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Bioactive acetogenins from the seeds of *Annona atemoya*

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Abstract

Six new annonaceous acetogenins, 12,15-*cis*-squamostatin-D (**1**), 12,15-*cis*-squamostatin-A (**2**), artemoin-A (**3**), artemoin-B (**4**), artemoin-C (**5**) and artemoin-D (**6**), along with eleven known ones, were isolated from the seeds of Formosan *Annona atemoya*. The structures of these isolates were established on the basis of mass and related spectral evidence. All of them, except for four linear annonaceous acetogenins, exhibited potent cytotoxicity against Hep G_2, Hep 2,2,15, KB, CCM_2 and CEM cancer cell lines.

Keywords: *Annona atemoya*; Annonaceae; 12; 15-*cis*-squamostatin-D; 12; 15-*cis*-squamostatin-A; Artemoin-A; Artemoin-B; Artemoin-C; Artemoin-D

1. Introduction

Annonaceous acetogenins have attracted much interest in recent years because of their wide range of biological activities and their special structures. More than 300 acetogenins, belonging to non-, mono, bis-, and tri-tetrahydrofuran (THF) subgroups, have been reported (Zafra-Polo, Figadère, Gallardo, Tormo & Cortes, 1998; Yazbak, Sinha & Keinan, 1998). Five cytotoxic annonaceous acetogenins, annotemoyin-1, annotemoyin-2, atemoyin (squamocin K), atemoyacin A (parviflorin, squamocin E) and atemoyacin B, have been isolated from the seeds of *A. atemoya* (*A. cherimolia* × *A. squamosa*) (Zeng et al., 1996). In our previous papers, several cytotoxic acetogenins were reported from *Annona reticulata* (Wu, Chang, Duh & Wang, 1992; Chang, Wu, Duh & Wang, 1993; Chen, Chang, Chiu, Wu & Wu, 1998), *A. montana* (Wu, Chang, Chen, Liang & Lee, 1994) and *Rollinia mucosa* (Chen, Chang, Yen & Wu, 1996) of annonaceous plants. As part of our continuous searching for novel bioactive agents from plants, a methanolic extract from the seeds of Formosan *A. atemoya* was found to show significant cytotoxic activities against Hep G_2, Hep 2,2,15, KB and CCM_2 cancer cell lines. Bioactivity-guided chromatographic fractionation of the active extract led to the isolation and characterization of six new compounds, 12,15-*cis*-squamostatin-D (**1**), 12,15-*cis*-squamostatin-A (**2**), artemoin-A (**3**), artemoin-B (**4**), artemoin-C (**5**) and artemoin-D (**6**), along with eleven known compounds, squamostatin-A (**7**) (Fujimoto et al., 1990), squamostatin-D (**8**) (Fujimoto et al., 1994), squamocin (**9**) (Born et al., 1990), neoannonin (**10**) (Sahai et al., 1994; Kawazu, Alcantara & Kobayashi, 1989), bullatacin (**11**) (Sahai et al., 1994; Kawazu et al., 1989; Duret, Hocquemiller, Laurens & Cave, 1995; Etse and Waterman, 1986), desacetyluvaricin (**12**) (Sahai et al., 1994), isodesacetyluvaricin (**13**) (Sahai et al., 1994), 12,15-*cis*-bullatanocin (**14**) (Gu, Zeng & McLaughlin, 1995), 12,15-*cis*-bullatalicin (**15**) (Gu et al., 1995), bullatanocin (**16**) (Gu et al., 1995), bullatalicin (**17**) (Gu et al., 1994). Their structures were determined by means of mass and spectral experiments before chemical derivatives were made. Herein, we report on the structural elucidation of new com-

