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

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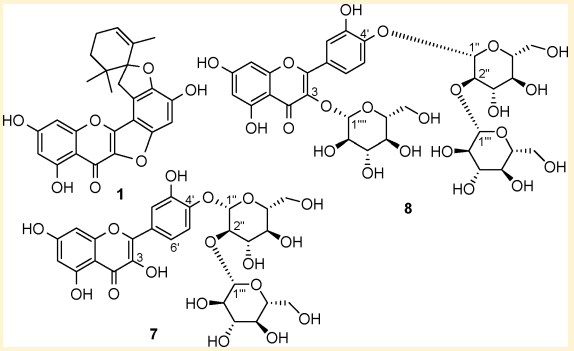
Supporting Information

ABSTRACT: Chemical investigation of the rhizomes of *Helminthostachys zeylanica* led to the isolation of eight new flavonoids including six cyclized geranylflavonoids, ugonins V−X (**1**−**3**), (10*R*,11*S*)-ugonin N (**4**), (10*R*,11*S*)-ugonin S (**5**), and ugonin Y (**6**), as well as two quercetin glucosides, quercetin-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**7**) and quercetin-3-*O*-*β*-D-glucopyranosyl-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**8**). The structures of these compounds were established by spectroscopic analyses and acid hydrolysis of the sugar moiety. Among the isolated compounds, **1**, **2**, **5**, **6**, ugonins J−S (**9**−**13**), ugonstilbene A (**14**), and ugonin L (**23**) were evaluated for their anti-inflammatory activity on lipopolysaccharide-induced nitric oxide (NO) production in microglial cells. Except for **1**, **5**, and **13**, all other compounds inhibited NO production with IC_{50} values of 6.2−10.1 μM and were more potent than the positive control, pyrrolidine dithiocarbamate. Compounds **1**, **2**, **5**, **6**, and **10**−**13** were tested for antiosteoporotic activities, and ugonin K (**10**) exhibited the highest inhibitory activity against RANKL-induced osteoclast differentiation in RAW264.7 cells with an IC_{50} value of 1.8 ± 0.2 μM.



The rhizomes of *Helminthostachys zeylanica* (L.) Hook. (Ophioglossaceae), a folk herbal medicine named "Daodi-Ugon" in Taiwan, have been used as an antipyretic and antiphlogistic agent or to treat bone fracture and contusion.[1] The previous studies revealed that this medicinal material is rich in prenylated flavonoids such as ugonins A−U.[2−7] Most of these compounds showed antioxidative[4] or anti-inflammatory activities.[5,6] Ugonins J and K also showed extracellular melanogenesis inhibitory activity,[8] and ugonin U displayed an immunomodulatory effect in human neutrophils via stimulation of phospholipase C.[7] Ugonstilbenes A and B[9] and ugonins G, E, J, K, and L have been tested for their antiosteoporotic activity. Ugonin K (0.5−10 μM) concentration-dependently increased alkaline phosphatase (ALP) activity of MC3T3-E1 cells without detectable cytotoxicity. Ugonin K also promotes osteoblastic differentiation and mineralization by activation of p38 MAPK- and ERK-mediated expression of Runx2 and osterix.[10] Moreover, ugonin K-stimulated osteogenesis involves estrogen-receptor-dependent activation of both the nonclassical Src and classical signaling pathways.[11] In this work, the chemical constituents of an *H. zeylanica* extract were investigated, and eight new flavonoids were isolated including six cyclized geranyl analogues, ugonins V−X (**1**−**3**), (10*R*,11*S*)-ugonin N (**4**), (10*R*,11*S*)-ugonin S (**5**), and ugonin Y (**6**), as well as two quercetin glucosides, quercetin-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**7**) and quercetin-3-*O*-*β*-D-glucopyranosyl-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**8**). In addition, the 14 known compounds ugonins J (**9**), K (**10**), M (**11**), O (**12**), S (**13**),[4,5] ugonstilbene A (**14**),[9] quercetin (**15**), quercetin-3-*O*-*β*-D-glucopyranoside (**16**), quercetin-4′-*O*-*β*-D-glucopyranoside (**17**), quercetin-3,4′-di-*O*-*β*-D-glucopyranoside (**18**), quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside (**19**),[12] quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-4′-*O*-*β*-D-glucopyranoside (**20**),[13] quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**21**),[13] and benzoic acid (**22**). The structures of the isolated compounds were elucidated on the basis of spectroscopic data, and the sugar portion was identified by high performance anion-exchange chromatography after acid hydrolysis. The absolute configurations of compounds **2**−**6** and ugonin L (**23**)[4]

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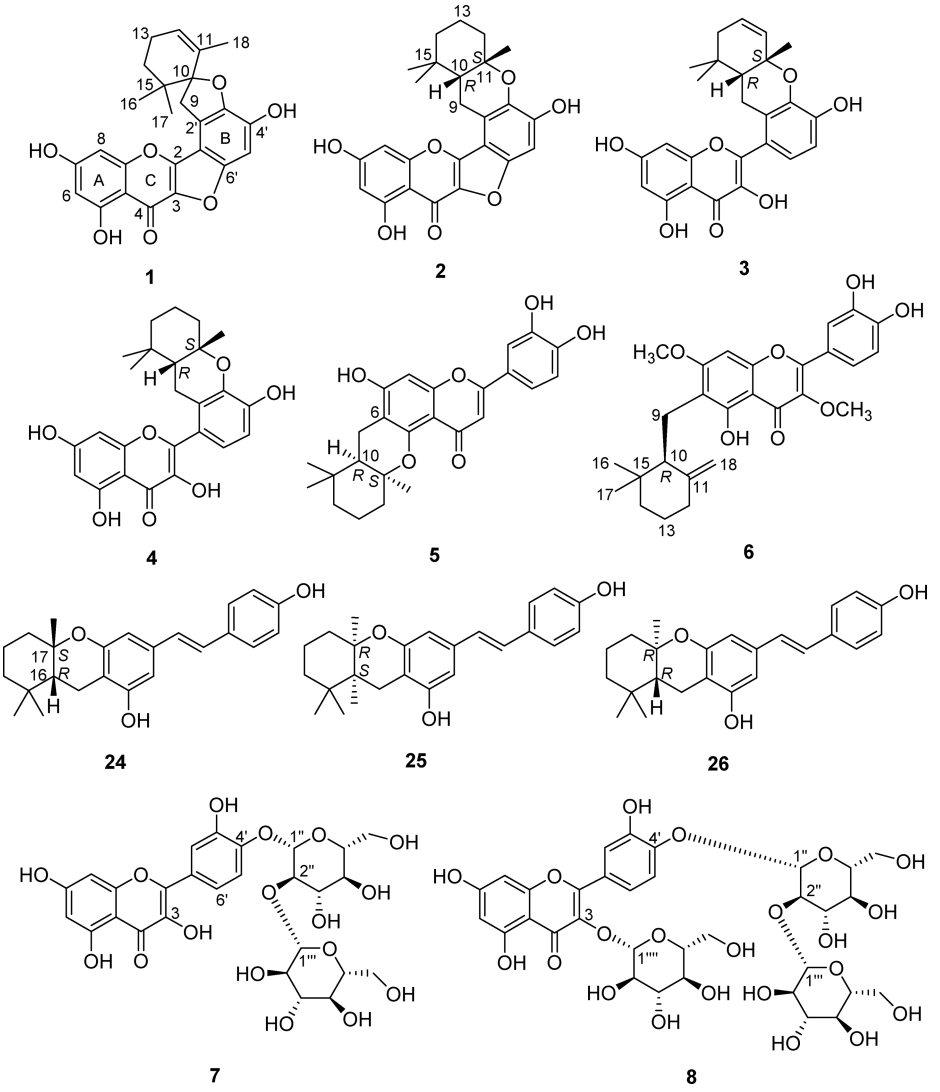
Chart 1



