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BULLATACIN AND BULLATACINONE: TWO HIGHLY POTENT BIOACTIVE ACETOGENINS FROM *ANNONA BULLATA*

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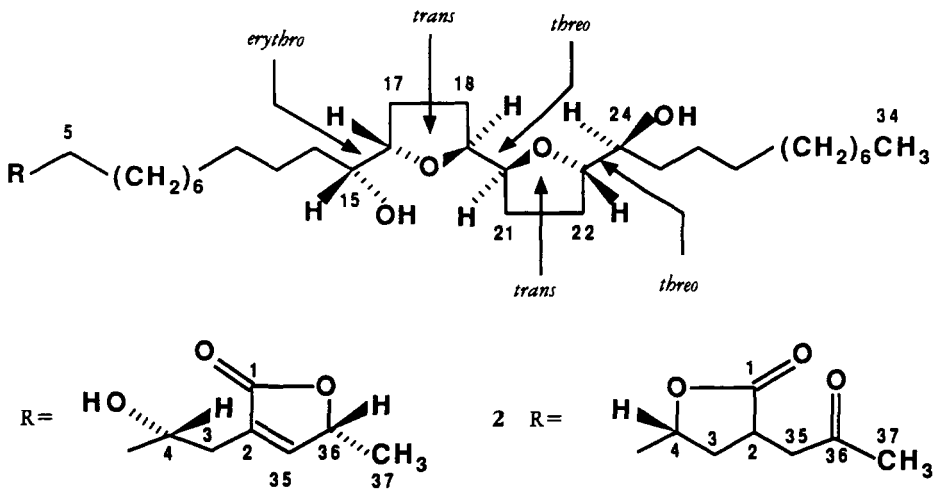
ABSTRACT.—Screening of crude extracts of the bark of *Annona bullata* showed cytotoxic and pesticidal activities. By monitoring with brine-shrimp lethality, two novel, extremely potent acetogenins, bullatacin [**1**] and bullatacinone [**2**], were isolated. Spectral and chemical methods identified bullatacin as a diastereomer of asimicin. Bullatacinone represents bullatacin with the lactone cleaved and reformed at the 4-OH. Compounds **1** and **2** show selective cytotoxicities in human tumor cell lines, and certain susceptible cells give ED_{50} values as low as 10^{-12}–10^{-15} μg/ml. Bullatacin was pesticidal at concentrations as low as 1 ppm, but bullatacinone lacked pesticidal activities. The known compounds liriodenine and (−)-kaur-16-en-19-oic acid were also isolated and were lethal to brine shrimp but were not significantly cytotoxic.

In recent years, our research has focused on the phytochemistry of the Annonaceae because of the cytotoxic, antitumor, pesticidal, and other bioactivities of the acetogenins that are present in certain members of this family (1–3). During the screening of plants in our laboratory, *Annona bullata* Rich. was found to have noteworthy activities in the BST (brine-shrimp lethality test), PD (crown-gall antitumor activity on potato discs), 9PS (murine leukemia cytotoxicity), 9KB (human nasopharyngeal carcinoma cytotoxicity), and 9ASK (astrocytoma reversal) bioassays; in addition, activities in several pesticidal tests showed promise. Even the crude EtOH extract of the bark gave significant results.

A. bullata is a tall tree native to Cuba. A new sesquiterpene, bullatantriol, has been recently isolated from the leaves (4), and the same research group has also reported the isolation of two known isoquinoline alkaloids, (*R*)-annonaine and liriodenine, from the leaves of trees collected near Havana, Cuba (5). These workers reported no indication of bioactivities attributable to these compounds. We obtained bark samples from the USDA Subtropical Horticulture Research Station in Miami, Florida (M-06983, PL-103519).

The residue of the EtOH extract (F001) of the bark was partitioned between H_2O and $CHCl_3$ to give residues F002 and F003, respectively, with F004 representing the interface residue. F003 was partitioned between hexane and 10% H_2O in MeOH to give F006 and F005, respectively. Results of the bioassays of F001–F006, summarized in Table 1, demonstrated that the activities had partitioned into F005.

F005 (BST LC_{50} 0.0025 μg/ml, 95% confidence interval 0.0050–0.0001) was chromatographed over Si gel using a gradient of hexane/$CHCl_3$/MeOH. Fractions were combined into pools according to similar appearance after analytical tlc, and the pools were bioassayed for lethality to brine shrimp (6). The most toxic pool, composed of fractions 40–51 (BST LC_{50} 0.0019 μg/ml, 0.0053–0.0002), was rechromatographed over Si gel using a gradient of $CHCl_3$/EtOAc/MeOH. Similar fractions were again formed into pools and assayed with the BST. The most toxic pool, from fractions 106–111 (BST LC_{50} 0.0015 μg/ml, 0.006–0.0003), formed fine white needles that were recrystallized from EtOAc to give bullatacin [**1**] (BST LC_{50} 0.00159 μg/ml, 0.0124–0.0008). Upon standing, pool 17–40 (BST LC_{50} 0.0046 μg/ml, 0.100–0.0001) formed a solid precipitate that was recrystallized from EtOAc to give bullatacinone [**2**]



(BST LC_{50} 0.003 μg/ml, 0.009–0.000). An additional amount of **2** was obtained by cc of pool 31–39 (BST LC_{50} 0.0089 μg/ml, 0.11–32.93) from the initial column.

Table 2 summarizes the biological activities of **1**, **2**, and their derivatives. Bullatacin [**1**] is extremely active in all these cell lines from 10^{-12} to 10^{-15} μg/ml; bullatacinone ranges from 5×10^{-12} μg/ml in human colon adenocarcinoma to 10^{-3} μg/ml in human lung carcinoma. This suggests that bullatacinone has more selective

TABLE 1. Bioactivities of Initial Fractions from *Annona bullata*.

Extracts	Bioactivity Assay							
	BST[a] LC_{50} μg/ml (95% Confidence Interval)	9PS[b] ED_{50}, μg/ml	9KB[c] ED_{50}, μg/ml	Insecticidal Activity[d] (% mortalities)				Protein Kinase C % Displacement (100 μg/ml)
				ML 1 ppm	2SSM 5000 ppm	MA 5000 ppm	BFL 1%	
F001	0.0062 (0.0120–0.0009)	$<10^{-2}$	20	100	0	0	100	11%
F002	>100	>10	>10	0	0	0	0	—[e]
F003	0.0030 (0.0036–0.0010)	$<10^{-2}$	10^{-1}–10^{-2}	100	90	90	100	67%
F004	5.400 (8.38–1.53)	2.11	>10	0	0	0	0	—
F005[f]	0.0025 (0.0050–0.0001)	$<10^{-2}$	$<10^{-5}$	100	80	80	100	68%
F006	>100	4.10	>10	0	70	80	0	—

[a]Brine shrimp lethality test.

[b]A chemically-induced murine lymphocytic leukemia.

[c]Human nasopharyngeal carcinoma.

[d]ML=mosquito larvae, 2SSM=two-spotted spider mite, MA=melon aphid, BFL=blowfly larvae; no activity was detected against corn root worm, southern army worm, and *Haemonchus contortus*.

