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1α,3β,5β-Trihydroxy-24-methylenecholestan-6-one: a novel steroid from a soft coral *Sinularia gibberosa*

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Abstract

A novel steroid, 1α,3β,5β-trihydroxy-24-methylenecholestan-6-one (gibberoketosterol) (**1**), along with four known steroids, was isolated from the lipophilic extracts of a Taiwanese soft coral *Sinularia gibberosa*. The structure of the new metabolite was determined on the basis of extensive spectral analyses and chemical reaction. The relative stereochemistry of gibberoketosterol was established by the NOESY experiments and analysis of the pyridine-induced deshielding effect of the axial hydroxy groups. Gibberoketosterol is the first example of 1α,3β,5β-trihydroxy-6-oxosteroids isolated from natural sources and was found to exhibit a moderate cytotoxicity against the growth of Hepa59T/VGH cancer cells.

Keywords: *Sinularia gibberosa*; 1α,3β,5β-Trihydroxy-24-methylenecholestan-6-one; Gibberoketosterol; Soft coral; Cytotoxicity

1. Introduction

Marine organisms, including soft corals, have been well-recognized as a natural source of 3β-hydroxy sterols and their oxygenated analogues [1]. Previous chemical investigations on the steroidal contents of the soft coral species belonging to genus *Sinularia*, have led to the isolation and identification of varieties of polyoxygenated steroids [2–8]. Some of these compounds have been shown to exhibit cytotoxic activity against the growth of the various cancer cell lines [6]. In our current chemical investigation on *Sinularia gibberosa*, we have succeeded in isolating a new oxygenated steroid along with four known steroids (Fig. 1) from the organic extracts. This paper deals with the isolation and structure elucidation of the new metabolite. The new steroid, 1α,3β,5β-trihydroxy-24-methylenecholestan-6-one (gibberoketosterol) (**1**), is the first example of natural 1α,3β,5β-trihydroxy-6-oxosteroids. The structure of **1** was deduced by a series of 2D-NMR experiments (^{1}H-^{1}H COSY, HMQC, HMBC, and NOESY) and by careful analysis of the pyridine-induced deshielding shifts effect exerted by axial hydroxy groups of this metabolite. Cytotoxicity of metabolites **1**, **4**, and **5** against Hepa59T/VGH (human liver carcinoma) cancer cells also is reported.

2. Experimental

2.1. General methods

Melting points were determined using a Fisher–Johns melting point apparatus. Optical rotations were measured on a Jasco DIP-1000 digital polarimeter. IR spectra were recorded on a Jasco FT-5300 infrared spectrophotometer. EIMS was obtained with a VG Quattro GC/MS spectrometer. HRMS spectra were recorded on a Finnigan MAT-95XL mass spectrometer. The NMR spectra were recorded on a Bruker AVANCE DPX300 FT-NMR at 300 MHz for ^{1}H and 75 MHz for ^{13}C or on a Varian Unity INOVA 500 FT-NMR at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively, in $CDCl_3$ using TMS as internal standard, unless otherwise indicated. Si gel (Merck; 230–400 mesh) was used for column chromatography. Precoated Si gel plates (Merck, Kieselgel 60 F-254; 0.2 mm) were used for analytical TLC.

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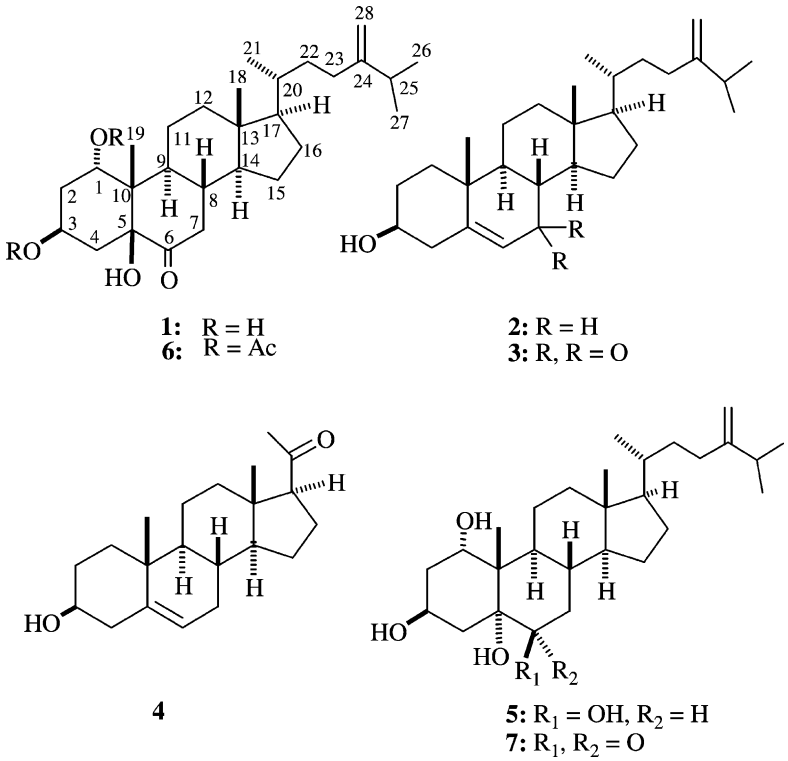

