Novel Abietane Diterpenoids and Aromatic Compounds from *Cladonia rangiferina* and Their Antimicrobial Activity against Antibiotics Resistant Bacteria

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From *Cladonia rangiferina* were isolated two novel abietane diterpenoids, hanagokenols A (1) and B (2). Also in this investigation, four known abitetane diterpenoids (3—6), four known labdane diterpenoids (7—10), one known isopimarane diterpenoid (11), and six known aromatic compounds were isolated. These structures were elucidated primarily through extensive NMR experiments. Hanagokenol A (1) was a unique abietane diterpene having an ether linkage between C-6 and C-18 of sugiol. Hanagokenol B (2) is also a unique secoabietane diterpene, having γ-lactone which occurred by cleavage and subsequently oxidation between C-6/C-7 of 12-hydroxydehydroabietinol. Furthermore, all the isolated compounds (1—17) were tested for the antimicrobial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE).

Key words *Cladonia rangiferina*; Cladoniaceae; secoabietane; depside; methicillin-resistant *Staphylococcus aureus*; vancomycin-resistant Enterococci

In the course of our research program aimed at the discovery of biologically active compounds from fungi,[1)] we have initiated the chemical study of the Japanese lichen, *Cladonia rangiferina* (L.) WEB. (Cladoniaceae). The lichen, *C. rangiferina*, is widely distributed in southern Japan and which grows on the ground and rocks from high mountains to low lands.[2)] An earlier chemical constituent study of this lichen resulted in no report of the isolation and the biological activity. In our investigation, two new abietane diterpenoids, called hanagokenols A (**1**), and B (**2**), along with 15 known compounds including abietane,[3—5)] labdane,[6—8)] isopimarane[9)] diterpenoids, monocyclic aromatic compound,[10,11)] depside,[12,13)] and dibenzofuran[14,15)] were isolated from *C. rangiferina*. We describe here the isolation, purification, and structural elucidation of the unique abietane diterpenes, **1** and **2**, primarily by extensive NMR experiments, and the antimicrobial activities of all the isolated compounds against methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant Enterococci (VRE). *C. rangiferina* was milled and exhaustively extracted with AcOEt at room temperature for 8 weeks. The AcOEt extract was fractionated into seven fractions by column chromatography (silica gel), followed by repeated separations of their seven portions by chromatography over silica gel. Reversed-phase silica gel furnished hanagokenols A (**1**), and B (**2**), obtuanhydride (**3**),[3)] sugiol (**4**),[4)] 5,6-dehydrosugiol(**5**),[4)] montbretol (**6**),[5)] *cis*-communic acid (**7**),[6)] imbricatoloic acid (**8**),[7)] 15-acetylimbricatoloic acid (**9**),[8)] junicedric acid (**10**),[8)] 7α-hydroxysandaracopimaric acid (**11**),[9)] β-resorylic acid (**12**),[10)] atranol (**13**),[11)] barbatic acid (**14**),[12)] homosekikaic acid (**15**),[13)] didymic acid (**16**),[14)] and condidymic acid (**17**).[15)]

Hanagokenol A (**1**), $[\alpha]_D^{25}$ +185.9°, was obtained as an amorphous solid and was considered to have the molecular formula of $C_{20}H_{26}O_3$ based on the high-resolution electron ion mass spectrum (HR-EI-MS) of the molecular ion at *m*/*z* 314.1864. The IR spectrum of **1** showed absorption bands at 3370 (OH), 1690 (C=O), and 1610 (aromatic) cm^{-1}. The presence of an aromatic ring was supported by the UV data (λ_{max} 217, 236 and 288 nm). The 20 carbon signals observed in the ^{13}C-NMR spectrum and distortionless enhancement by polarization transfer (DEPT) experiment (Table 1) revealed the presence of a ketone at δ 195.7 (s); a benzene ring at δ 111.3 (d), 124.0 (s), 127.3 (d), 134.8 (s), 155.3 (s), and 161.5 (s); two oxygenated carbons at δ 77.1 (d), and 84.1 (t), which requires that **1** should contain four rings. The ^{1}H-NMR spec-

