Oxidative Metabolism of Ambrox and Sclareolide by *Botrytis cinerea*

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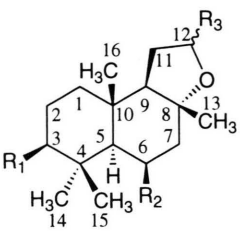
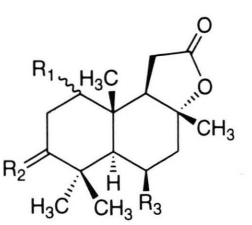
Ambrox, Sclareolide, *Botrytis cinerea*

Ambrox (**1**), a perfumery diterpene, was oxidatively metabolised by a plant pathogenic fungus *Botrytis cinerea* in a *xenobiotic* fashion to afford a major product, i.e., 1β-hydroxy-8-epiambrox (**13**) (60%) along with three minor metabolites 3β-hydroxyambrox (**2**), sclareolide (**5**) and 3β-hydroxysclareolide (**7**). Sclareolide (**5**), a cytotoxic diterpenoidal lactone was fermented with the same fungus to yield 3β-hydroxysclareolide (**7**) (59%) as a major metabolite together with two minor metabolites characterised as 1-ketosclareolide (**15**), and 3β,14-dihydroxysclareolide (**16**).

Introduction

Botrytis cinerea is a gray mold which causes diseases of many commercial plants (Agrios, 1988). The fungus produces botrydial and related terpenoids responsible for enhancing the pathogenicity of the fungus (Collado *et al.*, 1995, 1996; Rebordinos *et al.*, 1996). Metabolism of some clovanes, caryophyllene oxide and patchoulol sesquiterpenes by the said fungus have been reported in the literature (Collado *et al.*, 1998; Duran *et al.*, 1999; Aleu *et al.*, 1999). We have previously reported on metabolism of many prenylated flavonoids and related phytoalexins by *Botrytis cinerea* (Farooq and Tahara, 1999). Fermentation of ambrox (**1**) by *Cephalosporium aphidicola* has been reported to produce 3β-hydroxyambrox (**2**), 3β,12-dihydroxyambrox (**3**) and 3β,6β-dihydroxyambrox (**4**). On one occassion, 3β-hydroxy-8-epiambrox was also isolated as an example of epimerisation of ambrox at C-8 by *C. aphidicola* (Hanson and Truneh, 1996). In continuation of our studies on metabolism of natural products by *Botrytis cinerea* (AHU 9424) and to see the flexibility of metabolic system of the fungus, we fermented ambrox (**1**) for 10 days to yield a major metabolite 1β-hydroxy-8-epiambrox (**13**) (60%) and three minor metabolites, i.e., 3β-hydroxyambrox (**2**), sclareolide (**5**) and 3β-hydroxysclareolide (**7**). Sclareolide (**5**) which showed anticancer activities against breast (MCF-7), colon (CKCO-1), lung (H-1299) and skin (HT-144) human cancer cell lines has previously been fermented by *Curvularia lunata, Mucor plumbeus*, *Cephalosporium aphidicola* to yield various oxidised metabolites **6–11** in low chemical yields (Hanson and Truneh, 1996; Atta-ur-Rahman *et al.*, 1997; Aranda *et al.*, 1991). The effect of the lactone moiety toward the metabolism was marked by incubating sclareolide (**5**), a cytotoxic diterpenoidal lactone with *Botrytis cinerea* (AHU 9424). The metabolites 3β-hydroxysclareolide (**7**), 1-ketosclareolide (**15**), and 3β,14-dihydroxysclareolide (**16**) thus obtained revealed that the lactone functionality of the diterpene could cause hydroxylations at 1β, 3β and 14 positions.





