Secobotrytriendiol and Related Sesquiterpenoids: New Phytotoxic Metabolites from *Botrytis cinerea*

Rosa Durán-Patrón, 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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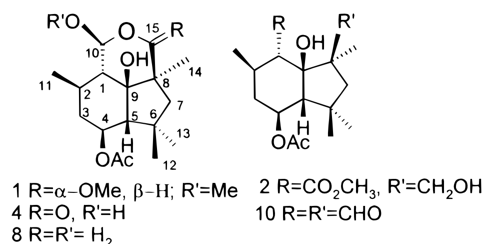
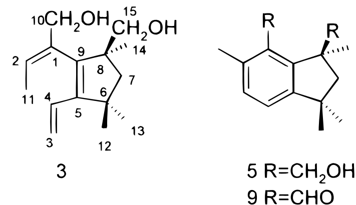
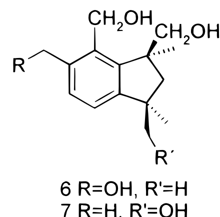
Six new sesquiterpenoid metabolites (**1**, **3−7)** have been isolated from *Botrytis cinerea*. Their structures were elucidated by means of MS and extensive NMR studies. The phytotoxic activities of these new products have been evaluated.

In the course of investigations of the phytopathogen *Botrytis cinerea,* and its pathogenic role, we have studied the production kinetics of the phytotoxic metabolites botrydial, dihydrobotrydial, and related compounds.[1−7] The biosynthetic pathway of these characteristic metabolites, which are phytotoxic to tobacco and grapes,[6,11] has been investigated.[8] The visible phytotoxic effect of botrydial, dihydrobotrydial, and other derivatives in vitro provides circumstantial evidence for their putative role in the pathogenicity of the fungus.

In addition to known compounds, we have found seven additional metabolites (**1**−**7**). In this paper we describe their isolation, structure elucidation, and phytotoxic activity. One of these metabolites, secobotrytriendiol (**3**), exhibits a previously undescribed carbon skeleton, which may be a result of biodegradation of botrydial.







Results and Discussion

B. cinerea 2100 was grown by inoculating and shaking a Czapeck−Dox medium for 3 days. The fermentation broth was extracted with ethyl acetate as described previously.[1−6] Chromatography of the extract on Si gel, followed by final purification by means of HPLC, led to the isolation of 15α-methoxy-*O*-methyl dihydrobotrydial (**1**), methyl botryoloate (**2**), secobotrytriendiol (**3**), dihydrobotrydialone (**4**), dehydrobotrydienol (**5**), 11-hydroxydehydrobotrydienol (**6**), and 12-hydroxydehydrobotrydienol (**7**).

Compound **1** was a colorless oil; HRMS data indicated the molecular formula $C_{19}H_{32}O_6$. The compound's ^{1}H NMR spectrum showed signals very similar to those of *O*-methyldihydrobotrydial (**8**), isolated previously from *Botrytis squamosa.*[7] In fact, the only differences between the ^{1}H NMR spectra of **1** and **8** were the absence of signals characteristic of H-15 in **1**, as well as the presence of a signal at δ 4.86 (s, 1H, H-15). COSY experiments showed a correlation between this signal and one corresponding to 3H, δ 3.49, indicating that **1** was the 15-methoxy derivative of **8**. The stereochemistry at C-15 was confirmed by NOE experiments. These showed an enhancement for the H-15 signal when the signal assigned to H-1 was irradiated, which is only possible in the case of an α orientation of the methoxyl group.

Compound **2** was a colorless oil. Its spectroscopic data were identical to those described in the literature for the methyl ester of botryoloic acid. This compound was previously obtained from the acidic fraction of a fermentation broth of *B. cinerea* after treatment with CH_2N_2.[4] This is the first time that **2** has been isolated from the fermentation broth without previous treatments.

