A New Cytotoxic Polyhydroxysterol from Soft Coral *Sarcophyton trocheliophorum*

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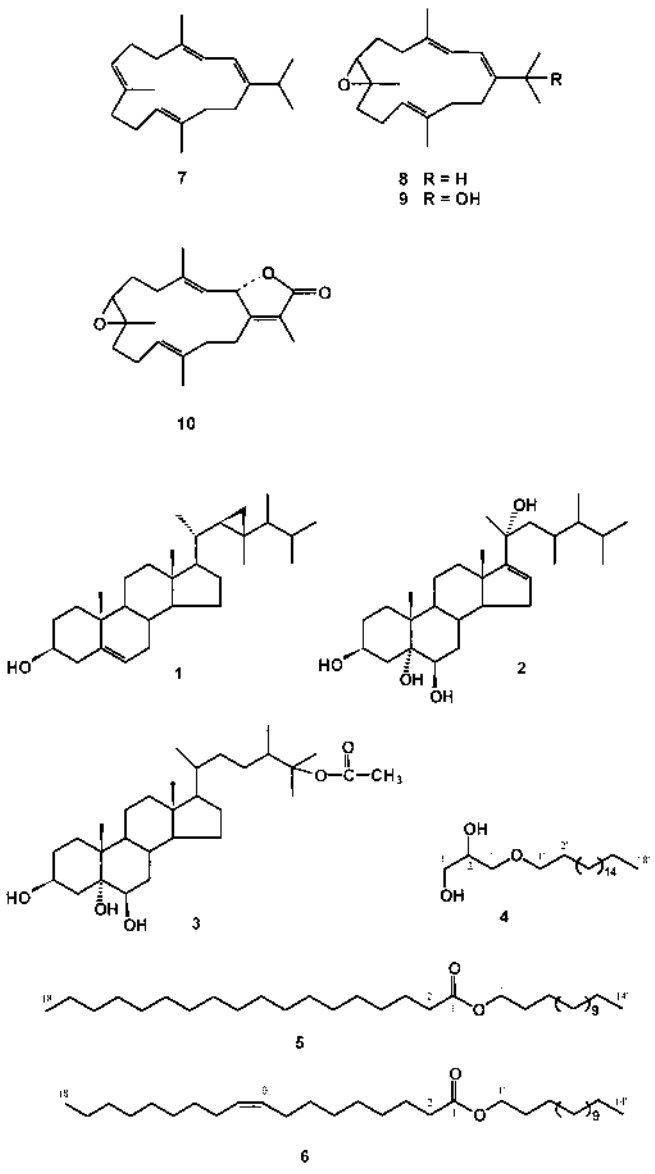
A new cytotoxic polyhydroxysterol, 23,24-dimethylcholest-16(17)-*E*-en-3β,5α,6β,20(*S*)-tetraol (2), together with nine known compounds was isolated from the soft coral *Sarcophyton trocheliophorum*. Their structures were determined by spectroscopic methods. Compound 2 showed potent growth inhibitory activity against human HL60 leukemia, M14 skin melanoma, and MCF7 breast carcinoma cells with EC_{50} values of 2.8, 4.3, and 4.9 μg/ml, respectively, and exhibited minimal toxicity to normal human peripheral blood lymphocytes.

