Bioactive Cembrane Diterpenoids of *Anisomeles indica*

Yu-Li Chen,[†] Yu-Hsuan Lan,[‡] Pei-Wen Hsieh,[†] Chin-Chung Wu,[†] Shu-Li Chen,[†] Chiao-Ting Yen,[†] Fang-Rong Chang,*[,†,⊥] Wen-Chun Hung,[§] and Yang-Chang Wu*[,†,§,⊥]

Graduate Institute of Natural Products, College of Pharmacy, Kaohsiung Medical University, Kaohsiung 807, Taiwan, Faculty of Pharmacy, College of Pharmacy, China Medical University, Taichung, 404, Taiwan, National Sun Yat-Sen University−Kaohsiung Medical University Joint Research Center, Kaohsiung 804, Taiwan, and Center of Excellence for Environmental Medicine, Kaohsiung Medical University, Kaohsiung 804, Taiwan, Republic of China

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Five new cembrane-type diterpenoids with a *trans*-fused α-methylene-γ-lactone (**1**−**5**), a new flavonoid glucoside (**6**), and 17 known compounds were isolated from a methanol extract of *Anisomeles indica*. The structures of **1**−**6** were elucidated by spectroscopic analysis, and the absolute configuration of compound **1** was determined using the modified Mosher's method. Compound **8** (4,5-epoxovatodiolide) exhibited cytotoxicity against a small panel of human cancer cell lines. Additionally, compounds **4** and **7** (ovatodiolide) exhibited selective antiplatelet aggregation activities toward collagen, while compounds **4**, **5**, and **8** showed inhibitory effects on antiplatelet aggregation induced by thrombin.

Eight species of *Anisomeles*, belonging to the plant family Labiatae, are distributed throughout the southern and tropical regions of Asia. Only one species, *A. indica* (L.) Kuntze, is found in Taiwan.[1] According to the *Dictionary of Chinese Crude Drugs* "Zhong-yao-da-ci-dian",[2] the dried whole plant of *A. indica* has been used for the treatment of gastrointestinal disease and hypertension in mainland China. In previous studies, many chemical constituents were identified from this plant, including terpenoids,[3–9] flavonoids,[6,10–13] steroids,[7] and some miscellaneous substances.[5,7] Of these, ovatodiolide (**7**), the major component of *A. indica,*[8,9] belonging to the cembrane class of diterpenoids, has exhibited several biological activities, such as cytotoxicity[8,9] and antihypertensive[14] and anti-HIV effects.[15] In addition, an ethanolic extract of *A. indica* showed an inhibitory effect on the growth of *Helicobacter pylori*.[16]

In an ongoing study of the biologically active constituents from natural sources, a methanolic extract of *A. indica* was found to show cytotoxicity against four human cancer cell lines and exhibited antiplatelet aggregation effects. Bioassay-directed fractionation led to the isolation of five new cembrane-type diterpenoids (**1**−**5**) and one new flavonoid glucoside (**6**), along with 17 known compounds, including two cembrane-type diterpenoids, ovatodiolide (**7**)[17] and 4,5-epoxovatodiolide (**8**);[18] six flavonoids, apigenin,[19] terniflorin,[20] 5,8,4′-trihydroxy-7,3′-dimethoxyflavone,[20] anisofolin A,[20] anisofolin B,[20] and prunin-6″-*p*-coumarate;[21] four triterpenoids, maslinic acid,[22] 3-*O*-*trans*-*p*-coumaroylmaslinic acid,[23] hederagenin,[23] and arjunolic acid;[24] and five benzenoids,[25] *p*-hydroxybenzoic methyl ester, *p*-hydroxybenzoic acid, *p*-hydroxycinnamic methyl ester, 3,4-dihydroxycinnamic methyl ester, and anisovatodside. We report herein on the structure elucidation of the new compounds (**1**−**6**) and on the cytotoxic and antiplatelet aggregation effects of compounds **1**−**8**.

Results and Discussion

The molecular formula of compound **1** was established by HRESIMS (*m*/*z* 367.1523 $[M + Na]^+$) as $C_{20}H_{24}O_5$, requiring nine degrees of unsaturation. The IR spectrum revealed the presence of hydroxyl (3484 cm^{-1}) and α-methylene-γ-lactone (1754 and 1661 cm^{-1}) moieties.[5–7] The ^{13}C NMR spectrum of **1** (Table 1) exhibited 20 carbon signals, including one methyl, seven methylenes, six methines, and six quaternary carbons. The NMR signals at δ_C 170.2 (s), 140.4 (s), 120.8 (t)/δ_H 6.28 and 5.56 (each 1H, d, $J = 2.8$ Hz), 79.3 (d)/δ_H 4.87 (1H, dd, $J = 9.6$, 7.2 Hz), and 45.8 (d)/δ_H 2.59 (1H, m) indicated the presence of an α-methylene-γ-lactone ring in **1**.[26–28] Furthermore, the signals at δ_C 109.0 (CH_2)/δ_H 5.03, 5.50 (each 1H, s) and the signals at δ_C 134.7 (s), 129.3 (d)/δ_H 5.44 (1H, d, 9.2 Hz) were assigned to a terminal methylene and trisubstituted double bonds, respectively. In addition, the NMR signals at δ_C 173.5 (s), 149.2 (d)/δ_H 7.27 (1H, s), 134.2 (s), and 79.7 (d)/δ_H 5.15 (1H, m) were suggestive of an α,β-unsaturated lactone ring.[26–28] On the basis of previous characteristic features, the remaining one degree of unsaturation was attributed to the 14-membered ring, and the skeleton of **1** was assigned as a cembrane-type diterpenoid.[8,9] Additionally, an oxymethine signal at δ_C 70.3 (d)/δ_H 3.94 (1H, d, $J = 9.2$ Hz) was assigned at C-5 because the HMBC spectrum of **1** showed correlations with C-4 (δ_C 153.1), C-18 (δ_C109.0), and three other methylenes (δ_C 32.8, 31.8, and 23.8). Further analysis of the COSY, HMQC, and HMBC spectra (Figure 1) established the NMR assignments for **1**.

