Cytotoxic Sterols from the Soft Coral *Nephthea erecta*

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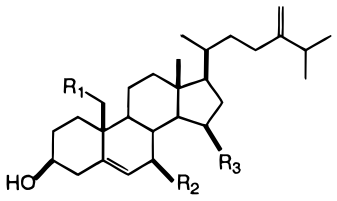
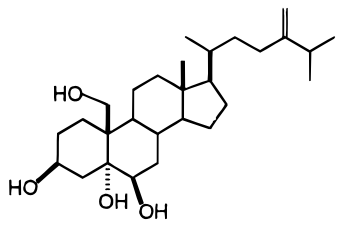
Two new cytotoxic sterols, 24-methylcholesta-5,24(28)-diene-3β,15β,19-triol (**1**) and 24-methylcholesta-5,24(28)-diene-3β,19-diol-7-one (**2**), as well as four cytotoxic sterols, 24-methylcholesta-5,24(28)-diene-3β,19-diol (**3**), 24-methylcholesta-5,24(28)-diene-3β,19-diol-7β-monoaetate (**4**), 24-methylcholesta-5,24(28)-diene-3β,7β,19-triol (**5**), and 24-methylcholesta-24(28)-ene-3β,5α,6β,19-tetraol (**6**), have been isolated from the soft coral *Nephthea erecta*. The structures of compounds **1** and **2** were determined by spectral analysis.

In our continuing search to discover bioactive substances from marine organisms, a sample of the soft coral *Nephthea erecta* Kükenthal (Nephtheidae) was investigated. Dichloromethane extracts of the soft coral showed significant cytotoxicity in cell cultures of A549 (human lung adenocarcinoma), HT-29 (human colon adenocarcinoma), KB (human epidermoid carcinoma), and P-388 (murine lymphocytic leukemia) cell lines as determined by standard procedures.[1,2] Bioassay-guided fractionation resulted in the isolation of two new cytotoxic sterols, 24-methylcholesta-5,24(28)-diene-3β,15β,19-triol (**1**) and 24-methylcholesta-5,24(28)-diene-3β,19-diol-7-one (**2**), as well as the four known sterols, 24-methylcholesta-5,24(28)-diene-3β,19-diol (**3**), 24-methylcholesta-5,24(28)-diene-3β,19-diol-7β-monoacetate (**4**), 24-methylcholesta-5,24(28)-diene-3β,7β,19-triol (**5**), and 24-methylcholesta-24(28)-ene-3β,5α,6β,19-tetraol (**6**), which were newly observed to have cytotoxic activity.



1 R_1 = OH R_2 = H R_3 = OH
2 R_1 = OH R_2 = O R_3 = H
3 R_1 = OH R_2 = H R_3 = H
4 R_1 = OH R_2 = OAc R_3 = H
5 R_1 = OH R_2 = OH R_3 = H



