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NEW ANTI-TMV ISOCHROMENES FROM *NICOTIANA TABACUM*-DERIVED ENDOPHYTIC FUNGUS *ASPERGILLUS VERSICOLOR*

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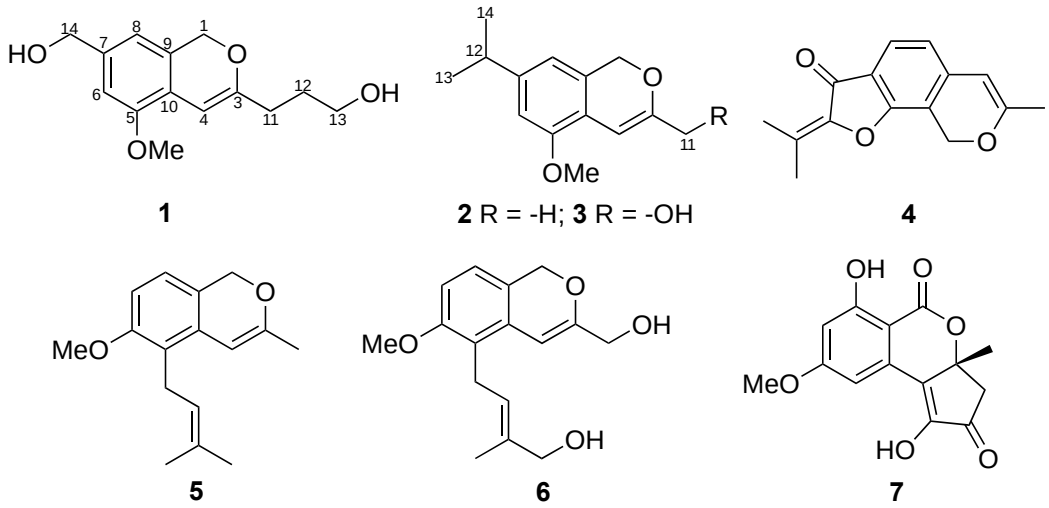
Abstract – Three new isochromenes, versicolols G-I (**1**-**3**), together with four known analogues (**4**-**7**) were isolated from the fermentation products of a *Nicotiana tabacum*-derived endophytic fungus *Aspergillus* versicolor. Their structures were elucidated by spectroscopic methods, including extensive ^{1}H, ^{13}C, and 2D-NMR techniques. Compounds **1**-**3** were also tested for their anti-tobacco mosaic virus (anti-TMV) activities, and the results revealed that compound **1** exhibited high anti-TMV activity with inhibition rate of 41.6%, and this rate are higher than that of positive control. Compounds **2** and **3** also showed potential anti-TMV activities with inhibition rates of 22.8 and 26.5%, respectively.

Endophytic fungi are prospective producers of an abundant source of bioactive compounds with potential for exploitation in a wide variety of medical areas.[1,2] Among them, the *Aspergillus* is an important fungal genus containing economically important species, as well as pathogenic species of animals and plants.[3,4] They also can produce a number of structurally complicated molecules with various biological activities.[5,6] In our previous works, some bioactive metabolites, such as, diterpenoids,[7] alkaloids,[8] butyrolactones,[9-11] isocoumarins,[12-14] and the like, had been isolated from the genus of this fungus. Particularly, some isocoumarins, such as oryzaeins B[14] with inhibition rate of 30.6%, versicoumarins A[15] with inhibition rate of 28.6%, versicolols D[16] with inhibition rate of 24.6%, have potential anti-tobacco mosaic virus (anti-TMV) activities.

The isocoumarins are an important class of naturally occurring coumarins isomers with a reversed lactone moiety, which are abundantly distributed in natural sources and are being extracted from different plants,

molds, lichens, fungi and bacteria strains.[17] As one of the characteristic components of *Aspergillus* fungi, isochromenes also attracts much attention from chemists and biologists due to their diverse structures and biological properties.[18,19] With the aim of continuously explore bioactive metabolites from *Aspergillus* fungi species, an investigations were carried out on the cultures of the endophytic fungi *Aspergillus versicolor* obtained from *Nicotiana tabacum*. As a result, three new isochromenes (**1**-**3**), together with four known analogues (**4**-**7**) were obtained. This paper describes the elucidation of the structures of these three new compounds, and a preliminary evaluation of their anti-TMV activities.