Table 1. ^{1}H- and ^{13}C-NMR Assignments for Compounds **1** and **2**

Positions	**1**		**2**	
	^{1}H δ (mult. *J* in Hz)	^{13}C δ (mult.)	^{1}H δ (mult. *J* in Hz)	^{13}C δ (mult.)
1α	1.26 (ddd, 13.2, 13.2, 4.4)	38.4 (t)	1.70 (m)	38.5 (t)
1β	1.98 (ddd, 13.2, 3.0, 3.0)		1.98 (m)	
2α	1.64 (m)	20.3 (t)	1.75 (m)	18.8 (t)
2β	1.50 (m)		1.86 (m)	
3α	1.26 (ddd, 13.5, 13.5, 3.8)	35.1 (t)	1.57 (ddd, 12.6, 12.6, 5.2)	33.8 (t)
3β	1.57 (ddd, 13.5, 3.3, 3.3)		1.80 (m)	
4		40.5 (s)		42.7 (s)
5	2.00 (d, 14.3)	56.8 (d)	3.25 (s)	56.6 (d)
6	4.67 (d, 14.3)	77.1 (d)		177.3 (s)
7		195.7 (s)		173.7 (s)
8		124.0 (s)		123.9 (s)
9		155.3 (s)		143.8 (s)
10		38.6 (s)		38.8 (s)
11	7.06 (s)	111.3 (d)	6.96 (s)	115.7 (d)
12		161.5 (s)		154.7 (s)
13		134.8 (s)		132.8 (s)
14	8.40 (s)	127.3 (d)	7.34 (s)	128.8 (d)
15	3.60 (sept, 6.9)	27.7 (d)	3.15 (sept, 6.9)	26.7 (d)
16	1.35 (d, 6.9)	23.0 (q)	1.23 (d, 6.9)	22.2 (q)
17	1.36 (d, 6.9)	23.0 (q)	1.24 (d, 6.9)	22.2 (q)
18a	3.46 (d, 6.9)	84.1 (t)	3.92 (d, 8.0)	81.8 (t)
18b	3.76 (d, 6.9)		4.03 (d, 8.0)	
19	1.09 (s)	18.6 (q)	1.30 (s)	20.5 (q)
20	1.20 (s)	22.3 (q)	1.61 (s)	20.7 (q)

Measurements were performed in C_5D_5N at 600 MHz for ^{1}H- and 125 Hz for ^{13}C-NMR. ^{13}C multiplicities were established by DEPT pulse sequences.

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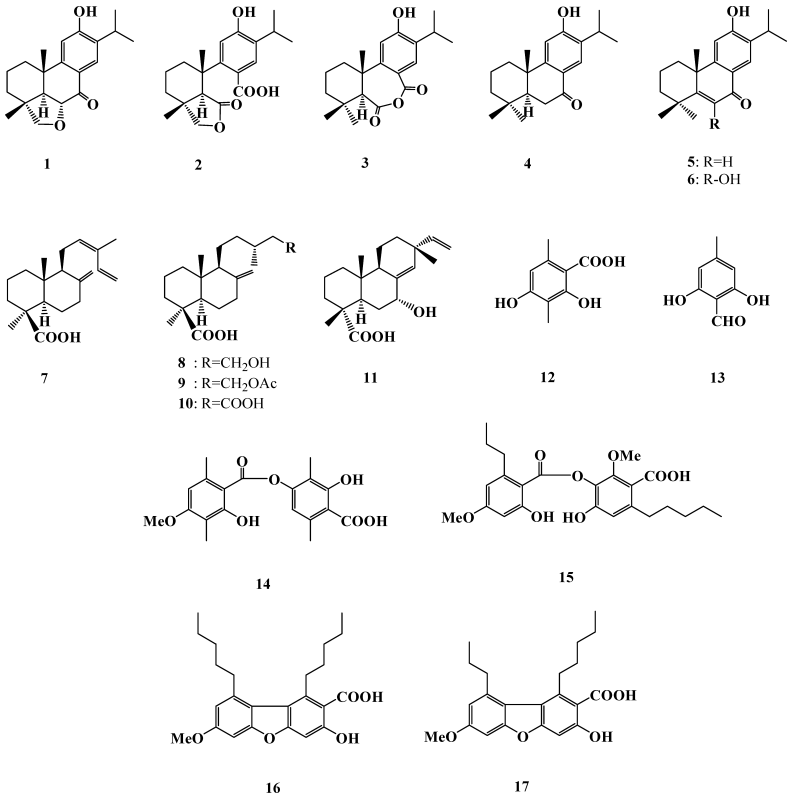

