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Bioassay and Ultraperformance Liquid Chromatography/Mass Spectrometry Guided Isolation of Apoptosis-Inducing Benzophenones and Xanthone from the Pericarp of *Garcinia yunnanensis* Hu

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Bioassay and ultraperformance liquid chromatography/photodiode array/mass spectrometry (UPLC/PDA/MS) guided isolation of the apoptosis-inducing active metabolites on HeLa-C3 cells from the pericarp of *Garcinia yunnanensis* (Guttiferae) yielded five active compounds, including the new garciyunnanins A (**1**) and B (**2**). The structures of the compounds were elucidated by comprehensive nuclear magnetic resonance and mass spectrometry analysis. Garciyunnanin B (**2**), featured with a natural tetracyclic xanthone skeleton derived from a polyisoprenylated benzophenone, is structurally interesting since it can be seen as an evidence of the previously described cyclization of garcinol by 2,2-diphenyl-1-picrylhydrazyl (DPPH). Garciyunnanin A (**1**) contains a 3-monohydroxy benzophenone skeleton, which is rarely found in *Garcinia* species. Both new compounds induce HeLa-C3 cells into apoptosis after 72 h of incubation at 15 μM. It is noteworthy that oblongifolin C (**4**), the major constituent of this plant, has proved to be the most active one among the isolates for inducing apoptotic cell death in cervical cancer derived HeLa-C3 sensor cells.

KEYWORDS: ***Garcinia yunnanensis*; apoptosis-inducing activity; UPLC/MS; benzophenones; xanthones**

INTRODUCTION

Garcinia is a plant genus of the Guttiferae family native of Asia, Southern Africa, and Polynesia, with a total of 21 species distributed in China (*1*). The fruits of many plants in this genus are edible such as *G. mangostana*, *G. subfalcata*, *G. esculenta*, and *G. yunnanensis*, etc. (*1*). A number of polyisoprenylated benzophenones and polyisoprenylated xanthones have been isolated from *Garcinia* plants to date in which many are purified from the fruits with garcinol being the best representative (*2*−*7*). Garcinol, a polyisoprenylated benzophenone purified from *G. indica* fruits and other species, has been reported to possess important biological activities such as antibiotic and antiulcer activities (*8*) and the ability to suppress colonic aberrant crypt foci (ACF) formation and inhibit histone acetyltransferases (HATs), which modulate gene expression (*2*), ability of induction of apoptosis through cytochrome *c* release, and activation of caspases in human leukemia HL-60 cells (*9*), and anti-inflammatory (*10, 11*) and anticarcinogenic properties (*10, 11*). Aiming at identifying structurally interesting and bioactive metabolites from the *Garcinia* species, we have started a series of studies on the chemical structures and pharmaceutical properties of several *Garcinia* species including *G. xipshuanbannaensis* (*12*), *G. kola* (*13*), *G. lancilimba* (*14*), *G. xanthochymus* (*15*), and *G. hanburyi* (*16*−*19*). Analytical studies of the chemical constituents of *Garcinia* plants using high-speed countercurrent chromatography (HSCCC), ultraperformance liquid chromatography/mass spectrometry (UPLC/MS), and high-performance

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liquid chromatography (HPLC) were also carried out in our laboratory (*20*−*23*).

Ultraperformance liquid chromatography/photodiode array/mass spectrometry (UPLC/PDA/MS) is a powerful tool for the phytochemical analysis since it can quickly separate the compounds with high efficiency and provide on-line UV and MS structural information (*21, 24*). All the xanthones and benzophenones isolated from *Garcinia* plants in our laboratory have been analyzed using the UPLC/PDA/MS equipment. On the basis of these analytical results, we have constructed a database containing the uniform UPLC method and MS information of 20 benzophenones and 36 xanthones obtained from *Garcinia* plants. To accelerate the discovery of new and bioactive compounds, an apoptotic assay and UPLC/PDA/MS guided isolation process were developed. In this method, each step of the extraction and fractionation process was guided by the UPLC/PDA/MS analysis and the cell-based apoptotic assay using HeLa-C3 cells that contain a fluorescent caspase sensor. This method enabled us to focus on the fractions and compounds with clear apoptotic effects. More importantly, for compounds with positive apoptotic effects and previously known chemical structures, their identities can be easily determined by comparing their UPLC/PDA/MS profiles with those in the database.

In this study, we used this combinative method to isolate anticancer compounds with apoptotic activities from *G. yunnanensis* Hu, a key protected plant distributed in the southwestern part of the Yunnan province in China. The fruits of this plant, called "Xiaogulangguo" by local people, are sweet and tasty (*1*). During our initial apoptotic activity survey, different parts of this plant, including the twig, leaves, and fruit, were studied. The results indicated that the acetone-soluble fraction of the fruit displayed the highest apoptotic activity against HeLa-C3 cells. Further investigations on the different extracts of the pericarps and seeds indicated that the acetone extract of pericarp was more effective than others. We report herein the bioassay and UPLC/PDA/MS guided isolation, structural elucidation, and apoptotic activity evaluation of the metabolites from the pericarps of *G. yunnanensis*.

MATERIALS AND METHODS

Instrumentation. Optical rotations were measured with a Horiba SEPA-300 high-sensitivity polarimeter (Horiba, Kyoto). Ultraviolet absorption spectra were recorded by a UV-2401 PC spectrophotometer (Shimadzus, Kyoto). IR spectra were obtained from a Bio-Rad FtS-135 spectrometer (Bio-Rad, California). NMR spectra were measured on a Bruker AV-400 spectrometer with TMS as the internal standard. Mass spectrometry was performed on a Waters Q-TOF Premier (Micromass MS Technologies, Manchester, U.K.) equipped with an electrospray ionization (ESI) source in positive ion mode. Column chromatography (CC) was performed with silica gel 60 (200−300 mesh, Merck), Sephadex LH-20, and reversed-phase C-18 silica gel (250 mesh, Merck). Precoated thin-layer chromatography (TLC) sheets of silica gel 60 GF254 were used. An Agilent 1100 series equipped with Alltima C-18 column (4.6 mm × 250 mm) was used for HPLC analysis, and the preparative Alltima C-18 column (22 mm × 250 mm) was used in the sample preparation.

