A Cytotoxic 5α,8α-Epidioxysterol from a Soft Coral *Sinularia* Species

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A new sterol, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6-en-3*β*-ol (**1**), as well as two known sterols, numersterol A (**2**) and pregnenolone (**3**), have been isolated from a soft coral *Sinularia* sp. The structure of metabolite **1** was determined by spectral analysis. Cytotoxicity of sterols **1**−**3** toward various cancer cell lines is also reported.

Soft corals have been well-recognized as marine organisms containing large quantities of secondary metabolites that exhibit varying degrees of biological activities.[1] Several cytotoxic compounds have been isolated previously from soft corals collected along the coast of Taiwan.[2−8] In connection with our continuing investigations of bioactive substances from marine organisms, a soft coral *Sinularia* species was selected for study, as the EtOAc extract of this organism was found to exhibit significant cytotoxicity against P-388 (mouse lymphocytic leukemia) and KB (human nasopharyngeal carcinoma) cells. Initial study of the crude extract of this organism has led to the isolation of a novel cytotoxic hydroperoxysterol and five known compounds.[9] Further investigation on the chemical constituents of this organism resulted in the isolation of a new cytotoxic sterol, (22*R*,23*R*,24*R*)-5α,8α-epidioxy-22,23-methylene-24-methylcholest-6-en-3*β*-ol (**1**), and two known cytotoxic sterols, numersterol A (**2**) and pregnenolone (**3**).

Compound **1** was isolated from the fraction eluted with hexanes−EtOAc (3:1) as a white powdery solid, mp 159−160 °C, $[\alpha]^{26}_D$ +35° (*c* 0.1, $CHCl_3$). The HREIMS of **1** established a molecular formula of $C_{29}H_{46}O_3$, implying seven degrees of unsaturation. This sterol was further recognized as a 5α,8α-epidioxy sterol by the presence of the characteristic signals for H-6 and H-7 at *δ* 6.25 (d, *J* = 8.4 Hz) and 6.51 (d, *J* = 8.4 Hz), respectively, in the ^{1}H NMR spectrum.[10] Four protons appeared at *δ* 0.13 (2H, m), 0.30 (1H, m), and 0.54 (1H, m) ppm, indicating the presence of a cyclopropyl group in the molecule. Two singlets, which appeared at *δ* 0.76 (3H) and 0.90 (3H) ppm, were attributed to C-18 and C-19 methyls, respectively. Four doublets at *δ* 0.86 (3H, *J* = 6.9 Hz), 0.88 (3H, *J* = 6.9 Hz), and 0.91 (6H, *J* = 6.3 Hz) ppm were due to the presence of C-28, C-27, C-21, and C-26 methyls, respectively. The above data suggested that **1** is a peroxysteroid containing a 22,23-methylene-24-methyl moiety in the side chain. By comparison of the proton shifts of H_3-21, H_3-26, H_3-27, and H_3-28 with those of the four synthetic demethylgorgosterol isomers,[11] it was suggested that the stereochemistry of **1** at side chain should be assigned as 22*R*, 23*R*, and 24*R* (Figure 1). The assignment of the carbon shifts of **1** (Table 1) was based on the comparison of these data with those of the tetracyclic system of 5α,8α-epidioxyergost-6-en-3*β*-ol[12] and those of the side chain carbons of stoloniferone-*d*.[13] The presence of a peroxide was further confirmed by the mass fragment ion, which showed peak at *m*/*z* 410 $[M - O_2]^+$, presumably *via* a retro-Diels−Alder fragmentation. Based on the above data, the structure of **1** was then established as (22*R*,23*R*,24*R*)-22,23-methylene-24-methylcholest-6-en-5α,8α-epidioxy-3*β*-ol. It was finally found that **1** is the deacetyl derivative of a known steroid, **4**.[14] A structurally similar compound, **5**, isolated previously from a *Lobophytum* sp. soft coral, has been designated as 22,23-methylene-24-methylcholest-6-en-5α,8α-epidioxy-3*β*-ol.[15] However, the stereochemistry of **5** at C-22, C-23, and C-24 has not been characterized, and the ^{13}C NMR data were not assigned and showed some discrepancies with those of

