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Phytotoxic and Antimicrobial Metabolites from *Paraphaeosphaeria* sp. QTYC11 Isolated from the Gut of *Pantala flavescens* Larvae

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Abstract: A new lunatoic acid C (**1**) along with eight known compounds were isolated from *Paraphaeosphaeria* sp. QTYC11 residing in the gut of *Pantala flavescens* larvae. They were determined on the basis of extensive spectroscopic analysis and by comparison of the corresponding data reported previously. Compounds **1** showed good phytotoxic activities, extremely potent antifungal activities and good antibacterial activities respectively.

Keywords: *Paraphaeosphaeria* sp.; natural products; antifungal activity; antibacterial activity; phytotoxic activity.

1. Introduction

Microorganisms keep interaction between different insect species help shape the biological complexity and diversity of the planet. The class insects have 900 000 known and 2-30 million predicted species that occupied a diverse range of ecological niches, often facilitated by their endosymbionts [1]. Such genetically tractable microorganisms undergo coevolution with the insect by producing small molecules, supporting nutrient or protection [2]. Irrespective of their role in the complicated systems, microbes associated with the insect can be regarded as a huge reservoir for pharmaceutically agents [3,4]. In our previous study, two microorganisms *Daldinia eschscholzii* and *Pestalotiopsis* sp. HC02 have been isolated from mantis (*Tenodera aridifolia*) and grasshopper (*Chondracris rosee*), respectively. Further chemical isolation revealed several new metabolites, which had potential to be used as immunosuppressants and herbicides [5,6]. With the interest to search for new bioactive metabolites from the insect gut microbiota, we isolated one endosymbiont and identified

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it as *Paraphaeosphaeria* sp. QTYC11, which was isolated from the gut of *Pantala flavescens* larvae. The ethyl acetate extract from the culture filtrate of the QTYC11 exhibited good phytotoxic activity against the radical growth of *Echinochloa crusgalli* and *Amaranthus retroflexus*, and antifungal activities to several plant pathogens as well as antibacterial activities against a panel of food spoiling microorganisms. Subsequently, one lunatoic acid C (**1**) together with 8 known biosynthetic relatives was isolated and identified from the ethyl acetate crude extract. Herein we reported the details of the isolation, structure elucidation, and biological activities of all the tested metabolites are presented.

2. Materials and Methods

2.1. Microorganism Material

According to the methods detailed elsewhere [7], the strain QTYC11 was isolated from the gut of *P. flavescens* larvae collected from the river near by the Zhejiang Normal University (29°00'17.37"N, 119°29'54.84"E), PR China, in August 2012. The title fungus QTYC11 was identified according to the morphological characteristics and 5.8S rDNA sequence to those of standard record. Preliminary identification of QTYC11 sequences were compared with those available in GenBank by using BLAST to determine its phylogenetic affiliation. Phylogenetic analysis was performed using the MEGA v5.0 after multiple alignments of data by CLUSTAL-X software [8]. Phylogenetic tree based on the neighbor-joining analysis was constructed by the model of (NJ) Kimura-2-parameter and gamma distribution [9]. Bootstrap analysis was used to evaluate the tree topology of the neighbor-joining data by performing 1,000 replicates. The phylogenetic tree (Fig. **1**) inferred from the ribosomal DNA ITS sequences indicated that the QTYC11 closely related to *Paraphaeosphaeria* sp. DQ092509 with the ITS sequence similarity of 99.7%. Therefore, QTYC11 was identified as *Paraphaeosphaeria* sp. The fungal strain QTYC11 was deposited in the China Center for Type Culture Collection as CCTCC M 2013435 and the GenBank accession number was KJ469553.

