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Chemical Investigation of a Co-Culture of A*spergillus fumigatus* D and *Fusarium oxysporum* R1

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Abstract: Chemical investigation of a co-culture of two endophytic fungi *Aspergillus fumigatus* D and *Fusarium oxysporum* R1 from two traditional medicinal plants, *Edgeworthia chrysantha* Lindl. and *Rumex madaio* Makino, led to isolation of a new amide **1** and six known compounds, including neovasinin (**2**), neovasifuranone B (**3**), N-(2-phenylethyl)acetamide (**4**), α-linolenic acid (**5**), α-elaeostearic acid (**6**), palmitoleic acid (**7**). On the basis of extensive spectroscopic analysis including 1D and 2D NMR, HR-ESI-MS and optical rotation measurement as well as comparison of literature data, chemical structure of **1** was unambiguous elucidated as (*S*, *E*)-methyl-2-(2,4-dimethylhex-2-enamido)acetate. Bioassay results indicated that none of these compounds exhibited strong inhibitory effect on three human pathogens *Escherichia coli*, *Staphyloccocus aureus* and *Candida albicans* with MIC values of ⩾25 μM.

Keywords: Endophytic fungus; *Aspergillus fumigatus* D; *Fusarium oxysporum* R1; co-culture; amide; antimicrobial effect.

1. Introduction

During the past almost two decades, phytochemical investigation of endophytic microbes had been worldwide carried out and resulted in isolation of more than 500 new functional chemicals, which exerted significant impact on development of new therapeutic agents and pesticides [1-2]. Recently, however, the speed of discovering new secondary metabolites from endophytes has been slowing down owing to consistent employment of conventional cultivation and isolation procedure. Microorganisms have potential biosynthetic gene clusters (BGCs) to make more compounds, which were left untapped under standard laboratory conditions [3-4]. A growing number of evidences indicated that the strategy of one strain many compounds (OSMAC) is a simple and powerful tool to enhance chemical diversity of microbes, such as changing medium composition and cultivation status, co-cultivation with other strain(s), adding enzyme inhibitor(s) and MSM biosynthetic precursor(s) [5]. Co-culture system can effectively alter individual microbial cellular physiology and access chemical diversity [6-10].

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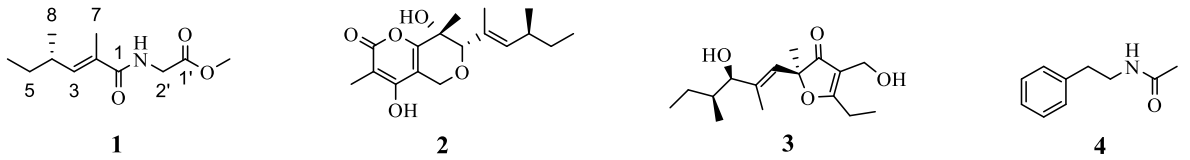
In our continuous search for structurally unique and bioactive secondary metabolites from endophytic microbes, chemical study of a rice medium co-culture of two endophytic strains *Aspergillus fumigatus* D and *Fusarium oxysporum* R1 from two traditional medicinal plants, *Edgeworthia chrysantha* Lindl. and *Rumex madaio* Makino respectively, resulted in isolation of one new amide **1** and three known compounds neovasinin (**2**), neovasifuranone B (**3**), *N*-(2-phenylethyl)acetamide (**4**) (Figure 1) and three well known fatty acids *α*-linolenic acid (**5**), *α*-elaeostearic acid (**6**) and palmitoleic acid (**7**). Interestingly, all these compounds were not detected in their axenic cultures [11-15]. Herein the isolation and structural elucidation of these compounds as well as antimicrobial effects are reported.



1 **2** **3** **4**

Figure 1. Chemical structures of compounds **1-4** from a co-culture of endophytic strains D and R1

2. Materials and Methods

2.1. General

All NMR experiments were run on a Bruker Avance DRX600 spectrometer (Bruker, Fällande, Switzerland) equipped with a 5 mm triple resonance (HCN) cold probe, using TMS as an internal standard. Optical rotation was detected on a JASCO P-2000 polarimeter (JASCO, Fukuoka, Japan). Melting points were recorded on an XRC-1 apparatus (Sichuan University Science and Education Instrument Factory, Chengdu, China). Ultraviolet (UV) spectrum was taken on Hitachi-UV-3000 spectrometer (Hitachi, Tokyo, Japan), and FT-IR spectrum was performed on Nexus 870 spectrometer (Thermo-Nicolet, Madison, WI, USA). ESI-MS and HR-ESI-MS data were obtained from an Agilent 6210 LC/TOF-MS spectrometer (Agilent Technologies, Santa Clara, CA, USA). Purification of all compounds was performed on Essentia LC-16P apparatus (Essentia, San Diego, CA, USA) equipped with a semi-preparative column (Phenomenex Hydro-RP, 250 mm × 10 mm, 4 μm, Torrance, CA, USA) or an analytical column (Phenomenex Hydro-RP, 250 mm × 4.6 mm, 4 μm, Torrance, CA, USA). Acetonitrile (Merck, Darmstadt, Germany), methanol (Merck) and H_2O used in HPLC system were of chromatographic grade, and all other chemicals were analytical.

