Novel Cytotoxic Oxygenated C29 Sterols from the Colombian Marine Sponge *Polymastia tenax*

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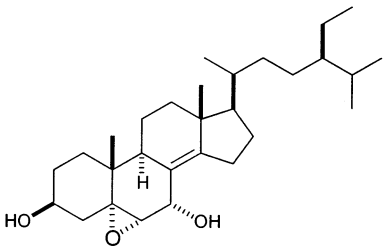
Three new sterols, 5α,6α-epoxy-24*R**-ethylcholest-8(14)-en-3*β*,7α-diol (**1**), 5α,6α-epoxy-24*R**-ethylcholest-8-en-3*β*,7α-diol (**2**), and 3*β*-hydroxy-24*R**-ethylcholesta-5,8-dien-7-one (**3**), have been isolated from the marine sponge *Polymastia tenax*, collected in the Colombian Caribbean, and their structures established on the basis of extensive NMR and MS studies. Compounds **1** and **2** showed antiproliferative activity toward A-549, HT-29, H-116, MS-1, and PC-3 tumor cells in the range 0.5−10 *μ*g/mL.

Steroids isolated from marine sponges are unique in having unusual functionalization and structures.[1,2] Specimens belonging to the *Polymastia* genus (order Hadromerida, family Polymastiidae) have proved to be a rich source of novel steroids. For example, the Norwegian sponge *P. boteliformis* yielded the first examples of naturally occurring steroids with a side chain containing an amide linkage between a carboxyl group on the steroid and the amino functionality of an α-amino acid.[3] More recently, the new sterols 3*β*-hydroxystigmast-5-en-7-one and 24-methylenecholestan-3*β*,6*β*,9α,19-tetrol were isolated from the sponge *P. sobustia* collected from the South China Sea.[4]

With these precedents in mind and in continuation of our search for new bioactive steroids from marine organisms,[5] we focused our attention on the Colombian sponge *Polymastia tenax* (Pulitzer-Finali, 1986)[6] because of the cytotoxicity found in its methanolic extracts. In the work reported here, we isolated and characterized three new steroids having the same side chain but a different steroid nucleus, 5α,6α-epoxy-24*R**-ethylcholest-8(14)-en-3*β*,7α-diol (**1**), 5α,6α-epoxy-24*R**-ethylcholest-8-en-3*β*,7α-diol (**2**), and 3*β*-hydroxy-24*R**-ethylcholesta-5,8-dien-7-one (**3**), from the sterol-containing lipid fractions of the sponge *P. tenax*.



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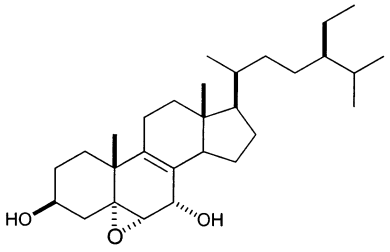

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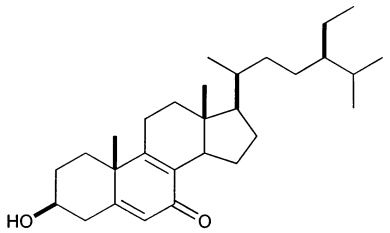

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Results and Discussion

Sponge specimens were collected from Punta de Betín, Bahía de Santa Marta, in the Colombian Caribbean (Colombia) and immersed in MeOH. The methanol extracts were partitioned between CH_2Cl_2/H_2O to yield a brown organic residue, which was subsequently partitioned between $MeOH/H_2O$ and solvents of increasing polarity according to our standard method.[7] The hexanes and CH_2Cl_2 extracts were submitted to repeated silica gel flash column chromatography to give several sterol-containing lipid fractions, which were finally purified by reversed-phase HPLC to yield compounds **1**−**3**.