1 **2** R = -H; **3** R = -OH **4** **5** **6** **7**

Figure 1. The structures of isochromenes and their analogues from fungus *A. versicolor*

The whole culture broth from the fermentation products of *A. versicolor* was extracted with EtOAc. The extract was subjected repeatedly to column chromatography on silica gel, MCI, RP-18 and preparative HPLC to afford compounds **1**-**7**, including three new isochromenes, versicolols G-I (1-**3**), along with four known analogues (**4**-**7**). The structures of compounds **1**-**7** were shown in Figure 1, and the and NMR data of **1**-**3** were listed in Table 1. The known compounds, ustusorane B (**4**),[20] versicolols B (**5**),[14] oryzaein D (**6**),[13] and altenusin D (**7**)[21] were identified by the comparison of their spectroscopic data with literatures.

Compound **1** was obtained as a pale-yellow gum. Its molecular formula $C_{14}H_{18}O_4$ was determined by its positive HRESIMS showed a peak at *m/z* 273.1012 $[M+Na]^+$ (calcd $C_{14}H_{18}NaO_4$ for 273.1103), indicating seven degrees of unsaturations. The IR spectrum showed the absorption bands for hydroxyl (3410 cm^{-1}) and aromatic group (1616, 1560, and 1462 cm^{-1}). The UV spectrum exhibited absorption bands at 215, 270, and 338 nm, also suggested the existence of aromatic chromophore. The ^1H and ^{13}C NMR spectrum of **1** (Table 1) showed the presence of a 1,2,3,5-tetrasubstituted benzene ring (C-5~C-10, H-6, and H-8), a hydroxypropyl group[14] (C-11~C-13,

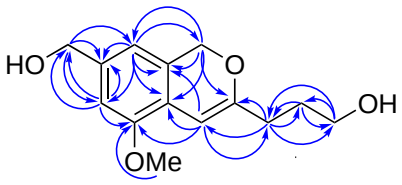


Figure 2. Key HMBC ( ) correlations of **1**

H_2-11, H_2-11~H_2-13'), a pair of double bond (C-3, C-4, and H-4), an oxidized methylene carbon (C-1 and H_2-1), a hydroxymethyl group (C-14 and H_2-14), and a methoxy group (δ_C 55.8 q and δ_H 3.77 s). According to above NMR data, the double bonds and oxidized methylene carbon should be incorporated with benzene ring to form an isochromene ring to support the seven degrees of unsaturations in the molecule.[13] In addition, the existence of isochromene core was supported by the HMBC correlations (Figure 2) from H-1 to C-3, C-8, C-9, C-10, from H-4 to C-3, C-5, C-9, C-10, and from H-8 to C-1, and the existence 3-hydroxypropyl group was also supported by the HMBC correlations from H_2-13 to C-11, C-12, from H_2-11 to C-12, C-13, and from H_2-12 to C-11, C-13.

Table 1. ^{1}H NMR and ^{13}C NMR data for compounds **1-3**

No.	Compound **1**		Compound **2**		Compound **3**	
	δ_C (m)	δ_H (m, *J*, Hz)	δ_C (m)	δ_H (m, *J*, Hz)	δ_C (m)	δ_H (m, *J*, Hz)
1	75.6 t	5.21 s	74.6 t	5.19 s	75.2 t	5.20 s
3	155.5 s		152.7 s		150.9 s	
4	96.7 d	5.84 s	96.4 d	5.84 s	96.8 d	5.87 s
5	156.9 s		156.9 s		156.5 s	
6	110.3 d	6.76 (d) 1.8	110.4 d	6.73 (d) 1.8	110.5 d	6.73 (d) 1.8
7	140.3 s		144.3 s		144.4 s	
8	117.7 d	6.82 (d) 1.8	118.6 d	6.89 (d) 1.8	118.5 d	6.90 (d) 1.8
9	136.3 s		135.3 s		135.0 s	
10	119.5 s		119.3 s		119.3 s	
11	31.5 t	2.26 (t) 7.6	20.7 q	1.93 s	62.2 t	4.33 s
12	28.4 t	1.62 m	31.9 d	3.06 m	31.9 d	3.04 m
13	64.2 t	3.55 (t) 6.6	23.1 q	1.52 (d) 6.8	23.1 q	1.53 (d) 6.8
14	66.5 t	4.65 s	23.1 q	1.52 (d) 6.8	23.6 q	1.53 (d) 6.8
-OMe	55.8 q	3.77 s	55.9 q	3.79 s	55.9 q	3.76 s