[e]A dash (—) indicates that tests were not conducted.

[f]F005 showed slight activity in astrocyte reversal (9ASK) and 78% inhibition of crown gall tumors on potato discs (PD).

TABLE 2. Bioactivities of Compounds **1** and **2** and their Derivatives.[a]

Compound	Assay				
	BST LC_{50}, μg/ml (95% Confidence Interval)	9PS ED_{50}, μg/ml	9KB ED_{50}, μg/ml	A-549 ED_{50}, μg/ml	HT-29 ED_{50}, μg/ml
Bullatacin [**1**]	0.00159 (0.0124–0.0008)	10^{-15}–10^{-16}	6.188×10^{-14}	1.25×10^{-13}	10^{-12}
Bullatacin triacetate	5.7 (17.12–2.84)	3.89×10^{-3}	6.85×10^{-7}	2×10^{-3}	$>10^{-1}$
Dihydro-bullatacin	0.0145 (0.0300–0.0100)	—[b]	—	$<10^{-6}$	3.33×10^{-5}
Bullatacinone [**2**]	0.0030 (0.0090–0.0000)	4.23×10^{-3}	$<10^{-12}$	10^{-3}	5×10^{-12}
Bullatacinone diacetate	>10	4.22×10^{-2}	5×10^{-3}	2.8×10^{-2}	10^{-1}

[a]Abbreviations: BST=Brine shrimp lethality test, 9PS=chemically-induced murine lymphocytic leukemia, 9KB=human nasopharyngeal carcinoma, A-549=human lung carcinoma, HT-29=human colon adenocarcinoma.

[b]Tests were not conducted.

cytotoxicities than bullatacin. Only a 7% displacement in a protein kinase C test at a concentration of 10 μM proved that bullatacin is not a phorbol ligand. A negative result in the protein tyrosine kinase test excluded a possible mechanism of action involving protein tyrosine kinase. The mode of action for the acetogenins is still unknown, but it is postulated to involve cell membranes.

To test further the selective specificity of the cytotoxic activities, both compounds were sent to the NIH, NCI at Bethesda, Maryland, for human tumor cell tests in a panel including leukemia, non-small cell lung cancer, small cell lung cancer, colon cancer, breast cancer, CNS cancer, melanoma, ovarian cancer, and renal cancer. These cytotoxicity results for bullatacin [**1**] are shown in Table 3, where IC_{50} is the concentration of the compound that was found to cause 50% inhibition of the growth of each cell line listed under "Cell." IC_{90} is the concentration of compound that was found to cause 90% inhibition of the growth of each cell line. Bullatacin shows interesting selective actions with a concentration range of at least six orders of magnitude across these cell lines. It is best active for certain CNS cancers, non-small cell lung cancers, leukemias, and ovarian cancers. Results of testing of bullatacinone [**2**] have not been provided to us at this time.

The pesticidal activities of bullatacin [**1**] and bullatacinone [**2**] are shown in Table 4. The results indicate that bullatacin is significantly toxic to cotton aphids (CA) at 1 ppm, southern corn rootworm (CRW) at 24 ppm, and two-spotted spider mites (2SSM) at 10 ppm. The lack of pesticidal activity for bullatacinone indicates that it did not contribute to the pesticidal activities of the initial EtOH extract.

The mol wt of bullatacin [**1**] was determined by analyses of the eims and cims of **1** and its trimethylsilyl and acetyl derivatives. Isobutane and NH_3 cims of **1** gave promi-

TABLE 3. NCI In Vitro Testing Results on Compound **1**.

Disease	Cell line	IC_{50} (μg/ml)	IC_{90} (μg/ml)
Leukemia	HOLT-4	1.41×10^{0}	4.72×10^{0}
	HL-60 TB	$<9.26 \times 10^{-4}$	—
	K562	$<9.26 \times 10^{-4}$	4.5×10^{0}
	P388/ADR	$<9.26 \times 10^{-4}$	2.56×10^{0}
	CCRF-CEM	8.94×10^{-5}	6.94×10^{-2}
	P388	$>9.24 \times 10^{-2}$	$>9.24 \times 10^{-2}$
Non-Small-Cell Lung Cancer	H522	2.46×10^{0}	$>9.24 \times 10^{0}$
	H125	2.40×10^{0}	$>9.26 \times 10^{0}$
	H23	2.38×10^{0}	9.21×10^{0}
	H460	2.87×10^{-5}	1.85×10^{-4}
	H322	$<9.26 \times 10^{-4}$	$>9.26 \times 10^{0}$
	EKV-X	1.15×10^{-4}	$>9.26 \times 10^{-2}$
	HOP-62	$<9.26 \times 10^{-4}$	5.13×10^{0}
	SK-MES-1	$>9.24 \times 10^{-2}$	$>9.24 \times 10^{-2}$
	A-549 (ATCC)	5.04×10^{-4}	$>9.24 \times 10^{-2}$
Small Cell Lung Cancer	H82	1.99×10^{0}	7.14×10^{0}
	H524	2.69×10^{0}	7.54×10^{0}
	H69	2.44×10^{0}	7.52×10^{0}
	H146	6.75×10^{0}	$>9.26 \times 10^{0}$
Colon Cancer	SW620	5.64×10^{-3}	5.66×10^{0}
	LOVO	9.15×10^{-4}	4.69×10^{0}
	DLD-1	2.76×10^{0}	8.62×10^{0}
	HCC-2998	4.05×10^{0}	$>9.26 \times 10^{0}$
	HT29	3.84×10^{-3}	$>9.24 \times 10^{-2}$
Breast Cancer	MCF7	$>9.24 \times 10^{-2}$	$>9.24 \times 10^{-2}$
CNS Cancer	TE-671	2.56×10^{-5}	2.74×10^{-3}
	U251	$<9.24 \times 10^{-6}$	4.37×10^{-5}
	SNB-19	$>9.26 \times 10^{-1}$	$>9.26 \times 10^{-1}$
	SNB-44	4.42×10^{0}	$>9.26 \times 10^{0}$
	SNB-75	3.35×10^{0}	$>9.26 \times 10^{0}$
Melanoma	SK-MEL5	$<9.26 \times 10^{-4}$	5.22×10^{0}
	RPMI-7951	2.73×10^{0}	7.71×10^{0}
	MALME-3M	2.82×10^{0}	7.67×10^{0}
	LOX	$<9.26 \times 10^{-4}$	3.76×10^{0}
	SK-MEL2	3.25×10^{0}	8.64×10^{0}
Ovarian Cancer	A2780	3.77×10^{-5}	6.50×10^{-4}
	OVCAR-8	2.57×10^{0}	7.65×10^{0}
	OVCAR-5	8.80×10^{0}	$>9.26 \times 10^{0}$
	OVCAR-4	$>9.24 \times 10^{-2}$	$>9.24 \times 10^{-2}$
	OVCAR-3	$>9.24 \times 10^{-2}$	$>9.24 \times 10^{-2}$
Renal Cancer	A498	4.24×10^{-5}	3.71×10^{-3}
	A704	1.36×10^{0}	8.00×10^{0}
	SN12-K1	2.24×10^{-5}	2.87×10^{-4}
	UO-31	1.90×10^{0}	$>9.26 \times 10^{0}$
	CAKI-1	3.94×10^{-3}	$>9.24 \times 10^{-2}$

