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Antiprotozoal Sesquiterpene Pyridine Alkaloids from *Maytenus ilicifolia*

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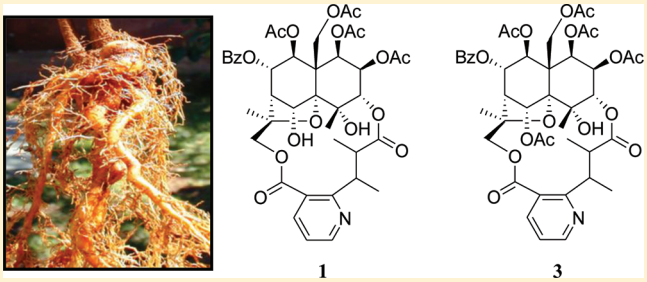
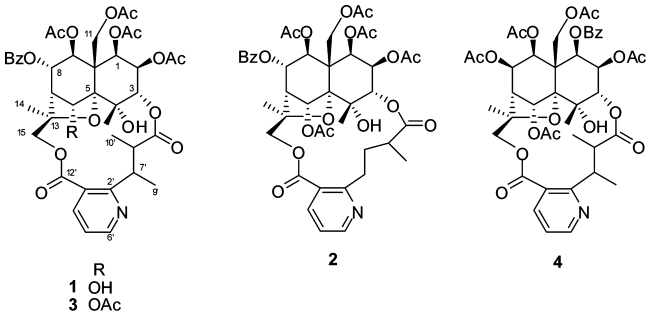
Supporting Information

ABSTRACT: As part of a bioprospecting program aimed at the discovery of antiprotozoal agents from the Brazilian flora, two new sesquiterpene pyridine alkaloids, ilicifoliunines A (**1**) and B (**2**), along with the known alkaloids aquifoliunine E-I (**3**) and mayteine (**4**), were isolated from the root bark of *Maytenus ilicifolia*. The structures of **1** and **2** were established on the basis of spectroscopic data interpretation. Alkaloid **3** displayed potent in vitro antiprotozoal activity against *Leishmania chagasi* and *Trypanosoma cruzi*, with IC_{50} values of 1.4 and 41.9 μM, respectively, as well as low cytotoxicity against murine peritoneal macrophages (IC_{50} of 1.8 mM).



Species of the plant family Celastraceae have long been used in traditional medicine.[1] For example, the stem bark and leaves of *Maytenus ilicifolia* Mart. ex Reiss., a plant that is native to Brazil and known locally as "espinheira-santa", have been employed for their purported anticancer and contraceptive properties in South America[2,3] and are also used in the treatment of gastric ulcers and inflammation, as well as in the reduction of vascular tension.[4−6] Diverse classes of secondary metabolites, including maytansinoids,[7] triterpenoids,[8] quinone methide triterpenoids,[9] catechins, epicatechins,[10] and flavonoid glycosides,[11] are reportedly responsible for the various biological activities associated with this genus.

A large number of highly oxygenated dihydro-β-agarofuran sesquiterpenoids and derivatives thereof have been isolated from members of the Celastraceae, and many are considered to be important chemotaxonomic indicators.[12] Members of this group of natural products are of particular interest because of their insect antifeedant,[13] insecticidal,[14] cytotoxic,[15] immunosuppressive,[16] anti-HIV,[17] and antitumor[18] activities, as well as their ability to reverse the P-glycoprotein-dependent multidrug resistance (MDR) phenotype of several human cancer cells.[19] The macrolide sesquiterpene pyridine alkaloids represent a major subclass of the group and are characterized by the presence of a dihydro-β-agarofuran moiety, the A and B rings of which are in the form of an axially dimethylated *trans*-decalin ring system, as well as a 1,3-diaxially fused $(CH_3)_2C-CO$ bridge constituting a tetrahydrofuran C ring and a pyridine unit.[20] The macrocyclic structure is formed by two ester linkages between the sesquiterpene moiety and one of the several pyridine dicarboxylic acids, such as evoninic acid, wilfordic acid, or edulinic acid, attached at positions C-3 and C-15.[21] As part of our continuing search for new bioactive compounds from species of the family Celastraceae,[22,23] the crude extract of *M. ilicifolia* root bark was selected for further chemical and biological investigations.



