First Discovery and Structure-Activity Relationship Study of Phenanthroquinolizidines as Novel Antiviral Agents against Tobacco Mosaic Virus (TMV)

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Abstract

A series of phenanthroquinolizidine alkaloids 1–24 were prepared and first evaluated for their antiviral activity against tobacco mosaic virus (TMV). The bioassay results showed that most of these compounds exhibited good to excellent in vivo anti-TMV activity, of which compounds 1, 2, 15 and 16 displayed significantly higher activity than (R)-antofine and commercial Ningnanmycin at the same test condition. The substituents on the phenanthrene moiety play an important role for maintaining high in vivo antiviral activity. The introduction of 6-hydroxyl, which is proposed to interact with TMV RNA, did increase anti-TMV activity. The 14αf-configuration was confirmed to be the preferred antiviral configuration for phenanthroquinolizidine alkaloids. Introduction of hydroxy group at 15-position of phenanthroquinolizidine alkaloids increased activity for S-configuration but decreased activity for R-configuration. Present study provides fundamental support for development and optimization of phenanthroquinolizidine alkaloids as potential inhibitors of plant virus.

Introduction

Plant viruses cause numerous diseases in a wide range of crop plant species and lead to an estimated $600 billion in annual losses worldwide [1]. Tobacco mosaic virus (TMV), the type member of Tobamovirus genus, 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 [2]. It is found that in certain fields 90–100% of the plants show mosaic or leaf necrosis by harvesting time. Therefore, this plant virus has the name “plant cancer” and is difficult to control.

To control viral plant diseases, many approaches have been used, including chemicals, breeding, cross-protection and transgenic plants. However, there are no control measures that can totally inhibit plant viruses after they have infected plants. Ningnanmycin (Figure 1), perhaps the most successful registered antiviral agent, displayed 56.0% in vivo curative effect at 500 μg/mL. Ribavirin (Figure 1) is another widely used plantviral inhibitor, its inhibitory effects are also less than 50% at 500 μg/mL. Because of the unsatisfactory cure rate (30–60%) of common antiviral agents (Ningnanmycin, Ribavirin, Virus A, et al.) and economic loss of tobacco, many efforts have been done to develop novel, potent and structure concise antiviral agents. Some chemicals, such as triazolyl compounds [3], pyrazole derivatives [4,5], cyanoacrylate derivatives [5,6], α-amino phosphonate derivatives [5,7], N-pyrimidin-5-yl)-N-phenylureas [8], and some natural products [9–11], have been found to possess antiviral activity. However, there are only a few reports on economically viable antiviral chemicals available for application in agriculture [12], so there still lies a great deal of scope for further research in this field.

Natural product-based agrochemicals offer advantages in that they can sometimes be specific to a target species and often have unique modes of action with little mammalian toxicity. Another benefit is their ability to decompose rapidly, thereby reducing their risk to the environment [13,14]. An additional advantage is the natural products can be a candidate that possesses the desirable biological activities.

Phenanthroquinolizidine alkaloids are a small group of alkaloids existing in the Laurusaceae, Vitaceae and Urticaceae family of plants. Only five of these alkaloids ([R]-cryptopleurine (2), (R)-boehmeriasin A (5), boehmeriasin B (18), cryptopleuridine and (4αR,15βR)-15-hydroxycryptopleurine (19), Figure 1) have been isolated by now [15–18]. It has been reported that these alkaloids possess unique and interesting biological properties including vesicant [15], antimicrobial [19], antiviral [20,21] and anti-tumor [22–24] activities. To date, most of the studies have been focused on anti-tumor activity in medicinal formulation. However, there have been no reports about the anti-TMV activity of phenanthroquinolizidine alkaloids in pesticide formulation.

In our previous work, the phenanthroindolizidine alkaloid (−)-antofine (Figure 1) was isolated from the aerial parts of Cynanchum komarovii and was first found to have good antiviral activity against TMV in vitro [25]. Further antiviral mechanism studies revealed...
that antofine has favorable interaction with origin of TMV RNA (oriRNA), likely exerting its virus inhibition by binding to oriRNA and interfering with virus assembly initiation [26]. Although phenanthroquinolizidine and phenanthroindolizidine are structure analogs, their biological properties are different [22]. Did the phenanthroquinolizidine alkaloids possess anti-TMV activity? Based on the above findings, a series of phenanthroquinolizidine alkaloids 1–24 were prepared and systematically evaluated for their antiviral activity against TMV.

