Eurysterols A and B, Cytotoxic and Antifungal Steroidal Sulfates from a Marine Sponge of the Genus *Euryspongia*

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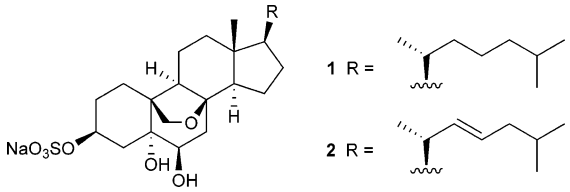
Two new steroidal sulfates, eurysterols A (**1**) and B (**2**), were isolated from an undescribed marine sponge of the genus *Euryspongia* collected in Palau. The structures of the new compounds were assigned by NMR spectroscopic data interpretation. Compounds **1** and **2** showed cytotoxicity against human colon carcinoma (HCT-116) cells with IC_{50} values of 2.9 and 14.3 μg/mL, respectively, and exhibited antifungal activity against amphotericin B-resistant and wild-type strains of *Candida albicans* with MIC values, in turn, of 15.6 and 62.5 μg/mL.

Sponges of the genus *Euryspongia* (order Dictyoceratida, family Dysideidae) have been shown to contain various types of secondary metabolites, including secosteroids,[1] hydroquinones,[2] sesquiterpene quinones,[3] and furanoterpenoids.[4,5] As part of an interest in the discovery of antifungal and cytotoxic natural products, we examined a cytotoxic crude methanol extract of a previously undescribed sponge of the genus *Euryspongia* collected in Palau. Using cytotoxicity against HCT-116 human colon carcinoma cells as a guide, two new moderately cytotoxic steroidal sulfates, eurysterols A (**1**) and B (**2**), were isolated. In this paper, we describe the isolation, structure elucidation, and biological activities of these new steroidal sulfates.



The *Euryspongia* sp. sponge was collected by hand using scuba at a depth of 37 m from a reef slope, west of the lighthouse at Koror, Palau. The frozen sample (187.6 g) was defrosted and extracted exhaustively with MeOH. The MeOH extract was then dried in vacuo, and the extract was fractionated by partition on HP-20SS resin, subsequently eluting with acetone/water mixtures to obtain 20%, 40%, 60%, 80%, and 100% acetone fractions. The 60% acetone fraction (64.1 mg), which contained all of the antifungal and cytotoxic activity, was further fractionated by reversed-phase C_{18} HPLC to yield eurysterols A (**1**, 5.5 mg) and B (**2**, 2.1 mg).

The molecular formula for eurysterol A (**1**) was determined as $C_{27}H_{45}O_7SNa$ by high-resolution MALDIFTMS (*m*/*z* 559.2688, $[M + Na]^+$) and from its NMR data. The LRMS spectra showed ions that analyzed for $[M - Na]^-$ and $[2M - Na]^-$ at *m*/*z* 513 and 1049, which suggested the presence of Na in this polar compound. Initial analysis by ^1H and ^{13}C NMR methods, including interpretation of the DEPT and HSQC spectroscopic data, revealed that the molecule **1** is steroidal. Analysis of the IR spectroscopic data showed broad absorption bands at 1216 and 1072 cm^{-1}, both of which suggested the presence of a sulfate group.[6]

