Occurrence of 5α-Cholesta-7,24-dien-3β-ol and 23-Dehydrolophenol in the Bean Lipids of *Vanilla madagascariensis*

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ABSTRACT: Eleven 4-desmethylsterols, four 4,4-dimethylsterols, and twelve 4-methylsterols were identified in *Vanilla madagascariensis* beans. The 4-desmethylsterol fraction was characterized by a high level of 5α-cholesta-7,24(25)-dien-3β-ol (35.3%). The 4-methylsterol fraction was characterized by a high level of 31-norcycloartenol (57.5%) and the presence of 23-dehydrolophenol (9.4%). Cycloartenol (72.9%), cyclosadol (12.7%), parkeol (9.7%), and 24-dehydrotirucallol (4.7%) were identified in the 4,4-dimethylsterol fraction.
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KEY WORDS: 5α-Cholesta-7,24-dien-3β-ol, 23-dehydrolophenol, 4-desmethylsterols, GC–MS, 4-methylsterols, Orchidaceae, triterpene alcohols, *V. madagascariensis*, Vanilla beans.

Among the various orchids belonging to the genus *Vanilla*, *V. madagascariensis* is native to the west coast of Madagascar (1). The plant has a very long stem (4 to 8 m) and grows on shrubs or trees. The fruit (or bean) of *V. madagascariensis* is cylindrical, 15 to 20 cm long × 1 cm diameter, but has no vanilla flavor like *V. fragrans* or *V. tahitensis* after drying and curing. Previous work on the chemistry of oleaginous plants has been reported (2–6). An extensive examination of the lipid fraction of *V. madagascariensis* revealed that the 4-desmethylsterol, 4-methylsterol, and 4,4-dimethylsterol fractions represented more than 50% of the unsaponifiable lipids. To our knowledge, 23-dehydrolophenol is described for the first time, and 5α-cholesta-7,24-dien-3β-ol is described for the first time in a plant. The latter sterol has been identified as a major sterol of the male hamster reproductive tract (7). 5α-Cholesta-7,24(25)-dien-3β-ol was characterized as a minor sterol of the marine animal chiton (*Liophura japonica*) (8). The biosynthesis of 5α-cholesta-7,24(25)-dien-3β-ol in a yeast homogenate has been achieved (9–12). *In vitro* studies of lipid production by rat renal papillae showed that the sterol fraction contained demosterol, lathosterol, and 5α-cholesta-7,24(25)-dien-3β-ol (13,14).

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EXPERIMENTAL PROCEDURES

Vanilla bean materials. Vanilla madagascariensis (native species from Madagascar) was collected in the Antalaha area (northeast of Madagascar) during the seventh month after pollination of flowers. The green beans were submitted to the same drying and curing processes as commercial vanilla beans for developing characteristic aroma and flavor, but this species does not contain vanilla aroma.

Extraction of unsaponifiable. Neutral lipids were obtained from crushed beans (40 g) by Soxhlet extraction with pentane (150 mL, 16 h) (14% yield based on dry material). The unsaponifiable fraction was obtained according to Itoh *et al.* (15). Water (40 mL) was added to the reaction mixture (10 mL), and unsaponifiable matter was extracted with three 30-mL vol of diisopropyl ether. The organic layer was washed with three 4-mL vol of a 10% aqueous carbonate solution, then washed with distilled water to neutrality. After drying and evaporation, the unsaponifiable matter was obtained at a yield of 20.8% (mass percentage expressed with respect to neutral lipid fraction).

