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FURAN-2-CARBOXYLIC ACIDS FROM THE LEAVES OF *NICOTIANA TABACUM* AND THEIR ANTI-TOBACCO MOSAIC VIRUS ACTIVITIES

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Abstract – Three new furan-2-carboxylic acids, 5-(4-hydroxy-2-methoxy-6-methyl- phenyl)-3-methylfuran-2-carboxylic acid (**1**), methyl 5-(4-hydroxy-2-methoxy-6- methylphenyl)-3-methylfuran-2-carboxylate (**2**), and 5-(3-hydroxy-4-methoxy-5- methylphenyl)-3-methylfuran-2-carboxylic acid (**3**), together with two known furan-2-carboxylic acids (**4** and **5**) were isolated from the leaves of *Nicotiana tabacum*. Their structures were determined by means of HRESIMS and extensive 1D and 2D NMR spectroscopic studies. Compounds **1**-**5** were tested for their anti-TMV activities. The results showed that compounds **3** and **4** exhibited high anti-TMV activities with inhibition rates of 29.8 and 30.3%. These rates are close to that of positive control. The other compounds also showed potential activities with inhibition rates in the range of 22.8%~25.4%, respectively.

Nicotiana tabacum, tobacco is an important economic crop originating from South America.[1,2] Its leaves are used as a raw material for the tobacco industry, aerial plant as an insecticide, and also as anesthetic, diaphoretic, sedative, and emetic agents in Chinese folklore medicine.[1-3] In previous literatures, many new bioactive compounds, such as, sesquiterpenes,[4-6] alkaloids,[7,8] lignans,[9,10] flavonoids,[11-14] phenylpropanoids,[15,16] chromanones,[17,18] biphenyls,[19] phenolic amides,[20] isocoumarins,[21] were isolated from *Nicotiana tabacum*.

In our continuing endeavor to discover new bioactive natural products, an investigation of the leaves of Yunyan 85 (a variety of *N. tabacum* widely cultivited in China) was undertaken. In continuing efforts to utilize *N. tabacum* and identify bioactive natural products, the phytochemistry investigation of the leaves of Yunyan 85 (a variety of *N. tabacum*) led to the isolation of three new (**1**-**3**) and two known (**4** and **5**) furan-2-carboxylic acids. This paper deals with the isolation, structural elucidation, and their anti-TMV activities of these compounds.

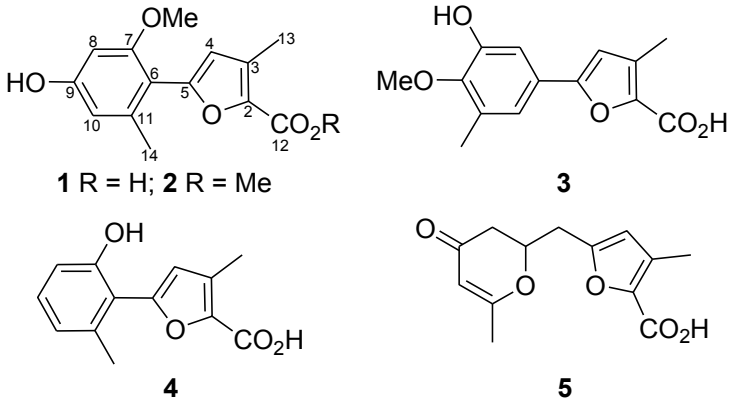

Figure 1. Furan-2-carboxylic acids from the leaves of *Nicotiana tabacum*

A 95% aq. MeOH extract prepared from the leaves of tobacco was subjected repeatedly to column chromatography on silica gel and preparative HPLC to afford three new furan-2-carboxylic acids, 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-methylfuran-2-carboxylic acid (**1**), methyl 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-methylfuran-2-carboxylate (**2**), and 5-(3-hydroxy-4-methoxy-5-methyl-phenyl)-3-methylfuran-2-carboxylic acid (**3**), together with two known furan-2-carboxylic acids (**4** and **5**). The structures of the compounds **1**-**5** were as shown in Figure 1, and the ^{1}H and ^{13}C NMR data of **1**-**3** were listed in Table 1. The known compounds, compared with literature, were identified as 5-(2'-hydroxy-6'-methylphenyl)-3-methylfuran-2-carboxylic acid (**4**),[22] and 5-((6'-methyl-4'-oxo-3',4'-dihydro-2*H*-pyran-2'-yl)methyl)-3-methylfuran-2-carboxylic acid (**5**).[22]

