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Original article

Two new diterpenoids from the endophytic fungus *Trichoderma* sp. Xy24 isolated from mangrove plant *Xylocarpus granatum*

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ABSTRACT

Two new harziane diterpenoids, named (9*R*,10*R*)-dihydro-harzianone (**1**) and harzianelactone (**2**), were isolated from the endophytic fungus *Trichoderma* sp. Xy24 by using various column chromatography techniques. Their structures were determined on the basis of extensive spectroscopic (HR-ESI-MS, 1D NMR, 2D NMR and CD) analyses. Among them, **1** was the reductive product of harzianone and **2** was the Baeyer–Villiger monooxygenase catalyzed oxidation product of harzianone. Compound **1** exhibited cytotoxic activity against HeLa and MCF-7 cell lines with IC_{50} values of 30.1 μmol/L and 30.7 μmol/L, respectively.

1. Introduction

Trichoderma spp. are widely used as biocontrol microbes against plant pathogens for their production of a wide range of antibiotic substances [1,2]. Their secondary metabolites exhibit multiple biological properties, such as immune system suppressor, plant growth promoter and regulator, anti-aging agents and so on [3]. Previously, an endophytic fungus was isolated and identified as *Trichoderma* sp. from a mangrove plant *Xylocarpus granatum* by our research group and a known harziane diterpene harzianone was isolated from the ethyl acetate (EtOAc) extract of the mycelia and filtrate [4]. Harzianone contained a unique tetracyclic scaffold with fused four-, five-, six-, and seven-membered carbon rings and exhibited 82.6% lethality in the brine shrimp toxicity assay [5]. To the best of our knowledge, there are only seven members of harziane tetracyclic diterpene family reported, including harzianone [5], harziandione [6], four harziane-related diterpenes [7] and a diterpenoid lactone [8]. Considering the structural novelty and biological activity of this kind of compounds, 60 L fermentation of *Trichoderma* sp. Xy24 was performed for the search of this kind of compounds. Consequently, two new harziane diterpenoids, namely, (9*R*,10*R*)-dihydroharzianone (**1**) and harzianelactone (**2**) (Fig. 1) were obtained. Biogenetically, **1** was the reductive product of harzianone and **2** was the Baeyer–Villiger monooxygenase catalyzed oxidation product of harzianone. Herein, we report the detailed isolation, structure elucidation and biological activity of the two new compounds.

2. Experimental

2.1. General experimental procedures

Optical rotations were measured on a Perkin-Elmer Model-343 digital polarimeter. The CD and UV spectra were recorded on a JASCO J-815 spectropolarimeter. IR spectra were acquired on a Nicolet 5700 FT-IR microscope spectrometer (FT-IR Microscope Transmission). 1D and 2D NMR spectra were obtained at 600 MHz for ^{1}H NMR and 150 MHz for ^{13}C NMR on a VNOVA SYSTEM-600 spectrometer. Chemical shifts (δ) are given in ppm, and coupling constants (J) are given in hertz (Hz). HR-ESI-MS data were measured using an Agilent Technologies 6520 Accurate Mass Q-TOF LC/MS spectrometer. Column chromatography (CC) was carried out with silica gel (200–300 mesh, Qingdao Marine Chemical Inc. Qingdao, PR China). Semi-preparative HPLC was performed on a Shimadzu HPLC instrument equipped with a Shimadzu RID-10A detector and a Grace Adsorbosphere C18

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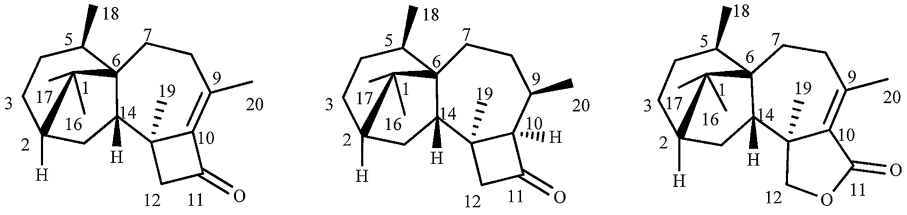