Compound **1** was isolated as a white solid. The steroid nature of this compound was deduced from a combination of ^{13}C NMR and DEPT-135 spectra, which showed that the compound had 29 carbon atoms, including five quaternary carbons (two olefinic, indicating the presence of a fully substituted double bond), eight methines, ten methylenes, and six methyl groups. The presence of three oxygenated methine carbons [δ_C 68.6 (d), 65.1 (d), and 61.3 (d)] and one oxygenated quaternary carbon [δ_C 67.7 (s)], two of which suggested the presence of an epoxy group, indicated the presence of three oxygen atoms. This information, along with the sodiated pseudomolecular ion $[M + Na]^+$ at *m*/*z* 467 in the (+)-LRFABMS of **1**, indicated a molecular formula of $C_{29}H_{48}O_3$ and thus the existence of six degrees of unsaturation. This molecular formula was corroborated by HRFABMS, which showed the $[M + Na]^+$ peak at *m*/*z* 467.3518.

The ^1H NMR spectrum contained signals for six methyl groups at δ_H 0.93 (3H, d, J = 6.6 Hz, H_3-21), δ_H 0.86 (3H, s, H_3-19), δ_H 0.85 (6H, s, H_3-18 and t, J = 7.0 Hz, H_3-29), δ_H 0.82 (3H, d, J = 6.8 Hz, H_3-27), and δ_H 0.80 (3H, d, J = 6.8 Hz, H_3-26), typical of a C29 steroid. The ^1H NMR spectrum also showed a broad multiplet at δ_H 3.91 (1H, m), indicating the existence of the characteristic hydroxyl group at C-3, and a proton signal at δ_H 3.15 (1H, d, J =

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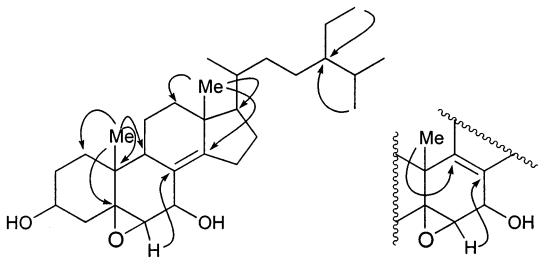

1 2

Figure 1. Selected HMBC correlations (^{1}H→^{13}C) observed for compounds **1** and **2**.

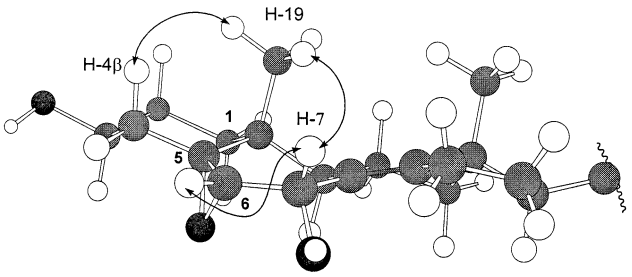

Figure 2. Selected NOESY correlations observed for compound **1**.

3.5 Hz, H-6), which helps to confirm the existence of a trisubstituted epoxide group. Furthermore, a ^{1}H−^{1}H COSY experiment showed that the epoxide proton was coupled with an allylic oxymethine proton observed at δ_H 4.41 (1H, m, $W_{1/2}$ = 9 Hz, H-7). The absence of signals for olefinic protons in the ^{1}H NMR spectrum corroborated the presence of a fully substituted double bond. The remaining proton and carbon chemical shifts of **1** were found to be coincident with those of a 24-ethylcholesterol derivative bearing an epoxide and hydroxyl groups and a fully substituted double bond. The locations of these groups were established by 2D NMR experiments (gHMQC, ^{1}H−^{1}H gCOSY, gHMBC) on **1**. The ^{13}C−^{1}H long-range correlations between the methyl protons H_3-19 (δ_H 0.86) and the quaternary epoxide carbon C-5 (δ_C 67.7 s) indicated the presence of this group between the C-5 and C-6 positions in the B ring of the steroid system. The HMBC correlations between the olefinic quaternary carbon C-8 at δ_C 125.0 (s) and the epoxide H-6 proton (δ_H 3.15 d), as well as between C-14 at δ_C 152.6 (s) and the methyl protons H_3-18 (δ_H 0.85 s), allowed us to locate the double bond between the C-8 and C-14 positions (see Figure 1).

