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OXYGENATED CLEROSTEROLS ISOLATED FROM THE MARINE ALGA *CODIUM ARABICUM*

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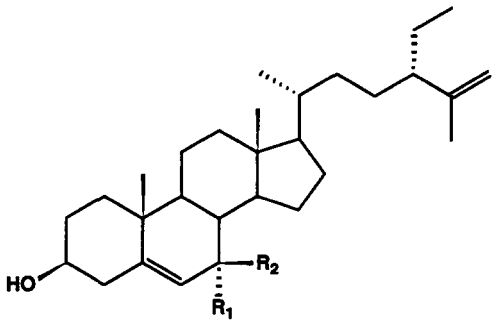
ABSTRACT.—Clerosterol [**1**], (24*S*)-24-ethyl-3-oxocholesta-4,25-dien-6β-ol [**2**], (24*S*)-24-ethyl-5α-hydroperoxycholesta-6,25-dien-3β-ol [**3**], (24*S*)-24-ethyl-7-oxocholesta-5,25-dien-3β-ol [**4**], (24*S*)-24-ethyl-7α-hydroperoxycholesta-5,25-dien-3β-ol [**5**], and (24*S*)-24-ethylcholesta-5,25-dien-3β,7α-diol [**6**], were isolated from the marine green alga *Codium arabicum*. A portion of steroid **5** was epimerized to (24*S*)-24-ethyl-7β-hydroperoxycholesta-5,25-dien-3β-ol [**8**]. LAH reduction of an inseparable mixture of **5** and **8** yielded diol **6** and (24*S*)-24-ethylcholesta-5,25-dien-3β,7β-diol [**7**]. Among these compounds, sterols **2**, **3**, and **5** were isolated for the first time from a natural source. Metabolites **2–6** showed significant cytotoxicity toward various cancer cell lines.

Marine green algae of the genus *Codium* (Codiaceae) have been found to be an important source of clerosterol [**1**] (1,2) and its oxygenated derivatives (3). Acyclic diterpenoids also have been isolated from *C. decorticatum* (4). As several reports have revealed that some oxygenated sterols possess interesting biological activities (5–8), and because the organic extract of *Codium arabicum* Kützing exhibited 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. This is the first report of a chemical investigation of *C. arabicum*. In the present study, we have isolated the known clerosterol [**1**], (24*S*)-24-ethyl-7-oxocholesta-5,25-dien-3β-ol [**4**] (3), and (24*S*)-24-ethylcholesta-5,25-dien-3β,7α-diol [**6**] (3), in addition to three novel compounds, (24*S*)-24-ethyl-3-oxocholesta-4,25-dien-6β-ol [**2**], (24*S*)-24-ethyl-5α-hydroperoxycholesta-6,25-dien-3β-ol [**3**], and (24*S*)-24-ethyl-7α-hydroperoxycholesta-5,25-dien-3β-ol [**5**]. We also report herein the cytotoxicity of these six sterols toward various cancer cell lines.

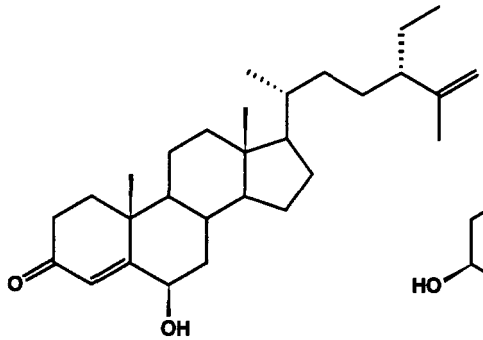
RESULTS AND DISCUSSION

The organism *C. arabicum*, collected in April 1993, along the coast of Penghu, Taiwan, was freeze-dried and extracted with EtOAc. The crude extract was triturated with hexane to afford a hexane-soluble fraction, and a hexane-insoluble but EtOAc-soluble fraction. Both fractions were shown to exhibit a similar activity against KB cells (ED_{50} values of 14–20 μg/ml) and P-388 cells (ED_{50} values of 5–7 μg/ml), and various sterols were isolated by Si gel cc of the hexane fraction. The major and least polar sterol obtained was found to be (24*S*)-24-ethylcholesta-5,25-dien-3β-ol (clerosterol) [**1**], isolated previously from *C. fragile* (1), *C. iyengaii* (2), and *C. decorticatum* (3). The structure of **1** was confirmed by comparison of the mp, $[\alpha]_D$, and spectral data of **1** with those reported previously (1,9). Moreover, by comparison of the physical and spectral data, the acetate of **1** was found to be identical with (24*S*)-24-ethylcholesta-5,25-dien-3β-yl acetate, which has been converted previously into the known (24*S*)-24-ethylcholest-5-en-3β-yl acetate (1).

