Solanidane and iminosolanidane alkaloids from *Solanum campaniforme*

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ABSTRACT

From the leaves of *Solanum campaniforme* (Solanaceae), eight solanidane alkaloids were isolated, four of which contain a *p*-hydroxyphenylethylamine unit. Their structures were established as: 22 β ,23 β -epoxy-solanida-1,4-dien-3-one; 22 α ,23 α -epoxy-10-epi-solanida-1,4,9-trien-3-one; 22 α ,23 α -epoxy-solanida-4-en-3-one; 22 β ,23 β -epoxy-solanida-4-en-3-one; (*E*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22 α ,23 α -epoxy-solanida-1,4,9-trien-3-imine; (*E*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22 α ,23 α -epoxy-solanida-1,4-dien-3-imine; (*Z*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22 α ,23 α -epoxy-solanida-1,4,9-trien-3-imine and (*Z*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22 α ,23 α -epoxy-solanida-1,4-dien-3-imine. All structures were determined using spectroscopic techniques, such as 1D and 2D NMR, and HRESIMS. The cytotoxicity and the antiophidic activities of the alkaloids were evaluated. The alkaloids did not show any cytotoxicity, but inhibited the main toxic actions of *Bothrops pauloensis* venom.

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1. Introduction

Plants belonging to the genus *Solanum* (Solanaceae) have been intensively investigated in terms of their chemical and/or biological properties (Lu et al., 2011; Chauhan et al., 2011; Silva, 2008; Pandurangan et al., 2010, 2011). In this context, several reviews have also been published (Li et al., 2011; Milner et al., 2011; Wang et al., 2011). The economic importance of the genus *Solanum* is also striking, including its ability to produce steroidal alkaloids many of which are of therapeutic interest (Nino et al., 2009). Due the medicinal importance of these plants in traditional medicine in the northeast region of Brazil, efforts have been dedicated to investigation of some species from the Solanaceae, especially those of the genera *Acnistus* and *Solanum* (Rocha et al., 2012; Pinto et al., 2011; Veras et al., 2004). A preliminary study of *Solanum campaniforme* resulted in the isolation of three major solanidane alkaloids from the EtOAc fraction of the EtOH extract, these being active against the *Bothrops pauloensis* venom (Torres et al., 2011). The study was then extended herein to the dichloromethane fraction of the same EtOH extract, from which were isolated several new minor solanidane alkaloids.

2. Results and discussion

Various chromatographic procedures, including open silica gel column, Sephadex LH-20, C-18 SPE cartridge and HPLC of the dichloromethane fraction of the EtOH extract from leaves of *S. campaniforme* afforded eight (**1–8**) new alkaloids (Fig. 1). Their structures and relative stereochemistries were elucidated using spectroscopic analyses, including NMR (1D and 2D) techniques and HRESIMS. Compounds **1–8** were isolated as dark resins which showed positive alkaloid-tests with the Dragendorff reagent.

The IR spectrum of compound **1** contained absorption bands consistent with a conjugated carbonyl (ν 1680 cm⁻¹) and an olefinic double bond (ν 1618 cm⁻¹), besides absorptions for carbon–nitrogen and carbon–oxygen stretching bands (ν 1212–1039 cm⁻¹). Its molecular formula C₂₇H₃₇NO₂ was established by HRESIMS which gave a quasimolecular ion [M+H]⁺ at *m/z* 408.2895 (calcd. 408.2897). Its ¹H NMR spectrum displayed two doublets at δ 7.28 (H-1) and 6.21 (H-2), both with characteristic *cis* coupling constants (9.8 Hz), a singlet at δ 6.06 (s, H-4) for an isolated olefinic proton, as well as signals at δ 4.37 (br d, *J* = 7.2 Hz, H-16), 3.16 (d, *J* = 12.4 Hz, H-23), and 3.04 (br d, *J* = 11.0 Hz, H-26) indicating presence of a heteroatom (nitrogen and/or oxygen) in the structure. In addition, signals for four methyl groups were observed at δ 1.00 (s, Me-18), 1.26 (s, Me-19), 1.10 (d, *J* = 6.2 Hz, Me-21) and 1.01 (d, *J* = 6.2 Hz, Me-27), respectively, beside a series of resonances for methine and methylene protons. The ¹³C NMR-CPD and DEPT spectra displayed signals for 27 carbon atoms,

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Fig. 1. Chemical structures of compounds 1–8, isolated from *S. campaniforme*.

