A new sesquiterpene from the entomogenous fungus *Phomopsis amygdali*

Xiaoli Ma\(^a\), Wenshu Wang\(^abc\)*, Erwei Li\(^b\), Fenghua Gao\(^a\), Liangdong Guo\(^b\) and Yunfei Pei\(^b\)

\(^a\)College of Life and Environmental Sciences, Minzu University of China, Beijing 100081, P.R. China; \(^b\)State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, P.R. China; \(^c\)Beijing Engineering Research Center of Food Environment and Health, Minzu University of China, Beijing 100081, P.R. China

(Received 15 February 2015; final version received 25 May 2015)

A new sesquiterpene, (+)-S-1-methyl-abscisic-6-acid (1), together with five known compounds, (+)-S-abscisic acid (2), fusicoccin J (3), 3α-hydroxyfusicoccin J (4), (R)-5-hydroxymethylmellein (5) and 4-hydroxyphenethyl acetate (6) was isolated from the fermentation extract of *Phomopsis amygdali*, an entomogenous fungus isolated from *Call midge*. Their structures were determined mainly by analysis of MS and NMR spectroscopic data. Compounds 1–6 were tested for antimicrobial activity against three plant pathogenic fungi: *Gibberella zeae*, *Verticillium albo-atrum*, and *Fusarium nivale*, and two bacteria: *Escherichia coli* and *Pseudomonas aeruginosa* 2033E. As a result, compounds 1–4 displayed antibacterial activity against Gram-negative *P. aeruginosa* 2033E, and the minimum inhibition concentration (MIC value) of 1–4 is 30 μg/mL, 58 μg/mL, 26 μg/mL, and 26 μg/mL, respectively.

**Keywords:** entomogenous fungus; *Phomopsis amygdali*; (+)-S-1-methyl-abscisic-6-acid; antimicrobial activity; *Pseudomonas aeruginosa* 2033

1. Introduction

Entomogenous fungi are associated with insects, other arthropods and even nonarthropod microinvertebrates mainly as pathogens or parasites (Humber 2009). Due to the capability of producing various secondary metabolites with the diverse structural features and interesting biological activities, entomogenous fungi have been increasingly investigated (Bandani & Butt 1999; Yang et al. 2006; Hu & Li 2007; Chen et al. 2008; Zhang et al. 2010). Some entomogenous fungal metabolites exhibited potent antimicrobial activity. For example, epicoccin A isolated from *Epicoccum nigrum* showed antibacterial activity against *Bacillus subtilis* (Zhang et al. 2007). Hirsutellide A isolated from *Hirsutella kobayasii* showed antymycobacterial activity.

*Corresponding author. Email: wangws@muc.edu.cn

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against *Mycobacterium tuberculosis* H37Ra with an MIC value of 6–12 µg/mL (Vongvanich et al. 2002). Cordycommunin isolated from *Ophiocordyceps communis* BCC 16475, showed antimycobacterial activity against *M. tuberculosis* H37Ra with an MIC value of 15 µM (Haritakun et al. 2010). Dustanin and 3β-acetoxy-15α, 22-dihydroxyhpane isolated from *Aschersonia tubulata* showed antimycobacterial activity with an MIC value of 12.5 µg/mL (Boonphong et al. 2001).

In order to screen new antibacterial secondary metabolites from entomogenous fungi, we initiated a chemical study of *Phomopsis amygdali*, which was isolated from Call midge. Bioassay-directed the fractionation of the EtOAc extract prepared from the solid-substrate fermentation products, and from which a new sesquiterpene (+)-S-1-methyl-abscisic-6-acid (1) and five known compounds, (+)-S-abscisic acid (2), fusicoccin J (3), 3α-hydroxyfusicoccin J (4), (R)-5-hydroxymethylmellein (5) and 4-hydroxyphenethyl acetate (6), were finally isolated. Details of the isolation, structure elucidation, and antimicrobial activity of compounds 1–6 are reported herein.