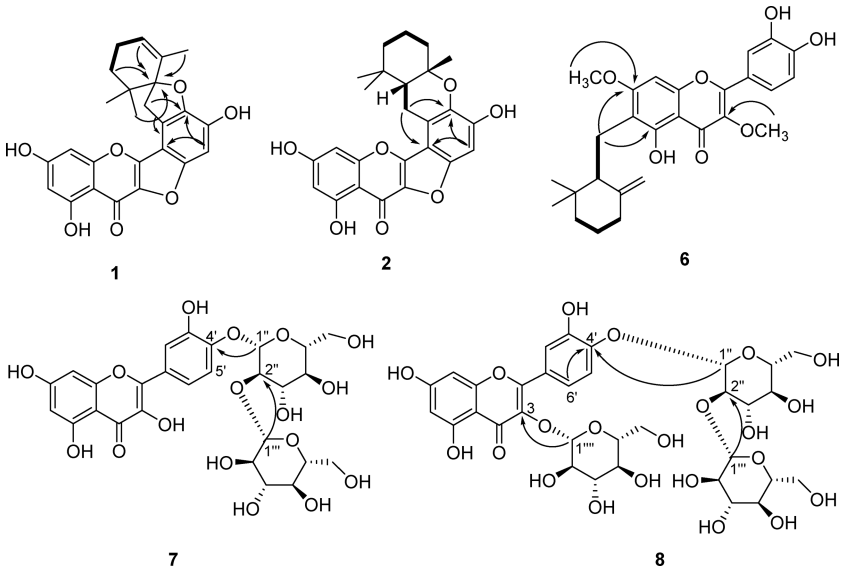


Figure 1. COSY (bold lines) and key HMBC (arrows) correlations of **1**, **2**, and **6**–**8**.

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were determined by comparison of their NOE, specific rotation, and electronic circular dichroism (ECD) data with those of synthesized compounds.[14,15] Compounds **1**, **2**, **5**, **6**, **9**−**14**, and **23**[4] were evaluated for their anti-inflammatory activity on lipopolysaccharide (LPS)-induced nitric oxide (NO) production in microglial cells. In addition, compounds **1**, **2**, **5**, **6**, and **10**−**13** were also tested for their antiosteoporotic activities.

RESULTS AND DISCUSSION

An ethanol extract of *H. zeylanica* rhizomes was repeatedly chromatographed to yield eight new flavonoids: ugonins V−X (**1**−**3**), (10*R*,11*S*)-ugonin N (**4**), (10*R*,11*S*)-ugonin S (**5**), ugonin Y (**6**), and two quercetin glucosides, quercetin-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**7**) and quercetin-3-*O*-*β*-D-glucopyranosyl-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**8**).

Compounds **1**−**8** were obtained as yellow powders. The spectroscopic data for **1**−**6** revealed that they possessed the characteristics of cyclized geranylflavonoids similar to those isolated from the same source.[2−7] Using HRESIMS (*m*/*z* 434.1374) and ^{13}C NMR data, the molecular formula of **1** was determined to be $C_{25}H_{22}O_7$. In the ^{1}H NMR spectrum of **1**, signals corresponding to the following were observed: a cyclized geranyl group comprising three methyl singlets at δ_H 0.89, 1.03 (H-16, 17), and 1.60 (H-18), two pairs of aliphatic methylene protons at δ_H 2.02 (2H, m, H-13) and 1.56 (2H, m, H-14), a benzylic methylene at δ_H 3.36 (1H, d, J = 17.4 Hz, H-9) and 3.69 (1H, d, J = 17.4 Hz, H-9), and an olefinic methine at δ_H 5.44 (1H, br s, H-12).[4] In addition, three aromatic proton signals resonated at δ_H 6.23 (1H, d, J = 1.8 Hz, H-6), 6.51 (1H, d, J = 1.8 Hz, H-8), and 6.96 (1H, s, H-5′), and a hydrogen-bonded hydroxy group at δ_H 12.77 (5-OH). H-6 and H-8 were assigned on the basis of HMBC cross-peaks between 5-OH and C-5 (δ_C 162.3), C-4a (δ_C 103.9), and C-6 (δ_C 99.1), the latter also correlating to H-8 (Figure 1). Further, H-5′ was assigned on the basis of its correlations with quaternary C-1′ (δ_C 105.0) and three oxygenated carbons at δ_C 147.1, 149.4, and 151.4, which indicated the presence of a pentasubstituted B-ring. Both C-1′ and the carbon at δ_C 147.1 showed correlations with H_2-9, suggesting the cyclized geranyl group to be located at C-2′ (δ_C 117.5) and HO-3′. The carbon resonating at δ_C 147.1 was assigned to C-3′. In the ^{13}C NMR spectrum, an aliphatic oxygenated tertiary carbon at δ_C 94.1 was assigned to C-10 on the basis of the HMBC cross-peaks of H-12, H_2-14, H_3-16, H_3-17, and H_3-18 with this carbon. Because C-10, C-3′, and C-3 (δ_C 133.9) were assigned referring to C-3 (δ_C 135.0) of broussofluorenone A,[16] C-6′ (δ_C 149.4 or 151.4) was oxygenated, and **1** had only 22 hydrogens, the ether linkages were deduced to be between C-10 and HO-3′ to form a dihydrofuran ring and between HO-3 and C-6′ to form a furan ring. Accordingly, the structure of **1**, ugonin V, was defined as 5,7,4′-trihydroxyspiro[2,6,6-trimethylcyclohex-2-ene-1,2-furo-[5,4:4,5]benzofuro[3,2-*b*]-4*H*-chromen]-4-one. The C-10 absolute configuration of this compound has not been determined yet.

