Constituents of Chinese Propolis and Their Antiproliferative Activities

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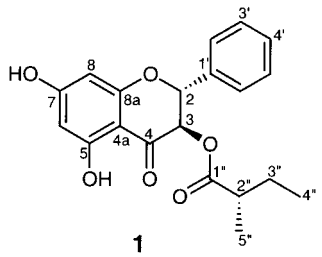
Two new flavonoids, 3-*O*-[(*S*)-2-methylbutyroyl]pinobanksin (**1**) and 6-cinnamylchrysin (**2**), were isolated from the EtOAc-soluble fraction of the MeOH extract of Chinese propolis, along with 12 known compounds (**3**−**14**). The structures of the isolated compounds were elucidated on the basis of spectroscopic and chemical analyses. The isolated compounds were tested for their antiproliferative activity toward five different cancer cell lines. Benzyl caffeate (**13**) and phenethyl caffeate (**14**) showed potent antiproliferative activity toward tested cell lines with a selective activity toward colon 26-L5 carcinoma cell line (EC_{50} values: **13**, 1.01; **14**, 0.30 μM).

Introduction

Propolis (sometimes referred to "bee glue"), a sticky material that honeybees collect from buds and exudates of plants, is used in the construction and adaptation of bee hives. There is a long history of the use of propolis for various purposes dating back to at least 300 BC,[1] and even now in the 21st century, it is used in home remedies and personal products. Propolis has also gained popularity as a health food in various parts of world, including the United States, Japan, and the European Union, where it is claimed to improve human health and prevent diseases such as inflammation, heart disease, diabetes, and even cancer. Several biological activities such as anticancer, antioxidant, antiinflammatory, antiseptic, antimycotic, bacteriostatic, astringent, choleric, spasmolytic, and anaesthetic properties have been reported for propolis and its constituents.[2−4] Due to these purported beneficial effects, there is a renewal of interest in the composition and biological activities of propolis. The composition of propolis depends on the vegetation of the area in which it is collected. Propolis from Europe, North and South America, Asia, and Africa differ in their composition because of the differences in the local vegetation. More than 300 constituents have been identified so far from propolis, among which phenolic compounds such as flavonoids and cinnamic acid derivatives have been reported as major constituents of propolis from the temperate zone.[2,5]

In our previous studies, we examined the ability of Brazilian propolis to protect β-cells against the toxicity of streptozotocin in rats[6] and isolated four potent antihepatotoxic dicaffeoylquinic acids,[7] a new prenylated chromane derivative,[8] and two novel benzofuran derivatives.[9] In other continuing work on propolis, we evaluated the quality of propolis from Brazil, Peru, The Netherlands, and China, based on the hepatoprotective, antiproliferative, and free radical scavenging activities and LCMS analysis.[10,11] In this study, we observed that an extract of Chinese propolis possessed potent antiproliferative activity.[10] However, there are only a few reports on the constituents of Chinese propolis,[12−15] and no report has been found on antiproliferative constituents. Thus, we examined the constituents of Chinese propolis and isolated two new flavonoids (**1**, **2**), together with 12 known compounds (**3**−**14**). In this paper, we report the isolation and structure elucidation of the two new flavonoids and antiproliferative activities of the isolated compounds toward murine B16-BL6 melanoma, human HT-1080 fibrosarcoma, human lung A549 adenocarcinoma, human cervix HeLa adenocarcinoma, and murine colon 26-L5 carcinoma cell lines.



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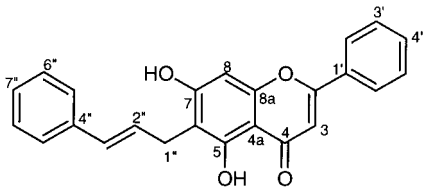

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Results and Discussion

Chinese propolis was successively extracted with H_2O, MeOH, and $CHCl_3$. The MeOH extract, having the strongest antiproliferative activity (EC_{50} values: 8.1 and 6.7 μg/mL against murine B16-BL6 melanoma and human cervix HeLa adenocarcinoma cell lines, respectively), was partitioned into EtOAc-soluble and EtOAc-insoluble fractions. The EtOAc-soluble fraction (EC_{50}: 7.9 and 6.5 μg/mL against B16-BL6 and HeLa cell lines, respectively) showed antiproliferative activity similar to the MeOH extract and thus was subjected to further chemical analysis to give two new flavonoids, namely, 3-*O*-[(*S*)-2-methylbutyroyl]pinobanksin (**1**) and 6-cinnamylchrysin (**2**), together with 12 known compounds: chrysin (**3**),[16] galangin (**4**),[17] izalpinin (**5**),[18] apigenin (**6**),[16] techtochrysin (**7**),[19] pinostrobin (**8**),[20] pinocembrin (**9**),[16] isoferulic acid (**10**),[21] 3,4-dimethoxycinnamic acid (**11**),[21] benzyl ferulate (**12**),[2] benzyl caffeate (**13**),[22] and phenethyl caffeate (**14**).[23] Among these, compounds **11**, **12**, and **14** were found for the first time from Chinese propolis.

