Microbial Transformation of Sclareolide

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The microbial transformation of sclareolide (**1**) by *Curvularia lunata* afforded five oxidized metabolites identified as 3-ketosclareolide (**2**), 1β-hydroxysclareolide (**3**), 3β-hydroxysclareolide (**4**), 1α,3β-dihydroxysclareolide (**5**), and 1β,3β-dihydroxysclareolide (**6**). Fermentations of **1** with *Aspergillus niger* also produced metabolites **2**−**6**. Metabolites **2**−**5** were obtained by fermention with *Gibberella fujikuroii,* while fermentation with *Fusarium lini* afforded metabolites **4** and **5**.

Sclareolide (**1**) is a minor constituent of *Arnica angustifolia*[1] and *Sideritis nutans*[2] and has been synthesized by the oxidation of sclareol, by ozonization of 12α-hydroxy-13-epinanoyl oxide[3−5] and by the degradation of a manoyl oxide derivative.[6] The microbiological oxidation of sclareolide (**1**) by *Mucor plumbeus* has afforded 3-ketosclareolide (**2**) (3.2%), 1β-hydroxysclareolide (**3**) (2.5%), and 3β-hydroxysclareolide (**4**) (7.9%).[7] Compound **1** showed cytotoxicity against breast (MCF-7), colon (CKCO-1), lung (H-1299), and skin (HT-144) human cancer cell lines.

The microbial transformation of sclareolide (**1**) by *Curvularia lunata* was therefore planned to obtain some chemically interesting and biologically active derivatives, which resulted in the production of 3-ketosclareolide (**2**) (7.3%), 1β-hydroxysclareolide (**3**) (8.8%), and 3β-hydroxysclareolide (**4**) (11.5%), as well as the new compounds 1α,3β-dihydroxysclareolide (**5**) and (13.3%) and 1β,3β-dihydroxysclareolide (**6**) (6.2%). The metabolites were identified by their ^{1}H- (Table 1) and ^{13}C-NMR data (Table 2). We describe the biotransformation of sclareolide (**1**) as an extension of our previous work.[8]

The crude organic extract obtained by incubation of sclareolide (**1**) with *Curvularia lunata* for 10 days was chromatographed over Si gel to yield metabolites **2**−**6**. Compound **2** had the molecular formula $C_{16}H_{24}O_3$, as determined by HREIMS. Its ^{1}H-NMR spectrum (Table 1) was very similar to that of **1**, while its ^{13}C-NMR spectrum displayed a ketonic signal at δ 215.4. The IR spectrum also showed absorption at 1700 cm^{-1}. The position of the carbonyl was established on the basis of HMBC correlations of the new carbonyl with H-2 (δ 2.52, 2.49).

Compound **3** has the molecular formula $C_{16}H_{26}O_3$, which was deduced from HREIMS. The ^{1}H-NMR data (Table 1) of **3** were also similar to those of **1**, but had a geminal proton to a new hydroxyl group resonating at δ 3.29 as a double doublet. The coupling constants (J = 11.1, 4.7 Hz) of this signal indicated that the hydroxyl group should be equatorial and situated either at C-1, C-3, or C-7. Position C-1 was confirmed for the new hydroxyl group on the basis of HMBC couplings of the proton resonating at δ 3.29 (H-1) with a quaternary carbon resonating at δ 36.4 (C-10). The ^{13}C-NMR data of **3** displayed a methine signal at δ 79.2 for C-1.

The HREIMS of compound **4** exhibited the molecular formula $C_{16}H_{26}O_3$. The ^{1}H-NMR spectrum of **4** was distinctively similar to that of **1** (Table 1) having an additional signal of a downfield proton geminal to a new hydroxyl group, which resonated at δ 3.22 as a double doublet (J = 11.8, 5.5 Hz). Again, the coupling constants suggested the presence of an equatorial OH either at C-1, C-3, or C-7. Position C-3 was confirmed for the new OH on the basis of HMBC couplings of the proton resonating at δ 3.22 (H-3) with the tertiary carbon resonating at δ 55.3 (C-5) and the lack of any coupling between the proton resonating at δ 3.22 (H-3) with carbons at δ 38.4 (C-10) or at δ 86.8 (C-8). The ^{13}C-NMR data of **4** showed a methine signal at δ 78.6 for C-3.

