Biotransformation of (–)-α-Pinene by *Botrytis cinerea*

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(–)-α-Pinene, *Botrytis cinerea*, Microbial Transformation

(–)-α-Pinene (**1**), a major constituent of many aromatic plants was biotransformed by the plant pathogenic fungus, *Botrytis cinerea* to afford three new metabolites, characterized as 3β-hydroxy-(–)-β-pinene (10%) (**3**), 9-hydroxy-(–)-α-pinene (12%) (**4**), 4β-hydroxy-(–)-α-pinene-6-one (16%) (**5**) by physical and spectroscopic methods. A known metabolite verbenone (**2**) was also obtained.

Introduction

Several hundred individual compounds with various skeletons of the C10 representatives of the terpenoid family of natural products, called monoterpenes have been reported (Devs and Yadeer, 1982). Monoterpenes are mostly produced by aromatic plants as constituents of essential oils and accumulated in various types of highly specialized secretory organs, *e.g.* glandular trichosomes, resin ducts *etc.* (Fahn, 1979). Research on various chemical and biological aspects of monoterpenes was stimulated mainly due to their commercial importance in flavor and fragrance industry (Croteau, 1988).

Botrytis cinerea is a gray mould, harmful to many commercial plants and crops (Agrios, 1998). The pathogenicity of the fungus is attributed to the production of botrydial and related terpenoids (Collado *et al.*, 1995, 1996; Rebordinos *et al.*, 1996). Oxidation of some clovanes, caryophyllene oxide and patchoulol sesquiterpenes by the fungus have been reported in the literature (Collado *et al.*, 1998; Duran *et al.,* 1999; Aleu *et al.*, 1999). We have previously reported on the metabolism of many prenylated flavonoids and related phytoalexins, some steroids, sesqui- and diterpenes by this fungus (Farooq and Tahara, 1999; Farooq and Tahara, 2000a, b, c). A comprehensive review was recently published on the biotransformation reactions of *B. cinerea* (Aleu and Collado, 2001).

Fungal transformations of flavor and fragrance monoterpenoids have been of paramount interest for the last two decades despite the fact of difficulty in handling such compounds due their volatility and toxicity to the fungi (Abraham *et al.*, 1985; van der Werf *et al.*, 1998). Oxidation of pinane-derivatives and menthol by *Cephalosporium aphidicola* has been achieved (Farooq and Hanson, 1995; Atta-ur-Rahman *et al.*, 1998). Microbial transformations by *Armillariella mellea* (honey fungus) of (–)-α-pinene and (–)-β-pinene, important flavor and fragrance constituents of many essential oils have previously been reported (Draczynska *et al.*, 1985). Other previous work on the biotransformation of pinenes can be found (Noma and Asakawa, 2000; Demmyttenaere, 2000; Yoo *et al.*, 2001; and references cited herein).

Since we have been working on the microbial hydroxylations of a variety of industrially important products, (–)-α-pinene (**1**) was fermented by *B. cinerea* to yield the oxidized metabolites (**2–5**) (See Fig. 1).

Experimental

General

The purity of the metabolites was checked on Merck Kieselgel 60 F_{254} 0.2 mm thick TLC plates and the spots were viewed under 254 and 365 nm UV and spraying with EtOH-H_2SO_4 (1:1, v/v) or

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anisaldehyde-H_2SO_4 spray reagent. The LiChroprepDIOL column (40–63 μm mesh, Art 13973) was used for column chromatography. A Yanaco MP-S3 micro-melting point apparatus was used to take the melting points which are uncorrected. A Jasco DIP 370 polarimeter was used for measuring the optical rotations. 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.

Fermentation, extraction and purification

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) to prepare the liquid medium for *B. cinerea* (AHU 9424). 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 (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 sulfate and concentrated *in vacuo* to afford a brown gum (630 mg) which was absorbed on an equal quantity of silica gel and chromatographed, where the elution with EtOAc:*n*-hexane (1:4, v/v) gave a colorless oily oxidized metabolite identified as 3β-hydroxy-(–)-β-pinene (**3**) (33 mg). Further elution yielded the major metabolite as a colorless oil identified as verbenone (**2**) (56 mg) and then 9-hydroxy-(–)-α-pinene (**4**) (27 mg). Elution with EtOAc:*n*-hexane (2:3, v/v) gave the metabolite 4β-hydroxy-(–)-α-pinen-6-one (**5**) (40 mg) (See Table I, for yields).

*3-β-Hydroxy-(–)-β-pinene (**3**)* was obtained as a colorless oil: $[\alpha]_D^{27}$: –59.0 ° ($CHCl_3$, *c* 0.1), FDMS, *m/z* 152; EIMS, *m/z* 152 (6), 137 (35), 121 (15), 107 (35), 93 (100), 81 (38), 77 (32), 69 (28), 55 (28), 43 (52); HREIMS, *m/z* 152.1192 ($C_{10}H_{16}O$ requires 152.1201); IR ν_{max} (cm^{-1}): 3434, 1675; for ^{1}H-NMR ($CDCl_3$, δ) and ^{13}C-NMR ($CDCl_3$, δ) see Table II.

