) was used for the subsequent isolation of insecticidal compounds.

Pretreatment and Isolation. The ethyl acetate fraction (2 g) was dissolved in a small amount of ethyl acetate and then transferred onto a chromatography column filled with 75 g of dry silica gel. Methylene chloride (850 mL) and ethyl alcohol (300 mL) were used as elution solvents. After removal of the solvent, the methylene chloride eluant gave 600 mg of residue that was proved to be the lethal fraction to mosquito larvae. This portion was repeatedly chromatographed on silica gel columns with a linear gradient of 20:1 to 2:1 hexane−ethyl acetate. The eluate was monitored with TLC, and eluates containing each pure compound were combined. Removal of the solvents from the combined fractions gave pure compounds **1** (420 mg), **2** (21 mg), **3** (3 mg), **4** (4 mg), and **5** (16 mg).

4-Ethoxymethylphenol (1). Yellowish oil. EI-MS (*m*/*z*, %): 152 (M^+, 40), 135 (M^+-OH, 1.5), 123 (M^+- C_2H_5, 12), 107 (M^+-OC_2H_5, 100), 95 (13), 77 (24), 65 (4), 51 (8), 41 (11). Acetate: 194 (M^+, 12), 152 (M^+-42, 85), 135 (5), 123 (18), 107 (100), 94 (14), 77 (17), 65 (4), 51 (4), 43 (15).^{1}H NMR($CDCl_3$): δ 7.50 (1H, br, OH), 7.18 (2H, d, J = 8.5 Hz, aromatic protons), 6.34 (2H, d, J = 8.5 Hz, aromatic protons), 4.45 (2H, s, Ar-CH_2OEt), 3.60 (2H, q, J = 7Hz, OCH_2Me), 1.25 (3H, t, J = 7 Hz, CH_3).

4-Butoxymethylphenol (2). Yellowish oil. EI-MS (*m*/*z*, %): 180 (M^+, 35), 163 (M^+-OH, 0.5), 123 (15), 107 (M^+-$CO_2C_2H_5$, 100), 95 (13), 77 (24), 65 (4), 51 (8), 41 (12). Acetate: 222 (M^+, 8), 180 (M^+-42, 65), 163 (1), 149 (2), 123 (12), 107 (100), 95 (8), 78 (10), 77 (11), 65 (1), 52 (2), 43 (9), 41 (4). ^{1}H NMR($CDCl_3$): δ 7.18 (2H, d, J = 8.5 Hz, aromatic protons), 6.73 (2H, d, J = 8.5 Hz, aromatic protons), 6.26 (1H, s, OH), 4.43 (2H, s, Ar-CH_2O), 3.50 (2H, t, J = 7 Hz, protons italicized in O*C*$H_2$$CH_2CH_2CH_3$), 1.59 (2H, quintet, CH_2), and 1.39 (2H, sextet, CH_2), 0.90 (3H, t, J = 7 Hz, CH_3). ^{13}C NMR($CDCl_3$): δ 156.2, 131.2,130.0, 115.9 (all aromatic carbons), 73.2, 70.7 (carbons italicized in $C$$H_2OC$$H_2$$CH_2CH_2CH_3$), 32.4 ($CH_2$-O$CH_2$$C$$H_2$$CH_2CH_3$), 20.0 ($CH_2OCH_2CH_2$$C$$H_2$$CH_3$), and 14.6 ($CH_3$).

Vanillin (3). White crystals; mp 80−81 °C. (Lit. value: 82 °C, *8*). EI-MS (*m*/*z*, %): 152 (M^+, 100), 151 (M^+-H, 98), 137 (M^+-CH_3, 5), 123 (14), 109 (15), 93 (2), 81(17), 77 (4), 65 (5), 53 (7), 41 (1). Acetate: 194 (M^+, 8), 152 (M^+-42, 100), 137 (4), 123 (6), 109 (7), 95 (1), 79 (4), 63 (5), 51 (6), 43 (16). ^{1}H NMR ($CDCl_3$): δ 9.70 (1H, s). 7.40 (2H, m, aromatic protons), 7.01 (1H, d, J = 8.5 Hz, aromatic protons), 6.30 (1H, s, OH), 3.90 (3H, s, OCH_3).

