Cardenolides from *Pergularia tomentosa* Display Cytotoxic Activity Resulting from Their Potent Inhibition of Na^+/K^+-ATPase

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Two new cardenolide glycosides (**1** and **2**), along with six known cardenolide glycosides (**3**−**8**), have been isolated from the roots of *Pergularia tomentosa*. In order to investigate their potential anticancer activity, these compounds were tested in an in vitro growth inhibitory assay (a MTT colorimetric assay), including six different human cancer cell lines, and for their ability to inhibit Na^+/K^+-ATPase activity, in addition to the morphologic changes induced in human cancer cell lines (using computer-assisted phase-contrast microscopy). The data revealed that these cardenolides displayed marked cytotoxic activity. The results obtained suggest that structural characteristics of the cardenolides studied, with the A/B rings of the steroidal skeleton *trans* fused and containing a single sugar in a unique "dioxanoid" attachment, confer on them specific cytotoxic properties that are distinct from those displayed by classic cardenolides such as digoxin.

Cardiotonic steroids (cardiac glycosides) represent a group of compounds that share the capacity to bind to the extracellular surface of the main ion transport protein in the cell, the membrane-inserted sodium pump (Na^+/K^+-ATPase).[1] Cardiotonic steroids, comprising cardenolides and bufadienolides, are characterized by the large number known, the diversity of structure exhibited, a potential for chemical modification, and their wide use in cardiology for congestive heart failure management. Over the past few years, there has been a marked increase in the number of reports of cardiotonic steroid-induced anticancer effects.[1,2] Cardiac glycoside-mediated effects are linked to their Na^+/K^+-ATPase binding characteristics. We reported recently that overexpressed Na^+/K^+-ATPase α subunits are newly important anticancer targets.[1,3] Cardiotonic steroids are thus promising new anticancer agents with novel mechanisms of action.[1,3]

Generally, in cardiac glycosides from *Digitalis* and *Strophanthus* plant species (such as digoxin, digitoxin), rings A/B and C/D are *cis* fused, while rings B/C are *trans* fused. Such ring fusion gives the aglycon nucleus of these cardiac glycosides a characteristic "U" shape.[1,4,5] In cardiac glycosides produced by plants from the milkweed family, Asclepiadaceae (such as calactin, uscharin, and 2″-oxovoruscharin), the A/B rings are *trans* fused, resulting thus in rather flat structures. The steroidal skeleton can be substituted at position 3 by a sugar moiety (glycoside), leading to the chemical classification of subfamilies such as glycosylated cardenolides or glycosylated bufadienolides (depending on the nature of the lactone moiety). Whereas cardiac glycosides from *Digitalis* and *Strophanthus* species contain sugar units linked through the 3*β*-OH of the steroid aglycon (single link), such compounds produced by plants from the Asclepiadaceae contain a single sugar in a unique "dioxanoid" attachment (doubly linked; Figure 1).[1,4−10] The consequences of these structural differences on the Na^+/K^+-ATPase binding of these compounds have been reported previously[9−11] and indicate the markedly more potent binding (particularly to Na^+/K^+-ATPase α1 subunits) of the *trans*−*trans*−*cis* cardiotonic steroids such as 19-hydroxy-2″-oxovoruscharin (UNBS1450).

The family Asclepiadaceae is a very rich source of the cardenolide cardiac glycosides. *Pergularia tomentosa* L., a climbing to

