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Short Report

Dinor Casearin X, a New Cytotoxic Clerodane Diterpene from *Casearia sylvestris*

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A new clerodane-type diterpene, named dinor casearin X, and four known casearins (A, B, G and J) were isolated from leaves of *Casearia sylvestris* (Salicaceae). These compounds were evaluated for cytotoxic activity against five human cancer cell lines (A2058, HL-60, HCT, MCF-7 and HeLa) as well as against a murine melanoma cell line (B16F10-Nex2). Among these compounds, dinor casearin X exhibited the highest cytotoxic activity against HL-60 cells with IC_{50} of 0.51 ± 0.11 μg mL^{-1}, whereas casearin A exhibited the highest cytotoxic activity against HCT cells (IC_{50} 1.84 ± 0.14 μg mL^{-1}).

Keywords: *Casearia sylvestris*, cytotoxic activity, dinor casearin X

Introduction

Casearia sylvestris Swartz (Salicaceae), popularly known as "guaçatonga", is geographically distributed throughout Latin America,[1] where it has been used by native communities to treat several diseases.[2] The use of this plant in traditional medicine and subsequent scientific investigations have highlighted the importance of *C. sylvestris* extracts due to their antiulcer, antiinflammatory, antiophidian and antitumor properties.[3] Chemically, *C. sylvestris* extracts are rich in clerodane-type diterpenes, known as casearins and casearvestrins.[3,4] Casearins A-F have been described as antitumor compounds, with casearin C exhibiting the highest cytotoxicity against V-79 cells *in vitro*.[5] Moreover, casearin B exhibited chemoprotective effect against DNA damage.[6] Other studies reported the occurrence of antitumoral casearins G-R,[7] DNA-damaging casearins S and T,[8] and cytotoxic casearins U and V.[9] Cytotoxic effects of casearin X were demonstrated in leukemia cells where it triggered apoptosis.[10,11] Furthermore, cytotoxic casearvestrins A-C have also been isolated in *C. sylvestris*.[4]

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As a part of our continuous study aiming at the discovery of novel bioactive compounds from *C. sylvestris*,[12,13] this work reports the isolation and characterization of casearins A, B, G and J, as well as a new derivative named dinor casearin X, which displayed cytotoxic activity against the human leukemia cell line HL-60.

Experimental

General experimental procedures

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra of compounds **1**-**4** were recorded at 300 and 75 MHz, respectively, in a Bruker Ultrashield 300 Avance III spectrometer. ^{1}H and ^{13}C NMR spectra of compound **5** were recorded in a Bruker AIII-500 (500 MHz for ^{1}H) spectrometer and in a Bruker Avance III Ultrashield Plus spectrometer (150 MHz for ^{13}C) with cryo-probe of 5 mm. CD_3OD (Aldrich) was the solvent and the residual resonance peaks at δ_H 3.3 (^{1}H) and δ_C 49.0 (^{13}C) were used as internal standard. Optical rotation was recorded in a Schmidt+Haensch polartronic H100 (automatic high resolution circular). Fourier transform infrared (FTIR) spectrum was recorded on a Shimadzu Prestige-21 FTIR

spectrometer. High resolution electrospray ionization mass spectra (HRESIMS) were obtained in a Bruker Daltonics ultrOTOFq (ESI/time-of-flight (TOF), positive mode). High performance liquid chromatography (HPLC) analysis was performed in a Dionex Ultimate 3000 chromatograph, using a Luna Phenomenex RP-18 column (3 μm, 150 × 5 mm) and an UV-diode array detector (DAD). Silica gel (Merck, 230-400 mesh) and Sephadex LH-20 (Sigma-Aldrich) were used for the column chromatographic (CC) separation, while silica gel 60 PF_{254} (Merck) was used for analytical and preparative thin-layer chromatography (TLC).

