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Anti-inflammatory Flavonoids from the Rhizomes of *Helminthostachys zeylanica*

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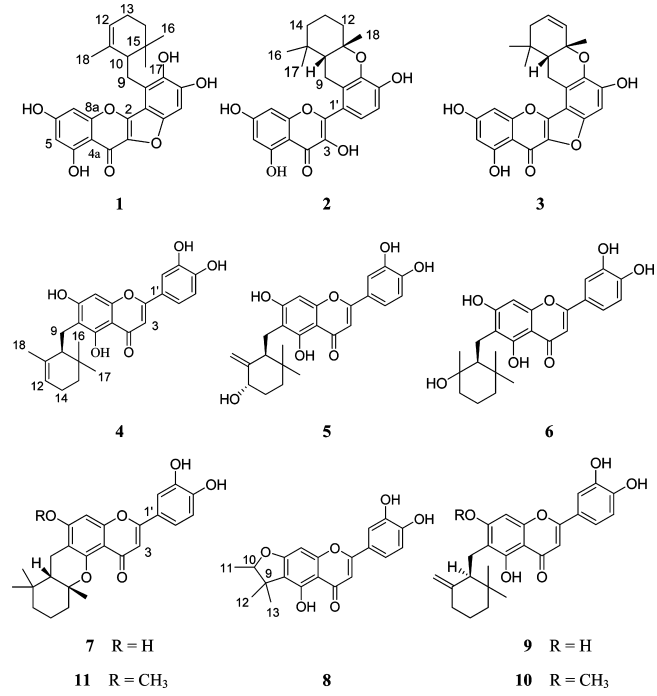
Eight new prenylated flavonoids, ugonins M−T (**1**-**8**), together with five known compounds, ugonins J−L (**9**−**11**), 5,4′-dihydroxy-4″,4″-dimethyl-5″-methyl-5″*H*-dihydrofurano[2″,3″:6,7]flavanone, and quercetin, were isolated and purified from the rhizomes of *Helminthostachys zeylanica*. The structures of the new isolates were elucidated by spectroscopic and chemical methods. Compounds **1**, **3**, **5**, **7**, **8**, and **11** showed inhibition of superoxide anion generation and elastase release by human neutrophils in response to formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (FMLP/CB).

In Chinese traditional medicine, the roots of the fern *Helminthostachys zeylanica* (L.) Hook. (Ophioglossaceae), known as "Ding-Di-U-Gon", have been used for centuries in the treatment of inflammation and various hepatic disorders. This medicinal fern is also used as a folk medicine in India and Sri Lanka. The plant has been shown to exhibit anti-inflammatory, antiphlogistic, antipyretic, and hepatoprotective activities.[1,2] Flavonoids with prenyl or geranyl groups have been reported to have specific bioactivities, such as cytotoxic activity against the P-388 cell line,[3] inhibitory activity toward protein tyrosine phosphatase-1B (PTP1B),[4] and strong antioxidant effects.[5] The flavonoids, ugonins A−L, as well as three cyclized geranyl stilbenes, ugonstiblenes A−C, have been identified from *H. zeylanica*.[6−9]

Neutrophils are active phagocytes that act as a crucial component of innate immunity. In response to diverse stimuli, activated neutrophils secrete a series of cytotoxins, such as superoxide anion ($O_2^{\bullet-}$), a precursor of other reactive oxygen species, and granule proteases.[10,11] Preliminary bioactivity screening indicated that the ethyl acetate extract of *H. zeylanica* has a potent inhibitory effect on human neutrophil-generated superoxide anions in the presence of formyl-L-methionyl-L-leucyl-L-phenylalanine/cytochalasin B (FMLP/CB) at 10 μg/mL. This prompted us to investigate the bioactivity of the secondary metabolites isolated from *H. zeylanica.* Herein, we report on eight new flavonoids, ugonins M−T (**1**−**8**), along with five known flavonoids, ugonins J (**9**),[8] K (**10**),[8] and L (**11**),[8] 5,4′-dihydroxy-4″,4″-dimethyl-5″-methyl-5″*H*-dihydrofurano[2″,3″:6,7]flavanone,[12] and quercetin,[13,14] as constituents of the roots of *H. zeylanica.* The inhibitory effects of these isolates on superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB were evaluated.



Results and Discussion

The ethyl acetate-soluble extract of the rhizomes of *H. zeylanica* was fractionated by column chromatography and purified by reversed-phase semipreparative HPLC to obtain eight new compounds (**1**−**8**), together with five known substances. The ^{13}C NMR data indicated that compounds **1**−**7** each gave a total of 25 resonances, in which 15 were attributed to a flavonoid part and 10 resonances to a cyclized geranyl group. Compound **8** exhibited a total of 20 resonances, with 15 due to a flavonoid core and five resonances to an isoprenyl group. The ^{1}H NMR spectra of compounds **1**−**6** and **8** (Table 1) revealed the presence of a signal in the region δ_H 12−13 for a hydroxy group in DMSO-d_6 or acetone-d_6 (Table 1). The UV bands at 331−363 and 262−274 nm, as well as the IR absorptions at ca. 3400 (O−H) and 1640 (C═O) cm^{-1}, were supportive of compounds **1**−**8** being flavones, as in the case of compounds **9**−**11**.[15]

Compound **1** was isolated and purified as a yellow powder. The HRFABMS showed a $[M + H]^+$ ion at *m*/*z* 437.1604 (calcd for $C_{25}H_{25}O_7$, 437.1600), consistent with the molecular formula, $C_{25}H_{24}O_7$, and corresponding to 14 degrees of unsaturation. The ^{13}C NMR and HSQC spectra of **1** indicated the presence of 14 quaternary carbons, five methines, three methylenes, and three methyl groups (Table 2). Fifteen ^{13}C NMR resonances were found at chemical shift values similar to those of 3,5,7,3′,4′,6′-hexahydroxyflavone.[16] The remaining 10 resonances resulted from a cyclized geranyl group. Two proton signals at δ_H 6.44 (1H, s) and

