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Metabolites from *Aspergillus fumigatus*, an Endophytic Fungus Associated with *Melia azedarach*, and Their Antifungal, Antifeedant, and Toxic Activities

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Supporting Information

ABSTRACT: Thirty-nine fungal metabolites **1**−**39**, including two new alkaloids, 12β-hydroxy-13α-methoxyverruculogen TR-2 (**6**) and 3-hydroxyfumiquinazoline A (**16**), were isolated from the fermentation broth of *Aspergillus fumigatus* LN-4, an endophytic fungus isolated from the stem bark of *Melia azedarach*. Their structures were elucidated on the basis of detailed spectroscopic analysis (mass spectrometry and one- and two-dimensional NMR experiments) and by comparison of their NMR data with those reported in the literature. These isolated compounds were evaluated for in vitro antifungal activities against some phytopathogenic fungi, toxicity against brine shrimps, and antifeedant activities against armyworm larvae (*Mythimna separata* Walker). Among them, sixteen compounds showed potent antifungal activities against phytopathogenic fungi (*Botrytis cinerea*, *Alternaria solani*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Fusarium solani*, *Fusarium oxysporum* f. sp. *niveum*, *Fusarium oxysporum* f. sp. *vasinfectum*, and *Gibberella saubinettii*), and four of them, 12β-hydroxy-13α-methoxyverruculogen TR-2 (**6**), fumitremorgin B (**7**), verruculogen (**8**), and helvolic acid (**39**), exhibited antifungal activities with MIC values of 6.25−50 μg/mL, which were comparable to the two positive controls carbendazim and hymexazol. In addition, of eighteen that exerted moderate lethality toward brine shrimps, compounds **7** and **8** both showed significant toxicities with median lethal concentration (LC_{50}) values of 13.6 and 15.8 μg/mL, respectively. Furthermore, among nine metabolites that were found to possess antifeedant activity against armyworm larvae, compounds **7** and **8** gave the best activity with antifeedant indexes (AFI) of 50.0% and 55.0%, respectively. Structure−activity relationships of the metabolites were also discussed.

KEYWORDS: *endophyte, Aspergillus fumigatus, indole diketopiperazine, toxicity, antifeedant activity, antiphytopathogenic fungi*

INTRODUCTION

Natural products have been a continuous source of new lead compounds, and chemical entities in the agrochemical and pharmaceutical industries. Ever since the antibiotic drug penicillin was identified from *Penicillium* fungi in 1929, chemists have been engaged in the discovery of novel bioactives from microbial metabolites. Despite a focused interest on synthetic products, bioactive natural products retain an immense impact on modern medicine and agriculture. From the 22500 biologically active compounds that have been obtained so far from microbes, 38% are produced by fungi.[1]

Endophytic fungi are microorganisms that live in the inter- and intracellular spaces of the tissues of apparently healthy host plants without causing apparent symptoms of disease, and most of them even have a beneficial effect on the host organisms.[2] Often a single woody plant will harbor dozens or even hundreds of fungal endophytes. Their specific ecology, metabolism, and bioactivities make them an important source for structurally novel bioactive natural products, such as cytochalasan alkaloids with efficient inhibition of actin filaments,[3] or jesterone with selective antimycotic activity against phytopathogens.[4]

Melia azedarach Linn (Meliaceae), also known as Chinaberry or Persian lilac tree, is a deciduous tree and has long been recognized for its insecticidal properties. This plant produces a class of highly oxygenated tetranortriterpenoids, also known as limonoids,[5] which display potential insect resistance. In our continuous screening for biologically active secondary metabolites from plant endophytic fungi,[6,7] we investigated chemical constituents produced by the anti-phytopathogenic fungus *Aspergillus fumigatus* LN-4, an endophyte associated with *M. azedarach*. In this study we report the isolation and structural elucidation of thirty-nine compounds from this endophyte, including two previously unreported alkaloids, and determine their brine shrimp toxicity and antifungal and antifeedant activities.

MATERIALS AND METHODS

General Experimental Procedures. Melting points were obtained on an XRC-1 apparatus (Beijing Tech Instrument Co., China) and uncorrected. Optical rotations were measured on a Rudolph Autopol III automatic polarimeter (Rudolph Research Analytical, USA). Ultraviolet (UV) spectra were obtained on a UV−vis Evolution 300 spectrometer (Thermo Scientific, USA). Infrared (IR) spectra were measured with a Bruker Tensor 27 spectrophotometer (BrukerOptics, Germany) in KBr pellets. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Avance III spectrometer (Unity Plus 500 MHz) (Bruker BioSpin, Rheinstetten,

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Germany) with TMS as internal standard. Electrospray ionization mass spectrometry (ESIMS) data were obtained on a Thermo Fisher LTQ Fleet instrument spectrometer (Thermo Scientific, USA). High resolution electrospray ionization mass spectrometry (HRESIMS) data were recorded on an Agilent 6520 Accurate-Mass Q-TOF LC/MS spectrometer (Agilent Technologies, USA). Thin-layer chromatography (TLC) and preparative thin-layer chromatography (PTLC) was performed on silica gel 60 F_{254} (Qingdao Marine Chemical Ltd., China). Column chromatography (CC) was performed over silica gel (200−300 mesh, Qingdao Marine Chemical Ltd.), Sephadex LH-20 (GE Healthcare, Uppsala, Sweden), and silica gel C18-reversed phase (RP-18) (Merck, Darmstadt, Germany). Fractions were monitored by TLC, and spots were visualized by spraying with 5% H_2SO_4 in ethanol, followed by heating. All other chemicals used in this study are of analytical grade.

