Mono-tetrahydrofuran Annonaceous Acetogenins from *Annona squamosa* as Cytotoxic Agents and Calcium Ion Chelators

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Eight new mono-tetrahydrofuran (THF)-type annonaceous acetogenins, squafosacins B, C, F, and G (**1**−**4**), squadiolins A−C (**5**−**7**), and *cis*-annotemoyin-1 (**8**), as well as eight known annonaceous acetogenins, glabranin, annotemoyins-1 and -2, bullatencin, *cis*-bullatencin, and uvariamicins-I, -II, and -III, were isolated from the seeds of *Annona squamosa* by HPLC. The structures of all new isolates were elucidated by using spectroscopic and chemical methods. Squadiolins A (**5**) and B (**6**) showed ng/mL potency against human Hep G2 hepatoma cells and significant cytotoxic activity against human MDA-MB-231 breast cancer cells. Squafosacin B (**1**) also exhibited significant cytotoxic activity against human Hep G2 and 3B hepatoma and MCF-7 breast cancer cells. In addition, the chelation of mono-THF acetogenins with calcium ions was investigated using isothermal titration calorimetry.

Plants of the family Annonaceae are regarded as important economic crops worldwide. In Taiwan, *Annona squamosa* is grown for its edible fruit. Annonaceous plants have also been widely investigated in pharmaceutical research during the last two decades because of the many antifungal and cytotoxic chemical constituents found in the leaves and bark.[1,2] Annonaceous acetogenins are bioactive secondary metabolites found primarily in annonaceous plants. More than 500 annonaceous acetogenins have been isolated from this plant family,[3] mostly from the seeds and stem bark. According to our literature survey, 56 annonaceous acetogenins have been found thus far from the seeds, leaves, and stem bark of *A. squamosa*. Acetogenins, which are naturally occurring polyketides, share similar carbon skeletons, but differ in the number, position, and configuration of their oxygen-containing moieties. Most acetogenins from *A. squamosa* are bis-tetrahydrofuran (THF) types (either adjacent or nonadjacent); fewer mono-THF-type compounds have been identified.

Annonaceous acetogenins exhibit potent anticancer, cytotoxic, antiparasitic, insecticidal, and immunosuppressive effects. During the past decade, medicinal chemists worldwide have exerted much effort to elucidate the mechanisms of action of these compounds, including inhibition of NADH oxidoreductase in the mitochondrial respiratory electron chain,[4] induction of programmed cell death (apoptosis),[5] and complexation with Ca^{2+} and Mg^{2+} as ionphores.[6–9] In specific examples, annonacin (a mono-THF acetogenin) causes apoptotic death of T24 tumor cells by inducing Bax expression and enhancing caspase-3 activity,[10] whereas bullatacin (a bis-THF acetogenin) induces apoptosis in Hep 2.2.15 cells in a time-dependent manner by decreasing both cAMP and cGMP levels.[11]

The abundance of plant material, particularly the seeds, and the associated significant cytotoxicity prompted us to investigate the chemical components from *A. squamosa*. Herein, we report 16 mono-THF annonaceous acetogenins from the seeds of this plant, including eight new compounds, squafosacins B, C, F, and G (**1**–**4**), squadiolins A−C (**5**–**7**), and *cis*-annotemoyin-1 (**8**), as well as eight known compounds, glabranin, [12] annotemoyin-1,[13] annotemoyin-2,[13] uvariamicin-I,[14,15] uvariamicin-II,[14,15] uvariamincin-III,[14] bullatencin,[16] and *cis*-bullatencin.[17] All structures were elucidated from spectroscopic data and chemical methods. The isolated compounds were screened for cytotoxic activity against various cancer cell lines. We also evaluated the interaction between selected mono-THF acetogenins and calcium ions by using NMR techniques and isothermal titration calorimetry (ITC), which is a useful tool for obtaining information on ligand binding with metal ions.

Results and Discussion

The bioactive *n*-hexane extract of *A. squamosa* L. was chromatographed on Si gel using reversed-phase preparative HPLC and recycle HPLC to give 16 pure mono-THF acetogenins as colorless, waxy solids. Squafosacin B (**1**), as well as squadiolins A (**5**), B (**6**), and C (**7**), were isolated and purified from fraction F16, together with the known glabranin. Squafosacin C (**2**) was separated and purified from fraction F15. Squafosacins F (**3**) and G (**4**) and *cis*-annotemoyin-1 (**8**), together with the known annotemoyin-1, annotemoyin-2, bullatencin, *cis*-bullatencin, and uvariamicins-I, -II, and -III, were isolated and purified from fraction F12. The eight known compounds were isolated from this plant for the first time. In addition, uvariamicins-I, -II, and -III were separated and purified individually in this study, while they were isolated previously only as a mixture.[14,15]

All of the isolated compounds possess a terminal α,β-unsaturated γ-lactone ring moiety (normal form), as indicated by UV absorption at 214−218 nm and IR bands at 1744−1752 cm^{-1}. In compounds **1**−**6** and **8**, ^1H and ^{13}C NMR spectra showed the presence of a methyl group at δ_H 1.38 (d, J = 7.0 Hz, 3H)/δ_C 19.2, two methines at δ_H 4.97 (qd, J = 7.0, 1.6 Hz, 1H)/δ_C 77.4 and δ_H 6.97 (q, J = 1.6 Hz, 1H)/δ_C 148.9, two quaternary carbons at δ_C 134.3 and 173.9, and a triplet methylene at δ 2.26 (t, J = 8.4 Hz, 2H)/δ_C 25.4. These resonances confirmed the presence of an α,β-unsaturated γ-lactone ring without a hydroxy group at C-4. Slight differences in the spectra of compound **7** [methine at δ_H 7.18 (q, J = 1.0 Hz, 1H)/δ_C 151.9, the quaternary carbons at δ_C 131.5 and 174.3, and an ABX system for a methylene function at δ 2.39 (dd, J = 15.2, 8.4 Hz, 1H) and 2.52 (dd, J = 15.2, 3.2 Hz, 1H)] indicated the presence of an α,β-unsaturated γ-lactone ring with a hydroxy group at C-4.[18] The absolute configuration of the sp^3 methine in the γ-lactone moiety (C-34/36) was determined from the CD spectra, in which a

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Table 1. ^{1}H NMR Chemical Shifts of Compounds **1**−**8** in $CDCl_3$ (measured at 400 MHz)[a]

