Cytotoxic Isoprenylated Flavans of *Broussonetia kazinoki*

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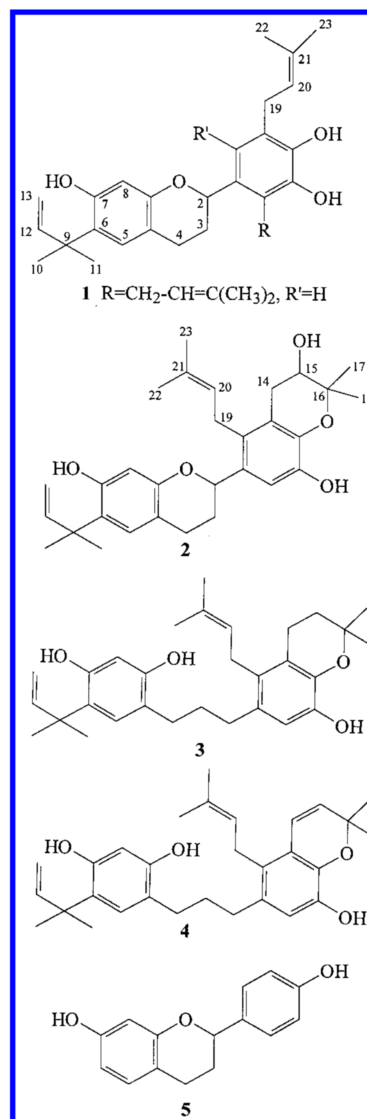
Two new prenylflavans [kazinols Q (**1**) and R (**2**)] and five known compounds [kazinols D (**3**), K (**4**), and H, 7,4′-dihydroxyflavan (**5**), and oleanolic acid] were isolated from the root bark of *Broussonetia kazinoki*. The cytotoxic activity of **1**−**5** was evaluated against several different cell lines.

Natural prenylflavonoids from plants in the Moraceae grown in Taiwan have shown potent inhibition against human hepatoma PLC/PRF/5 and epidermoid carcinoma KB cells in vitro.[1] In a continued search for bioactive constituents from plants in this family, two new prenylated flavans named kazinols Q (**1**) and R (**2**) and five known compounds, kazinols D (**3**), K (**4**), and H, 7,4′- dihydroxyflavan (**5**), and oleanolic acid, were isolated from *Broussonetia kazinoki* Sieb. et Zucc. (Moraceae). In the present paper, the structure characterization of **1** and **2** and the cytotoxic effects of **1**−**5** against a small cancer line panel are reported.

The HREIMS of **1** indicated a molecular ion peak at *m*/*z* 462.2752, which corresponded to a molecular formula $C_{30}H_{38}O_4$. Its IR spectrum showed absorption bands for hydroxyl groups (3500, 3483, and 3351 cm^{-1}) and aromatic rings (1619 and 1593 cm^{-1}), and the UV spectrum exhibited absorption maxima similar to those of kazinol E.[2,3] The ^1H NMR spectrum of **1** showed the presence of two 3,3-dimethylallyl groups and a 1,1-dimethylallyl group at δ 1.67, 1.69, 1.79, 1.81 (each 3H, s), 3.39 (4H, br d, $J = 6.4$ Hz, H-14 and -19), 5.00 (1H, t, $J = 6.4$ Hz, H-15) and 5.14 (1H, t, $J = 6.4$ Hz, H-20), and 1.43 (6H, s), 5.28 (1H, d, $J = 10.8$ Hz, H-13*E*), 5.35 (1H, d, $J = 17.6$ Hz, H-13*Z*), and 6.19 (1H, dd, $J = 17.6$, 10.8 Hz, H-12), as well as five aliphatic, three aromatic, and two phenolic hydroxyl proton signals at δ 1.91−2.13 (2H, m, H-3), 2.72−2.96 (2H, m, H-4), 5.10 (1H, dd, $J = 10.8$, 2.0 Hz, H-2); 6.40 (1H, s, H-8), 6.93 (1H, s, H-6′), and 6.94 (1H, s, H-5); and 5.43 (1H, br s, OH) and 5.69 (1H, br s, OH-7), respectively. This evidence and the UV spectrum showing bathochromic shifts upon addition of NaOMe and NaOAc−H_3BO_3 suggested that **1** is a prenylated 7,3′,4′-trihydroxyflavan.[3]

