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Article

Cytotoxic Alkaloids from *Hippeastrum solandriflorum* Lindl.

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One new alkaloid, 2α-10bα-dihydroxy-9-*O*-demethylhomolycorine, in addition to seven others known alkaloids, and 5-(hydroxymethyl)furfural, piscidic acid and eucomic acid, were isolated from the bulbs of *Hippeastrum solandriflorum*. The structures of all compounds were determined using nuclear magnetic resonance (NMR) spectroscopic techniques: ^{1}H NMR and ^{13}C NMR, heteronuclear single quantum coherence (HSQC), heteronuclear multiple bond correlation (HMBC), nuclear Overhauser effect spectroscopy (NOESY), and also the high-resolution electrospray ionization mass spectrometry (EI-HRMS). The cytotoxic activity of all alkaloids was evaluated against three human cancer cell lines (HCT-116, HL-60, OVCAR8 and SF-295) showing IC_{50} values ranging from 0.01 to 35.7 μM.

Keywords: *Hippeastrum solandriflorum*, Amaryllidaceae alkaloids, cytotoxic activity

Introduction

The Amaryllidaceae, the 4th of the 20 most important alkaloid-containing plant families, comprising approximately 1100 perennial bulbous species, represented by 85 genera.[1] Known for their beautiful flowers, Amaryllidaceae plants have been extensively investigated, particularly due to the structural diversity of their compounds and the broad spectrum of biological activities.[2] The main particularity of the plants from this family is the production of a large and peculiar group of isoquinoline alkaloids, most of which have never been found in any other plant family.[1] So far, more than 500 alkaloids have been isolated and evaluated through their pharmacological properties, particularly as: antiviral, antitumor, antioxidant, antimalarial, anti-inflammatory and cytotoxic.[3-5] Two of these alkaloids, lycorine and narciclasine, which were isolated from several Amaryllidaceae genera including *Hipeastrum*, have been extensively investigated due their anticancer properties.[6-8]

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Hippeastrum is a known ornamental genus, consisting of approximately 70 species, predominantly distributed in Latin America, especially in Brazil, with ca. 30 cataloged species.[9] Previous investigations carried out with *Hippeastrum* species have shown that this genus is a prolific source of alkaloids, particularly of the lycorine (pyrrolo[de]phenanthridine) and tazettine (2-benzopyrano[3,4-c] indole) types.[10]

In our continuing efforts searching for bioactive and/or novel secondary metabolites from plants of the Northeast Brazilian flora, *H. solandriflorum* was investigated in order to find out new anticancer compounds. In this paper, we report the isolation and characterization of a new homolycorine type alkaloid (**1**) (Figure 1) from the bulbs of *H. solandriflorum*, a species widely found in Ceará State. Additionally, seven known alkaloids (**2**-**8**) as well as their antiproliferative properties and three phenolic compounds (**9**-**11**), are also reported.

Results and Discussion

The chemical investigation of the ethanol extract from bulbs of *H. solandriflorum* allowed the isolation of eight

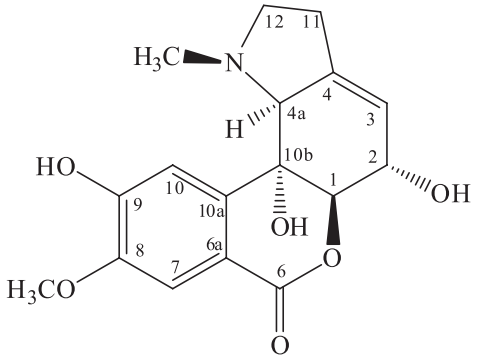

Figure 1. Structure of the compound **1** isolated from *H. solandriflorum.*

alkaloids, including a new one. In addition, three known phenolic compounds were also isolated (Figure 1). The chemical characterization was performed by analysis of their nuclear magnetic resonance (NMR) (in one and two dimensions), Fourier transform infrared (FTIR) spectroscopies and high-resolution electrospray ionisation mass spectrometry (EI-HRMS) spectral data.