The molecular formula of compound **3** ($C_{15}H_{24}O_2$) was obtained by HRMS and corroborated by ^{13}C NMR data. The IR absorption and ^{13}C NMR signals at δ 68.3 and 70.3 indicated two hydroxymethyl groups. The ^{13}C NMR spectrum exhibited signals for six double-bond carbons, one methylene, two methyne, and four quaternary carbons. The spectral data indicated that **3** was an unsaturated monocyclic sesquiterpenoid with three double bonds and two hydroxymethyl groups. The proposed structure was corroborated by homonuclear and heteronuclear 2D NMR correlation experiments. A 2D ^{1}H−^{13}C-shift correlation helped determine the sequence CH_3−CH=CH_2OH, which was then correlated with the fragment C=C−CH=CH_2 by means of a long-range HETCOR experiment. The orientation of the double bonds was inferred on the basis of NOE experiments. Irradiation of the signal at δ 5.40 (H-3) led to enhancement of the signals at δ 1.34 and 1.33 (H-12 and H-13), thus indicating the orientations for C-3,C-4 and C-5,C-9, which are given in the formula of **3**. Irradiation at δ 5.73 (H-2) enhanced the signals at δ 4.12 and 4.29

* To whom correspondence should be addressed. Tel.: 34-956-016371 or 34-956-016368. Fax: 34-956-016288. E-mail: rosario.hernandez@uca.es// isidro.gonzalez@uca.es.

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(H_2-10). These results indicated that the configuration of the C-1,C-2 double bond was *E*. Compound **3** thus shows a skeleton previously unknown in *B. cinerea*.

Comparison of the spectroscopic data of compound **4** with those of other compounds previously isolated from *B. cinerea* indicated that **4** was the *O*-demethyl derivative of β-*O*-methyldihydrobotrydialone.[5] This conclusion was supported by a significant downfield shift of H-7, the IR absorption band at 1735 cm^{-1}, and the presence of a signal at δ 170.4 in the ^{13}C NMR (C-15), all of which confirm that **4** is indeed the dihydrobotrydialone.

Compounds **5**, **6**, and **7** were colorless oils, and the IR and ^1H and ^{13}C NMR spectra indicated structures similar to that of dehydrobotrydienal (**9**). The ^1H NMR spectrum of **5** showed two doublets (δ 7.12 and 7.02), which, together with six aromatic and two hydroxymethyl signals in the ^{13}C NMR spectrum, indicated a dehydrobotrydienal (**9**) analogue. The proposed structure was confirmed by treating **5** with PCC, which afforded dehydrobotrydienal (**9**), a natural product previously isolated from *B. squamosa*.[12] The absence of methyl signals in the ^1H and ^{13}C NMR spectra of compounds **6** and **7**, along with the presence of new hydroxymethyl resonances, indicated that these compounds were hydroxymethyl derivatives of **5**. The hydroxymethyl groups of **6** and **7** were assigned to C-11 and C-12, respectively, after comparing the ^{13}C NMR spectra with that of compound **5**. The stereochemistry at C-6 in **7** was determined to be *R* based on the deshielding effect observed for the H-7β resonance (from δ 2.21 in **5** to δ 2.48 in **7**). This signal had previously been assigned to H-7β due to the enhancement observed when the H-15 signal was irradiated in the NOE experiment.

Phytotoxicity assays were carried out using methodology previously described.[11] Compounds **1**−**3** and **5**−**6** were phytotoxic. 11-Hydroxydehydrobotrydienol (**6**) was active at 250 ppm, while compounds **2**, **3**, and **5** were active at 500 ppm. In contrast, **1** was active only at 1000 ppm.

Experimental Section

General Experimental Procedures. 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. ^1H and ^{13}C NMR measurements (δ in ppm) were obtained on Varian Gemini 200 and Varian Unity 400 NMR spectrometers with TMS as internal reference. MS 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 a UV−vis detector (L 4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Si gel 60 F_{254}, 0.25 mm thick. Si gel (SDS, 60−200 μmesh or 40−60 μmesh) was used for column chromatography. Purification by means of HPLC was accomplished using 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).

Organism and Culture Conditions. The culture of *B. cinerea* 2100 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 in 40 flasks (500 mL), each containing 160 mL of Czapeck−Dox medium consisting of 5% glucose, 0.1% yeast extract, 0.05% KH_2PO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot 7H_2O$, and 0.001% $FeSO_4 \cdot H_2O$. A HCl solution was used to adjust the pH of the medium to 7.0. Each flask was then inoculated with 40 mL of a suspension of a 48-h-old culture. The cultures were incubated for 3 days on an orbital shaker at 250 rpm.

