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ANTIVIRAL ISOQUINOLINES FROM THE CIGAR TOBACCO DERIVED ENDOPHYTIC FUNGI *Aspergillus fumigatus*

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*With the aim of screening more antiviral activity metabolites, in this study, two new (**1** and **2**), together with five known (**3–7**), isoquinolines were isolated from the cigar tobacco-derived endophytic fungi* Aspergillus fumigatus*. Their structures were determined by means of HR-ESI-MS and extensive 1D and 2D NMR spectroscopic studies. Interestingly, the anti-TMV activities test revealed that compounds **1** and **2** showed potential anti-TMV activities with inhibition rates of 31.2 and 28.5%, respectively.*

Keywords: isoquinolines, fungi *Aspergillus fumigatus*, anti-tobacco mosaic virus (anti-TMV) activity.

Endophytic fungi are defined as fungi inside the healthy tissues of the host plants, typically causing no apparent symptoms of disease [1, 2]. It is estimated that there are over one million endophytic fungi existing in nature, and these fungi are also important components of plant micro-ecosystems [3].

Among the numerous existing endophytic fungi, *Aspergillus* strains constitute one of the most prolific sources of secondary metabolites with diverse chemical classes and interesting biological activities [4, 5]. In our previous works, some bioactive metabolites, such as alkaloids [6–9], diterpenoids [10], butyrolactones [11, 12], isocoumarins [13, 14], anthraquinones [15], and the like, have been isolated from the genus of this fungus. As a characteristic chemical component of *Aspergillus*, alkaloids are very important molecules, not only for chemical reasons but also for their diverse biological properties [16, 17]. In addition, some isoquinolines have also been reported as coming from the fungi *Aspergillus* [18–20].

Cigar tobacco is an important economic crop that is widely grown around the world [21]. The unique microbial population and rich microbial species in the fermentation process of Yunnan cigar tobacco provide a new source for the discovery of bioactivity metabolites. In the course of our ongoing research on the unique compounds from endophytes of tobacco, chemical investigations were carried out on the culture broth of the endophytic fungi *Aspergillus fumigatus* obtained from cigar tobacco. As a result, two new (**1** and **2**) and five known isoquinolines (**3–7**) were isolated from the EtOAc extract of its fermentation on a solid rice medium. Herein, we report on the isolation and structure elucidation of new compounds and their anti-TMV activity.

The whole culture broth of *A. fumigatus* was extracted with EtOAc. The extract was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9 with saturated Na_2CO_3 aq. and extracted again with EtOAc. The EtOAc-soluble alkaloidal materials were subjected repeatedly to column chromatography on silica gel, MCI, RP-18 and preparative HPLC to afford compounds **1–7**, including two new isoquinolines, 7-(4-hydroxybenzyl)-6-methoxy-3-methylisoquinolin-8-ol (**1**) and 4-((6-methoxy-3-methylisoquinolin-7-yl)methyl)phenol (**2**), along with five known ones (**3–7**).

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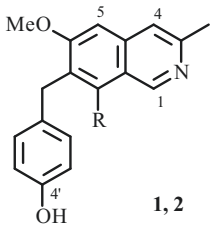
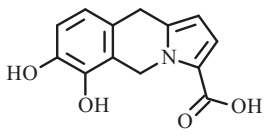
TABLE 1. ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR Data of Compounds **1** and **2** ($CDCl_3$, δ, ppm, J/Hz)