Compound **1**, isolated as an optically active white powder, was determined to have the molecular formula as $C_{17}H_{19}NO_6$ (9 degrees of unsaturation) by analysis of its EI-HRMS (*m/z*: 334.1272 for $[M + H]^+$, calcd.: 334.1285) and the ^{13}C NMR spectra. The FTIR spectrum showed absorption bands for hydroxyl group at 3461 cm^{-1}, for conjugated carboxyl ester at 1719 cm^{-1} and double bonds at 1673-1447 cm^{-1}, as well as absorption bands for carbon-oxygen and carbon-nitrogen at 1247-1034 cm^{-1}.

The ^1H NMR spectrum showed two singlets at δ_H 7.30 (H-10) and 7.57 (H-7) for aromatic protons *para*-positioned, a broad singlet at δ_H 5.96 (H-3) indicating a trissubstituted double bond, two singlets at δ_H 4.61 (H-1) and 4.07 (H-2) of oxymethine protons, as well as, a singlet at δ_H 4.36 (H-4a) of an azomethine proton. In the heteronuclear single quantum coherence (HSQC) spectrum the latest three proton signals showed correlations with the carbons at δ_C 83.4 (C-1), 72.3 (C-4a) and 68.1 (C-2), respectively. In addition, signals at δ_H 3.94 (s), for a methoxyl (MeO-8), and at δ_H 2.62 (s) for a *N*-methyl (Me-N) were also observed in the ^1H NMR spectrum. These data were consistent with a structure belonging to homolycorine type alkaloids of the Amaryllidaceae.[11,12] Further examination of the ^{13}C NMR and distortionless enhancement by polarization transfer (DEPT) 135 spectral data of **1** (Table 1) showed signals consistent with those of the homolycorine skeleton alkaloids,[12,13] corroborating with the ^1H NMR spectrum. The comparative analysis of the ^{13}C NMR chemical shifts revealed that **1** shared high structural similarity to 2α-hydroxy-9-*O*-demethylhomolycorine.[13] Nevertheless, the signal of C-10b (δ_C 67.3) of **1** showed to be strongly unshielded when compared to the corresponding in 2α-hydroxy-9-*O*-demethylhomolycorine (δ_C 40.0).[13] This significant difference ($\Delta\delta$ 27.3 ppm), was easily justified by the hydroxylation of that carbon. This proposition was confirmed by the heteronuclear multiple bond correlation (HMBC) spectrum through the long-range correlation between H-10 (δ_H 7.30) with C-10b (δ_C 67.3). The determination of the relative stereochemistry of compound **1** was accomplished through the nuclear Overhauser effect (NOE) spectra (Figure 2). In CD_3OD (Table 1) the dipolar coupling between H-1 (δ_H 4.61) and H-4a (δ_H 4.36), in addition to the NOE spectra of H-2 (δ_H 4.07) and the CH_3-N (δ_H 2.62) permitted to infer that the relative stereochemistry of H-4a is indeed opposite to H-4a of that of 2α-hydroxy-9-*O*-demethylhomolycorine.[12] In order to confirm the stereochemistry of **1**, a new experiment was done with the protonated alkaloid (**1**) in DMSO-d_6. In addition to the above mentioned NOE's were observed NOE's of the hydroxyl at 10b (δ_H 6.66) with both H-1 (δ_H 4.50) and H-10 (δ 7.20). Thus, the structure of **1** was established as *rel*-(1*S*,2*S*,4a*R*,10b*S*)2,10b-dihydroxy-9-*O*-demethylhomolycorine.

In addition to isolation of a new alkaloid named *rel*-(1*S*,2*S*,4a*R*,10b*S*)2,10b-dihydroxy-9-*O*-

