Two new paeciloxocins from a mangrove endophytic fungus *Paecilomyces* sp.

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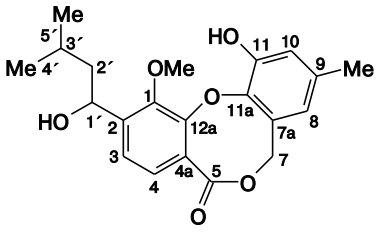
Paeciloxocins A and B (2-(1-hydroxy-3-methylbutyl)- and 2-(1-acetoxy-3-methylbutyl)-11-hydroxy-9-methyl-1-methoxy-5*H*,7*H*-dibenzo[*b*,*g*]-1,5-dioxocin-5-ones), *viz.*, two new metabolites, were isolated from the mangrove fungus *Paecilomyces* sp. collected from the Taiwan Strait. Their structures were elucidated by spectral methods. Paeciloxocin A exhibited strong cytotoxicity against the hepG2 cell line.

Key words: mangroves, endophytic fungus, metabolites, paeciloxocins, cytotoxicity.

Marine natural products continue to be a source of significant molecular structures that serve as a stimulus to seed further research.[1] A large variety of new bioactive compounds have recently[2] been isolated from marine fungi, mainly from the mangrove fungi.[3—5] Recently, we have embarked on a study of the metabolites of marine fungi including those found on mangroves from the South China Sea, and this has yielded a number of interesting compounds.[6—12] The mangrove fungus *Paecilomyces* sp., which was collected from the bark of a mangrove, was found to produce rich secondary metabolites,[13] in particular, paeciloxocins A (**1**) and B (**2**). In the primary bioassays, compound **1** exhibited strong antitumor activity and mild antomicrobial activity. In the present work, the isolation and identification of compounds **1** and **2** are described in detail.



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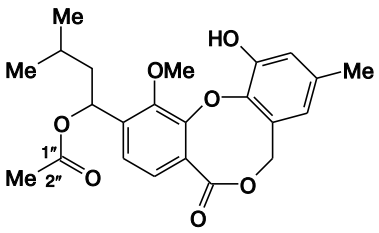

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The air-dried and finely pulverized mycellium was extracted with 70% EtOH to give the crude extract. The combined extracts were suspended in water and extracted successively with petroleum ether, ethyl acetyl acetate, and *n*-butyl alcohol. The ethyl acetate fraction was separated by column chromatography on silica gel and obtained many bioactive compounds, including two new compounds **1** and **2**.

Compound **1** was isolated as a shallow yellow oil. An analysis by high-resolution electron ionization mass spectrometry (HREIMS) provided the molecular formula $C_{21}H_{24}O_6$ (found *m*/*z* 372.1582 $[M]^+$, calculated 372.1567). It was calculated from the mass spectrometry and NMR data that compound **1** had ten units of unsaturation. In the ^1H NMR spectrum, one phenolic hydroxyl group gives a signal at δ_H 6.18 (br.s, 1 H). This compound contains two aromatic *meta*-protons with δ_H 6.85 (d, 1 H, J = 1.5 Hz) and 6.37 (d, 1 H, J = 2.0 Hz) and two aromatic *ortho*-protons with δ_H 7.56 (d, 1 H, J = 8.5 Hz) and 6.87 (d, 1 H, J = 8.5 Hz). In addition, the ^1H NMR spectrum of compound **1** (Table 1) reveals signals from one methoxy group at δ_H 3.98 (s, 3 H), three methyl groups at δ_H 2.24 (s, 3 H), 0.99 (d, 3 H, J = 6.5 Hz), and 0.96 (d, 3 H, J = 7.0 Hz), two methine groups at δ_H 5.07 (dd, 1 H, J = 4.5 Hz, J = 9.0 Hz), 1.68 (m, 1 H), one methylene group at δ_H 1.79 (ddd, 1 H, J = 4.5 Hz,

