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Article

Natural Products from the Gorgonian *Lophogorgia punicea*: Isolation and Struture Elucidation of an Unusual 17-Hydroxy Sterol

Rosângela de A. Epifanio[a]*, Lenize F. Maia[b], Angelo C. Pinto[b], Ingo Hardt[d], and William Fenical[d]

[a]*Instituto de Química, Universidade Federal Fluminense, 24020-150 Niterói - RJ, Brasil; e-mail: gqorose@vm.uff.br*

[b]*Núcleo de Pesquisas de Produtos Naturais, Universidade Federal do Rio de Janeiro, 21940-000 Rio de Janeiro - RJ, Brazil*

[c]*Instituto de Química, Universidade Federal do Rio de Janeiro, 21945-970 Rio de Janeiro - RJ, Brazil*

[d]*Scripps Institution of Oceanography, UCSD, 92093-0236, La Jolla, CA, USA*

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Este trabalho relata o primeiro estudo sobre a composição química da gorgônia *Lophogorgia punicea*, coletada no litoral sudeste do Brasil. A análise da mistura de esteróides monohidroxilados feita através de CGAR-EM revelou a presença de 9 derivados do esqueleto colestano contendo de 26 a 29 carbonos, bastante comuns em invertebrados marinhos. O estudo mais detalhado dos extratos brutos desta gorgônia, proporcionou o isolamento e a identificação por métodos espectroscópicos de hidroquinona e de um esteróide poliidroxilado denominado punicina (**1**). Punicina é um esteróide inédito com um padrão de oxigenação no carbono C-17 raro em organimos marinhos.

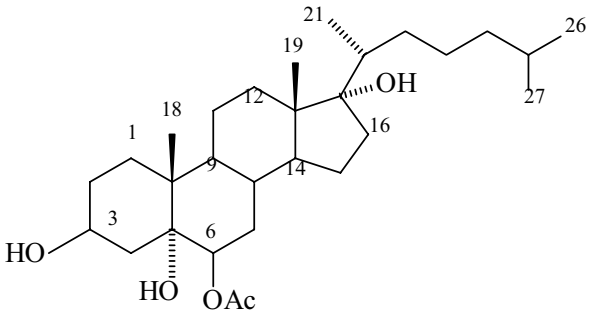
The natural products chemistry of the gorgonian *Lophogorgia punicea,* collected on the Southeast Brazilian coast, were investigated for the first time. Gas chromatography - mass spectral analysis of the monohydroxylated sterol mixture revealed the presence of nine common C_{26} to C_{29} cholestane derivatives. A detailed study of the crude extract of *Lophogorgia punicea* led to the isolation of hydroquinone and a new polyhydroxylated sterol named punicin (**1**). The structure of punicin was determined by interpretation of spectral data including 2D NMR and HRFABMS. Punicin is an unprecedented sterol possessing a C-17 hydroxyl group, a feature uncommon in marine organisms.

Keywords: *Gorgonacea, Octocorallia,* Lophogorgia punicea*, 17-hydroxysterol*

Introduction

Gorgonian corals (Octocorallia, Gorgonacea) are marine invertebrates found in all tropical and subtropical seas[1]. They are known to be a rich source of secondary metabolites which can play an important role in chemical defense interactions[2] and also possess unique pharmacological activities[3,4]. Compounds such as, terpenes, acetogenins, mono- and poly-hydroxylated steroids are commonly found in many gorgonian octocorals[5,6]. Most of the gorgonians that inhabit shallow waters in the tropical Atlantic Ocean contain dinoflagellate endosymbionts known as zooxanthellae, which contribute to the nutrition of the host and also produce sterols such as gorgorsterol, 4-methylsterols and derivatives[7,8]. These sterols are biosynthesized by the dinoflagellate, but the microalgae do not appear to be involved in the production of other secondary compounds.[9] Species of the genus *Lophogorgia* (Gorgonacea, Gorgoniidae) are known to contain C_{26} to C_{30}

monohydroxysterols[7,10,11], a polyoxygenated sterol (5,8-epoxysterol)[10], furanocembranolide diterpenes[12-14] and one bicyclogermacrane sesquiterpene[15]. The gorgonian *Lophogorgia punicea* (Milne-Edwards & Haime, 1857), a common species in Brazilian and Florida Keys waters[1], has not been examined previously. In this work we wish to report the identification of nine known monohydroxysterols, benzohydroquinone and a new polyoxygenated cholestane derivative (**1**) with an unprecedented steroidal structure.



