The Journal of Antibiotics (2011) 64, 723–727
© 2011 Japan Antibiotics Research Association All rights reserved 0021-8820/11 $32.00
www.nature.com/ja

ORIGINAL ARTICLE

Phthalide derivatives with antifungal activities against the plant pathogens isolated from the liquid culture of *Pestalotiopsis photiniae*

Xiao-Long Yang[1,2], Su Zhang[2], Qiong-Bo Hu[3], Du-Qiang Luo[1] and Yan Zhang[2]

Three new phthalide derivatives (1-3) named 5-(3′-methyl-2′-butenyl)-2-hydroxy-3-methoxy-4-methylbenzoic acid (1), 5-(3′-carboxyl-3′-methyl-2*E*-allyloxy)-3-methoxy-4-methylphthalide (2) and 5-(3′,3′-dimethylallyloxy)-2-methoxycarbonyl-3-methoxy-4-methylbenzoic acid (3) together with six known phthalide derivatives named 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide (4), zinnimidine (5), 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide (6), 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalic acid (7), zinniol anhydride (8) and porriolide (9) were isolated from the liquid culture of the plant endophytic fungus *Pestalotiopsis photiniae* isolated from the Chinese Podocarpaceae plant *Podocarpus macrophyllus*. Their structures were elucidated by extensive spectroscopic analysis. Compounds 1–9 displayed significant antifungal activities against three plant pathogens.
The Journal of Antibiotics (2011) **64,** 723–727; doi:10.1038/ja.2011.82; published online 14 September 2011

Keywords: antifungal activity; *Pestalotiopsis photiniae*; *Podocarpus macrophyllus*; phthalide derivatives; plant pathogen

INTRODUCTION

Fungi of the genus *Pestalotiopsis* (Amphisphaeriaceae) are known as endophytes of tropical higher plants, which are common in their distribution, and many are saprobes, while others are either pathogenic or endophytic to living plants.[1] Since the discovery of the anticancer agent taxol from an endophytic fungal strain of the genus *Pestalotiopsis*,[2–3] interest in searching for bioactive compounds from this fungal genus has increased considerably. Up to date, about 300 species of the genus *Pestalotiopsis* have been recorded in China, but only about 10% of these species referred to chemical investigation led to the isolation of a variety of bioactive metabolites.[4–9]

Our previous investigation of *Pestalotiopsis photiniae* grown in the liquid culture has led to the isolation of several compounds, such as dihydroberkleasmin A,[10] 10-norparvulenone[11] and zinniol-related phytotoxins.[12] Further to search for the minor active components, the fungus *P. photiniae* was refermented in a larger scale using the liquid fermentation culture. Its ethyl acetate extract displayed significant antifungal activities against three plant pathogens, *Fusarium graminearum*, *Botrytis cinerea* and *Phytophthora nicotianae*. Bioassay-directed fractionation of this extract has led to the isolation of nine phthalide derivatives (**1**-**9**) including three new ones (**1**-**3**). Compounds (**1**-**9**) displayed significant antifungal activities against the above three plant pathogens (Figure 1). Details of the isolation, structure elucidation and antifungal activity of these compounds are reported herein.

MATERIALS AND METHODS

General experimental procedures

Optical rotations: Perkin-Elmer 341 spectropolarimeter (Perkin-Elmer, Boston, MA, USA). IR spectra: Perkin-Elmer 577 spectrometer; KBr pellets; in cm^{-1}. NMR spectra: Bruker AM-600 spectrometer (Bruker, Bremen, Germany); δ in p.p.m., *J* in Hz; Me_4Si as internal standard. FT-MS spectra: Bruker apex-ultra 7.0T spectrometer in *m/z*. Column chromatography (CC): silica gel (200 ~ 300 mesh, Yantai Zhi Fu chemical, People's Republic of China), TLC: silica gel GF_{254} plates (Yantai Zhi Fu chemical, People's Republic of China) and Sephadex LH-20 gel (25 ~ 100 μm, GE Healthcare, Uppsala, Sweden).

Fungal material and cultivation conditions

Pestalotiopsis photiniae was isolated from the branch of *Podocarpus macrophyllus* in Hainan, People's Republic of China, in April 2008, and identified by Professor Jing-Ze Zhang, Institute of Biotechnology, Zhejiang University, which was assigned the accession number L328 in the culture collection at College of Life Science, Hebei University. The fungal strain was cultured on slants of potato dextrose agar (modified) at 28 °C for 7 days, and then inoculated into 500 ml Erlenmeyer flask containing 100 ml of modified potato dextrose agar medium (glucose 20 g, potato (peeled) 200 g, KH_2PO_4 3 g, $MgSO_4$ 1.5 g, citric acid 0.1 g and thiamin hydrochloride 10 mg in 1.0 l deionized H_2O). The final pH of the media was adjusted to 6.5 before sterilization. After 7 days of incubation at 28 °C on rotary shakers at 150 rpm, 25 ml of culture liquid were transferred as seed into each 1000 ml Erlenmeyer flask containing 250 ml of modified potato dextrose agar medium, and fermentation was carried out on a shaker for 30 days.