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Table 1. ^{1}H NMR Chemical Shifts of Compounds **1**−**8** (*J* in Hz)

position	**1**[a]	**2**[a]	**3**[c]	**4**[a]	**5**[c]	**6**[c]	**7**[b]	**8**[a]
3				6.53, s	6.65, s	6.64, s	6.41, s	6.55, s
5								
6	6.24, s	6.22, d (1.6)	6.27, d (2.0)					
8	6.44, s	6.37, s	6.56, d (2.0)	6.47, s	6.48, s	6.44, s	6.64, s	6.18, s
9	3.30, m	3.32, d (14.2)		2.82, dd (13.8, 6.0)	2.85, dd (13.8, 10.1)	2.84, dd (14.9, 7.2)	2.80, dd (16.8, 4.8)	
	3.04, dd (12.0, 12.0)	2.27, m	3.39, d (5.0)	2.61, dd (13.8, 7.2)	2.69, dd (13.8, 3.5)	2.55, dd (14.9, 2.2)	2.36, dd (16.8, 13.6)	
10	2.27, dd (12.0, 6.0)	1.54, m	1.98, dd (5.0, 5.0)	2.24, dd (7.2, 6.0)	2.58, m	1.58, dd (7.2, 2.2)	1.64, m	4.56, q (6.5)
11								1.42, d (6.5)
12	5.14, s	1.45, m	5.88, m	5.24, s	3.70, s	1.31, m	1.64 (2H), m	1.34, s
		1.31, m				1.14, m		
13	2.16, m	1.67 (2H), m	5.85, dd (10.0, 2.0)	2.03, m	1.74, m	1.45, m	1.67 (2H), m	1.62, s
	2.03, m				1.34, m			
14 a	1.28, m	2.28, m	2.05, d (17.7)	1.74, m	1.41, m	1.70, m	1.52, m	
b		1.54, m	1.92, dd (17.7, 5.7)	1.16, m		1.38, m	1.38, m	
16	0.93, s	0.91(3H), s	0.75, s	0.88, s	0.81, s	0.81, s	0.97 (3H), s	
17	1.11, s	1.30 (3H), s	1.09, s	0.97, s	1.04, s	0.95, s	1.05 (3H), s	
					5.05, s			
18	1.01, s	1.52 (3H), s	1.32, s	1.48, s	5.01, s	1.22, s	1.26 (3H), s	
2′				7.37, d (8.4)	7.39, brs	7.38, brs	7.47, d (2.0)	7.39, d (1.8)
5′	6.88, s	6.80, d (8.4)	6.97, s	6.89, d (8.4)	6.90, d (8.0)	6.89, d (8.0)	6.99, d (8.4)	6.92, d (8.3)
6′		6.90, d (8.4)		7.36, brs	7.41, dd (8.0, 2.0)	7.40, dd (8.0, 2.0)	7.36, dd (8.4, 2.0)	7.36, dd (8.3, 1.8)
hydroxy group	12.77, s[d]	12.86, s[d]	12.83, s	13.32, s[d]	13.45, s	13.54, s	13.36, s[b]	

[a] Measured in CD_3OD (**1** and **4** measured at 600 MHz, **2** measured at 400 MHz, **8** measured at 500 MHz). [b] Measured in Me_2CO-d_6 (400 MHz). [c] Measured in DMSO-d_6 (500 MHz). [d] Measured in DMSO-d_6 (400 MHz).

Table 2. ^{13}C NMR Chemical Shifts of Compounds **1**−**8**

position	**1**[a]		**2**[a]		**3**[c]		**4**[a]		**5**[c]		**6**[c]		**7**[b]		**8**[a]	
2	135.0	qC	160.3	qC	133.3	qC	166.0	qC	164.1	qC	163.6	qC	160.3	qC	166.2	qC
3	153.2	qC	134.6	qC	150.4	qC	103.8	CH	103.2	CH	102.6	CH	106.3	CH	104.4	CH
4	171.0	qC	180.3	qC	168.9	qC	183.9	qC	182.2	qC	181.7	qC	177.1	qC	184.1	qC
4a	105.4	qC	104.5	qC	103.9	qC	105.0	qC	103.9	qC	103.2	qC	107.8	qC	106.2	qC
5	164.0	qC	161.7	qC	162.3	qC	160.4	qC	159.3	qC	157.7	qC	154.2	qC	154.5	qC
6	100.2	CH	98.2	CH	99.0	CH	114.5	qC	111.8	qC	113.3	qC	107.1	qC	114.7	qC
7	165.1	qC	164.2	qC	163.4	qC	164.1	qC	162.7	qC	162.6	qC	159.6	qC	166.8	qC
8	95.4	CH	93.2	CH	94.7	CH	94.1	CH	93.8	CH	93.9	CH	93.8	CH	95.5	CH
8a	158.9	qC	157.3	qC	157.1	qC	157.3	qC	155.4	qC	155.1	qC	157.6	qC	163.9	qC
9	30.5	CH_2	22.8	CH_2	19.1	CH_2	25.7	CH_2	20.5	CH_2	17.8	CH_2	17.7	CH_2	45.1	qC
10	49.6	CH	55.6	CH	42.0	CH	48.8	CH	49.2	CH	55.4	CH	46.6	CH	92.2	CH
11	137.8	qC	83.4	qC	73.6	qC	139.0	qC	152.1	qC	73.6	qC	77.8	qC	14.7	CH_3
12	121.9	CH	41.1	CH_2	127.9	CH	121.0	CH	72.5	CH	41.8	CH_2	39.4	CH_2	22.1	CH_3
13	24.5	CH_2	20.3	CH_2	130.6	CH	24.3	CH_2	33.5	CH_2	19.9	CH_2	19.4	CH_2	26.7	CH_3
14	30.7	CH_2	41.2	CH_2	41.0	CH_2	31.4	CH_2	39.8	CH_2	42.5	CH_2	41.1	CH_2		
15	33.9	qC	35.6	qC	32.1	qC	33.8	qC	36.3	qC	35.1	qC	33.0	qC		
16	27.2	CH_3	20.3	CH_3	20.5	CH_3	27.6	CH_3	29.8	CH_3	31.8	CH_3	19.8	CH_3		
17	29.1	CH_3	33.5	CH_3	30.0	CH_3	28.1	CH_3	21.0	CH_3	20.7	CH_3	31.2	CH_3		
18	24.9	CH_3	15.9	CH_3	26.4	CH_3	24.4	CH_3	105.4	CH_2	22.6	CH_3	19.0	CH_3		
1′	110.6	qC	122.7	qC	106.6	qC	123.8	qC	122.1	qC	121.6	qC	123.2	qC	123.9	qC
2′	122.7	qC	130.6	qC	114.4	qC	114.1	CH	113.8	CH	113.1	CH	112.7	CH	114.3	CH
3′	143.8	qC	142.7	qC	139.9	qC	147.3	qC	146.2	qC	145.6	qC	145.3	qC	147.4	qC
4′	152.5	qC[d]	146.9	qC	151.0	qC	150.9	qC	150.1	qC	149.7	qC	148.2	qC	151.2	qC
5′	96.5	CH	111.8	CH	96.4	CH	116.7	CH	116.5	CH	115.9	CH	115.5	CH	117.0	CH
6′	151.5	qC[d]	119.7	CH	151.2	qC	120.2	CH	119.4	CH	118.8	CH	118.3	CH	120.4	CH

[a] Measured in CD_3OD (**1** and **4** measured at 150 MHz, **2** measured at 100 MHz, **8** measured at 125 MHz). [b] Measured in Me_2CO-d_6 (100 MHz). [c] Measured in DMSO-d_6 (125 MHz). [d] Assignments are interchangeable.