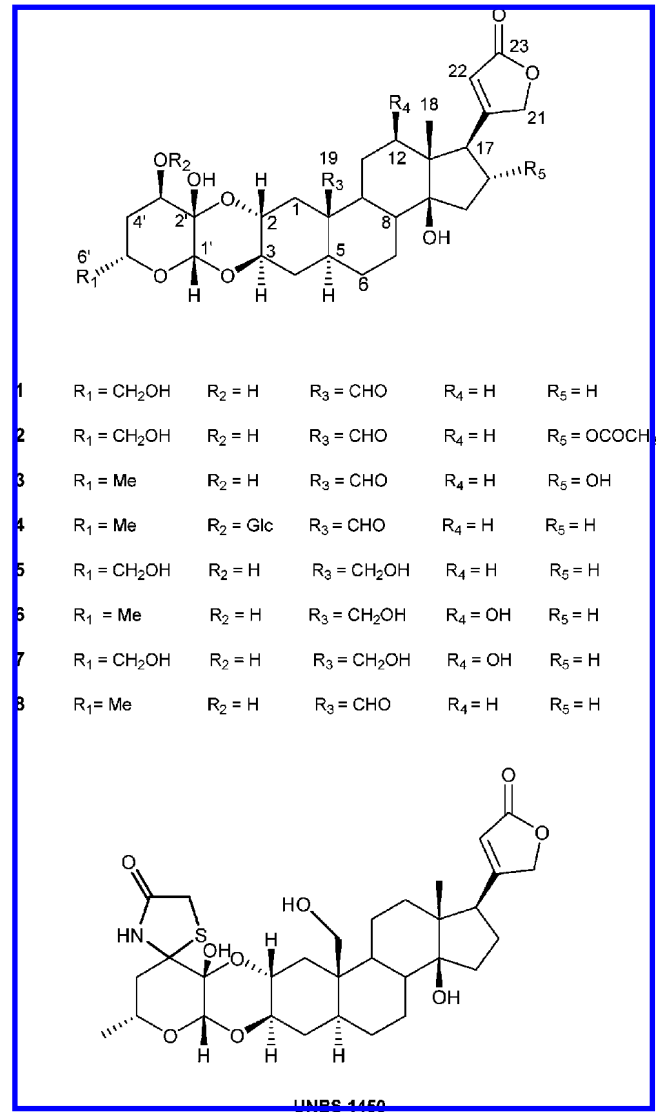

Figure 1. Chemical structures of cardenolides isolated from *P. tomentosa* and UNBS1450.

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semierect perennial herb,[12] belongs to the milkweed family[13] and has been reported as having molluscicidal[14] and antifungal[11] activities. The roots have been found applicable for the treatment of bronchitis, constipation, and skin disease, and as an abortive agent.[15] *P. tomentosa* is a rich source of cardenolide glycosides such as desglucouzarin, coroglaucigenin, and uzarigenin in the leaves[7,13,16] and uzarigenin, pergularoside, ghalakinoside, and calactin and their derivatives in the roots.[7]

Cardenolides of *P. tomentosa* are characterized by a sugar moiety linked to the 2α- and 3β-positions of the aglycon by hemiacetal and acetal functions, respectively, generating a "dioxanoid" structure.[17] The unique sugar unit is represented by 4,6-dideoxyhexosulose and a modified form of latter, 4-deoxyhexosulose.[13] The former sugar unit has been reported only in ghalakinoside and dehydroxyghalakinoside,[7,13] cardenolides found only in *P. tomentosa*. We recently reported the isolation and characterization of three new cardenolide glycosides along with the known cardenolide glycosides ghalakinoside and calactin from the roots of *P. tomentosa*.[7] Within the framework of an investigation aiming to test the ability of double-linked cardenolides to interfere with the Na^+/K^+-ATPase pump resulting in cytotoxic activity, two new cardenolide glycosides (**1** and **2**), along with several previously described[7] (**3**−**8**), have been isolated from the roots of *P. tomentosa*. The structures **1** and **2** were elucidated via the application of spectroscopic methods including 1D- (^1H and ^{13}C) and 2D-NMR (DQF-COSY, HSQC, HMBC, and TOCSY) experiments as well as ESIMS analysis.

In order to investigate their potential anticancer activity, these compounds were tested (i) in an in vitro growth inhibitory assay (the MTT colorimetric assay) including six different human cancer cell lines, (ii) for their ability to inhibit Na^+/K^+-ATPase activity, and (iii) for the morphologic changes they induce in human cancer cell lines, as evidenced using cellular imaging devices that rely on computer-assisted phase-contrast microscopy.[18,19]

Results and Discussion

The roots of *P. tomentosa* were extracted with 80% EtOH, and the obtained extract was fractionated over Sephadex LH-20. Fractions containing cardenolide glycosides were chromatographed by RP-HPLC to yield two new compounds (**1** and **2**) and six known compounds (**3**−**8**) (see Experimental Section).

