JOURNAL OF NATURAL PRODUCTS

Volume 72, Number 7 July 2009

Full Papers

Cytotoxic Triterpenoids from the Stems of *Microtropis japonica*

I-Hsiao Chen,[†] Mei-Chin Lu,[†] Ying-Chi Du,[†] Ming-Hong Yen,[†] Chin-Chung Wu,[†] Yung-Husan Chen,[†] Chih-Sheng Hung,[†] Shu-Li Chen,[†] Fang-Rong Chang,*[,†,‡,§] and Yang-Chang Wu*[,†,‡,§]

Graduate Institute of Natural Products, School of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan, Republic of China, National Sun Yat-Sen University−Kaohsiung Medical University Joint Research Center, Kaohsiung 804, Taiwan, Republic of China, and Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung 807, Taiwan, Republic of China

Received October 30, 2008

Bioassay-guided fractionation of a methanol extract obtained from stems of *Microtropis japonica* led to the isolation of six new ursane-type triterpenoids (**1**−**6**) and a new 2,3-*seco*-oleanane-type triterpenoid (**7**), together with seven known compounds. The structures of the new compounds were elucidated using spectroscopic data analysis. Among the known compounds isolated, the main component, **8** (ursolic acid), was active for HL60 cells, and its effects on histone hyperacetylation and the inhibition of histone deacetylase (HDAC) activity were investigated.

Chemical studies carried out on the Celastraceae family of plants have revealed the occurrence of important bioactive secondary metabolites, such as sesquiterpenoids and triterpenoids. Triterpenoids of the Celastraceae family have been found to have a variety of biological effects, such as antihyperlipidemic,[1,2] antiinflammatory,[3] antimicrobial,[4] antitumor promotion,[5] antiulcer,[6] hepatoprotective,[1,2] and hypoglycemic activities.[7]

In an ongoing search for bioactive compounds from Celastraceous plants, the MeOH extract of stems of *Microtropis japonica* (Franchet & Savatier) Hall. f. was selected for investigation. From previous studies, several dihydroxyagarofuranoid sesquiterpenes from *M. japonica* were found to exhibit cytotoxic and antituberculosis activities.[8,9] Compounds isolated in the present studies included seven new triterpenoids, **1**−**7**, as well as seven known compounds, ursolic acid (**8**),[10] 1β,2β,6α,15-tetraacetoxy-9α-benzoyloxydihydro-β-agarofuran (**9**),[11] 1β,2β,6α-triacetoxy-9α,15-dibenzoyloxydihydro-β-agarofuran (**10**),[12] 1β,2β,6α-triacetoxy-9α-benzoyloxydihydro-β-agarofuran (**11**),[13] 1β,2β,6α-triacetoxy-8α,9α,15-tribenzoyloxydihydro-β-agarofuran (**12**),[8] 1β,3β,6α,8α-tetraacetoxy-9α,15-dihydro-β-agarofuran (**13**),[14] and 1β,6α,8α-triacetoxy-2β,9α,15-tribenzoyloxydihydro-β-agarofuran (**14**).[8]

Results and Discussion

Compound **1** was obtained as a white, amorphous powder. Its IR spectrum showed absorption bands for hydroxy (3410 cm^{-1}), olefinic (1652 cm^{-1}), and *gem*-dimethyl (1382 cm^{-1}) groups. The molecular formula was determined as $C_{31}H_{52}O_3$ on the basis of the HRESIMS (495.3814 $[M + Na]^+$, calcd 495.3817). The ^{1}H NMR spectrum exhibited an olefinic proton at δ_H 5.53 (1H, d, J = 3.6 Hz), three methine protons at δ_H 3.51 (m), 3.85 (dd, J = 8.7, 3.6 Hz), and 4.57 (dd, J = 12.6, 4.8 Hz), six tertiary methyl protons at δ_H 1.11, 1.14 (×2 CH_3), 1.15, 1.27, and 1.35, and two secondary methyl protons at δ_H 0.95 (3H, d, J = 6.0 Hz) and 1.00 (3H, d, J = 6.0 Hz). In addition, the ^{13}C NMR spectrum displayed three oxygenated methines at δ 65.4, 76.6, and 77.9 and eight methyl signals at δ_C 16.6, 17.5, 17.8, 18.5, 21.6, 23.0, 23.9, and 28.9. Accordingly, **1** was suggested to be based on an urs-12-ene skeleton and to contain a methoxy group, similar to 3β,28-dihydroxy-11α-methoxyurs-12-ene, except for the presence of an additional oxymethine and the absence of a methylene.[15] The additional oxymethine was then assigned at C-16 as a result of the low-field-shifted signals of δ_C 60.5 (C-18) and HMBC correlations of H-18 (δ_H 1.69) and H-15 (δ_H 2.04)/C-16 (δ_C 65.4). The H-3 and H-16 protons were assigned with α-axial orientations because of the NOE correlations between H-3 and H-5 and between H-16 and H-27,

* To whom correspondence should be addressed. Tel: +886-7-312-1101, ext. 2197. Fax: +886-7-311-4773. E-mail: yachwu@kmu.edu.tw; aaronfrc@kmu.edu.tw.

[†] Kaohsiung Medical University.

[‡] National Sun Yat-Sen University−Kaohsiung Medical University Joint Research Center.

[§] Center of Excellence for Environmental Medicine.

10.1021/np800694b CCC: $40.75
Published on Web 06/17/2009

respectively. Meanwhile, the configuration of C-16 was also supported by the coupling constant of H-16α (J = 12.6, 4.8 Hz). The methoxy group was determined as being α-oriented by the correlations between H-26 and H-11 on the basis of the NOESY spectrum of **1** (Figure S1, Supporting Information). Furthermore, the NOE effect between H-12/H-18 clearly established ring E as being a *cis*-fused ring. Thus, the structure of **1** was determined as 11α-methoxyurs-12-ene-3β,16β-diol.

