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Three New 9,11-Secosterols from the Formosan Soft Coral *Sinularia leptoclados*

Shi-Yie Cheng,[1] Hwa-Pyng Chen,[1] Shang-Kwei Wang,*[2] and Chang-Yih Duh*[1,3]

[1]Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Kaohsiung 804, Taiwan

[2]Department of Microbiology, Kaohsiung Medical University, Kaohsiung 807, Taiwan

[3]Asia-Pacific Ocean Research Center, National Sun Yat-sen University, Kaohsiung 804, Taiwan

Received February 14, 2011; E-mail: yihduh@mail.nsysu.edu.tw

Chemical investigations of the Formosan soft coral *Sinularia leptoclados* resulted in the characterization of three new 9,11-secosterols, designated as leptosterols A–C (**1**–**3**), and six known 9,11-secosterols **5**–**10**. Additionally, a cyclized 9,11-secosterol enol-ether **4** was obtained by allowing **5** to stand in $CDCl_3$ overnight during NMR experiments. The structures of **1**–**4** were elucidated through extensive spectroscopic analyses. The cytotoxicity against selected cancer cells and antiviral activity against human cytomegalovirus (HCMV) of **1**–**10** were evaluated in vitro.

9,11-Secosterols have been obtained from various marine organisms such as soft corals,[1–8] gorgonians,[1,2,9,10] sponges,[2,11–16] an ascidian *Aplidium conicum*,[17] and a mollusk *Trimusculus costatus*.[18] These types of steroids were reported to exhibit various biological activities including cytotoxic,[3,4,8,9,16,17] antihistaminic,[13] anti-inflammatory,[9] antimicrobial,[14] antiproliferative,[6,9] and ichthyotoxic properties.[14] In the course of our ongoing search for bioactive substances from marine organisms, chromatographic fractionation of the Formosan soft coral *Sinularia leptoclados* (Ehrenberg, 1834) resulted in the isolation of three new 9,11-secosterols, designated as leptosterols A–C (**1**–**3**) and six previously characterized 9,11-secosterols, comprising 3β,11-dihydroxy-9,11-secocholest-5-en-9-one (**5**), 3β,11-dihydroxy-9,11-secogorgost-5-en-9-one (**6**), 3β,11-dihydroxy-24-methylene-9,11-secocholest-5-en-9-one (**7**), 3β-hydroxy-11-acetoxy-24-methylene-9,11-secocholest-5-en-9-one (**8**), (24*S*)-3β,11-dihydroxy-24-methyl-9,11-secocholest-5-en-9-one (**9**), and (24*S*)-11-acetoxy-3β-hydroxy-24-methyl-9,11-secocholest-5-en-9-one (**10**),[7] while **4** is an artifact obtained subsequently by allowing **5** in $CDCl_3$ during the process of the NMR experiments. Compound **5** was fully transformed into **4**, suggesting acid-catalyzed cyclization and dehydration occurred during the NMR experiments. The details of the isolation and structure elucidation of these compounds are discussed in the paper. Furthermore, compounds **1**–**10** were evaluated in vitro for cytotoxicity against P-388 (mouse lymphocytic leukemia), A-459 (human lung carcinoma), and HT-29 (human colon adenocarcinoma) cancer cell lines, as well as antiviral activity against HCMV.

Results and Discussion

The sliced bodies of the Formosan soft coral *S. leptoclados* were exhaustively extracted with acetone. The combined acetone extracts were concentrated to a brown gum, which was partitioned between H_2O and EtOAc. The EtOAc-soluble portion was concentrated under reduced pressure and the residue was fractionated by silica gel 60 and Sephadex LH-20 column chromatography. The resulting fractions were further purified by RP-18 HPLC to yield compounds **1**–**3** and **5**–**10** (Figure 1). Of these, compounds **5**–**10** are known, and were confirmed by comparison of their spectroscopic data with literature values.[7]

