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Article

Ixorine, a New Cyclopeptide Alkaloid from the Branches of *Ixora brevifolia*

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The isolation and structure determination of new cyclic peptide alkaloid ixorine, along with five known constituents frangulanine, syringaresinol, cinnamtannin B-1, daucosterol and mannitol from the branches of *Ixora brevifolia* are described. The cyclic peptide frangulanine is being described for the first time in the Rubiaceae family. The structures were elucidated on their spectral data basis, mainly one- (^{1}H, ^{13}C, DEPT) and two-dimensional (COSY, NOESY, HSQC and HMBC) nuclear magnetic resonance (NMR) and by comparison with data from the literature. The mixture of two cyclopeptide alkaloids showed weak activity against *Leishmania amazonensis*.

Keywords: *Ixora brevifolia*, Rubiaceae, cyclopeptide alkaloids, *Leishmania*

Introduction

The genus *Ixora* belongs to the Rubiaceae family and has approximately 350 species, mostly native to tropical Asia and Africa.[1] Chemical studies carried out with species of this genus indicated a diversity of secondary metabolites such as iridoids, flavonoids, triterpenes, proanthocyanidins and olygopeptides.[2-6]

A great number of species of genus *Ixora* is used in Indian traditional medicine for dysentery, ulcers, cancer pulmonary problems, anemia and urinary diseases.[7-9] Biological studies of these species showed antioxidant, antitumor and antimicrobial activities.[9-11]

Ixora brevifolia Benth. is a plant popularly known in Brazil as "ixora-arbórea" and can be found in Brazilian Cerrado. The study of the bark of this species led to the isolation of the steroid β-sitosterol glycoside, seven pentacyclic triterpenes: lupeol, 3β-lupeonil-eicosanate, 3β-lupeonil-estearate, 3β-lupeonil-palmitate, α-amyrin, β-amyrin, 30-hydroxyfriedelan-3-one, and the flavonoid quercetin. The same study reported the antifungal activity of extracts and fractions from this plant.[12]

Our continuing interest in the research on bioactive constituents of native plants of the Brazilian Cerrado led us to investigate bioactive metabolites of *Ixora brevifolia* branches. Chemical investigation of its methanolic extract resulted in the isolation and identification of two alkaloids, including a new cyclopeptide alkaloid (**1**), named ixorine, and the known cyclopeptide alkaloid frangulanine (**2**), the lignin syringaresinol (**3**), the proanthocyanidin cinnamtannin B-1 (**4**) (Figure 1), along with daucosterol and mannitol. The new and known cyclopeptide alkaloids are being described for the first time in the Rubiaceae family. In addition to the chemical study, several bioassays were performed, as antibacterial, antifungal and antiprotozoal.

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Experimental

General experimental procedures

Chromatography columns were carried out on silica gel 60 (Merck, 70-230 mesh). Analytical thin layer chromatography (TLC) was performed on precoated silica gel plates (TLC Silica gel 60F_{254} from Merck). The fractions and compounds were detected in TLC by UV light (254 and 366 nm) and by spraying with *p*-anisaldehyde-H_2SO_4 solution, followed by heating at 150 °C or by spraying with Dragendorff solution.

Nuclear magnetic resonance (NMR) experiments were recorded on a VARIAN-MERCURY plus spectrometer operating at 300 and 75 MHz for ^{1}H and ^{13}C, respectively. The optical rotations were measured on PerkinElmer 341 and 343 digital polarimeters. The high resolution electrospray ionization mass spectrometry (HRESIMS) were acquired using a ESI-Q-TOF-MS (WATERS) spectrometer. MS analysis was performed on a GC-MS (Thermo-Finnigan, Focus DSQ II) with a quadrupole mass analyzer, using electron impact ionization mode (70 eV).

Plant material

The branches of the plant were collected in May 2010, on Samambaia campus of the Federal University of Goiás (Goiânia, GO, Brazil). A voucher specimen has been deposited in the Herbarium at Universidade Federal de Goiás, Brazil, under the registration number 45523.