The ^1H NMR spectrum of **1** measured in CD_3OD (Table 1) demonstrated three methyl signals [δ 0.84 (3H, s), 0.87 (6H, d, J = 6.4 Hz), and 0.92 (3H, d, J = 6.4 Hz)]. A portion of the side chain of the molecule was assembled by analysis of HMBC correlations (Figure 1) from two methyl signals at δ 0.87 and 0.92 (Me-21 to C-17, C-20, C-22; Me-26 and 27 to C-24, C-25). Oxygenation at C-3 (δ 76.6), C-5 (δ 77.3), and C-6 (δ 75.6) in rings A and B was deduced from the ^{13}C NMR chemical shifts, which were in the downfield region. A sulfate group was placed at C-3 on the basis of the low-field resonance of H-3 (δ 4.63) and C-3 (δ 76.6). The major part of a tetracyclic steroidal backbone could be assembled through the interpretation of COSY, HMBC, and HSQC-TOCSY NMR correlations. Analysis of the COSY and HSQC-TOCSY data allowed the assignment of the C-1/C-2/C-3/C-4, C-6/C-7, C-9/C-11/C-12, and C-17/C-20/C-21/C-22/C-23/C-24/C-25/C-26/C-27 connectivities. Interpretation of the HSQC and HMBC NMR data suggested that an ether bridge was present involving the A-ring bridgehead methyl group and C-8. HMBC correlations from H-19a to C-1, C-5, C-10 and from H-19b to C-5, C-8, C-9, C-10 supported this assignment. The carbon ring skeleton borne by **1** was previously known from the literature. The main steroidal skeleton is similar to abscisterol D, which has been isolated from *Cryptosporiopsis abietina* and found to be useful as a herbicide.[7]

The relative stereochemistry of the 3β-sulfate, 5α-hydroxyl, and 6β-hydroxyl groups in the molecule **1** was determined by interpretation of the ROESY NMR spectroscopic data and by analysis of vicinal coupling constants. Protons H-3α (δ 4.63) and H-4β (δ 2.23) were both placed in axial positions on the basis of their large vicinal coupling constant, $J_{3,4}$ = 11.6 Hz. This configuration was also confirmed by ROESY NMR spectroscopic correlations (Figure 2) between H-3α and H-1α (δ 1.85) and between H-4β and H-19b (δ 4.33). The stereochemistry of the 6β-hydroxyl group was established by a ROESY correlation between H-6α (δ 3.42) and H-4α (δ 2.05). The assignment of stereochemistry at C-20 as *R** was indicated by ROESY correlations from Me-21 to H-12β and H-12α. Hence, the structure of eurysterol A (**1**) was elucidated as 5α-cholestan-8,19-epoxy-3β,5,6β-triol-3-sulfate.

The structure of eurysterol B (**2**) was also elucidated on the basis of the interpretation of its 2D NMR data. The correlation patterns of the COSY and HMBC NMR spectroscopic data were found to be similar to **1**. A molecular formula of $C_{27}H_{43}O_7SNa$ for **2** was established by HRESITOFMS (*m*/*z* 557.2523, $[M + Na]^+$) and only differed from **1** by having one additional degree of unsaturation. The ^1H NMR spectrum of **2** was almost superimposable on that of **1** except for the presence of the two additional olefinic proton signals at δ 5.20 and 5.30, respectively. The geometry of the double bond was established as *trans* on the basis of an observed vicinal coupling constant of 15.2 Hz. HSQC and HMBC NMR correlation

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Table 1. ^{1}H and ^{13}C NMR Spectroscopic Data (CD_3OD) for Eurysterols A (**1**) and B (**2**)