The EtOH extract of the root bark of *M. ilicifolia* was subjected to liquid−liquid partitioning, which yielded a bioactive dichloromethane-soluble fraction that was chromatographed over RP-18, followed by HPLC-UV separation. This procedure afforded four compounds (**1**−**4**), namely, two new sesquiterpene alkaloids, designated as ilicifoliunines A (**1**) and B (**2**), and the known alkaloids aquifoliunine E-I (**3**) and mayteine (**4**). The structure elucidation of compounds **1** and **2** was carried out by means of 2D NMR spectroscopic techniques, including HMQC, HMBC, COSY, and NOESY experiments.

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Table 1. NMR Spectroscopic Data for 1 and 2 in $CDCl_3$[a]

	ilicifoliunine A (1)		ilicifoliunine B (2)	
position	δ_C (mult.)	δ_H[b] (mult., J in Hz)	δ_C (mult.)	δ_H[b] (mult., J in Hz)
1	72.2 (CH)	5.67 d (3.5)	72.6 (CH)	5.65 d (3.0)
2	68.2 (CH)	5.32 dd (3.0, 3.5)	69.0 (CH)	5.12 t (3.0)
3	74.6 (CH)	4.75 d (3.0)	75.4 (CH)	4.87 d (3.0)
4	72.4 (C)		69.8 (C)	
HO-4		5.70 brd (1.5)		4.77 brd (0.5)
5	93.2 (C)		94.0 (C)	
6	76.8 (C)	5.32 d (2.0)	74.5 (C)	6.63 s
HO-6		6.14 d (2.0)		
7	51.2 (CH)	2.77 d (3.0)	49.9 (CH)	2.61 d (3.5)
8	74.1 (CH)	5.68 dd (3.0, 10.0)	74.2 (CH)	5.70 dd 3.5, 9.8)
9	73.9 (CH_2)	5.91 d (10.0)	74.1 (CH)	5.83 d (9.8)
10	50.9 (C)		51.4 (CH)	
11a	60.5 (CH_2)	4.80 d (13.0)	60.6 (CH_2)	4.73 d (13.0)
11b	60.5 (CH_2)	4.68 d (13.0)	60.6 (CH_2)	4.62 d (13.0)
12	23.8 (CH_3)	1.89 d (1.0)	23.3 (CH_3)	1.51 d (1.0)
13	85.9 (C)		85.8 (C)	
14	20.4 (CH_3)	1.85 brs	19.1 (CH_3)	1.73 brs
15a	70.7 (CH_2)	6.10 brd (12.0)	70.2 (CH_2)	5.64 d (12.0)
15b	70.7 (CH_2)	3.66 d (12.0)	70.2 (CH_2)	3.73 d (12.0)
2′	165.7 (C)		163.7 (C)	
3′	124.4 (C)		124.5 (C)	
4′	138.1 (CH)	8.11 dd (2.0, 8.0)	138.7 (CH)	8.19 dd (2.0, 8.0)
5′	121.1 (CH)	7.26 dd (5.0, 8.0)	121.1 (CH)	7.18 dd (5.0, 8.0)
6′	151.6 (CH)	8.71 dd (2.0, 5.0)	152.9 (CH)	8.66 dd (2.0, 5.0)
7′a	36.0 (CH)	4.80 q (7.0)	33.5 (CH_2)	3.79 ddd (8.0, 8.5, 13.5)
7′b			33.5 (CH_2)	2.94 ddd (5.5, 8.0, 13.5)
8′a	45.2 (CH)	2.58 q (7.0)	33.1 (CH_2)	2.00 m
8′b			33.1 (CH_2)	2.17 m
9′	11.4 (CH_3)	1.43 d (7.0)	38.6 (CH_3)	2.40 dqd (2.5, 7.0, 10.0)
10′	9.5 (CH_3)	1.20 d (7.0)	18.3 (CH_3)	1.16 d (7.0)
11′	173.7 (C)		174.9 (C)	
12′	168.7 (C)		166.9 (C)	
CH_3CO-1	20.4	1.86 s	20.5	1.79 s
CH_3*C*O-1	169.1		169.2	
CH_3CO-2	20.9	1.84 s	20.9	2.07 s
CH_3*C*O-2	168.4		168.6	
CH_3CO-6			21.4	2.11 s
CH_3*C*O-6			169.7	
CH_3CO-9	20.5	2.17 s	20.7	1.82 s
CH_3*C*O-9	169.8		169.9	
CH_3CO-11	21.3	2.29 s	21.2	2.28 s
CH_3*C*O-11	169.9		170.0	
COPh	165.5		165.4	
ipso	129.1		128.9	
o-COPh	129.6 (CH)	7.95 dd (1.0, 8.0)	129.7 (CH)	7.86 dd (1.0, 8.0)
m-COPh	128.6 (CH)	7.45 t (8.0)	128.7 (CH)	7.36 t (8.0)
p-COPh	133.5 (CH)	7.58 tt (1.0, 8.0)	133.7 (CH)	7.51 tt (1.0, 8.0)

