Nigerasterols A and B, Antiproliferative Sterols from the Mangrove-Derived Endophytic Fungus *Aspergillus niger* MA-132

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Two new 6,8(14),22-hexadehydro-5α,9α-epidioxy-3,15-dihydroxy sterols, nigerasterols A and B (**1** and **2**, resp.), together with two known cyclopentapeptides, malformins A_1 and C (**3** and **4**, resp.), were identified in the culture extract of *Aspergillus niger* MA-132, an endophytic fungus isolated from a fresh healthy sample of the mangrove plant *Avicennia marina*. The structures and relative configurations of these compounds were established on the basis of extensive spectroscopic analyses. The absolute configuration of compound **1** was determined by application of a modified version of *Mosher*'s method. Nigerasterols A and B (**1** and **2**, resp.) represent the first 5,9-epidioxy-sterol compounds of marine origin, and they displayed potent activity against tumor cell lines HL60 and A549 in a preliminary bioassay.

Introduction. – Sterols with a 5,8-epidioxy moiety are well-known metabolites from marine organisms, such as corals, sponges, and marine-derived fungi, as well as terrestrial macrofungi [1–5]. In contrast, sterols with the 5,9-epidioxy motif were rarely reported. To date, only five 5,9-epidioxy-sterols have been isolated from several edible mushrooms, but none of them was extracted from marine organisms. During our ongoing search for bioactive metabolites from marine-derived fungi [6–12], we have investigated the chemical constituents of *Aspergillus niger* MA-132, an endophytic fungus isolated from a fresh healthy sample of the mangrove plant *Avicennia marina*. As a result, eight new α-pyrone derivatives, nigerapyrones A–H, were identified and reported [13]. The extract from a further fermentation of this fungus, using different incubation temperature and rotational speed, showed weak antibacterial activity against *Staphylococcus aureus*, and antiproliferative activity against HL60 and A549 cell lines. Bioassay-guided fractionation of the extract has led to the identification of two new 5α,9α-epidioxy-sterols, **1** and **2**, and of the two known cyclopentapeptides malformins A_1 and C (**3** and **4**, resp.). The structures of these compounds were elucidated using spectroscopic methods. Compounds **1** and **2**, which represent the first examples of 5,9-epidioxy-sterols isolated from marine organisms, displayed potent antiproliferative activities against HL60 and A549 cell lines, while compounds **3** and **4** showed weak antibacterial activities against *S. aureus*. Here, we describe the isolation, structure elucidation, and evaluation of biological activity of compounds **1**–**4** (*Fig. 1*).

Results and Discussion. – 1. *Structure Elucidation*. Repeated column chromatography and semi-preparative HPLC procedures on the AcOEt extract from the mass

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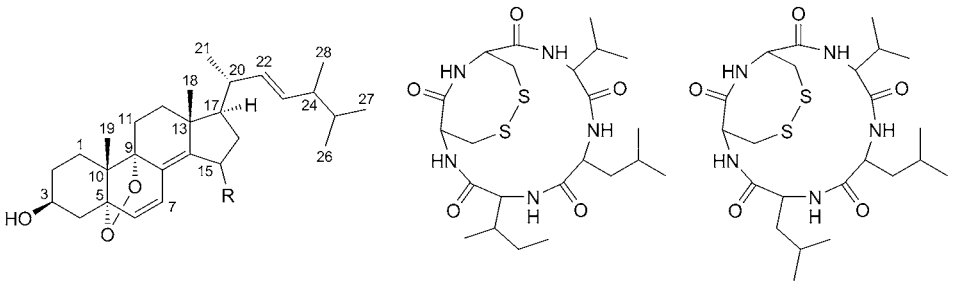