nent peaks at *m/z* 623 and 640, which suggested a molecular weight of 622. The isobutane cims of the acetyl derivative showed a prominent peak at *m/z* 749, indicating that **1** has three exchangeable protons and a mol wt of 622, in agreement with the results for the underivatized material. The eims of the trimethylsilyl derivative of **1** showed peaks at *m/z* 823 and 838, which corresponded to the $[M - Me]^+$ and $[M]^+$ of a trimethylsilyl derivative of **1**. From these results, it was concluded that **1** has a mol wt of 622 and that it has three exchangeable protons. The ir spectrum exhibited a prominent absorption at 3430 cm^{-1}, consistent with the presence of hydroxyl groups. The high resolution cims gave an $[MH]^+$ at *m/z* 623.4847 corresponding to the molecular

TABLE 4. Pesticidal activities of Compounds **1** and **2**.[a]

Compound	ppm	Pest/percent control				
		CA	ML	SAW	CRW	2SSM
Bullatacin [**1**]	0.5	—[b]	0	—	—	—
	1	80	0	—	—	—
	6	—	—	—	20	—
	10	80	80	0	—	20
	24	—	—	—	80	—
	100	80	—	0	—	30
	400	90	—	0	—	20
Bullatacinone [**2**] . . .	0.5	—	0	—	—	—
	1	—	0	—	—	—
	6	—	—	—	0	—
	10	0	0	0	—	0
	24	—	—	—	0	—
	100	0	—	0	—	0
	400	0	—	0	—	0

[a]Abbreviations: CA=cotton aphid, ML=mosquito larvae, SAW=southern army worm, CRW=corn root worm, 2SSM=two-spotted spider mite.

[b]A dash (—) indicates that tests were not conducted.

formula $C_{37}H_{66}O_7$ (calcd 623.4887). The only other molecular composition consistent with this mass, $C_{44}H_{63}O_2$, was discounted because it is inconsistent with the presence of three hydroxyl groups.

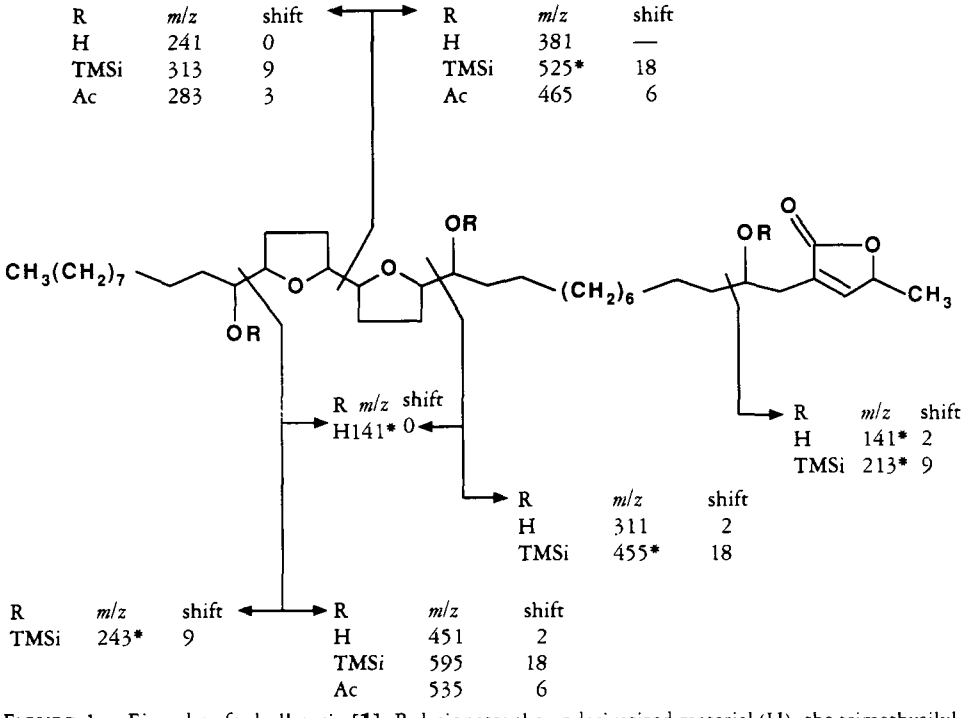


FIGURE 1. Eims data for bullatacin [**1**]. R designates the underivatized material (H), the trimethysilyl derivative (TMSi), or the acetyl derivative (Ac). "Shift" indicates the change in mass observed for hydrogenation of the lactone in the underivatized material, for the d_9 TMSi derivative, or for the d_3 acetyl derivative. The elemental compositions of fragments marked with an asterisk were confirmed through exact mass determination.

TABLE 5. ^{1}H-nmr Assignments and Comparisons of Compounds **1** and **2** with known Acetogenins.[a]

Proton	Bullatacin [**1**] (470 MHz, $CDCl_3$)		Bullatacin [**1**] (470 MHz, C_6D_6)		Bullatacinone [**2**] (470 MHz, C_6D_6)	
2	—		—		2.71 dddd	2,3a (9.34) 2,3b (9.34) 2,35b (3.42) 2,35a (9.34)
3a	2.50 dddd	3a,3b (15.0) 3a,4 (4.0) 3a,35 (1.5) 3a,36 (1.1)	2.30 dddd	3a,3b (15.0) 3a,4 (4.0) 3a,35 (1.5) 3a,36 (1.1)	1.70 ddd	3a,3b (12.82) 3a,4 (3.38) 3a,2 (9.34)
3b	2.36 ddt	3b,3a (15.0) 3b,4 (8.0) 3b,35 (1.4)	2.20 ddt	3b,3a (15.0) 3b,4 (8.0) 3b,35 (1.4)	1.40 ddd	
4	3.80 m		3.71 br tt		4.05 m	
5	1.3–2.0		1.3–2.1		1.3–2.0	
6–13	1.25 br s		1.25 br s		1.25 br s	
14	1.35 m		1.3–2.1		1.40, 1.55	
15	3.38 tt	15,16 (8.0) 15,14 (2.0)	3.45 ttt	15,16 (8.0) 15,14 (2.0) 15,17 (1.0)	3.49 tt	15,16 (8.06) 15,14 (2.38)
16	3.83 m		3.85 ddd	16,17a (8.0) 16,17b (6.9)	3.85 ddd	16,17a (8.06) 16,17b (6.96)
17,18	1.3–2.0		1.3–2.1		1.65–1.35	
19[b]	3.92 m		3.89 m		3.89 m	
20[b]	3.83 m		3.67 ddd	20,21a (6.0) 20,21b (1.0) 20,19 (15.0)	3.66 ddd	20,21a (5.96) 20,21b (1.00) 20,19 (14.93)
21,22	1.3–2.0		1.3–2.1		1.52, 1.42	
23[c]	3.92 m		3.99 dt	23,24 (8.5) 23,22 (2.5)	3.95 dd	23,24 (8.4) 23,22 (2.5)
24[c]	3.83 m		3.89 m		3.89 m	
25	1.3–2.0		1.3–2.1		2.10, 1.60	
26–33	1.25 br s		1.25 br s		1.25 br s	
34	0.85 t	34,33 (6.81)	0.9 t	34,33 (6.81)	0.90 t	34,3 (7.05)
35a	7.17 d	35,36 (1.70)	6.25 d	35,36 (1.70)	1.93 dd	35a,35b (18.31) 35a,2 (9.34)
35b					2.53 dd	35b,2 (3.42)
36	5.05 ddq	36,37 (6.83)	4.23 ddq	36,37 (6.83)	—	
37	1.41 d		0.80 d		1.55 s	

