Cytotoxic Prenylflavanones from Taiwanese Propolis

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Received July 11, 2002

Two new prenylflavanones, propolin A (**2**) and propolin B (**3**), were isolated and characterized from Taiwanese propolis. Both compounds were found to have cytotoxic properties *against* three cancer cell lines. DNA content analyses and DNA fragmentation indicated that propolin A (**2**) *efficiently* induced apoptosis in cancer cell lines, but *had* no effect on the cell cycle program. Furthermore, both propolin A (**2**) and B (**3**) are potential antioxidant agents and show strong scavenging effects against most types of free radicals.

Propolis is a natural honeybee product, a resinous material gathered by honeybees from the buds and barks of certain trees and plants, and is used inside the hives.[1] Propolis has long been used as a crude medicine and folk remedy chiefly in Europe.[2] The material contains various chemical components and exhibits a broad spectrum of biological activities, including antitumor,[3] antioxidant,[4] antibacterial,[5] antiviral,[6] antifungal,[7] and antiinflammatory activities.[7]

More than 200 constituents have been identified from propolis; however, they do not yet account for the various biological activities of propolis.[5] The mixture of propolis consists of wax, flavonoids, and phenolic conpounds, such as cinnamic acids and their derivatives, as well as various aldehydes and ketones.[10] Caffeic acid phenethyl ester (CAPE) (**1**) was one of the components isolated and identified to show antitumor and antioxidant properties.[8,11]

In the present study, two components of a local Taiwanese propolis were isolated and characterized. Their structures were elucidated mainly by NMR spectral evidence and found to be two unreported prenylflavanones, which were given the trivial names propolin A (**2**) and propolin B (**3**). Both propolins inhibit human melanoma, C6 glioma, and HL-60 cell proliferation through induction of apoptosis.

Results and Discussion

Compound **2** was isolated through repeated chromatographies of the 95% ethanol extract of the propolis glue under the guidance of human melanoma proliferation. Final purification of the active fraction was achieved by HPLC on a reversed-phase column. The total content of the active component, propolin A (**2**), was roughly 3% of the propolis glue. The ^{1}H NMR (Table 1) displayed absorptions of three methyl groups at δ_H 1.12 (s, 6H) and 1.64 (s, 3H), four methylenes at δ_H 1.35, 1.42, 1.95, and 3.46, and three mutually coupled protons at δ_H 2.60, 3.10, and 5.47. In addition, four other olefinic protons at δ_H 5.878, 5.882, 6.72, and 6.87 were also observed. Obviously, the last two were isolated adjacent protons on an aromatic ring. The ^{13}C-DEPT NMR (Table 2) confirmed three methyl groups, five methylenes, six methines at δ_C 77.8, 96.2, 97.1, 113.6, 118.7, and 124.7, and 11 quaternary carbons. Among the quaternary carbons, the one at δ_C 71.5 was obvious of the sp^3-type.

Table 1. ^{1}H NMR Assignments of Propolin A (**2**)

position		δ_H (*J*, Hz)	HMBC correlations (C #)	NOESY correlations (H #)
2β	CH	5.47 (dd, 13.3, 2.7)	6′, [4], [1′][a]	10″, 3β, 6′ [3α], [1″],
3β	CH_2	2.60 (dd, 17.2, 2.7)	4, [10]	3α, 2β
3α		3.10 (dd, 17.2, 13.3)	2, 4, [1′]	3β, 6′, [2β]
6	CH	5.878(d, 2.0)	5, 7, 8, [4]	
8	CH	5.882 (d, 2.0)	6, 7, 9, 10, [4]	
5′	CH	6.72 (d, 8.3)	1′, 3′, [6′]	6′
6′	CH	6.87 (d, 8.3)	2′, 4′, [2], [3′]	3α, 2β, 5′
1″	CH_2	3.46 (d, 6.3)	2′, 4′, 2″, 3″,[5″]	2″, 4″, [2β]
2″	CH	5.12 (t, 6.4)	2′, 1″, [2″], 4″, 5″	1″, 4″, 6″, [2β]
4″	CH_3	1.64 (s)	2″, 3″, 5″	1″, [2β]
5″	CH_2	1.95 (t, 7.3)	2″, 3″, 4″, 6″, 7″	1″, 6″, [4″]
6″	CH_2	1.42 (m)	5″, 7″, [8″]	5″, 7″, 9″, 10″, [4″]
7″	CH_2	1.35 (m)	5″, 6″, 8″, 9″	5″, 6″, 9″, 10″, [4″]
9″	CH_3	1.12 (s)	7″, 8″, 10″	6″, 7″
10″	CH_3	1.12 (s)	7″, 8″, 10″	6″, 7″

