

## NEW ISOCHROMENES FROM THE FERMENTATION PRODUCTS OF ENTOPHYTIC FUNGUS *ASPERGILLUS VERSICOLOR* AND THEIR ANTI-TOBACCO MOSAIC VIRUS ACTIVITIES

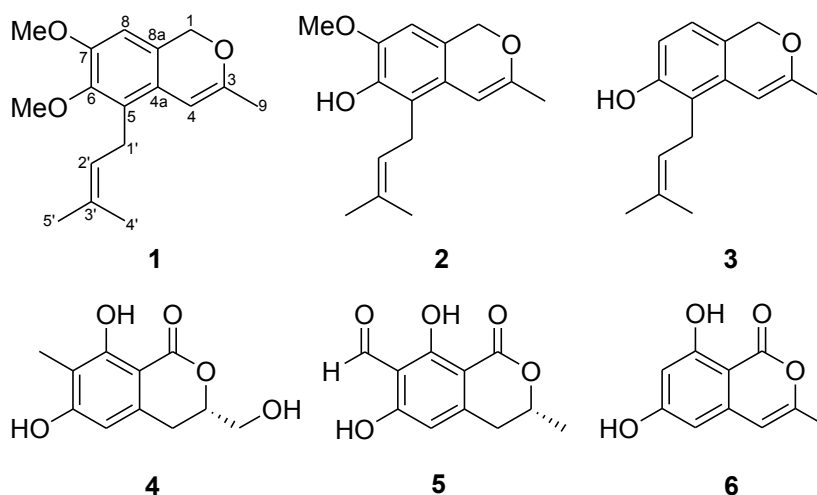
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**Abstract** – Three new isochromenes, 6,7-dimethoxy-3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromene (**1**), 7-methoxy-3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromen-6-ol (**2**), 3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromen-6-ol (**3**), together with three known isocoumarins (**4-6**) were isolated from the fermentation products of an 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 **1-3** and **6** were evaluated. The results revealed that compounds **1-3** and **6** showed anti-TMV activity with inhibition rates of 26.8 and 25.4, 24.2 and 18.6% at the concentration of 20  $\mu$ M, respectively.

Bioactive secondary metabolites from endophytic fungi, isolated from higher plants, are a major focus of natural product research.<sup>1</sup> *Aspergillus*, a genus of filamentous fungi, is famous for its medical and commercial importance.<sup>2</sup> Among them, *Aspergillus versicolor*, a well-studied specie, has attracted particular attention as a prolific source of secondary metabolites with diverse structures and biological properties.<sup>3-9</sup> Isocoumarins are an important class of natural products and are known to exhibit a wide range of pharmacological activities, such as antibacterial and antifungal,<sup>10,11</sup> cytotoxicity,<sup>12,13</sup> antiviral,<sup>14,15</sup> antioxidative,<sup>16</sup> and anti-inflammatory<sup>17</sup> properties. Motivated by a search for new bioactive

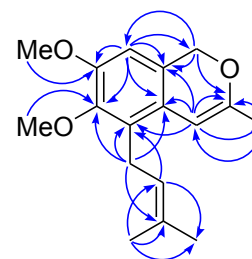
metabolites from endophytic fungus, our further efforts on the fermentation products from an endophytic strain of *A. versicolor* led to the isolation and identification of three new isochromenes (**1-3**) and three known (**4-6**) isocoumarins. The new compounds were elucidated by means of spectroscopic methods, while the known compounds were identified by comparison with data in the literature. Compounds **1-3** and **6** were also evaluated for their anti-tobacco mosaic virus (anti-TMV) activities.



**Figure 1.** The structures of compounds **1-6**

A 70% aq. acetone extract prepared from fermentation products of the endophytic fungus *A. versicolor* was subjected repeatedly to column chromatography on silica gel, RP-18 and preparative HPLC to afford compounds **1-6**, including three new isochromenes, 6,7-dimethoxy-3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromene (**1**), 7-methoxy-3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromen-6-ol (**2**), 3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromen-6-ol (**3**), together with three known isocoumarins (**4-6**). The structures of the compounds **1-6** were as shown in Figure 1, and the  $^1\text{H}$  and  $^{13}\text{C}$  NMR data of compounds **1-3** were listed in Table 1. The known compounds, compared with literature, were identified as periplanetin D (**4**),<sup>13</sup> periplanetin A (**5**),<sup>13</sup> and saccharonol A (**6**).<sup>15</sup>

