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Article

A New Germacrane Sesquiterpene from the Brazilian Endemic Gorgonian *Phyllogorgia dilatata* Esper

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Visando a caracterização de substâncias de defesa contra predadores, a reinvestigação dos produtos naturais do octocoral *Phyllogorgia dilatata* Esper (Gorgonacea, Gorgoniidae) revelou a presença de novos metabólitos para esta espécie. Neste trabalho descreve-se o isolamento e a elucidação estrutural de três substâncias: o carotenóide peridinina (**1**), pigmento característico de dinoflagelados, caracterizando a presença de simbiontes nos tecidos dessa gorgônia; o diterpeno fagorrepelente 11β,12β-epoxipukalídeo (**2**), previamente descrito para uma outra espécie de octocoral; e um novo sesquiterpeno natural, (*E*) germacra-1(10),4(15),7(11)-trien-5-ol-8-ona (**3**).

A bioassay-guided re-investigation of natural products from the Brazilian endemic octocoral *Phyllogorgia dilatata* Esper (Gorgonacea, Gorgoniidae), collected in Rio de Janeiro State, has revealed three further compounds from this species: the dinoflagellate pigment peridinin (**1**), the icthyodeterrent diterpene 11β,12β-epoxypukalide (**2**), both previously reported from other octocoral species, and the new natural sesquiterpene (*E*) germacra-1(10),4(15),7(11)-trien-5-ol-8-one (**3**). Spectroscopic analysis and comparison with literature data allowed the structure elucidation of **1**-**3**.

Keywords: Phyllogorgia dilatata, *peridinin, germacrane, epoxypukalide*

Introduction

Marine octocorals of the order Gorgonacea are recognized to be a rich source of structurally unique and biologically active terpenoids[1,2] that can play important roles in ecological interactions. Most of the gorgonians that inhabit shallow waters are known to live in symbiotic relationship with intracellular algae, called zooxanthellae, important for the nutrition of the colony, carrying out photosynthesis and transferring organic compounds to the host[3-5]. Compounds such as peridinin, gorgosterol and 4-methyl sterols already isolated from octocorals are known to be biosynthesized by symbionts, but little is known about whether zooxanthellae or the host are involved in the production of other metabolites[6-8].

The extensive Brazilian coast (more than 7,500 km) is rich in marine coral species[9], but assessments of their natural products have been rare and restricted to very few publications[10]. One of the rare examples of Brazilian marine invertebrates already studied is the gorgonian *Phyllogorgia dilatata* Esper (Gorgonacea, Gorgoniidae) an endemic organism, distributed along the southwest Atlantic from the Ceará until to the state of Rio de Janeiro, Brazil[9]. Previous studies of its hexane extract reported the isolation of one sterol[11] and two nardosinane sesquiterpenes[12,13].

During our studies searching for secondary metabolites from Brazilian marine invertebrates involved in chemical defense against natural predators, we found that the crude organic extract of *P. dilatata* significantly reduced the consumption of bites by co-occurring fishes[14]. In this paper we wish to report the isolation and structure elucidation of a new germacrane sesquiterpene (**3**) from *P. dilatata*, along with two already known compounds, peridinin (**1**) and 11β,12β-epoxypukalide (**2**).

Results and Discussion

Phyllogorgia dilatata Esper (Octocorallia, Gorgonacea, Gorgoniidae) was collected at Tartaruga beach, Armação dos Búzios, Rio de Janeiro State in December 1995 by snorkel diving. The gorgonian colonies were air dried for ca 2 h and kept frozen until extraction.

