Studies on Constituents from *Chamaecyparis pisifera* and Antibacterial Activity of Diterpenes

Du XIAO,[a] Masanori KUROYANAGI,*[,b] Tomio ITANI,[b] Hiromichi MATSUURA,[c] Manabu UDAYAMA,[c] Masatoshi MURAKAMI,[d] Kaoru UMEHARA,[d] and Nobuo KAWAHARA[e]

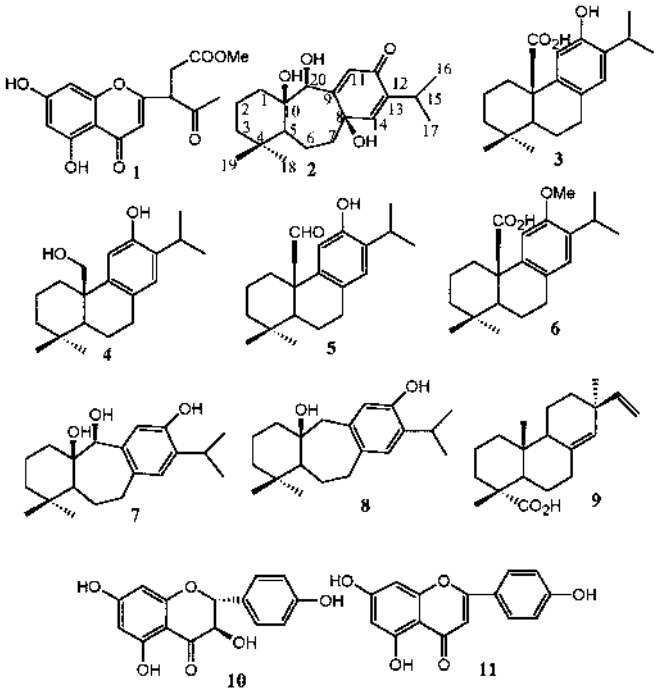
College of Forestry and Horticulture, Sichuan Agricultural University,[a] Yaan, Sichuan 625014, P.R. China, School of Bioresources, Hiroshioma Prefectural University,[b] 562 Nanatsuka, Shobara, Hiroshima 727–0023, Japan, Healthcare Research Institute, Wakunaga Pharmaceutical Co., Ltd.,[c] 1624 Shimokodachi, Koda-cho, Takata-gun, Hiroshima 739–1195, Japan, School of Pharmaceutical Sciences, University of Shizuoka,[d] 52–1 Yada, Shizuoka 422–8526, Japan, and National Institutes of Health Sciences,[e] 1–18–1 Kamiyoga, Setagaya, Tokyo 158–8501, Japan.
Received May 28, 2001; accepted July 13, 2001

In the course of our research for biologically active constituents from coniferous plants, a chromone derivative (1) and an abietane derivative (2) were isolated along with several diterpenes from *Chamaecyparis pisifera*. Structures of the new compounds were determined to be 5,7-dihydroxy-2-(1-acetyl-2-methoxycarbonylethyl)-chromone and *rel*-(8*R*,10*R*,20*S*)-8,10,20-trihydroxy-9(10→20)-*abeo*-abieta-9,13-dien-12-one by means of spectral methods including two-dimensional NMR experiments. Some of these abietane-type compounds isolated from this plants showed antibacterial activity against the gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*.

Key words 2-substituted chromone; 9(10→20)-*abeo*-abietane; *Chamaecyparis pisifera*; Pinaceae; antibacterial activity; diterpene

Plants have been used as crude drugs in medical systems worldwide for therapy of disease since ancient times and plants produce numerous secondary metabolites, some of which have been used as medicines. Herbal plants have been predominantly used as crude drugs and resources for the isolation of biologically active compounds. Studies on the screening of useful chemicals from woody plants have been fewer than those from herbal plants. Recently, woody plants have received attention as medicinal resources because of the development of some antitumor agents derived from their constituents, such as taxol[1)] and camptothecin.[2)] In the course of our studies on the isolation of biologically active constituents from woody plant resources, we studied the antibacterial activity of methanol extracts of about 30 coniferous plants. Antibacterial rearranged lanostane-type triterpenes were reported from *Abies sachalinensis*.[3)] Some of the methanol extracts showed potent antibacterial activities. Isolation of antibacterial constituents from *Chamaecyparis pisifera* (Japanese name *sawara*, Pinaceae), which methanol extract had indicated antibacterial activity against the gram-positive bacteria *Staphylococcus aureus* and *Bacillus subtilis*, were carried out as described in the Experimental section. Several types of abietane and 9(10→20)-*abeo*-abietane-type diterpenes were reported from *C. pisifera*.[4—7)] From the methanol extract of the plants, the novel chromone derivative (**1**) and 9(10→20)-*abeo*-abietane type diterpene (**2**) were isolated along with the known abietane-type diterpenes pisiferic acid (**3**),[7)] pisiferol (**4**),[7)] pisiferal (**5**),[7)] *O*-methylpisiferic acid (**6**),[7)] pisiferdiol (**7**),[6,7)] pisiferanol (**8**),[7)] and 8(14),15-pimaradiene-19-oic acid (**9**),[8)] and the flavonoids aromadendrin (**10**)[9)] and apigenin (**11**)[10)] as described in the Experimental section.

