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# Some key metabolic intermediates in the biosynthesis of botrydial and related compounds

Rosa Durán-Patrón, Ana Julia Colmenares, Rosario Hernández-Galán and Isidro G. Collado*

*Departamento de Química Orgánica, Facultad de Ciencias, Universidad de Cádiz, República Saharaui s/n, Apdo. 40, 11510 Puerto Real, Cádiz, Spain*

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**Abstract**—The isolation of three key intermediates from a culture of *Botrytis cinerea* sheds further light on the biosynthetic pathway to botrydial (**2**), and clarifies and establishes the stereochemistry of the oxidative steps which affect the probotryane skeleton to lead to the dialdehyde (**2**). The structures of these three new metabolites have been determined by means of extensive spectroscopic methods. © 2001 Elsevier Science Ltd. All rights reserved.

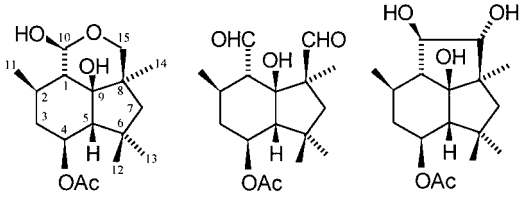
## 1. Introduction

*Botrytis cinerea* is well known as the source of characteristic metabolites with botryane skeleton, principally dihydrobotrydial (**1**) and botrydial (**2**).[1] Previous results obtained by our research group have shown that these metabolites are responsible for the typical lesions of the fungal infection and that they play an important role in the pathogenicity of the organism in vivo.[2–5] In particular, botrydial (**2**) has shown phytotoxic activity both in vitro[3] and in planta[6] on tobacco and bean leaves at a concentration of only 1 ppm, while the closely related compound dihydrobotrydial (**1**) shows similar activity at 250 ppm. Compound **2** has also exhibited interesting antibiotic activity against *Bacillus subtillus* and *Phytium debaryanum* at 100 ppm, as well as high cytotoxic activity against tumoral and non-tumoral cells, whereas dihydrobotrydial (**1**) was inactive in these cases.[1,4]

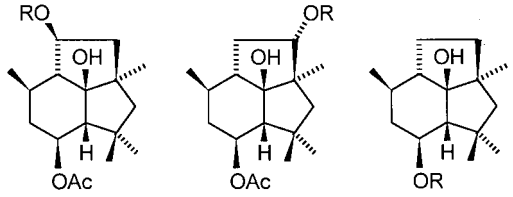
Much research has been done on the biosynthetic pathway to botrydial's (**2**) basic skeleton, which is a bicyclic, non-isoprenoid sesquiterpene system. After conducting biosynthetic studies with $^{13}$C-labelled substrates, Hanson and co-workers[7–12] were able to describe the way in which farnesyl pyrophosphate is folded to generate the carbon skeleton of the major sesquiterpenoid, dihydrobotrydial (**1**). However, none of the proposed intermediates[7–12] have ever been isolated; thus, the possibility that botrydial (**2**) is produced via an alternative biosynthetic pathway remains open. The detection of any of these metabolic intermediates in the culture broth would ultimately lead to a better understanding of the biosynthetic route to botrydial (**2**) and related compounds. The isolation of the tricyclic alcohol **3** by our research group[13] confirmed the last step in the botrydial (**2**) biosynthesis.

The high biological activity shown by botrydial (**2**) as a cytotoxin, as well as the fact that recent results suggest that (**2**) is associated with the pathogenic process of *B. cinerea*, prompted us to redouble our efforts to determine the biosynthetic route to botrydial (**2**).

This paper describes culture experiments designed to isolate the metabolic intermediates in the first steps of the biosynthesis of botrydial (**2**). We describe the isolation and structure elucidation of three new key intermediate




**1** **2** **3**




**4** R=H
**4a** R=Ac

**5** R=H
**5a** R=Ac

**6** R=H
**6a** R=Ac

*Keywords*: *Botrytis cinerea*; metabolic intermediate; biosynthesis; probotryane; botrydial.

