Isolation and Structure of Neoannonin, a Novel Insecticidal Compound from the Seeds of *Annona squamosa*†

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Two insecticidal compounds were isolated from the seeds of *Annona squamosa*. One was identified as annonin (squamocin) and the other was characterized as a novel dihydroxy-bistetrahydrofuran fatty acid lactone (acetogenin) with 35 carbons. These two compounds were toxic to eggs, larvae and adults of the fruitfly.

The seeds of *Annona squamosa* have been known to show insecticidal activity as well as vermicidal and abortifacient properties. In the Philippines, a coconut oil extract of this seed has been used to get rid of lice on the scalp.[2] In the course of our search for insect development inhibitors from plant sources, we investigated this seed for its active principles. Through activity-guided fractionation using the Drosophila feeding method[3] as the bioassay system, we isolated two insecticidal compounds (**1** and **2**). Compound **1** has already been identified as annonin[4] (squamocin).[5] In this paper, we report the isolation and structural elucidation of **2**, which we have named neoannonin.

The ethyl acetate extract of the defatted seeds showed pronounced activity. Chromatographic separation of this extract gave the two active compounds, compound **1** being obtained from the more polar fraction than that for **2**. After several rechromatographic steps, **1** was obtained as a viscous oil and **2** as a white waxy solid.

On the Drosophila feeding test, **1** and **2** showed strong ovicidal and larvicidal activity

Table I. EFFECT OF COMPOUNDS **1** AND **2** IN THE "Drosophila Feeding Test" (*D. melanogaster*)

Compd.	μg/2 g of diet	Day							
		0	2	4	6	8	10	12	14
1	250	10E	$6E_D4L_{vs}$	0	0	0	0	0	0
	125	10E	$4E_D5L_{vs}1L_D$	0	0	0	0	0	0
	62.5	10E	$2E_D8L$	8L	8L	8P	1P7A	1P7A	$1A_D7A$
2	280	10E	$5E_D5L_{vs}$	0	0	0	0	0	0
	140	10E	$3E_D5L_{vs}2L$	2L	$2L_D$	0	0	0	0
	70	11E	2E9L	$1E_D10L$	10L	10P	10A	10A	10A
Control		10E	10L	10L	10P	10P	10A	10A	10A

A = adult; E = egg; L = larva; P = pupa; D = dead; vs = very small.

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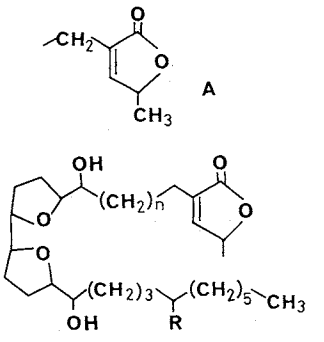
at 125~140 μg/2 g of diet. Thirty to forty percent of the Drosophila eggs turned brownish and did not hatch. The surviving very small larvae died a few days after hatching (Table I). By the dry film method, these compounds were found toxic against Drosophila adults at LD_{50} 62.5 μg after 48 hr.

Compound **1** had physical and spectral properties identical to those of annonin, which was isolated from the same plant and patented by a German company.[4] Just recently, the same compound from this plant has been reported by Fujimoto *et al.* as a cytotoxic compound, which was named squamocin. Its structure[5] was elucidated as a trihydroxybistetrahydrofuran fatty acid γ-lactone (**1**).



1 R = OH, n = 11
2 R = H, n = 9

Compound **2**, neoannonin, had a molecular weight conclusively established as 578 by FAB-MS (glycerin) *m/z* 579 ($M^+ + H$) and CI-MS (isobutane) *m/z* 579 ($M^+ + H$) analyses, and by its molecular formula, $C_{35}H_{62}O_6$. Its IR spectrum showed bands typical of hydroxyl (3425~3375 cm^{-1}), α,β-unsaturated-γ-lactone carbonyl (1760 cm^{-1}), and *n*-alkyl chain (720 cm^{-1}). The partial structure A was easily recognized from its ^{1}H-NMR signals [δ 1.38 (3H, d, J=6.8 Hz), 2.23 (2H, ddt, J=1.7, 1.7, 7.0 Hz), 4.97 (1H, dtq, J=1.7, 1.7, 6.8 Hz) and 6.96 (1H, dt, J=1.7, 1.7 Hz)] and ^{13}C-NMR signals [CH_3, δ 19.21; CH_2, 25.16; CH, 77.39; C=, 134.31; CH=, 148.83; and C=O, 174.00], which were also observed in **1**. The two 1H signals at δ 3.37 and 3.80~3.87 in compound **2** were shifted downfield to δ 4.84 (2H) by acetylation, suggesting the presence of two secondary hydroxyl groups in **2**. The signals of the remaining four protons attached to methine carbons bearing oxygen [δ 3.80~3.87 m (2H), 3.88~3.93 m (2H)] in combination with their ^{13}C-NMR signals (δ 82.28, 82.49, 82.79, 83.26) were assignable to two ether groups. In addition, the presence of a terminal methyl [δ_H 0.85 (3H, t, J=7.0 Hz) and δ_C 14.11] was observed, and 22 methylenes were confirmed from its ^{13}C-NMR spectrum.