Compound **1** was obtained as a pale yellow gum. Its molecular formula  $\text{C}_{17}\text{H}_{22}\text{O}_3$  was determined by positive HRESIMS at  $m/z$  297.1460  $[\text{M}+\text{Na}]^+$  (calcd  $\text{C}_{17}\text{H}_{22}\text{NaO}_3$  for 297.1467), indicating 7 degree of unsaturation. The IR spectrum showed the absorption bands of hydroxyl ( $3412$



**Figure 2.** Key HMBC ( $\curvearrowright$ ) correlations of (**1**)  $\text{cm}^{-1}$ ) and aromatic groups ( $1610$ ,  $1562$ ,  $1475$   $\text{cm}^{-1}$ ), respectively. The  $^1\text{H}$  NMR spectrum (Table 1) showed signals assignable to two methoxy group at  $\delta_{\text{H}}$  3.82 (3H, s, 6-OMe) and 3.84 (3H, s, 7-OMe); a

prenyl group at  $\delta_{\text{H}}$  3.28 (2H, d,  $J = 7.2$  Hz, H<sub>2</sub>-1'), 5.25 (1H, t,  $J = 7.2$  Hz, H-2'), 1.57 (3H, s, H<sub>3</sub>-4'), and 1.78 (3H, s, H<sub>3</sub>-5'); one oxidated methylene groups at  $\delta_{\text{H}}$  5.17 (2H, s, H<sub>2</sub>-1); one methyl group at  $\delta_{\text{H}}$  2.03 (3H, s, H<sub>3</sub>-9); and two additional olefinic protons at  $\delta_{\text{H}}$  6.35 (1H, s, H-4), 6.67 (1H, s, H-8). In the <sup>13</sup>C NMR and DEPT NMR spectra (Table 1), there were signals for five methyls (two oxygenated), two methylenes (one oxygenated), three methines, and seven quaternary carbons. These data were closely related to these of versicolol B.<sup>12</sup> The difference was due to the disappearance of a hydroxy group at C-7 in versicolol B and appearance of a methoxy group in **1**. The HMBC correlations (Figure 2) of H-1 with C-3, C-4a, C-8, and C-8a; of H-4 with C-3, C-4a, C-5, C-8a, and C-9; of H-8 with H-1; of H-9 with C-3 and C-4, also supported the 3-methylisochromene skeleton of compound **1**.<sup>12</sup> The HMBC correlations of H-1' ( $\delta_{\text{H}}$  3.28) with C-5 ( $\delta_{\text{C}}$  132.7), C-6 ( $\delta_{\text{C}}$  154.0), and C-4a ( $\delta_{\text{C}}$  129.4), and of H-2' ( $\delta_{\text{H}}$  5.25) with C-5 ( $\delta_{\text{C}}$  132.7) indicated that the prenyl group was attached to C-5. The attachment of two methoxy groups at C-6 and C-7 was supported by the HMBC correlations of two methoxy protons ( $\delta_{\text{H}}$  3.82 and 3.84) with C-6 ( $\delta_{\text{C}}$  154.0) and C-7 ( $\delta_{\text{C}}$  151.7), respectively. Thus, the structure of **1** was established as 6,7-dimethoxy-3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromene.

**Table 1.** <sup>1</sup>H and <sup>13</sup>C NMR data for compounds **1-3** (Data obtained in CDCl<sub>3</sub>)

No.	<b>1</b>		<b>2</b>		<b>3</b>	
	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (m, <i>J</i> , Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (m, <i>J</i> , Hz)	$\delta_{\text{C}}$	$\delta_{\text{H}}$ (m, <i>J</i> , Hz)
1	73.6 t	5.17 s	73.7 t	5.20 s	73.1 t	5.17 s
3	158.0 s		158.1 s		158.3 s	
4	99.5 d	6.35 s	98.4 d	6.27 s	98.5 d	6.37 s
4a	129.4 s		130.2 s		138.4 s	
5	132.7 s		134.8 s		133.8 s	
6	154.0 s		148.5 s		153.7 s	
7	151.7 s		153.0 s		115.1 d	6.49 (d) 8.2
8	110.5 d	6.67 s	111.8 d	6.43 s	126.7 d	6.93 (d) 8.2
8a	127.4 s		128.0 s		123.0 s	
9	19.1 q	2.03 s	19.3 q	2.01 s	19.0 t	2.04 s
1'	26.7 t	3.28 (d) 7.2	26.9 t	3.32 (d) 7.1	27.0 t	3.28 (d) 7.1
2'	123.2 d	5.25 (t) 7.2	123.4 d	5.29 (t) 7.1	123.4 d	5.28 (t) 7.1
3'	133.4 s		133.1 s		133.2 s	
4'	17.1 q	1.57 s	17.2 q	1.57 s	17.2 t	1.52 s
5'	25.4 q	1.78 s	25.2 q	1.75 s	25.4 q	1.79 s
6-OMe	61.0 q	3.82 s				
7-OMe	55.9 q	3.84 s	56.1 q	3.85 s		
Ar-OH				10.03 s		10.18 s