4-Hydroxy-2-methoxycinnamaldehyde (4). Yellowish powder. EI-MS (*m*/*z*, %): 178 (M^+, 100), 177 (M^+-H, 30), 163 (M^+-CH_3, 10) 161 (M^+-OH, 21), 147 (M^+-OCH_3, 38), 135 (44), 124 (16), 118 (16), 107 (30), 89 (16), 77 (26), 63 (10), 55 (4), 51 (12). Acetate: 220 (M^+, 36), 178 (M^+-42, 100), 163 (85), 145 (5), 134 (7), 17 (3), 105 (2), 89 (5), 77 (6), 63 (1), 51 (3), 43 (35). ^{1}H NMR (acetone-d_6): δ 9.65 (1H, d, J = 6 Hz), 7.50−7.70 (3H, m, aromatic protons), 6.80−6.90 (2H, m, olefinic protons), 3.85 (3H, s, OCH_3).

3,4-Dihydroxyphenylacetic acid (5). Yellow crystals; mp 126−127 °C (Lit. value: 129 °C, *8*). EI-MS (*m*/*z*, %): 168 (M^+, 40), 151 (M^+-OH, 1), 139 (5), 123 (M^+-COOH, 100), 111 (6), 94 (10), 77 (14), 65 (12), 51 (16). Acetate: 252 (M^+, 8), 220 (M^+-42, 35), 168 (M^+-42 × 2, 100), 151 (4), 139 (5), 123 (55), 111 (9), 93 (10), 77 (3), 65 (6), 55 (5), 43 (38). ^{1}H NMR (acetone-d_6): δ 7.0−8.50 (2H, br, OH×2), 6.60−6.70 (2H, m, aromatic protons), 6.39 (1H, s, aromatic proton), 3.15 (2H, s, Ar-CH_2 CO).

Acetylation of Compounds 1−5. One milligram of each compound was dissolved in pyridine and then a few drops of acetic anhydride were added to that solution. The resulting reaction was kept at room temperature overnight until acetylation was completed (monitored by TLC). Excess acetic anhydride and pyridine were removed under a stream of nitrogen. The residue was extracted with methylene chloride and subjected to mass spectrometry. The mass spectrum of the acetate was compared with that of each original compound and molecular weight increase was calculated.

General Spectroscopic Procedures. H (300 MHz) NMR spectra were measured on a General Electric QE-300Mz NMR spectrometer, and the C-13 spectrum of 4-butoxymethylphenol was obtained on a General Electric Omega-500Mz NMR spectrometer. Deuterated chloroform or deuterated acetone was used as solvent, and tetramethylsilane (TMS) was used as internal standard. Mass spectra were recorded on a Hewlett-Packard HP-5973 mass selective detector in the EI mode at 70 eV.

Mosquito Larvae Bioassay Screening Protocol. Mosquito eggs, *Culex pipiens*, were purchased from Carolina Biological Supply Company, Burlington, North Carolina. After purchase, the eggs were transferred into a culturing beaker with distilled water, into which a pinch of powdered milk was added. Within a short time, larvae were produced from the eggs. The larvae were then allowed to grow until they were approximately 7 days old, when the bioassays were conducted. Flat-bottom multiwell polystyrene cell culture plates with 24 wells were used for performing the bioassay. Bioassay solutions containing predetermined amounts of extracts were prepared in water for water-soluble samples or prepared in water with the presence of a trace amount of dimethyl sulfoxide (DMSO) for the ethyl acetate extract and the pure compounds isolated. In each case that DMSO was employed to prepare the solution, the same volume of DMSO was also added to each water control well. Five 7-day old larvae and a sample solution were then transferred into each well. The survivors of mosquito larvae were counted from 0.5 h to 24 h later at room temperature. The same protocol was used for controls containing distilled water with or without the presence of DMSO. All bioassays were performed in triplicate. It was found that the crude extract was lethal to the larvae and the extract was partitioned into ethyl acetate, butanol, and water fractions. Each fraction was then screened for bioactivity against mosquito larvae. The ethyl acetate fraction at the concentration of 1.0−2.0 mg/mL displayed a very strong toxicity to mosquito larvae, resulting in total lethality to larvae within only a few hours. Larvae exposed to either the butanol or water fraction showed little or no activity when used at the same concentration. Tests with the water fraction indicated that it might even have been slightly beneficial to larvae.

