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Characterization of anti-TMV indole alkaloid and isocoumarin derivatives from *Aspergillus versicolor* YNCA0363

Hua-Yin Liu[1,2], Yue-Yu Ma[2], Zhen-Jie Li[3], Xue-Mei Li[3], Yin-Ke Li[2,3], Wei-Guang Wang[2], Min Zhou[2], Qiu-Fen Hu[2,3*] and Feng-Xian Yang[1,2*]

Abstract

Background Tobacco mosaic virus (TMV) is a harmful plant pathogen that causes a decline in the quality and yield of many economic crops. Natural products are important potential sources of biopesticides for the prevention and treatment of TMV. This study focuses on the discovery of anti-TMV active compounds from *Aspergillus versicolor* and investigates their activities against TMV.

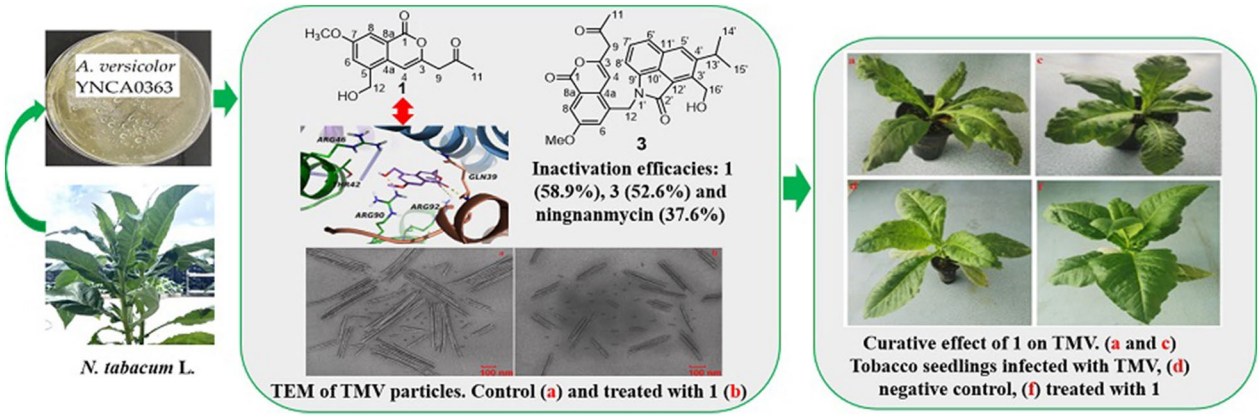
Results In this study, four isocoumarins 7-methoxy-3-(2-oxopropy)-5-hydroxymethyl-isocoumarin (**1**), 7-methyl-3-(2-oxopropy)-5-hydroxymethyl-isocoumarin (**2**), oryzaein A (**4**) and oryzaein B (**5**), two indole alkaloids aspergilline F (**6**) and aspergilline G (**7**), and one indole alkaloid and isocoumarin hybrid aspergillactone A (**3**) were isolated from *Nicotiana tabacum*-derived *A. versicolor* YNCA0363. Among them, compounds **1–3** are new isolates, compound **3** represents the first example of indole alkaloid and isocoumarin connected by C(12)-N(1′) bond. The inactivation efficacies for compounds **1**, **2** and **3** were 58.9, 43.8 and 52.6% at the concentration of 50 μg/mL, respectively, which were significantly higher than that of positive control, ningnanmycin. The protective effects of these three compounds ranged from 48.6 to 62.3%, which were significantly higher than that of positive control. At the same time, the content of TMV-CP was also significantly lower than that of positive control, and compound **1** was the lowest. The curative efficacy for compound **1** was also much better than that of positive control. Transmission electron microscopy (TEM) showed that compound **1** could directly destroy viral particles into small fragments. The results of molecular docking showed that the binding ability of compounds **1**, **3**, **2** to TMV-CP protein decreased in turn, which was consistent with the results of activities assays.

Conclusion Compounds **1–3** from *A. versicolor* showed potent antiviral activities against TMV including inactivation, protective and curative effects. Compound **1** can directly destroy the virus particles to achieve the effect of anti-TMV. In addition, compounds **1–3** can bind to TMV-CP protein in molecular docking experiments. The above experimental results show that TMV-CP was an important target for active indole alkaloid and isocoumarin derivatives to fracture TMV particle. The results provided evidence that indole alkaloid and isocoumarin derivatives from *A. versicolor* have the potential to control TMV.

Keywords *Aspergillus versicolor*, Anti-TMV activity, Isocoumarin, Indole alkaloid

*Correspondence:
Qiu-Fen Hu
huqf_tg@163.com
Feng-Xian Yang
yangfengxian90@163.com
Full list of author information is available at the end of the article

Graphical Abstract



Background

Plant viruses reduce the yield and quality of crops worldwide and cause huge economic losses [1]. The economic losses caused by plant viruses are as high as $ 30 billion per year [2]. The fact is that TMV is a very important plant virus, it can infect more than 885 species of plants belonging to 165 families, and can survive in dead plants for 100 years [3]. Because plants have not developed an immune system, it is difficult for plants to inhibit the proliferation of viruses [4, 5]. In addition, the anti-plant virus chemicals currently in use are economically unaffordable, so the prevention and treatment of plant viral diseases is very difficult [6]. In tobacco plants, virus-free plants [7], induction of cross-protection [8–10] and cultivation of resistance plants [10, 11] have been used as preventive measures to prevent TMV disease. In fact, once it is infected by a virus, no pesticide can completely eradicate the virus [12]. Therefore, it is a very urgent task to find new effective virus inhibitors.