These spectral characteristics of **1** indicated that it belongs to the familiar class of bioactive bistetrahydrofuran acetogenins, which include uvaricin (7), desacetyluvaricin (8), rollinicin (9), isorollinicin (9), rollinone (10), asimicin (1,2), cherimoline (11), dihydrocherimoline (11), rolliniastatin (12), and 14-hydroxy-25-desoxyrollinicin (13). These bis-ring compounds characteristically contain 37 carbons and two long hydrocarbon chains, one of which terminates with a γ-lactone. Recently, compounds have been found bearing single tetrahydrofuran rings and containing only 35 contiguous carbons (3,14). Hydroxyl, acetoxyl, and/or carbonyl groups are usually found along the hydrocarbon chains. A positive response to Kedde's reagent (15) suggested the presence of an α,β-unsaturated γ-lactone. A strong ir absorption at 1750 cm^{-1} and a uv absorption maximum at 215.5 nm (ϵ=7974) in EtOH supported the presence of this functionality.

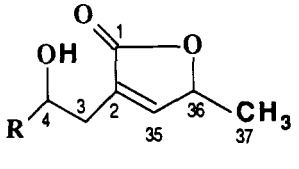
The structure of fragment **A**, which contains the lactone ring and one of the three

TABLE 5. Continued

Proton	Rolliniastatin (12)[d] (300 MHz, $CDCl_3$)	Asimicin (1)[e] (470 MHz, $CDCl_3$)	Asimicin (1)[e] (470 MHz, C_6D_6)
2	—	—	—
3a	2.50 dddd 3a,3b (15.1) 3a,4 (3.5) 3a,35 (1.5) 3a,36 (1.6)	2.51 dddd 3a,3b (15) 3a,4 (3.5) 3a,35 (1.5) 3a,36 (1.7)	2.35
3b	2.36 dddd 3b,4 (8.1) 3b,35 (1.2) 3b,36 (1.5)	2.38 ddt 3b,4 (8.0) 3b,35 (1.5) 3b,36 (1.5)	2.27
4	3.85 m	3.86 m	3.77
5	1.45	1.55 m	1.4–1.5
6–13	1.25	1.25 br s	1.35
14	1.50 m	1.55 m	1.55
15	3.38 m	3.37 br q	3.45
16	3.85 m	3.79–3.85 m	3.86
17,18	1.7–1.9 m	1.6–2.0 m	1.4–1.8
19,20	3.85 m	3.79–3.89 m	3.73
21,22	1.7–1.9 m	1.6–2.0 m	1.4–1.8
23,24	3.85 m	3.79–3.89 m (23) 3.37 br q (24)	3.86 (23) 3.45 (24)
25	1.45 m	1.55 m	1.55
26–33	1.25 br s	1.25 br s	1.35
34	1.85 t	0.86 t	0.90
35	7.16 ddd	7.17 q	6.35
36	5.02 dddq	5.06 qq	4.3
37	1.40 d	1.41 d	0.86

[a]Some coupling constants were obtained by decoupling experiments.
[b,c]Assignments may be interchangable.
[d]Data from Pettit *et al.* (12).
[e]Data from Rupprecht *et al.* (1).

hydroxyl groups, was elucidated by high field ^{1}H-nmr (Table 5), ^{13}C-nmr (Table 6), and ms (Figure 1) spectral analyses. Selective ^{1}H-^{1}H decoupling in the 470 MHz (C_6D_6) spectrum permitted the assignment of ^{1}H signals at δ 6.25 (d, C-35), 4.23 (ddq, C-36), 0.80 (d, C-37), which in addition to ^{13}C-nmr signals at δ 174.51 (s, C-1), 151.7 (d, C-35), 131.11 (s, C-2), 77.88 (d, C-36), and 19.06 (q, C-37) indicated the expected α,β-unsaturated γ-lactone. The ABB′ system in the ^{1}H-nmr and ^{1}H-^{1}H de-



A

coupling experiments showed coupling between the protons on C-3 at δ 2.20 (ddt, H-3b) and 2.30 (dddd, H-3a) to a single proton at C-4 (br tt, δ 3.71), establishing the presence of an hydroxyl at C-4 as was first recognized in asimicin (1). In addition, there is a peak in the cims at *m/z* 141, exact mass 141.0550 (calcd 141.0552); this peak corresponded to the fragment $C_7H_9O_3$, representing cleavage between C-4 and C-5, and helped to confirm the structure of fragment **A**. Also, the eims of the trimethylsilyl derivative of **1** had a prominent peak at *m/z* 213.0950, which is consistent with fragment

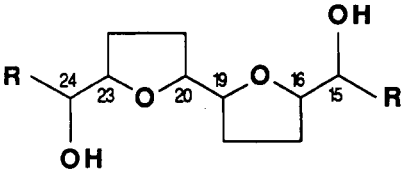
TABLE 6. ^{13}C-nmr ($CDCl_3$) Assignments and Comparisons.

Carbon	Bullatacin (50 MHz) [1]	Bullatacinone (50 MHz) [2]	Rolliniastatin[b]	Asimicin[c]
C-1	174.51 s	178.73 s	174.5 s	174.6 s
C-2	131.11 s	44.18 d	131.1 s	131.1 s
C-3[a]	33.23 t	34.41 t	33.2 t	33.4 t
C-4	69.91 d	78.86 d	69.9 d	69.9 d
C-5[a]	37.34 t	36.67 t	37.4 t	37.5 t
C-6[a]	25.99 t	25.99 t	26.0 t	25.6 t
C-7–12[a]	29.92 t	29.56 t	29.5 t	29.6 t
	29.28 t	29.37 t	29.3 t	29.3 t
C-13[a]	25.54 t	25.22 t	25.7 t	25.6 t
C-14[a]	33.23 t	33.19 t	34.1 t	33.6 t
C-15	74.08 d	74.10 d	74.0 d	74.1 d
C-16	83.20 d	83.26 d	83.0 d	83.1 d
C-17[a]	28.91 t	28.92 t	28.7 t	28.4 t
C-18[a]	28.37 t	28.37 t	27.8 t	28.4 t
C-19	82.44 d	82.44 d	81.1 d	81.8 d
C-20	82.17 d	82.19 d	81.0 d	81.8 d
C-21[a]	28.91 t	28.92 t	27.8 t	28.4 t
C-22[a]	28.37 t	28.37 t	28.4 t	28.4 t
C-23	82.75 d	82.77 d	83.0 d	83.1 d
C-24	71.34 d	71.26 d	71.8 d	74.1 d
C-25[a]	32.38 t	32.37 t	32.8 t	33.4 t
C-26[a]	25.54 t	25.59 t	25.5 t	25.6 t
C-27–31[a]	29.49 t	29.56 t	29.6 t	29.6 t
	29.28 t	29.25 t	31.9 t	29.3 t
C-32[a]	31.83 t	31.85 t	31.9 t	31.9 t
C-33	22.62 t	22.63 t	22.6 t	22.7 t
C-34	14.10 q	14.05 q	14.1 q	14.1 q
C-35	151.70 d	35.42 t	151.7 d	151.6 d
C-36	77.88 d	205.44 s	77.9 d	77.9 d
C-37	19.06 q	24.46 q	19.1 q	19.1 q

