Caryophyllene-type sesquiterpenoids and α-furanones from the plant endophytic fungus Pestalotiopsis theae

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Introduction

Caryophyllene-type sesquiterpenoids are a group of structurally unique secondary metabolites characterized by the bicyclo[7.2.0]undecane ring system, flexibly rearranged to various polycyclic derivatives through oxidation and cyclization [1-3]. They have been isolated frequently from various fungi origins such as Cytospora [4], Hansfordia [5], Ascomyceta [6], Puliaria [7] and members of the genus Pestalotiopsis [8-10]. Some of caryophyllene analogues are found with a wide range of biological functions including cytotoxic [11], immunosuppressive [12], analgesic [13] and antimicrobial activities [14]. Their attractive structures and profound bioactivities have attracted much attention from synthetic chemists [15-18].

Fungi have contributed significantly to drug development as valuable sources of lead compounds with inspiring novel structures [16-19]. Endophytic fungi, which inhabit normal tissues of healthy plants, are rich resources of bioactive secondary metabolites with a broad range of structural diversity [20-21]. The widely distributed endophytic fungi, Pestalotiopsis spp., has attracted much attention owing to the discovery of structurally diverse and biologically active secondary metabolites [22-25], including the anticancer drug, paclitaxel, which was isolated from P. guepinii and P. microspore [26-27]. In our search for structurally unique and bioactive natural products from the endophytic fungi, a strain of P. theae (N635), isolated from the branches of the tea plant Camellia sinensis (Theaceae) in the suburb of Hangzhou, China, was grown in different solid-substrate fermentation cultures. Chemical studies of the resulting crude extracts had afforded two cytotoxic spiroketal and their putative biosynthetic precursors [28], and nine cytotoxic and antioxidant polyketides [29]. Since the HPLC fingerprint of the remaining fractions revealed the presence of metabolites with different UV absorption spectra, further chemical investigations of these fractions were carried out. Two new caryophyllene-type sesquiterpenoids, pestathenols A (1) and B (2) and one new α-furanone, pestatheranone A (6), along with five known compounds (3–5, 7 and 8) have been isolated from the crude extract of the plant endophytic fungus Pestalotiopsis theae. Their structures were unambiguously established by extensive spectroscopic analyses. The absolute configuration of the 5,6-diol moiety in 1 was assigned using Snatzke’s method. Compounds 1 and 2 showed weak cytotoxicity against HeLa cell line.

