Cytotoxic Guanidine Alkaloids from *Pterogyne nitens*△

Luis Octávio Regasini,† Ian Castro-Gamboa,† Dulce Helena Siqueira Silva,† Maysa Furlan,† Eliezer Jesus Barreiro,‡ Paulo Michel Pinheiro Ferreira,§ Cláudia Pessoa,§ Letícia Veras Costa Lotufo,§ Manoel Odorico de Moraes,§ Maria Claudia Marx Young,⊥ and Vanderlan da Silva Bolzani*,†

Institute of Chemistry, Nucley of Bioassay, Biosynthesis and Ecophysiology of Natural Products, NuBBE, São Paulo State University, UNESP, Araraquara, SP, Brazil, CP 355, CEP 14801-970, LASSBio, Laboratório de Avaliação e Síntese de Substâncias Bioativas, Centro de Ciências da Saúde, Faculdade de Farmácia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil, CP 68.006, CEP 21944-910, LOE, Laboratório de Oncologia Experimental, Departamento de Fisiologia e Farmacologia, Universidade Federal do Ceará, Fortaleza, CE, Brazil, CP 12.200, CEP 60430-270, and Seção de Bioquímica e Fisiologia de Plantas, Instituto de Botânica, São Paulo, SP, Brazil, CP 4009, CEP 01061-970

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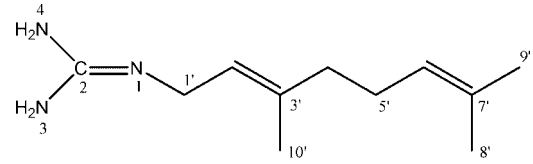
As part of a bioprospecting program aimed at the discovery of potential anticancer drugs, two new guanidine-type alkaloids, nitensidines D and E (**1**, **2**), and the known pterogynine (**3**), pterogynidine (**4**), and galegine (**5**), were isolated from the leaves of *Pterogyne nitens*. The structures of **1** and **2** were established on the basis of spectroscopic data interpretation. These compounds were tested against a small panel of human cancer cell lines. Compound **2** exhibited cytotoxicity for HL-60 (human myeloblastic leukemia) and SF-245 (human glioblastoma) cells.

Pterogyne nitens Tul. (Fabaceae, Caesalpinioideae), popularly known as "bálsamo", "yvira-ró", "cocal", and "amendoinzeiro", is a native tree common in South America. It is used as an ornamental due to its attractive flowers and fruits.[1,2] Medicinal uses of this species have not been reported frequently, although aqueous preparations from stem bark have been used by local Paraguayan populations in the therapy of ascariasis.[3] Previous phytochemical studies with the leaves and fruits of *P. nitens* have demonstrated the presence of several guanidine alkaloids, which exhibited cytotoxic activity toward the DNA-repair-deficient yeast mutant RS 321 and moderate cytotoxicity against CHO Aux B1 cells,[4] and phenolic compounds with myeloperoxidase inhibitory and radical-scavenging activities.[5−7] In our continuing search for new natural product-based anticancer agents from Brazilian Cerrado and Atlantic Forest plants, and their associated microorganisms,[8,9] the ethanol extract of *P. nitens* was selected for further investigation due to its cytotoxic activity in the MTT assay. This extract was subjected to liquid−liquid partitioning, yielding a bioactive *n*-butanol-soluble fraction with IC_{50} 60 μg/mL against HL-60 (leukemia) cells. The *n*-butanol fraction was chromatographed using silica gel and Sephadex LH-20 followed by RP-18 to afford five guanidine alkaloids (**1**−**5**).