The relative stereochemistry of compound **1** was elucidated by NOESY, coupling constant analysis, and comparison of its spectroscopic data with those of known 3,7-dihydroxy-5,6-epoxysteroid analogues. The axial−axial coupling constant found between H-3 at δ_H 3.91 (m) and the vicinal proton H-4β at δ_H 2.13 (dd, $J_{H3-H4\beta}$ = 11.5 Hz and $J_{H4\beta-H4\alpha}$ = 12.9 Hz) indicated the equatorial disposition of the OH at C-3 on the β face. The α-orientation of the hydroxyl group at C-7 was easily deduced from the NOESY correlation between H-7 at δ_H 4.41 (m) and H_3-19 methyl protons at δ_H 0.86 (s), which in turn showed a NOESY correlation with the H-4β proton at δ_H 2.13. These data showed that all of these protons were on the same β face of the ring (see Figure 2).

The experimentally observed coupling constant of 3.5 Hz between H-6β and H-7, which is close to the *J* value found for the corresponding protons (*J* = 3.0 Hz) in 3β,7α-dihydroxy-5α,6α-epoxycholest-8(14)-ene isolated from the sponge *Ircinia fasciculate*,[8] and the strong NOE correlation observed between these protons were consistent with a 5α,6α-epoxy configuration. Finally, the 24*R** relative stereochemistry of the ethyl group at C-24 was deduced by comparison of carbon and proton signals due to the side chain of **1** with the corresponding signals from a series of sterols having a similar configuration at C-24 (δ_C 45.83).[5a,9] The structure of compound **1** was thus established as 5α,6α-epoxy-24*R**-ethylcholest-8(14)-en-3β,7α-diol.

Compound **2** was also isolated as a white solid. The molecular formula of **2** was established as $C_{29}H_{48}O_3$ by HRFABMS, which showed the pseudomolecular ion $[M + Na]^+$ at *m/z* 467.3497 and indicated that compound **2** must be an isomer of compound **1**. Comparison of the ^{1}H and ^{13}C NMR data of compound **1** with those of **2** (see Tables 1 and 2) showed that both compounds share the same side chain and a similar steroid skeleton nucleus (including the hydroxyl and epoxide functionalities at the same positions), but differed in the location of the fully substituted double bond. A gHMQC experiment allowed us to assign all the protons to their corresponding carbon atoms. The location of the fully substituted double bond between carbons C-8 and C-9 was established with the help of a gHMBC experiment, which showed cross correlations between the olefinic quaternary carbon C-8 at δ_C 126.8 (s) and the epoxide H-6 proton (δ_H 3.31 d, *J* = 2.5 Hz), and also between C-9 at δ_C 134.5 (s) and the methyl protons H_3-19 (δ_H 1.14 s) (see Figure 1). The downfield shift of the C-19 methyl group (δ_H 1.14 s in **2** versus δ_H 0.86 s in **1**) was in accordance with the presence of a double bond between the C-8 and C-9 positions. NOESY experiments on **2** revealed the same relative stereochemistry as in **1**. Furthermore, the ^{1}H and ^{13}C NMR data of compound **2** for the fragment C-1/C-21 were coincident with those of melithasterol A (isolated from the soft coral *Melithaea ocracea*),[10,11] meaning that these two compounds share the same steroid nucleus and confirming the proposed relative stereochemistry of the 5α,6α-epoxy-3β,7α-dihydroxy groups in **2**. Consequently, the chemical structure of compound **2** was determined as 5α,6α-epoxy-24*R**-ethylcholest-8-en-3β,7α-diol.

The HRFABMS of compound **3** showed the pseudomolecular ion at *m/z* 427.3575 ($[M + H]^+$), which indicated the molecular formula $C_{29}H_{46}O_2$. The C29 steroid nature of this compound was deduced from its ^{13}C NMR and DEPT-135 spectra, which showed that the compound had 29 carbon atoms, including six quaternary carbons, seven methines, ten methylenes, and six methyl groups.