Fungal Material. The endophytic fungal strain LN-4 was isolated from the healthy stem bark of *Melia azedarach* L., which was collected at Yangling, Shaanxi province, China, in July 2005. The species was identified based on the phylogenetic taxonomy with sequence alignment of ITS and had a genetic closeness of over 98% to the *Aspergillus fumigatus* strain. This strain was thus defined as *A. fumigatus* LN-4 and was deposited at the College of Science, Northwest A&F University.

Cultivation. The producing strain was cultured on a plate of potato dextrose agar (PDA) at 28 ± 0.5 °C for 21 days. Then one piece (size 7 mm^2) of mycelium was inoculated aseptically to 100 mL Erlenmeyer flasks each containing 30 mL of PD liquid medium, and the seed liquids were incubated at 28 ± 0.5 °C for 3 days on a rotary shaker at 120 rpm. A suspension (200 μL) of the strain was inoculated aseptically to 500 mL Erlenmeyer flasks each containing 125 mL of SP solid medium consisting of sucrose 20 g/L, peptone 30 g/L, KH_2P0_4 0.5 g/L, $MgSO_4$ 0.5 g/L, and agar 15 g/L. The flask cultures were incubated at 28 ± 0.5 °C for 21 days.

Extraction, Isolation, and Identification of Metabolites. The strain cultures (30 L) were ultrasonically extracted four times with MeOH. The solvent was removed under reduced pressure to give a crude extract. The extract was dissolved in 90% MeOH/H_2O (2 L) and treated four times with petroleum ether to give 3.5 g of residue. The remaining layer was adjusted to 50% aqueous methanol and partitioned with chloroform. The chloroform soluble portion (22.5 g) was subjected to an RP-18 column and eluted with MeOH/H_2O (1:9 to 9:1) to give five fractions, 1−5.

Fraction 1 (MeOH/H_2O, 1:9) was chromatographed over silica gel ($CHCl_3$/MeOH, 10:1−1:1) and repeatedly purified by Sephadex LH-20 ($CHCl_3$/MeOH, 1:1, or MeOH) to afford compounds **22** (20 mg), **24** (1.5 g), **25** (70 mg), **26** (20 mg), **27** (50 mg), **28** (5 mg), **30** (55 mg), **32** (5 mg), **33** (20 mg), **34** (75 mg), and **35** (5 mg).

Fraction 2 (MeOH/H_2O, 3:7) was subjected to repeated CC on silica gel ($CHCl_3$/acetone, 10:1−0:1) and Sephadex LH-20 ($CHCl_3$/MeOH, 1:1, or MeOH) to give compounds **29** (110 mg), **31** (15 mg), **37** (6 mg), and **38** (6 mg).

Fraction 3 (MeOH/H_2O, 1:1) was purified by CC on Sephadex LH-20 (MeOH), PTLC (petroleum ether/acetone, 1:1; $CHCl_3$/acetone, 2:1), and silica gel ($CHCl_3$/acetone, 10:1) to give compounds **2** (30 mg), **3** (68 mg), **5** (15 mg), **6** (50 mg), **10** (50 mg), **19** (18 mg), **20** (4 mg), and **36** (10 mg).

Fraction 4 (MeOH/H_2O, 7:3) was subjected to CC on RP-18 silica gel (MeOH/H_2O, 4:6) and Sephadex LH-20 ($CHCl_3$/MeOH, 1:1, or MeOH), followed by purification with PTLC ($CHCl_3$/acetone, 5:1; $CHCl_3$/MeOH, 20:1) to yield compounds **1** (150 mg), **4** (33 mg), **9** (10 mg), **11** (7 mg), **12** (40 mg), **13** (10 mg), **14** (15 mg), **15** (30 mg), **16** (14 mg), **17** (25 mg), **18** (5 mg), **21** (10 mg), and **23** (120 mg).

Fraction 5 (MeOH/H_2O, 9:1) was purified by RP-18 (MeOH/H_2O, 3:7−6:4), PTLC (petroleum ether/acetone, 2:1) and repeated CC on silica gel ($CHCl_3$/MeOH, 100:1) to give compounds **7** (40 mg), **8** (13 mg), and **39** (50 mg).

Cyclotryprostatin A (2). White amorphous powder. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.78 (3H, s, H-23), 1.93 (1H, m, H-7b), 1.93 (1H, m, H-8b), 2.02 (1H, m, H-8a), 2.02 (3H, s, H-24), 2.39 (1H, m, H-7a), 3.50 (1H, m H-9b), 3.64 (1H, m, H-9a), 3.80 (3H, s, H-OMe), 4.26 (1H, dd, *J* = 10.8, 6.2 Hz, H-6), 5.08 (1H, s, H-13), 5.59 (1H, d, *J* = 10.0 Hz, H-21), 6.57 (1H, d, *J* = 9.5 Hz, H-3), 6.77 (1H, dd, *J* = 9.0, 2.0 Hz, H-17), 6.83 (1H, d, *J* = 2.0 Hz, H-19), 7.43 (1H, d, *J* = 8.5 Hz, H-16), 8.05 (1H, s, H-1). ^{13}C NMR (125 MHz, $CDCl_3$): δ 18.2 (C-24), 21.9 (C-8), 26.0 (C-23), 29.8 (C-7), 45.7 (C-9), 49.0 (C-3), 55.8 (C-OMe), 59.8 (C-6), 68.6 (C-13), 85.9 (C-12), 95.4 (C-19), 107.3 (C-14), 109.6 (C-17), 118.5 (C-16), 120.9 (C-15), 123.5 (C-21), 133.4 (C-2), 136.9 (C-20), 137.9 (C-22), 156.5 (C-18), 165.7 (C-5), 166.9 (C-11). ESI-MS (positive) *m/z* 434.2 $[M + Na]^+$. These data were identical to those recorded in the literature.[8]

12β-Hydroxyverruculogen TR-2 (5). White amorphous powder. Mp: 202−204 °C. $[\alpha]_D^{19.2}$: +58.5 (*c* 0.1, MeOH). UV (MeOH): λ_{max} (log ε) = 258 (3.71), 295 nm (3.75). IR (KBr): ν_{max} = 3364, 1663, 1466, 1423, 1158, 1029, 804 cm^{-1}. ESIMS: *m/z* 452.2 $[M + Na]^+$. HRESIMS: *m/z* 428.1823 $[M - H]^-$ (calcd for $C_{22}H_{26}N_3O_6$ 428.1827). ^{1}H and ^{13}C NMR data are shown in Table 1.