Table 1. ^{13}C NMR Data of Compounds **1**−**5** (in C_5D_5N, 100 MHz)[a]

position	1	2	3	4	5
1	45.8 (CH)	45.3 (CH)	43.8 (CH)	47.7 (CH)	47.3 (CH)
2	31.3 (CH_2)	30.8 (CH_2)	32.8 (CH_2)	24.7 (CH_2)	23.5 (CH_2)
3	31.8 (CH_2)	31.9 (CH_2)	26.7 (CH_2)	39.4 (CH_2)	35.3 (CH_2)
4	153.1 (qC)	148.2 (qC)	147.5 (qC)	71.8 (qC)	83.4 (qC)
5	70.3 (CH)	83.4 (CH)	200.2 (qC)	141.6 (CH)	136.7 (CH)
6	32.8 (CH_2)	28.3 (CH_2)	33.8 (CH_2)	123.6 (CH)	126.0 (CH)
7	23.8 (CH_2)	23.6 (CH_2)	20.6 (CH_2)	27.9 (CH_2)	28.5 (CH_2)
8	134.2 (qC)	133.7 (qC)	133.1 (qC)	134.0 (qC)	133.8 (qC)
9	149.2 (CH)	149.5 (CH)	149.7 (CH)	147.0 (CH)	147.0 (CH)
10	79.7 (CH)	79.8 (CH)	79.2 (CH)	78.8 (CH)	79.0 (CH)
11	40.7 (CH_2)	40.6 (CH_2)	41.4 (CH_2)	41.6 (CH_2)	41.3 (CH_2)
12	134.7 (qC)	135.0 (qC)	135.5 (qC)	134.7 (qC)	135.1 (qC)
13	129.3 (CH)	129.0 (CH)	127.7 (CH)	129.1 (CH)	128.7 (CH)
14	79.3 (CH)	78.8 (CH)	78.0 (CH)	79.8 (CH)	79.0 (CH)
15	170.2 (qC)	170.1 (qC)	170.3 (qC)	170.7 (qC)	170.5 (qC)
16	140.4 (qC)	140.1 (qC)	140.7 (qC)	140.9 (qC)	140.4 (qC)
17	120.8 (CH_2)	120.8 (CH_2)	121.2 (CH_2)	120.4 (CH_2)	120.3 (CH_2)
18	109.0 (CH_2)	111.7 (CH_2)	126.5 (CH_2)	30.6 (CH_3)	24.2 (CH_3)
19	173.5 (qC)	173.4 (qC)	173.4 (qC)	173.3 (qC)	173.2 (qC)
20	19.3 (CH_3)	19.4 (CH_3)	18.8 (CH_3)	19.0 (CH_3)	19.2 (CH_3)

[a] All assignments were confirmed by the DEPT, $^1H-^1H$ COSY, and HMQC spectra.

* To whom correspondence should be addressed. Tel: (+886)-(0)7-312-1101, ext. 2197. Fax: (+886)-(0)7-311-4773. E-mail: yachwu@kmu.edu.tw (Y.-C.W.). Tel: (+886)-(0)7-312-1101, ext. 2162. Fax: (+886)-(0)7-311-4773. E-mail: aaronfrc@kmu.edu.tw (F.-R.C.).

[†] Graduate Institute of Natural Products, Kaohsiung Medical University.

[‡] China Medical University.

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

[⊥] Center of Excellence for Environmental Medicine, Kaohsiung Medical University.

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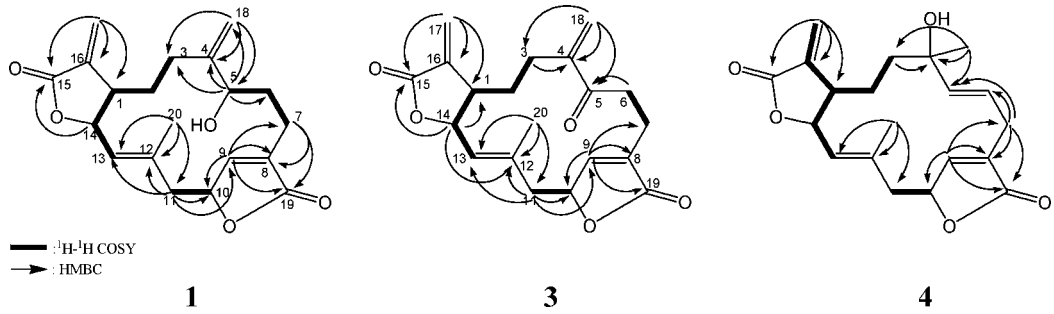


Figure 1. ^{1}H−^{1}H COSY and HMBC correlations of **1**, **3**, and **4**.

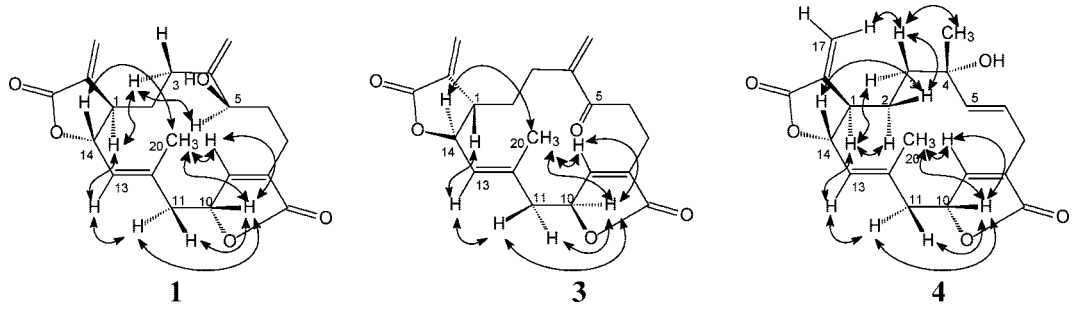


Figure 2. Key NOESY correlations of **1**, **3**, and **4**.

