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BULLATALICIN, A NOVEL BIOACTIVE ACETOGENIN FROM *ANNONA BULLATA* (ANNONACEAE)

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ABSTRACT: Bullatalicin, a novel bioactive acetogenin having two nonadjacent tetrahydrofuran rings, has been isolated from the bark of *Annona bullata* (Annonaceae). Its structure has been elucidated from chemical and spectral data. This compound showed potent and selective cytotoxic activities for certain human tumor cell lines with ED_{50} values as low as 10^{-7} mcg/ml.

INTRODUCTION

Over the past few years, fractionations of extracts of plants in the Annonaceae, monitoring with cytotoxic, antitumor, pesticidal, and antimicrobial activities, has led to the isolation of several biologically potent linear acetogenins, with two adjacent tetrahydrofuran (THF) rings. These include uvaricin (1), desacetyluvaricin (2), rollinicin and isorollinicin (3), rollinone (4), 14-hydroxy-25-desoxy-rollinicin (5), cherimoline and dihydrocherimoline (6), asimicin (7), rolliniastatin I (8), rolliniastatin II (9), squamocin (10), annonin I, annonin VI, annonin VII (11), and 4-hydroxy-25-desoxyneorollinicin (12), as well as bioactive single THF ring acetogenins such as annonacin (13) and goniothalamicin (14).

In our previous studies of the bark of *A. bullata* Rich. (Annonaceae), a species native to Cuba, searching for natural antitumor compounds, we isolated two extremely potent adjacent bis-THF ring acetogenins, named bullatacin and bullatacinone, two less-active known compounds [liriodenine and (-)-kaur-16-en-19-oic acid] (15), and a selectively cytotoxic diterpene, 16α-hydroxy-(-)-kauranoic acid (16). The present paper deals with the isolation, structure determination, and bioactivities of another novel potent acetogenin, bullatalicin, which has two tetrahydrofuran rings separated by four carbons.

RESULTS AND DISCUSSION

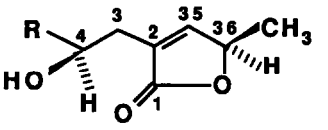
The EtOH extract of the bark was partitioned following a standard scheme to give the bioactive fraction FOO5 (15). FOO5 was chromatographed over Si gel using gradient elution. Fractions were combined into pools according to similar appearance after analytical tlc, and the pools were bioassayed for lethality to brine shrimp (BST) (17). The second most active pool, comprised of fractions 51-60 (BST LC_{50} = $2.58x10^{-2}$ ppm, 95% confidence interval $4x10^{-2}/2x10^{-2}$), was subjected to another Si gel column using gradient elution. Fractions 89-93 from this column yielded a white precipitate which was washed with EtOAc and recrystallized from MeOH to give a white powder (**1**).

Bullatalicin (**1**) is an amorphrous solid with m.p. 120-121°. The molecular weight of bullatalicin at 638 was obvious from cims (isobutane) m/z 639 (MH^+), 677 ($MC_3H_3^+$); cims (ammonia) m/z 639 (MH^+), 656 (MNH_4^+). Hr cims (isobutane) showed MH^+ 639.4828 corresponding to the molecular formular $C_{37}H_{66}O_8$ (cal. 639.4836). Sequential loss of four molecules of H_2O from the MH^+ in cims (methane) indicated the presence of four hydroxyl groups. A broad IR absorption at 3428 cm^{-1}, cims (isobutane) of a tetra-acetate derivative at m/z 807 (MH^+), and a tetra-TMS derivative at m/z 911 ($M\text{-}CH_3^+$) confirmed the presence of the four hydroxyl groups.

The ms and nmr spectral characteristics of **1** showed that it belongs to the familiar class of bioactive bis-THF acetogenins. The structure of a terminal α,β-unsaturated γ-lactone ring and one of the four hydroxy groups (fragment A) was readily recognized as a similar fragment found in bullatacin (1). Strong ir absorptions at 1748 and 1726 cm^{-1} (C=O) and a uv band at 212 nm (ε = 7270) substantiated the presence of the α,β-unsaturated lactone.

