

Article

# Metabolites with Insecticidal Activity from *Aspergillus fumigatus* JRJ111048 Isolated from Mangrove Plant *Acrostichum speciosum* Endemic to Hainan Island

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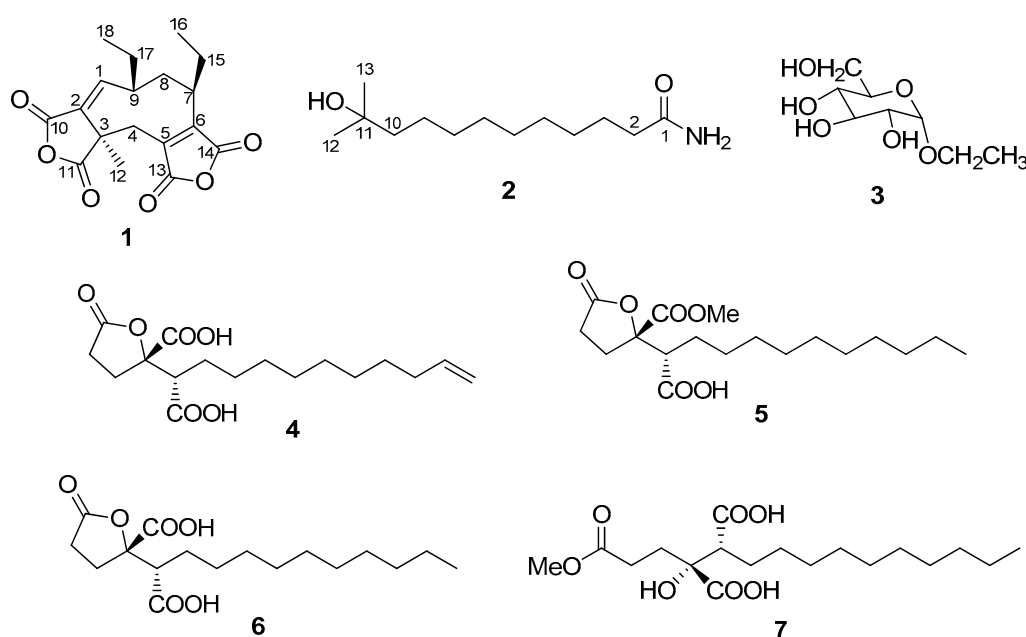
**Abstract:** Fungi residing in mangroves are considered to be a bank of novel bioactive natural products. In the screening for bioactive metabolites from mangrove-derived fungi, the ethyl acetate extract of the fermentation broth of *Aspergillus fumigatus* JRJ111048, a fungus isolated from the leaves of the mangrove plant *Acrostichum speciosum* endemic to Hainan island, was found to possess insecticidal activity against *Spodoptera litura*. Bioactivity-guided isolation led to the discovery of seven metabolites 1–7, including one new anhydride derivative aspergide (1), one new lipid amide 11-methyl-11-hydroxydodecanoic acid amide (2), and five known compounds;  $\alpha$ -ethyl glucoside (3), spiculisporic acid B (4), spiculisporic acid C (5), spiculisporic acid (6), and secospiculisporic acid B (7). Their structures were established by NMR spectroscopic and MS analyses, and by comparison of previously reported data. Insecticidal activity against *S. litura* and antifungal activity of these compounds were investigated. As a result, the new compound 1 showed potent insecticidal activity against newly hatched larvae of *S. litura*, and compound 4 displayed weak antifungal activity against *Candida albicans*.

**Keywords:** *Acrostichum speciosum*; mangrove endophyte; *Aspergillus fumigatus*; anhydride; fatty acid amide; insecticidal activity; antifungal activity

## 1. Introduction

Marine-derived fungi, such as species of the genus *Aspergillus* (Trichocomaceae), are a prolific resource for discovering structurally diverse and biologically active secondary metabolites [1–4]. Owing to their unique ecological environments, mangrove plants, growing in the saline coastal habitats in the tropics and subtropics, are considered to be a promising source of novel endophytic fungi such as *Aspergillus* spp., which could produce versatile bioactive natural products with novel structures [5–7]. In our continuous search for new bioactive natural products from plant endophytic fungi [8–12], and as part of a program to investigate the chemical and biological diversity of the