Fig. 1. Steroids isolated from *Sinularia gibberosa*.

2.2. Organism

The soft coral *S. gibberosa* Tixier-Durivault (0.6 kg, wet weight) was collected by hand via SCUBA along the coast of Kenting, Taiwan, in December 2000, at a depth of 10–15 m, and stored in a freezer until extraction. A voucher sample was deposited at the Department of Marine Resources, Sun Yat-Sen University (specimen no. NHSC 002).

2.3. Extraction and isolation

The organism was exhaustively extracted with EtOH. The EtOH extract was filtered and concentrated under reduced pressure and the aqueous residue was partitioned with *n*-hexane, followed by dichloromethane to provide *n*-hexane fraction (4 g) and dichloromethane fraction (11 g). The *n*-hexane fraction was chromatographed on Si gel 60 using acetone–*n*-hexane (stepwise, 0–100% acetone) to yield 20 fractions. Fraction 6 eluted with 5% acetone was further purified by normal phase MPLC using *n*-hexane–dichloromethane gradient (3:2 to 0:1) to yield **3** (10.0 mg). Fraction 14 eluted with 20% acetone was further purified by normal phase HPLC using dichloromethane–MeOH (98:2) to yield **2** (3.0 mg). The dichloromethane fraction was fractionated by normal phase MPLC and elution was performed with *n*-hexane–EtOAc (stepwise, 0–100% EtOAc) to yields 36 fractions. Fraction 26 eluted with 25% EtOAc was further purified by normal phase HPLC using *n*-hexane–EtOAc (2:1) to afford **4** (34 mg). Fraction 30 eluted with 40% EtOAc was further chromatographed by normal phase HPLC using dichloromethane–MeOH (99:1 to 95:5, gradient) to afford **1** (18 mg). Fraction 36 eluted with EtOAc yielded **5** (17 mg) after being purified by recrystallization from EtOAc.

2.3.1. Gibberoketosterol (*1*)

White solid: mp, 140–141 °C (EtOAc); $[\alpha]_D^{25}$, −0.7° (*c* 0.38, $CHCl_3$). IR (neat) ν_{max} (cm^{-1}): 3570 (br), 3015, 2936, 2872, 1707, 1219. ^1H NMR (500 MHz, $CDCl_3$) and ^{13}C NMR (125 MHz, $CDCl_3$), see Table 1. ^1H NMR (300 MHz, pyridine-d_5) δ_H: 4.89 (1H, dd, J = 14.0, 4.0 Hz, H-1), 4.87 (1H, s, H-28), 4.85 (1H, s, H-28), 4.48 (1H, brs, H-3), 1.39 (3H, s, 19-Me), 1.06 (6H, d, J = 6.6 Hz, 26-Me, 27-Me), 0.96 (3H, d, J = 6.6 Hz, 21-Me), 0.66 (3H, s, 18-Me). ^{13}C NMR (75 MHz, pyridine-d_5) δ_C: 211.5 (s, C-6), 156.7 (s, C-24), 106.7 (t, C-28), 85.1 (s, C-5), 69.8 (d, C-1), 68.3 (d, C-3), 56.7 (d, C-14), 56.3 (d, C-17), 49.8 (s, C-10), 43.7 (d, C-9), 42.7 (t, C-7), 42.3 (s, C-13), 40.2 (t, C-12), 39.2 (t, C-2), 38.2 (t, C-4), 37.6 (d, C-8), 36.0 (d, C-20), 35.0 (t, C-22), 34.1 (d, C-25), 31.4 (t, C-23), 28.1 (t, C-16), 24.4 (t, C-15), 24.2 (t, C-11), 22.2 (q, C-27), 22.0 (q, C-26), 18.8 (q, C-21), 14.6 (q, C-19), 12.2 (q, C-18). ^1H NMR (300 MHz, DMSO-d_6) δ_H: 5.02 (1H, s, OH), 4.69 (1H, s, H-28), 4.62 (1H, s, H-28), 4.56 (1H, d, J = 5.0 Hz, OH), 4.43 (1H, d, J = 6.8 Hz, OH), 3.98 (2H, m, H-1, H-3), 3.98 (1H,