Results and Discussion

Chemistry

The alkaloids 1–9 [27], 18 [28], 19–24 [27] and acids 25a–f [24] were prepared according to the corresponding reported procedures. Phenanthroquinolizidine alkaloids 10–17 were synthesized by using a practical synthetic approach involving coupling of the phenanthrene ring with 2-bromopyridine through a nucleophilic substitution reaction and subsequent reduction of the resulting pyridyl ketone as key steps.

As shown in Figure 2, 2-bromopyridine was treated with n-BuLi at –78°C to afford 2-lithiopyridine, which reacted smoothly with 25a–f to give pyridyl ketones 26a–f, respectively. By using PtO2 as the catalyst, the hydrogenation of 26a–e proceeded smoothly in the solution of acetic acid and trifluoroacetic acid (v/v 5:1) at 5 atm to give 27a–e, respectively. It should be noted that pyridyl ketone 26f could not been reduced under above reduction condition. The reduction of 27a–e in Et3SiH/TFA at reflux afforded 28a–e, which were then converted into phenanthroquinolizidine alkaloids 10–14, respectively, by using the Pictet–Spengler cyclomethylation.

The hydrogenation of 26f was carried out at several different conditions. The best result is that treatment of compound 26f with PtO2 in the solution of acetic acid and trifluoroacetic acid (v/v 4:1) at 50 atm gave the carbonyl group reduced intermediate 29 in 53% yield. Reduction of 29 using the similar procedure for the preparation of 27a–e gave the key intermediate 30 in 92% yield, which was converted into phenanthroquinolizidine alkaloid 15 by using the Pictet–Spengler cyclomethylation (Figure 3).

In order to further evaluate the effect of 6-hydroxy group on antiviral activity, which is proposed to interact with TMV RNA [26], phenanthroquinolizidine alkaloids 16 and 17 were designed and synthesized. Phenanthrene-9-carboxylic acid 34 was synthesized by employing 2-(3,4-dimethoxyphenyl)acetic acid and 4-hydroxybenzaldehyde as the starting materials (Figure 4). Ester 33 was obtained by the conventional four steps previously reported in the literature [29]. Then hydrolysis of ester 33 gave acid 34, which was treated with 2-lithiopyridine to afford the desired ketone 35. The catalytic hydrogenation of 35 to give 38 was carried out under two different procedures. Treatment of compound 35 with Pd/C as the catalyst in refluxing methanol at 1 atm gave the alcohol 36 in 85% yield. Reduction of 36 using the Et3SiH/TFA system afforded the desired product 37, which was further reduced using PtO2 as the catalyst in acetic acid and trifluoroacetic acid (v/v 5:1) at 10 atm gave 38 in 82% yield. For another method, treatment of ketone 35 with Pd/C as the catalyst afforded 28a–e, which were then converted into phenanthroquinolizidine alkaloids 10–14, respectively, by using the Pictet–Spengler cyclomethylation.

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Figure 2. Synthesis of compounds 10–14.
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Figure 3. Synthesis of compound 15.
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in refluxing acetic acid at 1 atm gave the key intermediate 38 in 59% yield. Then amino and phenol protection and Pictet–Spengler cyclomethylenation proceeded sequentially to give 40, which deprotection afforded phenanthroquinolizidine alkaloid 16 in 69% yield (Figure 4). Treatment of pyridine 37 with cyclopropylmethyl bromide obtained 41, which was reduced using PtO₂ as the catalyst in ethanol and conc. HCl (v/v 20:1) at 50 atm to afford the key intermediate 42. The Pictet–Spengler cyclomethylenation of 42 gave phenanthroquinolizidine alkaloid 17 in 80% yield (Figure 5).

Phytoxic Activity

The phenanthroquinolizidine alkaloids 1–24 were first tested for their phytoxic activity against the test plant, and the results indicated that these natural product-based compounds showed no phytoxic activity at 500 μg/mL.

Antiviral Activity

In Vitro And In Vivo

The commercial plant virucide Ningnanmycin, perhaps the most successful registered anti-plant viral agent, Ribavirin and (R)-antofine were used as the controls.

In order to investigate the substituent effects of phenanthrene moiety, a series of racemic phenanthroquinolizidine alkaloids 1, 4, 7, and 10–18 were designed, synthesized and evaluated for their antiviral activity (Table 1). To our delight, all the racemic phenanthroquinolizidine alkaloids displayed higher antiviral activity than Ribavirin, of which cryptopleurine (1) and alkaloid 16 exhibited significantly higher activity than (R)-antofine and commercial Ningnanmycin, thus emerged as novel potent lead compounds for anti-TMV agents. The in vitro activity and in vivo activity of each alkaloid are about similar, which indicates that these alkaloids possess a good biological availability.

