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Eight new cytotoxic annonaceous acetogenins from the seeds of *Annona squamosa*

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[ABSTRACT] Eight new annonaceous acetogenins, squamotin A–D (**1**–**4**), annosquatin IV–V (**5** and **6**), muricin O (**7**) and squamosten B (**8**), together with four known ones (**9**–**12**) were isolated from the seeds of *Annona squamosa*. Their structures were elucidated by chemical methods and spectral data. The inhibitory activities of compound **1**–**9** against three multidrug resistance cell lines were evaluated. All tested compounds showed strong cytotoxicity.

[KEY WORDS] *Annona squamosa*; Annonaceous acetogenins; Cytotoxicity; Multidrug resistance

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Introduction

Annona squamosa Linn., a species of the genus *Annonaceae*, had been used as folkloric medicine in many tropical and subtropical regions [1]. Srikaya, the fruit of *A. squamosa*, is extensively used to prepare candies, ice creams and beverages in tropics. Seeds of *A. squamosa* are mostly regarded as wastes and throw away. However, seeds accumulate toxic or health-beneficial compounds [2]. Seeds of *A. squamosa* were reported with several biological activities, such as anticancer [3], anti-parasitic [4] and anti-fungal [5]. Previous phytochemical and pharmacological investigations on seeds showed that annonaceous aecetogenins (ACGs) are the main bioactive compounds [6]. In our previous work, three new ACGs were isolated from the seeds of *A. squamosa*, and showed promising anticancer activities [1, 7]. In this study, the phytochemical investigation of *A. squamosa* seeds led to the isolate of eight new ACGs squamotin A–D (**1**–**4**), annosquatin IV–V (**5** and **6**), muricin O (**7**), squamosten B (**8**) along with four known ones gigantecin (**9**) [8], neoannonin (**10**) [9], squamocin-I (**11**) [10], squamostatin D (**12**) [11], whose structural elucidation and cytotoxicity against multidrug resistance cancer of compound **1**–**9** were reported herein.

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These authors have no conflict of interest to declare.

Result and discussion

Compound **1** had the molecular formula of $C_{37}H_{66}O_7$ from HR-ESIMS data (*m/z* 623.4921 $[M + H]^+$, calcd. for 623.4881). A positive reaction to Raymond's reagent indicated the presence of a terminal *α*, *β*-unsaturated *γ*-lactone. In its NMR data, the signals at δ_H 7.00 (1H, d, *J* =1.7 Hz, H-35), 5.01 (1H, dq, *J* = 6.8, 1.8 Hz, H-36), 1.42 (3H, d, *J* = 6.8 Hz, H-37) and δ_C 173.9 (C-1), 134.4 (C-2), 148.9 (C-35), 77.4 (C-36), 19.2 (C-37) testified the existence of the terminal lactone. The proton signal at δ_H 2.28 (2H, t, *J* = 7.6 Hz, H-3) suggested the absence of a hydroxyl group at the C-4 position [3]. The signals at δ_H 3.83–3.97 (5H, m, H-26, 25, 22, 21, 18), 3.39–3.44 (1H, m, H-17) and δ_C 83.3 (C-18), 82.8 (C-25), 82.5 (C-22), 82.2 (C-21), 74.1 (C-17), 71.4 (C-26) are characteristic of the presence of two adjacent bistetrahydrofuran rings flanked by two OH groups. The relative stereochemistry of them was determined to be *th/t/th/t/er* (*threo/trans/threo/trans/erythro*) by comparison of NMR data with a series of bullutacin-type compounds [12-13]. The bis-THF moiety was located from C-17 to C-26 by carefully analysis of the ESIMS fragment ions at *m/z* 417, 293, 265, 177 (Fig. 2). The third hydroxyl group was appeared in NMR spectrum at δ_H 3.58–3.61 (1H, m, H-10) and δ_C 71.8 (C-10). The location of this hydroxyl group was confirmed by the ESI-MS/MS fragment ion at *m/z* 377 (Fig. 2).