Compound **2**, $C_{25}H_{24}O_7$ ([M]$^+$ 436.1534), differed from ugonin O[5] in the cyclized geranyl group. The DEPT spectra of **2** displayed two aliphatic methylene carbons (δ_C 39.5, C-12; δ_C 18.6, C-13) instead of two olefinic methine carbons (δ_C 127.9, C-12; δ_C 130.6, C-13) in ugonin O. These two pairs of methylene proton signals resonated at δ_H 2.13 (1H, m, H-12b), 1.62 (1H, td, J = 3.5, 13.8 Hz, H-12a) and δ_H 1.88 (1H, m, H-13b), 1.45 (2H, m, H-13a, overlapped with H-14b) in the ^{1}H NMR spectrum of **2**. In addition, three methyl proton signals resonated at δ_H 0.67, 1.04 (H_3-16, 17) and 1.40 (H_3-18). As in the case of **1**, the cyclized geranyl group linked at C-2′ was assigned on the basis of the presence of HMBC cross-peaks (Figure 1) of H_2-9 (δ_H 3.40) and H-5′ (δ_H 6.94) with C-1′ (δ_C 108.3) and C-3′ (δ_C 141.0). An oxygenated aliphatic carbon signal (δ_C 77.8) corresponding to C-11 was observed, suggesting the presence of an ether linkage between C-11 and HO-3′ to form a dihydropyran ring. By comparing the methyl proton chemical shifts (H_3-16 and H_3-17) of the cyclized geranyl group in synthesized racemic *trans*-ugonin L (δ_H 0.95, 1.02), racemic *cis*-ugonin L (δ_H 0.62, 0.96), and racemic *cis*-ugonin S (δ_H 0.66, 0.97),[17] the *cis*-fused geranyl group showed one high-field methyl signal near δ_H 0.65. For **2**, the presence of the signal at δ_H 0.67 was in accordance with this characteristic, and the 10,11-*cis*-configuration was deduced for **2**, which was also supported by the NOE effect between H-10 and H_3-18. The (10*R*,11*S*)-absolute configuration of compound **2** was established by comparing its negative specific rotation ($[\alpha]^{25}_D$ −134) with that of synthesized (16*R*,17*S*)-ugonstilbene B (**24**, $[\alpha]^{25}_D$ −27).[14] Furthermore, both ECD spectra of **2** [+1.38 (210), +0.40 (221), +1.06 (227)] and **24** [−0.81 (210), −8.20 (221), −6.37 (227)] displayed negative Cotton effects (CEs) between 210 and 227 nm. In contrast, (16*S*,17*R*)-ugonstilbene B (**25**, $[\alpha]^{25}_D$ +36)[14] displayed positive CEs [+4.13 (210), +7.45 (221), +5.46 (227)]. Accordingly, the structure of **2**, ugonin W, was defined as (10*R*,11*S*)-5,7,4′-trihydroxy-(5,5,8a-trimethyl-5,6,7,8,8a,9a-hexahydro)xanthen-[4,3:4,5]furo[3,2-*b*]-4*H*-chromen-4-one.

The spectroscopic characteristics of **3** ($C_{25}H_{24}O_7$, $[\alpha]^{25}_D$ −162) closely resembled those of ugonin I,[4] except for the absence of a methoxy group at C-3. In the ^{1}H NMR spectrum of **3**, three methyl proton signals of the cyclized geranyl group resonated at δ_H 0.79, 0.98 (H_3-16, 17), and 1.35 (H_3-18). A pair of *o*-coupled doublets at δ_H 6.77 (H-5′, J = 8.4 Hz) and 7.02 (H-6′, J = 8.4 Hz) corresponding to the protons of ring B were also observed. The presence of a cyclized geranyl group linked to C-2′ and HO-3′ was deduced from HMBC cross-peaks (Figure 1) between H_2-9 (δ_H 3.00, 3.12) and C-1′ (δ_C 121.7) and C-3′ (δ_C 142.1). The physical data of **4** ($C_{25}H_{26}O_7$, $[\alpha]^{25}_D$ −12) and **5** ($C_{25}H_{26}O_6$, $[\alpha]^{25}_D$ −31) closely resembled those of ugonins N and S,[5] except that the ^{1}H NMR spectra of **4** and **5** showed one shielded H_3-16 (or H_3-17) signal (**4**: δ_H 0.68; **5**: δ_H 0.66) compared to those of ugonins N (δ_H 0.91) and S (δ_H 0.97), suggesting that **4** and **5** could be stereoisomers of ugonins N and S. Similar to ugonin W (**2**), the structures of compounds **3**−**5** were deduced to have the (10*R*,11*S*)-absolute configurations on the basis of the NOE effect between H-10 and H_3-18 and negative specific rotations. Thus, the structure of **3**, ugonin X, was defined as (10*R*,11*S*)-3,5,7,4′-tetrahydroxy-5″,5″,8″a-trimethyl-4″a,5″,6″,8″a-tetrahydro-4*H*-chromen-[3″,2″:2′,3′]flavone, and compounds **4** and **5** as (10*R*,11*S*)-ugonins N and S, respectively. Ugonins N (δ_H 0.91, 1.30), S (δ_H 0.97, 1.05),[5] and L (δ_H 0.93, 1.01)[4] showed deshielded signals for H_3-16 and H_3-17, suggesting their 10,11-*trans* relative configurations.[17] Furthermore, the (10*R*,11*R*) absolute configurations of *trans*-ugonins L, N, and S were determined by comparing their positive specific rotations ($[\alpha]^{25}_D$ +82,[4] +15,[5] +71, respectively) with that of synthesized 4a-*epi*-ugonstilbene B (**26**, $[\alpha]^{25}_D$ +49).[15] Besides, (10*R*,11*R*)-ugonins S (**13**) and L (**23**) showed characteristic positive CEs between 210 and 227 nm in their ECD spectra.

