The Microbial Oxidation of (−)-β-Pinene by Botrytis cinerea§

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

a International Centre for Chemical Sciences, H. E. J. Research Institute of Chemistry, University of Karachi, Karachi-75270, Pakistan
b Division of Applied Biosciences, Graduate School of Agriculture, Hokkaido University, Sapporo 060-8589, Japan
c Medicinal and Aromatic Plant and Drug Research Centre (TBAM), Anadolu University, 26470-Eskişehir, Turkey. Fax +90 (222) 3350127. E-mail: fdemirci@anadolu.edu.tr
* Author for correspondence and reprint requests

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(−)-β-pinene, a flavor and fragrance monoterpenoid is an important constituent of essential oils of many aromatic plants. It was oxidized by a plant-pathogenic fungus, Botrytis cinerea to afford four metabolites characterized as (−)-6α-hydroxy-β-pinene, (−)-4β,5β-dihydroxy-β-pinene, (−)-2β,3β-dihydroxypinane, and (−)-4β-hydroxy-β-pinene-6-one by detailed spectroscopic studies along with other known metabolites.

Introduction

The microbiological oxidations of flavor and fragrance monoterpeneoids have less frequently been studied due to the difficulty in handling because of their volatility and toxicity to the fungi (Abraham et al., 1985). Monoterpene oxidations using pinane derivatives and menthol by Cephalosporium aphidicola have been achieved in our previous work (Farooq and Hanson, 1995; Atta-ur-Rahman et al., 1998; Farooq et al., 2002). Microbial transformations of (−)-α-pinene and (−)-β-pinene (1), two important flavor and fragrance constituents of many essential oils have previously been reported and reviewed by various researchers and groups (Draczynska et al., 1985; Yoo et al., 2001; Noma and Asakawa, 2000; Demyttenaere, 2000; van Dyk et al., 1998; Savithiry, 1998; Ishida, 1981; Shukla, 1965).

Botrytis cinerea is a pathogenic fungus which causes diseases to commercial crops (Agrios, 1998). The production of botryidal and related terpenoidal phytotoxins enhances the pathogenicity of the fungus (Collado et al., 1995, 1996; Reberdinos et al., 1996). However, the microbiological oxidation of some clovanes, caryophyllene oxide and patchulol sesquiterpenes have previously been achieved by fermentation with B. cinerea (Collado et al., 1998; Duran et al., 1999; Aleu et al., 1999). The fungus B. cinerea has recently been subjected to a review by Aleu and Collado (2001), due to its versatile application in microbial transformations.

We have been interested in the microbial oxidation of different classes of biologically active natural compounds and have reported metabolism of many prenylated flavonoids and related phytalexins, some steroids and sesqui- and diterpenes using this competent fungus (Farooq and Tahara, 1999; 2000a, b).

Here we report the microbiological oxidation of (−)-β-pinene (1), an important natural product for flavor and fragrance industry by B. cinerea to yield oxidized compounds which could be used as flavor and fragrance compounds in food industry or as chiral auxiliaries in asymmetric syntheses.

Materials and Methods

General experimental procedures

Merck Kieselgel 60 F254 0.2 mm thick TLC plates were used to check the purity of the metabolites while the spots were viewed under 254 and 365 nm UV and spraying with EtOH–H2SO4 (1:1) or anisaldehyde–H2SO4 spray reagent. LiChro-prepDIOL (40–63 µm mesh, Art. 13973) was used
for column chromatography. A Jasco DIP 370 polarimeter was used for the measurement of the optical rotations. The IR spectra and the mass spectra were recorded in CHCl₃ using a Perkin-Elmer 2000 FTIR while the mass spectra were recorded on a Jeol JMS-SX 102 mass spectrometer. 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 67.8 MHz.

**Culture medium**

The liquid medium for the Botrytis 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) into 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.

