Antifungal and Cytotoxic Activity of Withanolides from *Acnistus arborescens*

Vincent Roumy,*[,†,∞] Murielle Biabiany,[†,∞] Thierry Hennebelle,[†] El Moukhtar Aliouat,[‡] Muriel Pottier,[‡] Henry Joseph,[⊥] Sami Joha,[§] Bruno Quesnel,[§] Racha Alkhatib,[†] Sevser Sahpaz,[†] and François Bailleul[†]

Laboratoire de Pharmacognosie, EA4481 (GRIIOT), Faculté des Sciences Pharmaceutiques et Biologiques, Université Lille Nord de France (Lille 2), F-59006 Lille Cedex, France, EA3609 (BDPEE), Faculté des Sciences Pharmaceutiques et Biologiques, Université Lille Nord de France (Lille 2) and Institut Pasteur de Lille, F-59019, Lille Cedex, France, Phytobôkaz, Gros morne Dolé, F-97113 Gourbeyre, France, and Unité 837 INSERM, Institut de Recherche sur le Cancer de Lille, Place de Verdun, F-59045, Lille Cedex, France

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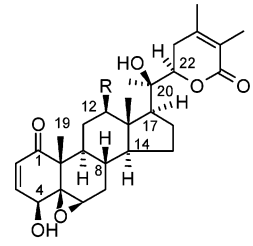
Three compounds were isolated from *Acnistus arborescens,* a tree commonly used in South and Central America in traditional medicine against several infectious diseases, some of which are caused by fungi. Bioassay-guided fractionation of a MeOH extract of leaves, based on its anti-*Pneumocystis carinii* activity, led to the isolation of compounds **1**−**3**. Mono- and bidimensional NMR analyses enabled identification of two new withanolides, (20*R*,22*R*)-5β,6β-epoxy-4β,12β,20-trihydroxy-1-oxowith-2-en-24-enolide (**1**) and (20*R*,22*R*)-16β-acetoxy-3β,4β;5β,6β-diepoxy-12β,20-dihydroxy-1-oxowith-24-enolide (**2**), and withanolide D (**3**). Antifungal activity on 13 fungi responsible for human infections (five dermatophytes, one nondermatophyte mold, six yeasts, and *Pneumocystis carinii*) was examined. Cytotoxicity of these compounds was also evaluated *in vitro*.

Acnistus arborescens (L.) Schlecht (Solanaceae) is a tree that is common from South to Central America.[1] Withanolides previously isolated from this species, including withacnistin, withaferin A, withaphysalins, withanolide D, and its derivatives, have been used medicinally (against infections due to bacteria, fungi, malaria, leishmaniasis, etc.). Considering the traditional antifungal use (in Guadeloupe and the Caribbeans) and the potential application of withanolides as anticancer compounds, this work focused on the bioassay-guided purification of such compounds and assessment of their antifungal and cytotoxic activities.[2,3] Further investigation of *A. arborescens* leaves was considered to be important due to the medicinal uses, its widespread occurrence, and the ease of collection. The present study describes the isolation and structural elucidation of two new withanolides (**1** and **2**), along with complementary spectroscopic data for withanolide D (**3**).[4,5] These structures were established on the basis of 1D and 2D NMR and mass spectrometry analyses. Biological activities of these compounds were evaluated on fungi responsible for dermatological infections and on *Pneumocystis carinii* (rat-associated species). *Pneumocystis* spp. are important opportunistic pathogens causing lethal pneumonia in immunocompromised hosts such as patients with AIDS and those receiving chemotherapy or immunosuppressive drugs for organ transplantation or certain other pathologies.[6,7] The cytotoxic activity of this plant and some of its withanolide derivatives has been evaluated previously on tumor cell lines, including BC-1, Lu1, Col2, KB y KB-V1, and LNCaP.[9]

The defatted MeOH extract of the leaves of *A. arborescens* was prepared by percolation, and it exhibited moderate inhibitory activity against *P. carinii*. Bioactivity-guided fractionation of the extract led to the major compound **3** and two minor compounds (**1** and **2**). The structure of compound **3** was elucidated by comparing its NMR, MS, and optical rotation data with reported values.[10,11]