Chart 1

trum of **1** showed two tertiary methyl signals at δ 1.09, and 1.20, an isopropyl group at δ 1.35 and 1.36 (each d, J=6.9 Hz), 3.60 (sept, J=6.9 Hz) and two aromatic protons at δ 7.06 (s), and 8.40 (s), AX type signals at δ 2.00 (d, J=14.3 Hz) and 4.67 (d, J=14.3 Hz), and AB type signals at δ 3.46 (d, J=6.9 Hz) and 3.76 (d, J=6.9 Hz). These data suggested that **1** was an abietane-type diterpene, as compounds **3**—**6**.[3—5] The gross structure of **1** was determined by analysis of the NMR data including heteronuclear multiple quantum coherence (HMQC), heteronuclear multiple bond correctivity (HMBC), and rotating frame nuclear Overhauser effect spectroscopy (ROESY) experiments. The HMBC spectrum (Fig. 1) of **1** showed long-range correlation from the AX type protons (δ 4.67, 2.00), which was assigned to H-6 and H-5, respectively, to the ketone at δ 195.7 assigned to C-7, and then from the H_2-18b (δ 3.76), which was correlated with the oxygenated carbon at δ 84.1 by HMQC spectrum, to C-5 (δ 56.8) and C-6 (δ 77.1), indicating that **1** had an oxolane formed between C-6 and C-18 in sugiol (**4**).[4] The stereochemistry of **1** was deduced by ROESY experiment (Fig. 2) and also coupling constant. The α-substituted group at C-6 could be assigned from the NOEs between H-6 (δ 4.67) and H_3-20 (δ 1.29), and between H-5 (δ 2.00) and H_2-18a (δ 3.46), and the observed large coupling constant (J=14.3 Hz) between H-5 and H-6. Thus, from the above findings and the biosynthesis considerations, **1** was shown to be 12-hydroxy-7-oxo-6α,18-epoxy-8,11,13-abietatrien and termed hanagokenol A.

Hanagokenol B (**2**), $[\alpha]_D^{25}$ −14.4°, was obtained as an amorphous solid and considered to have the molecular formula of $C_{20}H_{26}O_5$ based on the HR-EI-MS of the molecular ion at m/z 346.1768 $[M]^+$, which suggested the presence of

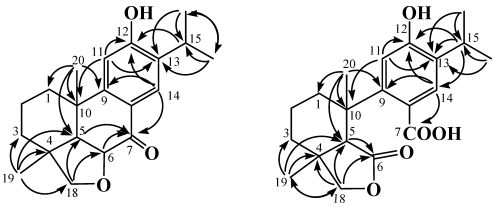

Fig. 1. HMBC Correlations of Hanagokenols A (**1**) and B (**2**)

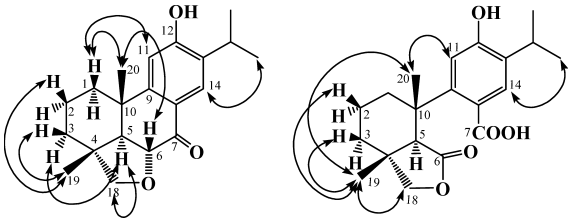

Fig. 2. ROESY Correlations of Hanagokenols A (**1**) and B (**2**)

eight degrees of unsaturation. The IR spectrum of **2** showed absorption bands at 3370 (OH), 1760, 1705 (C=O), 1610 and 1590 (aromatic) cm^{-1}. The presence of an aromatic ring was supported by the UV data (λ_{max} 233 and 287 nm). The 20 carbon signals observed in the ^{13}C-NMR spectrum (Table 1) and DEPT experiment showed the presence of two carbonyls at δ 173.7 (s) and 177.3 (s); a benzene ring at δ 115.7 (d), 123.9 (s), 128.8 (d), 132.8 (s), 143.8 (s), 154.7 (s), and one oxygenated methylene carbon at δ 81.8 (t), suggesting that the structure of **2** was similar to that of **1**, except for the presence of two carbonyl signals. Its NMR data (Table 1) showed an isopropyl group at δ 1.23, 1.24 (each d, J=6.9

Table 2. Antibacterial Activities against *Staphylococcus aureus* COL (MRSA) and *E. fuecium* (Van A) (VRE) of the Compounds Isolated from *Cladonia rangiferina*

