C_{35} Terpenoids from the Bark of *Calocedrus macrolepis* var. *formosana* with Activity against Human Cancer Cell Lines

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Ferrugicadinol (**1**) and ferrugieudesmol (**2**), two new compounds with a unique C_{35} terpene skeleton, were isolated from the bark of *Calocedrus macrolepis* var. *formosana*. Their structures were elucidated principally from spectroscopic evidence. The two new C_{35} terpenes showed cytotoxicity against human oral epidermoid carcinoma KB cells with IC_{50} values ranging from 11 to 14 μM.

Calocedrus macrolepis var. *formosana* (=*C. formosana*) is an endemic plant and grows at elevations from 300 to 2000 m in the central and northern mountains of Taiwan. It is a member of the Cupressaceae and widely used as a building material. Previous investigations on the chemical constituents of the heartwood of *C. formosana*[1−3] have resulted in the isolation of lignans and terpenoids. From the leaves of *C. formosana*,[4−7] 40 known components, including lignans, terpenes, and fatty acids, as well as four new constituents have been reported previously. Thirty years ago, only one paper indicated the elucidation of one new compound from the bark of *C. formosana* (old name: *Libocedrus formosana*).[8] Recently, we also reported some interesting dehydroabietane diterpene dimers.[9] Interestingly, our preliminary study showed that the EtOAc-soluble fraction of the acetone extract of the bark of *C. formosana* possessed cytotoxicity against cancer cells. With this information, we continued our effort to identify the active components toward neoplastic cells. In this paper, we report two new C_{35} terpenes, ferrugicadinol (**1**) and ferrugieudesmol (**2**), which are members of a new class of C_{35}-type skeletons composed of diterpene and sesquiterpene units. Notably, these two compounds exhibit antitumoral cytotoxic activity.

The IR spectrum of ferrugicadinol (**1**) suggested the presence of aromatic (3057, 1617, 1504 cm^{-1}) and hydroxy (3371 cm^{-1}) groups. Its molecular formula, $C_{35}H_{54}O_2$, was determined on the basis of HREIMS (*m/z* 506.4078, calcd for $[M]^+$ 506.4126). A detailed 2D NMR analysis, including COSY, HMQC, and HMBC experiments, resulted in the structure of **1** (Figure 1), a combination of α-cadinol and ferruginol. According to the analysis of ^1H NMR data (Table 1) and HMBC correlations (Figure 2), the compound was found to contain one set of dehydroabietane signals as follows: δ_H 0.88, 0.90, 1.12 (3H each, s, Me-19, Me-18, Me-20), 1.21, 1.23 (3H each, d, J = 7.4 Hz, Me-16, Me-17), 3.08 (1H, sep, J = 7.4 Hz, H-15), 6.59, 6.86 (1H each, s, H-11, H-14). One exchangeable phenolic proton at δ_H 4.58 (1H, s) was observed. The resonance at δ_H 2.14 (1H, br d, J = 11.5 Hz, H-1$_\beta$) is characteristic of a dehydroabietane[10,11] moiety. Comparison of the ^1H and ^{13}C NMR data (Table 1) with those of ferruginol[12] led to the assignment of the partial structure of **1** as a ferruginol with a substituent at C-7. The ^1H and ^{13}C NMR (Table 1) data of the second moiety were similar to those of α-cadinol[13] and assigned as follows: δ_H 1.12 (3H, s, Me-14′), 0.72, 0.88 (3H each, d, J = 6.9 Hz, Me-12′, Me-13′), 2.09 (1H, m, H-11′), 5.53 (br s, H-5′), 2.24 (1H, br d, J = 15.7 Hz, H$_\beta$-3′), 2.02 (1H, m, H$_\alpha$-3′). The presence of NOESY correlations (Figure 3) between H-6′/Me-14′ and H-6′/Me-12′, along with the broad singlet of the olefinic proton (H-5′),[14,15] allowed the establishment of this moiety as a *trans*-fused α-cadinol derivative. Comparing the NMR data of **1** with α-cadinol, we found that H-15′ changed from methyl to methylene protons, suggesting a substituent attached to C-15′. Furthermore, the methylene protons at H$_2$-15′ had a COSY correlation with H-7, while the HMBC correlations (Figure 2) with C-6, C-7, C-8, C-3′, C-4′, and C-5′ indicated the direct connection between C-7 of ferruginol and C-15′ of α-cadinol. Irradiation at δ_H 2.18 (H-15′) collapsed H-7 to a broad doublet (J = 6.2 Hz). The NOESY correlations (Figure 3) between H$_2$-15′/H-5 and H-7/H-14 confirmed H-7 to have a β-quasi-equatorial orientation and C-15′ of the α-cadinol moiety to be linked at C-7 in α-quasi-axial orientation. The structure of ferrugicadinol (**1**) was unambiguously established as shown in Figure 1.

