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Cytotoxic withanolides from *Acnistus arborescens*

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Abstract

Three cytotoxic withanolides, two with new structures, were isolated from the leaves of *Acnistus arborescens* and their structures determined by a combination of 1D and 2D NMR, mass spectral, and molecular modeling studies. Dereplication analysis of the ethyl ether extract was useful for evaluating the components showing significant cytotoxic activity.

Keywords: *Acnistus arborescens*; Solanaceae; Withanolides; Dereplication; Cytotoxicity and quinone reductase assays

1. Introduction

The withanolides comprise a group of complex steroidal lactones which occur in the Solanaceae family. They are of considerable phytochemical, chemotaxonomic and biological interest (Kirson and Glotter, 1981; Glotter, 1991). The leaves of *Acnistus* sp. have been used to treat liver and spleen diseases, as well as cancerous growths (Nittala et al., 1981). Kupchan et al. (1969) determined that the cytotoxic activity of *A. arborescens* (L.) Schlecht. (Solanaceae) from Costa Rica against KB cells activity was due to the withanolides, particularly withaferin A. Withacnistin was also isolated from this plant, but no anticancer data were described (Kupchan et al., 1969). More recently, the withanolides were shown to display immunosuppressive activity (Luis et al., 1994; Habtermariam, 1997). Ecologically, the withanolides exhibit activity as feeding deterrents, as insecticides and ecdysteroid antagonists, and appear to be significant as a part of the chemical defense armamentarium of solanaceous plants (Baumann and Meier, 1993; Ray and Gupta, 1994; Dinan et al., 1996; Mareggiani et al., 2000).

A. arborescens has two popular names in Brazil: *marianeira* and *esporão de galo falso*, and contains an unusual withanolide **1** carrying an acetoxy substituent at C-7 (Barata et al., 1970). Plant populations of *A. arborescens* from Costa Rica and Brazil have not been studied regarding their genotypes and differences in the composition of the steroidal lactones in the leaves (Kupchan et al., 1969). In this paper, we report on the isolation and spectroscopic characterization of **1**, and two new withanolides **2** and **3**, and an evaluation of their cytotoxic activity. In addition, based on previous tests conducted with withanolides (Kennelly et al., 1997), potential to induce phase II enzymes in cell culture was assessed.

2. Results and discussion

In the course of a continuing search for anticancer agents from terrestrial plants, the ethyl ether extract of the dried leaves of *A. arborescens* was evaluated and showed activity (ED_{50} 0.25 μg/ml) in the human epidermoid carcinoma (KB) test system (Likhitwitayawuid et al., 1993). In order to evaluate whether the extract was likely to contain new or known active metabolites, it was subjected to a HPLC/ESMS/bioassay/database dereplication technique (Cordell and Shin, 1999; Shin et al., 1999; Zani et al., 2000). Fig. 1 displays the HPLC chromatogram monitored at 254 nm (A), the negative total ion chromatogram (B), the extracted ion chromatograms at m/z 469, 527, 569 and 585 amu (C), and the activity profile of the separated ether extract in the KB cell line (D). From the cytotoxic activity profile, the