Compounds[a)]	Inhibition zone (mm)	
	Staphylococcus aureus COL (MRSA)	*E. fuecium* (Van A) (VRE)
Hanagokenol A (**1**)	8	−
Hanagokenol B (**2**)	11	15
Obtuanhydride (**3**)	13	−
Sugiol (**4**)	20	−
5,6-Dehydrosugiol (**5**)	−	−
Montbretol (**6**)	13	±
cis-Communic acid (**7**)	±	−
Imbricatoloic acid (**8**)	±	−
15-Acetyl-imbricatoloic acid (**9**)	±	±
Junicedric acid (**10**)	14	−
7α-Hydroxysandaracopimaric acid (**11**)	15	10
β-Resorylic acid (**12**)	±	−
Atranol (**13**)	14	−
Barbatic acid (**14**)	18	17
Homosekikaic acid (**15**)	20	23
Ddymic acid (**16**)	28	22
Condidymic acid (**17**)	17	22
EM[b)]	9	−
TC[c)]	−	8

a) 100 mg/disk (mm). *b*) EM, erythromycin (100 μg/ml) as reference for MRSA strain. *c*) TC, tetracycline (100 μg/ml) as reference for VRE strain.

Hz), and 3.15 (sept, J=6.9 Hz), an oxygenated methylene at δ 3.92, 4.03 (each d, J=8.0 Hz), in addition to the signals at δ 6.96 (s), and 7.34 (s) attributed to a 1,2,4,5-tetrasubstituted benzene ring. The above spectral data suggested **2** to be a secoabietane diterpene.[16] The gross strucuture of **2** was determined by analysis of the NMR data, including HMQC, HMBC, and ROESY experiments. The HMBC experiment (Fig. 1) of **2** showed the long-range correlations from H-11 (δ 6.96) to C-12, and C-13; from H-14 (δ 7.34) to C-7 (δ 177.3), and C-12; and from H-15 (δ 3.15) to C-12, C-13 and C-14. Thus, the aromatic moiety of **2** was deduced to be 1-carboxy-4-hydroxy-5-isopropyl-2-substituted benzene. Additional HMBC correlations from H_3-19 (δ 1.30) to C-3, C-4, C-5, and C-18 (δ 81.8); from H_3-20 (δ 1.61) to C-1, C-5, C-9, and C-10; from H-5 (δ 3.25) to C-6 (δ 177.3); from H-11 (δ 6.96) to C-10; and from H_2-18b (δ 4.03) to C-4, C-5, C-6, and C-19 established the γ-lactone ring between C-6/C-18 in 6,7-*seco*-12-hydroxydehydroabietinol.[17] Furthermore, NOEs (Fig. 2) between H_3-19 (δ 1.30) and H-2β (δ 1.86), between H_3-19 and H-3β (δ 1.80), and between H_3-19 and H_3-20 (δ 1.61), confirmed the α-orientations of H-5 and H_2-18. From the above findings and the biosynthesis considerations, the structure of hanagokenol B was established to be that shown as **2**.

The isolation of diterpenoids, for example, labdane and isopimarane diterpenes[18,19] from lichen is very rare. To the best of our knowledge, this is the first report of abietane diterpenes (**1**—**6**) from lichen. Also, the abietane anhydride derivative, obtuanhydride (**3**) was obtained for the second time as a natural product. Seventeen compounds isolated from *C. rangiferina* were evaluated for their antimicrobial activity by the disk-diffusion test for MRSA and VRE.[20] Among those tested, compounds 1 and 2 showed mild activity. Depside derivatives, (**14**, **15**) and benzofuran derivatives, (**16**, **17**) showed good correlation activities against MRSA and VRE strains (Table 2).

Experimental

General Experimental Procedures Optical rotations were taken on a JASCO DIP-1400 digital polarimeter; IR spectra were measured on a JASCO FT/IR-5300 instrument and UV spectra were recorded with a Shimadzu UV-6000 spectrophtometer. NMR spectra were recorded on a Varian UNITY 600 spectrometer. The chemical shifts are given in δ (ppm) in C_5D_5N or $CDCl_3$ solution, using tetramethylsilane (TMS) as an internal standard. NMR experiments included ^{1}H–^{1}H COSY, HMQC, HMBC, and ROESY. Coupling constants (J values) are given in hertz (Hz). HR-EI-MS were measured on a JEOL JMS-700 MS station. Kiesegel 60 (230—400 mesh, Merck) was used for column chromatography, and silica gel 60F-254 (Merck) for TLC. HPLC was carried out on a JASCO-PU 1580 instrument using a COSMOSIL C18 P-MS (4.6×150 mm and 20×250 mm) column.