Compound **2**, named ferrugieudesmol, was isolated as an oil. HREIMS revealed **2** to have the formula $C_{35}H_{54}O_2$. The IR spectrum showed absorption bands for hydroxy (3366 cm^{-1}), disubstituted terminal olefinic (1646, 885 cm^{-1}), and aromatic (3073, 1615, 1504 cm^{-1}) functionalities. Comparison of the ^1H and ^{13}C NMR data (Table 1) of **2** with **1** as well as 2D NMR (including HMQC, HMBC, COSY, and NOSEY methods) analysis revealed the structure of ferrugieudesmol (**2**) as a C−C linkage dimer of diterpene and sesquiterpene units. The diterpene moiety is a ferruginol, and it showed signals of three singlet methyl groups, an aromatic isopropyl group, two *para*-phenyl protons, and a typical H$_\beta$-1 proton of dehydroabietane. The ^1H NMR (Table 1) data of the diterpene moiety of compound **2** were quite similar to those of **1**. A signal at δ_H 3.20 (1H, d, J = 8.6 Hz) was assigned to be at benzylic C-7, and the evidence also revealed a substituent linking at C-7. The second set of NMR signals were similar to those of eudesmane-type β-eudesmol:[16] δ_H 0.84, 1.26, and 1.26 (3H each, s, Me-14′, Me-12′, Me-13′), 4.43 and 4.67 (br s each, H$_2$-15′). Comparing the NMR data of **2** with β-eudesmol, we found that H-1′ changed from a methylene to a methine proton, suggesting a substituent attached to C-1′. The HMBC correlations (Figure 4) from H-7 to C-1′, C-2′, and C-10′ led to the assignment of the

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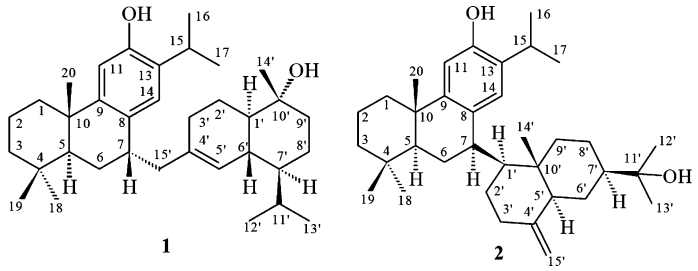

Figure 1. Structures of ferrugicadinol (**1**) and ferrugieudesmol (**2**).

Table 1. NMR Data (500 MHz, $CDCl_3$) for Compounds **1** and **2**

	1			**2**		
position	δ_C	δ_H (*J* in Hz)	HMBC[a]	δ_C	δ_H (*J* in Hz)	HMBC[a]
1	38.7	2.14, br d (11.5) 1.34, m	3, 5	38.8	2.16[b] 1.35, m	3, 5
2	19.4	1.60, m 1.72, m		19.3	1.59, m 1.70, m	4, 10
3	41.7	1.20, m 1.45, m		42.1	1.19, m 1.43, m	
4	33.4			34.0		
5	44.8	1.38, d (12.4)	7, 18, 19, 20	46.2	1.37[b]	1, 7, 18, 19, 20
6	22.0	1.61, td (12.4, 6.2) 1.68, d (12.4)	5, 10	21.6	1.64, m 1.90, m	7, 1′
7	35.3	2.90, td (7.5, 6.2)	5, 6, 8, 14, 4′, 15′	33.0	3.20, d (8.6)	5, 6, 1′, 2′, 10′
8	137.1			131.4		
9	148.6			149.9		
10	37.9			37.7		
11	110.8	6.59, s	8, 10, 13	109.9	6.62, s	8, 10, 13
12	150.8			150.6		
13	131.5			131.2		
14	127.1	6.86, s	7, 9, 12, 15	126.6	7.06, s	7, 9, 12, 15
15	27.0	3.08, sep (7.4)	12, 13, 14, 16, 17	27.2	3.13, sep (6.9)	12, 13, 14, 16, 17
16	22.7	1.21, d (7.4)	13, 15, 17	22.6	1.25, d (6.9)	13, 15, 17
17	22.5	1.23, d (7.4)	13, 15, 16	22.8	1.25, d (6.9)	13, 15, 16
18	33.8	0.88, s	3, 4, 5, 19	33.3	0.95, s	3, 4, 5, 19
19	21.6	0.90, s	3, 4, 5, 18	21.3	0.86, s	3, 4, 5, 18
20	25.0	1.12, s	1, 5, 9, 10	24.4	1.03, s	1, 5, 9, 10
1′	50.2	1.28, m	2′, 9′, 10′	58.7	1.85, m	
2′	22.9	1.29, m 2.08, m		24.8	1.31, m 1.63, m	6, 7, 14′
3′	28.3	2.24, br d (15.7) 2.02, m	1′, 5′, 15′	37.2	1.89[b] 2.26, br d (11.5)	15′
4′	131.6			150.9		15′
5′	125.0	5.53, br s	3′, 15′	51.6	1.88, m	14′, 15′
6′	39.8	1.80, m	1′, 4′, 10′	25.0	1.15, m 1.55, m	
7′	46.6	1.06, br d (11.5)	6′, 8′, 9′, 11′ 12′, 13′	49.0	1.42, m	12′, 13′
8′	21.7	1.12, m 1.61, m		22.6	1.78, m	
9′	42.2	1.46, m 1.80, m		39.6	2.16[b] 1.35, m	14′
10′	72.5			39.8		
11′	26.0	2.09 m	7′, 12′, 13′	73.0		12′, 13′
12′	21.5	0.72 d (6.9)	7′, 11′, 13′	27.0	1.26, s	7′, 11′
13′	15.0	0.88 d (6.9)	7′, 11′, 12′	27.3	1.26, s	7′, 11′
14′	20.8	1.12 s	1′, 10′	13.9	0.84, s	1′, 5′, 9′
15′	46.7	2.18 d (7.5)	6, 8, 3′, 4′, 5′	105.1	4.43, br s 4.67, br s	3′, 4′, 5′

