Biotransformation of Two Cytotoxic Terpenes, α-Santonin and Sclareol by *Botrytis cinerea*

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Two cytotoxic terpenes, α-santonin (**1**) and sclareol (**3**) were biotransformed by a plant pathogenic fungus *Botrytis cinerea* to produce oxidized metabolites in high yields. α-Santonin (**1**) on fermentation with the fungus for ten days afforded a hydroxylated metabolite identified as 11β-hydroxy-α-santonin (**2**) in a high yield (83%), while sclareol (**3**) was metabolized to epoxysclareol (**4**) (64%) and a new compound 8-deoxy-14,15-dihydro-15-chloro-14-hydroxy-8,9-dehydrosclareol (**5**) (7%), representing a rare example of microbial halogenation.

Introduction

Bio-oxidation of saturated carbon atoms of steroids, terpenoids and other natural products by fungi has frequently been achieved (Holland, 1982). Microbial hydroxylations of many kinds of terpenoids have been studied in order to yield derivatives with fragrance, flavor, and pharmacological properties, and to use as asymmetric synthons and chiral auxiliaries (Atta-ur-Rahman *et al.*, 1997).

A gray mold plant pathogenic fungus *Botrytis cinerea* causes diseases of many commercial plants (Agrios, 1988). The fungus produces botrydial type terpenoidal secondary metabolites which are believed to enhance the pathogenicity of the fungus (Collado *et al.*, 1995; 1996; Rebordinos *et al.*, 1996). Metabolism of some clovanes, caryophyllene oxide and patchoulol sesquiterpenes by *B. cinerea* have been reported in the literature (Collado *et al.*, 1999; Duran *et al.*, 1999; Aleu *et al.*, 1999).

α-Santonin (**1**) which is strongly anthelmintic sesquiterpene has previously been chemoselectively reduced to 1,2-dihydro-α-santonin (4.6%) and hydroxylated to 11β-hydroxy-α-santonin (11.7%) by *Cunninghamella blakesleeana*, *Streptomyces aureofaciens* and *Aspergillus niger* in poor yields (Atta-ur-Rahman *et al.*, 1998; Iida, 1988).

*Sclareol (***3**), a cytotoxic diterpene has previously been hydroxylated by *Cephalosporium aphidicola* (Hanson *et al.*, 1994). We have previously reported metabolism of many prenylated flavonoids and related phytoalexins by *B. cinerea* where epoxidation of the prenyl side chain occured frequently (Farooq and Tahara, 1999). Recently we have started to explore the versatility of epoxidases of *B. cinerea* by incubating with versatile natural products possessing acyclic and cyclic double bonds in biologically active terpenes and steroids. α-Santonin (**1**) and sclareol (**3**) were therefore fermented with *B. cinerea* where epoxidation of only sclareol (**3**) occurred and hence proved that the epoxidases of *B. cinerea* work efficiently for acyclic double bonds and not for the cyclic double bonds of terpenes and steroids as reported by us recently (Farooq and Tahara, 2000a; 2000b). It is therefore concluded that the fungus metabolises natural compounds by epoxidation of the acyclic double bond or hydroxylation of the ring systems of the natural products lacking acyclic double bond.

Experimental

General

The Merck silica gel 60 mesh 230–400 was used for column chromatography, and purity of the samples was checked on Merck silica TLC plates. The spots were viewed under 254 and 366 nm UV light and spraying with EtOH- H_2SO_4 (1:1). The melting points were determined on a Yanaco MP-

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S3 micro melting point apparatus and are uncorrected. The optical rotations were measured on a JASCO DIP-370 polarimeter. The IR spectra were recorded in $CHCl_3$ on a Perkin-Elmer 2000 FTIR. The ^{1}H- and 2D-NMR spectra were recorded on a Bruker-AMX 500 spectrometer. The ^{13}C-NMR spectra were recorded on a JEOL EX-270 spectrometer while collecting at 67.8 MHz or on a Bruker AMX500 spectrometer collecting at 125 MHz. The mass spectra were recorded on a JEOL JMS-SX 102A mass spectrometer.

