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# Novel Polyketides Produced by the Endophytic Fungus *Aspergillus Fumigatus* from *Cordyceps Sinensis*

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**Abstract:** Five new polyketides, including two pairs of enantiomers and a racemate, were isolated from the fermentation broth of *Aspergillus fumigatus*, an endophytic fungus isolated from *Cordyceps sinensis*. Their structures were identified using one-dimensional (1D) and two-dimensional (2D) NMR experiments, and the absolute configurations of the enantiomers were confirmed using electronic circular dichroism (ECD) calculations. Compounds **1a** and **2a** exhibited inhibitory activity against the MV4-11 cell line in vitro, with $IC_{50}$ values of 23.95 μM and 32.70 μM, respectively.

**Keywords:** *Aspergillus fumigatus*; *Cordyceps sinensis*; isochromanes; chiral resolution; ECD calculation; cytotoxicity

## 1. Introduction

*Aspergillus fumigatus* (*A. fumigatus*) is an omnipresent saprophytic fungus normally residing in the soil or decaying organic matter [1], and it has the ability to produce secondary metabolites that meet its survival requirements under various environmental conditions [2]. Previous chemical investigations revealed the constituents to be terpenes [3,4], phenolics [5], diketopiperazine [6], and other nitrogen compounds [7,8], which exhibit a variety of biological activities. For instance, fumagillin, which is a typical secondary metabolite from *A. fumigatus*, has the capacity to inhibit angiogenesis in tumor cells [9].

With the purpose of searching compounds with novel structures and bio-activities from endophytes of traditional Chinese medicine (TCM), *A. fumigatus*, as an endophytic fungus of *Cordyceps sinensis*, was chosen, and three new isochromanes were isolated from its fermentation broth (Figure 1). These polyketides were presumed to be the mixtures of enantiomers due to their approximate-to-zero optical rotation. Chiral resolution was further applied to two of these racemates, and it yielded two pairs of enantiomers. The absolute configurations of these enantiomers were further verified using quantum-chemical electronic circular dichroism (ECD) calculations. Details of the isolation, structure identification, and cytotoxicity evaluation of these new compounds are reported herein.

