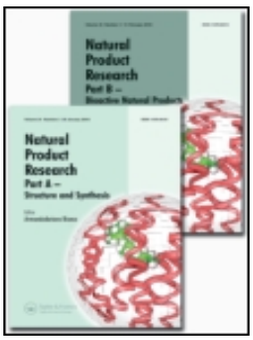

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A new phenol compound from endophytic *Phomopsis* sp. DC01

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The strain DC01 was isolated from the branch tissue of *Daphniphyllum longeracemosum* and determined to be a member of *Phomopsis* according to the ITS sequence analysis. The extracts from the PDA solid fermentation media of *Phomopsis* sp. DC01 were purified and three compounds including one new phenol compound were obtained. The new compound was identified to be (*E*)-7-(2-hydroxy-4-(hydroxymethyl)phenyl)-2-methyloct-6-enoic acid (1) based on 1-D NMR, 2-D NMR and HR–ESI data.

Keywords: *Phomopsis*; ITS sequence; endophytic; phenol

1. Introduction

Plant endophytes are a group of microorganisms, including fungi and bacteria, which not only live within plant internal tissues or organs without causing any apparent symptoms or diseases in the host plants, but also serve as important sources of bioactive compounds, presumably due to the symbiotic relationship with their hosts (Tan & Zou, 2001). In the course of our study of the chemical constituents from the endophytic microorganisms of plants, a series of new compounds were previously isolated (Li, Yang, Zeng, Zou, & Zhao, 2010; Zhao, Fan, Li, Zhu, & Shen, 2005; Zhao, Li, & Shen, 2006). In this article, we investigate the secondary metabolites from *Phomopsis* sp. DC01 – an endophytic fungal strain isolated from the surface-sterilised branch of *Daphniphyllum longeracemosum* Rosenth., which is an evergreen tree mainly distributed in Yunnan Province, China (Zhen & Min, 1980), and a series of novel daphniphyllines have been isolated from *D. longeracemosum* (Di et al., 2006; Li, He, Di, Tian, & Hao, 2006). Herein, we describe the isolation and identification of the strain DC01, and the isolation and structural elucidation of three compounds (**1**–**3**) (Figure 1) including one new phenol compound.

2. Results and discussion

2.1. *Identification of strain DC01*

The nucleotide sequences for the ITS1-5.8S rRNA-ITS4 region of the endophytic fungi DC01 was registered in the GenBank database with the accession number JF705873, and the strain was determined to be *Phomopsis* sp. according to the Internal Transcribed Spacer (ITS) analysis.

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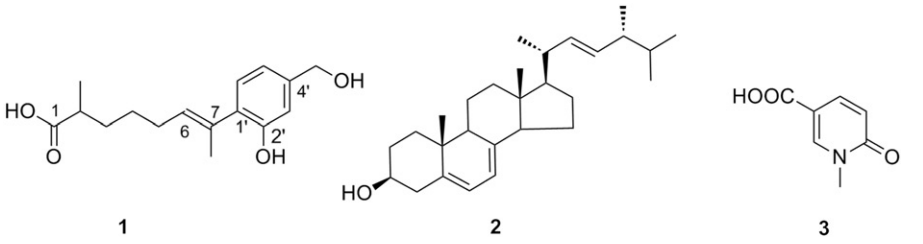

1 2 3

Figure 1. The structures of compounds **1**–**3**.

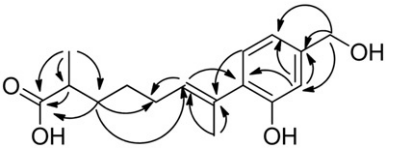

Figure 2. Key HMBC correlations of compound **1**.

