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ACETOGENINS FROM SEEDS OF *ANNONA RETICULATA*†

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Key Word Index—*Annona reticulata*; Annonaceae; seeds; *cis-/trans*-isomurisolenin; bullatacin; cytotoxicity.

Abstract—Chromatography of an ethyl acetate extract of seeds of *Annona reticulata* led to the isolation of a new cytotoxic γ-lactone acetogenin, *cis-/trans*-isomurisolenin, along with six known cytotoxic acetogenins, annoreticuin, annoreticuin-9-one, bullatacin, squamocin, *cis-/trans*-bullatacinone and *cis-/trans*-murisolinone. Structures of these compounds were established by means of mass and related spectral experiments. Some of the compounds isolated, showed potent cytotoxicities against Hep. 2,2,15, Hep. G_2, KB and CCM2, four cancer cell-lines. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

Annonaceous acetogenins have attracted much attention as potential anticancer agents in recent years [1]. Their mechanism of action is *via* inhibition of NADH ubiquinone oxido-reductase (complex I) in mitochondrial electron transport systems, and inhibition of NADH oxidase in the plasma membranes of tumour cells [2]. A number of cytotoxic acetogenins had been isolated from the stems and barks of *Annona reticulata* [3–5]. In previous papers, we reported the presence of some cytotoxic acetogenins from *A. reticulata* [3, 4], *A. montana* [6] and *Rollinia mucosa* [7]. As part of our continuing investigation on the acetogenins of Formosan Annonaceous plants, a new cytotoxic acetogenin, *cis-/trans*-isomurisolin (**1**), and six known cytotoxic compounds, annoreticuin (**2**) [4], annoreticiun-9-one (**3**) [4], bullatacin (**4**) [8], squamocin (**5**) [9], *cis-/trans*-bullactacinone (**6**) [10] and *cis/trans*-murisolinone (**7**) [11], have been isolated from the seeds of *A. reticulata*. Structures were determined by spectroscopic methods, before making certain derivatives. We report herein, the structural elucidation of **1** and the cytotoxicities of **1**, **3**, **6** and **7**.

RESULTS AND DISCUSSION

cis-/trans-Isomurisolenin (**1**) was obtained mixed with *cis-/trans*-murisolinone (**7**). The ^{1}H and ^{13}C NMR spectral data of **1** were similar to those of **7**, except for the signals of the double bond. The M_r of **1** was indicated by the peak at *m/z* 579 $[M+H]^+$ in the FAB mass spectrum, the HREI mass spectrum giving *m/z* 579.4597 for $[M+H]^+$, corresponding to the molecular formula, $C_{35}H_{62}O_6$. The IR spectrum showed strong absorptions at 1760 cm^{-1}, for a γ-lactone carbonyl, and at 1710 cm^{-1}, for a ketone carbonyl. The signals at δ 4.40 (*m*) and 4.55 (*m*), with combined integrations for one proton on a carbon attached to a lactone oxygen, in the ^{1}H NMR spectrum, suggested the presence of a mixture of (2,4-*cis* and 2,4-*trans*)-diastereoisomers of a ketolactone ring moiety [5, 8]. The ^{1}H NMR spectrum (Table 1), signals pairs at δ 3.04 and 3.00, 2.61 and 2.23, 1.40 and 1.96, 2.56 and 2.61, 3.08 and 3.00, and 2.20 and 2.20 were assigned to H-2, H-3a, H-3b, H-33a, H-33b and H-35, respectively. In the ^{13}C NMR spectrum (Table 2), signal pairs at δ 178.2 and 178.7, 36.7 and 34.5, 79.3 and 78.9, 43.7 and 44.2, 205.6 and 205.6, and 29.3 and 29.3 were assigned to C-1, C-2, C-4, C-33, C-34 and C-35 determinable by HETCOR, respectively. This indicated the presence of the mixture of (2,4-*cis* and -*trans*)-isomers [12, 13].

The remaining part of the structure of **1** exhibited identical ^{1}H and ^{13}C NMR signals for a long aliphatic chain bearing a mono-THF ring and two OH groups [3, 4]. In the FAB mass spectrum, a series of peaks at *m/z* 579, 561 and 543, arising from the successive losses of two molecules of H_2O were observed and confirmed the presence of the two OH groups. The OH groups were also identified by the broad IR absorption band at 3400 cm^{-1} and by the acetate methyl signal at δ 2.07 (6H,*s*) in the ^{1}H NMR spectrum of the diacetate derivative (**1a**).