among which there was one carbonyl at δ 189.0 (C-3), four olefinic resonances at δ 173.5 (C-5), 159.5 (C-1), 127.8 (C-2) and 124.2 (C-4), as well as signals for sp^3 nitrogenated [δ 72.2 (C-16) and 45.9 (C-26)] and oxygenated [δ 62.7 (C-23)] carbon atoms (Lawson et al., 1997). These data were in agreement with a structure of a solanidane alkaloid similar to that of 22 α ,23 α -epoxy-solanida-1,4-dien-3-one (**1a**) previously isolated from *S. campaniforme* (Torres et al., 2011). A detailed comparison of the ^1H and ^{13}C NMR spectroscopic data of **1**, to those of **1a**, clearly established differences that justified a β -position for the epoxy ring in contrast to the previously 22 α ,23 α -epoxy-solanidane alkaloid (**1a**) [**1**: $\delta_{\text{H}}/\delta_{\text{C}}$ 1.10 (H-21), 3.16 (H-23), 1.97 and 1.53 (2H-24)/16.3 (C-21), 95.8 (C-22), 62.7 (C-23) and 41.5 (C-24); **1a**: $\delta_{\text{H}}/\delta_{\text{C}}$ 1.27 (H-21), 3.12 (H-23), 2.16 and 1.38 (2H-24)/19.0 (C-21), 93.7 (C-22), 60.1 (C-23) and 45.6 (C-24)]. A NOESY experiment supported this proposition through the dipolar interactions of H-23 (δ_{H} 3.16), with Me-21 (δ_{H} 1.10) and H-16 (δ_{H} 4.37), and the Me-21 (δ_{H} 1.10) with H-17 (δ_{H} 1.29). Additional important spatial interactions are depicted in Fig. 2. Accordingly, structure **1** was established as the new 22 β ,23 β -epoxy-solanida-1,4-dien-3-one, a configurational isomer of **1a**.

Compound **2** had a molecular formula of $\text{C}_{27}\text{H}_{35}\text{NO}_2$, as determined by analysis of the HRESIMS spectrum through its quasimolecular ion $[\text{M}+\text{H}]^+$ at m/z 406.2757 (calcd. 406.2741). Detailed analyses of the 1D- and 2D-NMR spectra indicated that **2** possessed a structural profile similar to **1** and **1a**, but particularly with the 22 α ,23 α -epoxy-solanida-1,4,9-trien-3-one (**2a**), which differs only by the 19 α -methyl orientation ($\delta_{\text{H}}/\delta_{\text{C}}$ 1.18/24.9), uncommon to solanidane alkaloids. The higher shielding ($\Delta\delta_{\text{C}} = 5.4$ ppm) of C-6 (δ 27.9) and the concomitant deshielding ($\Delta\delta_{\text{C}} = 4.2$ ppm) of C-8 (δ 41.9) compared to the same carbons for **2a** (δ 33.3 and 37.7, respectively) can be associated with the γ -gauche effect caused by α -orientation of the 19-Me group. The configuration ascribed as 19 α -Me was also supported by the NOESY experiment which showed interactions of H-6 α (δ 1.59) with Me-19 (δ 1.18) and H-14 (δ 1.40) (see Fig. 2). Thus, structure **2** was established as the new solanidane alkaloid 22 α ,23 α -epoxy-10-*epi*-solanida-1,4,9-trien-3-one.

Compound **3** had a molecular formula of $\text{C}_{27}\text{H}_{39}\text{NO}_2$, which was determined by the HRESIMS spectrum from its quasimolecular ion $[\text{M}+\text{H}]^+$ at m/z 410.3057 (calcd. 410.3054). Comparative analysis of the 1D- and 2D-NMR spectra established that **3** shared a high structural similarity to **1a**. Its ^1H and ^{13}C NMR spectra contained signals for just one carbon–carbon double bond [δ 175.0 (C-5) and 124.4 (C-4)] and two additional methylene sp^3 carbons [δ 36.9 (C-1) and 34.8 (C-2)]. Deshielding of the carbonyl signal at δ 202.4 (C-3) compared to **1** [δ 189.0 (C-3)] and **2** [δ 189.9 (C-3)] allowed inference of A ring containing just an α,β -conjugated system and not a cross-conjugated system as observed for **1** and **2**. The complete structure of **3**, including its relative stereochemistry, was deduced by interpretation of its NMR spectroscopic data, including the NOESY spectrum, and comparative analyses with analogous compounds. Thus, structure **3** was established as the new alkaloid 22 α ,23 α -epoxy-solanida-4-en-3-one.

Compound **4**, from its HRESIMS spectrum, gave a quasimolecular ion peak $[\text{M}+\text{H}]^+$ at m/z 410.3059 (calcd. 410.3054) indicating the same molecular formula as **3** ($\text{C}_{27}\text{H}_{39}\text{NO}_2$), suggesting they were stereoisomers. An accurate analysis of the 1D- and 2D-NMR spectra, indicated that **4** differed from **3** solely by the configuration of the epoxy ring, as observed for **1** and **1a**. The β -position adopted by the epoxy moiety was supported by the chemical shift of the methine protons H-20 (δ 2.07) and H-25 (δ 2.07), which were both α -oriented and from the ^{13}C NMR chemical shifts of the C-22 (δ 95.8) and C-23 (δ 62.8), both showing a deshielding higher than 2 ppm, as compared to the same carbons in **3** (Table 2). On the other hand, C-21 (δ 16.4) and C-24 (δ 41.5) were shielded by more than 2.5 ppm. The dipolar interactions observed in the NOESY experiment of H-23 (δ 3.16) with H-16 (δ 4.39) and the Me-21 (δ 1.1) constitute additional support to establish the relative stereochemistry. Thus, the final structure of **4** is 22 β ,23 β -epoxy-solanida-4-en-3-one.

