Four New Lactones from *Botrytis cinerea*

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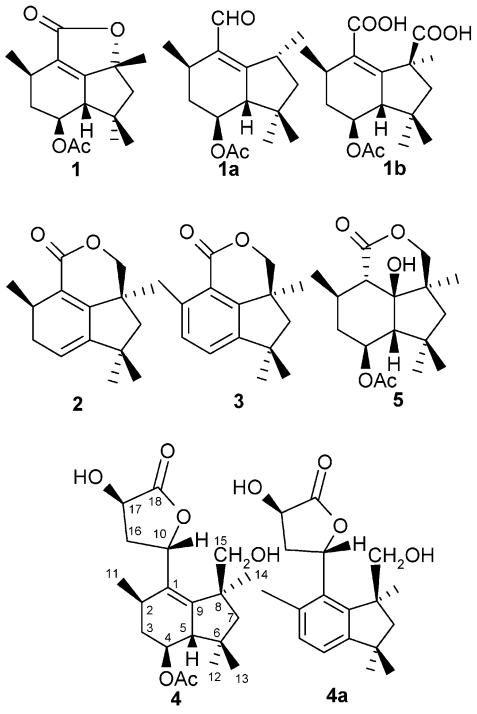
Received March 13, 2002

Four new lactones (**1**−**4**) have been isolated from *Botrytis cinerea*. Their structures were elucidated by interpretation of spectral data, mainly ^{1}H and ^{13}C NMR, including two-dimensional analysis (HOMOCOSY, HMQC, and HMBC). The phytotoxic activities of these new natural products have been evaluated. Compounds **1**−**3** were inactive, while **4** showed a phytotoxic effect when tested up to 250 ppm.

Botrytis cinerea is well known as the source of characteristic metabolites with the botryane skeleton, principally dihydrobotrydial and botrydial.[1−3] Recently, it has been reported that botrydial is a pathogenicity factor for *B. cinerea*, and this compound was detected in ripe fruits of *Capsicum annuum* which had been wound-inoculated with a conidial suspension of *B. cinerea*, as well as in leaves of *Phaseolus vulgaris* and *Arabidopsis thaliana*.[4] In the course of our experiments to determine the putative role of the toxins excreted by this fungus, we have isolated four new lactones (**1**−**4**) with the botryane skeleton. In the present paper, we describe their isolation and structure elucidation by the means of their spectroscopic data, mainly ^{1}H and ^{13}C NMR, including two-dimensional analysis (HOMOCOSY, HMQC, and HMBC). In addition, their phytotoxic activities have been evaluated.



Botrytis cinerea was grown on a Czapeck-Dox medium in an orbital shaker for 5 days. Under these conditions, four new lactones were produced, norbotrydialone acetate (**1**), 10-oxodihydrobotry-1(9),4(5)-diendial (**2**), 10-oxodehydrodihydrobotrydial (**3**), and 4β-acetoxytetrahydrobotryslactone (**4**).

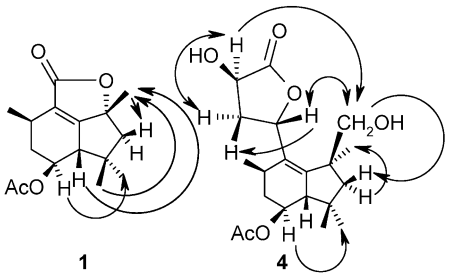


Figure 1. Selected NOE correlations for compounds **1** and **4**.

