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Antiviral isocoumarins from the roots and stems of *Nicotiana tabacum*

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ABSTRACT

Three new isocoumarin derivatives, tabaisocoumarins A–C (**1**–**3**), together with three known phenolic compounds (**4**–**6**), were isolated from the roots and stems of *Nicotiana tabacum*. Their structures were elucidated by spectroscopic methods, including extensive 1D- and 2D NMR techniques. Compounds **1**–**6** were evaluated for their anti-tobacco mosaic virus (anti-TMV) activities at the concentration of 20 μM. Compound **2** exhibited anti-TMV activity with inhibition rate of 33.8%, which is higher than that of positive control, ningnanmycin. The other compounds also showed potential anti-TMV activities with inhibition rates in the range of 18.7–28.5%, respectively.

1. Introduction

Nicotiana tabacum, tobacco, is a stout herbaceous plant in the Solanaceae (nightshade family) that originated in the tropical Americas (South America, Mexico, and the West Indies) and now cultivated worldwide as the primary commercial source of tobacco, which is smoked or chewed as a drug for its mild stimulant effects (Kuang and Lu, 2005; Hu et al., 2006). In addition, *N. tabacum* is also used as insecticides, anesthetics, diaphoretics, sedatives, and emetic agents in Chinese folklore medicines because of its containing many useful chemical compounds (Kuang and Lu, 2005; Rodgman and Perfetti, 2008). Previous investigation of this species led to the discovery of a number of new compounds that showed various bioactivities, such as anti-HIV-1, anti-TMV, and cytotoxicity by our groups (Tan et al., 2011; Chen et al., 2012a,b, 2013; Gao et al., 2012; Mou et al., 2012). In continuing efforts to the phytochemistry research on the roots and stems of K326 (a variety of *N. tabacum*) led to the isolation of three new isocoumarin derivatives (**1**–**3**) and three known phenolic compounds (**4**–**6**) (Fig. 1). This paper deals with the isolation, structural elucidation, and anti-TMV activities of these compounds.

2. Results and discussion

A 70% aq. acetone extract prepared from the roots and stems of *N. tabacum* was partitioned between EtOAc and H_2O. The EtOAc layer was subjected repeatedly to column chromatography on Si gel, RP-18 and preparative HPLC to afford compounds **1**–**6**, which included three new isocoumarin derivatives, named tabaisocoumarins A–C (**1**–**3**), together with three known phenolic compounds, 5-hydroxy-7-methoxy-3-(1-hydroxyethyl)phthalide (**4**) (Takenaka et al., 2011), angelicoin A (**5**) (Jens et al., 2012), and cichorin A (**6**) (Hidayat et al., 2011). The structures of compounds **1**–**6** are shown in Fig. 1, and the ^{1}H and ^{13}C NMR data of compounds **1**–**3** are listed in Table 1.

Compound **1**, obtained as white solid, had a molecular formula $C_{16}H_{18}O_4$ as established by the quasimolecular ion peak observed using HRESIMS measurement at *m*/*z* 297.1108 $[M + Na]^+$ (calcd for 297.1103), suggesting 8 degrees of unsaturation. The UV spectrum showed absorption maxima at 210, 268, 297, and 335 nm, and the IR spectrum showed absorption bands at 3423, 1718, 1604, 1566, and 1493 cm^{-1}, indicating the presence of hydroxy group(s), an aromatic ring and an unsaturated lactone. Its ^{1}H NMR spectrum exhibited signals for a 1,2,3,4-tetrasubstituted benzene ring at

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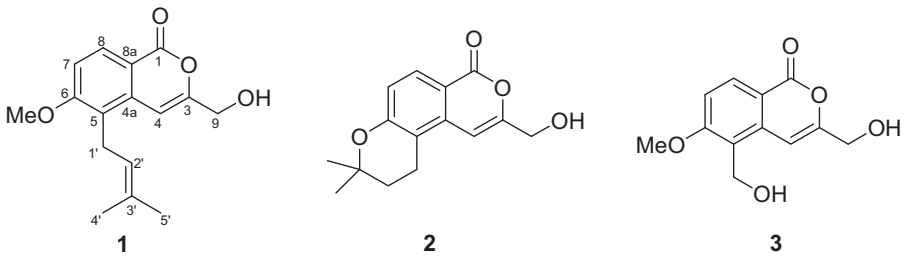

1 2 3

Fig. 1. Structures of new isocoumarins from *Nicotiana tabacum* (**1**–**3**).

