A New Bistetrahydrofuran Acetogenin from the Roots of *Annona salzmanii*[1]

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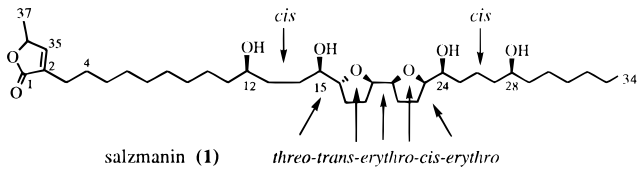
A new bistetrahydrofuran acetogenin, salzmanin (**1**), was isolated from the MeOH extract of *Annona salzmanii*, in addition to the known compounds, squamocin, almunequin, bullatalicin, and annonacin. The structure of **1** was elucidated by spectroscopic methods, including LSIMS−MS technique, and confirmed by a chemical transformation. The cytotoxic activity of **1** and squamocin was investigated.

The Annonaceous acetogenins are only encountered in a few members of the Annonaceae family. These polyketide-derived natural products have received much interest in recent years due to their significant antitumor, cytotoxic, antiparasitic, immunosuppressive, and insecticidal activities.[2,3] In a continuation of studies on this family, we have investigated the acetogenins from the roots of *Annona salzmanii* D. C. (Annonaceae).[4] Alkaloid components have been previously reported from this plant.[5] The present study has led to the isolation and structure elucidation of salzmanin **(1)**, a new bistetrahydrofuran (bis-THF) acetogenin, together with the known squamocin,[6] almunequin,[7] bullatalicin,[8] and annonacin.[9]



salzmanin **(1)** *threo-trans-erythro-cis-erythro*

Results and Discussion

Salzmanin **(1)** was isolated as a transparent oil from the MeOH extract of the roots by liquid−liquid partition followed by usual chromatographic methods including preparative HPLC. The structure was determined by ^{1}H and ^{13}C NMR (COSY, HOHAHA, HMBC, and HMQC), and MS (LSIMS−MS) on the native compound. The molecular weight of **1**, established by LSIMS as 638 from the $[M + Li]^+$ ion observed at *m/z* 645, is in agreement with the molecular formula $C_{37}H_{66}O_8$.

A weak UV absorption at 208.0 nm, and a strong one at 1749 cm^{-1} in the IR spectrum, indicated the presence of an α,β-unsaturated γ-lactone moiety, characteristic for acetogenins of subtype 1.[2,3] This structural feature was confirmed by typical resonances in the ^{1}H and ^{13}C NMR spectra (Table 1), also indicating the absence of OH group at C-4.[2,3] The presence of an adjacent bis-THF system was deduced from the ^{1}H NMR signals at δ 3.88 (2H), 3.79 (1H), and 3.75 (1H) for **1**, assigned to four oxymethine protons, in agreement with their ^{13}C NMR signals at δ 83.2 (1C), 82.7 (2C), 81.7 (1C).[10]

Two hydroxymethine groups flanking the bis-THF system were observed at δ 3.36 and 3.75 in the ^{1}H−^{1}H COSY spectrum, and at δ 74.1 and 71.3 in the ^{13}C NMR spectrum. Two further oxymethine protons appeared at δ 3.52 in the ^{1}H NMR. Their ^{13}C NMR resonances at δ 71.5 and 71.6 were indicative of isolated hydroxy groups in the aliphatic chain.[11,12]

The position of substituents on the aliphatic chain was further determined by MS.[2,3] The high-energy collision-induced dissociation (CID) spectrum of the $[M + Li]^+$ ion displayed the typical fragmentation pattern of lithiated acetogenins.[13] Two pairs of fragment ion peaks at *m/z* 221/291 and *m/z* 345/415 were assigned easily to fragmentations across two adjacent THF rings (ions Y_1-Y_2 and B_1-B_2 respectively, according to Das and Laprévote[13]), indicating the position of the bis-THF system along the alkyl chain. The *m/z* values of these fragments accounted for the presence of two hydroxy groups between the THF and the terminal lactone and of two other hydroxy groups on the methyl-terminal side chain, their locations being deduced from careful scrutiny of the CID spectrum. Two series of fragment ion peaks were, indeed, attributed to charge-remote fragmentations of the alkyl chain from the $[M + Li]^+$ precursor ion at *m/z* 645. Among them, the diagnostic fragment ions at *m/z* 559 and 529 were indicative of a hydroxy group at the C-28 position. The location of the three remaining OH groups at C-12, C-15, and C-24 was obtained by a similar way.

