ANNONACEOUS ACETOGENINS FROM THE SEEDS OF *Annona squamosa*

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Annona squamosa L. is native to tropic America. In 1980, *A. squamosa* began to be planted in Taitung, now it is cultivated in many tropic areas of China, such as Guang Dong, Yun Nan, Fu jian, Hai Nan, Hong Kong, and so on [1]. Because each part of *A. squamosa* can be used as folk medicine and has significant curative effects, a number of phytochemical studies have been carried out on this plant [2–4]. Many annonaceous acetogenins have been isolated from Annona plants [5]. Some exhibit potent anticancer, cytotoxic, antiparasitic, insecticidal, or immunosuppressive activity [6].

The abundance of plant material, particularly seeds, and the associated significant pharmacological effects prompted us to investigate the chemical components of *A. squamosa.* Herein, we report the isolation of uvarigrandin A (**1**), squamocin (**2**), motrilin (**3**), bullatacin (**4**), neo-expoxyrolin (**5**), neo-desacetyluvaricin (**6**), annonareticin (**7**), epoxyrolin B (**8**), murisolin (**9**), squamostatin C (**10**), annoglaxin (**11**), squamin-A (**12**), erythritol (**13**), D,L-threitol (**14**), D-mannitol (**15**), stigmasterol (**16**), heptadecanoic acid (**17**), nonadecanoic acid (**18**), and stearic acid (**19**) from *A. squamosa* seeds and the structure elucidation of these crystalline substances by physical and chemical properties and spectral analysis.

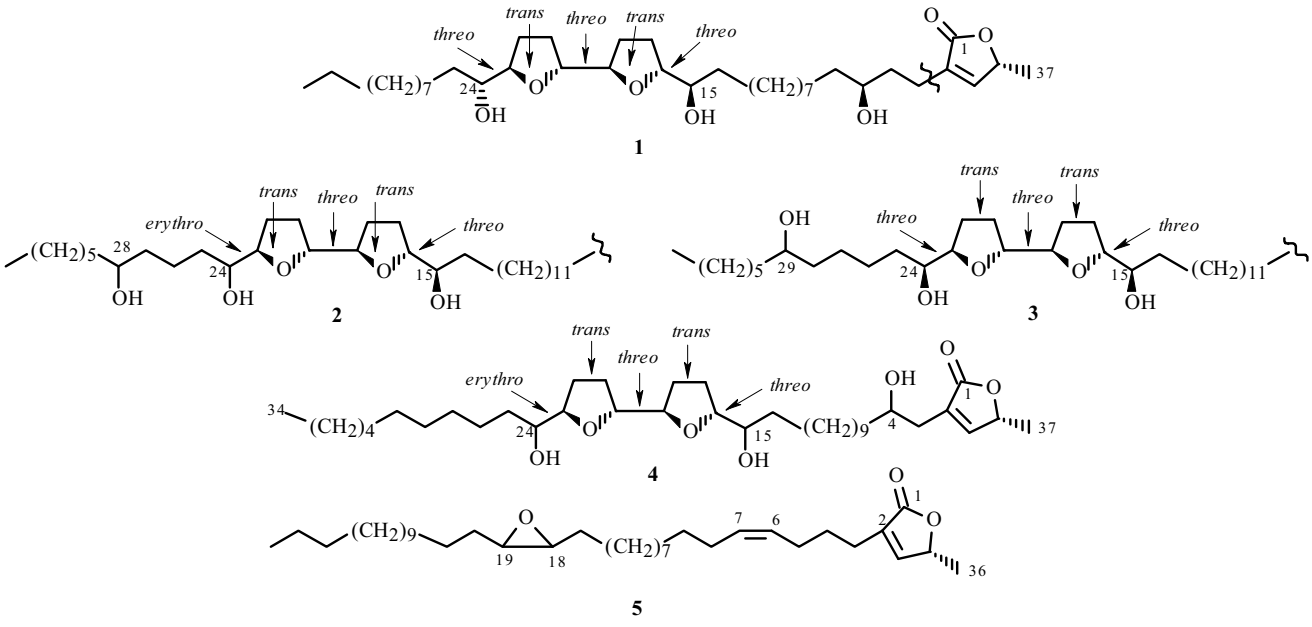

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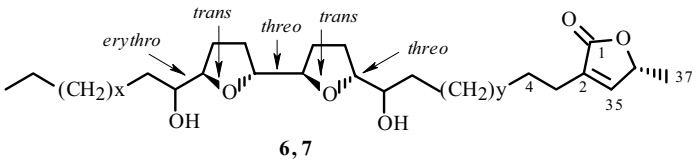
TABLE 1. Anti-Tumor Effects of **2–4**