C atom	**1**		**2**	
	δ_H	δ_C	δ_H	δ_C
1	9.22 (s)	142.6 (CH)	8.86 (s)	146.9 (CH)
3	–	150.4 (C)	–	150.7 (C)
4	7.73 (s)	118.1 (CH)	7.49 (s)	118.7 (CH)
5	6.77 (s)	98.4 (CH)	7.07 (s)	106.8 (CH)
6	–	165.9 (C)	–	164.8 (C)
7	–	113.3 (C)	–	128.6 (C)
8	–	152.6 (C)	7.36 (s)	126.5 (CH)
9	–	116.6 (C)	–	123.2 (C)
10	–	138.0 (C)	–	136.8 (C)
11	2.65 (s)	23.9 (CH_3)	2.66 (s)	24.0 (CH_3)
1′	–	132.4 (C)	–	132.5 (C)
2′, 6′	7.01 (d, J = 8.8)	129.2 (CH)	6.99 (d, J = 8.8)	129.4 (CH)
3′, 5′	6.53 (d, J = 8.8)	116.0 (CH)	6.53 (d, J = 8.8)	116.2 (CH)
4′	–	156.7 (C)	–	156.9 (C)
7′	4.11 (s)	25.4 (CH_2)	4.42 (s)	32.1 (CH_2)
MeO-6	3.84 (s)	56.2 (CH_3)	3.83 (s)	56.4 (CH_3)
8-OH	10.67 (s)		–	
4′-OH	10.11 (s)		10.11 (s)	

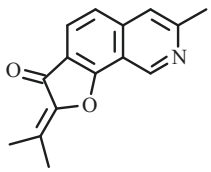


1, 2

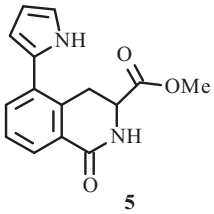
1: R = OH; **2:** R = H



3



4



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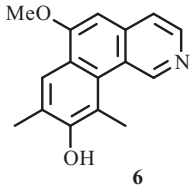
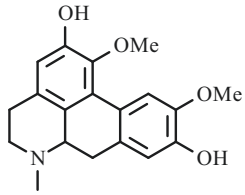


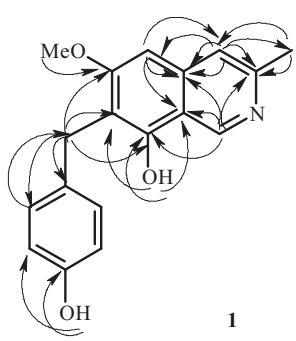
6



7

The structures of compounds **1–7** are shown in Fig. 1, and the NMR data of **1** and **2** are listed in Table 1. The new compounds were confirmed via a search through the newly updated Sci-finder database (an electronic database for chemical structure published by the American Chemical Society). The known compounds, 5,10-dihydropyrrolo[1,2-*b*]isoquinoline-6,7-diol (**3**) [22], TMC-120 A (**4**) [23], marinamide methyl ester (**5**) [24], puniceusine G (**6**) [20], and boldine (**7**) [25], were identified via comparison of their spectroscopic data with the literature.

Compound **1** was isolated as a pale-yellow gum, and its molecular formula was determined to be $C_{18}H_{17}NO_3$ by HR-ESI-MS *m/z* 318.1113 $[M + Na]^+$ (calcd for $C_{18}H_{17}NNaO_3$, 318.1106), indicating 11 degrees of unsaturation. Strong absorption bands accounting for hydroxy (3409 cm^{-1}), and aromatic (1618, 1462, and 1367 cm^{-1}) groups can be observed in its IR spectrum. Its UV spectrum showing max absorption at 215, 252 and 355 nm, suggested the existence of an aromatic structure. Its ^{1}H, ^{13}C, and DEPT NMR data (Table 1) showed resonances for 18 carbons and 17 hydrogen atoms, including a 1,4-disubstituted benzene ring (C-1′–C-6′, H_2-2′, 6′, H_2-3′, 5′), a 1,2,3,4,5-pentasubstituted benzene ring (C-5–10, H-5), one *N*-bearing aromatic methine (C-1 and H-1), one -CH=C(N-)CH_3 moiety (C-3, C-4, C-11, H-4, and H_3-11), one methylene (C-7′, H_2-7′), two phenolic hydroxyl groups (δ_H 10.67 s and 10.11 s), and one methoxy group (δ_C 56.2 q, δ_H 3.84 s). In further analysis of the preceding NMR data, the *N*-bearing aromatic methine and -CH=C(N-)CH_3 moiety is presumed to be incorporated with the benzene ring to form a 3-methylisoquinoline moiety to support the existence of 11 unsaturations.



1

Fig. 1. Key HMBC correlations of **1**.