	eurysterol A (**1**)				eurysterol B (**2**)			
position	δ_C		δ_H	*J* (Hz)	δ_C		δ_H	*J* (Hz)
1α	26.4	CH_2	1.85 m		26.4	CH_2	1.86 m	
1*β*			1.56 m				1.56 m	
2α	31.8	CH_2	2.13 m		31.8	CH_2	2.13 m	
2*β*			1.18 m				1.18 m	
3	76.6	CH	4.63 tt	11.6, 4.4	76.6	CH	4.63 tt	11.6, 4.4
4α	39.9	CH_2	2.05 m		39.9	CH_2	2.04 m	
4*β*			2.23 dd	13.6, 11.6			2.23 dd	13.6, 11.6
5	77.3	C			77.4	C		
6	75.6	CH	3.42 dd	4.0	75.6	CH	3.41 dd	4.0
7α	42.6	CH_2	1.87 m		42.6	CH_2	1.87 m	
7*β*			1.87 m				1.87 m	
8	85.5	C			85.5	C		
9	46.5	CH	2.03 m		46.6	CH	2.02 m	
10	50.3	C			50.3	C		
11α	21.6	CH_2	1.39 m		21.6	CH_2	1.39 m	
11*β*			1.41 m				1.42 m	
12α	40.0	CH_2	1.14 m		40.0	CH_2	1.17 m	
12*β*			2.04 m				2.03 m	
13	42.8	C			42.7	C		
14	54.8	CH	1.32 m		54.9	CH	1.34 m	
15α	21.6	CH_2	1.66 m		21.6	CH_2	1.62 m	
15*β*			1.49 m				1.49 m	
16α	28.8	CH_2	1.87 m		29.3	CH_2	1.69 m	
16*β*			1.30 m				1.27 m	
17	57.8	CH	1.09 m		57.6	CH	1.13 m	
18	12.6	CH_3	0.84 s		12.8	CH_3	0.85 s	
19a	72.2	CH_2	3.63 d	8.4	72.2	CH_2	3.63 d	8.4
19b			4.33 d	8.4			4.33 d	8.4
20	36.7	CH	1.39 m		41.2	CH	2.03	
21	19.3	CH_3	0.92 d	6.4	21.4	CH_3	1.00 d	6.8
22	37.2	CH_2	1.01 m		139.1	CH	5.20 dd	15.2, 8.4
23	25.0	CH_2	1.37 m		127.4	CH	5.30 dt	15.2, 6.8
24	40.7	CH_2	1.14 m		43.1	CH_2	1.83 m	
25	29.2	CH	1.52 m		29.8	CH	1.57 m	
26	23.0	CH_3	0.87 d	6.4	22.7	CH_3	0.87 d	6.8
27	23.2	CH_3	0.87 d	6.4	22.8	CH_3	0.87 d	6.8

data indicated the exact position of the double bond at C-22 and C-23 in the steroidal side chain. Therefore, eurysterol B (**2**) was defined as (22*E*)-5α-cholest-22-en-8,19-epoxy-3*β*,5,6*β*-triol-3-sulfate.

Marine organisms such as sponges and starfish are often found to contain sulfated secondary metabolites. Among these, a group of steroidal sulfates derived from marine sponges has been shown

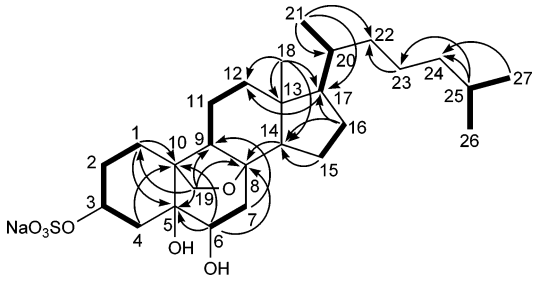

Figure 1. Selected COSY (bold lines) and HMBC (arrows) NMR correlations observed for **1**.

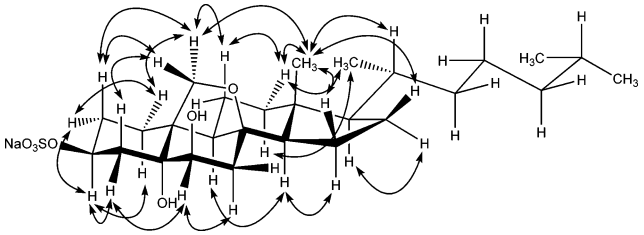

Figure 2. Assigned stereochemistry of **1** from selected ROESY NMR correlations.

Table 2. Cytotoxic and Antifungal Activities of **1** and **2**

	HCT-116 inhibition	*C. albicans* (WT)	*C. albicans* (AmBR)
compd	IC_{50} (*μ*g/mL)	MIC (*μ*g/mL)	MIC (*μ*g/mL)
1	2.9	15.6	15.6
2	14.3	62.5	62.5

to exhibit diverse biological activities including anticancer,[8] antiviral,[9] antifungal,[10] antibacterial,[11] HIV-inhibition,[12] and antifouling effects.[13] Several of these compounds were also discovered to possess selective inhibitory activities against the specific cellular targets in significant cellular pathways, such as halistanol sulfate and sokostrasterol sulfate, which were identified as inhibitors of endoglucanase.[14]

The isolation of steroidal sulfates from marine sponges of the genus *Euryspongia* has not been reported previously. Eurysterols A (**1**) and B (**2**) were tested for their cytotoxic activities against the HCT-116 human colon tumor cell line and for inhibition against wild-type and amphotericin B-resistant strains of *C. albicans*. The results are shown in Table 2. Despite their structural similarities, **1** and **2** showed somewhat different biological activities. Eurysterol A (**1**) was more active in both the cytotoxicity and fungal inhibition assays with an IC_{50} value of 2.9 *μ*g/mL and MIC value of 15.6 *μ*g/mL, respectively. The presence of the double bond in **2** reduced activities in both assays when compared to **1**.