[a] Square brackets signify weak correlations.

Table 2. ^{13}C NMR Assignments of Propolin A (**2**) and Propolin B (**3**)

carbon no.	**2** δ_C	**3** δ_C	carbon no.	**2** δ_C	**3** δ_C
2	77.8	80.7	1″	25.4	29.2
3	43.7	44.1	2″	124.7	123.9
4	198.2	197.7	3″	135.8	137.0
5	165.2[a]	164.8	4″	16.2	16.1
6	97.1	97.0	5″	41.2	41.3
7	168.3	168.5	6″	23.7	23.7
8	96.2	96.2	7″	44.3	44.3
9	165.5[a]	165.4	8″	71.5	71.4
10	103.2	103.4	9″	29.1	29.1
1′	129.7	130.9	10″	29.2	29.2
2′	128.3	111.9(CH)			
3′	144.5	146.1			
4′	146.5	144.6			
5′	113.6	129.7(4 °C)			
6′	118.7	119.8			

[a] Data interchangeable.

The subsequent HMQC and HMBC spectra (Tables 1 and 2) clearly demonstrated the presence of a C_{10}-geranyl group on a catechol ring. Further interpretation of all NMR data assisted by a series of mass spectral data and the result from elemental analysis suggested a flavanone skeleton of structure **2**. Although both the EIMS and the positive-ion FABMS showed a possible $[M]^+$ of 424, the negative-ion FABMS and the ESIMS both indicated *m*/*z*

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10.1021/np0203180 CCC: $25.00
Published on Web 03/29/2003

Table 3. ^{1}H NMR Assignments of Propolin B (**3**)

position		δ_H (J, Hz)	HMBC correlations (C #)	NOESY correlations (H #)
2β	CH	5.23 (dd, 13.0, 3.0)	1′, 2′, 6′, [4][a]	3α, 3β, 2′, 6′
3β	CH_2	2.68 (dd, 17.2, 3.0)	4, [10]	3α, 2β
3α		3.03 (dd, 17.2, 13.0)	2, 4, 5′	3β, 2β, 2′, 6′
6	CH	5.86(d, 1.9)	5, 7, 8, 9, 10	
8	CH	5.88 (d, 1.9)	5, 6, 7, 9, 10	
2′	CH	6.76 (d, 1.5)	2, 4′, 6′, [5′]	3α, 2β
6′	CH	6.68 (d, 1.5)	2′, 4′, [2], [3′]	3α, 2β, 1″, 2″
1″	CH_2	3.33 (d, 6.6)	4′, 5′, 6′, 2″, 3″, [2′], [5″], [6″]	6′, 2″, 4″
2″	CH	5.33 (t, 7.0)	1″, 6″, 4″, [1′]	1″, 6″, [7′]
4″	CH_3	1.70 (s)	2″, 3″, 6″	1″
5″	CH_2	2.03 (t, 6.8)	2″, 3″, 4″, 5″, 6″	6″, 7″
6″	CH_2	1.50 (m)	3″, 5″, 7″, [8″]	5″, 7″, 9″, 10″
7″	CH_2	1.41 (m)	5″, 6″, 8″, 9″, 10″	5″, 6″, 9″, 10″
9″	CH_3	1.36 (s)	7″, 8″, 10″	6″, 7″
10″	CH_3	1.36 (s)	7″, 8″, 9″	6″, 7″

[a] Square brackets signify weak correlations.

