Cytotoxic Withaphysalins from the Leaves of *Acnistus arborescens*

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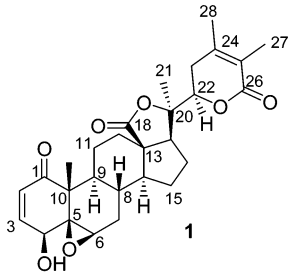
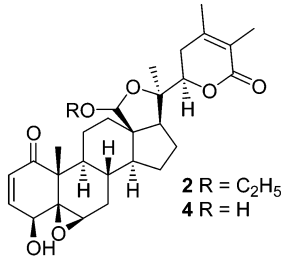
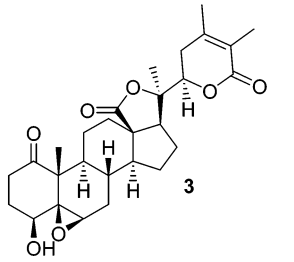
Three withaphysalins, *rel*-(17*S*,20*R*,22*R*)-5*β*,6*β*:18,20-diepoxy-4*β*-hydroxy-1,18-dioxowitha-2,24-dienolide-(withaphysalin M) (**1**), *rel*-(17*S*,20*R*,22*R*)-5*β*,6*β*:18,20-diepoxy-4*β*-hydroxy-18-ethoxy-1-oxowitha-2,24-dienolide (withaphysalin F ethyl ether, withaphysalin O) (**2**), and *rel*-(17*S*,20*R*,22*R*)-5*β*,6*β*:18,20-diepoxy-4*β*-hydroxy-1,18-dioxowitha-24-enolide (withaphysalin N) (**3**), were isolated from the leaves of *Acnistus arborescens*. The structures were deduced from 1D (^{1}H NMR, ^{13}C NMR, DEPT-^{13}C NMR) and 2D (COSY, HMQC, HMBC) NMR analysis and the relative stereochemical assignments based on 1D NOESY correlations and analysis of coupling constants. Compounds **1** and **2** displayed potent cytotoxic activity against a panel of human cancer cell lines.

The withasteroids comprise a class of naturally occurring C_{28}-steroidal lactones structurally based on the ergostane skeleton. These compounds are generally polyoxygenated, and this profusion of oxygen functions has led to several natural modifications of the carbocyclic skeleton, as well as of the side chain, resulting in compounds with complex structural features classified as withanolides, withaphysalins, physalins, ixocarpalactones, perulactones, and acnistins.[1,2] The withanolides are the most abundant and have been isolated from various genera of the family Solanaceae, i.e., *Physalis*,[3] *Acnistus*,[4] *Withania*,[5] *Datura*,[6] *Discopodium*,[7] *Iochroma*,[8] *Dunalia*,[9] *Exodeconus*,[10] *Trechonaetes*,[11] and *Vassobia*.[12] These compounds have also been found in *Ajuga parviflora* (Lamiaceae)[13,14] and in soft coral.[15] The withaphysalins are characterized by oxygenation at the C-18 methyl group to the level of alcohol, aldehyde, or acid, which, in the latter two cases, can lead to cyclization with the C-20 hydroxy group to afford a hemiacetal or a lactone. They are more limited in their distribution and have thus far been shown to be present in the genera *Dunalia*,[16] *Iochroma*,[8] *Physalis*,[17−20] and *Vassobia*,[12] all of which were previously being classified as members of the genus *Dunalia*. The withaphysalins have been evaluated for several biological activities, including antibacterial, antileishmanial, antitrypanosomal,[21] and induction of quinone reductase[22] activity.

Phytochemical studies on *Acnistus arborescens* (Solanaceae) have led to the isolation of several withanolides,[23,24] which are regarded as characteristic constituents of the plants of the genus *Acnistus*.[25−27] However, there are no previous reports on the isolation of withaphysalins from *Acnistus* species. The medicinal uses of *A. arborescens*[19] prompted an investigation of its secondary metabolites. In this paper, the isolation and structural elucidation of two new withaphysalins, **1** and **3**, from the leaves of *A. arborescens* are reported. The plant is an annual herb growing in the region of northeast Brazil and is popularly known as *marianeira* and *esporão de galo falso*. A third compound (**2**), possessing an ethoxy group at C-18, was also isolated. It is considered to be an artifact, probably formed by reaction of the intermediate natural product **4** (withaphysalin F) with the extraction solvent.