Fermentations and extraction

Botrytis cinerea (AHU 9424) was grown on potato dextrose agar and incubated at 25 °C for three days. Fermentation medium for *B. cinerea* (2 liters) was prepared by mixing glucose (80 g), yeast extract (2 g), anhydrous 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). Two days-old suspension cultures were inoculated into media flasks and incubation was continued for a further two days on a rotary shaker at 28 °C. A clear ethanolic solution (10 ml) of the substrate (500 mg) was evenly distributed to the culture flasks and fermentations were carried out for further 10 days. The mycelium was filtered, and washed with ethyl acetate. The filtrate and washings were extracted with ethyl acetate (2 l × 3) and the organic layer thus obtained was washed with brine, dried over anhydrous sodium sulfate and evaporated on a rotary evaporator to yield a brown gummy material which was adsorbed on an equal quantity of silica gel and subjected to column chromatography.

Fermentation of α-santonin (**1**)

The brown gum (1.3 g) obtained by fermenting **1** was chromatographed with elution by EtOAc:*n*-hexane (1:1) to recover the starting material (278 mg) while further elution with EtOAc:*n*-hexane (1:1) afforded 11β-hydroxy-α-santonin (**2**) (197 mg; 37% based on the initial substrate and 83% based on the transformed substrate. The product was identified by compring the physical and spectroscopic data with reported compound.

Metabolite **2**: m. p. 267–269 °C, $[\alpha]_D^{20}$ = −60°; MeOH; *c* 0.01, HRMS: calcd. for $C_{15}H_{18}O_4$ 262.1205, found 262.1227 (lit. m. p. = 273–275 °C, $[\alpha]_D^{20}$ = −71°; MeOH; *c* 0.085, Iida, 1988). We hereby report the ^{13}C NMR and complete ^{1}H NMR assignments for the first time. ^{1}H NMR (C_5D_5N, 500 MHz) see Table II. ^{13}C NMR δ_C (C_5D_5N, 67.8 MHz) see Table II.

Fermentation of sclareol (**3**)

Fermentation and extraction of **3** were carried out in exactly the same way as that for **1**, and the brown gum (1.2 g) obtained was chromatographed, eluting with EtOAc:*n*-hexane (1:4) to yield epoxysclareol (**4**; 336 mg; 64%) which was identified by comparison of the spectroscopic data with the literature values.

Metabolite **4**: m. p. 128–129 °C, $[\alpha]_D^{20}$ = −9°; MeOH; *c* 0.01, (Hanson *et al.*, 1994). Further elution with EtOAc:*n*-hexane (1:1) yielded colourless, amorphous material 8-deoxy-14,15-dihydro-15-chloro-8,9-dehydrosclareol (**5**) (38 mg; 7%) mp 148–150 °C. $[\alpha]_D^{20}$ −125° (*c* 0.08, MeOH). IR ($CHCl_3$): 3369, 1660, 1651. ^{1}H NMR (C_5D_5N, 500 MHz) see Table II. ^{13}C NMR δ_C (C_5D_5N, 125 MHz) see Table II. FDMS *m/z* (rel. int.): 344 [(M+2)$^+$] (36), 342 (M$^+$) (100). HRMS *m/z* 342.2359, ($C_{20}H_{35}O_2Cl$ requires 342.2328. EIMS *m/z* (rel. int.): 344 [(M+2)$^+$] (7), 342 [M$^+$] (22), 327 (M$^+$-15) (9), 309 (8), 291 (4), 245 (28), 204 (22), 191 (100), 189 (33), 175 (7), 162 (11), 149 (12), 135 (13), 121 (27), 119 (12), 109 (17), 107 (13), 95 (24), 69 (11), 43 (8).

Fermentation of epoxysclareol (**4**)

Normal fermentation medium for *B. cinerea* (AHU 9424) (200 mL) was prepared by mixing glucose (8 g), yeast extract (0.2 g), anhydrous KH_2PO_4 (1 g), $MgSO_4$ (0.1 g), $NaNO_3$ (0.4 g), $FeSO_4$ (2 mg) and $ZnSO_4$ (1 mg), with distilled water (200 mL).

