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Original Article

Intraspecific variation of meroditerpenoids in the brown alga *Stypopodium zonale* guiding the isolation of new compounds

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ABSTRACT

Intraspecific variation on meroditerpenoids production by the brown marine alga *Stypopodium zonale* at four different populations along the Brazilian coast was analyzed using Principal Component Analysis over high-performance liquid chromatography profiles from algae extracts. The ordination of the samples by the similarities of their chromatographic traits showed the existence of three chemotypes: (i) the populations Búzios and Abrolhos which were characterized by the presence of atomaric acid (**1**), (ii) the population Atol das Rocas which contained the compound stypoldione (**2**), and (iii) the population Marataízes which was characterized by other peaks that guided the isolation of three new meroditerpenoids stypofuranlactone (**3**), 10,18-dihydroxy-5′a-desmethyl-5′-acetylatomaric acid (**4**), and the 10-keto-10-deisopropyliden-5′a-desmethyl-5′-acetylatomaric acid (**5**) together with the known compound the 10-keto-10-deisopropyliden-atomaric acid (**6**). The structures and relative stereochemistry of **3**, **4** and **5** were elucidated by NMR and MS techniques. The observed chemical differences among populations of *S. zonale* can be related to its geographic distribution and can open an avenue to the discovery of new compounds in algae.

Introduction

The term meroterpenoids is applied to describe natural products of mixed biosynthetic origin, which are partially derived from terpenoids. The prefix mero (from Greek *merus*) means "part, partial or fragment" (Geris and Simpson, 2009). Therefore, a number of secondary metabolites can be described as meroterpenoids, which assembles a huge range of structural diversity, often linked with varied functionalities, arising from intra- and intermolecular ring closures and/or rearrangements of the terpene chains (Geris and Simpson, 2009; Macías et al., 2014). This class of natural products is widely distributed among marine organisms, but they are particularly abundant in marine brown algae, microorganisms and sponge (Vallim et al., 2005; Menna et al., 2013).

Within the marine brown algae, order Fucales and the genus *Stypopodium* (Dictyotales) have been recognized as a rich source of structurally unique, biologically and ecologically active meroditerpenoids (Valls et al., 1993; Mesguiche et al., 1997; Dorta et al., 2003; Soares et al., 2007; Mendes et al., 2011) that have been named by some authors as stypols (Macías et al., 2014). Some stypols, such as stypoldiol, stypoldione and epitaondiol, have been tested for their cell proliferation inhibitory activity in different cell lines (White and Jacobs, 1983; Sabry et al., 2005; Pereira et al., 2011), thus making them promising candidates as anticancer drugs. Stypoldiol and stypoldione have remarkable antioxidant properties through radical-scavenging together with structurally related compounds, taondiol and isoepitaondiol (Nahas et al., 2007). In addition to the variety of biological activity, the ecological function as defensive chemicals against herbivory of these meroditerpenoids has been documented (Gerwick and Fenical, 1981; Pereira et al., 2004). Interestingly, the analyses of meroditerpenoids composition and concentration in *Stypopodium* revealed an intra-specific variation among geographical locations (Gerwick and Fenical, 1981; Gerwick et al., 1985; Soares et al., 2003; Pereira et al., 2004).

Inter- and intrapopulational chemical differences in marine organisms (or chemotypes) have been described in several studies (*e.g.* Machado et al., 2014), and these variations are explained by combination of genetic (Wright et al., 2004), developmental (Paul

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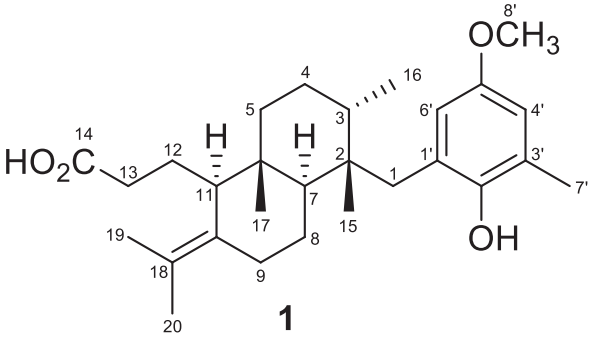
and Van Alstyne, 1988), and environmental (Sudatti et al., 2011) factors.

Here we investigated the spatial variability of the meroditerpenoids in *Stypopodium zonale* (Lamouroux) Papenfuss (Dictyotales), the unique specie of *Stypopodium* genus described to Brazil (Guiry and Guiry, 2015). Principal Component Analysis (PCA) of the chemical profiles was employed in this study to access the chemical variability among the populations and guided the isolation of three new compounds (**3**–**5**), in addition to other three known meroditerpenoids (**1**, **2** and **6**). The structures were elucidated based on spectroscopic and spectrometric analysis including ^{1}H and ^{13}C NMR, ^{1}H–^{1}H COSY, HSQC, HMBC, ROESY and HREIMS.