* Corresponding author. Tel.: +34-956-016-372; fax: +34-956-01-6288/6193; e-mail: isidro.gonzalez@uca.es

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metabolites, **4**–**6**, which confirm the proposed configuration for the probotryane skeleton and complete the biosynthetic route to botrydial (**2**) from farnesyl pyrophosphate.

## 2. Results and discussion

The isolation of natural products from the fermentation broth of *B. cinerea* was carried out as described below in Section 3. Two experiments were conducted under different conditions in an attempt to obtain metabolites coming from different steps of the biosynthetic route to botrydial (**2**). In the first experiment, *B. cinerea* 2100 was grown in a orbital shaker on a Czapeck–Dox medium for 5 days. Under these conditions, two new metabolic intermediates were isolated: 4β-acetoxyprobotryane-9β,10β-diol (**4**) and 4β-acetoxyprobotryane-9β,15α-diol (**5**). In the second experiment, *B. cinerea* was incubated for 3 days, using a 48-h old culture as inoculum. Under these latter conditions a new intermediate, probotryane-4β,9β-diol (**6**), as well as compounds **4** and **5** were isolated.

Compound **4** was obtained as a colorless oil with the molecular formula $C_{17}H_{28}O_4$, as deduced from high-resolution mass spectral data. The spectroscopic data of this compound (see Section 3) were similar to those of **3**.[13] However, the $^1$H NMR spectrum showed a change in the multiplicity of the H-10 signal at $\delta_H$ 4.48 from dd in **3** to ddd in **4**. This signal was correlated in the COSY experiment with the signal corresponding to H-1, as well as with two new signals at $\delta_H$ 1.82 and 1.96, which were in turn correlated with each other. Furthermore, the $^{13}$C NMR spectrum showed the presence of only two signals corresponding to carbon atoms attached to hydroxyl groups ($\delta_C$ 82.8 (d) and 97.1 (s)). These data suggest that compound **4** is a deoxy derivative of **3** at C-15.

The proposed structure for compound **4** was confirmed after acetylation of the C-10 hydroxyl group in **4** to afford the monoacetate **4a**. The $^1$H NMR spectrum of **4a** showed a new acetate signal at $\delta_H$ 2.03, as well as a deshielding of the H-1, H-10, and H-15β signals. The stereochemistry *S* at C-10 was assigned first by comparison of **4** with **3** and later confirmed by means of NOE experiments. Irradiation of the H-10 signal caused enhancement of the H-2, H-14, and H-15α signals, both in the natural product (**4**) and in the acetyl derivative (**4a**), supporting the proposed stereochemistry for the hydroxyl group at C-10.

With the aid of high-resolution mass spectral data, together with that of $^{13}$C NMR (17 signals; see Section 3), the molecular composition of compound **5** was determined to be $C_{17}H_{28}O_4$. This compound is thus an isomer of 4β-acetoxyprobotryane-9β,10β-diol (**4**). The spectroscopic data of compounds **4** and **5** were very similar; however, the $^1$H NMR spectrum of **5** showed the signal at $\delta_H$ 4.6 as a triplet. This signal was correlated in the COSY experiment with two new signals at $\delta_H$ 2.71 and 1.65 (ddd, each 1H), which were in turn correlated with each other as well as with the signal corresponding to H-1. The triplet was therefore assigned to H-15. A HETCOR experiment showed a correlation between the signal corresponding to this proton and one at $\delta_C$ 75.1, indicating that the hydroxyl group in compound **5** is located at C-15. This was corroborated by the downfield shift of the H-10α and H-15 signals in the $^1$H NMR spectrum of the acetyl derivative (**5a**). The stereochemistry at C-15 was determined as *S* with the aid of NOE experiments: so, the irradiation of the signal assigned to H-10β produced enhancement of the signals at H-10α, H-1, and H-15 while irradiation of the H-15 signal enhanced the H-10β and H-7β signals.