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Table 1. ^{1}H NMR Spectroscopic Data (500 and 600 MHz) for Ugonins 1−6 (J in Hz)

position	1[b,c]	2[a,d]	3[b,d]	4[a,d]	5[b,d]	6[a,d]
3					6.30, s	
6	6.23, d (1.8)	6.29, d (2.0)	6.26, br s	6.26, d (2.0)		
8	6.51, d (1.8)	6.60, d (2.0)	6.40, br s	6.40, d (2.0)	6.55, s	6.67, s
9	3.36, d (17.4)	3.40 (2H), d (4.5)	3.00, d (18.0)	2.85, br d (18.0)	2.76, dd	2.75, dd
	3.69, d (17.4)		3.12, dd	3.11, dd	(8.4, 18.0)	(4.0, 13.0)
			(8.4,18.0)	(8.0, 18.0)	2.84, br d (18.0)	2.94, dd
						(12.0, 13.0)
10		1.73, t (4.5)	1.86, m	1.55, br d (8.0)	1.58, br d (7.8)	2.29, dd
						(4.0, 12.0)
12	5.44, br s	1.62, td	5.78, d (10.2)	1.55, m	1.53, td	1.96, m
		(3.5, 13.8)		2.09, m	(4.2, 13.8)	2.48, ddd
		2.13, m			2.14, m	(5.5, 13.0, 13.0)
13	2.02 (2H), m	1.45 m	5.83, ddd	1.39, m	1.42, m	1.49, m
		1.88, m	(1.8, 5.4, 10.2)	1.85, m	1.94, m	1.59, m
14	1.56 (2H), m	1.38, m	1.86, m	1.31, m	1.33, m	1.26, m
		1.45, m	2.00, br d (18.0)	1.39, m	1.42, m	1.67, ddd
						(4.0, 13.0, 13.0)
16 (17)	0.89 (3H), s	0.67 (3H), s	0.79 (3H), s	0.68 (3H), s	0.66 (3H), s	0.93 (3H), s
	1.03 (3H), s	1.04 (3H), s	0.98 (3H), s	0.89 (3H), s	0.97 (3H), s	1.06 (3H), s
18	1.60 (3H), s	1.40 (3H), s	1.35 (3H), s	1.25 (3H), s	1.22 (3H) s	4.15, d (1.5)
						4.41, d (1.5)
2′					7.38, d (2.4)	7.10, d (2.0)
5′	6.96, s	6.94, s	6.77, d (8.4)	6.76, d (8.5)	6.95, d (8.4)	6.98, d (8.5)
6′			7.02, d (8.4)	7.02, d (8.5)	7.31, dd	7.59, dd
					(2.4, 8.4)	(2.0, 8.5)
5-OH	12.77, s	12.95, s	12.23, br s	12.21, br s		13.06, s
3-OCH_3						3.84 (3H), s
7-OCH_3						3.93 (3H), s

[a]500 MHz. [b]600 MHz. [c]Measured in DMSO-d_6. [d]Measured in acetone-d_6.

The spectroscopic characteristics of **6** with the molecular formula $C_{27}H_{30}O_7$ closely resembled those of ugonin K (**10**)[4] except for an additional methoxy group in **6**. The HMBC correlation (Figure 1) between the methoxy proton signal at δ_H 3.84 and C-3 (δ_C 139.5) placed the methoxy group at C-3. The (10*R*) absolute configuration of **6** was based upon the negative CEs between 285 and 320 nm in the ECD spectrum in contrast to the carbonyl derivative of ugonin K with a 10*S* configuration (280, 300, 335 nm: $\Delta\varepsilon$ 0.00, +0.68, +0.15, respectively).[4,18] Therefore, the structure of **6**, ugonin Y, was defined as (10*R*)-5,3′,4′-trihydroxy-3,7-dimethoxy-6-(6,6-dimethyl-2-methylenecyclohexylmethyl)flavone.

Compounds **7** and **8** were obtained as yellow, amorphous powders, and their ^{1}H and ^{13}C NMR spectroscopic features showed the characteristics of quercetin glucosides. The ^{1}H NMR spectrum of **7**, with the molecular formula $C_{27}H_{30}O_{17}$, displayed a broad singlet at δ_H 12.39 attributed to 5-OH; a pair of *m*-coupled doublets (J = 1.8 Hz) at δ_H 6.18 and 6.44 assigned to H-6 and H-8, respectively; and three signals at δ_H 7.24 (1H, d, J = 9.0 Hz, H-5′), 7.62 (1H, dd, J = 2.4, 9.0 Hz, H-6′), and 7.69 (1H, d, J = 2.4 Hz, H-2′) attributed to the protons of the ABX-type B-ring. In addition, signals corresponding to two β-linked six-membered hexosyl derivatives were observed, and two anomeric proton signals resonated at δ_H 4.60 (J = 7.8 Hz, H-1‴) and 5.01 (J = 7.8 Hz, H-1″) with their HMQC-associated carbon signals observed at δ_C 99.8 (C-1″) and 104.0 (C-1‴), respectively. The remaining sugar signals were assigned through analyses of 2D COSY, TOCSY, HMQC, and HMBC spectroscopic data. The 1→2 linkages of the two hexosyl moieties were deduced on the basis of the HMBC cross-peaks between H-1‴ and C-2″ (Figure 1). Furthermore, the H-1″ signal showed an HMBC cross-peak with a carbon signal at δ_C 146.5 (2 × C, C-3′, 4′) that also correlated with H-2′ (δ_H 7.69), H-5′ (δ_H 7.24), and H-6′ (δ_H 7.62). Subsequently, on the basis of the presence of the NOE effect between H-5′ and H-1″, this sugar moiety was determined to be attached at C-4′. The acid hydrolysis of **7** revealed the presence of quercetin and a sugar moiety. The sugar was identified as D-glucose by high-performance anion-exchange chromatography[19] and the positive specific rotation ($[\alpha]^{25}_D$ +30). On the basis of the above data, the structure of **7** was determined to be quercetin-4′-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

The ^{1}H and ^{13}C NMR spectra of **8** with the molecular formula $C_{33}H_{40}O_{22}$ displayed an additional set of glucose signals compared to **7**. Three anomeric proton signals resonated at δ_H 4.60 (J = 8.4 Hz, H-1‴), 5.02 (J = 7.8 Hz, H-1″), and 5.49 (J = 7.2 Hz, H-1⁗) with their HMQC-related carbon signals at δ_C 99.8 (C-1″), 100.7 (C-1⁗), and 104.0 (C-1‴), respectively. In the HMBC spectrum of **8** (Figure 1), cross-peaks between H-1‴ and C-2″ (δ_C 81.6), as well as between H-1″ and C-4′ (δ_C 147.2), were observed similar to **7**, indicating the presence of a 1→2 linkage between two the glucosyl moieties at C-4′. In addition, the correlation between H-1⁗ and C-3 (δ_C 133.8) suggested that the third glucosyl unit was linked at C-3. Like **7**, the sugar moiety was deduced as D-glucose. Therefore, the structure of **8** was determined to be quercetin-3-*O*-β-D-glucopyranosyl-4′-*O*-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside.