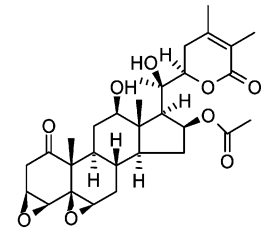
Compound **1** was isolated as a white, amorphous solid. The molecular formula was determined to be $C_{28}H_{39}O_7$ from the molecular ion peak at *m/z* 487.2678 $[M + H]^+$ in the HRESIMS. The NMR signals of the carbons and protons located on the steroidal framework closely resembled those of the known **3** except for the C-12 environment. On the basis of the interpretation of the JMOD spectral data in comparison with **3** (in DMSO-d_6, Table 2), compound **1** contained four methyl groups, five methylenes, 10 methines, and nine quaternary carbon atoms, two of which were carbonyl groups, consistent with the presence of an α,β-unsatured lactone (δ_C 166.5) and an α,β-unsatured ketone (δ_C 202.2). HSQC and HMBC correlations (in DMSO-d_6) of some hydroxy-bearing carbons displayed the chemical shift of their protons ($\delta_{H\text{-}6}$ 3.21; $\delta_{H\text{-}12}$ 3.36; $\delta_{H\text{-}4}$ 3.58) and indicated an epoxy function at C-5, C-6 and an additional secondary OH group at C-12 (δ_C 75.8) in comparison with **3**. Selective TOCSY experiments (Table 1) supported the OH at C-12. The relative configuration of **1** was established on the basis of relevant correlations observed in the NOESY experiment in $CD_3OD-CDCl_3$ (1:1) and was supported by comparison with the NMR data of **3** (withanolide D). The coupling constants of H-22 (δ_H 4.80, J = 3.0, 13.0 Hz) and the NOE correlation of H-22 with H_{eq}-23 (2.18, m) (and none with H_{ax}-23) compared with the reported values for **3** made it possible to define the configuration at C-22 as *R*, as usually reported in other withanolides.[9]

Compound **2** was isolated as a white, amorphous solid. The molecular formula was determined to be $C_{30}H_{41}O_9$ from the molecular ion peak at *m/z* 545.2735 $[M + H]^+$ in the HRESIMS. The NMR data (in DMSO-d_6, Table 2) differed from those of compound **1** mainly in the absence of olefinic protons from the α,β-unsatured ketone and additional signals of an acetate functionality (δ_H 1.95; δ_C 21.7 and 170.5). The presence of an acetate was supported by an $[M - 60]^+$ fragment ion in the ESIMS spectrum. The ^{1}H NMR spectrum exhibited signals of five methyl groups (δ_H



1. R = OH

3. R = H



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* To whom correspondence should be addressed. Tel/Fax: +33 320964955. E-mail: vincent.roumy@univ-lille2.fr.
† Lille 2 University.
‡ Lille 2/Institut Pasteur Lille.
§ IRC Lille.
⊥ Phytobôkaz.
∞ These authors contributed equally to this work

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Table 1. NMR Spectroscopic Data ($CD_3OD-CDCl_3$, 1:1) for Compounds **1** and **2**

