

## ORIGINAL ARTICLE

# Pestalols A–E, new alkenyl phenol and benzaldehyde derivatives from endophytic fungus *Pestalotiopsis* sp. AcBC2 isolated from the Chinese mangrove plant *Aegiceras corniculatum*

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Five alkenyl phenol and benzaldehyde derivatives, pestalols A–E (1–5), as well as seven known compounds (6–12), were isolated from endophytic fungus *Pestalotiopsis* sp. AcBC2 derived from the Chinese mangrove plant *Aegiceras corniculatum*. Their structures were determined by spectroscopic analyses. Compounds 2 and 3 showed cytotoxicity against a panel of 10 tumor cell lines. Compounds 1–5, 8, 9, 11, and 12 showed inhibitory activities against Influenza A virus subtype (H<sub>3</sub>N<sub>2</sub>) and Swine Flu (H<sub>1</sub>N<sub>1</sub>) viruses. Compound 2 also showed inhibitory activity against tuberculosis.

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**Keywords:** *Aegiceras corniculatum*; anti-tuberculosis; antiviral; benzaldehyde; cytotoxicity; endophytic fungi; *Pestalotiopsis* sp. AcBC2

## INTRODUCTION

Endophytes are microorganisms that spend the whole or part of their life cycle colonizing tissues of their host plants without causing apparent symptoms of disease. There is supposed to be an equilibrium between microorganism virulence and plant defense. In this balanced system, the host plant provides nutrients to the endophytes, and in return, the endophytes produce bioactive substances to enhance the growth and competitiveness of the host in its natural habitat. Thus endophytes have been identified as a prolific source of biologically active small molecules. For example, numerous secondary metabolites with novel structures and biological activities have been characterized from mangrove-derived endophytic fungi.<sup>1</sup> Investigation of the secondary metabolites of microorganisms isolated from mangrove plants residing in niche environments may lead to novel biologically active compounds.<sup>2</sup>

*Aegiceras corniculatum* is a shrub or small tree mangrove of the Myrsinaceae family, with a wide distribution in coastal and estuarine areas of southern China.<sup>3</sup> As part of our ongoing efforts toward the discovery of novel and biologically active natural products from

endophytic fungi isolated from the marine mangrove of the Chinese sea coasts, we have investigated the chemical constituents of an endophytic fungal strain *Pestalotiopsis* sp. AcBC2 that was isolated from the inner tissue of stems of the mangrove plant *A. corniculatum* collected from Nansha mangrove wetland, Guangdong province, China. This paper describes the isolation, structure elucidation and bioactivities of the isolated compounds.

## RESULTS AND DISCUSSION

Fermentation of *Pestalotiopsis* sp. AcBC2 was performed in flask liquid culture, was grown in potato-dextrose broth (PDB) media. The EtOAc extracts from the culture broth and the mycelium were fractionated by repeated column chromatography on silica gel, reversed-phase silica gel C<sub>18</sub> and Sephadex LH-20, as well as by preparative TLC, to afford 12 metabolites (1–12) (Figure 1). The gross structures of the compounds were elucidated with the aid of COSY, HMQC and HMBC experiments.

Pestalol A (1) was isolated as a yellowish oil. The ESIMS spectrum of 1 displayed a pseudomolecular ion peak at m/z 311 [M + Na]<sup>+</sup> for

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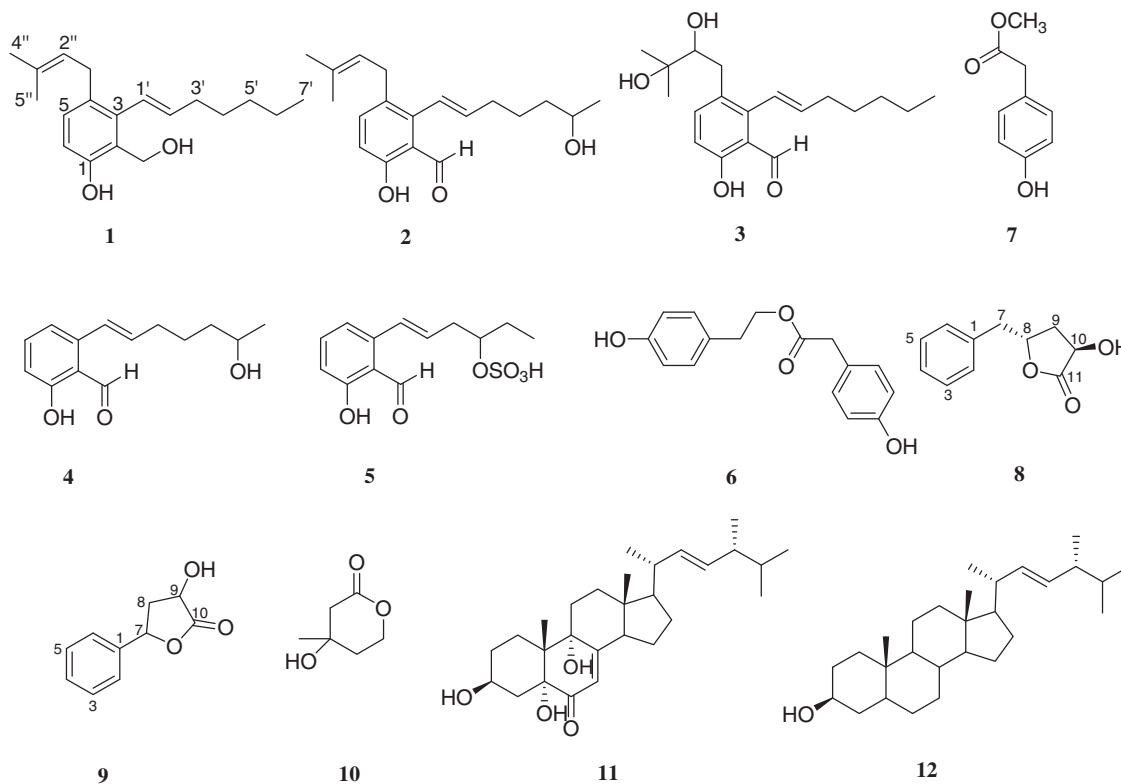
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the monosodium salt of the molecule. The molecular formula was established as  $C_{19}H_{28}O_2$  on the basis of HRESIMS, which is consistent with  $6^\circ$  of unsaturation. A tetrasubstituted benzene unit was recognized from two doublets at  $\delta_H$  7.01 (1H, d,  $J=8.0$  Hz, H-5) and 6.73 (1H, d,  $J=8.0$  Hz, H-6) in the  $^1H$  NMR spectrum (Table 1).