[1]Key Laboratory of Pharmaceutical Chemistry and Molecular Diagnosis of Ministry of Education and College of Life Science, Hebei University, Baoding, PR China; [2]College of Pharmaceutical Science, Hebei University, Baoding, PR China and [3]College of Natural Resources and Environment, South China Agricultural University, Guangzhou, PR China
Correspondence: Dr D-Q Luo, Key Laboratory of Pharmaceutical Chemistry and Molecular Diagnosis of Ministry of Education and College of Life Science, Hebei University, Baoding 071002, PR China
E-mail: Duqiangluo@163.com
or Dr Q-B Hu, College of Natural Resources and Environment, South China Agricultural University, 483#, Wushan Road, Tianhe, Guangzhou 510642, PR China.
E-mail: hqbscau@126.com
Received 5 April 2011; revised 21 July 2011; accepted 12 August 2011; published online 14 September 2011

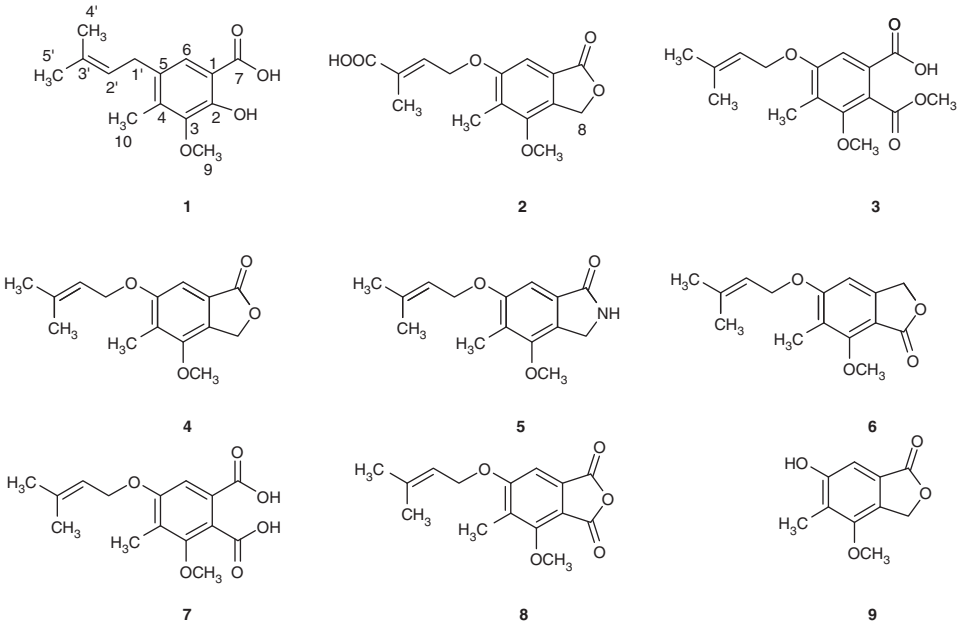


Figure 1 The structures of compounds **1**-**9**.

Extraction and isolation

The culture broth (60 l) was extracted three times with ethyl acetate (with each soaking for 2 days), and the organic layer was concentrated *in vacuo* to yield a brown oily residue (18.0 g). This residue was subjected to a silica gel CC with a gradient elution of petroleum ether/acetone (100:0, 98:2, 95:5, 90:10, 80:20 and 50:50 (v/v)) to obtain six fractions (Fr.) 1–6. Fr. 4 (2.3 g) eluted with petroleum ether/acetone (90:10) was repeatedly purified by CC (silica gel; petroleum ether/acetone 50:1 (v/v)) and Sephadex LH-20 (chloroform/methanol, 1:1) to afford compound **4** (10 mg). Fr. 5 (3.0 g) eluted with petroleum ether/acetone (80:20) was further fractionated by silica gel CC using petroleum ether/acetone gradient elution (from 20:1 to 1:1, v/v) to obtain six Fr., 5.1–5.6. The Fr. 5–2 (210 mg) eluted with petroleum ether/acetone (10:1, v/v) was further purified by repeated CC (silica gel; petroleum ether/ ethyl acetate 8:1 (v/v)), Sephadex LH-20 (chloroform/methanol, 1:1) and preparative TLC (petroleum ether/ethyl acetate, 1:4) to afford compounds **5** (5 mg), **7** (7 mg) and **8** (11 mg). Compounds **1** (2 mg) and **6** (4 mg) were obtained from the Fr. 5–3 (150 mg) eluted with petroleum ether/acetone (8:1, v/v) after repeated CC (silica gel; chloroform/acetone 20:1 (v/v)), Sephadex LH-20 (chloroform/methanol, 1:1) and preparative TLC (chloroform/acetone, 6:1). The Fr. 5–4 (160 mg) eluted with petroleum ether/acetone (6:1, v/v) was subsequently purified by repeated CC (silica gel; petroleum ether/ethyl acetate 12:1 (v/v)), Sephadex LH-20 (acetone) and preparative TLC (petroleum ether/acetone, 1:1) to afford compounds **2** (4 mg), **3** (3 mg) and **9** (8 mg).

