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# The Limonoids and Their Antitobacco Mosaic Virus (TMV) Activities from *Munronia unifoliolata* Oliv.

Yong-hui Ge,[†,‡] Kai-xing Liu,[†,‡] Jian-xin Zhang,[†] Shu-zhen Mu,[†] and Xiao-jiang Hao*[,†]

[†]The Key Laboratory of Chemistry for Natural Product of Guizhou Province and Chinese Academy of Science, Guiyang 550002, People's Republic of China

[‡]Center for Research and Development of Fine Chemicals, Guizhou University, Guiyang 550025, People's Republic of China

Supporting Information

**ABSTRACT:** Five new limonoids, named munronoids K−O (**1**−**5**), together with three known limonoids were isolated from *Munronia unifoliola* Oliv. These limonoids were involved in the skeletons of evodulone, gedunin, and peieurianin types of limonoids, and their structures were established on the basis of spectroscopic data. Compound **5** featuring a $\gamma$-lactone ring instead of the $\beta$-substituted furan ring was found in the peieurianin type for the first time. The antitobacco mosaic virus (anti-TMV) activities of compounds **1**−**8** were also evaluated with half-leaf, enzyme-linked immunosorbent assay, and Western blot methods, and limonoids **1**, **5**, and **8** showed stronger anti-TMV treatment activities than the positive control ningnanmycin. Six compounds (**1**−**5** and **8**) exhibited infection inhibition activities against TMV.

**KEYWORDS:** *tobacco mosaic virus (TMV), coat protein, Munronia unifoliolata Oliv., limonoids*

## INTRODUCTION

Tobacco mosaic virus (TMV) is a prevalent plant pathogen all over the world and has the widest host range of over 885 plant species in 65 families. Most TMVs have infected a number of economically important crops and have induced general mosaic symptoms to cause significant economic losses worldwide.[1] However, there have been few effective anti-TMV agents to be discovered up to now. On the other hand, plants have evolved multiple mechanisms to selectively suppress pathogens by the production of secondary metabolites with antimicrobial and/or antivirus. Therefore, it is a challenge to discover new natural anti-TMV agents from plants.

Limonoids are a big group of natural products in plants and have attracted great interest as challenging projects for their complicated structures and diverse bioactivities.[2,3] Until now, about 1300 limonoids with more than 35 carbon frameworks have been isolated from four families (Meliaceae, Rutaceae, Simaroubaceae, and Cneoraceae). Despite their significant ecological effects such as insecticidal and antifeedant activities, there have been no reports for their activity against TMV by the other research groups.

The discovery of an anti-TMV agent from plants is an effective way, and some new natural anti-TMV agents have been reported by our group since 2007, such as secopregnane steroids,[4] cinchnaglycoside C,[5] 7-deoxy-*trans*-dihydronarciclasine,[6] 3-acetonyl-3-hydroxyoxindole,[7] $\beta$-carbolines,[8] and quassinoids.[9] Recently, we first discovered that some limonoids isolated from *Munronia unifoliola* Oliv. also showed anti-TMV activity by primary screening,[10] which attracted us to further investigate the limonoids and their anti-TMV activity from this plant. In the continuous research, five new limonoids, named munronoids K−O (**1**−**5**), together with three known limonoids mombasol (**6**),[11] diacetylvilasinin (**7**),[12] and nymania (**8**),[13] were isolated from *M. unifoliola* Oliv., which is mainly distributed in the southwest of China.[14] We herein report the isolation, structural elucidation, and anti-TMV activities of all limonoids as above.

## MATERIALS AND METHODS

**General Experimental Procedures.** Optical rotations were determined with a JASCO DIP-370 Digital Polarimeter. IR spectra were recorded on a Bio-Rad FTS-135 spectrometer, KBr discs, in $cm^{-1}$. One- and two-dimensional NMR spectra were obtained on an INOVA-400 MHz NMR spectrometer with tetramethylsilane (TMS) as an internal standard; electrospray ionization−mass spectrometry (ESI-MS) and high-resolution (HR) ESI-MS spectra were measured with a Finnigan MAT 90 instrument and VG Auto Spec-3000 spectrometer, respectively. Semipreparative high-performance liquid chromatography (HPLC) was performed on a Waters column (i.d. 10−100 mm), developed with $CH_3OH/H_2O$ (40:60 to 58:42, 25 min) (flow rate, 2.0 mL/min; detection, UV, 230 nm) at 25 °C. Column chromatography was performed on silica gel (50−90 $\mu$m; Qingdao Marine Chemical Inc.), MCI gel (CHP20P, 75−150 $\mu$m, Mitsubishi Chemical Industries Ltd.), $C_{18}$ reversed-phase silica gel (20−45 $\mu$m; Merck, Darmstadt, Germany), and Sephadex LH-20 (40−70 $\mu$m; Amersham Pharmacia Biotech AB, Uppsala, Sweden). Thin-layer chromatography (TLC) plates were precoated with silica gel $GF_{254}$ (Qingdao Haiyang Chemical Plant, Qingdao, People's Republic of China). Spots on chromatograms were detected by spraying with 10% $H_2SO_4$−EtOH. Leaf disks were kept in a RXZ280B culture chamber (Ningbo, Zhejiang, China). Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out using a Bio-Rad electrotransfer system (Bio-Rad, Hercules, CA).