Column chromatography (CC). The unsaponifiable extract (0.525 g) was fractionated by CC (46 × 3 cm) over alumina gel [Aluminoxid 90 II-III, Merck (Darmstadt, Germany) 1097, 70–230 mesh ASTM, 3 to 6% hydration, 200 g) with hexane (200 mL), hexane/benzene (50:50, vol/vol, 200 mL), hexane/benzene (20:80, vol/vol, 200 mL), hexane/diethyl ether (80:20, vol/vol, 200 mL), hexane/diethyl ether (60:40, vol/vol, 600 mL), and hexane/diethyl ether (50:50, vol/vol, 800 mL). Fractions (25 mL) were collected and separated by thin-layer chromatography (TLC) on precoated plates (5 × 10 cm, silica gel 60 F_{254}, 0.25 mm; Merck), developed in $CHCl_3$-Et_2O (90:10, vol/vol). Spots were visualized by phosphomolybdic acid spray reagent. Tubes 26 to 33 contained 4,4-dimethylsterols (R_f, 0.47 to 0.54). Tubes 42 to 49 contained 4-methylsterols (R_f, 0.44 to 0.46). Tubes 50 to 57 contained 4-desmethylsterols (R_f, 0.32 to 0.41).

Liquid chromatography (LC) analysis. The 4-methylsterol fraction obtained by CC was then fractionated by LC before gas chromatography (GC) analysis. A 4 × 250 mm Lichrosorb (Merck) RP8 5 μm column was used, according to Bianchini *et al.* (16). The liquid chromatograph was equipped with a

SPD-2A Shimadzu (Thouzart et Matignon, Paris, France) spectrophotometer and an Analprep 93 pump (Thouzart et Matignon, Paris, France). Samples (20 μL) were eluted with acetonitrile-water (90:10, vol/vol) at a flow rate of 1 mL/min.

GC and GC–mass spectrometry (GC–MS). The 4,4-dimethylsterol, 4-methylsterol, and 4-desmethylsterol fractions (1 mg) were acetylated with acetic anhydride (0.1 mL) and pyridine (0.1 mL) according to NFT 60-232 official method (17) before GC analysis. A Girdel 30 (Girdel, Paris, France) gas chromatograph, equipped with a flame ionization detector (FID), was used. The column was an OV 17 glass capillary column (25 m × 0.36 mm i.d., 0.30 μm) and operated at a column temperature of 260°C. Detector and inlet temperatures were 290 and 280°C respectively. Hydrogen was used as carrier gas at an inner pressure of 0.9 bar. An alternate column was an OV 1 glass capillary column (25 m × 0.31 mm i.d., 0.15 μm) and operated at a column temperature of 260°C. Detector and inlet temperatures were 290 and 280°C, respectively. Hydrogen was used as carrier gas at an inner pressure of 0.5 bar. The injection volume averaged 1 μL of a 0.5% solution of crude mixtures in hexane. GC–MS analysis was conducted with 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} mm Hg) and a Sidar (Strasbourg, France) data computer. The GC column was an OV 1701 fused capillary column (50 m × 0.36 mm, 0.30 μm phase thickness). The column temperature was 280°C, carrier gas, helium (2 bars); ion source, 270°C; ionizing voltage, 70 eV.

RESULTS AND DISCUSSION

Among the sterols contained in the unsaponifiable matter of *V. madagascariensis* beans, we studied the 4-desmethylsterol fraction. It represented 28.1, 5.8, and 0.8% w/w of the unsaponifiable portion, lipid, and bean weights, respectively. Eleven 4-desmethylsterols were analyzed by GC and GC–MS. The composition of the desmethylsterol fraction is given in Table 1. Identification of 5α-cholesta-7,24(25)-dien-3β-ol was accomplished by using relative retention times (RRT) and MS. As shown in Table 2, the influence of the side chain on RRT is the same in the Δ^5 and Δ^7 sterol series. Therefore, the ratio RRT (Δ^5/Δ^7) for sterols with the same side chain is constant (0.84). Results were consistent with literature values (18,19). However, the sterol with RRT 1.42 should be 5α-cholesta-7,24(25)-dien-3β-ol because the RRT of desmosterol is 1.20. This structure was confirmed by GC–MS of its acetate, which showed fragmentation patterns that were consistent with published data (20,21). The molecular ion peak was present at *m/z* 426 (8.7). Mass peak at *m/z* 313 (100) indicated a Δ^7 and a $\Delta^{24(25)}$ double bond. Other prominent fragments were observed at *m/z* 213 (17.3), 351 (15.4), 342 (15.4), 411 (9.8), and 255 (8.6).

