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Biphenyls from *Nicotiana tabacum* and their anti-tobacco mosaic virus

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ABSTRACT

Five new biphenyls, tababiphenyls A–E (**1**–**5**), together with five known ones (**5**–**10**), were isolated from the leaves of *Nicotiana tabacum*, of which compound **1** possessed a seldom reported 6-carbons unit in biphenyls. Their structures were established on the basis of extensive spectroscopic analyses. All compounds were tested for their anti-tobacco mosaic virus (anti-TMV) activities. The results showed that compounds **3** and **5** exhibited high anti-TMV activities with inhibition rate of 48.4% and 32.1%, respectively, which were higher than that of positive control (ningnanmycin). The other compounds also showed potential anti-TMV activities with inhibition rates in the range of 18.6–28.7%, 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 [1,2]. In addition, *N. tabacum* is also used as insecticides, anesthetics, diaphoretics, sedatives, and emetic agents in Chinese folklore medicines because it contains many useful chemical compounds [1,3]. Previous investigation of this species led to the discovery of a number of new compounds by our groups, which were found to exhibit various bioactivities, such as anti-HIV-1, anti-TMV, and cytotoxicity [4–9]. In continuing efforts to utilize *N. tabacum* and identify bioactive natural products, the phytochemical investigation of the leaves of Honghua Dajinyuan (a variety of *N. tabacum*) led to the isolation of five new (**1**–**5**) and five known (**6**–**10**) biphenyls, of which compound **1** possessed a seldom reported 6-carbons unit (C-7, C-8, C-9, C-10, C-11, C-12) in biphenyls. This paper deals with the isolation, structural elucidation, and anti-TMV activity of these compounds (Fig. 1).

2. Experimental

2.1. General experimental procedures

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

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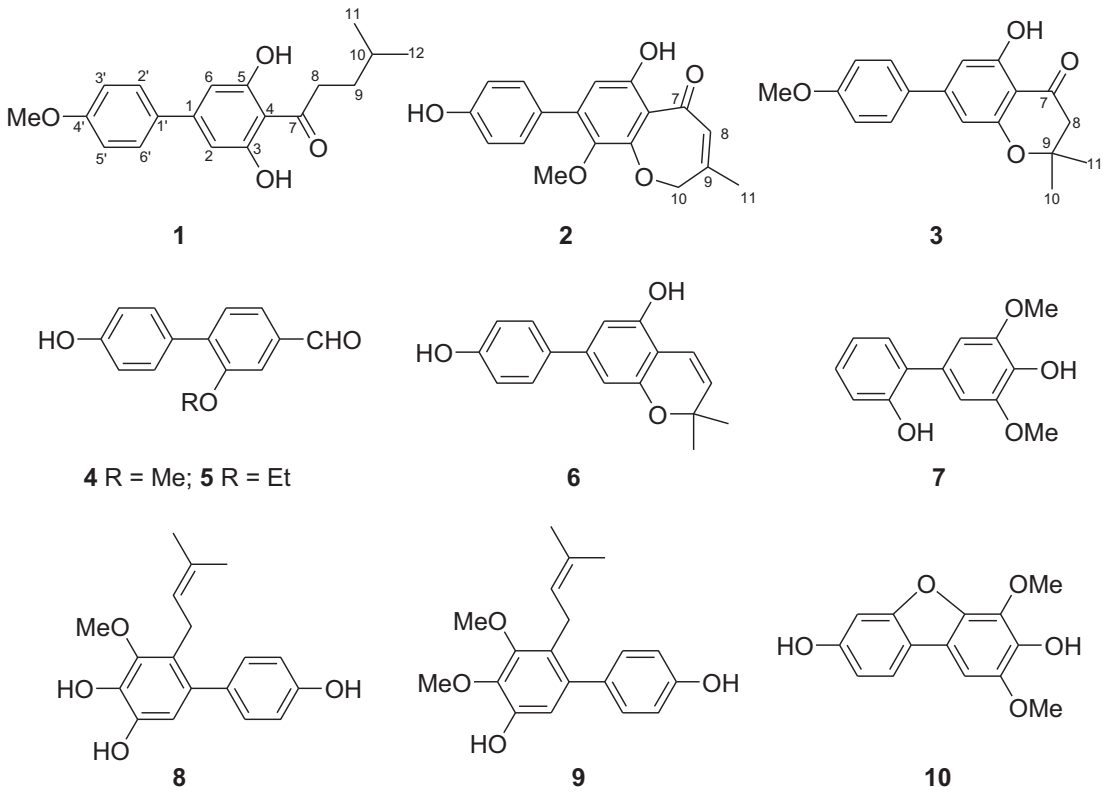

