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Structure and cytotoxicity of new polyhydroxylated sterols from the Caribbean gorgonian *Plexaurella grisea*

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Abstract

The gorgonian *Plexaurella grisea* contains the new steroids 9-hydroxygorgosterol (**1**), 9,11α,14-trihydroxygorgosterol (**2**), 5β,6β-epoxyergost-24(28)-ene-3β,7β-diol (**3**), ergost-24(28)-ene-3β,5α,6β,7β-tetrol (**4**), an unseparable 1:1 mixture of the epimers (25*R*) and (25*S*)-26-acetoxy-3β,5α-dihydroxyergost-24(28)-en-6-one (**5**/**6**), and seven related, known compounds (**7**-**13**). The structures of these new compounds were defined by spectroscopic analysis. All the compounds (**1**–**13**) isolated from *P. grisea* were tested against P 388, A 549, and HT 29 tumor cell lines. Compounds **3**, **5**/**6**, and **12** exhibited selective activity against the HT 29 cell line (ED_{50} = 0.1 μg/ml). © 2001 Elsevier Science Inc. All rights reserved.

Keywords: Sterols; Gorgonian; *Plexaurella grisea*; Cytotoxicity

1. Introduction

Marine organisms have been shown to be an extraordinarily rich source of new sterols displaying unconventional nuclear structures and side chains, as well as unusual oxygenation patterns of the A-D rings. Furthermore, many of the structural features of marine sterols have no counterpart among steroidal metabolites from terrestrial plants and animals. The biosynthetic origin of the complex mixtures of sterols often found in marine invertebrates is complicated by the fact that there are diverse contributing sources: de novo biosynthesis, assimilation of sterols produced by symbiont organisms, and assimilation or modification of dietary sterols [1–3].

Octocorals (class Alcyonaria) are recognized as one of the most prolific sources of sterols in the marine environment. In particular, organisms of the Orders Gorgonacea and Alcyonacea are characterized by their high content of polyhydroxylated sterols. In addition, a distinctive feature often found in sterols of Octocorals is the presence of a cyclopropane ring in the side chain [2,3].

In our search for new bioactive compounds from marine organisms, we obtained specimens of the Caribbean gorgonian *Plexaurella grisea* whose extract showed in vitro cytotoxicity against the tumor cell lines P 388, A 549, and HT 29 (ED_{50} = 2.5 μg/ml in all cases). Previous studies of this gorgonian had led to the isolation of acyclic sesquiterpenes [4,5] and several sterols [6]. In this paper, we report the isolation, structure, and cytotoxicity of six new polyhydroxylated sterols (**1**–**6**), together with seven known related sterols (**7**–**13**) from the gorgonian *P. grisea* collected at Punta Cana (Dominican Republic). The known compounds **8** and **9** have been encountered for the first time in natural sources.

2. Experimental procedures

2.1. General methods

Optical rotations were measured in $CHCl_3$ or CH_3OH solutions 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 C_5D_5N as solvent. Proton chemical shifts were referenced to the residual C_5H_5N signal at δ 8.71, and ^{13}C NMR spectra were referenced to the center peak of C_5D_5N at δ 149.9. NOE experiments, ^1H-^1H correlation spectroscopy (COSY), heteronuclear multiple quantum cor-

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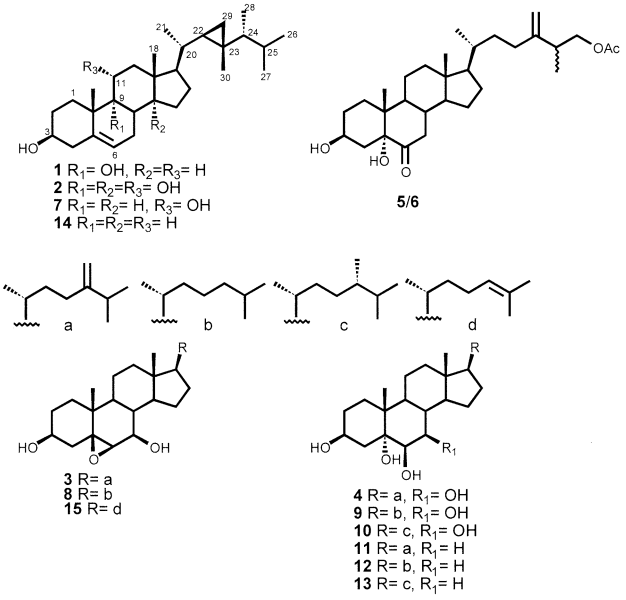
relation (HMQC), and heteronuclear multiple bond coherence (HMBC) were performed using standard VARIAN pulse sequences. Low resolution mass spectra were recorded on a Finnigan Voyager GC8000^{top} spectrometer. High resolution mass spectra (HRMS) were obtained on a VG Autospec spectrometer. Column chromatography 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 or LiChrosorb RP 18 columns using a differential refractometer detector.