Plant material

Leaves of *Casearia sylvestris* were collected from a single tree in the Atlantic Forest area of São Paulo City, SP, Brazil (coordinates 23°53'08.86'' S, 46°40'10.45'' W), in October 2012. Botanical identification was carried out by PhD Roseli Buzanelli Torres from Instituto Agronômico de Campinas (IAC), Campinas-SP, Brazil. A voucher specimen (IAC 55272) has been deposited in the IAC herbarium.

Extraction and isolation

Leaves of *C. sylvestris* (290 g) were dried, powdered and exhaustively extracted with MeOH to obtain 11.1 g of crude extract. After cytotoxic evaluation, the active MeOH extract was ressuspended in MeOH:H_2O (2:1) and partitioned using hexane, CH_2Cl_2 and EtOAc. Part of active hexane phase (6.4 g) was subjected to separation over silica gel CC and eluted with increasing amounts of EtOAc in hexane (9:1 to 1:9) to obtain 23 fractions (A1-A23), in which A11-A13 displayed cytotoxic activity. Fraction A11 (380 mg) was chromatographed over Sephadex LH-20, eluted with MeOH and purified by preparative TLC on SiO_2 (hexane:EtOAc, 4:1) to afford **3** (77 mg) and **4** (51 mg). Compound **5** (1 mg) was purified by semi-preparative RP-18 HPLC, eluted with MeCN:H_2O (64:36, flow rates 3.7 mL min^{-1}, UV 218 nm) from the active fraction A11-3-4 (20 mg). Fraction A12 (300 mg) was also subjected to CC over Sephadex LH-20, eluted with MeOH to yield **2** (12 mg). Fractionation of A13 (526 mg) over Sephadex LH-20 (MeOH as eluent) followed by preparative TLC on SiO_2 (hexane:EtOAc, 7:3) afforded **1** (43 mg).

Dinor casearin X ((1*R**,3*S**,5*S**,6a*R**,7*S**,8*S**,10*R**,10a*R**)-1-(acetyloxy)-3,5,6,6a,7,8,9,10-octahydro-10-hydroxy-7,8-dimethyl-7-[(1*E*)-butenone]naphtho[1,8a-c]furan-3,5-diyl dibutanoate) (**5**)

Amorphous white solid; $[\alpha]_D^{20}$ +0.20° (*c* 0.02, MeOH); HRESIMS $[M + Na]^+$ calcd.: 543.2570; found: 543.2564; IR (KBr) ν_{max} / cm^{-1} 3445, 2955, 2918, 2918, 2850, 1723, 1617; ^1H and ^{13}C NMR see Table 1.

Cytotoxicity assays

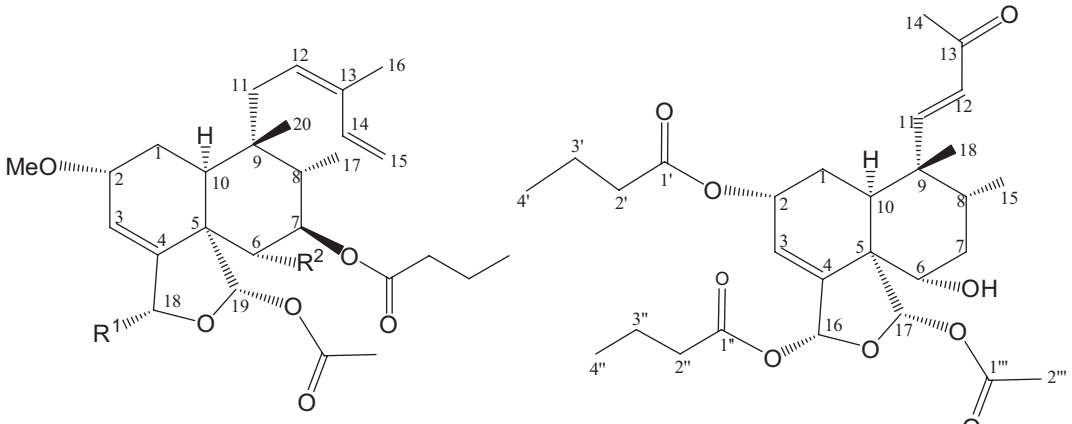
The murine melanoma cell line B16F10 was originally obtained from the Ludwig Institute for Cancer Research (São Paulo, Brazil). The melanotic B16F10-Nex2 subline, which is characterized by low immunogenicity and moderate virulence, was identified at the Experimental Oncology Unit (UNIFESP). Human melanoma (A2058), leukemia (HL-60), colon cancer (HCT) and breast cancer (MCF-7) cell lines were obtained from the Ludwig Institute for Cancer Research. Human cervical carcinoma (HeLa) was acquired from PhD Hugo Pequeno Monteiro (UNIFESP).

