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Biotransformation of the fungistatic sesquiterpenoids patchoulol, ginsenol, cedrol and globulol by *Botrytis cinerea*

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Abstract

The antifungal activity of natural sesquiterpenoids patchoulol, ginsenol, cedrol and globulol against the fungus *Botrytis cinerea* has been determined. The diminishing of the effect after 3–6 days suggests that a mechanism of detoxification is present. Biotransformation of these fungistatic compounds has been investigated as a method of studying this mechanism.

Keywords: Biotransformation; *Botrytis cinerea*; Patchoulol; Ginsenol; Cedrol; Globulol

1. Introduction

Botrytis species are significant pathogens implicated in many diseases of flowers, fruits and vegetables. In particular, *Botrytis cinerea* attacks economically important crops such as lettuce, carrots, tobacco, strawberries and grapes [1]. Moreover, *B. cinerea* has begun to develop a tolerance to commercial fungicides, thus provoking an increase in the quantities of fungicides used with the consequent additional problems of toxic fungicidal residues as well as serious economic losses due to the decreased quality of wines produced from treated grapes [2].

Over the last few years we have undertaken a research programme directed toward the rational design of fungicides for *Botrytis* infections of commercial crops based on biosynthetic principles. Botrydial and structurally related compounds are characteristic metabolites of *Botrytis* spp. Botrydial, the biosynthesis of which has been investigated [3], is a bicyclic non-isoprenoid sesquiterpene isolated from cultures of *B. cinerea* [4]. Although the precise role of these metabolites in the fungal physiology is unknown, our results have shown that they are responsible for the typical lesions of the fungal infection and that they play an important role in the expression of the phytotoxicity of the organism and its subsequent development [5].

We are currently exploring the inhibition of the biosynthesis of these key secondary metabolites by analogues of botrydial precursors in order to develop a rational means of controlling the fungus and its pathogenic effects. In the course of our research we have tested the fungicidal activity of different sesquiterpenoids and found that patchoulol (**1**), gin-

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senol (**2**), cedrol (**3**) and globulol (**4**) displayed antifungal activity against *B. cinerea.* However, the effect diminished after a 6-day incubation period with the fungus. The aim of the work described here was to study the biotransformation of these compounds by *B. cinerea* as part of the fungal detoxification mechanism.

2. Results and discussion

The antifungal properties of the compounds tested were established against the growth of *B. cinerea* using the "poisoned food" technique [6,7] (Experimental section); the commercial fungicide Euparen® was used as a standard. Patchoulol (**1**) displayed inhibitory activity at 40 and 60 ppm for 1 and 3 days, respectively, and total inhibition at 80 and 100 ppm for 4 days. Above 140 ppm, **1** exhibited total inhibition of the fungus for 6 days. The acetate of **1** was completely devoid of activity [8]. Ginsenol (**2**) was active above 60 ppm and gave total inhibition at 180 ppm for 4 days. It is worth noting that the acetyl and O-methyl derivatives were inactive, which indicates that the hydroxyl group plays an important role in the inhibitory mechanism [9]. Cedrol (**3**) displayed some inhibitory activity above 160 ppm (Fig. 1).

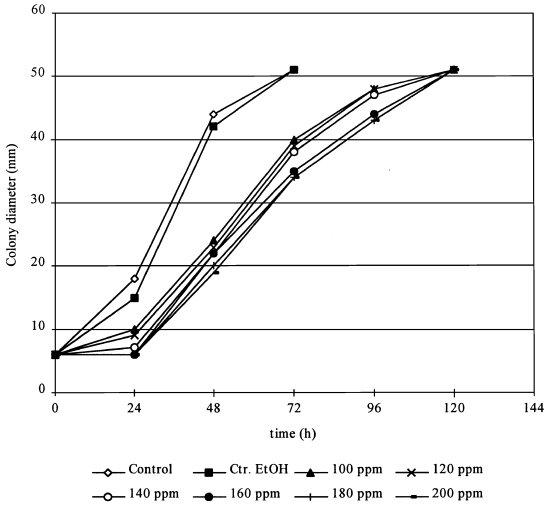


Fig. 1. Inhibition assay for compound **3**.