From the quasimolecular ion $[\text{M}+\text{H}]^+$ at m/z 525.3487 [$[\text{M}+\text{H}]^+$ (calcd. 525.3476 for) observed in the HRESIMS spectrum, the molecular formula $\text{C}_{35}\text{H}_{44}\text{N}_2\text{O}_2$ of compound **5** was determined. The ^1H and ^{13}C NMR spectroscopic data of **5** were partially similar to those of **2a** (Torres et al., 2011), albeit with additional signals

Fig. 2. Key NOESY correlations of compounds 1–4.

Table 1

¹H NMR spectroscopic data for compounds 1–8 (500 MHz in MeOD).

No.	1	2	3	4	5	6	7	8
1	7.28 d (10.0)	7.02 d (9.8)	2.07, 1.69	2.07, 1.69	7.60 d (10.2)	7.38 d (10.0)	7.60 d (10.0)	7.41 d (9.7)
2	6.21 d (10.0)	6.19 d (9.8)	2.48, 2.30	2.46, 2.30	6.61 d (10.2)	6.55 d (10.0)	6.61 d (10.0)	6.68
4	6.06 s	6.07 s	5.70 s	5.71 s	6.46 s	6.45 s	6.27 s	6.25 s
6	2.59, 2.39	1.94, 1.59	2.51, 2.33	2.48, 2.30	2.85 dt (13.3, 4.4); 2.58 d (13.3)	2.67, 2.54	2.77, 2.45	2.56
7	2.03, 1.09	1.96, 1.58	1.89, 1.05	1.91, 1.05	2.30, 1.12 m	2.08, 1.09	2.24, 1.15	2.38
8	1.88	2.02	1.69	1.77	2.45 m	1.86	2.38	1.82
9	1.10	–	1.07	1.00	–	1.0	–	0.88
11	1.79	5.91 br s	1.61, 1.54	1.60, 1.53	5.49 d (5.3)	1.83, 1.69	5.55 s	1.80, 1.73
12	1.97, 1.29	2.16; 2.05	1.82; 1.08	1.97; 1.30	2.07 dd (17.0, 5.3); 1.83 br d (17.0)	1.81, 1.12	2.06, 1.82	1.86
14	1.01	1.40	0.96	1.01	1.20	0.94	1.20	0.99
15	2.11, 1.29	2.38, 1.40	2.26, 1.38	2.27, 1.30	2.40, 1.40	2.25, 1.40	2.34, 1.44	2.21, 1.37
16	4.37 dd (7.2)	4.72 br s	4.67 br s	4.39 br s	4.68 m	4.67 s	4.69 br s	4.66 s
17	1.29	1.41	1.21 d (6.2)	1.30	1.28	1.23	1.32	1.23
18	1.00 s	0.90 s	1.00 s	0.96 s	0.94 s	0.94 s	0.93 s	0.99 s
19	1.26 s	1.18 s	1.26 s	1.25 s	1.45 s	1.28 s	1.44 s	1.26 s
20	2.10	2.30	2.30	2.07	2.27	2.25	2.27	2.30
21	1.10 d (6.2)	1.35 d (7.3)	1.30 d (7.0)	1.10 d (5.8)	1.31 d (7.2)	1.31 d (7.0)	1.32 d (7.2)	1.32 d (7.2)
23	3.16 d (12.4)	3.18 d (6.5)	3.14 d (6.0)	3.16 d (12.4)	3.18 d (6.6)	3.15 d (6.4)	3.17 d (6.7)	3.15 d (6.0)
24	1.97 1.53 t (13.4)	1.89, 1.38	1.89, 1.35	1.97, 1.53	1.88 br dd (12.9); 1.36 br dd (12.9)	1.89, 1.31	1.87, 1.36	2.01, 1.37
25	2.10	2.30	2.30	2.07	2.28 m	2.25 m	2.27 m	2.28 m
26	3.04 br d (11.0) 2.64	3.26 d (9.0) 2.77 t (11.5)	3.25 d (9.6); 2.75 t (11.9)	3.04 d (11.4); 2.65 t (12.2)	3.26 br d (12.1, 3.0) 2.77 t (12.1)	3.26, 2.77	3.27, 2.77	3.27, 2.76
27	1.01	1.00 d (6.4)	1.00 d (6.4)	1.01 d (6.6)	1.00 d (6.6)	1.00	1.00 d (6.6)	1.01
<i>p</i> -Hydroxyphenylethylamine unit								
2'/6'					7.00 d (8.3)	7.00 d (7.6)	7.00 d (8.3)	7.01 d (7.9)
3/5'					6.67 d (8.3)	6.68 d (7.6)	6.67 d (8.3)	6.66
7'					2.94 m	2.95, 2.91	2.94 m	2.98, 2.89
8'					3.92 t (6.2)	3.91	3.92 t (6.2)	3.99, 3.91

^aOverlapped ¹H NMR signals are reported without designating multiplicity. The chemical shifts were determined through the HSQC correlations.

related to a *p*-hydroxy-ethylamine moiety and some variations for the chemical shifts of the ring A of the solanidane nucleus, (Table 2). The imino and benzyl methylene protons at δ 3.92 (t, J = 6.2 Hz, 2H-8') and 2.94 (m, 2H-7'), correlated in the HSQC spectrum with the carbons at δ 47.6 and 35.5, respectively, displayed long range correlations with the carbons of the aromatic ring (C-1') and of ring A (C-3) of the solanidane nucleus (Fig. 3). This

indicated the presence of an imino system, thereby explaining the shielding of the carbon atoms at δ 115.3 (C-2), 176.5 (C-3), 115.9 (C-4) and 165.5 (C-5) when compared with the equivalent carbons of 2a, caused by the smaller electronegativity of the nitrogen atom compared to oxygen (Torres et al., 2011). In the NOESY experiment, the dipolar interaction between the olefinic proton (H-2) and the imino-methylene protons (2H-8') allowed inference

Fig. 3. Key NOESY and HMBC correlations of compounds 5–8.