UPLC Analysis. UPLC was performed using a Waters ACQUITY UPLC system (Waters Corp., MS), equipped with a binary solvent delivery system, autosampler, and a PDA detector. The chromatography was performed on a Waters ACQUITY BEH C_{18} column (100 mm × 2.1 mm, 1.7 μm, Waters Corp., Irel mL/min.) The column and autosampler were maintained at 35 and 10 °C, respectively. The mobile phase consisted of (A) 0.1% formic acid in water and (B) acetonitrile containing 0.1% formic acid. The UPLC eluting conditions were optimized as follows: isocratic at 75% B (0−0.5 min), linear gradient from 75% to 85% B (0.5−3 min), isocratic at 85% B (3−6 min), linear gradient from 85% to 95% B (6−7 min), isocratic at 95% B (9−12 min), and linear gradient from 95% to 75% B (12−13 min). The flow rate was 0.5 mL/min.

Table 1. ^{13}C and ^{1}H NMR Data (100 and 400 MHz, CD_3OD) for **1** and **2**

	garciyunnanin A (**1**)		garciyunnanin B (**2**)	
	δ_C	δ_H	δ_C	δ_H
1	194.3		195.8	
2	120.0		120.6	
3	191.7		177.1	
4	69.8		66.4	
5	51.2		51.6	
6	42.0	1.84, m	39.4	1.79, m
7	42.8	2.01, m	43.7	2.09, m
		1.45, t (12.8)		1.49, m
8	64.4		66.4	
9	209.5		207.2	
10	199.1		173.9	
11	140.9		118.3	
12	116.2	7.04, s	151.2	
13	158.5		104.1	6.81, s
14	120.5	6.94, overlap	155.3	
15	129.8	7.15, t (7.8)	147.3	
16	121.3	6.95, overlap	109.5	7.44, s
17	26.6	2.68, m	26.6	3.00, m
		2.64, m		2.87, m
18	121.7	4.82, m	120.8	4.70, m
19	134.5		135.2	
20	26.2	1.63, s	26.1	1.49, s
21	16.1	1.67, s	18.6	1.82, s
22	16.1	0.81, s	18.2	0.90, s
23	37.4	1.68, m	37.4	1.92, m
				1.56, m
24	25.2	1.97, m	23.7	1.80, m
25	125.6	5.05, m	124.7	4.92, m
26	132.3		133.1	
27	26.0	1.66, s	26.1	1.59, s
28	18.2	1.59, s	17.8	1.21, s
29	30.1	2.08, m	30.1	1.98, m
		1.78, m		1.76, m
30	123.7	5.03, m	123.3	4.92, m
31	134.4		138.4	
32	25.9	1.68, s	40.7	1.96, m
33	18.3	1.57, s	16.5	1.54, s
34	31.4	2.45, m	31.3	2.52, m
35	121.1	5.10, m	120.7	4.96, m
36	135.1		135.2	
37	26.2	1.69, s	26.1	1.59, s
38	17.9	1.66, s	18.2	1.69, s
39			27.5	2.52, m
40			125.1	5.02, m
41			132.3	
42			25.9	1.64, s
43			17.6	1.56, s

Plant Material. The pericarp of *Garcinia yunnanensis* Hu were collected in Luxi of Dehong prefecture, Yunnan province, China in 2006. The plant material was identified by Dr. Chunfeng Qiao. A herbarium sample (CMED-0477) was deposited in the Hong Kong Jockey Club Institute of Chinese Medicine.

Extraction and Isolation. The air-dried and powdered pericarp (9.0 kg) was extracted with acetone (20 L) at room temperature for three times. The extracted solution was evaporated under reduced pressure to yield a dark green residue (1.2 kg). The residue was chromatographed on silica gel eluted by $CHCl_3$, EtOAc, and acetone sequentially. The $CHCl_3$ fraction was tested to be the active fraction and evaporated in vacuum to give a residue (750 g), part of which (400 g) was subjected to silica gel column eluted with a gradient hexane/acetone system (100:0 to 0:100, v/v). Four fractions (I−V) were obtained on the basis of TLC analysis. The first three fractions were all tested to have apoptotic effects against HeLa-C3 cells. Fraction I was purified by reversed-phase C-18 silica gel to afford **4** (oblongifolin C, 5.0 g). Fraction II was separated using silica gel and eluted with gradient petroleum ether/acetone system (10:0 to 0:10) to produce four subfractions. The result of caspase activity

