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Unusual polyoxygenated sterols from a Philippines sponge *Xestospongia* sp.

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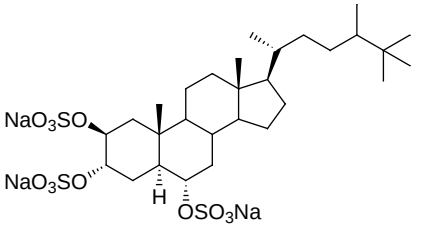
Abstract—Two novel sulfated sterols, ibisterol sulfates B (**3**) and C (**4**), and an unprecedented non-sulfated sterol, 4β,5β-epoxy-2β,3α,12β,22*S*-tetrahydroxy-14α-methylcholest-7,9(11)-dien-6,24-dione (**5**) have been isolated from a *Xestospongia* sp. collected in the Philippines. The structures were elucidated using spectroscopic data. These sterols were found to be inhibitors of HIV-1 integrase. © 2001 Elsevier Science Ltd. All rights reserved.

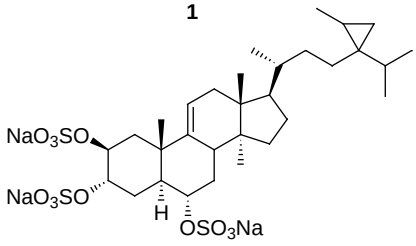
1. Introduction

Marine sponges are known to produce a variety of interesting and unconventional steroids,[1] of which polyoxygenated steroids have received the greatest amount of attention due to their remarkable biological and pharmacological activities.[2] In particular, sulfated sterols have been examined for their potential as inhibitors of HIV.[3] Halistanol sulfate (**1**), the most commonly isolated sulfated sterol from sponges, was originally isolated from *Halichondria* cf. *moorei* sp.[4] and was most recently reported from an Indo-Pacific species of *Haliclona*.[5] Another interesting sponge sterol, ibisterol sulfate (**2**), which was isolated from a *Topsentia* sp., contained an additional methyl group at C-14 and a $\Delta^{9(11)}$ double bond in the steroidal nucleus.[6] Both sulfated sterols inhibited HIV-1 and were cytotoxic.[3,6]

As part of an ongoing investigation to isolate biologically active marine metabolites, extracts of a collection of Philippines marine invertebrates were screened for cytotoxicity in a 25 cell-line panel. The methanolic extracts of a *Xestospongia* sp. showed some selectivity in the initial screen. Herein we describe the isolation of the major metabolites, the sulfated sterols ibisterol sulfate B (**3**) and C (**4**) and a minor non-sulfated metabolite, 4β,5β-epoxy-2β,3α,12β,22*S*-tetrahydroxy-14α-methylcholest-7,9(11)-dien-6,24-dione (**5**). Although it was later determined that they were not responsible for the selective cytotoxicity of the crude extract, the ibisterol sulfates exhibited mild inhibition of HIV-1 integrase.



1



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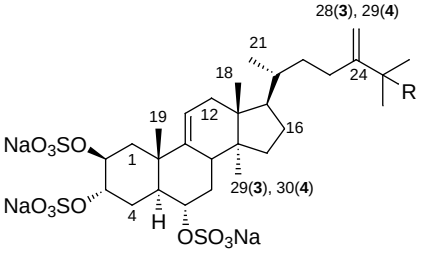

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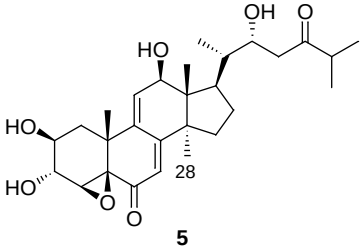
4 R = Me



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Keywords: marine natural products; HIV-1 integrase; cytotoxicity; *Xestospongia* sp; sterol.