5-(3′-Methyl-2′-butenyl)-2-hydroxy-3-methoxy-4-methylbenzoic acid (**1**): isolated as white powder. IR (KBr) ν_{max}: 3406 (OH), 1699 (C=O) and 1557 (C=C) cm^{-1}. ^1H (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): see Table 1. Positive ion HRAPCIMS $[M+H]^+$ *m/z* 251.12781 (calcd for $C_{14}H_{19}O_4$, 251.12779).

5-(3′-Carboxyl-3′-methyl-2E-allyloxy)-3-methoxy-4-methylphthalide (**2**): isolated as white powder. IR (KBr) ν_{max}: 3227 (OH), 1740, 1780 (C=O) and 1618 (C=C) cm^{-1}. ^1H (600 MHz, $CDCl_3$+CD_3OD) and ^{13}C NMR (150 MHz, $CDCl_3$+CD_3OD): see Table 1. Positive ion HRESIMS $[M+H]^+$ *m/z* 293.10185 (calcd for $C_{15}H_{17}O_6$, 293.10196).

5-(3′,3′-Dimethylallyloxy)-2-methoxycarbonyl-3-methoxy-4-methylbenzoic acid (**3**): isolated as white powder. IR (KBr) ν_{max}: 3431 (OH), 1680, 1720 (C=O) and 1600 (C=C) cm^{-1}. ^1H (600 MHz, $CDCl_3$) and ^{13}C NMR (150 MHz, $CDCl_3$): see Table 1. Positive ion HRESIMS $[M+H]^+$ *m/z* 309.13338 (calcd for $C_{16}H_{21}O_6$, 309.13326).

5-(3′,3′-Dimethylallyloxy)-3-methoxy-4-methylphthalide (**4**): isolated as white powder. ^1H (600 MHz, $CDCl_3$) δ 7.08 (1H, s and H-6), 5.83 (2H, s and H-8), 3.88 (3H, s and H-9), 2.20 (3H, s and H-10), 4.56 (2H, d, *J*=6.7 Hz and H-1), 5.47 (1H, t, *J*=6.7, 12.6 Hz and H-2), 1.80 (3H, s and H-4) and 1.75 (3H, s and H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ 127.9 (s, C-1), 124.7 (s, C-2), 159.2 (s, C-3), 125.6 (s, C-4), 152.8 (s, C-5), 101.8 (d, C-6), 171.1 (s, C-7), 68.3 (t, C-8), 59.2 (q, C-9), 9.7 (q, C-10), 65.8 (t, C-1), 119.3 (d, C-2), 138.1 (s, C-3), 25.7 (q, C-4) and 18.3 (q, C-5). It was identified as *5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide* by comparison of the spectral data with the literature.[14]

Zinnimidine (**5**): isolated as white powder. ^1H (600 MHz, $CDCl_3$) δ 7.10 (1H, s and H-6), 4.60 (2H, s and H-8), 3.87 (3H, s and H-9), 2.23 (3H, s and H-10), 4.58 (2H, d, *J*=6.7 Hz and H-1), 5.18 (1H, t, *J*=6.7, 12.6 Hz and H-2), 1.80 (3H, s and H-4) and 1.74 (3H, s and H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ 130.8 (s, C-1), 126.2 (s, C-2), 158.7 (s, C-3), 123.9 (s, C-4), 153.7 (s, C-5), 101.2