Fig. 1. *Chemical structures of compounds* **1**–**4**

growth of the fungus *A. niger* MA-132 resulted in the isolation of four compounds, **1**–**4**. Compound **1** was isolated as a colorless amorphous powder. The low-resolution EI-MS of **1** displayed a molecular-ion peak at *m*/*z* 442 (M^+), and the molecular formula was determined as $C_{28}H_{42}O_4$ on the basis of HR-EI-MS, implying eight degrees of unsaturation. The ^{1}H-NMR spectrum of **1** (*Table 1*) indicated the presence of six Me groups (Me(18, 19, 21, 26, 27, and 28)), four olefinic H-atoms (δ(H) 5.62 (*d*, *J* = 9.5, H–C(6)), 6.75 (*d*, *J* = 9.5, H–C(7)), 5.19 (*dd*, *J* = 15.3, 7.8, H–C(22)), and 5.24 (*dd*, *J* = 15.3, 7.2, H–C(23))), and two O-bearing CH H-atoms (δ(H) 3.96–4.01 (*m*, H–C(3)) and 4.71 (br. *d*, *J* = 6.5, H–C(15)). The ^{13}C-NMR, along with the DEPT spectra (*Table 1*), revealed the presence of 28 C-atoms, including six quaternary C-atoms (with two O-bearing), ten CH (with four olefinic and two O-bearing), six CH_2, and six Me groups. The presence of six Me signals due to two tertiary at δ(H) 0.85 (Me(18)) and 1.01 (Me(19)) and four secondary at δ(H) 1.04 (*d*, *J* = 6.7, Me(21)), 0.84 (*d*, *J* = 6.8, Me(26)), 0.82 (*d*, *J* = 6.8, Me(27)), and 0.92 (*d*, *J* = 6.8, Me(28)) indicated that compound **1** was a sterol of the ergostane series [14].

In the ^{1}H- and ^{13}C-NMR spectra, signals of one of the two O-bearing CH groups (δ(H) 3.96–4.02 (H–C(3)) and δ(C) 66.2 (C(3))) were typical for a 3β-hydroxy-*A*/*B*-*trans*-sterol [14]. The olefinic H-atom signals at δ(H) 5.19 (H–C(22)) and 5.24 (H–C(23)) were characteristic for the C(22)=C(23) bond, while those of the two O-bearing quaternary C-atoms at δ(C) 85.4 (C(5)) and 86.5 (C(9)) indicated the presence of an epidioxy group [14].

The structure of **1** was deduced from exhaustive analyses of the ^{1}H,^{1}H-COSY and HMBC spectra. The ^{1}H,^{1}H-COSY (*Fig. 2*) spectrum indicated the presence of five spin systems including CH_2(1) to CH_2(4), H–C(6) to H–C(7), CH_2(11) to CH_2(12), H–C(15) to H–C(17), and H–C(20) to Me(28). The HMBC spectrum (*Fig. 2*) revealed full sets of possible ^3J correlations from H–C(6) to C(4), C(8), and C(10), from H–C(7) to C(5), C(9), and C(14), from H–C(15) to C(13) and C(17), from Me(18) to C(12), C(14), and C(17), from Me(19) to C(1), C(5), and C(9), from Me(21) to C(17) and C(22), from Me(26) and Me(27) to C(24), and from Me(28) to C(23) and C(25). Based on all these data, the constitutional formula of **1** was deduced as 5,9-epidioxyergosta-6,8(14),22-triene-3,15-diol.

Table 1. *^1H- and ^{13}C-NMR Data* (500 and 125 MHz, resp.) *of* **1** *and* **2**. In $CDCl_3$, δ in ppm, J in Hz. Assignments were corroborated by $^1H,^1H$-COSY, HSQC, and HMBC experiments.

