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Biotransformation of the fungistatic sesquiterpenoid isoprobotryan-9α-ol by *Botrytis cinerea*

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Abstract

The antifungal activity of isoprobotryan-9α-ol (**1**) against the plant pathogen *Botrytis cinerea* has been determined. The biotransformation of this fungistatic sesquiterpenoid by *B. cinerea* gave the 12-, 14- and 15-hydroxyderivatives (**2**–**4**) as the major metabolites. The diminishing of the effect with time suggests that a mechanism of detoxification is present.

Keywords: Biotransformation; *Botrytis cinerea*; Sesquiterpenoid; Isoprobotryan-9α-ol

1. Introduction

The *Botrytis* species belong to the most geographically widespread group of plant pathogens and saprophytes [1]. Grey mould, a grapevine disease caused by the fungus *Botrytis cinerea* results in crop losses and low quality wines from infected grapevines in many wine-producing countries [2].

The widespread use of chemical fungicides to prevent or eliminate grey mould has resulted in both the appearance of highly resistant strains in *B. cinerea* populations, which very quickly develop resistance to benzimidazoles and dicarboximides [3], and the contamination of soil and water. Consequently, there is interest in the development of novel antifungal agents with activity against this microorganism.

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 some related compounds with the botryane skeleton are characteristic metabolites of *Botrytis* spp. [4]. Our results have shown that these metabolites are toxins [5] which reproduce the symptoms of grey mould on detached grapevine and tobacco leaves and that they play an important role in the expression of the phytotoxicity of the organism and its subsequent development [6].

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, selective environment friendly fungicides have been proposed as a more rational use of prophylactic treatments [7–9]. Thus, we have assessed the fungicidal activity of different sesquiterpenoids related to the metabolites of *B. cinerea* in order to inhibit the formation of phytotoxic botrylane metabolites by the fungus. Subsequently, isoprobotryan-9α-ol (**1**), which we had obtained by a novel rearrangement of isocaryophyllene [10], displayed antifungal activity

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against *B. cinerea*. The aim of the work described in this paper was to study the biotransformation of isoprobotryan-9α-ol (1) by *B. cinerea* as a part of the fungal detoxification mechanism.

2. Results and discussion

The antifungal properties of the compound tested were established against the growth of *B. cinerea* using the "poisoned food" technique [10,11] (see Section 3); the commercial fungicide Euparen® was used as a standard. Isoprobotryan-9α-ol (**1**) displayed inhibitory activity above 40 ppm and total inhibition at 100 ppm for 3 days. Above 140 ppm, **1** exhibited total inhibition of the fungus for 5 days (Fig. 1). The acetate of **1** was completely devoid of activity, which indicates that the hydroxyl group plays an important role in the inhibitory mechanism. However, the inhibitory effect of **1** diminished with time suggesting that the fungus possessed a detoxification mechanism.

In order to study this mechanism, **1** was incubated on surface cultures of *B. cinerea* on a Czapek-Dox medium for 3 days at a concentration of 60 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. In addition to the starting material, from the biotransformation of isoprobotryan-9α-ol (**1**), three compounds (**2**–**4**) were isolated. We identified these metabolites **2**–**4** as isoprobotryan-12,9α-diol (**2**), isoprobotryan-14,9α-diol (**3**) and isoprobotryan-15,9α-diol (**4**). Compounds **2**–**4** are described here for the first time.

The locations of the additional oxygen functions on the methyl groups were established after analysis of the spectroscopic data. Compounds **2**–**4** had the molecular formula $C_{15}H_{26}O_2$, as deduced from their mass spectra (M^+ at m/z 238). The absence of methyl group signals in the ^{1}H and ^{13}C NMR spectra assigned to C-12, C-14 and C-15, and the appearance of new hydroxymethyl resonances (δ_H 3.45 and 3.69; δ_C 71.6 for **2**; δ_H 3.32 and 3.34; δ_C 74.5 for **3**; δ_H 3.60 and 3.64; δ_C 69.7 for **4**) suggested that these compounds were hydroxylated at C-12, C-14 and C-15. The stereochemistry of these products was confirmed taking into consideration the nuclear Overhauser enhancement observed for the ^{1}H NMR signals.

