

Two new ambuic acid analogs from *Pestalotiopsis* sp. cr013Jin Xie<sup>a,b</sup>, Jing Li<sup>a,b,c</sup>, Yin-He Yang<sup>a</sup>, Yu-Hui Chen<sup>b</sup>, Pei-Ji Zhao<sup>a,\*</sup><sup>a</sup> State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650204, China<sup>b</sup> Life Science College, Southwest Forestry University, Kunming 650224, China<sup>c</sup> University of Chinese Academy of Sciences, Beijing 100049, China

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## ABSTRACT

*Pestalotiopsis* is a highly prolific genus with a plethora of diverse secondary metabolites. *Pestalotiopsis* sp. cr013 was isolated from aeciospores of *Cronartium ribicola* and subjected to phytochemical investigation based on its special environment. To understand the secondary metabolites of strain *Pestalotiopsis* sp. cr013, two new compounds **1** and **2** were isolated from solid fermentation products of the fungus. Two new structures were elucidated by extensive spectroscopic analyses, including 1D- and 2D-NMR, and HR-ESI-MS experiments. Compound **1** exhibited weak cytotoxicity activity against five cancer cell lines, with IC<sub>50</sub> values of 18.99 μM (HL-60), 17.68 μM (SMMC-7721), 18.28 μM (A-549), 21.67 μM (MCF-7) and 12.27 μM (SW480).

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## 1. Introduction

*Pestalotiopsis* species are widely distributed throughout the world (Maharachchikumbura et al., 2011). Some species of this genus may cause disease in a variety of plants (Hopkins and McQuilken, 2000; Taylor et al., 2001; Keith et al., 2006). In recent years, *Pestalotiopsis*, a highly prolific genus with a plethora of diverse secondary metabolites, has gained considerable attention (Xu et al., 2010). Some of the products of *Pestalotiopsis* are potential drug lead compounds for the treatment of human diseases and the control of plant diseases. For example, torreyanic acid was isolated from *Pestalotiopsis microspora* colonizing *Torreya taxifolia* plants, and it showed 5–10-fold stronger activity in cell lines that are sensitive to protein kinase C (PKC) antagonists than other human cell line (A549) (Lee et al., 1996). Chloropestolide A, a highly functionalized novel skeleton, was isolated from *Pestalotiopsis fici*, and it exhibited significant inhibitory effects against the growth of two human cancer cell lines, with GI<sub>50</sub> values of 0.7 (HeLa) and 4.2 (HT29) μM (Liu et al., 2009). Pestalachlorides B, from endophytic fungus *Pestalotiopsis adusta*, was reported to exhibit remarkable antifungal activities against the plant pathogen fungus *Gibberella zeae*, with an IC<sub>50</sub> value of 1.1 μM (Li et al., 2008). To search for new active structural compounds, the mycoparasite fungal strain *Pestalotiopsis* sp. cr013, isolated from plasmogamospore parasitiz-

ing *Pinus armandii*, was investigated, and two new ambuic acid analogs were obtained. This report describes two new structures and their activity.

## 2. Results and discussion

The nucleotide sequences for the ITS1–5.8S rDNA–ITS4 region of the endophytic fungi cr013 was registered in the GenBank database with the accession number KM652628, and the strain was determined to be *Pestalotiopsis* sp. according to the ITS analysis.

Compound **1** was obtained as a colorless amorphous material. The HR-ESI-MS data indicated a molecular formula of C<sub>18</sub>H<sub>24</sub>O<sub>5</sub> based on the [M]<sup>+</sup> ion signal at *m/z* 320.1616 ([M]<sup>+</sup>, calc. 320.1624). The <sup>13</sup>C NMR and DEPT spectra (Table 1) revealed five quaternary carbons (δ<sub>C</sub> 197.0, 171.2, 155.8, 132.7 and 65.6), six methines (δ<sub>C</sub> 136.4, 142.5, 130.3, 122.2, 65.7 and 59.4), five methylenes (δ<sub>C</sub> 31.3, 34.7, 29.7, 32.8 and 23.7) and two methyls (δ<sub>C</sub> 14.5 and 13.0). According to the <sup>1</sup>H NMR (Table 1), a singlet methyl signal (δ<sub>H</sub> 1.88 (3H, s)) and a triplet methyl signal (δ<sub>H</sub> 0.93 (3H, t, 6.9)) were also present, which suggested that compound **1** was an ambuic acid analog (Li et al., 2001; Ding et al., 2009). The detail structure was confirmed by 2D-NMR. The HMBC experiment (Table 1) showed that the proton at δ<sub>H</sub> 6.87 (H-3) correlated with the carbons at δ<sub>C</sub> 171.2 (C-1), 132.7 (C-2), 31.3 (C-4), 65.6 (C-5) and 13.0 (C-18); the protons of methyl at δ<sub>H</sub> 1.88 (H-18) with carbons at δ<sub>C</sub> 171.2 (C-1), 132.7 (C-2) and 136.4 (C-3); the protons of methylene δ<sub>H</sub> 3.19 and 2.71 (H-4) with carbons at δ<sub>C</sub> 171.2 (C-1), 132.7 (C-2), 136.4 (C-3),

