Antibacterial Novel Phenolic Diterpenes from *Podocarpus macrophyllus* D. Don

Kimihiko Sato,[a] Keito Sugawara,[a] Hirono Takeuchi,[a] Hyun-Sun Park,[b] Toshiyuki Akiyama,[b] Tetsuo Koyama,[b] Yutaka Aoyagi,[a] Koichi Takeya,*[,a] Takanori Tsugane,[c] and Susumu Shimura[c]

[a] *School of Pharmacy, Tokyo University of Pharmacy & Life Science; 1432–1 Horinouchi, Hachioji, Tokyo 192–0392, Japan:* [b] *The Kochi Prefectural Makino Botanical Garden; 4200–6 Godaisan, Kochi, Kochi 781–8125, Japan: and* [c] *Department of Basic Research, Lotte Central Laboratory Co., Ltd.; 3–1–1 Numakage, Minami-ku, Saitama 336–8601, Japan.* Received July 31, 2008; accepted September 24, 2008; published online September 30, 2008

Two new sempervirol type diterpenes, inumakiols A, B, and six new totarol type diterpenes, inumakiols C—H, were isolated from a methanolic extract of bark of *Podocarpus macrophyllus* (Podocarpaceae), along with one known abietane, two known totarol type diterpenes, and one known totarol type diterpene dimer. The structures of the new compounds were elucidated by the spectroscopic methods. Some of them possessed antibacterial activity against oral pathogenic microorganisms with minimum inhibitory concentration (MIC) values ranging from 3.1 to 25 ppm.

Key words *Podocarpus macrophyllus*; diterpene; antibacterial activity; totarol type diterpene; sempervirol type diterpene

Podocarpus macrophyllus D. Don (Podocarpaceae) (Japanese name: *Inumaki*) is a dioecious evergreen tree distributed in the subtropical areas of south eastern China, Taiwan, and Japan. From this plant, flavonoids, bisflavonoids, norditerpenoids, and ecdysons have been obtained[1—4] and from the leaves of *Podocarpus macrophyllus* var. *maki* (Japanese name: *Rakanmaki*), norditerpenes and totarols having cytotoxic activities against P388 murine leukemia cells have been reported by us.[5—7] In the present study, from the bark of *Podocarpus macrophyllus* D. Don, we isolated 8 new diterpenes along with known ones. This paper describes the isolation and structural elucidation of these new diterpenes and their antibacterial activities against oral pathogenic microorganisms.

Results and Discussion

By sequential Diaion® HP-20 column chromatography (H_2O–MeOH) and silica gel column chromatography ($CHCl_3$–MeOH) and the following repeated reversed-phase HPLC (MeCN–H_2O, MeOH–H_2O), a hot methanol extract of the bark of *Podocarpus macrophyllus* D. Don gave 8 new diterpene compounds (**1**—**8**) along with known lambertic acid (**9**),[8] 4β-carboxy-19-nortotarol (**10**),[8] totaradiol (**11**),[8] and macrophyllic acid (**12**).[9]

Compound **1** was isolated as a colorless amorphous solid with $[\alpha]_D$ +262.6° ($CHCl_3$) and mp 124—126 °C. The molecular formula was determined to be $C_{20}H_{28}O_3$ from the $[M^+ + Na]$ ion peak at *m*/*z* 339.1927 in HR-ESI-MS, implying the presence of 7 degrees of unsaturation. The IR absorption spectrum showed bands of carbonyl (1694 cm^{-1}) and hydroxyl groups (3376 cm^{-1}). The ^1H- and ^{13}C-NMR spectra of **1** (Table 1) showed the presence of an isopropyl group [δ_C 28.0 (d), 23.2 (q), 23.4 (q) and δ_H 3.70 (m), 1.42 (d), 1.46 (d)], an aromatic ring [δ_C 153.5 (s), 139.8 (s), 133.9 (s), 133.5 (s), 123.7 (d), 115.4 (d)], two methyl carbons [δ_C 29.2 (q) and 24.0 (q)] attached to quaternary carbons, and a carboxyl carbonyl carbon [δ_C 180.0 (s)]. These data and the molecular formula suggested that **1** was an isomer of a known diterpene, 4β-carboxy-19-nortotarol (**10**) obtained also from this material. The heteronuclear multiple bond coherence (HMBC) correlations observed between the hydrogens H-15, H16, H-17 and the carbon C-12, and between the hydrogens H-14, H15, H-16 and the carbon C-13 indicated that the isopropyl group was at C-12 and the phenolic hydroxyl group at C-13. Further HMBC correlations between the hydrogens H-3 and H-18 and the carbonyl carbon C-19 showed that the carboxyl group was at C-4. The nuclear Overhauser effect (NOE)s between the methine proton H-5 and both the methyl protons H-18 and H-1α, respectively, and between the methyl protons H-20 and H-1β implied that the ring junction of A/B was *trans* and the stereochemistry of the methyl group attached to C-4 was α-oriented (Fig. 2). Hence, **1** was concluded to be 13-hydroxy-12-isopropyl-8,11,13-podocarpatrien-19-oic acid and was named inumakiol A. Only very few 12-isopropyl diterpenes are known and **1** was shown to be an analogue of such 12-isopropyl diterpene, sempervirol,[10] whose methyl group at C-4 is replaced by a carboxy group (Fig. 1).

2 was isolated as a colorless amorphous solid with $[\alpha]_D$ +11.0° ($CHCl_3$) and mp 228—233 °C. The molecular formula was determined to be $C_{20}H_{28}O_3$ from the $[M^+ + Na]$ ion peak at *m*/*z* 339.1895 in HR-ESI-MS, implying the presence of 7 degrees of unsaturation. The IR absorption spectrum showed bands of carbonyl (1646 cm^{-1}) and hydroxyl groups (3432 cm^{-1}). The ^1H- and ^{13}C-NMR spectra of **2** showed the presence of an isopropyl group [δ_C 28.5 (d), 22.7 (q), 22.6 (q) and δ_H 3.73 (1H, septet, 6.9), 1.42 (3H, d, 6.9), 1.45 (3H, d, 6.9)], an aromatic ring [δ_C 154.3 (s), 148.3 (s), 142.8 (s), 130.1 (s), 122.3 (d), 112.4 (d)], two methyl groups [δ_C 27.3 (q) and 24.3 (q)] attached to quaternary carbons, a keto carbonyl carbon [δ_C 198.6 (s)], and a hydroxyl methylene group [δ_C 64.3 (t), δ_H 4.04 (1H, dd, 10.8, 5.2) and 3.88 (1H, dd, 10.8, 5.2)] (Table 1). The HMBC correlations observed between the hydrogens H-15, H16, H-17 and the carbon C-12 and between the hydrogen H-14 and the carbon C-13 indicated that the isopropyl group was at C-12 and the phenolic hydroxyl group at C-13. Further HMBC correlations between the hydrogen H-5 and the carbonyl carbon C-7, and between the hydrogens H-19 and H-18 and the carbon C-4 implied that C-7 bore carbonyl oxygen and that the hydroxy methyl