Purified casearins **1**-**5** were resuspended in dimethyl sulfoxide (DMSO) at a final concentration of 10 mg mL^{-1}, and next diluted in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Invitrogen) containing 10% fetal calf serum at concentrations ranging from 100 to 0 μg mL^{-1}. The media were then incubated with 1×10^4 cells in a 96-well plate. After 18 h of incubation, cell viability was measured using the cell proliferation kit 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; thiazol blue) (Sigma).[14] Readings were made on a plate reader at 570 nm. All experiments were performed in triplicate. Doxorubicin (positive control) was purchased from Sigma.

Results and Discussion

Bioactivity guided chromatographic fractionation of the hexane phase obtained from the MeOH extract of *C. sylvestris* leaves led to the isolation of known casearins A (**1**),[5] B (**2**),[5,15] G (**3**)[7,8] and J (**4**)[7] as well as one new derivative named dinor casearin X (**5**) (Figure 1). Identification was carried out by analysis of their spectral data and comparison with those reported in the literature.[5,7,8,15]

Dinor casearin X (**5**) was isolated as an amorphous white solid, with a molecular formula $C_{28}H_{40}O_9$, as determined from the HRESIMS (positive mode) adduct ion $[M + Na]^+$ at *m/z* 543.2564 (calcd. 543.2570). The IR spectrum showed the presence of one carbonyl ketone conjugated to a double bound at 1617 cm^{-1} and one hydroxyl broad band at 3445 cm^{-1}. The ^{13}C NMR spectrum exhibited 28 signals (Table 1), several of which were similar to casearins **1**-**4**, suggesting a substructure containing the decalinic system (rings A and B) and diacetalic ring C. Analysis of the heteronuclear multiple-bond correlation (HMBC) spectrum indicated the partial structure formed by the side chain containing one α,β-unsaturated system (double bond at



1 $R^1 = CH_3CO_2$, R^2 = OH (α) - casearin A
2 $R^1 = CH_3CO_2$, $R^2 = CH_3CO_2$(α) - casearin B
3 $R^1 = CH_3CO_2$, R^2 = H - casearin G
4 $R^1 = n\text{-}C_3H_7CO_2$, R^2 = OH(α) - casearin J

5 Dinor casearin X

Figure 1. Structures of casearins isolated from *C. sylvestris*.

