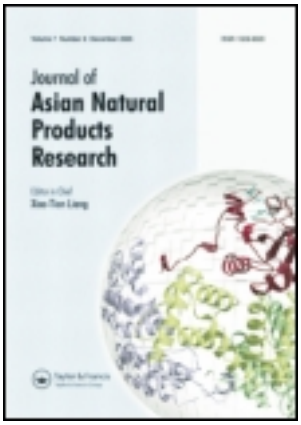

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Two new compounds from an endophytic fungus *Aspergillus* sp. HS-05

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Two new compounds, *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine methyl ester (**1**) and methyl 4-methoxy-3-(3′-hydroxy-2′-methyl)propionyloxy-benzoate (**2**), were isolated from EtOAc extract of the fermentation broth of the endophytic fungus *Aspergillus* sp. HS-05. Their structures were elucidated by NMR, IR, UV, MS, and CD methods. Compounds **1** and **2** showed no anticancer activities against HL-60 cell lines (both of $IC_{50} > 100\ \mu M$) *in vitro.*

Keywords: *Aspergillus* sp.; endophytic fungus; structure elucidation; anticancer activity

1. Introduction

Endophyte is one which resides in the tissues beneath the epidermal cell layers and causes no apparent harm to the host. They form inconspicuous infections within tissues of healthy plants for all or nearly all their life cycle [1]. The relationship between endophytes and host plants is complex. The host plant protects and feeds the endophyte, in return the endophyte produces bioactive metabolites to enhance the growth and competitiveness of the host and to protect it from herbivores and plant pathogens [2]. They have proven to be promising sources of new and biologically active natural products, which are of interest for specific medicinal or agrochemical applications [3]. Novel antibiotics, antimycotics, immunosuppressants, and anticancer agents have been found from endophytes [4]. It is estimated that there might be as many as one million different endophyte species. However, only a handful of them have been described [5]. Accordingly, it is necessary to further investigate the metabolites of endophytes.

The endophyte HS-05 was isolated from the leaves of *Huperzia serrata* collected in Changbai Mountain, Jinlin province of China in August 2008. Chemical studies on the ethyl acetate extract of the fermentation broth of HS-05 afforded two new compounds. We report here the isolation and structure elucidation of these compounds, as well as their anticancer activities.

2. Results and discussion

Compound **1** was obtained as a white amorphous solid. The molecular formula was determined to be $C_{19}H_{19}NO_5$ by HR-ESI-MS at *m/z* 340.1185 $[M - H]^-$. IR absorptions were observed at 3388, 1734, 1652, 1606, 1514, and 1442 cm^{-1}, which indicated the presence of a hydroxyl

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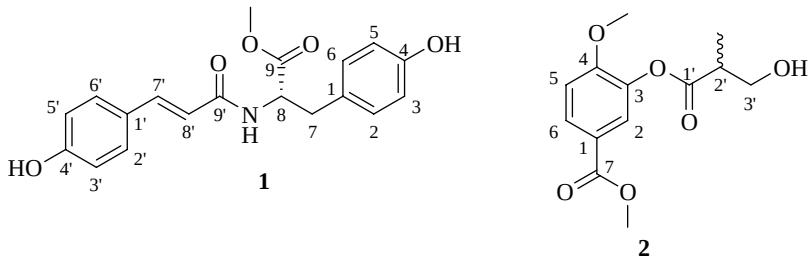
group, two carbonyl groups, and a benzene ring. The UV spectrum showed absorption maxima at 286 and 224 nm. The ^{1}H NMR spectrum showed two phenolic hydroxyl signals at δ 9.80 (1H, br s) and 9.30 (1H, br s), which were assigned to 4′-OH and 4-OH, respectively. Two pairs of aromatic protons at δ 7.37 (2H, d, $J = 8.4$ Hz, H-2′, 6′), 6.78 (2H, d, $J = 8.4$ Hz, H-3′, 5′) and two *trans*-olefin protons at δ 7.28 (1H, d, $J = 15.6$ Hz, H-7′), 6.45 (1H, d, $J = 15.6$ Hz, H-8′) indicated the existence of a *p*-hydroxycinnamoyl (group **1a**) as shown in Figure 2, which was supported by the ^{13}C NMR signals at δ 165.5, 159.2, 139.7, 129.5 × 2, 125.8, 117.8, and 115.9 × 2. It was further confirmed by the HMBC correlations of H-2′ and 6′ with C-7′, C-4′ and H-7′ with C-8′ and C-9′. The other two pairs of aromatic protons at δ 7.00 (2H, d, $J = 8.4$ Hz, H-2, 6), 6.65 (2H, d, $J = 8.4$ Hz, H-3, 5), together with corresponding carbon signals at δ 130.1 × 2, 115.2 × 2, exhibited the presence of 1,4-disubstituted phenyl ring. According to the HMBC correlations of the proton signals at δ_H 7.00 (H-2, 6) and 8.39 (—NH—) with the carbon signal at δ_C 36.2 (C-7) and the correlation of the proton signals at δ_H 2.90 (H-7), 4.48 (H-8), 3.59 (—OMe) with the carbonyl signal at δ_C 172.5 (C-9), the L-tyrosine-methyl ester (moiety **1b**) was established (Figure 2). The conjunction of moiety **1a** with moiety **1b** was deduced by the HMBC correlations of both the proton signals at δ_H 4.48 (H-8) and 8.39 (—NH—) with the cinnamoyl carbonyl signal at δ_C 165.5 (C-9′). The stereochemistry of compound **1** at C-8 was determined by its CD spectrum. The CD spectrum of compound **1** exhibited a negative Cotton effect at 304 nm and a positive Cotton effect at 220 nm similar to that of the methyl ester of *N*-[4′-methoxy-*trans*-cinnamoyl]-3-(4-methoxyphenyl)-L-alanine reported in the literature [6]. Therefore, compound **1** was finally elucidated as *N*-[4′-hydroxy-(*E*)-cinnamoyl]-L-tyrosine methyl ester.