Compound **1** was obtained as a pale yellow gum, and its (+)HRESIMS gave a quasimolecular ion at *m/z* 285.0731 $[M+Na]^{+}$. These datas, coupled with ^{13}C NMR spectroscopic data, established the molecular formula of **1** as $C_{14}H_{14}O_5$. The IR spectrum of **1** exhibited absorption bands for $-CO_2H$ (br. 3020 cm^{-1}), $-CO_2$ (1692 cm^{-1}) and aromatic functionality (1610, 1584 cm^{-1}). The ^{1}H NMR spectrum of **1** (Table 1) exhibited signals attributable to one methoxy group (δ_H 3.81 s), one $-CO_2H$ (δ_H 10.04) groups, three olefinic methines (δ_H 6.19 s, 6.40 s, and 6.29 s), two methyl (δ_H 1.87 s and 2.40 s) groups, and one phenolic hydroxy group (δ_H 10.60 s). The ^{13}C NMR spectrum (DEPT) indicated the presence of an ester or carboxyl carbon (δ_C 166.9 s), two methyl (δ_C 8.7 q and 20.1 q), one methoxy group (δ_C 55.8 q), three

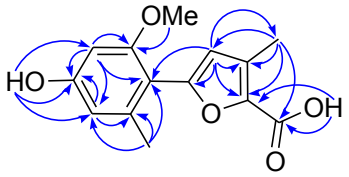

Figure 2. Key HMBC (↷) correlations of **1**

olefinic methine (δ_C 105.8 d, 100.6 d and 108.1 d) carbons, four *sp*2 oxygen-bearing quaternary olefinic carbons (δ_C 162.1 s, 156.5 s, 160.5 s, and 155.2 s), and three *sp*2 quaternary olefinic carbons (δ_C 101.7 s, 113.1 s, and 136.7 s). The HMBC correlations (Figure. 2) from H_3-13 (δ_H 1.87) to C-2 (δ_C 162.1), C-3 (δ_C 101.7), C-4 (δ_C 105.8), and C-12 (δ_C 166.9); from H-4 (δ_H 6.19) to C-2 (δ_C 162.1), C-3 (δ_C 101.7), C-5 (δ_C 156.5); and from CO_2H (δ_H 10.04) to C-2 (δ_C 162.1) indicated the presence of 3-methylfuran-2-carboxylic acid in **1**.[22] The long-range correlations from H-4 (δ_H 6.19) to C-6 (δ_C 113.1) indicated the attachment of a substituted benzene ring to C-5 of the 3-methylfuran-2-carbocyclic acid core. The HMBC correlations of H_3-14 (δ_H 2.40) with C-6 (δ_C 113.1), C-10 (δ_C 108.1), and C-11 (δ_C 136.7) indicated this methyl groups at C-11; the methoxy group located at C-7 was supported by the HMBC correlation of the methoxy protons (δ_H 3.81) with C-7 (δ_C 160.5); the phenolic hydroxy group located at C-9 was supported by the HMBC correlations of the phenolic hydroxy proton (δ_H 10.60) with C-8 (δ_C 100.6), C-9 (δ_C 155.2), and C-10 (δ_C 108.1). Thus, the structure of **1** was determined to be 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-methylfuran-2-carboxylic acid.