1: $R_1 = R_2 = R_3 = H$
2: $R_1 = OH$, $R_2 = R_3 = H$
3: $R_1 = R_3 = OH$, $R_2 = H$
4: $R_1 = R_2 = OH$, $R_3 = H$

5: $R_1 = R_3 = H$, $R_2 = H_2$
6: $R_1 = R_3 = H$, $R_2 = O$
7: $R_1 = R_3 = H$, $R_2 = \alpha$-H, β-OH
8: $R_1 = H$, $R_2 = \alpha$-H, β-OH, $R_3 = OH$
9: $R_1 = \beta$-OH, $R_2 = H_2$, $R_3 = H$
10: $R_1 = \beta$-OH, $R_2 = \alpha$-H, β-OH, $R_3 = H$
11: $R_1 = \alpha$-OH, $R_2 = \alpha$-H, β-OH, $R_3 = H$

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Experimental

General

The silica gel 60 ASTM mesh 230–400 was used for column chromatography while purity of the samples was checked on Merck Kieselgel 60 F_{254}, 0.2 mm thick TLC plates. The spots were viewed under 254 and 365 nm UV light and spraying with EtOH-H_2SO_4 (1:1 v/v). The melting points were determined on a Yanaco MP-S3 micro melting point apparatus and are uncorrected. The IR spectra were recorded in $CHCl_3$ on a Perkin-Elmer 2000 FTIR. The mass spectra were recorded on a JEOL JMS-SX 102 A mass spectrometer and a JASCO DIP-370 polarimeter was used for measuring the optical rotations. The ^{13}C NMR spectra were recorded on a JEOL EX-270 spectrometer while collecting at 68 MHz. The ^1H and 2D-NMR spectra were recorded on a Bruker AMX500 spectrometer.

Fermentations and extraction

Medium for *Botrytis cinerea* (2 liters) was prepared by mixing glucose (80 g), yeast extract (2 g), KH_2PO_4 (10 g), $MgSO_4$ (1 g), $NaNO_3$ (4 g), $FeSO_4$ (20 mg) and $ZnSO_4$ (10 mg), with distilled water (2 liters). The medium was evenly distributed among 10 flasks of 500 ml capacity (200 ml in each) and autoclaved. Each flask was innoculated with a mycelial suspension of *Botrytis cinerea* (1 ml) and incubated on a reciprocal shaker for three days at 120 rpm, a clear ethanolic solution (10 ml) of the substrate (500 mg) was evenly distributed to the 10 culture flasks (50 mg/200 ml). Fermentation was carried out for further 10 days. The mycelium was filtered, washed with water and ethyl acetate, and the broth thus obtained was extracted with ethyl acetate (6 liters). The organic layer was washed with brine and dried over anhydrous sodium sulfate and concentrated *in vacuo* to afford a brown gum which was adsorbed on an equal quantity of silica gel and chromatographed.

Metabolism of ambrox (**1**)

The brown gum (1.4 g) obtained by fermentation of ambrox (**1**) was chromatographed over a silica gel column where elution with EtOAc-*n*-hexane (3:7 v/v) afforded oxidised metabolite 3β-hydroxyambrox (**2**) (35 mg) which was identified by comparison of spectroscopic data with the literature values (Hanson and Truneh, 1996). Further elution with EtOAc-*n*-hexane (3:7 v/v) gave colourless crystalline material identified as sclareolide (**5**) (13 mg) by comparing its spectroscopic data with reported values (Hanson and Truneh, 1996). Elution with EtOAc-*n*-hexane (4:6 v/v) afforded colourless amorphous material 3β-hydroxysclareolide (**7**) (22 mg) as identified by comparing the spectroscopic data with the reported values (Hanson and Truneh, 1996).

Elution with EtOAc-*n*-hexane (1:1) gave 1β-hydroxy-8-epiambrox (**13**) (319 mg) as a colourless amorphous material. mp 211–213 °C. $[\alpha]_D^{27}$: −20.0°(*c* = 0.033, $CHCl_3$). IR ($CHCl_3$): 3342 and 3300 cm^{-1}. ^1H NMR (C_2D_6SO, 500 MHz) see Table II. ^{13}C NMR (C_2D_6SO, 68 MHz) see Table III. FDMS, *m/z* (rel. int.): 252 [M^+] (100). HREIMS *m/z* 252.2117, ($C_{16}H_{28}O_2$ requires 252.2090). EIMS *m/z* (rel. int.): 252 [M^+] (11), 237 [M^+-15] (100), 219 (31), 201 (10), 193 (19), 175 (29), 163 (14), 152 (10), 139 (22), 135 (75), 123 (72), 107 (39), 95 (42), 81 (37), 69 (34), 55 (29), 43 (41).

