

PRENYLATED ISOCOUMARINS FROM THE FERMENTATION PRODUCTS OF THE ENDOPHYTIC FUNGUS *Aspergillus versicolor* AND THEIR ANTI-TOBACCO MOSAIC VIRUS ACTIVITIES

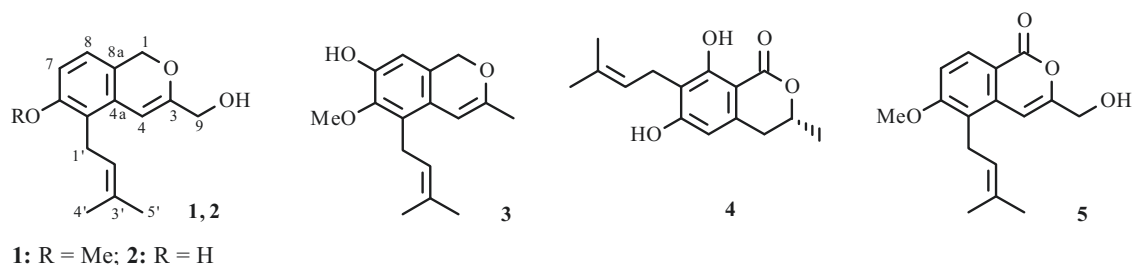
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Two new prenylated isocoumarins, versicolols C and D (**1** and **2**), together with three known isocoumarins (**3–5**), were isolated from the fermentation products of the endophytic fungus *Aspergillus versicolor*. Their structures were elucidated by spectroscopic methods, including extensive 1D and 2D NMR techniques. The anti-tobacco mosaic virus (anti-TMV) activities of compounds **1** and **2** were evaluated. The results revealed that compounds **1** and **2** showed moderate anti-TMV activity with inhibition rates of 22.4 and 24.6%, respectively.

Keywords: *Aspergillus versicolor*, isocoumarins, anti-TMV activity.

Bioactive secondary metabolites from endophytic fungi, isolated from higher plants, are a major focus of natural product research [1]. *Aspergillus*, a genus of filamentous fungi, is famous for its medical and commercial importance [2]. Among them, *Aspergillus versicolor*, a well-studied species, has attracted particular attention as a prolific source of secondary metabolites with diverse structures and biological properties [3–7]. Isocoumarins are an important class of natural products and are known to exhibit a wide range of pharmacological activities, such as antibacterial and antifungal [8], cytotoxicity [9, 10], antiviral [11, 12], antioxidative [13], and anti-inflammatory [14] properties. Here, our further efforts on the fermentation products of the endophytic fungus *A. versicolor* led to the isolation and identification of two new (**1** and **2**) and three known (**3–5**) prenylated isocoumarins. The new compounds were elucidated by means of spectroscopic methods, while the known compounds were identified by comparison with data in the literature. Compounds **1** and **2** were also evaluated for their anti-tobacco mosaic virus (anti-TMV) activity.

A 70% aq. acetone extract prepared from the fermentation products of the endophytic fungus *A. versicolor* was subjected repeatedly to column chromatography on silica gel, Sephadex LH-20, RP-18, and preparative HPLC to afford compounds **1–5**, including two new isocoumarins, versicolols C and D (**1** and **2**), together with three known isocoumarins (**3–5**). The ¹H and ¹³C NMR data of compounds **1** and **2** are listed in Table 1. The known compounds, compared with the literature, were identified as versicolols A (**3**) [9], angelicoin A (**4**) [15], and tabaisocoumarin A (**5**) [12].



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TABLE 1. ^1H (400 MHz) and ^{13}C (100 MHz) NMR Data for Compounds **1** and **2** (δ , ppm, J/Hz)