Natural products are mainly a class of compounds derived from animals, plants and microorganisms [13–18]. They are often used to control diseases in crops due to their environmental safety, low toxicity, and very effective against pathogens [19–21], but are safe to non-target organisms [6]. The endophytic fungi have played an important role in the history of drug discovery and chemical leads with promising biological activities [22]. Among the most dominated fungal genera, *Aspergillus* has been described as a rich source for a plethora of bioactive compounds with diverse chemical structures and biological activities [23]. *Aspergillus* is one of the most familiar filamentous fungi belonging to Ascomycetes (family Trichocomaceae) [24]. Different endophytic *Aspergillus* species have proved their ability to produce plenty of secondary metabolites including butenolides, alkaloids, terpenoids, coumarin, cytochalasins, phenalenones, r-terphenyls, xanthones, sterols, diphenyl ether and anthraquinone derivatives with diverse biological activities, such as anti-cancer, antifungal, anti-bacterial, anti-viral, anti-inflammatory, antitrypanosomal and antileishmanial activities [25]. Of course, *Aspergillus* also produces some important toxins, including aflatoxins, which are harmful to human and animal health [26]. Among these secondary metabolites, some alkaloids [27] and isocoumarins [28, 29] obtained from *Aspergillus* fungi in our laboratory have excellent anti-TMV activity. Previous studies on the chemical constituents of *A. versicolor* YNCA0363 found a series of new indole alkaloids with anti-TMV activity [30]. To explore more analogues with better anti-TMV activity, the strain was re-fermented and its subsequent chemical constituents were studied. In addition to their antiviral activities including protective, inactivation and curative activities against TMV, we studied the mode of action of compound **1** on TMV particles. The results of this study provide scientific evidence for using the compounds from the strain as a powerful tool for plant TMV virus control.

Materials and methods

General experimental procedures

One- and two-dimensional (1D and 2D) nuclear magnetic resonance (NMR) spectroscopic data were recorded on a DRX-500 NMR spectrometer in $CDCl_3$ with TMS serving as internal standard. Infrared (IR) measurements were carried out on a Bio-Rad FTS185. Ultraviolet (UV) spectra were recorded in MeOH on a Shimadzu UV-1900 spectrophotometer. Electrospray ionization mass spectrometry (ESIMS) and high resolution

ESIMS (HRESIMS) data were measured on an Agilent 1290UPLC/6540 Q-TOF mass spectrometer. Column chromatography (CC) was performed using silica gel (200–300 mesh, Qingdao Marine Chemical, Inc., Qingdao, China), Lichroprep RP-18 gel (40–63 μm, Merck, Darmstadt, Germany) and Sephadex LH-20 (Sigma-Aldrich, Inc, USA). Preparative high performance liquid chromatography (HPLC, Zorbax PrepHT GF C_{18} 21.2 mm × 25 cm) utilized isocratic elution conditions with a flow rate of 10 mL/min on an Agilent 1260 HPLC system operating at room temperature equipped with a photodiode array detector. Thin-layer chromatography (TLC) was performed on silica gel GF_{254} plates (Qingdao Marine Chemical, Inc., Qingdao, China) to monitor compounds with a UV detector at 254 nm combined with 15% H_2SO_4 in ethanol (EtOH) as a chromogenic agent. The morphology of TMV particles was observed by transmission electron microscopy (FEI Tecnai Bio, USA).

Isolation of fungi and viruses, and fermentation of fungi

The source, identification, storage, and fermentation of *A. versicolor* YNCA0363 see our previous literature [30]. A total of 80 Erlenmeyer flasks were made for this study. TMV was purified through the Gooding method [31] diluted with 0.01 M phosphate buffered saline (PBS) to achieve a 25 μg/mL or 50 μg/mL concentration, and then stored at − 80 ℃ refrigerator of Yunnan Academy of Tobacco Agricultural Sciences.

Extraction and isolation of compounds 1–7

The fermentation products were extracted with 70% aq. Me_2CO (4 × 15 L) at room temperature and filtered. The filtrate was concentrated to a small volume under reduced pressure until Me_2CO free, and extracted with an equal volume of EtOAc three times to obtain an EtOAc extract. The EtOAc extracts were combined and concentrated to dryness under reduced pressure to obtain a crude product of 63.8 g. The crude extract was subjected to CC (SiO_2, 200–300 mesh) by eluting stepwise with a $CHCl_3$/MeOH gradient system (25:1, 10:1, 5:1, 2:1, 1:1, v/v), to give seven fractions A–G (Fr.A–Fr.G). Fr.C (10:1, 17.1 g) was further separated by CC on silica gel with $CHCl_3$/Me_2CO (9:1, 8:2, 7:3, 6:4, 1:1, v/v) as mobile phase, affording eight subfractions (Fr.C1–C8). Subfraction Fr.C3 (200.0 mg) was further purified by preparative HPLC using isocratic elution with MeOH/H_2O mixtures (63:37, v/v) to afford compounds **7** (13.5 mg) and **6** (17.0 mg). Fr.D (10:1, 13.6 g) was subjected to RP-18 CC using a gradient with decreasing solvent polarity consisting of mixtures of MeOH/H_2O (25:75, 50:50, 75:25, 100:0, v/v), to afford nine subfractions (Fr.D1–D9). Subfraction Fr.D3 (3.0 g) were submitted to CC (Sephadex LH-20) by eluting with $CHCl_3$/MeOH to yield subfractions Fr.D3-1–14. Preparative HPLC purification of Fr.D3-8 (150.0 mg) using MeCN/H_2O isocratic elution (36:64, v/v) yielded compounds **4** (21.5 mg) and **2** (11.5 mg). Fr.D5 (1.3 g) was further separated on Sephadex LH-20 CC with MeOH as mobile phase, affording Fr.D5-8 (118.0 mg). Preparative HPLC purification of Fr.D5-8 using MeOH/H_2O isocratic elution (60:40, v/v) yielded compounds **1** (8.0 mg) and **5** (19.3 mg). Fr.D6 (1.0 g) was subjected to Sephadex LH-20 CC with $CHCl_3$/MeOH as mobile phase to yield subfractions Fr.D6-1–16, Fr.D6-4 was purified by preparative HPLC using isocratic elution with MeOH/H_2O mixtures (65:35, v/v) to afford compounds **3** (7.0 mg).

7-Methoxy-3-(2-oxopropy)-5-hydroxymethyl-isocoumarin (1)

Obtained as a pale yellow gum; UV (MeOH) λ_{max} (log ε) 215 (4.05), 276 (3.68), 338 (3.80) nm; IR (KBr) ν_{max} 3409, 3075, 2942, 2869, 1735, 1668, 1618, 1564, 1482, 1348, 1132, 1076, 868 cm^{-1}; ESIMS *m/z* (positive ion mode) 285 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 285.0738 $[M+Na]^+$ (calcd $C_{14}H_{14}NaO_5$ for 285.0733).

