cis-Annonacin and (2,4)-cis-and trans-Isoannonacins: Cytotoxic Monotetrahydrofuran Annonaceous Acetogenins from the Seeds of Annona cherimolia

Mi-Hee Woo, Soon-Ok Chung and Dal-Hwan Kim

Department of Pharmacy, College of Pharmacy, Catholic University of Taegu-Hyosung, Kyongsan, Korea

cis-Annonacin (1) and the mixture of (2,4)-cis-and trans-isoannonacins (2 and 3), three known mono-tetrahydrofuran annonaceous acetogenins, have been isolated from the seeds of Annona cherimolia by the use of the brine shrimp lethality test (BST) for bioactivity directed fractionation. Their structures were elucidated based on spectroscopic and chemical methods. 1 showed potent cytotoxicities in the brine shrimp lethality test (BST) and among six human solid tumor cell lines with notable selectivity for the pancreatic cell line (PaCa-2) at about 1,000 times the potency of adriamycin. The mixture of 2 and 3 is over 10,000 times cytotoxic as adriamycin in the pancreatic cell line (PaCa-2). All of the compounds are about 10 to 100 times as cytotoxic as adriamycin in the prostate cell line (PC-3).

INTRODUCTION

Most acetogenins are bioactive and offer exciting potential new antitumor, immunosuppressive, pesticidal, antiprotozoal, antifeedant, anthelmintic, and antimicrobial agent (Morre et al., 1995). Used in traditional medicine as insecticide and parasiticide (Barriga, 1974), Annona cherimolia Mill. (Annonaceae) is a tropical tree native to Peru (Fies, 1959), now cultivated for its edible fruits in the south of Spain.

From the seeds, we reported the isolation and structural elucidation of three novel bioactive acetogenins (annocherin, (2,4)-cis-and trans-annocherinones) (Woo et al., 1999). As part of our continuing efforts to find new potential anticancer and pesticidal constituents, we have isolated three known acetogenins, cis-annonacin (1) and the mixture of (2,4)-cis- and trans-isoannonacins (2 and 3), from an EtOH extract of the seeds of this plant, guided by brine shrimp lethality test (BST) (Meyer et al., 1982; McLaughlin, 1991). Their structures were identified as mono-THF acetogenins by NMR and MS spectroscopic techniques and chemical methods.

Correspondence to: Mi-Hee Woo, Department of Pharmacy, College of Pharmacy, Catholic University of Taegu-Hyosung, Kyongsan, 712-702, Korea.
E-mail: woomh@cuth.cataegu.ac.kr

MATERIALS AND METHODS

Instruments and reagents

Melting points were determined on a Yanaco micro melting point apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. IR spectra were measured on a JASCO FT/IR-300E spectrophotometer. UV spectra were obtained on a Shimadzu UV-1601PC spectrophotometer. NMR spectroscopy was taken on a Varian VXR 500S spectrophotometer in $CDCl_3$ using TMS as an internal standard. Mass spectra was recorded on a Quattro II spectrometer. For TLC, silicagel 60 F_{254} (EM 5717) glass plates (0.25 mm) were used and visualized by spraying with 5% phosphomolybdic acid in MeOH and subsequent heating. HPLC was carried out with a Rainin HPLC instrument using the Dynamax software system and a silica gel column (250 × 21 mm) equipped with a Rainin UV-1 detector at 230 nm. For preparation of tetra-TMSi derivative, N,O-bis-(trimethylsilyl)acetamide (BSA) and pyridine in silylation grade were purchased from the Aldrich Company.

Plant material

The seeds of Annona cherimolia. were obtained in 1996 from fruits grown commercially in plantations

in southern California and purchased from Hurov Botanicals and Seeds located in Chula Vista, California.