The ^{13}C NMR spectral assignments (Table 1) of **1** were made by performing ^1H-decoupled, DEPT, and 2D $^1H-^{13}C$ correlation experiments (Figure 1). The chemical shift values of the carbon atoms of the A and C rings were similar to those of the corresponding carbon atoms of kazinol E,[2] and the C-1 carbons of the two 3,3-dimethylallyl groups were observed to resonate at δ 25.9 and 27.3, respectively. These data and a positive Gibb's test for **1** clearly indicated that the two 3,3-dimethylallyl groups were attached at C-2′ and C-5′,[4] respectively. Therefore, kazinol Q (**1**) was characterized as 7,3′,4′-trihydroxy-6-(1,1-dimethylallyl)-2′,5′-di-(3,3-dimethylallyl)-flavan (**1**). The HMBC spectrum (Figure 1) also supported this structural assignment.



The HREIMS of **2** indicated a molecular ion peak at *m*/*z* 478.2714, which corresponded to a molecular formula $C_{30}H_{38}O_5$. Its IR spectrum showed absorption bands for hydroxyl groups (3475 cm^{-1}) and aromatic rings (1636 and 1606 cm^{-1}), and the UV spectrum showed absorption maxima similar to those of **1**. The ^1H NMR spectrum of **2** indicated the presence of a 3,3-dimethylallyl group; a 1,1-

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Table 1. ^{13}C NMR Data for 1 and 2 (100 MHz in $CDCl_3$)[a]

carbon	**1**[b]	**2**[b]
2	74.8	74.7
3	29.9	29.6
4	25.6	25.6
4a	113.2	113.6
5	126.8	126.8
6	124.8	124.8
7	153.7[c]	153.8[c]
8	105.6	105.6
8a	155.2[c]	155.2[c]
9	39.8	39.8
10	27.2	27.2
11	27.2	27.2
12	148.3	148.3
13	113.7	113.1
14	25.9	29.7
15	123.8	70.2
16	131.1	76.7
17	17.9[d]	21.3
18	25.6[e]	24.8
19	27.3	26.9
20	122.2	122.8
21	133.8	132.2
22	18.0[d]	18.1
23	25.7[e]	25.5
1′	132.0	131.9
2′	126.5	110.7
3′	142.1[f]	143.6
4′	142.0[f]	139.4
5′	129.8	118.0
6′	111.1	129.7

[a] The number of protons directly attached to each carbon was verified by DEPT experiments. [b] Signals obtained by ^{1}H−^{1}H COSY, HMQC, HMBC, and NOESY techniques. [c−f] Assignments with same superscript in each column may be interchanged.

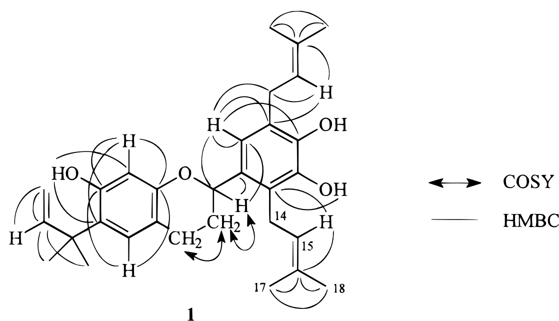

Figure 1. C/H long-range correlations obtained from HMBC spectra and selected COSY spectra of **1**.

dimethylallyl group; a 2,2-dimethyl chroman group; two methylene protons at δ 2.70 (1H, dd, J = 17.2, 6.4 Hz) and 2.97 (1H, dd, J = 17.2, 5.2 Hz); a methine proton signal at δ 3.85 (1H, dd, J = 6.4, 5.2 Hz); five aliphatic proton signals at δ 2.02−2.08 (2H, m, H-3), 2.74−2.96 (2H, m, H-4), and 5.09 (1H, dd, J = 10, 2.8 Hz, H-2); two phenolic hydroxyl groups at δ 5.51 and 5.68; and three aromatic proton signals at δ 6.39, 6.94, and 6.99. The EIMS of **2** showed a molecular ion peak at m/z 478 $[M]^+$ and significant fragments at m/z 461 $[M - H_2O + H]^+$, 287, and 191, and suggested that **2** is a flavan [5] with an alcoholic hydroxyl group.[6] In the ^{13}C NMR spectrum of **2**, the chemical shift values of C-2 to C-13, C-1′, C-3′, C-4′, and C-19 to C-23 were similar to those of the corresponding data for kazinol H.[2] In addition, **2** gave a negative Gibb's test, and its UV spectrum showed a bathochromic shift only upon addition of NaOMe but not on addition of $NaOAc-H_3BO_3$.[3] The above evidence clearly indicated that **2** is a hydrated form of kazinol H. Analysis of the COSY 90 and HMQC spectra of **2** established the connectivity of C-14 to C-15. In the HMBC spectrum (Figure 2), a carbon signal resonating at

