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Bioactivity-Guided Synthesis Accelerates the Discovery of 3-(Iso)quinolinyl-4-chromenones as Potent Fungicide Candidates

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ABSTRACT: Fungal infections could cause tremendous decreases in crop yield and quality. Natural products, including flavonoids and (iso)quinolines, have always been an important source for lead discovery in medicinal and agricultural chemistry. To promote the discovery and development of new fungicides, a series of 3-(iso)quinolinyl-4-chromenone derivatives was designed and synthesized by the active substructure splicing principle and evaluated for their antifungal activities. The lead optimization was guided by bioactivity. The bioassay data revealed that the 3-quinolinyl-4-chromenone **13** showed significant *in vitro* activities against *S. sclerotiorum*, *V. mali*, and *B. cinerea* with EC_{50} values of 3.65, 2.61, and 2.32 mg/L, respectively. The 3-isoquinolinyl-4-chromenone **25** exhibited excellent *in vitro* activity against *S. sclerotiorum* with an EC_{50} value of 1.94 mg/L, close to that of commercial fungicide chlorothalonil (EC_{50} = 1.57 mg/L) but lower than that of boscalid (EC_{50} = 0.67 mg/L). For *V. mali* and *B. cinerea*, 3-isoquinolinyl-4-chromenone **25** (EC_{50} = 1.56, 1.54 mg/L) showed significantly higher activities than chlorothalonil (EC_{50} = 11.24, 2.92 mg/L). In addition, *in vivo* experiments proved that compounds **13** and **25** have excellent protective fungicidal activities with inhibitory rates of 88.24 and 94.12%, respectively, against *B. cinerea* at 50 mg/L, while the positive controls chlorothalonil and boscalid showed inhibitory rates of 76.47 and 97.06%, respectively. Physiological and biochemical studies showed that the primary action of mechanism of compounds **13** and **25** on *S. sclerotiorum* and *B. cinerea* may involve changing mycelial morphology and increasing cell membrane permeability. In addition, compound **13** may also affect the respiratory metabolism of *B. cinerea*. This study revealed that compounds **13** and **25** could be promising candidates for the development of novel fungicides in crop protection.

KEYWORDS: *natural product, flavonoid, (iso)quinoline, fungicidal activity, structure−activity relationship*

INTRODUCTION

Fungal infection could cause tremendous decreases in crop yield and quality, which threatens worldwide food security.[1,2] Treating crops with fungicides has been one of the most effective ways against plant diseases. Nevertheless, widespread use and misuse of chemical fungicides have caused more and more resistance in the fungi.[3,4] Meanwhile, green fungicides with high efficiency and low toxicity are needed to reduce the negative impact on the environment. Using natural products as lead compounds to develop new fungicides is one of effective solutions to these problems.[5,6] Natural products with novel structures, as a source for new pesticides, not only can provide unique modes of action[7] but also have good environmental compatibility, which is important for development of green fungicides.[8]

Natural flavonoids have been found in many plants, such as tea,[9] ginkgo,[10] *Hippophae rhamnoides*,[11] *Flos Sophorae Immaturus*, etc.[9,12] Furthermore, flavonoids exhibit a variety of biological activities including antitumor,[13,14] anti-inflammatory,[15] antioxidation,[16] anticarcinogenic,[17] anti-HIV,[18] insecticidal,[19] and fungicidal effects (Figure 1).[20,21] Containing a valuable structural core, flavonoids have always been used as lead compounds in the development of medicines and pesticides.[22]

Meanwhile, (iso)quinolines, one of the largest classes of alkaloids, take a great proportion of the active natural products.[23] The first quinoline was isolated from the bark of the Cinchona tree in 1820.[24] To date, there are more than 150 (iso)quinoline alkaloids isolated,[25] which show a wide range of biological effects, such as antitumor,[26] antimalarial,[27] antibacterial,[28] and antifungal activities.[29] Also, (iso)quinolines have been identified as a pharmacophore in many complex natural products, which exhibit highly antifungal activities. They have been considered as potential active groups and a functional scaffold reasonably in agrochemical discovery. For instance, the quinoline skeleton has been imbedded in the marketed fungicides tebufloquin[30] and quinofumelin (Figure 1).[31,32]

