The Antibacterial Activity of Compounds Isolated from Oakmoss against *Legionella pneumophila* and Other *Legionella* spp.

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Oakmoss is a natural fragrance ingredient exhibiting highly specific, potent antibacterial activity against *Legionella pneumophila*, a causative agent of severe water-bone pneumonia. In the present study, the antibacterial activity of individual compounds isolated from oakmoss was investigated against *L. pneumophila* and other *Legionella* spp. A total of 18 known compounds and two minor novel compounds (*i.e.*, 3-methoxy-5-methylphenyl-2,4-dihydroxy-6-methylbenzoate (compound 9) and 8-(2,4-dihydroxy-6-(2-oxoheptyl)-phenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one (compound 20)) were purified from oakmoss. The minimum inhibitory concentrations (MICs) against clinical and environmental isolates of *L. pneumophila*, *L. bozemanii*, *L. micdadei*, *L. longbeachae*, and *L. dumoffii* for 11 of the 20 compounds were less than 100 μg/mL (range 0.8–64.0 μg/mL). Novel compounds 9 and 20 exhibited potent antibacterial activity against *L. pneumophila* strains (MIC ranges of 1.3–8.0 μg/mL and 3.3–13.3 μg/mL, respectively) and also against four other *Legionella* species (MIC ranges of 0.8–8.0 μg/mL and 3.3–21.3 μg/mL, respectively). Time–kill assays indicated that compounds 9 and 20 kill bacteria at a concentration equivalent to 2×MIC after 1 h and 6 h co-incubations, respectively. While oakmoss and the purified components exhibited antibacterial activity against *Legionella* spp., they were not active against other Gram-negative and -positive bacteria such as *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Staphylococcus aureus*.

Key words oakmoss; antibacterial activity; *Legionella pneumophila*; *Legionella* spp.

Oakmoss is derived from the lichen *Evernia prunastri* (L.) Arch., which grows throughout central and southern Europe. Oakmoss and *E. prunastri* extracts are composed of a variety of carbonyl, phenolic, acidic and depside compounds.[1,2] Several oakmoss components, including usnic acid, atranol and atranorin, possess antimicrobial activity against various Gram-positive and Gram-negative bacteria.[3,4] However, to date there have been no reports concerning the antibacterial activity of these components against *Legionella pneumophila* strains. We previously demonstrated that 101 natural and synthetic fragrance ingredients exhibit antimicrobial activity against representative Gram-positive and Gram-negative bacteria, as well as various fungi.[5] We then investigated 41 of these ingredients for antibacterial activity against *L. pneumophila*. Birch tar oil and oakmoss, both of which are multicomponent ingredients used in natural fragrances, exhibited potent antibacterial activity against *L. pneumophila*[6]; however, the chemical structures of the antibacterial components have not been determined.

L. pneumophila is a Gram-negative bacterium that is widely distributed in natural water environments, including hot springs, as well as in artificial water systems such as air conditioning equipment, fountains, public baths and spas. Contamination of artificial water systems by this bacterium may lead to outbreaks of legionellosis, a severe form of pneumonia that is also referred to as Legionnaires' disease or non-pneumonic Pontiac fever. While disinfection of water supplied through artificial water systems is crucial to avoid outbreaks of legionellosis, the standard disinfection method, chlorination, has a number of significant drawbacks associated with the presence of chlorine gas. Specifically, these drawbacks include water discolouration, pH-induced damage to circulation facilities, alteration of aqueous metal ion levels and variation in the residual chlorine concentration in disinfected water. Consequently, the development of novel disinfectants for use in public water systems is considered a high priority, if not a necessity.

In the present study, we isolated and identified the components of oakmoss and investigated their antibacterial activity against clinical and environmental isolates of *L. pneumophila* and other *Legionella* spp. We also assessed the potential of the antibacterial components of oakmoss to serve as a new type of disinfectant for public water systems.

MATERIALS AND METHODS

Fragrance Ingredients and Antibiotics Absolute Mousse De Chene Selecta (OM, Charabot, Grasse, France), Oakmoss Absolute AT 086 (OMAT, H. Reynaud and Fils, Monterun Les Bains, France) and cinnamic aldehyde (CA) were provided by Ogawa and Co., Ltd. (Chiba, Japan) and stored at 4°C until use. Chlorhexidine gluconate (CHG, Wako Pure Chemical Ind., Ltd., Osaka, Japan) was used as a reference for testing the antibacterial activity of oakmoss components.

Bacterial Strains The following bacterial strains were used in the study: *L. pneumophila* JCM7571 (Philadelphia 1 clinical isolate), *L. pneumophila* JBCC005 (environmental isolate), *L. pneumophila* GTC00748 (serogroup 6), *L. bozemanii* GTC09140, *L. micdadei* IID3044, *L. longbeachae* IID3046 and *L. dumoffii* IID3047. *L. pneumophila* GTC00748 and *L. bozemanii* were provided by the Genetic Information Genetic Resource Center of Human Pathogens at Gifu University and *L. micdadei*, *L. longbeachae*, and *L. dumoffii* were purchased from the Pathogenic Microbes Repository Unit, Tokyo University. *Escherichia coli* JCM5491, *Pseudomonas aeruginosa* JCM6119, *Bacillus subtilis* NBRC3134 and *Staphylococcus*

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aureus JCM2413 were used to compare the antibacterial activity of each component.