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**Table 1**NMR data of compounds **1** and **2** (in CD<sub>3</sub>OD, *J* in Hz).

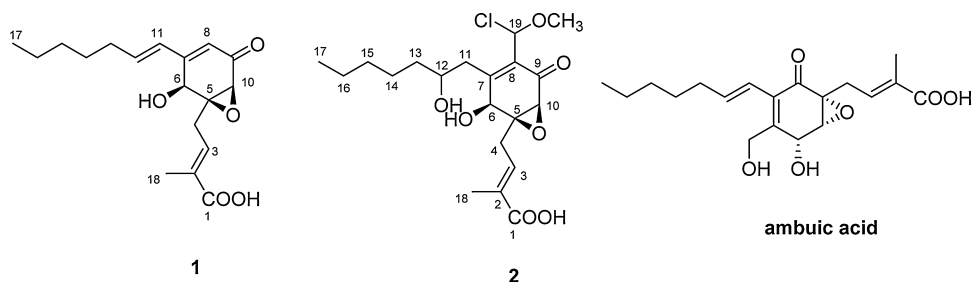
Position	<b>1</b>			<b>2</b>		
	<sup>1</sup> H	<sup>13</sup> C	HMBC	<sup>1</sup> H	<sup>13</sup> C	HMBC
1	–	171.2, s	–	–	171.5, s	–
2	–	132.7, s	–	–	133.0, s	–
3	6.87 (1H, td, 7.5, 1.4)	136.4, d	C-1, C-2, C-4, C-5, C-18	6.84 (1H, td, 7.6, 1.1)	136.1, d	C-1, C-2, C-4, C-5, C-18
4	3.19 (1H, dd, 15.6, 7.7)	31.3, t	C-1, C-2, C-3, C-5, C-10	3.18 (1H, dd, 7.8, 15.5)	31.3, t	C-2, C-3, C-5, C-10
	2.71 (1H, dd, 15.6, 7.6)	–	C-1, C-2, C-3, C-5, C-10	2.56 (1H, dd, 7.8, 15.5)	–	C-2, C-3, C-5, C-10
5	–	65.6, s	–	–	65.5, s	–
6	4.75 (1H, s)	65.7, d	C-4, C-5, C-7, C-8, C-9 (w), C-10, C-11	4.21 (1H, s)	68.0, d	C-5, C-7, C-8, C-9 (w), C-10, C-11,
7	–	155.8, s	–	–	155.9, s	–
8	5.79 (1H, d, 1.2)	122.2, d	C-5, C-7, C-10, C-11	–	127.7, s	–
9	–	197.0, s	–	–	193.3, s	–
10	3.32 (1H, t, 1.2)	59.4, d	C-5, C-8, C-9	3.32 (1H, m)	59.9, d	C-4, C-5, C-8, C-9, C-19 (w)
11	6.18 (1H, d, 16.2)	130.3, d	C-5, C-7, C-8, C-13	2.58 (1H, dd, 3.4, 18.9)	34.0, t	C-7, C-8, C-12
	–	–	–	2.04 (1H, dd, 11.1, 18.9)	–	C-7, C-8, C-9 (w), C-12, C-13
12	6.51 (1H, dt, 7.1, 16.2)	142.5, d	C-7, C-8, C-13, C-14	3.96 (1H, m)	67.2, d	–
13	2.25 (2H, dt, 7.2, 7.2)	34.7, t	C-7, C-11, C-12, C-14, C-15	1.60 (2H, m, overlap)	36.4, t	Overlap
14	1.51 (1H, dt, 14.5, 7.3)	29.7, t	Overlap	1.60 (1H, m, overlap)	26.5, t	Overlap
	1.37 (1H, m, overlap)	–	Overlap	1.44 (1H, m)	–	C-15
15	1.51 (1H, dt, 14.5, 7.3)	32.8, t	Overlap	1.38 (2H, m, overlap)	33.1, t	Overlap
	1.37 (1H, m, overlap)	–	Overlap	–	–	–
16	1.37 (2H, m, overlap)	23.7, t	Overlap	1.38 (2H, m, overlap)	23.9, t	Overlap
17	0.93 (3H, t, 6.9)	14.5, q	C-15, C-16	0.94 (3H, t, 6.9)	14.6, q	C-15, C-16
18	1.88 (3H, s)	13.0, q	C-1, C-2, C-3	1.87 (3H, s)	13.0, q	C-1, C-2, C-3
19	–	–	–	4.98 (1H, s)	95.4, d	C-7, C-8, C-9 (w), C-6, 19-OCH <sub>3</sub>
19-OCH <sub>3</sub>	–	–	–	3.43 (3H, s)	55.0, q	C-19