Table 1. Percent Mortality of Mosquito Larvae Exposed to Compounds 1−5 at Different Time Intervals

	0.5 h	3 h	5 h	10 h	24 h
distilled H_2O	0	0	0	0	0
compound **1**					
0.1 mg/mL	0	0	0	11	50
0.3 mg/mL	0	7	47	53	73
0.5 mg/mL	0	27	61	100	100
compound **2**					
0.05 mg/mL	0	0	0	17	50
0.1 mg/mL	0	67	73	83	100
0.2 mg/mL	10	100	100	100	100
0.4 mg/mL	60	100	100	100	100
compound **3**					
1.0 mg/mL	0	0	11	50	100
2.0 mg/mL	0	15	39	94	100
compound **4**					
0.6 mg/mL	0	0	17	33	67
1.0 mg/mL	0	0	33	50	100
compound **5**					
0.4 mg/mL	0	0	0	0	0
1.0 mg/mL	0	0	0	0	17

RESULTS AND DISCUSSION

Phenolic Compounds Isolated and Their Biological Activity against Mosquito Larvae. The mortality of mosquito larvae exposed to compounds **1**−**5** is listed in Table 1. In 10 h, compound **1** killed half of the tested mosquito larvae at the concentration of 0.3 mg/mL and killed all the larvae at the concentration of 0.5 mg/mL. Compound **2** killed 67% of the larvae at a concentration of 0.1 mg/mL and killed all the larvae at the concentration of 0.2 mg/mL in 3 h. At 1.0 mg/mL of compound **3** half of the larvae were killed within 10 h and all the larvae were killed in 24 h. The toxicity of compound **4** to the larvae was similar to that of compound **3** when the same concentration was used. Compound **5** exhibited no toxicity to the larvae when the concentration was below 1.0 mg /mL.

Phenolic and aromatic compounds were the most abundant ingredients in the ethyl acetate extract of *Vanilla fragrans* leaves and stems. Many naturally occurring phenolic compounds are known to possess biological activity in animals, microorganisms, and plants (*9*). Compounds **1**−**5** are phenolic derivatives. However, the biological activities of these ingredients have not yet received attention. 4-Ethoxymethylphenol (**1**) was the most abundant component, which was obtained at a 21% yield. But vanillin (**3**) and 4-hydroxy-2-methoxycinnamaldehyde (**4**) were found to exist in the extract in only small quantities. It has been reported that vanillin does not exist in the free form in freshly harvested vanilla beans (*10*). The present investigation, however, revealed that it might exist in fresh leaves and stems, even if only in trace amounts. It cannot be discounted that vanillin may be present as a breakdown product of another compound. Compounds **4** and **5** were isolated from *Vanilla* species for the first time. Compound **2** has not been reported as a naturally occurring compound to this date.

Compound **1** was proven to be a biologically active component that displayed 100% mortality to mosquito larvae in 10 h at the tested concentration of 0.5 mg/mL in water. Compound **2** was the most active of these compounds, displaying considerable mortality to mosquito larvae within 24 h at the concentration between 0.05 and 0.1 mg/mL. As an example of the toxicity of compound **2** to mosquito larvae, a solution of 0.2 mg/mL of compound **2** killed 100% of all larvae tested within only 3 h. Vanillin (**3**) and compound **4** were aromatic aldehydes, and it was interesting that they exhibited similar activity toward the larvae. They resulted in the death of the tested larvae in relatively concentrated solution, compared with solutions using compounds **1** or **2**. A solution of 1.0 mg/mL of compound **3** or **4** caused death of the larvae during 20 to 24 h. Unexpectedly, compound **5** did not have an effect on mosquito larvae within long periods of time, even at the concentration of 1.0 mg/mL. The mechanism by which toxicity to mosquito larvae is conferred is not known. Following exposure to compound **4** or **5** for a few hours, the inner transparent bodies of the larvae changed to the yellow color of the corresponding compounds. The larvae exposed to compounds **1** or **2** for a short time appeared shrunken after death. These observations, although open to interpretation, suggest the larvae were not repelled by those phenolic compounds, some of which are bitter tasting to human beings (*11*), indicating that antifeedant activity was not a factor.

Identification of Compounds. Compound **1** (420 mg) was a yellowish oil at room temperature. The mass spectrum of compound **1** indicated M^+ at *m*/*z* 152, suggesting a molecular formula as $C_9H_{12}O_2$. The major fragment ions at *m*/*z* 123, 107, 95, 77, 65, and 51 indicated the presence of a substituted benzene skeleton in the molecule. The significant ions at *m*/*z* 123 (M^+-29, 12%) and 107 (M^+-29-16, 100%) suggested the existence of an ethoxyl group in the molecular structure. In the mass spectra, the M^+ of its acetate was observed at *m*/*z* 194 (42 mass units higher than that of compound **1**), which showed that there was a free hydroxyl group in this molecule. In the ^{1}H NMR of compound **1**, characteristic proton signals of *p*-substituted benzene were observed at δ 7.18 (2H, d, J = 8.5 Hz) and 6.34 (2H, d, J = 8.5 Hz). Other signals at δ 7.50 (1H, broad s), 4.45 (2H, s) 3.60 (2H, q, J = 7 Hz), and 1.25 (3H, t, J = 7 Hz) reinforced the existence of a free hydroxyl group and an ethoxymethyl group in the molecule. On the basis of the evidence mentioned above, the structure of compound **1** was identified as 4-ethoxymethyl-phenol. This compound was also reported to be isolated from fresh *Gastrodia elata* (*12*).