Groups	Dosage	Average tumor weight (X±S), g	Inhibitory rate against tumor (IR), %
Negative	10 mL/kg	1.544±0.41	
[a]5-Fu	10 mg/kg	1.023±0.25	33.74
2, low-dose group	30 μg/kg	0.335±0.08	78.30*
2, high-dose group	60 μg/kg	0.330±0.08	77.96*
3, low-dose group	30 μg/kg	0.521±0.06	66.26*
3, high-dose group	60 μg/kg	0.309±0.18	79.98*
4, low-dose group	30 μg/kg	0.575±0.12	62.76*
4, high-dose group	60 μg/kg	0.164±0.04	89.38*

[a]Standard positive.

*P<0.01 as compared to standard positive groups.



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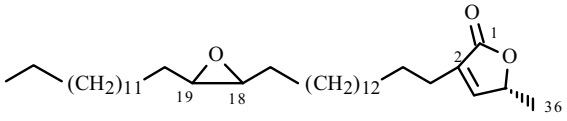
6: x = 7, y = 9; **7:** x = 8, y = 8



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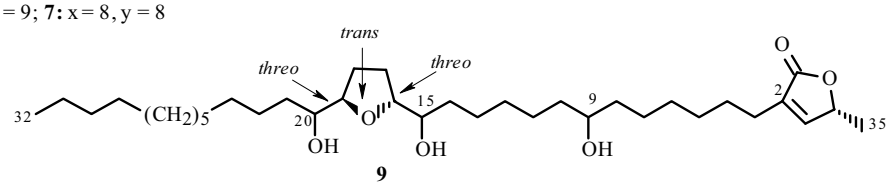

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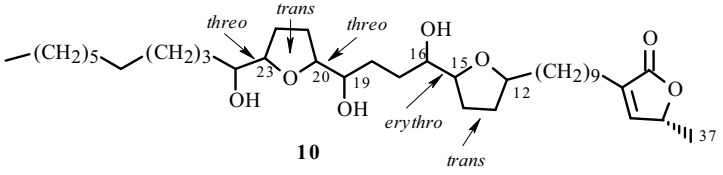

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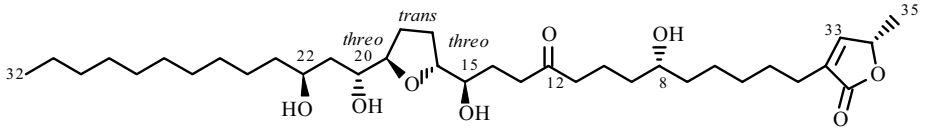

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The seeds of A. *squamosa* herb were collected from Hainan Province, China. The material was identified by Prof. Chen Jianwei (College of Pharmacy, Nanjing University of Chinese Medicine), dried, and (15 kg) macerated ordinally with petroleum (Pet) three times in 3 days in each extraction at room temperature. The mixture was filtered under reduce pressure on filter paper (middle speed for quantitative analysis), and a red solution was obtained. The residue, after removing the solvent in vacuo, was extracted with 95% EtOH four times in 8 days at room temperature. The extract was suspended in water and partitioned to provide $CHCl_3$ and *n*-BuOH solution fractions. The solutions obtained above were all concentrated under reduced pressure at 50–60°C until the solvents were thoroughly removed. The Pet extract was further separated by silica gel (200–300 mesh) column chromatography using Pet–EtOAc (100:1–1:2) as eluent. *neo*-Epoxyrolin (**5**, 20 mg), *neo*-desacetyluvaricin (**6**, 30 mg), annonareticin (**7**, 21 mg), epoxyrolin B (**8**, 24 mg), murisolin B (**9**, 17 mg), squamostatin C (**10**, 30 mg), annoglaxin (**11**, 25 mg), stigmasterol (**16**, 55 mg), heptadecanoic acid (**17**, 98 mg), nonadecanoic acid (**18**, 105 mg), and stearic acid (**19**, 50 mg) were obtained. In addition, the $CHCl_3$ and *n*-BuOH extracts were further separated by silica gel (200–300 mesh) column chromatography using Pet–EtOAc (150:1–1:5) and EtOAc–MeOH (200:1–1:6) as eluents, respectively. Uvarigrandin A (**1**, 15 mg), squamocin (**2**, 29 mg), motrilin (**3**, 10 mg), bullatacin (**4**, 20 mg), squamin-A (**12**, 8 mg), erythritol (**13**, 21 mg), DL-threitol (**14**, 25 mg), and D-mannitol (**15**, 22 mg) were obtained.