Table 2

 ^{13}C NMR spectroscopic data for compounds 1–8 (125 MHz in MeOD).

No.	1	2	3	4	5	6	7	8
1	159.5	160.1	36.9	36.9	165.1	166.1	162.1	163.2
2	127.8	127.3	34.8	34.8	115.3	115.3	119.9	120.1
3	189.0	189.9	202.4	202.4	176.5	178.8	180.4	182.3
4	124.2	123.2	124.4	124.4	115.9	116.2	111.8	112.0
5	173.5	170.8	175.0	175.0	165.6	165.4	165.4	165.2
6	33.9	27.9	34.0	34.0	33.4	34.5	34.5	35.4
7	35.2	36.1	33.3	33.3	36.6	35.5	36.6	35.2
8	36.3	41.9	36.6	36.5	37.5	36.2	37.5	36.2
9	54.2	140.7	55.9	55.4	144.3	55.8	144.8	56.3
10	45.5	42.7	40.2	40.1	50.4	48.7	50.4	48.3
11	23.9	127.3	21.9	22.0	123.4	23.9	123.2	24.0
12	40.9	40.7	38.1	41.8	40.7	37.8	40.6	38.0
13	43.2	43.0	43.7	43.0	42.7	44.0	42.7	44.0
14	53.6	51.3	54.0	54.0	51.1	53.4	51.1	53.1
15	34.3	35.0	34.4	34.4	35.5	34.6	35.5	34.6
16	72.2	69.8	70.0	72.3	69.8	69.8	69.8	69.9
17	62.0	58.2	58.8	62.1	58.4	58.6	58.3	58.6
18	15.5	15.4	15.4	15.4	15.3	15.5	15.3	15.5
19	19.2	24.9	17.8	17.8	26.9	18.8	26.9	18.8
20	27.6	27.9	27.6	27.6	27.7	27.9	27.9	27.6
21	16.3	18.8	19.0	16.4	18.8	19.0	18.8	19.1
22	95.8	93.8	93.7	95.8	93.8	93.8	93.8	93.8
23	62.7	60.0	60.1	62.8	60.0	60.1	60.1	60.1
24	41.5	45.4	45.5	41.5	45.5	45.5	45.5	45.5
25	27.0	28.2	28.1	27.1	28.2	28.1	28.2	28.1
26	45.9	52.1	52.1	46.0	52.1	52.2	52.2	52.2
27	18.3	18.2	18.2	18.3	18.3	18.3	18.3	18.3
<i>p</i> -Hydroxyphenylethylamine								
1'					128.8	128.7	128.8	128.9
2'/6'					131.6	131.8	131.8	132.0
3'/5'					116.8	116.7	116.8	116.8
4'					158.1	158.1	158.0	158.0
7'					35.5	35.6	35.6	35.7
8'					47.6	47.4	47.6	47.4

of an *E* configuration to the carbon–nitrogen double bond (Fig. 3). The above data were thus consistent with the structure of **5** being (*E*)-*N*-[8'(4-hydroxyphenyl)ethyl]-22 α ,23 α -epoxy-solanida-1,4,9-trien-3-imine, a natural unknown imino-solanidane alkaloid.

Compound **6** gave a molecular formula of $\text{C}_{35}\text{H}_{46}\text{N}_2\text{O}_2$, as determined through the HRESIMS spectrum of its quasimolecular ion $[\text{M}+\text{H}]^+$ at m/z 527.3673 (calcd. 527.3632), this being two atomic mass units lower than **5**. Comparative analysis of the ^1H and ^{13}C

NMR spectroscopic data of **6** with those of **5** (Tables 1 and 2) showed that **6** differs from **5** via two carbon–carbon double bonds located in ring A. A detailed analysis of the HSQC, HMBC and NOESY experiments allowed establishment of both the structure and relative stereochemistry of **6** as the (*E*)-*N*-[8'(4-hydroxyphenyl)ethyl]-22 α ,23 α -epoxy-solanida-1,4-dien-3-imine.

Compound **7**, like **5**, also showed a molecular ion peak $[\text{M}+\text{H}]^+$ at m/z 525.3487 (calc. 525.3476) indicating the same molecular

formula $C_{35}H_{44}N_2O_2$. Comparative analysis of the ^{13}C NMR spectroscopic data of **7** with those of **5**, established that, except for C-2, C-3 and C-4 which differ by ~ 4 ppm, all chemical shifts are similar. Deshielding of C-2 (δ 119.9) and C-3 (δ 180.4), and shielding of C-4 (δ 111.8) compared to the same carbons of **5** (δ 115.3, 176.5 and 115.9, respectively) indicated a *Z* configuration for the carbon–nitrogen double bond. This was confirmed through the dipolar interaction between the olefinic proton H-4 and the nitrogenated methylene protons (2H-8') that were clearly observed in the NOESY experiment. Thus, structure **7** was determined as the (*Z*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22 α ,23 α -epoxy-solanida-1,4,9-trien-3-imine, a configurational stereoisomer of **5**.