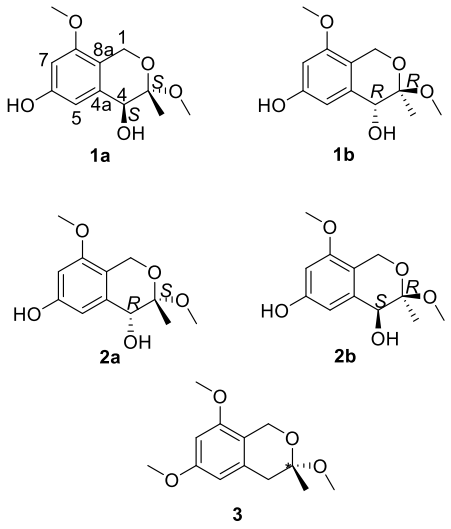


**Figure 1.** The structures of compounds obtained from *Aspergillus fumigatus*.

## 2. Results and Discussion

Compound **1** (Figure 1) was obtained as a yellow gum. The molecular formula of **1** was established using HRESIMS as $C_{12}H_{16}O_5$ (found 263.0897, calculated for $[M + Na]^+$ 263.0890). The infrared (IR) spectrum showed intense absorption bands of hydroxy at $\nu_{max}$ 3397.5 $cm^{-1}$ (OH), and 1613.2 $cm^{-1}$ and 1371.6 $cm^{-1}$ (phenyl), as well as a methylene band at 2932.1 $cm^{-1}$. The $^1H$ NMR spectrum displayed two aromatic proton signals at $\delta$ 6.38 (1H, d, $J$ = 2.2 Hz, H-5) and 6.33 (1H, d, $J$ = 2.2 Hz, H-7), two methoxyl signals at $\delta$ 3.76 (3H, s, 8-OMe) and 3.30 (3H, s, 3-OMe), one oxymethylene signal at $\delta$ 4.46 (1H, d, $J$ = 15.3 Hz, H-1) and 4.41 (1H, d, $J$ = 15.3 Hz, H-1), and one methine signal at $\delta$ 4.00 (1H, s, H-4) and 1.46 (3H, s, 3-Me). The $^{13}C$ NMR and DEPT spectra of Compound **1** exhibited 12 carbon signals, including six aromatic carbons at $\delta$ 158.4 (C-8), 157.2 (C-6), 137.3 (C-4a), 114.2 (C-8a), 109.0 (C-5), and 99.0 (C-7), a quaternary carbon at $\delta$ 101.5 (C-3), one methine carbon at $\delta$ 70.4 (C-4), one methylene carbon at $\delta$ 60.7 (C-1), two methoxyl carbons at $\delta$ 55.8 (8-OMe) and 49.8 (3-OMe), and a methyl signal at $\delta$ 19.1 (C-3). The HMBC correlations of H-1/C-8 and C-3, H-5/C-4, 3-Me/C-3 and C-4, 3-OMe/C-3, and 8-OMe/C-8, as well as the NOESY correlations of H-7/8-OMe confirmed the presence of an isochromane scaffold, and the primary structure of **1** was established as 3,8-dimethoxy-3-methylisochromane-4,6-diol (Figure 2). The NOESY correlations of H-4/3-OMe indicated that the relative configuration of **1** should be 3R*,4R* (Figure 3). Compound **1** was presumed to be a mixture of enantiomers, as its optical rotation was approximate to zero. Further chiral HPLC analysis confirmed the presence of a pair of anticipated enantiomers. Subsequent chiral resolution was applied, and two enantiomers, **1a** and **1b**, were obtained successfully. The ECD experiment and ECD calculation of **1** were conducted to determine its absolute configuration. The calculated ECD spectra of 3S, 4S-**1** fitted the experimental spectrum of **1a** nicely, while the calculated ECD spectra of 3R, 4R-**1** matched the experimental spectra of **1b** quite well, allowing the absolute configurations of **1a** and **1b** to be determined as 3S, 4S and 3R, 4R, respectively.

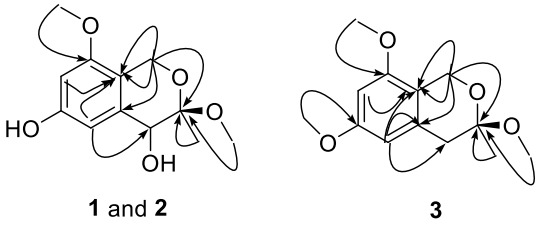


**Figure 2.** Key HMBC correlations of Compounds **1**–**3**.

Compound **2** (Figure 1) was obtained as a yellow gum. It was assigned the same molecular formula, $C_{12}H_{16}O_5$, as **1** on the basis of HRESIMS. The NMR spectra of **2** were similar to those of **1**, which indicated that it was an epimer of **1**. Both the changes in chemical shift of H-4 ($\delta$ 4.36), C-4 ($\delta$ 72.6), and C-3 ($\delta$ 99.5), and the absence of NOESY correlation of H-4/3-OMe indicated its relative configuration should be 3R*,4S*. Based on its optical rotation, chiral separation was applied, and it successfully produced a pair of enantiomers. The ECD experiment and ECD calculation of **2b** were conducted to determine its absolute configuration. The results (Figure 4) indicated that the calculated ECD curve of 3R, 4S-**2** was similar to the experimental ECD spectrum of (+)-**2** (**2b**), which designated the configuration of (+)-**2** as 3R, 4S-3,8-dimethoxy-3-methylisochromane-4,6-diol. On the other hand, (−)-**2** was assigned to be 3S, 4R-3,8-dimethoxy-3-methylisochromane-4,6-diol (**2a**) accordingly.

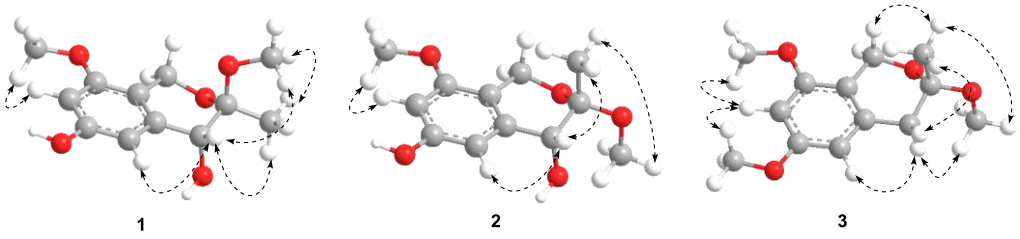


**Figure 3.** The key NOESY correlations of Compounds **1**–**3**.

Compound **3** (Figure 1) was isolated as a yellow gum. Its molecular formula was established as $C_{13}H_{18}O_4$ on the basis of the HRESIMS through the pseudo-molecular ion peak at $m/z$ 261.1107 $[M + Na]^+$ (calculated for 261.1097). The $^1H$ and $^{13}C$ NMR spectra indicated that **3** possessed a similar structure to **1** and **2** except for a methylene ($\delta_H$: 2.85, d, $J$ = 16.4 Hz, 2.73, d, $J$ = 16.4 Hz; $\delta_C$: 39.8) instead of a methine group at C-4, and the presence of another methoxyl ($\delta_H$: 3.78; $\delta_C$: 55.7). The HMBC correlation of 6-Me/C-6 readily located this methoxyl at C-6. Thus, **3** was elucidated to be 3,6,8-trimethoxy-3-methylisochromane.