Nitensidine D (**1**) was isolated as colorless oil and gave an alkaloid-positive test when sprayed with Dragendorff's and Sakaguchi's reagents. Additionally, evidence for a guanidine alkaloidal type came from its molecular formula, which was established as $C_{11}H_{21}N_3$ by HRESIMS. The ^{1}H NMR spectrum of **1** (Table 1) exhibited typical resonances for a terpenoid moiety, due to the presence of three vinyl methyl signals at δ 1.55, 1.61, and 1.63, two olefinic protons at δ 5.07 and 5.17, and three methylenes at δ 1.97, 2.03, and 3.63. These data were consistent with the presence of a geranyl moiety. Additionally, a broad singlet at δ 8.85 was observed, integrating for four hydrogens, which was compatible with two amino groups of a guanidine structure. The geranyl moiety was confirmed from the ^{13}C NMR spectrum (Table 1), due to signals at δ 130.9 and 138.3 (two quaternary olefinic carbons), δ 119.6 and 123.8 (two methine olefinic carbons), δ 38.5, 38.9, and 25.9 (three methylene carbons), and δ 16.0, 17.5, and 25.4 (three methyls). The guanidine moiety was also evident from the signal at δ 157.6, characteristic of an imine functionality (Table 1). These observations and additional NMR experiments (DEPT 135, gHMQC, gCOSY, NOESY, and gHMBC) allowed us to establish a guanidine alkaloid structure for **1**, with the same structural pattern for those alkaloids previously reported.[4] The isolate **1** was fully characterized as shown, and its structure was in agreement with a tautomeric form (Figure 1). This proposal was supported by the ^{1}H NMR data, in particular, due to the multiplicity observed for the signal displayed by the allyl hydrogen (H-1′) of the geranyl moiety. This signal appeared as a clear doublet at δ 3.63 (2H, J = 6.0 Hz), which is compatible with a guanidine nucleus geranylated at N-1. The natural guanidines occurring in plants or animals show a variety of substitution patterns, with most of these including the



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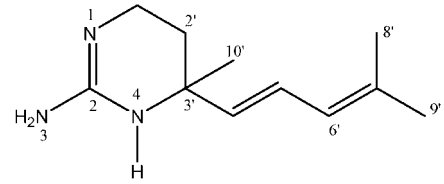

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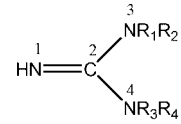

3 $R_1 = R_2 = H$; $R_3 = R_4 =$ 

4 $R_2 = R_3 = H$; $R_2 = R_4 =$ 

5 $R_1 = R_2 = R_3 = H$; $R_4 =$ 

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* To whom correspondence should be addressed. Phone: + 55 16 33016660. Fax: 55 16 33227932. E-mail: bolzaniv@iq.unesp.br.

† São Paulo State University.

‡ Universidade Federal do Rio de Janeiro.

§ Universidade Federal do Ceará.

⊥ Instituto de Botânica.

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Table 1. ^{1}H NMR (500 MHz), ^{13}C NMR (125 MHz), and gHMBC Data for Compounds **1** and **2** in DMSO-d_6

	nitensidine D (**1**)			nitensidine E (**2**)		
position	δ_C, mult.	δ_H *J* in Hz)	gHMBC	δ_C, mult.	δ_H *J* in Hz)	gHMBC
2	157.6, qC		1′	154.1, qC		1′
3		8.85, brs			7.98, brs	
4		8.85, brs			7.68, brs	
1′	38.5, CH_2	3.63, d (6.0)	2′	35.1, CH_2	2.92, ddd (12, 14, 4); 3.15, ddd (12, 4, 3.5)	
2′	119.6, CH	5.17, t (6.0)	1′, 4′, 10′	31.9, CH_2	1.61, m; 1.79, ddd (13.5, 4, 3.5)	4′, 10′
3′	138.3, qC		1′, 4′, 5′, 10′	52.4, qC		1′, 4′, 5′, 10′
4′	38.9, CH_2	1.97, m	5′, 10′	135.4, CH	5.52, d (15.0)	2′, 10′
5′	25.9, CH_2	2.03, m	4′	125.2, CH	6.20, dd (15.0, 10.5)	
6′	123.8, CH	5.07, t (7.0)	4′, 5′, 8′, 9′	123.9, CH	5.81, d (10.5)	4′, 8′, 9′
7′	130.9, qC		5′, 8′, 9′	135.0, qC		5′, 8′, 9′
8′	17.5, CH_3	1.55, s	6′, 9′	18.0, CH_3	1.69, s	6′, 9′
9′	25.4, CH_3	1.63, s	8′, 9′	25.5, CH_3	1.73, s	6′, 8′
10′	16.0, CH_3	1.61, s	2′, 4′	27.6, CH_3	1.26, s	2′, 4′