**Plant Material.** The whole plant of *M. unifoliolata* Oliv. was collected in Xiuwen, Guizhou Province, People's Republic of China, and was identified by Prof. Zhi-min Fu of Guiyang College of

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Table 1. $^1$H NMR Data of Limonoids 1−5 (400 MHz) in $CDCl_3$[a]

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 4.67 (1H, brs) | 4.72 (1H, d, $J$ = 5.2) | 4.74 (1H, brs) | 4.73 (1H, brs) | 6.88 (1H, d, $J$ = 12.8) |
| 2 | 3.08 (2H, d, $J$ = 3.2) | 3.15 (2H, d, $J$ = 4.8) | 3.19(2H, d, $J$ = 2.8) | 2.15 (2H, m) | 6.29 (1H, d, $J$ = 12.8) |
| 3 | | | | 4.94 (1H, brs) | |
| 5 | 2.34 (1H, t, $J$ = 3.2) | 2.49 (1H, t, $J$ = 9.6) | 2.52 (1H, t, $J$ = 11.6) | 2.88 (1H, t, $J$ = 12.8) | 3.31 (1H, d, $J$ = 8.4) |
| 6 | 2.06 (1H, m)<br>1.97 (1H, m) | 2.01 (1H, *)<br>1.93 (1H, m) | 2.02 (1H, *)<br>2.00 (1H, m) | 4.03 (1H, d, $J$ = 12.0) | 2.31 (2H, m) |
| 7 | 5.24 (1H, brs) | 5.20 (1H, brs) | 5.26 (1H, brs) | 4.36 (1H, brs) | |
| 9 | 2.62 (1H, m) | 2.73 (1H, m) | 2.72 (1H, m) | 3.15 (1H, d, $J$ = 10.0) | 3.08 (1H, d, $J$ = 5.2) |
| 11 | 1.05 (1H, m)<br>2.02 (1H,*) | 1.19 (1H, m)<br>2.10 (1H, m) | 1.19 (1H,*)<br>2.15 (1H, m) | 1.56 (1H, m)<br>1.87 (1H, m) | 5.59 (1H, m) |
| 12 | 4.99 (1H, t, $J$ = 8.4) | 5.01 (1H, t, $J$ = 8.0) | 5.03 (1H, t, $J$ = 8.4) | 4.60 (1H, brs) | 5.83 (1H, d, $J$ = 10.4) |
| 15 | 5.44 (1H, brs) | 5.48 (1H, brs) | 5.49 (1H, brs) | *b* | 3.87 (1H, brs) |
| 16 | 2.34 (2H, m) | 2.40 (2H, m) | 2.38 (2H, m) | 4.96 (1H, *) | 2.21 (1H, m)<br>2.31 (1H, m) |
| 17 | 2.94 (1H, t, $J$ = 9.6) | 2.96 (1H, t, $J$ = 9.6) | 2.96 (1H, t, $J$ = 9.2) | 3.42 (1H, m) | 3.01 (1H, m) |
| 18 | 0.87 (3H, *) | 0.97 (3H, s) | 0.94 (3H, s) | 1.75 (3H, s) | 0.95 (3H, s) |
| 19 | 1.20 (3H, s) | 1.21 (3H, s) | 1.22 (3H, s) | 0.96 (3H, s) | 0.95 (3H, s) |
| 21 | 7.13 (1H, s) | 7.19 (1H, s) | 7.19 (1H, s) | 7.27 (1H, s) | 4.72 (2H, d, $J$ = 4.8) |
| 22 | 6.17 (1H, brs) | 6.23 (1H, brs) | 6.23 (1H, brs) | 6.42 (1H, brs) | 7.14 (1H, s) |
| 23 | 7.29 (1H, brs) | 7.34 (1H, brs) | 7.32 (1H, brs) | 7.31 (1H, brs) | |
| 28 | 1.59 (3H, s) | 1.51 (3H, s) | 1.49 (3H, s) | 1.19 s (3H, s) | 1.55 (3H, s) |
| 29 | 1.40 (3H, s) | 1.40 (3H, s) | 1.40 (3H, s) | 3.58 (2H, d, $J$ = 7.6) | 1.26 (3H, s) |
| 30 | 1.14 (3H, s) | 1.17 (3H, s) | 1.19 (3H, s) | 1.34 (3H, s) | 5.36 (1H, m)<br>5.23 (1H, m) |
| 1- | 2.02 (3H, s) | 2.09 (3H, s) | 2.05 (3H, s) | | |
| 3- | | | | 1.96 (3H, s) | |
| 7- | 1.87 (3H, s) | 2.04 (3H, s) | | | 3.69 (3H, s) |
| 11- | | | | | 2.12 (3H, s) |
| 12- | | 1.93 (3H, s) | 1.92 (3H, s) | 3.05 (3H, s) | 1.86 (3H, s) |
| 2′ | 3.99 (1H, m) | | 6.86 (1H, d, $J$ = 6.8) | | |
| 3′ | 1.78 (1H, m) | | | 6.93 (1H, m) | |
| 4′ | 1.25 (2H, *) | | 1.84 (3H, s) | 1.82 (3H, d, $J$ = 7.2) | |
| 5′ | 0.89 (3H, t, $J$ = 7.6) | | 1.88 (3H, s) | 1.94 (3H, s) | |
| 6′ | 0.97 (3H, d, $J$ = 6.8) | | | | |

[a]*Overlapped, without denoting multiplicity. [b]Not detectable.

Traditional Chinese Medicine. A voucher specimen (1868) was deposited at the Herbarium of Guizhou.

**Extraction and Isolation.** The powder of a dried whole plant of *M. unifoliolata* Oliv. (5.0 kg) was extracted three times with 95% EtOH. The extracts were combined, concentrated, and suspended in $H_2O$. The water layer was then extracted with petroleum ether (PE) and EtOAc. The EtOAc extracts (322 g) were subjected to silica gel column chromatography, eluted with PE/acetone (from 1:0 to 2:1), yielding seven fractions (fractions 1−7), and sequentially eluted with $CHCl_3$/MeOH (from 10:1 to 0:1), yielding six fractions (A1−6). Fraction A3 (16 g) was first applied to MCI gel (eluted with MeOH/$H_2O$/acetone from 9:1:0 to 0:0:1) and then to a reversed $C_{18}$ column chromatography (eluted with MeOH/$H_2O$ from 3:7 to 1:0), yielding five fractions (B1−5). Fraction B1 (334 mg) was purified on Sephadex LH-20 followed by silica gel (eluted with $CHCl_3$/acetone from 20:1 to 8:1), yielding **1** (25 mg), **2** (101 mg), and **3** (22 mg). Fraction B5 (233 mg) was applied to Sephadex LH-20 (eluted with $CHCl_3$/MeOH, 1:1), yielding two fractions (C1 and C2). C1 (98 mg) was purified by semipreparative HPLC to yield **4** (33 mg) and **5** (36 mg).