[a]Recorded at 500 and 125 MHz for ^1H and ^{13}C NMR, respectively. [b]Multiplicity of signals is given as follows: s, singlet; d, doublet; dd, doublet of doublets; ddd, doublet of doublets of doublets; dqd, doublet of quartets of doublets; t, triplet; m, multiplet.

Ilicifoliunine A (**1**) was isolated as an amorphous, white solid, and it gave an alkaloid-positive TLC test when sprayed with Dragendorff's reagent. The molecular formula was established as $C_{41}H_{47}NO_{17}$ by HRMS (*m*/*z* 826.2905 $[M + H]^+$, calcd for $C_{41}H_{48}NO_{17}$, 826.2922). The IR spectrum showed absorption bands at 3425 and 1754 cm^{-1}, typical of hydroxy and ester carbonyl groups, respectively. The ^1H NMR spectrum of **1** (Table 1) exhibited, among other signals, resonances for four acetyl groups at δ 1.86, 1.84, 2.17, and 2.29, in addition to three signals at δ 7.45 (t, J = 8.0 Hz), 7.58 (tt, J = 1.0 and 8.0 Hz), and 7.95 (dd, J = 1.0 and 8.0 Hz), which were attributed to a benzoyl group. Proton assignments around the bicyclic skeleton were determined mainly on the basis of interpretation of a COSY contour map. The spin system derived from H-1α, H-2α, H-3β, H-8β, and H-9α was readily recognized, because the 1H doublet at δ 5.67, assigned to H-1α ($J_{1\alpha,2\alpha}$ = 3.5 Hz), showed a cross-peak with the 1H doublet of doublets at δ 5.32, ascribed to H-2α ($J_{2\alpha,1\alpha}$ = 3.5 Hz, $J_{2\alpha,3\beta}$ = 3.0 Hz), which, in turn, exhibited a cross-peak with the 1H doublet at δ 4.75, corresponding to H-3β ($J_{3\beta,2\alpha}$ = 3.0 Hz). The 1H doublet of doublets at δ 5.68 attributed to H-8β ($J_{8\beta,7\beta}$ = 3.0, $J_{8\beta,9\alpha}$ = 10.0 Hz) showed a cross-peak with the 1H doublet at δ 2.77 (H-7β). The relative configuration of H-8β was deduced from a 1D NOESY difference experiment and coupling constant. In this experiment, irradiation of the methine resonance at δ 5.32 (H-6, d, J = 2.0 Hz) caused enhancement of the signal at δ 5.68 (H-8β, dd, J = 3.0 and 10.0 Hz), while irradiation of the methyl resonance at δ 1.85 (H-14, brs) gave rise to enhancement of the signal at δ 5.91, hence corroborating the axial orientation of H-9 (Figure 1). The sets of methylenic protons at δ 4.68, 4.80 (d, $J_{11a,11b}$ = 13.0 Hz) and at δ 3.66 (d, $J_{15a,15b}$ = 12.0 Hz) and 6.10 (brd, J = 12.0 Hz) were assigned to H-11 and H-15, respectively. The presence of an esterified evoninic acid moiety in the macrocycle was suggested by two doublets at δ 1.20 (J = 7.0 Hz) and 1.43 (J = 7.0 Hz), ascribed to the two secondary methyl groups H-10′ and H-9′, respectively, which displayed cross-peaks with a pair of resonances at δ 2.58 (q, $J_{8',10'}$ = 7.0 Hz) and 4.80