Fermentation medium enriched by chloride ion was prepared by mixing the ingredeients of normal medium into 200 ml distilled water containing NaCl (20 mg) while that enriched with bromide ion was prepared by mixing the ingredients of normal medium into 200 ml distilled water enriched with NaBr (20 mg). Pre-established suspensions cultures of of *B. cinerea* were inoculated into the three flasks and fermentation was carried out for a further two days. A clear ethanolic solution (1 ml) of the substrate **4** (50 mg) was distributed

to each culture flask and fermentations were continued for 10 days. The EtOAc extracts obtained were spotted on tlc which showed conversion of epoxysclareol to 8-deoxy-14,15-dihydro-15-chloro-14-hydroxy-8,9-dehydrosclareol (**5**) as compared with standard sample from fermentation of sclareol. The extracts were combined, chromatographed and the product obtained was identified as **5** (18 mg, 20%). The epoxysclareol (**3**) (64 mg) was also recovered.

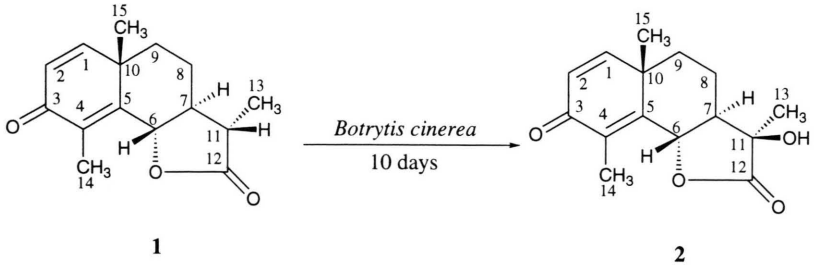
Results and Discussion

The metabolite, 11β-hydroxy-α-santonin (**2**) (Scheme 1) obtained in a high yield by fermentation of a-santonin (**1**) with *B. cinerea* (Table I) was identified by comparing the physical and spectroscopic data with literature (Iida, 1988). We report here the complete ^{1}H NMR and ^{13}C NMR data for the first time. The known metabolite epoxysclareol (**4**) (Scheme 2) which was obtained by fermentation of **3** by *B. cinerea* was identified by comparison of the physical and spectroscopic data with the literature values (Hanson *et al.,* 1994). The new metabolite 8-deoxy-14,15-dihydro-15-chloro-14-hydroxy-8,9-dehydrosclareol (**5**) was characterised by complete physical and spectroscopic data. The FDMS of **5** displayed a molecular mass at *m/z* 342. The exact molecular mass was found to be 342.2358 corresponding to the molecular formula $C_{20}H_{35}O_2Cl$ (calcd. 342.2328). The IR spectrum showed absorpations at 3369, 1660 and 1651 cm^{-1}. It was, therefore, anticipated that the chlorination of saturated carbon might have occurred. The complete ^{1}H and ^{13}C NMR values were assigned through a combination of HMQC, HMBC, COSY, and NOESY spectra (Table II) which helped in deducing the structure of **5** as 8-deoxy-14,15-dihydro-15-chloro-14-hydroxy-8,9-dehydrosclareol. ^{1}H NMR spectrum of **5** exhibited signals for H-14 (δ 3.57, dd, $J_{14,15a}$ = 9.5 Hz, $J_{14,15b}$ = 11.1 Hz) and H-15a (δ 3.67, dd, $J_{15a,14}$= 9.5 Hz, $J_{15a,15b}$ = 13.1 Hz), H-15b (δ 3.76, dd, $J_{15b,14}$ = 11.1 Hz, $J_{15b,15a}$ = 13.1 Hz). The ^{13}C NMR spectrum showed a methine resonance at δ 76.6 and a methylene resonance at δ 47.6 for C-14, and C-15, respectively. Two quaternary olefinic signals resonating at δ 140.1 and 126.5 were ascribed to C-8 and C-9, respectively. HMBC spectrum showed important correlations between H-7 (δ 1.55)/C-8 (δ 140.1), H-11 (δ 2.02, 2.12)/C-9 (δ 126.5), H-14 (δ 3.57)/C-13 (δ 74.4), H-16 (δ 1.19)/C-13 (δ 74.4), and H-15a,b (δ 3.67, 3.76)/C-14 (δ 76.6). The COSY spectrum displayed diagnostic interactions between H-15a,b (δ 3.67, 3.76) and H-14 (δ 3.57) while NOESY spectrum had correlations of CH_3-16 oriented at C-13 a position (δ 1.19) with H-14 (δ 3.57). The ^{1}H and ^{13}C NMR assignments of **5** were compared with the values of 14,15-dihydro-14-hydroxy-15-chlorosclareol obtained by fermentation of sclareol with *Cephalosporium aphidicola* (Hanson and Truneh, 1996). It was deduced that the sclareol was transformed to epoxysclareol which was further transformed to **5** by C-8,9-dehydration, opening of epoxide to afford 14,15-dihydroxy derivative followed by immediate enzyme-