7-Methyl-3-(2-oxopropy)-5-hydroxymethyl-isocoumarin (2)

Obtained as a pale yellow gum; UV (MeOH) λ_{max} (log ε) 215 (4.08), 274 (3.70), 335 (3.85) nm; IR (KBr) ν_{max} 3405, 3062, 2951, 2872, 1738, 1665, 1622, 1569, 1477, 1354, 1149, 1064, 850 cm^{-1}; ESIMS *m/z* (positive ion mode) 269 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 269.0780 $[M+Na]^+$ (calcd $C_{14}H_{14}NaO_5$ for 269.0784).

Aspergillactone A (3)

Obtained as a pale yellow gum; UV (MeOH) λ_{max} (log ε) 215 (3.84), 265 (3.61), 336 (3.54) 360 (3.64) nm; IR (KBr) ν_{max} 3412, 3154, 3070, 2964, 2875, 1740, 1694, 1669, 1625, 1578, 1461, 1350, 1132, 1084, 918 cm^{-1}; ESIMS *m/z* (positive ion mode) 508 $[M+Na]^+$; HRESIMS (positive ion mode) *m/z* 508.1731 $[M+Na]^+$ (calcd $C_{29}H_{27}NNaO_6$ for 269.0784).

Anti-TMV activities assays

A 50 μg/mL concentration TMV solution was used in this study. *Nicotiana glutinosa* (five- to six-leaf stage) was used for in vivo inactivation and protection activities against TMV, while *N. tabacum* cv. HD (six- to seven-leaf stage), which is a commonly grown tobacco variety (China), was used for curative activities against TMV. Tested compounds and ningnanmycin were dissolved in 1% DMSO to a concentration of 1 mg/mL and then diluted with sterile distilled water to 50 μg/mL concentration in the inactivation and protective assays, while in curative assay compound **1** and ningnanmycin have a concentration of 100 μg/mL. The equal concentration of

1% DMSO and sterile distilled water solution was used as a negative control (CK^-), while the new cytosine nucleoside peptide antiviral agent ningnanmycin ($C_{16}H_{25}N_7O_8$, CAS#: 156,410–09-2) was used as a positive agent.

Inactivation assay

To determine the inactivation activities against TMV, the half-leaf method was used as mentioned earlier [32, 33]. Briefly, the tested compound and ningnanmycin solutions were mixed with an equal volume of TMV solution (50 μg/mL). After 30 min, 20 μL of the mixture was inoculated onto the left side of the *N. glutinosa* leaves, while equal concentration of 1% DMSO, sterile distilled water and virus mixture was inoculated onto the right side as a control via gentle rubbing with a hair-brush. Three replicates were performed for each treatment. The numbers of local lesions were documented 5 days after inoculation, and the rates of inhibition were computed:

$$\text{Inhibition rate (\%)} = \left[(C - T)/C\right] \times 100\%. \quad (1)$$

Here, C represents the mean number of local lesions observed in the control, while T represents the mean number of lesions observed in the treatment.

Protective assay

Compounds, ningnanmycin, equal concentration of 1% DMSO and sterile distilled water solutions (1 mL/leaf) were smeared on the whole tobacco leaves (at least 5 leaves), respectively. After 6 h, 100 μL of TMV (50 μg/mL) were inoculated onto the whole leaves via gentle rubbing with a hair-brush. After 30 min, each inoculated leaf was washed with sterile distilled water. The number of local lesions was recorded 5 days after inoculation. Formula I was used to calculate the inhibition rates.

Curative assay

In China, flue-cured tobacco (*N. tabacum* cv. HD) is the most widely cultivated tobacco, accounting for more than 95% of the tobacco planting area. To better apply the laboratory results to the actual planting process, we selected flue-cured tobacco for curative assay. Tobacco seedlings at the 6–7 leaf stage that had been infected with TMV were selected, and the growth and the degree of infection were basically the same. (We mainly selected plants infected with TMV under natural conditions and with similar infection levels (through the size of the mosaic area on the leaves and the degree of leaf distortion), to conduct curative assay.) The leaves were evenly brushed with 100 μg/mL compound **1** and ningnanmycin using a fine brush, brushed again after 10 days (1 mL/leaf), and observed after 20 days. The equal concentration of 1% DMSO and sterile distilled water solution was used as a negative control (CK^-). The curative effect was recorded by taking photos. Furthermore, we employed western blot analysis to measure the tobacco mosaic virus capsid protein (TMV-CP) levels on days 5, 10, and 20 following treatment with compound **1** and ningnanmycin. This was done in order to more clearly observe the changes in TMV content after treatment with compound **1** and ningnanmycin.

Western blot analysis of TMV-CP

Western blots were carried out as previously described, with key details noted here [23, 34]. Anti-β-actin mouse monoclonal antibody (Plant) (CW0264M) and primary antibody against TMV-CP (SRA57400/10) were purchased from Beijing Com Win Biotech Co., Ltd. and American Agdia Corporation, respectively. The remaining reagents used in the SDS-PAGE and western blot experiments were purchased from Solarbio Science & Technology Co., Ltd., Beijing, China. The pretreatment methods used in this part for plants and compounds were the same as those in protective assay and curative assay. The tobacco leaves (0.1 g) were ground in the 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). After centrifuging the lysates, the supernatants were quantitated, dissolved with 5×sample loading buffer, and boiled for 7 min. Protein extracts were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% nonfat milk and incubated overnight with the primary antibodies (of TMV and β-actin) at 4℃ and then incubated for 1 h at room temperature with fluorescent-labeled secondary antibodies (anti-rabbit IgG) conjugated to horseradish peroxidase. The membranes were then incubated with Pierce ECL substrate (Thermo Scientific) and proteins of interest were visualized by chemiluminescent detection on an Image Quant LAS 4000 mini (GE Healthcare). Band densities were determined by using an Image J software (NIH, Bethesda, MD, United States) and normalized against that of actin for total protein.