6.24 (1H, s) were assigned to H-8 and H-6, corresponding with the HMBC cross-peaks between δ_H 6.44/δ_C 100.2 (C-6), 105.4 (C-4a), 158.9 (C-8a), and 165.1 (C-7) and δ_H 6.24/δ_C 95.4 (C-8), 105.4 (C-4a), 164.0 (C-5), and 165.1 (C-7), indicating the presence of a flavone with two hydroxy groups at C-5 and C-7 (Figure 1). The singlet signal at δ_H 6.88 was attributed to the C-5′ position according to the HMBC correlations between δ_H 6.88/δ_C 152.5 (C-4′), 151.5 (C-6′), 143.8 (C-3′), and 110.6 (C-1′) (Figure S1, Supporting Information), suggesting the B ring as having five substitutions. In addition, an olefinic methine signal at δ_H 5.14 (1H, s), three sets of methylene signals at δ_H 3.30 (1H, m) and 3.04 (1H, dd, J = 12.0, 12.0 Hz), 2.16 (1H, m), and 2.03 (1H, m), and 1.28 (2H, m), three methyl signals at δ_H 1.11, 1.01, and 0.93 (each 3H, s), and two quaternary carbons at δ_C 137.8 and 33.9 suggested the presence of a cyclized geranyl group located at C-2′, due to the HMBC correlations between δ_H 3.30, 3.04 (H_2-9)/δ_C 143.8 (C-3′), 122.7 (C-2′), 110.6 (C-1′), and 49.6 (C-10). Two clear NOESY correlations between δ_H 2.27 (H-10)/0.93 (Me-16) and 1.01 (Me-18) indicated H-10 as having a pseudoaxial orientation (Figure 1). Altogether, 11 degrees of unsaturation were attributed to the flavone skeleton and two degrees to the cyclized geranyl group. The remaining one degree of unsaturation was consistent with the presence of a furan moiety, supported by the ^{13}C NMR resonances at δ_C 135.0 (C-2), 153.2 (C-3), 110.6 (C-1′), and 151.5 (C-6′). Thus, the structure of **1** was determined as 5,7,3′,4′-tetrahydroxy-[2′(2,6,6-trimethylcyclohex-2-enyl)methyl]furano[1′,6′:2,3]-4*H*-chromeno-4-one and was named ugonin M.

Compound **2** was isolated as a yellow powder. The HRFABMS showed a $[M + H]^+$ ion at *m*/*z* 439.1750 (calcd for $C_{25}H_{27}O_7$, 439.1757), consistent with the molecular formula, $C_{25}H_{26}O_7$, from which 13 degrees of unsaturation were deduced. The ^{13}C NMR and DEPT data of **2** showed 13 quaternary carbons, four aromatic

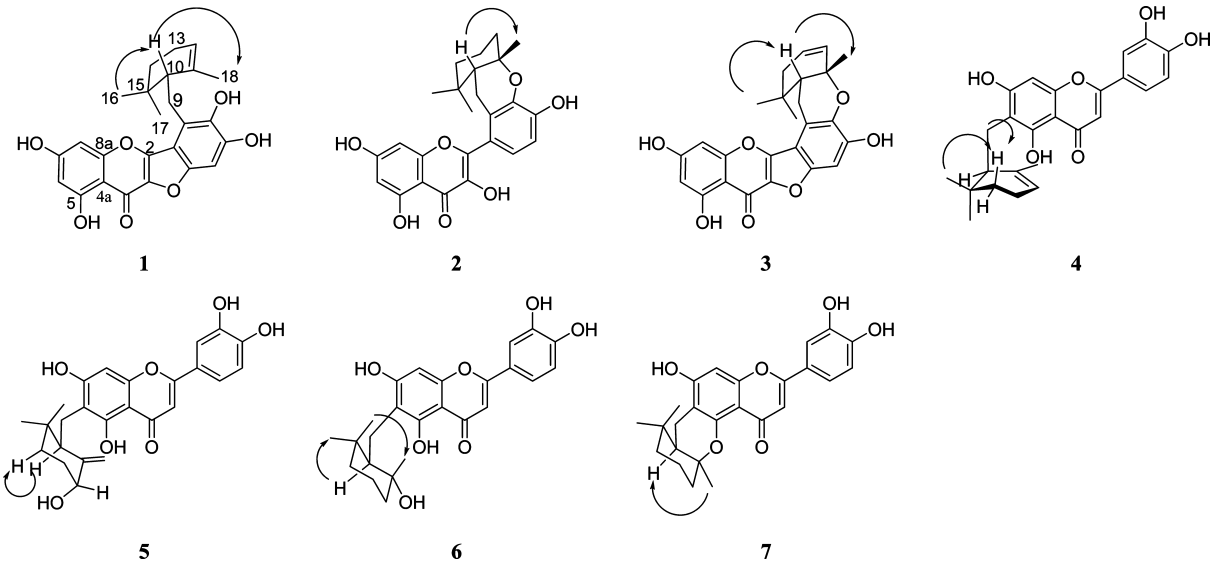

Figure 1. Selected NOESY correlations of compounds **1**−**7**.