C-11 and carbonyl group at C-13) attached at C-9. The proposed structure was confirmed by correlations of the hydrogen signal at δ_H 7.05 (H-11) with carbon resonances at δ_C 41.5 (C-8), 41.0 (C-9), 25.8 (C-18), 35.7 (C-10) and 200.1 (C-13). Additional cross peaks between the signal at δ_H 6.15 (H-12) with those at δ_C 41.0 (C-9) and δ_C 200.1 (C-13), and between the signal at δ_H 2.20 (H-14) with the resonance of the carbonyl group at δ_C 200.1 (C-13), confirmed the connectivity of the carbon side chain at C-9 (Figure 2). *Trans* configuration of the double bond at C-11 was confirmed by the coupling constant of doublets assigned to H-11 and H-12 (*J* 16.5 Hz) (Table 1).[16] Additionally, the ^{13}C NMR spectrum displayed signals at δ_C 169.9, 173.0 and 173.3, which were assigned to one acetate and two butanoate groups, respectively.[10] The acetate group was attached to C-17 of the diacetalic ring C, as deduced from the HMBC correlations between the signal at δ_H 6.31 (H-17) and that at δ_C 169.9 (C-1'''). Furthermore, the signal at δ_H 6.31 (H-17) exhibited correlations with those at δ_C 145.7 (C-4), 53.4 (C-5), 71.5 (C-6) and 95.0 (C-16) (Figure 2). The assignment of the structure was straightforward with exception of the butyrate groups, since HMBC correlations between H-2, or H-16, and the carbonyl groups were not observed, in accordance with previously reported data.[17,18] However, the downfield chemical shift of C-2 (δ_C 66.3) and C-16 (δ_C 95.9) indicated that the butanoate groups were attached at C-2 and C-16 of the A ring.[10,17] Spectral analysis of heteronuclear single quantum coherence (HSQC) indicated the correlations δ_H 7.05 (H-11)/δ_C 153.3 (C-11), δ_H 6.15 (H-12)/δ_C 130.6 (C-12), and δ_H 2.2 (H-14)/δ_C 23.5 (C-14), which confirmed the partial structure of the side chain. An additional cross peak between the signal at δ_H 1.17 (H-7) with that at δ_C 36.2 (C-7) suggested that there was no substituent in this carbon, a similar pattern previously observed for casearin X,[10] caseanigrescen D,[17] and argutin A.[19] Thus, considering that the structure of **5** differs from that of casearin X with regards to its C-9 side chain, which is shortened by two carbons, the name dinor casearin X was proposed for this substance.

The relative configuration at the stereogenic centers of **5** was suggested from the comparison of its ^{13}C NMR spectral data and hydrogen coupling constants with those reported in the literature.[20-22] The *cis* configuration of the A/B ring junction was deduced from chemical shift of Me-18 (δ_C 25.8).[10,21] *Cis* clerodane diterpenes that are not substituted at C-7 show chemical shifts around δ_C 15 and 26 for Me-17 and Me-20, respectively, when these groups are in a *trans* relationship,[5] whereas values

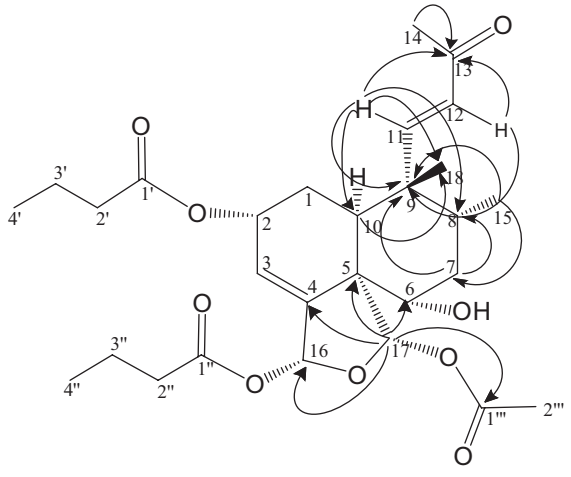


Figure 2. Key HMBC correlations (H → C) observed for compound **5**.

Table 1. ^{1}H and ^{13}C NMR (δ, CD_3OD, 500 and 150 MHz, respectively) data of dinor casearin X (**5**)

Position	δ_H (m, *J* / Hz)	δ_C
1	a: 2.03 (m, 1H) b: 2.11 (m,1H)	26.3
2	5.41 (br s, 1H)	66.3
3	5.96 (d, 1H, *J* 4.0)	120.9
4	–	145.7
5	–	53.4
6	3.61 (t, 1H, *J* 10.0)	71.5
7	1.17 (m, 2H)	36.2
8	1.88 (m, 1H)	41.5
9	–	41.0
10	2.30 (dd, 1H, *J* 7.5, 14.5)	35.7
11	7.05 (d, *J* 16.5)	153.3
12	6.15 (d, 1H, *J* 16.5)	130.6
13	–	200.1
14	2.20 (s, 3H)	23.5
15	0.89 (d, 3H, *J* 7.0)	15.1
16	6.71 (t, 1H, *J* 1.8)	95.9
17	6.31 (s, 1H)	97.4
18	0.88 (s, 3H)	25.8
1'	–	173.2
2'	2.35 (t, 2H, *J* 7.0)	35.7
3'	1.64 (m, 2H)	18.3
4'	0.97 (t, 3H, *J* 7.0)	12.5
1''	–	173.0
2''	2.33 (t, 2H, *J* 7.0)	35.7
3''	1.62 (m, 2H)	18.0
4''	0.92 (t, 3H, *J* 7.0)	12.4
1'''	–	169.9
2'''	1.80 (s, 3H)	20.0