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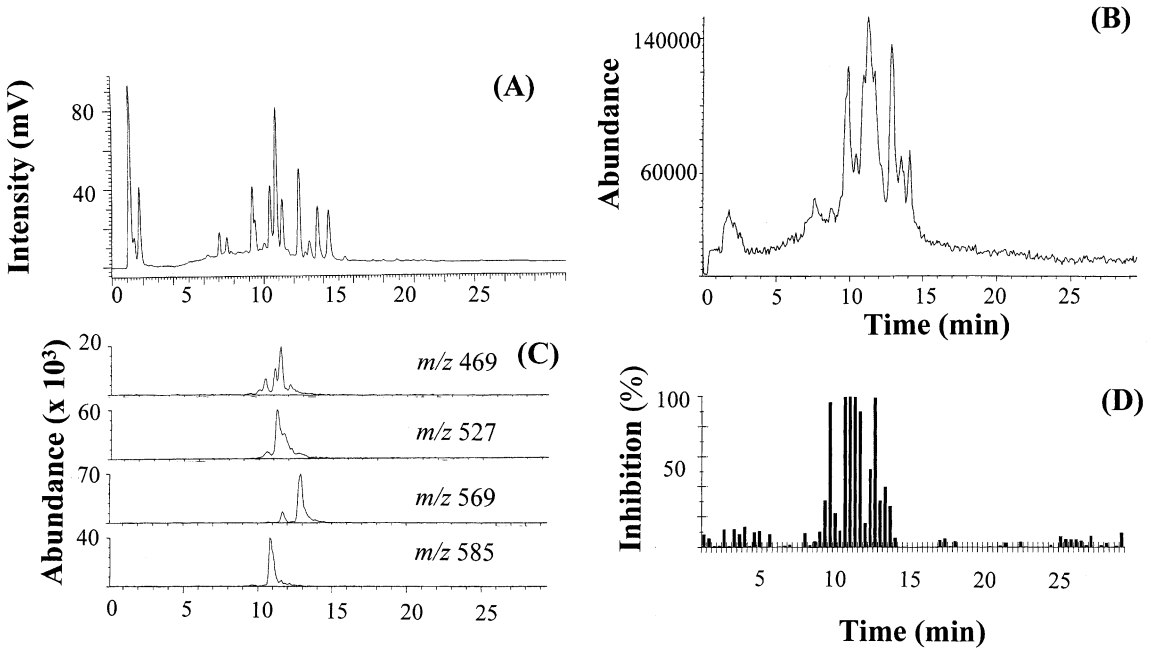
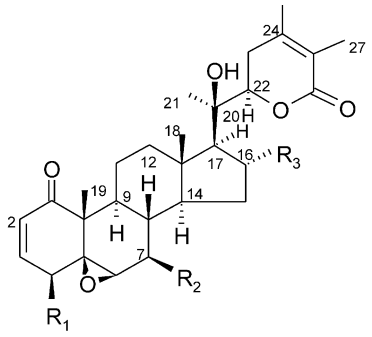


Fig. 1. The HPLC chromatogram at UV 254 nm (A), the negative total ion chromatogram (B), the extracted ion chromatogram at *m/z* 469, 527, 569, 585 (C), and the cytotoxicity profile against the KB carcinoma of the nasopharynx test system in vitro (D) of the CH_2Cl_2 extract of *Aescenso arborescens*.

retention times for the areas related to strong activity were at 9.3, 11.0, and 12.4 min. The masses corresponding to these retention times were *m/z* 468 and 486 (9.3 min), *m/z* 470, 528 and 586 (11.0 min) and *m/z* 570 (12.4 min). When these masses were compared with those of known withanolides it was found that *m/z* 468 could correspond to withanicandrin, *m/z* 486 could be 14α-hydroxy-withanolide D or nine isomers, *m/z* 470 could correspond to withaferin A or 10 isomers, *m/z* 528 could correspond to withanolide D or one isomer, and *m/z* 570 could correspond to 4,27-diacetoxy-27-hydroxy-withanolide D or one isomer. There are no known withanolides with mass 586 amu. Consequently, it was considered that the regions of activity at 11.0 and 12.4 min would yield new active metabolites.

Successive column and preparative layer chromatographic separations of the ethereal extract of *Acnistus arborescens* afforded the following compounds: 7β-acetoxy-4β,20*R*-dihydroxy-5β,6β-epoxy-1-oxo-witha-2,24-dienolide (**1**), 7β,16α-diacetoxy-4β,20*R*-dihydroxy-5β,6β-epoxy-1-oxo-witha-2,24-dienolide (**2**), and 7β,16α-diacetoxy-5β,6β-epoxy-20*R*-hydroxy-1-oxo-witha-2,24-dienolide (**3**). Compound **1** was described previously (Barata et al., 1970) and its detailed spectral data are reported for the first time. The withanolides **2** and **3** are new compounds.

Withanolide **1**, a colorless solid with mp 151–153 °C, $C_{30}H_{40}O_8$, M^+ *m/z* 528, has a base peak at *m/z* 126 ($C_7H_{10}O_2$) due to the C20–C22 bond cleavage, and an important fragment at *m/z* 169 due to fission between C17 and C20. Compound **1** showed characteristic UV absorption at 210 nm for an unsaturated lactone chromophore. The IR spectra of **1**, **2**, and **3** each showed absorptions at ca. 3450, 1715, 1697 and 1652 cm^{-1} assignable to hydroxy, unsaturated lactone, six-membered cyclic ketone, and C=C functions, respectively. The NMR spectral properties of withanolide **1** are summarized in Table 1. The ^{13}C NMR spectra of **1**, **2**, and **3** were analyzed by comparison with literature data on related withanolides (Kupchan et al., 1969; Nittala and Lavie, 1981; Vande Velde and Lavie, 1981; Alfonso et al., 1991, 1993; Habtemariam et al., 1993; Kennelly et al., 1997). The complete spectral data were interpreted from the 1D and 2D correlation spectra, with specific input from molecular modeling regarding through-space