Metabolism of sclareolide (**5**)

The brown gum (1.7 g) obtained by fermentation of sclareolide (**5**) (500 mg/2000 ml) was chromatographed over a silica gel column. Elution with EtOAc:*n*-hexane (4:6) yielded an oxidized metabolite characterised as 1-ketosclareolide (**15**) (17 mg). Further elution with EtOAc-*n*-hexane (4:6) gave a colourless amorphous oxidised material, i.e, 3β-hydroxysclareolide (**7**) (314 mg) identified by comparing the spectroscopic data with those of the literature (Hanson and Truneh, 1996). Elution with pure EtOAc afforded colourless crystalline substance 3β,14-dihydroxysclareolide (**16**) (96 mg).

1-Ketosclareolide (**15**) was obtained as a colourless amorphous material. mp 191–192 °C. $[\alpha]_D^{27}$: +124.6° (*c* = 0.064, $CHCl_3$). IR ($CHCl_3$): 1753 and 1696 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz) see Table II. ^{13}C NMR ($CDCl_3$, 68 MHz) see Table III. FDMS *m/z* (rel. int.): 264 [M^+] (100). HREIMS *m/z* 264.1732, ($C_{16}H_{24}O_3$ requires 264.1726). EIMS *m/z* (rel. int.): 264 [M^+] (100), 249 [M^+-15] (32), 231 (12), 222 (24), 193 (21), 181 (16), 163 (25), 152 (47), 139 (45), 121 (47), 109 (48), 93 (24), 83 (32), 67 (42), 55 (30), 43 (57).

3β,14-dihydroxysclareolide (**16**) was obtained as colourless cubes. mp 186–187 °C. $[\alpha]_D^{27}$: +65.3° (*c* = 0.08, $CHCl_3$). IR ($CHCl_3$): 3482, 3319 and 1755 cm^{-1}. ^1H NMR ($CDCl_3$, 500 MHz) see Table II. ^{13}C NMR ($CDCl_3$, 68 MHz) see Table III. FDMS *m/z* (rel. int.): 282 [M^+] (3), 264 [M^+-H_2O] (100). HREIMS *m/z* 264.1733, ($C_{16}H_{26}O_4-H_2O$ requires 264.1726) EIMS *m/z* (rel. int.): 264 [M^+-H_2O] (78), 249 (58), 233 (100), 221 (24), 207 (24), 191 (62), 173 (51), 161 (29), 153 (41), 147 (79), 133 (33), 121 (50), 107 (52), 93 (62), 81 (43), 67 (33), 55 (32), 43 (75).