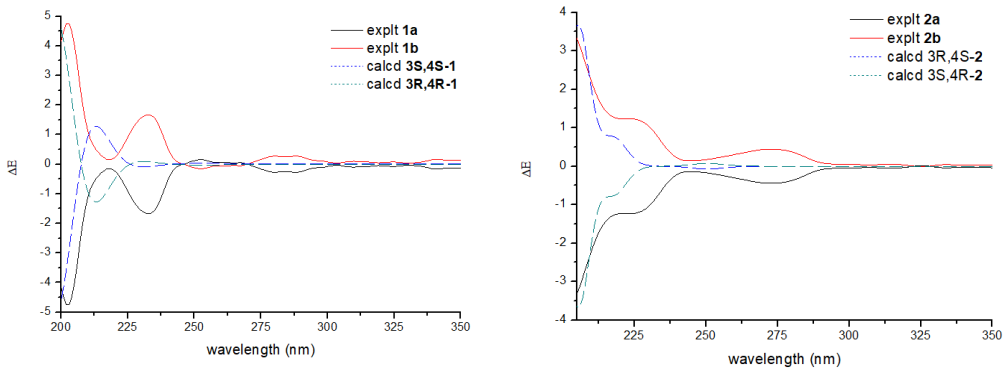


**Figure 4.** Experimental electronic circular dichroism (ECD) spectra of Compounds **1** and **2** and their calculated curves.

The MTT method was applied to evaluate the cytotoxicity of these compounds against MDA-ME-231 and MV4-11 cancer cell lines. Compounds **1a** and **2a** showed moderate growth inhibition against the MV4-11 cell line with $IC_{50}$ values of 23.95 μM and 32.70 μM, respectively.

## 3. Materials and Methods

### *3.1. General Experimental Procedures*

The UV spectra were measured on a PerkinElmer Lambda 35 UV-VIS spectrophotometer (PerkinElmer, Waltham, MA, USA). The IR spectra were recorded on a PerkinElmer Spectrum One

Fourier-transform IR (FT-IR) spectrometer, (PerkinElmer, Waltham, MA, USA). The ECD spectra were obtained on a JASCO (Oklahoma City, OK, USA) J-810 spectrometer. The optical rotations were measured on a JASCO (Oklahoma City, OK, USA) P-1020 polarimeter. The NMR spectra were recorded on a Bruker (Billerica, MA, USA) 400 spectrometer, for one-dimensional (1D) and two-dimensional (2D) NMR. The HRESIMS data were recorded on a Bruker (Billerica, MA, USA) Micro TOF-Q II mass spectrometer. Preparative HPLC was performed on a Hanbon Sci. & Tech. (Huaian, Jiangsu, China) NP7000 serials instrument equipped with a Hanbon Sci. & Tech. (Huaian, Jiangsu, China) NU3000 serials UV detector, using a Kromasil 100-5-$C_{18}$ column (10 × 250 mm, 5μm; Akzo Nobel Pulp and Performance Chemicals AB, Bohus, Sweden) for normal separation, and a column Chiralpak IC column (4.6 × 250 mm, 5 μm; Chiral Technologies, West Chester, PA, USA) for chiral resolution. Column chromatography (CC) was performed on a silica gel (200–300 mesh; Qingdao Marine Chemical Inc., Qingdao, China) and a Sephadex LH-20 (GE-Healthcare Bio-Sciences AB, Uppsala, Sweden). All solvents used were of analytical grade.