Compound **2** was obtained as a white powder, and its molecular formula, $C_{31}H_{50}O_4$, was established from the HRESIMS (m/z 509.3607 $[M + Na]^+$, calcd 509.3608) and the ^{13}C NMR and DEPT spectra. The ^1H NMR spectrum of **2** exhibited signals due to five tertiary methyls [δ_H 0.98, 1.13, 1.21 (×2), and 1.28], two secondary methyls [δ_H 0.98 (d, J = 6.0 Hz) and 1.23 (d, J = 6.0 Hz)], a hydroxymethylene [δ_H 3.76 (dd, J = 10.2, 2.4 Hz) and 4.08 (dd, J = 10.2, 2.4 Hz)], and an oxygenated methine [δ_H 4.56 (d, J = 10.2 Hz)]. The ^{13}C NMR spectrum indicated **2** to be constituted by 31 carbons including a cyclic ketone (δ_C 217.1), two olefinic carbons (δ_C 117.0 and 144.8), an oxygenated carbon (δ_C 77.0), a methylene carbon (δ_C 68.3), and a methoxy carbon (δ_C 51.5). This spectroscopic evidence suggested that **2** is an urs-12-en-3-one derivative possessing a tetrasubstituted double bond and a methoxy moiety.[16,17] The ^{13}C NMR chemical shifts assigned to the A ring carbon atoms of **2** correlated well with those of known 23-hydroxyurs-12-en-3-one derivatives but are different from those of their 24-hydroxy analogues.[16,17] From previous work, the chemical shift of a C-23 hydroxymethylene group, ca. δ_C 68−71, is downfield of that of a C-24 hydroxymethylene group shown around δ_C 63−66 in this compound series.[18] The connectivity of the protonated carbons (C-1 to C-2; C-5 to C-6; C-6 to C-7; C-9 to C-11; C-15 to C-16; C-18 to C-19; C-20 to C-21) was determined by analysis of the $^1H-^1H$ COSY spectrum of **2**. The HMBC correlations from the methoxy proton (δ_H 3.32) to C-11 and from H-11 (δ_H 4.56) to the methoxy carbon (δ_C 51.5), C-9 (δ_C 45.6), C-10 (δ_C 37.9), C-12 (δ_C 144.8), and C-13 (δ_C 117.0) showed the presence of this methoxy group at C-11. A cyclic ketone located at C-3 was confirmed by HMBC correlations of H-24 (δ_H 1.13) with C-3 and of H-2 (δ_H 2.65 and 2.73) with C-3. Furthermore, the 2D NMR and mass spectrometric data suggested the hydroxy group to be attached to the quaternary carbon signal at δ_C 144.8 (C-12). In particular, the signal of a hydrogen bond at δ_H 8.49 resulted from a dipole−dipole force between an electronegative atom (O-CH_3) at C-11 and a hydrogen atom bonded to oxygen (O-H) at C-12. Finally, the key NOESY correlations of **2** and its relative configuration were determined as shown in Figure S2 (Supporting Information). Thus, compound **2** was assigned as 12,23-dihydroxy-11α-methoxyurs-12-en-3-one.

Compound **3**, obtained as a powder, gave a molecular ion peak at m/z 509.3607 $[M + Na]^+$ (calcd for $C_{31}H_{50}O_4$, 509.3604) in the HRESIMS. The ^{13}C NMR signals of **3** were found to be closely related to those of compound **2** except for the signals corresponding to ring A. Thus, compound **3** was found to differ from compound **2** in the presence of an aldehyde and the absence of a cyclic ketone group. The NMR signals at δ_H 4.49 and δ_C 145.6 and 117.3 indicated the presence of a 12-hydroxy-11α-methoxy unit in **3**. Hydrogen bonding between the C-12 hydroxy proton to the oxygen atom of the C-11 methoxy group was evident. In addition, the ^1H NMR spectrum showed a singlet proton signal at δ_H 9.70 consistent with the occurrence of an aldehyde proton, which, in conjunction with the ^{13}C NMR spectroscopic evidence, suggested that a formyl group could best be accommodated at the C-23 position.[19] In order to determine the orientation of the aldehyde group, a NOESY experiment (Figure 1) was carried out. A NOE interaction between the aldehyde proton and H-3α confirmed that the aldehyde group is α-equatorial and located at C-23. Other NOEs were shown from H-3 (δ_H 4.18) to H-5 (δ_H 1.56) and the aldehydic proton (δ_H 9.70).

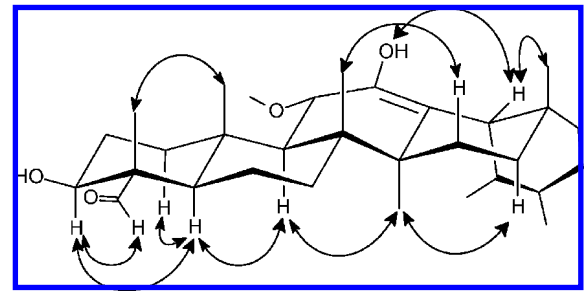


Figure 1. Key NOESY correlations of **3**.

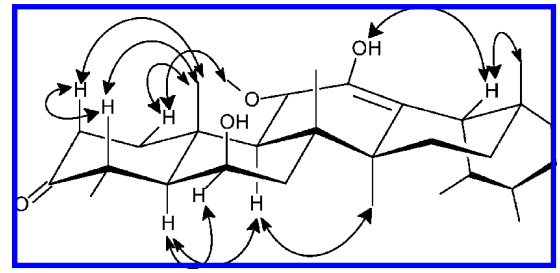


Figure 2. Key NOESY correlations of **6**.

Thus, compound **3** was assigned as 3β,12-dihydroxy-11α-methoxyurs-12-en-23-al.

Compound **4** was found to have the molecular formula $C_{30}H_{46}O_4$ on the basis of the HRESIMS molecular ion at m/z 493.3294 ($[M + Na]^+$, calcd for 493.3297), accounting for eight degrees of unsaturation. In the ^1H NMR spectrum, signals were observed for an AB system of methylene protons on a carbon bonded to an oxygen at δ_H 3.29 (1H, d, J = 7.2 Hz) and 3.70 (1H, d, J = 7.2 Hz), a carbinol proton at δ_H 4.09 (1H, m), an aldehyde proton at δ_H 9.64 (1H, s), four tertiary methyl groups at δ_H 0.88, 1.10, 1.29, and 1.39, and two secondary methyl groups at δ_H ca. 0.91 and 1.09 (d, J = 5.2 Hz). On the basis of the molecular formula and NMR spectroscopic data analysis, it was concluded that compound **4** is a triterpene on the basis of the ursane skeleton[15,19] with an aldehyde group and six rings, of which one is an ether bridge between the CH_2 at δ_C 77.2 and the quaternary carbon at δ_C 89.3, as shown clearly in the ^{13}C NMR spectrum. HMBC correlations from H-28 (δ_H 3.29) to C-17 (δ_C 42.7) and C-18 (δ_C 55.0) as well as H-18 (δ_H 2.22) to C-13 (δ_C 89.3) and C-14 (δ_C 45.9) indicated that compound **4** has a 13β,28-epoxyurs-12-one structure, with one aldehyde, one hydroxy group, and one carbonyl group.[15,19] The hydroxy and aldehyde functionalities were placed on the A ring at C-3 and C-23 in the same manner as for compound **3**. Furthermore, the relative configuration of **4** was assigned on the basis of NOESY correlations (Figure S3, Supporting Information). The correlations between H-3 and H-5 confirmed the α-axial orientation of H-3, and the aldehyde group was assigned with a 4α-equatorial configuration. Thus, compound **4** was established as 13β,28-epoxy-3β-hydroxy-12-oxoursan-23-al. 13β,28-Epoxy-12-oxoursane derivatives have been reported previously from *M. fokienensis*.[15]