Compound **1**, mp 237−239 °C, $[\alpha]_D^{20}$ +56° (*c* 0.33, pyridine), was isolated as an amorphous powder, whose molecular formula was established as $C_{28}H_{34}O_7$ from ^{13}C NMR spectral data and HREIMS ([M]$^+$ at *m*/*z* 482.2312, calcd 482.2305). The IR spectrum showed the presence of hydroxyl (ν_{max} 3402 cm^{-1}) and carbonyl (ν_{max} 1745 and 1685 cm^{-1}) groups consistent with the presence of lactone and α,*β*-unsaturated ketone moieties. On the basis of the interpretation of ^{13}C and DEPT spectral data, the com-

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Table 1. ^{13}C and ^{1}H NMR Spectral Data for Compounds **1**, **2**, and **3**[a]

	1		**2**		**3**	
atom	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H
1	203.1		202.6		211.2	
2	132.6	6.44, d, 9.8	132.7	6.23, d, 10.0	32.2	2.90, m
						2.77, m
3	145.7	7.25, dd, 9.8, 6.3	142.2	6.95, dd, 10.0, 5.8	32.3	2.32
						1.37
4	70.7	4.03, d, 6.3	70.3	3.79, d, 5.8	73.1	3.83, br s
5	64.7		64.3		67.3	
6	60.4	3.26, br s	62.9	3.28, br s	57.3	3.24, br s
7	32.3	2.16	31.7	2.26, dt, 14.6, 3.0, Hβ	27.9	1.80
		1.25, dd, 11.9, 14.5		1.39, Hα		1.22, m
8	29.1	2.70, dq, 10.5, 4.0	31.3	1.54, m	28.8	2.66, m
9	44.2	1.09	44.1	1.10, dt, 11.2, 4.9	42.8	1.33, m
10	48.9		48.2		51.2	
11	22.1	2.24	24.5	1.86, Hα	21.8	2.18
		2.12		1.44, Hβ		1.64
12	35.1	2.25	35.2	2.51, dt, 12.6, Hβ	35.1	2.32
		1.54		1.37, Hα		1.60
13	55.8		57.9		55.7	
14	56.9	1.15	56.8	1.18	56.0	1.31
15	28.0	1.70	26.6	1.60	27.2	2.23
		1.15		1.18		
16	25.9	1.86	25.9	1.69, dd, 14.0, 4.6	25.9	1.95
		1.62		1.44		1.66
17	52.5	2.14	56.8	1.98, br d, 9.8	52.3	2.12, m
18	178.2		106.7	4.71, s	177.9	
19	17.7	1.98, s	17.7	1.38, s	15.8	1.91, s
20	84.6		84.9		84.5	
21	22.8	1.45, s	21.4	1.44, s	22.7	1.50, s
22	79.5	4.53, dd, 13.1, 3.3	81.1	4.45, dd, 13.3, 3.0	79.3	4.59, d, 12.1
23	31.3	2.32	31.8	2.41, dd, 13.3, 15.7, Hβ	31.2	2.38
		2.05, dd, 17.4, 2.3		2.02, dd, 15.7, 3.0, Hα		2.10
24	148.8		148.0		148.6	
25	122.4		122.8		122.3	
26	165.6		166.3		165.4	
27	12.9	1.91, br s	12.9	1.90, s	12.8	1.97, s
28	20.4	1.77, s	20.8	1.95, s	20.2	1.82, s
29			64.2	3.87, qd, 9.5, 7.1		
				3.43, qd, 9.5, 7.1		
30			15.6	1.19, t, 7.1		
HO-4				2.55, br s		

[a] All assignments were based on DEPT, COSY, HMQC, HMBC, and NOESY experiments.

pound contained four methyl groups, six methylenes, and nine methines (including two olefinic at δ_C 145.7 and 132.6, and three carbinolic at δ_C 70.7, 60.4, and 79.5). Nine quaternary carbon atoms, of which two are sp^2 (δ_C 148.8 and 122.4) and three are carbonyl groups, consistent with the presence of a γ-lactone (δ_C 178.2), an α,β-unsaturated lactone (δ_C 165.6), and an α,β-unsaturated ketone (δ_C 203.1), were also detected.