Phytotoxicity Assays. Solutions of **1**−**3** and **5**−**7** were prepared by dissolving the compounds in acetone and then adding water containing 0.1% Tween 80 in order to yield concentrations of 1000, 500, and 250 ppm. The final volume of acetone in each case was 40%. Sterilized leaves from *Nicotiana tabacum* were cut into circles measuring 1 cm in diameter, which were then placed in Petri dishes (25 circles per dish) containing Whatman paper wetted with sterile H_2O. The different dilutions (10 μL each) were placed on each of the circles, and the plates were kept at 25−28 °C for 7 days. Assays were carried out in duplicate. Controls consisted of the same mixture of acetone, H_2O, and Tween 80 as that used for dissolving the purified metabolites. Qualitative results were obtained by comparing the number of circles affected with the total. Quantitative results were obtained by comparing the surface area affected with the total surface area.

Extraction and Isolation. The broth (8 L) was acidified to pH 2.0 with 1M HCl solution, saturated with NaCl, and extracted with EtOAc. The EtOAc extract was washed with a $NaHCO_3$ solution and H_2O and dried over anhydrous $NaSO_4$. Evaporation of the solvent at reduced pressure gave a yellow oil that was separated by column chromatography on Si gel, with an increasing gradient of ethyl acetate in petroleum ether, followed by final purification by means of HPLC to afford **1** (9.4 mg), **2**[4] (2.1 mg), **3** (9.1 mg), **4** (1.3 mg), **5** (6.2 mg), **6** (9 mg), and **7** (5 mg).

15α-Methoxy-*O*-methyldihydrobotrydial (1): colorless oil, $[\alpha]^{24}_D$ +102° (*c* 1, $CDCl_3$); IR (film) ν_{max} 3507, 2942, 2875, 1742, 1472, 1453, 1390, 1243, 1107 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.94 (3H, d, J_{11-2} = 6.3 Hz, H-11), 1.05 (1H, m, H-3β), 1.08 (3H, s, H*-13), 1.11 (3H, s, H-14), 1.24 (1H, d, $J_{7\alpha-7\beta}$ = 12.8 Hz, H-7_α), 1.24 (3H, s, H*-12), 1.54 (1H, dd, J_{1-2} = 13.4 Hz, J_{1-10} = 1.4 Hz, H-1), 1.80 (1H, m, H-2), 1.89 (1H, d, J_{5-4} = 10.9 Hz, H-5), 2.00 (1H, d, H-7β), 2.01 (3H, s, CH_3−COO), 2.04 (1H, ddd, $J_{3\alpha-4}$ = 4.9 Hz, $J_{3\alpha-2}$ = 3.3 Hz, $J_{3\alpha-3\beta}$ = 12.3 Hz, H-3"), 3.45 (3H, s, CH_3−O on C-10), 3.49 (3H, s, CH_3-O on C-15), 3.64 (1H, s, OH), 4.95 (1H, d, J_{10-1} = 1.4 Hz, H-10), 4.86 (1H, s, H-15), 5.10 (1H, ddd, $J_{4-3\alpha}$ = 4.9, $J_{4-3\beta}$ = 11.3, and J_{4-5} = 10.9 Hz, H-4), (* = interchangeable); ^{13}C NMR ($CDCl_3$, 50 MHz) δ 9.8 (t, C-3), 19.2 (q, C-14), 20.1(q, C-11), 21.4 (q, CH$_3$−CO), [a]27.2 (q, C-13), 29.10 (d, C-2), [a]35.6 (q, C-12), 39.2 (s, C-6), 49.3 (s, C-8), 50.4 (t, C-7), 55.0 (d, C-1), 56.8 (q, O-CH$_3$), 59.9 (d, C-5), 72.8 (d, C-4), 86.0 (s, C-9), 102.8 (d, C-15), 170.5 (s, CH_3-CO), ([a] = interchangeable); EIMS *m*/*z* (rel int) 324 $[M - CH_3OH]^+$ (1), 264 $[M - CH_3OH - AcOH]^+$ (2), 151 (50), 126 (38), 85 (100); HREIMS 324.1931 (calcd for $C_{18}H_{28}O_5$ $[M - CH_3OH]^+$ 324.1937).