Compound **8**, was a stereoisomer of **6** as indicated by its HRESIMS spectrum through the molecular ion peak $[M+H]^+$ at m/z 527.3678 (calc. 527.3632). Comparative analysis of the 1H and ^{13}C NMR spectroscopic data of **8** with those of **6** established several similarities except for the chemical shift values of C-1 to C-4, which differed significantly (2.9–4.2 ppm). Deshielding of C-2 (δ 120.1) and C-3 (δ 182.3) and relative shielding of C-1 (δ 163.2) and C-4 (δ 112.0) to the same carbons of **6**, supported a *Z* configuration for the carbon–nitrogen double bond instead of *E*, as determined for compound **6**. The *Z* configuration was confirmed through the dipolar interaction between the olefinic proton H-4 (δ 6.25) and the nitrogen methylene protons 2H-8' (δ 3.99/3.91) as observed in the NOESY experiment. Thus, structure **8** was (*Z*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22 α ,23 α -epoxy-solanida-1,4-dien-3-imine.

As the previously reported solanidane alkaloids isolated from *S. campaniforme* were able to inhibit some enzymatic and toxic actions of *B. pauloensis* venom, compounds **1**, **3–5**, and **7–8**, as well as the ethanol crude extract and the dichloromethane fraction, were submitted to the same activity test. Both the crude extract and the CH_2Cl_2 crude fraction inhibited proteolytic activity of azocasein (Fig. 4), and also inhibited hemorrhagic and skin necrotizing activities, these being the most important related toxic actions of the bothropic venoms in general (see Fig. 5a and b, respectively).

When the alkaloids were assayed individually, no inhibition of proteolytic activity in vitro was observed, except for compound **8** which showed $\sim 15\%$ inhibition. However, the in vivo tests showed a significant decrease in the hemorrhagic area when the venom was incubated with **1**, **3** and **8**. The necrotic areas were also smaller after treatment with **3–5**, and **8** (Fig. 6a and b). These results suggest a synergistic or additive action of the alkaloids, and the involvement of the three major alkaloids previously isolated from the EtOAc fraction (Torres et al., 2011) that were also found in the CH_2Cl_2 fraction. This can explain the greater inhibitions obtained using the crude extract and the CH_2Cl_2 fraction.

Fig. 4. Inhibition of proteolytic activity of *B. pauloensis* venom by the *S. campaniforme* crude extract (EESC) and CH_2Cl_2 fraction (EESC-D). Data were expressed as mean $\pm$ SEM and analyzed by ANOVA with Dunnett's post test. *** p < 0.001 in comparison with BpV group, # p < 0.05 between the treated groups.

Fig. 5. In vivo inhibition of the (a) hemorrhagic, and (b) necrotizing activities of *B. pauloensis* venom by the *S. campaniforme* crude extract (EESC) and CH_2Cl_2 fraction (EESC-D). Data were expressed as mean $\pm$ SEM and analyzed by ANOVA with Dunnett's post test. *** p < 0.001, ** p < 0.01 and * p < 0.05 comparing with the BpV group.

The proteolytic enzymes found in snake venoms cause local hemorrhage and necrosis through disruption of the protein arrangements in the basal membrane of capillaries and micro vessels. These damaged vessels enable blood cells to come into the extravascular space thereby causing an inflammatory reaction, and also by interrupting the oxygen supply. These factors together result in cellular death and tissue necrosis, mainly in the skin (Gutiérrez and Rucavado, 2000; Gutiérrez et al., 2005).

Another important group of toxins implicated in local tissue damage of bothropic envenomation is the phospholipase A_2 group, which is divided into enzymatically active Asp49-phospholipases A_2 (Asp49-PLA $_2$) and their homologues, as well as the enzymatically inactive Lys49-PLA $_2$ myotoxins. These toxins cause massive local myonecrosis with increasing plasma creatine kinase activity, but without generating rhabdomyolysis (Montecucco et al., 2008). Inhibition of these toxins is very important, since traditional treatment of victims with antiphidic sera are not able to fully neutralize them, leading to deforming sequelae and permanent incapacity (Lomonte et al., 2009).

In this work, both the crude extract and CH_2Cl_2 fraction inhibited the elevation of plasma CK activity in mice injected intramuscularly with *B. pauloensis* venom (Fig. 7). Alkaloids **1–4** also inhibited an increase in CK activity (Fig. 8), but when analyzed in the light of previous studies, with other antiphidic solanidane alkaloids also from *S. campaniforme* (Torres et al., 2011), one can speculate about their synergistic action, since none of them, separately, has any activity comparable to the crude extract.