The new sterol **2** was isolated from the fraction eluted with hexane-EtOAc (6:1). Its molecular formula, $C_{28}H_{46}O_2$, was established by hrms, and its ir spectrum exhibited a broad OH stretch at 3484 cm^{-1} and a conjugated carbonyl stretch at 1686 cm^{-1}. Its ^1H-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), as well as the olefinic



	R_1	R_2
1	H	H
4	O	O
5	OOH	H
6	OH	H
7	H	OH
8	H	OOH



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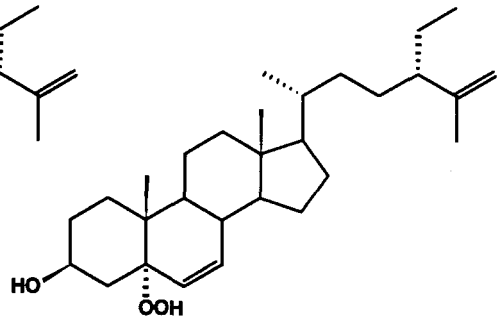

3

methylene proton resonances as broad singlets at δ 4.65 and 4.73. A triplet at δ 0.80 (J=7.2 Hz) was due to the 29-methyl group. A doublet at δ 0.91 (J=6.3 Hz) was due to the 21-methyl group. Three singlets of the H_3-18, H_3-19, and H_3-27 methyls appeared at δ 0.73, 1.38, and 1.56, respectively. The ^{13}C-nmr spectrum of **2** also showed the presence of 29 carbon signals in the molecule, including one carbonyl carbon (δ 200.36, C-3), four olefinic carbons (δ 111.39, C-26; 126.35, C-4; 147.53, C-25; 168.36, C-5), one oxygen-bearing methine carbon (δ 73.33, C-6), and five methyl carbons (δ 12.05, C-29; 12.51, C-18; 17.80, C-27; 18.61, C-21; 19.51, C-19). On the basis of all the spectral data obtained, and by comparison of these data with those of 3-oxostigmast-4-en-6β-ol (10), the structure of sterol **2** was established as (24*S*)-24-ethyl-3-oxocholesta-4,25-dien-6β-ol.

Further elution with hexane-EtOAc (5:1) led to the isolation of sterol **3**. Its ir spectrum showed stretching frequencies at 3436 and 3216 cm^{-1} due to hydroxyl and hydroperoxyl groups, respectively (11). Its ^{1}H-nmr spectrum showed a broad singlet at δ 7.06 which confirmed the presence of the hydroperoxyl group. The H-6 and H-7 olefinic protons appeared as two double doublets of an AB system at δ 5.60 (J=2.4 and 9.6 Hz) and 5.83 (J=1.8 and 9.6 Hz). The olefinic methylene protons (H_2-26) appeared as broad singlets at δ 4.64 and 4.73. A multiplet at δ 4.12 was due to the H-3 proton. The 29-methyl protons were observed at δ 0.80 (J=7.2 Hz) as a triplet. A doublet at δ 0.90 (J=6.3 Hz) was due to the 21-methyl group. Three singlets of the H_3-18, H_3-19, and H_3-27 methyls appeared at δ 0.68, 0.95, and 1.57, respectively. The ^{13}C-nmr (Table 1) spectrum of **3** also indicated the presence of 29 carbon signals in the molecule. The DEPT spectrum exhibited five methyl, eleven methylene, and nine methine signals. The remaining four signals in the broad-band spectrum were attributed to the quaternary carbon atoms. The molecular ion (*m/z* 444) of **3** was not observed in the eims, but instead a peak at *m/z* 426, representing the dehydration of **3**, was observed. From the above data, the molecular formula of **3** was deduced to be $C_{29}H_{48}O_3$. Also, by comparison of the ^{1}H-