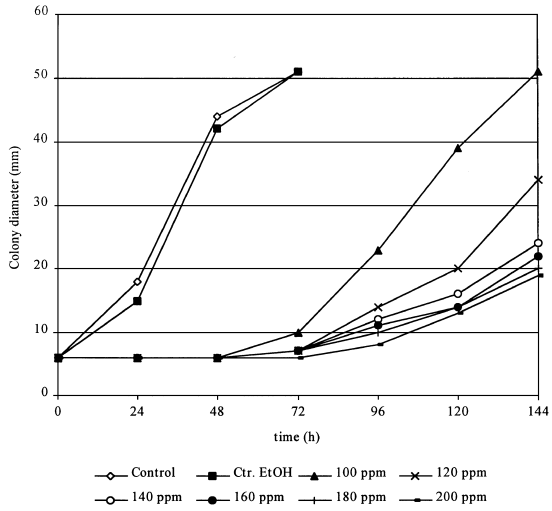
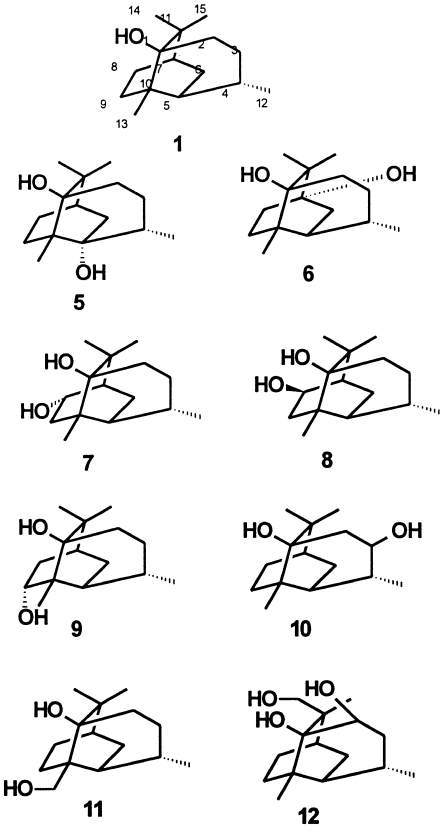


Fig. 2. Inhibition assay for compound **4**.

Globulol (**4**) displayed inhibitory activity from 100 ppm, showing total inhibition for 2 days, while at 200 ppm, total inhibition of fungus growth for 3 days was observed (Fig. 2). The acetyl derivatives of these compounds were inactive. These results show that *B. cinerea* has the ability to degrade the test compounds, depending on their concentration in the test, as the fungus begins to grow after 3–6 days, a fact which suggests that a detoxification mechanism is present.

In order to study this mechanism, **1**, **2**, **3** and **4** were incubated separately on surface cultures of *B. cinerea* on a Czapek–Dox medium for 3 days at a concentration of 150 ppm. The metabolites, which were not present in the controls, were detected by TLC. These metabolites were extracted with ethyl acetate and separated by means of column chromatography. From the biotransformation of **1**, eight compounds (**5**–**12**) were isolated [8]. Of these, Teisseire [10], using **1** with other microorganisms, had previously reported the formation of **5**, **6**, **8** and **9**. We have now obtained these products, along with compounds **7**, **10**, **11** and **12**, from the biotransformation by *B. cinerea* [8] (Scheme 1). In addition, compounds **13**–**18** (Scheme 2) were isolated from ginsenol (**2**) [9]. From compound **3**, six compounds (**19**–**24**) were isolated and their structures determined by means of spectral data. We identified

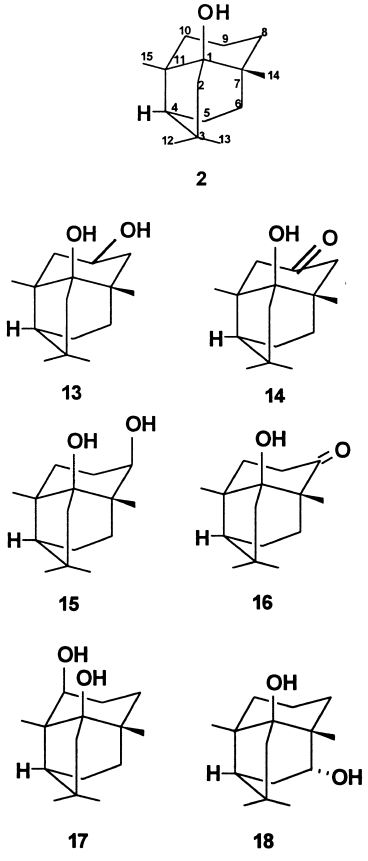


Scheme 1.