*Munronoid K (**1**).* A white amorphous solid; $[\alpha]_D^{25}$ +32.5 ($c$ 0.28, $CHCl_3$). UV $\lambda_{max}$ (MeOH): 229 nm. IR (KBr) $\nu_{max}$ 3520, 2964, 1728, 1373, 1230, 1026 cm$^{-1}$. Positive-ion ESI-MS $m/z$ 665 [M + Na]$^+$. HR-ESI-MS peak at $m/z$ 665.3318 [M + Na]$^+$; calcd, 665.3301. For $^1$H and $^{13}$C NMR data, see Tables 1 and 2.

*Munronoid L (**2**).* White powder; $[\alpha]_D^{25}$ +77.9 ($c$ 0.62, $CHCl_3$). UV $\lambda_{max}$ (MeOH): 228 nm. IR (KBr) $\nu_{max}$ 3531, 2925, 1728, 1374, 1241 cm$^{-1}$. Positive-ion ESI-MS $m/z$ 593 [M + Na]$^+$. HR-ESI-MS peak at $m/z$ 593.2728 [M + Na]$^+$; calcd, 593.2726. For $^1$H and $^{13}$C NMR data, see Tables 1 and 2.

*Munronoid M (**3**).* White powder; $[\alpha]_D^{25}$ +67.9 ($c$ 0.42, $CHCl_3$). UV $\lambda_{max}$ (MeOH): 233 nm. IR (KBr) $\nu_{max}$ 2932, 1727, 1372, 1243, 1026 cm$^{-1}$. Positive-ion ESI-MS $m/z$ 633 [M + Na]$^+$. HR-ESI-MS peak at $m/z$ 633.3039 [M + Na]$^+$; calcd, 633.3037. For $^1$H and $^{13}$C NMR data, see Tables 1 and 2.

*Munronoid N (**4**).* A white amorphous solid; $[\alpha]_D^{25}$ −22.5 ($c$ 0.68, $CHCl_3$). UV $\lambda_{max}$ (MeOH): 236 nm. IR (KBr) $\nu_{max}$ 3563, 2925, 1732, 1386, 1268, 1055 cm$^{-1}$. Positive-ion ESI-MS $m/z$ 621 [M + Na]$^+$. HR-ESI-MS peak at $m/z$ 621.3021 [M + Na]$^+$; calcd, 621.3039. For $^1$H and $^{13}$C NMR data, see Tables 1 and 2.

*Munronoid O (**5**).* A white amorphous solid; $[\alpha]_D^{25}$ +98.3 ($c$ 0.21, $CHCl_3$). UV $\lambda_{max}$ (MeOH): 227 nm. IR (KBr) $\nu_{max}$ 3543, 2923, 1739, 1640, 1371, 1267, 1060 cm$^{-1}$. Positive-ion ESI-MS $m/z$ 609 [M + Na]$^+$. HR-ESI-MS peak at $m/z$ 587.2509 [M + H]$^+$; calcd, 587.2492. For $^1$H and $^{13}$C NMR data, see Tables 1 and 2.

*Mombasol (**6**).* A white amorphous solid was identified by comparison of MS and NMR ($^1$H NMR and $^{13}$C NMR) spectroscopic data with those reported in the literature.[11]

*Diacetylvilasinin (**7**).* A white amorphous solid was identified by comparison of MS and NMR ($^1$H NMR and $^{13}$C NMR) spectroscopic data with those reported in the literature.[12]

*Nymania (**8**).* A white amorphous solid was identified by comparison of MS and NMR ($^1$H NMR and $^{13}$C NMR) spectroscopic data with those reported in the literature.[13]

**Anti-TMV Assays.** *Preparation of Screening Materials.* TMV (U1 strain) was obtained from the Wuhan Institute of Virology, Chinese Academy of Sciences, People's Republic of China. The virus was multiplied in *Nicotiana tabacum* cv. K326 and purified as described by Gooding and Hebert.[15] The concentration of TMV was determined as 18.2 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 30 μg/mL with 0.01 M 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 could be conducted when the plants grew to the 4−5 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 a negative control. The commercial antiviral agent ningnanmycin was used as a positive control.

**Half-Leaf Method[16].** *TMV Infection Inhibition Activities.* The virus was inhibited by mixing it with the solution of compound. After 30 min, the mixture was inoculated on the left side of the

