Alkaloid and Anthraquinone Derivatives Produced by the Marine-Derived Endophytic Fungus *Eurotium rubrum*

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Cultivation of the fungal strain *Eurotium rubrum*, an endophytic fungus that was isolated from the inner tissue of the semi-mangrove plant *Hibiscus tiliaceus*, resulted in the isolation of one new dioxopiperazine alkaloid, 12-demethyl-12-oxo-eurotechinulin B (**1**), and one new anthraquinone derivative, 9-dehydroxyeurotinone (**2**), together with ten known compounds including variecolorin J (**3**), eurotechinulin B (**4**), variecolorin G (**5**), alkaloid E-7 (**6**), cryptoechinuline G (**7**), isoechinulin B (**8**), 7-isopentenylcryptoechinuline D (**9**), 2-*O*-methyl-9-dehydroxyeurotinone (**10**), emodin (**11**), and emodic acid (**12**). The structures of the isolated compounds were determined by extensive analysis of their spectroscopic data as well as by comparison with literature reports. Some of the purified compounds were evaluated for antibacterial, antifungal, and cytotoxic activities.

Introduction. – Mangrove and semi-mangrove plants are mostly distributed in tropical and subtropical coastal regions of the world. Because of their special ecosystems that straddles the land and the sea, mangrove plants are found to be a rich source of microorganism species including mutualistic fungal endophytes [1][2]. Our previous chemical study of the liquid fermentation culture of *Eurotium rubrum*, an endophytic fungal obtained from the semi-mangrove plant *Hibiscus tiliaceus*, has afforded a variety of structurally interesting metabolites [3–5]. This fungus was reinvestigated by using the solid rice fermentation and, as a result, the two new metabolites **1** and **2** and the ten known compounds **3**–**12** with diverse molecular structures were isolated and identified (*Fig. 1*). This article describes the isolation, structure elucidation, antimicrobial activity, and cytotoxicity of these compounds.

Results and Discussion. – *Isolation and Structure Elucidation*. The fermented rice substrate was extracted repeatedly with AcOEt to afford a crude extract, which was further purified by a combination of column chromatography (CC) on silica gel (SiO_2), *Sephadex LH-20*, and reversed-phase SiO_2, to yield the two new metabolites **1** and **2**, besides the ten known compounds **3**–**12**.

Compound **1** was obtained as a colorless amorphous powder. The IR spectrum showed absorption bands for amide (3439, 1734, and 1687 cm^{-1}) and aromatic (1604 cm^{-1}) moieties in the molecules. The molecular formula was determined as $C_{28}H_{33}N_3O_3$ on the basis of the negative-ion-mode HR-ESI-MS. The ^{1}H-NMR spectrum (*Table 1*) exhibited signals attributed to three NH groups, eight aromatic