	compound **1**			compound **2**		
position	δ_c, mult.	δ_H (*J* in Hz)	selective TOCSY (150 ms)	δ_C	δ_H (*J* in Hz)	selective TOCSY (150 ms)
1	201.8, qC			209.6, qC		
2	132.1, CH	6.17 d (10.0)		39.1, CH_2	3.01 dd (5.9, 15.2) 2.59 m	
3	142.3, CH	6.97 dd (5.9, 10.0)		77.2, CH	3.72 m	4, 2
4	69.7, CH	3.73 m		74.8, CH	3.50 m	
5	63.8, qC			64.5, qC		
6	62.3, CH	3.23 d (1.7)	7, 8, 9, 14, 11, 12, 15	60.5, CH	3.23 d (13.1)	7, 8, 9, 14, 11
7	30.6, CH_2	2.13 dd (2.9, 14.2) 1.26 t (14.2)		30.6, CH_2	2.12 m 1.30 m	6, 8, 9, 11, 14, 12, 15
8	28.3, CH	1.40 m		30.4, CH	1.59 m	
9	43.0, CH	1.08 m	11, 8, 12, 7, 14	41.3, CH	1.35 s	
10	47.3, qC			50.0, qC		
11	30.4, CH_2	1.35 m 2.03 dt (4.6, 13.5)		30.9, CH_2	2.01 m 1.37 m	
12	76.7, CH	3.36 dd (4.6, 11.2)	11, 9, 8, 7, 14, 6	76.7, CH	3.56 dt (4.3, 10.9)	
13	47.4, qC			48.4, qC		
14	54.6, CH	0.92 m (7.4, 11.4)	8, 15, 7, 16, 9, 17, 6, 11, 12	51.6, CH	1.28 s	
15	22.9, CH_2	1.67 m		33.6, CH_2	1.58 m, H_a 1.82 m, H_b	
16	24.1, CH_2	1.78 m		75.5, CH	5.22 dt (3.2, 7.7)	17, 15, 14, 8
17	59.9, CH	1.90 t (10.9)		62.7, CH	2.37 t (7.7)	16, 15, 14, 8
18	8.7, CH_3	0.76 s		10.0, CH_3	0.76 s	
19	17.3, CH_3	1.38 s		15.8, CH_3	1.28 s	
20	74.9, qC			74.7, qC		
21	23.2, CH_3	1.18 s		21.9, CH_3	1.18 s	
22	79.0, CH	4.80 dd (3.0, 13.0)	23	79.4, CH	4.99 ddd (3.2, 13.2)	
23	32.6, CH_2	2.18 m 2.58 t (16.2)		31.3, CH_2	2.11 dd (3.2, 17.2) 2.65 dd (13.2, 17.2)	
24	149.0, qC			149.6, qC		
25	122.0, qC			121.6, qC		
26	166.0, qC			166.0, qC		
27	12.3, CH_3	1.87 s		12.4, CH_3	1.85 s	
28	20.6, CH_3	1.93 s		20.0, CH_3	1.95 s	
29				171.1, qC		
30				20.6, CH_3	2.01 s	

0.68, 1.08, 1.75, 1.92, 1.95) and no olefinic proton signals. HSQC and HMBC correlations indicated two epoxy groups (at C-3,C-4 and C-5,C-6) and established the acetate functionality at C-16. Selective TOCSY (Table 1) of H-16 (δ_H 5.22) with H-17 (δ_H 2.37), H-15 (δ_H 1.58, 1.82), H-14 (δ_H 1.28), and H-8 (δ_H 1.59) supported the proposed structure. The relative configuration of **2** was established following the same methodology as for **1**. NOE correlations of H-4 with H-3 and H-6; H-12 with H-14 and H-17; and H_a-15 with H-16 and H-14, with the observed H-22 (δ_H 4.99) coupling constants ($J = 3.2$, 13.2 Hz), as well as NOE correlation of H-22 with H_{eq}-23 as observed for **3** again indicated the *R* configuration at C-22.[9]

Configuration of the two new compounds was supported by data on other withanolides from *A. arborescens*, the main differences being an epoxide at C-3/C-4, acetylation at C-16, and hydroxylation at C-12.[3] The difference in chemical shift observed for H-22 could be explained by the presence of an OH group at C-12, interactions with the analyzing solvent, and the presence of hydrogen bonds.

The antifungal activity of light petroleum and MeOH extracts from *A. arborescens* was assayed against 13 fungi: five dermatophytes (*Microsporum canis*, *Trichophyton interdigitale*, *T. mentagrophytes*, *T. tonsurans*, *T. rubrum*) one nondermatophyte mold (responsible for onychomycosis: *Scytalidium dimidiatum*), six yeasts (*Candida albicans*, *C. glabrata*, *C. kefyr*, *C. krusei*, *C. parapsilosis*, *Malassezia* sp.), and *Pneumocystis carinii,* an atypical fungal microorganism. Growth-inhibitory activity was observed only for the MeOH extract against *P. carinii* (41% growth inhibition at 1 μg/mL) but not for other fungi or extracts (at 50 μg/mL). Bioassay-guided purification of this extract resulted in identification of **3** (withanolide D) (Table 3) as the most active compound. The intensity and the specificity of this anti-*Pneumocystis* activity is interesting, as *Pneumocystis* pneumonia remains a serious disease for immunocompromised patients and, to date, no drugs have been more clinically effective than trimethoprim−sulfametoxazol or pentamidine isethionate. Moreover, this was the first time anti-*Pneumocystis* activity has been observed in withanolides. The activity was observed at 13 concentrations, and EC_{50} was 0.084 ± 0.020 μM for pentamidine and 0.27 ± 0.04 μM for withanolide D (Table 3). Pentamidine is considered to be the most active commercially available drug *in vitro* to cure *P. carinii* pneumonia, but it is used only in cases of fungal resistance against the trimethoprim−sulfametoxazol treatment.[12−14] In spite of the toxicity previously described for withanolide D (LC_{50}: 1.10 ± 0.06 μM on MCF-7; LC_{50}: 1.30 ± 0.05 μM on HeLa cancer cell lines), anti-*Pneumocystis* activity remains significant and specific in comparison with reference drugs (only 3 times less active than pentamidine).[12]

