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Diverse Secondary Metabolites Produced by Marine-Derived Fungus *Nigrospora* sp. MA75 on Various Culture Media

by **Zhuo Shang**[a])[b]), **Xiao-Ming Li**[a]), **Chun-Shun Li**[a]), and **Bin-Gui Wang***[a])

[a]) Key Laboratory of Experimental Marine Biology, Institute of Oceanology, Chinese Academy of Sciences, Qingdao 266071, P. R. China (phone: +86-532-82898553; fax: +86-532-82880645; e-mail: wangbingui@gmail.com)

[b]) Graduate School of the Chinese Academy of Sciences, Beijing 100049, P. R. China

Bioassay-guided isolation of a fungal strain *Nigrospora* sp. MA75, an endophytic fungus obtained from the marine semi-mangrove plant *Pongamia pinnata*, which was fermented on three different culture media, resulted in the isolation and identification of seven known compounds, **2**, **3**, and **5**–**9**, from a medium containing 3.5% NaCl, while a new compound, 2,3-didehydro-19α-hydroxy-14-epicochlioquinone B (**10**) was obtained from the medium containing 3.5% NaI. In addition, two new griseofulvin derivatives, 6-*O*-desmethyldechlorogriseofulvin (**1**) and 6′-hydroxygriseofulvin (**4**), were isolated and identified from the rice solid medium. Dechlorogriseofulvin (**2**) and griseofulvin (**3**) were the major components in fermentation extracts of all these culture media, while compounds **1** and **4**, **5** and **6**, and **10** were only present in the extract of respective culture medium. The structures of these compounds were elucidated by detailed spectroscopic analysis, and the absolute configuration of **1** was determined by CD measurement. Compounds **9** and **10** exhibited antibacterial activities toward five tested bacterial strains, while compounds **5**, **6**, and **8** selectively inhibited MRSA, *E. coli*, and *S. epidermidis*, and compound **3** showed moderate activity against *V. mali* and *S. solani*. Moreover, compound **10** potently inhibited the growth of MCF-7, SW1990, and SMMC7721 tumor cell lines with IC_{50} values of 4, 5, and 7 μg/ml, respectively.

Introduction. – In recent years, marine-derived fungi have attracted considerable attention due to their ability to produce structurally unique and biologically active secondary metabolites [1]. We have recently reported a variety of secondary metabolites produced by marine-derived fungi [2–5]. Recently, the whole-genome sequencing programs have revealed that the biosynthetic potential of microorganisms has been greatly underexplored, mainly relying on traditional approaches [6]. Considering that every single biosynthesis step can be influenced by different environmental factors, either at the transcriptional level, at the translational level, or/and at the enzyme level, *Zeeck* and co-workers raised an OSMAC (One Strain MAny Compounds) approach. That is, systematic alteration of easy accessible cultivation parameters (*e.g.*, media composition, pH value, temperature, addition of enzyme inhibitors, oxygen-supply level, and culture vessel) would probably influence the structure and number of secondary metabolites from a single microorganism [7]. In the past few years, several novel bioactive compounds from fungi have been reported by applying this approach [8–11].

To efficiently utilize the marine fungal sources and increase the number of novel bioactive metabolites available from a single fungal strain, we have attempted to

explore the chemical diversity of marine-derived fungi by changing the cultivation parameters. In a preliminary HPLC screening, an endophytic fungus *Nigrospora* sp. MA75, which was obtained from stem of the semi-mangrove plant *Pongamia pinnata*, was found to be able to produce diverse secondary metabolites in different culture media. Furthermore, the crude extracts of the fungal strain exhibited antimicrobial activity. Cultivation of the fungal strain in three different media resulted in the isolation and characterization of ten compounds, **1**–**10** (*Fig. 1*), including three new ones, **1**, **4**, and **10**. From a medium containing 3.5% NaCl, two griseofulvin derivatives, **2** and **3**, two hydronaphthalenone derivatives, **5** and **6**, two xanthones, **7** and **8**, as well as griseophenone C (**9**) were obtained, while the medium containing 3.5% NaI produced a new cochlioquinone derivative, **10**. Meanwhile, two new griseofulvin derivatives, **1** and **4**, were characterized from rice solid medium. Compounds **2** and **3** were the common components in different culture media, while compounds **1**, **4**, **5**, **6**, and **10** solely existed in single culture medium. Here, we describe the isolation, structure elucidation, and biological activity of these compounds.

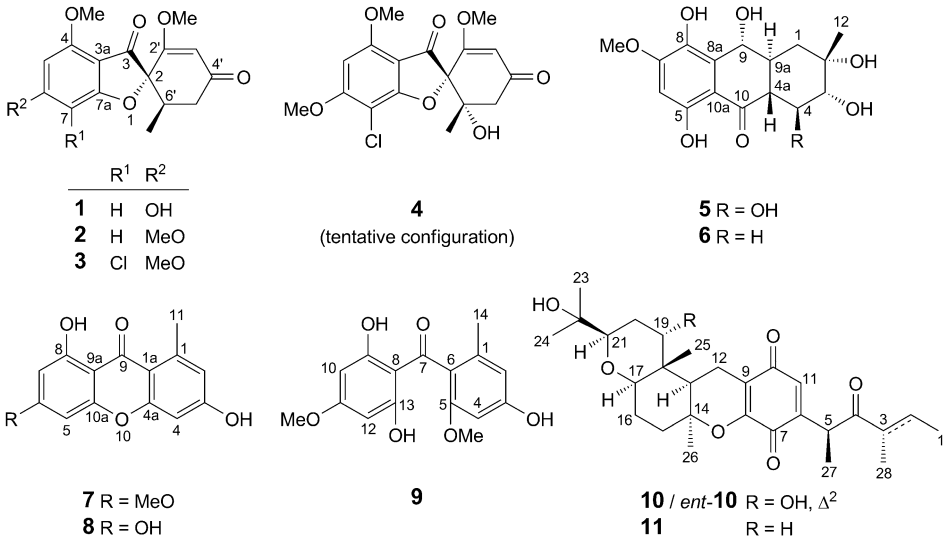
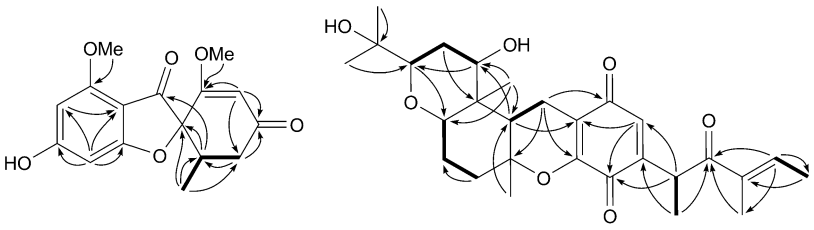