The relative stereochemistry around the bis-THF rings was determined by comparing the ^{1}H and ^{13}C NMR signals of **1** and the ^{1}H NMR data of its tetraacetate (**2**) (Table 1) with those of model compounds of known relative stereochemistry.[14,15] The comparison suggested that the relative configurations at C-15/C-16 and C-23/C-24 were different, according to the chemical shifts observed for H-15 or H-24 at δ 3.36 (*threo*) and 3.75 (*erythro*) for **1.**

To determine the relative configurations between C-12/C-15 and C-24/C-28, the formaldehyde acetal derivatives (**3**, **4**) were prepared from **1**.[16] The derivative **3** shows an ion peak at *m/z* 651 $[M + H]^+$ in the CIMS (CH_4^+) spectrum, in agreement with the molecular formula $C_{38}H_{66}O_8$ of a monoacetal derivative. The position of the acetal substituent was deduced from the fragment ions observed in the EIMS (Figure 1).

The downfield shifts (δ 3.62 and 3.58) of two hydroxymethine protons (H-24 and H-28, respectively) and the appearance of two doublets at δ 5.10 and 4.57 (J = 7.4) in

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Table 1. NMR Data ($CDCl_3$) of **1** and Its Tetraacetate (**2**), Monoacetal (**3**), and Bisacetal (**4**) Derivatives

	^{1}H NMR (400 MHz)				^{13}C NMR (50 MHz)
position	1	2	3	4	1
1					176.1
2					134.0
3	2.26, t[a]	2.26, t[a]	2.26, t[a]	2.26, t[a]	25.0
4	1.56 m	1.55 m	1.56 m	1.56 m	25.2−29.4[h]
5−10	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	25.2−29.4[h]
11	1.40 m	1.48−1.52 m	1.40 m	1.62−1.67 m	37.5
12	3.52 m	4.80 m	3.53 m	3.66 m	71.5
13	1.35 m	1.48−1.52 m	1.35 m	1.80 m	37.3
14	1.40 m	1.48−1.52 m	1.40 m	1.80 m	31.8
15	3.36 m	4.85 m	3.36 m	3.66 m	71.3
16	3.79 m	3.98 m	3.79 m	3.89 m	82.7
17	1.50;1.90 m	1.50;1.90 m	1.50;1.91 m	1.50;1.91 m	25.2−29.4[h]
18	1.50;1.90 m	1.50;1.90 m	1.50;1.91 m	1.50;1.91 m	25.2−29.4[h]
19	3.75 m	3.81 m	3.75 m	3.79 m	82.7
20	3.88 m	3.89 m	3.89 m	3.89 m	81.7
21	1.82;1.85 m	1.82;1.85 m	1.82;1.85 m	1.82;1.85 m	25.2−29.4[h]
22	1.82;1.85 m	1.82;1.85 m	1.82;1.85 m	1.82;1.85 m	25.2−29.4[h]
23	3.88 m	3.95 m	3.95 m	3.95 m	83.2
24	3.75 m	4.90 m	3.62 m	3.62 m	74.1
25	1.35 m	1.48−1.52 m	1.44−1.50 m	1.44−1.50 m	31.8
26	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	21.9[i]
27	1.39 m	1.48−1.52 m	1.62−1.67 m	1.62−1.67 m	37.5
28	3.52 m	4.80 m	3.58 m	3.58 m	71.6
29	1.39 m	1.48−1.52 m	1.62−1.67 m	1.62−1.67 m	37.5
30	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	25.2−29.4[h]
31	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	25.2−29.4[h]
32	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	31.8
33	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	1.20−1.30 m	22.5[i]
34	0.87, t[b]	0.87, t[b]	0.87, t[b]	0.87, t[b]	14.0
35	6.97, d[c]	6.98, d[c]	6.97, d[c]	6.97, d[c]	148.9
36	4.97, dq[d]	4.97, dq[d]	4.97, dq[d]	4.97, dq[d]	77.3
37	1.43, d[e]	1.43, d[e]	1.43, d[e]	1.43, d[e]	19.1
OAc-12		2.03, s			
OAc-15		2.04, s			
OAc-24		2.07, s			
OAc-28		2.02, s			
38a			5.10, d[f]	5.10, d[f]	
38b			4.57, d[f]	4.57, d[f]	
39a				4.82, d[g]	
39b				4.79, d[g]	

[a] J = 6.5 Hz. [b] J = 6.5 Hz. [c] J = 1.5 Hz. [d] J = 6.7 Hz and J = 1.7 Hz. [e] J = 6.7 Hz. [f] J = 1.5 Hz. [g] J = 1.5 Hz. [h] 25.2, 25.7, 25.8, 27.4, 28.5, 28.9, 29.1, 29.3, 29.4. [i] Signals may be interchanged.