position	1	2	3	4	5	6	7	8
3	2.260 t(7.2)	2.262 (m)	2.256 t(7.2)	2.243 t(7.6)	2.255 t(8.0)	2.260 t(8.4)	2.392 dd(15.2, 8.4) 2.515 dd(15.2, 3.2)	2.260 bt(5.6)
4	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.20–1.70	1.25–1.65	3.86 m	1.25–1.54
5	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.20–1.70	1.25–1.65	1.25–1.60	1.25–1.54
6–9	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.20–1.70	1.25–1.65	1.25–1.60	1.25–1.54
10	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.20–1.70	1.25–1.65	1.25–1.60	1.25–1.54
11	1.25–1.64	3.59 m	1.26–1.62	1.23–1.63	1.20–1.70	1.25–1.65	1.25–1.60	1.25–1.54
12	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.20–1.70	3.87 m	3.87 m	1.25–1.54
13	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.20–1.70	1.64, 1.98	1.59, 2.02	1.25–1.54
14	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.25 m	1.64, 1.98	1.59, 2.02	1.25–1.54
15	3.43 m	1.25–1.61	3.40 m	1.23–1.63	3.42 m	3.79 m	3.771 q(7.2)	1.25–1.54
16	3.84 m	1.25–1.61	3.80 m	1.23–1.63	3.42 m	3.45 m	3.391, m	1.25–1.54
17	1.95, 1.64	3.41 m	1.99, 1.68	1.23–1.63	1.25 m	1.25–1.65	1.25–1.60	3.42 m
18	1.95, 1.64	3.80 m	1.99, 1.68	1.23–1.63	1.39 m	1.25–1.65	1.25–1.60	3.82 m
19	3.84 m	2.01, 1.64	3.80 m	3.37 m	3.42 m	3.45 m	1.25–1.60	1.94, 1.74
20	3.43 m	1.98, 1.85	3.88 m	3.80 m	3.86 m	3.45 m	1.25–1.60	1.94, 1.74
21	1.25–1.64	3.80 m	1.26–1.62	2.00, 1.63	1.60, 2.01	1.25–1.65	3.391 m	3.82 m
22	1.25–1.64	3.41 m	1.26–1.62	1.98, 1.84	1.86 m	1.25–1.65	3.391 m	3.42 m
23	1.25–1.64	1.25–1.61	1.26–1.62	3.80 m	3.86 m	3.45 m	1.25–1.60	1.25–1.54
24	1.25–1.64	1.25–1.61	1.26–1.62	3.86 m	3.80 m	3.45 m	1.25–1.60	1.25–1.54
25	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.46 m	1.25–1.65	1.25–1.60	1.25–1.54
26	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.60 m	1.25–1.65	1.25–1.60	1.25–1.54
27	3.43 m	1.25–1.61	1.26–1.62	1.23–1.63	1.42 m	1.25–1.65	1.25–1.60	1.25–1.54
28	3.43 m	1.25–1.61	1.26–1.62	1.23–1.63	3.58 m	1.25–1.65	1.25–1.60	1.25–1.54
29	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.42 m	1.25–1.65	1.25–1.60	1.25–1.54
30	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.27–1.70	1.25–1.65	1.25–1.60	1.25–1.54
31	1.25–1.64	1.25–1.61	1.26–1.62	1.23–1.63	1.27–1.70	1.25–1.65	1.25–1.60	1.25–1.54
32	1.25–1.64	0.876 t(6.4)	0.872 t(6.8)	1.23–1.63	1.25 m	1.25 m	0.872 t(6.8)	0.875 t(6.4)
33	1.25–1.64	6.985 d(1.6)	6.981 d(1.2)	1.23–1.63	1.24 m	1.22 m	7.182 d(1.0)	6.983 d(1.6)
34	0.875 t(6.8)	4.990 qd(6.8, 1.6)	4.996 qd(6.8,1.6)	0.860 t(6.4)	0.874 t(7.2)	0.875 t(6.8)	5.053 qd(6.8, 1.0)	4.991 qd(6.8, 1.6)
35	6.984 d(1.2)	1.405 d(6.8)	1.398 d(6.8)	6.973 d(1.6)	6.973 q(1.6)	6.984 d(1.8)	1.428 d(6.8)	1.403 d(6.8)
36	5.00 qd(6.8,1.6)			4.984 qd(6.8, 1.6)	4.972 qd(7.0, 1.6)	4.991 qd(6.8, 1.8)		
37	1.403 d(6.8)			1.385 d(6.8)	1.396 d(7.0)	1.403 d(6.8)		

[a] Chemical shifts are in δ values.

positive $\pi-\pi^*$ Cotton effect ($\Delta\epsilon > 0$) indicated an *S*-configuration.[19,20] The oxygen-bearing moieties of the individual purified acetogenins were further determined by analysis of ^{1}H and ^{13}C NMR signals and EIMS fragments as described below.

In the ^{1}H and ^{13}C NMR spectra of compound **1**, two sets of signals at δ_H 3.84 (2H)/δ_C 82.6 and δ_H 3.40 (2H)/δ_C 74.0 and 74.6, in addition to signals at δ_H 1.95 (2H) and 1.64 (2H)/δ_C 28.8, indicated the presence of one THF ring with two flanking hydroxy groups in a relative *threo*/*trans*/*threo* configuration (Tables 1 and 2).[21,22] In addition, a signal at δ_H 3.40 (2H)/δ_C 74.5 and 74.4 indicated the presence of a vicinal diol group in a *threo* configuration.[23,24] An $[M]^+$ peak at *m*/*z* 624.4942 in the HRFABMS of compound **1** was consistent with the molecular formula $C_{37}H_{68}O_7$. Close examination of the EIMS fragmentation of **1** indicated that the THF ring was located between C-16/C-19 based on a base peak at *m*/*z* 295 and a peak at *m*/*z* 347 (C-19/C-20 cleavage − H_2O), and the vicinal diol was present at C-27/C-28 according to EIMS peaks at *m*/*z* 473 (C-27/C-28 cleavage − 2 H_2O) and 461 (C-26/C-27 cleavage − H_2O) (Scheme 1). Thus, the structure of **1** was fully determined as shown in Figure 1, and the compound has been given the name squafosacin B.

In the ^{1}H and ^{13}C NMR spectra of compound **2**, two sets of signals at δ_H 3.80 (2H)/δ_C 82.8 and 82.6 and δ_H 3.41 (2H)/δ_C 74.1, together with independent signals at δ_H 1.99 (2H) and 1.68 (2H)/δ_C 28.7, indicated the presence of a mono-THF ring with two flanking hydroxy groups in a relative *threo*/*trans*/*threo* configuration, as also found in compound **1** (Tables 1 and 2).[21,22] In contrast to compound **1**, a signal at δ_H 3.59 (1H)/δ_C 71.4 indicated the presence of an isolated hydroxy group in compound **2**.[25] An $[M + H]^+$ peak at *m*/*z* 581.4773 in the HRFABMS of compound **2** indicated a molecular weight of 580 and molecular formula of $C_{35}H_{64}O_6$. EIMS peaks at *m*/*z* 209 (C-10/C-11 cleavage) and 221 (C-11/C-12 cleavage − H_2O) suggested that the hydroxy group was at C-11, and the base peak at *m*/*z* 303 (C-17/C-18 cleavage − 2 H_2O) indicated that the THF ring was located between C-18/C-21. Thus, compound **2** has the structure shown in Figure 1 and has been named squafosacin C.

Compounds **3** and **4** were isolated and purified from fraction F12, together with seven known compounds. The ^{1}H and ^{13}C NMR spectra of compounds **3** and **4** indicated that both compounds have the same oxygen-bearing subunit, a THF moiety flanked with two hydroxy groups in *threo* and *erythro* configurations on the basis of signals at δ_H 3.85 (3H)/δ_C 83.2, 82.1, and 71.8 and δ_H 3.40 (1H)/δ_C 74.3 (Tables 1 and 2).[21,22] The two compounds were differentiated by analysis of their HRFABMS/HRESIMS and EIMS fragmentation. An $[M + H]^+$ peak at *m*/*z* 565.4761 in the HRFABMS of compound **3** indicated a molecular weight of 564 and molecular formula of $C_{35}H_{64}O_5$, whereas an $[M + Na]^+$ peak at *m*/*z* 615.4965 in the HRESIMS of compound **4** indicated a molecular weight of 592 and molecular formula of $C_{37}H_{68}O_5$. Close examination of EIMS fragmentation in both compounds located the THF moiety between C-16 and C-19 in compound **3** [base peak at *m*/*z* 295 (C-15/C-16 cleavage)] and between C-20 and C-23 in compound **4** [base peak at *m*/*z* 351 (C-19/C-20 cleavage)] (Scheme 1).

