RESEARCH ARTICLE

Chaetominine, (+)-alantrypinone, questin, isorhodoptilometrin, and 4-hydroxybenzaldehyde produced by the endophytic fungus Aspergillus sp. YL-6 inhibit wheat (Triticum aestivum) and radish (Raphanus sativus) germination

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Five phytotoxic substances with allelopathic activity were isolated from endophytic fungi Aspergillus sp. YL-6 habited in Pleioblastus amarus. The chemical structures of these substances were determined as chaetominine (1), (+)-alantrypinone (2), questin (3), isorhodoptilometrin (4), and 4-hydroxybenzaldehyde (5) by nuclear magnetic resonance and mass spectrometry data. The potential allelopathic effects of compounds 1–5 were evaluated on wheat (Triticum aestivum) and radish (Raphanus sativus). Under lab condition, at concentrations of 10 and 20 ppm, compounds 1–5 inhibit the germination and growth of the two tested seeds completely. An idole-3-acetic acid (IAA) derivative, (+)-alantrypinone (2) displayed the best inhibitory effects on radish seeds among these tested compounds with the similar activity as the positive control glyphosate, a broad-spectrum systemic herbicide. In the further evaluation of compounds 1–5, Questin (3), an anthraquinone derivative, can inhibit shoot and root elongation of wheat, the inhibitory effects assessed were similar to the positive control glyphosate.

Keywords: Pleioblastus amarus; Aspergillus sp. YL-6; herbicides; allelochemicals

Introduction

Allelopathy is a process by which plants, including microorganisms, produce special chemicals that can be either beneficial or harmful to other (or even their own) neighboring organisms (Hiroshi et al. 2014). Pleioblastus amarus is widely distributed in southern China and is used in traditional medicine for the treatment of inflammation, fever, and the prevention of senility (Sun et al. 2014). Some biological activity experiments have shown that P. amarus leaf extracts have remarkable immunomodulatory properties (Wang et al. 2004). Interestingly, little weeds grow around P. amarus and this plant rarely suffers from diseases and insect pests. The possible reason of these interesting phenomena might be attributed to the allelochemicals which are produced by the plant directly or by its endophytic fungi.

Endophytic fungi produce a large number of bioactive products possessing structural diversities such as alkaloids, steroids, terpenoids, isocoumarin derivatives, quinones, phenols, and lactones (Verma et al. 2009). Furthermore, the plethora of substances is seemingly produced as part of a defense strategy, as well as contributing to various physiological functions of the host plants (Strobel et al. 2004). Because of the ecological roles of endophytes, the chemical components in the endophytic fungi become attractive and sustainable for agricultural pharmacology. In our ongoing research of P. amarus, we are interested to explore the endophytic fungi and their potential contribution on the defense strategy of their host plant.

In our previous assay, we found a strain from P. amarus, named Aspergillus sp. YL-6, exhibited inhibitory effects on seeds growth. The aim of this study, therefore, was to determine the allelochemicals present in the plant endophytic fungi Aspergillus sp. YL-6 associated with P. amarus on the basis of a bioassay-guided fractionation. Five active compounds were obtained and their structures were identified to be chaetominine (1), (+)-alantrypinone (2), questin (3), isorhodoptilometrin (4), and 4-hydroxybenzaldehyde (5) by nuclear magnetic resonance (NMR) and mass spectrometry (MS) data.

Materials and methods

General procedure

NMR spectra were recorded on a Bruker AVANCE III (500 MHz) instrument. Chemical shifts were calculated using solvent residual as the internal standard. Electrospray ionization mass spectrometry was conducted with a Thermo LCQ Fleet instrument. Column chromatography (CC) was performed on silica gel (90–150 μm;
Plant samples

Stem tissues of *P. amarus* were collected from Zhejiang A & F University. Samples were brought to the laboratory in separate sterile polythene bags and processed within four hours of collection.

