Long-Chain γ-Pyrones in Epicuticular Wax of Two *Vanilla* Bean Species: *V. fragrans* and *V. tahitensis*

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Analyses of the neutral lipids from *Vanilla* species before saponification resulted in the identification of a new product family in this genus: long-chain γ-pyrone compounds with an aliphatic chain containing a cis double bond at the $n-9$ position. These compounds represent 7–8% of the neutral lipids in mature beans. Using NMR and gas chromatography/mass spectrometry, three γ-pyrones have been identified, including 2-(10-nonadecenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one, 2-(12-heneicosenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one, and 2-(14-tricosenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one. The major constituent was 2-(14-tricosenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one, which represented 70.3% of the γ-pyrone fraction. The variability of this compound family has been studied in relation to bean maturity in *V. fragrans* and *V. tahitensis* beans. This compound family has not been found either in leaves or in stems or in *V. madagascariensis* beans.

INTRODUCTION

Among the orchid species belonging to the *Vanilla* genus, two species are cultivated for their beans' aroma, *V. fragrans* and *V. tahitensis*. Natural vanilla aroma is composed of a large number of volatile substances, and >200 have been identified (Klimes and Lamparsky, 1976). In recent studies, we have presented our results on the composition of the neutral lipid fraction, which also contributes to the vanilla aroma. We have studied the composition of the unsaponifiable matter in neutral lipid fraction of vanilla beans and more particularly the composition of the hydrocarbon fraction (Ramaroson-Raonizafinimanana et al., 1997), 4-demethylsterol and triterpene alcohol fractions (Ramaroson-Raonizafinimanana et al., 1998). However, the study of the lipophilic fraction after saponification does not allow to us characterize all of the compound families that can be encountered in vanilla beans. Recently, we have characterized three new γ-pyrones in the lipophilic fraction before saponification. γ-Pyrones are versatile intermediates in organic chemistry for the synthesis of biologically important molecules. For this purpose, several methods have been developed for dihydropyran-4-one ring construction (Evans and Nelson, 1996) and more particularly for 2-functionalized 2,3-dihydropyran-4-ones (Peterson and Kirchhoff, 1990). The 2-functionalized 2,3-dihydropyran-4-ones encountered were synthetic products and were 2,5- or 5,6-disubstituted (Zawacki and Crimmins, 1996; Evans and Nelson, 1996).

Hyperbrasilone, a γ-pyrone, has been isolated from a dichloromethane extract of stems and roots of *Hyericum brasiliense* (Rocha et al., 1994). A 2,6-disubstituted 2,3-dihydropyran-4-one, called erinapyrone B, has been isolated from *Hericum erinaceum* (Noda et al., 1996). The sum of the γ-pyrone fraction from aerial parts of *Gentianopsis barbata* was studied by Tankhaeva and Nikolaeva (1989). In this paper, we present the isolation and characterization of three new 2,6-disubstituted 2,3-dihydropyran-4-ones from vanilla beans.

MATERIALS AND METHODS

Vanilla Bean Materials. *V. fragrans* beans (12 samples) were collected in Comoros, Madagascar, Hawaii, and Indonesia. *V. tahitensis* beans (four samples) were collected in Tahiti. *V. madagascariensis* beans (native species from Madagascar, one sample) were collected in the Antalaha area (northeastern Madagascar). Mature vanilla beans were collected during the ninth month after pollination. Immature vanilla beans were collected between the fifth and the seventh months after pollination. Commercial vanilla beans have undergone a drying and curing processes during which their characteristic aroma and flavor are developed.

Neutral Lipid Extraction. Neutral lipids were obtained from crushed beans (40 g) using Soxhlet extraction with pentane (150 mL, 16 h).

Column Chromatography. The neutral lipid extract (0.4 g) was fractionated by column chromatography (CC; 25 × 2.4 cm) over silica gel (silica gel 60, 230–400 mesh ASTM, 50 g, Merck 9385) using *n*-hexane/Et₂O (90:10 v/v, 600 mL). Fractions (20 mL) were collected, and each was checked using thin-layer chromatography (TLC) on precoated plates (5 × 10 cm, silica gel 60 F₂₅₄, 0.25 mm, Merck 5729), eluent *n*-hexane/Et₂O (90:10 v/v). Spots were visualized using the phosphomolybdic acid spray reagent. Tubes 21–25 contained γ-pyrones (approximate R_f values of 0.05–0.10). γ-Pyrone fractions represented 52.5–55.5% of the neutral lipid fraction.