Recently, We have developed a general approach for the synthesis of 3-(iso)quinolinyl 4-chromenones,[33] which displayed obvious antifungal activities during our preliminary biological screening. Motived by this, in the present study, we attempted to further investigate flavonoid-based antifungal agents. Quercetin was taken as the lead compound for systematic structural optimization. Attracted by the known fungicidal activities of (iso)quinoline, we expected that the antifungal effects of the lead compound could be further

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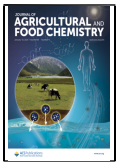

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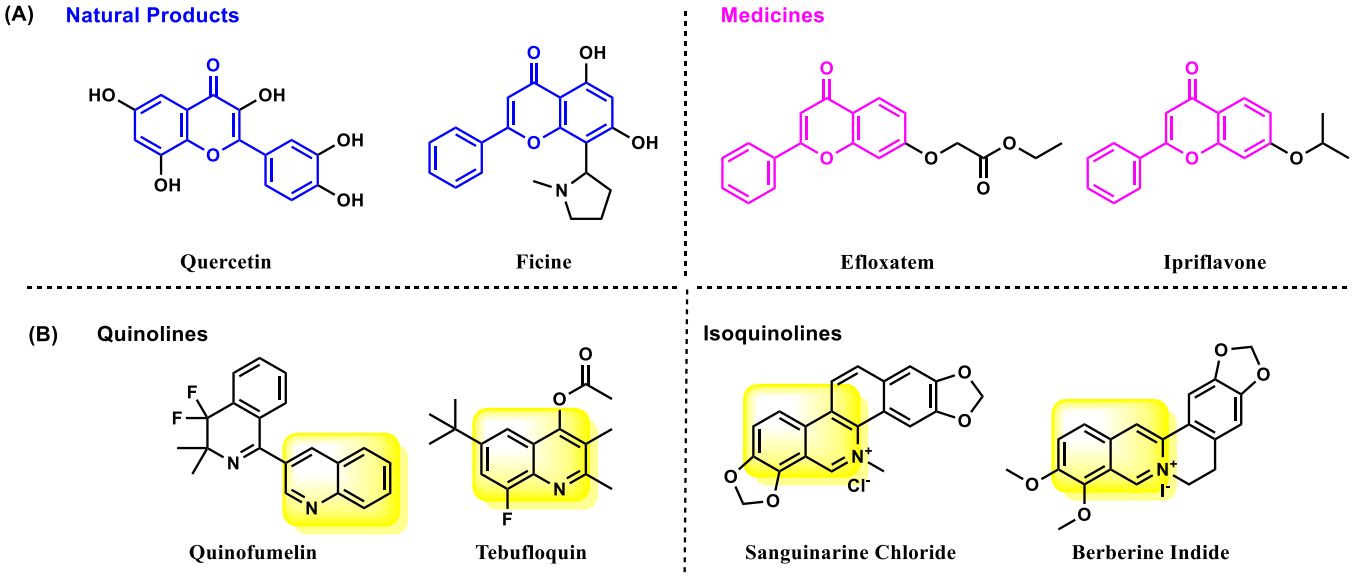

Figure 1. Chemical structures of flavonoids (A) and (iso)quinolines (B) with biological activities.