Oxidation of neoannonin with lead tetraacetate gave two aldehyde fragments, one having a molecular weight of 266 based on its EI-MS spectrum. Its ^{1}H-NMR spectrum revealed the proton signals of partial structure A and an aldehydic proton at δ 9.74 (t, J=1.6 Hz). These data complemented by the EI-MS fragments [*m/z* 29, CHO^+; 43, $C_2H_3O^+$; 55, $C_3H_3O^+$; 81, $C_6H_{11}O^+ - H_2O$; 149, $M^+ - C_6H_{11}O^+ - H_2O$; 112, $C_6H_7O_2^+ + H$; 97, $C_5H_5O_2^+$] strongly suggested that it was α-(ω-formyldecyl)-α,β-angelica lactone. This aldehyde fragment is smaller by two methylenes than the fragment similarly obtained from **1**. The other aldehyde fragment with a peculiar sweet smell was identified as undecanal by GC analysis. These findings therefore suggested that neoannonin should have a bistetrahydrofuran ring, which is flanked by these two terminal groups.

This structure was confirmed by the fragmentation pattern observed in the EI-MS spectrum of **2** as shown in Fig. 1. Configurations at the chiral centers remain unsolved.

Besides annonin (squamocin), many mem-

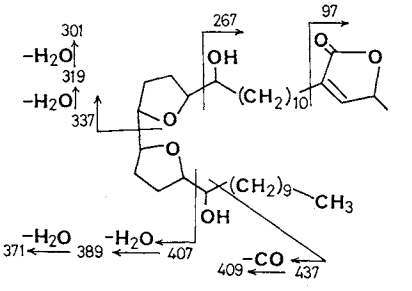


Fig. 1. Fragmentation Pattern in the EI-MS of **2**.

bers of the growing family of Annonaceous acetogenins have been previously reported such as uvaricin,[6] desacetyluvaricin,[7] rollinicin,[8] isorollinicin,[8] rollinone,[9] 14-hydroxy-25-desoxy-rollinicin,[10] cherimoline,[11] dihydrocherimoline,[11] asimicin[12] and rolliniastatin,[13] which characteristically contain 37 carbons, a γ-lactone moiety, bistetrahydrofuran ring and a few hydroxyl groups on a long alkyl chain. Neoannonin is the first example of this type of compound having 35 carbons.

Experimental

Analytical methods. Optical rotation was measured with a JASCO DIP-360. ^{1}H- and ^{13}C-NMR spectra were recorded in $CDCl_3$ on a Varian VXR-500 instrument. IR spectra were taken on a Hitachi EPI-G3 spectrometer, UV spectra on a Shimadzu UV-3000, and mass spectra on a JEOL JMS-D300. GC analysis was undertaken on a Shimadzu GC-4CM instrument, and thin-layer chromatography was carried out on Merck silica gel 60 F_{254}. Annonin and neoannonin on TLC were detected with a phosphomolybdic acid (PMA) spray reagent.

Bioassay.

Feeding method. The eggs of *Drosophila melanogaster* were used in the bioassay. Under aseptic conditions, the sample was placed in a petri dish ($\phi = 3$ cm), mixed with two grams of diet (a mixture of 20 g of dry yeast powder, 40 g of honey, 3 g of agar, 0.3 g of *n*-butyl-*p*-hydroxybenzoate and 250 g of water), seeded with 10 newly laid eggs and kept on a 16-hr-light-8-hr-dark cycle at 25°C and 93% R.H. The developmental state of the insect was precisely observed under a microscope, compared with that of the control and recorded every other day for 2 weeks.

Dry film method. A methanol solution of the test sample was applied in the cover of the petri dish ($\phi = 3$ cm) and was freed from solvent. The bottom of the dish was fitted with filter paper and moistened with water (150 μl). In the bottom of each petri dish, 10 flies anaesthetized at 3°C for 10 min were placed and were kept at 25°C. The number of survivors was counted after 48 hr and compared to that in the control experiment.