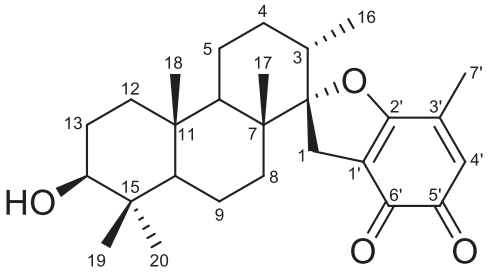
Materials and methods

General experimental procedures

Optical rotations were measured on a Perkin-Elmer model 241 polarimeter (Perkin-Elmer, USA) using a Na lamp at 25 °C. IR spectra were obtained with a Perkin-Elmer 1650/FTIR spectrometer in $CHCl_3$ solutions. HREIMS spectrum was taken on a Waters Micromass Autospec mass spectrometer (Waters, EUA). ^{1}H NMR, ^{13}C NMR, HSQC, HMBC and ^{1}H–^{1}H COSY spectra were measured employing a Varian Unity 300 instrument operating at 300 MHz for ^{1}H NMR and at 75 MHz for ^{13}C NMR. Two-dimensional NMR spectra



1



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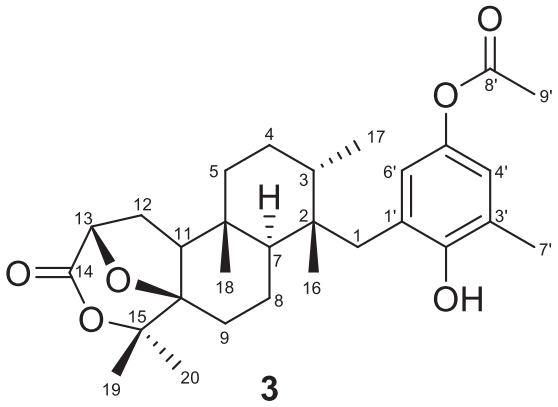

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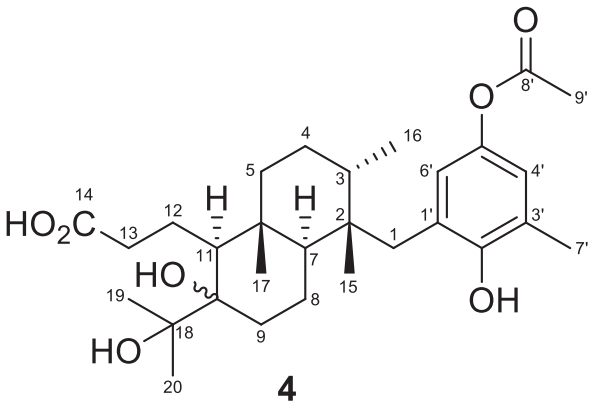

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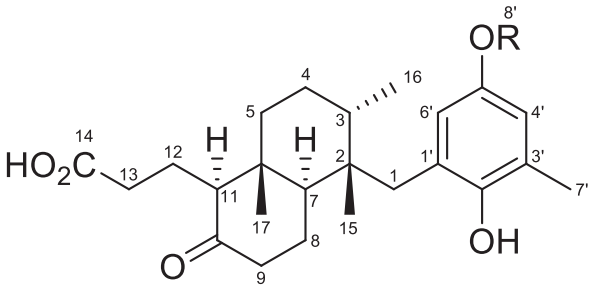

5 R=$COCH_3$
6 R=CH_3

were acquired with the standard Bruker DRX pulse sequence operating at 400 MHz for ^{1}H NMR. The 2D ROESY experiment was performed with mixing time of 300 ms, with 16 scans at 25 °C. Silica gel (Merck, 70–230 and 230–400 mesh) was used in column chromatography. Thin layer chromatography was carried out with silica gel 60 GF254 (Merck). Once developed, spots on the plates were visualized by spraying with 2% ceric sulphate in sulfuric acid, followed by gentle heating.

Algal material

Specimens of *Stypopodium zonale* (Lamouroux) Papenfuss (Dictyotales) were collected by snorkeling and SCUBA diving at four distinct areas of the Brazilian coast: Forno inlet, Búzios, Rio de Janeiro State (22° 45′ S, 41° 52′ W), at the depths of the 3–6 m in April, 2002; Marataízes, Espírito Santo State (21° 02′ S, 40° 49′ W), at the depths of the 13–15 m in April, 2002; Atol das Rocas, Rio Grande do Norte state (03° 51′ S, 33° 40′ W), at 6–10 m in June 2002 and Abrolhos, Bahia state (14° 46′ S, 39° 01′ W), at 10–12 m in April 1995. The algae were washed in seawater to eliminate associated organisms and air-dried. Voucher specimens (Búzios: HRJ 8643; Marataízes: HRJ 5543; Abrolhos: HRJ 5593 and Atol das Rocas: HRJ 8678) were deposited at the Herbarium of the Rio de Janeiro State University, Brazil (HRJ).