Compound **1** was isolated as a white amorphous powder, showing a pseudomolecular ion peak at m/z 453.3343 $[M + Na]^+$ in the HR-ESI-MS consistent with the molecular formula $C_{28}H_{46}O_3$ (calcd for 453.3344), requiring six degrees of unsaturation. The presences of an oxymethylene and a ketocarbonyl carbon were confirmed by the ^{1}H NMR (Table 1) [δ_H 3.87 (m, H-11a) and 3.70 (m, H-11b)] and ^{13}C NMR (Table 2) [δ_C 59.4 (CH_2) and 217.7 (qC)] data, as well as from the IR absorption at 3349 and 1713 cm^{-1}. The diagnostic NMR signals of a 9,11-secosterol were confirmed by the ^{1}H–^{1}H COSY correlation from H_2-11 to H_2-12 as well as HMBC correlations from H_3-18 to C-12, C-13, C-14, and C-17; from H_3-19 to C-1, C-5, C-9, and C-10. The NMR features of **1** were analogous to those of **9**,[7] except for the presence of an *E* double bond [δ_H 5.23 (1H, dd, $J = 15.2$, 8.0 Hz) and 5.19 (1H, dd, $J = 15.2$, 7.2 Hz); δ_C 134.4 (CH) and 132.8 (CH)] in the side chain. The relative stereochemistries at C-3, C-8, C-10, C-13, C-14, C-17, and C-20 in **1** were found to be the same as those of **9** (Figure 2). The absolute configuration of C-24 was determined to be *R* by comparison with the reported ^{13}C NMR data of acanthovagasteroid D, which was isolated from the gorgonian *Acanthogorgia vagae* Aurivillius.[19,20] It is suggested that the C-28 resonance appears at δ_C 17.6 $\pm$ 0.1 in the 24*R* epimer, while a 0.4 ppm downfield shift should be observed in the 24*S* epimer (δ_C 18.0).[20] On the basis of the above-mentioned findings, the structure of leptosecosterol A (**1**) was satisfactorily consistent with the structure shown as (24*R*)-3β,11-dihydroxy-24-methyl-9,11-secocholest-5,22*E*-dien-9-one.

(24*R*)-11-Acetoxy-3β-hydroxy-24-methyl-9,11-secocholest-5,22*E*-dien-9-one (**2**) appeared as a white amorphous powder

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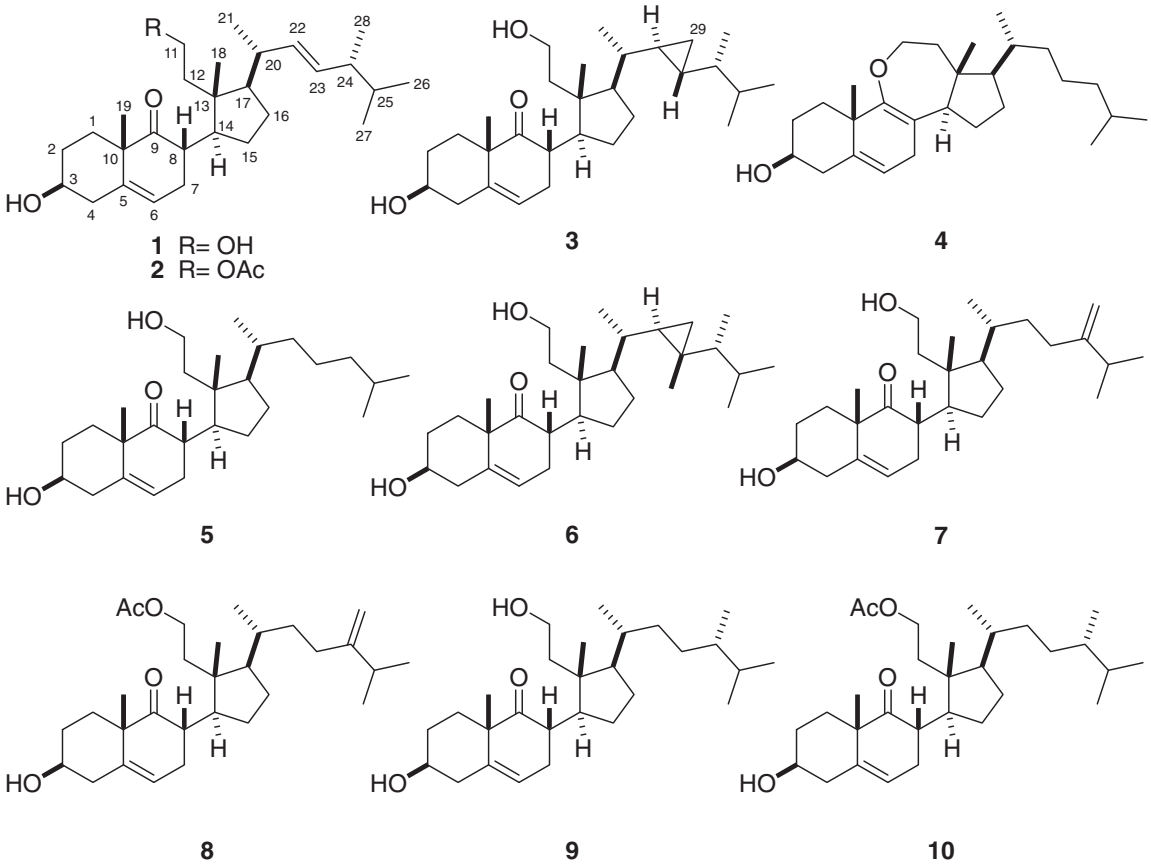