Fig. 6. In vivo inhibition of (a) hemorrhagic, and (b) necrotizing activities of *B. pauloensis* venom by alkaloids isolated from *S. campaniforme*. Data were expressed as mean $\pm$ SEM and analyzed by ANOVA with Dunnett's post test. * $p < 0.05$ in comparison with the BpV group.

Fig. 7. In vivo inhibition of myotoxicity induced by intramuscular injection of *B. pauloensis* venom by the *S. campaniforme* crude extract (EESc) and CH_2Cl_2 fraction (EESc-D). Data were expressed as mean $\pm$ SEM and analyzed by ANOVA with Dunnett's post test. *** $p < 0.001$ comparing with the BpV group.

Fig. 8. In vivo inhibition of myotoxicity induced by intramuscular injection of *B. pauloensis* venom by the alkaloids isolated from *S. campaniforme*. Data were expressed as mean $\pm$ SEM and analyzed by ANOVA with Dunnett's post test. * $p < 0.05$ comparing with the BpV group.

3. Conclusion

The genus *Solanum* is a prolific source of steroidal alkaloids like pregnane, piperidylpregnane, solanocapsin, solanidane and spiro-solane derivatives, including their glycosides. By far, solanidane and spiro-solane derivatives are the most common chemomarkers to the genus, particularly those with either an oxygen or nitrogen function at C-3. Although rare, imine derivatives are also known (Ripperger, 1998). In the case of *S. campaniforme*, both types of the most common chemomarkers were found, but a larger amount of solanidanes (11) versus spiro-solanes (4) were isolated (Torres et al., 2012), in opposition to the current trend shown in the literature where the spiro-solanes have a major occurrence. In this work, eight new solanidane alkaloids in small amounts were isolated from the dichloromethane fraction of the EtOH extract of leaves of *S. campaniforme*. All compounds showed a similar skeletal profile with minor differences as regards the presence of additional double bonds, specifically the imine *Z/E* configuration and C-10 epimerization. The imine solanidane derivatives 5–8, were probably produced by nucleophilic addition of tyramine to the carbonyl (C-3) followed by an elimination reaction; tyramine was previously isolated from this plant in significant amounts.

4. Experimental

4.1. General experimental procedure

One-dimensional (^1H , ^{13}C , DEPT) and two-dimensional (COSY, NOESY, HSQC and HMBC) NMR experiments were recorded on a Bruker DRX-500 spectrometer operating at 500 MHz for ^1H and 125 MHz for ^{13}C , using standard pulse sequences supplied by the manufacturer. Chemical shifts are given in ppm, referenced to the residual undeuterated solvent peak (δ_{H} 3.31, ppm for ^1H) and δ_{C} 49.1 ppm for the ^{13}C central peak of CD_3OD . High resolution electrospray ionization mass spectra (HRESIMS) were acquired using a LCMS-IT-TOF (SHIMADZU) spectrometer. Positive ion mass spectra were recorded in the (m/z 300–700 Da) range, using a potential of 4.0 kV on the capillary and He as collision gas. IR spectra (KBr pellets) were recorded using a FT-LA 2000–102, ABB-BOMEM spectrometer, in the range from 4000 to 400 cm^{-1} . Optical rotations were measured on a Perkin-Elmer 341 digital polarimeter. HPLC analyses were 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 ($4.6 \times 250\text{ mm}$). The eluting mobile phase consisted of H_2O with TFA (0.2%; solvent A) and MeOH or CH_3CN (solvent B) with a flow rate 4.72 ml/min and the chromatogram was monitored from 210 to 350 nm.

Column chromatography (CC) steps were carried out on either silica gel 60 (70–230 mesh, Vetec or 230–400 mesh, Merck), Sephadex LH-20 or SPE C-18 cartridges (Strata C18-E, 20 g/60 mL, 55 μm , 70 Å) from Phenomenex. TLC was performed on precoated silica gel aluminium sheets (kieselgel 60 F_{254} , 0.20 mm, Merck). Fractions and pure compounds were monitored by TLC, and the spots were visualized by either the Dragendorff reagent solution or by spraying with vanillin/perchloric acid/EtOH solution and heating ($\sim 100^\circ\text{C}$).

4.2. Plant material

Leaves of *S. 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 (N $^\circ$ 41038) has been deposited at the Herbário Prisco Bezerra (EAC) of the Universidade Federal do Ceará.