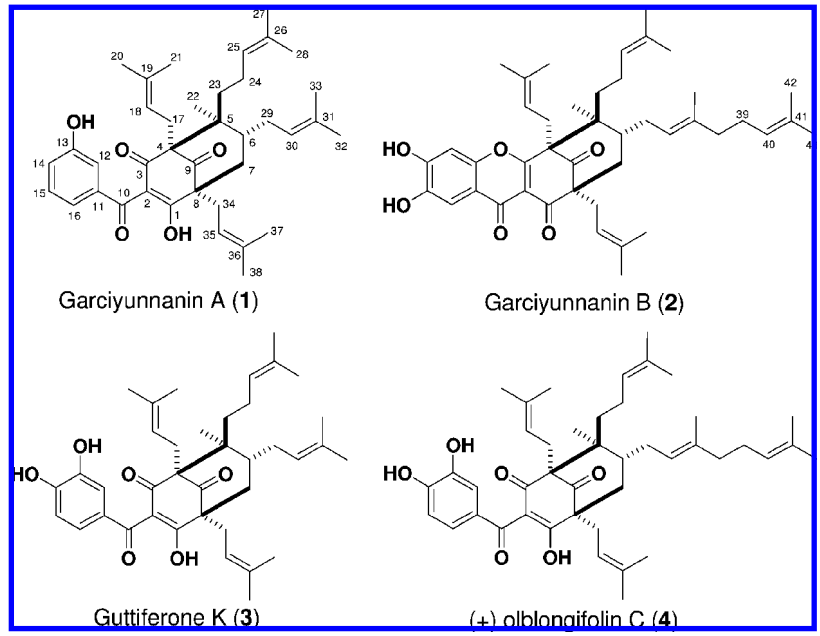

Figure 1. Structures of the new compounds (**1** and **2**) and two major constituents (**3** and **4**) of *Garcinia yunnanensis*.

assay indicated that the second and third subfractions were positive. The second subfraction was separated by preparative HPLC on an Alltima C-18 column eluted with CH_3CN in 0.1 acetic acid (0.1% acetic acid/CH_3CN, 5/95) to yield **3** (guttiferone K, 3.0 g) and **7** (oblongifolin D, 5.0 mg). The third subfraction was also separated by preparative HPLC (CH_3CN/H_2O, 85/15) to afford **4** (oblongifolin C, 6.0 mg). Fraction III was chromatographed on reversed-phase silica gel eluted with a gradient of CH_3OH/H_2O (from 8:2 to 10:0) to give five subfractions in which the second and the third subfractions demonstrated apoptotic activities. The second subfraction was then chromatographed over preparative HPLC on an Alltima C-18 column (CH_3CN/H_2O, 9/1) to give **1** (garciyunnanin A, 7.0 mg), **5** (oblongifolin A, 5.0 mg), and **8** (diethylhexyl phthalate, 10.0 mg). The third subfraction was further separated in a similar way, eluted with CH_3CN/H_2O (8:2) to give **2** (garciyunnanin B, 6.0 mg) and **6** (oblongifolin B, 9.0 mg).

Physical data for garciyunnanin A (**1**) are the following: yellow oil; $[\alpha]_D^{26.2}$ −3.0° ($CHCl_3$; *c* 0.11); IR (KBr) υ_{max} 3424, 2965, 2925, 2856, 1727, 1670, 1623, 1581, 1446, 1378, 1287, 1116, 1074, 997 cm^{-1}; UV (CH_3OH) λ_{max} (log ε) 204.2 (1.72), 262.0 (1.31) nm; ^1H and ^{13}C NMR spectral data, see **Table 1**; positive HRESIMS *m/z* 587.3742 [M + H]$^+$, (calcd for $C_{38}H_{50}O_5$, 587.3736).

Physical data for garciyunnanin B (**2**) are the following: yellow oil; $[\alpha]_D^{26.2}$ +18.1° ($CHCl_3$; *c* 0.12); IR (KBr) υ_{max} 3425, 2967, 2923, 1734, 1687, 1620, 1465, 1390, 1291, 1146, cm^{-1}; UV (CH_3OH) λ_{max} (log ε) 204.5 (1.77), 263.0 (1.44), 287.0 (1.32), 369.5 (1.11) nm; ^1H and ^{13}C NMR spectral data, see **Table 1**; positive HRESIMS *m/z* 669.4146 [M + H]$^+$, (calcd for $C_{43}H_{57}O_6$, 669.4155).

Bioassay. All the tested samples were dissolved in dimethyl sulfoxide (DMSO) to make stock solutions. The concentration of each stock is at least 1000 times higher than the working concentration. HeLa-C3 cells, which can detect apoptotic cell death involving caspase activation, were cultured in minimum essential medium (MEM) containing 10% fetal bovine serum, 100 U/mL penicillin, 100 mg/mL streptomycin, in a 5% CO_2 humidity incubator at 37 °C. In order to test a sample with apoptotic activity, one well of a 96-well plate was seeded with 7500 HeLa-C3 cells suspended in 100 μL of culture medium as a sample well. Another well of the plate was added with only 100 μL of culture medium as a background well. After 12 to ∼16 h of incubation, the plate was read by a Perkin-Elmer Victor plate reader with excitation wavelength at 440 ± 10 nm and emission wavelength at 486 ± 8 nm for CFP and 535 ± 8 nm for YFP to obtain the data for time point "0 h". Then immediately, the old medium was removed and 100 μL of freshly prepared culture medium containing the testing sample at a designed working concentration was added for both the sample well and the corresponding background well. Culture medium containing 0.1% DMSO was used as a negative control, whereas 500 nM paclitaxel was used as a positive control. After that, the plate was read repeatedly at indicated time points. The data acquisition duration was up to 72 h. The YFP/CFP emission ratio was then calculated. If the YFP/CFP emission ratio was decreased down below 3, then a significant apoptosis was considered to occur. All the screening of samples was conducted in triplicate, and all experiments were repeated for three times.