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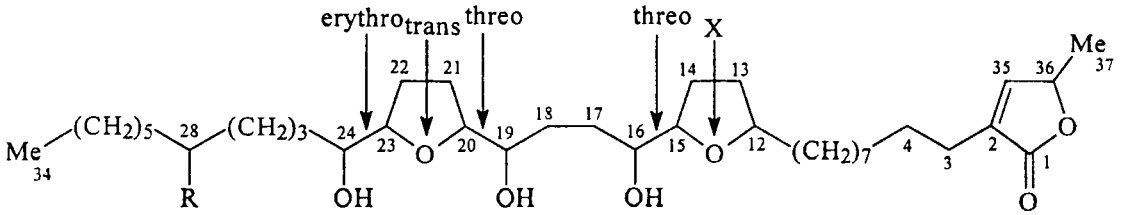
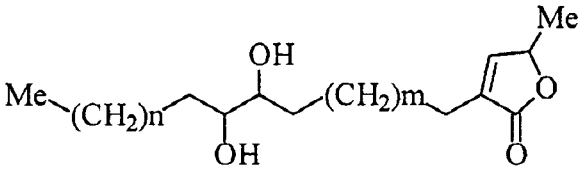


12,15-*cis*-squamostatin-D (**1**); R=H, X=cis
12,15-*cis*-squamostatin-A (**2**); R=OH, X=cis
squamostatin-A (**7**); R=OH, X=trans
squamostatin-D (**8**); R=H, X=trans



artemoin-A (**3**); (m=18, n=9)
artemoin-B (**4**); (m=16, n=11)
artemoin-C (**5**); (m=14, n=13)
artemoin-D (**6**); (m=12, n=15)

Scheme 1. The structures of 12,15-*cis*-squamostatin-D (**1**), 12,15-*cis*-squamostatin-A (**2**), squamostatin-A (**7**), squamostatin-D (**8**), artemoin-A (**3**), artemoin-B (**4**), artemoin-C (**5**) and artemoin-D (**6**).

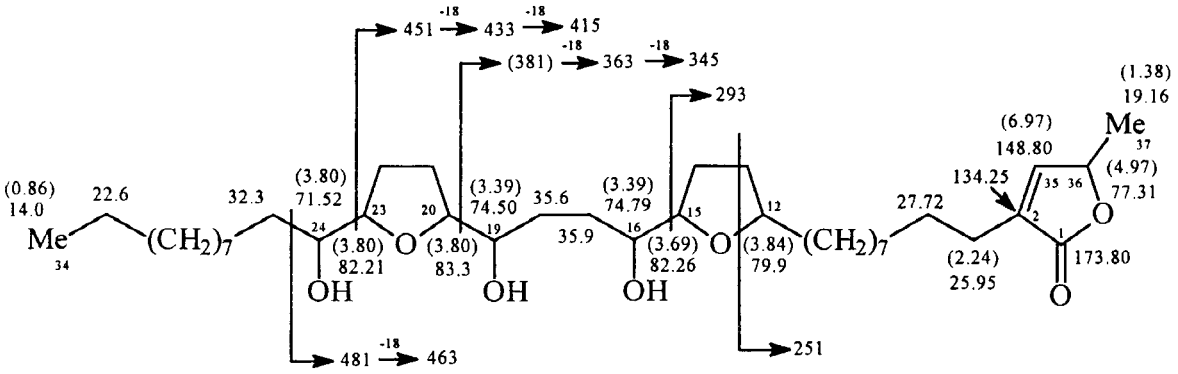
pounds and the cytotoxicities of the active constituents (Scheme 1).

2. Results and discussion

12,15-*Cis*-squamostatin-D (**1**) and squamostatin-D (**8**) (Fujimoto et al., 1994) were obtained as a mixture (2:3) initially. The chemical shifts of oxygen-bearing carbons in the ^{13}C NMR (at δ 68–74) indicated the existence of a mixture of two steric isomers. Because of their chemical similarity, purification of these compounds was difficult. The mixture was, however, subjected to semi-preparative HPLC, **1** AND **8** [HPLC (Hypersil BDS-C18 column, 250 × 20 mm, i.d.)] eluted with MeOH–H_2O (8:2, flow rate 2 ml/min, detection at 225 nm), which gave separation of both with retention times of 600 min and 580 min, respectively. The HR-FAB mass spectrum exhibited an $[M+H]^+$ ion at m/z 623.4892 (calcd 623.4887) corresponding to the molecular formula $C_{37}H_{66}O_7$. The IR spectrum of **1** showed characteristic absorption of an α,β-unsaturated γ-lactone at ν 1750 cm^{-1} and a hydroxyl group at ν 3450 cm^{-1}. The presence of a methylated α,β-unsaturated γ-lactone moiety was confirmed by ^1H NMR resonance peaks at δ 6.97 (1H, *d*, J=1.2 Hz, H-35), 4.97 (1H, *qd*, J=6.8, 1.2 Hz, H-36) and 1.38 (3H, *d*, J=6.8 Hz, H-37), and ^{13}C NMR resonance peaks at δ 173.80 (C-1), 134.25 (C-2), 148.80 (C-35), 77.31 (C-36) and 19.16 (C-37). That **1** was not hydroxylated at position 4 was established by the H-3 signal at δ 2.24 (2H, *t*, J=7.2 Hz, H-3) correlating with the ^{13}C NMR spectral resonance at δ 25.95 in the HETCOR spectrum of **1** (Sahai et al., 1994).

