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ORIGINAL PAPER

Bioactive secondary metabolites from *Nigrospora* sp. LLGLM003, an endophytic fungus of the medicinal plant *Moringa oleifera* Lam.

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Abstract An endophytic fungus was isolated from the root of the medicinal plant *Moringa oleifera* Lam. Based on analyzing the rDNA sequence, the fungus was identified as *Nigrospora* sp. This is the first report of the isolation of endophytic *Nigrospora* from *M. oleifera*. By bioassay-guided fractionation, four antifungal secondary metabolites were isolated from liquid cultures of the fungus *Nigrospora* sp. LLGLM003, and their chemical structures were determined to be griseofulvin (**1**), dechlorogriseofulvin (**2**), 8-dihydroramulosin (**3**) and mellein (**4**) on the basis of spectroscopic analyses. Compound **2**, **3** and **4** were isolated from *Nigrospora* sp. for the first time. In vitro antifungal assay showed that griseofulvin displayed clear inhibition of the growth of 8 plant pathogenic fungi. Dechlorogriseofulvin and mellein exhibited only weak antifungal activities, whereas 8-dihydroramulosin displayed no antifungal activities.

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Introduction

An endophytic fungus is a microorganism that colonized living, internal tissues of its host plants, without causing any apparent symptoms of disease (Strobel 2002). There are approximately 300,000 different plant species on earth. Generally, each individual plant is host to many endophytes, thus providing a rich reservoir of endophytic fungi (Schulz et al. 2002; Strobel 2003; Strobel et al. 2004; Zhang et al. 2006). Many endophytic fungi have proven to be a rich source of bioactive secondary metabolites which are of interest for proper medicinal or agrochemical applications (Strobel and Daisy 2003).

Since 2002, we have started a survey of the diversity of fungal endophytes distributed in China and its secondary metabolites with antifungal and antitumor activities. We have isolated many bioactive metabolites, including cercosporamide along with new α-tetralone derivative (3*S*)-3, 6, 7-trihydroxy-α-tetralone from endophytic *Phoma* sp. of *Arisaema erubescens* (Wang et al. 2011), trichodermin from endophytic *Trichoderma* sp. of *Taxus mairei* (Chen et al. 2008), etc. This paper reports the isolation and bioactive metabolites of an endophytic fungus *Nigrospora* sp. LLGLM003 from the medicinal plant *Moringa oleifera* Lam. *M. oleifera*, a small deciduous tree, is the most widely known species of *Moringaceae* family and widely distributed in Asia Minor, Africa, the Indian subcontinent (Morton 1991). Traditionally, its root, bark, pod, leave are used in medicine for the treatment of a variety of human ailments whereas pods and young leaves are used as vegetables (Siddhuraju and Becker 2003). There are few

studies on secondary metabolites of endophytic fungi isolated from *M. oleifera* (Ke et al. 2006). The crude extract of the endophytic fungus *Nigrospora* sp. LLGLM003 showed interesting antifungal activities. Investigation of the extract led to the isolation of four active compounds. Their structures were identified by spectroscopic data. Their antifungal activities were also evaluated.

Materials and methods

Isolation and purification of the endophytic fungi

The endophytic fungus strain LLGLM003 was isolated from a root of *M. oleifera*. collected in Xiamen municipality, Fujian Province, People's Republic of China. The general isolated procedures adopted followed the method described by preceding researchers (Gu 2005). Specifically, the roots of *M. oleifera* were washed softly with running tap water, then sterilized with 75% ethanol for 30 s and 1.5% sodium hypochlorite for 10 min, then rinsed in sterile water for three times. The sterilized root tissue were then cut into pieces of 0.5 cm length and six of them placed on a plate containing potato dextrose agar (PDA) supplemented with penicillin (100 μg/ml) and streptomycin sulphate (100 μg/ml) to prevent bacterial growth, and incubated in the dark at 25°C. After 1 week, the emerging hyphae from segments were cut and transferred into new PDA Petri dish for purification. The isolated endophytic fungi were stored by covering a culture on PDA slants with sterile liquid paraffin at 25°C.

