Cytotoxic Oxygenated Desmosterols of the Red Alga *Galaxaura marginata*

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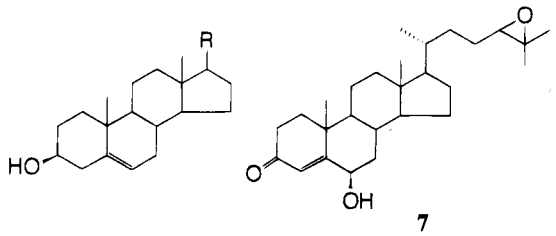
Received June 20, 1995[⊗]

Desmosterol (**1**), 24,25-epoxycholesterol (**2**), 24-hydroperoxycholesta-5,25-dien-3β-ol (**3**), 25-hydroperoxycholesta-5,23(*E*)-dien-3β-ol (**4**), cholesta-5,25-diene-3β,24-diol (**5**), and 24,25-epoxy-6β-hydroxycholest-4-en-3-one (**7**) were isolated from the marine red alga *Galaxaura marginata*; sterols **3**, **4**, and **7** were isolated for the first time from a natural source. Sterols **3**–**7** exhibited significant cytotoxicity toward serveral cancer cell lines.

Red algae have been known to be important sources of cholesterol and desmosterol (**1**)[1–7] in the marine environment. Several oxidized products of **1** also have been reported.[1,2,6,7] As some oxygenated steroids have been shown to exhibit cytotoxic properties,[8–10] and because the organic extract of *Galaxaura marginata* (Ellis and Solander) Lamouroux, a member of the family Galaxauraceae, exhibited significant cytotoxicity against KB (human nasopharyngeal carcinoma) and P-388 (mouse lymphocytic leukemia) cells, we initiated a study to investigate the bioactive metabolites of this organism. In the present study, we have isolated desmosterol (**1**), 24(*R*/*S*),25-epoxycholesterol (**2**), and cholesta-5,25-diene-3β,24ξ-diol (**5**), in addition to three novel compounds, 24ξ-hydroperoxycholesta-5,25-dien-3β-ol (**3**), 25-hydroperoxycholesta-5,23(*E*)-dien-3β-ol (**4**), and 24(*R*/*S*),25-epoxy-6β-hydroxycholest-4-en-3-one (**7**) from *G. marginata*. The structures of these compounds were identified based on spectroscopic methods. The structures of sterols **3** and **4** were further confirmed by chemical transformations into known compounds. This report deals with the isolation, structure determination, and cytotoxicity of these compounds.



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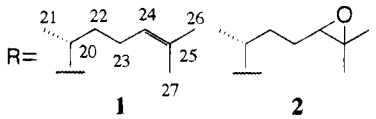
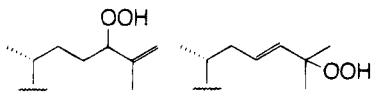


1 **2**



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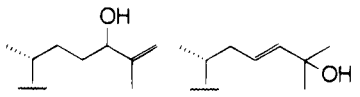

5 **6**

Results and Discussion

The organism *G. marginata*, collected in March 1994, along the coast of Kenting, Taiwan, was freeze-dried and extracted with EtOAc. Evaporation of the extract yielded a dark-green residue, which was triturated with hexane repeatedly to afford a hexane-soluble fraction and a hexane-insoluble but EtOAc-soluble fraction. Both fractions exhibited significant activity against P-388 and KB cells. Sterols **1**–**5** and **7** were isolated from Si gel CC of the hexane fraction. ^{1}H-NMR and ^{13}C-NMR spectroscopic analyses indicated that compounds **1**–**5** possessed a normal steroid ring structure. Mass spectra of **1**–**5** exhibited peaks in all spectra at *m*/*z* 273 [M^+ − side chain]; 271 [M^+ − (side chain + 2H)]; 255 [M^+ − (side chain + H_2O)]; 253 [M^+ − (side chain + 2H + H_2O)]; 231 [M^+ − (side chain and part of ring D + H)]; and 213 [M^+ − (side chain + part of ring D + H + H_2O)] and provided further confirmation for the presence of a normal ring skeleton in sterols **1**–**5**.[5] The spectral data and melting point of **1** were in full agreement with those reported previously.[10,11] Sterol **2** was found to be an inseparable mixture of 24*R* and 24*S* epimers of 24,25-epoxycholesterol, by careful comparison of its ^{13}C-NMR spectrum with those reported previously.[12]