		R_1	R_2	R_3
1	7ß-Acetoxywithanolide D	OH	OAc	H
2	7ß,16α-Diacetoxywithanolide D	OH	OAc	OAc
3	4-Deoxy-7ß,16α-diacetoxywithanolide D	H	OAc	OAc
4	Withanolide D	OH	H	H
5	7ß-Hydroxywithanolide D	OH	OH	H

Table 1
^{13}C and ^{1}H NMR resonances for withanolides **1**, **2**, and **3**

	Withanolide **1**		Withanolide **2**		Withanolide **3**	
C	^{13}C chemical shifts in ppm	^{1}H chemical shifts in ppm (*J* in Hz)	^{13}C chemical shifts in ppm	^{1} H chemical shifts in ppm (*J* in Hz)	^{13}C chemical shifts in ppm	^{1}H chemical shifts in ppm (*J* in Hz)
1	201.13		201.56		202.4	
2	132.33	6.20 *d* (10.0)	132.33	6.20 *d* (9.9)	129.3	6.05 *dd* (2.7; 10.0)
3	141.57	6.96 *dd* (5.9; 10.0)	142.00	6.96 *dd* (5.9; 9.9)	144.2	6.85 *ddd* (2.7; 10.0; 5.0)
4α	69.30	3.80 *d* (5.9)	69.18	3.80 *dd* (5.8; 1.1)	32.5	1.95 *dd* (19.2; 5.0)
4β						2.98 *dt* (19.2; 2.7)
5	67.02		66.86		64.9	
6	62.41	3.34 *d* (1.8)	62.34	3.40 *d* (1.8)	63.8	3.38 *d* (1.2)
7	74.56	4.84 *dd* (9.3, 1.8)	74.32	4.80 *dd* (1.8; 9.5)	74.6	4.84 *dd* (9.5; 1.2)
8	34.06	1.82 (9.3)	33.34	1.83 (9.5)	33.8	1.91 (9.5)
9	43.32	1.21	42.89	1.28	43.5	1.42
10	46.87		46.64		47.8	
11	22.12	1.48, 1.86	21.75	1.51; 1.94	23.7	2.12
12	39.47	1.20, 1.98	39.45	1.36, 1.95	40.2	1.39; 2.02
13	43.52		44.13		43.5	
14	55.52	1.10	52.56	1.39 *d* (2.0)	52.7	1.40 *d* (2.0)
15	25.55	1.45	35.89	1.34; 2.11	35.9	1.32; 2.06
16	29.69	1.25	75.57	5.40 *dd* (7.7; 7.4)	75.8	5.36 *dd* (7.7; 7.2)
17	53.82	1.39	59.08	1.75	59.3	1.72
18	13.45	0.89	14.28	0.91	14.7	0.93
19	17.18	1.44	17.14	1.45	15.4	1.29
20	74.96		74.49		74.7	
21	20.80	1.27	20.27	1.29	20.6	1.31
22	80.82	4.18 *dd* (3.4; 13.4)	80.62	4.20 *dd* (3.7; 13.2)	80.8	4.30 *dd* (3.4; 13.2)
23	31.48	2.06, 2.41	31.03	2.26; 2.38	31.4	2.27; 2.38
24	148.82		148.93		148.8	
25	121.99		122.37		122.3	
26	165.96		166.07		166.0	
27	12.46	1.88	12.23	1.89	12.7	1.90
28	20.55	1.94	20.56	1.96	20.9	1.97
7-OAc	171.25		170.72[a]		171.6[a]	
7-OAc	21.52	2.11	21.21[b]	2.10	21.7[b]	2.10
16-OAc			170.49[a]		170.3[a]	
16- OAc			21.14[b]	1.96	21.5[b]	1.95

[a] Assignments may be interchanged.
[b] Assignments may be interchanged.

