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Design, synthesis and anti-ToCV activity of novel 4(3*H*)-quinazolinone derivatives bearing dithioacetal moiety

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ABSTRACT: Tomato chlorosis virus (ToCV) has caused great harm to the production of tomato worldwide. To develop efficient anti-ToCV agents, some novel 4(3*H*)-quinazolinone derivatives containing dithioacetal were designed and synthesized, and their anti-ToCV activities were evaluated by microscale thermophoresis (MST) using ToCV coat protein (ToCV-CP) as a new target. The results showed that some compounds had strong binding capacity to ToCV-CP. In particular, compounds **C5** and **C22** have excellent binding capacity to ToCV-CP, with binding constant values of 0.24 and 0.25 μM, respectively. Additionally, reduced ToCV-CP gene expression levels of 81.05% and 87.59% could be achieved when tomato was treated with compounds **C5** and **C22**, respectively, which were obviously higher than those levels after Ningnanmycin (NNM) treatment (43.88%) and lead compound Xiangcaoliusuobingmi (XCLSBM) treatment (63.56%). Therefore, this work indicates that 4(3*H*)-quinazolinone derivatives containing dithioacetal moiety can be used as novel anti-ToCV agents.

KEYWORDS: *Tomato chlorosis virus, 4(3H)-quinazolinone, dithioacetal, MST, coat protein, anti-ToCV activity*

INTRODUCTION

Tomato chlorosis virus (ToCV), a plant RNA virus transmitted by whitefly, was first discovered in the United States in 1998, and it belongs to the family *Closteroviridae*, genus *Crinivirus.*[1,2] ToCV has a wide range of hosts; it not only infects tomato, pepper, potato and other *Solanaceae* plants but also infects 25 other plant species from eight different families. After being infected with ToCV, the tomato mainly exhibited developmental delay, leaf brittleness, interveinal chlorosis, and limited necrotic flecking.[3-6] In recent years, ToCV has occurred in more than 20 countries around the world and caused enormous economic losses.[7-11] Currently, effective agents to control this plant virus disease are still lacking, resulting in a serious decline in tomato quality and yield. Therefore, the development of efficient anti-ToCV agents is urgently needed.

The genome of ToCV consists of two genomic positive-sense RNAs, RNA1 and RNA2, with a total genome length of 16.8 kb,[12,13] encoding four and nine open reading frames (ORFs) [14,15]. RNA2 encodes multiple functional proteins, of which, the coat protein (CP) and the minor coat protein (mCP) are mainly involved in virus assembly, coating, and transporting. Coat proteins are essential for the intercellular translocation of this type virus.[16] The loss of CP can prevent the virus from forming a complete viable virion.[17,18] Therefore, it is an important pathway for development of anti-ToCV agents using ToCV-CP as a target protein.[19]

In our previous work, we discovered and reported the anti-plant virus activity of the dithioacetal structure, which has good antiviral activity against tobacco mosaic virus

(TMV), potato virus Y (PVY), cucumber mosaic virus (CMV) and ToCV.[19-21] In addition, we established an efficient and convenient screening method for anti-ToCV agents using ToCV-CP as a target protein and some glycoside-containing dithioacetal derivatives were synthesized with good antiviral activity against ToCV.[19] As an important class of nitrogen-containing heterocyclic compounds, 4(3*H*)-quinazolinones are widely found in natural products with a range of significant biological activities. Our group found that some 4(3*H*)-quinazolinone derivatives showed good antiviral activity against CMV and TMV.[22-24] However, the biological activity of this class of compounds against ToCV has not been reported. In this work, a series of 4(3*H*)-quinazolinone derivatives containing dithioacetal (Figure 1) were designed, synthesized, and evaluated for their anti-ToCV activities *in vitro* and *in vivo*. The findings will provide guidance for the design, synthesis and development of anti-ToCV agents.