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Results and Discussion

Colonies of *Lophogorgia punicea* were collected at Mangaratiba and Angra dos Reis Bay, Rio de Janeiro State, during July 1992 and March 1994, respectively.

The crude acetone extract of *Lophogorgia punicea* obtained in the first collection, was chromatographed on silica gel to furnish a fraction, eluted with hexane:ethyl acetate (1:1), containing a mixture of sterols (0.26% of dry weight gorgonian). Gas chromatographic analysis of this mixture indicated the presence of nine monohydroxysterols (Table 1), comprised of C_{26} to C_{29} cholestane derivatives.

The monohydroxysterols identified in the extract of *Lophogorgia punicea* were previously found in the species *L. platycados* (C_{27})[11], *L. cuspidata*, *L. alba* (C_{27} to C_{29})[12] and *L. subcompressa* (C_{26} to C_{30})[10], which also contained the C_{30} sterol gorgosterol. The sterol composition of *L. punicea* was found to be almost identical, including the relative concentrations, to the Pacific species *L. cuspidata* and *L. alba*, which lack zooxanthellae, as well as gorgosterol and 4-methyl sterols[11]. The absence of such sterols in *L. punicea* extract might suggest that this gorgonian does not contain the zooxanthellae responsible for their biosynthesis. From the same extract we isolated and identified hydroquinone (1 x10^{-2}% of dry weight gorgonian), a natural antioxidant already reported as a natural product from several organisms.

The crude extract of the second collection of *L. punicea* was chromatographed on silica gel yielding a fraction rich in a new compound (**1**). The new compound, named punicin, was further purified by reversed phase HPLC to yield 25.9 mg (1 x 10^{-2} of dry weight) of an amorphous optically active powder which showed $[\alpha]_D^{25}$ = -31.0 (c = 1.0, $CHCl_3$) and m.p. 132-134 °C. Punicin (**1**) showed an $[M+Na]^+$ ion at *m/z* 501.3521 (calcd 501.3556, Δ + 3.5 mmu) in the HR-FABMS, matching the molecular formula $C_{29}H_{50}O_5$. The mass spectra also exhibited diagnostic fragments at *m/z* 461, 443, 383 and 365, suggesting successive losses of OH, H_2O, AcOH and H_2O from the molecular ion $[M]^{+\cdot}$ 478, respectively. The presence of hydroxyl and ester funtionalities was also suggested by IR absortion bands at 3481 and 1718 cm^{-1}. On the basis of ^{13}C and DEPT-NMR experiments, it was deduced that one carbonyl, four quaternary carbons, seven methines, eleven methylenes and six methyl groups were present in the molecule (see Table 1). The ^1H and ^{13}C-NMR spectra indicated that punicin con-

Table 1. High Resolution GC-MS analysis of the monohydroxysterols from the Brazilian gorgonian *Lophogorgia punicea.*

Sterols[a,b]	Characteristic mass fragments	RR_t	Rc
24-*nor*cholesta-5,22-dien-3β-ol	70 $[M]^{+\cdot}$ 355, 352, 337, 300, 271, 255, 213, 111	0.92	3.49
27-*nor*-24-methylcholesta-5,22-dien-3β-ol	384 $[M]^{+\cdot}$ 369, 366, 351, 300, 273, 271, 255, 213, 111, 69, 55	0.96	2.37
cholesta-5,22-dien-3β-ol	384 $[M]^{+\cdot}$ 366, 351, 300, 271, 255, 213, 111, 69, 55	0.98	16.03
cholest-5-en-3β-ol[c]	386 $[M]^{+\cdot}$ 371, 368, 353, 301, 275, 255, 231, 213	1.00	33.89
ergosta-5,22-dien-3β-ol[c]	398 $[M]^{+\cdot}$ 380, 365, 337, 300, 271, 255, 213	1.02	16.83
ergosta-5,24(28)-dien-3β-ol[d]	398 $[M]^{+\cdot}$ 383, 314, 299, 271, 213	1.04	19.43
24-methylcholest-5-en-3β-ol[c,d]	400 $[M]^{+\cdot}$ 385, 382, 341, 315, 271, 255, 229, 213	1.04	d
stigmasta-5,22-dien-3β-ol[c]	412 $[M]^{+\cdot}$ 395, 379, 351, 300, 271, 255, 213	1.06	3.31
stigmasta-5,24(28)dien-3β-ol	412 $[M]^{+\cdot}$ 397, 379, 368, 314, 315, 300, 296, 281, 255, 231	1.10	4.65

