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Eudesmanolides from *Wedelia trilobata* (L.) Hitchc. as Potential Inducers of Plant Systemic Acquired Resistance

Yating Li,[†,‡] Xiaojiang Hao,[†] Shifei Li,[†] Hongping He,[†] Xiaohui Yan,[§] Yongdui Chen,[#] Jiahong Dong,[#] Zhongkai Zhang,[#] and Shunlin Li*[,†]

[†]State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, Kunming 650201, Yunnan, China

[‡]University of Chinese Academy of Sciences, Beijing 100049, China

[§]Key Laboratory of Forest Disaster Warning and Control of Yunnan Province, College of Forestry, Southwest Forestry University, Kunming 650224, Yunnan, China

[#]Yunnan Academy of Agricultural Sciences, Kunming 650023, Yunnan, China

Supporting Information

ABSTRACT: Ten eudesmanolides (**1**−**10**) including five new ones (**1**−**4** and **6**) were isolated from the whole plant of *Wedelia trilobata* (L.) Hitchc., a notoriously invasive weed in South China. As main constituents of *W. trilobata*, eight of these eudesmanolides were tested for their antitobacco mosaic virus (TMV) activities by the conventional half-leaf and leaf-disk method along with Western blot analysis. All of the tested compounds, at 10 μg/mL, showed strong antiviral activities in the pretreated tobacco plants with inhibition rates ranging from 46.7% to 76.5%, significantly higher than that of the positive control, ningnanmycin (13.5%). Their potential of inducing systemic acquired resistance (SAR) was also evaluated, and compounds **1** and **8** showed excellent induction activities. Furthermore, it was found that different concentrations of compound **1** promoted phenylalanine ammonia-lyase (PAL) activity in tobacco plants. To our knowledge, this is the first report that eudesmanolides could induce resistance in tobacco plants against the viral pathogen TMV.

KEYWORDS: *eudesmanolides, Wedelia trilobata (L.) Hitchc., tobacco mosaic virus, systemic acquired resistance*

INTRODUCTION

The plant disease caused by tobacco mosaic virus (TMV) is found worldwide. TMV is known to infect members of 65 plant families, and at least 885 individual species, and cause dramatic losses in agriculture and horticulture all over the world.[1] Traditional antiviral agents play a critical role in integrated disease management by killing or controlling target virus directly, sometimes causing adverse effects to the environment, and often leading to antiviral resistance as well. Therefore, it is necessary to discover new environmentally benign methods for plant protections.[2,3] Localized treatment of plants with specific biotic or abiotic agents can result in the development of enhanced resistance against pathogens in the whole plant. Resistance induced by such treatments is generally referred to as systemic acquired resistance (SAR).[4] The identification of new chemicals capable of inducing SAR was considered useful for developing new plant protection agents.[5]

In finding an effective way to protect plants from TMV infection, natural products from plants have been proved to be a rich resource. A series of natural products from plants with anti-TMV activities have been reported by our group since 2007, such as secopregnane steroids,[6] cinchnaglycoside C,[7] 7-deoxy-*trans*-dihydronarciclasine,[8] 3-acetonyl-3-hydroxyoxindole,[5] β-carbolines,[9] quassinoids,[10] and limonoids.[11] In our ongoing studies to discover anti-TMV active compounds from plants, *Wedelia trilobata* (L.) Hitchc. has attracted our attention. *W. trilobata* is a notoriously invasive weed in wide tropical and subtropical areas.[12] In southern China, this creeping, mat-forming perennial herb has caused significant damage to farmlands, forests, and orchards.[13,14] Studies have shown that *W. trilobata* has a strong allelopathic potential on neighboring native plants.[15,16] The major chemical constituents of *W. trilobata* are *ent*-kaurane diterpenes, sesquiterpene lactones, and triterpenes with a variety of biological activities, such as antibacterial, antitumor, hepatoprotective, and central nervous system depressant activity.[17] To date, most of the studies have been focused on anticancer activity of sesquiterpene lactones in medicinal formulation. However, relatively little is known about the antiviral activity in plant protection for these kinds of compounds.

Chen et al.[18] reported that the ethanolic extracts from *Wedelia chinensis* significantly suppressed TMV replication in the infected leaves. It is also found that parthenolide, one of the sesquiterpene lactones, isolated from *Parthenium hysterophorus* showed significant inhibition against TMV in vitro.[19] Considering that sesquiterpene lactones are the main constituents of *W. trilobata*, we systematically investigated the sesquiterpene lactones from *W. trilobata* in an attempt to find more effective compounds against TMV. Herein, we report the isolation and structural elucidation of new compounds, as well as anti-TMV and phenylalanine ammonia-lyase (PAL) activities

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Table 1. ^{1}H NMR Spectroscopic Data of Compounds 1−4 and 6 in $CDCl_3$

