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Antiophidic Solanidane Steroidal Alkaloids from *Solanum campaniforme*

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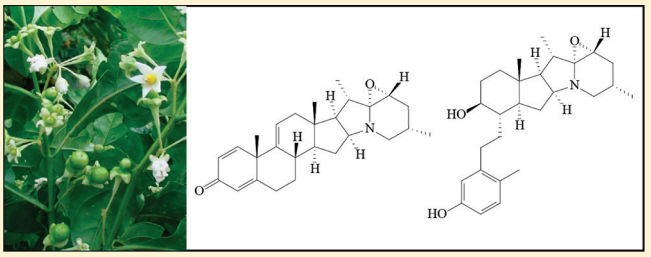
Supporting Information

ABSTRACT: Three new solanidane alkaloids bearing a 22,23-epoxy ring (**1**−**3**) and four known compounds were isolated from leaves of *Solanum campaniforme*. The structures were determined using spectroscopic techniques, including 1D and 2D NMR, and HRESIMS experiments. The antiophidic activity of the alkaloids was tested against *Bothrops pauloensis* venom. Compounds **1**−**3** completely inhibited myotoxicity without inhibiting phospholipase A_2 activity of the venom, while hemorrhage and skin necrosis were significantly reduced in the presence of alkaloids **1** and **2**.



Solanum is recognized as the major and most complex genus of the Solanaceae family and includes several species of medicinal and economical interest.[1] Plants of the genus *Solanum* produce a variety of bioactive compounds, with steroidal glycosides and glycoalkaloids being the most characteristic.[2,3] Previous investigations of these secondary metabolites have reported leishmanicidal,[2] anticancer,[4] cytotoxic,[5] antiinflamatory,[6] antinociceptive,[7] and antimicrobial activities.[8]

Snakebite is a neglected health problem in tropical regions, mainly in developing countries of South America, Africa, and Asia, and is an important cause of morbidity and mortality.[9] Although serum antivenom administration is clinically effective in systemic envenoming, even when antivenom is correctly administrated, local tissue damage neutralization mainly from *Bothrops* species envenoming is not achieved. Several secondary metabolites are able to bind peptide enzymes and toxins, such as snake venom metalloproteases (SVMP) and secretory phospholipases A_2 (sPLA$_2$), and interfere with their catalytic and pharmacological activities.[10] Despite the variable and poor immunogenic profile of these toxins, they usually have a well-conserved active site, which plays an important role in tissue damage in the area where bothropic envenomation occurs.[11,12] Thus, this has motivated the search for alternative neutralizing agents from plants as natural sources, with the aim of obtaining novel therapeutic tools to complement and improve the actions of conventional serum therapy.[13−15]

In continuation of our search for bioactive compounds from the genus *Solanum*, *Solanum campaniforme* Roemer & Schultes (Solanaceae) was investigated. The present paper reports the isolation and characterization of the three new solanidane steroidal alkaloids **1**−**3** from leaves of *S. capaniforme*. Four known compounds were also isolated. The antiophidic activity of alkaloids **1**−**3** was tested through inhibition of myotoxicity, hemorrhage, and skin necrosis induced by *Bothrops pauloensis* venom, the most prominent local tissue toxic effects of bothropic envenomation.[12]

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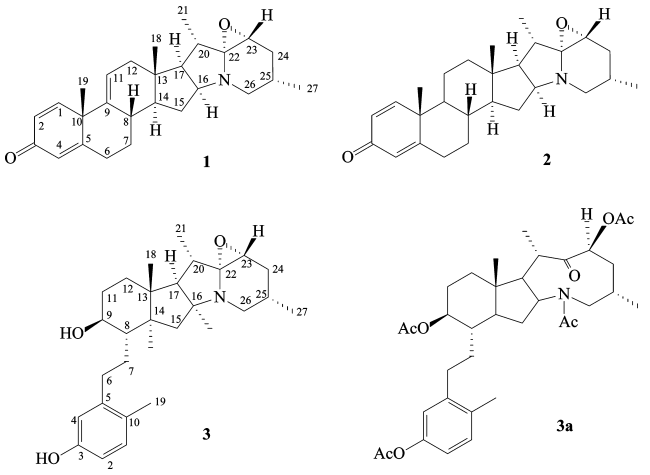

RESULTS AND DISCUSSION

Alkaloids **1**–**3** were isolated from an EtOH extract of *S. campaniforme* leaves using chromatographic fractionation techniques (open silica gel column, Sephadex LH-20, C-18 SPE cartridge, and HPLC). The structures were elucidated using spectroscopic analyses, including NMR (1D and 2D) techniques and HRESIMS.

Compound **1** was isolated as a white powder, and its IR spectrum showed absorption bands at ν_{max} 1662 and 1618 cm^{-1}, indicating cross-conjugated carbonyl and olefinic double bonds, respectively. The positive HRESIMS spectrum exhibited a quasi molecular ion, $[M + H]^+$, at m/z 406.2743 (calcd 406.2741), corresponding to the molecular formula $C_{27}H_{35}NO_2$ in accordance with 11 double-bond equivalents. Details of the molecular structure of alkaloid **1** were obtained by analysis of its ^1H and ^{13}C NMR spectra (Tables 1 and 2) and from the observed ^1H-COSY, HSQC, and HMBC correlations.