The new sterol **3** was isolated from the fraction eluted with hexane-EtOAc (6:1). The HRMS of **3** afforded the molecular formula $C_{27}H_{44}O_3$. The ^{1}H NMR spectrum of **3** showed a hydroperoxyl proton as a broad singlet at δ 7.83.[13,14] A proton geminal with the hydroperoxyl group, which appeared as a triplet at δ 4.27 (J = 6.3 Hz), was due to the 24-methine group. The olefinic methylene protons (H_2-26) were observed as broad singlets at δ 5.01 and 5.02. The 27-methyl protons appeared as a singlet at δ 1.73, indicating that the group is on an olefinic double bond. The ^{13}C-NMR spectrum of **3** showed the presence of 27 carbons, which were assigned as shown in Table 1. The DEPT spectrum exhibited four methyl, eleven methylene, and eight methine signals. The remaining four signals in the broad-band spectrum were attributed to the quaternary carbon atoms. Four olefinic carbons (δ 114.56, C-26; 121.66, C-6; 140.76, C-5; 143.65, C-25) and two oxygen-bearing methine carbons (δ 71.81, C-3; 90.37, C-24) were further identified. The unusual downfield shift of C-24 also suggested the presence of a hydroperoxyl group attached to this carbon atom.[13,15] On the basis of the above data, the structure of sterol **3** was determined as 24-hydroperoxycholesta-5,25-dien-3β-ol. A sterol having a hydroperoxyl group in the side chain has been isolated

[⊗] Abstract published in *Advance ACS Abstracts*, December 1, 1995.

0163-3864/96/3259-0023$12.00/0

Table 1. ^{13}C-NMR Chemical Shifts of Sterols **3**–**7**[a]

	compound					
					7	
position	3	4	5	6	24*R*	24*S*
C-1	37.25	37.24	37.25	37.25	37.12	37.12
C-2	31.89	31.89	31.89	31.67	34.27	34.27
C-3	71.81	71.80	71.81	71.81	200.32	200.32
C-4	42.29	42.30	42.30	42.32	126.37	126.37
C-5	140.76	140.75	140.75	140.77	168.30	168.30
C-6	121.66	121.65	121.70	121.67	73.30	73.30
C-7	31.66	31.65	31.67	31.67	38.55	38.55
C-8	35.49	36.00	35.60	36.09	29.74	29.74
C-9	50.11	50.09	50.10	50.10	53.60	53.60
C-10	36.50	36.49	36.51	36.51	37.98	37.98
C-11	21.08	21.06	21.08	21.07	21.00	21.00
C-12	39.76	39.69	39.76	39.69	39.61	39.61
C-13	42.35	42.38	42.32	42.36	42.56	42.56
C-14	56.74	56.71	56.75	56.72	55.89	55.89
C-15	24.26	24.30	24.27	24.32	24.14	24.14
C-16	28.15	28.23	28.20	28.22	28.17	28.17
C-17	55.83	55.87	55.88	55.80	56.03	56.03
C-18	11.85	11.91	11.86	11.91	12.03	12.03
C-19	19.39	19.39	19.40	19.40	19.52	19.52
C-20	31.74	31.65	31.39	31.89	35.55	35.64
C-21	18.56	18.73	18.72	18.67	18.68	18.55
C-22	27.02	39.14	28.20	38.82	32.55	32.34
C-23	29.69	130.64	31.41 76.35	125.49	25.44	25.72
C-24	90.37	134.48	77.35	139.41	64.75	64.90
C-25	143.65	82.28	147.79	70.77	58.40	58.11
C-26	114.56	24.35	110.86	29.92	24.94	24.94
C-27	16.92	24.35	17.60	29.92	18.75	18.62

[a] The chemical shifts of **3**–**6** were determined at 75 MHz, and those of **7** were determined at 100 MHz, in $CDCl_3$. The values are in ppm downfield from TMS.