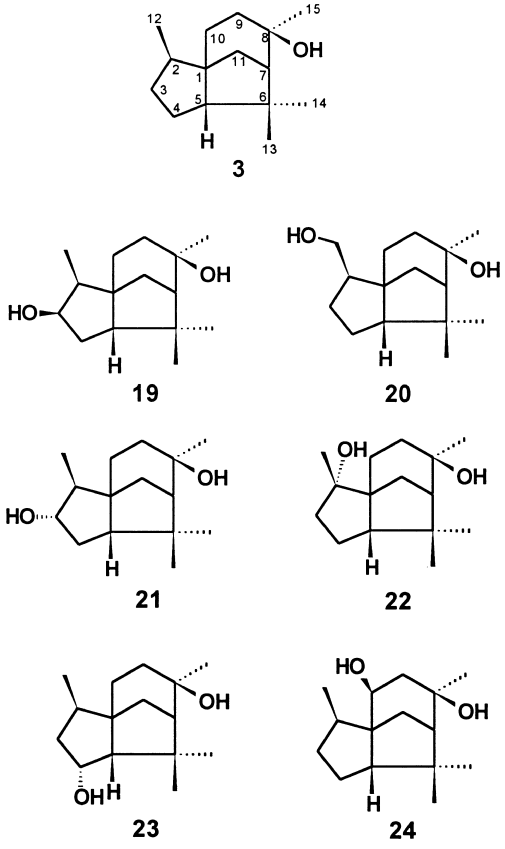
metabolites **19**–**21** as 3β-hydroxycedrol (**19**), 12-hydroxycedrol (**20**) and 3α-hydroxycedrol (**21**), using spectroscopic data [11]. Compounds **22**, **23** and **24** are described here for the first time (Scheme 3). Finally, globulol (**4**) was shown to be hydroxylated by *B. cinerea* on C-14, one of the methyl groups geminal to the cyclopropane ring. The resulting compound was identified as 14-hydroxyglobulol (**25**) after studying its spectroscopic data (Scheme 4). This compound had been previously reported by Hanson et al. [12], as obtained in the biotransformation of globulol (**4**) by the fungus *Cephalosporium aphidicola*. The fact that this hydroxylation occurs adjacent to a cyclopropane ring is significant.

The locations of the additional oxygen functions were established after analysis of the changes in the ^{13}C NMR spectra (Table 1). Compounds **22**, **23** and **24** had the molecular formula $C_{15}H_{26}O_2$, as deduced from their mass spectra (M^+ at m/z 238). The downfield shift of neighbouring carbon signals in these compounds from the corresponding positions in **3** (C-1, C-3 and C-12 for **22**, C-3 and C-5 for **23**, and C-1 and C-9 for **24**; Table 1) indicated that additional hydroxyl groups were located at C-2 in **22**, at C-4 in **23** and at C-10 in **24**. The stereochemistry of the hydroxyl groups was assigned taking into consideration the nuclear Overhauser enhancement observed for the ^{1}H NMR signals. In addition, in **24** a downfield shift of H-5 was observed, which suggests a β-stereochemistry of the hydroxyl group. Homo- and heteronuclear 2D-correlation experiments corroborated the proposed structures.

In the course of these biotransformation experiments, several effects on the growth of the fungus *B.*

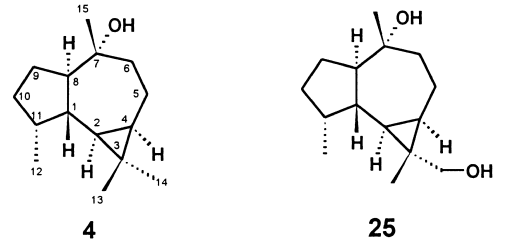


Scheme 2.



Scheme 3.

cinerea were observed. First, growth of the mycelium was inhibited when the substrate was added to the broth. Second, examination of the broth extracts showed that phytotoxic botryane metabolites were not present in the early stages of the fermentation when the fungistatic compounds were still present. The fact that the acetates of **1**, **2**, **3** and **4** had no effect on mycelial growth indicates the importance of the tertiary alcohol for the expression of the biological activity of these compounds. The poor recovery of the products from the biotransformations may be related to the further biodegradation of these compounds by Baeyer–Villiger oxidation and further fragmentation of the ring system. These results give an indication of the structural modifications which may be necessary if substrates of this type are to be further developed as selective fungal control agents for *B. cinerea*.



Scheme 4.