δ_H 6.76 (d, J = 8.4 Hz) and 7.69 (d, J = 8.4 Hz), other two olefinic protons at δ_H 5.12 (t, J = 6.9 Hz) and 6.50 (s), two methyl group at δ_H 1.57 (s) and 1.78 (s), one methoxy groups at δ_H 3.81, two methylene groups (including an oxygenated one) at δ_H 4.36 (s) and 3.40 (d, J = 6.9 Hz). The ^{13}C NMR spectrum of **1** showed eleven sp^2 signals containing four methine carbons and seven quaternary carbons (one carbonyl carbon), three methyl carbons including a methoxy group, two methylene carbons (one oxygenated) (Table 1). The ^{1}H and ^{13}C NMR signals were further assigned with the help of HSQC and HMBC experiments (Table 1, Fig. 2). These spectral data were closely related to those of 6-hydroxy-3-hydroxymethyl-8-methoxyisocoumarin (Takenaka et al., 2011) isolated from the spore-derived mycobionts of *Graphis proserpens*. The major differences were the presences of the 1,2,3,4-tetrasubstituted benzene ring and an dihydropyrano unit (C-1′–C-5′) being verified by the ^{1}H–^{1}H COSY and HMBC data in compound **1** while the presence of an 1,3,4,5-tetrasubstitued benzene ring and absence of dihydropyrano unit in compound 6-hydroxy-3-hydroxymethyl-8-methoxyisocoumarin (Fig. 2), which may be in virtue of the additional dihydropyrano unit and the different positions of the substituted benzene. The HMBC correlations from H-8 to C-1, C-4a, and C-8a, from H-7 to C-5 and C-6, from 6-OMe to C-6, together with the ^{1}H–^{1}H COSY correlations between H-7 and H-8 indicated that the 6-OMe was located at C-6; While the dihydropyrano unit was located at C-5 on account of the HMBC correlations from H-1′ to C-5 and C-6 (Fig. 2). Other 2D data further confirmed the deduction (Fig. 2). Thus, the structure of **1** was established as shown, and was given the name of tabaisocoumarin A.

Tabaisocoumarin B (**2**) was isolated as white solid and it gave an $[M + Na]^+$ peak at m/z 283.0942, consistent with a molecular formula of $C_{15}H_{16}O_4$. The IR spectrum exhibited absorptions of hydroxy (3425 cm^{-1}), aromatic ring (1608, 1578, and 1495 cm^{-1}), and unsaturated lactone groups (1716 cm^{-1}). The data of ^{1}H NMR were correlated with those of ^{13}C NMR with the help of HSQC spectrum (Table 1). Its ^{1}H and ^{13}C NMR spectroscopic data were similar to those of **1**, which suggested that **2** was structurally related to **1**. The marked differences between them were the absence of an methoxy group and an double bond (C-2′═C-3′) and the presence of additional signals of one methylene group and one oxygenated quaternary carbon in compound **2**. The HMBC correlations from H_3-4′ and H_3-5′ to C-6, C-2′ and C-3′, the ^{1}H–^{1}H COSY correlation between H-1′ and H-2′, combined with the chemical shift of C-3′ (δ_C 73.7) showed that C-3′ and C-6 was linked to each other through an oxygen atom (Fig. 2). Other 2D data further confirmed the whole structure of **2**.

Tabaisocoumarin C (**3**) was assigned a molecular formula of $C_{12}H_{12}O_5$ as supported by the HRESIMS (m/z 259.0587 $[M + Na]^+$), corresponding to seven degrees of unsaturation. Strong absorption bands accounting for hydroxy (3426 cm^{-1}), aromatic groups (1608, 1562, and 1476 cm^{-1}) and unsaturated lactone groups (1721 cm^{-1}) were observed in the IR spectrum. Its ^{1}H and ^{13}C NMR spectroscopic data were similar to those of compound **1**, except for the presence of an oxygenated methylene and the absence of an dihydropyrano unit. The HMBC correlations from H_2-1′ to C-4a, C-5, and C-6 suggested that the dihydropyrano unit in **1** was replaced as an oxygenated methylene (Fig. 2). Other ^{1}H–^{1}H COSY and HMBC correlations further verified the deduction. Accordingly, compounds **3** was formulated as shown.