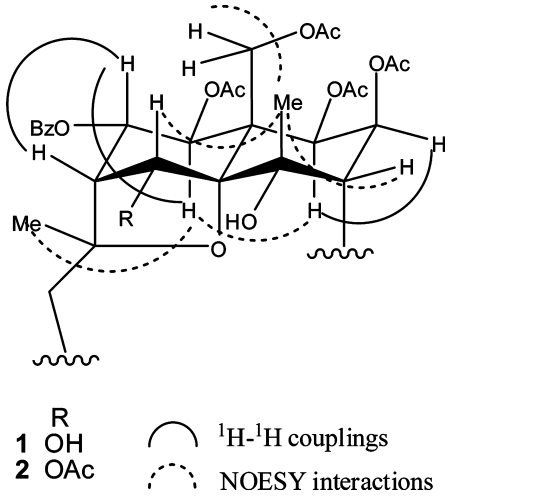

Figure 1. Major ^1H–^1H couplings and NOESY interactions of **1** and **2**.

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(q, $J_{7',9'}$ = 7.0 Hz) assigned to H-8′ and H-7′, respectively. Signals at δ 7.26 (dd, J = 5.0 and 8.0 Hz), 8.11 (dd, J = 2.0 and 8.0 Hz), and 8.71 (dd, J = 2.0, 5.0 Hz) corresponded to H-5′, H-4′, and H-6′, respectively, indicating the presence of a 2,3-disubstituted pyridine unit.

The linkages of acetyl groups to the sesquiterpene unit in **1** were elucidated using the $^{1}H-^{13}C$ HMBC experiment. The four methyl group singlets at δ 1.86, 1.84, 2.17, and 2.29 showed correlations to the four acetoxy carbonyl resonances at δ 169.1, 168.4, 169.8, and 169.9, respectively. The attachment of these acetoxy groups at C-1, C-2, C-9, and C-11 on the sesquiterpene unit was established by defined cross-peaks between the proton signals at δ 5.67 (H-1α), 5.32 (H-2α), 5.91 (H-9α), 4.68, and 4.80 (H-11) and the acetoxy carbonyl resonances. The aromatic *ortho* protons at δ 7.95 (J = 1.0 and 8.0 Hz) exhibited a cross-peak with the signal at δ 165.5, which was attributed to the carbonyl of the benzoyl substituent at position C-8. The attachment of the benzoyl group was deduced with the aid of a 1D NOESY experiment (Figure 1), in which irradiation of the broad singlet ressonance at δ 1.85 (H-14) caused enhancement of the signal δ 7.95 (*ortho*). Also, this attachment was confirmed by $^{1}H-^{13}C$ HMBC experiment, which indicated a cross-peak between proton signals at δ 5.68 (H-8) and 165.5 (COPh). There is a cross-peak between the proton signal at δ 5.91 (H-9α) and the carbon signal at δ 169.8 (9-CH_3CO). The signals at δ 2.58 (H-8′), 4.80 (H-7′), and 1.43 (H-9′) displayed cross-peaks with the aromatic carbon signal at δ 165.7, which was assigned to C-2′. The respective correlations between the proton signals at δ 6.10 (H-15a and H-15b) and 4.75 (H-3) with carbon signals at δ 173.7 (C-11′) and 168.7 (C-12′) proved the attachment of the evoninate moiety at C-3 and C-15.

The ^{1}H NMR analysis of **1** in $CHCl_3/D_2O$ indicated the presence of two free hydroxy groups. This spectrum displayed two signals at δ 5.70 (brd, J = 1.5 Hz) and 6.14 (d, J = 2.0 Hz), which exhibited time-dependent reductions in their magnitude signals. The location of these groups was elucidated by an HMBC experiment. The cross-peaks observed between the hydroxy group at δ 5.70 and C-12 (δ 23.8) and C-4 (δ 72.4), as well as the cross-peak between the hydroxy signal at δ 6.14 and C-6 (δ 76.8), corroborated the positions of these groups at C-4 and C-6, respectively.