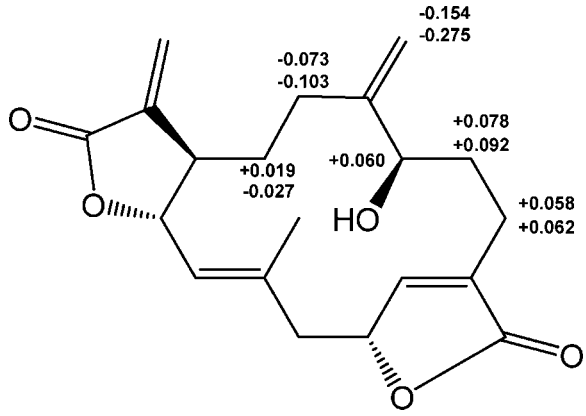


Figure 3. ^{1}H NMR chemical shift differences $\Delta\delta$ ($\delta_S - \delta_R$) in ppm for the MTPA ester of **1**.

In the NOESY spectrum (Figure 2), the correlations between H-1 and H-13, H-14, and H-20 were obtained, but no correlation was evident between H-1 and H-14, indicating the α-methylene-γ-lactone ring to be *trans*. The *E* configuration of the olefin at C-12/C-13 was supported by the chemical shift value of the methyl carbon signal for C-20 (δ_C 19.3), which appeared in the high-field area in the ^{13}C NMR spectrum.[7] Furthermore, the absolute configuration of **1** was determined by preparing Mosher esters and from a NOESY experiment.[29,30] First, the OH-5 group was converted to (*S*)-(−)- and (*R*)-(+)-*R*-methoxy-*R*-(trifluoromethyl)phenylacetyl (MTPA) ester derivatives, respectively. Distribution of the positive and negative δ values of the MTPA esters established the C-5 chiral center as having an *R* configuration (Figure 3). Finally, the NOE correlations between H-5/H-3α, H-3α/H-1, H-1/H-13, and H-13/H-11α indicated that those protons (H-1, H-3α, H-5, H-13, and H-11α) were oriented on the same side. In contrast, other correlations between H-14/H-20, H-20/H-10, and H-10/H-11 suggested the protons (H-14, H-9, H-10, H-11β, and H-20) were positioned on the opposite side of H-1 (Figure 2). Additionally, the CD spectrum of **1** showed two positive Cotton effects at ca. 245 and 220 nm, and these also suggested the stereochemistry at C-10 to be *R*.[31] Therefore, the structure of **1** [4(18)-methylene-5β-hydroxyovatodiolide] was determined to be (1*R*,5*R*,8*Z*,10*R*,12*E*,14*S*)-5-hydroxycembra-4(18),8,12,16-tetraene-15,14;19,10-diolide.

The HRESIMS of **2** gave a pseudomolecular ion at *m*/*z* 383.1472 [M + Na]$^+$ (calcd 383.1471), corresponding to $C_{20}H_{24}O_6$, with one more oxygen atom than **1**. The IR spectrum also revealed the presence of an α-methylene-γ-lactone (1749 and 1655 cm^{-1}). The NMR spectra were similar to **1** (Tables 1 and 2) except for the chemical shifts of C-5/H-5, which shifted downfield to δ_C 83.4 and δ_H 4.20, relative to **1** (δ_C 70.3 and δ_H 3.94). Therefore, the hydroxyl group attached at C-5 in **1** was substituted by a hydroperoxyl group in **2**, and this was confirmed by a reduction reaction using compound **2** and triphenylphosphine to yield compound **1**.[32,33] The stereochemistry of **2** was determined by CD and NOESY experiments. The results suggested the stereochmistry at C-10 also to be in the *R* form,[31] and the structure of **2** [4(18)-methylene-5β-hydroperoxyovatodiolide] was established as (1*R*,5*R*,8*Z*,10*R*,12*E*,14*S*)-5-hydroperoxycembra-4(18),8,12,16-tetraene-15,14;19,10-diolide.

Compound **3** was obtained as a colorless gum. Its molecular formula was established as $C_{20}H_{22}O_5$ by HRESIMS (*m*/*z* 365.1364 [M + Na]$^+$) and NMR data (Tables 1 and 2). Similar to **1** and **2**, the IR and NMR spectra of **3** also revealed the characteristic signals of an α-methylene-γ-lactone group. On comparing the NMR data of **3** with **1**, the signal at δ_H 3.94 (H-5) in **1** was absent. Also, the oxymethine signal (δ_C 70.3, C-5) in **1** was shifted to δ_C 200.2 in **3**, suggesting a ketone group at C-5 in **3**. The above observations and the very similar NOESY correlations (Figure 2) for both **1** and **3** revealed that they have the same configurations at C-1 and C-10, respectively (Figure 2). Furthermore, the CD spectrum of **3** showed a negative Cotton effect at ca. 240 nm and a positive Cotton effect at ca. 220 nm and suggested the stereochemistry at C-10 is *S*.[34] Thus the structure of **3** [4(18)-methylene-5-oxovatodiolide] was elucidated as (1*S*, 8*Z*,10*S*,12*E*,14*R*)-5-oxocembra-4(18),8,12,16-tetraene-15,14;19,10-diolide.