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The crude organic extract of the gorgonian was partitioned between different solvents furnishing non-polar (hexane), medium polarity (ethyl acetate) and polar fractions (water). The hexane and the polar fractions did not show any deterrent activity against generalist fishes, when incorporated into artificial food pellets in their natural volumetric concentration[14]. The presence of the known *P. dilatata* sesquiterpenes in the nonpolar fraction was deduced by the ^{1}H-NMR analysis of the total fraction as well as of semi-purified compounds[14]. On the other hand, the crude extract and the ethyl acetate fraction significantly inhibited the consumption of treated food pellets in comparison with controls[14]. These results indicated that the bioactive metabolites were not those previously isolated by Kelecom *et. al.* from the apolar extract of *P. dilatata*[12,13] and prompted us to investigate the medium polarity fraction in order to identify the icthyodeterrent compounds. Purification of the ethyl acetate fraction by a series of chromathographic steps yielded three pure compounds.

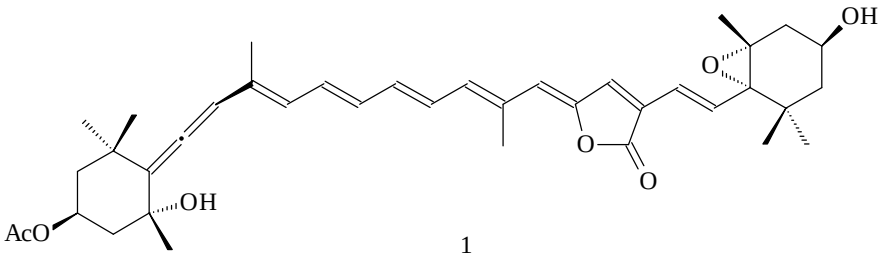
Compound **1** was obtained as an orange-red oil (7.6 x 10^{-4}% of dry weight gorgonian). Spectroscopic analysis of **1** (UV, IR, ^{1}H and ^{13}C-NMR) revealed that it was the known pigment called peridinin[6,15], a carotenoid unique to dinoflagellates, found in more than 75% of the investigated species, and proposed as an important taxonomic marker for symbiont determination in octocorals[16]. The presence of **1** in the crude extract reveals unambiguously for the first time the presence of a dinoflagellate in *P. dilatata* tissues.

Compound **2** was obtained as white fine needles (4.7 x 10^{-3}% of dry weight gorgonian), mp 211-213 °C. LREIMS showed the molecular ion $[M]^{+\bullet}$ at m/z 388 consistent with the molecular formula $C_{21}H_{24}O_7$. The IR absorptions at ν_{max} 1765 and 1710 cm^{-1} indicated the presence of at least two carbonyl functions in the molecule. Analysis of 1D and 2D NMR spectra, including ^{1}H-^{1}H-COSY, ^{1}H-^{13}C-HETCOR and n.O.e. experiments, led to the conclusion that compound **2** was 11β,12β-epoxypukalide, previously isolated from *Leptogorgia setacea*[18]. With the exception of the assignments for CH_2-13 (δ_C 23.8; δ_H 1.48, m, and 2.57, dd, J = 10.5 and 15.0 Hz) and CH_2-14 (δ_C 26.6; δ_H 1.43, m, and 1.66, m), all the carbon and hydrogen NMR spectral data obtained are identical to those proposed by Ksebati *et. al.*[17] The ^{1}H-^{1}H and ^{1}H-^{13}C long range correlation spectra as well as nuclear Overhauser enhancements experiments, allowed us to unambiguously assign the chemical shifts for all carbons and hydrogens for **2**. The cross peaks observed between C-14 (δ_C 26.6)/H-2 (δ_H 3.00, dd, J = 5.1 and 18.4 Hz; and δ_H 3.02, dd, J = 10.0 and 18.4 Hz), C-15 (δ_C 145.3)/H-14 (δ_H 1.43, m), and C-12 (δ_C 62.4)/H-13 (δ_H 2.57, dd, J = 10.5 and 15.0 Hz) in the 2D ^{1}H-^{13}C long range correlation and between H-1 (δ_H 3.48, ddt, J = 2.1, 5.1 and 10.0 Hz)/H-14 (δ_H 1.66, m) in the 2D ^{1}H-^{1}H COSY spectrum showed that the carbon chemical shifts previouslyproposed[18] for CH_2-13 and CH_2-14 should be exchanged.

