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ARTICLE

Tobacco Mosaic Virus (TMV) Inhibitors from *Picrasma quassioides* Benn

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To investigate natural inhibitors against tobacco mosaic virus (TMV) from plants, 10 known β-carboline alkaloids and one quassinoid have been isolated from MeOH extract of the wood of *Picrasma quassioides* Benn. These compounds were screened for their inhibitory activities against tobacco mosaic virus (TMV). The activity of each compound against TMV infection and replication was tested using a half-leaf assay method, a leaf-disk method, and Western blotting analyses. All of the β-carboline alkaloids showed moderate anti-TMV activities and exhibited synergistic effects when combined with the quassinoid nigakilactone B (**11**). To our knowledge, this is the first report on anti-TMV activity of β-carbolines and their synergistic effects against TMV when combined with a quassinoid.

KEYWORDS: Tobacco mosaic virus (TMV); *Picrasma quassioides* Benn.; β-carboline; synergistic effects

INTRODUCTION

Viral plant diseases are found worldwide and are serious threats to modern agriculture. Tobacco mosaic virus (TMV) is one of the most well-studied plant viruses, but its control remains a challenge (*1*). There are no chemical treatments that fully protect plants from TMV infection, nor are there any that eliminate TMV from infected plant tissues under field conditions. Plants have evolved to produce secondary metabolites with antimicrobial activities to selectively suppress pathogens (*2*). Therefore, selection of natural products with anti-TMV activity could be an effective method to control this plant disease.

In our ongoing studies on naturally occurring anti-TMV active compounds from plants (*3*−*5*), *Picrasma quassioides* Benn. (Simaroubaceae) has attracted our attention. *P. quassioides* (Chinese name: kumu) is widely distributed throughout western and southern China. It is seldom infected by viruses or pests (*6*), which suggests that it has an effective chemical defense system. A number of quassinoids and β-carboline alkaloids have been isolated from this plant (*7*−*12*). The quassinoid isolated from *Brucea javanica* (Simaroubaceae) exhibited significant inhibitory activity against TMV (*13*). The β-carboline alkaloids were shown to have significant antiviral activity, although they have not yet been tested against TMV (*14*−*16*). Therefore, it is conceivable that the β-carboline alkaloids are part of a suite of chemical defenses that protect against infection by viruses including TMV.

In this study, 10 known β-carboline alkaloids and a quassinoid were isolated from the wood of *P. quassioides*. Structures of the compounds were elucidated by comparison of their MS and NMR data with those reported in the literature. The crude extracts and all of the compounds have been screened for their inhibitory activities against TMV. The synergistic effects against TMV of β-carboline and quassinoid were observed. This is the first report of a synergistic effect between naturally occurring anti-TMV compounds.

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MATERIALS AND METHODS

General Experimental Procedures. ^{1}H and ^{13}C NMR spectra were acquired on Bruker DRX-500 and AM-400 spectrometers (Massachusetts) 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). 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), and sephadex LH-20 (40−70 μm) (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Thin-layer chromatography was conducted on precoated silica gel plates GF_{254} (Qingdao Haiyang Chemical Co., Qingdao, China). Spots on chromatograms were detected by spraying with Dragendorff's reagent and 10% H_2SO_4-EtOH. Plants and plant leaves kept in a LRH-250-G culture chamber (Zhujiang, Guangzhou, China). Absorbance values of ELISA assays were measured using an ELXx808 ELISA plate reader (Bio-TEK, Winooski,VT). SDS-PAGE and Western blotting were carried out using a Bio-Rad electrotransfer system (Bio-Rad, Hercules, CA).

Plant Materials. The wood of *P. quassioides* was collected in Kunming, Yunnan province, China, in October 2007. The specimen was identified by Professor Deding Tao of Kunming

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Institute of Botany (KIB), Chinese Academy of Sciences (CAS). A voucher specimen (NO.200710213) has been deposited in the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany.