Since the isochromene skeleton was determined, the positions of substituents (hydroxypropyl, hydroxymethyl, and methoxy groups) also can be determined by further analysis of its HMBC data (Figure 2). The HMBC correlations from the H_2-11 to C-3, C-4, from H-4 to C-11 established that the 3-hydroxypropyl group was located at C-3. The methoxy group located at C-5 was clearly indicated by the HMBC correlations from methoxy proton (δ_H 3.77 s) to C-5. Furthermore, the hydroxymethyl located at C-7 can also be determined by the HMBC correlations from H_2-14 to C-6, C-7, C-8, and from H-6 and H-8 to C-7. On the basis of above evidence, the structure of **1** was established as 3-(7-(hydroxymethyl)-5-methoxy-1*H*-isochromen-3-yl)propan-1-ol, and gave the trivial name of versicolol G.

7-Isopropyl-5-methoxy-3-methyl-1*H*-isochromene (**2)** was also obtained as pale-yellow gum with a molecular formula of $C_{14}H_{18}NaO_2$, according to the ion peak of (*m/z* 241.1210, $[M+Na]^+$) in the HRESIMS. The UV and IR spectra of **2** were highly similar to those of **1**. The chemical shift differences in NMR resulted from the disappearance of 3-hydroxypropyl and hydroxymethyl groups, and appearance of a isopropyl[22] (C-12~C-14, H-12, H_6-13,14) and methyl gCH3roup (C-11 and H_3-11) in **2**. These changes indicated that the hydroxypropyl and hydroxymethyl groups in **1** was converted into isopropyl and methyl groups in **2**. The HMBC correlation from H-12 to C-6, C-7, C-8, from H_3-11 to C-3, C-4, from H-4 to C-11 also supported the isopropyl group located C-7, and the methyl group located at C-3, respectively. In addition, the positions of methoxy group can also be determination by the HMBC correlations between the methoxy proton (δ_H 3.79 s) and C-5. The structure of **2** was therefore defined, and gave the trivial name of versicolol H.

(7-Isopropyl-5-methoxy-1*H*-isochromen-3-yl)methanol (**3**) was obtained as a pale-yellow gum and showed a quasi-molecular ion at *m/z* 257.1148 $[M+Na]^+$ in the HRESIMS (calcd *m/z* 257.1154), corresponding to the molecular formula of $C_{14}H_{18}O_3$. The ^1H and ^{13}C NMR spectra of **3** were also highly similar to those of **2**. The obvious chemical shift differences resulted from the methyl group in **2** was replaced by a hydroxymethyl group in **3**. The position of the hydroxymethyl group at C-3 can be determination by the HMBC correlations from H_2-11 to C-3, C-4, from H-4, to C-11. In addition, the positions of isopropyl and methoxy groups can also be determined by further analysis of their HMBC correlations. Thus, the structure of **3** was determined as shown, and gave the trivial name of versicolol I.

Since certain of the isocoumarins derivatives exhibit potential anti-virus activities.[12,13,15,16,23] Compounds **1**-**3** were evaluated for their anti-TMV activities. The anti-TMV activities were tested by half-leaf method,[8,24] using 20 μM of ningnanmycin as a positive control. The results showed that compound **1** exhibited high anti-TMV activity with inhibition rate of 41.6% at the concentration of 20 μM, and this rate are higher than that of positive control. Compounds **2** and **3** also showed potential activities with inhibition rates of 22.8 and 26.5% at the concentration of 20 μM, respectively.

EXPERIMENTAL

General Experimental Procedures. UV spectra were obtained using a Shimadzu UV-1900 spectrophotometer. A Bio-Rad FTS185 spectrophotometer was used for scanning IR spectra. ^1H, ^{13}C, and 2D-NMR spectroscopic data were recorded on a DRX-500 NMR spectrometer with TMS as internal standard. ESIMS and HRESIMS analyses were measured on Agilent 1290 UPLC/6540 Q-TOF mass spectrometer. Chemical shifts (δ) are expressed in ppm with reference to the TMS signal. Semi-preparative HPLC was performed on an Agilent 1260 preparative liquid chromatograph with Zorbax PrepHT GF C_{18} (2.12 mm×25 cm) or Venusil MP C_{18} (2.0 mm×25 cm) columns. Column