[a] HMBC correlations are from proton(s) to the indicated carbons. [b] Overlapped with other signal(s).

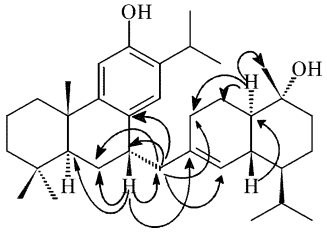

Figure 2. Key HMBC correlations of **1**.

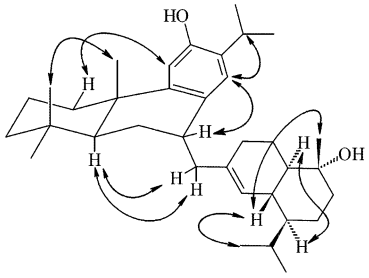

Figure 3. NOESY correlations of **1**.

structure of **2** as the connection between C-1′ of a β-eudesmol moiety and C-7 of a ferruginol moiety. Thus, the C_{35} skeleton could be established. The coupling constant ($J = 8.6$ Hz) of H-7 as well as the NOESY correlation of H-5/H-7 indicated H-7 to be in an α-quasi-axial orientation. Due to steric congestion by the β-quasi-equatorial substituent, H-14 resonated at δ_H 7.06, which is lower

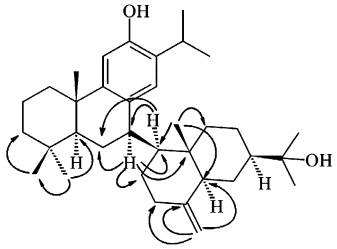

Figure 4. Key HMBC correlations of **2**.

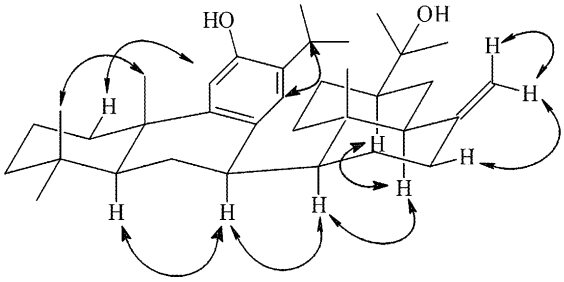

Figure 5. NOESY correlations of **2**.

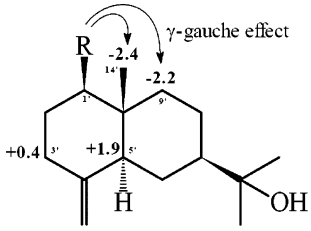

Figure 6. γ-Gauche effect.

than that of the corresponding proton (δ_H 6.81) of ferruginol. Furthermore, the NOESY correlations (Figure 5) from H-5′ to H-1′ and H-7′ indicated that all three protons were in an α-axial orientation. In Figure 6, C-14′ and C-9′ were in a γ-gauche relationship with C-7 of the ferruginol moiety, which causes the ^{13}C NMR signals of C-14′ and C-9′ to shift to higher field than those of the corresponding carbons of β-eudesmol. C-3′ and C-5′ were in a γ-anti-relationship with C-7, which led to the signals of C-3′ and C-5′ of **2** to shift to lower field. These findings confirmed that the ferruginol moiety is connected to C-1′ of the eudesmol moiety in a β-equatorial orientation.

