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Reticulatacin: A new bioactive acetogenin from *Annona reticulata* (Annonaceae)

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ABSTRACT: A new bioactive monotetrahydrofuran acetogenin, reticulatacin, containing 37 carbons, has been isolated from a 95% EtOH extract of the bark of *A. reticulata* by directing the fractionation with brine shrimp lethality. Another previously reported highly potent antitumor, adjacent ring bistetrahydrofuran acetogenin, bullatacin, has also been isolated in large quantity. Two known diterpenes, (-)-kau-16-en-19-oic acid and methyl 16β,17-dihydro-(-)-kauran-19-oate and a known alkaloid, liriodenine, have also been isolated. Some of these compounds showed selective cytotoxic activities for certain human tumor cell lines.

INTRODUCTION

Since the discovery in 1982 of uvaricin, the first Annonaceous acetogenin, this class of potent bioactive compounds has been rapidly expanding, and this progress has been recently reviewed by Rupprecht et al. (1). *Annona reticulata* Linnaeus is a popular tropical fruit and has the common names of custard apple or bullock's heart (2). Previous phytochemical studies of *A. reticulata* have resulted in the isolation of a number of alkaloids (3) as well as a series of diterpenes (4-6). In 1986, an adjacent bistetrahydrofuran acetogenin, 14-hydroxy-25-deoxyrollinicin, was reported (7), and a revision of this structure has been suggested (1). Another sterically undefined adjacent ring bistetrahydrofuran acetogenin, annonareticin, has also been reported from the bark of *A. reticulata* but without extensive experimental details (8). The present paper reports the bioactivity-directed (9) isolation, structural elucidation, and biological activities of a novel monotetrahydrofuran acetogenin, reticulatacin; in addition, a known potent adjacent ring bistetrahydrofuran acetogenin, bullatacin (10), two known diterpenes, (-)-kau-16-en-19-oic acid and methyl 16β,17-dihydro-(-)-kauran-19-oate, and a known alkaloid, liriodenine, were isolated.

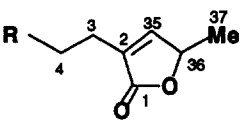
RESULTS AND DISCUSSION

The 95% EtOH extract of the dried bark of *A. reticulata* was partitioned through a standard scheme (See experimental). The most active fraction tested by brine shrimp lethality tests (9) (BST LC_{50} = 1.2 ppm) was subjected

to column chromatograghy on silica gel using hexane : CH_2Cl_2 : MeOH gradient elution. Fractions were combined into pools according to similar TLC appearance and BST results. The most active pool (BST LC_{50} < 1 ppm) was subjected to another silica gel column eluted with a gradient of hexane:EtOAc:MeOH in increasing polarity. Colorless crystalline needles were obtained from one fraction which were identified as (-)-kau-16-en-19-oic acid. Two subsequent applications of radial chromatography (Chromatotron) over silica gel and two chromatographic columns over silica gel were further used to purify the fractions from the second column to yield a new compound (**1**), a white amorphous powder of bullatacin, large crystals of methyl 16β,17-dihydro-(-)-kauran-19-oate, and yellow needles of liriodenine. The known compounds were identified by spectral analyses and/or direct comparisons with reference compounds (10).

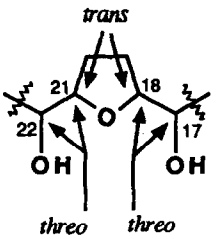
Compound **1** formed fine white needle-like crystals with m.p. 80-80.5°C. Its molecular weight at 592 was shown from CIMS (isobutane): 593 (MH^+) (100%); HR CIMS (isobutane) showed MH^+ 593.5145 corresponding to the molecular formular $C_{37}H_{68}O_5$ (calc. 593.5145). An IR absorption at 3439.5 cm^{-1} and the sequential loss of two molecules of water from the MH^+ in the CIMS (isobutane) at 575 ($MH^+ - H_2O$) (82%) and 557 (575 - H_2O) (18%) indicated the presence of two hydroxyl groups. CIMS (isobutane) of the diacetate derivative of **1** at m/z 677 (MH^+) (17%), 617 (MH^+ - AcOH) (100%), and 557 (617 - AcOH) (12%) confirmed this assignment..

