BULLACIN: A NEW CYTOTOXIC ANNONACEOUS ACETOGENIN FROM *ANNONA BULLATA*

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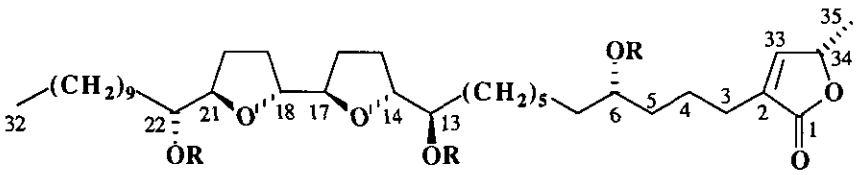
Abstract- Using the brine shrimp lethality test (BST) to direct fractionation, two additional Annonaceous acetogenins, bullacin (**1**) and parviflorin (**2**), were isolated from the ethanol extract of the bark of *Annona bullata* Rich. Their absolute stereochemistries were revealed by the use of Mosher's methodology. Compound (**1**) is a new adjacent bis-tetrahydrofuran (THF) acetogenin possessing a relatively rare C_{35} skeleton and an unprecedented C-6 hydroxyl group. The absolute configuration of **1** was assigned as 6*S*, 13*R*, 14*R*, 17*R*, 18*R*, 21*R*, 22*R*, and 34*S*. Compound (**2**) is a known acetogenin but is new to this plant. The absolute configuration of **2** was concluded to be 4*R*, 13*R*, 14*R*, 17*R*, 18*R*, 21*R*, 22*R*, and 34*S*. This is the first paper in which the absolute stereochemistry of a new acetogenin is published with the new structure. **1** showed 10 to 1000 times the cytotoxic potency of adriamycin when tested in human tumor cell lines.

Annonaceous acetogenins have attracted considerable interest in the last few years due to their potent bioactivities. About 90 acetogenins had been reported through 1992.[1,2] Among these, 23 have been previously isolated by our group from the bark of *Annona bullata* Rich., a tree indigenous to Cuba. These include adjacent bis-tetrahydrofuran (THF), non adjacent bis-THF, and mono-THF acetogenins,[3] but all have a C_{37} skeleton. Lethality in a test using brine shrimp larvae (BST) is a simple and efficient, bench-top, bioassay for fractionation and isolation of Annonaceous acetogenins and other antitumor and pesticidal compounds.[4,5] By further BST activity-directed fractionation, bullacin (**1**), a new Annonaceous acetogenin, and parviflorin (**2**),

a known acetogenin,[6] have now been isolated and characterized. Both are relatively rare C_{35} adjacent bis-THF acetogenins, and bullacin (**1**) is the first acetogenin to be reported with a hydroxyl group at the C-6 position. In acetogenins like **1** and **2** that have three hydroxyl groups, there are eight chiral centers which may form 256 isomers. Consequently in elucidating the structures of such acetogenins, efforts must be made to assign the stereochemistries. For some time, only the relative stereochemistries around the THF ring(s) of many acetogenins were reported after being determined by comparisons of the ^1H and ^{13}C nmr chemical shifts of the natural products and their acetates with those of model compounds.[7] Recently, however, the absolute stereochemistries of nine acetogenins were determined using Mosher's methodology.[8] In the present paper, we used Mosher's methods,[9] as modified by Ohtani *et al.*,[10] to determine the absolute stereochemistries of bullacin (**1**) and parviflorin (**2**).



	1	**1a**	**1b**	**1c**	**1d**	**1e**
R	**H**	**Ac**	**TMS**	***d*-TMS**	**(*S*)-MTPA**	**(*R*)-MTPA**

Bullacin (**1**) was obtained as a whitish wax, $[\alpha]_D$ +15.6° (c 0.3, $CHCl_3$). The molecular formula was established to be $C_{35}H_{62}O_7$ by HR-CIms [obsd 595.4541 (MH^+), calcd 595.4574]. The ^1H and ^{13}C nmr spectral data of **1** (Table 1) showed that it is an adjacent bis-THF Annonaceous acetogenin.[1,2] The presence of an α, β-unsaturated γ-lactone was indicated by the proton signals at δ 7.05 (H-33), 5.00 (H-34), 1.41 (H-35), and the carbon signals at δ 173.8 (C-1), 149.1 (C-33), 134.0 (C-2), 77.5 (C-34), and 19.2 (C-35). The presence of this functionality was also supported by the ir carbonyl absorption band at 1755 cm^{-1} and by uv absorption at λ_{max} (MeOH) 213.3 nm (log ε 3.75).[1,2]