Gas Chromatography (GC). A Girdel 30 gas chromatograph equipped with a flame ionization detector (FID) was used for compound separations with an OV-1 fused silica capillary column (25 m × 0.31 mm i.d.) (phase thickness, 0.15 μm; column temperature, 160–280 °C, 3 °C min⁻¹). Detector

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Table 1. Neutral Lipid Content[a] of Commercial and Green *Vanilla* Beans Investigated

origin	V. fragrans		V. tahitensis		V. madagascariensis
	comm	green	comm	green	comm
Tahiti			9.3	2.2[b]−4.2	
Comoros	13.3[b]				
Hawaii	11.5				
Reunion	10.8	2.8[c]			
Madagascar	10.3[d]				14.0
Indonesia	8.6				

[a] Weight percentage based on dry material (± 0.5%). [b] Mean value from two samples. [c] Mean value from three samples. [d] Mean value from four samples.

and inlet temperatures were 300 and 295 °C, respectively. Hydrogen was used as a carrier gas at an inner pressure of 0.5 bar (3 mL/min, split 60 mL/min). The injections averaged 1 μL of a 0.5% solution in *n*-hexane of crude mixtures. For quantitative analyses, the injections averaged 1 μL of a solution prepared as follows: To 1 mL of the solution A [raw extract (200 mg) in *n*-hexane (100 mL, 2 mg/mL)] was added 0.25 mL of the solution B [octacosane (25 mg) in *n*-hexane (100 mL, 0.25 mg/mL)] used as internal standard. Retention indices (I_R) on OV-1 were calculated using the formula

$$IR = 100N + 100n(t'_{R(X)} - t'_{R(N)})/(t'_{R(N+n)} - t'_{R(N)})$$

where $t'_{R(X)}$ is the relative retention time of the substance and $t'_{R(N)}$ and $t'_{R(N+n)}$ are the relative retention times of *n*-paraffin hydrocarbons of carbon number N and $(N + n)$ that are, respectively, smaller and larger than $t'_{R(X)}$.

GC/Mass Spectrometry (GC/MS). Combined GC/MS was carried out on a Girdel gas chromatograph linked to a Ribermag R-10-10B mass spectrometer equipped with a quadrupole mass analyzer (15.6 mm i.d. × 350 mm, 10^{-6} mmHg) and coupled with a Sidar data computer. The GC column was an OV-1701 fused silica capillary column (50 m × 0.32 mm, 0.30 μm phase thickness). The column temperature was 100−280 °C, 3 °C min^{-1}; the carrier gas, helium (2 bar, 4 mL/min, split 80 mL/min); the ion source temperature, 270 °C; and the ionizing voltage, 70 eV.

Infrared (IR) Spectroscopy and Ultraviolet (UV) Spectroscopy. IR spectra were recorded on a Perkin-Elmer Model 457 infrared spectrophotometer. UV spectra were recorded on a DU-20 Beckman ultraviolet spectrophotometer.

Nuclear Magnetic Resonance (NMR) Spectroscopy. All spectra were recorded on a Bruker AM-200 spectrometer. The NMR spectra were measured as solutions in chloroform-*d* in 5 mm o.d. tubes for ^{13}C and ^{1}H. Tetramethylsilane was used as internal standard in both measurements. Resonance multiplicities for ^{13}C were established via the acquisition of DEPT spectra (Doddrell et al., 1982) obtained for proton pulses $P = 90°$ (CH only) and $P = 135°$ (CH and CH$_3$ differentiated from CH$_2$).