Compound **1** was obtained as an amorphous white solid, and its molecular formula, $C_{29}H_{40}O_{10}$, was deduced by HRMALDITOFMS analysis (*m/z* 549.6298 $[M + H]^+$, calcd for $C_{29}H_{41}O_{10}$, 549.6292). Its IR spectrum exhibited absorption bands for hydroxy (3438 cm^{-1}), carbonyl (1750 cm^{-1}), and olefinic (1635 cm^{-1}) groups. The UV spectrum was consistent with the presence of an α,β-unsaturated carbonyl group (λ_{max} 218 nm). The ^1H NMR spectrum of the aglycon portion of **1** showed characteristic signals of a butenolactone ring at δ 5.98 (1H, d, $J = 1.6$ Hz), 5.05 (1H, dd, $J = 18.2$, 1.6 Hz), and 4.94 (1H, dd, $J = 18.2$, 1.6 Hz), as well as a singlet proton at δ 10.07 (1H, s) and a methyl signal at δ 0.85 (3H, s), indicating a cardenolide skeleton with an aldehyde function. The ^{13}C NMR spectrum of **1** showed 29 carbon signals, of which 23 could be assigned to the aglycon moiety and six to a sugar portion. The ^{13}C NMR chemical shifts of all the hydrogenated carbons could be assigned unambiguously from the HSQC spectrum. In particular, the analysis of the ^{13}C NMR spectrum on the basis of the HSQC correlations confirmed clearly in the aglycon moiety the occurrence of an aldehyde carbon (δ 209.3) along with signals typical of a butenolactone ring: a carbonyl group (δ 177.2), an olefinic quaternary function (δ 178.3), an olefinic methine (δ 117.7), and a primary alcoholic function (δ 74.9). The ^1H and ^{13}C NMR chemical shifts of the aglycon moiety of **1** were superimposable on those of the known compound calactin (**8**).[13] Additionally, the ^1H NMR spectrum of **1** displayed a signal corresponding to an anomeric proton at δ 4.75 (1H, s), which suggested that **1** is a doubly linked glycoside. Instead of the signal corresponding to the methyl signal at δ 1.15 (3H, d) in calactin (**8**), a signal centered at δ 3.60 (2H, m), corresponding to a CH_2OH group at C-5, was observed. This was confirmed by the signal in the ^{13}C NMR spectrum at δ 65.5 instead of δ 20.9 for the Me group in calactin (**8**). On the basis of 1D-TOCSY, DQF-COSY, HSQC, and HMBC NMR experiments, the occurrence of a 4-deoxyhexosulose unit was identified. The relative configurations of C-3′ and C-17 were established by analysis of ^1H NMR coupling constants. The *J* coupling constant value of H-3′ (δ 3.61, t, $J = 2.0$ Hz) indicated an equatorial position and thus an α-orientation of H-3′.[20] The relative configuration of H-17 was assigned as α on the basis of coupling constants of the signal at δ 2.86 (1H, dd, $J = 5.7$, 3.1 Hz), in line with data published for 17β-cardenolides.[21] On the basis of this evidence, the structure of compound **1** was established as the new compound 6′-hydroxycalactin.

Compound **2**, obtained as a colorless amorphous solid, displayed a molecular formula, $C_{31}H_{42}O_{12}$, as determined from its HRMALDITOF mass spectrum (*m/z* 607.2755 $[M + H]^+$, calcd for $C_{31}H_{43}O_{12}$, 607.2763). The ^1H NMR spectrum of **2** in comparison to that of **1** showed the presence of a signal at δ 5.29 (1H, td, $J =$ 8.0, 4.0 Hz), corresponding to a secondary alcohol group, and a signal at δ 2.10 (3H, s), ascribable to an acetate group. The NMR data of **2** were in good agreement with those of **1** except for the presence of a secondary alcohol function located at C-16, as shown by the HMBC correlations between the proton signal at δ 5.29 and the carbon resonances at δ 39.0 (C-15), 58.9 (C-17), and 49.9 (C-13) and by the COSY correlation between the proton signals at δ 5.29 and 2.74 (H-17). The downfield shifts of the C-16 resonances (δ_H 5.29, δ_C 79.9) suggested esterification at this position. The HMBC correlation between the proton signal at δ 5.29 (H-16) and the carbon resonance at δ 172.7 ($COCH_3$) confirmed the location of an acetoxy function at C-16. The relative configuration of H-16 was established by analysis of ^1H NMR coupling constants. The *J* coupling constant value of H-17 (δ 2.74, d, $J = 4.0$ Hz) suggested a β orientation for H-16 and hence an α-orientation for the acetoxy function.[22] Thus, the structure of **2** was established as the new compound 6′-hydroxy-16α-acetoxycalactin.