Methyl botryolate (2): colorless oil (lit.[4] 123−125 °C), $[\alpha]^{26}_D$ +36.5° (*c* 2, ethyl acetate); spectroscopic data agreed with those previously reported;[4] ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.8 (q, C-11), 21.5 (q, CH$_3$−COO), [a] 21.7 (q, C-13), [a] 27.8 (q, C-12), 29.2 (d, C-2), 35.9 (q, C-14), 36.5 (s, C-6), 38.7 (t, C-3), 49.7 (s, C-8), 51.5 (q, COO-CH$_3$), 52.2 (t, C-7), 60.9 (d, C-1), 63.9 (d, C-5), 67.7 (t, C-15), 72.9 (d, C-4), 88.3 (s, C-9), [b]170.4 (s, CH_3-COO), [b]173.7 (s, C-10), ([a,b] = interchangeable).

Secobotrytriendiol (3): colorless oil; $[\alpha]^{28}_D$ +53.8° (*c* 4.2, $CDCl_3$); IR (film) ν_{max} 3384, 2943, 2884, 1660, 1379 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.98 (3H, s, H-14), 1.33 (3H, s, H*-13), 1.34 (3H, s, H*-12), 1.52 (3H, dd, J_{11-2} = 6.8 and $J_{11-10'}$ = 1.2 Hz, H-11), 1.62 (1H, d, $J_{7\alpha-7\beta}$ = 13.0 Hz, H-7α), 2.08 (1H, d, $J_{7\beta-7\alpha}$ = 13.0 Hz, H-7β),3.27 (1H, d, $J_{15-15'}$ = 11.2 Hz, H-15), 3.56 (1H, d, $J_{15'-15}$ = 11.2 Hz, H-15′), 4.12 (1H, d, $J_{10-10'}$ = 12.9 Hz, H-10), 4.29 (1H, dt, $J_{10'-10}$ = 12.9 and $J_{10'-11}$ = 1.2 Hz, H-10′), 5.06 (1H, dd, $J_{3-3'}$ = 1.7 and J_{3-4} = 11.8 Hz, H-3), 5.40 (1H, dd, $J_{3'-3}$ = 1.7 and $J_{3'-4}$ = 18.3 Hz, H-3′), 5.73 (1H, c, J_{2-11} = 6.8 Hz, H-2), 6.20 (1H, dd, J_{4-3} = 11.8 and $J_{4-3'}$ = 18.3 Hz, H-4), (* = interchangeable); ^{13}C NMR (100 MHz) δ 14.7 (q, C-11), 24.0 (q, C-14), [a]29.2 (q, C-13), [a]30.7 (q, C-12), 45.2 (s, C-6), 52.3 (t, C-7), 52.5 (s, C-8), 68.3 (t, C-10), 70.3 (t, C-15), 115.3 (t, C-3), 126.6 (d, C-2), 131.1 (d, C-4), 140.0 (s, C-9), 148.6 (s, C-5), ([a] = interchangeable); EIMS *m*/*z* (rel int) 236 $[M]^+$ (2), 218 $[M - H_2O]^+$ (5), 205 $[M - CH_2OH]^+$ (82), 187 $[M - CH_2OH - H_2O]^+$ (36), 172 (70), 159 (59), 145 (100); HREIMS 205.1591(calcd for $C_{14}H_{21}O$ $[M - CH_2OH]^+$ 205.1592).