Withanolide D (**3**) and compound **1** were active against tyrosine kinase inhibitor-resistant myeloid leukemia cells, K562R (imatinib-resistant human leukemic cells), with IC_{50} values of 1.23 μM (for withanolide D) and 1.54 μM (for compound **1**), and DA1-3b/M2 (imatinib- and dasatinib-resistant murine leukemic cells) with IC_{50} 0.52 and 0.93 μM, respectively. Interestingly, the toxicity of these compounds was lower for healthy human and mouse control cells (IC_{50} 7.24 for **3** and 9.71 μM for **1**) on bone marrow cells from healthy volunteers, and IC_{50} values were 1.37 μM for both active compounds on hematopoietic mouse stem cells EML-C1. The

Table 2. NMR Spectroscopic Data (DMSO-d_6) for Withanolide D and Compounds **1** and **2**

	withanolide D (**3**)		compound **1**		compound **2**	
position	δ_C, mult.	δ_H (J in Hz)	δ_C	δ_H (J in Hz)	δ_C	δ_H (J in Hz)
1	202.3, qC		202.2		209.3	
2	131.8, CH	6.13 d (9.9)	131.6	6.15 d (10.0)	40.0	2.68 m
						2.62 m
3	145.7, CH	6.92 dd (6.2, 9.9)	145.9	7.11 dd (6.1, 10.0)	77.9	3.55 m
4	69.1, CH	3.54 d (6.2)	69.0	3.58 dd (6.1, 4.1)	73.4	3.30 m
5	63.7, qC		63.5		63.6	
6	59.3, CH	3.19 s	59.5	3.21 br, s	57.5	3.11 br s
7	31.2, CH_2	1.27 m	30.6	2.13 dd (2.9, 14.2)		2.04 m
		2.01 dt (2.4, 4.0)		1.26 t (14.2)	30.6	1.22 m
8	29.3, CH	1.30 m	28.3	1.40 m	27.4	1.20 m
9	43.9, CH	0.76 m	42.7	0.86 m	40.9	1.23 m
10	47.7, qC		47.3		49.7	
11	21.0, CH_2	1.28 m	30.34	1.37	40.4	1.10 m
		1.48 m		1.32		
12	40.0, CH_2	1.08 m	75.8	3.36 dt	76.4	3.28 br s
		1.83 m		(4.7, 11.2)		
13	42.4, qC		47.4		48.6	
14	56.2, CH	0.93 m	54.9	0.92 q (7.6, 11.2)	51.8	1.19 m
15	24.1, CH_2	1.02 m	22.9	1.67 m	33.7	1.47 dt (5.5, 13.9), H_a
		1.53 m				1.71 m, H_b
16	21.8, CH_2	1.52 m	24.1	1.78 m	74.1	5.24 br q (7.2)
		1.76 m				
17	54.4, CH	1.53 m	57.1	2.05 t (11.2)	62.3	2.17 m
18	13.7, CH_3	0.75 s	8.7	0.67 s	10.2	0.68 s
19	16.4, CH_3	1.25 s	16.6	1.27 s	14.8	1.08 s
20	74.4, qC		73.9		73.7	
21	21.0, CH_3	1.12 s	21.8	1.05 s	23.1	1.09 s
22	81.3, CH	4.05 dd (3.5, 13.2)	80.0	4.98 dd (3.3, 13.0)	80.1	4.79 br d (13.6)
23	31.4, CH_2	2.20 dt (3.5, 16.9)	31.2	2.18 m	30.6	2.16 m
		2.38 dd (13.2, 16.9)		2.54 br t (16.2)		2.42 m
24	150.9, qC		149.0		150.3	
25	120.6, qC		122.0		120.8	
26	166.3, qC		166.5		166.3	
27	12.6, CH_3	1.76 s	12.7	1.78 s	12.7	1.75 s
28	20.6, CH_3	1.91 s	20.6	1.93 s	20.5	1.92 s
29					170.5	
30					21.7	1.95 s
OH-4		5.61 br, s		5.66 d (4.1)		
OH-12				4.76 d (4.7)		4.81 m
OH-20		4.32 s		4.60 s		4.44 m

