Sesquiterpenes from the Mycelial Cultures of *Dichomitus squalens*

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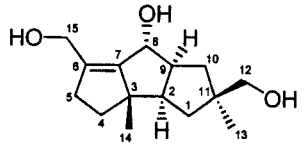
Three new sesquiterpenes including a rearranged hirsutane, dichomitol (**1**), an aromadendrane, 2β,13-dihydroxyledol (**2**), and a 1,10-seco-2,3-seco-aromadendrane, dichomitone (**3**), were isolated from mycelial solid cultures of *Dichomitus squalens*. Their structures were elucidated by spectroscopic methods, and their nematicidal activities against *Bursaphelenchus xylophilus* were assessed.

Dichomitus squalens is a commonly found white-rot Basidiomycete fungus. Its ligninolytic properties and laccases have been widely investigated.[1−4] During an ongoing screening for biologically active Basidiomycetes growing in South China, we have found that the mycelial cultures of this fungus possess potent nematicidal activity against *Bursaphelenchus xylophilus*. This prompted us to investigate its secondary metabolites. Three new sesquiterpenes, dichomitol (**1**), 2β,13-dihydroxyledol (**2**), and dichomitone (**3**), were isolated. Their structures were established by spectroscopic means. This paper reports the isolation, structure elucidation, and nematicidal activities of these new compounds.

The fungus was isolated from tissue culture of the fruiting bodies of *D. squalens* collected in Dinghu Mountain, Guangdong, China. The mycelia were grown on solid cultures for 13 days at 28 °C. The EtOH extract of the mycelial cultures was fractionated, and the $CHCl_3$-soluble fraction was separated by silica gel and Sephadex LH-20 column chromatography to afford **1**−**3**.



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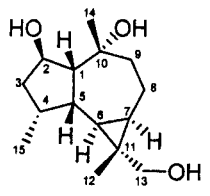

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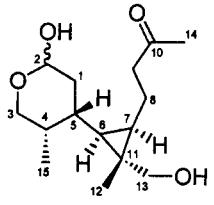

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Dichomitol (**1**) had the molecular formula $C_{15}H_{24}O_3$ by combined analysis of its HRTOFMS, ^{13}C NMR, and DEPT data. The IR spectrum exhibited absorptions at 3357 cm^{-1} for hydroxyl groups. The ^1H NMR spectrum (Table 1) exhibited signals for 21 nonexchangeable protons, including two singlets at δ 0.99 (3H, H-13) and 1.07 (3H, H-14) for two tertiary methyl groups, a broad doublet at δ 4.13 (1H, J = 7.5 Hz, H-8) for an oxygenated methine, and two broad doublets at δ 4.22 and 4.20 (each 1H, J = 12.5 Hz, H_2-15) and two doublets at δ 3.48 and 3.46 (each 1H, J = 11.0 Hz, H_2-12) for two oxygenated methylenes. The ^{13}C NMR (Table 1) and DEPT spectra indicated the presence of two methyl groups (δ 22.7, C-13; δ 20.3, C-14), six methylenes, two oxygenated (δ 72.1, C-12; δ 59.0, C-15), and three methines, one oxygenated (δ 74.3, C-8), as well as four quaternary carbons, of which two were olefinic (δ 145.8, C-7; δ 129.1, C-8). The COSY spectrum and HMQC experiment revealed partial structures shown by bold lines in Figure 1. Further connectivities were deduced from the HMBC spectrum (Figure 1). The HMBC correlations from H-12 and H-13 to C-1, C-10, and C-11 indicated connectivity of C-11 to C-1 and C-10, with C-13 and C-12 attached to C-11. The correlations from H-15 to C-5, C-6, and C-7 and from H-14 to C-2, C-3, C-4, and C-7 indicated connectivity of C-5 to C-6 and C-3 to C-2, C-7, and C-4 with C-14 attached to C-3. Thus, the gross structure of **1** was derived as shown in Figure 1. The relative configuration of **1** was determined from the NOESY spectrum (Table 1). The presence of mutual NOE correlations between H-13, H-2, and H-9, and between H-14 and H-8, and the absence of the correlations between H-14 and H-2 indicated that

