

HETEROCYCLES, Vol. 102, No. 6, 2021, pp. 1167 - 1174. © 2021 The Japan Institute of Heterocyclic Chemistry
Received, 20th February, 2021, Accepted, 22nd March, 2021, Published online, 26th March, 2021
DOI: 10.3987/COM-21-14435

ANTI-TOBACCO MOSAIC VIRUS ISOINDOLIN-1-ONES FROM THE STEMS OF *NICOTIANA TABACUM*

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Abstract –Three new (**1-3**) and three known (**4-6**) isoindolin-1-ones were isolated from the stems of *Nicotiana tabacum*. Their structures were determined by means of HRESIMS and extensive 1D and 2D NMR spectroscopic studies. Compounds **1-3** were tested for their anti-tobacco mosaic virus (anti-TMV) activity. The results showed that compounds **1-3** showed high anti-TMV activity with inhibition rates of 42.6, 46.7 and 38.5% at the concentration of 20 μ M, respectively. These rates are higher than that of ningnanmycin, a positive control.

Nicotiana tabacum L. (Tobacco) is a robust annual little branched herb in the Solanaceae (nightshade family). It is found only in cultivation, where it is the most commonly grown of all plants in the *Nicotiana* genus, and its leaves are commercially grown in many countries to be processed into tobacco.¹⁻³ *N. tabacum* had also been used in traditional medicine in China, India, and some African countries in the treatment of various pathologies, such as rheumatic swelling, skin diseases, painful piles, stings.² Previous phytochemical studies of *N. tabacum* have shown the presence of sesquiterpenoids,^{5,6} flavonoids,^{7,8} alkaloids,^{9,10} furans,^{11,12} coumarins,^{13,14} and the like.

In recent years, gene editing technology has been widely used in crop variety improvement.¹⁵ Because the secondary metabolism of plant would be affected by the change of gene, the gene editing mutants should be provided a new sample source for discovery of new active metabolites.^{16,17} In continuing our efforts to utilize *N. tabacum* and identify bioactive natural products from *N. tabacum*, the phytochemistry investigation of the stems of YNZY-1-12 (a mutant tobacco for gene editing with alkaloid metabolic pathway cultivated by Yunnan China Tobacco Industry Co., Ltd) led to the isolation of three new (**1-3**) and three known (**4-6**) isoindolin-1-ones. Their structures were determined by means of HRESIMS and extensive 1D and 2D NMR spectroscopic studies. Compounds **1-3** were tested for their anti-tobacco

mosaic virus (anti-TMV) activity. The structure elucidation of these compounds and a preliminary evaluation of their anti-TMV are reported in this manuscript.