TABLE 1. ^{13}C-Nmr Chemical Shifts of Sterols **2**, **3**, **5**, and **6**.[a]

Carbon	Compound			
	2	**3**	**5**	**6**
C-1	37.11	28.54	36.76	37.00
C-2	34.27	30.45	31.36	31.34
C-3	200.36	66.98	71.41	71.35
C-4	126.35	35.75	42.20	42.01
C-5	168.36	84.30	148.84	146.21
C-6	73.33	129.00	119.92	123.87
C-7	38.56	136.27	78.49	65.34
C-8	29.73	39.06	37.11	37.51
C-9	53.61	43.82	43.54	42.26
C-10	37.98	38.37	37.42	37.39
C-11	20.99	20.82	20.89	20.70
C-12	39.61	39.89	39.03	39.17
C-13	42.51	43.63	42.34	42.14
C-14	55.88	53.50	49.04	49.41
C-15	24.14	23.80	24.42	24.28
C-16	28.11	28.29	28.11	28.19
C-17	56.06	55.88	55.58	55.71
C-18	12.51	12.49	11.30	11.61
C-19	19.51	15.23	18.20	18.68
C-20	35.52	35.58	35.46	35.47
C-21	18.61	18.54	18.69	18.24
C-22	33.63	33.61	33.64	33.64
C-23	29.40	29.47	29.25	29.24
C-24	49.52	49.49	49.52	49.50
C-25	147.53	147.53	147.55	147.54
C-26	111.39	111.39	111.35	111.37
C-27	17.80	17.77	17.83	17.79
C-28	26.52	26.52	26.52	26.51
C-29	12.05	12.05	12.05	12.05

[a]Chemical shifts were determined at 75.3 MHz in $CDCl_3$. The values are in ppm downfield from TMS.

nmr spectral data with those of (24*R*)-24-ethyl-5α-hydroperoxycholest-6-en-3β-ol (12), the structure of sterol **3** was established as (24*S*)-24-ethyl-5α-hydroperoxycholesta-6,25-dien-3β-ol. Moreover, the DEPT spectrum of **3** showed that the chemical shifts of C-4, C-9, and C-10 appeared at δ 35.75, 43.82, and 38.37, respectively, which are different from those reported previously (δ 43.8, C-4; 39.4, C-9; 35.7, C-10) for (24*R*)-24-ethyl-5α-hydroperoxycholest-6-en-3β-ol (12), and suggest the need for revision of the data reported previously.

The next sterol isolated was identified as (24*S*)-24-ethyl-7-oxocholesta-5,25-dien-3β-ol [**4**], obtained previously from *C. decorticatum* (3). The spectral data (^{1}H-, ^{13}C-nmr, ir, and ms) were in full agreement with those reported (3).

Further elution with hexane-EtOAc (4:1) yielded sterol **5**. The eims showed a peak at *m/z* 426 for $(M-H_2O)^+$. The ^{1}H-nmr spectrum of **5** showed a broad singlet at δ 7.64 which revealed the presence of a hydroperoxyl group. Two olefinic methylene protons again gave signals at δ 4.64 and 4.73 as broad singlets. An olefinic proton resonance in the ^{1}H-nmr spectrum appeared as a double doublet at δ 5.72 (J=5.1 and 1.5 Hz) and was ascribed to H-6. The hydroperoxyl and hydroxyl-bearing methines showed signals at δ 4.16 (t, J=5.1 Hz, H-7) and 3.62 (m, H-3), respectively. A triplet at δ 0.80 (J=7.2 Hz) was assigned to the 29-methyl protons. A doublet at δ 0.91 (J=6.3 Hz) was due

to the 21-methyl group. Three singlets of the H_3-18, H_3-19, and H_3-27 methyls appeared at δ 0.65, 0.99, and 1.57, respectively. The ^{13}C-nmr spectrum of **5** displayed 29 carbons. The DEPT spectrum showed five methyl, eleven methylene, and nine methine signals. The remaining four signals in the broad-band spectrum were due to the quaternary carbon atoms. The molecular formula of **5** was thus formulated as $C_{29}H_{48}O_3$. By comparison of its spectral data (^{1}H- and ^{13}C-nmr) with those reported for 24-ethylcholest-5,25-dien-3β-7α-ol [**6**] (3), the structure of **5** was identified as (24*S*)-24-ethyl-7α-hydroperoxycholesta-5,25-dien-3β-ol.