position	1[a]	2[a]	3[b]	4[a]	6[b]
1	4.88 (1H, dd, 4.6, 9.5)	4.89 (1H, dd, 4.3, 10.7)	3.91 (1H, dd, 4.5, 11.4)	3.95 (1H, dd, 4.7, 11.3)	5.48 (1H, t, 9.0)
2a	1.52 (1H, *[c])	1.52 (1H, *)	1.64 (1H, *)	1.70 (1H, *)	1.78 (1H, *)
2b	2.04 (1H, m)	2.03 (1H, m)	1.75 (1H, m)	1.80 (1H, m)	2.15 (1H, *)
3a	1.52 (1H, *)	1.51 (1H, *)	1.43 (1H, m)	1.51 (1H, m)	1.74 (1H, *)
3b	1.66 (1H, m)	1.66 (1H, m)	1.65 (1H, *)	1.70 (1H, *)	1.92 (1H, m)
5	1.47 (1H, d, 2.3)	1.47 (1H, d, 1.9)	1.43 (1H, d, 2.4)	1.46 (1H, d, 2.5)	1.97 (1H, d, 11.0)
6	5.47 (1H, t, 2.8)	5.46 (1H, t, 3.2)	5.52 (1H, t, 3.2)	5.52 (1H, *)	5.93 (1H, dd, 9.2, 11.0)
7	3.19 (1H, br d, 2.5)	3.18 (1H, dd, 3.5, 6.1)	3.26 (1H, dd, 3.6, 6.0)	3.26 (1H, br d, 2.6)	3.44 (1H, m)
8	5.45 (1H, br s)	5.40 (1H, br s)	5.48 (1H, dd, 1.8, 3.5)	5.51 (1H, *)	4.88 (1H, dd, 2.8, 9.7)
9	4.40 (1H, br s)	4.38 (1H, t, 2.1)	4.63 (1H, t, 2.1)	4.66 (1H, br s)	3.63 (1H, br s)
13a	5.78 (1H, s)	5.77 (1H, s)	5.81 (1H, s)	5.83 (1H, s)	5.60 (1H, d, 2.8)
13b	6.60 (1H, s)	6.61 (1H, s)	6.62 (1H, s)	6.64 (1H, s)	6.27 (1H, d, 2.8)
14	1.26 (3H, s)	1.26 (3H, s)	1.23 (3H, s)	1.31 (3H, s)	1.39 (3H, s)
15	1.37 (3H, s)	1.37 (3H, s)	1.29 (3H, s)	1.34 (3H, s)	1.27 (3H, s)
1-OAc	1.98 (3H, s)	1.97 (3H, s)	–	–	–
1-ester	–	–	–	–	2.57 (1H, sept, 7.0)
	–	–	–	–	1.22 (3H, d,7.0)
	–	–	–	–	1.23 (3H, d, 7.0)
6-ester	2.59 (1H, sept, 7.0)	2.58 (1H, sept, 7.0)	5.66 (a, 1H, s)	2.64 (1H, sept, 7.0)	2.57 (1H, sept, 7.0)
	1.19 (3H, d,7.0)	1.18 (3H, d, 7.0)	6.13 (b, 1H, s)	1.25 (3H, d, 7.0)	1.17 (3H, d, 7.0)
	1.22 (3H, d, 7.0)	1.22 (3H, d, 7.0)	1.96 (3H, s)	1.28 (3H, d, 7.0)	1.18 (3H, d, 7.0)
8-ester	6.10 (1H, q, 7.1)	6.75 (1H, q, 7.1)	6.10 (1H, q, 7.2)	6.14 (1H, q, 7.3)	–
	1.75 (3H, br s)	1.70 (3H, br s)	1.76 (3H, br s)	1.82 (3H, br s)	–
	1.86 (3H, d, 7.1)	1.71 (3H, *)	1.87 (3H, dd, 1.2, 7.2)	1.92 (3H, dd, 1.3, 7.3)	–

[a]Recorded at 400 MHz. [b]Recorded at 600 MHz. [c]The asterisk indicates overlapping, without denoting multiplicity.

of the compounds. Furthermore, this study first demonstrated that these kinds of compounds enhanced resistance against TMV as potential inducers of plant systemic acquired resistance.

MATERIALS AND METHODS

General Experimental Procedures. 1D and 2D NMR spectra were carried out on either a Bruker AM-400 or a DRX-500 or an Avance III-600 spectrometer (Karlsruhe, Germany) with TMS as an internal standard. MS were measured on a Waters HPLC-Thermo Finnigan LCQ Advantage ion trap mass spectrometer (Milford, PA). Optical rotation was determined on a Horiba SEPA-300 polarimeter (Horiba, Tokyo, Japan). UV spectroscopic data were measured on a Shimadzu-210A double-beam spectrophotometer. IR spectra of samples in KBr discs were recorded on a Bruker-Tensor-27 spectrometer with KBr pellets. Column chromatography (CC) (Qingdao Haiyang Chemical Co., Qingdao, China) was carried out on silica gel G (100−200 mesh, 200−300 mesh), silica gel H (10−40 μm), Sephadex LH-20 (40−70 μm, Amersham Pharmacia Biotech AB, Uppsala, Sweden), and Lichroprep RP-18 gel (20−45 μm, Merck). HPLC preparation was performed on an Agilent 1200 series instrument equipped with a quaternary pump, a vacuum degasser, an autosampler, a thermostatted column compartment, and a diode array detector. Thin-layer chromatography (TLC) was conducted on precoated silica gel plates GF 254 (Qingdao Haiyang Chemical Co., Qingdao, China). TLC spots were visualized by heating silica gel plates sprayed with 10% H_2SO_4 in EtOH. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out using a Bio-Rad electrotransfer system (Bio-Rad, Hercules, CA).

X-ray Diffraction. Crystal data were obtained on a Bruker APEX CCD detector employing graphite monochromated Cu Kα radiation (λ = 1.54178 Å) at 100(2) K and operating in the φ/ω scan mode. The crystal structure was solved by the direct method using the program SHELXS-97 (G. M. Sheldrick, SHELXS-97: Program for X-ray Crystal Structure Solution, University of Gottingen, Germany, 1997) and subsequent Fourier difference techniques, and refined anisotropically by full-matrix least-squares on F^2 using SHELXL-97. All non-hydrogen atoms were refined anisotropically. The hydrogen atom positions were geometrically idealized and allowed to ride on their parent atoms.