Table 1 ^1H (600 MHz) and ^{13}C NMR (150 MHz) data for compounds **1–3**

	1		*2*		*3*	
No.	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1		131.5 (s)		125.9 (s)		125.6 (s)
2		152.9 (s)		128.6 (s)		124.7 (s)
3		157.8 (s)		153.1 (s)		158.2 (s)
4		122.2 (s)		125.9 (s)		128.7 (s)
5		126.8 (s)		158.6 (s)		159.8 (s)
6	6.85 (s)	112.4 (d)	7.03 (s)	101.3 (d)	7.32 (s)	109.4 (d)
7		175.0 (s)		172.1 (s)		171.2 (s)
8			5.44 (s)	69.0 (t)		169.3 (s)
9	3.55 (s)	60.3 (q)	3.91 (s)	59.0 (q)	3.80 (s)	62.3 (q)
10	2.16 (s)	9.5 (q)	2.21 (s)	9.4 (q)	2.21 (s)	9.7 (q)
1′	3.41(d, 4.8)	26.0 (t)	4.80 (br. s)	66.0 (t)	4.60 (d, 6.6)	65.5 (t)
2′	5.01(br. s)	124.5 (d)	6.94 (br. s)	136.0 (d)	5.47 (br. s)	119.2 (d)
3′		130.9 (s)		130.4 (s)		138.9 (s)
4′	1.25 (s)	17.7 (q)		169.4 (s)	1.80 (s)	25.8 (q)
5′	1.57 (s)	25.4 (q)	1.93 (s)	12.6 (q)	1.77 (s)	18.3 (q)
8-OCH_3					3.92 (s)	52.7 (q)

[a]Compounds **1** and **3** were measured in $CDCl_3$; compound **2** was measured in $CDCl_3$+CD_3OD.

(d, C-6), 171.2 (s, C-7), 43.9 (t, C-8), 59.8 (q, C-9), 9.6 (q, C-10), 65.7 (t, C-1), 119.6 (d, C-2), 137.8 (s, C-3), 25.8 (q, C-4) and 18.3 (q, C-5). It was identified as zinnimidine by comparison of the spectral data with the literature.[14]

5-(3′,3′-Dimethylallyloxy)-3-methoxy-4-methylphthalide (**6**): isolated as white powder. ^{1}H (600 MHz, $CDCl_3$) δ 6.62 (1H, s and H-6), 5.18 (2H, s and H-7), 4.03 (3H, s and H-9), 2.15 (3H, s and H-10), 4.59 (2H, d, *J*=6.6 Hz and H-1), 5.49 (1H, t, *J*=6.6, 13.2 Hz and H-2), 1.81 (3H, s and H-4) and 1.75 (3H, s and H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ 109.5 (s, C-1), 148.1 (s, C-2), 163.6 (s, C-3), 120.8 (s, C-4), 157.8 (s, C-5), 99.5 (d, C-6), 68.7 (t, C-7), 169.0 (s, C-8), 62.1 (q, C-9), 8.7 (q, C-10), 65.8 (t, C-1), 118.9 (d, C-2), 138.6 (s, C-3), 25.8 (q, C-4) and 18.3 (q, C-5). It was identified as 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide by comparison of the spectral data with the literature.[14]

5-(3′,3′-Dimethylallyloxy)-3-methoxy-4-methylphthalic acid (**7**): isolated as white powder. ^{1}H (600 MHz, CD_3OD) δ 7.32 (1H, s and H-6), 3.78 (3H, s and H-9), 2.18 (3H, s and H-10), 4.63 (2H, d, *J*=6.6 Hz and H-1), 5.47 (1H, t, *J*=6.6, 12.0 Hz and H-2), 1.79 (3H, s and H-4) and 1.78 (3H, s and H-5). ^{13}C NMR (150 MHz, CD_3OD) δ 127.9 (s, C-1), 126.7 (s, C-2), 159.3 (s, C-3), 125.5 (s, C-4), 157.2 (s, C-5), 109.8 (d, C-6), 172.0 (s, C-7), 168.4 (s, C-8), 66.5 (q, C-9), 9.7 (q, C-10), 62.6 (t, C-1), 120.7 (d, C-2), 139.3 (s, C-3), 25.8 (q, C-4) and 18.3 (q, C-5). It was identified as 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalic acid by comparison of the spectral data with the literature.[15]