Compound **1** showed the molecular formula $C_{15}H_{14}O_7$, from the pseudo molecular ion, *m/z* 307.0822 $[MH]^+$, $C_{15}H_{15}O_7$ from the high-resolution (HR)-FAB-MS. The IR spectrum of **1** showed the absorption bands at 3088 cm^{-1} (aromatic or olefinic H), 1730 cm^{-1} (ester carbonyl), 1723 cm^{-1} (carbonyl), and 1642 cm^{-1} (characteristic of a flavone carbonyl). The ^{1}H-NMR spectrum of **1** showed the presence of the characteristic protons on the A- and C-ring of a typical flavone, (δ 6.18 [1H, d, J=2.4 Hz, H-6], δ 6.31 [1H, d, J=2.4 Hz, H-8], δ 6.42 [s, H-3]), and a chelated hydroxy group at C-5 (δ 12.62 [s]). However, the ^{1}H-NMR spectrum of **1** showed the presence of an acetyl methyl (δ 2.24 [3H, s]), a carboxymethyl (δ 3.59 [3H, s]), a methylene (δ 2.86 [1H, dd, J=6.6, 16.8 Hz], δ 3.06 [1H, dd, J=8.4, 16.8 Hz]) and a methine (δ 4.32 [1H, dd, J=6.6, 8.4 Hz]) instead of proton signals for the B-ring of flavone. The ^{13}C-NMR spectrum of **1** showed the presence of typical carbon signals for the A- and C-ring of a flavone (δ 93.9, 99.0, 103.7, 109.6,



* To whom correspondence should be addressed. e-mail: kuroyang@bio.hiroshima-pu.ac.jp

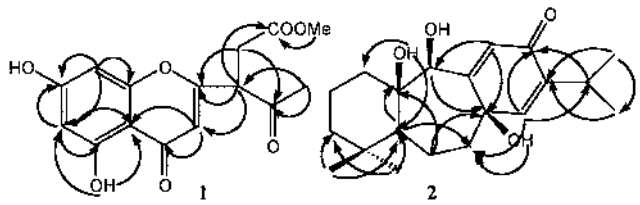


Fig. 1. Selected HMBC Correlations of **1** and **2**

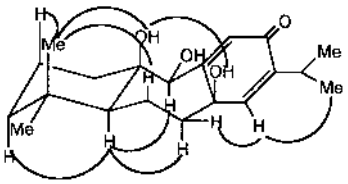


Fig. 2. NOE Correlations by NOESY Experiment on **2**

157.6, 161.4, 164.3, 164.8, 181.5) with 5,7-dihydroxy groups along with carbomethoxy (δ 171.2, 51.7), a carbonyl (δ 202.5), a methylene (δ 32.1), and a methine (δ 52.7) groups instead of the flavone B-ring as detailed in the Experimental section. From these data, **1** was considered to have a 2-substituted 5,7-dihydroxychromone structure. These NMR data indicated that **1** should be substituteded by a 1-acetyl-2-methoxycarbonyl-ethyl instead of the flavone B-ring.

This structure was confirmed by the heteronuclear multiple-bond connectivity (HMBC) experiment (Fig. 1). The 5′-methyl protons showed correlations with the C-3′ and C-4′ carbons. The carbomethoxy methyl protons showed correlation with the C-1′ carbon. The H-2′ methylene protons showed the correlations with the C-3′, C-4′, and C-2 carbons. The H-3′ methine proton showed correlations with the C-4′, C-5′, C-2, and C-3 carbons. Thus the structure of **1** was determined to be 5,7-dihydroxy-2(1-acetyl-2-methoxycarbonylethyl)-benzpyran-1-one and called sawarachromone.