RR_t = Relative retention times of the free sterols relative to cholest-5-en-3β-ol; Rc = Relative concentration expressed as percent of total sterols identified in the mixture (area of peak/total area of sterol peaks); [a]Nomenclature used was based on Ref. 30; [b]Identified by Mass Spectra and comparison with literature data; [c]Identified by co-injection and co-elution with authentic samples; [d]Sterols in mixture.

tains one secondary and two tertiary hydroxy groups [signals at δ 67.2 (CH) and 4.09 (1H, m), δ 75.2 (C) and 86.4 (C)]. The presence of an acetyl group, suggested by MS and IR data, was confirmed by NMR signals at δ 170.6 (C) and 21.4 (CH_3) and δ 2.06, (3H, s). The remaining five methyl groups and four unsaturations suggested a cholestane skeleton, which was confirmed by comparison with literature data[16] and by 2-dimensional NMR experiments (HMQC, HMBC and ^{1}H-^{1}H COSY).

All the ^{1}H-NMR resonances could be correlated to their corresponding carbons by direct 2D ^{1}H-^{13}C heterocorrelation methods (HMQC, Table 2). The positions of the hydroxyl and acetoxyl moieties on the cholestane ring were deduced by ^{1}H-^{1}H COSY and long range ^{1}H-^{13}C heterocorrelation (HMBC) NMR experiments. The ^{1}H-NMR signal at δ 4.72 (1H, bs, H-6) exhibited, in addition to ^2J correlation with the carbon at δ 31.4 (C-7), long range ^3J cross-peaks with the carbons at δ 170.6 (C-28), 38.4 (C-10) and 31.0 (C-8), confidently placing the acetoxyl group at C-6. The α-equatorial position of H-6 was defined by its typical coupling constants and comparison with literature data[17,18]. The typical sterol hydroxyl group was placed at C-3 (δ 4.09) by analysis of the ^{1}H-^{1}H couplings with the multiplets at δ 1.50 and 1.84 (H-2), and δ 1.58 and 1.82 (H-4). This assignment was confirmed by HMBC correlations of the C-3 carbon (δ 67.2) with the C-2 and C-4 protons, as well as those at δ 1.43 and 1.59 (H-1). The same experiments revealed that one of the tertiary hydroxyl groups was at C-5 (δ 75.2) by the conectivities with the multiplets at δ 1.58, and 1.82 (H-4) and the singlet at δ 1.15 (H-19). The hydroxylation pattern in rings A and B was confirmed by the low-field shift of the H-3 protons (multiplet at δ 4.09), due to the 1,3-diaxial interaction between 3α-H and 5β-OH, indicative of 3β-hydroxysterols bearing a 5α-hydroxyl group[19,20].

The position of the remaining tertiary hydroxy group was established by HMBC correlations between the signal at δ 86.4 (C-17) with the proton resonances at δ 1.65 (H-15b, H-16a), 0.77 (H-18) and 0.89 (H-21). The hydroxy group at C- 17 was observed to generate a downfield shift of the β carbon signals corresponding to C-13 (+5.1 ppm), C-16 (+9.8 ppm) and C-20 (+4.0 ppm) when compared to cholestane and androstane derivatives[17]. On the other hand, the corresponding NMR signals for C-12, C-15, C-21 and C-22 were shifted upfield (from -1.1 to -7.7 ppm) due to the C-17 hydroxyl γ-gauche shielded effect. The hydroxyl group at C-17 was placed in the α-position based upon ^{13}C resonance effects observed between the epimeric pair of 17-androstanols, in comparison with cholestane and androstrane hydrocarbonic skeletons[16]. The presence of 17α-hydroxyl group in **1** and in 17α-androstanol gave rise to a larger shielding effect at C-12 (-7.7 and -7.4 ppm, respectively), than that observed for 17β-androstanol (-2.1 ppm). In addition, the C-18 methyl group was also affected by the orientation of the hydroxyl group at C-17. In **1** the C-18 carbon was shifted downfield (+2.4 ppm) in comparison with the unsubstituted skeleton, whereas between the epimeric pair 17α-androstanol/17β-androstanol and androstane, the resonance differences are -0.4 and -5.4 ppm, respectively.

These comprehensive NMR measurements provided the data needed to make all carbon and proton assignments, which fully defined the structure of punicin as **1** (lacking absolute stereochemistry).

From the seven *Lophogorgia* species chemically investigated[3-10], only *L. platycados* is known to possess a polyoxygenated sterol, clionasterol endoperoxide (5,8-epoxisterol)[4].