Fig. 1. Structures of harzianone, (9*R*,10*R*)-dihydro-harzianone (**1**) and harzianelactone (**2**).

column (250 mm × 10 mm, i.d., 5 μm) by eluting with mixtures of CH_3OH and H_2O or CH_3CN and H_2O. Analytical TLC was carried out on pre-coated silica gel GF_{254} plates (Qingdao Marine Chemical Industry, Qingdao, China), and spots were visualized under UV light or by spraying with10% H_2SO_4 in 90% EtOH followed by heating at 120 °C.

2.2. Fungal material and fermentation

The fungal strain *Trichoderma* sp. Xy24 was isolated from the leaves, stems and peels of mangrove plant *X. granatum* collected in Sanya district, Hainan province of China. It was identified as *Trichoderma* sp. Xy24 according to the morphological and molecular (ITS1-5.8S-ITS2 rDNA sequence) analyses by our research group [9]. The strain was deposited at the Institute of Materia Medica, Chinese Academy of Medical Sciences.

The fungal strain was maintained on slants of modified potato dextrose agar (PDA) medium (potato 200 g, glucose 20 g, distilled water 1 L, KH_2PO_4 3 g, $MgSO_4$ 0.75 g, vitamin B_1 10 mg, agar 8.0 g, pH 6.0; the media were autoclaved at 115 °C for 30 min) at 4 °C. Seed cultures were performed in Erlenmeyer flasks (250 mL) containing 100 mL of PDA liquid medium on a shaker at 150 rpm at 25 °C for 2 days, after that 5 mL seed cultures were inoculated into each 1000 mL flask with 300 mL medium and cultivated for 14 days (150 rpm, 25 °C).

2.3. Extraction and isolation

The cultures (60 L) were filtered under reduced pressure to afford the filtrate and mycelia. The filtrate was applied to an Amberlite XAD-16 macroporous adsorbent resin column by eluting with H_2O and 90% EtOH successively and then gained the residue (30.0 g) under reduced pressure. The dried mycelia were extracted with methanol by the ultrasonic extraction method to afford 92.0 g of residue. The residues of the two parts were combined for further separation based on their identical TLC profiles. The combined extract (122.0 g) was partitioned with petroleum ether. The petroleum ether extract was evaporated under reduced pressure to yield 30.0 g of residue, which was subjected to silica gel column chromatography (CC) eluting with a petroleum ether–EtOAc gradient (100:0, 50:1, 20:1, 10:1, 5:1, 3:1, 1:1, 1:3, 0:100) to give nine fractions based on TLC analysis.

Fraction 2 (5.2 g) was initially subjected to silica gel CC eluting with a detailed petroleum ether–EtOAc gradient (100:0, 200:1, 100:1, 50:1, 10:1, 3:1) to give six fractions (Fr2.1–Fr2.6). Fr2.2 (185.3 mg) was further separated by reversed-phase semi-preparative HPLC with CH_3CN/H_2O (85:15, v/v) at 3 mL/min to give **1** (10.3 mg, t_R 28.9 min). Fr2.3 (79.2 mg) was further separated by reversed-phase semi-preparative HPLC with CH_3OH/H_2O (90:10, v/v) at 3 mL/min to give 2 (2.4 mg, t_R 21.8 min).

(9R, 10R)-Dihydro-harzianone (**1**): colorless oil; $[\alpha]_D^{20}$ −48.6 (*c* 0.19, MeOH); CD (MeOH) 304 (Δε − 1.04) nm; IR (cm^{-1}): ν_{max} 3021, 2933, 1775, 1741, 1461, 1383, 1198, 1135, 1007, 979, 937, 925; ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data see Table 1; HR-ESI-MS *m/z* 289.2518 $[M + H]^+$ (calcd. for $C_{20}H_{33}O$, 289.2526).

