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BIOACTIVE ANNONACEOUS ACETOGENINS FROM *ROLLINIA MUCOSA*

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Key Word Index—*Rollinia mucosa*; Annonaceae; Annonaceous acetogenins; BST; anti-cancer drugs.

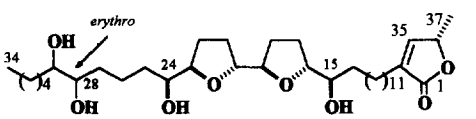
Abstract—Two new bioactive Annonaceous acetogenins, rollitacin (**1**) and rollinacin (**2**), along with one known acetogenin, javoricin, were isolated from the ethanolic extract of the leaves of *Rollinia mucosa*. Compounds **1** and **2** exhibited selective inhibitory effects among six human solid tumour cell lines. The structural elucidations of **1** and **2** were achieved by various spectroscopic analyses and chemical derivatizations.

INTRODUCTION

Rollinia mucosa (Jacq.) Baill. (Annonaceae) is a tropical fruit tree indigenous to the West Indies and Central America. Plant materials of this, or closely related species, have been used for the traditional medical treatment of tumours in the West Indies and Indonesia [1]. We speculate that the active principles may be Annonaceous acetogenins, which are known to have anti-cancer activities [2–6].

Our laboratory has investigated the bioactive leaves of *R. mucosa* and, so far, has identified 20 bioactive Annonaceous acetogenins [7–14]; among these, 13 are new. These include three new *mono*-THF ring acetogenins, rollinecins A and B [7] and muricatetrocin C [8]; the first nonadjacent bis-*cis*-THF ring acetogenin, 12,15-*cis*-sylvaticin [9]; six new adjacent bis-THF ring acetogenins, 12-hydroxy-bullatacins A and B [10], and rollidecins A–D [8, 11, 12]; the first tetrahydropyran (THP) ring acetogenins, mucocin [13] and muconin [14]; and the first hydroxyl-substituted THF ring acetogenin, mucoxin [14].

Our ongoing research on this species has now yielded three additional acetogenins, of which two are new. Javoricin is a known mono-THF ring acetogenin originally isolated from *Annona muricata* [15]. Rollitacin (**1**, Fig. 1) resembles bullatacin [16, 17] by sharing the latter's hydroxyl flanked adjacent bis-THF ring unit, however, it is a 4-deoxy derivative and bears a distinctive *erythro* vicinal diol near the aliphatic end of the molecule; the diol position differentiates **1** from bullatetrocin [18], a very similar acetogenin recently isolated from *Asimina triloba*. Rollinacin (**2**, Fig. 1) is unique in that its adjacent bis-THF ring system is flanked on one side by a hydroxyl at a distance of two C-C bonds away; it represents one of the first examples of annonaceous acetogenins bearing such a structural feature [19].



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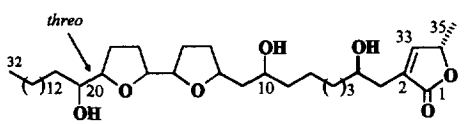

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Fig. 1. The chemical structures of rollitacin (**1**) and rollinacin (**2**).

RESULTS AND DISCUSSION

The ethanolic extract of the leaves of the title species was partitioned between two immiscible solvent systems (see Experimental) to enrich the bioactive components, as evidenced by sequentially decreasing brine shrimp lethality (BST) LC_{50} values [20, 21]. The resulting fraction (F005) was repeatedly flash-chromatographed through open silica gel columns to yield bioactive fraction pools, which were then subjected to

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Table 1. NMR δ (J, Hz) values for **1** and **2** (500 MHz for ^{1}H and 75 MHz for ^{13}C, in $CDCl_3$, TMS reference)

