

TWO NEW ANTIFUNGAL INDOLE ALKALOIDS FROM AN ENDOPHYTIC FUNGI *Aspergillus felis* OBTAINED FROM CIGAR TOBACCO

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With the aim of screening more bioactivity metabolites, in this study, two new indole alkaloids, felisindoles A and B (1 and 2), were isolated from the cigar tobacco-derived endophytic fungi Aspergillus felis. Their structures were determined by means of HR-ESI-MS and extensive 1D and 2D NMR spectroscopic data. Interestingly, the anti-fungi activity assay revealed that compounds 1 and 2 showed potential anti-G. cichoracearum (the main pathogen of tobacco powdery mildew disease) activity with inhibition rates of 72.6% ± 5.2, and 61.7% ± 5.0, respectively, compared to the negative control. However, these rates are lower than that of the positive control (with an inhibition rate of 93.2% ± 5.5).

Keywords: indole alkaloids, fungi *Aspergillus felis*, anti-fungal activity.

Fungal diseases have a serious effect on the growth and yield of crops. Once infected by pathogenic fungi, the crops will cause a large reduction in production, slow growth and death, and finally lead to incalculable economic loss [1, 2]. Tobacco powdery mildew disease was caused by powdery mildew fungi, *Golovinomyces cichoracearum* (DC.) V. P. Gelyuta [3]. This disease causes serious problems in the commercial production of tobacco. In the case of foliar diseases such as powdery mildew, the application of fungicide spray may be the main measure to control the disease in most crops [4, 5]. In recent years, natural products have attracted increasing attention from plant protection scholars. The endophytic fungi are wonderful producers of various secondary metabolites [6, 7], and scientists have been increasing their interest in studying endophytic fungi as potential producers of novel environment-friendly botanical fungicides with high efficiency [8, 9].

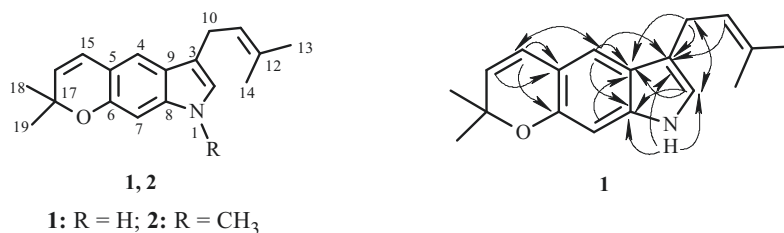
Among the numerous existing endophytic fungi, *Aspergillus* strains constitute one of the most prolific sources of secondary metabolites with diverse chemical classes and interesting biological activities [10, 11]. In our previous works, some bioactive metabolites, such as, alkaloids [12–15], diterpenoids [16], butyrolactones [17, 18], isocoumarins [19, 20], anthraquinones [21], and the like, had been isolated from the genus of this fungus. As characteristic chemical components of *Aspergillus*, indole alkaloids are very important molecules, not only for chemical reasons, but also for their significant antifungal activity [22, 23].

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 [14, 15]. In the course of our ongoing research on the unique compounds from endophytes of cigar tobacco, the chemical investigations were carried out on the culture broth of the endophytic fungi *Aspergillus felis* obtained from cigar tobacco. As a result, two new indole alkaloids (1 and 2) were isolated from the EtOAc extract during its fermentation on a solid rice medium. Herein, we report on the isolation and structure elucidation and the antifungal activity of two compounds.

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

C atom	1		2	
	δ_{H}	δ_{C}	δ_{H}	δ_{C}
2	7.09 (s)	121.9 (CH)	6.58 (s)	123.6 (CH)
3	–	116.6 (C)	–	112.9 (C)
4	6.87 (s)	120.6 (CH)	7.39 (s)	120.3 (CH)
5	–	115.8 (C)	–	115.4 (C)
6	–	153.8 (C)	–	153.5 (C)
7	6.53 (s)	98.4 (CH)	6.42 (s)	98.9 (CH)
8	–	137.7 (C)	–	138.3 (C)
9	–	119.5 (C)	–	119.0 (C)
10	3.45 (d, J = 7.8)	24.6 (CH_2)	3.42 (d, J = 7.8)	24.5 (CH_2)
11	5.41 (t, J = 7.8)	123.2 (CH)	5.39 (t, J = 7.8)	123.1 (CH)
12	–	132.2 (C)	–	132.4 (C)
13	1.80 (s)	17.6 (CH_3)	1.76 (s)	17.4 (CH_3)
14	1.83 (s)	25.7 (CH_3)	1.80 (s)	25.4 (CH_3)
15	6.67 (d, J = 9.8)	118.2 (CH)	6.67 (d, J = 9.8)	118.1 (CH)
16	5.84 (d, J = 9.8)	128.6 (CH)	5.85 (d, J = 9.8)	128.5 (CH)
17	–	80.4 (C)	–	80.5 (C)
18,19	1.57 (s)	28.1 (CH_3)	1.56 (s)	28.3 (CH_3)
20	–	–	3.56 (s)	35.2 (C)
NH	8.23 (s)	–	–	–

Fig. 1. The structure alkaloids **1**, **2** and key HMBC correlations of **1**.