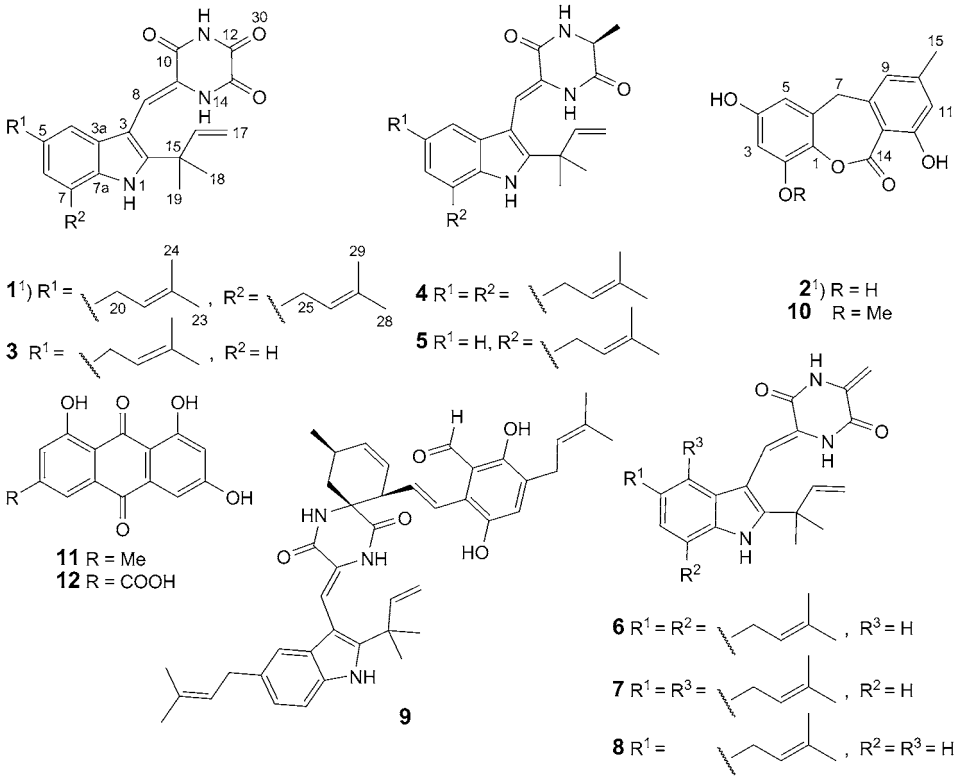

Fig. 1. *Compounds* **1**–**12**, *isolated from the endophytic fungus* Eurotium rubrun

and olefinic H-atoms, and two aliphatic CH_2 and six Me groups (*Table 1*). In the ^{13}C-NMR and DEPT spectra, 28 C-atoms including six Me, three CH_2, and six CH groups, and 13 quaternary C-atoms were observed (*Table 1*). Detailed analysis of the ^{1}H- and ^{13}C-NMR spectra revealed that **1** might be a dioxopiperazine alkaloid derivative containing a 1*H*-indole moiety. Comparison of the ^{1}H- and ^{13}C-NMR spectral data of **1** with those of eurotechinulin B (**4**) [3] revealed that the structures of these two compounds are very similar. However, the Me(30) resonating at δ(C) 20.8 and the CH group at δ(C) 51.6 (C(12)) in the ^{13}C-NMR spectrum of **4** disappeared in that of **1**. Instead, a quaternary C-atom resonating at δ(C) 152.5 (C(12)) was present in that of **1**. According to the molecular formula, one more O-atom and one more degree of unsaturation were present in **1** as compared to **4**. The above observation implied that Me–C(12) of **4** was replaced by a C(12)=O group in **1**. The lower-field-shifted H–C(8) of **1** (δ(H) 7.39) implied that H–C(8) was influenced by the deshielding effect of the C=O group, which suggested the C=C bond at C(8) to have (*Z*)-geometry. Based on the above spectral evidence (see also *Fig. 2* for the HMBC), the structure of **1** was established, and this compound was named 12-demethyl-12-oxoeurotechinulin B[1]).

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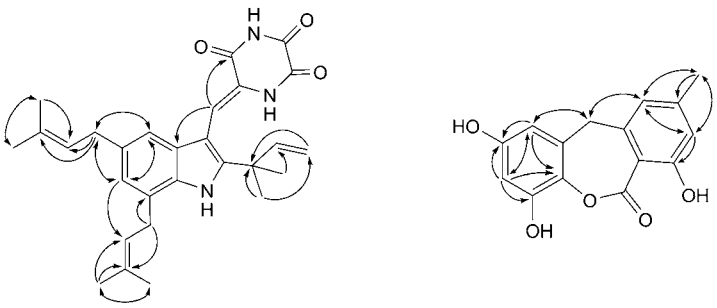
[1]) Trivial atom numbering; for systematic names, see *Exper. Part.*



1 **2**

Fig. 2. *Selected HMBC* (H → C) *features of* **1** *and* **2**

Table 1. *^{1}H- and ^{13}C-NMR Data* ((D$_6$)acetone, 500 and 125 MHz, resp.) *of Compound* **1**. δ in ppm, J in Hz.