Plant Material. The whole plant of *Wedelia trilobata* (L.) Hitchc was collected in Simao, Yunnan Province, China, in August 2011. The specimen was identified by Yu Chen of Kunming Institute of Botany (KIB), Chinese Academy of Sciences (CAS). A voucher specimen (H20110805) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany.

Extraction and Isolation. Dried powder of the whole plant of *W. trilobata* (9 kg) was extracted with MeOH (three times under reflux for 4, 4, and 3 h). The solvent was removed under reduced pressure to give a residue (1020 g, 11.3%), which was suspended with water and then extracted with petroleum ether, chloroform, EtOAc, and *n*-BuOH successively. The extracts were evaporated under vacuum to afford the corresponding extracts of petroleum ether (200 g), chloroform (90 g), EtOAc (90 g), and *n*-BuOH (380 g). The chloroform extract was separated with a silica gel G column (100−200 mesh, 10 × 120 cm, 0.65 kg), eluted with petroleum ether/EtOAc (v/v = 9:1, 7:3, 6:4, 1:1, 0:1, each 10 L), to give five fractions (1−5). Fraction 3 (5.13 g) was extensively chromatographed over columns of silica gel and Sephadex LH-20 ($CHCl_3$−MeOH, 1:1, 3.2 × 140 cm) to afford compounds **1** (10.0 mg), **2** (5.5 mg), **7** (17.3 mg), and **8** (9.6 mg). After the same chromatographic process, fraction 4 (5.57 g) gave compounds **3** (0.8 mg), **4** (18 mg), and **5** (4.7 mg). By silica gel G column (100−200 mesh, 20 × 150 cm, 1.0 kg) eluted with petroleum ether and EtOAc (v/v = 9:1, 7:3, 6:4, 1:1, 0:1, each 20 L), the petroleum ether extract of *W. trilobata* also gave five fractions (1−5). Fraction 3 (12.13 g) and 4 (15.05 g) were subjected respectively to RP-18 silica gel column chromatography (20−45 μm, 49 × 460 mm, 440 g), eluted with MeOH−H_2O (v/v = 3:7, 5:5, 7:3, 8:2, 9:1, each 10 L), and Sephadex LH-20 ($CHCl_3$−MeOH, 1:1, 1.8 × 120 cm), to give compounds **6** (1.8 mg), **9** (13.5 mg), and **10** (31.0 mg).

*Wedelolide C (**1**).* Colorless columnar crystals, mp 221−223 °C; $[\alpha]_D^{26}$ = −10.1° (*c* 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 212.6 (4.0) nm; IR (KBr) ν_{max} 3459, 2976, 1739, 1387, 1241, 1146, 1134, 1041

cm^{-1}; positive-ion ESI-MS m/z 515 $[M + Na]^+$, 1007 $[2 M + Na]^+$; HR-EIMS m/z 492.2359 M^+ (calcd for $C_{26}H_{36}O_9$, 492.2359); ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Tables 1 and 2. Weight isolated: 10.0 mg.

Table 2. ^{13}C NMR Spectroscopic Data of Compounds 1−4 and 6 in $CDCl_3$

position	1[a]	2[a]	3[b]	4[a]	6[b]
1	73.0 d	73.0 d	70.6 d	70.3 d	69.0 d
2	24.1 t	24.0 t	28.2 t	27.9 t	22.2 t
3	41.0 t	41.0 t	41.6 t	41.3 t	36.0 t
4	70.8 s	70.8 s	71.3 s	71.0 s	72.5 s
5	43.6 d	43.6 d	43.8 d	43.5 d	56.2 d
6	73.8 d	73.8 d	74.7 d	74.1 d	73.6 d
7	43.9 d	43.9 d	44.2 d	44.0 d	42.6 d
8	64.3 d	64.2 d	64.7 d	64.5 d	75.0 d
9	81.4 d	81.2 d	81.9 d	81.9 d	71.0 d
10	43.3 s	43.4 s	44.6 s	44.4 s	42.5 s
11	131.8 s	132.0 s	132.3 s	132.1 s	136.2 s
12	162.8 s	162.7 s	163.6 s	163.4 s	170.0 s
13	133.4 t	133.3 t	133.6 t	133.2 t	123.6 t
14	25.1 q	25.1 q	25.3 q	25.1 q	23.0 q
15	15.4 q	15.4 q	14.6 q	14.3 q	25.8 q
1-OAc	21.2, 169.8	21.2, 169.8	–	–	–
1-ester	–	–	–	–	18.9 q
	–	–	–	–	19.1 q
	–	–	–	–	34.5 d
	–	–	–	–	177.7 s
6-ester	18.5 q	18.5 q	18.7 q	18.5 q	19.2 q
	19.1 q	19.0 q	127.6 t	19.0 q	19.3 q
	34.5 d	34.5 d	136.0 s	34.5 d	34.8 d
	176.2 s	176.2 s	167.0 s	176.3 s	176.1 s
8-ester	15.9 q	14.5 q	16.1 q	15.8 q	–
	20.4 q	11.8 q	20.6 q	20.3 q	–
	126.8 s	127.6 s	127.1 s	126.9 s	–
	140.8 d	139.6 d	140.8 d	140.3 d	–
	166.6 s	166.7 s	166.9 s	166.5 s	–

[a]Recorded at 100 MHz. [b]Recorded at 150 MHz.