2.2. Extraction and isolation procedure

Specimens of *Plexaurella grisea* Kunze (Plexauridae) were collected by hand using apneal diving in a lagoon zone near Punta Cana (Dominican Republic) and immediately frozen. The frozen tissue was lyophilized, and the freeze-dried material was extracted with MeOH. The MeOH solution was concentrated to leave an oily residue (23.0 g) that was dissolved in MeOH (500 ml) and subsequently extracted using a modified Kupchan partition [7] as follows. The H_2O content (% v/v) of the MeOH extract was adjusted prior to sequential partitioning against hexane (10% v/v) and $CHCl_3$ (40% v/v). The hexane soluble material (9.38 g) was chromatographed on a SiO_2 column using solvents of increasing polarity from hexane to hexane/Et_2O (3:7) and finally with $CHCl_3$/MeOH (8:2). Fractions eluted with hexane/Et_2O (3:7) yielded, after separation on reversed phase HPLC using MeOH/H_2O (8:2), 9-hydroxygorgosterol (**1**) (4.0 mg, 0.001% yield) and 11α-hydroxygorgosterol (**7**, 2.3 mg, 0.0006% yield). Fractions from the general chromatography eluted with $CHCl_3$/MeOH (8:2) were further separated on SiO_2 column using mixtures of increasing polarity from $CHCl_3$/MeOH (98:2) to $CHCl_3$/MeOH (1:1). Repeated separations of the fractions obtained with $CHCl_3$/MeOH (97:3) on reversed phase HPLC using mixtures of MeOH/H_2O, MeCN/H_2O, or MeOH/MeCN/H_2O (3.75:3.75:2.5) yielded the mixture of isomers (25*R*) and (25*S*)-26-acetoxy-3β,5α-dihydroxyergost-24(28)-en-6-one (**5**/**6**, 28.8 mg, 0.008%), 9,11α,14-trihydroxygorgosterol (**2**, 16.7 mg, 0.004%), 5β,6β-epoxyergost-24(28)-ene-3β,7β-diol (**3**, 6.2 mg, 0.002%), and 5β,6β-epoxycholestane-3β,7β-diol (**8**, 4.5 mg, 0.001%). Fractions eluted with $CHCl_3$/MeOH (9:1) yielded, in order of elution after purification on reversed phase HPLC (MeOH/MeCN/H_2O 5.5:4:0.5) or (MeOH/H_2O 9:1), the compounds cholestane-3β,5α,6β-triol (**12**, 56.4 mg, 0.015%), ergost-24(28)-ene-3β,5α,6β-triol (**11**, 54.5 mg, 0.015%), (24*S*)-ergostane-3β,5α,6β-triol (**13**, 43.0 mg, 0.011%), ergost-24(28)-ene-3β,5α,6β,7β-tetrol (**4**, 50.6 mg, 0.013%), cholestane-3β,5α,6β,7β-tetrol (**9**, 20.6 mg, 0.005%), and (24*S*)-ergostane-3β,5α,6β,7β-tetrol (**10**, 6.7 mg, 0.002%). Final purification of each compound was accomplished by HPLC using different mixtures of MeOH/H_2O.



2.2.1. 9-Hydroxygorgosterol (*1*)

Amorphous solid. $[\alpha]_D$ −41.3° (*c* 0.08, $CHCl_3$), EIMS *m/z* (assignment, relative intensity) 442 (M^+, 1), 424 (M^+-H_2O, 22), 271 (M^+-H_2O-$C_{11}H_{21}$, 5), 253 (M^+-$2H_2O$-$C_{11}H_{21}$, 9). HREIMS *m/z* (assignment) 442.3803 $(M)^+$, $C_{30}H_{50}O_2$ requires 442.3811. IR (film) ν_{max} 3396, 1095 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.2.2. 9,11α,14-Trihydroxygorgosterol (*2*)

Amorphous solid. $[\alpha]_D$ −15.0° (*c* 0.1, MeOH), EIMS *m/z* (assignment, relative intensity) 474 (M^+, 5), 456 (M^+-H_2O, 52), 439 [$(M+H)^+$-$2H_2O$, 59], 421 [$(M+H)^+$-$4H_2O$, 100]. HREIMS *m/z* (assignment) 474.3733 $(M)^+$, $C_{30}H_{50}O_4$ requires 474.3709. IR (film) ν_{max} 3400, 1120 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.2.3. 5β,6β-Epoxyergost-24(28)-ene-3β,7β-diol (*3*)

Amorphous solid. $[\alpha]_D$ +48.9° (*c* 0.09, MeOH). EIMS *m/z* (assignment, relative intensity) 430 (M^+, 7), 413 [$(M+H)^+$-H_2O, 48], 395 [$(M+H)^+$-$2H_2O$, 34]. HREIMS *m/z* (assignment) 430.3407 $(M)^+$, $C_{28}H_{46}O_3$ requires 430.3470. IR (film) ν_{max} 3325, 1641, 1143, 1089 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

2.2.4. Ergost-24(28)-ene-3β,5α,6β,7β-tetrol (*4*)

Amorphous solid. $[\alpha]_D$ +30.0° (*c* 0.12, MeOH). EIMS *m/z* (assignment, relative intensity) 448 (M^+, 4), 433