The  $^1H$  NMR spectrum also featured two vinylic methyl singlet at  $\delta_H$  1.71 (3H, s, H-4'') and 1.68 (3H, s, H-5'') and a trisubstituted olefinic triplet at 5.18 (1H, t,  $J=7.0$  Hz, H-2'') and  $\delta_H$  3.22 (2H, d,  $J=7.0$  Hz, H-1''); these signals were proposed to be isoprenyl group attached to the benzene ring. Signals characteristic of an alkyl chain were observed



**Figure 1** Chemical structures of compounds 1–12. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

**Table 1**  $^1H$  NMR (500 MHz) spectral data of compounds 1–5

No	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>a</sup>	5 <sup>a</sup>
4				6.89 d (8.0)	6.89 d (8.0)
5	7.01 d (8.0)	7.30 d (8.5)	7.47 d (8.5)	7.42 t (8.0)	7.42 t (8.0)
6	6.73 d (8.0)	6.80 d (8.5)	6.79 d (8.5)	6.83 d (8.0)	6.84 d (8.0)
7	4.95 s	10.1 s	10.1 s	10.1 s	10.3 s
1'	6.30 d (16.0)	6.55 d (16.0)	6.72 d (16.0)	6.86 d (16.0)	6.88 d (16.0)
2'	5.50 dt (16.0, 6.5)	5.67 dt (16.0, 7.0)	5.72 dt (16.0, 7.0)	6.09 dt (16.0, 7.0)	6.11 dt (16.0, 7.0)
3'	2.22 m	2.33 m	2.36 q (7.0)	2.29 m	2.45 m
					2.36 m
4'	1.46 m	1.66 m	1.57 m	1.69 m	3.60 m
		1.54 m		1.53 m	
5'	1.33 m	1.53 m	1.40 m, 1.25 m	1.52 m	1.55 m
6'	1.35 m	3.84 m	1.41 m	3.84 m	0.95 t (6.5)
7'	0.91 t (6.5)	1.22 d (6.5)	0.95 t (7.0)	1.22 d (6.0)	
1''	3.22 d (7.0)	3.24 d (7.0)	3.02 dd (14.0, 1.5) 2.50 dd (14.0, 10.0)		
2''	5.18 t (7.0)	5.16 t (7.0)	3.47 dd (10.5, 2.0)		
4''	1.71 s	1.72 s	1.26 s		
5''	1.68 s	1.69 s	1.23 s		
1-OH	11.8 s	11.7 s		11.8 s	11.8 s

<sup>a</sup>Measured in  $CDCl_3$ .

<sup>b</sup>Measured in  $CD_3OD$ .

at the upfield region of the  $^1\text{H}$  NMR spectrum: the presence of a primary methyl group at  $\delta_{\text{H}}$  0.91 (3H, t,  $J=6.5$  Hz, H-7'), and four methylene protons at  $\delta_{\text{H}}$  1.33 (2H, m, H-5'), 1.35 (2H, m, H-6'), 1.46 (2H, m, H-4') and 2.22 (2H, m, H-3'). A disubstituted double bond was indicated by signals at  $\delta_{\text{H}}$  6.30 (1H, d,  $J=16.0$  Hz, H-1') and 5.50 (1H, dt,  $J=16.0, 6.5$  Hz, H-2'); the geometry was determined to be *E* on the basis of the large coupling constant of the respective protons.<sup>4</sup> An isolated oxygenated methylene at  $\delta_{\text{H}}$  4.95 (2H, s, H-7) was observed; the signals must be methylene protons of phenylmethanol. In addition, the presence of a phenolic hydroxyl proton was observed at  $\delta_{\text{H}}$  11.8 (OH-1).

The  $^{13}\text{C}$  NMR spectrum (Table 2) of **1** exhibited 19 carbon signals attributable to three methyls, six  $\text{sp}^3$  methylenes (one oxygenated), five  $\text{sp}^2$  methines and five  $\text{sp}^2$  quaternary carbon atoms according to the DEPT spectrum. Detailed analysis of the one- and two-dimensional NMR spectral data resulted in the elucidation of three substructures for pestalol A (**1**). The two terminal vinylic methyl resonances ( $\delta_{\text{C}}$  17.8, C-5'';  $\delta_{\text{C}}$  25.7, C-4'') the two olefinic carbon signals ( $\delta_{\text{C}}$  123.5, C-2'' and  $\delta_{\text{C}}$  131.6, C-3'') and  $\delta_{\text{C}}$  31.5 (C-1'') were observed in the  $^{13}\text{C}$  NMR spectrum. These observations confirmed the isoprenyl group was joined to phenyl ring. In the  $^1\text{H}$ - $^1\text{H}$  COSY spectrum of **1** (Figure 2), one of the olefinic proton at  $\delta_{\text{H}}$  6.30 (H-1') correlated to the other olefinic proton resonating at  $\delta_{\text{H}}$  5.50 (H-2'). The latter was revealed by COSY to be connected to a methylene group at  $\delta_{\text{H}}$  2.22 (H-3'). The correlations from H<sub>2</sub>-4' to H<sub>2</sub>-5' and H<sub>2</sub>-3', from H<sub>2</sub>-5' to H<sub>2</sub>-6' and from H<sub>2</sub>-6' to H<sub>3</sub>-7', revealed contiguous sequence of the proton signals from H-1' to H-7'. This led to the elucidation of the substructure comprised of C-1'-C-7'. The presence of this substructure was further confirmed by HMBC correlations. In addition, the cross-peak between a two-proton doublet at  $\delta_{\text{H}}$  3.22 ( $J=7.0$  Hz, H-1'') and a one-proton broad olefinic triplet at  $\delta_{\text{H}}$  5.18 ( $J=7.0$  Hz, H-2'') in the  $^1\text{H}$ - $^1\text{H}$  COSY revealed the presence of a coupling system containing only H-1'' and H-2''. Furthermore, HMBC correlations from H-1'' to the olefinic quaternary carbon signal appearing at  $\delta_{\text{C}}$  131.6 (C-3'') and from H-2'' to a