In the course of this biotransformation experiment, several effects on the growth of the fungus *B. cinerea* were observed. First, growth of the mycelium was inhibited when the substrate was added to the broth. Second, examination of the broth extract showed that phytotoxic botryane metabolites were not present

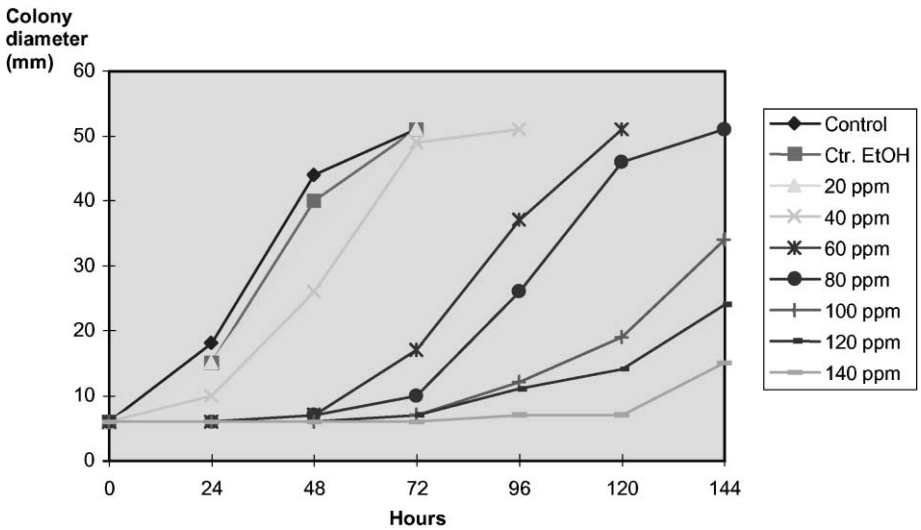
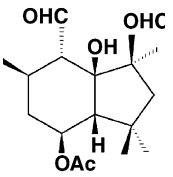


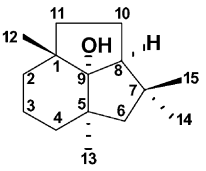
Fig. 1. Inhibition assay for compound **1**.

in the early stages of the fermentation when the fungistatic compound was still present. These results seem to indicate that the biosynthesis of botrydial and its derivatives is inhibited. The inhibition of the biosynthesis of these toxins could reduce the virulence of the fungus, disabling one of its mechanisms of infection.

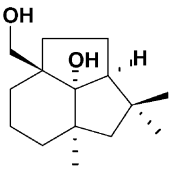
The fact that the acetate of isoprobotryan-9α-ol (**1**) had no effect on micelial growth indicates the importance of the tertiary alcohol for the expression of the biological activity of this compound. The low recovery of the products from the biotransformation may be related to their further biodegradation by fragmentation of the ring system.



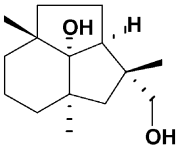
Botrydial



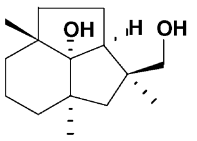
1



2



3



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3. Experimental section

3.1. 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. ^{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 an 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) was used for column chromatography. Purification by HPLC was accomplished using a silica gel column (Hibar 60, 7 m, 1 cm wide, 25 cm long).

3.2. Microorganism and antifungal assays

The culture of *B. cinerea* employed in this work, *B. cinerea* (UCA 992), was obtained from grapes of Domecq vineyard, Jerez de la Frontera, Cádiz, Spain. This culture of *B. cinerea* is deposited in the Universidad de Cadiz, Facultad de Ciencias Mycological Herbarium Collection (UCA). Bioassays were performed by measuring inhibition of radial growth on agar medium in a Petri dish. Test compound was dissolved in ethanol to give a final compound concentration of 20–140 mg l^{-1}. Solutions of test compound were added to glucose–malt–peptone–agar medium (61 g of glucose–malt–peptone–agar per litre, pH 6.5–7.0). The final ethanol concentration was identical in control and treated cultures. The medium was poured in 6 cm diameter sterile plastic Petri dishes and a 5 mm diameter micelial 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 litre of distilled water), 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 dissolved in ethanol was added to each flask and the 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 sodium chloride, acidified (pH 2) and extracted with ethyl acetate. The extracts were separated into