Table 1
^{1}H NMR data (400 MHz, C_5D_5N)[a] for **1, 2, 3, 4,** and **5/6**

	1	**2**	**3**	**4**	**5/6**
1	1.61 (dt, 13.2, 3.2); 2.40 (ddd, 14.0, 13.6, 3.6)	2.35 (m); 2.75 (m)	1.31 (m); 1.96 (m)	1.61 (m); 2.16 (m)	1.61 (m); 2.12 (dd, 13.4, 3.8)
2	1.85 (m); 2.17 (m)	1.96 (m); 2.17 (m)	1.69 (m); 2.03 (m)	2.06 (m); 2.28 (m)	1.88 (ddd, 12.8, 11.2, 4.8); 2.18 (m)
3	3.84 (m)	3.92 (m)	4.00 (m)	4.84 (m)	4.68 (m)
4	2.67 (m); 2.71 (m)	2.75 (m)	1.87 (m); 2.48 (dd, 13.6, 11.6)	2.34 (m); 2.93 (dd, 12.4, 11.6)	2.37 (dd, 12.8, 12.0); 2.61 (dd, 13.8, 4.6)
6	5.51 (bd, 2.4)	5.62 (d, 4.8)	3.47 (s)	4.13 (d, 3.6)	
7	1.89 (m); 2.08 (m)	1.96 (m); 2.87 (dd, 15.7, 13.0)	3.79 (dd, 8.0, 7.6)	4.42 (brd, 3.6)	2.24 (dd, 12.8, 4.4); 3.12 (dd, 12.8, 12.4)
8	1.85 (m)	2.13 (dd, 11.6, 5.2)	1.87 (m)	2.03 (m)	1.74 (m)
9			0.86 (m)	2.03 (m)	2.32 (ddd, 14.2, 11.6, 3.6)
11	1.76 (m); 1.85 (m)	4.52 (m)	1.42 (m)	1.52 (m); 1.61 (m)	1.27 (m); 1.51 (m)
12	1.85 (m)	2.23 (dd, 12.4, 5.2); 2.48 (t, 12.0)	1.08 (m); 1.96 (m)	1.23 (m); 2.06 (m)	1.16 (m); 1.97 (m)
14	1.89 (m)		1.19 (m)	1.45 (m)	1.19 (m)
15	1.15 (m); 1.61 (m)	1.52 (m); 2.35 (m)	1.75 (m); 2.43 (m)	1.33 (m); 1.88 (m)	0.99 (m); 1.43 (m)
16	1.40 (m); 2.08 (m)	1.52 (m); 1.79 (dd, 10.4, 8.8)	1.31 (m); 1.92 (m)	1.81 (m); 2.36 (m)	1.19 (m); 1.79 (m)
17	1.33 (m)	2.39 (m)	1.14 (m)	1.14 (m)	1.11 (m)
18	0.76 (s)	0.95 (s)	0.62 (s)	0.78 (s)	0.61 (s)
19	1.24 (s)	1.43 (s)	1.15 (s)	1.60 (s)	0.94 (s)
20	1.10 (m)	1.11 (m)	1.41 (m)	1.45 (m)	1.39 (m)
21	1.10 (bs)	1.11 (s)	0.99 (d, 6.4)	1.01 (d, 6.4)	0.94 (d, 5.2)
22	0.16 (m)	0.19 (m)	1.24 (m); 1.62 (m)	1.25 (m); 1.66 (m)	1.19 (m); 1.61 (m)
23			1.96 (m); 2.18 (m)	1.95 (m); 2.16 (m)	1.97 (m); 2.18 (m)
24	0.21 (m)	0.14 (m)			
25	1.52 (m)	1.49 (m)	2.27 (sept, 6.8)	2.24 (m)	2.55 (m)
26	0.85 (d, 6.4)	0.82 (d, 6.4)	1.04 (d, 6.8)	1.04 (d, 6.8)	4.07 (ddd, 10.8, 7.2, 0.8)/4.09 (ddd, 10.8, 7.2, 0.8); 4.27 (ddd, 10.8, 6.4, 1.6)/4.28 (ddd, 10.8, 6.4, 1.2)
27	0.96 (d, 6.8)	0.94 (d, 7.6)	1.05 (d, 6.8)	1.05 (d, 6.8)	1.10 (dd, 7.2, 1.2); 1.11 (dd, 7.2, 1.2)
28	0.98 (d, 6.8)	0.89 (d, 6.8)	4.83 (brd, 1.2); 4.84 (brs)	4.83 (brs) 4.84 (brs)	4.91 (s), 4.94 (d, 1.2)
29	−0.11 (dd, 5.6, 4.4); 0.45 (dd, 9.2, 4.4)	−0.11 (dd, 5.6, 4.4); 0.43 (dd, 9.2, 4.4)			
30	0.91 (s)	0.92 (s)			
OH	6.12 (bs); 4.21 (s)	5.90 (d, 3.2); 6.36 (d, 3.2); 6.91 (brs)	6.10 (brd, 8.0); 6.35 (brd, 3.2)	5.56 (brs); 5.96 (s); 6.24 (brs)	6.20 (brs); 7.28 (brs)
$OCOCH_3$					2.03 (s)

[a] *J* in Hz. Assignments aided by COSY and HMQC experiments.

$[(M+H)^+-H_2O$, 60], 415 $[(M+H)^+-2H_2O$, 12]. HREIMS *m/z* (assignment) 433.3333 $(M-CH_3)^+$, $C_{27}H_{45}O_4$ requires 433.3318, 413.3390 $(M-OH-H_2O)^+$, $C_{28}H_{45}O_2$ requires 413.3420. IR (film) ν_{max} 3430, 1644, 1078 cm^{-1}. ^{1}H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

*2.2.5. (25R) and (25S)-26-Acetoxy-3β,5α-dihydroxyergost-24(28)-en-6-one (**5/6**)*

Amorphous solid. $[\alpha]_D$ −12.2° (*c* 0.09, MeOH). EIMS *m/z* (assignment, relative intensity) 489 $[(M+H)^+$, 14], 471 $[(M+H)^+-H_2O$, 24], 453 $[(M+H)^+-2H_2O$, 26]. HREIMS *m/z* (assignment) 489.3579 $(M)^+$, $C_{30}H_{49}O_5$

