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ORIGINAL ARTICLE

Synthesis and evaluation of imidazo[1,2-a]quinoxaline derivatives as potential antifungal agents against phytopathogenic fungi

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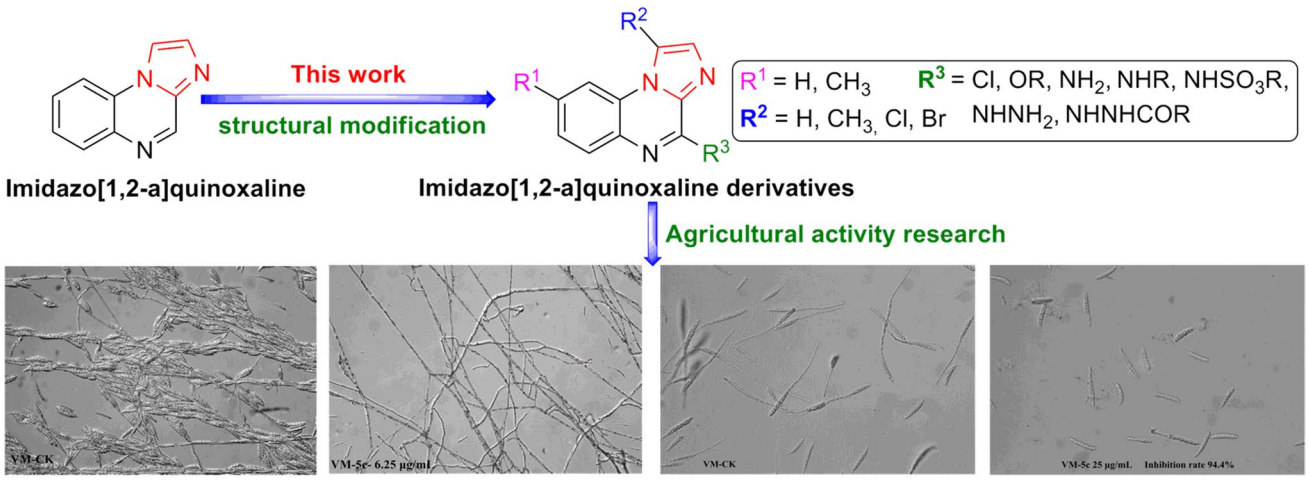
Abstract

To discover novel and effective potential agricultural antifungal agents, various kinds of imidazo[1,2-a]quinoxaline derivatives were designed, and synthesized from available and inexpensive reagents. Their antifungal activities were first evaluated against ten typical phytopathogenic fungi. The in vitro antifungal activity showed that some compounds exhibited more obvious broad-spectrum fungicidal activity than the two commercially-available fungicides chlorothalonil and hymexazol. *Valsa mali and Botrytis cinerea* strains exhibited the highest susceptibility with EC_{50} values of 1.4–27.0 μg/mL to more than ten compounds. Compounds **5c** and **5f** showed the most promising inhibitory effects against *Valsa mali* (EC_{50} = 5.6 μg/mL) *and Fusarium solani* (EC_{50} = 5.1 μg/mL), respectively. Preliminary studies on the mechanism of action indicated that the imidazo[1,2-a]quinoxaline skeleton likely exerted its antifungal effects by disrupting hyphal differentiation, spore germination, and germ tube growth. Moreover, the cell experiment results indicated that these target compounds possessed good safety to BV2 cells. Overall, compounds **5c** and **5f** can be considered candidate compounds against specific fungi for further detailed research. This study can provide a theoretical basis for the application of imidazo[1,2-a]quinoxaline scaffolds as novel fungicides in agriculture.

Graphical abstract



Effect of compound **5c** on the mycelial growth and spore germination of *V. mali*

Keywords imidazo[1,2-a]quinoxaline · Design · Synthesis · Antifungal activity

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Introduction

Plant diseases caused by phytopathogenic fungi are the primary factor causing global agricultural losses, which can seriously affect the normal growth, transportation, and storage of grains, vegetables, and fruits [1]. Taking the filamentous fungi *Botrytis cinerea and Fusarium graminearum* as examples, *Botrytis cinerea* is the major fungal species that triggers gray mold, which can infect over 200 plant species and cause considerable yield loss during agricultural production [2]. *Fusarium graminearum* is commonly found on wheat, barley, and corn, infected kernels appear shrunken and have white-to-pink colored mold, which leads to a reduction in grain yield and nutritional value by producing various mycotoxins [3]. Currently, the application of chemical fungicides has given a satisfactory effect on the prevention and control of these devastating diseases. Nevertheless, the long-term overuse and misuse of a single chemical fungicide are responsible for the disadvantages of resistance, residue, and resurgence ("3R" problems) [4]. Therefore, it is urgent to search for small molecules with high efficiency and low toxicity to expand the selection range of fungicides.

Nitrogen-containing heterocyclic compounds are currently considered a promising framework for the development of new pesticides [5, 6], especially compounds containing quinoxaline rings that exhibit various biological properties including antitumor, antimalarial, antiviral, antibacterial, and anti-inflammatory [7, 8]. Meanwhile, imidazoquinoxaline has attracted much attention owing to its striking antitumor [9–12], antiepileptic (LU 73068) [13], and antiallergic (Dazoquinast) activity [14], while there are rare reports on the utilization of the imidazoquinoxaline backbone as a pesticide. Therefore, to explore the application value of the imidazolequinoxaline skeleton in the prevention and control of plant diseases, this paper adopts homologous derivative and molecular hybridization strategy to design and synthesize various kinds of imidazo[1,2-a]quinoxaline derivatives via available and inexpensive reagents, and their antifungal properties against ten phytopathogenic fungi of agricultural relevance were evaluated firstly (Fig. 1). Finally, the antifungal mechanism of the compounds with excellent inhibitory activity was preliminarily explored by observing the mycelial morphology changes and spore germination via electron microscopy.

Results and discussion

Chemistry

Initially, the construction method of the imidazo[1,2-a]quinoxaline skeleton is shown in Scheme 1. 2,3-dichloroquinoxaline (**1a**) was reacted with 2,2-dimethoxyethanamine,