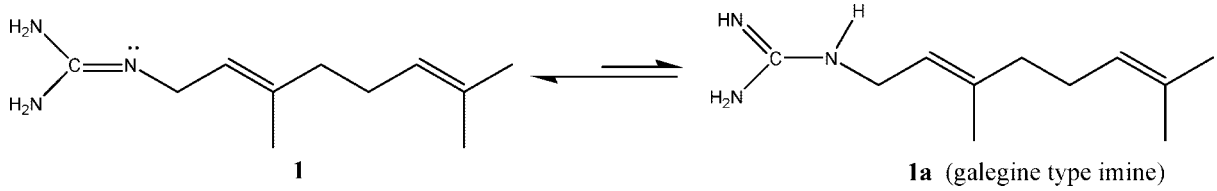
imine feature of galegine (**5**). However, the occurrence of the new guanidine-type alkaloid **1** can be explained by taking into account the imine tautomerism (Figure 1).

Nitensidine E (**2**) was isolated as a colorless oil. The molecular formula was determined to be $C_{11}H_{19}N_3$ by HRESIMS (*m/z* 194.2900 $[M + H]^+$, calcd for $C_{11}H_{20}N_3$, 194.2888), suggesting four degrees of unsaturation, with one additional unsaturation when compared with nitensidine D. The ^{1}H and ^{13}C NMR spectra of compound **2** (Table 1) indicated structural characteristics somewhat different from those of **1** and the other guanidines isolated previously from *P. nitens*.[4] Considering that **2** possesses four unsaturations and showed positive Dragendorff's and Sakaguchi's tests, an alkaloid structure was deduced for this compound. The ^{1}H NMR spectrum (Table 1) exhibited the presence of a set of typical resonances at δ 5.52 (d, $J = 15.0$ Hz), 5.81 (d, $J = 10.5$ Hz), and 6.20 (dd, $J = 15.0$, 10.5 Hz), characteristic of a sp^2-conjugated system, which could be attributed to an *E*-diene unit based on the coupling constants shown for these hydrogens. The ^{13}C NMR data (Table 1) showed resonances for three methine sp^2 carbons at δ 135.4, 125.2, and 123.9 and for one quaternary sp^2 carbon at δ 135.0. Additionally, both the ^{1}H and ^{13}C NMR spectra showed the presence of two vinyl methyl groups [δ 1.69 (18.0), 1.73 (25.5)] and a tertiary methyl group at δ 1.26 (27.6). These data revealed the presence of a C_{10} alkyl chain, which could be attributed to a monoterpene-type unit. The structural evidence from the spectroscopic data obtained allowed us to elucidate compound **2** with a guanidine alkaloid structure. Additionally, the mutually coupled methylenes [δ 1.61 (1H, m), 1.79 (1H, ddd, $J = 13.5$, 4.0, 3.5 Hz), and 2.92 (1H, ddd, $J = 12.0$, 14.0, 4 Hz), and δ 3.15 (1H, ddd, $J = 12.0$, 4.0, 3.5 Hz)] would be compatible with a cyclic guanidine structure for **2**. Indeed, this proposal was confirmed from the gHMBC spectrum. The location of the methyl group C-10′ was assigned at C-3′ due to the observed gHMBC correlations between the methyl protons at H-10′ and C-2′, C-3′, and C-4′. Further, gHMBC cross-peaks between H-4′ and C-2′, C-10′, C-6′; H-1′ and C-2, C-3′; H-2′ and C-10′, C-4′; and H-6′ and C-8′, C-9′ completed the assignment of a cyclic guanidine-type alkaloid and its substituents. Also, a C_{10} alkyl chain composed of an *E*-diene unit was confirmed through NOESY correlations observed for H-4′ and H-6′. The conformation adopted by this molecule was evaluated in a preliminary manner employing semiempirical calculations (Hamiltonian as AM1 and PM3 and ab initio HF/6-31G**), and a plausible conformation obtained for compound **2** with the methyl group at C-3′ in the β-position is shown in Figure S1, Supporting Information. In Figure S2 (Supporting Information) we show a biogenetic proposal for the formation of the new guanidine derivatives **1** and **2**.