Table I. Percentage yields of the metabolites of α-santonin (**1**) and sclareol (**3**).

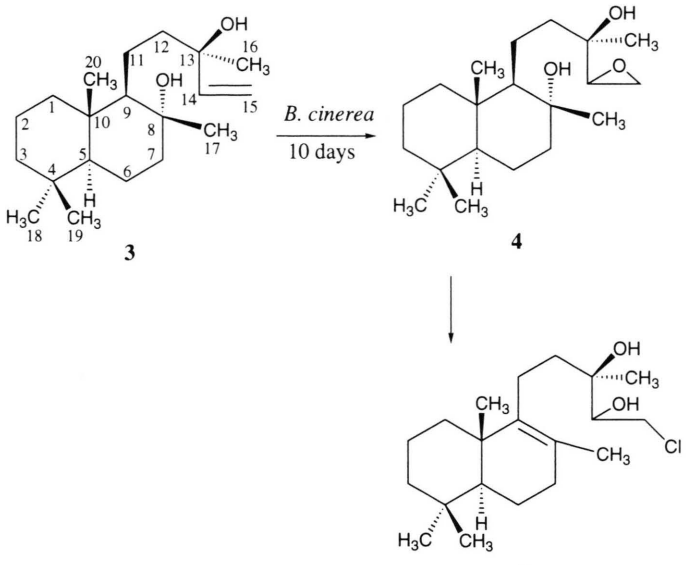
Substrate	Product	% Yield
α-Santonin (**1**)	11β-hydroxy-α-santonin (**2**)	83*
Sclareol (**3**)	epoxysclareol (**4**)	64
	8-deoxy-14,15-dihydro-15-chloro-14-hydroxy-8,9-dehydro-sclareol (**5**)	7

Substrate amount = 500 mg.
* The transformation yield was calculated to be 83% because 278 mg of the unchanged substrate was recovered.



1 **2**

Scheme 1. Hydroxylation of α-santonin (**1**) biocatalysed by *Botrytis cinerea* to yield 11β-hydroxy-α-santonin (**2**) (83%).



Scheme 2. Biotransformation of sclareol (**3**) by *Botrytis cinerea*.

Table II. ^{13}C- and ^{1}H NMR chemical shift assignments of metabolites **2** and **5**.

C	δ_C (ppm)		δ_H (ppm), (*J*=Hz)	
	2	**5**	**2**	**5**
1	125.8	34.1	6.30, d, (9.9)	1.96, m
2	155.7	19.4	6.60 (9.9)	1.66, m
3	185.9	37.4	–	1.81, m
4	152.0	33.7	–	–
5	128.1	52.3	–	1.12, dd, (2.2, 11.0)
6	80.2	21.9	5.41, d, (12.2)	2.08, m, 2.13, m
7	56.7	40.0	1.88, dt (3.4, 12.2)	1.55, dd (3.0, 6.6), 1.62, dd (5.9, 8.4)
8	37.6	140.1	1.37, dt, (4.2, 12.2, H-$8_{ax.}$), 1.74 dt (4.0, 6.6, H-$8_{eq.}$)	–
9	17.6	126.5	2.09, dd (4.2, 12.2, H-$9_{ax.}$),	–
9			1.72, dd, (2.0, 4.2, H-$9_{eq.}$)	
10	41.6	39.5	–	–
11	72.4	20.5	–	2.12, m, 2.02, m
12	177.5	42.2	–	1.15, dd, (4.7, 6.6), 1.39, dd (3.3, 5.2)
13	21.3	74.4	1.59, s	–
14	11.3	76.6	2.31, s	3.57, dd, (9.6, 11.1)
15	24.6	47.6	1.41, s	3.67, dd, (9.5, 13.1), 3.76, dd, (11.1, 13.1)
16	–	22.1	–	1.19, s
17	–	33.7	–	1.57, s
18	–	22.0	–	0.88, s
19	–	19.8	–	0.83, s
20	–	19.5	–	0.95, s