Table 1
^{1}H and ^{13}C NMR data for compound **1**

C/H	δ_H (ppm)[a]	^{13}C[b]
1	4.25 dd (13.0, 4.0)[c]	70.7 (d)[d]
2α	1.96 td (13.5, 3.0)	37.5 (t)
2β	2.09 m	
3	4.20 brs	67.7 (d)
4α	2.34 dd (14.5, 3.5)	37.3 (t)
4β	1.62 brd (14.5)	
5		83.7 (s)
6		211.5 (s)
7α	2.24 t (14.5)	41.2 (d)
7β	2.43 dd (14.5, 5)	
8	1.79 dd (12.5, 5.0)	37.4 (d)
9	1.99 dd (11.0, 5.0)	43.3 (d)
10		49.1 (s)
11α	2.20 m	23.6 (t)
11β	1.47 brdd (12.5, 3.0)	
12α	1.21 dd (14.5, 3.5)	39.7 (t)
12β	2.02 dt (13.0, 4.0)	
13		42.4 (s)
14	1.25 m	56.8 (d)
15α,β	1.54 m	24.2 (t)
16α	1.30 td (12.0, 5.0)	27.7 (t)
16β	1.86 dd (11.0, 5.0)	
17	1.16 t (9.5)	56.0 (d)
18	0.66 s	11.9 (q)
19	0.97 s	13.4 (q)
20	1.42 m	35.6 (d)
21	0.94 d (7.0)	18.5 (q)
22α	1.54 m	34.5 (t)
22β	1.14 m	
23α	2.10 brd (11.0)	30.9 (t)
23β	1.89 m	
24		156.7 (s)
25	2.21 m	33.8 (d)
26	1.02 d (7.0)	21.8 (d)
27	1.03 d (7.0)	22.0 (q)
28	4.72 s, 4.65 s	106.0 (t)
5-OH	4.50 s	

[a] Spectra recorded at 500 MHz in $CDCl_3$ at 25 °C.
[b] Spectra recorded at 125 MHz in $CDCl_3$ at 25 °C.
[c] *J* values (in Hz) in parentheses.
[d] Multiplicity deduced by DEPT and indicated by usual symbols. The values are in ppm downfield from TMS.

m, H-1), 0.97 (3H, d, J = 6.6 Hz, 27-Me), 0.96 (3H, d, J = 6.6 Hz, 26-Me), 0.90 (3H, d, J = 6.4 Hz, 21-Me), 0.81 (3H, s, 19-Me), 0.59 (3H, s,18-Me). ^{13}C NMR (75 MHz, DMSO-d_6) δ_C: 210.8 (s, C-6), 156.0 (s, C-24), 106.7 (t, C-28), 83.6 (s, C-5), 72.5 (d, C-1), 66.7 (d, C-3), 55.9 (d, C-17), 55.8 (d, C-14), 48.4 (s, C-10), 42.4 (s, C-13), 42.1 (d, C-9), 41.7 (t, C-7), 39.4 (t, C-12), 37.7 (t, C-2), 37.1 (d, C-8), 36.6 (t, C-4), 35.2 (d, C-20), 34.3 (t, C-22), 33.2 (d, C-25), 30.6 (t, C-23), 27.6 (t, C-16), 24.0 (t, C-15), 23.3 (t, C-11), 22.0 (q, C-27), 21.8 (q, C-26), 18.6 (q, C-21), 13.9 (q, C-19), 11.9 (q, C-18). HREIMS (*m/z*): 446.3385 $[M]^+$. $C_{28}H_{46}O_4$ requires 446.3398. EIMS (70 eV) (*m/z*): 447 [0.2, $(M+H)^+$], 446 [0.2, $(M)^+$], 428 [0.2, $(M–H_2O)^+$], 410 [0.2, $(M–2H_2O)^+$], 400 [0.4], 382 [1.0], 347 [2.3], 316 [3.5], 298 [3.6], 69 [100].