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24-Methylcholesta-5,24(28)-diene-3β,15β,19-triol (**1**), [α] −28.2° (*c* 0.34, MeOH), had the molecular formula $C_{28}H_{46}O_3$ as deduced from HREIMS, ^{13}C NMR, and DEPT spectra. The IR spectrum showed the presence of a hydroxyl group (3340 cm^{-1}) and a terminal methylene group (1640, 890 cm^{-1}). The presence of a primary hydroxy group and two secondary hydroxyl groups was shown by the ^{1}H NMR [3.62 (1H, d, J = 11.7 Hz), 3.85 (1H, d, J = 11.7 Hz), 3.56 (1H, m), 4.16 (1H, m)] and ^{13}C NMR (CD_3OD) [δ 63.7 (t), 70.8 (d), 72.5 (d)] spectra. The ^{1}H and ^{13}C NMR spectra also showed signals due to four methyl groups [^{1}H NMR 1.01 (3H, s), 0.96 (3H, d, J = 6.3 Hz), 1.02 (3H, d, J = 6.6 Hz), 1.04 (3H, d, J = 6.9 Hz)], a terminal methylene group [^{1}H NMR 4.66 (1H, br s), 4.72 (1H, br s), ^{13}C NMR 107.1 (t), 157.9 (s)], and a trisubstituted double bond [^{1}H NMR 5.74 (1H, t, J = 2.4 Hz), ^{13}C NMR 126.4 (d), 138.3 (s)]. This evidence suggested that **1** was a monohydroxy derivative of 24-methylcholesta-5,24(28)-diene-3β,19-diol[3] as shown by common ^{1}H and ^{13}C NMR signals for the A, B, and C rings and the side chain. The extra secondary hydroxyl group, on the D-ring, caused a γ-substitutent effect (−3.2 ppm) at C-8 (δ 30.2) and a δ_1-hydroxy substituent effect (+3.2 ppm) at C-18 (δ 15.4) as compared with **3** (C-8, δ 33.4; C-18, 12.2). The pyridine-induced deshielding at 18-H_3 [$\Delta\delta = \delta$(py-d_5) − δ($CDCl_3$) = + 0.40 ppm)] indicated that the 15-OH was β.[4] These results indicate unambiguously that compound **1** is 24-methylcholesta-5,14(28)-diene-3β,-15β,19-triol. The HMBC and NOESY spectra (Figure 1) of **1** confirmed this, showing the expected correlations.

24-Methylcholesta-5,24(28)-diene-3β,19-diol-7-one (**2**) was obtained as an amorphous colorless solid whose molecular formula, $C_{28}H_{44}O_3$, was established by HREIMS and ^{13}C NMR spectra. The UV spectrum of **2** indicated the presence of an α,β-unsaturated carbonyl group (241 nm, log ϵ = 4.56). The IR spectrum of **2** showed the presence of a terminal double bond (1650 and 890 cm^{-1}), hydroxyl group (3340 cm^{-1}), and α,β-unsaturated carbonyl group (1690 cm^{-1}). The presence of a primary and a secondary hydroxyl group was shown by ^{1}H NMR [δ 3.88 (1H, d, J = 11.4 Hz), 4.03 (1H, d, J = 11.4 Hz), 3.73 (1H, m)] and ^{13}C NMR [δ 70.4 (d), 63.8 (t)] spectra. ^{1}H and ^{13}C NMR spectra of **2** also showed signals due to four methyl groups [δ_H 0.73 (3H, s), 0.95 (3H, d, J = 6.6 Hz), 1.02 (3H, d, J = 6.6 Hz), 1.03 (3H, d, J = 6.9 Hz); δ_C 12.3 (q), 18.9 (q), 21.9 (q), 22.0 (q)], a

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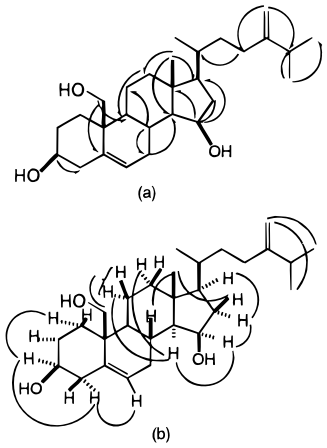

Figure 1. (a) HMBC correlations of compound **1**. (b) NOEST correlations of compound **1**.

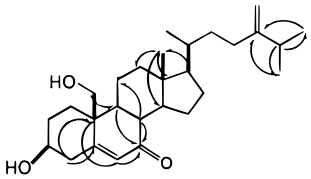

Figure 2. HMBC correlations of compound **2**.

terminal methylene [δ_H 4.66 (s), 4.71 (s); δ_C 106.0 (t), 156.8 (s)], and a trisubstituted olefin containing an α,β-unsaturated ketone [δ_H 5.93 (s); δ_C 160.0 (s), 129.6 (d), 202.6 (s)]. These spectral data suggested that **2** was a monohydroxy derivative of 3β-hydroxy-24-methylcholesta-5,24(28)-diene-7-one,[5] and the two compounds showed common ^{1}H and ^{13}C NMR signals for the C- and D-rings and for C-2 to C-4. The hydroxyl group on C-19 caused a γ-substitutent effect [−3.3 ppm at C-1 (δ 33.1), −5.4 ppm at C-5 (δ 160.0)] and a δ_1-hydroxy substituent effect (+3.5 ppm) at C-6 (δ 129.6), as compared with 3β-hydroxy-24-methylcholesta-5,24(28)-dien-7-one (C-1, δ 36.4; C-5, δ 165.4, C-6, 126.1). These results indicate unambiguously that compound **2** is 24-methylene-cholesta-5-ene-3β,19-diol-7-one. The HMBC spectrum confirmed the structure of **2** (Figure 2).