The molecular formula of compound **5** was assigned as $C_{30}H_{46}O_4$ on the basis of the HRESIMS molecular ion at m/z 493.3294 ($[M + Na]^+$, calcd for 493.3296). Comparison of the NMR spectroscopic data with those of **4** revealed that **5** possesses a similar structure except in ring A. The chemical shift of the hydroxymethylene carbon of **5** was observed at δ_C 68.3, similar to compound **2**, and it was consequently assigned to C-23. From the HMBC spectrum, the location of the carbonyl group at C-3 was established from the following correlations (H-23/C-3; H-24/C-3, C-4, C-5, C-23). Accordingly, compound **5** was determined as 13β,28-epoxy-23-hydroxyursane-3,12-dione.

Compound **6** was obtained as a white powder, with its molecular formula determined as $C_{30}H_{48}O_4$ on the basis of the HRESIMS

Table 1. ^{1}H NMR Spectroscopic Data for Compounds **1**−**3** and **6** (in C_5D_5N, 600 MHz)

proton	**1**	**2**	**3**[a]	**6**
1	1.52 (m, α)	1.93 (m, α)	2.50 (dt, 13.5, 3.5, α)	1.57 (m, α)
	2.12 (dt, 13.8, 3.6, *β*)	2.72 (m, *β*)	1.10 (m, *β*)	2.80 (ddd, 12.9, 6.6, 2.4, *β*)
2	1.91 (m, α)	2.73 (m, α)	2.21 (2H, m)	2.42 (ddd, 14.4, 5.4, 2.4, α)
	1.98 (m, *β*)	2.65 (m, *β*)		2.65 (td, 14.4, 6.6, *β*)
3	3.51 (m, α)		4.18 (m)	
4				3.17 (dq, 12.0, 6.6)
5	ca. 0.95	2.50 (dd, 12.0, 2.4)	1.56 (m)	1.33 (m)
6	1.59 (m, α)	1.56 (m, α)	1.10 (m)	4.42 (t, 3.0)
	1.42 (m, *β*)	1.46 (m, *β*)	1.55 (m)	
7	1.58 (m, α)	1.58 (m, α)	1.26 (m)	1.86 (m)
	1.33 (m, *β*)	1.36 (m, *β*)	1.55 (m)	1.76 (m)
9	1.86 (d, 9.0)	2.22 (d, 10.2)	2.17 (d, 10.0)	2.22 (d, 10.2)
11	3.85 (dd, 8.7, 3.6)	4.56 (d, 10.2)	4.49 (d, 10.0)	4.80 (d, 10.2)
12	5.53 (d, 3.6)			
15	1.65 (dd, 13.8, 4.8, α)	1.01 (m, α)	1.02 (m, α)	1.08 (m)
	2.04 (m, *β*)	1.82 (td, 13.8, 4.8, *β*)	1.78 (td, 13.5, 4.5, *β*)	2.01 (dd, 13.2, 4.8)
16	4.57 (dd, 12.6, 4.8, α)	2.07 (td, 13.2, 4.8, α)	2.10 (td, 13.5, 4.5, α)	2.11 (td, 13.2, 4.8, α)
		0.85 (m, *β*)	0.86 (m, *β*)	0.85 (m, *β*)
18	1.69 (d, 12.0)	2.86 (dd, 10.8, 1.2)	2.83 (d, 10.0)	2.88 (dd, 11.4, 1.2)
19	1.53 (m)	1.53 (m)	1.56 (m)	1.10 (m)
20	0.95 (m)	1.08 (m)	1.09 (m)	1.55 (m)
21	1.50 (m)	1.33 (m)	1.43 (2H, m)	1.42 (2H, m)
	1.55 (m)	1.42 (m)		
22	1.19 (m)	1.43 (m)	1.41 (m)	1.41 (m)
	2.63 (dt, 13.2, 3.0)	1.48 (m)	1.51 (m)	1.47 (m)
23	1.27 (s)	3.76 (dd, 10.2, 2.4)	9.70 (s)	1.39 (d, 6.6)
		4.08 (dd, 10.2, 2.4)		
24	1.11 (s)	1.13 (s)	1.45 (s)	
25	1.14 (s)	1.21 (s)	1.19 (s)	1.83 (s)
26	1.14 (s)	1.21 (s)	1.14 (s)	1.79 (s)
27	1.35 (s)	1.28 (s)	1.37 (s)	1.36 (s)
28	1.15 (s)	0.98 (s)	0.97 (s)	0.98 (s)
29	1.00 (d, 6.0)	1.23 (d, 6.6)	1.28 (d, 6.5)	1.28 (d, 6.6)
30	0.95 (d, 6.0)	0.98 (d, 6.0)	1.00 (d, 6.5)	1.00 (d, 6.6)
$OC\underline{H_3}$	3.34 (s)	3.32 (s)	3.45 (s)	3.38 (s)

[a] Measured in C_5D_5N, 500 MHz.