Fig. 1. *Chemical structures of the isolated compounds* **1**–**10** *and the reference compound* **11**

Results and Discussion. – 1. *Structure Elucidation*. Bioassay-guided isolation of the AcOEt extract derived from three different culture media resulted in the isolation of ten compounds, **1**–**10**. Compound **1** was obtained as colorless needles. The IR spectrum displayed absorption bands at 3196 and 1670 cm^{-1}, characteristic for OH and C=O functionalities, respectively. The molecular formula was determined as $C_{16}H_{16}O_6$ by HR-ESI-MS (positive-ion mode; *m*/*z* 305.1021 ($[M+H]^+$); calc. for $C_{16}H_{17}O_6^+$, 305.1025), which was in agreement with the ^{1}H- and ^{13}C-NMR data (*Table 1*). The ^{1}H-NMR spectrum exhibited signals of two aromatic H-atoms at δ(H) 6.07 (*s*, H–C(5)) and 6.23 (*s*, H–C(7)), of one olefinic H-atom at δ(H) 5.54 (*s*, H–C(3′)), of one isolated CH_2 group at δ(H) 3.06 (*dd*, $J = 17.0$, 13.4, H_a–C(5′)) and 2.43 (*dd*, $J = 17.0$, 4.8,

H_b–C(5′)), and of one CH group at δ(H) 2.71–2.79 (*m*, H–C(6′)). In addition, a Me *doublet* group and two MeO *singlet*s were also observed. The ^{13}C-NMR and DEPT spectra (*Table 1*) revealed the presence of 16 C-atoms including those of three Me, one CH_2, and four CH groups, as well as eight quaternary C-atoms. The general features of the NMR data of **1** (*Table 1*) closely resembled those of dechlorogriseofulvin (**2**), an antifungal compound isolated from *Xylaria* sp. F0010, an endophytic fungus of *Abies holophylla* [12]. However, one of the three MeO groups in **2** was absent in **1** according to the NMR spectrum. The observed HMBCs from two MeO groups to C(4) and C(2′) confirmed their positions (*Fig. 2*). The ^2J correlations from H–C(7) to C(6) and C(7a), as well as ^3J correlations from H–C(7) to C(3a) and C(5), and from H–C(5) to C(3a) and C(7) supported the above deduction. The structure of **1** was, therefore, recognized as the 6-*O*-demethylation product of dechlorogriseofulvin (**2**). It should be noted that the NMR signals for H–C(5) and H–C(7) for **2**, as well as for its congener griseofulvin (**3**) were assigned reversely in [12]. Based on 2D-NMR experiments, the NMR data of **2** and **3** were fully assigned as compiled in *Table 1*.



1 **10**

Fig. 2. *Key COSY correlations and HMBCs of compounds* **1** *and* **10**

The absolute configuration of **1** was established on the basis of the CD experiment and optical rotation as compared with those of griseofulvin (**3**), whose absolute configuration was determined by CD spectrum [13]. The CD spectrum of **1** exhibited *Cotton* effects at 295.2 ($\Delta\varepsilon = +26.0$), 267 (+2.9), 236.3 (+32.8), 218.7 (−55.5), and 195.0 (+2.7), which were very similar to those of **3** [13]. In addition, compounds **1** and **3** had similar optical rotations, $[\alpha]_D^{20}$ of +332 for **1** *vs.* +370 for **3**. This evidence indicated that **1** has the same absolute configuration as **3**, that is, (2*S*,6′*R*). It should be mentioned that all the naturally occurring griseofulvin derivatives possess the (*S*)-configuration at C(2) [14–16].

Compound **4** was obtained as a white amorphous powder. The IR spectrum displayed the absorption bands for OH group (3399 cm^{-1}), α,β-unsaturated C=O group (1701 cm^{-1}), and C=C bonds (1617 cm^{-1}). Its molecular formula was established as $C_{17}H_{17}ClO_7$ on the basis of HR-ESI-MS data (positive-ion mode; *m*/*z* 369.0747 ($[M+H]^+$); calc. for $C_{17}H_{18}ClO_7^+$, 369.0741). The ^{1}H- and ^{13}C-NMR spectra indicated the presence of a pentasubstituted benzene ring, three MeO groups, and one Me group. The general features of the ^{1}H- and ^{13}C-NMR data of **4** (*Table 1*) closely resembled those of griseofulvin (**3**) [12]. However, the CH signals at δ(H) 2.76–2.84 (H–C(6′)) and δ(C) 35.5 (C(6′)) for **3** were absent in the NMR spectra of **4**. Instead, an O-bearing

Table 1. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp.) *of* **1**–**4**. δ in ppm, J in Hz. Assignments were corroborated by ^{1}H,^{1}H-COSY, HSQC, and HMBC experiments.