Table 1
^{13}C NMR data of compounds **3**, **22**, **23**, **24** (100 MHz, $CDCl_3$)

C	3	22	23	24
1	54.1s	57.6s	52.8s	58.2s
2	41.1d	79.6s	38.6d	44.5d
3	37.0t	41.3t	46.7t	40.1t
4	25.3t	21.5t	73.5d	26.3t
5	56.5d	53.5d	65.1d	52.5d
6	43.4s	45.0s	41.3s	43.0s
7	61.0d	59.1d	61.4d	61.0d
8	75.3s	74.9s	74.8s	74.2s
9	35.3t	35.6t	34.7t	45.3t
10	31.6t	30.0t	31.6t	72.1d
11	42.0t	36.6t	42.7t	40.1t
12	15.6q	24.3q	15.3q	14.7q
13	27.6q	27.5q	28.2q	27.3q
14	28.9q	28.4q	28.5q	29.3q
15	30.2q	30.4q	30.2q	31.4q

3. Experimental section

3.1. General experimental procedures

Melting points were measured with a Reichert–Jung Kofler block (uncorrected). Optical rotations were determined with a Perkin-Elmer 241 polarimeter in $CHCl_3$. IR spectra were recorded on a Perkin-Elmer 881 spectrophotometer. ^{1}H and ^{13}C NMR measurements were obtained on Varian Gemini 200 and Varian Unity 400 NMR spectrometers with $SiMe_4$ as internal reference. Mass spectra were recorded on a VG 12-250 spectrometer 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 Kiesegel 60 F_{254}, 0.2 mm thick. Silica gel (Merck) type 9385 was used for column chromatography. Purification by means of HPLC was accomplished using a silica gel column (Hibar 60, 7 m, 1 cm wide, 25 cm long).

3.2. Microorganism and antifungal assays

B. cinerea (UCA 992), was obtained from grapes from the Domecq vineyards, Jerez de la Frontera, Cádiz, Spain. This culture is deposited in the Universidad de Cádiz, Facultad de Ciencias Mycological Herbarium Collection (UCA). Compounds **1**, **2**, **3** and **4** were tested on *B. cinerea* with the "poisoned food technique". Test compounds were dissolved in ethanol to give a final compound concentration of 50 to 200 ppm. Solutions of the test compounds were added to glucose–malt–peptone–agar medium (61 g of glucose–malt–peptone–agar per liter, pH 6.5–7.0). The final EtOH concentration was identical for control and test experiments. The medium was poured into 6-cm-diameter sterile plastic Petri dishes, and a 5-mm-diameter mycelial disc of *B. cinerea* cut from an actively growing culture was placed in the centre of the agar plate. Inhibition of radial growth was measured for 6 days.

3.3. General culture conditions

B. cinerea (UCA 992) was grown on surface culture in Roux bottles at 25°C for 3 days on a Czapek–Dox medium (150 ml per flask) comprising (per liter of distilled H_2O), glucose (40 g), yeast extract (1 g), potassium dihydrogen phosphate (5 g), sodium nitrate (2 g), magnesium sulphate (0.5 g), ferrous sulphate (10 mg) and zinc sulphate (5 mg). The substrate was dissolved in EtOH (250 μl) and then added to each flask, after which fermentation continued for a further period of 3 days. The mycelium was filtered and washed with brine and ethyl acetate. The broth was saturated with NaCl, acidified to pH 2 and extracted with ethyl acetate. The extracts were separated into acidic and neutral fractions with aqueous sodium bicarbonate. The acidic fraction was recovered in ethyl acetate. The extracts were dried over anhydrous Na_2SO_4, the solvent was evaporated and the residues were chromatographed on silica gel 60 (70–230 mesh) with an increasing gradient of ethyl acetate to petroleum ether.