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J = 9.0 Hz, J = 13.0 Hz), 1.48 (ddd, 1 H, J = 4.5 Hz, J = 9.0 Hz, J = 13.0 Hz), and one methylene group attached to the oxygen atom at δ_H 5.08 and 5.07 (d, 1 H each, J = 9.5 Hz). The ^{13}C NMR spectrum exhibits 13 signals of the sp^2-hybridized carbon atoms (six C=C bonds, one C=O bond) corresponding to seven of ten units of unsaturation, which confirms the presence of two unconjugated aromatic rings. Thus, the third ring should be lactone according to the presence of the band at 1750 cm^{-1} in the IR spectrum. Signals of four carbon atoms attached to the oxygen atoms at δ_C 154.1, 151.1, 147.3, and 141.3 can be deduced from the ^{13}C NMR spectrum (see Table 1). The single hydroxy group, one methyl group, one methoxy group, one methine group, and one lactone moiety together with four olefinic hydrogen atoms account for ten of possible twelve substituents in the aromatic rings. Thus, both rings have to be connected *via* an oxygen atom.

An analysis of the $^1H—^1H$ COSY spectrum revealed the presence of the 1-hydroxy-4-methylbutyl moiety attached to the benzene ring having the *ortho*- and *meta*-protons. After correlating all carbon atoms with their directly bonded hydrogen atoms by measuring the 2D $^1H—^{13}C$ NMR spectrum (HMQC), the structure was further elaborated by the interpretation of the $^1H—^{13}C$ long-range correlations (HMBC, Fig. 1). The long-range correlations between the H(8), H(10), and 9-CH_3 atoms to C(9) connected C(8), C(10), and the methyl group to C(9). Moreover, H(10) showed the long-range correlation with C(11), establishing the molecular moiety from C(8) to C(11). The long-range correlations between OCH_2-7 and C(5), C(7a), C(8) and C(11a), H(4) and C(2), C(4a), C(5) and C(12a) allowed one to determine the remained part of the molecular structure: two aromatic rings should be connected through two pairs of the quaternary C(11a) and C(12a), C(4a) and C(7a) carbon atoms. The long-range correlations between the H(3) and C(1), C(2), OMe(1) and C(1) atoms made it possible to construct the molecular moiety from C(1) to C(4). The remained H(1′) atom showed long-range correlations between C(1), C(2), and C(3), indicating the attachment of the 1-hydroxy-4-methylbutyl substituent to the C(2) atom of the benzene ring. On the basis of the above mentioned results, the structure of 2-(1-hydroxy-3-methylbutyl)-11-hydroxy-1-methoxy-9-methyl-5*H*,7*H*-dibenzo-[*b*,*g*]-1,5-dioxocin-5-one, named paeciloxocin A, was ascribed to compound **1**.

Table 1. ^1H and ^{13}C NMR spectra and HMBC correlations for compound **1** (in $CDCl_3$)

Atom, group	δ_H (J/Hz)	δ_C	COSY	HMBC (H→C)
1	—	154.1	—	—
2	—	136.7	—	—
3	7.56 (d, 1 H, J = 8.5)	130.8	—	1, 1′
4	6.87 (d, 1 H, J = 8.5)	117.6	H(3)	2, 4a, 5, 12a
4a	—	119.4	—	—
5	—	167.6	—	—
7	5.08 (d, 1 H, J = 9.5); 5.07 (d, 1 H, J = 9.5)	69.1	—	5, 7a, 8
7a	—	125.7	—	—
8	6.37 (d, 1 H, J = 2.0)	120.5	H(10)	7, 10, 11a, 9-Me
9	—	134.8	—	—
10	6.85 (d, 1 H, J = 1.5)	117.8	—	8, 11, 11a, 9-Me
11	—	147.3	—	—
11a	—	141.3	—	—
12a	—	151.1	—	—
1′	5.07 (dd, 1 H, J = 4.5, J = 9.0)	66.6	—	—
2′	1.79 (ddd, 1 H, J = 4.5, J = 9.0, J = 13.0); 1.48 (ddd, 1 H, J = 4.5, J = 9.0, J = 13.0)	47.7	H(1′), H(3′)	1′, 4′, 5′
3′	1.68 (m, 1 H)	25.1	H(1′)	5′
4′	0.99 (d, 3 H, J = 6.5)	23.6	H(3′)	2′, 3′, 5′
5′	0.96 (d, 3 H, J = 7.0)	22.0	H(3′)	2′, 3′, 4′
1-OMe	3.98 (s, 3 H)	62.4	—	1
9-Me	2.24 (s, 3 H)	20.6	—	8, 9, 10
11-OH	6.18 (br.s, 1 H)	—	—	—
1′-OH	3.64 (br.s, 1 H)	—	—	—

