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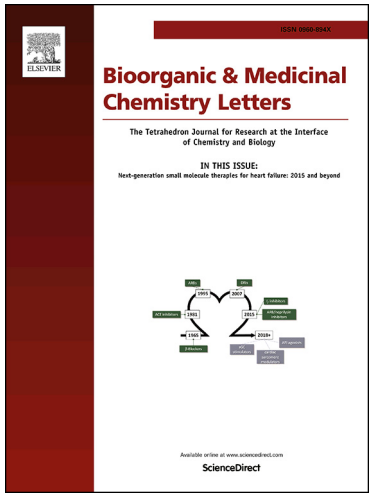

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Pyrazolo[3,4-*d*]pyrimidine derivatives containing a Schiff base moiety as potential antiviral agents

Yan-Yan Wang[a,#], Fang-Zhou Xu [a,#], Yun-Ying Zhu [b], Baoan Song [a,*] , Dexia Luo [a], Gang Yu [a], Shunhong Chen [a], Wei Xue [a], Jian Wu [a], *

Corresponding author footnote

* To whom correspondence should be addressed. Tel.:/fax: +86(851) +86(851)88292090. E-mails: basong@gzu.edu.cn, jwu6@gzu.edu.cn

[#]. The authors are equally contribution to this work.

Current address:

a. State Key Laboratory Breeding Base of Green Pesticide and Agricultural Bioengineering, Key Laboratory of Green Pesticide and Agricultural Bioengineering, Ministry of Education, Research and Development Center for Fine Chemicals, Guizhou University, Guiyang 550025, China.

b. School of Chemical Engineering, Guizhou Institute of Technology, Guiyang 550001, China.

ABSTRACT

A series of pyrazolo[3,4-d]pyrimidine derivatives containing a Schiff base moiety were synthesized, characterised, and evaluated for their activity against tobacco mosaic virus (TMV). Biological assays indicated that several of the derivatives exhibited significant activity against TMV. In particularly, compounds **5y** and **5aa** displayed excellent inactivating activity against TMV, with half maximal effective concentration (EC_{50}) values of 70.3 and 53.65 μg/mL, respectively, which were much better than that of ribavirin (150.45 μg/mL), and **5aa** was superior to ningnanmycin (EC_{50} = 55.35 μg/mL). Interactions of compounds **5y** and **5aa** with TMV coat protein (TMV-CP) were investigated using microscale thermophoresis and molecular docking. Compounds **5y** and **5aa** displayed strong binding capability to TMV-CP with dissociation constant (K_d) values of 22.6 and 9.8 μM, respectively. These findings indicate that pyrazolo[3,4-d]pyrimidine derivatives containing a Schiff base may be potential antiviral agents.

Keywords: Pyrazolo[3,4-d]pyrimidine; Schiff base; Antiviral activity; Molecular docking.

Virus in plants, known as plant cancer, are very difficult to control once plants have been infected, and give rise to serious damage. As an example, tobacco mosaic virus (TMV) is the most persistent plant virus and can survive in dry plant debris for up to 100 years.[1] More than 885 individual species in 65 plant families can be infected by this virus,[1,2] and U.S. $100 million is lost each year globally due to plant diseases caused by TMV. Hence, the development of novel anti-viral molecules against TMV has attracted an increasing amount of attention.

Pyrazolo[3,4-d]pyrimidine are fused heterocyclic ring systems that structurally resemble purines and play a crucial roles in drug discovery due to their broad spectrum of biological properties.[3] And interestingly, the Schiff base is also a highly efficient pharmacophore, which is an ideal sub-structure for developing agrochemicals and medicines,[4-6] previous studies by our group [6d] also reported that several Schiff bases contain quinazolinone derivatives that have excellent anti-TMV activity.

Considering the above mentioned information and the purpose for discovery novel antiviral agents for plant. In current study, we sought to incorporate the sub-structure unit of a Schiff base with the backbone of pyrazolo[3,4-d]pyrimidine, by replacing the quinazolinone backbone with pyrazolo[3,4-d]pyrimidine based on previous works (Figure 1).[6d] Then we derived the structure by introducing different types of moieties, which we hypothesised would result in pyrazolo[3,4-d]pyrimidines containing a Schiff base substructure with good anti-plant virus activity. Based on this hypothesis, a series of novel pyrazolo[3,4-d]pyrimidine derivatives containing a Schiff base was designed and synthesized. Biological assay revealed that most of the synthesized compounds exhibited excellent anti-TMV activity.