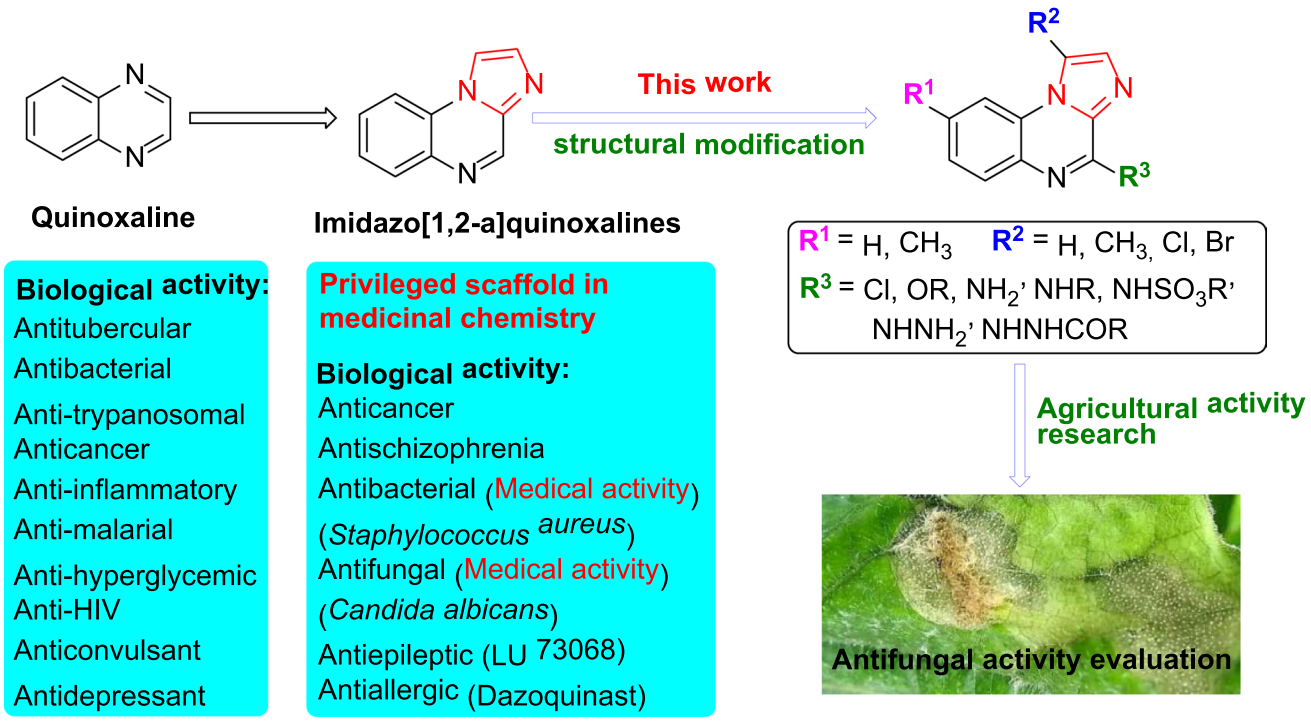
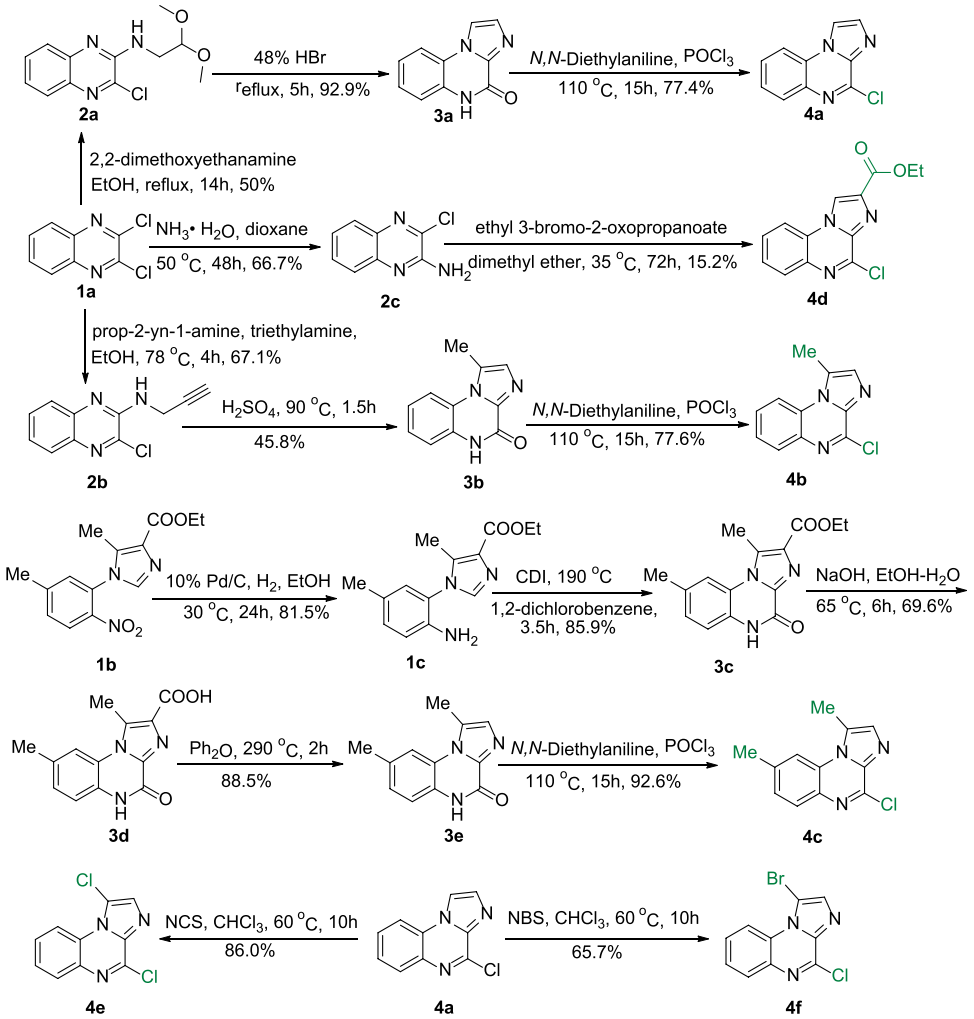


Fig. 1 The design strategy of target compounds in this work



Scheme 1 Synthetic route for the preparation of compounds **4a–f**

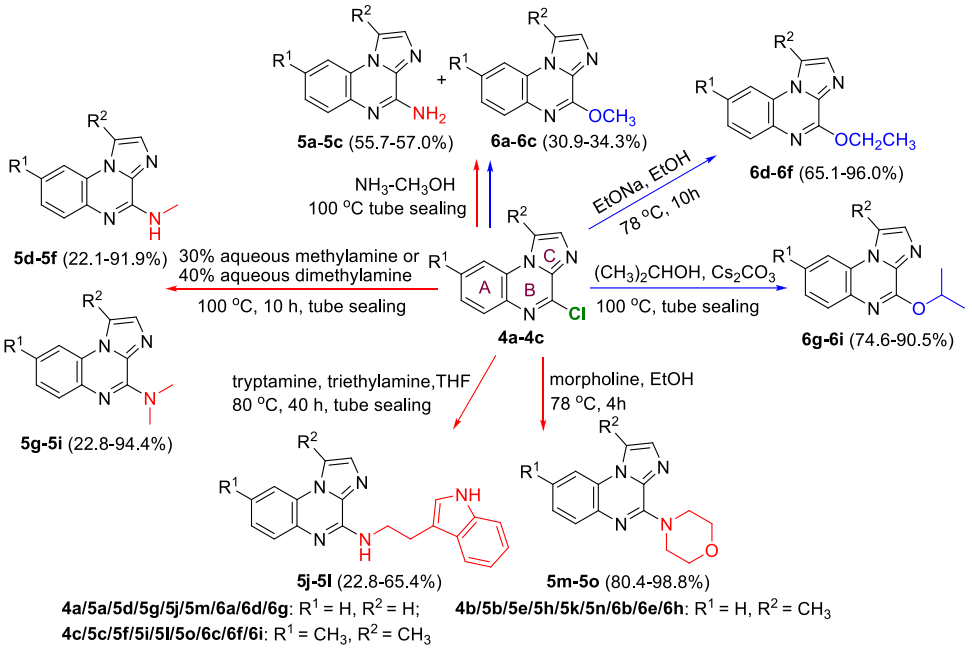
prop-2-yn-1-amine or ammonium hydroxide in different solvents to obtain the respective 2-amino-3-chloroquinoxaline (**2c**) and its derivatives (**2a** and **2b**), followed by treatment of compounds **2a** and **2b** with strong acids (HBr and H_2SO_4) to afford cyclization products **3a** and **3b**, which were converted to key intermediate compounds **4a, b** via chlorination with $POCl_3$ [15]. Meanwhile, compound **4d** can be obtained directly by condensation of compound **2c** with ethyl 3-bromo-2-oxopropanoate, but the resulting yield was relatively low (15.2%) [14]; the preparation procedure of compound **4c** includes reduction, cyclization, hydrolysis, decarboxylation and chlorination five synthetic steps [16]. Chlorinated (**4e**) and brominated (**4f**) derivatives were synthesized by reacting intermediate **4a** with *N*-chlorosuccinimide (NCS) and *N*-bromosuccinimide (NBS), respectively [17].