The ^1H NMR spectrum displayed two doublets with a characteristic cis coupling constant (δ 7.44 (d, J = 9.5 Hz, H-1) and 6.24 (d, J = 9.5 Hz, H-2)) and signals at δ 6.05 (s, H-4) and 5.61 (br s, H-11) for two additional olefinic protons. The signals at δ 4.69 (br s, H-16), 3.26 (Ha-26), and 2.77 (m, Hb-26) in the HSQC spectrum correlated with the carbons at δ 69.8 and 52.1 and were assigned to protons attached to nitrogenated carbons (C-16 and C-26, respectively). The signal at δ 3.18 (d, J = 5.5 Hz), correlating with the carbon at δ 60.1 (C-23), was attributed to an epoxy oxymethine proton. In addition to a series of signals for methine and methylene protons the ^1H NMR spectrum of **1** also showed signals for four methyl groups, two of which were attached to quaternary carbons at δ 1.47 (s, 3H-19) and 0.95 (s, 3H-18) and other two linked to methine carbons at δ 1.31 (d, J = 7.0 Hz, 3H-21) and 1.0 (d, J = 6.0 Hz, 3H-27).

The ^{13}C NMR CPD and DEPT spectra exhibited 27 carbon signals comprising 4 methyl, 6 methylene (1 nitrogenated, δ 52.1 (C-26)), 11 methine (4 olefinic, δ 158.6 (C-1), 127.4 (C-2), 124.0 (C-4), and 121.8 (C-11); 1 oxygenated, δ 60.1 (C-23); 1 nitrogenated, δ 69.8 (C-16)) and 6 non-hydrogenated carbon,s including 1 carbonyl at δ 188.8 (C-3) and 2 olefinic at δ 171.5 (C-5) and 145.0 (C-9). The spectroscopic data and functionalities were compatible with a steroidal alkaloid. In the HSQC experiment, correlation between the proton signal at δ 4.69 (br s, H-16) with the carbon at δ 69.8 indicated a solanidane type alkaloid.[16,17] Comparison of the ^{13}C NMR data of **1** with those related to rings E and F of leptinidine, a well-known solanidane alkaloid bearing an OH at C-23, showed a good match, considering the differentiation aspects. However, a noticeable difference was observed in the chemical shifts of C-22 (δ 93.8) and C-23 (δ 60.1) of **1** in comparison to those of leptinidine (C-22 (δ 78.2) and C-23 (64.9)).[17] In order to explain the chemical shift features at δ 93.8 (with a deshielding shift of 15.6 ppm) and 60.1 (with a shielding shift of 3.8 ppm), an epoxy ring was postulated to involve these carbons. This was confirmed by the HMBC experiment, particularly through the key correlations of 3H-21 to C-22, as well as of H-20 to C-16, C-22, and C-23. Similarly, the long-range correlations of H-1 to C-3, C-5, and C-10 and of 3H-19 to C-1 and C-5 supported a 1,4-cyclohexadien-3-one system. In addition, HMBC correlation of 3H-19 to C-10 and of H-11 to C-10 supported a double bond at C-9/C-11. Different from the case for the spirosolane-type alkaloids (the most characteristic compounds produced by *Solanum* species), in the solanidane alkaloids the side chain is cyclized, forming a fused bicyclic ring system with a bridged nitrogen, and the orientation of both H-22 and H-25 is usually α and β, respectively.[18] In fact, the NOE correlations of H-23 with Me-18/H-20/H-25 and of H-16 with Me-21 indicated an α orientation of the methyl groups 21 and 27. Thus, the structure

Table 1. ^1H NMR Data for 1–3 (MeOD) and 3a ($CDCl_3$)[a]