Lichen Material *Cladonia rangiferina* (L.) Web. from Naka-cho, Tokushima, was collected in March, 2004 and identified by Akinori Kawamata, a chief researcher from Ehime Prefectural Science Museum. A voucher specimen (TB 3101) has been deposited in the Herbarium of the Department of Pharmacognosy, Tokushima Bunri University, Tokushima, Japan.

Extraction and Isolation *C. rangiferina* (280 g) was exhaustively extracted with AcOEt at room temperature for 8 weeks. The AcOEt extract was evaporated under a vacuum to yield a brown residue (10.4 g), which was subjected to silica gel column chromatography with hexane–AcOEt–MeOH (1 : 9 : 0→0 : 10 : 3) to afford fractions 1—7. Fraction 2 (0.1 g) was purified by preparative HPLC (78—100% MeOH, flow rate 8 ml/min) to yield *cis*-communic acid (**7**, 38.6 mg). Fraction 3 (0.65 g) was passed through silica gel with hexane–AcOEt (1 : 1→1 : 5) and purified by preparative HPLC (73—100% MeOH, flow rate 8 ml/min) to afford 5,6-dehydrosugiol (**5**, 5.0 mg), monbretol (**6**, 8.8 mg), acetylimbricatoloic acid (**9**, 7.9 mg), and β-resoncylic acid (**12**, 11.5 mg). Fraction 4 (0.51 g) was passed through silica gel with hexane–AcOEt (3 : 7→0 : 10) and purified by preparative HPLC (70—100% MeOH, flow rate 8 ml/min) to yield obtuanhydride (**3**, 3.8 mg), sugiol (**4**, 18.7 mg), atranol (**13**, 9.1 mg), barbatic acid (**14**, 12.9 mg), homosekikaic acid (**15**, 78.0 mg), didymic acid (**16**, 12.9 mg), and condidymic acid (**17**, 11.5 mg). Fraction 5 (0.39 g) was purified by preparative HPLC (70% MeOH, flow rate 8 ml/min) to afford imbricatoloic acid (**8**, 5.2 mg), and junicedric acid (**10**, 4.6 mg). Fraction 6 (0.43 g) was passed through silica gel with hexane–AcOEt (7 : 3→0 : 10) and purified by preparative HPLC (70—100% MeOH, flow rate 8 ml/min) to afford hanagokenols A (**1**, 2.8 mg), B (**2**, 2.9 mg), and 7α-hydroxy-sandaracopimaric acid (**11**, 2.3 mg).

Hanagokenol A (**1**): An amorphous solid; $[\alpha]_D^{25}$ +185.9° (c=0.18, MeOH); FT-IR (dry film) cm^{-1}: 3370, 1690, 1610; UV λ_{max} (MeOH) nm (log ε): 217 (4.90), 236 (4.57), 288 (4.57); ^{1}H- and ^{13}C-NMR see Table 1; HR-EI-MS m/z 314.1864 (Calcd for $C_{20}H_{26}O_3$: 314.1882).

Hanagokenol B (**2**): An amorphous solid; $[\alpha]_D^{25}$ −14.4° (c=0.29, MeOH); FT-IR (dry film) cm^{-1}: 3370, 1760, 1705, 1610, 1590, 1030; UV λ_{max} (MeOH) nm (log ε): 212 (4.11), 233 (4.17), 287 (4.00); ^{1}H- and ^{13}C-NMR see Table 1; HR-EI-MS m/z 346.1768 (Calcd for $C_{20}H_{26}O_5$: 346.1781).

Preparation of Bacterial Cells *Staphylococcus aureus* COL (MRSA), and *E. fuecium* (VanA) (VRE) strains were from laboratory stock cultures. After culturing all strains on Mueller-Hinton agar (Difco, Detroit, MI, U.S.A.), the cells were resuspended in Mueller-Hinton broth (Difco) to give 10^5 colony-forming units/ml, the resuspended cells were then incubated.

Determination of Antimicrobial Activity During extraction and purification, disk-diffusion tests were performed with Whatman AA disks (6 mm) containing the test compounds (**1**—**17**, each 10 mg/ml), positive control sample (erythromycin and tetracyline, each 100 mg/ml), and DMSO as a control. The disks were placed on Mueller-Hinton agar inoculated with 10^5 colony-forming units/ml of MRSA and VRE. The zone of inhibition was determined after incubation at 37 °C for 24 h. A disk that contained DMSO showed no zone of inhibition.

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