Compound **6** was obtained as an oily material, the molecular formula of which, $C_{15}H_{26}O_2$, was deduced from mass spectral (*m/z* 238) and $^{13}$C NMR spectroscopic data. The IR absorption at 3370 cm$^{-1}$ and $^{13}$C NMR signals at $\delta_C$ 84.4 (s) and 68.2 (d) indicated the presence of two hydroxyl groups in the molecule. Its $^{13}$C NMR spectrum exhibited signals for four methyl ($\delta_C$ 14.6, 30.2, 22.6, and 30.5), four methylene ($\delta_C$ 39.9, 58.7, 40.5, and 42.4), and four methyne groups ($\delta_C$ 63.2, 34.7, 68.2, and 56.9), as well as three quaternary carbons ($\delta_C$ 45.6, 52.3, and 84.4). Moreover, the $^1$H NMR spectrum showed two signals (m) at $\delta_H$ 2.03 and 1.28 and four signals corresponding to four methyl groups at $\delta_H$ 0.94 (d), 0.94 (s), 1.10 (s), and 1.20 (s). These data suggest that **6** is a saturated tricarbocyclic sesquiterpenoid of the botryane type, displaying both a tertiary and a secondary hydroxyl group. The signal at $\delta_H$ 4.00 (br. t, 1H) was assigned to proton H-4, geminal to the secondary hydroxyl group. Acetylation of this hydroxyl group with acetic anhydride-pyridine formed the monoacetate **6a**, for which the $^1$H NMR spectrum showed an acetate signal at $\delta_H$ 2.00. A deshielding of H-4 was also evident in this spectrum. The proposed structure was corroborated by homonuclear and heteronuclear 2D NMR correlation experiments. A 2D $^1$H–$^1$H-shift correlation suggested the presence of the fragment C-11 (3H) C-2 (1H) C-3 (2H) C-4 (1H) C-5 (1H), in addition to a geminal C-7 (2H).

The isolation of compounds **4**–**6** with botryane skeleton completes the biosynthetic pathway to botrydial (**2**), and clarifies and establishes the stereochemistry of the oxidative steps which affect the probotryane skeleton. The oxidative cleavage of the C-10 and C-15 bond in **3** would lead to the dialdehyde **2**.

Interestingly, none of the compounds obtained from the fermentation of *B. cinerea* under different culture conditions have carbon skeletons directly deriving from the proposed carbocation (**9**, **10**) in the first steps of the biosynthetic route to botrydial (**2**). On the other hand, when *B. cinerea* cultures were fed with (4*E*,8*R*)-caryophyll-4(5)-en-8-ol,[14] a synthetic compound analogous to the carbocation at C-8 (**10**), a significant number of caryophyllenic derivatives were obtained, but neither botrydial (**2**) nor any of its derivatives were detected. These results can be accommodated by the proposal that the fungal enzymatic system directly transforms farnesyl pyrophosphate into probotryane-4β,9β-diol (**6**) via the proposed carbocations. According to studies carried out by Hanson et al.[10] using $H_2{}^{18}O$, the 9-hydroxyl group in intermediate **6** arises from water.

The structure of compounds **4** and **5** indicates that the probotryane skeleton is hydroxylated in two steps to give the