Identification of the endophytic fungal strain LLGLM003

The fungal strain LLGLM003 was incubated in PDA medium at 25°C in darkness for 6–8 days. Mycelia were scraped from the plate and grounded to a powder under liquid nitrogen. Fungal DNA was extracted using the Multisource Genomic DNA Miniprep Kit (Axygen Bioscience, Inc.) following the manufacturer's instructions. Primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al. 1990) were used to amplify a fragment of rDNA internal transcribed spacer (ITS) regions 1 and 2. Each 50-μl reaction contained 1× polymerase chain reaction (PCR) buffer, 2.5 mM Mg^{2+}, 100 μM dNTPs, 0.5 μM each primer, 10 ng total DNA, and 2 U Taq polymerase. PCR thermocycling was carried out at 94°C for 3 min, followed by 35 cycles of 94°C for 40 s, 54°C for 50 s, and 72°C for 60 s, followed by a final extension at 72°C for 10 min. The PCR products were purified using a DNA gel extraction kit (Axygen Incorpo-ration, China) and sequenced on an ABI 3730 sequencer (Applied Bio-systems, USA). The sequence was submitted to GenBank (accession no.JN387909) and analyzed by BLAST. Phylogenetic analysis was done by neighbor-joining in MEGA 4, using 1,000 bootstrap replicates.

Fermentation and metabolites isolation

The fungal strain *Nigrospora* sp. LLGLM003 was grown in 1,000 ml Erlenmeyer flasks containing 500 ml of potato dextrose broth (PDB), and then incubated in the dark on a rotary shaker (150 rpm; 25°C; 7 days). After filtration, the culture filtrate (totaling 20 l) was extracted three times with equal volumes of ethyl acetate (EtOAc). The EtOAc layer was evaporated to dryness (3.57 g) and mixed with 7 g silica gel, dried at 50°C, and then loaded on a silica gel column (40 × 1,000 mm) containing 210 g silica gel (200–300 mesh). The column was eluted with petroleum ether: ethyl acetate (4:1, 2:1, 1:1, and 1:2, v/v) firstly, and then the eluent was changed into chloroform: methanol (15:1, v/v). Five fractions were collected and labeled F1, F2, F3, F4, F5, which were detected in antifungal assay with *Botrytis cinerea* as target microorganism. Fractions F1, F4, F5 with antifungal activity were purified thereafter. The F1 fraction (140 mg) was purified by CC over SephadexLH-20 using a mixture of methanol and chloroform in a ratio of 1 to 1 as eluent to give compounds **3** (35 mg) and **4** (18 mg). The F4 (89 mg) fraction was subjected to the same purification method as the F1 fraction to give compound **2** (43 mg). The F5 (500 mg) fraction was subjected to crystallization in petroleum ether: ethyl acetate (2:1, v/v) to give compound **1** (450 mg).

Spectral measurements

To elucidate the chemical structures of four antifungal compounds, they were analyzed using mass and nuclear magnetic resonance (NMR) spectrometry. Mass spectra were recorded on a Bruker Esquire 3000^{plus} mass spectrometer. All NMR data were collected on a Bruker AMX-500(500 MHz) NMR spectrometer using TMS as internal standard, the chemical shifts were expressed in δ (ppm) and coupling constants J in Hz. Melting points were determined on a Beijing X4 micro melting point apparatus and were uncorrected. Silica gel (200–300 mesh) for column chromatography was produced by Qingdao Marine Chemical Factory, Qingdao, China. SephadexLH-20 was produced by Pharmacia Biotech, Sweden.