Compound **1** has the molecular formula $C_{16}H_{22}O_4$, as deduced from HRMS and ^{13}C NMR data. The ^{13}C NMR spectrum showed 16 signals arising from five methyls, two methylenes, three methines, and six quaternary carbon atoms. The spectroscopic data of this compound were similar to those of norbotrydial acetate (**1a**), a norsesquiterpenoid aldehyde previously isolated from *B. cinerea* for which the stereochemistry has already been established.[5] The main differences in the ^{1}H NMR spectra of the two compounds were the absence of the signals corresponding to H-8 and H-10 in **1** and a change in both the ^{1}H NMR chemical shift and multiplicity of the C-8 methyl signal from δ 1.28 (d) in **1a** to δ 1.58 (s) in **1**. The signals corresponding to methine groups, at δ 27.8, 48.9, and 72.0, were assigned to C-2, C-5, and C-4, respectively, by analysis of the HMBC, HMQC, and ^{1}H−^{1}H COSY data. The signal at δ 86.9 (C) correlated with the signal at δ 1.58 (H_3-14) in the HMBC experiment, indicating that C-8 is substituted by the oxygen atom of a lactone. The remaining 15 carbons were assigned to a botrydial-type sesquiterpenoid on the basis of the spectroscopic features of **1**. The shift observed in the signal of C-10 in **1a** from δ 190.4 (CH) to δ 174.3 (C) in **1** was consistent with the proposed structure in which the lactone is located at C-10. The β-disposition of the methyl group at C-8 was assigned by means of NOE experiments (Figure 1). Irradiation of the H-4 proton caused enhancement of the H_3-13 and H-7 signals at δ 0.99 and 1.49 ppm, respectively, which resulted in its assignment as α, while irradiation of the H-5 (β) signal enhanced the signals for H_3-12 and H_3-14. In addition, when the H-7β proton was irradiated, the signal assigned to H_3-14 was enhanced. The lactone ring in compound **1** may be formed by decarboxylative oxygenation of the 1,5-dicarboxylic derivative (**1b**).

The IR and ^{1}H and ^{13}C NMR spectra of compounds **2** and **3** indicated that these two compounds were similar.

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10.1021/np020107v CCC: $22.00
Published on Web 09/24/2002

The absence in the ^{1}H NMR spectra of signals characteristic of H-10, together with IR absorption bands at 1714 and 1725 cm^{-1} and the presence of ^{13}C NMR signals at δ 165.4 and 164.0, respectively, indicated that **2** and **3** are unsaturated δ-lactones. The presence of six aromatic carbon signals in the ^{13}C NMR spectrum of compound **3**, in addition to the downfield chemical shift of the methyl group on C-2 at 2.61 ppm (s) in the ^{1}H NMR spectrum, indicated that **3** was an aromatized analogue of 10-oxodihydrobotrydial (**5**).[6] The remaining signals in the ^{13}C NMR spectrum along with the chemical shift and coupling constants appearing in the ^{1}H NMR spectrum were consistent with the structure shown for compound **3**.

The ^{13}C NMR spectrum of compound **2** showed four alkene carbon signals at δ 120.1 (C, C-1), 121.5 (CH, C-4), 145.9 (C, C-5), and 158.9 (C, C-9), while in the ^{1}H NMR spectrum, the methyl group on C-2 appeared as a doublet at 0.87 ppm. The remaining 11 carbons were assigned to a botryane-type sesquiterpenoid on the basis of the spectroscopic features of **2**, which were closely related to those of the lactone **5**,[6] the stereochemistry of which has been well established by means of chemical transformation from dihydrobotrydial.[2,3,6] These data are consistent with the structure of 10-oxodihydrobotry-1(9),4(5)-diendial proposed for **2**. Stereochemistry was proposed by analogy to known compounds in this series.

The molecular formula of compound **4** was established as $C_{20}H_{30}O_6$ with the aid of HRMS and the ^{13}C NMR data (20 signals, see Experimental Section). The ^{1}H NMR spectrum of **4** showed signals similar to those for H-10, H_2-16, and H-17 of botryslactone (**4a**), a compound previously isolated from *Botrytis squamosa*.[7] The occurrence in the ^{13}C NMR spectrum of two alkene carbon signals at δ 133.7 (C, C-1) and 147.1 (C, C-9), as well as the presence in the ^{1}H NMR spectrum of a methyl group signal appearing as a doublet (δ 1.08, J = 7.0 Hz) and a proton signal at δ 5.09 (dd, J = 9.8 and 4.9 Hz), characteristic of H_3-11 (β-disposition) and H-4 (α), respectively, indicated that **4** possesses a botryane-type skeleton similar to that of botryenalol.[8] The stereochemistry for compound **4** was proposed by comparison of the proton signals and their coupling constants with those described for **4a**[7] and botryenalol[8] and was supported by the results of a set of NOE experiments (see Figure 1).