interactions in the lowest energy state. Comparison with withanolide D (**4**) and 7β-hydroxy withanolide D (**5**) (Barata et al., 1970) revealed that withanolide **1** possessed an additional acetate functionality (δ_H 2.11, δ_C 21.52, 171.25) compared with **4**. This group was placed at C-7 based on a dd ($J = 9.3$, 1.8 Hz) at δ 4.84 which correlated with a carbon resonance at δ 74.56. Couplings of this proton were to a doublet at δ 3.34 (H-6, $J = 1.8$ Hz) and a multiplet at δ 1.82 (H-8). The stereochemistry at C-7, and the configurations at C-4 and C-6 were established through a combination of NOESY and NOE difference experiments, together with an augmented MM2 force field program (Cache 4.4 for Windows, 2001). The angles between H-6 and H-7 in the β-isomer (63.5°) and the α-isomer (50.4°) do not permit a distinction to be made based on the small coupling constant. Similarly, NOE effects are anticipated for H-6α with either H-7α (2.63 Å) or H-7β (2.54 Å). However, the angle between the H-7 and H-8β in the β-isomer (171.0°) and the α-isomer (54.8°) are significantly distinct. Thus, the *J*-value of 9.3 Hz between these two protons suggests a 7β-stereochemistry for the acetoxy substituent. In the 7β-isomer an NOE is anticipated, and observed, for the interaction of a H-7α and H-9α (2.56 Å), but not for the 7α-isomer, where H-7β to H-9α is 3.75 Å. Consequently, the acetoxy substituent at C-7 has the β-configuration. The stereochemistry at C-4 was also evaluated through the NOESY experiment. H-6α is anticipated to have a NOE effect with H-4α (2.31 Å), but not H-4β (3.52 Å). Since an NOE correlation was observed, the hydroxy substituent at C-4 has the β-configuration. It is stated that "all the withanolides have the 22*R* configuration" (Glotter, 1991). Consequently, assignment of this stereochemistry is rarely determined unambiguously (Ma et al., 1999), only occasionally discussed (Khan et al., 1999), and usually ignored (Habtemarian and Gray, 1998; Kennelly et al., 1997). The calculated coupling constants with H_2-23 (Cache 4.4 for

Windows, 2001) are quite different for the two H-22 isomers (0.5–4 Hz and 9–13.8 Hz for H-22α, and 2.5–7 Hz and 2–5 Hz for H-22β). Thus, the observed coupling constants ($J = 3.4$, 13.4 Hz) define H-22 as α and the center as *R*. The proton H-22α (δ 4.18) showed NOE correlations with the H_2-23 protons at δ 2.06 and 2.41.

The assignment of the methine carbons of C-14 and C-17 was made through HMQC correlations which established the corresponding protons and carbons as δ 1.10/55.52 and δ 1.39/53.82. A distinction between them was made from the correlation of the resonance at δ 1.10 with a methylene group (H_2-15) at δ 1.45. Therefore, compound **1** is assigned the structure 7β-acetoxywithanolide D (7β-acetoxy-4β,20*R*-dihydroxy-5β,6β-epoxy-1-oxo-witha-2,24-dienolide).

Withanolide **2,** mp 163–166 °C, $C_{32}H_{42}O_{10}$, M^+ *m*/*z* 586, has an unusual base peak at *m*/*z* 401 $(M-125)^+$ and a very intense ion at *m*/*z* 126. The molecular ion of **2** was 58 mass units greater than that of **1**, suggesting that **2** has an additional acetoxy group. The location and configuration of this second acetoxy group (δ_H 1.96; δ_C 21.14, 171.72) in **2** was determined by ^1H–^1H COSY, long-range ^1H–^{13}C (HMBC), HSQC, NOESY, and difference NOE experiments, which were also used to make complete ^1H and ^{13}C NMR spectral assignments. The hydroxy group at C-4 and the acetoxy substituent at C-7 were both deduced to have the β-configuration in the same manner as for withanolide **1**, where significant NOESY correlations were observed between H-6α and H-4α, and H-7α and H-9α. Coupling constant values defined the C-22 stereochemistry as *R*.