Pos.	Rollitacin (**1**) ^{1}H	^{13}C	Rollinacin (**2**) ^{1}H	^{13}C
1		173.8		174.6
2		134.3		131.2
3	2.26 *tt* (7.8, 1.6)	25.2	2.40*m*, 2.53*m*	33.4
4	1.20–1.50*m*	28.4–29.7	3.84*m*	70.0
5	1.20–1.40*m*	28.4–29.7	1.40–1.60*m*	37.3
6–8	1.20–1.40*m*	28.4–29.7	1.20–1.40*m*	25.3–29.7
9	1.20–1.40*m*	28.4–29.7	1.40–1.60*m*	37.3
10	1.20–1.40*m*	28.4–29.7	3.82*m*	71.7
11	1.20–1.40*m*	28.4–29.7	1.50–1.60	42.4
12	1.20–1.40*m*	28.4–29.7	4.22*m*	80.5
13	1.20–1.40*m*	25.2–29.7	1.55*m*, 2.15*m*	28.1–31.9
14	1.50–1.70*m*	30.6–33.2*m*	1.80*m*, 1.93*m*	28.1–31.9
15	3.40*m*	74.2	3.97*m*	80.9*
16	3.86*m*	83.4	3.92*m*	81.0*
17	1.75–1.98*m*	28.4–29.7	1.80*m*, 1.93*m*	28.1–31.9
18	1.75–1.98*m*	28.4–29.7	1.80*m*, 1.93*m*	28.1–31.9
19	3.86*m*	82.9	3.83*m*	82.8
20	3.93*m*	82.6	3.38*m*	74.4
21	1.75–1.98*m*	28.4–29.7	1.45*m*	32.8–34.3
22	1.75–1.98*m*	28.4–29.7	1.20–1.40*m*	28.1–31.9
23	3.93*m*	82.2	1.20–1.40*m*	28.1–31.9
24	3.86*m*	71.5	1.20–1.40*m*	28.1–31.9
25	1.40–1.70*m*	30.6–33.2*m*	1.20–1.40*m*	28.1–31.9
26–27	1.20–1.70*m*	25.2–33.2	1.20–1.40*m*	28.1–31.9
28	3.60*m*	74.6*	1.20–1.40*m*	28.1–31.9
29	3.60*m*	74.7*	1.20–1.40*m*	28.1–31.9
30	1.20–1.70*m*	30.6–33.2*m*	1.20–1.40*m*	28.1–31.9
31	1.20–1.40*m*	28.4–29.7	1.20–1.40*m*	22.7
32	1.20–1.40*m*	28.4–29.7	0.88*t*(7.0)	14.1
33	1.20–1.40*m*	22.7	7.19*m*	151.8
34	0.88*t*(7.0)	14.1	5.06*m*	78.0
35	6.98*m*	148.9	1.43*d*(6.9)	19.1
36	4.99*m*	77.4		
37	1.41*d*(6.9)	19.2		

* Exchangeable within the same columns.

normal and reverse phase HPLC chromatography to yield the above three pure compounds. The molecular formulae for **1**, **2** and javoricin were established as $C_{37}H_{66}O_8$, $C_{35}H_{62}O_7$ and $C_{35}H_{64}O_7$, respectively, by their individual HRFABMS or HRCIMS [MH^+] m/z values (see Experimental). By comparing their ^{1}H and ^{13}C NMR data (Table 1) with those of known acetogenins [2–4], it was readily recognizable that **1** had the α,β-unsaturated γ-lactone without the 4-OH group, while **2** had the same terminal lactone unit, but with a 4-OH functionality. The identification of the known compound as javoricin was suggested by the complete agreement of its ^{1}H and ^{13}C NMR spectroscopic data and the EIMS spectrum of its TMS derivative with those published for the original [15]. The possibility that this compound might be arianacin [15], the C-12 epimer of javoricin, was excluded because its intramolecular cyclic formaldehyde acetal clearly showed the diagnostic NOESY pattern indicating a *cis*-1,4-diol at the C-12/15 position [9].

Rollitacin (**1**) possessed an adjacent bis-THF ring and four hydroxyl groups as evidenced by its molecular formula, ^{1}H NMR data and COSY spectrum. The hydroxyl flanked adjacent bis-THF ring moiety of **1** was identified as of the bullatacin type by the close match of its ^{1}H and ^{13}C NMR δ values from C-15 to C-24 with those of bullatacin at the same region [16, 17]. The two protons resonating at δ 3.60 indicated an *erythro* vicinal diol [3], whose position was determined to be at C-28/29 by the EIMS spectrum of its tetra-TMS derivative (Fig. 2). Because this diol was relatively far away from the terminal methyl group, it did not cause any noticeable ^{1}H or ^{13}C NMR downfield shifts of the latter.