*Wedelolide D (**2**).* Amorphous, white powder, $[\alpha]_D^{26} = -21.5$ (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 211.8 (4.0) nm; IR (KBr) ν_{max} 3440, 2929, 1738, 1385, 1250, 1130 cm^{-1}; positive-ion ESI-MS m/z 515 $[M + Na]^+$, 1007 $[2 M + Na]^+$, HR-EIMS m/z 492.2362 M^+ (calcd for $C_{26}H_{36}O_9$, 492.2359); ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Tables 1 and 2. Weight isolated: 5.5 mg.

*Wedelolide E (**3**).* Amorphous, white powder, $[\alpha]_D^{23} = -16.5$ (c 0.14, MeOH); UV (MeOH) λ_{max} (log ε) 210.0 (4.2) nm; IR (KBr) ν_{max} 3438, 2930, 1718,1632, 1385, 1296, 1154, 1048 cm^{-1}; positive-ion ESI-MS m/z 471 $[M + Na]^+$, 919 $[2 M + Na]^+$, HR-EIMS m/z 448.2099 M^+ (calcd for $C_{24}H_{32}O_8$, 448.2097); ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz), see Tables 1 and 2. Weight isolated: 0.8 mg.

*Wedelolide F (**4**).* Colorless crystals, mp 167−169 °C; $[\alpha]_D^{25} = -7.4$ (c 0.1, MeOH); UV (MeOH) λ_{max} (log ε) 213.6 (4.2) nm; IR (KBr) ν_{max} 3452, 2964, 1719, 1390, 1262, 1149, 1084, 1031, 803 cm^{-1}; positive-ion ESI-MS m/z 473 $[M + Na]^+$, 923 $[2 M + Na]^+$, HR-EIMS m/z 450.2261 M^+ (calcd for $C_{24}H_{34}O_8$, 450.2254); ^1H NMR ($CDCl_3$, 400 MHz) and ^{13}C NMR ($CDCl_3$, 100 MHz), see Tables 1 and 2. Weight isolated: 18 mg.

*Wedetrilide A (**6**).* Colorless oil, $[\alpha]_D^{20} = +24.8$ (c 0.07, MeOH); UV (MeOH) λ_{max} (log ε) 206.6 (3.5) nm; IR (KBr) ν_{max} 3443, 2974, 2929, 1732, 1470, 1386, 1271, 1196, 1158, 1078, 943 cm^{-1}; positive-ion ESI-MS m/z 461 $[M + Na]^+$, 899 $[2 M + Na]^+$, HR-EIMS m/z 438.1929 M^+ (calcd for $C_{23}H_{34}O_8$, 438.2254); ^1H NMR ($CDCl_3$, 600 MHz) and ^{13}C NMR ($CDCl_3$, 150 MHz), see Tables 1 and 2. Weight isolated: 1.8 mg.

Assays of Anti-TMV Activity. *Preparation of Screening Materials.* TMV (U1 strain) was obtained from the Yunnan Key Laboratory of Agricultural Biotechnology, Yunnan Academy of Agricultural Sciences, P. R. China. The virus was multiplied in *Nicotiana tabacum* cv. K326 and purified as described by Gooding and Hebert.[20] The concentration of TMV was determined as 3 mg/mL with an ultraviolet spectrophotometer [virus concentration = (A_{260} × dilution ratio)/$E_{1cm}^{0.1\%,260nm}$]. The purified virus was kept at −20 °C and was diluted to 50 μg/mL with 0.01 M phosphate-buffered saline (PBS) before use.

Nicotiana glutinosa and *N. tabacum* cv. K326 plants were cultivated in an insect-free greenhouse. *N. glutinosa* was used as a local lesion host, and *N. tabacum* cv. K326 was used to determine systemic TMV infection. The experiments were conducted when the plants grew to the 5−6-leaf stage.

The tested compounds were dissolved in dimethyl sulfoxide (DMSO) and diluted with distilled H_2O to the required concentrations. The solution of equal concentration of DMSO was used as negative control (CK), and ningnanmycin, a commercial antiviral agent known to impart its action by destruction of the coat protein of TMV and inducing plant host resistance, was used as a positive control. The healthy plants were used as mock for assays with *N. tabacum* cv. K326.

Half-Leaf Method.[21] *Protective Effect of Compounds against TMV in Vivo.* The compound solution was smeared on the left side, whereas the solvent served as the control on the right side of growing *N. glutinosa* leaves of the same ages. The leaves were then inoculated with the virus after 6 h. A brush was used to inoculate the leaves, which were previously scattered with silicon carbide, and each side was smeared with 100 μL of TMV of 50 μg/mL. The leaves were then washed with water and rubbed softly along the nervature once or twice. The local lesion numbers were recorded 3−4 days after inoculation. Three repetitions were conducted for each compound.

Inactivation Effect of Compounds against TMV in Vivo. The virus was inhibited by mixing it with the compound solution at the same volume for 30 min. The mixture was then inoculated on the left side of the leaves of *N. glutinosa*, whereas the right side of the leaves was inoculated with the mixture of solvent and the virus for control. The local lesion numbers were recorded 3−4 days after inoculation. The experiment was repeated three times with each compound.

Curative Effect of Compounds against TMV in Vivo. Growing leaves of *N. glutinosa* of the same ages were selected. The TMV (concentration of 50 μg/mL) was inoculated on the whole leaves. Then the leaves were washed with water and dried. Thirty minutes later, the compound solution was smeared on the left side, and the solvent was smeared on the right side for control. The local lesion numbers were recorded 3−4 days after inoculation. Three repetitions were conducted for each compound. The inhibition rate of the compound was then calculated according to the following 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.