secondary metabolites from the endemic mangrove plants-associated fungi, we found a series of novel meroterpenes, sesquiterpenes, and glycoside and furan derivatives with diverse bioactivities [13–16]. During our ongoing investigation on bioactive secondary metabolites from mangrove-derived fungi, the ethyl acetate extract of the fermentation broth of *Aspergillus fumigatus* JRJ111048, a fungus isolated from the leaves of the mangrove plant *Acrostichum speciosum* endemic to Hainan island, was found to possess insecticidal activity against *Spodoptera litura*. Further isolation by bioactivity-guided fractionation led to the discovery of seven metabolites (1–7), including one new anhydride derivative aspergide (1), one new fatty acid amide 11-methyl-11-hydroxydodecanoic acid amide (2), and five known compounds;  $\alpha$ -ethyl glucoside (3) [17], spiculisporic acid B (4) [18], spiculisporic acid C (5) [18], spiculisporic acid (6) [19], and secospiculisporic acid B (7) [20] (Figure 1), the structures of which were identified by NMR spectroscopic and MS analyses, as well as by comparison of previously reported data in the literature. In this paper, we report the isolation and structure elucidation of two new compounds, (1) and (2), as well as the insecticidal and antifungal activity of these isolated compounds.



**Figure 1.** The chemical structures of compounds 1–7 isolated from *A. fumigatus* JRJ11104.

## 2. Results

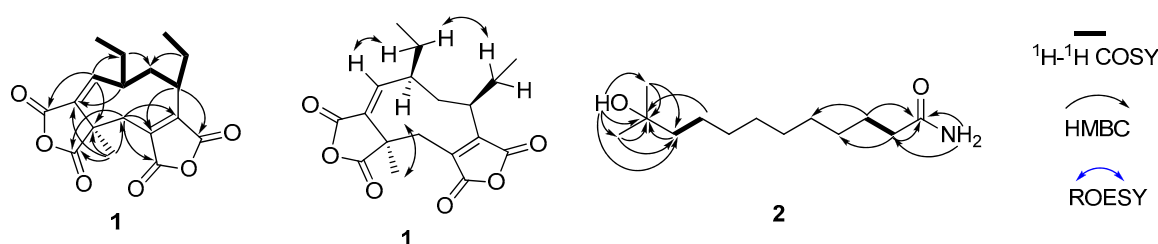
Aspergide (1) was isolated as a white powder. The molecular formula of 1 was determined as  $C_{18}H_{20}O_6$  (pointing to nine degrees of unsaturation) on the basis of the HR-ESI-MS, giving an  $[M - H]^-$  ion at  $m/z$  331.11842, and the  $^1H$  and  $^{13}C$  NMR data (Table 1). The  $^1H$  NMR spectrum of 1 in  $CDCl_3$  showed the presence of three methyl groups ( $\delta_H$  0.81, 1.07, and 1.49), one olefinic proton ( $\delta_H$  7.00), four methylene groups ( $\delta_H$  3.28 and 2.66; 2.87 and 1.98; 1.86 and 1.14; and 1.67 and 1.50), and two aliphatic methine protons ( $\delta_H$  2.12 and 2.10). The  $^{13}C$  NMR and DEPT135 spectra of 1 showed the presence of 18 carbon resonances, including four carbonyl carbons ( $\delta_C$  173.7, 165.3, 164.5, and 163.7), four aromatic or double-bond carbons ( $\delta_C$  150.1, 148.4, 140.5, and 131.9), one quaternary carbon ( $\delta_C$  48.6), three methyl carbons ( $\delta_C$  12.4, 12.8, and 20.3), four aliphatic methylene carbons ( $\delta_C$  31.7, 28.4, 26.0, and 21.4), and two aliphatic methine carbons ( $\delta_C$  48.1 and 43.9). The complete assignments for 1 were deduced by detailed analyses of HSQC,  $^1H$ - $^1H$  COSY, HMBC, and ROESY (in Supplementary Materials). The  $^1H$ - $^1H$  COSY correlations of H-1 ( $\delta_H$  7.00)/H-9 ( $\delta_H$  2.10)/H<sub>2</sub>-8 ( $\delta_H$  2.87 and 1.98); of H-9/H<sub>2</sub>-17 ( $\delta_H$  1.67 and 1.50)/H<sub>3</sub>-18 ( $\delta_H$  0.81); of H<sub>2</sub>-8/H-7 ( $\delta_H$  2.12); and of H-7/H<sub>2</sub>-15 ( $\delta_H$  1.86 and 1.14)/H<sub>3</sub>-16 ( $\delta_H$  1.07) revealed a spin system, as shown in Figure 2. The HMBC correlations from H-1 to C-17, C-3, and C-10; from H<sub>3</sub>-12 ( $\delta_H$  1.49) to C-2, C-3, C-4 and C-11; from H-7 to C5