* To whom correspondence should be addressed. e-mail: takeyak@ps.toyaku.ac.jp

Table 1. ^{1}H- and ^{13}C-NMR Data of Compounds **1**—**8**

Position	**1**[a]		**2**[a]		**3**[a]		**4**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.50 (1H, m) 2.40 (1H, m)	40.4 (t)	1.53 (1H, m) 2.37 (1H, d, 12.4)	38.6 (t)	2.54 (1H, d, 13.7) 3.23 (1H, dd, 13.7, 1.7)	56.5 (t)	1.22 (1H, m) 2.26 (1H, d, 12.4)	40.3 (t)
2	1.69 (1H, m) 2.40 (1H, m)	20.9 (t)	1.58 (1H, m) 1.81 (1H, m)	19.2 (t)		207.9 (s)	1.57 (1H, m) 2.35 (1H, m)	20.9 (t)
3	1.14 (1H, ddd, 12.8, 12.8, 4.0) 2.56 (1H, d, 12.8)	38.5 (t)	1.06 (1H, ddd, 13.9, 13.9, 3.5) 2.16 (1H, d, 13.9)	36.1 (t)	2.27 (1H, d, 13.7) 3.44 (1H, dd, 13.7, 1.7)	51.8 (t)	1.09 (1H, ddd, 13.1, 13.1, 3.9) 2.55 (1H, d, 13.1)	38.4 (t)
4		44.2 (s)		38.7 (s)		47.4 (s)		43.7 (s)
5	1.64 (1H, dd, 12.0, 2.3)	53.6 (d)	2.02 (1H, dd, 12.5, 5.3)	50.6 (d)	2.13 (1H, d, 11.9)	51.1 (d)	2.47 (1H, d, 12.8)	45.6 (d)
6	2.40 (1H, m) 2.40 (1H, m)	22.0 (t)	3.04 (1H, m) 3.04 (1H, m)	36.8 (t)	2.38 (1H, dddd, 13.3, 12.9, 12.9, 5.5) 2.57 (1H, dd, 12.9, 5.5)	22.0 (t)	2.74 (1H, m) 2.91 (1H, d, 14.3)	32.7 (t)
7	2.80 (1H, m) 2.87 (1H, m)	32.2 (t)		198.6 (s)	2.83 (1H, m) 3.15 (1H, dd, 16.7, 5.5)	30.2 (t)	5.49 (1H, s)	65.5 (d)
8		133.9 (s)		130.1 (s)		134.3 (s)		136.6 (s)
9		139.8 (s)		148.3 (s)		138.1 (s)		140.3 (s)
10		38.7 (s)		38.2 (s)		42.4 (s)		39.2 (s)
11	7.38 (1H, s)	123.7 (d)	7.42 (1H, s)	122.3 (d)	7.04 (1H, d, 8.6)	124.4 (d)	7.16 (1H, d, 8.5)	124.4 (d)
12		133.5 (s)		142.8 (s)	7.01 (1H, d, 8.6)	115.6 (d)	7.19 (1H, d, 8.5)	117.3 (d)
13		153.5 (s)		154.3 (s)		155.4 (s)		155.7 (s)
14	6.91 (1H, s)	115.4 (d)	8.01 (1H, s)	112.4 (d)		131.7 (s)		134.0 (s)
15	3.70 (1H, m)	28.0 (d)	3.73 (1H, sept, 6.9)	28.5 (d)	3.49 (1H, br s)	28.3 (d)	4.25 (1H, m)	28.5 (d)
16	1.42 (3H, d, 7.0)	23.2 (q)	1.42 (3H, d, 6.9)	22.7 (q)	1.71 (3H, d, 7.3)	20.8 (q)	1.86 (3H, d, 7.0)	21.3 (q)
17	1.46 (3H, d, 7.0)	23.4 (q)	1.45 (3H, d, 6.9)	22.6 (q)	1.63 (3H, d, 7.3)	20.6 (q)	1.72 (3H, d, 7.0)	21.2 (q)
18	1.41 (3H, s)	29.2 (q)	1.18 (3H, s)	27.3 (q)	1.57 (3H, s)	28.4 (q)	1.55 (3H, s)	29.1 (q)
19		180.0 (s)	3.88 (1H, dd, 10.8, 5.2) 4.04 (1H, dd, 10.8, 5.2)	64.3 (t)		179.1 (s)		180.4 (s)
20	1.48 (3H, s)	24.0 (q)	1.30 (3H, s)	24.3 (q)	1.48 (3H, s)	25.2 (q)	1.40 (3H, s)	23.1 (q)
7-OH							6.47 (1H, br s)	
13-OH	10.9 (1H, s)		11.77 (1H, s)		10.9 (1H, s)		10.8 (1H, s)	
19-OH			5.99 (1H, dd, 10.8, 5.2)					

Table 1. (Continued)