**Incubation of (-)-β-pinene (1) with B. cinerea**

Each flask was inoculated with a mycelia suspension of B. cinerea (1 ml) and incubated on a reciprocal shaker for three days at 120 rpm at room temperature (20–25 °C). (-)-β-pinene (1) (200 mg) was diluted using ethanol (5 ml) and was evenly distributed in the 5 culture flasks (40 mg/200 ml). Substrate controls were composed of sterile medium to which the substrate was added and incubated without microorganisms. Culture controls consisted of fermentation blanks in which the microorganisms were grown under identical conditions but without the addition of substrate. All were fermented for a further 10 days. The mycelia were filtered, washed with water and EtOAc, and the broth thus obtained was successively extracted with EtOAc. The organic layer was washed with brine and dried over anhydrous sodium sulfate and concentrated in vacuo to afford a brown gum (802 mg), which was adsorbed on an equal quantity of silica gel and chromatographed.

**Chromatography**

The elution of the adsorbed constituents with EtOAc:n-hexane (3:7, v/v) afforded the metabolites: (-)-6α-hydroxy-β-pinene (2) (49 mg) as a colourless oil. Elution with EtOAc:n-hexane (2.3, v/v) yielded the metabolite (-)-4β,5β-dihydroxy-β-pinene (3) (25 mg). Further elution with EtOAc:n-hexane (2:3, v/v) gave the metabolite (-)-2β,3β-dihydroxypinane (4) (29 mg) as amorphous material. The metabolite eluted with the same system was characterised as (-)-4β-hydroxy-β-pinene-6-one (5) (22 mg).

(-)-6α-hydroxy-β-pinene (2). A colourless oil. [α]°D: -70.0° (CHCl₃, c 0.1); IR (CHCl₃) νmax (cm⁻¹): 3342, 1674, 1555; ¹H-NMR (CDCl₃, 500 MHz, δ) 5.11 (2H, brs, H-10), 3.98 (1H, t, J = 1.2 Hz, H-6β), 2.35 (2H, m, H-4), 2.26 (2H, m, H-3), 2.08 (1H, br s, H-1), 2.00 (1H, m, H-5), 1.29 (3H, s, H-8), 0.83 (3H, s, H-9); ¹³C-NMR (CDCl₃, 67.5 MHz, δ) 39.9 (C-1), 147.6 (C-2), 41.0 (C-3), 43.4 (C-4), 38.0 (C-5), 65.9 (C-6), 50.5 (C-7), 25.8 (C-8), 26.9 (C-9), 124.3 (C-10); FDMS, m/z 152 [M⁺] (100%); EIMS, m/z 152 (12), 137 (7), 121 (24), 109 (30), 93 (48), 79 (100), 67 (21), 55 (22), 41 (29); HREIMS, m/z 152.1175 (calculated for C₁₀H₁₂O₂, 152.1202).

(-)-4β,5β-dihydroxy-β-pinene (3). Colourless oil. [α]°D: +21.0° (CHCl₃, c 0.1); IR (CHCl₃) νmax (cm⁻¹): 3678, 1665, 1556; ¹H-NMR (CDCl₃, 500 MHz, δ) 5.04 (1H, brs, H-10), 3.42 (1H, t, J = 3.2 Hz, H-6α), 1.98 (2H, d, J = 3.2 Hz, H-3), 1.81 (1H, dd, J = 8.9, 11.9 Hz, H-1β), 1.61 (3H, s, H-9), 1.53 (3H, s, H-8), 1.21 (2H, m, H-6); ¹³C-NMR (CDCl₃, 67.5 MHz, δ) 41.0 (C-1), 140.5 (C-2), 31.2 (C-3), 69.3 (C-4), 68.9 (C-5), 28.6 (C-6), 47.8 (C-7), 17.8 (C-8), 25.7 (C-9), 122.0 (C-10); FDMS, m/z 168 [M⁺] (100%); EIMS, m/z 168 (15), 153 (20), 139 (100), 125 (64), 109 (56), 91 (33), 69 (84), 55 (39), 43 (85), 41 (59); HREIMS, m/z 168.1198 (calculated for C₁₀H₁₆O₂, 168.1151).