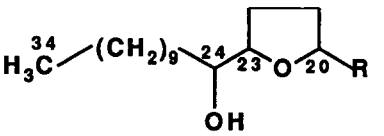
The ^{1}H-nmr signals (500 MHz, C_6D_6) (Table 1) at ppm: 6.240 (d, H35), 4.235 (qq, H36), 0.808 (d, H37) and ^{13}C-nmr signals (50 MHz, CD_3OD) at ppm: 176.40 (C1), 131.471 (C2), 154.310 (C35), 79.714 (C36) and 19.154 (C37) also showed the expected α,β-unsaturated γ-lactone. The ABB' system in the ^{1}H-nmr at ppm: 2.300 (dddd, H3a), 2.200 (ddt, H3b), 3.708 (m, H4) and ^{13}C-nmr chemical shifts at ppm: 33.315 (C3) and 70.370 (C4) established the presence of an hydroxyl at C4. In addition, a peak at m/z 144 in cims (isobutane) of **1**, a peak at m/z 183 in the cims (isobutane) of the **1** tetra-acetate derivative, and a peak at m/z 213 in the eims of the **1** TMS derivative (Figure 1) supported the structure of fragment A.

The similar ^{1}H-nmr and ^{13}C-nmr signals of fragment A with those signals of bullatacin suggested the same stereochemistry in this fragment which was previously determined as 4S and 36R (15). In addition, the cd spectrum of **1** gave a negative Cotton effect, the same as bullatacin, asimicin and rolliniastatin which also confirmed the above stereochemical assignments for fragment A.



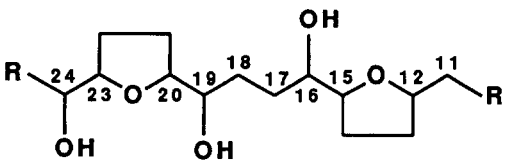
(A)

1 has one more hydroxyl group than bullatacin. Comparisions of ms data (Figure 1) of 1, 1 acetate, and the 1 TMS derivative with those of bullatacin, bullatacin acetate, and the bullatacin TMS derivative (15) [peaks at m/z 171 (24/23 left), 469 (24/23 right), 241 (20/19 left) in cims (isobutane) of 1, peaks at m/z 213 (24/23 left), 593 (24/23 right), 283 (20/19 left), 524 (20/19 right) in cims (methane) of 1 acetate, peaks at m/z 243 (24/23 left), 683 (24/23 right), 313 (20/19 left), and 613 (20/19 right) in eims of 1 TMS derivative] suggested the presence of fragment B.



(B)

The fact that the peak at m/z 397 (20/19 right) of cims (methane) of 1 was not evident but there were strong peaks at 379 (397 - H_2O, 24%), and 361 (379 - H_2O, 12%) suggested that there might be a hydroxyl group adjacent to this THF ring instead of the second adjacent THF ring as in bullatacin. If the second THF ring of bullatacin were to be open biosynthetically, because there is a fourth hydroxyl group in compound 1, the hydroxyl group at C15 might then form a second THF ring with the fourth hydroxyl group at C12. This would leave 1 with two nonadjacent THF rings as in fragment C.



(C)

To test this proposition, the cims, ^{1}H-nmr, and ^{13}C-nmr data were carefully examined. As expected, the cims (methane) peaks at m/z 339 (17/16 right), and 309 (16/15 right) for 1, the cims peaks at m/z 381 (17/16 right), 455 (16/15 left), and 351 (16/15 right) for 1 tetra-acetate, the eims peaks at 483 (17/16 right), 545 (16/15 left), and 381 (16/15 right) for the 1 TMS derivative were evident. In addition, the cims peak at m/z 329 (16/15 left) was not evident, but peaks for its loss of H_2O at m/z 311 (329 - H_2O, 9%) and 293 (329 - H_2O, 8%) supported the presence of the hydroxyl group