Ilicifoliunine B (**2**) was obtained as an amorphous, white solid, and it tested positively with Dragendorff's reagent. This compound exhibited spectroscopic features very similar to those of **1** (Table 1), except for its macrocycle, which was formed from esterification of the dihydro-β-agarofuran sesquiterpene moiety with wilfordic acid, in addition to the presence of five acetyl groups. The ^{13}C NMR and DEPT spectra revealed the presence of a methyl carbon at δ 18.3 (C-10′), two methylenes at δ 33.5 (C-7′) and 33.1 (C-8′), and a methine carbon at δ 38.6 (C-9′). The ^{1}H NMR and COSY spectra evidenced methyl hydrogens at δ 1.16 (d, J = 7.0 Hz, CH_3-10′), two pairs of diastereotopic methylenes at δ 2.00 and 2.17 (each m, H-8′), and 3.79 (ddd, J = 8.0, 8.5, and 13.5 Hz, H-7′a) and 2.94 (ddd, J = 5.5, 8.0, and 13.5 Hz, H-7′b), and a methine proton at δ 2.40 (dqd, J = 2.5, 7.0, and 10.0 Hz, H-9′). The HMBC spectrum displayed five cross-peaks between the singlets at δ 1.79, 1.82, 2.07, 2.11, and 2.28 with signals at δ 169.2, 169.9, 168.6, 169.7, and 170.0, corroborating the presence of five acetyl substituents. The linkages of the acetyl substituents were mainly established by HMBC. The proton signals at δ 5.65 (H-1), 5.12 (H-2), 6.63 (H-6), 5.83 (H-9), and 4.62 (H-11) were correlated with the carbonyl carbons of acetyl groups at δ 169.2, 168.6, 169.7, 169.9, and 170.0, respectively.

The structures of the previously known alkaloids aquifoliunine E-I (**3**)[22] and mayteine (**4**)[24] were confirmed by 1D NMR, 2D NMR, and mass espectrometric analysis, as well as by comparison with previously reported data.

Although a broad spectrum of biological activities has been described for sesquiterpene pyridine alkaloids, few studies have dealt with their antiparasitic capacity.[25] All isolates obtained were evaluated for their biological activity against *Leishmania amazonensis*, *L. chagasi*, and *Trypanosoma cruzi*. Mean 50% inhibitory concentration values (IC_{50}) were determined using MTT colorimetric assays, and the results are summarized in Table 2.

Among the tested compounds, alkaloid **3** was found to be active against *L. chagasi* and *T. cruzi*, with IC_{50} values of 1.4 and 41.9 μM, respectively. These data indicate the antiprotozoal potency of **3**, as compared to the positive controls pentamidine (IC_{50} = 5.1 μM) and benznidazole (IC_{50} = 42.7 μM), drugs that are currently employed for the treatment of leishmaniasis and trypanosomiasis, respectively.[26] In our previous biological investigations, **3** has a demonstrated weak and selective effect in a mechanism-based DNA-modifying yeast assay, suggesting its cytotoxic activity.[22] Alkaloid **1** displayed potent antitrypanosomal activity, with an IC_{50} value of 27.7 μM. However, this compound was inactive against both *Leishmania* species. Compounds **2** and **4** did not exhibit activity against the protozoan species tested at 100 μM. Taken together, these results suggested preliminary structural features correlated with antiprotozoal activity, such as the number and position of the ester groups on the dihydro-β-agarofuran sesquiterpene unit. Additional studies involving other analogues are necessary to provide conclusive structure–activity relationship data.