Compound **2** was isolated as a shallow yellow oil. The results of fast atom bombardment mass spectrometry (FAB-MS) showed the molecular formula $C_{23}H_{26}O_7$. This suggests that compound **2** has eleven units of unsaturation. The ^{13}C NMR spectrum contains 23 carbon signals, five of which correspond to the CH_3 groups, two signals cor-

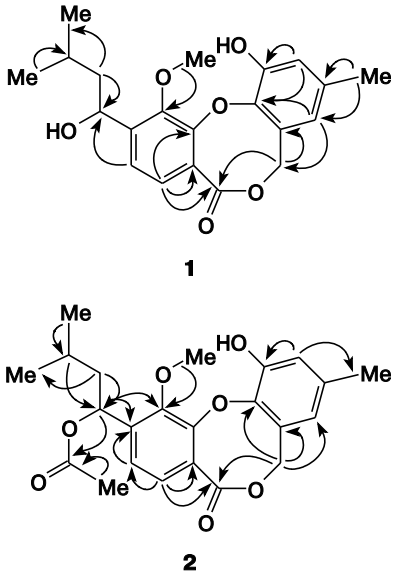

Fig. 1. Principal HMBC correlations (H with C) for compounds **1** and **2**.

respond to the CH_2 groups, six signals correspond to the CH groups, and ten signals correspond to the quaternary C atoms, which was determined by the DEPT spectra. The general shape of the ^{1}H NMR spectrum of compound **2** is analogous to that for compound **1**. The spectrum of compound **2** also exhibits two signals from the aromatic *meta*-protons with δ_H 6.79 (d, 1 H, J = 1.5 Hz) and 6.30 (d, 1 H, J = 1.5 Hz) and two signals from the aromatic *ortho*-protons with δ_H 7.38 (d, 1 H, J = 8.5 Hz) and 6.85 (d, 1 H, J = 8.5 Hz). In addition, the following signals were detected: of one methoxy group at δ_H 3.98 (s, 3 H); four methyl groups at δ_H 2.18 (s, 3 H), 2.02 (s, 3 H), 0.90 (d, 3 H, J = 6.5 Hz), and 0.90 (d, 3 H, J = 6.5 Hz); two methine groups at δ_H 6.08 (dd, 1 H, J = 4.5 Hz, J = 9.5 Hz) and 1.58 (m, 1 H); one methylene group attached to the oxygen atom with δ_H 5.06 (d, 1 H, J = 14.5 Hz) and 4.96 (d, 1 H, J = 14.5 Hz); one methylene group at δ_H 1.71 (ddd, 1 H, J = 4.5 Hz, J = 9.0 Hz, J = 14.0 Hz), and 1.43 (ddd, 1 H, J = 4.5 Hz, J = 8.5 Hz, J = 13.5 Hz). The ^{1}H and ^{13}C NMR spectra (Table 2, see Fig. 1) confirmed that the structure of compound **2** resembles structure **1** but has two carbon atoms more (C(1″), δ_C 170.2; C(2″), δ_C 20.9) and one hydroxy group less (1′-OH, δ_H 3.64). The further interpretation of the structure of compound **2** was based on the long-range correlations in its HMBC spectrum (see Table 2). The structure of 2-(1-acetoxy-3-methylbutyl)-11-hydroxy-1-methoxy-9-methyl-5*H*,7*H*-dibenzo[*b*,*g*]-1,5-dioxocin-5-one, named paeciloxocin B, was ascribed to compound **2** on the basis of these results. The structures of compounds **1** and **2** are closely related to that of janthinone, which was identified as a fungal metabolite from the endophytic fungus *Penicillium janthinellum* isolated from the fruits of *Melia azedarach*.[14]