Dihydrobotrydialone (4): white solid; ^1H NMR ($CDCl_3$, 400 MHz) δ 0.99 (3H, s, H*-12), 1.14 (3H, s, H*-13), 1.20 (3H, d, J_{11-2} = 6.8 Hz, H-11), 1.37 (1H, m, $J_{3\beta-4}$ = 11.7 Hz, H-3β), 1.34 (1H, d, J_{5-4} = 3.2 Hz, H-5), 1.43 (3H, s, H-14), 1.46 (1H, dd, J_{1-2} = 1.7, J_{1-10} = 8.5 Hz, H-1), 1.61 (1H, d, $J_{7\alpha-7\beta}$ = 14.6

Hz, H-7α), 1.86 (1H, d, $J_{7\beta-7\alpha}$ = 14.6 Hz, H-7β), 2.03 (3H, s, C*H*$_3$−COO), 2.10 (1H, m, H-3α), 2.13 (1H, m, H-2), 4.85 (1H, ddd, $J_{4-3\alpha}$ = 11.7, $J_{4-3\beta}$ = 11.7, and J_{4-5} = 3.2 Hz, H-4), 5.07 (1H, d, J_{10-1} = 8.5 Hz, H-10), (* = interchangeable); ^{13}C NMR (100 MHz) δ [a]18.2 (q, C-11), 21.3 (q, *C*H$_3$−COO), [b]25.0 (q, C-13), [b]25.1 (q, C-14), 28.0 (d, C-2), [a]33.6 (q, C-12), 36.6 (t, C-3), 38.5 (s, C-6), 50. 6 (t, C-7), 52.8 (d, C-1), 63.2 (d, C-5), 71.6 (d, C-4), 91.8 (s, C-9), 99.2 (d, C-10), 170.4 (s, C-15), 170.4 (s, CH$_3$−*C*OO), ([a,b] = interchangeable); EIMS *m*/*z* (rel int) 236 [M − AcOH − 2 × CH$_3$]$^+$ (7), 221 [M − AcOH − 3 × CH$_3$]$^+$ (3), 193 (24), 150 (34), 135 (91), 109 (52), 95 (100); HREIMS 236.1414 (calcd for $C_{14}H_{20}O_3$ [M − AcOH − 2 × CH$_3$]$^+$ 236.1412).

Dehydrobotrydienol (5): colorless oil, $[\alpha]^{27}_D$ −26.7° (*c* 0.6, ethyl acetate); IR (film) ν_{max} 3362, 2960, 2925, 1655, 1474, 1460, 1383, 1042, 824 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 1.29 (3H, s, H-12), 1.29 (3H, s, H-13), 1.38 (3H, s, H-14), 1.78 (1H, d, $J_{7\alpha-7\beta}$ = 13.2 Hz, H-7α), 2.21 (1H, d, $J_{7\beta-7\alpha}$ = 13.2 Hz, H-7β), 2.42 (3H, s, H-11), 3.63 (1H, d, $J_{15-15'}$ = 11.3 Hz, H-15), 3.92 (1H, d, $J_{15'-15}$ = 11.3 Hz, H-15′), 4.72 (1H, d, $J_{10-10'}$ = 11.4 Hz, H-10), 4.78 (1H, d, $J_{10'-10}$ = 11.4 Hz, H-10′), 7.02 (1H, d, J_{4-3} = 7.7 Hz, H-4), 7.12 (1H, d, J_{3-4} = 7.7 Hz, H-3); ^{3}C NMR (CDCl$_3$, 100 MHz) δ 18.9 (q, C-11), 26.3 (q, C-14), [a]31.1 (q, C-12), [a]32.2 (q, C-13), 40.8 (s, C-6), 50.3 (s, C-8), 54.0 (t, C-7), 58. 7 (t, C-10), 71.0 (t, C-15), 123.0 (d, C-4), 130.4 (d, C-3), 134.4 (s, C-1), 136.4 (s, C-2), 144.0 (s, C-9), 152.3 (s, C-5) ([a] = interchangeable); EIMS *m*/*z* (rel int.) 234 [M]$^+$ (0.4), 216 [M − H$_2$O]$^+$ (0.7), 203 [M − CH$_2$OH]$^+$ (7), 185 [M − CH$_2$OH − H$_2$O]$^+$ (100); HREIMS 199.1484 (calcd for $C_{15}H_{19}$ [M − H$_2$O − OH]$^+$ 199.1487).