position	1	2	3	3a
1	7.44 d (9.5)	7.25 d (10.0)	6.84 d (8.1)	7.10 d (8.0)
2	6.24 d (9.5)	6.17 d (10.0)	6.44 br d (8.1)	6.80 dd (8.0, 2.0)
4	6.05 s	6.02 s	6.55 br s	6.79 d (2.0)
6	2.70 d (13.5)	2.56 dt	2.53 m	2.55
	2.46 d (13.5)	2.37 d (13.0)	2.43 m	2.48
7	2.28	1.98 br d (12.0)	1.63	1.50
	1.20	1.04	1.63	1.50
8	2.38	6.02 s	1.49	1.80
9		1.05	3.31 m	4.67 m
11	5.61 br s	1.78	1.63	1.80
				1.55
12	2.07 dd (17.0)	1.80	1.64	1.78
	1.80 d (17.0)	1.02	1.05	1.30
14	1.16	0.90 m	1.15	1.35
15	2.38 m	2.16	2.25	2.38
	1.50 m	1.38	1.41	1.43 dt (13.5, 4.0)
16	4.69 br s	4.62 br s	4.65 m	4.70 br s
17	1.30	1.17 d (6.5)	1.17 t (7.2)	1.66 dd (11.5, 6.0)
18	0.95 s	0.95 s	0.94 *s*	0.99 s
19	1.47 s	1.27 s	2.16 br s	2.25 s
20	2.27	2.20 m	2.25	2.40
21	1.31 d (7.0)	1.27 *s*	1.26 d (7.3)	1.09 d (7.0)
23	3.18 d (5.5)	3.12 d (6.5)	3.09 br d (6.6)	4.65
24	1.90	2.16 m	1.82	2.50
	1.35	1.38	1.26	2.30
25	2.26	2.16	2.25	2.45
26	3.26	1.83 m	3.22 dd	3.87 dd (13.5, 4.0)
	2.77 m	1.36 m	2.70 t (12.3)	3.64 dd (13.5, 5.0)
27	1.0 d (6.0)	0.96 d (6.5)	0.94 s	1.06 d (6.5)
Ac-N				2.21 s
AcO-3				2.28 s
AcO-9				2.04 s
AcO-23				2.04 s

[a] δ values are given in ppm and J values (in parentheses) in Hz. Overlapped ^1H NMR signals are reported without designating multiplicity. The chemical shifts were determined through HSQC correlations.

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Table 2. ^{13}C NMR Data for 1−3 (MeOD) and 3a ($CDCl_3$)[a]

position	1	2	3	4
1	158.6	159.2	131.9	131.0
2	127.4	127.8	113.6	118.9
3	188.8	188.8	156.5	149.0
4	124.0	124.2	116.8	121.7
5	171.5	173.6	143.9	142.7
6	33.3	33.4	31.3	29.4
7	36.2	35.0	32.4	30.0
8	37.7	36.4	44.1	40.1
9	145.0	54.8	75.3	76.0
10	47.9	45.6	127.7	133.5
11	121.8	23.5	32.4	27.6
12	40.6	38.0	36.9	37.7
13	42.6	43.0	44.3	42.7
14	51.6	51.6	50.9	50.2
15	35.5	34.6	34.9	35.5
16	69.8	69.9	70.3	63.6
17	58.4	58.7	58.4	56.7
18	15.2	15.5	15.4	12.8
19	28.0	19.3	19.0	18.9
20	27.8	27.6	27.7	37.3
21	18.8	19,0	18.8	16.2
22	93.8	93.7	93.6	206.9
23	60.1	60.1	60.1	71.9
24	45.5	45.6	45.4	47.3
25	28.1	28.1	28.1	28.1
26	52.1	52.2	52.1	51.3
27	18.2	18,3	18.3	18.7
Ac-N				22.8
AcO-3				21.3
AcO-9				21.5
AcO-23				21.5

[a] δ values are given in ppm.

of **1** was established as the new solanidane alkaloid 22α,23α-epoxy-solanida-1,4,9-trien-3-one.

Compound **2**, a white powder, had an IR spectrum similar to that of compound **1**, with absorption bands for conjugated carbonyl (ν_{max} 1657 cm^{-1}) and olefinic double bonds (ν_{max} 1606 cm^{-1}). The positive HRESIMS spectrum exhibited a quasi molecular ion, $[M + H]^+$, at m/z 408.2897 (calcd 408.2897), corresponding to the molecular formula $C_{27}H_{37}NO_2$, and one double-bond equivalent less than found for **1**. The ^1H and ^{13}C NMR spectra of **2** (Tables 1 and 2) were also similar to those of **1**. Comparison of the ^{13}C NMR spectra of **2** and **1** showed that the main difference was the appearance of additional sp^3 carbon signals (δ 54.8 (C-9) and 23.5 (C-11)) in **2** and the absence of signals near δ 145.0 and 121.0 corresponding to the double bond at C-9/C-11 in **1**. Analysis of the HMBC and NOESY experiments established the structure and relative configuration of **2** as 22α,23α-epoxy-solanida-1,4-dien-3-one.