previously from the marine brown alga *Turbinaria conoides*[16] and the tunicate *Ciona intestinalis*.[13]

Further elution with hexane-EtOAc (5:1) led to the isolation of sterol **4**, $C_{27}H_{44}O_3$ (HRMS). The ^{1}H-NMR spectrum of **4** showed a broad singlet at δ 7.57, which also confirmed the presence of a hydroperoxyl group.[13,14] Three olefinic methine protons were observed. A doublet at δ 5.35 (J = 5.4 Hz) was due to the H-6 olefinic proton, while a doublet at δ 5.51 (J = 16.2 Hz) and a double triplet at δ 5.68 (J = 16.2, 7.2 Hz) were assigned to the H-24 and H-23 olefinic protons, respectively. The large coupling constant (J = 16.2 Hz) indicated the presence of a *trans*- disubstituted double bond, probably located at C-23 (24). The 6H singlet at δ 1.34 could be rationalized as due to two methyl groups (CH_3-26 and CH_3-27) attached to an oxygen-bearing quaternary carbon (C-25). The ^{13}C-NMR spectrum of **4** also showed the presence of 27 carbons, which were assigned as shown in Table 1. The DEPT spectrum indicated the presence of five methyl, nine methylene, and nine methine signals. The remaining four signals in the broad-band spectrum were due to the quaternary carbon atoms. Four olefinic carbons, including three methine and one quaternary carbons, were observed at δ 121.65 (C-6), 130.64 (C-23), 134.48 (C-24), and 140.75 (C-5). Two oxygen-bearing carbons, which appeared at δ 71.80 (CH) and 82.28 (C), were assigned to C-3 and C-25, respectively. The unusual downfield shift of C-25 also indicated the presence of a hydroperoxyl group attached to this carbon atom. On the basis of the above data, and by comparison of the ^{1}H-NMR spectrum with that of cholesta-5,23(*E*)-dien-3β,25-diol (**6**),[10] the structure of this sterol was elucidated as 25-hydroperoxycholesta-5,23(*E*)-dien-3β-ol (**4**).

The molecular structures of hydroperoxysterols **3** and **4** were further confirmed by chemical transformation to the corresponding known compounds. Reactions of **3** and **4** with triphenylphosphine[15] gave the reduced products cholesta-5,25-dien-3β,24-diol (**5**) and cholesta-5,23(*E*)-dien-3β,25-diol (**6**), respectively. Steroid **5** was also obtained directly from the algal extract, eluting after **4** on CC. The spectral data of **5** and **6**, whether generated from chemical transformation or from the alga, were in full agreement with those reported previously.[10]

The new sterol **7** was isolated from the fraction eluted with hexane-EtOAc (3:1), and the molecular formula $C_{27}H_{42}O_3$ was established from its HRMS. Its IR spectrum exhibited a broad OH stretch at 3472 cm^{-1} and a conjugated carbonyl stretch at 1688 cm^{-1}. Its ^{1}H-NMR spectrum showed an olefinic proton as a sharp singlet at δ 5.82 (H-4) and a proton geminal with the hydroxyl group as a narrow signal at δ 4.35 (H-6). A doublet at δ 0.93 (J = 6.3 Hz) was due to the 21-methyl group. Four singlets of the H_3-18, H_3-19, H_3-26, and H_3-27 methyls appeared at δ 0.75, 1.38, 1.31, and 1.27, respectively. A triplet at δ 2.68 (1H, J = 4.8 Hz), together with the downfield shift of the signals of the H_3-26 and H_3-27 methyls, indicated the presence of a 24,25-epoxy moiety. The ^{13}C-NMR spectrum of **7** (Table 1) showed the presence of 27 carbons in the molecule, including one carbonyl carbon (δ 200.32, C-3), two olefinic carbons (δ 126.37, C-4; 168.30, C-5), and five methyl carbons (δ 12.03, C-18; 18.68, 18.75, C-21 and C-27; 19.52, C-19; 24.94, C-26). On the basis of all the spectral data, and by comparison of these data with those of 3-oxostigmast-4-en-6β-ol,[17] the structure of **7** was determined as 24,25-epoxy-6β-hydroxycholest-4-en-3-one. Sterol **7**, like epoxide **2**, was found to be an inseparable mixture of 24*R* and 24*S* epimers by analysis of its ^{13}C-NMR spectrum.