The multiplicity and chemical shift of H-16 were changed from a methylene multiplet at ca. δ 1.25 to a methine doublet of doublets at δ 5.40, with a concomitant shift in the ^{13}C from δ 29.69 in **1** to δ 75.57 in **2**. From the ^1H–^1H COSY, the two ^2J correlations were with the protons at δ 2.11 (H-15) and δ 1.75 (H-17). The latter resonance showed, by the HSQC technique, a ^1J correlation with C-17 at δ 59.08 (Table 2); the latter attribution being confirmed by the HMBC spectrum. The three protons of the methyl group at C-18 showed a ^2J correlation with C-13 and two other ^3J correlations with C-14 and C-17, and the three protons of the methyl group at C-21 also demonstrated a ^3J correlation with C-17. Thus, C-17 is a methine group and C-16 is substituted by the acetoxy residue. Establishment of the C-16 stereochemistry merits particular discussion. A number of withanolides substituted at C-16 from Solanaceae plants have been isolated recently. Three 16α-substituted derivatives were isolated from *Exodeconus maritumus* (Gil et al., 1997), two 16β-substituted derivatives were obtained from tomatillos (*Physalis philadelphica*) (Kennelly et al., 1997), three 16α-substituted compounds from *Discopodium penninervum* (Habtemariam et al., 1993), and a 16α- isomer from *Iochoma coccineum* (Alfonso et al., 1991), *Hyoscyamus niger* (Ma et al., 1999), and *Salpichroa origanifolia* (Tettamanzi et al., 2001). The C-16 hydroxy group stereochemistry was deduced through the observation of a NOE between H-14α and H-16α (Kennelly et al., 1997), by X-ray crystallography (Alfonso et al., 1991; Ma et al., 1999), and by a NOE between H_3-18β and H-16β (Habtemariam et al., 1993; Gil et al., 1997; Tettamanzi et al., 2001).

Table 2
^1H–^{13}C HSQC direct correlation (^1J) and ^1H–^{13}C HMBC long-range correlations (^2J and ^3J) in withanolide **2**

Proton	$\delta^{13}C$		
	^1J	^2J	^3J
H-2	132.33		69.18 (C-4)
H_3-18	14.28	44.13 (C-13)	52.56 (C-14); 59.08 (C-17)
H_3-19	17.14	46.64 (C-10)	42.89 (C-9); 66.86 (C-5); 201.56 (C-1)
H_3-21	20.27	74.49 (C-20)	59.08 (C-17); 80.62 (C-22)
H-28	12.23	122.37 (C-25)	148.93 (C-24); 166.07 (C-26)
7-OCO$\underline{CH_3}$	21.14	170.49	
16-OCO$\underline{CH_3}$	21.21	171.72	

Molecular modeling studies on withanolide **2** examining the lowest energy conformation indicated that there are four NOE effects which are potentially useful in defining the stereochemistry at C-16. There is (i) the NOE between H-14α and H-16α (3.064 Å) (H-14α to H-16β is 3.769 Å), (ii) between H-17α and H-16α (2.302 Å) (H-17α to H-16β is 3.047 Å), (iii) between H-22α and H-16α (2.888 Å) (H-22α to H-16β is 3.709 Å), and (iv) between H_3-18β to H-16β (2.526 Å) (H_3-18β to H-16α is 4.599 Å). The observation of NOE effects for H-16 and H_2-15 (α and β) do not distinguish the H-16 stereochemistry, and a NOE with H-22 does not distinguish the H-16 stereochemistry unless H-22 is first defined. In the case of withanolide **2**, NOE effects were observed for H-16 (δ 5.40) with five other protons at ca. δ 0.91, 1.32, 1.72, 2.06, and 4.08, corresponding to H_3-18β, H-15, H-17α, H-15, and H-22α, respectively. Given the prior discussion, the observation of a NOE between H-16 and both H_3-18β (δ 0.91) and H-22α seemed impossible. This phenomenon was observed previously, but was not explained (Habtemariam et al., 1993). In order to establish the utility of the NOE effects for the determination of the stereochemistry at C-16, energy calculation studies for the isomers in which C-22 has the *R* configuration and the C-16 has either an α or β configuration. The results are shown in Table 3 for two of the lower energy conformations of the isomers and are labeled as A–D. The slightly higher energy conformation represents one in which the C-17 to C-20 bond is rotated, forcing the lactone ring away from the plane of the steroid nucleus. The data show that in conformations A and B (16β-H) NOE effects between both H_3-18β and H-22α are possible (and anticipated). In neither conformation with a 16α-H (C or D) can this occur. The