Fig. 1. The structures of compounds **1**–**10**.

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.

2.2. Plant material

The variety of *Nicotiana tabacum* L studied is Honghuadajinyuan. Its leaves were collected from Yuxi County, Yunnan Province, PR China, in September 2011.

2.3. Extraction and isolation

The plant material of *N. tabacum* (5.0 kg) was ground and exhaustively extracted with Me_2CO-H_2O (V/V = 7:3, 3 × 15 L) at room temperature. The solvent was evaporated in vacuo, and the crude extract was dissolved in H_2O and partitioned with EtOAc. The EtOAc portion (165 g) was chromatographed on a silica gel column (200–300 mesh, 15 × 120 cm, 1.2 kg), eluting with a $CHCl_3$–MeOH gradient system (20:1, 9:1, 8:2, 7:3, 6:4, 5:5, and 0:1), to give seven fractions A–G. Fraction B (18.7 g) was decolorized by MCI gel (8 × 50 cm) firstly, and then further separation of fraction B by silica gel column chromatography (200–300 mesh, 8 × 50 cm), eluted with $CHCl_3$/$(CH_3)_2CO$ (9:1–2:1), yielded mixtures B1–B6. B2 (2.1 g) were repeatedly chromatographed on silica gel (a, 200–300 mesh, 3 × 35 cm, petroleum ether–Me_2CO, 12:1, 9:1, 6:1, and 2:1, each 0.9 L; b, 200–300 mesh, 1.5 × 35 cm, $CHCl_3$–Me_2CO, 30:1, 20:1, 15:1, 10:1, each 0.6 L) and semi-preparative HPLC (45% MeOH–H_2O, flow rate 12 mL/min) to yield **1** (9.0 mg), **4** (6.5 mg), **5** (7.2 mg), and **7** (10.0 mg). Fraction B-3 (2.8 g) were chromatographed on silica gel (a, 200–300 mesh, 3 × 35 cm, petroleum ether–Me_2CO, 10:1, 8:1, 4:1, and 2:1, each 1.2 L; b, 200–300 mesh, 1.5 × 35 cm, $CHCl_3$/Me_2CO, 30:1, 20:1, 12:1, 6:1, each 0.8 L), further over an semi-preparative HPLC column (40% MeOH/H_2O, flow rate 12 mL/min) to yield **2** (3.5 mg), **3** (4.9 mg), **6** (15.0 mg), **8** (2.0 mg) and **9** (13.6 mg). Fraction B3 (5.4 g) was chromatographed on a silica gel column (200–300 mesh, 4 × 50 cm), eluted with $CHCl_3$–Me_2CO (20:1, 15:1, 9:1, 6:1 and 2:1, each 1.5 L), followed by semi-preparative HPLC (40% MeOH-H2O) to yield **10** (11.6 mg).