The structure of **5** was further confirmed by transformation to a known compound. Reaction of **5** with LAH converted it into the known (24*S*)-24-ethylcholesta-5,25-dien-3β,7α-diol [**6**] (3). In addition, **6** was isolated from the crude extract of *C. arabicum* by elution with hexane-EtOAc (1:1). The spectral data of **6** (^{13}C-nmr data, see Table 1), whether generated by chemical conversion or from natural sources, were in full agreement with reported data (3).

A previous study showed that (24*S*)-24-ethylcholesta-5,25-dien-3β,7β-diol [**7**], an epimer of **6**, could also be isolated from the extract of *C. decorticatum* (3). We did not detect the presence of **7** in *C. arabicum*, but we observed that the 7α-hydroperoxy sterol **5** could be epimerized to the 7β-hydroperoxy sterol **8** to form an inseparable mixture when it was stored either in solution or in the solid state. When **5** was stored in a refrigerator (0–10°), it slowly rearranged to **8** until the ratio of **5** and **8** reached 3:1 (estimated from integration of the ^{1}H-nmr spectrum). The ^{1}H-nmr spectrum of **8** showed a hydroperoxyl proton as a broad singlet at δ 7.53. The H-6 signal of **8** appeared as a broad peak at δ 5.58, and the two singlets of the H_3-18 and H_3-19 methyls appeared at δ 0.68 and 1.04, respectively. By comparison of these data with those of 7β-hydroperoxycholest-5-en-3β-ol (11), the structure of **8** was identified as (24*S*)-24-ethyl-7β-hydroperoxycholesta-5,25-dien-3β-ol. The inseparable mixture of **5** and **8** (3:1) was subjected to reaction with $LiAlH_4$ to yield **6** and its 7β-hydroxyl epimer [**7**] (3) in a ratio also near to 3:1. The above transformation not only further confirmed the structure of **8**, but also supported the previous assumption that the epimerization of 7α-hydroperoxy steroids leads to the formation of 7β-hydroperoxyl epimers (11).

We also report here the cytotoxicity of **1** and its oxygenated derivatives **2–6** against various cancer cell lines. The cytotoxicity of these compounds is given in Table 2, which shows that **1** exhibited significant activity against P-388 cells and was the most active against A-549 cells among compounds **1–6**. However, **1** was inactive against the growth of KB and HT-29 cells. The oxidized products **2–6** all showed significant activity against the growth of the four indicated cancer cell lines, indicating that oxidation increases the activity of clerosterol [**1**].

TABLE 2. Cytotoxicity of Sterols **1–6**.[a]

	ED_{50} (μg/ml)			
	Cell line			
	P-388	KB	A-549	HT-29
1	1.7	>50	0.3	43.1
2	0.7	1.3	2.1	0.3
3	0.5	1.0	1.1	0.9
4	0.8	1.0	1.2	2.0
5	0.4	1.0	0.5	1.0
6	0.2	1.1	0.5	0.6

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

EXPERIMENTAL

GENERAL EXPERIMENTAL PROCEDURES.—Mps were determined using a Fisher-Johns melting-point apparatus and are uncorrected. The ir spectra were measured on a Hitachi I-2001 infrared spectrophotometer. ^{1}H- and ^{13}C-nmr spectra were recorded with a VXR-300/5 FT-nmr spectrometer at 300 MHz and 75.3 MHz, respectively, in $CDCl_3$ using TMS as internal standard. Eims spectra were obtained with a VG Quattro GC/MS spectrometer at 30 or 70 eV. Hrms spectra were recorded on a JMX-HX 110 mass spectrometer. Si gel 60 (Merck, 230–400 mesh) was used for cc. Precoated Si gel plates (Merck, Kieselgel 60 F_{254}, 0.20 mm) were used for analytical tlc.

PLANT MATERIAL.—The marine green alga *C. arabicum* was collected along the coast of Penghu, Taiwan. A voucher specimen was deposited in the Department of Marine Resources, National Sun Yat-Sen University.