Transmission electron microscopy

TMV (25 μg/mL) was mixed with an equal volume of compound **1** (250 μg/mL, sterile distilled water as solvent) solution at 25 °C for 60 min, with a mixture of 25 μg/mL TMV and same volume sterile distilled water as a control. Subsequently, the mixture was placed on a carbon-coated grid and negatively stained with 0.01 mL 2% phosphotungstic acid for 1 min. The direct effect of compound **1** on TMV could be observed by the morphological changes of TMV particles [35].

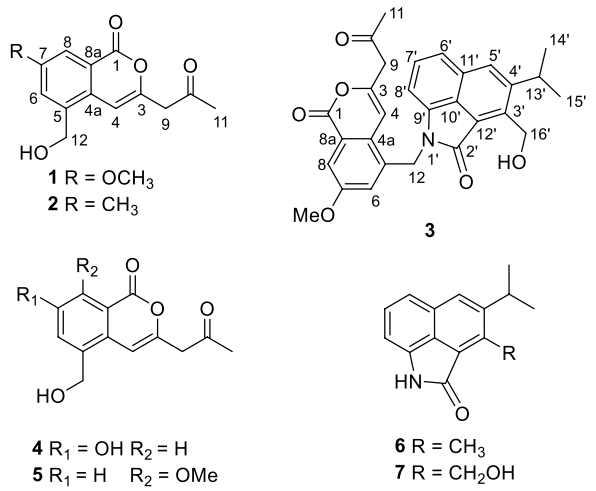


Fig. 1 Structures of compounds **1**–**7**

Table 1 ^{1}H and ^{13}C NMR data of compounds **1** and **2** (δ in ppm, data obtained in $CDCl_3$, 500 MHz)

No.	1		2	
	δ_C, type	δ_H, mult (*J* in Hz)	δ_C, type	δ_H, mult (*J* in Hz)
1	161.2, C		161.5, C	
3	152.9, C		152.6, C	
4	102.3, CH	6.35, s	102.6, CH	6.36, s
4a	126.0, C		131.2, C	
5	135.0, C		133.3, C	
6	116.5, CH	6.90, d, 2.2	131.8, CH	7.36, d, 2.2
7	160.8, C		138.2, C	
7-OMe	56.0, C	3.80, s		
7-CH_3			23.9, CH_3	2.35, s
8	112.3, CH	7.63, d, 2.2	128.8, CH	7.67, d, 2.2
8a	130.6, C		128.0, C	
9	47.3, CH_2	3.63, s	47.1, CH_2	3.62, s
10	203.3, C		203.5, C	
11	30.0, CH_3	2.15, s	30.2, CH_3	2.15, s
12	63.6, CH_2	4.64, s	63.0, CH_2	4.63, s
12-OH		4.96, br s		4.97, br s

Molecular docking

In the current study, molecular docking calculations were executed using AutoDock Vina software [25–27]. The protein sequence was obtained from the NCBI database, and the 3D crystal protein structure was obtained from RCSB PDB database (PDB code: 2OM3). The ligand structures were generated by chem3D. To prepare for molecular docking calculations, the pdbqt files for the proteins and ligands were created based on the AutoDock protocol, with all docking parameters set to their default values, except for the maximum number of energy evaluations (eval) and the number of genetic algorithm (GA) runs. The docking grid was made to binding site (6.70, 55.21, 4.01) for the receptor with a grid size of 40 Å * 40 Å * 40 Å. The grid spacing value was adjusted to 0.375 Å. Gasteiger atomic partial charges were assigned for all investigated ligands.

Results and discussion

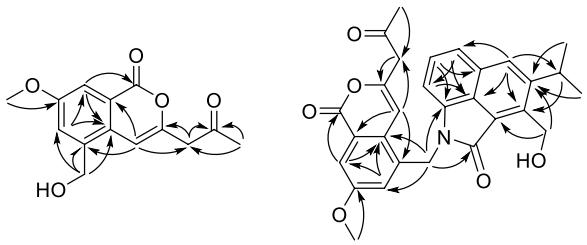
Structural elucidation

As a part of our ongoing investigation of the *N. tabacum*-derived microorganisms, a 70% aq. Me_2CO extract prepared from the fermentation products of the endophytic fungus *A. versicolor* YNCA0363 was partitioned with EtOAc. The EtOAc soluble materials were subjected repeatedly to CC on silica gel, Sephadex LH-20, preparative HPLC to afford three undescribed compounds (**1**–**3**), together with four known compounds (**4**–**7**). Their structures are shown in Fig. 1, and the ^{1}H and ^{13}C NMR data of new compounds are listed in Tables 1 and 2. The known compounds, were identified as oryzaein A (**4**), oryzaein B (**5**) [28], aspergilline F (**6**) and aspergilline G (**7**) [27] by compared with literatures. To the best of our knowledge, compound **3** represents the first example of indole alkaloid and isocoumarin connected by C(12)-N(1′) bond.

Compound **1** was isolated as a pale yellow gum. Its molecular formula $C_{14}H_{14}O_5$ was established by HRESIMS, requiring eight degrees of unsaturation. The IR absorptions revealed the presence of hydroxyl (3409 cm^{-1}), carbonyl (1735 cm^{-1}), and aromatic or olefinic (1668 cm^{-1}) functionalities. The ^{1}H NMR spectrum showed characteristic signals of a 1,2,3,5-tetra substituted benzene moiety, one trisubstituted olefinic proton at δ_H 6.35 (s, H-4), two methylenes at δ_H 4.64 (s, H_2-12) and 3.63 (s, H_2-9), two methyls at δ_H 3.80 (s, H_3-7-OCH_3) and 2.15 (s, H_3-11), and an active proton at δ_H 4.96 (br s, 12-OH). The ^{13}C NMR and DEPT data exhibited 14 carbon signals, consisting of two methyls, two methylenes (of which one was oxygenated), one olefinic and two aromatic methines, and seven quaternary carbons (including two oxygenated ones and two carbonyls) (Table 1). 1D NMR data and the HMBC correlations between H-4 with C-5 and C-8a, H-6 with C-8 and C-4a, H-8 with C-1, C-6 and C-4a, H_2-9 with C-10 and C-11, and H_2-11 with C-9 and C-10, indicated that compound **1** had an isocoumarin skeleton with a 2-oxopropyl group, a hydroxymethyl group, and a methoxy group. The locations of these three substituents on the isocoumarin nucleus were confirmed at C-3, C-5, and C-7 based on the HMBC correlations from H_2-9 to C-3 and C-4, H_2-12 to C-4a, C-5 and C-6, and H_3-7-OCH_3 to C-7, respectively. The HMBC correlations from active proton at δ_H 4.96 to C-12 and C-5 indicate that there was