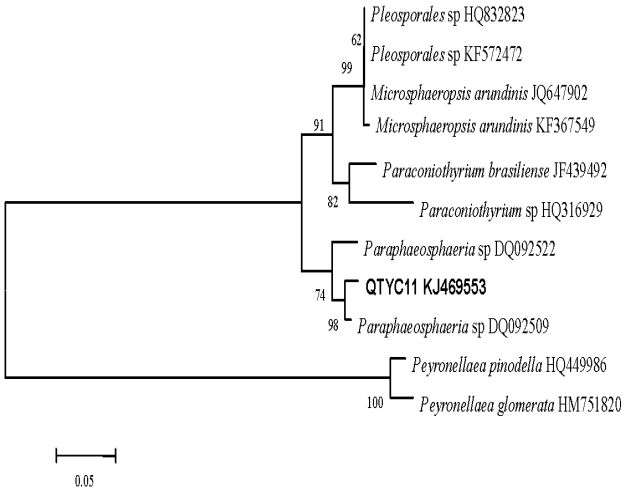

Figure 1. Phylogenetic tree of the QTYC11 based on 5.8S and ITS regions sequences

2.2. Fermentation and Isolation

The fresh mycelium grown on Malt Extracts Agar medium at 28°C for 4 d was inoculated into 250 mL Erlenmeyer flask containing 100 mL of ME medium (20 g of malt extract, 20 g of sucrose, 1 g of peptone, 1 L of distilled H_2O). After 3 d of incubation at 28°C on rotary shakers at 180

rpm, 15 mL of culture liquid were transferred as seed into each 1000 mL Erlenmeyer flask containing 350 mL of ME medium, and cultivation was carried out at 28°C for 7 d.

The filtrate of the cultural broth (50 L) was extracted with ethyl acetate (6 × 50 L). Evaporation of menstruum *in vacuo* gave a yellow oily residue (32.6 g), which was subjected to column chromatography over silica-gel (SiO_2; 200-300 mesh; Qingdao Marine Chemical Ltd, Qingdao, China) eluting with CH_2Cl_2/MeOH mixtures of a stably growing polarity (100:0, 100:1, 100:2, 100:4, 100:8 v/v) to afford five parts (F1,3.2 g; F2, 4.6 g; F3, 8.9 g; F4, 2.3 g; F5, 2.3 g). Compound **5** (1530 mg) was crystallized from the MeOH solution of subfraction F1. The fraction of F2 was purified by Sephadex LH-20 using a CH_2Cl_2/MeOH mixture (1:1) as the eluent to give the compound **9** (9 mg). Fraction (F3) was further fractionated over silica gel using CH_2Cl_2/MeOH (100:2) to give five subfractions (R1-R5), compound **1** (1356 mg), compound **4** (10.6 mg) and compound **6** (30.3 mg) were crystallized from the MeOH solution of subfractionR1, R3, R4 respectively. R2 was further chromatographed over silica gel (CH_2Cl_2/MeOH, 100:0-100:2) to give compound **7** (15 mg), **8** (6 mg). F4 (CH_2Cl_2/MeOH, 100:8) was repeatedly purified on Sephadex LH-20 (MeOH) and detected by TLC to yield compound **3** (56 mg). The remaining F5 was purified by Sephadex LH-20 to give the compound **2** (13 mg).