The biosynthetic origin of carbons C-16, C-17, and C-18 in compounds **4** and **4a** is unknown. However, in previous work with *B. cinerea*, our group has isolated various macrolides, which may be derived from condensation with propionate.[9] In a similar fashion, the carbons in question may arise from the condensation of a unit of propionate and the corresponding botryane derivative.

In bioassays with *Phaseolus vulgaris* plants, when purified botrydial and related compounds were applied to the leaf surface, they were found to be phytotoxic, reproducing the symptoms of the plant disease.[10,11] To determine the activity of the new compounds, phytotoxicity assays were carried out using previously described methodology.[10,11] The results showed that compounds **1**−**3** were inactive at levels of 1000 ppm, while **4** showed phytotoxic effects when tested up to concentration levels of 250 ppm.

Experimental Section

General Methods and Organism and Culture Conditions. These were identical to those previously described.[12]

Extraction and Isolation. The broth (29 L) was saturated with NaCl and extracted with EtOAc. The EtOAc extract was washed with H_2O and then dried over anhydrous $NaSO_4$. Evaporation of the solvent at reduced pressure gave 25.5 g of a yellow oil. Fractionation of the extract was carried out by means of column chromatography (35 × 6 cm) on silica gel, eluting with 14 × 1000 mL of petroleum ether/ethyl acetate mixtures with increasing percentage composition of ethyl acetate to give 14 fractions. Compounds **1**−**3** eluted in fraction 3 (petroleum ether/ethyl acetate, 85:15) were obtained by further purification using silica gel chromatography under similar conditions to give 10 fractions (A−J). Final purification of fraction F (petroleum ether/ethyl acetate, 80:20) was carried out by semipreparative HPLC (hexane/ethyl acetate, 90:10; 2.8 mL min^{-1}) to afford norbotrydialone acetate (**1**) (5 mg), 10-oxodihydrobotry-1(9),4(5)-diendial (**2**) (2 mg), and 10-oxodehydrodihydrobotrydial (**3**) (4 mg). Further purification of fraction 8 (petroleum ether/ethyl acetate, 60:40) by semipreparative HPLC (hexane/ethyl acetate, 40:60; 3 mL min^{-1}) led to the isolation of pure 4β-acetoxytetrahydrobotryslactone (**4**) (4 mg).

Norbotrydialone acetate (1): white solid; mp 70−72 °C, $[\alpha]^{25}_D$ −54° (*c* 5.0 mg/mL, $CHCl_3$); IR (film) ν_{max} 2961, 1726, 1227 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.99 (3H, s, H-13), 1.19 (3H, d, J_{11-2} = 6.8 Hz, H-11), 1.33 (3H, s, H-12), 1.36 (1H, m, H-3β), 1.49 (1H, d, $J_{7\alpha-7\beta}$ = 13.2 Hz, H-7α), 1.58 (3H, s, H-14),1.87 (1H, d, $J_{7\beta-7\alpha}$ = 13.2 Hz, H-7β), 2.05 (3H, s, COCH_3), 2.17 (1H, ddd, $J_{3\alpha-4}$ = 3.4, $J_{3\alpha-2}$ = 4.9 and $J_{3\alpha-3\beta}$ = 12.6 Hz, H-3α), 2.65 (1H, m, H-2), 2.71 (1H, dd, J_{5-2} = 3.0 and J_{5-4} = 8.1 Hz, H-5), 4.75 (1H, ddd, $J_{4-3\beta}$ = 11.7, $J_{4-3\alpha}$ = 3.4 Hz, and J_{4-5} = 8.1 Hz, H-4); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.3 (CH_3, C-11), 21.2 (CH_3, CH_3CO), 24.0 (CH_3, C-14), 27.3 (CH_3, C-13), 27.8 (CH, C-2), 33.4 (CH_3, C-12), 37.6 (CH_2, C-3), 45.0 (C, C-6), 48.9 (CH, C-5), 51.3 (CH_2, C-7), 72.0 (CH, C-4), 86.9 (C, C-8), 128.8 (C, C-1), 170.1 (C, CH_3*C*O−), 171.4 (C, C-9), 174.2 (C, C-10); EIMS *m*/*z* 279 $[M + H]^+$ (3), 235 (5), 218 $[M - CH_3\text{-}COOH]^+$ (25), 203 (22), 193 (6), 180 (100); HREIMS *m*/*z* 279.1609 (calcd. for $C_{16}H_{23}O_4$ $[M + H]^+$, 279.1596).