Extracts preparation

Adult and well-developed specimens of *S. zonale* from Búzios (115 g alga dry weight, dw), Marataízes (12.8 g dw), Abrolhos (173 g dw) and Atol das Rocas (106 g dw) were air-dried, milled and extracted three times with CH_2Cl_2 (using 100 ml of solvent to each g of dw) at room temperature. The solvent was removed in vacuum to yield a dark green tar in all cases (19 g, 16.5% dw; 1.8 g, 14.2% dw; 31 g, 17.9% dw; 12 g, 12.9% dw, respectively). Individual specimens ($n = 3$) from each population were independently extracted in an identical fashion and had their chemical profiles analyzed by HPLC-DAD.

HPLC-DAD chemical profiles

The crude extracts and pure compounds (**1**) and (**2**) were analyzed by high-performance liquid chromatography (HPLC) and carried out on a VP-ODS Shim-pack column (5 μM, 250 mm × 4.6 mm) using a Shimadzu HPLC chromatograph equipped with a Diode Array Detector (DAD, Shimadzu, Japan) and a coupled system consisting of a vacuum degasser, pump LC-20AT, and detectors SPD-M20A and CBM-20A. A binary gradient elution at flow rate 1.0 ml/min was employed using water (pH 3.0 with phosphoric acid) as solvent A and acetonitrile as solvent B, as follows: 60–100% B at 0–25 min. The eluent remained at 100% acetonitrile during 5 min, then changed directly to the initial gradient conditions during 10 min for the column re-conditioning between two sequential injections. The UV–Vis absorbance spectra were registered. Crude extracts were diluted in acetonitrile to a final concentration of 10 mg/ml and filtered in a 0.45 μM PTFE syringe filter (Millipore, USA) prior injection. An aliquot of 20 μl of filtrate solution was injected for HPLC analysis.

Principal Component Analysis (PCA)

The first step of the data preparation for the PCA analysis was the removal of the first 5 min of the retention time (t_R) from the two-dimensional chromatogram matrices from each extract sample from specimens of *S. zonale* obtained by the HPLC-DAD system, since it contains a very low peak resolution caused by the strong overlapping signal from polar compounds. The chromatograms were then baseline aligned and corrected for the time shift of peaks among them using the Correlation Warping Algorithm (COW), as described by Nielsen et al. (1998), using the software "COW tool" (http://www2.biocentrum.dtu.dk/mycology/analysis/cow/). Data from wavelength of 280 nm and t_R between 5 and 27 min were extracted from each of the two-dimensional chromatograms and placed in a matrix containing all samples. Each chromatogram was normalized by the highest peak found in each sample in other to enhance the proportional information among high peaks. The matrix with all chromatograms was then mean centered before running the PCA routine, which was conducted using the R language and environment (http://www.R-project.org) with the installed package "ChemometricsWithR" (Wehrens, 2011).

Isolation and identification of the meroditerpenes

To obtain the pure standards of the known compounds **1** and **2** previously described by Soares et al. (2003), the extracts from specimens collected at Búzios and Atol das Rocas were fractionated. Following the method used by Soares et al. (2003), part of the extract of the specimens collected at Búzios (2.1 g) was submitted to an acidic–basic extraction. The acid fraction (198 mg) was fractionated on silica gel vacuum liquid chromatography to yield 8 mg of **1**. The extract obtained from algae collected at Atol das Rocas (2.5 g) was eluted in a column chromatography over silica gel with an increasing gradient of hexane, dichloromethane (CH_2Cl_2) and ethyl acetate (EtOAc) to provide a slightly impure fraction of compound **2**. This fraction was purified by vacuum liquid chromatography over silica gel and eluted with hexane: EtOAc (1:1, v/v) to yield **2** (6 mg).

The extract (1.8 g) from Marataízes was fractionated by silica gel vacuum liquid chromatography using a stepwise gradient solvent system of increasing polarity starting from 100% hexane to 100% methanol (twelve fractions, A–M). The fraction eluted with 15% EtOAc in CH_2Cl_2 (fraction G, 247 mg) was further purified with silica gel column chromatography using a stepwise gradient of methanol (MeOH) in CH_2Cl_2 to afford **3** (12.0 mg). The fraction eluted with 50% EtOAc in CH_2Cl_2 (fraction I, 126 mg) was re-chromatographed using silica gel column chromatography with EtOAc 20% in CH_2Cl_2 to yield 4 mg of the compound **4**. Finally, the fraction eluted with 5% MeOH in EtOAc (91 mg, fraction L) was purified with silica gel column chromatography eluted with increasing amount of EtOAc in CH_2Cl_2 to give 38 mg of mixture of **5** and **6**.