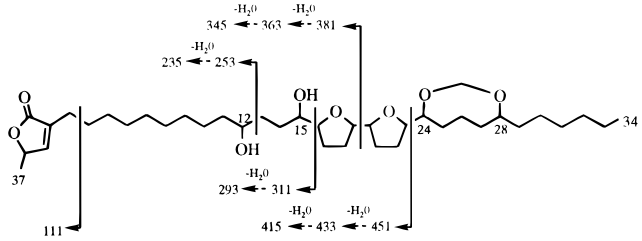

Figure 1. Fragment ions observed in the EIMS of **3**.

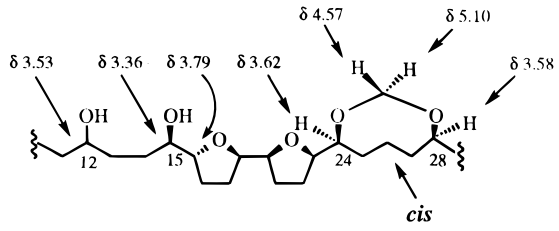

Figure 2. Relative configuration of the C-24 and C-28 OH groups in **1**, according to the ^{1}H NMR data of the acetal derivative (**3**).

the ^{1}H NMR of **3** (Figure 2) confirmed the formation of a cyclic acetal. The configuration between the two OH-groups at C-24 and C-28 in **1** was subsequently determined to be cis, a trans configuration resulting in a unique multiplet near δ 4.70 for the acetonide, as observed for dioxo-1,3-cycloheptane.[17]

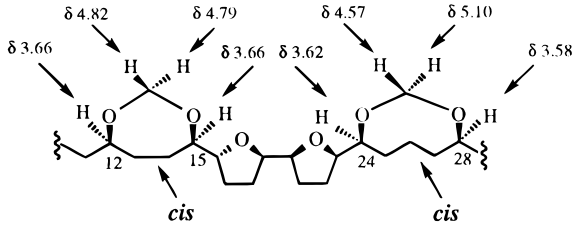

Figure 3. Relative configuration of the C-12/C-15 and C-24/C-28 OH groups in **1**, according to the ^{1}H NMR data of its bis acetal (**4**).

The bisacetal derivative **4** displayed a $[M + H]^+$ peak at m/z 663 in the CIMS (CH_4^+), in agreement with the molecular formula $C_{39}H_{66}O_8$. Four hydroxymethine protons (H-15/H-12 and H-24/H-28) were observed in the ^{1}H NMR spectrum of **4**, along with four doublets at δ 4.82/4.79 (J = 5.4) and 5.10/4.57 (J = 7.4). These NMR data were consistent with the postulated bisacetal structure and indicative of a cis configuration of both dioxo nuclei at C-12/C-15 and C-24/C-28 (Figure 3).[17]

The isolation of the 24,28-formaldehyde acetal derivative (**3**) was crucial for the determination of the relative configuration of the adjacent bis-THF system. From the chemical shifts at δ 3.36 (H-15) and 3.79 (H-16), indicating a *threo* stereochemistry between C-15 and C-16, it was possible to deduce the *erythro* configuration between C-23 and C-24 in **1**. With the relative configurations at C-15/C-

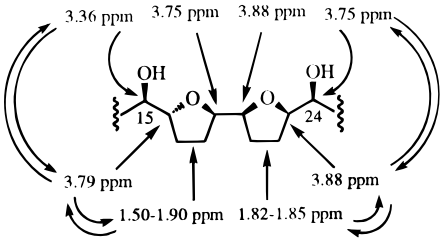

Figure 4. Relative configuration between C-15 and C-24 in **1** according to the 2D NMR (COSY DQF) data.

16 and C-23/C-24 already determined, the complete stereochemistry of **1** between C-15 and C-24 was deduced from the δ values observed in the 2D NMR (COSY DQF) spectrum of **1** (Figure 4).