Results and Discussion

Chromatographic separation of the EtOAc extract of P. theae cultured in solid rice medium, including semipreparative HPLC purification, led to the isolation of compounds 1–8, three of which were new. Pestathenol A (1) was obtained as yellow oil. The molecular formula C_{37}H_{50}O_{4} with six degrees of unsaturation, was determined by its HR-ESI-MS data (m/z 375.1777 [M +
The $^1$H and $^{13}$C NMR (Table 1) together with the HSQC spectrum of 1, revealed the presence of five methyls, three methylenes (one oxygenated), three methines (two oxygenated), four $sp^3$ quaternary carbons with one oxygenated, two olefinic carbons (two protonated), and two carboxylic carbons ($\delta_C$ 170.5 and 171.0, respectively). These data accounted for all $^1$H and $^{13}$C resonances except for two exchangeable protons, and suggested that 1 was a tricyclic compound. Interpretation of the $^1$H–$^1$H COSY NMR data (Fig. 2) defined three isolated spin-systems of C-2–C-3, C-6–C-7, and C-9–C-10–C-11 subunits of structure 1. HMBC correlations from the geminal methyls from H$_2$-13 and H$_2$-14 to C-3, C-4, and C-5, from H-2 and H$_2$-3 to C-4, and C-5 revealed the connections of C-4 to C-3, C-5, C-13, and C-14, along with the connection of C-2 to C-5, completing the cyclobutane moiety. Other correlations from H-2 to C-1 and C-11, H-11 to C-1 and C-8, and from H$_2$-12 to C-1, C-2, C-8 and C-11 indicated that C-2, C-8, C-11 and C-12 were all attached to the $sp^3$ quaternary carbon C-1. Further correlations from H-7 to C-1, C-8, C-9 and C-15, and from H-9 to C-1, C-8 and C-15, as well as from H$_2$-15 to C-1, C-7, C-8 and C-9 implied that C-7, C-9 and C-15 were all attached to the $sp^3$ quaternary carbon C-8 to form the cyclopentene ring. Key HMBC cross-peaks from the olefinic protons H-6 and H-7 to C-5 and from H-2 to C-6 revealed that C-5 was located between C-2 and C-6, permitted completion of the cyclohexane ring. Fusion of the cyclobutane moiety with the cyclohexane subunit at C-2/C-5 formed a bicyclo[4.2.0]octane skeleton, which fused to the cyclopentene ring at C-1/C-8 to complete the octahydro-1H-cyclobuta[e]indene core structure of 1. In addition, the cross-peaks from H-9 and H$_2$-17 to the carboxylic carbon C-16 ($\delta_C$ 170.5), and from H$_2$-12 and H$_2$-19 to the carboxylic carbon C-18 ($\delta_C$ 171.0) established the locations of these acetyl groups at C-9 and C-12, respectively. The two exchangeable protons were located at C-5 and C-6, respectively, by default, which partially supported by the chemical shift values for C-5 ($\delta_C$ 81.5) and C-6 ($\delta_C$ 68.4). Collectively, the planar structure of 1 was determined as a C-12 acetoxylated punctaporin P (3).

The relative configuration of 1 was deduced by analysis of the $^1$H–$^1$H coupling constants and NOESY data (Fig. 3), as well as by comparison with those of 3. The C-10/C-11 olefin was assigned the Z-geometry based on the $J$ value of 6.0 Hz.

<table>
<thead>
<tr>
<th>No.</th>
<th>$\delta_H$ ($J$ in Hz)</th>
<th>$\delta_C$</th>
<th>$\delta_H$ ($J$ in Hz)</th>
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**Fig. 1** Chemical structures of compounds 1–8
The absolute configuration of the 5,6-diol moiety in 1 was determined to be 1S, 2R, 5S, 6S, 8S, 9R.

The molecular formula of pestathenol B (2) was determined to be C_{16}H_{20}O_{6} (six degrees of unsaturation) by HR-ESI-MS (m/z 375.1781 [M + Na]+), which is same with that of 1. The NMR data of 2 (Table 1) revealed nearly identical structural features to those of 1, except that the oxymethine proton signal (H-6) was shifted downfield (δ 5.28 in 1 vs. 4.06 in 2). In addition, the oxymethene proton signals (H_{2}-12) were shifted upfield (δ_{H} 3.53/3.76 in 1 vs. 4.44/4.58 in 2), indicating that the exchangeable proton located at C-6 was replaced by an acetyl unit (δ_{C} 2.14/21.4, 170.8) and the acetyl unit located at C-12 was replaced by the exchangeable proton. This was further confirmed by HMBC correlation from H-6 to the carboxylic carbon (δ_{C} 170.8). The relative and absolute configurations of 2 were deduced as shown by analogy to 1 (Fig. 3), which was further confirmed by comparison of its CD data with those of 1 (Fig. 5).
Pestatheranone A (6) was assigned the molecular formula C_{12}H_{16}O_{3} (4 degrees of unsaturation) on the basis of HR-ESI-MS data (m/z 211.1330 [M + H]^+). Analysis of the ^1H, ^13C and HSQC NMR data (Table 2) revealed two methyl groups, six methylene groups (one oxygenated), two olefinic carbons, one carboxylic carbon (δ_C 175.6), and one ketone carbon (δ_C 209.9). Interpretation of the ^1H–^1H COSY NMR data of 6 established two isolated proton spin-systems of C-10–C-11–C-12 and C-6–C-7–C-8 (Fig. 2). HMBC correlations from H_2-5 and H_2-13 to C-2, C-3 and C-4 completed the α, β-unsaturated lactone moiety with C-13 attached to C-3. HMBC cross-peaks from H_2-7, H_2-8, H_2-10 and H_2-11 to the ketone carbon C-9 (δ_C 209.9) revealed that C-9 was located between C-8 and C-10. Therefore, the planar structure of 6 was assigned as shown.