Figure 1. Structures of compounds **1**–**10**.

like **1**. Careful inspection of the 2D NMR spectroscopic data of **2** led to the establishment of the same nucleus as that of **1**. The NMR spectroscopic data (Tables 1 and 2) of **2** were analogous to those of **1**, except for NMR signals due to the additional acetoxy group in **2** and the corresponding downfield shift of the H_2-11 signals from δ_H 3.87 and 3.70 in **1** to 4.20 and 4.17 in **2**. The location of the acetoxy group was identified by the crucial HMBC correlations from the methylene protons (H_2-11) to the carbonyl carbon of 11-OAc, securing the structure of **2**, which was designated as leptosterol B.

The molecular formula of 3β,11-dihydroxy-23-demethyl-9,11-secogorgost-5-en-9-one (**3**) was determined to be $C_{29}H_{48}O_3$ from the $[M + Na]^+$ ion peak at m/z 467.3504 in HR-ESI-MS, accounting for six degrees of unsaturation. The NMR spectroscopic data (Tables 1 and 2) of **3** resembled those of **6**, except for the absence of the methyl at C-23. The NMR data (Tables 1 and 2) revealed that the presence of a 1,2-disubstituted cyclopropane-containing [δ_H 0.15 (2H, m, H-29), 0.35 (1H, m, H-22), and 0.51 (1H, m, H-23); δ_C 10.5 (CH_2, C-29), 24.4 (CH, C-22), and 23.9 (CH, C-23)] of **3** was very compatible with a previously characterized sterol, stoloniferone-d. The configurations of C-22, C-23, and C-24 in the side chain were determined as all *R* by comparison of the ^{13}C NMR data with those of stoloniferone-d, whose stereochemistry has been unambiguously established by X-ray crystallographic analysis.[21] According to the aforementioned observations, the structure of leptosterol C (**3**) was determined unambiguously.

Compound **4** was obtained subsequently by allowing **5** to stand in $CDCl_3$ during the NMR experiments. A molecular formula of $C_{27}H_{44}O_2$ for **4** was determined by the HR-ESI-MS, indicating six degrees of unsaturation. The NMR data (Tables 1 and 2) of **4** revealed the presence of an oxepene moiety [δ_H 4.02 (1H, dt, $J = 12.4$, 4.0 Hz, H-11α) and 3.65 (1H, td, $J = 12.4$, 2.0 Hz, H-11β); δ_C 68.0 (CH_2, C-11), 114.9 (qC, C-8), and 156.7 (qC, C-9)], which was further confirmed by the ^{1}H–^{1}H COSY between H_2-11 and H_2-12, together with the crucial HMBC correlations from H_2-11 to C-9, C-12, and C-13; from H-14 to C-8, C-9, C-12, and C-13 (Figure 3). In addition, stellattasterenol obtained from the sponge *Eurypongia arenaria* was previously reported to possess the oxepene moiety in ring C.[16] Therefore, the structure of **4** was established.

The structures of six additional 9,11-secosterols were confirmed by comparing their physical and spectroscopic data with those of metabolites isolated from a soft coral *Sinularia*, leading to the identification of **5**–**10**.[7] It is worthwhile to mention that H-8 in **6** was previously reported to be β-oriented by CD spectral data and X-ray analysis.[7] Accordingly, H-8 is also β-oriented in **1**–**3** due to a negative Cotton effect around λ_{max} 280–295 nm in the CD spectra (Figure 4).[7]

As noted in the introduction, a large number of unique 9,11-secosterols and their analogs exhibited remarkable biological activities. These isolated 9,11-secosterols **1**–**3** and **5**–**10** in the present study were evaluated in vitro for cytotoxicity against P-388, A-459, and HT-29 cancer cell lines using the MTT assay, and antiviral activity against HCMV. Preliminary