A strong IR absorption at 1752.3 cm^{-1} (C=O) and a UV band at 215 nm ($\varepsilon = 6.48 \times 10^3$) suggested the presence of an α,β-unsaturated lactone. The ^{1}H-NMR signals (500 MHz, $CDCl_3$) at ppm 6.90 (q, H35), 4.97 (qq, H36), and 1.38 (d, H37) and ^{13}C-NMR signals (125 MHz, $CDCl_3$) at ppm 173.82 (C1), 134.25 (C2), 148.77 (C35), 77.40 (C36), and 19.26 (C37) confirmed the presence of the α,β-unsaturated lactone. The lack of the characteristic H3a and H3b peaks in the ^{1}H-NMR spectra of **1** (1) and the appearance of a characteristic peak at 2.24 ppm (t, H3) showed the absence of a hydroxyl group at position 4. Therefore, fragment **A** was substantiated.



(A)

The integration for four protons in the range of 3.38-3.78 ppm suggested the presence of only four oxygenated methine protons. Two of these are the methine protons on the carbon with the secondary hydroxyl groups, and the other two methine protons analogous to those of the single tetrahydrofuran ring in annonacin (11) indicated the presence of a single tetrahydrofuran ring in the molecule. The homonuclear COSY spectrum in $CDCl_3$ of **1** showed crosspeaks for the two protons on the tetrahydrofuran ring with two methine protons on the carbon with hydroxyl groups which indicated that the two hydroxyl groups are adjacent to the tetrahydrofuran ring as shown in fragment **B**. To determine the stereochemistry around the tetrahydrofuran ring, ^{1}H-NMR and ^{13}C-NMR data for **1** and ^{1}H-NMR data for the diacetate of **1** were examined according to the methods reviewed in reference 1. The ^{13}C-NMR signals at 74.03 ppm for C17 and C22 suggested the stereorelationships between 17/18 as threo and 21/22 as threo, respectively. The ^{1}H-NMR (500 MHz, $CDCl_3$) signals at 3.38 ppm for H17 and H22 of **1** and the ^{1}H-NMR signals at 4.86 ppm for H17 and H22 of **1** diacetate supported the above assignments. The trans relation between positions 18 and 21 was obtained from the ^{1}H-NMR signals at 3.98 ppm for H18 and H21 of **1** diacetate (1).



(B)

The placement of fragments **A** and **B** along the hydrocarbon chain was made possible by MS data as shown in figure 1. This compound is unique among the monotetrahydrofuran ring acetogenins in that it has a different length between the lactone ring and the tetrahydrofuran ring and lacks a hydroxyl group at the 4 position.

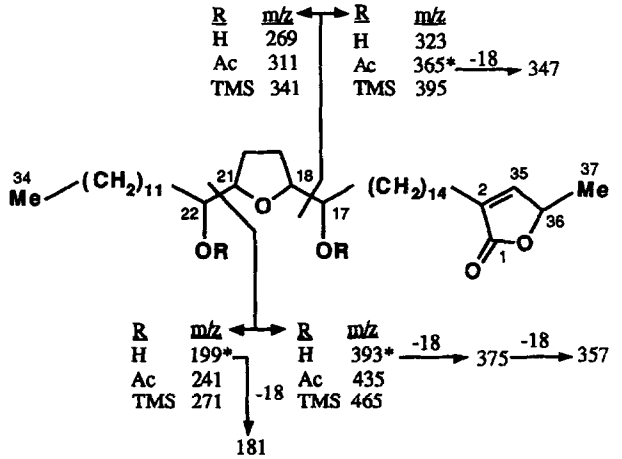
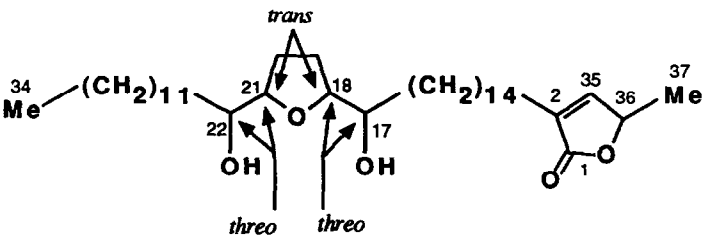


Figure 1. MS data for reticulatacin (**1**). "R" designates: the underivatized material (H), the acetyl derivative (Ac), or the trimethylsilyl derivative (TMS). Asterisks * indicate that peaks cannot be seen, but the corresponding peaks formed by consecutive loss of one or two molecules of water were evident.