From their correlations with H-24 and H-23, the methylene protons at C-21 and C-22 were assigned to signals at δ 1.82 and 1.85, indicating a cis stereochemistry for the C-20/C-23 THF ring.[18] In the same way, the protons at C-17 and C-18 were assigned to signals at δ 1.50 and 1.90, diagnostic values for a C-16/C-19 THF ring of trans stereochemistry.[18]

The relative configuration between C-19 and C-20 was then determined by the two distinct NMR signals at δ 3.75 and 3.88, in agreement with the *erythro* configuration.[19] If the relative stereochemistry was *threo*, as usually observed in the bis-THF acetogenins, the two protons should be deshielded and appear as a unique signal resonance between δ 3.85 and 3.89 in **1**.[19]

The absolute configuration of the C-36 chiral center of **1** was determined by the simple enzymatic method developed by Duret et al.[20] The results indicated that salzmanin **(1)** has the usual 36*S* absolute configuration.

The cytotoxic activity of salzmanin **(1)** was investigated [21] and compared with that of squamocin, which differs in the stereochemistry of the THF rings and the position of one OH group. The two acetogenins display significantly higher activity on cancer cell lines (KB, $ED_{50} = 10^{-4}$ μg/mL) compared with normal cells (VERO, $ED_{50} = 10^{-2}$ μg/mL). The comparison reveals a strong enhancement of the cytotoxic activity of salzmanin (0.1×10^{-4} μg/mL) versus squamocin (4×10^{-4} μg/mL), probably related to the presence of a hydroxy group at C-12.

Experimental Section

General Experimental Procedures. CIMS and EIMS were obtained on a Nermag R1010 C spectrometer. UV spectra were recorded on a Philips PU 8700 series UV/vis spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded at 200 and 50 MHz, respectively, on a Bruker AC-200 P spectrometer, and the ^{1}H−^{1}H (COSY−DQF, HOHAHA) and ^{1}H−^{13}C (HMQC and HMBC) correlation spectra at 400 MHz, on a Bruker ARX-400 spectrometer. Optical rotations were determined using a Schmidt−Haensch Polartronic I polarimeter. HPLC was carried out with a Millipore−Waters (Milford, MA) system equipped with a Waters 484 spectrophotometer. MS−MS spectra were obtained from a Zabspec-T five-sector tandem spectometer (Micromass, VG organic, Manchester, UK), using the experimental conditions described earlier.[22]

Plant Material. Roots of *Annona salzmanii* were collected in September 1996, João Pessoa, Paraiba, Brazil. Voucher specimens are deposited at the "Prof. Lauro Pires Xavier" herbarium, no. 23.158, and identified by Prof. Carlos Alberto B. de Miranda of the Department of Sciences of Nature, University of Paraiba, Brazil.

Extraction and Separation. The dried and pulverized roots (3 kg) were percolated with MeOH. The concentrated MeOH extract was added with H_2O (10%) and extracted with hexane to yield the hexane extract (40 g). The aqueous MeOH fraction was then extracted with CH_2Cl_2 to yield 30 g of extract, 25 g of which were fractionated by column chromatography (Si gel 60 M, 230−400 mesh), eluting with a CH_2Cl_2−MeOH (99:1 to 60:40) gradient. Four bis-THF γ-lactone acetogenins were obtained: salzmanin (**1**), squamocin, almunequin, and bullatalicin, along with one mono-THF acetogenin, annonacin. The five crude acetogenins were purified by HPLC, using a μ-Bondapak C_{18} prepacked column [10 μm, 25 × 100 mm] and UV detection at 214 nm. Elution with MeOH−H_2O (80:20), flow rate 9 mL/min, afforded **1** (25.5 mg, $t_R = 18.1$ min); purification of squamocin and almunequin was achieved with a MeOH−H_2O (82:18) mixture at 10 mL/min, affording squamocin (30 mg, $t_R = 64.7$ min) and almunequin (11 mg, $t_R = 42.3$ min); elution with MeOH−H_2O (78:22) at 10 mL/min afforded pure bullatalicin (11 mg, $t_R = 53.5$ min); a MeOH−H_2O (85:15) eluent at 10 mL/min afforded annonacin (4.5 mg, $t_R = 30.9$ mg).

