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BULLATACIN, BULLATACINONE, AND SQUAMONE, A NEW BIOACTIVE ACETOGENIN, FROM THE BARK OF *ANNONA SQUAMOSA*

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ABSTRACT.—Activity-directed fractionation of the stem bark of *Annona squamosa*, monitoring with brine shrimp lethality, led to the isolation of the highly bioactive acetogenins bullatacin [**1**] and bullatacinone [**2**], thus demonstrating a new abundant plant source for these potent compounds. A new keto-monotetrahydrofuran acetogenin with a ketolactone terminus, as first seen in bullatacinone [**2**], was also isolated, characterized by spectral analyses, and named squamone [**3**]. The cytotoxicities of **3** were increased significantly by reduction of the two keto groups to hydroxyls, and the tetrahydrosquamone [**7**] and bullatacinone [**2**] both showed selective cytotoxicities to MCF-7 human breast carcinoma. Liriodenine and (−)-kaur-16-en-19-oic acid were also isolated.

Annona squamosa L. (Annonaceae) is the most widely grown of the tropical/subtropical *Annona* fruit trees; it is commonly called sugar apple and is an item of considerable commerce. A number of insecticidal and medicinal uses, including antitumor uses, employing various parts of the plant, have been tabulated (1). A patent has been granted for a chemically uncharacterized insecticide, named annonin, isolated from the seeds (2). Annonin has an identical mol wt (622) to asimicin and its diastereomers, bullatacin [**1**], and the rolliniastatins (3–6), but is spectrally unidentical to these known acetogenins. However, annonin may be identical with squamosin, a stereochemically undefined 15,24,28-trihydroxy-bis-tetrahydrofuran acetogenin recently described from the seeds (7).

During our screening for antitumor plants, extracts of the bark of *A. squamosa* exerted a high degree of lethality to nauplii in the brine shrimp test (BST) (8) and in our panel of three human tumor cell lines. The EtOH extract (F001) of the bark was partitioned to give F005, as previously described (4), in which the bioactivity was concentrated. Successive Si gel columns, monitoring with the BST, led to the known highly bioactive acetogenins bullatacin [**1**] and bullatacinone [**2**] (4), thus demonstrating a new, more readily available plant source for these potent compounds. In addition, a novel, but less active, acetogenin, which we have named squamone [**3**], was isolated and characterized. The known compounds liriodenine and (−)-kau-16-en-19-oic acid were also found as in *A. bullata* (4).

Squamone [**3**] was obtained as a waxy colorless solid with mp 89°. The hrcims gave an $[MH]^+$ ion at *m/z* 595.4521, consistent with a molecular formula of $C_{35}H_{62}O_7$. The presence of hydroxyl moieties was obvious by the loss of H_2O in the cims and a broad absorption in the ir at 3517 cm^{-1}. The presence of only two hydroxyls was further suggested by two resonances (δ 74.04 for C-20 and 73.92 for C-15) in the region for hydroxyl-bearing carbons in the ^{13}C-nmr spectrum, by a multiplet for two protons (H-15 and H-20) at δ 3.33 in the ^1H-nmr spectrum, and by the formation of a diacetate and a di-trimethylsilyl (TMSi) derivative.

The ^{13}C-nmr resonances at δ 82.65 (C-16) and 82.60 (C-19) and their correspond-

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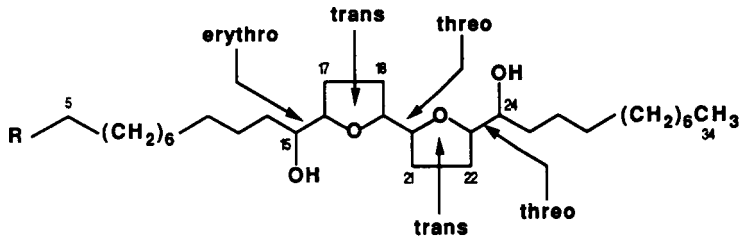
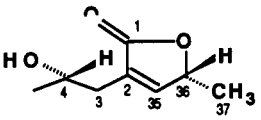


