Detoxification of Terpinolene by Plant Pathogenic Fungus *Botrytis cinerea*

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Detoxification of an antifungal monoterpene terpinolene (**1**) by the plant pathogenic fungus *Botrytis cinerea* afforded hydroxlyated metabolites 2,3-dihydro-3β,6β-dihydroxy-terpinolene (**2**) (39%) and 2,3-dihydro-1α,3α-dihydroxy-terpinolene (**3**) (20%), respectively. Terpinolene showed good levels of antifungal activity while both the metabolites were inactive against another plant pathogenic fungus *Cladosporium herbarum.*

Introduction

Monoterpenoid hydrocarbons are volatile constituents of the essential oils produced by many aromatic and medicinal plants, in various types of highly specialised secretory structures such as glandular trichomes and resin ducts (Fahn, 1979; Tavera, 1999). The ecological significance of these secondary metabolites is well documented and it is known that they play a defensive role against herbivores, phytophagous insects and microbial pathogens (Croteau, 1992; Himejima *et al.*, 1992).

The phytopathogenic fungus *Botrytis cinerea* has been reported to metabolise a variety of natural products. We have previously reported the fungal detoxification of many phytoalexins of *Lupinus* species by *B. cinerea* as summarised in a recent review paper (Farooq and Tahara, 1999; Atta-ur-Rahman *et al.*, 1999). Furthermore, the biotransformation of the monoterpene α-pinene by *B. cinerea* was also studied (Farooq *et al.*, 2002).

The four major stages of complex eco-chemical interactions of phytopathogenic fungi and plants are: 1) Production of antifungal secondary metabolites by plants to ward-off the pathogenic fungi. 2) Detoxification of the antifungal plant metabolites by phytopathogenic fungi in order to invade the plants. 3) Production of phytotoxic metabolites by plant pathogenic fungi. 4) Detoxification of phytotoxic metabolites by plants (Farooq and Tahara, 1999).

Terpinolene is a monoterpenic constituent of some essential oils of various fir and pine species, as well as plants such as *Manilla elemi*, *Nectranda elaiophora*, and *Dacrydium colensoi* (Burdock, 1995). It displays antifungal activity against various pathogens (Himejima *et al.*, 1992).

Hence, it seemed interesting to study the detoxification of antifungal monoterpene terpinolene by plant pathogenic fungus *B. cinerea* as an exemplary eco-chemical interaction of the aromatic plants and phytopathogenic fungi.

This present work resulted in isolation and characterisation of dihydroxylated monoterpenes with no antifungal property, which suggested that the plant pathogenic fungus, *B. cinerea* can survive the deleterious effects of antifungal eco-chemicals of aromatic plants through enzymatic detoxfication and can therefore cause infections to aromatic plants unless they adopt some other mechanism of ecological survival.

Methods and Materials

General

The metabolites were purified by column chromatography (LiChroprepDIOL column, 40–63 μm mesh, Art 13973), while Merck Kieselgel 60 F_{254} 0.2 mm thick TLC plates were used to check the purity and the spots were viewed under 254 and 365 nm UV and spraying with EtOH-H_2SO_4 (1:1,

v/v) or anisaldehyde-H_2SO_4 spray reagent. Optical rotations were taken on a Jasco DIP 370 polarimeter. The IR spectra and the mass spectra were recorded in $CHCl_3$ using a Perkin-Elmer 2000 FTIR and a Jeol JMS-SX 102 mass spectrometer, respectively. The ^{1}H- and 2D-NMR spectra were recorded on a Bruker AMX500 while the ^{13}C-NMR spectra were recorded on a Jeol EX-270 spectrometer at 67.5 MHz.

Detoxification of Terpinolene (**1**)

Liquid medium for *B. cinerea* (AHU 9424) was prepared by mixing glucose (40 g), yeast extract (1 g), KH_2PO_4 (5 g), $MgSO_4$ (0.5 g) $NaNO_3$ (2 g), $FeSO_4$ (10 mg) and $ZnSO_4$ (5 mg) were mixed in distilled water (1 l). The medium was evenly distributed among 5 culture flasks of 500 ml capacity (200 ml in each) and autoclaved for 15 min at 121 °C. Each flask was inoculated with a mycelial suspension of *B. cinerea* (1 ml) and incubated on a reciprocal shaker for three days at 120 rpm at room temperature. A clear solution in ethanol (5 ml) of the substrate terpinolene (200 mg) was also distributed among the 5 culture flasks (40 mg/200 ml) and fermented for further 10 days. The mycelium was filtered, washed with water and EtOAc, and the broth obtained was successively extracted with EtOAc. The organic layer was washed with brine and dried over anhydrous sodium sulphate and concentrated *in vacuo* to afford a brown gum (780 mg) which was adsorbed on an equal quantity of silicagel and chromatographed where the elution with EtOAc:*n*-hexane (1:1, v/v) gave a colourless oily metabolite identified as 2,3-dihydro-3β,6β-dihydroxy-terpinolene (**2**) (98 mg, 39%). Further elution using the same solvent system yielded the metabolite as a colourless oil identified as 2,3-dihydro-1α,3α-dihydroxy-terpinolene (**3**) (51 mg, 20%).

