New *ent*-Kaurane Diterpenoids with Anti-Platelet Aggregation Activity from *Annona squamosa*

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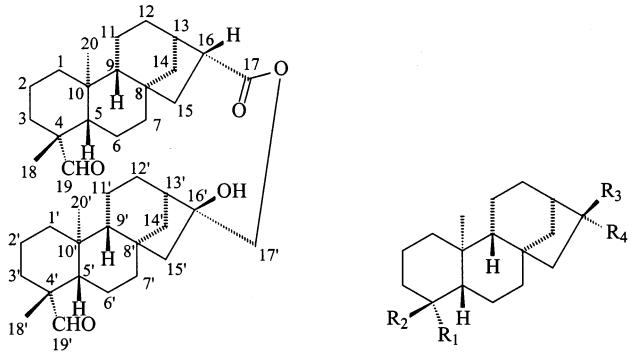
A phytochemical investigation on the stems of *Annona squamosa* led to the isolation of six new *ent*-kaurane diterpenoids, annomosin A (16*β*-hydroxy-19-al-*ent*-kauran-17-yl 16*β*-hydro-19-al-*ent*-kauran-17-oate) (**1**), annosquamosin C (16α-hydro-17-hydroxy-19-nor-*ent*-kauran-4α-ol) (**2**), annosquamosin D (16*β*-acetoxy-17-hydroxy-19-nor-*ent*-kauran-4α-ol) (**3**), annosquamosin E (16*β*-hydroxy-17-acetoxy-19-nor-*ent*-kauran-4α-formate) (**4**), annosquamosin F (16*β*-hydroxy-17-acetoxy-18-nor-*ent*-kauran-4*β*-hydroperoxide) (**5**), and annosquamosin G (16*β*,17-dihydroxy-18-nor-*ent*-kauran-4*β*-hydroperoxide) (**6**), along with 14 known *ent*-kaurane diterpenoids. The structures of **1**−**6** were elucidated by spectroscopic data interpretation. Compound **1** is the first dimeric *ent*-kaurane derivative to have been reported from a plant in the family Annonaceae. Certain useful NMR data were generalized to determine the stereochemistry of C-16 among the *ent*-kaurane diterpenoids investigated. *ent*-Kaur-16-en-19-oic acid (**9**) and 16α-hydro-19-al-*ent*-kauran-17-oic acid (**17**) showed complete inhibitory effects on rabbit platelet aggregation at 200 *μ*M.

The tetracyclic diterpenoid *ent*-kaurane is regarded as an intermediate in the biogenesis of the gibberellin plant growth hormones.[1] The biological activity of medicinal plants, such as exhibiting antimicrobial, antiinflammatory, cardiovascular, diuretic, anti-HIV, and cytotoxic effects, can sometimes be attributed in part to the presence of *ent*-kaurane diterpenoid constituents.[2−5] As part of our systematic phytochemical investigation of the genus *Annona*, nearly 40 *ent*-kaurane diterpenoids have been isolated from the unripe fruits of *Annona glabra*[6] and *A. squamosa*[4] and from the stems of *A. glabra*[7] and *A. cherimola*,[8,9] and some of these exhibited interesting biological activities.[6]

Annona squamosa L. (Annonaceae) was introduced to Taiwan during the 17th century.[10] Today, the plant is in large-scale cultivation in southern and southeastern Taiwan as an edible fruit. In Taiwan, the powdered seeds of *A. squamosa* have been used as a fish poison or an insecticide, the crushed leaves employed in the treatment of ulcers and wounds, and the bark given as a tonic against diarrhea.[11] In the present investigation, six new *ent*-kauranes [annomosin A (**1**) and annosquamosin C−G (**2**−**6**)] and 14 known *ent*-kauranes [4α-hydroxy-19-nor-*ent*-kauran-17-oic acid (**7**),[12] 19-nor-*ent*-kaurane-4α,16*β*,17-triol (annosquamosin B) (**8**),[4] *ent*-kaur-16-en-19-oic acid (**9**),[4] 16α-hydro-*ent*-kauran-17,19-dioic acid (**10**),[13] 16*β*-hydro-*ent*-kauran-17,19-dioic acid (**11**),[4] 17-hydroxy-16α-*ent*-kauran-19-oic acid (**12**),[4] 17-hydroxy-16*β*-*ent*-kauran-19-oic acid (**13**),[4] 16α,17-dihydroxy-*ent*-kauran-19-oic acid (**14**),[4] 16*β*,-17-dihydroxy-*ent*-kauran-19-oic acid (**15**),[4] 16*β*-hydroxy-17-acetoxy-*ent*-kauran-19-oic acid (**16**),[14] 16α-hydro-19-al-*ent*-kauran-17-oic acid (**17**),[15] 17-hydroxy-16*β*-*ent*-kauran-19-al (**18**),[4] 16α,17-dihydroxy-*ent*-kauran-19-al (**19**),[16] and 16*β*,17-dihydroxy-*ent*-kauran-19-al (**20**)[4]] have been isolated from a MeOH-soluble extract of the stems of this plant. The structures of **1**−**6** were elucidated on the basis of their spectral properties. Some of the known *ent*-kauranes, namely, **10**/**11**, **12**/**13**, **14**/**15**, and **19**/**20**, were isolated as pairs of diastereomers. Certain useful NMR observations concerning the stereochemistry at C-16 of the *ent*-kaurane diterpenoids were made.

Compound **9** has been reported previously as an inhibitor of malondialdehyde generation in rat platelets in response to thrombin,[17] so the isolated *ent*-kauranes were examined for inhibitory effects in rabbit platelet aggregation. The isolation and biological activity of the *ent*-kauranes **1**−**20** (Chart 1) from *A. squamosa* stems are reported herein.