Finally, compounds **1**–**6** were tested for their anti-TMV activity. The inhibitory activity of compounds **1**–**6** against TMV replication were tested using the half-leaf method (Hu et al., 2013). Ningnanmycin, a commercial product for plant disease in China, was used as a positive control. The antiviral inhibition rates of compounds **1**–**6** at the concentration of 20 μM were listed in

Table 1
^{1}H (500 MHz) and ^{13}C NMR (125 MHz) data of compounds **1**–**3** in C_5D_5N.

Position	Compound **1**		Compound **2**		Compound **3**	
	δ_C	δ_H (m, J, Hz)	δ_C	δ_H (m, J, Hz)	δ_C	δ_H (m, J, Hz)
1	161.2 s		161.3 s		161.2 s	
3	157.0 s		156.8 s		157.2 s	
4	106.0 d	6.50 s	105.7 d	6.49 s	106.0 d	6.48 s
4a	139.1 s		137.3 s		136.8 s	
5	132.4 s		129.9 s		116.6 s	
6	163.1 s		58.8 s		163.0 s	
7	114.1 d	6.76 (d) 8.4	114.9 d	6.91 (d) 8.4	115.1 d	6.81 (d) 8.4
8	127.6 d	7.69 (d) 8.4	126.7 d	7.81 (d) 8.4	130.5 d	7.86 (d) 8.4
8a	121.9 s		122.0 s		122.2 s	
9	62.0 t	4.36 s	62.1 t	4.33 s	62.4 t	4.35 s
1′	27.2 t	3.40 (d) 6.9	21.8 t	2.69 (t) 6.8	52.8 t	4.87 (d) 9.2
2′	124.2 d	5.12 (t) 6.9	33.1 t	1.79 (t) 6.8		
3′	133.7 s		73.7 s			
4′	17.7 q	1.57 q	26.4 q	1.64 q		
5′	25.9 q	1.78 q	26.4 q	1.64 q		
6-OMe	56.0 q	3.81 s			56.0 q	3.80 s
1′-OH						4.47 br s

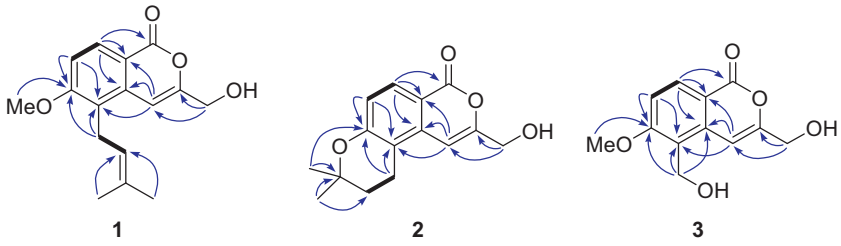

Fig. 2. Key HMBC (↷) and ^{1}H–^{1}H COSY (▬) correlations of **1**–**3**.

Table 2. The results showed that compound **2** exhibited high anti-TMV activity with inhibition rate of 33.8%. The inhibition rate is higher than that of positive control. The other compounds also showed potential anti-TMV activities with inhibition rates in the range of 18.7–28.5%, respectively.

3. Experimental

3.1. General

UV spectra were obtained using a Shimadzu UV-2401A spectrophotometer. A Tenor 27 spectrophotometer was used for scanning IR spectroscopy with KBr pellets. 1D- and 2D NMR spectra were recorded on DRX-500 spectrometers with TMS as internal standard. Unless otherwise specified, chemical shifts (δ) were expressed in ppm with reference to the solvent signals. HRESIMS was performed on an API QSTAR time-of-flight spectrometer, or a VG Autospec-3000 spectrometer, respectively. Preparative HPLC was performed on a Shimadzu LC-8A preparative liquid chromatograph with a ZORBAX PrepHT GF (21.2 mm × 25 cm, 7 μm) column or a Venusil MP C_{18} (20 mm × 25 cm, 5 μm) column. Column chromatography was performed with Si gel (200–300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany) and MCI gel (75–150 μm, Mitsubishi Chemical Corporation, Tokyo, Japan). The fractions were monitored by TLC, and spots were visualized by heating Si gel plates sprayed with 5% H_2SO_4 in EtOH.

3.2. Plant material

The roots and stems of *N. tabacum* L was collected from Yuxi County, Yunnan Province, PR China, in September 2011. The specimen was identified by Prof. Guangyu Yang and a voucher specimen (No. KIB 2011-08-11) has been deposited at key laboratory of tobacco chemistry of yunnan province, China tobacco yunnan industrial Co.