Key words Soft coral; *Sarcophyton trocheliophorum*; polyhydroxysterol

A variety of constituents, such as sterols, terpenoids, and alicyclic compounds have been isolated from soft coral species of the *Sarcophyton* genus.[1)] Some of these compounds have been shown to have cytotoxic,[2)] cancer chemopreventative,[3,4)] and anti-inflammatory potential.[5)] The hexane soluble extract of the soft coral *Sarcophyton trocheliophorum* Von Marenzeller showed growth inhibitory activity against human tumor cells. In this paper, we report the isolation and structure determination of a new cytotoxic polyhydroxysterol, 23,24-dimethylcholest-16(17)-*E*-ene-3β,5α,6β,20(*S*)-tetraol (**2**), together with nine known compounds, gorosten-5(*E*)-3β-ol (**1**), 24-methylcholestane-3β,5α,6β,25-tetraol 25-monoacetate (**3**), 2,3-dihydroxypropyl octadecyl ether (**4**), tetradecyl octadecanoate (**5**), tetradecyl-9-*Z*-octadecenoate (**6**), isoneocembrene A (**7**), 7,8-epoxy-1(*E*),3(*E*),11(*E*)-cembratriene (**8**), 7,8-epoxy-1(*E*),3(*E*),11(*E*)-cembratrien-15-ol (**9**), and sarcophine (**10**) from the hexane soluble fraction of the soft coral *Sarcophyton trocheliophorum* (Alcyoniidae) and their cytotoxic activity on human tumor cells.



Compound **2** was obtained as colorless amorphous powder. The molecular formula was determined as $C_{29}H_{50}O_4$ by HREIMS (M^+ 462.3716, Calc. 462.3709). The ^{13}C NMR and DEPT spectra showed twenty nine signals including characteristic signals of eight methylene carbons, eight methine carbons, five quaternary carbons, one olefinic carbon, and seven methyl carbons. In the ^1H NMR spectrum of compound **2**, four methyl groups appeared as four doublets (J=6.6, 7.9, 7.2, and 7.2 Hz) at δ 0.75, 0.77, 0.86, and 0.88 and three methyl groups appeared as three singlets at δ 0.98, 1.20, and 1.36, respectively. These data indicated the presence of the partial structure of the side chain of 23,24-dimethyl cholesterol derivatives. It was further confirmed in the mass spectrum by the fragmentation pattern with peaks at m/z 349 [$M-C_8H_{17}$], 331 [$M-C_8H_{17}-H_2O$], 313 [$M-C_8H_{17}-2H_2O$], and 295 [$M-C_8H_{17}-3H_2O$], which corresponded to the cleavage of the side chain of 23,24-dimethyl cholesterol derivatives. Furthermore, the ^1H–^1H COSY and HMBC spectra of this compound also gave good information for supporting the assignment of the side chain. There were long-range correlations between C-24 (δ 45.89) with Me-26 (δ 0.77),

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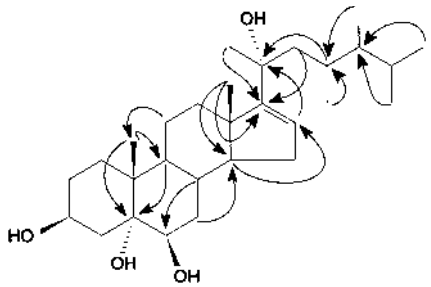