The IR spectrum of compound **3** displayed an intense absorption band at ν_{max} 3391 cm^{-1} (OH) and additional bands at ν_{max} 1608, 1500, and 1458 cm^{-1}. The positive HRESIMS spectrum of **3** showed a quasi molecular ion, $[M + H]^+$, at m/z 426.3003 (calcd 426.3003), corresponding to the molecular formula $C_{27}H_{39}NO_3$ with 9 double-bond equivalents. The ^1H and ^{13}C NMR spectra of **3** showed signals compatible with those of rings D−F of solanidane alkaloids. However, additional signals for an oxymethine (δ 3.31/75.3 (H-9/C-9)) and a trisubstituted aromatic ring (δ 6.84 (d, J = 8.1 Hz, H-1), 6.44 (br d, J = 8.1 Hz, H-2), and 6.55 (br s, H-4), corresponding to an AMX system, and carbon signals at δ 156.5 (C-3), 143.9 (C-5), 131.9 (C-1), 127.7 (C-10), 116.8 (C-4), and 113.6 (C-2)) (Tables 1 and 2) were present. The HMBC spectrum showed correlations of the methylene protons 2H-6 with C-10, C-5, and C-7, as well as, of the methylene protons 2H-7 with C-9 and C-14, indicating linkage of the 2-(5-hydroxy-2-methylphenyl)ethyl moiety through an ethyl group at C-8 of the C ring. The relative configuration of **3** was established by interpretation of the NOESY spectrum. The β orientation inferred for the OH at C-9 was determined by the dipolar interaction between H-9 and H-14, while the ethylmethylphenol subunit was α equatorial due to NOE correlations between H-8 and 3H-18. Thus, compound **3** was established to be 3,9β-hydroxy-22α,23α-epoxy-9(10)-*seco*-solanida-1,3,5(10)-triene, a new rearranged solanidane alkaloid formed by the opening of the B ring through the scission of the C-9/C-10 linkage, with a concomitant aromatization of the A ring. This type of unusual "dienone-phenol rearrangement" was previously observed during the synthesis of mometasone furoate leading to a byproduct similar to **3**.[19]

In order to confirm the suggested structure of **3**, it was submitted to acetylation with pyridine/acetic anhydride. The unexpected tetracetylated derivative **3a**, in accordance with its ^1H and ^{13}C NMR spectra (Tables 1 and 2), was obtained. Interestingly, an additional carbonyl carbon signal was observed at δ 206.9 (C-22). The HRESIMS spectrum of **3a** exhibited a pseudo molecular ion, $[M + Na]^+$, at m/z 634.3370 (calcd 634.3350), indicating the molecular formula $C_{35}H_{49}NO_8$. Analysis of the **3a** NMR spectral data verified the acetylation of the OH groups at C-3 and C-9 and the tertiary amino group. The relative configuration of **3a** was determined by the observed NOE between H-23 and H-17 and of this with H-14. Thus, the structure of the tetraacetylated derivative **3a** was established, consistent with and supporting the structure of **3**.

In addition to alkaloids **1**−**3**, the four known compounds caffeic acid,[20] caffeic acid ethyl ester,[20] tyramine,[21] and kaempferol-3-rutinoside[22] were isolated. Compounds **1**−**3** were tested in vitro for their antiproliferative effects against four cell lines (HL-60, SF-295, MDA-MB-435, and HCT-8), since such activity had previously been published for soladinane alkaloids.[23,24] Compounds **1**−**3** were inactive.

Antiophidic activity of compounds **1**−**3** was also screened for inhibition of the main toxic effects of bothropic envenomation, myotoxicity, hemorrhage, and skin necrosis: the first promoted by phospholipases A_2 (Asp49 PLA2) and phospholipases A_2 homologues (Lys49 PLA_2) myotoxins and the other two promoted by metalloproteases (SVMP).[12] The antimyotoxic activity of compounds **1**−**3** against *B. pauloensis* venom is shown in Figure 1.

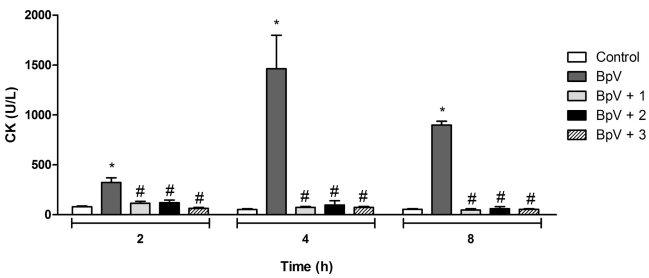

Figure 1. Antimyotoxic activity of compounds **1**−**3** against *B. pauloensis* venom. The plasma creatine kinase activity is shown as units per liter. Results are expressed as mean ± SEM with the significance level set at $p < 0.05$. legend: (*) significance compared with control group; (#) significance compared with BpV group (positive control).

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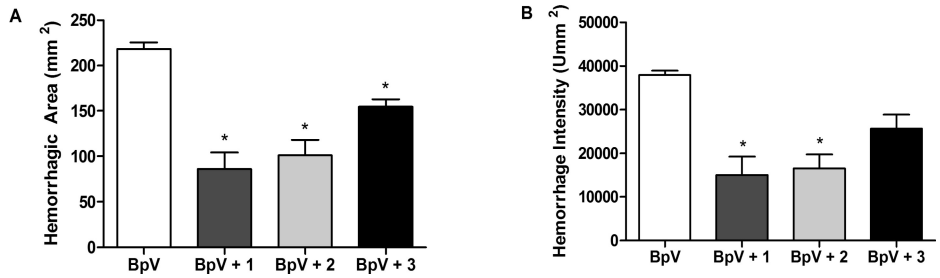

Figure 2. Antihemorrhagic activity of compounds **1**−**3** against *B. pauloensis* venom: (A) hemorrhagic area calculated in mm^2; (B) hemorrhage intensity in arbitrary units/mm^2. Results str expressed as mean ± SEM with the significance level set at (*) $p < 0.05$.