Cryptopleurine (1) exhibited significantly higher activity than alkaloid 7, which indicated that the removal of methoxyl at 7-

Figure 4. Synthesis of compound 16.
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position of the phenanthrene moiety is beneficial for maintaining excellent antiviral activity. Alkaloids 4 and 7 displayed a similar activity, which indicated that the removal of methoxyl at 2-position of the phenanthrene moiety is tolerant. The 6-position of the phenanthrene moiety, which is proposed to interact with TMV RNA [26], really is a sensitive site: removal of 6-O methyl increased the antiviral activity (antiviral activity: \(16 > 1\); 18 > 2), which indicated that the 6-position may be a hydrogen donor site; replacement of 6-O methyl with 6-O cyclopropyl methyl significantly decreased the antiviral activity (antiviral activity: \(1 > 17\)), which indicated that the steric effect is also a very important factor. Instead of 2,3-dimethoxyl or 6,7-dimethoxyl with methylenedioxy is tolerant (antiviral activity: \(7 \approx 10 \approx 11 > 12\)). However, replacement of 2,3-dimethoxyl or 6,7-dimethoxyl with ethylendioxy significantly increased the anti-TMV activity, especially for 6,7-dimethoxyl (antiviral activity: \(15 > 13 > 14 > 7\)).

Further to investigated the effect of \(14a\)-chirality on anti-TMV activity, a series of chiral phenanthroquinolizidine alkaloids 2, 3, 5, 6, 8 and 9 were prepared and evaluated for their antiviral activity (Table 2). The \((R)\)-phenanthroquinolizidine alkaloids exhibited significantly higher activity than their enantiomers (antiviral activity: \(2 > 3; 5 > 6; 8 > 9\)). The structure difference between \((R)\)-cryptopleurine (2) and \((R)\)-antofine lies in the “E” ring. \((R)\)-Antofine with a 5-member “E” ring displayed inhibition activity of 42.7% inactivation effect, 46.0% curative effect and 45.5% protection effect at 500 \(\mu\)g/mL. However, \((R)\)-cryptopleurine (2) with a 6-member “E” ring exhibited significantly higher \(\text{in vivo}\) antiviral activity than \((R)\)-antofine even at 100 \(\mu\)g/mL. The results further indicated that although phenanthroquinolizidine and phenanthroindolizidine are structure analogs, their biological properties are different [22]. For \((R)\)-phenanthroquinolizidine alkaloids: removal of 7-methoxyl increased antiviral activity (antiviral activity: \(2 > 8\)); removal of 2-methoxyl decreased antiviral activity (antiviral activity: \(5 > 6\)). However, the \((S)\)-phenanthroquinolizidine alkaloids displayed about similar moderate antiviral activity (antiviral activity: \(3 > 6 \approx 9\)).

In order to investigated the effect of 15-hydroxy on antiviral activity, a series of 15-hydroxyphenanthroquinolizidine alkaloids 19-24 were prepared and evaluated for their antiviral activity (Table 3). The bioassay results showed that the 15-hydroxyphenanthroquinolizidine alkaloids also displayed good to excellent \(\text{in vivo}\) antiviral activity, of which alkaloids 19, 20, 22 and 24 exhibited significantly higher antiviral activity than Ribavirin, and alkaloids 19 and 22 displayed higher \(\text{in vivo}\) activity than Ningnannycin at 100 \(\mu\)g/mL. The introduction of hydroxy group at 15-position significantly increased antiviral activity for \((S)\)-phenanthroquinolizidine alkaloids (antiviral activity: \(2 > 19; 5 > 21, 8 > 23\), but decreased antiviral activity for \((R)\)-phenanthroquinolizidine alkaloids (antiviral activity: \(20 > 3; 22 > 6, 24 > 9\)). The \((S,S)\)-enantiomers 22 and 24 exhibited significantly higher antiviral activity than their corresponding enantiomers 21 and 23. However, the \((S,S)\)-enantiomer 20 showed a lower activity than its enantiomer 19. For \((14a,S,15a)\)-phenanthroquinolizidine alkaloids: removal of 7-methoxyl decreased antiviral activity (antiviral activity: \(20 < 24\)); removal of 2-methoxyl increased antiviral activity (antiviral activity: \(22 > 24\)). For \((14a,R,15a)\)-phenanthroquinolizidine alkaloids, removal of 7-methoxyl or 2-methoxyl all increased antiviral activity (antiviral activity: \(19 > 21 > 23\)).

Materials and Methods

Synthesis

Reagents were purchased from commercial sources and were used as received. All anhydrous solvent were dried and purified by standard techniques just before use. Reaction progress was monitored by thin-layer chromatography on silica gel GF\(_{254}\) with detection by UV. Please see supporting information for the melting points, \(^1\)H NMR spectra, \(^1\)C NMR spectra, elemental analyses or high-resolution mass spectra of the synthesized compounds 10–17 and 26–42.