chromatography was performed using silica gel (200-300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China), 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 versicolor* YNCA-20-25 was isolated from the leave of cigar tobacco, collected from Yingjiang County, Dehong Prefecture, Yunnan Province, in 2020. The strain was identified by one of 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 100 Fernbach flasks (500 mL) each containing 300 mL of medium (glucose 5%; peptone 0.15%; yeast 0.5%; KH_2PO_4 0.05%; $MgSO_4$ 0.05% in 1 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. versicolor* was extracted four times with EtOAc (4×10 L) at room temperature and filtered. The crude extract (108 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 fraction A (9:1, 12.5 g) by silica gel column chromatography, eluted with $CHCl_3$-acetone (9:1, 8:2, 7:3, 6:4, 1:1) again, yielded mixtures A1-A5. Fraction A2 (8:2, 2.68 g) was subjected to RP-18 column chromatography and HPLC (MeOH/H_2O 50:50-80:20 gradient) to give **4** (21.8 mg)and **5** (18.0 mg). Fraction A3 (7:3, 2.87 g) was subjected to RP-18 column chromatography and HPLC (MeOH/H_2O 40:60-75:25 gradient) to give **2** (23.5 mg) and **3** (20.2 mg), and **6** (18.5 mg). The further separation of fraction B (8:2, 9.26 g) by silica gel column chromatography, eluted with $CHCl_3$-acetone (8:2, 7:3, 6:4, 1:1), yielded mixtures B1-B4. Fraction B3 (6:4, 2.38 g) was subjected to RP-18 column chromatography and HPLC (MeOH/H_2O 30:70-40:60 gradient) to give **1** (18.2 mg) and **7** (15.8 mg).

Anti-TMV Assays. The anti-TMV activities were tested using the half-leaf method according our previous literature,[24,25] and 20 μM of Ningnanmycin ($C_{16}H_{25}N_7O_8$, CAS#: 156410-09-2, a commercial new cytosine nucleoside peptide antibiotics for plant viral diseases in China, with inhibition rate of 34.2%) was used as a positive control. The virus was inhibited by mixing with the solution of tested compounds (20 μM in DMSO). 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.

3-(7-(Hydroxymethyl)-5-methoxy-1*H*-isochromen-3-yl)propan-1-ol (1): $C_{14}H_{18}O_4$, Obtained as a pale yellow gum; UV (MeOH) λ_{max} (log ε) 215 (3.84), 270 (3.68), 338 (3.57) nm; IR (KBr) ν_{max} 3410, 3052, 2938, 2870, 1648, 1616, 1560, 1462, 1373, 1241, 1166, 1067, 832 cm^{-1}; ^1H and ^{13}C NMR (500 and 125 MHz, in $CDCl_3$ see Table 1; ESIMS *m/z* (positive ion mode) 273 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 273.1012 $[M+Na]^+$ (calcd $C_{14}H_{18}NaO_4$ for 273.1103).

7-Isopropyl-5-methoxy-3-methyl-1*H*-isochromene (2): $C_{14}H_{18}O_2$, Obtained as a pale yellow gum; UV (MeOH) λ_{max} (log ε) 215 (3.79), 264 (3.64), 332 (3.52) nm; IR (KBr) ν_{max} 3062, 2943, 2865, 1646, 1615, 1554, 1470, 1384, 1239, 1156, 1062, 809 cm^{-1}; ^1H and ^{13}C NMR (500 and 125 MHz, in $CDCl_3$ see Table 1; ESIMS *m/z* (positive ion mode) 241 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 241.1210 $[M+Na]^+$ (calcd $C_{14}H_{18}NaO_2$ for 241.1204).

(7-Isopropyl-5-methoxy-1*H*-isochromen-3-yl)methanol (3): $C_{14}H_{18}O_3$, Obtained as a pale yellow gum; UV (MeOH) λ_{max} (log ε) 215 (4.01), 266 (3.73), 334 (3.67) nm; IR (KBr) ν_{max} 3392, 3057, 2938, 2862, 1614, 1550, 1463, 1376, 1245, 1159, 1058, 835 cm^{-1}; ^1H and ^{13}C NMR (500 and 125 MHz, in $CDCl_3$ see Table 1; ESIMS *m/z* (positive ion mode) 257 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 257.1148 $[M+Na]^+$ (calcd $C_{14}H_{18}NaO_3$ for 257.1154).

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