Vanilla madagascariensis was characterized by high contents of 5α-cholesta-7,24(25)-dien-3β-ol (35%), desmosterol (14%), β-sitosterol (12%), stigmasterol (11%), ergosta-7,24(28)-dien-3β-ol (9%), and 24-methylene cholesterol (9%). Zymosterol, cholesterol, campesterol, and Δ^5 and Δ^7 avenasterol were found in lower amounts.

Vanilla madagascariensis was also distinguished by a high content in 4-methylsterols, which accounted for 20.9, 4.3, and 0.6% w/w of the unsaponifiable matter, lipid, and bean weights, respectively. Identification was based on comparison of their RRT with those found in the literature (18,19) and GC-MS of their acetate derivatives. The composition of the 4-methylsterol fraction is given in Table 3. Among the twelve 4-methylsterols identified, 31-norcycloartenol was the major constituent (57.5%). A new sterol, 23-dehydrolophenol, was identified for the first time to our knowledge and represented 9.4% of the 4-methylsterol fraction. MS data of its acetate derivative (4-methyl-5α-cholesta-7,23-dien-3β-ol-acetate) exhibited a mass peak at *m/z* 327 (100), which is characteristic of the lophenol family. The molecular ion peak was observed at *m/z* 440 (14.3). Fragmentation ions at 356 (15.4), 296 (1.9), and 281 (1.2) indicated a Δ^{23} double bond. Other prominent fragments were observed at *m/z* 227 (19.0), 425 (18.7), 365 (6.2), 269 (9.4), and 267 (5.9).

Gramisterol (9.2%) coeluated with 24-methylen-23-dehydrolophenol, and 4-methylergost-8(14)-en-3β-ol, which represented 4.7% of this fraction, coeluated with obtusifoliol.

TABLE 1
4-Desmethylsterol Composition[a] of *Vanilla madagascariensis* Beans

RRT[b]	4-Desmethylsterol	Relative composition (%)[a]
1.00	Cholest-5-en-3β-ol (cholesterol)	3.0[c]
1.20	Cholesta-5,24-dien-3β-ol (desmosterol)	14.3
1.27	5α-Cholesta-8,24-dien-3β-ol (zymosterol)	4.3
1.32	24-Methylcholest-5-en-3β-ol (campesterol)	0.9
1.36	24-Methylenecholest-5-en-3β-ol (24-methylene cholesterol)	8.9
1.42	5α-Cholesta-7,24(25)-dien-3β-ol	35.3
1.44	24-Ethylcholesta-5,*trans*-22-dien-3β-ol (stigmasterol)	11.2
1.60	24-Methylen-5α-cholest-7-en-3β-ol (ergosta-7,24(28)-dien-3β-ol)	9.1
1.63	24-Ethylcholest-5-en-3β-ol (β-sitosterol)	11.8
1.80	24(*Z*)-Ethylidencholest-5-en-3β-ol (Δ^5-avenasterol)	1.2
2.14	24(*Z*)-Ethylidene-5α-cholest-7-en-3β-ol (Δ^7-avenasterol)	Trace

[a]Determined on OV 17 capillary column.
[b]Relative retention time (RRT) expressed against cholesterol acetate on OV 17 glass capillary column at 260°C.
[c]Relative percentage determined without standard cholesterol added in the fraction.

TABLE 2
Influence of the Δ^5 or Δ^7 Double-Bond Position on Sterol RRT[a]

Δ^5-Sterol	Δ^5 RRT	Δ^7-Sterol	Δ^7 RRT	RRT (Δ^5/Δ^7)
Stigmasterol	1.43	Spinasterol	1.70	0.84
24-Methylene cholesterol	1.35	Ergosta-7,24(28)-dien-3β-ol	1.61	0.84
Δ^5-Avenasterol	1.81	Δ^7-Avenasterol	2.15	0.84
24E-Ethylidencholest-5-en-3β-ol (fucosterol)	1.72	28-Isoavenasterol	2.04	0.84
Desmosterol	1.20	5α-Cholesta-7,24(25)-dien-3β–ol	1.42	0.84

[a]Relative retention time (RRT) expressed against cholesterol acetate on OV17 glass capillary column at 260°C.