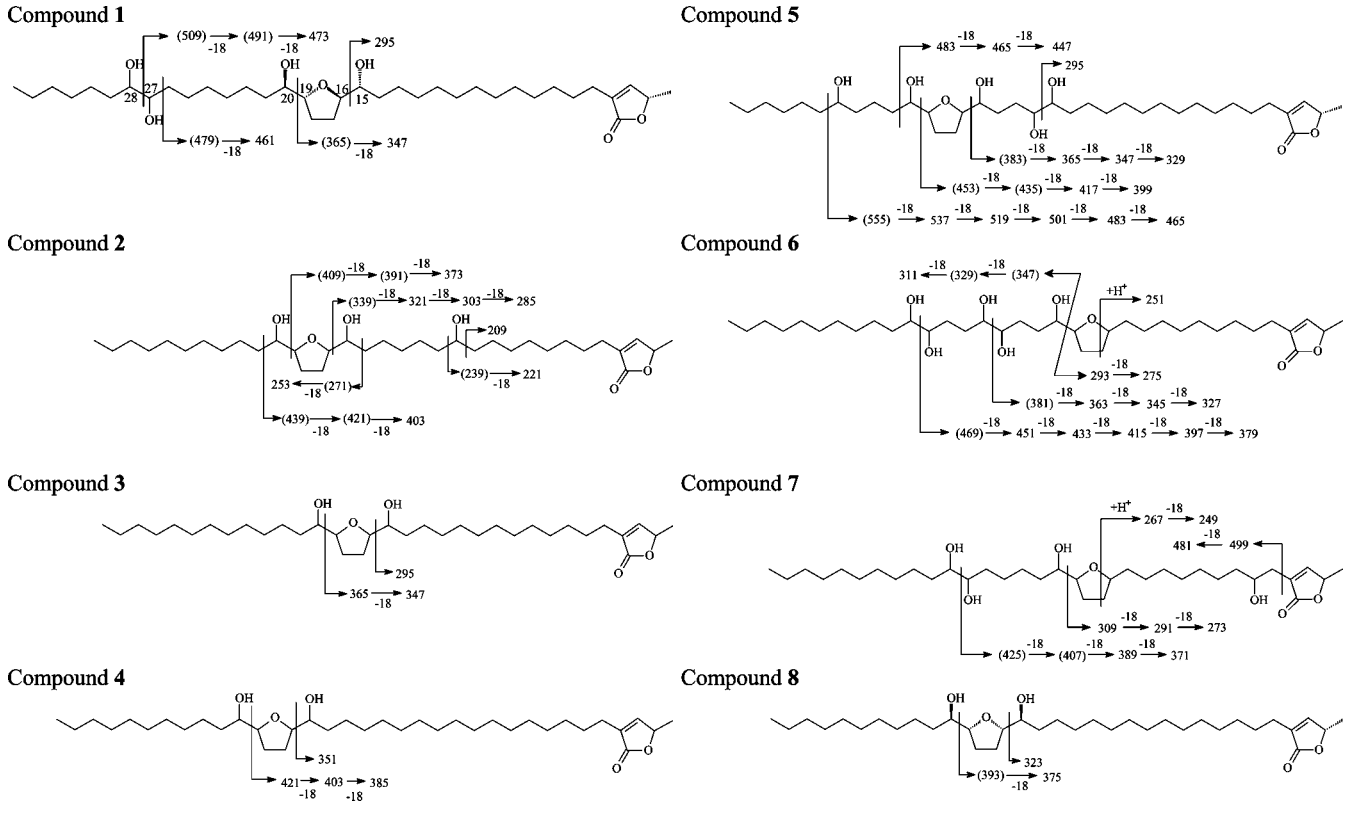
The relative configuration of the OH−THF−OH moiety could be either *threo*/*trans*/*erythro* or *threo*/*cis*/*erythro* and could not be determined from the complex proton signals between δ_H 2.00 and 1.64. Accordingly, to confirm the ring's relative configuration and determine the absolute configuration of the carbinol stereogenic centers, the (*S*)- and (*R*)-Mosher ester derivatives of compounds **3** and **4** were prepared by the modified methodology of Kinghorn et al.[26] ^{1}H−^{1}H COSY spectra of these MTPA derivatives showed correlations between H-15/H-16 and H-19/H-20. Thus, it was

Table 2. ^{13}C NMR Chemical Shifts (δ values) of Compounds **1**−**8** in $CDCl_3$ (measured at 100 MHz)

position	1	2	3	4	5	6	7	8
1	173.94	173.93	173.93	173.77	173.95	173.89	174.30	173.90
2	134.33	134.31	134.30	134.29	134.30	134.32	131.50	134.36
3	25.15	25.16	25.27	25.30	25.15	25.16	33.59[b]	25.15
4	27.38	27.38	27.37	27.40	27.37	27.41	69.97	27.38
5	25.7–30.0	25.7–29.7	25.6–29.7	25.5–29.6	29.7–29.8	28.9–29.6	37.35	25.7–29.6
6	25.7–30.0	25.7–29.7	25.6–29.7	25.5–29.6	29.7–29.8	28.9–29.6	25.50[c]	25.7–29.6
7–9	25.7–30.0	25.7–29.7	25.6–29.7	25.5–29.6	29.7–29.8	28.9–29.6	29.3–29.7	25.7–29.6
10	25.7–30.0	37.23[a]	25.6–29.7	25.5–29.6	29.7–29.8	26.20	26.11	25.7–29.6
11	25.7–30.0	71.38	25.6–29.7	25.5–29.6	29.7–29.8	35.59	35.53	25.7–29.6
12	25.7–30.0	37.36[a]	25.6–29.7	25.5–29.6	29.7–29.8	79.48	79.33	25.7–29.6
13	25.7–30.0	25.7–29.7	25.6–29.7	25.5–29.6	25.42[a]	32.33[b]	31.89[b]	25.7–29.6
14	33.48[a]	25.7–29.7	33.22[a]	25.5–29.6	31.83[b]	28.39	28.40	25.7–29.6
15	74.04[b]	25.7–29.7	74.35	25.5–29.6	74.48	81.78	81.88	25.7–29.6
16	82.65	33.35[b]	83.21	25.5–29.6	74.59	74.41[a]	74.12[a]	34.11
17	28.77	74.11	28.58	25.5–29.6	31.83[b]	31.90[b]	33.30[b]	74.38
18	28.77	82.77[c]	25.56	33.25[a]	32.42[b]	32.33[b]	25.67[c]	82.66
19	82.50	28.72	82.12	74.31	74.26	74.41[a]	25.67[c]	28.11
20	74.29[b]	28.35	71.51	82.19	83.05	74.55[a]	33.10[b]	28.11
21	34.18[a]	82.62[c]	32.53[a]	28.59	28.62	31.90[b]	74.40[a]	82.66
22	25.7–30.0	74.11	25.6–29.7	25.57	25.64	32.33[b]	74.54[a]	74.38
23	25.7–30.0	32.49[b]	25.6–29.7	83.24	82.19	74.55[a]	32.39[b]	34.11
24	25.7–30.0	25.7–29.7	25.6–29.7	71.57	71.55	74.70[a]	25.67[c]	25.7–29.6
25	25.7–30.0	25.7–29.7	25.6–29.7	32.59[a]	33.45[b]	33.52[b]	29.3–29.7	25.7–29.6
26	33.48	25.7–29.7	25.6–29.7	25.5–29.6	21.90	25.68	29.3–29.7	25.7–29.6
27	74.45	25.7–29.7	25.6–29.7	25.5–29.6	37.20[c]	28.9–29.6	29.3–29.7	25.7–29.6
28	74.49	25.7–29.7	25.6–29.7	25.5–29.6	71.83	28.9–29.6	29.3–29.7	25.7–29.6
29	31.90	25.7–29.7	25.6–29.7	25.5–29.6	37.51[c]	28.9–29.6	29.3–29.7	25.7–29.6
30	25.7–30.0	31.90	31.90	25.5–29.6	25.70[a]	28.9–29.6	31.89[b]	31.89
31	25.7–30.0	22.61	22.69	25.5–29.6	29.7–29.8	28.9–29.6	22.66	22.66
32	31.90	14.11	14.11	31.99	31.83[b]	31.90[b]	14.08	14.10
33	22.67	148.88	148.86	22.66	22.61	22.67	151.87	148.83
34	14.11	77.42	77.41	14.08	14.08	14.09	78.01	77.64
35	148.88	19.21	19.20	148.76	148.90	148.88	19.07	19.20
36	77.42			77.64	77.43	77.32		
37	19.20			19.21	19.20	19.20		