Position	**5**[a]		**6**[a]		**7**[a]		**8**[b]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	1.39 (1H, m) 2.29 (1H, d, 13.4)	40.5 (t)	1.70 (1H, m) 2.25 (1H, d, 12.4)	37.4 (t)	1.54 (1H, ddd, 13.4, 13.4, 3.7) 2.26 (1H, d, 13.4)	39.4 (t)	1.48 (1H, ddd, 13.0, 13.0, 3.0) 2.29 (1H, m)	38.3 (t)
2	1.60 (1H, d, 13.4) 2.38 (1H, m)	20.9 (t)	1.70 (1H, m) 2.32 (1H, d, 13.3)	20.6 (t)	1.66 (1H, m) 2.32 (1H, m)	20.6 (t)	2.06 (1H, m) 2.18 (1H, m)	29.5 (t)
3	1.14 (1H, ddd, 13.4, 13.4, 3.5) 2.57 (1H, d, 13.4)	38.3 (t)	1.09 (1H, ddd, 13.3, 13.3, 3.8) 2.56 (1H, d, 13.3)	38.0 (t)	1.08 (1H, ddd, 13.1, 13.1, 3.7) 2.50 (1H, d, 13.1)	38.4 (t)	3.67 (1H, d, 11.4)	80.0 (d)
4		43.7 (s)		43.4 (s)		44.2 (s)		43.1 (s)
5	2.22 (1H, d, 13.7)	45.7 (d)	2.37 (1H, m)	51.3 (d)	2.08 (1H, dd, 13.3, 6.4)	49.6 (d)	1.42 (1H, d, 12.5)	50.5 (d)
6	2.38 (1H, m) 2.91 (1H, d, 13.7)	24.8 (d)	7.07 (1H, m)	121.6 (d)	3.20 (1H, dd, 18.4, 6.4) 4.20 (1H, dd, 18.4, 12.9)	40.4 (t)	1.75 (1H, m) 2.06 (1H, m)	20.1 (t)
7	4.65 (1H, s)	75.3 (d)	7.14 (1H, d, 2.8)	123.0 (d)		202.9 (s)	2.77 (1H, m) 3.03 (1H, dd, 16.9, 6.1)	30.0 (t)
8		134.4 (s)		131.9 (s)		134.7 (s)		133.0 (s)
9		140.6 (s)		139.3 (s)		146.6 (s)		141.3 (s)
10		39.2 (s)		38.9 (s)		38.8 (s)		37.7 (s)
11	7.12 (1H, d, 8.5)	124.3 (d)	7.07 (1H, m)	131.6 (d)	7.19 (1H, d, 8.5)	122.9 (d)	7.08 (1H, d, 8.5)	123.0 (d)
12	7.16 (1H, d, 8.5)	117.7 (d)	7.07 (1H, m)	115.6 (d)	7.27 (1H, d, 8.5)	120.8 (d)	7.01 (1H, d, 8.5)	115.2 (d)
13		155.5 (s)		155.6 (s)		156.6 (s)		155.1 (s)
14		134.0 (s)		130.3 (s)		135.0 (s)		131.2 (s)
15	3.50 (1H, m)	28.9 (d)	3.83 (1H, m)	28.5 (d)	4.39 (1H, m)	29.2 (d)	3.41 (1H, br s)	28.1 (d)
16	1.75 (3H, d, 6.9)	21.0 (q)	1.64 (3H, d, 7.0)	21.7 (q)	1.85 (3H, d, 7.0)	21.2 (q)	1.66 (3H, d, 7.0)	20.7 (q)
17	1.83 (3H, d, 6.9)	21.1 (q)	1.66 (3H, d, 7.0)	21.5 (q)	1.76 (3H, d, 7.0)	21.6 (q)	1.61 (3H, d, 7.0)	20.6 (q)
18	1.49 (3H, s)	29.1 (q)	1.44 (3H, s)	28.5 (q)	1.33 (3H, s)	28.4 (q)	1.59 (3H, s)	23.6 (q)
19		181.7 (s)		179.8 (s)		180.2 (s)	3.77 (1H, dd, 10.9, 7.6) 4.60 (1H, d, 10.9)	64.2 (t)
20	1.39 (3H, s)	22.9 (q)	1.31 (3H, s)	20.6 (q)	1.30 (3H, s)	22.6 (q)	1.25 (3H, s)	26.5 (q)
7-OCH_3	3.51 (3H, s)	55.2 (q)						
3-OH							6.72 (1H, d, 3.6)	
13-OH			10.96 (1H, br s)				10.75 (1H, s)	
19-OH							5.41 (1H, d, 7.6)	

a) ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data were measured in pyridine-d_5. *b*) ^{1}H (600 MHz) and ^{13}C (150 MHz) NMR data were measured in pyridine-d_5.

group was at C-4. The NOE correlations noted between the hydroxy methylene protons H-19 and the methyl protons H-20, and between the methyl protons H-18 and the methine proton H-5 implied that the hydroxy methylene group at C-4 and the methyl group C-18 were β- and α-oriented and that the ring junction of A/B was *trans* (Fig. 2). Consequently, the structure of **2** was concluded to be 13,19-dihydroxy-12-isopropyl-7-oxo-8,11,13-podocarpatriene and was named inumakiol B. It has the same carbon skeleton as **1**, and is also a sempervirol type diterpene (Fig. 1).

3 was isolated as colorless needles with $[\alpha]_D$ +41.9° ($CHCl_3$) and mp 142—143 °C. The molecular formula was determined to be $C_{20}H_{26}O_4$ from the $[M^+ + Na]$ ion peak at *m/z* 353.1730 in HR-ESI-MS implying the presence of 8 degrees of unsaturation. The IR absorption spectrum showed bands of carbonyl (1705, 1698 cm^{-1}) and hydroxyl groups (3358 cm^{-1}). The ^1H- and ^{13}C-NMR spectra of **3** showed the presence of an isopropyl group [δ_C 28.3 (d), 20.8 (q), 20.6 (q) and δ_H 3.49 (1H, br s), 1.71 (3H, d, 7.3), 1.63 (3H, d, 7.3)], an aromatic ring [δ_C 155.4 (s), 138.1 (s), 134.3 (s), 131.7 (s), 124.4 (d), 115.6 (d)], two methyl groups [δ_C 28.4 (q) and 25.2 (q)] attached to quaternary carbons, a ketone carbonyl group [δ_C 207.9 (s)], and a carboxy carbonyl group [δ_C 179.1 (s)] (Table 1). The HMBC correlations observed between the hydrogens H-15, H16, H-17 and the carbon C-14 and between the hydrogen H-11 and the carbon C-13 indicated that the isopropyl group was at C-14, and the phenolic hydroxyl group at C-13. Further HMBC correlations observed between the hydrogens H-3, H-5, and H-18 and the carbonyl carbon C-19 and between the hydrogens H-1 and H-3 and the carbon C-2 showed that the carboxyl group was present at C-4, and that C-2 bore a keto carbonyl oxygen, respectively. The NOE between the methyl protons H-18 and the methine proton H-5 implied that the methyl group at C-4 and the carboxyl group at C-4 were α- and β-oriented, respectively. On the other hand, the NOEs between H-1β and the methyl protons H-20 and between H-1α and the methine proton H-5 implied that the ring junction of A/B was *trans* (Fig. 2). Consequently, the structure of **3** was concluded to be 13-hydroxy-14-isopropyl-2-oxo-8,11,13-podocarpatrien-19-oic acid and was named inumakiol C (Fig. 1).