On the basis of the NMR and MS spectroscopic data comparison with those reported in the literatures, in addition to the specific rotation, the other five compounds were identified as punctaporins P (3), O (4), R (5) [3], ficifuranone B (7) [30] and decaestricitene D (8) [31].

Compounds 1–8 were tested for cytotoxicity against HeLa (human cervical carcinoma cell line), MCF-7 (human breast cancer cell line), HepG2 (human hepatoma cell line), and ACHN (human renal carcinoma cell line). Only compounds 1 and 2 showed cytotoxic to HeLa cell line, with IC_{50} values of 78.2 and 88.4 μmol·L^{-1}, respectively, whereas the corresponding positive control cisplatin showed IC_{50} value of 21.1 μmol·L^{-1}. While compounds 3–8 did not show detectible inhibitory effects on the cell lines tested at 100 μmol·L^{-1}.

Biogenetically, β-caryophyllene, derived from farnesyl diphosphate could be the biosynthetic intermediate for compounds 1–5, first via cycloaddition, oxidation and dehydration, and then followed by a series of methylation or acetylation to form 1–5 (Scheme 1). However, compounds 6–8 could be biosynthesized by highly reducing polyketide synthases from one molecule of acetyl-CoA and four molecules of malonyl-CoA, via reactions including oxidation, reduction, dehydration, methylation, and esterification (Scheme 1). Compound 7 could be the oxidation product of compound 6. In summary, two new caryophyllene-type sesquiterpenoids and one new α-furanone, along with five known compounds have been isolated from the crude extract of the fungus P. theae. The discovery of these secondary metabolites further expanded the structural diversity of the natural products produced by the fungal genus Pestalotiopsis.

**Experimental**

**General procedures**

Optical rotations were measured on an Anton Paar MCP 200 Automatic Polarimeter and UV data were obtained on a Thermo Genesys-10S UV/Vis spectrophotometer. IR data were recorded using a Nicolet IS5 FT-IR spectrophotometer. ^1H and ^13C NMR data were acquired with Bruker Avance-500 spectrometer using solvent signals (acetone-d_6: δ_H 2.05/δ_C 29.8, 206.1; CDCl_3: δ_H 7.26/δ_C 77.2 ppm) as references. The HSQC and HMBC experiments were optimized for 145.0 and 8.0 Hz, respectively. ESI-MS and HR-ESI-MS data were obtained using an Agilent Accurate-Mass-Q-TOF LC/MS 6520 instrument equipped with an electrospray ionization (ESI) source. The fragmentor and capillary voltages were kept at 125 and 3500 V, respectively. Nitrogen was supplied as the nebulizing and drying gas. The temperature of the drying gas was set at 300 °C. The flow rate of the drying gas and the pressure of the nebulizer were 10 L·min^{-1} and 10 psi, respectively. All MS experiments were performed in positive ion mode. Full-scan spectra were acquired over a scan range of m/z 100–1000 at 1.03 spectra/s. HPLC separations were performed on an Agilent 1260 instrument equipped with a variable-wavelength UV detector.

**Fungal material**

The culture of P. theae (N635) was isolated from Camel- lia sinensis (Theaceae) in Hangzhou, China. The isolate was identified based on sequence analysis of the ITS region of the rDNA (GenBank Accession No. KF641183). Firstly, the strain was cultured on potato dextrose agar (PDA) at 25 °C.
for 10 days. Secondly, agar plugs were cut into small pieces (about 0.5 × 0.5 × 0.5 cm³) under aseptic conditions, and every five pieces were inoculated into an Erlenmeyer flask (250 mL) containing 50 mL of media (0.4% glucose, 1% malt extract, and 0.4% yeast extract) with final pH 6.5. The flasks inoculated with the media were used as seed cultures after incubating at 25 °C on a rotary shaker at 170 rpm for 5 days.