EXTRACTION AND ISOLATION.—The bodies of the marine green alga were freeze-dried. The dried plant material (830 g) was minced and extracted exhaustively with EtOAc. After removal of solvent *in vacuo*, the oily residue (9.60 g) was triturated with hexane. The hexane layer (6.10 g) was found to exhibit significant cytotoxicity against the P-388 cell line with an ED_{50} value of 6.8 μg/ml and against the KB cell line with an ED_{50} value of 19.9 μg/ml. Cc of the hexane layer was undertaken using hexane and hexane/EtOAc mixtures of increasing polarity. Finally, the column was washed with pure EtOAc. Sterol **1** was eluted with hexane-EtOAc (7:1), hydroxyenone **2** with hexane-EtOAc (6:1), hydroperoxide **3** with hexane-EtOAc (5:1), hydroxyenone **4** and hydroperoxide **5** with hexane-EtOAc (4:1), and finally diol **6** with hexane-EtOAc (1:1).

(24S)-24-Ethyl-3-oxocholesta-4,25-dien-6β-ol [**2**].—White powdery solid (1.7 mg); mp 194°; ir (KBr) ν max 3484, 1686, 1648, 892 cm^{-1}; ^{1}H nmr δ 5.82 (1H, s, H-4), 4.73 (1H, s, H-26), 4.65 (1H, s, H-26), 4.35 (1H, br s, H-6), 1.56 (3H, s, Me-27), 1.38 (3H, s, Me-19), 0.91 (3H, d, J=6.3 Hz, Me-21), 0.80 (3H, t, J=7.2 Hz, Me-29), 0.73 (3H, s, Me-18); ^{13}C-nmr data, see Table 1; eims *m/z* [M]$^+$ 426 (13), 408 (5), 343 (27), 313 (9), 285 (10), 267 (9), 227 (5); hrms *m/z* found 426.3502, calcd 426.3499 for $C_{29}H_{46}O_2$.

(24S)-24-Ethyl-5α-hydroperoxycholesta-6,25-dien-3β-ol [**3**].—White powdery solid (8.4 mg); $[\alpha]^{24}$D −9.1° (c=0.07, $CHCl_3$); mp 152–153°; ir (KBr) ν max 3436, 3216, 1678, 1644, 890 cm^{-1}; ^{1}H nmr δ 7.06 (1H, s, OOH), 5.83 (1H, dd, J=1.8 and 9.6 Hz, H-6), 5.60 (1H, dd, J=2.4 and 9.6 Hz, H-7), 4.73 (1H, s, H-26), 4.64 (1H, s, H-26), 4.12 (1H, m, H-3), 1.57 (3H, s, Me-27), 0.95 (3H, s, Me-19), 0.90 (3H, d, J=6.3 Hz, Me-21), 0.80 (3H, t, J=7.2 Hz, Me-29), 0.68 (3H, s, Me-18); ^{13}C-nmr data, see Table 1; eims *m/z* [M−H_2O]$^+$ 426 (2), 410 (7), 396 (1), 343 (1), 313 (1), 273 (2), 229 (2); hrms *m/z* found 426.3499, calcd 4444. 3605 for $C_{29}H_{48}O_3$, 426.3499 for $C_{29}H_{46}O_2$.

(24S)-24-Ethyl-7α-hydroperoxycholesta-5,25-dien-3β-ol [**5**].—White powdery solid (16.3 mg); $[\alpha]^{24}$D −87.6° (c=0.14, $CHCl_3$); mp 127–128°; ir (KBr) ν max 3444, 3392, 1678, 1646, 892 cm^{-1}; ^{1}H nmr δ 7.64 (1H, br s, OOH), 5.72 (1H, dd, J=4.8 and 1.5 Hz, H-6), 4.73 (1H, s, H-26), 4.64 (1H, s, H-26), 4.16 (1H, t, J=5.1 Hz, H-7), 3.62 (1H, m, H-3), 1.57 (3H, s, Me-27), 0.99 (3H, s, Me-19), 0.91 (3H, d, J=6.3 Hz, Me-21), 0.80 (3H, t, J=7.2 Hz, Me-29), 0.65 (3H, s, Me-18); ^{13}C-nmr data, see Table 1; eims *m/z* [M−H_2O]$^+$ 426 (3), 410 (17), 396 (5), 343 (2), 267 (2), 227 (2); hrms *m/z* found 426.3492, calcd 444.3605 for $C_{29}H_{48}O_3$, 426.3499 for $C_{29}H_{46}O_2$.

