



# Article Discovery of cysteine and its derivatives as novel antiviral and antifungal agents

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## 1. The detailed bio-assay procedures

# 1.1. Phytotoxic Activity.

The growing 5–6 leaf stage tobaccos (*Nicotiana tabacum var Xanthi nc*) were selected. The compound solution (500  $\mu$ g/mL) was smeared on the leaves and calculated the number of lesions after 0, 3, 7 and 10 days respectively. [1] There are three replicates for each compound.

## 1.2. Antiviral Biological Assay

# 1.2.1. Purification of Tobacco Mosaic Virus.

Using Gooding's method [2], the upper leaves of *Nicotiana tabacum* L. inoculated with TMV were selected and ground in phosphate buffer and then filtered through double-layer pledget. The filtrate was centrifuged at 10000 g, treated with PEG twice, and centrifuged again. The whole experiment was processed at 4 °C. Absorbance value was estimated at 260 nm by ultraviolet spectrophotometer

*Virus concn* = ( $A_{260} \times dilution \ ratio$ )  $E / \frac{0.1\%}{1 cm}$ 

# 1.2.2. Antiviral Activity of Compounds against TMV in Vitro.

In vitro activity of the synthesized compounds against TMV was performed by the conventional half-leaf method. [1,3] Fresh leaf of the 5-6 growth stage of tobacco inoculated by the juice-leaf rubbing method (concentration of TMV is  $5.88 \times 10^{-2} \,\mu g/mL$ ) was cut into halves along the main vein. The halves were immersed into the solution of different concentrations (see **Table 1**) of the compounds and double distilled water for 20 min, respectively, and then cultured at 25 °C for 72 h. Every concentration for each compound was replicated at least three times.

#### 1.2.3. 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 *N. tabacum* L. leaves of the same ages. The leaves were then inoculated with the virus after 12 h. A brush was dipped in TMV of  $6 \times 10^{-3}$  mg/mL to inoculate the leaves, which were previously scattered with silicon carbide. The leaves were then washed with water and rubbed softly along the nervature once or twice. The local lesion numbers appearing 3–4 days after inoculation were counted. [1,4] There are three replicates for each compound.

## 1.2.4. Inactivation Effect of Compounds against TMV in Vivo.

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 *N. tabacum* L., 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. [1,4] There are three replicates for each compound.

# 1.2.5. Curative Effect of Compounds against TMV in Vivo.

Growing leaves of *N. tabacum* L. of the same ages were selected. TMV (concentration of  $6.0 \times 10^{-3}$  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. [1,4] There are three replicates for each compound. The in vitro and in vivo inhibition rates of the compound were then calculated according to the following formula ("av" means average, and controls were not treated with compound).

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

# 1.3.1. In Vitro Antifungal Bioassay.

The fungicidal activities of compounds were evaluated in mycelial growth tests conducted in artificial media against 14 plant pathogens at a rate of 50  $\mu$ g/mL. Each test compound was dissolved in a suitable amount of acetone and diluted with water containing 0.1% TW-80 to a concentration of 500  $\mu$ g/mL. To each petri dish was added 1 mL of the test solution and 9 mL of culture medium to make a 50  $\mu$ g/mL concentration of the test compound, while in another petri dish was added 1 mL distilled water containing 0.1% TW-80 and 9 mL of culture medium as a blank control. A 4 mm diameter of hyphal growth was cut using a hole puncher on a growing fungal culture and the hyphae were moved to the petri dish containing the test compound. Each assay was performed three times. The dishes were stored in controlled environment cabinets (24 ± 1 °C) for 4 days, after which the diameter of mycelial growth was measured and the percentage inhibition was calculated using the following equation: *Percentage inhibition* (%) = (averaged diameter of mycelia in blank controls – averaged diameter of mycelia in medicated tablets)/(averaged diameter of mycelia in blank controls – 4 mm) × 100.[5]

# 1.3.2. In Vivo Antifungal Activity

The in vivo antifungal activity was evaluated by two methods. [5] In the two methods, azoxystrobin was evaluated as the positive controls at the same condition. Aqueous DMF (1%) containing 0.1% Tween 80 was set up as the blank control. Each tested compound was dissolved in DMF and then suspended in the distilled water (containing 0.1% Tween 80) to give the tested concentration of 200 mg·L<sup>-1</sup>. Three replicates were done for each compound. The inhibition percentage was expressed as the mean of values obtained in three independent experiments.