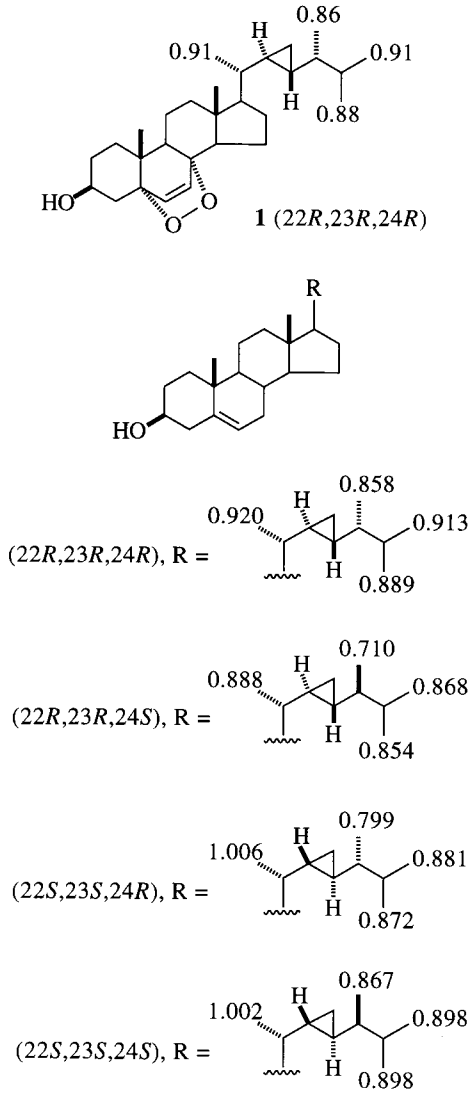

Figure 1. ^{1}H NMR Chemical shifts of the side-chain methyl groups of **1** and synthetic isomers of demethylgorgosterols.[11]

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Table 1. ^{13}C NMR Chemical Shifts of Sterols **1**−**3**

	compound		
position	**1**[a]	**2**[b]	**3**[c]
C-1	39.3	75.9	38.3
C-2	30.1	39.2	32.5
C-3	66.5	64.8	71.1
C-4	51.1	41.9	42.3
C-5	79.5	79.2	142.4
C-6	130.8	76.4	121.4
C-7	135.4	35.3	32.8
C-8	82.2	31.5	32.6
C-9	34.7	41.5	52.6
C-10	36.9	44.0	37.4
C-11	20.8	21.7	21.9
C-12	39.6	41.3	39.8
C-13	44.8	41.7	44.4
C-14	51.3	57.6	57.7
C-15	28.4	25.3	23.5
C-16	23.4	29.3	39.6
C-17	57.8	57.6	64.0
C-18	12.4	12.6	13.6
C-19	18.5	17.3	19.9
C-20	39.5	34.9	208.5
C-21	19.1	19.1	31.5
C-22	24.2	36.0	
C-23	25.1	32.1	
C-24	45.0	157.9	
C-25	32.8	37.0	
C-26	18.1	22.4	
C-27	20.6	22.3	
C-28	15.7	106.9	
C-29	10.5		

[a] The chemical shifts were determined at 75 MHz in $CDCl_3$. [b] 75 MHz in CD_3OD. [c] 75 MHz in acetone-d_6, respectively. The values are in ppm downfield from TMS.

Table 2. Cytotoxic Data of Sterols **1**−**3**[a]

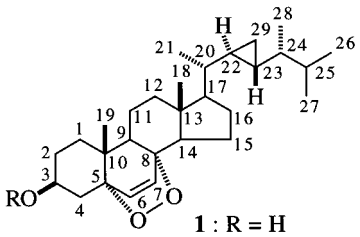
	cell lines ED_{50} (μg/mL)			
compound	P-388	KB	A549	HT-29
1	0.4	2.1	2.7	1.4
2	8.3	1.9	10.8	1.5
3	7.8	>50	8.6	0.7