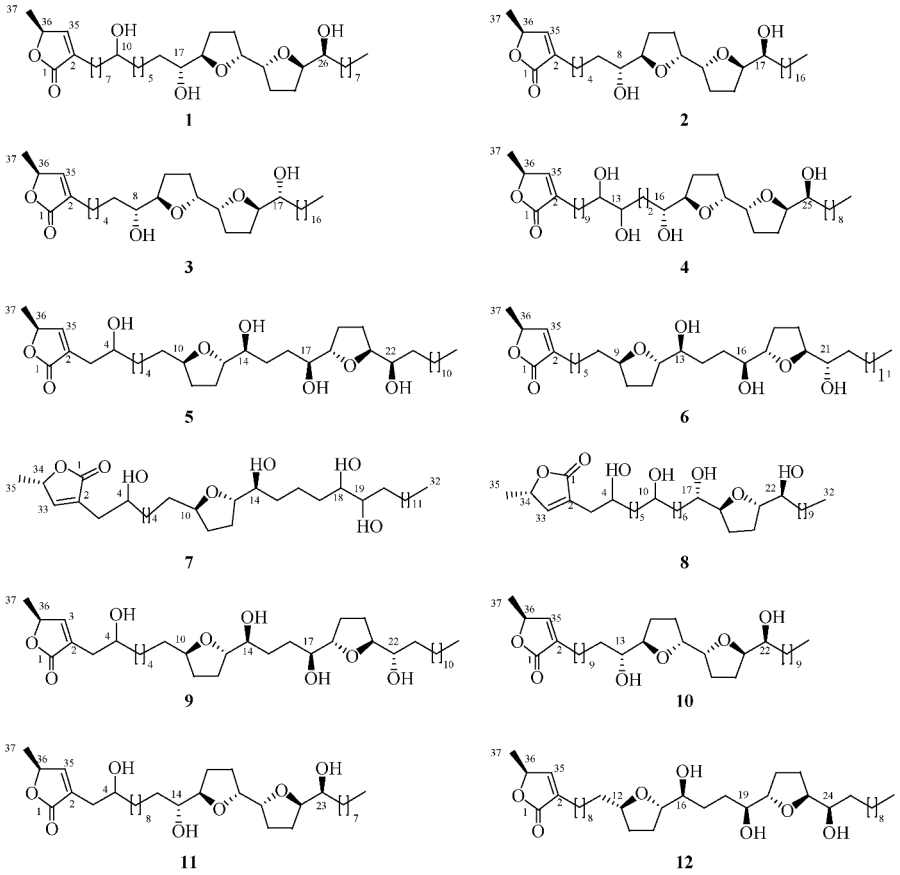
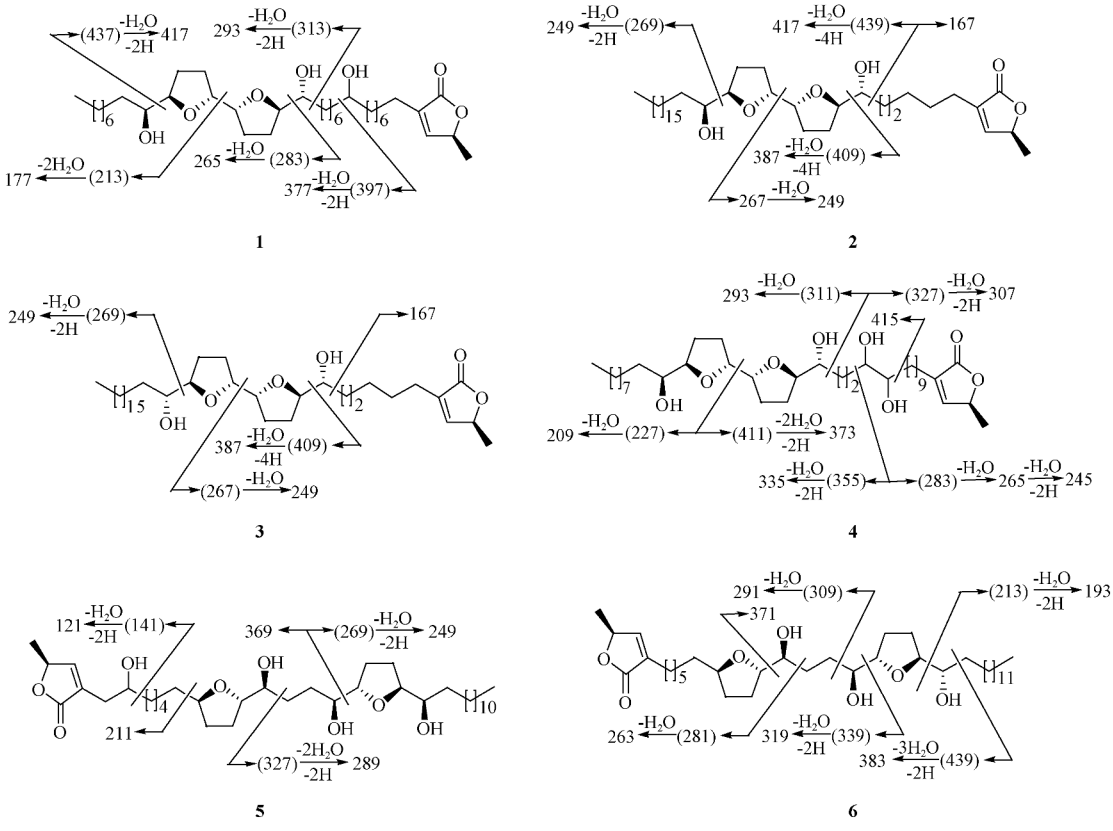