Isolation and Structure Determination of OM and OMAT Compounds A total of 2.24 g of OM was dissolved in a small volume of MeCN–MeOH (10 : 1) and subjected to gel filtration chromatography using a Sephadex LH-20 column (30×500 mm, GE Healthcare Japan Co., Ltd., Tokyo, Japan) eluted with MeCN–MeOH (10 : 1) at a flow rate of 2 mL/min. The effluent was monitored at 280 nm using a spectrophotometric detector (SPD-2A, Shimadzu Co., Ltd., Kyoto, Japan) and separated into seven fractions. The fractions were concentrated to dryness, resulting in the following yields: fraction A (514 mg), fraction B (316 mg), fraction C (218 mg), fraction D (260 mg), fraction E (763 mg), fraction F (311 mg) and fraction G (117 mg). The fractions were subjected to further fractionation by solid-phase separation and/or HPLC. For solid-phase separation, either a Sep-pak octadecylsilica (ODS) cartridge (0.5 g, classic, Nihon Waters K. K., Tokyo, Japan) or a Bond Elut phenyl cartridge (0.5 g, Varian Technologies Japan Ltd., Tokyo, Japan) was used as the solid-phase adsorbent, and the adsorbed compounds were eluted with stepwise increases of MeOH in water (20, 40, 60, 80, 100%; 10 mL each). The effluents containing objective compounds were concentrated to dryness, dissolved in MeOH and subjected to HPLC using an LC-6A HPLC system (Shimadzu) equipped with a Capcell Pak C18 MG II column (10×250 mm, Shiseido Co., Ltd., Tokyo, Japan). The solvent (4 mL/min) varied from 25–80% MeCN in water depending on the samples applied, and the eluate was monitored at 280 nm using a spectrophotometric detector.

Fraction A obtained by LH-20 gel filtration chromatography was further fractionated by solid-phase separation on a Sep-pak ODS cartridge. The effluents produced using 20 and 40% MeOH in water were combined and subjected to HPLC using 25% MeCN in water to yield compound **2**. The effluents produced using 60, 80 and 100% MeOH in water were also combined and subjected to HPLC using 60% MeCN in water to yield compounds **11**, **14**, **15** and **16**.

Fraction B was fractionated by solid-phase separation on a Bond Elut phenyl cartridge. The effluent produced using 20% MeOH in water was subjected to HPLC using 35% MeCN in water to yield compounds **3**, **6** and **7**. The effluent produced using 40% MeOH in water was subjected to HPLC using 45% MeCN in water to yield compounds **6**, **7** and **8**. The effluents produced using 60 and 80% MeOH in water were combined and subjected to HPLC using 65% MeCN in water to yield compounds **10**, **11**, **13**, **15** and **17**.

Fraction C was fractionated by solid-phase separation on the Sep-pak ODS cartridge. The effluents produced using 20 and 40% MeOH in water were combined and subjected to HPLC using 38% MeCN in water to yield compounds **2**, **3**, **6** and **7**. The effluents produced using 60 and 80% MeOH in water were combined and subjected to HPLC using 45% MeCN in water to yield compounds **6**, **7**, **8** and **9**.

Fractions D, E and G were directly subjected to HPLC by stepwise elution with 35% MeCN in water for 35 min followed by 65% for 15 min (fraction D), 35% for 35 min followed by 75% for 25 min (fraction E) and 50% for 15 min followed by 80% for 15 min (fraction G), respectively. Compounds **2**, **3**, **4**, **6**, **7**, **8**, **9**, **12** and **19**, compounds **1**, **3**, **17**, **18** and **19** as well as compounds **5** and **14** were isolated from the respective fractions.

HPLC separation of fraction F using 70% MeCN in water yielded compounds **1** and **14**.

Compound **20** was isolated from OMAT. A total of 210 g of OMAT was subjected to silica gel column chromatography using hexane–EtOAc (10 : 1) and $CHCl_3$–MeOH (100 : 0, 10 : 1, 0 : 100) as eluates. The fraction eluted with 10 : 1 $CHCl_3$–MeOH was further fractionated on a silica gel column eluted with $CHCl_3$–EtOH. A fraction eluted with 25 : 1 $CHCl_3$–EtOH was dried (23.7 g) and subjected to HPLC using a Capcell Pak C18 MGII column eluted with 60% MeCN in water, yielding compound **20** (78.5 mg).

Electron impact mass spectrometry (EI-MS) was performed using a JMS-700 instrument (JEOL, Tokyo, Japan). Optical rotation was recorded using a DIP-1000 digital polarimeter (Jasco, Tokyo, Japan), and UV and IR spectra were obtained using a V-530 UV/VIS spectrophotometer and FTIR-410 spectrometer (Jasco), respectively. For NMR spectroscopy, samples were dissolved in one of the following solvents: $(CD_3)_2CO$, $CDCl_3$, or CD_3OD, and ^1H- and ^{13}C-NMR spectra were recorded for each sample using either a JEOL α-500 spectrometer (JEOL) or a Varian NMR System 400 spectrometer (Varian Technologies Japan, Ltd.).