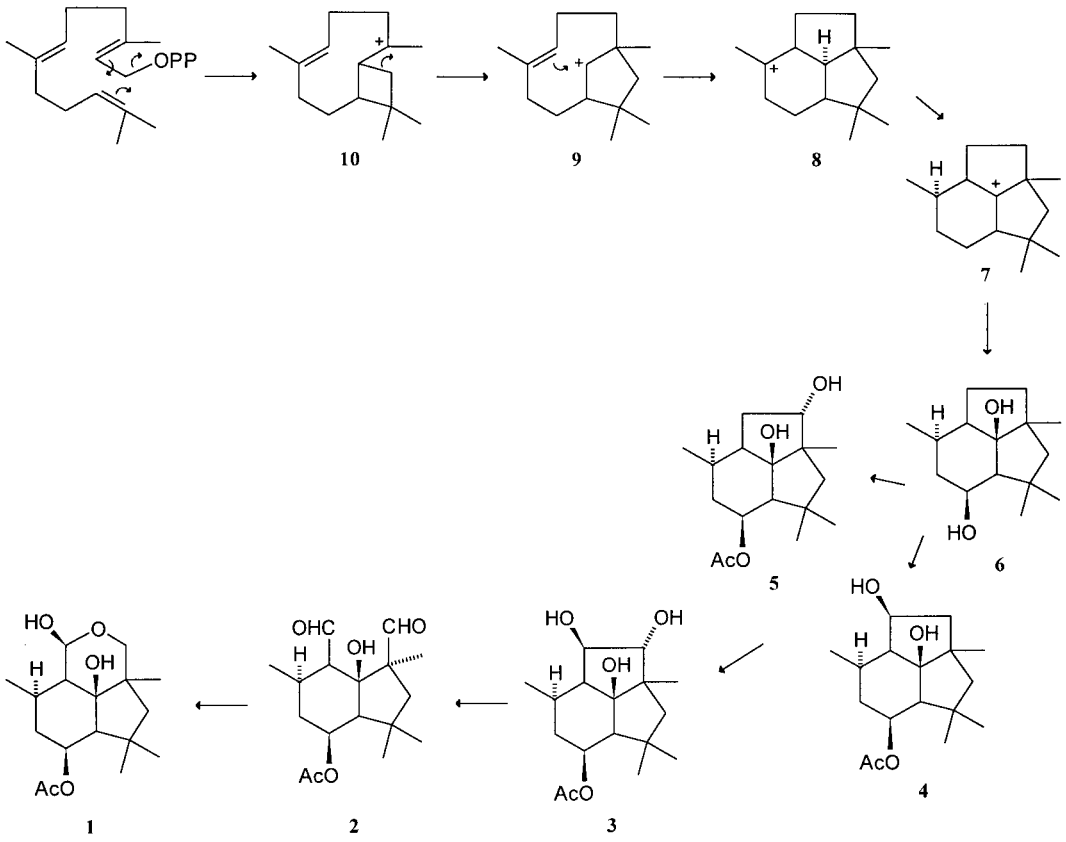


**Figure 1.** Biosynthetic pathway to botrydial (**2**) and related compounds.

key intermediate **3**, which in turn is oxidized to generate the dialdehyde **2**. The subsequent reduction of the aldehyde at C-15, which proceed with the *re* stereospecificity typical of a microbial dehydrogenase,[8,10,12] leads to the hemiacetal **1**. These results confirm the folding and rearrangements proposed thus far for farnesyl pyrophosphate and complete the biosynthetic pathway to botrydial (**2**) as represented in Fig. 1.

## 3. Experimental

### 3.1. General methods

Melting points were measured with a Reichert–Jung Kofler block and are uncorrected. Optical rotations were determined with a Perkin–Elmer 241 polarimeter. IR spectra were recorded on a Perkin–Elmer 881 spectrophotometer. $^{1}H$ and $^{13}C$ NMR measurements ($\delta$ in ppm) were obtained on Varian Gemini 200 and Varian Unity 400 NMR spectrometers with $SiMe_4$ as internal reference. Mass spectra were recorded on VG 12-250 and VG-Autospec spectrometers at 70 eV. HPLC was performed with a Hitachi/Merck L-6270 apparatus equipped with an UV–Vis detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 $F_{254}$, 0.25 mm thick. Silica gel (Merck) was used for column chromatography. Purification by means of HPLC was carried out with a Si gel column (LiChrospher Si-60, 10 μm, 1 cm wide, 25 cm long or 5 μm, 0.4 cm wide, 25 cm long).

### 3.2. Organism and cultural conditions

The culture of *B. cinerea* 2100 employed was obtained from the Colección Española de Cultivos Tipo (CECT), Facultad de Biología, Universidad de Valencia, Spain. The fungus was grown at 24–26°C and 250 rpm in 500 ml Erlenmeyer flasks containing Czapeck–Dox medium. Two different types of experiments were conducted. In the first, 133 Erlenmeyer flasks, each containing 200 ml of culture medium, were inoculated with $10^7$ fresh conidia/flask and incubated for 5 days. In the second experiment, 40 ml of a suspension of a 48-h old culture was used as inoculum. The organism was then grown in 40 Erlenmeyer flasks, each containing 160 ml of culture medium, and incubated for 3 days.