Fig. 1 Structures of annonaceous acetogenins 1–12



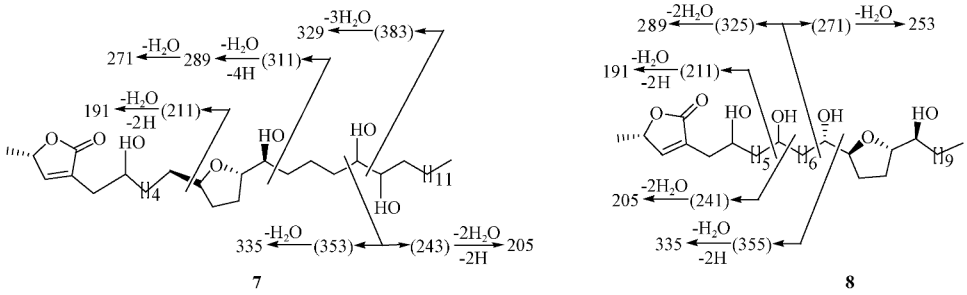


Fig. 2 Diagnostic ESIMS^2 fragment ions (*m/z*) of compounds 1–8

Compound **2** was isolated as a white waxy. The molecular formula was established as $C_{37}H_{66}O_6$ by HR-ESIMS (*m/z* 607.5068 $[M + H]^+$, calcd. for 607.4932). The positive reaction of Raymond's reagent suggested the presence of a terminal lactone. Comparision of its NMR data (Tables 1 and 2) with those of **1**, indicated that the relative of bis-THF unit of **2** is closely similar to **1**. The only difference was the absence of a hydroxyl group (δ_C 71.8 and δ_H 3.58–3.61) in **2**. The placement of bis-THF unit was located at C-8 to C-17 by analysis of the ESI-MS^2 fragments at *m/z* 417, 387, 267, 249, 167 (Fig. 2).

Table 1 ^{13}C NMR spectral data of compounds 1–8 (101 MHz in CDCl3)

Position	1	2	3	4	5	6	7	8
1	173.9	173.9	173.9	173.9	174.6	173.9	174.7	174.7
2	134.4	134.4	134.4	134.3	131.2	134.3	131.1	131.1
3	25.2	25.2	25.2	25.2	33.4	25.2	33.3	33.4
4	27.4	27.4	27.4	27.4	70.0	27.4	69.9	69.9
5	24.6–29.7	24.5–29.7	25.7–29.7	22.7–29.7	37.4	22.7–33.5	37.4	25.5–29.7
6	24.6–29.7	24.5–29.7	25.7–29.7	22.7–29.7	22.7–29.7	22.7–33.5	25.5–29.9	25.5–29.7
7	24.6–29.7	33.4	33.5	22.7–29.7	22.7–29.7	22.7–33.5	25.5–29.9	25.5–29.7
8	24.6–29.7	74.1	74.1	22.7–29.7	22.7–29.7	35.7	25.5–29.9	25.5–29.7
9	37.3	83.2	83.2	22.7–29.7	35.6	79.4	25.5–29.9	37.3
10	71.8	28.4	28.4	22.7–29.7	79.3	22.7–33.5	79.4	71.5
11	37.5	29.2	29.2	31.9	32.6	22.7–33.5	28.4	37.4
12	24.6–29.7	82.3	81.8	74.2	28.4	82.0	32.4	25.5–29.7
13	24.6–29.7	82.5	81.8	74.6	82.0	74.1	81.8	25.5–29.7
14	24.6–29.7	28.9	28.9	31.9	74.5	22.7–33.5	74.5	25.5–29.7
15	24.6–29.7	25.7	25.7	32.9	22.7–29.7	22.7–33.5	35.5	25.5–29.7
16	33.3	82.8	83.2	74.2	22.7–29.7	74.3	25.5–29.9	33.3
17	74.1	71.4	74.1	83.5	74.6	82.7	33.5	74.3
18	83.3	32.5	33.5	28.5	83.3	22.7–33.5	74.3	82.5
19	28.4	24.5–29.7	25.7–29.7	29.0	28.6	22.7–33.5	74.5	28.8
20	29.2	24.5–29.7	25.7–29.7	82.0	25.2	82.7	25.5–29.9	28.8
21	82.2	24.5–29.7	25.7–29.7	82.7	82.2	74.5	25.5–29.9	82.8
22	82.5	24.5–29.7	25.7–29.7	29.0	71.6	32.4	25.5–29.9	74.1
23	28.9	24.5–29.7	25.7–29.7	25.6	32.4	22.7–33.5	25.5–29.9	33.4
24	25.7	24.5–29.7	25.7–29.7	82.8	22.7–29.7	22.7–33.5	25.5–29.9	25.5–29.7
25	82.8	24.5–29.7	25.7–29.7	71.4	22.7–29.7	22.7–33.5	25.5–29.9	25.5–29.7
26	71.4	24.5–29.7	25.7–29.7	32.9	22.7–29.7	22.7–33.5	25.5–29.9	25.5–29.7
27	32.4	24.5–29.7	25.7–29.7	22.0–29.7	22.7–29.7	22.7–33.5	25.5–29.9	25.5–29.7
28	24.6–29.7	24.5–29.7	25.7–29.7	22.0–29.7	22.7–29.7	22.7–33.5	25.5–29.9	25.5–29.7
29	24.6–29.7	24.5–29.7	25.7–29.7	22.0–29.7	22.7–29.7	22.7–33.5	25.5–29.9	25.5–29.7
30	24.6–29.7	24.5–29.7	25.7–29.7	22.0–29.7	22.7–29.7	22.7–33.5	31.9	31.9
31	24.6–29.7	24.5–29.7	25.7–29.7	22.0–29.7	22.7–29.7	22.7–33.5	22.7	22.7
32	31.9	31.9	31.9	31.9	22.7–29.7	22.7–33.5	14.1	14.1
33	22.6	22.7	22.7	22.6	22.7–29.7	22.7–33.5	151.9	151.9
34	14.0	14.1	14.1	14.1	14.1	14.1	78.0	78.0
35	148.9	148.8	148.8	148.9	151.8	148.9	19.1	19.1
36	77.4	77.4	77.4	77.4	78.0	77.4	-	-
37	19.2	19.2	19.2	19.2	19.1	19.2	-	-