methyl carbon signal at  $\delta_{\text{C}}$  25.7 (C-4'') as well as from both H-4'' and H-5'' to C-2'' ( $\delta_{\text{C}}$  123.5) clearly indicated the presence of a isoprenyl substructure in **1**. The two doublets at  $\delta_{\text{H}}$  7.01 (H-5) and 6.73 (H-6) proton signal in the aromatic region, as well as the remaining six carbon NMR signals resonating at  $\delta_{\text{C}}$  154.6 (C-1), 122.7 (C-2), 137.4 (C-3), 131.7 (C-4), 129.1 (C-5) and 114.8 (C-6) revealed the presence of a tetrasubstituted benzene ring system.

The connection of the substructures in **1** was accomplished by the analysis of key HMBC correlations. The cross-peaks observed from the isolated oxygenated methylene proton at  $\delta_{\text{H}}$  4.95 (2H, s, H-7) to C-2 and C-1 and from the 1-OH proton to C-1, C-2 and C-6 enabled placement of the oxygenated methylene and one hydroxyl group at C-2 and C-1, respectively. The HMBC correlations between H-1'' and C-3, C-4 and C-5 indicated the connection of the isoprenyl substituent to C-4. The HMBC correlations from H-1' to C-2 and C-4 and from H-2' to C-3 indicated the connection of C-1' ( $\delta_{\text{C}}$  126.0) at C-3. The HMBC correlations from H-6 to C-2 and C-4 and from H-5 to C-1 and C-3 were also observed. From the above deductions, the structure of compound **1** was assigned as pestalol A.

Pestalol B (**2**) was obtained as a yellow oil. The ESIMS spectrum of **2** showed a pseudomolecular ion peak at  $m/z$  325  $[\text{M} + \text{Na}]^+$ , which was in agreement with the monosodium salt formula  $\text{C}_{19}\text{H}_{26}\text{O}_3\text{Na}$ . Both the  $^1\text{H}$  and  $^{13}\text{C}$  NMR (Tables 1 and 2) spectral data of **2** were similar to those of **1**, but several differences were observed, including the carbinol group at C-7 and the methylene at C-6' of **1** being replaced by aldehyde group and an oxygenated methine, respectively, which in turn replaced by an aldehyde proton at  $\delta_{\text{H}}$  10.1 (1H, s, H-7) and an oxygenated methane proton at  $\delta_{\text{H}}$  3.84 (1H, m, H-6') in the lower field, respectively, and supported by the HMBC correlations from H-7 to C-1 and C-2, from H-6' to C-4' and from H-7' to C-6'. In addition, the  $^1\text{H}$  NMR spectrum of **2** revealed the presence of a phenolic hydroxyl proton at  $\delta_{\text{H}}$  11.7 (1H, s, H-1). In addition, the presence of an oxygenated proton signal at  $\delta_{\text{H}}$  3.84 (H-6'), and an aliphatic methyl doublet at  $\delta_{\text{H}}$  1.22 (3H, d,  $J=6.5$  Hz, H-7') were also present in the  $^1\text{H}$  NMR spectrum. The connection of the substructures in **2** was accomplished by the analysis of key HMBC correlations. The cross-peaks observed from the aldehyde proton ( $\delta_{\text{H}}$  10.1, s, H-7) to C-1 and C-3 enabled placement of the aldehyde. The methylene protons at  $\delta_{\text{H}}$  1.53 (H-5') also displayed a correlation to an oxygenated methine proton appearing at  $\delta_{\text{H}}$  3.84 (H-6'). The latter displayed a further cross-peak with the doublet methyl signal at  $\delta_{\text{H}}$  1.22 (3H, d,  $J=6.5$  Hz, H-7'). This led to the elucidation of the substructure comprised of C-1' through C-7' with a hydroxyl substitution at C-6'. The presence of this substructure was further confirmed by HMBC correlations. The absolute configuration at C-6' remained to be determined.

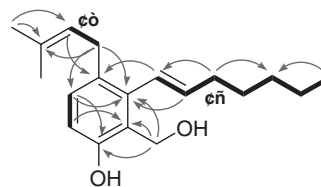
Pestalol C (**3**) was found to be a yellow oil. The ESIMS displayed a pseudomolecular ion peak at  $m/z$  343  $[\text{M} + \text{Na}]^+$ , the molecular formula was established as  $\text{C}_{19}\text{H}_{28}\text{O}_4$  on the basis of HRESIMS data.