1 R=



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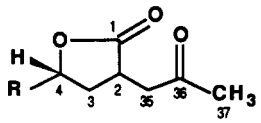
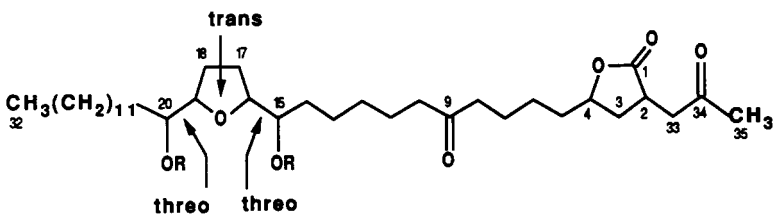


ing ^{1}H nmr resonances at δ 3.62 (2H) were directly analogous to similar signals in annonacin (9) and goniothalamicin (10), indicating the presence of a single tetrahydrofuran moiety. The placement of the two hydroxyls alpha to the tetrahydrofuran ring was established by ^{1}H-^{1}H COSY experiments that linked the two proton signals at δ 3.62 (for H-15 and H-20) to two proton signals at 3.33 (for H-16 and H-19). The subsequent downfield shift of the signal at δ 3.33 to two multiplets of one proton each at 4.84 and 4.87 in the ^{1}H nmr of the acetate confirmed this assignment.

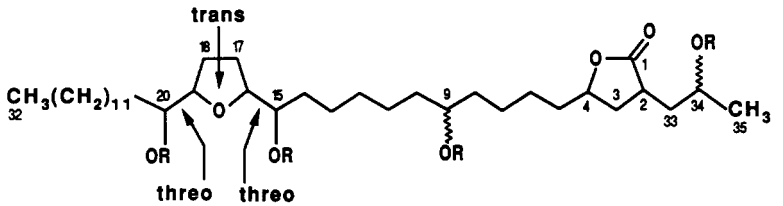
Certain aspects of the ^{1}H-nmr spectra of squamone [**3**] were quite similar to the spectra of bullatacinone [**2**] (4). A characteristic signal at δ 1.54 (s, 3H, H-35) suggested a terminal methyl ketone as was first observed in the acetogenins with bullatacinone [**2**]. ^{1}H-nmr (C_6D_6) signals at δ 2.69 (dddd, 1H, H-2, J = 3.47, 9.35, 9.35, 9.35), 2.51 (dd, 1H, H-33a, J = 3.47, 18.41), 1.912 (dd, 1H, H-33b, J = 9.35, 18.41), and 4.00 (dddd, 1H, H-4, J = 3.38, 4.48, 8.24, 8.24) specifically corresponded to fragment C of bullatacinone [**2**] (4). This was further substantiated by a 2D ^{1}H-^{1}H COSY experiment (C_6D_6) that showed connectivities between H-4/H-33a and H-33b, H-4/H-5, H-2/H-33a and H-33b, and H-2/H-35a and H-35b, indicative of a γ-lactone. These assignments were confirmed in the ^{13}C nmr by resonances at δ 205.4 (C-34), 178.00 (C-1), 78.56 (C-4), 44.21 (C-33), 35.36 (C-2), 25.01 (C-35), and 33.50 (C-3). In addition, the eims of **3** was dominated by the acetyl ion at *m/z* 43 that originated from the cleavage of the bond between C-33 and C-34.

The carbonyl peak for C-34 at δ 205.44 was apparent in the ^{13}C-nmr spectra of **3**,



3 R=H
4 R=Ac
5 R=TMSi
6 R=TMSi-d_9

but another carbonyl peak (δ 210.81, C-9) was also evident. Two triplets at δ 2.07 (H-10) and 1.94 (H-8) in the ^{1}H nmr (C_6H_6) were indicative of methylene protons adjacent to a keto group located somewhere along the hydrocarbon chain. The positions of the tetrahydrofuran ring and the keto group along the chain were determined by careful analysis of the cims and eims fragments of **3** and its acetate derivative **4**, TMSi derivative **5**, and its deutero-TMSi derivative **6**. Also, reduction of the two carbonyls of **3** produced a diastereomeric mixture of derivatives with hydroxyls at C-9 and C-34, and this reduced product (tetrahydrosquamone) **7** afforded a TMSi derivative **8**, which was



7 R=H
8 R=TMSi

helpful in placing the internal keto group at C-9. Exact mass measurements by hrms of selected key fragments substantiated their identifications. [Tetrahydrosquamone was homogeneous on tlc (three systems) but presumably consists of a mixture of diastereomers due to non-stereoselective reaction of the two carbonyl groups. There was not sufficient material available for a more detailed investigation of this point.]