10-Oxodihydrobotry-1(9),4(5)-diendial (2): amorphous solid; $[\alpha]^{25}_D$ +25° (*c* 0.6 mg/mL, $CHCl_3$); IR (film) ν_{max} 2958, 1714, 1074, 1042 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.87 (3H, d, J_{11-2} = 7.1 Hz, H-11), 1.17 (3H, s, H-13), 1.27 (3H, s, H-12), 1.35 (3H, s, H-14), 1.53 (1H, d, $J_{7\alpha-7\beta}$ = 13.1 Hz, H-7α), 1.72 (1H, d, $J_{7\beta-7\alpha}$ = 13.1 Hz, H-7β), 2.24 (1H, ddd, $J_{3\alpha-2}$ = 1.2, $J_{3\alpha-3\beta}$ = 17.6 and $J_{3\alpha-4}$ = 6.4 Hz, H-3α), 2.60 (1H, ddd, $J_{3\beta-2}$ = 9.8, $J_{3\beta-3\alpha}$ = 17.6 and $J_{3\beta-4}$ = 2.8 Hz, H-3β), 2.79 (1H, ddq, $J_{2-3\alpha}$ = 9.8, $J_{2-3\beta}$ = 1.2 and J_{2-11} = 7.1 Hz, H-2), 3.98 (1H, d, $J_{15\alpha-15\beta}$ = 10.0 Hz, H-15α), 4.21 (1H, d, $J_{15\beta-15\alpha}$ = 10.0 Hz, H-15β), 5.72 (1H, dd, $J_{4-3\beta}$ = 2.8 and $J_{4-3\alpha}$ = 6.4 Hz, H-4); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 18.1 (CH_3, C-11), 23.2 (CH_3, C-14), 23.3 (CH, C-2), 31.1 (CH_3, C-13), 31.2 (CH_3, C-12), 31.8 (CH_2, C-3), 39.1 (C, C-6), 41.8 (C, C-8), 51.2 (CH_2, C-7), 78.8 (CH_2, C-15), 120.1 (C, C-1), 121.5 (CH, C-4), 145.9 (C, C-5), 158.9 (C, C-9), 165.4 (C, C-10); EIMS *m*/*z* 232 $[M]^+$ (10), 217 $[M - CH_3]^+$ (9), 202 $[M - 2\times CH_3]^+$ (4), 187 $[M - 3\times CH_3]^+$ (18), 159 $[M - 3\times CH_3\text{-}CO]^+$ (68), 131 (100); HREIMS *m*/*z* 232.1458 (calcd for $C_{15}H_{20}O_2$, 232.1463).

10-Oxodehydrodihydrobotrydial (3): colorless oil; $[\alpha]^{28}_D$ +87° (*c* 3.9 mg/mL, $CHCl_3$); IR (film) ν_{max} 2963, 1725, 1256, 1091 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.31 (3H, s, H-13), 1.44 (3H, s, H-12), 1.50 (3H, s, H-14), 1.84 (1H, d, $J_{7\alpha-7\beta}$ = 13.0 Hz, H-7α), 1.96 (1H, d, $J_{7\beta-7\alpha}$ = 13.0 Hz, H-7β), 2.61 (3H, s, H-11), 4.12 (1H, d, $J_{15\alpha-15\beta}$ = 10.1 Hz, H-15α), 4.35 (1H, d, $J_{15\beta-15\alpha}$ = 10.1 Hz, H-15β), 7.14 (1H, d, J_{4-3} = 7.8 Hz, H-4), 7.20 (1H, d, J_{3-4} = 7.8 Hz, H-3); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.3 (CH_3, C-11), 24.7 (CH_3, C-14), 30.7 (CH_3, C-13, C-12), 40.8 (C, C-6), 45.1 (C, C-8), 52.1 (CH_2, C-7), 79.2 (CH_2, C-15), 119.6 (C, C-1), 127.3 (CH, C-4), 131.7 (CH, C-3), 139.6 (C, C-2), 147.0 (C, C-9), 151.4 (C, C-5), 164.0 (C, C-10); EIMS *m*/*z* 230 $[M]^+$ (100), 215 $[M - CH_3]^+$ (71), 200 $[M - 2\times CH_3]^+$ (69); HREIMS *m*/*z* 230.1300 (calcd for $C_{15}H_{18}O_2$, 230.1307).