The mono-THF ring with the usual OH group on

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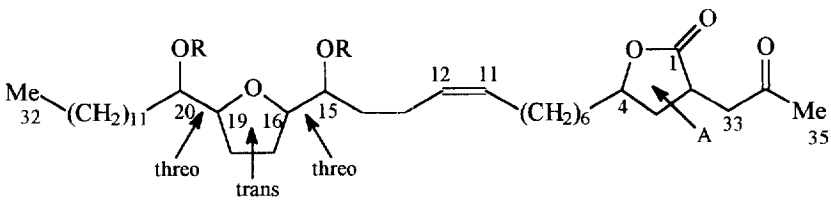


1	A *cis / trans*,	R = H
1a	A *cis / trans*,	R = Ac

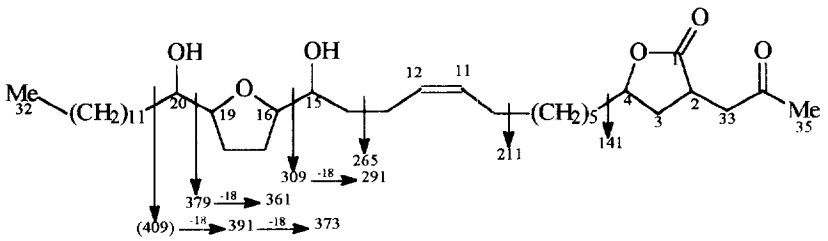
Table 1. ^{1}H NMR Spectral data of compound **1** (400 MHz in $CDCl_3$)

	1 (*cis*)	**1a** (*cis*)	**1** (*trans*)	**1a** (*trans*)
1	—	—	—	—
2	3.04 *m*	3.04 *m*	3.0 *m*	3.0 *m*
3a	2.61	2.61	2.23	2.23
	ddd (12.3, 9.4, 5.6)		*ddd* (12.9, 9.6, 3.4)	
3b	1.40 *m*	1.40 *m*	1.96 *m*	1.96 *m*
4	4.40	4.40	4.55	4.55
	dddd (10.7, 7.4, 5.4, 5.4)		*dddd* (8.3, 8.2, 5.7, 3.2)	
5–9	1.26–1.74 *m*	1.26–1.74 *m*	1.26–1.74 *m*	1.26–1.74 *m*
10	2.01 *m*	2.01 *m*	2.01 *m*	2.01 *m*
11, 12	5.36, 5.38	5.36, 5.38	5.36, 5.38	5.36, 5.38
13	2.15 *m*	2.15 *m*	2.15 *m*	2.15 *m*
14	1.26–1.74 *m*	1.26–1.74 *m*	1.26–1.74 *m*	1.26–1.74 *m*
15	3.40 *m*	4.85 *m*	3.40 *m*	4.85 *m*
16	3.80 *m*	3.98 *m*	3.80 *m*	3.98 *m*
17a, 18a	1.60–1.67 *m*	1.60–1.67 *m*	1.60–1.67 *m*	1.60–1.67 *m*
17b, 18b	1.85–2.05 *m*	1.85–2.05 *m*	1.85–2.05 *m*	1.85–2.05 *m*
19	3.80 *m*	3.98 *m*	3.80 *m*	3.98 *m*
20	3.40 *m*	4.85 *m*	3.40 *m*	4.85 *m*
21–31	1.26–1.74 *m*	1.26–1.74 *m*	1.26–1.74 *m*	1.26–1.74 *m*
32	0.88 *t* (6.9)	0.88 *t* (6.9)	0.88 *t* (6.9)	0.88 *t* (6.9)
33a	2.56	2.56	2.61	2.61
	dd (15.3, 8.6)		*dd* (19.5, 10.6)	
33b	3.08	3.08	3.0	3.0
	dd (18.5, 3.5)		*dd* (18.5, 3.4)	
34	—	—	—	—
35	2.20 *s*	2.20 *s*	2.20 *s*	2.20 *s*
OAc-15	—	2.07 *s*	—	2.07 *s*
OAc-16	—	2.07 *s*	—	2.07 *s*

J (in Hz) in parentheses.

each side was indicated in **1** (*cis-/trans*) by ^{1}H NMR chemical shifts at δ 3.40 (H-15 and H-20) and δ 3.80 (H-16 and H-19) and ^{13}C NMR signals at δ 74.0 and 74.0 (C-15 and C-20), δ 82.6 and 82.5 (C-16 and C-19), respectively. Moreover, the proton signals for H-15 and H-20 at δ 3.40 in **1** were shifted downfield to δ 4.85 in **1a** supporting the presence of OH groups. The placement of the mono-THF ring was shown to be at C-16 from the diagnostic fragments in *m/z* 309 and 379 in the EI mass spectrum (Scheme 1). The HREI mass spectrum gave an ion at *m/z* 309.2082 for $C_{18}H_{29}O_4$ which confirmed the position of the mono-THF ring at C-16. The relative configuration in this THF ring moiety was established as *threo-trans-threo* by comparing the ^{1}H and ^{13}C NMR data of **1** and its diacetate derivative **1a** with those of model compounds of known relative sterochemistry [5, 8].

