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Fumigaclavine I, a new alkaloid isolated from endophyte *Aspergillus terreus*

SHEN Li[1, 3*], ZHU Li[1], LUO Qian[1], LI Xiao-Wen[1], XI Ju-Qun[1, 2], KONG Gui-Mei[2], SONG Yong-Chun[4]

[1] *Medical College, Yangzhou University, Yangzhou 225001, China;*

[2] *Jiangsu Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Treatment of Senile Diseases, Yangzhou 225001, China;*

[3] *Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225009, China;*

[4] *Institute of Functional Biomolecules, Nanjing University, Nanjing 210046, China*

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[ABSTRACT] The present study was designed to isolate and purify chemical constituents from solid culture of endophyte *Aspergillus terreus* LQ, using silica gel column chromatography, gel filtration with Sephadex LH-20, and HPLC. Fumigaclavine I (**1**), a new alkaloid, was obtained, along with seven known compounds, including fumigaclavine C (**2**), rhizoctonic acid (**3**), monomethylsulochrin (**4**), chaetominine (**5**), spirotryprostatin A (**6**), asperfumoid (**7**), and lumichrome (**8**). The structure of compound **1** was elucidated by various spectroscopic analyses (UV, MS, 1D and 2D NMR). The *in vitro* cytotoxicity of compound **1** was determined by MTT assay in human hepatocarcinoma cell line SMMC-7721, showing weaker cytotoxicity, compared with cisplatin, a clinically used cancer chemotherapeutic agent.

[KEY WORDS] Endophyte; *Aspergillus terreus*; Chemical constituent; Ergot alkaloid

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Introduction

Aspergillus terreus is a common fungus widely used in chemical and pharmaceutical industries. It is a main strain used for producing the important chemical intermediate Itaconic acid [1]. Lovastatin, namely mevinolin, an important cholesterol-lowering drug, was first isolated from *A. terreus* [2] and is still commercially produced by *A. terreus* in submerged fermentation [3]. Furthermore, *A. terreus* from special eco-environment has been proven to be a rich source of bioactive natural products [4-8]. In our continuous research on the chemical constituents of endophytic fungus, separation and purification of solid culture of endophyte *A. terreus* LQ led to the isolation of a new alkaloid, named fumigaclavine I (**1**), together with seven known metabolites identified as fumigaclavine C (**2**) [9], rhizoctonic acid (**3**) [10], monomethylsulochrin (**4**) [10], chaetominine (**5**) [11-12], spirotryprostatin A (**6**) [13], asperfumoid (**7**) [14] and lumichrome (**8**) [15]. In addition, the *in vitro* cytotoxicity of compound **1** was determined in human hepatocarcinoma cell line SMMC-7721. It is hoped that our study will facilitate the research and development of active constituents of *A. terreus* in the future.

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[*Corresponding author] Tel: 86-514-87992233, E-mail: shenli@yzu.edu.cn

These authors have no conflict of interest to declare.

Results and Discussion

Characterization of compound 1

Fumigaclavine I (**1**) was isolated as white powder, and its molecular formula was deduced as $C_{23}H_{30}N_2O_4$ from the quasi-molecular ion at *m/z* 399.228 2 (Calcd. for $C_{23}H_{31}N_2O_4$ 399.228 4) in positive HR-ESI mass spectra, implying that it