Compound **2** was obtained as a colorless oil, with $[\alpha]_D^{20} + 73.2$ (in MeOH). The molecular formula was determined to be $C_{13}H_{16}O_6$ by HR-ESI-MS at *m/z* 291.0846 $[M + Na]^+$. The IR absorptions were observed at 3430, 1761, 1718, 1613, 1513, and 1439 cm^{-1}, which indicated the presence of a hydroxyl group, two ester carbonyl groups, and a benzene ring. The UV spectrum showed absorption maxima at 256 and 228 nm. The ^{1}H NMR signals at δ 7.96 (1H, dd, $J = 8.7$, 2.0 Hz, H-6), 7.75 (1H, d, $J = 2.0$ Hz, H-2), and 7.00 (1H, d, $J = 8.7$ Hz, H-5) were readily attributed to a 1,3,4-trisubstituted phenyl ring (moiety **2a**) as shown in Figure 1, which was supported by the ^{13}C NMR signals at δ 154.6, 139.1, 129.2, 124.4, 123.1, and 111.6. In addition, two methoxyl signals [δ_H 3.90 (3H, s, 4-OMe), 3.89 (3H, s, 7-OMe)], a methyl signal [δ_H 1.34 (3H, d, $J = 7.2$ Hz, 2′-Me)], a hydroxymethylene (—C*H*$_2$OH) group [δ_H 3.85 (2H, d, $J = 2.6$ Hz, H-3′)], a methine proton [δ_H 2.98 (1H, m, H-2′)], and two ester carbonyl



1 **2**

Figure 1. The structures of compounds **1** and **2**.

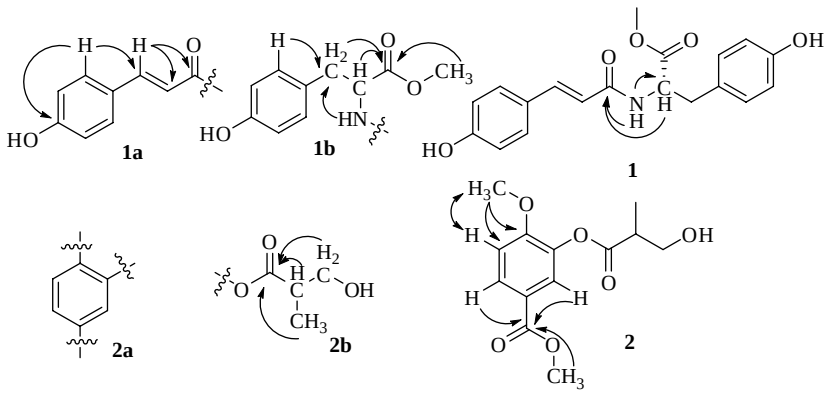


Figure 2. The key HMBC (H → C) and NOE (H ↔ H) correlations of the moieties of compounds **1** and **2**.