(495.3450 $[M + Na]^+$, calcd 495.3447). In the ^{1}H NMR spectrum, characteristic hydrogen bonding of the 11α-methoxy and 12-hydroxy groups was evident. The ^{1}H NMR signals of the C−E rings were closely comparable to those of compounds **2** and **3**. The ^{13}C NMR spectrum showed 30 carbon signals comprised of one methoxy, seven methyls, seven methylenes, eight methines, and seven quaternary carbons. This indicated that compound **6** has a nor-triterpene skeleton and bears a methoxy group. The positions of a ketone group at C-3 and a hydroxy group at C-6 were established from long-range ^{1}H−^{13}C correlations in the HMBC spectrum between H-1 (δ_H 2.80) and C-2 (δ_C 38.2)/C-3 (δ_C 213.3)/C-5 (δ_C 55.0)/C-10 (δ_C 37.5)/C-25 (δ_C 16.0) and between H-7 (δ_H 1.86) and C-6 (δ_C 66.8)/C-9 (δ_C 45.2)/C-26 (δ_C 19.9). The placement of only one methyl group (δ_H 1.39, d, J = 6.6 Hz; δ_C 11.8, q, assigned for H-23) connected to C-4, instead of two methyl groups as commonly found at C-4 of usual ursane triterpenes, was proved by ^{1}H−^{13}C correlations between H-4 (δ_H 3.17) and C-3 (δ_C 213.3)/C-5 (δ_C 55.0)/C-23 (δ_C 11.8); between H-5 (δ_H 1.33) and C-4 (δ_C 43.2)/C-10 (δ_C 37.5)/C-25 (δ_C 16.0); and between H-23 (δ_H 1.39) and C-4 (δ_C 43.2)/C-5 (δ_C 55.0).[20] A 6*β*-hydroxy-24-norursane backbone was established by examination of the NOESY spectrum of **6** (Figure 2). The NOE correlations between H-5/H-6 and H-4/H-25 as well as the coupling constant of H-6 (t, J = 3.0 Hz) and H-4 (dq, J = 12.0, 6.6 Hz) indicated that both H-6 and 23-methyl are α-equatorially oriented. Thus, compound **6** was assigned as 6*β*,12-dihydroxy-11α-methoxy-24-norurs-12-en-3-one.

Compound **7** was obtained as a white, amorphous powder. The molecular formula was established as $C_{31}H_{50}O_5$ by HRESIMS (*m*/*z* 525.3556 $[M + Na]^+$, calcd 525.3552). Signals for two carbonyls (δ_C 175.0 and 180.4) and an olefinic group (δ_H 5.30/δ_C 123.2 and 145.2) were observed from the ^{1}H and ^{13}C NMR spectra. Seven degrees of unsaturation were implied by the molecular formula, which, together with the presence of two carbonyls and one olefinic group, suggested that **7** is based on a tetracyclic system. The NMR data (Tables 2 and 3) indicated that this compound belongs to the olean-12-ene family of triterpenoids.[21] In the HMBC spectrum (Figure S5, Supporting Information), the signals at δ_H 1.97 (H-16) and 2.31 (H-18) correlated with the signal at δ_C 69.1, the methyl proton signal at δ_H 1.10 (H-25) correlated with signals at δ_C 42.8 (C-1), 49.4 (C-5), and 39.8 (C-9), the signals at δ_H 1.42 (H-23) and 1.43 (H-24) correlated with the signals at δ_C 180.4 (C-3), 47.1 (C-4), and 49.4 (C-5), the methoxy proton signal at δ_H 3.74 (−OCH_3) correlated with the carboxyl carbon signal at δ_C 180.4 (C-3), and the signals at δ_H 2.68 (2H, H-1) correlated with the signal at δ_C 175.0 (C-2). On this basis, compound **7** was proposed as being cleaved between C-2 and C-3 and containing two carboxylic acid functions.[22] In general, ring-A-opened triterpenoids normally have a cleavage between C-3 and C-4, while 2,3-*seco*-triterpenoids, such as compound **7**, are rare. Thus, compound **7** was elucidated as 28-hydroxy-2,3-*seco*-olean-12-ene-2,3-dioic acid 3-methyl ester.

The cytotoxic effects of the new compounds **1**−**7** and of the known compounds obtained were evaluated against the human hepatoma (HepG2 and Hep3B), breast cancer (MCF-7 and MDA-MB-231), lung cancer (A549), gingival cancer (Ca9-22), and human leukemia cancer (HL60) cell lines, using a MTT assay. According to the results obtained, 12,23-dihydroxy-11α-methoxyurs-12-en-3-one (**2**) was found to be weakly active against HepG2 and HL60 cells with IC_{50} values of 7.7 and 5.1 μg/mL, respectively. Moreover, the main component, ursolic acid (**8**), showed cytotoxic activity against Ca9-22 and HL60 cells with IC_{50} values of 5.9 and 8.7 μg/mL, respectively (Table S1, Supporting Information). Ursolic acid (**8**) is known to exhibit a wide range of biological activities, including antiproliferative and cytotoxic activities, and inhibition of lipoxygenase and cyclooxygenase.[23−26] Since Bax, Bcl-2, and PARP are important regulators in the process of cell death, we assayed the expression of these regulating proteins with western

Table 2. ^{1}H NMR Spectroscopic Data for Compounds **4**, **5**, and **7** (in C_5D_5N, 500 MHz)

proton	**4**[a]	**5**	**7**
1	1.50 (m)	1.45 (m)	2.68 (2H, m)
	1.52 (m)	1.71 (m)	
2	1.88 (2H, m)	2.55 (m)	
		2.66 (ddd, 17.0, 8.0, 3.5)	
3	4.09 (m, α)		
4			
5	1.44 (dd, 12.0, 2.4)	2.38 (m)	3.02 (m)
6	1.06 (2H, m)	1.58 (2H, m)	1.60 (2H, m)
7	1.01 (m)	1.28 (m)	1.35 (m)
	1.17 (m)	1.57 (m)	1,79 (m)
9	1.84 (dd, 13.2, 4.8)	1.92 (dd, 13.5, 4.5)	3.30 (m)
11	2.52 (dd, 17.2,4.8)	2.54 (m)	1.97 (m)
	2.72 (dd, 17.2, 13.2)	2.76 (dd, 17.0, 13.5)	2.15 (dt, 17.5, 4.5)
12			5.30 (brs)
15	1.01 (2H, m)	1.02 (2H, m)	1.08 (m)
			1.98 (m)
16	1.17 (2H, m)	1.94 (2H, m)	1.51 (m)
			1.97 (m)
18	2.22 (d, 10.8)	2.20 (d, 11.5)	2.31 (dd, 14.0, 4.5)
19	1.68 (m)	1.69 (m)	1.09 (m)
			1.81 (m)
20	ca. 0.91	ca. 0.93	
21	1.25 (m)	1.24 (m)	1.24 (m)
	1.38 (m)	1.38 (m)	1.42 (m)
22	1.24 (m)	1.24 (m)	1.69 (m)
	1.48 (m)	1.50 (dd, 8.5, 2.5)	1.98 (m)
23	9.64 (s)	3.71 (d, 10)	1.42 (s)
		4.09 (d, 10)	
24	1.39 (s)	1.08 (s)	1.43 (s)
25	0.88 (s)	0.93 (s)	1.10 (s)
26	1.29 (s)	1.40 (s)	1.04 (s)
27	1.10 (s)	1.03 (s)	1.43 (s)
28	3.29 (d, 7.2)	3.30 (d, 7.0)	3.88 (d, 10.0)
	3.70 (d, 7.2)	3.73 (d, 7.0)	3.60 (d, 10.0)
29	1.09 (d, 5.2)	1.09 (d, 6.5)	0.91 (s)
30	ca. 0.91	0.91 (d, 5.5)	0.96 (s)
$OC\underline{H}_3$			3.74 (s)

[a] Measured in C_5D_5N, 400 MHz.