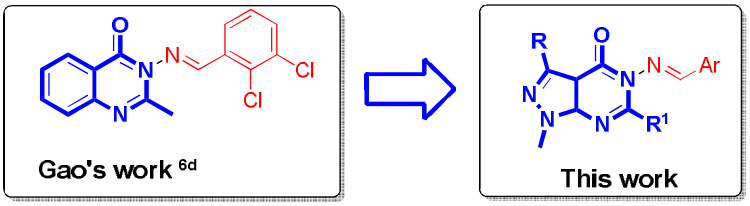
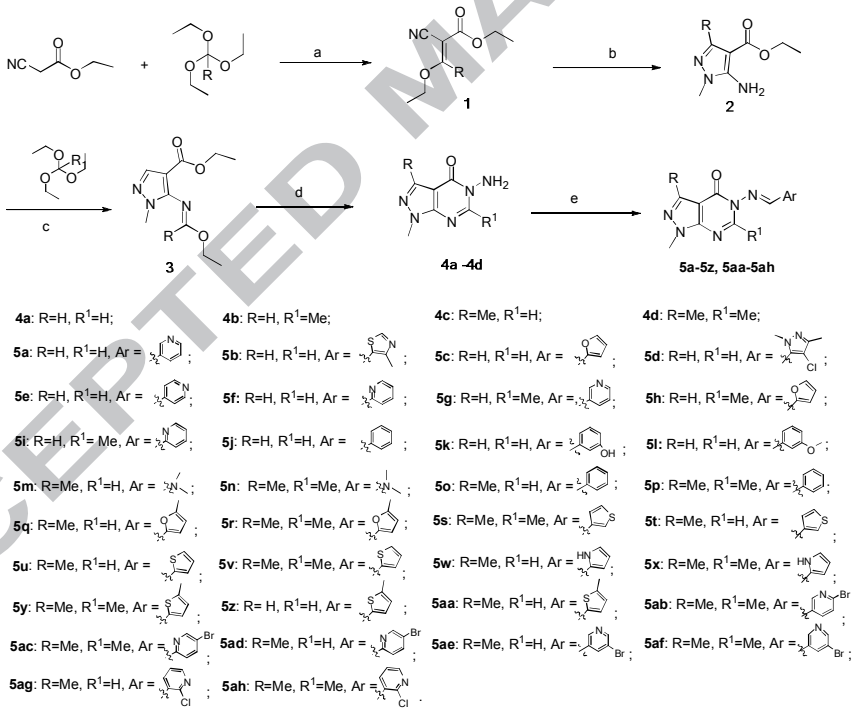


Figure 1. Design of pyrazolo[3,4-d]pyrimidine derivatives containing a Schiff base moiety

The synthesis protocol of pyrazolo[3,4-d]pyrimidines (**5**) is depicted in Scheme 1. All intermediates (**1**–**4**) can be easily synthesized according to references, [7-9] and compound **5** was prepared by the reaction of intermediate **4** with different aromatic aldehyde (or hemiacetal) at reflux in glacial acetic acid with excellent yield. The details of the protocols and spectroscopic data of the synthesized compounds are included in the Supplementary Data.



Scheme 1 Reagents and conditions. (a) reflux, Ac_2O; (b) 40% methylhydrazine, EtOH, 80°C; (c) triethoxyethane/triethoxymethane, reflux; (d) 80%$N_2H_4 \cdot H_2O$, EtOH, r.t; (e) 1,1-dimethoxy-N,N-dimethylmethanamine/aromatic aldehyde, Acetic acid, reflux.

The antiviral activity of pyrazolo[3,4-d]pyrimidine derivatives against TMV was evaluated using the half-leaf spot method [10] (Table 1). Compound **III-31**,[6d]

commercial ningnanmycin and ribavirin were selected as the positive controls. Interestingly, the results indicated intermediate **4** showed good to excellent activity in three models (curative, protection, and inactivation). In particular, the curative activities of **4b** and **4c** were slightly better than that of ningnanmycin. And the protective effects of the intermediates were equivalent to that of ningnanmycin, they also showed moderate inactivation activity at 500 μg/mL. And it could conclude that activities of intermediates **4a**–**4d** could be decreased by introducing a methyl at the 6 position of pyrazolo[3,4-d]pyrimidine, but could be increased by a methyl at the 3 position; thus, the active order was **4b** < **4a** < **4d** <**4c**. The above active data of intermediates **4a**–**4d** led us to conclude that pyrazolo[3,4-d]pyrimidine is an effective pharmacophore. However, the activity of this pharmacophore could be impacted by introducing different types of groups. Generally, compounds with a thiazole (**5b**), pyrazole (**5d**), or pyrrole (compounds **5w** and **5x**) showed low activity, but introduction of a pyridine ring (compounds **5g**, **5i**, **5ab**, **5ad**, **5ae**, **5af**, **5ag**) and 5-methylthiophen-2-yl (**5y, 5z, 5aa**) resulted in excellent anti-TMV activity (curative activity). For instance, compound **5ad** showed 61.4% activity against TMV, and curative activity of compounds **5y** and **5aa** was greater than 62%, which were at the same levels as that of commercial ningnanmycin. Introduction of furan-2-yl (**5h**, **5q**, **5r**) and benzyl (**5o**, **5p**) also led to good anti-TMV activity, the curative activity of them was similar as that of positive control **III-31**. In addition, most of the synthesized compounds had significant protective effects against TMV and were much better than that of ribavirin (50.4%), as well as compounds **5a**, **5g–5j**, **5o, 5t**, **5u**, **5w**, **5y**, **5aa**, and **5ab** showed better inactivation activity than ribavirin (73.2%) and compound **III-31** (78.2%) at 500 mg L^{-1}. In particularly, inactivation effects of compounds **5y** and **5aa** approached the inhibition rate of ningnanmycin.