Fungal material

The samples were rinsed gently in running tap water to remove dusts and debris. The stem was cut into segments (0.5–1 cm). The samples were dipped in 75% ethanol for 1 minute, immersed in 4% sodium hypochlorite for 3 minutes and then rinsed in sterile distilled water for 30 s. The sterilized tissue samples were plated on potato dextrose agar (PDA) medium contained in Petri dishes, amended with chloramphenicol (150 mg/L). The Petri dishes were sealed using parafilm and incubated at 26°C at 12-h light/dark (L/D) cycle. The Petri dishes were amended with chloramphenicol (150 mg/L). The Petri dishes were monitored every day to check the growth of endophytic fungal colonies from the segments.

The hyphal tips which grew out from the segments were isolated and subcultured on PDA medium. The pure cultures were maintained on PDA slants. The fungal material within four hours of collection.

Cultivation

After growing on PDA medium at 28°C for seven days, the fungus was inoculated in liquid medium containing: CaCl₂ 0.5 g, KH₂PO₄ 0.1 g, KCl 0.05 g, MgSO₄·7H₂O 0.1 g, glucose 20.0 g, peptone 15.0 g in 1000 ml H₂O.

The pH was adjusted to 6.0 before autoclaving. Fermentation was carried out in 1000 ml flasks each containing 200 ml medium on a rotary shaker at 150 rpm at 28°C for seven days.

Extraction and isolation

The culture broth (30 L) of *Aspergillus* sp. YL-6 was filtered to obtain the mycelium and water phase. The mycelium was dried at 50°C, and smashed directly with ultrasonic extraction by ethyl acetate and followed by acetone for three times. These two parts were combined and dried after TLC, then defatted with cyclohexane after being dissolved in methanol to get a crude extract (6.2 g). The crude extract was then subjected to a silica gel column and eluted with a chloroform/MeOH mixture (100:1, 50:1, and 20:1) to give three fractions (Fr.1–Fr.3). Fr.1 was further purified on Sephadex LH-20, silica gel, and preparative TLC to afford 2 (11 mg). Fr.2 was further chromatographed by CC on RP-18, Sephadex LH-20, silica gel, and prepared TLC to yield compounds 1 (8.2 mg) and 5 (6.8 mg). Fr.3 was rechromatographed on a Sephadex LH-20 column, and further purified by prepared TLC to give compounds 3 (12 mg) and 4 (9.5 mg).

**Chaetominine (1)**

White powder. ¹³C NMR (125 MHz, DMSO-d₆) δc: 171.8 (C-10), 165.3 (C-17), 159.8 (C-15), 147.3 (C-24), 146.6 (C-23), 138.6 (C-9), 136.6 (C-4), 134.5 (C-21), 129.7 (C-7), 127.1 (C-20), 127.0 (C-22), 126.3 (C-19), 125.3 (C-6), 124.8 (C-5), 121.0 (C-18), 114.2 (C-8), 82.3 (C-2), 76.3 (C-3), 59.4 (C-11), 49.9 (C-14), 38.1 (C-13), 13.9 (C-12). 1H NMR (500 MHz, DMSO-d₆) δh: 8.29 (1H, brs, H-24), 8.19 (1H, brd, J = 7.5 Hz, H-19), 7.87 (1H, td, J = 7.5, 1.5 Hz, H-21), 7.70 (1H, brd, J = 8.0 Hz, H-22), 7.59 (1H, brd, J = 7.5 Hz, H-20), 7.52 (1H, brd, J = 8.0 Hz, H-8), 7.50 (1H, brd, J = 7.0 Hz, H-5), 7.44 (1H, 7d, J = 7.5, 1.0 Hz, H-7), 7.26 (1H, td, J = 7.5, 1.0 Hz, H-6), 6.72 (1H, d, H-10), 5.93 (1H, brs, H-14), 5.62 (1H, s, H-2), 4.62 (1H, q, J = 7.0 Hz, H-11), 1.61 (1H, d, J = 6.5 Hz, H-12), 2.55 (1H, dd, J = 13.0, 3.0 Hz, H-13α), 2.94 (1H, t, J = 13.0 Hz, H-13β).