Compound **1** showed no optical rotation, and therefore **1** must be a racemate. Sawarachromone has very a novel structure of 2-substituted chromone and might be derived from flavone by means of oxidative ring cleavage of the flavone B-ring. Examples of natural chromone derivatives other than flavone derivatives are rare. Some 2-substituted chromone derivatives were reported from Cape aloe.[11] Assignments of ^{1}H- and ^{13}C-NMR spectra of **1** were confirmed by heteronuclear multiple-quantum coherence (HMQC) and HMBC experiments.

Compound **2** had the molecular formula $C_{20}H_{30}O_4$ based on the pseudo molecular ion, 335.2216 ($C_{20}H_{31}O_4$), of the HR-FAB-MS. The IR spectrum of **2** showed the absorption bands at 3407 cm^{-1} (hydroxy), 2959 cm^{-1} (aliphatic CH, CH_2, CH_3), 1668 cm^{-1} (cross-conjugated carbonyl). The ^{1}H-NMR spectrum of **2** showed the presence of two singlet methyl groups (δ 0.87 [3H, s], 0.89 [3H, s]), two doublet methyl groups (δ 1.02 [3H, d, J=7.0 Hz], 1.04 [3H, d, J=7.0 Hz]), two olefin protons (δ 6.27 [1H, br s], 6.52 [1H, d, J=1.0 Hz]), a secondary methine proton (δ 4.50 [1H, br s]), and a methine proton (δ 2.83 [1H, hep., J=7.0 Hz]). The ^{13}C-NMR spectrum of **2** showed the presence of 20 carbons including a cross-conjugated carbonyl (δ 186.2), two olefin groups (δ 126.6, 141.7, 146.6, 162.8), a secondary alcohol group (δ 75.8), and two tertiary alcohol groups (δ 70.6, 74.7). These NMR data indicated that the Me-20 signal had disappeared. From *Chamaecyperis* sp. plants, 9(10→20)-*abeo*-abietan derivatives have been isolated.[4—7] These data indicated that **2** should have a 9(10→20)-*abeo*-abieta skeleton. The 9(10→20)-*abeo*-abieta skeleton and positions of the hydroxy groups, carbonyl group, and olefin groups were confirmed by the HMBC experiment (Fig. 1). The H-11 olefin proton showed cross peaks with the C-8 carbinol carbon, C-20 carbinol carbon, and C-12 carbonyl carbon. The H-20 carbinol proton showed the cross peaks with the C-5 methine carbon, C-8 carbinol carbon, and C-11 olefin carbon. The two doublet methyl protons showed a cross peak with the C-13 olefine carbon. The H-15 methine proton showed the cross peaks with the C-12 carbonyl carbon and C-14 olefine carbon. The hydroxy proton at C-10 showed cross peaks with the C-1 and C-5 carbons. The hydroxy proton at C-8 showed a cross peak with the C-7 carbon. The relative configuration of **2** was studied from the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum (Fig. 2). H-14 showed NOEs with Me-15 and H-7β. H-5 showed NOEs with H-7α, H-20, and H-3α. OH-10 showed NOEs with OH-8 and Me-19. Me-19 showed NOEs with OH-10, H-6β, and H-2β. Based on these finding the relative configuration of **2** was determined. The absolute stereochemistry of **2** was not confirmed from the circular dichroism (CD) spectrum ($[\theta]_{352}$ +3026), but was assumed to be the same as that of pisiferdiol (**7**), for which the absolute configuration was determined.[6] Thus the structure of **2** was deduced to be *rel*-(8*R*,10*R*,20*S*)-8,10,20-trihydroxy-9-(10→20)-*abeo*-abieta-9,13-dien-12-one and called as sawaradienone. Many B-*homo* abietane-type diterpenes have been isolated from coniferous plants as characteristic constituents. Assignments of the ^{1}H- and ^{13}C-NMR spectra of **2** were confirmed by the HMQC and HMBC experiments.

Table 1. MIC[a] (μg/ml) of the Constituents from *C. pisifera* against *Staphylococcus aureus* and *Bacillus subtilis*

Compound	*S. aureus*	*B. subtilis*
1	100<	100<
2	100<	100<
3	25	25
4	25	25
5	25	25
6	12.5	12.5
7	100	50
8	25	25
9	100<	50
10	100<	100<

a) Minimum inhibitory concentration.