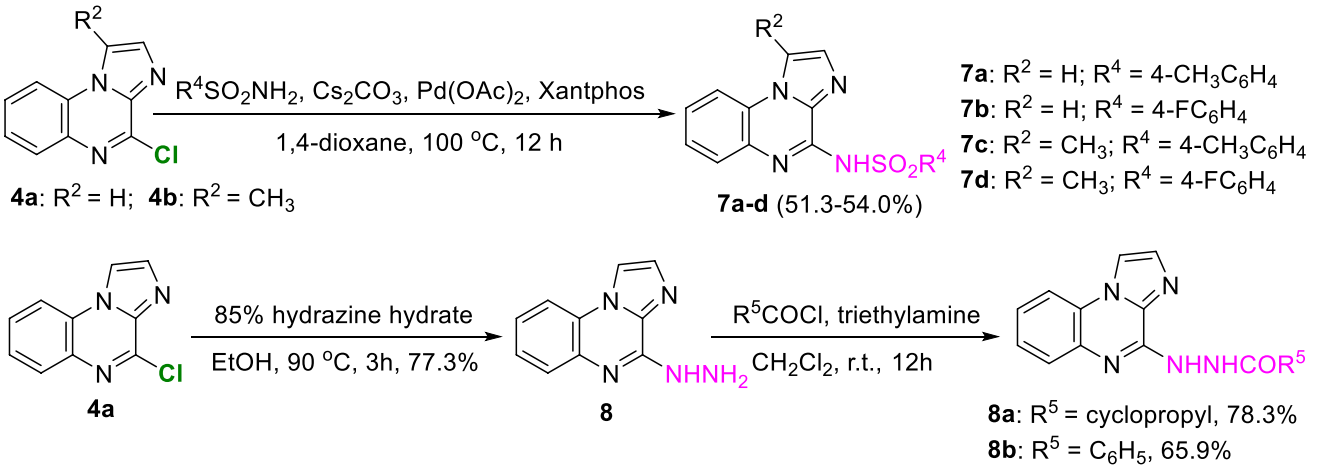
To investigate the effect of substituents on the antifungal activity, the B ring of imidazo[1,2-a]quinoxalines (**4a–c**) was modified by the introduction of the corresponding amine, ether, sulfonamide, and hydrazine functionalities as depicted in Schemes 2, 3. Compounds **5a–c** and **6a–c** (by-products) were obtained by ammonolysis of intermediate **4a–c** with NH_3–CH_3OH at 100 °C, and methylamino (**5d–f**), dimethylamino (**5g–i**), and tryptamino (**5j–l**) substituted compounds were also formed under similar conditions (100/80 °C, tube sealing). Compounds **5m–o** were prepared by replacing the chlorine atom of compounds **4a–c** with morpholine. In addition, etherification of compounds **4a–c** with various alcohols or phenols under basic conditions afforded ether derivatives **6d–i**. Finally, as depicted in Scheme 3, direct coupling of compounds **4a, b** with various sulfonamides in $Pd(OAc)_2$/xantphos/Cs_2CO_3/1,4-dioxane system at 100 °C gave compounds **7a–d**. Compound **4a** was hydrolyzed with 85% hydrazine hydrate and subsequently acylated with acyl chloride to afford **8a, b**. Spectroscopic data (HRMS, ^{1}H NMR, and ^{13}C NMR) of the target compounds were consistent with their structures.

Antifungal activity

Ten phytopathogenic fungi, including *Fusarium solani* (FS), *Fusarium oxysporum* (FO), *Botryosphaeria dothidea* (BD),

Scheme 2 Synthetic route for the preparation of target compounds **5a–o** and **6a–i**





Scheme 3 Synthetic route for the preparation of compounds **7a–d** and **8a–b**

Fusarium graminearum (FG), *Sclerotinia sclerotiorum* (SS), *Valsa mali* (VM), *Alternaria alternata* (AA), *Pyricularia oryzae* (PO), *Alternaria brassicae* (AB), and *Botrytis cinerea* (BC), that frequently occur in the Chinese agroecosystem were selected as the test strains. The in vitro antifungal activities of the target compounds were investigated through the classical mycelium linear growth rate method [18]. Hymexazol (Hym) and chlorothalonil (Chl), two commercial fungicides served as positive controls.

The results of the preliminary antifungal activity of all the target compounds are summarized in Table 1. The obtained data revealed that the synthesized compounds displayed good to excellent antifungal activity at 50 μg/mL. Among them, eight compounds (including compounds **4b**, **4c**, **4e**, **5c**, **5f**, **6e**, **6 h**, and **7d**) showed satisfactory antifungal effects against *F. solani* with inhibition rates of > 80%, which was superior to the positive controls hymexazol (63.9%) and chlorothalonil (76.6%); eleven compounds exhibited better antifungal activity (> 50%) against *F. oxysporum* than hymexazol (46.2%), especially compounds **4e** (73.9%), **5c** (84.1%), and **7d** (72.1%), which gave higher activity than chlorothalonil (69.2%). Toward *B. dothidea*, compounds **4e** (81.9%), **5a** (98.4%), **6 g** (91.9%), and **7d** (89.6%) exerted more promising antifungal effects than the two commercial fungicides. For *F. graminearum*, seven compounds (**4b**, **4c**, **4e**, **5c**, **5f**, **5i,** and **7c**) displayed slightly higher antifungal activity (75.8–86.7%) than chlorothalonil (70.7%) and hymexazol (63.8%). Regretfully, only five compounds **4e**,

Table 1 Fungicidal activity of the target compounds against ten phytopathogenic fungi (50 μg/mL)