Compound **3** was identified as 16α-hydroxycalactin[23] and is being reported for the first time in a plant of the *Pergularia* genus. The other known compounds were identified as 3′-*O*-α-D-glucopyranosylcalactin (**4**),[7] 12-dehydroxyghalakinoside (**5**),[7] 6-dehydroxyghalakinoside (**6**),[7] ghalakinoside (**7**),[13] and calactin (**8**).[13]

The antiproliferative effects of the cardenolides extracted from the roots of *P. tomentosa* were assessed by a cytotoxic MTT assay on six human cancer cell lines from different histological types.[24] As presented in Table 2, the cardenolides from *P. tomentosa* were found to be very potent antiproliferative agents; the most potent substance was calactin (**8**). This activity is only slightly dependent on cell type, in sharp contrast with certain other compounds recently tested.[6] All eight cardenolides displayed IC_{50} values 10 times lower for A549 cells than in the five other cancer cell lines of different origin. In line with this, it is worth noting that another cardenolide (UNBS1450, Figure 1) bearing similar structural specificities (i.e., *trans* A/B rings and a doubly linked sugar) to the present cardenolides extracted from *P. tomentosa* showed the most potent in vitro growth inhibition of A549 NSCLC cells ($IC_{50} = 0.008$ μM),[6] and an orthotopic mouse model bearing A549 NSCLC cells was the most UNBS1450-responsive in vivo preclinical model.[10,11,25,26] Since cardenolide-mediated effects are linked to Na^+/K^+-ATPase binding characteristics, their ability to induce anticancer effects may depend on their structural specificity. As indicated above, in cardenolides bearing a single-linked sugar and A/B *cis* fused rings, the aglycon nucleus is present in a characteristic "U" shape, while in cardiac glycosides produced by plants from the milkweed family (Asclepiadaceae), sugars are doubly linked and the A/B rings are *trans* fused, resulting in rather

Table 1. NMR Spectroscopic Data (600 MHz, CD_3OD) for Compounds **1** and **2**[a]