The existence of adjacent bis-THF rings with a flanking hydroxyl group at each side (fragment A, Figure 1) was suggested by the proton resonances at δ 3.38 (H-13, H-22), 3.83 (H-14, H-21), and 3.87 (H-17, H-18), and carbon resonances at δ 74.1 (C-13), 74.0 (C-22), 83.2 (C-14), 83.1 (C-21), and 81.8 (C-17, C-18).[1,2] The EIms ions of **1**, its tri-trimethylsilyl (**1b**) and tri-deutero-trimethylsilyl (**1c**) derivatives (Figure 3) indicated that this subunit was located at C-13 through C-22; thus, **1** is two carbons shorter between the THF rings and the

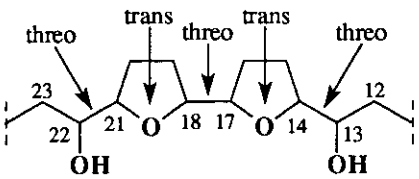


Figure 1. Fragment A of **1**.

lactone subunit than most reported C_{37} adjacent bis-THF acetogenins.[1,2] The ^1H nmr spectrum of **1** showed two symmetrical peaks at δ 3.38 and 3.83 to 3.87 which indicated that **1** , like asimicin[11] and parviflorin,[6] likely had a pseudo-symmetrical configuration in fragment A, and, indeed, its relative stereochemistries were subsequently determined to be threo/trans/threo/trans/threo from C-13 through C-22 (Figure 1), by comparing the ^1H and ^{13}C nmr chemical shifts of this subunit of **1** and its acetate (**1a**) with those of model compounds of known relative stereochemistries.[7]

Three successive losses of H_2O (m/z 18) from the molecular ion in the CIms (m/z at 577, 559, 541) suggested that there were three hydroxyl groups in **1**. This conclusion was also supported by the EIms of **1b** and **1c** (Figure 3). There was, thus, another hydroxyl group in **1** besides the two that flank the THF rings. The ^1H nmr resonances of the lactone protons and H-3 in **1** showed slight upfield shifts, compared with those of compounds

Table 1. The ^1H (500 MHz) and ^{13}C (125 MHz) Nmr Data of **1** and **1a** ($CDCl_3$)

	^1H of **1**	^{13}C of **1**	^1H of **1a**
1	-	173.8	-
2	-	134.0	-
3	2.29	23.6	2.28
4	1.65	29.8 - 25.2	1.55
5	1.46	36.8	1.54
6	3.62 m	71.5	4.87
7	1.46	37.6	1.54
8 - 11	1.70 - 1.21	29.8 - 25.2	1.40 -1.21
12, 23	1.41	33.4,	1.52
13, 22	3.38 m	74.1, 74.0	4.87
14, 21	3.83 m	83.2, 83.1	3.99
15, 20	1.98, 1.62	29.8 - 25.2	1.95, 1.55
16, 19	1.98, 1.67	29.8 - 25.2	1.95, 1.78
17, 18	3.87 m	81.8	3.91
24-29	1.70 - 1.21	29.8 - 25.2	1.40 -1.21
30	1.70 - 1.21	31.9	1.40 -1.21
31	1.70 - 1.21	22.7	1.40 -1.21
32	0.88 t	14.2	0.88
33	7.05	149.1	7.01
34	5.00	77.5	5.00
35	1.40 d	19.2	1.41
6-OAc	-	-	2.05
13, 21-OAc	-	-	2.08