[a]Assignments of similar signals may be interchanged.
[b]Data from Pettit *et al.* (12).
[c]Data from Rupprecht *et al.* (1).

A having added one trimethylsilyl group. Dihydrobullatacin was then prepared by catalytic reduction of **1**, and its ms (Figure 1) and ^{1}H-nmr (see Experimental) spectral data also supported the structure of fragment **A**.

The structure of fragment **B**, which contains the two tetrahydrofuran rings and the remaining two hydroxyl groups, was elucidated by essentially the same techniques. The ^{13}C-nmr (50 MHz) spectrum shows four resonances at δ 83.20 (d, C-16), 82.75 (d, C-23), 82.44 (d, C-19), and 82.17 (d, C-20) due to oxygen-bearing carbons, as well as resonances at 74.08 (d, C-15), 71.34 (d, C-24), and 69.91 (d, C-4) corresponding to three hydroxylated carbons. These ^{13}C-nmr resonances and their corresponding ^{1}H-



B

nmr resonances were directly analogous to similar signals of asimicin (1), uvaricin (7), and rolliniastatin (12), indicating the common presence of a bistetrahydrofuran moiety as illustrated in fragment **B**. Exact mass measurement of a second peak at *m/z* 141 (141.0909) corresponded to fragment $C_8H_{13}O_2$ (calcd 141.0916). It is proposed that this peak represents cleavage between C-15 and C-16 and between C-23 and C-24. This assignment, which is substantiated by the eims of the trimethylsilyl and acetyl derivatives of **1** (Figure 1), confirmed that there is no substituent on the bistetrahydrofuran ring system. The placement of the remaining two hydroxyls was based on ^{1}H-^{1}H decoupling experiments. Decoupling at δ 3.45, 3.67, 3.85, 3.89, and 3.99 linked the two-proton signal at 3.89 (C-24 and C-19) and the one-proton signals at 3.67 (C-20) and 3.99 (C-23) as well as 3.85 (C-16) and 3.45 (C-15). The subsequent downfield shift of the two signals for C-15 and C-24 to δ 4.88 and 4.93, respectively, in the ^{1}H nmr of the triacetate derivative of **1** (Table 7) confirmed these assignments.

Subtracting fragments **A** and **B**, the remainder, $C_{20}H_{41}$, of the structure of bullatacin [**1**] belongs to the unsubstituted alkyl chain, typical of the acetogenins. This was corroborated by multiple CH_2 resonances between δ 1.2 and 1.5 in the ^{1}H nmr (C_6D_6) and δ 20–40 in the ^{13}C nmr (Tables 5 and 6). Having determined the structures of the two major functional units of bullatacin [**1**] as fragments **A** and **B**, it was then necessary to place these along the hydrocarbon chain. This was accomplished through analysis of the ms fragmentation patterns of **1** and its trimethylsilyl, acetate, and hydrogenation derivatives. For example, fragments corresponding to cleavage of the C-19–C-20 bond between the two tetrahydrofuran rings were evident in these spectra as illustrated in Figure 1. These assignments were substantiated by noting predictable shifts in masses as the double bond in the lactone was hydrogenated or when deuterated derivatives were prepared. The elemental compositions of ions marked with an asterisk in Figure 1 were deduced from their exact mass measurements. This approach has been used previously to determine the structures of similar acetogenins (3,14). Thus, the pattern of contiguous atoms in the structure of **1** was established. The absolute value two dimensional homonuclear correlated spectrum (2D COSY, 200 MHz) of bullatacin [**1**] confirmed the proton assignments (Table 5) and the proton-proton connectivities except for those of the long aliphatic -CH_2- chain.

Surprisingly, this carbon skeleton of bullatacin [**1**] is the same as that of asimicin (1) and rolliniastatin (12). However, the mp, co-tlc, ^{1}H nmr, and, most importantly, the bioactivities are different. The compounds are stereoisomers at one or more of their eight chiral centers. Because **1** yielded crystals that were not suitable for X-ray diffraction studies and because the acetogenins are difficult to convert into crystalline derivatives suitable for X-ray analysis (12), other methods were used to predict the stereochemistry.

First, the relative configuration of six of the eight chiral centers, those on the bistetrahydrofuran ring system and its two adjacent hydroxyl bearing carbons, was obtained by comparing the ^{1}H nmr ($CDCl_3$) spectral signals of bullatacin acetate with those of a recently published series of synthetic dibutylated diacetate bistetrahydrofuran models and uvaricin and uvaricin acetate; stereochemical information could then be extracted from "iterative and synergistic" analysis of very small differences in the high-field proton chemical shifts (16,17).

The acetate of bullatacin was prepared and analyzed by ^{1}H nmr at 470 MHz ($CDCl_3$) (Table 7). The signals were assigned by comparison with **1** and asimicin (1). The chemical shifts of **1**-acetate were then compared with those published for the model compounds, uvaricin and uvaricin acetate (16). Suprisingly, the signals at δ 4.93 (1H, C-24), 4.88 (1H, C-15), 4.0 (2H, m, C-16 and C-23), 3.90 (2H, C-19 and C-20), and the acetate methyl signals at δ 2.09 (s, 3H) and 2.06 (s, 3H) showed exactly the same

TABLE 7. ^{1}H-nmr Assignments and Comparison of Acetate Derivatives.