Leaf-Disk Method. Systemic Acquired Resistance Screening. Three pots of healthy whole *N. tabacum* cv. K326 plant at 5−6-leaf stage were chosen for the screening of one compound. A 200 μL amount of target compound or 200 μL of solvent (control solution) was softly smeared on the lower leaves (the third to fourth leaves from the top) of one plant. After 24 h, the treated leaves were inoculated with TMV. The plants were cultured in the green house for 3 d of further cultivation, and then 10 leaf disks without major veins were cut from the treated leaf surface with a diameter of 1 cm. A week later, the same size of 10 leaf disks were cut from the newly grown leaves of the inoculated plants. The leaf disks were kept at −80 °C before Western blot analysis.

SDS-PAGE and Western Blot Analysis of TMV Coat Protein (CP). SDS-PAGE was performed as described previously.[22] Briefly, leaf disks from the leaf-disk method were ground in protein loading buffer (40

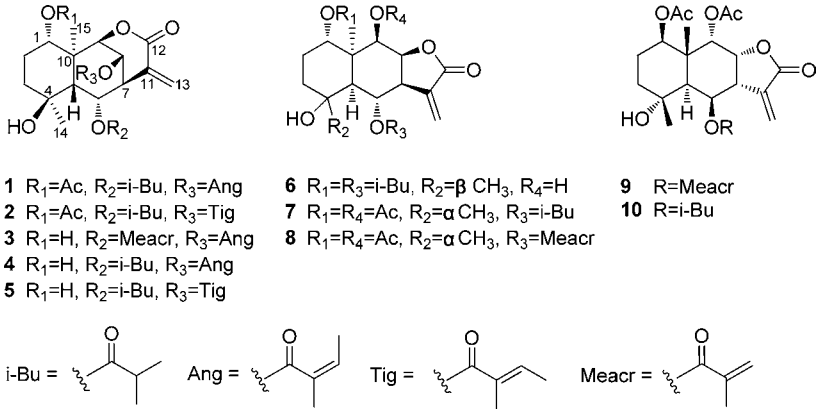

Figure 1. Structures of compounds **1**−**10** from *Wedelia trilobata*.

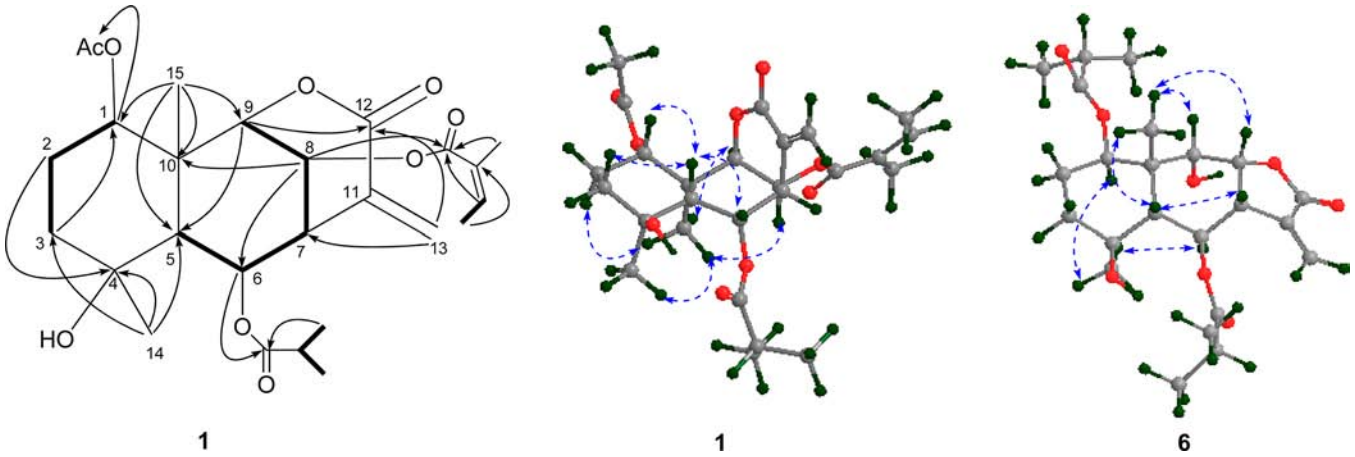

Figure 2. Selected 2D NMR correlations of **1** and **6** (^{1}H−^{1}H COSY: bold, HMBC: solid arrow, H→C, ROESY: dashed arrow).

g/L SDS, 10 mL/L *β*-ME, 200 mL/L glycerin, 2 g/L bromophenol blue, 0.1 mol/L Tris-HCl, pH 6.8), and then 5 *μ*L of sample and 3 *μ*L of marker (Blue Plus Protein Marker, 16−94 kDa, Beijing, China) were loaded on a polyacrylamide gel (5% stacking gel, 12.5% separating gel). Samples were run in duplicate. After SDS-PAGE, TMV protein bands were transferred at 200 mA for 45 min onto nitrocellulose membrane (0.2 *μ*m) using an electrotransfer system (Bio-Rad, Hercules, CA). The membrane was washed in TBST (1 mol/L Tris-HCl, pH 7.5; 1 mol/L NaCl; 0 0.05% Tween-20) and blocked with 5% nonfat milk powder in TBST for 1 h at 37 °C. The membrane was washed three times for 15 min with TBST and reacted with a mixture of 1:5000 alkaline phosphatase-conjugated antirabbit IgG (Sigma, St. Louis, MO) and 1:8000 polyclonal antibodies of TMV for 1 h at 37 °C. After it was washed three times for 15 min with TBST, the membrane was incubated in substrate buffer (1 mol/L Tris-HCl, pH 9.5; 1 mol/L NaCl; 1 mol/L MgCl) with 330 *μ*L/mL NBT and 165 *μ*L/mL BCIP for 3−5 min in the dark until the bands of the CP were clear.