	1		**2**	
position	δ_C, mult.	δ_H (J in Hz)	δ_C, mult.	δ_H (J in Hz)
1	36.7, CH_2	2.41, dd (12.5, 4.8) 1.13, t (12.5)	36.7, CH_2	2.49, (12.5, 4.4) 1.19, (12.5)
2	70.1, CH	3.89, m	70.1, CH	3.89, m
3	72.1, CH	4.03, m	72.3, CH	4.02, dt (10.9, 4.4)
4	34.3, CH_2	1.67, m 1.42, m	34.2, CH_2	1.66, m 1.40, m
5	44.2, CH	1.59, m	44.1, CH	1.59, m
6	28.3, CH_2	2.27, m	28.5, CH_2	2.25, m 1.68, m
7	28.3, CH_2	2.05, m 2.27, m	28.3, CH_2	2.06, m
8	43.2, CH	1.62, m	43.1, CH	1.62, m
9	49.4, CH	1.46, m	49.4, CH	1.51, m
10	53.9, qC		53.7, qC	
11	22.8, CH_2	1.73, m 1.31, m	22.5, CH_2	1.80, m 1.33, m
12	40.1, CH_2	1.53, m 1.48, m	40.3, CH_2	1.67, m
13	50.6, qC		49.9, qC	
14	85.7, qC		85.2, qC	
15	32.5, CH_2	2.12, m 1.72, m	39.0, CH_2	2.24, d (8.0)
16	27.6, CH_2	2.19, m 1.90, m	79.9, CH	5.29, dt (8.0, 4.0)
17	51.5, CH	2.86, dd (5.7, 3.1)	58.9, CH	2.74, d (4.0)
18	15.7, CH_3	0.85, s	15.8, CH_3	0.83, s
19	209.3, CH	10.07, s	209.1, CH	10.07, s
20	178.3, qC		174.9, qC	
21	74.9, CH_2	5.05, dd (18.2, 1.6) 4.94, dd (18.2, 1.6)	75.2, CH_2	4.98, brd (1.5)
22	117.7, CH	5.98, t (1.6)	118.4, CH	5.98, brd (1.5)
23	177.2, qC		176.2, qC	
1′	95.3, CH	4.75, s	97.3, CH	4.50, s
2′	92.8, qC		92.6, qC	
3′	73.7, CH	3.61, t (2.0)	72.7, CH	3.61, t (2.0)
4′	32.5, CH_2	1.85, m 1.65, m	34.2, CH_2	1.66, m
5′	71.4, CH	3.70, m	73.7, CH	3.65, m
6′	65.5, CH_2	3.60, m	65.5, CH_2	3.61, m
$OCOCH_3$			172.7, qC	
$OCOCH_3$			20.9, CH_3	2.06, s

[a] Assignments were confirmed by HSQC and HMBC experiments.

Table 2. Antiproliferative Activity for Cancer Cell Lines Induced by Cardenolides from *Pergularia tomentosa*[a]

compound	PC3	BxPC3	LoVo	A549	MCF-7	U373
1	0.3	0.6	0.9	0.08	0.5	0.4
2	1.2	4.4	6.4	0.8	4.1	3.4
3	1.0	2.3	4.4	0.4	3.8	2.6
4	0.2	0.5	0.4	0.2	0.3	0.4
5	0.04	0.3	0.2	0.04	0.4	0.1
6	0.05	0.3	0.3	0.05	0.3	0.4
7	0.08	0.3	0.3	0.08	0.3	0.4
8	0.02	0.08	0.05	0.02	0.04	0.2

[a] Data presented as IC_{50} values (μM). The SEM values are not reported for the sake of clarity. Additionally, it should be noted that the highest SEM value calculated was less than 10% of its mean value.

flat structures.[1,4−10] The consequences of these structural differences on the Na^+/K^+-ATPase binding have been reported previously.[9−11]

Accordingly, we aimed to assess the effect on Na^+/K^+-ATPase activity of the eight cardenolides (**1**−**8**) under study. For this purpose, a colorimetric assay was used involving Na^+/K^+-ATPase extracted from porcine cerebral cortex (Sigma-Aldrich, Bornem, Belgium) containing three isoforms of the α subunit (α1−3). The color intensity was proportional to the release of phosphate ions, which is a direct indicator of ATP hydrolysis and, therefore, of sodium pump activity. In Table 3, the data are reported as IC_{50} values of Na^+/K^+-ATPase inhibition (i.e., the concentration at which 50% of the Na^+/K^+-ATPase activity is inhibited by the tested compound), ranging from 0.29 to 1.7 μM (average values for

Table 3. Na^+/K^+-ATPase Activity Inhibition Induced by Cardenolides from *Pergularia tomentosa*[a]

compound	IC_{50} (μM)[a]
1	0.5
2	1.2
3	1.7
4	0.5
5	0.4
6	0.7
7	0.3
8	0.4

[a] SEM values are not reported for the sake of clarity. Additionally, it should be noted that the highest SEM value calculated was less than 4% of its mean value.

UNBS1450: 0.13−0.20 μM). As shown in Figure S7 of the Supporting Information, there was a good correlation (Spearman rank order correlations, $R = 0.763650$, $p = 0.027433$) between the antiproliferative activity (represented by median IC_{50} obtained by means of the MTT assay, see Table 2) and the inhibition of the Na^+/K^+-ATPase (IC_{50} of Na^+/K^+-ATPase inhibition as reported in Table 3).