The presence of an isolated double bond in **1** was determined by the proton signals at δ 5.36 and 5.38 in the ^{1}H NMR spectrum and the carbon signals at δ 128.9 and 130.7 in the ^{13}C NMR spectrum, respectively. When the protons were selectively decoupled at δ 2.01 (H-10) (which showed correlation cross-peaks with the protons at δ 5.36 in the COSY spec-



Scheme 1. Diagnostic EI mass spectral peaks for compound **1**. Peaks in parentheses were not observed or very weak.

Table 2. ^{13}C NMR Spectral data of compound **1** (100 MHz in $CDCl_3$)

	1 (*cis*)	**1** (*cis*)
1	178.2	178.7
2	36.67	34.47
3	25.2–31.8	25.2–31.8
4	79.3	78.9
5	35.57	35.45
6–8	25.2–31.8	25.2–31.8
9	31.89	31.89
10	28.75	28.75
11–12	128.9, 130.7	128.9, 130.7
13	27.21	27.21
14	33.26	33.26
15	74.0	74.0
16	82.6[a]	82.6[a]
17, 18	28.75	28.75
19	82.5[b]	82.5[b]
20	74.0	74.0
21	33.45	33.45
22–30	25.2–31.8	25.2–31.8
31	22.6	22.6
32	14.0	14.0
33	43.7	44.2
34	205.6	205.6
35	29.3	29.3

[a,b] Interchangeable within same column.

trum), the proton at δ 5.36 became a doublet with $J = 11.2$ Hz. The proton signals at δ 5.38 and δ 5.36 were compared with those of olefinic protons in *cis*- and *trans*-9-octadecenoic acid. The result indicated the presence of an isolated *cis*-double bond [7, 14, 15].

The position of the double bond in **1** was also determined by analyses of the fragments in EI mass spectrum. Diagnostic fragments at *m/z* 211, 265 and 309 (formed by cleavage between C-15 and C-16 possessing a ketolactone tail and a double bond), confirmed the position of the THF moiety at C-16–C-19 and the double bond at C-11 and C-12. This pattern of THF moiety and double bond is ready to form a *bis*-tetrahyrofuran acetogenin and is consistent with the biosynthetic pathway [9, 10, 12]. From the above discussion, *cis*-/*trans*-isomurisolenin (**1**) is a new acetogenin which possesses a typical C_{35} skeleton with a monotetrahydrofuran group and an unsaturated aliphatic chain.

Annoreticuin (**2**) [4], annoreticuin-9-one (**3**) [4], bullatacin (**4**) [8], squamocin (**5**) [9] and *cis*-/*trans*-bullatacinone (**6**) [9] were also isolated as white amorphous or colourless oil-like substances and their structures were established by IR, mass ^1H and ^{13}C NMR studies and, finally, by TLC comparison with authentic samples (except compound **6**).

cis-/*trans*-Isomurisolenin (**1**), annoreticuin-9-one (**3**), *cis*-/*trans*-bullatacinone (**6**) and *cis*-/*trans*-murisolinone (**7**), exhibited significant cytotoxicities against Hep 2,2,15 (human hepatoma cell transfected HBV), Hep. G_2 (human hepatoma cell), KB (human nasophryngeal carcinoma) and CCM2 (human colon tumor cell) cell culture systems (Table 3).

EXPERIMENTAL

General

UV spectra were obtained in EtOH, IR spectra (in KBr), ^1H NMR (400 MHz), ^{13}C NMR (100 MHz), HETCOR, COSY and DEPT spectra (all in $CDCl_3$) were obtained on a Varian spectrometer. Silica gel 60 (Merck, 230–400 mesh) was used for CC and, pre-

Table 3. Cytotoxicity of compounds **1**, **3**, **6** and **7**

Compound	ED_{50} $\mu g/ml^{-1}$ Hep. 2,2,15	Hep. G_2	KB	CCM2
1 and **7**	1.38×10^{-1}	4.70×10^{-2}	1.00	8.50×10^{-2}
3	5.40×10^{-3}	9.10×10^{-4}	3.10	1.00×10^{-2}
6	1.38×10^{-1}	7.90×10^{-2}	4.80×10^{-1}	8.00×10^{-3}

Hep. 2,2,15 (human hepatoma cell transfected HBV), Hep. G_2 (human hepatoma cell), KB (human nasopharyngeal carcinoma) and CCM2 (human colon tumor cell).

coated silica gel (Merck, Kieselgel 60 F-254) for TLC. Compounds were detected by spraying with Kedde's reagent or 50% H_2SO_4 followed by heating.