Chart 1



1

	R_1	R_2	R_3	R_4
2	OH	CH_3	CH_2OH	H
3	OH	CH_3	$OCOCH_3$	CH_2OH
4	OCHO	CH_3	OH	CH_2OCOCH_3
5	CH_3	OOH	OH	CH_2OCOCH_3
6	CH_3	OOH	OH	CH_2OH
7	OH	CH_3	COOH	H
8	OH	CH_3	OH	CH_2OH
9	COOH	CH_3	$=CH_2$	
10	COOH	CH_3	COOH	H
11	COOH	CH_3	H	COOH
12	COOH	CH_3	CH_2OH	H
13	COOH	CH_3	H	CH_2OH
14	COOH	CH_3	CH_2OH	OH
15	COOH	CH_3	OH	CH_2OH
16	COOH	CH_3	OH	CH_2OCOCH_3
17	CHO	CH_3	COOH	H
18	CHO	CH_3	H	CH_2OH
19	CHO	CH_3	CH_2OH	OH
20	CHO	CH_3	OH	CH_2OH

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Table 1. ^{1}H NMR (400 MHz), ^{13}C (100 MHz) NMR, and HMBC Data of **1** in $CDCl_3$

position	δ_H (*J* in Hz)	δ_C	HMBC
1	0.79, td (13.2, 4.4)	39.8, t	C-3, C-20
	1.84, m		
2	1.50, m	18.3, t	
3	1.00, m	34.2, t	
	2.12, br d (7.6)		
4		48.4, s	
5	1.15, m	56.5, d	C-1, C-4, C-10, C-19, C-20
6	1.85, m	19.6, t	
7	1.46, m	41.7, t	C-8
8		44.9, s	
9	1.04, m	54.5, d	C-8, C-12
10		39.4, s	
11	1.50, m	18.7, t	C-12
12	1.52, m	30.9, t	
13	2.47, br s	41.1, d	
14	1.23, m	38.3, t	C-8, C-9, C-12
	1.87, m		
15	1.69, m	44.7, t	C-8, C-9, C-17
16	2.67, t (7.6)	45.4, d	C-12, C-13, C-15, C-17
17		177.6, s	
18	0.99, s	24.3, q	C-3, C-4, C-5, C-19
19	9.72, s	205.9, s	C-3, C-4
20	0.85, s	16.2, q	C-1, C-5, C-9, C-10
1′	0.79, td (13.2, 4.4)	39.7, t	C-3′, C-20′
	1.84, m		
2′	1.50, m	18.3, t	
3′	1.00, m	34.1, t	
	2.12, br d (7.6)		
4′		48.4, s	
5′	1.15, m	56.5, d	C-1′, C-4′, C-10′, C-19′, C-20′
6′	1.72, m	20.4, t	
7′	1.58, m	40.9, t	C-8′
8′		43.6, s	
9′	1.12, m	55.5, d	C-8′
10′		39.3, s	
11′	1.58, m	18.5, t	C-12′
12′	1.48, m	26.6, t	C-9′
	1.78, m		
13′	2.00, m	41.3, d	
14′	1.10, m	38.4, t	C-12′, C-15′, C-16′
	1.95, m		
15′	1.48, m	52.4, t	C-9′, C-16′, C-17′
16′		78.8, s	
17′	3.88, d (11.2)	71.0, t	C-13′, C-15′, C-16′, C-17
	4.02, d (11.2)		
18′	0.97, s	24.3, q	C-3′, C-4′, C-5′, C-19′
19′	9.72, s	205.8, s	C-3′, C-4′
20′	0.87, s	16.3, q	C-1′, C-5′, C-9′, C-10′

Results and Discussion

The MeOH extract of the stems of *A. squamosa* was partitioned with H_2O and $CHCl_3$. The $CHCl_3$ solution was extracted with 3% HCl to remove alkaloids, and the neutral $CHCl_3$ extract was subjected to repeated column chromatography and HPLC (see Experimental Section) to give the individual *ent*-kaurane diterpenes.

Compound **1**, obtained as white needles, was assigned the molecular formula $C_{40}H_{60}O_5$ by HREIMS. The degree of unsaturation calculated from the molecular formula was 11. The IR spectrum showed the presence of a hydroxyl group (3519 cm^{-1}) and an ester carbonyl group (1718 cm^{-1}). The ^{1}H NMR spectrum of **1** showed four tertiary methyl groups at δ 0.85 (3H, s), 0.87 (3H, s), 0.97 (3H, s), and 0.99 (3H, s), two aldehyde groups at δ 9.72 (2H, s), and one oxymethylene group at δ 3.88 and 4.02 (each 1H, d, J = 11.2 Hz), in which the oxymethylene protons were shifted downfield because of formation of an ester. ^{13}C NMR and DEPT experiments revealed the presence of 40 carbons: 19 methylenes (including one oxymethylene at δ 71.0), seven methines, four methyls, and 10 quaternary carbons (including two aldehyde carbonyls at δ 205.8 and 205.9 and one ester carbonyl at δ 177.6). The above data indicated

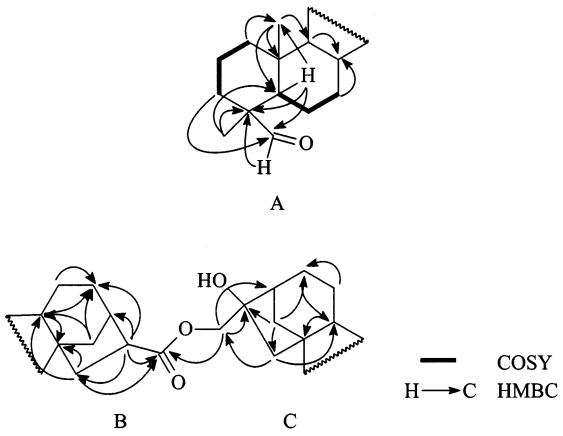

Figure 1. COSY and HMBC correlations of the partial moieties of **1**.