4 was isolated as a colorless amorphous solid with $[\alpha]_D$ +74.4° (MeOH) and mp 135—136 °C. The molecular formula was determined to be $C_{20}H_{28}O_4$ from the $[M^+ + Na]$ ion peak at *m/z* 355.1889 in HR-ESI-MS, implying the presence of 7 degrees of unsaturation. The IR absorption spectrum showed bands of carbonyl (1694 cm^{-1}) and hydroxyl groups (3362 cm^{-1}). The spectral data and the molecular formula suggested that **4** had the same carbon skeleton as **3**. The HMBC correlations observed between the hydrogens H-15, H-12, H-7 and the carbon C-14 and between the hydrogens H-11, H-12, H-15 and the carbon C-13 indicated that the isopropyl group was at C-14 and the phenolic hydroxyl group at C-13. Other HMBC correlations noted between the hydrogens H-5 and H-6 and the carbon C-7 and between the hydrogen H-7 and the carbons C-8, C-9, C-5, and C-14 showed that the hydroxyl group was present at C-7. The NOEs between H-5 and the methyl protons H-18 and H-1α implied

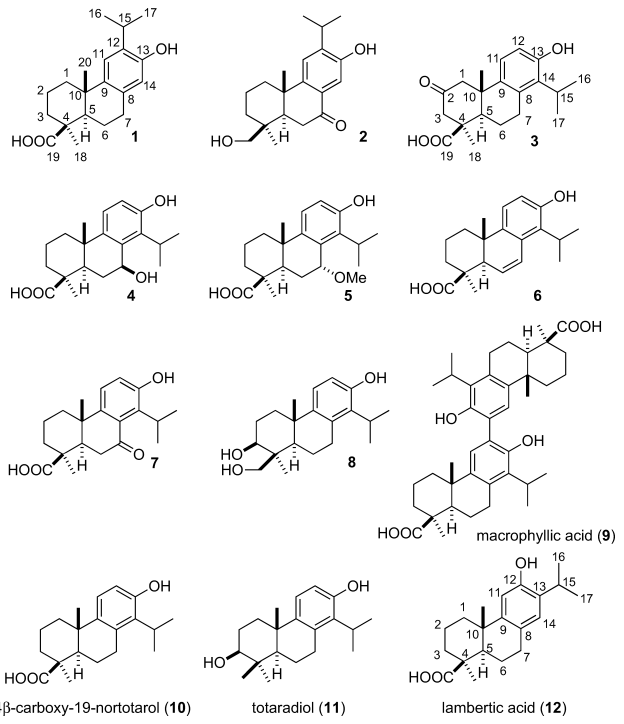

Fig. 1. New and Known Phenolic Diterpenes Isolated in the Present Study from Bark of *Podocarpus macrophyllus* D. Don

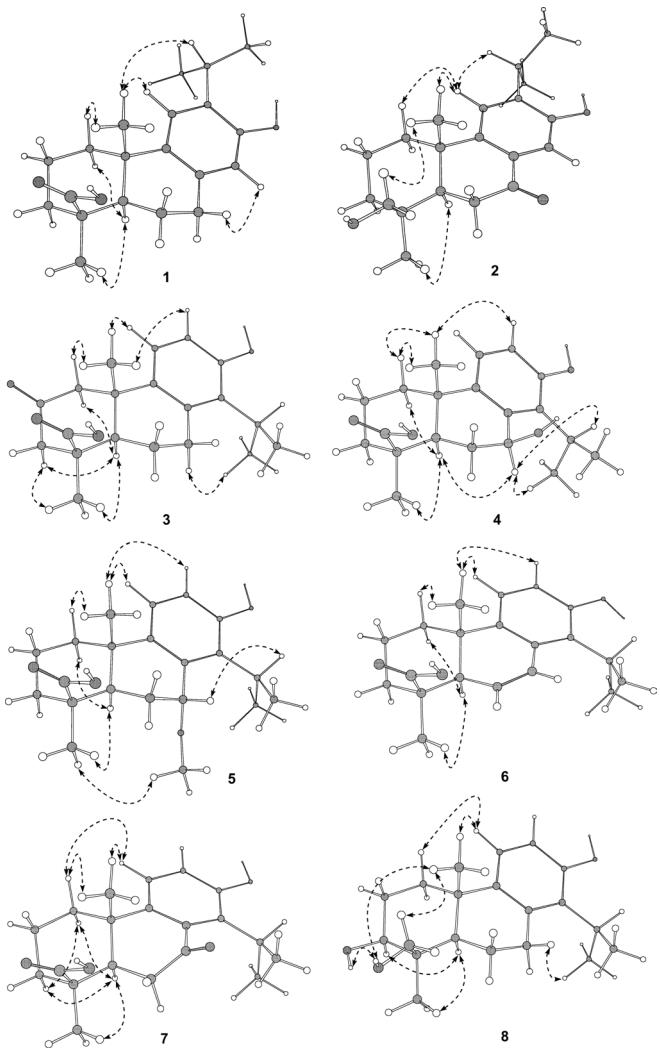

Fig. 2. Selected NOESY Correlations for Compounds **1**—**8**

that the methyl group and the carboxy group at C-4 were α- and β-oriented, respectively. The NOE between the methine protons H-7 and H-5 implied that the hydroxyl group at C-7 was β-oriented. On the other hand, The NOE between H-1β and the methyl protons H-20 implied that the ring junction of A/B was *trans*. Thus, the structure of **4** was concluded to be (7*S*)-7,13-dihydroxy-14-isopropyl-8,11,13-podocarpatrien-19-oic acid and was named inumakiol D (Fig. 1).