### 2. Results and discussion

(+)-S-1-Methyl-abscisic-6-acid (1) (Figure 1), colourless powder; [α]$_D^{25}$ + 273.0 ($c = 0.10$, MeOH): it was assigned a molecular formula of C$_{15}$H$_{20}$O$_4$ (six degrees of unsaturation) on the basis of high-resolution electrospray ionisation mass spectrometry, HR ESI-MS $m/z$: 265.1433 [M + H]$^{+}$ (calculated for C$_{15}$H$_{21}$O$_4$, 265.1440). The presence of hydroxyl groups at 3412 cm$^{-1}$ and carbonyl groups at 1658 cm$^{-1}$ was observed in IR spectrum. Its $^1$H NMR data revealed the presence of four methyl groups at $δ_H$: 1.03 (s, H-9'), 1.07 (s, H-8'), 1.89 (brs, H-7'), 2.31 (brs, H-1); a methylene at $δ_H$: 2.18 (d, $J = 16.8$ Hz, H-5'a) and 2.57 (d, $J = 16.8$ Hz, H-5'b); two singlet olefinic protons at $δ_H$: 5.83 (s, H-3') and 5.88 (s, H-2); and a couple of trans-double bond protons at $δ_H$: 6.42 (d, $J = 15.7$ Hz, H-5) and 6.58 (d, $J = 15.7$ Hz, H-4). In the low field of $^{13}$C NMR spectrum, an α, β-unsaturation ketone carbon at $δ_C$: 197.3 (C-4') and a carboxylic carbon at $δ_C$: 168.0 (C-6) were observed. Furthermore, there were six olefinic carbons accounting for three double bond groups, $δ_C$: 162.8 (C-2'), 152.4 (C-3), 137.2 (C-5), 134.4 (C-4), 127.4 (C-3') and 120.5 (C-2), as well as an oxygenated quaternary carbon at $δ_C$: 79.8 (C-1') in its $^{13}$C NMR spectrum. All the NMR data of compound 1 was structurally similar to abscisic acid (Jadulco et al. 2002; Zheng et al. 2002; del Refugio Ramos et al. 2004), except that the $^1$H NMR chemical shifts of H-3', H-4 and H-5, and the $^{13}$C NMR chemical shifts of C-2, C-3, C-4 and C-5 ($δ_C$: 120.5, 152.4, 134.4, 137.2 vs 118.6, 151.1, 128.6, 138.4) in 1 were different from those of abscisic acid. Actually, interpretation of the $^1$H–$^1$H COSY NMR data of 1 revealed the presence of two isolated spin-system, which were C-1–C-2 and C-4–C-5. In the HMBC spectrum of 1, correlations from H$_3$-1, H-2 to C-6, from H-4 to C-2, C-3, C-5 and C-1', from H-5 to C-3, C-4

![Figure 1. Chemical structures of compounds 1–6.](image-url)
and C-1’, indicating that the carboxylic carbon C-6 (δC: 168.0) was attached to C-3, but not attached to C-2 in abscisic acid. All these data suggested that compound 1 possess the same 2-methylenbut-3-enoic acid moiety as found in heliolactone (Ueno et al. 2014). They both have two trans-double bond protons, and a methyl group at C-6 was oxidised and methylated to a methoxycarbonyl group. Therefore, the planar structure of 1 was proposed.

The optical rotation of compound 1 ([α]D25 + 273.0, c = 0.10, MeOH) was positive, which is in agreement with that of (+)-S-abcisic acid ([α]D20 + 411.1, c = 1.0, EtOH) (Hampson et al. 1992). Thus, 1 was assigned as (+)-S-1-methyl-abcisic-6-acid (Figure 1).