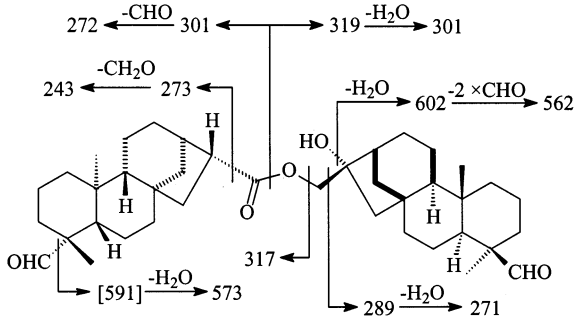

Figure 2. EIMS fragmentation (*m*/*z* values) of **1**.

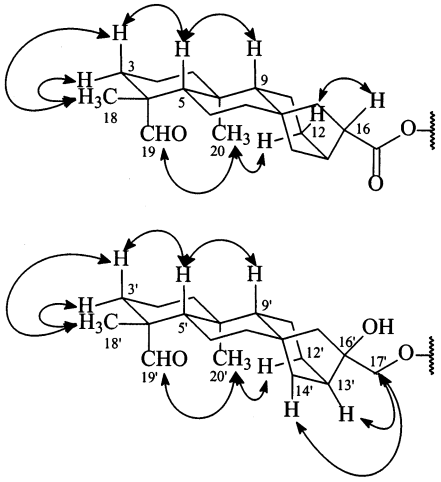

Figure 3. Key NOESY correlations of the two monomers of **1**.

that **1** is a dimeric diterpene. On analysis of the ^{1}H−^{1}H COSY, HMQC, and HMBC spectra (Table 1 and Figure 1), two sets of the moiety A could be proposed, which were similar to the A/B ring system of known *ent*-kauran-19-al (**17**−**20**) derivatives.[4,15,16] Moieties B and C (Figure 1) could also be established. A key HMBC correlation of the oxymethylene protons and an ester carbonyl carbon indicated that moieties B and C were linked. Moreover, moieties B and C were connected with two moieties of A, respectively, by HMBC correlations of H-7/C-8, H-12/C-9, and H-20/C-9 (H-7′/C-8′, H-12′/C-9′, and H-20′/C-9′). On analysis of the data available and by considering the previous literature, the A−B moiety and the A−C moiety of **1** were determined to be 19-al-*ent*-kauran-17-oic acid and 16,17-dihydroxy-*ent*-kauran-19-al units, respectively.[4,15] These data suggested that compound **1** is a novel dimeric *ent*-kaurane diterpenoid. This proposal was further confirmed by the EIMS fragmentation pattern (Figure 2).

Table 2. ^{1}H NMR Data of Compounds **2**−**6** (*J* in Hz)[a]

position	2	3	4	5	6
1	0.73, td (12.4, 4.8)	0.79, td (12.4, 4.4)	0.85, td (13.2, 3.6)	0.83, td (12.4, 4.4)	0.82, m
	1.61, m	1.76, m	1.90, m	1.76, m	1.73, m
2	1.50, m	1.54, m	1.64, m	1.65, m	1.62, m
			1.93, m	1.94, m	2.49, m
3	0.97, m	1.69, m	1.29, td (14.6, 4.4)	1.68, m	1.88, m
	1.91, m	1.95, br d (13.2)	2.60, br d (14.6)		2.12, m
5	0.97, m	1.23, m	0.89, m	1.46, m	1.80, m
6	2.15, m	1.64, m	1.50, m	1.58, m	1.56, m
		2.44, m	1.70, m		
7	1.49, m	1.50, m	1.44, m	1.39, m	1.38, m
9	1.31, m	1.39, m	1.11, m	1.20, m	1.29, m
11	1.50, m	1.38, m	1.44, m	1.26, m	1.30, m
		2.26, m	1.64, m	1.68, m	2.00, m
12	1.53, m	1.59, m	1.58, m	1.54, m	1.59, m
	1.70, m	2.23, m	1.80, m	1.78, m	2.21, m
13	2.34, br s	2.27, m	2.06, m	2.00, m	2.45, m
14	1.48, m	1.23, m	1.09, m	1.03, m	1.22, m
	1.67, m	2.06, dd (12.0, 1.6)	2.08, m	1.95, m	1.88, m
15	1.08, m	1.78, m	1.49, m	1.46, m	1.58, m
	1.55, m				1.79, m
16	2.42, m				
17	3.95, d (7.6)	4.28, d (11.2)	3.91, d (11.2)	3.91, d (11.2)	3.77, d (10.8)
		4.34, d (11.2)	4.04, d (11.2)	4.04, d (11.2)	3.86, d (10.8)
18	1.27, s	1.31, s	1.51, s	1.09, s	1.26, s
19	0.91, s	1.04, s	1.11, s	1.02, s	1.03, s
OH	5.90, br s	5.20, br s			6.40, br s
		6.11, br s			
OOH					12.44, br s
OCHO			8.07, s		
$OCOCH_3$		1.94, s	2.11, s	2.11, s	

[a] Spectra recorded in pyridine-d_5 (**2**, **3**, and **6**) or $CDCl_3$ (**4** and **5**) solution at 400 MHz.