3.3. Extraction and isolation

The air-dried and powdered roots and stems of *N. tabacum* (6.0 kg) were extracted four times with 70% aqueous acetone (3 × 12 L) at room temperature and filtered. The solvent was evaporated in vacuo, and the crude extract was dissolved in H_2O and partitioned with EtOAc. The EtOAc partition (240 g) was applied to silica gel (200–300 mesh) column chromatography, eluting with a $CHCl_3$– $(CH_3)_2CO$ gradient system (10:0, 9:1, 8:2, 7:3, 6:4, 5:5, 0:10), to give six fractions A–F. Further separation of fraction B (9:1, 32.4 g) by silica gel column chromatography, eluted with $CHCl_3$ –$(CH_3)_2CO$ (15:1–2:1), yielded mixtures B1–B6. Fraction B1 (15:1, 3.8 g) was subjected to silica gel column chromatography using petroleum ether-acetone and semi-preparative HPLC (55% MeOH–H_2O, flow rate 12 mL/min) to give **6** (5.8 mg). Fraction B2 (10:1, 6.4 g) was subjected to silica gel column chromatography using petroleum ether-acetone and semi-preparative HPLC (52% MeOH–H_2O, flow rate 12 mL/min) to give **1** (11.6 mg), **2** (9.6 mg), and **3** (15.4 mg). Fraction B3 (6:1, 5.2 g) was subjected to silica gel column chromatography using $CHCl_3$–$(CH_3)_2CO$ and semi-preparative HPLC (49% MeOH–H_2O, flow rate 12 mL/min) to give **4** (9.2 mg) and **5** (18.5 mg).

3.3.1. Tabaisocoumarin A (*1*)

Obtained as white solid; UV (MeOH) λ_{max} (log ε) 210 (4.22), 268 (3.92), 297 (3.73), 335 (3.70) nm; IR (KBr) ν_{max} 3423, 3072, 2930, 2862, 1730, 1718, 1604, 1566, 1493, 1380, 1215, 1134, 1073, 868, 758 cm^{-1}; ESIMS *m/z* (positive ion mode) 297 $[M + Na]^+$; HRESIMS (positive ion mode) *m/z* 297.1108 $[M + Na]^+$ (calcd $C_{16}H_{18}NaO_4$ for 297.1103).

3.3.2. Tabaisocoumarin B (*2*)

Obtained as white solid; UV (MeOH) λ_{max} (log ε) 210 (4.18), 260 (3.86), 292 (3.68), 322 (3.65) nm; IR (KBr) ν_{max} 3425, 3069, 2932, 2855, 1732, 1716, 1660, 1608, 1578, 1495, 1384, 1218, 1130, 1079, 864, 757 cm^{-1}; ESIMS *m/z* (positive ion mode) 283 $[M + Na]^+$; HRESIMS (positive ion mode) *m/z* 283.0942 $[M + Na]^+$ (calcd $C_{15}H_{16}NaO_4$ for 283.0946).

3.3.3. Tabaisocoumarin C (*3*)

Obtained as white solid; UV (MeOH) λ_{max} (log ε) 210 (4.03), 255 (3.76), 280 (3.65), 316 (3.64) nm; IR (KBr) ν_{max} 3426, 3057, 2922, 2850, 1732, 1721, 1608, 1562, 1476, 1357, 1209, 1138, 1067, 865, 774 cm^{-1}; ESIMS *m/z* (positive ion mode) 259 $[M + Na]^+$; HRESIMS (positive ion mode) *m/z* 259.0587 $[M + Na]^+$ (calcd $C_{12}H_{12}NaO_5$ for 259.0582).

3.4. Anti-TMV assay

The Anti-TMV activities were tested using the half-leaf method, and ningnanmycin (Hu et al., 2013), a commercial product for plant disease in China, was used as a positive control.

Table 2
TMV infection inhibition activity of compounds **1**–**6**.

Compounds	Inhibition rates (%)	Compounds	Inhibition rates (%)
1	24.6 ± 3.1	**5**	24.8 ± 2.8
2	33.8 ± 3.3	**6**	28.5 ± 3.0
3	20.4 ± 2.5	Ningnanmycin	31.5 ± 3.0
4	18.7 ± 2.2		

All results are expressed as mean ± SD; $n = 3$ for all groups.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytol.2014.11.008.

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