By direct comparison of the ^1H and ^{13}C NMR spectra, the structure of **1** was found to be similar to that of 12,15-*cis*-bullatalicin (**15**) (Gu et al., 1995) except for substitution at the C-4 position. Two signals for H-16 and H-19 at *ca* δ 3.40 (1H each) and another signal for H-24 at *ca* δ 3.80 (1H) were used to assign the relative stereochemistries of C-15/C-16, C-19/C-20 as *threo*, and C-23/C-24 as *erythro*. The methylene protons of C-21 and C-22 at *ca* δ 1.98 and 1.64 suggested that the relative configurations of the C-20/C-23 THF ring of **1** should be *trans* (Fujimoto et al., 1994; Gu et al., 1994).

The ^1H and ^{13}C NMR spectral data of **1** were compared with those of **8** (Fujimoto et al., 1994); the C-12 signal at δ 79.9 indicated the *cis* configuration of a THF ring with a single flanking hydroxyl group (the *trans* type shows the C-12 value at δ 79.2) (Fujimoto et al., 1994; Gu et al., 1995). The signal for H-15 resonating at δ 3.78 in **8** was shifted upfield to δ 3.69 in **1**. This assignment was confirmed by single- and double-relayed COSY spectra. The difference between **1** and **8** has to be the configuration of the C-12/15 THF ring. The unusual chemical shift of H-15 was comparable with that of H-15 in 12,15-*cis*-bullatanocin (**14**) and

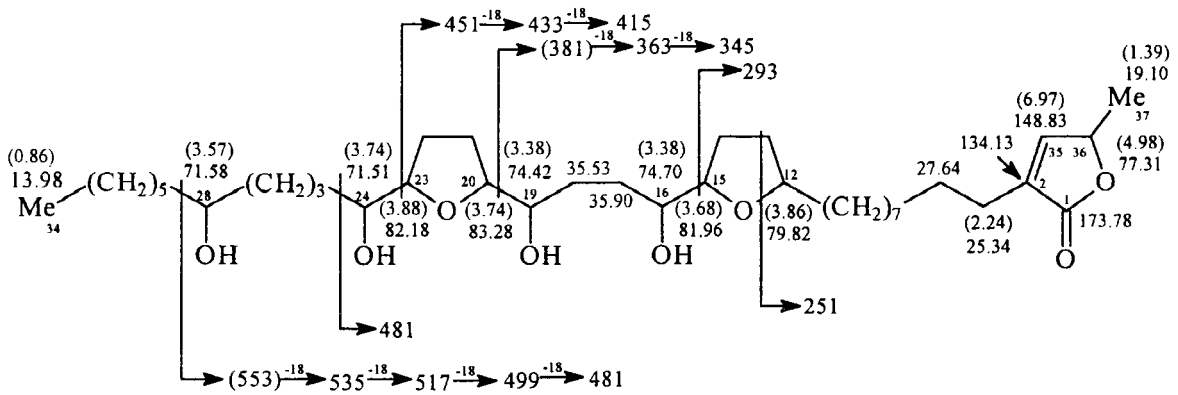


Scheme 2. The ^{1}H and ^{13}C NMR spectral data and diagnostic EIMS fragment ions of 12,15-*cis*-squamostatin-D (**1**). EIMS peaks in parentheses were not observed.