Position	**1**		**2**	
	δ(H)	δ(C)	δ(H)	δ(C)
1	2.00–2.04 (*m*, H_α), 1.46–1.50 (*m*, H_β)	27.5 (*t*)	2.03–2.05 (*m*, H_α), 1.46–1.48 (*m*, H_β)	27.5 (*t*)
2	1.98–2.00 (*m*, H_α), 1.48–1.52 (*m*, H_β)	31.2 (*t*)	1.88–1.90 (*m*, H_α), 1.50–1.53 (*m*, H_β)	31.3 (*t*)
3	3.96–4.01 (*m*)	66.2 (*d*)	3.98–4.03 (*m*)	66.2 (*d*)
4	2.23 (*ddd*, J = 14.1, 4.7, 1.6, H_α), 1.58–1.62 (*m*, H_β)	35.8 (*t*)	2.22–2.25 (*m*, H_α), 1.60–1.63 (*m*, H_β)	35.8 (*t*)
5		85.4 (*s*)		85.3 (*s*)
6	5.62 (*d*, J = 9.5)	131.0 (*d*)	5.67 (*d*, J = 9.4)	131.8 (*d*)
7	6.75 (*d*, J = 9.5)	128.7 (*d*)	6.64 (*d*, J = 9.4)	126.9 (*d*)
8		131.8 (*s*)		131.0 (*s*)
9		86.5 (*s*)		86.2 (*s*)
10		51.4 (*s*)		51.5 (*s*)
11	1.94–2.00 (*m*)	23.4 (*t*)	1.92–1.95 (*m*)	23.3 (*t*)
12	1.43–1.47 (*m*, H_α), 1.93–2.00 (*m*, H_β)	33.7 (*t*)	1.42–1.45 (*m*, H_α), 1.88–1.92 (*m*, H_β)	33.7 (*t*)
13		44.0 (*s*)		42.7 (*s*)
14		154.6 (*s*)		151.3 (*s*)
15	4.71 (br. *d*, J = 6.6)	69.6 (*d*)	4.73 (*dd*, J = 8.2, 6.3)	69.8 (*d*)
16	1.78–1.81 (*m*, H_α), 1.73–1.76 (*m*, H_β)	39.6 (*t*)	2.27–2.30 (*m*, H_α), 1.43–1.46 (*m*, H_β)	39.1 (*t*)
17	1.54–1.58 (*m*)	52.9 (*d*)	1.20–1.22 (*m*)	53.5 (*d*)
18	0.85 (*s*)	18.2 (*q*)	1.07 (*s*)	18.2 (*q*)
19	1.01 (*s*)	16.3 (*q*)	1.03 (*s*)	16.2 (*q*)
20	2.07–2.11 (*m*)	38.5 (*d*)	2.16–2.19 (*m*)	39.2 (*d*)
21	1.04 (*d*, J = 6.7)	21.3 (*q*)	1.02 (*d*, J = 7.2)	21.2 (*q*)
22	5.19 (*dd*, J = 15.3, 7.8)	134.7 (*d*)	5.19 (*dd*, J = 15.3, 7.8)	134.5 (*d*)
23	5.24 (*dd*, J = 15.3, 7.2)	133.0 (*d*)	5.24 (*dd*, J = 15.3, 8.6)	133.1 (*d*)
24	1.84–1.87 (*m*)	42.9 (*d*)	1.83–1.86 (*m*)	42.9 (*d*)
25	1.44–1.47 (*m*)	33.1 (*d*)	1.44–1.48 (*m*)	33.1 (*d*)
26	0.84 (*d*, J = 6.8)	19.7 (*q*)	0.84 (*d*, J = 6.8)	19.7 (*q*)
27	0.82 (*d*, J = 6.8)	20.0 (*q*)	0.82 (*d*, J = 6.8)	20.0 (*q*)
28	0.92 (*d*, J = 6.8)	17.6 (*q*)	0.92 (*d*, J = 6.8)	17.6 (*q*)

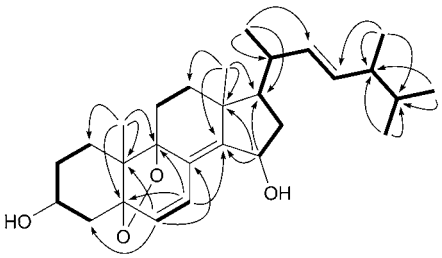

Fig. 2. *Key COSY* (—) *and HMB* (H → C) *correlations of compound* **1**

The relative configuration of **1** was determined by analysis of the H-atom coupling constants and by a NOESY experiment, as well as by comparison with literature data. The coupling constant for the olefinic H-atoms ($d, J(6,7) = 9.5$) indicated (Z)-geometry for the C(6)=C(7) bond, while the (E)-geometry for the C(22)=C(23) bond was deduced from the large coupling constant (d, $J(22,23) = 15.3$). The observed NOESY correlations H–C(3)/H_α–C(4) and H_β–C(4)/Me(19) suggested that compound **1** was a 3β-hydroxy-*A*/*B*-*trans*-sterol (*Fig. 3*) and thus, indicating the 5α,9α orientation of the 5,9-epidioxy group [14]. The observed cross-peaks in the NOESY spectrum, *i.e.*, Me(18)/H–C(15), Me(19)/H–C(20), and H–C(17)/Me(21) allowed the assignment of the relative configuration of compound **1**. Thus, the structure for compound **1** was established as (3β,5α,15α,22E)-5,9-epidioxyergosta-6,8(14),22-triene-3,15-diol, named nigerasterol A.