Fig. 1. Selected HMBC Correlations in Compound **2**

Me-27 (δ 0.75), Me-28 (δ 0.86), and Me-29 (δ 0.88), C-23 (δ 29.59) with Me-28 and Me-29, and C-22 (δ 49.09) with Me-21 (δ 1.36) and Me-29 in the HMBC spectrum. The ^{1}H NMR spectrum also showed an olefinic proton at δ 5.47 which appeared as a broad singlet and two secondary carbinol methines at δ 3.55 (1H, br) and 4.08 (1H, br m). The broad methine multiplet at δ 4.08 had the normal complexity for the 3α-carbinol proton of an A/B *trans*-steroid. This unusually downshifted signal is typical of 3β-hydroxysterols bearing a 5α-hydroxyl group.[6,7] Two double doublets at δ 2.05 (J=11.2 and 12.5 Hz) and, 1.76 (J=12.5 and 3.9 Hz), mutually coupled and coupled with the 3α-proton at δ 4.08, were assigned, respectively, to the 4-H_{ax} and 4-H_{eq} protons next to the C-5 substituted position.[8] The downshift of the C-19 methyl group signal at δ 1.20 was indicative of the β-orientation of the C-6 hydroxyl group.[9] These data suggested the 3β,5α,6β-trihydroxyl substitutions of compound **2**. This partial structure was further confirmed by observation of the long-range correlations between C-3 (δ 67.63) with H_{ax}-4 (δ 2.05) and H-1 (δ 1.40) and C-5 (δ 76.78) with Me-19 (δ 1.23) and H-6 (δ 3.55) in the HMBC spectrum. Likewise, the HMBC spectrum permitted the location of another hydroxylated quaternary carbon (δ 75.97) at C-20 and a double bond (δ 123.69 and 160.90) at C-16(17) due to the unambiguous correlations between C-20 (δ 75.97) with H-16 (δ 5.47), Me-21 (δ 1.36), and H_2-22 (δ 1.47 and 1.58) and C-17 (δ 160.90) with Me-18 (δ 0.98), Me-21 (δ 1.36), and H_2-15 (δ 1.89 and 2.02) (Fig. 1). The relative stereochemistry of compound **2** was elucidated on the basis of NOESY correlations. The NOESY spectrum showed cross-peaks of H-4β/Me-19 and H-8/Me-19 which confirmed a *trans*-junction between rings A and B and the 3β,5α,6β-oriented substitutions.[10] NOESY correlations for H-16/Me-21, H-15β/Me-21, Me-18/Me-21, and H-12β/Me-21 suggested that the relative stereochemistry at C-20 was *S* configuration. From the above data, the structure of compound **2** was concluded to be 23,24-dimethylcholest-16(17)-*E*-ene-3β,5α,6β,20(*S*)-tetraol. The ^{1}H- and ^{13}C-NMR data of compound **2** were assigned by analysis of DEPT, ^{1}H, ^{1}H COSY, ^{1}H, ^{13}C COSY, HMQC, and HMBC spectral data.

The other nine compounds, gorosten-5(*E*)-3β-ol (**1**),[11] 24-methylcholestane-3β,5α,6β,25-tetraol 25-monoacetate (**3**),[12,13] 2,3-dihydroxypropyl octadecyl ether (**4**),[14] tetradecyl octadecanoate (**5**),[15] tetradecyl-9-*Z*-octadecenoate (**6**),[15] isoneocembrene A (**7**),[16] 7,8-epoxy-1(*E*),3(*E*),11(*E*)-cembratriene (**8**),[17] 7,8-epoxy-1(*E*),3(*E*),11(*E*)-cembratrien-15-ol (**9**),[18] and sarcophine (**10**)[18] were identified by comparing their MS, ^{1}H-, and ^{13}C-NMR spectral data with those reported in the literature. The assignment of ^{13}C-NMR data of compound **3** was different from the data reported in the literature[12] and is listed in Table 1.

Table 1. ^{13}C-NMR Spectral Data of Compounds **2** and **3**[a] (125 MHz, $CDCl_3$)

C	**2**	**3**[b]	C	**2**	**3**
1	32.21, t[c]	32.41, t	16	123.69, d	24.16, t
2	30.82, t	30.88, t	17	160.90, s	55.95, d
3	67.63, d	67.66, d	18	18.39, q	12.19, q
4	40.72, t	40.78, t	19	16.76, q	16.89, q
5	76.78, s	76.12, s	20	75.97, s	36.24, d
6	75.93, d	76.09, d	21	29.65, q	18.97, q
7	34.28, t	34.57, t	22	49.09, t	34.69, t
8	28.89, d	30.25, d	23	29.59, d	27.79, t
9	45.99, d	45.89, d	24	45.89, d	41.98, d
10	38.54, s	38.34, d	25	30.94, d	85.96, s
11	21.19, t	21.20, t	26	20.91, q	14.52, q
12	36.26, t	39.96, t	27	15.78, q	23.36, q
13	47.76, s	42.78, s	28	11.59, q	22.94, q
14	57.33, d	55.99, d	29	21.49, q	
15	30.85, t	28.14, t	OAc		170.49, s

a) δ in ppm. *b*) Data assigned by analysis of ^{1}H, ^{1}H COSY, HMQC, and HMBC spectral data. *c*) Multiplicities determined by DEPT sequences.