Table 3. *In Vitro* Biological Activities of Compounds **1**−**3** on *P. carinii* and Leukemic Cells

	IC_{50} (μM)				
	fungus	leukemic cells		healthy marrow cells	
compound	*P. carinii*	K562R	DA1-3b/M2	BM cells[a]	EML-C1
withanolide D (**3**)	0.27 ± 0.04	1.23 ± 0.07	0.52 ± 0.04	7.24 ± 0.10	1.37 ± 0.05
1	2.0 < < 20.0	1.54 ± 0.08	0.93 ± 0.03	9.71 ± 0.55	1.37 ± 0.09
2	>100	30.0 ± 1.5	28.30 ± 0.85	>100	15.3 ± 0.6
pentamidine	0.084 ± 0.020	n.t.[b]	n.t.	n.t.	n.t.
camptothecin (nM)	n.t.	2.0 ± 0.2	30.0 ± 0.5	n.t.	n.t.

[a] Bone marrow cells from healthy volunteers. [b] Nontested compounds.

greater activity on murine cells can be explained by the acute sensitivity of these cells. These results proved the selective activity of withanolide D and compound **1** *in vitro*, but did not justify complementary biological investigations.

Such biological and structural data led us to assume that the double bond between C-2 and C-3, the hydroxylation at C-4, and the methylene group at C-12 are significant elements contributing to the biological activity and specificity of withanolides. Withanolides from different Solanaceae genera (*Withania*, *Datura*, *Acnistus*) have previously been studied for their antitumor activity, but the high toxicity of those compounds did not allow clinical trials. Withanolides have been reported previously from leaves of *A. arborescens*; however, this is the first report of compounds **1** and **2** and of antifungal activities of withanolides against *P. carinii*.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a Perkin-Elmer 343 polarimeter. UV spectra were recorded on a Biochrom WPA Lightwave II UV−visible spectrometer. IR spectra were recorded on a Thermo Nicolet Avatar 320 FT-IR spectrometer. NMR spectra were recorded in CD_3OD−$CDCl_3$ (1:1) and DMSO-d_6 on a Bruker Avance 500 spectrometer in the Laboratoire d'Application de RMN (LARMN), Université de Lille 2. Mass spectra (ESIMS^2) were performed on a PE Sciex API 3000 triple quadrupole mass spectrometer equipped with an ionspray-turbo source (Perkin-Elmer Sciex). HRESIMS spectra were acquired with a Thermo Scientific Orbitrap Exactive mass spectrometer (Bremen, Germany), using a heated electrospray. Chromatographic separations were carried out by HPLC using a Shimadzu LC-10 AS pump and SCL-10A detector with a semipreparative RP18 column (Silica Upti-prep Strategy, 100 Å, 5 μm RP, 25 × 1 cm Interchrom, Interchim, Montluçon, France). MPLC was performed

on a Büchi system composed of a C-605 pump module and a Büchi column (Büchi 460 × 35 mm packed with Merck silica gel 60 H; Darmstadt, Germany). Fractions were monitored by TLC (silica gel 60 F_{254}; Merck; Darmstadt, Germany).

Plant Materials. The leaves of *A. arborescens* were collected in December 2007 at Gourbeyre in Guadeloupe (Lesser Antilles). A voucher specimen is deposited in the French Institute of the Agricultural Research Centre (INRA) at Petit-bourg (Guadeloupe), number 5601.