65.6 (C-5) and 59.4 (C-10); and the proton at  $\delta_{\text{H}}$  4.75 (H-6) with carbons at  $\delta_{\text{C}}$  155.8 (C-7), 122.2 (C-8), 65.6 (C-5), 59.4 (C-10) and 31.3 (C-4). Together with the correlations between H-10 and C-9, C-8 and C-5, these findings indicated that the compound contained a 4-(2-hydroxy-5-oxo-7-oxabicyclo[4.1.0]hept-3-en-1-yl)-2-methylbut-2-enoic acid unit. A heptene group located at C-7 was identified based on other correlations (Table 1). In the COSY spectrum, three branches were deduced to be C-3–C-4 (–branch), –C-11–C-12–C-13 (–branch) and –C-15–C-16–C-17 (–branch) from a complete interpretation of the key cross-peaks (H-3/H-4; H-11/H-12/H-13; H-15/H-16/H-17). The NOESY experiment showed NOE interactions between H-4, H-6 and H-10, supporting the relative configurations of C-5, C-6 and C-10 (Fig. 2). In the <sup>1</sup>H NMR spectrum, the coupling constant of H-11 and H-12 was 16.2 Hz, revealing that the double bond at C-11 was in the *E* configuration. For the relative configuration of ambuic acid was similar with compound **1** and optical rotation of compound **1** ( $[\alpha]_{\text{D}} -129$  (c 0.17, MeOH)) have opposite sign from ambuic acid ( $[\alpha]_{\text{D}} +106$  (c 0.1, MeOH)) from the literature (Ding et al., 2009), the absolute configuration of compound **1** are shown in Fig. 1.

Compound **2** was obtained as a colorless powder. The HR-ESI-MS data indicated a molecular formula of C<sub>20</sub>H<sub>19</sub><sup>35</sup>ClO<sub>5</sub> based on the  $[\text{M} + \text{Na}]^+$  ion signal at *m/z* 439.1503 (calc. 439.1500). The MS and NMR spectroscopic data for compound **2** were very similar to those for compound **1** except that two extra carbons ( $\delta_{\text{C}}$  95.4 and 55.0) appear in compound **2**. For natural abundance of isotope <sup>37</sup>Cl

(24.47%) was about one third of <sup>35</sup>Cl (75.53%) and the quasi molecular ion *m/z* 441  $[\text{M} + 2 + \text{Na}]^+$  signal of one-third intensity was observed in addition to *m/z* 439  $[\text{M} + \text{Na}]^+$  of compound **2** in ESI-MS experiment, this MS experiment result implied presence a chloro group in compound **2**. And then the chemical shift of oxygenated methine  $\delta_{\text{C}}$  95.4 ( $\delta_{\text{H}}$  4.98) shifted to low-field, indicated a electronegative group (chloro group) was connected with the oxygenated methine. The 2D-NMR data (Table 1) revealed that the H-19 ( $\delta_{\text{H}}$  4.98 (1H, s)) of methine correlated with the carbons at  $\delta_{\text{C}}$  155.9 (C-7), 127.7 (C-8), 193.3 (C-9) (w), 68.0 (C-6) and 55.0 (19-OCH<sub>3</sub>), which indicated a chloro-methoxy-methyl group located at the C-8 position (Fig. 1). The correlations between H-11 ( $\delta_{\text{H}}$  2.58 and 2.04) of the methylene group and the carbons at  $\delta_{\text{C}}$  155.9 (C-7), 127.7 (C-8) and 67.2 (C-12) and between H-10 ( $\delta_{\text{H}}$  3.32) and the carbons at  $\delta_{\text{C}}$  31.3 (C-4), 65.5 (C-5), 127.7 (C-8), 193.3 (C-9) and 95.4 (C-19) showed that the 11-double bond was hydrated and that one hydroxyl group was located at C-13. The other main HMBC correlations were almost the same as those of compound **1** (Table 1). The NOESY experiment showed NOE interactions between H-4, H-6 and H-10, supporting the relative configurations of C-5, C-6 and C-10 (Fig. 2). Based on the above data, compound **2** was elucidated to be as shown in Fig. 1.