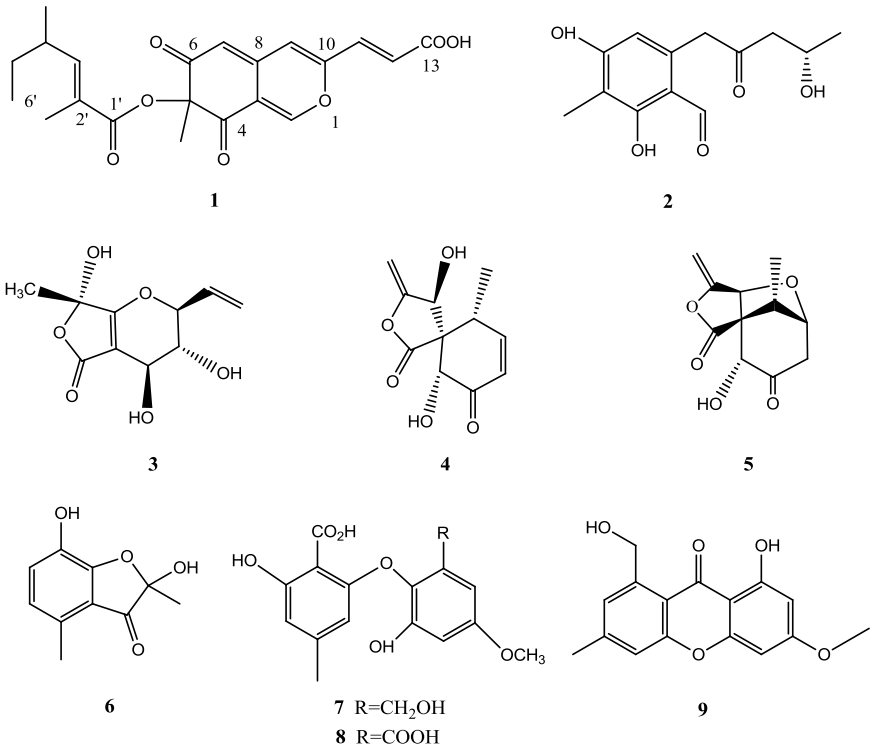

Figure 2. Structures of Compounds **1-9**

3. Results and Discussion

3.1. Structure elucidation

Compound **1** was obtained as orange powder and its molecular formula $C_{21}H_{22}O_7$ was deduced from HR-ESI-MS (409.1259 $[M+Na]^+$, calculated for, 409.1258) and ^{13}C NMR spectra. The ^1H NMR (Table 1) of **1** indicated the presence of four methyls (δ_H 0.88, 1.02, 1.60 and 1.87), one methylene (δ_H 1.40), one methine (δ_H 2.44), six olefinic methines (δ_H 5.78, 6.54, 6.55, 6.73, 7.23 and 7.94). The ^{13}C NMR spectra (Table 1) of **1** showed 21 carbon signals in conjunction with DEPT spectrum indicated

the presence of four methyls, one methylenes, six olefinic methines, four quaternary olefinic carbons, one oxygenated quaternary carbon, one ester carbonyl and two enone carbonyls. The bicyclic bis-enone structure was established from the two and three-bond HMBC correlations of H-2 to C-3 (δ_C 114.9), C-4 (δ_C 193.4), C-8 (δ_C 140.8), and C-10 (δ_C 152.5); H-7 to C-3 (δ_C 114.9), C-5 (δ_C 84.0); H-9 to C-3, C-7 (δ_C 110.8), C-10, and C-11 (δ_C 135.2); H-11 to C-9, C-10, and C-13 (δ_C 169.5); H-12 to C-10 and C-13; and H_3-14 (δ_H 1.60) to C-5 and both enone carbonyls C-4 and C-6 (δ_C 192.9). The acyl moiety of **1** was clarified as follows: a triplet methyl at δ_H 0.88(J=7.4 Hz), one doublet methyls at δ_H 1.02 (J=6.6 Hz) , 1.19 (J=7 Hz), one single methyls δ_H 1.87 and the ^{13}C NMR spectra indicated the presence of two olefinic carbons, one ester carbonyl and HMBC correlations of H-3' to C-1' (δ_C 167.7) and C-2' (δ_C 124.9); H-4' to C-2', C-3' (δ_C 151.0), C-6' (δ_C 11.9) and C-8' (δ_C 19.4); H-5' to C-4' (δ_C 35.1); H-6' to C-4' and C-5' (δ_C 29.5); H-7' to C-1', C-2', C-3'; H-8' to C-3' thus establishing the structure as a derivative of lunatoic acid A [10].