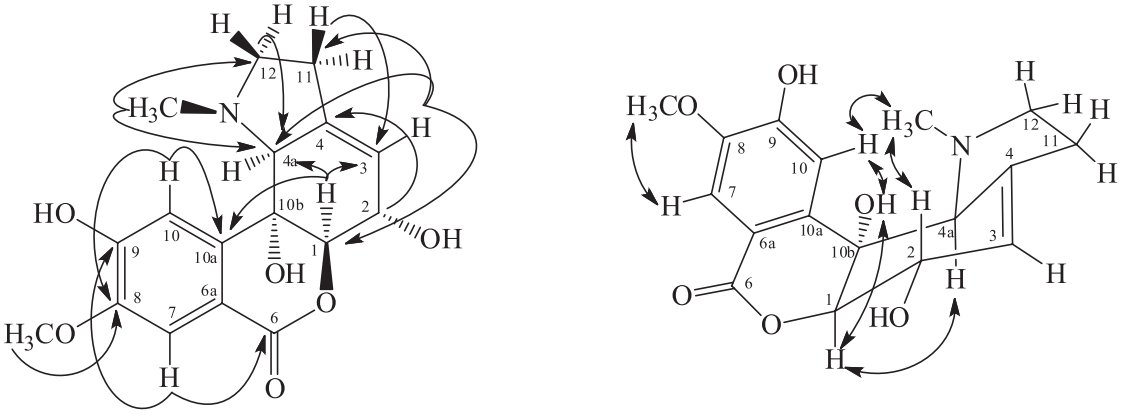

Figure 2. HMBC (→) and NOESY (↔) correlations for compounds **1**.

Table 1. ^{1}H NMR (300 MHz, CD_3OD) and ^{13}C NMR (75 MHz, CD_3OD) data of compound **1**

Position	**1**		
	δC	δ_H	δ_H[a]
1	83.4	4.61 (s)	4.50 (s)
2	68.1	4.07 (s)	4.05 (m)
3	123.7	5.96 (br s)	5.83 (s)
4	135.5	–	–
4a	72.3	4.36 (s)	4.24 (s)
6	165.5	–	–
6a	115.3	–	–
7	114.3	7.57 (s)	7.41 (s)
8	154.5	–	–
9	150.1	–	–
10	112.3	7.30 (s)	7.20 (s)
10a	140.3	–	–
10b	67.3	–	–
11	28.2	2.91 (m) / 2.80 (m)	2.76 (m) / 2.58 (m)
12	57.7	3.75 (m) / 3.31 (m)	3.58 (m) / 3.19 (m)
8-OMe	56.8	3.94 (s)	3.86 (s)
H_3C-N	43.4	2.62 (s)	2.44 (s)
2-OH	–	–	5.50 (s)
9-OH	–	–	9.69 (s)
10b-OH	–	–	6.66 (s)
$H_3C–N^+H$[a]	–	–	10.58 (s)

[a]^{1}H NMR spectrum of **1** (protonated) in DMSO-d_6.

demethylhomolycorine seven known ones, pseudolycorine (**2**),[14] narcissidine (**3**),[15] sanguinine (**4**),[16] 11-hydroxyvittatine (**5**),[17] galanthamine *N*-oxide (**6**),[16,18] galanthamine (**7**),[16] and narciclasine (**8**),[19] besides three phenolic compounds, 5-(hydroxymethyl)furfural (**9**),[20] piscidic acid (**10**)[21] and eucomic acid (**11**).[22] Although lycorine has been previously isolated from *Hippeastrum solandriflorum*,[10] has not been isolated in this work.

The overall cytotoxic effect of all isolated alkaloids (**1**-**8**) was assessed by the [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] (MMT) assay against the HCT-116 (colon adenocarcinoma), HL-60 (leukemia), OVCAR-8 (ovarian carcinoma) and SF-295 (glioblastoma) cell lines. The data on Table 2 show the high cytotoxicity of narciclasine (**8**) against the four cell lines tested, with IC_{50} values ranging from 0.01 to 0.09 μM. This result is in accordance with other reports, which found a mean IC_{50} value of 0.05 μM for **8** against several other cancer cell lines.[23] Besides **8**, only compound **2** can be considered as highly cytotoxic, with IC_{50} values on the 1μM range. In general, HCT-116 was the most sensitive cell line, to which five of the eight tested compounds presented IC_{50} values below 50 μM.

Data are presented as IC_{50} values in μM and as the 95% confidence interval obtained by nonlinear regression for all of the cell lines from two independent experiments, performed in duplicate, after 72 h incubation.

Conclusions

A total of eleven compounds were isolated from *H. solandriflorum*, among which eight were alkaloids. These finds are in agreement with the chemistry produced by plants of the Amaryllidacea family, a prolific source of alkaloids.

