A new bioactive diterpenoid from *Pestalotiopsis adusta*, an endophytic fungus from *Clerodendrum canescens*

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**ABSTRACT**

Bioassay-guided fractionation of the culture extract of *Pestalotiopsis adusta*, an endophytic fungus isolated from the medicinal plant *Clerodendrum canescens*, led to the isolation of one new, (10$^S$)-12,16-epoxy-17(15→16)-abeo-3,5,8,12,15-abietapentaen-2,7,11,14-tetraone (1), and four known diterpenoids, teuvincenone F (2), uncinatone (3), coleon U (4), coleon U-12-methyl ether (5). These structures were identified by using spectroscopic methods, including UV, MS, 1D and 2D NMR experiments. This is the first report of these compounds being isolated from a *Pestalotiopsis* species. The cytotoxic activities of the compounds were evaluated, and compounds 1 and 3 demonstrated cytotoxic activities against the HL-60 tumour cell line (IC$_{50}$ < 20 μM).

1. Introduction

Endophytic fungi are increasingly being recognised as an important source of bioactive natural products (Meng et al. 2015; Siriwardane et al. 2015). Since discovery of the
anticancer agent taxol from an endophytic fungal strain of the genus *Pestalotiopsis*, interest in bioactive compounds from this fungal genus has increased considerably (Xu et al. 2010; Yang et al. 2012). Previous chemical investigations of *Pestalotiopsis* spp. led to the discovery of various bioactive natural products, such as diterpenoids, flavonoid glycosides, polyketides and terpenoids (Subban et al. 2013; Bharitkar et al. 2015; Nandi et al. 2015; Yang et al. 2015; Yue et al. 2015). As part of our ongoing efforts to isolate and identify bioactive substances from plants and fungi, we noted that *Pestalotiopsis adusta*, an endophytic fungus from *Clerodendrum canescens* contained cytotoxic substances. In this report, we describe the isolation, structure elucidation and cytotoxic activities of the five compounds from *P. adusta*. Our research showed that the endophytic *P. adusta* is a potentially significant resource of natural products.

2. Results and discussion

Compound 1 was obtained as yellowish needles, it was assigned the molecular formula \( \text{C}_{20}\text{H}_{16}\text{O}_{5} \) (13° of unsaturation) by HRESIMS \((m/z\ 335.0920 \ [M - H]^{-}\), Calcd for \( \text{C}_{20}\text{H}_{15}\text{O}_{5}^{-}\, 335.0919\)). The absorption bands in the UV spectrum (224, 268, 489 nm) exhibited the presence of a benzoquinone moiety. In the IR spectrum, a carbonyl signal was observed at 1724 cm\(^{-1}\) in addition to the absorption peaks at 1675 and 1617 cm\(^{-1}\) for the \( \text{p} \)-quinone moiety. The \(^{13}\text{C}\) NMR and DEPT spectra of 1 showed the presence of 20 carbon atoms: four methyls, one methylene, two methines, four carbonyl groups and nine quaternary carbons. Its \(^1\text{H}\) and \(^{13}\text{C}\) NMR spectra were almost identical with those of teuvincenone F (Cuadrado et al. 1992). In fact, the only differences were consistent with the presence in compound 1 of a keto carbonyl at 182.3 ppm in the \(^{13}\text{C}\) NMR spectrum allowed it to be assigned to C-7 and thus conjugated to the \( \text{p} \)-quinone moiety rather than being an isolated oxo group. Two quinone carbonyls were observed at 179.3 and 173.5 ppm, and the four downfield carbon signals at \( \delta_{\text{C}} 129.6, 154.5, 160.9, 129.2\) ppm were assignable to olefinic carbon atoms as members of the quinone ring. The \(^1\text{H}\) NMR spectrum of 1 showed the presence of two olefinic protons at \( \delta_{\text{H}} 6.66 \) and 6.51, and four methyl singlets at \( \delta_{\text{H}} 2.48, 2.02, 2.20 \) and 1.70, assigned to C-17, C-18, C-19 and C-20, respectively. Coupled resonances at \( \delta_{\text{H}} 2.51 \) and 3.93 were assigned to a methylene at C-1. The presence of four methyl singlets in the \(^1\text{H}\) NMR and 20 carbon signals in the \(^{13}\text{C}\) NMR spectrum suggested that 1 possessed an abietane diterpenoid structure. Our assignments were supported by HMBC data that showed correlations from H-17 to C-15, from H-15 to C-12, and C-16. The negative absorption at 305 nm in the CD spectrum showed that the structure had the same abietane absolute configuration as mandarone A (Fan et al. 1999). Therefore, the structure of 1 was elucidated as (105)-12,16-epoxy-17(15→16)-abeo-3,5,8,12,15-abietapentaen-2,7,11,14-tetraone.

