Detoxification of Terpinolene by Plant Pathogenic Fungus *Botrytis cinerea*

Afgan Farooqa,b, M. Iqbal Choudharya Atta-ur-Rahmana, Satoshi Taharab, K. Hüsnü Can Başerc and Fatih Demircic*

a International Centre for Chemical Sciences, H. E. J. Research Institute of Chemistry, University of Karachi, 75270-Karachi, Pakistan
b Division of Applied Biosciences, Graduate School of Agriculture, Hokkaido University, 060-8589 Sapporo, Japan
c Medicinal and Aromatic Plant and Drug Research Centre (TBAM), Anadolu University, 26470-Eskişehir, Turkey. Fax: +90 222 35 01 27. E-mail: fdemirci@anadolu.edu.tr
* Author for correspondence and reprint requests

Z. Naturforsch. 57c, 863–866 (2002); received April 30/May 22, 2002

Terpinolene, Detoxification, Plant Pathogenic Fungi

Detoxification of an antifungal monoterpene terpinolene (1) by the plant pathogenic fungus *Botrytis cinerea,* afforded hydroxylated 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,* *Nectandra 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 detoxification 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 (*LiChroprep*DIOL column, 40–63 µm mesh, Art 13973), while Merck Kieselgel 60 F254 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-H₂SO₄ (1:1,
H-6

(CDCl₃, 500 MHz, recorded in CHCl₃ using a Perkin-Elmer 2000 FTIR and a Jeol JMS-SX 102 mass spectrometer, respectively. The ¹H- and ²D-NMR spectra were recorded on a Bruker AMX500 while the ¹³C-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), KH₂PO₄ (5 g), MgSO₄ (0.5 g) NaNO₃ (2 g), FeSO₄ (10 mg) and ZnSO₄ (5 mg) were mixed in distilled water (1 l). The medium was evenly distributed among 5 culture flasks of 500 ml capacity (200 ml 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):
[α]₂₀^D = −12° (CHCl₃, c 0.1), IR ν₂₅₀ (in CHCl₃, cm⁻¹): 3412 (OH), 1520 (C = C); ¹H-NMR (CDCl₃, 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); ¹³C-NMR (CDCl₃, 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 (C₁₀H₁₈O₂ 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):
[α]₃₀^D = +3° (CHCl₃, c 0.1), IR ν₂₅₀ (in CHCl₃, cm⁻¹): 3609 (OH), 3408 (OH), 1517 (C = C); ¹H-NMR (CDCl₃, 500 MHz, δ): 4.55 (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); ¹³C-NMR (CDCl₃, 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 (C₁₀H₁₈O₂ – H₂O, 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 KH₂PO₄ (350 mg), Na₂HPO₄·H₂O (150 mg), KNO₃ (200 mg), MgSO₄·7 H₂O (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...
fair yield (Fig. 1). The optical rotations of both metabolites indicated the presence of chiral centres. The IR ($\nu_{\text{max}}$) 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 13C-NMR spectrum of 2 exhibited resonances for 10 carbons while the DEPT spectra proved the presence of 3 methyl, 2 methylene, 3 methine and 2 quaternary carbons. Two CH-OH signals featured at $\delta$ 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 ($\delta$ 1.92)/H-1 ($\delta$ 4.35) and H-3 ($\delta$ 3.83) while $\alpha$-stereochemistry of both hydroxyl groups was established due to coupling patterns and the absence of any NOESY correlations of 10$\alpha$-CH$_3$ ($\delta$ 1.15) with 1$\beta$-H ($\delta$ 4.35) and 3$\beta$-H ($\delta$ 3.83).

The TLC bioautography of terpinolene showed inhibitory zone of 19 mm at a concentration of 10 $\mu$g/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.

Acknowledgement

The authors would like to thank Dr. Alexander Pauli for his AMICBASE-Database search of the antimicrobial monoterpene terpinolene.