Zinniol anhydride (**8**): isolated as white powder. ^{1}H (600 MHz, $CDCl_3$) δ 7.15 (1H, s and H-6), 4.16 (3H, s and H-9), 2.20 (3H, s and H-10), 4.68 (2H, d, *J*=6.6 Hz and H-1), 5.47 (1H, t, *J*=6.6, 12.6 Hz and H-2), 1.78 (3H, s and H-4) and 1.82 (3H, s and H-5). ^{13}C NMR (150 MHz, $CDCl_3$) δ 139.6 (s, C-1), 128.3 (s, C-2), 160.9 (s, C-3), 112.1 (s, C-4), 157.9 (s, C-5), 103.1 (d, C-6), 164.5 (s, C-7), 163.5 (s, C-8), 66.4 (q, C-9), 9.7 (q, C-10), 62.3 (t, C-1), 118.2 (d, C-2), 131.9 (s, C-3), 25.8 (q, C-4) and 18.4 (q, C-5). It was identified as zinniol anhydride by comparison of the spectral data with the literature.[16]

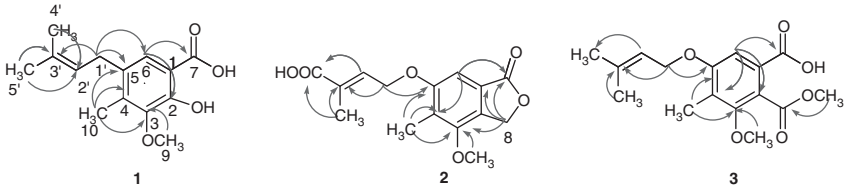
Porriolide (**9**): isolated as white powder. ^{1}H (600 MHz, CD_3OD) δ 6.93 (1H, s and H-6), 5.47 (2H, s and H-8), 3.91 (3H, s and H-9) and 2.16 (3H, s and H-10). ^{13}C NMR (150 MHz, CD_3OD) δ 127.6 (s, C-1), 124.9 (s, C-2), 159.6 (s, C-3), 125.6 (s, C-4), 154.7 (s, C-5), 105.3 (d, C-6), 173.8 (s, C-7), 70.1 (t, C-8), 59.6 (q, C-9) and 9.8 (q, C-10). It was identified as porriolide by comparison of the spectral data with the literature.[17]

Antifungal assays MIC of the compounds (**1**-**9**) against fungi were determined in a 96-well microtitre dish using a method modified from Nair *et al.*[13]. The inocula used in this test were a zoospore suspension (10^6 zoospores ml^{-1}) of *Phytophthora nicotianae*, spore suspensions of *Fusarium graminearum* and *Botrytis cinerea* (10^6 spores ml^{-1}). To each well 10 μl of suspension containing 1 μl of potato dextrose broth (PDB, Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) was added amended with compounds **1**–**9** in the range 0–50 mg l^{-1}, and ketoconazole was used as the positive control. The inoculated well plates were incubated at 28(±1) °C on a rotary shaker at 120 rev min^{-1}. The inhibitory effects of the compounds on the growth of the test microorganisms were evaluated after incubation for 2–4 days. The lowest concentration of the compounds that completely inhibited the growth of the microorganism was considered to be minimum inhibitory.

RESULTS AND DISCUSSION

Compound **1** was obtained as white powder. HRAPCIMS of **1** indicated a molecular formula of $C_{14}H_{18}O_4$ ($[M+H]^+$ at *m/z* 251.12781, calcd for $C_{14}H_{19}O_4$, 251.12779) with six degrees of unsaturation. This was corroborated by the ^{13}C NMR (DEPT) (Table 1), which displayed 14 signals for the carbons including four methyl carbons (δ_C 60.3 (q), 25.4 (q), 17.7 (q) and 9.5 (q)), one carboxyl group (δ_C 175.0 (s)), one trisubstituted double bond (δ_C 124.5 (d), 130.9 (s)), one pentasubstituted aromatic group (δ_C 157.8 (s), 152.9 (s), 131.5 (s), 126.8 (s), 122.2 (s) and 112.4 (d)) and one methylene carbon (δ_C 26.0 (t)). Analysis of the ^{1}H NMR spectrum (Table 1) indicated the presence of four methyl signals including one methoxy group (δ_H 3.55 (s), 2.16 (s), 1.57 (s) and 1.25 (s)), one olefinic proton signal (δ_H 5.01 (br.s), one aromatic proton signal (δ_H 6.85 (s)) and one methylene proton signal (δ_H 3.41 (d, *J*=4.8 Hz)). The connectivity of the protons and C-atoms was established by the ^{1}H, ^{13}C HSQC spectrum. Further interpretation of HMBC spectrum showed the following long-range correlations (Figure 2): from H-6 to C-2 and C-7, from H-9 to C-3, from H-10 to C-3, C-4 and C-5, from H-1′ to C-5, C-6 and C-3′, from H-2′ to C-5, C-1′, C-4′ and C-5′, from H-4′ to C-2′ and from H-5′ to C-2′ and C-3′. The above spectral evidences led to the establishment of **1** as 5-(3′-methyl-2′-butenyl)-2-hydroxy-3-methoxy-4-methylbenzoic acid.

