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Terrusnolides A-D, new butenolides with anti-inflammatory activities from an endophytic *Aspergillus* from *Tripterygium wilfordii*

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ABSTRACT

Terrusnolides A−D (**1–4**), four butenolides were isolated from an endophytic Aspergillus from Tripterygium wilfordii. The structures of **1–4** were established by comprehensive spectroscopic analyses and electronic circular dichroism (ECD) calculation. It is interesting that **1** was a butenolide derived by a triple decarboxylation, while **2–4** were the metabolites with 4-benzyl-3-phenyl-5H-furan-2-one motif possessing an isopentene group fused to the benzene ring. *In vitro* anti-inflammatory effects of these isolates were evaluated in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages. **1–4** exhibited excellent inhibitory effects on the production of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and nitric oxide (NO) in LPS-induced macrophages, comparable with the positive control (indomethacin). Those results indicated that, terrusnolides A−D might serve as new potential natural remedies for the treatment of inflammation.

1. Introduction

Endophytes were defined as microorganisms that inhabit internal tissues of plants but causing no apparent disease symptoms [1,2]. Endophytic secondary metabolites possess comprehensive biological activities and complicated structures, have been identified as a major source for the medicinal, agricultural, or industrial applications [3–5].

Butenolides are a class of fungal metabolites belonging to lignans, which are normally characterized by a 4-benzyl-5-phenyl segment, and rare possess the 4-benzyl-3-phenyl-5H-furan-2-one motif [6]. Since the first compound (microperfuranone) had been obtained from *Anixiella micropertusa* in 1998, only 18 natural products equipped with the 4-benzyl-3-phenyl-5H-furan-2-one motif have been reported to date [7]. Notably, these butenolides equipped with 4-benzyl-3-phenyl-5H-furan-2-one motif exhibited interesting biological activities, such as glycogen synthase kinase-3β (GSK-3β) inhibitory activity, antifungal, and vasodilatory activity [8].

Previously, we have isolated two new meroterpenoids with outstanding beta-secretase 1 (BACE1) inhibitory activities *in vitro* and *in vivo* from *Aspergillus terreus* [9]. In our ongoing search for bioactive metabolites from the genus of *Aspergillus*, four new butenolides, namely terrusnolides A-D (**1–4**) were isolated from an endophytic *Aspergillus* from *Tripterygium wilfordii* (Fig. 1). Compound **1** was a butenolide derived by a triple decarboxylation (Scheme 1). Furthermore, compounds **2–4** were the 4-benzyl-3-phenyl-5H-furan-2-one derivatives with an isopentene group fused to the benzene ring. Compounds **1–4** were tested for their inhibitory effects on the production of interleukin-1β (IL-1β), tumor necrosis factor-α (TNF-α), and nitric oxide (NO) in lipopolysaccharide (LPS)-induced macrophages. Those compounds exhibited excellent inhibitory effects on the production of IL-1β, TNF-α and NO in LPS-induced macrophages, comparable with the positive control (in-domethacin). Herein, we report the isolation, structure elucidation, as well as anti-inflammatory activities of these isolated butenolides.

2. Experimental

2.1. General experimental procedures

Extensive NMR spectra were obtained from a Bruker AM-400

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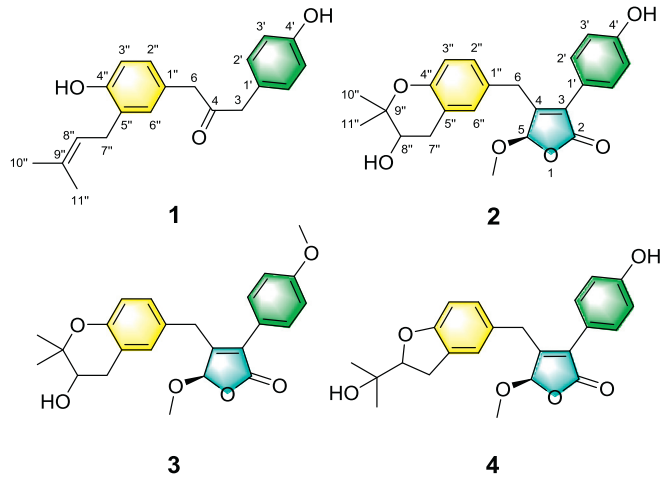