Extraction and Isolation. Powdered leaves of *A. arborescens* (610 g) were defatted by percolation with light petroleum followed by extraction with MeOH. The dried MeOH extract (129 g) was then partitioned between H_2O (800 mL) and EtOAc (2 × 800 mL), and the residual aqueous phase was further extracted with an EtOAc−MeCN (1:1; 2 × 800 mL) mixture to obtain fractions F_1 (3.5 g) and F_2 (4.5 g). Each fraction was subjected to MPLC on silica gel, eluted with a MeOH−CH_2Cl_2 gradient (0:100−20:80). Fractionations were monitored by TLC with visualization under UV (254 and 365 nm) and mainly revealed with Dragendorff reagent. Five fractions were obtained from F_1 (A to E), and fractions B and D were separated by successive MPLC on silica gel then isocratic semipreparative RP18-HPLC using MeOH−H_2O (65:35) to yield **3** (25 mg) and MeOH−H_2O (60:40) to give **1** (8 mg). Fraction G, obtained from F_2 by MPLC on silica gel, was also subjected to isocratic semipreparative RP18-HPLC [MeOH−H_2O (57:43)] to yield **2** (7 mg).

(20*R*,22*R*)-5*β*,6*β*-Epoxy-4*β*,12*β*,20-trihydroxy-1-oxowith-2-en-24-enolide (1): white, amorphous solid; $[\alpha]^{24}_D$ +4.1 (*c* 0.7, MeOH); UV (MeOH) λ_{max} (log ε) 218 (3.66) nm; IR (film) ν_{max} 3338, 2966, 1682, 1096, 1025 cm^{-1}; ^1H NMR and ^{13}C NMR ($CDCl_3$−CD_3OD, 1:1; or DMSO-d_6) data, Tables 1 and 2; ESIMS-MS *m*/*z* 487 $[M + H]^+$ (6), 469 (35), 451 (62), 433 (26), 415 (8), 301 (13), 283 (10), 265 (4), 169 (100), 125 (3); HREIMS (positive) *m*/*z* 487.2678 ($[M + H]^+$ calcd for $C_{28}H_{39}O_7$, 487.2690).

(20*R*,22*R*)-16*β*-Acetoxy-3*β*,4*β*;5*β*,6*β*-diepoxy-12*β*,20-dihydroxy-1-oxowith-24-enolide (2): white, amorphous solid; $[\alpha]^{24}_D$ −1.16 (*c* 0.6, MeOH); UV (MeOH) λ_{max} (log ε) 223 (3.52) nm; IR (film) ν_{max} 3260, 2960, 1705, 1696, 1684 cm^{-1}; ^1H NMR and ^{13}C NMR ($CDCl_3$−CD_3OD, 1:1; or DMSO-d_6) data, Tables 1 and 2; EIMS-MS *m*/*z* 545 $[M + H]^+$ (33), 527 (45), 509 (30), 491 (8), 485 (13), 467 (93), 449 (100), 431 (52), 413 (22), 395 (4), 299 (74), 281 (30), 169 (37), 153 (12), 125 (4); HRESIMS (positive) *m*/*z* 545.2735 ($[M + H]^+$ calcd for $C_{30}H_{41}O_9$, 545.2745).

Biological Materials. Fungal strains were isolated by Mycology Laboratories in Regional University Hospitals in Guadeloupe, Martinique, and France. Medium was composed of $RPMI_{1640}$ buffered at pH 7 with MOPS; two antifungal standards (griseofulvine, Sigma; fluconazole, Pfizer) at concentrations of 0.13 to 64.0 mg/mL in microplates were used. Dermatophytes and moist aqueous suspensions were diluted in $RPMI_{1640}$ medium (Gibco/BRL, Eggenstein, Germany) at (1−5) × 10^3 cfu/mL. The yeast inoculum was standardized in water at 0.5 McFarland using a densitometer and diluted in $RPMI_{1640}$ medium to obtain 10^4 cfu/mL. Antifungal activity was determined after distributing the inoculi in 96-well plates (100 μL) and adding 100 μL of culture medium containing extract at various concentrations (50 to 500 μg/mL). The agar diffusion method was used for *Malassezia* sp., and the mixture $RPMI_{1640}$−extract was incorporated into a Sabouraud agar medium preinoculated before cooling in a Petri dish. After 48 h (for yeast and moist) or 7 days (for dermatophytes) of incubation, cell growth was visually estimated.