The whole culture broth of *A. felis* 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 with EtOAc again. The EtOAc-soluble alkaloidal material was subjected repeatedly to column chromatography on silica gel, MCI, RP-18 and preparative HPLC to afford two new indole alkaloids, felisindoles A and B (**1** and **2**). The structures of compounds **1** and **2** were shown in Fig. 1, and their NMR data were listed in Table 1. In addition, the new compounds were confirmed by the search of the newly updated Sci-finder database (an electronic database for chemical structure published by the American Chemical Society).

Compound **1** was isolated as a pale yellow gum, and its molecular formula was determined to be $\text{C}_{18}\text{H}_{21}\text{NO}$ by molecular ion (m/z 290.1528 $[\text{M} + \text{Na}]^+$, calcd for $\text{C}_{18}\text{H}_{21}\text{NaNO}$, 290.1521) in HR-ESI-MS, indicating 9 degrees of unsaturation. Strong absorption bands accounting for amino (3392 cm^{-1}), and aromatic (1618 , 1576 , and 1482 cm^{-1}) groups can be observed in its IR spectrum. Its UV spectrum showed the max absorption at 215, 286, and 335 nm also suggesting the existence of an aromatic structure. The ^1H NMR and ^{13}C spectra data (Table 1) showed signals assignable to the presence of a 1,2,4,5-tetrasubstituted benzene ring (C-4–C-9, H-4, and H-7), a pair of double bond (C-2, C-3, and H-2), a prenyl group (C-10–C-14, H_2 -10, H-11, H_3 -13, and H_3 -14), [24], and a *gem*-dimethylchromene moiety ($-\text{CH}=\text{CH}-\text{C}(\text{CH}_3)_2-\text{O}-$; C-15–C-19; H-15, H-16, and H_6 -18,19) [25]. According to the preceding NMR data, the double bonds and nitrogen atom should be incorporated with the benzene ring to form an indole nucleus to support the 9 degrees of unsaturation [18]. The existence of an indole core was also supported by the HMBC correlations (Fig. 1) from $-\text{NH}$ (δ_{H} 8.23) to C-2, C-3, C-7, C-8, C-9, from H-2 to C-8, C-9, from H-4 to C-3, C-8, and from H-7 to C-9.

As the indole skeleton was determined, the positions of the substituents (prenyl group and *gem*-dimethylchromene moiety) can be considered as substituents, and their position can also be determined by further analysis of its HMBC data (Fig. 1). The HMBC correlations from the H_2 -10 to C-2, C-3, C-9, from H-11 to C-3 established that the prenyl group was located at C-3.

Long-range correlations from H-15 to C-4, C-5, and C-6, and from H-16 to C-5 were observed, leading us to conclude that the *gem*-dimethylchromene moiety was fused at C-5 and C-6, and C-15 was linked to a benzene ring. On the basis of the above evidence, the structure of **1** was established as shown and it was given the trivial name of felisindole A, and the systematic name of 2,2-dimethyl-6-(3-methylbut-2-en-1-yl)-2,8-dihydropyrano[3,2-*f*]indole.

Felisindole B (**2**) was also obtained as a pale yellow gum, and its molecular formula was determined to be C₁₉H₂₃NO by HR-ESI-MS (*m/z* 304.1684 [M + Na]⁺). The ¹H and ¹³C spectral data of **2** depict a similar structure to compound **1**. The obvious chemical shift differences resulted from the disappearance of an aminohydrogen (δ_H 8.23) resonance, and the appearance of an *N*-methyl group (C-20, and H₃-20). This change indicated that the hydrogen on the nitrogen atom of the indole ring should be replaced by a methyl group in compound **2**. In addition, the position of a methyl group on the nitrogen atom can also be determined by further analysis of its HMBC correlations. Therefore, the structure of **2** was determined and given the systematic name of 2,2,8-trimethyl-6-(3-methylbut-2-en-1-yl)-2,8-dihydropyrano[3,2-*f*]indole.

Since some of the indole alkaloids exhibit potential antifungal activity [22, 23], and the fungi, *G. cichoracearum* (DC.) is the main pathogen of tobacco powdery mildew disease, in this manuscript, compounds **1** and **2** were tested for their anti-*G. cichoracearum* activity.