Table 2 ^{1}H and ^{13}C NMR data of compound **3** (δ in ppm, data obtained in $CDCl_3$, 500 MHz)

3					
No.	**δ_C, type**	**δ_H, mult (*J* in Hz)**	**No**	**δ_C, type**	**δ_H, mult (*J* in Hz)**
1	161.7, C		2′	166.0, C	
3	152.6, C		3′	136.7, C	
4	102.6, CH	6.37, s	4′	137.7, C	
4a	127.4, C		5′	129.4, CH	8.13, s
5	134.6, C		6′	121.4, CH	7.53, d, 7.8
6	118.2, CH	6.88, d, 2.2	7′	130.9, CH	7.65, like t, 7.8
7	159.5, C		8′	115.8, CH	7.05, d, 7.8
7-OMe	55.9, C	3.79, s	9′	139.9, C	
8	112.3, CH	7.62, d, 2.2	10′	126.3, C	
8a	129.8, C		11′	132.2, C	
9	46.4, CH_2	3.63, s	12′	124.2, C	
10	203.2, C		13′	31.6, CH	2.84, q, 6.8
11	30.1, CH_3	2.15, s	14′/15′	23.5, $2CH_3$	1.40, d, 6.8
12	43.8, CH_2	4.14, s	16′	57.0, CH_2	5.24, s
			16′-OH		4.97, br s



1 **3**

Fig. 2 Key HMBC correlations of compounds **1** and **3**

a hydroxyl group at C-12 (Fig. 2). Therefore, the structure of **1** was established as 7-methoxy-3-(2-oxopropy)-5-hydroxymethyl-isocoumarin.

Compound **2**, obtained as a pale yellow gum, was assigned the molecular formula $C_{14}H_{14}O_4$ by HRESIMS, requiring same degrees of unsaturation as compound **1**, a molecular mass 16 Da less than that of **1**. Comparison of their NMR spectra revealed that a methoxyl group in compound **1** was replaced with a methyl group (δ_H 2.35; δ_C 23.9) in compound **2**, consistent with C-7 was shifted upfield from 160.8 to 138.2, while methoxy signals changed to methyl signal. The structure of compound **2** was therefore assigned as 7-methyl-3-(2-oxopropy)-5-hydroxymethyl-isocoumarin.

Compound **3** was obtained as a pale yellow gum and its molecular formula was established as $C_{29}H_{27}NO_6$ based on HRESIMS data, which corresponded to 17 units of unsaturation. An inspection of the ^{1}H and ^{13}C NMR data for compound **3** (Table 2) revealed that this compound composed of co-occurring compound **1** and known metabolite aspergilline G (**7**) [27]. Two substructures were connected by C-12 and N-1′, which can be deduced from the HMBC correlations between H_2-12 with C-2′ and C-9′. Thus, the structure of compound **3** was established, and given the trivial name of aspergillactone A.

Inactivation, protective and curative activities

Since many isocoumarin and indole alkaloid derivatives have been reported to exhibit potential anti-TMV properties [27, 28, 36], new compounds **1**–**3** were tested for their anti-TMV activities. As a result, antiviral activity of compound **2** with inactivation efficacy of 43.8% ± 3.5 was slightly better than that of positive control, ningnanmycin (37.6% ± 3.8), a widely used commercial antiviral agent in China, and mainly inhibited polymerization of the TMV coat protein to restrain spread of the virus in the host and promote tobacco resistance against TMV [37], at a concentration of 50 μg/mL. Interestingly, compounds **1** and **3** exhibited potent antiviral activities against TMV with inhibition rates of 58.9% ± 4.0 and 52.6% ± 3.9 at a concentration of 50 μg/mL, respectively, and these rates are higher than that of positive control, these findings are showed in Additional file 1: Fig. S11. These findings indicated that compounds **1**–**3** exhibited higher efficacies than ningnanmycin.

Given that the inactivation effects of compounds **1**–**3** were higher than that of positive control, we selected these compounds for further protection activity studies.