2.2. Biological Material

Two endophytic strains D and R1 were respectively isolated from two traditional medicinal plants *Edgeworthia chrysantha* Lindl. [16] and *Rumex madaio* Makino [17], and identified as *Aspergillus fumigatus* and *Fusarium oxysporum* according to their morphological characteristics and 18*S* rDNA gene sequence, which had been reported before[18]. And these two strains were preserved in China General Microbiological Culture Collection Centre and coded as Nos. 17762 and 17763, respectively.

2.3. Fermentation and Isolation

Endophytic strains D and R1 were inoculated separately into on slants of potato dextrose agar (PDA) for 7 days at 28 ℃. A proper amount of each fungal colony was inoculated into 500-mL Erlenmeyer flask, which contained 250 mL potato dextrose broth (PDB), and shaken for 7 days at 200 rpm and 28 ℃, respectively. Then these seed cultures were simultaneously and respectively transferred into a 1000-mL Erlenmeyer flask with sterilized rice (160 g) and tap water (320 mL) followed by co-cultivation at 28 ℃ for 30 days. 100 flasks of the rice medium co-culture were collected and extracted with ethyl acetate for 3 times. The organic layer was obtained by filtration

through gauze and concentrated under vacuum at 40 ºC. The crude extract (approx. 90 g) was dissolved in 100 mL of methanol to obtain a suspension, which was respectively extracted with n-hexane and dichloromethane. The dichloromethane extraction layer was concentrated under reduced pressure to yield a crude extract (27.09 g) and then subjected to chromatography on a silica-gel (200 mesh) column eluted sequentially with dichloromethane and ethanol (100:0, 99:1, 98:2, 95:5, 90:10, 50:50; v/v) to obtain six fractions A-F. And the n-hexane extraction layer was evaporated at 25 °C in vacuum to yield the extract (45.49 g) followed by separation on a preparative HPLC column (Phenomenex Gemini-NX C18, 50 mm × 21.2 mm, 5 μm) to afford six fractions G-L according to our reported procedure [19].

Compound **4** (3.6 mg, t_R = 15.5 min) was purified from fraction A using a semi-preparative HPLC column with 30% CH_3CN/H_2O with a flow rate of 3.0 mL/min at 210 nm. Fraction B was subjected to HPLC with an analytical column to afford compound **1** (4.1 mg, t_R = 13.0 min) with 65% CH_3OH/H_2O and compound **3** (39.7mg, tR = 13.5 min) with 40% CH_3OH/H_2O, which the flow rate was set as 1.0 mL/min at 210 nm. While a large amount (3624.4 mg, t_R = 13.0 min) of compound **2** was purified from fraction C with 65% CH_3OH/H_2O using the semi-preparative HPLC column. Fraction J was also separated on the semi-preparative HPLC column with 87% CH_3CN/H_2O to yield **5** (46.5 mg, t_R = 7.8 min), **6** (91 mg, t_R = 7.9 min) and **7** (45.4 mg, t_R = 14.0 min).

2.4. Antimicrobial Assay

Antimicrobial test was carried out using three human pathogenic strains, *Escherichia coli* 25922, *Staphyloccocus aureus* ATCC 25923 and *Candida albicans* ATCC 10231, which were purchased from China Center for Type Culture Collection (CCTCC) by 96-well plate gradient dilution method [20-21]. The tested fungus was cultured in Sabouraud medium at 150 rpm, 28 ºC for 48 hours, while the bacteria cultured in Luria-Bertani medium (LB) was incubated at 37 ºC for 24 hours at the same rotation speed, and the indicator strain was diluted with fresh medium with a cell density of 10^5-10^6 cfu/mL. All tested compounds were accurately weighed and dissolved with dimethyl sulphoxide (DMSO) while ampicillin and amphotericin B were used as the positive controls. A suitable volume of sample and microbial suspension was pipetted into a 96-well plate, which the first well had a volume of 200 μL and a concentration of 100 μM. After pipetting evenly, 100 μL was taken from the first hole and added to the next hole, and so on. The corresponding sample concentration per well was as follows: 100, 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39, 0.195 μM. Three sets of parallel experiments were performed in each group, and the absorbance value of the indicated culture medium was detected by enzyme marker (Molecular Devices m5, MD, US). The minimum inhibitory concentration (MIC) was the lowest concentration of each compound that inhibited the growth of pathogenic microorganisms.