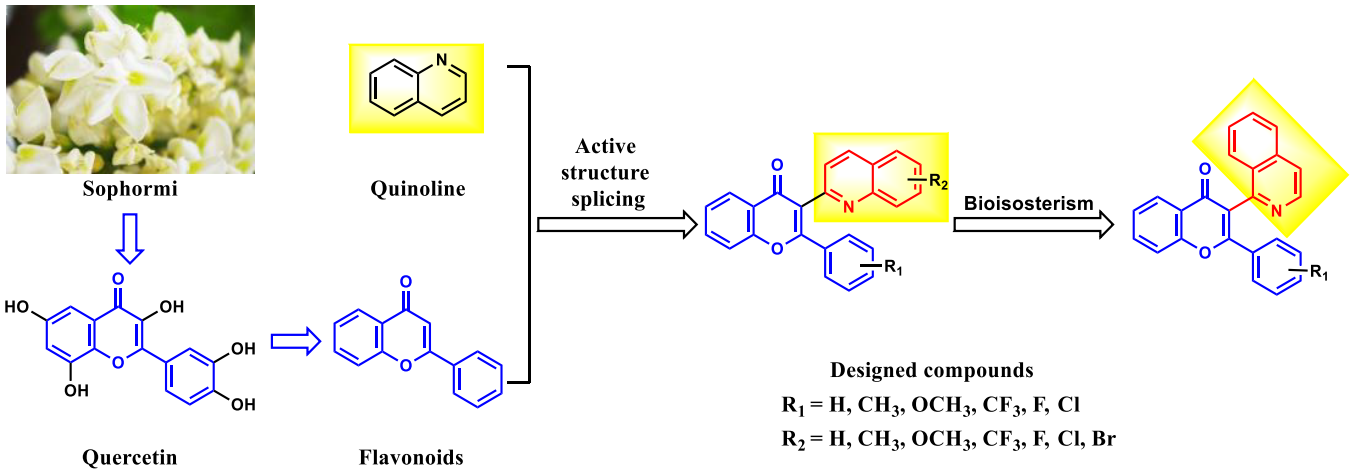

Figure 2. Design of the target compounds.

improved by introducing (iso)quinoline to the flavonoid skeleton through an active substructure splicing strategy. With this in mind, series of novel 3-(iso)quinolinyl-4-chromenone derivatives were designed and synthesized (Figure 2). The fungicidal activities of these novel compounds were evaluated to validate the idea, and the structure−activity relationships (SARs) were analyzed. The results of biological activity assays indicate that many of target compounds exhibit excellent fungicidal activities, which initially verified the rationality of our design strategy. To explore the preliminary mechanisms of the action of 3-(iso)quinolinyl-4-chromenone derivatives, the morphological changes of the mycelia cell wall and the antifungal effects on the permeability of the mycelia cell membrane and respiratory metabolism of fungi were examined.

MATERIALS AND METHODS

Instruments and Chemicals. All chemicals (reagent grade) were purchased from commercial sources (Energy, Shanghai, China). All the ^{1}H and ^{13}C NMR spectra were measured on a Bruker AV-400, AV-500, or AV-600 spectrometer with $CDCl_3$, CD_2Cl_2, or DMSO-d_6 as the solvent and tetramethylsilane as the internal standard. Chemical shifts were reported in ppm (δ). Melting points (mp) were recorded on a WRS-1B melting point apparatus (Jingsong, Shanghai, China) and were measured without correction. High-resolution mass spectrometry (HRMS) was recorded using an electrospray ionization (ESI) technique. Thin-layer chromatography (TLC) was performed on a silica gel 60 GF254 plate. The silica gel (size of 200−300 mesh) used for the column chromatography was purchased from Qingdao Haiyang Chemistry Plant (China). Microscopic morphology of fungal hyphae was observed by a scanning electron microscope (Hitachi, S-3400 N, Tokyo, Japan). The conductivity was measured by a CON510 Eutech/Oakton conductometer (OAKTON Instruments, Waltham). Dissolved oxygen was measured by a JPB-607A dissolved oxygen meter (INESA Scientific Instrument Co., Shanghai, China).

Fungi. *Colletotrichum orbiculare* (*C. orbiculare*), *Fusarium oxysporum* (*F. oxysporum*), *Sclerotinia sclerotiorum* (*S. sclerotiorum*), *Physalospora piricola* (*P. piricola*), *Valsa mali* (*V. mali*), *Altenaria alternariae* (*A. alternariae*), and *Botrytis cinerea* (*B. cinerea*) were provided by the College of Plant Protection, Northwest A&F University.