Compound **5** had a molecular formula $C_{16}H_{26}O_4$, which was determined by HREIMS. The ^{1}H-NMR data of **5** (Table 1) had two downfield signals ascribed to two protons geminal to hydroxyl groups resonating at δ 3.57 (t, J = 3.0 Hz) and at 3.76 (dd, J = 12.0, 4.8 Hz). The coupling constants (J = 3.0 Hz) suggested that the OH should be either at C-1, C-3, or C-7 with an axial orientation, while the coupling constants (J = 12.0, 4.8 Hz) indicated that the second OH might have C-1, C-3, or C-7 equatorial orientation. The ^{13}C-NMR spectra of the compound showed two methine carbon signals at δ 71.7 and 72.5. The C-1 position for one of the new hydroxyl groups was determined by the HMBC couplings of the proton geminal to hydroxyl group resonating at δ 3.57 (H-1) and the quaternary carbon resonating at δ 36.4 (C-10). Position C-3 was confirmed for the other hydroxyl group on the basis of HMBC couplings of the proton resonating at δ 3.76 (H-3) with tertiary carbon resonating at δ 47.1 (C-5) and the lack of any coupling between the proton resonating at δ 3.76 with carbons at δ 36.4 (C-10) or at 86.5 (C-8).

The HREIMS of the compound **6** exhibited the molecular formula $C_{16}H_{26}O_4$. The ^{1}H-NMR spectrum of **6** (Table 1) showed signals for two protons geminal to two new hydroxyl groups featured at δ 3.25 (dd, J = 11.8, 4.6 Hz) and 3.34 (dd, J = 11.5, 4.4 Hz). The coupling constants were suggestive of an equatorial orientation of OH at either of the C-1, C-3, or C-7 positions. The ^{13}C-NMR spectra of the compound showed two methine carbon signals at δ 75.9 and 76.4. The position for the hydroxyl group at C-1 was determined by the HMBC couplings of the proton geminal to hydroxyl group resonating at δ 3.25 (H-1) and the quaternary carbon resonating at δ 38.4 (C-10). The configuration of the hydroxyl group at C-3 was confirmed on the basis of HMBC couplings of the proton resonating at δ 3.34

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Table 1. ^{1}H-NMR Assignments of Sclareolide (**1**) and Its Metabolites (**2**−**6**) in $CDCl_3$ (J = Hz)

proton	1	2	3	4	5	6
H-1α	1.38, m	1.70, m	3.29, dd (11.1, 4.7)	2.04, m		3.25, dd (11.8, 4.6)
H-1β	1.04, m	1.58, m		1.63, m	3.57, t (3.0)	
H-2α	1.45, m	2.52, m	1.61, m	1.66, m	1.99, m	2.56, m
H-2β	1.45, m	2.49, m	1.58, m	1.59, m	1.83, m	1.90, m
H-3α	1.40, m		1.40, m	3.22, dd (11.8, 5.5)	3.76, dd (12.0, 4.8)	3.34, dd (11.5, 4.4)
H-3β	1.18, m		1.18, m			
H-5α	1.04, dd (12.7, 2.6)	1.62, dd (12.6, 2.9)	1.62, m	0.97, dd (10.4, 2.8)	1.64, m	0.84, dd (12.1, 3.0)
H-6α	1.32, m	1.85, m	1.86, m	1.33, m	1.99, m	2.00, m
H-6β	1.28, m	1.80, m	1.82, m	1.38, m	1.83, m	1.82, m
H-7α	2.06, m	2.15, m	1.68, m	1.42, m	1.92, m	2.26, m
H-7β	2.06, m	2.06, m	1.65, m	1.16, m	1.60, m	1.86, m
H-9α	1.95, dd (14.4, 6.5)	1.98, dd (14.7, 6.5)	1.96, dd (13.8, 7.3)	1.85, dd (13.9, 7.3)	2.51, m	2.62, m
H-11α	2.38, dd (16.4, 6.6)	2.44, dd (16.6, 6.9)	2.57, dd (14.8, 6.2)	2.37, dd (16.2, 6.5)	2.04, m	1.98, m
H-11β	2.21, dd (16.4, 6.7)	2.28, dd (16.6, 6.9)	2.54, dd (14.8, 6.2)	2.18, dd (16.2, 6.6)	2.01, m	1.90, m
H-13	1.32, s	1.35, s	1.31, s	1.29, s	1.30, s	1.27, s
H-14	0.90, s	1.10, s	0.89, s	0.97, s	1.02, s	0.95, s
H-15	0.87, s	1.03, s	0.85, s	0.86, s	0.89, s	0.89, s
H-16	0.82, s	1.00, s	0.80, s	0.77, s	0.79, s	0.76, s