In the NOESY spectrum of **1** (Figure 3), a correlation between H-12 and H-16 confirmed that the stereochemistry of H-16 was *β*. The stereochemistry of OH-16′*β* was established by the NOE correlations between H-17′/H-13′ and H-17′/H-14′. On the other hand, the two aldehyde groups in C-19 and C-19′ were confirmed unambiguously to be in an axial orientation from the NOEs between Me-18/H-3*β* and Me-18′/H-3′*β*, respectively. Thus, the structure of **1** was determined to be 16*β*-hydroxy-19-al-*ent*-kauran-17-yl 16*β*-hydro-19-al-*ent*-kauran-17-oate and has been named annomosin A.

In previous reports on plants of the Annonaceae, dimeric diterpenoids have been characterized as composed of one *ent*-kaurane monomer and one labdane monomer and were isolated only from *Xylopia* species.[18−20] Annomosin A (**1**) is the first dimer of two *ent*-kaurane monomeric units isolated from a plant in the Annonaceae. This type of dimeric *ent*-kaurane has only been isolated previously from the traditional Chinese medicine "Bei-Mu", prepared from the bulbs of *Fritillaria thunbergii* (Liliaceae) and related species.[21,22]

Compound **2** was isolated as a white powder. The HREIMS and DEPT spectra established the molecular formula of **2** as $C_{19}H_{32}O_2$, which indicated four degrees of unsaturation. The IR spectrum of **2** exhibited the presence of a free hydroxyl group (3436 cm^{-1}). The ^{1}H NMR spectrum (Table 2) showed two tertiary methyls at *δ* 0.91 and 1.27 and one oxymethylene at *δ* 3.95. In the ^{13}C NMR (Table 3) and the DEPT spectra, compound **2** revealed 19 carbons, including two methyls, 10 methylenes, four methines, and three quaternary carbons. These data were similar to those of annosquamosin B (19-nor-*ent*-kaurane-4α,16*β*,17-triol) (**8**),[4] indicating that **2** has a 19-nor-*ent*-kauran-4α-ol skeleton.

In its other NMR features, the H-17 signal at *δ* 3.95 (2H, d, $J = 7.6$ Hz) and C-17 signal at *δ* 63.2 of **2** were different from those of **8** [δ_{H-17} 3.78 and 3.88 (each 1H, d, $J = 11.0$ Hz); δ_{C-17} 69.1], indicating that compound **2** possesses a 16-hydro-17-hydroxy-*ent*-kaurane skeleton.[4] The different C-16 signals of **2** [*δ* 43.7 (d)] and **8** [*δ* 78.3(s)] also suggested this feature. The structure proposed was confirmed by a mass spectral peak appearing an *m*/*z* 261, which indicated the facile loss of CH_2OH. In the NOESY spectrum (Figure 4), a significant correlation between H-11 and H-17 confirmed the stereochemistry of the CH_2OH group to be *β*. On the other hand, the hydroxyl group in C-4 was determined to be in an axial orientation by the NOE correlation between Me-18 and H-3*β*. Thus, the structure of **2** (annosquamosin C) was determined to be 16α-hydro-17-hydroxy-19-nor-*ent*-kauran-4α-ol.

Table 3. ^{13}C NMR Data of Compounds **2**−**6**[a]

position	2	3	4	5	6
1	39.9, t	40.2, t	41.3, t	40.8, t	41.5, t
2	19.2, t	20.2, t	18.5, t	18.8, t	19.4, t
3	40.7, t	43.7, t	36.3, t	35.3, t	36.3, t
4	71.1, s	71.0, s	84.3, s	84.8, s	83.2, s
5	57.3, d	57.4, d	56.1, d	50.4, d	50.9, d
6	19.7, t	19.3, t	19.5, t	19.0, t	19.6, t
7	41.9, t	41.9, t	39.6, t	39.1, t	39.7, t
8	44.2, s	44.1, s	43.5, s	43.6, s	43.8, s
9	57.8, d	58.0, d	56.3, d	56.7, d	57.4, d
10	39.8, s	40.1, s	39.3, s	40.0, s	40.2, s
11	19.9, t	19.4, t	17.6, t	18.8, t	19.3, t
12	26.2, t	27.3, t	26.6, t	26.6, t	27.5, t
13	37.6, d	42.4, d	41.4, d	41.4, d	41.8, d
14	43.4, t	38.6, t	38.3, t	38.3, t	38.6, t
15	44.5, t	53.6, t	52.5, t	52.4, t	53.5, t
16	43.7, d	77.8, s	78.5, s	78.5, s	79.7, s
17	63.2, t	72.0, t	71.2, t	71.2, t	70.5, t
18	23.2, q	23.5, q	26.3, q	18.1, q	18.8, q
19	17.3, q	17.3, q	17.3, q	17.2, q	17.5, q
OCHO			160.6, d		
$OCOCH_3$		171.1, s	171.3, s	171.4, s	
$OCOCH_3$		20.8, q	20.9, q	20.9, q	

[a] Spectra recorded in pyridine-d_5 (**2**, **3**, and **6**) or $CDCl_3$ (**4** and **5**) solution at 100 MHz.