Squamocin, motrilin, and bullatacin were assayed for inhibitory effects on the growth of hepatoma 22 in mice using the live test method. A freshly prepared solution of squamocin, motrilin, and bullatacin with different concentrations in 0.1 M citrate buffer, pH 4.5, was injected intraperitoneally to mice (inoculated HepS cell suspension 1×10^8/mL) every day for 7 days, respectively, 24 h after stopping administration, the liver tissues were weighed. The data are expressed as mean ±S.D. Statistical comparisons were performed by one-way analysis of variance followed by Duncan's multiple range test. The results were considered statistically significant if the *p*-values were 0.05 or less.

Compound 1. $C_{37}H_{66}O_7$. White waxy solid ($CHCl_3$), mp 31–33°C, $[\alpha]_D^{20}$ +16.2° (*c* 0.8 $CHCl_3$), ESI-MS *m/z:* 645 $[M+Na]^+$, 623 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.99 (1H, br.s, H-35), 5.00 (1H, qd, J = 6.45, H-36), 1.41 (3H, d, J = 6.45, H-37), 3.80 (4H, m, H-16, 19, 20, 23), 3.40 (2H, m, H-5, 24); ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 173.84 (C=O), 134.30 (C-2), 148.82 (C-35), 77.42 (C-36), 19.09 (C-37), 83.26 (C-23), 83.25 (C-16), 82.66 (C-19), 82.19 (C-20), 74.32 (C-24), 74.02 (C-15).

This crystal was identified as uvarigrandin A, with NMR data in agreement with [7].

Compound 2. $C_{37}H_{66}O_7$. White needles (EtOH), mp 30–31°C, $[\alpha]_D^{20}$ +18.0° (*c* 0.25 $CHCl_3$) ESI-MS *m/z:* 645 $[M+Na]^+$, 623 $[M+H]^+$. ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.99 (1H, d, J = 2.23, H-35), 5.06 (1H, qd, J = 5.16, H-36), 1.42 (3H, d, J = 6.44, H-37), 3.90 (5H, m, H-16, 19, 20, 23, 24), 3.39 (1H, m, H-15), 3.83 (1H, m, H-28); ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 174.60 (C=O), 131.17 (C-2), 151.76 (C-35), 77.95 (C-36), 19.09 (C-37), 74.08 (C-15), 71.29 (C-24), 69.96 (C-28), 83.26 (C-16), 82.79 (C-19), 82.50 (C-20), 82.27 (C-23).

This crystal was identified by NMR as squamocin, with spectral data in agreement with [8].

Compound 3. $C_{37}H_{66}O_7$. White waxy solid ($CHCl_3$), mp 31–32°C, $[\alpha]_D^{20}$ +18.2° (*c* 0.054 MeOH). ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 6.99 (1H, br.s, H-35), 5.02 (1H, qd, H-36), 1.41 (3H, d, J = 6.45, H-37), 3.82 (5H, m, H-16, 19, 20, 23, 24), 3.40 (1H, m, H-15), 3.83 (1H, m, H-29); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 175.70 (C=O), 134.31 (C-2), 148.85 (C-35), 77.42 (C-36), 19.20 (C-37), 74.34 (C-15), 74.04 (C-24), 71.48 (C-29), 83.22 (C-16), 82.63 (C-19), 82.63 (C-20), 82.11 (C-23).

This crystal was identified by NMR as motrilin, with spectral data in agreement with [9].

Compound 4. $C_{37}H_{66}O_7$. White waxy solid ($CHCl_3$), mp 77–78°C, ESI-MS *m/z:* 623 $[M+H]^+$, 645 $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 7.19 (1H, d, J = 2.23, H-35), 5.07 (1H, dq, J = 6.87, H-36), 1.42 (3H, d, J = 6.89, H-37), 3.85 (6H, m, H-4, 16, 19, 20, 23, 24), 3.40 (1H, m, H-15); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 174.60 (C=O), 131.16 (C-2), 151.76 (C-35), 76.58 (C-36), 19.07 (C-37), 74.09 (C-15), 71.25 (C-24), 69.94 (C-4), 83.24 (C-16), 82.49 (C-19), 82.26 (C-20), 82.77 (C-23).