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Table 1. ^{1}H NMR data for ibisterol sulfate B (**3**) and C (**4**)

C #	ibisterol sulfate B (**3**) δ_C	δ_H	mult, *J* (Hz)	ibisterol sulfate C (**4**) δ_C	δ_H	mult, *J* (Hz)
1α	37.7	1.83	dd, 14, 4	37.7	1.63	m
1β		2.25	d, 14		2.18	m
2	75.7	4.90	br s	75.8	4.91	br s
3	75.5	4.75	d, 3	75.6	4.75	d, 3
4α	25.6	2.34	d, 14	25.6	2.39	m
4β		1.82	m		1.83	m
5	43.6	1.71		43.6	1.73	m
6	79.0	4.47	dt, 5, 12	79.0	4.42	dt, 4, 12
7α	35.5	1.47	t, 12	35.5	1.88	m
7β		2.23	d, 14		2.23	m
8	41.4	2.44	d,15	41.4	2.44	m
9	145.9			146.2		
10	40.2			40.2		
11	118.1	5.40	s	118.1	5.38	br d, 6
12α	38.5	2.11	m	38.6	2.18	m
12β		1.97	t, 6		1.92	m
13	45.7			45.7		
14	48.2			48.3		
15	35.0	1.39	m	35.0	1.40	m
16α	32.5	2.22	m	29.1	2.14	m
16β		1.93	t, 6		1.85	m
17	52.3	1.69	m	52.5	1.67	m
18	15.2	0.70	s, 3 H	15.2	0.70	s, 3 H
19	22.8	1.20	s, 3 H	22.8	1.22	s, 3 H
20	37.5	1.79	d, 3	37.9	1.37	m
21	19.0	0.94	d, 3 H, 6	19.2	0.95	d, 3 H, 6
22	36.4	1.65	t, 4	37.7	1.80	m
23	29.1	1.86	m	29.6	1.39	m
24	157.8			160.2		
25	35.0	1.52	m	37.9		
26	22.4	1.01	d, 3 H, 7	30.0	1.05	s, 9 H
27	22.6	1.04	d, 3 H, 7	30.0	1.05	s, 9 H
28	107.0	4.67	s	30.0	1.05	s, 9 H
		4.71	s			
29	18.9	0.80	s, 3 H	106.9	4.65	s
					4.85	s
30				18.9	0.82	s, 3 H

2. Results and discussion

The dark gray *Xestospongia* sp. was collected in the Philippines and immediately frozen. When the frozen sponge was extracted with methanol, an off-white precipitate formed and this was removed by filtration. The sulfated sterols were isolated from the precipitate using chromatography on ToSoHaas TSK support (1:1 MeOH/H_2O) to obtain two pure fractions that contained ibisterol sulfate B (**3**) and ibisterol sulfate C (**4**), respectively. The crude methanol extract was partitioned between ethyl acetate and water, and the organic partition was further separated on C_{18} RP-HPLC with a gradient of methanol and water to obtain a minor metabolite, the non-sulfated sterol, 4β,5β-epoxy-2β,3α,12β,22*S*-tetrahydroxy-14α-methylcholest-7,9(11)-dien-6,24-dione (**5**). The structures of the novel sulfated sterols were elucidated by interpretation of spectral data and comparison to those of the known compound, ibisterol sulfate (**2**), to which they are closely related.