5 was isolated as colorless needles with $[\alpha]_D$ +86.8° (pyridine) and mp 255—257 °C. The molecular formula was determined to be $C_{21}H_{30}O_4$ from the $[M^+ + Na]$ ion peak at *m/z* 369.2029 in HR-ESI-MS implying the presence of 7 degrees of unsaturation. The IR absorption spectrum showed bands of carbonyl (1695 cm^{-1}) and of hydroxyl groups (3346 cm^{-1}). ^{1}H-NMR, ^{13}C-NMR, and distortionless enhancement by polarization transfer (DEPT) spectra of **5** indicated that **5** was a methyl ether of **4** (Table 1). The HMBC correlations observed between the methyl protons of the methoxy group and the carbon C-7 indicated that the methoxyl group was at C-7. The NOE between H-5 and the methyl protons H-18 implied that the methyl group at C-4 and the carboxyl group at C-4 were α- and β-oriented, respectively. The NOE between the methyl protons of the methoxy group at C-7 and the methyl group at C-4 implied that the methoxy group at C-7 was α-oriented. Further, the NOEs between the methine proton H-5 and both H-1α and the methyl protons H-18 and between the methyl protons H-20 and H-1β implied that the ring junction of A/B was *trans* (Fig. 2). Finally, the crystals from methanol were subjected to X-ray crystallographic analysis,[11] which showed the structure of **5** to be (7*R*)-13-hydroxy-14-isopropyl-7-methoxy-8,11,13-podocarpatrien-19-oic acid and was named inumakiol E (Figs. 1, 3).

6 was isolated as a colorless amorphous solid with $[\alpha]_D$ −10.6° ($CHCl_3$) and mp 250—252 °C. The molecular formula was determined to be $C_{20}H_{26}O_3$ from the $[M^+ + H]$ ion peak at *m/z* 315.1946 in HR-ESI-MS, implying the presence of 8 degrees of unsaturation. The IR absorption spectrum showed bands of carbonyl (1694 cm^{-1}) and hydroxyl groups (3390 cm^{-1}). The ^{1}H-NMR, ^{13}C-NMR, and DEPT spectral data of **6** were quite similar to those of **4** and **5**, excepting for the chemical shift values of C-6 and C-7 signals in the ^{13}C-NMR spectral data, suggesting that **4**, **5**, and **6** had the same basic structure (Table 1). The HMBC correlations observed between the hydrogen H-5 and the carbon C-7, and between the hydrogen H-6 and the carbons C-8 and C-18 indicated that **6** had Δ^6 in the molecule. The NOE between H-5 and the methyl protons H-18 implied that the methyl and the carboxyl groups at C-4 were α- and β-oriented, respectively. On the other hand, the NOEs between the methine proton H-5 and both H-1α and the methyl protons H-18 and between the methyl protons H-20 and H-1β implied that the ring junction of A/B was *trans* (Fig. 2). Consequently, the structure of **6** was concluded to be 13-hydroxy-6,8,11,13-podocarpatetraen-19-oic acid and was named inumakiol F (Fig. 1).

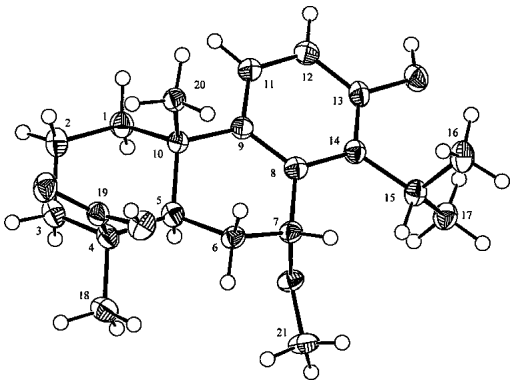

Fig. 3. Ortep Representation for Inumakiol E (**5**)

7 was isolated as a colorless amorphous solid with $[\alpha]_D$ −12.0° (MeOH) and mp 122—123 °C. The molecular formula was determined to be $C_{20}H_{26}O_4$ from the $[M^+ + H]$ ion peak at *m/z* 331.1909 in HR-ESI-MS, implying the presence of 8 degrees of unsaturation. The IR absorption spectrum showed bands of carbonyl (1694, 1657 cm^{-1}) and of hydroxyl groups (3366 cm^{-1}). The ^{1}H-NMR, ^{13}C-NMR, and DEPT spectral data of **7** were quite similar to those of **6**, excepting for the data due to C-6, C-7, C-8, and C-9 (Table 1). The HMBC correlations observed between the hydrogen H-6 and the carbon C-7 indicated that C-7 bore carbonyl oxygen. The NOE between H-5 and the methyl protons H-18 implied that the methyl and the carboxyl groups at C-4 were α- and β-oriented, respectively. On the other hand, the NOEs between the methyl protons H-20 and H-1β and between the methine proton H-5 and H-1α implied that the ring junction of A/B was *trans* (Fig. 2). Consequently, the structure of **7** was concluded to be 13-hydroxy-14-isopropyl-7-oxo-8,11,13-podocarpatrien-19-oic acid and was named inumakiol G (Fig. 1).

8 was isolated as colorless amorphous solid with $[\alpha]_D$ +34.4° (MeOH) and mp 118—120 °C. The molecular formula was determined to be $C_{20}H_{30}O_3$ from the $[M^+ + Na]$ ion peak at *m/z* 341.2062 in HR-ESI-MS, implying the presence of 6 degrees of unsaturation. The IR absorption spectrum showed bands of hydroxyl groups (3441, 3213 cm^{-1}). The ^{1}H- and ^{13}C-NMR spectra of **8** indicated the presence of an isopropyl group [δ_C 28.1 (d), 20.7 (q), 20.6 (q) and δ_H 3.41 (1H, br s), 1.66 (3H, d, 7.0), 1.61 (3H, d, 7.0)], an aromatic ring [δ_C 155.1 (s), 141.3 (s), 133.0 (s), 131.2 (s), 123.0 (d), 115.2 (d)], two methyl groups [δ_C 26.5 (q) and 23.6 (q), δ_H 1.25 and 1.59 (each 3H, s)] attached to quaternary carbons, and a hydroxymethyl group [δ_C 64.2 (t), δ_H 4.60 (1H, d, 10.9) and 3.77 (1H, dd, 10.9, 7.6)]. The HMBC correlations between the hydrogens H-3, and H-5 and the carbon C-18, and between the hydrogens H-1 and H-2 and the carbon C-3 showed that the hydroxyl groups were present at C-3 and C-19. Further HMBC correlations observed between the hydrogens H-12 and H-7 and the carbon C-14, and between the hydrogens H-11 and H-12 and the carbon C-13 and the NOEs between the hydrogens H-16 or H-17 and H-7, and between H-1 and H-11 indicated that the isopropyl group was at C-14 and the phenolic hydroxyl group at C-13. The NOEs between the hydrogens H-3 and H-5, H-19 and H-20, and H-5 and H-18 implied that the methyl group at C-4 and the hydroxy group at C-3 were α- and β-oriented, respectively (Fig. 2). The ring junction of A/B was also *trans*. Consequently, the structure of **8** was (3*S*)-14-isopropyl-3,13,19-trihydroxy-8,11,13-podocarpatriene and was named inumakiol H (Fig. 1).