Proton	Bullatacin triacetate (470 MHz, $CDCl_3$)		Bullatacinone diacetate (470 MHz, $CDCl_3$)		Asimicin triacetate[a] (300 MHz, $CDCl_3$)	Uvaricin acetate[b] (300 MHz, $CDCl_3$)
H-2	—		3.02 ddd	2,3a (12.82) 2,3b (9.34) 2,35a (9.34) 2,35b (3.42)	—	—
H-3a	2.52 dddd		1.74 m		2.53	2.62 ddd
H-3b	2.58 ddt		2.00 m		2.53	
H-4	5.11 dddd	4,3a (4.0) 4,3b (8.0) 4,5a (10.1) 4,5b (1.8)	4.53 dddd		5.09	1.25 m
H-5	1.5–2.0		1.5–2.0		1.5–1.9	1.25 m
H-6–H-13	1.25 br s		1.25 br s		1.25	1.25 m
H-14	1.5–2.0		1.5–2.0		1.5–1.9	1.75–1.9
H-15	4.88 dt	15,16 (8.0) 15,14 (2.0)	4.80 dt	15,16 (8.0) 15,14 (2.0)	4.85	4.86 ddd
H-16	4.00 ddd		4.00 ddd		3.98	3.98 ddd
H-17, 18	1.5–2.0		1.5–2.0		1.5–1.9	1.75–1.9
H-19,20	3.9 br t		3.9 br t		3.9	3.89 br t
H-21,22	1.5–2.0		1.5–2.0		1.5–1.9	1.75–1.9
H-23	4.00 ddd		4.00 ddd		2.98	3.98 ddd
H-24	4.93 ddd	24,25a (3.0) 24,25b (2.8)	4.93 ddd	24,25a (3.0) 24,25b (2.8)	3.83	4.92 ddd
H-25	1.5–2.0		1.5–2.0		1.5–1.9	1.75–1.9
H-26–H-33	1.28 br s		1.28 br s		1.25	1.25 m
H-34	0.89 t	34,33 (7.05)	0.89 t	34,33 (7.05)	0.87	0.878 t
H-35	7.09 d	35,36 (1.29)	2.66 dd	35a,35b (18.3) 35a,2 (9.34)	7.06	6.98 qq
			3.04 dd	35b,2 (3.42)		
H-36	5.02 qq		—		5.01	4.99 dtq
H-37	1.41 d	36,37 (7.05)	2.20 s		1.42	1.40 d
4-OAc	2.04 s		—		2.01	—
15-OAc	2.06 s		2.05 s		2.06	2.074 s
24-OAc	2.09 s		2.08 s		2.06	2.046 s

[a]Data from Rupprecht *et al.* (1).
[b]Data from Hoye and Suhadolnik (16).

shifts as uvaricin acetate (16). This suggested that the configuration of fragment **B** is *threo, trans, threo, trans, erythro,* the same as in uvaricin, or *erythro, trans, threo, trans,* and *threo.* However, differences in the ^{1}H nmr ($CDCl_3$, 200 MHz) of bullatacin compared with that of uvaricin indicate the probable configuration of bullatacin to be *erythro, trans, threo, trans, threo* as illustrated for **1**. Similarly, we have determined that asimicin is *threo, trans, threo, trans,* and *threo.* From X-ray data rolliniastatin is reported to be *threo, cis, threo, cis,* and *erythro* (12).

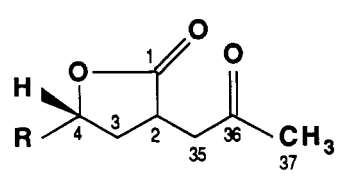
Bullatacin [**1**] has one more hydroxyl group than uvaricin (7); this hydroxyl is at C-4 and adds a new chiral center. The eighth and final chiral center is at C-36. To propose the stereochemistry at C-4 and C-36, we compared nmr spectral data with those in the literature for rolliniastatin (12). The ^{1}H nmr and the ^{13}C nmr of **1** (Tables 5 and 6) for the signals for fragment **A** were essentially the same as reported for rolliniastatin (12). This suggested that the stereochemistry of fragment **A** is the same as in rolliniastatin in which the configuration has been determined by X-ray as 4(*S*) and 36(*R*). Furthermore, essentially identical cd curves for rolliniastatin, asimicin, and bullatacin suggest their stereochemical identity in this region. From the above data, we conclude that the structure of bullatacin is as illustrated in **1**.

Bullatacinone [**2**] gave negative results with Kedde's reagent (15) indicating that there is no α,β-unsaturated γ-lactone ring. Weak uv end absorption at a λ maximum of 203.5 nm ($\epsilon = 3799$) showed a lack of the conjugated unsaturation usually found in these acetogenins.

Prominent peaks in the NH_3 (*m/z* 623 and 640) and isobutane (*m/z* 623 and 661) cims of bullatacinone [**2**] indicated that it has a mol wt of 622. High resolution cims gave *m/z* 623.4839, corresponding to a molecular formula of $C_{37}H_{66}O_7$ (calcd 623.4889), which is identical to bullatacin [**1**].

The 470 MHz ^{1}H nmr (Table 5) of bullatacinone [**2**] in C_6D_6 looked quite similar to that of **1** with an obvious presence of two tetrahydrofuran rings with adjacent hydroxyls as in fragment **B** of **1**. To determine the stereochemistry of fragment **B** of **2**, the diacetate of **2** was made, and the ^{1}H nmr (470 MHz) ($CDCl_3$) spectrum was compared with that of **1**-acetate (Table 7). The comparison showed that the bistetrahydrofuran ring of **2**, together with the two adjacent hydroxyl groups at C-15 and C-24, have exactly the same configuration as that of **1**, i.e., *erythro, trans, threo, trans,* and *threo.* The ^{13}C nmr of **2** (Table 6) showed two carbonyl carbons and an absence of the vinyl carbons seen in **1**.

The ei and cims of bullatacinone [**2**] and the **2**-trimethylsilyl derivative and exact mass measurements of selected fragments (Figure 2) showed that the alkyl chain was the same length as in **1**. The observation that the **2**-trimethylsilyl derivative in cims did not show the *m/z* 213 typical of the **A** fragment of **1** suggested the lack of a 4-OH. The ir spectrum of **2** at 1770 and 1715 cm^{-1} suggested two carbonyl groups, of which one corresponds to a lactone ring. In the ^{1}H nmr (470 MHz), a characteristic signal at δ 2.20 (s, 3H) in $CDCl_3$ suggested a terminal methyl ketone. It seemed likely that this compound has the lactone formed with the 4-OH, leaving a carbonyl at C-36 as shown in fragment **C**. To test this proposition, **1** was converted into **2** by treating with base to



C

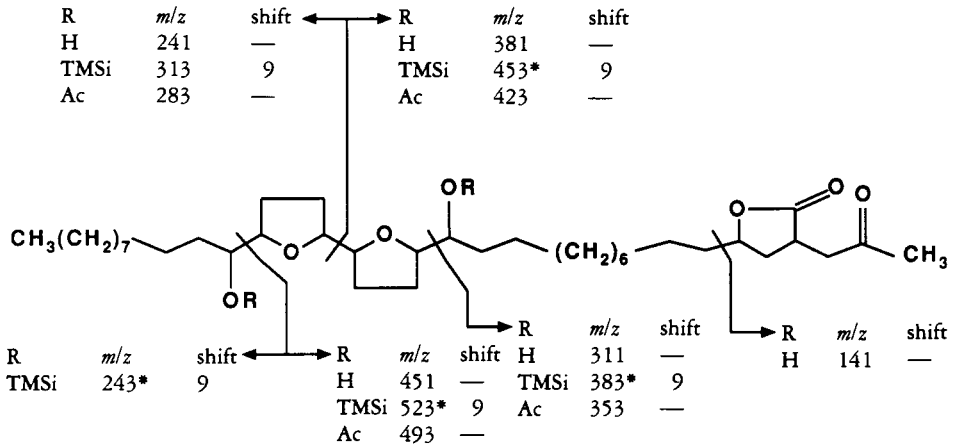


FIGURE 2. Eims data for bullatacinone [**2**]. R designates the underivatized material (H), the trimethylsilyl derivative (TMSi), or the acetyl derivative (Ac). "Shift" indicates the change in mass observed for the d_9 TMSi derivative. The elemental compositions of fragments marked with an asterisk were confirmed through exact mass determination.

hydrolyze the lactone and to recyclize upon acidification. The reaction product showed two major components (tlc), and one was identical to **2** (four tlc systems). Resolution on a microcolumn of Si gel gave **2** which was identical (co-tlc, cims and ^{1}H nmr) with **2** isolated from the plant material.