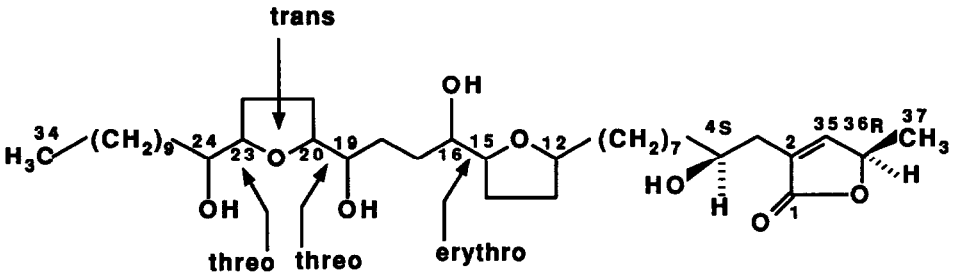
at C16. Exact mass measurements for the key ms fragments (Figure 1) confirmed the proposed cims assignments of fragment C.

^{1}H-Nmr showed that there are three protons attached to carbons with hydroxyl groups adjacent to THF rings. This also supported the presence of two non-adjacent THF rings because three such protons would not be possible with two adjacent THF rings. One of the protons on the THF oxygen-bearing carbons was shifted 0.25 ppm upfield, compared to that of bullatacin; this suggested the absence of an hydroxyl group on this side of the THF ring. One of the ^{13}C-nmr chemical shifts (80.919 ppm), for the four oxygen-bearing carbons on both THF rings (84.046, 83.908, 83.231, and 80.919 ppm) (Table 1), was found shifted about 3 ppm upfield compared with bullatacin. This further confirmed that there is no hydroxyl group attached to this side of the THF ring.

The determination of the relative stereochemistry of fragment C relies on ^{1}H-nmr and ^{13}C-nmr data for **1** and the ^{1}H-nmr data for **1** tetra-acetate. Comparisons were made for the ^{1}H-nmr hydroxyl methines of 16, 19, and 23 with those of annonastatin II which contains a single ring of the threo-trans-erythro configuration; the proton chemical shift for the erythro center in annonastatin II is at 3.82 ppm whereas in **1** it is at 3.64 ppm; this upfield shift indicates that the erythro stereocenters in **1** do not have an OH on the other side of the THF ring and therefore this erythro relationship must be at 15/16 (18). The chemical shifts for **1** at 3.437 and 3.381 ppm for H19 and H24 showed threo stereo relationships between 19/20 and 23/24. The same ^{13}C-nmr chemical shift for C19 and C24 in CD_3OD at 75.348 ppm confirmed the stereo relationships of 19/20 and 23/24 as threo. The upfield chemical shift of C16 at 73.981 ppm confirmed the stereo relationship of 15/16 as erythro (11). The ^{1}H-nmr signals of **1** tetra-acetate at 2.090 and 2.077 ppm for the 19 OAc and 24 OAc substantiated the stereo relationships of 19/20 and 23/24 as threo. The signal at 2.051 ppm for the 16 OAc further substantiated the stereo relationship of 15/16 as erythro. The ^{1}H-nmr signals of **1** tetra-acetate at 3.965 ppm for H19 and H23 indicated the trans relationship between positions 19 and 23 (19). The relative stereochemistry between positions 12 and 15 remains unknown.

The absolute value two dimentional homonuclear correlated spectrum (2D COSY, 500 MHz), and two dimentional heteronuclear shift correlation spectrum (2D HETCOR) confirmed the proton and carbon assignments in Table 1 and provided the proton-proton and proton-carbon connectivities.

From the above data, we concluded that the structure of bullatalicin is as illustrated in **1**. Concurrently gigantecin, a similar bioactive bistetrahydrofuran acetogenin, with a different placement of the nonadjacent THF rings and different stereochemistry on the THF rings, has been found in our laboratory from extracts of *Goniothalamus giganteus* Hook. f., Thomas (Annonaceae) (20).