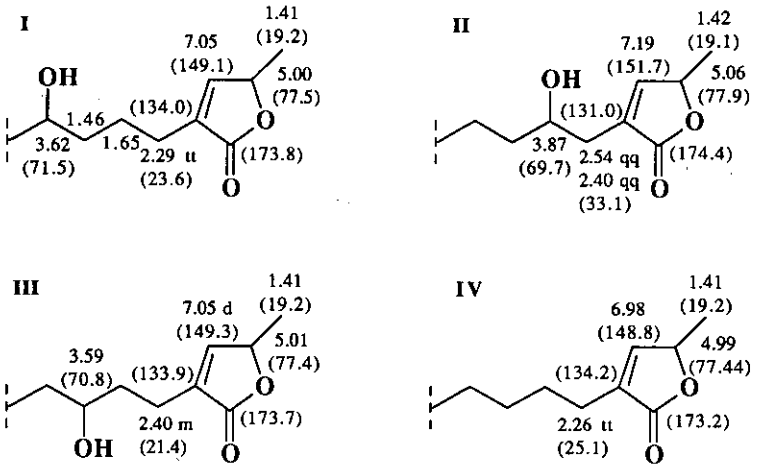


Figure 2. The comparisons of the ^{1}H and ^{13}C (in parentheses) nmr spectral data of fragment B of **1** (I) with those of compounds having a 4-OH group (II), a 5-OH group (III), and without any nearby hydroxyl group (IV).

having the lactone with a 4-OH group, and slight downfield shifts, compared with those of compounds having the lactone without any hydroxyl group nearby.[1,2] These ^{1}H nmr data were similar to those of compounds having the lactone with a 5-OH group except for H-3 (Figure 2).[1,2] Though these spectral differences were very small, they were quite reproducible. Thus, the third hydroxyl group was concluded not to be located at C-4 or C-5 but still to be near the lactone subunit. In the single relayed COSY spectrum of **1**, cross peaks between the signals at δ 2.29 (H-3) and 1.46 (H-5) and between the signals at δ 3.62 (H-6) and 1.65 (H-4) were observed. In the double relayed COSY spectrum, correlation between the signals at δ 3.62 (H-6) and 2.29 (H-3) was found. These results strongly suggested that the hydroxyl group was at the C-6 position (see fragment B, structure I, Figure 2). The EIms ions of **1**, **1b**, and **1c** (Figure 3) then confirmed this conclusion.

Because Mosher's methodology was recently demonstrated by Rieser *et al.*[8] to be a valuable method for the determination of the absolute stereochemistry of the carbinol chiral centers in acetogenins, the (*S*)- and (*R*)-methoxylfluoromethylphenylacetic acid (MTPA) esters (Mosher esters) of **1** (**1d** and **1e**) were prepared. Their proton chemical shifts for fragments A and B were carefully assigned according to the COSY spectra and are summarized in Tables 2 and 3, respectively. In Table 2, we first included the chemical shift changes for the methylene protons (H-15, 16, 19, and 20) of the THF rings compared to those in the paper of Rieser *et al.*,[8] as these data showed the expected changes and made a stronger case for the absolute stereochemical assignments.

Table 2. ^{1}H Chemical Shift Data for H(12) - H(23) from the (*S*)- and (*R*)- Per-MTPA Mosher Ester Derivatives of **1** (**1d** and **1e**) and **2** (**2a** and **2b**).

	1d	**1e**	$\Delta\delta_H$		**2a**	**2b**	$\Delta\delta_H$
MTPA config	*S*	*R*	$\delta_S - \delta_R$	MTPA config	*S*	*R*	$\delta_S - \delta_R$
H- 12/23	1.57	1.47	+ 0.10	H- 12/23	1.57	1.46	+ 0.11
13/22 (*R*)	5.02	5.02	*	**13/22 (*R*)**	5.02	5.02	*
14/21	3.95	4.00	- 0.05	14/21	3.95	4.00	- 0.05
15a/20a	1.83	1.94	- 0.11	15a/20a	1.81	1.94	- 0.13
15b/20b	1.44	1.55	- 0.11	15b/20b	1.43	1.55	- 0.12
16a/19a	1.72	1.92	- 0.20	16a/19a	1.69	1.92	- 0.23
16b/19b	1.66	1.84	- 0.18	16b/19b	1.64	1.83	- 0.19
17/18	3.78	3.93	- 0.15	17/18	3.78	3.93	- 0.15

* The carbinol chiral centers.