Table 2 ^{1}H NMR spectral data of new compounds (400 MHz in CDCl3, *J* in Hz)

Position	1	2	3	4	5	6	7	8
3	2.28 (t, 7.6)	2.28 (t, 7.8)	2.28 (t, 7.8)	2.26 (t, 7.4)	2.41 (dd, 15.2, 8.3), 2.54 (dd, 15.1, 3.4)	2.28 (t, 7.7),	2.40 (dd, 15.1, 8.2), 2.52 (dd, 15.1, 3.3)	2.40 (dd, 15.1, 8.2), 2.51 (dd, 15.1, 3.3)
4	1.27–1.56 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.26–1.57 (m)	3.80–3.92 (m)	1.28–1.75 (m)	3.80–3.89 (m)	3.77–3.85 (m)
5	1.27–1.56 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.26–1.57 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.48–1.74 (m)	1.26–1.74 (m)
6	1.27–1.56 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.26–1.57 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.26–1.74 (m)	1.26–1.74 (m)
7	1.27–1.56 (m)	1.45–1.68 (m)	1.45–1.60 (m)	1.26–1.57 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.26–1.74 (m)	1.26–1.74 (m)
8	1.27–1.56 (m)	3.39–3.44 (m)	3.39–3.44 (m)	1.26–1.57 (m)	1.28–1.75 (m)	1.49–1.75 (m)	1.26–1.74 (m)	1.26–1.74 (m)
9	1.59–1.70 (m)	3.83–3.98 (m)	3.83–3.95 (m)	1.26–1.57 (m)	1.28–1.75 (m)	3.80–3.92 (m)	1.26–1.74 (m)	1.46–1.74 (m)
10	3.58–3.61 (m)	1.79–2.04 (m)	1.62–1.72 (m)	1.26–1.57 (m)	3.80–3.92 (m)	1.96–2.04 (m), 1.63–1.75 (m)	3.80–3.89 (m)	3.58–3.64 (m)
11	1.59–1.70 (m)	1.79–2.04 (m)	1.96–2.02(m)	1.44–1.57 (m)	1.96–2.07 (m), 1.82–1.94 (m)	1.96–2.04 (m), 1.63–1.75 (m)	1.98–2.05 (m), 1.48–1.74 (m)	1.46–1.74 (m)
12	1.27–1.56 (m)	3.83–3.98 (m)	3.83–3.95 (m)	3.39–3.41 (m)	1.96–2.07 (m), 1.82–1.94 (m)	3.80–3.92 (m)	1.98–2.05 (m), 1.48–1.74 (m)	1.26–1.74 (m)
13	1.27–1.56 (m)	3.83–3.98 (m)	3.83–3.95 (m)	3.39–3.41 (m)	3.80–3.92 (m)	3.40–3.47 (m)	3.80–3.89 (m)	1.26–1.74 (m)
14	1.27–1.56 (m)	1.79–2.04 (m)	1.96–2.02 (m)	1.44–1.57 (m)	3.40–3.47 (m)	1.28–1.75 (m)	3.41–3.44 (m)	1.26–1.74 (m)
15	1.27–1.56 (m)	1.79–2.04 (m), 1.45–1.68 (m)	1.62–1.72 (m)	1.62–1.72 (m)	1.96–2.07 (m), 1.82–1.94 (m)	1.28–1.75 (m)	1.48–1.74 (m)	1.26–1.74 (m)
16	1.44–1.58 (m)	3.83–3.98 (m)	3.83–3.95 (m)	3.39–3.41 (m)	1.96–2.07 (m), 1.82–1.94 (m)	3.40–3.47 (m)	1.26–1.74 (m)	1.46–1.74 (m)
17	3.39–3.44 (m)	3.83–3.98 (m)	3.39–3.44 (m)	3.78–3.96 (m)	3.40–3.47 (m)	3.80–3.92 (m)	1.26–1.74 (m)	3.40–3.47 (m)
18	3.83–3.97 (m)	1.45–1.68 (m)	1.45–1.60 (m)	1.79–2.01 (m)	3.80–3.92 (m)	1.96–2.04 (m), 1.63–1.75 (m)	3.41–3.44 (m)	3.77–3.85 (m)
19	1.79–2.03 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.79–2.01 (m)	1.84–2.06 (m)	1.96–2.04 (m), 1.63–1.75 (m)	3.41–3.44 (m)	1.95–2.03 (m), 1.46–1.74 (m)
20	1.79–2.03 (m)	1.28–1.58 (m)	1.28–1.60 (m)	3.78–3.96 (m)	1.84–2.06 (m)	3.80–3.92 (m)	1.26–1.74 (m)	1.95–2.03 (m), 1.46–1.74 (m)
21	3.83–3.97 (m)	1.28–1.58 (m)	1.28–1.60 (m)	3.78–3.96 (m)	3.80–3.92 (m)	3.40–3.47 (m)	1.26–1.74 (m)	3.77–3.85 (m)
22	3.83–3.97 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.79–2.01 (m)	3.80–3.92 (m)	1.49–1.75 (m)	1.26–1.74 (m)	3.40–3.47 (m)