RESULTS AND DISCUSSION

The lipid content of various *Vanilla* beans samples (*V. fragrans*, *V. tahitensis*, and *V. madagascariensis*)
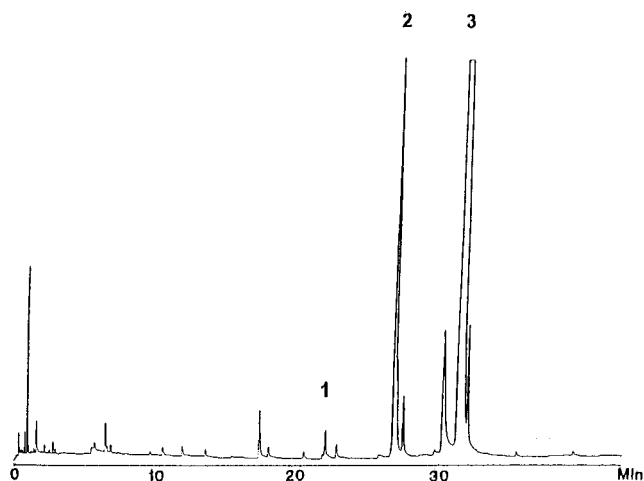
Figure 1. Gas chromatogram of lipidic neutral extract of γ-pyrone compounds **1**−**3** obtained by GC on OV-1 column at 160−280 °C, 3 °C min^{-1}.

obtained using *n*-pentane extraction varied from 8.6 to 14.0% for commercial beans and from 2.2 to 4.2% for green beans (Table 1). TLC of the neutral lipid fraction, eluent *n*-hexane/Et$_2$O (90:10 v/v), revealed the presence of a polar fraction (approximate R_f value of 0.05−0.10). This fraction was isolated by CC over silica using *n*-hexane/Et$_2$O and represented 54% (mean value) of the neutral lipid fraction. GC analysis of this fraction showed three major compounds, **1**−**3** (Figure 1).

Structure Determination. Identification of compounds **1**−**3** was achieved by UV, IR, and GC/MS analyses and NMR. The UV spectrum of the mixture of compounds **1**−**3** showed a wavelength absorption with $\nu_{max} = 260$ nm, which is consistent with the presence of an enone group. The IR spectrum showed absorptions at 1640 and 1688 cm^{-1} characteristic of a pyrone moiety. Mass spectra of compounds **1**−**3** showed molecular ion peaks at *m/z* 376 (2.5%), 404 (15.1%), and 432 (23.0%), respectively. A fragmentation ion at *m/z* 111 (100, 100, and 95%) indicated the loss of an aliphatic chain, in agreement with a pyrone moiety. Compounds **1**−**3** were then γ-pyrones with an enone group, disubstituted by a methyl group and a long aliphatic chain having 19, 21, and 23 carbon atoms. Furthermore, characteristic fragments of monounsaturated hydrocarbons were observed at *m/z* 69 (11.1, 36.6, and 50.0% for compounds **1**−**3**, respectively), *m/z* 55 (21.1, 59.2, and 82.8%), *m/z* 43 (22.6, 60.9, and 84.9%), and *m/z* 41 (12.2, 29.4, and 40.7%). The absence of an absorption at 967 cm^{-1}, characteristic of a trans-disubstituted bouble bond, is in agreement with a cis-disubstituted double bond located in the fatty chain (Madison et al., 1982). The occurrence of a fragment at *m/z* 125 (1.0, 3.2, and 3.8%, respectively, in **1**, **2**, and **3**) corresponding to a C$_9$H$_{17}$
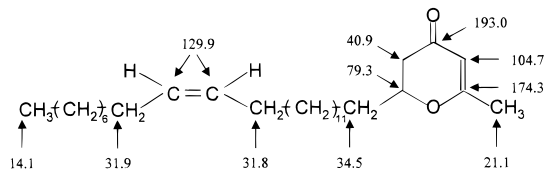
Table 2. Relative Compositions of the γ-Pyrone Fraction, Formulas, and Retention Indices of Compounds 1−3

compd	formula	I_R[a,b]	relative compos (%)[a]
1		2854	0.9
2		3077	16.1
3		3298	70.3

[a] Determined on a 25 m capillary column (OV-1). [b] Retention indices.
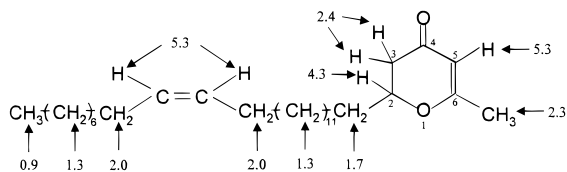
Figure 2. ¹H chemical shift of 2-(14-tricosenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one (**3**).