3.4. Biotransformations by B. cinerea

Test compounds (150 ppm per flask) were distributed over 10 flasks of *B. cinerea* and the fermentation was continued for 3 days. Following the general isolation procedure, a TLC of the acidic fraction revealed no differences between the test groups and control; this fraction was not studied further. Nine compounds were isolated from the neutral fraction of the patchoulol (**1**) fermentation [8]: recovered patchoulol (**1**) (62 mg); (5*R*)-5-hydroxypatchoulol (**5**) (11 mg); (7*S*)-7-hydroxypatchoulol (**6**) (47 mg); (8*R*)-8-hydroxypatchoulol (**7**) (28 mg); (8*S*)-8-hydroxypatchoulol (**8**) (3.6 mg); (9*R*)-9-hydroxypatchoulol (**9**) (4.3 mg); (3*R*)-3-hydroxypatchoulol (**10**) (1 mg); 13-hydroxypatchoulol (**11**) (1 mg); (2*S*)-2,14-dihydroxypatchoulol (**12**) (1.5 mg). Seven compounds were isolated from the neutral fraction of the ginsenol (**2**) fermentation [9]: ginsenol (**2**) (69 mg), 9β-hydroxyginsenol (**13**) (25 mg), 9-oxoginsenol (**14**) (5 mg), 8β-hydroxyginsenol (**15**) (25 mg); 8-oxoginsenol (**16**) (1.5 mg), 10β-hydroxyginsenol (**17**) (7 mg), 6α-hydroxyginsenol (**18**) (7.5 mg). From the neutral fraction of compound **3**, the following compounds were isolated: cedrol (**3**) (61 mg), 3β-hydroxycedrol (**19**) (26 mg), 12-hydroxycedrol (**20**) (29 mg), 3α-hydroxycedrol (**21**) (17 mg); 2α-hydroxycedrol (**22**) (2 mg), 4α-hydroxycedrol (**23**) (1.4 mg); 10β-hydroxycedrol (**24**) (4.5 mg). Finally, from the globulol (**4**) fermentation, globulol (**4**) (176 mg) and 14-hydroxyglobulol (**25**) (75 mg) were obtained.

2α-Hydroxycedrol (**22**): white solid; mp 85–86 C; $[\alpha]_D^{25} + 28$ (*c* 1.0, $CHCl_3$); IR υ_{max} (film) 3382, 2966, 1467, 1367, 1118, 944, 762 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 1.76 (1H, m, H-5), 1.47 (1H, m, H-7), 1.29 (3H, s, H-13), 1.27 (3H, s, H-15), 1.17 (3H, s, H-12), 1.04 (3H, s, H-14); ^{13}C NMR data, Table 1; EIMS *m/z* 238 [M^+] (4), 220 (53), 205 (29), 203 (19), 162 (65), 150 (100), 43 (94); HREIMS *m/z* 238.1930 [M^+] (calcd for $C_{15}H_{26}O_2$, 238.1932).

4α-Hydroxycedrol (**23**): white solid; mp 86–87 C; $[\alpha]_D^{25} + 33$ (*c* 1.0, $CHCl_3$); IR υ_{max} (film) 3340, 2967, 1649, 1458, 1350, 1127, 763 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 4.10 (1H, ddd, $J = 9.6$, 8.4 and 5.3 Hz, H-4), 2.08 (1H, ddd, $J = 11.4$, 5.7 and 5.6 Hz, H-3β), 1.72 (1H, m, H-2), 1.68 (1H, d, $J = 8.1$ Hz, H-5), 1.45 (1H, m, H-3α), 1.40 (3H, s, H-15), 1.24 (3H, s, H-14), 1.15 (3H, s, H-13), 0.91 (3H, d, $J = 7.1$ Hz, H-12). ^{13}C NMR data, Table 1; EIMS *m/z* 238 [M^+] (25), 220 (10), 205 (10), 203 (6), 162 (22), 151 (100), 93 (92), 43 (42); HREIMS *m/z* 238.1944 [M^+] (calcd for $C_{15}H_{26}O_2$, 238.1933).

10β-Hydroxycedrol (**24**): white solid; mp 83–84 C; $[\alpha]_D^{25} + 12$ (*c* 1.0, $CHCl_3$); IR υ_{max} (film) 3418, 2944, 1635, 1467, 1380, 1127, 1043, 770 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 3.77 (1H, dd, $J = 9.8$ and 6.2 Hz, H-10α), 2.12 (1H, dd, $J = 9.7$ and 7.6 Hz, H-5), 2.05 (1H, ddd, $J = 12.9$, 6.3 and 1.3 Hz, H-9α), 1.87 (1H, m, H-3), 1.70–1.56 (4H, m, H-2, H-4, H-7, H-9β), 1.36 (1H, m, H-4′), 1.32 (3H, s, H-13), 1.24 (3H, s, H-15), 1.13 (3H, d, $J = 6.8$ Hz, H-12); 1.03 (3H, s, H-14); ^{13}C NMR data, Table 1; EIMS *m/z* 238 [M^+] (6), 220 (20), 203 (70), 109 (65), 69 (100), 43 (88); HREIMS *m/z* 238.1977 [M^+] (calcd for $C_{15}H_{26}O_2$ 238.1933).

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