signals at δ_C 173.3 (C-1′), 166.0 (C-7) were observed from the ^{1}H and ^{13}C NMR spectra, respectively. The assignment of all protonated carbon was established by HSQC experiment. The structure assembly of **2** was mainly achieved using HMBC experiment (Figure 2). The HMBC correlations of the signals at δ 7.96 (H-6), 7.75 (H-2), and 3.89 (—OMe) with the carbonyl signal at δ 166.0 indicated the location of a —COOMe group at C-1. The location of another methoxyl group at C-4 was determined by the correlations of the proton signal at δ 3.90 (—OMe) with the carbons at δ 154.6 (C-4) and 111.6 (C-5), along with the NOESY correlation between the signal at δ 3.90 (—OMe) and the signal at 7.00 (H-5). The correlations of the signals at δ 3.85 (H-3′), 2.98 (H-2′), and 1.34 (2′-Me) with the other ester carbonyl signal at δ 173.3 indicated the presence of the moiety **2b**. The location of the moiety **2b** at C-3 was suggested by the chemical shift of C-3 (δ 139.1) and the molecular formula. Accordingly, compound **2** was assigned as methyl 4-methoxy-3-(3′-hydroxy-2′-methyl) propionyloxy-benzoate.

The cytotoxicities of compounds **1** and **2** against HL-60 (human leukemia) cell lines were tested by MTT assay. Neither of them showed anticancer activities ($IC_{50} > 100\,\mu M$).

3. Experimental

3.1 General experimental procedures

Optical rotations were measured on a Perkin-Elmer 241 polarimeter. A JASCO CD-2095 Chiral Detector was employed for CD determination using MeOH as solvent. UV spectra were measured on a Shimadzu UV-1601. IR spectra were taken on a Bruker IFS-55 infrared spectrophotometer. The NMR spectral data were recorded on Bruker AV-600 (600 MHz for ^{1}H and 150 MHz for ^{13}C) with TMS as the internal standard. The HR-ESI-MS data were obtained on a Varian QFT-ESI instrument. Chromatography was performed on silica gel (200–300 mesh; Qingdao Haiyang Chemical Factory, Qingdao, China), Sephadex LH-20 (Pharmacia, Piscataway, NJ, USA), and reversed-phase HPLC (Hitachi L-6000 pump and Hitachi L-7400 UV detector).

3.2 Strain isolation and cultivation

Strain HS-05 was isolated from the leaves of *H. serrata* collected in Changbai Mountain, Jinlin province of China in August 2008. The strain HS-05 was identified as *Aspergillus* sp. by Prof. Yixuan Zhang, and has

been deposited in the School of Traditional Chinese Materia Medica, Shenyang Pharmaceutical University.

The fresh mycelium grown on PDA medium was inoculated into 0.5 l Erlenmeyer flasks containing 0.15 l liquid medium (2% glucose, 2% maltose, 1% monosodium glutamate, 0.05% KH_2PO_4, 0.03% $MgSO_4{\cdot}7H_2O$, 0.3% yeast extract, tap water, pH 6.5). After 2 days of incubation on rotary shaker at 28°C at 180 rpm, a 7.5 ml culture liquid was transferred as a seed into each of 0.5 l Erlenmeyer flask containing 0.15 l of the same liquid medium. The flasks were subsequently incubated at the same conditions for 8 days.

3.3 *Extraction and isolation*

The fermentation broth of the strain HS-05 (about 80 l) was concentrated and extracted with ethyl acetate and *n*-butanol, successively. The EtOAc crude extract (24 g) was applied on a silica gel column, and eluted with $CHCl_3$–CH_3OH gradient (from 100:1 to 0:1) to afford 12 fractions. Fraction 4 (4.5 g) was subjected to silica gel column, eluted with petroleum ether–acetone (100:1–0:1), yielding five subfractions. Subfraction four gave compound **1** (4 mg) by HPLC (MeOH–H_2O 45:55). Fraction 3 (6 g) was then subjected to silica gel column chromatography, eluted with petroleum ether–EtOAc (100:1–0:1), yielding eight subfractions. Among these subfractions, subfraction five was then submitted to semi-preparative reverse-phase HPLC (C-18) by eluting with MeOH–H_2O (32:68) (3 ml/min) to yield compound **2** (5.5 mg).

3.3.1 *Compound* ***1***

White amorphous solid, $[\alpha]_D^{20}$ − 45.2 (in MeOH). UV(MeOH) λ_{max}: 286, 224 nm; IR (KBr) ν_{max} (cm^{-1}): 3388, 1734, 1652, 1606, 1514, 1442; CD(CH_3OH) $\Delta\varepsilon_{304}$ nm − 1.19, $\Delta\varepsilon_{202}$ nm + 6.72; ^1H and ^{13}C NMR spectral data, see Table 1; HR-ESI-MS *m/z*:

Table 1. ^1H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **1** (in DMSO-d_6).