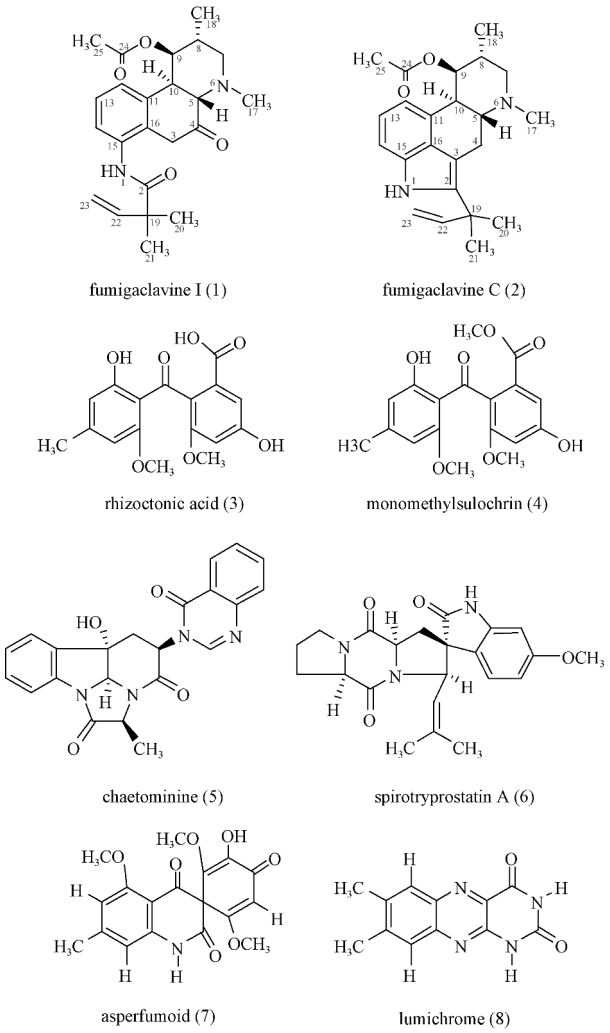


Fig. 1 Structures of compounds 1−8

possessed 10 degrees of unsaturation. The ^{1}H and ^{13}C NMR spectra of compound **1** displayed the coexistence of one labile proton, one 1, 2, 3-trisubstituted benzene, one double bond, one ketone, two ester or amide carbonyls, five methyls, two methylenes, four methines, and one quaternary carbon, which was confirmed by DEPT spectrum. Thus compound **1** was deduced as a tricyclic compound, in accordance with its degree of unsaturation. In ^{1}H-^{1}H COSY spectrum of compound **1**, the coupling sequences from H-5 through H-7 and between H-8 and H-18 were readily recognized. In addition, the observed correlations in the HMBC spectrum from H-7 to C-5, C-8, C-9, C-17 and C-18, from H-9 to C-5, C-7, C-8, C10 and C-24, from H-5 to C-7 and C-10, from H-25 to C-24 and C-9, from H-18 to C-7, C-8 and C-9, and from H-17 to C-5, C-7 and C-8 indicated the existence of a six-member ring (C5-N6-C7-C8-C9-C10) with an acetoxy group connected to C-9 in compound **1**. The correlations in the HMBC spectrum of compound **1** from H-5 to C-3 and C-4, from H-3 to C-4, C-5, C-10 and C-16, from H-10 to C-11, and from H-12 to C-10, in accordance with the correlation between H-10 and H-12 in ^{1}H-^{1}H COSY spectrum, disclosed that compound **1** possessed a fused tricyclic substructure. In the HMBC spectrum, the correlations from H-23 to C-2, C-19, C-20/C-21 and C-22, from H-22 to C-2, C-19 and C-20/C-21, from H-20/H-21 to C-19, C-22, C-23 and C-2, along with the correlation between H-23 and H-24 in COSY spectrum, indicated an isopentenyl attached at C-2. The rest signals of ^{1}H NMR and ^{13}C NMR were attributed to establish the gross structure of compound **1** (Fig. **1**). All ^{1}H and ^{13}C NMR spectral data (Table **1**) of compound **1** were assigned unambiguously through a set of 2D NMR spectroscopic experiments (HSQC, HMBC, ^{1}H-^{1}H COSY and NOESY spectra). Thus, the relative configuration of compound **1** was deduced by comparing NMR data with fumigaclavine C (**2**). The chemical shifts of compound **1** from C/H-5 through C/H-10 were very close to that of compound **2**. Furthermore, the NOE correlations between proton pairs H-9/H-10 and H-9/H-18 in compound **2** were also observed in compound **1**, while the absence of correlation between H-10 and H-5 in compound **2** was not found in compound **1** either. The aforementioned NMR data indicated that compounds **1** and **2** shared the same orientation of H-5, H-8, H-9 and H-10, *i.e.,* H-5 and H-8 were in α-position and H-9 and H-10 were in β-position [9]. Finally, the relative configuration of Compound **1** was determined as 5S^{*}, 8R^{*}, 9$S^{*}$and 10R^{*}.