RESULTS AND DISCUSSION

The pericarp of *G. yunnanensis* was extracted with acetone and then chromatographed on silica gel eluted with $CHCl_3$, EtOAc, and acetone sequentially. The $CHCl_3$ fraction was tested to be the best one by our cell-based caspase activity assay. The UPLC/PDA/MS analysis of the $CHCl_3$ fraction revealed eight compound peaks (**Figure 1**). Then, by comparing the retention time, UV, and MS information with that of authentic compounds in our database, the compound peaks **4**−**7** were identified to be oblongifolins A−D (*25*). The accuracy of these identifications was later confirmed by the isolation of the pure compounds as shown in the Extraction and Isolation Procedures section. However, compounds **1**, **2**, and **3**, with the [M + H]$^+$ ion peak at 587.3742, 669.4146, and 603.3686, respectively, were absent from the database. Further isolation and purification using a series of chromatographic separations finally yielded the bioactive components garciyunnanin A (**1**) and garciyunnanin B (**2**) together with guttiferone K (**3**) and diethylhexyl phthalate (**8**) (*26, 27*). In addition, compounds **4**−**7** were also isolated. Both new compounds can induce HeLa-C3 cells into apoptosis after 72 h of incubation at 15 μM. It is noteworthy that among the five active components (**1**−**5**) tested, the major constituent, oblongifolin C (**4**), was the most potent(**Figure 4**).

Compound **1** was obtained as a yellow oil; its molecular formula was determined to be $C_{38}H_{50}O_5$ by high-resolution. electrospray ionization mass spectroscopy (HRESIMS) (found [M + H]$^+$ 587.3742, calcd 587.3736) and ^{13}C NMR spectrum. The IR spectrum displayed bands for hydroxyl (3424 cm^{-1}) and carbonyl (1727, 1670, 1623 cm^{-1}) groups. The ^1H NMR spectrum (**Table 1**) displayed that **1** possessed a 1,3-disubstituted benzene ring, four olefinic protons, one methyl on sp^3 carbon, and eight vinyl methyl groups. The ^{13}C NMR spectrum of **1** exhibited the presence of six aromatic carbons, a conjugated

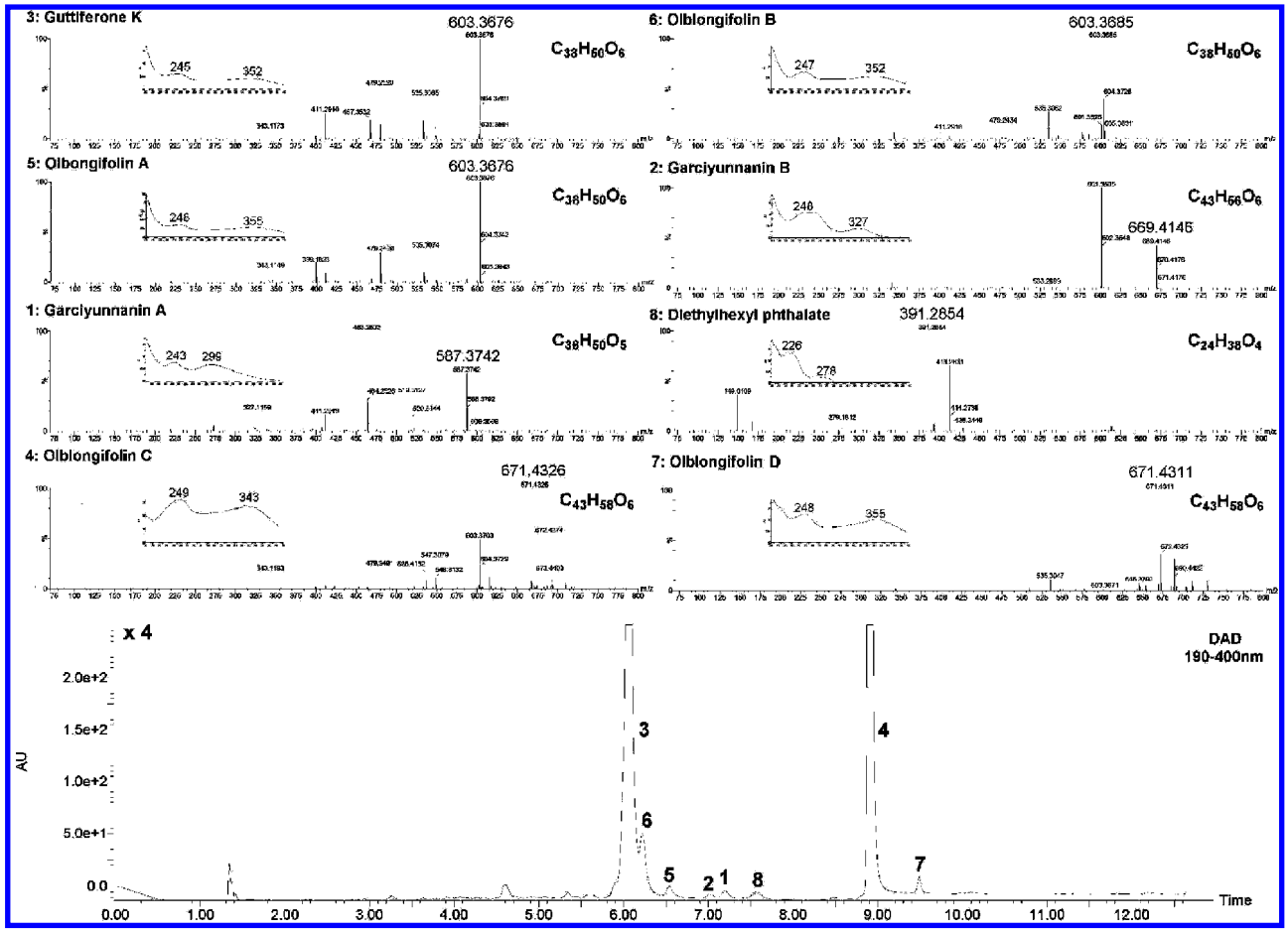

Figure 2. UPLC/PDA/MS analysis of the CH_3Cl part of the acetone extract together with the HRESIMS and UV information of the isolated compounds.