and C-14; and from H<sub>2</sub>-4 ( $\delta_{\text{H}}$  3.28 and 2.66) to C-5, C-6, C-11, and C-13 suggested the presence of a nine-membered ring, and revealed the positions of four carbonyl groups adjacent to C-2, C-3, C-5, and C-6. The relatively upfield chemical shifts of the four carbonyl carbons, in combination with the fact that only seven of the nine degrees of unsaturation were accounted for, supported the connectivity of C-10 to C-11 and of C-13 to C-14 through the remaining two oxygen atoms, forming two anhydride rings. The relative configuration of **1** was determined by inspection of the ROESY spectrum, which showed the correlations of H-9 with H<sub>3</sub>-12 and of H<sub>2</sub>-15 with H<sub>2</sub>-17, indicating that these two ethyl groups on C-7 and C-9 have same orientation, while the methyl group CH<sub>3</sub>-12 was on the other plane. However, the absolute stereochemistry of **1** has not been determined yet. Therefore, the structure of **1**, designated as aspergide, was elucidated as shown in Figure 1.

**Table 1.** <sup>1</sup>H and <sup>13</sup>C NMR spectroscopic data for compounds **1** and **2**.

No.	<b>1</b> <sup>a</sup>		<b>2</b> <sup>b</sup>	
	$\delta_{\text{H}}$ , mult. (J in Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ , mult. (J in Hz)	$\delta_{\text{C}}$
<b>1</b>	7.00, d (12.5)	150.1, CH		174.8, C
1-NH <sub>2</sub>			7.20, br s, 6.66, br s	
<b>2</b>		131.9, C	2.02, t (7.7)	35.6, CH <sub>2</sub>
<b>3</b>		48.6, C	1.47, m	25.5, CH <sub>2</sub>
<b>4</b>	3.28, d (13.0), 2.66, d (13.0)	31.7, CH <sub>2</sub>	1.20–1.27, m <sup>c</sup>	29.2 <sup>d</sup> , CH <sub>2</sub>
<b>5</b>		140.5, C	1.20–1.27, m <sup>c</sup>	29.3 <sup>d</sup> , CH <sub>2</sub>
<b>6</b>		148.4, C	1.20–1.27, m <sup>c</sup>	29.4 <sup>d</sup> , CH <sub>2</sub>
<b>7</b>	2.12, m, overlap	48.1, CH	1.20–1.27, m <sup>c</sup>	29.6 <sup>d</sup> , CH <sub>2</sub>
<b>8</b>	2.87, m, 1.98, m	28.4, CH <sub>2</sub>	1.20–1.27, m <sup>c</sup>	30.3 <sup>d</sup> , CH <sub>2</sub>
<b>9</b>	2.10, m, overlap	43.9, CH	1.28, m	24.3, CH <sub>2</sub>
<b>10</b>		163.7, C	1.31, m	44.2, CH <sub>2</sub>
<b>11</b>		173.7, C		69.2, C
11-OH			4.04, s	
<b>12</b>	1.49, s	20.3, CH <sub>3</sub>	1.05, s	29.8, CH <sub>3</sub>
<b>13</b>		165.3, C	1.05, s	29.8, CH <sub>3</sub>
<b>14</b>		164.5, C		
<b>15</b>	1.86, m, 1.14, m	21.4, CH <sub>2</sub>		
<b>16</b>	1.07 t (7.0)	12.4, CH <sub>3</sub>		
<b>17</b>	1.67, m, 1.50, m	26.0, CH <sub>2</sub>		
<b>18</b>	0.81, t (7.5)	12.8, CH <sub>3</sub>		

<sup>a</sup> Acquired in CDCl<sub>3</sub>; <sup>b</sup> Acquired in DMSO-*d*<sub>6</sub>; <sup>c</sup> overlapping signals; <sup>d</sup> Interchangeable signals.



