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Biotransformation of the fungistatic compound (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol by *Botrytis cinerea*

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Abstract

The biotransformation of the fungistatic agent (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**) by the phytopathogen *Botrytis cinerea* has been studied. The main reaction pathways involved hydroxylations on several positions as well as condensations with secondary metabolites of the fungus. The antifungal activity of compound **1** against *B. cinerea* has also been determined.

Keywords: Biotransformation; *Botrytis cinerea*; (*R*)-(+)-1-(4′-Chlorophenyl)propan-1-ol; Fungistatic

1. Introduction

The *Botrytis* species comprises potent pathogens implicated in many diseases of flowers, fruits, and vegetables. In particular, *Botrytis cinerea* attacks economically important crops such as carrots, grapes, lettuce, strawberries, and tobacco, producing various leaf spot diseases and powdery grey mildews [1].

Because the fungus develops a rapid tolerance to commercial fungicides, these compounds must be used in increasingly higher quantities. This in turn leads to the appearance of highly resistant strains in *B. cinerea* populations, as well as the contamination of both soil and water. Thus, interest in the development of novel antifungal agents against this microorganism is understandably keen.

Over the last few years we have undertaken a research programme directed toward the rational design of fungicides for *Botrytis* infections of commercial crops. In order to find substrates with antifungal properties against this fungus, we have undertaken a screening of compounds analogous to various phytoalexins [2,3]. In the course of our research, we have been able to prove the fungicidal activity against *B. cinerea* of various compounds with a skeleton similar to that of several active compounds. One such compound, (±)-1-(4′-chlorophenyl)propan-1-ol, exhibited high antifungal activity against the fungus.

In order to compare the activities of enantiomeric compounds, we obtained both *R* and *S* alcohol configurations and found that (*R*)-(+)-1-(4′-chlorophenyl)-propan-1-ol (**1**) [4,5], which had been obtained by means of an enzymatic transesterification reaction [6], displayed a higher antifungal activity against *B. cinerea* than its enantiomer.

Recently, there have been many reports on the degradation of plant anti-microbial compounds by phytopathogenic fungi via a variety of mechanisms; indeed, in many cases the degradation products have been shown to be less toxic to fungal growth than the

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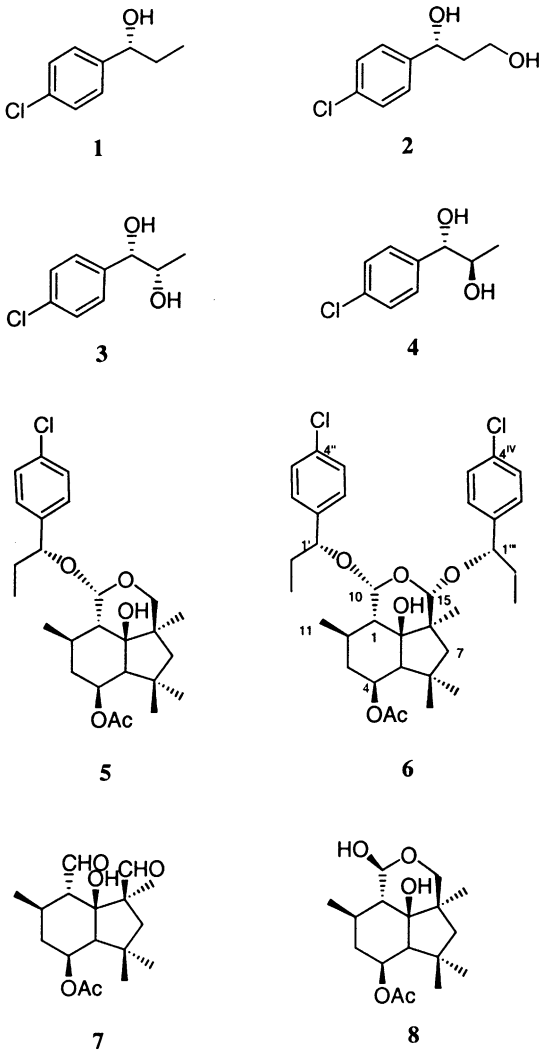
parent compounds [7]. Some fungi have even been reported to detoxify more than one plant compound. *B. cinerea* provides a good example of this ability, as isolates of this fungus have been found to detoxify the grapevine phytoalexin resveratrol [8].

The aim of the work described in this paper was to study the biotransformation of (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**) by *B. cinerea* in order to understand better the possible fungal detoxification mechanism. In this manner, we hope to gain insight into which structural modifications would be necessary if substrates of this type are to be further developed as selective fungal control agents for *B. cinerea*.