23	1.79–2.03 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.79–2.01 (m), 1.49–1.67 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.26–1.74 (m)	1.46–1.74 (m)
24	1.79–2.03 (m), 1.59–1.70 (m)	1.28–1.58 (m)	1.28–1.60 (m)	3.78–3.96 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.26–1.74 (m)	1.26–1.74 (m)
25	3.83–3.97 (m)	1.28–1.58 (m)	1.28–1.60 (m)	3.78–3.96 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.26–1.74 (m)	1.26–1.74 (m)
26	3.83–3.97 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.44–1.57 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.26–1.74 (m)	1.26–1.74 (m)
27	1.59–1.70 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.26–1.57 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.26–1.74 (m)	1.26–1.74 (m)
28–31	1.27–1.56 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.26–1.57 (m)	1.28–1.75 (m)	1.28–1.75 (m)	1.26–1.74 (m)	1.26–1.74 (m)
32	1.27–1.56 (m)	1.28–1.58 (m)	1.28–1.60 (m)	1.26–1.57 (m)	1.28–1.75 (m)	1.28–1.75 (m)	0.88 (t, 6.8)	0.88 (t, 6.8)
33	1.44–1.56 (m)	1.45–1.68 (m)	1.45–1.60 (m)	1.44–1.57 (m)	1.28–1.75 (m)	1.28–1.75 (m)	7.21 (d, 1.1)	7.21 (d, 1.2)
34	0.91 (t, 6.8)	0.90 (t, 6.7)	0.90 (t, 6.7)	0.90 (t, 6.7)	0.90 (t, 6.8)	0.90 (t, 6.7)	5.07 (dq, 6.8, 1.2)	5.06 (dq, 6.8, 1.2)
35	7.00 (d, 1.7)	7.00 (d, 1.0)	7.00 (d, 1.5)	7.00 (d, 1.5)	7.20 (d, 1.5)	7.01 (d, 1.7)	1.44 (d, 6.8)	1.43 (d, 6.8)
36	5.01 (qd, 6.8, 1.8)	5.01 (qd, 6.8, 1.3)	5.01 (qd, 6.8, 1.8)	5.01 (qd, 6.8, 1.8)	5.08 (dq, 6.8, 1.6)	5.02 (dq, 6.8, 1.9)	–	–
37	1.42 (d, 6.8)	1.42 (d, 6.8)	1.43 (d, 6.8)	1.41 (d, 6.8)	1.45 (d, 6.8)	1.43 (d, 6.8)	–	–

Compound **3**, a colorless oil, shared a molecular formula of $C_{37}H_{66}O_6$ with **2** (*m/z* 607.4879 $[M + H]^+$, calcd. 607.4932). A positive reaction to Raymond's reagent suggested the existence of a *γ*-lactone. The signals at δ_H 7.00 (1H, d, *J* = 1.5 Hz, H-35), 5.01 (1H, dq, *J* = 6.8, 1.8 Hz, H-36), 1.43 (3H, d, *J* = 6.8 Hz, H-37) and δ_C173.9 (C-1), 134.4 (C-2), 148.8 (C-35), 77.4 (C-36) and 19.2 (C-37) confirmed the presence of lactone. Signal at 2.28 (2H, t, *J* = 7.7 Hz, H-3) indicated there is no hydroxyl group at C-4 [3]. The signals at δ_H 3.83–3.95 (4H, m, H-9, 12, 13, 16), 3.38-3.43 (2H, m, H-8, 17) and those at δ_C 83.2 (C-9, 16), 81.8 (C-12, 13), 74.1 (C-8, 17) in the ^{1}H and ^{13}C NMR spectra are the characteristic of the presence of symmetrical two adjacent bis-THF unit with two flanking OH groups, and the relative stereochemistry was established as *th/t/th/t/th* (*threo/trans/threo/trans/threo*) by carefully comparison of NMR data with a series of Asimicin-type ACGs [14-15]. The position of bis-THF was determined on the basis of the ESIMSMS fragments (387, 249 and 167) (Fig. 2).