(1)

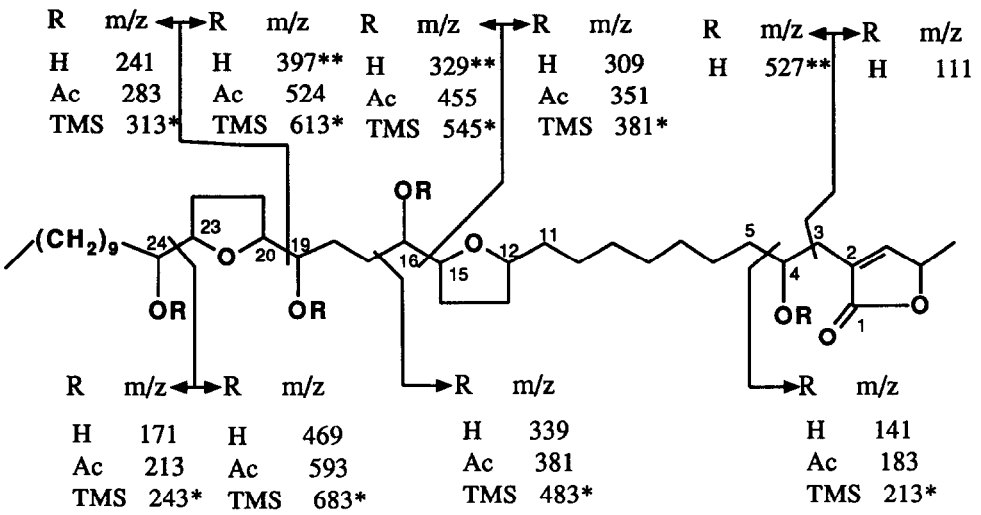


Figure 1. Ms data for bullatalicin (1). "R" designates: the underivatized material (H), the acetyl derivative (Ac), or the trimethylsilyl derivative (TMS). Exact mass measurements (within 3 mmu) confirmed the elemental compositions of the proposed fragments marked with *. Two asterisks ** indicates that peaks cannot been seen, but the corresponding peaks formed by consecutive loss of two molecules of water were evident.

Both bullatalicin and bullatalicin tetra-acetate showed potent bioactivities and selective cytotoxicities. Bullatalicin showed BST LC_{50} = 0.154 ppm (0.727/0.058), 63% inhibition of crown gall tumors on potato discs, 9KB ED_{50} > 10 mcg/ml, A549 ED_{50} = 2.34×10^{-7} mcg/ml, MCF-7 ED_{50} = 2.34 mcg/ml, HT-29 ED_{50} = 8.8×10^{-6} mcg/ml, 9ASK showed cytotoxicity with slight astrocyte reversal at 100 mcg/ml. Bullatalicin tetra-

acetate showed A459 $ED_{50} < 10^{-3}$ mcg/ml, MCF-7 $ED_{50} < 10^{-3}$ mcg/ml, and HT-29 ED_{50} = 1.35 mcg/ml. Interestingly, after peracetylation, the activity for MCF-7 (human breast carcinoma) increased at least 1000 fold; while the activity for HT-29 (human colon adenocarcinoma) decreased significantly. *In vivo* antitumor testing of these potent compounds is being pursued. In similar runs, adriamycin as a positive control gave A549 $ED_{50} = 1.56 \times 10^{-2}$ mcg/ml, MCF-7 $ED_{50} = 7.22 \times 10^{-2}$ mcg/ml, and HT-29 $ED_{50} = 3.95 \times 10^{-2}$ mcg/ml, showing non-selectivity.

EXPERIMENTAL

Plant Material. Bark of *A. bullata* Rich. (M-06983, PL-103509) was collected at the USDA Subtropical Horticulture Research Station, Miami, Florida. The material was authenticated by Edward Garvey of the USDA. The tree originated from seeds collected in Cuba in 1933 by Robert M. Grey of Harvard University. The dried bark was pulverized in a Wiley mill. CAUTION: Exposure to the dust caused facial edema in one of us (YHH), and two similar episodes during fractionation prompted extreme caution when handling the material and derived products.