Plant material

Seeds of *A. reticulata* were collected from Kaohsiung Hsein, Taiwan, in June, 1994. A voucher specimen is deposited in the Graduate Institute of Natural Products, Kaohsiung Medical College, Kaohsiung, Taiwan, Republic of China.

Extraction and isolation

Fresh seeds (120 g) 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. After concn, the hexane soln afforded extract A (16.01 g) as an oil, positive to Kedde's reagent. The MeOH soln afforded extract B (20.21 g) as a wax, also positive to Kedde's reagent. The residual aq. layer afforded extract C (4.42 g), negative to Kedde's reagent. The methanol extract B was separated by CC on silica gel 60 (EtOAc–hexane–Me_2CO, 10:10:1) giving a mixture of *cis-/trans*-isomurisolenin (**1**) and *cis-/trans*-murisolinone (**7**) (15 mg), which was further separated by CC on silica gel 60 ($CHCl_3$–MeOH, 20:1) by increasing polarity. Five tetrahyrofuran acetogenins, annoreticuin (**2**) (162 mg), annoreticuin-9-one (**3**) (46 mg), bullatacin (**4**) (15 mg), squamocin (**5**) (108 mg), *cis-/trans*-bullatacinone (**6**) (23 mg) were also isolated.

cis-/trans-Isomurisolenin (**1**) *cis-/trans*-murisolinone (**7**)

Waxy solid. $[\alpha]_D^{24}$ +32.89 ($CHCl_3$; *c* 0.31). IR ν_{max}^{film} cm^{-1}: 3500, 2910, 2860, 1760, 1710, 1460, 1250, 1050, 800. HREIMS $[M+H]^+$ *m/z*: 581.4789 (calcd 581.4781) for $C_{35}H_{64}O_6$ and HREIMS $[M+H]^+$ *m/z*: 579.4597 (calcd 579.4625) for $C_{35}H_{62}O_6$. ^1H NMR (400 MHz, $CDCl_3$): δ (numbering is given for *cis-/trans*-murisolinone): 4.55 (*trans*, 1H, *dddd*, *J* = 8.3, 8.2, 5.7, 3.2 Hz, H-4), 4.40 (*cis*, 1H, *dddd*, *J* = 10.7, 7.4, 5.4 and 5.4 Hz, H-4), 3.80 (2H, *m*, H-16, H-19), 3.40 (2H, *m*, H-15, H-20), 3.08–3.00 (2H, H-33b, H-2), 2.61–2.23 (2H, H-33a, H-3a), 2.20 (3H, *s*, H-35), 0.88 (3H, *t*, H-32). ^{13}C (100 MHz, $CDCl_3$): δ (*cis-/trans*-murisolinone): 205.6 (C-34), 178.7 (*trans*, C-1), 178.2 (*cis*, C-1), 82.6 (C-16 or C-19), 82.5 (C-19 or C-16), 79.3 (*cis*, C-4), 78.9 (*trans*, C-4), 74.0 (C-15 and C-20), 36.67 (*cis*, C-2), 34.47 (*trans*, C-2), 29.30 (C-35), 14.0 (C-32). FABMS *m/z*: 603 $[M+Na]^+$, 581 $[M+H]^+$. EIMS (probe) 30 eV *m/z*: 411, 393, 381, 375, 363, 311, 293, 281, 141. ^1H NMR (400 MHz, $CDCl_3$): δ (numbering is given for *cis-/trans*-isomurisolenin): 5.36, 5.38 (2H, H-11, H-12), 4.55 (*trans*, 1H, *dddd*, *J* = 8.3, 9.2, 5.7, 3.2 Hz, H-4), 4.40 (*cis*, 1H, *dddd*, *J* = 10.7, 7.4, 5.4 and 5.4 Hz, H-4), 3.80 (2H, *m*, H-16, H-19), 3.40 (2H, *m*, H-15, H-20), 3.08–3.00 (2H, H-33b, H-2), 2.61–2.23 (2H, H-33a, H-3a), 2.20 (3H, *s*, H-35), 0.88 (3H, *t*, H-32). ^{13}C (100 MHz, $CDCl_3$): δ (*cis-/trans*-isomurisolenin): 205.6 (C-34), 178.7 (*trans*, C-1), 178.2 (*cis*, C-1), 130.7 (C-11), 128.9 (C-12), 82.6 (C-16 or C-19), 82.5 (C-19 or C-16), 79.3 (*cis*, C-4), 78.9 (*trans*, C-4), 74.0 (C-15 and C-20), 36.7 (*cis*, C-2), 34.4 (*trans*, C-2), 29.3 (C-35), 14.0 (C-32). FABMS *m/z*: 601 $[M+Na]^+$, 579 $[M+H]^+$. EIMS (probe) 30 eV *m/z*: 391, 379, 373, 361, 309, 291, 265, 211, 141.