Compounds 2–6 were identified as abscisic acid (2) (Jadulco et al. 2002; Zheng et al. 2002; del Refugio Ramos et al. 2004), fusicoccin J (3) (Tajima et al. 2004), 3α-hydroxyfusicoccin J (4) (Tajima et al. 2004), 5-hydroxymethylmellein (5) (Kokubun et al. 2003) and 4-hydroxyphenethyl acetate (6) (Elbandy et al. 2008), respectively, by comparison of their NMR and MS spectroscopic data with those in the literatures. In addition, the positive optical rotation of 2 ([α]D25 + 335.0, c = 0.10, MeOH) was in agreement with (+)-S-abcisic acid ([α]D20 + 411.1, c = 1.0, ethanol) (Hampson et al. 1992), displayed 2 was (+)-S-abcisic acid, and the negative optical rotation of 5 ([α]D28 − 95.0, c = 0.10, MeOH) was in agreement with (R)-7-hydroxymellein ([α]D25 − 127.5, c = 0.06, CHCl3) (Oliveira et al. 2011), displayed 5 was (R)-5-hydroxymethylmellein.

Compounds 1–6 were tested for antimicrobial activity against three plant pathogenic fungi: Gibberella zeae, Verticillium albo-atrum and Fusarium nivale, and two bacteria: Escherichia coli and Pseudomonas aeruginosa 2033E. The positive controls were carbendazim and gentamicin, respectively. Compounds 1–4 displayed antibacterial activity against Gram-negative P. aeruginosa 2033E, and the minimum inhibition concentration (MIC value) of 1–4 is 30 μg/mL, 58 μg/mL, 26 μg/mL and 26 μg/mL, respectively. However, compounds 1–6 did not display antimicrobial activity against the three plant pathogenic fungi and E. coli with MIC over the concentration of 120 μg/mL.

3. Experimental

3.1. General

Optical rotations were measured on an Anton Paar MCP 200 Analytical Automatic Polarimeter (Anton Paar GmbH, Austria). 1H, 13C NMR, and 2D NMR data were acquired with Bruker Avance III 500 (Bruker Corporation, Germany). ESIMS and HR ESIMS data were obtained using an Agilent HPLC–QTOF/MS 6520 System (Agilent Technologies Inc., USA) instrument equipped with electrospray ionisation source. HPLC separations were performed on an Agilent 1100 instrument (Agilent, Santa Clara, CA, USA) equipped with a variable wavelength UV detector. IR data were recorded using a Nicolet IS5 FT-IR spectrophotometer (Thermo Scientific, USA). Column chromatography (CC) was performed using ODS (Qingdao Marine Chemical Factory, Qingdao, China) and Sephadex LH-20 (Pharmacia Biotech Ltd., Uppsala, Sweden).

3.2. Fungal material

The culture of P. amygdali (No. RAXL Y-6) was isolated from a Call midge collected from Ai Sha forest farm, Guang Xi Region, People’s Republic of China, in August 2012. The isolate was identified by Wang Lin based on morphology. The isolate was identified by one of the authors (Yunfei Pei) based on morphology and sequence (Genbank Accession No. AF102998) analysis of the ITS region of the rDNA. The strain has been preserved at the State Key Laboratory of Mycology, Institute of Microbiology, Chinese Academy of Sciences, China. The fungal strain was cultured on slants of potato dextrose agar (PDA) at 25°C for five days. Agar plugs were cut into small pieces (about 0.5 × 0.5 × 0.5 cm3) under aseptic conditions, and 15 pieces were used to inoculate three
Erlenmeyer flasks (250 mL), each containing 50 mL of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract; the final pH of the media was adjusted to 6.5 and sterilised by autoclave). Five flasks of the inoculated media were incubated at 25°C on a rotary shaker at 200 rpm for five days to prepare the seed culture. Fermentation was carried out in 20 Fernbach flasks (500 mL), each containing 80 g of rice. Distilled H2O (120 mL) was added to each flask, and the contents were soaked overnight before autoclaving at 15 psi for 30 min. After cooling to room temperature, each flask was inoculated with 30 mL of the spore inoculum and incubated at 25°C for 40 days.