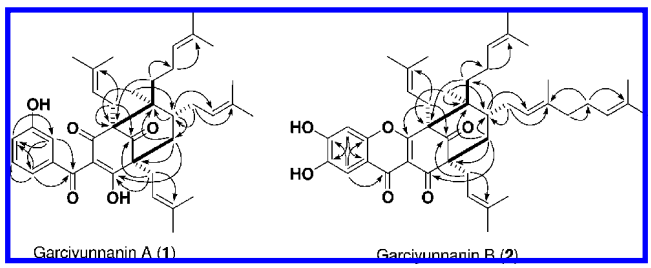

Figure 3. Key HMBC correlations of compounds **1** and **2**.

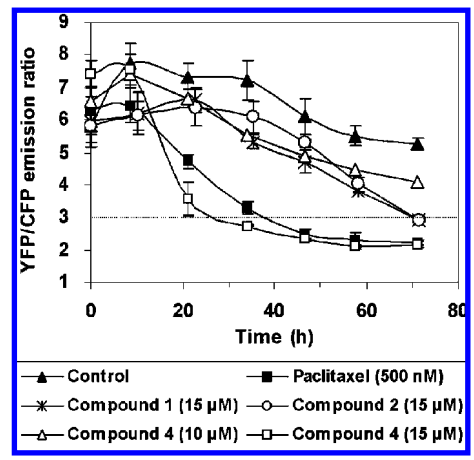

Figure 4. Time-dependent YFP/CFP emission ratio changes of **1**, **2**, and **4**.

carbonyl at δ_C 199.1 (C-10), a nonconjugated carbonyl at δ_C 209.5 (C-9), an enolized 1,3-diketone group (δ_C 194.3, C-1; δ_C 120.0, C-2; δ_C 191.7, C-3), two quaternary carbons at δ_C 69.8 (C-4) and 64.4 (C-8), and 25 other signals assignable to four isoprene units and another C5 unit. Considering a number of benzophenones have been isolated from *Garcinia* species, along with the spectrometric features discussed above, **1** could be ascribed to be a typical benzophenone derivative (*2, 25, 26*).

The ^1H and ^{13}C NMR data (**Table 1**) of **1** is very similar to those of guttiferone K (**3**), which was also isolated as one of the major constituents in this study (*26*). The only difference observed in the ^1H and ^{13}C NMR spectra was the presence of one more aromatic methine (δ_C 120.5; δ_H 6.94, overlap) in **1** instead of the quaternary aromatic carbon at δ_C 152.6 in **3**. In addition, the chemical shift value of C-13 was also downshifted from δ_C 146.5 in **3** to δ_C 158.5 in **1**. Therefore, compound **1** was deduced to be the 14-dehydroxyl derivative of guttiferone K (**3**). In the heteronuclear multiple bond correlation (HMBC) spectrum, the correlations from H-12 (δ_H 7.04, s) to C-10, C-13, C-14, and C-16, from H-14 (δ_H 6.94, overlap) to C-12, and C-15, and from H-16 (δ_H 6.95, overlap) to C-10, C-12, C-14, and C-15 confirmed that the aromatic ring in **1** was 1,3-disubstituted (**Figure 3**). In addition, the HMBC correlations between H-6/C-5, C-7, C-29, and C-30; H-7/C-1, C-5, C-6, and C-8; H-17/C-4, C-9, C-18, and C-19; H-22/C-4, C-5, C-6, and C-23; H-23/C-4, C-22, and C-24; H-24/C-23, C-25, and C-26; and H-29/C-5, C-30, and C-31 were also found, which further confirmed the core fragment (a 2,2-dimethylbicyclo[3,3,1]-nonane ring system) of **1** and the locations of four prenyl groups, respectively.

Scheme 1. Plausible Biogenetic Pathway of **2**

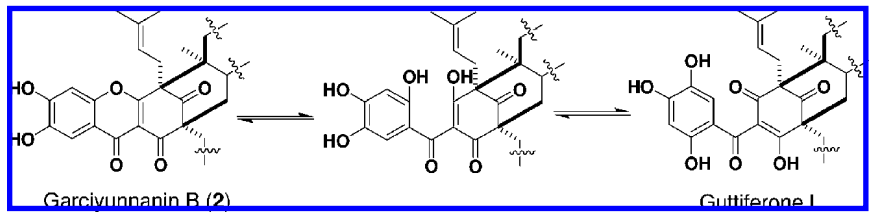

Table 2. Apoptosis-Inducing Effects of Compounds **1**−**8**[a]

compd	apoptosis-inducing effects at 20 μg/mL	apoptosis-inducing effects at 10 μg/mL
1	−	+
2	+	+
3	+	−
4	+	+
5	+	−
6	−	−
7	−	−
8	−	−

[a] "+" indicates the compound could decrease YFP/CFP emission ratio below 3 in 72 h.

The relative stereochemistry of **1** was deduced to be consistent with that of guttiferone K (**3**). The obvious nuclear Overhauser effect (NOE) correlations between CH_3-22 and CH_2-17/H-7α found in the nuclear Overhauser enhancement spectroscopy (NOESY) spectrum indicated that CH_3-22 must be in the α-orientation like CH_2-17. As mentioned in the literature (*2, 25*), the large coupling constant between H-7α (δ_H 1.45, t, $J = 12.8$ Hz) and H-6 further revealed the axial orientation for H-6 and H-7α together with the equatorial asset of the isoprenyl group at C-6. In addition, the ^{13}C NMR chemical shift of C-6 at δ_C 42.0 suggested that H-6 was β-oriented, since the signal of H-6 with α-orientation is located at a lower field (δ_C 46.0−48) (*26, 28*). On the basis of all the above evidence, the structure of **1** was established and named as garciyunnanin A.