4.3. Extraction and isolation

Dried leaves (3.14 kg) of *S. campaniforme* were macerated with EtOH (3 × 6 L) at room temperature for 24 h, and the combined EtOH solubles were concentrated under reduced pressure to afford the crude extract (347.8 g). The crude EtOH extract was next dissolved in a mixture of MeOH–H₂O (7:3, 300 mL) and partitioned successively with hexane, CH₂Cl₂, EtOAc and *n*-BuOH (5 × 200 mL, each), then concentrated to yield the fractions: hexane (72.6 g), CH₂Cl₂ (109.9 g), EtOAc (59.5 g) and *n*-BuOH (88.9 g), respectively. An aliquot of the CH₂Cl₂ fraction (50.0 g) was treated with a mixture of MeOH–H₂O (1:5) and kept in a refrigerator for 6 h to eliminate mainly chlorophyll. The material virtually free of chlorophyll (29.40 g) was separated by silica gel CC using CH₂Cl₂, EtOAc and MeOH mixtures of increasing polarity, to give four fractions: F-1 (1.1 g), F-2 (1.0 g), F-3 (20.7 g), and F-4 (3.0 g). The F-3 fraction (20.7 g), eluted with EtOAc–MeOH (1:1 v/v), was submitted to silica gel CC using as eluent gradients of EtOAc–MeOH (10:0 to 6:4) affording 38 fractions of 10 mL, which after TLC analyses were combined to provide five subfractions (1–17; 18–20; 21–25; 26–33 and 34–38). Subfraction 34–38 (6.4 g), containing a mixture of alkaloids, was subjected to silica gel CC using CH₂Cl₂–MeOH as mobile phase to give 41 subfractions. Subfraction 34–38/11–20 (632.8 mg) was purified through a SPE cartridge using MeOH–H₂O (5:5 to 8:2) as eluent. The fraction MeOH–H₂O 5:5 (221.1 mg) was further purified by HPLC analyses (MeOH–H₂O 52:48) to give compounds **1** (*t*_R 12.3 min, 8.1 mg), **2** (*t*_R 13.1 min, 4.0 mg), **3** (*t*_R 16.3 min, 18.0 mg) and **4** (*t*_R 18.1 min, 8.7 mg).

Subfraction 34–38/21–34 (3.3 g), after silica gel CC using increasing amounts of CH₂Cl₂–MeOH, afforded fraction 16–21 (406.7 mg), which was submitted to SPE chromatography using MeOH–H₂O (2:8 to 8:2) as eluent. The fraction MeOH–H₂O 2:8 (91.8 mg), composed of a mixture of alkaloids, was further purified by HPLC (CH₃CN–H₂O 25:75) to afford compounds **5** (*t*_R 9.5 min, 12.9 mg), **6** (*t*_R 10.1 min, 4.1 mg), **7** (*t*_R 10.6 min, 11.7 mg) and **8** (*t*_R 11.6 min, 11.2 mg), respectively.

4.4. Compound (**1**)

22β,23β-Epoxy-solanida-1,4-dien-3-one (**1**): Dark resin; [α]_D²⁵ + 5.4 (c 0.54, MeOH); For ¹H- (500 MHz, MeOD) and ¹³C NMR (125 MHz, MeOD) spectroscopic data, see Tables 1 and 2; positive HRESIMS: *m/z* 408.2895 [M+H]⁺ (calcd. 408.2897 for C₂₇H₃₈NO₂).

4.5. Compound (**2**)

22α,23α-Epoxy-10-epi-solanida-1,4,9-trien-3-one (**2**): Dark resin; [α]_D²⁵ + 44.1 (c 0.45, MeOH); For ¹H- (500 MHz, MeOD) and ¹³C NMR (125 MHz, MeOD) spectroscopic data, see Tables 1 and 2; positive HRESIMS: *m/z* 406.2757 [M+H]⁺ (calcd. 406.2741 for C₂₇H₃₆NO₂).

4.6. Compound (**3**)

22α,23α-Epoxy-solanida-4-en-3-one (**3**): Dark resin; [α]_D²⁵ + 8.6 (c 0.40, MeOH); For ¹H- (500 MHz, MeOD) and ¹³C NMR (125 MHz, MeOD) spectroscopic data, see Tables 1 and 2; positive HRESIMS: *m/z* 410.3057 [M+H]⁺ (calcd. 410.3054 for C₂₇H₄₀NO₂).

4.7. Compound (**4**)

22β,23β-Epoxy-solanida-4-en-3-one (**4**): Dark resin; [α]_D²⁵ – 21.2 (c 0.44, MeOH); For ¹H- (500 MHz, MeOD) and ¹³C NMR (125 MHz, MeOD) spectroscopic data, see Tables 1 and 2;

positive HRESIMS: *m/z* 410.3059 [M+H]⁺ (calcd. 410.3054 for C₂₇H₄₀NO₂).

4.8. Compound (**5**)

(*E*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22α,23α-epoxy-solanida-1,4,9-trien-3-imine (**5**): Dark resin; [α]_D²⁵ – 7.6 (c 0.46, MeOH); For ¹H- (500 MHz, MeOD) and ¹³C NMR (125 MHz, MeOD) spectroscopic data, see Tables 1 and 2; positive HRESIMS: *m/z* 525.3487 [M+H]⁺ (calcd. 525.3476 for C₃₅H₄₅N₂O₂).

4.9. Compound (**6**)

(*E*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22α,23α-epoxy-solanida-1,4-dien-3-imine (**6**): Dark resin; [α]_D²⁵ + 11.2 (c 0.47, MeOH); For ¹H- (500 MHz, MeOD) and ¹³C NMR (125 MHz, MeOD) spectroscopic data, see Tables 1 and 2; positive HRESIMS: *m/z* 527.3673 [M+H]⁺ (calcd. 527.3632 for C₃₅H₄₇N₂O₂).

4.10. Compound (**7**)

(*Z*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22α,23α-epoxy-solanida-1,4,9-trien-3-imine (**7**): Dark resin; [α]_D²⁵ + 16.1 (c 0.47, MeOH); For ¹H- (500 MHz, MeOD) and ¹³C NMR (125 MHz, MeOD) spectroscopic data, see Tables 1 and 2; positive HRESIMS: *m/z* 525.3487 [M+H]⁺ (calcd. 525.3476 for C₃₅H₄₅N₂O₂).