2,3-Dihydro-3β,6β-dihydroxy-terpinolene (**2**): $[\alpha]_D^{27}$: −12° ($CHCl_3$, *c* 0.1), IRν_{max} (in $CHCl_3$, cm^{-1}): 3412 (OH), 1520 (C = C); ^{1}H-NMR ($CDCl_3$, 500 MHz, δ) 3.79 (1H, dd, *J* = 5 and 6 Hz, H-6α), 3.52 (1H, dt, *J* = 7 and 11 Hz, H-3α), 2.21 (2H, m, H-1α,1β), 2.19 (2H, m, H-4α,4β), 1.95 (1H, m, H-2β), 1.16 (3H, s, H-9), 1.17 (1H, s, H-9), 0.99 (3H, d, *J* = 7 Hz, H-10); ^{13}C-NMR ($CDCl_3$, 67.5 MHz, δ) 33.3 (C-1), 34.4 (C-2), 72.5 (C-3), 39.2 (C-4), 116.3 (C-5), 70.9 (C-6), 141.1 (C-7), 19.8 (C-8), 19.8 (C-9), 16.9 (C-10); FDMS, *m/z* 170 [M$^+$]; HREIMS, *m/z* 170.1341 ($C_{10}H_{18}O_2$ requires 170.1307); EIMS, *m/z* 170 [M$^+$] (20), 152 [M$^+$−18] (34), 137 (28), 123 (15), 109 (54), 97 (36), 83 (13), 74 (100), 55 (24), 43 (71).

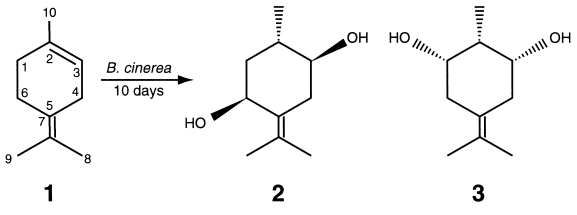
2,3-Dihydro-1α,3α-dihydroxy-terpinolene (**3**): $[\alpha]_D^{27}$: +3° ($CHCl_3$, *c* 0.1), IRν_{max} (in $CHCl_3$, cm^{-1}): 3609 (OH), 3408 (OH), 1517 (C = C); ^{1}H-NMR ($CDCl_3$, 500 MHz, δ) 4.35 (1H, dd, *J* = 7 and 11 Hz, H-1β), 3.83 (1H, dt, *J* = 7 and 10 Hz, H-3β), 2.51 (2H, m, H-6α,6β), 2.21 (2H, m, H-4α,4β), 1.92 (1H, m, H-2β), 1.48 (3H, s, H-8), 1.47 (3H, s, H-9), 1.15 (3H, d, *J* = 7 Hz, H-10); ^{13}C-NMR ($CDCl_3$, 67.5 MHz, δ) 66.5 (C-1), 35.8 (C-2), 71.0 (C-3), 44.6 (C-4), 115.5 (C-5), 40.3 (C-6), 136.2 (C-7), 20.3 (C-8), 20.3 (C-9), 16.8 (C-10); FDMS, *m/z* 170 [M$^+$]; HREIMS, *m/z* 152.1176 ($C_{10}H_{18}O_2-H_2O$, requires 152.1202); EIMS, *m/z* 152 [M$^+$−18] (48), 137 (28), 121 (28), 107 (19), 93 (39), 79 (84), 67 (24) 59 (83), 43 (100).

TLC-Bioautography Assay

The assay described by Homans & Fuchs (1970) was used to check the antifungal property of terpinolene (**1**) and its metabolites using *C. herbarum* (wild). The solutions of terpinolene and its metabolites **2** and **3** were prepared in EtOAc at a concentration of 1 mg/ml and 10 μl (10 μg/ml) of the solutions of the test compounds were charged on TLC plates, the spots were developed in EtOAc: *n*-hexane (1:1, v/v) and were marked under UV light. An autoclaved solution of KH_2PO_4 (350 mg), $Na_2HPO_4 \cdot H_2O$ (150 mg), KNO_3 (200 mg), $MgSO_4 \cdot 7\,H_2O$ (50 mg), and NaCl (50 mg) in distilled water (50 ml) was poured onto pre-established conidia of *C. herbarum* 10 ml of 30% (w/v) glucose solution in water was added in the conidial suspension and then sprayed over the TLC plate aseptically. The TLC plate was incubated at 25 °C under humid conditions (approx. 80%) for 72 h.