Compound 1

Griseofulvin, white solid, molecular formula $C_{17}H_{17}ClO_6$; m.p. 221–223°C; ESI–MS (m/z): 353 $[M + H]^+$; ^1H NMR

(500 MHz, $CDCl_3$, *J* in Hz): δ6.14 (1H, s, 5-H), δ5.55 (1H, s, 2′-H), δ4.04 (3H, s, 4-OCH_3), δ4.04 (3H, s, 6-OCH_3), δ3.63 (3H, s, 1′-OCH_3), δ3.04 (1H, dd, $J = 16.7$ Hz, 4.6, 4′-H), δ2.86 (1H, m, 5′-H), δ2.44 (1H, dd, $J = 16.0$ Hz, 3.8, 4′-H), δ0.97 (3H, d, $J = 6.8$ Hz, 5′-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ197.1 (C-3′), δ192.5 (C-3), δ169.5 (C-7a), δ164.6 (C-6), δ170.8 (C-1′), δ157.7 (C-4), δ105.1 (C-3a), δ104.8 (C-2′), δ97.2 (C-7), δ90.7 (C-2), δ89.4 (C-5), δ57.0 (4-OCH_3), δ56.7 (1′-OCH_3), δ56.4 (6-OCH_3), δ40.0 (C-4′), δ36.4 (C-5′), δ14.2 (5′-CH_3). The ^{1}H NMR and ^{13}C NMR data are consistent with the literature reported (Park et al. 2005).

Compound 2

Dechlorogriseofulvin, white solid, molecular formula $C_{17}H_{18}O_6$; m.p. 179–181°C; ESI–MS (m/z): 319 $[M + H]^+$ and 336 $[M + NH_4]^+$; ^{1}H NMR (500 MHz, $CDCl_3$, *J* in Hz): δ6.24 (1H, d, $J = 1.8$ Hz, 5-H), δ6.05 (1H, d, $J = 1.7$ Hz, 7-H), δ5.55 (1H, s, 2′-H), δ3.91 (3H, s, 1′-OCH_3), δ3.90 (3H, s, 6-OCH_3), δ3.63 (3H, s, 4-OCH_3), δ3.05 (1H, dd, $J = 16.8$ Hz, 13.4, 4′-H), δ2.75 (1H, m, 5′-H), δ2.41 (1H, dd, $J = 16.8$ Hz, 4.7, 4′-H), δ0.97 (3H, d, $J = 6.7$ Hz, 5′-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$): δ197.4 (C-3′), δ192.5 (C-3), δ176.0 (C-7a), δ171.3 (C-6), δ170.3 (C-1′), δ159.1 (C-4), δ104.7 (C-3a), δ104.3 (C-2′), δ93.3 (C-7), δ89.9 (C-2), δ88.5 (C-5), δ57.0 (4-OCH_3), δ56.6 (1′-OCH_3), δ56.1 (6-OCH_3), δ40.0 (C-4′), δ36.5 (C-5′), δ14.2 (5′-CH_3). The ^{1}H NMR and ^{13}C NMR data were identical with literature reported (Park et al. 2005).

Compound 3

8-dihydroramulosin, white solid, molecular formula $C_{10}H_{16}O_3$; m.p. 142–144°C; ESI–MS (m/z): 185 $[M + H]^+$ and 207 $[M + Na]^+$; ^{1}H NMR (500 MHz, $CDCl_3$, *J* in Hz): δ4.39 (1H, m, 3-H), δ2.23 (1H, ddd, $J = 14.1$ Hz, 6.4, 3.8, 4-H), δ1.23 (1H, m, 4-H), δ2.13 (1H, m, 4a-H), δ1.61 (1H, brd, $J = 12.7$ Hz, 5-H), δ0.92 (1H, qd $J = 13.0$ Hz, 3.2, 5-H), δ1.70 (1H, m, 6-H), δ1.23 (1H, m, 6-H), δ1.87 (1H, dt, $J = 13.0$ Hz, 4.0, 7-H), δ1.72 (1H, m, 7-H), δ3.61 (1H, dt, $J = 11.3$ Hz, 5.2, 8-H), δ2.95 (1H, brt, $J = 5.3$ Hz, 8a-H), δ1.31 (3H, d, $J = 6.2$ Hz, 9-H); ^{13}C NMR (125 MHz, $CDCl_3$): δ174.9 (C-1), δ73.4 (C-3), δ36.5 (C-4), δ31.9 (C-4a), δ30.9 (C-5), δ23.4 (C-6), δ32.8 (C-7), δ70.1 (C-8), δ44.2 (C-8a), δ20.6 (C-9). The ^{1}H NMR and ^{13}C NMR data are consistent with the literature reported (Stierle et al. 1998).