Results and Discussion

Our studies on metabolism of **1** with *Botrytis cinerea* (AHU 9424) for ten days showed the presence of three known minor metabolites **2**, **5** and **7** along with the major, new oxidised metabolite **13** (Scheme) (see Table I). The minor metabolites **2, 5** and **7** were isolated as colourless cubes and were identified as 3β-hydroxyambrox (**2**), sclareolide (**5**) and 3β-hydroxysclareolide (**7**) through comparison of the spectroscopic data with the literature values (Hanson and Truneh, 1996) while the major metabolite was characterised through detailed physical and spectroscopic studies. The EIMS of **13** displayed a molecular ion peak at *m/z* 252 which was confirmed by recording the FDMS and introduction of an oxygen function was hence anticipated. The HREIMS of the metabolite showed exact molecular wieght at *m/z* 252.2117 corresponding to the molecular formula $C_{16}H_{28}O_2$. The IR spectrum of **13** showed a hydroxy absorption at 3342 cm^{-1}. The ^{13}C NMR spectrum of **13** exhibited resonances for 16 carbons while DEPT spectra revealed the presence of 4 methyl, 6 methylene, 3 methine and 3 quaternary carbons. A lowfield hydroxy-bearing methine signal resonating at δ 76.8 proved the hydroxylation of a methylene carbon. The C-1 position of the newly introduced hydroxyl was established due to the γ-upshifts of C-3, C-5 and C-9 carbons compared to the 3β-hydroxyambrox and was further confirmed by the HMBC correlations of H-1 (δ 2.97) with C-10 (δ 37.5) and C-2 (δ 26.9). The epimerisation of ambrox at C-8 results in the upfield shift of all the carbons except C-9 (Hanson and Truneh, 1996). The ^{13}C NMR data of **13** was compared with that of 3β-hydroxy-8-epiambrox. It was therefore concluded that epimerisation of the ambrox (**1**) might have afforded 8-epiambrox (**12**) which could go hydroxylation to afford 1β-hydroxy-8-epiambrox (**13**). The epimerisation was further confirmed by the NOESY correlations of CH_3-13 (δ 1.00) with H-5α (δ 0.77) and H-9α (δ 0.92). The ^1H NMR spectrum of **13** displayed an 1H, dd at δ 2.97 due to a proton on the hydroxyl-bearing methine and the coupling pattern (dd, $J_{1ax,2ax}$ = 10.9, $J_{1ax,2eq}$ = 4.7 Hz) showed the axial orientation of H-1 and hence equatorial (β) orientation of newly introduced hydroxyl group. The β stereochemistry of 1-OH was further confirmed by NOESY correlations of H-1α (axial) (δ 2.97) and H-5α (axial) (δ 0.77). The possibility of the metabolite being 3β-hydroxy-8-epiambrox was ruled out on the basis that all the spectroscopic and physical data did not match the reported values especially the γ-upshift of C-9 due to hydroxylation at C-1. The metabolite characterised as 1β-hydroxy-8-epiambrox (**13**) is difficult to obtain by chemical ways and can be obtained by fementation of **1** with *Botrytis cinerea* in a good yield. The complete ^1H NMR and ^{13}C NMR chemical shifts of **13** were unambiguously assigned by a combination of Broad Band, DEPT, HMQC, HMBC, COSY and NOESY spectra and are summarised in Tables II and III, respectively.

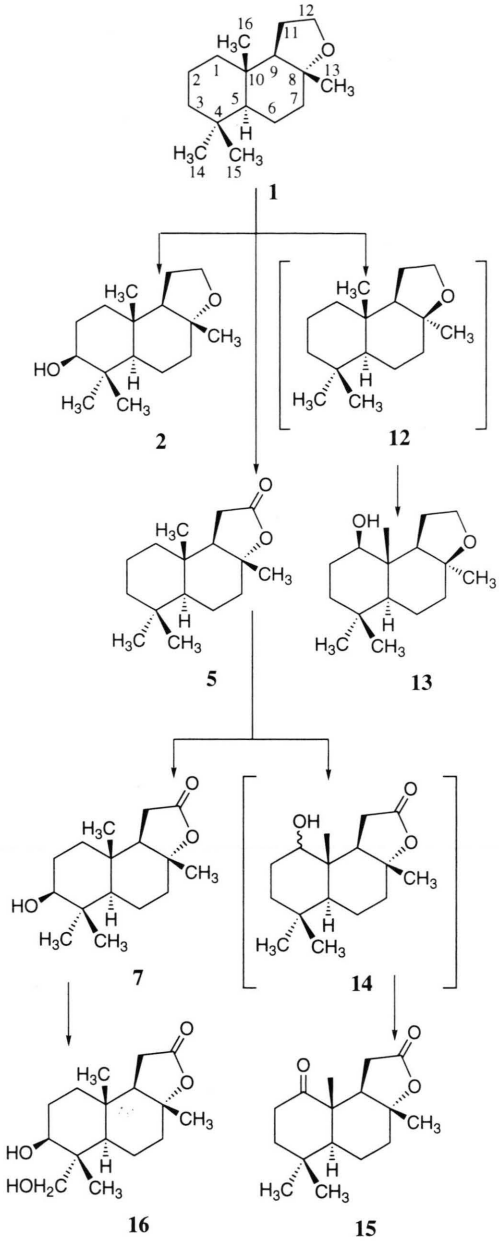
Fermentation of sclareolide (**1**) by *Botrytis cinerea* yielded 3β-hydroxysclareolide (**7**) (59%) as a major metabolite along with 1-ketosclareolide (**15**), and 3β,14-dihydroxysclareolide (**16**) (see Table I). The known metabolite **7** was identified by comparing the spectroscopic data with the literature values (Hanson and Truneh, 1996).