### 3.3. Extraction and isolation

The broth (26.6 and 8 l in the first and second experiments, respectively) was acidified to pH 2.0 with HCl, saturated with NaCl, and extracted with EtOAc. The EtOAc extract was washed with $NaHCO_3$ and $H_2O$ and then dried over anhydrous $NaSO_4$. Evaporation of the solvent at reduced pressure gave a yellow oil that was separated by means of column chromatography on silica gel, with an increasing gradient of ethyl acetate in petroleum ether. Final purification was carried out with HPLC. 4β-Acetoxyprobotryane-9β,10β-diol (**4**) (8.0 mg) and 4β-acetoxyprobotryane-9β,15α-diol (**5**) (4.2 mg) were obtained in the first experiment. The second experiment gave probotryane-4β,

9β-diol (**6**) (1.4 mg), as well as compounds **4** (11.4 mg) and **5** (19.5 mg).

**3.3.1. 4β-Acetoxyprobotryane-9β,10β-diol (4).** Colorless oil, $[\alpha]^{26}_{D}=+19°$ ($c=1$ mg ml$^{-1}$, ethyl acetate); IR (film) $\nu_{max}$ 3421, 2961, 1736, 1241, 1024 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 1.02 (3H, d, $J_{11\text{-}2}=6.4$ Hz, H-11), 1.11 (3H, d, $J_{14\text{-}15\beta}=1.2$ Hz, H-14), 1.12 (3H, s, H-13), 1.12 (1H, m, H-3β superimposed), 1.18 (1H, d, $J_{7\alpha\text{-}7\beta}=12.0$ Hz, H-7α), 1.25 (1H, dd, $J_{1\text{-}2}=11.1$ Hz and $J_{1\text{-}10}=2.0$ Hz, H-1), 1.28 (3H, s, H-12), 1.65 (1H, m, H-2), 1.75 (1H, d, $J_{5\text{-}4}=10.2$ Hz, H-5), 1.82 (1H, ddd, $J_{15\beta\text{-}10}=6.7$ Hz, $J_{15\beta\text{-}14}=1.2$ Hz, and $J_{15\beta\text{-}15\alpha}=11.7$ Hz, H-15β), 1.92 (1H, ddd, $J_{3\alpha\text{-}2}=J_{3\alpha\text{-}4}=3.8$ Hz, H-3α), 1.96 (1H, dd, $J_{15\alpha\text{-}10}=6.7$ Hz and $J_{15\alpha\text{-}15\beta}=11.7$ Hz, H-15α), 1.97 (1H, d, $J_{7\beta\text{-}7\alpha}=12.0$ Hz, H-7β), 2.01 (3H, s, C*H*$_3$–COO), 4.48 (1H, ddd, $J_{10\text{-}1}=2.0$ Hz and $J_{10\text{-}15\alpha}=J_{10\text{-}15\beta}=6.7$ Hz, H-10), 5.01 (1H, ddd, $J_{4\text{-}3\alpha}=3.8$ Hz, $J_{4\text{-}3\beta}=11.2$ Hz, and $J_{4\text{-}5}=10.2$ Hz, H-4); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 20.9 (q, C-11), 21.4 (q, *C*H$_3$COO), 27.4 (q, C$^*$-13), 29.0 (q, C$^*$-14), 33.3 (d, C-2), 36.5 (q, C-12), 39.8 (t, C-3), 45.4 (t, C-15), 46.9 (s, C-6), 49.4 (t, C-7), 55.9 (s, C-8), 56.9 (d, C-5), 61.1 (d, C-1), 72.9 (d, C-4), 82.8 (d, C-10), 97.1 (s, C-9), 170.6 (s, CH$_3$*C*OO), ($^*$=interchangeable); EIMS *m/z* (rel. int.) 236 [M−AcOH]$^+$ (22), 221 [M−AcOH–CH$_3$]$^+$ (20), 218 [M−AcOH–H$_2$O]$^+$ (28), 203 [M−AcOH–H$_2$O–CH$_3$]$^+$ (50), 180 (100); HREIMS calcd for $C_{15}H_{24}O_2$ [M−AcOH]$^+$236.1776, found 236.1777.