7-Methoxy-3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromen-6-ol (**2**) was obtained as pale yellow gum

with the molecular formula  $C_{16}H_{20}O_3$  determined by its HRESIMS at  $m/z$  283.1316  $[M+Na]^+$  (calcd 283.1310). Analyses of the  $^1H$  and  $^{13}C$  NMR spectra (Table 1) revealed that the structure of **2** was very similar to these of **1**, except for the replacement of a methoxy group into a phenolic hydroxy group in **2** (Table 1). The phenolic hydroxy group located at C-6 also supported by the HMBC correlations of the phenolic hydroxy proton at ( $\delta_H$  10.03) with C-5 ( $\delta_C$  134.8), C-6 ( $\delta_C$  148.5), and C-7 ( $\delta_C$  153.0). The structure of **2** was therefore determined.

Compound **3** was also obtained as a pale yellow gums and showed a quasi-molecular ion at  $m/z$  253.1213  $[M+Na]^+$  in the HRESIMS (calcd  $m/z$  253.1204), corresponding to the molecular formula  $C_{15}H_{18}O_2$ . The  $^1H$  and  $^{13}C$  NMR spectra of **3** were similar to those of **2**. The chemical shift differences resulted from the disappearance of a methoxy resonance ( $\delta_C$  55.9,  $\delta_H$  3.83) and appearance of an aromatic proton signal in **3**. This indicated that the methoxy group at C-7 in **2** was converted into an aromatic proton in **3**. The HMBC correlations of the phenolic hydroxy proton ( $\delta_H$  10.18, s) with C-5 ( $\delta_C$  133.8), C-6 ( $\delta_C$  153.7), and C-7 ( $\delta_C$  115.1) supported the phenolic hydroxy group located at C-6. In addition, the typical proton signals of H-7 ( $\delta_H$  6.49, d,  $J = 8.2$ ) and H-8 ( $\delta_H$  6.93, d,  $J = 8.2$ ) also supported this substituent pattern. Thus, the structure of 3-methyl-5-(3-methylbuten-2-yl)-1*H*-isochromen-6-ol (**3**) was established.

Since some isocoumarins are known to exhibit potential anti-virus activities,<sup>14,15</sup> compounds **1-3** and **6** were tested for their anti-TMV activities. The anti-TMV activities were tested by half-leaf method, using ningnanmycin (a commercial product for plant disease in China, with inhibition rate of 31.6%) as a positive control.<sup>18,19</sup> The results revealed that compound **1-3** and **6** showed anti-TMV activity with inhibition rates of 26.8 and 25.4, 24.2 and 18.6% at the concentration of 20  $\mu M$ , 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 or 500 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  $\mu m$ , Qingdao Marine Chemical Inc., Qingdao, China). Second separate was performed on an Agilent 1100 HPLC (Agilent Technologies, Englewood, CO, USA) equipped with ZORBAX- $C_{18}$  (21.2 mm  $\times$  250 mm, 7.0  $\mu m$ ) column and DAD detector.

**Fungal material.** The culture of *Aspergillus versicolor* was isolated from the rhizome of *Paris polyphylla* var. *yunnanensis*, collected from Tonghai, Yunnan, People's Republic of China, in 2013. The strain was identified by one of authors (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 100 Fernbach flasks (500 mL) each containing 100 g of rice and 120 mL of distilled H<sub>2</sub>O. 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 (128 g) was applied to silica gel (200–300 mesh) column chromatography, eluting with a CHCl<sub>3</sub>-Me<sub>2</sub>CO 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, 12.6 g) by silica gel column chromatography, eluted with petroleum ether-EtOAc (7:3, 6:4, 1:1, 4:6, 3:7, 2:8), yielded mixtures C1-C6. Fraction C2 (6:4, 3.25 g) was subjected to preparative HPLC (50% MeOH, flow rate 12 mL/min) to give **1** (10.7 mg), **2** (12.2 mg), and **3** (14.6 mg). Fraction C4 (3:7, 1.48 g) was subjected to preparative HPLC (35% MeOH, flow rate 12 mL/min) to give **4** (14.2 mg) **5** (18.6 mg), and **6** (15.2 mg).