acidic and neutral fractions with aqueous sodium hydrogen carbonate. The acidic fraction was recovered in ethyl acetate. The extracts were dried over anhydrous sodium sulphate, the solvent was evaporated and the residues were chromatographed on silica gel with an increasing gradient of ethyl acetate to petroleum ether.

3.4. Biotransformation by B. cinerea

B. cinerea was grown on static culture on Czapeck-Dox medium. The substrate (isoprobotryan-9α-ol (**1**), 60 ppm per bottle) in ethanol was distributed over 10 Roux bottles after 2 days growth. After 6 days, the fermentation broth was extracted with ethyl acetate and the residue was chromatographed on silica gel column and HPLC. From neutral fractions following compounds were isolated: recovered isoprobotryan-9α-ol (**1**) (10 mg), isoprobotryan-12,9α-diol (2) (1 mg), isoprobotryan-14,9α-diol (3) (2 mg), isoprobotryan-15,9α-diol (4) (0.5 mg).

3.5. Isoprobotryan-9α-ol (*1*)

White solid, mp 32–35 °C; $[\alpha]_D$ ($CHCl_3$, $c = 0.9$) + 8°; IR υ_{max} (film) 3474, 2950, 1443, 1373; ^{1}H NMR (400 MHz, $CDCl_3$) 0.98 (3H, d, $J = 0.9$ Hz, H-13), 1.01 (3H, s, H-12), 1.02 (3H, s, H-15), 1.04 (3H, s, H-14), 1.32 (1H, m, H-3), 1.40 (2H, m, H-10), 1.60 (1H, m, H-2), 1.67 (1H, m, H-3′), 1.77 (2H, m, H-11), 1.88 (1H, ddd, $J = 14.1$ Hz, $J = 14.1$ Hz, $J = 3.6$ Hz, H-2′), 1.89 (1H, d, $J = 13.6$ Hz, H-6α), 2.13 (1H, d, $J = 13.6$ Hz, H-6β), 2.67 (1H, dd, $J = 12.4$ Hz, $J = 8.0$ Hz, H-8); ^{13}C NMR data (Table 1); EIMS *m/z* 222 [M^+] (11), 207 [$M^+ - 15$] (3), 204 [$M^+ - 18$] (3), 189 [$M^+ - 15$–18] (2), 151 (23), 125 (52), 40 (100); HREIMS *m/z* 222.1988 [M^+] (calcd. for $C_{15}H_{26}O$, 222.1983).

3.6. Isoprobotryan-12,9α-diol (*2*)

White solid 74–76 °C; $[\alpha]_D$ ($CHCl_3$, $c = 0.6$) +33.3°; IR υ_{max} (film) 3315, 2924, 1458, 1042; ^{1}H NMR (400 MHz, $CDCl_3$) 1.06 (3H, s, H-13), 1.10 (3H, s, H-14α), 1.13 (3H, s, H-15β), 1.42 (1H, m, H-10), 1.88 (1H, d, $J = 13.3$ Hz, H-6α), 2.26 (1H, d, $J = 13.3$ Hz, H-6β), 2.71 (1H, dd, $J = 12.2$ Hz, $J = 8.3$ Hz, H-8), 3.45 (1H, d, $J = 10.3$ Hz, H-12), 3.69 (1H, d, $J = 10.3$ Hz, H-12′); ^{13}C NMR data, Table 1; EIMS *m/z* 238 [M^+] (17), 221 [$M^+ + 1$-18] (64), 207 [$M^+ - 31$] (100), 203 [$M^+ + 1$–18–18] (37), 196 (15), 189 (43), 181 (21); HREIMS *m/z* 238.1925 [M^+] (calcd. for $C_{15}H_{26}O_2$, 238.1933).