The planar structure of rollinacin (**2**) was established by its EIMS and that of its tri-TMS derivative (Fig. 3). The placement of the OH-10 was unusual because all the bis-THF ring acetogenins reported to date have their THF rings tightly flanked by hydroxyls. The EI fragment ion at m/z 241 of **2** was verified by HREIMS to confirm further this unusual structural feature. Accordingly, the 1,3-oxygenation

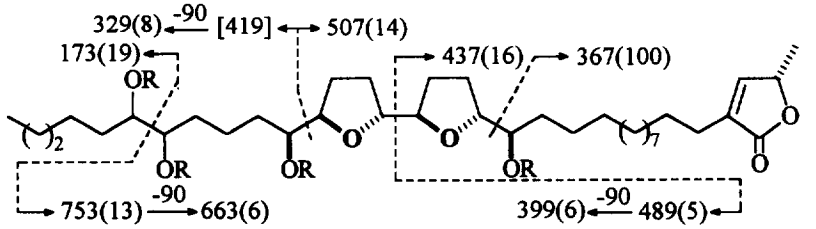

Fig. 2. The EIMS fragment ions (shown in *m/z* values with the percentage intensities in parentheses) of the tetra-TMS derivatives (*R* = TMS) of **1**.

Table 2. Bioactivities[a] of rollitacin (**1**) and rollinacin (**2**)

	BST[b] LC_{50}	A-549[c] ED_{50}	MCF-7[d] ED_{50}	HT-29[e] ED_{50}	A-498[f] ED_{50}	PC-3[g] ED_{50}	PACA-2[h] ED_{50}
1	4.3×10^{-1}	1.6	2.5×10^{-4}	4.6×10^{-3}	1.5	1.1	3.0
2	3.5	4.6×10^{-2}	1.6×10^{-1}	2.1	1.6	2.5	2.0
Adr.[i]	—	7.8×10^{-3}	7.6×10^{-2}	4.9×10^{-2}	7.8×10^{-3}	3.3×10^{-2}	1.1×10^{-3}

[a]All results were reported in mg ml^{-1}, and all samples were tested in the same run in each cytotoxicity bioassay c–h; [b]brine shrimp lethality test; [c]human lung carcinoma; [d]human breast carcinoma; [e]human colon adenocarcinoma; [f]human renal carcinoma: [g]human prostatic adenocarcinoma; [h]human pancreatic carcinoma; [i]adriamycin was used as the standard positive control.

pattern at the C-10/12 position was also suggested by its COSY spectrum in which the signal at δ 4.22 (H-12) was not correlated with any oxygenated moiety in the region from δ 3.36 to 3.98; this same proton, however, showed a cross peak with the oxymethine (H-10) at δ 3.83 in the single-relayed COSY spectrum. The assignments of ^1H and ^{13}C NMR spectroscopic data for **2** were aided by its ^1H-^{13}C HMQC spectrum. Since neither synthetic nor natural model compounds for this new acetogenin type are available, it was not possible, as yet, to establish the relative stereochemical relationships between the THF rings (at the C-15/16 position) and between the OH-10 and the C-12/15 THF ring. Also, since the C-19/16 THF oxymethines were too close in their ^1H δ values, a NOESY experiment of **2** was predicted to be inadequate to indicate whether this THF ring was *cis* or *trans*. The relative configuration at the C-20/19 position, however, could be confidently assigned as *threo* [22].

Compounds **1** and **2** were significantly active in the brine shrimp lethality test (BST) [20, 21] and hence, were further tested against our panel of six human solid tumour cell lines [9]; the LC_{50} and ED_{50} values of these tests are summarized in Table 2. It appeared that **1** was quite selective against the MCF-7 human breast cancer cells and HT-29 human colon cancer cells; **2** also showed moderate selectivity against the A-549 human lung tumor cells. The annonaceous acetogenins are active *in vivo* as antitumor agents and act as potent inhibitors of ATP production *via* the blockage of mitochondrial complex I and as inhibitors of the plasma membrane NADH oxidase of cancerous cells [23, 24]. These compounds are also effective against multiple drug resistant (MDR) tumour cells [25], suggesting excellent potential for development into clinically useful products.

