Artigo

ABIETATRIENES DITERPENOIDS FROM *Sagittaria montevidensis* SSP *Montevidensis*

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The antimicrobial properties of the hexane, hexane/EtOAc and methanol fractions of the fresh petioles of *Sagittaria montevidensis* ssp *montevidensis* (Alismataceae) were evaluated against fungi and Gram-negative and Gram-positive bacteria. A new abietatriene-type diterpenoid, 3β,7α–dihydroxi-abieta-8,11,13-triene and the known 3β-hydroxy-abieta-8,11,13-trien-7-one were isolated from the most active fraction tested and the structures of these compounds were elucidated by data including IR, EIMS, and 1D and 2D NMR spectra.

Keywords: *Sagittaria*; abietatrienes; antimicrobial activity.

INTRODUCTION

The genus *Sagittaria* (Alismataceae) comprises 25 species of herbaceous aquatic plants that are distributed throughout the Americas in tropical regions. No evidence has been found for the use of *Sagittaria montevidensis* ssp *montevidensis* in traditional medicine. However, others *Sagittaria* species are known to produce antibacterial compounds such as clerodane, pimarane, labdane and rosane-type diterpenoids.[1,2] During of our survey of the active substances from Alistamaceae species,[3-6] fractions of different polarity deriving from the crude methanolic extract of *S. montevidensis* ssp *montevidensis* were tested against a representative set of fungi and bacteria strains. Chromatographic treatment of the most active fraction led to the isolation of a new diterpenoid abietatriene derivative, 3β,7α–dihydroxi-abieta-8,11,13-triene (**1**), along with a known diterpenoid, 3β-hydroxy-abieta-8,11,13-trien-7-one (**2**). The structures of the compounds were determined by spectroscopic analysis including EIMS, IR, and 1D and 2D ^{1}H, ^{13}C NMR data and also by comparison of its NMR data with those of related compounds.

EXPERIMENTAL

General experimental procedures

1D and 2D ^{1}H, ^{13}C NMR spectra were acquired on a Varian Mercury plus BB spectrometer, operating at 300.059 MHz (^{1}H) and 75.458 MHz (^{13}C) for a $CDCl_3$ solution using TMS as an internal standard. Mass spectrometry was performed on a Shimadzu GC-MS QP 2000A, 70 eV.

Plant material

The plant material was collected in Curitiba, Paraná, Brazil and authenticated by Dr. M. do C. Amaral (IB-UNICAMP). A voucher specimen (# UEC 115194) was deposited in the Herbarium of the Instituto de Biologia, Universidade Estadual de Campinas, Campinas-SP.

Extraction and isolation

Fresh petioles (86.0 g) of *S. montevidensis ssp montevidensis* were extracted with MeOH. After removal of the solvent by vacuum, the residue (32.0 g) was partitioned between EtOAc and H_2O. The EtOAc (6.0 g) extract was subjected to column chromatography on a silica gel 60 to give *n*-hexane, *n*-hexane/EtOAc (95:5, 90:10, 80:20, 70:30, 60:40, 50:50) and MeOH fractions. These subfractions were named subfractions A, B, C, D, E, F, G and H, respectively. The fraction E eluted with *n*-hexane/EtOAc 70:30 (602.0 mg) was further purified by column chromatography (CC) on silica gel 60 using an *n*-hexane-EtOAc gradient solvent system to obtain compounds **1** (12.0 mg) and **2** (40.0 mg).

*3β-hydroxy-abieta-8,11,13-trien-7-one (**2**)*[7,8]