Table 1. ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) Data for Compounds 5 and 6[a]

	5		6	
no.	δ_H	δ_C	δ_H	δ_C
1(NH)	9.47, s		9.72, s	
2		134.7 s		135.1 s
3	5.95, d (5.0)	47.5 d	6.04, d (7.3, 3.0)	47.6 d
5		165.7 s		167.0 s
6	4.34, dd (10.1, 6.2)	59.9 d	4.32, d (10.4, 6.3)	59.8 d
7a	2.40, m	29.8 t	2.44, t (5.5)	29.6 t
7b	1.90, m		1.93, m	
8a	2.01, m	22.0 t	2.05, m	21.9 t
8b	1.94, m		1.93, m	
9a	3.73, m	45.7 t	3.71, dd (14.4, 6.0)	45.7 t
9b	3.59, m		3.63, t (9.5)	
11		166.9 s		165.9 s
12		85.7 s		84.8 s
13	5.02, s	68.9 d	4.70, s	76.8 d
14		106.2 s		103.9 s
15		120.7 s		122.3 s
16	7.39, d (8.5)	118.3 d	7.38, d (8.6)	118.3 d
17	6.74, d (8.5)	109.6 d	6.75, dd (8.6, 1.3)	109.6 d
18		156.4 s		156.1 s
19	6.79, s	95.4 d	6.84, d (1.3)	95.3 d
20		136.5 s		136.4 s
21a	2.27, dd (15.0, 7.4)	49.3 t	2.29, dd (15.1, 7.4)	49.4 t
21b	2.04, m		2.05, m	
22		71.1 s		71.1 s
23	1.27, s	32.2 q	1.27, s	32.2 q
24	1.45, s	28.6 q	1.48, s	28.6 q
12-OH			4.52, s	
13-OMe/OH	5.59, s		3.31, s	56.6 q
18-OMe	3.78, s	55.8 q	3.78, s	55.7 q

[a]In $CDCl_3$; δ in ppm, *J* in Hz. Assignments were confirmed by ^{1}H−^{1}H COSY, HSQC, HMBC.

12β-Hydroxy-13α-methoxyverruculogen TR-2 (6). White amorphous powder. Mp: 208−210 °C. $[\alpha]_D^{24.7}$: +32.4 (c 0.1, MeOH). UV (MeOH): λ_{max} (log ε) = 259 (3.80), 295 nm (3.83). IR (KBr): ν_{max} = 3440, 1661, 1465, 1423, 1160 cm^{-1}. ESIMS: *m/z* 444.1 $[M + H]^+$, 466.2 $[M + Na]^+$. HRESIMS: *m/z* 466.1967 $[M + Na]^+$ (calcd for $C_{23}H_{29}N_3NaO_6$ 466.1949). ^{1}H and ^{13}C NMR data are shown Table 1.