Fig. 1. Structures of compounds **1**–**4**.

instrument with tetramethylsilane as an internal standard. The ^{1}H and ^{13}C NMR chemical shifts of the solvent peaks for methanol-d_4 (δ_H 3.31 and δ_C 49.0) were referenced, and for $CDCl_3$ (δ_H 7.24 and δ_C 77.23). High-resolution electrospray ionization mass spectra (HRESIMS) data were recorded in the positive ion mode on a Thermo Fisher LC-LTQ-Orbitrap XL spectrometer. UV, ECD data, and optical rotations were recorded on a Varian Cary 50, a JASCO-810 CD spectrometer instrument, and a PerkinElmer 341 polarimeter, respectively. FT-IR spectra were detected by a Bruker Vertex 70 instrument. Semi-preparative HPLC separations were accomplished on an Agilent 1220 instrument with a reversed-phased C_{18} column (10 × 250 mm; 5 μm), equipped with an Ultimate 3000 pump, Ultimate 3000 DAD operated by Chromeleon software (version 6.80) and Ultimate 3000 autosamper injector. Column chromatography (CC) was performed by silica gel (100–200 mesh, 200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, and People's Republic of China), Lichroprep RP-C_{18} gel (40–63 μm, Merck, Darmstadt, Germany) and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Sweden). Thin-layer chromatography was used by a RP-C_{18} F_{254} and silica gel 60 F_{254} plates, and spots were visualized by spraying heated silica gel plates with 10% H_2SO_4 in EtOH.

2.2. Fungal material, cultivation, extraction and isolation

The strain, *Aspergillus* sp. was isolated from the root of *Tripterygium wilfordii*, which collected in Wuhan, China, in 2014. According to the sequence analysis of the ITS region of the rDNA, combined with morphology of this fungus, the strain was identified as a *Aspergillus* species, and its sequence data have been submitted to the GenBank with accession no. MF962867. This strain has been preserved in the culture collection of Tongji Medical College, Huazhong University of Science and Technology. The *Aspergillus* sp. strain was cultured on potato dextrose agar (PDA) at 28 °C for 5 days to prepare the seed cultures, and then cut into small pieces (roughly 0.3 cm × 0.3 cm × 0.3 cm) and inoculated into 200 × 1000 mL sterilized Erlenmeyer flasks (each containing 300 g rice and 300 mL distilled water). The flasks were incubated at 28 °C for 30 days, then the growth of fungus was stopped by adding 500 mL ethanol to each flask, followed by extracting with ethanol for five times. At last, these extracts were mixed together and then the organic solvent was evaporated to dryness under reduced pressure, given a dark brown crude extract of 580 g.

The ethanol extracts (580 g) were performed to silica gel chromatography column (CC), and eluted with CH_2Cl_2/MeOH (30:1–1:2), to afford seven fractions (Fr.1–Fr.7). The Fr.3 was separated with RP-C_{18} CC (MeOH–H_2O, 10%–100%) to obtain six subfractions (Fr.3.1–Fr.3.6), and then subfraction Fr. 3.4 was subjected to a Sephadex LH-20 CC ($CHCl_3$-MeOH, 1:1), given three parts (Fr. 3.4a–Fr. 3.4c). Further separations on the second part (Fr. 3.4b) by ODS (MeOH-H_2O, 20%–100%) yielded four mixtures (A–D). Mixture C was purified by repeated semi-preparative HPLC (MeOH–H_2O, 65:35, *v*/v; 2 mL/min and MeCN–H_2O, 40:60, v/v; 2 mL/min) to yield compounds **1** (7 mg) and **4** (12 mg). Fr.4 was loaded onto Sephadex LH-20 CC ($CHCl_3$-MeOH, 1:1) to give six subfractions (Fr.4.1-Fr.4.6). By the methods of silica gel CC (CH_2Cl_2–MeOH, 20:1, *v*/v) and repeated semi-preparative HPLC (MeOH–H_2O, 63:37, v/v; 2 mL/min), Fr.4.2 was finally separated to yield compounds **2** (6 mg) and **3** (7 mg).