When the venom was administrated alone, plasma creatine kinase (CK) activity increased after 2, 4, and 8 h, returning to normal levels 24 h after administration. When the venom was preincubated with each alkaloid separately, plasma CK activity remained at normal levels. Phospholipases A_2 are the main toxins involved in neurotoxicity and myotoxicity of Viperiadae venoms.[12] They are subdivided into two major groups, the Asp49 $sPLA_2$s, which are catalytically active and exert their neurotoxic and myotoxic effects through the hydrolysis of sn-2 bonds in membrane phospholipids to release free fatty acids and lisophospholipids,[25] and the Lys49 $sPLA_2$s homologues, which are neither catalytically active or neurotoxic and probably exert their myotoxicity through the C-terminal region.[26,27] In order to investigate to role of Asp49 $sPLA_2$s in the myotoxicity of *B. pauloensis* venom, the ability of these alkaloids to inhibit the phospholipase A_2 activity of the whole venom was assayed using 4-nitro-3-octanoyloxybenzoic acid (4N3OBA) as substrate. Compounds **1**−**3** had no effect on the enzymatic activity of the crude venom (results not shown). These results indicated that compounds **1**−**3** do not inhibit myotoxicity of *B. pauloensis* venom through inhibition of Asp49 catalytic activity, pointing to the role of Lys49 PLA_2 inhibition.[28] The antihemorrhagic and antinecrotizing effects of compounds **1**−**3** are shown in Figures 2 and 3, respectively. The extent of the

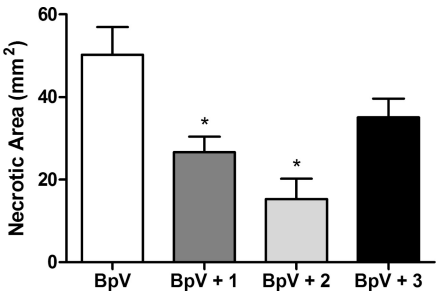

Figure 3. Antinecrotizing activity of compounds **1**−**3** against *B. pauloensis* venom. The necrotic skin area is shown in mm^2. Results are expressed as mean ± SEM with the significance level set at (*) $p < 0.05$.

hemorrhagic area (Figure 2A) as well as the intensity of the hemorrhage (Figure 2B) clearly decreased when the venom was preincubated with alkaloids **1** and **2**. Inhibition of the skin necrotic lesion was also significantly affected by alkaloids **1** and **2** (Figure 3). SVMP are the main toxins involved in bleeding and skin necrosis, both related to their ability to degrade proteins of the extracellular matrix.[12] Hence, the antihemorrhagic and antinecrotizing effects of alkaloids **1** and **2** are probably due to interaction of these compounds with metals or metalloproteases present in *B. pauloensis* whole venom.[29]

EXPERIMENTAL SECTION

General Experimental Procedures. Melting points (mp), not corrected, were determined using a digital MQ APF-301 apparatus. Optical rotations were measured on a Perkin-Elmer 341 digital polarimeter. IR (4000−400 cm^{-1}) spectra (KBr pellets) were recorded using an ABB-BOMEM FT-LA 2000-102 spectrometer. High-resolution electrospray ionization mass spectra (HRESIMS) were acquired using a LCMS-IT-TOF (Shimadzu) spectrometer. ^1H (500 MHz) and ^{13}C (125 MHz) NMR spectra were performed on a Bruker DRX-500 spectrometer. HPLC analysis was carried out using a UFLC (Shimadzu) system equipped with a SPD-M20A diode array UV−vis detector and a Phenomenex C-18 column, 5 μm (4.6 × 250 mm). The mobile phase consisted of H_2O (TFA 0.3%) and MeOH with a 4.72 mL/min flow rate, and the chromatograms were acquired at 242 nm. Column chromatography (CC) was carried out on silica gel 60 (70−230 mesh, Vetec; 230−400 mesh, Merck), Sephadex LH-20, or SPE C-18 cartridges (Strata C18-E, 20 g/60 mL, 55 μm, 70 Å). TLC was performed on precoated silica gel aluminum sheets (Kieselgel 60 F_{254}, 0.20 mm, Merck). Fractions and pure compounds were monitored by TLC, and spots were visualized using Dragendorff reagent or by heating (~100 °C) the plates sprayed with vanillin/perchloric acid/EtOH solution.

Plant Material. Leaves of *Solanum campaniforme* were collected in Guaramiranga County, Ceará State, Brazil, in October 2007 and identified by Professor Edson P. Nunes of the Departamento de Biologia, Universidade Federal do Ceará. A voucher specimen (No. 41038) has been deposited at the Herbário Prisco Bezerra (EAC) of the Universidade Federal do Ceará.