Figure 1

MATERIALS AND METHODS

Chemicals. Analytically pure reagents used in the experiments did not require further drying or purification.

Instruments. ^{1}H NMR and ^{13}C NMR spectra of the compounds were obtained using a Bruker DPX 400 MHz (Bruker, Germany) and a JEOL-ECX500 MHz (JEOL, Tokyo, Japan) in $CDCl_3$ or DMSO-d_6 solution. HRMS was performed with a Thermo Scientific Q Exactive (Thermo Scientific, USA). The melting points of the compounds were measured using WRX-4 equipment.

General Procedure for the Synthesis of the Intermediates A1-A3 and B1-B9. Intermediates **A1-A3** were synthesized according to the methods reported in the literature.[25] Intermediates **A1-A3** (2.35 mmol), substituted p-hydroxybenzaldehyde (2.35 mmol) and CH_3CN (20 mL) were added to a 50 mL three-necked flask, with K_2CO_3 (2.82 mmol) as an acid-binding agent. The mixing system was stirred at room temperature for 10 minutes, and KI (0.23 mmol) was then added. The mixture was allowed to react under reflux for 8 to 12h. After the reaction completed (as monitored by TLC), the solvent was removed under vacuum, the residue was then diluted with water, dichloromethane (50 mL×3) was added for extraction, and the two layers were separated. The organic layer was dried and concentrated to obtain the crude products, which were recrystallized with anhydrous ethanol to obtain the intermediates **B1-B9**.

Figure 2

General Procedure for Preparation of the Title Compounds C1-C27. The intermediates **B1-B9** (1.34mmol) were added to a round bottom flask containing substituted mercaptan (2.68 mmol) and $NaHSO_4 \cdot SiO_2$ (1.34 mmol) in CH_2Cl_2 solvent (10 mL). Upon reaction completion, the solvent was removed under vacuum, the residue was dissolved with water, dichloromethane (30 mL×3) was added for extraction, and the two layers were separated. The collected dichloromethane layer was evaporated, and then purified by flash chromatography with ethyl acetate /petroleum ether (1:3, v/v) to obtain the title Compounds **C1-C27** (Figure 2).

Purification of Tomato Chlorotic Virus Coat Protein (ToCV-CP). The ToCV-CP was cloned, expressed and purified according to the method described in

the literature.[19]

Evaluation of Anti-ToCV Activity *in vitro*. Binding of compounds with ToCV-CP was analyzed through microscale thermophoresis (MST) to obtain Kd values by methods described in the literature.[26,27] Meanwhile, NNM, the lead compound XCLSBM and Ribavirin (RIB) were used as positive controls. All tests were duplicated three times.

Evaluation of Anti-ToCV Activity in vivo. We used tomato samples (Shouguang, Shandong) infected with ToCV to start *in vivo* experiments. All of the samples were confirmed to be infected with ToCV by polymerase chain reaction (PCR). The PCR primer sequences used are shown in Table 1. Anti-ToCV activity was then assessed *in vivo* according to the previously described method.[19] The primers used in quantitative real-time PCR are shown in Table 1. A MiniBEST Plasmid Purification Kit 4.0 (Takara) was used to extract the total RNA of samples, and a Goldenstar RT6 cDNA Synthesis Kit (Tsingke) was used when the extracted total RNA was reverse transcribed into cDNA. The quantitative real-time PCR was performed using the Fast qPCR mix SYBR Green kit (Tsingke).

RESULTS AND DISCUSSION

Chemistry. Figure 2 shows the synthetic routes of 4(3*H*)-quinazolinone derivatives bearing dithioacetal groups. Methanol was used as a solvent, sodium was added, substituted anthranilic acid and chloroacetonitrile as raw materials, and the mixture was stirred at room temperature for 4-6 h to obtain intermediates **A1**-**A3**. The second step of the reaction used acetonitrile as a solvent, K_2CO_3 as a catalyst, intermediates

A1-A3 and substituted 4-hydroxybenzaldehyde under reflux for 8-12 h to obtain intermediates **B1-B9**. Finally, with dichloromethane as a solvent and $NaHSO_4 \cdot SiO_2$ as a catalyst, intermediates **B1-B9** and substituted mercaptan were reacted at room temperature to obtain the target compounds **C1-C27**. The chemical structures of these compounds were identified by HRMS, and NMR (Supporting Information).