Synthesis. *General Procedure for the Preparation of Intermediates **1a−h**.* *n*-Butyllithium (2.0 mL, 5.0 mmol, 2.5 M in hexane) was added dropwise to the solution of alkyne (5 mmol) in dry THF (30 mL) at −78 °C. The solution was stirred at this temperature under argon for 1 h. After that, salicylaldehyde (2.27 mmol) was added using a syringe at the same temperature; the solution was stirred for 2 h at −78 °C. Then, the reaction was quenched by the

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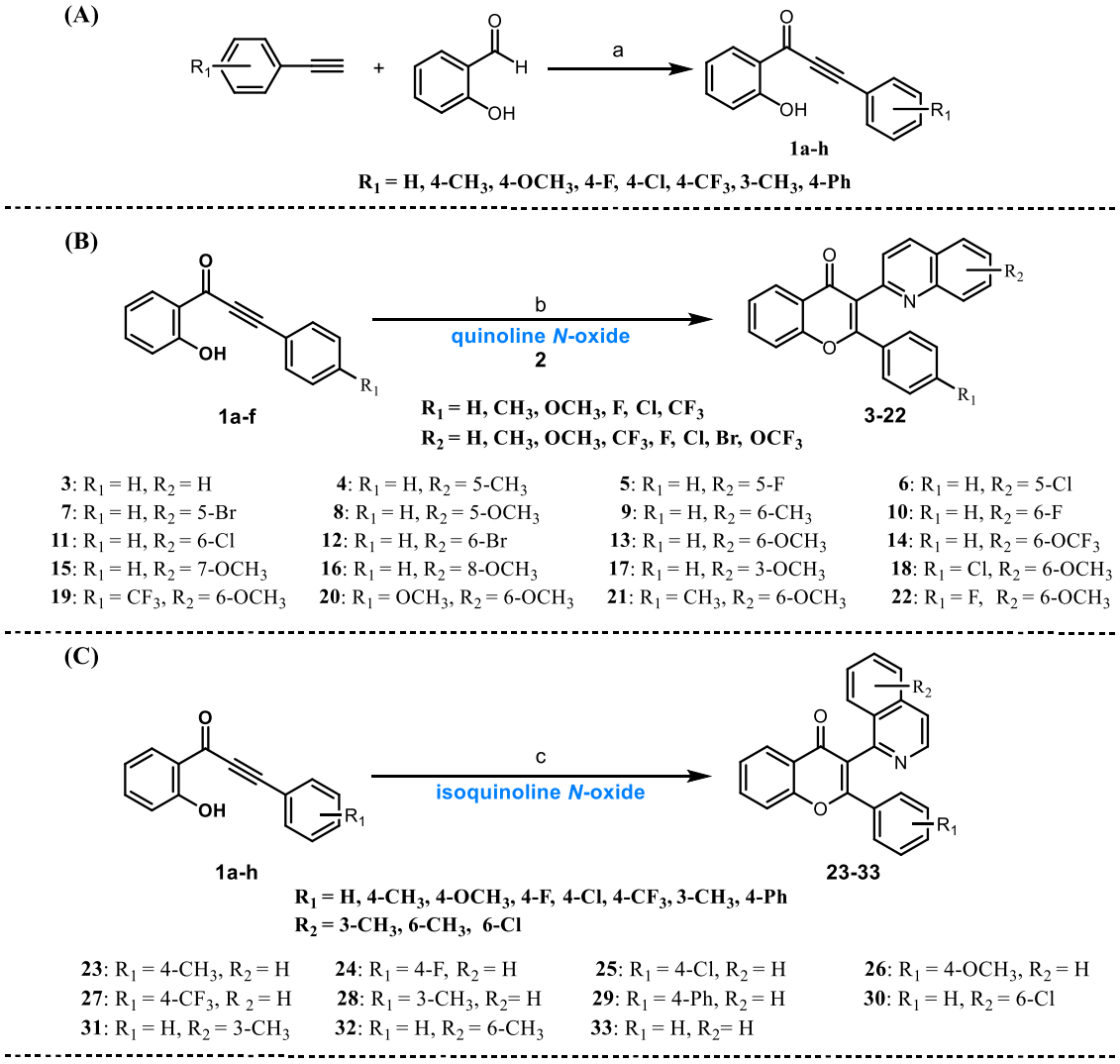