The structure of all the compounds was elucidated based on the spectral data (optical rotation, IR, EIMS and/or HREIMS, 1D and 2D NMR). The known compounds were identified comparing these spectroscopic data with the literature (Mori and Koga, 1992; Wessels et al., 1999; Dorta et al., 2003) and, when available, by direct comparison with the original purified standards.

Results and discussion

Marine algae of the *Stypopodium* genus are a rich source of meroditerpenoids, which very often display potent biological activities in addition to their ecological roles (Valls et al., 1993; Mesguiche et al., 1997; Dorta et al., 2003; Soares et al., 2007; Mendes et al., 2011). These compounds have been used as chemotaxonomic markers to distinguish the representatives of the genus within the Dictyotales (Vallim et al., 2005). The presence of these compounds in *Stypopodium* can be useful for dereplication purposes helping on the detection of new compounds among a plethora of known compounds.

Here we compared four populations of *S. zonale* collected along of Brazilian coastline according to their HPLC-DAD data. The ordination of the populations scores by the first two components (CP1 and CP2), which explained 97% of all chromatographic data

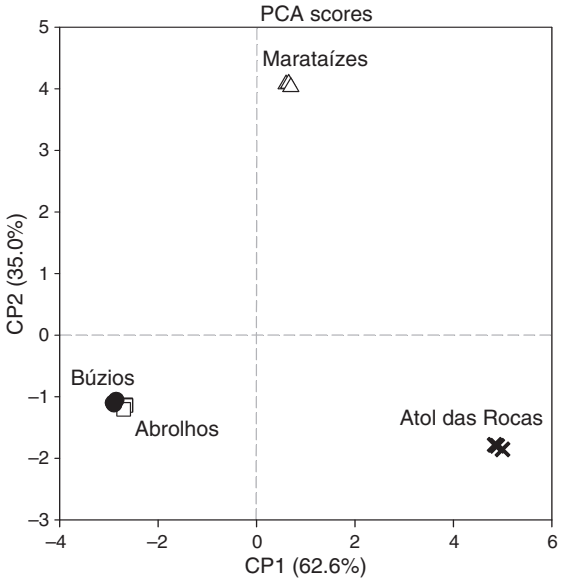

Fig. 1. Ordination of the first two PCA scores (CP1 and CP2) obtained from the analysis of chemical profiles from four different populations of *Stypopodium zonale*.

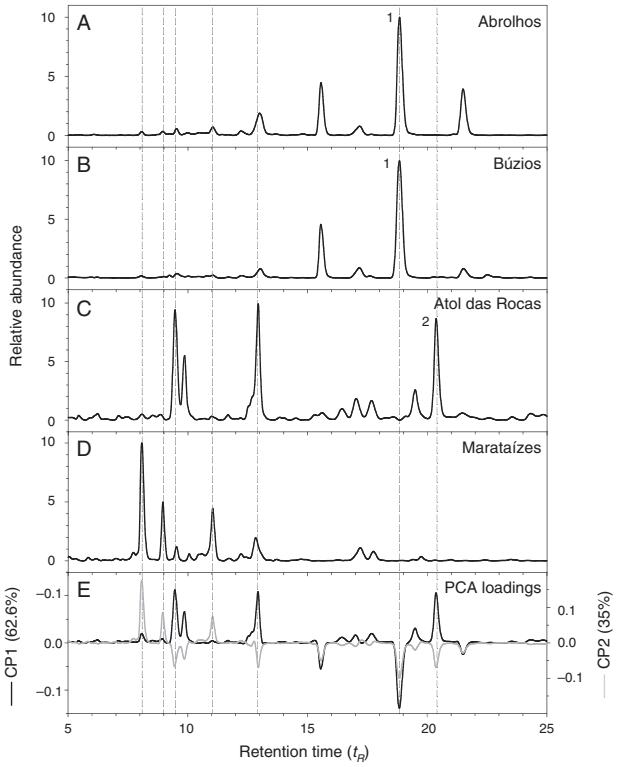

Fig. 2. Mean chemical profiles ($n = 3$) of four different populations of *Stypopodium zonale* and the PCA loadings plotted against the retention time. Each chemical profile was normalized by its maximal peak intensity in other to enhance the relative difference among peaks. (**1**) and (**2**) mark the peaks of atomaric acid and stypoldione respectively.

variation, revealed that the samples from Abrolhos and Búzios sites were grouped closely together at the negative side of CP1 (Fig. 1). This is a result of their similar chromatograms (Fig. 2A and B), different only by presence of one peak (retention time, $t_R = 22.5$) in the extracts from Abrolhos specimens of *S. zonale*. These two populations differ from the other populations mostly by the presence of a peak at $t_R = 18.9$ min, which had the strongest loading on sample variation (CP1, Fig. 2E). In contrast, the samples from Atol das Rocas, placed at the positive side of CP1 (Fig. 1), do not have this peak and were mostly characterized by three peaks (t_R's = 9.5, 12.9 and 20.4 min) according to the PCA loading (Fig. 2C and E). The extract of samples from Marataízes had a weak correlation with the other three and it was completely separated from them at CP2 (Fig. 1), with the strongest peak loadings at t_R's = 8, 9 and 11 min (Fig. 2D and E), not observed in other populations.