3.3. Extraction and isolation
The fermented material was extracted repeatedly with EtOAc, and the organic solvent was evaporated to dryness under vacuum to afford the crude extract (13 g), which was fractionated by ODS using MeOH–water (MeOH/water 5:95 → 100:0) gradient elution, getting a fraction of 1–20. The fraction 7 eluted with 35% MeOH was separated by Sephadex LH-20 CC eluting with MeOH. The resulting subfractions were purified by semipreparative RP HPLC (Agilent Zorbax SB-C18 column; 5 μm; 9.4 × 250 mm; 40% MeOH in H2O for 5 min; followed by 40–48% over 40 min; 2 mL/min) to afford compounds 1 (4.9 mg, tR = 23 min) and 2 (11.8 mg, tR = 25 min). The fraction 12 eluted with 60% MeOH was separated by Sephadex LH-20 CC eluting with CH2Cl2–MeOH (CH2Cl2/MeOH 1:1). The resulting subfractions were purified by semipreparative RP HPLC (Agilent Zorbax SB-C18 column; 5 μm; 9.4 × 250 mm; 36% acetonitrile in H2O for 5 min; followed by 36–43% over 50 min; 2 mL/min) to afford compounds 3 (6.9 mg, tR = 23 min) and 4 (4.2 mg, tR = 30 min). The fraction 5 eluted with 25% MeOH was separated by Sephadex LH-20 CC eluting with MeOH to afford compound 5 (5 mg). The fraction 6 eluted with 30% MeOH was separated by Sephadex LH-20 CC eluting with MeOH. The resulting subfractions were purified by semipreparative RP HPLC (Agilent Zorbax SB-C18 column; 5 μm; 9.4 × 250 mm; 35% MeOH in H2O isocratic elution) to afford compound 6 (17.6 mg, tR = 30 min).

3.4. (±)-S-1-Methyl-6-acid (1)
Colourless powder; [α]D25 + 273.0 (c = 0.10, MeOH); HR ESI-MS m/z: 265.1433 [M + H]⁺ (calculated for C15H21O4, 265.1440). 1H NMR (500 MHz, acetone-d6) δH: 6.58 (d, J = 15.7 Hz, H-4), 6.42 (d, J = 15.7 Hz, H-5), 5.88 (s, H-2), 5.83 (s, H-3α), 2.57 (d, J = 16.8 Hz, H-5β), 2.31 (brs, H-1), 2.18 (d, J = 16.8 Hz, H-5′a), 1.89 (brs, H-7′), 1.07 (s, H-8′), 1.03 (s, H-9′). 13C NMR (125 MHz, acetone-d6) δC: 197.3 (C-4′), 168.0 (C-6), 162.8 (C-2′), 152.4 (C-3), 137.2 (C-5), 134.4 (C-4), 127.4 (C-3′), 120.5 (C-2), 79.8 (C-1′), 50.3 (C-5′), 42.2 (C-6′), 24.7 (C-9′), 23.5 (C-8′), 19.1 (C-7′), 14.0 (C-1).

3.5. Antimicrobial activity assay
The antimicrobial activity assays were carried out in the 96-well sterilised microplates using a micro-dilution method (Zhou et al. 2014). All experiments were repeated three times. MICs were determined as the lowest concentrations that produce complete growth inhibition of the tested microorganisms.

Supplementary material
Supplemental data for this article can be accessed at http://dx.doi.org/10.1080/14786419.2015.1055742

Disclosure statement
No potential conflict of interest was reported by the authors.
Funding
This work was supported by the National Natural Science Foundation of China [grant number 31200260]; the First-class University and the First-rate Discipline Construction Projects of Minzu University of China [grant number YLD01013]; the Fundamental Research Funds for the Central Universities [grant number Z2014044] together with 111 Project [grant number B08044]; and the National College Students’ Innovation and Entrepreneurship Training Program [grant number GCCX2014110026].

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