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ORIGINAL PAPER

Bioactive metabolites from *Penicillium* sp., an endophytic fungus residing in *Hopea hainanensis*

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Abstract The metabolites of endophytic fungus *Penicillium* sp. from the leaf of *Hopea hainanensis* were reported for the first time. By bioassay-guided fractionation, the EtOAc extract of a solid-matrix steady culture of this fungus afforded six compounds, which were identified through a combination of spectral and chemical methods (IR, MS, ^{1}H- and ^{13}C-NMR) to be monomethylsulochrin (**1**), rhizoctonic acid (**2**), asperfumoid (**3**), physcion (**4**), 7,8-dimethyl-iso-alloxazine (**5**) and 3,5-dichloro-*p*-anisic acid (**6**). Compounds **2**, **3** and **6** were obtained from *Penicillium* sp. for the first time. All of the six isolates were subjected to in vitro bioactive assays including antifungal action against three human pathogenic fungi *Candida albicans*, *Trichophyton rubrum* and *Aspergillus niger* and cytotoxic activity against the human nasopharyngeal epidermoid tumor KB cell line and human liver cancer HepG2 cell line. As a result, compounds **2**–**4** and **6** inhibited the growth of *C. albicans* with MICs of 40.0, 20.0, 50.0 and 15.0 μg/ml, respectively and the compound **6** showed growth inhibition against *A. niger* with MICs of 40.0 μg/ml. In addition, compounds **1**–**3** and **6** exhibited cytotoxic activity against KB cell line with IC_{50} value of 30.0, 20.0, 20.0, 5.0 μg/ml, respectively and against HepG2 cell line with IC_{50} value of 30.0, 25.0, 15.0, 10.0 μg/ml, respectively.

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Introduction

An increase in the number of people in the world having health problems caused by various cancers, drug-resistant bacteria, parasitic protozoans, and fungi is a cause for alarm (Wagenaar et al. 2000). Investigating the secondary metabolites of microorganisms isolated from unusual or specialized ecology niches increases the chance of finding bioactive compounds. Endophytes, which commonly present in almost all plants, are being recognized a potential source of useful medicinal compounds (Tan and Zou 2001). Continuing our work on the characterization of structurally novel and/or biologically active metabolites from endophyte cultures (Shu et al. 2004; Ye et al. 2005; Wang et al. 2007), we found that the EtOAc extract of the solid-substrate culture of *Penicillium* sp., which was isolated from the normal leaves of *Hopea hainanensis* (Dipterocarpaceae) endemic to Hainan island, China, showed antifungal and cytotoxic activity. The aim of the present work was to systemically isolate and identify bioactive secondary metabolites from the title fungus. The literature to date showed no information on the chemistry and biology of any endophytes from *Hopea hainanensis* except our group (Wang et al. 2006).

Materials and methods

Isolation and culture of the endophytic fungi

The endophytic fungal strain *Penicillium* sp. was isolated from healthy leaves of *Hopea hainanensis* collected in

August 2003 from Hainan Island, China, according to the procedure described elsewhere (Wang et al. 2007). Specifically, the leaves of *H. hainanensis* were washed with running tap water, sterilized with 75% ethanol for 1 min and 2.5% sodium hypochlorite for 15 min, then rinsed in sterile water for three times and placed in water agar (WA) medium supplemented with antibiotics (penicillin, 125 μg/ml and streptomycin sulphate, 100 μg/ml) and cultivated at 28 ± 1°C according to the methodology described by Bills (1996). The isolated endophytic fungi were numbered and preserved on potato dextrose agar (PDA) slants at 4°C. The endophytic fungus was identified according to the characteristics of morphology of the culture and spores and the mechanism of spore production, which was reinforced by the sequence of its 18S rDNA that gave a 99% sequence similarity to those accessible at the BLASTN of *Penicillium* sp. The fresh mycelium of the strain was cultured on PDA medium at 28°C for 4 days and further fermented on solid medium following the protocol of Liu et al. (2004).