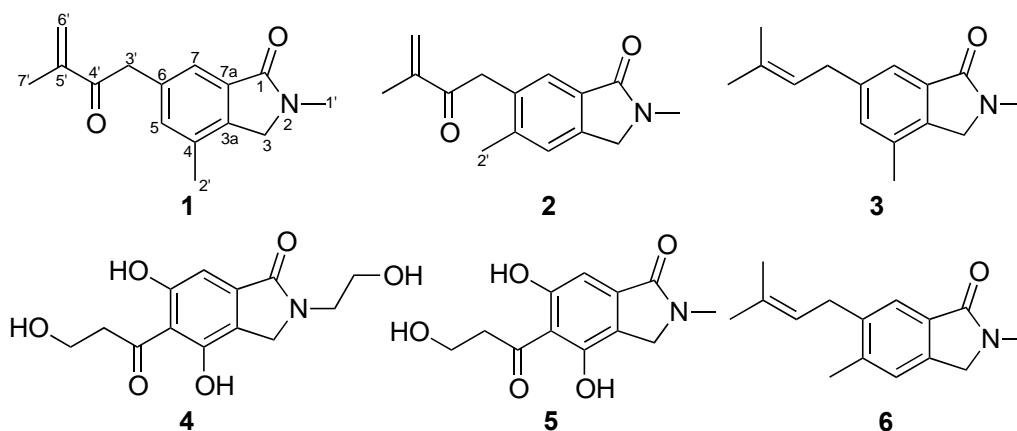


Figure 1. Isoindolin-1-ones from the stems of *N. tabacum*

A 95% aq. ethanol extract prepared from stems of *N. tabacum* was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9.0 with saturated Na_2CO_3 aq., and extracted with EtOAc again. The EtOAc-soluble alkaloid materials were subjected repeatedly to column chromatography and preparative HPLC to afford three new isoindolin-1-ones, 2,4-dimethyl-6-(3-methyl-2-oxobut-3-enyl)isoindolin-1-one (**1**), 2,5-dimethyl-6-(3-methyl-2-oxobut-3-enyl)isoindolin-1-one (**2**) and 2,4-dimethyl-6-(3-methylbut-2-enyl)isoindolin-1-one (**3**), together with three known isoindolin-1-ones (**4-6**). Their structures of the compounds **1-6** were as shown in Figure 1, and the ^1H and ^{13}C NMR data of compounds **1-3** were listed in Table 1. The known compounds, compared with literatures, were identified as 4,6-dihydroxy-2-(2-hydroxyethyl)-5-(3-hydroxy-1-oxopropyl)isoindolin-1-one (**4**),⁹ 4,6-dihydroxy-5-(3-hydroxy-1-oxopropyl)-2-methylisoindolin-1-one (**5**),⁹ and 2,5-dimethyl-6-(3-methylbut-2-enyl)isoindolin-1-one (**6**).¹⁰

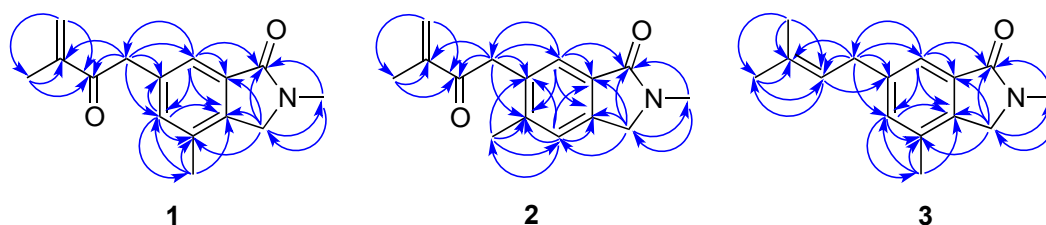


Figure 2. Key HMBC (\curvearrowright) correlations of **1 - 3**

Compound **1** was obtained as a yellow gum. Its positive HRESIMS gave a quasimolecular ion at m/z 266.1150 $[\text{M}+\text{Na}]^+$. These data established the molecular formula of **1** as $\text{C}_{15}\text{H}_{17}\text{NO}_2$, with eight degree of

unsaturations. The IR spectrum of **1** exhibited absorption bands for carbonyl (1688 and 1660 cm^{-1}) and aromatic functionality (1615, 1547, and 1469 cm^{-1}). Its UV spectrum showed the maximum absorption at 308 and 262 nm also supported the existences of aromatic functionality. The ^1H , ^{13}C , and HSQC NMR data (Table 1) of **1** showed resonances due to a 1,2,3,5-tetrasubstituted benzene ring (C-4~C-7, C-3a and C-7a, H-5 and H-7), an acyl carbonyl (C-1), a 3-methyl-2-oxobut-3-enyl group (C-3'~C-7', H₂-3', H₂-6', H₃-7'), a *N*-methylene (C-3, H₂-3), and a *N*-methyl (C-1', H₃-1'). In addition, the benzene ring, acyl carbonyl, and *N*-methylene should be formed a isoindolin-1-one nucleus^{9,10} to meet the six degree of unsaturations, and these deductions were supported by the HMBC correlations (Figure 2) from H₂-3 to C-1, C-3a, C-4, C-7a, from H-7 to C-1, and from H-5 to C-3a.

Since the nucleus and the main substituent groups were determined, the positions of substituent groups can also be determined by the further analysis of its HMBC correlations. The HMBC correlations of H₂-3' with C-5, C-6, C-7, and of H-5 and H-7 with C-3' indicated that the 3-methyl-2-oxobut-3-enyl group was attached to C-6. The location of the methyl group was assigned to C-4 position on the basis of HMBC correlations of the methyl proton signal (H₃-2', δ_{H} 2.22) with C-4, C-5, C-3a, of H-5 with C-2'. Finally, the other methyl group (C-1') linked to nitrogen-atom (N-2) was confirmed by the HMBC correlations of H₃-1' (δ_{H} 2.85) with C-1 and C-3. Therefore, compound **1** was assigned as shown in Figure 1 and given the system name of 2,4-dimethyl-6-(3-methyl-2-oxobut-3-enyl)isoindolin-1-one.