442 should be the molecular ion. A LC-MS of the impure sample with electrospray ionization also confirmed a $[M]^+$ of 442. HRMS of a pure sample of compound **2** indicated its molecular composition as $C_{25}H_{30}O_7$.

A closely related compound, nymphaeol B (**4**) from *Hernandia nymphaefolia*, has been reported.[12] The ^{13}C NMR data (in acetone-d_6) of **4** matched well with our data (in CD_3OD, Table 2). The absolute configuration of propolin A (**2**) was assumed the same as that of nymphaeol B (**4**) since they both showed very similar CD spectra [328.5 nm ($\Delta\epsilon$ +0.74), 286 nm ($\Delta\epsilon$ −5.96)].[12] Another C_{10}-geranyl flavanone, tanariflavanone A, with similar substitution pattern on ring B was also reported from the fallen leaves of *Macaranga tanarius* and showed inhibition of radicle growth of lettuce seedlings. [9]

A minor component, propolin B (**3**), of roughly one-third the quantity of the major one, **2**, with similar bioactivities was also isolated. Propolin B revealed ^{1}H and ^{13}C NMR spectra (Tables 2 and 3) very similar to those of propolin A (**2**). The only obvious differences were signals of the two aromatic protons at δ_H 6.68 (d, J = 1.5 Hz) and 6.76 (d, J = 1.5 Hz) on the catechol ring. The small coupling constants indicated a *meta*-relationship of these two protons instead of an *ortho*-relationship (d, J = 8.3 Hz, Table 1) in propolin A (**2**). The HMBC correlations as shown in Table 3 indeed supported structure **3**, with the geranyl group shifted from C-2′ to C-5′. The key NOE observations of both **2** and **3** as depicted in Figure 1 confirmed the substitution pattern. In the NOESY spectrum of propolin A (**2**), both H-1″ and H-10″ correlated with H-2β, and such correlations were not observed in propolin B (**3**). On the other hand, H-1″ showed a cross-peak with H-6′ in propolin B (**3**), indicating their proximity in space. A C_5-prenylated flavanone, sigmoidin B (**5**), with the same substitution pattern on ring B as that of propolin B (**3**) has been reported.[13] Sigmoidin B was isolated from the medicinal plant *Erythrina sigmoidea* with significant antibacterial activity.

Dose−response studies of cytotoxicity effects of propolins A (**2**) and B (**3**) on cultured cancer cell lines of human melanoma, C6 glioma, and HL-60 were conducted. Treatment of propolins for 24 h inhibited growth of all three cancer cell lines in a concentration-dependent manner. The results revealed that values of IC_{50} for propolin A (**2**) were respectively 6.0, 3.5, and 7.5 μg/mL, against human melanoma, C6 glioma, and HL-60. Corresponding IC_{50} values for propolin B (**3**) were 7.5, 4.0, and 7.5 μg/mL, respectively. Furthermore, when the cells were exposed to propolins A (**2**) and B (**3**) for 24 h at concentration above 15 μg/mL, all cells were damaged.

