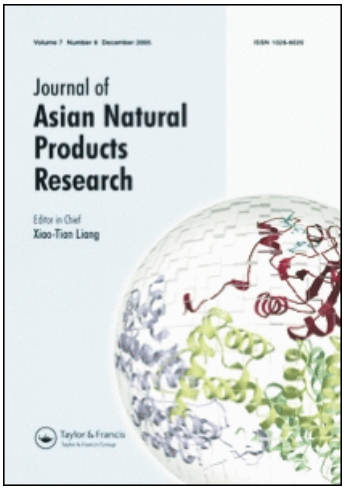

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Harzianums A and B produced by a fungal strain, *Hypocrea* sp. F000527, and their cytotoxicity against tumor cell lines

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A new compound, harzianum B (**1**), was isolated from the culture broth of a fungal strain, *Hypocrea* sp. F000527, together with a known trichothecene, harzianum A (**2**). Its structure was determined by spectroscopic analyses including HRFAB-MS and various ^{1}H NMR and ^{13}C NMR spectral data. Harzianum B (**1**) was characterised as (*E*,*Z*,*E*)-2′, 4′, 6′-octatriendioic acid esterified on the 4β-hydroxyl group of trichodermol. Harzianums A (**2**) and B (**1**) showed cytotoxicity against several human cancer cell lines.

Keywords: Harzianum B; *Hypocrea* sp; Cytotoxicity; Tumor cell lines

1. Introduction

Mycotoxins are secondary metabolites produced by microfungi that are capable of causing diseases and death in humans and other animals [1,2]. Various genera of toxigenic fungi are capable of producing such diverse mycotoxins as the aflatoxins, rubratoxins, ochratoxins, fumonisins, and trichothecenes. Out of these toxins, trichothecenes, which are mainly produced by fungal species of *Fusarium*, *Myrothecium* and *Trichoderma*, are a well-studied class of sesquiterpene-based mycotoxins and are potent cytotoxins to eukaryotic cells [3]. Trichothecenes are also known to be potent inhibitors of protein and/or DNA synthesis and can impact actively dividing tissues with the immune system being particularly susceptible [3,4]. To date, only a few trichothecenes have been found in *Trichoderma* species but no precedent in *Hypocrea* spp. In our search for cytotoxic compounds from fungal metabolites, two trichothecene compounds, harzianums A (**2**) and B (**1**) were isolated from a fungal strain, which was identified as *Hypocrea* sp. F000527 [5] (figure 1). In this paper, we

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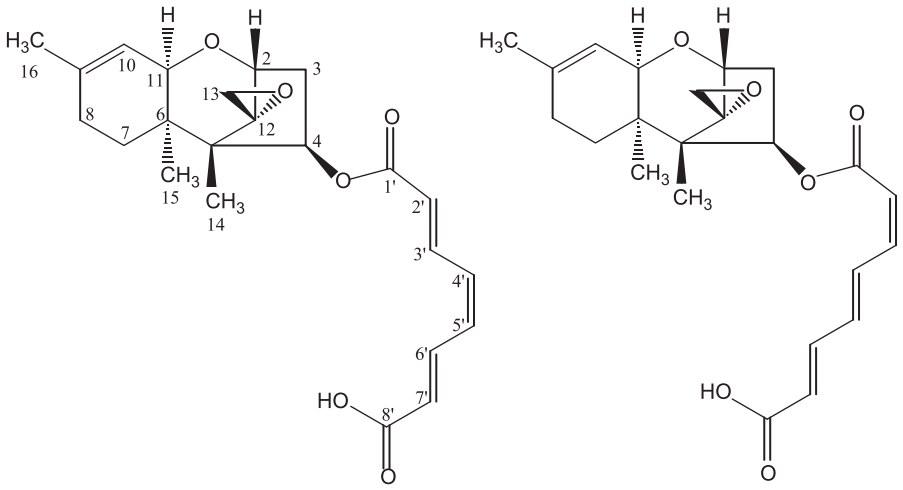

Figure 1. Chemical structures of harzianums A (**2**) and B (**1**).

describe the structure elucidation of these two compounds and their cytotoxicity against tumor cell lines.