**Table 2**  $^{13}\text{C}$  NMR (125 MHz) spectral data of compounds 1–5

No	1 <sup>a</sup>	2 <sup>a</sup>	3 <sup>b</sup>	4 <sup>a</sup>	5 <sup>a</sup>
1	154.6	161.1	162.4	162.7	162.8
2	122.7	118.7	120.0	117.3	117.3
3	137.4	142.3	144.6	143.4	143.4
4	131.7	130.9	131.0	118.8	118.8
5	129.1	137.6	140.5	137.1	137.1
6	114.8	115.8	116.2	116.4	116.4
7	61.8	197.5	198.9	195.6	195.6
1'	126.0	124.1	125.7	124.7	124.8
2'	137.5	141.5	143.3	138.6	138.6
3'	33.3	33.4	34.4	33.3	29.7
4'	29.0	25.4	30.0	25.2	72.7
5'	31.9	38.8	23.6	38.7	36.1
6'	22.5	67.8	32.8	67.9	9.9
7'	14.0	23.6	14.4	23.6	
1''	31.5	31.2	35.2		
2''	123.5	122.4	79.6		
3''	131.6	132.7	73.8		
4''	25.7	25.7	25.2		
5''	17.8	17.9	23.6		

<sup>a</sup>Measured in  $\text{CDCl}_3$ .

<sup>b</sup>Measured in  $\text{CD}_3\text{OD}$ .



**Figure 2** Key HMBC and  $^1\text{H}$ - $^1\text{H}$  COSY correlations of pestalol A **1**. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

Detailed comparison of the  $^1\text{H}$  and  $^{13}\text{C}$ -NMR spectral data (Tables 1 and 2) of **3** with those of **1** revealed that the structures of these two compounds were very similar, except that the two olefinic carbon signals of C-2'' ( $\delta_{\text{C}}$  123.5) and C-3'' ( $\delta_{\text{C}}$  131.6) in the  $^{13}\text{C}$  NMR of **1** were replaced by two oxygenated carbon signals at  $\delta_{\text{C}}$  79.6 (C-2'') and 73.8 (C-3''), respectively, in **3**. This observation was supported by the fact that one trisubstituted olefinic proton at  $\delta_{\text{H}}$  5.18 (t,  $J=5.5$  Hz, H-2'') was replaced by one oxygenated methine proton ( $\delta_{\text{H}}$  3.47, dd,  $J=10.5, 2.0$  Hz, H-2'') and confirmed by the HMBC correlations from H-5'' to C-2'' and C-3'' as well as from H-2'' to C-4. The carbinol group at C-7 of **1** was replaced by an aldehyde group in **3** (aldehyde proton at  $\delta_{\text{H}}$  10.1 (1H, s, H-7)), which was supported by the HMBC correlations from H-7 to C-1 and C-2. Thus the planar structure of **3** was established, while the absolute configurations at C-2'' remained to be determined.

Pestalol D (**4**) was isolated as a yellow oil. The ESIMS displayed a pseudomolecular ion peak at  $m/z$  257  $[\text{M} + \text{Na}]^+$ , the molecular formula of **4** was determined as  $\text{C}_{14}\text{H}_{18}\text{O}_3$  by analysis of HRESIMS and its NMR spectra. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data (Tables 1 and 2) of **4** were similar to those of **2**. These data revealed the presence of a phenolic hydroxyl proton at  $\delta_{\text{H}}$  11.8 (1H, s, OH-1) and an aldehyde proton at  $\delta_{\text{H}}$  10.1 (1H, s, H-7) in the lower field, as well as an oxygenated methine at C-6'. The only difference is the absence of an isoprenyl group at C-4 in **4**. The correlations from H-4 to H-5 to H-6 in the  $^1\text{H}$ - $^1\text{H}$  COSY spectrum of **4** supported this deduction. Based on the above evidences, the structure of **4** was assigned. The absolute configuration at C-6' remained to be determined.

Pestalol E (**5**) was isolated as a yellow oil. The ESIMS displayed a pseudomolecular ion peak at  $m/z$  301  $[\text{M} + \text{H}]^+$ , the molecular formula was established as  $\text{C}_{13}\text{H}_{16}\text{O}_6\text{S}$  on the basis of the HRESIMS. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR data (Tables 1 and 2) showed a close similarity to those of **4**. The differences were observed with regard to the substituted position of hydroxyl instead of sulfonic group and the length of side chain, in which one methylene was missing and the sulfonic group was attached to C-4'. This deduction was supported by the  $^1\text{H}$ - $^1\text{H}$  COSY connectivities of H-1'/H-2'/H<sub>2</sub>-3'/H-4'/H<sub>2</sub>-5'/H<sub>3</sub>-6' and the HMBC correlation of H<sub>3</sub>-6' ( $\delta_{\text{H}}$  0.95, t,  $J=6.5$ , Hz) to C-4'. The absolute configuration at C-4' remained to be determined. There are presumed to be close biogenetic relationships among compounds **1**–**5**, a biosynthetic route involving polyketide pathway is proposed in Figure 3.

The known compounds, 4-hydroxyphenethyl 2-(4-hydroxyphenyl) acetate (**6**),<sup>5</sup>  $p$ -hydroxyphenylacetic acid methyl ester (**7**),<sup>6</sup> *trans*-harzialactones A (**8**),<sup>7</sup> F (**9**),<sup>8</sup> 3-hydroxy-3-methyl- $\delta$ -lactone (**10**),  $3\beta$ ,  $5\alpha$ ,  $9\alpha$ -trihydroxy-7, 22-en-ergost-6-one (**11**)<sup>9</sup> and  $3\beta$ -hydroxy-sterol (**12**),<sup>10</sup> have been identified by comparison with those reported data.

The antiproliferative effect of 12 compounds were evaluated in a panel of human cancer cell lines representing colon, lung, prostate, ovarian, breast, cervical, pancreatic and melanoma malignancies. Cells were exposed to a serial of compounds for 72 h, and cell viability was measured by CCK8 assay. Of the 12 compounds, compounds **2** and **3** displayed antiproliferative effects at range of 23.4–42.5  $\mu\text{M}$  against 10 human tumor cell lines (Table 3).