Table 1. ^1H and ^{13}C NMR Data and NOESY Correlations for **1** in $CDCl_3$[a]

position	^1H	^{13}C	NOESY
1β	1.44 dd (12.0, 11.0)	36.0	1α, 10β, 12, 14
1α	1.35 ddd (12.0, 8.0, 1.5)		1β, 2, 12, 13
2	2.40 td (11.0, 8.0)	45.5	1α, 4α, 9, 13
3		45.9	
4β	1.85 m	36.1	4α, 5α, 5β, 14
4α	1.87 m		2, 4β, 5α
5β	2.78 m	25.1	4β, 5α, 14, 15
5α	2.64 m		4α, 4β, 5β, 15
6		129.1	
7		145.8	
8	4.13 br d (7.5)	74.3	9, 10β, 14
9	2.33 m	50.5	2, 8, 10α, 13
10β	1.73 br dd (11.0, 7.5)	40.8	1β, 8, 10α, 12
10α	1.27 t (11.0)		9, 10β, 12, 13
11		45.1	
12	3.48 d (11.0), 3.46 d (11.0)	72.1	1α, 1β, 10α, 10β, 13
13	0.99 s	22.7	1α, 2, 9, 10α, 12
14	1.07 s	20.3	1β, 4β, 5β, 8
15	4.22 br d (12.5), 4.20 br d (12.5)	59.0	5α, 5β

[a] Chemical shifts (δ) in ppm; coupling constants (parentheses) given in Hz.

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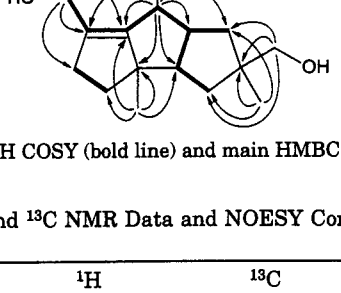

Figure 1. $^1H-^1H$ COSY (bold line) and main HMBC (arrow) correlations of **1**.

Table 2. ^1H and ^{13}C NMR Data and NOESY Correlations for **2** in $CDCl_3$[a]

position	^1H	^{13}C	NOESY
1	1.98 dd (8.8, 6.4)	64.0	4, 5, 14
2	4.34 td (8.8, 4.5)	73.6	3α, 6, 14
3β	1.70 m	42.0	3α, 4
3α	1.89 m		2,·3β, 4, 6, 15
4	2.32 m	35.2	1, 3α, 3β, 5, 15
5	2.08 dt (9.8, 6.4)	39.0	1, 4, 6, 8β, 12
6	0.36 t (9.8)	20.6	2, 3α, 5, 7, 13, 15
7	0.76 m	23.6	6, 8α, 8β, 13
8β	1.47 m	18.6	5, 7, 8α, 9β, 12
8α	1.73 m		7, 8β, 9α, 9β
9β	1.70 m	39.9	8α, 8β, 9α, 14
9α	1.83 m		8α, 9β, 14
10		74.4	
11		26.1	
12	1.09 s	11.3	5, 8β, 13
13	3.43 d (11.0), 3.25 d (11.0)	73.4	6, 7, 12
14	1.42 s	31.0	1, 2, 9α, 9β
15	0.93 d (7.0)	16.3	3α, 4, 6

[a] Chemical shifts (δ) in ppm; coupling constants (parentheses) given in Hz.

H-2, H-9, 8-OH, and 13-Me were at the same side and in α-orientations, while 14-Me and 12-CH_2OH were in β-orientations. In conclusion, the structure of **1** was established as depicted.

The structure of **1** is interesting because C-15 is attached to C-6 instead of C-4 in other hirsutane sesquiterpenes.[5] It is presumably derived from a ceratopicane derivative through a biogenetic Wagner–Meerwein rearrangement.[6] This is the first example to be assigned with such a hirsutane skeleton.