2.3.2. Acetylation of *1*

A solution of gibberoketosterol (**1**) (13 mg, 0.032 mM) in pyridine (0.25 ml) was added with Ac_2O (0.25 ml) and the mixture was stirred at RT for 36 h. After evaporation of excess reagent, the residue was separated by column chromatography on silica gel to give 1,3-diacetyl derivative **6** (CH_2Cl_2–MeOH = 98:2, 11 mg, 0.022 mmol, 69%), as fine needles.

2.3.3. 1,3-Diacetate (*6*)

The mp: 71–72 °C; $[\alpha]_D^{27}$ −33.5° (*c* 0.5, $CHCl_3$). IR (neat) ν_{max} (cm^{-1}): 3479 (br), 3030, 2926, 2874, 1732, 1710, 1215. ^{1}H NMR (300 MHz, $CDCl_3$) δ_H: 5.51 (1H, dd, J = 9.3, 6.6 Hz, H-1), 5.20 (1H, brs, H-3), 4.72 (1H, s, H-28), 4.66 (1H, s, H-28), 2.45 (1H, dd, J = 14.8, 4.5 Hz, H-7b), 2.26 (1H, m, H-7α), 2.24 (2H, m, H-4α, H-25), 2.09 (3H, s, 3-OAc), 2.07 (3H, m, H-11α, H-12β, H-23α), 2.06 (1H, m, H-2β), 2.04 (3H, s, 1-OAc), 2.00 (1H, m, H-2α), 1.99 (1H, m, H-23β), 1.91 (1H, dd, J = 11.1, 3.7 Hz, H-9), 1.85 (1H, m, H-16β), 1.78 (2H, brd, J = 14.8 Hz, H-4β, H-8), 1.60 (2H, m, H_2-15), 1.55 (1H, m, H-22α), 1.52 (1H, m, H-11β), 1.39 (1H, m, H-20), 1.29 (1H, m, H-16α), 1.26 (1H, m, H-14), 1.22 (1H, m, H-12α), 1.15 (1H, dd, J = 11.1, 3.4 Hz, H-17), 1.15 (1H, m, H-22β), 1.03 (6H, d, J = 6.3 Hz, 26-Me, 27-Me), 0.96 (3H, s, 21-Me), 0.83 (3H, s, 19-Me), 0.67 (3H, s, 18-Me). ^{13}C NMR (75 MHz, $CDCl_3$) δ_C: 211.5 (s, C-6), 171 (s, CO of 3-acetate), 170.1 (s, CO of 1-acetate), 156.7 (s, C-24), 106.1 (t, C-28), 81.8 (s, C-5), 72.6 (d, C-1), 68.8 (d, C-3), 56.9 (d, C-14), 56.1 (d, C-17), 48.7 (s, C-10), 43.5 (d, C-9), 42.4 (s, C-13), 41.4 (t, C-7), 39.7 (t, C-12), 37.2 (d, C-8), 35.7 (d, C-20), 35.0 (t, C-4), 34.6 (t, C-22), 33.9 (d, C-25), 31.0 (t, C-23), 31.0 (t, C-2), 27.8 (t, C-16), 24.3 (t, C-15), 23.1 (t, C-11), 22.0 (q, C-27), 21.9 (q, C-26), 21.5 (q, CH_3 of 1-acetate), 21.4 (q, CH_3 of 3-acetate), 18.6 (q, C-21), 12.9 (q, C-19), 12.0 (q, C-18).

2.4. Cytotoxicity assay

Hepa59T/VGH cells were purchased from the American Type Culture Collection (ATCC). Cytotoxicity assay of the test compounds **1**, **4**, and **5** was performed using 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric method [9,10].

