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Isolation and structure elucidation of new cytotoxic steroids from the gorgonian *Leptogorgia sarmentosa*☆

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Abstract

The gorgonian *Leptogorgia sarmentosa* contains three new steroids, (20S)-20-hydroxycholestane-3,16-dione (**1**), (16S, 20S)-16,20-dihydroxycholestan-3-one (**2**), and (20S)-20-hydroxycholest-1-ene-3,16-dione (**3**) together with a known related compound (**4**). Their structures were defined by spectroscopic analysis. The new steroids exhibited significant cytotoxicity against four tumor cell lines (ED_{50} = 1 μg/ml). © 2000 Elsevier Science Inc. All rights reserved.

Keywords: Gorgonian; *Leptogorgia sarmentosa*; Cytotoxicity

1. Introduction

Octocorals (class Alcyonaria) are marine organisms whose chemistry is dominated by the presence of mevalonic acid derived metabolites [1]. In particular, those of the orders Alcyonacea (soft corals) and Gorgonacea (gorgonians) are among the most renowned producers of marine polyhydroxylated sterols, which are often the major steroids contained in the organism [2].

In the development of our research project focusing on the discovery of new cytotoxic metabolites from marine organisms of the south coast of Spain we obtained specimens of the gorgonian *Leptogorgia sarmentosa*. Previous studies of mediterranean specimens of *L. sarmentosa* yielded a series of polyoxygenated steroids that uncommonly present oxidation both at C-16 and C-20 [3–5]. Related C-16, C-20 oxygenated cholestanes have also been described from specimens of the anthozoan *Antipathes subpinnata* [6,7]. In the present paper we report the isolation, structure elucidation and cytotoxicity of three new steroids (**1**-**3**) (Fig. 1) together with the known steroid (**4**) from the gorgonian *L. sarmentosa* collected in Gibraltar Strait (Cádiz, Spain). The new steroids (**1**-**3**) are related to previous metabolites described from *L. sarmentosa* [4], but differ either in the level of hydrogenation of ring A (**1**, **2**) or in the location of ring A carbon-carbon double bond (**3**). Steroid **3** presents a Δ^1-3-keto conjugated moiety unusual among marine steroids.

2. Experimental procedures

2.1. General method

Optical rotation was measured in $CHCl_3$ solution on a Perkin–Elmer 241 polarimeter and IR spectra were recorded on a Genesis Series FT IR Mattson spectrophotometer. ^1H and ^{13}C NMR spectra were recorded at 400 and 100 MHz, respectively, on a Varian Unity 400 spectrometer using $CDCl_3$ as solvent. Proton chemical shifts were referenced to the residual $CHCl_3$ signal at δ 7.26 and ^{13}C NMR spectra were referenced to the center peak of $CDCl_3$ at δ 77.0. NOE experiments, ^1H-^1H correlation spectroscopy (COSY) and heteronuclear multiple quantum correlation (HMQC) were performed by using standard VARIAN pulse sequences. High resolution mass spectra (HRMS) were obtained by chemical ionization on a VG Autospec spectrometer. Col-

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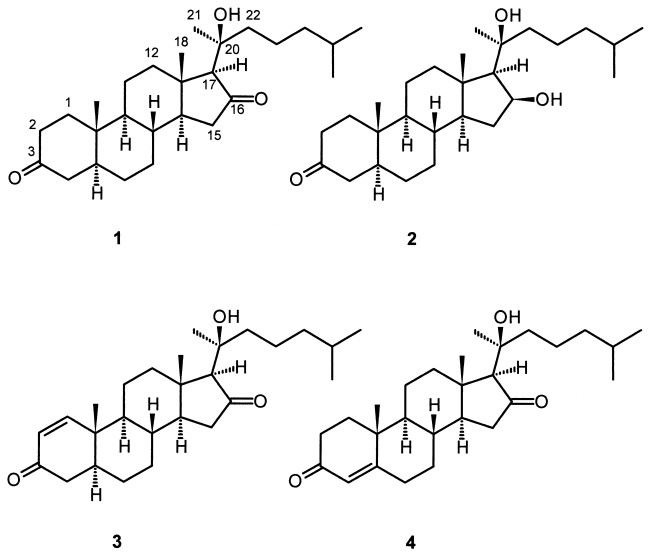

Fig. 1. Steroids isolated from *Leptogorgia sarmentosa.*

umn chromatography was carried out on Merck Silica gel 60 (70–230 mesh). HPLC separations were performed on a LaChrom–Hitachi apparatus equipped with LiChrosorb Si 60 columns using a differential refractometer detector.