Figure 3. (A) General synthesis of **1a-h**. Reagents and conditions: (a) *n*-BuLi, THF, −78 °C; MnO_2, acetone. (B) General synthesis of **3**−**22**. Reagents and conditions: (b) quinoline *N*-oxide, DMF, 140 °C, HCl. (C) General synthesis of **23**−**33**. Reagents and conditions: (c) isoquinoline *N*-oxide, DMF, 140 °C, HCl.

addition of aqueous saturated NH_4Cl (20 mL). The phases were separated and the aqueous phase was extracted with EtOAc (3 × 10 mL). The combined organic layers were washed with brine (30 mL) and dried over Na_2SO_4. The resulting liquid was concentrated under a reduced pressure. Then, the crude material was dissolved in 30 mL of acetone, and activated MnO_2 (15.0 g, 172.5 mmol) was added to the mixture. Then, the reaction mixture was stirred for 12 h at room temperature, filtered through a pad of celite, concentrated under a reduced pressure, and purified by flash chromatography on silica gel (petroleum ether:ethyl acetate = 60:1) to afford the product.

General Procedure for the Preparation of Compounds ***3*****−****22***. Intermediate **1** (1 mmol) and quinoline *N*-oxide (1.5 mmol) are dissolved in 1.5 mL of DMF in a 20 mL sealed tube; then, 25 μL of 12 M HCl solution was added into the mixture quickly. The reaction mixture was stirred at 140 °C in a dry block heater for 2 h. After that, the solvent was removed, and the crude product was purified via column chromatography on silica gel (Gradient elution: petrol ether:ethyl acetate = 15:1 to dichloromethane:methanol = 100:1) to give the corresponding product.

General Procedure for the Preparation of Compounds ***23*****−****33***. Intermediate **1** (1 mmol) and isoquinoline *N*-oxide (1.5 mmol) are dissolved in 1.5 mL of DMF in a 20 mL sealed tube; then, 25 μL of 12 M HCl solution was added into the mixture quickly. The reaction mixture was stirred at 140 °C in a dry block heater for 2 h. After that, the solvent was removed, and the crude product was purified via column chromatography on silica gel (Gradient elution: petrol ether:ethyl acetate = 15:1 to dichloromethane:methanol = 100:1) to give the corresponding product.

Biological Assay. *In Vitro Fungicidal Activities.* All synthesized compounds were screened for their *in vitro* antifungal activities against *C. orbiculare*, *F. oxysporum*, *S. sclerotiorum*, *P. piricola*, *V. mali*, *A. alternariae*, and *B. cinerea* at the concentration of 50 mg/L for the preliminary screening according to a mycelia growth inhibition method, with quercetin, chlorothalonil, tebuconazole, procymidone, and boscalid as positive controls.

In Vivo Fungicidal Activities against B. cinerea. The *Cucumis sativus Linn.* leaves of rape were collected from the Key Laboratory of Botanical Pesticide R&D in Northwest A&F University. For the protective activity assay, healthy leaves of *Cucumis sativus Linn.* were sprayed with the target compounds (50 mg/L), respectively, and then cultivated at 25 °C for 24 h before inoculation with *B. cinerea*. Chlorothalonil and boscalid were used as the positive controls.

Effects of Compounds ***13*** *and* ***25*** *on Mycelial Morphology of S. sclerotiorum and B. cinerea.* *S. sclerotiorum* and *B. cinerea* were cultured in a 90 mm culture dish. When the mycelia grew to 70 mm in diameter, fresh fungus dishes (5 mm in diameter) were made from the edge of the colonies. The mycelia were inoculated on potato dextrose agar (PDA) medium plates containing no compound (negative control) and compounds **13** and **25** with a concentration of 5 mg/L,

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respectively. Then, the mycelia were cultured at 25 °C for 2 days. The mycelia cell wall structure of *S. sclerotiorum* at the top of each treated colonies were selected and observed under an S-3400 N scanning electron microscope (SEM) (Hitachi, Ltd., Tokyo, Japan).