The antibacterial activity of the constituents isolated from *C. pisifera* was tested against *S. aureus* and *B. subtilis.* Of the tested compounds, abietane-type derivatives **3**—**6**, and 9-(10→20)-*abeo*-abietan-type derivatives **7** and **8** had antibacterial activity, but **1**, **2**, **9** and **10** 100 μg/ml did not show activity against *S. aureus* and **1**, **2** and **9** 100 μg/ml did not show activity against *B. subtilis*, as shown in Table 1. These results indicated that the aromatic C-ring and phenolic hydroxyl or methoxyl groups are important for the antibacterial activity.

Experimental

Melting points were recorded on a Yanagimoto micro melting point apparatus and are uncorrected. IR spectrum data were recorded on a Perkin Elmer GX-FT-IR spectrometer. HR-FAB-MS data were measured on a JEOL HX110 mass spectrometer. ^{1}H- and ^{13}C-NMR data were measured on a JEOL α-500 (500, 125 MHz) with tetramethylsilane as an internal standard. $[\alpha]_D$ values were recorded on a JASCO P-1010 polarimeter at 20 °C. Analytical and preparative HPLC was carried out on a reversed-phase column (YMC R-ODS-5A packed column).

Extraction and Isolation Leaves of *C. pisifera* (4 kg), collected at Tenryu, Shizuoka, Japan, in September 1997, were extracted with methanol under reflux to give a methanol extract, which was fractionated between ethyl acetate (EtOAc) and water to give an EtOAc-soluble fraction (190 g) and a water layer. The water layer was extracted with *n*-BuOH to give an *n*-BuOH-soluble fraction (60 g) and a water layer. Of these three fractions, the EtOAc fraction showed the most potent antibacterial activity against the gram-positive bacteria. Therefore that fraction was chromatographed on a SiO_2 column using a gradient $CHCl_3$–MeOH solvent system to give nine fractions. Of these, fractions 3, 4, and 5 showed antibacterial activity. Fraction 3 (37.3 g) was chromatographed on a SiO_2 column and HPLC using an octadecyl silica (ODS) column successively to give **4** (282 mg), **5** (565 mg), **8** (465 mg) and **9** (51 mg). Fraction 4 (39.1 g) was chromatographed on a SiO_2 column and HPLC using an ODS column successively to give **2** (44 mg), **3** (7.4 g), **6** (30 mg), and **7** (7.9 g). Fraction 5 (12 g) was chromatographed on a SiO_2 column and HPLC using an ODS column successively to give **1** (20 mg), **3** (89 mg), **7** (210 mg), **8** (89 mg), and **11** (10 mg).

Sawarachromone (1) Fine crystals, mp 191—195 °C (MeOH); HR-FAB-MS *m/z* 307.0822 $[MH]^+$ (Calcd 307.0818, $C_{15}H_{15}O_7$); $[\alpha]_D$ ±0° (*c*=0.03, MeOH); IR ν cm^{-1} (KBr) 3088, 3018, 2925, 1730, 1723, 1642, 1168; ^{1}H-NMR ($CDCl_3$) δ: 2.24 (3H, s, Me-5′), 2.86 (1H, dd, *J*=6.6, 16.8 Hz, H-2′), 3.06 (1H, dd, *J*=8.4, 16.8 Hz, H-2′), 3.59 (3H, s, COMe), 4.32 (1H, dd, *J*=6.8, 8.4 Hz, H-3′), 6.18 (1H, d, *J*=2.4 Hz, H-6), 6.31 (1H, d, *J*=2.4 Hz, H-8), 6.42 (1H, s, H-3), 10.87 (1H, s, OH at C-7), 12.62 (1H, s, OH at C-5); ^{13}C-NMR ($CDCl_3$) δ: 28.6 (C-5′). 32.1 (C-2′), 51.7 (COO<u>Me</u>), 52.7 (C-3′), 93.9 (C-8), 99.0 (C-6), 103.7 (C-10), 109.6 (C-3), 157.6 (C-9), 161.4 (C-5), 164.3 (C-7), 164.8 (C-2), 171.2 (C-1′), 181.5 (C-4), 202.5 (C-4′).