The ^{1}H NMR spectrum (Table 1) exhibited signals for two coupled olefinic protons at δ_H 6.44 (d, $J = 9.8$ Hz) and 7.25 (dd, $J = 9.8$ and 6.3 Hz) assignable to the H-2 and H-3 vicinal protons, respectively. In addition to these signals, the ^{1}H NMR spectrum displayed a carbinyl hydrogen signal at δ_H 4.03 (d, $J = 6.3$ Hz) which showed connectivity in the COSY spectrum with the hydrogen signal at δ_H 7.25, indicating that **1** bears a hydroxyl group at C-4. The signals at δ_C 64.7 (C) and 60.4 (CH) indicated that the compound also possesses an epoxy function in the molecule, which was located in the C-5/C-6 positions, supported by HMBC correlations from H-3 to C-5, and H-4 to C-5 and C-6. The appearance of a signal as a double doublet at δ_H 4.53 ($J = 13.1$ and 3.3 Hz) and two vinylic methyl signals at δ_H 1.91 and 1.77 in the ^{1}H NMR spectrum, the chemical shift at δ_C 165.6 in the ^{13}C NMR spectrum, and the mass spectral peak at m/z 125 indicated the presence of the α,β-unsaturated lactone side chain of the withasteroids.[2] The presence of the C-21 methyl signal as a singlet (δ_H 1.45), which showed a correlation with the resonance at δ_C 84.6 (C-20) in the HMBC spectrum, and the absence of the C-18 methyl signal, in conjunction with the presence of a carbonyl carbon signal at δ_C 178.2, led to the conclusion that the compound contains the methyl-18,20-γ-lactone moiety characteristic of the withaphysalins. The relative stereochemistry of **1** was established on the basis of relevant correlations observed in the NOESY experiment. The presence of NOE correlations between H-4, H-6, and H-9 supported the proposed β-orientation of the C-4-hydroxyl group, as well as the epoxy ring. This also was confirmed by analogy with the NMR data of known 4β-hydroxy-5β,6β-epoxywithanolides.[8,12,16,21,24,28] Thus, the structure of the new withaphysalin was established as *rel*-(17*S*,20*R*,22*R*)-5β,6β:18,20-diepoxy-4β-hydroxy-1,18-dioxowitha-2,24-dienolide (withaphysalin M) (**1**).

Compound **2**, mp 263−266 °C, $[\alpha]_D^{20}$ +96° (*c* 0.05, $CHCl_3$), was obtained as a white amorphous powder. In the ^{1}H and ^{13}C NMR spectra most of the signals were similar to those of **1** (Table 1). The main difference was the conspicuous absence of the signal corresponding to the lactone carbonyl carbon (C-18) and the presence of additional signals attributable to an ethoxy group: δ_C 64.2/δ_H 3.87 (dq, $J = 7.1$ and 9.5 Hz) and 3.43 (dq, $J = 7.1$ and 9.5 Hz); δ_C 15.6/δ_H 1.19 (t, $J = 7.1$ Hz). The chemical shifts at δ_C 106.7/δ_H 4.71 (CH) were also indicative of a lactol at the C-18 position.[12] Thus, the structure of **2**, an 18,20-hemiacetal-type withaphysalin, was established as *rel*-(17*S*,20*R*,22*R*)-5β,6β:18,20-diepoxy-4β-hydroxy-18-ethoxy-

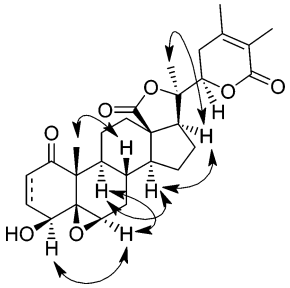

Figure 1. Selected NOE correlations for **1** and **3**.