The eims of the TMSi derivative **5** (mol wt 738) produced intense ions at *m/z* 271.2460 (calcd 271.2457), 467.2826 (calcd 467.2829), 341.2873 (calcd 341.2876), and 397.2409 (calcd 397.2410) which served to locate the presence of the two hydroxyl groups at C-15 and C-20 relative to the tetrahydrofuran ring (Figure 1). The TMSi spectrum also showed an intense ion at *m/z* 541 indicative of alpha cleavage (adjacent to a carbonyl) between C-8 and C-9, but the exact mass for this peak suggested that it originated from TMSi derivatization of the enol at C-9 and cleavage between C-19 and C-20. The hrcims (isobutane) of tetrahydrosquamone [**7**] gave an $[MH]^+$ at *m/z* 599.4873 (calcd 599.4887) for $C_{35}H_{64}O_7$. Eims of the TMSi derivative of **7** produced the expected ions at *m/z* 271, 615.3931 (calcd 615.3933), 341, and 545.3513 (calcd 545.3514), positioning the tetrahydrofuran ring and determining the location of the reduced carbonyl at C-9 by the presence of ions at *m/z* 271.1725 (calcd 271.1730), 615, and 373.2229 (calcd 373.2230) (Figure 2).

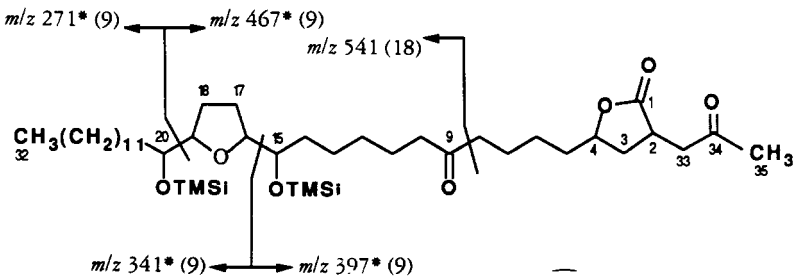


FIGURE 1. Eims fragmentation of the TMSi (**5**) and TMSi-d_9 (**6**) derivatives of squamone [**3**]; numbers in parentheses indicate the shift in mass units seen with the TMSi-d_9 derivative; the elemental compositions of fragments marked with an asterisk (*) were confirmed through exact mass measurements, and all agree within 3 mmu with the calculated values.

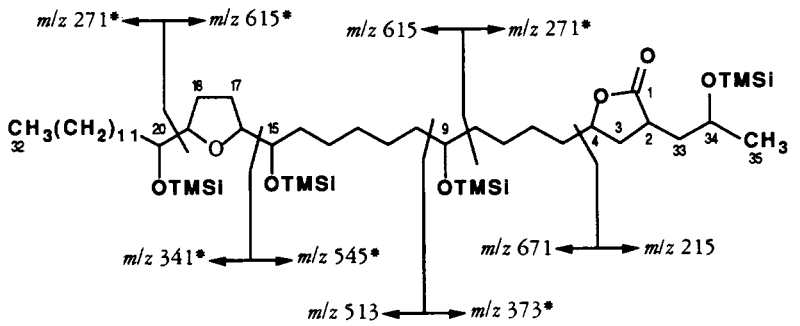


FIGURE 2. Eims fragmentations of the TMSi derivative **8** of tetrahydrosquamone [7]; the elemental compositions of the fragments marked with an asterisk (*) were confirmed through exact mass measurements, and all agree within 3 mmu with the calculated values.

The relative stereochemistry of the tetrahydrofuran ring and the two adjacent hydroxyls at C-15 and C-20 was deduced by close examination of the nmr data. In the spectra of squamone [**3**], the ^{13}C-nmr chemical shifts of C-15 and C-20 at δ 73.92 and 74.04 clearly showed a threo relationship between C-15/C-16 and a threo relationship between C-29/C-20 (11). These assignments are based on the ^{13}C-nmr chemical shifts of a pair of model monotetrahydrofuran compounds with adjacent hydroxyl groups of the threo and erythro configuration (11). In squamone diacetate [**4**], the ^{1}H-nmr chemical shifts of H-16 and H-19 at δ 3.975 indicated the trans relationship for H-16/H-19 (12). The trans relationship for H-16/H-19 is in agreement with the same assignment in bullatacin [**1**] (4) and is based on the methods of Hoye and Suhadolnik (12). Thus, the relative configuration of threo, trans, threo from C-15 to C-20 is evident. However, the stereochemistry of the chiral centers at C-2 and C-4 remains unsolved; squamone [**3**] does not exhibit significant absorption in the cd spectrum.