**3.3.2. 4β,10β-Diacetoxyprobotryan-9β-ol (4a).** Alcohol **4** (2.5 mg) was dissolved in pyridine (250 μl) and $Ac_2O$ was added. The reaction mixture was stirred for 17 h at room temperature. The solvent was evaporated at reduced pressure to afford **4a** (2.1 mg). White solid, mp 95–97°C, $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 0.96 (3H, d, $J_{11\text{-}2}=6.4$ Hz, H-11), 1.13 (1H, m, H-3β), 1.14 (3H, s, H-13), 1.16 (3H, s, H-14), 1.19 (1H, d, $J_{7\alpha\text{-}7\beta}=12.1$ Hz, H-7α), 1.29 (3H, s, H-12), 1.39 (1H, dd, $J_{1\text{-}2}=11.2$ Hz and $J_{1\text{-}10}=2.5$ Hz, H-1), 1.69 (1H, m, H-2), 1.79 (1H, d, $J_{5\text{-}4}=10.4$ Hz, H-5), 1.91 (1H, m, H-3α), 1.91 (1H, m, H-15β superimposed), 1.99 (1H, d, $J_{7\beta\text{-}7\alpha}=12.1$ Hz, H-7β), 1.99 (1H, m, H-15α superimposed), 2.01 (3H, s, C*H*$_3$–COO), 2.03 (3H, s, C*H*$_3$–COO), 5.02 (1H, ddd, $J_{4\text{-}3\alpha}=3.9$ Hz, $J_{4\text{-}3\beta}=10.8$ Hz, and $J_{4\text{-}5}=10.4$ Hz, H-4), 5.39 (1H, ddd, $J_{10\text{-}1}=2.5$ Hz and $J_{10\text{-}15\alpha}=J_{10\text{-}15\beta}=7.0$ Hz, H-10); EIMS *m/z* (rel. int.) 278 [M−AcOH]$^+$ (0.5), 263 [M−AcOH–CH$_3$]$^+$ (1), 260 [M−AcOH–H$_2$O]$^+$ (2), 218 [M−2×AcOH]$^+$ (46), 200 [M−2×AcOH–H$_2$O]$^+$ (26), 144 (100).

**3.3.3. 4β-Acetoxyprobotryane-9β,15α-diol (5).** White solid, mp 107–109°C, $[\alpha]^{27}_{D}=+13°$ ($c=2.7$ mg ml$^{-1}$, ethyl acetate); IR (film) $\nu_{max}$ 3509, 2955, 2869, 1713, 1455, 1363, 1255 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 0.86 (3H, d, $J_{11\text{-}2}=6.4$ Hz, H-11), 1.03 (1H, m, H-3β), 1.15 (3H, s, H-13), 1.18 (1H, d, $J_{7\alpha\text{-}7\beta}=11.8$ Hz, H-7α), 1.19 (3H, s, H-14), 1.27 (1H, m, H-1), 1.27 (3H, s, H-12 superimposed), 1.56 (1H, d, $J_{5\text{-}4}=10.4$ Hz, H-5), 1.65 (1H, ddd, $J_{10\alpha\text{-}1}=4.4$ Hz, $J_{10\alpha\text{-}10\beta}=13.9$ Hz and $J_{10\alpha\text{-}15}=7.6$ Hz, H-10α), 1.72 (1H, m, H-2), 1.92 (1H, d, $J_{7\beta\text{-}7\alpha}=11.8$ Hz, H-7β), 1.92 (1H, ddd, $J_{3\alpha\text{-}2}=J_{3\alpha\text{-}4}=4.0$ Hz and $J_{3\alpha\text{-}3\beta}=12.1$ Hz, H-3α superimposed), 2.00 (3H, s, C*H*$_3$–COO), 2.71 (1H, ddd, $J_{10\beta\text{-}1}=9.0$ Hz, $J_{10\beta\text{-}10\alpha}=13.9$ Hz, and $J_{10\beta\text{-}15}=7.6$ Hz, H-10β), 4.60 (1H, t, $J_{15\text{-}10\alpha}=J_{15\text{-}10\beta}=7.6$ Hz, H-15), 5.12 (1H, ddd, $J_{4\text{-}3\alpha}=4.0$ Hz, $J_{4\text{-}3\beta}=11.1$ Hz, and $J_{4\text{-}5}=10.4$ Hz, H-4); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 20.9 (q, C-14), 20.9 (q, C-11 superimposed), 21.4 (q, *C*H$_3$COO), 27.4 (q, C-13), 34.6 (d, C-2), 36.4 (q, C-12), 39.7 (t, C-3), 41.2 (t, C-10), 47.6 (s, C-6), 47.7 (t, C-7), 49.0 (d, C-1), 57.3 (s, C-8), 57.7 (d, C-5), 73.1 (d, C-4), 75.1 (d, C-15), 98.1 (s, C-9), 170.4 (s, CH$_3$*C*OO); EIMS *m/z* (rel. int.) 236 [M−AcOH]$^+$ (20), 218 [M−AcOH–H$_2$O]$^+$ (37), 203 [M−AcOH–H$_2$O–CH$_3$]$^+$ (39), 159 (100); HREIMS calcd for $C_{15}H_{24}O_2$ [M−AcOH]$^+$ 236.1776, found 236.1775.