Position	**1**[a])		**2**[a])		**3**[b])		**4**[a])	
	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)	δ(H)	δ(C)
C(2)		89.6 (*s*)		89.9 (*s*)		90.1 (*s*)		92.9 (*s*)
C(3)		192.4 (*s*)		192.4 (*s*)		191.1 (*s*)		190.6 (*s*)
C(3a)		103.2 (*s*)		104.4 (*s*)		104.0 (*s*)		104.9 (*s*)
C(4)		159.7 (*s*)		159.1 (*s*)		157.5 (*s*)		158.1 (*s*)
H–C(5)	6.07 (*s*)	93.8 (*d*)	6.04 (*d*, *J*=1.8)	93.3 (*d*)	6.50 (*s*)	91.3 (*d*)	6.16 (*s*)	90.0 (*d*)
C(6)		169.7 (*s*)		170.4 (*s*)		164.4 (*s*)		164.8 (*s*)
H–C(7)	6.23 (*s*)	91.7 (*d*)	6.22 (*d*, *J*=1.8)	88.6 (*d*)		95.2 (*s*)		97.7 (*s*)
C(7a)		175.8 (*s*)		176.1 (*s*)		168.5 (*s*)		169.2 (*s*)
C(2′)		173.0 (*s*)		171.3 (*s*)		170.2 (*s*)		167.9 (*s*)
H–C(3′)	5.54 (*s*)	104.3 (*d*)	5.53 (*s*)	104.8 (*d*)	5.60 (*s*)	104.6 (*d*)	5.57 (*s*)	104.1 (*d*)
C(4′)		199.0 (*s*)		197.3 (*s*)		195.4 (*s*)		195.1 (*s*)
CH_2(5′)	3.06 (*dd*, *J*=17.0, 13.4), 2.43 (*dd*, *J*=17.0, 4.8)	39.9 (*t*)	3.05 (*dd*, *J*=16.8, 13.4), 2.39 (*dd*, *J*=16.8, 4.7)	40.1 (*t*)	2.67 (*dd*, *J*=16.6, 13.3), 2.35 (*dd*, *J*=16.6, 4.8)	39.8 (*t*)	3.18 (*d*, *J*=16.6), 2.61 (*d*, *J*=16.6)	46.0 (*t*)
H–C(6′)	2.71–2.79 (*m*)	36.5 (*d*)	2.70–2.78 (*m*)	36.6 (*d*)	2.76–2.84 (*m*)	35.5 (*d*)		74.5 (*s*)
Me–C(6′)	0.95 (*d*, *J*=6.7)	14.2 (*q*)	0.96 (*d*, *J*=6.7)	14.2 (*q*)	0.80 (*d*, *J*=6.6)	13.7 (*q*)	1.20 (*s*)	23.1 (*q*)
*Me*O–C(4)	3.85 (*s*)	55.9 (*q*)	3.90 (*s*)	56.1 (*q*)	3.94 (*s*)	56.5 (*q*)	3.99 (*s*)	56.4 (*q*)
*Me*O–C(6)			3.89 (*s*)	56.1 (*q*)	4.04 (*s*)	57.5 (*q*)	4.04 (*s*)	57.1 (*q*)
*Me*O–C(2′)	3.62 (*s*)	56.7 (*q*)	3.62 (*s*)	56.6 (*q*)	3.62 (*s*)	56.9 (*q*)	3.63 (*s*)	56.7 (*q*)

[a]) Recorded in $CDCl_3$. [b]) Recorded in (D_6)DMSO.

quaternary C-atom signal at δ(C) 74.5 (C(6′)) was present in the ^{13}C-NMR of **4**. Moreover, the *double doublet* signals for the isolated CH_2 group at δ(H) 2.67 (*dd*, J = 16.6, 13.3, H_a–C(5′)) and 2.35 (*dd*, J = 16.6, 4.8, H_b–C(5′)) in **3** were replaced by the *doublet*s at 3.18 (*d*, J = 16.6, H_a–C(5′)) and 2.61 (*d*, J = 16.6, H_b–C(5′)) in **4**. Accordingly, the chemical shifts of C(2), C(5′), and C(7′) in **4** were moved downfield within a range of 2.8 to 9.4 ppm (*Table 1*). The above finding suggested the presence of an OH substituent at C(6′) of **4**. This conclusion was also supported by the HMBCs from H–C(5′) and H–C(7′) to C(6′). The absolute configuration at C(2) was tentatively assigned as (*S*) (same as that in **1** and **3**), since all reported griseofulvin derivatives from natural resources possess this configuration [14–16]. Considering the big difference in optical rotation of **4** ($[\alpha]_D^{20} = -75$) compared to those of **1** and **3**, the absolute configuration at C(6′) of **4** was tentatively assigned as (*S*), differing from (*R*) in **1** and **3**. The structure of **4** was, therefore, characterized and named as 6′-hydroxygriseofulvin. However, to obtain convincing evidence for the assignment of absolute configuration of **4**, further efforts such as synthesis, computational modeling, or crystallization of its chemical derivatives for X-ray crystallographic analysis, should be conducted in future.

Compound **10** was isolated as a yellow amorphous powder. The IR spectrum exhibited bands characteristic for OH groups (3440 cm^{-1}), conjugated C=O groups (1637 cm^{-1}), and C=C bonds (1610 cm^{-1}). The molecular formula was determined as $C_{28}H_{38}O_7$ by HR-ESI-MS (positive-ion mode; *m*/*z* 509.2512 ($[M+Na]^+$), calc. for $C_{28}H_{38}NaO_7^+$, 509.2515). The ^{1}H- and ^{13}C-NMR data were found similar to those of 14-epicochlioquinone B (**11**; *Fig. 1*) [17], except for an additional CH–O signal at δ(C) 69.2 (C(19)) in **10** (*Table 2*), which replaced the CH_2 signal at δ(C) 37.0 (C(19)) in **11** [17]. Accordingly, two isolated H-atom signals of CH_2(19) at δ(H) 1.91 and 1.12 in **11** were replaced by an O-bearing CH signal at δ(H) 3.87 (br. *s*, H–C(19)) in **10**. The observed HMBCs from H–C(13), H–C(20), and Me(25) to C(19), and from H–C(19)

Table 2. *^{1}H- and ^{13}C-NMR Data* (500 and 125 MHz, resp.) *of* **10** *in $CDCl_3$, δ in ppm, J in Hz.* Assignments were corroborated by ^{1}H,^{1}H-COSY, HSQC, and HMBC experiments. For atom numbering, see *Fig. 1*.