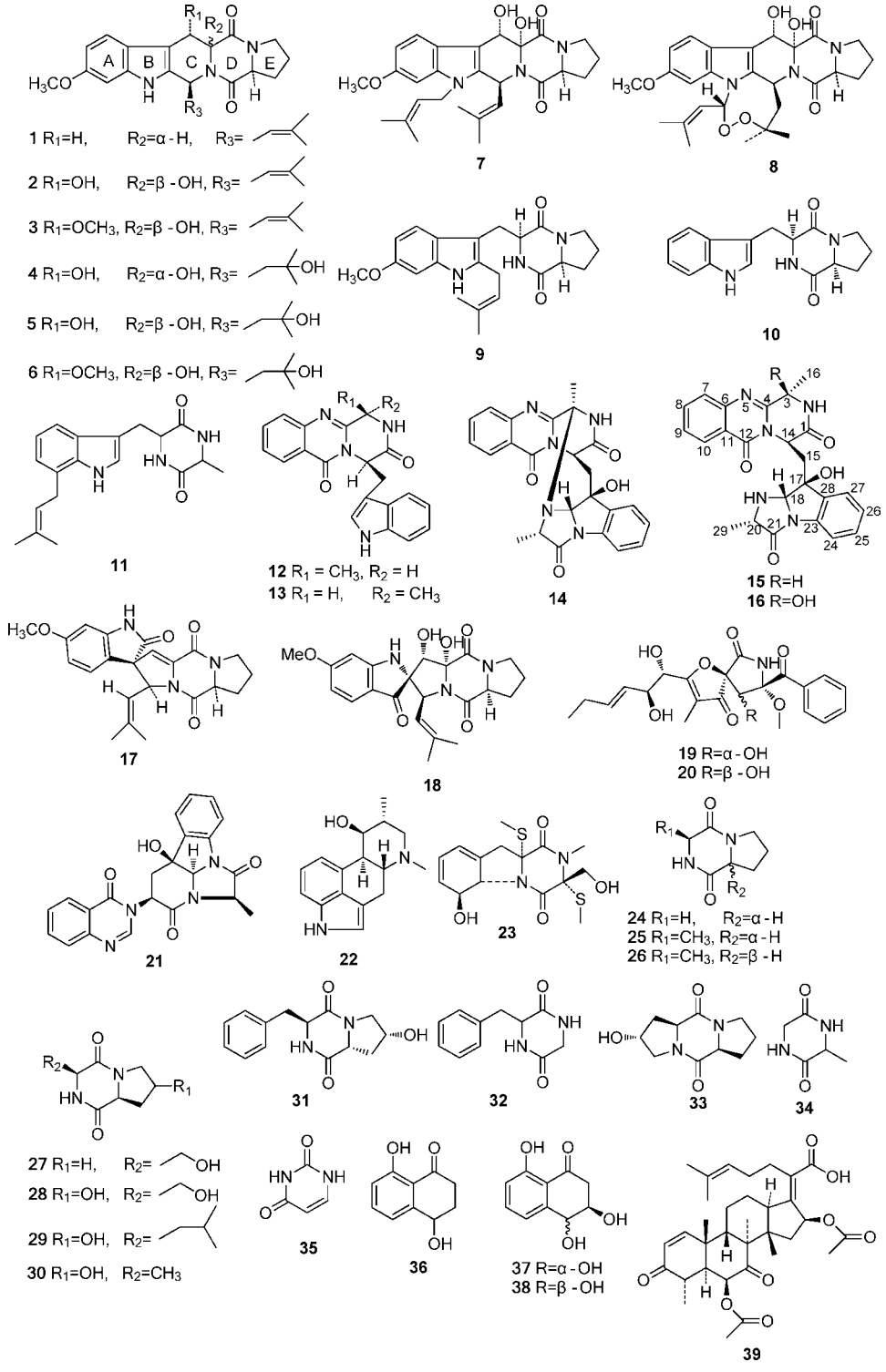

Figure 1. Structures of metabolites **1**−**39** isolated from *A. fumigatus*.

3-Hydroxyfumiquinazoline A (16). Pale yellow amorphous powder. Mp: 170−172 °C. $[\alpha]_D^{18.7}$: −62.9 (c 0.1, MeOH). IR (KBr): ν_{max} = 3351, 1681, 1606, 1488, 1391, 1125, 758, 697 cm^{-1}. ^{1}H NMR (500 MHz, $CDCl_3$): δ 1.24 (3H, d, J = 7.0 Hz, H-29), 2.03 (3H, s, H-16), 2.64 (1H, m, H-15b), 2.72 (1H, m, H-15a), 4.03 (1H, d, J = 5.5 Hz, H-20), 5.38 (1H, s, H-18), 5.87 (1H, t, J = 7.0 Hz, H-14), 7.09 (1H, t, J = 7.5 Hz, H-26), 7.23 (1H, d, J = 7.5 Hz, H-25), 7.43 (1H, d, J = 7.5 Hz, H-9), 7.47 (1H, d, J = 8.0 Hz, H-24), 7.54 (1H, d, J = 7.5 Hz, H-27), 7.64 (1H, d, J = 8.0 Hz, H-7), 7.72 (1H, t, J = 7.5 Hz, H-8), 8.14 (1H, d, J = 8.0 Hz, H-10). ^{13}C NMR (125 MHz, $CDCl_3$): δ 17.2 (C-29), 26.5 (C-16), 39.3 (C-15), 53.1 (C-14), 59.5 (C-20), 80.7 (C-17), 80.9 (C-3), 86.2 (C-18), 114.8 (C-24), 120.4 (C-11), 124.9 (C-27), 125.6 (C-26), 126.7 (C-10), 127.8 (C-9), 127.9 (C-7), 129.9 (C-25), 134.9 (C-8), 136.0 (C-23), 138.3 (C-28), 146.6 (C-6), 150.0 (C-4), 160.6 (C-12), 169.9 (C-21), 173.3 (C-1). ESIMS (positive): m/z 462.6 $[M + H]^+$, 484.2 $[M + Na]^+$.

Antifungal Bioassay. The test phytopathogenic fungi used in this study were *Botrytis cinerea*, *Alternaria solani*, *Alternaria alternata* (Fries) Keissler, *Colletotrichum gloeosporioides*, *Fusarium solani*, *Fusarium oxysporum* f. sp. *niveum*, *Fusarium oxysporum* f. sp. *vasinfectum*, and

Gibberella saubinettii. All the fungi were isolated from infected plant organs at the Northwest A&F University.

Antifungal activity was assessed by the microbroth dilution method in 96-well culture plates using a potato dextrose (PD) medium.[9] The test compounds were made up to 2 mg/mL in DMSO. Two commercial fungicides, carbendazim and hymexazol (Aladdin chemistry Co. Ltd.), were used as positive control, and the solution of equal concentration of DMSO was used as a negative control. The tested fungi were incubated in the PD medium for 18 h at 28 ± 0.5 °C at 150 rpm, and spores of different microorganism concentrations were diluted to approximately 1×10^6 CFU with PD medium. Test compounds (10 μL) were added to 96-well microplates, and 90 μL of PD medium was added. Serial dilutions were made in the 96-well round-bottom sterile plates in triplicate in 50 μL of PD medium, and then 50 μL of the fungal suspension was added. After incubation for 48 h at 28 ± 0.5 °C, minimum inhibitory concentration (MIC) was taken as the lowest concentration of the test compounds in the wells of the 96-well plate in which no microbial growth could be observed.