	δ(H)	δ(C)		δ(H)	δ(C)
H–N(1)	9.59 (br. *s*)		C(15)		40.3 (*s*)
C(2)		146.3 (*s*)	H–C(16)	6.17 (*dd*, J = 17.5, 10.5)	146.0 (*d*)
C(3)		104.8 (*s*)	CH_2(17)	5.16 (*d*, J = 17.5)	112.9 (*t*)
C(3a)		127.4 (*s*)		5.14 (*d*, J = 10.5)	
H–C(4)	7.13 (*s*)	117.1 (*d*)	Me(18)	1.58 (*s*)	28.1 (*q*)
C(5)		133.6 (*s*)	Me(19)	1.58 (*s*)	28.1 (*q*)
H–C(6)	6.84 (*s*)	123.4 (*d*)	CH_2(20)	3.34 (br. *d*, J = 7.0)	35.2 (*t*)
C(7)		124.9 (*s*)	H–C(21)	5.32 (*t*, J = 7.2)	125.5 (*d*)
C(7a)		135.5 (*s*)	C(22)		131.9 (*s*)
H–C(8)	7.39 (*s*)	117.2 (*d*)	Me(23)	1.74 (*s*)	25.8 (*q*)
C(9)		125.5 (*s*)	Me(24)	1.72 (*s*)	18.0 (*q*)
C(10)		160.6 (*s*)	CH_2(25)	3.55 (br. *d*, J = 7.0)	30.4 (*t*)
H–N(11)	10.83 (br. *s*)		H–C(26)	5.42 (*t*, J = 7.2)	123.3 (*d*)
C(12)		152.5 (*s*)	C(27)		133.5 (*s*)
C(13)		157.3 (*s*)	Me(28)	1.78 (*s*)	25.8 (*q*)
H–N(14)	8.91 (br. *s*)		Me(29)	1.77 (*s*)	17.9 (*q*)

Compound **2** was obtained as a colorless amorphous powder. The IR absorptions at 3416, 1646, and 1621 and 1568 cm^{-1} indicated the presence of OH, C=O, and aromatic moieties, respectively. The molecular formula was established as $C_{15}H_{12}O_5$ by negative-ion-mode HR-ESI-MS, which was in agreement with the ^{1}H- and ^{13}C-NMR data (*Table 2*). The ^{13}C-NMR and DEPT spectra displayed the presence of one Me, one CH_2, and four aromatic CH groups, and of nine quaternary C-atoms. The ^{1}H-NMR and HSQC spectra revealed the presence of three phenolic OH groups at δ(H) 10.08 (1 H) and 8.38 (2 H), four aromatic proton signals at δ(H) 6.73 (*d*, J = 0.9, H–C(9)), 6.71 (*d*, J = 0.9, H–C(11)), 6.33 (*d*, J = 2.3, H–C(3)), and 6.34 (*d*, J = 2.3, H–C(5)), one aliphatic CH_2 group at δ(H) 3.90 (CH_2(7)), and one Me group at δ(H) 2.28 (Me(15)) (*Table 2*). Detailed comparison of the 1D- and 2D-NMR data of **2** with those of 2-*O*-methyl-9-dehydroxyeurotinone (**10**) [4] revealed that the structures of these compounds are very similar. However, the signal at δ(H) 3.72 (MeO) and at δ(C) 55.6 for the MeO group of **10** disappeared in the NMR spectra of **2**. Instead, one more phenolic

OH signal was observed in the ^{1}H-NMR spectrum of **2**. The molecular formula as well as the observed HMBCs of **2** (*Fig. 2*) indicated that an OH group replaced in **2** the MeO group at C(2) of **10**.