[a] Assignments may be interchanged. [b] Assignments may be interchanged. [c] Assignments may be interchanged.

Scheme 1. EIMS Fragmentation (*m*/*z* values) of Annonaceous Acetogenins Purified from Seeds of *A. squamosa*



concluded that compounds **3** and **4** have the same relative configuration, as well as a 15*S*/16*S*-19*R*/20*S* or 15*S*/16*R*-19*S*/20*S* absolute configuration, which also confirmed the *cis* configuration of the THF ring (Table 3). On the basis of these data, the structures

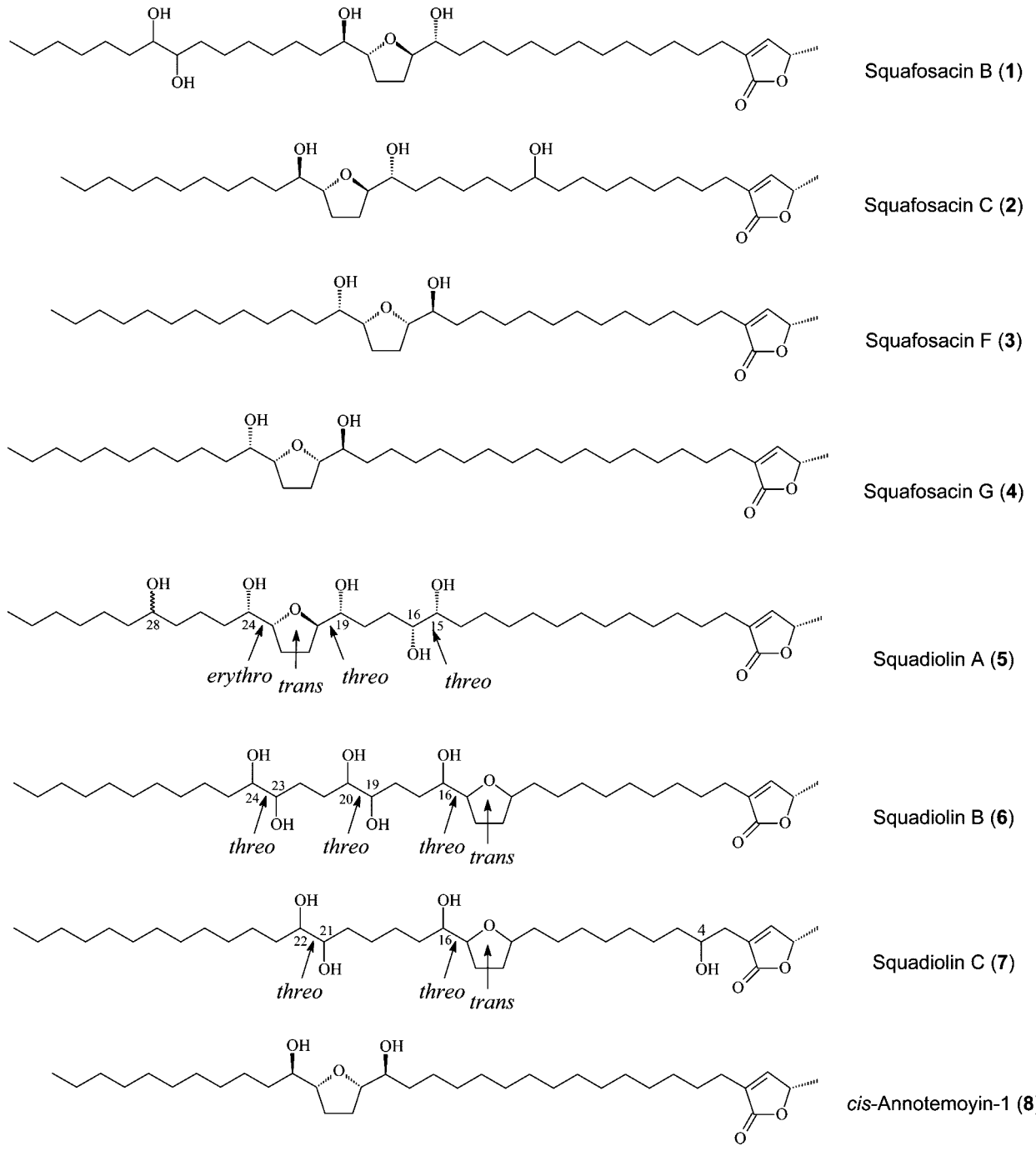


Figure 1. Mono-THF annonaceous acetogenins, **1**−**8**, isolated from seeds of *A. squamosa*.

Table 3. ^{1}H NMR Data of the (*S*)- and (*R*)-Mosher Esters of **3** and **4**

	3					**4**			
proton	15	16	19	20	proton	19	20	23	24
S-MTPA	5.378	4.157	4.218	5.556	*S*-MTPA	5.375	4.157	4.250	5.533
R-MTPA	5.327	4.012	4.190	5.625	*R*-MTPA	5.321	4.003	4.188	5.627
$\Delta\delta_{S-R}$	0.051	0.145	0.028	−0.069	$\Delta\delta_{S-R}$	0.054	0.154	0.062	−0.086
config.	*S*			*S*	config.	*S*			*S*

of **3** and **4** were fully elucidated, and the compounds have been named squafosacins F and G, respectively.