Spore inoculum was prepared by suspension in sterile, distilled H₂O, resulting in a final spore/cell suspension of 1 × 10⁹/mL. Thirdly, each Fernbach flask (500 mL) containing 80 g of rice and 120 mL of distilled H₂O was then sealed, soaked overnight and autoclaved at 15 psi for 30 min. After cooling to room temperature, 5.0 mL of the spore inoculums obtained from liquid phase cultivation was added to each flask and incubated at 25 °C for 40 days.

Extraction and isolation

The fermented rice material was extracted several times with EtOAc (4 × 4.0 L), and the organic solvent was evaporated to dryness under vacuum steam to afford the crude extract (15 g), which was fractionated by silica gel vacuum liquid chromatography (VLC) using petroleum ether–EtOAc gradient elution. The fractions (1.2 g) eluted with 35%–40% EtOAc were combined and separated by ODS CC using MeOH–H₂O gradient elution. A 77 mg subfraction eluted with 60% MeOH was purified by RP HPLC (Agilent Zorbax SB-C₁₈ column; 5 μm; 9.4 × 250 mm; 65%–72% MeOH in H₂O for 30 min; 2.0 mL·min⁻¹) to afford 1 (3.8 mg, tᵣ 15.84 min) and 2 (1.3 mg, tᵣ 16.19 min). The fractions (1.9 g) eluted with 50%–60% EtOAc were combined and separated by ODS CC using MeOH–H₂O gradient elution. A 238 mg subfraction eluted with 50% MeOH was purified by RP HPLC to afford 3 (7.1 mg, tᵣ 14.1 min; 55%–70% MeOH in H₂O for 35 min; 2.0 mL·min⁻¹). A 154 mg subfraction eluted with 55% MeOH was separated by Sephadex LH-20 CC eluting with MeOH, and the resulting subfractions were purified by RP HPLC (40%–45% CH₃CN in H₂O for 30 min; 2.0 mL·min⁻¹) to afford 4 (3.6 mg, tᵣ 15.08 min) and 5 (1.2 mg, tᵣ 14.78 min). A 187.7 mg subfraction eluted with 20% MeOH was separated by Sephadex LH-20 CC eluting with MeOH, and the resulting subfractions were purified by RP HPLC (5%–30% CH₃CN in H₂O for 30 min; 2.0 mL·min⁻¹) to afford 7 (2.0 mg, tᵣ 15.02 min). The fraction (1.2 g) eluted with 30% EtOAc was combined and separated by ODS CC using MeOH–H₂O gradient elution. A 108.3 mg subfraction eluted with 50% MeOH was separated by Sephadex LH-20 CC eluting with MeOH, and the resulting subfractions were purified by RP HPLC (50%–70% MeOH in H₂O for 35 min; 2.0 mL·min⁻¹) to afford 6 (1.5 mg, tᵣ 11.95 min). The fraction (1.5 g) eluted with 80% EtOAc was combined and purified by RP HPLC (25%–50% MeOH in H₂O for 30 min; 2.0 mL·min⁻¹) to afford 8 (5.9 mg, tᵣ 10.62 min).