Terrusnolide A ***(1)***: Yellow oil, UV (MeOH) λ_{max} (log ε) = 219 nm (4.15), 244 nm (3.97), 278 nm (3.74); IR ν_{max} = 3432, 2919, 2850, 1787, 1711, 1631, 1540, 1454 cm^{-1}; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data please see Table 1; HRESIMS $[M + Na]^+$ *m/z* 311.1642 (calcd for $C_{23}H_{24}O_6Na$, 311.1647).

Terrusnolide B ***(2)****:* Yellow oil, $[\alpha]_D^{20}$ -16.1 (*c* = 0.2 mg/mL, MeOH); UV (MeOH) λ_{max} (log ε) = 201 nm (4.48), 284 nm (3.84); CD (MeOH) λ_{max} ($\Delta\varepsilon$): 210 nm (+ 9.66) and 282 nm (+ 7.39); IR ν_{max} = 3405, 3057, 2979, 2935, 1748, 1661, 1611, 1588, 1516 cm^{-1}; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data please see Table 1; HRESIMS $[M + Na]^+$ *m/z* 419.1483 (calcd for $C_{23}H_{24}O_6Na$, 419.1471).

Terrusnolide C ***(3)***: Colorless oil, $[\alpha]_D^{20}$ -103.3 (*c* = 1.6 mg/mL, MeOH); UV (MeOH) λ_{max} (log ε) = 201 nm (4.30), 282 nm (3.62); CD (MeOH) λ_{max} ($\Delta\varepsilon$): 207 nm (+ 5.00) and 280 nm (+3 0.72); IR ν_{max} = 3477, 2975, 2934, 2840, 1761, 1664, 1609, 1513, 1496 cm^{-1}; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data please see Table 1; HRESIMS $[M + Na]^+$ *m/z* 433.1539 (calcd for $C_{24}H_{26}O_6Na$, 433.1627).

Terrusnolide D ***(4)***: Colorless oil, $[\alpha]_D^{20}$ -131.0 (*c* = 1.0 mg/mL, MeOH); UV (MeOH) λ_{max} (log ε) = 201 nm (4.28), 286 nm (3.68) nm); CD (MeOH) λ_{max} ($\Delta\varepsilon$): 206 nm (+ 13.60) and 283 nm (+ 10.65); IR ν_{max} = 3406, 2976, 2936, 1753, 1661, 1611, 1589, 1516 cm^{-1}; for ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) data please see Table 1; HRESIMS $[M + Na]^+$ *m/z* 419.1471 (calcd for $C_{23}H_{24}O_6Na$, 419.1471).

2.3. Cell culture and treatment of compounds

RAW 264.7 cells were obtained from the Boster Biological Technology Co., Ltd. (Wuhan, China) and maintained in DMEM containing 10% fetal bovine serum (FBS) (Gibco BRL Co, Grand Island, NY, USA) at 37 °C in humidified incubator containing 5% CO_2. All tested compounds were dissolved in DMSO (DMSO concentration was < 0.25% in assay).

2.4. Cytotoxicity assay

The cytotoxicity of the compounds against RAW264.7 macrophage was evaluated by the 2-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay [10]. Briefly speaking, RAW264.7 cells were seeded at a density of 1 × 10^4 cells/wells in 96-well plates, incubated for 12 h and then treated with compounds at final concentration of 100 μM. After 24 h of incubation, 20 μL MTT (5 mg/mL) was added and the plates were incubated for another 4 h. The formed formazan crystals were dissolved in 150 μL DMSO and absorbance was recorded at 570 nm on a microplate reader (BioTek Instruments, Inc., USA).

2.5. NO, TNF-α and IL-1β inhibition assay

This experiment was conducted in the light of published procedures [11]. In brief, RAW264.7 cells were seeded to a 96-well plate with a concentration of 1 × 10^5 cells/well in the solution of 10% FBS DMEM, then incubated at 37 °C in 5% carbon dioxide overnight. Then, the RAW264.7 cells were incubated for 1 h with compounds and indomethacin (positive control) in different concentrations prior to add the LPS. 20 h after the management of LPS, the liquid supernatant was collected. Then, the content of NO in the supernatant was detected by