The proton and carbon spectral data of compound **3** indicated a number of structural similarities between this compound and **1** and **2**. The ^{1}H and ^{13}C NMR spectra showed the presence of the same side chain and a similar steroid skeleton nucleus, including a hydroxyl functionality at C-3. However, there were significant differences in the substitution pattern of the B ring. The absence of signals in the range 62−67 ppm in the ^{13}C NMR spectra of compound **3** indicated that it does not bear either epoxide or hydroxyl groups in the B ring. Instead, a signal with the chemical shift of a ketone carbonyl carbon at δ_C 186.3 (s), along with four olefinic carbons at 161.5 (s) 161.0 (s), 134.0 (s), and 126.8 (d), in the ^{13}C NMR spectrum of **3** suggested the presence of a double α,β-unsaturated ketone functionality in the B ring of this compound. A gHMBC experiment allowed us to determine the positions of the α,β-unsaturated ketone, which showed cross-correlations between the following signals: the C-19 methyl protons at δ_H 1.34 (s) and the C-1 (δ_C 34.6 t), C-10 (δ_C 41.8 s), C-5 and C-9 (δ_C 161.5 and 161.0 s) carbons, and between the olefinic

Table 1. ^{1}H NMR (500 MHz) Chemical Shifts (ppm) and Selected HMBC and NOESY Correlations for Compounds **1**−**3** in $CDCl_3$

	1			**2**			**3**	
C	δ_H mult (*J* in Hz)	HMBC	NOESY	δ_H mult (*J* in Hz)	HMBC	NOESY	δ_H mult (*J* in Hz)	HMBC
1	α: 1.40 m			α: 1.62 m			1.38 m/1.27 m	
	β: 1.70 m			*β*: 1.82 m				
2	α: 1.93 m			α: 1.97 m		H-3	1.97 m/1.72 m	
	β: 1.52 m			*β*: 1.60 m		H-19		
3	3.91 m		H-4α	3.95 m		H-2α, H-4α	3.67 m	
4	α: 1.41 m		H-3, H-6	α: 1.44 m		H-3, H-6	2.60 m/	
	β: 2.13 dd (12.9, 11.5)		H-19	*β*: 2.18 m		H-19	2.54 dd (11.0, 1.4)	
6	3.15 d (3.5)	C: 8	H-4α, H-7	3.31 d (2.5)	C: 8	H-4α, H-7	6.04 d (1.4)	C: 10
7	4.41 m ($W_{1/2}$ = 9)		H-6, H-15, H-19	4.23 m ($W_{1/2}$ = 9.7)		H-6, H-19		
9	2.34 m							
11	1.57 m/1.48 m			2.14 m				
12	1.92 m/1.15 m			1.97 m/1.41 m				
14				2.15 m			2.40 m	
15	2.64 m/2.34 m		H-7	2.04 m/1.14 m			2.61 m/1.43 m	
16	1.92 m/1.40 m			1.67 m/1.30 m				
17	1.21 m			1.15 m			1.21 m	
18	0.85 s	C: 12, 13, 14, 17		0.57 s	C: 12, 13, 14, 17		0.64 s	C: 12, 13, 14, 17
19	0.86 s	C: 1, 5, 9, 10	H-4*β*, H-7	1.14 s	C: 1, 5, 9, 10	H-2*β*, H-4*β*, H-7	1.34 s	C: 1, 5, 9, 10
20	1.45 m			1.38 m			1.41 m	
21	0.93 d (6.6)	C: 17, 20, 22		0.93 d (6.6)	C: 17, 20, 22		0.96 d (6.6)	C: 17, 20, 22
22	1.03 m/1.30 m			1.32 m/0.91 m				
23	1.04 m/1.32 m			1.34 m/0.96 m				
24	0.92 m			0.92 m			0.92 m	
25	1.67 m			1.62 m			1.69 m	
26	0.80 d (6.8)	C: 24, 25, 27		0.80 d (6.8)	C: 24, 25, 27		0.81 d (6.9)	C: 24, 25, 27
27	0.82 d (6.8)	C: 24, 25, 26		0.82 d (6.8)	C: 24, 25, 26		0.83 d (6.9)	C: 24, 25, 26
28	1.32 m/1.15 m	C: 29		1.32 m/1.15 m	C: 29		1.32 m/1.15 m	
29	0.85 t (7.0)	C: 24, 28		0.85 t (7.0)	C: 24, 28		0.86 t (7.4)	C: 24, 28