Macrophyllic acid (**9**) isolated is a known compound with the spectral and physical data as reported.[10] Since the structure of **9** was determined only on the basis of spectral data

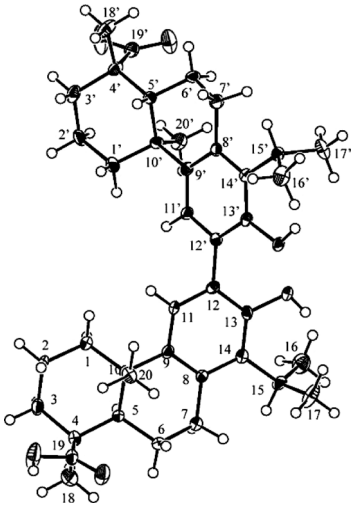

Fig. 4. Ortep Representation for Macrophyllicacid (**9**)

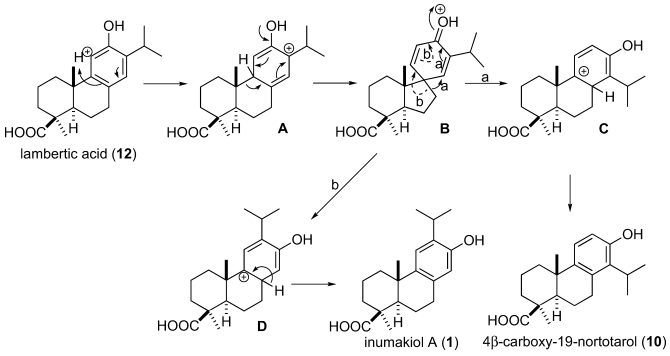

Chart 1. Plausible Biogenetic Synthetic Pathway of Semperviol and Totarol Type Diterpenes from Ferruginol Type Diterpenes

and chemical evidences, just for confirmation of the proposed structure, the crystals of **9** from MeOH were prepared and subjected to X-ray crystallographic analysis.[12] The proposed structure was identical to a crystallographically refined structure of **9** (Fig. 4).

In this study from the bark of *Podocarpus macrophyllus* D. Don, we isolated 8 new (compounds **1**—**8**) and 4 known (compounds **9**—**12**) diterpenes of sempervirol, totarol, and ferruginol types. Those are congeners having an isopropyl group at C-12, C-13, or C-14 in the ring C, and may be considered to be biogenetically related to each other, as shown in Chart 1.[13] Namely, protonation of lambertic acid (**12**) gives an intermediate cation **A**, which, after rearrangement of C-10 to C-8, gives **B**. Subsequent rearrangement (b) of C-7 to C-9 in the cation **B** gives **D**, which by elimination of proton gives an inumakiol A (**1**) type diterpene having an isopropyl group at C-12. On the other hand, rearrangement (a) of C-7 to C-14 in the cation **B** produces 4β-carboxy-19-nortotarol (**10**) with the isopropyl group at C-14 type diterpene *via* **C**.

All the compounds isolated were assayed for their antibacterial activities against eight oral pathogenic microorganisms, *Streptococcus mutans*, *S. sobrinus*, *S. pyogenes*, and *Staphylococcus aureus* (aerobic conditions), *Actinomyces viscosus*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Actinobacillus actinomycetemcomitans* (anaerobic conditions). The results are shown in Table 2 (See Experimental). Compounds **2**, **6**, **8**, **9**, **10**, and **12** showed antibacterial activities, of them, macrophyllic acid (**9**), a totarane diterpene dimer, being the most active.

Table 2. Antibacterial Activity (MIC; ppm) against Oral Pathogenic Microorganisms of Compounds **1**—**12**

Compounds	MIC (ppm)							
	S. m.[a]	*S. s.*[b]	*A. v.*[c]	*P. g.*[d]	*F. n.*[e]	*A. a.*[f]	*S. a.*[g]	*S. p.*[h]
1	—	—	—	—	—	—	—	—
2	—	—	25	25	—	50	50	—
3	—	—	—	50	—	—	—	—
4	—	—	—	—	—	—	—	—
5	—	—	—	—	—	—	—	—
6	—	—	—	25	50	50	50	—
7	—	—	—	50	50	—	—	—
8	—	—	—	25	—	—	50	—
9	6.3	12.5	6.3	3.1	—	—	3.1	—
10	—	50	50	25	50	25	25	—
11	—	—	—	—	—	—	—	—
12	—	—	—	50	—	50	50	—

Thymol was used as reference gave MIC of 100—200 ppm to those bacteria. —: MIC >50 ppm. *a*) *Streptococcus mutans* MT8148R. *b*) *Streptococcus sobrinus* 6715. *c*) *Actinomyces viscosus* ATCC15987. *d*) *Porphyromonas gingivalis* ATCC33277. *e*) *Fusobacterium nucleatum* JCM 8532. *f*) *Actinobacillus actinomycetemcomitans* ATCC29522. *g*) *Streptococcus aureus* IID671. *h*) *Streptococcus pyogenes*.