Assay of PAL Activity. *N. tabacum* cv. K326 plants were sprayed with different concentrations of compound **1**, and a solution of equal concentration of DMSO was used as negative control. Leaves were harvested 6 and 24 h, respectively, after spraying and used for quantitation of the PAL activity. PAL activity was determined in three replicate samples by an adaptation of the methods of Zucker,[23] and McCallum and Walker.[24] PAL was extracted from 0.5 g of pretreated fresh leaves with 0.5 mL of 10% poly(vinylpyrrolidone) (PVP) and 2 mL of 0.05 M borate buffer, pH 8.8, containing 5 mM *β*-mercaptoethanol. The reaction mixture consisted of 1 mL of L-phenylalanine (0.02 M), 1 mL of crude enzyme (diluted to the proper concentration with borate buffer), and enough water to bring the total volume to 4 mL. The reaction was carried out at room temperature (20 °C). PAL activity was measured by determining the absorbance of *trans*-cinnamic acid at 290 nm over 2 min after the addition of phenylalanine. The results obtained were expressed as micromoles of *trans*-cinnamic acid per gram of fresh weight per hour.

RESULTS AND DISCUSSION

Dried powder of whole plant of *W. trilobata* was extracted with MeOH, suspended with water, and then extracted with petroleum ether, chloroform, EtOAc, and *n*-BuOH successively. At a concentration of 1 mg/mL, the petroleum ether and the chloroform extracts showed a protective effect against TMV with inhibition rates of 53.5% and 53.4%, respectively. Further separation of the two extracts afforded five new compounds, wedelolides C−F (**1**−**4**) and wedetrilide A (**6**) (Figure 1), together with five known compounds, identified as wedelolide A (**5**),[25,26] 1*β*,9*α*-diacetoxy-4*α*-hydroxy-6*β*-isobutyroxyprostatolide (**7**),[27] 1*β*,9*α*-diacetoxy-4*α*-hydroxy-6*β*-methacryloxyprostatolide (**8**),[27] and trilobolides 6-*O*-methacrylate (**9**)[26−28] and 6-*O*-isobutyrate (**10**),[26−29] by comparison of their spectroscopic data with the published data.

Structure Elucidation of New Compounds. Compound **1** was obtained as colorless columnar crystals, $[\alpha]_D^{26} = -10.1°$ (*c* 0.10, MeOH). The molecular formula, $C_{26}H_{36}O_9$, was deduced from the HREIMS molecular ion M^+ together with NMR data (Tables 1 and 2) and indicated nine degrees of unsaturation. The ^{13}C NMR spectrum ($CDCl_3$) showed four ester carbonyl signals at *δ* 162.8, 166.7, 169.8, and 176.2, and four vinyl carbon signals at *δ* 126.8, 132.0, 133.4 (CH_2), and 140.7 (CH), taking

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into account six unsaturations. Hence, the three remainders were attributed to three saturated rings. The ^{1}H and ^{13}C NMR and HSQC spectra indicated the presence of seven methyls, two methylenes, six methines, and two quaternary carbons. Detailed 2D NMR ($^{1}H-^{1}H$ COSY, HSQC, and HMBC experiments) (Figure 2) data revealed the presence of an acetate group, an isobutyrate group, an angeloyloxy group, and a hydroxy group, consistent with the molecular formula of **1**. Comparison of the NMR data of **1** with those of **5** suggested that they were structural analogues with the same basic skeleton. The locations of those substituents mentioned above were designated on the basis of HMBC correlations. The acetate group, isobutyrate group, and angeloyloxy group were located at C-1, C-6, and C-7, respectively, based on the correlations of H-1, H-6, and H-7 to the corresponding ester carbonyls. The remaining group, hydroxy, was only placed at C-4 due to its chemical shift (δ 70.8) although there was no direct HMBC correlation evidence. The relative stereochemistry of compound **1** was assigned by the ROESY spectrum. CH_3-15 protons gave interactions with CH_3-14, H-8, and H-2a protons. All of these protons were situated on the same side of the molecule as shown in the X-ray crystallographic data, whereas the axial H-5 interacted with axial H-1b and H-3b, situating it on the other side. The equatorial H-7 showing a cross-peak with vinylidene proton H-13a was found in a spatially closed position to each other. The results revealed a trans chair−chair junction of rings A and B (Figure 1). The absolute stereochemistry of compound **1** was determined by means of X-ray diffraction experiment with Cu Kα radiation, which confirmed the structure of **1** as above.

Compound **2** has the same molecular formula $C_{26}H_{36}O_9$ as that of **1** based on HREIMS. The ^{1}H and ^{13}C NMR spectroscopic data of **2** were closely similar to those of **1** except for bearing a tigloyloxy group instead of an angeloyloxy group at C-8 position, as shown in the Tables 1 and 2. This assignment was determined by the strong ROESY correlations between the two methyls attributed to the tigloyloxy group in **2**.

Compound **3**, amorphous solid, $[\alpha]_D^{23} = -16.5$ (c 0.14, MeOH). The molecular formula, $C_{24}H_{32}O_8$, was deduced from HREIMS molecular ion M^+ together with NMR data (Tables 1 and 2) and indicated nine degrees of unsaturation. The ^{13}C spectrum presented similar signals of two methine and four oxymethine carbons in the δ 60−85 ppm area, the same as those of compound **1**. Detailed comparison of the NMR data of **3** with those of **1** suggested that they were structural analogues. The main difference was that compound **3** lacked the acetyl group and isopropyl signal of **1**, while possessing a methacryl group at the O-6 position.