Compound **4** was obtained as colorless oil. The molecular formula was determined as $C_{37}H_{66}O_8$ by HR-ESIMS data (*m/z* 639.4963 $[M + H]^+$, calcd. for 639.4831). The NMR data (Tables 1 and 2) analysis showed its structure was closely related to that of **1**, with the only difference being at δ_C 74.2, 74.6 and δ_H 3.39–3.41 (2H, m) for **4**, indicating a fourth hydroxy in **4**, and a existence of a vicinal diol group by comparison with that of glabranin [16] and 4-acetyl gigantetrocin A [17]. The location of vicinal diol group was determined by carefully analysis of ESIMSMS data (*m/z* 415, 335, 265, 245) (Fig. 2). The THF rings were located at C-16 to C-25, on basis of the fragment ions at *m/z* 373, 307, 293, 209 (Fig. 2).

Compound **5** displayed a molecular formula of $C_{37}H_{66}O_8$ by HR-ESIMS (*m/z* 639.4980 $[M + H]^+$, calcd. 639.4831). The presence of two nonadjacent bis-THF rings with three flanking OH groups was determined by ^{1}H NMR signals at δ_H 3.80–3.92 (6H, m. H-4, 10, 13, 18, 21, 22), 3.40–3.47 (2H, m, H-14, 17) and ^{13}C NMR signals at δ_C 83.3 (C-18), 82.2 (C-21), 82.0 (C-13), 79.3 (C-10), 74.6 (C-14), 74.5 (C-17), 71.6 (C-22). The signals at δ_H 7.20 (1H, d, *J* =1.5 Hz, H-35), 5.08 (1H, dq, *J* = 6.8, 1.6z Hz, H-36), 1.45 (3H, d, *J* = 6.8 Hz, H-37) and those at δ_C 174.6 (C-1), 131.2 (C-2), 151.8 (C-35), 78.0 (C-36), 19.1 (C-37) were characeristic of a terminal *α*, *β*-unsaturated *γ*-lactone. ^{1}H NMR signals at 2.41 (1H, dd, *J* = 15.2, 8.3 Hz, H-3) and 2.54 (1H, d , *J* = 15.1, 3.4 Hz, H-3) indicated the presence of a hydroxyl at C-4 [18]. The THF rings with flanking hydroxyls were located at C-10 to C-22 (Fig. 2), on the basis of ESIMSMS fragments at 369, 289, 249 and 211. The relative stereochemistry across the THF rings and flanking hydroxyls of **5** was assigned as *t/th-th/t/er* (*trans*/*threo-threo/trans/erythro*) by comparison of NMR data with a series of bullatalicin-type compounds [3, 19].

Compound **6** was isolated as a white waxy with molecular formula of $C_{37}H_{66}O_7$, on basis of its HR-ESIMS fragment (*m/z* 623.4789 $[M + H]^+$, calcd for 623.4881). The NMR data (Tables 1 and 2) displayed characteristic signals for a terminal unsaturated lactone. The signal at 2.28 (2H, t, *J* = 7.8 Hz, H-3) indicated an absence of hydroxyl at C-4 [3]. NMR data at δ_H 3.80–3.92 (4H, m. H-9, 12, 17, 20), 3.40–3.47 (3H, m, H-13, 16, 21) and those at δ_C 82.7 (C-17, 20), 82.0 (C-12), 79.4 (C-9), 74.5 (C-21), 74.3 (C-16) 74.1 (C-13) indicated the presence of two nonadjacent bis-THF rings with three flanking hydroxyl groups, and the relative stereochemistry of those was *t/th-th/t/th* (*trans/threo-threo/trans/threo*) by comparison of NMR data with a series of bullatanocin-type compounds [20-21]. The bis-THF rings and the flanking OH groups were located at C-9 to C-21 on the basis of MS^2 fragments at *m/z* 383, 371, 319, 291, 263 and 193 (Fig. 2).