The structures of the known compounds were determined by comparison of their physicochemical and spectral data with literature values, and they were identified as teuvincenone F (2) (Cuadrado et al. 1992), uncinatone (3) (Tian et al. 1993), coleon U (4) (Carreiras et al. 1990), and coleon U-12-methyl ether (5) (Carreiras et al. 1990), respectively (Figure 1).

The cytotoxic activities of 1–5 are summarised in Table 1. All showed some degree of cytotoxic activities, and compounds 1 and 3 exhibiting moderate cytotoxic activities (IC...
values of 12.54 ± 1.18 and 15.66 ± 2.01, respectively) against HL-60 tumour cell line, comparable to with those observed for positive control (IC$_{50}$ 9.20 ± 1.02 μM).

3. Experimental

3.1. General

Optical rotations were measured on a Perkin–Elmer 241 automatic polarimeter (Perkin Elmer, Massachusetts, USA), and UV data were obtained on a Shimadzu UV-2550 spectrophotometer (Shimadzu, Kyoto, Japan), and IR data on a Nicolet 380 FT-IR spectrophotometer (Thermo Fisher Scientific, USA); Electron-capture dissociation spectra were recorded at room temperature with a Jasco J-815 spectropolarimeter (Jasco, Tokyo, Japan); NMR spectra were recorded on a Bruker AVANCE III 500 spectrometer (Bruker, Bremen, Germany), using TMS as internal standard; HRESIMS data were obtained on an Agilent 6210 TOF–MS mass spectrometer; column chromatography (CC), silica gel (Qing Dao Hai Yang Chemical Group Co., Qingdao, China; 200–300 mesh), Sephadex LH-20; high-performance liquid chromatography (HPLC) (Amersham Biosciences, GE Healthcare Life Science, USA), Waters 1525 semi-preparative

Table 1. Cytotoxicity of compounds isolated from P. adusta.

| Compounds | IC$_{50}$ (μM) | Compounds | IC$_{50}$ (μM) | Compounds | IC$_{50}$ (μM) |
|-----------|----------------|-----------|----------------|-----------|----------------|
| 1         | 12.54 ± 1.18   | 3         | 15.66 ± 2.01   | 5         | 66.41 ± 7.29   |
| 2         | 25.06 ± 2.89   | 4         | 57.60 ± 4.98   | Cisplatin | 9.20 ± 1.02    |

Note: IC$_{50}$ is the concentration that affords 50% inhibition of the growth.
HPLC system (Waters Co. Ltd, Milford, MA, USA) coupled with a Waters 2996 photodiode array detector. A Kromasil C18 preparative HPLC column (250 × 10 mm, 5 μm) was used; TLC (Merck, Darmstadt, Germany) silico-gel F254 plates visualisation under UV light, and by spraying with 10% aq. H$_2$SO$_4$.

3.2. Identification of *C. canescens* and fungal isolation

Fresh, healthy stems of *C. canescens* were collected in September 2012 from the mountain of South Yandang, Zhejiang Province, People’s Republic of China. The plant was identified by Dr Chunhui Dai in Zhejiang Academy of Traditional Chinese Medicine. Voucher specimens (201206) have been deposited in the Key Laboratory for Genetic Improvement and Quality Control of Medical Plants of Zhejiang Province. The plant stems were washed three times in sterilised distilled water to remove dust and debris and dried on sterile filter paper. The clean material was cut into small pieces (about 0.5 × 0.5 × 0.5 cm$^3$). Sterile conditions were maintained for the isolation of endophytes and all the work was performed in a laminar flow hood to avoid contamination. Surface sterilisation of the samples was achieved with 95% ethanol for 1 min, 10% sodium hypochlorite for 10 min and 70% ethanol for 2 min, and then, the stems were allowed to dry in a sterile environment. The tissues were placed on isolation media (potato dextrose agar; PDA) in Petri dishes supplemented with 200 mg/L chloramphenicol and 200 mg/L streptomycin to suppress bacterial growth, and incubated at room temperature (27 °C) until the outgrowth of endophytes was observed. Single fungal colonies were removed and transferred onto sterile potato dextrose agar (PDA: cooking water from 200 g potatoes/L, added with glucose 40 g/L, and agar 20 g/L) and potato sucrose agar (PSA: potato cooking water (200 g potatoes/L), sucrose 40 g/L, agar-agar 20 g/L) or M2 agar (malt extract 10 g/L, yeast extract 4 g/L, glucose 4 g/L, and agar-agar 15 g/L) and periodically checked for purity. Each isolate was kept in a slant agar tube for future investigations.