(-)-2β,3β-dihydroxypinane (4). A colourless amorphous material. [α]°D: -45.0° (CHCl₃, c 0.1); IR (CHCl₃) νmax (cm⁻¹): 3408; ¹H-NMR (CDCl₃, 500 MHz, δ) 3.73 (1H, dd, J = 7.3, 10.8 Hz, H-3α), 2.05 (1H, m, H-6α), 2.0 (1H, m, H-1), 1.90 (1H, m, H-5), 1.60 (3H, s, H-9), 1.53 (3H, s, H-8), 1.43 (1H, d, J = 7.3 Hz, H-4α), 1.02 (1H, m, H-4β), 0.94 (1H, m, H-6β), 1.86 (3H, s, H-10); ¹³C-NMR (CDCl₃, 67.5 MHz, δ) 38.6 (C-1), 73.4 (C-2), 68.0 (C-3), 36.5 (C-4), 38.3 (C-5), 30.5 (C-6), 48.0 (C-7), 17.4 (C-8), 25.8 (C-9), 20.0 (C-10); FDMS, m/z 170 [M⁺] (100%); EIMS, m/z 170 [M⁺] (2) [155 (4), 139 (38), 127 (11), 109 (39), 95 (47), 81 (100), 67 (29), 55 (29), 41 (43); HREIMS, m/z 170.1344 (calculated for C₁₀H₁₆O₂, 170.1307).
A colourless amorphous material: $[\alpha]_{D}^{22} = -31.0^\circ$ (CHCl$_3$, c 0.1); IR (CHCl$_3$) $\nu_{max}$ (cm$^{-1}$): 3411, 1716, 1669, 1558; $^1$H-NMR (CDCl$_3$, 500 MHz, $\delta$): 5.05 (2H, brs, H-10), 3.47 (1H, d, $J = 4.2$, H-1), 1.86 (1H, s, H-3), 1.78 (1H, d, $J = 8.2$ Hz, H-5), 1.61 (3H, s, H-9), 1.53 (3H, s, H-8); $^{13}$C-NMR (CDCl$_3$, 125 MHz, $\delta$): 138.1 (C-2), 41.0 (C-3), 72.5 (C-4), 38.0 (C-5), 45.3 (C-7), 19.3 (C-8), 25.7 (C-9), 124.4 (C-10); FDMS, m/z 166 [M$^+$] (100%); EIMS, m/z 166 [M$^+$] (70), 121 (33), 109 (50), 93 (64), 79 (54), 69 (63), 59 (70). 

The FDMS and EIMS of metabolite 3 displayed molecular ion peak at m/z 168 while exact molecular mass was determined to be 168.1198, which corresponds to molecular formula C$_{10}$H$_{16}$O$_2$ as determined by HREIMS. Introduction of two hydroxyl functions was hence anticipated. The IR spectrum of 3 showed hydroxyl absorption at 3678 cm$^{-1}$. Resonance for 10 carbons were observed in the $^{13}$C NMR spectrum of 3, and the DEPT spectra showed the presence of 2 methyl, 3 methylene, 2 methine, and 3 quaternary carbons. 

Results and Discussion

Four oxidised metabolites were detected by TLC analysis of the fermentation broth of (−)-β-pinene (1) and plant pathogenic fungus, B. cinerea. The metabolites were isolated as described in the experimental section and characterised through detailed spectroscopic MS, IR, 1D- and 2D-NMR analyses as (−)-6α-hydroxy-β-pinene (2), (−)-4β,5β-dihydroxy-β-pinene (3), (−)-2β,3β-dihydroxy-β-pinene (4) and (−)-4β-hydroxy-β-pinene-6-one (5), see Scheme 1.