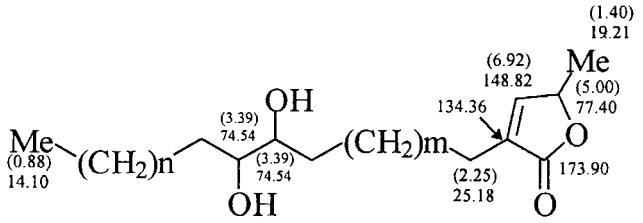
12,15-*cis*-bullatalicin (**15**) (Gu et al., 1995), which also possesses a *cis* THF ring at C-12/C-15 with a single flanking hydroxyl. H-15 of **14** and **15** resonates at δ 3.72 and the configurations of the THF rings have been determined to be *cis* (Duret et al., 1995). Thus, the configuration of the C-12/C-15 THF ring in **1** was proposed to be *cis* and not *trans* as in **8**, and the relative stereochemistry of the non-adjacent bis-THF subunit (C-12–C-24) of **1** was determined to be *cis-threo-threo-trans-erythro* (Fujimoto et al., 1994; Gu et al., 1995; Shi, Zeng, Gu, MacDougal & McLaughlin, 1995). The carbon skeleton and placement of the THF rings of **1** were determined on the basis of FAB and EI mass spectra analysis (Scheme 2). Based on the above discussion, the structure of 12,15-*cis*-squamostatin-D was determined as **1**.

12,15-*Cis*-squamostatin-A (**2**) was obtained as a colorless oil mixed with squamostatin-A (**7**) (Fang, Gu, Rieser, Hui & McLaughlin, 1993) (3:1). Various semi-preparative HPLC columns were employed to separate this sample in various solvent systems, without success. The IR spectrum also showed the characteristic absorptions of an α,β-unsaturated γ-lactone (1750 cm^{-1}, C=O) and OH (3450 cm^{-1}) functional groups. The HR-FAB mass spectrum exhibited an $[M+H]^{+}$ ion at m/z 639.4838 (calcd 639.4834) corresponding to the molecular formula $C_{37}H_{66}O_8$. ^{1}H NMR and ^{13}C NMR data of mixtures of **2** and **7** were similar to those of mixtures of **1** and **8** except for an additional oxymethine resonance. The location and placement of the THF rings of **2** and **7** were determined by FAB and EI mass spectral analyses (Scheme 3). ^{1}H and ^{13}C NMR data (Scheme 3) suggested that the methylated α,β-unsaturated-γ-lactone moiety of **2** should be the same as that of **1** (Sahai et al., 1994).

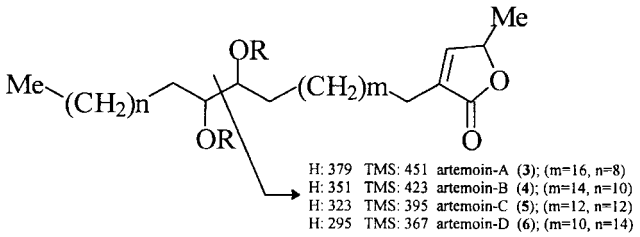
^{1}H and ^{13}C NMR data of **2** were compared with those of **7** (Fang, Gu et al., 1993). The C-12 signal at δ 79.9 in the ^{13}C NMR of **2** indicated the *cis* configuration of a THF ring with a single flanking hydroxyl as in compound **1** (Fujimoto et al., 1994; Gu et al., 1995). The H-15 signal resonating at δ 3.78 in **7** was shifted upfield to δ 3.68 in **2**. This assignment was confirmed by single- and double-relayed COSY spectra. The difference between **2** and **7** has to be the configuration of the C-12/15 THF ring. The structure of **2** was similar to the non-adjacent bis-THF acetogenin **1** in



Scheme 3. The ^{1}H and ^{13}C NMR spectral data and diagnostic EIMS fragment ions of 12,15-*cis*-squamostatin-A (**2**). EIMS peaks in parentheses were not observed.