Table 3. ^{13}C NMR Spectroscopic Data for Compounds **1**−**7** (in C_5D_5N, 150 MHz)

carbon	**1**	**2**	**3**[a]	**4**[b]	**5**[c]	**6**	**7**[c]
1	40.6	38.7	39.8	38.1	38.0	42.4	42.8
2	28.5	36.7	28.0	27.0	36.6	38.2	175.0
3	77.9	217.1	71.8	71.6	216.9	213.3	180.4
4	39.7	53.2	57.2	56.3	53.2	43.2	47.1
5	55.8	47.6	48.3	47.4	47.1	55.0	49.4
6	18.8	20.0	21.7	20.5	19.7	66.8	21.8
7	33.8	33.4	34.0	32.5	32.7	39.7	32.8
8	43.4	42.8	43.8	42.4	42.4	42.3	40.6
9	52.5	45.6	48.6	48.7	48.2	45.2	39.8
10	38.5	37.9	38.0	36.0	36.9	37.5	42.2
11	76.6	77.0	77.6	37.3	38.0	77.5	24.7
12	125.0	144.8	145.6	209.4	209.7	145.0	123.2
13	142.7	117.0	117.3	89.3	90.0	116.6	145.2
14	44.2	40.9	41.3	45.9	46.5	41.4	43.3
15	37.0	27.6	28.2	26.2	26.8	27.6	26.5
16	65.4	28.0	28.4	26.3	26.8	28.0	23.4
17	39.1	33.7	34.1	42.7	43.2	33.6	38.1
18	60.5	47.5	47.9	55.0	55.4	47.5	43.2
19	39.6	41.4	41.9	37.9	38.5	40.0	47.3
20	39.8	40.3	40.5	40.7	41.2	41.4	31.7
21	31.1	31.6	32.1	31.5	32.1	31.6	35.1
22	35.9	42.2	42.7	34.8	35.4	42.2	32.2
23	28.9	68.3	207.7	207.2	68.3	11.8	28.3
24	16.6	18.0	10.3	9.4	18.1		24.7
25	17.5	16.3	17.8	15.8	15.9	16.0	20.0
26	18.5	18.2	18.8	18.6	18.6	19.9	17.5
27	23.9	23.8	24.5	17.7	17.8	24.2	26.4
28	23.0	28.9	29.4	77.2	77.7	28.9	69.1
29	17.8	17.4	17.3	19.2	19.6	17.5	33.8
30	21.6	21.5	22.0	19.8	20.3	21.6	24.3
$OC\underline{H}_3$	54.6	51.5	53.0			51.6	52.4

[a] Measured in C_5D_5N, 125 MHz. [b] Measured in C_5D_5N, 100 MHz. [c] **AUTHOR**: Please supply footnote c.

blotting to better understand the molecular basis for the cytotoxic effect of ursolic acid (**8**). Western blotting indicated that treatment of **8** induced increases of Bax and PARP cleavage in HL60 cells, whereas the expression of Bcl-2 was not changed (Figure S20, Supporting Information). Recently, histone hyperacetylation has become an important issue epigenetically. Histone deacetylases (HDACs) have been demonstrated to be associated with oncogenic transformation by mediating the function of transcription factors in certain forms of hematologic malignancies.[27] It is now becoming clear that histone deacetylase (HDAC) inhibitors offer a promising treatment for cancer therapy.[28,29] We then investigated whether the levels of histone acetylation are involved in the process of cell death induced by ursolic acid (**8**). Trichostatin A, a potent inhibitor of HDAC, enhances histone H3 acetylation in leukemia cells.[30] Treatment with ursolic acid (**8**) and trichostatin A increased histone H3 acetylation in HL60 cells at 20 μg/mL (Figure S21, Supporting Information). A growing body of evidence has suggested that functional inhibition of HDACs can alter the cellular protein level of histone deacetylase and influence the balance of acetylation and deacetylation of core histones.[31,32] We next addressed whether the increased acetylation of histone H3 induced by **8** was involved in inhibition of HDAC activity. As shown in Figure S21 (Supporting Information), the activities of HDAC 1, 3, 4, 5, and 6 decreased profoundly with ursolic acid (**8**) (0, 5, 10, and 20 μg/mL) treatment. These results demonstrate for the first time that treatment with this compound induces cell death partially through increasing acetylation of histone H3 and inhibition of HDAC activity.