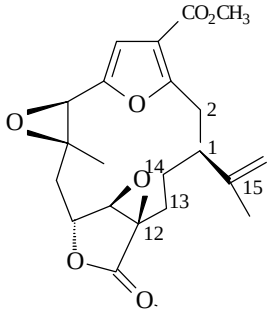
Although cembranolide diterpenes are common in gorgonians possessing symbionts, there is evidence that they are produced by the host. In fact, *Leptogorgia setacea* (Gorgonacea, Gorgoniidae), from which **2** was previously isolated, lack zooxanthellae as do other octocorals that yield cembranolides[17,18], suggesting that both *P. dilatata and L. setacea* can biosynthesize this diterpene.

Compound **3** was obtained as a pale yellow oil (3.6 x 10^{-3}% of dry weight gorgonian) The LREIMS of this compound showed an $[M]^{+\bullet}$ ion at *m/z* 234 consistent with the molecular formula $C_{15}H_{22}O_2$. The mass spectra also exhibited a diagnostic fragment at *m/z* 216, suggesting the loss of H_2O from the molecular ion $[M]^{+\bullet}$. The IR absorption bands at ν_{max} 3432 and 1724 cm^{-1} indicated the presence of hydroxyl and ketone funtionalities. Based on HBBD and DEPT ^{13}C-NMR experiments, it was deduced that one carbonyl, four quaternary carbons, two methines, five methylenes and three methyl groups were present in the structure. The ^{1}H and ^{13}C (HBBD and DEPT) NMR spectra confirmed the presence of one secondary hydroxyl group by the signals at δ_C 74.8 (CH-5) and δ_H 3.86 (1H, t, J = 7.0 Hz, H-5). The ^{13}C-NMR spectra also revealed seven sp^2 carbon signals at δ_C 112.3 (CH_2-15), 127.8 (CH-1), 130.3 (C-10), 133.9 (C-7), 134.4 (C-11), 151.5 (C-4) and 206.0 (C-8). The presence of an exomethylidene was deduced, by the singlets at δ_H 4.97 and 5.12 (1H each, H-15ab). These data were in accordance with a decacyclic sesquiterpene skeleton for **3**, which were confirmed by two dimensional NMR experiments [homonuclear ^{1}H-^{1}H and heteronuclear ^{1}H-^{13}C ($^1J_{CH}$ and $^{2,3}J_{CH}$) correlation spectroscopy].

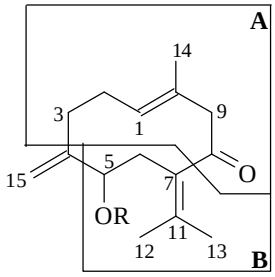
All the ^{1}H-NMR resonances of **3** were assigned to their corresponding carbon partner signals by direct 2D ^{1}H-^{13}C spectra ($^1J_{CH}$) analysis (Table). Homonuclear ^{1}H-^{1}H and long range ($^{2,3}J_{CH}$) ^{1}H-^{13}C 2D spectra allowed us to build partial structures for **3**. The ^{1}H-NMR signal at δ_H 5.33 (bt, J = 8.0 Hz, H-1) exhibited an allylic coupling with the hydrogens at δ_H 1.61 (d, J = 1.2 Hz, 3H-14) and a vicinal coupling with the hydrogens at δ_H 2.20 (m, 2H-2), which in turn was coupled with the signal at δ_H 2.25 (m, 2H-3). ^2J and ^3J ^{1}H-^{13}C couplings between the methylene hydrogens (δ_H 3.17, d, J = 15.9 Hz, H-9a, and 3.22, d, J = 15.9 Hz, H-9b) and the carbons at δ_C 127.8 (C-1), 130.3 (C-10) and 206.0 (C-8) allowed us to build the partial structure **A** (Fig. 1). In addition, the cross peaks, observed in the ^{1}H-^{1}H COSY spectra, between the hydrogens at δ_H 2.69 (d, J = 7.0 Hz, 2H-6) with those at δ_H 3.86 (t, J = 7.0 Hz, H-5) and 1.79 (s, 3H-12), together with the long range couplings observed in the 2D ^{1}H-^{13}C spectra between the carbons at



1



2



3 R = H
4 R = Ac

Figure 1. A and B = partial structures for **3**, deduced by 2D NMR experiments.