2.2. *Structure determination of compounds 1–3*

Compound **1** was obtained as a colourless powder. The HR–ESI–MS data indicated a molecular formula of $C_{16}H_{22}O_4$ based on the $[M+Na]^+$ pseudo-molecular ion signal at m/z 301.1419 (calcd 301.1415), with 6° of unsaturation. The ^{1}H-NMR spectrum of **1** revealed the presence of the following fragments: one tertiary methyl at δ_H 1.96 (3H, s), one secondary methyl at δ_H 1.23 (3H, d, $J = 7.0$ Hz); and a tri-substituted double bond function at δ_H 5.51(1H, t, $J = 7.1$ Hz) and three substituent benzene rings at δ_H 6.88 (1H, s), δ_H 6.85 (1H, d, $J = 7.7$ Hz) and δ_H 7.04 (1H, d, $J = 7.7$ Hz).

Structure of **1** was established by detailed HMQC and HMBC experiments. The HMBC data showed correlations (Figure 2) between H-2 (δ_H 2.54) and the carbon at δ_C 181.7 (C-1), between H-3 (δ_H 1.64 and 1.85) and the carbons at δ_C 181.7 (C-1), 39.0 (C-2), 26.1 (C-5), 130.3 (C-6) and 17.1 (2-CH_3), between H-5 (δ_H 2.29) and the carbons at δ_C 39.0 (C-2), 33.0 (C-3), 130.3 (C-6) and 133.1 (C-7), between H-6 (δ_H 5.51) and the carbons at δ_C 17.8 (7-CH_3) and 26.1 (C-5), which suggested the 2-methyloct-6-enoic acid part unit. And this substituent connected with benzene ring on the basis of HMBC correlations from H-6 (δ_H 5.51) to C-1′ (δ_C 130.5), and the hydroxymethyl group was placed at C-4′ (δ_C 141.0) on the basis of correlations from 4′-CH_2 (δ_H 4.60) to C-3′ (δ_C 114.1), C-4′ (δ_C 141.0) and C-5′ (δ_C 118.7). And according to the ROESY experiments, the 6(7) double bound was determinated to be *E*-configuration for no NOE correlation between the 7-CH3 and H-6. So, the compound **1** was determined to be (*E*)-7-(2-hydroxy-4-(hydroxymethyl)phenyl)-2-methyloct-6-enoic acid.

Compounds **2**–**3** were determined as ergosterol (**2**) (Zhou & Nes, 2000) and 1,6-dihydro-1-methyl-6-oxo-3-pyridinecarboxylic acid (**3**) (Buurman & van der Plas, 1986) on the basis of their NMR data and comparison with the data given in references.

3. Experimental

3.1. *Spectroscopic measurements and chromatography*

NMR 1-D/2-D experiments were carried out on Bruker AM-400 and Bruker DRX-500 spectrometers. Mass spectra were recorded on a Finnigan LCQ-Advantage and VG

Auto-Spec-3000 mass spectrometer. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. UV spectra were measured on Shimadzu 2401PC spectrophotometer, λ_{max} log (ε) in nm. IR spectra were measured on a Paragon 1000 PC spectrometer. Column chromatography was carried out on silica gel (200–300 mesh; Qingdao Marine Chemical Factory, Qingdao, PR China.) and Sephadex LH-20 (Pharmacia). Thin-layer chromatography was performed on silica gel (Si gel G; Qingdao Marine Chemical Factory, Qingdao, China). A Waters series HPLC 996 (Waters Corporation) was used, and the samples were purified by an XterraTM RP-C18 7 μm 7.8 × 300 mm^2 column (Waters Corporation).

3.2. *Isolation of endophytic fungus*

The branch tissue of *D. longeracemosum* was collected at Kunming Botanical Garden, Kunming Institute of Botany, The Chinese Academy of Sciences, Yunnan, China, in August 2009. A voucher specimen (no. KUN0404996) was identified by Prof. Xu Gong and deposited at the Herbarium of the Kunming Institute of Botany, Chinese Academy of Sciences. The plant materials were washed by running tap water and successively sterilised with 75% ethanol for 1 min and 0.1% corrosive sublimate for 5 min, then rinsed in sterile water for five times and cut into small pieces. These small pieces were incubated at 26°C on PDA media (potato 200 g, dextrose 20 g, agar 15 g, distilled water 1000 mL) and cultured until colony or mycelium appeared surrounding the segments. After culturing about 2 weeks, a strain named DC01 appeared and was isolated from the sterilised branch, which was then was deposited in Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, China.