Brine Shrimp Bioassay. Brine shrimp (*Artemia salina*) eggs (Artemia cysts, Weifang Anjing Sea Aquaculture Co., Ltd., China) were hatched in artificial seawater prepared from commercial sea salt (Bosskasbio Chemistry Technolgy Co., Ltd., China) 25 g/L. After 48 h incubation at 28 ± 0.5 °C, nauplii were used in subsequent experiments. The tested compounds **1**–**39** and a positive control toosendanin (Shanghai Standard Biotech Co. Ltd., China) were dissolved in DMSO to 2 mg/mL and 10, 8, 6, 4, 2, 1, 0.5 μL added to 96-well microplates (Nunc, Denmark), and then a suspension of nauplii containing 15–20 organisms (190, 192, 194, 196, 198, 199, 199.5 μL) was added to each well respectively. Three repetitions were conducted for each compound. After being incubated at 28 ± 0.5 °C for 24 h, the microplates were then examined under the microscope to count dead nauplii and total numbers of brine shrimp in each well.[6] The corrected mortality rate was calculated according to the following formula:

$$\text{corrected mortality rate } (\%) = [(T - C)/(1 - C)] \times 100\%$$

where T is the mortality rate of the treatment and C is the mortality rate of the negative control.

Analysis of the data was performed by probit analysis on SPSS 16.0 (SPSS Inc., USA) to determine the median lethal concentration (LC_{50}).

Antifeedant Bioassay. The third instar larvae of armyworm (*Mythimna separata* Walker) were reared indoors, and larvae of the same size, health, and starvation for 24 h were selected. The tested compounds were dissolved in acetone to 10 mg/mL, and 1 μL of this solution was placed on fresh wheat leaves (0.5 cm ×0.5 cm) and then dried at room temperature. Toosendanin as positive control was made up to 10 mg/mL in acetone. Leaves treated with acetone alone were used as a control. Moistened filter paper (9 cm in diameter) was placed at the bottom of a Petri dish (9 cm in diameter) to maintain humidity. Ten treated leaves were kept in each dish, and every 10 larvae were raised in it. Three repetitions were conducted for each compound. The experiment was conducted under laboratory conditions (25 ± 2 °C) with 12L:12D photoperiod and 65–80% rh. Progressive consumption of leaf area by the larvae was recorded after 48 h. Antifeedant index (AFI) was assessed by the area of the leaf consumed using the following formula:[10]

$$\text{AFI} = (C - T)/C \times 100\%$$

where T is the area consumed on the treated disk and C is the area consumed on the control disk.

RESULTS AND DISCUSSION

Extraction and Isolation. The fungal strain was cultured on SP medium at 28 °C for 5 days. The extract of the culture was partitioned between chloroform and 50% MeOH/H_2O. The chloroform extract was repeatedly purified by RP-18 gel, silica gel, and Sephadex LH-20 column chromatography to afford two new natural products, 12β-hydroxy-13α-methoxyverruculogen TR-2 (**6**) and 3-hydroxyfumiquinazoline A (**16**) together with 37 known compounds (Figure 1).

Structure Elucidation. 12β-Hydroxyverruculogen TR-2 (**5**) was obtained as a white amorphous powder and its molecular formula $C_{22}H_{27}N_3O_6$ determined by HRESIMS. Comparison of its ^{1}H and ^{13}C NMR spectra with those of cyclotryprostatin A (**2**)[8] suggested that compound **5** had the same core framework as compound **2**. A difference was that an isobutene group at position C-3 in **2** was replaced by a 2-methylpropan-2-ol group in **5** (Table 1). Although 12β-hydroxyverruculogen TR-2 (**5**) was isolated previously from *A. fumigatus*,[11] its NMR data was not reported. As a result, full assignments of the NMR data of **5** were achieved by means of 2D NMR experiments, and the key COSY and HMBC correlations are shown in Figure 2. Biogenetically, compound

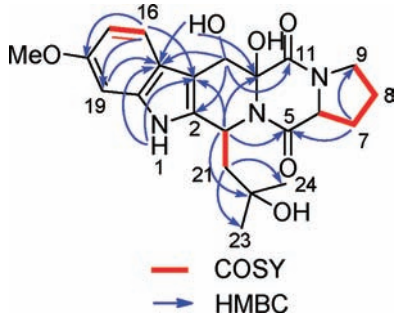

Figure 2. Key HMBC and ^{1}H–^{1}H COSY correlations of **5**.

5 is derived from compound **2** due to their coexistence. Thus, **5** was established as shown in Figure 1. Compound **5** was previously isolated as a cytotoxic agent from *A. fumigatus*, which existed in the gastrointestinal tract of the saltwater fish *Pseodolabrus japonicas*.[11]

12β-Hydroxy-13α-methoxyverruculogen TR-2 (**6**) had a molecular formula $C_{23}H_{29}N_3O_6$ determined by HRESIMS. Its ^{1}H NMR spectrum (Table 1) showed signals due to a 1,2,4-trisubstituted benzene ring (δ 7.40, 6.78, 6.86), two tertiary methyls (δ 1.27, 1.48), two methoxyls (δ 3.31, 3.78), and two exchangeable protons (δ 3.31, 4.52) along with signals due to several methylene and methine groups. Its ^{1}H and ^{13}C NMR data (Table 1) were similar to those of **5**, except that C-11/C-14 upfield shifted and C-5/C-13/C-15 downfield shifted in **6**. It indicated that the compound is a methylated derivative of the alcohol **5** (Figure 1). It was further confirmed by analyses of ^{1}H–^{1}H COSY correlations of H-16/H-17, H-3/H-21, H-6/H-7, H-7/H-8, and H-8/H-9 as well as of HMBC correlations of H-1/C-14, 15, 20, H-16/C-14, 18, C-20, H-3/C-5, 12, 14, 22, H-7/C-5, 9, H-13/C-2, 11, 15, and H-13/MeO-13 and MeO-18/C-18 (Figure 3). NOESY correlations of H-3/H-6 and H-6/MeO-13 implied the relative configuration of H-3, H-6, and MeO-13 in **6** to have the same α-orientation (Figure 3). Further, the NOEs suggested a spatial proximity of H-13β to HO-12, which requires the hydroxyl group at C-12 with β-orientation. Thus, the structure of **6** was elucidated.