From the above data we concluded that the structure of squamone is as illustrated for **3** with the absolute and certain relative stereochemistries remaining undefined. Biological activities of the isolated bullatacin [**1**], bullatacinone [**2**], and squamone [**3**] and the prepared tetrahydrosquamone [**7**] (stereochemistry undefined) are presented in Table 1. It is noteworthy that bullatacinone [**2**] showed strong selective activity against the MCF-7 breast carcinoma and that tetrahydrosquamone [**7**] showed one hundred times the activity of squamone in the MCF-7 cell line. The presence of hydroxyls in place of the keto groups may enhance the bioactivity of the keto-acetogenins.

TABLE 1. Bioactivities of Bullatacin [**1**], Bullatacinone [**2**], Squamone [**3**], and Tetrahydrosquamone [**7**] (ED_{50} μg/ml).

Compound	Bioactivity Assay			
	BST[a]	A-549[b]	MCF-7[c]	HT-29[d]
Bullatacin	0.002	8.99×10^{-12}	>10	$<10^{-12}$
Bullatacinone	0.003	1.44×10^{-4}	$<10^{-7}$	$<10^{-3}$
Squamone	2.1	1.34	2.14	1.50
Tetrahydrosquamone	2.7	1.41×10^{-1}	9.89×10^{-3}	2.98×10^{-1}
Adriamycin	not tested	6.96×10^{-3}	2.90×10^{-2}	3.01×10^{-2}

[a]Brine shrimp lethality test.
[b]Human lung carcinoma.
[c]Human breast carcinoma.
[d]Human colon adenocarcinoma.

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Instruments used and derivative preparations were the same as described previously (4) with the exception that a Varian 500 was used for most ^{1}H-nmr spectra. The extracts, fractions, and isolated compounds were routinely evaluated for lethality to brine shrimp larvae (8). Cytotoxicity tests were performed at the Cell Culture Laboratory, Purdue Cancer Center, using standard protocols for A-549 (human lung carcinoma), MCF-7 (human breast carcinoma), and HT-29 (human colon adenocarcinoma).

PLANT MATERIAL.—Stem bark of *A. squamosa* was purchased from United Chemical and Allied Products, 10 Clive Row, Calcutta-1, India, May 4, 1988.

EXTRACTION AND ISOLATION.—The dried pulverized bark (2.28 kg) was extracted by macerating three times in excess 95% EtOH. The EtOH residue (F001) (209 g) was partitioned between $CHCl_3$-H_2O

TABLE 2. Nmr Data For Squamone [**3**] And Its Derivatives, Squamone Diacetate [**4**] and Tetrahydrosquamone [**7**].

Atom	Compound			
	3		**4**	**7**
	^{13}C nmr[a]	^{1}H nmr[b]	^{1}H nmr[c]	^{1}H nmr[d]
1	178.67	—	—	—
2	35.36	2.69 dddd 2,33a (9.15) 2,33b (3.47) 2,3a (9.15) 2,3b (9.15)	3.05	2.85 m
3a	22–38	1.65 m	1.2–1.8 m	1.2–1.8 m
3b		1.65 m		
4	78.56	3.995 dddd (3.38, 4.85) (8.24, 8.24)	4.56 m	4.55 m
5–7	22–38	1.2–1.7	1.2–1.8 m	1.2–1.8 m
8	42.75	1.94 t 7,8 (7.24)	2.41 t (6.7)[e]	1.2–1.8 m
9	210.81	—	—	3.60 m
10	42.41	2.07 t 10,11 (7.24)	2.37 t (6.7)[e]	1.2–1.8 m
11–14	22–38	1.2–1.7	1.2–1.8 m	1.2–1.8 m
15	73.92	3.33 m	4.87 m[f]	3.41 m
16	82.65	3.62 dt 15,16 (2.11) 16,17 (6.32)	3.98 m[g]	3.80 m
17	33.55	1.45–1.70 m	1.2–1.8 m	1.2–1.8 m
18	34.47	1.45–1.70 m	1.2–1.8 m	1.2–1.8 m
19	82.60	3.62 dt 19,18 (6.32) 19,20 (2.11)	3.98 m[g]	3.80 m
20	74.04	3.33 m	4.84 m[f]	3.41 m
21–31	22–38	1.2–1.7	1.2–1.8	1.2–1.8 m
32	14.11	0.90 t	0.9 t	0.9 t
33a	44.21	1.912 dd 33a, 33b (18.4) 33a, 2 (9.15)	2.68 dd	1.2–1.8 m
33b		2.510 dd 33a, 33b (18.4) 33b, 2 (3.47)	3.04 m	
34	205.44	—	—	3.9
35	23.30	1.54 s	2.20 s	1.2–1.8 m
150Ac	—	—	2.07 s	—
200Ac	—	—	2.07 s	—