Ibisterol sulfate B (**3**) has the molecular formula $C_{29}H_{45}O_{12}S_3Na_3$, which was established from the NMR data (Table 1) and the HRMS peak at *m/z* 727.1882 ([M-Na]$^-$, Δ +1.4 mmu). This formula requires six degrees of unsaturation, consisting of four rings and two double bonds. The IR spectrum contained a strong band at 1230 cm^{-1}, consistent with the presence of sulfate groups. The structure of ibisterol sulfate B was established using a combination of homonuclear and heteronuclear two-dimensional NMR experiments. The ^{1}H NMR spectrum contained three methyl singlets (δ 0.70, 0.80, 1.20), three methyl doublets (δ 0.94, 1.01, 1.04), three oxymethine signals (δ 4.47, 4.75, 4.90), a trisubstituted olefinic proton (δ 5.40) and a 1,1-disubstituted methylene signal (δ 4.67, 4.71). The presence of six methyl groups assisted in elucidating the structure by providing a number of strong HMBC correlations that established most of the ring system. Rings A and B were assigned using the HMBC correlations from Me-19 to C-1, C-5, C-9 and C-10, H-7 to C-6 and C-8, and Me-29 to C-8. The COSY correlations from H-1α and H-1β through H-2, H-3, H-4β and H-4α to H-5 completed the structural assignment of the A/B ring system. Rings C and D were assembled using the HMBC correlations from Me-18 to C-12, C-13, C-14 and C-17, and Me-29 to C-8, C-13, C-14, and C-15. The position of the $\Delta^{9(11)}$ double bond was confirmed by the HMBC correlations from H-11 to C-12, C-8 and C-13. The only carbon without a clear assignment was C-16, which is notoriously difficult to assign, but upon close examination, a weak HMBC correlation could be observed from H-17 to C-16. The structure of the side chain was established using HMBC correlations from the methyl groups at either end; from Me-21 to C-20, C-17, and C-22, and from Me-26/27 to C-24, C-25, and each other. HMBC correlations from H-28 to both C-23 and C-25 completed the assignment.

Table 2. ^{1}H NMR data for 4β,5β-epoxy-2β,3α,12β,22*S*-tetrahydroxy-14α-methylcholesta-7,9(11)-dien-6,24-one (**5**).

C#	δ_C	δ_H	mult, *J* (Hz)	HMBC	ROESY
1α	36.3	1.43	t, 12	C-3, C-9	Me-19
1β		2.00	m	C-3	H-11, Me-19
2	71.2	3.34	m		
3	72.9	3.55	d, 8	C-2, C-5	H-1α, H-5
4	67.0	2.93	s	C-2	H-4
5	66.8				
6	195.7				
7	121.9	5.92	s	C-5, C-9, C-14	H-15, H-15′
8	165.4				
9	138.5				
10	40.4				
11	138.4	6.11	br s	C-8, C-10, C-13	H-2, H-12
12	74.0	4.42	br s	C-9, C-18	H-11, H-17, Me-28
13	52.1				
14	54.5				
15α	32.3	1.53	m		
15β		1.91	m		
16α	27.2	1.95	m		
16β		1.69	m		
17	49.8	1.97	m		
18	10.8	0.72	s, 3 H	C-12, C-13, C-14,C-17	H-15β, H-16β, Me-211
19	27.2	1.23	s, 3 H	C-1,C-5, C-9, C-10	H-1α, H-1β
20	42.1	1.71	m		
21	14.1	1.16	d, 3 H, 7	C-17, C-20, C-21	H-17, H-23α
22	70.9	4.30	ddd, 9, 3, 2		H-16β, H-16α(weak)
23α	42.6	2.64	dd, 13, 9	C-22, C-24	Me-21
23β		2.48	dd, 13, 2	C-24	H-17
24	217.0				
25		2.77	hept, 7	C-26, C-27	
26	18.3	1.08	d, 3 H, 7	C-24, C-25, C-27	H-25
27	18.4	1.10	d, 3 H, 7	C-24, C-25, C-26	H-25
28	25.6	1.14	s, 3 H	C-8, C-13, C-14, C-15	H-14, H-15α, H-17

The relative stereochemistry of the ibisterol sulfates was determined using NOESY correlations and the interpretation of coupling constant data. The A/B *trans* ring junction was suggested by the presence of large (J=12 Hz) axial-axial coupling between H-5 and H-6, and confirmed by the presence of NOE correlations between Me-19 and H-4β, H-6, and H-8. The C/D *trans* ring junction was suggested by the NOE correlations observed from Me-29 to H-17 and H-12α, and the correlations between Me-18 to H-8 and H-12β resulting in the conformation shown for Me-18 and Me-29. The relative stereochemistry of the sulfate groups was determined to be 2β, 3α and 6α due to the presence of a typical equatorial-equatorial coupling between H-2 and H-3, and the aforementioned NOE correlations. This stereochemical assignment for the three sulfate groups is consistent with that of other sulfated sterols previously reported from marine organisms.[7]