Among the isolated compounds, **1**, **2**, **5**, **6**, **9**−**14**, and **23** were evaluated for their anti-inflammatory activity on LPS-

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Table 2. ^{13}C NMR Spectroscopic Data (125 and 150 MHz) for Ugonins 1−6

position	1[b,c]	2[a,d]	3[b,d]	4[a,d]	5[b,d]	6[a,d]
2	146.1, C	151.9, C	149.3, C	149.4, C	161.0, C	156.6, C
3	133.9, C	134.9, C	137.5, C	137.5, C	107.6, CH	139.5, C
4	169.2, C	170.6, C	176.8, C	176.8, C	176.7, C	179.6, C
4a	103.9, C	105.6, C	104.4, C	104.4, C	108.9, C	106.2, C
5	162.3, C	164.2, C	162.5, C	162.5, C	156.7, C	159.2, C
6	99.1, CH	99.8, CH	99.4, CH	99.4, CH	107.3, C	113.1, C
7	163.7, C	164.0, C	165.8, C	165.8, C	160.0, C	164.8, C
8	94.8, CH	95.6, CH	94.6, CH	94.6, CH	94.7, CH	90.4, CH
8a	157.2, C	158.6, C	158.5, C	158.5, C	158.3, C	155.9, C
9	35.5, CH_2	21.8, CH_2	20.6, CH_2	22.0, CH_2	19.0, CH_2	21.7, CH_2
10	94.1, C	44.3, CH	44.4, CH	44.7, CH	43.4, CH	53.6, CH
11	135.8, C	77.8, C	74.5, C	76.6, C	76.8, C	149.0, C
12	123.1, CH	39.5, CH_2	131.6, CH	39.5, CH_2	39.7, CH_2	30.2, CH_2
13	22.1, CH_2	18.6, CH_2	128.7, CH	18.7, CH_2	18.8, CH_2	24.0, CH_2
14	32.4, CH_2	42.0, CH_2	42.1, CH_2	42.1, CH_2	42.1, CH_2	35.5, CH_2
15	36.8, C	34.7, C	33.2, C	34.7, C	34.6, C	35.2, C
16 (17)	22.2, CH_3	21.6, CH_3	21.5, CH_3	21.8, CH_3	21.7, CH_3	28.3, CH_3
	22.7, CH_3	32.4, CH_3	30.7, CH_3	32.6, CH_3	32.5, CH_3	28.5, CH_3
18	17.6, CH_3	26.8, CH_3	27.5, CH_3	27.1, CH_3	27.1, CH_3	109.4, CH_2
1′	105.0, C	108.3, C	121.7, C	122.0, C	124.5, C	123.1, C
2′	117.5, C	115.9, C	122.9, C	123.3, C	113.7, CH	116.3, CH
3′	147.1, C	141.0, C	142.1, C	142.8, C	146.3, C	145.8, C
4′	151.4, C	152.1, C	148.5, C	148.8, C	149.1, C	150.5, C
5′	97.8, CH	96.9, CH	112.8, CH	112.7, CH	116.5, CH	116.2, CH
6′	149.4, C	152.3, C	123.0, CH	123.1, CH	119.2, CH	122.1, CH
OCH_3 (3)						60.1
OCH_3 (7)						56.4

[a]125 MHz. [b]150 MHz. [c]Measured in DMSO-d_6. [d]measured in acetone-d_6.

induced NO production in microglial cells, and the results are shown in Table 3. Except compounds **1**, **5**, and **13**, all the other compounds inhibited NO production with IC_{50} values of 6.2−10.1 μM and were more potent than the NF-kB inhibitor pyrrolidine dithiocarbamate (PDTC), used as the positive control, with an IC_{50} value 10.8 μM. In addition, the antiosteoporotic activities of compounds **1**, **2**, **5**, **6**, and **10**−**13** were investigated through the increase in osteoblast differentiation (osteoblastogenesis) or decrease in osteoclast differentiation (osteoclastogenesis).[20] The results revealed that none of them had considerable osteoblastogenetic activity, except ugonin K (**10**) at 10 μM (data not shown). Nevertheless, all tested compounds except **13** displayed the ability to suppress RANKL-induced osteoclast differentiation in RAW264.7 cells with IC_{50} values of 1.8−4.6 μM (Table 4); moreover, ugonin K (**10**) exhibited the greatest inhibitory activity with an IC_{50} value of 1.8 ± 0.2 μM. In these experiments, genistein was used as the positive control.

The present and previous[5] studies show that *H. zeylanica* rhizomes contain a rich array of anti-inflammatory constituents, which may support its traditional use as an antipyretic and antiphlogistic agent. The considerable antiosteoporotic activities of the constituents of *H. zeylanica* rhizomes may offer

Table 3. Inhibition of LPS-Induced Nitric Oxide (NO) Production in Microglial Cells by Compounds 1, 2, 5, 6, 9−14, and 23[a]

compound	IC_{50} (μM) in LPS-induced NO production
1	18.0 ± 4.8
2	7.0 ± 0.4
5	19.7 ± 5.2
6	6.7 ± 0.9
9	6.5 ± 0.5
10	8.1 ± 1.3
11	10.1 ± 1.1
12	7.7 ± 0.4
13	24.1 ± 6.1
14	6.2 ± 0.1
23	10.1 ± 0.6
PDTC	10.8 ± 1.3

[a]Data were calculated as 50% inhibitory concentration (IC_{50}). Values are expressed as mean ± SEM (n = 3). Pyrrolidine dithiocarbamate (PDTC, a NF-kB inhibitor) was used as a positive control.

Table 4. Effects of Compounds 1, 2, 5, 6, and 10−13 on the Differentiation of Primary Cultured Osteoclasts[a]

compound	IC_{50} (μM) in TRAP activity
1	4.5 ± 0.2
2	3.5 ± 0.3
5	4.6 ± 0.4
6	3.2 ± 0.3
10	1.8 ± 0.2
11	2.5 ± 0.2
12	3.3 ± 0.4
13	>10
genistein	11.4 ± 0.2

[a]Data were calculated as 50% inhibitory concentration (IC_{50}). Values are expressed as mean ± SEM (n = 3). Genistein was used as a positive control.

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evidence for another use of this herbal drug, i.e., to treat bone fracture and contusions.