The molecular formula of **4** was established as $C_{20}H_{24}O_5$ by HRESIMS (*m*/*z* 367.1522 [M + Na]$^+$), implying nine degrees of unsaturation. The IR spectrum of **4** indicated the presence of hydroxyl (3482 cm^{-1}) and α-methylene-γ-lactone (1745 and 1664 cm^{-1}) moieties. The NMR data of **4** indicated the presence of a 1,2-disubstituted double bond [δ_C141.6 (d) and 123.6 (d)/δ_H 6.03 (2H, m)], a methyl [δ_C30.6/δ_H 1.49 (3H, s)], a quaternary

Table 2. ^{1}H NMR Data for Compounds **1**−**5** (in C_5D_5N, 400 MHz)

position	1	2	3	4	5
1	2.59 m	2.59 m	2.41 m	2.60 m	2.60 m
2	1.72 2H, m	1.68 m 1.82 m	1.53 m 1.77 m	1.70 m 1.83 m	1.69 2H, m
3	2.09 m 2.45 m	2.06 m 2.38 m	2.18 m 2.86 m	1.52 m 2.14 ddd (13.2, 9.6, 7.2)	1.57 m 2.28 m
5	3.94 d (9.2)	4.20 dd (9.2, 2.4)		6.03 m	5.94 m
6	1.93 m 2.58 m	2.20 m 2.48 m	2.71 ddd (18.0, 5.6, 3.2) 3.77 ddd (18.0, 12.0, 3.2)	6.03 m	5.94 m
7	2.59 m 2.75 m,	2.52 m 2.75 m	2.43 m 2.86 m	2.93 m 3.10 m	2.93 dd (14.0, 6.0) 3.14 dd (14.0, 1.6)
9	7.27 s	7.25 s	7.13 s	7.12 s	7.11 s
10	5.15 m	5.15 m	5.09 m	5.12 m	5.13 m
11	2.25 dd (14.0, 4.0) 2.82 dd (14.0, 3.2)	2.26 dd (14.8, 4.4) 2.83 dd (14.8, 3.6)	2.31 dd (14.0, 4.8) 2.54 dd (14.0, 4.8)	2.27 dd (14.0, 4.0) 2.71 dd (14.0, 4.8)	2.24 dd (14.0, 4.00) 2.75 dd (14.0, 1.6)
13	5.44 d (9.2)	5.42 d (10.0)	5.18 d (10.0)	5.40 d (9.2)	5.37 d (9.2)
14	4.87 dd (9.6, 7.2)	4.99 dd (10.0, 6.8)	4.95 dd (10.0, 4.8)	4.75 t (9.2)	4.73 t (9.2)
17	5.56 d (2.8) 6.28 d (2.8)	5.54 d (2.4) 6.27 d (2.4)	5.58 d (3.0) 6.25 d (3.0)	5.63 d (2.8) 6.26 d (2.8)	5.63 d (2.8) 6.26 d (2.8)
18	5.03 s 5.50 s	5.13 d (1.2) 5.40 d (1.2)	5.80 s 6.10 s	1.49 s	1.58 3H, s
20	1.65 3H, d (1.2)	1.77 3H, d (1.6)	1.78 3H, d (1.2)	1.58 3H, d (1.6)	1.56 3H, d (1.2)

oxygenated carbon (δ_C 71.8, s), and two allylic methylene protons (δ_H 2.93 and 3.10). Analysis of the ^{1}H/^{13}C NMR, COSY, HMQC, and HMBC spectra was used to establish the structure of **4**. The *E* configuration of the 19,20-double bond was determined by the chemical shift value of C-20 (δ_C 19.0).[7] Furthermore, the IR absorption (970 cm^{-1}) and the connection between H-6/H-7 and H-5/H-2 in the NOESY spectrum were consistent with the *E* configuration of a 5,6-double bond. The relative configurations of the four chiral centers at C-1, C-4, C-10, and C-14 in **4** were determined from the NOESY spectrum (Figure 2). The results indicted the same configurations at C-1 and C-10 in the structure of **4**. The CD spectrum and NOE correlations of **4** were similar to **1** and **2**. On this basis, the structure of **4** (4α-hydroxy-5-en-ovatodiolide) was established as (1*R*,8*Z*,10*R*,12*E*,14*S*)-4-hydroxycembra-5,8,12,16-tetraene-15,14;19,10-diolide.

Compound **5** showed a pseudomolecular ion peak in the HRESIMS (*m*/*z* 383.1470 [M + Na]$^+$), corresponding to the molecular formula $C_{20}H_{24}O_6$. Comparison of the NMR data of **5** with those of **4** (Tables 1 and 2) suggested only a structural difference confined to C-4. It was found that C-4 of **5** was shifted downfield ($\Delta\delta_C$ +11.6 ppm) relative to that of **4**, a shift similar to that shown for **2** relative to **1** ($\Delta\delta_C$ +13.1 and $\Delta\delta_H$ +0.26 ppm). Therefore, compound **5** was deduced as the 4-hydroperoxy derivative of **4** and the isomer of **2**. This conclusion was confirmed by reduction of **5** with triphenylphosphine to **4**. Additionally, the CD spectrum of **5** was similar to **1** and **2** and suggested the stereochemistry at C-10 to be *R*.[31,34] Therefore, the structure of **5** (4-hydroperoxy-5-en-ovatodiolide) was established as (1*R*,4*S*,5*E*,8*Z*,10*R*,12*E*,14*S*)-4-hydroperoxycembra-5,8,12,16-tetraen-15,14;19,10-diolide.

Compound **6** was isolated as a yellow powder with the molecular formula $C_{30}H_{26}O_{12}$, as deduced from the HRFABMS (*m*/*z* 579.1511 [M + H]$^+$). The IR spectrum revealed the presence of the carbonyl group of an α-pyrone (1654 cm^{-1}) and a hydroxyl group (3354 cm^{-1}). The UV and NMR spectroscopic data suggested that **6** belongs to the flavone type, with apigenin, D-glucose, and *cis*-*p*-coumaryl moieties.[35,36] In the ^{1}H NMR spectrum, the coupling constant (J = 7.2 Hz) of H-1″ (δ_H 5.81, d) determined the β-configuration of D-glucose, and the coupling constant (J = 12.8 Hz) of H-2‴ and H-3‴ (δ_H 6.10 and 6.79) was consistent with *cis*-olefinic protons in the α,β-unsaturated carbonyl system of the coumaryl moiety. From the HMBC spectrum (Figure 4), long-range correlations between H-1″ (δ_H 5.81) and C-7 (δ_C 163.8, s), H_2-6 (δ_H 5.15 and 4.90), and C-1‴ (δ_C 166.6, s) were found, indicating their relationships. Thus, the structure of **6** was assigned as apigenin 7-*O*-β-D-(6″-*cis*-*p*-coumaroyl) glucoside.