Table 3
Lowest energy levels for the C-16 isomers of withanolide **2**

Energy kcal/mol		Calculated interproton distance (Å)		
		H-16 to H-18	H-18 to H-22[a]	H-16 to H-22[a]
16α-OAc/16β-H				
	A 72.49	2.53	4.46[b]	2.54
	B 76.46	3.00	4.65[b]	2.43
16α-H/16β-OAc				
	C 68.89	3.89[b]	4.39[b]	2.42
	D 71.15	3.94[b]	4.45[b]	2.99

[a] H-22 is assumed to have the *R*-configuration.
[b] Indicates no NOE effect anticipated from these interactions.

data also show in that *both* C-16 isomers can show a NOE between H-16 and H-22α (A, B, C and D). Thus, it is the NOE correlations within the rigid steroid framework (i.e. of H-16 with either H_3-18β or H-14α) which define the C-16 stereochemistry. From the observed correlation of H-16 with H_3-18β it was concluded that the 16-acetoxy substituent had an α-configuration. Hence the structure of **2** was established as 7β,16α-diacetoxywithanolide D (7β,16α-diacetoxy-4β,20*R*-dihydroxy-5β,6β-epoxy-1-oxo-witha-2,24-dienolide).

Withanolide **3**, $C_{30}H_{40}O_8$ (M^+ *m*/*z* 570), is a colorless solid, mp 132–137 °C. Analysis of **3** by mass spectrometry showed important ions at *m*/*z* 385, 325, 263, 160, and 126. The only difference from withanolide **2** was the absence of a hydroxyl group at C-4, as demonstrated by ^{1}H NMR (Table 1) and DEPT experiments, which now showed H-4α at δ 1.95 and H-4β at δ 2.98. The geminal coupling constant of the 4-H_2 in withanolide **3** was rather large (J = 19.2 Hz), which is known (Nittala et al., 1981) to occur because of the orbital system of the 2-en-1-one function, and the orientation of an electronegative group at C-5. The maximum effect is obtained when the angle (ϕ_1) formed by the *p* orbital and the plane (P) bisects Hα- C(4)-Hβ at 90°. A large effect is also obtained when the angle (ϕ_2) existing between the C(5)-O bond and the plane (P) is 90°, as occurs for a 5β,6β-epoxide. Withanolide **3** shows vicinal coupling constants for 3-H (δ 6.85) of 5.0 Hz with the 4α-H (δ 1.95), and of 2.7 Hz with the 4β-H (δ 2.98). The latter proton shows a NOESY correlation with H_3-19 at δ 1.29. H-2 is now a doublet of doublets and shows correlations with 3-H (J = 10 Hz) and the 4β-H (J = 2.7 Hz), but not the 4α-H, as observed previously (Habtemariam et al., 1993). The ^{13}C NMR spectra of **3** were similar to those of **2**, except for the changes induced by the elimination of the 4-hydroxy group (δC**2**- δC**3**: C-1,-0.8; C-2, +3.0; C-3,-2.2; C-4, +26.7; C-5, +2.0; C-6,-1.5; C-10,-1.2; C-19, +1.7).

The acetoxy substituent at C-7 (δ_H 2.10; δ_C 21.5, 170.3) was deduced to have the β-configuration in the same manner as for withanolide **1**. The location and stereochemistry of the second acetate group (δ_H 1.95; δ_C 21.7, 171.6) in withanolide **3** were established using the NOESY and NOE difference technique. These data showed very similar correlations between H-16 (δ 5.36) and H-22α (δ 4.30), H-17α (δ 1.72), the two H-15 protons (δ 1.32 and 2.06), and H_3-18β (δ 0.93) as those of withanolide **2**. Thus, the structure of the new withanolide **3** is established as 4-deoxy-7β,16α-diacetoxywithanolide D (7β,16α-diacetoxy-5β,6β-epoxy-20*R*-hydroxy-1-oxo-witha-2,24-dienolide). This appears to be the first report of withanolides bearing oxygenation at both C-7 and C-16.