The two new C_{35}-type compounds, **1** and **2**, were evaluated for their cytotoxic activity against human oral epidermoid carcinoma KB cells. After treating cells for 72 h, **1** and **2** exhibited IC_{50} values of 14.1 ± 0.1 and 11.4 ± 3.6 μM, respectively. This result demonstrated that **1** and **2** exhibited significant antitumoral cytotoxic activity. However, the potency of these two compounds was slightly weaker than that of VP-16 (IC_{50} value of 2.0 μM), a clinically used anticancer drug.

Experimental Section

General Experimental Procedures. Melting points were determined with a Yanagimoto micromelting point apparatus and are uncorrected. IR spectra were recorded on a Perkin-Elmer 983 G spectrophotometer. ^{1}H and ^{13}C NMR spectra were performed on a Bruker Avance 500 NMR spectrometer. EIMS, HREIMS, and optical rotations were recorded on JEOL JMS-HX 300 and JEOL SX-102 mass spectrometers and JASCO DIP-180 digital polarimeter, respectively. Extracts were chromatographed on silica gel (Merk 70−230 mesh, 230−400 mesh, ASTM) and purified with a semipreparative normal-phase HPLC column (250 × 10 mm, 7 μm, LiChrosorb Si 60) on an LDC Analytical-III.

Plant Material. The bark of *C. macrolepis* var. *formosana* was collected in Nan-Tou, Taiwan (1998). The plant was identified by Dr. Shang-Tzen Chang, a Professor in the Department of Forestry. A voucher specimen (voucher no. 223133) has been deposited in the Herbarium of the Department of Botany of National Taiwan University, Taipei, Taiwan.

Extraction and Isolation. The dried bark of *C. macrolepis* (16 kg) was extracted with Me_2CO (140 L) at room temperature (7 days × 2). After removal of Me_2CO, H_2O was added to bring the total volume to 1 L. This suspended phase was extracted with EtOAc (1 L × 3). Evaporation of the combined EtOAc layers afforded a black syrup (734 g), which was purified by means of silica gel chromatography and repeated HPLC (normal phase on Lichrosorb Si 60), using a hexane−EtOAc gradient solvent system. Compounds **1** (3.5 mg) and **2** (9.8 mg) were eluted with 25% EtOAc in hexane solvent systems.

Ferrugicadinol (1): gum; $[\alpha]_D^{29}$ −4.0 (*c* 0.25, $CHCl_3$); IR (KBr) ν_{max} 3371, 3057, 1617, 1504, 1460, 1414, 1386, 1373, 1265, 1239, 1165, 1122, 738 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 231 (3.85), 283 (3.63) nm; ^{1}H and ^{13}C NMR, Table 1; EIMS *m/z* 506 $[M]^+$ (1), 488 (1), 285 (100), 243 (4), 229 (5), 215 (5), 201 (10), 189 (15), 159 (4); HREIMS *m/z* 506.4078 (calcd for $C_{35}H_{54}O_2$ 506.4126).

Ferrugieudesmol (2): oil; $[\alpha]_D^{23}$ +12.2 (*c* 0.85, MeOH); IR(KBr) ν_{max} 3366, 3073, 1646, 1615, 1504, 885 cm^{-1}; UV (MeOH) λ_{max} (log ϵ) 224 (3.80), 281 (3.34) nm; ^{1}H and ^{13}C NMR, Table 1; EIMS *m/z* 506 (M^+, 2), 488 (3), 285 (100), 201 (10), 189 (16); HREIMS *m/z* 506.4112 (calcd for $C_{35}H_{54}O_2$ 506.4126).

Anticancer Cytotoxicity Assay. Human oral epidermoid carcinoma KB cells were maintained in RPMI-1640 medium supplemented with 5% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. The cells in logarithmic growth phase were seeded at a density of 1 × 10^4 cells/mL/well in a 24-well plate. After 24 h, the cells were exposed to various concentrations of the test drugs for 3 days. At the end of the incubation period, cells were fixed and stained with 50% EtOH containing 0.5% methylene blue for 30 min. The plates were washed five times with H_2O and allowed to air-dry. The resulting colored residue was dissolved in 1% *N*-lauroylsarcosine, and the optical density was read at 570 nm using an Emax Precision microplate reader (Molecular Devices Corporation, CA). Each point represents the average of at least two independent experiments run in triplicate.

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