2β,13-Dihydroxyledol (**2**) was established as having a molecular formula of $C_{15}H_{26}O_3$ by its HRTOFMS, EIMS, and NMR (^1H, ^{13}C, and DEPT) data. The IR spectrum exhibited absorptions at 3365 cm^{-1} for hydroxyl groups. The ^1H NMR spectrum (Table 2) showed signals indicating the presence of two tertiary methyl groups at δ 1.09 and 1.42, a secondary methyl group at δ 0.93, as well as two doublets at δ 3.43 and 3.25 corresponding to two protons of an isolated oxygenated methylene, and a multiplet at δ 4.34 for an oxygenated methine proton. The ^{13}C NMR (Table 2) and DEPT spectra indicated three methyl groups, four methylenes, six methines, and two quaternary carbons, of which a methylene, a methine, and a quaternary carbon were oxygenated. From the $^1H-^1H$ COSY, HMQC, and HMBC spectra, an aromadendrane structure as shown in Figure 2 could be readily derived. The presence of the NOE correlations between H-1/H-5, H-1/H-4, H-1/H-14, H-2/H-14, H-2/H-6, H-3α/H-6, H-4/H-5, H-6/H-7, H-12/H-5, H-13/H-6, H-13/H-7, and H-15/H-6 in the NOESY spectrum (Table 1), as well as the proton coupling constants, $J_{1,5}$ = 6.4 Hz, $J_{4,5}$ = 6.4 Hz, $J_{5,6}$ = 9.8 Hz, and $J_{6,7}$ = 9.8 Hz, in the ^1H NMR spectrum indicated that the relative configuration of **2** was the same as that of ledol.[7] The β-orientation of 2-OH was derived from the NOESY correlations of H-2 with H-6 and H-3α and from the 8.8 Hz coupling constant between H-1 and H-2.[8] Compound **2** was thus elucidated as shown.

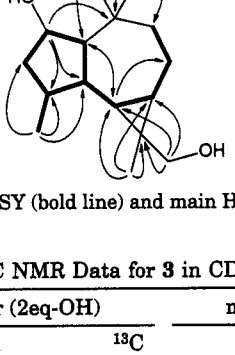

Figure 2. $^1H-^1H$ COSY (bold line) and main HMBC (arrow) correlations of **2**.

Table 3. ^1H and ^{13}C NMR Data for **3** in $CDCl_3$[a]

	major (2eq-OH)		minor (2ax-OH)	
position	^1H	^{13}C	^1H	^{13}C
1eq	1.64 m	35.0	1.48 m	32.6
1ax	1.47 m		1.73 m	
2	4.68 dd (9.0, 2.0)	96.3	5.22 br s	91.7
3ax	3.69 dd (11.5, 2.0)	71.3	4.10 dd (11.0, 2.5)	65.8
3eq	3.75 dd (11.5, 1.5)		3.37 dd (11.0, 2.5)	
4	1.59 m	31.2	1.68 m	31.9
5	1.55 m	33.1	1.90 m	27.7
6	0.56 t (10.0)	26.4	0.50 t (10.0)	26.2
7	0.60 m	22.8	0.60 m	22.9
8	1.76 m, 1.48 m	18.3	1.80 m, 1.52 m	18.4
9	2.51 m	44.1	2.51 m	44.1
10		209.0		209.4
11		24.5		24.4
12	1.08 s	10.9	1.10 s	11.0
13	3.20 d (11.0), 3.45 d (11.0)	73.4	3.17 d (11.0), 3.46 d (11.0)	73.6
14	2.17 s	30.0	2.17 s	29.9
15	1.04 d (7.0)	12.0	1.02 d (7.0)	11.4

[a] Chemical shifts (δ) in ppm; coupling constants (parentheses) given in Hz.

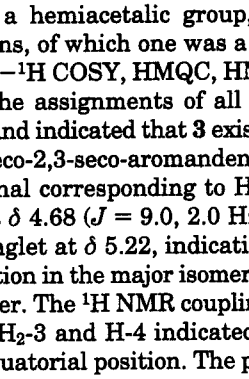

Figure 3. $^1H-^1H$ COSY (bold line) and main HMBC (arrow) correlations of **3**.