δ_C 133.9 (C-7) and 134.4 (C-11) and the methyl hydrogens at δ_H 1.79 (s, 3H-12) and 1.83 (s, 3H-13) led us to build the partial structure **B** (Figure). The *E* geometry of the double bond in **A** was deduced by the nuclear Overhauser enhancements observed for the hydrogens at δ_H 3.17 and 3.22 (d, *J* = 15.9 Hz, H-9a and H-9b) when the signal at δ_H 5.33 (bt, *J* = 8.0 Hz, H-1) was irradiated. This geometry is also consistent with the chemical shift observed for the methyl group (δ_C 17.4, C-14) and confirmed by literature data for other similar germacrane derivatives[19,20].

The extensive NMR measurements and the UV absorption for the chromophore in **3** at λ_{max} 249.5 nm (log ε 3.17, calcd 250 nm) provided the data, which fully defined the structure shown in **3**.

In order to confirm the proposed structure, compound **3** was acetylated by treatment with acetic anhydride in presence of pyridine. The incorporation of the acyl group deshielded the H-5 hydrogen (+ 1.13 ppm) and the carbons C-3 (+ 1.6 ppm), C-5 (+ 1.9 ppm), C-7 (+ 1.2 ppm) and C-15 (+ 2.7 ppm) and shielded C-4 (- 2.6 ppm) and C-6 (- 3.4 ppm), confirming the proposed structure (Table 1).

A literature search about germacrane sesquiterpenes revealed that this is the first time **3** has been isolated from natural sources. Although this compound has been once proposed as a product from the photosensitized oxygenation of germacrone, the published spectral data are inconclusive for its structure[21].

Germacrane sesquiterpenes are common in terrestrial plants and algae, and a few of them have been also obtained from octocorals. In addition to **3** from *P. dilatata*, the majority of these sesquiterpenes have been found in the families Nephtheidae, Gorgoniidae, Xeniidae, and Plexauridae[1,2]. Furanocembranolides, on the other hand, as a particular class of cembranes, are exclusive to octocorals. They have been found only in the families Gorgoniidae and Alcyonidae[1,2].

Whereas octocoral secondary metabolites have been extensively studied for their medical potential, their ecological functions began to be investigated only a decade ago, showing that ancepsenolides, sesquiterpenes and diterpenes can be important for survival of the species in areas of high levels of predation pressure[22]. In a parallel study about feeding deterrent properties of Brazilian octocoral species, we found that among *P. dilatata* lipophilic metabolites, only the diterpene **2** reduced the consumption of artificial diets by co-occurring fishes relative to controls[14].

This study led to the isolation of three compounds not previously reported for *Phyllogorgia dilatata*. One of them, the pigment peridinin (**1**), confirmed the presence of zooxanthellae in symbiosis with this octocoral. The diterpene 11β,12β-epoxypukalide (**2**), probably produced by the gorgonian and not by the symbiont, is a potent feeding deterrent compound against co-occurring fishes[14]. During this investigation, we also identified a new natural sesquiterpene, (*E*) germacra-1(10),4(15),7(11)-trien-5-ol-8-one (**3**), not previously reported from natural sources and for the first time structurally assigned by spectroscopic methods.

Table 1. NMR ($CDCl_3$, 300 MHz) data of **3** and the acetylated derivative **4**.