C atom	1		2	
	δ_{C}	δ_{H}	δ_{C}	δ_{H}
1	73.7 t	5.19 s	73.4 t	5.15 s
3	156.0 s	–	156.2 s	–
4	98.5 d	6.22 s	98.3 d	6.20 s
4a	136.5 s	–	136.9 s	–
5	132.3 s	–	133.3 s	–
6	156.9 s	–	155.0 s	–
7	114.0 d	6.59 (d, J = 8.2)	115.4 d	6.50 (d, J = 8.2)
8	125.1 d	6.82 (d, J = 8.2)	125.5 d	6.74 (d, J = 8.2)
8a	127.6 s	–	127.9 s	–
9	62.4 t	4.29 s	62.8 t	4.28 s
1'	22.8 t	3.37 (d, J = 7.1)	22.5 t	3.31 (d, J = 7.2)
2'	124.2 d	5.33 (t, J = 7.1)	123.2 d	5.29 (t, J = 7.2)
3'	133.1 s	–	133.0 s	–
4'	17.8 q	1.54 s	17.2 q	1.54 s
5'	25.5 q	1.75 s	25.2 q	1.73 s
6-OMe	56.0 q	3.82 s	–	–
6-OH	–	–	–	9.22 s

Compound **1** was obtained as a pale yellow gum. Its molecular formula $\text{C}_{16}\text{H}_{20}\text{O}_3$ was determined by positive HR-ESI-MS at m/z 283.1303 $[\text{M} + \text{Na}]^+$ (calcd for $\text{C}_{16}\text{H}_{20}\text{NaO}_3$, 283.1310), indicating seven degrees of unsaturation. The IR spectrum showed the absorption bands of hydroxy (3417 cm^{-1}) and aromatic groups (1615 , 1546 , 1487 cm^{-1}). The ^1H NMR spectrum (Table 1) showed signals assignable to a methoxy group at δ 3.82 (1H, s, 6-OMe); a prenyl group at δ 3.37 (2H, d, J = 7.1 Hz, H-1'), 5.33 (1H, t, J = 7.2 Hz, H-2'), 1.54 (3H, s, H₃-4'), and 1.75 (3H, s, H₃-5'); two oxygenated methylene groups at δ 5.19 (2H, s, H-1) and 4.29 (2H, s, H-9); and three additional olefinic protons at δ 6.22 (1H, s, H-4), 6.59 (1H, d, J = 8.2, H-7), and 6.82 (1H, d, J = 8.2, H-8). In the ^{13}C NMR and DEPT NMR spectra (Table 1), there were signals for three methyls (one oxygenated), three methylenes (two oxygenated), four methines, and six quaternary carbons. These data were closely related to versicolol B [9], an unusual prenylated isocoumarin, except that the methyl group at C-3 in versicolol B was converted into an oxygenated methylene group in **1**. This structural assignment was further supported by the further analysis of its HMBC correlations. Thus, compound **1** was named as versicolol C.

Versicolol D (**2**) was also obtained as a pale yellow gum with the molecular formula $\text{C}_{15}\text{H}_{18}\text{O}_3$ determined by its HR-ESI-MS at m/z 269.1162 $[\text{M} + \text{Na}]^+$ (calcd 269.1154). Analysis of the ^1H and ^{13}C NMR spectra (Table 1) revealed that the structure of **2** was very similar to that of **1**, except for the replacement of a methoxy group by a phenolic hydroxyl group in **2**. The phenolic hydroxy group located at C-6 was also supported by the HMBC correlations of the phenolic hydroxy proton at δ (9.22, s) with C-5 (δ 133.3), C-6 (δ 155.0), and C-7 (δ 115.4). The structure of **2** was therefore determined.

Since some isocoumarins are known to exhibit potential antivirus activities [11, 12], compounds **1** and **2** were tested for their anti-TMV activities. The anti-TMV activities were tested using the half-leaf method [16], using ningnanmycin (a commercial product for plant disease in China) as a positive control. Compounds **1** and **2** showed high anti-TMV activity with inhibition rates of 22.4 and 24.6% at the concentration of 20 μM , respectively.

EXPERIMENTAL

General. UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer (Shimadzu, Kyoto, Japan). IR spectra were obtained in KBr disc on a Bio-Rad Win infrared spectrophotometer (Bio-Rad, Hercules, CA, USA). ESI-MS were measured on a VG Auto Spec-3000 MS spectrometer (VG, Manchester, UK). ^1H , ^{13}C , and 2D NMR spectra were recorded on Bruker 400 instrument (Bruker, Karlsruhe, Germany) with TMS as internal standard. Column chromatography was performed on silica gel (200–300 mesh) or on silica gel H (10–40 μm , Qingdao Marine Chemical Inc., Qingdao, China). The second separation was performed on an Agilent 1100 HPLC (Agilent Technologies, Englewood, CO, USA) equipped with a ZORBAX-C₁₈ (21.2 mm \times 250 mm, 7.0 μm) column and DAD detector.