*9-Hydroxy-(–)-α-pinene (**4**)* was obtained as a colorless oil. $[\alpha]_D^{27}$: –35.0 ° ($CHCl_3$, *c* 0.1), FDMS, *m/z* 152; EIMS, *m/z* 152 (6), 137 (13), 121 (25), 107 (44), 93 (100), 81 (47), 77 (42), 69 (43), 55 (51), 43 (68); HREIMS, *m/z* 152.1211 ($C_{10}H_{16}O$ requires 152.1201); IR ν_{max} (cm^{-1}): 3411, 1521; for ^{1}H-NMR ($CDCl_3$, δ) and ^{13}C-NMR ($CDCl_3$, δ) see Table II.

*4β-Hydroxy-(–)-α-pinen-6-one (**5**)* was obtained as a colorless oil. $[\alpha]_D^{27}$: –36.0 ° ($CHCl_3$, *c* 0.1), FDMS, *m/z* 166; EIMS, *m/z* 166 (4), 151 (8), 137 (6), 125 (3), 123 (9), 119 (5), 108 (100), 94 (34), 79 (12), 69 (10), 55 (10), 43 (20); HREIMS *m/z* 166.0908 ($C_{10}H_{14}O_2$ requires 166.0994); IR ν_{max} (cm^{-1}): 3379, 1713,1554; for ^{1}H-NMR ($CDCl_3$, δ) and ^{13}C-NMR ($CDCl_3$, δ) see Table II.

Results and Discussion

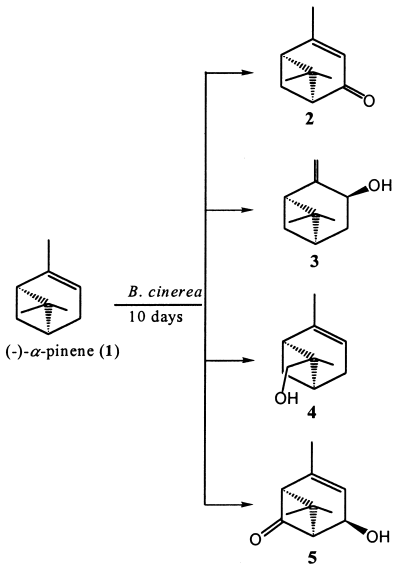
Our studies on the microbial metabolism of (–)-α-pinene (**1**) with *B. cinerea* for ten days showed the presence of three new metabolites **3–5** along with the major known oxidized metabolite **2**. The metabolites were isolated as colorless oils by column chromatography as presented in the experimental section. The known metabolite was identified as verbenone (**2**) by comparing the physical and spectroscopic data with the literature values (Bates and Thalacker, 1968). The new metabolites were characterized as 3β-hydroxy-(–)-β-pinene (**3**); 9-hydroxy-(–)-α-pinene (**4**), 4β-hydroxy-(–)-α-pinen-6-one (**5**) (Fig. 1).

The FDMS and EIMS of metabolite **3** had a molecular ion peak at *m/z* 152. The molecular formula $C_{10}H_{16}O$ of the compound **3** was deduced by recording the HREIMS (exact molecular weight at *m/z* 152.1192). The IR spectrum showed a hy-

Table I. The microbial oxidation of (–)-α-pinene (**1**) by *B. cinerea.*

Substrate*	Metabolite	Yield (%)
(–)-α-pinene (**1**)	Verbenone (**2**)	25
	3β-Hydroxy(–)-β-pinene (**3**)	10
	9-Hydroxy-α-pinene (**4**)	12
	3β-Hydroxy-(–)-α-pinen-6-one (**5**)	16

* Concentration: 40 mg/200 ml liquid medium.



Compounds

(–)-α-pinene (**1**)
verbenone (**2**)
3β-hydroxy-(–)-β-pinene (**3**)
9-hydroxy-(–)-α-pinene (**4**)
4β-hydroxy-(–)-α-pinen-6-one (**5**)

Fig. 1. Microbial transformation of (–)-α-pinene (**1**) by *B. cinerea*.