Figure 3

Anti-ToCV Activity of Compounds *in vitro*. The results of MST experiments are shown in Table 2, which indicates that the target compounds of this series have good binding affinity to ToCV-CP, with most of the compounds binding at the micromolar level. In particular, compounds **C5** and **C22** showed excellent binding capacity to ToCV-CP, with Kd values of 0.24 and 0.25 μM, which are significantly better than that of RIB (15.62 μM) and slightly superior to those of NNM (0.44 μM) and lead compound XCLSBM (0.36 μM). Compounds **C9**, **C10**, **C12**, and **C19** showed good binding capacity to ToCV-CP, with Kd values of 0.52, 1.63, 1.66, and 1.02 μM, respectively, which are preferred over that of RIB, but second to those of NNM and lead compound XCLSBM (Figure 3).

Structure-activity relationships (SARs). The *in vitro* binding affinities of ToCV-CP with target compounds showed that when R_2 was a chlorine atom (electron-withdrawing group), these compounds had the worst anti-ToCV activity; for example, **C10 > C13 > C16** (H > 2-OCH_3 > 2-Cl); **C11 > C14 > C17** (H > 2-OCH_3 > 2-Cl); **C12 > C15 > C18** (H > 2-OCH_3 > 2-Cl); **C20 > C23 > C26** (H > 2-OCH_3 > 2-Cl); **C5 > C2> C8** (2-OCH_3 > H > 2-Cl); **C22 > C19 > C25** (2-OCH_3 > H > 2-Cl);

however, this rule is not followed by all compounds. For example, **C9 > C3 > C6** (2-Cl > H > 2-OCH_3) and **C27 > C21 > C24** (2-Cl > H> 2-OCH_3). The changes of the substituents of R_1 have a slight effect on the anti-ToCV activity, as fluorine, chlorine and bromine atoms are all electron-withdrawing groups; for example, **C19 > C10 > C1** (6-Br > 6-Cl > 6-F), but **C20 > C2 > C11** (6-Br > 6-F > 6-Cl).

Figure 4

Anti-ToCV Activity of Compounds C5 and C22 *in vivo*. After confirming tomato infection of ToCV by polymerase chain reaction (PCR), the PCR reaction produced an expected site amplicon (774 bp), as shown in Figure 4. The side branches of tomato infected with ToCV were cut off and soaked it in tap water for 5 days, and then situated in a 50 μg/mL compound solution for 7 days. The result showed that the expression levels of the ToCV-CP gene in different compound treatment groups showed large differences. As shown in Figure 5, 81.05% and 87.59% reductions in ToCV-CP gene expression levels could be achieved in the groups treated with compounds **C5** and **C22** in the tomato sample, respectively, which were obviously superior to the water (CK) treatment, the NNM treatment (43.88%) and the lead compound XCLSBM treatment (63.56%) counterparts. The results of in vivo experiments showed that the expression level of ToCV-CP in tomato can be significantly reduced by compounds **C5** and **C22**, which have certain controlling effects on tomato chlorotic virus.

Figure 5

In summary, twenty-seven novel 4(3*H*)-quinazolinone derivatives bearing

dithioacetal moiety were designed and synthesized. The results showed that most of the compounds had good binding capacity to ToCV-CP. In particular, compounds **C5** and **C22** had excellent Kd to ToCV-CP, with values of 0.24 and 0.25 μM, respectively. The *in vivo* experiments further indicate that the ToCV-CP gene expression levels are reduced when tomatoes are treated with compounds **C5** and **C22**. Our present work indicated that novel 4(3*H*)-quinazolinone derivatives containing dithioacetal moiety can be used as novel anti-ToCV agents to control this plant virus disease.