The potent trypanocidal compounds **1** and **3** were selected for cytotoxic activity tests in mammalian cells, using murine peritoneal macrophages (Table 2). The results revealed that these two sesquiterpene pyridine alkaloids are not toxic at the concentrations at which they display antitrypanosomal activity

Table 2. Antiprotozoal and Cytotoxic Activity Data for Alkaloids[a]

	antiprotozoal activity (IC_{50})[b]				
compound	*Leishmania amazonensis*	*Leishmania chagasi*	*Trypanosoma cruzi*	cytotoxicity (IC_{50})[b]	SI[e]
1	>100	>100	27.7	1282	46.2
3	>100	1.4	41.9	1847	44.0
benznidazole[c]	nt[d]	nt[d]	42.7	1141	26.7
pentamidine[c]	7.0	5.1	nt[d]	nt[d]	

[a]Alkaloids **2** and **4** were found to be inactive against all the tested parasites. [b]Values are expressed in μM and obtained by nonlinear regression. [c]Positive control. [d]Not tested. [e]Selectivity index obtained from the ratio between IC_{50} for murine peritoneal macrophages and IC_{50} for *Trypanosoma cruzi*.

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(IC_{50} of 1282 and 1847 μM), but their cytotoxicity was similar to that of benznidazole (IC_{50} of 1141 μM). The latter was included for comparison purposes, because it is the only drug available for the treatment of Chagas disease. Knowing that selectivity is a crucial parameter for the development of antiprotozoal agents,[27] the cytotoxic activity was correlated with the antitrypanosomal activity by calculating the selectivity index (SI), SI = IC_{50} (murine peritoneal macrophages)/IC_{50} (*Trypanosoma cruzi*). The SI values for **1**, **3**, and benznidazole were 46.2, 44.0, and 26.7, respectively, demonstrating that the two isolated alkaloids are more selective than the standard drug. In conclusion, the pyridine sesquiterpene alkaloids **1** and **3** isolated from *M. ilicifolia* may be useful as prototype compounds for the development of new antiparasitic drugs. Further work to evaluate the mechanism of action of these compounds is currently in progress.

EXPERIMENTAL SECTION

General Experimental Procedures. Optical rotations were obtained by means of a Perkin-Elmer polarimeter equipped with a sodium lamp, using a sample cell volume of 1 mL, in $CHCl_3$. IR spectra were measured on a Perkin-Elmer 1600 FT-IR spectrometer using KBr disks. 1D (^{1}H, ^{13}C, DEPT 90°, DEPT 135°, and NOESY) and 2D (^{1}H−^{1}H COSY, HMQC, and HMBC) NMR experiments were recorded on a Varian INOVA 500 spectrometer (11.7 T) at 500 MHz (^{1}H) and 125 MHz (^{13}C), using $CDCl_3$ as internal standard. Positive-ion high-resolution mass spectra were acquired on an HRMS ultrOTOFQ-ESI-TOF instrument (Bruker Daltonics), using MeOH/H_2O (1:1) as solvent and a cone voltage of 40 V. Analytical HPLC was performed on a Varian Pro Star 230 using a Phenomenex C_{18} column (250 mm × 4.6 mm, 5 μm). Column chromatography was conducted over reversed-phase silica gel 230−400 mesh (Merck). TLC analyses were accomplished using silica gel 60 (>230 mesh) and precoated silica gel 60 PF_{254}. Spots on TLC plates were visualized under UV light and by spraying with Dragendorff's reagent. Preparative HPLC was carried out on a Varian Prep-Star 400 system employing a Phenomenex C_{18} (250 mm × 21.2 mm, 10 μm) preparative column.

Plant Material. The root bark of *Maytenus ilicifolia* was collected in the city of Ribeirão Preto (São Paulo State, Brazil, at 21°11′56.1″ S; 47°46′42.2″ W) in March 2006. The plant was identified by Rita Maria de Carvalho. A voucher specimen (HPM-BR 0059) has been deposited in the Herbarium of the University of Campinas, São Paulo, Brazil.