From the above data, we concluded that the structure of reticulatacin is as illustrated in structure **1** with the stereochemistry at position 36 remaining undefined (12). NMR assignments in table 1 were confirmed by the absolute value two dimensional homonuclear correlated spectrum (2D COSY, 500 MHz) and the two dimensional heteronuclear shift correlation spectrum (2D HETCOR).



reticulatacin (**1**)

Table 1. NMR data (ppm, Hz) for reticulatacin (1) and its diacetate.

	reticulatacin (1)		reticulatacin diacetate
	^{1}H-NMR 500 MHz, $CDCl_3$	^{13}C-NMR 125 MHz, $CDCl_3$	^{1}H-NMR 500 MHz, $CDCl_3$
1	-	173.82	-
2	-	134.25	-
3	2.24 t (7.66)	25.20	2.39 tt(7.14,1.65)
4	1.51 m	27.42	1.55 m
5-15	1.18-1.60	22.73-31.95	1.23-1.63
16	1.38 m	33.50	1.23-1.63
17	3.38 ddd(6.65,6.65,4.21)	74.03	4.86 ddd(5.22,5.22,7.96)
18	3.78 ddd(6.35,6.35,7.63)	82.61	3.98 ddd(6.32,6.32,5.86)
19a	1.96 m*	28.77	1.95 m*
19b	1.66 m*		1.55 m*
20a	1.96 m*	28.77	1.95 m*
20b	1.66 m*		1.55 m*
21	3.78 ddd(6.35,6.35,7.63)	82.61	3.98 ddd(6.32,6.32,5.86)
22	3.38 ddd(6.65,6.65,4.21)	74.03	4.86 ddd(5.22,5.22,7.96)
23	1.38 m	33.50	1.23-1.63
24-33	1.18-1.60	22.73-31.95	1.23-1.63
34	0.86 t(6.99)	14.17	0.88 t(6.99)
35	6.97 q(1.56)	148.77	7.00 q(1.56)
36	4.97 qq(6.83,1.77)	77.40	5.00 qq(6.77,1.74)
37	1.38 d(6.5)	19.26	1.41 d(6.77)
17 OAc	-	-	2.08 s
22 OAc	-	-	2.08 s

* indicates that assignments may be interchangeable in the same column.

The moderate bioactivities of reticulatacin (1) (table 2) are consistent with those of other monotetrahydrofuran ring acetogenins (1). **1** showed good activities in the BST assay but only marginal activities against three cancer cell lines including human lung carcinoma (A-549), human breast carcinoma (MCF-7), and human colon adenocarcinoma (HT-29). The diacetate of **1** showed better activity in the BST assay. However, the activities against the three human tumor cell lines decreased after peracetylation. Bullatacin is by far the most potent compound found in this plant; bullatacin showed BST LC_{50} = 0.0063 ppm (0.0105/0.0038), and in the human cancer cell lines showed values $ED_{50} < 10^{-3}$ μg/ml (exact numbers from dilutions are given in reference 10). Interestingly, the two diterpenes, methyl 16α,17-dihydro-(-)-kauran-19-oate and (-)-kau-16-en-19-oic acid, also showed good activities in the BST assay. Methyl 16α,17-dihydro-(-)-kauran-19-oate is weakly toxic to the cells of human breast carcinoma (MCF-7), but not active against the two other human cancer cell lines. (-)-Kau-16-en-19-oic acid showed marginal activity on all three human tumor cell lines. The activities of the alkaloid, liriodenine, have been previously reported (10). The cytotoxicities of adriamycin were determined in similar runs and are shown as a positive control.

Table 2. Biological activities of compounds isolated from *A. reticulata*

	BST LC_{50},ppm	A-549 ED_{50}, μg/ml	MCF-7 ED_{50}, μg/ml	HT-29 ED_{50}, μg/ml
1	27.32	3.49	2.91	4.66
1 diacetate	2.34	23.19	>100	>100
bullatacin	0.0063	$<10^{-5}$	$<10^{-5}$	$<10^{-3}$
methyl 16α,17-dihydro-(-)-kauran-19-oate	53.96	>10	8.85	>10
(-)-kau-16-en-19-oic acid	28.64	25.64	24.76	31.70
adriamycin	-	1.6×10^{-2}	1.68×10^{-1}	3.02×10^{-2}

EXPERIMENTAL

Plant Material. Bark of *Annona reticulata* Linnaeus (Annonaceae) was purchased from United Chemical & Allied Products, 10 Clive Road, Calcutta-1, India.