2, 11β-Hydroxy-α-santonin
5, 8-deoxy-14,15-dihydro-15-chloro-14-hydroxy-8,9-dehydrosclareol (**5**).

based nucleophilic displacement of hydroxyl group by a chlorine atom. The epoxy sclareol was hence fermented with the same fungus using three media, i.e, normal, containing NaBr (0.01%), and NaCl (0.01%). The metabolite **5** was the only product obtained in all the three fermentations (20%). We have previously demonstrated that the fungus epoxidizes the double bond of prenyl chain of prenylated isoflavones followed by opening of the epoxide to yield a diol. In present studies, it could be anticipated that the fungus also possesses an enzyme responsible for displacement of one of the hydroxyl groups of the vicinal diol by a chloro group and lacks any enzyme which could replace hydroxyl by a bromine atom.

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Agrios G. N. (1988), Plant Pathology, 3rd ed. Academic Press, San Diego, 403–407.

Aleu J., Hanson J. R., Hernandez-Galan R. and Collado I. G. (1999), Biotransformation of the fungistatic sesquiterpenoid patchoulol by *Botrytis cinerea*. J. Nat. Prod. **62**, 437–440.

Atta-ur-Rahman, Farooq A. and Choudhary M. I. (1997), Microbial transformations of sclareolide. J. Nat. Prod. **60**, 1038–1040.

Atta-ur-Rahman, Choudhary M. I., Shaheen F., Rauf A. and Farooq A. (1998), Microbial transformations of some bioactive natural products. Nat. Prod. Lett. **12**, 12–16.

Collado I. G., Hernandez-Galan R., Duran-Patron R. and Cantoral J. M. (1995), Metabolites from a shake culture of *Botrytis cinerea*. Phytochemistry **38**, 647- 650.

Collado I. G., Hernandez-Galan R., Prieto V., Hanson J. R. and Rebordinos L. G. (1996), Biologically active sesquiterpenoid metabolites from the fungus *Botrytis cinerea*. Phytochemistry **41**, 513–517.

Collado I. G., Hanson J. R., Macias-Sanchez A. and Mobbs D. (1999), The biotransformation of some clovanes by *Botrytis cinerea*. J. Nat. Prod. **61**, 1348–1351.

Duran R., Corrales E., Hernandez-Galan R. and Collado I. G. (1999), Biotransformation of caryophylene oxide by *Botrytis cinerea*. J. Nat. Prod. **62**, 41–44.

Farooq A. and Tahara S. (1999), Fungal metabolism of flavonoids and related phytoalexins. Curr. Top. Phytochem. **2**, 1–33.

Farooq A. and Tahara S. (2000a), Oxidative metabolism of ambrox and sclareolide by *Botrytis cinerea*. Z. Naturforsch. **55c**, 341–346.

Farooq A. and Tahara S. (2000b), Biotransformation of testosterone and pregnenolone catalyzed by the fungus *Botrytis cinerea*. J. Nat. Prod. **63**, 489–491.

Hanson J. R., Hitchcock P. B., Nasir H. and Truneh A. (1994), The biotransformation of the diterpene sclareol by *Cephalosporium aphidicola*. Phytochemistry **36**, 903–906.

Holland H. L. (1982), Oxidations of steroids. Chem Soc. Rev. 371–399 .

Iida M., Mikani A., Yamakawa K. and Nishitani K. (1988), Hydroxylation of α-santonin by *Aspergillus niger*. J. Ferment. Technol. **66**, 51–55 .

Rebordinos L. G., Cantoral M. J., Prieto M. V., Hanson J. R. and Collado I. G. (1996), The phytotoxic activity of some metabolites of *Botrytis cinerea*. Phytochemistry **42**, 383–387.