Position	δc	δ_H (*J*, Hz)	HMBC (H → C)
1	127.3	–	
2	130.1	7.00 (1H, d, 8.4)	C-3, 4, 6, 7
3	115.2	6.65 (1H, d, 8.4)	C-1, 4, 5
4	156.2	–	
5	115.2	6.65 (1H, d, 8.4)	C-1, 3, 4
6	130.1	7.00 (1H, d, 8.4)	C-2, 4, 5, 7
7	36.2	2.90 (2H, m)	C-1, 2, 8, 9
8	54.3	4.48 (1H, m)	C-1, 7, 9
9	172.5	–	
1′	125.8	–	
2′	129.5	7.37 (1H, d, 8.4)	C-3′, 4′, 6′, 7′
3′	115.9	6.78 (1H, d, 8.4)	C-1′, 4′, 5′
4′	159.2	–	
5′	115.9	6.78 (1H, d, 8.4)	C-1′, 3′, 4′
6′	129.5	7.37 (1H, d, 8.4)	C-2′,4′, 5′, 7′
7′	139.7	7.28 (1H, d, 15.6)	C-1′, 2′, 8′, 9′
8′	117.8	6.45 (1H, d, 15.6)	C-1′, 9′
9′	165.5	–	
9-OCH_3	52.0	3.59 (3H, s)	C-9
—NH—	–	8.39 (1H, d, 7.8)	C-7, 8, 9′
4-OH	–	9.30 (1H, br s)	
4′-OH	–	9.80 (1H, br s)	

Table 2. ^{1}H NMR (600 MHz) and ^{13}C NMR (150 MHz) spectral data of compound **2** (in $CDCl_3$).

Position	δc	δ_H (*J*, Hz)	HMBC (H → C)
1	123.1	–	
2	124.4	7.75 (1H, d, 2.0)	C-1, 3, 4, 6, 7
3	139.1	–	
4	154.6	–	
5	111.6	7.00 (1H, d, 8.7)	C-1, 3, 4
6	129.2	7.96 (1H, dd, 8.7, 2.0)	C-2, 7
7	166.0	–	
7-OCH_3	52.1	3.89 (3H, s)	C-1, 7
4-OCH_3	56.2	3.90 (3H, s)	C-4, 5
1′	173.3	–	
2′	42.1	2.98 (1H, m)	C-1′, 3′, C-(2′-CH_3)
3′	64.8	3.85 (2H, d, 2.6)	C-1′, 2′, C-(2′-CH_3)
2′-CH_3	13.3	1.34 (3H, d, 7.2)	C-1′, 2′, 3′

340.1185 $[M - H]^-$ (calcd for $C_{19}H_{18}NO_5$, 340.1190).

3.3.2 *Compound* **2**

Colorless oil, $[\alpha]_D^{20} + 73.2$ (in MeOH). UV(MeOH) λ_{max}: 256, 228 nm; IR (KBr) ν_{max} (cm^{-1}): 3430, 1761, 1718, 1613, 1513, 1439; ^{1}H and ^{13}C NMR spectral data, see Table 2; HR-ESI-MS *m/z*: 291.0846 $[M + Na]^+$ (calcd for $C_{13}H_{16}O_6Na$, 291.0839).

3.4 *Cytotoxicity of compounds 1 and 2*

RPMI-1640 medium (Gibco, New York, NY, USA) contained 100 U/ml penicillin, 100 mg/ml streptomycin, 1 mmol glutamine, and 10% heat-inactivated fetal bovine serum (Gibco). Human leukemia HL-60 cells (American Type Culture Collection, Rockville, MD, USA) were cultured in the above medium at a density of 5×10^4 cells/ml at 37°C under an atmosphere of 5% CO_2. Cell growth inhibition assay was performed as reported previously. The compounds were dissolved in DMSO, and the amount of DMSO was controlled lower than 0.1% in the final concentration. Cells were incubated with various drug concentrations for 3 days. The number of cells was determined by hemocytometer, and its viability was determined using trypan blue staining. The growth inhibitory ability of the new compounds was calculated and expressed using the IC_{50} value (half-inhibitory concentration). 5-Fluorouracil (5-FU) and 0.1% DMSO were used as a positive control and a negative control, respectively.

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