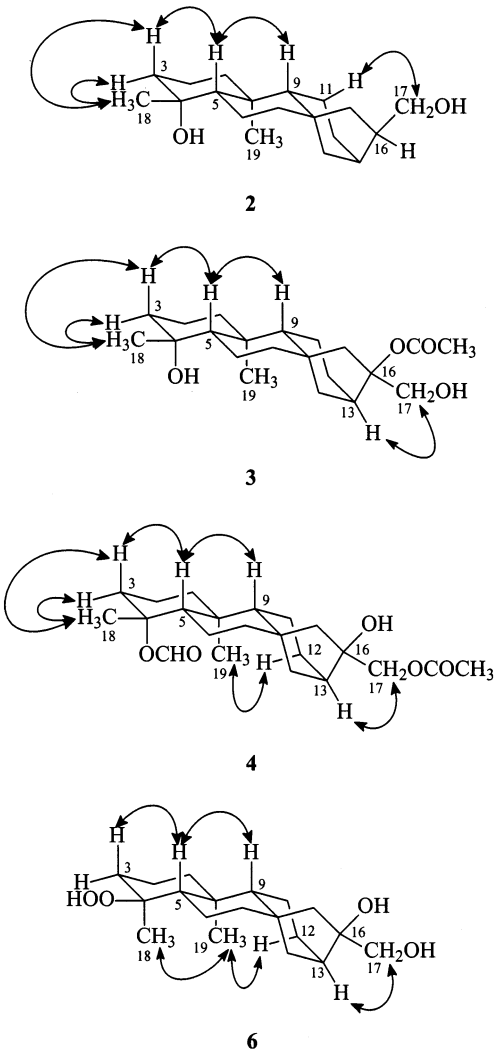

Figure 4. Key NOESY correlations of **2**, **3**, **4**, and **6**.

Compound **3** was assigned the molecular formula $C_{21}H_{34}O_4$, from its HREIMS, indicating five degrees of unsaturation. Its IR spectrum showed the presence of a hydroxyl group (3430 cm^{-1}) and an ester carbonyl group (1710 cm^{-1}). The ^1H NMR spectrum (Table 2) of **3** showed signals of three methyls at δ 1.04 (3H, s), 1.31 (3H, s), and 1.94 (3H, s) and one oxymethylene at δ 4.28 and 4.34 (each 1H, d, $J = 11.2$ Hz). The ^{13}C NMR (Table 3) and the DEPT spectra indicated that **3** contains 21 carbons, including three methyls, 10 methylenes, three methines, and five quaternary carbons. These data were similar to those of **2** and **8**,[4] indicating that **3** is also a 19-nor-*ent*-kauran-4α-ol diterpene with an acetoxy group.

The proton signal at δ 1.94 (s) and the carbon signals at δ 20.8 (q) and 171.1 (s) were attributable to an acetoxy group, which was attached to C-16 (δ 77.8). The proton signals of H-17 (δ 4.28 and 4.34) were shifted downfield because of the anisotropic effect of the C-16 acetoxy group. The NOESY correlation between H-17 and H-13 (Figure 4) proved the 16β-acetoxy stereochemistry. Thus, compound **3**, annosquamosin D, was established as 16β-acetoxy-17-hydroxy-19-nor-*ent*-kauran-4α-ol.

Compound **4**, obtained as a white powder, was assigned the molecular formula $C_{22}H_{34}O_5$ by HREIMS, representing six degrees of unsaturation. The IR spectrum showed a hydroxyl absorption at 3415 cm^{-1} and a strong ester carbonyl absorption at 1722 cm^{-1}. The ^1H NMR spectrum (Table 2) exhibited three tertiary methyls at δ 1.11 (3H, s), 1.51 (3H, s), and 2.11 (3H, s), one broad doublet at δ 2.60 (1H, $J = 14.6$ Hz), two doublets at δ 3.91 and 4.04 (each 1H, $J = 11.2$ Hz), and one singlet at δ 8.07 (1H). The characteristic signal at δ 8.07 (s) and the carbon signal at δ 160.6 (d) were attributable to a formic ester moiety,[23] which was substantiated by the EIMS fragment at *m*/*z* 332 for $[M - CHOOH]^+$. The two doublet protons at δ 3.91 and 4.04, one methyl at δ 2.11, and the carbon signals at δ 71.2 (t), 171.3 (s), and 20.9 (q) indicated the presence of a $-CH_2OCOCH_3$ moiety. The ^{13}C NMR spectrum (Table 3) and a DEPT experiment on **4** indicated a total of 22 carbons, as three methyls, 10 methylenes, four methines, and five quaternary carbons. On the basis of these observations, compound **4** was assigned as a 16-hydroxy-17-acetoxy-nor-*ent*-kaurane diterpenoid[14] with a formic ester moiety. The C-4 signal at δ 84.3 suggested that this position was substituted to form the formic ester. The key NOESY correlation between Me-18 and H-3β suggested that the Me-18 and the formic ester unit are in β-equatorial and α-axial orientations, respectively. The 16β-hydroxy stereochemistry was established by a NOE correlation (Figure 4) between H-13 and H-17. Thus, the structure of **4**, annosquamosin E, was determined as 16β-hydroxy-17-acetoxy-19-nor-*ent*-kauran-4α-formate. The 19-nor-*ent*-kauran-4-formate moiety has been synthesized via the Baeyer–Villiger rearrangement of *ent*-kaurane-19-al.[24] This is the first time that a 19-nor-*ent*-kauran-4-formate analogue has been isolated from a natural source.