Table 2. $^{13}C$ NMR Data of Limonoids 1−5 (100 MHz) in $CDCl_3$

| | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| 1 | 70.8 | 70.7 | 70.6 | 70.7 | 148.0 |
| 2 | 34.7 | 34.6 | 34.8 | 27.6 | 122.3 |
| 3 | 169.9 | 170.0 | 170.0 | 71.5 | 166.5 |
| 4 | 85.4 | 85.2 | 85.8 | 42.6 | 83.5 |
| 5 | 43.8 | 43.7 | 43.8 | 38.4 | 50.0 |
| 6 | 26.0 | 26.0 | 25.2 | 73.9 | 34.8 |
| 7 | 75.4 | 73.6 | 73.5 | 73.3 | 173.5 |
| 8 | 41.3 | 41.4 | 41.2 | 40.6 | 136.2 |
| 9 | 37.1 | 36.6 | 37.0 | 34.7 | 53.1 |
| 10 | 44.0 | 43.8 | 44.1 | 46.0 | 46.1 |
| 11 | 25.0 | 25.0 | 26.0 | 31.4 | 71.0 |
| 12 | 76.5 | 76.7 | 76.8 | 97.9 | 74.3 |
| 13 | 51.0 | 50.8 | 51.1 | 53.8 | 45.8 |
| 14 | 155.2 | 155.6 | 155.7 | 144.4 | 70.9 |
| 15 | 122.0 | 121.7 | 121.9 | 139.0 | 59.7 |
| 16 | 36.5 | 36.4 | 36.6 | 76.6 | 30.2 |
| 17 | 49.7 | 49.8 | 49.9 | 46.7 | 38.9 |
| 18 | 14.8 | 14.8 | 15.2 | 16.0 | 13.2 |
| 19 | 28.2 | 28.2 | 27.8 | 16.0 | 22.7 |
| 20 | 124.1 | 124.6 | 124.3 | 128.6 | 131.6 |
| 21 | 140.1 | 140.5 | 140.1 | 138.8 | 69.8 |
| 22 | 111.4 | 111.8 | 111.5 | 110.4 | 148.2 |
| 23 | 142.1 | 142.4 | 142.0 | 142.7 | 173.1 |
| 28 | 23.5 | 23.3 | 23.7 | 19.6 | 22.3 |
| 29 | 34.3 | 34.2 | 34.4 | 77.9 | 30.2 |
| 30 | 14.9 | 14.9 | 14.8 | 20.6 | 121.4 |
| 1- | 20.6<br>169.9 | 20.7<br>169.9 | 20.6<br>169.5 | | |
| 3- | | | | 20.2<br>170.2 | |
| 7- | 21.2<br>170.7 | 20.8<br>170.2 | | | 52.3 |
| 11- | | | | | 20.3<br>170.4 |
| 12- | | 21.3<br>170.4 | 21.3<br>170.8 | 53.8 | 20.5<br>169.0 |
| 1′ | 173.8 | | 166.4 | 166.7 | |
| 2′ | 75.5 | | 137.6 | 129.3 | |
| 3′ | 38.2 | | 128.4 | 136.5 | |
| 4′ | 22.6 | | 14.4 | 14.2 | |
| 5′ | 11.6 | | 12.0 | 11.9 | |
| 6′ | 15.2 | | | | |

leaves of *N. glutinosa*, whereas the right side of the leaves was inoculated with the mixture of DMSO solution and the virus as a 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.

*TMV Treatment Activities.* Growing leaves of *N. glutinosa* were mechanically inoculated with purified TMV (30 $\mu$g/mL). After 2 h, the solution of compounds was inoculated on the left side of the leaves of *N. glutinosa*, whereas the right side of the leaves was inoculated with the DMSO as a 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.

*Leaf-Disk Method (**6**).* Growing leaves of *N. tabacum* cv. K326 were mechanically inoculated with equal volumes of TMV (30 $\mu$g/mL). After 72 h, 1 cm diameter leaf disks were removed. The leaf disks were floated on solutions of compounds or ningnanmycin and solutions of DMSO as a negative control. Disks of healthy leaves were floated on DMSO solution as a mock. All leaf disks were kept in a culture chamber at 28 °C for 48 h, and then, the TMV concentration in the leaf disk was determined by enzyme-linked immunosorbent assay (ELISA). The inhibition rate of TMV was calculated as follows: (1 − TMV concentration of treatment/TMV concentration of negative control) × 100%. The TMV concentration was calculated from a virus standard curve constructed using $OD_{405}$ values of TMV at concentrations of 3.75, 1.87, 0.93, 0.46, and 0.23 $\mu$g/mL. Three replicates were tested for each sample.

*ELISA.* ELISA was performed as described by Wang et al.[17] The polyclonal antibodies of TMV and rabbit polyclonal antibody against TMV were prepared to construct the ELISA. The antigen solution consisted of leaf disks ground in 0.05 mol/L coating buffer ($Na_2CO_3$, 1.59 g; $NaHCO_3$, 2.93 g; and $H_2O$, 1000 mL). In each 96-well plate, wells were coated with 100 $\mu$L of polyclonal antibody in 0.05 M sodium carbonate (pH 9.7) for 12 h at 4 °C, and then, the solution was removed, and the wells were washed three times with phosphate buffer containing 0.5% Tween 20 (PBST). Then, the polyclonal antibodies of TMV were diluted by PBST/PVP 1:200, the wells of each 96-well plate were coated with 100 $\mu$L of solution for 1 h at 37 °C, and then, the wells were washed three times with PBST. Then, 100 $\mu$L of mixed solution of monoclonal antibody and peroxidase-conjugated secondary antibody (goat antimouse IgG) (Sigma, St. Louis, MO) in PBS was added. Plates were then incubated for 2 h at 37 °C, before washing a further three times with PBST. Plates were developed by adding 100 $\mu$L of 1 mg/mL on-nitrophenyl phosphate disodium (pNPP) in substrate buffer. They were incubated until the color reaction, and then, the absorbance value was measured at 405 nm using an ELISA plate reader.