Extraction and Isolation. Air-dried wood chips of *Picrasma quassioides* Benn. (15.0 kg) were exhaustively extracted with MeOH (four times under reflux for 4, 3, 3, and 2 h). The solvent was removed under reduced pressure to give a residue (2200.0 g, 14.7%), which was separated by 4.0 kg silica gel G (100−200 mesh) on a 600 mm × 250 mm i.d. column to give three fractions: EtOAc (A, 1100 g, 7.3%), $CHCl_3$/MeOH (1:1) (B, 840.0 g, 5.6%), and *n*-BuOH (C, 220.0 g, 1.5%). Fraction A was further separated into six fractions (Fr. A1−A6) on a silica gel G column (650 mm × 100 mm i.d., 200−300 mesh) and eluted with PE/Me_2CO and $CHCl_3$/MeOH, depended on TLC results by spraying with Dragendorff's reagent or spraying with 10% H_2SO_4-EtOH and heated to 120 °C. Fr. A3 showed most spots of target compounds, which was purified by Sephadex LH-20 ($CHCl_3$−MeOH = 1:1) (2000 mm × 30 mm i.d.), then subjected to on a silica gel G column (600 mm × 50 mm i.d., 200−300 mesh) and eluted with PE and EtOAc (100:1−10:1), and finally repeatedly purified by LH-20 ($CHCl_3$−MeOH = 1:1 or MeOH) (2000 mm × 15 mm i.d.) to give compounds **2** (190.0 mg), **3** (293.0 mg), **4** (37.0 mg), **5** (39.0 mg), **7** (424.0 mg), **8** (17.0 mg), and **11** (27.0 mg). Fraction B was subjected to repeated column chromatography (CC) on silica gel eluted with $CHCl_3$/MeOH (80:1−1:1) and purified over Sephadex LH-20 with $CHCl_3$/MeOH (1:1) (2000 mm × 15 mm i.d.) to give compounds **1** (19.0 mg), **6** (383.0 mg), **9** (20.0 mg), and **10** (19.0 mg).

β-Carboline-1-yl-4,8-dimethoxy-*β*-carboline-1-ylethyl ketone (**1**): yellow prisms ($CHCl_3$-MeOH), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*17*).

4-Hydroxy-1-methoxycarbonyl-*β*-carboline (**2**): yellow needle crystals ($CHCl_3$-MeOH), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*18*).

1-Methoxycarbonyl-*β*-carboline (**3**): yellow needle crystals (acetone), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*17*).

4,8-Dimethoxy-1-vinyl-*β*-carboline (**4**): yellow needle crystals (MeOH), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*19*).

1-Ethyl-8-methoxy-*β*-carboline (**5**): yellow crystals ($CHCl_3$), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*20*).

5-Methoxycanthin-6-one (**6**): yellow needle crystals (MeOH), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*21*).

4.5-Dimethoxycanthin-6-one (**7**): yellow needle crystals ($CHCl_3$), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*22*).

4-Hydroxy-5-methoxycanthin-6-one (**8**): yellow needle crystals ($CHCl_3$), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*10*).

1,2,3,4-Tetrahydro-1,3,4-trioxo-*β*-carboline (**9**): yellow prisms (MeOH−H_2O), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*11*).

8-Methoxy-1,2,3,4-tetrahydro-1,3,4-trioxo-*β*-carboline (**10**): orange prisms (MeOH−H_2O), identified by comparing the MS and NMR (^{1}H NMR, ^{13}C NMR) data with those reported in the literature (*11*).

Nigakilactone B (**11**): white prisms ($CHCl_3$−MeOH); $[\alpha]_D$ = +9.6° (*c* = 0.35, MeOH), identified by comparison of MS and NMR (^{1}H NMR, ^{13}C NMR) spectroscopic data with those reported in the literature (*23*).

ANTI-TMV ASSAYS

Preparation of Screening Materials. TMV (strain U1) was propagated and maintained in the systemic host *Nicotiana tabacum* cv. K_{326}, and purified as described by Gooding (*24*). The concentration of TMV was determined as 16 mg/mL by an ultraviolet spectrophotometer [virus concentration = (A_{260} × dilution ratio)/$E_{1\,cm}^{0.1\%,260\,nm}$]. The purified virus was kept at −20 °C and was diluted to 32 *μ*g/mL with 0.01 M PBS before use.

Nicotiana glutinosa and *N. tabacum* cv.K_{326} plants were cultivated in a disease-free greenhouse. *N. glutinosa* was used to determine systemic TMV infection, and *N. tobacum* was used as a local lesion host. Plants with similar leaf-size and weight were used for each experiment.