**Figure 2.** The key two-dimensional (2D) NMR correlations of **1** and **2**.

The molecular formula of 11-methyl-11-hydroxyldodecanoic acid amide (**2**) was found to be C<sub>13</sub>H<sub>27</sub>NO<sub>2</sub> by HR-ESI-MS ([M + H]<sup>+</sup> at *m/z* 230.2113), which, in combination with the <sup>1</sup>H and <sup>13</sup>C NMR data (Table 1), dictated one unsaturation equivalent. Analysis of the <sup>1</sup>H, <sup>13</sup>C, and HSQC spectra of **2** revealed the presence of two methyl groups [CH<sub>3</sub>-12 and CH<sub>3</sub>-13 ( $\delta_{\text{H}}$  1.05;  $\delta_{\text{C}}$  29.8)], nine aliphatic methylene groups ( $\delta_{\text{H}}$  1.20–2.02;  $\delta_{\text{C}}$  24.3–44.2), three exchangeable protons, one carbonyl group ( $\delta_{\text{C}}$  174.8), and one oxygenated quaternary carbon ( $\delta_{\text{C}}$  69.2). The planar structure of **2** was elucidated by comprehensive analyses of its two-dimensional (2D) NMR data (Figure 2), revealing a structure containing a long alkyl chain. Two terminal methyl groups were deduced by the HMBC

correlations from H<sub>3</sub>-12 and H<sub>3</sub>-13 to C-10 ( $\delta_C$  44.2) and C-11 ( $\delta_C$  69.2). The exchangeable proton of 11-OH ( $\delta_H$  4.04) was assigned to C-11 through interpretation of the key HMBC correlations from 11-OH to C-10, C-11, C-12, and C-13. In addition, HMBC correlations from 1-NH<sub>2</sub> ( $\delta_H$  7.20, 6.66) to the amide carbonyl carbon C-1 ( $\delta_C$  174.8) and C-2 ( $\delta_C$  35.6) established the amino group at the C-1 position. The alkyl chain composed of overlapping methylene protons was deduced from the COSY correlations among the methylene protons along with the clear HMBC correlations from H<sub>2</sub>-2 ( $\delta_H$  2.02) to C-1 and C-4 and from H<sub>2</sub>-3 ( $\delta_H$  1.47) to C-1 and C-5. Thus, the planar structure of **2** was identified as 11-methyl-11-hydroxydodecanoic acid amide (Figure 1).

Compounds **1–7** were assessed in in vivo assays against newly hatched larvae of *S. litura* by mixing the sample into an artificial diet. As a result, compound **1** displayed potent insecticidal activity against newly hatched larvae of *S. litura* at the concentration of 20  $\mu\text{g/mL}$ , and caused obvious effects such as reduced larval growth when compared with the untreated controls. Also, the pupae weight was reduced and the morphology of the adult was affected. At the larval stage, the treated groups showed mortality of  $40.00 \pm 10.00\%$ ,  $46.67 \pm 5.77\%$ ,  $60.00 \pm 0.00\%$ , and  $76.67 \pm 5.77\%$ , while the positive controls (azadirachtin) showed mortality of  $86.67 \pm 5.77\%$ ,  $93.33 \pm 5.77\%$ ,  $100.00 \pm 0.00\%$ , and  $100.00 \pm 0.00\%$  at 7th, 10th, 14th, and 20th days, respectively (Table 2). The antifungal activities of these compounds were also tested in vitro towards *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. cubense race 4, and *C. albicans* ATCC10231 by the filter paper disc diffusion method. In the assays, only compound **4** showed weak antifungal effects on *C. albicans* with diameters of inhibition zones of 8.6 mm at the concentration of 20 mg/mL, as compared to the positive control ketoconazole with an inhibition diameter of 29.2 mm.

**Table 2.** Insecticidal activity of compound 1.

Samples	Mortality Rate (%)			
	7 d	10 d	14 d	20 d
Compound 1	$40.00 \pm 10.00$	$46.67 \pm 5.77$	$60.00 \pm 0.00$	$76.67 \pm 5.77$
Azadirachtin	$86.67 \pm 5.77$	$93.33 \pm 5.77$	$100.00 \pm 0.00$	$100.00 \pm 0.00$
Control	$0.00 \pm 0.00$	$3.33 \pm 5.77$	$3.33 \pm 5.77$	$6.67 \pm 5.77$