Position	δ(H)	δ(C)	Position	δ(H)	δ(C)
Me(1)	1.88 (*d*, *J* = 6.5)	15.0 (*q*)	CH_2(15)	2.28–2.31 (*m*), 1.64–1.67 (*m*)	36.3 (*t*)
H–C(2)	6.91 (*q*, *J* = 6.5)	138.5 (*d*)	CH_2(16)	1.80–1.84 (*m*), 1.56–1.60 (*m*)	23.4 (*t*)
C(3)		136.9 (*s*)	H–C(17)	3.63 (*dd*, *J* = 12.0, 3.4)	76.1 (*d*)
C(4)		200.7 (*s*)	C(18)		41.1 (*s*)
H–C(5)	4.62 (*q*, *J* = 6.9)	37.8 (*d*)	H–C(19)	3.87 (br. *s*)	69.2 (*d*)
C(6)		146.5 (*s*)	CH_2(20)	1.91–1.96 (*m*), 1.47–1.49 (*m*)	30.3 (*t*)
C(7)		181.0 (*s*)	H–C(21)	3.53 (*dd*, *J* = 12.3, 2.3)	78.3 (*d*)
C(8)		153.2 (*s*)	C(22)		71.6 (*s*)
C(9)		119.0 (*s*)	Me(23)	1.12 (*s*)	23.8 (*q*)
C(10)		186.6 (*s*)	Me(24)	1.18 (*s*)	26.2 (*q*)
H–C(11)	6.53 (*s*)	133.5 (*d*)	Me(25)	0.69 (*s*)	12.0 (*q*)
CH_2(12)	2.39 (*dd*, *J* = 19.7, 8.4), 2.50–2.57 (*m*)	15.5 (*t*)	Me(26)	1.22 (*s*)	27.2 (*q*)
H–C(13)	2.21 (br. *d*, *J* = 8.3)	35.0 (*d*)	Me(27)	1.27 (*d*, *J* = 6.9)	16.3 (*q*)
C(14)		79.8 (*s*)	Me(28)	1.79 (*s*)	11.5 (*q*)

to C(21), as well as the ^{1}H,^{1}H-COSY correlation from H–C(19) to H–C(20) confirmed the presence of the OH group at C(19) in **10**. On the other hand, the H-atom signals at δ(H) 1.73/1.39 (H–C(2)) and 2.73 (H–C(3)) in **11** [17] were replaced by an olefinic *quartet* at δ(H) 6.91 (H–C(2)) in **10** (*Table 2*). The presence of a C(2)=C(3) bond was also supported by the HMBCs from H–C(2) to Me(1), Me(28), and C(4) (*Fig. 2*).

The relative configuration of **10** was determined by NOESY experiment as well as by analysis of H,H coupling constants. The NOE correlations Me(26)/H–C(13), H–C(13)/H–C(17), and H–C(17)/H–C(21) indicated that these H-atoms were *cis*-oriented and in α-configuration, while NOE correlation Me(25)/H–C(19) revealed that the two groups were β-oriented (*Fig. 3*). In addition, the large vicinal coupling constants $J(H_{ax}$–C(16), H–C(17)) of 12.0 Hz and $J(H_{ax}$–C(20), H–C(21)) of 12.3 Hz indicated that both H–C(17) and H–C(21) are axially oriented (*Fig. 3*). On the basis of these findings, the structure of compound **10** was deduced as 2,3-didehydro-19α-hydroxy-14-epicochlioquinone B.

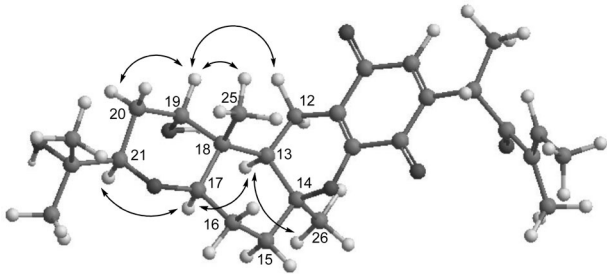


Fig. 3. *Key NOESY correlations* (arrows) *and the 3D computer modeling of* **10**

Attempts to determine the absolute configuration of **10** by using the modified *Mosher*'s method failed, possibly due to the degradation of **10** under the reaction conditions.

In addition to the new compounds **1**, **4**, and **10**, seven known compounds, *i.e.*, dechlorogriseofulvin (**2**) [12], griseofulvin (**3**) [12], tetrahydrobostrycin (**5**) [18][19], 4-deoxytetrahydrobostrycin (**6**) [18][19], 3,8-dihydroxy-6-methoxy-1-methylxanthone (**7**) [20], 3,6,8-trihydroxy-1-methylxanthone (**8**) [20], and griseophenone C (**9**) [21], were also isolated and identified.