ASSOCIATED CONTENT

Supporting information

Characterization data, ^{1}H and ^{13}C NMR spectra, and HRMS for the title compounds **C1−C27** are shown in the Supplementary Information. The data of the interactions between **C1−C27** and ToCV-CP are also listed in the Supporting Information.

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NOTES

The authors declare no competing financial interest.

ABBREVIATIONS

^{1}H NMR, ^{1}H nuclear magnetic resonance; ^{13}C NMR, ^{13}C nuclear magnetic resonance; HRMS, High-resolution mass spectrometry; NNM, Ningnanmycin; RIB, Ribavirin; XCLSBM, Xiangcaoliusuobingmi; ToCV, tomato chlorosis virus; CP, coat protein; MST, microscale thermophoresis; ToCV-CP, Tomato chlorosis virus coat protein.

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299 **Figure Information**

300 **Figure 1**. Design of the title compounds.

301 **Figure 2**. Synthesis route of the title compounds **C1-C27**.

302 **Figure 3**. Microscale thermophoresis (MST) results of the title compounds.

303 **Figure 4**. Results of samples tested *in vivo* through polymerase chain reaction (PCR).

304 **Figure 5**. Effects of compounds on the inhibition of ToCV-CP levels in tomato *in*

305 *vivo*.

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Table 1. Primers used in this study

Primer	Sequence (5'-3')	Purpose
ToCP F	ATGGAGAACAGTGCCGTTGC	ToCV identification[a]
ToCP R	TTAGCAACCAGTTATCGATGC	
qToCP-F2	TAGATGATGGCGTAGATGAC	ToCV qPCR[b]
qToCP-R2	CTAGTGGAGTGTACCTTCAAT	
qActin-F	TGCCATTCTCCGTCTTGACT	Tomato reference genes[b]
qActin-R	TGCAGTCTCGAGTTCCTGTT	

[a] Primer sequences were reported by Segev *et al.*[10]

[b] Primer sequences were provided by Zhou Tao of China Agricultural University Friendship.

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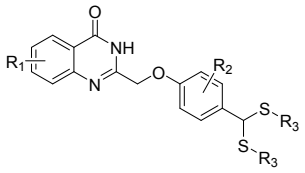
342 **Table 2.** Compound structures and their binding constants (Kd) with the ToCV coat
343 protein

Compound				Kd value (μM)
	R_1	R_2	R_3	
C1	6-F	H	CH_2CH_3	3.49±2.27
C2	6-F	H	$CH_2CH_2CH_3$	5.67±2.92
C3	6-F	H	$CH(CH_3)_2$	3.03+1.42
C4	6-F	2-OCH_3	CH_2CH_3	15.44±5.99
C5	6-F	2-OCH_3	$CH_2CH_2CH_3$	0.24±0.07
C6	6-F	2-OCH_3	$CH(CH_3)_2$	33.95±20.84
C7	6-F	2-Cl	CH_2CH_3	11.56±7.00
C8	6-F	2-Cl	$CH_2CH_2CH_3$	59.65±23.49
C9	6-F	2-Cl	$CH(CH_3)_2$	0.52±0.09
C10	6-Cl	H	CH_2CH_3	1.63±0.73
C11	6-Cl	H	$CH_2CH_2CH_3$	9.98+3.08
C12	6-Cl	H	$CH(CH_3)_2$	1.66±0.54
C13	6-Cl	2-OCH_3	CH_2CH_3	10.43±6.81
C14	6-Cl	2-OCH_3	$CH_2CH_2CH_3$	67.60±30.87
C15	6-Cl	2-OCH_3	$CH(CH_3)_2$	28.74±10.69
C16	6-Cl	2-Cl	CH_2CH_3	15.22±3.64
C17	6-Cl	2-Cl	$CH_2CH_2CH_3$	73.11±52.75
C18	6-Cl	2-Cl	$CH(CH_3)_2$	42.91±29.93
C19	6-Br	H	CH_2CH_3	1.02+0.39
C20	6-Br	H	$CH_2CH_2CH_3$	4.82±3.13
C21	6-Br	H	$CH(CH_3)_2$	25.50±8.79
C22	6-Br	2-OCH_3	CH_2CH_3	0.25±0.06
C23	6-Br	2-OCH_3	$CH_2CH_2CH_3$	42.69±22.76
C24	6-Br	2-OCH_3	$CH(CH_3)_2$	48.65±32.42
C25	6-Br	2-Cl	CH_2CH_3	9.64±8.79
C26	6-Br	2-Cl	$CH_2CH_2CH_3$	44.61±24.51
C27	6-Br	2-Cl	$CH(CH_3)_2$	10.71±4.15
ningnanmycin[a]				0.44±0.26
Ribavirin[a]				15.62±7.15
XCLSBM[b]				0.36±0.24