Tababiphenyl A (**1**): yellow gum; UV (MeOH) λ_{max} (log ε) 212 (4.27), 268 (3.83), 309 (3.70) nm; IR (KBr) ν_{max} 3418, 2931, 2839, 1668, 1605, 1582, 1468, 1357, 1183, 1067, 976, 827 cm^{-1}; ^1H and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz); see Table 1. Positive ESIMS *m/z* 337 $[M + Na]^+$; positive HRESIMS *m/z* 337.1411 $[M + Na]^+$ (calcd for $C_{19}H_{22}NaO_4$, 337.1416).

Tababiphenyl B (**2**): yellow gum; UV (MeOH) λ_{max} (log ε) 210 (4.38), 280 (3.78), 335 (3.26) nm; IR (KBr) ν_{max} 3423, 2925, 2876, 1639, 1604, 1563, 1459, 1387, 1201, 1147, 985, 836 cm^{-1}; ^1H and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz); see Table 1. Positive ESIMS *m/z* 335 $[M + Na]^+$; positive HRESIMS *m/z* 335.0899 $[M + Na]^+$ (calcd for $C_{18}H_{16}NaO_5$, 335.0895).

Tababiphenyl C (**3**): yellow gum; UV (MeOH) λ_{max} (log ε) 213 (4.26), 267 (3.82), 332 (3.38) nm; IR (KBr) ν_{max} 3430,

Table 1
^{13}C NMR and ^{1}H NMR spectroscopic assignments of compounds **1**–**3**[a].

No.	**1**		**2**		**3**	
	δ_C	δ_H (m, *J*, Hz)	δ_C	δ_H (m, *J*, Hz)	δ_C	δ_H (m, *J*, Hz)
1	144.8 s		133.1 s		144.2 s	
2	107.2 d	6.54 s	143.0 s		108.2 d	6.49 s
3	162.1 s		154.1 s		155.9 s	
4	112.0 s		113.8 s		112.3 s	
5	162.1 s		156.0 s		161.8 s	
6	107.2 d	6.54 s	107.2 d	6.40 s	109.7 d	6.61 s
7	204.8 s		193.4 s		192.5 s	
8	41.3 t	2.97 (t) 7.1	128.0 d	6.51 s	48.2 t	2.55 s
9	32.0 t	1.69, m	153.9 s		80.9 s	
10	27.8 d	1.86, m	72.0 t	4.42 s	20.1 q	1.52 q
11,12	22.0 q	0.98 (d) 6.8	22.1 q	2.01 s		
1′	132.9 s		128.7 s		130.8 s	
2′,6′	130.7 d	7.66 (d) 8.6	131.0 d	7.62 (d) 8.8	131.6 d	7.65 (d) 8.8
3′,5′	116.9 d	6.84 (d) 8.6	116.4 d	6.83 (d) 8.8	116.0 d	6.86 (d) 8.8
4′	160.3 s		158.0 s		160.8 s	
2-OMe			61.0 q	3.79 s		3.93 s
4′-OMe	55.9 q	3.82 s			55.9 q	
Ar-OH		9.23 s		9.71 s		10.53 s

[a] Spectra of **1**–**3** were recorded in $CDCl_3$, and all chemical shifts (δ) were in ppm.

2923, 2872, 1675, 1604, 1542, 1480, 1435, 1350, 1138, 962, 871 cm^{-1}; ^{1}H and ^{13}C NMR data ($CDCl_3$, 500 and 125 MHz); see Table 1. Positive ESIMS *m/z* 321 $[M + Na]^+$; positive HRESIMS *m/z* 321.1106 $[M + Na]^+$ (calcd for $C_{18}H_{18}NaO_4$, 321.1103).

Tababiphenyl D **(4):** white powder; UV (MeOH) λ_{max} (log ε): 215 (4.13), 272 (3.86), 318 (2.87) nm; IR (KBr) ν_{max}: 3378, 2916, 2855, 1712, 1604, 1527, 1439, 1382, 1320, 1256, 1162, 1058, 895, 763 cm^{-1}; ^{1}H and ^{13}C NMR (($CDCl_3$, 500 and 125 MHz)); see Table 2; Positive ESIMS *m/z* 251 $[M + Na]^+$; positive HRESIMS *m/z* 251.0680 $[M + Na]^+$ (calcd $C_{14}H_{12}NaO_3$ for 251.0684).