Scheme 4. The ^{1}H and ^{13}C NMR spectral data of artemoin-A (**3**), artemoin-B (**4**), artemoin-C (**5**) and artemoin-D (**6**).



Scheme 5. Diagnostic EIMS fragmentation of artemoin-A (**3**), artemoin-B (**4**), artemoin-C (**5**) and artemoin-D (**6**).

comparison with spectral data. The relative stereochemistry of the non-adjacent bis-THF subunit (C-12–C-24) of **2** was determined to be *cis-threo-threo-trans-erythro* again. However, minor differences in the ^{1}H NMR, ^{13}C NMR and mass spectra were observed between **1** and the mixture of **2** and **7**. A multiplet proton at δ 3.57 in the mixture of **2** and **7**, correlating with a methine carbon signal at δ 71.58, was characteristic of one more hydroxyl group in the alkyl chain. The position of the hydroxyl group was confirmed by the EIMS of **2** and **7**, which sowed a cleavage between C-28 and C-29 and successive losses of H_2O (Scheme 3). The additional hydroxyl group was determined to be at position 28 in **2** and **7**. Thus, the structure of 12,15-*cis*-squamostatin-A was determined as **2**.

Artemoin-A (**3**), artemoin-B (**4**), artemoin-C (**5**) and artemoin-D (**6**) were obtained in a mixture as a colorless oil. The molecular formula was deduced as $C_{35}H_{66}O_4$ by the HR-FAB mass spectrum which gave an $[M+H]^+$ ion at m/z 551.5035 (calcd 551.5041). The existence of OH groups was indicated by an IR hydroxy absorption at 3400 cm^{-1}, two successive losses of H_2O from the $[M+H]^+$ in the mass spectra and the preparation of di-TMSi (trimethylsilyl) derivatives. Like other annonaceous acetogenins, the presence of the methyl substituted α,β-unsaturated γ-lactone was suggested by the IR carbonyl absorption (1750 cm^{-1}) and the corresponding resonances in the ^{1}H- and ^{13}C-NMR spectra (Scheme 4) of **3–6**. However, the lack of a THF ring in the aliphatic chain was indicated by the absence of any corresponding THF either proton and carbon signals in the NMR spectra (Scheme 4).

The presence of the diol moiety was also indicated by ^{1}H-NMR signals at δ 3.40 for two carbinol methine protons, and two ^{13}C-NMR signals for oxygenated carbons overlapping at δ 74.54. The carbon signal of the isolated hydroxyl group was typically displayed at δ 70–72 (Rupprecht, Hui & McLaughlin, 1990; Fang, Rieser, Gu, Zhao & McLaughlin, 1993). To determine the relative configuration of the diol, acetonide derivatives of **3–6** were prepared. The ^{1}H-NMR signals for the diol at δ 3.58, and signals for the non-equivalent acetonyl methyl protons at δ 1.37 and δ 1.38, suggested a *trans* assignment for the dioxolane ring (the methyl protons of a *cis* dioxolane ring show two well-separated singlet peaks at δ 1.43 and δ 1.33, and the methine protons of the acetonide appeared at δ 4.03 and δ 4.00) (Doong, Tsai, Schinazi, Liotta & Cheng, 1991). Thus, the configuration of the diol was determined to be *threo*, since the *trans* configuration of the diol in **3–6** could only be derived from a vicinal diol with a *threo* configuration (Scheme 4).

Table 1
Cytotoxicities of compounds **1**, **8–12**

Compound	ED_{50} μg/ml				
	Hep G_2[b]	Hep 2,2,15[a]	KB[c]	CCM_2[d]	CEM[e]
1	2.20×10^{-4}	3.10×10^{-3}	4.05×10^{-4}	Non-test	Non-test
8	1.50×10^{-4}	1.50×10^{-3}	3.90×10^{-4}	Non-test	Non-test
9	8.80×10^{-4}	1.50×10^{-3}	2.70×10^{-1}	1.60×10^{-2}	149
10	1.10×10^{-4}	1.26×10^{-4}	1.46×10^{-4}	10.9	520
11	9.70×10^{-5}	1.11×10^{-4}	1.17×10^{-4}	1.41×10^{-1}	169
12	1.02×10^{-4}	1.18×10^{-4}	1.35×10^{-4}	23.5	100
Adriamycin	5.00×10^{-2}	4.50×10^{-1}	Non-test	Non-test	Non-test

[a] Hep 2,2,15 (human hepatoma cell *trans*fected HBV).
[b] Hep G_2 (human hepatoma cell).
[c] KB (human nasopharyngeal carcinoma).
[d] CCM_2 (human colon tumor cell).
[e] CEM (T-lymphoblastoid cell).