Extraction and isolation

The seeds of *Annona cherimolia* (8 kg) were extracted with 95% ethanol (4 times). The EtOH extract (F001, BST LC_{50} 1.33 × 10⁻¹ μg/ml, 700 g) was partitioned between H_2O (F002, BST LC_{50} 2.79 × 10² μg/ml, 300 g) and CH_2Cl_2 (F003, BST LC_{50} 1.19 × 10⁻² μg/ml, 400 g), and F003 was partitioned between 10% H_2O in MeOH (F005, BST LC_{50} 1.13×10⁻² μg/ml, 250 g) and n-hexane (F006, BST LC_{50} 1.78 μg/ml 150 g). F005 (250 g) was subjected to open column chromatography over silica gel (2.8 kg) eluted with a gradient of hexane/$CHCl_3$/MeOH. Fractions (F-1 to F-17) were collected and pooled according to their similar TLC patterns. The BST active pool F-12 (130 g) was further resolved on another silica gel (1.5 kg) open column, eluted with a gradient of hexane/$CHCl_3$/MeOH. Fractions were collected into 13 pools (A to M) on the basis of TLC patterns. Further purification of the most bioactive BST fraction (H) was carried out by HPLC eluted with 85% acetonitrile in water to afford 1 and the mixture of 2 and 3.

cis-Annonacin (1): white amorphous powder, mp 77-78°C, [α]$_D^{25}$: +10° (c 0.17, $CHCl_3$), UV λ_{max}^{MeOH} nm (log ε) : 215 (4.0), v IR(cm⁻¹) : 3395 (OH), 1724 (C=O α, β-unsaturated γ-lactone), ¹H-NMR see Table I.

Tetra-TMSi derivative (1a) of *cis*-annonacin: Mass (EI) m/z: Fig. 1.

(2,4)-*cis*- and *trans*-isoannonacins (2 and 3): white amorphous powder, mp 95-96°C, [α]$_D^{20}$:+20° (c 0.07, $CHCl_3$), UV λ_{max}^{MeOH} nm (log ε) : 205 (3.8), IR v (cm⁻¹) : 3414 (OH), 1752 (C=O of ketolactone), ¹H-NMR see Table I.

Tri-TMSi derivatives (2a and 3a) of (2,4)-*cis*- and *trans*-isoannonacins: Mass (EI) m/z: Fig. 2.

Preparation of TMSi-derivatives

Approximately 10 μg of 1, and the mixture of 2 and 3 were separately treated with 0.2 μl of pyridine and 2 μl of *N,O-bis*-(trimethylsilyl)acetamide (BSA) for 5 hrs to give a 1a and the mixture of 2a and 3a.

Bioassay

The extracts, fractions, and isolated compounds were routinely evaluated for lethality to brine shrimp lethality test (BST) (Meyer et al., 1982; McLaughlin, 1991).

MTT cytotoxicity tests

Seven-day in vitro MTT cytotoxicity tests against human tumor cell lines were carried out at the Cell Culture Laboratory, Purdue Cancer Center, using standard protocols for A-549 (human lung carcinoma), MCF-7 (human breast carcinoma), HT-29 (human colon adenocarcinoma), A-498 (human kidney carcinoma), PC-3 (human prostate adenocarcinoma), and MIA PaCa-2 (human pancreatic carcinoma) with adriamycin as a positive control.