3.3. *Identification of DC01 by amplification of the 5.8S rRNA gene*

The total DNA of DC01 was extracted by wrapper methods (Sambrook & Russell, 2001). PCR was performed in a total volume of 50 μL using Primers ITS1 (5′> TCC GTA GGT GAA CCT GCG G <3′) and ITS4 (5′> TCC TCC GCT TAT TGA TAT GC <3′). Amplification reaction mixture contains 100 ng DNA template, 0.5 μM primers, 0.2 mM dNTP, 1 × Ex-Taq buffer (Takara) and 1.25 U of Ex-Taq (Takara). The reaction mixture was incubated in a thermal cycler (Eppendorf) as follows: 10 min of predenaturation; then, 25 cycles of 1 min of denaturation at 94 C, annealing at 54 C for 1 min and elongation for 1.5 min; 10 min of additional extension at 72 C. The 600 pb PCR products were recovered by gel purification using UNIQ-10 column DNA gel extraction kit (Shanghai Sangon Biotechnology Co., Ltd) and ligated into pUCm T vector (Sangon). The competent *Escherichia coli* JM109 was prepared and plasmids were transformed into it by standard method (Sambrook & Russell, 2001). Three randomly picked clones were sequenced by ABI 3700 sequencer for insert fragment. The 5.8S *rRNA* partial sequence was assembled using Vector NTI software and blasted against the latest GenBank database using BLASTn.

3.4. *Extraction and isolation*

The strain was cultured on PDA solid medium for 10 L. After being cultivated for 3 weeks, the cultures were exhaustively extracted five times by EtOAc–MeOH–AcOH (80 : 15 : 5, v/v/v) to obtain extracts. The extracts (17 g) were chromatographed on silica gel column (silica gel G, 80 g) and eluted with petroleum ether/acetone (10 : 1 to 7 : 3) and then chloroform/methanol (20 : 1 to 1 : 1) to afford six fractions (Fr-1 to Fr-6). Fr-1 (1.2 g) was subjected on silica gel column (silica gel G, 40 g) and eluted with petroleum ether/chloroform (20 : 1 to 7 : 3) to give two fractions (Fr-1-1 and Fr-1-2). Fr-1-2 (21 mg) was subjected on Sephadex LH-20 eluting with chloroform/methanol (1 : 1) and then was

purified on HPLC with isocratic elution (5% methanol, 0.3% (v/v) formic acid) at a flowrate of 2 mL min^{-1} to obtain compound **2** (6.7 mg). Fr-2 (1.7 g) was subjected on silica gel column (silica gel G, 60 g) and eluted with petroleum ether/ethyl acetate (20 : 1 to 3 : 2) to afford three fractions (Fr-2-1 to Fr-2-3). Fr-2-3 (121 mg) was subjected on Sephadex LH-20 eluting with chloroform/methanol (1 : 1) and then was purified on HPLC with isocratic elution (98% methanol, 0.3% (v/v) formic acid) at a flowrate of 2 mL min^{-1} to obtain compound **3** (11 mg). Fr-3 (2.1 g) was chromatographed on silica gel column (silica gel G, 80 g) and eluted with chloroform/methanol (20 : 1 to 7 : 3) to produce three fractions (Fr-3-1 to Fr-3-3). Fr-3-2 (272 mg) was subjected on silica gel column (silica gel G, 15 g) eluting with chloroform/methanol (20 : 1 to 8 : 2) and was further purified on HPLC with isocratic elution (90% methanol, 0.3% (v/v) formic acid) at a flowrate of 2 mL min^{-1} to afford compound **1** (7 mg).