The tested compounds were dissolved in DMSO, and diluted with distilled H_2O to the required concentration. The final concentration of DMSO was 25 *μ*L/mL, and at this concentration did not adversely affect the plants. We used the commercial antiviral agent Ningnanmycin (EC_{50} = 55.6 *μ*g/mL) as a positive control. Ningnanmycin is a successful registered anti-TMV agent available in China, however, its mechanism of action is not yet clear (*25*).

Assay of Compounds' TMV Replication Inhibition Activities: Half-Leaf Method (*5*). Growing leaves of *N. glutinosa* were mechanically inoculated with purified TMV (32 *μ*g/mL). After 2 h, each leaf was cut along the main vein. One half of the leaf was immersed into a solution of the crude extract or compound, and the other was immersed into 25 *μ*L/mL DMSO solution as the negative control. A healthy leaf was immersed into 25 *μ*L/mL DMSO solution as mock. Leaves were kept in a culture chamber at 25 °C for 48 h, and then the rate of TMV inhibition was calculated as follows: (1 − average number of local lesions of treatment/average number of local lesions of negative control) × 100%. Three replicates were tested for each sample.

Assay of Compounds' TMV Replication Inhibition Activities: Leaf-Disk Method (*5*). Growing leaves of *N. tabacum* cv.K_{326} were mechanically inoculated with equal volumes of TMV (32 *μ*g/mL). After 6 h, 1 cm diameter leaf discs were removed. Discs were floated on solutions of crude extracts or compounds and on 25 *μ*L/mL DMSO solution as the negative control. Discs of healthy leaves were floated on 25 *μ*L/mL DMSO solution as mock. All leaf discs were kept in a culture chamber at 25 °C for 48 h, and then the TMV concentration in the leaf disk was determined by TAS-ELISA. The inhibition rate of TMV was calculated as follows: (1−TMV concentration of treatment/TMV concentration of negative control) × 100%. TMV concentration was calculated from a virus standard curve constructed using OD_{405} values of TMV at concentrations of 3.2, 1.6, 0.8, 0.4, and 0.2 *μ*g/mL. Three replicates were tested for each sample. We also evaluated the inhibition rate of compounds at concentrations of 10, 20, 40, and 80 *μ*g/mL, then constructed a concentration-inhibition curve for each compound to determine the 50% inhibition rate (EC_{50}).

Assay For Inhibition Of TMV Infection. The crude extracts and compounds were mixed with TMV to the required concentration. After 30 min, the mixture was mechanically inoculated onto the left side of leaves of *N. glutinosa* as the treatment, while the right side of the leaves was inoculated with a mixture of 25 *μ*L/mL DMSO solution and TMV as the negative control. Leaves were

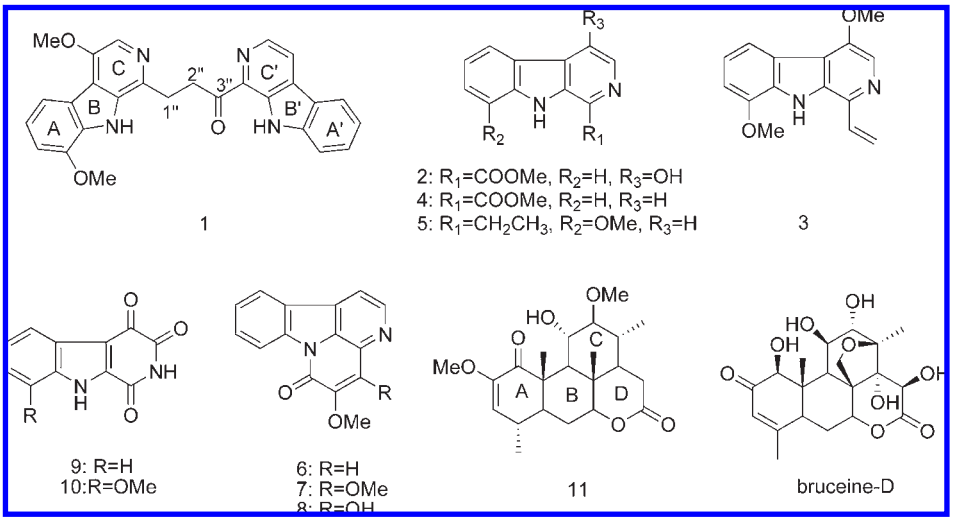

Figure 1. Structures of compounds **1**−**11**.

inoculated with only 25 μL/mL DMSO as mock. Plants were kept in a culture chamber at 25 °C for 5−6 days, then the number of local lesions on the two leaf halves were compared. The TMV inhibition rate was calculated as follows: (1 − average number of local lesions of treatment/average number of local lesions of negative control) × 100%. Three replicates were tested for each sample.