Compound **2** was obtained as white powder that gave a quasi-molecular ion peak at $[M+H]^+$ *m/z* 293.10185 in the HRESIMS (positive) consistent with a molecular formula of $C_{15}H_{16}O_6$ (calcd for $C_{15}H_{17}O_6$, 293.10196), requiring eight degrees of unsaturation. The ^{1}H NMR (Table 1) spectrum of **2** contained signals of three methyl signals including one methoxy group (δ_H 3.91 (s), 2.21 (s) and 1.93 (s)), one aromatic proton signal (δ_H 7.03 (s)), which showed the presence of pentasubstituted aromatic ring, one olefinic proton signal (δ_H 6.94 (s)), which implied the presence of trisubstituted double bond and two oxygenated methylene proton signals (δ_H 5.44 (s) and 4.80 (br. s)). From the ^{13}C NMR data, two carboxyl groups (δ_C 172.1 (s) and 169.4 (s)), three methyl signals including one methoxy group (δ_C 59.0 (q), 12.6 (q) and 9.4 (q)) and two oxygenated methylene carbon signals (δ_C 69.0 (t) and 66.0 (t)) were observed. The remaining carbon signals (δ_C 158.6 (s), 153.1 (s), 136.0 (d), 130.4 (s), 128.6 (s), 125.9×2 (s) and 101.3 (d)) could be assigned to one pentasubstituted aromatic ring and one trisubstituted double bond. The connectivity of the protons and C-atoms was established by the ^{1}H, ^{13}C HSQC spectrum. Comparison of the NMR data of **2** with those of **5**,[14] a known compound firstly isolated from *Alternaria porri*, revealed that both compounds possess the same substitution pattern. The distinct differences between **2** and **5** are that: the chemical shifts value at C-4′ of **2** (δ_C 169.4 (s)) were absent in **5** (δ_C 25.8 (q)). In addition, the chemical shift value at C-2′ (δ_C 136.0 (d)) in **2** was shifted downfield compared with **5** (δ_C 119.2 (d)) affected by conjugative effect. The chemical shift value at C-5′ (δ_C 12.6 (q)) in **2** was shifted upfield compared with **5** (δ_C 18.3 (q)) owing to shielding effect caused by carboxyl group at C-3′ in **2**. Further interpretation of HMBC spectrum showed the following long-range correlations (Figure 2): from H-6 to C-4 and C-7, from H-8 to C-1, C-3 and C-7, from H-9 to C-3, from H-10 to C-3, C-4 and C-5, from H-1′ to C-5 and C-3′, from H-2′ to C-4′ and from H-5′ to C-4′. The configuration of the double



1 2 3

Figure 2 Selected HMBC correlations of compounds **1**-**3**.

bond at C-2 was assigned to be *E*-orientation because Me-5 characteristic upfield resonance at δ_C 12.6 (q) and no NOE correlation observed between H-2 and H-5. In addition, the chemical shift at C-2′ (δ_H 6.94 (1H, br. s); δ_C 136.0 (d)) in **1** were shifted downfield comparison chemical shifts with **4** (δ_H 5.47 (1H, t, J=6.7, 12.6 Hz); δ_C 119.3 (d)) owing to deshielding effect of carboxyl group at C-3′. In light of the evidences mentioned above, the structure of **2** was finally established as 5-(3′-carboxyl-3′-methyl-2*E*-allyloxy)-3-methoxy-4-methylphthalide (**2**).

Compound **3** had the molecular formula $C_{16}H_{20}O_6$, as established from positive ion HRESIMS $[M+H]^+$ *m/z* 309.13338 (calcd for $C_{16}H_{21}O_6$, 309.13326). The ^{1}H NMR (Table 1) spectrum exhibited one aromatic resonance at δ_H 7.32 (s), which was indicative of a pentasubstituted phenyl ring, and one olefinic proton signal (δ_H 5.47 (br. s)) exhibited the presence of trisubstituted double bond. In addition, the remaining signals in ^{1}H NMR spectrum assigned to five methyl signals including two methoxy groups (δ_H 3.92 (s), 3.80 (s), 2.21 (s), 1.80 (s) and 1.77 (s)), and one oxygenated methylene proton signal (δ_H 4.60 (d, J=6.6 Hz)). The 16 carbon signals in the ^{13}C NMR spectrum were sorted by DEPT experiment into five methyls (involving two methoxy groups), two carboxyl carbons, eight olefinic carbons and one oxygenated methylene carbon. The connectivity of the protons and C-atoms was established by the ^{1}H, ^{13}C HSQC spectrum. By careful analysis of NMR data, we found that the NMR spectral data of **3** was the same as **7** except for one additional methoxy group. The additional methoxy group was located at C-8 deduced from the long-range correlation from H_3-(8-OCH_3) to C-8. Further interpretation of HMBC spectrum showed the following long-range correlations (Figure 2): from H-6 to C-2, C-4 and C-7, from H-9 to C-3, from H-10 to C-3 and C-5, from H-1′ to C-5 and C-3′ and from H-2′ to C-4′ and C-5′. Accordingly, the structure of **3** was elucidated as 5-(3′,3′-dimethylallyloxy)-2-methoxycarbonyl-3-methoxy-4-methylbenzoic acid.