The ^{1}H and ^{13}C NMR spectra of compound **5** showed the characteristic signals for an α,β-unsaturated γ-lactone moiety without a hydroxy group at C-4 as mentioned above (Tables 1 and 2). Seven oxygen-bearing methines [δ_H 3.41–3.43 (m, 3H)/δ_C 74.3, 74.5, and 74.6, δ_H 3.58 (m, 1H)/δ_C 71.8; δ_H 3.80 (m, 1H)/δ_C 71.6; and δ_H 3.83–3.88 (m, 2H)/δ_C 82.2 and 83.1] were also observed. Four of these methines [δ_H 3.41–3.43 (one of three H), 3.80 (1H), and 3.83–3.88 (2H)] could be assigned to a THF ring with two flanking hydroxy groups in a relative *threo*/*trans*/*erythro* configuration, according to empirical rules.[22,27] The one proton signal at δ_H 3.58 indicated the presence of a free hydroxy group, and the remaining two proton signals at δ_H 3.41–3.43 were assigned as a vicinal diol group in a *threo* configuration.[23,24]

Table 4. *In Vitro* Cytotoxicity Data of Acetogenins

compound		cytotoxicity IC_{50} (μM)[a]				
		MDA-MB-231	MCF7	A549	HepG2	Hep 3B
squafosacin B	(**1**)	17.04	0.96	0.66	0.71	0.72
squafosacin C	(**2**)	18.10	26.36	20.97	18.95	13.97
squafosacin F	(**3**)	−[b]	35.07	−	−	−
squafosacin G	(**4**)	−	29.75	−	−	−
squadiolin A	(**5**)	0.63	2.83	<0.20[a]	5.60×10^{-3}	3.52
squadiolin B	(**6**)	0.28	5.47	2.75	1.1×10^{-2}	6.98
squadiolin C	(**7**)	14.45	3.62	8.11	3.48	−
cis-annotemoyin-1	(**8**)	−	30.71	−	−	−
bullatencin		−	−	−	−	31.44
cis-bullatencin		−	−	−	−	30.57
uvariamicin-I		−	28.34	−	−	−
uvariamincin-II		−	8.11	−	−	−
uvariamicin-III		−	3.31	−	−	−
positive controls						
doxorubicin		0.18	0.90	0.86	0.64	0.59
paclitaxel		7.03×10^{-2}	1.17×10^{-2}	7.0×10^{-3}	4.68×10^{-2}	0.13

[a] MDA-MB-231: human Caucasian breast adenocarcinoma cell; MCF-7: human breast adenocarcinoma cell; A549: human lung carcinoma cell; Hep G2: human hepatoma cells; Hep3B: human Negroid hepatocyte carcinoma. [b] −: inactive, $IC_{50} > 36$ μM.

The structure of compound **5** was fully elucidated, with the exception of the configuration at C-28, by MS examination. In the ESIMS spectrum of **5**, $[M + H]^+$ and $[M + Na]^+$ peaks appeared at *m/z* 641.7 and 663.8, respectively, indicating a molecular weight of 640 and molecular formula of $C_{37}H_{68}O_8$. A base-peak ion at *m/z* 347 and ions for serial losses of water (*m/z* 365–347–329), together with three additional series of fragment ions (*m/z* 417–399, 483–465–447, and 537–519–501–483–465) and a significant ion peak at *m/z* 295, suggested that the 1,2-diol group was located at C-15/C-16, the THF ring at C-20/C-23, and the free hydroxy group at C-28. The base-peak ion (*m/z* 347) was formed by cleavage at C-19/C-20, consistent with prior reports.[28] Furthermore, a TOCSY cross-peak between the proton signals at δ_H 3.80 (H-24) and 3.58 (H-28) also confirmed this assignment. The structure for compound **5**, which has been named squadiolin A, is shown in Figure 1.

In addition to signals for an α,β-unsaturated γ-lactone moiety, the ^{1}H and ^{13}C NMR spectra of compound **6** showed signals for seven oxymethine protons located at δ_H 3.87 (m, 1H)/δ_C 79.5, δ_H 3.79 (m, 1H)/δ_C 81.8, and δ_H 3.45 (m, 5H)/δ_C 74.3, 74.4, 74.6, 74.6, and 74.7. Carbon signals at δ_C 81.8 and 79.5 and proton signals at δ_H 3.87 and 3.45 (one of five protons) were characteristic of the presence of a THF ring with one flanking hydroxy group in a *threo* configuration.[29] Analysis of the ^{1}H NMR spectrum suggested that proton resonances at δ_H 1.98 (2H) and 1.64 (2H) corresponded to the two methylene groups of the THF ring in a *trans* configuration.[30] The remaining four proton signals at δ_H 3.45 were assigned to two sets of *threo* 1,2-diol groups.[23,24]

The locations of the oxygen-bearing moieties of compound **6** were determined from the ESIMS and EIMS data. $[M + H]^+$ and $[M + H + Na]^+$ peaks at *m/z* 641.7 and 664.0, respectively, in the ESIMS spectrum of **6** indicated that the molecular weight is 640, indicating the molecular formula $C_{37}H_{68}O_8$. In the EIMS spectrum, three major series of fragments (*m/z* 293–275, 363–345–327, 451–433–415–397–379) established the locations of the THF ring at C-12/C-15 and the two sets of diol groups at C-19/C-20 and C-23/C24. The base-peak ion at *m/z* 311 and an ion at *m/z* 251 also confirmed these assignments. The assigned structure of **6** is shown in Figure 1. The compound has been given the name squadiolin B.

The ^{1}H and ^{13}C NMR spectra of compound **7** showed signals similar to those of compounds **1**–**6** (Tables 1 and 2). However, unlike **1**–**6**, compound **7** has a hydroxy group at C-4 (δ_H 3.87/δ_C 70.0), as indicated by the presence of the signals of a methylene at δ_H 2.39 (dd, $J = 15.2$, 8.4 Hz, 1H) and 2.52 (dd, $J = 15.2$, 3.2 Hz, 1H), rather than a triplet signal of a methylene at δ_H 2.26 (t, $J = 8.4$, 2H).[18] In addition, signals were observed at δ_H 3.87 (m, 2H)/δ_C 79.3, δ_H 3.77 (m, 1H)/δ_C 81.9, and δ_H 3.39 (m, 3H)/δ_C 74.5, 74.4, 74.1, which were consistent with a THF ring with one flanking hydroxy group in a *threo* configuration, as also found in compound **6**,[29] and a *threo* 1,2-diol group.[23,24]

Ion peaks at *m/z* 597.7 $[M + H]^+$ and 619.7 $[M + Na]^+$ in the ESIMS spectrum of compound **7** indicated a molecular weight of 596 and molecular formula of $C_{35}H_{64}O_7$. In the EIMS spectrum, three major series of fragments (*m/z* 267–249, 309–291–273, and 389–371) revealed that the THF ring should be located between C-12/C-15 and the diol group at C-21/C-22. Thus, together with the aforementioned α,β-unsaturated γ-lactone ring, the structure of **7** was determined and the compound was named squadiolin C.

Compound **8** was isolated from the same fraction as uvariamicin-I, bullatencin, and *cis*-bullatencin and has similar ^{1}H and ^{13}C NMR spectra to those of uvariamicin-I. In addition, the EIMS spectrum of **8** is extremely similar to those of annotemoyin-1, annotemoyin-2, and uvariamicin-II. Examination of the ^{1}H and ^{13}C NMR spectra showed that, unlike annotemoyin-1, the THF ring of **8** has a *cis* configuration. The THF methylene proton signals [1.94 (2H) and 1.74 (2H)] were also different from those of annotemoyin-1, and the methylene carbon signals were slightly but clearly shifted from δ_C 28.7 in annotemoyin-1 to 28.1 in **8**.[31]

The molecular ion peak at *m/z* 565 $[M + H]^+$ in the EIMS spectrum of compound **8** indicated a molecular weight of 564 and molecular formula of $C_{35}H_{64}O_5$. In the EIMS spectrum, the base peak at *m/z* 323 and two major series of fragments (*m/z* 323–305–287, 375–357) revealed that the THF ring should be located between C-18/C-21. Thus, compound **8** was determined to have the structure shown in Figure 1 and has been named *cis*-annotemoyin-1.