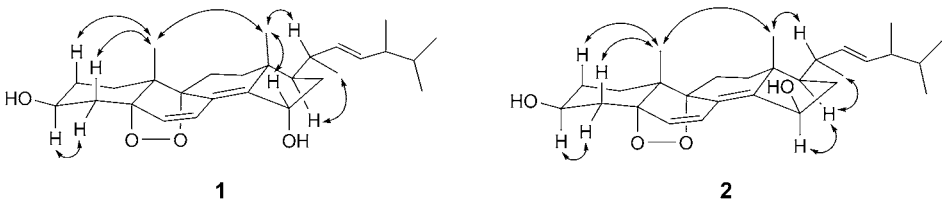

Fig. 3. *Key NOESY* (H ↔ H) *correlations of compounds* **1** *and* **2**

The absolute configuration of **1** was determined by application of a modification of *Mosher*'s method [15]. Acylation of **1** with (–)-(*R*)- and (+)-(*S*)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride (MTPA-Cl) furnished 3,15-bis-MTPA esters **1s** and **1r**, respectively (*Fig. 4*). The ^{1}H-NMR signals of the two MTPA esters were assigned on the basis of their COSY spectra, and the $\Delta\delta_{H(S-R)}$ values were then calculated (*Fig. 4*). The results indicated that the absolute configurations at C(3) and C(15) were both (*S*) (*Fig. 4*). Therefore, the absolute configurations at C(5), C(9), C(10), C(13), C(17), and C(20) were assigned as (*S*), (*R*), (*S*), (*R*), (*R*), and (*R*), respectively.

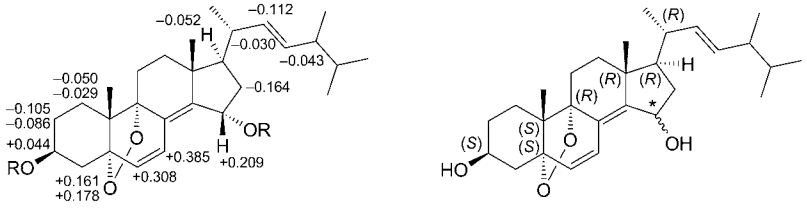

Fig. 4. *Values of* $\Delta\delta_{H(S-R)}$ (measured in $CDCl_3$) *of the MTPA esters of compound* **1**, *and the absolute configuration of* **1** *and* **2**

Compound **2**, obtained as a colorless amorphous powder, exhibited a molecular-ion peak at m/z 442 (M^+) in the EI-MS. Its molecular formula was determined as $C_{28}H_{42}O_4$, same as for **1**, on the basis of HR-EI-MS. Detailed comparison of the 1D- and 2D-NMR

data suggested that **1** and **2** are epimers. However, some correlations were different in the NOESY spectra. The correlation Me(18)/H–C(15) in the NOESY spectrum of **1** disappeared in that of **2**. Instead, a NOE correlation H–C(15)/H–C(17) was observed (*Fig. 3*). Additionally, cross-peaks in the NOESY spectrum from H–C(3) to H_α–C(4), from H_β–C(4) to Me(19), from Me(18) to Me(19) and H–C(20), and from H–C(17) to Me(21) were also observed. The relative configuration of **2** was, therefore, determined as (3*β*,5*α*,15*β*,22*E*)-5,9-epidioxyergosta-6,8(14),22-trien-3,15-diol, the *C(15)*-epimer of **1**. This compound was named nigerasterol B. Because it was obtained in a very small amount, after bioassay, the available sample was not sufficient to determine the absolute configuration. However, nigerasterols A and B (**1** and **2**, resp.) seem to be derived from the same biosynthetic precursor, (3*β*)-24-methylcholestan-3-ol [16], therefore, the absolute configurations at C(3), C(5), C(9), C(10), C(13), C(15), C(17), and C(20) for **2** were tentatively assigned as to be (*S*), (*S*), (*R*), (*S*), (*R*), (*R*), (*R*), and (*R*), respectively.

In addition to the new sterols **1** and **2**, two known cyclopentapeptides, malformins A_1 and C (**3** and **4**, resp.), were also isolated. The structures of these two compounds were identified by comparison of their physicochemical, and ^{1}H- and ^{13}C-NMR data with those reported in the literature [17–19].

2. *Antimicrobial Activity.* The antimicrobial activities of compounds **1**–**4** against two bacteria and four plant-pathogen fungi were evaluated. Nigerasterols A and B (**1** and **2**, resp.) did not display potent antimicrobial activity. However, malformins A_1 and C (**3** and **4**, resp.) exhibited weak antibacterial activities against *Staphylococcus aureus* with a clear inhibition zone of 9.0 and 8.5 mm diameter, respectively, at a concentration of 20 μg/disk, while chloramphenicol showed a 20.0 mm diameter of inhibition zone at the same concentration.