2. Results and discussion

The antifungal properties of compound **1** were established against the growth of *B. cinerea* using the "poisoned food" technique [9,10] (Section 3); the commercial fungicide Euparen® was used as a standard. (*R*)-(+)-1-(4′-Chlorophenyl)propan-1-ol (**1**) displayed inhibitory activity above 25 ppm and total inhibition for 3 days at 125 ppm. Above 150 ppm, **1** exhibited total inhibition of the fungus for 5 days (Fig. 1). The acetate of **1** was completely devoid of activity, a fact that 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 has its own detoxification mechanism.

In order to study this mechanism, compound **1** was incubated for 3 days at a concentration of 150 ppm on surface cultures of *B. cinerea* on a Czapeck–Dox medium. The metabolites, which were not present in the controls, were detected by means of TLC. These metabolites were extracted with ethyl acetate and separated by means of column chromatography. In addition to the starting material, the biotransformation of (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**) afforded five compounds: (*R*)-(+)-1-(4′-chlorophenyl)propan-1,3-diol (**2**), *threo*-1-(4′-chlorophenyl)propan-1,2-diol (**3**), *erythro*-1-(4′-chlorophenyl)propan-1,2-diol (**4**), and two new compounds, **5** and **6**, which are formed by reaction of **1** with the major metabolites from the fungus, botrydial (**7**) and dihydrobotrydial (**8**) [11]:



The location of an additional oxygen function on the linear chain in compounds **2**–**4** was established after analysis of the spectroscopic data. Compounds **2**–**4** all had the molecular formula $C_9H_{11}O_2Cl$ as deduced from their mass spectra (M^+ at *m*/*z* 186). The absence in the ^1H and ^{13}C NMR spectra of methyl group signals in compound **2**, as well as the appearance of new hydroxymethyl resonances (δ_H 3.81 and δ_C 61.2) suggested that this compound was hydroxylated at C-3.

Likewise, the ^1H and ^{13}C NMR spectral data for compounds **3** and **4** displayed new hydroxymethine resonances at δ_H 3.81 (1H, qd) and 4.00 (1H, m), respectively, indicating that both compounds are products of hydroxylation at C-2 methylene groups. The

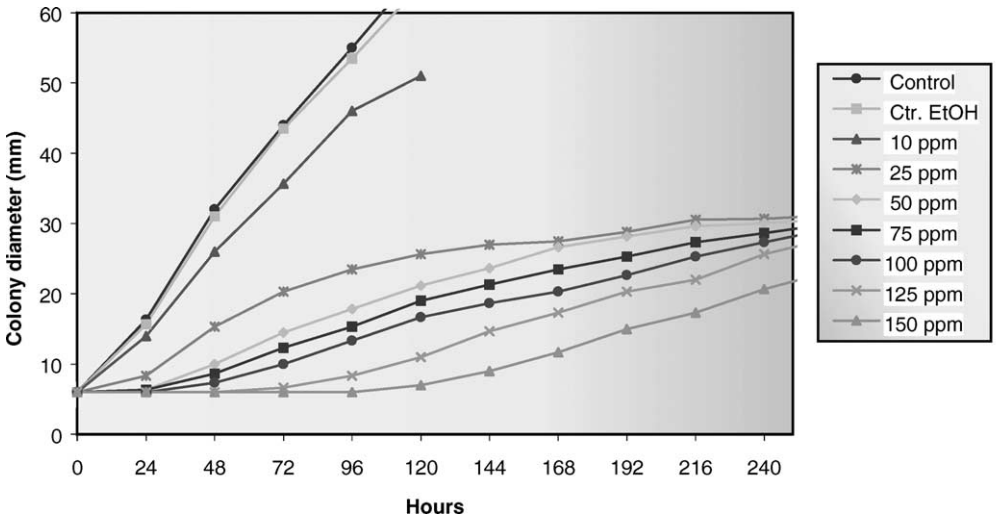


Fig. 1. Inhibition assay for compound **1**.

spectroscopic data for **3** and **4** were identical to those described in the literature for the diasteromers *threo* and *erythro*-1-(4′-chlorophenyl)propan-1,2-diol [12].

In addition, antifungal assays of the compounds **2**–**4** against *B. cinerea* were performed. These products have been shown to be less toxic to fungal growth than (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**). This fact confirms that *B. cinerea* has a mechanism to detoxify compound **1** by hydroxylating positions C-2 and C-3.