The analyses of the $\Delta\delta_H$ (δ_S - δ_R) of H-12 to H-23 (Table 2) showed negative results on the ring side and positive results on the chain side. According to Mosher's assumptions,[9,10] only the *R* configurations of C-13 and C-22 could have the protons on the rings more highly shielded and the protons on the chain less highly shielded in the (*S*)-MTPA derivative, and conversely in the (*R*)-MTPA ester. Thus, the *R* configuration was assigned for the carbinol chiral centers at C-13 and C-22. These are exactly the same as those of asimicin;[11] this was not unexpected, for the ^{1}H and ^{13}C nmr data of fragment A in **1** were in close agreement with those of asimicin.[11] Since the relative stereochemical data around the bis-THF rings of **1** were already in hand, all the other chiral centers in fragment A were automatically assigned as shown in the structure of **1**. The $\Delta\delta_H$ in

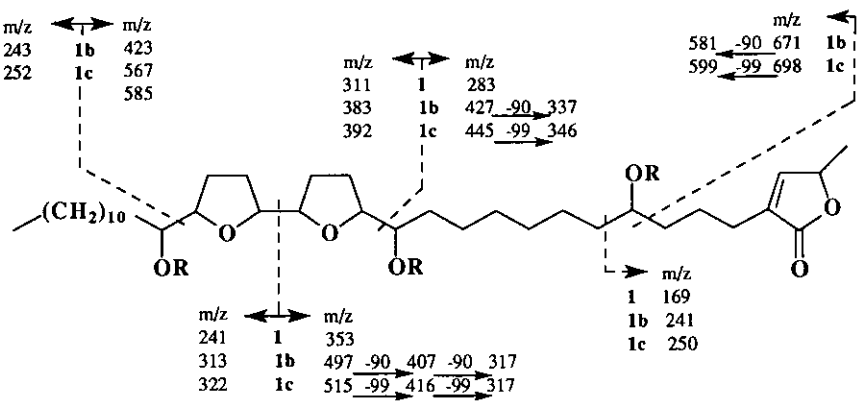


Figure 3. The diagnostic EIms fragment ions of bullacin(**1**), its tri-trimethylsilyl derivative (**1b**), and tri-deutero-trimethylsilyl derivative (**1c**).

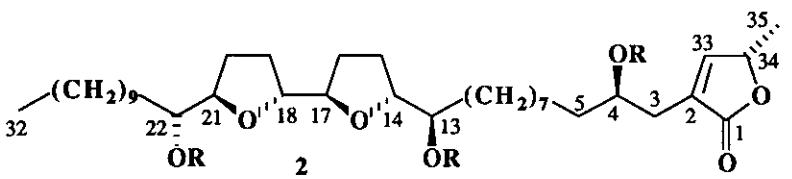
Table 3. The ^1H Chemical Shift Data for Fragment A from the (*S*)- and (*R*)- Per-MTPA Mosher Ester Derivatives of **1** (**1d** and **1e**) and **2** (**2b** and **2c**).

	1d	**1e**	$\Delta\delta_H$		**2a**	**2b**	$\Delta\delta_H$
MTPA config	*S*	*R*	$\delta_S - \delta_R$	MTPA config	*S*	*R*	$\delta_S - \delta_R$
H- 35	1.39	1.39	0	H- 35	1.28	1.31	- 0.03
34	4.97	4.96	+ 0.01	34	4.86	4.92	- 0.06
33	6.95	6.86	+ 0.09	33	6.72	6.97	- 0.25
3	2.27	2.18	+ 0.09	3a	2.59	2.67	- 0.08
4	1.54	1.43	+ 0.11	3b	2.54	2.60	- 0.06
5	1.67	1.55	+ 0.12	**4 (*R*)***	5.31	5.38	**- 0.07**
6 (*S*)*	5.08	5.07	**~ 0**	5	1.65	1.60	+ 0.05
7	1.57	1.67	- 0.10				

* The carbinol chiral centers.