Ibisterol sulfate C (**4**) is a homologue of ibisterol sulfate B (**3**) and has the molecular formula $C_{30}H_{47}O_{12}S_3Na_3$, which was established by NMR data (Table 1) and a HRMS peak at *m/z* 741.2035 ($[M\text{-}Na]^-$, Δ +1.0 mmu). The IR spectrum contained a strong band at 1250 cm^{-1}, consistent with the presence of sulfate groups. The ^{1}H NMR spectrum contained a large methyl singlet (9 H) in place of two of the methyl doublets in ibisterol sulfate B. These data required a *t*-butyl terminus on the sterol side chain. All other spectral data, particularly the long-range C-H correlations, indicated that the remainder of the molecule is identical to ibisterol sulfate B (**3**).

4β,5β-Epoxy-2β,3α,12β,22*S*-tetrahydroxy-14α-methylcholest-7,9(11)-dien-6,24-dione (**5**) had a molecular formula of $C_{28}H_{40}O_7$, which was established from NMR data (Table 2) and the HRMS peak at *m/z* 489.2847 ($[M+H]^+$, Δ−0.5 mmu). This formula requires nine degrees of unsaturation consisting of five rings, two double bonds, and two carbonyl groups. The ^{1}H NMR spectrum contained three methyl singlets (δ 0.70, 1.14, 1.23), three methyl doublets (1.08, 1.10, 1.16), five oxymethine signals (2.93, 3.34, 3.55, 4.30, 4.42), and two olefinic signals (5.92, 6.11). As with the ibisterol sulfates A and B, the ring system was established using the HMBC correlations from the six methyls. The highly substituted nature of this sterol provided strong HMBC correlations to complete the ring assignments, but the proximity of the carbon signals for C-11 (δ 138.4) and C-9 (138.5), and for C-4 (67.0) and C-5 (66.8) made assignments difficult. The placement of the epoxide was confirmed by the observation of HMBC correlations from H-2 to C-4 and from H-7 to C-5. The remainder of the ring system was established using HMBC correlations from H-3 to C-5, H-4 to C-3, and H-2 to C-3. The double bonds in rings B and C can be assigned using the correlations from H-7 to C-10 and C-13, and H-11 to C-8, C-10 and C-13.

The stereochemistry of **5** was determined using a combination of coupling constant data and ROESY data. In determining the stereochemistry of ring A, the coupling constants for the H-3 signal, $J_{2,3}$=8 Hz and $J_{3,4}\approx$0 Hz, indicated that H-2 and H-3 were axial and that H-4 was orthogonal to H-3, conditions that are met by both the 4β,5β-epoxy-2β,3α-dihydroxy or 4α,5α-epoxy-2α,3β-dihydroxy conformations. The 4β,5β-epoxy-2β,3α-dihydroxy conformation,

which gives rise to an A/B-*cis* ring junction, was assigned on the basis of ROESY correlations from Me-19 to both H-1α and H-1β but not to H-2. The ROESY correlations from Me-28 to H-12α and H-17 confirm the C/D-*trans* ring junction, similar to that in the ibisterol sulfates. The stereochemistry of the side chain was deduced from the coupling constants for H-22 (J=9, 3, 2 Hz) and analysis of the ROESY data. Models of both the 22(*S*) and 22(*R*)-hydroxy isomers were minimized using PCModel™ and the intensities of the ROESY crosspeaks compared with the proton-proton distances obtained from the minimized structures. Nearly all of the correlations, even those from H-22 to H-16β and H-16α, were reasonable for either isomer but the strong correlation between H-23β and H-17 could only be explained for the 22(*S*)-hydroxy isomer.