Table 2. ^{1}H and ^{13}C NMR and HMBC correlations for compound **2** (in $CDCl_3$)

Atom, group	δ_H (J/Hz)	δ_C	COSY	HMBC (H→C)
1	—	154.5	—	—
2	—	134.8	—	—
3	7.38 (d, 1 H, J = 8.5)	130.6	H(4)	1, 2, 4a, 1′
4	6.85 (d, 1 H, J = 8.5)	117.7	—	2, 3, 4a, 5, 12a
4a	—	119.8	—	—
5	—	167.3	—	—
7	5.06 (d, 1 H, J = 14.5); 4.96 (d, 1 H, J = 14.5)	68.9	—	5, 7a, 8, 11a
7a	—	125.9	—	—
8	6.30 (d, 1 H, J = 1.5)	120.5	H(10)	7, 10, 11a, 9-Me
9	—	133.8	—	—
10	6.79 (d, 1 H, J = 1.5)	117.7	—	8, 11, 11a, 9-Me
11	—	147.5	—	—
11a	—	141.3	—	—
12a	—	151.6	—	—
1′	6.08 (dd, 1 H, J = 4.5, J = 9.5)	68.7	H(2′)	1, 2, 3, 2′, 3′, 1″
2′	1.71 (ddd, 1 H, J = 4.5, J = 9.0, J = 14.0); 1.43 (ddd, 1 H, J = 4.5, J = 8.5, J = 13.5)	45.1		1′, 4′, 5′
3′	1.58 (m, 1 H)	24.8	H(2′), H(4′)	1′, 4′, 5′
4′	0.90 (d, 3 H, J = 6.5)	22.9	—	3′
5′	0.90 (d, 3 H, J = 6.5)	21.7	—	2′
1″	—	170.2	—	—
2″	2.02 (s, 3 H)	20.9	—	1″
1-OMe	3.98 (s, 3 H)	62.4	—	—1
9-Me	2.18 (s, 3 H)	20.6	H(8), H(10)	8, 9, 10
11-OH	7.00 (br.s, 1 H)	—	—	—

Compound **1** exhibited significant cytotoxicity against hepG2 (IC_{50} = 1 μg mL^{-1}), whereas compound **2** had no cytotoxicity (IC_{50} = 65 μg mL^{-1}). Compound **1** inhibits the growth of *Curvularia lunata* (Walker) Boedijn and *Candida albicans* ATCC 10231 with inhibition zones of 12 and 10 mm, respectively. Compound **2** showed no antimicrobial activity. As for inhibitory activity against acetylcholinesterase *in vitro*, the both new substances are inactive.

Experimental

^{1}H and ^{13}C NMR spectra were obtained for $CDCl_3$ solutions on a Varian Inova 500NB spectrometer (^{1}H, 500 MHz; ^{13}C, 125 MHz) using Me_4Si as an internal standard. Mass spectra were measured on a VG ZAB-HS two-focus mass spectrometer and a VG-ZAB mass spectrometer. IR spectra were recorded on a Bruker EQUINOX 55 spectrometer. UV spectra were obtained on a Shimadzu UV-2501PC spectrophotometer. Optical rotation was measured on a Horiba SEPA-300 polarimeter.

Isolation of the fungal strain. The fungal strain *Paecilomyces* sp. was isolated from the saprophytic bark of mangrove from the Taiwan Strait. The color of mycelium was yellow, and it was plated on a potato—dextrose agar medium. The fungus is stored at the School of Chemistry and Chemical Engineering, Sun Yat-Sen (Zhongshan) University, Guangzhou, P. R. China.