Seven cembrane-type diterpenoids (**1**−**5**, **7**, and **8**) were subjected to cytotoxicity and antiplatelet aggregation assays. Only compound **7** exhibited moderate cytotoxicity against all of the lung (A-549), breast (MDA-MB-231 and MCF-7), and liver (Hep G2 and Hep 3B) cancer cell lines. Additionally, diterpenoids **1**−**5**, **7**, and **8**, as well as the positive controls, aspirin, were also subjected to an antiplatelet aggregation assay. The results (Table 4) showed that **4** and **7** exhibited selective activities toward collagen with IC_{50} values of 41.9 ± 7.1 and 19.7 ± 6.7 μM, respectively. In contrast to **1** and **7**, compounds **4**, **5**, and **8** showed selective activities toward thrombin with IC_{50} values of 20.0 ± 6.2, 11.9 ± 5.3, and 4.8 ± 0.4 μM, respectively. With the presence of an epoxy group at C-4/

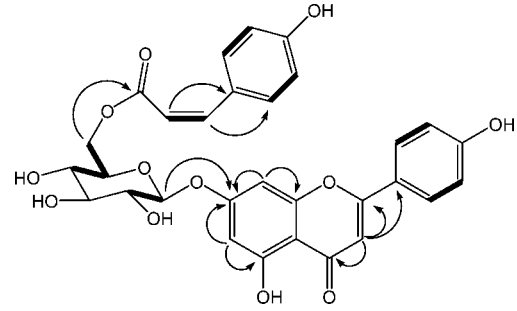


Figure 4. Selected ^{1}H−^{1}H COSY and HMBC correlations of **6**.

Table 3. Cytotoxicity (IC_{50} μM) of Compounds **1**−**8**[a,b]

	Hep G2	Hep 3B	A549	MDA-MB-231	MCF-7
3	10	>10	>10	9.8	>10
4	10	>10	9.9	>10	>10
7	10	7.4	9.6	9.5	9.5
8	10	9.3	8.8	>10	>10
doxorubicin	0.82	0.48	0.40	0.07	0.65

[a] A compound is considered active when the IC_{50} is <10 μM. Compounds **1**, **2**, and **5** were not active. [b] Human cancer cell lines used were Hep G2 (liver), Hep 3B (liver), A549 (lung), MDA-MB-231 (breast), and MCF-7 (breast).

Table 4. Antiplatelet Aggregation Activity (IC_{50} μM) of Compounds **1**−**8**[a]

	thrombin (0.05 U/mL)	collagen (10 μg/mL)
1	100	41.9 ± 7.1
4	20.0 ± 6.2	>100
5	11.9 ± 5.3	>100
7	>100	19.7 ± 6.7
8	4.8 ± 0.4	>100
aspirin	>100	153.2 ± 12.0

[a] A compound is considered active when the IC_{50} is >100 μM. Compounds **2** and **3** were not active.

C-5, the antiplatelet effect was increased. In addition, since compounds **4** and **5** showed greater potency than compounds **1**−**3** and **7**, a C-4 hydroxyl or hydroperoxyl moiety seems to be required for an inhibitory effect on platelet aggregation induced by thrombin.

Experimental Section

General Experimental Procedures. Melting points were determined using a Yanaco micromelting point apparatus and are uncorrected. Optical rotations were measured with a JASCO P-1020 digital polarimeter. The UV spectra were obtained on a JASCO V-530 UV/vis spectrophotometer, and IR spectra were measured on a Perkin-Elmer 2000 FT-IR spectrophotometer. CD spectra were measured on a Jasco J-810 spectrometer. 1D (^{1}H, ^{13}C, DEPT) and 2D (COSY, HMQC, HMBC, NOESY) NMR spectra using C_5D_5N and $CDCl_3$ as solvents were obtained on a Varian Unity Plus 400 or a G200 NMR spectrometer. Chemical shifts are reported in parts per million (δ), and coupling constants (J) are expressed in Hz and were internally referenced to the solvent signals in C_5D_5N (^{1}H, δ_H 7.21; ^{13}C, δ_C 123.5) and $CDCl_3$ (^{1}H, δ_H 7.26; ^{13}C, δ_C 77.0). Low-resolution EIMS were measured on a JEOL JMS-SX/SX 102A mass spectrometer or Quattro GC-MS spectrometer having a direct inlet system. LRESIMS and HRESIMS were obtained on a Bruker Daltonics APEX II 30e spectrometer. LRFABMS were recorded on a VG Biotech Quattro 5022 spectrometer, and HRFABMS on a Finnigan/Thermo Quest MAT 95XL spectrometer. A JASCO PU-1575 pump, a JASCO UV-1575 detector, a Thermo Hypersil ODS 5 μm (250 × 4 mm i.d.), and a preparative ODS 5 μm (250 × 10 mm i.d.) column were used for HPLC, with detection at 208 nm.

Plant Material. Whole plants of *A. indica* were purchased at a Chinese drugstore in Taipei, Taiwan, in April 2005 and identified by Shing-Ginn Lee, the Taitung District Agriculture Research and Extension Station, Taitung, Taiwan. The samples were authenticated and deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Taiwan (KMU-AS-01).

Extraction and Isolation. The dry whole plants (21.6 kg) were extracted three times with MeOH overnight at room temperature to give 476 g of crude extract. The extract was partitioned between *n*-hexane and 90% MeOH (1:1) to give an *n*-hexane extract (35 g) and a 90% MeOH extract. The MeOH extract was further partitioned with EtOAc and H_2O, and then the aqueous layer was extracted with *n*-BuOH to yield EtOAc (165 g), *n*-BuOH (48 g), and H_2O (204 g) extracts.