UV, IR, ^{1}H NMR, ^{13}C NMR, and MS data of the known annonaceous acetogenins were compared with published values of glabranin,[12] annotemoyin-1,[13] annotemoyin-2,[13] uvariamicin-I,[14,15] bullatencin,[16] *cis*-bullatencin,[17] uvariamicin-II,[14,15] and uvariamincin-III[14] to verify these structures.

Compounds **1**−**8**, as well as five of the known compounds, were evaluated in three-day MTT cytotoxicity bioassays against five human cancer cell lines, MDA-MB-231 (Caucasian breast adenocarcinoma), MCF-7 (breast adenocarcinoma), A549 (lung carcinoma), HepG2 (hepatoma), and Hep3B (Negroid hepatocyte carcinoma) (Table 4). Compounds **5** and **6** generally showed cytotoxic activity against all five human cancer cell lines, especially against HepG2 with ng/mL IC_{50} values. Compound **1** was significantly potent against MCF7, A549, HepG2, and Hep3B cells, compound **7** against MCF7 and HepG2, and uvariamicin-III against MCF7. The remaining compounds were inactive.

From a structure–activity relationship (SAR) viewpoint, the number of hydroxy groups affected the potency in this series of mono-THF acetogenins. Compounds with a greater number of

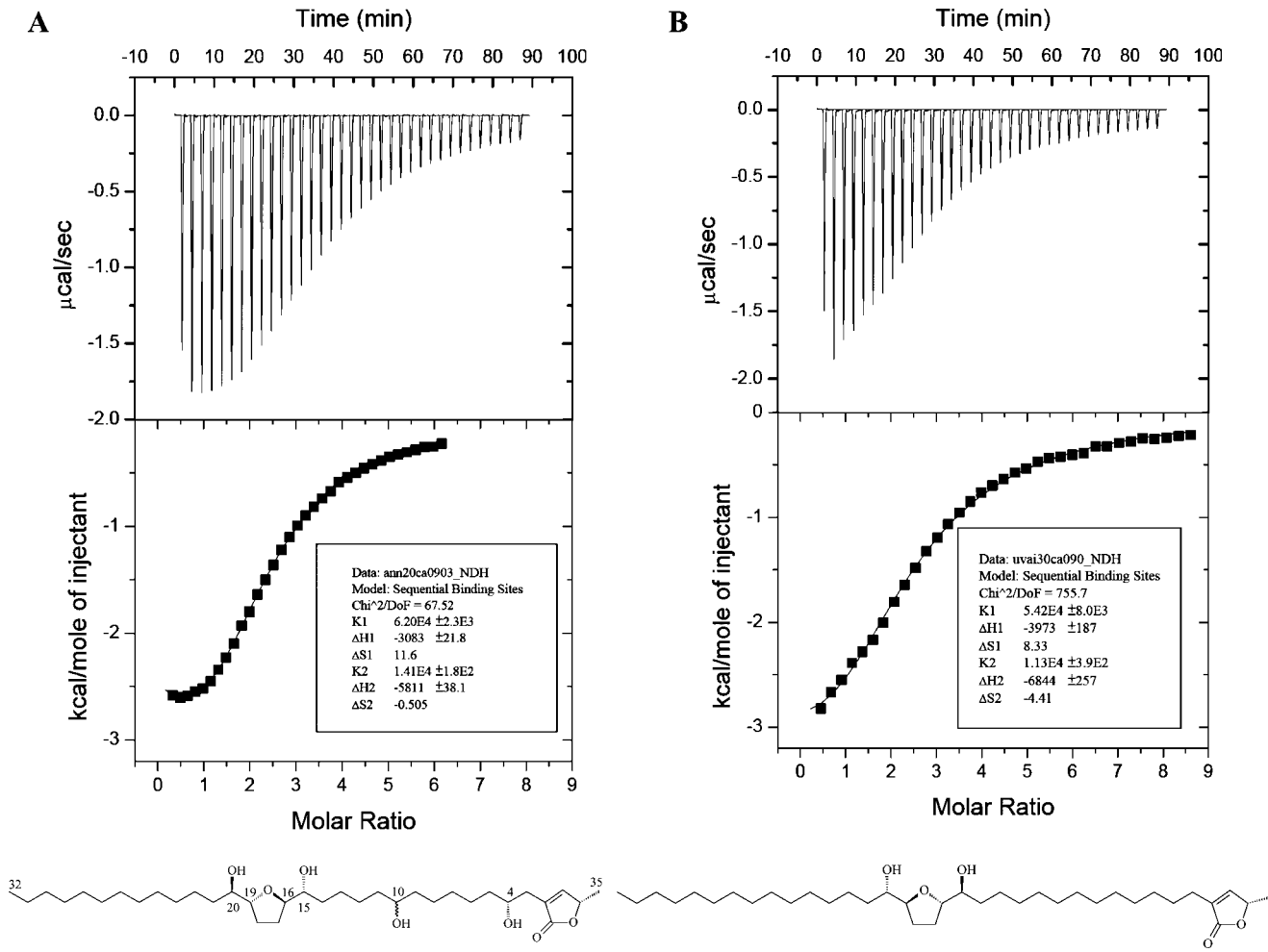


Figure 2. Binding isotherm for the titration of $Ca(ClO_4)_2$ (0.07 and 0.046 mM, respectively) with 8 μL injections of annonacin (2.0 mM, A) and uvariamicin-I (1.86 mM, B) at 25 °C.

hydroxy groups were generally more potent. For example, among compounds with two hydroxy groups flanking the THF ring, the rank order of potency against all five tested cell lines was **5** (with three additional OH groups) > **1** (two OH groups) > **2** (one OH group). For compounds with one hydroxy group adjacent to the THF ring, compound **6** (four additional OH groups) was more active than **7** (three OH groups).

Annonaceous acetogenins have been found to act as calcium ionophores.[6] Our previous study also proposed direct evidence to support the hypothesis that acetogenins show greater inhibition of cancer cell growth when Ca^{2+} ions are present in the bioassays.[32] Peyrat and co-workers tried to observe the interaction between Ca^{2+} ions and acetogenins by using NMR techniques. Their results showed the stoichiometry of a mono-THF acetogenin complexed with Ca^{2+} ions.[9] We tried to clarify the interaction between mono-THF acetogenins and Ca^{2+} by using isothermal titration calorimetry (ITC), which is an extremely powerful and highly sensitive technique for measuring the heats of interaction of reacting species in dilute solution. Interestingly, we found that the mono-THF acetogenins, annonacin and uvariamicin-I, interacted with Ca^{2+} by an exothermic process, indicating the formation of the acetogenin−calcium complex (Figure 2). NMR data suggested that the hydroxy groups adjacent to the THF moiety should play a key role in the chelation process. The chelating processes between acetogenins and Ca^{2+} are under further investigation.