Extraction and isolation

The dried powdered branches (676.5 g) were exhaustively extracted by maceration at room temperature with MeOH and concentrated under vacuum to yield the crude extract (CE, 91.8 g). During the solvent removal, it was observed the formation of a precipitate, which filtered with vacuum and washed with MeOH. It was identified as mannitol (2.2 g). A portion of CE (50.7 g) was dissolved with a mixture of H_2O/MeOH (7/3, v/v) and then successively partitioned with different solvents to give *n*-hexane (HF, 3.0 g), $CHCl_3$ (CF, 0.8 g), EtOAc (EAF, 10.2 g), *n*-BuOH (BF, 15.3 g) and the remaining hydromethanolic (HMF, 19.7 g) fractions. The CF fraction (0.4 g) was subjected to a silica gel column using a gradient of *n*-hexane, EtOAc and MeOH to yield 201 fractions (10 mL each), which after TLC analysis were pooled together to 30 fractions (CF1 to CF30). The CF22 fraction afforded **3** [(+)-syringaresinol, (9.7 mg)]. Fractions CF23 and CF24 showed precipitates, which were washed with acetone and provided **2** (frangulanine, 3.7 mg) and a mixture of **1** and **2** (ixorine, 5.6 mg), respectively. The EAF fraction (203.3 mg) was chromatographed on a Sephadex LH-20 column eluted with MeOH to give twenty sub-fractions (EAF-1 to EAF-20). Sub-fraction EAF-13 (36.0 mg) afforded **4** (cinnamtannin B-1). The hexane fraction (1.21 g) was subjected to column chromatography (CC) on silica gel with mixtures of $CHCl_3$ and MeOH in order of increasing polarity ($CHCl_3$, $CHCl_3$/MeOH 5-95%), providing the substance daucosterol (26.0 mg).

A portion of CE (18.2 g) was dissolved in H_2O/MeOH 7/3 and acidified with a solution of hydrochloric acid (HCl, 2 mol L^{-1}) to pH 2-3. The acidic solution was exhaustively extracted with diethyl ether (Et_2O) to yield the acidic ether fraction (AEF, 1.70 g). The aqueous solution was made basic with ammonium hydroxide (pH 8-9) and extracted with Et_2O to yield the basic ether fraction (BEF, 43.0 mg) and basic aqueous fraction (BAF, 15.5 g). The BEF (43.0 mg) was purified using silica gel column chromatography, which was eluted with chloroform containing increasing amounts of methanol (up to 50%). It provided 123 fractions (8 mL each), which were pooled together to 21 fractions after TLC analysis (BEF1 to BEF21). BEF6 provided **2** (frangulanine, 2.6 mg) and BEF9, after acetone washing, provided **1** (ixorine, 0.8 mg). Fractions BEF7 (0.6 mg) and BEF8 (1.1 mg) afforded a mixture of these two substances.

Antileishmanial activity assay

Promastigote forms of *L. amazonensis* (MHOM/BR/75/Josefa strain) were maintained by weekly transfers in Warren's medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) at 25 °C in a tissue flask. For the experiments, promastigotes (1×10^6 parasites mL^{-1}) were inoculated in a 24-well plate containing Warren's medium supplemented with 10% inactivated fetal bovine serum with different concentrations of isolated compound, and incubated at 25 °C for 72 h. The cell density for each concentration was determined by counting in a hemocytometer (Improved Double Neubauer). Controls containing 0.5% dimethyl sulfoxide (DMSO from Sigma) and medium alone were also included. The 50% inhibitory concentration (IC_{50}) was determined by logarithm regression analysis of the data obtained.[13]

Ixorine (**1**)

HRESIMS *m/z*, calcd.: $C_{30}H_{40}N_4O_4$ $[M + H]^+$: 521.3044; found: 521.3049; $[\alpha]_D^{20} = -292.3$ (*c* 0.001, $CHCl_3$); ^{1}H NMR (300 MHz, $CDCl_3$) and ^{13}C NMR (75 MHz, $CDCl_3$), see Table 1.

Frangulanine (**2**)

^{1}H NMR (300 MHz, $CDCl_3$) δ 0.69 (d, 3H, *J* 6.6 Hz, CH_3-32), 0.75 (d, 3H, *J* 6.3 Hz, CH_3-31), 0.86 (d, 3H, *J* 6.6 Hz, CH_3-26), 0.87 (t, 3H, *J* 7.5 Hz, CH_3-25), 0.94 (d, 3H, *J* 6.8 Hz, CH_3-19), 1.20 (d, 3H, *J* 6.8 Hz, CH_3-18), 1.23 (m, 1H, H-29a), 1.30 (m, 1H, H-24a), 1.37 (m, 1H, H-30), 1.45 (m, 1H, H-24b), 1.61 (m, 1H, H-29b), 1.71 (m, 1H, H-23), 1.93 (m, 1H, H-17), 2.11 (s, 6H, CH_3-27, CH_3-28), 2.58 (d, 1H, *J* 3.6 Hz, H-22), 3.94 (m, 1H, H-7), 4.41 (dd, 1H, *J* 10.4, 7.5 Hz, H-4), 4.88 (dd, 1H, *J* 7.5, 2.1 Hz, H-3), 5.85 (d, 1H, *J* 7.2 Hz, H-6), 6.35 (m, 1H, H-9), 6.36 (d, 1H, *J* 7.5 Hz, H-11), 6.53 (dd, 1H, *J* 9.2, 7.5 Hz, H-10), 6.97