### 3. Materials and Methods

#### 3.1. General Experimental Procedures

Optical rotations were measured in MeOH on a Rudolph Autopol III automatic polarimeter. The UV spectrum was recorded on a Shimadzu UV-2550 spectrophotometer. The IR spectra were obtained on a Nicolet 380 FT-IR instrument. The HR-ESI-MS spectra were acquired using an Agilent 1260 Infinity liquid chromatography coupled to a 6230 time of flight. One-dimensional (1D) and 2D NMR spectra were conducted on a Bruker AvanceIII 500 MHz NMR spectrometer at 500 MHz for <sup>1</sup>H NMR and 125 MHz for <sup>13</sup>C NMR or Bruker Avance III Ultrashield 700 MHz NMR spectrometer at 700 MHz for <sup>1</sup>H NMR and 175 MHz for <sup>13</sup>C NMR. The chemical shifts were given in  $\delta$  (ppm) and referenced to the solvent signal (CDCl<sub>3</sub>,  $\delta_H$  7.26,  $\delta_C$  77.1; DMSO-*d*<sub>6</sub>,  $\delta_H$  2.50,  $\delta_C$  39.5). Column chromatography (CC) was performed on silica gel (200–300 mesh, Qingdao Marine Chemical Inc., Qingdao, China), Octadecylsilyl (40–70  $\mu\text{m}$ , Fuji Silysia Chemical Ltd., Nagoya, Japan), and Sephadex LH-20 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). HPLC was carried out on a Varian semi-preparative HPLC system (Woburn, MA, USA) with an Alltima C18 column (250  $\times$  10.0 mm, 5  $\mu\text{m}$ , CH<sub>3</sub>OH-H<sub>2</sub>O, 2 mL/min) for purification. Thin-layer chromatography (TLC) was performed with silica gel GF254 (Marine Chemical Inc., Qingdao, China). All chemicals used in this study were of analytical grade and HPLC grade.

### 3.2. Fungal Material and Cultivation

The fungus *A. fumigatus* (JRJ111048) was isolated by one of the authors (Z.G.) from a healthy leaf of endemic mangrove plant *A. speciosum*, collected from the natural mangrove forest of Dongzhaigang in Haikou, Hainan Province, China, in October 2013, which was identified by Dr. Shoubai Liu, School of the Tropical Agriculture and Forestry, Hainan University. The isolate was identified as *A. fumigatus* on the base of its morphological characteristics and partial 18S rDNA gene sequence data (GenBank accession number MF817615) by a National Center for Biotechnology Information Basic Local Alignment Search Tool search. A voucher specimen, ITBBF14155, was deposited at the Key Laboratory of Biology and Genetic Resources of Tropical Crops, Ministry of Agriculture, Institute of Tropical Bioscience and Biotechnology, Chinese Academy of Tropical Agriculture Sciences, Haikou, China. The strain was grown on a Potato Dextrose Agar medium plate at 28 °C for 5 days. Then six small agar plugs (0.5 × 0.5 cm<sup>2</sup>) with mycelia were inoculated into each 1-L Erlenmeyer flask, each containing 300 mL Malt Extract liquid media (consisting of 10 g/L malt extract, 20 g/L sucrose, 2 g/L peptone, and deionized water). Fermentation was conducted on a rotary shaker with 180 rpm at 28 °C for 12 days.

### 3.3. Extraction and Isolation

The filtrate of fermented broth (35 L) was harvested and extracted four times with ethyl acetate at room temperature. The organic solvent was removed under reduced pressure to afford a crude extract (6.8 g). Then the extract was fractionated by silica gel (200–300 mesh) CC eluting with a gradient of CHCl<sub>3</sub>-MeOH (*v/v*, 100:0, 100:1, 100:2, 100:4, 100:8, 100:16, 100:32, 0:100) to give eight fractions (Fr.1–8). Fraction 2 (432 mg) was subjected to ODS CC with MeOH-H<sub>2</sub>O (*v/v*, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 100:0) to give seven subfractions (Fr.2.1–Fr.2.7), and then Fr.2.4 (62 mg) was further purified by Sephadex LH-20 CC using MeOH as an eluent to afford compound **1** (7.6 mg). Fraction 4 (360 mg) was separated by Sephadex LH-20 CC eluting with MeOH to give five subfractions (Fr.4.1–Fr.4.5), and Fr.4.3 (55 mg) was further purified by semipreparative HPLC (CH<sub>3</sub>CN-H<sub>2</sub>O, 75%) to yield **2** (6.2 mg). Fraction 5 (753 mg) was isolated by ODS CC using MeOH-H<sub>2</sub>O (*v/v*, 30:70, 40:60, 50:50, 60:40, 70:30, 80:20, 100:0) as an eluent to give seven subfractions (Fr.5.1–Fr.5.7), and purification of the resulting Fr.5.6 (226 mg) by semipreparative HPLC (CH<sub>3</sub>OH-H<sub>2</sub>O, 70%) afforded **4** (11.5 mg), **5** (9.3 mg), **6** (18.7 mg), and **7** (21.2 mg). Fraction 5.4 (73 mg) was purified by Sephadex LH-20 CC, and eluted with MeOH to produce **3** (6.2 mg).