Compd.	Inhibition rate ± SD (%)									
	FS	*FO*	*BD*	*FG*	*SS*	*VM*	*AA*	*PO*	*AB*	*BC*
4a	31.7 ± 1.1	27.5 ± 1.2	**57.7 ± 0.9**	44.7 ± 1.1	**61.0 ± 2.0**	**54.5 ± 0.6**	28.1 ± 0.8	9.3 ± 1.1	30.5 ± 2.1	*98.5 ± 0.7*
4b	*86.4 ± 1.3*	**58.3 ± 1.3**	**66.2 ± 0.8**	*86.7 ± 0.6*	**62.5 ± 1.0**	*89.5 ± 0.6*	**61.2 ± 1.0**	**48.7 ± 2.1**	**68.5 ± 1.5**	**80.5 ± 0.6**
4c	*87.1 ± 1.3*	**55.8 ± 1.3**	29.0 ± 0.8	*78.9 ± 0.6*	**40.7 ± 1.3**	*89.5 ± 0.6*	**60.1 ± 1.5**	46.2 ± 1.2	**57.1 ± 0.9**	**66.7 ± 0.6**
4d	–	13.9 ± 2.3	40.3 ± 1.3	22.7 ± 1.1	36.3 ± 1.1	26.3 ± 1.1	28.6 ± 1.0	30.8 ± 1.2	42.6 ± 0.9	33.3 ± 2.5
4e	*88.9 ± 1.0*	*73.9 ± 2.0*	*81.9 ± 1.0*	*83.3 ± 0.1*	**67.4 ± 1.5**	*87.3 ± 0.7*	*68.0 ± 0.1*	**71.8 ± 1.2**	**69.4 ± 1.0**	**90.2 ± 0.7**
4f	31.1 ± 1.0	21.7 ± 1.0	–	27.5 ± 1.8	–	28.2 ± 0.7	24.0 ± 0.9	15.4 ± 0.2	28.3 ± 0.1	64.8 ± 0.7
5a	30.6 ± 1.3	25.2 ± 1.3	*98.4 ± 0.8*	**69.3 ± 1.1**	32.5 ± 0.6	80.3 ± 1.1	42.9 ± 2.1	28.2 ± 2.1	48.1 ± 2.3	**50.6 ± 0.6**
5b	2.5 ± 0.6	–	34.4 ± 2.4	–	23.9 ± 2.0	22.7 ± 0.7	23.4 ± 1.1	20.9 ± 2.2	44.0 ± 0.9	28.4 ± 2.8
5c	*85.2 ± 1.0*	*84.1 ± 0.7*	**57.1 ± 0.6**	*80.3 ± 1.5*	**58.5 ± 1.2**	*96.1 ± 0.8*	*67.9 ± 0.7*	**53.7 ± 1.5**	43.3 ± 0.7	31.8 ± 0.2
5d	38.9 ± 0.6	19.4 ± 1.3	37.1 ± 0.8	**64.0 ± 0.6**	40.0 ± 0.6	*76.3 ± 0.6*	**51.2 ± 1.7**	30.8 ± 1.2	38.9 ± 0.9	**65.4 ± 1.5**
5e	**58.3 ± 1.3**	41.7 ± 1.3	33.9 ± 1.3	16.0 ± 0.6	**52.5 ± 0.6**	*90.8 ± 0.6*	**51.1 ± 1.0**	46.2 ± 0.9	**63.0 ± 0.9**	**56.8 ± 1.0**
5f	*85.7 ± 1.3*	**52.4 ± 1.9**	10.6 ± 2.0	*80.9 ± 1.3*	28.6 ± 1.3	*92.4 ± 0.7*	45.3 ± 1.8	23.7 ± 2.5	27.8 ± 2.3	**78.4 ± 0.6**
5g	5.6 ± 1.3	8.3 ± 1.3	17.7 ± 2.4	41.3 ± 0.1	28.8 ± 0.6	42.1 ± 1.1	38.8 ± 1.7	33.3 ± 0.2	**50.0 ± 0.9**	**51.9 ± 1.5**
5h	33.3 ± 3.5	36.1 ± 2.3	17.7 ± 0.8	58.7 ± 0.6	28.8 ± 1.0	**64.5 ± 0.6**	46.9 ± 0.8	43.6 ± 1.2	24.1 ± 0.9	**76.5 ± 1.5**
5i	**58.5 ± 1.0**	48.2 ± 0.6	21.2 ± 0.9	*75.8 ± 2.6*	**68.7 ± 1.2**	*71.2 ± 1.2*	*68.1 ± 1.4*	**55.1 ± 1.3**	43.3 ± 1.1	**91.1 ± 0.8**
5j	**50.0 ± 2.4**	25.6 ± 2.2	**70.3 ± 2.2**	37.8 ± 1.9	**62.0 ± 2.0**	**54.5 ± 1.4**	**63.8 ± 2.0**	**58.1 ± 0.7**	**60.1 ± 0.9**	**85.1 ± 0.1**
5k	22.2 ± 0.9	30.6 ± 2.3	43.5 ± 0.8	30.7 ± 1.1	38.8 ± 0.6	48.7 ± 0.6	49.0 ± 1.7	43.6 ± 1.2	**59.3 ± 2.3**	**66.7 ± 1.0**
5l	53.3 ± 1.2	10.9 ± 0.1	44.7 ± 2.0	33.3 ± 0.9	4.3 ± 1.0	38.0 ± 1.8	30.0 ± 0.9	23.1 ± 1.2	32.6 ± 1.0	50.7 ± 1.3
5m	27.8 ± 1.3	27.8 ± 2.3	*77.4 ± 0.5*	45.3 ± 1.1	46.3 ± 0.6	**53.9 ± 1.1**	**59.2 ± 1.7**	48.7 ± 1.2	**59.3 ± 0.9**	**77.8 ± 2.5**
5n	–	22.2 ± 2.9	16.1 ± 1.3	26.7 ± 0.6	35.0 ± 0.6	30.3 ± 1.1	40.8 ± 2.5	30.8 ± 1.2	48.1 ± 0.9	**56.8 ± 0.6**
5o	28.9 ± 0.2	26.1 ± 1.0	–	27.5 ± 1.8	–	31.0 ± 0.3	26.1 ± 0.9	20.5 ± 1.2	17.4 ± 1.1	42.3 ± 0.7
6a	**61.0 ± 1.1**	23.1 ± 1.2	30.8 ± 0.9	5.8 ± 0.9	31.1 ± 0.9	34.7 ± 0.7	30.8 ± 0.9	14.6 ± 1.1	27.5 ± 0.7	**96.4 ± 0.1**
6b	**61.1 ± 1.1**	38.5 ± 1.2	32.7 ± 0.9	–	17.6 ± 0.6	**63.9 ± 0.7**	48.1 ± 0.9	26.8 ± 1.1	**60.8 ± 0.9**	**58.9 ± 0.8**
6c	**54.4 ± 1.2**	42.8 ± 1.3	42.3 ± 0.9	46.5 ± 0.9	37.8 ± 1.0	**63.5 ± 0.9**	38.5 ± 0.4	29.1 ± 0.3	25.9 ± 0.3	**90.2 ± 0.1**
6d	**68.9 ± 1.1**	42.5 ± 2.0	**57.4 ± 0.8**	**60.8 ± 2.2**	43.9 ± 2.0	**57.1 ± 1.1**	38.0 ± 1.7	33.3 ± 1.9	34.8 ± 0.8	*98.6 ± 1.2*
6e	*84.4 ± 1.0*	**54.3 ± 1.0**	14.9 ± 2.0	**68.6 ± 1.8**	–	*71.8 ± 0.7*	48.0 ± 0.9	41.0 ± 1.2	41.3 ± 0.1	**56.3 ± 0.7**
6f	**57.1 ± 2.7**	**52.4 ± 1.1**	42.6 ± 2.0	**60.3 ± 0.7**	**57.1 ± 0.7**	*75.8 ± 1.2*	45.3 ± 1.8	31.6 ± 1.2	**51.9 ± 0.9**	**90.5 ± 0.6**
6g	**72.7 ± 2.1**	**56.4 ± 3.2**	*91.9 ± 0.6*	**55.7 ± 0.6**	**59.5 ± 0.6**	*68.6 ± 1.7*	**53.8 ± 0.9**	41.5 ± 1.1	**60.8 ± 0.9**	**84.4 ± 0.8**
6h	*83.0 ± 1.3*	**60.5 ± 1.1**	**58.3 ± 1.7**	**59.7 ± 2.9**	48.6 ± 0.7	*77.8 ± 0.7*	**61.7 ± 1.5**	45.9 ± 2.2	**58.7 ± 1.8**	**80.3 ± 1.3**
6i	42.2 ± 3.1	32.6 ± 0.1	12.8 ± 1.0	33.3 ± 0.9	–	35.2 ± 0.2	24.0 ± 0.9	20.5 ± 1.2	10.9 ± 0.1	43.7 ± 1.3
7a	15.0 ± 1.2	9.3 ± 1.1	12.5 ± 0.7	31.1 ± 1.3	36.6 ± 2.8	27.3 ± 0.7	36.2 ± 2.0	32.6 ± 1.1	48.0 ± 2.8	58.2 ± 3.5
7b	8.3 ± 1.3	8.3 ± 1.3	24.2 ± 0.8	24.0 ± 0.6	17.5 ± 0.9	18.4 ± 1.1	34.7 ± 1.0	28.2 ± 1.2	31.5 ± 1.5	45.7 ± 1.5
7c	**73.9 ± 1.2**	**53.5 ± 1.1**	**65.6 ± 2.2**	*78.4 ± 1.3*	**70.1 ± 0.7**	65.2 ± 2.9	*74.5 ± 0.4*	**69.8 ± 0.1**	**68.0 ± 0.2**	**85.5 ± 0.2**
7d	*80.9 ± 1.0*	*72.1 ± 1.1*	*89.6 ± 0.3*	**56.9 ± 1.7**	**72.9 ± 1.8**	*70.8 ± 0.7*	*74.5 ± 1.0*	**70.3 ± 1.3**	**71.7 ± 1.5**	**88.7 ± 1.1**
8a	–	2.8 ± 1.3	11.3 ± 1.3	9.3 ± 0.6	21.3 ± 0.6	18.4 ± 0.6	51.0 ± 1.0	15.4 ± 1.2	44.4 ± 1.5	–
8b	14.9 ± 1.0	7.0 ± 1.1	–	20.8 ± 2.1	8.6 ± 0.7	16.7 ± 0.7	23.4 ± 1.0	13.5 ± 1.3	32.6 ± 0.6	38.0 ± 0.7
Chl	76.6 ± 2.0	69.2 ± 1.2	72.6 ± 0.8	70.7 ± 0.6	81.1 ± 1.1	66.7 ± 0.7	49.9 ± 0.9	49.4 ± 4.4	45.7 ± 1.0	98.2 ± 0.8
Hym	63.9 ± 2.3	46.2 ± 2.1	21.2 ± 0.9	63.8 ± 1.2	75.6 ± 1.2	29.2 ± 0.7	65.6 ± 1.0	82.1 ± 1.2	86.4 ± 1.7	72.7 ± 3.1

