Novel Microbial Transformations of Sclareolide

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Received July 12, 2006; accepted October 13, 2006

Fungal catalysis of sclareolide (1) using *Mucor plumbeus* (ATCC 4740), *Cunninghamella blakesleeana* (ATCC 9245), *Cunninghamella echinulata* (ATCC 9244), *Curvularia lunata* (ATCC 12017) and *Aspergillus niger* (ATCC 1004), was performed. *Cunninghamella blakesleeana* (ATCC 9245) metabolized compound 1 to afford O^6-sclareolide (2), 3β,6α-dihydroxysclareolide (3), 9-hydroxysclareolide (4), along with three known metabolites, 1β,3β-dihydroxysclareolide (5), 3-oxosclareolide (6) and 3β-hydroxysclareolide (7). Biotransformation experiments of compound 1 with *Cunninghamella echinulata* (ATCC 9244) also yielded two new compounds, 5-hydroxysclareolide (8), and 7β-hydroxysclareolide (9) along with two known compounds 5 and 7. Spectroscopic methods were used to establish the structures of compounds 2—9. Compounds 2—9 exhibited modest acetylcholinesterase inhibitory activity.

Key words O^6-sclareolide; 9-hydroxysclareolide; 5-hydroxysclareolide; 3β,6α-dihydroxysclareolide; 7β-hydroxysclareolide

Microorganisms such as bacteria and fungi are capable to perform regio- and stereo-selective reactions on organic compounds. The different possible microbial reactions include oxidation, reduction, and degradative reactions.[1)] Many of these reactions allow the production of new compounds that would be extremely difficult to synthesize chemically.[2)] For instance hydroxylation at inactivated methylene is a very common microbial reaction.[3)] The results obtained from microbial transformations are quite often similar to those obtained from mammal biotransformations.[4)] The parallel relationship between mammals and microbial transformations is due to the fact that many fungi, such as the filamentous fungus *Cunninghamella*, utilize the enzyme cytochrome P-450 monoxygenase in the metabolism of xenobiotics.[5)] This enzyme system is analogous to the enzyme system used by mammals, allowing microbial transformations to be used successfully as *in vitro* models for mammalian drug metabolism.[6)] A lead bioactive compounds can also be generated through microbial transformations of natural products. The metabolic products often have improved bioactivity with minimal toxicity compared to the parent natural product.[7)]

In our continuing effort to discover new bioactive natural products, we decided to do biotransformation studies of sclareolide (**1**) using whole cell cultures of different fungi. Sclareolide (**1**) is a minor constituent of the vascular plant *Arnica angustifolia* and is commercially available.[8)] Compound **1** has shown modest cytotoxcity against breast (MCF-7), colon (CKCO-1), lung (H-1299) and skin (HT-144) human cancer cell lines.[8)] For this project, we screened five different fungi, namely *Mucor plumbeus* (ATCC 4740), *Cunninghamella blakesleeana* (ATCC 9245), *Cunninghamella echinulata* (ATCC 9244), *Curvularia lunata* (ATCC 12017) and *Aspergillus niger* (ATCC 1004), for their capability to metabolize compound **1**. During these biotransformation experiments, we discovered that *Cunninghamella blakesleeana* (ATCC 9245) metabolized compound **1** into O^6-sclareolide (**2**), 3β,6α-dihydroxysclareolide (**3**) and 9-hydroxysclareolide (**4**), along with three known metabolites, 1β,3β-dihydroxysclareolide (**5**), 3-oxosclareolide (**6**) and 3β-hydroxysclareolide (**7**). Biotransformation experiments of compound **1** with *Cunninghamella echinulata* (ATCC 9244) also yielded two new compounds, 5-hydroxysclareolide (**8**), and 7β-hydroxysclareolide (**9**) along with two known compounds **5** and **7**. NMR spectroscopic methods were used to establish the structures of compounds **2**—**9**. Compounds **2**—**9** were found to exhibit modest acetylcholinesterase inhibitory activity. To the best of our knowledge, biotransformation of **1** into **2** is a novel ether-forming microbial reaction. In this paper we report the biotransformations of **1** and structure elucidation of compounds **2**—**9** as well as their bioactivity data.