**(+)-Alantrypine (2)**

White powder. ¹³C NMR (125 MHz, DMSO-d₆) δc: 169.6 (C-1), 159.8 (C-12), 154.8 (C-4), 147.1 (C-6), 135.3 (C-18), 135.2 (C-20), 134.5 (C-8), 127.9 (C-7, C-9), 127.8 (C-25), 126.8 (C-10), 122.8(C-22), 120.6 (C-11), 119.8 (C-23), 118.6 (C-24), 112.2 (C-21), 106.0 (C-17), 55.1 (C-3), 54.6 (C-14), 26.1 (C-15), 18.8 (C-16). ¹H NMR (500 MHz, DMSO-d₆) δh: 11.22 (1H, s, H-19-NH), 9.68 (1H, s, 2-NH), 8.15 (1H, dd, J = 8.0, 1.5 Hz, H-10), 7.82 (1H, ddd, J = 7.5, 7.0, 1.5 Hz, H-8), 7.65 (1H, d, J = 8.0 Hz, H-7), 7.54 (1H, ddd, J = 8.5, 7.0, 1.5 Hz, H-9), 7.41 (1H, d, J = 8.0 Hz, H-24), 7.38 (1H, d, J = 8.0 Hz, H-21), 7.13 (1H, ddd, J = 8.0, 7.0, 1.5 Hz, H-22), 7.00 (1H, ddd, J = 8.0, 7.0, 1.5 Hz, H-23), 5.71 (1H, t, J = 3.0 Hz), 3.44 (1H, dd, J = 17.0, 2.5 Hz, H-15α), 3.24 (1H, dd, J = 17.0, 2.5 Hz, H-15β), 2.13 (3H, s, H-16).

**Questin (3)**

Yellow powder. ¹³C NMR (125 MHz, DMSO-d₆) δc: 186.9 (C-9), 182.6 (C-10), 164.6 (C-6), 163.9 (C-8), 162.2 (C-1), 146.7 (C-3), 137.3 (C-11), 132.5 (C-14), 124.6 (C-2), 119.6 (C-4), 114.9 (C-13), 112.5 (C-12), 107.5 (C-5), 105.5 (C-7), 56.8 (C-8-OMe), 21.8 (C-3- Me). ¹H NMR (500 MHz, DMSO-d₆) δh: 13.26 (1H, s, H-1- OH), 11.19 (1H, brs, H-3-OH), 7.45 (1H, d, J = 1.5 Hz, H-4), 7.22 (1H, d, J = 2.0 Hz, H-5), 7.14 (1H, d, J = 1.5 Hz, H-2), 6.86 (1H, d, J = 2.0 Hz, H-7), 3.92 (3H, s, H-8-OMe), 2.40 (3H, s, H-3-Me).
**Isorhodoptilometrin (4)**

Yellow powder. $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$: 190.1 (C-9), 182.0 (C-10), 164.9 (C-8), 163.2 (C-6), 161.6 (C-1), 150.5 (C-3), 135.7 (C-10a), 133.1 (C-4a), 125.3 (C-2), 121.5 (C-4), 114.2 (1a), 112.8 (9a), 109.3 (C-5), 108.4 (C-7), 76.0 (C-2’), 45.6 (C-1’), 23.9 (C-3’).

$^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$: 7.57 (1H, $d$, $J = 1.5$ Hz, H-4), 7.21 (1H, $d$, $J = 1.5$ Hz, H-2), 7.14 (1H, $d$, $J = 2.5$ Hz, H-5), 6.61 (1H, $d$, $J = 2.5$ Hz, H-7), 3.91 (1H, $m$, H-2’), 2.74 (2H, $m$, H-1’), 1.11 (3H, $d$, $J = 6.5$ Hz, H-3’).