Extraction and isolation of secondary metabolites

The biomass of the fungal strain (1.2 kg, incompletely dried) was collected after cultivation on solid medium for 30 days, extracted with methanol for four times and concentrated in vacuo to give a brown residue (150 g), which was suspended in water (750 ml). The suspension was extracted successively with petroleum ether (750 ml × 3), EtOAc (750 ml × 3) and *n*-butanol (750 ml × 3). Concentration of the EtOAc fraction in vacuo gave a brown bioactive residue (60 g), which was subjected to chromatography over silica gel column (300 g, 200–300 mesh) eluting with a chloroform–methanol gradient (1:0, 100:1, 50:1, 20:1, 10:1, 5:1, 0:1) to obtain seven fractions (F-1: 10 g, F-2: 15 g, F-3: 10 g, F-4: 5 g, F-5: 5 g, F-6: 5 g and F-7: 8 g). The antifungal activities of these fractions were tested by a modified paper-disk method (Bauer et al. 1966). F-1, F-6 and F-7 were shown no antifungal activity. The bioactive F-2 was re-chromatographed over a silica gel column eluted using a $CHCl_3$/MeOH gradient ($CHCl_3$:MeOH = 100:0, 100:0.5 and 100:1), with the active part separated further by gel filtration over Sephadex LH-20 with $CHCl_3$/MeOH (1:1) to yield monomethylsulochrin (**1**) (68.8 mg), rhizoctonic acid (**2**) (9.5 mg) and asperfumoid (**3**) (7.4 mg). Repeated chromatography of another bioactive F-3 on a silica gel (100 g) column eluted with $CHCl_3$/MeOH mixtures (100:0, 100:1, 100:2 and 100:4) followed by gel filtration over Sephadex LH-20 (MeOH) gave 7, 8-dimethyl-iso-alloxazine (**5**) (10.2 mg). In addition, F-4 and F-5 were fractionated further on an ODS column using gradient elution (100% water–100% MeOH) to give 3, 5-dichloro-*p*-anisic acid (**6**) (7.0 mg) and physcion (**4**) (11.5 mg).

Analytical methods

Melting points were determined on a Boetius micromelting point apparatus, and were uncorrected. IR spectra were recorded in KBr disks on Nexus 870 FT-spectrometer, v in cm^{-1}. All NMR data were collected on a Bruker DRX500 spectrometer with ^1H and ^{13}C nuclei observed at 500 and 125 MHz, respectively, and the chemical shifts were expressed in δ (ppm) relative to SiMe4 (the internal standard) with coupling constants J in Hz. EI–MS was recorded on a VG-ZAB-HS instrument and ESI–MS was recorded on a Mariner System 5304 instrument. Silica gel (200–300 mesh) for column chromatography was produced by Qingdao Marine Chemical Factory, Qingdao, China and ODS silica gel was from Nacalai Tesque, Kyoto, Japan. Sephadex LH-20 was from Pharmacia Biotech, Sweden.

Compound **1**

Monomethylsulochrin, molecular formula $C_{18}H_{18}O_7$, mp 197–198°C, ESI–MS: *m/z* 347 $[M + H]^+$ and *m/z* 369 $[M + Na]^+$, ^1H-NMR (500 MHz, $CDCl_3$, J in Hz): δ7.01 (1H, d, J = 2.0, H-6), δ6.66 (1H, d, J = 2.0, H-4), δ6.45 (1H, br s, H-3′), δ6.06 (1H, br s, H-5′), δ3.69 (3H, s, $COOCH_3$), δ3.68 (3H, s, H-8), δ3.37 (3H, s, H-9′), δ2.28 (3H, s, H-7′), δ12.9 (1H, br s, 2′-OH). The spectral data are in full agreement with those reported by Ma et al. (2004).