Compound **7**, a white waxy, gave a molecular formula of $C_{35}H_{64}O_7$, deduced from HR-ESIMS fragment at *m/z* 597.4621 $[M + H]^+$ (calcd. 597.4725). The NMR data at δ_H 7.21 (1H, d, *J* = 1.1 Hz, H-33), 5.07 (1H, dq, *J* = 6.8, 1.2 Hz, H-34), 1.44 (3H, d, *J* = 6.8 Hz, H-35) and those at δ_C 174.7 (C-1), 131.1 (C-2), 151.9 (C-33), 78.0 (C-34), 19.1 (C-35) were characeristic of a terminal *α*, *β*-unsaturated *γ*-lactone. ^{1}H NMR signals at 2.40 (1H, dd, *J* = 15.1, 8.2 Hz, H-3) and 2.52 (1H, d , *J* = 15.1, 3.3 Hz, H-3) indicated the presence of hydroxyl at C-4 [18]. NMR data (Tabs 1 and 2) indicated the presence of a THF ring with one flanking hydroxyl and two other OH groups. The relative configuration of THF ring and flanking hydroxyl was assigned as *th/t* (*threo/trans*) [16, 22-23]. The position of them was located at C-10 to C-14 by analysis of the fragment ions at *m/z* 289 and 191 (Fig. 2). The proton signal at δ_H 3.41– 3.44 (3H, m) and the carbon signals at δ_C 74.3 and 74.5 were suspected to represent a 1,2-diol group [16-17]. The position of two adjacent hydroxyls was assigned by carefully analysis on MSMS data (*m/z* 335, 329, 205) (Fig. 2).

Compound **8** had a molecular formula of $C_{35}H_{64}O_7$ based on the HR-ESIMS ($[M + H]^+$ *m/z* 597.4636, calcd. 597.4725). The NMR data (Tables 1 and 2) of **8** suggested the presence of a terminal *α, β*-unsaturated γ-lactone and a presence of a hydroxyl at C-4 [18]. NMR signals at δ_H 3.77–3.85 (3H, m, H-4, 18, 21), 3.58–3.64 (1H, m, H-10), 3.40–3.47 (2H, m, H-17, 22) and those at δ_C 82.8 (C-21), 82.5 (C-18), 74.3 (C-22), 74.1 (C-17), 71.5 (C-10) indicated the existence of a THF ring with two flanking OH groups in a *th/t/th* (*threo*/*trans*/*threo*) configuration [23-24] and a fourth hydroxyl. The location was at C-17 to C-22, on the basis of ESIMS^2 data *m/z* 335, 289, 253. The position of fourth OH group was determined by *m/z* 205 and 191 (Fig. 2).

Compounds **1**–**9** were evaluated for their anticancer activities against three MDR cancer cell lines including liver cancer SMMC 7721/Adr, breast cancer MCF-7/Adr and lung cancer A549/T (Table 3). All tested compounds showed promising cytotoxicity against these MDR cancer cell lines, and cytotoxicity of them is much stronger than Adriamycin against SMMC 7721/Adr.

Table 3 The cytotoxicity of compound 1–9 from *A. squamosa*

Sample	IC_{50} (μmol·L^{-1})		
	SMMC 7721/Adr	MCF-7/Adr	A549/T
squamotin A (**1**)	2.56	12.42	7.44
squamotin B (**2**)	8.54	22.55	5.23
squamotin C (**3**)	14.10	54.97	11.04
squamotin D (**4**)	1.43	2.88	7.27
annosquatin IV (**5**)	5.68	4.71	6.13
annosquatin V (**6**)	7.88	2.07	3.21
muricin O (**7**)	5.43	5.78	8.37
squamosten B (**8**)	5.40	4.03	10.06
gigantecin (**9**)	15.45	20.58	36.33
Adr	1815	9.99	7.96

Materials and Methods

General experimental procedures

NMR spectroscopic data were collected on a Bruker AV-400 spectrometer with deuterated chloroform ($CDCl_3$) as solvents. HR-ESIMS were measured on an ESI-Q-TOF-MS (Triple TOF 5600, AB Sciex). Optical rotations were recorded in EtOH on a Rudolph AUTOPOL III high sensitive polarimeter (America). The Ultraviolet spectra were measured on a HP 8451A diode array spectrophotometer. Silica gel (100–200 mesh, 200–300 mesh) (Qingdao marine Chemical Ltd., Qingdao, China), ODS-A (YMC Co., Japan) was used for column chromatography. Preparative HPLC were performed on Shimazu LC-20A (Shimazu, Japan) with a Waters ODS column (250 mm × 19 mm i.d., 5 μm, Part No. 186004021, Waters, America). Unless otherwise specified, all chemicals and solvents were purchased from Nanjing Chemical Reagent Co., Ltd. (Nanjing, China)

Plant material

A. squamosa seeds were collected in Lincang City of Yunnan province in China, and identified by Prof. CHEN Jian-Wei (Nanjing University of Chinese Medicine). Sample was authenticated and deposited in Pharmaceutical Institute of Nanjing University of Chinese Medicine (No. 20150124).