Table 1. Inhibitory effects of the test compounds against TMV at 500 μg/mL

Compd.	Curative activity (%)	Protection effect (%)	Inactivation effect (%)
4a	58.4±2.9	60.6±0.9	68.7±2.3
4b	33.5±3.1	52.8±0.8	64.3±1.3
4c	64.9±1.7	63.2±2.7	74.2±2.4
4d	62.8±3.2	62.6±2.4	62.2±2.5
5a	48.0±1.9	33.7 ±1.7	83.4 ±2.4
5b	37.1±3.2	49.9±2.1	60.5±1.4
5c	30.2±2.3	44.1±1.1	54.5±2.5
5d	43.3±3.9	41.5±2.1	74.2±1.1
5e	50.4±0.5	45.7±1.2	65.5±1.3
5f	37.1±1.3	45.8±1.8	58.9±2.7
5g	56.8 ±2.1	23.1±2.5	85.2±3.1
5h	55.9±1.4	54.2±1.5	84.0±3.3
5i	56.5±2.3	40.3±1.1	80.1±1.6
5j	47.8±4.2	52.2±1.9	82.5±2.3
5k	42.2±2.4	50.8±2.3	64.8±2.5
5l	32.3±2.1	42.6±1.3	42.1±2.3
5m	29.2±3.9	39.4±2.7	63.5±3.1
5n	32.7±1.9	60.1±1.5	72.9±1.3
5o	52.1±2.5	65.1±1.6	88.9±0.9
5p	55.8±1.7	64.8±4.9	76.4±2.4
5q	54.2±0.9	63.5±1.7	64.1±2.3
5r	55.6±1.9	62.5±1.4	78.0±2.1
5s	45.7±2.2	56.9±1.6	62.4±3.1
5t	57.2±0.9	62.8±1.4	89.6±1.8
5u	51.9±2.2	63.4±3.1	84.5 ± 2.3
5v	42.6±2.8	51.9±1.5	67.4±2.5
5w	32.8±1.3	46.6±2.1	82.2±1.7
5x	14.2±1.3	22.3±1.4	34.8±2.3
5y	62.7±2.4	64.0±1.6	92.1±0.7
5z	59.4±0.9	62.0±1.9	72.8±2.1
5aa	62.7±0.3	62.0±1.9	94.6±2.1
5ab	58.5±1.3	65.2±1.9	85.0±2.1
5ac	22.7±1.2	22.8±1.3	32.9±3.1
5ad	61.4±1.7	57.6±1.2	76.2±2.4
5ae	59.6±0.9	62.0±1.2	67.1±2.3
5af	57.1±1.3	59.1±2.3	65.0±1.5
5ag	58.8±1.6	34.6±1.5	75.5±1.9
5ah	47.5±0.9	43.5±1.4	51.9±2.7
III-31[a]	54.3 ± 2.9	63.2 ± 1.8	78.2± 1.5
ningnanmycin	62.6±2.3	64.2±2.1	93.5±1.7
ribavirin	37.9±1.9	50.4±2.3	73.2±2.2

a. Compound in reference [6d]

The half maximal effective concentration (EC_{50}) shown in Table 2 further revealed that the synthesized compounds displayed good anti-TMV activity. In

particularly, compounds **5y** and **5aa** showed the best curative activity, which were much better than commercial ningnanmycin. And their EC_{50} values for inactivation activity were 70.3 and 53.8 μg/mL, respectively, which were much lower than that of ribavirin, and compound **5aa** showed the best inactivation activity, which was superior to that of ningnanmycin (EC_{50} = 55.35 μg/mL).

Table 2**.** The EC_{50} (μg/mL) of the title compounds against TMV

Compd.	Curative activity	Protection activity	Inactivation activity
4a	505.2± 4.25	383.1± 2.44	/
4c	354.2± 2.42	559.8± 4.45	257.8± 2.21
4d	315.6± 2.33	355.5± 3.12	/
5g	481.4± 4.25	/	165.9± 2.62
5h	480.7± 5.34	282.4± 2.11	195.6± 2.33
5o	659.24± 4.24	380.94± 3.32	181.3± 2.32
5p	485.29± 3.67	340.5± 3.12	157.25± 2.45
5q	401.1± 2.56	365.7± 3.31	364.1± 2.65
5r	337.2± 2.33	280.5± 2.13	/
5t	400.6± 4.31	301.6± 3.16	109.5± 3.11
5u	402.4± 3.14	353.5± 3.23	144.3± 2.46
5y	272.8± 2.31	306.1± 3.41	70.3± 2.43
5z	392.9± 4.12	367.6± 2.12	301.1± 3.18
5aa	283.5± 2.23	296.8± 2.15	53.8± 1.43
5ab	400.6± 5.12	313.2± 2.12	155.5± 2.15
5ad	317.2± 1.34	412.7± 2.15	167.2± 1.34
5ae	400.4± 4.13	274.4± 3.32	329.7± 2.15
5af	427.2± 4.43	432.1± 2.54	328.5 ± 3.01
5ag	446.4± 2.31	/	398.3 ± 2.15
ningnanmycin	324.51± 3.15	165.95± 2.15	55.35 ± 1.45
ribavirin	/	435.99± 4.23	124.05 ± 3.5