General Procedure for the Synthesis of Methanones 26a–f

To a stirred mixture of 2-bromopyridine (80 mmol) in THF (300 mL) was added \(n\)-BuLi (32 mL, 80 mmol, 2.5 M in hexane solution) at \(-78^\circ\text{C}\) over 30 min. The brown solution was stirred at \(-78^\circ\text{C}\) for 4 h and the acid 25 (20 mmol) was added at once. The

![Figure 5. Synthesis of compound 17.](Image 300x400)

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mixture was stirred at –78°C for 2 h and then warmed to room temperature for 6 h, then quenched with saturated aqueous NH₄Cl (300 mL) and extracted with CH₂Cl₂ (3×200 mL). The organic layer was dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was added dissolved in methanol (50 mL), and the precipitate was filtered and washed with methanol (50 mL) to give methanone 26.

**General Procedure for the Synthesis of Alcohol Amines 27a–e**

A mixture of 26 (5 mmol), acetic acid (150 mL), trifluoroacetic acid (30 mL) and PdO₂·H₂O (50 mg) was stirred for 36 h at a pressure of 50 atm under hydrogen. The mixture was filtered, and the filtrate was evaporated to dryness in vacuo. The residue was treated with aqueous NaOH and extracted with CH₂Cl₂ (3×100 mL). The combined organic extracts were dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with CH₂Cl₂/CH₃OH (12:1) as eluant to afford 27.

**General Procedure for the Synthesis of Piperidines 28a–e**

A mixture of 27 (5 mmol), trifluoroacetic acid (120 mL) and triethylsilane (6 mmol) was refluxed for 8 h. The reaction mixture was concentrated to dryness under reduced pressure. The residue was treated with aqueous NaOH and extracted with CH₂Cl₂ (3×100 mL). The combined organic layer was dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with CH₂Cl₂/CH₃OH (10:1) as eluant to afford 28.

**Table 1. In vitro and in vivo anti-TMV activity of racemic phenanthroquinolizidine alkaloids 1, 4, 7, and 10–18.**

| Compd. | Conc. (µg/mL) | In vitro | In vivo |
|--------|--------------|----------|---------|
|        |              | Inhibition ratio (%) | Inactivation effect (%) | Curative effect (%) | Protection effect (%) |
| 1      | 500          | 75.4±2   | 73.1±1  | 71.5±2  | 73.8±1  |
|        | 100          | 48.2±3   | 43.6±2  | 38.3±1  | 53.6±2  |
| 4      | 500          | 41.4±5   | 45.0±3  | 45.9±4  | 42.5±8  |
|        | 100          | 20.3±2   | 27.6±1  | 20.2±4  | 16.3±3  |
| 7      | 500          | 48.7±5   | 46.1±6  | 44.2±3  | 39.6±2  |
|        | 100          | 20.3±3   | 25.2±7  | 27.1±4  | 20.2±5  |
| 10     | 500          | 46.9±2   | 41.8±8  | 42.7±6  | 38.7±3  |
|        | 100          | 22.3±1   | 10.3±4  | 15.2±5  | 9.6±3   |
| 11     | 500          | 47.8±2   | 44.3±4  | 39.2±3  | 49.3±7  |
|        | 100          | 22.4±4   | 9.8±3   | 12.6±2  | 18.2±6  |
| 12     | 500          | 46.4±2   | 43.2±1  | 40.8±1  | 48.2±4  |
|        | 100          | 28.3±5   | 20.1±1  | 25.3±3  | 26.7±6  |
| 13     | 500          | 58.2±2   | 57.2±3  | 52.2±7  | 56.5±4  |
|        | 100          | 30.1±1   | 22.1±5  | 20.1±6  | 26.2±4  |
| 14     | 500          | 50.5±3   | 52.1±5  | 46.6±7  | 49.2±1  |
|        | 100          | 23.2±2   | 29.7±6  | 25.1±3  | 19.7±1  |
| 15     | 500          | 70.3±2   | 68.2±1  | 66.6±1  | 64.3±2  |
|        | 100          | 45.7±1   | 38.2±2  | 42.3±1  | 40.6±3  |
| 16     | 500          | 72.4±2   | 73.6±1  | 77.3±3  | 76.3±2  |
|        | 100          | 57.6±2   | 51.4±1  | 55.2±1  | 55.6±2  |
| 17     | 500          | 43.1±1   | 40.2±3  | 48.3±4  | 51.6±3  |
|        | 100          | 25.2±3   | 20.1±3  | 28.1±2  | 28.4±4  |
| 18     | 500          | 66.6±7   | 55.4±3  | 61.4±5  | 60.2±6  |
|        | 100          | 36.8±1   | 32.3±3  | 40.3±10 | 37.3±6  |
| (l)-Antofine | 500  | 52.8±1   | 42.7±2  | 46.0±1  | 45.5±3  |
|        | 100          | 27.4±2   | 15.3±2  | 19.4±1  | 20.4±4  |
| Ribavirin        | 500  | 41.0±3   | 33.8±2  | 36.7±1  | 38.9±3  |
|        | 100          | 19.6±5   | 9.8±2   | 8.2±1   | 7.5±2   |
| Ningnanmycin     | 500  | 70.2±1   | 68.5±1  | 56.0±2  | 66.6±1  |
|        | 100          | 23.9±2   | 37.7±3  | 18.9±1  | 25.2±2  |