2. Results and discussion

Compound harzianum B (**1**) was obtained as a colourless oil. Its molecular formula $C_{23}H_{28}O_6$ was determined by HRFAB-MS. The ^{1}H NMR and ^{13}C NMR spectra of harzianum B showed a pattern quite similar to those of harzianum A [6], except for six signals of olefinic protons (table 1, figure 1). The ^{1}H NMR spectrum of harzianum B showed the expected signals for trichothecene skeleton: a vinylic methyl (δ_H 1.72, 3H, s, H-16), an olefinic proton (δ_H 5.42, d, $J = 5.5$ Hz, H-10), a 1,1-disubstituted epoxide AB system (δ_H 2.85, 1H, d, $J = 3.9$ Hz, H-13β; δ_H 3.14, 1H, d, $J = 3.9$ Hz, H-13α), two oxygen-bearing methines (δ_H 3.85, 1H, d, $J = 5.0$ Hz, H-2β; δ_H 3.63, 1H, d, $J = 5.4$ Hz, H-11α), and two methyls (δ_H 0.73, 3H, s, H-14; δ_H 0.96, 3H, s, H-15). One proton at δ_H 5.66 (dd, $J = 7.5$ and 3.0 Hz, H-4α), which correlated to C-4 at δ_C 75.5 in HMQC, showed long-range correlation with a carbonyl at δ_C 166.1 in HMBC spectrum, indicating an ester linkage at C-4. Other important correlations between H-2 with C-4 and C-11, H-11 with C-2, C-5, and C-9, CH_3-16 with C-8 and C-10, H-3′ with C-1′, H-6′ with C-8′ were observed in HMBC (figure 2). The UV spectrum ($\lambda_{max} = 306$, $\log \varepsilon$ 4.36) was indicative of a triene moiety. The coupling constants of olefinic protons suggested geometric arrangements between each pair of the triene moiety: H-2′ and H-3′, *trans* ($J = 15.4$ Hz); H-4′ and H-5′, *cis* ($J = 10.0$ Hz); H-6′ and H-7′ *trans* ($J = 15.4$ Hz); and H-3′ and H-4′, H-5′ and H-6′, vicinal ($J = 10.0$, and 10.0 Hz, respectively) [7]. These were supported by observation of the NOE enhancement between each pair of H-2′ and H-4′, H-3′ and H-6′, and H-5′ and H-7′, respectively. Consequently, the structure of harzianum B was assigned as *trans–cis–trans* stereochemistry for 2′,4′,6′-octatrienedioic acid esterified on the 4β hydroxyl group of trichodermol.

Table 1. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compounds 1 and 2 ($CDCl_3$) (δ ppm, J in Hz).

No.	***1***		***2***	
	^{13}C	^{1}H	^{13}C	^{1}H
2β	79.1	3.85 d (5.0)	79.1	3.87 d (5.4)
3α	36.6	2.58 dd (15.3, 7.5)	36.8	2.59 dd (15.6, 7.5)
3β		2.04 ddd (15.3, 8.5, 4.5)		2.08 ddd (15.6, 5.2, 3.6)
4α	75.4	5.66 dd (7.5, 3.0)	75.2	5.66 dd (7.8, 3.3)
5	49.2		49.2	
6	40.4		40.4	
7α	24.4	1.43 m	24.4	1.44 m
7β		1.94 m		1.94 m
8α	27.9	1.99 m	28.0	1.99 m
8β		1.99 m		1.99 m
9	140.2		140.2	
10	118.5	5.42 d (5.5)	118.5	5.42 d (4.2)
11α	70.5	3.63 d (5.5)	70.6	3.64 d (5.4)
12	65.5		65.5	
13α	47.8	3.14 d (3.9)	47.9	3.16 d (3.9)
13β		2.85 d (3.9)		2.86 d (3.9)
14	5.9	0.73 s	6.1	0.733 s
15	16.0	0.96 s	16.0	0.97 s
16	23.2	1.72 s	23.2	1.72 s
1′	166.1		165.6	
2′	123.6	6.03 d (15.4)	121.4	5.87 d (11.4)
3′	142.8	7.34 dd (15.4, 10.0)	142.6	6.66 t (11.4)
4′	136.7	6.66 d (10.0)	136.5	7.95 dd (15.0, 11.5)
5′	137.8	6.66 d (10.0)	137.3	6.57 dd (15.0, 11.4)
6′	144.7	7.40 dd (15.4, 10.0)	145.6	7.51 dd (15.3, 11.4)
7′	125.1	6.09 d (15.4)	123.3	6.02 d (15.3)
8′	170.9		170.9	

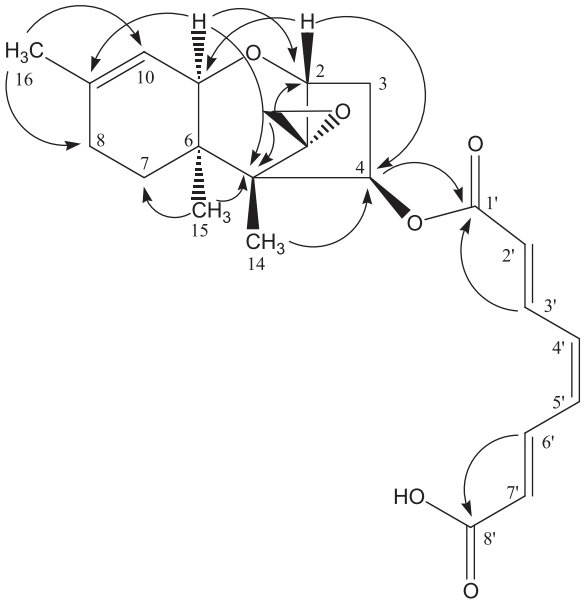

Figure 2. Key HMBC correlations of harzianum B (**1**).