TMV coat protein (TMV-CP) plays a vital role in the translation of mRNAs,

transcription of tRNA, and self-assembly of TMV particles, and may be a target protein for the screening of anti-TMV agents.[10] Due to the excellent inactivation activity of compounds **5y** and **5aa**, we speculated that they would interact well with TMV-CP. Hence, the binding interaction between **5y** (or **5aa**) and TMV-CP was investigated using microscale thermophoresis (MST). As shown in Figure 3, compound **5aa** showed strong binding with TMV-CP. MST measurements showed that **5aa** bond to TMV-CP with a dissociation constant (K_d) of 9.8 ± 2.6 μM, which was much lower than that of ribavirin (K_d = 64.7 ± 2.12 μM) and similar to that of ningnanmycin (K_d = 9.75 ± 0.55 μM). In addition, compound **5y** displayed good binding capability with TMV-CP (K_d = 22.6 ± 2.7 μM), which was much better than that of ribavirin. These results demonstrated that both **5y** and **5aa** interacted with TMV-CP.

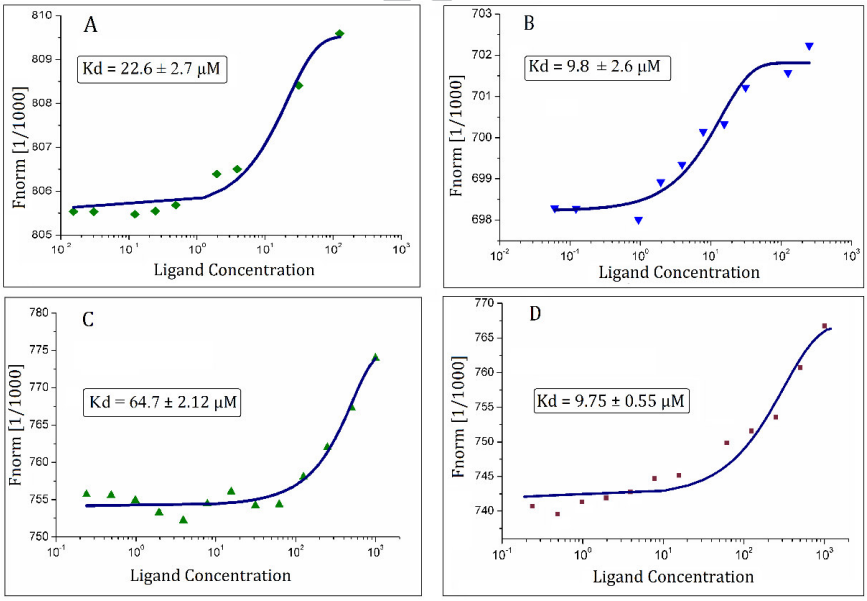


Figure 2. Microscale thermophoresis (MST) results of compounds **5y** (A), **5aa** (B), ribavirin (C), and ninnanmycin (D).

Molecular docking studies for **5y** and **5aa** with TMV-CP (PDB code: 1EI7) [11] indicated that the two compounds were embedded well in the pocket (including residues of TYR139, PHE12, ALA74, SER147, VAL75, THR136, ARG134, etc)

between the two subunits of TMV-CP (Figure. 3). Reports have demonstrated these residues played a vital roles in the self-assembly of TMV particles.[11-14] Such as TYR139 and PHE12 are the components of hydrophobic cluster of aromatic side-chains, and play an important role in a continuous belt of hydrophobic interactions encircling each ring of the disk assembly.[13] As well as Arg134, SER147, ALA74, VAL75 showed strong interactions with another subunit of TMV-CP.[11] Figure. 3 indicated the actions of the conventional hydrogen bond, π-Sulfur, π-Alkyl, and Alkyl existed between both molecules and the above residues in TMV-CP (more details can be found in Supporting information). For example, ALA-74 had strong hydrogen bond with **5y** (2.42 Å) and **5aa** (2.41 Å), and pyrimidine showed an π-Alkyl interaction with residue VAL-75, as well as an π-Alkyl interaction between the methyl group and residue PHE-12. These interactions between small molecules and TMV-CP are likely to weaken the interaction of two sub-unit of TMV-CP, thereby preventing the self-assembly of TMV particle.