*Effects of Compounds **13** and **25** on Cell Membrane Permeability of S. sclerotiorum, V. mali, and B. cinerea.* The tested strains were cultured on a PDA plate at 25 °C for 72 h. Then, the strains were placed in a potato dextrose broth (PDB) culture medium and cultured by shaking (25 °C, 120 rpm) for 72 h. The mycelia were filtered and washed with distilled water and put into a centrifugal tube, and then 10 mL of compounds **13** and **25** (25 mg/L) and distilled water were added into the centrifugal tube, respectively. Finally, the mycelia were oscillated (120 rpm) in a water bath at a constant temperature of 28 °C at different times. The conductivity was measured by a CON510 Eutech/Oakton conductometer (OAKTON Instruments, Waltham). The negative control was mycelia with distilled water. The conductivity of compounds **13** and **25** were determined at 0, 30, 60, 120, 180, 240, and 300 min and finally boiled (dead treatment) to determine the conductivity. The relative permeability was calculated for each measurement, and then the permeability of cell membranes was compared according to the conductivity. Each experiment was run in triplicate.

*Effects of Compounds **13** and **25** on the Respiratory Metabolism of B. cinerea.* The respiratory oxygen consumption rate of *B. cinerea* was measured by the oxygen electrode method.[34]

A total of 500 mg of fresh mycelia were added into 10 mL of medium solution (9 mL of phosphate buffer solution with a concentration of 1 M, pH = 7.2 and 0.2 mL of glucose solution with a concentration of 2%). After stirring for 10 min, dissolved oxygen was measured by a JPB-607A dissolved oxygen meter (INESA Scientific Instrument Co., Shanghai, China).

Then, compounds **13** and **25**, and boscalid (20 mg/L) were added. Respiration rates of mycelia (O_2, μmol/g min) were calculated from the change of oxygen content in the medium. The inhibition rates of mycelia respiration (IR_r) were calculated according to the respiration rates of mycelia before and after adding test agents with the following equation:

$$IR_r(\%) = (R_0 - R_1)/R_0$$

where R_0 and R_1 are respiration rates of mycelia before and after adding tested agents.

RESULTS AND DISCUSSION

Chemistry Synthesis. The synthetic route for compounds **1a–h** was described in Figure 3A. The target compounds can be divided into two series: (1) 3-quinolinyl-4-chromenones (Figure 3B); (2) 3-isoquinolinyl-4-chromenones (Figure 3C). The chemical structures of all prepared compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS.

Fungicidal Activities and SAR Discussion. A bioactivity-guided synthesis was applied for the discovery of antifungal candidates. All compounds were evaluated against seven fungi including *C. orbiculare*, *F. oxysporum*, *S. sclerotiorum*, *P. piricola*, *V. mali*, *A. alternariae*, and *B. cinerea*. This biologically indicating method is handleable and intuitionistic. The results generated from this tactic are easy to interpret with a significant value (Figure 4).

Initially, 3-quinolinyl-4-chromenones **3–14** were synthesized and evaluated for their antifungal activities. As can be seen from Table 1, most of the synthesized compounds exhibited fair to good antifungal activities against *S. sclerotiorum*, *V. mali*, and *B. cinerea*. Among them, compounds **8** (R_2 = 5-OCH_3) and **13** (R_2 = 6-OCH_3) displayed over 96% inhibitory activity, which indicated that the methoxy group at the quinoline moiety may be crucial for their significant *in vitro* antifungal bioactivity. The higher biological activity of the methoxy group may be related to its electron-donating effect.[35]

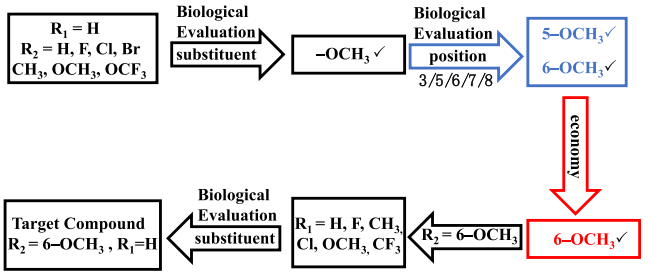

Figure 4. Bioactivity guided synthesis of compounds **3–22**.