Table 1. ^{1}H NMR and ^{13}C NMR data **(**in C_5D_5N**)** of 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)
2	162.1 s		163.5 s		162.7 s	
3	101.7 s		102.6 s		103.0 s	
4	105.8 d	6.19 s	105.3 d	6.14 s	105.8 d	6.21 s
5	156.5 s		157.3 s		156.9 s	
6	113.1 s		112.4 s		125.4 s	
7	160.5 s		160.2 s		111.3 d	6.53 d (1.8)
8	100.6 d	6.40 s	100.2 d	6.38 s	146.1 s	
9	155.2 s		155.4 s		152.5 s	
10	108.1 d	6.29 s	108.4 d	6.28 s	127.2 s	
11	136.7 s		136.9 s		124.1 d	6.68 d (1.8)
12	166.9 s		162.2 s		167.3 s	
13	8.7 q	1.87 s	8.9 q	1.89 s	9.0 q	1.85 s
14	20.1 s	2.40 s	20.3 s	2.39 s	18.2 s	2.39 s
7-OMe	55.8 q	3.81 s	56.0 q	3.80 s		
9-OMe					61.4 q	3.86 s
12-OMe			52.4 s	4.04 s		
-C(O)-OH		10.04 s				10.05 s
Ar-OH		10.60 s		10.59 s		10.68 s

Compound **2** was also obtained as a pale yellow gum and showed a quasi-molecular ion at *m/z* 299.0900 $[M+Na]^+$ in the HRESIMS (calcd *m/z* 299.0895), corresponding to the molecular formula $C_{15}H_{16}O_5$. The ^{1}H and ^{13}C NMR spectra of **2** were similar to those of **1**. The chemical shift differences resulted from the carboxyl group in **1** was converted into a methoxycarbonyl group in **2**. The HMBC correlation of the phenolic hydroxy proton (δ_H 10.59) with C-8 (δ_C 100.2), C-9 (δ_C 155.4), and C-10 (δ_C 108.4) indicated

that the phenolic hydroxy group was located at C-9. In addition, the positions of the methoxy group and methyl group can also be determined by further analysis of its HMBC correlations. The structure of **2** was therefore defined, and gives the semi-systematic name of methyl 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-methylfuran-2-carboxylate.

5-(3-Hydroxy-4-methoxy-5-methylphenyl)-3-methylfuran-2-carboxylic acid (**3**) was also isolated as a pale yellow gum, and its molecular formula was determined as 285.0746 through HRESIMS analysis (pseudomolecular ion $[M+Na]^+$ at *m/z* 285.0739). Comparisons of the ^1H and ^{13}C NMR data of **3** with those of **1** (Table 1) indicated that both compounds have identical skeletons, the differences was due to the substituents positions variation on the aromatic ring. A phenolic hydroxy group located at C-8 was supported by the HMBC correlation of phenolic hydroxy proton (δ_H 10.68) with C-7, C-8, and C-9. A methoxy group located C-9 was confirmed by the HMBC correlation of methoxy proton (δ_H 3.86) with C-9. Finally, the HMBC correlations of methyl proton (δ_H 2.39) with C-6, C-10, and C-11 supported the methyl group located at C-10. Thus, the structure of **3** was determined as shown.

Compounds **1-5** 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 32.2%) as a positive control.[23,24] The results showed that compound **3** and **4** exhibited high anti-TMV activities with inhibition rates of 29.8 and 30.3% at the concentration of 20 μM. These rates are close to that of positive control. The other compounds also showed potential activities with inhibition rates in the range of 22.8%~25.4% at the concentration of 20 μM, respectively.

Table 2. TMV Infection inhibition activities of compounds **1-5**

Compounds	Inhibition rate (%)	IC_{50} (μM)	Compounds	Inhibition rates (%)	IC_{50} (μM)
1	22.8 ± 3.2	75.4	**4**	30.3 ± 3.3	44.7
2	25.4 ± 3.0	66.2	**5**	24.2 ± 3.0	68.9
3	29.8 ± 3.5	52.1	ningnanmycin	32.2 ± 3.4	38.2

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

EXPERIMENTAL

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, and the chemical shifts (δ) were expressed in ppm. HRESIMS was performed on an API QSTAR time-of-flight spectrometer and 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 silica gel (200–300 mesh, Qing-dao Marine Chemical, Inc., Qingdao, China). The fractions were monitored by TLC, and spots were visualized by heating Si gel plates sprayed with 5% H_2SO_4 in EtOH.