To investigate the anti-influenza virus effects of the compounds, we tested the anti-proliferative activity of the compounds in influenza virus-infected Madin–Darby canine kidney (MDCK) cells (Table 4). Of the 12 compounds, 7 compounds displayed potency to different extents against the replication of A/HK/8/68 ( $\text{H}_3\text{N}_2$ ) virus and A/WSN/33 ( $\text{H}_1\text{N}_1$ ), compound **12** is the most potency, with  $\text{IC}_{50}$  of 4.7  $\mu\text{M}$  for virus A/HK/8/68 ( $\text{H}_3\text{N}_2$ ) and  $\text{IC}_{50}$  of 2.2  $\mu\text{M}$  for A/WSN/33

( $\text{H}_1\text{N}_1$ ). Compounds **6**, **7** and **10** did not show significant effect on both the replication of virus strains at the tested concentration.

To investigate the anti-tuberculosis effects of the compounds, we also tested the anti-tuberculosis activity of the compounds in the bioluminescence assay. Figure 4 shows the bioluminescence intensity of tuberculosis for 3 days after adding different compounds. Compound **2** shows inhibition to tuberculosis compared with dimethyl sulfoxide control with the INH (isoniazid) and RIF (rifampin) as positive drugs.

## EXPERIMENTAL PROCEDURE

### General experimental procedures

MS data were acquired using a Thermo DSQ mass spectrometer (Thermo, Finnigan, Baton Rouge, LA, USA).  $^1\text{H}$  and  $^{13}\text{C}$  NMR and 2D NMR data were obtained with a Bruker Avance 500 spectrometer (Bruker, Karlsruhe, Germany), with tetramethylsilane (TMS,  $\delta$  0.0 p.p.m.) as the internal standard. The optical rotations were recorded on a 341 polarimeter (Perkin-Elmer, Waltham, MA, USA). Column chromatography (CC) was performed with silica gel (100–200 mesh; 300–400 mesh; Jiangyou Silica Gel Development, Inc., Yantai, China) and Sephadex LH-20 (Pharmacia, Uppsala, Sweden). TLC (0.1–0.2 mm or 0.3–0.4 mm) was carried out with precoated silica gel plates (GF-254, Jiangyou Silica Gel Development, Inc.). Positive controls: Oseltamivir phosphate ( $\geq 97\%$ , HPLC), isoniazid ( $\geq 97\%$ , HPLC), rifampicin ( $\geq 97\%$ , HPLC), and taxol ( $\geq 97\%$ , HPLC) were purchased from Sigma-Aldrich Company Ltd (Dorset, UK).