Method 1: Healthy tomato were sprayed with a solution of the tested compound at 200 mg·L<sup>-1</sup> and evaporated under an ambient environment (~28 °C), which were washed and treated with 75% aqueous ethanol in advance. The epidermis of the fruits was punctured with an inoculating needle ( $\emptyset$ , 4.5 mm) and then the pathogen was inoculated with 4 mm plugs of corresponding pathogens. The fruits were assessed after cultivation at 25 °C for 4 days. The control efficacy of the target compounds was calculated as (*averaged diameter of mycelia in blank controls—averaged diameter of mycelia in medicated tablets*) / (*averaged diameter of mycelia in blank controls*—4 mm) × 100.

Method 2: Rape (*Sclerotinia sclerotiorum*) seedlings at the two-leaves stage were used to assay fungicidal activity. The tested compounds at the concentration of 200 mg·L<sup>-1</sup> was sprayed on the surface of the rape leaves and evaporated under an ambient environment (~28 °C). After air drying, the rape leaves were inoculated by spraying the mycelial suspension of corresponding pathogens. Three days later, the symptoms were examined. The control efficacy of the target compounds was calculated as (*averaged disease index of blank controls*—*averaged disease index of drug treated*)/(*averaged disease index of blank controls*) × 100.

# 2. Mode of Action Studies

## 2.1. In vitro TMV rod assembly inhibition

TMV purification was performed according to the instructions by Leberman and Hao. [6,7] TMV RNA was purified by RNA pure virus kit (CoWin Biosciences) and TMV capsid protein (TMV CP) was isolated using glacial acetic acid as described by Fraenkel-Conrat. [8] Before assembly, 20S CP Disk was prepared by incubating 20 mg/mL TMV CP in 0.1 M phosphate buffer (pH 7.0) at 20 °C for 12 h. After incubation, in vitro TMV reconstitution reactions were performed by adding 5  $\mu$ L of phosphate buffer (0.1 M, pH 7.0), 4  $\mu$ L of 20S Disk (2 mg/mL) and 1  $\mu$ L of TMV RNA (200 ng/ $\mu$ L). The assembly reaction mixture was incubated at 20 °C for 12 h and could be then transferred into the copper grid for transmission electron microscop (TEM) assay. The assembly reaction mixture (5  $\mu$ L) was mixed with 5  $\mu$ L 0.1 M phosphate buffer (pH 7.0) and dropped onto the copper film waiting for 5 minutes. After the incubation, the droplet was removed by filter paper and negatively stained by 2% phosphotungstic acid (pH 7.0) for three minutes. After removing the staining agent, the copper

was placed at 37  $^{\circ}$ C for 2 h for drying. The morphology of the reconstituted TMV rods was imaged at 200 keV on a CCD camera. For the inhibition tests with the compounds, in vitro TMV reconstitution inhibition reactions were performed by adding 4.8 µL of phosphate buffer (0.1 M, pH 7.0), 4 µL of 20S Disk (2 mg/mL), 1 µL of TMV RNA (200 ng/µL) and 0.2 µL of DMSO or the compound (10 µM). All treatments were repeated over time to validate the results. [6]

# 2.2. In vitro 20S CP Disk assembly inhibition

For the inhibition tests with the compounds, TMV CP was first adjusted to 20.1 mg/mL with 0.1 M phosphate buffer (pH 7.0). In vitro 20S CP Disk assembly reactions were performed by adding 9.8  $\mu$ L TMV CP (20.1 mg/mL) and 0.2  $\mu$ L DMSO or the compound (10  $\mu$ M). The assembly reaction was incubated at 20 °C for 12 h. The morphology of the 20S CP Disk was imaged via TEM at 200 keV on a CCD camera. All treatments were repeated over time for confirmation. [6]

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ppm (t1)



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ppm (t1)

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![](_page_21_Picture_3.jpeg)

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