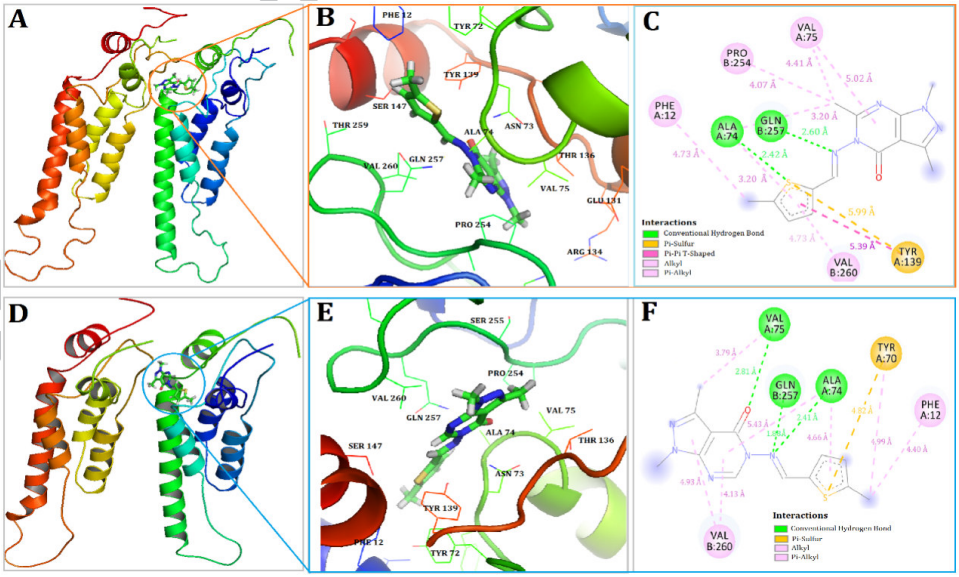


Figure 3. Molecular docking studies of compounds **5y** (**A**, **B**, and **C**) and **5aa** (D, E, F). **A**, **B**, **D,** and **E** were depicted using PyMol soft, while 2D diagram **C** and **F** were drawn by Discovery Studio 4.5.

In summary, a series of pyrazolo[3,4-d]pyrimidine derivatives containing a

Schiff base moiety was synthesized. The results of the bioactive tests for anti-TMV activity revealed that several of the target compounds exhibited considerable antiviral activity *in vivo*. In particular, compounds **5y** and **5aa** showed prominent inactivation activity against TMV. Structure–activity relationship analysis showed that introduced 5-methylthiophen-2-yl enhanced the anti-TMV activity. Furthermore, the MST results revealed that compound **5aa** had strong binding capability with TMV-CP, and the results of the molecular docking studies were consistent with the experimental results (inactivation activity). These findings indicate that pyrazolo[3,4-d]pyrimidine with a Schiff base may be a potential lead structure for developing novel anti-TMV agents.

Acknowledgements

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at…..

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Table 1. Inhibitory effects of the test compounds against TMV at 500 μg/mL

Compd.	Curative activity (%)	Protection effect (%)	Inactivation effect (%)
4a	58.4±2.9	60.6±0.9	68.7±2.3
4b	33.5±3.1	52.8±0.8	64.3±1.3
4c	64.9±1.7	63.2±2.7	74.2±2.4
4d	62.8±3.2	62.6±2.4	62.2±2.5
5a	48.0±1.9	33.7 ±1.7	83.4 ±2.4
5b	37.1±3.2	49.9±2.1	60.5±1.4
5c	30.2±2.3	44.1±1.1	54.5±2.5
5d	43.3±3.9	41.5±2.1	74.2±1.1
5e	50.4±0.5	45.7±1.2	65.5±1.3
5f	37.1±1.3	45.8±1.8	58.9±2.7
5g	56.8 ±2.1	23.1±2.5	85.2±3.1
5h	55.9±1.4	54.2±1.5	84.0±3.3
5i	56.5±2.3	40.3±1.1	80.1±1.6
5j	47.8±4.2	52.2±1.9	82.5±2.3
5k	42.2±2.4	50.8±2.3	64.8±2.5
5l	32.3±2.1	42.6±1.3	42.1±2.3
5m	29.2±3.9	39.4±2.7	63.5±3.1
5n	32.7±1.9	60.1±1.5	72.9±1.3
5o	52.1±2.5	65.1±1.6	88.9±0.9
5p	55.8±1.7	64.8±4.9	76.4±2.4
5q	54.2±0.9	63.5±1.7	64.1±2.3
5r	55.6±1.9	62.5±1.4	78.0±2.1
5s	45.7±2.2	56.9±1.6	62.4±3.1
5t	57.2±0.9	62.8±1.4	89.6±1.8
5u	51.9±2.2	63.4±3.1	84.5 ± 2.3
5v	42.6±2.8	51.9±1.5	67.4±2.5
5w	32.8±1.3	46.6±2.1	82.2±1.7
5x	14.2±1.3	22.3±1.4	34.8±2.3
5y	62.7±2.4	64.0±1.6	92.1±0.7
5z	59.4±0.9	62.0±1.9	72.8±2.1
5aa	62.7±0.3	62.0±1.9	94.6±2.1
5ab	58.5±1.3	65.2±1.9	85.0±2.1
5ac	22.7±1.2	22.8±1.3	32.9±3.1
5ad	61.4±1.7	57.6±1.2	76.2±2.4
5ae	59.6±0.9	62.0±1.2	67.1±2.3
5af	57.1±1.3	59.1±2.3	65.0±1.5
5ag	58.8±1.6	34.6±1.5	75.5±1.9
5ah	47.5±0.9	43.5±1.4	51.9±2.7
III-31[a]	54.3 ± 2.9	63.2 ± 1.8	78.3± 1.5
ningnanmycin	62.6±2.3	64.2±2.1	93.5±1.7
ribavirin	37.9±1.9	50.4±2.3	73.2±2.2