Tababiphenyl E **(5)**: white powder; UV (MeOH) λ_{max} (log ε): 210 (4.38), 275 (3.81), 315 (2.86) nm; IR (KBr) ν_{max}: 3372, 2918, 2857, 1710, 1606, 1520, 1443, 1377, 1325, 1258, 1169, 1054, 892, 768 cm^{-1}; ^{1}H and ^{13}C NMR (($CDCl_3$, 500 and 125 MHz)); see Table 2; positive ESIMS *m/z* 265 $[M + Na]^+$; positive HRESIMS *m/z* 265.0846 $[M + Na]^+$ (calcd $C_{15}H_{14}NaO_3$ for 265.0841).

2.4. Anti-MTV assay

TMV (U1 strain) was obtained from the Key Laboratory of Tobacco Chemistry of Yunnan Province, Yunnan Academy of Tobacco Science, PR China. The virus was multiplied in Nicotiana tabacum cv. K326 and purified as described [10]. The concentration of TMV was determined as 20 mg/mL with a UV spectrophotometer [virus concentration = (A_{260} × dilution ratio) / $E_{1\,m}^{0.1\,\%,260nm}$]. 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_2O to the required concentrations. A solution of equal concentration of DMSO was used as a negative control. The commercial antiviral agent ningnanmycin was used as a positive control.

For the half-leaf method [11], 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 or 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. Results and discussion

Powdered leaves and stems of *N. tabacum* were extracted with 70% aqueous acetone. The filtrate was concentrated and partitioned between H_2O and EtOAc. The EtOAc fraction was

Table 2
^{1}H NMR and ^{13}C NMR Assignments of Compounds **4** and **5**[a].

No.	4		5	
	δ_C	δ_H (m, *J*, Hz)	δ_C	δ_H (m, *J*, Hz)
1	129.7 s		129.8 s	
2	157.8 s		155.6 s	
3	114.3 d	6.76 (d) 1.8	114.3 d	6.71 (d) 1.8
4	136.9 s		136.3 s	
5	121.4 d	7.02 (dd) 1.8, 8.2	122.1 d	7.06 (dd) 1.8, 8.2
6	127.6 d	7.41 (d) 8.2	127.4 d	7.42 (d) 8.2
7	191.1 d	9.80 s	191.0 d	9.81 s
1′	128.4 s		129.0 s	
2′,6′	131.0 d	7.63 (d) 8.8	130.6 d	7.65 (d) 8.8
3′,5′	116.5 d	6.89 (d) 8.8	116.1 d	6.84 (d) 8.8
4′	158.3 s		158.5 s	
1″	56.1 q	3.93 s	64.7 t	4.18 m
2″			14.7 q	1.46 (t) 7.2
Ar-OH	10.57 s			10.53 s

[a] Spectra of **4** and **5** were recorded in CD_3Cl.

dried under reduced pressure, and then submitted to silica gel, MCI, RP-18 gel column chromatography (CC), and semi-preparative HPLC to yield five new compounds (**1**–**5**) and five known ones. The ^{1}H and ^{13}C NMR spectroscopic data of **1**–**5** are listed (Tables 1, 2)