3-Hydroxyfumiquinazoline A (**16**) was obtained as a light yellow powder. It showed quasimolecolar ion peaks at m/z 462.2 $[M + H]^+$ and 484.2 $[M + Na]^+$, indicating a molecular weight of 461. The ^{13}C NMR and DEPT spectra of compound **16** showed resonances for 24 carbon atoms, including two methyls, one methylene, three methines, two 1,2-disubstituted

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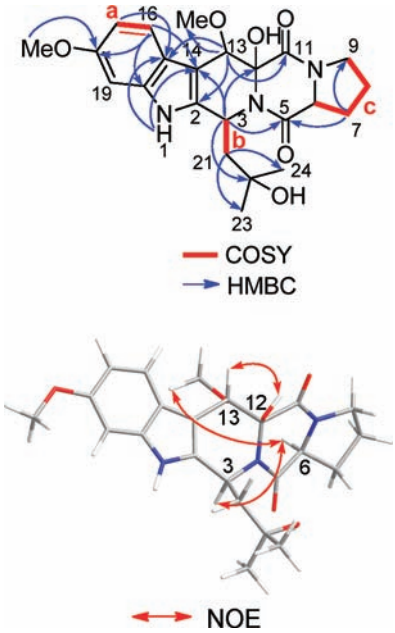

Figure 3. Key HMBC, ^{1}H−^{1}H COSY and NOE correlations of **6**.

benzene rings, three amide groups, and two oxygen-bearing sp^3 quaternary carbons. The NMR spectroscopic data of **16** were identical with those of 3-hydroxyfumiquinazoline A, a semisynthetic compound derived from fumiquinazoline D (**14**).[12] The unambiguous assignments of the ^{1}H- and ^{13}C NMR data and the relative configuration of **16** were based on COSY, HSQC, HMBC, and NOESY experiments. Thus, the structure of **16** was determined (Figure 1), which was isolated as a natural product from the *Aspergillus* genus for the first time.

The other known metabolites were identified by means of spectroscopic data as fumitremorgin C (**1**),[13] cyclotryprostatins A (**2**) and B (**3**),[8] verruculogen TR-2 (**4**),[14] fumitremorgin B (**7**), verruculogen (**8**),[15,16] tryprostatin A (**9**),[16] cyclo-L-tryptophyl-L-proline (**10**),[16] terezine D (**11**),[13] fumiquinazolines F, G, D, and A (**12**−**15**),[12] 6-methoxyspirotryprostatin B (**17**),[13] spiro [5*H*,10*H*-dipyrrolo[1,2-*a*:1′,2′-*d*]pyrazine-2-(3*H*),2′-[2*H*]indole]-3′,5,10(1′*H*)-trione (**18**),[16] pseurotin A (**19**), pseurotin A$_1$ (**20**),[17] tryptoquivaline O (**21**),[18] fumigaclavine B (**22**),[13] bisdethiobis(methylthio)gliotoxin (**23**),[19] cyclo-(Pro-Gly) (**24**),[20] cyclo-(Pro-Ala) (**25**),[20] cyclo-(D-Pro-L-Ala) (**26**),[21] cyclo-(Pro-Ser) (**27**),[20] cyclo-(Ser-*trans*-4-OH-Pro) (**28**),[22] cyclo-(Leu-4-OH-Pro) (**29**),[23] cyclo-(Ala-*trans*-4-OH-Pro) (**30**),[24] cyclo-(*cis*−OH-D-Pro-L-Phe) (**31**),[25] cyclo-(Gly-Phe) (**32**),[20] cyclo-(Pro-*trans*-4-OH-Pro) (**33**),[26] cyclo-(Gly-Ala) (**34**),[20] uracil (**35**),[27] 4,8-dihydroxy-1-tetralone (**36**),[28] *trans*-3,4-dihydro-3,4,8-trihydroxynaphthalen-1(2*H*)-one (**37**), *cis*-3,4-dihydro-3,4,8-trihydroxynaphthalen-1(2*H*)-one (**38**),[29] and phytotoxic nordammarane triterpenoid helvolic acid (**39**),[13] respectively.

Four fumiquinazolines (**12**−**15**) have been isolated before from an *A. fumigatus* strain separated from the marine fish *Pseudolabrus japonicus*,[12] and two spiro indole diketopiperazine alkaloids including methoxyl spirotryprostatin B (**17**) and spiro[5*H*,10*H*-dipyrrolo[1,2-*a*:1′,2′-*d*]pyrazine-2(3H),2′-[2*H*]-indole]-3′,5,10(1′*H*)-trione (**18**), as well as one 1-oxa-7-azaspiro[4.4]non-2-ene-4,6-dione pseurotin A$_1$ (**20**), had previously been reported from a marine-derived fungus *A. fumigatus*.[13,16,17] These alkaloid metabolites were first reported from a plant endophytic *A. fumigatus* in this study.