Dichomitone (**3**) had a molecular formula of $C_{15}H_{26}O_4$ by HRTOFMS. The IR spectrum indicated the presence of hydroxyl groups (3400 cm^{-1}) and a carbonyl group (1707 cm^{-1}). The ^1H and ^{13}C NMR spectra exhibited two sets of signals, with the signals of one set (set A) at slightly higher intensity and those of another set (set B) at lower intensity. Each set of signals indicated the presence of three methyl groups, five methylenes, two oxygenated, and four methines including a hemiacetalic group,[9] as well as two quaternary carbons, of which one was a carbonyl. Careful analysis of the $^1H-^1H$ COSY, HMQC, HMBC, and NOESY data facilitated the assignments of all ^1H and ^{13}C NMR signals (Table 3) and indicated that **3** existed as an isomeric mixture of 1,10-seco-2,3-seco-aromandendranes (Figure 3). The ^1H NMR signal corresponding to H-2 in set A was a double doublet at δ 4.68 (J = 9.0, 2.0 Hz), and that in set B was a broad singlet at δ 5.22, indicating that 2-OH was in equatorial position in the major isomer and axial position in the minor isomer. The ^1H NMR coupling constants (1.5–2.5 Hz) between H_2-3 and H-4 indicated that H-4 in both isomers was in equatorial position. The presence of mutual NOE interactions between H-2, H-3ax, and H-5 in set A and between H-3ax and H-5 in set B, and the absence of

interactions between H-15 and H-3ax, respectively, and H-5 in both sets in the NOESY spectrum indicated the axial position of H-5 and the chair form of the pyran ring in both isomers. The presence of the mutual NOE interactions between H-13, H-6, and H-7, as well as the 9.0 Hz coupling constant between H-6 and H-7 in both sets, indicated that H-6, H-7, and 13-CH_2OH were on the same side of the cyclopropane ring in both isomers.[7] The relative configuration of **3** was assumed to be as shown on the basis of co-occurrence with **2**. The NOESY data provided no information about its relative configuration. An equilibrium ratio of 5:4 between both isomers in $CHCl_3$ at room temperature was deduced from the NMR signal intensity of both isomers. Compound **3** is the first example of 1,10-seco-2,3-seco-aromandendrane sesquiterpenes.

In an assessment of nematicidal activity against *Bursaphelenchus xylophilus*, compound **2** exhibited potency with an LC_{50} of 35.6 μg/mL, while compounds **1** and **3** showed no activity.

Experimental Section

General Experimental Procedures. Optical rotations were obtained on a Perkin-Elmer 343 polarimeter with MeOH as solvent. The IR spectra were measured in KBr on a WQF-410 FT-IR spectrophotometer. The ^1H (500 MHz), ^{13}C (125 MHz), and 2D NMR spectra were recorded in $CDCl_3$ on a Bruker AV-500 instrument using TMS as an internal reference. HRTOFMS data were obtained on an API QSTAR mass spectrometer in negative-ion mode. EIMS were collected on a Micromass Platform EI 200 GC/MS instrument at 70 eV by direct inlet. For column chromatography, silica gel 60 (200–300 mesh, Qingdao Marine Chemical Ltd., Qingdao, China), Develosil ODS (10 μm, Nomura Chemical Co. Ltd., Japan), and Sephadex LH-20 were used. TLC was performed on precoated plates (Kieselgel 60GF_{254}, Merck) with detection effected by exposure to I_2 vapor and spraying with H_2SO_4 (10%) in EtOH followed by heating.

Producing Fungus. Fruiting bodies of *Dichomitus squalens* (Karst.) Reid were collected in Dinghu Mountain, Guangdong, China, in May 2002, and authenticated by one of the authors (T.L.). The mycelia were isolated from tissue plugs of a young fruiting body. A voucher specimen (DH0082) and mycelial culture (SC0197) are deposited in the culture collection of South China Botanical Garden, Chinese Academy of Sciences, Guangzhou, China. For maintenance on agar slants and submerged cultures, the fungus was grown on MEA medium.

Fermentation. The mycelia of *D. squalens* grown on MEA plates were used to inoculate 10 500 mL Erlenmeyer flasks containing 100 mL of YMG medium (glucose 0.4%, malt extract 1.0%, yeast extract 0.4%, pH 5.5). The flasks were incubated on a rotary shaker for 5 days at 28 °C with shaking at 120 rpm. The cultures were transferred into 10 5000 mL flasks containing 1000 mL of YMG medium and 500 g of wheat grains, and the cultivation was carried out in the stationary phase at 28 °C for 13 days.