In addition to **1** and **2**, three known compounds were isolated and identified as pterogynine[10] (**3**), pterogynidine[4] (**4**), and galegine[11] (**5**). Compound **5** was first isolated from *Verbesina enceloiodes* (Asteraceae) and later identified as a toxic and antidiabetic component from *Galega officinalis* (Goat's Rue).[12] Galegine was a template for the design of metformin and other biguanide-type hypoglycemic drugs.[13] The identification of compounds **3**−**5** was based on ^{1}H and ^{13}C NMR and MS data analysis and by comparison with authentic material available in our laboratory.[4,10,11]

Although a broad spectrum of biological activities has been described for guanidine alkaloids belonging to marine sources, few studies were reported from higher plants and of the potential anticancer activity of this class of natural products.[14−16] All isolates were evaluated for their cytotoxic activity, using a MTT assay, evidencing a key role of the prenyl substitution pattern in the level of activity. The cytotoxic activity of compounds **1**−**5** was evaluated *in vitro* against HL-60 (human myeloblastic leukemia), HCT-8 (human colon carcinoma), MDA-MB-435 (human melanoma), and SF-295 (human glioblastoma). Mean concentration inhibitory (IC_{50}) values were determined using the National Cancer Institute (NCI) protocol, with slight modifications, and the results are shown in Table 2. In human cells, nitensidine E (**2**) was the most active compound, showing activity for the different cell lines (Table 2). Therefore, guanidine alkaloids **2** may be useful as a prototype compound for the development of new antineoplastic drugs. Further work to evaluate the mechanism of action of the guanidine alkaloids characterized in this study is currently in progress.

The structural features of nitensidines D (**1**) and E (**2**) appear less common than the guanidine alkaloids already reported from other higher plants. It is noteworthy that the majority of these alkaloids contain isoprenyl groups. Only one alkaloid having a similar geranyl side chain has been previously isolated from *Fontainea pancheri* (Euphorbiaceae).[17] In some cases, the isopentenyl groups have undergone cyclization to produce hexahydroimidazo-pyrimidines, typical of *Alchornea* species (Euphorbiaceae).[18,19] The structure of nitensidine E (**2**) constitutes the first report of the



1 **1a** (galegine type imine)

Figure 1. Iminic tautomeric forms of guanidine-type alkaloids **1** and **1a**.

Table 2. Cytotoxic Activity of Compounds **1**−**5** Isolated from *Pterogyne nitens* against Human Tumor Cell Lines[a,b]

	cell line			
compound	HL-60 (leukemia)	HCT-8 (colon)	MDA-MB-435 (melanoma)	SF-295 (glioblastoma)
2	3.6	>5	>5	4.9
doxorubicin	0.02	0.04	0.47	0.25

[a] Data are presented as IC_{50} values given in μg/mL obtained by nonlinear regression. [b] Compounds **1** and **3**−**5** were inactive (IC_{50} > 5 μg/mL) for all cell lines used.

natural occurrence of a cyclic monoterpene derivative on a guanidine moiety.

To date, the isolation and characterization of prenylated guanidine alkaloids has been reported only within a limited number of plant genera.[20−23] Furthermore, terpenoid-like alkaloids may have some taxonomic significance for Fabaceae and Euphorbiaceae, since their occurrence is most common among these taxa.

Experimental Section

General Experimental Procedures. 1D (^{1}H, ^{13}C, DEPT 90°, and DEPT 135°) and 2D (^{1}H−^{1}H gCOSY, gHMQC, and gHMBC) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^{1}H) and 125 MHz (^{13}C), using DMSO-d_6 as an internal standard. Positive-ion high-resolution mass spectra were recorded on an HRMS ultrOTOFQ-ESI-TOF instrument (Bruker Daltonics). IR spectra were measured on a Perkin-Elmer 1600 FT-IR spectrometer using KBr disks. Optical rotations were measured on a Polamat A Carl Zeiss Jena polarimeter. Analytical HPLC was performed on a Varian Pro Star 230 using a Phenomenex C_{18} column (250 mm × 4.6 mm). Column chromatography was performed over reversed-phase silica gel 230−400 mesh (Merck). TLC was performed using Merck silica gel 60 (>230 mesh) and precoated silica gel 60 PF_{254} plates. Spots on TLC plates were visualized under UV light and by spraying with anisaldehyde-H_2SO_4 reagent followed by heating at 120 °C, as well as by using Dragendorff's and Sakaguchi's reagents. Preparative HPLC was performed on a Varian Prep-Star 400 system using a Phenomenex C-18 (250 mm × 21.2 mm) preparative column. Sephadex LH-20 was purchased from Pharmacia Biotech, Sweden.