Triple Antibody Sandwich ELISA (TAS-ELISA). TAS-ELISA was performed as described by Wang et al. (*26*). A mouse monoclonal antibody and a rabbit polyclonal antibody against TMV were prepared to construct the TAS-ELISA. In each 96-well plate, wells were coated with 100 μL polyclonal antibody in 0.05 M sodium carbonate (pH 9.7) for 3 h at 37 °C, then the solution was removed and wells were washed three times with phosphate buffer containing 0.5% Tween 20 (PBS). The antigen solution consisted of leaf discs ground in coating buffer (15 mol/L Na_2CO_3, 35 mol/L $NaHCO_3$, pH 9.6). The antigen solution was added to the well (100 μL per well) and incubated for 3 h at 37 °C, then the solution was removed and wells were washed four times with phosphate buffer containing 0.5% Tween 20 (PBS). Blocking solution (200 μL of 1% bovine serum albumin (BSA) in PBS) was added to each well and the plate was incubated for 40 min at 37 °C. The wells were then washed three times with PBS before addition of 100 μL mixed solution of monoclonal antibody and peroxidase-conjugated secondary antibody (goat antimouse IgG) (Sigma, St. Louis, MO) in PBS. Plates were then incubated for 3 h at 37 °C, before washing a further three times with PBST. Plates were developed by adding 200 μL of 1 mg/mL 4-nitrophenyl phosphate bis-(cyclohexylammonium) salt in 10% diethanolamine. The absorbance value was measured at 405 nm using an ELISA plate reader (Bio-TEK, Winooski,VT).

SDS-PAGE and Western Blot Analysis Of TMV Coat Protein (CP). SDS-PAGE was performed as described by Sambrook (*27*). Briefly, leaf discs from the leaf-disk method were ground in protein loading buffer (40 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), then 5 μL sample and 3 μL marker were loaded on a polyacrylamide gel (12.5% 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 μm) using an electrotransfer system (Bio-Rad, Hercules, CA). The membrane was washed in TBS (1 mol/L Tris-HCl, pH 7.5; 1 mol/L NaCl; 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 TBS, 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. Then, after washing three times for 15 min with TBS, 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 μL/mL NBT and 165 μL/mL BCIP for 3−5 min in the dark at 25 °C.

RESULTS AND DISCUSSION

Air-dried wood chips of *P. quassioides* were extracted with MeOH and the extract was separated by column chromatography (CC) on silica gel into three fractions, EtOAc (A, 1100.0 g, 7.3%), $CHCl_3$-MeOH (1:1) (B, 840.0 g, 5.6%), and *n*-BuOH (C, 220.0 g, 1.5%). Compounds **2** (190.0 mg), **3** (293.0 mg), **4** (37.0 mg), **5** (39.0 mg), **7** (424.0 mg), **8** (17.0 mg), and **11** (27.0 mg) were isolated from fraction A, while **1** (19.0 mg), **6** (383.0 mg), **9** (20.0 mg), and **10** (19.0 mg) were obtained from fraction B. (**Figure 1**).

TMV Inhibition Activities of Crude Extracts. We tested inhibition activities against TMV of crude extracts of *P. quassioides* using the half-leaf method and the leaf-disk method. Extracts at a concentration of 2 mg/mL showed no inhibition activity. It is possible that this lack of inhibition activity was due to the very low concentration of active compounds in the crude extracts.

TMV Inhibition Activities of Compounds. The inhibitory activities of compounds **1**−**11** against TMV replication were tested using two approaches. First, the half-leaf method was used to test each compound's antiviral activity in the local lesion host, *N. glutinosa*. Then, the leaf-disk method was used to evaluate each compound's antiviral activity in the systemic infection host, *N. tabacum* cv.K_{326}.