The anti-fungi activity was tested according to previous literatures [26, 27], and chlorothalonil was used as a positive control. The results revealed that compounds **1** and **2** showed potential anti-*G. cichoracearum* activity with inhibition rates of 72.6% ± 5.2, and 61.7% ± 5.0, respectively, compared to the negative control. However, these rates are lower than that of positive control (with an inhibition rate of 93.2% ± 5.5).

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 an internal standard. ESI-MS and HR-ESI-MS analyses were measured on an 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 C₁₈ (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 and visualized by spraying with 5% H₂SO₄ in ethanol and heating.

Fungal Material. The culture of *Aspergillus felis* YATAS-20-35 was isolated from the leaves of cigar tobacco, which were collected from the fermentation plant of Yuanjiang County, Yuxi Prefecture, Yunnan Province in 2020. The strain was identified by one of authors (Dr. W. L. Yang) 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%; KH₂PO₄ 0.05%; urea 0.1%; MgSO₄ 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. felis* 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 Na₂CO₃ aq. and extracted with EtOAc again. The crude extract (54.6 g) was applied to silica gel column chromatography, eluting with a CHCl₃–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, 6.84 g) by silica gel column chromatography, eluted with CHCl₃–(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–H₂O, 40:60–80:20 gradient) and HPLC to give **1** (23.5 mg) and **2** (25.7 mg).

Felisindole A (1). C₁₈H₂₁NO, obtained as a pale-yellow gum. UV (MeOH, λ_{max}, nm) (log ε): 215 (3.92), 286 (3.70), 335 (3.62). IR (KBr, ν_{max}, cm⁻¹): 3392, 3062, 2935, 1618, 1576, 1482, 1339, 1270, 1165, 1057, 868. ¹H (500 MHz) and ¹³C (125 MHz) NMR data, see Table 1. ESI-MS *m/z* 290 [M + Na]⁺; HR-ESI-MS *m/z* 290.1528 [M + Na]⁺ (calcd for C₁₈H₂₁NaNO, 290.1521).

Felisindole B (2). C₁₉H₂₃NO, obtained as a pale-yellow gum. UV (MeOH, λ_{max}, nm) (log ε): 215 (3.90), 288 (3.74), 338 (3.65). IR (KBr, ν_{max}, cm⁻¹): 3054, 2946, 1618, 1562, 1446, 1362, 1237, 1143, 1058, 920. ¹H (500 MHz) and ¹³C (125 MHz) NMR data, see Table 1. ESI-MS *m/z* 304 [M + Na]⁺; HR-ESI-MS *m/z* 304.1684 [M + Na]⁺ (calcd for C₁₉H₂₃NNaO, 304.1677).

Anti-fungi Activity Assays. The anti-fungi activity activities were tested according to previous literature [26, 27]. The fungi, *Golovinomyces cichoracearum* (DC.) V. P. Gelyuta was obtained from a diseased tobacco plant in the greenhouse at Kunming, P. R. China, and it was morphologically identified by Dr. W. L. Yang (Yunnan Academy of Tobacco Agricultural Sciences). The fungi were propagated on the sterile tissue culture bottle seedlings using *N. tabacum* cv. HD (a tobacco variety widely cultivated in China) as the host. In addition, another batch of tissue culture seedlings was cultured, and tobacco plants at the stage of 4–5 true leaves were used in the antifungal assay.

The propagated fungi were collected from tobacco leaves on the ultra-clean workbench, and prepared to a conidial suspension of the pathogen (1 × 10⁵ conidia/mL) in 0.1% of Tween-20 aqueous solution. This pathogen solution was sprayed on sterile seedlings of tobacco plants at the stage of 4–5 true leaves (the sprayed amount is 1.0 mL per plant). After 6 h, waiting for the moisture on the tobacco leaves to be evaporated, 250 μg/mL of the tested compound solution (dissolved in 0.1% of Tween-20 aqueous) was evenly coated on the tobacco leaves using a fine brush. The cap of the tissue culture bottle was resealed, and the tobacco was cultivated under the tissue culture lamp. After 7 days, the tobacco seedlings were tacked out, and the leaves of similar size and growth position were selected in order to count the infected spot area. Tween-20 aqueous solution (0.1%) was used as a negative control, and chlorothalonil (C₈C₁₄N₂, CAS NO. 1897-45-6, a commercial antifungal pesticide for powdery mildew diseases in China) was used as a positive control. The inhibition rates were calculated according to the formula:

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

where C is the average infected spot area of the negative control and T is the infected spot area of the treatment. All results are expressed as the average of three parallel treatments.

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