Table 2. ^{13}C NMR (125 MHz) Chemical Shifts (δ_C mult in ppm) for Compounds **1**−**3** in $CDCl_3$

C	**1**	**2**	**3**
1	32.1 t	30.1 t	34.6 t
2	31.1 t	30.8 t	30.6 t
3	68.6 d	68.5 d	71.9 d
4	39.5 t	39.1 t	41.8 t
5	67.7 s	65.6 q	161.5[a] s
6	61.3 d	62.5 d	126.8 d
7	65.1 d	67.1 d	186.3 s
8	125.0 s	126.8 s	134.0 s
9	38.6 d	134.5 s	161.0[a] s
10	35.8 s	37.9 s	41.8 s
11	18.9 t	23.3 t	24.7[b] t
12	36.6 t	35.7 t	35.6 t
13	43.1 s	42.1 s	42.4 s
14	152.6 s	49.5 d	48.3 d
15	24.9 t	23.8 t	24.6[b] t
16	26.6 t	28.7 t	29.3 t
17	56.6 d	53.6 d	53.3 d
18	17.9 q	11.0 q	11.7 q
19	16.5 q	22.8 q	23.7 q
20	34.9 d	36.6 d	36.6 d
21	18.9 q	18.7 q	18.9 q
22	33.6 t	33.7 t	33.8 t
23	26.2 t	26.4 t	26.5 t
24	46.0 d	46.0 d	46.0 d
25	28.9 d	28.9 d	28.9 d
26	19.0 q	18.9 q	18.9 q
27	19.5 q	19.5 q	19.6 q
28	22.9 t	22.9 t	23.0 t
29	12.3 q	12.2 q	12.3 q

[a,b] These assignments may be interchanged.

proton H-6 at δ_H 6.04 and the quaternary C-10 carbon at δ_C 41.8. On the basis of these data, compound **3** was determined to be 3*β*-hydroxy-24*R**-ethylcholesta-5,8-dien-7-one.

The new steroids **1** and **2** exhibited significant cytotoxic activity against human lung carcinoma (A-549), human colon carcinomas (HT-29 and H-116), mice endothelial (MS-1), and human prostate carcinoma (PC-3) cell lines. In an in vitro assay both compounds provided, as a starting point for further bioassays, a range of IC_{50} values of 0.5−10 *μ*g/mL (see results in Table 3). Some selective cytotoxicity was observed against MS-1 cells, as can be seen from the results in Table 3, where values of 0.5−1 *μ*g/mL were observed for both compounds.

Table 3. Cytotoxicity of Compounds **1** and **2**

	LC_{50} *μ*g/mL				
compd	A-549	HT-29	H-116	MS-1	PC-3
1	5−10	1−5	1−5	0.5−1	1−5
2	1−5	1−5	1−5	0.5−1	1

Experimental Section

General Experimental Procedures. Optical rotations were measured in CH_2Cl_2 using a JASCO DIP-1000 polarimeter with a sodium lamp operating at 589 nm. NMR spectra were recorded at 500/125 MHz (^{1}H/^{13}C) (AMX-Bruker spectrometer) and 200/50 MHz (^{1}H/^{13}C) (Bruker AC-200 NMR spectrometer) using $CDCl_3$ as solvent and internal standard. Carbon multiplicities were determined using DEPT-135. Atom connectivities were determined using gHMQC, gHMBC, and gCOSY data. NOESY experiments were carried out using a mixing time of 0.8 s. ESMS and AP_CIMS were obtained using a Thermoquest Navigator spectrometer. (+)-LRFABMS were measured on a VG-Quattro spectrometer, while (+)-HRFABMS were measured on a Trisector EBE spectrometer from Micromass Instruments using thioglycerol with 1% NaI as matrix. Semipreparative HPLC was performed using *μ*-Bondapak C_{18} columns (250 × 10 mm) with RI detection.