Extraction and Isolation. Dried leaves (3.14 kg) of *S. campaniforme* were extracted with EtOH (3 × 10 L) at room temperature for 24 h, and the resulting solution was concentrated under reduced pressure to give the crude extract (348 g). This extract was dissolved in a MeOH−H_2O mixture (7:3, 300 mL) and partitioned with hexane, CH_2Cl_2, EtOAc, and *n*-BuOH (5 × 200 mL each), to give the following fractions: hexane (72.6 g), CH_2Cl_2 (109.9 g), EtOAc (59.5 g), and n-BuOH (88.9 g). The EtOAc fraction was separated by CC using EtOAc−MeOH (10:0, 8:2, 5:5, 3:7, and 0:10; 500 mL each) to give five fractions (A_1, 2.1 g; A_2, 19.8 g; A_3, 27.6 g; A_4, 4.4 g; A_5, 2.8 g). Fraction A_2 (19.78 g) was chromatographed over silica gel using EtOAc−MeOH (10:0, 8:2, 5:5, and 0:10; 300 mL each) to give four fractions (B_1, 4.1 g; B_2, 4.5 g; B_3, 7.9 g; B_4, 1.1 g). Fraction B_1 (4.1 g), after chromatography over silica gel using increasing amounts of CH_2Cl_2/acetone, yielded caffeic acid ethyl ester (21.5 mg) and caffeic acid (1.43 g). Fraction B_2 (4.5 g) was chromatographed over silica gel using EtOAc/MeOH mixtures with increasing polarity to give five subfractions ($B_{2.1}$−$B_{2.5}$). Subfraction $B_{2.2}$ (2.3 g), after flash CC over silica gel using a gradient of CH_2Cl_2−MeOH, afforded kaempferol-3-rutinoside (9.7 mg). Subfraction $B_{2.3}$ (0.8 g), after two Sephadex LH-20 columns using MeOH, provided tyramine (200 mg).

Fraction B_3 (7.9 g) was chromatographed using EtOAc/MeOH mixtures with increasing amounts of MeOH (10:0, 9:1, 8:2, 6:4, 4:6, and 0:10) to afford 58 fractions of 30 mL each, which were analyzed by TLC and pooled together to 7 fractions ($B_{3.1}$−$B_{3.7}$). Fraction $B_{3.5}$ (4.57 g), which showed a positive test after spraying with the

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Dragendorff reagent, was submitted to a Sephadex LH-20 column using MeOH as eluent to obtain 43 10 mL fractions. After TLC analyses these were combined to seven subfractions (1/7, 8/9, 10/11, 12/13, 14/15, 16/30, and 31/43). Subfractions 8/9 and 10/11 were both composed of a mixture of alkaloids. Subfraction 8/9 (1.15 g) was purified with a SPE C-18 cartridge using MeOH–H_2O (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, and 10:0; 50 mL each) as eluent. Subfraction MeOH–H_2O 3:7 (250 mg) was submitted to semipreparative HPLC analysis using MeOH–H_2O (5.7:4.3) to yield compounds **1** (127 mg) and **2** (34 mg). Subfraction 10/11 (1.38 g) was subjected to Sephadex LH-20 CC using MeOH as the mobile phase to give 36 subfractions, which after TLC analysis were grouped into six subfractions (1–6). Subfraction 3 (565 mg) was purified with a SPE C-18 cartridge using MeOH–H_2O (1:9, 2:8, 3:7, 4:6, 5:5, 6:4, and 10:0; 50 mL each) to afford compound **3** (194 mg).

22α,23α-Epoxysolanida-1,4,9-trien-3-one (1): white powder; mp 165.2–166.3 °C; $[\alpha]^{25}_D$ = +13° (*c* 0.04, MeOH); IR (KBr) ν_{max} 2964, 2883, 1662, 1618, 1461, 1201, 1135, 1039 cm^{-1}; ^1H (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Tables 1 and 2; positive HRESIMS *m/z* 406.2743 $[M + H]^+$ (calcd for $C_{27}H_{36}NO_2$, 406.2741).

22α-23α-Epoxysolanida-1,4-dien-3-one (2): white powder; mp 168.9–170.7 °C; $[\alpha]^{25}_D$ = +50° (*c* 0.04, MeOH); IR (KBr) ν_{max} 2964, 2852, 1679, 1657, 1606, 1461, 1203, 1134, 1043 cm^{-1}; ^1H (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Tables 1 and 2; positive HRESIMS *m/z* 408.2897 $[M + H]^+$ (calcd for $C_{27}H_{38}NO_2$, 408.2897).

3,9β-Hydroxy-22α,23α-epoxy-9(10)-seco-solanida-1,3,5(10)-triene (3). yellowish powder; mp 167.8–168.3 °C; $[\alpha]^{25}_D$ = −21° (*c* 0.04, MeOH); IR (KBr) ν_{max} 3391, 2939, 1608, 1500, 1458, 1259, 1041, 876 cm^{-1}; ^1H (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Tables 1 and 2; positive HRESIMS *m/z* 426.3003 $[M + H]^+$ (calcd for $C_{27}H_{40}NO_3$, 426.3003).