The previously known compounds, 24-methylcholesta-5,24(28)-diene-3β,19-diol (**3**),[3] 24-methylcholesta-5,24-(28)-diene-3β,19-diol-7β-monoacetate (**4**),[6] 24-methyl-cholesta-5,24(28)-diene-3β,7β,19-triol (**5**),[6] and 24-meth-ylcholesta-24(28)-ene-3β,5α,6β,19-tetraol (**6**)[7] were iden-tified by their $[\alpha]_D$, IR, UV, ^{1}H NMR, ^{13}C NMR, and mass spectra.

Compounds **1** and **3**−**6** exhibited significant cytotox-icity toward the growth of A549, HT-29, KB, and P-388 cells. Compound **2** exhibited significant cytotoxicity toward the growth of P-388 and HT-29 cells. The cytotoxicity values for compounds **1**−**6** are presented in Table 3.

Experimental Section

General Experimental Procedures. Melting points were determined using a Yanagimoto micro-melting point apparatus and are reported uncorrected. Optical rotations were determined on a JASCO DIP-181 pola-rimeter. UV spectra were obtained on a Shimadzu UV-160A spectrophotometer, and IR spectra were recorded on a Hitachi 26−30 spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded with a Varian Unity Plus 400 NMR spectrometer at 400 and 100.6 MHz, respec-tively, in $CDCl_3$ using TMS as internal standard unless otherwise stated. EIMS were obtained with a JEOL JMS−SX/SX 102A mass spectrometer at 70 ev. Si gel 60 (Merck, 230−400 mesh) was used for column chro-matography, precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.50 mm) were used for preparative TLC.

Table 1. ^{1}H NMR Data of **1**−**3**

	compound			
proton	**1**[a]	**1**[b]	**2**[a]	**3**[a]
H-1α	0.98 m		1.13 m	
H-1β	2.15 m		2.02 m	
H-2α	2.17 m		1.80 m	
H-2β	1.76 m		1.46 m	
H-3	3.56 m	4.00 m	3.73 m	3.56 m
H-4α	2.26 m		2.51 m	
H-4β			2.33 m	
H-6	5.76 m	6.29 br s	5.93 s	5.74 m
H-7	1.87 m			
H-8	2.14 m		2.83 m	
H-9	0.93 m		1.60 m	
H-11α	1.56 m		1.54 m	
H-11β	1.69 m		1.68 mn	
H-12α	1.09 m		1.99 m	
H-12β	1.98 m		1.98 m	
H-14	0.76 m		1.12 m	
H-15	4.16 m	4.48 br s	1.19 m	
H-15			1.20 m	
H-16α	2.37 m		1.18 m	
H-16β	1.29 m		1.17 m	
H-17	1.09 m		1.04 m	
H-18	1.01 s	1.41 s	0.73 s	0.73 s
H-19	3.62 (d, 11.7)[c]	4.02 (d, 10.8)	3.88 (d, 11.7)	3.60 (d, 11.7)
	3.85 (d, 11.7)	4.29 (d, 10.8)	4.03 (d, 11.7)	3.82 (d, 11.7)
H-20	1.54 m		1.33 m	
H-21	0.96 (d, 6.3)	1.02 (d, 6.3)	0.95 (d, 6.6)	0.94 (d, 6.3)
H-22	1.15 m		1.36 m	
H-23	1.41 m, 1.76 m		1.97 m, 1.76 m	
H-26	1.02 (d, 6.6)	1.04 (d, 6.6)	1.02 (d, 6.6)	1.02 (d, 6.9)
H-27	1.04 (d, 6.9)	1.06 (d, 6.9)	1.03 (d, 6.9)	1.03 (d, 6.9)
H-28	4.66 br s, 4.72 br s	4.86 br s, 4.88 br s	4.66 br s, 4.71 br s	4.65 br s, 4.71 br s

[a] Chemical shifts were determined at 400 MHz in CD_3OD. The values are in ppm downfield from TMS. [b]Chemical shifts were determined at 300 MHz in pyridine-d_5. The values are in ppm downfield from TMS. [c] J values in Hz are in parentheses.