3.3. Identification of the endophytic fungus

The strain was identified to be *P. adusta* based on sequence analysis of the ITS region of the ribosomal DNA. The sequence data have been deposited at GenBank (Accession number KF011509). A voucher strain has been stored at the Key Laboratory for Genetic Improvement and Quality Control of Medical Plants of Zhejiang Province.

3.4. Fermentation, extraction and isolation

The fungus isolated CCA-1 was cultured on slants of PDA at 27 °C for 7 days. Agar plugs were cut into small pieces under aseptic conditions and 200 pieces were used to inoculate 100 Erlenmeyer flasks, each containing 100 mL potato dextrose broth (0.4% glucose, 1% malt extract and 0.4% yeast extract); the final pH of the media was adjusted to 6.5 and sterilised by autoclave. 100 flasks of the inoculated media were incubated at room temperature (27 °C) using a rotary shaker for 14 days. Following incubation, the mycelia and solid rice media were extracted three times with EtOAc and the filtrate was concentrated in a rotating evaporator under reduced pressure to afford a dark brown gum (30.5 g). A portion of the extract (25.0 g) was fractionated by CC on silica gel to give four fractions (F$_1$–F$_4$), eluted with dichloromethane–methanol (CH$_2$Cl$_2$–MeOH) mixtures of increasing polarity. Fraction F$_2$ which eluted with
CH₂Cl₂–MeOH (80:20), was chromatographed by silica gel column chromatography eluting with n-hexane–EtOAc with increasing polarity to give three subfractions (F₂₆–F₂₉). F₂₆ (20 mg) was applied to a Sephadex LH-20 column (CH₂Cl₂–MeOH, 50:50) to afford the new compound \(1\) (10.5 mg) and \(2\) (12.7 mg). F₂₈ was further purified by Sephadex LH-20 (CH₂Cl₂–MeOH, 30:70) and preparative HPLC (MeOH–H₂O, 80:20) to give \(3\) (8.2 mg) and \(4\) (14.1 mg). F₂₉ was applied to Sephadex LH-20 eluting with CH₂Cl₂–MeOH (20:80) to afford \(5\) (18.2 mg).

Compound \(1\): yellowish needles. HPLC purity grade 97%. \([\alpha]^{22}_D\) 120.1° \((c = 0.1,\ CHCl₃)\). \(^1\)H NMR (500 MHz, CDCl₃) \(\delta\): 2.51 (1H, m, H-1α), 3.93 (1H, m, H-1β), 6.67 (1H, s, H-6), 6.51 (1H, s, H-15), 2.49 (3H, s, H-17), 2.02 (3H, s, H-18), 2.20 (3H, s, H-19), 1.70 (3H, s, H-20). \(^13\)C NMR (125 MHz, CDCl₃) \(\delta\): 45.5 (C-1), 193.9 (C-2), 136.6 (C-3), 144.8 (C-4), 158.3 (C-5), 126.2 (C-6), 182.3 (C-7), 129.6 (C-8), 154.5 (C-9), 42.6 (C-10), 179.3 (C-11), 160.9 (C-12), 129.2 (C-13), 173.5 (C-14), 104.0 (C-15), 149.2 (C-16), 13.6 (C-17), 11.6 (C-18), 16.8 (C-19), 26.4 (C-20). HRESIMS: 335.0920 ([M – H]⁻, Calcd 335.0919).

3.5. **Cytotoxicity assay**

The inhibitory effects of the compounds against HL-60 cells were determined using a MTT assay to measure viable cells using cisplatin as a positive control and activity is expressed as percentage inhibition relative to the negative control (He et al. 2012). The dose resulting in 50% inhibition of cell growth (IC₅₀) was calculated by NDST software.

4. **Conclusion**

A new (1) and four known abietane diterpenoids, teuvincenone F (2), uncinatone (3), coleon U (4), coleon U-12-methyl ether (5) have been isolated from the endophytic fungus *P. adusta*. These structures were identified by using spectroscopic methods. All the isolated compounds were evaluated for cytotoxicity against HL-60 tumour cell line, and compound (1) and (3) exhibited moderate cytotoxic activities with IC₅₀ value < 20 μM.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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