**4-Hydroxybenzaldehyde (5)**

White powder. $^{13}$C NMR (125 MHz, DMSO-$d_6$) $\delta_C$: 191.4 (C-1), 163.8 (C-5), 132.6 (C-3 and C-7), 128.9 (C-2), 116.3 (C-4 and C-5). $^1$H NMR (500 MHz, DMSO-$d_6$) $\delta_H$: 10.61 (1H, $brs$, H-5-OH), 9.79 (1H, $s$, H-1-CHO), 7.76 (2H, $d$, $J = 9.0$ Hz, H-3 and H-7), 6.94 (2H, $d$, $J = 8.5$ Hz, H-4 and H-6).

**Seed germination and seedling growth bioassay**

The seeds of two herbaceous plants, wheat (*Triticum aestivum*) and radish (*Raphanus sativus*), were used for the allelopathic bioassay. The procedures were conducted according to the reported protocol (Kuang et al. 2014). The plant seeds were imbibed in deionized water for two hours, soaked in 0.3% KMnO$_4$ for 15 minutes, and washed with sterile water until they were colorless. The compounds, positive control and blank solvent acetone, were placed in a 9-cm Petri dish lined with a Whatman No. 1 filter paper to final concentrations of 1.25, 2.5, 5, 10, and 20 ppm. After the evaporation of acetone, the plant seeds were sown in the Petri dish and irrigated with deionized water. Three repeats were conducted. The Petri dishes were placed in an illuminated growth chamber held at 25 ± 1°C, 80 ± 2% RH and a 12/12 h L/D photoperiod for 90 hours.

![Figure 1. Structures of compounds 1–5 (Chaetominine (1), (+)-Alantrypinone (2), Questin (3), Isorhodoptilometrin (4), and 4-Hydroxybenzaldehyde (5)).](image)

The germination rates were calculated according to Equation 1. Allelopathic effects (response index [RI]) were calculated according to Equation 2 (Zhang et al. 2013).

Germination rate (%) = \( \frac{\text{(number of germinated seeds)}}{\text{(total number of seeds)}} \) \( \times \frac{1}{\text{T}} \) (Equation 1)

where $T$ is the length of the treatment, $C$ is the length of the blank control, and RI is the RI. The RI values range from $+1$ to $-1$, with positive values indicating stimulation and negative values indicating inhibition relative to the controls. The RI was considered to be superior to the T/C statistical method and easy to interpret because it is simply the proportional reduction of the treatment relative to the control (Bruce & Richardson 1998).

Germination rates and RIs were expressed as means ± standard deviation (SD) for three replicates. All of the statistical differences were compared by SPSS software using one-way analysis of variance at an $\alpha = 0.05$ significance level.

**Results and discussion**

**Identification of allelochemicals**

Chemical investigations of *Aspergillus* sp. YL-6 crude extracts led to the isolation of five compounds (Figure 1) by multiple chromatographic procedures: chaetominine (1) (Jiao et al. 2006), (+)-alantrypinone (2) (Larsen et al. 1998), questin (3) (Kimura et al. 1983), isorhodoptilometrin (4) (Ren et al. 2006), and 4-hydroxybenzaldehyde (5) (Liu et al. 2009). Their structures were identified on the basis of comparison of their NMR and MS data with those reported compounds.
Allelopathic activity against radish (R. sativus) and wheat (T. aestivum) was evaluated to obtain information on the herbicidal effect of the tested compounds. The inhibition increased with the concentration of these compounds. The root and shoot growth of the tested weeds were suppressed by increasing concentrations of these compounds. The root inhibition was more sensitive to the concentration of 5 ppm. For the inhibition on shoot elongation, compound 2 showed a significant higher inhibition on the germination of radish completely at the concentration of 10 ppm and 20 ppm. The inhibition increased with the concentration up to 10 ppm all the tested compounds could completely inhibit the germination of radish, compounds 1, 2, 4, and 5 did not show significant differences compared with glyphosate from the concentration of 1.25 ppm to 5 ppm.