Sawaradienone (2) Colorless viscous oil; HR-FAB-MS *m/z* 335.2216 $[MH]^+$ (Calcd 335.2222, $C_{20}H_{31}O_4$); IR ν cm^{-1} (KBr); 3407, 2959, 1668, 1629, 1389, 996; ^{1}H-NMR ($CDCl_3$) δ: 0.87 (3H, s, Me-19), 0.89 (3H, s, Me-18), 0.90 (1H, over-lap, H-6), 1.02 (3H, d, *J*=7.0 Hz, Me-16 or 17), 1.04 (3H, d, *J*=7.0 Hz, Me-17 or 16), 1.17 (1H, br d, *J*=9.5 Hz, H-5), 1.25 (1H, dt, *J*=4.2, 13.2 Hz, H-3α), 1.31 (1H, dt, *J*=3.0, 13.2 Hz, H-1), 1.45 (1H, br d, *J*=13.2 Hz, H-3β), 1.46 (1H, over-lap, H-6), 1.5 (1H, over-lap, H-2α) 1.70 (1H, br t, *J*=13.2 Hz, H-7), 1.78 (1H, tq, *J*=3.0, 13.2 Hz, H-2β), 1.95 (1H, br s, OH at C-10), 2.2 (1H, over-lap, H-1), 2.22 (1H, br d, *J*=13.2 Hz, H-7), 2.83 (1H, br hep, *J*=7.0 Hz, H-15), 3.49 (1H, br s, OH at C-8), 4.50 (br s, H-20), 6.27 (1H, br s, H-11), 6.52 (1H, d, *J*=1.0 Hz, H-14); ^{13}C-NMR ($CDCl_3$) δ: 18.2 (C-2), 19.9 (C-6), 21.3 (C-16 or 17), 21.5 (C-18), 21.7 (C-17 or 16), 25.6 (C-15), 32.2 (C-19), 34.7 (C-4), 36.7 (C-1), 40.6 (C-7), 41.8 (C-3), 54.8 (C-5), 70.6 (C-8), 74.7 (C-10), 75.8 (C-20), 126.6 (C-11), 141.7 (C-14), 146.6 (C-13), 162.8 (C-9), 186.2 (C-12); CD $[\theta]_{352}$ +3026, $[\theta]_{280}$ +16026, $[\theta]_{238}$ −10680 ($c=1.3\times10^{-4}$ M, MeOH).

Antibacterial Activity Antibacterial activity tests were carried out against the gram-positive bacteria *S. aureus* and *B. subtilis*, by the dilution alga plate methods as described in previous reports.[12)]

Acknowledgments Authors thank Dr. M. Uchida (School of Pharmaceutical Sciences, University of Shizuoka) for FAB-MS measurement and Mr. M. Takase (Showa College of Pharmaceutical Sciences) for HR-FAB-MS measurement. This work was supported by the Hiroshima Prefectural Government.

References

1) Borman S., *Chem. Engng. News*, **69**, 11—18 (1991).
2) Wall M. E., Wani M. C., Cook C. E., Palmer K. H., McPhail A. T., Sim G. A., *J. Am. Chem. Soc*., **88**, 3888—3890 (1966).
3) Kuroyanagi M., Sugiyama K., Kanazawa M, Kawahara N., *Chem. Pharm. Bull*., **48**, 1917—1920 (2000).
4) Fukui H., Koshimizu K., Egawa H., *Agric. Biol. Chem*., **42**, 1419—1423 (1978).
5) Yatagai M., Takahashi T., *Phytochemistry*, **19**, 1149—1151 (1980).
6) Ahn J. W., Wada K., Maruo S., *Agric. Biol. Chem*., **48**, 3177—3179 (1984).
7) Hasegawa S., Kojima T., Hirose Y., *Phytochemistry*, **24**, 1545—1551 (1985).
8) Nagashima F., Tanaka H., Takaoka S., Asakawa Y., *Phytochemistry*, **45**, 353—363 (1997).
9) Lee I.-K., Kim C.-J., Song K.-S., Kim H.-M., Yoo I-D., *J. Nat. Prod*., **58**, 1614—1617 (1995).
10) Christiansen K., Boll P. M., *Tetrahedron Lett*., **1966**, 1293—1297.
11) Speranza G., Fontana G., Zanzola S., Meo A. D., *J. Nat. Prod*., **60**, 692—694 (1997).
12) Kuroyanagi M., Arakawa T., Hirayama Y., Hayashi T., *J. Nat. Prod*., **62**, 1595—1599 (1999).