Compounds **1** and **2** were assayed for antifungal activity (*Gaeumannomyces graminis*, *Fusarium moniliforme*, *Verticillium cinnabarinum* and *Pyricularia oryzae*) and antibacterial activity (*Pseudomonas solanacearum*, *Alternaria alternate*, *Staphylococcus*

**Fig. 1.** The structures of compounds **1**, **2** and ambuic acid.

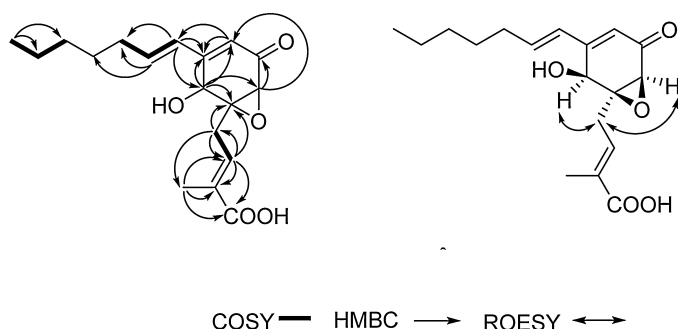


Fig. 2. HMBC and key NOESY correlations of compounds 1.

*aureus*, *Salmonella typhimurium* ATCC 6539). However, neither showed any inhibition activity to the tested microorganisms at 100  $\mu\text{g}/\text{disk}$ . The nematocidal activity results indicated that neither compound had activity against *Panagrellus redivivus* and *Caenorhabditis elegans*. The cytotoxicity activity showed that compound 1 had weak cytotoxicity activity against five cancer cell lines, with  $\text{IC}_{50}$  values of 18.99  $\mu\text{M}$  (HL-60), 17.68  $\mu\text{M}$  (SMMC-7721), 18.28  $\mu\text{M}$  (A-549), 21.67  $\mu\text{M}$  (MCF-7) and 12.27  $\mu\text{M}$  (SW480), while the control of MW300 showed cytotoxicity activity against five cancer cell lines, with  $\text{IC}_{50}$  values of 1.28  $\mu\text{M}$  (HL-60), 6.72  $\mu\text{M}$  (SMMC-7721), 6.15  $\mu\text{M}$  (A-549), 16.33  $\mu\text{M}$  (MCF-7) and 12.86  $\mu\text{M}$  (SW480).

### 3. Experimental

#### 3.1. General

UV spectra were measured on a Shimadzu UV-2401PC spectrophotometer, and  $\lambda_{\text{max}}$  (log  $\epsilon$ ) values are reported in nm. NMR experiments were carried out on Bruker Avance 600 NMR spectrometers with tetramethylsilane (TMS) as an internal standard. ESI-MS and HR-ESI-MS were recorded on a Finnigan LCQ-Advantage mass spectrometer and a VG Auto-Spec-3000 mass spectrometer. Optical rotations were measured on a Jasco DIP-370 digital polarimeter. Column chromatography was carried out on silica gel (G, 200–300 mesh and GF254) (Qingdao Marine Chemical Factory, Qingdao, China) and Sephadex LH-20 (Pharmacia). Precoated silica gel GF254 plates (Qingdao Marine Chemical Factory, Qingdao, China) were used for thin layer chromatography (TLC).

#### 3.2. Fungal material

The plasmogamospores, which had parasitized on *Pinus armandii*, were collected at Kunming, Yunnan Province, People's Republic of China, in August 2013. The plasmogamospores were incubated on distilled filter paper at 25  $^{\circ}\text{C}$  and cultured until colonies or mycelia appeared. After culturing for approximately 7 days, a strain was isolated from the plasmogamospore, identified as *Pestalotiopsis* sp. (strain number cr013) and deposited at Kunming Institute of Botany, Chinese Academy of Science, Kunming, China. *Pestalotiopsis* sp. cr013 (30 L) was cultured on a dish containing improved Fries culture agar ( $\text{KH}_2\text{PO}_4$  1 g,  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g, NaCl 0.1 g,  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  0.13 g, saccharose 20.0 g, ammonium tartrate 5.0 g, yeast extract 1.0 g,  $\text{NH}_4\text{NO}_3$  1.0 g, distilled water 1 L, agar 15.0 g, natural pH) at 25  $^{\circ}\text{C}$  for 21 days.