3-*O*-[(*S*)-2-Methylbutyroyl]pinobanksin (**1**) was obtained as a yellow amorphous solid, having $[\alpha]^{25}_{D}$ +24.5° (*c* 0.1,

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Table 1. ^{1}H and ^{13}C NMR Data for Compounds **1** (in $CDCl_3$) and **2** (in DMSO)[a]

	1			**2**		
position	δ_H	δ_C	HMBC[a]	δ_H	δ_C	HMBC[b]
2	5.36 d (12.0)	81.5	3, 2′, 6′		162.7	3, 2′, 6′
3	5.85 d (12.0)	71.9	2	6.90 s	105.0	
4		191.7	2, 3		181.7	3
4a		101.9	6, 8, 5-OH		103.7	3, 8, 5-OH
5		164.2	6, 5-OH		158.6	1″, 5-OH
6	6.04 d (2.2)	97.3	8, 5-OH		109.3	1″, 8, 5-OH
7		165.1	6, 8		162.1	8, 1″
8	6.00 d (2.2)	95.8	6	6.57 s	93.2	
8a		162.5	8		155.4	8
5-OH	11.49 br s			13.09 s		
7-OH				10.86 s		
1′		135.0	2, 3′, 5′		130.7	3, 3′, 5′
2′, 6′	7.47 m	127.6	2, 3′, 4′, 5′	8.03 d (6.4)	126.1	3′, 4′, 5′
3′, 5′	7.41 m	128.7	2′, 4′, 6′	7.56 m	128.8	2′, 4′, 6′
4′	7.39 m	129.6	2′, 3′, 5′, 6′	7.56 m	131.5	2′, 3′, 5′, 6′
1″		175.1	3, 2″, 3″, 5″	3.45 d (5.1)	25.1	3″
2″	2.36 qt (7.1, 6.9)	40.7	3″, 4″, 5″	6.31 dt (16.1, 5.1)	127.5	1″, 3″
3″	1.29 dqd (7.6, 14.3, 14.3)	26.4	2″, 4″, 5″	6.37 d (16.1)	129.4	1″, 5″, 9″
	1.47 dqd (7.6, 14.3, 14.3)					
4″	0.60 t (7.6)	10.9	2″, 3″		137.1	2″, 6″, 8″
5″	1.05 d (6.8)	16.4	2″, 3″	7.31 d (7.3)	125.5	3″, 7″
6″				7.25 dd (7.6, 7.3)	128.2	5″, 7″
7″				7.15 br t (7.6)	126.5	5″, 9″
8″				7.25 dd (7.6, 7.3)	128.2	5″, 7″
9″				7.31 d (7.3)	125.5	3″, 7″

[a] The ^{1}H and ^{13}C NMR spectra were measured at 100 and 400 MHz, respectively, and coupling constants (parentheses) are in Hz. [b] ^{1}H correlating with ^{13}C resonance.

$CHCl_3$) and molecular formula $C_{20}H_{20}O_6$ (HRFABMS). The IR spectrum of **1** shows absorption bands corresponding to hydroxyl (3350 cm^{-1}) and ester carbonyl (1735 cm^{-1}) groups. The ^{1}H NMR spectrum of **1** (Table 1) displays signals for a monosubstituted benzene ring, *meta*-coupled aromatic protons ($J = 2.2$ Hz), vicinally coupled oxygenated methines ($J = 12.0$ Hz), and a 1-methylpropyl group, together with a signal for a hydrogen-bonded phenolic proton (δ 11.49, s). The ^{13}C NMR spectrum shows 20 carbon signals including those corresponding to the above groups, as well as ketone (δ 191.7) and ester (δ 175.1) carbonyl carbons (Table 1). These data suggested **1** is a flavonol having a 2-methylbutyroyl group. The analyses of the COSY, HMQC, and HMBC spectra (Table 1) indicated the flavonol to be 3,5,7-trihydroxyflavonol (pinobanksin).[24] The location of the 2-methylbutyroyl group was determined to be at C-3, on the basis of a low-field shift of H-3 (**1**, δ 5.85; pinobanksin,[24] δ 4.54) and the HMBC correlation between the ester carbonyl carbon (δ 175.1) and the protons H-3, H-2″, H-3″, and H-5″. Alkaline hydrolysis of **1** with 1 N KOH gave 2-methylbutyric acid and pinobanksin. The absolute configuration of pinobanksin was determined to be 2*R*,3*R* on the basis of the $[\alpha]^{25}_D$ value (+16.5°, MeOH; lit.[24] $[\alpha]_D$ +15°, MeOH). On the other hand, the absolute configuration of 2-methylbutyric acid was determined to be *S* by GC analysis of its methyl ester with a chiral column. Thus, compound **1** was identified as 3-*O*-[(*S*)-2-methylbutyroyl]pinobanksin. To the best of our knowledge, this is the first report of the presence of a (*S*)-2-methylbutyroyl group in flavonoids.