The known compounds isolated from the *Paraphaeosphaeria* sp. QTYC11 were identified as 2,4-Dihydroxy-6-((R)-4-hydroxy-2-oxopentyl)-3- methylbenzaldehyde (**2**)[11], massarilactone D (**3**)[12] 6 - epi - 5' - hydroxymycosporulon (**4**) [13], massarilactone E (**5**) [14] enalin A (**6**) [15], barceloneic acid A (**7**), barceloneic acid B (**8**) [16] and 1-Hydroxy-8-(hydroxymethyl)-3-methoxy-6-methyl-9H-xanthen-9-one (**9**) [17] by comparison of their spectroscopic data with literature values.

Table 1. ^{1}H NMR, ^{13}C NMR and HMBC data for compound **1**
(at 600 MHz in acetone-d_6, δ in ppm, J in Hz).

Position	δ_C	δ_H, mult, J in Hz	HMBC
2	153.4	7.94 (1H, *s*)	C-3, 4, 8, 10
3	114.9		
4	193.4		
5	84.0		
6	192.9		
7	110.8	5.78 (1H, *s*)	C-3, 5
8	140.8		
9	116.6	6.55 (1H, *s*)	C-3, 7, 10, 11
10	152.5		
11	135.2	7.23 (1H, *d*, *J* =15.6)	C-9, 10, 13
12	123.6	6.54 (1H, *d*, *J* =15.6)	C-10, 13
13	169.5		
14	21.8	1.60 (3H, *s*)	C-4, 5, 6
1'	167.7		
2'	124.9		
3'	151.0	6.73(1H dd, *J* =10.1, *J* =1.2)	C-1', 2'
4'	35.1	2.44 (1H, *m*)	C-2', 3', 6', 8'
5'	29.5	1.40 (2H, *m*)	C-4', 5'
6'	11.9	0.88 (3H, *t*, *J* =7.3)	C-4', 5'
7'	12.4	1.87 (3H, s	C-1', 2', 3'
8'	19.4	1.02 (3H, d, *J* =6.6)	C-3'

3.2. Phytotoxic and Antimicrobial activity

The phytogrowth inhibitory activity was evaluated on seedling of *A. retroflexus* and *E. crusgalli* using a *Petri*-dish bioassay, which was performed as described [18]. Antimicrobial assays were performed as described [19-21].

Compounds **1** and **6** showed good activities against the radical growth of *A. retroflexus* with the IC_{50} values of 10.2 and 27.6 μg/mL respectively. The metabolites **1** and **4** had good phytotoxic activities against the radical growth of *E. crusgalli* with IC_{50} values of 5.9 and 16.0 μg/mL respectively, and compound **1** showed moderate post-emergence activity for *E. crusgalli* with 46.0% decrease in dry wight. The ingredient **1** showed extremely potent antifungal activities against the *Valsa mali* (IC_{50} = 3.3 μg/mL), *Gibberella sanbinetti* (IC_{50} = 1.7 μg/mL) and *Fusarium oxysporum* f. sp. *cucumerinum* (IC_{50} = 4.2 μg/mL), which were comparable to those of referenced cycloheximide with IC_{50} values of 0.3, 3.3 and 4.9 μg/mL, respectively. The compound **1** also showed good antibacterial activities against *Staphylococcus aureus* and *Bacillus subtilis* with the inhibition zone of

17.1 and 28.3 mm, respectively. Based on this result, a patent had been applied in China (number of the patent application 201310729506.1).

Interestingly, lunatoic acid A was first reported with strong antifungal activity but bacterial growth was not affected at all [22]. Compound **1** as a derivative of lunatoic acid A was different from lunatoic acid A by an extra double bond on the fatty acid moiety of the molecule. However, lunatoic acid C exhibited moderate antibacterial activities against *S. aureus* and *B. subtilis*. It was obvious that the extra double bond on the fatty acid moiety of the molecule contribute to the antibacterial activities of family lunatoic acid. Whether the incorporation of an extra double bond on the fatty acid moiety into natural pharmacophore could improve antibacterial activity of medicines needed deep research.

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Supporting Information

Supporting Information accompanies this paper on http://www.acgpubs.org/RNP

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