This deduction can be confirmed by the HMBC correlations from H-1 to C-3/C-8/C-9/C-10, from H-4 to C-3/C-5/C-9/C-10, from H-5 to C-4, from H_3-11 to C-3/C-4, from H-4 to C-11, and the comparison of the NMR data of C-1–11 with known compounds TMC-120 A [23] and ampullosine [26]. In addition, the presence of a methylene in the molecule indicated that the 3-methylisoquinoline moiety and the other benzene ring are presumed to be connected by methylene (C-7′), which was also supported by the existence of HMBC correlations between H_2-7′ and two benzene rings (from H_2-7′ to C-6/C-7/C-8, and from H_2-7′ to C-1′/C-2′, 6′).

Since the skeleton of the compound was determined, the positions of substituents (phenolic hydroxy and methoxy groups) can also be determined by further analysis of its HMBC data. The HMBC correlations from the methoxy protons (δ 3.84) to C-6 indicated that the methoxy group was located at C-6. In addition, the presence of two phenolic hydroxy groups located at C-8 and C-4′ was supported by the HMBC correlations from one phenolic hydroxyl proton (δ_H 10.67 s) to C-7/C-8/C-9, and from another phenolic hydroxy proton (δ_H 10.11 s) to C-4′/C-3′, 5′, respectively. Thus, the structure of **1** was established and given the systematic name 7-(4-hydroxybenzyl)-6-methoxy-3-methylisoquinolin-8-ol.

4-((6-Methoxy-3-methylisoquinolin-7-yl)methyl)phenol (**2**) was also obtained as a pale-yellow gum, and its molecular formula was determined to be $C_{18}H_{17}NO_2$ by HR-EI-MS *m/z* 302.1164 $[M + Na]^+$. The ^1H and ^{13}C spectral data of **2** depict a similar structure to compound **1**. The obvious chemical shift differences resulted from the disappearance of a phenolic hydroxyl resonance and the appearance of an aromatic proton signal (H-8), indicating that there is no substituent at C-8. In addition, the position of a phenolic hydroxyl group at C-4′ and a methoxy group at C-6 can also be determined by further analysis of its HMBC correlations. Therefore, in this way, the structure of **2** was determined.

Since certain isoquinolines exhibit potential antiviral activity [27–29], compounds **1** and **2** were tested for their anti-TMV activities. The anti-TMV activities were tested by the half-leaf method, using ningnanmycin ($C_{16}H_{25}N_7O_8$, CAS#: 156410-09-2, a commercial product for plant disease in China, with an inhibition rate of 33.2%) as a positive control [30, 31]. The results revealed that compounds **1** and **2** showed potential anti-TMV activities with inhibition rates of 31.2 and 28.5%, respectively, rates that are close to that of the positive control.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-1900 spectrophotometer. A Bio-Rad FTS185 spectrophotometer was used for scanning IR spectra. 1D and 2D NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as the internal standard. ESI-MS and HR-ESI-MS analyses were measured on Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. Preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF (2.12 cm × 25 cm) or Venusil MP C_{18} (2.0 cm × 25 cm) columns. Column chromatography was performed using silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany), Sephadex LH-20 (Sigma-Aldrich, Inc., USA), or MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). Column fractions were monitored by TLC visualized by spraying with 5% H_2SO_4 in ethanol and heating.

Fungal Material. The culture of *Aspergillus fumigatus* YATAS-20-32 was isolated from the leaves of cigar tobacco, which was collected from the fermentation plant of Yuanjiang County, Yuxi Prefecture, Yunnan Province in 2020. The strain was identified by one of the authors (Dr. Yin-Ke Li) based on the analysis of the ITS sequence. It was cultivated at room temperature for 7 days on potato dextrose agar at 28°C. Agar plugs were inoculated into 250-mL Erlenmeyer flasks each

containing 100 mL potato dextrose broth and cultured at 28°C on a rotary shaker at 180 rpm for five days. Large-scale fermentation was carried out in 100 Fernbach flasks (1.0 L) each containing 500 g of rice and 300 mL nutrient solution (glucose 5%; peptone 0.15%; yeast 0.5%; KH_2PO_4 0.05%; urea 0.1%; $MgSO_4$ 0.05% in 1.0 L of deionized water; pH 6.5 before autoclaving). Each flask was inoculated with 5.0 mL of cultured broth and incubated at 27°C for 20 days.