6-Cinnamylchrysin (**2**) was isolated as a yellow amorphous solid with the molecular formula $C_{24}H_{18}O_4$, and its IR spectrum shows the presence of hydroxyl (3125 cm^{-1}) and carbonyl (1640 cm^{-1}) groups. The ^{1}H NMR spectrum of **2** shows signals for two phenyl groups, two isolated olefins, a *trans*-olefin ($J = 16.1$ Hz) coupled with a methylene, and two phenolic protons (Table 1). The ^{13}C NMR spectrum shows 24 carbon signals including a carbonyl carbon signal (δ 181.7) (Table 1). These data and analyses of the COSY, HMQC, and HMBC spectra indicated **2** to be a chrysin derivative having a cinnamyl group at C-6 or C-8. The location of the cinnamyl group was determined to be at C-6 from the HMBC correlation of H-1″ with C-5, C-6, and C-7. Thus, compound **2** was determined to be 6-cinnamylchrysin. Cinnamic acid and its derivatives are widely distributed in the plant kingdom, and there are several reports of the attachment of the cinnamyl group with other classes of compounds.[5,25,26] However, this is the first report of a flavone with a cinnamyl moiety from propolis.

Table 2. Antiproliferative Activity of the Isolated Compounds (EC_{50} Values in μM)

compound	B16-BL6	HT-1080	A549	HeLa	colon 26-L5
1	185	143	160	116	78.9
2	>270	>270	>270	>270	>270
3	74.4	94.9	233	111	109
4	26.8	35.7	93.6	79.0	19.2
5	>350	>350	>350	106	30.3
6	31.6	36.6	101	92.7	25.0
7	226	358	>370	>370	273
8	111	156	>370	202	128
9	68.0	92.4	229	108	75.6
10	>515	>515	>515	>515	>515
11	>480	>480	>480	>480	>480
12	63.7	46.7	92.8	51.1	46.4
13	9.78	9.74	35.0	2.33	1.01
14	6.79	9.50	27.9	2.36	0.30
5-fluorouracil	1.23	1.38	3.61	0.23	0.31
doxorubicin HCl	0.11	0.02	0.04	0.02	0.02

The isolated compounds were tested for their antiproliferative activity toward murine B16-BL6 melanoma, human HT-1080 fibrosarcoma, human lung A549 adenocarcinoma, human cervix HeLa adenocarcinoma, and murine colon 26-L5 carcinoma cell lines (Table 2). Benzyl caffeate (**13**) and phenethyl caffeate (**14**) showed potent antiproliferative activity toward all cell lines, especially toward colon 26-L5 carcinoma cell line (EC_{50} values: **13**, 1.01; **14**, 0.30 μM), with EC_{50} values identical to that of 5-fluorouracil. Isoferulic acid (**10**) and 3,4-dimethoxycin-

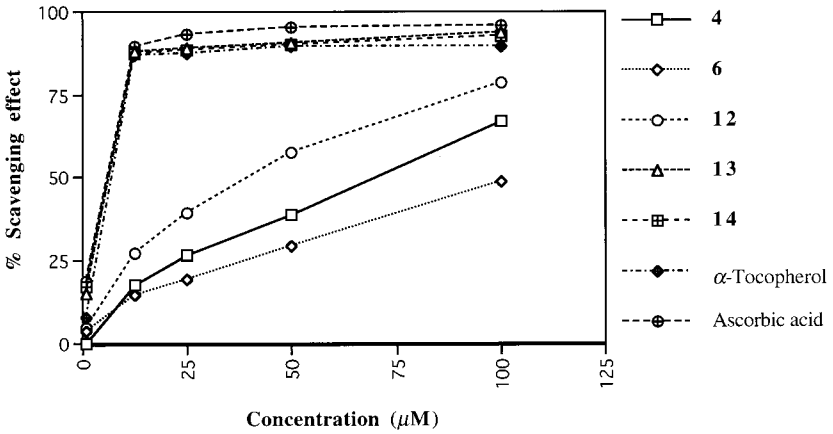

Figure 1. DPPH free radical scavenging activity of isolated compounds.