Table 2. ^{13}C-NMR Assignments of Compounds **5** and **6** in $CDCl_3$

carbon	multiplicity	5 δ (ppm)	6 δ (ppm)
1	CH	71.7	75.9
2	CH_2	34.7	37.0
3	CH	72.5	76.4
4	−C−	39.6	41.5
5	CH	47.1	52.7
6	CH_2	20.7	19.9
7	CH_2	38.1	38.4
8	−C−	86.5	85.9
9	CH	58.5	58.9
10	−C−	36.4	38.4
11	CH_2	28.5	28.6
12	−C−	176.4	177.4
13	CH_3	27.7	27.9
14	CH_3	21.3	21.5
15	CH_3	15.7	15.1
16	CH_3	14.8	11.2

(H-3) with the tertiary carbon resonating at δ 52.7 (C-5) and the lack of any coupling between the proton resonating at δ 3.34 with carbons at δ 38.4 (C-10) or at 85.9 (C-8). The 1β- and 3β-positions of the new hydroxyl groups were further confirmed from the HOHAHA spectrum recorded at 100 ms, which exhibited correlations between protons resonating at δ 3.25 and 3.34.

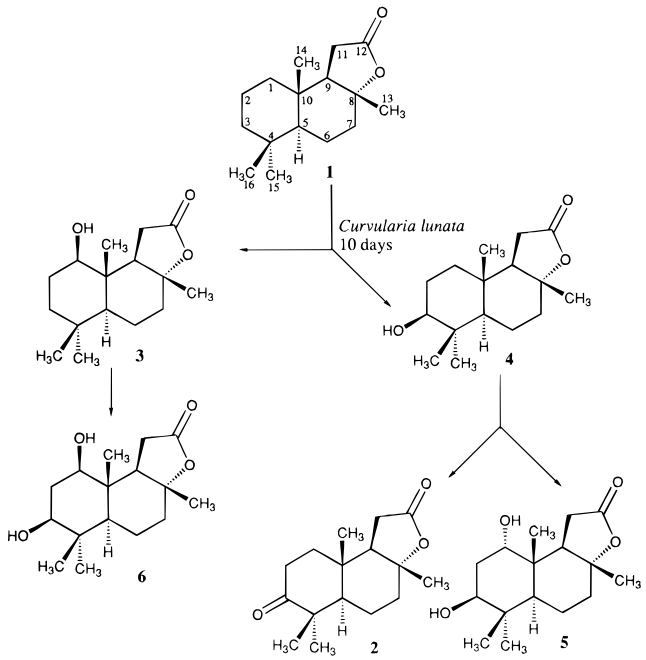
A time-dependent study of the formation of the metabolites was carried out in which one flask was harvested daily. The metabolites were examined by TLC, which showed that the microbial hydroxylation of sclareolide (**1**) initially resulted in the formation of 1β-hydroxysclareolide (**3**) and 3β-hydroxysclareolide (**4**). 1β-Hydroxysclareolide (**3**) underwent further hydroxylation to give 1β,3β-dihydroxysclareolide (**6**), while 3β-hydroxysclareolide (**4**) was oxidized to afford 3-ketosclareolide (**2**) and 1α,3β-dihydroxysclareolide (**5**) (Scheme 1).

Four other fungal species were screened for the microbial transformation of sclareolide (**1**). All the five metabolites obtained with *Curvularia lunata* were also obtained with *Aspergillus niger*. Microbial transformation of **1** by *Gibberella fujikuroii* yielded **2**−**5**, while compounds **4** and **5** were only produced by *Fusarium lini*. No transformation of **1** was observed with *Pleurotus ostreatus*.

Experimental Section

General Experimental Procedures. The ^{1}H-NMR spectra were recorded on a Bruker AMX 500 spectrometer at 500 MHz, while the ^{13}C-NMR spectra were recorded on the same instrument at 125 MHz. The mass spectra were recorded on a Varian MAT 112S mass spectrometer. HREIMS were recorded on a JEOL-JMS HX 110 mass spectrometer. The IR spectra were recorded on a JASCO IRA-1 IR spectrophotometer, and UV spectra were recorded on a Pye Unicam PU 8700 UV−vis spectrometer. A Polatronic D polarimeter was used for measuring the optical rotations. The melting points were taken on a Buchi 535 melting point apparatus and are uncorrected. The purity of the samples was checked on TLC (Si gel G_{254} precoated plates), and flash chromatography on Si gel was used in order to obtain the pure metabolites.