Punicin (**1**) is an unprecedented sterol possessing a hydroxyl group at C-17. A sterol of this type is unknown from organisms of the Phylum Cnidaria and not especially common in marine organisms overall. Cholestane derived sterols oxygenated at C-17 have previously been found in dimeric steroidal alkaloids from the marine worm *Cephalodiscus gilchrist*[21-25], in metabolites from the tunicate *Ritterella tokioka*[26-28] and in a steroid sulfate from the marine sponge *Echinoclathria subhispida*[29].

Experimental

General procedures

The monohydroxylated sterol fraction was analysed by HRGC on a SE-54 glass capilary column (24 m x 30 mm, d*f* = 0.25 mm) using H_2 as carrier gas and temperature programming from 60 to 290 °C at 8 °C/min. The same chromatographic conditions were used for HRGC-MS analysis on a HP 5987 A, using a linear scanning (50-500 DA, 1.87 s dec^{-1}) and EI (70 eV) ionization. Co-injection of the authentic samples with the sterol fraction was performed insuring that the addition of standards enhanced the height of the target peaks by 20%. Reversed-phase (C_8) HPLC was carried out on a semi-preparative column (i.d. 9.4 mm) using a Waters 510 pump and a 410 refractometer. IR spectra (film, $CHCl_3$) were measured on a Perkin-Elmer Model 1600 FTIR spectrometer. FABMS and HRFABMS measurements were obtained with a VG-ZAB-E mass spectrometer. Optical rotation was measured on a Rudolf Autopol III (c = 1.0, $CHCl_3$). Corrected melting points were observed with a Thomas Hoover capillary apparatus. NMR spectra were recorded using a Varian Unity NMR spectrometer at 500 MHz for ^{1}H ($CDCl_3$ and C_6D_6) and 2D experiments (^{1}H-^{1}H-COSY, C_6D_6; HMQC, $CDCl_3$ and C_6D_6; and HMBC, $CDCl_3$) and at 50 MHz for ^{13}C-NMR and DEPT experiments ($CDCl_3$ and C_6D_6).

Table 2. NMR Data (500 MHz) for Punicin (1).

# C/H	δ ^{13}C (m)[a,c]	δ^{1}H (m)[a,d]	HMBC[a,e] #H	δ ^{1}H (m)[b]	1H-^{1}H-COSY[b] #H
1a	31.9 (t)	1.43 (m)	19	1.40 (m)	1b, 2a
b		1.59 (m)		1.75 (m)	1a
2a	30.6 (t)	1.50 (m)	1ab	1.58 (m)	1a, 2b
b		1.84 (m)		1.93 (m)	1a, 2a
3	67.2 (d)	4.09 (m)	1ab, 2ab, 4ab	4.12 (m)	2ab, 4ab
4a	40.5 (t)	1.58 (m)	3	1.68 (m)	3
b		1.82 (m)		1.91 (m)	3
5	75.2 (s)		4ab, 19		
6	76.0 (d)	4.72 (bs)		4.99 (bs)	7ab
7a	31.4 (t)	1.57 (m)	6, 8	1.73 (m)	6, 7b
b		1.71 (m)		1.88 (m)	6, 7a
8	31.0 (d)	1.64 (m)	6	1.90 (m)	7a, 9
9	44.9 (d)	1.34 (m)	19	1.58 (m)	11a
10	38.4 (s)		1b, 4a, 6, 19		
11a	20.9 (t)	1.34 (m)		1.30 (m)	9, 11b, 12ab
b		1.48 (m)		1.48 (m)	11a, 12ab
12a	32.5 (t)	1.68 (m)	18	1.45 (m)	11ab, 12b
b		1.68 (m)		1.64 (m)	11ab, 12a
13	47.7 (s)		15b, 16ab, 18		
14	50.3 (d)	1.78 (m)	12, 16b, 18	1.90 (m)	15
15a	23.4 (t)	1.05 (m)		1.64 (m)	14
b		1.65 (m)		1.64 (m)	
16a	38.1 (t)	1.65 (m)	15a	1.67 (m)	
b		1.80 (m)		1.67 (m)	
17	86.4 (s)		16a, 15b, 18, 21		
18	14.6 (q)	0.77 (s)		0.70 (s)	
19	16.4 (q)	1.15 (s)		1.19 (s)	
20	39.8 (d)	1.64 (m)	16b, 21	1.56 (m)	21, 22
21	13.9 (q)	0.89 (d, J = 7.0 Hz)		0.95 (d, J = 7.0 Hz)	20
22a	32.4 (t)	1.68 (m)	21	1.23 (m)	22b
b				1.76 (m)	20, 22a, 23b
23a	25.6 (t)	1.13 (m)	25	1.16 (m)	23b
b		1.42 (m)		1.46 (m)	22b, 23a
24a	39.3 (t)	1.13 (m)	25, 26	1.18 (m)	23b, 24b, 25
b		1.13 (m)		1.27 (m)	23b, 24a, 25
25	28.0 (q)	1.53 (m)	26	1.54 (m)	24ab, 26, 27
26	20.9 (q)	0.86 (d, J = 6.5 Hz)		0.92 (d, J = 7.0 Hz)	25
27	22.7 (q)	0.87 (d, J = 6.5 Hz)	26	0.92 (d, J = 7.0 Hz)	25
28	170.6 (s)		6, 29		
29	21.4 (q)	2.06(s)		1.70 (s)	