The origin of oxidized sterols such as **2**–**7** has been questioned for a long time.[2] As the work was carried out with a freshly freeze-dried organism rather than on air-dried material in order to avoid possible autoxidation and because desmosterol was not transformed into oxidized products in amounts detectable by ^{1}H-NMR spectra or by TLC when exposed to air for 2 weeks in the solid state or in solution (EtOAc), it is possible that these oxysterols could be biosynthesized from desmosterol via photosensitized oxygenation or a related mechanism.[2] However, the possibility that these oxydesmosterols are artifacts arising from the autoxidation of **1** cannot be completely ruled out.

The cytotoxicities of sterols **1**, **5**, and **6** toward hepatoma cells have been reported.[10] Diol **6** was found to be active against both HTC and ZHC cells. We report here the cytotoxicities of desmosterol (**1**) and its oxygenated derivatives **3**–**7** against other cancer cell lines for the first time. The biological activity of the epimeric mixture of **2** was not tested, as it was contaminated with a small amount of inseparable impurities. The cytotoxicities of these compounds are shown in Table 2, which shows that desmosterol (**1**) was not cytotoxic to P-388, KB, A-549, and HT-29 cell lines. However, the oxidized products **3**–**7** all exhibited significant cytotoxicity against the four specified cancer cell lines, indicating that oxidation increases the activity of desmosterol.

Table 2. Cytotoxicity of Sterols **1** and **3–7**

compound	cell lines ED_{50} (μg/mL)			
	P-388	KB	A-549	HT-29
1	33.53	>50	>50	>50
3	0.26	0.33	0.64	0.43
4	0.22	1.41	1.68	1.27
5	0.36	0.67	1.61	0.37
6	0.15	0.61	1.08	0.60
7	0.75	0.30	3.14	0.87

[a] For significant activity of pure compounds, an ED_{50} value of ≤ 4.0 μg/mL is required. See Geran *et al.*[19]

Experimental Section

General Experimental Procedures. Melting points were determined using a Fisher-Johns melting points apparatus and were uncorrected. The IR spectra were measured on a Hitachi I-2001 infrared spectrophotometer. ^{1}H-NMR and ^{13}C-NMR spectra were recorded with VXR-300/5 FT NMR at 300 MHz 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 at 70 eV. HRMS spectra were recorded on a JMX-HX 110 mass spectrometer. Silica gel 60 (Merck, 230–400 mesh) was used for CC. Precoated silica gel plates (Merck, Kieselgel 60 F-254, 0.20 mm) were used for analytical TLC.

Plant Material. The marine alga *G. marginata* was collected along the coast of Kenting, located in the southernmost tip of Taiwan. A voucher specimen was deposited in the Department of Marine Resources, National Sun Yat-Sen University.

Extraction and Separation. The marine red alga (12.2 kg fresh wt) was collected in February and March 1994, and was freeze-dried. The freeze-dried plant material (2.72 kg) was minced and extracted exhaustively with EtOAc. The organic extract was evaporated to dryness and the dark oily residue (20.20 g) was triturated with hexane. The hexane layer (18.56 g) was found to exhibit significant cytotoxicity against P-388 cell line with ED_{50} of 3.71 μg/mL, and KB cell line with ED_{50} of 3.46 μg/mL. CC of the hexane layer was undertaken using hexane and hexane-EtOAc mixtures of increasing polarity. Sterol **1** was eluted with hexane-EtOAc (10:1), epoxysterol **2** with hexane-EtOAc (8:1), hydroperoxysterol **3** with hexane-EtOAc (6:1), hydroperoxysterol **4** with hexane-EtOAc (5:1), diol **5** with hexane-EtOAc (4:1), and ketosterol **7** with hexane-EtOAc (3:1).