3. *Antiproliferative Activity.* The antiproliferative activities against tumor cell lines HL60 and A549 were also determined (*Table 2*). Nigerasterol B (**2**) displayed potent activity against the tumor cell line HL60 with an IC_{50} value of 1.50 μM, while nigerasterol A (**1**) displayed stronger activity with an IC_{50} value of 0.30 μM. Both **1** and **2** displayed potent activities against A549 cell line with IC_{50} values of 1.82 and 5.41 μM, respectively. Preliminary structure–activity relationship revealed that compound **1**, which has an *α*-OH at C(15), displayed stronger activity than that of compound **2** with a *β*-OH group at C(15).

Table 2. IC_{50} *Values for Compounds* **1** *and* **2** *against Two Tumor Cell Lines*

Compound	$IC_{50} \pm$ SD [μM]	
	HL60	A549
1	0.30 ± 0.01	1.82 ± 0.01
2	1.50 ± 0.01	5.41 ± 0.02
Adriamycin	0.11 ± 0.01	0.43 ± 0.01

Financial support from the *Natural Science Foundation of China* (30910103914 and 31270403) and the *Ministry of Science and Technology of China* (2010CB833800) is gratefully acknowledged.

Experimental Part

General. Column chromatography (CC): commercial silica gel (SiO_2; 100–200 and 200–300 mesh; *Qingdao Haiyang Chemical Factory*) and *Sephadex LH-20* gel (*Pharmacia*). TLC: precoated SiO_2 plates (GF_{254}; *Qingdao Haiyang Chemical Factory*). Anal. HPLC: *Dionex P680* HPLC System with a *P680* pump, an *ASI-100* automated sample injector, a *TCC-100* column oven, a *UV-DAD 340U* detector, and a *Dionex Acclaim ODS* column (4.6 × 250 mm, 5 μm). Semi-prep. HPLC: *Dionex UltiMate U3000* system with an *Elite ODS-BP* column (10 × 300 mm, 10 μm) with UV detection. Optical rotation: *Optical Activity AA-55* polarimeter. UV Spectra: *Gold Spectrumlab 54* UV/VIS spectrophotometer (*Shanghai Lengguang Tech. Co.*); λ_{max} (log ε) in nm. NMR Spectra: *Bruker Avance 500* spectrometer (500 (^{1}H) and 125 MHz (^{13}C)); δ in ppm rel. to Me_4Si as internal standard, *J* in Hz. Low- and high-resolution EI-MS: *API QSTAR Pulsar 1* mass spectrometer; in *m*/*z*.

Fungal Strain and Identification. The fungus *Aspergillus niger* MA-132 was obtained from a fresh healthy sample of the mangrove plant *Avicennia marina* collected in Hainan, China, in August 2004. The fungus was identified to be the most similar (99%) to the *Aspergillus niger* [13], by analysis of the ITS region of the rDNA, as described in [20]. The strain was deposited with the Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences.

Fermentation, Extraction, and Isolation. *Aspergillus niger* MA-132 was incubated on a rotary shaker. The incubation temp. and rotational speed were changed from 28° and 160 rpm [13] to 30° and 180 rpm, resp. In addition to those of the previously isolated nigerapyrones A–H [13], some new peaks were detected in the HPLC profiles. The mass growth of the fungus was thereafter accomplished by incubation at 30° on a rotary shaker (180 rpm) for 7 d in 60 × 500-ml conical flasks containing liquid medium (200 ml/flask) composed of dextrose (20 g/l), malt extract (5 g/l), peptone (10 g/l), and seawater; pH 7.2–7.4. The whole fermented cultures (12 l) were filtered to separate the mycelia from the broth. The former was partitioned three times with acetone/H_2O 4:1. The acetone soln. was evaporated under reduced pressure to afford an aq. soln., which was then extracted three times with AcOEt. The AcOEt soln. was concentrated under reduced pressure to give an extract (0.6 g). The extract was fractionated by vacuum liquid chromatography (VLC) on SiO_2 (different solvents of increasing polarity from petroleum ether (PE) to MeOH) to yield eight fractions, *Frs. 1–8*, based on TLC analysis. *Fr. 3* (200 mg) was further purified by CC (SiO_2; $CHCl_3$/MeOH 1:0 → 1:1) and by semi-prep. HPLC (*Elite ODS-BP* column, 10 μm; 10 × 300 mm; 80% MeOH/H_2O, 3 ml/min) to afford **3** (t_R 22.0 min; 8.0 mg) and **4** (t_R 20.1 min; 20.2 mg). *Fr. 5* (120 mg) was purified by CC (*Sephadex LH-20*; MeOH) to afford two subfrations. *Fr. 5.1* (60.0 mg) was further purified by semi-prep. HPLC (90% MeOH/H_2O; 3 ml/min) to yield **1** (t_R 15.3 min; 4.0 mg) and **2** (t_R 18.0 min; 2.0 mg).