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points were determined on a Yanaco MP-I3 micro melting point apparatus, and the thermometer was used without correction. Optical rotations were measured on a JASCO P-2000 polarimeter. UV spectra were recorded on a Hitachi U-3310 UV/vis spectrometer. The ECD spectra were recorded on a JASCO J-715 spectrometer. IR spectra were recorded on a Nicolet Avatar 320 FT-IR spectrometer. ^{1}H NMR and ^{13}C NMR spectra were measured with a Varian Unity Inova 500 MHz or Varian VNMRS 600 MHz FT-NMR spectrometer. EIMS spectra were obtained using a Finnigan Focus GC & DSQ II GC-MS spectrometer at 70 eV. ESIMS spectra were acquired on a Finnigan MAT LCQ mass spectrometer. HRESIMS spectra were recorded on a Finnigan MAT 95S mass spectrometer or a Q Exactive Focus mass spectrometer.

Plant Material. The rhizomes of *H. zeylanica* (L.) Hook. were purchased in Taipei, Taiwan, in June 2010. The plant was identified by comparison with the voucher specimen (NRICM-99-003) already deposited at the herbarium of the National Research Institute of Chinese Medicine, Republic of China.

Extraction and Isolation. The rhizomes of *H. zeylanica* (10.8 kg) were extracted with EtOH (3 × 80 L) at 50 °C for 24 h. The concentrated EtOH extract (280.5 g) was partioned between EtOAc and H_2O as well as between H_2O and butanol. The EtOAc extract (215.5 g) was subjected to a silica gel column (70−230 mesh, 10 × 60 cm, Merck) eluting with gradient solvent systems of *n*-hexane−acetone (10:1, 5:1, 2:1), CH_2Cl_2−acetone (10:1, 5:1), CH_2Cl_2−MeOH (10:1, 5:1), and MeOH to yield 87 fractions (3 L each). From fractions 15−27, *n*-hexane−acetone (5:1) eluate, the CH_2Cl_2-undissolved precipitate was obtained and identified as ugonin K (**10**, 2.5 g). The filtrate was repeatedly chromatographed over silica gel columns eluting with *n*-hexane−acetone (3:1) and CH_2Cl_2−acetone (30:1) and Sephadex LH-20 columns eluting with MeOH−H_2O (3:1) to afford ugonins V (**1**, 11.6 mg), W (**2**, 97.8 mg), and X (**3**, 2.5 mg), (10*R*,11*S*)-ugonin N (**4**, 4.2 mg), Y (**6**, 21.0 mg), and O (**12**, 74.6 mg), and ugonstilbene A (**14**, 126.7 mg). The same treatment as above for fraction 31 gave ugonins M (**11**, 114.1 mg) and J (**9**, 208.4 mg). Fractions 42−55, CH_2Cl_2−acetone (10:1−5:1) eluate, were repeatedly purified by silica gel columns eluting with CH_2Cl_2−MeOH (40:1−20:1) and Sephadex LH-20 columns eluting with MeOH−H_2O (3:1) to afford (10*R*,11*S*)-ugonin S (**5**, 20.0 mg) and ugonin S (**13**, 22.5 mg). The *n*-BuOH extract (24.9 g) was subjected to a 75C_{18} OPN column (4.6 × 60 cm, Cosmosil) eluting with H_2O−MeOH (80:20−20:80) to yield 50 fractions (250 mL each). The combined fractions 1−5, H_2O−MeOH (80:20−60:40) eluate, was chromatographed through a 75C_{18} OPN column (H_2O−MeOH, 60:40) and further purified by a Sephadex LH-20 column (2.6 × 36 cm) eluting with MeOH−H_2O (1:1) to give benzoic acid (**22**, 3.7 mg) and quercetin-3,4′-di-*O*-*β*-D-glucopyranoside (**18**, 37.7 mg). Using the same separation methods as fractions 1−5, the combined fractions 6−11 gave quercetin (**15**, 6.7 mg), quercetin-3-*O*-*β*-D-glucopyranoside (**16**, 18.4 mg), quercetin-4′-*O*-*β*-D-glucopyranoside (**17**, 28.9 mg), quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranoside (**19**, 32.6 mg), and quercetin-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**7**, 22.6 mg). The H_2O extract (24.3 g) was concentrated and treated with MeOH to afford MeOH-soluble (12.6 g) and MeOH-insoluble (1.75 g) portions. The former was repeatedly chromatographed on Sephadex LH-20 columns eluting with MeOH−H_2O (3:1) to afford quercetin-3-*O*-*β*-D-glucopyranosyl-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**8**, 16.4 mg) and quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-4′-*O*-*β*-D-glucopyranoside (**20**, 16.7 mg). The latter was dissolved in MeOH−H_2O (3:1) and chromatographed over Sephadex LH-20 columns eluting with MeOH−H_2O (1:1−3:1) to afford **20** (39.0 mg) and quercetin-3-*O*-*β*-D-glucopyranosyl-(1→4)-*β*-D-glucopyranosyl-4′-*O*-*β*-D-glucopyranosyl-(1→2)-*β*-D-glucopyranoside (**21**, 38.7 mg).

*Ugonin V (**1**):* yellow powder; mp 174−176 °C; $[\alpha]^{25}_D$ +24 (*c* 0.4, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.51), 264 (4.15), 292 (3.84), 362 (4.31) nm; IR (KBr) ν_{max} 3363 (OH), 2923, 1657, 1634, 1611, 1460, 1364, 1291, 1221, 1001 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) see Table 1; ^{13}C NMR (DMSO-d_6, 150 MHz) see Table 2; ESIMS *m*/*z* 435.23 $[M + H]^+$; HRESIMS *m*/*z* 434.1374 $[M]^+$ (calcd for $C_{25}H_{22}O_7$, 434.1366).

*Ugonin W (**2**):* yellow powder; mp 145−147 °C; $[\alpha]^{25}_D$ −134 (*c* 1.0, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.57), 260 (4.23), 351 (4.27) nm; IR (KBr) ν_{max} 3399 (OH), 2929, 1650, 1609, 1454, 1377, 1332, 1262, 1213, 1160, 1009, 825 cm^{-1}; ECD (*c* 4.59 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) +1.38 (210), +0.40 (221), +1.06 (227), −0.80 (264), −0.16 (275), −1.53 (304), −0.22 (332), −0.85 (366); ^{1}H NMR (acetone-d_6, 500 MHz) see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz) see Table 2; ESIMS *m*/*z* 435.25 $[M - H]^-$; HRESIMS *m*/*z* 436.1534 $[M]^+$ (calcd for $C_{25}H_{24}O_7$, 436.1522).