EXPERIMENTAL

Instruments. Mp measured on a Fisher–Johns apparatus and the thermometer used without corr. OR: Perkin–Elmer 241 polarimeter; IR: Perkin–Elmer 1600 FTIR spectrophotometer; UV: Beckman DU-7 UV spectrophotometer. NMR: Varian-500S spectrometer (^1H at 500 MHz, ^{13}C at 125.75 MHz) using $CDCl_3$ as solvent and TMS as ref. LREIMS: Finnigan

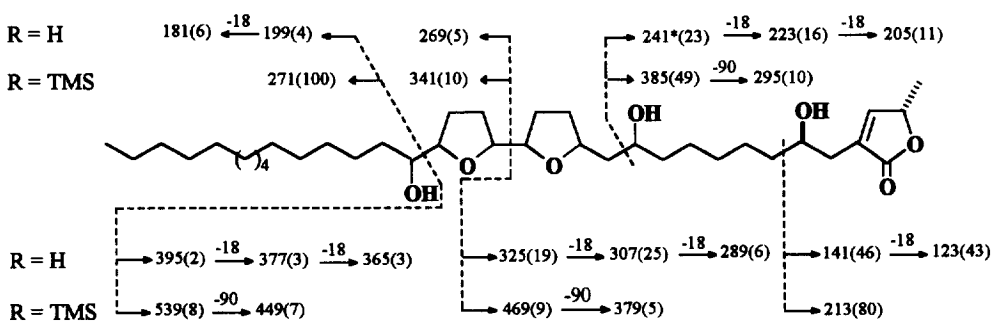

Fig. 3. The EIMS fragment ions (shown in *m/z* values with the percentage intensities in parentheses) of **2** (*R* = H) and its tri-TMS derivative (*R* = TMS). *Confirmed with HREIMS (see Experimental).

4000 spectrometer; HRFABMS and EIMS of TMS derivatives: Kratos MS 50 spectrometer. A Rainin system equipped with Dynamax software and a Rainin UV-1 detector (set at 222–235 nm) was used for all normal phase (using a 250 × 21 mm silica gel column) and reverse-phase (using a 250 × 21 mm C18 column) HPLC sepns.

Plant material. The leaves of *Rollinia mucosa* (Jacq.) Bail., were collected in the conservatory of the Missouri Botanical Garden, St. Louis, Missouri; the associated plant identification numbers are: MBG no. 891568, voucher *Sherman* 285 (MO).

Extraction and purification procedures. The oven-dried (< 50°) pulverized leaves (1630 g) were extracted exhaustively with 95% EtOH (8 l × 3) and CH_2Cl_2 (8 l × 2 then 5 l × 2) at room temp and condensed under vacuum to yield a combined extract F001 (144 g; BST LC_{50} 2.1 μg ml^{-1}); F001 was partitioned between H_2O (1.5 l) and $CHCl_3$ (3 l × 3) to yield the H_2O soluble fr. (F002) (6 g; BST LC_{50} 25 μg ml^{-1}), the $CHCl_3$ soluble fr. (F003) (120 g; BST LC_{50} 0.9 μg ml^{-1}) and an insoluble interface (F004) (5 g). F003 was further partitioned between hexane (1 l) and 10% aq. MeOH (2 l × 2) to yield the MeOH soluble fr. (F005) (71 g; BST LC_{50} 0.7 μg ml^{-1}) and the hexane soluble fr. (F006) (33 g; BST LC_{50} > 1000 μg ml^{-1}). 68 g of F005 was fractionated on an open column (i.d. = 9 cm, packed with 1.1 kg of 60–200 mesh silica gel) using a $CHCl_3$–MeOH gradient elution; 29 frs collected. Frs F-17 to F-26 were pooled (6.8 g; BST LC_{50} 0.12 μg ml^{-1}) and further fractionated on a second open column (i.d. = 5 cm, packed with 600 g of 60–200 mesh silica gel) using a hexane–acetone gradient elution. Of the 60 frs collected, the frs F-(17,26)-8 to F-(17,26)-17 were subjected to repetitive normal phase HPLC (hexane–THF–MeOH, gradient elution) to yield **3**; the frs F-(17,26)-48 to F-(17,26)-54 were subjected to repetitive normal and reverse-phase HPLC (H_2O–MeCN, gradient elution) to yield **1** and javoricin.