around δ_C 16 and 18 for Me-17 and Me-20, respectively, characterize a *cis* relationship.[7,10] Considering that in the structure of dinor casearin X there is no substituent at C-7, the carbon resonances of Me-15, and Me-18 indicated their *trans* relationship. The carbon chemical shift for C-2 was determined to be δ_C 66.3, which is consistent with a C-2 substituent having an α-orientation. When the substituent has β-orientation the chemical shift is around δ_C 70.[17,19,20,23,24] Moreover, the *J* values of 7.5 and 14.0 Hz for the coupling H-10/H-1 revealed that H-10 has an axial orientation.[21]

The crude MeOH extract from the leaves of *C. sylvestris* and its respective hexane partition phase displayed cytotoxic effects on B16F10-Nex2, A2058, HL-60, HCT, MCF-7, and HeLa tumor cell lines. Bioguided fractionation afforded compounds **1**-**5** as being responsible for the activity (Table 2). Based on the IC_{50} values casearins **1**, **2** and **4** showed cytotoxic activities for all tested cell lines, while casearin G (**3**) exhibited cytotoxic activity only for HL-60, HeLa and HCT. Dinor casearin X (**5**) was the less active compound against the tested cell lines in comparison with casearins **1**-**4**, except for the HL-60 cell line, thus indicating a probable selective activity of this compound. Therefore, studies upon the mechanism of action of this new compound on HL-60 cells must be addressed in order to eventually propose dinor casearin X as a lead anti-leukemic agent.

Conclusions

One novel clerodane diterpene, named dinor casearin X, was isolated from leaves of *Casearia sylvestris*. Dinor casearin X reduced cell viability of the HL-60 tumor cell line, indicating a selective cytotoxic activity of this compound when compared to that displayed against other cells lines (A2058, HCT, MCF-7 and HeLa). These findings suggest that dinor casearin X could be considered as a lead anti-leukemic agent.

Table 2. Cytotoxic activity of compounds **1**-**5** against human tumor cell lines

Line	IC_{50} / (μg mL^{-1})					
	1	**2**	**3**	**4**	**5**	Dox
B16F10-Nex2	4.18 ± 0.36	6.05 ± 0.12	3.37 ± 0.42	5.98 ± 0.49	11.07 ± 1.14	0.02 ± 0.03
A2058	2.99 ± 0.12	14.96 ± 0.41	3.37 ± 0.44	9.38 ± 0.28	13.10 ± 1.13	0.04 ± 0.01
HL-60	4.27 ± 0.51	1.07 ± 0.23	22.55 ± 1.58	3.28 ± 0.35	0.51 ± 0.11	0.06 ± 0.01
HCT	1.84 ± 0.14	7.62 ± 0.11	23.71 ± 2.54	4.67 ± 0.31	25.45 ± 7.46	1.16 ± 0.74
MCF-7	21.28 ± 0.62	5.12 ± 1.13	nd	23.71 ± 1.39	29.60 ± 3.63	0.20 ± 0.09
HeLa	4.67 ± 0.11	23.20 ± 0.57	23.51 ± 1.19	4.78 ± 0.18	10.50 ± 1.50	0.30 ± 0.07

Dox: Doxorubicin; nd: not determined.

Supplementary Information

Supplementary data are available free of charge at http://jbcs.sbq.org.br as PDF file.

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