Compound **2**, named garciyunnanin B, was isolated as yellow oil. The HRESIMS (*m/z* 669.4146 $[M + H]^+$, calcd 669.4155) indicated that **2** has a molecular formula of $C_{43}H_{56}O_6$, which was supported by the ^{13}C NMR spectrum (**Table 1**). Analysis of the ^1H and ^{13}C NMR of **2** exhibited the presence of a 1,2,4,5-tetrasubstituted benzene ring, a conjugated carbonyl carbon at δ_C 173.9 for C-10, a 2,2-dimethylbicyclo[3,3,1]-nonane ring system, and five prenyl groups. Careful comparison of the NMR data of **2** with another major constituent of this plant, (+)-oblongifolin C (**4**) (*25*), indicated that they are very similar to each other except for the signals for the aromatic ring and the enolized 1,3-diketone group due to C-1, C-2, and C-3. Different from the trisubstituted benzene ring in **4**, the aromatic ring in **2** was determined to be a 1,2,4,5-tetrasubstituted benzene ring based on the analysis of its ^1H and ^{13}C NMR data. Two singlets (δ_H 6.81 and 7.44) found in the aromatic region of the ^1H NMR spectrum were assignable to H-13 (δ_H 6.81) and H-16 (δ_H 7.44), respectively, which can be confirmed by the HMBC correlations from both H-13 and H-16 to C-10, C-11, C-12, C-14, and C-15 (**Figure 3**). An ether bridge between C-3 or C-1 and C-12 through an oxygen atom was determined by MS and NMR data analysis, which can be seen as the most interesting characteristic of its structure. Because the enolized 1,3-diketone can undergo keto−enol tautomerism, the formation of the epoxyl group could occur between C-12 and C-3 or between C-12 and C-1. In (+)-oblongifolin C (**4**), C-3 of the enolized 1,3-diketone group was a carbonyl and C-1 was substituted by a hydroxyl group, whereas in **2**, the carbonyl group (δ_C 195.8) was assigned to C-1 based on its HMBC correlations with H-7 together with the HMBC correlations from H-17 to the olefinic carbon at δ_C 177.1 (C-3), C-4, and C-5. The relative stereochemistry of C-4,

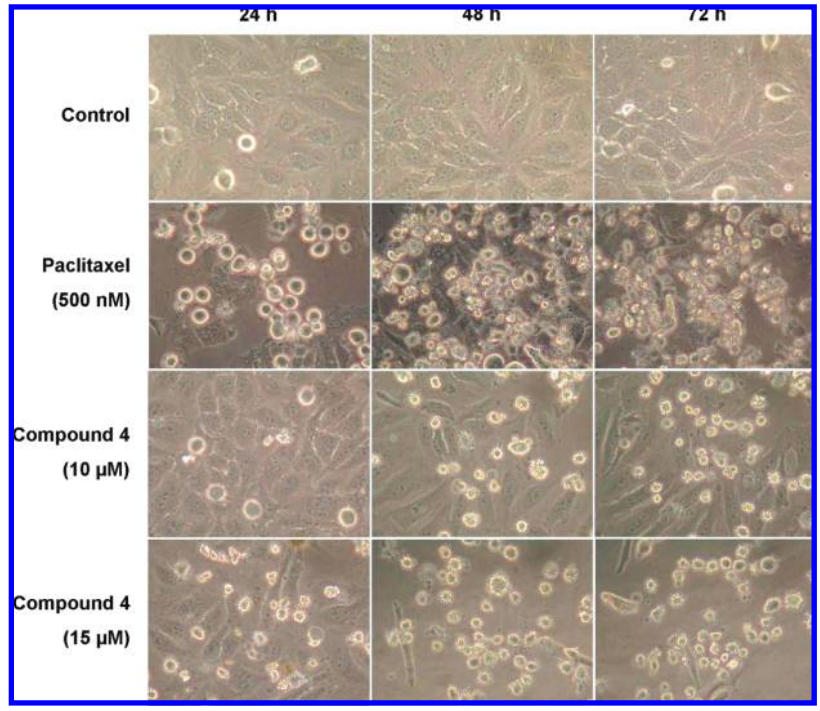

Figure 5. Cell morphology changes during the course of compound **4** treatment.

C-5, C-6, C-7, and C-8 were deduced to be the same with those of **1** based on the comparative analysis of the ^{1}H and ^{13}C NMR data with those of **1** and oxyguttifereone K (*3, 26*), together with the NOE correlations between CH_3-22 and CH_2-17/H-7α in the NOESY spectrum. Therefore, the structure of **2** was determined and named as garciyunnanin B.

As mentioned above, the most interesting characteristic of the structure of garciyunnanin B (**2**) is the epoxy linkage between C-3 and C-12, which was the second example of natural xanthone possessing this tetracyclic skeleton. Just as the isolation of the first tetracyclic xanthone, oxyguttiferone K, the natural formation of garciyunnanin B (**2**) also confirmed the previously described cyclization of garcinol by 2,2-diphenyl-1-picrylhydrazyl (DPPH) (*3, 10, 11*). From a biogenetic view, garciyunnanin B (**2**) should derived from a 12,13,14-trihydroxy benzophenone compound such as guttiferone L (**Scheme 1**) (*26*). As to garciyunnanin A (**1**), it is also noteworthy since its 12-monohydrxyl polyprenylated benzophenone skeleton is seldom found in *Garcinia* species.

All the isolated *G. yunnanensis* extracts and compounds were tested for their apoptotic activities using genetically engineered HeLa-C3 cells that can produce a fluorescent biosensor capable of detecting caspase-3 activation (*29*). These cells can emit green light under normal growth conditions and change to blue light when caspase-3 is activated during apoptosis to cleave the sensor protein inside the cells. This color change allows one to use a fluorescent plate reader to directly detect the activation level of caspase-3 in HeLa-C3 cells during the course of the compound treatment in a noninvasive way (*30*).