2.2. Extraction and isolation procedure

Specimens of *L. sarmentosa* Linné (Gorgoniidae) were collected by hand using SCUBA at Gibraltar Strait in November 1995 and immediately frozen. The frozen tissue was extracted with acetone at room temperature. After filtration the acetone solution was evaporated under reduced pressure. After addition of water the residue was extracted three times with Et_2O. The organic layers were combined and the solvent was evaporated yielding a brown oily residue (0.47 g) that was chromatographed on a SiO_2 column using solvents of increasing polarities from hexane to Et_2O and, subsequently, chloroform-methanol (8:2). The fraction eluted with hexane/Et_2O (1:1) was subjected to HPLC separation [hexane/EtOAc (85:15)] to give compounds **1** (2.4 mg) and **4** (1.7 mg). Fractions of the general chromatography eluted with hexane/Et_2O (3:7) were subjected to preparative-TLC plate (0.5 mm) using hexane/Et_2O (3:7) and subsequent separation by HPLC [hexane/EtOAc (8:2)] to afford, in order of elution, compounds **3** (1 mg) and **2** (1.7 mg).

2.3. (20S)-20-Hydroxycholestane-3,16-dione (1)

White solid. $[\alpha]_D$ −47.3° (*c* 0.11, $CHCl_3$), CIMS *m/z* (assignment, relative intensity) 417 [$(M + H)^+$, 17)], 399 [$(M + H)^+ - H_2O$, 34)], 331 ($M^+ - C_6H_{13}$, 99). HRCIMS *m/z* (assignment) 417.3353 $(M + H)^+$, $C_{27}H_{45}O_3$ requires 417.3369. IR (film) V_{max} 3500, 1727, 1717 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) are listed in Tables 1 and 2, respectively.

Table 1
^1H NMR data (400 MHz, $CDCl_3$)[a] for **1**, **2**, and **3**

Position	1[b]	2[b]	3[c]
1	1.38 (1H, m)	1.34 (1H, m)	7.12 (1H, d, 10.3)
	2.02 (1H, m)	2.01 (1H, m)	
2	2.33 (1H, m)	2.27 (1H, m)	5.88 (1H, d, 10.3)
	2.40 (1H, m)	2.39 (1H, m)	
4	2.11 (1H, m)	2.08 (1H, m)	2.25 (1H, dd, 17.7, 4.6)
	2.28 (1H, m)	2.27 (1H, m)	2.38 (1H, dd, 17.7, 14.1)
5	1.55 (1H, m)	1.50 (1H, m)	1.94 (1H, m)
6	1.38 (2H, m)	1.34 (2H, m)	
7	1.64 (1H, m)	1.70 (1H, m)	1.66 (1H, m)
	1.00 (1H, m)	0.90 (1H, m)	1.06 (1H, m)
8	1.60 (1H, m)	1.50 (1H, m)	1.54 (1H, m)
9	0.92 (1H, m)	0.73 (1H, ddd, 12.1, 10.2, 5.1)	
11	1.50 (1H, m)	1.44 (1H, m)	
	1.64 (1H, m)	1.54 (1H, m)	
12	1.50 (1H, m)	1.16 (1H, m)	1.54 (1H, m)
	2.11 (1H, m)	2.13 (1H, m)	2.16 (1H, m)
14	1.44 (1H, m)	0.84 (1H, m)	1.48 (1H, m)
15	1.88 (1H, dd, 18.5, 13.5)	1.26 (1H, m)	1.90 (1H, dd, 18.5, 14.1)
	2.28 (1H, m)	2.24 (1H, m)	2.28 (1H, dd, 18.5, 7.4)
16		4.59 (1H, m)	
17	2.20 (1H, s)	1.20 (1H, m)	2.21 (1H, s)
18	0.92 (3H, s)	1.16 (3H, s)	0.95 (3H, s)
19	1.02 (3H, s)	1.03 (3H, s)	1.05 (3H, s)
21	1.25 (3H, s)	1.28 (3H, s)	1.26 (3H, s)
22	1.52 (2H, m)	1.54 (1H, m)	
		1.77 (1H, m)	
23	1.38 (2H, m)	1.34 (2H, m)	1.37 (2H, m)
24	1.15 (2H, m)	1.20 (2H, m)	1.15 (2H, m)
25	1.52 (1H, m)	1.54 (1H, m)	1.54 (1H, m)
26	0.87 (3H, d, 6.6)	0.88 (3H, d, 6.6)	0.88 (3H, d, 6.6)
27	0.87 (3H, d, 6.6)	0.88 (3H, d, 6.6)	0.88 (3H, d, 6.6)