The EIMS of metabolite 2 showed a molecular ion peak at m/z 152, which was confirmed by recording the FDMS. The HREIMS of the metabolite showed exact molecular weight at m/z 152.1175 corresponding to the molecular formula C$_{10}$H$_{16}$O$_2$. A strong absorption at 3342 cm$^{-1}$ in the IR spectrum of 2 indicated the introduction of a hydroxyl group in the substrate by B. cinerea. The $^{13}$C NMR spectrum of 2 exhibited resonances for 10 carbons whereas the DEPT spectra revealed the presence of 2 methyl, 3 methylene, 3 methine and 2 quaternary carbons. A hydroxy-bearing methine signal featured at $\delta$ 65.9 proved the hydroxylation of a methylene carbon. The $^1$H NMR spectrum of 2 displayed a signal at $\delta$ 3.98 (t, $J_{\beta\beta, SP.1\beta} = 1.2$ Hz, H-6β). The position of the newly introduced hydroxyl was established (C-6) due to HMBC correlations of H-6β ($\delta$ 3.98) with C-2 ($\delta$ 147.5), C-10 ($\delta$ 124.3), C-1 ($\delta$ 39.9) and C-5 ($\delta$ 38.0). The COSY interaction of H-6β ($\delta$ 3.98) with H-5β ($\delta$ 2.00) and H-1β ($\delta$ 2.08) further proved the C-6 position of the new hydroxyl group. The stereochemistry of H-6β was established due to the coupling pattern (δ 3.98, t, $J_{\beta\beta, SP.1\beta} = 1.2$ Hz). Which is in accordance with the Karplus Equation due to the dihedral angle of 100°. The NOESY spectrum was recorded to confirm the stereochemistry of H-6β which showed correlations of H-6β with H-5β and H-1β. The complete $^1$H and $^{13}$C NMR chemical shifts of 2 were unambiguously assigned by combination of Broad Band, DEPT, HMQC, HMBC, COSY and NOESY spectra.

The microbial transformation products and percentages

Scheme 1. Microbial transformation of (−)-β-pinene (1) by B. cinerea.
δ 69.3 proved the hydroxylation of a methylene carbon while a low field quaternary signal featured at δ 68.9. It was therefore concluded that one hydroxyl group has been introduced into a methylenic carbon while other into a methine carbon. The 1H NMR spectrum of 3 had a signal at δ 3.42 (dd, J_{4α,3α} = 6.7 Hz, J_{4α,3β} = 10.9 Hz, H-4α). The C-5 position of the newly introduced hydroxyl was established due to the HMBC correlations of H_{2-6} (δ 1.21) with C-5 (δ 68.9). The C-4 position of the second hydroxyl was established based on the HMBC correlation of C-5 (δ 68.9) with H-4 (δ 3.42), and COSY correlations between H-3 (δ 1.98), as well as H-4 (δ 3.42). The stereochemistry of the 4β-OH was deduced due to the coupling pattern of H-4α (τ, J_{4α,3α,β} = 3.2 Hz), and NOESY correlations between H-4α (δ 3.42) as well as CH_{3-8} (δ 1.53).

The EIMS of metabolite 4 displayed a molecular ion peak at m/z 170 as confirmed by the FDMS. The HREIMS of the metabolite showed exact molecular weight at m/z 170.1344 which corresponds to the molecular formula C_{10}H_{14}O_{2}. The IR spectrum of 4 showed a hydroxyl absorption at 3408 cm⁻¹. The 13C NMR spectrum displayed signals for 10 carbons whereas the DEPT spectra revealed the presence of 3 methyl, 2 methylene, 3 methine, and 3 quaternary carbons. Two characteristic signals featured at δ 72.5 and 209.6 due to a hydroxyl-bearing methine (C-4) and a ketonic carbon (C-6). As shown in the 1H NMR spectrum a signal resonated due to H-4α at δ 3.47 (dd, J_{4α,3α} = 4.2 Hz, J_{4α,3β} = 5.9 Hz, J_{4β,3β} = 8.2 Hz, H-4α). The position of the newly introduced hydroxyl group was established using the HMBC correlation data of H-4α (δ 3.47) with C-3 (δ 41.0) and C-5 (δ 38.0) and COSY correlations of H-4α (δ 3.47) with H-3 (δ 2.14) and H-5 (δ 1.78). The NOESY spectrum showed correlations between H-4α (δ 3.47) and H-5 (δ 1.78) which confirmed the α-stereochemistry of H-4 as suggested by multiplicity pattern as well. Hence β-orientation of newly introduced OH at C-4 was inferred. The HMBC correlations between C-6 (δ 209.6) with H-1 (δ 1.86) and H-5 (δ 1.78) helped in assigning the C-6 position of the newly introduced ketone group.

Further biotransformation work on monoterpenes and pinenes are still under progress.