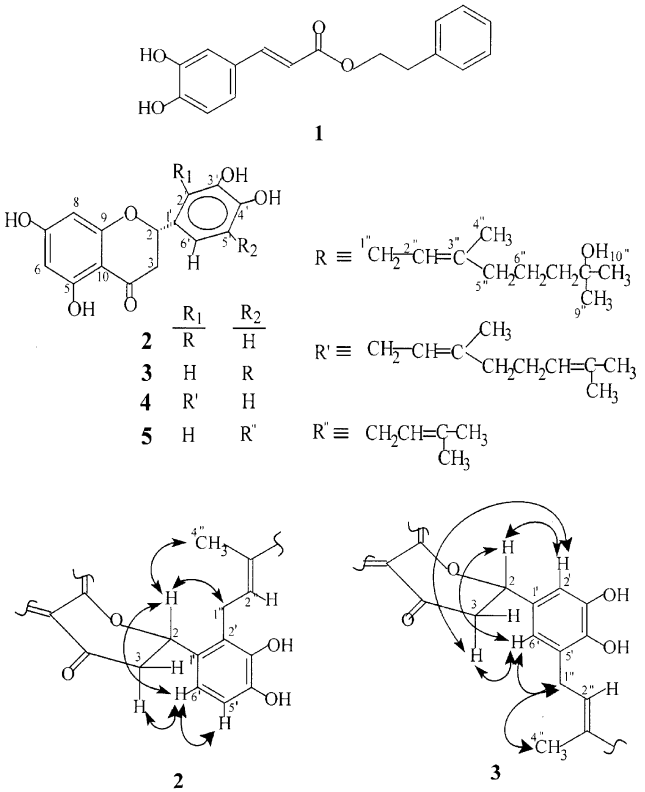

Figure 1. Key NOEs observed in compounds **2** and **3**.

Physiological cell death is usually characterized by apoptotic morphology, including chromatin condensation, DNA fragmentation, internucleosomal degradation of DNA, cell shrinkage, membrane blebbing, nuclear pyknosis, and apoptotic body formation. Observation of any of the above phenomena represents a universal and exquisitely efficient cellular suicide pathway. Apoptosis not only plays a key role in the development and maintenance of tissue homeostasis but is also an effective mechanism by which harmful cells can be eliminated.

We were interested in exploring how propolin A (**2**) affects apoptosis. After treatment of HL-60 cells at various concentrations for 24 h, the genomic DNA from cells was subjected to agarose gel electrophoresis. When HL-60 cells were exposed to propolin A (**2**) at concentrations below 5 μg/mL, the DNA fragmentation ladder, as observed by microscopy, was as shown in Figure 2. The results showed

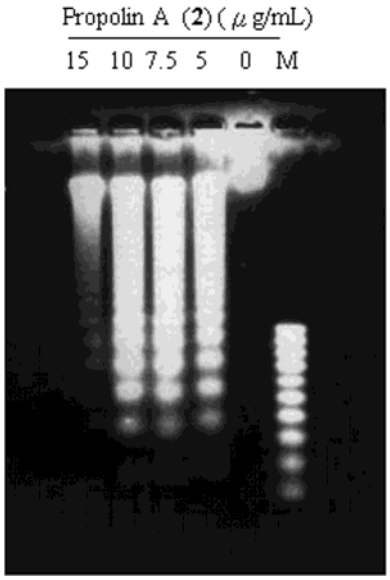

Figure 2. Apoptosis evidence of internucleosomal DNA fragmentation induced by propolin A (**2**). After being treated with **2** at various concentrations (0, 5.0, 7.5, 10.0, and 15.0 μg/mL) for 24 h, the internucleosomal DNA gragments from HL-60 cells were analyzed by agarose gel electrophoresis.

that the cytotoxic effect of HL-60 cancer cells induced bypropolin A (**2**) was triggered by apoptotic effects on cancer cell lines.

To investigate the induction of a sub-G1 cell population, the DNA content of human melanoma cells treated with propolin A (**2**) at various times was analyzed by flow cytometry. The percentages of apoptotic human melanoma cells observed after 0, 3, 6, 12, and 24 h were as follows: 13.8% [left column in Figure 3, with propolin A (**2**)] vs 11.07% [right column in Figure 3, without propolin A (**2**)], 15.01% vs 11.45%, 15.22% vs 5.26%, 23.93% vs 7.56%, and 40.95% vs 3.94%. A sub-G1 (sub-2N) DNA peak, which has been suggested to be the apoptotic DNA, was detected. However, the cell damage was not attributable to the cell cycle program and not arrested at any cell cycle phases. These results indicated that propolin A (**2**) induced apoptosis efficiently in cancer cell lines, but had no effect on the cell cycle program. All bioactivity data demonstrated that both propolin A and B showed cytotoxic effects on three cancer cell lines, and the effect was due to induced apoptosis.