| Compound | Germination rate | Root elongation (RI) | Shoot elongation (RI) |
|----------|------------------|----------------------|----------------------|
|          | 1.25 ppm | 2.5 ppm | 5 ppm | 10 ppm | 20 ppm | 1.25 ppm | 2.5 ppm | 5 ppm | 10 ppm | 20 ppm | 1.25 ppm | 2.5 ppm | 5 ppm | 10 ppm | 20 ppm |
| 1        | 0.92 ± 0.08 a   | 0.82 ± 0.12 a       | 0.38 ± 0.06 bc      | 0 ± 0 c             | 0 ± 0 c             | 0.04 ± 0.03 a       | -0.31 ± 0.06 c      | -1 ± 0 c           | -1 ± 0 b          | -1 ± 0 b          | -0.02 ± 0.06 ab | -0.19 ± 0.05 bc | -1 ± 0 b | -1 ± 0 b | -1 ± 0 b |
| 2        | 0.58 ± 0.15 bc  | 0.43 ± 0.06 cd     | 0.08 ± 0.06 cd      | 0 ± 0 c             | 0 ± 0 c             | -0.20 ± 0.06 b      | -0.18 ± 0.08 ab    | -0.08 ± 0.05 a      | -1 ± 0 b          | -1 ± 0 b          | -0.15 ± 0.01 bc | -0.13 ± 0.01 ab  | -0.47 ± 0.07 a | -1 ± 0 b | -1 ± 0 b |
| 3        | 0.65 ± 0.13 bc  | 0.62 ± 0.21 b      | 0.40 ± 0.25 b      | 0 ± 0 c             | 0 ± 0 c             | -0.21 ± 0.07 ab    | -0.12 ± 0.09 ab    | -0.30 ± 0.03 b      | -1 ± 0 b          | -1 ± 0 b          | -0.20 ± 0.07 c  | -0.24 ± 0.05 bc  | -0.45 ± 0.02 a | -1 ± 0 b | -1 ± 0 b |
| 4        | 0.50 ± 0.13 c   | 0.52 ± 0.10 bc     | 0.23 ± 0.06 bcd    | 0 ± 0 c             | 0 ± 0 c             | -0.35 ± 0.05 b     | -1 ± 0 b           | -1 ± 0 b          | -1 ± 0 b          | -1 ± 0 b          | 0.02 ± 0.02 a   | -0.04 ± 0.10 a  | -0.48 ± 0.13 a | -1 ± 0 b | -1 ± 0 b |
| 5        | 0.72 ± 0.06 b   | 0.55 ± 0.15 bc     | 0.45 ± 0.13 b      | 0 ± 0 c             | 0 ± 0 c             | -0.13 ± 0.07 ab    | -0.12 ± 0.09 ab    | -0.30 ± 0.03 b      | -1 ± 0 b          | -1 ± 0 b          | -0.40 ± 0.06 d  | -0.34 ± 0.07 c  | -0.47 ± 0.04 a | -1 ± 0 b | -1 ± 0 b |
| gp       | 0.92 ± 0.03 a   | 0.85 ± 0.09 a      | 0.80 ± 0.05 a      | 0.75 ± 0.05 b      | 0.73 ± 0.08 b      | -0.02 ± 0.05 ab    | -0.10 ± 0.01 ab    | -0.21 ± 0.07 ab    | -0.21 ± 0.07 a    | -0.45 ± 0.01 a    | -0.19 ± 0.04 c  | -0.26 ± 0.02 bc  | -0.33 ± 0.03 a  | -0.13 ± 0.04 a | -0.16 ± 0.12 a |
| ck       | 0.95 ± 0 a      | 0.95 ± 0 a         | 0.95 ± 0 a         | 0.95 ± 0 a         | 0.95 ± 0 a         | 0.95 ± 0 a         | 0.95 ± 0 a         | 0.95 ± 0 a         | 0.95 ± 0 a        | 0.95 ± 0 a        | 0.95 ± 0 a      | 0.95 ± 0 a      | 0.95 ± 0 a      | 0.95 ± 0 a | 0.95 ± 0 a |

Note: gp standards for glyphosate and ck for the blank control.