(dd, 2H, *J* 8.7, 2.1 Hz, H-14, H-15), 7.05 (dd, 1H, *J* 8.7, 2.1 Hz, H-13), 7.10 (dd, 1H, *J* 8.7, 2.1 Hz, H-16), 7.32 (d, 1H, *J* 10.4 Hz, H-20); ^{13}C NMR (75 MHz, $CDCl_3$) δ 12.6, 15.3, 15.4, 20.5, 21.8, 23.1, 24.6, 28.6, 29.3, 34.0, 40.3, 43.2, 52.9, 55.5, 73.1, 81.7, 118.0, 122.2, 122.9, 126.0, 130.3, 131.7, 132.0, 156.3, 168.2, 171.7, 172.6.

Results and Discussion

Cyclopeptide alkaloids ixorine (**1**) and frangulanine (**2**), lignin syringaresinol (**3**), proanthocyanidin cinnamtannin B-1 (**4**) (Figure 1), along with daucosterol and mannitol were isolated from the branches extract of *Ixora brevifolia*. The structures of known compounds were identified and elucidated using a combination of spectroscopic techniques (^{1}H, ^{13}C NMR and 2D NMR) and by comparisons with literature data.[5,14-17]

The structure of the new compound **1** was elucidated by spectrometric methods, including 1D and 2D NMR experiments and HRESIMS.

Ixorine (**1**) was obtained as a white amorphous powder with positive test with Dragendorff's reagents, with levogire optical rotation. Its molecular formula $C_{30}H_{40}N_4O_4$ was determined by HRESIMS through the ion at *m/z* 521.3049 $[M + H]^+$. In ^{13}C NMR spectra (Table 1), 30 carbons signals were observed, compatible with the suggested molecular structure, which were consistent with six methyl at δ 15.2, 17.6, 20.4, 21.0, 43.1, 43.1, one methylene at δ 37.1, seventeen methine groups (with saturated carbons at δ 81.7, 75.2, 55.6, 55.3, 29.3 and 27.8) and six quaternary carbons (including three carbonyl carbons at δ 172.4, 171.6 and 167.2).

The ^{1}H NMR spectrum of **1** showed the presence of two methyl doublets (H-18 and H-19) at δ 1.25 ($J_{18,17}$ = 7.2 Hz) and 1.00 ($J_{19,17}$ = 6.6 Hz), two methine protons (H-3 and H-4) as double doublets at δ 4.91 ($J_{3,4}$ = 8.0; $J_{3,17}$ = 2.1 Hz) and at δ 4.44 ($J_{4,20}$ = 9.0; $J_{4,3}$ = 8.0 Hz) of the β-hydroxyleucine unit, which was confirmed by HMBC correlations between C-3 (δ 81.7) and H-18 and H-19, C-5 (δ 171.6) and H-6 (δ 5.94, NH) and by COSY correlation between H-4 and H-20 (δ 6.94, NH). The presence of a *p*-oxigenated *Z*-styrylamine group was indicated by a multiplet at δ 6.56 (H-10), a doublet at δ 6.40 ($J_{11,10}$ = 6.6 Hz, H-11) and aromatic signals at δ 6.92 (m, H-16), 7.03 (m, H-13 and H-15), and 7.15 (m, H-14), the H-13 showed correlation with C-11 (δ 118.4) and H-14 with C-12 (δ 131.8) in HMBC spectra. The methyl doublets of *N,N*-dimethyl valine (H-24 and H-25) appeared at δ 1.05 ($J_{24,23}$ = 6.9 Hz) and 0.93 ($J_{25,23}$ = 6.8 Hz) and the methine proton (H-22) of this moiety appeared as doublet at δ 2.40 (J = 4.2 Hz), which presented a correlation with a singlet at δ 2.14 (H-26, H-27) in COSY experiment. The HMBC experiment showed correlations between C-21 (δ 172.4) and H-22 and H-23 (δ 2.05) for this moiety. The methine and methylene proton (H-7 and H-28) of phenylalanine moiety were observed as a multiplet at δ 4.30 and two double doublet at δ 3.07 ($J_{28,28'}$ = 13.5; $J_{28,7}$ = 6.0 Hz) and 2.76 ($J_{28',28}$ = 13.8; $J_{28',7}$ = 4.5 Hz), respectively, which presented correlations with C-29 (δ 135.7) and the latter presented a correlation with C-8 (δ 167.2) in HMBC experiment.