Table 1. ^1H and ^{13}C NMR Data of compounds **1-3** (CDCl_3 , δ in ppm, J in Hz)

No.	Compound 1		Compound 2		Compound 3	
	δ_{C}	δ_{H} (m, J in Hz)	δ_{C}	δ_{H} (m, J in Hz)	δ_{C}	δ_{H} (m, J in Hz)
1	167.8 s		167.4 s		168.0 s	
3	42.6 t	4.20 s	45.0 t	4.25 s	44.2 t	4.23 s
3a	134.6 s		142.3 s		132.7 s	
4	136.9 s		128.4 d	6.70 s	135.4 s	
5	133.4 d	6.86 d (2.2)	138.1 s		132.1 d	6.87 d (2.2)
6	135.6 s		134.2 s		138.1 s	
7	122.2 d	7.41 d (2.2)	130.6 d	7.44 s	121.9 d	7.39 d (2.2)
7a	132.0 s		124.0 s		130.8 s	
1'	33.6 q	2.85 s	33.0 q	2.84 s	33.6 q	2.86 s
2'	20.2 q	2.12 s	19.7 q	2.10 s	20.4 q	2.17 s
3'	43.5 t	4.57 s	37.4 t	4.45 s	32.6 t	3.42 d (6.8)
4'	200.7 s		201.3 s		123.8 d	5.35 t (6.8)
5'	144.3 s		144.7 s		133.9 s	
6'	123.4 t	6.22, 5.88 s	122.9 t	6.20, 5.91 s	16.9 q	1.54 s
7'	17.6 q	1.98 s	18.4 q	1.95 s	25.8 q	1.79 s

2,5-Dimethyl-6-(3-methyl-2-oxobut-3-enyl)isoindolin-1-one (**2**) was also obtained as pale yellow gum, and showed quasi molecular ion at m/z 266.1163 $[M+Na]^+$ in the HRESIMS (calcd m/z 266.1157 for $C_{15}H_{17}NNaO_2$), corresponding to the molecular formula of $C_{15}H_{17}NO_2$. The 1H and ^{13}C NMR spectra of **2** were highly similar to those of **1**. The obvious chemical shift differences resulted from the proton signals on benzene ring. The typical signals (6.86 (d) 2.2 and 7.41 (d) 2.2) in **1** replaced by (6.70 s and 7.44 s) in **2** indicated that the substituent positions had been varied on benzene ring, and **2** should be a 2,5,6-disubstituted isoindolin-1-one. In addition, the 3-methyl-2-oxobut-3-enyl group attached to C-6 and two methyl groups attached C-5 and N-2 were also be supported by the HMBC correlations from $H_{2-3'}$ with C-5, C-6, C-7, from $H_{3-1'}$ to C-1 and C-3, and from $H_{3-2'}$ to C-4, C-5, and C-6, respectively. Thus, the structure of **2** was established as shown.

Compound **3**, a yellow gum, which showed a quasi-molecular ion at m/z 252.1368 $[M+Na]^+$ in the HRESIMS (calcd m/z 252.1364 for $C_{15}H_{19}NNaO$), corresponding to the molecular formula $C_{15}H_{19}NO$. Its 1H and ^{13}C NMR spectroscopic data were also similar to those of **1**, which suggested that compound **3** was structurally related to **1**. The marked differences between them were due to the disappearance of a 3-methyl-2-oxobut-3-enyl group signals, and appearance of a prenyl signal (C-3'~C-7', $H_{2-3'}$, $H_{4-4'}$, $H_{3-6'}$, and $H_{3-7'}$). This indicated that the 3-methyl-2-oxobut-3-enyl group in **1** was substituted by a prenyl group in **3**. The 2D HMBC data of two or three-bond correlations from $H_{2-3'}$ to C-5, C-6, C-7, and from H-5 and H-7 to C-3' revealed that the prenyl group located at C-6. In addition, the positions of the other substituent can also be determination by further analysis of its HMBC correlations. The structure of 2,4-dimethyl-6-(3-methylbut-2-enyl)isoindolin-1-one (**2**) was therefore defined as shown.