Acetylation of Compound 3. Acetic anhydride (1 mL) was added to a solution of compound **3** (20.0 mg) in pyridine (0.5 mL). The reaction mixture was stirred at room temperature for 24 h and after the usual workup afforded compound **3a** (28.9 mg): powder, mp 111.7–112.9 °C; $[\alpha]^{25}_D$ = −86° (*c* 0.02, $CHCl_3$); IR (KBr) ν_{max} 2955, 2874, 1761, 1730, 1612, 1496, 1445, 1369, 1242, 1032, 908 cm^{-1}; ^1H (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$) data, see Tables 1 and 2; positive HRESIMS *m/z* 634.3370 $[M + Na]^+$ (calcd for $C_{35}H_{49}NO_8Na$, 634.3350).

Inhibition of Myotoxic Activity. Male Swiss mice (18–22 g, *n* = 6) were injected in the right gastrocnemius muscle with 50 μL of a solution containing 50 μg of *Bothrops pauloensis* venom (BpV). Inhibition studies were performed by injecting 50 μL of a mixed solution composed of 50 μg of BpV and 50 μg of each alkaloid, dissolved in 1% DMSO in PBS (pH 7.2). Prior to the injections, the mixtures containing BpV and the inhibitors were preincubated for 30 min at 37 °C. Negative controls received 50 μL of 1% DMSO–PBS alone. After 2, 4, 8, 24, and 48 h, blood was collected by retroorbital puncture. Plasma creatine kinase activity was determined using a commercial kit CK-UV (Labtest, Brazil) according to the manufacturer's instructions. Activity was expressed in U/L, with one unit corresponding to the production of 1 μmol of NADH per minute at 37 °C.

Antiphospholipase A_2 Activity. The sPLA2 activity of whole venom was measured in 96-well plates, using 4-nitro-3-octanoyloxybenzoic acid (4N3OBA, Biomol, USA) as substrate. Enzyme activity, expressed as the initial velocity of the reaction (V_0), was calculated on the basis of the increase in absorbance after 20 min. All assays were performed with absorbance at 425 nm using a SpectraMax 340 multiwell plate reader (Molecular Devices, Sunnyvale, CA).[14] After the addition of BpV (1 mg/mL) or BpV preincubated with each compound (1:1, w:w) to the buffer (Tris-HCl 0.1 M, Ca^{2+} 0.01 M, pH 8.0), the reaction mixture was incubated for up to 40 min at 37 °C and the absorbance read at 5 min intervals.

Antihemorrhagic and Antinecrotizing Activity. Male Swiss mice (18–22 g, *n* = 10) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), shaved, and injected in the dorsal skin with 50 μL of a solution containing 50 μg of BpV. For neutralization assays, 50 μL of a mixed solution composed of 50 μg of BpV and 50 μg of each alkaloid, dissolved in 1% DMSO in PBS (pH 7.2), was used. Prior to the injections, the mixtures containing BpV and the inhibitors were preincubated for 30 min at 37 °C. Negative controls received 50 μL of 1% DMSO–PBS alone. After 2 h, five animals were killed, the dorsal skins were removed, and the inner surfaces were examined and photographed. The images were analyzed using ImageTool 3.00 software (University of Texas Health Science Center, San Antonio, TX; http://ddsdx.uthscsa.edu/dig/itdesc.html). The intensity of the hemorrhagic area was determined by the equation $I = A \times (N - H)$, where *I* is the intensity of the hemorrhagic area, *A* the hemorrhagic area measured in mm^2, *N* the average color intensity in non-hemorrhagic skin, and *H* is the average color intensity in the hemorrhagic area, as described by Esmeraldino and co-workers.[30] After 72 h, the five other animals were killed and the same procedure was done. Necrotic areas were expressed in mm^2.

Statistical Analysis. Results were reported as the mean ± SEM of replicate experiments. The significance of differences between means was assessed by analysis of variance, followed by a Dunnett's test when several experimental groups were compared with the control group. The confidence limit for significance was 5%.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Figures giving HRESIMS and ^1H and ^{13}C NMR spectra of compounds **1**–**3** and **3a**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ REFERENCES

(1) Wink, M. *Phytochemistry* **2003**, *64*, 3–19.
(2) Nino, J.; Correa, Y. M.; Mosquera, O. M. *Pharm. Biol.* **2009**, *47*, 255–259.
(3) Ikeda, T.; Tsumagari, H.; Honbu, T.; Nohara, T. *Biol. Pharm. Bull.* **2003**, *26*, 1198–1201.
(4) Kodoru, S.; Grierson, D. S.; Van De Venter, M.; Afolayan, A. J. *Pharm. Biol.* **2007**, *45*, 613–618.
(5) Sun, L.; Zhao, Y.; Li, X.; Yuan, H.; Cheng, A.; Lou, H. *Toxicol. in Vitro* **2010**, *24*, 1504–151.
(6) Emmanuel, S.; Ignacimuthu, S.; Perumalsamy, R.; Amalraj, T. *Fitoterapia* **2006**, *77*, 611–612.
(7) Pandurangan, A.; Khosa, R. L.; Hemalatha, S. *J. Asian Nat. Prod. Res.* **2010**, *12*, 691–695.
(8) Fenner, R.; Betti, A. H.; Mentz, L. A.; Rates, S. M. K. *Rev. Bras. Cienc. Farm.* **2006**, *42*, 369–394.
(9) Harrison, R. A.; Hargreaves, A.; Wagstaff, S. C.; Faragher, B.; Lalloo, D. G. *PLoS Negl. Trop. Dis.* **2009**, *3*, e569.
(10) Mors, W. B.; Nascimento, M. C.; Pereira, B. M. R.; Pereira, N. A. *Phytochemistry* **2000**, *55*, 627–642.
(11) Correa-Netto, C.; Teixeira-Araujo, R.; Aguiar, A. S.; Melgarejo, A. R.; De-Simone, S. G.; Soares, M. R.; Foguel, D.; Zingali, R. B. *Toxicon* **2010**, *55*, 1222–1235.
(12) Gutiérrez, J. M.; Rucavado, A.; Chaves, F.; Díaz, C.; Escalante, T. *Toxicon* **2009**, *54*, 958–975.
(13) Caron, E. J.; Manock, S. R.; Maudlin, J.; Koleski, J.; Theakston, R. D.; Warrell, D. A.; Smalligan, R. D. *Toxicon* **2009**, *54*, 779–83.