Isolation and Quantitation of *P. carinii*. Athymic Lou nu/nu rats (Pasteur Institute, Lille, France) were administered dexamethasone (Merck Sharp & Dohme Chibret, Paris, France) at a final concentration of 1 mg/L in their drinking water for 2 weeks. Then, after isoflurane anesthesia, the rats were inoculated with 10 × 10^6 cryopreserved parasites following a nonsurgical endotracheal method.[13] Dexamethasone administration was maintained for approximately 7 weeks to obtain highly *P. carinii*-infected animals. At the end of the immunosuppression period the rats were sacrificed and *P. carinii* was recovered from their lungs as previously described.[14] *In vitro* pharmacodynamic properties were determined using the Hill equation (E_{max} sigmoid model). *In vitro* susceptibility studies were performed using the broth microdilution technique.[20] Final drug concentrations ranged from 1.1 × 10^{-5} to 212.3 μM for each compound. All the experiments were carried out in 24-well plates with a final volume of 2 mL of Dulbecco's modified Eagle's medium (DMEM) (Bio-Whittaker, Boehringer Ingelheim, Brussels, Belgium) supplemented with 10% fetal calf serum (FCS) (Gibco BRL, Life Technologies Inc.) containing a final inoculum of 1.0 × 10^6 organisms per mL. Plates were then incubated for 4 days in an atmosphere of 5% CO_2 at 37 °C. One drug-free control was included in each assay. Five microliter smears were obtained from each suspension. *P. carinii* organisms were stained with RAL-555 and were microscopically quantified. All susceptibility assays were in triplicate.[15−17] Extracts, withanolides, or pentamidine isothionate (Sigma Chemical Co., St Louis, MO) were dissolved in 100% DMSO to produce a 10 mg/mL stock solution. The drug stock solutions were then diluted in DMEM supplemented with 10% heat-inactivated FCS to produce the required drug concentrations.

Cytotoxic Activity. The imatinib-resistant human chronic myeloid leukemia K562 cell line (K562R) and imatinib- and dasatinib-resistant leukemic mouse DA1-3b $p210^{BCR-ABL}$ cell line (DA1-3b/M2) have been described previously.[18−20] EML-C1, hematopoietic mouse stem cells (ATCC, Manassas, VA), were cultured according to ATCC instructions. Bone marrow cells were obtained from three healthy volunteers (BM cells).[21,22] Donors gave informed consent in accordance with the Declaration of Helsinki. K562R, DA1-3b/M2, and BM cells were cultured in RPMI 1640 medium supplemented with 1% glutamine, 1% penicillin/streptomycin, and 10% fetal calf serum.[23]

Cell toxicity was analyzed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) kit (Promega, Madison, WI). Briefly, cells were seeded at a density of 10^4 cells per well in a 96-well plate and treated with drug or DMSO (vehicle). MTT (20 μL) was added to each well for the last 4 h of 72 h cultures. Absorbance was measured at 490 nm using a SpectraMaxPlus 384 spectrophotometer (Molecular Devices). All experiments were performed in triplicate. Camptothecin was used as the positive control.