Bioassays. The extracts, fractions, and isolated compounds were routinely evaluated for lethality to brine shrimp larvae (BST) (9). Cytotoxicity tests were made at the Purdue Cell Culture Laboratory, Purdue Cancer Center, using standard protocols for A-549 (human lung carcinoma) (13), MCF-7 (human breast carcinoma) (14), and HT-29 (human colon adenocarcinoma) (15).

Instrumentation. Mp determinations were made on a Mettler FP5 and are uncorrected. Optical rotation determinations were made on a Perkin Elmer 241 polarimeter. CD spectra were obtained on a JASCO Model J600 Circular Dichroism Spectrometer. IR spectra were obtained neat on a Perkin-Elmer 1420. UV spectra were taken on a Beckman DU-7. ^{1}H-NMR, 2D-COSY, ^{13}C-NMR, APT, DEPT, and HETCOR spectra were obtained on a Varian VXR-500S and referenced to $CDCl_3$. LRMS were performed on a Finnigan 4000. Exact mass measurements were obtained on a Kratos MS50 though peak matching.

Isolation of the compounds. The pulverized bark (9.5 kg) was exhaustively extracted with 95% EtOH and vacuum evaporated to yield 700 g of FOO1. The EtOH extract (FOO1) was partitioned between H_2O-$CHCl_3$ (1:1) to give residues 130 g (FOO2) and 420 g (FOO3), respectively, with 145 g (FOO4) representing the insoluble interface residue. The $CHCl_3$ residue (FOO3) was further partitioned between hexane-90% aqueous MeOH to yield 177 g of hexane soluble residue (FOO6) and 243 g of MeOH soluble residue (FOO5). The BST of residues FOO1-FOO6 showed that bioactivity was concentrated in FOO5 (LC_{50} = 1.2 ppm). FOO5 (100 g) was applied to a column of silica gel (230-400 mesh) packed in a hexane slurry, and the column was eluted in a gradient of hexane:CH_2Cl_2:MeOH in increasing polarity. 1.5-2 liter fractions were collected, and the fractions were pooled according to similar TLC appearance and BST activity. The most active pool (BST LC_{50} < 1 ppm) (25 g) was subjected to another column of silica gel elucidated with hexane:EtOAc:MeOH in increasing polarity. 250 ml fractions were collected and the fractions were again pooled by their appearance on TLC and tested on BST. From fraction 6, 100 mg of colorless needles were obtained which were identified as (-)-kau-16-en-19-oic acid. The active pool 2 (BST LC_{50} = 0.81 ppm), pool 3 (BST LC_{50} = 1.5 ppm), pool 5 (BST LC_{50} > 1 ppm), and pool 6 (BST LC_{50} > 1 ppm) were rechromatographed over silica gel on two Chromatotron plates and two columns to yield 145 mg of fine needles which were identified as reticulatacin (**1**), 260 mg of white amorphous powder which was identified as bullatacin, 110 mg of large crystals which was identified as methyl 16β,17-dihydro-(-)-kauran-19-oate, and yellow needles which was identified as liriodenine.

Characterization of **1**. Mp 80-80.5°C. $[\alpha]_D$ = +26.0 (c = 0.005 g/ml, $CHCl_3$); CIMS (isobutane) m/z: 593 (MH^+) (100%), 575 ($MH^+ - H_2O$) (82%), 557 ($MH^+ - 2H_2O$) (18%); EIMS (figure 1) m/z: 97 (cleaved between 2/3, ion at right) (4.3%), 323 (17/18 ion at right) (100%), 269 (17/18 ion at left) (3.2%), 375 (393 - H_2O) (38%), 357 (375 - H_2O) (6%), 181 (199 - H_2O) (0.6%); HR CIMS (isobutane) 593.5145 corresponding to molecular formula $C_{37}H_{68}O_5$ (calc. 593.5145); ^{1}H-NMR (table 1); ^{13}C-NMR (table 1); UV (EtOH) λ_{max} 215 nm ($\varepsilon = 6.84 \times 10^3$); IR (neat) cm^{-1}: 3419.5 (OH), 2917.9, 2849.9 (CH), 1752.3 (CO). CD (c = 0.02 mg/ml, abs. EtOH): $[\theta]_{300}$, 0; $[\theta]_{264.0}$, -2405.31; $[\theta]_{250.0}$, -2423.80; $[\theta]_{240.4}$, -1964.73; $[\theta]_{227}$, 0; $[\theta]_{207.8}$, 15601.18; $[\theta]_{206.8}$, 16093.30; $[\theta]_{205.0}$, 16731.95; $[\theta]_{203.0}$, 17032.99; $[\theta]_{200.8}$, 16855.41.