Standards of the meroditerpenes atomaric acid (Soares et al., 2003) (**1**) and stypoldione (Soares et al., 2003) (**2**) were analyzed by HPLC in the same way as the extracts. The major chromatographic peak in Fig. 2A and B ($t_R = 18.9$ min) was identified as the atomaric acid (**1**) in Búzios and Abrolhos extracts. In the chromatogram of Atol das Rocas (Fig. 2C), the peak at $t_R = 20.4$ min was identified as stypoldione (**2**). To unambiguously assign these peaks to the atomaric acid (**1**) and stypoldione (**2**), the extracts from Búzios and Atol das Rocas were fractionated and the compounds were identified by the comparison of their spectroscopic data with the literature (Mori and Koga, 1992; Wessels et al., 1999). Neither of these compounds was identified in the chromatogram of the Marataízes samples, which were quite different from the other populations as shown by PCA analysis. ^{1}H NMR data of this extract indicated the presence of different meroditerpenes. Then, in order to evaluate the extract of Marataízes for the presence of new secondary metabolites, it was fractionated and three new compounds (**3**–**5**) in addition of the known compound **6** were isolated and identified.

Compound **3** was obtained as an optically active yellow oil ($[\alpha]_D^{26}$ +6.36°, *c* 0.04, CH_2Cl_2). The ^{1}H and ^{13}CNMR data (Table 1) combined with the $[M]^+$ peak at *m/z* 484.2713 (calc. for $C_{29}H_{40}O_6$, 484.2814) in the HREIMS suggested a molecular formula of $C_{29}H_{40}O_6$, indicating that this compound possesses 10 degrees of unsaturation. The IR spectrum displayed strong absorption bands for a hydroxyl group at 3435 cm^{-1} and two carbonyl group at 1749 and 1740 cm^{-1}. The ^{13}C NMR spectrum showed signals for all 29 carbons: seven methyl groups, six methylene groups (all sp^3), six methine groups (4 sp^3 and 2 sp^2) and ten quaternary carbons (4 sp^3 and 6 sp^2). Among the quaternary carbons, two of them were resonating at δ_C carbonyl groups of ester or lactone functionalities at δ_C 172.3 and δ_C 169.9. The ^{1}H NMR spectrum showed signals for two meta-coupled aromatic protons at δ_H 6.75 (d, 2.7) and δ_H 6.72 (d, 2.7) that together with the characteristic aromatic carbon signals indicated the presence of a tetrasubstituted phenyl ring. In the up-field region signals appear for five methyl groups at δ_H 1.46 (s), δ_H 1.43 (s), δ_H 1.18 (d, 6.9), δ_H 0.98 (s) and δ_H 0.80 (s). These signals, in addition to the benzylic methylene group at δ_H 3.18 (d, 13.5) and δ_H 2.12 (d, 13.5), suggested a diterpene moiety of the compound **3**. Additionally, the analysis of the ^{1}H NMR spectrum also indicated an aromatic methyl group at δ_H 2.19 (s), an acetyl group at δ_H 2.26 (s) and a deshielded methine resonance at δ_H 4.80 (dd, 1.2 and 4.2) that, by HSQC, corresponded to an oxygen-bonded methine.

The inspection of the ^{1}H–^{1}H COSY spectrum allowed us to connect the proton at δ_H 4.80 (H-13) to the methylene group at δ_H 1.48 (H-12) and this to the methine group at δ_H 1.93 (H-11). HMBC correlation of the proton at δ_H 4.80 (H-13) with the carbon at δ_C 81.3 (C-10) established an oxygen bridge between these carbons (at δ_C 81.3 and 79.9). The proton at δ_H 4.80 (H-13) also showed HMBC cross correlation to carbon at δ_H 41.4 (C-11), which combined with further ^{1}H–^{1}H COSY correlations established the five-membered ring fragment containing a heteroatom. Finally, the HMBC correlations of δ_H 1.43 (H-19) to the quaternary carbons at δ_C 81.3 (C-10), 89.1 (C-15), and the methyl group at δ_C 26.4 (C-20) in addition to the second carbonyl group at δ_C 172.3 (C-14) allowed to establish the unusual structure of **3**. Interpretation of ^{1}H–^{1}H COSY, HSQC and HMBC experiments assigned all of ^{1}H and ^{13}C NMR signals (Table 1) to the compound **3**.