a. Compound in reference [6d]

Table 2. The EC_{50} (μg/mL) of the title compounds against TMV

Compd.	Curative activity	Protection activity	Inactivation activity
4a	505.2± 4.25	383.1± 2.44	/
4c	354.2± 2.42	559.8± 4.45	257.8± 2.21
4d	315.6± 2.33	355.5± 3.12	/
5g	481.4± 4.25	/	165.9± 2.62
5h	480.7± 5.34	282.4± 2.11	195.6± 2.33
5o	659.24± 4.24	380.94± 3.32	181.3± 2.32
5p	485.29± 3.67	340.5± 3.12	157.25± 2.45
5q	401.1± 2.56	365.7± 3.31	364.1± 2.65
5r	337.2± 2.33	280.5± 2.13	/
5t	400.6± 4.31	301.6± 3.16	109.5± 3.11
5u	402.4± 3.14	353.5± 3.23	144.3± 2.46
5y	272.8± 2.31	306.1± 3.41	70.3± 2.43
5z	392.9± 4.12	367.6± 2.12	301.1± 3.18
5aa	283.5± 2.23	296.8± 2.15	53.8± 1.43
5ab	400.6± 5.12	313.2± 2.12	155.5± 2.15
5ad	317.2± 1.34	412.7± 2.15	167.2± 1.34
5ae	400.4± 4.13	274.4± 3.32	329.7± 2.15
5af	427.2± 4.43	432.1± 2.54	328.5 ± 3.01
5ag	446.4± 2.31	/	398.3 ± 2.15
ningnanmycin	324.51± 3.15	165.95± 2.15	55.35 ± 1.45
ribavirin	/	435.99± 4.23	124.05 ± 3.5

Figures/Scheme Captions

Figure. 1. Design of pyrazolo[3,4-d]pyrimidine derivatives containing a Schiff base moiety

Scheme 1. Reagents and conditions. (a) reflux, Ac_2O; (b) 40% methylhydrazine, EtOH, 80 ^oC; (c) triethoxyethane/triethoxymethane, reflux; (d) 80%$N_2H_4 \cdot H_2O$, EtOH, r.t; (e) 1,1-dimethoxy-N,N-dimethylmethanamine/aromatic aldehyde, Acetic acid, reflux.

Figure. 2. Microscale thermophoresis (MST) results of compounds **5y** (A), **5aa** (B), ribavirin (C), and ninnanmycin (D).

Figure. 3. Molecular docking studies of compounds **5y** (**A**, **B**, and **C**) and **5aa** (**D**, **E**, **F**). **A**, **B**, **D,** and **E** were depicted using PyMolsoft, while 2D diagram **C** and **F** were drawn by Discovery Studio 4.5.

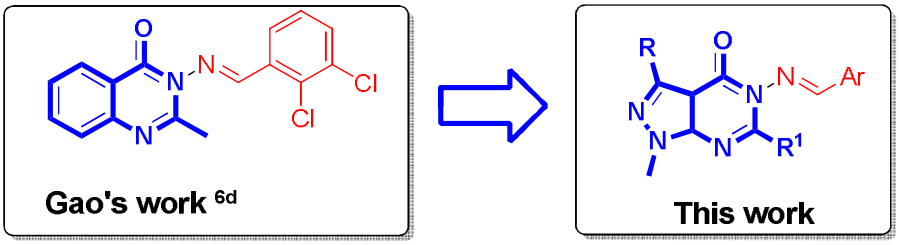
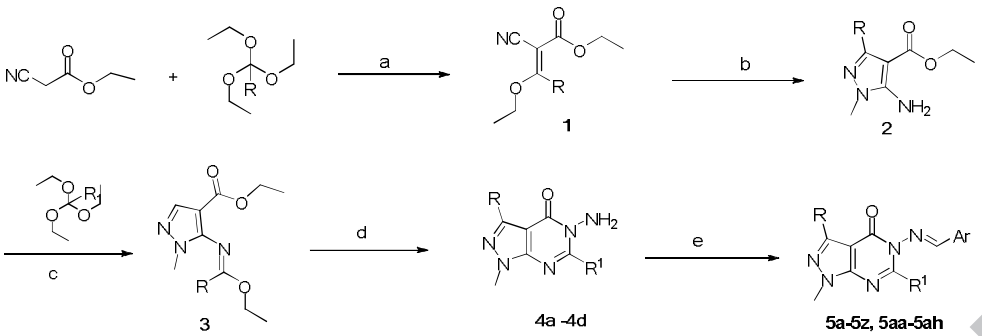