The EtOAc extract was separated on a Celite 545 column (0.02−0.1 mm) eluting with *n*-hexane, EtOAc, and MeOH to give three fractions, respectively. The EtOAc fraction (72.5 g) was further chromatographed on silica gel (230−400 mesh) and eluted with a gradient of $CHCl_3$/MeOH to give 12 fractions (A1−A12). Fractions A2−A4 (29.8 g) were combined, recrystallized from MeOH, and filtered to afford **7** (9.63 g). The mother liquid was concentrated under reduced pressure and chromatographed on silica gel with a gradient of $CHCl_3$/MeOH to give seven fractions (B1−B7). Fraction B2 was purified on a silica gel column to afford **8** (32.6 mg) and *p*-hydroxycinnamic methyl ester (89.6 mg). A part of fraction B4 (4.3 g) was chromatographed on Sephadex LH-20 with $CHCl_3$/MeOH (1:1) and further purified using ODS HPLC (MeCN/H_2O, 30:70) to give **1** (26.0 mg, t_R 41.5 min, 5 mL/min), **2** (39.0 mg, t_R 66.0 min, 5 mL/min), **4** (15.3 mg, t_R 34.6 min, 5 mL/min), **5** (28.1 mg, t_R 56.8 min, 5 mL/min), and **3** (8.2 mg, t_R 90.2 min, 5 mL/min). Subfraction B6 was chromatographed on silica gel eluting with $CHCl_3$/EtOAc (5:1) to yield *p*-hydroxybenzoic methyl ester. Fraction A6 (1.2 g) was chromatographed on silica gel with a gradient of $CHCl_3$/MeOH to give six fractions (C1−C6). Subfraction C2 was separated using ODS HPLC (MeOH/H_2O, 80:20) to give masmalic acid (29.4 mg, t_R 21.5 min, 3 mL/min), heteragenin (19.0 mg, t_R 34.2 min, 3 mL/min), and 3-*O*-*trans*-*p*-coumaroylmaslinic acid (5.3 mg, t_R 48.0 min, 3 mL/min). Subfraction C3 was chromatographed on silica gel eluting with a gradient of $CHCl_3$/MeOH (20: 1) to give apigenin (15.8 mg) and 3,4-dihydroxycinnamic methyl ester (12.8 mg). Fraction A8 (3.3 g) was chromatographed on silica gel eluting with a gradient of $CHCl_3$/MeOH to give three fractions (D1−D3). Fraction D1 was chromatographed on silica gel eluting with a gradient of $CHCl_3$/MeOH (20:1) to give 5,8,4-trihydroxy-7,3′-dimethoxyflavone (6.0 mg). Fraction D2 (1.0 g) was further chromatographed on silica gel eluting with $CHCl_3$/acetone (10:1) and purified using ODS HPLC (MeCN/H_2O, 35: 65) to afford **8** (21.8 mg, t_R 64.9 min, 3 mL/min). Fraction D3 (800 mg) was recrystallized from MeOH and filtered to afford anisofolin A (370.0 mg). The mother liquid was concentrated under reduced pressure and purified using ODS HPLC (MeCN/H_2O, 40:60) to afford anisofolin B (15.2 mg, t_R 64.9 min, 3 mL/min) and prunin-6″-*p*-coumarate (29.6 mg, t_R 25.9 min, 3 mL/min). Fraction A9 (2.0 g) was chromatographed on silica gel eluting with a gradient of $CHCl_3$/MeOH to give four fractions (E1−E4). Fraction E2 was further separated over a silica gel column eluting with a gradient of $CHCl_3$/MeOH (1:1) to also yield four fractions (F1−F4). Fraction F2 was purified using ODS HPLC (MeOH/H_2O, 70:30) to afford arjumolic acid (18.9 mg, t_R 25.1 min, 3 mL/min). Fraction F4 was recrystallized from *n*-hexane and filtered to afford *p*-hydroxybenzoic acid (47.5 mg). Fraction A11 (2.8 g) was recrystallized from MeOH and filtered to afford terniflorin (840.9 mg).

4-Methylene-5*β*-hydroxyovatodiolide (1): white powder; mp 164−167 °C; $[\alpha]^{25}_D$ +36.4 (*c* 0.07, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 210 (4.28) nm; IR (neat) ν_{max} 3484, 2925, 1754, 1661 cm^{-1}; CD (MeOH) nm (mdeg) 242 (1.70), 222 (3.91) nm; ^{13}C NMR and ^{1}H NMR, see Tables 1 and 2, respectively; HRESIMS *m*/*z* 367.1523 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_5Na$, 367.1521).

4-Methylene-5*β*-hydroperoxyovatodiolide (2): white powder; mp 152−156 °C. $[\alpha]^{24}_D$ +33.3 (*c* 0.15, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 210 (4.09) nm; IR (neat) ν_{max} 3405, 2926, 1749, 1655 cm^{-1}; CD (MeOH) 240 ($\Delta\epsilon$, +0.27), 222 ($\Delta\epsilon$, +1.39) nm; ^{13}C NMR and ^{1}H NMR, see Tables 1 and 2, respectively; HRESIMS *m*/*z* 383.1472 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_6Na$, 383.1471).

4-Methylene-5-oxovatodiolide (3): colorless gum; $[\alpha]^{25}_D$ +23.1 (*c* 0.06, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 210 (4.15) nm; IR (neat) ν_{max} 2926, 1753, 1674 cm^{-1}; CD (MeOH) 240 ($\Delta\epsilon$, −0.30), 220 ($\Delta\epsilon$, +1.96) nm; ^{13}C NMR and ^{1}H NMR, see Tables 1 and 2, respectively; HRESIMS *m*/*z* 365.1364 $[M + Na]^+$ (calcd for $C_{20}H_{22}O_6Na$, 365.1365).