methines, one methine, four methylenes, and three methyl groups (Table 2), similar to ugonin H.[8] Two proton signals at δ_H 6.90 (1H, d, $J = 8.4$ Hz) and 6.80 (1H, d, $J = 8.4$ Hz) were assigned as the *ortho*-coupled proton signals on the B ring. The signals at δ_H 6.37 (1H, s, H-8) and 6.22 (1H, d, $J = 1.6$ Hz, H-6), with their corresponding HMBC correlations, suggested a flavone with hydroxy groups at C-5 and C-7. In addition, the cyclized geranyl group was located at C-2′ and C-3′ according to the HMBC correlations between δ_H 3.32 and 2.27 (H_2-9) and δ_C 142.7 (C-3′), 130.6 (C-2′), 122.7 (C-1′), 83.4 (C-11), and 55.6 (C-10). In contrast to the ^{1}H NMR data of ugonin H,[8] two methylene signals at δ_H 1.45 and 1.31 (H_2-12) and 1.67 (H_2-13) were apparent in compound **2**, instead of the olefinic proton signals at δ_H 4.92 of ugonin H, indicating that the cyclized geranyl group is saturated. Moreover, a NOESY correlation between δ_H 1.54 (H-10) and δ_H 1.52 (H_3-18) demonstrated a *cis*-configuration between H-10 and H_3-18 (Figure 1). Thus, the structure of **2** (ugonin N) was determined as 3,5,7-trihydroxy-2-(5-hydroxy-1,1,4a-trimethyl-2,3,4,4a,9,9a-hexahydro-1*H*-xanthen-8-yl)-4*H*-chromen-4-one.

Compound **3** was isolated and purified as a yellow powder. The HRFABMS showed a $[M + H]^+$ ion at *m*/*z* 435.1435 (calcd for $C_{25}H_{23}O_7$, 435.1444), corresponding to the molecular formula, $C_{25}H_{22}O_7$, representing 15 degrees of unsaturation. The ^{13}C NMR and HSQC spectra of **3** showed the presence of 14 quaternary carbons, three aromatic methines, three methines, two methylenes, and three methyl groups (Table 2). The intramolecular hydrogen-bond signal at δ_H 12.83 and the aromatic proton signals at δ_H 6.97, 6.56, and 6.27 indicated that **3** has a similar skeleton to compound **1**. The HMBC correlations between the signals at δ_H 3.39 (H_2-9) and the ^{13}C NMR resonances at δ_C 32.1 (C-15), 42.0 (C-10), 73.6 (C-11), 106.6 (C-1′), 114.4 (C-2′), and 139.9 (C-3′) suggested that the cyclized geranyl group is located at the C-2′ position. In contrast to the ^{1}H NMR spectrum of **1**, a pair of olefinic proton signals at δ_H 5.88 (H-12) and 5.85 (H-13) appeared for **3**, instead of the signal at δ_H 5.14, and a methyl group at δ_H 1.32 in **3** was shifted downfield from δ_H 1.01, consistent with the cyclized geranyl group of **3** being cyclized between C-3′ and C-11 by an ether linkage, as supported by the MS data and the number of degrees of unsaturation. The NOESY correlations between δ_H 1.98 (H-10)/1.32 (H_3-18) and 1.09 (H_3-17) indicated a *cis*-configuration between H_3-18 and H-10 (Figure 1). Thus, the structure of **3** (ugonin O) was determined as 5,7,4′-trihydroxy-2′-(2,6,6-trimethyl-3-cyclohexenylmethyl)furano[1′,6′:2,3]-4*H*-chromeno[2″,3″:2′,3′]flavone.

Compound **4** was obtained as a yellow powder, with a molecular formula of $C_{25}H_{26}O_6$ based on the $[M + H]^+$ ion at *m*/*z* 423.1803 (calcd for $C_{25}H_{27}O_6$, 423.1807) by HRFABMS, showing 13 degrees of unsaturation. The ^{13}C NMR and HSQC spectra of **4** displayed 12 quaternary carbons, five aromatic methines, one olefinic methine, one methine, three methylenes, and three methyl groups. In the ^{1}H NMR spectrum of **4**, three proton signals at δ_H 7.37 (1H, d, $J = 8.4$ Hz), 7.36 (1H, brs), and 6.89 (1H, d, $J = 8.4$ Hz) were attributed to the protons of an ABX-type B ring. Two methine signals at δ_H 6.53 (1H, s) and 6.47 (1H, s), as well as the HMBC correlations between δ_H 6.53 and δ_C 105.0 (C-4a), 123.8 (C-1′), 166.0 (C-2), and 183.9 (C-4), and δ_H 6.47 and δ_C 105.0 (C-4a), 114.5 (C-6), 157.3 (C-8a), and 160.4 (C-5), indicated the methines to be located at the C-3 and C-8 positions. The signal at δ_H 5.24 (1H, s) for a methine group and three signals at δ_H 1.48 (3H, s), δ_H 0.97 (3H, s), and δ_H 0.88 (3H, s) for methyl groups indicated that **4** has the same cyclized geranyl group as that of **1**. The linkage site of the cyclized geranyl group was assigned at C-6 by the proton signals at δ_H 2.82 and 2.61 (H_2-9), which correlated with δ_C 33.8 (C-15), 48.8 (C-10), 114.5 (C-6), 139.0 (C-11), 160.4 (C-5), and 164.1 (C-7) in the HMBC spectrum. The proton signal at δ_H 1.74 (H-14a) showed clear NOESY correlations with signals at δ_H 2.82 and 2.61 (H_2-9) and 0.97 (H_3-17) and indicated the 1,3-diaxial orientations of C-9 and H-14a (Figure 1). The CD spectra of **4** showed similar characteristics to that of ugonin K (Table S2, Supporting Information), suggesting that **4** has the *S*-configuration at the C-10 position. Thus, compound **4** (ugonin P) was determined as 5,7,3′,4′-tetrahydroxy-6-(2,6,6-trimethyl-2-cyclohexenylmethyl)flavanone.

Compounds **5** and **6** were each isolated as a yellow powder. The HRFABMS of **5** showed a $[M + H]^+$ ion at *m*/*z* 439.1768 (calcd for $C_{25}H_{27}O_7$, 439.1757; $C_{25}H_{26}O_7$), from which 13 degrees of unsaturation were deduced. Altogether, 12 quaternary carbons, five aromatic methines, two methines, four methylenes, and two methyl groups were evident from the ^{13}C NMR and DEPT spectra of **5**. The ^{1}H NMR signal at δ_H 13.45 for a hydroxy group, signals at δ_H 6.65 (s) and 6.48 (s), and the ABX system signals at δ_H 7.41 (dd, $J = 8.0$, 2.0 Hz), 7.39 (brs), and 6.90 (d, $J = 8.0$ Hz) indicated that **5** has the same flavone moiety as that of **4**. In contrast to the ^{1}H NMR chemical shifts of **4**, however, two signals at δ_H 5.05 (s, 1H) and 5.01 (s, 1H) and a signal at δ_H 3.70 of **5** suggested that a terminal methylene group and an oxygen-bearing methine group are present in the cyclized geranyl part of the molecule. The HMBC correlations between the methylene signals at δ_H 2.85 and 2.69 (H_2-9) and ^{13}C NMR resonances at δ_C 49.2 (C-10), 111.8 (C-6), 159.3 (C-5), and 162.7 (C-7) indicated the cyclized geranyl group to be linked to the C-6 position. In addition, the HMBC correlation between δ_H 5.01 and δ_C 72.5 suggested that the hydroxy group