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### Table 2. *In vitro* and *in vivo* anti-TMV activity of chiral phenanthroquinolizidine alkaloids 2, 3, 5, 6, 8 and 9.

| Compd. | Conc. (µg/mL) | *In vitro* | *In vivo* |
|--------|---------------|------------|-----------|
|        |               | Inhibition ratio (%) | Inactivation effect (%) | Curative effect (%) | Protection effect (%) |
| 2      | 500           | 72.5±1     | 76.3±3     | 76.2±2     | 65.4±1     |
|        | 100           | 55.6±2     | 52.4±1     | 50.1±1     | 41.8±2     |
| 3      | 500           | 51.2±4     | 46.2±2     | 43.5±2     | 47.1±3     |
|        | 100           | 25.3±1     | 17.8±5     | 13.4±3     | 22.2±6     |
| 5      | 500           | 51.8±4     | 45.6±1     | 49.4±1     | 47.2±2     |
|        | 100           | 27.6±3     | 18.9±2     | 26.2±1     | 27.9±1     |
| 6      | 500           | 42.7±3     | 30.1±2     | 36.4±2     | 46.6±5     |
|        | 100           | 20.0±1     | 16.2±3     | 25.9±3     | 18.0±2     |
| 8      | 500           | 64.6±3     | 61.5±1     | 60.0±1     | 57.8±5     |
|        | 100           | 33.3±1     | 27.2±2     | 25.3±1     | 35.6±3     |
| 9      | 500           | 45.2±5     | 40.1±3     | 41.8±1     | 36.9±2     |
|        | 100           | 10.3±3     | 21.3±12    | 22.4±7     | 18.5±9     |
| (R)-Antofine | 500      | 52.8±1     | 42.7±2     | 46.0±1     | 45.5±3     |
|        | 100           | 27.4±2     | 15.3±2     | 19.4±1     | 20.4±4     |
| Ribavirin | 500         | 41.0±3     | 33.8±2     | 36.7±1     | 38.9±3     |
|        | 100           | 19.6±5     | 9.8±2      | 8.2±1      | 7.5±2      |
| Ningnanmycin | 500    | 70.2±1     | 68.5±1     | 56.0±2     | 66.6±1     |
|        | 100           | 23.9±2     | 37.7±3     | 18.9±1     | 25.2±2     |

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### Table 3. *In vitro* and *in vivo* anti-TMV activity of 15-hydroxyphenanthroquinolizidine alkaloids 19–24.

| Compd. | Conc. (µg/mL) | *In vitro* | *In vivo* |
|--------|---------------|------------|-----------|
|        |               | Inhibition ratio (%) | Inactivation effect (%) | Curative effect (%) | Protection effect (%) |
| 19     | 500           | 52.0±1     | 53.7±2     | 57.3±4     | 58.9±2     |
|        | 100           | 28.8±1     | 40.4±3     | 35.4±2     | 31.0±1     |
| 20     | 500           | 51.7±3     | 51.2±2     | 53.4±2     | 50.0±1     |
|        | 100           | 28.7±4     | 18.7±3     | 17.5±1     | 23.4±2     |
| 21     | 500           | 46.4±5     | 44.9±3     | 46.7±4     | 45.2±1     |
|        | 100           | 18.5±2     | 17.0±2     | 20.2±4     | 21.7±5     |
| 22     | 500           | 65.3±3     | 60.3±1     | 56.7±2     | 63.8±3     |
|        | 100           | 40.0±2     | 37.9±1     | 32.3±2     | 38.5±3     |
| 23     | 500           | 35.9±1     | 39.6±2     | 40.3±3     | 42.7±2     |
|        | 100           | 16.6±3     | 5.8±2      | 13.1±4     | 10.2±2     |
| 24     | 500           | 52.5±5     | 57.6±3     | 55.7±2     | 52.4±4     |
|        | 100           | 34.0±3     | 26.0±1     | 26.8±2     | 32.6±4     |
| (R)-Antofine | 500     | 52.8±1     | 42.7±2     | 46.0±1     | 45.5±3     |
|        | 100           | 27.4±2     | 15.3±2     | 19.4±1     | 20.4±4     |
| Ribavirin | 500         | 41.0±3     | 33.8±2     | 36.7±1     | 38.9±3     |
|        | 100           | 19.6±5     | 9.8±2      | 8.2±1      | 7.5±2      |
| Ningnanmycin | 500   | 70.2±1     | 68.5±1     | 56.0±2     | 66.6±1     |
|        | 100           | 23.9±2     | 37.7±3     | 18.9±1     | 25.2±2     |