[a]$CDCl_3$, 50 MHz.
[b]C_6D_6, 500 MHz, *J* values (in Hz) in parentheses.
[c]$CDCl_3$, 200 MHz, *J* values (in Hz) in parentheses.
[d]$CDCl_3$, 500 MHz.
[e,f,g]Signals with the same superscript may be interchanged.

(1:1), and the $CHCl_3$ residue (F003) (185 g) was partitioned between hexane-10% H_2O in MeOH (1:1) to afford the aqueous MeOH residue (F005) (90 g) and the hexane residue (F006) (80 g). The BST showed that the bioactivity was concentrated in F005 (LC_{50} 1.95 ppm, 95% confidence interval 0.07–4.91), and this fraction was the most cytotoxic: A-549 ED_{50} 1.02×10^{-2} μg/ml, MCF-7 $ED_{50} > 10$ μg/ml, and HT-29 ED_{50} 3.96×10^{-3} μg/ml.

F005 (90 g) was applied to a column of Si gel 60 (230–400 mesh) packed in a hexane slurry, and the column was developed with a gradient of hexane/$CHCl_3$/MeOH, collecting 110 fractions of 200–400 ml each. Activity in the BST was found in fractions 40–94 which were eluted with $CHCl_3$-MeOH (100:2–100:7). These fractions were combined into five pools on the basis of similar tlc patterns [Si gel plates, $CHCl_3$-MeOH (12:1), phosphomolybdic acid spray]. After bioassays in the BST and in the human tumor cells, the most active pools (60–70 and 71–80) were further resolved by Si gel cc ($CHCl_3$/MeOH gradient). From pool 60–70 bullatacin [**1**] (80 mg) and bullatacinone [**2**] (28 mg) were obtained; from pool 71–80 compound **3** (16 mg) was isolated.

BULLATACIN [**1**].—White wax: mp 69–70° [lit. (4) mp 69–70°]; co-tlc with reference bullatacin; $[\alpha]_{589}^{25°} = +17$; ir and ^{13}C nmr identical to those reported (4).

BULLATACINONE [**2**].—White needles: mp 79° (from $CHCl_3$) [lit. (4) 90.5–90.7° (from EtOAc)]; $[\alpha]_{589}^{25°} = +16$; co-tlc with reference bullatacinone; ir and ^{13}C nmr identical to those reported (4).

SQUAMONE [**3**].—White wax: mp 88°; $[\alpha]_{189}^{25°} = +3.5$; hrcims exact mass *m/z* $[MH]^+$ 595.4521 (calcd 595.4557) for $C_{35}H_{62}O_7$; uv λ max (log ε) 204 (3.12) (MeOH); ir ν max (KBr) cm^{-1} 3518, 2912, 2851, 1743, 1707, 1702, 1466, 1405, 1369, 1287, 1164, 1067, 1000, 953, 723; ^1H and ^{13}C nmr see Table 2; eims of TMSi derivatives see Figure 1.

SQUAMONE ACETATE [**4**].—The diacetate of **3** was prepared in the usual way using Ac_2O and pyridine: oil; cims *m/z* $[MH]^+$ 678; ^1H nmr (200 MHz, $CDCl_3$) δ 4.84 (m, 1H) and 4.87 (m, 1H) for H-15 and H-20 (Table 2).

TETRAHYDROSQUAMONE [**7**].—Compound **3** (3 mg) and 0.1 mg of $NaBH_4$ were mixed with constant stirring in *i*PrOH (3 ml) and refluxed for 30 min. The mixture was cooled at room temperature, and 1.5 ml of 5% NaOH was added with vigorous stirring. The alkaline solution was acidified to pH 2 with 10% HCl and extracted three times with 5-ml portions of CH_2Cl_2. The residue of **7** gave mp 65°; hrcims *m/z* $[MH]^+$ 599.4873 (calcd 599.4887) for $C_{35}H_{64}O_7$; eims of TMSi derivative **8** see Figure 2; ^1H nmr see Table 2.

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