Results and Discussion

Our first biotransformed product, O^6-sclareolide was obtained as a colorless gum. Its UV spectrum showed terminal absorption indicating the lack of a conjugated π system. The

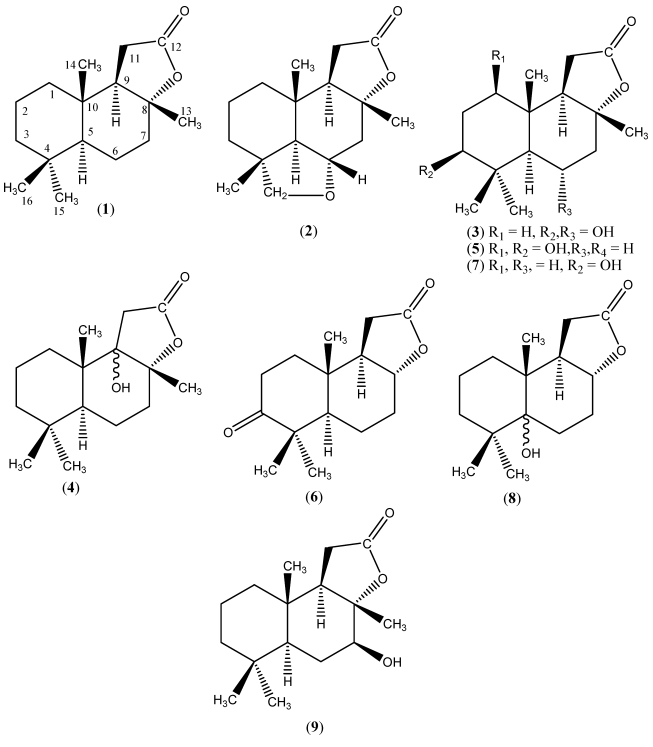


Fig. 1. Structures of Compounds **1**—**9**

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IR spectrum displayed intense absorption bands at 2918 (CH), 1755 (C=O) and 1112 (C–O) cm^{-1}. The high-resolution electron-impact mass spectrum (HR-EI-MS) of **2** showed molecular ion peak at *m/z* 264.1722 corresponding to the molecular formula $C_{16}H_{24}O_3$ (Calcd 264.1725). This suggested the presence of five degrees of unsaturation in compound **2**.

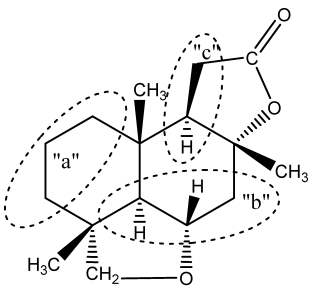
The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of **2** showed the resonance of three three-proton singlets at δ 1.30, 0.92 and 0.88 due the C-13, C-14 and C-16 methyl protons, respectively. Two AB doublets at δ 3.65 and 3.71 (J=10.5 Hz) were assigned to the C-15 methylene protons. The C-6 methine proton appeared as a doublet of double doublets at δ 3.75 (J_1=12.0 Hz, J_2=11.0 Hz, J_3=3.5 Hz). The downfield chemical shift values of C-15 methylene and C-6 methine protons indicated the presence of geminal oxygen functionality. A combination of COSY-45° and TOCSY spectra showed the presence of three isolated spin systems in compound **2**. The first spin system "a" was traced from the C-6 methine proton (δ 3.75), which showed cross-peaks with the C-5 methine (δ 2.13) and C-7 methylene (δ 1.92, 1.54) protons. The second spin system "b" started from the C-1 methylene proton (δ 1.82, 1.42) and showed cross-peaks with the C-2 methylene protons (δ 2.15, 1.61). The latter exhibited vicinal couplings with the C-3 methylene protons (δ 1.88, 1.16). The third spin system "c" was traced from the C-9 methine proton (δ 1.98), which exhibited vicinal couplings with the C-11 methylene protons (δ 2.42, 1.80). The spin systems "a—c" are shown in Fig. 2.

The ^{13}C-NMR spectrum ($CDCl_3$, 125 MHz) of **2** showed the resonance of all sixteen carbons in compound **2**. The DEPT spectrum was also recorded to establish the multiplicity of each carbon signal and it revealed the presence of three methyl, six methylene, three methine carbons in compound **2**. Subtraction of the DEPT spectrum from the broadband ^{13}C-NMR spectrum indicated the presence of four quaternary carbon atoms. Two downfield aliphatic signals at δ 60.1 and 68.9 were due to the C-15 and C-6 carbons, respectively. Their low field resonances were due to the presence of geminal oxygen functionality. The C-12 resonated at δ 176.2. The HSQC spectrum was also recorded to determine ^{1}H/^{13}C one-bond shift correlations of compound **2**. Complete ^{13}C-NMR chemical shift assignments of **2** and ^{1}H/^{13}C one-bond shift correlations of compound **2**, as determined from HSQC spectrum are shown in Table 1.