Yellowish oil: RMN- ^{1}H δ (300 MHz, $CDCl_3$): 7.80 (d, 2.1 Hz, H-14), 7.40 (dd, 8.2; 2.1 Hz, H-12), 7.28 (d, 8.2 Hz, H-11), 3.36 (dd, 4.8, 10.8 Hz, H-3), 2.90 (sept, 6.0 Hz, H-15), 2.73 (d, 11.4 Hz, H-6β), 2.72 (d, 6.6 Hz, H-6α), 2.40 (m, H-1), 1.86 (dd, 11.4 and 6.6, H-5), 1.82-1.90 (m, H-2), 1.24 (d, 6.0 Hz, H-16), 1.24 (d, 6.0 Hz, H-17), 1.24 (s, H-20), 1.05 (s, H-19), 0.97 (s, H-18). NMR ^{13}C (75 MHz; $CDCl_3$): 199.7 (C-7), 153.0 (C-9), 147.0 (C-13), 132.7 (C-12), 130.5 (C-8), 125.0 (C-14), 123.9 (C-11), 78.0 (C-3), 48.4 (C-5), 38.7 (C-4), 37.4 (C-10), 36.1 (C-1), 35.7 (C-6), 33.5 (C-15), 27.4 (C-2), 27.3 (C-19), 23.7 (C-20), 23.6 (C-17), 23.2 (C-16), 14.8 (C-18).

Microorganisms used and growth condition

The fractions above were assayed against Gram-positive and Gram-negative bacteria by a broth microdilution assay to determine the minimal inhibitory concentrations (MICs) as described below. The assays were performed with *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 15442, *Bacillus subtilis* ATCC 6623, and *Staphylococcus aureus* ATCC 25923 obtained from the American Type Culture Collection (ATCC, Rockville, MD). For the antifungal assay, a single clinical isolate of each species (*Candida albicans*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*), obtained from vaginal mucosa, was selected for testing. The bacteria were maintained in Tryptic Soy Agar (Difco). The yeast was maintained in Sabouraud Dextrose Agar (Difco).

Antifungal susceptibility testing

The MICs of all the fractions and reference antifungal compounds

† This paper is dedicated to our colleague and friend Clara M. A. Tanaka *in memoriam*

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were determined by microdilution techniques in RPMI 1640 (Sigma Chemical Co., St. Louis, MO) for yeast.[9] Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard (10^6 colony-forming units [CFU] m/L) and diluted to a 1:10 ratio for the broth microdilution procedure. Microtiter trays were incubated at 37º C, and the MICs were recorded after 24 h of incubation. Two susceptibility endpoints were recorded for each isolate. The MIC was defined as the lowest concentration of compounds at which the tested microorganism does not demonstrate visible growth. Nystatin (Sigma Chemical Co., St. Louis, MO, USA) was included in the test as a control. When the MIC was equal or smaller than 100 µg/mL, the antimicrobial activity was considered significant. If the fractions displayed a MIC from 100 to 500 µg/mL, the antimicrobial activity was considered moderate; from 500 to 1000 µg/mL, the antimicrobial activity was considered weak; over 1000 µg/mL, the fractions were considered inactive.[10]

Antibacterial susceptibility testing

The MICs of all the extracts and reference antibiotics (tetracycline, vancomycin, and penicillin - Sigma Chemical Co., St. Louis, MO, US) were determined by microdilution techniques in Mueller-Hinton broth (Difco) according to CLSI.[9] Inoculates were prepared in the same medium at a density adjusted to a 0.5 McFarland turbidity standard (10^8 colony-forming units [CFU])/mL] and diluted 1:10 for the broth microdilution procedure. Microliter plates were incubated at 37 ºC and the MICs were recorded after 24 h of incubation. Two susceptibility endpoints were recorded for each isolate. The MIC was defined as the lowest concentration of compounds at which the tested microorganism did not demonstrate visible growth.

RESULT AND DISCUSSION

The results of the preliminary screening for antimicrobial activity of all fractions are summarized in Table 1, which shows that six among ten fractions demonstrated significant activity against at least one of the microorganisms tested. However, the most active fraction was hexane/EtOAc (70:30, fraction E) with an MIC = 31.2 µg/mL against *C. krusei*. Therefore, it was purified by sequential chromatographic techniques, with the metabolites **1** and **2** (Figure 1) as the major constituents. Compound **2** was identified as 3β-hydroxy-abieta-8,11,13-trien-7-one by comparison of the IR, ^{1}H NMR, and ^{13}C NMR spectra with those already published. We now report the characterization of **1.**