occurs at C-12. The proton signal at δ_H 2.58 (H-10) showed a clear NOESY correlation to that at δ_H 1.41 (H_2-14), indicating 1,3-diaxial orientations of these protons, but not to that at δ_H 3.70, thus showing that the hydroxy group at C-12 is in the axial orientation (Figure 1). Thus, the structure of **5** (ugonin Q) was assigned as 5,7,3′,4′-tetrahydroxy-6-(6,6-dimethyl-3-hydroxyl-2-methylenecyclohexylmethyl)flavone.

The HRFABMS of **6** showed a $[M + H]^+$ ion at *m/z* 441.1940 (calcd for $C_{25}H_{29}O_7$, 441.1936), consistent with the molecular formula, $C_{25}H_{28}O_7$, representing 12 degrees of unsaturation. Altogether, 12 quaternary carbons, five aromatic methines, one methine, four methylenes, and three methyl groups were present in the ^{13}C NMR and DEPT spectra of **6**. The proton hydrogen-bond signal and aromatic methine signals, as well as the ABX system signals, indicated **6** to have the same flavone moiety as **4** and **5** (Table 1). However, the quaternary carbon at δ_C 73.6 (C-11) substituted by a hydroxy group in **6** makes the cyclized geranyl group different from those of compounds **4** and **5**. The assignments and location of the cyclized geranyl group at C-6 were also confirmed by the HMBC correlations (Figure S1, Supporting Information). In the NOESY experiment, correlations between δ_H 0.95 (H_3-17) and 1.22 (H_3-18) and δ_H 1.58 (H-10) and 0.81 (H_3-16) indicated 1,3-diaxial orientations of the C-17 and C-18 methyl groups, and H-10 and the hydroxy group at C-11 were assigned in equatorial orientations (Figure 1). Thus, the structure of **6** (ugonin R) was determined as 5,7,3′,4′-tetrahydroxy-6-(2″,6″,6″-trimethyl-2-hydroxylcyclohexylmethyl)flavone.

Compound **7**, also a yellow amorphous powder, showed in the HRFABMS a $[M + H]^+$ ion at *m/z* 423.1815 (calcd for $C_{25}H_{27}O_6$, 423.1807), consistent with the molecular formula, $C_{25}H_{26}O_6$ (13 degrees of unsaturation). The ^{13}C NMR and HSQC spectra of **7** showed 12 quaternary carbons, five aromatic methines, one methine, four methylenes, and three methyl groups. Comparison with the ^1H NMR data of **6** and **7** indicated that both these compounds have a similar structure, but there is no intramolecular hydrogen bond in **7**. The location of the cyclized geranyl group at C-6 in **7** was assigned on the basis of HMBC correlations (Figure S1, Supporting Information). The cyclized geranyl group of **7** was further cyclized from C-11 to C-5 by an ether linkage, as confirmed by comparison with those of ugonin L (**11**).[8] A NOESY correlation between δ_H 1.64 (H-10) and δ_H 1.26 (H_3-18) indicated a *cis*-configuration between H-10 and H_3-18 (Figure 1). Thus, the structure of **7** (ugonin S) was assigned as 4″a,5″,6″,7″,8″,8″a-hexahydro-7,3′,4′,-trihydroxy-5″,5″,8″a-trimethyl-4*H*-chromeno[2″,3″:5,6]flavone.

Compound **8**, a yellow amorphous powder, exhibited in the HRFABMS a $[M + H]^+$ ion at *m/z* 355.1177 (calcd for $C_{20}H_{19}O_6$, 355.1182; $C_{20}H_{18}O_6$; 12 degrees of unsaturation). Analysis of the ^{13}C NMR spectra showed 11 quaternary carbons, five aromatic methines, one methine, and three methyl groups. In the ^1H NMR spectrum of **8**, the ABX system signals at δ_H 7.39 (1H, d, $J = 1.8$ Hz), 7.36 (1H, dd, $J = 8.3$, 1.8 Hz), and 6.92 (1H, d, $J = 8.3$ Hz) were attributed to ring B. Resonances at δ_H 4.56 (1H, q, $J = 6.5$ Hz), 1.62 (3H, s), 1.42 (1H, d, $J = 6.5$ Hz), and 1.34 (3H, s) indicated the presence of a 2,3-dihydro-2,3,3-trimethylfuran ring.[10] In the HMBC spectrum, the signal at δ_H 6.55 showed correlations with resonances at δ_C 106.2 (C-4a), 123.9 (C-1′), 166.2 (C-2), and 184.1 (C-4), and the signal at δ_H 6.18 showed correlations with resonances at δ_C 106.2 (C-4a), 114.7 (C-6), 163.9 (C-8a), and 166.8 (C-7), suggesting that the methines should be located at the C-3 and C-8 positions (Figure S1, Supporting Information). The 2,3-dihydro-2,3,3-trimethylfuran ring was located at C-6 since the proton signals at δ_H 1.62 (H-11) and 1.34 (H-12) correlated with δ_C 114.7 (C-6). Thus, compound **8** was determined to be 4″,5″-dihydro-5,3′,4′-trihydroxy-4″,4″,5″-trimethylfurano[2″,3″:6,7]flavones and named ugonin T.

The spectroscopic (UV, IR, ^1H NMR, ^{13}C NMR, and MS) data of the known isoprenylated or general flavonoids were compared with published values of ugonins J (**9**),[8] K (**10**),[8] L (**11**),[8] 5,4′-dihydroxy-4″,4″-dimethyl-5″-methyl-5″*H*-dihydrofurano[2″,3″:6,7]flavanone,[12] and quercetin[13] to identify these compounds.