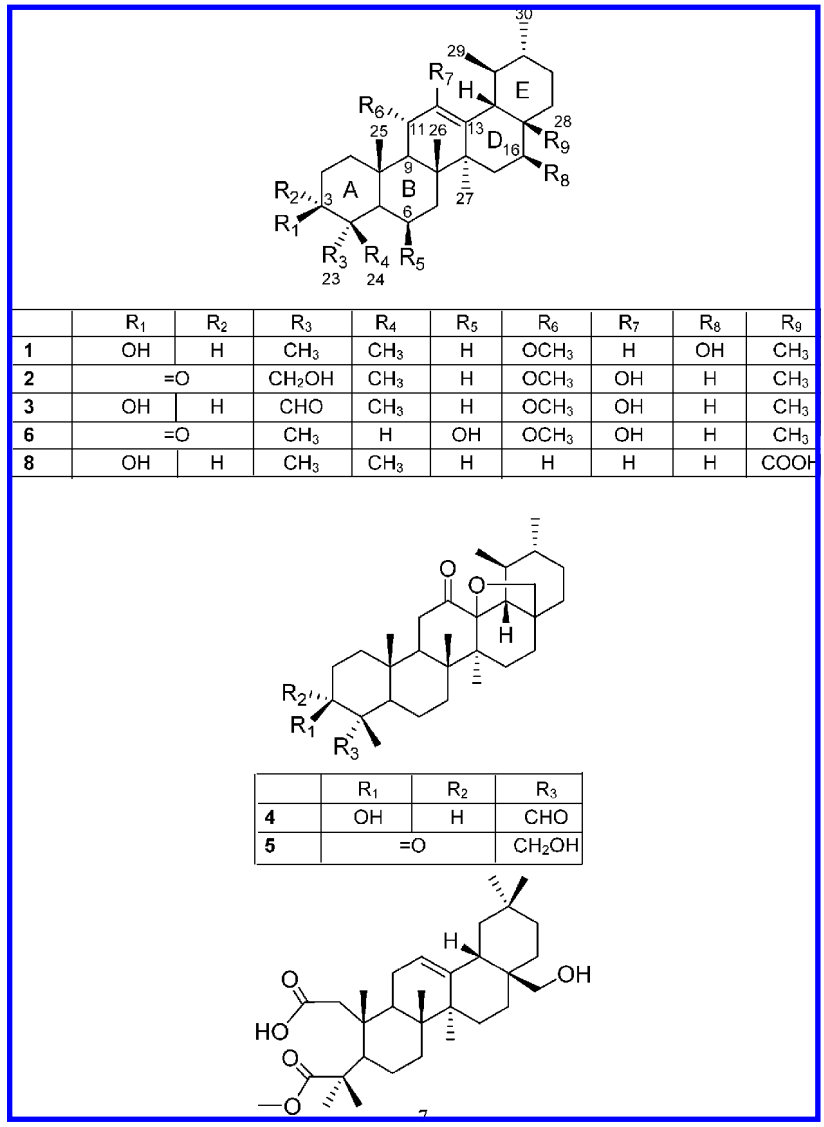
Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. The IR spectra were obtained on a Mattson Genesis II spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on Varian VNMR-600, Varian INOVA 500, Varian Unity Plus 400, or Varian Gemini 200 NMR spectrometers. Chemical shifts (δ) are reported in parts per million, and coupling constants (J) are expressed in hertz. LREIMS and LRESIMS were measured on a VG Biotech Quattro 5022 mass spectrometer. HRESIMS were measured on a Bruker Daltonics APEX Π mass spectrometer. Silica gel 60 (Merck, 230−400 mesh) and Sephadex LH-20 were used for column chromatography, while TLC analysis was carried out on silica gel GF_{254} precoated plates with detection using 50% H_2SO_4 followed by heating on a hot plate. HPLC was performed with a Hitachi L-7100 pump and D-7000 interface equipped with a Bischoff RI detector using ODS (Hypersil, 250 × 4 mm; Hypersil, 250 × 10 mm) columns.

Plant Material. The dried stems of *Microtropis japonica* were collected from Hen-Chun, Ping-Tong County, Taiwan, in February 2007, and identified by one of the authors (M.-H.Y.). A voucher specimen (Microtropis-02) was deposited at the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. The dried stems (8.0 kg) of *M. japonica* were extracted four times with MeOH overnight at room temperature to give a crude extract. This MeOH extract was partitioned between EtOAc and H_2O to produce an EtOAc-soluble fraction. Using hexane and 80% MeOH, the EtOAc-soluble fraction was divided into hexane and aqueous MeOH layers. The aqueous MeOH layer (64.5 g) was chromatographed on silica gel using mixtures of hexane−EtOAc of increasing polarity as eluants to afford 19 fractions. Among them, fractions 1 and 3−11 showed more than 50% inhibition toward hepatoma cell lines at a concentration of 20 μg/mL. Fraction 3 (2.46 g) was purified on Sephadex LH-20 with $CHCl_3$−MeOH (1:3) to give five subfractions. Fraction 3-3 (1.02 g) was further chromatographed on silica gel, eluting with mixtures of $CHCl_3$−MeOH, to afford nine subfractions. Fraction 3-3-4 (54.1 mg) was subjected to passage over an ODS HPLC column (250 × 10 mm, Hypersil, MeOH−H_2O, 90:10) to give **2** (7.1 mg, t_R 20.5 min, flow rate 2 mL/min) and **5** (0.73 mg, t_R 17.8 min, flow rate 2 mL/min). Fraction 3-3-7 (110.3 mg) was chromatographed on reversed-phase silica gel with MeOH to produce

Chart 1



	R_1	R_2	R_3	R_4	R_5	R_6	R_7	R_8	R_9
1	OH	H	CH_3	CH_3	H	OCH_3	H	OH	CH_3
2	=O		CH_2OH	CH_3	H	OCH_3	OH	H	CH_3
3	OH	H	CHO	CH_3	H	OCH_3	OH	H	CH_3
6	=O		CH_3	H	OH	OCH_3	OH	H	CH_3
8	OH	H	CH_3	CH_3	H	H	H	H	COOH

	R_1	R_2	R_3
4	OH	H	CHO
5	=O		CH_2OH

seven subfractions. Fraction 3-3-7-4 (14.4 mg) was subjected to passage over an ODS HPLC column (250 × 10 mm, Hypersil, MeOH−H_2O, 87:13) to give **1** (1.16 mg, t_R 24.6 min, flow rate 2 mL/min) and **6** (3.05 mg, t_R 26.7 min, flow rate 2 mL/min). Fraction 3-3-7-5 (15.94 mg) was further separated using an ODS HPLC column (250 × 10 mm, Hypersil, MeOH−H_2O, 90:10) to afford **3** (2.7 mg, t_R 23.8 min, flow rate 2 mL/min) and **4** (2.1 mg, t_R 19.4 min, flow rate 2 mL/min). Fraction 3-3-8 (47.9 mg) was chromatographed on reversed-phase silica gel with MeOH to furnish eight fractions. Further, fraction 3-3-8-3 (7.8 mg) was subjected to passage over an ODS HPLC colum (250 × 10 mm, Hypersil, MeOH−H_2O, 85:15) to give **7** (1.9 mg, t_R 17.4 min, flow rate 2 mL/min). The isolation procedures of known compounds are available in the Supporting Information.

11α-Methoxyurs-12-ene-3*β*,16*β*-diol (1): white, amorphous solid; $[\alpha]^{25}_D$ +0.9 (*c* 0.1, MeOH); IR (neat) ν_{max} 3410 (OH), 2925, 2856, 1652, 1457, 1382 cm^{-1}; ^1H NMR (C_5D_5N, 600 MHz) and ^{13}C NMR (C_5D_5N, 150 MHz), see Tables 1 and 3; ESIMS *m/z* 495 [M + Na]$^+$; HRESIMS *m/z* 495.3814 [M + Na]$^+$ (calcd for $C_{31}H_{52}O_3Na$, 495.3817).

12,23-Dihydroxy-11α-methoxyurs-12-en-3-one (2): white, amorphous solid; $[\alpha]^{26}_D$ +7.1 (*c* 0.2, MeOH); IR (neat) ν_{max} 3439 (OH), 2924, 1694, 1646, 1455, 1378 cm^{-1}; ^1H NMR (C_5D_5N, 600 MHz) and ^{13}C NMR (C_5D_5N, 150 MHz), see Tables 1 and 3; ESIMS *m/z* 509 [M + Na]$^+$; HRESIMS *m/z* 509.3607 [M + Na]$^+$ (calcd for $C_{31}H_{50}O_4Na$, 509.3608).