Harzianelactone (**2**): colorless solid, $[\alpha]_D^{20}$ +7.5 (*c* 0.35, MeOH); IR (cm^{-1}): ν_{max} 3028, 2933, 2926, 2896, 1740, 1649, 1478, 1382, 1254, 1167, 1031, 976, 910, 779; UV (MeOH) λ_{max} (log ε): 233.0 (0.67) nm; ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz) data see Table 1; HR-ESI-MS *m/z* 303.2310 $[M + H]^+$ (calcd. for $C_{20}H_{31}O_2$, 303.2319).

3. Results and discussion

Compound **1** was obtained as colorless oil. Its molecular formula was determined to be $C_{20}H_{32}O$ by HR-ESI-MS at *m/z* of 289.2518 $[M + H]^+$ (calcd. for $C_{20}H_{33}O$, 289.2526), corresponding to 5 degrees of unsaturation. The IR spectrum showed absorption band for carbonyl group at 1775 cm^{-1}. The ^1H NMR (Table 1) spectrum and HSQC data displayed three methyl singlets at δ_H 0.92 (s, 3H), 1.02 (s, 3H) and 1.44 (s, 3H); two methyl doublets at δ_H 1.06 (d, 3H, *J* = 7.5 Hz) and 1.09 (d, 3H, *J* = 7.5 Hz). The ^{13}C NMR and DEPT spectra showed 20 carbon resonances (Table 1), which consisted of four quaternary carbons (δ_C 213.4, 51.3, 46.3 and 36.0, including one carbonyl carbon), five methine carbons (δ_C 74.4, 51.5, 44.1, 32.6 and 30.8), six methylene carbons (δ_C 63.8, 29.1, 28.2, 26.3, 26.3 and 25.1), and five methyl carbons (δ_C 27.0, 26.3, 22.8, 21.0 and 17.5). The NMR data were closely related to those of harzianone, except for the replacement of a methyl singlet (δ_H 2.07, s) in harzianone by a methyl doublet (δ_H 1.09, d, *J* = 7.5 Hz) in compound **1**, and the appearance of two additional methine protons at δ_H 2.28 (m, 1H) and δ_H 3.01 (dd, 1H, *J* = 6.3 Hz, 4.8 Hz) in the ^1H NMR spectrum of compound **1**. The ^{13}C NMR spectrum of compound **1** displayed two methine carbons at δ_C 74.4 (appearing in lower field affected by carbonyl negative shielding effect, as well as C-12 at δ_C 63.8) and 32.6 in the replacement of two olefinic carbons at δ_C 150.2 (C-10) and δ_C 146.6 (C-9) in harzianone. All the data above indicated that compound **1** was the reductive product of harzianone. The structure of compound **1** was supported by 2 D NMR ^1H–^1H COSY and HMBC correlations shown in Fig. 2.

The configuration of **1** was deduced by NOEs (Fig. S10 in Supporting information) and the CD spectrum (Fig. S13 in Supporting information). The irradiation of H-16 enhanced H-14 and H-17, the irradiation of H-20 enhanced H-14 and H-16, and the irradiation of H-19 enhanced H-5 and H-10. These observations indicated that H-16, H-17, H-14 and H-20 were *syn*-oriented, while H-19, H-5 and H-10 were on the opposite side. The negative Cotton curve at 304 nm arising from carbonyl $n \rightarrow \pi^*$ transition in the CD spectrum of compound **1** suggested *R* configuration for C-10 by the ketone octant rule [10,11]. Thus, the absolute configuration of **1** was assigned as 2*S*, 5*R*, 6*R*, 9*R*, 10*R*, 13*S*, 14*S*, and the structure of **1** was determined as (9*R*,10*R*)-dihydroharzianone, a reductive product of harzianone.