Tababiphenyl A (**1**) was obtained as yellow gum and its molecular formula was determined to be $C_{19}H_{22}O_4$, by HREIMS experiment (*m/z* 337.1411 $[M + Na]^+$), requiring nine degrees of unsaturation. The IR spectrum showed absorption bands of hydroxy (3418 cm^{-1}), carbonyl (1668 cm^{-1}), and aromatic (1605, 1582, and 1468 cm^{-1}) groups. The ^{1}H NMR data (Table 1) displayed characteristic signals for a 1,4-disubstituted benzene ring [δ_H 7.66 (2H, d, J = 8.6 Hz, H-2′,6′), 6.84 (2H, d, J = 8.6 Hz, H-3',5')], a symmetrically 1,3,4,5-tetrasubstituted benzene ring [δ_H 6.54 (2H, s, H-2,6), one methoxyl group [δ_H 3.82 (3H, s, 4'-OMe)], and two methyl groups [δ_H 0.98 (6H, d, J = 6.8 Hz, H-11,12)]. The ^{13}C NMR and DEPT data (Table 1) further supported the presence of the characteristic signals. In addition, NMR signals for two methylenes (δ_C 41.2 and 32.0), one methine (δ_C 27.8) and one carbonyl carbon (δ_C 204.8), were observed. The two methylenes [δ_H 2.97 (2H, t, J = 7.1 Hz, H-8), 1.69 (2H, m, H-9)], one methines [δ_H 1.86 (1H, m, H-10)], and two methyls [δ_H 0.98 (6H, d, J = 6.8 Hz, H-11,12)] were ascribed to an isopentene skeleton group by ^{1}H–^{1}H COSY (Fig. 2) and HSQC spectra. The isopentene skeleton group was linked to C-4 in benzene ring by carbonyl carbon (C-7) on the basis of the HMBC correlations from H-8 to C-7 and C-4. Besides, the HMBC correlations from hydroxyl hydrogen (C-3) to C-3, C-2, and C-4, from hydroxyl hydrogen (C-5) to C-4, C-5, and C-6, showed that two hydroxyl groups were located at C-3 and C-5, respectively (Fig. 2). Besides, the HMBC correlation from the 4′-OMe to C-4′ and the ^{1}H–^{1}H COSY correlations between H-2′ and H-3′, H-4′ and H-5′ indicated that the methoxyl group was lacated at C-4′ (Fig. 2). The two benzene rings were linked by C-1 and C-1′, which were determined by the HMBC correlations from H-2′ and H-2 to C-1′, C-1, respectively (Fig. 2).

Tababiphenyl B (**2**) was obtained as yellow gum and it was assigned the molecular formula of $C_{18}H_{16}O_5$ from the molecular ion peak at *m/z* 335.0899 $[M + Na]^+$ in positive HRESIMS. The IR spectrum indicated that **1** possessed hydroxy (3423 cm^{-1}), conjugated carbonyl (1639 cm^{-1}), and aromatic (1604, 1563, and 1459 cm^{-1}) functional groups. The ^{1}H NMR data (Table 1) displayed signals for a 1,4-disubstituted benzene ring [δ_H 7.62 (2H, d, J = 8.8 Hz, H-2′,6′), 6.83 (2H, d, J = 8.6 Hz, H-3′,5′)], two allyl protons [δ_H 6.40 (1H, s, H-6), 6.51 (1H, s, H-6)], one methoxyl group [δ_H 3.79 (3H, s, 2-OMe)], one methyl groups [δ_H 2.01 (3H, s, H-11)], one oxygenated methylene group [δ_H 4.42 (2H, s, H-10)] and two hydroxyl group [δ_H 9.71 (1H, s, H-5), 5.02 (1H, s, H-4′)]. The ^{13}C NMR and DEPT spectra (Table 1) exhibited the fifteen sp2 carbon groups (six methines, and nine quaternary carbons including one carbonyl carbon), two methyl (one methoxyl), and one oxygenated methylene (Table 2). Analyses of ^{1}H–^{1}H COSY, HSQC, and HMBC spectra suggested that it was a biphenyl derivatives. HMBC correlations (Fig. 2) from H-10 and H-11 to C-9, C-6, from H-8 to C-7, C-9, C-10, and C-11 suggested the presence of the isopentene skeleton fragment, which was linked to C-4 and C-3 by C-7 and oxygen atom according to the HMBC correlation from H-8 to C-4, from H-10 to C-3, respectively. The methoxy group was located at C-2 on the basis of the HMBC correlation from 2-OMe to C-2, and C-5 was hydroxylated deduced from the HMBC correlations from hydroxyl hydrogen to C-5, C-6, and C-4. The ^{1}H–^{1}H COSY correlations between H-2′ and H-3′, H-4′ and H-5′, combined with the chemical shift of C-4′, indicated that one hydroxyl group was lacated at C-4′. The two benzene rings were linked by C-1 and C-1′, which were determined by the HMBC correlations from H-2′ and H-6 to C-1′, C-1, respectively (Fig. 2).