Comparison of the physicochemical properties with reported data allowed identifying the compounds (**4-9**) as 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalide (**4**),[14] zinnimidine (**5**),[14] 5-(3′,3′-dimethyl allyloxy)-3-methoxy-4-methylphthalide (**6**),[14] 5-(3′,3′-dimethylallyloxy)-3-methoxy-4-methylphthalic acid (**7**),[15] zinniol anhydride (**8**)[16] and porriolide (**9**).[17]

Antifungal activities

Compounds (**1–9**) were evaluated for antifungal activities against three plant pathogenic fungi, and the results are shown in Table 2. Compounds (**1–9**) displayed significant antifungal activities against three fungal strains including *Fusarium graminearum*, *Botrytis cinerea* and *Phytophthora nicotianae*, with MIC values from 50.0–3.1 μg ml^{-1} (the positive control ketoconazole showed MIC values are 3.1 μg ml^{-1}).

Table 2 Antifungal activities against three plant pathogenic fungi of compounds (1–9)

	MIC (μg ml^{-1})									
Fungal strain	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*ketoconazole*
Fusarium graminearum	6.3	6.3	3.1	6.3	6.3	3.1	3.1	6.3	3.1	3.1
Botrytis cinerea	12.5	6.3	25	12.5	6.3	25	3.1	50	25	3.1
Phytophthora nicotianae	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	6.3	3.1

In summary, we have reported the isolation, identification and biological study of nine metabolites from *Pestalotiopsis photiniae*. All metabolites were structurally related to zinniol, and three of them were identified as new compounds (**1-3**), whereas six compounds (**4–9**) have already been reported as being produced by a number of *Alternaria* species, including *A. porri*, *A. tagetica* and *A. solani*, or synthesized as the major intermediates. Compounds **4**, **6** and **8** were first reported as the main products resulting from the oxidation of zinniol.[16] Compound **4** has also been reported previously as a natural compound from *A. porri* and *A. solani*, which exhibited no particular inhibitory activity toward lettuce and stone-leek seedlings.[16–18] Compounds **6** and **8** displayed no significant phytotoxic activities using leaf-spot assay on marigold leaves.[16] Compound **5** was firstly isolated from *A. cichorii* which was inactive toward Russian knapweed and the lettuce shoot.[12,19] Compound **7** was the key intermediate of total synthesis of some phthalides.[15] According to experimental results reported by R. Suemitsu *et al.*, compound **9** inhibited the root elongation of both lettuce and stone-leek seedlings by 53.3 and 48.5% at 400 p.p.m., respectively, the toxicity of **9** was rather weak compared with that of porritoxin, which inhibited lettuce seedling growth at a concentration of 10 p.p.m..[17] In this paper, compounds (**1-9**) were evaluated in antifungal activity assay, and the results indicated that compounds (**1-9**) displayed significant antifungal activities against three fungal strains.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This work was supported by the programs for New Century Excellent Talents in University (NCET-09-0112), the Key Project of Chinese Ministry of Education and the Key Applied Basic Research Programs of Hebei Province (0996030917D) and National Natural Science Foundation of China (31071701), Hebei Province Science Fund for Distinguished Young Scholars (C2011201113), National Department Public Benefit Research Foundation (201003065) and Project supported by the National High Technology Research and Development Program of China (Grant no. 2011AA10A204).