	3		**4**	
#C/H	δ ^{13}C(m)[a]	δ ^{1}H (m, *J* in Hz)[b]	δ ^{13}C(m)	δ ^{1}H (m, *J* in Hz)
1	127.8 (CH)	5.33 (1H, bt, 8.0)	128.1 (CH)	5.39 (1H, bt, 8.0)
2	28.0 (CH_2)	2.20 (2H, m)	28.0 (CH_2)	2.28 - 2.39 (2H, m)
3	33.5 (CH_2)	2.25 (2H, m)	35.1 (CH_2)	2.28-2.39 (2H, m)
4	151.5 (C)		148.9 (C)	
5	74.8 (CH)	3.86 (1H, t, 7.0)	76.7 (CH)	4.99(1H, dd, 4.5
6a	37.2 (CH_2)	2.69 (2H, d, 7.0)	33.8 (CH_2)	2.64 (1H, m)
b				2.69 (1H,m)
7	133.9*(C)		135.1*(C)	
8	206.0 (C)		205.7 (C)	
9a	55.0 (CH_2)	3.17 (1H, d, 15.9)	55.3 (CH_2)	3.17 (1H, d, 11.4)
b		3.22 (1H, d, 15.9)		3.27 (1H, d, 11.4)
10	130.3 (C)		130.8 (C)	
11	134.4* (C)		133.8*(C)	
12	23.0 (CH_3)	1.79 (3H, s)	22.9 (CH_3)	1.79 (3H, d, 0.9)
13	21.1 (CH_3)	1.83 (3H, s)	21.2 (CH_3)	1.80 (3H, d, 0.9)
14	17.4 (CH_3)	1.61 (3H, d, 1.2)	17.2 (CH_3)	1.60 (3H,s)
15a	112.3 (CH_2)	4.97 (1H, s)	115.0 (CH_2)	5.04 (1H, s)
b		5.12 (1H, s)		5.22 (1H, s)
COCH$_3$			170.2 (C)	
COCH$_3$			21.1 (CH_3)	2.01 (3H, s)

a) Number of H bonded to C deduced by DEPT experiments; b) Assignments aided by direct ^{1}H-^{13}C HETCOR; *Values may be interchanged.

Experimental

General experimental procedures

The melting points were determined using a Fisher Johns melting point apparatus and are uncorrected. Optical rotations were measured on a Perkin Elmer 243 B Polarimeter. UV spectra were obtained on a Shimadzu UV-160A spectrophotometer. ^{1}H and ^{13}C-NMR spectra were recorded in a Varian-Unity Plus 300 NMR spectrometer. LREIMS spectra were obtained through HRGC-MS analysis (SE-54 glass capillary column, 24 m x 30 mm, d*f* = 0.25 µm) on a Hewlett Packard 5890 A, using linear scanning (50-500 DA, 1.87 s dec^{-1}) and EI (70 eV) ionization. HPLC was carried out on a semi-preparative column using a Waters 510 pump, a 410 refractometer and a 486 UV. Isolation procedures were monitored by employing thin-layer chromatography (tlc) on pre-coated silica gel plates (Merck, Kieselgel 60 F-254).

Collection

Phyllogorgia dilatata Esper (Octocorallia, Gorgonacea, Gorgoniidae) was collected at Tartaruga beach, Armação dos Búzios, Rio de Janeiro State, Brazil (22° 45' S; 41° 51' W) in December 1995 by free diving between 3 and 5 meter depths. Colonies were air dried for ca 2 h and kept frozen until laboratory analyses. A voucher specimen (PD1295) was deposited at IQ-UFF.

Extraction and isolation of compounds **1 - 3**

P. dilatata (3.44 cm^3, 3326 g of dry weight gorgonian) was cut into small pieces and extracted with a mixture of methanol:dichloromethane 1:1 (once) and dichloromethane (twice). The extracts were combined and evaporated under reduced pressure yielding 368 g of a brownish gum (11.06% of dry weight gorgonian). Partition of the crude extract between hexane/methanol and ethyl acetate/water, afforded non-polar (hexane, 260 g), medium polarity (ethyl acetate, 11 g) and polar (aqueous, 90 g) fractions.