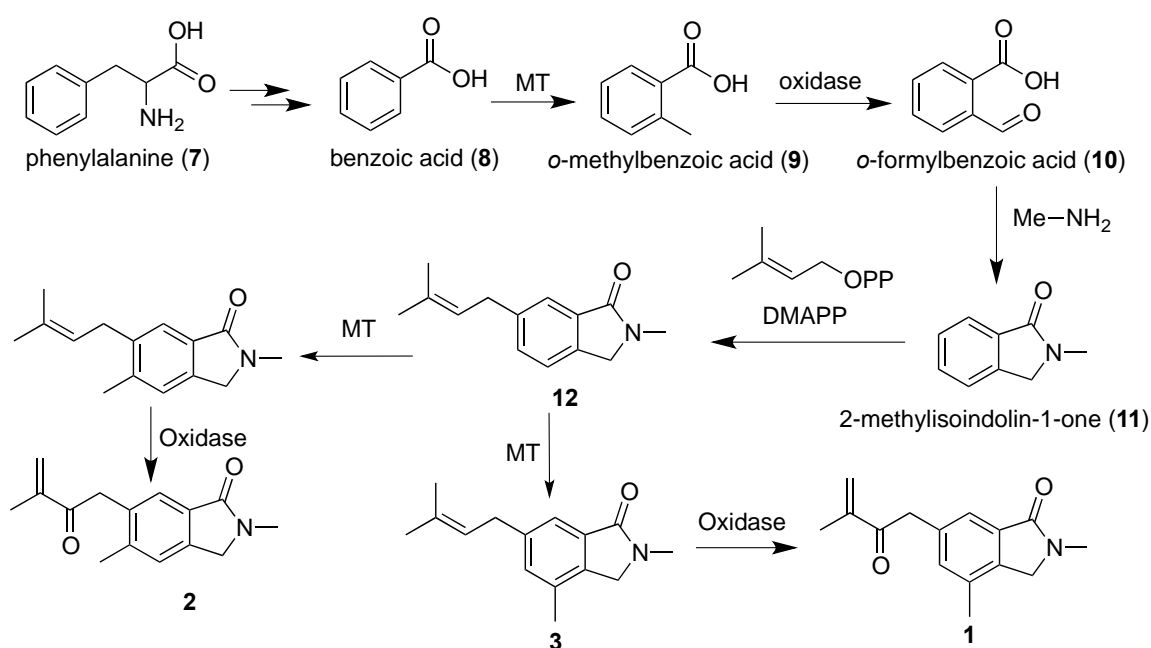


Figure 3. Hypothetical biogenetic pathway of **1** - **3**

The possible biogenetic pathway of **1-3** is proposed as shown in Figure 3. In plants, benzoic acid (**8**) is biosynthesized from phenylalanine (**7**) metabolic pathway. Benzoic acid could be methylated into *o*-methylbenzoic acid (**9**) by methyltransferase (MT), and subsequently could be oxidized into *o*-formylbenzoic acid (**10**) by oxidase, which reacts with methanamine to give 2-methylisoindolin-1-one (**11**). Compound **11** reacts with dimethylallyl pyrophosphate (DMAPP) to afford key presumed intermediate (**12**). Finally, compound **12** is transformed into isoindoline derivatives **1-3** by methyltransferase and/or oxidase.

Since certain of the chromone derivatives from *Nicotiana tabacum* exhibited potential anti-TMV activity,^{9,10} compounds **1-3** 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.^{19,20} The results revealed that compounds **1-3** showed anti-TMV activities with inhibition rates of 42.6, 46.7, and 38.5% at the concentration of 20 μM , respectively. These rates are higher than that of positive control.

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 \times 25 cm, 7 μm) column or a Venusil MP C₁₈ (20 mm \times 25 cm, 5 μm) column. Column chromatography was performed with Si 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% H₂SO₄ in EtOH.