344 [a]Commercially available agrichemicals were used as controls.

345 [b]Lead compound was reported by Zhang *et al*.[20]

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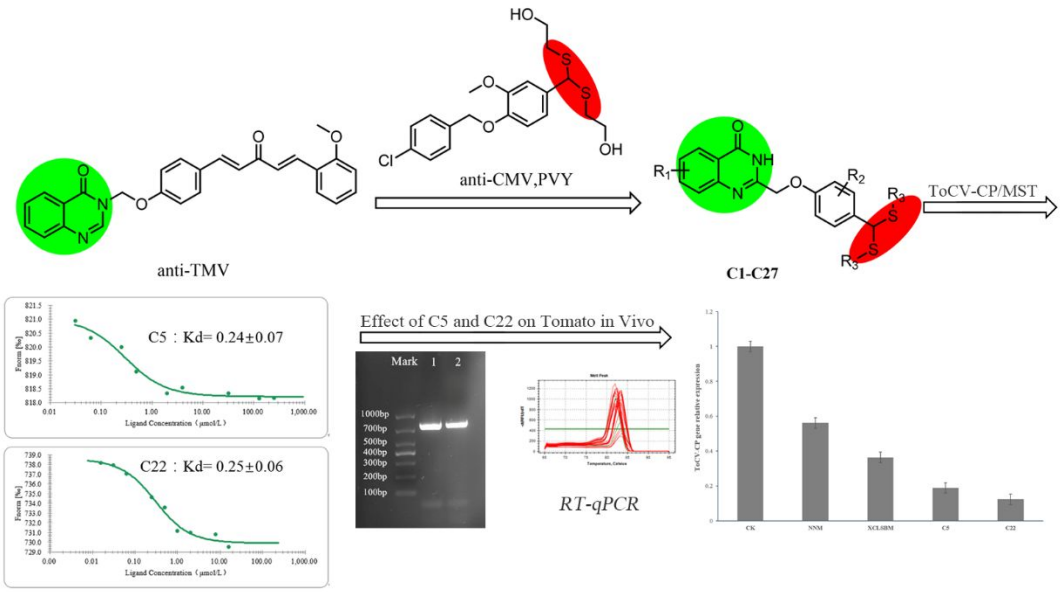
HO
S
S
OH
Cl
O
O
anti-CMV,PVY
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NH
N
O
R1
R2
R3
C1-C27
ToCV-CP/MST
C5 : Kd= 0.24±0.07
C22 : Kd= 0.25±0.06
Ligand Concentration (μmol/L)
Effect of C5 and C22 on Tomato in Vivo
Mark 1 2
1000bp
700bp
500bp
400bp
300bp
200bp
100bp
RT-qPCR
CK
NNM
C5
C22