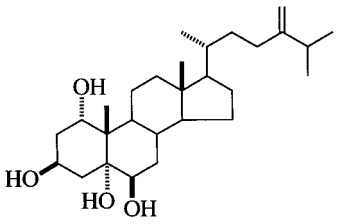
[a] For significant activity of pure compounds, an ED_{50} value of ≤4.0 μg/mL is required. See Geran et al.[20]

1 and the related compounds,[12,13] particularly at C-29, the cyclopropyl methylene carbon, which should show a peak at about δ 10.5 ppm. In contrast, the most upfield signal of the eight methylene carbons of **5** was reported to appear at δ 23.3 ppm. Thus, peroxysterol **1** is regarded as a new compound.

The previously known compounds, numersterol A (**2**) and pregnenolone (**3**), were identified by their physical (mp and optical rotation) and spectral [IR, MS, ^1H and ^{13}C NMR (Table 1)] data. The polyhydroxylated sterol **2** was isolated previously from the South China Sea soft coral *Sinularia numerosa*.[16] The occurrence of pregnenolone (**3**) in a sponge *Haliclona rubens* has been reported;[17] however, this is the first observation for the existence of **3** in a soft coral.

To find the future biomedical potential for the above steroids, cytotoxicity testing was performed. The cytotoxicity of these compounds toward a limited panel of cancer cell lines is shown in Table 2. A sterol with a structure similar to **1**, 5α,8α-epidioxyergost-6-en-3β-ol, has been shown to exhibit cytotoxicity toward MCF-7 breast and Walker 256 carcinomasarcoma cell lines.[12] Our present study also shows that **1** exhibited significant cytotoxicity toward the growth of P-388, KB, A549, and HT-29 cells. The results of these two investigations indicated that steroids containing a 5α,8α-epidioxy functional group may warrant further antitumor studies in the future. Compound





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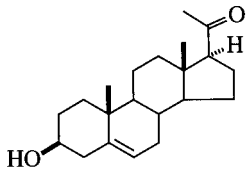

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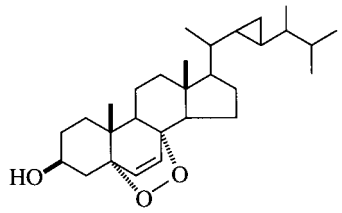

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2 exhibited selective cytotoxicity against KB and HT-29 cells. Compound **3** also was found to show significant activity exhibiting the growth of HT-29 cells.

Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher−Johns melting points apparatus and were uncorrected. Optical rotations were measured on a JASCO DIP-1000 digital polarimeter. The IR spectra were measured on a Horiba FT-720 infrared spectrophotometer. ^1H and ^{13}C NMR spectra were recorded on a Varian VXR-300/5 FT NMR spectrometer at 300 and 75 MHz, respectively, in $CDCl_3$ using TMS as internal standard, unless otherwise indicated. EIMS spectra were obtained with a VG QUATTRO GC−MS spectrometer. HREIMS spectra was recorded on a JMX-HX 110 mass spectrometer. Si gel 60 (Merck, 230−400 mesh) was used for column chromatography. Precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.20 mm) were used for analytical TLC.

Animal Material. The soft coral *Sinularia* sp. was collected by hand using scuba at the South Bay, Kenting, located in the southernmost tip of Taiwan, in June 1995, at a depth of 4 m and was immediately stored in a freezer until extraction. The colony morphology and the shape of the sclerites in both the surface and interior of the lobe indicate that the sample is a soft coral of the genus *Sinularia*.[18] A voucher specimen was deposited in the Department of Marine Resources, National Sun Yat-Sen University (specimen no. KTSC-105).

Extraction and Separation. The soft coral (3.3 kg fresh wt) was collected and freeze-dried. The freeze-dried material

(0.8 kg) was minced and extracted exhaustively with EtOAc (8 L × 5). The organic extract was evaporated to dryness, and the oily residue (8.3 g) was found to exhibit cytotoxcicity against the P-388 cell line, with an ED_{50} of 12.8 μg/mL, and the KB cell line, with an ED_{50} of 15.4 μg/mL. The extract was separated by Si gel column chromatography. Sterol **3** was eluted with hexanes−EtOAc (4:1), **1** with hexanes−EtOAc (3:1), and **2** with EtOAc−EtOH (3:1).