Pestathenol A (1)

Yellow oil; [α]₂⁰D −79.2 (c 0.38, MeOH); CD ε₂⁰₃⁰ −9.07, ε₂¹₅ −32.13, ε₃⁰⁹⁹ +0.20 (MeOH; c 6.0 × 10⁻³); UV (MeOH)
\( \lambda_{\text{max}} \) (log e) 204 (3.48); IR (neat) \( \nu_{\text{max}} \) 3420 (br), 2938, 1736, 1469, 1378, 1242, 1144, 1083, 1028 cm\(^{-1}\); ¹H NMR (acetone-\( d_6 \), 500 MHz), ¹³C NMR (acetone-\( d_6 \), 125 MHz) see Table 1; HR-ESI-MS \( m/z \) 375.1777 [M + Na]\(^{+} \) (Calcd. for \( \text{C}_{10}\text{H}_{23}\text{O}_{3}\text{Na}, 375.1778 \)).

**Pestathenol B (2)**

Yellow oil; [\( \alpha \)]\( _{D} \)^{20} −75.0 (c 0.12, MeOH); CD \( \Delta \varepsilon_{320} \) +9.21, \( \Delta \varepsilon_{220} \) −28.4, \( \Delta \varepsilon_{220} \) +2.10 (MeOH; c 5.7 × 10\(^{-3} \)); UV (MeOH) \( \lambda_{\text{max}} \) (log e) 204 (3.87), 319 (2.75); IR (neat) \( \nu_{\text{max}} \) 2927, 2935, 1736, 1607, 1463, 1373, 1244, 1028 cm\(^{-1}\); ¹H NMR (acetone-\( d_6 \), 500 MHz), ¹³C NMR (acetone-\( d_6 \), 125 MHz) see Table 1; HR-ESI-MS \( m/z \) 375.1781 [M + Na]\(^{+} \) (Calcd. for \( \text{C}_{10}\text{H}_{23}\text{O}_{3}\text{Na}, 375.1778 \)).

**Pestatheranone A (4)**

Colorless oil; UV (MeOH) \( \lambda_{\text{max}} \) (log e) 205 (3.51); IR (neat) \( \nu_{\text{max}} \) 2960, 2531, 2158, 1729, 1680, 1539, 1454, 1374, 1261, 1031 cm\(^{-1}\); ¹H NMR (CDCl\(_3\), 500 MHz), ¹³C NMR (CDCl\(_3\), 125 MHz) see Table 2; HR-ESI-MS \( m/z \) 211.1330 [M + H]\(^{+} \) (Calcd. for \( \text{C}_{12}\text{H}_{15}\text{O}_{2}, 211.1329 \)).

**Absolute configuration of the 5,6-Diol functionalities in 1**

HPLC grade DMSO was dried with 4 Å molecular sieves. According to the published procedure, mixtures of 1 : 1.3 diol/Mo\(_4\)O\(_9\)(OAc)\(_4\) for I were subjected to CD measurements at concentration of 0.5 mg mL\(^{-1}\). The first CD spectrum was recorded immediately after mixing, and its time evolution was monitored until stationary (about 10 min after mixing). The inherent CD was subtracted. The observed signs of the diagnostic bands at around 330 nm in the induced CD spectrum were correlated to the absolute configuration of the 5,6-diol moiety.

**MTS assay**

The assay plate was read at 490 nm using a microplate reader. The assay was run in triplicate. In a 96-well plate, each well was plated with (2–5) × 10\(^4\) cells (depending on the cell multiplication rate). After cell attachment overnight, the medium was removed, and each well was treated with 100 \( \mu \)L of medium containing 0.1% DMSO, or appropriate concentrations of the test compounds and the positive control cisplatin (100 mmol·L\(^{-1}\) as stock solution of a compound in DMSO and serial dilutions; the test compounds showed good solubility in DMSO and did not precipitate when added to the cells). The plate was incubated at 37 \( ^\circ \)C for 48 h in a humidified, 5% CO\(_2\) atmosphere. Proliferation was assessed by adding 20 \( \mu \)L of MTS (Promega) to each well in the dark, followed by incubation at 37 \( ^\circ \)C for 90 min. The assay plate was read at 490 nm using a microplate reader. The assay was run in triplicate \[1\].

**References**


[22] Yang XL, Zhang JZ, Luo DQ. The taxonomy, biology and