Table 2. *^{1}H- and ^{13}C-NMR Data* ((D$_6$)acetone, 500 and 125 MHz, resp.) *of Compound* **2**. δ in ppm, J in Hz.

	δ(H)	δ(C)		δ(H)	δ(C)
C(1)		133.0 (*s*)	C(10)		147.9 (*s*)
C(2)		149.5 (*s*)	H–C(11)	6.71 (*d*, J = 0.9)	117.1 (*d*)
H–C(3)	6.33 (*d*, J = 2.3)	133.3 (*d*)	C(12)		163.2 (*s*)
C(4)		156.8 (*s*)	C(13)		109.9 (*s*)
H–C(5)	6.34 (*d*, J = 2.3)	106.2 (*d*)	C(14)		169.8 (*s*)
C(6)		135.6 (*s*)	Me(15)	2.28 (*s*)	21.6 (*q*)
CH$_2$(7)	3.90 (*s*)	38.2 (*t*)	OH	8.38 (*s*)	
C(8)		144.6 (*s*)	OH	8.38 (*s*)	
H–C(9)	6.73 (*d*, J = 0.9)	121.1 (*d*)	OH	10.08 (*s*)	

In addition to the new compounds **1** and **2**, the ten known related compounds **3**–**12** were also isolated and identified. By comparison of their NMR data with those reported in the literature, the structures of these compounds were identified as variecolorin J (**3**) [6], eurotechinulin B (**4**) [3], variecolorin G (**5**) [6], alkaloid E-7 (**6**) [6][7], cryptoechinuline G (**7**) [6][8], isoechinulin B (**8**) [6][9], 7-isopentenylcryptoechinuline D (**9**) [10][11], 2-*O*-methyl-9-dehydroxyeurotinone (**10**) [4], emodin (**11**) [12], and emodic acid (**12**) [13][14].

Antimicrobial Activity. Compounds **1**–**3**, **6**–**8**, and **11** were investigated for the antibacterial activity against *Staphylococcus aureus* and *Escherichia coli*, and the antifungal activity against *Fusarium oxysporium* f. sp. *vasinfectum*, *Alternaria brassicae*, and *Physalospora piricola*. Compound **2** showed weak antibacterial activity against *E. coli* with an inhibition zone of 7.0 mm at 100 μg/disk compared to amphotericin B with an inhibition zone of 11.0 mm at 20 μg/disk.

Cytotoxic Activity. Compounds **1**–**7**, **10**, and **11** were evaluated for their cytotoxic activity against seven tumor cell lines of MCF-7, SW1990, HepG2, NCI-H460, SMMC-7721, Hela, and Du145. Compounds **1**, **2**, **5**, **6**, and **11** displayed cytotoxic activities against one or two of these cell lines (*Table 3*).

Table 3. *Cytotoxic Activity Against Seven Tumor Cell Lines*

	IC_{50} [μg/ml]						
	MCF-7	SW1990	HepG2	NCI-H460	SMMC-7721	Hela	Du145
1	–	–	–	–	30	–	–
2	–	25	–	–	–	–	–
5	–	–	20	22	–	20	–
6	20	20	–	–	20	30	–
11	–	–	–	–	–	–	15

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Experimental Part

General. Column chromatography (CC): silica gel (SiO_2, 200–300 mesh; *Qingdao Haiyang Chemical Group Co.*), *Lobar LiChroprep RP-18* (40–63 μm; *Merck*), and *Sephadex LH-20* (*Pharmacia*). TLC: precoated SiO_2 *GF-254* plates (*Qingdao Haiyang Chemical Group Co.*). Optical rotation: *AA-55* digital polarimeter (*Optical Activity Ltd.*). UV Spectra: *Lengguang-Gold-Spectrumlab-54* UV/VIS spectrophotometer; λ_{max} (log ε) in nm. IR Spectra: *Nicolet-NEXUS-470* IR spectrophotometer; $\tilde{\nu}$ in cm^{-1}. NMR Spectra: *Bruker-Avance-500* spectrometer; at 500 (^1H) and 125 MHz (^{13}C); δ in ppm, J in Hz. Low- and high-resolution ESI-MS: *VG-Autospec-3000* spectrometer; in m/z.