*SDS-PAGE and Western Blot Analysis of TMV Coat Protein (CP).* SDS-PAGE was performed as described by Sambrook et al.[18] Briefly, leaf disks from the leaf-disk method were ground in 5 × protein loading buffer (10% SDS, 5% $\beta$-ME, 50% glycerin, 0.5% bromophenol blue, and 250 mM Tris-HCl, pH 6.8), and then, 5 $\mu$L of sample and 3 $\mu$L of marker were loaded on a polyacrylamide gel (5% stacking gel and 12% separating gel). Samples were run in duplicate. After SDS-PAGE, TMV protein bands were transferred at 90 mA for 1 h onto a polyvinylidene fluoride membrane (0.46 $\mu$m, washed by MeOH to active) using an electrotransfer system (Bio-Rad). The membrane was washed in TBST (20 mM Tris-HCl, pH 8.0; 150 nM NaCl; and 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, each time for 3 min with TBST, and reacted with a mixture of 1:30000 alkaline phosphadase-conjugated antirabbit IgG (Sigma) and 1:200 polyclonal antibodies of TMV for 2 h at 37 °C. After it was washed three times, each time for 3 min with TBST, the membrane was incubated in substrate buffer (Tris-HCl, 12.1 g, pH 9.5; NaCl, 5.84 g; MgCl, 10.2 g; and $H_2O$, 800 mL) with 330 $\mu$L/mL NBT and 165 $\mu$L/mL BCIP for 3−5 min in the dark until the bands of the CP were clear.

## RESULTS AND DISCUSSION

**Structure Elucidation of New Compounds.** Munronoid K (**1**) has the molecular formula $C_{36}H_{50}O_{10}$ on the basis of the $[M + Na]^+$ ion peak at $m/z$ 665.3318 (calcd, 665.3301) in the HR-ESI-MS. The IR spectrum indicated the presence of ester (1728 $cm^{-1}$) groups. The $^1H$ and $^{13}C$ NMR data suggested the presence of a $\beta$-substituted furan ring, two acetyls, five methyls, one double bond, and one carbonyl carbon of lactone (Tables 1 and 2). These data were indicative of an evodulone type


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limonoid for **1**. The NMR spectra data of **1** were similar to those of carapolide I,[19] with the only difference being the addition of a 2-hydroxy-3-methylpentanoate at C-12 in **1**. As compared with carapolide I, the observed significant downfield shifts of C-12 ($\delta_C$ 76.5) in the $^{13}C$ NMR spectrum indicated that **1** has an oxygen-containing group in C-12. The exception being the presence of an additional 2-hydroxy-3-methylpentanoate group in **1**, which was located at C-12 ($\delta_C$ 76.5) according to the $^1H-^1H$ correlation spectroscopy (COSY) correlations of H-11 ($\delta_H$ 1.05 m, 2.02 overlapped)/H-12 ($\delta_H$ 4.99, t, $J$ = 8.4 Hz) and the heteronuclear multiple bond correlation (HMBC) correlation between H-12 and the carbonyl carbon of 2-hydroxy-3-methylpentanoate group ($\delta_C$ 173.8, s), the HMBC correlations of H-1/C-1-OAc and H-7/C-7-OAc identified two acetoxyl groups at C-1 and C-7, respectively. The planar structure of **1** was thus established as shown in Figure 1.

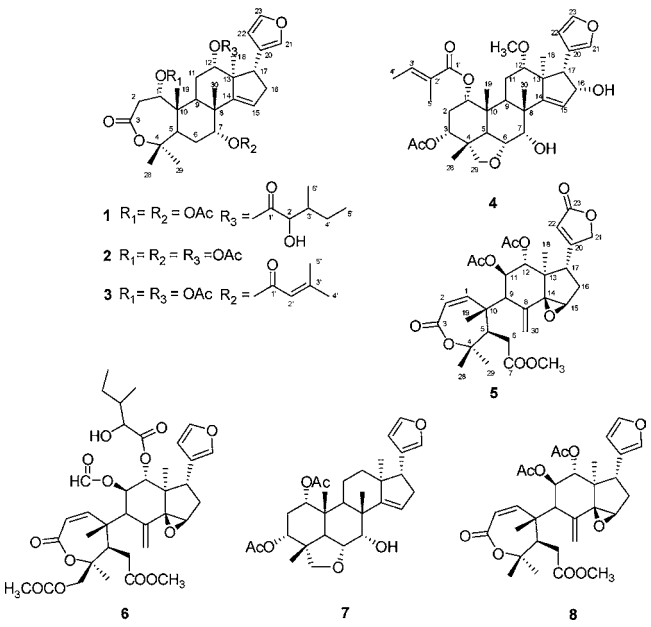


**Figure 1.** Structures of limonoids **1**−**8**.

The relative configuration of **1** was deduced from its rotating frame Overhauser effect spectroscopy (ROESY) correlations. As shown in Figure 3, the observed correlations of $H_3$-29/H-5, H-5/H-9, and H-9/$H_3$-18 indicated that $H_3$-29, H-5, H-9, and $H_3$-18 were assigned as $\alpha$-oriented. The cross-peaks from $H_3$-28 to $H_3$-19, $H_3$-19 to H-7, H-1 to H-12/H-19, H-12 to H-17, and H-30 to H-17 indicated the $\beta$-orientations of $H_3$-28, $H_3$-19, H-1, H-17, H-12, and $H_3$-30, which therefore determined the $\alpha$-orientation of 1-OAc, 7-OAc, and 12-(2-hydroxy-3-methylpentanoate) groups, accordingly.

The molecular formula of munronoid L (**2**) was determined as $C_{32}H_{42}O_9$ on the basis of the $[M + Na]^+$ ion peak at $m/z$ 593.2728 (calcd, 593.2726) in the HR-ESI-MS. The $^1H$ and $^{13}C$ NMR data of **2** were similar to those of **1**. The only difference was the presence of an acetyl at C-12 in **2** instead of the 2-hydroxy-3-methylpentanoate group in **1**. This structural variation was supported by HMBC correlation of H-12 to C-12-OAc ($\delta_C$ 170.4). The HMBC correlations of H-1/C-1-OAc and H-7/C-7-OAc identified two acetoxyl groups at C-1 and C-7, respectively. The planar structure of **2** was thus established as shown in Figure 1. The relative configuration of **2** was suggested to be the same as that of **1** on the basis of similar $^1H$ and $^{13}C$ NMR data and a ROESY spectrum.