Determination of Minimum Inhibitory and Minimum Bactericidal Concentrations Minimum inhibitory concentrations (MICs) were determined using a modification of the broth microdilution method,[7] which is based on the standard method employed by the Clinical and Laboratory Standards Institute (CLSI).[8] Given the susceptibility of *L. pneumophila* and other *Legionella* spp. to detergents, Tween 80 was not used in the experiments. The two types of oakmoss (OM and OMAT) and their purified components were dissolved in dimethyl sulfoxide (DMSO) to a concentration of 12.8 mg/mL and then diluted to a final concentration of 512 μg/mL in buffered yeast extract broth (1% yeast extract, 1% *N*-(2-acetamide)-2-aminoethanesulfonic acid, 0.025% iron(II) diphosphate and 1% L-cysteine) supplemented with 0.1% potassium α-ketoglutarate (BYE-α). Samples were serially diluted with BYE-α as described previously.[6] Each diluted sample (50 μL) was mixed with 50 μL of bacterial suspension adjusted to 6.0×10^5 colony forming units (CFU)/mL with BYE-α and incubated at 37°C for 48 h. To determine the MICs against other bacterial strains, bacterial suspensions were adjusted to 1.0×10^6 CFU/mL with Mueller Hinton II broth (Becton Dickinson, Sparks, MD, U.S.A.). A 50 μL volume of each bacterial suspension was mixed with serially diluted oakmoss or one of its components (50 μL) prepared according to the microdilution method[7] and incubated at 35°C for 20 h. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of test sample producing no visible colony formation upon subculturing on buffered charcoal yeast extract agar supplemented with 0.1% α-ketoglutarate (BCYE-α, Becton Dickinson).

Time–Kill Assays Time–kill assays were performed using *L. pneumophila* strain JCM7571 according to the standard CLSI method[9] with slight modifications.[6] The resulting CFU data are expressed as means±standard deviation based on the CFU values obtained from six plates.

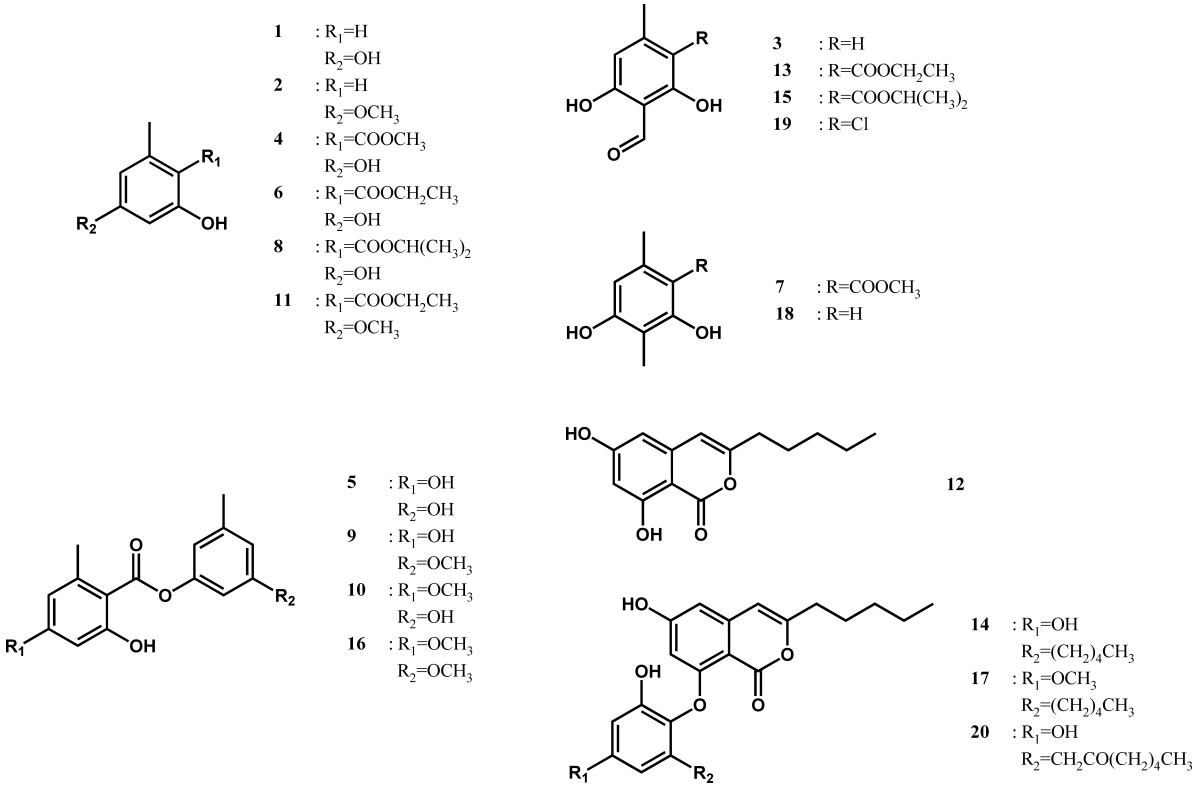

Fig. 1. Structures of the Compounds Isolated from OM and OMAT

RESULTS

Yield and Structural Identification of Purified Compounds In the present study, we purified 20 compounds from two types of oakmoss (OM and OMAT). The chemical structures of the purified compounds were defined based on ^{1}H- and ^{13}C-NMR data and their molecular weights were determined using EI-MS. The chemical structures of the oakmoss compounds were classified into three chemical groups: 12 compounds were classified as phenol derivatives, four were classified as didepside derivatives, and four were classified as isochromen derivatives (Fig. 1). Compounds **9** and **20** were novel components. The IUPAC name with common name, yield, and molecular weight of each of the 20 isolated compounds are summarized in Table 1.