On the basis of our previous results, the emission ratio of YFP (yellow fluorescent protein)/CFP (cyan fluorescent proteins) is usually between 6 and 8 in normal cells, and this ratio will decrease to a value below 3 if a compound can activate caspase-3 and kill cancer cells. Therefore, any compound that can reduce the YFP/CFP emission ratio to a value below 3 will be considered positive in activating apoptosis. As shown in **Table 2**, among the eight tested compounds, **2**, **3**, **4**, and **5** were positive in activating apoptosis in HeLa-C3 cells at a concentration of 20 μg/mL. At 10 μg/mL, compounds **2** and **4** were still positive, whereas **3** was not, suggesting that **3** is less potent than **2** and **4**. It is worth mentioning that compound **1** tested positive for apoptosis at 10 μg/mL, but not at 20 μg/mL. We have examined cell morphology changes under compound **1** treatment at 20 μg/mL and found that those cells displayed typical features of necrotic cell death, such as prominent nuclear membrane, cell round-up, and membrane rupture. Since HeLa-C3 cells can only specifically detect apoptosis but not necrosis, a negative result was expected for compound **1** at higher concentration.

The investigation on the time-dependent kinetics of caspase activation for compounds **1**, **2**, and **4** indicated that **4** could fully activate caspase-3 within 30 h of compound addition at 15 μM (= 9.5 μg/mL), while **1** and **2** required 72 h to reduce the YFP/CFP emission ratio to a level just below 3 (**Figure 4**). This result suggests that compound **4** is the most potent apoptotic inducer among the tested compounds from *G. yunnanensis*. We further examined the apoptotic effect of compound **4** at a lower concentration and found that, at 10 μM, it can partially activate caspase (**Figure 4**). In addition, cell morphology observation (**Figure 5**) showed that many cells became detached and died off at 48 h after the treatment of compound **4**, which further supports the results of our caspase activity assay.

LITERATURE CITED

(1) Li, Y. H. In *Flora Reipublicae Popularis Sinicae*; Wu, Z. Y., Ed.; Science Press: Beijing, 1990; Vol. 50 (2), pp 89−110.

(2) Ciochina, R.; Grossman, R. B. Polycyclic polyprenylated acylphloroglucinols. *Chem. Rev.* **2006**, *106*, 3963–3986.

(3) Masullo, M.; Bassarello, C.; Suzuki, H.; Pizza, C.; Piacente, S. Polyisoprenylated benzophenones and an unusual polyisoprenylated tetracyclic xanthone from the fruits of *Garcinia cambogia*. *J. Agric. Food Chem.* **2008**, *56*, 5205–5210.

(4) Baggett, S.; Protiva, P.; Mazzola, E. P.; Yang, H.; Ressler, E. T.; Basile, M. J.; Weinstein, I. B.; Kennelly, E. J. Bioactive benzophenones from *Garcinia* xanthochymus fruits. *J. Nat. Prod.* **2005**, *68*, 354–360.

(5) Cuesta-Rubio, O.; Padron, A.; Velez-Castro, H.; Pizza, C.; Rastrelli, L. Aristophenones A and B. A new tautomeric pair of polyisoprenylated benzophenones from *Garcinia aristata*. *J. Nat. Prod.* **2001**, *64*, 973–975.

(6) Hussain, R. A.; Owegby, A. G.; Parimoo, P.; Waterman, P. G. Kolanone, a novel polyisoprenylated benzophenone with antimicrobial properties from the fruit of *Garcinia kola*. *Planta Med.* **1982**, *44*, 78–81.

(7) Xu, Y. J.; Yip, S. C.; Kosela, S.; Fitri, E.; Hana, M.; Goh, S. H.; Sim, K. Y. Novel cytotoxic, polyprenylated heptacyclic xanthonoids from Indonesian *Garcinia gaudichaudii* (Guttiferae). *Org. Lett.* **2000**, *2*, 3945–3948.

(8) Yamaguchi, F.; Saito, M.; Ariga, T.; Yoshimura, Y.; Nakazawa, H. Free radical scavenging activity and antiulcer activity of garcinol from *Garcinia indica* fruit rind. *J. Agric. Food Chem.* **2000**, *48*, 2320–2325.

(9) Pan, M. H.; Chang, W. L.; Lin-Shiau, S. Y.; Ho, C. T.; Lin, J. K. Induction of apoptosis by garcinol and curcumin through cytochrome *c* release and activation of caspases in human leukemia HL-60 cells. *J. Agric. Food Chem.* **2001**, *49*, 1464–1474.

(10) Sang, S.; Pan, M. H.; Cheng, X.; Bai, N.; Stark, R. E.; Rosen, R. T.; Lin-Shiau, S. Y.; Lin, J. K.; Ho, C. T. Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol and their antitumor activities. *Tetrahedron* **2001**, *57*, 9931–9938.

(11) Sang, S.; Liao, C. H.; Pan, M. H.; Rosen, R. T.; Lin-Shiau, S. Y.; Lin, J. K.; Ho, C. T. Chemical studies on antioxidant mechanism of garcinol: analysis of radical reaction products of garcinol with peroxyl radicals and their antitumor activities. *Tetrahedron* **2002**, *58*, 10095–10102.

(12) Han, Q. B.; Yang, N. Y.; Tian, H. L.; Qiao, C. F.; Song, J. Z.; Chang, D. C.; Chen, S. L.; Luo, K. Q.; Xu, H. X. Xanthones with growth inhibition against HeLa cells from *Garcinia xipshuanbannaensis*. *Phytochemistry* **2008**, *69*, 2187–2192.

(13) Han, Q. B.; Lee, S. F.; Qiao, C. F.; He, Z. D.; Song, J. Z; Sun, H. D.; Xu, H. X. Complete NMR assignment of the antibacterial biflavonoid GB1 from *Garcinia kola*. *Chem. Pharm. Bull.* **2005**, *53*, 1034–1036.