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organic layer was dried over anhydrous MgSO₄, filtered and concentrated in vacuo to afford alkaloids 10-14.

Synthesis of 2-[(2,3-Ethynedioxy-6,7-dimethoxyphenanthren-9-y1)methyl]pyridine (29)
A mixture of 26f (0.3 mmol), acetic acid (15 mL), trifluoroacetic acid (3.8 mL) and PO₂H₂O (3 mg) was stirred for 24 h at a pressure of 50 atm under hydrogen. The mixture was filtered, and the filtrate was evaporated to dryness in vacuo. The residue was treated with aqueous NaOH and extracted with CH₂Cl₂ (5×20 mL). The combined organic extracts were dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with CH₂Cl₂/EtOAc (6:1) as eluant to give 29 (0.1 g, 53%) as a white powder.

Synthesis of 2-[(2,3-Ethynedioxy-6,7-dimethoxyphenanthren-9-y1)methyl]piperidine (30)
A mixture of 29 (5 mmol), acetic acid (150 mL), trifluoroacetic acid (30 mL) and PO₂H₂O (10 mg) was stirred for 24 h at a pressure of 50 atm under hydrogen. The mixture was filtered, and the filtrate was evaporated to dryness in vacuo. The residue was treated with aqueous NaOH and extracted with CH₂Cl₂ (5×100 mL). The combined organic extracts were dried over anhydrous Na₂SO₄, filtered, and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with CH₂Cl₂/CH₃OH (2:1) as eluant to give 30 (1.8 g, 92%) as a white powder.

Synthesis of 6,7-Ethynedioxy-2,3-dimethoxyphenanthro[9,10-b]quinolizidine (15)
Compound 15 was synthesized from 33 (3 mmol) by using a similar procedure for the synthesis of alkaloids 10-14, white powder.

Synthesis of (E)-Methyl 2-(3,4-dimethoxyphenyl)-3-(4-hydroxyphenyl)acrylate (31)
A mixture of 2-(3,4-dimethoxyphenyl)acetic acid (0.1 mol), 4-hydroxybenzaldehyde (0.1 mol), acetic anhydride (40 mL), and triethylamine (20 mL) was refluxed for 20 h with the exclusion of moisture. The solution was cooled to room temperature, then water (60 mL) was added, and the mixture was stirred for 1 h. The mixture was then poured into aqueous potassium carbonate (70.0 g in 160 mL water) and refluxed until nearly all the gummy material was dissolved. The solution obtained was cooled, extracted with ether (3×30 mL), and carefully acidified with concentrated hydrochloric acid (pH 4–5) to produce a white precipitate, filtered and dried to give acid 310 (3.8 g, 98%) as a white powder.

Synthesis of Methyl 3-(benzoyloxy)-6,7-dimethoxyphenanthrene-9-carboxylic acid (34)
A solution of ester 33 (0.01 mol) and KOH (0.05 mol) in H₂O (140 mL) and CH₃OH (140 mL) was refluxed for 8 h, and cooled to room temperature. The solution obtained was cooled, extracted with ether (3×100 mL), and carefully acidified with concentrated hydrochloric acid (pH 4–5) to produce a white precipitate, filtered and dried to give acid 34 (3.8 g, 98%) as a white powder.

Synthesis of 3-(Benzyloxy)-6,7-dimethoxyphenanthrene-9-yl)(pyridin-2-yl)methanol (35)
Compound 35 was synthesized from 34 (20 mmol) by using a similar procedure for the synthesis of methanol 26, yellow powder (3.4 g, 60%).

Synthesis of (3-Hydroxy-6,7-dimethoxyphenanthren-9-yl)(pyridin-2-yl)methanone (36)
A mixture of methanone 35 (4 mmol) and 10% Pd/C (0.1 g) in CH₃OH (600 mL) was refluxed for 24 h under hydrogen. The solution was cooled to room temperature, filtered and concentrated in vacuo. The residue was recrystallized from CH₂Cl₂ and petroleum ether to give alcohol 36 (1.2 g, 83%) as a white powder.