Using high-resolution EI-MS, we determined the molecular formula of compound **9** to be $C_{16}H_{16}O_5$ based upon the molecular ion peak at *m/z* 288.1020. The UV spectrum in MeCN showed absorption peaks at λ_{max} (ε) 219 (19800), 268 (16200) and 302 (6180) nm, and the specific optical rotation was $[\alpha]_D$ −0.16° (*c*=0.1, MeCN, 27.8°C). The IR spectrum showed absorption peaks at ν_{max} 3324, 2926, 1661, 1458, 1300 and 1169 cm^{-1}, indicating the presence of hydroxyl and carbonyl groups.

The ^{1}H- and ^{13}C-NMR data are presented in Table 2. In the ^{13}C-NMR spectrum (125 MHz, CD_3OD), 16 signals arising from two methyl carbons (δ_C 24.8, 21.5), five methine carbons (δ_C 102.0, 106.1, 113.1, 113.6, 115.7), seven quaternary carbons (δ_C 105.2, 144.7, 144.7, 152.3, 162.0, 165.0, 166.6), one methoxy carbon (δ_C 55.9) and one carbonyl carbon (δ_C 171.5) were assigned. In the ^{1}H-NMR spectrum (500 MHz, CD_3OD), eight signals arising from two benzylic protons (δ_H 2.24, s; 2.47, s), five aromatic protons (δ_H 6.06, d, *J*=2.4 Hz; 6.18, d, *J*=2.4 Hz; 6.48, s; 6.51, s; 6.59, s) and one methoxy proton (δ_H 3.69, s) were assigned. The results of ^{1}H–^{13}C heteronuclear multiple bond connectivity (HMBC) experiments revealed ^{1}H–^{13}C long-range couplings from protons to carbon atoms, as shown in Table 2. Based on these data, compound **9** was identified as 3-methoxy-5-methylphenyl-2,4-dihydroxy-6-methylbenzoate; this compound has not been previously reported.

The molecular formula of compound **20** was determined to be $C_{27}H_{32}O_7$ based on the molecular ion peak at *m/z* 468.2176 observed in the high-resolution EI-MS spectrum. The UV spectrum in MeCN showed absorption peaks at λ_{max} (ε) 211 (14799), 246 (34300), 279 (7670) and 324 (5160) nm, and the specific optical rotation was $[\alpha]_D$ +5.84° (*c*=0.1, MeCN, 27.8°C). The IR spectrum showed absorption peaks at ν_{max} 3405, 2958, 2931, 2857, 1697, 1600, 1463, 1359 and 1164 cm^{-1}. The ^{1}H- and ^{13}C-NMR data for compound **20** are presented in Table 3. In the ^{13}C-NMR spectrum (100 MHz, $(CD_3)_2CO$), 27 signals arising from two methyl carbons (δ_C 14.2, 14.1), nine methylene carbons (δ_C 44.3, 42.5, 33.7, 31.9, 31.8, 27.2, 23.9, 23.0, 23.0), five methine carbons (δ_C 109.9, 104.8, 103.6, 103.2, 102.0), nine quaternary carbons (δ_C 164.5, 162.8, 159.0, 156.2, 151.1, 142.9, 133.8, 130.8, 102.5) and two carbonyl carbons (δ_C 207.0, 159.3) were assigned. In the ^{1}H-NMR spectrum (400 MHz, $(CD_3)_2CO$), 16 signals arising from two methyl protons (δ_H 0.92, t, *J*=7.0, 7.0 Hz; 0.82, t, *J*=7.4, 7.4 Hz), nine methylene protons (δ_H 3.54, s; 2.48, t, *J*=7.6, 7.6 Hz; 2.38, t, *J*=7.4, 7.4 Hz; 1.68, m; 1.39, m; 1.38, m; 1.36, m; 1.19, m; 1.10, m) and five aromatic protons (δ_H 6.47, s; 6.46, s; 6.33, s; 6.27, s; 6.08, s) were assigned. The results of ^{1}H–^{13}C HMBC experiments revealed ^{1}H–^{13}C long-range couplings from protons to carbon atoms, as shown in Table 3. Based on these