The cytotoxic activity of these metabolites was evaluated in a panel of human cancer cell lines. Each of the compounds was very broadly active with ED_{50} values in the 0.1–1 μg/ml range. The presence of a 7β-acetoxy group (withanolides **1**, **2**, and **3**), the presence of a 16α-acetoxy group (withanolides **2** and **3**), and the absence of the 4β-hydroxy group (withanolide **3**) had essentially no effect on the cytotoxic activity. This is in contrast to the 17-oxygenated withanolides, where introduction of 16-oxygenation (hydroxy or acetoxy) enhanced the cytotoxicity (Habtemariam, 1997).

The withanolides were also evaluated for their cancer chemopreventive activity as inducers of phase II detoxification enzymes, using the quinone reductase system in cultured murine hepatoma 1c1c7 cells (Song et al., 1999), and the results are shown in Table 4. Previous studies (Kennelly et al., 1997) indicated that some of the withanolides from *Physalis philadelphica* were very active in this assay and showed high levels of selectivity. Each of the withanolides from *A. arborescens* was very potent as a monofunctional inducer of quinone reductase (CD value), but their selectivity (CI value) was marginal.

These experiments indicate the power of the HPLC/ESMS/bioassay/database dereplication strategy (Cordell and Shin, 1999) to identify those active extracts which are likely to yield novel cytotoxic compounds (Zani et al., 2000).

3. Experimental

3.1. General

The IR spectra were recorded on a Perkin Elmer 1600 (FTIR Series) spectrophotometer. HPLC-electrospray/MS chromatograms and spectra for dereplication were measured on a Hewlett Packard 5989B mass spectrometer coupled with a 59987A electrospray interface and a Hitachi HPLC L-7100 system. The EI were recorded on VG Autospec High Resolution Mass Spectrometer (Micromass Company). The ^{1}H and ^{13}C NMR spectra were recorded on Bruker Avance spectrometers operating at 500 and 300 MHz. Column chromatography was

Table 4
Induction of quinone reductase activity by withanolides (**1**–**3**) from *Acnistus arborescens* and 4′-bromoflavone[a]

Sample tested	CD (μg/ml)	IC_{50} (μg/ml)	CI
Withanolide **1**	0.21	0.45	2.1
Withanolide **2**	0.21	0.45	2.1
Withanolide **3**	0.3	0.62	2.1
4′-Bromoflavone	0.003	>20	>6,667

[a] Quinone reductase (QR) activity was determined with the Hepa 1c1c7 murine hepatoma cell line. CD, concentration required to double QR activity; IC_{50}, concentration inhibiting cell growth by 50%; CI, chemoprevention index (IC_{50}/CD). 4′-Bromoflavone was used as a positive control.

conducted on silica gel, 230–400 mesh. Thin layer chromatography was performed on precoated silica gel GF-254 chromatoplates (20×20 cm, 0.2 mm thick E. Merck). Visualization of the TLC plates was achieved at 254 and 366 nm, and the MeOH/sulfuric acid (1:1) spray reagent was used for detection. Molecular modeling studies were performed with Cache 4.4 for Windows (Oxford Molecular Ltd.).

3.2. Plant material

Acnistus arborescens leaves were collected in May 1997 at the Rio de Janeiro Botanical Garden, and were identified by Jorge Tamashiro from the Botany Department of the State University of Campinas where the specimen sample is deposited in the herbarium.

3.3. Extraction and isolation

Powdered leaves of *Acnistus arborescens* (100 g) were defatted with hexane in a Soxhlet apparatus followed by successive extractions with ether and ethanol. The ether fraction was evaporated and the residue evaluated biologically (Section 3.5) and through the dereplication technique (Section 3.4). Chromatography over silica gel with solvents of increasing polarity afforded a fraction eluted with hexane–EtOAc (3:2) which was chromatographed by preparative TLC, eluting twice with a mixture of hexane/EtOAc (1:1). Withanolides **1** (25 mg) and **2** (12 mg) were obtained after crystallization from a mixture of hexane/EtOAc (3:2) yielding two colorless, microcrystalline powder substances. The fraction eluted with hexane/EtOAc (7:3) was chromatographed by prep TLC eluting with hexane/EtOAc (7:3) to afford withanolide **3** (3 mg).