The combined use of 1D (^{1}H and ^{13}C NMR, DEPT) and 2D (COSY and HSQC) spectra allowed attributable unambiguous assignments of all protons and carbons of the amino acids units and the presence of a *p*-oxygenated *Z*-styrylamine group (Table 1).

The relative stereochemistry for **1** was proposed from the ^{1}H NMR coupling constants and NOESY analysis (Figure 2) and is in agreement with that of frangulanine (**2**).[14] The vicinal coupling constants of 8.0 Hz of the methines H-3/H-4 and ^{13}C NMR resonances

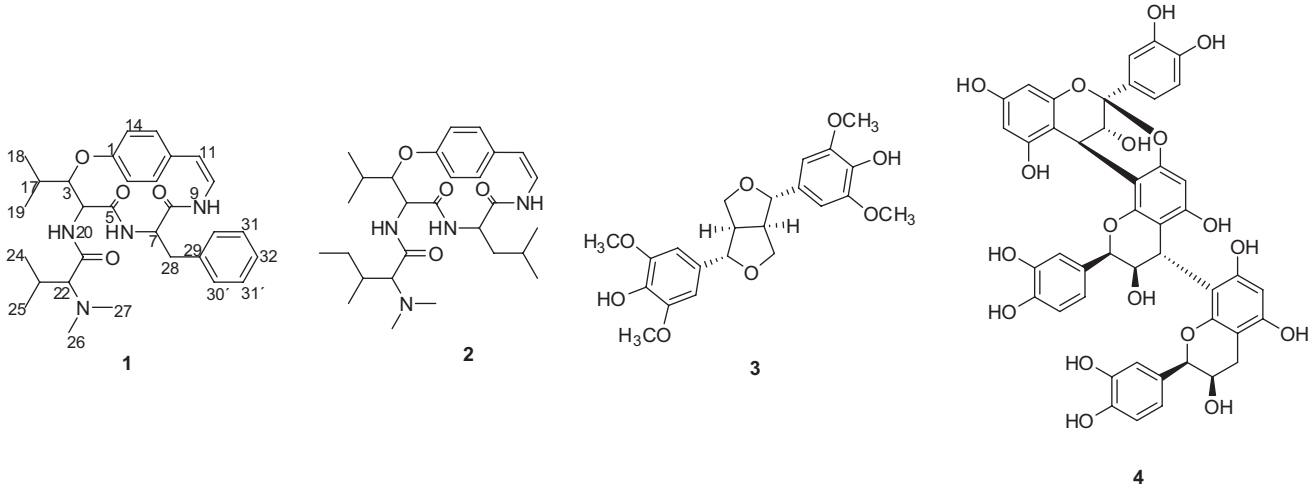

Figure 1. Structures of compounds**1**-**4** isolated from the branches of *I. brevifolia*.

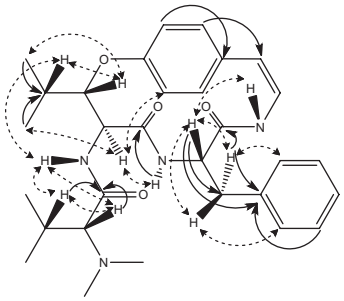

Figure 2. Selected key HMBC (^{1}H ⟶ ^{13}C) and NOESY (^{1}H ⟷ ^{1}H) correlations for ixorine (**1**).

of C-3 and C-4 (δ 81.7 and 55.6) indicate a *L-erythro* configuration for β-substituted leucine moiety, as reported for cyclopeptide alkaloids.[18-20] In addition, H-3 showed correlation with H-17 and H-18 in the NOESY spectra, while the H-4 exhibited correlation with H-19 and NH-6, and the latter did not showed interaction with H-7, which is consistent with the coupling constant of 6.3 Hz of the H-6, due coupling with H-7. These evidences are also in agreement with *L-erythro* configuration of β-hidroxyleucine of ixorine.[21] The coupling constants of the H-4 ($J_{4,20}$ = 9.0; $J_{4,3}$ = 8.0 Hz) show that the relative orientation of H-4 and H-20 are also in opposite faces. The correlations of H-20 with H-17, H-22, and H-23, observed in NOESY spectra, indicate that these protons are co-facially oriented.