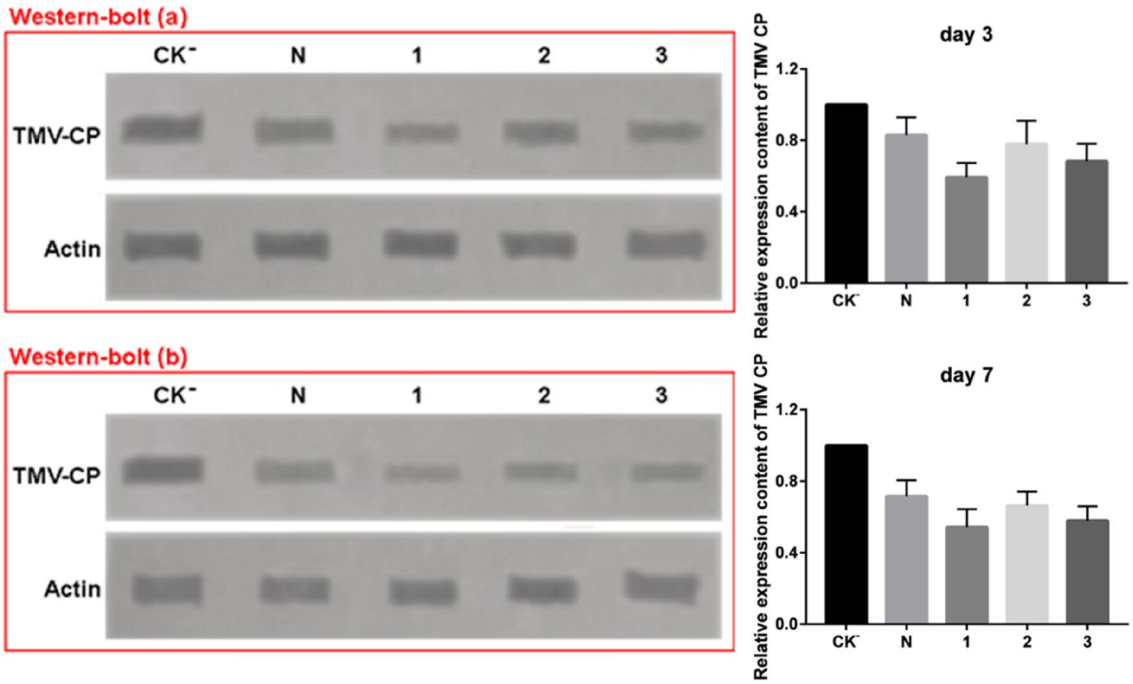


Fig. 3 Analysis of accumulation of TMV-CP. Western blot analysis and relative expression content of TMV-CP in the inoculated leaves treated with 50 μg/mL of compounds **1**–**3** on day 3 (**a**) and day 7 (**b**), respectively. CK.⁻, negative control; N, ningnanmycin, actin serve as internal references. Each value is express as the mean ± SD ($n = 3$)

The results indicated that compounds **1**, **2** and **3** exhibited notable protective effects at a concentration of 50 μg/mL, with inhibition rates of 62.3% ± 4.7, 48.6% ± 3.2 and 54.5% ± 4.5, respectively (Additional file 1: Fig. S12). These rates were better than that of ningnanmycin, which had an inhibition rate of 39.5% ± 4.2.

To verify the inhibitory effect of compounds **1**–**3** on viruses in protective effect assay, the analysis of TMV-CP was conducted by western blot (Fig. 3). In Fig. 3a, on day 3 after inoculation, the bands intensities indicated that the accumulation of TMV-CP were significantly less than that of in CK⁻ when the tobacco leaves treated with compounds **1**, **2** and **3**. In addition, the bands intensities of compounds **1**, **2** and **3** were also less than that of ningnanmycin, and compound **1** had the lowest band. The results were agreed with the inhibition rates obtained from protective assay. In Fig. 3b (on day 7 after inoculation), the trends for bands intensities of TMV-CP are agreed with that observed on day 3. However, the bands intensities for compounds **1**, **2** and **3** on day 7 were less than that observed on day 3. The above results indicated that pretreatment with compounds **1**–**3** could reduce the content of TMV-CP protein in tobacco leaves and improve the resistance of tobacco to TMV infection.

The experimental results of the above studies combined with previous literature reports found that compounds **1**–**3** and **7** have higher inactivation and protective effects than compounds **4**–**6** [27, 28]. It can be seen that 3′-hydroxymethyl in indole alkaloids, 7-methoxy or 7-methyl substitution in isocoumarins may enhance their antiviral effects, 7-methoxy is better than 7-methyl.

From the above results, compound **1** had the strongest inactivation effect and protective effect on TMV. To better apply the laboratory results to the actual flue-cured tobacco planting process, we selected the 6–7 leaf stage flue-cured tobacco (*N. tabacum* cv. HD, China's main flue-cured tobacco cultivar) to carry out the curative effects experiment of compound **1** and ningnanmycin on TMV. From the experimental results, it can be seen that the negative control group was seriously infected with TMV, the lesions were very obvious, the growth trend was slow, and the leaves were obviously deformed (Fig. 4d). This may have a serious impact on the yield and quality of tobacco leaves. However, after treatment with ningnanmycin, the lesions were significantly reduced, the disease was significantly improved, and the leaf growth tended to be normal (Fig. 4e). After treatment with compound **1**, the lesions were obviously further reduced compared with those after treatment with ningnanmycin. There was only a small amount of lesion observed on the newly grown leaves. The growth of tobacco plants also had a tendency to return to