Scheme 1 Microbial Transformation of Sclareolide (**1**) Using *Curvularia lunata*



Preparation of Fermentation Media. *Aspergillus niger*. The medium for *Aspergillus niger* (ATCC 10549) was prepared by mixing the following ingredients in distilled H_2O (2 L): tartaric acid (70.0 g), sucrose (10.0 g), KH_2PO_4 (4.0 g), $MgSO_4 \cdot 2H_2O$ (2.0 g), NH_4NO_3 (4.0 g), Zn $(OAc)_2$ (0.06 g); 0.04 N NaOH was used to adjust the pH to 6.0.

Curvularia lunata, Fusarium lini, Pleurotus ostreatus,* and *Gibberella fujikuroi. The medium for *Curvularia lunata* (NRRL 2178), *Fusarium lini* (NRRL 68751), and *Pleurotus ostreatus* (NRRL 4590) was prepared by mixing the following ingredients into distilled H_2O (4.0 Ls): glucose (40.0 g), glycerol (40.0 g), peptone (20.0 g), yeast extract (20.0 g), KH_2PO_4 (20.0 g), and NaCl (20.0 g).

Substrate Incubation. The fungi were grown by shake culture (100 mL medium) in 250-mL conical flasks and incubated for 2 days on a shake table at 29 °C. Sclareolide (**1**) (1.0 g) was dissolved in acetone (20 mL), and the resulting solution was evenly distributed among 40 conical flasks having well-established shake cultures, and the fermentation was carried for 10 days more. The mycelium was filtered and washed with EtOAc (1 L), and the broth obtained was extracted with EtOAc (12 L).

Extraction and Isolation. The EtOAc extract was dried over anhydrous Na_2SO_4 and concentrated *in vacuo* to afford a brown gum (3 g) that was adsorbed on an equal quantity of Si gel and subjected to chromatography on a column containing 30 g of Si gel. Elution with 10% EtOAc−90% petroleum ether (40−60 °C) afforded the starting material (**1**) (64 mg). Elution with 20% EtOAc−80% petroleum ether (1 L) afforded a white crystalline solid identified as 3-ketosclareolide (**2**) (87 mg): mp 183−184 °C (lit.[7] mp 184−186 °C); ^{1}H-NMR ($CDCl_3$, 500 MHz), see Table 1. Elution with 30% EtOAc−70% petroleum ether (1 L) afforded a white crystalline solid identified as 1β-hydroxysclareolide (**3**) (93 mg): mp 192−193 °C. (lit.[7], 191−193 °C); ^{1}H-NMR ($CDCl_3$, 500 MHz), see Table 1. Elution with 50% EtOAc−50% petroleum ether (1 L) gave 3β-hydroxysclareolide (**4**) (122 mg): mp 178 °C (lit.[7], 173−175 °C); ^{1}H-NMR ($CDCl_3$, 500 MHz), see Table 1.

Elution with 75% EtOAc−25% petroleum ether afforded 1α,3β-dihydroxysclareolide (**5**) (160 mg) as white needles: mp 245−245 °C; $[\alpha]^{25}_D$ +66° (*c* 0.5, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3550, 3380, 2950, 1755 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 219 (2.5); HREIMS, obsd *m*/*z* 282.1871, $C_{16}H_{26}O_4$, calcd *m*/*z* 282.1831; FAB positive *m*/*z* 283; EIMS *m*/*z* 267 (3) $[M - 15]^+$, 249 (4), 231 (5), 193 (8), 167 (18), 151 (12), 133 (10), 107 (27), 81 (32), 69 (34), 55 (100%); ^{1}H-NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 125 MHz), see Table 2. Elution with pure EtOAc (1 L) yielded a white crystalline powder identified as 1β,3β-dihydroxysclareolide (**6**) (70 mg): mp 233−234 °C, $[\alpha]^{25}_D$ −75° (*c* 0.5, MeOH); IR ($CHCl_3$)ν_{max} 3420, 3350, 2925,1760 cm^{-1}; UV (MeOH) λ_{max} (log ϵ): 234 (3.2); HREIMS, obsd *m*/*z* 282.1875, $C_{16}H_{26}O_4$, calcd *m*/*z* 282.1831; FAB positive *m*/*z* 283; EIMS *m*/*z* 267 (19) $[M - 15]^+$, 249 (24), 207 (5), 193 (13), 177 (6), 151 (15), 133 (13), 119 (6), 105 (4), 91 (4), 79 (6), 77 (3), 67 (9), 55 (100); ^{1}H-NMR ($CDCl_3$, 500 MHz), see Table 1; ^{13}C-NMR ($CDCl_3$, 125 MHz), see Table 2.

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