Table 1. Compounds Isolated from OM and OMAT

Label	Compound IUPAC name (Common name)	Weight of isolated compound (mg)	Yield (%, w/w)
From OM (2.24 g)			
1	5-Methylbenzene-1,3-diol (orcinol)	197.8	8.03
2	3-Methoxy-5-methylphenol (*O*-methylorcinol)	61.9	2.76
3	2,6-Dihydroxy-4-methylbenzaldehyde (atranol)	19.7	0.88
4	Methyl 2,4-dihydroxy-6-methylbenzoate (methyl orsellinate)	1.3	0.06
5	3-Hydroxy-5-methylphenyl-2,4-dihydroxy-6-methylbenzoate (lecanorin)	8.4	0.38
6	Ethyl 2,4-dihydroxy-6-methylbenzoate (ethyl orsellinate)	35.4	1.58
7	Methyl 2,4-dihydroxy-3,6-dimethylbenzoate (atraric acid)	231.1	10.32
8	Isopropyl 2,4-dihydroxy-6-methylbenzoate (isopropyl orsellinate)	2.1	0.09
9	3-Methoxy-5-methylphenyl-2,4-dihydroxy-6-methylbenzoate	2.0	0.09
10	3-Hydroxy-5-methylphenyl-2-hydroxy-4-methoxy-6-methylbenzoate	13.5	0.60
11	Ethyl 2-hydroxy-4-methoxy-6-methylbenzoate (everninate)	51.7	2.31
12	6,8-Dihydroxy-3-pentyl-1*H*-isochromen-1-one (olivetonide)	2.1	0.09
13	Ethyl 3-formyl-2,4-dihydroxy-6-methylbenzoate (haematommate)	8.4	0.38
14	8-(2,4-Dihydroxy-6-pentylphenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one	21.7	0.97
15	Isopropyl 3-formyl-2,4-dihydroxy-6-methylbenzoate	22.7	1.01
16	3-Methoxy-5-methylphenyl-2-hydroxy-4-methoxy-6-methylbenzoate	6.2	0.28
17	8-(2-Hydroxy-4-methoxy-6-pentylphenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one	7.9	0.35
18	2,5-Dimethylbenzene-1,3-diol (*β*-orcinol)	1.6	0.07
19	3-Chloro-2,6-dihydroxy-4-methylbenzaldehyde (chloroatranol)	5.3	0.24
From OMAT (210 g)			
20	8-(2,4-Dihydroxy-6-(2-oxoheptyl)phenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one	78.5	0.04

data, compound **20** was identified as 8-(2,4-dihydroxy-6-(2-oxoheptyl)phenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one. As with compound **9**, this compound has not been previously reported.

Antibacterial Activity of Oakmoss Compounds against *L. pneumophila* and Other *Legionella* spp. Data regarding the antibacterial activity (MIC and MBC) of oakmoss and each of its isolated components are presented in Table 4. The antibacterial activity of each compound was compared with that of chlorhexidine gluconate and cinnamic aldehyde, the latter of which is a well-known natural fragrance ingredient with potent antibacterial activity.[10–12] All 20 compounds isolated from oakmoss exhibited antibacterial activity against *L. pneumophila* strains JCM7571, GTC00748 and JBCC005, with MICs ranging from 0.8 to 256.0 μg/mL and MBCs ranging from 1.7 to greater than 256.0 μg/mL. The isochromen derivatives (compounds **12**, **14**, **17**, **20**) and didepside derivatives (compounds **5**, **9**, **10**, **16**) displayed relatively high antibacterial activity against *L. pneumophila* strains. The MICs of these compounds were lower than that of cinnamic aldehyde. In particular, the antibacterial activity of compounds **12** and **14** as determined by MIC and MBC was nearly equal to that of the general disinfectant chlorhexidine gluconate. All 20 compounds exhibited antibacterial activity against *L. bozemani*, *L. micdadei*, *L. longbeachae* and *L. dumoffii*, with MICs ranging from 0.1 to 256.0 μg/mL and MBCs ranging from 0.5 to greater than 256.0 μg/mL. The MIC and MBC values for the isochromen (compounds **12**, **14**, **17**, **20**) and didepside derivatives (compounds **5**, **9**, **10**, **16**) against these organisms were high compared with those for chlorhexidine gluconate.

We also examined the 20 compounds isolated from oakmoss for antibacterial activity against other Gram-negative (*E. coli* JCM5491 and *P. aeruginosa* JCM6119) and Gram-positive (*B. subtilis* NBRC134 and *S. aureus* JCM2413) organisms. However, despite exhibiting antibacterial activity against *L. pneumophila*, none of these compounds showed any antibacterial activity against any of the other bacterial strains tested; all of the MICs obtained were higher than 256 μg/mL (data not shown). Our data indicate that the antibacterial activity of oakmoss and its components is specific against *Legionella* spp.

The antibacterial effect of compounds **9** and **20** were also examined using a time-kill assay (Fig. 2). When *L. pneumophila* JCM7571 was exposed to the unfractionated oakmoss sample at concentrations equivalent to 1×MIC and 2×MIC, the number of CFUs decreased rapidly to 10^{-2} to 10^{-3} of the control value; however, the number of CFUs did not decline to below the limit of detection, even after a 48 h coincubation. Conversely, administration of compound **9** at a concentration equivalent to 2×MIC resulted in a rapid reduction in the number of CFUs, which declined to below the limit of detection after a 1 h co-incubation, even though the bactericidal activity at a 1×MIC level was limited. Thus, compound **9** was shown to be bactericidal.