The HMBC spectrum of **2** was used to assign ^{13}C-NMR chemical shift assignments of quaternary carbon atoms and to determine the position of substitution of different functional groups. The C-15 methylene protons (δ 3.71, 3.65) showed cross-peaks with the C-4 (δ 35.0), C-5 (δ 56.1) and C-6 (δ 68.9). The C-6 methine proton (δ 3.75) showed long-range hetero-nuclear couplings with the C-5 (δ 56.1) C-7 (δ 39.6), C-8 (δ 86.1), C-10 (δ 37.4) and C-15 (δ 60.1). These HMBC interactions indicated the presence of an ether bond between C-6 and C-15. The presence of an ether linkage between C-6 and C-15 was also inferred from the ^{1}H, ^{13}C and mass spectral data. The ^{1}H-NMR spectrum of this metabolite featured downfield resonance of C-6 methine (δ 3.75) and C-15 methylene (δ 3.71, 3.65) protons. The ^{13}C-NMR spectrum of **2** also showed low-field resonances of C-6 and C-15 at δ 68.9 and 60.1, respectively. These downfield chemical shift values suggested the presence of an oxygen functionality at C-6 and C-15, respectively. The HR-EI-MS provided the molecular formula $C_{16}H_{24}O_3$ suggesting the presence of three oxygen atoms. Two oxygen atoms were due to the lactone moiety and the third oxygen could only be present in the form of an ether bridge between C-6 and C-15. Additionally, the molecular formula suggested the presence of five double bond equivalents in compound **2**. Four of them were accounted for by the presence of ring A, B and a lactone substituted at C-8 and C-9 in this metabolite. By incorporating a tetrahydrofuran ring in structure **2** could only satisfy the remaining fifth degree of unsaturation of **2**. The presence of an ether bond between C-6 and C-15 was also confirmed by recording the ^{1}H-NMR spectrum of **2** in pyridine-d_5. The ^{1}H-NMR spectrum (C_5D_5N, 300 MHz) of **2** showed the resonance of C-6 methine proton at δ 3.79 while C-15 methylene protons appeared at δ 3.68 and 3.76. It has already been reported in the literature that a pronounced shift of *ca.* 0.2 ppm was observed in the case of protons adjacent to a hydroxyl group, when the ^{1}H-NMR spectrum was recorded in pyridine-d_5.[9] The chemical shift of H-6 and H_2-15 remains nearly unaffected in pyridine-d_5. This further suggested the presence of an ether linkage between C-6 and C-15. Important HMBC interactions of compound **2** are shown in Fig. 3.

The stereochemistry at various chiral centers was established with the aid of a NOESY spectrum and ^{1}H–^{1}H coupling constant data. The NOESY spectrum showed a *cis* relationship between H_3-13, H_3-14 and H_3-16. The C-13 methyl proton (δ 1.30) showed an NOE with the C-14 methyl protons (δ 0.92), which further showed spatial couplings with the C-16 methyl protons (δ 0.88). After making sure that C-13, C-14 and C-16 methyl group have β orientations, as reported in compound **1**,[8,10] the NOESY spectrum was used to establish stereochemistry at C-6. The C-6 methine proton (δ 3.75) showed NOE with the C-14 (δ 0.92) and C-16 (δ 0.88) methyl protons. This NOE data helped us to propose a β-orientation for H-6 and an α-orientation for C-6/O bond. This was further confirmed from the ^{1}H–^{1}H coupling constant data. H-6 resonated at δ 3.75 as a doublet of double doublets ($J_{6\beta,5\alpha}$=12.0 Hz, $J_{6\beta,7\alpha}$=11.0 Hz, $J_{6\beta,7\beta}$=3.5 Hz). The *trans* diaxial couplings between H-6β/H-5α (J=12.0 Hz) and H-6β/H-7α (J=11.0 Hz) further suggested the β-orientation of H-6. Based on these spectroscopic studies, structure **2** was assigned to this novel metabolite.

Our second metabolite, 3β,6α-dihydroxysclareolide (**3**), was isolated as a colorless gummy material from the liquid



(**2**)

Fig. 2. Spin Systems "a—c" of Compound **2** Obtained from COSY-45° and TOCSY Spectra

Table 1. ^{13}C-NMR Chemical Shift Assignments of Compounds and ^{1}H/^{13}C One-Bond Shift Correlations of **2**—**4** and **8**, **9**, as Determined from Their HSQC Spectra