Bold: the inhibition rate was over 50%; Italic: the inhibition rate was over positive control

5c, **5i**, **7c**, and **7d** possessed comparable antifungal activities (67.9–74.5%) to hymexazol (65.6%) against *A. alternata*, and no noticeable inhibitory efficacies were observed for all compounds toward *S. sclerotiorum, P. oryzae*, *and A. brassicae* in comparison with the positive control hymexazol. However, it is worth mentioning that eighteen compounds demonstrated satisfactory inhibitory effects (> 60%) against *V. mali*, and thirteen compounds revealed better antifungal activity (> 80%) against *B. cinerea* than hymexazol (72.7%), especially the inhibition rates of compounds **4a**, **4e**, **5i**, **6a**, **6c**, **6d**, and **6f** reached over 90%, which could almost completely suppress the growth of mycelium. Furthermore, the preliminary structure–activity relationship of these compounds is summarized in Fig. 2.

Inspired by the preliminary antifungal activity results, the median effective concentration (EC_{50}) values of some

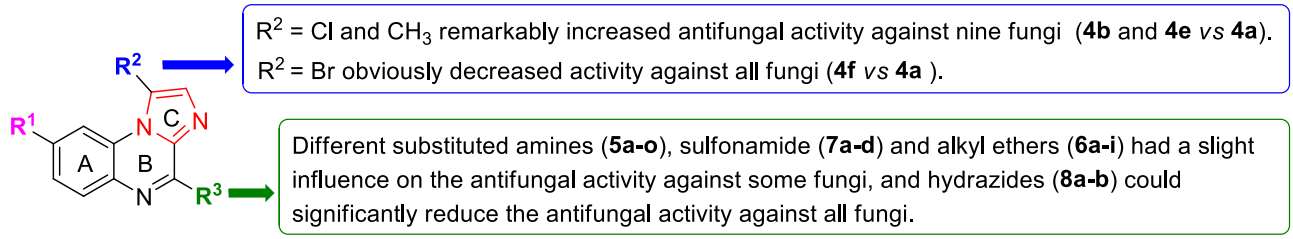


Fig. 2 The preliminary structure–activity relationships of the compounds against tested fungi

selected compounds were further determined at six different concentrations (50, 25, 12.5, 6.25, 3.125, and 1.5625 μg/mL). As displayed in Fig. 3, many compounds exhibited obvious inhibitory effects against the eight tested fungi. For instance, compound **5f** (5.1 μg/mL) exhibited the best antifungal activity against *F. solani*; compounds **4c**, **7c**, and **7d** had anti-*A. brassicae* EC_{50} values of 15.4, 18.9, and 19.7 μg/mL, respectively, superior to hymexazol (26.6 μg/mL) and chlorothalonil (> 50 μg/mL); the EC_{50} range values of compounds **4b**, **4e**, **5a**, **6 g**, **7c**, and **7d** against *B. dothidea* were 10.3–24.9 μg/mL, which was better than hymexazol (> 50 μg/mL) but lower than chlorothalonil (7.9 μg/mL); the inhibitory effects of **4e** (28.8 μg/mL) and **7c** (23.7 μg/mL) on *P. oryzae* were equivalent to that of hymexazol (24.9 μg/mL). For *A. alternata*, eight compounds possessed more pronounced antifungal activity than chlorothalonil, especially compound **7c** (11.2 μg/mL), which was better than hymexazol (16.7 μg/mL). Regarding the *F. graminearum* strain, seven compounds exhibited higher activity than hymexazol, but failed to exceed chlorothalonil. Furthermore, it is fortunate that twelve compounds demonstrated more promising potential in controlling *V. mali*, especially compounds **5c** and **5f** with EC_{50} values lower than 6.0 μg/mL; thirteen compounds (1.4–15.2 μg/mL) displayed obvious antifungal activity against *B. cinerea*, particularly compounds **4a**, **4e**, **6a**, **6c**, and **6d** exhibited 3.0–8.5 folds more potent activities than hymexazol. In addition, the concentration-dependent suppression of the mycelial growth of *F.solani*, *V.mali*, and *B. cinerea* by compounds **5f** or **6a** could also be clearly observed in Fig. 4.