3*β*,12-Dihydroxy-11α-methoxyurs-12-en-23-al (3): white, amorphous solid; $[\alpha]^{25}_D$ +4.7 (*c* 0.3, MeOH); IR (neat) ν_{max} 3440 (OH), 2923, 2860, 1727, 1454, 1375 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz), see Tables 1 and 3; ESIMS *m/z* 509 [M + Na]$^+$; HRESIMS *m/z* 509.3607 [M + Na]$^+$ (calcd for $C_{31}H_{50}O_4Na$, 509.3604).

13*β*,28-Epoxy-3*β*-hydroxy-12-oxoursan-23-al (4): white, amorphous solid; $[\alpha]^{25}_D$ +4.6 (*c* 0.2, MeOH); IR (neat) ν_{max} 3439 (OH), 2925, 2866, 1712, 1458, 1374 cm^{-1}; ^1H NMR (C_5D_5N, 400 MHz) and ^{13}C NMR (C_5D_5N, 100 MHz), see Tables 2 and 3; ESIMS *m/z* 493 [M + Na]$^+$; HRESIMS *m/z* 493.3294 [M + Na]$^+$ (calcd for $C_{30}H_{46}O_4Na$, 493.3297).

13*β*,28-Epoxy-23-hydroxyursane-3,12-dione (5): white, amorphous solid; $[\alpha]^{25}_D$ −2.7 (*c* 0.02, MeOH); IR (neat) ν_{max} 3433 (OH), 2915, 2851, 1730, 1699, 1454, 1376 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz), see Tables 2 and 3; ESIMS *m/z* 493 [M + Na]$^+$; HRESIMS *m/z* 493.3294 [M + Na]$^+$ (calcd for $C_{30}H_{46}O_4Na$, 493.3296).

6*β*,12-Dihydroxy-11α-methoxy-24-norurs-12-en-3-one (6): white, amorphous solid; $[\alpha]^{25}_D$ +2.5 (*c* 0.3, MeOH); IR (neat) ν_{max} 3457 (OH), 2925, 2855, 1703, 1455, 1376 cm^{-1}; ^1H NMR (C_5D_5N, 600 MHz) and ^{13}C NMR (C_5D_5N, 150 MHz), see Tables 1 and 3; ESIMS *m/z* 495 [M + Na]$^+$; HRESIMS *m/z* 495.3450 [M + Na]$^+$ (calcd for $C_{30}H_{48}O_4Na$, 495.3447).

28-Hydroxy-2,3-*seco*-olean-12-ene-2,3-dioic acid 3-methyl ester (7): white, amorphous solid; $[\alpha]^{25}_D$ +3.7 (*c* 0.19, MeOH); IR (neat) ν_{max} 3426 (OH), 2922, 2858, 1720, 1557, 1457, 1387 cm^{-1}; ^1H NMR (C_5D_5N, 500 MHz) and ^{13}C NMR (C_5D_5N, 125 MHz), see Tables 2 and 3; ESIMS *m/z* 525 [M + Na]$^+$; HRESIMS *m/z* 525.3556 [M + Na]$^+$ (calcd for $C_{31}H_{50}O_5Na$, 525.3552).

Bioassay Materials. Medium, fetal calf serum (FCS), trypan blue, penicillin G, and streptomycin were obtained from Gibco BRL (Gaithersburg, MD). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethylsulfoxide (DMSO), ribonuclease (RNase), and propidium iodide (PI) were from Sigma-Aldrich (St. Louis, MO). Histone H3, acetyl-histone H3, H3K9, and H3K18 were purchased from Upstate Biotechnology (Temecula, CA). Antibodies against HDAC1, -3, -4, -5, and -6 were purchased from Cell Signaling Technologies (Beverly, MA), Bax, Bcl-2, PARP, and tubulin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), and antimouse and rabbit 1gG peroxidase-conjugated secondary antibody were from Pierce (Rockford, IL).

Cytotoxicity Assay. Test compounds were assayed for cytotoxicity against human hepatoma (HepG2 and Hep3B), breast cancer (MCF-7 and MDA-MB-231), lung cancer (A549), gingival cancer (Ca9-22), and human leukemia cancer (HL60) cell lines, using the MTT method performed according to a previously published protocol.[30,33]

Histone Fraction from Acid Extraction. Histone isolation was performed as described previously.[34] The cells were harvested by centrifugation at 700*g* and washed once with ice-cold PBS. The pellet was resuspended in 1 mL of lysis buffer (10 mM Tris-HCl pH 6.5, 50 mM sodium disulfite, 10 mM $MgCl_2$, 10 mM sodium butyrate, 8.6% sucrose, 1% Triton X-100) and centrifuged at 1000*g*. After three washes in lysis buffer, the pellet was resuspended in 10 mM Tris-HCl pH 7.4 and 13 mM EDTA. The pellet was then resuspended in cold distilled water, and H_2SO_4 was added to 0.4 N concentration. The sample was centrifuged at 10000*g* for 5 min after incubation on ice for 1 h. From the supernatant, total histones were precipitated with 10× volume of acetone at −20 °C overnight. The precipitated histones were collected by centrifugation, dried, and resuspended in distilled water. The protein content was measured by the Bio-Rad protein assay kit (BioRad Laboratories GmbH, Munich, Germany).

Western Blotting Analysis. Cell lysates were prepared by treating the cells for 30 min in RIPA lysis buffer (1× PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM sodium orthovanadate, 100 μg/mL phenylmethylsulfonyl fluoride, and 30 μg/mL aprotinin) (all chemicals were from Sigma), as described previously.[30] The lysates were centrifuged at 20000*g* for 30 min, and the protein concentration in the supernatant was determined using the Bio-Rad protein assay kit. Equal amounts of proteins were separated respectively by 7.5, 10, or 12% of SDS-polyacrylamide gel electrophoresis, and then these were electrotransferred to a PVDF membrane. The membrane was blocked with a solution containing 5% nonfat dried milk TBST buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween 20) for 1 h and washed with TBST buffer. The protein expressions were monitored by immunoblotting using specific antibodies. These proteins were detected by an enhanced chemiluminescence kit (Pierce, Rockford, IL).

Acknowledgment. We gratefully acknowledge the financial support for this project from the KMU-EM-97-2.1.a & b, the National Science Council, and National Science Technology Program/Biotechnology and Pharmaceuticals, Taiwan.