Chemical derivatization procedures. (1) Formation of the intramolecular formaldehyde acetals of javoricin [9]: a mixture of DMSO and TMSCl (molar ratio 1.2:1) was prepd in 2 ml of benzene and placed in a refrigerator without stirring for 2 hr to allow the formation of white crystals. The benzene was decanted and the crystals were washed twice with CH_2Cl_2. These crystals were added stepwise to a 0.5 ml $CHCl_3$ soln containing 5 mg of javoricin at room temp (usually a large excess of the crystals was added). The reaction was monitored by TLC at intervals of 2 hr and was quenched with H_2O after 10 hr. After workup by extractions with 5% $NaHCO_3$ aq. soln, the reaction mixt. was purified by normal phase HPLC. The yield was *ca* 50%, with most of the unreacted javoricin recovered.

(2) Per-TMS derivatives of **1**, **2** and javoricin: starting acetogenins (50–80 μg) were treated with *N,O*-bis(trimethylsilyl)-acetamide (20 μl) and heated at 70° for 30 min to yield their respective per-TMS derivatives. These derivatives were kept in the freezer (up to 1 week) for the EIMS analyses.

Bioassays. The brine shrimp (*Artemia salina* Leach) test (BST) was performed as modified [20, 21] to determine LC_{50} values in μg ml^{-1} for each partition fr. and chromatographic column pool. Seven-day MTT *in vitro* cytotoxicity tests against human tumour cell lines were carried out on **1** and **2** at the Purdue Cancer Center, using standard protocols and as previously referenced [9], for A-549, MCF-7, HT-29, A-498, PC-3 and PACA-2 with adriamycin as the positive control (Table 2).

Rollitacin (**1**). White waxy solid (11 mg; % yield 7.4×10^{-4}); IR $\nu^{\text{dry film}}$ cm^{-1}. 3348 (br OH), 2924, 1743, 1668, 1071; λ_{max}^{MeOH} nm (log ε): 222 nm (3.54); $[\alpha]_D^{23}$ +15.7° ($CHCl_3$; *c* 0.62); HRCIMS MH$^+$ ion *m/z* 639.4848 (calcd 639.4836), corresponding to $C_{37}H_{69}O_8$; ^{1}H and ^{13}C NMR, Table 1; LREIMS of its *tetra*-TMS derivative **2a**, Fig. 2.

Rollinacin (**2**). Oil (3 mg; % yield 1.8×10^{-4}); IR ν^{film} cm^{-1}: 3446 (br OH), 2938, 1731, 1670, 1079; UV λ_{max}^{MeOH} nm (log ε): (3.52); HRFABMS MH$^+$ ion *m/z* 595.4586 (calcd 595.4574), corresponding to $C_{35}H_{63}O_7$; ^{1}H and ^{13}C NMR, Table 1; LREIMS of **3** and its *tri*-TMS derivative **3a**, Fig. 2; HREIMS of **3**, fragment $C_{13}H_{21}O_4$ *m/z* 241.1447 (calcd 241.1440).

Javoricin. White waxy solid (12 mg); IR ν^{film} cm^{-1}. 3450 (br OH), 2930, 1745, 1671, 1075; UV λ_{max}^{MeOH} nm (log ε): 219 (3.50); $[\alpha]_D^{23}$ +12.0° ($CHCl_3$; *c* 0.5800); HRFABMS MH$^+$ ion *m/z* 597.4742 (calcd 597.4730), corresponding to $C_{35}H_{65}O_7$. NMR data of this compound were found to be the same as those previously reported [15].

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