Fungal Material. The endophytic fungus *Eurotium rubrum* G2 was isolated from a sample of the mangrove plant *Hibiscus tiliaceus* LINN. that was collected from Hainan Island, China, in August 2004. Fungal identification was carried out by a method reported previously [15], and the sequence data derived from the fungal strain was submitted and deposited at *GenBank*, *NIH*, with accession No. EU001331. A BLAST search result showed that the sequence was the most similar (99%) to the sequence of *Eurotium rubrum* (compared to accession No. AY373891.1). The strain is preserved at the Institute of Oceanology, Chinese Academy of Sciences.

Fermentation, Extraction, and Isolation. For chemical investigations, the fungal strain was statically fermented at r.t. for 30 d on sterilized solid medium containing rice (100 g), peptone (0.6 g), and sea water (100 ml) in 1-l *Fernbach* flasks (× 100). The fermented rice substrate was extracted repeatedly with AcOEt, and the solvent was evaporated: 90 g of crude extract. The extract was subjected to CC (SiO_2, petroleum ether/AcOEt 1:0 → 1:1 and $CHCl_3$/MeOH 20:1 → 0:1): *Fractions 1–10*. *Fr. 2* was further purified by CC (SiO_2, petroleum ether/AcOEt 50:1 and then *RP-18*, MeOH): **4** (6.4 mg). *Fr. 3* was fractionated by CC (SiO_2, petroleum ether/AcOEt 65:1 → 10:1): **3** (3.2 mg), **9** (19.3 mg), and **10** (4.2 mg). *Fr. 4* was subjected to CC (SiO_2, petroleum ether/AcOEt 50:1 → 10:1, then *Sephadex LH-20*, $CHCl_3$/MeOH 1:1, then *Sephadex LH-20*, MeOH, and finally *RP-18*, MeOH/H_2O 1:1): **1** (18.2 mg), **2** (11.1 mg), **11** (30.3 mg), and **12** (9.5 mg). *Fr. 5* was subjected to CC (SiO_2, petroleum ether/AcOEt 50:1 → 10:1, then *RP-18*, MeOH/H_2O 0 → 100, and then *Sephadex LH-20*, $CHCl_3$/MeOH 1:1): **6** (20.1 mg) and **7** (20.1 mg). *Fr. 6* was further purified by CC (SiO_2, petroleum ether/AcOEt 50:1 → 10:1, then *RP-18*, MeOH/H_2O 1:1, and then *Sephadex LH-20* MeOH): **5** (7.3 mg) and **8** (19.3 mg).

*12-Demethyl-12-oxo-eurotechinulin B (=(6Z)-6-{[2-(1,1-Dimethylprop-2-en-1-yl)-5,7-bis(3-methylbut-2-en-1-yl)-1*H*-indol-3-yl]methylene}piperazine-2,3,5-trione*; **1**): Colorless amorphous powder. $[\alpha]_D^{25} = -12.5$ ($c = 0.08$, MeOH). UV (MeOH): 227 (4.46), 277 (4.19), 393 (3.97). IR (KBr): 3439, 3082, 2971, 2925, 1734, 1687, 1604, 1436, 1375, 1321, 1233. ^1H- and ^{13}C-NMR: *Table 1*. ESI-MS (neg.): 458.2488 ($[M-H]^-$, $C_{28}H_{32}N_3O_3^-$; calc. 458.2444).