Table 2
^{13}C NMR data (100 MHz, C_5D_5N)[a] for **1, 2, 3, 4** and **5/6**

	1	2	3	4	5/6
1	29.6 (t)	30.9 (t)	37.6 (t)	33.4 (t)	30.7 (t)
2	32.7 (t)	32.6 (t)	32.1 (t)	32.6 (t)	31.8 (t)
3	70.6 (d)	70.9 (d)	68.7 (d)	67.1 (d)	66.8 (d)
4	43.9 (t)	44.5 (t)	43.3 (t)	43.1 (t)	37.6 (t)
5	139.9 (s)	139.8 (s)	66.8 (s)	76.8 (s)	80.3 (s)
6	121.4 (d)	121.2 (d)	69.1 (d)	79.5 (d)	213.6 (s)
7	27.8 (t)	23.5 (t)	74.6 (d)	73.0 (d)	42.2 (t)
8	35.2 (d)	36.5 (d)	38.1 (d)	39.5 (d)	37.7 (d)
9	73.7 (s)	77.7 (s)	50.6 (d)	44.8 (d)	44.8 (d)
10	43.2 (s)[b]	44.2 (s)	34.8 (s)	38.6 (s)	43.3 (s)
11	27.3 (t)	69.3 (d)	22.5 (t)	22.0 (t)	21.9 (t)
12	36.1 (t)	40.8 (t)	40.1 (t)	40.9 (t)	40.1 (t)
13	42.6 (s)[b]	48.6 (s)	43.3 (s)	43.9 (s)	42.9 (s)
14	50.1 (d)	82.6 (s)	56.3 (d)	56.5 (d)	56.5 (d)
15	24.6 (t)	27.8 (t)	28.0 (t)	27.9 (t)	24.1 (t)
16	28.8 (t)	32.7 (t)	29.0 (t)	29.2 (t)	28.3 (t)
17	58.1 (d)	52.5 (d)	55.6 (d)	55.8 (d)	56.1 (d)
18	11.4 (q)	16.9 (q)	12.0 (q)	12.6 (q)	12.2 (q)
19	23.0 (q)	22.4 (q)	17.4 (q)	17.6 (q)	14.2 (q)
20	35.7 (d)	35.4 (d)	36.0 (d)	36.2 (d)	35.8/35.9 (d)
21	21.6 (q)	21.6 (q)	19.1 (q)	19.1 (q)	18.7/18.8 (q)
22	32.5 (d)	32.2 (d)	35.1 (t)	35.2 (t)	34.6/34.7 (t)
23	26.0 (s)	26.0 (s)	31.4 (t)	31.4 (t)	31.5/31.7 (t)
24	50.9 (d)	50.9 (d)	156.7 (s)	156.8 (s)	151.9/152.0 (s)
25	32.3 (d)	32.6 (d)	34.1 (d)	34.1 (d)	39.1/39.2 (d)
26	21.7 (q)[c]	21.7 (q)[b]	22.2 (q)[b]	22.2 (q)[b]	68.1/68.2 (t)
27	22.4 (q)[c]	22.4 (q)[b]	22.0 (q)[b]	22.0 (q)[b]	17.0/17.1 (q)
28	15.7 (q)	15.7 (q)	106.7 (t)	106.7 (t)	109.5/109.7 (t)
29	21.5 (t)	21.4 (t)			
30	14.4 (q)	14.5 (q)			
$OC\underline{O}CH_3$					170.8 (s)
$OCOC\underline{H}_3$					20.9 (q)

[a] Assignments aided by HMQC and HMBC experiments.
[b–c] Values with the same superscript in the same column may be interchanged.

requires 489.3580. IR (film) ν_{max} 3394, 1741, 1709, 1230, 1072 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) data are listed in Tables 1 and 2, respectively.

3. Results and discussion

Specimens of *P. grisea* from Punta Cana (Dominican Republic) were collected by hand and immediately frozen. The frozen material was freeze-dried and subsequently, extracted following the Kupchan method [7]. Column chromatography of the more polar material from the hexane extract, followed by HPLC separation using reversed phase of the selected fractions, allowed for isolation of the new sterols **1** (4.0 mg), **2** (16.7 mg), **3** (6.2 mg), **4** (50.6 mg), and an unseparable mixture of compounds **5**/**6** (28.8 mg, ratio 1:1) together with the following known compounds, whose structures were elucidated by comparison with the data previously reported: **7** (2.3 mg) [8], **8** (4.5 mg) [9], **9** (20.6 mg) [9], **10** (6.7 mg) [10], **11** (54.5 mg) [11], **12** (56.4 mg) [12], and **13** (43.0 mg) [6].

Compound **1** was isolated as an amorphous powder with a molecular formula $C_{30}H_{50}O_2$ as indicated by the high resolution mass measurement. Its ^{13}C NMR spectrum (Table 2) accounted for the thirty carbons in its molecular formula and presented two signals at δ 139.9 (s) and 121.4 (d) in the olefinic carbon region, indicating the presence of a trisubstituted double bond, and two signals at δ 73.7 (s) and 70.6 (d) that were assigned to carbons bearing tertiary and secondary hydroxyl groups, respectively. The ^1H NMR spectrum (Table 1) presented seven signals at δ 0.76 (3H, s), 0.85 (3H, d, J = 6.4 Hz), 0.91 (3H, s), 0.96 (3H, d, J = 6.8 Hz), 0.98 (3H, d, J = 6.8 Hz), 1.10 (3H, br s), and 1.24 (3H, s), which were assigned to seven methyl groups. This spectroscopic evidence suggested a pentacyclic structure, either steroidal or triterpenic, for compound **1.** The upfield region of the ^1H NMR spectrum presented, in addition to the methyl proton signals, two signals at δ −0.11 (1H, dd, J = 5.6 and 4.4 Hz) and 0.45 (1H, dd, J = 9.2 and 4.4 Hz), which in the HMQC spectrum, correlated to a common

methylene signal at δ 21.5 (t), and a multiplet at δ 0.16 (1H, m), which correlated to a signal at δ 32.5 (d). Together with a singlet in the ^{13}C NMR spectrum at δ 26.0, these findings were indicative of the presence of a trisubstituted cyclopropane ring [13,14]. Based on this structural feature and on the seven methyl signals, it was proposed that compound **1** was a dihydroxylated steroid bearing a side chain like that of gorgosterol (**14**).