Annosquamosin F (**5**) was obtained as a white powder, and the molecular formula $C_{21}H_{34}O_5$ was assigned by HREIMS. Five degrees of unsaturation were determined. The IR spectrum showed a free hydroxyl group absorption at 3415 cm^{-1} and a strong ester carbonyl absorption at 1722 cm^{-1}. The ^1H NMR spectrum (Table 2) exhibited three tertiary methyls at δ 1.02 (3H, s), 1.09 (3H, s), and 2.11 (3H, s, $-CH_2OCOCH_3$) and two doublets at δ 3.91 and 4.04 (each 1H, $J = 11.2$ Hz, $-CH_2OCOCH_3$). The ^{13}C NMR spectrum (Table 3) and a DEPT experiment on **5** showed a total of 21 carbons, as three methyls, 10 methylenes, three methines, and five quaternary carbons. These data suggest that **5** belongs to the group of compounds with a 16β-hydroxy-17-acetoxy-nor-*ent*-kaurane moiety.[14] The 16β-hydroxy stereochemistry was established by the NOESY correlation between H-13 and H-17. These NMR data were very close to those of **4**, except for the signals for Me-18 (**4**, δ_H 1.51, δ_C 26.3; **5**, δ_H 1.09, δ_C 18.1), the C-5 signal (**4**, δ 56.1; **5**, δ 50.4), and the disappearance of the resonance due to the C-4 OCHO group. On analysis of the EIMS, the fragments at *m*/*z* 333 $[M - OOH]^+$ and 315 $[M - OOH - H_2O]^+$ suggested a hydroperoxide group attached to the C-4 position. The γ-gauche effect of the hydroperoxide group results in a shielding effect on the C-5 (δ 50.4) signal.[25] There was a NOE correlation between the two upfield tertiary methyls which were affected by the anisotropic effect of the *ent*-kaurane A ring.[26] Both confirmed the hydroperoxide group being in a β-equatorial orientation. Thus, the structure of **5** was determined as 16β-hydroxy-17-acetoxy-18-nor-*ent*-kauran-4β-hydroperoxide.

Annosquamosin G (**6**) was obtained as a white powder, and the molecular formula $C_{19}H_{32}O_4$ was assigned by HREIMS, indicating four degrees of unsaturation. The IR spectrum exhibited a free hydroxyl group absorption at 3415 cm^{-1}. The ^1H NMR spectrum (Table 2) exhibited two tertiary methyls (δ 1.03 and 1.26), two doublets (δ 3.77 and 3.86, each 1H, $J = 10.8$ Hz, $-CH_2OH$), and two broad singlets (δ 6.40, 1H, −OH, δ 12.44, 1H, −OOH). The ^{13}C NMR spectrum (Table 3) and a DEPT experiment on **6**

Table 4. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compounds **10** and **11**[a]

	10		**11**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
13	2.74, br t (4.8)	39.5	2.79, br d (3.6)	41.6
15		40.8		45.2
16	3.16, dt (12.0, 6.0)	45.3	2.93, d (5.6)	46.2
17		176.9		179.7

[a] Spectra recorded in pyridine-d_5.

showed a total of 19 carbons, as two methyls, 10 methylenes, three methines, and four quaternary carbons [including two oxygenated carbons at δ 79.7 (C-16) and 83.2 (C-4)]. These results indicated that **6** possesses a 16,17-dihydroxy-nor-*ent*-kaurane skeleton[4] along with a hydroperoxide group. The hydroperoxide group was confirmed by the EIMS fragments at m/z 273 $[M - H_2O - OOH]^+$ and 255 $[M - 2 \times H_2O - OOH]^+$.

The γ-gauche effect of the hydroperoxide group caused a shielding effect on the C-5 signal (δ 50.9),[25] and a NOE correlation between the two upfield methyls (δ 18.8 and 17.5) supported the β-equatorial orientation of the hydroperoxide group (Figure 4). The 16β-hydroxy stereochemistry was also established by the NOE correlation between H-13 and H-17 (Figure 4). Thus, the structure of **6** was determined as 16β,17-dihydroxy-18-nor-*ent*-kauran-4β-hydroperoxide.

This is the first time that the nor-*ent*-kaurane hydroperoxides have been isolated. In previous studies, norditerpene alcohols, formates, and hydroperoxides were produced by the autoxidation of the diterpene aldehydes.[27] The nor-*ent*-kaurane diterpenoids **2**−**8** can be derived from autoxidation and Baeyer−Villiger oxidation of the corresponding aldehydes.

The known compounds **7**−**20** were identified on the basis of comparison of their spectral and physical data with authentic compounds and values reported in the literature. Among them, four pairs of diastereomeric *ent*-kaurane diterpenes were identified: compounds **10**/**11**, **12**/**13**, **14**/**15**, and **19**/**20**. However, there have been few publications describing systematic investigations by NMR of this type of compounds. Crucial NMR shift values are listed in Tables 4−7 to help distinguish these diastereomers. In Table 4, the H-16 ^{1}H NMR resonance signals of **10** and **11** are significantly different, because the anisotropic effect of the *ent*-kaurane C-ring made the H-16α shift downfield. It also caused the ^{13}C NMR chemical shift of the 17-carboxylic acid to shift upfield. On the other hand, the anisotropic effect of the carboxylic acid affected the resonance of C-15 (by about 5 ppm). The differences observed offer important evidence to distinguish this type of diastereomer. In the NOESY spectrum of **10**, correlations between H-16α and H-13 or H-16α and H-14 proved its stereochemistry. The H-16β stereochemistry of **11** was confirmed by the NOE correlation between H-16β and H-11.

The key NOE correlation between H-17 and H-11 suggested the stereochemistry of 17-hydroxy-16α-*ent*-kauran-19-oic acid (**12**). The anisotropic effect of the *ent*-kaurane C-ring shielded the H-17 signal of **12** and deshielded the H-17 signal of **13** (see Table 5).