Carbon No.	Compounds									
	2		**3**		**4**		**8**		**9**	
	^{1}H δ	^{13}C δ	^{1}H δ	^{13}C δ	^{1}H δ	^{13}C δ	^{1}H δ	^{13}C δ	^{1}H δ	^{13}C δ
1	1.82 (m) 1.42 (m)	48.2	1.81 (m) 1.40 (m)	48.1	1.84 (m) 1.45 (m)	47.9	1.82 (m) 1.43 (m)	48.5	1.79 (m) 1.44 (m)	48.2
2	2.15 (m) 1.61 (m)	26.8	2.17 (m) 1.59 (m)	27.0	2.12 (m) 1.63 (m)	26.9	2.16 (m) 1.60 (m)	26.9	2.13 (m) 1.62 (m)	26.6
3	1.88 (m) 1.16 (m)	51.4	3.33 (m) —	72.2	1.87 (m) 1.14 (m)	51.7	1.85 (m) 1.18 (m)	51.3	1.86 (m) 1.17 (m)	51.6
4	—	35.0	—	34.5	—	34.9	—	34.3	—	34.6
5	2.13 (d, J=12.0 Hz)	56.1	2.10 (m)	55.8	2.17 (m)	56.3	—	75.8	2.15 (m)	56.2
6	3.75 (ddd, J=12.0, 11.0, 3.5 Hz)	68.9	3.88 (ddd, J=9.7, 9.6, 3.3 Hz)	69.8	1.88 (m) 1.25 (m)	27.1	1.85 (m) 1.23 (m)	27.0	1.86 (m) 1.28 (m)	27.3
7	1.92 (m) 1.54 (m)	39.6	1.91 (m) 1.53 (m)	39.7	1.78 (m) 1.44 (m)	38.2	1.77 (m) 1.43 (m)	38.3	4.01 (dd, J=12.0, 3.5 Hz)	69.8
8	—	86.1	—	86.0	—	97.3	—	86.2	—	94.6
9	1.98 (m)	58.9	1.97 (m)	60.0	—	76.5	1.95 (m)	58.7	2.00 (m)	60.1
10	—	37.4	—	37.6	—	39.6	—	40.1	—	37.3
11	2.42 (m) 1.80 (m)	28.9	2.40 (m) 1.79 (m)	28.8	2.46 (m) 1.85 (m)	32.7	2.39 (m) 1.81 (m)	28.8	2.43 (m) 1.80 (m)	28.8
12	—	176.2	—	176.3	—	176.1	—	176.5	—	176.1
13	1.30 (s)	21.8	1.31 (s)	21.7	1.31 (s)	22.0	1.29 (s)	21.8	1.31 (s)	21.7
14	0.92 (s)	21.5	0.93 (s)	21.2	0.91 (s)	21.7	0.91 (s)	21.3	0.89 (s)	21.5
15	3.71 3.65 (d, J=10.5 Hz)	60.1 —	0.94 (s)	16.1 —	0.93 (s)	15.5 —	0.94 (s)	15.8 —	0.93 (s)	15.6
16	0.88 (s)	16.0	0.89 (s)	15.8	0.85 (s)	15.9	0.86 (s)	16.1	0.85 (s)	15.9

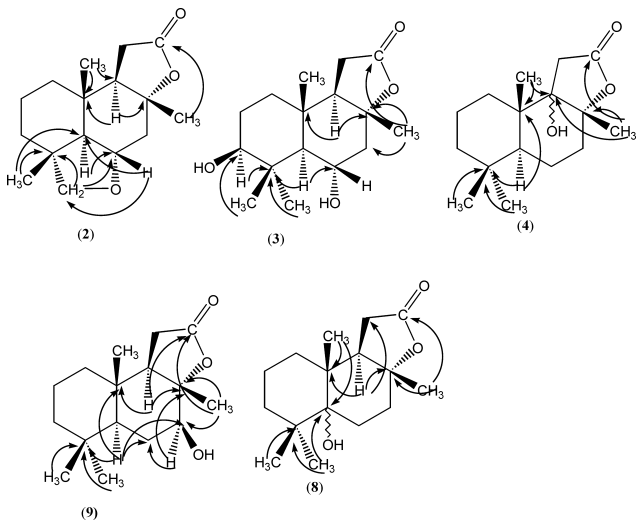


Fig. 3. Selected HMBC Interactions Observed in Compounds **2**—**4** and **8**, **9**

culture of *C. blakesleeana*. The UV spectrum of compound **3** was identical to that of compound **2** indicating the presence of similar chromophore. The IR spectrum showed the presence of intense absorption bands at 3423 (OH) and 1758 (C=O) cm^{-1}. The HR-EI-MS of **3** showed the molecular ion peak at m/z 282.1828 corresponding to the molecular formula $C_{16}H_{26}O_4$ (Calcd 282.1831) and indicated the presence of four degrees of unstaturation in compound **3**. This mass spectral data suggested that *C. blakesleeana* has performed hydroxylation reactions at two carbon atoms. The ^{1}H-NMR spectrum ($CDCl_3$, 300 MHz) of **3** showed two additional downfield signals at δ 3.88 (ddd, J_1=9.7 Hz, J_2=9.6 Hz, J_3=3.3 Hz) and 3.33 (m) further confirming the presence of two hydroxyl groups in this new metabolite. The multiplicity of these signals suggested that these hydroxyl groups might be substituted at C-3 and C-6. The presence of C-3/OH and C-6/OH were confirmed from the COSY-45° spectrum, which showed the vicinal couplings of C-3 methine proton (δ 3.33) with the C-2 methylene protons (δ 2.17, 1.59). The latter showed cross-peaks with the C-1 methylene protons (δ 1.81, 1.40). The C-6 methine proton (δ 3.88) exhibited COSY-45° interactions with the C-5 methine (δ 2.10) and C-7 methylene protons (δ 1.91, 1.53).