droxyl absorption at 3434 cm^{-1} and an olefinic signal at 1675 cm^{-1}. The ^{13}C-NMR spectrum displayed resonances for 10 carbons while the DEPT spectra showed the presence of 2 methyl, 3 methylene, 3 methine and 2 quaternary carbons. The C-3 position of the newly introduced hydroxyl was established due to the HMBC correlations of H-3 (δ 4.35) with C-1 (δ 51.6) and C-5 (δ 39.9), and COSY correlations of H-3 (δ 4.35) and H-4 (δ 1.12 and 1.5, doublet). The NOESY spectrum displayed correlations between H-3 and H-8 (δ 0.57) and therefore proved the α-stereochemistry of H-3 and hence β-orientation of newly introduced hydroxyl at position 3. This observation is consistent with the coupling pattern of the H-3 signal at δ 4.35 (d, J= 7.6 Hz) according to the Karplus equation. The epimerisation of the olefinic bond from C-3 to C-10 was proved because of the appearance of two olefinic signals in the ^{1}H-NMR spectrum at δ 4.35 and δ 4.92. The ^{13}C-NMR spectrum of **3** had a methylene signal at δ 128.7. The metabolite was hence characterized as 3β-hydroxy(–)-β-pinene (**3**).

EIMS of the metabolite 9-hydroxy(–)-α-pinene (**4**) showed a molecular ion peak at *m/z* 152 as confirmed by FDMS due to the introduction of an oxygen atom. The molecular formula of the metabolite was deduced as $C_{10}H_{16}O$ by recording the HREIMS which displayed the exact molecular mass as at *m/z* 152.1211. A hydroxyl absorption at 3411 cm^{-1} and an olefinic signal at 1521 cm^{-1} in the IR spectrum of **4** suggested that a hydroxylation product was formed. The ^{13}C-NMR spectrum showed signals for 10 carbons while DEPT spectra displayed the presence of 2 methyl, 4 methylene, 2 methine and 2 quaternary carbons. A methylene signal at δ 62.6 suggested the hydroxylation of a methyl group. The C-9 position of the newly introduced hydroxyl function was deduced because of the HMBC correlations of C-7 (δ 46.9) with H-9 (δ 3.56 and 3.66).

Table II. The ^{1}H and ^{13}C NMR chemical shift assignments* of new metabolites of (–)-α-pinene (**1**).

C	H	**3**		**4**		**5**	
		δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	β	2.44, t (5.4)	51.6, d	1.76, t (3.8)	46.8, d	1.22, s	39.4, d
2		–	155.9, s	–	148.4, s	–	133.0, s
3		4.35, d (7.6)	67.0, d	5.16, brs	121.5, d	5.50, d (5.4)	125.5, d
4	α	1.50, m	34.5, t	1.68, m	33.3, t	4.14, dd (5.4, 9.9)	80.2, t
	β	1.12, m		1.47, m		–	–
5	β	1.92, m	39.9, d	1.73, m	46.8, d	1.64, d (9.9)	43.3, d
6	α	2.31, m	28.0, t	2.21, m	35.5, t	–	209.0, s
	β	1.20, m		1.80, m		–	
7		–	40.4, s	46.9, s		57.0, s	
8		0.57, s	22.0, q	0.92, s	25.8, q	1.09, s	27.1, q
9		1.21, s	26.0, q	3.66 m, 3.56 m	62.6, t	1.13, s	27.2, q
10		4.92 s, 4.74 s	128.7, t	0.71, s	19.8, q	0.75, s	19.2, q

* values are given in ppm (coupling constant, J = Hz).

The FDMS and EIMS of the metabolite **5** displayed a molecular ion peak at *m/z* 166 and the HREIMS displayed the exact molecular mass at *m/z* 166.1908 corresponding to the molecular formula $C_{10}H_{14}O_2$ indicating the introduction of a hydroxyl and a ketone group. The IR spectrum showed absorptions at 3379 (OH), 1713 (C=O) and 1554 cm^{-1} (C=C). The ^{13}C-NMR spectrum exhibited resonances for 10 carbons while the presence of 3 methyl, 4 methine and 2 quaternary carbon atoms were deduced by DEPT spectra. A low field hydroxyl-bearing methine signal resonating at δ 80.2 along with a quaternary carbon signal at δ 209.6 proved the hydroxylation of a methylene and the oxidation of a methylene carbon into a ketone function. The C-4 position of the newly introduced hydroxyl was established due to the HMBC correlations of C-4 (δ 80.2) with H-3 (δ 5.5) and H-5 (δ 1.64). The COSY spectrum showed correlations of H-4 (δ 4.14, d) with H-5 (δ 1.64) and H-3 (δ 5.50). The β-stereochemistry of 4-OH was established on the basis of the coupling pattern of H-4α (dd, $J_{4\alpha,3}$ = 5.3, $J_{4\alpha,5\beta}$ = 9.9 Hz) and the NOESY correlations of H-4α (δ 4.14) with CH_3-8 (δ 1.22). The position 6 of keto function was deduced from HMBC correlations of H-1 (δ 1.22) and H-5 (δ 1.64) with C-6 (δ 209.0). This new metabolite was thus characterized as 4β-hydroxy-(–)-α-pinen-6-one (**5**).

Further microbial transformation studies are still ongoing with pinenes and other monoterpenes.

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