[a] *J* in Hz.
[b] Assignments aided by COSY and HMQC experiments.
[c] Assignments aided by a COSY experiment and by comparison with the data of compound **1**.

2.4. (16S, 20S)-16,20-Dihydroxycholestan-3-one (2)

White solid. $[\alpha]_D$ +14.4° (*c* 0.16, $CHCl_3$), CIMS *m/z* (assignment, relative intensity) 419 [$(M + H)^+$, 13)], 333 ($M^+ - C_6H_{13}$, 88), 316 ($M^+ - C_6H_{14}O$, 100). HRCIMS *m/z* (assignment) 419.3497 $(M + H)^+$, $C_{27}H_{47}O_3$ requires 419.3525. IR (film) V_{max} 3400, 1700 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) are listed in Tables 1 and 2, respectively.

2.5. (20S)-20-Hydroxycholest-1-ene-3,16-dione (3)

White solid. $[\alpha]_D$ −48.3° (*c* 0.06, $CHCl_3$), UV (MeOH) λ 228 nm (ε = 4900). CIMS *m/z* (assignment, relative intensity) 415 [$(M + H)^+$, 9], 396 ($M^+ - H_2O$, 5), 381 ($M^+ - H_2O$-CH_3, 10), 329 ($M^+ - C_6H_{13}$, 90). HRCIMS *m/z*

Table 2
^{13}C NMR data (100 MHz, $CDCl_3$) for **1**, **2**, and **3**

Position	1[a]	2[a]	3[b]
1	38.2 (CH_2)	38.5 (CH_2)	157.4 (CH)
2	38.0 (CH_2)	38.2 (CH_2)	127.7 (CH)
3	211.5 (C)	211.9 (C)	193.5 (C)
4	44.5 (CH_2)	44.7 (CH_2)	40.9 (CH_2)
5	46.5 (CH)	46.7 (CH)	44.2 (CH)
6	28.6 (CH_2)	28.8 (CH_2)	27.4 (CH_2)
7	31.7 (CH_2)	31.5 (CH_2)	31.3 (CH_2)
8	33.8 (CH)	34.4 (CH)	34.1 (CH)
9	53.5 (CH)	53.9 (CH)	49.8 (CH)
10	35.7 (C)	35.7 (C)	39.0 (C)
11	21.0 (CH_2)	21.1 (CH_2)	21.1 (CH_2)[c]
12	39.4 (CH_2)	40.5 (CH_2)	39.3 (CH_2)[d]
13	42.8 (C)	43.1 (C)	43.0 (C)
14	50.8 (CH)	54.3 (CH)	50.9 (CH)
15	39.4 (CH_2)	37.4 (CH_2)	39.2 (CH_2)[d]
16	221.6 (C)	74.1 (CH)	221.0 (C)
17	71.4 (CH)	60.3 (CH)	71.4 (CH)
18	14.7 (CH_3)	15.1 (CH_3)	14.8 (CH_3)
19	11.5 (CH_3)	11.5 (CH_3)	13.1 (CH_3)
20	74.0 (C)	*	73.9 (C)
21	25.4 (CH_3)	26.9 (CH_3)	25.4 (CH_3)
22	42.4 (CH_2)	44.4 (CH_2)	42.4 (CH_2)
23	20.9 (CH_2)	22.4 (CH_2)	20.7 (CH_2)[c]
24	39.5 (CH_2)	39.6 (CH_2)	39.5 (CH_2)
25	28.1 (CH)	27.9 (CH)	28.1 (CH)
26	22.6 (CH_3)[c]	22.6 (CH_3)[c]	22.7 (CH_3)
27	22.7 (CH_3)[c]	22.7 (CH_3)[c]	22.7 (CH_3)

* Overlapped with the $CDCl_3$ signal.
[a] Assignments aided by ATP and HMQC experiments.
[b] Assignments made by comparison with the data of compound **1**.
[c,d] Values with the same superscript in the same column may be interchanged.