Table 1. ^{1}H NMR Spectroscopic Data of **1**–**4**[a)]

H#	**1**	**2**	**3**	**4**
1	α: 1.51 m; β: 1.83 m	α: 1.50 m; β: 1.84 m	α: 1.50 m; β: 1.84 m	α: 1.34 m; β: 1.99 m
2	α: 1.95 m; β: 1.53 m	α: 1.92 m; β: 1.50 m	α: 1.95 m; β: 1.53 m	α: 1.88 m; β: 1.57 m
3	3.50 m	3.51 m	3.51 m	3.56 m
4	α: 2.41 m; β: 2.26 m	α: 2.41 m; β: 2.26 m	α: 2.43 m; β: 2.26 m	α: 2.34 m; β: 2.25 m
6	5.48 d (5.6)[b)]	5.48 d (5.6)	5.49 d (6.0)	5.34 br s
7	α: 2.02 m	α: 2.04 m	α: 2.04 m	α: 2.69 d (19.2)
	β: 2.44 m	β: 2.39 m	β: 2.44 m	β: 2.61 d (19.2)
8	3.03 dt (12.4, 6.8)	2.98 dt (12.4, 6.0)	3.04 dt (12.4, 6.8)	
11	a: 3.87 m	a: 4.20 m	a: 3.88 m	α: 4.02 dt (12.4, 4.0)
	b: 3.70 m	b: 4.17 m	b: 3.70 m	β: 3.65 td (12.4, 2.0)
12	a: 1.66 m; b: 1.36 m	α: 1.73 m; β: 1.48 m	α: 1.72 m; β: 1.32 m	α: 1.71 m; β: 1.99 m
14	2.61 m	2.46 m	2.64 m	2.87 t (10.4)
15	α: 1.57 m; β: 1.30 m	α: 1.57 m; β: 1.32 m	α: 1.56 m; β: 1.30 m	α: 1.35 m; β: 1.63 m
16	α: 1.70 m; β: 1.33 m	α: 1.67 m; β: 1.34 m	α: 2.00 m; β: 1.45 m	α: 1.83 m; β: 1.35 m
17	1.71 m	1.67 m	1.76 m	1.35 m
18	0.68 s	0.71 s	0.67 s	0.77 s
19	1.38 s	1.36 s	1.39 s	1.30 s
20	2.16 m	2.19 m	0.94 m	1.40 m
21	1.03 d (6.8)	1.04 d (6.8)	0.92 d (6.8)	0.94 d (6.4)
22	5.23 dd (15.2, 8.0)	5.26 dd (15.2, 8.0)	0.35 m	1.37 m; 1.02 m
23	5.19 dd (15.2, 7.2)	5.20 dd (15.2, 7.2)	0.51 m	1.35 m; 1.13 m
24	1.86 m	1.84 m	0.54 m	1.13 m
25	1.47 m	1.45 m	1.64 m	1.52 m
26	0.81 d (6.8)	0.81 d (6.8)	0.86 d (6.8)	0.87 d (6.8)
27	0.83 d (6.8)	0.84 d (6.8)	0.89 d (6.8)	0.87 d (6.8)
28	0.90 d (6.8)	0.91 d (6.8)	0.90 d (6.8)	
29			0.15 m	
11-OAc		2.02 s		

a) Spectra were measured in $CDCl_3$ (400 MHz). b) J values (in Hz) in parentheses.

cytotoxicity screening revealed that **2** and **4** exhibited significant cytotoxicty against P-388 with ED_{50} of 3.7 and 3.2 $\mu g\,mL^{-1}$, respectively. The other tested metabolites were not cytotoxic to P-388, A549, and HT-29 cell lines. The anticancer agent mithramycin was used as the positive control and exhibited ED_{50} of 0.06, 0.08, and 0.07 μM against P-388, HT-29, and A549 cells, respectively. The results for antiviral activity revealed that **1**–**10** did not exhibit activity against HCMV.