Experimental

General Experimental Procedures Optical rotations were measured on a JASCO DIP-360 automatic digital polarimeter, IR spectra on a JASCO FT/IR 620 spectrophotometer, and Mass spectra on VG AutoSpec E and Micromass LCT (Manchester, U.K.) spectrometers. NMR spectra were obtained on a Brucker DRX-500 and AV-600 spectrometer at 300K in C_5D_5N. The chemical shifts (δ) of proton signals are given in ppm relative to the resonances of residual $C_5D_4\underline{H}N$ at 7.19 ppm and those of carbon signals are given in ppm relative to the resonances at 135.5 ppm for C_5D_5N and those of residual $CDCl_3$ at 7.26 ppm and those of carbon signals are given in ppm relative to the resonance at 77.6 ppm for $CDCl_3$. Elemental analysis was carried out by using an Elemental Vario EL (Hanau, Germany) and a Mettler DL70ES elemental analyzer. Silica gel (Merck Kiesel gel 60, 70—230 μm, Kanto silica gel N 60, 63—210 μm) and Diaion® HP-20 (Mitsubishi Chemical) were used for column chromatography and precoated Kieselgel 60 F_{254} (0.25 mm thick, Merck), RP-18 $F_{254}S$ (0.25 mm thick, Merck) plates for TLC, in which the spots were visualized by spraying of 10% H_2SO_4 solution, followed by heating. Preparative HPLC was carried out on a JASCO PU-986 equipped with a UV-970 UV detector (λ 220 nm) and Inertsil PREP-ODS column (10 μm, 20×250 mm), by using MeOH/H_2O or MeCN/H_2O at a flow rate of 10 ml/min. X-ray single-crystal analysis was taken on a Mac Science DIP diffractometer with Mo$K\alpha$ radiation (λ= 0.71073 Å).

Plant Material The bark of *Podocarpus macrophyllus* D. Don was collected in Kochi, Japan, in Nobember 2004. The botanical identification was made by K. Takeya, Professor of Plant Chemistry of Tokyo University of Pharmacy and Life Science. A voucher specimen (08JCP18) has been deposited in the herbarium of Tokyo University of Pharmacy and Life Science.

Extraction and Isolation Air-dried bark of *Podocarpus macrophyllus* D. Don (3.2 kg) was extracted with hot MeOH (3×45 l). The combined MeOH extract was concentrated and the residue (284 g) was subjected to Diaion HP-20 resin column chromatography (10 cm×40 cm) eluting with H_2O, 50% MeOH, 80% MeOH, 100% MeOH, and acetone (each 5 l). The fraction eluted with 80% MeOH was concentrated to give the residue (10.4 g), which was subjected to silica gel column chromatography (solvent system: $CHCl_3$–MeOH=19 : 1) to give two fractions fr. 21 (635 mg) and fr. 22 (896 mg). Fr. 21 was purified by ODS-HPLC (solvent system: H_2O–MeCN=60 : 40) to give compounds **5** (45.2 mg), **6** (10.3 mg), **1** (3.8 mg), and **10** (54 mg). Fr. 22 was purified by ODS-HPLC (solvent system: H_2O–MeOH=70 : 30 and then H_2O–MeCN=45 : 55) to give compounds **4** (7.1 mg), **3** (4.1 mg), **8** (4.3 mg), and **7** (4.0 mg). The fraction eluting with 100% MeOH in HP-20 resin column chromatography was concentrated (18.4 g) and subjected to silica gel column chromatography (solvent system: $CHCl_3$ and then $CHCl_3$: MeOH= 19 : 1) to give two fractions fr. 31 (392 mg) and fr. 32 (1440 mg). Fr. 31 was

purified by using silica gel column chromatography (solvent system: hexane : AcOEt=8 : 1) to give compound **10** (1015 mg), and frs. 311 (54 mg) and 312 (336 mg). Fr. 311 was purified by ODS-HPLC (solvent system: H_2O–MeCN=50 : 50) to give compounds **2** (14.2 mg) and **11** (8.2 mg). Fr. 312 was also purified by ODS-HPLC (solvent system: H_2O–MeCN=62 : 38) to give compound **9** (72 mg). Fr. 32 was subjected to repeated ODS-HPLC (solvent system: H_2O–MeCN=25 : 75 and then 40 : 60) to give compounds **12** (8 mg) and **1** (3.8 mg).

Inumakiol A (**1**): A colorless amorphous solid, mp 124—126 °C (MeOH). $[\alpha]_D^{24}$ +262.6° (c=0.10, MeOH); IR (film) 3376 (OH), 1694 (C=O) cm^{-1}; HR-ESI-MS *m/z*: 339.1927 [Calcd for $C_{20}H_{28}O_3Na$ 339.1936 (M^++Na)]. ^{1}H- and ^{13}C-NMR, see Table 1.

Inumakiol B (**2**): A colorless amorphous solid, mp 228—233 °C (MeOH). $[\alpha]_D^{24}$ +11.0° (c=0.05, $CHCl_3$); IR (film) 3432 (OH), 1646 (C=O) cm^{-1}; HR-ESI-MS *m/z*: 339.1895 [Calcd for $C_{20}H_{28}O_3Na$ 339.1936 (M^++Na)]. ^{1}H- and ^{13}C-NMR, see Table 1.

Inumakiol C (**3**): A colorless needles, mp 142—143 °C (MeOH). $[\alpha]_D^{24}$ +41.9° (c=0.10, $CHCl_3$); IR (film) 3358 (OH), 1705, 1698 (each C=O) cm^{-1}; HR-ESI-MS *m/z*: 353.1730 [Calcd for $C_{20}H_{26}O_4Na$ 353.1729 (M^++Na)]. ^{1}H- and ^{13}C-NMR, see Table 1.

Inumakiol D (**4**): A colorless amorphous solid, mp 135—136 °C (MeOH). $[\alpha]_D^{24}$ +74.4° (c=0.10, MeOH); IR (film) 3362 (OH), 1694 (C=O) cm^{-1}; HR-ESI-MS *m/z*: 355.1889 [Calcd for $C_{20}H_{28}O_4Na$ 355.1885 (M^++Na)]. ^{1}H- and ^{13}C-NMR, see Table 1.

Inumakiol E (**5**): A colorless needles, mp 255—257 °C (MeOH). $[\alpha]_D^{24}$ +86.8° (c=0.29, pyridine); IR (film) 3346 (OH), 1695 (C=O) cm^{-1}; HR-ESI-MS *m/z*: 369.2029 [Calcd for $C_{21}H_{30}O_4Na$ 369.2042 (M^++Na)]. ^{1}H- and ^{13}C-NMR, see Table 1.