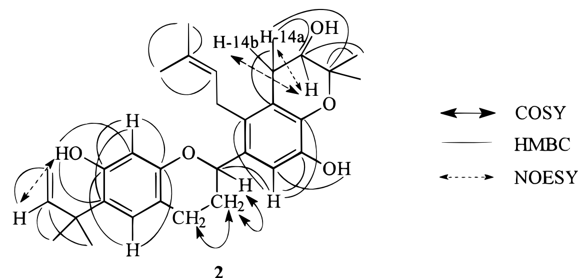

Figure 2. C/H long-range correlations obtained from HMBC spectra, selected COSY spectra, and NOESY interactions of **2**.

δ 70.2 (Table 1) was correlated with the two methyl groups at C-16 and the methylene proton signals at δ 2.70. The methylene proton signals at δ 2.70 and 2.97, the methine proton signal at δ 3.85, and the aromatic proton signals at δ 6.39, 6.94, and 6.99 were assigned to H-14a, H-14b, H-15, H-8, H-2′, and H-5, respectively. The coupling constants (6.4 and 5.2 Hz) between H-14a and H-15 and between H-14b and H-15 clearly indicated that the hydroxyl group at C-15 occupies an axial position.[7] Consequently, kazinol R (**2**) was characterized as 7,3′-dihydroxy-6-(1,1-dimethylallyl)-4′,5′-(2,2-dimethyl-3-hydroxy-chromano)-6′-(3,3-dimethylallyl)-flavan (**2**). The ^{13}C NMR assignments (Table 1) of **2** were made by performing ^{1}H-decoupled, DEPT, 2D ^{1}H−^{13}C correlation experiments (Figure 2), and by comparison with the corresponding data of kazinol H.[2]

The chemical shift values of C-3 and C-4 of kazinols E and H, reported in the literature,[2] should be revised according to those of C-3 and C-4 of **1** and **2** shown in Table 1.

The cytotoxic effects of the constituents isolated from this plant against a number of cancer cell types were studied. The results are listed in Table 2. Compound **1** showed modest cytotoxic effects against T24, human hepatoma PLC/PRF/5 and 212 cells, respectively, while **3** showed cytotoxic effects against PLC/PRF/5 and HT3 cells. Cisplatin and actinomycin D were used as positive controls. The above results indicated that the 3′,4′-dihydroxy group of flavan derivatives may be responsible for the weak cytotoxicity observed and that the reduction of the chromene ring in **4** increased the cytotoxic effects.

Experimental Section

General Experimental Procedures. Melting points are reported uncorrected. The optical rotation was obtained on a JASCO model DIP-370 digital polarimeter. UV spectra were obtained on a JASCO model 7800 UV−vis spectrophotometer, and IR spectra were recorded on a Hitachi model 260−30 spectrophotometer. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR spectra were recorded on a Varian Unity-400 spectrometer, and MS were obtained on a JMS−HX 100 mass spectrometer.

Plant Material. Roots of *Broussonetia kazinoki* were collected at Taichung Hsien, Taiwan, in August 1995. A voucher specimen is on deposit at the Department of Medicinal Chemistry, School of Pharmacy, Kaohsiung Medical College.

Extraction and Isolation. The root bark of *B. kazinoki* (320 g) was chipped and extracted with Me_2CO at room temperature. The Me_2CO extract (43 g) was chromatographed on Si gel. Elution with cyclohexane−EtOAc (4:1) yielded **1** (31 mg, 0.07%); cyclohexane−EtOAc (3:2) yielded **3** (23 mg, 0.05%) and **4** (25 mg, 0.06%); cyclohexane−EtOAc (1:1) yielded kazinol H (13 mg, 0.03%) and 7,4′-dihydroxyflavan (**5**) (18 mg, 0.04%); and cyclohexane−EtOAc (2:3) yielded oleanolic acid (18 mg, 0.04%) and kazinol R (**2**) (10 mg, 0.02%). The known compounds, **3**−**5**, kazinol H, and oleanolic acid were identified by spectroscopic methods and by comparison with authentic samples or reported data.[2,3,8]