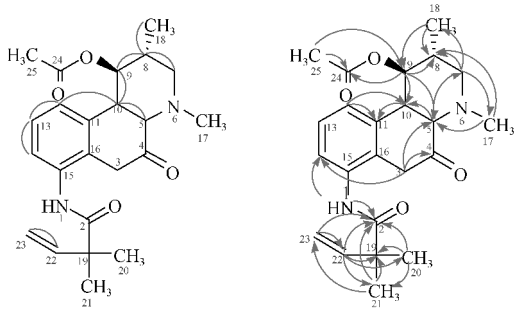


Fig. 2 Key ^{1}H-^{1}H COSY (⌒), and HMBC (↷) correlations of compound 1

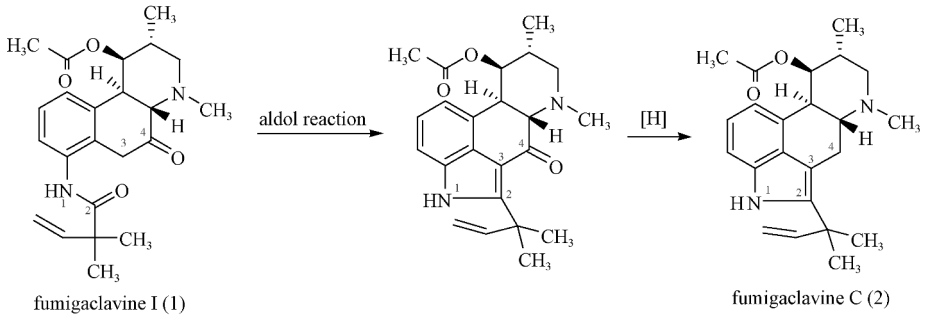
Investigations of the biosynthesis of ergot alkaloids by feeding experiments and with crude enzyme extracts have clearly revealed that the tetracyclic ergoline moiety in ergot alkaloids is derived from L-tryptophan and dimethylallyl diphosphate (DMAPP) [16]. L-tryptophan is converted to biosynthetic intermediate chanoclavine-I as well as two isomers chanoclavine-Ⅱ and isochanoclavine-I through a five-step reaction including prenylation, methylation, decarboxylation, cyclization and hydroxylation [9], and then transformed to fumigaclavine C and other ergot alkaloids. However, the isolation of compound **1** from *A. terreus* LQ indicated that there might be a new biosynthesis pathway for ergot alkaloid, not from L-tryptophan to agroclavine or festuclavine (**Scheme 1**).

Cytotoxicity of compound 1

In the present study, fumigaclavine I (**1**) was evaluated for its *in vitro* cytotoxicity to the human hepatocarcinoma cell line SMMC-7721 by the MTT method. The result showed that compound **1** was slightly cytotoxic to the

Table 1 ^{1}H and ^{13}C NMR data of fumigaclavine I (1) and fumigaclavine C (2) in $CDCl_3$ (δ in ppm and J in Hz)