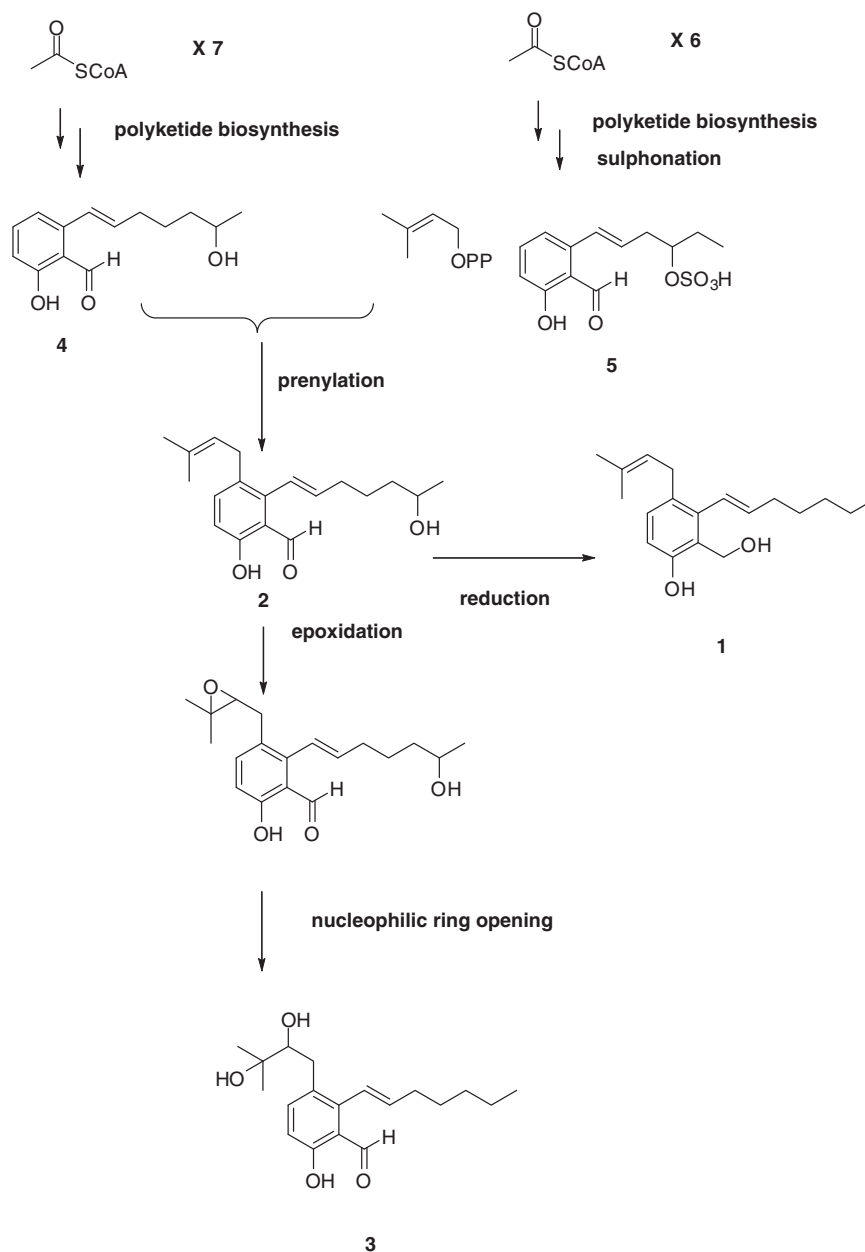
### Fungal strain

An endophytic strain AcBC2 was isolated from the inner stems of a Chinese mangrove plant *A. corniculatum* collected in Nansha mangrove wetland, Guangdong province, China. On PDA, it showed floccose colonies, the surface at first white then beige and the reverse appeared tawny. It was identified by use of a molecular biological protocol by DNA amplification and sequencing of the ITS region as our previous report.<sup>11</sup> The ITS region sequence of strain AcBC2 (525 bp, accession number is JN989552) was amplified by PCR, and results from DNA sequencing showed that it shares significant homology to those of several species of *Pestalotiopsis*, with sequence identifies ranging from 100% to 99%. The phylogenetic tree constructed using the neighbor-joining method revealed the evolutionary relationship of strain AcBC2 to a group of *Pestalotiopsis* species (Figure 5). Therefore, strain AcBC2 clearly belongs to the member of the genus *Pestalotiopsis*, designated as *Pestalotiopsis* sp. AcBC2. A voucher specimen (registration number: AcBC2) has been deposited in the CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China.

### Fermentation and extraction

Strain AcBC2 stored on PDA medium (dextrose 20 g, crude sea salt 10 g, potato infusion 1000 ml, agar 15 g) slants at 4 °C was inoculated on PDA plates and incubated for 7 days at 25 °C. Plugs of the cultures were cut and transferred into 500-ml Erlenmeyer flasks containing 100 ml of liquid seed medium (dextrose 20 g, crude sea salt 10 g, potato infusion 1000 ml) and incubated at 25 °C for 48 h on a rotating shaker (180 r.p.m.). Production medium of solid rice in 1000-ml flasks (commercial rice 200 g, crude sea salt 10 g and distilled water 200 ml) was inoculated with 10-ml seed solution. Then flasks were incubated at 25 °C under static stations and daylight. After 35 days, cultures from 15 flasks were harvested for the isolation of substances.

The culture medium containing the mycelium was cut into small pieces, mixed with ethyl acetate (EtOAc, 3000 ml) and steeped for 1 day. The content was filtered under vacuum using a Buchner funnel (Shanghai Heqi Glassware Co., Ltd., Shanghai, China (Mainland)) and the extraction with EtOAc was repeated until exhaustion. The combined filtrates were washed with 5000 ml water to eliminate remaining sugar and starch. The organic phase was collected and evaporated (40 °C) to remove EtOAc. The residue (30 g) was obtained, chromatographed on silica gel (0.6 kg, 200–300 mesh,  $10 \times 100 \text{ cm}^2$ ) and eluted with a gradient of petroleum ether (PE)–Acetone (1:0 (11), 30:1(21),



**Figure 3** Proposed biosynthetic relationships of compounds 1–5.

**Table 3** Anti-proliferative activities of the compounds against 10 tumor cell lines (IC<sub>50</sub>, μM)<sup>a</sup>

Cell lines	MCF-7	BT474	A549	DU145	H1975	SK-BR-3	K562	MOLT-4	U937	BGC823
<b>2</b>	42.5	>50	40.2	38.3	27.7	47.1	36.2	26.9	25.1	32.8
<b>3</b>	>50	39.1	>50	38.0	26.8	>50	>50	23.4	29.7	>50
Taxol	0.0090	0.0084	0.0131	0.0064	0.0026	0.0034	0.0039	0.0020	0.0019	0.0043

<sup>a</sup>All the IC<sub>50</sub> values are the mean of least three independent experiments.

**Table 4** Compound IC<sub>50</sub> values (μM) against influenza viruses determined by cytopathic effect inhibition assay<sup>a</sup>

Compounds	1	2	3	4	5	6	7	8	9	10	11	12	Oseltamivir
H <sub>3</sub> N <sub>2</sub>	22.6	27.7	42.6	48.0	>50	>50	>50	>50	>50	>50	>50	4.7	0.0034
H <sub>1</sub> N <sub>1</sub>	18.9	28.7	40.0	48.0	39.2	>50	>50	37.6	38.0	>50	36.9	2.2	0.0075

<sup>a</sup>All the IC<sub>50</sub> values are the mean of least three independent experiments; influenza viruses, A/HK/8/68 (H<sub>3</sub>N<sub>2</sub>) and A/WSN/33 (H<sub>1</sub>N<sub>1</sub>).

20:1(21), 10:1(1.51), 5:1(1.51), 1:1(21), 0:1(21)) to yield nine fractions (Fr. 1–9). Fr. 4 (6.6 g) was chromatographed on a Sephadex LH-20 column ( $3 \times 160 \text{ cm}^2$ ) using MeOH to produce six fractions (Fr. F41–46). Fr. 44(2.4 g) was further separated by silica gel column chromatography ( $3 \times 60 \text{ cm}^2$ , 80 g, 200–300 mesh) and eluted with PE–EtOAc (v/v from 4:1 to 2:1, then pure EtOAc, each 1 l), resulting in four fractions (Fr. 441–444). A further separation of Fr. 441 (50 mg) yielded **1** (5.1 mg) by preparative TLC using  $\text{CHCl}_3$ –MeOH (16:1) as developers. A further separation of Fr. 442 (60 mg) yielded **2** (8.0 mg) by preparative TLC using  $\text{CHCl}_3$ –Acetone (9:1) as developers. A further separation of Fr. 444 (550 mg) yielded **4** (4.2 mg) by preparative TLC using  $\text{CHCl}_3$ –Acetone (9:4) as developers. Fr. 46 (3 g) was further separated by silica gel column chromatography ( $3.5 \times 60 \text{ cm}^2$ , 100 g, 200–300 mesh) and eluted with PE–EtOAc (4:1, 2 l), resulting in three fractions (Fr. 461–463). A further separation of Fr. 463 (550 mg) yielded **5** (3.5 mg) by preparative TLC using  $\text{CHCl}_3$ –Acetone (9:1) as developers. Fr. 5 (2.4 g) was further separated by silica gel column chromatography ( $3.5 \times 60 \text{ cm}^2$ , 100 g,