### *3.2. Fungal Material*

The *A. fumigatus* strain was separated from *Cordyceps sinensis* collected in Xiahe county, China in May 2017. The fungus was identified using morphological observation and sequence (GenBank acccession No. MG519287) analyses of the ITS region of recombinant DNA (rDNA). The identified strain was inoculated into 24,500 mL Erlenmeyer flasks, each containing 200 mL of potato dextrose agar (PDA) at room temperature, agitated on an orbital shaker at 200 rpm for seven days to produce the seed culture. The fermentation was carried out in one hundred and twenty 1000 mL Fernbach flasks, each containing 10 mL of seed culture and 400 mL of medium (soluble starch 0.8%, peptone 0.5%, NaCl 0.2%, $CaCO_3$ 0.2%, $MgSO_4 \cdot 7H_2O$ 0.05%, and $K_2HPO_4$ 0.05%), and was incubated at 25 °C on a rotary shaker at 200 rpm for 15 days.

### *3.3. Fractionation and Isolation*

The culture was filtered to separate the mycelia and the broth. The broth was firstly extracted with petroleum ether, followed by ethyl acetate. The ethyl acetate solution was concentrated to a brown residue (9.3 g). The crude extract was fractionated using liquid chromatography on a silica gel (6 × 30 cm) with a gradient elution of $CHCl_3$/MeOH. The fractions eluted with a ratio of 75:25 were combined, (1.08 g) and were further subjected to Sephadex LH-20 column chromatography (4 × 180 cm; mobile phase $CHCl_3$/MeOH, 1:1), which afforded three sub-fractions (Fr.1–Fr.3). Fr.3 (91 mg) was purified using a preparative HPLC with a reversed-phase column (5u 100 A; 10 × 250 mm; mobile phase MeOH/$H_2O$, 65:35) to furnish Compounds **1** (3.0 mg), **2** (1.9 mg), and **3** (2.9 mg). Compound **1** was further separated using HPLC with a Chiralpak IC column (mobile phase n-hexane/isopropanol, 85:15) to yield Compounds **1a** (0.33 mg, $R_t$ 10.4 min) and **1b** (0.42 mg, $R_t$ 20.6 min), while Compound **2** was further separated under the same conditions to give Compounds **2a** (0.16 mg, $R_t$ 8.1 min) and **2b** (0.26 mg, $R_t$ 28.5 min).

#### 3.3.1. 3R,4S-3,8-Dimethoxy-3-methylisochromane-4,6-diol (**1a**)

Yellow gum, $[\alpha]_D^{20}$ = +167.9 (c = 0.02, MeOH), IR (KBr): 3397.5, 2932.1, 1613.2, 1371.6, 1055.3 $cm^{-1}$; $\lambda_{max}$ 207.3 (3.74), 281.5 (2.72); $^1H$ ($CD_3OD$, 400 MHz) and $^{13}C$ NMR ($CD_3OD$, 100 MHz) spectroscopic data (see Table 1 and Supplementary Material); HRESIMS *m*/*z* 263.0897 $[M + Na]^+$ (calculated for $C_{12}H_{16}O_5Na^+$, 263.0890).

**Table 1.** The $^{1}$H (400 MHz) and $^{13}$C (100 MHz) NMR data ($\delta$ in ppm, multiple $J$ in Hz) of Compounds **1**–**3**.

| Position | **1** [a] | | **2** | | **3** | |
|---|---|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | 4.41, d, 15.3 Hz<br>4.46, d, 15.3 Hz | 60.7 | 4.56, d, 15.2 Hz<br>4.43, d, 15.2 Hz | 60.6 | 4.65, d, 15.1 Hz<br>4.44, d, 15.1 Hz | 60.2 |
| 2 | - | - | - | - | - | - |
| 3 | - | 101.5 | - | 99.5 | - | 98.8 |
| 4 | 4.00, s | 70.4 | 4.36, s | 72.6 | 2.85, d, 16.4 Hz<br>2.73, d, 16.4 Hz | 39.8 |
| 4a | - | 137.3 | - | 138.6 | - | 134.4 |
| 5 | 6.38, d, 2.2 Hz | 109.0 | 6.63, d, 2.1 Hz | 105.5 | 6.33, d, 2.1 Hz | 105.5 |
| 6 | - | 157.2 | - | 158.5 | - | 161.0 |
| 7 | 6.33, d, 2.2 Hz | 99.0 | 6.28, d, 2.1 Hz | 97.9 | 6.33 d, 2.1 Hz | 96.9 |
| 8 | - | 158.4 | - | 156.8 | - | 157.4 |
| 8a | - | 114.2 | - | 114.6 | - | 115.2 |
| 3-Me | 1.46, s | 19.1 | 1.49, s | 20.5 | 1.44, s | 23.4 |
| 3-OMe | 3.30, s | 49.8 | 3.31, s | 49.1 | 3.28, s | 48.8 |
| 6-OMe | - | - | - | - | 3.78 | 55.7 |
| 8-OMe | 3.76, s | 55.8 | 3.75, s | 55.8 | 3.72 | 55.8 |

[a] Compounds **1**–**3** were measured in $CD_3OD$.