The analysis of the correlations observed in the COSY spectrum allowed us to confirm the presence in sterol **1** of the usual secondary hydroxyl at C-3 as well as a C-5,C-6 double bond. The tertiary hydroxyl group was located on C-9 as determined by the correlations observed in the HMBC spectrum between the carbon signal at δ 73.7 (s) and the signals at δ 1.76 (1H, m) and 1.24 (3H, s), which were assigned to H-11 and Me-19, respectively. Finally, the ROESY spectrum indicated a relative stereochemistry with respect to both the steroidal nucleus and the side chain, which was similar to that exhibited by gorgosterol (**14**) and therefore, the structure of 9-hydroxygorgosterol was proposed for compound **1**.

Compound **2** was isolated as an amorphous solid with a molecular formula $C_{30}H_{50}O_4$, as indicated by the high resolution mass measurement. Its ^{1}H NMR spectrum was similar to that of 9-hydroxygorgosterol (**1**); thus, the trisubstituted cyclopropane ring signals were present at δ 0.43 (1H, dd, J = 9.2 and 4.4 Hz), 0.19 (1H, m), and −0.11 (1H, dd, J = 5.6 and 4.4 Hz), indicating that compound **2** belonged to the gorgosterol family. Furthermore, the ^{13}C NMR spectrum of **2** exhibited signals at δ 82.6 (s), 77.7 (s), 70.9 (d), and 69.3 (d). Since these latter two signals were correlated in the HMQC spectrum with proton signals at δ 3.92 (1H, m) and 4.52 (1H, m), respectively, it was concluded that compound **2** possessed two secondary and two tertiary hydroxyl groups.

With the aid of the COSY spectrum, the signal at δ 3.92 was assigned to a proton geminal to a hydroxyl group at C-3, and the signal at δ 5.62 (1H, d, J = 4.8 Hz) was determined to be due to the presence of a C-5,C-6 double bond. Because these signals exhibited correlations in the COSY spectrum similar to those shown by compound **1,** this region of the molecule must share identical functionalities and stereochemistry in both compounds **1** and **2**.

The three remaining hydroxyl groups were located at C-9, C-11, and C-14 with the aid of COSY and HMBC (J = 9 Hz) spectra as follows. The signal of a proton geminal to hydroxyl group at δ 4.52 was exclusively coupled in the COSY spectrum with the methylene proton signals at δ 2.48 (1H, t, J = 12.0 Hz) and 2.23 (1H, dd, J = 12.4 and 5.2 Hz). Furthermore, in the HMBC spectrum, the signal at δ 2.48 was correlated with the Me-18 signal at δ 16.9 (q), while the signal at δ 2.23 was correlated with two singlets of quaternary carbons bearing oxygen at δ 82.6 and 77.7. This evidence allowed us to assign the signals at δ 2.48 and 2.23 to the methylene protons at C-12 and defined the presence of a secondary alcohol at C-11 and two tertiary alcohols at C-9 and C-14.

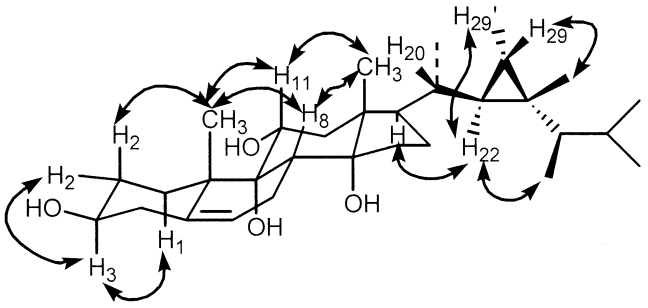


Fig. 1. Selected ROESY correlations observed for compound **2**.

An uncommon feature of the ^{1}H NMR spectra of compounds **1** and **2** recorded in C_5D_5N was the Me-21 signal that appeared in both cases as a singlet (δ 1.10 for **1** and δ 1.11 for **2**) rather than the doublet that should be expected by coupling to H-20. This feature has been previously described [15] in a 9,11-secosterol possessing a gorgosterol-like side chain, and it has been attributed to the fact that Me-21 and H-20 are accidentally isochronous. However, this isochronism can be avoided by recording the spectrum in CD_3OD. In this solvent, the Me-21 signal appeared as a doublet at δ 1.02 (3H, d, J = 6.0 Hz) for **1** and at 1.04 (3H, d, J = 6.4 Hz) for **2**.

On the basis of a gorgosterol-like skeleton, the hydroxyl groups at C-9 and C-14 must be axial. On the other hand, the mutual correlations observed in the ROESY spectrum between the H-11 signal and the Me-18 and Me-19 signals also required an axial orientation for H-11 and therefore, an α orientation for the C-11 hydroxyl group. The ROESY spectrum additionally confirmed the α orientation of H-3 and thus, β stereochemistry of the hydroxyl group at C-3. This rationale defined the structure of 9,11α,14-trihydroxy gorgosterol for compound **2** (Fig. 1).