The ^{13}C-NMR spectrum ($CDCl_3$, 75 MHz) of **3** showed the resonance of all sixteen carbon atoms. Two downfield signals at δ 72.2 and 69.8 were due to the C-3 and C-6 carbon atoms, respectively. Their downfield chemical shift values were due to the presence of geminal hydroxyl groups. Complete ^{13}C-NMR chemical shift assignments of compound **3** and ^{1}H/^{13}C one-bond connectivity of all hydrogen-bearing carbon atoms of **3**, as determined from HSQC spectrum, are shown in Table 1. H-3 (δ 3.33) showed HMBC interactions with the C-4 (δ 34.5), C-15 (δ 16.1) and C-16 (δ 15.8). H-6 (δ 3.88) exhibited long-range heteronuclear couplings with the C-5 (δ 55.8) and C-7 (δ 39.7). These HMBC interactions further confirmed the presence of a hydroxyl group at C-3 and C-6, respectively. Important HMBC interactions of **3** are presented in Fig. 3. The NOESY spectrum was used to estab-

lish stereochemistry at C-3/OH and C-6/OH. H-3 (δ 3.33) showed NOE with the C-5 methine (δ 2.10) and C-15 methyl protons (δ 0.94). H-5 has been reported to have α-orientation in compound **1**.[8,10] This NOE data led us to assign α-stereochemistry for H-3 and β-stereochemistry for C-3/OH. H-6 exhibited NOE with H_3-13 (δ 1.31), and H_3-14 (δ 0.89). This NOE helped us to assign β-stereochemistry for H-6 and α-orientation for C-6/OH. These spectroscopic studies led us to establish structure **3** for this new metabolite.

9-Hydroxysclareolide (**4**) was purified as a light yellow colored gum from the fermentation flask of *C. blakesleeana*. The UV and IR spectra of compound **4** were identical to those of compound **3** indicating the presence of similar functional groups. The HR-EI-MS showed molecular ion peak at *m/z* 266.1879 and provided molecular formula $C_{16}H_{26}O_3$ (Cacld 266.1882) indicating the presence of four double bond equivalents in **4**. The ^{1}H-NMR spectrum ($CDCl_3$, 500 MHz) of compound **4** was similar that of compound **1** and it was hard to determine anything else about the biotransfromed structure **4** from the ^{1}H-NMR spectrum alone. A combination of ^{13}C and DEPT spectra suggested the occurrence of a hydroxyl group at a quaternary carbon atom. In compound **1**, there are two methine carbon atoms (C-5, C-9). This spectral data suggested the occurrence of a microbial hydroxylation either at C-5 or C-9. In the ^{13}C-NMR spectrum, two aliphatic downfield signals at δ 76.5 and 97.3 were observed. The signal at δ 97.3 was assigned to the C-8 carbon atom. The C-8 resonated at δ 86.7 in the parent sclareolide (**1**). This downfield resonance of C-8 in compound **4** suggested the presence of a hydroxyl group at C-9. The position of C-9 hydroxyl groups was further confirmed from the HMBC spectrum, in which C-11 methylene (δ 2.46, 1.85), C-13 (δ 1.31) and C-14 (δ 0.91) methyl protons showed cross-peaks with the C-9 (δ 76.5). This HMBC data favors the substitution of a hydroxyl group at C-9. The stereochemistry of all chiral centers in compound **4** was also found to be identical to that of compound **1**, except for C-9 hydroxyl group. Our attempts to develop good crystals of compound **4** were not successful and this led us to remain the stereochemistry of C-9/OH unsolved. This spectral data led us to characterize this new metabolite as 9-hydroxysclareolide (**4**).

From the incubation experiments of compound **1** with *C. blakesleeana*, we have also isolated three known metabolites, 1β,3β-dihydroxysclareolide (**5**), 3-oxosclareolide (**6**) and 3β-hydroxysclareolide (**7**). Previously, these compounds were obtained during the microbial transformation of compound **1** by using *Curvulria lunata*.[8,10] During our screening experiments, we also observed the formation of compounds **5**—**7** during the biocatalysis of **1** with *C. lunata*. The ^{1}H and ^{13}C and mass spectral data of compounds **5**—**7** were identical to those of 1β,3β-dihydroxysclareolide, 3-oxosclareolide and 3β-hydroxysclareolide, reported in the literature.[8] This led us to characterize compounds **5**—**7** as 1β,3β-dihydroxysclareolide (**5**), 3-oxosclareolide (**6**) and 3β-hydroxysclareolide (**7**), respectively.