RESULTS AND DISCUSSION

Compound 1 was isolated as a white amorphous powder. Its molecular weight was suggested by the peak at m/z 597 [M+H]⁺ in the FABMS. The presence of four hydroxyl groups was indicated by the losses of four molecules of H_2O (m/z 18) from the [M+H]⁺ in the FABMS spectrum and by a broad absorption in the IR spectrum at 3395 cm⁻¹. An IR absorption at 1734 cm⁻¹, UV λ max at 215 nm and the ¹H-NMR spectrum peak (Table I) at δ 1.41 (H-35), 2.38 (H-3a), 2.51 (H-3b), 3.82 (H-4), 5.01 (H-34), and 7.18 (H-33) suggested the presence of an α,β-unsaturated γ-lactone ring with a 4-OH moiety (Rupprecht et al., 1990). Mass fragmentation analysis of the tetra-TMSi derivative (1a) demonstrated that the four OH groups were located at C-4, C-10, C-15, and C-20, as shown in Fig. 1. The presence of a
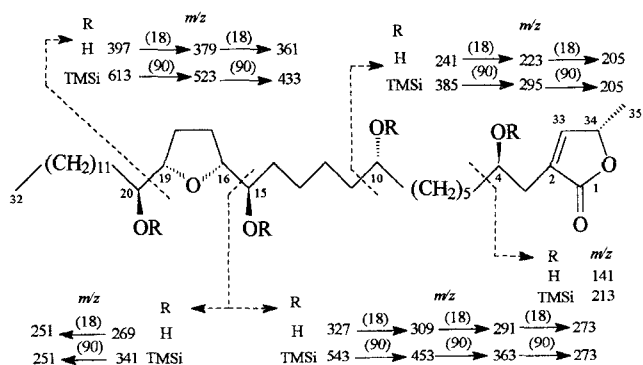
Fig. 1. Diagnostic EIMS fragmetations of **1** and its tetra-TMSi(**1a**) derivative.
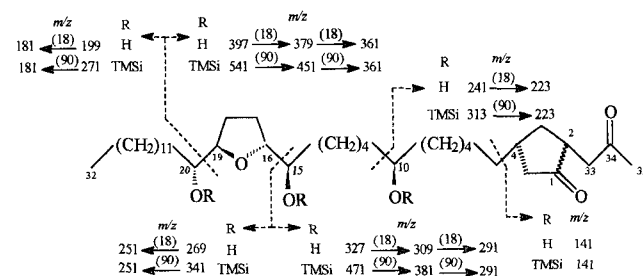
Fig. 2. Diagnostic EIMS fragmetations of **2** and **3** and its tetra-TMSi (the mixture of **2a** and **3a**) derivative.

Table. I. ^{1}H-NMR spectral data of **1**, **2** and **3** (CDCl$_3$)

No.	^{1}H-NMR (500 MHz)		
	1	**2**	**3**
1	-	-	-
2	-	3.05 m	3.02 m
3a	2.38 ddt (15.1, 8.0, 1.4)	1.41 m	1.99 m
3b	2.51 ddd (15.1, 4.0, 1.4)	2.60 ddd (12.3, 9.4, 5.6)	2.20 ddd (12.9, 9.6, 3.4)
4	3.82 m	4.39 dddd (12.3, 9.5, 4.3, 3.0)	4.55 dddd (8.3, 8.2, 5.7, 3.2)
5a	1.1-1.6 m	1.56 m	1.49 m
5b	1.1-1.6 m	1.68 m	1.56 m
6-9	1.1-1.6 m	1.1-1.6 m	1.1-1.6 m
10	3.59 m	3.60 m	3.60 m
11-14	1.1-1.6 m	1.1-1.6 m	1.1-1.6 m
15	3.42 m	3.41 m	3.41 m
16	3.82 m	3.80 m	3.80 m
17a	1.87 m	1.68 m	1.68 m
17b	1.98 m	1.99 m	1.99 m
18a	1.87 m	1.68 m	1.68 m
18b	1.98 m	1.99 m	1.99 m
19	3.82 m	3.80 m	3.80 m
20	3.42 m	3.41 m	3.41 m
21-31	1.1-1.6 m	1.1-1.6 m	1.1-1.6 m
32	0.88 t (7.0)	0.88 t (7.0)	0.88 t (7.0)
33a	7.18 q (1.5)	2.61 dd (18.0, 9.0)	2.68 dd (18.0, 9.0)
33b	-	3.10 dd (18.5, 3.5)	3.04 dd (18.0, 3.0)
34	5.01 qq (7.0, 1.5)	-	-
35	1.41 d (7.0)	2.19 s	2.19 s

Table II. Cytotoxicity against human solid tumor cell lines for **1** and the mixture of **2** and **3**

Compound	BST LC50 (µg/ml)	Human cancer cell line (ED50, µg/ml)					
		A-549	MCF-7	HT-29	A-498	PC-3	MIA PaCa-2
1	2.30	1.16	9.25	3.39×10^{-2}	3.16×10^{-2}	6.77×10^{-3}	4.39×10^{-5}
2 and **3**	6.03 5 10^{-2}	1.88×10^{-3}	2.83	5.29×10^{-4}	1.62×10^{-3}	4.21×10^{-4}	5.22×10^{-7}
adriamycin	Not tested	3.03×10^{-3}	1.32×10^{-1}	6.06×10^{-3}	3.47×10^{-3}	5.02×10^{-2}	1.69×10^{-2}