9-Dehydroxyeurotinone (*=2,4,7-Trihydroxy-9-methyldibenz[*b,e*]oxepin-6(11*H*)-one*; **2**): White amorphous powder. $[\alpha]_D^{25} = -30.8$ ($c = 0.13$, MeOH). UV (MeOH): 202 (4.52), 215 (4.41), 256 (3.80), 310 (3.54). IR (KBr): 3416, 1646, 1621, 1568, 1487, 1353, 1312, 1254, 1198. ^1H- and ^{13}C-NMR: *Table 2*. ESI-MS (neg.): 271.0656 ($[M-H]^-$, $C_{15}H_{11}O_5^-$; calc. 271.0607).

Antimicrobial Assays. The antimicrobial assay toward *Staphylococcus aureus*, *Escherichia coli*, and *Aspergillus niger* was carried out by using the filter-paper method [16]. Chloramphenicol and amphotericin B were used as antibacterial and antifungal positive controls, resp.

Cytotoxicity Assays. The cytotoxic assay toward seven tumor cell lines including MCF-7 (human-breast-cancer cell line), SW1990 (human-cholangiocarcinoma cell line), HepG2 (human-hepatoma cell line), NCI-H460 (human-nonsmall-cell-lung-cancer cell line), SMMC-7721 (human-hepatoma cell line), Hela (human-cervical-cancer cell line), and DU145 (human-prostate-cancer cell line) were carried out by the method reported in [17].

REFERENCES

[1] P. Lin, *J. Xiamen Univ.* **2001**, *40*, 592.
[2] A.-R. Liu, X.-P. Wu, T. Xu, *Chin. J. Appl. Ecol.* **2007**, *18*, 912.
[3] D.-L. Li, X.-M. Li, T.-G. Li, H.-Y. Dang, B.-G. Wang, *Helv. Chim. Acta* **2008**, *91*, 1888.
[4] D.-L. Li, X.-M. Li, B.-G. Wang, *J. Microbiol. Biotechnol.* **2009**, *19*, 675.
[5] D.-L. Li, X.-M. Li, T.-G. Li, H.-Y. Dang, P. Proksch, B.-G. Wang, *Chem. Pharm. Bull.* **2008**, *56*, 1282.
[6] W.-L. Wang, Z.-Y. Lu, H.-W. Tao, T.-J. Zhu, Y.-C. Fang, Q.-Q. Gu, W.-M. Zhu, *J. Nat. Prod.* **2007**, *70*, 1558.
[7] S. Inoue, J. Murata, N. Takamatsu, H. Nagano, Y. Kishi, *Yakugaku Zasshi* **1977**, *97*, 576.
[8] G. Gatti, R. Cardillo, C. Fuganti, *Tetrahedron Lett.* **1978**, *19*, 2605.
[9] H. Nagasawa, A. Isogai, A. Suzuki, S. Tamura, *Tetrahedron Lett.* **1976**, *17*, 1601.
[10] G. Gatti, R. Cardillo, C. Fuganti, D. Ghiringhelli, *J. Chem. Soc., Chem. Commun.* **1976**, 435.
[11] S. Inoue, N. Takamatsu, K. Hashizume, Y. Kishi, *Yakugaku Zasshi* **1977**, *97*, 582.
[12] Y. Dong, C. J. Chen, S. M. Li, G. D. Zhao, *J. Inner Mongolia Univ.* **2009**, *40*, 239.
[13] J. L. Bloomer, K. W. Stagliano, J. A. Gazzillo, *J. Org. Chem.* **1993**, *58*, 7906.
[14] K. A. Alvi, B. Nair, C. Gallo, D. Baker, *J. Antibiot.* **1997**, *50*, 264.
[15] 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.
[16] C.-C. Shen, W.-J. Syu, S.-Y. Li, C.-H. Lin, G.-H. Lee, C.-M. Sun, *J. Nat. Prod.* **2002**, *65*, 1857.
[17] R. J. Bergeron, P. F. Cavanaugh, S. J. Kline, R. G. Hughes, G. T. Elliott, C. W. Porter, *Biochem. Biophys. Res. Commun.* **1984**, *121*, 848.

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