(assignment) 415.3200 $(M + H)^+$, $C_{27}H_{43}O_3$ requires 415.3212. IR (film) V_{max} 3500, 1727, 1686 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) are listed in Tables 1 and 2, respectively.

3. Results and discussion

Frozen specimens of *L. sarmentosa* from Gibraltar Strait were extracted with acetone. After evaporation of the solvent the aqueous residue was diluted with water and extracted with Et_2O. Evaporation of the solvent yielded a residue (0.47 g) that was chromatographed to afford **1** (2.4 mg), **2** (1.7 mg), and **3** (1 mg) together with the known compound **4** (1.7 mg) whose structure was elucidated by comparison with the data previously reported [4].

Compound **1** was isolated as a white solid. The molecular formula, $C_{27}H_{44}O_3$, was obtained from the high resolution CI mass spectrum. The ^{13}C NMR spectrum accounted for the 27 carbons of the molecular formula and indicated the presence of five methyls, eleven sp^3 methylenes, six sp^3 methines, three sp^3 quaternary carbons and two carbonyl carbons. The IR spectrum exhibited absorptions at 3500, 1727, and 1717 cm^{-1} indicating the presence of hydroxyl and carbonyl groups.

The upfield region of the ^{1}H NMR spectrum of compound **1** exhibited a signal at δ 0.87 (6H, d, J = 6.6 Hz) attributable to the two methyl groups of an isopropyl moiety, two signals at δ 1.02 (3H, s) and δ 0.92 (3H, s) assigned to two methyl groups attached to two quaternary carbons, and a signal at δ 1.25 (3H, s) that was correlated in the HMQC spectrum with the carbon signal at δ 25.4 (CH_3) assigned to a methyl group bonded to a fully substituted carbon atom bearing oxygen. These methyl signals could be accommodated by a C-20 hydroxylated cholestane skeleton, and thus, the ^{13}C NMR singlet at δ 74.0 (C) was assigned to C-20.

A cholestane skeleton accounts for four unsaturations of the molecular formula. The two unsaturations remaining were due to the presence of two carbonyl groups that gave rise to the ^{13}C NMR singlets at δ 221.6 (C), typical of a ketone in a five membered ring, and at δ 211.5 (C), attributable to a ketone either acyclic or in a six membered ring. However the possibility of an acyclic ketone in the side chain was discarded upon observation of the peak in the CI mass spectrum at *m/z* 331 (99%) due to the fragment arising by cleavage between C-20 and C-22 with loss of the saturated moiety C_6H_{13}. The carbonyl signal at δ 211.5 (C) was assigned to a ketone at C-3 both by analyses of COSY and HMQC spectra and by comparison of the ring A resonances of compound **1** with those reported for the 5α-cholestan-3-one [8]. The cyclopentanone carbonyl group was located at C-16 upon observation in the ^{1}H NMR spectrum of a signal at δ 2.20 (1H, s) assigned to a proton vicinal to the carbonyl. The singlet multiplicity of this signal indicated that this proton must be located in a methine group vicinal both to the cyclopentanone carbonyl and to two fully substituted carbon atoms. Furthermore, this proton signal at δ 2.20 was correlated in the HMQC spectrum with a ^{13}C NMR signal at δ 71.4 (CH) which is strongly deshielded by the presence of the vicinal hydroxyl and carbonyl groups at C-20 and C-16, respectively. The structure of compound **1** was defined therefore as 20-hydroxycholestane-3,16-dione.