2. *Chemical Profiles of Crude Extracts from Various Culture Media*. Crude extracts of *Nigrospora* sp. MA75 fermented in three different culture media as well as all isolated compounds were analyzed by HPLC (gradient: 0–5 min, 20% MeOH; 5–40 min, 20–80% MeOH; 40–50 min, 100% MeOH, 1.0 ml/min, 25°). By comparison of retention time t_R and UV absorption of each chromatographic peak, compounds **1**–**6**, **8**, and **10** were identified in the HPLC profiles of crude extracts, except for compounds **7** and **9**, which were relatively minor in quantity (*Fig. 4*). Compounds **2** and **3** were common and major components in the crude extracts of this fungal strain in three culture media, while compound **8** exclusively occurred in extracts of culture medium containing 3.5% NaCl and rice solid medium. Interestingly, compounds **5** and **6** were only produced in culture medium containing 3.5% NaCl, while compounds **1** and **4** were only detected in rice solid medium. Additionally, in the fermentation extract of

the fungus in culture medium with 3.5% NaI, compound **10** was found as the major component, whereas in the other two culture media it was not detected. The production of compound **10** was effected by adding of iodide ion, which might be playing as a trigger to activate a mixed polyketide–terpenoid biosynthetic pathway in this fungal strain. On the other hand, the iodide ion may also affect on cryptic gene clusters encoding biosynthetic enzymes to catalyze the synthesis of this compound, and the molecular mechanism of iodide ion in the fungal strain should be further investigated. In summary, the results from our experiments demonstrated that changing the fermentation media is an effective way to diversify the metabolites of a single fungal strain.

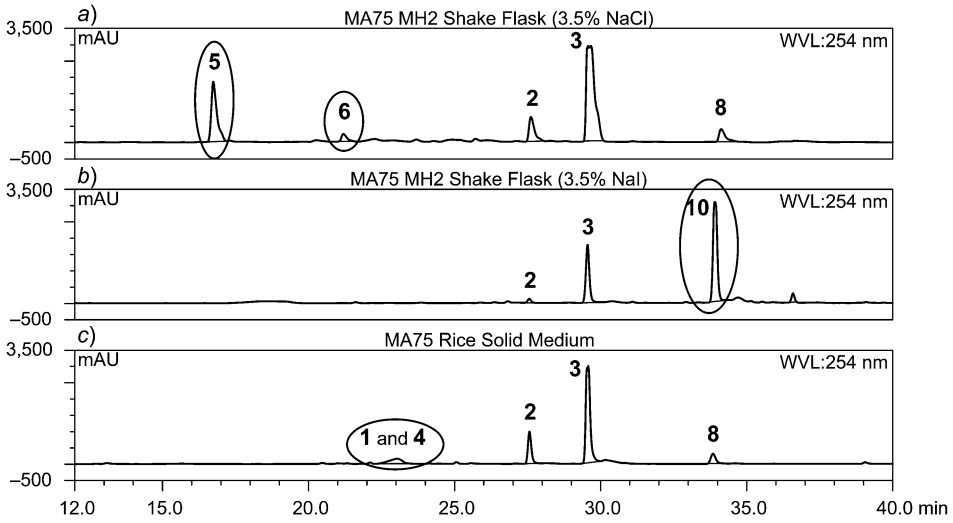


Fig. 4. *HPLC Profiles of crude extracts from three different culture media.* Major compounds were assigned, and the peaks of compounds occuring in only one medium were circled with ellipses in the profiles. *a*) The medium with 3.5% NaCl; *b*) the medium with 3.5% NaI; *c*) rice solid medium.

3. *Antimicrobial Activity.* The antimicrobial activity of compounds **1**–**10** against five bacterial and three fungal strains were evaluated, and the *MIC* values were collected in *Table 3*. The results revealed that compound **10** exhibited remarkable antibacterial activity against all the tested bacteria (MRSA, *E. coli*, *P. aeruginosa*, *P. fluorescens*, and *S. epidermidis*) with *MIC* values of 8, 4, 4, 0.5, and 0.5 μg/ml, respectively. Among these, the activity toward *E. coli*, *P. fluorescens*, and *S. epidermidis* was stronger than that of the positive control (ampicillin, with *MIC* values of 8, 4, and 4 μg/ml, resp.). Compound **9** was also a broad-spectrum antibiotic which strongly inhibited MRSA, *E. coli*, *P. aeruginosa*, and *P. fluorescens* with *MIC* values of 0.5, 2, 0.5, and 0.5 μg/ml, respectively. Compound **5** exhibited significant activity toward MRSA and *E. coli* (*MIC* 2 and 0.5 μg/ml, resp.), while its analog, compound **6**, only showed activity against *E. coli* (*MIC* 4 μg/ml). This finding indicated that the OH group at C(4) might be important for the activity against MRSA. Among the griseofulvin derivatives, only compound **3** showed moderate activity against *V. mali* and *S. solani*, both with *MIC* values of 16 μg/ml. Griseofulvin (**3**) is an antifungal antibiotic and is now used for the treatment of

mycotic diseases of human, veterinary, and plant systems [22]. However, other members of griseofulvin family have been reported to exhibit much weaker or no antifungal activities [23][24], because the activity of griseofulvin should be strictly related to both its planar structure and spatial configuration. Compared to griseofulvin (**3**), the much weaker antifungal activity of its derivatives, *i.e.*, **1**, **2**, and **4**, further confirmed this conclusion.

Table 3. MIC *Values* [μg/ml] *of Compounds* **1**–**10** *against Five Bacteria and Three Fungi*

Tested microorganisms	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	Ampicillin[a])	Nystatin[b])	Amphotericin B[b])
MRSA	na[c])	na	na	na	2	na	na	na	0.5	8	8	–	–
E. coli	na	na	na	na	0.5	4	na	32	2	4	8	–	–
P. aeruginosa	na	na	na	na	na	na	na	na	0.5	4	2	–	–
P. fluorescens	na	na	na	na	na	na	na	na	0.5	0.5	4	–	–
S. epidermidis	na	na	na	na	na	na	na	0.5	16	0.5	4	–	–
C. albicans	na	na	na	na	na	na	na	na	na	64	–	2	–
V. mali	na	na	16	na	na	na	na	na	na	na	–	–	4
S. solani	na	128	16	na	na	na	na	na	na	na	–	–	16

[a]) Antibacterial positive control. [b]) Antifungal positive controls. [c]) na: No activity (*MIC* > 256 μg/ml).