Table 3. Inhibitory Effects of Compounds **1**−**11** on Superoxide Anion Generation and Elastase Release by Human Neutrophils in Response to FMLP/CB

compound[c]	superoxide anion IC_{50} (μM)[a]	elastase IC_{50} (μM)[a]
1	4.1 ± 1.18	1.6 ± 0.33
3	3.9 ± 1.07	3.4 ± 0.50
5	2.8 ± 1.28	0.49 ± 0.27
6	>10	4.56 ± 0.32
7	2.2 ± 0.07	1.9 ± 0.52
8	0.25 ± 0.01	1.2 ± 0.13
10	2.0 ± 0.04	>10
11	5.8 ± 1.41	3.8 ± 0.08
DPI[b]	1.0 ± 0.35	
PMSF[b]		95 ± 25

[a] Concentration necessary for 50% inhibition (IC_{50}). Results are presented as mean ± SEM ($n = 3$). [b] Diphenyleneiodonium (DPI, a NADPH oxidase inhibitor) and phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitor) were used as the positive controls in the generation of superoxide anion and release of elastase, respectively. [c] Compounds **2**, **4**, and **9** were inactive ($IC_{50} > 10$ μM) in both test systems.

Compounds **1**−**11** were evaluated for their inhibitory effects on superoxide anion generation and elastase release by human neutrophils in response to FMLP/CB (Table 3). With the exception of compounds **2**, **4**, **6**, and **9**, these isolated flavonoids showed bioactivity against FMLP/CB-induced superoxide anion generation by human neutrophils. Compound **8** was the most active inhibitor of FMLP/CB-induced superoxide anion generation (IC_{50} value, 0.25 μM). The bioactivity of these uncyclized prenylated flavonoids against FMLP/CB-induced superoxide anion generation by human neutrophils, however, is too random to apply any formal structure−activity relationship (SAR) analysis.

In the FMLP/CB-induced elastase release assay (Table 3), compound **5** was the most effective inhibitor (IC_{50} value, 0.49 μM). The 3D-superimposed modeling of the chromanone core showed that the cyclization between rings B and C is important for the potency of compounds **1**−**3** in inhibiting elastase release (Figure S2, Supporting Information). Moreover, compounds **7** and **11** were more potent than compounds **4** and **9**, revealing that the ether-linked cyclization between the geranyl group and ring A is important for their activity (Figure S3, Supporting Information). Finally, compounds with a hydroxy group substituted at the geranyl group (**5** and **6**) were more potent than those without a hydoxy group (**4**, **9**, and **10**) (Figure S4, Supporting Information).

Treatment of human neutrophils with all compounds except for compounds **2** and **9**, up to a test concentration of 10 μM, did not cause any marked increase in LDH release, an enzyme considered a marker for cell toxicity (Figure S5, Supporting Information).[17,18] In addition, these isolates were also evaluated for their cytotoxicity toward two human cancer cell lines, HepG2 and MCF-7. The results showed that none of these isolates were cytotoxic toward these human cancer cell lines ($IC_{50} > 10$ μM).

Experimental Section

General Experimental Procedures. Melting points were measured on a Yanaco MP-500D melting point apparatus and are uncorrected. Optical rotations were measured on a JASCO P-1020 polarimeter. UV spectra were recorded on a Hitachi U-2800 UV−vis spectrophotometer. IR spectra were taken on a Shimadzu IR Prestige-21 FT-IR spectrometer. CD spectra were obtained on a JASCO J-715 spectropolarimeter. 1D and 2D NMR spectra were recorded with Bruker 400 AV, Bruker 500 AVII, and Varian Unity 600 NMR spectrometers. HRFABMS were measured with a Finnigan/Thermo Quest MAT 95XL spectrometer, and ESIMS/MS were obtained on a Bruker HCT ultra PTM Discovery system. Sephadex LH-20 (Amersham Biosciences) was used for column

chromatography; precoated Si gel plates (Merck, silica gel 60 F_{254}) were used for analytical TLC. HPLC was performed on a Hitachi L-2310 apparatus equipped with a Hitachi L-2420 UV−vis detector. Discovery C_{18} 5 μm (250 × 4.6 mm i.d.) and semipreparative C_{18} 5 μm (250 × 10 mm i.d.) columns were used for analytical and preparative purposes, respectively. Molecular models are built using Discovery Studio v.2.0 (Accelrys Inc., San Diego, CA). The performance of energy minimization on a molecule was done with the CHARm force field and a smart minimizer algorithm.

Plant Material. The dried rhizomes of *H. zeylanica* (10 kg) were purchased from Taichung City, Taiwan, in October 2000. The plant was identified by Dr. Hsien-Cheh Chang, School of Chinese Medicine, China Medical University, Taichung, Taiwan. A voucher specimen (NP-HZ-200010) was deposited in the Graduate Institute of Pharmaceutical Chemistry, Taichung, Taiwan.

Extraction and Isolation. The rhizomes of *H. zeylanica* (10 kg) were pulverized and extracted with methanol (15 × 10 L). After removing the solvent, the MeOH extract (416 g) was partitioned successively with *n*-hexane (10 × 1 L), $CHCl_3$ (10 × 1 L), and EtOAc (10 × 1 L).