As mentioned above, apart from the diols **2**–**4**, two new compounds, **5** and **6**, were isolated. Many of the signals in their ^{1}H and ^{13}C NMR spectra were similar to those of dihydrobotrydial (**8**). However, the signals corresponding to the hydroxyl group on C-10 were absent in both compounds while the signals assigned to H-10 were more deshielded. Moreover, signals corresponding to a 1-(4′-chlorophenyl)propoxy group were present in the ^{1}H and ^{13}C NMR spectra of **5** and **6**, indicating that the 10-hydroxy group of dihydrobotrydial (**8**) had been etherificated with a 1-(4′-chlorophenyl)propan-1-ol group. NOE interactions observed between H-1, H-11, and H-10 indicated the β orientation for H-10. In addition, the ^{1}H and ^{13}C NMR spectra of compound **6** gave evidence for the presence of another 1-(4′-chlorophenyl)propoxy group while a signal at δ 4.93 (1H, s) corresponding to H-15 in its ^{1}H NMR spectrum helped locate that group on C-15. This was confirmed by an observable downfield shift of the H-7α and H-7β signals. NOE experiments revealed an enhancement of the signals corresponding to H-1, H-11 and H-1‴ upon irradiation of H-15, which must thus have a β orientation.

Both compounds **5** and **6** could be considered products from the chemical reaction between the major secondary metabolites of *B. cinerea*, botrydial (**7**) and dihydrobotrydial (**8**), and the starting material of the biotransformation, **1**. To confirm this, we treated (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**) with **7** and **8** separately in the culture broth under the same conditions as those of the biotransformation, but without the fungus. In both cases, the corresponding condensation compounds were obtained.

The fact that neither compound **5** nor **6**, nor the acetate of (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**) had any effect on mycelial growth indicates the importance of the secondary alcohol of **1** for the expression of the antifungal activity against *B. cinerea*. The low recovery of the products from the biotransformation may be related to the condensation of the starting material **1** with the secondary metabolites of the fungus.

3. Experimental

3.1. General experimental procedures

Optical rotations were determined with a Perkin-Elmer 241 polarimeter. IR spectra were recorded on a Mattson Genesis spectrophotometer, series FTIR^{TM}.

^{1}H and ^{13}C NMR measurements were obtained on Varian Gemini 200 and Varian Unity 400 NMR spectrometers with $SiMe_4$ as the internal reference. Mass spectra were recorded on a GC-MS Thermoquest spectrometer, model Voyager, and a VG Autospec-Q spectrometer. 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 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

The culture of *B. cinerea* employed in this work, *B. cinerea* 2100, was obtained from the "Centro Español de cultivos tipos" (CECT), Facultad de Biologia, Universidad de Valencia, Spain, where a culture of this strain is deposited. Bioassays were performed by measuring inhibition of radial growth on an agar medium in a Petri dish. Test compound was dissolved in ethanol to give final compound concentrations ranging from 10 to 150 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 both control and treated cultures. The medium was poured into sterile plastic Petri dishes measuring 6 cm in diameter 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 10 days.

3.3. Biotransformation by B. cinerea

B. cinerea 2100 was grown as a surface culture in Roux bottles at 25 °C on a Czapeck–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 (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**) was dissolved in ethanol and then distributed over 10 Roux bottles after 2 days' growth (150 ppm per bottle). Fermentation continued for 3 more days, after which time the mycelium was filtered and then washed with brine and ethyl acetate. The broth was extracted three times with ethyl acetate and the extract was dried over anhydrous sodium sulphate. The solvent was then evaporated and the residue was chromatographed first on a silica gel column and then with HPLC with an increasing gradient of ethyl acetate to petroleum ether. The following compounds were isolated from the fractions: recovered (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**) (79 mg), (*R*)-(+)-1-(4′-chlorophenyl)propan-1,3-diol (**2**) (52 mg), *threo*-1-(4′-chlorophenyl)propan-1,2-diol (**3**) (16 mg), *erythro*-1-(4′-chlorophenyl)propan-1,2-diol (**4**) (20 mg), 10α-[1′-(4″-chlorophenyl)propoxy]-10-epidihydrobotrydial (**5**) (29 mg), and 10α,15α-bis [1-(4′-chlorophenyl)propoxy]-10-epidihydrobotrydial (**6**) (5 mg).