1 Suryanarayanan, T. S., Senthilarasu, G. & Muruganandam, V. Endophytic fungi from *Cuscuta reflexa* and its host plants. *Fungal Divers.* **4,** 117–123 (2000).
2 Strobel, G. A., Hess, W. M., Ford, E., Sidhu, R. S. & Yang, X. Endophytic fungi in grasses and woody plants. *J. Ind. Microbiol.* **17,** 417–423 (1996).
3 Li, J. Y., Strobel, G. A., Hess, W. M. & Ford, E. Endophytic taxol-producing fungi from bald cypress, *Taxodium distichum*. *Microbiology* **142,** 2223–2226 (1996).
4 Liu, L., Liu, S. C., Chen, X. L., Guo, L. D. & Che, Y. S. Pestalofones A–E, bioactive cyclohexanone derivatives from the plant endophytic fungus *Pestalotiopsis fici*. *Bioorg. Med. Chem.* **17,** 606–613 (2009).
5 Ding, G., Li, Y., Fu, S. B., Liu, S. C., Wei, J. C. & Che, Y. S. Ambuic acid and torreyanic acid derivatives from the endolichenic fungus *Pestalotiopsis sp. J. Nat. Prod.* **72,** 182–185 (2009).
6 Xu, J. *et al*. Chromones from the endophytic fungus *Pestalotiopsis sp.* isolated from the Chinese mangrove plant *Rhizophora mucronata*. *J. Nat. Prod.* **72,** 662–665 (2009).
7 Ding, G., Zheng, Z. H., Liu, S. C., Zhang, H., Guo, L. D. & Che, Y. S. Photinides A-F, cytotoxic benzofuranone-derived ã-lactones from the plant endophytic fungus *Pestalotiopsis photiniae*. *J. Nat. Prod.* **72,** 942–945 (2009).
8 Liu, L. *et al*. Chloropestolide A, an antitumor metabolite with an unprecedented spiroketal skeleton from *Pestalotiopsis fici*. *Org. Lett.* **11,** 2836–2839 (2009).
9 Liu, L., Liu, S. C., Niu, S. B., Guo, L. D., Chen, X. L. & Che, Y. S. Isoprenylated chromone derivatives from the plant endophytic fungus *Pestalotiopsis fici*. *J. Nat. Prod.* **72,** 1482–1486 (2009).
10 Yang, X. L., Zhang, S., Zhu, H. J. & Luo, D. Q. Dihydroberkleasmin A: a new eremophilane sesquiterpenoid from the fermentation broth of the plant endophytic fungus *Pestalotiopsis photiniae*. *Molecules* **16,** 1910–1916 (2011).
11 Fukami, A. *et al.* A new antiinfluenza virus antibiotic, 10-norparvulenone from *Microsphaeropsis sp.* FO-5050. *J. Antibiot.* **53,** 1215–1218 (2000).
12 Andrea, S., Joseph, H. & Gary, S. Zinniol-related phytotoxins from *Alternaria cichorii*. *Phytochemistry* **32,** 1145–1149 (1993).

13 Ding, G., Jiang, L. H., Guo, L. D., Chen, X. L., Zhang, H. & Che, S. Y. Pestalazines and pestalamides, bioactive metabolites from the plant pathogenic fungus *Pestalotiopsis theae*. *J. Nat. Prod.* **71,** 1861–1865 (2008).
14 Rikisaku, S., Keiichiro, O., Yoshiyuki, M. & Shigemi, N. Zinnimidine and 5-(3, 3-dimethylallyloxy)-7-methoxy-6-methylphthalide from *Alternaria porri*. *Phytochemistry* **38,** 495–497 (1995).
15 Hariprakasha, H. K. & Subbarao, G. S. R. Synthesis of some naturally occurring phthalides from *Alternaria species*. *Indian J. Chem. Sect. B Org. Chem. Incl. Med. Chem.* **37,** 851–856 (1998).
16 Gamboa-Angulo, M. M., Escalante-Erosa, F., García-Sosa, K., Alejos-González, F., Delgado-Lamas, G. & Peña-Rodríguez, L. M. Natural zinniol derivatives from *Alternaria tagetica*. Isolation, synthesis, and structure-activity correlation. *J. Agric. Food Chem.* **50,** 1053–1058 (2002).
17 Suemitsu, R., Ohnishi, K., Horiuchi, M., Morikawa, Y., Sakaki, Y. & Matsumoto, Y. Structure of porriolide, a new metabolite from *Alternaria porri*. *Biosci. Biotechnol. Biochem.* **57,** 334–335 (1993).
18 Gamboa-Angulo, M. M., Alejos-González, F. & Peña-Rodríguez, L. M. Homozinniol, a new phytotoxic metabolite from *Alternaria solani*. *J. Agric. Food Chem.* **45,** 282–285 (1997).
19 Horiuchi, M. *et al*. A novel isoindoline, porritoxin sulfonic acid, from *Alternaria porri* and the structure-phytotoxicity correlation of its related compounds. *Biosci. Biotechnol. Biochem.* **67,** 1580–1583 (2003).