Position	Fumigaclavine I (**1**)		Fumigaclavine C (**2**)	
	δ_H	δ_c	δ_H	δ_c
1-NH	12.24 (s)		7.73 (s)	
2		176.3		136.7
3	3.19 (dd, 4.2, 16.8) 2.55 (dd, 13.2, 16.8)	45.0		106.1
4		200.9	3.51 (dd, 3.0, 13.2) 2.65 (m)	28.0
5	2.67 (m)	59.1	2.65 (m)	61.7
7	2.62 (m) 2.58 (m)	56.5	2.72 (m) 2.65 (m)	57.8
8	2.12 (m)	33.1	2.10 (m)	33.1
9	5.54 (s)	71.0	5.66 (s)	71.4
10	3.21 (d, 10.8)	41.4	3.32 (d, 9.0)	39.3
11		143.3		129.2
12	6.94 (d, 8.4)	119.3	6.71 (d, 7.2)	112.9
13	7.50 (t, 8.4)	135.6	7.04 (t, 7,8)	122.3
14	8.68 (d, 8.4)	118.7	7.08 (d, 7,8)	107.7
15		142.4		132.1
16		118.9		128.0
17	2.30 (s)	42.5	2.45 (s)	43.5
18	1.30 (d, 7,2)	16.6	1.32 (d, 7.8)	16.7
19		47.0		39.1
20	1.42 (s)	24.7	1.52 (s)	27.4
21	1.42 (s)	24.7	1.52 (s)	27.3
22	6.13 (dd, 10.8, 17.4)	142.5	6.10 (dd, 10.8, 17.4)	145.7
23	5.37 (d, 17.4) 5.32 (d, 10.8)	114.7	5.14 (d, 6.6) 5.12 (s)	111.9
24		170.5		170.9
25	1.89 (s)	21.0	1.89 (s)	21.2



Scheme 1 A putative biosynthetic route from fumigaclavine I (1) to fumigaclavine C (2)

SMMC-7721 cells with the growth inhibition rate being 20.3% at 10 μg·mL^{-1}, while the positive control cisplatin obviously inhibited the growth of SMMC-7721 cells with the IC_{50} value being 7.62 μg·mL^{-1}.

Endophyte *A. terreus* LQ could produce several alkaloids with various structures and bioactivities, including a new ergot alkaloid-like compound, which implied that LQ is a potential producer. In summary, the OSMAC (One Strain Many Compounds) approach is a simple and effective means in the research of microbial metabolites and *A. terreus* LQ may produce much more new and/or bioactive compounds by using this method in further research.

Experimental

General

The optical rotation was determined in CH_3OH on a W22-2B automatic polarimeter (Shanghai Precision Scientific Instrument Co., Ltd., Shanghai, China). The UV spectrum was acquired on a U-3900 spectrophotometer (Hitachi, Ltd., Tokyo, Japan). The NMR spectra were recorded on an AVANCE 600 NMR spectrometer (Bruker Co., Karlsruhe, Germen) using tetramethylsilane (TMS) as internal standard. The HR-ESI mass spectra were taken on UHR-TOF maXis MS instrument, Bruker Corporation, Karlsruhe, Germen and Mariner Mass 5304 instrument (Applied Biosystems, California, USA). The HPLC was performed on Agilent 1260 HPLC (Agilent Technologies Inc., California, USA) with Sinochrom ODS-AP column (4.6 mm × 250 mm, 5 μm) and Elite P200Ⅱ HPLC with Kromasil C_{18} column (4.6 mm × 250 mm, 5 μm), Dalian Elite Analytical Instruments Co., Ltd, Dalina, China. Thermo Forma CO_2 incubator was purchased from Thermo Fisher Scientific (Ohio, USA). The ELISA plate reader was from Bio-Tek Instruments, Inc., Vermont, USA. Silica gel (200−300 mesh) for column chromatography and silica GF_{254} for TLC were produced by Qingdao Marine Chemical Company, Qingdao, China. Sephadex LH-20 was purchased from Pharmacia Biotech., Uppsala, Sweden. HPLC-grade methanol was provided by Tedia Company, Inc., Ohio, USA. Deuterium reagent was provided by Sigma-Aldrich Co., LLC., Missouri, USA. Fetal bovine serum was produced by Shanghai Luoshen Biotechnology Co., Ltd., Shanghai, China. DEME medium and 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) was purchased from Gibco Co., New York, USA. The 96-well plates were supplied by Corning Costar Inc., New York, USA. Cisplatin was provided by Jiangsu Hansoh Pharmaceutical, Lianyungang, China. Human hepatocarcinoma cell line SMMC-7721 was supplied by Medical College of Yangzhou University, Yangzhou, China.