3.3.1. (R)-(+)-1-(4′-Chlorophenyl)propan-1,3-diol (2)

Colourless oil, $[\alpha]_D^{20}$ ($CDCl_3$, $c = 0.4$): +13.2; IR ν_{max} (film): 3362, 2956, 2890, 1491, 1091, 1051, 827 cm^{-1}; ^{1}H NMR (200 MHz, $CDCl_3$): δ 1.89 (2H, dt, H-2), 3.23 (2H, brs, OH), 3.81 (2H, t, $J_{3,2} = 5.4$ Hz, H-3), 4.90 (1H, dd, $J_{1-2\alpha} = 7.8$ Hz, $J_{1-2\beta} = 4.6$ Hz, H-1), 7.29 (2H, d, H arom.), 7.31 (2H, d, H arom.); ^{13}C NMR (50 MHz, $CDCl_3$): δ 40.2 (t, C-2), 61.2 (t, C-3), 73.4 (d, C-1), 127.0 (d, 2C arom.), 128.5 (d, 2C arom.), 133.1 (s, 1C arom.), 142.7 (s, 1C arom.); EIMS *m/z*: 188 ($M^+ + 2$, 6), 186 (M^+, 21), 143 (31), 141 (100), 115 (5), 113 (13), 77 (40); HREIMS *m/z* 186.044113 [M^+] (calculated for $C_9H_{11}O_2Cl$, 186.044757).

3.3.2. 10α-[1′-(4″-Chlorophenyl)propoxy]-10-epidihydrobotrydial (5)[1]

White solid, $[\alpha]_D^{20}$ ($CDCl_3$, $c = 0.8$): +96.7; IR ν_{max} (film): 3514, 2962, 2937, 1489, 1362, 1241, 1091, 1004, 824 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.65 (3H, d, $J_{11-2} = 6.1$ Hz, H-11), 0.91 (3H, t, $J_{3'-2'} = 7.3$ Hz, H-3′), 1.00 (1H, m, H-3β), 1.09 (3H, s, H-14), 1.11 (1H, d, $J_{7\alpha-7\beta} = 11.4$ Hz, H-7α), 1.23 (3H, s, H-13^a), 1.26 (3H, s, H-12^a), 1.59 (1H, d, $J_{1-2} = 12.6$ Hz, H-1), 1.67 (2H, m, H-2′), 1.80 (1H, m, H-2), 1.87 (1H, d, $J_{7\alpha-7\beta} = 11.4$ Hz, H-7β), 1.91 (1H, d, $J_{5-4} = 11.0$ Hz, H-5), 2.00 (1H, m, H-3α), 2.01 (3H, s, $\underline{C}H_3COO$), 3.22 (1H, d, $J_{15\alpha-15\beta} =$

[1] Assignments (a–e in superscript) may be interchanged.

10.5 Hz, H-15α), 4.06 (1H, d, $J_{15\beta-15\alpha}$ = 10.5, H-15β), 4.15 (1H, s, OH), 4.62 (1H, t, $J_{1'-2'}$ = 6.3 Hz, H-1′), 4.69 (1H, s, H-10), 5.04 (1H, ddd, $J_{4-3\alpha}$ = 4.8, $J_{4-3\beta}$ = 11.1, J_{4-5} = 11.0, H-4), 7.19 (2H arom., d, J = 8.4 Hz), 7.29 (2H arom., d, J = 8.4 Hz); ^{13}C NMR (50 MHz, $CDCl_3$): δ 10.4 (c, C-3′), 19.9 (c, C-11), 21.4 (c, $\underline{C}H_3COO$), 25.0 (c, C-14), 27.3 (c, C-13), 28.7 (d, C-2), 30.9 (t, C-2′), 35.6 (c, C-12), 38.7 (s, C-6), 39.9 (t, C-3), 45.5 (s, C-8), 50.2 (t, C-7), 54.7 (d, C-1), 59.5 (d, C-5), 67.6 (t, C-15), 72.7 (d, C-4), 78.4 (d, C-1′), 82.4 (s, C-9), 93.9 (d, C-10), 128.4 (d, 2C arom.), 128.6 (d, 4C arom.), 133.5 (s, C arom.), 139.5 (s, C arom.), 170.5 (s, $CH_3\underline{C}OO$); EIMS *m/z*: 406 (M^+ + 2-60, <0.1), 404 (M^+-60, <0.1), 388 (M^+ + 2-60-18, <0.1), 386 (M^+-60-18, 0.23), 296 (M^+ + 2-170, 3), 294 (M^+-170, 28), 234 (86), 152 (100); HREIMS *m/z* 387.208101 [M^+ + 1-OAc-OH] (calculated for $C_{24}H_{32}O_2Cl$, 387.209083).