4.11. Compound (**8**)

(*Z*)-*N*-[8'-(4-hydroxyphenyl)ethyl]-22α,23α-epoxy-solanida-1,4-dien-3-imine (**8**): Dark resin; [α]_D²⁵ + 6.0 (c 0.47, MeOH); For ¹H- (500 MHz, MeOD) and ¹³C NMR (125 MHz, MeOD) spectroscopic data, see Tables 1 and 2; positive HRESIMS: *m/z* 527.3678 [M+H]⁺ (calcd. 527.3632 for C₃₅H₄₇N₂O₂).

4.12. Antiophidic assays

4.12.1. Animals and venom

Male Swiss mice (18–22 g) obtained from the Animal Facilities of the Federal University of Ceará were maintained in acrylic cages at 22 ± 2 °C, with a light/dark cycle of 12 h according to Guide for the Care and Use of Laboratory Animals of the National Institute of Health (NIH). All protocols were approved by the Ethics Committee of Animal Research of the Federal University of Ceará. *B. pauloensis* snake venom was kindly provided by Prof. M. H. Toyama of São Paulo State University (UNESP).

4.12.2. Experimental groups

The antiophidic tests were performed to evaluate the ethanol extract of *S. campaniforme* (EESc), the dichloromethane fraction (EESc-D) and the alkaloids **1**, **3–5**, and **7–8** isolated from that fraction.

4.12.3. Effect on the proteolytic activity of the venom

Proteolytic activity was tested on azocasein using 96 well plates as described by Rucavado et al. (2008). The *B. pauloensis* venom (BpV) or mixtures containing BpV and the alkaloids or extracts (1:1; w:w) were diluted in the reaction buffer (25 mM Tris, 150 mM NaCl, 5 mM CaCl₂, pH 7.4) at 1 mg/mL. Ten microliters of this solution were incubated with an azocasein solution (100 μL; 5 mg/mL in the reaction buffer) at 37 °C for 60 min, after what the reaction was stopped by the addition 5% CCl₃CO₂H (200 μL), and centrifuged at 2205 g for 5 min. An aliquot of the supernatant (100 μL) was transferred to another plate, to which was added 0.5 M NaOH (100 μL). The absorbance was read at

450 nm. Proteolytic activity was normalized considering the venom alone as 100% of activity.

4.12.4. Inhibition of myotoxicity

The capability of neutralizing the myotoxicity induced by *B. pauloensis* venom was assayed according to Torres et al. (2011). Male Swiss mice (18–22 g, $n = 6$) were injected in the right gastrocnemius muscle with PBS (50 μ L; pH 7.4) containing *B. pauloensis* venom (BpV) (50 μ g). Inhibition studies were performed by injecting 50 μ L of a mixed solution composed of BpV (50 μ g) and each alkaloid or extract (50 μ g), dissolved in 1% DMSO in PBS (pH 7.4). Prior to the injections, mixtures containing BpV and alkaloids were pre-incubated for 30 min at 37 °C. Control groups were injected with 1% DMSO in PBS (50 μ L), containing or not containing each alkaloid. After 4 h, the blood was collected by retro-orbital puncture in heparinized tubes and centrifuged for separation of the plasma. Creatine kinase (CK) activity was determined using a commercial kit CK-UV (Labtest, Brazil) as described in the manufacturer's instructions. CK activity was expressed in U/L, with one unit corresponding to production of NADH (1 μ mol) per minute at 37 °C. The values of the control groups were subtracted from the corresponding treated group. No statistical differences were observed among control groups.

4.12.5. Antihemorrhagic and antinecrotizing activities

Antihemorrhagic and antinecrotizing activities of alkaloids were assessed as described by Esmeraldino et al. (2005), and modified by Torres et al. (2011). Briefly, male Swiss mice (18–22 g, $n = 12$) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.), and had the dorsal skin shaved and injected with 50 μ L of a solution containing 50 μ g of BpV. For neutralization assays, 50 μ L of a mixed solution composed of 50 μ g of BpV and 50 μ g of each alkaloid or extract, dissolved in 1% DMSO in PBS (pH 7.4), were used. Prior to the injections, the mixtures containing BpV and the alkaloids were pre-incubated for 30 min at 37 °C. The control groups were injected with 50 μ L of 1% DMSO in PBS, containing or not containing each alkaloid. After 2 h, six animals were killed. The dorsal skins were removed, and inner surfaces were examined and photographed. Images were analyzed using the ImageTool 3.00 software (University of Texas Health Science Center, San Antonio, TX; <http://ddsdx.uthscsa.edu/dig/itdesc.html>), with hemorrhagic area measured in mm². After 72 h, the other six animals were killed and the same procedure was done. Necrotic areas were also expressed in mm².

4.13. Statistical analysis

Results were expressed as the mean $\pm$ SEM, and analyzed by Two-Way ANOVA followed by a Dunnett's test when several experimental groups were compared with the control group. The confidence limit for significance was set at $p < 0.05$.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.phytochem.2013.09.007>.

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