EIMS of the metabolite 1-ketosclareolide (**15**) showed a molecular ion peak at *m/z* 264 as confirmed by FDMS due to the introduction of an oxygen atom. The molecular formula of the me-

Table I. Percentage yields of the metabolites of ambrox (**1**) and sclareolide (**5**).

Substrate*	Metabolite	Yield (%)**
Ambrox (**1**)	3β-hydroxyambrox (**2**)	7
	sclareolide (**5**)	2
	3β-hydroxysclareolide (**7**)	4
	1β-hydroxy-8-epiambrox (**13**)	60
Sclareolide (**5**)	1-ketosclareolide (**15**)	3
	3β-hydroxysclareolide (**7**)	59
	3β,14-dihydroxysclareolide (**16**)	17

* Concentration : 50 mg/200 ml medium.
** When 500 mg of each substrate (**1** or **5**) was used.



Scheme: Oxidative metabolism of ambrox (**1**) and sclareolide (**5**).

tabolite was deduced as $C_{16}H_{24}O_3$ by recording the HREIMS which exhibited exact molecular mass at *m/z* 264.1732. A ketonic absorption at 1696 cm^{-1} in the IR spectrum of **15** suggested that the newly introduced oxygen could be as a keto group. The ^{13}C NMR spectrum of **15** exhibited resonances for 16 carbons while DEPT spectra revealed the presence of 4 methyl, 5 methylene, 2 methine and 5 quaternary carbons. A quaternary signal at δ 216.6 proved the oxidation of a methylene carbon to a ketonic carbon. The C-1 position of the newly introduced keto function was deduced because of the HMBC correlations of C-1 (δ 216.6) with H_2-2 (δ 2.51, 2.57) and CH_3-16 (δ 1.03). Since the spectroscopic data varied from that of 3-ketosclareolide specially the downfield shift of C-10 and C-16 due to the oxygenation of C-1, therefore the possibility of 3-ketoscalreolide was ruled out. The ^{13}C- and ^{1}H NMR chemical shifts of all the carbons and protons were assigned by recording Broad Band, DEPT, HMQC, HMBC, COSY and NOESY spectra (see Tables II and III). The metabolite **15** might have been formed by the C-1 hydroxylation of sclareolide (**5**) to yield an intermediate **14** followed by oxidation of the C-1 alcohol to the C-1 keto function (Scheme).

The FDMS and EIMS of **16** displayed a weak molecular ion peak at *m/z* 282 (3%) and an intense peak at *m/z* 264 (100%) due to the loss of a water molecule from molecular ion which is common in dihydroxysclareolide derivatives (Atta-ur-Rahman *et al.*, 1997). The molecular mass was therefore anticipated to be 282 due to the introduction of two hydroxyl functions. The HREIMS of **16** displayed the exact molecular mass at *m/z* 264.1733 corresponding to the intense dehydrated ion $[C_{16}H_{26}O_4-H_2O]^+$. Two hydroxyl absorptions at 3482 and 3319 cm^{-1} were observed in the IR spectrum of **16** due to the introduction of two hydroxyl groups. The ^{13}C NMR spectrum of **16** exhibited resonances for 16 carbons while DEPT spectra revealed the presence of 3 methyl, 6 methylene, 3 methine and 4 quaternary carbons. A low-field hydroxyl-bearing methine signal resonating at δ 76.5 along with a methylene signal at δ 70.6 proved the hydroxylation of a methylene and a methyl carbons. The C-3 position of the newly introduced hydroxyl was established by comparison of the ^{13}C NMR data with that of 3β-hydroxysclareolide (**7**) and due to the HMBC correlations of C-3 (δ 76.5) and H-2 (δ 1.73), C-4 (δ 42.3) and H-3 (δ 3.69). The β stereochemistry (equatorial) of 3-OH was established on the basis of coupling pattern of 3α-H (dd, $J_{3ax,2eq}$ = 5.5, $J_{3ax,2ax}$ = 11.5 Hz) and the NOESY correlations of H-3α (δ 3.69)