Experimental

General Experimental Procedures. Optical rotations were determined with a JASCO P1020 digital polarimeter. Ultraviolet (UV) and infrared (IR) spectra were obtained on JASCO V-650 and JASCO FT/IR-4100 spectrophotometers, respectively. The NMR spectra were recorded on a Varian MR 400 NMR spectrometer at 400 MHz for ^{1}H and 100 MHz for ^{13}C or on a Varian Unity INOVA 500 FT-NMR spectrometer at 500 MHz for ^{1}H and 125 MHz for ^{13}C, respectively. Chemical shifts are expressed in δ (ppm) referring to the solvent peaks δ_H 7.27 and δ_C 77.0 for $CDCl_3$, respectively, and coupling constants are expressed in Hz. ESI-MS were recorded by ESI FT-MS on a Bruker APEX II mass spectrometer. Silica gel 60 (Merck, Germany, 230–400 mesh) and LiChroprep RP-18 (Merck, 40–63 μm) were used for column chromatography. Precoated silica gel plates (Merck, Kieselgel 60 F_{254}, 0.25 mm) and precoated RP-18 F_{254s} plates (Merck) were used for thin-layer chromatography (TLC) analysis. High-performance liquid chromatography (HPLC) was carried out using a Hitachi L-7100 pump equipped with a Hitachi L-7400 UV detector at 220 nm together with a semi-preparative reversed-phase column (Merck, Hibar LiChrospher RP-18e, 5 μm, 250 $\times$ 25 mm).

Animal Material. The Formosan soft coral *Sinularia leptoclados* was collected by hand using SCUBA from the inner coral reef at a depth of around 6–8 m in Dongsha Atoll in April 2007. The sample was immediately frozen after collection and stored in a freezer for 2 months until extraction. This soft coral was identified by Prof. Chang-Feng Dai, Institute of Oceanography, National Taiwan University, Taiwan. A voucher specimen (TS-21) was deposited in the Department of Marine Biotechnology and Resources, National Sun Yat-sen University, Taiwan.

Extraction and Isolation. The freeze-dried soft coral *S. leptoclados* (3.0 kg) was chopped into small pieces and extracted with acetone for 24 h at room temperature. The quantity of solvent used for each extraction (2.0 L) was at least three times the amount of the soft coral material used. The combined extracts were concentrated in vacuo (under 30 °C) to obtain a dry crude extract, which was suspended in water and extracted with EtOAc. The EtOAc phase was evaporated to dryness in vacuo to give a brown residue (25.0 g). The resulting EtOAc residue was subjected to Si-60 column chromatography

Table 2. ^{13}C NMR Spectroscopic Data of **1**–**4**[a)]

C#	1	2	3	4
1	31.0 (CH)[b)]	31.1 (CH_2)	31.1 (CH_2)	34.1 (CH_2)
2	30.8 (CH_2)	30.8 (CH_2)	30.8 (CH_2)	31.3 (CH_2)
3	71.4 (CH)	71.5 (CH)	71.4 (CH)	72.0 (CH)
4	40.6 (CH_2)	40.7 (CH_2)	40.6 (CH_2)	41.0 (CH_2)
5	140.4 (qC)	140.3 (qC)	140.4 (qC)	138.2 (qC)
6	121.5 (CH)	121.4 (CH)	121.5 (CH)	118.6 (CH)
7	33.1 (CH_2)	32.3 (CH_2)	33.0 (CH_2)	30.3 (CH_2)
8	43.8 (CH)	42.7 (CH)	43.5 (CH)	114.9 (qC)
9	217.7 (qC)	215.3 (qC)	217.6 (qC)	156.7 (qC)
10	48.4 (qC)	48.0 (qC)	48.4 (qC)	39.4 (qC)
11	59.4 (CH_2)	61.5 (CH_2)	59.4 (CH_2)	68.0 (CH_2)
12	40.2 (CH_2)	36.4 (CH_2)	40.2 (CH_2)	46.1 (CH_2)
13	45.5 (qC)	45.3 (qC)	45.6 (qC)	42.5 (qC)
14	42.1 (CH)	41.7 (CH)	41.6 (CH)	50.3 (CH)
15	24.6 (CH_2)	24.0 (CH_2)	24.4 (CH_2)	24.1 (CH_2)
16	24.7 (CH_2)	24.9 (CH_2)	26.7 (CH_2)	26.9 (CH_2)
17	49.4 (CH)	49.9 (CH)	50.2 (CH)	57.0 (CH)
18	17.6 (CH_3)	17.5 (CH_3)	17.2 (CH_3)	12.1 (CH_3)
19	22.8 (CH_3)	23.0 (CH_3)	22.9 (CH_3)	21.5 (CH_3)
20	38.1 (CH)	38.2 (CH)	38.4 (CH)	35.4 (CH)
21	21.9 (CH_3)	22.0 (CH_3)	18.8 (CH_3)	19.6 (CH_3)
22	134.4 (CH)	134.3 (CH)	24.4 (CH)	35.9 (CH_2)
23	132.8 (CH)	132.9 (CH)	23.9 (CH)	24.2 (CH_2)
24	43.0 (CH)	43.1 (CH)	44.8 (CH)	39.5 (CH_2)
25	33.1 (CH)	33.1 (CH)	32.8 (CH)	28.0 (CH)
26	19.7 (CH_3)	19.7 (CH_3)	18.5 (CH_3)	22.6 (CH_3)
27	20.0 (CH_3)	20.0 (CH_3)	20.7 (CH_3)	22.8 (CH_3)
28	17.5 (CH_3)	17.6 (CH_3)	15.7 (CH_3)	
29			10.5 (CH_2)	
11-OAc		171.2 (qC)		
		21.2 (CH_3)		