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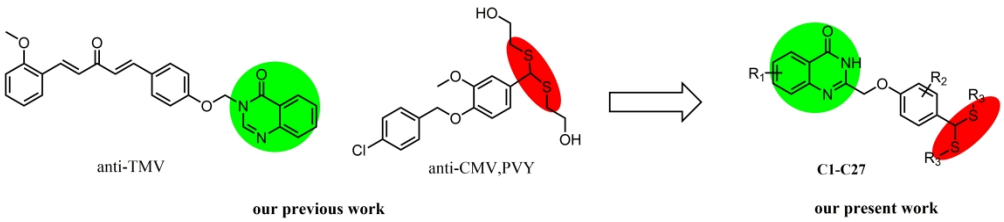
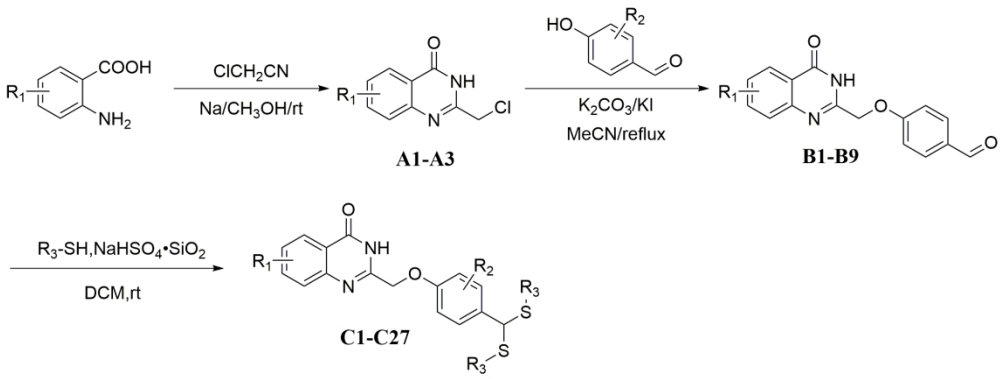


Figure 1. Design of the title compounds.

379x82mm (300 x 300 DPI)



C1:R_1=6-F R_2=H R_3=CH_2CH_3
C2:R_1=6-F R_2=H R_3=$CH_2CH_2CH_3$
C3:R_1=6-F R_2=H R_3=$CH(CH_3)_2$
C4:R_1=6-F R_2=2-OCH_3 R_3=CH_2CH_3
C5:R_1=6-F R_2=2-OCH_3 R_3=$CH_2CH_2CH_3$
C6:R_1=6-F R_2=2-OCH_3 R_3=$CH(CH_3)_2$
C7:R_1=6-F R_2=2-Cl R_3=CH_2CH_3
C8:R_1=6-F R_2=2-Cl R_3=$CH_2CH_2CH_3$
C9:R_1=6-F R_2=2-Cl R_3=$CH(CH_3)_2$
C10:R_1=6-Cl R_2=H R_3=CH_2CH_3
C11:R_1=6-Cl R_2=H R_3=$CH_2CH_2CH_3$
C12:R_1=6-Cl R_2=H R_3=$CH(CH_3)_2$
C13:R_1=6-Cl R_2=2-OCH_3 R_3=CH_2CH_3
C14:R_1=6-Cl R_2=2-OCH_3 R_3=$CH_2CH_2CH_3$
C15:R_1=6-Cl R_2=2-OCH_3 R_3=$CH(CH_3)_2$
C16:R_1=6-Cl R_2=2-Cl R_3=CH_2CH_3
C17:R_1=6-Cl R_2=2-Cl R_3=$CH_2CH_2CH_3$
C18:R_1=6-Cl R_2=2-Cl R_3=$CH(CH_3)_2$
C19:R_1=6-Br R_2=H R_3=CH_2CH_3
C20:R_1=6-Br R_2=H R_3=$CH_2CH_2CH_3$
C21:R_1=6-Br R_2=H R_3=$CH(CH_3)_2$
C22:R_1=6-Br R_2=2-OCH_3 R_3=CH_2CH_3
C23:R_1=6-Br R_2=2-OCH_3 R_3=$CH_2CH_2CH_3$
C24:R_1=6-Br R_2=2-OCH_3 R_3=$CH(CH_3)_2$
C25:R_1=6-Br R_2=2-Cl R_3=CH_2CH_3
C26:R_1=6-Br R_2=2-Cl R_3=$CH_2CH_2CH_3$
C27:R_1=6-Br R_2=2-Cl R_3=$CH(CH_3)_2$