Experimental

General experimental procedures

Optical rotations were measured on a Perkin-Elmer 341 digital polarimeter. FTIR spectra were obtained on a

Table 2. Cytotoxicity of compounds **1**-**8** on select tumor cell lines evaluated by the MTT assay after 72 h of exposure

	IC_{50} / μM[a]			
	HCT-116	HL-60	OVCAR8	SF-295
1	11.69 (8.10-16.87)	> 50	15.11 (6.41-35.66)	16.31 (11.96-22.24)
2	0.85 (0.71-1.02)	1.10 (0.97-1.26)	1.59 (1.28-1.97)	1.81 (1.54-2.11)
3	> 50	> 50	> 50	> 50
4	29.00 (16.97-49.57)	32.08 (19.83-52.02)	> 50	> 50
5	35.71 (24.05-53.01)	> 50	> 50	> 50
6	> 50	> 50	> 50	> 50
7	> 50	> 50	> 50	> 50
8	0.02 (0.01-0.03)	0.01 (0.02-0.01)	0.09 (0.07-0.10)	0.02 (0.02-0.03)
Doxorubicin	0.02 (0.02-0.04)	0.03 (0.02-0.04)	0.29 (0.18-0.40)	0.33 (0.22-0.43)

[a]confidence interval of 95%.

Perkin Elmer FT-IR 1000 spectrometer. EI-HRMS were acquired using a LCMS-IT-TOF (Shimadzu) spectrometer. ^{1}H NMR (500 or 300 MHz) and ^{13}C NMR (125 or 75 MHz) spectra were performed either on a Bruker DRX-500 or DPX 300 spectrometer. High performance liquid chromatography (HPLC) analysis was carried out using a UFLC (Shimadzu) system equipped with a SPD-M20A diode array UV-Vis detector and a Phenomenex C-18 column, 5 µm (4.6 × 250 mm). The Mobille phase consisted of H_2O (with trifluoroacetic acid 0.1% v/v) and CH_3CN with a 4.72 mL min^{-1} flow rate and the chromatograms were acquired at 210-400 nm. Chromatographic columns were performed in Sephadex LH-20 or solid phase extraction (SPE) C-18 cartridges (Strata C18-E, 20 g 60 mL^{-1}, 55 µm, 70 Å). Thin layer chromatography (TLC) was performed on precoated silica gel aluminium sheets (kieselgel 60 F_{254}, 0.20 mm, Merck), and the spots were visualized by the Dragendorff reagent or by heating (at ca. 100 °C) the plates sprayed with a vanillin/perchloric acid/EtOH solution.

Plant material

Bulbs of *H. solandriflorum* were collected in Russas County, Ceará State, Brazil, in February 2012, and identified by Dr. Luiz Wilson Lima-Verde of the Departamento de Biologia, Universidade Federal do Ceará. A voucher specimen (# 37956) has been deposited at the Herbário Prisco Bezerra (EAC) of the Universidade Federal do Ceará.

Extraction and isolation

Fresh bulbs (8.8 kg) of *H. solandriflorum* were extracted with EtOH (3 × 5.0 L) at room temperature for 24 h, and the resulting solution was concentrated under reduced pressure to give the crude extract (283.0 g).