*Preparation of the (*R*)- and (*S*)-MTPA Ester Derivatives of* **1** [15]. (+)-(*S*)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride (10 μl) and 4-(dimethylamino)pyridine (DMAP; 2 mg) were added to *nigerasterol* A (**1**; 1.5 mg), which was dissolved in dried pyridine (400 μl). The mixture was kept at r.t. for 12 h. The acylation product was purified by prep. TLC (SiO_2; $CHCl_3$/MeOH 20:1) to yield corresponding (*R*)-*Mosher* ester **1r**. Treatment of **1** (1.5 mg) with (*R*)-MTPA-Cl (10 μl) as described above gave the corresponding (*S*)-*Mosher* ester **1s**.

Nigerasterol A (=*(3*S*,5*S*,9*R*,10*S*,13*R*,15*S*,17*R*)-1,3,4,10,11,12,13,15,16,17-Decahydro-10,13-dimethyl-17-[(1*R*,2*E*)-1,4,5-trimethylhex-2-en-1-yl]-5,9-epidioxy-2*H*-cyclopenta[a]phenanthrene-3,15-diol*; *(3β,5α,15α,22*E*)-5,9-Epidioxyergosta-6,8(14),22-triene-3,15-diol*; **1**). Colorless amorphous powder. $[\alpha]_D^{20} = +12.5$ ($c = 0.40$, MeOH). UV (MeOH): 252 (4.34). ^{1}H- and ^{13}C-NMR: *Table 1*. EI-MS: 91 (100), 442 (40, M^+), 424 (50, $[M - H_2O]^+$), 409 (36, $[M - H_2O_2 + H]^+$). HR-EI-MS: 442.3070 (M^+, $C_{28}H_{42}O_4^+$; calc. 442.3083).

Nigerasterol B (=*(3*S*,5*S*,9*R*,10*S*,13*R*,15*R*,17*R*)-1,3,4,10,11,12,13,15,16,17-Decahydro-10,13-dimethyl-17-[(1*R*,2*E*)-1,4,5-trimethylhex-2-en-1-yl]-5,9-epidioxy-2*H*-cyclopenta[a]phenanthrene-3,15-diol*; *(3β,5α,15β,22*E*)-5,9-Epidioxyergosta-6,8(14),22-triene-3,15-diol*; **2**). Colorless amorphous powder. $[\alpha]_D^{20} = -5$ ($c = 0.30$, MeOH). UV (MeOH): 253 (4.45). ^{1}H- and ^{13}C-NMR: *Table 1*. EI-MS: 55 (100), 442 (34, M^+), 424 (36, $[M - H_2O]^+$), 408 (16, $[M - H_2O_2]^+$). HR-EI-MS: 442.3088 (M^+, $C_{28}H_{42}O_4^+$; calc. 442.3083).

Antimicrobial Activity. The isolated compounds were tested for their antimicrobial activities against two bacteria (*Staphylococcus aureus* and *Escherichia coli*) and four plant-pathogen fungi (*Alternaria brassicae*, *Fusarium oxysporum*, *Coniella diplodiella*, and *Physalospora piricola*), compared to chloramphenicol and amphotericin B, which were used as positive controls against bacteria and fungi, resp. [21].

Antiproliferative Activity. The antiproliferative activity for isolated compounds against two tumor cell lines was evaluated by SRB (=sulforhodamine B) and MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assays [22–24]. The tested tumor cell lines were HL60 (human promyelocytic leukemia) and A-549 (human lung carcinoma). Adriamycin was used as the positive control against tumor cell lines of HL60 and A549, and showed the IC_{50} values of 0.11 and 0.43 μM, resp.

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Received March 18, 2012