Bioassays. The extracts, fractions, and isolated compounds were routinely evaluated for lethality to brine shimp larvae (BST) (17). Occasional checks were made in the PD assay (% inhibition of crown gall tumors on potato discs) (21). Cytotoxicity tests were made at the Purdue Cell Culture Laboratory, Purdue Cancer Center, using standard protocols for 9KB (human nasopharyngeal carcinoma), 9ASK (astrocytoma reversal), A459 (human lung carcinoma), MCF-7 (human breast carcinoma), and HT-29 (human colon adenocarcinoma).

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 Spectropolarimeter. Ir spectra were obtained in KBr on a Perkin-Elmer 1420. Uv spectra were taken on a Beckman DU-7. ^{1}H-Nmr and 2D COSY spectra were obtained on a Varian VXR-500S (500 MHz). ^{13}C-Nmr were obtained on a Chemagnetics A-200 (50 MHz). 2D HETCOR were taken on a Varian XL-200. Lrms were performed on a Finnigan 4000. Exact mass measurements were obtained on a Kratos MS50 through peak matching.

Isolation of the compound. The pulverized bark (3.9 kg) was extracted as previously described (15); the EtOH extract of the bark (FOO1) was partitioned between H_2O and $CHCl_3$ to give residues FOO2 and FOO3, respectively, with FOO4 representing the interface residue. FOO3 was partitioned between hexane and 10% H_2O in MeOH to give FOO6 and FOO5, respectively. Results of the bioassays of FOO1-FOO6 demonstrated that the activities had partitioned into FOO5 (BST $LC_{50} = 2.5 \times 10^{-3}$ mcg/ml, 95% confidence interval $5 \times 10^{-3}/1 \times 10^{-4}$, 9PS $ED_{50} < 10^{-2}$ mcg/ml, 9KB $ED_{50} < 10^{-5}$ mcg/ml). FOO5 (80 g) was adsorbed onto 100 g of Celite and applied to a column of Si gel (3 kg) packed in a slurry of hexane. A gradient of hexane-$CHCl_3$-MeOH was used to elute the column, collecting 82 fractions of 100-200 ml each. Fractions were combined into pools according to their similar tlc patterns, weighed, and bioassayed by the BST. The second most active pool (fractions 51-60) (15 g) (BST $LD_{50} = 2.58 \times 10^{-2}$ ppm, 0.04/0.02) was subjected to another Si gel chromatography column (230-400 mesh, 600 g), eluting by a gradient of $CHCl_3$-EtOAc-MeOH. Fractions 89-93 yielded a white residue which was washed by EtOAc and recrystallized from MeOH to give a white amorphous powder (150 mg) of **1**.

Characterization of **1**. Mp 120-121°. $[\alpha]_D$ = +13.25 (c = 0.004 g/ml, abs. EtOH); cims (isobutane) m/z 639 (MH^+), 677 ($MC_3H_3^+$); cims (ammonia) m/z 639 (MH^+), 656 (MNH_4^+) (Figure 1); hr cims (isobutane) MH^+ 639.4828 for $C_{37}H_{67}O_8$ (calc. 639.4836); ^{1}H-nmr (Table 1); ^{13}C-nmr (Table 1); uv (EtOH) λ_{max} 212 nm (ε = 7270); ir (KBr) cm^{-1}, 3430 (OH), 1748, 1726 (C=O). CD spectrum of **1** (c, 0.023 mg/ml, abs. EtOH): $[\theta]_{247.0}$, 0.0;

Table 1. Nmr data for bullatalicin (1) and its tetra-acetate.