Figure 1 Design of pyrazolo[3,4-d]pyrimidine derivatives containing a Schiff base moiety



4a: R=H, R^1=H; **4b**: R=H, R^1=Me; **4c**: R=Me, R^1=H; **4d**: R=Me, R^1=Me;

5a: R=H, R^1=H, Ar = ; **5b**: R=H, R^1=H, Ar = ; **5c**: R=H, R^1=H, Ar = ; **5d**: R=H, R^1=H, Ar = ;

5e: R=H, R^1=H, Ar = ; **5f:** R=H, R^1=H, Ar = ; **5g**: R=H, R^1=Me, Ar = ; **5h**: R=H, R^1=Me, Ar = ;

5i: R=H, R^1= Me, Ar = ; **5j**: R=H, R^1=H, Ar = ; **5k**: R=H, R^1=H, Ar = ; **5l:** R=H, R^1=H, Ar = ;

5m: R=Me, R^1=H, Ar = ; **5n**: R=Me, R^1=Me, Ar = ; **5o**: R=Me, R^1=H, Ar = ; **5p**: R=Me, R^1=Me, Ar = ;

5q: R=Me, R^1=H, Ar = ; **5r**: R=Me, R^1=Me, Ar = ; **5s**: R=Me, R^1=Me, Ar = ; **5t**: R=Me, R^1=H, Ar = ;

5u: R=Me, R^1=H, Ar = ; **5v**: R=Me, R^1=Me, Ar = ; **5w**: R=Me, R^1=H, Ar = ; **5x**: R=Me, R^1=Me, Ar = ;

5y: R=Me, R^1=Me, Ar = ; **5z**: R= H, R^1=H, Ar = ; **5aa**: R=Me, R^1=H, Ar = ; **5ab**: R=Me, R^1=Me, Ar = ;

5ac: R=Me, R^1=Me, Ar = ; **5ad**: R=Me, R^1=H, Ar = ; **5ae**: R=Me, R^1=H, Ar = ; **5af**: R=Me, R^1=Me, Ar = ;

5ag: R=Me, R^1=H, Ar = ; **5ah**: R=Me, R^1=Me, Ar = .

Scheme 1 Reagents and conditions. (a) reflux, Ac_2O; (b) 40% methylhydrazine, EtOH, 80 °C; (c) triethoxyethane/triethoxymethane, reflux; (d) 80%$N_2H_4 \cdot H_2O$, EtOH, r.t; (e) 1,1-dimethoxy-N,N-dimethylmethanamine/aromatic aldehyde, Acetic acid, reflux.

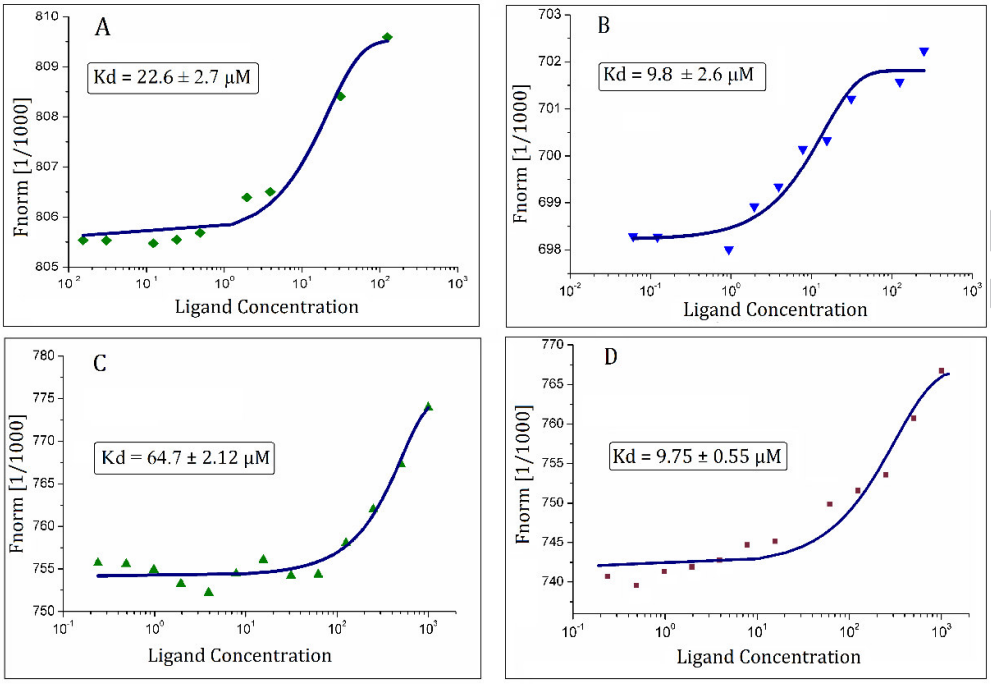


Figure 2. Microscale thermophoresis (MST) results of compounds **5y** (A), **5aa** (B), ribavirin (C), and ninnanmycin (D).