Aspergide (**1**): white powder;  $[\alpha]_D^{25} +290$  (*c* 0.05, MeOH); IR (neat)  $\nu_{\max}$  3444, 2080, 1633, 1017, 555 cm<sup>-1</sup>; UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ) 216 (4.05), 261 (3.45) nm; <sup>1</sup>H and <sup>13</sup>C NMR data see Table 1; HR-ESI-MS *m/z* 331.1184 [M – H]<sup>-</sup> (calcd. for C<sub>18</sub>H<sub>19</sub>O<sub>6</sub>, 331.1182).

11-Methyl-11-hydroxydodecanoic acid amide (**2**): white powder; IR (neat)  $\nu_{\max}$  3450, 3342, 3180, 2933, 2855, 1742, 1433, 1061, 651 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR data see Table 1; HR-ESI-MS *m/z* 230.2113 [M + H]<sup>+</sup> (calcd. for C<sub>13</sub>H<sub>28</sub>NO<sub>2</sub>, 230.2115).

### 3.4. Antifungal Activity Assay

The antifungal activities of these isolated compounds were evaluated against *Colletotrichum gloeosporioides*, *Fusarium oxysporum* f. sp. *cubense* race 4, and *C. albicans* ATCC10231 by using the agar diffusion method with 6-mm filter paper discs at the concentration of 20 mg/mL in accordance with the previous report [11], and ketoconazole was used as the positive control.

### 3.5. Insecticidal Activity Assay

*S. litura* eggs were collected from the leaves of lotus (*Nelumbo nucifera* G.) in Guilinyang, Meilan District, Haikou, P.R. China, where pesticides had not been applied. Then these eggs were fed daily with meridic diet until they reproduced more generations under 25 ± 1 °C and a relative humidity of 80%. The insecticidal activity was investigated by adding sample to the diet of the newly hatched larvae.

In the test, there were three groups, each containing 10 larvae. The tested compounds were dissolved in acetone at the concentration of 1 mg/mL. Meridic diet was pipetted into 12-well plates (1 mL/well), and then the tested compounds (20 µL/well) were added when they were solidified. Newly hatched larvae were raised under  $25 \pm 1$  °C and a relative humidity of 80%. In the tests, Azadirachtin was used as the positive control, while acetone was used as the blank control. The number of dead larvae was recorded on the 7th, 10th, 14th, and 20th day after treatment, respectively, and then the mortality was calculated. The corrected mortality (CM) was calculated by the following index:

$$CM = (T - C) \times 100 / (1 - C) \quad (1)$$

where T is the mortality percentage of the tested compounds, and C is the mortality percentage in the blank control.

#### 4. Conclusions

In summary, one new anhydride derivative aspergide (1), one new lipid amide 11-methyl-11-hydroxydodecanoic acid amide (2), along with five known compounds;  $\alpha$ -ethyl glucoside (3), spiculisporic acid B (4), spiculisporic acid C (5), spiculisporic acid (6), and secospiculisporic acid B (7), were isolated from the ethyl acetate extract of the fermentation broth of *A. fumigatus* JRJ111048, isolated from the leaves of *A. speciosum*, a mangrove plant endemic to Hainan island. Their chemical structures were established by NMR and MS analyses, and by comparison of previously reported data. Compound 4 displayed weak antifungal activity against *C. albicans*. The new compound 1 showed potent insecticidal activity against newly hatched larvae of *S. litura*, indicating that it could be used as a lead compound in insect-control agents or for managing the field population of *S. litura* pests in agriculture. These results indicate that mangrove-derived fungi are a prolific resource of new bioactive natural products.

**Supplementary Materials:** The NMR and MS spectra for 1 and 2 are available online at [www.mdpi.com/1660-3397/15/12/381/s1](http://www.mdpi.com/1660-3397/15/12/381/s1).

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**Author Contributions:** Zhikai Guo, Wenli Mei and Haofu Dai conceived and designed the experiments; Zhikai Guo and Cuijuan Gai performed the isolation of the fungus, and the fermentation, isolation, and characterization of the compounds; Caihong Cai, Liangliang Chen and Shoubai Liu performed the biological tests; Zhikai Guo, Cuijuan Gai, Yanbo Zeng, Wenli Mei and Haofu Dai analyzed the data; Jingzhe Yuan contributed to the acquirement and analyses of the NMR data; Zhikai Guo, Wenli Mei and Haofu Dai wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

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