Compound 4

Mellein, white solid, molecular formula $C_{10}H_{10}O_3$; m.p. 55–56°C; ESI–MS (m/z): 179 $[M + H]^+$; ^{1}H NMR (500 MHz, $CDCl_3$, *J* in Hz): δ11.03 (1H, s, OH), δ7.41 (1H, dd, $J = 8.5$ Hz, 0.5, 6-H), δ6.89 (1H, d, $J = 8.5$ Hz, 5-H), δ6.69 (1H, dd, $J = 0.5$ Hz, 8.5, 7-H), δ4.73 (1H, m, 3-H), δ2.93 (2H, d, $J = 7.5$ Hz, 4-H), δ1.53 (3H, d, $J = 6.0$ Hz, Me); ^{13}C NMR (125 MHz, $CDCl_3$): δ169.9 (C-1), δ116.2 (C-7), δ162.1 (C-8), δ108.2 (C-8a), δ139.3 (C-4a), δ117.9 (C-5), δ136.1 (C-6), δ76.1 (C-3), δ34.6 (C-4), δ20.8 (3-Me). The ^{1}H NMR and ^{13}C NMR data are in full agreement with literature reported (Dimitriadis et al. 1997).

Antifungal assay

The four metabolites dissolved in methanol were tested for fungistatic activity in vitro against 8 plant pathogenic fungi: *B. cinerea*, *Colletotrichum orbiculare, Fusarium oxysporum* f.sp. *cucumerinum*, *Fusarium oxysporum* f.sp. *melonis*, *Pestalotia diospyri*, *Pythium ultimum*, *Rhizoctonia solani* and *Sclerotinia sclerotiorum*. To this end, a series of different concentrations of the metabolite were diluted with melted PDA medium and poured into a Petri dish. After cooling, agar discs (5 mm in diameter) of test fungi were placed at the center of plates containing the fungistatic compound of 100, 50, 25, 10, 5, 2.5, and 1.25 μg/ml concentrations. Three replicate plates of each concentration for each fungus were incubated in the dark at 25°C for all test fungi. Plates containing media mixed with isometric methanol were included as control. The diameter of the growing colony was measured at least three times during the following days, and plotted against the incubation time. The part of the curve which showed a linear relationship was used to calculate the growth rate (millimeter per day). This was then plotted against the respective metabolite concentration. The effective concentrations (EC_{50}) are defined as the concentration of the test compound that allows the test organism to grow at 50% of its rate under the same conditions in the absence of the metabolite.

Results and discussion

In order to identify the fungus isolated from the root of miracle trees (*M. oleifera*), its internal transcribed spacer 1(ITS1), 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (ITS2) were sequenced and submitted to BLASTN at the NCBI nucleotide database. It was showed that similar sequences belong to *Nigrospora*. We chose some sequences located in *Trichosphaeriales* also with one from *Achroceratosphaeria potamia* as the out-group to be aligned with that of LLGLM003 using ClustalX1.8. The alignment was then subjected to neighbor-joining analysis, placing LLGLM003 within the clade including *Nigrospora sphaerica* and *Nigrospora oryzae* (Fig. 1), indicating that

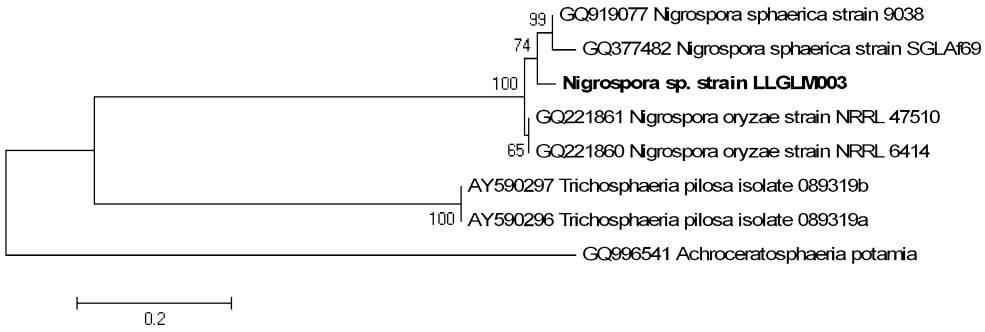

Fig. 1 Neighbor-joining tree based on ITS sequence

this isolate LLGLM003 is a *Nigrospora* sp. closely related to these taxa.