Table 1
^{13}C NMR data of compounds **1**–**4** (100 MHz, $CDCl_3$)[a]

C	**1**	**2**	**3**	**4**
1	37.9^0		38.2^0	38.2^0
2	37.1^-	35.8^-	36.8^-	37.0^-
3	20.3^-	20.9^-	20.1^-	19.9^-
4	38.3^-	39.1^-	38.4^-	38.5^-
5	56.9^0			
6	60.8^-	59.4^-	55.9^-	56.8^-
7	32.6^0		37.6^0	37.3^0
8	58.3^+	58.1^+	53.9^+	55.4^+
9	76.8^0		76.1^0	71.5^0
10	18.9^-	18.5^-	19.9^-	19.1^0
11	44.0^-	36.8^-	43.9^-	43.8^-
12	23.9^+	71.9^-	23.7^+	23.7^+
13	13.7^+	13.3^+	14.0^+	13.7^+
14	26.9^+	34.0^+	74.5^-	28.2^+
15	34.2^+	26.8^+	21.4^+	69.7^-

[a] Amplitude of signals in DEPT spectrum (Me or CH = +, CH_2 = −; quart C = 0).

3.7. Isoprobotryan-14,9α-diol (*3*)

Colourless oil, mp: 89–91 °C;$[\alpha]_D$ ($CHCl_3$, $c = 1.7$) +6.5°; IR υ_{max} (film) 3385, 2928, 1461, 1383, 1030; ^{1}H-RMN (400 MHz, $CDCl_3$) 0.97 (3H, s, H-12), 1.06 (3H, s, H-15β), 1.10 (3H, s, H-13), 1.77 (1H, dd, $J = 7.4$, $J = 13.8$ Hz, H-11), 1.84 (1H, dd, $J = 3.7$, $J = 13.8$ Hz, H-11′), 1.91 (1H, dd, $J = 3.6$, $J = 14.1$ Hz, H-2), 1.97 (1H, d, $J = 13.9$ Hz, H-6α), 2.02 (1H, d, $J = 13.9$ Hz, H-6β), 2.72 (1H, dd, $J = 13.3$ Hz, $J = 7.2$ Hz, H-8), 3.32 (1H, d, $J = 10.1$ Hz, H-14), 3.34 (1H, d, $J = 10.1$ Hz, H-14′); ^{13}C NMR data, Table 1; EIMS *m/z* 238 [M^+] (4), 223 (28), 220 [$M^+ - 18$] (8), 207 [$M^+ - 31$] (27), 202 [$M^+ -$ 18–18] (2), 196 (15), 190 (51), 165 (46), 147 (57), 125 (100); HREIMS *m/z* 238.1929 [M^+] (calcd. for $C_{15}H_{26}O_2$, 238.1933).

3.8. Isoprobotryan-15,9α-diol (*4*)

Colourless oil; $[\alpha]_D$ ($CHCl_3$, $c = 0.5$) +6°; IR υ_{max} (film) 3401, 2927, 1463, 1379, 1139, 1028; ^{1}H NMR

(400 MHz, $CDCl_3$) 0.97 (3H, s, H-12), 1.01 (3H, s, H-13), 1.12 (3H, s, H-14), 1.63 (1H, m, H-2), 1.75 (1H, m, H-10), 1.77 (1H, d, $J = 14.0$ Hz, H-6α), 1.90 (1H, ddd, $J = 3.5$, $J = 13.9$, $J = 14.4$ Hz, H-2′), 2.20 (1H, d, $J = 14.0$ Hz, H-6β), 2.66 (1H, dd, $J = 13.5$ Hz, $J = 7.0$ Hz, H-8), 3.60 (1H, d, $J = 10.7$ Hz, H-15), 3.64 (1H, d, $J = 10.7$ Hz, H-15′); ^{13}C NMR data, Table 1; EIMS *m/z* 238 [M^+] (14), 220 [$M^+ - 18$] (12), 207 [$M^+ - 31$] (26), 190 (34), 167 (36), 125 (100); HREIMS *m/z* 238.1920 [M^+] (calcd. for $C_{15}H_{26}O_2$, 238.1933).

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