3. Results and Discussion

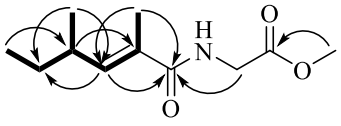
3.1. Structure Elucidation

Compound **1** was obtained as yellowish powder. It had a molecular formula of $C_{11}H_{19}NO_3$ requiring 3 degrees of unsaturation, which was deduced from the positive molecule-ion peak at m/z 236.1262 [M + Na^+] (calcd for $C_{11}H_{19}NO_3Na$: 236.1257) in the HR-ESI-MS. Its strong IR bands at 1746, 1661, 1625 and 1207 cm^{-1} indicated the presence of chelated carbonyl group(s). The ^{1}H NMR spectrum (table 1) displayed compound **1** possesses four methyl signals at δ_H 0.84 (*t*, *J* = 7.8, 7.2 Hz), 0.99 (*d*, *J* = 6.6 Hz), 1.87 (*d*, *J* = 1.2 Hz) and 3.77 (s), and an olefinic proton at δ_H 6.18 (*d*, *J* = 9.6 Hz) and an amide hydrogen at δ_H 6.34 (*brs*). Detailed analysis of its ^{13}C NMR data and DEPT-135 spectrum (table 1) exhibited eleven carbon signals, including four methyls (δ_C 12.01, 12.87, 19.95, 52.53), two methylenes (δ_C 29.83, 41.64), two methines (δ_C 34.78, 143.31) and three quaternary carbons (δ_C 128.90, 169.83, 170.91), which accounted for three double-bond equivalents in **1**. By careful inspection of the HMBC spectrum (Figure 2), the olefinic proton (H-3, δ_H 6.18) had remarkable HMBC correlations to C-1, 5, 7, 8, and the methoxyl (δ_H 3.77) showed only correlation to C-1′ (δ_C 170.91). On basis of other key HMBC correlations from H-4 to C-2, H-6 to C-4, H-7 to C-1

and C-3, H-8 to C-3 and C-5, and H-2′ to C-1 and C-1′ as well as no NOESY correlation between H-7 (δ_H 0.99) and H-8 (δ_H 1.87), the relative configuration of compound **1** was established as (*E*)-methyl-2-(2,4-dimethylhex-2-enamido)acetate, which possessed only one stereogenic centre at C-4. Considering its similar optical rotation ($[\alpha]_D^{25}$ = 11.7) with that ($[\alpha]_D^{25}$ = 17.7) of fusarester A, which was determined by electronic circular dichroism (ECD) calculation[22], the absolute configuration of the C-4 in the molecule of **1** was determined as *S*. Therefore, the chemical structure of compound **1** was unambiguously established as (*S*, *E*)-methyl-2-(2,4-dimethylhex-2-enamido)acetate.

Biogenetically, compound **1** could have similar biosynthetic pathway with those of neovasinin derivatives [23], and be derived from a triketide precursor via condensation of three acetyl CoA followed by methylation and amidation [24] (Figure S13).

By careful comparison of the ^{1}H, ^{13}C NMR and ESI-MS data with literature, compounds **2-7** were characterized as neovasinin [25], neovasifuranone B [26], *N*-(2-phenylethyl)acetamide [27], α-linolenic acid [28], α-elaeostearic acid [29], palmitoleic acid [30], respectively.



1

COSY HMBC

Figure 2. Key HMBC and ^{1}H–^{1}H COSY correlations of compound **1**

Table 1. ^{1}H NMR (600 MHz), ^{13}C NMR (151 MHz), HMBC and ^{1}H-^{1}H COSY data ($CDCl_3$) for compounds **1** (δ in ppm, *J* in Hz)

Position	^{1}H	^{13}C	HMBC	^{1}H-^{1}H COSY
1		169.83 (C)	3, 7, 2′	
2		128.90 (C)	4	
3	6.18 (1H, d, *J* = 9.6)	143.31 (CH)	7, 8	4, 7
4	2.39 (1H, m)	34.78 (CH)	6	3
5	1.35 (2H, m)	29.83 (CH_2)	8	6
6	0.84 (3H, t, *J* = 7.8, 7.2)	12.01 (CH_3)		5
7	1.87 (3H, d, *J* = 1.2)	12.87 (CH_3)		3
8	0.99 (3H, d, *J* = 6.6)	19.95 (CH_3)		4
1′		170.97 (C)	OCH_3	
2′	4.09 (2H, d, *J* = 5.4)	41.64 (CH_2)		
OCH_3	3.77 (3H, s)	52.53 (CH_3)		
NH	6.34 (1H, br s)			

3.2. Antimicrobial Activity

Bioassay results indicated that none of these compounds from the co-culture medium of strains D and R1 had potent antimicrobial activity against three human pathogens *E. coli* 25922, *S. aureus* ATCC 25923 and *C. albicans* ATCC 10231 owing to their MIC values of no less than 25 μM (Table S1).

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Supporting Information

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