Plant Material. The tobacco (YNZY-1-12, a gene editing mutant with alkaloid metabolic pathway, which cultivated by Yunnan China Tobacco Industry Co., Ltd) was planted in Greenhouse in Shilin County, Kunming, Yunnan Province. After the leaves had been picked at the mature stage, the stems had been collected as samples for phytochemical study. A voucher specimen (Ynni-19-10-018) has been deposited in Key Laboratory of Chemistry in Ethnic Medicinal Resources, Yunnan Minzu University, P. R. China.

Extraction and Isolation. The air dried tobacco stems (5.2 kg) were crushed to 30 mesh, and extracted with 95% aq. EtOH, and the extract was partitioned between EtOAc and 3% tartaric acid. The aqueous layer was adjusted to pH 9 with saturated Na₂CO₃ aq., and extracted with EtOAc again. The EtOAc-soluble alkaloid materials (68.4 g) were applied to silica gel (200–300 mesh) column

chromatography, eluting with CHCl₃/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 B (9:1, 7.68 g) by silica gel column chromatography, eluted with CHCl₃/Me₂CO (9:1-2:1), yielded sub-fractions B1–B7. Sub-fraction B1 (9:1, 2.42 g) was subjected to silica gel column chromatography using petroleum ether/acetone, and then semi-preparative HPLC (68% MeOH/H₂O, flow rate 20 mL/min) to give **1** (8.6 mg), **2** (11.8 mg), **3** (10.2 mg), and **6** (14.6 mg). Sub-fraction B3 (7:3, 4.26 g) was subjected to another silica gel column chromatography using petroleum ether/acetone, and then semi-preparative HPLC (68% MeOH/H₂O, flow rate 20 mL/min) to give **4** (6.2 mg) and **5** (8.6 mg).

Anti-TMV Assays. The anti-TMV activities were tested using the half-leaf method,^{19,20} and ningnanmycin (2% water solution), a commercial product for plant disease in China, was used as a 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 *Nicotiana 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.

2,4-Dimethyl-6-(3-methyl-2-oxobut-3-enyl)isoindolin-1-one (1): Obtained as yellow gum; UV (MeOH) λ_{max} (log ϵ) 212 (4.32), 262 (3.65), and 308 (3.28) nm; IR (KBr) ν_{max} 3086, 2925, 1688, 1660, 1615, 1547, 1469, 1354, 1252, 1146, 1058, 940, and 864 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; positive ESIMS m/z 266 [M+Na]⁺, positive HRESIMS m/z 266.1150 [M+Na]⁺ (calcd for C₁₅H₁₇NNaO₂, 266.1157).

2,5-Dimethyl-6-(3-methyl-2-oxobut-3-enyl)isoindolin-1-one (2): Obtained as yellow gum; UV (MeOH) λ_{max} (log ϵ) 212 (4.20), 260 (3.65), and 305 (3.18) nm; IR (KBr) ν_{max} 3075, 2936, 1690, 1662, 1612, 1560, 1462, 1347, 1258, 1162, 1053, 952, and 849 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; positive ESIMS m/z 266 [M+Na]⁺, positive HRESIMS m/z 266.1163 [M+Na]⁺ (calcd for C₁₅H₁₇NNaO₂, 266.1157).

2,4-Dimethyl-6-(3-methylbut-2-enyl)isoindolin-1-one (3): Obtained as yellow gum; UV (MeOH) λ_{max} (log ϵ) 212 (4.15), 256 (3.65), and 294 (3.32) nm; IR (KBr) ν_{max} 3086, 2938, 1668, 1550, 1461, 1349, 1233, 1161, 1064, 830, and 758 cm⁻¹; ¹H and ¹³C NMR data, see Table 1; positive ESIMS m/z 252 [M+Na]⁺, positive HRESIMS m/z 252.1368 (calcd for C₁₅H₁₉NNaO, 252.1364).

ACKNOWLEDGEMENTS

This work was supported financially by the Foundation of Yunnan basic research program (2019FD086),

the National Natural Science Foundation of China (No. 21967021), and the Foundation of Yunnan Tobacco Industry Co. Ltd (2018JC06, 2018JC07).

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