24-Dehydrocholesterol (1): white powder (23.2 mg); $[\alpha]^{24}_D$ −39° (*c* 0.11, $CHCl_3$); mp 117–118 °C; IR (CCl_4) ν_{max} 3632, 3356, 1052 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.68 (3H, s, H-18), 0.94 (3H, d, *J* = 6.3 Hz, H-21), 1.01 (3H, s, H-19), 1.60 (3H, s, H-26), 1.68 (3H, s, H-27), 3.52 (1H, m, H-3), 5.09 (1H, t, *J* = 6.9 Hz, H-24), 5.35 (1H, d, *J* = 4.8 Hz, H-6); EIMS *m*/*z* [M]$^+$ 384 (6.25), 369 (7.0), 300 (10.0), 273 (4.7), 271 (31.4), 255 (6.8), 253 (7.0), 231 (11.3), 213(11.1), 145 (26.8), 105 (36.2), 69 (100).

(*R*/*S*),25-Epoxycholesterol (2): white powder (7.1 mg); ^{1}H NMR ($CDCl_3$) δ 0.69 (3H, s, H-18), 0.94 (3H, d, *J* = 6.3 Hz, H-21), 1.01 (3H, s, H-19), 1.27 (3H, s, H-27), 1.31 (3H, s, H-26), 2.68 (1H, t, *J* = 6.0 Hz, H-24), 3.52 (1H, m, H-3), 5.35 (1H, dd, H-6); EIMS *m*/*z* [M]$^+$ 400 (1.45), 382 (3.86), 273 (3.7), 271 (27.4), 255 (7.8), 253 (5.2), 231 (4.6), 213 (16.1), 145 (26.5), 105 (59.1), 69 (45.0).

24ξ-Hydroperoxycholesta-5,25-dien-3β-ol (3): white powder (52.0 mg); $[\alpha]^{24}_D$ −38° (*c* 0.20, $CHCl_3$); mp 186–188 °C; IR (KBr) ν_{max} 3400, 1056 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.67 (3H, s, H-18), 0.92 (3H, d, *J* = 6.3 Hz, H-21), 1.01 (3H, s, H-19), 1.73 (3H, s, H-27), 3.53 (1H, m, H-3), 4.27 (1H, t, *J* = 6.3 Hz, H-24), 5.01 (1H, s, Ha-26), 5.02 (1H, s, Hb-26), 5.35 (1H, d, *J* = 4.8 Hz, H-6), 7.83 (1H, br, −OOH); ^{13}C NMR see Table 1; EIMS *m*/*z* [M]$^+$ 416 (1.4), 400 (6.2), 398 (9.8), 382 (9.5), 330 (10.3), 273 (10.9), 271 (98.5), 255 (20.3), 253 (11.4), 213 (36.7), 145 (69.9), 105 (100.0); HRMS found 416.3284, calcd 416.3292 for $C_{27}H_{44}O_3$.

25-Hydroperoxycholesta-5,23(*E*)-dien-3β-ol (4): white powder (52.7 mg); $[\alpha]^{26}_D$ −41° (*c* 0.05, $CHCl_3$); mp 148–151 °C; IR (KBr) ν_{max} 3380, 1056, 972 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.69 (3H, s, H-18), 0.91 (3H, d, *J* = 6.3 Hz, H-21), 1.01 (3H, s, H-19), 1.34 (6H, s, H-26, H-27), 3.53 (1H, m, H-3), 5.35 (1H, d, *J* = 5.4 Hz, H-6), 5.51 (1H, d, *J* = 16.2 Hz, H-24), 5.68 (1H, dt, *J* = 16.2, 7.2 Hz, H-23), 7.57 (1H, br, −OOH); ^{13}C NMR see Table 1; EIMS *m*/*z* [M$^+$ − H_2O] 398 (0.7), 382 (2.3), 300 (3.5), 283 (6.0), 273 (1.8), 271 (16.3), 255 (3.1), 253 (3.9), 213 (6.7), 145 (17.8), 105 (36.9); HRMS found 416.3326, calcd 416.3292 for $C_{27}H_{44}O_3$; found 398.3162, calcd 398.3185 for $C_{27}H_{42}O_2$.