Biological Material. Specimens of *Polymastia tenax* were collected in April−May 2001 in Punta de Betín, Bahía de Santa Marta, in the Colombian Caribbean. Voucher samples are deposited at the Departamento de Química Fundamental, Universidade de A Coruña, under reference UNAL0101.

Extraction and Isolation. Specimens of the sponge were lyophilized (390 g dry wt) and extracted with MeOH (3 × 2.5 L), and the solvent was evaporated under reduced pressure. The crude extract was partitioned between CH_2Cl_2 and H_2O (1:1). The fraction soluble in CH_2Cl_2 was evaporated under reduced pressure and partitioned between 10% aqueous MeOH (400 mL) and hexanes (2 × 400 mL). Water was added to the polar fraction until the mixture became 50% aqueous MeOH, and this was extracted with CH_2Cl_2 (3 × 400 mL). After evaporation of the solvent, the separate combined organic layers yielded 0.74 g (hexanes) and 0.51 g (CH_2Cl_2) of residue.

The fraction that was soluble in hexanes was fractionated by flash column chromatography (silica gel 230−400 mesh, eluting with hexanes/EtOAc and EtOAc/MeOH mixtures of increasing polarity) to give several fractions. The fraction eluted with just EtOAc (76 mg) was purified by repeated reversed-phase HPLC with MeOH/H_2O (9:1) to afford compound **1** (3.5 mg) and compound **2** (1.5 mg). The viscous oil (0.74 g) obtained from the CH_2Cl_2 fraction was purified by flash column chromatography (eluting with CH_2Cl_2 and then with EtOAc/MeOH mixtures of increasing polarity) to give several fractions rich in steroids. The less polar fraction was purified by reversed-phase HPLC eluting with mixtures of MeOH/H_2O (9:1) to give compound **3** (1 mg). The more polar fraction was submitted to repeated reversed-phase HPLC eluting with MeOH/H_2O (1:1) to give further quantities of compounds **1** (6 mg) and **2** (3 mg).

5α,6α-Epoxy-24*R-ethylcholest-8(14)-en-3*β*,7α-diol (1):** amorphous white solid; $[\alpha]^{25}_D$ −69.0° (*c* 2.25 × 10^{-3}, CH_2Cl_2); UV (MeOH) λ_{max} (log ϵ) 248 (3.92) and 206 (3.96) nm; ^{1}H and ^{13}C NMR, see Tables 1 and 2; (+)-HRFABMS *m*/*z* 467.3518 $[M + Na]^+$ (calcd for $C_{29}H_{48}O_3Na$, Δ 1.7 mmu).

5α,6α-Epoxy-24*R-ethylcholest-8-en-3*β*,7α-diol (2):** amorphous white solid; $[\alpha]^{25}_D$ −47.3° (*c* 1.75 × 10^{-3}, CH_2Cl_2); ^{1}H and ^{13}C NMR, see Tables 1 and 2; (+)-HRFABMS *m*/*z* 467.3497 $[M + Na]^+$ (calcd for $C_{29}H_{48}O_3Na$, Δ 0.4 mmu).

3*β*-Hydroxy-24*R-ethylcholesta-5,8-dien-7-one (3):** amorphous colorless solid; $[\alpha]^{25}_D$ +9.1° (*c* 5 × 10^{-4}, CH_2Cl_2); ^{1}H and ^{13}C NMR, see Tables 1 and 2; (+)-HRFABMS *m*/*z* 427.3575 $[M + H]^+$ (calcd for $C_{29}H_{47}O_2$, Δ 0.1 mmu).

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