Extraction and isolation

The air-dried seeds (22.5 kg) were powdered and extracted as previously described [25]. As a result, 3.3 kg of supercritical fluid CO_2 extraction (SFE) was obtained. This extraction was passed through silica gel (200–300 mesh) with a gradient of increasing polarity with Pet-ether/EtOAc/MeOH, and the resulting fractions were analyzed by TLC on silica gel. As a result, twelve fractions (Fr 1–Fr 12) were obtained. Fr 7 (171.1 g) was loaded on silica gel column and eluted by Pet-ether/EtOAc/MeOH (100 : 1 : 0–0 : 10 : 1, and further purified on an ODS column (MeOH–H_2O, 95 : 5; 8 mL·min^{-1} and 220 nm detection) to yield **2** (209 mg), **3** (53 mg), **10** (37 mg). Fr 8 (60.0 g) was loaded on a silica gel column and eluted with Pet-ether/EtOAc/MeOH (2 : 1 : 0–0 : 25 : 1), and further purified on an ODS column (MeOH–H_2O, 9:1; 10 mL·min^{-1} and 220 nm detection) to yield **1** (18 mg), **6** (21 mg), **11** (537 mg) and **12** (164 mg). Fr 10 (23.7 g) was separated by octadecyl-silylated silica at a MPLC (medium-pressure liquid chromatography) and further purified on an ODS column; (MeOH–H_2O, 75 : 25; 8 mL·min^{-1} and 220 nm detection) to yield **4** (12 mg), **5** (21 mg), **7** (17 mg), **8** (31 mg) and **9** (22 mg).

Squamotin A (**1**): a white waxy, mp 60–62 °C; $[\alpha]_D^{29}$ + 16.3° (*c* 0.10, EtOH); IR (KBr) ν_{max} 3379, 2917, 2851 and 1743; ^{1}H and ^{13}C-NMR: showed in Tab 1 and Tab 2; HR-ESIMS *m/z* 623.4921 $[M + H]^+$ ($C_{37}H_{67}O_7$, calcd. 623.4881).

Squamotin B (**2**): a white waxy, mp 62–64 °C; $[\alpha]_D^{29}$ + 10.2° (*c* 0.13, EtOH); IR (KBr) ν_{max} 3421, 2917, 2850 and 1744; ^{1}H and ^{13}C-NMR: showed in Tables 1 and 2; HR-ESIMS *m/z* 607.5068 $[M + H]^+$ ($C_{37}H_{67}O_6$, calcd. 607.4932).

Squamotin C (**3**): colorless oil, $[\alpha]_D^{29}$ +0.3° (*c* 0.31, EtOH); IR (KBr) ν_{max} 3421, 2927, 2855 and 1757; ^{1}H and ^{13}C-NMR: showed in Tables 1 and 2; HR-ESIMS *m/z* 607.4879 $[M + H]^+$ ($C_{37}H_{67}O_6$, calcd. 607.4932).

Squamotin D (**4**): colorless oil; $[\alpha]_D^{29}$ +1.4° (*c* 0.21, EtOH); IR (KBr) ν_{max} 3425, 2927, 2855 and 1757; ^{1}H and ^{13}C-NMR: showed in Tables 1 and 2; HR-ESIMS *m/z* 639.4963 $[M + H]^+$ ($C_{37}H_{67}O_8$, calcd. 639.4831).

Annosquatin IV (**5**): white waxy, mp 109–111 °C; $[\alpha]_D^{29}$ + 4.0° (*c* 0.12, EtOH); IR (KBr) ν_{max} 3431, 2922, 2851 and 1751; ^{1}H and ^{13}C-NMR: showed in Tables 1 and 2; HR-ESIMS *m/z* 639.4980 $[M + H]^+$ ($C_{37}H_{67}O_8$, calcd. 639.4831).

Annosquatin V (**6**): white waxy, mp 66–68 °C; $[\alpha]_D^{29}$ + 8.0° (*c* 0.10, EtOH); IR (KBr) ν_{max} 3431, 2920, 2851 and 1740; ^{1}H and ^{13}C-NMR: showed in Tab 1 and Table 2; HR-ESIMS *m/z* 623.4789 $[M + H]^+$ ($C_{37}H_{67}O_7$, calcd. 623.4881)

Muricin O (**7**): white waxy, mp 82–84 °C; $[\alpha]_D^{29}$ +6.7° (*c* = 0.10, EtOH); IR (KBr) ν_{max} 3422, 2921, 2850 and 1752; ^{1}H and ^{13}C-NMR: showed in Tables 1 and 2; HR-ESIMS *m/z* 597.4621 $[M + H]^+$ ($C_{35}H_{65}O_7$, calcd. 597.4725).

Squamosten B (**8**): white waxy, mp 62–64 °C; $[\alpha]_D^{29}$ + 10.7° (*c* 0.08, EtOH); IR (KBr) ν_{max} 3431, 2921, 2851 and 1736; ^{1}H and ^{13}C-NMR: showed in Tables 1 and 2; HR-ESIMS *m/z* 597.4636 $[M + H]^+$ ($C_{35}H_{65}O_7$, calcd. 597.4725).

Anticancer activity assay

Compounds **1–9** were evaluated for their cytotoxicity against three MDR cancer cell lines (liver cancer SMMC 7721/Adr, breast cancer MCF-7/Adr and lung cancer A549/T), using the MTT method in 96-well plates. Adriamycin was used as a positive control.

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