Table 1 shows the ability of compounds **1**−**11** (at 50 μg/mL) to inhibit replication of TMV in *N. glutinosa,* with Ningnanmycin as the positive control. β-Carbolines **1**−**10** exhibited moderate inhibition activities against TMV replication, but were not more effective than Ningnanmycin. These ten compounds were further tested on *N. tabacum* cv. K_{326} and the concentration of TMV was assayed by TAS-ELISA (**Figure 2**). Inhibitory activities against TMV replication were calculated from the TMV standard curve. The inhibitory activities showed a similar trend but a greater magnitude compared to the *N. glutinosa* test, and 1-methoxycarbonyl-β-carboline (**3**) inhibited TMV replication more effectively than Ningnanmycin. The quassinoid nigakilactone B (**11**) slightly inhibited TMV replication in both *N. glutinosa* and *N. tabacum* cv.K_{326}.

To assess whether compounds **1**−**11** inhibit TMV replication in plants, Western blot analysis of TMV CP accumulation in the presence of compounds **1**−**11** (50 μg/mL) was carried out (**Figure 3A**). The results further confirmed that β-carbolines reduced accumulation of the TMV CP, and 1-methoxycarbonyl-β-carboline (**3**), 4, 5-Dimethoxycanthin-6-one (**7**) inhibited TMV accumulation more effectively than Ningnanmycin. Nigakilactone B (**11**) did not affect TMV CP accumulation. The quantity of TMV CP decreased with increasing concentrations of compounds in a dose-dependent manner (e.g., **Figure 3B**, compound **3**). Moreover, the EC_{50} values of compounds **1**, **3**, **7**, and **10** were determined to be 58.8, 40.4, 53.0, 62.4 μg/mL, respectively (**Table 2**). These values are relatively similar, and are higher than that of Ningnanmycin, except for 1-methoxycarbonyl-β-carboline (**3**). Because compound **1** is a dimer, we adjusted the EC_{50} units of aforementioned compounds from μg/mL to mM to compare their activities more logically. Compound **1** then showed the strongest inhibition activity against TMV.

Table 1. TMV Replication Inhibition Activities of Compounds on *N. glutinosa* and *N. tabacum* cv. K_{326}

	N. glutinosa. inhibition% ± SE		*N. tabacum* cv. K_{326}. inhibition% ± SE	
compound	alone (50 μg/mL)	mixed with **11** (25 μg/mL each)	alone (50 μg/mL)	mixed with **11** (25 μg/mL each)
1	39.4 ± 5.7	52.4 ± 3.7	28.9 ± 6.0	64.8 ± 4.5
2	41.7 ± 1.9	55.6 ± 1.5	45.7 ± 5.2	62.3 ± 4.9
3	47.4 ± 3.0	68.4 ± 1.9	60.4 ± 4.8	71.9 ± 6.4
4	38.5 ± 3.5	47.1 ± 2.5	39.7 ± 0.9	63.0 ± 3.5
5	25.0 ± 1.4	36.4 ± 6.1	20.9 ± 4.3	48.4 ± 2.7
6	40.0 ± 4.5	50.0 ± 5.0	27.5 ± 2.3	45.7 ± 2.9
7	33.3 ± 4.5	43.8 ± 3.5	42.6 ± 6.1	59.0 ± 2.5
8	35.7 ± 2.6	53.8 ± 6.0	25.6 ± 3.0	52.6 ± 3.2
9	29.4 ± 3.9	33.3 ± 3.2	29.5 ± 3.9	35.8 ± 2.2
10	39.5 ± 4.0	47.3 ± 1.5	35.1 ± 2.0	59.8 ± 5.6
11	6.7 ± 2.0		3.2 ± 1.7	
Ningnanmycin	54.5 ± 1.7	53.8 ± 1.8	51.0 ± 1.5	52.6 ± 2.1

The inhibition activities of compounds **1**−**11** (50 μg/mL) against TMV infection were also assayed in the local lesion host *N. glutinosa*. Inhibitory values for the 11 compounds were 45.5, 60.0, 71.4, 53.3, 45.5, 36.4, 58.3, 42.9, 44.4, 50.0, and 10.0%, respectively (**Table 3**).