Similar methods used to demonstrate the NOE correlation between H-17 and H-11 or H-17 and H-13 helped establish the stereochemistries of the 16,17-dihydroxy-*ent*-kaurane diterpenes. Relative to the 17-hydroxy-16α-*ent*-kaurane diterpenes, a 16α-hydroxy group results in the ^{1}H NMR chemical shift of the 17-oxymethene becoming more deshielded, but a 16β-hydroxy group makes the ^{13}C NMR shift of C-17 more deshielded. Diagnostic carbon signals of C-13 and C-16, especially C-13, are also key points in distinguishing this type of diastereomer (see Table 6). Similar results (Table 7) were observed from the NMR data measured in different solvents.

Table 5. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compounds **12** and **13**[a]

	12		**13**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
17	3.40, d (7.0)	67.4	3.72, d (7.0)	64.2

[a] Spectra recorded in $CDCl_3$.

Table 6. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compounds **14** and **15**[a]

	14		**15**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
13		45.9		41.7
16		81.7		78.8
17	4.07, d (11.2) 4.17, d (11.2)	66.9	3.78, d (11.2) 3.86, d (11.2)	70.4

[a] Spectra recorded in pyridine-d_5.

Table 7. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compounds **19** and **20**[a]

	19		**20**	
position	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C
13		45.6		40.6
16		81.7		79.8
17	3.63, d (11.2) 3.75, d (11.2)	66.3	3.39, d (11.2) 3.49, d (11.2)	69.7

[a] Spectra recorded in $CDCl_3$.

Table 8. Inhibitory Effects of Selected *ent*-Kauranes on the Aggregation of Washed Rabbit Platelets[a]

	percent inhibition (%)			
compound (200 μM)	AA (100 μM)	collagen (10 μg/mL)	PAF (1 ng/mL)	thrombin (0.05 U/mL)
3	4.9 ± 2.2	10.6 ± 5.9	9.3 ± 3.2	2.0 ± 1.2
7	4.5 ± 1.6	30.5 ± 5.7	13.3 ± 1.1	2.0 ± 1.2
8	8.7 ± 2.1	21.1 ± 3.2	8.5 ± 2.2	1.6 ± 0.9
9	24.8 ± 3.9	100	12.6 ± 3.3	5.6 ± 0.3
11	15.4 ± 2.8	56.9 ± 14.0	15.2 ± 0.8	6.0 ± 2.5
13	4.1 ± 1.3	23.8 ± 6.8	7.4 ± 0.6	2.8 ± 1.4
14	7.2 ± 2.0	2.3 ± 1.4	8.9 ± 0.5	0.4 ± 0.3
15	14.9 ± 3.8	8.6 ± 4.0	14.4 ± 3.4	6.8 ± 2.3
16	10.8 ± 5.1	30.1 ± 6.1	11.1 ± 2.6	8.0 ± 2.8
17	100	100	11.8 ± 2.4	6.8 ± 2.3
19	0.5 ± 0.4	4.9 ± 2.9	10.3 ± 1.7	3.2 ± 1.4
20	9.6 ± 2.1	17.6 ± 7.2	5.5 ± 3.7	0

[a] Values are presented as means ± SEM (n = 3 or 4).

In screening the isolated compounds for biological activity, their inhibitory effects were examined on platelet aggregation induced by arachidonic acid (AA), collagen, thrombin, and platelet-activating factor (PAF). Compound **17** showed complete inhibition of platelet aggregation induced by AA and collagen at 200 μM, and **9** showed complete inhibition of platelet aggregation induced by collagen at the same dose. Also at 200 μM, **11** showed moderate inhibition of platelet aggregation induced by collagen. The other compounds evaluated were much less active (Table 8).

Experimental Section

General Experimental Procedures. Melting points were determined using a Yanagimoto micro-melting point apparatus and are uncorrected. Optical rotations were measured with a JASCO DIP-370 digital polarimeter. The UV spectra were obtained on a Hitachi 200-20 spectrophotometer. IR spectra

were measured on a Mattson Genesis II spectrophotometer. ^{1}H NMR (400 MHz, using pyridine-d_5 and $CDCl_3$ as solvent for measurement), ^{13}C NMR, HETCOR, DEPT, and NOESY spectra were obtained on a Varian NMR spectrometer (Unity Plus). Low-resolution EIMS were collected on a JEOL JMS-SX/SX 102A mass spectrometer or Quattro GC/MS spectrometer having a direct inlet system. High-resolution EIMS were measured on a JEOL JMS-HX 110 mass spectrometer. Silica gel 60 (Merck, 230−400 mesh) was used for column chromatography. A preparative column (Develosil ODS-10) was used for preparative HPLC (Shimadzu LC-10AT VP). A diode array detector (Shimadzu SPD-M10A VP) was used for HPLC. The TLC spots were detected by spraying with 50% H_2SO_4 and then heating on a hot plate.

Plant Material. Fresh stems of *A. squamosa* were collected from Pingtung, Taiwan, and identified by Dr. Hsin-Fu Yen (National Museum of Natural Science, Taichung, Taiwan) in May 2000. A voucher specimen (Annona 6) is deposited in the Graduate Institute of Natural Products, Kaohsiung Medical University, Kaohsiung, Taiwan.