Tababiphenyl C (**3**) was obtained as yellow gum and it gave an $[M + Na]^+$ peak at *m/z* 321.1106 in the HRESIMS, consistent with a molecular formula of $C_{18}H_{18}O_4$. The IR spectrum exhibited absorptions of hydroxy (3430 cm^{-1}) and aromatic (1604, 1563, and 1459 cm^{-1}) groups. The data of ^{1}H NMR were assigned to that of ^{13}C NMR with the help of HSQC spectrum (Table 1), and the NMR data displayed signals for all 18 carbons and 17 protons, suggesting the presence of a 1,4-disubstituted benzene ring and a 1,3,4,5-tetrasubstituted benzene, a methoxy group (δ_C 55.9, δ_H 3.93). Analyses of the ^{1}H–^{1}H COSY, HSQC, and

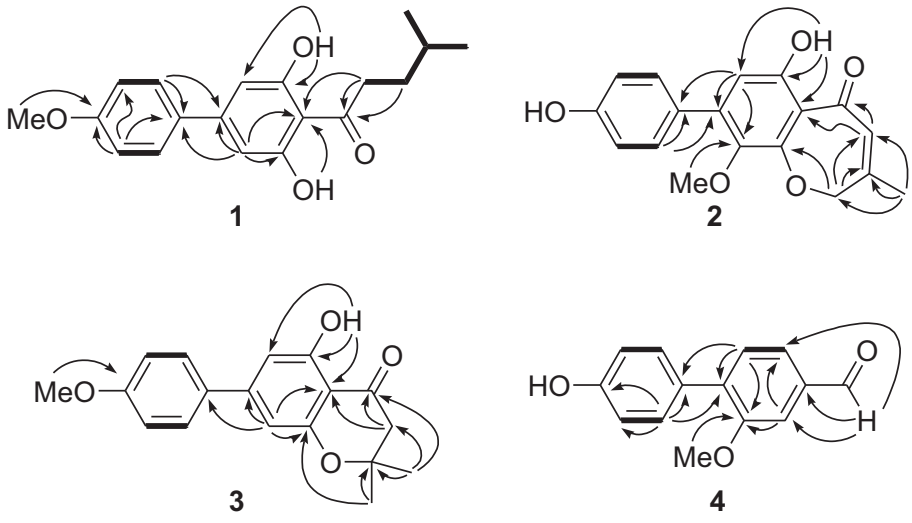

Fig. 2. Key HMBC (H → C) and ^{1}H–^{1}H COSY () correlations of **1**–**4**.

Table 3
TMV infection inhibition activities of compounds **1**–**10**.

Compounds	Inhibition rates (%)	Compounds	Inhibition rates (%)
1	22.5 ± 2.8	7	20.8 ± 2.4
2	25.6 ± 3.0	8	18.8 ± 2.5
3	48.4 ± 3.4	9	24.8 ± 2.6
4	28.7 ± 2.6	10	18.6 ± 2.3
5	32.1 ± 2.9	Ningnamycin	31.5 ± 3.0
6	26.8 ± 2.1		

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

HMBC spectra suggested that **3** had a biphenyl skeleton and was a derivative of clusiparalicoline C (**6**) [12]. The major differences between them were the additional existence of a methoxy group, a carbonyl group and a methylene but the disappearance of di-substituted double bonds (CH═CH) in **3**, which may be attributed to the changes in C-7, C-8 and C-4′. The deductions were further verified by the key HMBC correlations from H-8 to C-7, C-4, C-9, C-10 and C-11, from 4′-OMe to C-4′. Thus, the structure of **3** was determined.