The radical scavenging abilities of propolins A (**2**) and B (**3**) and CAPE (**1**) were tested with 1,1-diphenyl-2-picrylhydrazyl (DPPH) and the results are displayed in Table 4. The data indicated IC_{50} values of propolins A (**2**) and B (**3**) were approximately 5.0 and 9.0 μg/mL, respectively. These results suggested that both propolins A (**2**) and B (**3**) are potential antioxidant agents.

Experimental Section

General Experimental Procedures. The following mass spectrometers were used: EIMS, Finnigan MAT 95S; FABMS, JEOL SX 102A; HRMS, MAT-95XL; LC/MS, API-150EX from PE-SCIES; and LC/MS/MS, API-III from PE-SCIEX. Both ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) spectra were measured on a Bruker AMX-500 using CD_3OD as solvent. UV spectra were obtained on a Shimadzu UV-160A spectrophotometer and IR spectra on a Bio-Rad FTS-135 spectrophotometer. CD data were acquired on a Jasco T15. HPLC collections were performed on a reversed-phase (C18, 10 μm, 10 × 250 mm) column and eluted by a solvent system of 7:3 methanol/water.

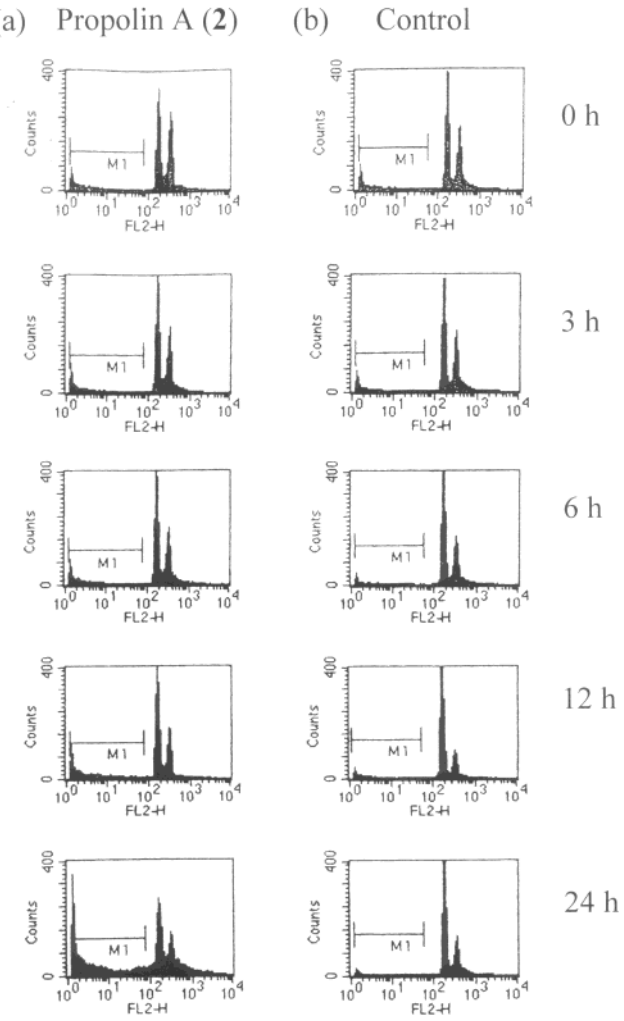

Figure 3. Observation of the increment of sub-G1 human melanoma cells induced by propolin A (**2**) by flow cytometry. Human melanoma cells were treated with (a) **2** at 15 μg/mL or (b) DMSO only as control for 0, 3, 6, 12, and 24 h. M1 represents apoptotic cells with a lower DNA content.

Extraction. Taiwanese propolis glue (245 g, mixture of the collection from hives located in the area of Bagwa Shan, Taiwan) was homogenized by stirring at 4 °C and washed with 0.7 L of deionized water three times. The residue was extracted with 95% ethanol three times. The filtered ethanol extract was evaporated to dryness under reduced pressure to furnish a brown powder, which was kept at −20 °C until further purification.