Silica gel (tlc grade) vacuum chromatography of the ethyl acetate fraction employing a gradient of 0-100% of ethyl acetate in hexane, followed by methanol afforded 13 fractions. Purification of fractions 5 (1,184.6 mg) and 6 (749.3 mg) (1:1 and 3:7 of hexane:ethyl acetate, respectively) yielded compounds **1**, **2** and **3**.

Reversed phase chromatography of 700 mg of fraction 6 using water:methanol (2:8) followed by silica Sep Pack (10 g) filtration with hexane:ethyl acetate (1:1) yielded 25.4 mg of an orange-red oil, $[\alpha]^{20}_{D}$ - 2.94 (*c* 0.0034, MeOH). UV, IR and ^{1}H and ^{13}C-NMR ($CDCl_3$, 300 MHz) data of **1** showed it to be identical to peridinin (**1**)[6,15].

Fraction 5 (1,184.6 mg) was recrystallized from methanol yielding 156.8 mg of white needles, mp 211-213 °C, identified as **2**[17].

*11*β,*12*β-*Epoxypukalide* (**2**)*:* $[\alpha]^{20}_{D}$ - 3.47 (*c* 0.0270, CH_2Cl_2); UV ($CHCl_3$) λ_{max} (log ε) 251,5 (3.75) nm; IR (film) ν_{max}: 3090, 2980, 2940, 1765, 1710, 1640, 1620, 1575, 1440, 1380, 1350, 1275, 1255, 1245, 1220, 1200, 1090, 1070, 880, 870, 770 cm^{-1}. ^{1}H and ^{13}C-NMR ($CDCl_3$, 300 MHz): with the exception of the data shown in "Results and Discussion" all the chemical shifts are identical to those published[17]. LREIMS (70 eV) *m/z* (rel. int.): $[M]^{+\bullet}$ 388 (4), 373 (2), 356 (5), 328 (3), 313 (2), 208 (11,4), 179 (14), 173 (11), 168 (20), 165 (14), 159 (10), 152 (14), 137 (22), 110 (16), 91 (20), 83 (32), 79 (32), 69 (30), 67 (29), 55 (48), 43 (100).

The remaining fraction 5 was purified by silica gel flash chromatography and normal phase semi-preparative HPLC, using 4:6 hexane:ethyl acetate to furnish 120 mg of a pale yellow oil (**3**).

(E) Germacra-1(10),4(15),7(11)-trien-5-ol-8-one (**3**)*:* $[\alpha]^{20}_{D}$ - 8,78 (*c* 0.0260, MeOH); UV ($CHCl_3$) λ_{max} (log ε) 249.5 (3.17); IR (KBr) ν_{max}: 3433, 3076, 2925, 2854, 1725, 1670, 1448, 1376, 1286, 1263, 1193, 1137, 1079, 1040, 904, 755 cm^{-1}; ^{1}H and ^{13}C-NMR ($CDCl_3$, 300 MHz): See Table and "Results and Discussion". LREIMS (70 eV) *m/z* (rel. int.): $[M]^{+\bullet}$ 234 (98), 220 (30), 216 (15), 201 (55), 191 (15), 177 (35), 175 (94), 173 (38), 163 (45), 162 (28), 159 (30), 150 (32), 149 (100), 136 (55), 124 (60), 123 (70), 122 (56), 121 (80), 119 (46), 109 (80), 107 (65), 105 (48), 93 (65), 91 (70), 79 (50), 67 (45), 53 (35), 41 (60).

(E) 5-Acetyl-germacra-1(10),4(15),7(11)-trien-8-one (4): A solution of 10 mg of 3 in 5 mL of dry acetic anhydride and 2 drops of pyridine was left at room temperature for two hours. After the usual work-up and purification of the resulting oil by silica gel chromatography with 9.5:0.5 hexane:ethyl acetate, 3.5 mg (30%) of pure 4 was obtained as a colorless oil. ^{1}H and ^{13}C-NMR ($CDCl_3$, 300 MHz): See Table.

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