3. Results and discussion

Gibberoketosterol (**1**) was isolated as translucent thin flakes, mp 140–141 °C. Its HREIMS exhibited a molecular ion peak at *m/z* 446.3385, corresponding to the molecular formula $C_{28}H_{46}O_4$ and six units of unsaturation. The IR spectrum showed absorption bands of hydroxy (3570 cm^{-1}, broad) and carbonyl (1707 cm^{-1}) groups. EIMS of **1** exhibited peaks at *m/z* 428 $[M–H_2O]^+$ and 410 $[M–2H_2O]^+$, suggesting the presence of at least two hydroxy groups in **1**. The ^{13}C NMR spectrum displayed 28 signals, which were

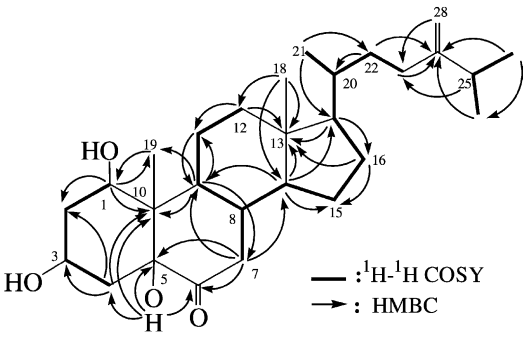

Fig. 2. ^{1}H-^{1}H COSY and key HMBC correlations for **1**.

identified by the assistance of a DEPT spectrum as 5 methyl, 10 methylene, 8 methine, and 5 quaternary carbons (Table 1). Above data suggested that **1** is a hydroxylated steroid. The presence of a ketone and one 1,1-disubstituted double bond could be deduced from signals appearing at δ 211.5 (s), 106.0 (t), and 156.7 (s). Three signals appearing at δ 67.7 (d), 70.7 (d), and 83.7 (s) were due to carbons bonded to an oxygen. Comparison of the ^{13}C NMR spectral data of **1** (in DMSO-d_6,) with those of the known 1,3,5-trihydroxylated sterone (**7**) suggested that **1** could be an isomer of **7** [5]. In the ^{1}H NMR spectrum of **1** (in $CDCl_3$; see Table 1), the signals appearing at δ 4.25 (dd, J = 13.0, 4.0 Hz) and 4.20 (1H, brs) were assigned to H-1 and H-3, respectively, by the assistance of ^{1}H-^{1}H COSY and HMBC spectra (Fig. 2). An additional signal at δ 4.50 (1H, s) was deduced to be the resonating peak of a tertiary hydroxy group attached to a quaternary carbon (δ 83.7). A 1,1-disubstituted double bond was further confirmed by ^{1}H NMR spectrum which showed signals at δ 4.72 (1H, s) and 4.65 (1H, s). ^{1}H-^{1}H COSY and HMBC spectral data further established three partial structures of sequential proton sets and long-range ^{1}H/^{13}C correlations, respectively (Fig. 2). On the basis of above observations, the planar structure of **1** was established as 1,3,5-trihydroxy-24-methylenecholestan-6-one.

The relative stereochemistry of **1** was determined on the basis of the NOESY experiment for **1** (Fig. 3), and the observed pyridine-induced solvent shifts [11]. In the NOESY spectrum of **1**, the axially oriented H-1 (δ 4.25, dd, J = 13.0, 4.0 Hz) exhibited a significant NOE with H_3-19, suggesting the α-orientation of C-1 hydroxy group. The β-configuration of C-3 hydroxy group was deduced owing to the lack of NOE correlations between H-3 and H-2β, which showed NOE interaction with H-1. Careful investigation on the NOESY spectrum of the diacetate **6**, obtained from the acetylation of **1**, revealed significant NOE correlations between H-3 and both of H-2α and H-4α, and between H-4α and H-9, and further confirmed the above results. Furthermore, the chemical shifts of carbons 2–5, 7, and 9–10 of **1** showed significant differences in comparison with the corresponding chemical shifts of 1α,3β,5α-trihydroxy-24-methylenecholestan-6-one (**7**) [5] (Table 2). Thus, **1** should be the 5-epimer of **7**. On the basis of the above results, the configuration of **1** could be established as shown in Fig. 2, reflecting the α-equatorial orientation of 1-OH, and the β-axial orientations of 3-OH and 5-OH. Moreover, the large pyridine-induced downfield shifts ($\Delta\delta = \delta CDCl_3 - \delta C_6D_5N$; see Table 3) experienced upon H-1 ($\Delta\delta = -0.64$ ppm) could be achieved only when both 3-OH and 5-OH are axially oriented on the same face of H-1 [11]. Finally, the α-downward orientation of ring A resulted from the β-orientation of 5-OH was further interpreted by the NOE interactions, observed between H-4α (δ 2.34, dd, J = 14.5, 3.5 Hz) and both H-9 (δ 1.99, dd, J = 11.0, 5.0 Hz) and H-7α (δ 2.24, t, J = 14.5 Hz). Therefore, the