Compound **2**

Rhizoctonic acid, molecular formula $C_{17}H_{16}O_7$, mp 230–232°C, ESI–MS: *m/z* 331 $[M - H]^+$, ^1H-NMR (500 MHz, Aceton-d_6, J in Hz): δ7.09 (1H, d, J = 1.9, H-6), δ6.74 (1H, d, J = 1.9, H-4), δ6.36 (1H, s, H-3′), δ6.23 (1H, s, H-5′), δ3.69 (3H, s, H-8), δ3.40 (3H, s, H-9′), δ2.26 (3H, s, H-7′), δ13.24 (1H, br s, 2′-OH), δ8.99 (1H, br s, 5-OH). The ^1H-NMR data are in full agreement with literature reported (Ma et al. 2004).

Compound **3**

Asperfumoid, molecular formula $C_{18}H_{17}NO_7$, m = 359. ESI–MS: *m/z* 360 $[M + 1]^+$; ^1H-NMR (500 MHz, DMSO-d_6, J in Hz): δ6.56 (1H, s, H-8′), δ6.51 (1H, s, H-6′), δ6.01 (1H, s, H-5), δ3.85 (3H, s, 5′-OCH_3), δ3.66 (3H, s, 6-OCH_3), δ3.37 (3H, s, 2-OCH_3), δ2.38 (3H, s, H-9′); ^{13}C-NMR (125 MHz, DMSO-d_6): δ193.5 (C-4′), δ181.3 (C-4), δ173.7 (C-2′), δ171.8 (C-6), δ167.4 (C-2), δ157.7 (C-5′), δ151.6 (C-7′), δ148.1 (C-3 and C-8a′), δ105.5 (C-8′), δ108.9 (C-4a′), δ105.4 (C-6′), δ102.8 (C-5), δ85.4 (C-1 and C-3′), δ57.9 (6-OCH_3), δ56.2 (5′-OCH_3), δ51.5 (2-OCH_3), δ22.9 (C-9′). The spectral data are consistent with the literature reported (Liu et al. 2004).

Compound **4**

Physcion, molecular formula $C_{16}H_{12}O_5$, ESI–MS: *m/z* 283[M − H]$^-$, ^{1}H-NMR (500 MHz, DMSO-d_6, *J* in Hz): δ7.45 (1H, br s, H-4), δ7.22 (1H, br s, H-5), δ7.15 (1H, br s, H-2), δ6.86 (1H, br s, H-7), δ3.91 (3H, s, H-12), δ2.40 (3H, s, H-11), δ13.24 (1H, br s, 1-OH), δ11.25 (1H, br s, 8-OH). The data were identical with reported by Lu et al. (1992).

Compound **5**

7,8-Dimethyl-iso-alloxazine, molecular formula $C_{12}H_{10}N_4O_2$; mp 279–281°C; ESI–MS: *m/z* 243 [M + H]$^+$, 265 [M + Na]$^+$ and 507 [2M + Na]$^+$; ^{1}H-NMR (500 MHz, DMSO-d_6, *J* in Hz): δ11.86 (1H, br s, 1-NH), δ11.77 (1H, br s, 3-NH), δ7.86 (1H, s, H-6), δ7.64 (1H, s, H-9), δ2.46 (3H, s, 8-CH_3), δ2.43 (3H, s, 7-CH_3); ^{13}C-NMR (125 MHz, DMSO-d_6): δ161.1 (C-4), δ150.3 (C-2), δ146.5 (C-4a), δ145.4 (C-10a), δ141.4 (C-5a), δ139.3 (C-8), δ138.3 (C-7), δ130.2 (C-6), δ129.1 (C-9a), δ125.8 (C-9), δ20.7 (8-CH_3), δ20.1 (7-CH_3). The ^{1}H- and ^{13}C-NMR data are consistent with the literature reported (Grande et al. 1977a, b).

Compound **6**

3,5-Dichloro-*p*-anisic acid, molecular formula $C_8H_6Cl_2O_3$, EI–MS: *m/z* 220 [M]$^+$ (100), 222 [M + 2]$^+$ (68). The ^{1}H-NMR data were reported by Manfred (2006) while the ^{13}C-NMR data were presented for the first time (Table 1).