In the case of compound **20**, co-incubation with *L. pneumophila* JCM7571 at a 2×MIC concentration also resulted in a rapid reduction in the number of CFUs to below the limit of detection. The number of CFUs remained above the limit of detection following administration of compound **20** at a concentration equivalent to 1×MIC, even after 24 h. These results indicate that both compounds **9** and **20** are bactericidal against *L. pneumophila*, although the bactericidal activity of

Table 2. ^{1}H- and ^{13}C-NMR Data for Novel Compound **9** in CD_3OD

Carbon No.	^{13}C Chemical shift (ppm)	^{1}H Chemical shift (ppm)	^{1}H–^{13}C Correlation observed in HMBC
1	105.2	—	
2	165.0	—	
3	102.0	6.06 (1H, d, *J*=2.4 Hz)	C-1, C-2, C-4, C-5
4	166.6	—	
5	113.1	6.18 (1H, d, *J*=2.4 Hz)	C-1, C-3, C-8
6	144.7	—	
7	171.5	—	
8	24.8	2.47 (3H, s)	C-1, C-5, C-6
1′	152.3	—	
2′	106.1	6.48 (1H, s)	C-1′, C-3′, C-6′
3′	162.0	—	
4′	113.6	6.59 (1H, s)	C-2′, C-6′
5′	144.7	—	
6′	115.7	6.51 (1H, s)	C-1′, C-2′, C-4′, C-7′
7′	55.9	3.69 (3H, s)	C-4′, C-5′, C-6′
8′	21.5	2.24 (3H, s)	C-3′

Table 3. ^{1}H- and ^{13}C-NMR Data for Novel Compound **20** in $(CD_3)_2CO$

Carbon No.	^{13}C Chemical shift (ppm)	^{1}H Chemical shift (ppm)	^{1}H–^{13}C Correlation observed in HMBC
1	159.3	—	
2	—	—	
3	159.0	—	
4	103.2	6.27 (1H, s)	C-3, C-4a, C-5, C-8a, C-9
4a	142.9	—	
5	104.8	6.46 (1H, s)	C-4, C-6
6	164.5	—	
7	102.0	6.08 (1H, s)	C-5, C-8, C-8a
8	162.8	—	
8a	102.5	—	
9	33.7	2.48 (2H, t, *J*=7.6, 7.6 Hz)	C-3, C-4, C-10, C-11
10	27.2	1.68 (2H, m)	C-3, C-11, C-12
11	31.8	1.39 (2H, m)	C-12
12	23.0	1.38 (2H, m)	C-11
13	14.2	0.92 (3H, t, *J*=7.0, 7.0 Hz)	C-11, C-12
1′	133.8	—	
2′	151.1	—	
3′	103.6	6.47 (1H, s)	C-1′, C-2′, C-4′, C-5′
4′	156.2	—	
5′	109.9	6.33 (1H, s)	C-1′, C-3′, C-4′, C-7′
6′	130.8	—	
7′	44.3	3.54 (2H, s)	C-1′, C-5′, C-6′, C-8′
8’	207.0	—	
9′	42.5	2.38 (2H, t, *J*=7.4, 7.4 Hz)	C-8′, C-10′, C-11′
10′	23.9	1.36 (2H, m)	C-8′, C-9′
11′	31.9	1.10 (2H, m)	C-12′
12′	23.0	1.19 (2H, m)	C-10′, C-11′, C-13′
13′	14.1	0.82 (3H, t, *J*=7.4, 7.4 Hz)	C-11′, C-12′

compound **20** is weaker than that of compound **9**.

DISCUSSION

We isolated and identified 20 compounds from oakmoss that exhibited antibacterial activity against *Legionella pneumophila* and other *Legionella* spp. Isolated compounds **9** and **20** were identified as 3-methoxy-5-methylphenyl-2,4-dihydroxy-6-methylbenzoate and 8-(2,4-dihydroxy-6-(2-oxoheptyl)phenoxy)-6-hydroxy-3-pentyl-1*H*-isochromen-1-one, respectively. Both of these compounds are novel and have not been previously reported. The total yield of the 19 compounds isolated from the OM type of oakmoss comprised approximately 30% of the starting material. Gel filtration chromatography of oakmoss extract on an LH-20 column resulted in the adsorption of a brownish material onto the gel which could not be eluted with 10% or higher concentrations of methanol in water. This material was believed to contain high-molecular

Table 4. MICs and MBCs of Compounds Isolated from Oakmoss with Antibacterial Activity against *Legionella* Species