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Supporting Information Available: ^1H and ^{13}C NMR spectra and anti-*Pneumocystis* activity of compounds **2** and **3**. This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) De la Cruz, H.; Vilcapoma, G.; Zevallos, P. *J. Ethnopharmacol.* **2007**, *111*, 287–294.
(2) Usubillaga, A.; Khouri, N.; Baptista, J.; Bahsas, A. *Rev. Lationoam. Quim.* **2005**, *33*, 121–127.
(3) Veras, M. L.; Bezerra, M. Z.; Lemos, T. A.; Uchoa, D.; Braz-Filho, R.; Chai, H.; Cordell, G.; Pessoa, O. D. *J. Nat. Prod.* **2004**, *67*, 710–713.
(4) Barata, L.; Mors, W. B.; Kirson, I. A. *An. Acad. Bras. Ciénc.* **1970**, *42*, 401–407.
(5) Gemeinholzer, B., Wink, M. *Solanaceae: Occurrence of Secondary Compounds versus Molecular Phylogeny, Advances in Taxonomy and Utilization*; Nijmegen University Press: Nijmegen, 2001; Vol. 5, pp 165−178.
(6) Dei-Cas, E. *Med. Mycol.* **2000**, *38*, 23–32.
(7) Aliouat-Denis, C. M.; Chabé, M.; Demanche, C.; Aliouat, E. M.; Viscogliosi, E.; Guillot, J.; Delhaes, L.; Dei-Cas, E. *Infect. Genet. Evol.* **2008**, *5*, 708–726.
(8) Dinan, L.; Harmatha, J.; Lafont, R. *J. Chromatogr. A* **2001**, *935*, 105–123.
(9) Minguzzi, S.; Barata, L.; Geun, Y. G.; Jonas, P. *Phytochemistry* **2002**, *59*, 635–641.
(10) Kupchan, S. M.; Anderson, W. K.; Bollinger, P.; Doskotch, R. W.; Smith, R. M.; Saenz-Renauld, J. A.; Schnoes, H. K.; Burlingaure, A. L.; Smith, D. H. *J. Org. Chem.* **1969**, *34*, 3858–3866.
(11) Kupchan, S. M.; Doskotch, R. W.; Bollinger, P.; McPhail, A. T.; Sim, G. A.; Renauld, J. A. *J. Am. Chem. Soc.* **1965**, *87*, 5805.
(12) Cordero, C. P.; Morantes, S. J.; Páez, A.; Rincón, J.; Aristizábal, F. A. *Fitoterapia* **2009**, *80*, 364–368.
(13) Roblot, F.; Godet, C.; Le Moal, G.; Garo, B.; Souala, M. F. *Eur. J. Clin. Microbiol. Infect. Dis.* **2002**, *7*, 523–531.
(14) Ambrose, H. E.; Keely, S. P.; Aliouat, E. M.; Dei-Cas, E.; Wakefield, A. E.; Miller, R F.; Stringer, J. R. *Microbiology* **2004**, *150*, 293–300.
(15) Aviles, P.; Aliouat, E. M.; Martinez, A.; Dei-cas, E.; Herreros, E. *Antimicrob. Agents Chemother.* **2000**, *44*, 1284–1290.
(16) Garry, S.; Nesslany, F.; Aliouat, E. M.; Haguenoer, J. M.; Marzin, D. *Mutat. Res.* **2003**, *538*, 19–29.
(17) Roux, P.; Lavrard, I.; Poirot, J. L.; Chouaid, C.; Denis, M.; Oliver, J. L.; Nigou, M.; Miltgen, M. *J. Clin. Microbiol.* **1994**, *32*, 2324–2326.

(18) Alkhatib, R.; Joha, S.; Cheok, M.; Roumy, V.; Idziorek, T.; Preudhomme, C.; Quesnel, B.; Sahpaz, S.; Bailleul, F.; Hennebelle, T. *Planta Med.* **2010**, *76*, 86–87.

(19) Alkhatib, R.; Hennebelle, T.; Joha, S.; Idziorek, T.; Preudhomme, C.; Quesnel, B.; Sahpaz, S.; Bailleul, F. *Phytochemistry* **2008**, *69*, 2979–2983.

(20) Liu, J.; Joha, S.; Idziorek, T.; Corm, S.; Hetuin, D.; Philippe, N.; Preudhomme, C.; Quesnel, B. *Leukemia* **2008**, *4*, 791–799.

(21) Mahon, F.X.; Deininger, M. W.; Schultheis, B.; Chabrol, J.; Reiffers, J.; Goldman, J. M.; Melo, J. V. *Blood* **2000**, *96*, 1070–1079.

(22) Aoyama, K.; Oritani, K.; Yokota, T.; Ishikawa, J.; Nishiura, T.; Miyake, K.; Kanakura, Y.; Tomiyama, Y.; Kincade, P. W.; Matsuzawa, Y. *Blood* **1999**, *93*, 2586–2594.

(23) Takemura, T.; Nakamura, S.; Yokota, D.; Hirano, I.; Ono, T.; Shigeno, K.; Fujisawa, S.; Ohnishi, K. *J. Biol. Chem.* **2010**, *285*, 6585–6594.

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