Effects of compounds on mycelial growth and spore germination [19]

To elucidate the preliminary mechanism of the antifungal activity of these compounds, light microscopy was used to investigate the influence of compound **5c** (6.25 μg/mL) on the hyphal growth and spore germination of *V. mali* (VM) fungi. As shown in Fig. 5, in the blank control, the mycelium had a smooth surface, much-branched and abundant attachment of spindle-shaped spores. In contrast, the mycelium of the compound **5c** treatment group appeared obvious shrinkage and no spore formation. Furthermore, inhibiting spore germination is an important means to prevent fungal regeneration and infection in plants. Figure 6 shows that the spore germination and germ tube elongation were both significantly suppressed in the presence of compound **5c** at different concentrations, and the inhibition rates at concentrations of 25, 12.5, and 6.25 μg/mL were 94.4%, 70.3%, and 54.1%, respectively. This phenomenon demonstrated that compound **5c** likely exerted antifungal effects by disrupting hyphal differentiation, spore germination, and germ tube growth.

Cell cytotoxicity

Finally, the cell cytotoxicity of the six compounds (**4a**, **4e**, **5c**, **6a**, **6c,** and **6d**) with promising antifungal activity against mouse microglia (BV2) cells was further investigated in vitro using the CCK-8 assay [20, 21]. From Fig. 7, we can see that the cell viability of the tested compounds was more than 82.7% on BV2 cells at the high concentration of 100 μg/mL, and the current results suggested that the tested compounds showed low toxicities.

Conclusions

In summary, thirty-six imidazo[1,2-a]quinoxaline derivatives were synthesized and evaluated for their fungicidal activity against ten common phytopathogenic fungi. The results showed that some compounds exhibited more excellent and broad-spectrum fungicidal activity in vitro than the positive controls chlorothalonil and hymexazol, particularly *V. mali and B. cinerea* strains exhibited the highest susceptibility with an EC_{50} values of 1.4–27.0 μg/mL for more than ten compounds. Among them, compounds **5c** and **5f** displayed the most promising antifungal activity against *V. mali* and *F. solani*, with an EC_{50} values of 5.6 and 5.1 μg/mL, respectively, which can be considered as the potential candidate compounds for controlling specific fungi. SAR analysis showed that the type of substituents on the imidazo[1,2-a]quinoxaline skeleton significantly effects the antifungal activity. Preliminary studies on the mechanism of action indicated that these compounds likely exerted their antifungal effects by disrupting hyphal

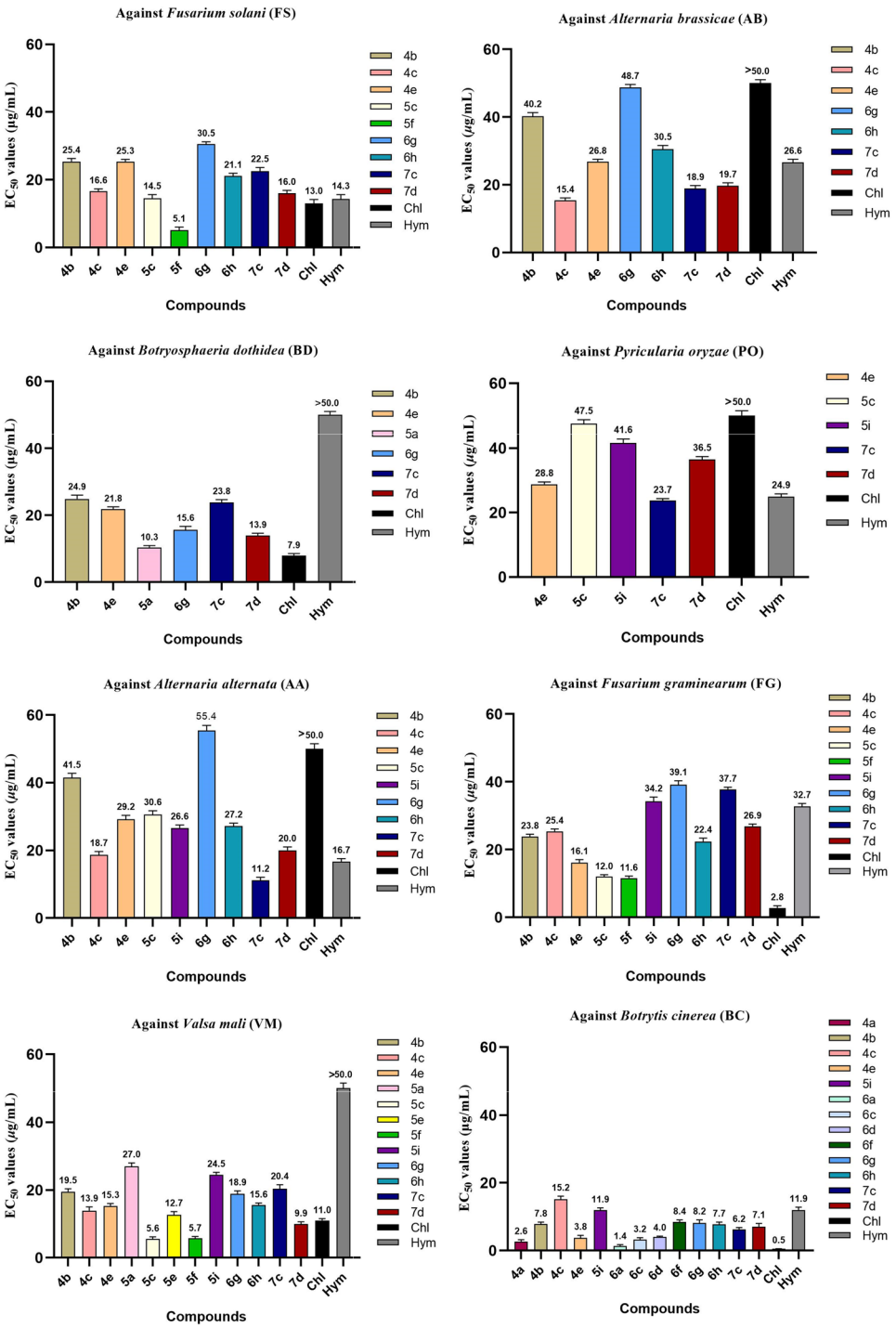


Fig. 3 EC_{50} values (μg/mL) of selected compounds against eight phytopathogenic fungi

differentiation, spore germination, and germ tube growth. Moreover, the cell experiment results indicated that the significantly bioactive compounds possessed good safety to BV2 cells. It is worth pointing out that this is the first report on the application of an imidazo[1,2-a]quinoxaline skeleton as agricultural antifungal agent, and further studies on the structural optimization and target exploration are still underway in our laboratory. Overall, our findings may provide a theoretical basis for the future utilization of imidazo[1,2-a]quinoxaline scaffolds as novel fungicides in agriculture.