3.3.3. 10α,5α-bis[1-(4′-Chlorophenyl)propoxy]-10-epidihydrobotrydial (*6*)[1]

White solid, $[\alpha]_D^{20}$ ($CDCl_3$, c = 0.2): +45.4; IR ν_{max} (film): 3468, 2961, 2931, 1464, 1362, 1244, 1087, 823 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$): δ 0.62 (3H, d, J_{11-2} = 6.3 Hz, H-11), 0.68 (3H, t, $J_{3'-2'}$ = 7.4 Hz, H-3′), 0.89 (3H, t, $J_{3'''-2'''}$ = 7.3 Hz, H-3‴), 0.97 (1H, m, H-3β), 1.09 (3H, s, H-14), 1.16 (3H, s, H-13[a]), 1.20 (3H, s, H-12[a]), 1.26 (1H, d, $J_{7\alpha-7\beta}$ = 11.6 Hz, H-7α), 1.51 (1H, dd, J_{1-2} = 12.5 Hz, H-1), 1.54 (2H, m, H-2′), 1.66 (2H, m, H-2‴), 1.78 (1H, m, J_{2-3} = 7.1 Hz, H-2), 1.83 (1H, d, $J_{7\alpha-7\beta}$ = 11.6 Hz, H-7β), 1.85 (1H, d, J_{5-4} = 11.0 Hz, H-5), 2.00 (1H, m, H-3α), 2.00 (3H, s, $\underline{C}H_3COO$), 3.61 (1H, s, OH), 4.59 (1H, t, $J_{1'''-2'''}$ = 6.7 Hz, H-1′), 4.68 (1H, t, $J_{1'''-2'''}$ = 6.5 Hz, H-1‴), 4.86 (1H, s, H-10), 4.93 (1H, s, H-15), 5.09 (1H, ddd, $J_{4-3\alpha}$ = 4.6, $J_{4-3\beta}$ = 11.1, J_{4-5} = 11.0, H-4), 7.18–7.33 (m, 8H arom.); ^{13}C NMR (50 MHz, $CDCl_3$): δ 10.1 (c, C^b-3‴), 10.2 (c, C^b-3′), 19.1 (c, C-14), 19.9 (c, C-11), 21.5 (c, $\underline{C}H_3COO$), 27.3 (c, C^c-13), 28.8 (d, C-2), 30.6 (t, C^d-2‴), 31.7 (t, C^d-2′), 35.6 (c, C^c-12), 38.8 (s, C-6), 39.8 (t, C-3), 49.8 (t, C-7′), 54.5 (d, C-1), 60.1 (d, C-5), 72.6 (d, C-4), 78.7 (d, C^e-1‴), 79.3 (d, C^e-1′), 96.2 (d, C-10), 97.2 (d, C-15), 128.4 (d, 4C arom.), 128.6 (d, 4C arom.), 133.5 (s, 2C arom.), 139.5 (s, 2C arom.), 170.5 (s, CH_3-$\underline{C}OO$); EIMS *m/z*: 462 (M^+-170, 3), 444 (M^+-170-18, 4), 384 (13), 294 (12), 234 (20), 216 (14), 204 (55), 152 (100); HREIMS *m/z* 463.226624 [M^+ + 1-$C_9H_{11}OCl$] (calculated for $C_{26}H_{36}O_5Cl$, 463.225127).

3.4. Treatment of (R)-(+)-1-(4′-chlorophenyl)-propan-1-ol (*1*) with botrydial (*7*) and dihydrobotrydial (*8*)

A mixture of dihydrobotrydial (**8**) (7 mg, 0.022 mmol) and (*R*)-(+)-1-(4′-chlorophenyl)propan-1-ol (**1**) (15 mg, 0.088 mmol) in 10 ml of Czapeck–Dox medium was stirred at room temperature for 72 h without *B. cinerea*. After extraction with ethyl acetate, purification on silica gel afforded the compound 10α-[1′-(4″-chlorophenyl)propoxy]-10-epidihydrobotrydial (**5**) (2 mg, 20%).

The same reaction was carried out with botrydial (**7**) (14 mg, 0.045 mmol) and (*R*)-(+)-1-(4′-chlorophenyl)-propan-1-ol (**1**) (32 mg, 0.188 mmol), affording compound 10α,15α-bis[1-(4′-chlorophenyl)propoxy]-10-epidihydrobotrydial (**6**) (3 mg, 11%) after 72 h.

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