11-Hydroxydehydrobotrydienol (6): colorless oil, $[\alpha^{27}_D$ −28.5° (*c* 2, ethyl acetate); IR (film) ν_{max} 3341, 2959, 2872, 1736, 1723, 1657, 1562, 1510, 1460, 1378, 1249, 1041, 834, 761 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 1.30 (s, 3H, H*-13), 1.31 (3H, s, H*-12), 1.38 (3H, s, H-14), 1.79 (1H, d, $J_{7\alpha-7\beta}$ = 13.1 Hz, H-7α), 2.25 (1H, d, $J_{7\beta-7\alpha}$ = 13.1 Hz, H-7β), 3.59 (1H, d, $J_{15-15'}$ = 11.7 Hz, H-15), 3.94 (1H, d, $J_{15'-15}$ = 11.7 Hz, H-15′), 4.56 (1H, d, $J_{11-11'}$ = 11.8 Hz, H-11), 4.76 (1H, d, $J_{10-10'}$ = 12.4 Hz, H-10), 4.80 (1H, d, $J_{10'-10}$ = 12.4 Hz, H-10′), 4.96 (1H, d, $J_{11'-11}$ = 11.8 Hz, H-11′), 7.09 (1H, d, J_{4-3} = 7.6 Hz, H-4), 7.21 (1H, d, J_{3-4} = 7.6 Hz, H-3), (* = interchangeable); ^{13}C NMR (CDCl$_3$, 50 MHz) δ 26.2 (q, C-14), [a]30.8 (q, C-12), [a]32.1 (q, C-13), 41.1 (s, C-6), 50.5 (s, C-8), 53.9 (t, C-7), 58.6 (t, C-10), 64.9 (t, C-11), 79.9 (t, C-15), 123.1 (d, C-4), 129.6 (d, C-3), 135.9 (s, C-1), 138.5 (s, C-2),145.1 (s, C-9), 155.2 (s, C-5) ([a] = interchangeable); EIMS *m*/*z* (rel int) 219 [M − CH$_2$OH]$^+$ (5), 202 [M − CH$_2$OH − OH]$^+$ (100), 157 [M − 3 ×CH$_2$OH]$^+$ (20); HREIMS 219.1387 (calcd for $C_{14}H_{19}O_2$ [M − CH$_2$OH]$^+$ 219.1385).

12-Hydroxydehydrobotrydienol (7): colorless oil, $[\alpha]^{25}_D$ +2° (*c* 2.2, ethyl acetate); IR (film) ν_{max} 3350, 2926, 2872, 1656, 1459, 1384, 1026, 912, 824, 735 cm^{-1}; ^{1}H NMR (CDCl$_3$, 400 MHz) δ 1.23 (3H, s, H-13), 1.30 (3H, s, H-14), 1.69 (1H, d, $J_{7\alpha-7\beta}$ = 13.7 Hz, H-7α), 2.41 (3H, s, H-11), 2.48 (1H, d, $J_{7\beta-7\alpha}$ = 13.7 Hz, H-7β), 3.52 (1H, d, $J_{12-12'}$ = 10.7 Hz, H-12), 3.66 (1H, d, $J_{12'-12}$ = 10.7 Hz, H-12′), 4.66 (1H, d, $J_{10-10'}$ = 11.7 Hz, H-10), 4.72 (1H, d, $J_{10'-10}$ = 11.7 Hz, H-10′), 6.97 (1H, d, J_{4-3} = 7.7 Hz, H-4), 7.14 (1H, d, J_{3-4} = 7.7 Hz, H-3); ^{13}C NMR (CDCl$_3$, 100 MHz) δ 19.0 (q, C-11), 26.8 (q, C-13), 28.0 (q, C-14), 46.5 (s, C-6), 49.3 (t, C-7), 50.4 (s, C-8), 58.4 (t, C-10), 71.7 (t, C-15), 71.7 (t, C-12), 122.9 (d, C-4), 134.9 (s, C-1), 130.7 (d, C-3), 137.3 (s, C-2), 145.7 (s, C-9), 147.3 (s, C-5); EIMS *m*/*z* (rel int) 219 [M − CH$_2$OH]$^+$ (0.3), 202 [M − CH$_2$OH − OH]$^+$ (78), 183 (100); HREIMS 219.1377 (calcd for $C_{14}H_{19}O_2$ [M − CH$_2$OH]$^+$ 219.1385).

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