Endophyte strain

Endophyte strain LQ was isolated from stem of rice and authenticated by Dr. SONG Yong-Chun as *Aspergillus terreus* according to the morphological character. The live culture of *A. terreus* LQ was kept at the Institute of Functional Biomolecules, Nanjing University (Nanjing, China).

Extraction and isolation

A. terreus LQ was cultured in solid substrate as detailed elsewhere [9]. The cultured biomass was extracted with $CHCl_3/CH_3OH$ (1 : 1) three times at room temperature to afford a crude extract (40 g) after *in vacuo* evaporation of the solvent. The crude extract was chromatographed on silica gel column that was eluted with a gradient of $CHCl_3/CH_3OH$ (100 : 0 → 0 : 100) to give 7 fractions. The precipitate crystallized from methanol solution of Fr. 1 (2.5 g) was purified by HPLC (Kromasil C_{18} column, MeOH : H_2O 60 : 40, 0.9 mL·min^{-1}) to provide compound **7** (7.5 mg, t_R = 8.4 min). Fr. 2 (1.7 g) was subjected to further column chromatography with Sephadex LH-20 ($CHCl_3/CH_3OH$ 1 : 1) and gave compound **4** (16.8 mg). Fr. 3 (2.6 g) was chromatographed by silica gel column chromatography with $CHCl_3$/MeOH gradient to give Fr. 3-1 and Fr. 3-1 was then divided into 3 subfractions (Fr. 3-1-1−Fr. 3-1-3) through gel filtration with Sephadex LH-20. Fr. 3-1-1 was purified by HPLC (Sinochrom ODS-AP column, MeOH : H_2O 70 : 30, 1.0 mL·min^{-1}, t_R = 35.01 min) to give compound **1** (10.0 mg) and Fr. 3-1-1-1 and the obtained Fr. 3-1-1-1 was further purified by HPLC (Kromasil C_{18} column, MeOH : H_2O 44 : 56, 1.0 mL·min^{-1}) to yield compound **6** (6.0 mg, t_R = 43.9 min). Fr. 3-1-3 was also purified by HPLC (Sinochrom ODS-AP column, MeOH : H_2O 45 : 55, 1.0 mL·min^{-1}) to give compound **8** (8.4 mg, t_R = 11.3 min). Fr. 4 (4.2 g) was separated by silica gel column chromatography with $CHCl_3/CH_3OH$ gradient (100 : 1→0 : 100) to give Fr. 4-1 and Fr. 4-2. Gel filtration of Fr. 4-1 over Sephadex LH-20 column eluting with $CHCl_3$/ CH_3OH (1 : 1) followed by HPLC (Sinochrom ODS-AP column, MeOH : H_2O 45 : 55, 0.8 mL·min^{-1}) to provide compound **5** (23.2 mg, t_R = 35.6 min). Gel filtration of Fr. 4-2 with Sephadex LH-20 column eluted with $CHCl_3/CH_3OH$ (1 : 1) gave Fr. 4-2-2 and Fr. 4-2-3; Fr. 4-2-2 and Fr. 4-2-3 were further purified by HPLC to yield compound **2** (Sinochrom ODS-AP column, MeOH : H_2O 65 : 35, 0.8 mL·min^{-1}, t_R = 34.83 min) and compound **3** (Sinochrom ODS-AP column, MeOH : H_2O 55 : 45, 0.8 mL·min^{-1}, t_R = 2.50 min).

Fumigaclavine I (**1**): white powder; $[\alpha]_D^{25}$ −82.9 (*c* 0.048, CH_3OH); UV (CH_3OH): λ_{max} (logε) = 238 (4.97), 265 (4.51), 336 (4.32) nm; ^1H and ^{13}C NMR (**Table 1**); HR-ESI-MS: *m/z* 399.228 2 $[M + H]^+$ (Calcd. for $C_{23}H_{31}N_2O_4$: 399.228 4).

Cytotoxicity assay

In vitro cytotoxicity of compound **1** was evaluated using the MTT method as described in our previously published paper [17].

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