Isolation and Purification of Cytotoxic Compounds. The brown powder from the ethanol extract was dissolved in methanol and applied to a Sephadex LH-20 column (Amersham Pharmacia Biotech AB, Uppsala, Sweden) using 95% ethanol as eluting solvent. All eluates including fractions from the follow-up chromatographies were assayed on human melanoma proliferation, and the active fractions were again chromatographed on a Sephadex LH-20 column using 95% ethanol to elute. The active fractions were then subjected to silica gel column chromatography (Kiesel gel 60, E. Merck, Darmstadt 1, Germany) using a solvent system of *n*-hexane/EtOAc. Purification of the most active fraction (*n*-hexane/EtOAc, 30:70) was carried out on reversed-phase preparative HPLC. Fractions of retention times at 16.5 and 22.0 min for propolins A (**2**) and B (**3**) were collected, respectively. The conditions were as follows: column, Luna Phenomenex (C18, 250 × 10 mm); solvent system, methanol/water (7:3); flow rate, 3 mL/min; detection, UV 280 nm.

Propolin A (2): slightly yellow powder from methanol/water (7:3); $[\alpha]_D$ −4.3 (*c* 0.13, CH_3OH); IR ν_{max} (KBr pellet)

Table 4. Antioxidant Activity of Propolin A (**2**), Propolin B (**3**), and CAPE (**1**) in the 1,2-Diphenyl-2-picrylhydrazyl (DPPH) Test[a]

propolin A (**2**)		propolin B (**3**)		CAPE (**1**)	
concentration (μg/mL)	free radical scavenged (%)	concentration (μg/mL)	free radical scavenged (%)	concentration (μg/mL)	free radical scavenged (%)
3.125	36 ± 3.6	3.125	18.3 ± 1.5	3.125	39.1 ± 3.1
6.25	72.2 ± 3.3	6.25	36.7 ± 3.1	6.25	85.5 ± 5.7
12.5	92.1 ± 3.8	12.5	85.7 ± 4.2	12.5	98.5 ± 4.9
25.0	99.5 ± 4.7	25.0	94.7 ± 5.4	25.0	99.8 ± 3.7

[a] Data are presented as means ± SEM, $n = 3$.

cm^{-1} 3560, 3480, 1639; UV (EtOH) λ_{max} nm (log ϵ) 213 (2.35), 227.5 (1.88), 288 (1.50), 330 (0.51); CD (MeOH) 328.5 nm ($\Delta\epsilon$ +0.74), 286 nm ($\Delta\epsilon$ −5.96); FABMS (negative mode) *m/z* 441 ($[M - H]^-$); FABMS (positive mode): *m/z* 425 ($[M + H]^+ - H_2O$); ESIMS *m/z* 465 ($[M + Na]^+$), 443 ($[M + H]^+$), 425 ($[M - H_2O + H]^+$); EIMS *m/z* (rel int) 442 ($[M]^+$, 6%), 424 (100), 406 (10), 342 (12), 300 (36), 256 (20), 153 (20); HREIMS *m/z* 442.1989 (calcd for $C_{25}H_{30}O_7$ 442.1992); ^{1}H and ^{13}C NMR, Tables 1 and 2.

Propolin B (3): slightly yellow liquid from methanol/water (7:3); $[\alpha]_D$ +7.7 (*c* 0.17, CH_3OH); IR ν_{max} (film) cm^{-1} 3547, 3470, 1639; UV (EtOH) λ_{max} nm (log ϵ) 213.5 (2.50), 229 (1.99), 288.5 (1.62), 330 (0.47); CD (MeOH) 304.5 nm ($\Delta\epsilon$ +1.97), 273 nm ($\Delta\epsilon$ −2.47); ESIMS *m/z* 465 ($[M + Na]^+$), 460 ($[M + NH_4]^+$), 443 ($[M + H]^+$), 425 ($[M - H_2O + H]^+$); HREIMS *m/z* 442.1989 (calcd for $C_{25}H_{30}O_7$ 442.1992); ^{1}H and ^{13}C NMR, Tables 3 and 2.