Salzmanin (1): transparent oil; $[\alpha]^{20}_D + 26°$ (*c* 1, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 208 (4.5) nm; IR ν_{max} film 3836, 3677, 2995, 2859, 1752 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 50 MHz), see Table 1; LSIMS−MS of the $[M + Li]^+$ (*m/z* 645) ion, lactone-containing fragment ions at *m/z* 629, 627 (645 − H_2O), 615, 601, 587, 573, 559, 529, 515, 501, 487, 457, 415 (B_2), 345 (B_1), 317, 273, 259; terminal methyl-containing ions at *m/z* 547, 533, 519, 505, 491, 477, 463, 449, 435, 421, 391, 377, 363, 333, 291 (Y_2), 221 (Y_1).

Tetraacetate of Salzmanin (2). **1** (5 mg) was acetylated with Ac_2O + pyridine (1:1), affording **2** (5.1 mg, 84.8%); CIMS (NH_4^+) *m/z* 807 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 1.

24,28-Formaldehyde Acetal and 12,15/24,28-bis-Formaldehyde Acetal Derivatives of Salzmanin (3, 4). To Me_3SiCl (1 mL) was added Me_2SO (1 mL), and the mixture was allowed to stand at room temperature for about 1 h until a white precipitate appeared. The excess of unreacted reagents was decanted, and the white precipitate quickly washed with 1 mL of CH_2Cl_2. A solution of **1** (8 mg) in CH_2Cl_2 (2 mL) and a excess of $(C_2H_5)_3N$ was added to this precipitate and stirred at room temperature for 12 h. The mixture was washed using 1% $NaHCO_3$ (5 mL) and H_2O (2 × 5 mL), and the CH_2Cl_2 layer was evaporated in vacuo. The product was purified by HPLC, using a μ-Bondapak C_{18} prepacked column [10 μm, 25 × 100 mm], eluted with MeOH−H_2O (93:7) (flow rate 10 mL/min, UV detection at 214 nm) affording **3** (2 mg, 24.5%, $t_R = 11.1$ min); CIMS (CH_4^+) *m/z* 651 $[M + H]^+$; EIMS, see Figure 1; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 1; **4** (4.0 mg, 49%, $t_R = 24.5$ min); CIMS (CH_4^+) *m/z* 663 $[M + H]^+$; ^{1}H NMR ($CDCl_3$, 400 MHz): see Table 1.

Squamocin: transparent oil; $[\alpha]^{20}_D + 10°$ (*c* 1, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 209 (4.7) nm; IR ν_{max} (film): 3678, 2994, 1750 cm^{-1}; CIMS (NH_4^+) *m/z* 623 $[M + H]^+$; for EIMS, ^{1}H and ^{13}C NMR data, see Fujimoto et al.[6]

Almunequin: white wax; $[\alpha]^{20}_D +18°$ (*c* 0.06, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 204.5 (4.6) nm; IR ν_{max} (film) 3867, 3677, 2995, 2859, 1751 cm^{-1}; CIMS (NH_4^+) *m/z* 639 $[M + H]^+$; for EIMS, ^{1}H and ^{13}C NMR, see Cortes et al. [7]

Bullatalicin: white wax, $[\alpha]^{20}_D + 6°$ (*c* 0.5, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 214 (4.6) nm; IR ν_{max} (film) 3372, 2995, 1748 cm^{-1}; CIMS (NH_4^+) *m/z* 639 $[M + H]^+$; for EIMS, ^{1}H and ^{13}C NMR, see Huy et al.[8]

Annonacin: transparent oil; $[\alpha]^{20}_D + 14.2°$ (*c* 0.04, $CHCl_3$); UV (EtOH) λ_{max} (log ϵ) 210 (4.2) nm; IR ν_{max} (film): 3294, 2889, 1749 cm^{-1}; CIMS (NH_4^+) *m/z* 597 $[M + H]^+$; for EIMS, ^{1}H and ^{13}C NMR, see McCloud et al. [9]

Biological Assays. Cytotoxic activity (ED_{50}, μg/mL) was determined according to the procedure described by Fleury et al.[21] against human nasopharyngeal carcinoma cells (KB) and monkey epithelioid renal cells (VERO). The compounds with ED_{50} values ≤ 10^{-1} μg/mL are considered active if the factor of selectivity is at least 2 $\log_{10}$ between KB and VERO cell lines. Salzmanin: 0.1×10^{-4} (KB), > 10^{-2} (VERO); squamocin: 4×10^{-4} (KB), 10^{-2} (VERO); vincaleucoblastine: 0.1×10^{-4} (KB), > 1 (VERO) used as reference compound.

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