The FAB mass spectrum of compounds **3**–**6** exhibited one pseudo-molecular ion at m/z 551 $[M+1]^+$ and an ion at m/z 573 $[M+Na]^+$. Moreover, four peaks appeared in the EIMS spectrum at m/z 379 (100%), 351 (47%), 323 (51%) and 295 (20%). This phenomenon suggested that four constitutional isomers were mixed together. The positions of the vicinal diol were determined to be at C-21/C-22 in **3**, C-19/C-20 in **4**, C-17/C-18 in **5** and C-15/C-16 in **6** by diagnostic fragments, respectively. The HREI mass gave ions at m/z 379.3191 for $C_{24}H_{43}O_3$ (calcd 379.3212), m/z 351.2903 for $C_{22}H_{39}O_3$ (calcd 351.2900), m/z 323.2575 for $C_{20}H_{35}O_3$ (calcd 323.2586) and m/z 295.2271 for $C_{18}H_{31}O_3$ (calcd 295.2273), which further confirmed the structures of **3**–**6** (Scheme 5).

Squamocin (**9**), neoannonin (**10**), bullatacin (**11**), mixtures of desacetyluvaricin (**12**) and isodesacetyluvaricin (**13**), and mixtures of 12,15-*cis*-bullatanocin (**14**), 12,15-*cis*-bullatalicin (**15**), bullatanocin (**16**) and bullatalicin (**17**) were also isolated as white amorphous or colorless oil-like substances and their structures were established by analyses of the IR, mass, ^1H and ^{13}C NMR spectra.

The cytotoxicities of **1**, **8**–**11** are shown in Table 1. The cytotoxicities of compounds **3**–**6** were lost due to a lack of THF ring systems (Wu et al., 1994; Chen et al., 1996).

3. Experimental

3.1. General

UV spectra were obtained in EtOH, IR spectra (KBr) were measured on a Hitachi 260-30 spectrophotometer. ^1H NMR (400 MHz), ^{13}C NMR (100 MHz), HETCOR, COSY, double-relayed COSY and DEPT spectra (all in $CDCl_3$) were obtained on a Varian Unity Plus 400 NMR spectrometer. FABMS and EIMS were collected on a Jeol JMS-SX/SX 102A mass spectrometer or Quattro GS/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, precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical TLC and precoated silica gel plated (Merck, Kieselgel 60 F-254, 0.50 mm) were used for preparative TLC. The spots were detected by spraying with Kedde's reagent or 50% H_2SO_4 and then heating on a hot plate. HPLC separations were carried out on Shimadzu and JASCO HPLC systems using reversed-phase columns (Hypersil BDS-C18, 250 × 20 mm, i.d.; and Develosil ODS, 250 × 20 mm, i.d.) with detection at 225 nm.

3.2. Plant material

Seeds of *Annona atemoya* were collected in Chia-Yi in Taiwan, June 1994. A voucher specimen (Anno. 14) is deposited in the Graduate Institute of Natural Products, Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