Table 2. Cytotoxicity of harzianums A (**2**) and B (**1**) on the tumor cell lines.

Compounds	*Toxicity to*		
	HeLa cell	*MCF-7 cell*	*HT1080 cell*
1	74.18 ± 0.55*	74.04 ± 1.17	15.63 ± 0.37
2	5.07 ± 0.18	10.13 ± 0.33	0.65 ± 0.03
Doxorubicin	0.24 ± 0.02	9.68 ± 0.21	0.48 ± 0.01

* Data are mean ± SD (IC_{50}, μg/ml) from two separate experiments.

Harzianums A and B were tested for their cytotoxicity against HeLa, MCF-7, and HT1080 cell lines. The results (IC_{50} values) are summarised in table 2. In comparison with harzianum B, harzianum A showed stronger cytotoxicity against a human fibrosarcoma cell line, HT1080, HeLa cells and MCF-7 cell lines. It seemed that the *cis–trans–trans* stereochemistry of harzianum A contributes this cytotoxicity. Furthermore, similar to harzianum A, harzianum B showed especially stronger toxicity against HT1080 cells with an IC_{50} value of 15.63 μg/ml.

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a JASCO DIP-370 polarimeter. Melting points were measured on an Electrothermal 9100 instrument and are uncorrected. UV spectra were obtained on a Milton Roy 3000 spectrometer. ^{1}H NMR, ^{13}C NMR, DEPT, HMQC, HMBC and NOESY spectra were recorded on Bruker DMX 600 NMR spectrometer with $CDCl_3$ as a solvent. HRFAB-MS was obtained on a JMS-HX110A/HX110A Tandem Mass spectrometer (JEOL). Preparative HPLC was carried out on a J'sphere ODS-H80 (150 × 20 mm, YMC). Fetal bovine serum, media and supplement materials for cell culture were purchased from Gibco-BRL (Gaithersberg, MD, USA).

3.2 Fungal isolation and fermentation condition

The fungal strain F000527 used in this study was isolated from a soil sample using dilution plating method. Identification of the strain was described as literature [5]. The fungal strain was cultured on wheat bran in order to evaluate its toxigenic potential. Wheat bran (200 g) as a solid medium was adjusted to about 45–50% moisture content in 500-ml Erlenmeyer flasks, and autoclaved at 115°C for 20 min. The cultures were placed in fermentation room at 28°C for 8 days prior to harvesting.

3.3 Extraction and isolation

The wheat bran was extracted with 50% acetone at room temperature twice. Acetone was evaporated and the residue was partitioned between H_2O and ethyl acetate. The ethyl acetate fraction was chromatographed on a silica gel column (6 × 40 cm) eluting with a step gradient of CH_2Cl_2/MeOH (100:1, 50:1, 30: 1, 20: 1, 10: 1, 5: 1, 1: 1, 100% MeOH, each 1.5 L) to give 11 fractions (Fr1–Fr11). Active fraction Fr7 was subjected to Sephadex LH-20

(3.5 × 100 cm) chromatography eluted with MeOH to give four fractions (Fr7-1–Fr7-4). Active subfraction Fr7-2 (156 mg) was subjected to semi-preparative HPLC (ODS-H80, 150 × 10 mm, YMC, Japan, $CH_3CN/H_2O = 30:70$, 0.05% TFA in both solvent, flow rate: 3 ml/min). Fractions eluted from 140 to 149 min yielded 52.4 mg of harzianum A and from 152–160 min yielded 27.2 mg of harzianum B.

3.3.1 Harzianum A (2). Colourless oil; $[\alpha]_D^{25} + 71.25$ (*c* 1.00, $CHCl_3$); UV λ_{max} (MeOH) (log ε) 205 (4.17), 306 (4.36) nm; ESI-MS *m/z* 399.4 $[M - H]^+$; ^{1}H NMR and ^{13}C NMR data: see table 1.

3.3.2 Harzianum B (1). Colourless oil; $[\alpha]_D^{25} + 27.94$ (*c* 0.3, $CHCl_3$); UV λ_{max}(MeOH) (log ε) 306 (4.36) nm; HR-FAB-MS *m/z* 401.1964 $[M + H]^+$ (calcd. for $C_{23}H_{29}O_6$, 401.1964); ^{1}H NMR and ^{13}C NMR data: see table 1.

3.4 Cytotoxicity assay

A cytotoxicity assay was carried out according to Denizot and Lang [8]. Each cell (concentration of 1×10^4) was seeded in each well containing 100 µl DMEM. Subsequently, various concentrations of samples were added. The cells were incubated for 48 h at 37°C in an atmosphere containing 5% (v/v) CO_2, then 10 µl FBS-free medium containing 5 mg/ml MTT solution was added to the wells. After 4 h of incubation at 37°C, the medium was discarded and the formazan blue, which formed in the cells, was dissolved by adding 100 µl DMSO. Optical density was measured at 570 nm using a microplate reader (Molecular Devices Co., Menlo Park, CA, USA). Doxorubicin was used as a positive control.

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