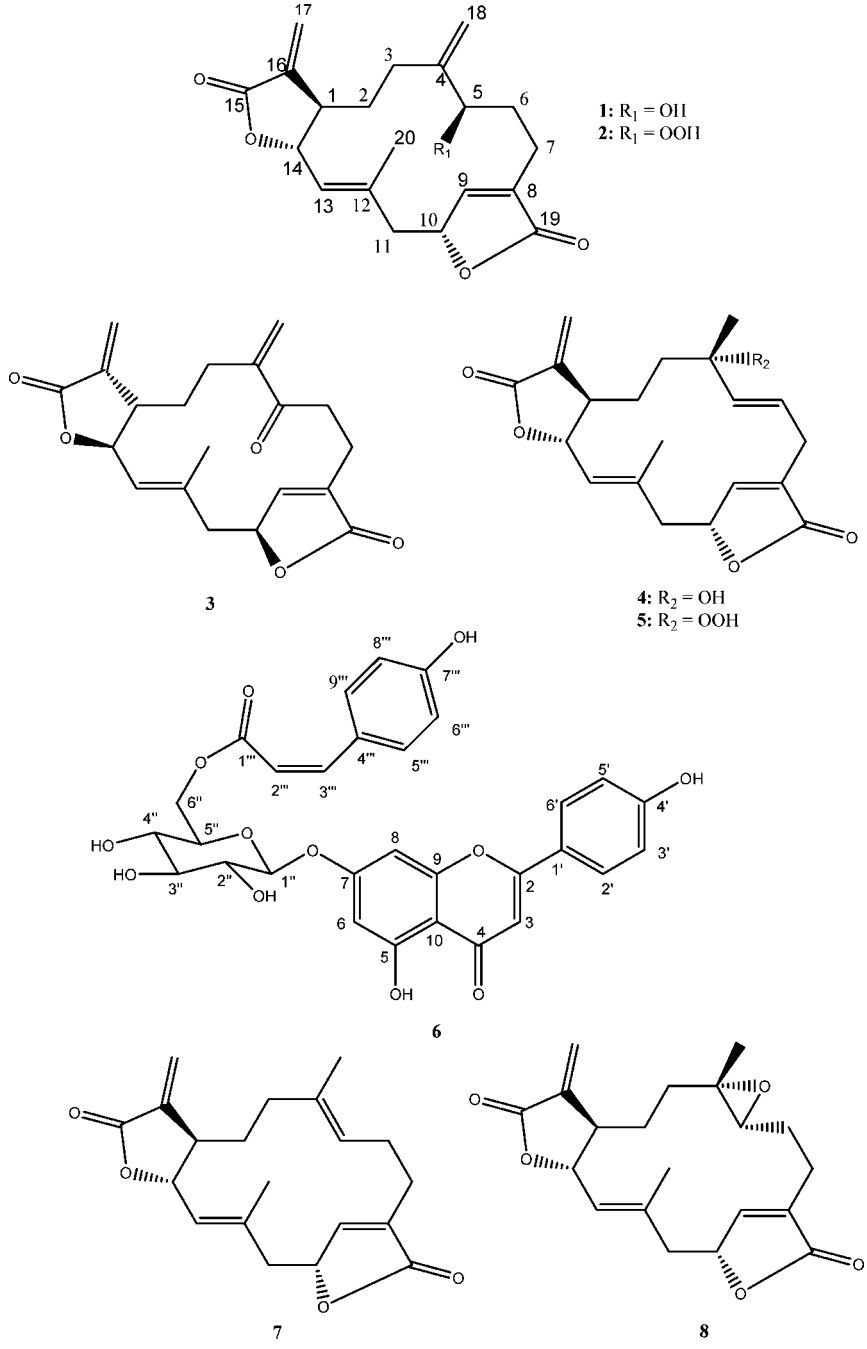
4α-Hydroxy-5-enovatodiolide (4): white powder; mp 156−160 °C; $[\alpha]^{24}_D$ +36.6 (*c* 0.13, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 210 (4.01) nm; CD (MeOH) 256 ($\Delta\epsilon$, −4.38), 240 ($\Delta\epsilon$, +5.39), 227 ($\Delta\epsilon$, +18.44), 220 ($\Delta\epsilon$, +13.10) nm; IR (neat) ν_{max} 3482, 2926, 1754, 1664, 1646, 970 cm^{-1}; ^{13}C NMR and ^{1}H NMR, see Tables 1 and 2, respectively; HRESIMS *m*/*z* 367.1522 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_5Na$, 367.1521).

4α-Hydroperoxy-5-enovatodiolide (5): white powder; mp 145−148 °C; $[\alpha]^{25}_D$ +19.0 (*c* 0.05, $CHCl_3$); UV ($CHCl_3$) λ_{max} (log ϵ) 210 (4.23) nm; CD (MeOH) 242 ($\Delta\epsilon$, +1.11), 221 ($\Delta\epsilon$, +5.99) nm; IR (neat) $_{max}$ 3389, 2927, 1749, 1661, 972 cm^{-1}; ^{13}C NMR and ^{1}H NMR, see Tables 1 and 2, respectively; HRESIMS *m*/*z* 383.1470 $[M + Na]^+$ (calcd for $C_{20}H_{24}O_6Na$, 383.1471).