4. *Cytotoxic Activity.* Compounds **1**–**10** were evaluated for their cytotoxic activities against seven tumor cell lines, and the IC_{50} values of active compounds were compiled in *Table 4*. Compound **10** displayed activities against MCF-7, SW1990, and SMMC7721 cell lines (IC_{50} values of 4, 5, and 7 μg/ml, resp.) as well as moderate activity against HepG2, NCI-H460, and DU145 cell lines (IC_{50} values of 20, 11, and 17 μg/ml, resp.). Among these, the cytotoxicity of **10** toward SW1990 cell line was stronger than that of positive control (fluorouracil, with an IC_{50} value of 16 μg/ml). According to literature reports, most members of cochlioquinone class are inhibitors of diacylglycerol kinase [25], mitochondrial NADH-ubiquinone reductase [26], and acyl-CoA:cholesterol acyltransferase (ACAT) [27]. They most likely possess cytotoxic activity by inhibiting the key enzymes for regulation of cancer cell proliferation. Compounds **1**, **6**, and **8** possessed selective activities against HepG2, HeLa, and HepG2 cell lines, respectively. However, the other compounds showed weak or no appreciable activities against the tested tumor cell lines.

Table 4. IC_{50} *Values* ([μg/ml]) *of Compounds* **1**, **6**, **8**, *and* **10** *against Seven Tumor Cell Lines*

Compounds	Tumor cell lines						
	MCF-7	SW1990	HepG2	NCI-H460	HeLa	DU145	SMMC7721
1	–	–	20	–	–	–	–
6	–	–	–	–	22	–	–
8	–	–	15	–	–	–	–
10	4	5	20	11	–	17	7
Fluorouracil[a])	4	16	14	1	14	0.4	2

[a]) Positive control.

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Experimental Part

General. Column chromatography (CC): commercial silica gel (SiO_2; 100–200 and 200–300 mesh; *Qingdao Haiyang Chemical Factory*), *Lobar LiChroprep RP-18 gel* (40–63 μm; *Merck*), and *Sephadex LH-20* gel (*Pharmacia*). TLC: Precoated silica gel plates (*GF-254*, *Qingdao Haiyang Chemical Factory*). Anal. HPLC: *Dionex P680* HPLC System comprising 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). Prep. HPLC: *Dionex UltiMate U3000* system with an *Agilent Prep RP-18* column (21.2 × 250 mm, 10 μm); UV detection. M.p.: *SGW X-4* micro-melting-point apparatus (*Shanghai Precision & Scientific Instrument Co.*). Optical rotations: *Optical Activity AA-55* polarimeter. UV Spectra: *Gold Spectrumlab 54* UV/VIS spectrophotometer (*Shanghai Lengguang Tech. Co.*); λ_{max} (log ε) in nm. IR Spectra: *JASCO FT/IR-4100 Fourier Transform* infrared spectrometer; $\tilde{\nu}_{max}$ in cm^{-1}. NMR Spectra: *Bruker Avance 500* spectrometer (^1H: 500 and ^{13}C: 125 MHz); chemical shifts δ in ppm, *J* in Hz. Low- and high-resolution (LR and HR, resp.) ESI-MS: *VG Autospec-3000* mass spectrometer; in *m*/*z*.

Fungal Strain and Identification. The endophytic fungus *Nigrospora* sp. MA75 was isolated from the stem of semi-mangrove plant *Pongamia pinnata*, which was collected from Guangxi Zhuang Autonomous Region of China in August 2010. Fungal identification was performed based on sequencing of the ITS regions [28]. The fungal strain grew slowly on potato dextrose agar at 28°. Color of the colony was white for the first 4 d, and then gradually became gray with black areas and eventually turned to black. Spores were generated after more than 10 d, and only a few spores can be observed by naked eyes. The sequence data of 5.8S rDNA and ITS regions derived from the fungal strain has been submitted to and deposited with the GenBank with accession No. HQ891662. The nucleotide BLAST search result showed that the sequence was most similar (98%) to the sequence of *Nigrospora* sp. (compared to accession No. HQ248210). The strain is preserved with the Institute of Oceanology, Chinese Academy of Sciences.

Culture Media and Method. The fungal strain *Nigrospora* sp. MA75 was cultured in three different media: *1*) A medium with 3.5% NaCl (6.0 g of mannitol, 6.0 g of maltose, 3.0 g of monosodium glutamate, 0.9 g of yeast extract, 3.0 g of glucose, 0.3 g of corn steep liquor, 0.09 g of $MgSO_4 \cdot 7\ H_2O$, 0.15 g of KH_2PO_4, 0.15 g of L-tryptophan, and 200 ml of dist. H_2O with 7.0 g of NaCl in 500-ml *Erlenmeyer* flasks; pH 6.5–7.0; 30 flasks) fermented at 28° under 130 rpm rotary flask shaker for 8 d. *2*) A medium with 3.5% NaI (the medium composition and culture method were the same as above except using NaI instead of NaCl in each flask; 10 flasks). *3*) Rice solid medium with 3.5% NaCl (70 g of rice, 0.2 g of corn steep liquor, 0.3 g of peptone, 0.1 g of monosodium glutamate, and 100 ml of dist. H_2O with 3.5 g of NaCl in 1-l *Erlenmeyer* flasks; pH 6.5–7.0; 30 flasks) statically fermented at 28° for 60 d.

Extraction. For shake-flask culture, the fermented broth and mycelium of *Nigrospora* sp. MA75 were separated by filtration and then exhaustively extracted with AcOEt and MeOH by ultrasonic processor, resp. Since the TLC and HPLC profiles of the two extracts were nearly identical, they were combined to afford the crude extract. For rice solid medium, the rice matrix and mycelium of *Nigrospora* sp. MA75 were soaked with AcOEt for three times and concentrated under vacuum to obtain crude extract.