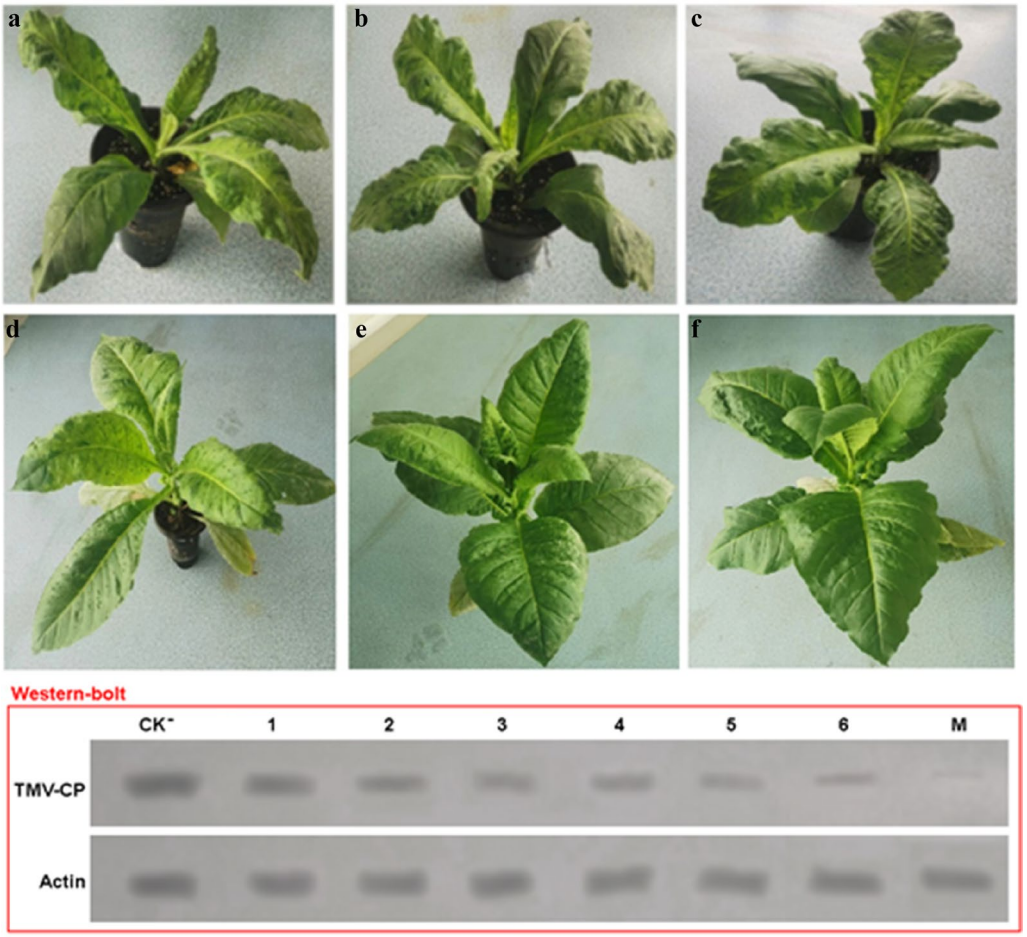


Fig. 4 The curative effects of compound **1** and ningnanmycin on TMV. Tobacco seedlings infected with TMV to be treated (**a** – **c**), negative control (treated with equal concentration of 1% DMSO and sterile distilled water solution) (**d**), treated with 100 μg/mL ningnanmycin (**e**) and compound **1** (**f**). Photos were taken 20 days after treatment. CK^- (negative control), 1 – 3 (5, 10, 20 days after treatment with ningnanmycin), 4 – 6 (5, 10, 20 days after treatment with compound **1**), M (healthy tobacco leaves)

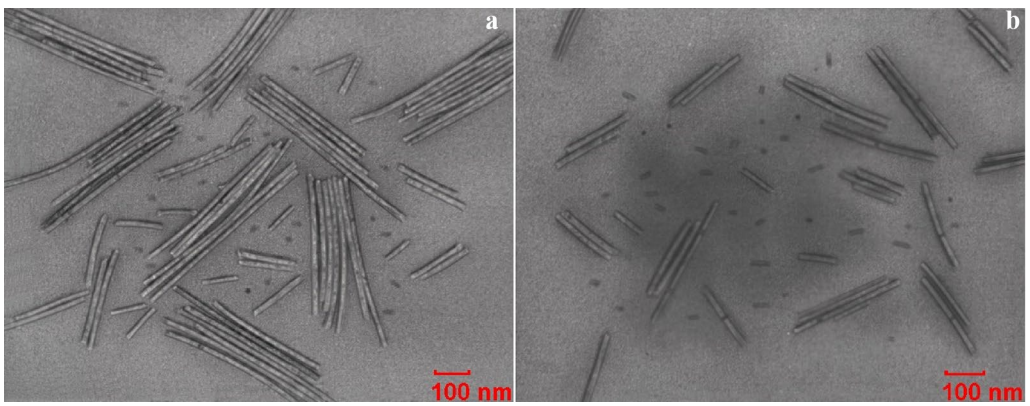


Fig. 5 The direct effect of compound **1** on TMV. 250 μg/mL compound **1** was mixed with TMV solution for 60 min (**b**), and the mixture was used to observe the morphology of TMV particles, whereas a mixture of sterile distilled water with TMV solution was used as a control (**a**)

normal (Fig. 4f). In addition, the content of TMV-CP protein was determined by western blot after 5, 10 and 20 days of treatment. The results showed that the content of TMV-CP protein in tobacco leaves decreased gradually with time after treatment with compound **1** and ningnanmycin, and the effect of compound **1** treatment was significantly better than that of ningnanmycin. The experimental results are consistent with the phenotypic observation. These results showed that compound **1** had an obvious curative effect on tobacco plants infected with TMV, and its curative effect was obviously better than that of ningnanmycin. The curative effect results indicated that compound **1** could significantly alleviate the impact of TMV on tobacco growth and effectively improve the yield and quality of tobacco infected with TMV.

Direct action of compound 1 on TMV particles

Among the tested compounds, compound **1** had the best inhibitory effect on TMV. Based on the above analysis, the inactivation effect was the most effective factor for the antiviral activities of these compounds against TMV [35]. Therefore, compound **1** was selected to further study its direct action on TMV particles and understand the inactivation mechanism of it on TMV through TEM. The results showed that TMV particles treated with compound **1** were seriously broken (Fig. 5b). It can be clearly seen that most of the TMV particles broke into small fragments from 300 nm to 10–250 nm. Due to the fusion phenomenon, the number of TMV particles is also reduced. However, there was no obvious aggregation of TMV particles treated with compound **1**. The above information indicates that compound **1** has a strong direct effect on TMV particles, which is the reason for its significant inactivation of TMV.

Molecular docking

To further understand the structure–activity relationship of compounds **1**–**3**, the molecular docking was conducted. The molecular weight of the protein (PDB code: 2OM3) is 27.63 kDa and it comprises 159 amino acids. The protein crystal structure shows that this protein is a polymer, in which the 5-mer can encapsulate a pocket, and the active pocket of the protein consists of Thr42-Arg46-Gln45-Arg90-Arg92, Asn33-Gln34-Gln39-Ala40-Gln38-Thr37, Asn127-Ser123-Val119-Asp116, forming a more hydrophilic pocket (Fig. 6). In compound **1**, ester carbonyl group formed hydrogen bond with Gln39, side chain carbonyl group formed hydrogen bond with Arg92, and methoxy group formed hydrophobic interaction with Arg90. Although compound **2** also has hydrogen bonds with Gln39 and Arg92, the hydrophobic interaction between methyl and Arg90 was significantly weaker than that of methoxy, so the activity was reduced. Compound **3** formed hydrogen bonds with Gln39, the side chain carbonyl group formed hydrogen bonds with Arg92, and