When screened in an HIV-integrase inhibition assay, the following IC_{50} values were obtained; halistanol sulfate (**1**) 0.4 μg/mL, ibisterol sulfate B (**3**) 2.3 μg/mL, ibisterol sulfate C (**4**) 1.8 μg/mL and epoxide (**5**) 26 μg/mL. Since negatively charged compounds generally do not enter cells efficiently, these polysulfated compounds are poor candidates for further development. However, they are interesting examples to add to the small group of 14α-methyl sterols that occur among the plethora of steroids from marine organisms.

3. Experimental

3.1. General methods

All solvents used in the isolation and purification of the compounds were distilled prior to use. All NMR experiments except the ^{13}C were run using a Varian Inova 300 MHz spectrometer. ^{13}C NMR experiments were recorded on a Varian Unity 500 MHz spectrometer. The carbon multiplicities for compounds **3**, **4**, and **5** were determined using the meHSQC experiment, in which methyls and methines are represented as positive peaks and methylenes as negative peaks. In addition a DEPT experiment was performed for compound **3** to confirm all assignments. Optical rotations were measured using a Rudulph Autopol III polarimeter at 589 nm. HRMS data was obtained from the Scripps Research Institute using an IonSpec FT mass spectrometer.

3.2. Animal material

The dark gray boring sponge *Xestospongia* sp. (order Haplosclerida, family Petrosiidae) was collected by hand using SCUBA (−5 m) off Boracay Island in the Philippines in May 1998, and was immediately frozen. A voucher specimen (collection # NCI 2853) has been deposited in the SIO Benthic Invertebrate Collection.

3.3. Isolation

The sponge (343 g wet weight) was kept frozen until extraction in MeOH (2×1 L). An off-white precipitate was isolated from the crude methanol extract. The precipitate was further partitioned with 1:1 MeOH and water on ToSoHaas TSK HW-40 gel yielding ibisterol sulfate B (**3**) and ibisterol sulfate C (**4**). The combined methanol extracts were partitioned between 1:1 ethyl acetate and water. The organic extract was further separated using RP-HPLC on a C_{18} Dynamax 60Å column with a gradient of 50% aqueous methanol to 100% methanol to obtain pure 4β,5β-epoxy-2β,3α,12β,22*S*-tetrahydroxy-14α-methylcholest-7,9(11)-dien-6,24-dione (**5**), which eluted at 19 min.

3.3.1. Ibisterol sulfate B (3). White powder; $[\alpha]_D$=+50° (*c* 0.28, MeOH); UV (MeOH) 270 nm (ε 2160); IR (film) 3460, 2930, 1645, 1230 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) see Table 1; ^{13}C NMR (100 MHz, CD_3OD) see Table 1; MALDI-HRMS *m/z* 727.1882 $[M-Na]^-$ (calcd for $C_{29}H_{45}O_{12}S_3Na_2$, 727.1868).

3.3.2. Ibisterol sulfate C (4). White powder; $[\alpha]_D$=+27° (*c* 0.29, MeOH); UV (MeOH) 272 nm (ε 4838); IR (film) 3480, 2950, 1640, 1250 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) see Table 1; ^{13}C NMR (100 MHz, CD_3OD) see Table 1; MALDI-HRMS *m/z* 741.2035 $[M-Na]^-$ (calcd. for $C_{30}H_{47}O_{12}S_3Na_2$, 741.2025).

3.3.3. 4β,5β-Epoxy-2β,3α,12β,22*S*-tetrahydroxy-14α-methylcholest-7,9(11)-dien-6,24-dione (5). White powder; UV (MeOH) 296 nm (ε unmeasurable); IR (film) 3400, 2960, 2900, 2340, 1660, 1070, 1025 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) see Table 2; ^{13}C NMR (100 MHz, CD_3OD) see Table 2; MALDI-HRMS *m/z* 489.2847 $[M+H]^+$ (calcd. for $C_{28}H_{41}O_7$, 489.2852).

Acknowledgements

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