Bioassay Guided Isolation. In the disk-diffusion test, both the crude extracts from the medium with 3.5% NaCl and 3.5% NaI displayed activities against *E. coli* and *V. mali*, while the crude extract from rice solid medium only showed mild activity against *V. mali*. For antimicrobial-compound isolation, the crude extract from the medium with 3.5% NaCl was subjected to silica-gel vacuum liquid chromatography (VLC) eluting with gradient solvent of petroleum ether (PE)/AcOEt (from 1:0 to 1:1) and $CHCl_3$/MeOH (from 20:1 to 0:1) to afford twelve fractions (*Frs. 1–12*). *Fr. 7* (1.0 g) showed mild activity against *V. mali* with 10-mm inhibition zone, and *Fr. 8* (1.9 g) exhibited significant activity against *E. coli* with 22-mm inhibition zone. *Fr. 7* was, therefore, further fractionated by CC (*Lobar LiChroprep* C_{18}; MeOH/H_2O 1:4–1:0) to yield five subfractions, *Frs. 7-1–7-5*. *Fr. 7-4*, which showed moderate activity against *V. mali*, was further purified by prep. HPLC (55% MeOH, 10.0 ml/min) to afford compounds **2** (28.4 mg), **3** (56.3 mg), and **9** (6.0 mg). By the same method, *Fr. 8* was fractionated into five subfractions,

Frs. 8-1–8-5, and *Fr. 8-3* was purified by prep. HPLC (35% MeOH, 10.0 ml/min) to yield compounds **5** (38.5 mg) and **6** (16.6 mg). In addition, *Fr. 5* (83.9 mg) was subjected to CC (*Sephadex LH-20*; $CHCl_3$/MeOH, 1:1) and then purified by prep. HPLC (60% MeOH, 10.0 ml/min) to yield compounds **7** (6.5 mg) and **8** (14.2 mg). The crude extract from the medium with 3.5% NaI was separated by CC (*Sephadex LH-20*; MeOH; and SiO_2; $CHCl_3$/MeOH 100:1–40:1) to yield compound **10** (8.0 mg). Following the procedures described above, the crude extract from rice solid medium was fractionated into twelve fractions, *Frs.1–12* by VLC, and *Fr. 7* was further purified by CC (*Lobar LiChroprep C_{18}*) to afford five subfractions, *Frs. 7-1–7-5*. *Frs. 7-2* and *7-3* were combined and separated by CC (SiO_2; $CHCl_3$/MeOH 100:1–10:1; and *Sephadex LH-20*; MeOH), and prep. TLC (PE/acetone 1:1) to yield compounds **1** (31.9 mg) and **4** (4.2 mg).

6-O-*Desmethyldechlorogriseofulvin* (=*(2*S*,6′*R*)-6-Hydroxy-2′,4-dimethoxy-6′-methyl-3*H*,4′*H*-spiro[1-benzofuran-2,1′-cyclohex[2]ene]-3,4′-dione*; **1**). Colorless needles (MeOH). M.p. 265–267°. $[\alpha]_D^{20} = +332$ ($c = 0.25$, MeOH). UV (MeOH): 208 (4.47), 231 (4.33), 250 (4.31), 289 (4.42). CD (MeOH): 295.2 (+ 26.0), 267 (+2.9), 236.3 (+32.8), 218.7 (−55.5), 195.0 (+2.7). IR (KBr): 3196, 1670, 1594, 1457, 1357, 1226, 1125, 1058, 832, 675. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 305.1021 ($[M+H]^+$, $C_{16}H_{17}O_6^+$; calc. 305.1025).

6′-Hydroxygriseofulvin (=*(2*S*,6′*S*)-7-Chloro-6′-hydroxy-2′,4,6-trimethoxy-6′-methyl-3*H*,4′*H*-spiro[1-benzofuran-2,1′-cyclohex[2]ene]-3,4′-dione*; **4**). White amorphous powder. M.p. 115–116°. $[\alpha]_D^{20} = -75$ ($c = 0.20$, MeOH). UV (MeOH): 209 (4.29), 232 (4.18), 291 (4.22). IR (KBr): 3399, 1701, 1617, 1590, 1470, 1354, 1219, 1142, 1102. ^{1}H- and ^{13}C-NMR: see *Table 1*. HR-ESI-MS: 369.0747 ($[M+H]^+$, $C_{17}H_{18}ClO_7^+$; calc. 369.0741).

19α-Hydroxy-2,3-didehydro-14-epicochlioquinone B (=*(1*S*,3*R*,4a*R*,6a*S*,12a*R*,12b*S*)-1,2,3,4a,5,6,6a,12,12a,12b-Decahydro-1-hydroxy-3-(2-hydroxypropan-2-yl)-6a,12b-dimethyl-9-[(2*S*,4*E*)-4-methyl-3-oxohex-4-en-2-yl]pyrano[3,2-*a*]xanthene-8,11-dione*; **10**). Yellow amorphous powder. $[\alpha]_D^{20} = +100$ ($c =$ 0.06, MeOH). UV (MeOH): 231 (4.09). IR (KBr): 3440, 2928, 1637, 1610, 1384, 1130, 1080, 701, 540. ^{1}H- and ^{13}C-NMR: see *Table 2*. HR-ESI-MS: 09.2512 ($[M+Na]^+$, $C_{28}H_{38}NaO_7^+$; calc. 509.2515).

Antimicrobial Activity. The isolated compounds were tested for their inhibitory activity against five bacteria, methicillin-resistant *Staphylococcus aureus* (MRSA), *Escherichia coli*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Staphylococcus epidermidis*, and three fungi, *Candida albicans*, *Valsa mali*, and *Stemphylium solani* by the broth-microdilution method [29].

Cytotoxicity Assay. The cytotoxic activities of the isolated compounds were evaluated by MTT (= 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) method as described in [30]. The tested tumor cell lines were MCF-7 (human breast adenocarcinoma), SW1990 (human pancreatic cancer), HepG2 (human hepatocellular liver carcinoma), NCI-H460 (human non-small cell lung cancer), HeLa (human epithelial carcinoma), DU145 (human prostate carcinoma), and SMMC7721 (human hepatocellular liver carcinoma).