Antifungal activities tests

Antifungal activities of compounds **1**–**6** were assayed in vitro using the three human pathogenic fungi including *Candida albicans*, *Tricophyton rubrum* and *Aspergillus niger* by the method outlined elsewhere (Wang et al. 2007).

Cytotoxicity assay

The in vitro cytotoxic activity was performed by the MTT [3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] colometric method (Wang et al. 2003) with some modification. Briefly, KB and HepG2 cells were grown in Roosevelt Park Institute Medium (RPMI) 1640 medium supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (50 μg/ml). Cells were harvested in the log phase of growth and seeded into 96-well plates (100 μl/well at a density of 2 × 10^5 cells/ml). After 24 h of incubation at 37°C, 5% CO_2 to allow cell attachment, cultures were exposed to various concentrations of the investigated compounds for 48 h. Finally, MTT solution (2.5 mg/ml in PBS) was added (40 μl/well). Plates were further incubated for 4 h at 37°C and the formazan crystals formed were dissolved by adding 150 μl/well of DMSO. Absorption was measured at 570 nm with an ELISA plate reader and the IC_{50} value was defined as the concentration at which 50% survival of cells was allowed.

Table 1 ^{1}H-(500 MHz) and ^{13}C-(125 MHz) NMR data of compound **6** (DMSO-d_6)

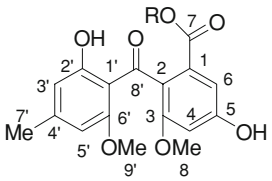
Position	δ_H (*J* in Hz)	δ_C (DEPT)	Position	δ_H (*J* in Hz)	δ_C (DEPT)
1		129.7 (C)	5		129.1 (C)
2	7.94 (*br s*)	130.4 (CH)	6	7.94 (*br s*)	130.4 (CH)
3		129.1 (C)	4-OCH_3	3.89 (*s*)	61.2 (CH_3)
4		155.5 (C)	COOH		165.4 (C)

Results and discussion

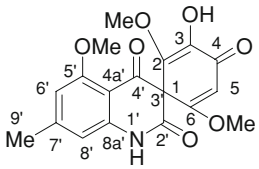
The EtOAc extract derived from the solid-substrate fermentation materials of the endophytic strain *Penicillium* sp. was chromatographed repeatedly on silica gel, Sephadex LH-20 and reversed-phase ODS column to yield six compounds (Fig. 1), which were identified as monomethylsulochrin (**1**), rhizoctonic acid (**2**), asperfumoid (**3**), physcion (**4**), 7,8-dimethyl-iso-alloxazine (**5**) and 3,5-dichloro-*p*-anisic acid (**6**), respectively according to spectral and physical data. And the compounds **2**, **3** and **6** were isolated from *Penicillium* sp. for the first time. In order to ascertain whether any of the six isolates obtained in this study was the component of the millet and yeast extract in the substrate, the EtOAc extract of the sterile medium treated equally but without inoculation of the microorganism was subjected to an LC–MS comparison, the result showed that all the isolates were indeed produced by the title endophytic fungus.

All six metabolites were tested in vitro for the antifungal activity against the human pathogenic fungi *C. albicans*, *T. rubrum* and *A. niger*, and the results were listed in Table 2. Compounds **2**–**4** and **6** inhibited the growth of *C. albicans* with MICs of 40.0, 20.0, 50.0 and 15.0 μg/ml, respectively and the compound **6** showed growth inhibition against *A. niger* with MICs of 40.0 μg/ml. The MICs of ketonazole used as a positive reference in the study against *C. albicans*, *T. rubrum* and *A. niger* were 10.0, 50.0 and 10.0 μg/ml, respectively. In addition, Cytotoxic activity of **1**–**6** was performed in vitro using human liver cancer cell line HepG2 and the human nasopharyngeal epidermoid tumor KB cell line as target. The results (Table 3) showed compounds **1**–**3** and **6** exhibited cytotoxic activity against KB cell line with IC_{50} value of 30.0, 20.0, 20.0, 5.0 μg/ml, respectively, and against HepG2 cell line with IC_{50} value of 30.0, 25.0, 15.0, 10.0 μg/ml, respectively, comparable to

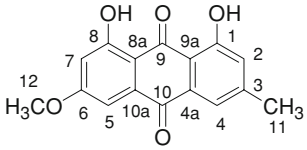
Fig. 1 Structure of the compounds **1–6** isolated from endophytic fungi *Penicillium* sp.