Figure 3. ¹³C chemical shift of 2-(14-tricosenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one (**3**).

Table 3. γ-Pyrones Composition[a] of *V. fragrans* Commercial Beans of Different Origins and Stages of Maturity

compd	Comoros immature[b]	Madagascar immature[b]	Madagascar mature[c]	Hawaii mature[c]	Indonesia mature[c]
2	1.5	1.9	1.4	1.6	0.8
3	5.8	6.5	5.0	5.6	2.6

[a] Weight percentage based on lipidic extract and determined on OV-1 capillary columns with octacosane as internal standard. [b] Beans were collected during the seventh month after pollination. [c] Mature beans were collected during the ninth month after pollination.

group was in agreement with an *n*−9 position of the double bond. The ¹H and ¹³C chemical shifts of **3** are given in Figures 2 and 3. The ¹H chemical shifts of **3** were consistent with 2,3-dihydro-γ-pyrones disubstituted by a methyl group at the C-6 position and by the fatty chain at the C-2 position. The ¹³C chemical shifts of **3** was consistent with published data for the pyrone moiety in 2,2,6-trimethyl-2,3-dihydro-4*H*-pyran-4-one (Boring and Sindelar, 1988). Structure elucidations were in agreement with 2-(10-nonadecenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one for compound **1**, 2-(12-heneicosenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one for compound **2**, and 2-(14-tricosenyl)-2,3-dihydro-6-methyl-4*H*-pyran-4-one for compound **3**.

Quantitative Analysis. The relative composition of the pyrone fraction and retention indices on OV-1 of compounds **1**−**3** are given in Table 2. Compound **3** was the major component and represented 70.3% of γ-pyrones. The two others compounds, **1** and **2**, were found in a slight concentration. A quantitative study of the γ-pyrones was achieved by GC analysis with octacosane as internal standard (retention index of 2800). We first studied the percentage of this compound family in the lipidic fraction from *V. fragrans* commercial beans of different origins and different stages of maturity. Results are summarized in Table 3. For all of the samples, compound **3** was the major component (2.6−5.8%, based on lipidic extract). Mature beans from Indonesia were characterized by a lower content of **2** and **3** (1.5 and 2.6%, respectively). For all of the samples including various countries of origins, the γ-pyrone percentages were higher in immature beans than in mature beans and the sums ranged from 7.3 to 8.4% and from 3.4 to 7.2%, respectively. These results were confirmed when we studied the lipidic fraction of green beans (mature and immature from Madagascar and Tahiti). The results obtained with green *V. fragrans* (from Madagas-

Table 4. γ-Pyrone Composition[a] of Mature and Immature *Vanilla* Green Beans

compd	*V. fragrans*[d] immature[b]	*V. fragrans*[d] mature[c]	*V. tahitensis*[e] immature[b]	*V. tahitensis*[e] mature[c]
2	2.0	1.3	0.6	0.5
3	9.0	6.3	5.5	5.2

[a] Weight percentage based on lipidic extract and determined on OV-1 capillary columns with octacosane as internal standard. [b] Beans were collected during the seventh month after pollination. [c] Mature beans were collected during the ninth month after pollination. [d] From Madagascar. [e] From Tahiti.

car) and *V. tahitensis* (from Tahiti) beans are given in Table 4. As in the case of commercial beans, **3** was the main γ-pyrone compound in green beans with a content ranging between 6.3 and 9.0% in *V. fragrans* and between 5.2 and 5.5% in *V. tahitensis*. Percentages were higher in immature beans than in mature beans. The decreasing γ-pyrone content during bean development shows that the curing and drying processes in commercial beans have a little influence on the γ-pyrone relative contents and percentages in lipidic vanilla bean extracts.

The occurrence of γ-pyrones in these extracts may play a biological role because such family compounds are known to have anti-inflammatory effects (Lin et al., 1996) and potent antiplatelet effects (Lin et al., 1995).

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