Compound **3** was obtained as an amorphous powder of molecular formula $C_{28}H_{46}O_3$, as indicated by the high resolution mass measurement. These data, together with a general inspection of the IR and NMR spectra, indicated that **3** was a polyoxygenated sterol. The ^{13}C NMR spectrum of **3** exhibited two signals at δ 156.7 (s) and 106.7 (t), which were due to olefinic carbon atoms. The latter signal at δ 106.7 was correlated in the HMQC spectrum with the signals at δ 4.83 (1H, br d, J = 1.2 Hz) and at δ 4.84 (1H, br s) that were assigned to a disubstituted terminal double bond. This bond must be located in the side chain of the sterol. These data, together with the signals of two methyls at δ 1.04 (3H, d, J = 6.8 Hz) and 1.05 (3H, d, J = 6.8 Hz), correlated in the COSY spectrum with an allylic methine signal at δ 2.27 (1H, sept, J = 6.8 Hz), and the doublet representing another methyl group at δ 0.99 (3H, d, J = 6.4 Hz) indicated that compound **3** possessed an ergostane side chain with a C-24,C-28 double bond. Complete assignments of the protons and of the nine carbon signals of the side

chain were made with the aid of COSY, HMQC, and HMBC spectra.

The remaining nineteen signals of the ^{13}C NMR spectrum were due to the tetracarbocyclic steroidal nucleus. Four of these signals appeared at δ 74.6 (d), 69.1 (d), 68.7 (d), and 66.8 (s), indicating the presence of three methines and a fully substituted carbon bearing oxygens. Since the molecular formula accounted for only three oxygen atoms, it was determined that two of the carbons mentioned must be attached to a common oxygen forming a cyclic ether ring. This ring would account for the sixth unsaturation of the molecule, whereas the other two carbons must be attached to two hydroxyl groups.

The location of these functionalities as well as the full assignments of the ^{1}H and ^{13}C NMR data were determined with the aid of COSY, HMQC, and HMBC spectra and by comparison of the spectroscopic data of compound **3** with those reported [16] for 5β,6β-epoxycholest-24-ene-3β,7β-diol (**15**), allowing us to conclude that both compounds shared identical functionalities and stereochemistry at the carbocyclic steroidal nucleus. The structure 5β,6β-epoxyergost-24(28)-ene-3β,7β-diol was therefore proposed for compound **3**.

Compound **4** was obtained as an amorphous powder. The molecular formula, $C_{28}H_{48}O_4$, obtained from the high resolution mass measurement, required five degrees of unsaturation. Like that of compound **3**, the ^{13}C NMR spectrum of **4** showed two olefinic carbon signals at δ 156.8 (s) and 106.7 (t). The triplet was correlated in the HMQC experiment with the ^{1}H NMR singlets at δ 4.84 (1H, br s) and 4.83 (1H, br s) that were assigned to a terminal disubstituted double bond in an ergostane side chain whose remaining ^{1}H and ^{13}C NMR signals were assigned with the aid of COSY, HMQC, and HMBC spectra. The ^{13}C NMR spectrum also presented four signals at δ 76.8 (s), 75.9 (d), 73.0 (d), and 67.1 (d), which were consistent with the presence of four hydroxyl groups, one of them tertiary, in the steroidal tetracarbocyclic system.

The ^{1}H NMR signal of compound **4** at δ 4.84 (1H, m) was assigned to the characteristic H-3 proton of a 3-hydroxysterol, since it was correlated in the COSY spectrum with the signals at δ 2.28 (1H, m) and 2.06 (1H, m) and with those at δ 2.93 (1H, dd, J = 12.4 and 11.6 Hz) and 2.34 (1H, m) which were assigned to the C-2 and C-4 methylene protons, respectively. These latter two signals did not exhibit any additional correlation, and therefore, the tertiary hydroxyl was located at C-5. The two remaining signals geminal to the hydroxyl groups at δ 4.42 (1H, br d, J = 3.6 Hz) and 4.13 (1H, d, J = 3.6 Hz) were mutually coupled, and since the signal at δ 4.42 was correlated in the COSY spectrum with a methine proton at δ 2.03 (1H, m), the presence of a –[CH(OH)CH(OH)CH]- subunit was defined. This subunit was located at C-6, C-7, and C-8 carbons with the aid of the HMBC correlations. In particular, the correlation observed between the signal at δ 4.13 and the C-5 singlet at δ 76.8 allowed for the identification of the proton signal as that of H-6 (Fig. 2).

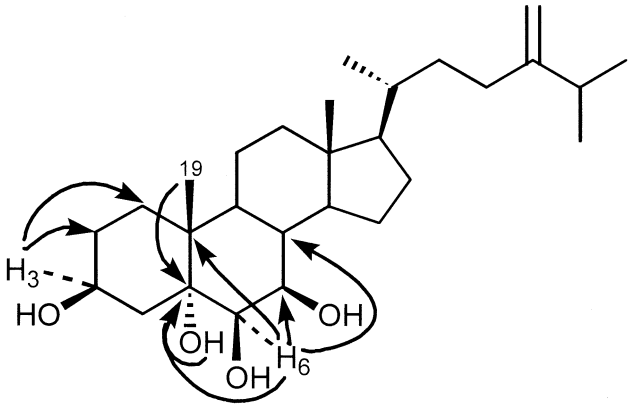


Fig. 2. Selected HMBC correlations observed for compound **4**.