*Ugonin X (**3**):* yellow powder; mp 130−132 °C; $[\alpha]^{25}_D$ −162 (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 207 (4.65), 254 (4.33), 299 (3.92), 349 (4.04) nm; IR (KBr) ν_{max} 3440 (OH), 2927, 1654, 1600, 1484, 1457, 1372, 1268, 1237, 1175, 1074 cm^{-1}; ^{1}H NMR (acetone-d_6, 600 MHz) see Table 1; ^{13}C NMR (acetone-d_6, 150 MHz) see Table 2; ESIMS *m*/*z* 435.12 $[M - H]^-$; HRESIMS *m*/*z* 436.1530 $[M]^+$ (calcd for $C_{25}H_{24}O_7$, 436.1522).

*(10R,11S)-Ugonin N (**4**):* yellow powder; mp 164−166 °C; $[\alpha]^{25}_D$ −12 (*c* 0.9, MeOH); UV (MeOH) λ_{max} (log ε) 207 (5.00), 254 (4.56), 295 (4.32), 346 (4.27) nm; IR (KBr) ν_{max} 3408 (OH), 2929, 2868, 1638, 1601, 1450, 1373, 1234, 1164, 1038, 833 cm^{-1}; ^{1}H NMR (acetone-d_6, 500 MHz) see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz) see Table 2; ESIMS *m*/*z* 437.30 $[M - H]^-$; HRESIMS *m*/*z* 437.1609 $[M - H]^-$ (calcd for $C_{25}H_{25}O_7$, 437.1595).

*(10R,11S)-Ugonin S (**5**):* yellow powder; mp 261−263 °C; $[\alpha]^{25}_D$ −31 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ε) 216 (4.54), 272 (4.23), 337 (4.31) nm; IR (KBr) ν_{max} 3429 (OH), 2943, 2838, 1634, 1600, 1573, 1461, 1353, 1294, 1264, 1156, 1082, 862 cm^{-1}; ECD (*c* 4.74 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) −2.9 (210), −9.86 (215), −1.85 (231), +3.59 (272), −3.97 (313), +1.57 (353), +0.04 (372); ^{1}H NMR (acetone-d_6, 600 MHz) see Table 1; ^{13}C NMR (acetone-d_6, 150 MHz) see Table 2; ESIMS *m*/*z* 423.00 $[M + H]^+$; HRESIMS *m*/*z* 423.1809 $[M + H]^+$ (calcd for $C_{25}H_{27}O_6$, 423.1802).

*(10R,11R)-Ugonin S (**13**):* $[\alpha]^{25}_D$ +71 (*c* 0.5, MeOH); ECD (*c* 4.74 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) +3.71 (206), +7.78 (214), +3.69 (225), +1.11 (239), +2.43 (262), + 0.22 (282), +1.95 (308), −0.32 (346); ^{1}H NMR (acetone-d_6, 600 MHz) δ 7.44 (1H, d, *J* = 1.8 Hz, H-2′), 7.34 (1H, dd, *J* = 1.8, 8.4 Hz, H-6′), 6.95 (1H, d, *J* = 8.4 Hz, H-5′), 6.59 (1H, s, H-8), 6.36 (1H, s, H-3), 2.77 (1H, dd, *J* = 4.8, 16.2 Hz, H-9a), 2.35 (1H, dd, *J* = 13.8, 16.2 Hz, H-9b), 2.07 (1H, m, H-12a), 1.67 (1H, m, H-12b), 1.64 (2H, m, H_2-13), 1.62 (1H, dd, *J* = 4.8, 13.8 Hz, H-10), 1.50 (1H, m, H-14a), 1.36 (1H, td, 4.8, 12.6 Hz, H-14b), 1.24 (3H, s, CH_3-18), 1.03 and 0.95 (each 3H, s, CH_3-16, 17); ^{13}C NMR (acetone-d_6, 150 MHz) δ 176.7 (C-4), 161.0 (C-2), 160.4 (C-7), 158.5 (C-8a), 155.3 (C-5), 149.0 (C-4′), 146.3 (C-3′), 124.4 (C-1′), 119.2 (C-6′), 116.4 (C-5′), 113.6 (C-2′), 108.9 (C-4a), 108.0 (C-6), 107.4 (C-3), 94.7 (C-8), 78.6 (C-11), 47.6 (C-10), 42.1 (C-14), 40.4 (C-12), 34.0 (C-15), 32.3 (C-17), 20.8 (C-16), 20.4 (C-13), 19.9 (C-18), 18.7 (C-9).

*Ugonin Y (**6**):* yellow powder; mp 208−210 °C; $[\alpha]^{25}_D$ +3 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ε) 203 (4.64), 264 (4.33), 292 (4.00), 363 (4.66) nm; IR (KBr) ν_{max} 3484 (OH), 2931, 2864, 1719, 1648, 1593, 1477, 1434, 1354, 1215, 1163, 1056, 810 cm^{-1}; ECD (*c* 4.29 × 10^{-5} M, MeOH) $\Delta\varepsilon$ (nm) −1.42 (260), −0.16 (280), −2.61 (300), −0.36 (331), −2.62 (369); ^{1}H NMR (acetone-d_6, 500 MHz) see Table 1; ^{13}C NMR (acetone-d_6, 125 MHz) see Table 2; ESIMS *m*/*z* 489.00 $[M + Na]^+$; HRESIMS *m*/*z* 466.2002 $[M]^+$ (calcd for $C_{27}H_{30}O_7$, 466.1992).

*Quercetin-4′-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (**7**):* yellow, amorphous powder; mp 216−218 °C; UV (MeOH) λ_{max} (log ε) 203 (4.79), 253 (4.43), 336 (4.40) nm; IR (KBr) ν_{max} 3412 (OH), 2921, 1655, 1618, 1597, 1507, 1462, 1368, 1254, 1209, 1168, 1070, 805 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 12.39 (1H, br s, 5-OH), 7.69 (1H, d, *J* = 2.4 Hz, H-2′), 7.62 (1H, dd, *J* = 2.4, 9.0 Hz, H-6′), 7.24 (1H, d, *J* = 9.0 Hz, H-5′), 6.44 (1H, d, *J* = 1.8 Hz, H-8), 6.18 (1H, d, *J* = 1.8 Hz, H-6), 5.01 (1H, d, *J* = 7.8 Hz, H-1″), 4.60 (1H, d, *J*