Extraction and Isolation. The shade-dried and powdered root bark of *M. ilicifolia* (670 g) was extracted with EtOH (750 mL, 3×), at room temperature. The crude extract was filtered and concentrated under reduced pressure, affording a brown residue (154 g). The concentrate was solubilized in MeOH/H_2O (1:4, 750 mL) and then partitioned successively with hexane (500 mL, 3×), hexane/EtOAc (4:1) (500 mL, 3×), and CH_2Cl_2 (500 mL, 3×). After removal of solvent, 4.8, 4.7, and 12.6 g of the resultant fractions were furnished, respectively. The CH_2Cl_2 fraction (10.0 g) was subjected to reversed-phase silica gel column chromatography (RP-18) and eluted with increasing amounts of CH_3OH in H_2O, affording 14 fractions (DCM-1−DCM-14), which were combined based on the analysis of their TLC visualized with Dragendorff's reagent. Fraction DCM-2 (0.400 g) was further purified by RP-HPLC-UV [MeCN/H_2O (6:4), 15 mL/min, 254 nm], yielding **4** (0.064 g, t_R = 28 min). Fraction DCM-3 (0.407 g) was submitted to RP-HPLC-UV separation [MeCN/H_2O (6:4), 12 mL/min, 254 nm], and the new alkaloid **1** (0.033 g, t_R = 32 min) was obtained. Fraction DCM-4 (0.400 g) was subjected to preparative RP-HPLC-UV separation [MeCN/H_2O (55:45), 16 mL/min, 254 nm], giving nine subfractions. Subfraction DCM-4-7 afforded **3** (0.062 g, t_R = 47 min). Subfraction DCM-4-8 was further chromatographed using RP-HPLC-UV [MeCN/H_2O (7:3), 12 mL/min, 254 nm] and furnished **2** (0.043 g, t_R = 24 min).

Antileishmanial Assay. An antileishmanial assay using promastigote forms of *Leishmania amazonensis* and *Leishmania chagasi* was performed using a MTT colorimetric method described by Muelas-Serrano and co-workers.[28] All experiments were accomplished using the *L. amazonensis* MPRO/BR/1972/M1841-LV-79 and *L. infantum* (syn. *L. chagasi*) MHOM/BR/1974/PP75 strains. Parasites were cultured in LIT medium supplemented with 10% heat-inactivated fetal calf serum. LIT medium was prepared by mixing of 10 mg/mL hemin (bovine, type I) (1 mL) with a solution containing NaCl (4.0 g), KCl (0.4 g), Na_2HPO_4 (8.0 g), glucose (2.0 g), liver infusion broth (5.0 g), and tryptose (5.0 g) at pH 7.0 (900 mL). The parasites were harvested at the end of the exponential growth phase (4-day-old culture forms) and seeded at 8 × 10^6 parasites/mL in 96-well microplates. Alkaloids **1**−**4** were dissolved in DMSO (stock solution, 16.7 mg/mL) and added to the parasite suspension, which furnished 10 concentrations ranging from 100 to 0.098 μg/mL, and an aliquot of 3.0 μL was added to the plate. Then, an aliquot of 97 μL of 8 × 10^6 parasites/mL (*L. amazonensis* or *L. chagasi*) was mixed with the sample solution. These 96-well tissue culture plates were maintained at 28 °C for 72 h. After this period, a 10 μL aliquot of 2.5 mg/mL 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide-phenazine methosulfate (MTT-PMS) solution was added to each well, and the plates were incubated for 75 min, in the dark, at 28 °C. Subsequently, a solution of 10% sodium dodecyl sulfate (SDS) (100 μL) was added to the previous solution and maintained at room temperature, in the dark, for 30 min. Absorbance of the samples was read at 490 nm. The assays were carried out in triplicate for each concentration, for 72 h at 28 °C. The 50% mean inhibitory concentration (IC_{50}) values of the test compounds and the positive control pentamidine isothionate were determined by calculating the percentage of cytotoxicity.[28]

Antitrypanosomal Assay. An antitrypanosomal assay employing *T. cruzi* epimastigotes was performed using the previously described MTT colorimetric method.[28] All the experiments were conducted on the Y-strain, which is considered a standard strain of type I. Parasites were grown axenically at 28 °C in liver-infusion tryptose (LIT) medium supplemented with 10% fetal calf serum and harvested during the exponential growth phase (7-day-old culture forms). Alkaloids **1**−**4** were dissolved in DMSO (stock solution 16.7 mg/mL) and added to the parasite suspension, leading to 10 concentrations ranging from 100 to 0.098 μg/mL. Next, *T. cruzi* (1 × 10^7 parasites/mL) was added to each test well, and the same volume of LIT medium (50 μL), with and without parasites, was added to the control wells in the absence of the test compounds. The plates were maintained at 28 °C for 72 h. After this period, an aliquot of 10 μL of the MTT-PMS solution (2.5 mg/mL) was added to each well, and the plates were incubated in the dark, at 28 °C, for 75 min. Subsequently, a solution of 10% SDS (100 μL) was added to the previous solution and maintained at room temperature, in the dark, for 30 min. Absorbance of the samples was read at 595 nm. All the assays were accomplished in triplicate. The mean inhibitory concentration (IC_{50}) values were established after 72 h of incubation. Benznidazole was used as positive control.