Plant material. The leaves of *N. tabacum* L (tobacco leaves) was collected from Dali County, Yunnan Province, P. R. China, in September 2014. The tobacco variety is Yunyan-85, which had widely cultivated in China. The identification of the plant material was verified by Prof. H. W. Yang (School of Tobacco, Yunnan Agriculture University).

Extraction and Isolation. The air-dried and powdered tobacco leaves (3.5 kg) were extracted with 95% MeOH, and the extract was partitioned between EtOAc. The EtOAc-soluble materials (92.7 g) were applied to silica gel (200–300 mesh) column chromatography, eluting with $CHCl_3$/MeOH gradient system (10:0, 9:1, 8:2, 7:3, 6:4, 5:5) to give six fractions A-F. Further separation of fraction C (8:2, 25.8 g) by silica gel column chromatography, eluted with $CHCl_3$/Me_2CO (8:2-2:1) yielded mixtures C1–C7. Fraction C4 (1:1, 0.82 g) was subjected to silica gel column chromatography using petroleum ether/acetone, and then semi-preparative HPLC (38% MeOH/H_2O, flow rate 12 mL/min) to give **2** (13.5 mg). Fraction C6 (3:7, 1.98 g) was subjected to silica gel column chromatography using petroleum ether/acetone, and then semi-preparative HPLC (30% MeOH/H_2O, flow rate 12 mL/min) to give **1** (11.8 mg), **3** (15.3 mg), **4** (16.0 mg), and **5** (14.7 mg).

Anti-TMV Assays. The anti-TMV activity was tested using the half-leaf method,[23,24] and ningnanmycin (2% water solution), a commercial product for plant disease in China, was used as positive control.

The virus was inhibited by mixing with the solution of tested compounds. 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:

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

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.

5-(4-Hydroxy-2-methoxy-6-methylphenyl)-3-methylfuran-2-carboxylic acid (1): $C_{14}H_{14}O_5$, obtained as pale-yellow gum; UV (MeOH), λ_{max} (log ε) 305 (2.82), 248 (3.22), 210 (3.50) nm; IR (KBr) λ_{max} 3418, 3020, 2934, 1692, 1610, 1584, 1235, 1118, 1052 cm^{-1}; ^1H NMR and ^{13}C NMR data (C_5D_5N, 500 and 125 MHz, respectively), Table 1; ESIMS (positive ion mode) *m/z* 285 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 285.0731 $[M+Na]^+$ (calcd 285.0739 for $C_{14}H_{14}NaO_5$).

Methyl 5-(4-hydroxy-2-methoxy-6-methylphenyl)-3-methylfuran-2-carboxylate (2): $C_{15}H_{16}O_5$, obtained as pale-yellow gum; UV (MeOH), λ_{max} (log ε) 312 (2.71), 246 (3.26), 210 (3.79) nm; IR (KBr) λ_{max} 3415, 2940, 1716, 1614, 1570, 1267, 1128, 1049 cm^{-1}; ^1H NMR and ^{13}C NMR data (C_5D_5N, 500 and 125 MHz, respectively), Table 1; ESIMS (postive ion mode) *m/z* 299 $[M+Na]^+$; HRESIMS (postive ion mode) *m/z* 299.0900 $[M+Na]^+$ (calcd 299.0895 for $C_{15}H_{16}NaO_5$).

5-(3-Hydroxy-4-methoxy-5-methylphenyl)-3-methylfuran-2-carboxylic acid (3): $C_{14}H_{14}O_5$, obtained as pale-yellow gum; UV (MeOH), λ_{max} (log ε) 306 (2.70), 248 (3.35), 210 (3.74) nm; IR (KBr) λ_{max} 3419, 3023, 2942, 1687, 1613, 1582, 1240, 1120, 1048 cm^{-1}; ^1H NMR and ^{13}C NMR data (C_5D_5N, 500 and 125 MHz, respectively), Table 1; ESIMS (postive ion mode) *m/z* 285 $[M+Na]^+$; HRESIMS (postive ion mode) *m/z* 285.0746 $[M+Na]^+$ (calcd 285.0739 for $C_{14}H_{14}NaO_5$).

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