**3.3.4. 4β,15α-Diacetoxyprobotryan-9β-ol (5a).** Alcohol **5** (2.6 mg) was dissolved in pyridine (250 μl) and $Ac_2O$ was added. The reaction mixture was stirred for 17 h at room temperature. The solvent was evaporated at reduced pressure to afford **5a** (1.6 mg). White solid, mp 110–112°C, $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 0.88 (3H, d, $J_{11\text{-}2}=6.5$ Hz, H-11), 1.05 (1H, q, $J_{3\beta\text{-}2}=J_{3\beta\text{-}4}=11.7$ Hz and $J_{3\beta\text{-}3\alpha}=12.2$ Hz, H-3β), 1.14 (3H, s, H-13), 1.14 (3H, s, H-14 superimposed), 1.19 (1H, d, $J_{7\alpha\text{-}7\beta}=12.3$ Hz, H-7α), 1.28 (3H, s, H-12), 1.31 (1H, ddd, $J_{1\text{-}2}=1.8$ Hz, $J_{1\text{-}10\alpha}=4.3$ Hz, and $J_{1\text{-}10\beta}=9.0$ Hz, H-1), 1.62 (1H, d, $J_{5\text{-}4}=10.4$ Hz, H-5), 1.72 (1H, m, H-2), 1.81 (1H, ddd, $J_{10\alpha\text{-}1}=4.3$ Hz, $J_{10\alpha\text{-}10\beta}=14.0$ Hz, and $J_{10\alpha\text{-}15}=7.7$ Hz, H-10α), 1.93 (1H, ddd, $J_{3\alpha\text{-}2}=J_{3\alpha\text{-}4}=4.0$ Hz and $J_{3\alpha\text{-}3\beta}=12.2$ Hz, H-3α), 2.01 (6H, s, 2×C*H*$_3$–COO), 2.10 (1H, d, $J_{7\beta\text{-}7\alpha}=12.3$ Hz, H-7β), 2.77 (1H, ddd, $J_{10\beta\text{-}1}=9.0$ Hz, $J_{10\beta\text{-}10\alpha}=14.0$ Hz, and $J_{10\beta\text{-}15}=7.7$ Hz, H-10β), 5.11 (1H, ddd, $J_{4\text{-}3\alpha}=4.0$ Hz, $J_{4\text{-}3\beta}=11.7$ Hz, and $J_{4\text{-}5}=10.4$ Hz, H-4), 5.28 (1H, t, $J_{15\text{-}10\alpha}=J_{15\text{-}10\beta}=7.7$ Hz, H-15); EIMS *m/z* (rel. int.) 278 [M−AcOH]$^+$ (8), 260 [M−AcOH–H$_2$O]$^+$ (8), 218 [M−2×AcOH]$^+$ (33), 200 [M−2×AcOH–H$_2$O]$^+$ (53),109 (100).