Extraction and Isolation. Fresh stems of *A. squamosa* (15 kg) were extracted repeatedly with MeOH at room temperature. The combined MeOH extract was evaporated under reduced pressure to yield a dark brown syrup (550 g). The syrup was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$ solution was extracted with 3% HCl to remove alkaloids, and then the neutral $CHCl_3$ solution was dried and evaporated to leave a brownish viscous residue (160 g). The residue was subjected to Si gel column chromatography and eluted with gradually more polar $CHCl_3$−MeOH mixtures; the eluents were combined into 22 fractions on the basis of TLC. Fractions 1−5 were further purified by recrystallization to give **9** (30 g). Fraction 7 was further purified by recrystallization to give **17** (3 g). The residue of fraction 7 was eluted with *n*-hexane−EtOAc (6:1) to give **1** (10 mg). Fraction 8 was further purified by recrystallization to give **7** (1 g). Fraction 9 was eluted with $CHCl_3$ to give **3** (7 mg), **8** (10 mg), and **10** (5 mg). Fraction 10, eluting with *n*-hexane−EtOAc (1:10), was further purified by RP-HPLC (MeOH−H_2O, 72:28) to give **4** (5 mg), **5** (6 mg), **11** (18 mg), **13** (8 mg), and **16** (9 mg). Fraction 11 was eluted with *n*-hexane−EtOAc (2:1) to give **12** (6 mg). Fraction 13, eluted with *n*-hexane−EtOAc (1:40), was further purified by recrystallization and repeated Si gel column chromatography to give **15** (20 mg). Fraction 18 was chromatographed on RP-HPLC using MeOH−H_2O (70:30) to give **2** (5 mg) and **6** (4 mg). Fraction 19 was chromatographed on RP-HPLC using MeOH−H_2O (80:20) to give **14** (7 mg), **18** (6 mg), **19** (18 mg), and **20** (15 mg).

16*β*-Hydroxy-19-al-*ent*-kauran-17-yl 16*β*-hydro-19-al-*ent*-kauran-17-oate (annomosin A) (1): white needles; mp 170−171 °C; $[\alpha]_{25}^{D}$ −49.3° (*c* 0.12, $CHCl_3$); IR (KBr) ν_{max} 3519, 1718 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; FABMS *m/z* 621 $[M + 1]^+$ (21); EIMS (30 eV) *m/z* 602 $[M - H_2O]^+$ (3), 573 $[M - CHO - H_2O]^+$ (3), 562 $[M - H_2O - 2\times CHO]^+$ (4), 319 (2), 317 (2), 301 (38), 289 (100), 273 (36), 272 (12), 271 (30), 243 (19); HREIMS *m/z* 602.4331 $[M - H_2O]^+$ (calcd for $C_{40}H_{58}O_4$, 602.4335).

16α-Hydro-17-hydroxy-19-nor-*ent*-kauran-4-α-ol (annosquamosin C) (2): white powder; mp 156−158 °C; $[\alpha]_{26}^{D}$ −47.6° (*c* 0.03, MeOH); IR (KBr) ν_{max} 3436 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS (30 eV) *m/z* 292 $[M]^+$ (4), 274 $[M - H_2O]^+$ (30), 256 $[M - 2\times H_2O]^+$ (6), 261 $[M - CH_2OH]^+$ (5); HREIMS *m/z* 274.2290 $[M - H_2O]^+$ (calcd for $C_{19}H_{30}O$, 274.2296).

16*β*-Acetoxy-17-hydroxy-19-nor-*ent*-kauran-4α-ol (annosquamosin D) (3): white powder; mp 169−171 °C; $[\alpha]_{26}^{D}$ −147.0° (*c* 0.02, MeOH); IR (KBr) ν_{max} 3430, 1710 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS (30 eV) *m/z* 332 $[M - H_2O]^+$ (1), 314 $[M - 2\times H_2O]^+$ (5); HREIMS *m/z* 332.2357 $[M - H_2O]^+$ (calcd for $C_{21}H_{32}O_3$, 332.2351).

16*β*-Hydroxy-17-acetoxy-19-nor-*ent*-kauran-4-yl formate (annosquamosin E) (4): white powder; mp 172−174 °C; $[\alpha]_{26}^{D}$ −26.8° (*c* 0.05, MeOH); IR (KBr) ν_{max} 3415, 1722 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS (30 eV) *m/z* 332 $[M - CHOOH]^+$ (1), 305 $[M - CH_2OAc]^+$ (43), 259 $[M - CH_2OAc - CHOOH]^+$ (70); HREIMS *m/z* 305.2104 $[M - CH_2OAc]^+$ (calcd for $C_{19}H_{29}O_3$, 305.2117).

16*β*-Hydroxy-17-acetoxy-18-nor-*ent*-kauran-4-hydroperoxide (annosquamosin F) (5): white powder; mp 144−146 °C; $[\alpha]_{26}^{D}$ −39.2° (*c* 0.05, MeOH); IR (KBr) ν_{max} 3415, 1722 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS (30 eV) *m/z* 333 $[M - OOH]^+$ (2), 315 $[M - H_2O - OOH]^+$ (18), 255 $[M - CH_2OAc - H_2O - OOH]^+$ (66); HREIMS *m/z* 315.2311 $[M - H_2O - OOH]^+$ (calcd for $C_{21}H_{31}O_2$, 315.2324).

16*β*,17-Dihydroxy-18-nor-*ent*-kauran-4-hydroperoxide (annosquamosin G) (6): white powder; mp 175−177 °C; $[\alpha]_{26}^{D}$ −74.4° (*c* 0.02, MeOH); IR (KBr) ν_{max} 3415 cm^{-1}; ^{1}H and ^{13}C NMR data, see Tables 2 and 3; EIMS (30 eV) *m/z* 293 $[M - CH_2OH]^+$ (15), 288 $[M - 2\times H_2O]^+$ (5), 275 $[M - CH_2OH - H_2O - OOH]^+$ (14), 273 $[M - H_2O - OOH]^+$ (56), 255 $[M - 2\times H_2O - OOH]^+$ (27); HREIMS *m/z* 306.2188 $[M - H_2O]^+$ (calcd for $C_{19}H_{30}O_3$, 306.2195).

Platelet Aggregation Assays. The platelet aggregation assays were carried out according to established protocols.[28]

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