Incubation of compound **1** with *C. echinulata* also yielded two new compounds, 5-hydroxysclareolide (**8**), and 7β-hydroxysclareolide (**9**) along with two known compounds, **5**—**7**. 5-Hydroxysclareolide (**8**) was isolated as colorless gum. Its UV, IR, ^{1}H, and mass spectral data were identical to those of compound **4** indicating that compound **8** had the same structure as that of **4**. The ^{13}C-NMR spectrum ($CDCl_3$, 75 MHz) of **8** was also nearly similar to that of compound **4** with the exception of resonance for C-8. The C-8 resonance remained unaffected in this ^{13}C-NMR spectrum and indicated that a hydroxylation reaction occurred at C-5 in this fermentation experiment. Two downfield signals at δ 86.2 and 75.8 were due to the C-8 and C-5 carbon atoms, respectively. The position of a hydroxyl group at C-5 was also confirmed from the HMBC spectrum, in which C-6 methylene (δ 1.85, 1.23) C-14 (δ 0.91), C-15 (δ 0.94) and C-16 (δ 0.86) methyl protons showed HMBC couplings with the C-5 (δ 75.8). A combination of ^{13}C and HMBC spectral data suggested the presence of a hydroxyl group at C-5. Complete ^{1}H- and ^{13}C-NMR spectral data of compound **8** is shown in Table 1. Important HMBC interactions of **8** are also presented in Fig. 3. The stereochemistry at all chiral centers in compound **8** was found to be same as reported for compound **1** with the exception of C-5. The stereochemistry at C-5 remains unsolved. Based on these spectroscopic studies, this metabolite was characterized as 5-hydroxyclareolide (**8**).

7β-Hydroxysclareolide (**9**) was isolated as colorless gum. Its UV and IR spectral data were identical to those of previously discussed compounds **3**—**8**. The mass spectrum showed the molecular ion peak at *m/z* 266.1884 ($C_{16}H_{26}O_3$, Cacld 266.1882). The ^{1}H-NMR spectrum ($CDCl_3$, 300 MHz) of **9** showed the resonance of C-7 methine proton at δ 4.01 which showed vicinal couplings with the C-6 methylene proton (δ 1.86, 1.28). The C-6 methylene protons in turn exhibited cross-peaks with the C-5 methine proton (δ 2.15). The ^{13}C-NMR spectrum also showed the presence of two downfield resonances at δ 94.6 and 69.8. These signals were attributed to the C-8 and C-7 carbon atoms, respectively. The downfield chemical shift value of C-8 again indicated to us that microbial hydroxylation had occurred at C-7. The position of hydroxyl group was further confirmed by recording HMBC spectrum of **9**. This spectrum showed the long-range heteronuclear couplings of H-5 (δ 2.15), H_2-6 (δ 1.86, 1.28), H-9 (δ 2.00) and H_3-13 (δ 1.31) with the C-7 (δ 69.8). A combination of ^{1}H, ^{13}C, COSY-45° and HMBC spectral data suggested the presence of a hydroxyl group at C-7. Complete ^{13}C-NMR chemical shift assignments and ^{1}H/^{13}C one-bond shift correlations of all hydrogen-hearing carbon atoms are presented in Table 1. The stereochemistry of C-7 hydroxyl group was established with the aid of a NOESY spectrum and ^{1}H–^{1}H coupling constant data. H-7 (δ 4.01) showed NOE with H-9 (δ 2.00) and H-5 (δ 2.15) suggesting a *cis* relationship between them. H-5 and H-9 have α-orientation in the parent compound and based on this we assumed α-stereochemistry for H-7 and β-orientation for C-7/OH. The ^{1}H–^{1}H coupling constant data of H-7 in the ^{1}H-NMR spectrum further supported this assumption. The C-7 methine proton resonated as a double doublet at δ 4.01 ($J_{6\beta,7\alpha}$=12.0 Hz, $J_{6\alpha,7\alpha}$=3.5 Hz). This *trans* diaxial coupling of H-7α/H-6β further suggested β-orientation of C-7 hydroxyl group. Based on these spectroscopic studies, this new metabolite was characterized as 7β-hydroxysclareolide (**9**). Additionally, during this fermentation experiment, two known metabolites **5** and **7** were also obtained.

Bioactivity of Compounds 1—9 Compounds **1**—**9** were screened against breast (MCF-7), colon (CKCO-1), lung (H-1299) and skin (HT-144) human cancer cell lines using MTT

assay[11] and found to exhibit modest cytotoxcity against the aforementioned cell lines. For breast cancer cell lines (MCF-7), the IC_{50} values of compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9** were found to be 48, 43, 35, 46, 28, 45, 55, 67 and 42 μM, respectively. Compounds **1**, **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9** exhibited cytotoxcity against lung cancer cell lines (H-1299) with IC_{50} values of 49, 55, 54, 44, 68, 34, 46, 39 and 24 μM, respectively. The extent of the bioactivity of biotransformed compounds (**2**—**9**) was found to be nearly the same as that of compound **1**. This indicated that substitution of oxygen in the form of a hydroxyl group or ether moiety does not have any role in this particular bioactivity.