Compound **4** possessed the molecular formula of $C_{24}H_{34}O_8$ as determined by HREIMS. Comparison of the ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **4** with those of **1** revealed that the only difference between the two compounds was the absence of the acetyl group at C-1 in **4**, and this was supported by its molecular formula, which showed 42 mass units less than that of **1**. This assignment was also confirmed by the upfield shift of ca.$\Delta\delta$ 0.93 of H-1 at δ H 3.91 (1H, dd, J = 4.5, 11.4 Hz) in the ^{1}H NMR spectrum of **4** compared with that of **1**.

The molecular formula of **6**, $C_{23}H_{34}O_8$, was established by analysis of the ^{13}C NMR and HREIMS data. The ^{1}H and ^{13}C NMR data (Tables 1 and 2) of **6** was closely similar to those of **7** and **8**, suggesting that these compounds had the same basic skeleton containing an 8,12-γ-lactone. Detailed 1D and 2D NMR ($^{1}H-^{1}H$ COSY, HSQC, and HMBC experiments) data revealed the presence of two isobutyrate groups and two hydroxy groups in **6** that were consistent with the molecular formula. The two isobutyrate groups were located at C-1 and C-5, respectively, confirmed by HMBC correlations of the H-1 and H-6 to the corresponding ester carbonyl of the isobutyrate group. The remaining two hydroxy groups were placed at C-4 and C-9, respectively, according to analysis of NMR spectra and compared with those of **7** and **8**. The relative stereochemistry of compound **6** was assigned by the ROESY spectrum (Figure 2). CH_3-15 protons gave interactions with H-5, H-8, and H-9 that indicated that these protons were situated on the same side of molecule with β-orientation as shown in Figure 2, while CH_3-14 protons interacted with H-1 and H-6, situated on the other side in α-orientation. Therefore, compound **6** was assigned and named wedetrilide A.

Activities of Anti-TMV and Inducing Plant Systemic Acquired Resistance. We tested inhibitory activities of compounds **1**, **2**, **4**, **5**, and **7**−**10** against TMV by using two approaches. First, the half-leaf method was used to test in vivo for anti-TMV activity in the local lesion host *N. glutinosa*. Second, the leaf-disk method was used to screen the SAR of each compound in the systemic infection host *N. tabacum* cv. K326. Ningnanmycin, a commercial antiviral product used for plant disease in China, was a positive control.

The antiviral inhibition rates of compounds **1**, **2**, **4**, **5**, and **7**−**10** at the concentration of 100 μg/mL tested by the half-leaf method are listed in Table 3. The result showed that except for compound **4**, all the other compounds showed obvious protective effects. Accordingly, we tested the protective effects of compounds **1**, **2**, **4**, **5**, and **7**−**10** at concentrations of 10, 50, 100, and 200 μg/mL, respectively. As shown in Table 4, all the compounds, at 10 μg/mL, showed inhibition rates ranging from 46.7% to 76.5%, significantly higher than that of the positive control, ningnanmycin (13.5%). However, the inhibition rates against TMV were negatively correlated with the concentration of these compounds. On the other hand, the results indicated that pretreatment with these compounds could increase the resistance of the host plant to TMV infection.

Table 3. Antiviral Activities against TMV on *N. glutinosa* in Vivo[a]

	inhibition rate (%)		
compound	curative effect	protective effect	inactivation effect
1	17.0 ± 2.4	41.3 ± 3.9	36.9 ± 1.2
2	35.4 ± 6.9	39.2 ± 5.5	4.0 ± 3.4
4	27.0 ± 4.1	33.8 ± 7.6	38.0 ± 6.3
5	24.4 ± 4.5	35.6 ± 6.8	25.0 ± 8.4
7	17.2 ± 3.7	40.4 ± 6.8	36.5 ± 6.9
8	25.7 ± 7.5	35.6 ± 5.1	10.4 ± 3.5
9	24.4 ± 6.1	38.8 ± 5.8	11.2 ± 7.3
10	23.7 ± 4.9	39.4 ± 8.1	9.9 ± 6.2
ningnanmycin	58.9 ± 3.3	52.1 ± 6.1	48.9 ± 3.6

[a]The concentrations of compounds were 100 μg/mL. All results are expressed as mean ± SD; n = 6 for all groups.

To assess whether the protective effects of compounds **1**, **2**, **4**, **5**, and **7**−**10** result from inducing SAR in the tobacco plants, the SAR screening of these eight compounds in the systemic infection host *N. tabacum* cv. K326 along with Western blot analysis of TMV coat protein (CP) was carried out (Figure 3). The bands of CP in Figure 3A showed that on the third day

Table 4. Protective Effects of Compounds on *N. glutinosa* at Different Concentrations[a]

	inhibition rate (%)			
compound	10 μg/mL	50 μg/mL	100 μg/mL	200 μg/mL
1	65.8 ± 4.6	42.1 ± 5.5	39.9 ± 8.6	21.3 ± 7.5
2	46.7 ± 7.6	41.5 ± 3.6	32.1 ± 5.4	7.5 ± 3.4
4	55.4 ± 5.0	44.4 ± 4.1	30.2 ± 8.0	13.3 ± 4.7
5	66.6 ± 6.4	48.5 ± 6.8	37.4 ± 5.3	15.9 ± 6.4
7	65.8 ± 3.5	63.9 ± 3.9	46.6 ± 5.8	34.1 ± 6.2
8	55.3 ± 7.2	35.4 ± 9.0	31.9 ± 5.5	11.3 ± 5.1
9	59.6 ± 6.5	43.8 ± 6.4	34.3 ± 7.4	9.6 ± 2.8
10	76.5 ± 7.0	53.9 ± 6.5	35.2 ± 8.4	14.7 ± 3.4
ningnanmycin	13.5 ± 3.2	55.3 ± 2.8	62.2 ± 5.6	81.2 ± 3.5

[a]All results are expressed as mean ± SD; $n = 6$ for all groups.