Table II. ^{1}H NMR spectral data of new metabolites **13**, **15** and **16**.

Hydrogen	**13** δ_H (ppm, J = Hz)	**15** δ_H (ppm, J = Hz)	**16** δ_H (ppm, J = Hz)
H-1	2.97, dd, (4.7, 10.9)	-----	1.22, m, 1.45, m
H-2	1.50, m, 1.54, m	2.51, m, 2.57, m	1.73, m
H-3	1.53, m, 1.55, m	1.61,m, 1.72, m	3.69, dd, (5.5, 11.5)
H-5α	0.77, d (11.5)	1.64, m	1.70, m
H-6	1.20, m, 1.54, m	1.55, m, 1.85, m	1.68, m
H-7	1.22, m, 1.70, m	2.13, m	1.71, m
H-9α	0.92, m	2.01, m	1.94, dd (6.7, 14.8)
H-11	1.25, m	2.29, m	2.41, dd (14.8, 16.2)
	1.55, m	2.46, m	2.24, dd (6.7, 16.2)
H-12	3.36, br. s	-----	-----
H-13	1.00, s	1.39, s	1.34, s
H-14	0.87, s	1.13, s	3.40, d, (10.3)
			3.69, d (10.3)
H-15	0.70, s	1.06, s	0.96, s
H-16	0.63, s	1.03, s	0.86, s

Table III. ^{13}C NMR spectral data of new metabolites **13**, **15** and **16**.

Carbon	**13** δ_C (ppm)	**15** δ_C (ppm)	**16** δ_C (ppm)
C-1	76.8 *d*	216.6 *s*	36.2 *t*
C-2	26.9 *t*	34.5 *t*	26.6 *t*
C-3	38.4 *t*	38.7 *t*	76.5 *d*
C-4	37.8 *s*	48.4 *s*	42.3 *s*
C-5	54.7 *d*	55.4 *d*	49.9 *d*
C-6	19.8 *t*	22.5 *t*	20.5 *t*
C-7	43.9 *t*	38.8 *t*	37.8 *t*
C-8	71.4 *s*	86.7 *s*	86.4 *s*
C-9	57.6 *d*	59.3 *d*	59.2 *d*
C-10	37.5 *s*	36.6 *s*	36.1 *s*
C-11	28.4 *t*	29.7 *t*	29.1 *t*
C-12	63.2 *t*	177.0 s	176.9 *s*
C-13	23.9 *q*	22.2 *q*	21.9 *q*
C-14	28.3 *q*	27.7 *q*	70.6 *q*
C-15	15.7 *q*	21.5 *q*	15.8 *q*
C-16	15.3 *q*	15.6 *q*	11.5 *q*

with H-1α (δ 1.22), H-5α (δ 1.70) and H-14α (δ 3.40). The C-14 hydroxylation was established by comparison of the carbon and proton data with those of the starting material where the methyl signals resonating at δ 33.1 and 1.13 respectively were missing while NOESY interactions between H-5α (δ 1.60) and H-3α (δ 3.69) were observed with hydroxyl-bearing methylene protons H_2-14 α (δ 3.40 and 3.69). NOESY correlations between CH_3-15 (δ 0.96) and CH_3-16 (δ 0.86) also proved the hydroxylation of CH_3-14. It was concluded that the metabolite **16** might have been formed by the further hydroxylation of the metablite **7** (Scheme).

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