The EtOAc extract (15.0 g) was applied to a Sephadex LH-20 column eluted with MeOH to yield three fractions (Et1−Et3). Fraction Et1 (1.2 g) was also applied to a Sephadex LH-20 column eluted with MeOH to yield four fractions (Et11−Et14). Fraction Et1-1 (95.2 mg) was separated by reversed-phase HPLC (MeOH−H_2O (0.05% TFA)−MeCN, 70:20:10; flow rate, 2 mL/min; UV detector, 300 nm) to obtain ugonin T (**8**, 3.4 mg, t_R 15.29 min), ugonin Q (**5**, 3.0 mg, t_R 19.19 min), and ugonin R (**6**, 2.5 mg, t_R 21.23 min). The $CHCl_3$-soluble layer (Et2C, 580 mg) of fraction Et2 (7.3 g) was applied to a Sephadex LH-20 column, eluted with MeOH, to yield nine fractions (Et2C1−Et2C9). Fraction Et2C4 (64.5 mg) was separated by reversed-phase HPLC (MeOH−H_2O (0.05% TFA)−MeCN, 70:20:10; flow rate, 2 mL/min; UV detector, 300 nm) to obtain 5,4′-dihydroxy-4″,4″-dimethyl-5″-methyl-5″*H*-dihydrofurano[2″,3″:6,7]flavanone (6.4 mg, t_R 13.42 min) and ugonin L (**11**, 9.9 mg, t_R 18.51 min). Fraction Et2C6 (156.3 mg) was purified by reversed-phase HPLC (MeOH−H_2O (0.05% TFA)−MeCN, 70:20:10; flow rate, 2 mL/min; UV detector, 300 nm) to obtain ugonin O (**3**, 4.4 mg, t_R 18.16 min). A portion (62.2 mg) of fraction Et2C7 (91.2 mg) was separated by reversed-phase HPLC (MeOH−H_2O (0.05% TFA)−MeCN, 75:15:10; flow rate, 2 mL/min; UV detector, 300 nm) to obtain ugonin M (**1**, 2.3 mg, t_R 12.48 min), ugonin J (**9**, 19.9 mg, t_R 18.20 min), ugonin P (**4**, 4.2 mg, t_R 20.68 min), and ugonin K (**10**, 5.1 mg, t_R 37.36 min). The $CHCl_3$-insoluble residue of fraction Et2 (Et2R) (3.17 g) was also applied to a Sephadex LH-20 column eluted with MeOH to yield 11 fractions (Et2R1−Et2R11). Fraction Et2R5 (151.1 mg) was purified by reversed-phase HPLC (MeOH−H_2O (0.05% TFA)−MeCN, 70:20:10; flow rate, 2 mL/min; UV detector, 300 nm) to obtain ugonin S (**7**, 9.5 mg, t_R 12.73 min). An aliquot (155.2 mg) of fraction Et2R8 (313.0 mg) was purified by reversed-phase HPLC (MeOH−H_2O (0.05% TFA)−MeCN, 70:20:10; flow rate, 2 mL/min; UV detector, 300 nm) to obtain ugonin N (**2**, 4.5 mg, t_R 18.08 min). Fraction Et2R11 (48.1 mg) was purified by reversed-phase HPLC (MeOH−H_2O (0.05% TFA)−MeCN, 60:30:10; flow rate, 2 mL/min; UV detector, 300 nm) to obtain quercetin (2.1 mg, t_R 8.60 min).

Ugonin M (1): yellow powder; mp 248−250 °C; $[\alpha]^{25}_D$ +79.6 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 363 (2.57), 262 (2.47) nm; +$AlCl_3$ 418, 271 nm; +$AlCl_3$/HCl 401, 296, 271 nm; IR (KBr) ν_{max} 3385, 2949, 2924, 2862, 1637, 1031, 1018 cm^{-1}; CD (*c* 1.15 × 10^{-4} M, MeOH) $\Delta\varepsilon$ (nm) −37.23 (206), 39.79 (212), −5.23 (265), 6.16 (284), −15.55 (311), −4.06 (368); ^{1}H NMR (CD_3OD, 600 MHz), see Table 1; ^{13}C NMR (CD_3OD, 150 MHz), see Table 2; (+)-ESIMS *m/z* 437.19 $[M + H]^+$ (75), 301.17 (100); (−)-ESIMS *m/z* 549.17 $[M + TFA - H]^-$ (36.8), 435.23 $[M - H]^-$ (100); (−)-ESIMS/MS (435.23) *m/z* 312.0; HRFABMS *m/z* 437.1604 $[M + H]^+$ (calcd for $C_{25}H_{25}O_7$, 437.4618).

Ugonin N (2): yellow powder; mp 222−224 °C; $[\alpha]^{25}_D$ +14.9 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ϵ) 347 (2.48), 257 (2.67) nm; +$AlCl_3$ 424, 271 nm; +$AlCl_3$/HCl 389, 345, 269 nm; IR (KBr) ν_{max} 3448, 3350, 2937, 2872, 1653, 1610, 1558, 1497, 1360, 1300, 1174 cm^{-1}; CD (*c* 2.28 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) −42.53 (203), 126.81 (213), −56.74 (254), 27.83 (281), −16.21 (307), −21.62 (349); ^{1}H NMR (CD_3OD, 400 MHz), see Table 1; ^{13}C NMR (CD_3OD, 100 MHz), see Table 2; (+)-ESIMS *m/z* 439.22 $[M + H]^+$ (71), 301.11 (100); (−)-ESIMS *m/z* 551.17 $[M + TFA - H]^-$ (100), 437.20 $[M - H]^-$ (53.3); HRFABMS *m/z* 439.1750 $[M + H]^+$ (calcd for $C_{25}H_{27}O_7$, 439.1757).

Ugonin O (3): yellow powder; mp 234−236 °C; $[\alpha]^{25}_D$ −19.8 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 363 (2.30), 264 (2.20) nm; +$AlCl_3$ 404, 298, 272 nm; +$AlCl_3$/HCl 400, 297, 273 nm; IR (KBr) ν_{max} 3455, 2954, 2854, 1635, 1541, 1456, 1012 cm^{-1}; CD (*c* 1.15 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) −12.69 (228), −4.45 (248), 1.10 (278), −10.04 (364), 1.02 (415); ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 2; (+)-ESIMS *m/z* 435.15 $[M + H]^+$ (100), 301.15 (34.4); (+)-ESIMS/MS (435.15) *m/z* 313.01; (−)-ESIMS *m/z* 547.22 $[M + TFA - H]^-$ (64.4), 433.38 $[M - H]^-$ (100); (−)-ESIMS/MS (433.38) *m/z* 311.02; HRFABMS *m/z* 435.1435 $[M + H]^+$ (calcd for $C_{25}H_{23}O_7$, 435.1444).

Ugonin P (4): yellow powder; mp 214−216 °C; $[\alpha]^{25}_D$ +55.3 (*c* 0.10, MeOH); UV (MeOH) λ_{max} (log ϵ) 343 (2.38), 274 (2.35) nm; +$AlCl_3$ 420, 275 nm; +$AlCl_3$/HCl 363, 286, 259 nm; IR (KBr) ν_{max} 3444, 3352, 2949, 2833, 1654, 1649, 1570, 1450, 1182 cm^{-1}; CD (*c* 1.42 × 10^{-4} M, MeOH) $\Delta\varepsilon$ (nm) 33.85 (216), 11.80 (238), 6.98 (272), 0.12 (290), 3.26 (343); ^{1}H NMR (CD_3OD, 600 MHz), see Table 1; ^{13}C NMR (CD_3OD, 150 MHz), see Table 2; (+)-ESIMS *m/z* 423.13 $[M + H]^+$ (17.8), 301.19 (100); (−)-ESIMS *m/z* 535.21 $[M + TFA - H]^-$ (100), 421.16 $[M - H]^-$ (79.1); (−)-ESIMS/MS (421.26) *m/z* 298.02; HRFABMS *m/z* 423.1803 $[M + H]^+$ (calcd for $C_{25}H_{27}O_6$, 423.1807).