Figure 2. Synthesis route of the title compounds C1-C27.

197x113mm (300 x 300 DPI)

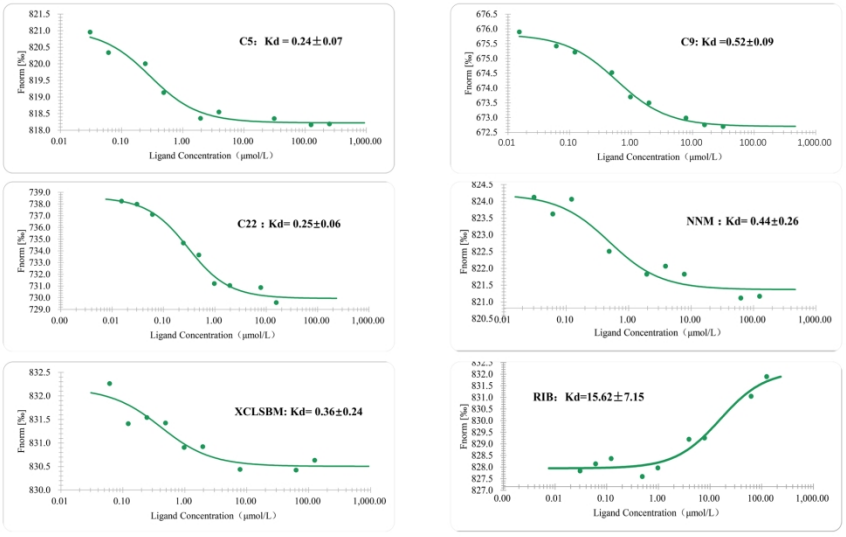


Figure 3. Microscale thermophoresis (MST) results of the title compounds.

279x165mm (200 x 200 DPI)

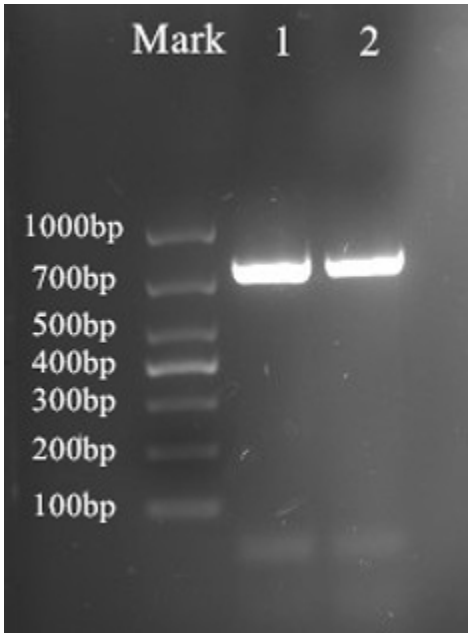


Figure 4. Results of samples tested in vivo through polymerase chain reaction (PCR).