Kazinol Q (1): colorless powder ($CHCl_3$−MeOH); mp 142 °C; $[\alpha]^{26}_{D}$ 4.8° (c 0.5, $CHCl_3$); Gibb's test (positive); UV (MeOH)

Table 2. Cytotoxicity of Prenylflavonoids Isolated from *B. kazinoki* (ED_{50} values in μg/mL)[a]

compound	cell lines*					
	PLC/PRF/5	T24	212	HT3	SiHa	CaSki
1	3.5	2.3	3.8	4.3	4.7	[c]
2	NS[b]	[c]	NS	9.3	9.3	8.2
3	3.3	NS	7.0	3.6	NS	[c]
4	NS	NS	NS	8.6	NS	[c]
5	NS	[c]	NS	11.6	8.9	17.4
cisplatin	5.3	[c]	1.3	[c]	[c]	[c]
actinomycin D	1.4×10^{-3}	1.5×10^{-3}	[c]	5.6×10^{-4}	8.1×10^{-4}	1.9×10^{-3}

[a] For significant activity of the pure compounds, an ED_{50} < 4.0 μg/mL is required. [b] NS, no significant activity of the pure compounds. [c] Not determined.

λ_{max} (log ϵ) 214 (3.54), 231 (sh) (3.08), 283 (2.68), 403 (2.27) nm; IR (KBr) ν_{max} 3483, 3425, 3351 (OH), 1691, 1593 (aromatic rings) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz), see text; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; EIMS (70 eV) *m/z* $[M]^+$ 462 (16), $[M - C_4H_8]^+$ 406 (2), $[M - C_5H_8 - 1]^+$ 393 (1), 272 (13), 271 (14), 258 (7), 216 (13), 215 (49), 191 (90); HREIMS *m/z* 462.2752 (calcd for $C_{30}H_{38}O_4$, 462.2770).

Kazinol R (2): yellow oil; $[\alpha]^{26}_D$ 18°(*c* 0.1, $CHCl_3$); Gibb's test (negative); UV (MeOH) λ_{max} (log ϵ) 213 (3.89), 238 (sh) (3.52), 286 (3.20), 403 (2.41) nm; IR ($CHCl_3$) ν_{max} 3475 (OH), 1636, 1606 (aromatic rings) cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) δ 1.34 (3H, s, Me-17 or 18), 1.36 (3H, s, Me-17 or 18), 1.42 (6H, s, Me-10 and 11), 1.67, 1.71 (each 3H, s, Me-23 and 22), 2.02−2.08 (2H, m, H-3), 2.74−2.96 (2H, m, H-4), 2.70 (1H, dd, J = 17.2, 6.4 Hz, H-14a), 2.97 (1H, dd, J = 17.2, 5.2 Hz, H-14b), 3.21 (1H, dd, J = 16.4, 6.4 Hz, H-19), 3.34 (1H, dd, J = 16.4, 6.4 Hz, H-19), 3.85 (1H, dd, J = 6.4, 5.2 Hz, H-15), 4.95 (1H, t, J = 6.4 Hz, H-20), 5.09 (1H, dd, J = 10, 2.8 Hz, H-2), 5.27 (1H, d, J = 10.4 Hz, H-13*E*), 5.34 (1H, d, J = 17.6 Hz, H-13*Z*), 5.51 (1H, br s, OH -3′), 5.68 (1H, br s, OH-7), 6.18 (1H, dd, J = 17.6, 10.4 Hz, H-12), 6.39 (1H, s, H-8), 6.94 (1H, s, H-5), 6.99 (1H, s, H-2′); ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 1; EIMS (70 eV) *m/z* $[M]^+$ 478 (29), $[M - H_2O + 1]^+$ 461 (2), 422 (4), 287 (7), 215 (15), 191 (66); HREIMS *m/z* 478.2714 (calcd for $C_{30}H_{38}O_5$, 478.2719).

Kazinol D (3): yellow oil; UV, IR (film), MS, and ^1H and ^{13}C NMR data were in agreement with literature values.[2]

Kazinol K (4): yellow oil; UV, IR (film), MS, and ^1H and ^{13}C NMR data were in agreement with literature values.[2]

7,4′-Dihydroxyflavan (5): white plates; UV, IR (KBr), MS, and ^1H and ^{13}C NMR data were in agreement with literature values;[9] HREIMS *m/z* 242.0953 (calcd for $C_{15}H_{14}O_3$, 242.0943).