Antifungal Activity against Phytopathogens. All 39 metabolites were tested in vitro for the antifungal activity against the phytopathogenic fungi *B. cinerea*, *A. solani*, *A. alternata*, *C. gloeosporioides*, *F. solani*, *F. oxysporum* f. sp. *niveum*,

Table 2. Inhibitory Effects of Compounds 1−39 on Phytopathogenic Fungi[a]

	phytopathogenic fungi							
compd	*B.c.*	*A.s.*	*A.a.*	*C.g.*	*F.s.*	*F.n.*	*F.v.*	*G.s.*
1	12.5	25	12.5	12.5	50	25	50	12.5
2	50	50	25	50	>100	50	100	50
3	12.5	12.5	12.5	12.5	25	12.5	25	12.5
4	12.5	12.5	6.25	12.5	25	12.5	25	12.5
5	25	25	25	25	50	25	50	25
6	6.25	6.25	6.25	6.25	25	12.5	25	6.25
7	6.25	6.25	6.25	12.5	100	25	50	12.5
8	6.25	12.5	6.25	6.25	50	12.5	25	6.25
12	12.5	25	12.5	12.5	50	25	50	12.5
13	12.5	25	12.5	12.5	50	50	50	12.5
14	25	25	25	25	>100	50	50	25
15	12.5	12.5	12.5	12.5	50	25	50	12.5
16	12.5	12.5	12.5	12.5	100	50	50	12.5
21	25	12.5	25	100	>100	>100	>100	50
36	12.5	12.5	12.5	12.5	50	25	50	12.5
39	6.25	12.5	6.25	6.25	50	12.5	25	6.25
carbendazim	12.5	12.5	6.25	6.25	25	12.5	25	6.25
hymexazol	12.5	12.5	12.5	12.5	50	12.5	25	12.5

B.c., *Botrytis cinerea*; *A.s.*, *Alternaria solani*; *A.a.*, *A. alternata* (Fries) Keissler; *C.g.*, *Colletotrichum gloeosporioides*; *F.s.*, *Fusarium solani*; *F.n.*, *F. oxysporum* f. sp. *niveum*; *F.v.*, *F. oxysporum* f. sp. *vasinfectum*; *G.s.*, *Gibberella saubinettii*. Compounds with MIC values >100 μg/mL were not shown.

[a]MIC: μg/mL.

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F. oxysporum f. sp. *vasinfectum*, and *G. saubinettii*, and the results are listed in Table 2. Compounds **1**−**8**, **12**−**16**, **21**, **36**, and **39** displayed varying degrees of antifungal activities against several plant pathogenic fungi, while the other compounds did not show antibiotic activities against any fungal species. Among the tested compounds, 12β-hydroxy-13α-methoxyverruculogen TR-2 (**6**), fumitremorgin B (**7**), verruculogen (**8**), and helvolic acid (**39**) (MICs = 6.25−50 μg/mL) all showed significant antifungal activities, which were comparable to those of two commercial fungicides carbendazim and hymexazol as the positive control.

Among the tested indole diketopiperazine alkaloids **1**−**8**, both fumitremorgin C (**1**) and cyclotryprostatin B (**3**) (MICs = 12.5 μg/mL) had higher inhibitory effects on *Botrytis cinerea*, *Alternaria alternata*, *Colletotrichum gloeosporiodes*, and *Gibberella saubinetti* than cyclotryprostatin A (**2**) (MICs = 50 μg/mL), while compound **3** (MICs = 12.5−25 μg/mL) exhibited more inhibitory effects on *Alternaria solani*, *Fusarium solani*, *F. oxysporum* f. sp. *niveum*, and *F. oxysporum* f. sp. *vasinfectum* than **1** (MICs = 25−50 μg/mL) and **2** (MICs = 50−100 μg/mL). Both verruculogen TR-2 (**4**) and 12β-hydroxy-13α-methoxyverruculogen TR-2 (**6**), which displayed significant growth inhibition against *A. alternata*, *F. solani*, *F. oxysporum* f. sp. *niveum*, and *F. oxysporum* f. sp. *vasinfectum* with MICs of 6.25−25 μg/mL, were more active than 12β-hydroxyverruculogen TR-2 (**5**) with MICs of 25−50 μg/mL; the order of antifungal activity of compounds **4**, **5**, and **6** against *B. cinerea*, *A. solani*, *C. gloeosporioides*, and *G. saubinettii* was **6** > **4** > **5**, with their MICs of 6.25, 12.5, and 25 μg/mL, respectively. These observations suggested that the introduction of a MeO group onto C-13 in these molecules gave higher activity (**3** and **6** vs **2** and **5**), regardless of the configuration of the OH group at C-12. Compound **6** (MIC = 6.25 μg/mL), with a 2-methylpropan-2-ol group at C-3, was higher in antifungal activities than **3** (MIC = 12.5 μg/mL), with an isobutenyl group at C-3, against *B. cinerea*, *A. solani*, *A. alternata*, *C. gloeosporioides*, and *G. saubinettii*. These results indicated that the 2-methylpropan-2-ol substiuent at C-3 on ring C of compounds **4**−**6** appears to be necessary for activity.

Fumitremorgin B (**7**) and verruculogen (**8**) also significantly inhibited the growth of both *B. cinerea* and *A. alternata* with the same MICs of 6.25 μg/mL, while **7** (MIC = 6.25 μg/mL) gave more potent activity on *A. solani* than **8** (MIC = 12.5 μg/mL). On the other hand, in the case of five plant pathogenic fungi, *C. gloeosporioides*, *F. solani*, *F. oxysporum* f. sp. *niveum*, *F. oxysporum* f. sp. *vasinfectum*, and *G. saubinettii*, compound **8**, possessing a peroxide bridge, with MICs of 6.25, 50, 12.5, 25, and 6.25 μg/mL, respectively, was 2-fold more active than **7**, indicating that this peroxide bridge is important for antifungal activity.

Among the five fumiquinazolines (**12**−**16**), fumiquinazoline D (**14**) weakly inhibited the growth of phytopathogenic fungi (MICs = 25−50 μg/mL) (Table 2), whereas fumiquinazolines F (**12**), G (**13**), A (**15**), and 3-hydroxyfumiquinazoline A (**16**) had good antifungal activities (MICs = 12.5−25 μg/mL) except *Fusarium* species, indicating the presence of a C−N bridge between C-3 and N-22 in compound **14** could be detrimental to activity.