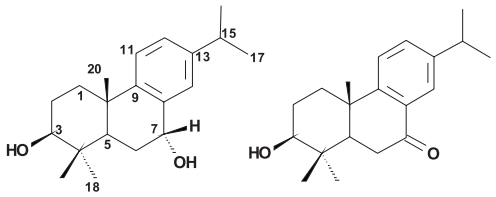

Figure 1. *Structure of abietatrienes **1** and **2***

Compound **1** $[\alpha]_D$ = +17 (MeOH, *c*.0.011)**,** was isolated as a yellowish oil. HRMS suggested a molecular formula of $C_{20}H_{30}O_2$, *m/z* 302.2232 (calculated *m/z* 302.2238). The IR spectrum showed absorption bands at ν_{max} 3400 and 1600 cm^{-1}, which could be attributed to hydroxyl groups and double bonds, respectively. The ^{1}H NMR (Table 2) spectrum showed signals for a trisubstituted aromatic ring at δ_H 7.19 (1H, d, *J*=1.8 Hz), 7.18 (1H, d, *J*=8.2 Hz) and 7.12 (1H, dd, *J*=8.2 and 1.8 Hz), oxymethine at 4.85 (1H, t, *J*= 3.0 Hz) and 3.35 (1H, dd, *J*=10.8 and 5.4 Hz), methylenes at 2.30 (1H, m), 2.00 (2H, ddd, *J*=9.0, *J*=5.7 and 3.0 Hz), 1.70-1.80 (2H, m) and 1.56 (1H, dd, *J*=13.2 and 5.4 Hz), a septet at 2.87 (1H, *J*=6.9 Hz) and methyl groups at 1.23 (6H, d, *J*=6.9 Hz), 1.13 (3H, s), 1.09 (3H, s) and 0.90 (3H, s). From the ^{13}C NMR (Table 2) and DEPT 20 carbons were detected and assigned by HMQC to a aromatic ring (δ_C 146.9, 146.8, 136.1, 127.9, 126.9 and 124.7), two oxygenated methine (δ_C 78.9 and 68.7), three methylene groups (δ_C 36.8, 28.5 and 28.2), two methines (δ_C 43.8 and 33.7), two sp^3 quaternary carbons (38.7 and 37.8) and finally four methyl signals at 28.1, 24.2, 24.1 (2x) and 15.7. Comparison of above data and those of 3β-hydroxy-abieta-8,11,13-trien-7-one **2**,[7,8] showed a good agreement except for the presence of an additional oxymethine group in the compound **1** replacing the carbonyl group observed in **2.** Analysis of the ^{1}H -^{1}H COSY experiment (Figure 2) showed correlations between the oxymethine at δ_H 4.85 (δ_C 68.7) and protons H-5, H-6 indicating that the extra hydroxyl group was possibly situated on C-7. This assumption was further confirmed by an HMBC experiment, where correlation of H-5 with the C-7 was observed. Additional HMBC correlations of C-3/H-18, H-19, H-1β and C-12/H-14, H-11 and a NOESY experiment, showing that the axial methyl protons at C-19 gave cross peaks with the 20-methyl protons and the H-2β, confirmed the structural similarities of **1** and **2**. The presence and location of the isopropyl group linked to quaternary aromatic carbon was supported by chemical shift and NOESY

Table 1. Minimum inhibitory concentrations MICs (µg/mL) of the fractions of *S. montevidensis spp montevidensis*

	A	B	C	D	E	F	G	H	References
Gram-positive bacteria									
Staphylococcus aureus ATCC 25923	1000	125	1000	125	62.5	125	250	>1000	0.00975[a]
Bacillus subtilis ATCC 6623	>1000	>1000	250	250	125	125	125	>1000	0.09[b]
Gram-negative bacteria									
Escherichia coli ATCC 25922	>1000	>1000	>1000	>1000	1000	1000	>1000	>1000	0.78[c]
Pseudomonas aeruginosa ATCC 15442	>1000	>1000	>1000	>1000	>1000	>1000	>1000	>1000	3.125[c]
Fungi									
Candida albicans	>1000	>1000	>1000	500	500	250	500	1000	1.0[d]
Candida parapsilosis	500	500	500	500	125	250	250	500	8.0[d]
Candida tropicalis	>1000	500	1000	250	125	250	250	>1000	8.0[d]
Candida krusei	500	62.5	250	62.5	31.2	62.5	62.5	500	4.0[d]

[a]Penicillin; [b]Vancomycin; [c]Tetracycline; [d]Nystatin data from experiments in triplicate, MIC defined as the lowest concentration for which no growth was observed in every tested well. Subfractions A-H were referred to as hexane, hexane/EtOAc 95:5, 90:10, 80:20, 70:30, 60:40, 50:50 mixtures and methanol subfractions, respectively.