The molecular formula of munronoid M (**3**) was determined as $C_{32}H_{42}O_9$ on the basis of the $[M + Na]^+$ ion peak at $m/z$ 633.3039 (calcd, 633.3037) in the HR-ESI-MS. Comparison of the 1D NMR data of **3** with those of **1** showed an overall similarity, except that the 3-methylbut-2-enoate group at C-7 in the former was replaced the 2-hydroxy-3-methylpentanoate group in the latter. The HMBC correlations from H-7 ($\delta_H$ 5.26, brs) to the carbonyl carbon ($\delta_C$ 166.4) of 3-methylbut-2-enoate group confirmed the above deduction. Therefore, the planar structure of **3** was thus established as shown in Figure 1. The relative configuration of **3** was suggested to be the same as that of **1** on the basis of similar $^1H$ and $^{13}C$ NMR data and a ROESY spectrum.

Munronoid N (**4**) has the molecular formula $C_{34}H_{46}O_9$ on the basis of the $[M + Na]^+$ ion peak at $m/z$ 621.3021 (calcd, 621.3039) in the HRESI-MS. The IR spectrum indicated the presence of hydroxyl (3563 $cm^{-1}$) and ester (1732 $cm^{-1}$) groups. The $^1H$ and $^{13}C$ NMR data suggested the presence of a furan ring, one acetyl, one methoxy, six methyls, two double bond, and one carbonyl carbon of ester group (Tables 1 and 2). These data were indicative of a gedunin type limonoid for **4**. The NMR spectra data of **4** were similar to those of meliavolkinin,[13] with the difference being the addition of a hydroxyl at C-16 and a methoxy at C-12 and a 2-methylbut-2-enoate group at C-1 in **4** replaced the methyl benzoate in meliavolkinin. As compared with meliavolkinin, the observed significant downfield shifts of C-12 ($\delta_C$ 97.9) in the $^{13}C$ NMR spectrum indicated that **4** has a methoxyl group, which was located at C-12 according to the $^1H-^1H$ COSY correlations of H-11 ($\delta_H$ 1.56, m, 1H, 1.87, m, 1H)/H-12 ($\delta_H$ 4.60, brs, 1H) and the HMBC correlation between H-12 and the carbon of methoxy ($\delta_C$ 53.8, $\delta_H$ 3.05, s, 3H). The exception being the presence of an additional hydroxyl in **4**, which was located at C-16 ($\delta_C$ 76.6) according to the HMBC correlation between H-16 ($\delta_H$ 4.96, overlapped) to C-14 and C-17. The 2-methylbut-2-enoate group located at C-1 because of the $^1H-^1H$ COSY correlations of H-1 ($\delta_H$ 4.73, brs, 1H)/H-2 ($\delta_H$ 2.15, m, 2H) and the HMBC correlation between H-1 and the carbonyl carbon of 2-methylbut-2-enoate group ($\delta_C$ 166.7). Moreover, the HMBC correlation of H-3/C-3-OAc was identified as an acetoxyl group at C-3. The planar structure of **4** was thus established as shown in Figure 1.

The relative configuration of **4** was deduced from the analysis of its ROESY correlations. As shown in Figure 3, the observed correlations of H-3/H-5 and H-1, H-1/H-6, H-7/H-5, H-1/H-12, H-12/H-17, and H-17/H-16 indicated that H-3, H-6, H-7, H-1, H-12, H-17, and H-16 were assigned as $\beta$-oriented, which then determined the $\alpha$-orientation of the oxygen-containing 5-membered ring between C-4 and C-6 and 1-2-methylbut-2-enoate, 3-OAc, 7-OH, 16-OH, 12-OMe, and furan ring, accordingly. The cross-peaks from $H_3$-18 to $H_3$-12-OMe determined the $\alpha$-orientation of $H_3$-18. The cross-peaks from $H_3$-28 to $H_3$-19 and from $H_3$-19 to $H_3$-30 indicated the $\beta$-orientations of $H_3$-28, $H_3$-19, and $H_3$-30, respectively. Therefore, the structure of **4** was established as shown in Figure 3.

The molecular formula $C_{29}H_{36}O_7$ was assigned to munronoid O (**5**) from its HR-ESI-MS peak at $m/z$ 587.2509 $[M + H]^+$ (calcd, 587.2492). The IR spectrum showed strong absorption bands at 1739 and 1267 $cm^{-1}$, suggesting the presence of carbonyl and ether functionalities. The $^1H$ and $^{13}C$ NMR spectra data (Tables 1 and 2) showed the presence of four tertiary methyls, a methoxy ($\delta_H$ 3.69 s 3H, $\delta_C$ 52.3), an