**3.3.5. Probotryane-4β,9β-diol (6).** Colorless oil, $[\alpha]^{25}_{D}=-10°$ ($c=1$ mg ml$^{-1}$, ethyl acetate); IR (film) $\nu_{max}$ 3370, 2957, 2920, 2854, 1460, 1375, 1018, 958 cm$^{-1}$; $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 0.94 (3H, d, $J_{11\text{-}2}=6.8$ Hz, H-11), 0.94 (3H, s, H-13 superimposed), 1.10 (3H, s, H-12), 1.20 (3H, s, H-14), 1.28 (1H, m, H-3β), 1.38 (1H, d, $J_{5\text{-}4}=9.5$ Hz, H-5), 1.49 (1H, d, $J_{7\beta\text{-}7\alpha}=13.3$ Hz, H-7β), 1.58 (1H, d, $J_{7\alpha\text{-}7\beta}=13.3$ Hz, H-7α), 1.62 (1H, m, H-1), 1.81 (1H, m, H-3α), 2.03 (1H, m, H-2), 4.00 (1H, br t, $J_{4\text{-}5}=9.5$ Hz, H-4); $^{13}$C NMR ($CDCl_3$, 100 MHz) $\delta$ 14.6 (q, C-11), 22.6 (q, C-13), 30.2 (q, C-12), 30.5 (q, C-14), 34.7 (d, C-2), 39.9 (t, C-3), 40.5 (t, C$^*$-10), 42.4 (t, C$^*$-15), 45.6 (s, C-6), 52.3 (s, C-8), 56.9 (d, C-5), 58.7 (t, C-7), 63.2 (d, C-1), 68.2 (d, C-4), 84.4 (s, C-9), ($^*$=interchangeable); EIMS *m/z* (rel. int.) 238 [M]$^+$ (3), 223 [M−CH$_3$]$^+$ (8), 205 [M−CH$_3$–H$_2$O]$^+$ (12), 187 [M−CH$_3$–2×H$_2$O]$^+$ (2), 165 (100).

**3.3.6. 4β-Acetoxyprobotryan-9β-ol (6a).** Alcohol **6** (1 mg), which had been dissolved in pyridine (1 ml), was treated with $Ac_2O$ (250 μl) and stirred for 22 h 30 min at room temperature. The reaction mixture was diluted with water and extracted with EtOAc (×3). The organic phase was washed with HCl 2 M, saturated $HNaCO_3$, and brine, and then dried and evaporated to afford **6a** (0.4 mg). Amorphous solid, $^1$H NMR ($CDCl_3$, 400 MHz) $\delta$ 0.91 (3H, s, H-13), 0.93 (3H, d, $J_{11\text{-}2}=6.8$ Hz, H-11), 0.95 (3H, s, H-12), 1.21 (3H, s, H-14), 1.30 (1H, ddd, $J_{3\beta\text{-}2}=1.1$ Hz,

$J_{3\beta\text{-}3\alpha}$=15.0 Hz and $J_{3\beta\text{-}4}$=2.5 Hz, H-3β), 1.50 (1H, d, $J_{7\beta\text{-}7\alpha}$=13.3 Hz, H-7β), 1.57 (1H, d, $J_{7\alpha\text{-}7\beta}$=13.3 Hz, H-7α), 1.64 (1H, d, $J_{5\text{-}4}$=7.1 Hz, H-5), 1.82 (1H, m, H-3α), 1.92–1.99 (1H, m, H-2), 2.00 (3H, s, C$H_3$–COO), 5.07 (1H, ddd, $J_{4\text{-}3\alpha}$=7.1 Hz, $J_{4\text{-}3\beta}$=2.5 Hz, and $J_{4\text{-}5}$=7.1 Hz, H-4); $^{13}$C NMR ($CDCl_3$, 100 MHz) δ 14.2 (q, C-11), 21.5 (q, *C*$H_3$COO), 22.5 (q, C-13), 29.4 (q, C-12), 30.3 (q, C-14), 35.1 (d, C-2), 37.0 (t, C-3), 40.1 (t, C*-10), 42.4 (t, C*-15), 53.1 (d, C-5), 58.5 (t, C-7), 63.1 (d, C-1), 70.7 (d, C-4), (*=interchangeable).

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