1-oxowitha-2,24-dienolide (withaphysalin F ethyl ether, withaphysalin O), which may be an artifact derived from the acetal **4**, during the extraction of the plant material with EtOH. Compound **4** was isolated previously as withaphysalin F.[12] Related compounds with a methyl ether at C-18 were isolated following processing involving the use of MeOH.[12]

Compound **3**, mp 295−299 °C, $[\alpha]_D^{20}$ −25° (*c* 0.4, pyridine), was also obtained as a white amorphous powder whose molecular formula was established as $C_{28}H_{36}O_7$ from ^{13}C NMR spectral data and HREIMS ($[M]^+$ at *m*/*z* 484.2444, calcd 484.2461). The IR spectrum showed the presence of hydroxyl (ν_{max} 3415 cm^{-1}) and carbonyl (ν_{max} 1753 and 1700 cm^{-1}) groups consistent with the presence of lactone and ketone functions, emphasizing the similarity between **3** and **1**. The ^1H and ^{13}C NMR spectral (Table 1) patterns of **3** were very similar to those of **1**, except for the signals at δ_C 32.2 and 32.3, which were directly connected, from the HMQC spectrum, to the pairs of methylene protons at δ_H 2.90, 2.77, and 2.32, 1.37, respectively, due to the absence of the double bond at C-2/C-3. The locations of the hydroxyl at C-4 (δ_C 73.1) and the epoxy group at C-5/C-6 (δ_C 67.3/57.3) were confirmed unambiguously from the HMBC spectrum, while the relative configurations were established from the NOESY experiment. The 1D and 2D NMR data and the EIMS analysis were consistent with the structure *rel*-(17*S*,20*R*,22*R*)-5*β*,6*β*:18,20-diepoxy-4*β*-hydroxy-1,18-dioxowitha-24-enolide (withaphysalin N) for **3**.

The ethanol extract of *A. arborescens* and the compounds **1**, **2**, and **3** were evaluated for their cytotoxic potential in a small panel of human cancer cell lines.[29] The ethanol extract was cytotoxic in the lung (Lu1, ED_{50} 1.5 μg/mL), hormone-dependent prostate (LNCaP, ED_{50} 4.4 μg/mL), and estrogen-dependent human breast (MCF-7, ED_{50} 4.2 μg/mL) cancer cell lines. Two of the compounds, **1** and **2**, were also highly cytotoxic, with ED_{50} values in the range 0.22−1.2 μg/mL for **1** and 0.16−1.0 μg/mL for **2**. Compound **3** on the other hand was inactive (ED_{50} range 9.3 to >20 μg/mL), suggesting that in this series of compounds the 2,3-unsaturated ketone moiety is an important pharmacophoric unit.

Experimental Section

General Experimental Procedures. Melting points were measured on a digital Mettler Toledo FP90 apparatus and were uncorrected. The optical rotations were measured on a Perkin-Elmer 341 digital polarimeter. IR spectra were recorded on a Perkin-Elmer 1000 FT-IR spectrometer. Mass spectral data (EIMS and RHEIMS) were acquired on a Shimadzu QP5050A and a JEOL GCMate II instrument, through direct probe and operating at 70 eV. All experiments were performed on a Bruker DRX-500 spectrometer equipped with a 5 mm inverse detection *z*-gradient probe. ^1H (500.13 MHz) and ^{13}C (125.77 MHz) NMR spectra were measured at 27 °C using pyridine-d_5 (**1** and **3**) and $CDCl_3$ (**2**). Chemical shifts are given on the δ scale and were referenced to residual pyridine [δ_H 8.74, 7.58, and 7.22 and δ_C 150.35, 135.91, and 123.87] and $CHCl_3$ [δ_H 7.27 and δ_C 77.23]. Si gel 60 (Merck, 70−230 mesh) was used for column chromatography, and precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.20 mm) were used for analytical chromatography.

Plant Material. The leaves of *A. arborescens* were collected in August 2000, in the locality Pico Alto, Guaramiranga Mountain, State of Ceará (northeastern Brazil). The plant was authenticated by Professor E. P. Nunes, and a voucher specimen (# 30.513) is deposited in the Herbarium Prisco Bezerra (EAC) of the Departamento de Biologia, Universidade Federal do Ceará.