Table 3, showing positive data on the lactone side and negative data for H-7, suggested that the configuration at C-6 was *S*, using the same arguments as explained above. The configuration at C-34 was assumed to be *S* based on the fact that the configuration of this chiral center (which is C-36 in the C_{37} acetogenins and C-34 in the C_{35} acetogenins) has been determined to be *S* in all the acetogenins whose absolute stereochemistries have been solved.[7] Thus, the structure of **1** is proposed to be 6*S*, 13*R*, 14*R*, 17*R*, 18*R*, 21*R*, 22*R*, and 34*S*, as a new C_{35} adjacent bis-THF acetogenin. This is the first C_{35} acetogenin from *A. bullata*. Until now, including **1**, only five of the C_{35} adjacent bis-THF acetogenins have been isolated, while more than 30 of the C_{37} adjacent bis-THF acetogenins have been reported.[1,2] This is the first time that a 6-OH group has been reported in Annonaceous acetogenins; however, a new C-35 acetogenin, with a mono-THF ring at C-15 to C-20, has recently been isolated from *Goniothalamus amuzon* and has a 6-OH group.[12] This is also the first paper in which the absolute stereochemistry of a new acetogenin is published with the new structure.

Compound (**2**) was also obtained as a whitish wax. All of its ^1H and ^{13}C nmr data were very similar to those of asimicin[11] and the same as those of parviflorin.[6] These data indicated that **2** has similar fragments A and B and relative stereochemistries as these two known compounds. Asimicin and parviflorin differ only in that the former has a C_{37} skeleton,[11] while the latter is a C_{35} acetogenin.[6] The HR-CIms of **2** [obsd 595.4553 (MH^+), calcd 595.4574] determined its molecular formula to be $C_{35}H_{62}O_7$ and suggested that **2** should be identical to parviflorin. Co-TLC of **2** and parviflorin on silica gel gave the same R_f values in $CHCl_3$-MeOH (9.5 : 0.5) and hexane-acetone (3 : 1) solvent systems. The EIms of **2** and its TMS derivative gave identical major



	2	2a	2b
R	H	(*S*)-MTPA	(*R*)-MTPA

fragmentation ions as those of parviflorin. Thus, **2** was concluded to be parviflorin, which was recently reported by our group from *Asimina parviflora*; however, in that paper only relative stereochemistry was suggested.[6] The relative stereochemistries around the bis-THF rings of **2** were already known to be exactly the same as fragment A (Figure 1).[6] Mosher's methodology was applied, and the pertinent ^{1}H nmr data from the (*S*)- and (*R*)-MTPA esters (**2a** and **2b**) are presented in Tables 2 and 3. The configuration at C-4 was assigned as *R* according to the data listed in Table 3; this is the same as all the other natural 4-hydroxylated acetogenins whose absolute stereochemistry at C-4 have been revealed. The $\Delta\delta_H$ for H-12 to H-23 (Table 2), again, showed negative on the ring side and positive on the chain side, the same as those of **1**. Thus, both carbinol chiral centers at C-13 and C-22 have the *R* configuration. The absolute configuration of **2** was, therefore, concluded to be 4*R*, 13*R*, 14*R*, 17*R*, 18*R*, 21*R*, 22*R*, and 34*S*.

1 and **2** were highly toxic to brine shrimp; both components also showed potent cytotoxicity to three human solid tumor cell lines (Table 4). This is another example in which the use of the simple BST for bioactivity-directed isolation has lead us to potent cytotoxic natural products with potential as antitumor agents. The Annonaceous acetogenins are potent inhibitors of NADH: ubiquinone oxidoreductase in mitochondrial electron transport systems,[13] and they may be useful in inhibiting the ATP-driven P-170 glycoprotein pump that is responsible for multidrug resistance.[2]

Table 4. Bioactivities of Bullacin(1) and Parviflorin (2).

Compound	BST[a] LC_{50} (μg/ml)	A-549[b] ED_{50} (μg/ml)	MCF-7[c] ED_{50} (μg/ml)	HT-29[d] ED_{50} (μg/ml)
1	7.0×10^{-2}	1.79×10^{-5}	1.00×10^{-5}	5.23×10^{-3}
2[e]	8.8×10^{-2}	1.27×10^{-15}	1.72	5.49×10^{-1}
Adriamycin[f]	8.0×10^{-2}	1.53×10^{-4}	1.38×10^{-2}	5.58×10^{-4}

a) Brine shrimp lethality test.[4,5] b) Human lung carcinoma.[14] c) Human breast carcinoma.[15]
d) Human colon adenocarcinoma.[16] e) Data from Ratnayake *et al.*[6] f) Positive control.

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