To determine the absolute stereochemistry of C-4, the cd curves of bullatacinone were compared with those of rubrenolide and rubrynolide (18), two 2,4-disubstituted-γ-butyrolactones as shown in Figure 3.

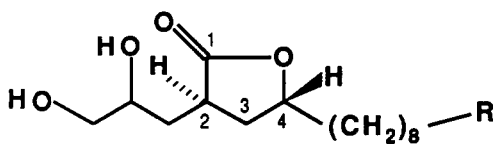


FIGURE 3. Structure of rubrenolide (R = −CH=CH$_2$) and rubrynolide (R = −C ≡ CH).

Compound **2** showed a negative Cotton effect whereas rubrenolide and rubrynolide gave positive Cotton effects. Application of the modified Hudson lactone rule (19) enabled the assignment of the *S* configuration to the chiral center at C-4. For the stereochemistry at position 2, the ^{13}C nmr showed three carbons (C-1, C-2, and C-4) as doublets. This suggested that bullatacinone is a mixture of two stereoisomers at C-2. ^{1}H nmr confirmed this proposal. From the ^{13}C nmr, the ratio of the two isomers at C-2 was estimated to be 2:1. The phase-sensitive 2D COSY spectrum (500 MHz) of bullatacinone confirmed the ^{1}H assignments and provided the ^{1}H-^{1}H connectivities in Table 5. From the above data, we conclude that bullatacinone is as illustrated in structure **2** and partially racemized at C-2.

Liriodenine (5) and (−)-kaur-16-en-19-oic acid (20) were also isolated. The former is active in the BST (LC_{50} 1.1 μg/ml, 0.87/0.01) but is inactive in the 9PS and 9KB cytotoxicity tests. The latter, which is a diterpene new to this species but known in several other Annonaceae (20), was active in the BST (LC_{50} 2.03 μg/ml, 60/1), slightly active in 9PS (ED_{50} 21 μg/ml) but inactive (ED_{50} > 10 μg/ml) in 9KB, A-549, and HT-29, with some cytotoxicity but no astrocyte reversal in 9ASK.

EXPERIMENTAL

PLANT MATERIAL.—Bark of *A. bullata* (M-06983, PL-103509) was collected at the USDA Subtropical Horticulture Research Station, ARS, 13601 Old Cutler Rd., Miami, Florida 33158. 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. Air-dried bark was pulverized through a 2-mm screen in a Wiley mill; 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 the derived products.

INSTRUMENTATION.—Mp determinations were made on a Mettler FP5 and are uncorrected. Optical rotation determinations were made on a Perkin-Elmer 241 polarimeter. Ir spectra were obtained in KBr on a Beckman IR-33 or a Perkin-Elmer 1420. Uv spectra were taken on a Beckman DU-7. Cd curves were obtained using a Cary Model 60 recording spectrophotometer with the Cary Model 6002 circular dichroism accessory. ^{1}H-nmr spectra were recorded on a Chemagnetics A-200 (200 MHz) and a Nicolet NTC-470 (470 MHz). ^{13}C-nmr spectra were obtained on a Chemagnetics A-200 (50 MHz). 2D-COSY was obtained on a Varian XL-200 and Varian VXR-500S. Eims and cims (isobutane and NH_3) were performed on a Finnigan 4000. Exact mass measurements were determined on a Kratos MS50 through peak matching. For microacetylations for ms, dry samples were treated overnight with 26 μl of plain or deutero Ac_2O-pyridine (10:3). For preparation of micro-trimethylsilyl derivatives for ms, dry samples were treated with 20 μl of plain or deutero *N,O*-bis-(trimethylsilyl)-acetamide (BSA)-pyridine (10:1) and heated at 70° for 30 min.

BIOASSAYS.—The extracts, fractions, and isolated compounds were routinely evaluated for lethality to brine shrimp larvae (BST) (6). Occasional checks in the potato disc (PD) assay (21) assured us that antitumor activity was present. Cytotoxicity tests were performed at the Purdue Cell Culture Laboratory, Purdue Cancer Center, using standard protocols for 9KB (human nasopharyngeal carcinoma) and 9PS (a chemically-induced murine lymphocytic leukemia) (22), 9ASK (astrocytoma reversal) (23), A-549 (human lung carcinoma) (24), and HT-29 (human colon adenocarcinoma) (25). Protein kinase C tests were performed by the National Institutes of Health, National Cancer Institute, and the tyrosine protein kinase tests were performed in house.

Isolated pure compounds were sent to the NIH, NCI, Bethesda, Maryland for testing in human cancer cell line panels including leukemia, non-small cell lung cancer, small cell lung cancer, CNS cancer, melanoma, ovarian cancer, and renal cancer.

Pesticidal bioassays were conducted at Eli Lilly Laboratories (Greenfield, Indiana) following standard procedures with eight indicator organisms: mosquito larvae (ML) *Aedes aegypti* (in the media), blowfly larvae (BFL) (1% in the diet), corn root worm (CRW) *Diabrotica undecimpunctata howardii* (in soil), two-spotted spider mite (2SSM) *Tetranychus urticae* (on foliage), southern army worm (SAW) *Spodoptera eridania* (on foliage), melon aphid (MA) (5000 ppm on foliage), cotton aphid (CA) *Aphis gossypii* (on foliage), and *Haemonchus contortus* (HC) (a nematode, 0.1% in the media).

EXTRACTION AND ISOLATION.—The pulverized bark (3.9 kg) was extracted by exhaustive percolation with 777 liters of EtOH. Vacuum evaporation left 380 g of syrupy residue (F001). F001 was partitioned between $CHCl_3$-H_2O (1:1), and the H_2O solubles were freeze-dried and labeled F002 (11 g). The $CHCl_3$ solubles were vacuum-evaporated to form F003 (181 g). The insoluble interface was air-dried (188 g) and labeled F004. Then F003 was partitioned between hexane-90% aqueous MeOH (1:1). The 90% MeOH fraction was vacuum-evaporated to a thick syrup (156 g) and labeled F005. The hexane residue (25 g) was labeled F006. The bioassay data (Table 1) clearly showed that the activity was concentrated in the 90% MeOH fraction (F005).

F005 (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 [$CHCl_3$-MeOH (9:1) on Si gel, phosphomolybdic acid spray], weighed, and bioassayed by the BST.