Compound **1**: Colourless powder. $[\alpha]_D^{23} = -5.79$ ($c = 0.16$, acetone). UV ($CHCl_3$) λ_{max} (log ε): 194.6 (5.16), 218.6 (5.28), 259.4 (5.27), 292 (4.92) and 327.4 (4.85). IR (KBr) ν_{max}: 3422, 2923, 1705 (s), 1640, 1620, 1463, 1421 and 1288 cm^{-1}. H-NMR (500 MHz, $CDCl_3$) δ: 2.54 (1H, m, H-2), 1.85 (1H, m, H-3A), 1.64 (1H, m, H-3A), 1.25 (2H, m, H-4), 2.29 (2H, m, H-5), 5.51 (1H, t, $J = 7.1$ Hz, H-6), 6.88 (1H, s, H-3′), 6.85 (1H, d, $J = 7.7$ Hz, H-5′), 7.04 (1H, d, $J = 7.7$ Hz, H-6′), 4.60 (2H, s, 4′-CH_2) and 1.23 (3H, d, $J = 7.0$ Hz, 2-CH_3), 1.96 (3H, s, 7-CH_3). ^{13}C-NMR (125 MHz, $CDCl_3$) δ: 181.7 (C-1, s), 39.0 (C-2, d), 33.0 (C-3, t), 29.7 (C-4, t), 26.1 (CH_2-5, t), 130.3 (CH-6, d), 133.1 (C-7, s), 130.5 (C-1′, s), 152.2 (C-2′, s), 114.1 (CH-3′, d), 141.0 (C-4′, s), 118.7 (CH-5′, d), 128.6 (CH-6, d), 64.9 (4′-CH_2, t), 17.1 (2-CH_3, q) and 17.8 (7-CH_3, q). ESI–MS: 301 ($[M + Na]^+$) and 579 ($[2M + Na]^+$). HR–ESI–MS: 301.1419 (($[M + Na]^+$), calcd 301.1415).

Compound **2**: Colourless powder. ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 0.62 (3H, s), 0.83 (3H, s), 0.81 (3H, t, $J = 6.4$ Hz), 0.92 (3H, d, $J = 6.8$ Hz), 0.94 (3H, s), 1.04 (3H, d, $J = 6.6$ Hz), 3.67 (1H, m), 5.25 (2H, m), 5.38 (1H, brm), and 5.57 (1H, brm). ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 141.4 (C), 139.8 (C), 135.6 (CH), 132.0 (CH), 119.6 (CH), 116.3 (CH), 70.5 (CH), 55.8 (CH), 54.6 (CH), 46.3 (CH), 42.8 (CH), 40.8 (CH_2), 40.4 (CH), 39.1 (CH_2), 38.4 (CH_2), 37.1 (C), 33.1 (C), 32.0 (CH_2), 28.3 (CH_2), 23.0 (CH_2), 21.1 (CH_3), 19.9 (CH_3), 19.6 (CH_3), 17.6 (CH_3), 16.3 (CH_3) and 12.1 (CH_3). EI–MS: 396 $[M]^+$.

Compound **3**: Colourless powder. ^{1}H-NMR (400 MHz, CD_3OD) δ: 3.60 (3H, s), 6.53 (1H, d, $J = 9.4$ Hz), 7.98 (1H, d, $J = 9.4$ Hz) and 8.42 (1H, s). ^{13}C-NMR (100 MHz, CD_3OD) δ: 38.6 (CH_3), 113.2 (C), 119.2 (CH), 141.3 (CH), 145.5 (CH), 165.4 (C) and 167.7 (C). ESI–MS: 154 ($[M + H]^+$).

4. Conclusions

In the course of searching new compounds from the endophytic microorganisms of plants, some endophytic fungal strains were isolated from the surface-sterilised branch of *D. longeracemosum*, which produce a series of new skeleton daphniphyllines (Di et al., 2006; Li et al., 2006). One new phenol and two known compounds were isolated from an endophytic *Phomopsis* sp. DC01. The new compound was determined to be (*E*)-7-(2-hydroxy-4-(hydroxymethyl)phenyl)-2-methyloct-6-enoic acid (**1**) by spectroscopic methods, including HR–ESI–MS and 2-D NMR experiments.

Supplementary material

The 1-D NMR and 2-D NMR, UV, IR and mass spectra of compound **1** are supplied in the supplementary data. This material is available online.

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