#### 3.3. Extraction and isolation

The solid fermentation products were cut into small pieces and extracted exhaustively with mixture of solvents (EtOAc/MeOH/HAc, 80:15:5, v/v/v) three times to obtain the crude extract. The

extract was dissolved in water and then re-extracted with equal volume EtOAc three times. The extract of the EtOAc section residue (30.2 g) was subjected to a silica gel G column (200–300 mesh) using a  $\text{CHCl}_3$ –MeOH (100:0–0:100) gradient solvent system to produce five fractions (Fr. 1–Fr.5). Fr.1 (9.223 g) was placed in a silica gel column (200–300 mesh) and eluted with a petroleum ether–EtOAc (10:1–6:4) and  $\text{CHCl}_3$ –MeOH (20:1–0:100) gradient solvent system to yield eleven sub-fractions (Fr. 1.1–Fr. 1.11). Fr. 1.6 was subjected to GF254 column using a petroleum ether–acetone (9:1) solvent system to produce eight sub-fractions (Fr. 1.6.1–Fr. 1.6.8). Fr. 1.6.6 was purified by GF254 column using  $\text{CHCl}_3$ –MeOH (100:1) with formic acid (0.3%) as the eluent to produce 1 (33.5 mg). Fr. 1.7 was subjected to GF254 column using a petroleum ether–acetone (100:7–8:2) gradient solvent system to give six sub-fractions (Fr. 1.7.1–Fr. 1.7.6). Fr. 1.7.6 was purified by Sephadex LH-20 (MeOH) and GF254 column using  $\text{CHCl}_3$ –MeOH (5:1) to produce 2 (3.5 mg).

#### 3.3.1. Compound 1

Colorless amorphous material;  $[\alpha]_D^{25} = -129.02$  ( $c = 0.17$ , MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 290 (4.25), 203 (4.26); NMR data see Table 1; ESI-MS: 321  $[\text{M} + \text{H}]^+$ ; HR-ESI-MS: 320.1616 ( $[\text{M}]^+$ , calc. 320.1624).

#### 3.3.2. Compound 2

Colorless powder;  $[\alpha]_D^{25} = -112.76$  ( $c = 0.14$ , MeOH); UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ): 245 (3.86), 210 (4.34); NMR data see Table 1; ESI-MS: 439  $[\text{M} + \text{Na}]^+$ , 441  $[\text{M} + 2 + \text{Na}]^+$ ; HR-ESI-MS: 439.1503 ( $[\text{M} + \text{Na}]^+$ , calc. 439.1500).

#### 3.4. Assay activities

The antifungal activity against phytopathogenic fungi (*G. graminis*, *F. moniliforme*, *V. cinnabarinum* and *P. oryzae*) and antibacterial activity (*P. solanacearum*, *A. alternate*, *S. aureus*, *S. typhimurium* ATCC 6539) were assessed using the disk diffusion method (Espinel-Ingroff et al., 1999). The individual compounds were dissolved in methanol and each was added 10  $\mu\text{L}$  (50 mg/mL) to a paper disk (6 mm in diameter) respectively. The dried paper discs were applied onto the surface of the assay plates seeded with the bacterial cells (fungi), which were incubated for 24 h at 37  $^{\circ}\text{C}$  (antifungal test at 28  $^{\circ}\text{C}$ ). The diameter of the resulting bacterial-free zone was then determined. The nematocidal activity against *P. redivivus* and *C. elegans* was obtained using a technique from the literature (Li et al., 2005). Each compound was diluted to different concentrations (400, 100 and 50 ppm) to assay for the nematocidal activity. Dilution was performed in an Eppendorf tube, with acetone used as a control. The numbers of active and inactive nematodes were counted at different times. Antitumor activity was measured by the microculture tetrazolium [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide, MTT, Sigma] assay (Su et al., 2013): Cells were treated with the indicated compounds at concentrations of 0.064, 0.32, 1.6, 8 and 40  $\mu\text{M}$  in 96-well plates. After 48 h, 0.1 mg MTT was added to each well, for a final concentration of 20%. Cells were then incubated at 37  $^{\circ}\text{C}$  for 4 h and the absorbance was measured at 595 nm by spectrophotometry. Five cancer cell lines were selected for testing (leukemia cell line HL-60, hepatocarcinoma cell line SMMC-7721, lung adenocarcinoma cell line A-549, breast cancer cell line MCF-7 and colon cancer cell line SW480), and MW300 used as a control.

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