Cytotoxicity Assay. An unspecific cytotoxic activity was determined according to the method of Mosmann, with minimal modifications.[29] The cytotoxicity experiments were performed as indicated by the ethical committee of Faculdade de Ciências Farmacêuticas, Univ. Estadual Paulista, Araraquara (protocol number 09/2009). Briefly, Balb/cisogenic mice were inoculated intraperitoneally with 3 mL of 3% sodium thioglycolate. The animals were sacrificed in a CO_2 chamber 3 days after inoculation. The peritoneal macrophages were extracted by injection of phosphate buffer (pH 7.2) followed by slight massage and suction using a syringe. The cells were transferred to a sterile tube placed in an ice bath, and the cellular suspension was prepared after centrifugation. The sediment cells were resuspended in RPMI 1640 medium with 5% fetal calf serum, 100 μg/mL streptomycin, 100 U/mL penicillin, and 5 × 10^{-2} M mercaptoethanol at a density of 5 × 10^6 cells/mL (stock suspension). The stock suspension (100 μL) and test solutions (100 μL) were dissolved in RPMI 1640 medium in a 96-well microtiter plate. All the treatments were performed in triplicate, and the IC_{50} values were determined. After incubation for 24 h at 37 °C and 7.5% CO_2, an MTT solution (2.5 mg/mL) (100 μL) was added to each well and incubated for 3 h under the same conditions. The optical density (590 nm)

of the formazan crystals dissolved in 2-propanol was measured and compared to that of the negative control (RPMI only). All treatments were carried out in triplicate, and the IC_{50} values were measured. The positive control for the antitrypanosomal assay (benznidazole) was also evaluated in the in vitro assay.

*Ilicifoliunine A (**1**):* amorphous solid; $[\alpha]^{24}_{D}$ +10.6 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 230 (3.61), 265 (3.24) nm; IR (KBr) ν_{max} 3425, 2928, 1754, 1565, 1225 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 826.2905 $[M + H]^+$ (calcd for $C_{41}H_{48}NO_{17}$, 826.2922).

*Ilicifoliunine B (**2**):* amorphous solid; $[\alpha]^{24}_{D}$ −1.1 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 231 (4.20), 266 (3.72) nm; IR (KBr) ν_{max} 3472, 2968, 1746, 1573, 1234 cm^{-1}; ^1H and ^{13}C NMR data, see Table 1; HRESIMS *m*/*z* 868.3020 $[M + H]^+$ (calcd for $C_{43}H_{50}NO_{18}$, 868.3027).

*Aquifoliunine E-I (**3**):* amorphous solid; $[\alpha]^{23}_{D}$ −5.0 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 231 (3.62), 264 (3.22) nm; IR (KBr) ν_{max} 3493, 2991, 1758, 1644, 1225 cm^{-1}; ^1H and ^{13}C NMR data; HRESIMS *m*/*z* 868.3010 $[M + H]^+$ (calcd for $C_{43}H_{50}NO_{18}$, 868.3027).

*Mayteine (**4**):* amorphous solid; $[\alpha]^{23}_{D}$ −21.1 (*c* 0.2, $CHCl_3$); UV (MeOH) λ_{max} (log ε) 231 (3.60), 265 (3.24) nm; IR (KBr) ν_{max} 3524, 2982, 1743, 1648, 1113 cm^{-1}; ^1H and ^{13}C NMR data; HRESIMS *m*/*z* 868.3010 $[M + H]^+$ (calcd for $C_{43}H_{50}NO_{18}$, 868.3027).

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Copies of ^1H and ^{13}C NMR as well as selected 2D NMR spectra for alkaloids **1** and **2**. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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