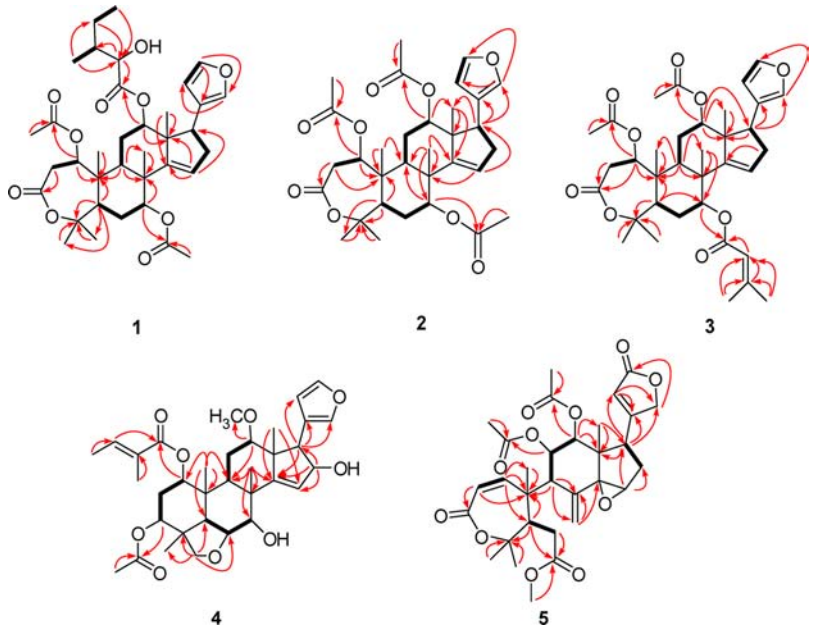


**Figure 2.** $^{1}H-^{1}H$ COSY (—) and selected HMBC (↷) of limonoids **1**−**5**.

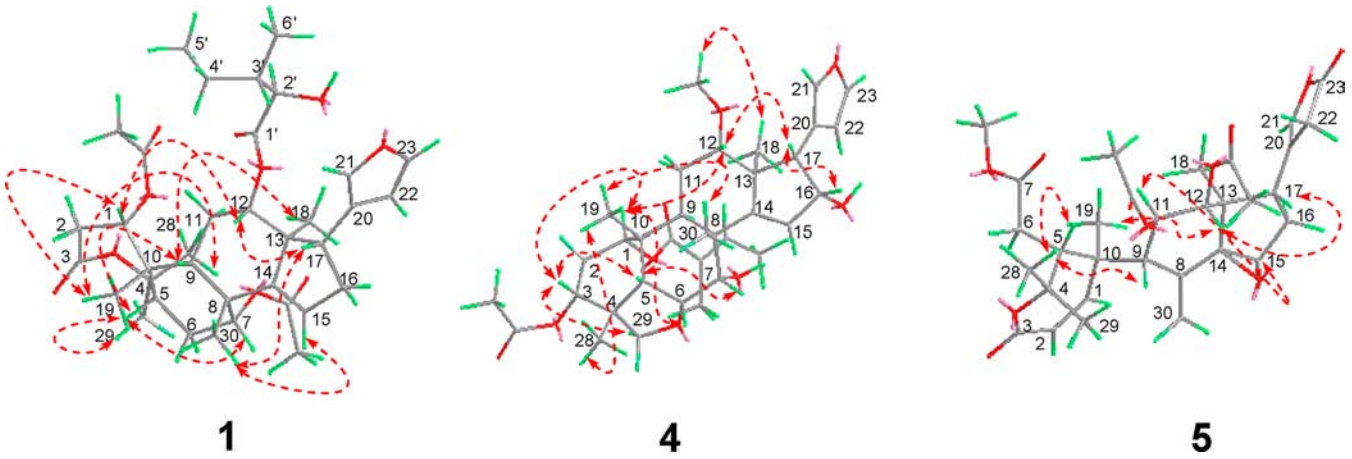


**Figure 3.** Selected ROESY (↷) correlations of limonoids **1**, **4**, and **5**.

**Table 3. Inhibitory Activities against TMV of Limonoids 1−8**

| compd | inhibition rate (%)[a] | inhibition rate (%)[b] |
|---|---|---|
| munronoid K (**1**) | 55.6 ± 3.3 | 67.2 ± 3.4 |
| munronoid L (**2**) | 34.6 ± 4.5 | 54.4 ± 3.9 |
| munronoid M (**3**) | 29.3 ± 6.6 | 45.7 ± 3.6 |
| munronoid N (**4**) | 24.5 ± 3.9 | 30.2 ± 3.2 |
| munronoid O (**5**) | 50.2 ± 4.8 | 64.2 ± 2.6 |
| mombasol (**6**) | 27.7 ± 7.9 | 39.3 ± 3.6 |
| diacetylvilasinin (**7**) | 17.2 ± 7.8 | |
| nymania (**8**) | 39.2 ± 2.4 | 55.4 ± 3.6 |
| ningnanmycin | 42.1 ± 5.4 | 56.5 ± 3.1 |

[a]Treatment rate on *N. glutinosa* in vivo with half-leaf method. The concentrations of compounds were 200 μg/mL. All results are expressed as mean ± SDs; $n = 3$ for all groups. [b]On *N. tabacum* cv. K326 with the DAS-ELISA method. The concentrations of compounds were 30 μg/mL. All results are expressed as mean ± SDs; $n = 4$ for all groups.

exocyclic methylene group ($\delta_H$ 5.23, 5.36, each 1H, $\delta_C$ 121.4), and a γ-lactone ring ($\delta_H$ 7.14, 1H, 4.72, 2H; $\delta_C$ 69.8, 131.6, 148.2, and 173.1), suggesting that compound **5** is a peieurianin type limonoid. However, the characteristic chemical shift of C-21 ($\delta_C$ 69.8), combined with the presence of a C-23 ketone established by the HMBC correlation of H-21 ($\delta_H$ 4.72)/C-23 ($\delta_C$ 173.1) and H-22 ($\delta_H$ 7.14 s)/C-21 and C-20 ($\delta_C$ 131.6) strongly indicated that **5** has a γ-lactone ring moiety. Comparison of the 1D NMR data of **5** with those of nymania (**8**) showed an overall similarity,[13] except that the common furan ring at C-17 in **8** was replaced by a γ-lactone ring group in **5**. The HMBC correlations from H-17 ($\delta_H$ 3.01, 1H, m) to C-20, C-21, and C-22 confirmed the above deduction. Therefore, the planar structure of **5** was fully established as shown in Figure 1. The relative configuration of **5** was suggested to be the same as that of nymania (**8**) on the basis of similar $^{1}H$ and $^{13}C$ NMR data and a ROESY spectrum.