It was noted that most of the tested compounds were more active toward four fungi, *B. cinerea*, *A. solani*, *A. Alternata*, and *G. saubinettii*, than toward *Fusarium* species.

In this study, seven indole-derived tremorgenic mycotoxins, belonging to the same class, including fumitremorgin C (**1**), cyclotryprostatins A (**2**) and B (**3**), verruculogen TR-2 (**4**), fumitremorgin B (**7**), verruculogen (**8**), and fumigaclavine B (**22**), were characterized from the culture of an endophytic fungus, although they had been isolated previously from various *A. fumigatus* strains in molded silage[30] and saltern,[31] from the plant *Cynodon dactylon*,[32] a holothurian,[15] and soil.[10,33] However, they were newly shown to be potential antifungal agents in the present study.

Brine Shrimp Toxicity. Brine shrimp lethality activity of compounds **1**−**39** were shown in Table 3. Compounds **1**, **3**−**10**, **12**, **13**, **15**, **16**, **21**, **23**, **30**, **36**, and **39** showed varying degrees of toxicities with LC_{50} values less than 81 μg/mL but gave lower activities than the positive control toosendanin, a triterpenoid from *M. azedarach*. Among the 13 indole alkaloids (**1**−**11**, **17**, **18**), both fumitremorgin B (**7**) and verruculogen (**8**) exhibited the best activity, with LC_{50} 13.6 and 15.8 μg/mL, respectively. Among the compounds **1**−**3**, both **1** and **3**, with respective LC_{50} values of 40.5 and 37.9 μg/mL, had higher toxicity than **2** (LC_{50} > 100 μg/mL). Replacement of 13-H in **1** with a methoxy group enhanced activity, while that of 13-H in **1** with one OH caused a great decrease in activity (**1** and **3** vs **2**). With respect to **4**−**6**, conversion of 12α-OH (**4**) to 12β-OH (**5**) caused a reduction in activity, as LC_{50} values increased from 26.9 to 73.2 μg/mL, whereas methylation of 13-OH in **5** to **6** (LC_{50} = 60.7 μg/mL) improved activity significantly. The results indicated the 12-αOH and 13-OMe groups may play an important role in the activity. Among the tested fumiquinazolines **12**−**16**, fumiquinazoline D (**14**) was found to be almost inactive, while the other compounds gave a moderate activity (LC_{50} = 39.7−80.8 μg/mL), indicating the C−N bridge from C-3 to N-22 can result in a loss in activity. This result, interestingly, was consistent with that of fumiquinazolines **12**−**16** that exhibited moderate cytotoxicity against cultured lymphocytic leukemia P388 cells.[20]

Table 3. Toxicity of Compounds 1−39 against Brine Shrimps

compd	LC_{50} (μg/mL)	compd	LC_{50} (μg/mL)
1	40.5	**21**	72.8
2	>100	**22**	>100
3	37.9	**23**	33.5
4	26.9	**24**	>100
5	73.2	**25**	>100
6	60.7	**26**	>100
7	13.6	**27**	>100
8	15.8	**28**	>100
9	44.8	**29**	>100
10	83.7	**30**	66.1
11	>100	**31**	>100
12	55.3	**32**	>100
13	78.8	**33**	>100
14	>100	**34**	>100
15	39.7	**35**	>100
16	80.8	**36**	52.7
17	>100	**37**	>100
18	>100	**38**	>100
19	>100	**39**	73.7
20	>100	toosendanin	<1

Antifeedant Activity against Armyworm. Antifeedant activity of compounds **1**−**39** against armyworm (*Mythimna separata*) larvae was examined. As shown in Table 4, compounds **1**, **7**, **8**, **12**, **14**−**16**, **18**, and **39** exhibited

Table 4. Antifeedant Activity of Compounds 1, 7, 8, 12, 14–16, 18, and 39[a]

compd	AFI (%)	compd	AFI (%)
1	15.0	**15**	45.0
7	50.0	**16**	7.5
8	55.0	**18**	5.0
12	30.0	**39**	7.5
14	10.0	toosendanin	97.5

[a]Antifeedant index: AFI.

antifeedant activities with antifeedant index (AFI) ranging from 5.0% to 55.0%. Like the brine shrimp toxicity, all compounds showed lower antifeedant rates than toosendanin. Among the 13 indole alkaloids (**1**–**11**, **17**, **18**), both compounds **7** and **8**, with an isoprenyl group at N-1, exhibited the best activity with AFI 50.0% and 55.0%, respectively. This suggested that the isoprenyl group at N-1 may be essential for the activity. Moreover, fumiquinazoline A (**15**) gave moderate activity with AFI 45.0%, which was much better than 3-hydroxyfumiquinazoline A (**16**) with AFI 7.5%. This indicated that the insertion of one hydroxyl group into C-3 of compound **15** led to a decrease in activity.

In the current study, we demonstrated for the first time that the several substances **3**, **6**, **7**, **8**, and **39** isolated from the endophytic fungus *A. fumigatus* might serve as the main components responsible for pronounced in vitro antifungal and antifeedant properties of this fungus. In addition, the occurrence of these bioactive metabolites, in particular antifungal substances, in *A. fumigatus* could be involved in protecting the host plant *M. azedarach* against invasion by parasite, insects, and pathogens. Further studies will be carried out in order to better understand the mechanism of action associated with antifungal and antifeedant effects. Some prenylated indole diketopiperazine alkaloids, such as 12β-hydroxy-13α-methoxyverruculogen TR-2 (**6**) and fumitremorgin B (**7**), as well as helvolic acid (**39**) could be considered as promising lead compounds for developing new fungicides.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

1D and 2D NMR, UV, and IR spectra of 12β-hydroxy-13α-methoxyverruculogen TR-2 (**6**). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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