Kazinol H: yellow oil; UV, IR (film), MS, and ^1H and ^{13}C NMR data were in agreement with literature values.[2]

Tumor Cell Growth Inhibition Assays. A microassay for cytotoxicity was performed using a MTT (3-[4,5-dimethylthiazol-2-yl]-5-[3-carboxymethoxymethoxyphenyl]-2-[4-sulfophenyl]-2*H*-tetrazolium bromide) assay.[9,10] Briefly, 1−3 × 10^3 cells/100 μL were seeded in 96-well microplates (Nunck, Roskilde, Denmark) and preincubated for 6 h to allow cell attachment. This medium was then aspirated, and 100 μL of fresh medium containing various concentrations of test drug were added to the cultures. The cells were incubated with each drug for 6 days. Cell survival was evaluated by adding 10 μL of tetrazolium salt solution (1 mg MTT/mL in PBS). After 4 h incubation at 37 °C, 100 μL DMSO was added to dissolve the precipitate of reduced MTT. Microplates were then shaken for 15 min, and the absorbance was determined at 550 nm with a multiwell scanning spectrophotometer (Dynex MR 5000, Chantilly, VA).

PLC/PRF/5 cells were established from a human hepatoma and known to produce HBs Ag continuously in culture fluids.[11] Human hepatoma PLC/PRF/5, T24 cells, human cervical carcinoma, HT-3, SiHa, and CaSki cells were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Grand Island, NY),[9,10] containing 10% fetal bovine serum (FBS; Gibco BRL), 2 mM L-glutamine, 100 units/mL penicillin, and 100 μg/mL streptomycin. The 212 cells (an inducible Ha-*ras* oncogene transformed NIH/3T3 cell line) were maintained in minimum essential alpha medium (MEM; Gibco BRL), containing 10% calf serum (Gibco BRL) and antibiotics.[12] For the microassay, the growth medium was supplemented with 10 mM HEPES buffer pH 7.3 and incubated at 37 °C in a CO_2 incubator.

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References and Notes

(1) Liou, S. S.; Shieh, W. L.; Cheng, T. H.; Won, S. J.; Lin, C. N. *J. Pharm. Pharmacol.* **1993**, *45*, 791−794.
(2) Ikuta, J.; Hano. Y.; Nomura, T. Kawakami, Y.; Sato, T. *Chem. Pharm. Bull.* **1986**, *34*, 1968−1979.
(3) Saini, K. S.; Ghosal, S. *Phytochemistry* **1984**, *23*, 2415−2421.
(4) Fukai, T.; Nomura, T. *Heterocycles* **1989**, *29*, 2379−2390.
(5) Ikuta, J.; Hano, Y.; Nomura, T. *Heterocycles* **1985**, *23*, 2835−2842.
(6) Fang, S. C.; Shieh, B. J.; Lin, C. N. *Phytochemistry* **1994**, *37*, 851−853.
(7) Silverstein, R. M.; Bassler, G. C.; Morrill, T. C. *Spectrometric Identification of Organic Compounds*, 4th ed.; John Wiley and Sons: New York, 1981; p 235.
(8) Coxon, D. T.; O'Neill, T. M.; Mansfield, J. W.; Porter, A. E. A. *Phytochemistry* **1980**, *19*, 889−891.
(9) Carmichael, J.; Mitchell, J. B.; DeGraff, W. G.; Gamson, J.; Gazdar, A. F.; Johnson, B. E.; Glatstein, E.; Minna, J. D. *Br. J. Cancer* **1988**, *57*, 540−547.
(10) Tsai, C. M.; Gazdar, A. F.; Venzon, D. J.; Steinberg, S. M.; Dedrick, R. L.; Mulshine, J. L.; Kramer, B. S. *Cancer Res.* **1989**, *49*, 2390−2392.
(11) Nakajima, Y.; Kuwata, T.; Nomita, Y.; Okuda, K. *Microbiol. Immunol.* **1982**, *26*, 705−712.
(12) Liu, H. S.; Scrable, H.; Villaret, D. B.; Lieberman, M. A.; Stambrook, P. J. *Cancer Res.* **1992**, *52*, 983−989.

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