An aliquot of this extract (88.0 g) was dissolved in a mixture of MeOH-H_2O (7:3 (v/v), 100 mL) and partitioned with CH_2Cl_2 (5 × 100 mL), EtOAc (5 × 100 mL) and n-BuOH (5 × 50 mL), to give the following fractions: CH_2Cl_2 (2.80 g), EtOAc (1.64 g), n-BuOH (1.54 g), and aqueous fraction (55.0 g). The latter fraction was acidified with diluted HCl (10%, v/v) and extracted with CH_2Cl_2 (3 × 100 mL) in order to remove the non-alkaloidal compounds. The aqueous solution was basified with 25% NH_4OH up to pH 9 and extracted with EtOAc (10 × 100 mL) to give extract A (760.8 mg) and subsequently with n-BuOH (2 × 50 mL) to give extract B (507.0 mg). Extract A was re-suspended in MeOH, leading to the formation of a precipitate (80.5 mg). This precipitate was submitted to a semi-preparative reverse HPLC analysis using CH_3CN-H_2O 15:85 (v/v) to yield pure compounds **1** (38.0 mg, t_R 5.8 min) and **2** (28.0 mg, t_R 4.2 min). The MeOH soluble material (679.0 mg) was submitted to a Sephadex LH-20 column using MeOH as eluent. 37 fractions of 8 mL were obtained, which were monitored by TLC (Dragendorff's reagent, UV light λ 254 nm) and combined according to their TLC profiles, yielding fractions A-G. Fraction D (274.0 mg) was purified through a SPE cartridge using MeOH-H_2O (5:5 to 10:0, v/v) as eluent, providing 44 subfractions of 5 mL each. Subfractions 4-8 and 19-37 composed of a mixture of alkaloids detected by Dragendorff's test, were selected for investigation. Subfraction 4-8 (73.0 mg) was purified through a SPE cartridge using MeOH-H_2O (5:5 to 10:0, v/v) as eluent. Fraction MeOH-H_2O 5:5 (v/v) (39.5 mg) was further purified by HPLC analyses (CH_3CN-H_2O 15:85, v/v) to give compound **3** (4.1 mg, t_R 12.3 min). Subfraction 19-37, was purified by semi-preparative HPLC (CH_3CN-H_2O 15:85, v/v) to afford compounds **4** (4.5 mg, t_R 4.0 min), **5** (6.4 mg, t_R 5.0 min) e **6** (6.2 mg, t_R 9.2 min). Fraction E (83.1 m g) was subjected to SPE cartridge using MeOH-H_2O (5:5, v/v) as eluent, resulting in 44 subfractions. Subfraction 12-22 (21.4 mg), showing positive Dragendorff's test, was submitted to HPLC analyses, using CH_3CN-H_2O (15:85, v/v) as mobile phase, and monitored at 210-400 nm, to afford the compound **7** (4.1 mg, t_R 6.1 min).

The CH_2Cl_2 and EtOAc fractions, obtained from the first partition, were combined (4.44 g) and fractionated on a silica gel column chromatography (CC) and eluted with pure or binary mixtures of n-hexane, CH_2Cl_2, EtOAc and MeOH to give 80 fractions (ca. 8 mL), which were monitored by TLC (Dragendorff's reagent, UV light λ 254 nm) and combined according to their TLC profiles, yielding fractions A-F. Fraction F (950.2 mg), after successive silica gel CC eluted with n-hexane, CH_2Cl_2, EtOAc and MeOH, pure or as binary mixtures, allowed to isolation of compounds **8** (4.5 mg), **9** (14.0 mg), **10** (5.6 mg) and **11** (5.0 mg).

2α-10bα-dihydroxy-9-*O*-demethylhomolycorine (**1**): white amorphous powder; melting point: 252.3-252.8; $[\alpha]_D^{20}$: +40.2 (*c* 0.14, MeOH); IV ν_{max} / cm^{-1} 3461, 2923, 1719, 1673, 1597, 1523, 1447, 1172, 1132, 1084; ^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) data, see Table 1; EI-HRMS ([M + H]$^+$) calcd.: 334.1285 for $C_{17}H_{20}NO_6$; found: 334.1272.

Cytotoxicity evaluation: MTT assay

Cytotoxicity was evaluated against four different human cancer cell lines provided by the National Cancer Institute U.S. (Bethesda, MD): HCT-116 (colon adenocarcinoma), HL-60 (leukemia), OVCAR-8 (ovarian carcinoma) and

SF-295 (glioblastoma). Cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mmol L^{-1} glutamine, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin at 37 °C under a 5% CO_2 atmosphere. Compounds (**1**-**8**) were tested at concentrations ranging from 0.001 to 50 μM during 72 h and the effect on cell proliferation was evaluated *in vitro* using the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-*2H*-tetrazolium bromide] assay, as described by Mosmann.[24] Doxorubicin was used as positive control. IC_{50} (the concentration that inhibits growth in 50%) values were calculated, along with the respective 95% of confidence interval (CI), by non-linear regression using the software GraphPad Prism 5.0.

Supplementary Information

Supplementary information, including ^{1}H NMR, ^{13}C NMR, COSY, HSQC, and HMBC spectra, as well as mass spectra (Figures S1-S29), are available free of charge at http://jbcs.sbq.org.br as a PDF file.

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