	L. pneumophila JCM7571		*L. pneumophila* GTC00748		*L. pneumophila* JBCC005		*L. bozemanii* GTC09140		*L. micdadei* IID3044		*L. longbeachae* IID3046		*L. dumoffii* IID3047	
	MIC[a)]	MBC[a)]	MIC[a)]	MBC[a)]	MIC[a)]	MBC[a)]	MIC[a)]	MBC[a)]	MIC[a)]	MBC[a)]	MIC[a)]	MBC[a)]	MIC[a)]	MBC[a)]
OM	10.7	32.0	16.0	96.0	8.0	21.3	16.0	24.0	26.7	32.0	3.3	7.4	16.0	16.0
OMAT	10.7	42.7	16.0	96.0	7.3	24.0	16.0	26.7	21.3	21.3	3.3	5.3	16.0	16.0
1	213.3	>256.0	256.0	>256.0	213.3	256.0	128.0	213.3	213.3	>256.0	53.3	53.3	170.7	170.7
2	213.3	>256.0	128.0	256.0	128.0	256.0	96.0	106.7	256.0	>256.0	53.3	53.3	170.7	170.7
3	128.0	234.7	128.0	234.7	53.3	117.3	128.0	170.7	128.0	170.7	53.3	53.3	106.7	170.7
4	106.7	213.3	85.3	234.7	53.3	106.7	64.0	74.7	64.0	64.0	13.3	13.3	42.7	64.0
5	8.0	16.0	8.0	42.7	8.0	16.0	4.0	8.0	8.0	16.0	2.0	2.0	5.3	10.7
6	32.0	64.0	42.7	128.0	26.7	36.3	32.0	32.0	32.0	32.0	6.7	13.3	53.3	53.7
7	21.3	53.3	26.7	106.7	12.0	23.7	24.0	32.0	21.3	26.7	6.7	6.7	21.3	42.7
8	26.7	32.0	32.0	85.3	8.0	29.3	16.0	32.0	32.0	32.0	6.7	13.3	26.7	42.7
9	8.0	16.0	5.3	37.3	1.3	16.0	1.5	5.3	8.0	16.0	0.8	0.8	5.3	8.0
10	4.0	8.0	4.0	21.3	1.3	12.7	1.0	1.0	6.7	10.7	0.8	1.0	1.0	1.3
11	106.7	>256.0	>256.0	NT[b)]	53.3	213.3	>256.0	NT[b)]	>256.0	NT[b)]	128.0	213.3	213.3	>256.0
12	1.7	8.0	1.0	8.0	0.8	7.2	0.1	0.5	1.0	1.3	0.7	0.8	1.0	4.0
13	16.0	>256.0	32.0	>256.0	13.3	170.7	26.7	53.3	128.0	192.0	13.3	13.3	21.3	21.3
14	2.0	3.0	1.5	1.7	0.8	2.0	1.8	2.7	2.0	2.0	1.7	1.7	1.0	1.3
15	8.0	256.0	24.0	256.0	8.0	181.3	13.3	13.3	32.0	32.0	8.0	8.0	8.0	10.7
16	8.0	32.0	7.3	>256.0	4.0	25.0	2.7	3.3	64.0	>256.0	1.0	1.3	1.3	2.7
17	6.7	21.3	42.7	>256.0	2.7	18.7	8.0	21.3	42.7	58.7	3.3	8.0	8.0	8.0
18	74.4	192.0	128.0	>256.0	26.7	96.0	192.0	256.0	128.0	>256.0	26.7	53.3	106.7	106.7
19	32.0	117.2	64.0	234.7	14.7	21.3	85.3	128.0	128.0	128.0	53.3	53.3	42.7	85.3
20	8.0	17.2	13.3	21.3	3.3	16.0	8.0	29.3	21.3	24.0	10.7	10.7	8.0	8.0
CHG[c)]	0.8	0.8	0.8	0.8	0.7	1.0	1.0	1.2	1.0	1.2	1.0	1.0	0.3	0.3
CA[d)]	48.0	64.0	64.0	96.0	14.7	21.3	64.0	64.0	58.7	64.0	26.7	32.0	32.0	53.3

a) Values (μg/mL) are means obtained from three independent experiments. *b*) NT: not tested. *c*) Chlorhexidine gluconate. *d*) Cinnamic aldehyde.

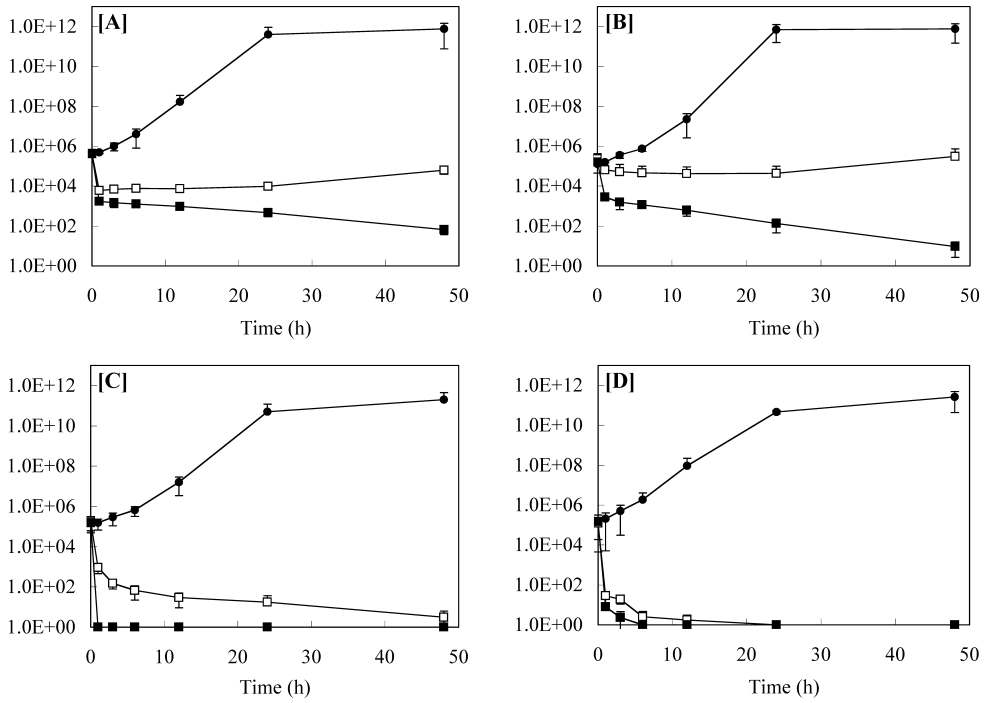

Fig. 2. Time–Kill Curves for *L. pneumophila* JCM7571 in the Presence of 1×MIC (□) and 2×MIC (■)
(A) OM, (B) OMAT, (C) compound **9**, and (D) compound **20**. Closed circles (●) represent the positive control.

weight substances, such as tar.