The largest and most toxic pool (P40–51, 25 g) was adsorbed onto 100 g of Celite and chromatographed over a column of 4.4 kg of Si gel packed as slurry in $CHCl_3$. A gradient of $CHCl_3$/EtOAc/MeOH was used to elute the column, collecting fractions of 100–200 ml. Pools were made after tlc and bioassayed in the BST. From the most toxic pool (P106–111), which had been eluted with $CHCl_3$-EtOAc (1:1), a white precipitate was obtained. Recrystallization from EtOAc gave fine white needles that were labeled as **1** (100 mg); this material was homogeneous in several tlc systems. From P17–40, which had been eluted with $CHCl_3$, another white precipitate appeared which was recrystallized from EtOAc to form a white homogenous solid (compound **2**) (5 mg).

Pool 31–39, from the first column, stood for some time. Orange crystals formed and were recrystallized from MeOH to yield yellow needles (150 mg); this material was identified (mp, ir, ci and eims) as liriodenine (5). The residue (5.7 g) of the mother liquor was chromatographed over Si gel at medium pres-

sure in a Michel-Miller column, eluted with a gradient of $CHCl_3$/MeOH. A white solid formed in pool 104–113; this was recrystallized from EtOAc to yield fine white needles (8 mg) of additional **2**.

Upon standing, pool 8–17 from the first chromatography column gave large colorless crystals. After recrystallization from MeOH, these were identified (uv, eims, cims, hrms, ^{1}H nmr, and ^{13}C nmr) as (−)-kaur-16-en-19-oic acid (20).

BULLATACIN [**1**].—Mp 69–70°; $[\alpha]_{589}^{23}$ +13.00, $[\alpha]_{578}^{23}$ +14.70, $[\alpha]_{546}^{23}$ +19.04, $[\alpha]_{436}^{23}$ +36.63, $[\alpha]_{365}^{23}$ +66.99 (c = 0.004, $CHCl_3$); cims (isobutane) m/z $[MH]^+$ 623; cims (NH_3) m/z $[M+NH_4-H_2O]^+$ 622, $[M+NH_4]^+$ 640; eims m/z $[M]^+$ 622; hr cims 623.4847 for $C_{37}H_{66}O_7$ (calcd 623.4889); eims see Figure 1; ^{1}H nmr see Table 5; ^{13}C nmr see Table 6; uv (EtOH) λ max 215.5 nm (ε = 7974); ir (KBr) cm^{-1} 3430 (hydroxyl), 1750 (carbonyl).

BULLATACIN ACETATE.—Compound **1** (10 mg) was treated overnight with Ac_2O in pyridine, and the acetate was recovered in the usual way: eims see Figure 1; ^{1}H nmr see Table 7.

2,35-DIHYDROBULLATACIN.—A small sample (3 mg) of bullatacin [**1**] was dissolved in 10 ml of absolute EtOH, palladium on activated carbon (0.3 mg) was added, and the suspension was flushed overnight with H_2. Tlc of the filtrate residue showed a single major spot with R_f higher than **1**. Eims and cims (isobutane and NH_3) indicated that the mol wt was 624, two mu higher than **1**; peaks at m/z 313 and 143 located the added protons in fragment **A**. ^{1}H nmr (200 MHz in C_6D_6) showed the absence of the vinyl proton at C-35 and an upfield shift of the proton signals at C-36.

BULLATACINONE [**2**].—Mp 90.5–90.7°; $[\alpha]_{589}^{23}$ +12.00, $[\alpha]_{578}^{23}$ +12.50, $[\alpha]_{546}^{23}$ +14.50, $[\alpha]_{436}^{23}$ +29.75, $[\alpha]_{365}^{23}$ +51.25 (c = 0.400, $CHCl_3$); cims (NH_3) m/z $[MH]^+$ 623, and $[MNH_4]^+$ 640]; cims (isobutane) m/z $[MH]^+$ 623, $[MH+38]^+$ 661; hr cims 623.4839 (calcd 623.4889) for $C_{37}H_{66}O_7$; eims see Figure 2; ^{1}H nmr see Table 5; ^{13}C nmr see Table 6; uv (EtOH) λ max 203.5 nm (ε = 3799); ir (KBr) absorption at 1770 and 1715 cm^{-1}.

BULLATACINONE ACETATE.—Compound **2** (10 mg) was acetylated in the usual way with Ac_2O/pyridine: ^{1}H nmr see Table 7; cims see Figure 2.

CONVERSION OF BULLATACIN [**1**] TO BULLATACINONE [**2**] (26).—Compound **1** (15 mg) was treated with 2% KOH in t-BuOH (1 ml) at room temperature for 24 h; the solution was acidified with 10% HCl to pH 1–2, set aside for 30 min, and partitioned between $CHCl_3/H_2O$. Two major components were evident in the $CHCl_3$ residue (tlc); these were resolved by micro-chromatography over Si gel in a Dispopipette ($CHCl_3$/EtOAc/MeOH gradient). One of the compounds was identical to **2** (co-tlc in four systems, eims, and ^{1}H nmr).

CD DETERMINATIONS.—Bullatacin [**1**] (c = 0.025, abs EtOH): $[\theta]_{265}$ 0.00°, $[\theta]_{260}$ −298.56°, $[\theta]_{250}$ −995.20°, $[\theta]_{240}$ −2189.44°, $[\theta]_{233}$ −2587.52°, $[\theta]_{230}$ −2348.67°, $[\theta]_{225}$ 0.00°, $[\theta]_{220}$ 4378.88°, and $[\theta]_{218}$ 7961.60°.

Bullatacinone [**2**] (c = 0.1, abs EtOH): $[\theta]_{250}$ 0.00°, $[\theta]_{240}$ −124.4°, $[\theta]_{235}$ −248.8°, $[\theta]_{230}$ −497.6°, $[\theta]_{225}$ −870.8°, $[\theta]_{220}$ −1555°, $[\theta]_{215}$ −1990.4°, $[\theta]_{212}$ −2177°, $[\theta]_{210}$ −1492.8°, $[\theta]_{207}$ −746.4°, $[\theta]_{205}$ 0.00°.

Rolliniastatin (c = 0.025; abs EtOH): $[\theta]_{265}$ 0.00°, $[\theta]_{260}$ −199.04°, $[\theta]_{250}$ −1393.28°, $[\theta]_{240}$ −2587.52°, $[\theta]_{235}$ −2786.56°, $[\theta]_{230}$ −2089.92°, $[\theta]_{225}$ 0.00°, $[\theta]_{220}$ 6369.28°.

Asimicin (c = 0.025; abs EtOH): $[\theta]_{265}$ 0.00°, $[\theta]_{260}$ −199.04°, $[\theta]_{250}$ −995.20, $[\theta]_{246}$ −2288.95, $[\theta]_{234}$ −2687.04°, $[\theta]_{230}$ −2388.48°, $[\theta]_{225}$ −59.712°, $[\theta]_{224}$ 0.00°, $[\theta]_{220}$ 4080.32°, and $[\theta]_{218}$ 7862.08°.

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