Table 2. Cytotoxicity of Compounds **1**—**10** (n=3—6)

Compound	Cell line EC_{50} (μg/ml)		
	HL60	M14	MCF7
1	54.1	20.3	30.2
2	2.8	4.3	4.9
3	19.6	13.2	34.5
4	46.9	20.1	29.8
5	76.8	17.2	30.2
6	53.1	10.4	26.3
7	37.2	74.6	72.8
8	34.2	>100	>100
9	63.8	>100	56.4
10	61.8	>100	>100

Constituents such as polyhydroxysterols and cembranoid diterpenes from soft corals are well known for their cytotoxic and cancer chemopreventative activities.[2—4] In this study, we examined the growth inhibitory activity of compounds **1**—**10** against human HL60 leukemia, M14 skin melanoma, and MCF7 breast carcinoma cells. As shown in Table 2, compound **2** showed the most potent growth inhibitory activity against human HL60, M14, and MCF7 cells with EC_{50} values of 2.8, 4.3, and 4.9 μg/ml, respectively, and in a dose-dependent manner. Compound **3** also exhibited strong growth inhibitory activity against these tested tumor cells. Compound **1**, however, showed less activity than either compound **2** or **3**. Of these sterols, compound **1** has only one hydroxyl group at C-3 position. Compounds **2** and **3** are polyhydroxysterols with the same 3β,5α,6β-oriented partial structure, but compound **2** has one more free hydroxyl group at C-20 position, whereas compound **3** has one acetyl group at C-25 position.

Triggered by our finding of the antitumor activity of compound **2**, we next investigated the effect of this compound on normal human peripheral blood lymphocytes (PBLs). Our data showed that compound **2** had minimal toxicity to normal human PBLs, Indicating its selective targeting of human tumor cell lines.

Data obtained from this study indicated that compound **2** showed potent inhibitory activity on human tumor cells and minimal toxicity to the normal human peripheral blood lymphocytes. Further investigations on this compound as a chemotherapeutic agent are in progress.

Experimental

General The optical rotation was obtained on a Perkin-Elmer 241 polarimeter. EIMS were obtained on a MACROMASS 7035E mass spectrometer at 70 eV. All spectra (^{1}H, ^{13}C, COSY, HMQC, NOESY, and HMBC) were recorded on a Bruker AMX 500 spectrometer (500 MHz for ^{1}H and 125 MHz for ^{13}C), and the chemical shifts are reported in ppm using TMS as an internal standard. TLC was performed on 0.25-mm Si gel (60 F_{254}, Merck) plates. Spots were visualized by spraying 10% H_2SO_4 aqueous and heating for 5 min.

Animal Materials The soft coral *Sarcophyton trocheliophorum* was collected in April 1998 from Pulau Hantu, an island south of Singapore, at a depth of 10 m. The voucher specimen (SC980401) is deposited in the Herbarium of Bioscience Center, Department of Biological Science, Faculty of Science, National University of Singapore, Singapore.

Extraction and Isolation The fresh material (2.5 kg, wet wt.) was sliced and percolated with acetone–H_2O (7 : 3) three times (8×3 l) at room temperature. The acetone was evaporated *in vacuo*. The aqueous portion was partitioned by hexane (4 l, 32 g) and EtOAc (2 l, 4 g). The hexane extract (32 g) was subjected to column chromatography over Si gel 60 and eluted by CH_2Cl_2, CH_2Cl_2–MeOH (8 : 2), CH_2Cl_2–MeOH (1 : 1), and CH_2Cl_2–MeOH–H_2O (1 : 1 : 0.1) solvent systems to afford four fractions, fractions A—D. Fraction A (5.6 g) was purified again by column chromatography over Si gel and eluted under gradient conditions with increasing amounts of EtOAc in hexane to afford **1** (30 mg), **5** (200 mg), **6** (150 mg), and **7** (20 mg). Fraction B (0.5 g) was further purified by repeated Si gel chromatography under the above conditions and/or preparative TLC to give **8** (12 mg), **9** (40 mg), and **10** (8 mg). Fraction D (1.2 g) was subjected to column chromatography over LiChroprep RP-18 (60—43 μm) and elution with ethanol–H_2O (6 : 4) to give **2** (6 mg), **3** (12 mg), and **4** (70 mg).