A series of nuclear Overhauser effect difference spectroscopy experiments were performed to define the relative stereochemistry in compound **4**. Irradiation of the H-6 signal at δ 4.13 produced enhancements of the H-7 and H-4_{eq} signals at δ 4.42 and 2.34, respectively, and of the hydroxyl proton singlets at δ 6.24, 5.96, and 5.56. These results were consistent with an equatorial orientation of H-6. Furthermore, irradiation of the Me-19 signal at δ 1.60 (3H, s) caused, among other enhancements, those of the H-4_{ax} and H-1_{eq} signals at δ 2.93 and 2.16 (1H, m), respectively, whereas irradiation of the Me-18 signal at δ 0.78 (3H, s) enhanced the H-8 signal at δ 2.03. The stereochemistry at C-7 could not be inferred from these nOe experiments; however, the coupling constants of the H-7 signals in the ^{1}H NMR spectrum recorded in CD_3OD [δ 3.69 (1H, dd, J = 10.0 and 4.0 Hz)] suggested an axial orientation of H-7. All of these results, together with the correlations observed in a ROESY spectrum, indicated a β orientation of the C-3, C-6, and C-7 hydroxyl groups, Me-18 and Me-19, and H-8. The results also indicated that the hydroxyl group at C-5 was oriented in the α face of the molecule. It was concluded that the structure of compound **4** was ergost-24(28)-ene-3β,5α,6β,7β-tetrol.

Compounds **5** and **6** were obtained as an unseparable mixture in a 1:1 ratio. The high resolution mass spectrum was consistent with a molecular formula $C_{30}H_{48}O_5$, which accounted for seven unsaturations. The IR spectrum exhibited absorptions at 3394, 1741, and 1709 cm^{-1}, indicating the presence of hydroxyl and carbonyl groups. A general inspection of the NMR spectra of the mixture **5**/**6** suggested that the compounds were steroids. The ^{13}C NMR spectrum exhibited thirty signals, nine of them doubled. Since the ^{13}C NMR signals at δ 170.8 (s) and 20.9 (q), together with the ^{1}H NMR signal at δ 2.03 (3H, s), indicated the presence of an acetoxyl group in the molecule, it was determined that compounds **5**/**6** possessed skeletons of twenty-eight carbon atoms.

In the ^{13}C NMR spectrum, the signals attributable to the

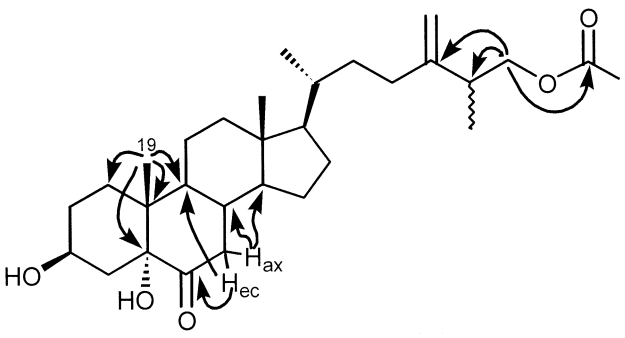


Fig. 3. Selected HMBC correlations observed for the mixture of compounds **5**/**6**.

side chain were doubled indicating that the structural difference between isomers **5** and **6** had to be located on this chain. In the COSY spectrum, the signals of two exomethylene protons at δ 4.94 (1H, d, J = 1.2 Hz) and 4.91 (1H, s) were coupled to the methylene proton signals at δ 2.18 (1H, m) and 1.97 (1H, m). However, the signal at δ 4.91 showed allylic coupling with a methine proton signal at δ 2.55 (1H, m), which was additionally coupled to the doubled signals of a methylene at δ 4.07 (1H, ddd, J = 10.8, 7.2, and 0.8 Hz)/4.09 (1H, ddd, J = 10.8, 7.2, and 0.8 Hz) and at δ 4.27 (1H, ddd, J = 10.8, 6.4, and 1.6 Hz)/4.28 (1H, ddd, J = 10.8, 6.4, and 1.2 Hz). These signals were attributable to the protons geminal to the acetoxyl group. This assignment was confirmed by the HMBC spectrum, which exhibited cross peaks between these signals and the carbonyl singlet at δ 170.8 that additionally was correlated with the methyl proton signal at δ 2.03. Furthermore, the allylic methine signal at δ 2.55 was correlated in the COSY spectrum with a methyl doubled signal at δ 1.10 (3H, dd, J = 7.2 and 1.2 Hz)/1.11 (3H, dd, J = 7.2, and 1.2 Hz). This spectroscopic evidence indicated that isomers **5** and **6** of the mixture each possessed a 24,28-unsaturated-26-acetoxyergostane side chain, but with opposite stereochemistry at C-25.

The nature and location of the three remaining oxygen functionalities on the tetracarbocyclic system were established as follows: The ^{13}C NMR signals at δ 213.6 (s) was assigned to a ketone on a six membered ring, whereas two signals at δ 80.3 (s) and 66.8 (d) indicated the presence of tertiary and secondary hydroxyl groups. The doublet at δ 66.8, which was correlated in the HMQC experiment with the signal at δ 4.68 (1H, m), was assigned to C-3. In the COSY spectrum, this latter signal exhibited correlations with the methylene signals at δ 2.61 (1H, dd, J = 13.8 and 4.6 Hz) and 2.37 (1H, dd, J = 12.8 and 12.0 Hz), which did not gave rise to any additional cross peak in the COSY spectrum, indicating the presence of a tertiary hydroxyl group at C-5. Finally, the ^{1}H NMR signals at δ 3.12 (1H, dd, J = 12.8 and 12.4 Hz) and 2.24 (1H, dd, J = 12.8 and 4.4 Hz) were assigned to the protons of a methylene α to the ketone carbonyl group. Furthermore, with the aid of COSY and HMQC spectra, the signals at δ_C 37.7/δ_H 1.74 and at δ_C 44.8/δ_H 2.32 were unequivocally assigned to two methines β and γ to the carbonyl, respectively. The HMBC cross peak between the Me-19 singlet at δ 0.94 and the γ-carbon signal at δ 44.8 allowed us to identify this carbon as C-9 and then to define the location of the carbonyl group at C-6 (Fig. 3).