Table 1
NMR spectroscopic data for stypofuranlactone (**3**), 10,18-dihydroxy-5′a-desmethyl-5′-acetylatomaric acid (**4**) and 10-keto-10-deisopropyliden-5′a-desmethyl-5′-acetylatomaric acid (**5**) in $CDCl_3$ (300.0 MHz).

Position	3			4			5		
	δ_C. m	δ_H (*J* in Hz)	HMBC[a]	δ_C, m	δ_H (*J* in Hz)	HMBC[a]	δ_C, m	δ_H (*J* in Hz)	HMBC[a]
1	38.4, CH_2	2.12, d (13.5) 3.18, d (13.5)	17, 1′, 2′, 6′	35.7, CH_2	2.31, d (13.8) 2.86, d (13.8)	2, 3, 17, 1′, 2′, 3′	35.8, CH_2	2.31, d (12.9) 3.03, d (12.9)	6′
2	39.8, C			40.0, C			40.6, C		
3	37.1, CH	1.75, m		35.5, CH	1.73, m		35.5, CH	1.75, m	
4	24.8, CH_2	1.31, m 1.82, m	16	25.3, CH_2	1.75, m	15	25.4, CH_2	1.42, m 1.82, m	
5	31.2, CH_2	1.37, d (6.6)		35.2, CH_2	1.34, m 1.65, m		31.8, CH_2	1.44, m 1.64, m	
6	35.1, C			38.9, C			42.8, C		
7	45.9, CH	2.46, m		46.8, CH	1.31, m		47.4, CH	1.99, d (10.2)	16, 17
8	18.0, CH_2	1.54, d (4.8) 2.03, m		19.1, CH_2	1.38, m		24.1, CH_2	1.62, m 2.03, m	
9	29.8, CH_2	1.85, d (9.3) 2.50, d (9.3)		28.7, CH_2	2.47, m 2.54, m		42.3, CH_2	2.36, m 2.40, m	
10	81.3, C			87.5, C			212.5, C		
11	41.4, C	1.93, ddd (1.2, 4.2, 13.2)		45.0, C	1.60, m		63.4, CH	2.24, m	
12	24.9, CH_2	1.48, d (4.2)		17.4, CH_2	1.89, d (12.3) 2.25, m		17.2, CH_2	1.62, m 1.93, m	
13	79.9, CH	4.80, dd (1.2, 4.2)	10, 11	35.1, CH_2	1.34, m		32.5, CH_2	2.44, m 2.49, m	
14	172.3, C			175.0, C			179.2, C		
15	89.1, C			20.2, CH_3	0.92, s	2, 3, 7	20.1, CH_3	0.91, s	2, 3
16	20.8, CH_3	0.98, s		16.3, CH_3	1.08, d (6.9)		16.4, CH_3	1.15, d (6.9)	
17	15.9, CH_3	1.18, d (6.9)		19.5, CH_3	1.07, s	6, 11	16.9, CH_3	0.78, s	5, 11
18	16.2, CH_3	0.80, s	5, 6, 7, 11	76.9, C					
19	22.7, CH_3	1.43, s	10, 15, 20	25.5, CH_3	1.29, s	10, 18			
20	26.4, CH_3	1.46, s		27.2, CH_3	1.27, s	10, 18, 19			
1′	130.0, C			126.8, C			126.7, C		
2′	150.2, C			150.3, C			150.5, C		
3′	126.7, C			123.7, C			123.7, C		
4′	120.9, CH	6.72, d (2.7)	3′, 6′, 7′	121.0, CH	6.73, d (2.7)	2′, 5′, 7′	121.0, CH	6.71, d (2.4)	2′, 5′, 7′
5′	143.6, C			143.1, C			143.0 (C)		
6′	121.3, CH	6.75, d (2.7)	3′, 4′, 6′	121.4, CH	6.81, d (2.7)	2′, 5′	121.4, CH	6.82, d (2.4)	2′, 4′, 5′
7′	16.6, CH_3	2.19, s	2′, 3′	16.3, CH_3	2.23, s	2′	15.9, CH_3	2.21, s	2′, 3′
8′	169.9, C			169.8, C			170.0, C		
9′	21.0, CH_3	2.26, s	8′	20.9, CH_3	2.26, s	8′	21.0, CH_3	2.26, s	8′
—OH					4.58, s			4.56, s	

[a] HMBC correlations are from proton(s) stated to the indicated carbon.

The relative stereochemistry of **3** was achieved by ROESY experiment (Fig. 3) along with biogenetic considerations (González et al., 1982). In the ROESY spectrum, correlations of 17-Me (δ_H 1.18) with H-7 (δ_H 2.46), H-11 (δ_H 1.93) and H-13 (δ_H 4.80), in addition to the correlation of the hydrogen H-13 (δ_H 4.80) and 20-Me (δ_H 1.46) indicated that these hydrogen atoms are spatially close, and according to Dorta et al. (2003), these were proposed in a α-configuration. On the other hand, ROESY correlations of H-3 (δ_H 1.75) and 16-Me (δ_H 0.98) and 18-Me (δ_H 0.80), and also the correlation of 18-Me (δ_H 0.80) and 16-Me (δ_H 0.98) and 19-Me (δ_H 1.43) suggested that all of them were β-oriented.