Animal Materials. The soft coral *N. erecta* was collected at Green Island, Taiwan, at a depth of 10 m in June 1994, and was stored in a freezer for 3 months until extraction. The voucher specimen (NSUGN-1003) was deposited in the Department of Marine Resources, National Sun Yat-sen University.

Extraction and Isolation. The bodies of the soft coral *N. erecta* were freeze-dried to give 566 g of a solid, which was extracted with CH_2Cl_2 (1 L × 3). After removal of solvent in vacuo, the residue (50 g) was chromatographed over Si gel 60 (open column, 60 cm × 3.5 cm) using $CHCl_3$ and $CHCl_3$−MeOH mixtures of increasing polarity. Elution by $CHCl_3$−MeOH (9:1) afforded fractions containing sterols **1** and **2**, which were separated by Si gel column chromatography (30 cm × 1 cm) using EtOAc−*n*−hexane (1:6 → 5:1) as eluting solvent system. Elution by $CHCl_3$−MeOH (97:3) af-forded fractions containing sterols **3** and **4**, which were separated by column chromatography over Si gel (30 cm × 1 cm) with CH_2Cl_2−Me_2CO (3:1 → 1:3) as eluting solvents. Elution by $CHCl_3$−MeOH (17:1) afforded fractions containing sterols **5** and **6**, which were sepa-rated by column chromatography over Si gel with *n*-hexanes−EtOAc (4:1→ 1:5) as eluting solvents. Com-

Table 2. ^{13}C NMR Data of **1**−**3**

carbon	compound **1**[a]	**2**[b]	**3**[b]
C-1	34.5 t	33.1 t	33.8 t
C-2	32.2 t	31.6 t	31.2t
C-3	70.8 d	70.4 d	71.5 d
C-4	43.1 t	42.1 t	42.3 t
C-5	138.3 s	160.0 s	135.5 s
C-6	126.4 d	129.6 d	127.4 d
C-7	32.8 t	202.6 s	32.0 t
C-8	30.2 d	46.9 d	33.4 d
C-9	52.7 d	50.3 d	50.4 d
C-10	43.6 s	43.2 s	41.5 s
C-11	23.2 t	21.9 t	21.8 t
C-12	42.8 t	39.2 t	40.4 t
C-13	43.6 s	43.5 s	42.6 s
C-14	63.6 d	51.3 d	57.6 d
C-15	72.5 d	26.1 t	24.1 t
C-16	42.2 t	28.6 t	28.2 t
C-17	57.8 d	54.7 d	55.9 d
C-18	15.4 q	12.3 q	12.2 q
C-19	63.7 t	63.8 t	62.7 t
C-20	36.9 d	35.7 d	35.7 d
C-21	19.4 q	18.9 q	18.7 q
C-22	36.1 t	34.7 t	34.7 t
C-23	32.0 t	31.0 t	31.0 t
C-24	157.9 s	156.8 s	156.9 s
C-25	35.1 d	33.8 t	33.8 d
C-26	22.6 q	21.9 q	21.8 q
C-27	22.5 q	22.0 q	22.0 q
C-28	107.1 t	106.0 t	106.0 t

[a] Chemical shifts were determined at 100.6 Hz in CD_3OD. The values are in ppm downfield from TMS. [b]Chemical shifts were determined at 100.6 Hz in $CDCl_3$. The values are in ppm downfield from TMS.