(14) Yang, N. Y.; Han, Q. B.; Cao, X. W.; Qiao, C. F.; Song, J. Z.; Chen, S. L.; Yang, D. J.; Yiu, H.; Xu, H. X. Two new xanthones isolated from the stem bark of *Garcinia lancilimba*. *Chem. Pharm. Bull.* **2007**, *55*, 950–952.

(15) Han, Q. B.; Qiao, C. F.; Song, J. Z.; Yang, N. Y.; Cao, X. W.; Peng, Y.; Yang, D. J.; Chen, S. L.; Xu, H. X. Cytotoxic prenylated phenolic compounds from the twig bark of *Garcinia xanthochymus*. *Chem. Biodiversity* **2007**, *4*, 940–946.

(16) Han, Q. B.; Yang, L.; Liu, Y.; Wang, Y. L.; Qiao, C. F.; Song, J. Z.; Xu, L. J.; Yang, D. J.; Chen, S. L.; Xu, H. X. Gambogic acid and epigambogic acid, C-2 epimers with novel anticancer effects from *Garcinia hanburyi*. *Planta Med.* **2006**, *72*, 281–284.

(17) Han, Q. B.; Yang, L.; Wang, Y. L.; Qiao, C. F.; Song, J. Z.; Sun, H. D.; Xu, H. X. A pair of novel cytotoxic polyprenylated xanthone epimers from gamboges. *Chem. Biodiversity* **2006**, *3*, 101–105.

(18) Han, Q. B.; Cheung, S.; Tai, J.; Qiao, C. F.; Song, J. Z.; Xu, H. X. Stability and cytotoxicity of gambogic acid and its

derivative, gambogoic acid. *Biol. Pharm. Bull.* **2005**, *28*, 2335–2337.

(19) Han, Q. B.; Wang, Y. L.; Yang, L.; Tso, T. F.; Qiao, C. F.; Song, J. Z.; Xu, L. J.; Chen, S. L.; Yang, D. J.; Xu, H. X. Cytotoxic polyprenylated xanthones from the resin of *Garcinia hanburyi*. *Chem. Pharm. Bull.* **2006**, *54*, 265–267.

(20) Song, J. Z.; Yip, Y. K.; Han, Q. B.; Qiao, C. F.; Xu, H. X. Rapid determination of polyprenylated xanthones in gamboge resin of *Garcinia hanburyi* by HPLC. *J. Sep. Sci.* **2007**, *30*, 304–309.

(21) Li, S. L.; Song, J. Z.; Han, Q. B.; Qiao, C. F.; Xu, H. X. Improved HPLC method for simultaneous determination of 12 cytotoxic caged xanthones in gamboges, a potential anticancer resin from *Garcinia hanburyi*. *Biomed. Chromatogr.* **2008**, *22*, 637–644.

(22) Han, Q. B.; Song, J. Z.; Qiao, C. F.; Wong, L.; Xu, H. X. Preparative separation of gambogic acid and its C-2 epimer using recycling high-speed counter-current chromatography. *J. Chromatogr., A* **2006**, *1127*, 298–301.

(23) Han, Q. B.; Song, J. Z.; Qiao, C. F.; Xu, H. X. Characterization and quantitation analysis of xanthones in gamboges. *Chin. J. Nat. Med.* **2006**, *4*, 210–214.

(24) Churchwell, M. I.; Twaddle, N. C.; Meeker, L. R.; Doerge, D. R. Improving LC−MS sensitivity through increases in chromatographic performance: Comparisons of UPLC−ES/MS/MS to HPLC−ES/MS/MS. *J. Chromatogr., B* **2005**, *825*, 134–143.

(25) Hamed, W.; Brajeul, S.; Mahuteau-Betzer, F.; Thoison, O.; Mons, S.; Delpech, B.; Hung, N. V.; Sevenet, T.; Marazano, C. Oblongifolins A−D, polyprenylated benzoylphloroglucinol derivatives from *Garcinia oblongifolia*. *J. Nat. Prod.* **2006**, *69*, 774–777.

(26) Cao, S.; Brodie, P. J.; Miller, J. S.; Ratovoson, F.; Birkinshaw, C.; Randrianasolo, S.; Rakotobe, E.; Rasamison, V. E.; Kingston, D. G. I. Guttiferones K and L, antiproliferative compounds of *Rheedia calcicola* from the Madagascar rain forest. *J. Nat. Prod.* **2007**, *70*, 686–688.

(27) Du, Q.; Shen, L.; Xiu, L.; Jerz, G.; Winterhalter, P. Di-2-ethylhexyl phthalate in the fruits of *Benincasa hispida*. *Food Addit. Contam.* **2006**, *23*, 552–555.

(28) Piccinelli, A. L.; Cuesta-Rubio, O.; Chica, M. B.; Mahmood, N.; Pagano, B.; Pavone, M.; Barone, V.; Rastrelli, L. Structural revision of clusianone and 7-epi-clusianone and anti-HIV activity of polyisoprenylated benzophenones. *Tetrahedron* **2005**, *61*, 8206–8211.

(29) Luo, K. Q.; Yu, V. C.; Pu, Y. M.; Chang, D. C. Application of the fluorescence resonance energy transfer method for studying the dynamics of caspase-3 activation during UV-Induced apoptosis in living HeLa cells. *Biochem. Biophys. Res. Commun.* **2001**, *283*, 1054–1060.

(30) Tian, H.; Ip, L.; Luo, H.; Chang, D. C.; Luo, K. Q. A high throughput drug screen based on fluorescence resonance energy transfer (FRET) for anticancer activity of compounds from herbal medicine. *Br. J. Pharmacol.* **2007**, *150*, 321–334.

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