(22*R*,23*R*,24*R*)-5α,8α-Epidioxy-22,23-methylene-24-methylcholest-6-en-3*β*-ol (1): white powder (1.7 mg); mp 159−160 °C; $[\alpha]^{26}_D$ +35° (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max} 3380, 1646, 1062 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.13 (2H, m, H_2-29), 0.30 (1H, m, H-22α), 0.54 (1H, m, H-23*β*), 0.54 (1H, m, H-24), 0.76 (3H, s, H_3-18), 0.86 (3H, d, *J* = 6.9 Hz, H_3-28), 0.88 (3H, d, *J* = 6.9 Hz, H_3-27), 0.90 (3H, s, H_3-19), 0.91 (6H, d, *J* = 6.3 Hz, H_3-21 and H_3-26), 3.98 (1H, m, H-3), 6.25 (1H, d, *J* = 8.4 Hz, H-6), 6.51 (1H, d, *J* = 8.4 Hz, H-7); ^{13}C NMR, see Table 1; EIMS (30 eV) *m*/*z* (rel int) 442 (1, M^+), 424 (3), 410 (7), 303 (1), 301 (3), 271 (3), 269 (1), 253 (5), 251 (9), 69 (100); HREIMS *m*/*z* 442.3472 (calcd for $C_{29}H_{46}O_3$ 442.3435).

Numersterol A (2): white powder (18.5 mg); mp 296−298 °C; $[\alpha]^{26}_D$ +5° (*c* 0.3, MeOH); IR (KBr) ν_{max} 3330, 1678, 1070, 890 cm^{-1}; ^1H NMR (300 MHz, CD_3OD) δ 0.69 (3H, s, H_3-18), 0.95 (3H, d, *J* = 6.3 Hz, H_3-21), 1.02 (6H, d, *J* = 6.9 Hz, H_3-26 and H_3-27), 1.11 (3H, s, H_3-19), 3.49 (1H, br s, H-6α), 3.61 (1H, br s, OH), 3.89 (1H, t, *J* = 3.9 Hz, H-1*β*), 4.30−4.50 (1H, m, H-3α), 4.65 (1H, s, H-28), 4.71 (1H, s, H-28); ^{13}C NMR, see Table 1; EIMS (30 eV) *m*/*z* (rel int) 433 [3, $(M - CH_3)^+$], 412 (8), 397 (5), 346 (12), 321 (18), 69 (100).

Pregnenolone (3): white powder (10.2 mg); mp 192−193 °C; $[\alpha]^{26}_D$ +27° (*c* 0.3, $CHCl_3$); IR (KBr) ν_{max} 3410, 1710, 1650 cm^{-1}; ^1H NMR (300 MHz, acetone-d_6) δ 0.62 (3H, s, H_3-18), 1.01 (3H, s, H_3-19), 2.06 (3H, s, H_3-21), 2.61 (1H, t, *J* = 9.0 Hz, H-17), 3.42 (1H, m, H-3), 3.80 (1H, d, *J* = 4.8 Hz, OH), 5.33 (1H, d, *J* = 4.5 Hz, H-6); ^{13}C NMR, see Table 1; EIMS (30 eV) *m*/*z* (rel int) 316 (2, M^+), 298 (2), 283 (2), 43 (100).

Cytotoxicity Testing. KB and P-388 cells were kindly provided by Prof. J. M. Pezzuto, University of Illinois at Chicago; A549 (human lung adenocarcinoma) and HT-29 (human colon adenocarcinoma) were purchased from the American Type Culture Collection. The cytotoxic activities of tested compounds **1**−**3** were assayed by a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[19] The cultured cells were treated at eight concentrations of pure test compounds ranging from 0.00064 to 50 μg/mL. All assays were performed in triplicate. The results were expressed as a percentage, relative to control incubations, and the effective dose required to inhibit cell growth by 50% (ED_{50}) was determined.

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