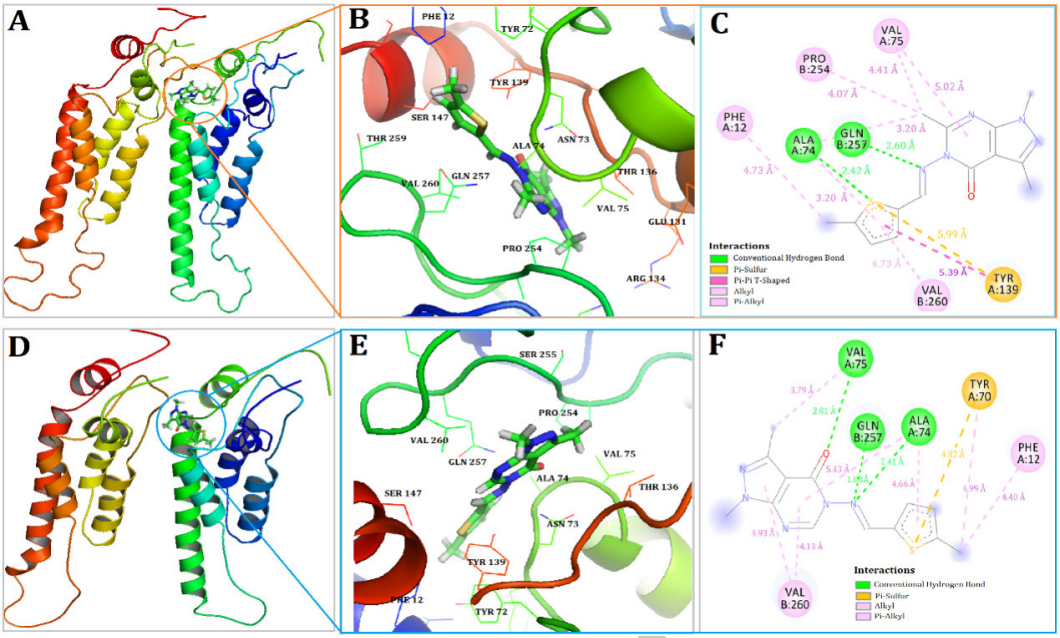
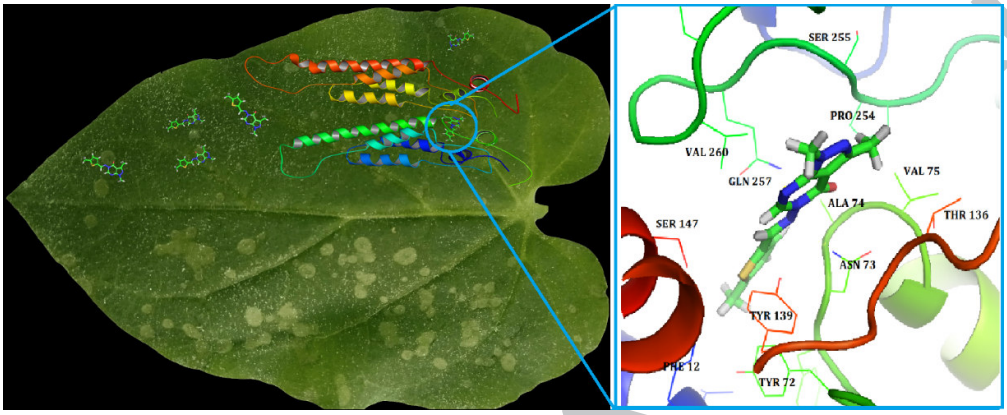


Figure 3. Molecular docking studies of compounds **5y** (**A**, **B**, and **C**) and **5aa** (D, E, F). **A**, **B**, **D,** and **E** were depicted using PyMol soft, while 2D diagram **C** and **F** were drawn by Discovery Studio 4.5.

A series of pyrazolo[3,4-d]pyrimidine derivatives containing Schiff base was synthesized. Compounds **5y** and **5aa** exhibited excellent antiviral activity against TMV. The interaction mode of **5y** and **5aa** with TMV-CP was conducted through molecular docking and microscale thermophoresis (MST) techniques.



Highlights

1. Pyrazolo[3,4-d]pyrimidines with a Schiff base moiety were synthesized.
2. Compounds **5y** and **5aa** exhibited excellent antiviral activity against TMV.
3. Interactions of **5y (5aa)** with TMV-CP were conducted using MST and docking.
4. Compound **5aa** showed strong combining capability with TMV-CP.