Thus, the structure of **3** was determined and named as stypofuranlactone, a derivative compound of the 5′a-desmethyl-5′-acetylatomaric acid, isolated of the *S. zonale* from Pacific, north Atlantic and Caribbean area (Gerwick et al., 1985; Dorta et al., 2003). Although molecules with this furan-lactone moiety have been obtained from plants and fungi (Macías et al., 2014), this is the first time that compounds with this moiety have been obtained from marine brown algae (MarinLit, 2015).

Compound **4** was isolated as a pale yellow oil ($[\alpha]_D^{26}$ +4.55°, *c* 0.02, CH_2Cl_2) with the molecular formula $C_{29}H_{44}O_7$, as determined by its HREIMS at *m/z* 504.3087 $[M]^+$ (calc. for $C_{29}H_{44}O_7$, 504.3081), 20 mass units more than compound **3**. The IR showed the absorption at 3434 cm^{-1} for hydroxyl group and at 1698 cm^{-1} for carbonyl group. The ^1H and ^{13}C NMR data for **4** (Table 1) were quite similar to those of **3**, except by absence of the oxymethine group, the difference observed in the chemical shifts to the two oxygenated quaternary carbons at δ_C 87.5 (C-10) and δ_C 76.9 (C-18), and to the geminal dimethyl groups at δ_H 1.27 (H-20) and δ_H 1.29 (H-19). In the HMBC spectrum, these two methyl groups showed cross-peaks with both oxygenated quaternary carbons at δ_C 87.5 (C-10) and δ_C 76.9 (C-18). These data indicated a hydroxyisopropyl moiety adjacent to the second hydroxy group bonded to the main backbone of **4**. The complete analysis of the 2D NMR experiments including ^1H–^1H COSY, HSQC and HMBC allowed the determination of the planar structure of the compound **4**, which was named as 10,18-dihydroxy-5′a-desmethyl-5′-acetylatomaric acid. The relative stereochemistry of all chiral centers of the compound **4**, except for C-10 (δ_C 87.5), was deduced by comparison of the ROESY correlations (Fig. 3) with those of the compound **3**.

The 10-keto-10-deisopropyliden-5′a-desmethyl-5′-acetylatomaric acid (**5**) and the 10-keto-10-deisopropyliden-atomaric acid (**6**) were isolated as a homogeneous mixture by TLC. The compound **5** was obtained as the major component in the mixture with **6**. The molecular formula of the major compound was determined to be $C_{26}H_{36}O_6$ by HREIMS (*m/z* $[M]^+$ 444.2511) and NMR data. Comparison of spectroscopic data of **5** (Table 1) with the literature (Gerwick et al., 1985) suggested that compound **5** was closely related to 5′a-desmethyl-5′-acetylatomaric acid, except by replacement of isopropylene group of 5′a-desmethyl-5′-acetylatomaric acid by a ketone group at δ_C 212.5 (C-10). The analysis of ^1H–^1H COSY, HSQC and HMBC spectra allowed

Table 2
Isolated and identified meroditerpenoids found in four populations of *Stypopodium zonale* along the Brazilian coast.

Meroditerpenoids	Populations			
	Abrolhos	Búzios	Atol das Rocas	Marataízes
Atomaric acid (**1**)	X	X		
Stypoldione (**2**)			X	
Stypofuranlactone (**3**)				X[a]
10,18-Dihydroxy-5′a-desmethyl-5′-acetylatomaric acid (**4**)				X[a]
10-Keto-10-deisopropyliden-5′a-desmethyl-5′-acetylatomaric acid (**5**)				X[a]
10-Keto-10-deisopropyliden-atomaric acid (**6**)				X

[a] New compounds.