The *Nigrospora* species are common endophytes in plants, previously isolated from *Taxus globosa* (Ruiz-Sanchez et al. 2010), *Rhizophora mucronata* (Xu et al. 2011), *Azadirachta indica* (Wu et al. 2009), *Smallanthus sonchifolins* (Lopes and Pupo 2011). The genus *Nigrospora* was also a rich source of biologically active secondary metabolites, such as phytotoxic and antibacterial nigrosporins (Tanaka et al. 1997), herbicidal lactones (Fukushima et al. 1998), phomalactone (Kim et al. 2001) and phytoxin epoxyexserohilone (Cutler et al. 1991). This is the first report of the isolation of endophytic *Nigrospora* from *M. oleifera*.

The endophytic fungus *Nigrospora* sp. LLGLM003 was selected for chemical and biological investigations because of the strong antifungal activity of the crude extract against the plant pathogenic fungi *B. cinerea* (EC_{50} = 6.09 μg/ml). The EtOAc extract of the fermentation broth was isolated and purified repeatedly by silica gel, SephadexLH-20 column and re-crystallization to yield four compounds (Fig. 2), which were identified as griseofulvin (**1**), dechlorogriseofulvin (**2**), 8-dihydroramulosin (**3**), mullein (**4**), respectively according to mass and NMR spectral data. So far as we know, compounds **2-4** were isolated from *Nigrospora* sp. for the first time.

The antifungal efficacy experiments of compounds **1**–**4** were tested in vitro against 8 plant pathogenic fungi and the results were listed in Table 1. Among them, compound **1** exhibited strong activity against *B. cinerea* and *C. orbiculare* with the EC_{50} of 0.20 and 0.49 μg/ml, respectively, and moderate activity against other test fungi. Compound **2** displayed weak activity against *B. cinerea*, *R. solani*, *C. orbiculare*, and *S. sclerotiorum* with the EC_{50} of 40.0, 43.9, 81.7 and 28.5 μg/ml, respectively, and inactive to other test fungi. But compound **3** showed inactive to all test