**Anti-TMV Assays.** TMV (U1 strain) was obtained from the Key Laboratory of Tobacco Chemistry of Yunnan Province, China Tobacco Yunnan Industrial Co., Ltd. The virus was multiplied in *Nicotiana tabacum* cv. K326 and purified as described.<sup>20</sup> The concentration of TMV was determined as 20 mg/mL with a UV spectrophotometer [virus concentration = (A<sub>260</sub> × dilution ratio) / E<sub>1cm</sub><sup>0.1%,260nm</sup>]. The purified virus was kept at -20 °C and was diluted to 32 μg/mL with 0.01 M PBS 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 H<sub>2</sub>O to the required concentrations. A solution of equal concentration of DMSO was used as negative control. The commercial antiviral agent ningnanmycin (purity > 98%) was used as a positive control.

For Half-Leaf Method,<sup>18,19</sup> the virus was inhibited by mixing with the solution of 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 3-4 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.

**6,7-Dimethoxy-3-methyl-5-(3-methylbuten-2-yl)-1H-isochromene (1):** C<sub>17</sub>H<sub>22</sub>O<sub>3</sub>, Obtained as a pale yellow gum; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 213 (3.87), 278 (3.72), 295 (3.30), 338 (3.64) nm; IR (KBr)  $\nu_{\text{max}}$  3412, 3069, 2972, 2855, 1610, 1562, 1475, 1352, 1142, 1064 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR (500 and 125 MHz, in CDCl<sub>3</sub> see Table-1; ESIMS *m/z* (positive ion mode) 297 [M+Na]<sup>+</sup>; HRESIMS (positive ion mode) *m/z* 297.1460 [M+Na]<sup>+</sup> (calcd C<sub>17</sub>H<sub>22</sub>NaO<sub>3</sub> for 297.1467).

**7-Methoxy-3-methyl-5-(3-methylbuten-2-yl)-1H-isochromen-6-ol (2):** C<sub>16</sub>H<sub>20</sub>O<sub>3</sub>, Obtained as a pale yellow gum; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 212 (3.92), 276 (3.67), 293 (3.28), 332 (3.58) nm; IR (KBr)  $\nu_{\text{max}}$  3418, 3064, 2967, 2861, 1608, 1557, 1479, 1359, 1140, 1068 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR (400 and 100 MHz, in CDCl<sub>3</sub> see Table-1; ESIMS *m/z* (positive ion mode) 283 [M+Na]<sup>+</sup>; HRESIMS (positive ion mode) *m/z* 283.1316 [M+Na]<sup>+</sup> (calcd C<sub>16</sub>H<sub>20</sub>NaO for 283.1310).

**3-Methyl-5-(3-methylbuten-2-yl)-1H-isochromen-6-ol (3):** C<sub>15</sub>H<sub>18</sub>O<sub>2</sub>, Obtained as a pale yellow gum; UV (MeOH)  $\lambda_{\text{max}}$  (log  $\epsilon$ ) 212 (3.89), 275 (3.75), 288 (3.25), 330 (3.62) nm; IR (KBr)  $\nu_{\text{max}}$  3422, 3073, 2971, 2856, 1610, 1553, 1468, 1353, 1152, 1074 cm<sup>-1</sup>; <sup>1</sup>H and <sup>13</sup>C NMR (400 and 100 MHz, in CDCl<sub>3</sub> see Table-1; ESIMS *m/z* (positive ion mode) 253 [M+Na]<sup>+</sup>; HRESIMS (positive ion mode) *m/z* 253.1213 [M+Na]<sup>+</sup> (calcd C<sub>15</sub>H<sub>18</sub>NaO<sub>2</sub> for 253.1204).

## ACKNOWLEDGMENTS

This research was financially supported by the National Natural Science Foundation of China (No. 21462051 and 31560099), the Scientific Research Fund Key Project of Education Department of Yunnan Province (No. 2013Z038), and the Foundation of Yunnan Province (No. 2012BA015 and Category of Industry Guide No. 2014-01).

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