Experimental Section

General Experimental Procedures. Optical rotations were measured using a Rudolph Autopol III polarimeter. UV spectra were obtained using a Perkin-Elmer Lambda Bio-20 spectrometer. Infrared spectra were recorded on a Perkin-Elmer 1600 spectrophotometer. COSY, HMBC, ROESY, NOESY, HSQC-TOCSY, and HSQC NMR

spectra were recorded on a Varian INOVA 300 MHz spectrometer, and ^{1}H, ^{13}C, and DEPT NMR spectra were recorded on a Varian Gemini 400 MHz spectrometer. High-resolution mass spectrometer measurements were obtained on IonSpec Ultima FTMS and Agilent ESI-TOF instruments at the Scripps Research Institute, La Jolla, CA. All solvents were distilled prior to being used.

Biological Material. The sponge, collection #00-317, was collected from a reef slope on Light House Reef, west of the lighthouse at Koror, Palau, from a depth of 37 m, in August 2000. It was kept frozen and stored at −20 °C until processed. In life, the sponge is composed of multiple conulose fingers, 12 cm tall, 10 mm diameter, that are fused at the base. The texture is firm but compressible, the sponge is elastic and tears quite easily, and the color is deep azure blue. The surface is closely conulose and granular. The skeleton is a reticulation of fine primary fibers that are packed with spicules and sand debris, and secondary reticulation of clear fibers with a granular pith region. The mesophyll has large eurpylous chambers. The sponge is an undescribed species of *Euryspongia* (order Dictyoceratida, family Dysideidae). A voucher specimen has been deposited at the Natural History Museum, London, UK (BMNH 2005.9.2.1).

Extraction and Purification. The sponge was lyophilized (22.2 g dry weight/ 187.6 g wet weight) before extraction with methanol (4 × 500 mL). The extracts were dried to obtain a dark green oil, which was fractionated by HP20SS column chromatography (acetone/water) to give five fractions. The fraction eluted with 60% acetone−water (64.1 mg) was further purified by reversed-phase HPLC (Dynamax C_{18} semipreparative, gradient from 40% to 100% MeOH, 3 mL/min) to obtain eurysterols A (**1**, 5.5 mg, 2.5 × 10^{-2}% dry weight) and B (**2**, 2.1 mg, 9.5 × 10^{-3}% dry weight).

Eurysterol A (1): colorless oil; $[\alpha]_D$ −15.4 (*c* 0.5, MeOH); UV (MeOH) λ_{max} (log ϵ) 258 nm (2.79); IR ν_{max} (KBr) 3449, 2952, 2868, 1216, 1072 cm^{-1}; NMR data, see Table 1; EIMS $[M + Na]^+$ *m*/*z* 559; HRMALDIFTMS $[M + Na]^+$ *m*/*z* 559.2688 (calcd for $C_{27}H_{45}O_7SNa_2$, 559.2681).

Eurysterol B (2): colorless oil; $[\alpha]_D$ −19 (*c* 0.2, MeOH); UV (MeOH) λ_{max} (log ϵ) 258 nm (2.59); IR ν_{max} (KBr) 3449, 2952, 2868, 1212, 1071 cm^{-1}; NMR data, see Table 1; EIMS $[M + Na]^+$ *m*/*z* 557; HRESITOFMS $[M + Na]^+$ *m*/*z* 557.2523 (calcd for $C_{27}H_{43}O_7SNa_2$, 557.2525).