[a]$CDCl_3$; [b]C_6D_6; [c]Recorded at 50 MHz, multiplicity deduced by DEPT experiments; [d]Assignments aided by HMQC experiments; [e]HMBC J_{CH}value = 8 Hz.

Collection

Gorgonians, *Lophogorgia punicea,* were collected in the southern part of the state of Rio de Janeiro, Brazil, by SCUBA in 1992 (Mangaratiba) and 1994 (Angra dos Reis bay) at a depth of 6-15 m. Animals were identified by Prof. Frederick Bayer, National Museum of Natural History Smithsonian Institution, Washington DC, USA.

Monohydroxysterols analysis

Fresh specimens (228.4 g dry weight) of *L. punicea* collected at Mangaratiba in 1992, were extracted with CH_2Cl_2 (3x), acetone (3x) and MeOH (3x). An aliquot (6 mg) of the acetone extract (1.78 g) was filtered through a silica gel Sep-Pak with hexane, hexane:EtOAc (1:1) and EtOAc. All fractions were submited to HRGC and HRGC-MS analysis, revealing the presence of the cholestane derivatives in the fraction eluted with hexane:EtOAc (1:1, 0.26% of dry weight gorgonian) (Table 1). Authentic samples of cholest-5-en-3β-ol, ergosta-5,22-dien-3β-ol, 24-methylcholest-5-en-3β-ol and stigmasta-5,22-dien-3β-ol were used to perform the co-injections.

Hydroquinone

Part of the above acetone crude extract (902 mg) was chromatographed over silica gel column using a solvent gradient of increasing polarity (pure hexane, hexane-EtOAc, and pure EtOAc) furnishing a solid, further purified by recrystalization from hexane: EtOAc (1:1) to give 5 mg of white needles ($1x10^{-2}$% of gorgonian dry weight). IR (film, $CDCl_3$) and ^{1}H-NMR ($CDCl_3$) spectral data of the isolated compound were shown to be identical to hydroquinone[31].

Punicin (1)

The animals collected in 1994 (Angra dos Reis bay, RJ) were stored frozen until extraction. After freeze-drying, gorgonians (1437.27 g) were extracted with MeOH:CH_2Cl_2 (1:2, 1x) and pure CH_2Cl_2 (2x). Silica gel vacuum-liquid chromatography of the combined crude extracts (32.6 g) employing a gradient of 0-100% EtOAc in isooctane furnished nine fractions. The fraction eluted with 1:4 isooctane:EtOAc (62 mg) was purified by reversed-phase HPLC (MeOH:H_2O, 9:1, 2 mL/min flow), yielding 25.9 mg punicin (**1**) (1.8 x 10^{-3}% of gorgonian dry weight) as an amorphous white solid, mp 132-134 °C: Punicin (**1**, 6β-acetoxycholestan-3β,5α,17α-triol) showed $[\alpha]_D^{25}$ = -31.0 (c = 1.0, $CHCl_3$); FABMS m/z (rel.int.): $[M]^{+\cdot}$ 478(3), 477 (6), 461(6), 443(11), 401 (32), 383(96), 365(36), 347(12), 154 (100); HRFABMS $[MNa]^+$ 501.3221 (calcd. 501.3556 for $C_{29}H_{50}O_5Na$). IR $\nu_{máx}$. (film, $CHCl_3$): 3481, 2947, 1720, 1466, 1376, 1263 and 1030 cm^{-1}. ^{1}H and ^{13}C-NMR: Table 2.

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