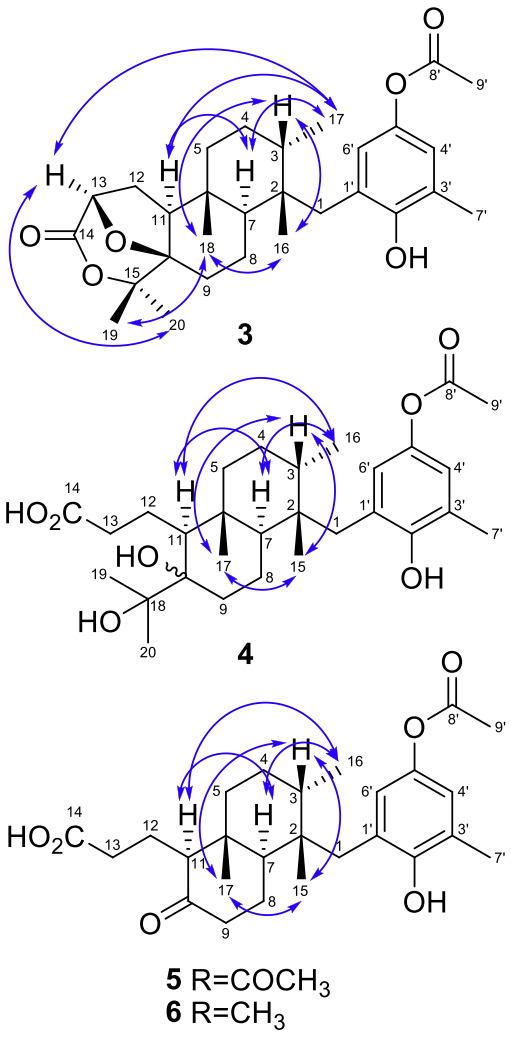

Fig. 3. Key ROESY correlations of the metabolites **3**–**5**.

assigning all of ^{1}H and ^{13}C NMR signals (Table 1) to the compound **5**. The minor compound in the mixture was identified as the 10-keto-10-deisopropyliden-atomaric acid (**6**) by analyses of the ^{1}H NMR and ^{13}C NMR data and comparison them with the literature (Dorta et al., 2003). By comparison of the ROESY correlations (Fig. 3) of the **5** and **6** compounds with those of the compound **3**, we could deduce that they have the same relative stereochemistry of **3**.

Table 2 shows the meroditerpenoids identified in the four studied populations, in which we could distinguish three chemotypes constituted by algae from (i) Abrolhos and Búzios, (ii) Atol das Rocas and (iii) Marataízes. In the last one, the new compounds (**3**), (**4**) and (**5**) were found. Chemical diversity among populations of *S. zonale* was reported in small and large spatial scale. Gerwick et al. (1985) isolated different compounds of this specie occurring at South Atlantic (Palao) and at the Caribbean Sea (Belize). Within the collection site of Belize, these authors have also found chemical diversity differences between populations from deep and shallow waters. Different and novel compounds of *S. zonale* were also found in other studies comparing populations from distant geographic locations (Dorta et al., 2002, 2003). Additionally, previous studies from our research group have demonstrated intraspecific variation in *S. zonale* from Brazilian populations. Collection campaigns in 1998/1999 showed the presence of atomaric acid (**1**) related to other populations from Archipelago of Abrolhos and Búzios, Rio de Janeiro State, as well as the presence of stypodione (**2**) in populations from Atol das Rocas and Archipelago of Fernando de Noronha (Soares et al., 2003; Pereira et al., 2004). These findings are congruent with the chemotypes found in this work, where the populations from Abrolhos and Búzios still showed the presence of atomaric acid and Atol das Rocas, the stypodione. This pattern was independent of the collection time, indicating a temporal conservation of the chemotypes found.

The differences observed using PCA on HPLC profiles among the samples clearly showed that the populations of *S. zonale* along the Brazilian coast could be unique in relation to secondary metabolite composition. The chemical diversity in algae could arrive from biotic and abiotic factors, and also by the interaction between them. Crossing experiments with species of *Laurencia* genus revealed that genetic variation was responsible for the diversity of halogenated metabolites in natural populations of *L. nipponica* (Masuda et al., 1997). Nevertheless, the intraspecific variations of halogenated compounds are related to algal sex and life history phases in the red alga *Asparagopsis armata*, which results in a selective herbivory by sea hare (Vergés et al., 2008), and stress the importance intraspecific variation on ecological and evolutionary consequences of plant–herbivore interactions. Furthermore, abiotic factors such as temperature (Sudatti et al., 2011), salinity (Kuwano et al., 1998), light exposure (Yuan et al., 2009), induction of chemical defenses against herbivores and space competition pressure (Amade and Lemée, 1998) are also related to intraspecific differences in chemical composition of marine organisms. Although the specific source of intraspecific variation was not considered here, the high chemical diversity of *Stypopodium zonale* is once more suggested to be related to geographic distribution.

Moreover, the application of analytical methods together with multifactorial statistics has proven to be a useful toll to ordinate samples by the similarities of their complex chromatographic traits, and guide the prioritization, which helps on isolation and identification of new meroditerpenes in *S. zonale*.

Author's contribution

ARS collected part of the biological material, performed the experiments, analyzed the data and wrote the paper. HMD was responsible for the PCA and wrote the paper. LT and VLT contributed with the NMR analyses. VLT and RCP supervised the phytochemical

work and did critical reading of the manuscript. All the authors have read the final manuscript and approved the submission.

Conflicts of interest

The authors declare no conflicts of interest.

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