a) Spectra were measured in $CDCl_3$ (100 MHz). b) Multiplicities are deduced by HSQC and DEPT experiments.

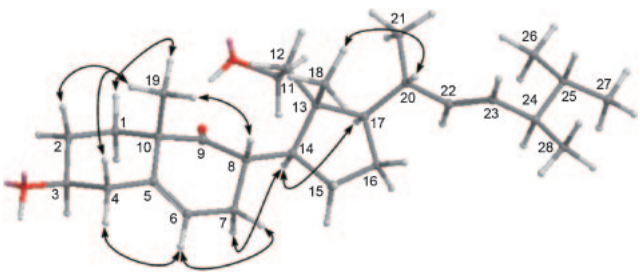

Figure 2. Selected NOESY correlations of **1**.

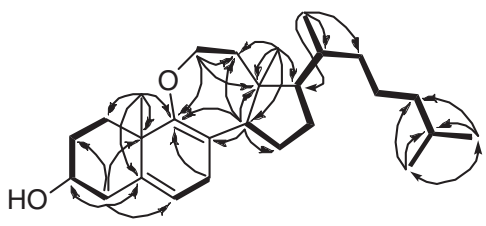

Figure 3. Selected ^{1}H–^{1}H COSY (—) and HMBC (→) correlations of **4**.

using a stepwise gradient mixture of *n*-hexane–EtOAc–MeOH for elution and separated into 42 fractions on the basis of TLC and ^{1}H NMR analyses. Fraction 18 (0.9 g) eluted with *n*-hexane–EtOAc (1:6) was chromatographed over Si-60 gel using *n*-hexane–EtOAc mixtures of increasing polarity for elution to afford five subfractions. A subfraction 18–2 (179 mg) eluted with *n*-hexane–EtOAc (1:2) was fractionated over Sephadex LH-20 (100% MeOH) to produce a mixture (50 mg) that was further purified by HPLC (RP-18) using 90% MeOH in H_2O as a mobile phase to give **2** (1 mg), **8** (10 mg), and **10** (8 mg). In addition, fraction 21 (0.5 g) eluted with 100% EtOAc was subjected to Si-60 column chromatography using *n*-hexane–EtOAc mixtures of increasing polarity for elution to afford six subfractions. A subfraction 21–3 (366 mg) eluted with *n*-hexane–EtOAc (1:4) was subjected to Si-60 column chromatography using *n*-hexane–EtOAc (1:2) to discover a subfraction (210 mg). In turn, the subfraction was fractionated over Sephadex LH-20 (100% MeOH) to produce a mixture (149 mg) that was further separated on HPLC (RP-18) with 90% MeOH in H_2O as a mobile phase to afford **1** (8 mg), **3** (3 mg), **5** (3 mg), **6** (8 mg), **7** (39 mg), and **9** (20 mg).

Leptosterol A (1): White amorphous powder; $[\alpha]_D^{25} = -60$ (*c* 0.1, $CHCl_3$); CD (1.47×10^{-4} M, MeOH): λ_{max} (θ) 206 (+12177), 220 (−7458), 292 (−9240) nm; IR (KBr): ν_{max} 3349, 2956, 2929, 1713, 1628, 1572, 1456, 1373, 1089, 1049, 824 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 2; HR-ESI-MS: *m*/*z* 453.3343 $[M + Na]^+$ (calcd for $C_{28}H_{46}O_3Na$, 453.3344).