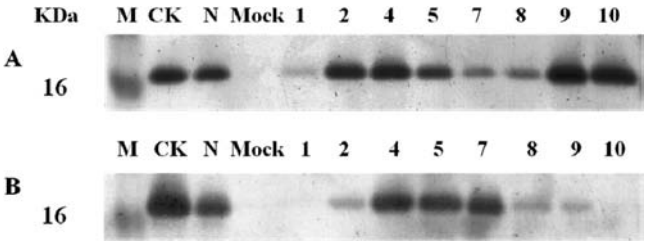

Figure 3. Western blot analysis. (A) Accumulation of TMV coat protein in the lower leaves treated with compounds **1**, **2**, **4**, **5**, and **7**–**10** (50 μg/mL) on the third day after inoculation; M, marker; N, ningnanmycin; CK, negative control. (B) Accumulation of TMV coat protein in newly grown leaves on the seventh day after inoculation.

after inoculation, accumulation of TMV in the treated leaves (with compounds **1**, **7**, and **8**) were less than that of negative control, while leaves treated with compounds **2**, **4**, **5**, **9**, and **10** showed strong bands of CP, which indicated low activities. Nevertheless, the bands of CP in Figure 3B showed different evidence of virus accumulation in newly grown leaves. With the treatment on lower leaves by compounds **1**, **2**, and **8**–**10**, the CP bands of newly grown leaves were undetected (**1**, **10**) or relatively weaker (**2**, **8**, and **9**) than that of negative control. It was possible that compounds **1**–**2**, **8**–**10** induced SAR in the plants to inhibit virus accumulation and movement. Among the tested compounds, **1** and **8** showed higher induction activities on both treated leaves and newly grown leaves.

As these results indicated the induced SAR-like disease resistance to TMV in tobacco plants (*N. tabacum* cv. K326), we tested one of the key molecular targets of the SAR signal pathway, phenylalanine ammonia-lyase (PAL), to further determine whether SAR was triggered. Several reports have suggested that PAL is a key regulatory enzyme in the biosynthesis of salicylic acid (SA) and the establishment of SAR.[30] Tobacco plants in which PAL activity has been epigenetically suppressed are unable to express SAR.[31] On the basis of the results of screening in vivo, wedelolide C, **1**, with good induction activity against TMV was chosen for further evaluating its potential of inducing SAR. The data in Figure 4 showed an increase in PAL activity in tobacco plants treated with compound **1** at concentrations of 0.01, 0.1, 1, 10, 100, and 200 μg/mL, and the PAL activity was basically negatively correlated with the concentration of compound **1**. Six hours after treatment, the PAL activity in plants treated with 0.1 μg/mL of compound **1** showed a 2.5-fold increase with respect to that in untreated plants, and 24 h after treatment, the

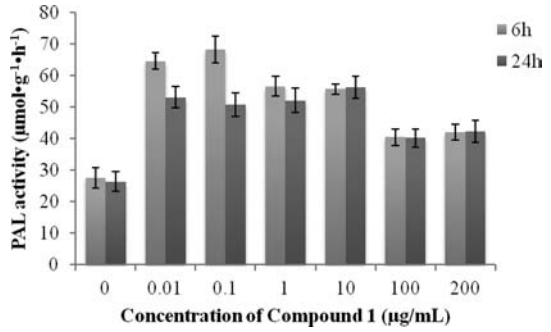

Figure 4. Enhancement of PAL activity in tobacco plants treated with compound **1**. All results are expressed as mean ± SD; $n = 3$ for all groups.

PAL activity tended to decrease a little but still exibited a 1.9-fold increase. These results suggested that compound **1** could promote the activity of PAL in the initial stage, which was responsible for the production of *trans*-cinnamic acid, an indirect precursor of SA, and then activated the plant SAR by triggering the SA biosynthesis pathway.

In summary, ten eudesmanolides, including δ- and γ-lactones, were isolated from the whole plant of *Wedelia trilobata* (L.) Hitchc. The in vivo studies indicated that all the tested compounds exhibited excellent protection effects against TMV compared with the commercial ningnanmycin. The present work also first demonstrated that these kinds of sesquiterpene lactones, especially compounds **1**, **2**, and **8**–**10**, could act as plant SAR inducers and that they had the potential to be developed into a novel type of plant protection agent, inducing the plant's inherent disease resistance mechanisms. Because sesquiterpene lactones are the major chemical constituents of the invasive alien species *W. trilobata*, which has caused significant damage to farmlands, forests, and orchards, we consider it significant to take full advantage of this noxious but resourceful plant as a resource for the development of new anti-TMV agents.

■ ASSOCIATED CONTENT

Supporting Information

^{1}H, ^{13}C, and 2D NMR, ESI-MS, and HREIMS spectra of new compounds **1**–**4** and **6**, and X-ray crystallographic data of compound **1**. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*Tel: +86-871-65223076. Fax: +86-871-65223070. E-mail: lisl@mail.kib.ac.cn.

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Notes

The authors declare no competing financial interest.

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