The *S* configuration at C-20 was proposed by comparison of Me-21 protons signal of compound **1** at δ 1.25 (3H, s) with those of (20*R)*- and (20*S)*-20-hydroxycholesterol [9,10]. The differences of Me-21 chemical shifts in these epimers have been attributed to the preferential conformation in 20-hydroxysterols about the 17(20) bond with Me-21 and C-22 of the side chain pseudoequatorially oriented to the rear of 17(20) bond and with the hydroxyl group oriented opposing Me-18. On this basis it has been reported that a mutual NOE enhancement between H-12*eq* and Me-21 signals indicates a 20*S* configuration [6]. Unlike compound **2** (below discussed) whose 20*S* configuration was supported by NOE measurements, the overlapping of signals in the upfield region of the ^{1}H NMR spectrum of **1** prevented NOE difference spectroscopy from being employed. However, because the new steroids of *L. sarmen-*

tosa **1**-**3** likely share a common biogenetic origin, an identical stereochemistry at C-20 should be expected.

Compound **2** was isolated as a white solid. The molecular formula, $C_{27}H_{46}O_3$, was obtained from the high resolution CI mass-measurement and indicated five degrees of unsaturation. The IR spectrum exhibited absorptions at 3400 and 1700 cm^{-1} indicating the presence of both hydroxyl and carbonyl groups in the structure of **2**.

A general inspection of the ^1H and ^{13}C NMR spectra of **2** together with the presence of the ^1H NMR signals at δ 1.28 (3H, s), 1.16 (3H, s), 1.03 (3H, s), and 0.88 (6H, d, J = 6.6 Hz) suggested that compound **2**, similarly to **1**, was a C-20 hydroxylated cholestane steroid. However the ^{13}C NMR spectrum of **2** lacked the cyclopentanone carbonyl signal at δ 221.6 (C) presenting, in turn, an additional doublet at δ 74.1 (CH) which was correlated in the HMQC spectrum with the proton signal at 4.59 (1H, m). These data indicated that compound **2** was one of the epimeric alcohols that would arise by reduction at C-16 of compound **1**. The chemical shift of Me-21 protons signal at δ 1.28 together with the NOE enhancement observed in this signal upon irradiation of H-12*eq* signal at δ 2.13 (1H, m) indicated an *S* configuration at C-20. Finally, the stereochemistry at C-16 was proposed as *S* based on the upfield shift of C-17 signal at δ 60.3 (CH) consistent with the presence of a hydrogen bond between C-16 and C-20 hydroxyl groups [4,5]. These data defined the structure of 16*S*,20*S*-dihydroxycholestan-3-one for compound **2**.

Compound **3** was the minor steroid isolated from *L. sarmentosa*. The molecular formula, $C_{27}H_{42}O_3$, was obtained from the high resolution CI mass measurement. Although the small amount available of compound **3** prevented better spectroscopic data from being obtained, the general structural similarities of **3** with the steroids **1** and **2** above described allowed its structural elucidation. Thus both ^1H and ^{13}C NMR resonances were very similar to those of compound **1**. However the analysis of the molecular formula of **3** indicated an additional degree of unsaturation. The ^1H NMR exhibited two mutually coupled signals at δ 7.12 (1H, d, J = 10.3 Hz) and 5.88 (1H, d, J = 10.3 Hz) assigned to the protons of a *cis*-disubstituted conjugated double bond whose carbons gave rise to the ^{13}C NMR signals at δ 157.4 (CH) and 127.7 (CH). The conjugated carbonyl signal appeared at δ193.5 (C) and the cyclopentanone carbonyl gave rise to the signal at δ 221.0 (C). Because the vinyl proton signals were exclusively coupled it was concluded that the conjugated carbonyl was located at C-3 and that compound **3** was the C-1, C-2 dehydroderivative of compound **1**.

The new steroids isolated from *L. sarmentosa* were tested in bioassays directed to detect in vitro cytotoxicity against several cancer cell lines. Compounds **1** and **2** showed significant, although non selective, cytotoxicity against P-388 suspension culture of mouse lymphoid neoplasm and the monolayer cultures of human lung carcinoma (A 549), human colon carcinoma (HT 29), and human melanoma (MEL 28) exhibiting ED_{50} values of 1 μg/ml, in all cases. Although the small amount isolated of **3** prevented this compound from being tested in its pure form, a fraction that contained **3** as the major component exhibited cytotoxicity against the four tumor cell lines above mentioned (ED_{50} = 1 μg/ml).

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