Ugonin Q (5): yellow powder; mp 236−238 °C; $[\alpha]^{25}_D$ −38.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 351 (2.27), 260 (2.28) nm; +$AlCl_3$ 422, 275 nm; +$AlCl_3$/HCl 358, 282 nm; IR (KBr) ν_{max} 3454, 2950, 2842, 1645, 1463, 1014 cm^{-1}; CD (*c* 1.14 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) −2.85 (238), 6.14 (267), −0.56 (297), 0.52 (312), −1.47 (346), 0.43 (411); ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 2; (+)-ESIMS *m/z* 439.21 $[M + H]^+$ (100); (−)-ESIMS *m/z* 551.16 $[M + TFA - H]^-$ (100), 437.32 $[M - H]^-$ (34.1); HRFABMS *m/z* 439.1768 $[M + H]^+$ (calcd for $C_{25}H_{27}O_7$, 439.1757).

Ugonin R (6): yellow powder; mp 220−222 °C; $[\alpha]^{25}_D$ −13.9 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 350 (1.92), 270 (1.92), 259 (1.92) nm; +$AlCl_3$ 420, 276 nm; +$AlCl_3$/HCl 363, 286 nm; IR (KBr) ν_{max} 3423, 3415, 1646, 1567, 1515, 1025 cm^{-1}; CD (*c* 1.14 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 0.74 (229), 3.47 (272), 0.68 (347), 0.76 (380), 1.37 (398); ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 125 MHz), see Table 2; (+)-ESIMS *m/z* 441.20 $[M + H]^+$ (100); (−)-ESIMS *m/z* 553.23 $[M + TFA - H]^-$ (100), 439.41 $[M - H]^-$ (58); (−)-ESIMS/MS (439.41) *m/z* 298.07 (100), 285.11 (32.8); HRFABMS *m/z* 441.1932 $[M + H]^+$ (calcd for $C_{25}H_{29}O_7$, 441.1936).

Ugonin S (7): yellow powder; mp 223−225 °C; $[\alpha]^{25}_D$ +37.5 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ϵ) 338 (2.58), 270 (2.28) nm; +$AlCl_3$ 465, 363, 307, 274 nm; +$AlCl_3$/HCl 416, 343, 272 nm; IR (KBr) ν_{max} 3446, 3059, 2939, 2870, 1645, 1610, 1558, 1188 cm^{-1}; CD (*c* 7.11 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 22.26 (214), 7.38 (268), −0.63 (285), 7.69 (313), −3.93 (352); ^{1}H NMR (Me_2O-d_6, 400 MHz), see Table 1; ^{13}C NMR (Me_2O-d_6, 100 MHz), see Table 2; (+)-ESIMS *m/z* 423.21 $[M + H]^+$ (100), 301.17 (12.9); (−)-ESIMS *m/z* 535.20 $[M + TFA - H]^-$ (100), 421.22 $[M - H]^-$ (35.8); (−)-ESIMS/MS (421.22) *m/z* 297.0; HRFABMS *m/z* 423.1815 $[M + H]^+$ (calcd for $C_{25}H_{27}O_6$, 423.1807).

Ugonin T (8): yellow powder; mp 202−204 °C; $[\alpha]^{25}_D$ −28.0 (*c* 0.05, MeOH); UV (MeOH) λ_{max} (log ϵ) 357 (2.48), 262 (2.55) nm; +$AlCl_3$ 429, 277 nm; +$AlCl_3$/HCl 395, 383, 280 nm; IR (KBr) ν_{max} 3423, 3415, 1646, 1567, 1515, 1025 cm^{-1}; CD (*c* 1.41 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) 0.74 (229), 3.47 (272), 0.68 (347), 0.76 (380), 1.37 (398); ^{1}H NMR (CD_3OD, 500 MHz), see Table 1; ^{13}C NMR (CD_3OD, 125 MHz), see Table 2; (+)-ESIMS *m/z* 355.14 $[M + H]^+$ (100); (−)-ESIMS *m/z* 467.16 $[M + TFA - H]^-$ (100), 353.26 $[M - H]^-$ (71.1); (−)-ESIMS/MS (433.38) *m/z* 311.02; HRFABMS *m/z* 355.1177 $[M + H]^+$ (calcd for $C_{20}H_{19}O_6$, 355.1182).

Ugonins J (**9**),[8] K (**10**),[8] and L (**11**),[8] 5,4′-dihydroxy-4″,4″-dimethyl-5″-methyl-5″*H*-dihydrofurano[2″,3″:6,7]flavanone,[12] and quercetin[13,14] were obtained as light yellow powders. Their MS and ^{1}H and ^{13}C NMR data were in accordance with published values.

Measurement of Superoxide Anion Generation.[19,20] Human neutrophils from the venous blood of healthy, adult volunteers (18−32 years old) were isolated with a standard method of dextran sedimentation prior to centrifugation in a Ficoll Hypaque gradient and hypotonic lysis of erythrocytes. Neutrophil superoxide anion generation was determined using superoxide dismutase (SOD)-inhibitory cytochrome

c reduction according to described procedures. Diphenyleneiodonium (DPI, a NADPH oxidase inhibitor) was used as the positive control in the generation of superoxide anion.

Measurement of Elastase Release.[19,20] Degranulation of azurophilic granules was determined by elastase release as described previously. Measurement of elastase release was carried out according to such described procedures. The results are expressed as the percentage of elastase release in the FMLP/CB-activated, drug-free control system. Phenylmethylsulfonyl fluoride (PMSF, a serine protease inhibitior) was used as the positive control in the release of elastase.

Lactate Dehydrogenase (LDH) Release.[17,20] LDH release was determined by a commercially available method (Promega, Madison, WI). Human neutrophils (6×10^5/mL) were equilibrated at 37 °C for 2 min and incubated with either control or test compounds for 15 min. Cytotoxicity was represented by LDH release in a cell-free medium as a percentage of the total LDH released. The total LDH released was determined by lysing cells with 0.1% Triton X-100 for 30 min at 37 °C.

Cytotoxicity.[21] Three-day bioassays against HepG2 and MCF-7 cell lines were carried out according to described procedures. Doxorubicin was used as the positive controls in the MTT assay (IC_{50} values, 1.6 and 2.8 μM, respectively).

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Supporting Information Available: UV and selective HMBC spectra of the eight new compounds; the 3D-superimposed modeling of compounds **1**−**5** and **7**; and the effect of compounds **1**−**11** on LDH release assay are available free of charge via the Internet at http://pubs.acs.org.

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