(14) Cotrim, C. A.; de Oliveira, S. C.; Diz Filho, E. B.; Fonseca, F. V.; Baldissera, L. Jr.; Antunes, E.; Ximenes, R. M.; Monteiro, H. S.; Rabello, M. M.; Hernandes, M. Z.; Toyama, D. O.; Toyama, M. H. *Chem. Biol. Interact.* **2011**, *15*, 9−16.
(15) Lomonte, B.; León, G.; Angulo, Y.; Rucavado, A.; Núñez, V. *Toxicon* **2009**, *54*, 1012−1028.
(16) Coelho, R. M.; Souza, M. C.; Giotto, M. H. S. *Phytochemistry* **1998**, *49*, 893−897.
(17) Lawson, D. R.; Green, T. P.; Haynes, L. W.; Miller, A. R. *J. Agric. Food Chem.* **1997**, *45*, 4122−4126.
(18) Chiesa, F. A. F.; Moyna, P. Alcaloides Esteroidales. In *Farmacognosia: da Planta ao Medicamento*, 5th ed.; Simões, C. M. O., Schenkel, E. P., Gosmann, G., Mello, J. C. P., Mentz, L. A., Petrovick, P. R., Eds.; Ed.a da UFRGS: Porto Alegre, 2004; pp 869−883.
(19) Draper, R. W.; Puar, M. S.; Vater, E. J.; McPhail, A. T. *Steroids* **1998**, *63*, 135−140.
(20) Jiang, Z. H.; Wang, J. R.; Li, M.; Liu, Z. Q.; Chau, K. Y.; Zhao, C.; Liu., L. *J. Nat. Prod.* **2005**, *68*, 397−399.
(21) Daneluttea, A. P.; Costantina, M. B.; Delgadob, G. E.; Braz-Filho, R.; Kato, M. J. *J. Braz. Chem. Soc.* **2005**, *16*, 1425−1430.
(22) Slimestad, R.; Fossen, T.; Verheul, M. J. *J. Agric. Food Chem.* **2008**, *56*, 2436−2441.
(23) Friedman, M.; Lee, K.-R.; Kim, H.-J.; Lee, I.-S.; Kozukue, N. *J. Agric. Food Chem.* **2005**, *53*, 6162−6169.
(24) Milner, S. E.; Brunton, N. P.; Jones, P. W.; Brien, N. M. O.; Collins, S. G.; Maguire, A. R. *J. Agric. Food Chem.* **2011**, *59*, 3454−3484.
(25) Corrêa, L. C.; Marchi-Salvador, D. P.; Cintra, A. C. O.; Sampaio, S. V.; Soares, A. M.; Fontes, M. R. M. *Biochim. Biophys. Acta* **2008**, *1784*, 591−599.
(26) Cintra-Francischinelli, M.; Pizzo, P.; Angulo, Y.; Gutiérrez, J. M.; Montecucco, C.; Lomonte, B. *Toxicon* **2010**, *55*, 590−596.
(27) Montecucco, C.; Ornella, R.; Caccin, P.; Rigoni, M.; Carli, L.; Morbiato, L.; Muraro, L.; Paoli, M. *Toxicon* **2009**, *54*, 561−564.
(28) Fernandes, C. A.; Marchi-Salvador, D. P.; Salvador, G. M.; Silva, M. C.; Costa, T. R.; Soares, A. M.; Fontes, M. R. *J. Struct. Biol.* **2010**, *171*, 31−43.
(29) Silva, J. O.; Fernandes, R. S.; Ticli, F. K.; Oliveira, C. Z.; Mazzi, M. V.; Franco, J. J.; Giuliatti, S.; Pereira, P. S.; Soares, A. M.; Sampaio, S. V. *Toxicon* **2007**, *50*, 283−291.
(30) Esmeraldino, L. E.; Souza, A. M.; Sampaio, S. V. *Phytomedicine* **2005**, *12*, 570−576.