namic acid (**11**), on the other hand, had no antiproliferative effect, indicating that the ester moiety is important for antiproliferative activity. The hydroxyl groups at the caffeic acid moiety might also contribute to the antiproliferative activity, since it was observed that benzyl ferulate (**12**), which has a methoxy group at C-3, showed weaker antiproliferative activity. Among the flavonoids, galangin (**4**) and apigenin (**6**) showed moderate antiproliferative activities toward B16-BL6, HT-1080, and colon 26-L5 cell lines, with EC_{50} values less than 40 μM. The remaining compounds, including both of the new flavonoids, possessed only weak or no antiproliferative activities. Phenethyl caffeate (**14**), also called CAPE, has been reported to have strong antitumor activity both in vitro and in vivo.[23] Moreover, CAPE (**14**) has been reported to have strong antioxidative activities,[27,28] which seem to correspond to its antiproliferative activity. Thus, as a measure of antioxidative effect, we also examined the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activities of **4**, **6**, **12**, **13**, and **14** (Figure 1), which showed a range of antiproliferative activities from mild to potent. Interestingly, **13** and **14**, both of which show potent antiproliferative activities, also strongly scavenge DPPH free radical (Figure 1). Thus, it appears that the antiproliferative activities of these caffeic acid derivatives may also be associated with their antioxidative properties.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-140 digital polarimeter. IR spectra were measured with a Shimadzu IR-408 spectrophotometer in a KBr disk or in $CHCl_3$ solution. HRFABMS measurements were performed on a JEOL JMS-700T spectrometer, and glycerol was used as a matrix. Gas chromatography was done on a Shimadzu GC-14AH gas chromatograph using a Chiraldex G-TA column with nitrogen as carrier gas. UV spectra were recorded on a Shimadzu UV-160A UV−visible spectrophotometer. The ^{1}H, ^{13}C, and 2D NMR spectra were taken on a JEOL JNM LA-400 spectrometer with tetramethylsilane (TMS) as an internal standard. Column chromatography was performed with silica gel 60 (Nacalai tesque, Inc., Kyoto, Japan). Analytical and preparative TLC was conducted on precoated Merck Kieselgel 60F_{254} and RP-18F_{254} plates (0.25 or 0.50 mm thickness).

Biological Material. Propolis used in this study was a gift from Saitama Bee-Keeping Co., Ltd. A voucher sample (TMPW 20925) is preserved in the Museum for Materia Medica, Analytical Research Center for Ethnomedicines, Institute of Natural Medicine, Toyama Medical and Pharmaceutical University, Toyama, Japan.

Extraction and Isolation. Chinese propolis (834 g) was extracted with H_2O (3 L, 2 h, 80 °C, ×2), and the insoluble portion was separated by filtration. The filtrate, on cooling, gave a fatty substance (145 g), and the remaining filtrate was lyophilized to give a H_2O extract (12.5 g). On the other hand, the residue was further extracted with MeOH (3 L, reflux, 2 h, ×2) and then by $CHCl_3$ (2 L, room temperature, ×2) to yield a MeOH extract (220 g) and a $CHCl_3$ extract (123 g), respectively. The MeOH extract (120 g) was further fractionated into EtOAc-soluble (115 g) and EtOAc-insoluble fractions (3.2 g). The EtOAc-soluble fraction (90.0 g) was subjected to silica gel column chromatography with a $CHCl_3$−MeOH gradient system to afford eight fractions: fraction 1: $CHCl_3$ eluate, 4.4 g; fraction 2: 1% MeOH−$CHCl_3$ eluate, 4.5 g; fraction 3: 2% MeOH−$CHCl_3$ eluate, 7.8 g; fraction 4: 3% MeOH−$CHCl_3$ eluate, 10.8 g; fraction 5: 5% MeOH−$CHCl_3$ eluate, 3.7 g; fraction 6: 10% MeOH−$CHCl_3$ eluate, 11.5 g; fraction 7: 20% MeOH−$CHCl_3$ eluate, 22.3 g; fraction 8: 30% MeOH−$CHCl_3$ eluate, 25.5 g. Repeated column chromatography of these fractions over silica gel, followed by preparative TLC, yielded the following compounds: fraction 1: **5** (33.0 mg), **7** (37.0 mg), and **8** (614 mg); fraction 2: **7** (168 mg) and **12** (740 mg); fraction 3: **1** (8.8 mg), **2** (30.5 mg), and **9** (57.8 mg); fraction 4: **3** (110 mg), **4** (333 mg), **11** (306 mg), and a mixture of **4** and **9** (550 mg); fraction 5: **3** (3.5 g); fraction 6: **3** (65.0 mg), **13** (9.9 mg), and **14** (27.3 mg); fraction 7: **6** (15.6 mg) and **10** (33.4 mg); fraction 8: **3** (120 mg) and **6** (12.5 mg).