Supporting Information Available: Spectroscopic data and additional information on known compounds, and ^1H and ^{13}C NMR spectra of new compounds **1**−**7** are available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) Liu, J. *J Ethnopharmacol.* **1995**, *49*, 57–68.
(2) Somova, L. O.; Nadar, A.; Rammanan, P.; Shode, F. O. *Phytomedicine* **2003**, *10*, 115–121.
(3) Ghezzi, P.; Dinarello, C. A. *J. Immunol.* **1988**, *140*, 4238–4244.
(4) Thiem, D. A.; Sneden, A. T.; Khan, S. I.; Tekwani, B. L. *J. Nat. Prod.* **2005**, *68*, 251–254.
(5) Feng, X.; Gao, Z.; Li, S.; Jones, S. H.; Hecht, S. M. *J. Nat. Prod.* **2004**, *67*, 1744–1747.
(6) de Andrade, S. F.; Comunello, E.; Noldin, V. F.; Monache, F. D.; Cechinel Filho, V.; Niero, R. *Arch. Pharm. Res.* **2008**, *31*, 41–46.
(7) Matsuda, H.; Murakami, T.; Yashiro, K.; Yamahara, J.; Yoshikawa, M. *Chem. Pharm. Bull.* **1999**, *47*, 1725–1729.
(8) Chen, J. J.; Yang, C. S.; Peng, C. F.; Chen, I. S.; Miaw, C. L. *J. Nat. Prod.* **2008**, *71*, 1016–1021.
(9) Chou, T. H.; Chen, I. S.; Peng, C. F.; Sung, P. J.; Chen, J. J. *Chem. Biodiversity* **2008**, *5*, 1412–1418.
(10) Miyase, T.; Melek, F. R.; El-Gindi, O. D.; Abdel-Khalik, S. M.; El-Gindi, M. R.; Haggag, M. Y.; Hilal, S. H. *Phytochemistry* **1996**, *41*, 1175–1179.
(11) González, A. G.; Nuñez, M. P.; Ravelo, A. G.; Luis, J. G.; Jiménez, I. A. *J. Nat. Prod.* **1990**, *53*, 474–478.
(12) Tsanuo, M. K.; Hassanali, A.; Jondiko, I. J. O.; Torto, B. *Phytochemistry* **1993**, *34*, 665–667.
(13) Kim, S. E.; Kim, H. S.; Hong, Y. S.; Kim, Y. C.; Lee, J. J. *J. Nat. Prod.* **1999**, *62*, 697–700.
(14) Chou, T. H.; Chen, I. S.; Sung, P. J.; Peng, C. F.; Shieh, P. C.; Chen, J. J. *Chem. Biodiversity* **2007**, *4*, 1594–1600.
(15) Chen, I. H.; Chang, F. R.; Wu, C. C.; Chen, S. L.; Hsieh, P. W.; Yen, H. F.; Du, Y. C.; Wu, Y. C. *J. Nat. Prod.* **2006**, *69*, 1543–1546.
(16) Zhang, A. L.; Ye, Q.; Li, B. G.; Qi, H. Y.; Zhang, G. L. *J. Nat. Prod.* **2005**, *68*, 1531–1535.
(17) Fourie, T. G.; Matthee, E.; Snyckers, F. O. *Phytochemistry* **1989**, *28*, 2851–2852.
(18) Lahlou, H. E.; Hirai, N.; Tsuda, M.; Ohigashi, H. *Phytochemistry* **1999**, *52*, 623–629.
(19) Durham, D. G.; Liu, X.; Richards, R. M. E. *Phytochemistry* **1994**, *36*, 1469–1472.
(20) Hart, N. K.; Lamberton, J. A.; Sioumis, A. A.; Suares, H. *Aust. J. Chem.* **1976**, *29*, 655–671.
(21) Yamagishi, T.; Zhang, D. C.; Chang, J. J.; McPhail, D. R.; McPhail, A. T.; Lee, K. H. *Phytochemistry* **1988**, *27*, 3213–3216.
(22) Duan, H.; Takaishi, Y.; Momota, H.; Ohmoto, Y.; Taki, T.; Tori, M.; Takaoka, S.; Jia, Y.; Li, D. *Tetrahedron* **2001**, *57*, 8413–8424.
(23) Kassi, E.; Papoutsi, Z.; Pratsinis, H.; Aligiannis, N.; Manoussakis, M.; Moutsatsou, P. *J. Cancer Res. Clin. Oncol.* **2007**, *133*, 493–500.
(24) He, X.; Liu, R. H. *J. Agric. Food Chem.* **2007**, *55*, 4366–4370.
(25) Dufour, D.; Pichette, A.; Mshvildadze, V.; Bradette-Hebert, M. E.; Lavoie, S.; Longtin, A.; Laprise, C.; Legault, J. *J. Ethnopharmacol.* **2007**, *111*, 22–28.
(26) Najid, A.; Simon, A.; Cook, J.; Chable-Rabinovitch, H.; Delage, C.; Chulia, A. J.; Rigaud, M. *FEBS Lett.* **1992**, *299*, 213–217.
(27) Rosato, R. R.; Grant, S. *Exp. Opin. Investig. Drugs.* **2004**, *13*, 21–38.
(28) Zhao, Y.; Tan, J.; Zhuang, L.; Jiang, X.; Liu, E. T.; Yu, Q. *Proc. Natl. Acad. Sci., U.S.A.* **2005**, *102*, 16090–16095.
(29) Dwarakanath, B. S.; Verma, A.; Bhatt, A. N.; Parmar, V. S.; Raj, H. G. *Indian J. Med. Res.* **2008**, *128*, 13–21.
(30) Lu, M. C.; Du, Y. C.; Chuu, J. J.; Hwang, S. L.; Hsieh, P. C.; Hung, C. S.; Chang, F. R.; Wu, Y. C. *Arch. Toxicol.* **2009**, *83*, 121–129.
(31) Kramer, O. H.; Zhu, P.; Ostendorff, H. P.; Golebiewski, M.; Tiefenbach, J.; Peters, M. A.; Brill, B.; Groner, B.; Bach, I.; Heinzel, T.; Gottlicher, M. *EMBO J.* **2003**, *22*, 3411–3420.
(32) Xu, Y.; Voelter-Mahlknecht, S.; Mahlknecht, U. *Int. J. Mol. Med.* **2005**, *15*, 169–172.
(33) Sladowski, D.; Steer, S. J.; Clothier, R. H.; Balls, M. *J. Immunol. Methods* **1993**, *157*, 203–207.
(34) Taplick, J.; Kurtev, V.; Lagger, G.; Seiser, C. *FEBS Lett.* **1998**, *436*, 349–352.

NP800694B