3.3. Extraction and isolation

Fresh seeds of *A. atemoya* (3 kg) were extracted repeatedly with EtOAc at room temp. The combined EtOAc extracts were evapd and partitioned to yield $CHCl_3$ and aq. extracts. The $CHCl_3$ layer (*ca* 40 g) after concn was partitioned between *n*-hexane and MeOH. The MeOH phase afforded a wax (140 g) positive to Kedde's reagent, implying the presence of annonaceous acetogenins. The MeOH phase was repeatedly subjected to CC on silica gel eluting with *n*-hexane, $CHCl_3$ gradient mixture of $CHCl_3$–MeOH system (99:1, 19:1, 9:1 and 4:1) and MeOH, and gave 30 frs. The frs were rechromatographed, followed by recrystallization in cold EtOAc (ice bath) or preparative TLC (MeOH–$CHCl_3$ 20:1 or *n*-hexane–EtOAc 2:1) to afford acetogenin **1** (20 mg), a mixt. of **2** and **7** (72 mg) (1:3), a mixture of **3**–**6** (20 mg), **8** (30 mg), **9** (4.2 g), **10** (80 mg), **11** (72 mg), a mixt. of **12** and **13** (43 mg) (4:1), and a mixture of **14**, **15**, **16** and **17** (49 mg). **1** and **8** were subjected into a Hepersil BDS-C18 column (250 × 20 mm i.d.) eluted with MeOH–H_2O 8:2 (flow rate 2 ml/min) in a HPLC system with detection at 225 nm, and they were separated after 600 min and 580 min, respectively. **2** and **7** could not be separated by various columns in different HPLC systems.

3.4. 12,15-Cis-squamostatin-D (*1*)

Colorless oil. $[\alpha]_D^{24}$ +19.90° ($CHCl_3$; *c* 0.62), IR ν_{max}^{Neat} cm^{-1}: 3400, 2910, 2850, 1750, 1205 and 750. HR-FABMS $[M+H]^+$ m/z: 623.4897 (calcd 623.4887) for $C_{37}H_{67}O_7$. ^1H NMR (400 MHz, $CDCl_3$) δ: 6.97 (1H, *d*, $J=1.2$ Hz, H-35), 4.97 (1H, *qd*, $J=6.8$, 1.2 Hz, H-36), 3.84–3.76 (4H, *m*, H-12, H-20, H-23, H-24), 3.69 (1H, *m*, H-15), 3.39 (2H, *m*, H-16, H-19), 1.38 (3H, *d*, $J=6.8$, H-37), 0.86 (3H, *t*, $J=6.4$, H-34), ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 173.80 (C-1), 148.80 (C-35), 134.25 (C-2), 83.30 (C-20), 82.21 (C-23), 82.26 (C-15), 79.90 (C-12), 77.31 (C-36), 74.50 (C-19), 74.79 (C-16), 71.52 (C-24), 19.16 (C-37), 14.04 (C-34). FABMS m/z: 645 $[M+Na]^+$, 623 $[M+H]^+$, EIMS (probe) 30 eV m/z: 481, 463, 451, 433, 415, 363, 345, 293 and 251.

3.5. 12,15-Cis-squamostatin-A (2) and squamostatin-A (7)

Colorless oil. IR ν_{max}^{Neat} cm^{-1}: 3400, 2910, 2850, 1750 and 1050. HR-EIMS $[M+H]^+$ *m/z*: 639.4838 (calcd 639.4834) for $C_{37}H_{67}O_8$. ^1H NMR (400 MHz, $CDCl_3$) δ (numbering is given for 12,15-*cis*-squamostatin-A/squamostatin-A): 6.97 (1H, *d, J* = 1.6 Hz, H-35), 4.98 (1H, *qd, J* = 6.8, 1.6 Hz, H-36), 3.88–3.74 (squamostatin-A, 5H, *m*, H-12, H-15, H-20, H-23, H-24), 3.88–3.74 (12,15-*cis*-squamostatin-A, 4H, *m*, H-12, H-20, H-23, H-24), 3.68 (12,15-*cis*-squamostatin-A, 1H, *m*, H-15), 3.57 (1H, *m*, H-28), 3.38 (2H, *m*, H-16, H-19), 1.39 (3H, *d, J* = 7.2, H-37), 0.86 (3H, *t, J* = 7.2, H-34), ^{13}C-NMR (100 MHz, $CDCl_3$) δ (squamostatin-A/12,15-*cis*-squamostatin-A): 173.78/173.78 (C-1), 148.83/148.83 (C-35), 134.13/134.13 (C-2), 83.28/83.28 (C-20), 82.18/82.18 (C-23), 81.96/81.96 (C-15), 79.24/79.82 (C-12), 77.31/77.31 (C-36), 74.42/74.42 (C-19), 74.33/74.70 (C-16), 71.58/71.58 (C-28), 71.51/71.51 (C-24), 19.10/19.10 (C-37), 13.98/13.98 (C-34). FABMS *m/z*: 661 $[M+Na]^+$, 639 $[M+H]^+$, EIMS (probe) 30 eV *m/z*: 535, 517, 499, 481, 451, 433, 415, 363, 345, 293 and 251.