The relative stereochemistry of **5**/**6** was established by using the ROESY spectrum and implied an all-*trans* fusion of the tetracarbocyclic system. The β orientation of the hydroxyl at C-3 was confirmed by the correlations observed between the H-3 signal and the H-1$_{ax}$, H-2$_{eq}$, and H-4$_{eq}$ protons. The axial orientation of the hydroxyl at C-5 was confirmed by the correlations observed between the hydroxyl proton signal at δ 7.28 and the H-4$_{eq}$ and H-7$_{ax}$ signals. It was proposed that the structures of the epimer constituents of the mixture were (25*R*) and (25*S*)-26-acetoxy-3β,5α-dihydroxyergostan-24(28)-en-6-one (**5** and **6**).

Both the new and the known steroids isolated from *P. grisea* (**1**–**13**) were tested in bioassays to detect in vitro cytotoxicity against the following cancer cell lines: P 388 suspension cultures of mouse lymphoid neoplasm, monolayer cultures of human lung carcinoma (A 549), and monolayer cultures of human colon carcinoma (HT 29). ED$_{50}$ values over 1 μg/ml in these tests were not reported. Cholest-3β,5α,6β,7β-tetrol (**9**) and cholest-3β,5α,6β-triol (**11**) were active against the A 549 and HT 29 cell lines with ED$_{50}$ values of 1 μg/ml. The new compounds 5β,6β-epoxyergost-24(28)-ene-3β,7β-diol (**3**), ergost-24(28)-ene-3β,5α,6β,7β-tetrol (**4**), and the mixture of C-25 epimers **5**/**6**, together with the known compound ergost-24(28)-ene-3β,5α,6β-triol (**12**), exhibited strong and selective cytotoxicity against the HT-29 cell line with an ED$_{50}$ = 0.1 μg/ml for compounds **3**, **5**/**6**, and **12** and an ED$_{50}$ = 0.25 μg/ml for compound **4**.

Acknowledgments

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References

[1] Baker BJ, Kerr RG. Biosynthesis of marine sterols. Top Curr Chem 1993;167:1–31.
[2] D'Auria MV, Minale L, Riccio R. Polyoxygenated steroids of marine origin. Chem Rev 1993;93:1839–95.
[3] Kerr RG, Baker BJ. Marine sterols. Nat Prod Rep 1991;8:465–97.
[4] Gopichand Y, Schmitz FJ, Schmidt PG. Marine natural products: two new acyclic sesquiterpene hydrocarbons from the gorgonian *Plexaurella grisea*. J Org Chem 1980;45:2523–6.
[5] Rueda A, Zubía E, Ortega MJ, Salvá J. New acyclic sesquiterpenes and norsesquiterpene from the Caribbean gorgonian *Plexaurella grisea*. J Nat Prod (in press).
[6] Pruna LB, Henriques RD, Huneck S, Schreiber K, Preiss A. Chemical studies of Cuban gorgonians. Part 10: Di- and trihydroxysteroids from *Plexaurella grisea*. Pharmazie 1984;39:117–20.

[7] Kupchan SM, Britton RW, Ziegler MF, Sigel CW. Bruceantin, a new potent antileukemic simaroubolide from *Brucea antidysenterica*. J Org Chem 1973;38:178–9.
[8] Anjaneyulu ASR, Krishna Murthy MVR, Gowry PM. Novel epoxy steroids from the Indian ocean soft coral *Sarcophyton crassocaule*. J Nat Prod 2000;63:112–8.
[9] Kawata M, Tohma M, Sawaya T, Kimura M. A new product of cholesterol by metal free autoxidation in aqueous dispersion. Chem Pharm Bull 1976;24:3109–13.
[10] Sjöstrand U, Bohlin L, Fisher L, Colin M, Djerassi C. Minor and trace sterols from marine invertebrates 28. A novel polyhydroxylated sterol from the soft coral *Anthelia glauca*. Steroids 1981;38: 347–54.
[11] Bortolotto M, Braekman JC, Daloze D, Tursch B. Chemical studies of marine invertebrates XVIII. Four novel polyhydroxylated steroids from *Sinularia dissecta*. Bull Soc Chim Belg 1976;85:27–34.
[12] Datta PK, Ray AK, Barua AK, Chowdhuri SK, Patra A. Isolation of a bioactive sterol from a sea pen, *Pteroeides esperi*. J Nat Prod 1990;53:1347–8.
[13] Hale RL, Leclercq J, Tursch B, Djerassi C, Gross Jr. RA, Weinheimer AJ, Gupta K, Scheuer PJ. Demonstration of a biogenetically unprecedented side chain in the marine sterol, gorgosterol. J Am Chem Soc 1970;92:2179–80.
[14] Ling NC, Hale RL, Djerassi C. The structure and absolute configuration of the marine sterol gorgosterol. J Am Chem Soc 1970;92: 5281–2.
[15] Morris LA, Christie EM, Jaspars M, van Ofwegen LP. A bioactive secosterol with an unusual A- and B-ring oxygenation pattern isolated from an Indonesian soft coral *Lobophytum* sp. J Nat Prod 1998;61: 538–41.
[16] Koljak R, Lopp A, Pehk T, Varvas K, Müürisepp AM, Järvin I, Samel N. New cytotoxic sterols from the soft coral *Gersemia fruticosa*. Tetrahedron 1998;54:179–86.