23,24-Dimethylcholest-16(17)-*E*-ene-3β,5α,6β,20(*S*)-tetraol (**2**): Colorless amorphous powder. A spot sprayed with 5% H_2SO_4 and heated for 5 min showed a light blue color. $[\alpha]_D^{20} = -14.1°$ (*c* 0.16, EtOH); IR (KBr) ν_{max} 3422 (OH), 2938, 1376, 1083 cm^{-1}. UV λ_{max} (log ε): 225 (0.26) nm. HREIMS 462.3716 (calcd for $C_{29}H_{50}O_4$, 462.3709); EIMS *m*/*z* 426 (M^+, 1.2), 349 (64), 331 (24), 313 (18), 295 (30), 280 (20), 271 (14), 269 (26), 229 (12), 133 (30), 95 (48), 43 (100); ^{1}H NMR (500 MHz, $CDCl_3$) δ 0.75 (3H, d, *J*=6.6 Hz, Me-27), 0.77 (3H, d, *J*=7.9 Hz, Me-26), 0.86 (3H, d, *J*=7.2 Hz, Me-29), 0.88 (3H, d, *J*=7.2 Hz, Me-28), 0.98 (3H, s, Me-18), 1.06 (1H, m, H-24), 1.20 (3H, s, Me-19), 1.31 (1H, m, H-23), 1.33 (1H, m, H-9), 1.36 (3H, s, Me-21), 1.61 (1H, m, H-12), 1.71 (1H, ddd, *J*=3.9, 11.8, 15.4 Hz, H-12), 1.76 (1H, dd, *J*=3.9, 12.5 Hz, H_{eq}-4), 1.96 (1H, m, H-8), 2.05 (1H, dd, *J*=11.2, 12.5 Hz, H_{ax}-4), 3.55 (1H, br s, H-6), 4.08 (1H, br m, H-3), 5.47 (1H, br s, H-16); ^{13}C NMR (125 MHz, $CDCl_3$) see Table 1.

Cytotoxicity Assays[19] Human promyelocytic leukemia cell line HL60, human skin melanoma cell line M14, and human breast carcinoma cell line MCF7 were obtained from ATCC (Rockville, MD) and maintained in culture in RPMI 1640 supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT) in an atmosphere of 5% CO_2 at 37 °C. Normal human PBLs were obtained from blood donated by healthy volunteers by ficoll hypaque density centrifugation. For HL60 cells and normal PBLs, 1×10^5 cells/well in a 96-well plate were exposed to different concentrations of compounds **1**—**10** for 24 h. M14 cells and MCF7 cells were plated in 96-well plates (2×10^4) for 24 h before the addition of compounds **1**—**10** with different concentrations. Medium was then aspirated and replaced with fresh RPMI 1640+10% FBS containing compounds **1**—**10** for 24 h. Equal concentration of DMSO was used in the control wells. Viability was determined by the MTT assay for HL60 cells and for PBLs, or by the crystal violet assay for M14 and MCF7 cells. For MTT assay, 10 μl of 5 mg/ml of MTT was added to each well and incubated for 4 h at 37 °C. Elution of the precipitate was performed with 200 μl of DMSO+10 μl of Tris–glycine buffer (0.1 M Tris, 0.1 M Glycine, pH 10.5, with 1 N NaOH). Cell viability was calculated from absorption values obtained at 570 nm using an automated enzyme-linked immunosorbent assay (ELISA) reader. For crystal violet assays, medium was aspirated and replaced for 10 min with 50 μl of 0.75% crystal violet in 50% ethanol, 0.25% NaCl, and 1.75% formaldehyde solution. Cells were then washed with water, air-dried, and the dye eluted with PBS+1% sodium dodecyl sulfate (SDS) solution. Cell viability was assessed by dye absorbance measured at 595 nm on an automated ELISA reader.

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