HCT-116 Assay. HCT-116 human adenocarcinoma cells were plated in the 96-well plates and incubated overnight at 37 °C in 5% CO_2/air. Compounds were applied to the plates in DMSO and serially diluted, then the plates were further incubated for another 72 h. At the end of this period, a CellTiter 96 Aq_{ueous} non-radioactive cell proliferation assay (Promega) was used to assess cell viability. Inhibition concentration (IC_{50}) values are determined from the bioreduction of MTS/PMS by living cells into a formazan product. MTS/PMS was first added to the sample wells, followed by incubation for 3 h. The positive and negative controls that were used in this assay consisted of etoposide (Sigma) and DMSO (solvent), respectively. The Molecular Devices Emax microplate reader, set to a 490 nm wavelength, was used to determine the quantity of the formazan product (in proportion to the number of living cells) in each well, and IC_{50} values were calculated by an analysis program, SOFTMax.

Antifungal Assay with *C. albicans*. The *C. albicans* strains ATCC 32354 (wild-type) and ATCC 90873 (amphotericin B-resistant) were purchased from American Type Culture Collection (ATCC). Inhibitory activity was determined by a liquid antifungal assay. *C. albicans* was incubated overnight at 37 °C in RPMI media and transferred to 96-well plates the following day. Compounds were added to the plates and serially diluted. The plates were then incubated for 14−16 h. Amphotericin B (Sigma) and DMSO (solvent) were used as positive and negative controls, respectively. Minimum inhibitory concentration (MIC) values were interpreted by the change in color from blue to pink of the media according to the indicator Alamar Blue. This liquid antifungal assay was developed by A. Kanjana-Opas.

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Supporting Information Available: NMR spectra of eurysterols A (**1**) and B (**2**). This material is available free of charge via the Internet at http://pubs.acs.org.

References and Notes

(1) van Altena, I. A.; Butler, A. J.; Dunne, S. J. *J. Nat. Prod.* **1999**, *62*, 1154−1157.
(2) Hallock, Y. F.; Cardellina, J. H., II; Boyd, M. R. *Nat. Prod. Lett.* **1998**, *11*, 153−160.
(3) Urban, S.; Capon, R. J. *Aust. J. Chem.* **1996**, *49*, 611−615.
(4) van Altena, I. A.; Miller, D. A. *Aust. J. Chem.* **1989**, *42*, 2181−2190.
(5) Clark, R. J.; Garson, M. J.; Brereton, I. M.; Kennedy, J. A. *J. Nat. Prod.* **1999**, *62*, 915−916.
(6) Pretsch, E.; Simon, W.; Seibl, J.; Clerc, T. *Tables of Spectral Data for Structure Determination of Organic Compounds*; Springer-Verlag: Berlin, 1989; p I230.
(7) Yada, H.; Sato, H.; Ichihara, A. *Tetrahedron Lett.* **1995**, *36*, 7471−7474.
(8) Li, H.; Matsunaga, S.; Fusetani, N.; Fujiki, H.; Murphy, P. T.; Willis, R. H.; Baker, J. T. *Tetrahedron Lett.* **1993**, *34*, 5733−5736.
(9) Sun, H. H.; Cross, S. S.; Gunasekera, M.; Koehn, F. E. *Tetrahedron* **1991**, *47*, 1185−1190.
(10) Tsukamoto, S.; Matsunaga, S.; Fusetani, N.; van Soest, R. W. M. *J. Nat. Prod.* **1998**, *61*, 1374−1378.
(11) Nakatsu, T.; Walker, R. P.; Thompson, J. E.; Faulkner, D. J. *Experientia* **1983**, *39*, 759−761.
(12) Bifulco, G.; Bruno, I.; Minale, L.; Riccio, R. *J. Nat. Prod.* **1994**, *57*, 164−167.
(13) Tsukamoto, S.; Kato, H.; Hirota, H.; Fusetani, N. *Biofouling* **1997**, *11*, 283−291.
(14) Zvyagintseva, T. N.; Makar'eva, T. N.; Stonik, V. A.; Elyakova, L. A. *Khim. Prir. Soedin.* **1986**, 71−77.

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