Annoreticuin (**2**)

White amorphous. $[\alpha]_D^{24}$ +10.5° ($CHCl_3$, *c* 0.02). IR ν_{max}^{film} cm^{-1}: 3450, 2950, 2850, 1745, 1480, 1320, 1090, 760. EIMS (probe) 70 eV *m/z*: 381, 361, 343, 327, 309, 291, 269, 255, 245, 227, 213, 197. Identified by direct comparison with authentic sample (TLC, UV, IR, EIMS, FABMS, NMR) and lit. [4].

Annoreticuin-9-one (**3**)

White amorphous. $[\alpha]_D^{24}$ +8.45° ($CHCl_3$, *c* 0.93). IR ν_{max}^{film} cm^{-1}: 3420, 2915, 2850, 1750, 1700, 1470, 1320, 1080, 1030, 960. Identified by direct comparison with authentic sample (TLC, UV, IR, EIMS, FABMS, NMR) and lit. [4].

Bullatacin (**4**)

$[\alpha]_D^{24}$ +17.46° ($CHCl_3$, *c* 0.13). IR ν_{max}^{film} cm^{-1}: 3460, 2910, 2840, 1740, 1620, 1450, 1310, 1060. EIMS (probe) 70 eV *m/z*: 452, 433, 415, 397, 381, 363, 345, 311, 293, 241, 223, 169, 141, 123. Identified by direct comparison with authentic sample (TLC, UV, IR, EIMS, FABMS, NMR) and lit. [8].

Squamocin (**5**)

Waxy solid. $[\alpha]_D^{24}$ +20.0 ($CHCl_3$, *c* 0.05). IR ν_{max}^{film} cm^{-1}: 3450, 2905, 2850, 1740, 1615, 1450, 1310, 1080. EIMS (probe) 70 eV *m/z*: 604, 569, 519, 483, 436, 399, 365, 347, 329, 311, 295, 267, 239, 195, 169. Identified by direct comparison with authentic sample (TLC, UV, IR, EIMS, FABMS, NMR) and lit. [9].

Cis-/trans-Bullactacinone (**6**)

Waxy solid. $[\alpha]_D^{24}$ +12.60° ($CHCl_3$, *c* 0.48). IR ν_{max}^{film} cm^{-1}: 1770, 1715. Characterized by spectral (TLC, UV, IR, EIMS, FABMS, NMR) analyses and comparison with lit. [10].

Bioassays

Human hepatoma cell (Hep. G2), Hep. G2 cell transfect with HBV (Hep. 2,2.15), human nasopharyngeal carcinoma cell (KB) and human colon tumor cell (CCM2) were cultured in RPMI 1640 and DMEM medium containing 10% FCS, 100 units ml^{-1}

penicillin and 100 μg ml^{-1} streptomycin. T-lymphoblastoid cells (CEM) (2×10^4 cells ml^{-1}) were seeded in 5 ml of RPMI 1640 medium supplemented with 5% FCS. All the cell lines were maintained in an incubator at 37° in humidified air containing 5% CO_2. Activity on the various cancer cells was assayed using the Methylene Blue colourimetric method [16, 17]. To measure the cytotoxicities of **1**, **3**, **6** and **7** on Hep G2, Hep 2,2,15 and KB cells, six different concns (0.0001 to 30 μg ml^{-1}) were added to 2 ml (5×10^4 cells ml^{-1}) culture medium. After 72 h, cells were stained with 5% Methylene Blue for 40 min, the plates washed $\times 3$ with H_2O followed by addition of 1% sacosyl to each well for 4 h. Finally, plates were read on an enzyme-linked immunosorbant microplate reader at a wavelength of 592 nm. IC_{50} is defined as 50% reduction of A in the control assay without drugs. Selective cytotoxicity was determined by the IC_{50} ratio of cancer cell vs CEM cell.

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