Withanolide **1**: (7β-acetoxywithanolide D, 7β-acetoxy-4β,20*R*-dihydroxy-5β,6β-epoxy-1-oxo-witha-2,24-dienolide). Mp: 151–153 °C (EtOAc); UV λ_{max} 217 nm (ε 15,000); IR ν_{max} cm^{-1}: 3448, 1700, 1686; EIMS *m*/*z* (rel. int.): 468 $[M–HOAc]^+$ (2), 403 $[M-125]^+$ (15), 385 $[M-125–H_2O]^+$ (12), 343 $[M-125–HOAc]^+$ (30), 325 $[M-125–HOAc–H_2O]^+$ (25), 169 (25), 126 (100); ^1H NMR spectral data, see Table 1; ^{13}C NMR spectral data, see Table 1.

Withanolide **2**: (7β,16α-diacetoxywithanolide D, 7β,16α-diacetoxy-4β,20*R*-dihydroxy-5β,6β-epoxy-1-oxo-witha-2,24-dienolide). Mp: 163–166 °C (EtOAc); IR ν_{max} cm^{-1}: 3430, 1720, 1707, 1680; EIMS *m*/*z* (rel. int.): 461 $[M-125]^+$ (20), 401 $[M-125–HOAc]^+$ (100), 341 $[M-125–2HOAc]^+$ (28), 323 (19), 281 (20), 169 (50), 126 (100); HR–EIMS *m*/*z* 586 (M^+, not detected); *m*/*z* 461.52175 (calc. for $C_{25}H_{33}O_8$: 461.52472); ^1H NMR spectral data, see Table 1; ^{13}C NMR spectral data, see Table 1, ^1H–^{13}C HMQC correlations, see Table 2.

Withanolide **3**: (4-deoxy-7β,16α-diacetoxywithanolide D, 7β,16α-diacetoxy-5β,6β-epoxy-20*R*-hydroxy-1-oxo-witha-2,24-dienolide). Mp: 132–137 °C (EtOAc); IR ν_{max} cm^{-1}: 3430, 1720, 1707, 1680; EIMS *m*/*z* (rel. int.): No M^+, 263 (95), 385 (65), 325 (32), 126 (40), and 169 (15); HR–EIMS *m*/*z* 570 (M^+, not detected); *m*/*z* 445.52364 (calc. for $C_{25}H_{33}O_7$: 445.52532); ^1H NMR spectral data, see Table 1; ^{13}C NMR spectral data, see Table 1.

3.4. Dereplication method using HPLC-electrospray MS

The cytotoxic ether extract (ED_{50} 0.25 μg/ml, KB system) from the leaves of *A. arborescens*, prepared as indicated above, was subjected to dereplication analysis, employing a previously published protocol, using standard chromatographic conditions, and the KB cytotoxicity assay to monitor activity (Cordell and Shin, 1999). Cytotoxic activity was observed at the times 9.3 min (*m*/*z* 468 and 486), 11.0 min (*m*/*z* 470, 528, and 586), and 12.4 min (*m*/*z* 470).

3.5. Evaluation of cytotoxic potential

The ether extract of the leaves of *A. arborescens* and the purified withanolides were evaluated according to standard procedures (Likhitwitayawuid et al., 1993) against the BC-1 (human breast cancer), Lu1 (human lung cancer), Col2 (human colon cancer), KB (human oral epidermoid carcinoma), KB-V1 [vinblastine-resistant KB cell line tested in the presence and absence of vinblastine (1 μg/ml)], and LNCaP (hormone-dependent human prostate cancer) cell lines. The cytotoxic data (ED_{50} values, in μg/ml) for the withanolides in the respective test systems were: withanolide **1**, 0.2, 1.3, 0.03, 0.4, 0.4, 0.4, 0.2; withanolide **2**, 0.3, 2.1, 0.08, 0.5, 0.4, 0.3, 0.2; withanolide **3**, 0.5, 0.3, 0.5, 0.1, 0.8, 1.0, 0.2.

3.6. Evaluation of quinone reductase activity

Using the modified quinone reductase assay (Kennelly et al., 1997; Song et al., 1999) the withanolides **1**, **2**, and **3** were evaluated for their ability to act as phase II enzyme inducers using cultured Hepa 1c1c7 mouse

hepatoma cells. Enzyme activity was expressed as a "CD" value, the concentration of sample required to double the specific activity of quinone reductase. IC_{50} values (half-maximal inhibitory concentration of cell viability) were determined and the chemoprevention index (CI) values were calculated by dividing the IC_{50} values by the CD values (Gerhäuser et al., 1997). 4′-Bromoflavone was used as the positive control (Song et al., 1999) and the data are summarized in Table 4.

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