4β-Acetoxytetrahydrobotryslactone (4): colorless oil; $[\alpha]^{25}_D$ +26° (*c* 1.0 mg/mL, $CHCl_3$); IR (film) ν_{max} 3398, 2926, 1767, 1731, 1245 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 0.89 (3H, s, H-13), 1.05 (3H, s, H-12), 1.08 (3H, d, J_{11-2} = 7.0 Hz, H-11), 1.39 (1H, d, $J_{7\alpha-7\beta}$ = 12.8 Hz, H-7α), 1.41 (3H, s, H-14), 1.59−1.74 (2H, m, H_2-3), 1.78 (1H, d, $J_{7\beta-7\alpha}$ = 12.8 Hz, H-7β), 2.02 (3H, s, CH_3CO-), 2.25 (1H, brd, J_{5-4} = 4.9 Hz, H-5), 2.31 (1H, ddd, $J_{16\beta-17}$ = 3.7, $J_{16\beta-10}$ = 7.6 and $J_{16\beta-16\alpha}$ = 13.9 Hz, H-16β),

2.48 (1H, m, H-2), 2.48 (1H, ddd, $J_{16\alpha-17} = 7.7$, $J_{16\alpha-10} = 7.6$ and $J_{16\alpha-16\beta} = 13.9$ Hz, H-16α), 3.38 and 3.43 (1H each, d, $J_{15\alpha-15\beta} = 10.5$ Hz, H_2-15) 4.51 (1H, dd, $J_{17-16\beta} = 3.7$ and $J_{17-16\alpha} = 7.7$ Hz, H-17), 5.09 (1H, dd, $J_{4-3} = 9.8$ and $J_{4-5} = 4.9$ Hz, H-4), 5.88 (1H, dd, $J_{10-16\alpha} = J_{10-16\beta} = 7.6$ Hz, H-10); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 21.2 (CH_3, C-11), 21.3 (CH_3, *C*H_3CO-), 23.4 (CH_3, C-13), 25.5 (CH_3, C-14), 28.3 (CH_3, C-12), 28.6 (CH, C-2), 35.3 (CH_2, C-16), 37.0 (CH_2, C-3), 39.0 (C, C-6), 46.1 (C, C-8), 54.0 (CH_2, C-7), 56.8 (CH, C-5), 68.5 (CH, C-17), 70.2 (CH, C-4), 72.0 (CH_2, C-15), 77.5 (CH, C-10), 133.7 (C, C-1), 147.1 (C, C-9), 170.1 (C, CH_3*C*O−), 174.2 (C, C-18); EIMS *m*/*z* 366 $[M]^+$ (1), 335 $[M - CH_2OH]^+$ (2), 306 $[M - CH_3COOH]^+$ (22), 288 (100); HREIMS *m*/*z* 335.1898 (calcd for $C_{19}H_{27}O_5$ $[M - CH_2OH]^+$, 335.1858).

Phytotoxicity Assay.[10,11] Sterilized leaf disks of *Phaseolus vulgaris* were treated with solutions of the compound to be tested at concentrations of 1000, 500, and 250 ppm. The leaf disks (20 circles of 1 cm diameter) were placed in Petri dishes containing Whatman paper wetted with sterile H_2O. Purified metabolites, 10 μL, dissolved in 40% aqueous Me_2CO containing aqueous Tween 80 (10 μL of Tween 80 in 100 mL of water) were placed on each of the circles, and the plates were kept at 25−28 °C. Controls consisted of the mixtures of Me_2CO-H_2O-Tween 80 used for dissolving the purified metabolites. Two experiments were carried out for each compound and concentration. Botrydial, used as control substance, caused severe chlorosis and cell collapse. Compounds **1**−**3** were inactive at maximum concentrations tested. Lactone **4** produced chlorotic lesions at all concentrations tested. At 250 ppm, chlorosis took place in 100% of the treatments affecting 10% of the treated surface.

Acknowledgment. This work was financed by projects from the European Commission (FAIR 5 PL97-3351) and DGICYT AGL2000-0635-C02-01.

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NP020107V