Synthesis of 2-[(3-Hydroxy-6,7-dimethoxyphenanthren-9-yl)methyl]pyridine (37)
A mixture of 36 (1 mmol), trifluoroacetic acid (60 mL) and triethylsilane (10 mL) was refluxed for 8 h. The reaction mixture was concentrated to dryness under reduced pressure. The residue was treated with aqueous NaOH and extracted with CH₂Cl₂ (3×50 mL). The combined organic layer was dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with CH₂Cl₂/EtOAc (2:1) as eluant to afford 37 (0.27 g, 79%) as a white powder.

Synthesis of 2-[(3-Hydroxy-6,7-dimethoxyphenanthren-9-yl)methyl]piperidine (38)
Method A. Compound 38 was synthesized from 37 (3 mmol) as a white powder (0.87 g, 82%) by using a similar procedure for the synthesis of compound 30 except for under hydrogen at a pressure of 10 atm.

Method B. A mixture of methanone 35 (4 mmol) and 10% Pd/C (0.16 g) in acetic acid (40 mL) was stirred at room temperature for 24 h, and then refluxed for 24 h under hydrogen. The mixture was cooled to room temperature, filtered and concentrated in vacuo. The residue was treated with aqueous NaOH and extracted with CH₂Cl₂/petroleum ether (1:3) as eluant to obtain 32 (4.9 g, 51%) as a yellow powder.
NaOH and extracted with CH₂Cl₂ (3×100 mL). The combined organic layer was dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with CH₂Cl₂/CH₃OH (12:1) as eluant to afford 38 (0.83 g, 59%) as a white powder.

### Synthesis of 2-[(3-Benzzyloxy)-6,7-dimethoxyphenanthren-9-yl]methyl)piperidine (39)

A mixture of 38 (1 mmol), (Boc)₂O (9.3 mmol) and saturated NaHCO₃ solution (1.5 mL) in THF (40 mL) was stirred at room temperature for 3 h, then extracted with CH₂Cl₂ (3×50 mL). The combined organic layer was washed with brine (2×50 mL), dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. The residue was dissolved in acetone. To the solution was added benzyl bromide (4.2 mmol) and K₂CO₃ (5.5 mmol). The mixture was refluxed for 3 h under nitrogen, and quenched with H₂O (50 mL), extracted with CH₂Cl₂ (3×50 mL). The combined organic layer was washed with brine (2×50 mL), dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. The residue was recrystallized from ether acetate and petroleum ether to give a white powder. A mixture of the powder in 1,4-dioxane (20 mL) was stirred for 45 min at 0°C under HCl gas atmosphere. The reaction mixture was concentrated in vacuo. The residue was treated with aqueous NaOH and extracted with CH₂Cl₂ (3×50 mL). The combined organic layer was washed with brine (2×50 mL), dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. The residue was recrystallized from ethanol to afford compound 39 (0.16 g, 36%) as a white powder.

### Synthesis of 6-Benzylxoy-2,3-dimethoxyphenanthro[9,10-b]quinolizidine (40)

Compound 40 was synthesized from 39 (3 mmol) by using a similar procedure for the synthesis of alkaloids 10–14, white powder (1.1 g, 80%).

### Synthesis of 6-Hydroxy-2,3-dimethoxyphenanthro[9,10-b]quinolizidine (16)

A mixture of compound 40 (0.08 mmol) and 10% Pd/C (0.1 g) in THF (50 mL) was stirred for 36 h under hydrogen in the dark. The mixture was filtered and concentrated in vacuo to afford alkaloid 16 (0.02 g, 69%) as a light pink powder.

### Synthesis of 2-[(3-Cyclopropylmethoxy-6,7-dimethoxyphenanthren-9-yl)methyl]piperidine (41)

A mixture of pyridine 37 (2.8 mmol) and Cs₂CO₃ (3.7 mmol) in DMF (20 mL) was stirred for 1 h at room temperature. To the result solution was added cyclopentylmethyl bromide (3.3 mmol). The mixture was stirred for 10 h at room temperature, and quenched with H₂O (100 mL), then extracted with CH₂Cl₂ (3×50 mL). The combined organic layer was washed with H₂O (6×50 mL) and brine (3×50 mL), dried over anhydrous MgSO₄, filtered and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with CH₂Cl₂/EtOAc (6:1) as eluant to afford 41 (1.0 g, 83%) as a white powder.