*Means (±SD) within columns followed by the same letter are not significantly different at p ≤ 0.05.
control glyphosate (RI values ranging from −0.47 to −0.58 at 1.25 to 5 ppm), which was stronger than the other compounds (RI values ranging from −0.08 to −0.30 at 1.25 to 5 ppm). In particular, at a lower concentration of 1.25 ppm, the RI of 3 was still up to −0.53. Our observations suggest that 3 could play an important role in plant growth regulation.

Questin (3), an anthraquinone derivative, was first found in a typical fungi *Penicillium frequentans* (Mahmoodian & Stickings 1964). Later it was discovered in many plants and microorganisms with various bioactivities, such as antimicrobial activities (Inamori et al. 1983; Du et al. 2014). Some antimicrobial activities of 3 may directly or indirectly affect the growth of other plants. Al Saadawi et al. (1983) pointed out that some organisms produce allelopathic chemicals that inhibited soil bacteria and changed the soil environment to their own advantage (Al Saadawi et al. 1983). Moreover, emodin, an isomer of 3 differs for the position of methoxy, also exhibited good phytotoxic activity on weed growth (Inoue et al. 1992), which means that the methoxylation of the hydroxyl on the aromatic ring would not be a necessary procedure for the weed growth inhibition activity. Further studies will be carried out to investigate the inhibition mechanism and ecological relevance of allelopathy with respect to the effective presence and persistence time.

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### Table 2. Allelopathic effects on wheat (*T. aestivum*) of compounds 1–8

| Compounds | Concentration (ppm) | Germination rate | Root elongation RI | Shoot elongation RI |
|-----------|---------------------|-----------------|-------------------|---------------------|
|           | 0.125 | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 | 8.0 | 12.5 | 20.0 | 0.125 | 0.25 | 0.5 | 1.0 | 2.0 | 4.0 | 8.0 | 12.5 | 20.0 |
| 1         | a     | a    | a    | a    | a    | a    | a    | a    | a    | b     | b    | b    | a    | a    | a    | a    | a    | a    |
| 2         | 0.35  | 0.45  | 0.55  | 0.65  | 0.75  | 0.85  | 0.95  | 1.05  | 1.15  | 0.35  | 0.45  | 0.55  | 0.65  | 0.75  | 0.85  | 0.95  | 1.05  | 1.15  |
| 3         | 0.95  | 0.8    | 0.7    | 0.6    | 0.5    | 0.4    | 0.3    | 0.2    | 0.1    | 0.95  | 0.8    | 0.7    | 0.6    | 0.5    | 0.4    | 0.3    | 0.2    | 0.1    |
| 4         | 1.25  | 1.1    | 1.0    | 0.9    | 0.8    | 0.7    | 0.6    | 0.5    | 0.4    | 1.25  | 1.1    | 1.0    | 0.9    | 0.8    | 0.7    | 0.6    | 0.5    | 0.4    |
| 5         | 1.5   | 1.4    | 1.3    | 1.2    | 1.1    | 1.0    | 0.9    | 0.8    | 0.7    | 1.5   | 1.4    | 1.3    | 1.2    | 1.1    | 1.0    | 0.9    | 0.8    | 0.7    |
| 6         | 1.75  | 1.6    | 1.5    | 1.4    | 1.3    | 1.2    | 1.1    | 1.0    | 0.9    | 1.75  | 1.6    | 1.5    | 1.4    | 1.3    | 1.2    | 1.1    | 1.0    | 0.9    |
| 7         | 2.0   | 1.9    | 1.8    | 1.7    | 1.6    | 1.5    | 1.4    | 1.3    | 1.2    | 2.0   | 1.9    | 1.8    | 1.7    | 1.6    | 1.5    | 1.4    | 1.3    | 1.2    |
| 8         | 2.25  | 2.1    | 2.0    | 1.9    | 1.8    | 1.7    | 1.6    | 1.5    | 1.4    | 2.25  | 2.1    | 2.0    | 1.9    | 1.8    | 1.7    | 1.6    | 1.5    | 1.4    |

Note: Means (SD) within columns followed by the same letter are not significantly different at p ≤ 0.05.
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