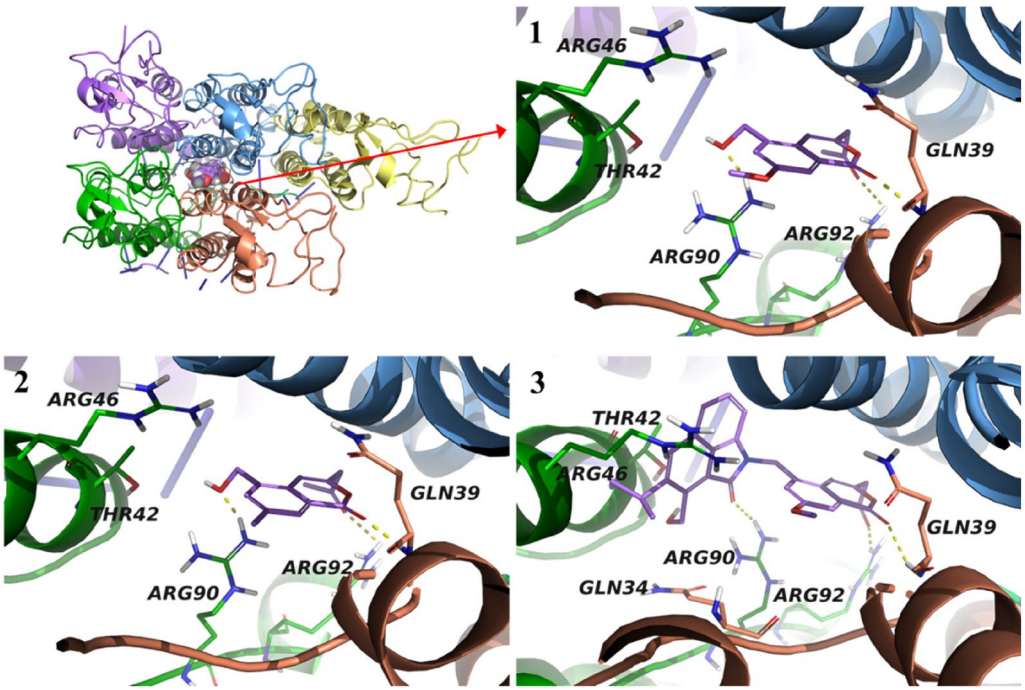


Fig. 6 Interactions between TMV-helicase and compounds **1**–**3** (sticks are used to represent key residues while dotted lines indicate H-bonds)

the indole ring formed new hydrogen bonds with Arg90. However, compound **3** had a relatively large indole ring side chain. When combined with protein, the loss of entropy was larger than that of compound **1**, so the activity of compound **3** was lower than that of compound **1**.

The results of molecular docking were consistent with the previous anti-TMV effect. It revealed that the isocoumarin nucleus can interact with TMV-CP proteins, which may be fundamental for its direct effect on TMV. In addition, 7-methoxy or 7-methyl substitution in isocoumarin may enhance its antiviral effect, and 7-methoxy substitution is superior to 7-methyl.

Conclusions

Three new compounds were isolated from *A. versicolor* YNCA0363 and evaluated for their anti-TMV activity. Among them, compound **3** is the first compound covalently bound by indole alkaloid and isocoumarin. Biological studies indicated that compounds **1 – 3** exhibited significant antiviral activities against TMV including inactivation, protective and curative effects. The inactivation effect and protective effect of compounds **1 – 3** were stronger than that of the positive control, ningnanmycin, especially compounds **1** and **3**. In addition, the content of TMV-CP protein was indeed reduced by pretreatment with compounds **1 – 3**, indicating that pretreatment of tobacco with these compounds can reduce the infection of TMV virus to tobacco. Compound **1** had obvious therapeutic effect on TMV, which was significantly stronger than the positive control. The molecular docking studies for compounds **1 – 3** may also reveal that isocoumarin nucleus is fundamental for anti-TMV activity and 7-methoxy or 7-methyl substitution in isocoumarin may enhance their antiviral activities, and 7-methoxy is better than 7-methyl. Further studies demonstrated that the mode of action on TMV particles for compound **1** involved the fracture of the TMV particles into small fragments combined with the fusion phenomena. This study provides new evidence that indole alkaloids and isocoumarins from *N. tabacum*-derived *Aspergillus* have the potential to be developed as antiviral agents to control TMV.

Abbreviations

UV	Ultraviolet
IR	Infrared radiation
NMR	Nuclear magnetic resonance
ESIMS	Electrospray ionization mass spectrometry
HRESIMS	High-resolution electrospray ionization mass spectrometry
TEM	Transmission electron microscopy
CC	Column chromatography
HPLC	High-performance liquid chromatography
TLC	Thin-layer chromatography
$CDCl_3$	Chloroform
KBr	Potassium bromide
TMS	Tetramethylsilane
MeOH	Methanol
EtOH	Ethanol
Fr.A–Fr.G	Fractions A–G
N. tabacum	*Nicotiana tabacum*
N. glutinosa	*Nicotiana glutinosa*
A. versicolor	*Aspergillus versicolor*
TMV	Tobacco mosaic virus
TMV-CP	Tobacco mosaic virus capsid protein
SDS-PAGE	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s40538-023-00514-4.

Additional file 1. Additional figures.

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Author contributions

F-XY: data curation, methodology, writing–review and editing. H-YL and Y-YM: data curation. Z-JL: validation. X-ML: formal analysis. Y-KL: validation. W-GW, MZ and Q-FH: conceptualization, project administration, funding acquisition, supervision.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. All data generated or analyzed during this study are included in this published article [and its Additional file 1]. Figure S1 – S10: ^{1}H, ^{13}C NMR, DEPT, HSQC and HMBC spectra of compounds **1 – 3**; Figure S11: Inactivation effects of ningnanmycin, compounds **1**, **2** and **3** on *N. glutinosa*; Figure S12: The protective effects of ningnanmycin and compound **1** on *N. glutinosa*. The possible biogenetic pathway of compounds **1**–**3** is included.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

This research has been confirmed for publication in the journal.

Competing interests

The authors have no conflicts of interest.

Author details

[1]Key Laboratory of Tropical Plant Resource and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming 650223, People's Republic of China. [2]Key Laboratory of Chemistry in Ethnic Medicinal Resources, State Ethnic Affairs Commission & Ministry of Education, Yunnan Minzu University, Kunming 650500, People's Republic of China. [3]Yunnan Key Laboratory of Tobacco Chemistry, China Tobacco Yunnan Industrial Co., Ltd., Kunming 650031, People's Republic of China.

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