BST (brine shrimp lethality test), A-549 (human lung carcinoma), MCF-7 (human breast carcinoma), HT-29 (human colon adenocarcinoma), A-498 (human kidney carcinoma), PC-3 (human prostate adenocarcinoma), MIA PaCa-2 (human pancreatic carcinoma), adriamycin (positive control standard)

mono-THF ring with two flanking hydroxyl groups was evident from the NMR spectral data. In the ^{1}H-NMR spectrum, a three proton multiplet is visible at δ 3.82 due to the two oxymethine protons of the THF ring as well as the carbinol methine proton at C-4. In the ^{1}H-NMR spectrum, a two proton multiplet is observed at 3.42 due to two carbinol methine protons indicating the presence of mono-THF ring with two flanking hydroxyl groups. The relative stereochemistry of C-15/C-16 and C-19/C-20 was defined by comparing the ^{1}H-NMR signals of H-15/H-20 at δ 3.43 and H-16/H-19 at δ 3.82 in **1** with those of model compounds of known relative stereo-chemistry (Rupprecht et al., 1990; Fang et al., 1993). These data suggested that the relative configurations between C-15/C-16 and C-19/C-20 were both threo (Laprevote et al., 1992). The ^{1}H-NMR signals at δ 1.98 and 1.87, corresponding to H-17a/H-18a and H-

17b/H-18b, are typical methylene proton signals of a cis-THF ring configuration (Harmange et al., 1992), whereas these are at δ 1.98 and 1.66 for the trans-THF ring configuration. Thus, the relative configuration shown in mono-THF ring is threo/cis/threo of the cis-annonacin type. Finally this compound was identified as cis-annonacin (Rieser et al., 1996).

The mixture of compounds **2** and **3** was obtained as a white amorphous powder. FABMS spectrometry gave a [M+H]$^+$ at m/z 597. The existence of three OH groups in the mixture of **2** and **3** were indicated by three successive losses of H$_2$O (m/z 18) from the [M+H]$^+$ in the FABMS and the preparation of the tri-TMSi (**2a**, **3a**) derivatives, which showed three successive losses of TMSiOH (m/z 90) in the EIMS. In the ^{1}H-NMR spectrum, the resonances at δ 4.55 and 4.39, with a combined integration for one proton, were assigned to H-4 and

suggested the presence of the (2,4)-cis- and trans-diastereoisomers at the ketolactone ring moiety, as it was typical with the ketolactones. The position of the THF rings as well as the hydroxyl groups along the hydro-carbon chain, were deduced from the EIMS of the TMSi derivative (the mixture of 2a and 3a). As shown in Fig. 2, the fragmentation patterns clearly indicated that the mono-THF ring was located at C-16 to C-19. The positions of the three hydroxyl groups were also suggested by the fragment ions and were located at C-10, C-15, and C-20. The stereochemistries at C-15/C-16 and C-19/C-20 in the mixture of 2 and 3 were concluded to be threo, and the stereochemistry of the THF ring was determined as trans by comparison with model compounds of known relative configuration, synthesized by both Harmange et al. (Harmange et al., 1992) and Fujimoto et al. (Fujimoto et al., 1994), as well as by comparisons with the reported data for annonacin (Alkofahi et al., 1988) and annomontacin (Jossang et al., 1991). So, this compound was identified as (2,4)-cis- and trans-isoannonacin (Xu et al., 1989).

Bioactivity data obtained with 1 and the mixture of 2 and 3 are summarized in Table II. 1 showed potent bioactivities in the brine shrimp lethality test (BST) and among six human solid tumor cell lines with notable selectivity for the pancreatic cell line (PaCa-2) at about 1,000 times the potency of adriamycin. The mixture of 2 and 3 is over 10,000 times cytotoxic as adriamycin in the pancreatic cell line (PaCa-2). All of the compounds are about 10 to 100 times as cytotoxic as adriamycin in the prostate cell line (PC-3). The acetogenins exert their effects through inhibition of mitochondrial electron transport (complex I) and inhibition of the plasma membrane NADH oxidase of cancer cells, and the resulting depletion of ATP thwarts ATP-dependent resistance mechanisms as discussed in the reviews (Zeng et al., 1996; Alali et al., 1999).