(24S)-24-Ethylcholesta-5,25-dien-3β,7β-diol [**7**].—To a stirred solution of the mixture of **5** and **8** (3:1, 11 mg) in dry THF (5 ml) was added excess $LiAlH_4$ at room temperature under N_2. The stirring was continued at room temperature for 2 h. Thereafter, H_2O (0.1 ml) was added followed by the addition of EtOAc (20 ml). The organic layer was filtered and evaporated. The crude product was separated by cc with the stepwise elution of hexane-EtOAc (2:1→1:1) to yield the known compound **7** (2.4 mg) as a white powdery solid. Further elution with hexane-EtOAc (1:1) yielded diol **6** (7.5 mg). The spectral data of **6** and **7** were in full agreement with those reported previously (3).

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 the American Type Culture Collection. Cytotoxicity tests were carried out according to standard protocols (13) in the following manner.

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's 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° in humidified air containing 5% CO_2.

The cytotoxic activities of tested compounds or fractions against the P-388, KB, A-549, and HT-29 cancer cell lines were assayed by a modification of the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] colorimetric method (14). For P-388 cells, 200-μl cultures were established at 1500 cells/well in 96-well tissue culture plates (Falcon). Compounds were dispensed subsequently to the established culture plate at eight concentrations in triplicate. After three days of incubation, P-388 cells were enumerated with MTT.

To measure the cytotoxic activities of purified compounds or crude fractions against KB, A-549, and HT-29 cells, each cell line was initiated at 1,000 cells/well in 96-well microtiter plates. Eight concentrations of test compounds encompassing a 128-fold range were added to each cell line. KB, A-549, and HT-29 cells were enumerated using MTT after exposure to test compounds for 3, 6, and 6 days, respectively. To each well 50 μl of 1 mg/ml MTT were added, and plates were incubated at 37° for a further 5 h. Formazan crystals were redissolved in DMSO (E. Merck) for 10 min with shaking, and the plate was read immediately on a microtiter plate reader (Titertek Mutiskan, Flow) 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 cells in the MTT assay.

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LITERATURE CITED

1. I. Rubinstein and L.J. Goad, *Phytochemistry*, **13**, 481 (1974).
2. V.U. Ahmad, R. Aliya, S. Perveen, and M. Shameel, *Phytochemistry*, **31**, 1429 (1992).
3. V.U. Ahmad, R. Aliya, S. Perveen, and M. Shameel, *Phytochemistry*, **33**, 1189 (1993).
4. R. Aliya and M. Shameel, *Botan. Marina*, **36**, 371 (1993).
5. K.P. Cheng, L. Bang, G. Ourisson, and J.P. Beck, *J. Chem. Res. (S)*, 84 (1979).
6. K.P. Cheng, L. Bang, G. Ourisson, and J.P. Beck, *J. Chem. Res. (M)*, 1101 (1979).
7. G. Schroeder, M. Rohmer, J.P. Beck, and R. Anton, *Phytochemistry*, **19**, 2213 (1980).
8. C.E. Heltzel, A.A.L. Gunatilaka, and D.G.I. Kingston, *J. Nat. Prod.*, **57**, 620 (1994).
9. W. Sucrow, *Chem. Ber.*, **99**, 2765 (1966).
10. M.D. Greca, P. Monaco, and L. Previtera, *J. Nat. Prod.*, **53**, 1430 (1990).
11. J.I. Teng, M.J. Kulig, L.L. Smith, G. Kan, and J.E. ven Lier, *J. Org. Chem.*, **38**, 119 (1973).
12. M.D. Greca, A. Fiorentino, A. Molinaro, P. Monaco, and L. Previtera, *Nat. Prod. Lett.*, **5**, 7 (1994).
13. R.I. Geran, N.H. Greenberg, M.M. MacDonald, A.M. Schumacher, and B.J. Abbott, *Cancer Chemother. Rep.*, **3** (3), 1 (1972).
14. T. Mosmann, *J. Immunol. Meth.*, **65**, 55 (1983).

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