This crystal was identified by NMR as bullatacin, with spectral data in agreement with [8].

Compound 5. $C_{36}H_{64}O_3$. White waxy powder ($CHCl_3$), mp 65–66°, $[\alpha]_D^{20}$ +16.2° (*c* 0.4, $CHCl_3$), ESI-MS *m/z* 545 $[M+H]^+$, 567 $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 6.98 (1H, d, J = 1.7, H-34), 4.97 (1H, dq, J = 6.8, 1.7, H-35), 1.39 (3H, d, J = 6.8, H-36), 5.38 (1H, d, t, J = 11.8, 6.2, H-6), 5.42 (1H, dt, J = 11.8, 6.2, H-7); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 173.83 (C=O), 134.29 (C-2), 148.81 (C-34), 77.34 (C-35), 56.55 (C-18), 57.29 (C-19), 128.09 (C-6), 131.10 (C-7).

This crystal was identified by NMR as neo-expoxyrolin, with spectral data in agreement with [10].

Compound 6. $C_{37}H_{66}O_6$. White crystals ($CHCl_3$), mp 68–69°C, ESI-MS *m/z* 629 $[M+Na]^+$, 645 $[M+K]^+$. ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 6.99 (1H, d, J = 1.3, H-35), 4.99 (1H, dq, J = 6.87, 1.3, H-36), 1.42 (3H, d, J = 6.9, H-37), 3.88 (5H, m, H-16, 19, 20, 23, 24), 3.39 (1H, m, H-15); ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 173.79 (C=O), 134.29 (C-2), 148.78 (C-35), 77.32 (C-36), 19.15 (C-37), 74.09 (C-15), 71.32 (C-24), 83.26 (C-16), 82.43 (C-19), 82.19 (C-20), 82.77 (C-23).

This crystal was identified by NMR as neo-desacetyluvaricin, with spectral data in agreement with [11].

Compound 7. $C_{37}H_{66}O_7$. White powder ($CHCl_3$), mp 72–73°C. ESI-MS *m/z:* 623 $[M+H]^+$, 645 $[M+Na]^+$, 661 $[M+K]^+$. ^{1}H NMR (500 MHz, $CDCl_3$, δ, ppm, J/Hz): 7.18 (1H, d, J = 1.4, H-35), 5.04 (1H, dq, J = 6.8, 1.4, H-36), 1.42 (3H, d, J = 6.8, H-37), 3.88 (6H, m, H-4, 15, 18, 19, 22, 23), 3.39 (1H, m, H-14); ^{13}C NMR (125 MHz, $CDCl_3$, δ, ppm): 174.52 (C=O), 134.26 (C-2), 151.69 (C-35), 77.34 (C-36), 19.14 (C-37), 83.22 (C-15), 82.45 (C-18), 69.88 (C-4), 82.16 (C-19), 82.76 (C-22), 71.34 (C-23), 74.06 (C-14).

This crystal was identified by NMR as annonareticin, with spectral data in agreement with [12].

Compound 8. $C_{36}H_{66}O_3$. White waxy powder ($CHCl_3$), mp 65–66°C. ESI-MS *m/z:* 547 $[M+H]^+$, 569 $[M+Na]^+$. ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 6.99 (1H, d, J = 1.26, H-34), 4.98 (1H, dq, J = 5.16, 1.71, H-35) 1.39 (3H, d, J = 6.8, H-36), 2.94 (1H, d, J = 5.16, H-18), 2.96 (1H, d, J = 5.16, H-19); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 173.82 (C=O), 134.29 (C-2), 148.80 (C-34), 77.33 (C-35), 56.69 (C-18), 57.29 (C-19).

This crystal was identified by NMR as epoxyrolin B, with spectral data in agreement with [10].

Compound 9. $C_{35}H_{64}O_6$. White waxy powder ($CHCl_3$), mp 62–63°C. ESI-MS *m/z:* 603 $[M+Na]^+$, 581 $[M+H]^+$. ^1H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 6.99 (1H, d, J = 1.4, H-33), 5.04 (1H, qd, J = 6.8, 1.4, H-34), 1.42 (3H, d, J = 6.8, H-35), 3.60 (1H, m, H-9), 3.40 (2H, m, H-15, 20), 3.83 (2H, m, H-16, 19); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 173.88 (C=O), 134.44 (C-2), 148.84 (C-33), 77.34 (C-34), 71.55 (C-9), 74.33 (C-15), 83.23 (C-16), 82.64 (C-19), 74.02 (C-20).