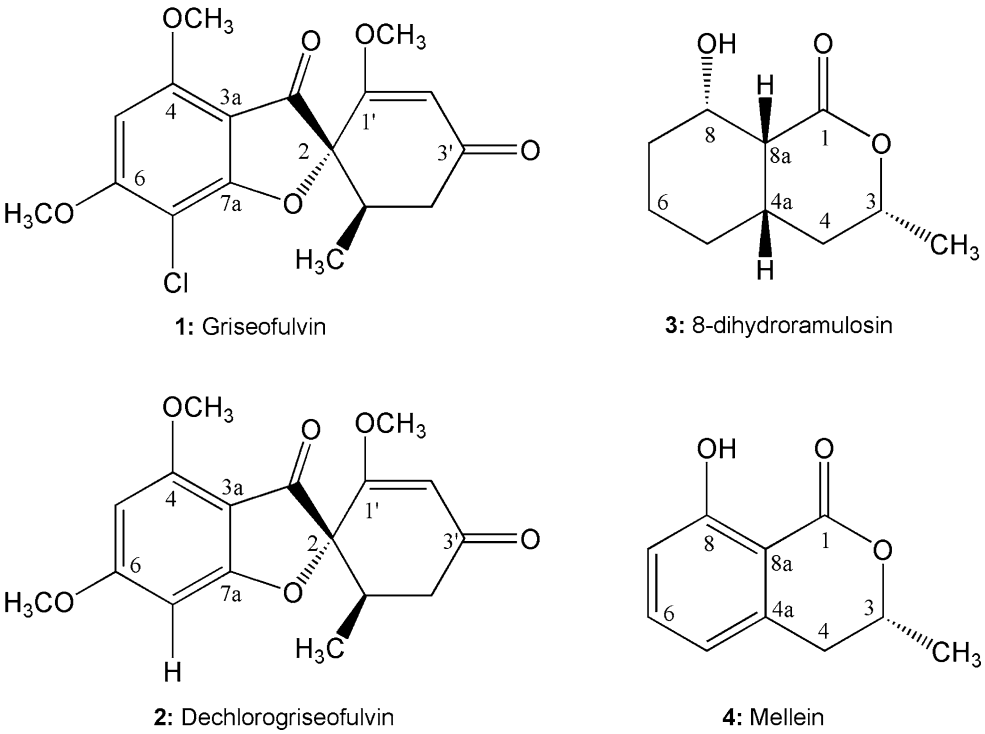

Fig. 2 Structure of the compounds **1-4**

Table 1 The antifungal activities of compounds **1**-**4** obtained from *Nigrospora* sp

Test plant pathogenic fungi	EC_{50} (μg/ml)			
	1	**2**	**3**	**4**
Botrytis cinerea	0.20	40.0	>100	49.2
Colletotrichum orbiculare	0.49	81.7	>100	>100
Fusarium oxysporum f.sp. *cucumerinum*	4.08	>100	>100	>100
Fusarium oxysporum f.sp. *melonis*	8.69	>100	>100	>100
Pestalotia diospyri	42.7	>100	>100	>100
Pythium ultimum	6.08	>100	>100	>100
Rhizoctonia solani	17.6	43.9	>100	>100
Sclerotinia sclerotiorum	5.78	28.5	>100	>100

fungi and compound **4** only exhibited weak activity against *B. cinerea* with the EC_{50} of 49.2 μg/ml.

Griseofulvin (**1**) has been used as an antifungal antibiotic for the treatment of diseases of humans caused by pathogenic fungus (Gupta et al. 1997; Knasmüller et al. 1997) and inflammatory diseases (Asahina et al. 2001). Many fungus of *Penicillium* sp. (Mantle et al. 1984), *Aspergillus versicolor* (Kingston et al. 1976) and *Xylaria* sp. (Park et al. 2005) have presently been considered as typical griseofulvin producing fungi. Although chemical synthesis of griseofulvin was reported by former researchers (Stork and Tomasz 1962; Danishefsky and Walker 1979), it is economically infeasible. Therefore, griseofulvin is commercially produced by fermentation processes instead of by chemical synthesis (Dasu et al. 2002a; Dasu et al. 2002b). In this aspect, the discovery of more fungal species that can produce griseofulvin is very important. Dechlorogriseofulvin (**2**) showed weak antifungal activity and was previously obtained from the endophytic fungi *Penicillium janthinellum* (Xue et al. 2006) and *Xylaria* sp. (Cafêu et al. 2005; Park et al. 2005).

Both 8-dihydroramulosin (**3**) and mellein (**4**) were isocoumarin derivatives and widely distributed in fungi. 8-dihydroramulosin (**3**) was formerly reported as metabolite of the endophyte *Canoplea elegantula* (Findlay et al. 1995) and *Botrytis* sp. (Stierle et al. 1998) and toxic to spruce budworm larvae (Findlay et al. 1995). Mellein (**4**) was previously isolated from many endophytic fungi, including *Botryosphaeria rhodina* (Rukachaisirikul et al. 2009), *Botryosphaeria mamane* (Pongcharoen et al. 2007), *Prumnopitys andina* (Schmeda-Hirschmann et al. 2005), *Plectophomella* sp., *Pezicula livida* and *Cryptosporiopsis malicorticis* (Krohn et al. 1997). The biological properties of mellein include: antibacterial activity (Krohn et al. 1997), antifungal activity (Höller et al. 1998), phytotoxic activity (Parisi et al. 1993) and bioactivity against the protease of hepatitis C virus (HCV) (Dai et al. 2001).

In conclusion, this is the first report on the isolation of endophytic *Nigrospora* from *M. oleifera*. The compounds from the strain LLGLM003 displayed strong or weak antifungal activity, which indicated that endophytic *Nigrospora* are promising sources of natural bioactive and novel metabolites and with great potential for further study.

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