Compounds **1**—**9** were also evaluated for acetylcholinesterase inhibitory activity. Compounds **2**, **3**, **4**, **5**, **6**, **7**, **8** and **9** exhibited modest acetylcholinesterase inhibition activity with IC_{50} values (inhibition of enzyme activity by 50%) of 60, 80, 45, 82, 38, 98, 82 and 42 μM, respectively. Compound **1** was found to be inactive in this bioassay. The moderate bioactivity of **3**—**9** may be due to the presence of hydroxyl group(s) at different carbon atoms. The bioactivity of compound **2** is hypothesized due to the presence of a tetrahydrofuran ring incorporated in its structure.

In summary, we have discovered two fungi, *Cunninghamella blakesleeana* (ATCC 9245) *Cunninghamella echinulata* (ATCC 9244) are capable of metabolizing sclareolide (**1**) to afford compounds **2**—**9**. The bioconversion of **1** in to **2** is a novel ether-forming microbial reaction. We are also planning to design an experiment to purify key enzyme(s) responsible for this reaction using microbial transformation as an assay.

Experimental

General Optical rotations were measured on a Hitachi Polatronic-D polarimeter. UV and IR spectra were recorded on Shimadzu UV-250 1 PC and Bomem Hartmann and Braun (MB Series) spectrophotometers, respectively. The ^{1}H-, ^{13}C-NMR, ^{1}H–^{1}H COSY, HSQC, HMBC and NOESY spectra were recorded on a Bruker Avance 300 or 500 spectrometer; chemical shifts are in ppm (δ) relative to $SiMe_4$ as internal standard, coupling constants J are in Hz. EI/CI and HR-EI-MS were measured on Hewlett Packard 5989B. Column chromatography was carried out on silica gel (200—400 mesh). Thin-layer chromatography was performed on Merck silica gel GF_{245} pre-coated plates. All chemical used in this research were purchased from Sigma Aldrich.

Microorganisms Fungi: *Mucor plumbeus* (ATCC 4740), *Cunninghamella blakesleeana* (ATCC 9245), *Cunninghamella echinulata* (ATCC 9244), *Curvularia lunata* (ATCC 12017) and *Aspergillus niger* (ATCC 1004) were purchased from ATCC and maintained on Potato Dextrose Agar and stored in a refrigerator at 4 °C.

Preparation of Media The soy broth medium was prepared by mixing the following ingredients in 1 l of distilled water:

Dextrose (20 g), yeast (5 g), sodium chloride (5 g), potassium hydrogen phosphate (5 g) and soy flour (5 g). The pH was adjusted to 7.0 before autoclaving.

Incubation Experiment Five fungi, *Mucor plumbeus* (ATCC 4740), *Cunninghamella blakesleeana* (ATCC 9245), *Cunninghamella echinulata* (ATCC 9244), *Curvularia lunata* (ATCC 12017) and *Aspergillus niger* (ATCC 1004) were screened for their capability to metabolize sclareolide (**1**) at an analytical scale. During these analytical experiments, we discovered the biotransformations of compound **1** into compounds **2**—**7** by using *C. blakesleeana* as a biocatalyst and *C. echinulata* metabolized compound **1** to afford two new compounds **8** and **9** along with two known metabolites **5** and **7**. Biotransformation experiments with these fungi were carried out on a preparative scale. Both analytical and preparative fermentation experiments were conducted by using standard two-stage fermentation experiments.[12—14] For the preparation of stage I liquid culture, ten 250 ml flasks (each containing 50 ml of liquid culture media) were inoculated with microorganisms to be screened and incubated on a shaker for 24 h. Stage II cultures was prepared by adding sclareolide (**1**) (500 mg) as a solution in ethanol having a concentration of 0.33 mg/ml to each flask having five different microorganisms. These fermentation experiments were also monitored by including two controls, a "culture control" and a "substrate control" to eliminate the possibility that metabolic products are not microbial secondary metabolites and that the culture media has not done any chemical transformation on the substrate. The culture control contained only fermentation blanks in which the microorganisms were grown under identical conditions but without substrates, while the substrate control consisted of the substrate in sterile liquid media. All these flasks were again placed on a shaker at room temperature. After 14 d, these culture media were extracted with ethyl acetate and the crude extracts concentrated under reduced pressure and analyzed by TLC.

The crude ethyl acetate extracts obtained from the liquid culture of *C. blakesleeana* was subjected to silica gel column chromatography using gradient elution with hexane–ethyl acetate (0—100%) and ethyl acetate–methanol (0—100%) to purify compounds **1** (42 mg), **2** (123 mg, 24.6% yield), **3** (65 mg, 13% yield) , **4** (10 mg, 2% yield), **5** (80 mg, 16% yield), **6** (140 mg, 28% yield), and **7** (40 mg, 8%). The crude ethyl acetate extract extracted from the culture broth of *C. echinulata* was subjected to preparative TLC using ethyl acetate–hexane (9 : 1) as mobile phase to purify compounds **1** (186 mg), **8** (102 mg, 20.4% yield), **9** (100 mg, 20% yield), **5** (25 mg, 5% yield) and **7** (87 mg, 17.4% yield).