ACKNOWLEDGEMENTS

REFERENCES CITED

Alali, F., Liu, X.-X., McLaughlin, J. L., Annonaceous acetogenins: Recent progress. J. Nat. Prod., 62, 504-540 (1999).

Alkofahi, A., Rupprecht, J. K., Smith, D. L., Chang, C.-J., McLaughlin, J. L., Goniothalamicin and annonacin: Bioactive acetogenins from Goniothalamus giganteus

(Annonaceae). Experientia, 44, 83-85 (1988).

Barriga, H. G., Flora Medicinal de Colombia. Vol. 1, Botánica, Bogatá, p. 340, (1974).

Fang, X. -P., Rieser, M. J., Gu, Z. -M., Zhao, G. -X., McLaughlin, J. L., Annonaceous acetogenins: an updated review. Phytochem. Anal., 4, 27-67 (1993).

Fies, R. E., "Annonaceae", in "Die Natürlichen Pflanzenfamilien", Engler, A., Prantl, K. (Eds.) 2nd ed., Dunker and Humbolt, Berlin, Vol.17, 1959.

Fujimoto, Y., Murasaki, C., Shimada, H., Nishioka, S., Kakinuma, K., Singh, S., Singh, M., Gupta, Y. K., Sahai, M., Annonaceous acetogenins from the seeds of Annona squamosa, non-adjacent bis-tetrahydrofuranic acetogenins. Chem. Pharm. Bull., 42, 1175-1184 (1994).

Harmange, J. C., Figadere, B., Cavé, A., Stereocontrolled synthesis of 2,5-linked monotetrahydrofuran units of acetogenins. Tetrahedron Lett. 33, 5749-5752 (1992).

Jossang, A., Dubaele, B., Cavé, A., Bartoli, M. H., Beriel, H., Annomontacin: une nouvelle acetogenines γ-lactone-monotetrahydrofurannique cytotoxique de l' Annona montana. J. Nat. Prod., 54, 967-971 (1991).

Laprevote, O., Girard, C., Das, B. C., Formation of gas-phase lithium complexes from acetogenins and their analysis by fast atom bombardment mass spectrometry. Tetrahedron Lett., 33, 5237-5240 (1992).

McLaughlin, J. L., "Methods in Plant Biochemistry", Hostettmann, K. (Ed.), Academic Press, London, Vol. 6, pp. 1-35, (1991).

Meyer, B. N., Ferrigni, N. R., Putnam, J. E., Jacobson, L. B., Nichols, D. E., McLaughlin, J. L., Brine shrimp: a convenient general bioassay for active plant constituents. Planta Med., 45, 31-34 (1982).

Morre, J. D., Decabo, R, D., Farley, C., Oberlies, N. H., McLaughlin, J. L., Mode of action of bullatacin, a potent antitumor acetogenin: inhibition of NADH oxidase activity of HELA and HL-60, but not liver, plasma membranes, Life Sci., 56, 343-348 (1995).

Rieser, M. J., Gu, Z.-M., Fang, X.-P., Zeng, L., Wood, K. V., McLaughlin, J. L., Five novel mono-tetrahydrofuran ring acetogenins from the seeds of Annona muricata, J. Nat. Prod., 59, 100-108 (1996).

Rupprecht, J. K., Hui, Y.-H., McLaughlin, J. L., Annonaceous acetogenins: a review. J. Nat. Prod. 53, 237-278 (1990).

Woo, M. H., Kim, D. H., Fotopoulos, S. S., McLaughlin, J. L., Annocherin and (2, 4)-cis- and trans-annocherinones: monotetrahydrofuran Annonaceous acetogenins with a C-7 carbonyl group from Annona cherimolia seeds. J. Nat. Prod., accepted (1999).

Xu, L. Z., Chang, C. J., Yu, J. G., Cassady, J. M., Chemistry and selective cytotoxicity of annonacin-10-one, iso-annonacin, and isoannonacin-10-one. Novel polyketides from Annona densicoma (Annonaceae). J. Org. Chem., 54, 5418-5421 (1989).

Zeng, L., Ye, Q., Oberlies, N. H., Shi, G., Gu, Z.-M., He, K., McLaughlin, J. L., Recent Advances in Annonaceous acetogenins. *Nat. Prod. Rep.*, 13, 275-306 (1996).