Fungal Material. The culture of *Aspergillus versicolor* 0456 was isolated from the leaves of *Nicotiana sanderae*, which was collected from Shilin, Yunnan Province, China, in 2014. The strain was identified by one of the authors (Dr. Gang Du) 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 200 Fernbach flasks (500 mL) each containing 100 g of rice and 120 mL of distilled H₂O. Each flask was inoculated with 5.0 mL of cultured broth and incubated at 25°C for 45 days.

Extraction and Isolation. The fermentation products were extracted four times with 70% acetone (4 × 10 L) at room temperature and filtered. The crude extract (226 g) was subject to silica gel (200–300 mesh) column chromatography eluted with a CHCl₃–(CH₃)₂CO gradient system (20:1, 9:1, 8:2, 7:3, 6:4, 5:5) to give six fractions A–F. The further separation of fraction C (8:2, 22.1 g) by silica gel column chromatography eluted with petroleum ether–EtOAc (8:2, 7:3, 6:4, 1:1, 1:2) yielded mixtures C1–C5. Fraction C2 (7:3, 5.08 g) was subjected to preparative HPLC (55% MeOH, flow rate 12 mL/min) to give **1** (13.6 mg), **3** (15.2 mg), and **5** (14.6 mg). Fraction C3 (6:4, 3.67 g) was subjected to preparative HPLC (50% MeOH, flow rate 12 mL/min) to give **2** (13.8 mg) and **4** (12.2 mg).

Anti-TMV Assays. TMV (U1 strain) was obtained from the Key Laboratory of Tobacco Chemistry of Yunnan Province, Yunnan Academy of Tobacco Science, China. The virus was multiplied in *Nicotiana tabacum* cv. K326 and purified as described. The concentration of TMV was determined as 20 mg/mL with a UV spectrophotometer [Virus concentration = ($A_{260} \times \text{dilution ratio}$)/ $E_{0.1\%, 260 \text{ nm/1 cm}}$]. Purified virus was kept at –20°C and was diluted to 32 µg/mL with 0.01 M PBS (phosphate buffer saline) before use.

Nicotiana glutinosa plants were cultivated in an insect-free greenhouse. *N. glutinosa* was used as a local lesion host. The experiments were conducted when the plants grew to the 5–6 leaf stage. The tested compounds were dissolved in DMSO and diluted with distilled H₂O to the required concentrations. A solution of equal concentration of DMSO was used as a negative control. The commercial antiviral agent ningnanmycin was used as a positive control.

For the half-leaf method, the virus was inhibited by mixing with the solution of the compound. After 30 min, the mixture was inoculated on the left side of the leaves of *N. glutinosa*, whereas the right side of the leaves was inoculated with the mixture of DMSO solution and the virus as control. The local lesion numbers were recorded 3 or 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.

Versicolol C (1). Obtained as a pale yellow gum, C₁₆H₂₀O₃. UV (MeOH, λ_{max} , nm) (log ϵ): 215 (3.96), 275 (3.69), 290 (3.22), 335 (3.52). IR (KBr, ν_{max} , cm⁻¹): 3417, 3084, 2963, 2870, 1615, 1546, 1487, 1359, 1131, 1078. For ¹H and ¹³C NMR (400 and 100 MHz, CDCl₃), see Table 1. ESI-MS (positive ion mode) m/z 283 [M + Na]⁺; HR-ESI-MS (positive ion mode) m/z 283.1303 [M + Na]⁺ (calcd for C₁₆H₂₀NaO₃, 283.1310).

Versicolol D (2). Obtained as a pale yellow gum, C₁₅H₁₈O₃. UV (MeOH, λ_{max} , nm) (log ϵ): 215 (3.98), 272 (3.62), 288 (3.28), 332 (3.46). IR (KBr, ν_{max} , cm⁻¹): 3452, 3069, 2970, 2865, 1612, 1550, 1479, 1363, 1145, 1070. For ¹H and ¹³C NMR (400 and 100 MHz, CDCl₃), see Table 1. ESI-MS (positive ion mode) m/z 271 [M + Na]⁺; HR-ESI-MS (positive ion mode) m/z 269.1162 [M + Na]⁺ (calcd for C₁₅H₁₈NaO₃, 269.1154).

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