Table 2
^{13}C NMR data for ring A and B carbons of **1** and **7**

C	**1**[a]	**7**[b]
1	72.5 (d)[c]	73.2 (d)
2	37.7 (t)	41.3 (t)
3	66.7 (d)	61.6 (d)
4	36.6 (t)	39.0 (t)
5	83.6 (s)	81.6 (s)
6	210.8 (s)	209.9 (s)
7	41.7 (t)	36.0 (t)
8	37.1 (d)	36.4 (d)
9	42.1 (d)	39.3 (d)
10	48.4 (s)	44.3 (s)

[a] Spectra recorded at 75 MHz in DMSO-d_6 at 25 °C.
[b] Spectra recorded at 100 MHz in DMSO-d_6 at 25 °C (see ref. [5]).
[c] Multiplicity deduced by DEPT and indicated by usual symbols. The values are in ppm downfield from TMS.

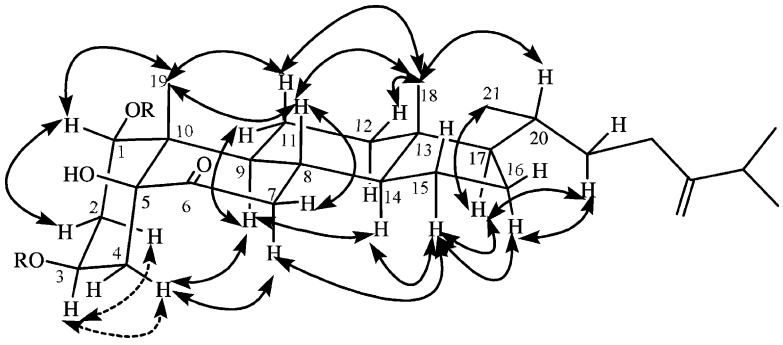

Fig. 3. Observed NOESY correlations for **1** (R = H; solid arrows) and **6** (R = Ac; dashed and solid arrows).

Table 3
Selected ^{1}H NMR chemical shifts of compounds **1** and **7** in $CDCl_3$ and pyridine-d_5

H	δ_H in $CDCl_3$		δ_H in C_5D_5N	
	1[a]	**7**[b]	**1**[c]	**7**[b]
H-1	4.25 dd (13.0, 4.0)[d]	4.00 brs	4.89 dd (14.0, 4.0)	4.19 brs
H-3	4.25 brs	4.33 m	4.48 brs	5.4–5.6 m
18-Me	0.66 3H, s	0.69 3H, s	0.66 3H, s	0.63 3H, s
19-Me	0.97 3H, s	0.74 3H, s	1.39 3H, s	0.88 3H, s

[a] Spectra recorded at 500 MHz at 25 °C.
[b] Spectra recorded at 400 MHz at 25 °C (see ref. [5]).
[c] Spectra recorded at 300 MHz at 25 °C.
[d] *J* values (in Hz) in parentheses.

structure of gibberoketosterol (**1**) could be established unambiguously as 1α,3β,5β-trihydroxy-24-methylenecholestan-6-one.

The known steroids **2**, **3**, **4**, and **5** were also isolated from the lipophilic fractions of *S. gibberosa* and identified by comparison of their physical characters (mp and $[\alpha]_D$) and spectral data with those of 3β-hydroxy-24-methylenecholest-5-en-7-one [12,13], 24-methylenecholesterol [14], pregnenolone [6], and numersterol [4], respectively.

Although the ketosteroid **2** was previously isolated from two sponge species [12,13] and a higher plant [15], it is worthwhile to mention that this is the first time of isolating this metabolite from a coral. In addition, steroids containing 1α,3β,5β-trihydroxy-6-oxo functionality have not been found before. Thus, gibberoketosterol (**1**) represents the first example of the natural 1α,3β,5β-trihydroxy-6-oxosteroids.

The cytotoxicity of metabolites **1**, **4**, and **5** against the growth of Hepa59T/VGH cancer cells was evaluated and the results showed that compounds **1**, **4**, and **5** possess moderate cytotoxicity against this cell line with ED_{50}'s 10.0, 9.3, and 6.8 μg/ml, respectively.

Acknowledgments

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