This crystal was identified by NMR as murisolin, with spectral data in agreement with [13].

Compound 10. $C_{37}H_{66}O_8$. White crystals ($CHCl_3$), mp 104–105°C, $[\alpha]_D^{20}$ +12.8° (*c* 0.08 $CHCl_3$). ^1H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 7.05 (1H, d, J = 1.61, H-35), 5.02 (1H, qd, J = 6.85, 1.61, H-36), 1.42 (3H, d, J = 6.85, H-37), 3.86 (5H, m, H-12, 15, 16, 20, 23), 3.42 (2H, m, H-19, 24); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 173.81 (C=O), 134.59 (C-2), 158.84 (C-35), 77.42 (C-36), 79.47 (C-12), 82.08 (C-15), 71.93 (C-16), 74.62 (C-19), 82.36 (C-20), 83.43 (C-23), 74.66 (C-24).

This crystal was identified by NMR as squamostatin C, with spectral data in agreement with [14].

Compound 11. $C_{35}H_{62}O_8$. White waxy powder ($CHCl_3$), mp 70–71°C, $[\alpha]_D^{20}$ +15.2° (*c* 0.3 $CHCl_3$), ESI-MS *m/z:* 649 $[M+K]^+$, 611 $[M+H]^+$. ^1H NMR (500 MHz, DMSO-d_6, δ, ppm): 6.99 (1H, d, J = 1.5, H-33), 5.02 (1H, dq, J = 6.9, 1.5, H-34), 1.43 (3H, d, J = 6.90, H-35), 2.26 (2H, m, H-3), 3.41 (1H, m, H-15), 3.81 (4H, m, H-16, 19, 20, 22), 3.60 (1H, m, H-8); ^{13}C NMR (125 MHz, DMSO-d_6, δ, ppm): 172.17 (C=O, C-1), 209.95 (C=O, C-12), 131.62 (C-2), 150.59 (C-33), 78.16 (C-34), 71.31 (C-8), 42.47 (C-11), 42.72 (C-13), 74.18 (C-15), 82.23 (C-16), 82.58 (C-19), 70.34 (C-20), 68.85 (C-22).

This crystal was identified by NMR as annoglaxin, with spectral data in agreement with [15].

Compound 12. $C_{39}H_{60}N_8O_{11}S$. White needles (EtOH), mp 215–216°C. IR (KBr, cm^{-1}): 3300, 3279, 1681, 1653, ESI-MS *m/z*: 849 $[M+H]^+$, 871 $[M+Na]^+$, 887 $[M+K]^+$ 736, 665, 566, 465, 408, Upon treating with 12 moL^{-1} HCl, a positive ninhydrin test was obtained. This crystal was further identified by NMR as squamin-A, with spectral data in agreement with [16].

Compound 13. $C_4H_{10}O_4$. Colorless crystals (EtOH), mp 121–122°C. This crystal was further identified as erythritol by NMR, with spectral data in agreement with [17].

Compound 14. $C_4H_{10}O_4$. Colorless crystals (EtOH), mp 88–89°C. This crystal was identified as DL-threitol by co-TLC with reference samples.

Compound 15. $C_6H_{14}O_6$. White needles (CH_3OH), mp 166–167°C. This crystal was identified by NMR as D-mannitol, with spectral data in agreement with [17].

Compound 16. $C_{29}H_{48}O$. White needles ($CHCl_3$), mp 160–161°C. This crystal was identified by NMR as stigmasterol, with spectral data in agreement with [18].

Compound 17. $C_{17}H_{34}O_2$. White needles ($CHCl_3$), mp 60–61°C. This crystal was identified by NMR as heptadecanoic acid, with spectral data in agreement with [18].

Compound 18. $C_{19}H_{38}O_2$. White crystals ($CHCl_3$), mp 73–74°C. This crystal was identified by NMR as nonadecanoic acid, with spectral data in agreement with [19].

Compound 19. $C_{18}H_{36}O_2$. White crystals ($CHCl_3$), mp 65–66°C. This crystal was identified by NMR as stearic acid, with spectral data in agreement with [20].

The anti-tumor activities of the compounds squamocin, motrilin, and bullatacin were evaluated against the growth of HepS tumor in mice. Squamocin, motrilin, and bullatacin all showed clear inhibitory effects on the growth of HepS tumor in mice (IR 78.30%, 79.98% and 89.38%, respectively) (Table 1).

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