Extraction and Isolation. The dried leaves of *A. arborescens* (2.2 kg) were percolated with cold EtOH (2 × 6 L), and the EtOH extract was evaporated under reduced pressure. The residue was dissolved in water (50 mL) and extracted successively with *n*-hexane, CH_2Cl_2, and EtOAc. The CH_2Cl_2 fraction (4.3 g) was subjected to chromatography over a Si gel column and eluted with *n*-hexane and a CH_2Cl_2−EtOAc gradient. The combined fractions (76 mg) eluted by CH_2Cl_2−EtOAc (8:2) were further rechromatographed over a Si gel column using EtOAc−CH_2Cl_2 (0 → 30%) as eluent to afford **1** (18 mg) and **2** (11 mg). A second collection of the plant material (4 kg) was acquired and subjected to the same procedure. The CH_2Cl_2 fraction (10 g) was then submitted to repeated column chromatography over Si gel and eluted with *n*-hexane and a CH_2Cl_2−EtOAc gradient to give **3** (39 mg), which was isolated by elution with CH_2Cl_2−EtOAc (8:2).

***rel*-(17*S*,20*R*,22*R*)-5*β*,6*β*:18,20-Diepoxy-4*β*-hydroxy-1,18-dioxowitha-2,24-dienolide (withaphysalin M) (1):** white powder, mp 237−239 °C; $[\alpha]_D^{20}$ +56° (*c* 0.33, py); IR ν_{max} (KBr) 3402, 2944, 2920, 1745, 1685, 1389, 1218, 1115 cm^{-1}; ^1H (py-d_5, 500 MHz) and ^{13}C (py-d_5, 125 MHz) NMR data, see Table 1; EIMS *m*/*z* (rel int) 482 (2), 464 (4), 437 (7), 359 (10), 183 (8), 151 (18), 125 (55); HREIMS *m*/*z* 482.2312 (M^+ calcd for $C_{28}H_{34}O_7$, 482.2305).

***rel*-(17*S*,20*R*,22*R*)-5*β*,6*β*:18,20-Diepoxy-4*β*-hydroxy-18-ethoxy-1-oxowitha-2,24-dienolide (withaphysalin F ethyl ether, withaphysalin O) (2):** white powder, mp 263−266 °C; $[\alpha]_D^{20}$ +96° (*c* 0.05, $CHCl_3$); IR ν_{max} (KBr) 3448, 2969, 2929, 1688, 1650, 1377, 1129, 1076, 1003 cm^{-1}; ^1H ($CDCl_3$, 500 MHz) and ^{13}C ($CDCl_3$, 125 MHz) NMR data, see Table 1; EIMS *m*/*z* (rel int) 512 (2), 468 (3), 421 (6), 388 (21), 325 (8), 157 (15), 125 (57).

***rel*-(17*S*,20*R*,22*R*)-5*β*,6*β*:18,20-Diepoxy-4*β*-hydroxy-1,18-dioxowitha-24-enolide (withaphysalin N) (3):** white powder, mp 295−299 °C; $[\alpha]_D^{20}$ −25° (*c* 0.4, py); IR ν_{max} (KBr) 3448, 2969, 2929, 1688, 1650, 1377, 1129, 1076, 1003 cm^{-1}; ^1H (py-d_5, 500 MHz) and ^{13}C (py-d_5, 125 MHz) NMR data, see Table 1; EIMS *m*/*z* (rel int) 484 (1), 465 (19), 450 (52), 405 (14), 358 (52), 329 (17), 293 (19), 267 (27), 152 (33), 125 (100); HREIMS *m*/*z* 484.2444 (M^+ calcd for $C_{28}H_{36}O_7$, 482.2461).

Evaluation of Cytotoxic Potential. The EtOH extract of the leaves of *A. arborescens* and the purified withaphysalins (**1**, **2**, and **3**) were evaluated according to standard procedures[29] against the Lu1 (human lung cancer), LNCaP (hormone-dependent human prostate cancer), and MCF7 (estrogen-dependent human breast cancer) cell lines. The EtOH extract displayed an ED_{50} against the three cell lines of 1.5, 4.4, and 4.2 μg/mL, respectively. The cytotoxicity data (ED_{50} in μg/mL) for the withaphysalins in the respective test systems were withaphysalin **1**, 0.22, 1.0, 1.2; withaphysalin **2**, 0.16, 0.9, 1.0; and withaphysalin **3**, 9.3, >20, 15.0.

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