200–300 mesh) and eluted with PE–EtOAc (4:1, 2.5 l), resulting in five fractions (Fr. 51–55). A further separation of Fr. 53 (150 mg) yielded **3** (2.5 mg) by preparative TLC using  $\text{CHCl}_3$ –Acetone (8:1) as developers. A further separation of Fr. 55 (135 mg) yielded **8** (2.8 mg) and **9** (4.5 mg) by preparative TLC using  $\text{CHCl}_3$ –EtOAc (8:1) as developers. Fr. 7 (2.9 g) was chromatographed on a Sephadex LH-20 column ( $3 \times 160 \text{ cm}^2$ ) using MeOH to produce seven fractions (Fr. F71–77), Fr. 72 (600 mg) was further separated by silica gel column chromatography ( $2.5 \times 40 \text{ cm}^2$ , 60 g, 200–300 mesh) and eluted with  $\text{CHCl}_3$ –Acetone (10:1, 1 l), resulting in four fractions (Fr. 721–724). A further separation of Fr. 722 (120 mg) yielded **11** (8.7 mg) and **12** (9.5 mg) by preparative TLC using  $\text{CHCl}_3$ –Acetone (8:1) as developers. A further separation of Fr. 74 (136 mg) yielded **10** (6.1 mg) by preparative TLC using PE–EtOAc (1:1) as developers. A further separation of Fr. 76 (146 mg) yielded **7** (5.5 mg) and **6** (2.5 mg) by preparative TLC using  $\text{CHCl}_3$ –Acetone (8:1) as developers.

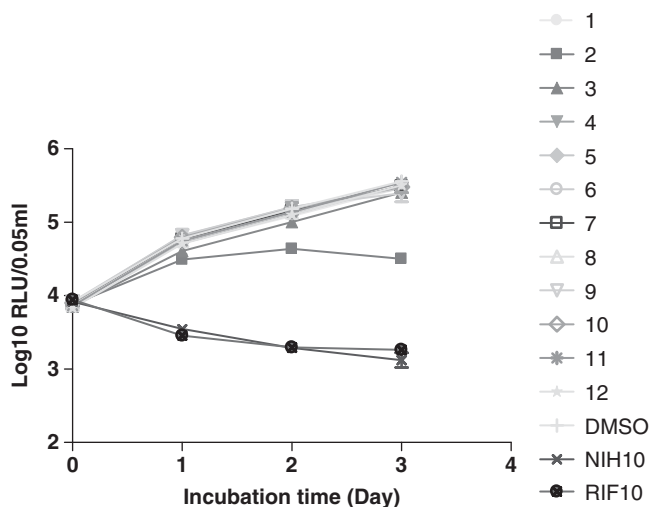
**Pestalol A (1):** Yellowish oil;  $[\alpha]_{\text{D}}^{20}$ : 0 (*c* 0.64,  $\text{CHCl}_3$ );  $^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 500/125 MHz), see Tables 1 and 2; ESIMS *m/z* 311  $[\text{M} + \text{Na}]^+$ ; HRESIMS *m/z* 311.1968  $[\text{M} + \text{Na}]^+$  (calcd for  $\text{C}_{19}\text{H}_{28}\text{O}_2\text{Na}$ , 311.1987).

**Pestalol B (2):** Yellow oil;  $[\alpha]_{\text{D}}^{20}$ : –6.3 (*c* 0.84,  $\text{CHCl}_3$ );  $^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 500/125 MHz), see Tables 1 and 2; ESIMS *m/z* 325  $[\text{M} + \text{Na}]^+$ ; HRESIMS *m/z* 325.1758  $[\text{M} + \text{Na}]^+$  (calcd for  $\text{C}_{19}\text{H}_{26}\text{O}_3\text{Na}$ , 325.1779).

**Pestalol C (3):** Yellow oil;  $[\alpha]_{\text{D}}^{20}$ : –69.3 (*c* 1.3, MeOH);  $^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CD}_3\text{OD}$ , 500/125 MHz), see Tables 1 and 2; ESIMS *m/z* 343  $[\text{M} + \text{Na}]^+$ ; HRESIMS *m/z* 343.1877  $[\text{M} + \text{Na}]^+$  (calcd for  $\text{C}_{19}\text{H}_{28}\text{O}_4\text{Na}$ , 343.1885).

**Pestalol D (4):** Yellow oil;  $[\alpha]_{\text{D}}^{20}$ : –5.6 (*c* 0.60,  $\text{CHCl}_3$ );  $^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 500/125 MHz), see Tables 1 and 2; ESIMS *m/z* 257  $[\text{M} + \text{Na}]^+$ ; HRESIMS *m/z* 257.1140  $[\text{M} + \text{Na}]^+$  (calcd for  $\text{C}_{14}\text{H}_{18}\text{O}_3\text{Na}$ , 257.1153).