Table 2. ^{1}H, ^{13}C NMR data of the compound **1** ($CDCl_3$), COSY and HMBC correlations

	^{13}C	^{1}H	COSY	HMBC
1	36.8	1β: 2.30 (dt, *J*=13.2 and 3.0); 1α:1.56 (ldd, *J*=5.4 and 13.2 Hz)		H-20
2	28.2	1.70-1.80 (m)	H-1β	H-1β
3	78.9	3.35 (dd, *J*=10.8 and 5.4 Hz)	H-2	H-18; H-19 and H-1β
4	38.7	-		H-18 and H-19
5	43.8	1.65 (dd, *J*=9.0 and 5.7 Hz)	H-6	H-20
6	28.5	2.00 (ddd, *J*= 9.0, 5.7 and 3.0 Hz)	H-5	
7	68.7	4.85 (bt, *J* =3.0 Hz)	H-6	H-5
8	136.1	-		
9	146.8	-		H-20
10	37.8	-		H-20
11	124.7	7.18 (1H, d, *J*=8.2 Hz)		
12	126.9	7.12 (1H, dd, *J*= 8.2 and 1.8 Hz)		H-11 and H-14
13	146.9	-		
14	127.9	7.19 (1H, d, *J*= 1.8 Hz)		
15	33.7	2.87 (1H, sep, *J*=6.9 Hz)	H-16 and H-17	H-16 and H-17
16	24.1[a]	1.23 (3H, d, *J*=6.9 Hz)		
17	24.2[a]	1.23 (3H, d, *J*=6.9 Hz)		
18	15.7	0.90 (3 H, s)		H-19
19	28.1	1.09 (3H, s)		H-18
20	24.1	1.13 (3H, s)		

[a] interchangeable signals

correlations of H-16 (H-17) with H-14. This assumption was further confirmed by a NOE experiment, where correlation of H-14 with the H-7 was observed.

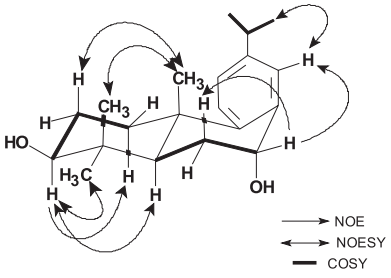

Figure 2. *COSY, NOESY and NOE correlations observed for compound* ***1***

The NOESY and NOE experiments as well as coupling constants established the relative configuration of the compound **1**. Proton H-3 ($J_{3ax,2eq}$=5.4 and $J_{3ax,2ax}$ =10.8 Hz) had an α-disposition as showed by its coupling constants. It was further supported by NOESY correlations of H-3 with H-5 and H-3 with Me-19. No NOESY correlation was observed between H-7 and H-5 or H-7 and H-19, but the coupling constant corresponding to H-7 (bt, *J* = 3.0 Hz) and the ^{13}C NMR chemical shift values[11] suggests an α-disposition of the hydroxyl group at the C-7. These configurations (Figure 2) were supported by an NOE spectrum that exhibited effects between H-3 (δ 3.35) with H-5 (δ 1.65) and H-1α (δ 1.56); H-7 (δ 4.85) with H-6β (δ 2.00) and H-14 (δ 7.19). Therefore, compound **1** was assigned to be 3β,7α –hydroxi-abieta-8,11,13-triene.

Finally, a literature survey revealed that abietane-type diterpenoids are recognized to inhibit Gram-positive bacteria and some fungi species with appreciable MIC values.[12] Therefore, compounds **1** and **2** may merit further experiments to evaluated their antimicrobial activities.

SUPPLEMENTARY MATERIAL

Available at http://quimicanova.sbq.org.br, in format .PDF, with access free.

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