Tababiphenyl D (**4**) was obtained as yellow gum and assigned a molecular formula of $C_{14}H_{12}O_3$ as supported by the HRESIMS (*m/z* 251.0680 $[M + Na]^+$), corresponding to nine degrees of unsaturation. Strong absorption bands accounting for hydroxy (3378 cm^{-1}) and aromatic groups (1604, 1527, and 1439 cm^{-1}) were observed in the IR spectrum. The ^1H NMR data (Table 1) exhibited signals for one AA′BB′-aromatic system at δ_H 7.63 (2H, d, $J = 8.8$ Hz, H-2′,6′), 6.89 (2H dd, J = 8.8 Hz, H-3′,5′), one ABX-aromatic system at δ_H 6.76 (1H, d, $J =$ 1.8 Hz, H-3), 7.41 (1H d, $J = 8.2$ Hz, H-6), 7.02 (1H, dd, J = 1.8, 8.2 Hz, H-5), one aldehyde signal at δH 9.89 (1H, s), one methoxy group at δ_H 3.93 (3H, s), and a hydroxy group at δ_H 10.57 (1H, s) (Table 2). The ^{13}C NMR and DEPT spectra exhibited fourteen carbon signals, including twelve sp^2 carbons (seven methines and five quaternary carbons) indicative of the presence of two benzene rings, one aldehyde carbon, and one methoxy group (Table 2). Analyses of ^1H–^1H COSY, HSQC, HMBC, and ROESY spectra suggested that it had a biphenyl skeleton. The hydroxyl group was deduced to be located at C-4′ by the ^1H–^1H COSY correlations (H-2′/H-3′ and H-5′/H-6′) and the HMBC correlations from 4′-OH to C-4′ (Fig. 2). The HMBC correlations of H-6 with C-1 and C-1′ and of H-2′ with C-1 and C-1′ revealed that the two benzene rings joined together through the band between C-1 and C-1′. The HMBC correlations from H-7 to C-3, C-4, and C-5 and the ^1H–^1H COSY correlation between H-5 and H-6 indicated that the aldehyde group was attached to C-4. Thus, the structure of **4** was established.

Tababiphenyl E (**5**) was obtained as yellow gum and had the molecular formula $C_{15}H_{14}O_3$ as revealed by its HRESIMS at m/z 265.0846 $[M + Na]^+$ (calcd $C_{15}H_{14}NaO_3$ for 265.0841). The NMR spectra of **5** were almost identical to that of **4**. The only difference was that the substituent group at C-2 changed from methoxy group to oxyethyl group, which was further confirmed by 2D NMR correlations.

The known compounds were identified as clusiparalicoline C (**6**) [12], 2′-hydroxyaucuparin (**7**) [13], doitungbiphenyl A (**8**) [14], doitungbiphenyl B (**9**) [14], and δ-cotonefuran (**10**) [13].

Compounds **1**–**10** were tested for their anti-TMV activities. The inhibitory activities of compounds **1**–**10** against TMV replication were tested using the half-leaf method [11,15]. Ningnanmycin, a commercial product for plant disease in China, was used as a positive control. The antiviral inhibition rates of compounds **1**–**10** at the concentration of 20 μM were listed Table 3. The results showed that compounds **3** and **5** exhibited high anti-TMV activity with inhibition rate of 48.4% and 32.1%, respectively, which were higher than that of positive control (ningnanmycin). The other compounds also showed potential anti-TMV activity with inhibition rates in the range of 18.6–28.7%, respectively.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.fitote.2014.08.013.

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