Compound **2** (21 mg) was also a yellowish oil. The M^+ at *m*/*z* 180 in the mass spectrum suggested its molecular formula as $C_{11}H_{16}O_2$. The presence of major fragment ions at *m*/*z* 123, 107, 95, 77, 65, and 51 was similar to that of compound **1**, which indicated that the compound was also a substituted benzene. In the mass spectrum of its acetate, the M^+ was observed at *m*/*z* 222, which confirmed the existence of a free hydroxyl group in molecule. The ^{1}H NMR of compound **2** was also similar to that of compound **1** except for two more groups of proton signals due to two more CH_2 groups, which were observed at δ 1.59 (2H, quintet) and 1.39 (2H, sextet). The signals of 1,4-disubstituted benzene appeared at δ 7.18 (2H, d, J = 8.5 Hz) and 6.73 (2H, d, J = 8.5 Hz). The signals at δ 6.26 (1H, broad s), 4.43 (2H, s), 3.50 (2H, t, J = 7Hz), and 0.90 (3H, t, J = 7 Hz) confirmed the existence of a butoxymethyl group in the molecule. Therefore, the structure of compound **2** was determined to be 4-butoxymethylphenol. The structure was reinforced by the observation of carbon signals in its carbon-13 spectrum at δ 156.2, 131.2, 130.0, 115.9 (all aromatic carbons), 73.2, 70.7 (two oxygenated carbons), 32.4, 20.0 (carbons of two CH_2 groups), and 14.6 (methyl carbon).

This is the first report of 4-butoxymethylphenol as a naturally occurring compound.

Compound **3** (3 mg) was white crystals. The mass spectrum of compound **3** gave M^+ at *m/z* 152, suggesting its molecular formula as $C_8H_8O_3$. In the mass spectrum of its acetate, M^+ was observed at *m/z* 194, which confirmed the existence of a free hydroxyl group in molecule. The compound was identified as vanillin by comparing its mass spectrum and ^1H NMR spectrum with that of an authentic sample.

Compound **4** (4 mg) was a yellowish powder. In the mass spectrum of compound **4**, the M^+ was observed at *m/z* 178, and fragment ions of substituted benzene appeared at *m/z* 51,55, 63, and 77, suggesting its molecular formula as $C_{10}H_{10}O_3$. The presence of a hydroxyl group and a methoxyl group were suggested in the molecule by the observation of signals at *m/z* 161 (M-OH, 21%), 163 (M-CH_3, 10%), and 147 (M-OCH_3, 38%). In the mass spectrum of its acetate, M^+ at *m/z* 220 reinforced the existence of a hydroxyl group. In the ^1H NMR spectrum of compound **4**, the presence of the methoxyl group and an aldehyde group were confirmed by the signal at δ 3.85 (3H, s) and 9.60 (1H, d, $J = 6$ Hz). The compound was finally identified as 4-hydroxy-2-methoxycinnamaldehyde by analysis of all the NMR signals and a comparison with that of related compounds (*8*, *13*). The existence of this compound in sunflower aroma has been reported (*14*).

Compound **5** (16 mg) was yellow crystals. The mass spectrum of compound **5** gave M^+ at *m/z* 168, suggesting its molecular formula as $C_8H_8O_4$. Signals due to a benzene ring were found at *m/z* 51, 65, and 77. The base peak was observed at *m/z* 123 (M^+-COOH), indicating the formation of a very stable fragment ion after the loss of a carboxyl group from the molecule. Two hydroxyls were confirmed after acetylation by observation of signals at *m/z* 252 (M^+), 210 (M^+-42), and 168 (M^+-42 $\times$ 2) in the mass spectrum. In the ^1H NMR spectrum of compound **5**, the presence of a carboxymethyl (CH_2-COOH) attached to a benzene ring was suggested by a signal at δ 3.14 (2H, s). The above-described evidence indicated that compound **5** is a trisubstituted benzene. The observation of identical signals of ^1H NMR between compound **5** and 3,4-dihydroxyphenylacetic acid revealed that they were same compound (*12*).

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