Acetylation of **1**. 15 mg of **1** was stirred with about 1 ml of anhydrous pyridine and 1 ml of acetic anhydride at room temperature overnight. Ice water was added and the mixture was partitioned with $CHCl_3$. After evaporation, the product mixture was purified by a micro-column to yield 12 mg of colorless oil of **1** diacetate. CIMS (isobutane) m/z: 677 (MH^+) (17%), 617 (MH^+ - AcOH) (100%), 557 (617 - AcOH) (12%), 650 (MH^+ - CO) (15%), 589 (650 - AcOH) (66%); EIMS (figure 1) m/z: 617 (MH^+ - AcOH) (48%) and 435 (21/22 ion at right) (100%), 407 (435 - 28) (37%), 375 (435 - AcOH) (51%), 357 (375 - H_2O) (46%), 347 (365 - H_2O) (23%), 323 (365 - 42) (9%), 311 (17/18 ion at left) (58%), 283 (311 - 28) (36%), 251 (311 - AcOH) (19%), 241 (21/22 ion at left) (5%), 223 (241 - H_2O) (12%), 181 (241 - AcOH) (3%); ^{1}H-NMR (table 1).

TMS derivatization. About 1 mg of **1** was treated with 20 µl of N,O-bis-(trimethylsilyl)-acetamide(BSA)-pyridine (10:1) and heated at 70°C for 30 min for EIMS determination (figure 1) m/z: 465 (21/22 ion at right) (35%), 395 (17/18 ion at right) (100%), 367 (395 - 28) (16%), 341 (17/18 ion at left) (12%), 271 (21/22 ion at left) (38%).

Identification of known compounds. Bullatacin is a white powder; yield 0.00274% from the dried bark; MS data and co-TLC in three solvent systems: $CHCl_3$:MeOH (9:1), CH_2Cl_2:MeOH (19:1) and hexane:EtOAc:MeOH (4:5:1) showed to be identical with bullatacin isolated from *A. bullata* (10). ^{1}H-NMR and ^{13}C-NMR are identical to those reported (1, 10). Thus, *A. reticulata* is an alternative plant source for bullatacin should its antitumor and pesticidal effects become commercially utilized.

Methyl 16β,17-dihydro-(-)-kauran-19-oate had mp 160-162°C; $[\alpha]_D$ = -72.8 (c = 0.005 mg/ml, abs. EtOH), UV (EtOH) λ_{max} 203 nm (ε = 474.59); IR (neat) cm^{-1}: 3848-2000 (COOH), 2843.4 (CH), 1721.7 (CO); CIMS (isobutane) m/z: 351 (MH^+) (50%), 333 ($MH^+ - H_2O$) (100%), 315 (333 - H_2O); HR CIMS (isobutane) gave 351.2527 corresponding to the molecular formula $C_{21}H_{34}O_4$ (calc. 351.2535). The fully decoupled ^{13}C-NMR

spectrum, APT, and DEPT resulted in the assignment of the ^{13}C chemical shifts of methyl 16α,17-dihydro-(-)-19-oate which are comparable to those of methyl 16β,17-dihydroxy-(-)-kauran-19-oate as previously reported (6). (-)-Kau-16-en-19-oic acid was isolated as colorless crystals; MS data, co-TLC, ^1H-NMR, and ^{13}C-NMR were identical to those of (-)-kau-16-en-19-oic acid isolated from *A. bullata* (10). Liriodenine was identified by MS and co-TLC with a reference sample (10).

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12. After this paper was submitted for publication, Hisham et al (*Tetrahedron Letters*, **1990**, *31*, 4649-4652) reported a compound named uvariamicin-II, as one component of a ternary mixture; this compound when compared with **1** has the same carbon skeleton and relative stereochemistry around the chiral centers associated with the tetrahydrofuran ring and the adjacent hydroxyl bearing carbons. However, the ^{13}C-NMR chemical shifts reported for the two oxygenated carbons on the tetrahydrofuran ring for the uvariamicin mixture are 74.34 and 74.05 ppm. Reticulatacin (**1**), on the other hand, has identical chemical shifts (74.03 ppm) for these two carbons. This suggests that reticulatacin (**1**) and uvariamicin-II are different compounds. Furthermore, there was no bioactivity reported for the mixture of the uvariamicin compounds, and the bioactivities presented herein for pure reticulatacin (**1**) may help in differentiating these compounds in the future.
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