Results and Discussion

TLC analysis of the broth obtained after incubation of **1** with *B. cinerea* for ten days revealed the presence of two polar metabolites which were isolated as oils by column chromatography in a



1 - Terpinolene
2 - 2,3-Dihydro-3β,6β-dihydroxy-terpinolene (98 mg, 39%)
3 - 2,3-Dihydro-1α,3α-dihydroxy-terpinolene (51 mg, 20%)

Fig. 1. Detoxification of terpinolene (**1**) by *B. cinerea.*

fair yield (Fig. 1). The optical rotations of both metabolites indicated the presence of chiral centres. The IR (ν_{max}) of **2** revealed the presence of a hydroxyl group at 3412 cm^{-1} and a tetra-substituted double bond at 1520 cm^{-1}. The FDMS and EIMS revealed the molecular ion peak at *m/z* 170 and introduction of two oxygen atoms coupled with reduction of one of the two double bonds of terpinolene was anticipated. The HREIMS of the metabolite showed exact molecular weight at *m/z* 170.1341 corresponding to the molecular formula $C_{10}H_{18}O_2$. The ^{13}C-NMR spectrum of **2** exhibited resonances for 10 carbons while the DEPT spectra revealed the presence of 3 methyl, 2 methylene, 3 methine and 2 quaternary carbons. Two downfield hydroxy-bearing methine signals resonating at δ 72.5 and 70.9 proved the introduction of two oxygen functions. The ^{1}H-NMR spectrum of 2 showed two signals at δ 3.52 (dt, $J_{3\alpha,2\beta}$ = 11 Hz, $J_{3\alpha,4\alpha,4\beta}$ = 7 Hz, H-3α) and δ 3.79 (dd, $J_{6\alpha,1\alpha}$ = 6 Hz, $J_{6\alpha,1\beta}$ = 5 Hz, H-6α). The C-3 and C-6 positions were established due to the COSY interactions of H-2 (δ 1.95)/H-3 (δ 3.52) and H-1 (δ 2.32)/H-6 (δ 3.79) while β-stereochemistry of the newly introduced 3-OH and 6-OH were based on the coupling constants and the NOESY correlations of H-3α and H-6α with CH_3-10α.

The IR of **3** showed the presence of two hydroxyl groups at 3609 and 3408 cm^{-1}, and a tetra-substituted double bond at 1517 cm^{-1}. The FDMS displayed a molecular ion peak at *m/z* 170 while EIMS showed a peak at *m/z* 152 corresponding to M^+-H_2O and the HREIMS had the exact molecular weight at *m/z* 152.1176 corresponding to the molecular formula $C_{10}H_{18}O_2-H_2O$. The ^{1}H-NMR spectrum showed two low-field methine signals at δ 4.35 (dd, $J_{1\beta,2\beta,6\beta}$ = 11 Hz, $J_{1\beta,6\alpha}$ = 7 Hz, H-1β) and at δ 3.83 (dt, $J_{3\beta,4\alpha,4\beta}$ = 7 Hz, $J_{2\beta,3\beta}$ = 11 Hz). The signals for 10 carbons were observed in the ^{13}C-NMR spectrum of **3** and the DEPT spectra proved the presence of 3 methyl, 2 methylene, 3 methine and 2 quaternary carbons. Two CH-OH signals featured at δ 66.5 and 71.0 were ascribed to CH-1 and CH-3, respectively. The C-1 and C-3 positions of the hydroxyl groups were established due to COSY interactions of H-2 (δ 1.92)/H-1 (δ 4.35) and H-3 (δ 3.83) while α-stereochemistry of both hydroxyl groups was established due to coupling patterns and the absence of any NOESY correlations of 10α-CH_3 (δ 1.15) with 1β-H (δ 4.35) and 3β-H (δ 3.83).

The TLC bioautography of terpinolene showed inhibitory zone of 19 mm at a concentration of 10 μg/ml while metabolites **2** and **3** did not show inhibition of germination of conidia of *C. herbarum* at the same concentration. It is a well documented fact that most of the xenobiotics are detoxified by hydroxylation through cytochrome P-450 oxidases by eukaryotic cells. As previous work, the metabolism of terpinolene in rabbits and various microorganisms has already been reported (Asakawa *et al.*, 1991; Abraham *et al.*, 1986). The present study therefore concludes that plant pathogenic fungus *B. cinerea* like *Aspergillus*, *Corynospora* and rabbits can detoxify terpinolene, possibly by cytochrome P-450 oxidases.

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