Apigenin 7-*O*-*β*-D-(6″-*cis*-*p*-coumaroyl) glucoside (6): yellow powder; $[\alpha]^{26}_D$ −112.8 (*c* 0.09, MeOH); UV (MeOH) λ_{max} (log ϵ) 210 (4.15), 225 (sh), 274 (4.27), 316 (4.40) nm; IR (neat) ν_{max} 3354, 1701, 1654, 1604, 1511 cm^{-1}; ^{1}H NMR (C_5D_5N, 400 MHz) δ 13.62 (1H, s, OH-5), 7.98 (2H, d, J = 8.8 Hz, H-5‴, H-9‴), 7.96 (2H, d, J = 8.8 Hz, H-2′, H-6′), 7.27 (2H, d, J = 8.8 Hz, H-3′, H-5′), 7.08 (1H, d, J = 1.6 Hz, H-8), 7.07 (2H, d, J = 8.8 Hz, H-6‴, H-8‴), 6.92 (1H, s, H-2), 6.83 (1H, d, J = 1.6 Hz, H-6), 6.79 (1H, d, J = 12.8 Hz, H-3‴), 6.10 (1H, d, J = 12.8 Hz, H-2‴), 5.81 (1H, d, J = 7.2 Hz, H-1″), 5.15 (1H, dd, J = 11.6, 1.6 Hz, H-6″b), 4.90 (1H, dd, J = 11.6, 7.2 Hz, H-6″a), 4.40 (3H, m, H-2′, H-3′, H-5′), 4.20 (1H, t, J = 9.2 Hz, H-4′); ^{13}C NMR (C_5D_5N, 100 MHz) δ 182.8 (C, C-4), 167.5 (C, C-1‴), 164.9 (C, C-2), 163.2 (C, C-7), 162.9 (C, C-4′), 162.6 (C, C-7‴), 161.4 (C, C-5), 157.8 (C, C-9), 145.6 (CH, C-3‴), 130.6 (2CH, C-5‴, C-9‴), 128.9 (C, C-2′), 123.8 (C, C-4‴), 122.0 (C, C-1′), 116.9 (2CH, C-3′, C-5′), 116.7 (2CH, C-6‴, C-8‴), 114.7 (CH, C-2‴), 106.6 (C, C-10), 103.9 (CH, C-3), 101.7 (CH, C-1″), 100.6 (CH, C-6), 95.4 (CH, C-8), 78.3 (CH, C-3″), 76.7 (CH, C-5″), 74.6 (CH, C-2″), 71.4 (CH, C-4″), 64.4 (CH_2, C-6″); HRFABMS *m*/*z* 579.1511 $[M + H]^+$ (calcd for $C_{30}H_{27}O_{12}$, 579.1513).

Preparation of (*S*)- and (*R*)-MTPA Esters of 1. Compound **1** (2.0 mg) in an NMR tube was added to a solution of (−)-MTPA chloride in C_5D_5N (0.5 mL) for reaction overnight at room temperature. Then, the spectroscopic data of (*S*)-MTPA ester **1a** in solution were measured directly. The same procedure was applied to obtain the (*R*)-MTPA ester **1b** from the reaction of (+)-MTPA chloride with **1** in C_5D_5N. ^{1}H NMR (C_5D_5N, 400 MHz) of **1a**: δ 6.299 (1H, d, J = 2.8 Hz, H-17), 5.539 (1H, d, J = 2.8 Hz, H-17), 5.464 (1H, d, J = 9.2 Hz, H-13), 5.246 (1H, s, H-10), 5.124 (1H, d, J = 8.4 Hz, H-5), 5.00 (1H, t, J = 9.2 Hz, H-14), 4.937 (1H, s, H-18), 4.920 (1H, s, H-18), 2.895 (1H, dd, J = 14.4, 4.0 Hz, H-11), 2.570 (1H, m, H-1), 2.520 (1H, m, H-7), 2.502 (1H, m, H-6), 2.316 (1H, dd, J = 14.4, 3.6 Hz, H-11), 2.298 (1H, m, H-7), 2.287 (1H, m, H-3), 2.082 (1H, m, H-6), 2.053 (1H, m, H-3), 1.841 (3H, d, J = 1.2 Hz, H-20), 1.785 (1H, m, H-2), 1.635 (1H, m,

Chart 1



H-2). ^{1}H NMR (C_5D_5N, 400 MHz) of **1b**: δ 6.308 (1H, d, J = 2.8 Hz, H-17), 5.543 (1H, d, J = 2.8 Hz, H-17), 5.457 (1H, d, J = 10.0 Hz, H-13), 5.234 (1H, m, H-10), 5.212 (1H, s, H-18), 5.074 (1H, s, H-18), 5.064 (1H, d, J = 8.0 Hz, H-5), 5.045 (1H, dd, J = 10.0, 8.4 Hz, H-14), 2.893 (1H, dd, J = 14.4, 3.6 Hz, H-11), 2.592 (1H, m, H-1), 2.458 (1H, m, H-7), 2.410 (1H, m, H-6), 2.390 (1H, m, H-3), 2.317 (1H, dd, J = 14.4, 3.6, H-11), 2.240 (1H, m, H-7), 2.126 (1H, m, H-3), 1.867 (3H, d, J = 1.6 Hz, H-20), 1.812 1.841 (1H, m, H-2), 1.616 1.841 (1H, m, H-2).

Reduction of Hydroperoxy Diterpenoids into Hydroxy Diterpenoids.[32,33] 4α-Hydroperoxy-5-enovatodiolide (**2**) (5.2 mg) was stirred with 25.8 mg of triphenylphosphine in 5 mL of diethyl ether for 4 h at room temperature. The solution was evaporated to dryness and measured, and its spectroscopic data were found to be in full agreement with those of the natural product **1**. The 1D NMR data of the product were found to be in agreement with those of the natural product **2**. Following the same procedure as **2**, reduction of 4-methylene-5β-hydroperoxyovatodiolide (**5**) (5.6 mg) yielded a reduced product, which afforded identical spectroscopic data to **4**.

Cytotoxicity Assays.[37] Compounds **1**−**8** and doxorubicin, which was included as a positive control, were assayed for cytotoxicity against the human cancer cell lines Hep G2 and Hep 3B (hepatoma), A549 (lung), and MDA-MB-231 and MCF-7 (breast) using the MTT method. Freshly trypsinized cell suspensions were seeded in 96-well microtiter plates at densities of 5000−10 000 cells per well, and test compounds were added from DMSO stock solutions. After 3 days in culture, attached cells were incubated with MTT (0.5 mg/mL, 1 h) and subsequently solubilized in DMSO. The absorbance was measured at 550 nm using a microplate reader. The IC_{50} is the concentration of agent that reduced cell growth by 50% under the experimental conditions. Results represent the mean of two to three separate experiments, each performed in triplicate (Table 3).

Antiplatelet Aggregation Assay.[38] Platelet aggregation was measured turbidimetrically with a light-transmission aggregometer. The platelet suspension from human blood was incubated with DMSO or test compounds **1**−**5**, **7**, and **8**, as well as the positive controls, aspirin, at 37 °C for 3 min under stirring (1200 rpm) prior to the addition of the platelet activators. The extent of platelet aggregation was measured

as the increase of light transmission at 5 min after the addition of inducers (thrombin and collagen).

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