Leptosterol B (2): White amorphous powder; $[\alpha]_D^{25} = -66$ (*c* 0.1, $CHCl_3$); CD (1.35×10^{-4} M, MeOH): λ_{max} (θ) 205 (+11748), 220 (−8019), 293 (−10725) nm; IR (KBr): ν_{max} 3376, 2956, 2923, 1737, 1713, 1634, 1572, 1459, 1370, 1049, 824 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 2; HR-ESI-MS: *m*/*z* 495.3452 $[M + Na]^+$ (calcd for $C_{30}H_{48}O_4Na$, 495.3450).

Leptosterol C (3): White amorphous powder; $[\alpha]_D^{25} = -56$ (*c* 0.1, $CHCl_3$); CD (1.43×10^{-4} M, MeOH): λ_{max} (θ) 204 (+9174), 220 (−6270), 293 (−8712) nm; IR (KBr): ν_{max} 3368, 2956, 2930, 1710, 1631, 1576, 1454, 1379, 1088, 1052, 822 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 2; HR-ESI-MS: *m*/*z* 467.3504 $[M + Na]^+$ (calcd for $C_{29}H_{48}O_3Na$, 467.3501).

9,11-Epoxy-9,11-secocholest-5,8(9)-dien-3β-ol (4): White powder; $[\alpha]_D^{25} = +37$ (*c* 0.1, $CHCl_3$); IR (KBr): ν_{max} 3394, 2956, 2937, 1666, 1637, 1463, 1373, 1044, 958, 797 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz), see Table 1; ^{13}C NMR ($CDCl_3$, 100 MHz), see Table 2; HR-ESI-MS: *m*/*z* 423.3239 $[M + Na]^+$ (calcd for $C_{27}H_{44}O_2Na$, 423.3236).

Cytotoxicity Assay. Cytotoxicity was determined against P-388 (mouse lymphocytic leukemia), HT-29 (human colon adenocarcinoma), and A-549 (human lung epithelial carcinoma) tumor cells using a modification of the MTT colorimetric method. The provision of the P-388 cell line was supported by J. M. Pezzuto, formerly of the Department of Medicinal Chemistry and Pharmacognosy, University of Illinois at Chicago. HT-29 and A-549 cell lines were purchased from the American Type Culture Collection. The experimental details of this assay were carried out according to a previously described procedure.[22,23]

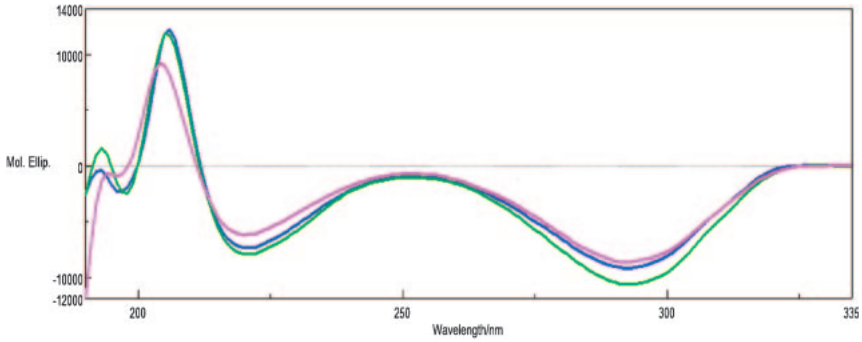

Figure 4. CD curves of **1** (blue curve), **2** (green curve), and **3** (pink curve).

Anti-HCMV Assay. To determine the effects of natural product upon HCMV cytopathic effect (CPE), confluent human embryonic lung (HEL) cells grown in 24-well plates were incubated for 1 h in the presence or absence of various concentrations of tested natural product. Then, cells were infected with HCMV at an input of 1000 pfu (plaque forming units) per well of a 24-well dish. Antiviral activity is expressed as IC_{50} (50% inhibitory concentration), or compound concentration required to reduce virus induced CPE by 50% after 7 days as compared with the untreated control. To monitor the cell growth upon treating with natural products, an MTT-colorimetric assay was employed.[24,25]

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Supporting Information

^{1}H and ^{13}C NMR spectra of compounds **1**–**4**. This material is available free of charge on the web at http://www.csj.jp/journals/bcsj/.

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