Fermentation, extraction, and isolation. The starting cultures were maintained on cornmeal sea water agar. Plugs of agar supporting mycellium growth were cut and transferred aseptically to a 250-mL Erlenmeyer flask containing 10 mL of the liquid medium (glucose (10 g L^{-1}), peptone (2 g L^{-1}), yeast extract (1 g L^{-1}), and sea salt (2 g L^{-1})). The flask was incubated at 28 °C on a rotary shaker for 5—7 days. The mycellium was aseptically transferred to 130 1-L Erlenmeyer flasks containing totally 70 L of the liquid medium. The flasks were incubated for 30 days at ~20 °C.

The air-dried finely pulverized mycelium (180 g) was extracted with 70% EtOH (3×400 mL) under reflux for 2 h. After EtOH removal by evaporation, the resulting residue (45 g) was suspended in water (300 mL) and then extracted successively

with petroleum ether (1×300 mL), ethyl acetate (1×300 mL), and *n*-butyl alcohol (1×300 mL). The ethyl acetate fraction (28 g) was subjected to column chromatography (SiO_2, gradient $CHCl_3$: MeOH 20 : 1→0 : 1). Six fractions were collected and analyzed by TLC. The fractions containing the desired product were repeatedly chromatographed. The elution of fraction *2* with a petroleum ether—ethyl acetate (0→30%) gradient gave 45 mg of paeciloxocin A (**1**) as a yellowish oil, $[\alpha]^{20}_D = +0.5°$ (*c* 1, $CHCl_3$). UV (MeOH), λ_{max}/nm (log ε): 226 (5.64), 281 (5.24). IR (KBr), ν_{max}/cm^{-1}: 3425, 1750, 1576, 1419, 1035. The NMR spectra are given in Table 1. FAB-MS, *m/z*: 373 $[M + H]^+$, 355, 299, 269, 163, 109, 69, 55, 43. HREIMS, *m/z*: 372.1582. Calculated *M* = 372.1567. The elution of fraction *3* with the petroleum ether—ethyl acetate (0→20%) gradient gave 110 mg of paeciloxocin B (**2**) as a yellow oil, $[\alpha]^{20}_D = -47°$ (*c* 1, $CHCl_3$). UV (MeOH), λ_{max}/nm (log ε): 228 (5.43), 283 (4.91). IR (KBr), ν_{max}/cm^{-1}: 3426, 1741, 1597, 1494, 1417, 1234, 1050. The NMR spectra are presented in Table 2. FAB-MS, *m/z*: 415 $[M + H]^+$, 355, 337, 269, 163, 154, 107, 69, 55, 43.

Cell growth inhibitory activity. Human lung cancer A549 and human liver cancer hepG2 cells were harvested and seeded in 96-well plates ($3.0 \cdot 10^3$ cells/well) in a final volume of 190 μL. The cytotoxic agent (10 μL) or compound vehicles were added to each well after 24 h of incubation. After 68 h, a solution of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide, 10 μL) was added to each well, and DMSO (100 μL) was added after 4 h. The concentration (IC_{50}) required to inhibit cell growth by 50% was calculated from the cytotoxicity curves using Bliss software.

Antimicrobial activity. Antimicrobial activity was determined using the agar diffusion test with paper disks (8 mm in diameter, thin, ADVANTEC). The following organisms were tests: *Staphyococcus aureus* ATCC27154, *Escherichia coli* ATCC 25922, *Curvularia lunata* (Walker) Boedijn, and *Candida albicans* ATCC 10231. An antibiotic paper disk was loaded with a sample under study and dried for 2 h *in vacuo* to remove the solvent. Then the disk was placed on the agar plate inoculated with test strains, and then they were incubated at 25 °C. Antimicrobial activity was estimated measuring the diameter of the inhibition zone formed on the agar.

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