Isolation of annonin (**1**) *and neoannonin* (**2**). The air-dried seeds (2.1 kg) of *Annona squamosa* collected at Nueva Ecija and Ilocos Sur, Philippines were ground, and successively extracted with *n*-hexane and ethyl acetate. The resulting extracts were subjected to the bioassay. Since the ethyl acetate extract (366 g) exhibited activity by the feeding test [minimum effective dose (MED), 17 mg], it was partitioned between *n*-hexane and 10% aqueous methanol. The organic constituent of the methanol layer (46 g, MED 1 mg) was retaken in ethyl acetate and fractionated on a silica gel (Wakogel C-100) column eluted with *n*-hexane–ethyl acetate–methanol in increasing polarity. Of the 17 fractions collected, active compound **1** was obtained from the tenth and eleventh fractions, and **2** from the sixth and seventh fractions. These fractions were subjected to medium-pressure column chromatography on Kieselgel 60, using benzene–benzene : acetone (6 : 4) as the eluent for **1** and *n*-hexane–*n*-hexane : acetone (3 : 1) for **2**.

Compound **1** (500 mg) was isolated by ODS column chromatography (Lichroprep RP 18 as packing material eluted with H_2O–CH_3OH in a gradient) as a viscous oil, $[\alpha]_D^{22}$ +14.9° ($c = 0.94$, MeOH), *Rf* 0.52 [benzene–acetone (6 : 4), v/v]. The ^{1}H- and ^{13}C-NMR spectral data of **1** were identical with those of annonin[4] (squamocin).[5]

Successive silica gel column chromatography yielded compound **2** (100 mg) as a white waxy solid, $[\alpha]_D^{22}$ +18.8° ($c = 1.35$, MeOH), *Rf* 0.41 [benzene–acetone (8 : 2), v/v]. UV λ(MeOH) nm (ε): 215 (1.2×10^4); IR ν_{max} (KBr) cm^{-1}: 3425, 3375, 2925, 2850, 1760, 1470, 1390, 1320, 1120, 1080, 1020, 960, 910, 790, 720, 630; FAB-MS (glycerin) *m/z*: 579 ($M^+ + H$); CI-MS (isobutane) *m/z*: 579 ($M^+ + H$), 561 ($579 - H_2O$), 543 ($579 - 2H_2O$), 525 ($579 - 3H_2O$), 407 ($C_{24}H_{39}O_5^+$), 337 ($C_{20}H_{33}O_4^+$), 311 ($C_{19}H_{35}O_3^+$), 267 ($C_{16}H_{27}O_3^+$), 241 ($C_{15}H_{29}O_2^+$); EI-MS *m/z* (rel. int., %): 524 ($M^+ - 3H_2O$, 12.3), 506 ($M^+ - 4H_2O$, 7.5), 437 ($C_{25}H_{41}O_6^+$, 3.5), 409 ($437 - CO$, 2.4), 407 ($C_{24}H_{39}O_5^+$, 4.7), 389 ($407 - H_2O$, 6.4), 371 ($407 - 2H_2O$, 13.2), 337 ($C_{20}H_{33}O_4^+$, 9.5), 319 ($337 - H_2O$, 37.7), 301 ($337 - 2H_2O$, 16.0), 267 ($C_{16}H_{27}O_3^+$, 100); ^{1}H-NMR δ ($CDCl_3$): 0.85 (3H, t, $J = 7.0$ Hz), 1.15 ~ 1.34 (28H, br. s), 1.35 ~ 1.37 (2H, m), 1.38 (3H, d, $J = 6.8$ Hz), 1.40 ~ 1.55 (2H, m), 1.56 ~ 1.66 (2H, m), 1.74 ~ 1.81 (2H, m), 1.82 ~ 1.91 (6H, m), 1.92 ~ 2.00 (2H, m), 2.23 (2H, ddt, $J = 1.7$, 1.7, 7.0 Hz), 3.37 (1H, dt, $J = 7.1$, 4.1 Hz), 3.80 ~ 3.87 (3H, m), 3.88 ~ 3.93 (2H, m), 4.97 (1H, dtq, $J = 1.7$, 1.7, 6.8 Hz), 6.96 (1H, dt, $J = 1.7$, 1.7 Hz); ^{13}C-NMR δ ($CDCl_3$): CH_3, 14.11, 19.21; CH_2, 22.67, 24.46, 25.16, 25.66, 26.04, 27.38, 28.35, 28.91, 28.97, 29.17, 29.29, 29.32, 29.50, 29.54, 29.60 (× 4), 29.67, 29.73, 31.89, 32.42, 33.32; CH, 71.28, 74.10, 77.39, 82.28, 82.49, 82.79, 83.26; CH=, 148.83; C=, 134.31; C=O, 174.00.