Recent literature suggests that there exist two pools of Na^+/K^+-ATPase within the plasma membrane, with distinct functions. One is the classic pool of the enzyme as an energy transducing ion pump, and the other the signal transducing pool is restricted to *caveolae*, forming the so-called Na^+/K^+-ATPase signalosome.[27−30] The binding of cardenolides to the sodium pump causes rapid modifications in the signalosome and affects multiple downstream signaling pathways pertinent to cancer. It is important to consider the sodium pump as a signal transducer able to mediate cardiac glycoside-induced effects in a compound-, concentration-, and cell type-specific manner. In line with this, in our previous study, we reported pro-apoptotic effects on Kaposi's sarcoma cells of compounds **4**−**8** isolated from *P. tomentosa* when assayed at 1 and 10 μM.[7] Literature scrutiny indicates there are cardiac glycosides that induce antiproliferative activities in the nM range without altering ion concentrations (as demonstrated for UNBS1450[9,31]) and therefore target the signal-involved Na^+/K^+-ATPase, in contrast to those that significantly alter intracellular ion concentrations when used, mostly at μM concentrations.[1,2,32] Furthermore, while earlier reports implied the mainly pro-apoptotic action of number of different cardiac glycosides tested when used at μM concentrations,[1,2,32−34] more recent articles have suggested pro-autophagic effects (at least for oleandrin and UNBS1450) if used at nM concentrations.[1−3,9,11,24,35] In line with this, and with the aim to further characterize the potential anticancer properties of cardenolides isolated from *P. tomentosa*, we used a cellular imaging assay to investigate the effects of these cadenolides on growth delay, cell death, and morphology modifications.

Cellular imaging is a video-microscopy technique enabling cell observation in time, which allows evaluating the effect of a given product on the morphology, the motility, the death, and proliferation of cells.[18] The cells are maintained alive in closed flasks containing buffered medium at a controlled temperature of 37 ± 0.1 °C during the time required for the experiment (in this case 72 h). Monitoring is conducted with a phase-contrast microscope coupled with a CCD camera. A control monitor placed between the camera and the computer allows visualization at all times of the cells, with a picture taken every 4 min. Movies are made by compressing all the pictures obtained.[19] For this assay, we chose two cell lines: a more responsive one (A549) and a less responsive one (U373), based on the results obtained by the MTT assay. Each compound was assayed at its respective IC_{50} value for the corresponding cell line (Table 2). The cellular imaging revealed growth delay/cell death induction with morphology modifications of NSCLC A549 and U373 glioblastoma cells observed during three days of treatment with cardenolides tested at their IC_{50} concentration. Figure S8 (Supporting Information) presents these characteristics as observed with three

representative compounds: one first reported in the present study (**2**), one first reported by us in our previous study[7] (**4**), and one known cardenolide (ghalakinoside) reported previously[7] (**7**). Also, this experiment enabled us to observe the absence of cell explosion and apoptotic features. Rather, the treated cells displayed rounded morphology, similar that observed with UNBS1450,[9−11,31] suggesting the impairment of cellular cytoskeleton.

As indicated by the IC_{50} values shown in Tables 2 and 3, compound **8** (calactin) was the most active compound, while compound **3**, which differs from calactin only by the occurrence of a further hydroxy group at C-16, exhibited weak cytotoxicity and Na^+/K^+-ATPase activity inhibition. Compounds **3** and **2**, being less active, are characterized by the presence of a hydroxy group and an acetoxy functionality at C-16, respectively. These results indicate the importance of the absence of substitution at C-16 to induce the inhibition of tumor cell proliferation and to maintain a certain level of inhibition of Na^+/K^+-ATPase activity.