	bullatalicin (1)			bullatalicin tetra-acetate	
	^{1}H-NMR 500 MHz, C_6D_6		^{13}C-NMR 50 MHz, CD_3OD	^{1}H-NMR 500 MHz, $CDCl_3$	
1	-		176.40	-	
2	-		131.471	-	
3a	2.300(dddd)	3a,3b(14.7) 3a,4(3.7) 3a,35(1.8) 3a,36(1.2)	33.315	2.568(dddd)	3a,3b(18.5) 3a,4(3.3) 3a,35(2.9) 3a,36(1.3)
3b	2.200(ddt)	3b,3a(14.7) 3b,4(8.2) 3b,35(1.2)		2.512(ddt)	3b,3a(18.5) 3b,4(7.8) 3b,35(1.3)
4	3.708(m)		70.370	5.101(dddd)	4,3a(3.3) 4,3b(7.8) 4,5a(5.1) 4,5b(8.1)
5	1.630, 1.390		38.293[a]	1.500-1.800	
6-10	1.29		31.024	1.29	
11	1.500, 1.680		33.078[b]	1.500-1.800	
12	3.800		80.919	3.856(m)	
13	1.500, 1.680		27.806[b]	1.200-2.000	
14	1.625, 1.375		27.233[b]	1.200-2.000	
15	3.708(m)		83.231	3.965(m)	
16	3.64		73.981	4.907(ddd)	(8.6, 4.2, 5.5)
17	1.773, 1.525		36.887[a]	1.500-1.800	
18	1.600(m)		34.502[a]	1.500-1.800	
19	3.437(t)	19,18(7.7)	75.348	4.822(m)	
20	3.800		84.046	3.965(m)	
21	1.500, 1.680		27.037[b]	1.200-2.000	
22	1.230, 1.680		26.733[b]	1.200-2.000	
23	3.708(m)		83.908	3.965(m)	
24	3.387(t)	24,25(7.3)	75.348	4.822(m)	
25	1.600(m)		33.938[a]	1.600(m)	
26-31	1.29		31.024	1.29	
32	1.29		29.306[b]	1.29	
33	1.29		23.744	1.29	
34	0.905(t)	34,33(7.1)	14.486	0.879(t)	34,33(7.2)
35	6.240(d)	35,36(1.3)	154.310	7.086(d)	35,36(1.6)
36	4.235(qq)	36,35(1.3) 36,37(6.7)	79.714	5.013(qq)	36,35(1.6) 36,37(6.9)
37	0.808(d)	37,36(6.8)	19.154	1.400(d)	37,36(6.9)
4OAc	-		-	2.027(s)	
16OAc	-		-	2.051(s)	
19OAc	-		-	2.090(s)	
24OAc	-		-	2.077(s)	

[a, b] Indicate that assignments of similar signals may be interchangable.

$[\theta]_{243.0}$, -0.37; $[\theta]_{235.2}$, 0.02; $[\theta]_{227.0}$, 2,78; $[\theta]_{218.0}$, 7.66; $[\theta]_{215.0}$, 9.84; $[\theta]_{213.3}$, 8.39; $[\theta]_{211.6}$, 7.83.

Acetylation. 6 mg of **1** was mixed with 0.5 ml of anhydrous pyridine and 1 ml of acetic anhydride at room temperature for 48 hrs. Ice water was added, and the mixture was partitioned with $CHCl_3$. The $CHCl_3$ layer was dried over anhydrous sodium carbonate and vacuum evaporated to give a white powder (single spot on tlc), cims (isobutane) m/z 807 (MH^+) (Figure 1); ^{1}H-nmr (Table 1).

TMS derivatization. Dry micro amount samples of **1** were treated with 20 mcl of N,O-bis-(trimethylsilyl)-acetamide (BSA)-pyridine (10:1) and heated at 70° for 30 min for ei and cims (isobutane) determinations (Figure 1). Exact mass measurements were

made for the key fragment elemental compositions: 243.2142 for $C_{14}H_{31}OSi$ (calc. 243.2144, 23/24 left), 683.4199 for $C_{35}H_{67}O_7Si_3$ (calc. 683.4195, 23/24 right), 313.2557 for $C_{18}H_{37}O_2Si$ (calc. 313.2563, 19/20 left), 613.3781 for $C_{31}H_{61}O_6Si_3$ (calc. 613.3776, 19/20 right), 483.3904 for $C_{24}H_{59}O_5Si_2$ (calc. 483.3901, 16/17 left), 545.3882 for $C_{28}H_{61}O_4Si_3$ (calc. 545.3878, 15/16 left), 381.2457 for $C_{21}H_{37}O_4Si$ (calc. 381.2461, 15/16 right), 213.0946 for $C_{10}H_{17}O_3Si$ (calc. 213.0947, 4/5 right).

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