Several of the compounds we identified in oakmoss and *E. prunastri* (L.) ACH. extracts have been identified previously, including atranol (compound **3**), chloroatranol (compound **19**), atraric acid (compound **7**), everninate (compound **11**), haematommate (compound **13**), orsellinates (compounds **4**, **6**, **8**) and lecanorin (compound **5**).[1–4] The antimicrobial activity of these compounds against various fungi and Gram-positive and Gram-negative bacteria has also been demonstrated in previous reports.[13,14] However, an examination of the antibacterial activity of these oakmoss components against *L. pneumophila* and *Legionella* spp. has not been reported. Although the antibacterial activity of the oakmoss compounds varied considerably, all 20 of the compounds we isolated were active against clinical and environmental isolates of *L. pneumophila* and other *Legionella* spp. While the reported MICs against Gram-negative bacteria such as *P. aeruginosa* and *E. coli* range between 30 and 1000 μg/mL for the compounds identified as phenol derivatives,[13,14] the MICs against *L. pneumophila* were lower, ranging between 8.0 and 213.3 μg/mL, which suggests that *L. pneumophila* is more susceptible to these compounds than are *P. aeruginosa* and *E. coli*. Atranorin is a didepside compound that exhibits antibacterial activity against Gram-positive bacteria such as *B. mycoides*, *B. subtilis* and *S. aureus*, and against Gram-negative bacteria such as *Enterobacter cloacae*, *E. coli* and *Klebsiella pneumoniae*,[4] with a reported MIC of 31 μg/mL. Although we were unable to isolate atranorin from oakmoss in the present study, four didepside derivatives (compounds **5**, **9**, **10**, **16**) were isolated. The MICs of these compounds against the three *L. pneumophila* strains and four other *Legionella* species strains tested ranged between 0.8 and 64.0 μg/mL, and were considerably lower than the previously reported MICs of these compounds against Gram-positive and Gram-negative bacteria. One possible explanation for this result could be the slight differences between the structures of the didepsides we isolated and that of atranorin.

Fujikawa *et al.*[15] reported that atraric acid and orsellinates isolated from *Parmelia* spp. and olivetonide (compound **12**) isolated from *Cetrelia* spp., possess antibacterial activity against *Lactobacillus* spp. Olivetonide and three additional isochromen derivatives (compounds **14**, **17**, **20**) that we isolated from oakmoss exhibited potent antibacterial activity against *L. pneumophila*, with olivetonide exhibiting the highest antibacterial activity against strains of *L. pneumophila* and other *Legionella* spp. Our results suggest that the antibacterial activity of oakmoss is due to the simultaneous actions of the 20 compounds that we isolated. However, neither oakmoss itself nor the 20 individual compounds exhibited antibacterial activity against other Gram-negative and Gram-positive bacteria tested.[6]

All Gram-negative bacteria possess an outer membrane that imparts a hydrophilic surface to the cell. Small hydrophilic compounds can traverse the outer membrane through porin proteins that form hydrophilic trans-membrane channels that exclude hydrophobic compounds. Thus, Gram-negative bacteria are relatively resistant to hydrophobic compounds.[16] The degree of hydrophobicity of the outer membrane depends on physicochemical properties of its protein, phospholipid and lipopolysaccharide components. The outer membrane of *Legionella* spp. is more hydrophobic than that of other Gram-negative bacteria.[17–19] In particular, the *O*-polysaccharide portion of *L. pneumophila* (serogroup 1) lipopolysaccharide is a homopolymer of 5-acetamide-7-acetamide-8-acetyl-3,5,7,9-tetradeoxy-L-*glycero*-D-*galacto*-nonnulosonic acid,[20–22] which completely lacks free hydroxyl groups and is therefore highly hydrophobic. In addition, the lipopolysaccharide contains a highly *O*-acetylated core structure[23] that is also hydrophobic.

These factors account for the greater hydrophobic character of the outer membrane of *Legionella* spp. relative to that of other Gram-negative bacteria and may explain its unusual permeability to hydrophobic compounds.[16–19] The exceptional hydrophobicity of the cell surface might be responsible for the higher susceptibility of *Legionella* spp. to hydrophobic oakmoss and its components compared to other Gram-negative bacteria.

In addition to its antibacterial activity, oakmoss is a causative agent of allergic contact dermatitis.[24–26] Allergens present in oakmoss reportedly include atranol and chloroatranol, which may be produced by the decomposition of didepsides during the preparation of oakmoss products. The removal of such allergens is essential for the safe use of oakmoss as a perfume or antiseptic. Thus, separated fractions and components that are free of any allergens may prove useful as disinfectants for public water systems.

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