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= 7.8 Hz, H-1‴), 3.72 (1H, br d, J = 10.2 Hz, H-6″b), 3.60 (1H, t, J = 8.4 Hz, H-2″), 3.50 (1H, t, J = 9.0 Hz, H-3″), 3.49 (1H, dd, J = 6.0, 10.2 Hz, H-6″a), 3.42 (2H, m, H-6‴), 3.39 (1H, dd, J = 1.8, 6.0, Hz,H-5″), 3.25 (1H, t, J = 9.6 Hz, H-4″), 3.19 (1H, t, J = 8.4 Hz, H-3‴), 3.17 (1H, m, H-5‴), 3.13 (1H, t, J = 9.0 Hz, H-4‴), 3.02 (1H, t, J = 8.4 Hz, H-2‴); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 176.0 (C-4), 164.6 (C-7), 160.7 (C-5), 156.3 (C-9), 146.5 (2 × C, C-3′, 4′), 145.7 (C-2), 136.4 (C-3), 125.4 (C-1′), 119.4 (C-6′), 115.9 (C-5′), 114.7 (C-2′), 104.0 (C-1‴), 102.9 (C-10), 99.8 (C-1″), 98.4 (C-6), 93.6 (C-8), 81.9 (C-2″), 77.0 (C-5″), 76.8 (C-5‴), 76.2 (C-3‴), 75.8 (C-3″), 74.4 (C-2‴), 69.6 (C-4‴), 69.3 (C-4″), 60.5 (C-6″), 60.4 (C-6‴); ESIMS m/z 625.00 $[M - H]^-$; HRESIMS m/z 626.1502 $[M]^+$ (calcd for $C_{27}H_{30}O_{17}$, 626.1483).

Acid Hydrolysis of ***7****.* Compound **7** (7.5 mg) was treated with 5 mL of 10% HCl at 100 °C for 8 h and gave quercetin and D-glucose ($[\alpha]^{25}_D$ +30, c 0.6, H_2O).

*Quercetin-3-O-β-D-glucopyranosyl-4′-O-β-D-glucopyranosyl-(1→2)-β-D-glucopyranoside (****8****):* yellow, amorphous powder; mp 210−212 °C; UV (MeOH) λ_{max} (log ε) 205 (4.76), 267 (4.48), 347 (4.33) nm; IR (KBr) ν_{max} 3432 (OH), 2913, 1655, 1609, 1512, 1360, 1205, 1168, 1074, 827 cm^{-1}; ^{1}H NMR (DMSO-d_6, 600 MHz) δ 12.54 (1H, br s, 5-OH), 7.64 (1H, dd, J = 2.4, 9.0 Hz, H-6′), 7.59 (1H, d, J = 2.4 Hz, H-2′), 7.20 (1H, d, J = 9.0 Hz, H-5′), 6.40 (1H, d, J = 1.8 Hz, H-8), 6.17 (1H, d, J = 1.8 Hz, H-6), 5.49 (1H, d, J = 7.2 Hz, H-1‴), 5.02 (1H, d, J = 7.8 Hz, H-1″), 4.60 (1H, d, J = 8.4 Hz, H-1‴), 3.73 (1H, br d, J = 11.1 Hz, H-6″b), 3.61 (1H, t, J = 10.8 Hz, H-2″), 3.58 (1H, br d, J = 8.4 Hz, H-6⁗b), 3.50 (1H, t, J = 10.8 Hz, H-3″), 3.47 (2H, m, H-6″b, 6‴a), 3.43 (2H, m, H-5″, 6″a), 3.34 (1H, m, H-6⁗a), 3.25 (1H, t, J = 9.0 Hz, H-4″), 3.20 (2H, m, H-2⁗, 3⁗), 3.17 (2H, m, H-3‴, 5‴), 3.12 (1H, t, J = 9.0 Hz, H-4‴), 3.07 (2H, m, H-4⁗, 5⁗), 3.02 (1H, t, J = 8.4 Hz, H-2‴); ^{13}C NMR (DMSO-d_6, 150 MHz) δ 177.4 (C-4), 165.0 (C-7), 161.2 (C-5), 156.5 (C-9), 155.2 (C-2), 147.2 (C-4′), 146.3 (C-3′), 133.8 (C-3), 124.7 (C-1′), 121.1 (C-6′), 115.9 (C-2′), 115.5 (C-5′), 104.0 (C-1‴), 103.8 (C-10), 100.7 (C-1⁗), 99.8 (C-1″), 98.9 (C-6), 93.8 (C-8), 81.6 (C-2″), 77.7 (C-5⁗), 77.0 (C-5″), 76.9 (C-5‴), 76.5 (C-3‴), 76.3 (C-3⁗), 75.7 (C-3″), 74.4 (C-2‴), 74.1 (C-2⁗), 70.0 (C-4⁗), 69.7 (C-4‴), 69.3 (C-4″), 61.0 (C-6⁗), 60.5 (2 × C, C-6‴, 6″); ESIMS m/z 787.00 $[M - H]^-$; HRESIMS m/z 788.1986 $[M]^+$ (calcd for $C_{33}H_{40}O_{22}$, 788.2011).

Microglial Cells Culture and Measurement of Nitric Oxide.[21] A murine microglial cell line (BV2) was cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco; Grand Island, NY, USA) supplemented with 5% fetal bovine serum (Hyclone; Logan, UT, USA). The production of NO was determined by measuring the accumulation of nitrite in the culture medium 24 h after stimulation with LPS (0.5 μg/mL) by the Griess reagent as in the previous report.[22]

Osteoclast Differentiation Assay.[23] The culture condition for RAW264.7 macrophages (American Type Culture Collection, Manassas, VA, USA) was described previously.[24] For differentiation, cells were cultured for 4−5 days in DMEM containing 100 ng/mL recombinant murine RANKL (defined as differentiation medium) in the absence or presence of tested compounds (1, 3, 10, and 20 μM). Osteoclast differentiation was analyzed by measuring tartrate-resistant acid phosphatase (TRAP) activity, an osteoclast-specific marker. After differentiation, osteoclasts were fixed and incubated with a reaction buffer containing p-nitrophenylphosphate to measure the TRAP activity as described previously.[23] TRAP activity measured in the presence of RANKL alone was defined as the control (100%). All results were expressed as relative to the control. The cell viability of all compound-treated cells is comparable to nontreated control cells (data not shown).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jnatprod.5b01164.

^{1}H, ^{13}C, 2D NMR, and HRESIMS spectra of new compounds **1**−**8** along with ^{1}H and ^{13}C NMR spectra of known compounds and the values of mean ± SD of Tables 3 and 4 (PDF)

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Notes

The authors declare no competing financial interest.

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