Inumakiol F (**6**): A colorless amorphous solid, mp 250—252 °C (MeOH). $[\alpha]_D^{24}$ −10.6° (c=0.10, $CHCl_3$); IR (film) 3390 (OH), 1694 (C=O) cm^{-1}; HR-ESI-MS *m/z*: 315.1946 [Calcd for $C_{20}H_{27}O_3$ 315.1960 (M^++H)]. ^{1}H- and ^{13}C-NMR, see Table 1.

Inumakiol G (**7**): A colorless amorphous solid, mp 122—123 °C (MeOH). $[\alpha]_D^{24}$ −12.0° (c=0.10, MeOH); IR (film) 3366 (OH), 1694, 1657 (C=O) cm^{-1}; HR-ESI-MS *m/z*: 331.1909 [Calcd for $C_{20}H_{27}O_4$ 331.1909 (M^++H)]. ^{1}H- and ^{13}C-NMR, see Table 1.

Inumakiol H (**8**): A colorless amorphous solid, mp 118—120 °C (MeOH). $[\alpha]_D^{24}$ +34.4° (c=0.10, MeOH); IR (film) 3441, 3213 (OH) cm^{-1}; HR-ESI-MS *m/z*: 341.2062 [Calcd for $C_{20}H_{30}O_3Na$ 341.2093 (M^++Na)]. ^{1}H- and ^{13}C-NMR, see Table 1.

Compounds **9**—**12** were identified each as lambertic acid (**9**),[8] 4β-carboxy-19-nortotarol (**10**),[8] totaradiol (**11**),[8] and macrophyllic acid (**12**)[9] by the comparison of their NMR data with those reported.

X-Ray Crystallographic Studies Crystal Data for Inumakiol E (**5**)[9]: $C_{21}H_{30}O_4$; FW 346.4605; colorless prisms, monoclinic, space group $P2_1$, unit cell dimensions a=10.0600(3) Å, b=16.9250(10) Å, c=11.9550(7) Å, V=2035.51(18) Å^3, Z=2; d_{calc}=1.183 Mg m^{-3}; μ (Mo$K\alpha$, λ=0.71073 Å)=0.21 mm^{-1}.

Crystal Data for Macrophyllic Acid (**9**)[11]: $C_{21}H_{30}O_4$; FW 346.4605; colorless prisms, triclinic, space group $P2_1$, unit cell dimensions a=10.0600(3) Å, b=16.9250(10) Å, c=11.9550(7) Å, V=2035.51(18) Å^3, Z=2; d_{calc}=1.183 Mg m^{-3}; μ (Mo$K\alpha$, λ=0.71073 Å)=0.21 mm^{-1}.

Antibacterial Assay The test organisms used were *Streptococcus mutans*, *S. sobrinus*, *S. pyogenes*, and *Staphylococcus aureus* (aerobic bacteria), *Actinomyces viscosus*, *Porphyromonas gingivalis*, *Fusobacterium nucleatum*, and *Actinobacillus actinomycetemcomitans* (anaerobic bacteria). The culture medium used was Brain heart infusion (BHI) broth for *S. mutans*, *S. sobrinus*, *S. pyogenes*, Tripticase soy (TSB) broth for *A. viscosus*, *P. gingivalis*, and *F. nucleatum*, Todd hewite (THB) broth for *A. actinomycetemcomitans*, and Nutrent yeast glucose (NYG) broth for *S. aureus*. 100 μl of culture broth containing 1—2×10^5 colony-forming units/ml was placed in each of the wells of a flat bottomed 96-well plate. To each well was added 100 μl of solutions of serial two fold dilutions of test substances (**1**—**12**) in PBS. The plates were then incubated at 37 °C for 24 h under the aerobic conditions for the aerobic bacteria, for 24 h under the anaerobic conditions for *A. viscosus*, *P. gingivalis*, and *A. actinomycetemcomitans*, and for 72 h under the anaerobic conditions for *F. nucleatum*. Then, the growth was assayed by the observation of the sediment film features. The sample concentration of the solution of the maximum dilution that gave the complete inhibition of the growth was taken as the minimum inhibitory concentration (MIC) and was recorded for each sample.

Acknowledgements This work was supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports, Science and Technology-Japan.

References and Notes

1) Saeki I., Sumitomo M., Kondo T., *Holzforschung*, **24**, 83—86 (1970).
2) Hayashi T., Kakisawa H., Ito S., Chen Y. P., Hsu H.-Y., *Tetrahedron Lett.*, **1972**, 3385—3388 (1972).
3) Kodama M., Kabuto C., Sunagawa M., Ito S., *Tetrahedron Lett.*, **1977**, 2909—2910 (1977).
4) Omoto T., Yoshida O., *Chem. Pharm. Bull.*, **28**, 1894—1899 (1980).
5) Park H.-S., Takahashi Y., Fukaya H., Aoyagi Y., Takeya K., *J. Nat. Prod.*, **66**, 282—284 (2003).
6) Park H.-S., Yoda N., Fukaya H., Aoyagi Y., Takeya K., *Tetrahedron*, **60**, 171—177 (2004).
7) Park H.-S., Kai N., Fukaya H., Aoyagi Y., Takeya K., *Heterocycles*, **63**, 347—357 (2004).
8) Campello J. P., Fonseca S. F., *Phytochemistry*, **14**, 243—248 (1975).
9) Bocks S. M., Cambie R. C., Takahashi T., *Tetrahedron*, **19**, 1109—1116 (1963).
10) Prasad J. S., Krishnamurty H. G., *Ind. J. Chem.*, *Sect. B*, **15B**, 397—398 (1977).
11) Crystallographic data for compound **5** has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication No. CCDC 696337. Copies of the data can be obtained, free of charge, on application to the Director, CCDC (e-mail: deposit@ccdc.cam.ac.uk).
12) Crystallographic data for compound **9** has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication No. CCDC 696823. Copies of the data can be obtained, free of charge, on application to the Director, CCDC (e-mail: deposit@ccdc.cam.ac.uk).
13) Bendall J. G., Cambie R. C., *Aust. J. Chem.*, **48**, 883—917 (1995).