REFERENCES

[1] M. E. Rateb, R. Ebel, *Nat. Prod. Rep.* **2011**, *28*, 290.
[2] S.-S. Gao, X.-M. Li, Y. Zhang, C.-S. Li, B.-G. Wang, *Chem. Biodiversity* **2011**, *8*, 1748.
[3] C.-S. Li, C.-Y. An, X.-M. Li, S.-S. Gao, C.-M. Cui, H.-F. Sun, B.-G. Wang, *J. Nat. Prod.* **2011**, *74*, 1331.
[4] S.-S. Gao, X.-M. Li, Y. Zhang, C.-S. Li, C.-M. Cui, B.-G. Wang, *J. Nat. Prod.* **2011**, *74*, 256.
[5] S.-S. Gao, X.-M. Li, C.-S. Li, P. Proksch, B.-G. Wang, *Bioorg. Med. Chem. Lett.* **2011**, *21*, 2894.
[6] K. Scherlach, C. Hertweck, *Org. Biomol. Chem.* **2009**, *7*, 1753.
[7] H. B. Bode, B. Bethe, R. Höfs, A. Zeeck, *ChemBioChem* **2002**, *3*, 619.
[8] O. E. Christian, J. Compton, K. R. Christian, S. L. Mooberry, F. A. Valeriote, P. Crews, *J. Nat. Prod.* **2005**, *68*, 1592.
[9] P. A. Paranagama, E. M. K. Wijeratne, A. A. L. Gunatilaka, *J. Nat. Prod.* **2007**, *70*, 1939.
[10] G. Yang, K. Yun, V. N. Nenkep, H. D. Choi, J. S. Kang, B. W. Son, *Chem. Biodiversity* **2010**, *7*, 2766.
[11] F.-Z. Wang, H.-J. Wei, T.-J. Zhu, D.-H. Li, Z.-J. Lin, Q.-Q. Gu, *Chem. Biodiversity* **2011**, *8*, 887.

[12] J.-H. Park, G.-J. Choi, S.-W. Lee, H.-B. Lee, K.-M. Kim, H.-S. Jung, K.-S. Jung, K.-Y. Cho, J.-C. Kim, *J. Microbiol. Biotechnol.* **2005**, *15*, 112.
[13] T. Takahashi, M. Yamaguchi, *J. Inclusion Phenom. Mol. Recognit. Chem.* **1991**, *10*, 283.
[14] W. A. C. Brown, G. A. Sim, *J. Chem. Soc.* **1963**, 1050.
[15] B. B. Jarvis, Y. Zhou, J. Jiang, S. Wang, W. G. Sorenson, E.-L. Hintikka, M. Nikulin, P. Parikka, R. A. Etzel, D. G. Dearborn, *J. Nat. Prod.* **1996**, *59*, 553.
[16] G. N. Belofsky, K. B. Gloer, J. B. Gloer, D. T. Wicklow, P. F. Dowd, *J. Nat. Prod.* **1998**, *61*, 1115.
[17] K. Lorenzen, T. Anke, U. Anders, H. Hindermayr, F. Hansske, *Z. Naturforsch., C* **1994**, *49*, 312.
[18] U. Sommart, V. Rukachaisirikul, Y. Sukpondma, S. Phongpaichit, J. Sakayaroj, K. Kirtikara, *Chem. Pharm. Bull.* **2008**, *56*, 1687.
[19] J. Xu, T. Nakazawa, K. Ukai, H. Kobayashi, R. E. P. Mangindaan, D. S. Wewengkang, H. Rotinsulu, M. Namikoshi, *J. Antibiot.* **2008**, *61*, 415.
[20] J. Mutanyatta, B. Matapa, D. Shushu, B. Abegaz, *Phytochemistry* **2003**, *62*, 797.
[21] A. Rhodes, B. Boothroyd, M. P. McGonagle, G. A. Somerfield, *Biochem. J.* **1961**, *81*, 28.
[22] V. Venkata Dasu, T. Panda, *Bioprocess Biosyst. Eng.* **1999**, *21*, 489.
[23] A. Mustafa, 'Chemistry of heterocyclic compounds, Vol. 29, Benzofurans', 1st edn., John Wiley & Sons, 1974, p. 420.
[24] Atta-ur-Rahman, 'Studies in natural products chemistry: bioactive natural products (Part L)', Vol. 32, 1st edn., Gulf Professional Publishing, 2005, p. 478.
[25] T. Machida, K. Higashi, H. Ogawara, *J. Antibiot.* **1995**, *48*, 1076.
[26] C.-H. Lim, H. Ueno, H. Miyoshi, H. Miyagawa, H. Iwamura, T. Ueno, *J. Pesticide Sci.* **1996**, *21*, 213.
[27] T. Fujioka, K. Yao, K. Hamano, T. Hosoya, T. Kagasaki, Y. Furukawa, H. Haruyama, S. Sato, T. Koga, Y. Tsujita, *J. Antibiot.* **1996**, *49*, 409.
[28] S. Wang, X.-M. Li, F. Teuscher, D.-L. Li, A. Diesel, R. Ebel, P. Proksch, B.-G. Wang, *J. Nat. Prod.* **2006**, *69*, 1622.
[29] C.-C. Shen, W.-J. Syu, S.-Y. Li, C.-H. Lin, G.-H. Lee, C.-M. Sun, *J. Nat. Prod.* **2002**, *65*, 1857.
[30] R. J. Bergeron, P. F. Cavanaugh Jr., S. J. Kline, R. G. Hughes Jr., G. T. Elliott, C. W. Porter, *Biochem. Biophys. Res. Commun.* **1984**, *121*, 848.

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