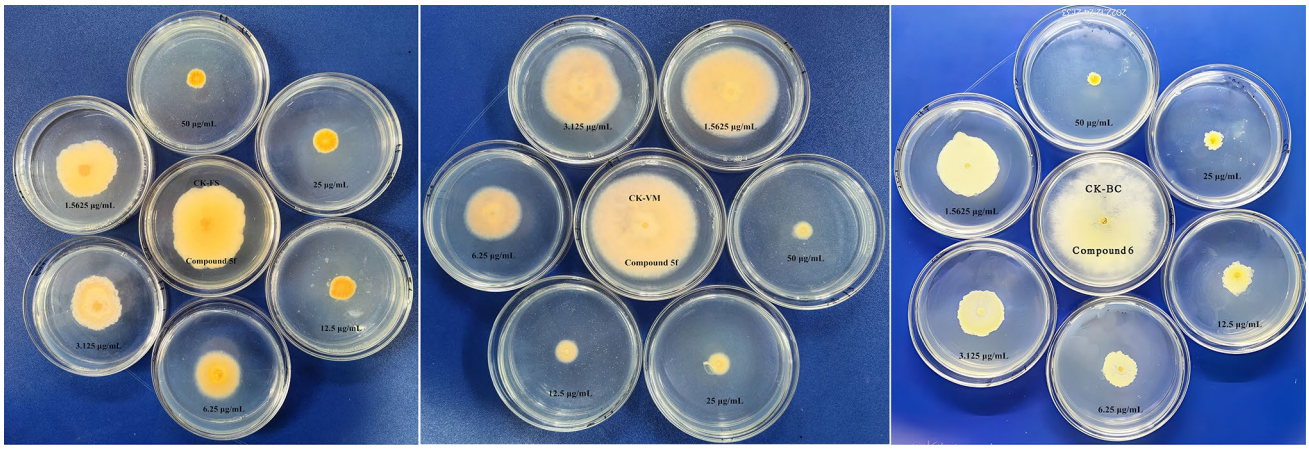


Fig. 4 Effects of compound **5f** or **6a** on the growth of *F.solani*, *V.mali*, and *B. cinerea* at different concentrations (CK: blank control group)

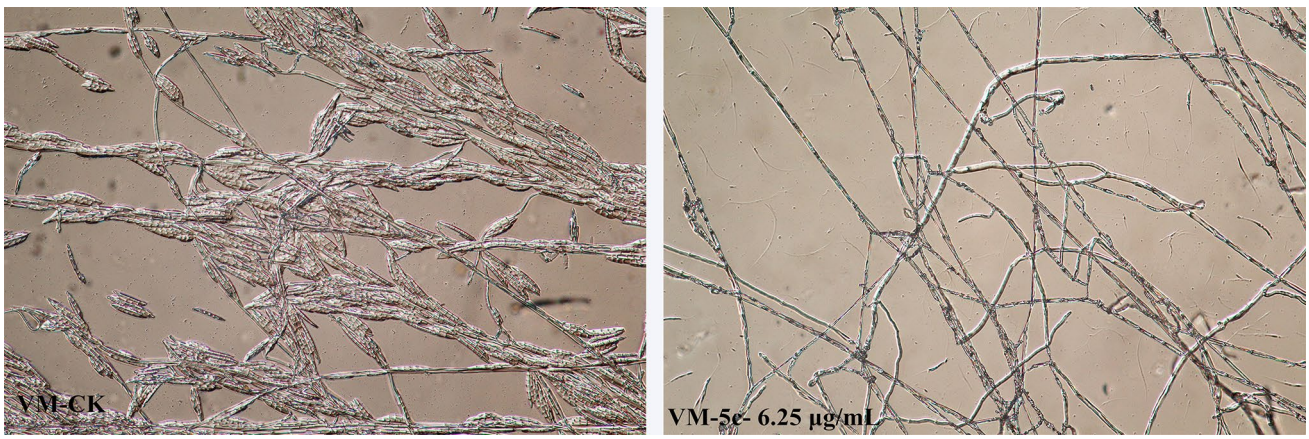


Fig. 5 Effects of compound **5c** on the mycelial morphology of *V. mali* at 6.25 μg/mL. (VM-CK represented the normal mycelial morphology of VM, and VM-**5c** represented the mycelial morphology of VM after treatment with compound **5c**)

Experimental

All starting materials were obtained from commercial sources and used without further purification. Melting points were determined by the X-4 digital display micro melting point apparatus (Beijing Tech Instrument Co., Ltd). ^{1}H NMR and ^{13}C NMR spectra were recorded on Bruker Avance NEO 600 MHz and 150 MHz instruments, respectively, using TMS as the internal standard and $CDCl_3$ or DMSO-d_6 as the solvent. High-resolution mass spectra (HRMS) were carried out with an APEX II Bruker 4.7 T AS instrument.

Synthesis

See the "Supporting information" section for the synthetic methods of the target compounds.

Antifungal activity and spore germination assay

Antifungal activity assay [18]

The target compounds were screened in vitro for their antifungal activity against ten phytopathogenic fungi (*Fusarium solani*, *Fusarium oxysporum*, *Botryosphaeria dothidea*, *Fusarium graminearum*, *Sclerotinia sclerotiorum*, *Valsa mali*, *Alternaria alternata*, *Pyricularia oryzae*, *Alternaria brassicae*, and *Botrytis cinerea*) by using the mycelial growth rate method. Potato dextrose agar (PDA) medium was prepared in the flasks and sterilized. The target compounds were dissolved in DMSO before mixing with PDA, and the concentration of test compounds in the medium was fixed at 50 μg/mL. The medium was then poured into sterilized Petri dishes. The mycelia disks (4 mm) were inoculated in the center of the Petri dishes (three replicates for each treatment) and incubated at 27 ± 1 °C for 4 days. DMSO