Some β-carboline alkaloids showed antiviral activity. Studies have shown that β-carboline derivatives interact with the RNA of the HIV virus and induce changes in RNA conformation that inhibit viral multiplication (*14*). Recently, it was reported that high-affinity binding of β-carboline derivatives to the RNA targets was controlled by the electrostatic interactions of the aromatic heterocyclic ring structures, optimized linker length, and electronic substituents on the β-carboline ring (*28*, *29*). Similarly, analysis of the structure−activity relationship showed that anti-TMV activities of the various β-carboline alkaloids from *P. quassioides* were influenced by their basic aromatic heterocyclic structures and electrostatic potentials of their amino groups. Together, these results suggest that β-carboline alkaloids affect TMV RNA replication by binding with its nucleic acids. However, the detailed mechanisms of this interaction remain unclear. Our results show that β-carboline alkaloids of *P. quassioides* not only inhibit TMV replication but also inhibit TMV infection. To the best of our knowledge, this is the first report on the anti-TMV activities of β-carbolines alkaloids.

The quassinoid nigakilactone B from *P. quassioides* showed only weakly inhibitory activities against TMV (**Tables 1**, **2**,

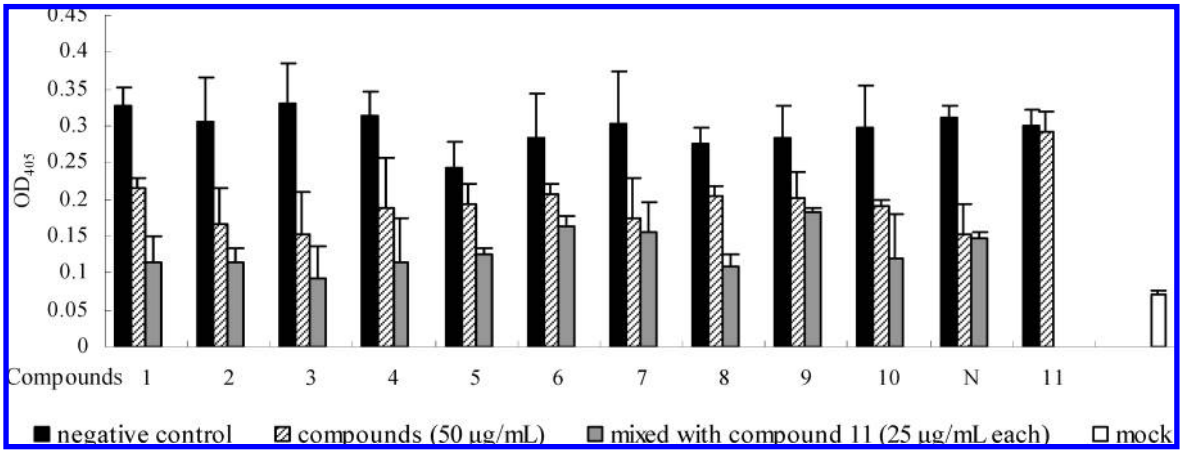

Figure 2. Inhibition activities of compounds against TMV replication in *N. tabacum* cv.K_{326} as determined by TAS-ELISA (SD < 10). N, Ningnanmycin.

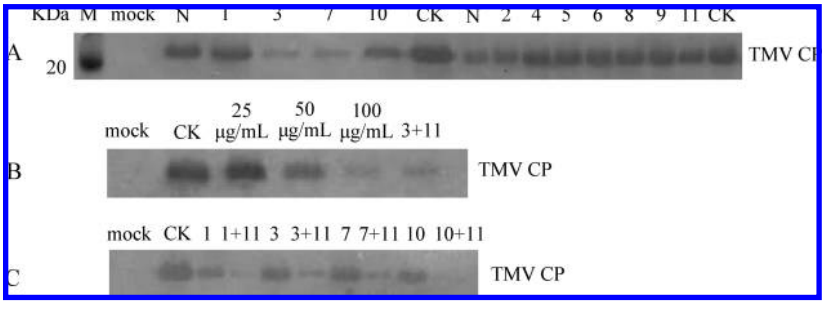

Figure 3. Western blot analysis. (A) Inhibition activities of compounds **1**−**11** (50 μg/mL) against TMV replication. (B) Dose-dependent inhibition of TMV replication (Compound **3**). (C) Synergistic effects of compounds **1, 3, 7, 10** with compound **11** (50 μg/mL). M, marker; N, Ningnanmycin; CK, negative control; 1 + 11, 3 + 11, 7 + 11, 10 + 11, compounds **1**, **3**, **5**, **7**, and **10** mixed with compound **11** (25 μg/mL each).