O^6-Sclareolide (**2**): 123 mg. Colorless gum. $[\alpha]_D^{25}$ −18° (c=1.8, $CHCl_3$). UV λ_{max} (MeOH): 226 nm. IR ν_{max} (KBr): 2918 (CH), 1755 (C=O), 1112 (C–O) cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: see Table 1. ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: see Table 1. HR-EI-MS m/z: 264.1722 (M^+, $C_{16}H_{24}O_3$, Calcd 264.1725). CI-MS: 263 (M^+−H). EI-MS m/z (rel. int.): 264 (10), 249 (13), 207 (5), 55 (100).

3β,6α-Dihydroxysclareolide (**3**): 65 mg. Light yellow colored gum. $[\alpha]_D^{25}$ −35° (c=0.39, $CHCl_3$). UV λ_{max} (MeOH): 226 nm. IR ν_{max} (KBr): 3423 (OH), 2910 (CH), 1758 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: see Table 1. ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: see Table 1. HR-EI-MS m/z: 282.1828 (M^+, $C_{16}H_{26}O_4$, Calcd 282.1831). CI-MS: 283 (M^++H). EI-MS m/z (rel. int.): 267 (15), 249 (20), 207 (8), 55 (100).

9-Hydroxysclareolide (**4**): 10 mg. Colorless gum. $[\alpha]_D^{25}$ −80° (c=0.6, $CHCl_3$). UV λ_{max} (MeOH): 224 nm. IR ν_{max} (KBr): 3428 (OH), 2906 (CH), 1756 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$, 500 MHz) δ: see Table 1. ^{13}C-NMR ($CDCl_3$, 125 MHz) δ: see Table 1. HR-EI-MS m/z: 266.1879 (M^+, $C_{16}H_{26}O_3$, Calcd 266.1882). CI-MS: 267 (M^++H). EI-MS m/z (rel. int.): 266 (8), 251 (15), 207 (11), 55 (100).

5-Hydroxysclareolide (**8**): 12 mg. Colorless gum. $[\alpha]_D^{25}$ −45° (c=0.5, $CHCl_3$). UV λ_{max} (MeOH): 225 nm. IR ν_{max} (KBr): 3431 (OH), 2900 (CH), 1755 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: see Table 1. ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: see Table 1. HR-EI-MS m/z: 266.1885 (M^+, $C_{16}H_{26}O_3$, Calcd 266.1882). CI-MS: 267 (M^++H). EI-MS m/z (rel. int.): 266 (6), 251 (13), 207 (7), 55 (100).

7β-Hydroxysclareolide (**9**): 10 mg. Colorless gum. $[\alpha]_D^{25}$ −64° (c=0.23, $CHCl_3$). UV λ_{max} (MeOH): 226 nm. IR ν_{max} (KBr): 3425 (OH), 2907 (CH), 1757 (C=O) cm^{-1}. ^{1}H-NMR ($CDCl_3$, 300 MHz) δ: see Table 1. ^{13}C-NMR ($CDCl_3$, 75 MHz) δ: see Table 1. HR-EI-MS m/z: 266.1884 (M^+, $C_{16}H_{26}O_3$, Calcd 266.1882). CI-MS: 267 (M^++H). EI-MS m/z (rel. int.): 266 (5), 251 (8), 207 (10), 55 (100).

Acetylcholinesterase (AChE) Inhibitory Activity For this bioassay, AChE (0.04 U/ml) and acetylthiocholine iodide (ATC) (75 mM) were prepared by dissolving in 0.1 M phosphate buffer (pH 8). 5,5-Dithiobis(2-nitrobenzoic acid) (DTNB) was made up in 10 ml of 0.1 M phosphate buffer (pH 7.0) containing 15 mg of $NaHCO_3$. Compounds **1**—**9** were dissolved in isopropanol and the concentration of sample solution was 5% (v/v). A control of similar concentration was also prepared.

The colorimetric method was used to determine the inhibition of AChE.[15] A solution of test compounds (**1**—**9**) (50 μl) and AChE (0.5 ml) were mixed in a test tube, and the tube was set on the incubator at 25 °C. DTNB (100 μl) and buffer (2.4 ml) were added to the test tube. The tube was incubated at 25 °C for 5 min as pre-incubation. The addition of ATC (40 μl) initiated the reaction and the mixture was then again incubated at 25 °C for 20 min. The absorbance at 412 nm was measured spectrophotometerically and all test and control (without sample) assays were corrected by blanks for nonenzymic hydrolysis. This assay was performed in triplicate.

Acknowledgements Natural Sciences and Engineering Research Council of Canada (NSERC) and Research Corporation, Tucson, AZ, U.S.A. provided financial support for this project and authors gratefully acknowledge this funding. We are also thankful to Dr. Kirk Marat, Department of Chemistry, The University of Manitoba, for his help in running two-dimensional

NMR experiments.

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