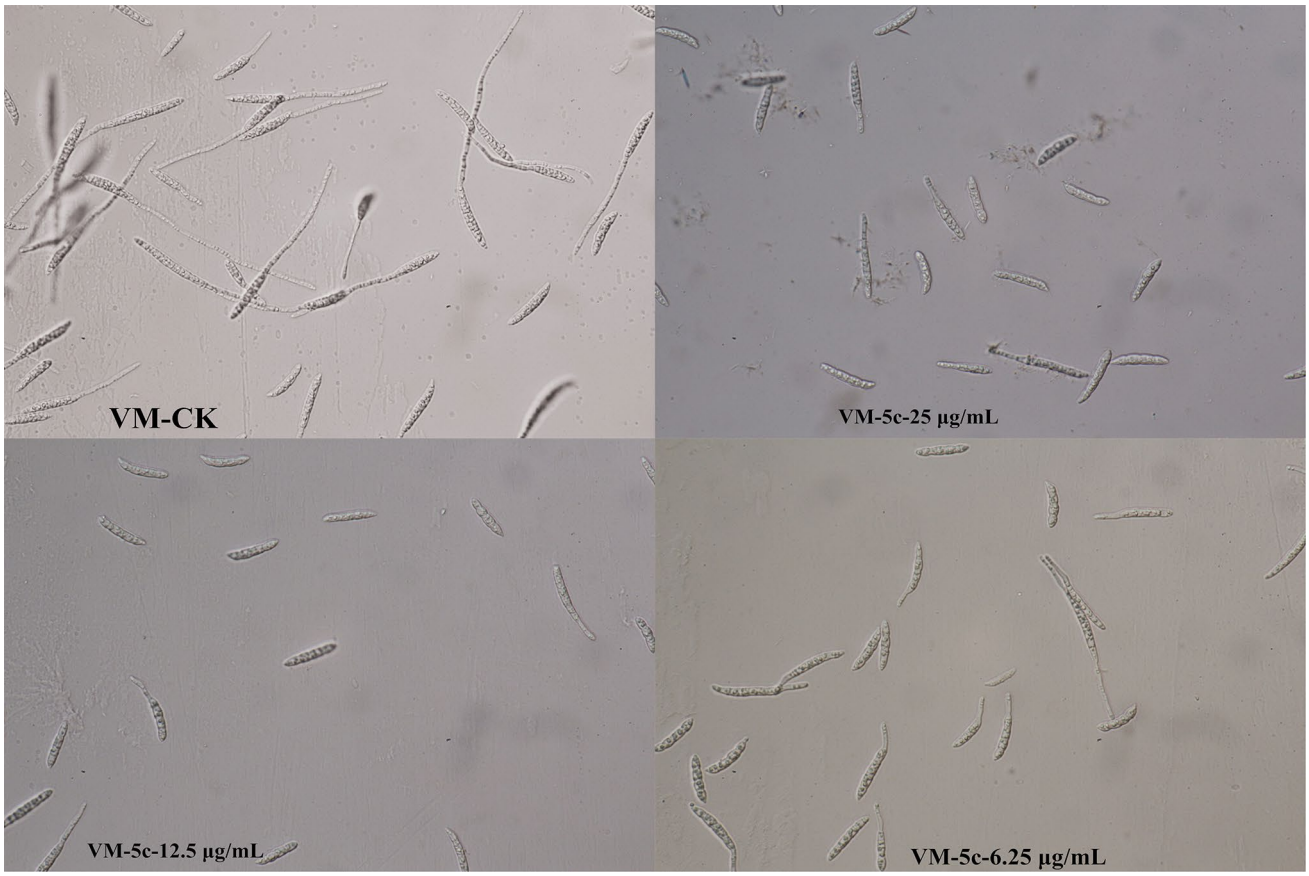
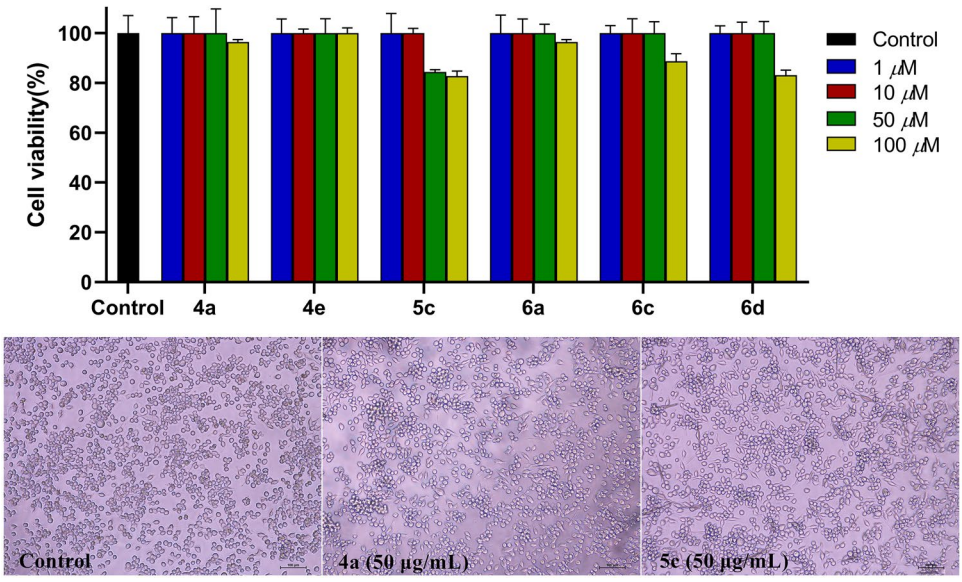


Fig. 6 Inhibitory effect of different concentrations of compound **5c** on the spore germination of *V. mali*. (400×; scale bar: 50 μm)

Fig. 7 In vitro cytotoxicity of some compounds to BV2 cells



without any compounds mixed with PDA served as a control (the final concentration of DMSO < 0.5%). Hymexazol and chlorothalonil were used as positive controls. The radial growth of the fungal colonies were measured and the data were statistically analyzed. The inhibitory rate was calculated by the following formula: inhibition rate (%) = (C–T) × 100/(C–4 mm), where C represents the diameter of fungi growth on untreated PDA, and T represents

the diameter of fungi on treated PDA. Finally, the linear regressions of inhibition rates (%) versus seven concentrations of some selected compounds, were obtained, and the EC_{50} values were calculated. Statistical analyses of the data were performed with GraphPad Prism 5.0.

Spore germination assay [19]

The *V. mali* was retrieved from the storage tube and cultured for 2 weeks at 27.5 °C on potato dextrose agar (PDA, Difco). Plates were then flooded with sterile distilled water, and conidia were scraped with a glass stick. Mycelial debris was removed by filtration through double-layer cheesecloth, and the spores were harvested and suspended in sterile distilled water containing 0.1% (v/v) Tween 20. Spores were counted using a hemocytometer and adjusted to 1.0×10^6 spores/mL.

Three concentrations (6.25 μg/mL, 12.5 μg/mL, and 25 μg/mL) of compound **5c** and the control (0.5% DMSO) were separately tested for spore germination of *V. mali*. The samples were inoculated with spore suspension of *V. mali* containing 1.0×10^6 spores/mL. Aliquots of 10 μL of prepared spore suspension were placed on 96-hole plate in six copies. 96-hole plate containing the spores was incubated in a moisture chamber at 25 °C for 48 h. Each hole was then observed under the microscope for spore germination. The spore-generated germ tubes were enumerated, and the percentage of spore germination was calculated.

Cytotoxicity activity [21]

The cytotoxicity of the target compounds was detected by Cell Counting CCK-8 kit (CCK-8 assay). CCK-8 was based on the water-soluble tetrazolium salt WST8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-dinitrophenyl)-2H-tetrazole monosodium salt). The BV2 cells were seeded at a density of 1.5×10^4 cells per well in the 96-hole plate and incubated at 37 °C in an atmosphere of 5% CO_2 for 24 h. After incubation, different concentrations of the target compounds were added and incubated for 24 h. 10 μL of CCK-8 reagent was added to each well and incubated for 1 h in the dark. The absorbance at 450 nm was measured by microplate reader. The untreated group was considered as the control. The data were analyzed by GraphPad Prism 5.0.

Supporting information

Spectral images of ^{1}H-NMR, ^{13}C-NMR and HRMS are provided in the Supporting Information Section.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11030-023-10739-y.

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Author contributions Lingling Fan, Yong Li, Bing Guo and Lei Tang designed the experiments; Taigui Ma and Xu Zhong synthesized the target compounds and analyzed the data; Judi Fan, Ya Yang and Wenjing Liu are in charge of bioactivity and cytotoxicity tests; Lingling Fan and Yong Li wrote the paper. All authors approved the final manuscript.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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