Table 2. EC_{50} Values of Selected Compounds Against TMV on *N. tabacum* cv.K_{326}

	Inhibition%					
concentration (μg/mL)	10 (μg/mL)	20 (μg/mL)	40 (μg/mL)	80 (μg/mL)	EC_{50} (μg/mL)	EC_{50} (mM)
1 (50 μg/mL)	15.6	25.9	39.9	62.5	58.8	0.131
1+**11** (25 μg/mL each)	26.2	38.6	61.9	89.3	35.3	0.078
3 (50 μg/mL)	21.0	27.6	61.5	79.0	40.4	0.179
3+**11** (25 μg/mL each)	34.8	43.2	70.5	90.4	26.0	0.115
7 (50 μg/mL)	12.0	24.5	48.9	64.9	53.0	0.189
7+**11** (25 μg/mL each)	29.5	38.5	56.4	78.6	36.4	0.130
10 (50 μg/mL)	11.9	20.8	30.6	63.7	62.4	0.257
10+**11** (25 μg/mL each)	29.3	37.3	50.1	68.1	44.4	0.183
Ningnanmycin (50 μg/mL)	12.3	24.2	41.3	60.6	59.8	0.135
Ningnanmycin+**11** (25 μg/mL each)	12.9	26.3	40.9	63.7	57.2	0.129

Table 3. TMV Infection Inhibition Activities of Compounds on *N. glutinosa*

compound	alone (50 μg/mL)	mixed with **11** (25 μg/mL each)
1	45.5 ± 1.3	68.3 ± 3.4
2	60.0 ± 5.1	69.2 ± 2.0
3	71.4 ± 5.7	73.3 ± 4.3
4	53.3 ± 6.7	58.3 ± 5.7
5	45.5 ± 2.8	50.0 ± 1.0
6	36.4 ± 1.4	58.3 ± 1.4
7	58.3 ± 5.6	61.1 ± 4.2
8	42.9 ± 1.3	66.7 ± 1.4
9	44.4 ± 3.7	52.1 ± 0.5
10	50.0 ± 6.7	55.9 ± 6.1
11	10.0 ± 4.2	
Ningnanmycin	60.0 ± 2.1	55.6 ± 2.8

Figures 2 and **3A**). However, the quassinoid bruceine-D (**Figure 1**) significantly inhibits TMV infection and replication (*13*). It is possible that nigakilactone B lacks some essential group that is required for anti-TMV activity. Comparing the structures of the two compounds, nigakilactone B lacks an epoxymethano bridge between C-8 and C-13 in ring C. It may be that this bridge is an essential requirement for anti-TMV activity of quassinoids.

Synergistic Effects Between β-Carboline Alkaloids And Quassinoids Against TMV. Although the quassinoid nigakilactone B from *P. quassioides* only weakly inhibited TMV, a synergistic anti-TMV effect was observed when it was combined with isolated β-carboline alkaloids. The inhibitory activity of the β-carboline alkaloids−nigakilactone B (**11**) mixture (25 μg/mL each) was significantly greater than the effect of the corresponding β-carboline (50 μg/mL) alone (**Tables 1**, **3**, **Figure 3B** and **C**). For example, compounds **1**, **3**, **7**, and **10** mixed with nigakilactone B (25 μg/mL each) showed significantly lower EC_{50} (mM) values, indicating that all had higher activities than the positive control, except compound **10** (**Table 2**). These results demonstrate the synergistic effects between isolated β-carboline alkaloids and the quassinoid nigakilactone B (**11**) against TMV.

This is the first report of a synergistic effect between naturally occurring anti-TMV compounds in plants. This synergistic effect between β-carboline alkaloids and a quassinoid suggests an interesting and unique mechanism that requires further investigation. It is likely that other secondary metabolites in plants have similar synergistic effects. As suggested by the screening hypothesis, plants generate the most diverse secondary metabolites at the lowest metabolic cost to economically defend themselves against disease (*30*, *31*). Therefore, such synergistic effects could be a useful strategy to reduce costs while protecting against a wider variety of pathogens. Thus, more attention should be focused on synergistic effects when selecting materials with various activities from plants.

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

LITERATURE CITED

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