**TMV Inhibitory Activities of Limonoids 1−8.** The inhibitory activities of limonoids **1**−**8** against TMV replication were tested using three approaches: (1) The compounds were assayed by the half-leaf method in *N. glutinosa*, for infection inhibition activities. (2) The ELISA method was used to evaluation of antiviral activity in the local lesion host *N. tabacum* cv. K326. (3) The Western blot analysis was used to verify the

Table 4. Infection Inhibitory Activities against TMV of Limonoids 1−8[a]

| | μg/mL | | | | | | |
|---|---|---|---|---|---|---|---|
| | 30 | 25 | 20 | 15 | 10 | 5 | $IC_{50}$ (μg/mL) |
| munronoid K (1) | 51.9 ± 2.9 | 45.7 ± 3.1 | 38.7 ± 2.7 | 28.6 ± 3.4 | 18.7 ± 3.6 | 6.5 ± 2.9 | 28.3 |
| munronoid L (2) | 47.7 ± 3.4 | 43.9 ± 2.6 | 35.8 ± 3.0 | 25.7 ± 2.4 | 15.3 ± 3.2 | 4.4 ± 2.2 | 34.6 |
| munronoid M (3) | 45.4 ± 4.6 | 42.9 ± 2.4 | 33.5 ± 2.4 | 22.6 ± 1.8 | 11.5 ± 2.2 | | 37.0 |
| munronoid N (4) | 52.0 ± 4.3 | 44.6 ± 2.5 | 37.9 ± 3.1 | 26.4 ± 2.9 | 16.5 ± 2.8 | 8.3 ± 3.4 | 27.9 |
| munronoid O (5) | 64.2 ± 3.6 | 51.4 ± 2.5 | 42.8 ± 3.4 | 33.4 ± 2.8 | 24.6 ± 3.3 | 11.2 ± 2.6 | 22.2 |
| mombasol (6) | 25.9 ± 2.4 | | | | | | |
| diacetylvilasinin (7) | 14.6 ± 4.9 | | | | | | |
| nymania (8) | 58.0 ± 4.3 | 49.8 ± 2.6 | 40.2 ± 3.0 | 29.8 ± 2.6 | 19.6 ± 3.6 | 8.9 ± 3.4 | 22.3 |
| ningnanmycin | 42.6 ± 3.5 | 38.3 ± 2.6 | 32.1 ± 2.6 | 30.3 ± 2.5 | 25.0 ± 3.8 | | 52.1 |

[a]Infection inhibition rate on *N. glutinosa* in vivo with the half-leaf method. The concentrations of compounds were 30 μg/mL. All results are expressed as mean ± SDs; $n = 3$ for all groups.

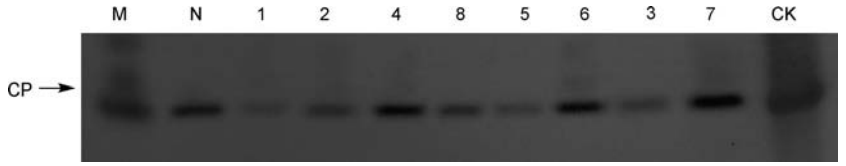


**Figure 4.** Western blot analysis. (A) Inhibition activities of compounds **1**−**8** (30 μg/mL) against TMV replication. M, marker; N, ningnanmycin; and CK, negative control.

inhibition on accumulation of TMV CP. Ningnanmycin, a commercial anti-TMV agent, was used as a positive control.

The treatment rates of compounds **1**−**8** at the concentration of 200 μg/mL tested by the half-leaf method are listed in Table 3. The results showed that all of the compounds exhibited inhibition activities against TMV. On *N. glutinosa* in vivo, compounds **1** and **5** showed higher inhibition rates than ningnanmycin. In the concentration of 200 μg/mL, the inhibitory values of **1**, **5**, and ningnanmycin were 55.6, 50.2, and 42.1%, respectively. In the same concentrations, **2**−**4**, **6**, and **8** showed moderate antiviral activities, with inhibitory values of 34.6 (**2**), 29.3 (**3**), 24.5 (**4**), 27.7 (**6**), and 39.2% (**8**), while **7** showed weak antiviral activities with inhibitory values of 17.2%.

The infection inhibition activities of compounds **1**−**8** at a concentration of 30 μg/mL tested by the half-leaf method are listed in Table 4. Except for compounds **6** and **7**, other compounds exhibited infection inhibition activities against TMV, with higher infection inhibition rates than ningnanmycin on *N. glutinosa* in vivo.

DAS-ELISA was performed as described by Wang et al.[17] Table 3 shows the ability of compounds **1**−**8** (at 30 μg/mL) to inhibit replication of TMV in *N. tabacum* cv. K326, with ningnanmycin (at 30 μg/mL) as the positive control. Except for **7**, all of the compounds exhibited inhibitory activities against TMV replication. In particular, **1** and **5** showed stronger activities than ningnanmycin, and **2** and **8** showed the obvious activities (for the value of $OD_{405}$ of different dosage and inhibition curves constructed using inhibition rates (%) and dosages of compounds, see the Supporting Information).

To assess whether these compounds inhibit TMV replication in systemic infection host *N. tabacum* cv. K326, the leaf-disk method along with Western blot analysis of TMV CP in the presence of 30 μg/mL compound was carried out (Figure 4). SDS-PAGE was performed as described by Sambrook.[18] The bands of CP were weak when treated with compounds **1** and **5**, which indicated strong activities, while when treated with compounds **2**, **3**, **8**, and ningnanmycin they showed moderate activities, and **4**, **6**, and **7** showed low activities. This result was in accordance with their inhibitory rates in those of the ELISA method, which further confirmed that limonoids from *M. unifoliolata* Oliv. could inhibit the accumulation of TMV CP in vitro.

## ■ ASSOCIATED CONTENT

### Ⓢ Supporting Information

Supporting figures. This material is available free of charge via the Internet at http://pubs.acs.org.

## ■ AUTHOR INFORMATION

### Corresponding Author

*Tel: (+86)0851-3804492. Fax: (+86)0851-3805081. E-mail: haoxj@mail.kib.ac.cn.

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### Notes

The authors declare no competing financial interest.

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