Although the 14-membered ring is the largest subgroup of cyclopeptide alkaloids, few studies of their biological activities have been reported due to the low amounts present in the plants of origin. The biological properties for this class of substances includes antibacterial, antifungal, antiplasmodial, antinociceptive and immunostimulant activities.[22]

The alkaloidal mixture **1** and **2** (CF24 fraction) were tested for antibacterial, antifungal and antiprotozoal activities. Antibacterial and antifungal activities were evaluated for the strains *Escherichia coli* ATCC 25922,

Table 1. NMR spectroscopic data (300 MHz, $CDCl_3$) for ixorine (**1**)

Position	δ_C	δ_H (mult., *J* in Hz)	HMBC	COSY
1	156.3	–	H-14, H-16	–
2	–	–	–	–
3	81.7	4.91 (dd, 8.0, 2.1)	H-4, H-18, H-19	H-4
4	55.6	4.44 (dd, 9.0, 8.0)	–	H-20 (NH)
5	171.6	–	H-6 (NH)	–
6	–	5.94 (d, 6.3, NH)	–	–
7	55.2	4.30 (m)	H-28	H-28
8	167.2	–	H-28	–
9	–	6.20 (sl, NH)	–	–
10	125.7	6.56 (m)	–	–
11	118.4	6.40 (d, 6.6)	H-13	–
12	131.8	–	H-14	–
13	131.7	7.03 (m)	–	H-14
14	121.9	7.15 (m)	–	–
15	122.7	7.03 (m)	–	H-16
16	130.2	6.92 (m)	–	–
17	29.3	2.00 (m)	H-18, H-19	H-18, H-19
18	20.4	1.25 (d, 7.2)	H-17, H-19	–
19	15.2	1.00 (d, 6.6)	H-17	–
20	–	6.94 (m, NH)	–	–
21	172.4	–	H-22, H-23	–
22	75.2	2.40 (d, 4.2)	H-24, H-25, H-26, H-27	H-26, H-27
23	27.8	2.05 (m)	H-24, H-25	H-24, H-25
24	21.0	1.05 (d, 6.9)	–	–
25	17.6	0.93 (d, 6.6)	–	–
26	43.1	2.14 (s)	H-27	–
27	43.1	2.14 (s)	–	–
28	37.1	2.76 (dd, 13.8, 4.5)/3.07 (m)	H-30, H-30'	H-28
29	135.7	–	H-7, H-28, H-31, H-31'	–
30, 30'	129.6	7.15 (m)	–	H-31, H-31'
31, 31'	129.0	7.34 (m)	–	–
32	127.4	7.24 (m)	–	H-30, H-30'

Pseudomonas aeruginosa (ATCC 27853), *Staphylococcus aureus* (ATCC 25923), *Candida albicans* (ATCC 10231). The minimum inhibitory concentrations were determined by micro dilution techniques in Mueller Hinton broth for bacteria, and RPMI 1640 for yeasts, described by the Clinical and Laboratory Standards Institute (CLSI).[23,24] However, no significant biological activities were observed.

Antiprotozoal activity was evaluated *in vitro* against promastigotes of *Leishmania amazonensis*. Treatment with concentrations 5.0, 10.0, 50.0 and 100.0 μg mL^{-1} of CP24 inhibited the parasite growth by 12.41, 24.13, 48.27 and 63.45%, respectively. The IC_{50} value was 54.16 μg mL^{-1}. Leishmaniasis is regarded as a neglected disease, and nothing was found in literature about assays with these or related cyclic peptides against this protozoal.

Conclusions

The phytochemical study of *Ixora brevifolia* Benth. has led to the isolation of two cyclopeptide alkaloids, ixorine and frangulanine, along four known constituents. Frangulanine, previously isolated in species of Rhamnaceae family,[14] is reported in Rubiaceae family for the first time. This kind of cyclic peptide alkaloids was isolated only in five species of this family: *Canthium anorldianum*, *Canthium euryoides*, *Feretia apodanthera*, *Plectronia odorata* and *Amaioua guianensis*[25,26] and all of these species belong to the subfamily Ixoroideae, as well as *Ixora brevifolia*.

Supplementary Information

1D and 2D NMR spectra for compounds **1**-**2** are available free of charge online at http://jbcs.org.br as a PDF file.

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