Plant Material. *Pterogyne nitens* leaves were collected at the Botanic Garden of São Paulo, São Paulo State, Brazil, in May 2003. A voucher specimen (SP204319) has been deposited in the herbarium of the Botanic Institute (São Paulo State, Brazil).

Extraction and Isolation. The shade-dried leaves (2.8 kg) of *P. nitens* were ground and defatted with hexane (2.0 L × 5, at room temperature, for five weeks) and exhaustively extracted by maceration with ethanol (4.0 L × 5) at room temperature. The ethanol extract was concentrated under reduced pressure (<40 °C) to yield 12.7 g of a syrup. The concentrate was then diluted with CH_3OH-H_2O (4:1) (3.5 L) and partitioned successively with EtOAc (5.0 L × 3) and *n*-butanol (5.0 L × 3). After removal of the solvent 3.7 and 5.9 g of extract were afforded, respectively. The *n*-butanol residue was subjected to gel permeation chromatography on a column of Sephadex LH-20 (2.5 g) in methanol to afford nine fractions, which were combined on the basis of their TLC visualized with Sakaguchi's[24] and Dragendorff's reagents, to yield an alkaloidal fraction (587 mg). Separation of this fraction on reversed-phase silica gel column chromatography (RP-18) by elution with increasing amounts of MeCN in H_2O afforded eight fractions (ALK-1−ALK-8) and guanidine **5** (18 mg). Fraction ALK-3 (121 mg) was subjected to repeated column chromatography on silica gel (230−400 mesh), eluted with $CHCl_3$−MeOH mixtures (ranging from 0 to 35% MeOH), to furnish the isoprenylated alkaloids **3** (27 mg) and **4** (12 mg). Fraction ALK-7 (62 mg) was further purified by RP-HPLC [MeCN−H_2O−AcOH (16:84:0.01), UV detection at 235 nm; flow rate 13 mL/min], affording the new geranylated alkaloids **1** (22 mg) and **2** (11 mg).

Nitensidine D (1): colorless oil; IR (KBr) ν_{max} 1652, 1618, 1012, 845 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 196.3052 $[M + H]^+$ (calcd for $C_{11}H_{22}N_3$, 196.3063).

Nitensidine E (2): colorless oil; $[\alpha]^{25}_D$ +140.0 (*c* 0.01, MeOH); IR (KBr) ν_{max} 1649, 1618, 1093, 839 cm^{-1}; ^{1}H and ^{13}C NMR data, see Table 1; HRESIMS *m/z* 194.2900 $[M + H]^+$ (calcd for $C_{11}H_{20}N_3$, 194.2888).

Cytotoxicity Assay. The cytotoxicity of the guanidine alkaloids **1**−**5** was tested against four tumor cell lines: HL-60 (human leukemia), HCT-8 (human colon carcinoma), MDA/MB-435 (human melanoma), and SF-295 (human glioblastoma) (National Cancer Institute, Frederick, MD). Cells were cultured in RPMI-1640 medium, supplemented with 10% fetal calf serum, 2 mM glutamine, 100 mg/mL streptomycin, and 100 U/mL penicillin at 37 °C with 5% CO_2. For experiments, cells were seeded in 96-well plates (10^5 cells/well for adherent cells or 0.3 × 10^6 cells/well for suspended cells in 100 μL of medium). After 24 h, the compounds (0.39−25 μg/mL) dissolved in DMSO (5%) were added to each well and incubated for three days. Control groups received the same amount of DMSO. Doxorubicin (0.01−0.58 μg/mL) was used as positive control. Growth of tumor cells was quantified by the ability of living cells to reduce the yellow dye 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) to a blue formazan product. At the end of 72 h incubation, the medium in each well was replaced by fresh medium (200 μL) containing 0.5 mg/mL MTT. Three hours later, the formazan product of MTT reduction was dissolved in DMSO, and absorbance was measured using a multiplate reader (Spectra Count, Packard, Ontario, Canada). Drug effect was quantified as the percentage of control absorbance of reduced dye at 590 nm. The IC_{50} values were obtained by nonlinear regression using the GRAPHPAD program (Institutive Software for Science, San Diego).[25]

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Supporting Information Available: Figures showing the possible conformation of **2**, other semiempirical conformation analysis, and biogenetic pathway for guanidine alkaloids. This information is available free of charge via the Internet at http://pubs.acs.org.

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