Table 3. Cytotoxicitiy[a] of **1**−**6** (n = 8)'

compound	cell line [ED_{50} (μg /mL)] A549	HT-29	KB	P-388
1	0.41	0.17	0.60	0.07
2	4.09	3.34	>50	0.40
3	1.76	1.31	1.10	0.45
4	0.81	0.87	0.38	0.42
5	0.69	0.72	0.58	0.24
6	0.81	0.93	0.39	0.34

[a] For significant activity of pure compounds, an ED_{50} of 4.0 μg/mL is required.[1]

pounds **3**−**6** were identified by comparison of their $[\alpha]_D$, IR, UV, ^1H NMR, ^{13}C NMR, and mass spectral data with literature data.[3,6,7]

24-Methylenecholesta-5-ene-3*β*,15*β*,19-triol (1): 6.8 mg; colorless prisms; mp 204−205 °C; $[\alpha]^{25}_D$ −28.8° (*c* 0.34, MeOH); UV (MeOH) λ_{max} (log ϵ) 208 (4.4) nm; IR (KBr) ν_{max} 3340, 1640, 890 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; EIMS *m/z* $[M]^+$ 430 (0.3), 412 (7), 382 (35), 364 (10), 349 (5), 321 (1), 298 (3), 265 (5), 239 (32), 225 (4),213 (8), 197 (12), 185 (25), 157 (16), 145 (40), 131 (34), 91 (52), 69 (100); HREIMS *m/z* 412.3338 $[M^+ - H_2O]$ (calcd for $C_{28}H_{44}O_2$, 412.3330).

24-Methylenecholesta-5-ene-3*β*,19-diol-7-one (2): 3.7 mg; colorless prisms; mp 165−167 °C; $[\alpha]^{25}_D$ −19.1° (*c* 0.13, MeOH); UV (MeOH) λ_{max} (log ϵ) 241 (4.56) nm; IR (KBr) ν_{max} 3340, 1690, 1040, 890 cm^{-1}; ^1H NMR, see Table 1; ^{13}C NMR, see Table 2; EIMS *m/z* $[M]^+$ 428 (2), 410 (28), 398 (9), 380 (6), 344 (2), 326 (19), 284 (7), 227 (10), 161 (21), 160 (42), 91 (82), 69 (99) 55 (100); HREIMS *m/z* 428.3293 (calcd for $C_{28}H_{44}O_3$, 428.3279).

Cytotoxicity Testing. KB and P-388 cells were donated by Prof. J. M. Pezzuto, University of Illinois at Chicago; A549 and HT-29 were purchased from the American Type Culture Collection. The P-388 cells were cultured in Fisher's medium supplemented with 10% heat-inactivated fetal calf serum (FCS). The KB cells were maintained in Basal Medium Eagle containing 10% heat-inactivated FCS. The A549 cell line was cultured in Eagle Minimum Essential Medium containing Earle's salts and supplemented with 0.1 mM of nonessential amino acids and 10% heat-inactivated FCS. The HT-29 cell lines were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium containing 10% heat-inactivated FCS. All the cell lines were maintained in an incubator at 37 °C in humidified air containing 5% CO_2. For routine cytotoxicity assay, all four cell lines were adapted to one single medium, RPMI 1640 supplemented with 10% FCS and 1 mM glutamate. To measure the cytotoxic activities of pure compounds or crude fractions against A549, HT-29, KB, and P-388, each cell line was initiated at 750, 750, 2000, and 1500 cells/well, respectively, in 96-well microtiter plates. Three to eight concentrations encompassing an 8- to 128-fold range were evaluated on each cell line. A549, HT-29, KB, and P-388 cells were enumerated using MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] after the exposure to test samples for 6, 6, 3, and 3 days, respectively. MTT 50 mL of 1 mg/mL was added to each well, and plates were incubated at 37 °C for a further 4 h. Supernatant was aspirated with a Dynatech automatic washer. Formazan crystals were redissolved in DMSO (Merck) for 10 min with shaking, and the plate was read immediately on a microtiter plate reader (Dynatech) at a wavelength of 540 nm. The ED_{50} value was defined as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cell in the MTT assay.[8]

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