### Synthesis of 2-[(3-Cyclopropylmethoxy-6,7-dimethoxyphenanthren-9-yl)methyl]piperidine (42)

A mixture of 41 (1 mmol), conc. HCl solution (5 mL), ethanol (100 mL) and PO₄₂⁻H₂O (0.1 g) was stirred for 36 h at a pressure of 50 atm under hydrogen. The mixture was filtered, and the filtrate was evaporated to dryness in vacuo. The residue was treated with aqueous NaOH and extracted with CH₂Cl₂ (3×100 mL). The combined organic extracts were dried over anhydrous Na₂SO₄, filtered and concentrated in vacuo. The residue was purified by flash column chromatography on silica gel with CH₂Cl₂/CH₃OH (12:1) as eluant to give 42 (0.3 g, 83%) as a white powder.

### Synthesis of 6-Cyclopropylmethoxy-2,3-dimethoxyphenanthro[9,10-b]quinolizidine (17)

Compound 17 was synthesized from 42 (3 mmol) by using a similar procedure for the synthesis of alkaloids 10–14, white powder (0.87 g, 80%).

### Antiviral Biological Assay

The anti-TMV activity of the synthesized pure compounds was tested using our previously reported method [30].

#### Antiviral activity of compounds against TMV in vitro

- Fresh leaf of the 5–6 growth stage of tobacco (Nicotiana tabacum var Xanthi nc) inoculated by the leaf-leaf rubbing method (concentration of TMV is 5.88×10⁻¹² µg/mL) was cut into halves along the main vein. The halves were immersed into the solution of 500 µg/mL of the compounds and double distilled water for 20 min, respectively, and then cultured at 25°C for 72 h. Each compound was replicated at least three times.

- **Protective effect of compounds against TMV in vivo**
  - The compound solution was smeared on the left side and the solvent serving as control on the right side of growing tobacco (Nicotiana tabacum var Xanthi nc) leaves of the same ages. The leaves were then inoculated with the virus after 12 h. A brush was dipped in TMV of 6×10⁻⁵ µg/mL to inoculate the leaves, which were previously scattered with silicon carbide. The leaves were then washed with water and rubbed softly along the nervation once or twice. The local lesion numbers appearing 3–4 days after inoculation were counted. There are three replicates for each compound.

#### Inactivation effect of compounds against TMV in vitro

The virus was inhibited by mixing with the compound solution at the same volume for 30 min. The mixture was then inoculated on the left side of the leaves of tobacco (Nicotiana tabacum var Xanthi nc), whereas the right side of the leaves was inoculated with the mixture of solvent and the virus for control. The local lesion numbers were recorded 3–4 days after inoculation. There are three replicates for each compound.

#### Curative effect of compounds against TMV in vivo

- Growing leaves of tobacco (Nicotiana tabacum var Xanthi nc) of the same ages were selected. TMV (concentration of 6.0×10⁻⁵ mg/mL) was dipped and inoculated on the whole leaves. Then the leaves were washed with water and dried. The compound solution was smeared on the left side, and the solvent was smeared on the right side for control. The local lesion numbers were then counted and recorded 3–4 days after inoculation. There are three replicates for each compound.

- **Inhibition rate (%)** = [(av local lesion no. of drug-treated) / av local lesion no. of control] × 100%.

### Conclusion

In summary, based on the literature report and our previous work, a series of phenanthroquinolizidine alkaloids 1–24 were designed, prepared and evaluated for their anti-TMV activity for the first time. The in vitro and in vivo antiviral bioassays showed that most of these alkaloids exhibited good to excellent in vivo anti-TMV activity, of which compounds 1, 2, 15 and 16 displayed significantly higher activity than (R)-antofine and commercial...
Ningnamycin at the same test condition. The *in vitro* activity and *in vivo* activity of each alkaloid are about similar, which indicates that these alkaloids possess a good biological availability. The introduction of 6-hydroxy, which is proposed to interact with TMV RNA, did increased anti-TMV activity. The 14α-introduction of 6-hydroxyl, which is proposed to interact with TMV RNA, did increased anti-TMV activity. The 14α-introduction of 6-hydroxyl, which is proposed to interact with TMV RNA, did increased anti-TMV activity.

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Supporting Information

Figure S1 *1H NMR and 13C NMR spectra of the synthesized compounds 10–17 and 26–42. *1H NMR spectra: 26a, 27a, 28a, 10, 17 and 29, 42. *13C NMR spectra: 26a, 27c, 28a, 28c, 28d, 10, 17 and 29, 42.

Text S1 Experimental data of the synthesized compounds 10–17 and 26–42.

Author Contributions

Conceived and designed the experiments: ZW AF QW. Performed the experiments: ZW AF MC LW QW. Analyzed the data: ZW AF YL QW. Contributed reagents/materials/analysis tools: ZW AF MC QW. Wrote the paper: ZW QW.