#### 3.3.2. 3R,4R-3,8-Dimethoxy-3-methylisochromane-4,6-diol (**1b**)

Yellow gum, $[\alpha]_D^{20} = -167.9$ (c = 0.01, MeOH), spectrometric (UV, IR, NMR, MS, and HRESIMS) data are the same as those of **1a**.

#### 3.3.3. 3S,4R-3,8-Dimethoxy-3-methylisochromane-4,6-diol (**2a**)

Yellow gum, $[\alpha]_D^{20} = -260.1$ (c = 0.01, MeOH), IR (KBr): 3421.1, 2940.8, 1623.6, 1384.6, 1061.2 cm$^{-1}$; $\lambda_{max}$ 211.1 (3.76), 282.2 (2.73); $^{1}$H ($CD_3OD$, 400 MHz) and $^{13}$C NMR ($CD_3OD$, 100 MHz) spectroscopic data (see Table 1); HRESIMS $m/z$ 263.0893 $[M + Na]^+$ (calculated for $C_{12}H_{15}O_5Na^+$, 263.0890).

#### 3.3.4. 3R,4S-3,8-Dimethoxy-3-methylisochromane-4,6-diol (**2b**)

Yellow gum, $[\alpha]_D^{20} = +260.1$ (c = 0.01, MeOH), spectrometric (UV, IR, NMR, MS, and HRESIMS) data are the same as those of **2a**.

#### 3.3.5. 3,6,8-Trimethoxy-3-methylisochromane (**3**)

Yellow gum, $[\alpha]_D^{20} = +0.9$ (c = 0.11, MeOH), IR (KBr): 3435.5, 2923.0, 1638.5, 1384.2, 1074.7 cm$^{-1}$; $\lambda_{max}$ 210.6 (3.85), 281.0 (2.97); $^{1}$H ($CD_3OD$, 400 MHz) and $^{13}$C NMR ($CD_3OD$, 100 MHz) spectroscopic data (see Table 1); HRESIMS $m/z$ 261.1107 $[M + Na]^+$ (calculated for $C_{12}H_{16}O_4Na^+$, 261.1097).

### *3.4. MTT Assay*

The MV4-11 and MDA-ME-231 cells (American Type Culture Collection, Manassas, VA, USA) were grown in DMEM or IMDM medium containing 10% FBS in 5% $CO_2$ at 37 °C. When the cells entered the exponential growth phase, they were seeded in a 96-well plate and incubated overnight. Afterward, media containing various concentrations of tested compounds from 3.125 μM to 100 μM were added to each well. Additionally, 0.1% DMSO was used as a blank control, while taxol was used as a positive control. After the incubation period (72 h at 37 °C), 20 μL/well of MTT reagent (5 mg/mL) was added, and the wells were incubated for 2–4 h, before 50 μL/well of 20 acidified SDS was added to lyse the cells. Finally, the absorbance was measured at 570 nm to evaluate the inhibition effects of the tested compounds on cell growth. All experiments were performed in triplicate.

## 4. Conclusions

Among the five new compounds obtained from a TCM-related strain *A. fumigatus*, two pairs of novel enantiomers were discovered. The MTT method was used to detect the cytotoxicity of these compounds against MDA-ME-231 and MV4-11 cells. Compounds **1a** and **2a** exhibited moderate cytotoxic activity against the MV4-11 cell line with $IC_{50}$ values of 23.95 μM and 32.70 μM, respectively.

**Supplementary Materials:** The following are available online, Pages 1–3: The computational details; Figures S1–S2: HPLC chromatograms of **1** and **2** using a Chiralpak IC column; Figures S3–S23: MS and NMR spectra of Compounds **1**–**3**.

**Author Contributions:** D.-L.G. and Y.D. conceived and designed the experiments; X.-H.L., M.-Y.J. and Y.-M.C., performed the experiments; D.-L.G., Z.-X.C., and Z.G. analyzed the data; D.F. contributed materials; Y.-C.G. and F.D. wrote the paper.

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**Conflicts of Interest:** No potential conflict of interest was reported by the authors.

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**Sample Availability:** Samples of the compounds **1a**, **1b**, and **3** are available from the authors.

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