3.6. Artemoin-A (3), artemoin-B (4), artemoin-C (5) and artemoin-D (6)

Colorless oil. IR ν_{max}^{Neat} cm^{-1}: 3400, 2900, 2850, 1750, 1460, 1305 and 1080. HR-EIMS $[M+H]^+$ *m/z*: 551.5035 (calcd 551.5041) for $C_{35}H_{66}O_4$); 379.3191 (calcd 379.3212 for $C_{24}H_{43}O_3$); 351.2903 (calcd 351.2900 for $C_{22}H_{39}O_3$); 323.2575 (calcd 323.2586 for $C_{20}H_{35}O_3$); 295.2271 (calcd 295.2273 for $C_{18}H_{31}O_3$). ^1H NMR (400 MHz, $CDCl_3$) δ: 6.92 (1H, *d, J* = 1.6 Hz, H-33), 5.00 (1H, *qd, J* = 6.8, 1.6 Hz), 1.40 (3H, *d, J* = 6.8 Hz, H-35), 0.88 (3H, *t, J* = 6.4 Hz, H-32), ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 173.90 (C-1), 148.82 (C-33), 134.36 (C-2), 77.40 (C-34), 19.21 (C-35), 14.10 (C-33), FABMS *m/z*: 551 $[M+H]^+$, 573 $[M+Na]^+$, EIMS (probe) 30 eV *m/z*: artemoin-A 379, artemoin-B 351, artemoin-C 323 and artemoin-D 295.

3.7. Bioassays

Human hepatoma cells (Hep G_2), Hep G_2 cells *trans*fect with HBV (Hep 2,2,15), human nasopharyngeal carcinoma cells (KB) and human colon tumor cells (CCM_2) were cultured in RPMI 1640 and DMEM medium containing 10% FCS, 100 units/ml penicillin and 100 μg/ml streptomycin. T-lymphoblastoid cells (CEM) (2×10^4 cells/ml) were seeded in 5 ml of RPMI 1640 medium supplemented with 5% FCS. All the cell lines were maintained in an incubator at 37°C in humidified air containing 5% CO_2. The drug activity on various cancer cells was assayed by methylene blue colorimetric method according to the process reported by Doong et al. (1991). To measure the cytotoxicities of **1**, **2** and **7**–**17** on Hep G_2, Hep 2,2,15, KB, CCM_2, CEM cells (Doong et al., 1991; Colman-Saizarbitoria et al., 1995), 11 different concentrations (0.0001 to 30 μg/ml) were added to 2.0 ml (5×10^4 cells/ml) culture medium. After 72 h, cells were stained with 5% methylene blue for 40 min, then the plates were washed with tap water three times following addition of 1% sacosyl to each well for 4 h. Finally, the plates were read immediately on an enzyme-linked immunosorbant microplate reader (ELISA Biokinetics Reader) at a wavelength of 592 nm (Elliott and Auersperg, 1993). The 50% inhibition concentration (IC_{50}) was defined as 50% reduction of absorbance in the control assay without isolated compounds or adriamycin. The results of the cytotoxicity of pure compounds are shown in Table 1.

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Appendix

Bioactive acetogenins from the seeds of *Annona atemoya*

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Six new annonaceous acetogenins, 12,15-*cis*-squamostatin-D, 12,15-*cis*-squamostatin-A, artemoin-A, artemoin-B, artemoin-C and artemoin-D, along with eleven known ones, were isolated from the seeds of Formosan *Annona atemoya*. The structures of these isolates were established on the basis of mass and related spectral evidence. All of them, except for four linear annonaceous acetogenins, exhibited potent cytotoxicity against Hep G_2, Hep 2,2,15, KB, CCM_2 and CEM cancer cell lines.

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