Acetylation of **2**. Compound **2** (19.4 mg) was acetylated with acetic anhydride/pyridine in a sealed vial at 60°C overnight. The reaction mixture was dried *in vacuo* and purified by silica gel column chromatography [ϕ 0.4 × 50 cm, Kieselgel 60, *n*-hexane–*n*-hexane : ethyl acetate (7 : 3) in gradient elution] to yield the diacetate (7.1 mg); IR ν_{max} (KBr) cm^{-1}: 3410, 2925, 2850, 1760 (broad), 1470, 1320, 1200, 1120, 1070, 1025, 950, 790, 720; ^{1}H-NMR δ ($CDCl_3$): 0.85 (3H, t, $J = 7.0$ Hz), 1.12 ~ 1.34 (28H, br. s), 1.38 (3H, d, $J = 6.8$ Hz), 1.49 ~ 1.61 (10H, m), 1.74 ~ 1.78 (2H, m), 1.87 ~ 1.95 (4H, m), 2.05 (6H, s), 2.23 (2H, ddt, $J = 1.7$, 1.7, 7.0 Hz), 3.88 (2H, dt, $J = 9.5$, 5.7 Hz), 3.96 (2H, dt, $J = 12.2$, 6.8 Hz), 4.84 (2H, dt, $J = 7.8$, 5.3 Hz), 4.97

(1H, dtq, $J=1.7$, 1.7, 6.8 Hz), 6.96 (br. d, $J=1.7$ Hz); CI-MS (isobutane) *m/z*: 663 ($M^+ + 1$); EI-MS *m/z* (rel. int., %): 602 ($M^+ - AcOH$, 8.8), 542 ($M^+ - 2AcOH$, 14.0), 524 ($542 - H_2O$, 9.6), 449 ($C_{26}H_{41}O_6^+$, 13.2), 431 ($449 - H_2O$, 25.3), 379 ($C_{22}H_{35}O_5^+$, 89.7), 371 ($431 - AcOH$, 35.5), 353 ($C_{21}H_{37}O_4^+$, 17.5), 319 ($379 - AcOH$, 100.0), 301 ($319 - H_2O$, 83.1), 293 ($353 - AcOH$, 35.9), 283 ($C_{17}H_{31}O_3^+$, 16.5), 223 ($283 - AcOH$, 25.3), 205 ($C_{18}H_{29}O_4^+ - AcOH - CO_2$, 16.0), 135 ($C_{13}H_{25}O_2^+ - AcOH - H_2O$, 34.8).

Lead tetraacetate oxidation of **2**. To a solution of 13.2 mg of lead tetraacetate in 500 μl of benzene was added 14.6 mg of neoannonin (**2**). The reaction mixture was stirred for 3 hr and allowed to stand for 24 hr at room temperature before it was partitioned between pH 7 phosphate buffer and ethyl acetate. The aqueous layer was extracted twice with ethyl acetate, and the combined organic layers were washed with brine, dried over anhydrous sodium sulfate and concentrated *in vacuo*. By TLC analysis using benzene–acetone (9.5 : 0.5) as the developing solvent, two major spots in the crude products were detected with 2,4-dinitrophenylhydrazine and PMA spray reagents. The crude products were separated by silica gel column chromatography [ϕ 0.4 × 100 cm, Kieselgel 60H, benzene–benzene : acetone (9.5 : 0.5) in gradient elution] to give two compounds, undecanal (0.75 mg) and α-(ω-formyldecyl)-α,β-angelica lactone (1.01 mg). Undecanal was identified by GC analysis at t_R 10.7 min (OV-1; ϕ 4 mm × 1.5 m, FID detector, col. temp. 110°C, N_2 carrier gas, 40 ml/min) with authentic undecanal prepared by pyridinium chlorochromate oxidation of 1-undecanol. α-(ω-Formyldecyl)-α,β-angelica lactone was characterized from its EI-MS *m/z* (rel. int., %): 266 (M^+, 12.0), 248 ($M^+ - H_2O$, 11.4), 238 ($M^+ - CO$, 28.3), 223 ($M^+ - C_2H_3O$, 29.6), 149 ($M^+ - C_6H_{11}O - H_2O$, 15.2), 112 ($C_6H_7O_2^+ + H$, 85.0), 97 ($C_5H_5O_2^+$, 43.8), 81 ($C_6H_{11}O^+ - H_2O$, 41.4), 71 ($C_4H_7O^+$, 40.5), 69 ($C_5H_5O_2^+ - CO$, 69.7), 57 ($C_3H_5O^+$, 73.1), 55 ($C_3H_3O^+$, 100.0), 43 ($C_2H_3O^+$, 88.6), 29 (CHO^+, 28.7), and ^{1}H-NMR ($CDCl_3$): δ 1.26 ~ 1.28 (10H, br. s), 1.39 (3H, d, $J=6.7$ Hz), 1.49 ~ 1.54 (4H, m), 1.60 (2H, p, $J=7.3$ Hz), 2.24 (2H, ddt, $J=1.7$, 1.7, 7.7 Hz), 2.40 (2H, dt, $J=7.3$, 1.6 Hz), 4.97 (1H, dtq, $J=1.7$, 1.7, 6.7 Hz), 6.95 (1H, dt, $J=1.7$, 1.7 Hz), 9.74 (1H, t, $J=1.6$ Hz).

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