Experimental Section

General Experimental Procedures. Optical rotations were measured on a JASCO DIP 1000 polarimeter. IR measurements were obtained on a Bruker IFS-48 spectrometer. NMR experiments were performed on a Bruker DRX-600 spectrometer at 300 K. All 2D-NMR spectra were acquired in CD_3OD (99.95%, Sigma-Aldrich), and standard pulse sequences and phase cycling were used for DQF-COSY, HSQC, HMBC, and TOCSY spectra. Exact masses were measured by a Voyager DE mass spectrometer. Samples were analyzed by matrix-assisted laser desorption ionization time-of-flight (MALDITOF) mass spectrometry. A mixture of analyte solution and α-cyano-4-hydroxycinnamic acid (Sigma) was applied to the metallic sample plate and dried. Mass calibration was performed with the ions from ACTH (fragment 18−39) at 2465.1989 Da and angiotensin III at 931.5154 Da as internal standard. ESIMS analyses were performed using a ThermoFinnigan LCQ Deca XP Max ion-trap mass spectrometer equipped with Xcalibur software. Column chromatography was performed over Sephadex LH-20 (Pharmacia). Semipreparative RP-HPLC was perfomed on a Knauer Smartline Pump 1000 system equipped with a differential refractometer K-2301, a μ-Bondapack column (300 × 7.8 mm i.d.), a Rheodyne injector, and Clarity Lite software. TLC was performed on silica gel F254 (Merck) plates, and reagent grade chemicals (Carlo Erba) were used throughout.

Plant Material. Fresh roots of *Pergularia tomentosa* were collected in Wadi Um Hebal, Egypt, in July 2006 and identified by Prof. Arafa I. Hamed. A voucher specimen (no. 10397) was deposited at the Herbarium of the Department of Botany of Aswan.

Extraction and Isolation. The dried and powdered roots of *P. tomentosa* (800 g) were extracted at room temperature with EtOH−H_2O (4:1) by maceration until exhaustion. The alcohol extract was concentrated under reduced pressure to a syrupy consistency (50 g). Part of the extract (2.5 g) was fractionated on Sephadex LH-20 (100 × 5 cm) using MeOH as mobile phase. Seventy fractions (8 mL) were obtained. The fractions containing cardenolide glycosides (14−25, 600 mg) were chromatographed by semipreparative HPLC, using MeOH−H_2O (11:9) as mobile phase (flow rate 2.0 mL/min), to yield compounds **6** (3.8, t_R = 12.2 min), **4** (12.0 mg, t_R = 16.1 min), and **8** (5.2 mg, t_R = 24 min); using MeOH−H_2O (1:1) as mobile phase (flow rate 2.0 mL/min), to yield compounds **7** (3.9 mg, t_R = 15.2 min), **5** (3.4 mg, t_R = 21.1 min), and **1** (1.7 mg, t_R = 25.0 min); and using MeOH−H_2O (3:7) as mobile phase (flow rate 2.0 mL/min) to yield compounds **3** (3.0 mg, t_R = 18.1 min) and **2** (1.7 mg, t_R = 25.5 min).

Compound 1: white, amorphous powder; $[\alpha]^{25}_D$ +25.3 (*c* 0.11, MeOH); UV (MeOH) λ_{max} (log ε) 218 (4.37) nm; IR (KBr) ν_{max} 3438, 1750, 1635 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; ESIMS *m/z* 549 $[M + H]^+$; HRMALDIMS *m/z* $[M + H]^+$ calcd for $C_{29}H_{41}O_{10}$ 549.6292, found 549.6298.

Compound 2: white, amorphous powder; $[\alpha]^{25}_D$ +16.1 (*c* 0.17, MeOH); UV (MeOH) λ_{max} (log ε) 213 (4.23) nm; IR (KBr) ν_{max} 3438, 1746, 1636 cm^{-1}; ^1H NMR (CD_3OD, 600 MHz) and ^{13}C NMR (CD_3OD, 150 MHz), see Table 1; ESIMS *m/z* 607 $[M + H]^+$, HRMALDIMS *m/z* $[M + H]^+$ calcd for $C_{31}H_{43}O_{12}$ 607.2763, found 607.2755.