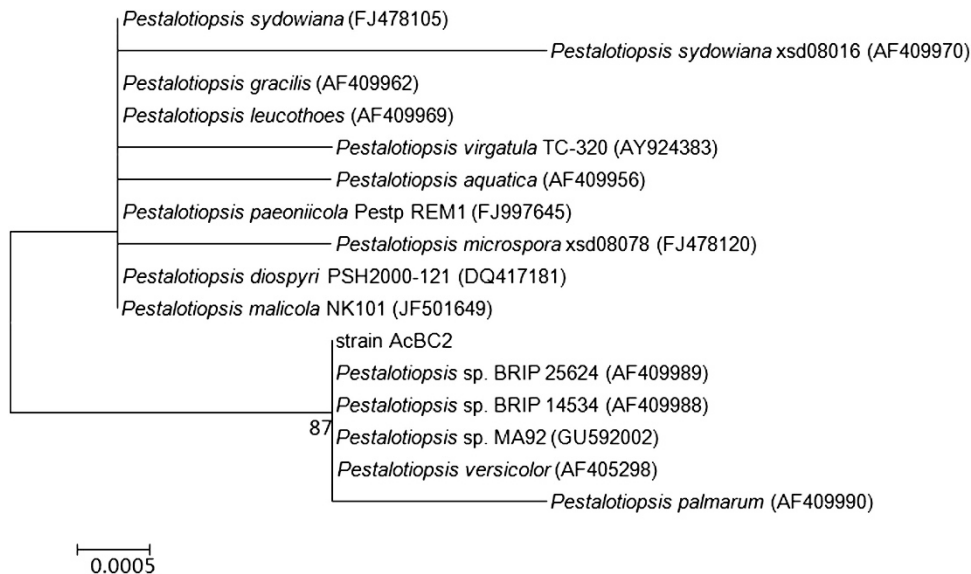
**Pestalol E (5):** Yellow oil;  $[\alpha]_{\text{D}}^{20}$ : –8.2 (*c* 0.08,  $\text{CHCl}_3$ );  $^1\text{H}$  and  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 500/125 MHz), see Tables 1 and 2; ESIMS *m/z* 301  $[\text{M} + \text{H}]^+$ ; HRESIMS *m/z* 301.0756  $[\text{M} + \text{H}]^+$  (calcd for  $\text{C}_{13}\text{H}_{17}\text{O}_6\text{S}$ , 301.0745).



**Figure 4** The figure shows the bioluminescence intensity of different compounds against tuberculosis for 3 days. The purple line refers to the effective compound **2** ( $50 \mu\text{M}$ ). INH, isoniazid, RIF, rifampin. A full color version of this figure is available at *The Journal of Antibiotics* journal online.

## Bioassays

**Cell proliferation and growth inhibition assay.** The NSCLC cell lines H1975 and A549 were purchased from ATCC (Manassas, VA, USA), the breast cancer cell lines MCF-7, SK-BR-3 and BT474 were obtained from Shanghai Institutes for Biological Sciences, CAS, Guangzhou, China; the prostate cancer cell lines DU145, the gastric cancer cell line BGC-823 and the leukemia cell lines K562, U937 and MOLT-4 were gifts from Dr K Ding, Guangzhou institutes of Biomedicine and Health, Chinese Academy of Sciences, Guangzhou, China. The cells were maintained at  $37^\circ\text{C}$  in a 5%  $\text{CO}_2$  incubator in RPMI 1640 (Gibco, Invitrogen, Carlsbad, CA, USA) or Dulbecco's modified Eagle's



**Figure 5** Neighbor-joining tree based on ITS region sequences, showing phylogenetic relationship between strain AcBC2 and related *Pestalotiopsis* species. Numbers at nodes indicate bootstrap values from 1000 replicates. GenBank accession numbers are given in parentheses. Bar, 0.05% sequence divergence.

medium (Gibco, Invitrogen) containing 10% fetal bovine serum (Gibco, Invitrogen). The DU145 was maintained in Ham's/F-12 medium (Gibco, Invitrogen) with 10% fetal bovine serum. Cell proliferation assay was determined by the CCK8 (Dojindo, Kumamoto, Japan) assay. Cells were seeded at a density of 800–1000 cells/well in 384 well plates and treated with various concentration of compounds or solvent control. After 72 h incubation, CCK8 reagent was added, and the absorbance was measured at 450 nm using Envision 2104 multi-label Reader (Perkin Elmer, Foster City, CA, USA). All experiments were repeated at least three times. Dose-response curves were plotted to determine the IC<sub>50</sub> values using Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

**MDCK cell-based anti-influenza inhibitor assay.** Cell-based anti-influenza virus inhibitor screening was based on the principle of cytopathic effect protection assay. In brief, MDCK cells cultured to approximately 90% confluence were detached with 0.25% Trypsin-EDTA (Invitrogen), washed and re-suspended in complete Eagle's minimum essential medium. In all,  $2.5 \times 10^4$  MDCK cells were plated in triplicate in a 96-well plate and incubated overnight at 37 °C in a humidified 5% CO<sub>2</sub> incubator. The confluent MDCK monolayers cells were rinsed twice with Hanks' solution devoid of serum, then the cells were treated with 50 µl medium with 1 mg ml<sup>-1</sup> TPCK and 0.3% bovine serum albumin and infected by a A/WSN/33 (H<sub>1</sub>N<sub>1</sub>) virus or A/Hong Kong/8/68(H<sub>3</sub>N<sub>2</sub>) virus at a multiplicity of infection of 0.01 plaque-forming units/cell. After 2 h incubation, serially diluted compounds were added. After 72 h incubation, the medium was removed and 50 µl medium containing 5 µl CCK8 reagent was added into each well followed by an additional 2-h incubation. The absorbance was measured at 450 nm using a UV-Star-Microplates Synergy HT plate reader (Greiner Bio-One, Frickenhausen, Germany). The IC<sub>50</sub> values were calculated by nonlinear regressions using GraphPad Prism 5.

**Anti-tuberculosis drug screening in vitro.** Autoluminescent *Mycobacterium tuberculosis* were inoculated in a 50-ml centrifuge tube containing 5 ml 7H9 with 0.1% Tween 80 and 10% OADC and then incubated at 37 °C. When the cultures reached an optical density at 600 nm of 0.3–1.0, the culture was diluted and 50 µl diluted H37Ra were inoculated in sterile 384-well plates, the relative light units of which should be between 10 000 and 50 000 and were recorded as the base luminescent at day 0. The compounds and the positive drug were added to the 384-well plates in triplicate by the Echo520 (Bucher Biotec, Basel, Switzerland) with the final concentration 50 µM. The luminescent value was detected for the following 3 days. The data were analyzed with the Excel (Microsoft, Redmond, WA, USA) and compared with dimethyl sulfoxide control to estimate the inhibition activity of the compounds.<sup>12</sup>

Supporting information: The NMR spectra of compounds 1–5 are available as Supplementary Information.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

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