3-*O*-[(*S*)-2-Methylbutyroyl]pinobanksin (1): yellow amorphous solid; $[\alpha]^{25}_D$ +24.5° (*c* 0.1, $CHCl_3$); IR ($CHCl_3$) ν_{max} 3350, 1735, 1635, 1460, 1145, 1085 cm^{-1}; HRFABMS *m*/*z* 357.1328 (calcd for $C_{20}H_{21}O_6$ $[M + H]^+$, 357.1341); ^{1}H and ^{13}C NMR, see Table 1.

6-Cinnamylchrysin (2): yellow amorphous solid; IR (KBr) ν_{max} 3125, 1640, 1610, 1580, 1350, 1300, 1240, 1175, 1090 cm^{-1}; HRFABMS *m*/*z* 371.1326 (calcd for $C_{24}H_{19}O_4$ $[M + H]^+$, 371.1343); ^{1}H and ^{13}C NMR, see Table 1.

Alkaline Hydrolysis of Compound 1. A solution of **1** (2.0 mg) in MeOH (1.0 mL) and 1 N KOH (1.0 mL) was stirred overnight at room temperature. The reaction mixture was neutralized with 1 N HCl and extracted with EtOAc (10 mL). The EtOAc layer was evaporated, and the residue was dissolved in MeOH (1.0 mL) and treated with excess CH_2N_2. After evaporation, the residue was dissolved in $CHCl_3$ (5.0 mL) and filtrated. An aliquot of the filtrate was analyzed by GC (column, Astec Chiraldex G-TA G0012-08, 30 m × 0.25 mm; column temperature, 50 °C; detector temperature, 250 °C; injection temperature, 250 °C), to give a peak at t_R 11.05. Standard (*R*)- and (*S*)-2-methylbutyrate gave one peak at t_R 9.99 and 10.92 min, respectively. The remaining filtrate was subjected to preparative TLC with 1% MeOH−$CHCl_3$ to give pinobanksin (0.2 mg) ($[\alpha]^{25}_D$ +16.5°, *c* 0.01, MeOH).

Antiproliferative Assay. All the cancer cell lines were maintained in Eagle's minimum essential medium (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), except for murine colon 26-L5 carcinoma cell line, which was maintained in RPMI (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Both of these media were supplemented with 10% fetal calf serum

(Gibco BRL Products, Gaithersburg, MD), 0.1% sodium bicarbonate (Nacalai tesque Inc., Kyoto, Japan), and 2 mM glutamine (Wako Pure Chemicals Ind., Ltd., Osaka, Japan).

Cellular viability in the presence and absence of experimental agents was determined using the standard 3-(4,5-dimethylthiazol-2-yl)-2,5-dimethyltetrazolium bromide (MTT; Sigma, St. Louis, MO) assays as described previously.[8,29−31] Briefly, exponentially growing cells were harvested, and a 100 μL suspension containing 2500 cells was plated in 96-well microtiter plates (Corning Incorporated, Costar, 3595). After 24 h of incubation at 37 °C under 5% CO_2 to allow cell attachment, the cells were treated with varying concentrations of test specimens in their respective medium (100 μL) and incubated for 72 h under the same conditions as above. After adding a solution of MTT for 2 h, the amount of formazan formed was measured spectrophotometrically at 550 nm using a Perkin-Elmer HTS-7000 Bio Assay Reader. Test specimens were dissolved in DMSO and then diluted by medium. DMSO less than 0.1% in the test solution had no effect. 5-Fluorouracil (Tokyo Kasei Kogyo Co., Ltd., Tokyo, Japan) and doxorubicin HCl (Kyowa Hakko Co., Ltd., Tokyo, Japan) were used as positive controls, and EC_{50} values were calculated from the mean values of data from four wells.

DPPH Radical Scavenging Assay. DPPH radical scavenging activity was measured according to the procedure described previously.[31] Briefly, samples dissolved in EtOH (500 μL) were mixed with an equal volume of DPPH solution (60 mM). The resulting solution was thoroughly mixed by vortex, and absorbance was measured at 520 nm after 30 min. The scavenging activity was determined by comparing the absorbance with that of the blank (100%) containing only DPPH and solvent.

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