61x83mm (96 x 96 DPI)

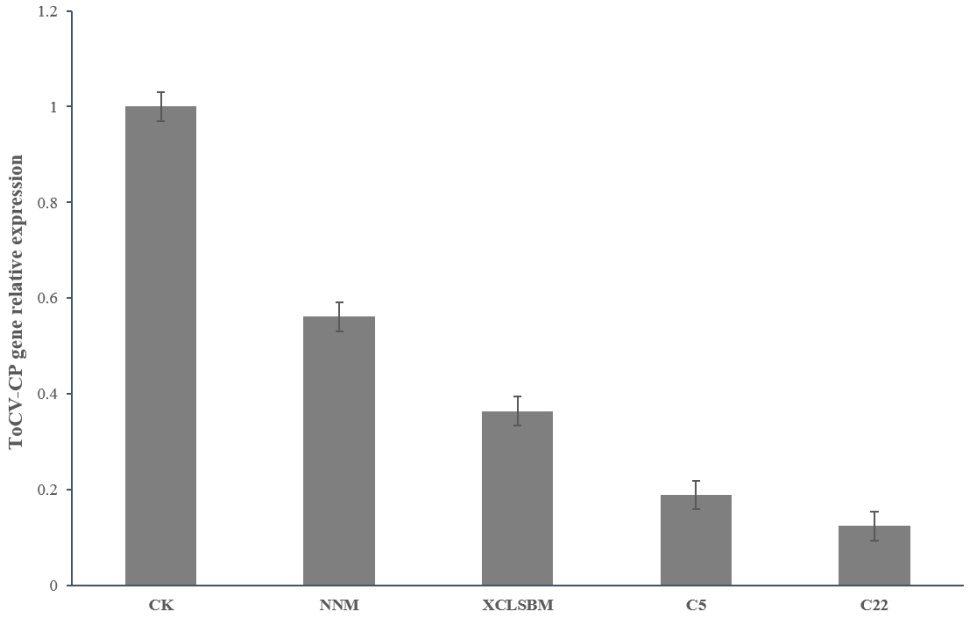
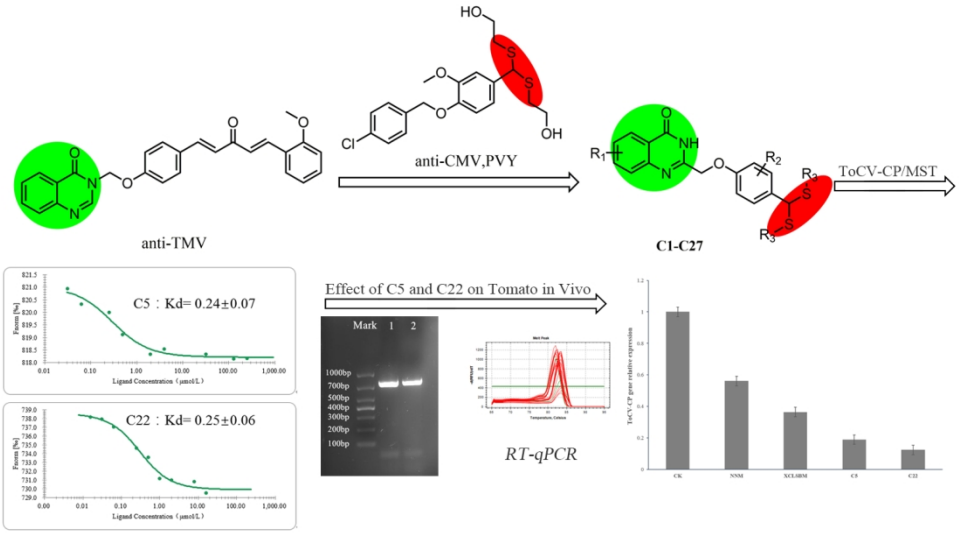


Figure 5. Effects of compounds on the inhibition of ToCV-CP levels in tomato in vivo.

192x122mm (150 x 150 DPI)


anti-TMV
anti-CMV,PVY
C1-C27
ToCV-CP/MST
C5 : Kd= 0.24±0.07
C22 : Kd= 0.25±0.06
Effect of C5 and C22 on Tomato in Vivo
Mark 1 2
1000bp
700bp
500bp
400bp
300bp
200bp
100bp
RT-qPCR

TOC Graphic