Extraction and Isolation. The whole culture broth of *A. fumigatus* was extracted four times with EtOH (4 × 25 L) at room temperature and filtered. The extract was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9 with saturated Na_2CO_3 aq. and extracted again with EtOAc. The crude extract (58.3 g) was applied to silica gel column chromatography, eluting with a $CHCl_3$–MeOH gradient system (9:1, 8:2, 7:3, 6:4, 5:5). Five fractions were obtained from the silica gel column and individually decolorized on MCI gel to yield fractions A–E. The further separation of Fr. A (9:1, 6.52 g) by silica gel column chromatography, eluted with $CHCl_3$–$(Me)_2CO$ (9:1, 8:2, 7:3, 6:4, 1:1), yielded mixtures subfractions A1–A5. Subfraction A2 (8:2, 1.85 g) was subjected to RP-18 column chromatography (MeOH–H_2O, 40:60–80:20 gradient) and HPLC to give **2** (14.4 mg), **4** (15.6 mg), **6** (12.8 mg), and **7** (10.4 mg). Subfraction A3 (7:3, 1.06 g) was subjected to RP-18 column chromatography (MeOH–H_2O, 30:70–70:30 gradient) and HPLC to give **1** (15.0 mg). The further separation of Fr. C (7:3, 9.64 g) by silica gel column chromatography, eluted with $CHCl_3$–$(Me)_2CO$ (7:3, 6:4, 1:1, 4:6, 3:7), yielded mixtures subfractions C1–C5. Subfraction C-2 (6:4, 1.22 g) was subjected to RP-18 column chromatography (MeOH–H_2O, 20:80–60:40 gradient) and HPLC to give **3** (15.5 mg) and **5** (13.8 mg).

7-(4-Hydroxybenzyl)-6-methoxy-3-methylisoquinolin-8-ol (1), $C_{18}H_{17}NO_3$, obtained as a pale-yellow gum. UV (MeOH, λ_{max}, nm) (log ε): 215 (4.10), 252 (3.81), 355 (3.62). IR (ν_{max}, cm^{-1}): 3409, 3076, 1618, 1462, 1367, 1355, 1273, 1132, 1055, 830. ^1H and ^{13}C NMR data (500 and 125 MHz), see Table 1. ESI-MS *m/z* 318 $[M + Na]^+$; HR-ESI-MS *m/z* 318.1113 (calcd for $C_{18}H_{17}NNaO_3$, 318.1106).

4-((6-Methoxy-3-methylisoquinolin-7-yl)methyl)phenol (2), $C_{18}H_{17}NO_2$, obtained as a pale-yellow gum. UV (MeOH, λ_{max}, nm) (log ε): 215 (4.13), 250 (3.79), 351 (3.65). IR (ν_{max}, cm^{-1}): 3402, 3080, 1615, 1468, 1372, 1343, 1279, 1138, 1062, 847. ^1H and ^{13}C NMR data (500 and 125 MHz), see Table 1. ESI-MS *m/z* 302 $[M + Na]^+$; HR-ESI-MS *m/z* 302.1164 (calcd for $C_{18}H_{17}NNaO_2$, 302.1157).

Anti-TMV Assays. The anti-TMV activities were tested using the half-leaf method [30, 31], and ningnanmycin, a commercial product for plant disease in China, was used as a positive control. The virus was inhibited by mixing with the solution of tested compounds. After 30 min, the mixture was inoculated on the left side of the leaves of *Nicotiana glutinosa*, whereas the right side of the leaves was inoculated with the mixture of DMSO solution and the virus as a control. The local lesion numbers were recorded 3–4 days after inoculation. Three repetitions were conducted for each compound. The inhibition rates were calculated according to the formula:

$$\text{Inhibition rate } (\%) = [(C - T)/C] \times 100\%,$$

where C is the average number of local lesions of the control and T is the average number of local lesions of the treatment. Ningnanmycin, a commercial virucide for plant disease in China, was used as a positive control.

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