Cell Culture and Cytotoxicity Assay. Human melanoma and C6 glioma cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco) containing 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. HL-60 cell culture was maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in RPMI 1640 media supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells, (1×10^5)/well, were cultured in a 12-well plate at various concentrations of propolin A (**2**) and propolin B (**3**) for 24 h. Treated cells were counted, and cell viability was determined by a trypan blue exclusion assay.

DNA Fragmentation for Detection of Cell Apoptosis. HL-60 cells in a 60 mm dish were treated with various concentrations of propolin A (**2**) for 24 h, collected with ice-cold PBS. After centrifugation (2000*g*) for 10 min at 4 °C, cells were washed with PBS and recentrifuged at (12 000 rpm) for 10 min at 4 °C. Cell pellets were resuspended in 0.1 mL of isolation buffer (10 mM EDTA, 50 mM Tris-HCl, pH 8.0, 0.5% SDS, 1 mg/mL proteinase K) and incubated 12 h at 50 °C and treated with RNase A (0.5μg/mL) for another 4 h at 37 °C. The lysate was centrifuged at 12 000 rpm for 10 min at 4 °C to separate the soluble fragmented DNA from the intact chromatin pellet. Fragmented DNA was extracted with phenol/chloroform/isoamyl alcohol (25:24:1), precipitated with ethanol, and analyzed by 1.8% agarose gel electrophoresis. Approximately 20 μg of DNA was loaded in each well, visualized under UV light, and photographed.

Flow Cytometry for Detection of Cell Apoptosis. Human melanoma cells (1×10^6) were cultured in 60 mm dishes and incubated for 12 h. Cells were then treated with 15 μg/mL of propolin A (**2**) for various times, trysinized, and collected with ice-cold PBS. The cells were resupended in 200 μL of PBS and fixed in 800 μL of iced 100% ethanol at −20 °C. After being left to stand overnight, the cell pellets were collected by centrifugation, resuspended in 1 mL of hypotonic buffer (0.5% Triton X-100 in PBS and 0.5 μg/mL RNase A), and incubated at 37 °C for 30 min. Then 1 mL of propidium iodide solution (50 μg/mL) was added, and the mixture was allowed to stand at 4 °C for 30 min. Fluorescence emitted from the propidium iodide/DNA complex was quantitated after exicitation of the fluorescent dye by FACScan cytometry (Becton Dickenson, San Jose, CA).

Stable Free Radical Scavenging Capacity. The free radical scavenging capacity of propolins A (**2**) and B (**3**) was measured with DPPH. The DPPH radical has a deep violet color due to its unpaired electron, and radical scavenging capability can be followed spectrophotometrically by absorbance loss at 517 nm when the pale yellow nonradical form is produced. A 0.5 mL aliquot of propolins (3.125, 6.25, 12.5, and 25.0 μg/mL, final concentration) dissolved in methanol was mixed with 0.5 mL of 0.15 mM DPPH solution (in methanol) in a cuvette, and the optical density change at 517 nm was followed for 1 min in a UV/VIS U-3210 spectrophotometer. The DPPH test was also performed with CAPE (**1**) (3.125, 6.25, 12.5, and 25.0 μg/mL, final concentration) as control. Percentage of radical scavenging capability was calculated as $100 \times (A_0 - A_i)/A_0$, where A_0 is the initial absorbance of DPPH, and A_i represents the absorbance with propolin A (**2**) and propolin B (**3**) or CAPE (**1**).

Acknowledgment. The authors wish to thank Dr. Shu-Wei Yang for the helpful suggestion on the propolin structure, Dr. Larry Heimark for the LC/MS/MS data, and Mr. Rong-Sheng Yang for the LC/MS data. All of them are researchers at Schering-Plough Research Institute, New Jersey. Thanks also go to Dr. Yun-Lian Lin, National Research Institute of Chinese Medicine, for the CD spectra taken. The present research was supported by the National Science Council, NSC 90-2320-B-002-163 and NSC 90-2320-B-002-164, by the National Health Research Institute, NHRI-EX91-8913 BL, and by the National Research Institute of Chinese Medicine, NRICM-9101.

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NP0203180