In summary, eight new mono-THF annonaceous acetogenins were isolated from the seeds of *A. squamosa*. Interestingly, compounds **5** and **6** exhibited significant potency against Hep G2 at ng/mL concentrations. The number of hydroxy groups and the polarity of the compounds could be factors affecting the cytotoxic activity. The chelating ability of mono-THF acetogenins with Ca^{2+} could be another key factor in the bioactivity. In addition, according to accepted hypotheses on biosynthesis,[33,34] squadiolin A (**5**) should be generated through the biogenetic pathway of squamocin-type acetogenins, whereas squadiolin B (**6**) should arise from the biogenetic pathway of rollidecin-type or squamostatin-A-type acetogenins (Scheme 2). Generally, mono-THF ring acetogenins show less *in vitro* cytotoxic activity than bis-THF ring acetogenins (either adjacent or nonadjacent). However, in this report, both compounds **5** and **6** showed excellent cytotoxicity against Hep G2 and were more active than other mono-THF acetogenins. We suspect that compounds **5** and **6** might be precursors to generate bis-THF acetogenins in the cancer cells during the bioassay.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. CD spectra were measured on a JASCO J-720 spectropolarimeter. The IR spectra were measured on a Hitachi 260-30 spectrophotometer. ^1H and ^{13}C NMR spectra (all in $CDCl_3$) were recorded with Varian Unity 400 NMR spectrometers, using TMS as the internal standard. LRFABMS and LREIMS were obtained with a JEOL JMS-SX/SX 102A mass spectrometer or a Quattro GC/MS spectrometer having a direct inlet system. HRFABMS were measured on a JEOL JMS-700 MStation (Okayama University of Science, Japan). Si gel 60 (Macherey-Nagel, 230–400 mesh) was used for column chromatography; precoated Si gel plates (Macherey-Nagel, SIL G-25 UV254, 0.25 mm) were used for analytical TLC. The spots were detected by spraying with Dragendorff's reagent, Kedde's reagent, or 50% H_2SO_4 aqueous solution and then heating on a hot

Scheme 2. Biogenetic Pathways of **5** (squamocin-type) and **6** (rollidecin/squamostatin-A-type) Acetogenins

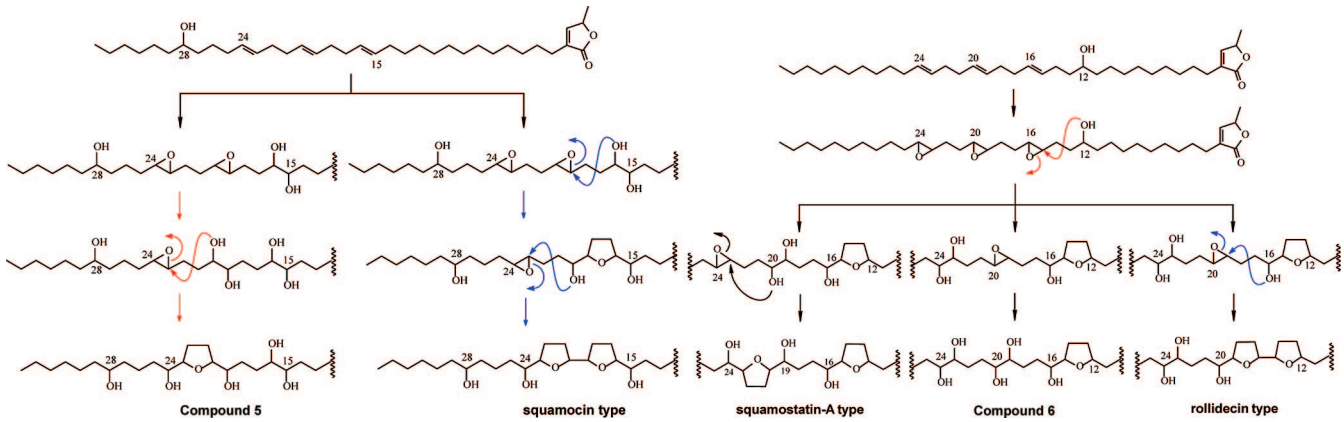


plate. HPLC was performed on a Shimadzu LC-10AT apparatus equipped with a Shimadzu SPD-10A UV–vis detector, Shimadzu LC-6AD apparatus with Shimadzu SPD-M10Avp diode array detector, and a JAI LC918 apparatus with RI detector. Analytical chromatography used Purosphere STAR RP-18 (250 × 4 mm i.d.) and Discovery C18 5 μm (250 × 4.6 mm i.d.) columns, and preparative chromatography used Purosphere preparative RP-18 (250 × 25 mm i.d.) and Discovery semipreparative C18 5μm (250 × 10 mm i.d.) columns.

Plant Material. The seeds of *A. squamosa* L. were collected from TaiDong County, Taiwan, in 2002. Voucher specimens (Annona-06b) were deposited in the Graduate Institute of Natural Products, Kaohsiung, Taiwan, Republic of China.

Extraction and Isolation. The seeds of *A. squamosa* (10 kg) were extracted with MeOH (5 L × 3). The MeOH extracts of the seeds were partitioned with $CHCl_3$ and water−MeOH (1:1) to yield $CHCl_3$ (295.0 g) and aqueous extracts (100.9 g). After removing solvent *in vacuo*, the $CHCl_3$ residue was partitioned using MeOH and *n*-hexane to yield MeOH (87.0 g) and *n*-hexane layers (208.0 g). The *n*-hexane layer was further separated into 18 fractions by column chromatography on Si gel with gradients of *n*-hexane−$CHCl_3$ (200:1, 100:1, 50: 1, 10:1, 2:1, 1:1, pure $CHCl_3$) and $CHCl_3$−MeOH (20:1, 10:1, 5:1).

Fraction 12 (340 mg), eluted with pure $CHCl_3$, was separated by column and RP-HPLC (Purosphere STAR RP-18, 250 × 25 mm, MeCN−H_2O, 98:2, flow rate 4 mL/min; RI detector) to provide eight subfractions. The fifth subfraction contained annotemoyin-1 (46.0 mg) and the seventh squafosacin G (**4**) (40.1 mg). The fourth subfraction was repurified by the same RP-recycle HPLC system to give annotemoyin-2 (27.4 mg) and squafosacin F (**3**) (10.7 mg). The sixth subfraction was isolated and purified by RP-HPLC (Discovery C18, 250 × 10 mm, MeCN−H_2O, 98:2, flow rate 2 mL/min; PDA detector) to give *cis*-annotemoyin-1 (**8**) (2.3 mg), uvariamicin-I (19.2 mg), bullatencin (11.9 mg), and *cis*-bullatencin (6.0 mg). The eighth subfraction was purified by recycle HPLC (Discovery C18, 250 × 10 mm, MeCN−H_2O, 98:2, flow rate 2 mL/min; RI detector) to give uvariamicin-II (6.9 mg) and uvariamicin-III (19.7 mg).

Fraction 15 (20.5 g), eluted with $CHCl_3$−MeOH (20:1), was isolated by column and HPLC (Purosphere STAR RP-18, 250 × 25 mm, MeCN−H_2O, 95:5, flow rate 3 mL/min; RI detector) to give four subfractions. The fourth subfraction was repurified by RP-recycle HPLC (Purosphere STAR RP-18, 250 × 25 mm, MeCN−H_2O, 95:5, flow rate 3 mL/min; RI detector) to give squafosacin C (**2**) (0.9 mg).

Fraction 16 (12.6 g), eluted with $CHCl_3$−MeOH (10:1), was subjected to column chromatography and HPLC (Purosphere STAR RP-18, 250 × 25 mm, MeCN−H_2O, 90:10, flow rate 3 mL/min; RI detector) to give 13 subfractions. The 10th subfraction was further purified by recycle HPLC (Purosphere STAR RP-18, 250 × 25 mm, MeCN−H_2O, 90:10, flow rate 3 mL/min; RI detector) to give squadiolin C (**7**) (17.8 mg). The 13th subfraction was also purified by RP-recycle HPLC (Purosphere STAR RP-18, 250 × 25 mm, MeCN−H_2O, 85:15, flow rate 3 mL/min; RI detector) to give glabranin (9.2 mg), squafosacin (**1**) (2.6 mg), squadiolin A (**5**) (8.0 mg), and squadiolin B (**6**) (5.8 mg).