Extraction and Isolation. The mycelial cultures of *D. squalens* were extracted with 95% EtOH three times at room temperature. The EtOH solution, after concentration in vacuo, was suspended in H_2O, and this aqueous suspension was sequentially extracted three times each with petroleum ether, $CHCl_3$, and EtOAc. The combined $CHCl_3$ solution, upon evaporation, yielded a deep brown syrup (2.5 g). This syrup was subjected to silica gel column chromatography, eluted with $CHCl_3$–MeOH mixtures of increasing polarities (98:2 to 80:20), to obtain six fractions (I–VI). Fraction IV, obtained on elution with $CHCl_3$–MeOH (92:8), was separated by silica gel column chromatography eluted with $CHCl_3$–MeOH (9:1) to afford subfractions IV-1 and IV-2. Subfraction IV-1 was further separated by reversed-phase C-18 column chromatography eluted with MeOH–H_2O (3:2) to obtain **1** (20 mg). Subfraction IV-2 was also applied to a reversed-phase C-18 column eluted with MeOH–H_2O (4:1) followed by purification on a Sephadex LH-20 column eluted with MeOH to afford **2** (11 mg). Fraction III, obtained on elution with $CHCl_3$–MeOH (95:5), was rechromatographed on a silica gel column eluted with petroleum ether–acetone (4:1) to give compound **3** (30 mg).

Dichomitin A (1): colorless oil, $[\alpha]^{20}_D$ +6.3° (*c* 0.168, MeOH); IR (KBr) ν_{max} 3357 and 1660 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), see Table 1; EIMS *m/z* 251 $[M - H]^+$ (2), 234 $[M - H_2O]^+$ (3), 233 (4), 232 (3), 217 (7), 203 (12), 187 (18), 159 (20), 105 (30), 91 (67), 79 (80), 55 (100); HRTOFMS *m/z* 251.1648 $[M - H]^-$ (calcd for $C_{15}H_{23}O_3$, 251.1647).

Dichomitin B (2): colorless oil, $[\alpha]^{20}_D$ −20.1° (*c* 0.035, MeOH); IR (KBr) ν_{max} 3365 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), see Table 2; EIMS *m/z* 236 $[M - H_2O]^+$ (2), 221 (4), 203 (10), 187 (8), 175 (22), 147 (34), 133 (35), 125 (62), 55 (100); HRTOFMS *m/z* 253.1804 $[M - H]^-$ (calcd for $C_{15}H_{25}O_3$, 253.1803).

Dichomitin C (3): colorless oil, $[\alpha]^{20}_D$ +14.3° (*c* 0.072, MeOH); IR (KBr) ν_{max} 3400 and 1707 cm^{-1}; ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), see Table 2; EIMS *m/z* 253 $[M - OH]^+$ (7), 242 (21), 235 (10), 221 (7), 217 (10), 177 (30), 159 (29), 97 (98), 57 (99), 55 (100); HRTOFMS *m/z* 269.1747 $[M - H]^-$ (calcd for $C_{15}H_{25}O_4$, 269.1752).

Nematicidal Activity. Nematicidal activity was determined in a microtiter plate assay as described previously.[10] The test organism was pine wood nematode (*Bursaphelenchus xylophilus*), which was supplied by College of Resources and Environment, South China Agriculture University, Guangzhou, China. The assay was performed in a 48-well microtiter plate. Each compound (2 mg) was dissolved in acetone (100 μL) and serially diluted with water to the concentrations of 2000, 200, and 20 μg/mL. Then 50 μL of each serial solution was added to the well containing 30–50 nematodes in 50 μL of water. Five percent of aqueous acetone was used as control. After incubation at 25 °C for 24 h in the dark, the numbers of live and dead nematodes were counted under a dissecting microscope. The average mortality of three replications at each concentration and control was calculated, and LC_{50}, which was defined as the concentration causing 50% mortality, was determined. The LC_{50} value for compound **2** was determined to be 35.6 μg/mL, and those for **1** and **3** were more than 1000 μg/mL.

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