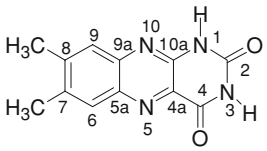
1: R=CH_3, monomethylsulochrin
2: R=H, rhizoctonic acid



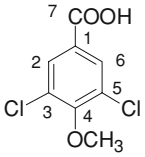
3: Asperfumoid



4: Physcion



5: 7, 8-dimethyl-iso-alloxazine



6: 3, 5-dichloro-p-anisic acid

Table 2 The antifungal MICs (μg/ml) of compounds **1–6** obtained from *Penicillium* sp.

	1	2	3	4	5	6	KCZ[a]
C. albicans	>100	40.0	20.0	50.0	>100	15.0	10.0
T. rubrum	>100	>100	>100	>100	>100	>100	50.0
A. niger	>100	>100	>100	>100	>100	40.0	10.0

[a] KCZ—ketoconazole used as positive control

Table 3 Cytotoxicity of compounds **1–6** on KB and HepG2 cell lines (data are presented as IC_{50} (μg/ml))

	1	2	3	4	5	6	5-Fluorouracil
KB cell	30.0	20.0	20.0	>50.0	>50.0	5.0	2.5
HepG2	30.0	25.0	15.0	>50.0	>50.0	10.0	2.5

that of 5-fluorouracil (2.5 μg/ml) co-assayed as a positive reference. Thereinto compounds **6** were as active to both cell lines as 5-fluorouracil was.

Both **1** and **2** were benzophenone derivatives, a type of metabolites previously reported from the fungi belonging to the genera *Aspergillus* (Turner 1965; Kiriyama et al. 1977; Inamori et al. 1983), *Penicillium* (Mahmoodian and Stickings 1964), *Rhizoctonia* (Ma et al. 2004) and *Oospora* (Curtis et al. 1966). The present characterization of **1** and **2** from the title fungus confirmed *Penicillium* sp. was an important source of benzophenone analogs. Some of this type of compounds have been shown to be anti-*Helicobacter pylori* (Ma et al. 2004), and to inhibit eosinophils, which may play important roles in allergic diseases such as asthma and atopic dermatitis (Ohashi et al. 1999). Compound **3** was isolated only from *Aspergillus fumigatus* by Liu et al. (2004), and obtained from *Penicillium* sp. for the first time. Compound **3** showed moderate antifungal and cytotoxic activity. Compound **4**, a compound was isolated as an antifungal agent from *Rheum emodi* (Agarwal et al. 2000) and as a tumor cell growth inhibitor from *Polygonum hypoleucum* (Kuo et al. 1997) and was obtained from the endophytic fungi *Aspergillus fumigatus* (Liu et al. 2004) and *Pleospora* sp. (Ge et al. 2005), which showed endophyte owned the similar biosynthetic pathway to plants and could produce the same metabolites as plants. Compound **6**, which showed strong antifungal and cytotoxic activity, was obtained previously mainly from *Bjerkandera* species (Henk et al. 1996; Silk et al. 2001). And any reports on Compound **6** isolated from *Penicillium* sp. have not been seen.

This is the first report on the secondary metabolites of *Penicillium* sp. from *Hopea hainanensis*. The study on endophyte from *H. hainanensis* had not been reported previously except our group (Wang et al. 2006). The compounds produced by our title fungus showed strong or moderate antifungal and cytotoxic activity, which confirmed the endophytic fungi were an important source of active natural products. Further research work on other endophytic fungi from *H. hainanensis* is in progress.

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