Squafosacin B (1): colorless, waxy solid; $[\alpha]^{21}_D$ +17.8 (*c* 0.17, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 214 (3.51) nm; IR (neat) ν_{max} 3424, 2849, 2919, 1750 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Tables 1 and 2; FABMS *m/z* 624 $[M]^+$; HRFABMS *m/z* 624.4942 (calcd for $C_{37}H_{68}O_7$, 624.4942); EIMS (70 eV) *m/z* 499, 473, 461, 295; CD (MeCN) $\Delta\epsilon$ > 0 (*c* 1.0 × 10^{-4}), $\Delta\epsilon$ (nm) n−π^* −0.06 (238), π−π^* +1.02 (209).

Squafosacin C (2): colorless, waxy solid; $[\alpha]^{18}_D$ –11.6 (*c* 0.06, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 216 (3.53) nm; IR (neat) ν_{max} 3398, 2924, 2855, 1752 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Tables 1 and 2; FABMS *m/z* 581 $[M + H]^+$; HRFABMS *m/z* 581.4773 (calcd for $C_{35}H_{65}O_6$, 581.5781); EIMS (70 eV) *m/z* 403, 373, 321, 303, 285, 253, 221, 209; CD (MeCN) $\Delta\epsilon$ > 0 (*c* 9.8 × 10^{-5}), $\Delta\epsilon$ (nm) n−π^* –0.15 (243), π−π^* +4.42 (214).

Squafosacin F (3): colorless, waxy solid; $[\alpha]^{21}_D$ +11.5 (*c* 0.34, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 214 (3.51) nm; IR (neat) ν_{max} 3417, 2923, 2853, 1744 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Tables 1 and 2; EIMS *m/z* 565 $[M + H]^+$;HRFABMS *m/z* 565.4761 (calcd for $C_{35}H_{65}O_5$, 565.4832); EIMS (70 eV) *m/z* 365, 347, 295; CD (MeCN) $\Delta\epsilon$ > 0 (*c* 1.2 × 10^{-4}), $\Delta\epsilon$ (nm) n−π^* –0.08 (242), π−π^* +3.57 (215).

Squafosacin G (4): colorless, waxy solid; $[\alpha]^{18}_D$ +11.7 (*c* 0.08, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 214 (3.69) nm; IR (neat) ν_{max} 3424, 2918,2850, 1746 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Tables 1 and 2; ESIMS *m/z* 615 $[M + Na]^+$; HRESIMS *m/z* 615.4965 (calcd for $C_{37}H_{68}O_5Na$, 615.4964); EIMS (70 eV) *m/z* 421, 403, 385, 351; CD (MeCN) $\Delta\epsilon$ > 0 (*c* 1.2 × 10^{-4}), $\Delta\epsilon$ (nm) n−π^* –0.13 (241), π−π^* +5.24 (215).

(*R*)- and (*S*)-MTPA Derivatives of 3 and 4. Compound **3** (2.0 mg) was transferred into an NMR tube and was dried completely under vacuum. Pyridine-d_5 (0.5 mL) and (*S*)-(+)-methoxy-α-(trifluoromethyl)phenylacetyl chloride (6 μL) were added immediately into the NMR tube under an N_2 gas stream, and the NMR tube was shaken carefully to evenly mix the sample and MTPA chloride. The NMR tube was permitted to stand at room temperature and monitored every 1 h by ^1H NMR. The reaction was found to be complete after 2 h. ^1H NMR (400 MHz, pyridine-d_5) data of the (*R*)-MTPA ester derivative (**3r**) of **3** were obtained directly and were assigned on the basis of the correlations of the ^1H−^1H COSY spectrum. The (*S*)-MTPA ester derivative (**3s**) was prepared in the same way using (*R*)-(−)-methoxy-α-(trifluoromethyl)phenylacetyl chloride. (*R*)- and (*S*)-MTPA derivatives (**4r** and **4s**) of **4** were also prepared in the same manner as described above.

Squadiolin A (5): colorless, waxy solid; $[\alpha]^{27}_D$ +21.3 (*c* 0.12, $CHCl_3$); IR (neat) ν_{max} 3580, 2935, 2870, 1750 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Tables 1 and 2; ESIMS *m/z* 641.7 $[M + H]^+$, 663.8 $[M + Na]^+$ (for $C_{37}H_{68}O_8$); EIMS (70 eV) *m/z* 399, 365, 347, 329, 295; CD (CH_3CN) $\Delta\epsilon_{240}$ −0.56.

Squadiolin B (6): colorless, waxy solid; $[\alpha]^{26}_D$ +15.6 (*c* 0.09, $CHCl_3$); IR (neat) ν_{max} 3560, 2928, 2865, 1760 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and^{13}C NMR ($CDCl_3$, 100 MHz) data, see Tables 1 and 2; ESIMS *m/z* 641.7 $[M + H]^+$ (for $C_{37}H_{68}O_8$); EIMS (70 eV) *m/z* 433, 415, 397, 379, 363, 345, 327, 311, 293, 275, 251; CD (CH_3CN) $\Delta\epsilon_{242}$ −0.43.

Squadiolin C (7): colorless, waxy solid; $[\alpha]^{26}_D$ +19.6 (*c* 0.21, $CHCl_3$); IR (neat) ν_{max} 3490, 2941, 2875, 1760 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Tables 1 and 2; ESIMS *m/z* 597.7 $[M + H]^+$, 619.7 $[M + Na]^+$ (for $C_{35}H_{64}O_7$); EIMS (70 eV) *m/z* 389 (82), 371 (11), 309 (33), 291 (20), 273 (24), 267 (100), 249 (43); CD (CH_3CN) $\Delta\epsilon_{241}$ −0.44.

***cis*-Annotemoyin-1 (8):** colorless, waxy solid; $[\alpha]^{21}_D$ +11.7 (*c* 0.17, $CHCl_3$); UV (MeOH) λ_{max} (log ϵ) 214(3.56) nm; IR (neat) ν_{max} 3410,

2919, 2850, 1739 cm^{-1}; ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz) data, see Tables 1 and 2; EIMS *m/z* 565 $[M + H]^+$; HREIMS *m/z* 565.4823 (calcd for $C_{35}H_{65}O_6$, 565.4832); EIMS (70 eV) *m/z* 375, 323; CD (MeCN) $\Delta\epsilon > 0$ (c 1.1×10^{-4}), $\Delta\epsilon$ (nm) $n-\pi^*$ –0.10 (237.4), $\pi-\pi^*$ +0.87 (212.7).

Glabranin,[12] annotemoyins-1 and -2,[13] uvariamicins-I, -II, and -III[14,15] bullatencin,[16] and *cis*-bullatencin[17] were obtained as colorless, waxy solids. Their MS and ^1H and ^{13}C NMR data were in accord with published values. Optical rotation, UV, and IR data can be found as Supporting Information.

Bioassays. The three-day bioassays against MDA-MB-231, MCF-7, A549, HepG2, and Hep3B cell lines were carried out according to described procedures.[35]

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Supporting Information Available: This material is available free of charge via the Internet at http://pubs.acs.org.

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