TABLE 3
4-Methylsterol Composition[a] of *Vanilla madagascariensis* Beans

RRT[b]	4-Methylsterol	Relative composition (%)[a]
1.22	4-Methylcholest-8-en-3β-ol	0.6
1.26	4α-Methyl-5α-cholesta-8,24-dien-3β-ol (4α-methylzymosterol)	3.7
1.28	4-Methyl-5α-cholest-7-en-3β-ol (lophenol)	5.1
1.30	4,14-Dimethyl-5α-cholesta-8,24-dien-3β-ol (31-norlanosterol)	1.9
1.32	31-Norcycloartanol	1.3
1.35	Unknown	1.9
1.36	4-Methyl-5α-cholesta-7,23-dien-3β-ol (23-dehydrolophenol)	9.4
1.46	31-Norcycloartenol	57.5
1.48	4,24-Dimethyl-5α-cholest-8(14)-en-3β-ol (4-methylergost-8(14)-en-3β-ol) + 4,14-Dimethyl-5α-cholest-8,24-dien-3β-ol (obtusifoliol)	4.7
1.52	4,14,24-Trimethyl-5α-cholest-8-en-3β-ol (24-dihydroobtusifoliol)	0.8
1.64	4-Methyl-24-methyliden-5α-cholest-7-en-3β-ol (gramisterol) + 4,24-Dimethyl-5α-cholesta-7,23-dien-3β-ol (24-methylen-23 dehydrolophenol)	9.2
1.68	24-Methyl-31-nor-23-dehydrocycloartenol	4.3
2.07	4-Methyl-24-ethyl-5α-cholesta-7-en-3β-ol (24-ethyllophenol)	0.1

[a]Determined on OV 1 capillary columns.
[b]Relative retention time (RRT) expressed against cholesterol acetate on OV1 glass capillary column at 260°C.

TABLE 4
4,4-Dimethylsterol Composition[a] of *Vanilla madagascariensis* Beans

RRT[b]	4,4-Methylsterol	Relative composition (%)[a]
1.23	4,4,14-Trimethylcholest-8-en-3β-ol (24-dehydrotirucallol)	4.7
1.77	4,4,14-Trimethylcholesta-9(11),24-dien-3β-ol (parkeol)	9.7
1.86	Cycloartenol	72.9
2.06	Cyclosadol	17.7

[a]Determined on OV 17 capillary columns.
[b]Relative retention time (RRT) expressed against cholesterol acetate on OV17 glass capillary column at 260°C.

Lophenol (5.1%), 24-methyl-31-nor-23-dehydrocycloartanol (or cycloeucalenol) (4.3%) and 4α-methylzymosterol (3.7%) were present with lower amounts of 4-methylcholest-8-en-3β-ol, 31-norlanosterol, cycloartanol, 24-dihydroobtusifoliol, and 24-ethyllophenol.

The 4,4-dimethylsterol fraction of *V. madagascariensis* represented 2.6, 0.5, and 0.07% w/w of the unsaponifiable matter, lipid, and bean weights, respectively. The composition is reported in Table 4. *Vanilla madagascariensis* was characterized by the presence of four 4,4-dimethylsterols, cycloartenol being the major constituent (72.9%). 24-Dehydrotirucallol, cyclosadol, and parkeol accounted for 4.7, 12.7, and 9.7% of the fraction, respectively.

Desmethylterol, methylsterol, and 4,4-dimethylsterols with a 24–25 double bond are the main compounds in these three families (53.9, 63.1, and 82.6%). The occurrence of Δ^5-sterols in plants rich in Δ^7-sterols suggests that the Δ^5-hydrogenase and the Δ^7-reductase exist, as is well known for plants rich in Δ^5-sterols (4). In conclusion, the co-occurrence of Δ^5- and Δ^7-sterols in this species may help to further clarify the biosynthesis of the phytosterol nucleus and evolution in higher plants.

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