Cancer Cell Lines. Cancer cell lines used in these studies were obtained from (i) the European Collection of Cell Cultures (ECACC, Sigma-Aldrich, Bornem, Belgium), U-373 (code 89081403) and BxPC-3 (code 93120816), and (ii) the Deutsche Sammlung von Mikroorganismen and Zellkulturen GmbH (DSMZ, Braunschweig, Germany), PC-3 (code ACC465), LoVo (code ACC350), A549 (code 107), and MCF-7 (code ACC115). Cells were maintained as previously described.[6]

Cytotoxicity Assays. Cells were seeded in flat-bottomed 96-well microwells with 100 μL of cell suspension per well (1000 and 5000 cells/well depending on cell type). Each cell line was seeded in its own cell culture medium. After a 24 h period of incubation at 37 °C the culture medium was replaced by 100 μL of fresh medium in which the substance to be tested was dissolved at the different concentrations required. The compounds were evaluated at nine concentrations ranging from 10^{-5} to 10^{-9} M by 1/2 log dilution steps. Each experimental condition was carried out in hexaplicate. After 72 h of incubation at 37 °C with the drug (experimental conditions) or without the drug (control), the medium was replaced by 100 μL of MTT at a concentration of 1 mg/mL dissolved in colorless RPMI. The plates were then incubated for 3 h at 37 °C and centrifuged at 400*g* for 10 min. The MTT was removed and the formazan crystals that formed were dissolved in 100 μL of DMSO. The plates were then shaken for 5 min and read on a spectrophotometer at two wavelengths (570 nm, the maximum formazan absorbance wavelength; 630 nm, the background noise wavelength).

The IC_{50} value was calculated with the formula

$$IC_{50} = (X2 - X1) \times (50 - Y1)/(Y2 - Y1)) + X1$$

where X1 is the higher used concentration that bordered the concentration that reduced the global cell growth by 50%, and X2 is the lower used concentration that bordered the concentration that reduced the global cell growth by 50%. Y1 is the percentage of viable cells at the X1 concentration, and Y2 is the percentage of viable cells at the X2 concentration.

Na^+/K^+-ATPase Activity Assay. The colorimetric 96-well microplate-based assay used Na^+/K^+-ATPase extracted from the porcine cerebral cortex (Sigma-Aldrich, Bornem, Belgium; working solution: 0.33 U/mL Tris pH 7.8 buffer) containing three isoforms of the α subunit. First, the enzyme was incubated with test compounds at different concentrations to allow potential interactions and/or bindings. Then, ATP was added and the hydrolysis reaction occurred at a rate dependent on the inhibition ability of the test molecules. The colorimetric quantitation was performed with Biomolgreen reagent (Tebu-Bio, Boechout, Belgium) and the color intensity measured on a microplate reader at 655 nm. The color intensity was proportional to the release of phosphate ions, which is a direct indicator of ATP hydrolysis and, therefore, of sodium pump activity. Ouabain (Acros Organics, Geel, Belgium) at 1 mM was considered to inhibit 100% sodium pump activity and was included in every test. The results were expressed as percent sodium pump activity (0% for ouabain at 1 mM). The data were expressed as IC_{50} of Na^+/K^+-ATPase inhibition (i.e., the concentration at which 50% of the Na^+/K^+-ATPase activity is inhibited by the tested compound). Four replicate determinations per concentration point were performed for each tested compound.

Cellular Imaging. Cells were plated in 25 cm^2 flasks in 7 mL of cell medium. The density of cell plating was established to obtain microscope fields (ocular 10×) that comprised ∼20 cells individualized at the start of the experiment (T = 0). The cells were incubated at 37 °C until the start of experiment. The culture medium was then removed using a vacuum pump and replaced by 7 mL of fresh medium in which the substance to be tested was diluted. The flasks were placed slightly inclined in the chamber for cellular imaging for 30 min to avoid liquid on the inside top of the flask. Each experiment lasted for 72 h. The experiments were performed in triplicate.

Supporting Information Available: ^1H, HSQC, and HMBC NMR spectra for compounds **1** and **2**; correlation of cardenolide (**1**−**8**)-mediated antiproliferative effects and Na^+/K^+-ATPase activity inhibition; the effects of tested cardenolides on A549 NSCLC and U373 glioblastoma cell proliferation and migration assessed by cellular imaging using videomicroscopy. This material is available free of charge via the Internet at http://pubs.acs.org.

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