Cholesta-5,25-diene-3β,24ξ-diol (5): white powder (1.7 mg); $[\alpha]^{26}_D$ −37° (*c* 0.05, $CHCl_3$); mp 193–195 °C; IR (KBr) ν_{max} 3292, 1050 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.68 (3H, s, H-18), 0.94 (3H, d, *J* = 6.3 Hz, H-21), 1.01 (3H, s, H-19), 1.72 (3H, s, H-27), 3.52 (1H, m, H-3), 4.01 (1H, t, *J* = 4.2 Hz, H-24), 4.83 (1H, s, Ha-26), 4.93 (1H, s, Hb-26), 5.35 (1H, d, *J* = 5.1 Hz, H-6); ^{13}C NMR see Table 1; EIMS *m*/*z* [M]$^+$ 400 (6.5), 382 (14.0), 273 (8.4), 271 (62.9), 255 (13.2), 253 (6.9), 231 (6.7), 213 (23.2), 145 (46.7).

24(*R*/*S*)-Epoxy-6β-hydroxycholest-4-en-3-one (7): white powder (2.6 mg); mp 205–208 °C; IR (CCl_4) ν_{max} 3616, 3472, 1688, 1264, 1020 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 0.75 (3H, s, H-18), 0.93 (3H, d, *J* = 6.3 Hz, H-21), 1.38 (3H, s, H-19), 1.27 (3H, s, H-27), 1.31 (3H, s, H-26), 2.68 (1H, t, *J* = 4.8 Hz, H-24), 4.35 (1H, brs), 5.82 (1H, s, H-4); ^{13}C NMR see Table 1; EIMS *m*/*z* [M]$^+$ 414 (12.3), 285 (16.2), 267 (15.2), 245 (6.9), 227 (9.4); HRMS found 414.3146, calcd 414.3136 for $C_{27}H_{42}O_3$.

Conversion of Hydroperoxyl Sterols into Hydroxy Sterols. A stirred solution of **3** (5 mg) in 3 mL of ethyl ether was treated with triphenylphosphine (10 mg) at room temperature for 4 h. The solution was evaporated to dryness and separated by CC with the elution of hexane–EtOAc (3:1) to give **5** (4.4 mg). By using a similar procedure, **4** (4 mg) was converted into **6** as a white powder (3 mg): $[\alpha]_D$ −41° (*c* 0.1, $CHCl_3$), mp 157–159 °C; IR (KBr) ν_{max} 3388, 1060, 970 cm^{-1}; ^{1}H NMR δ 0.68 (3H, s), 0.90 (3H, d, *J* = 5.5 Hz), 1.00 (3H, s), 1.31 (6H, s), 3.53 (1H, m), 5.35 (1H, m), 5.58 (2H, m); EIMS *m*/*z* 400 [M]$^+$, 382 [M$^+$ − H_2O], 367 [M$^+$ − (H_2O + CH_3)], 283 and 271. The above spectral data were identical with those reported.[10] The ^{13}C-NMR spectral data of **6** were listed in Table 1.

Cytotoxicity Testing. KB and P-388 cells were kindly provided by Prof. J. M. Pezzuto, University of Illinois at Chicago; A-549 (human lung adenocarcinoma) and HT-29 (human colon adenocarcinoma) were purchased from American Type Culture Collection.

P-388 cells were cultured in Fisher's medium supplemented with 10% heat-inactivated (56° for 30 min) fetal calf serum (FCS). The KB cells were maintained in Basal Medium Eagle (BME) containing 10% heat-inactivated FCS. A-549 cells were cultured in Eagle Minimum Essential Medium (EMEM) containing Earle's salts and supplemented with 0.1 mM of nonessential amino acids and 10% Heat-inactivated FCS. HT-29 cells 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.

The cytotoxic activities of tested compounds against P-388, KB, A-549, and HT-29 were assayed by a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method.[18] The cultured cells were treated at eight concentrations of pure test compounds ranging from 0.00064−50 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.

Acknowledgment. This work was supported by a grant from the National Science Council of the Republic of China (NSC-81-0418-B-110-511-BH) awarded to J.-H. Sheu.

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NP960016W