To confirm this, the compounds **15** (R_2 = 7-OCH_3), **16** (R_2 = 8-OCH_3), and **17** (R_2 = 3-OCH_3) were synthesized. Interestingly, they all show good biological activities against *S. sclerotiorum*, *V.mali*, and *B.cinerea*, which further proved that the methoxy group is a factor determining the biological activities of the target compounds. Comparing the biological activities of compounds **8**, **13**, **15**, **16**, and **17**, we found that the compounds with a methoxy group at the C5/C6 position of quinoline exhibited relatively higher activities. However, because the cost of the starting material 6-methoxyquinoline ($ 201/100 g, Energy) is much lower than that of 5-methoxyquinoline ($ 857/100 g, Energy), we chose compound **13** (R_2 = 6-OCH_3) as the lead compound for further optimization. Therefore, after R_2 is fixed as the 6-OCH_3 group, the influence of R_1 substituents at the 4-position of a phenyl ring on the antifungal activities was then investigated. The compounds **18**, **19**, **20**, **21**, and **22** were synthesized. The results show that compound **13** (R_1 = H) exhibited the best antifungal activity compared with the corresponding compounds.

In order to investigate whether compounds **3–22** possess a broad spectrum of bioactivity, we selected four more fungi (*C. orbiculare*, *F. oxysporum*, *P. piricola*, and *A. alternariae*) to test their antifungal activities. For *C. orbiculare*, compound **17** (R_2 = 3-OCH_3, 80.81%) showed an excellent inhibitory rate (about 80%), which is better than that of other compounds. In addition, compounds **6** (R_2 = 5-Cl, 72.22%) and **11** (R_2 = 6-Cl, 76.77%) displayed good antifungal activities against *C. orbiculare* (inhibition rate > 70%). For *F. oxysporum*, although the inhibition rates of all compounds are below 70%, compound **17** with a methoxy group (R_2 = 3-OCH_3) still showed the highest inhibition rate (67.62%), and compounds **15** (R_2 = 7-OCH_3, 57.90%) and **16** (R_2 = 8-OCH_3, 63.81%) showed moderate fungicidal activity compared with other compounds. For *P.piricola*, unfortunately, only compounds **3** (R_2 = H, 52.22%), **13** (R_2 = 6-OCH_3, 50.00%), and **21** (R_2 = 6-OCH_3, 55.21%) gave the inhibition rates over 50%.

To further optimize the leads, compounds **23–33** were designed by bioisosterism and evaluated for their antifungal effects against *S. sclerotiorum*, *V.mail*, and *B. cinerea* at 50 mg/L. As shown in Table 2, some of them displayed good fungicidal activity against specific fungi. Among them, compounds **23**, **24**, **25**, **28**, and **29** displayed good antifungal activities against *S. sclerotiorum* (inhibition rate > 80%) at 50 mg/L, which showed inhibition rates of 98.33, 98.06, 100, 84.44, and 98.33%, respectively. For the *V. mali*, compounds **23**, **24**, and **25** exhibited >90% inhibition rates. Notably, compounds **23–33** displayed good antifungal activities against *B. cinerea* (>80% inhibition rates). Of note, in the 3-isoquinolinyl-4-chromenone series, compound **25** showed the best antifungal activities against the seven fungi.

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Table 1. *In Vitro* Fungicidal Activities (Inhibition Rate) of the Compounds 3−22 (50 mg/L)[a,b,c,d,e,f,g,h,i]

compd[a]	*C.o*[b]	*F.o*[c]	*S.c*[d]	*P.p*[e]	*V.m*[f]	*A.a*[g]	*B.c*[h]
3	50.56 ± 1.10	34.44 ± 2.86	50.56 ± 1.22	52.22 ± 1.01	65.64 ± 1.12	65.63 ± 0.53	**90.01** ± 1.04
4	49.49 ± 0.88	30.48 ± 2.18	44.12 ± 2.25	<5	72.92 ± 1.38	59.90 ± 1.57	34.09 ± 2.23
5	61.11 ± 1.75	52.38 ± 0.83	**93.14** ± 1.30	42.53 ± 3.77	**93.75** ± 0.52	60.94 ± 1.56	37.12 ± 1.52
6	72.22 ± 0.87	38.10 ± 0.83	61.27 ± 0.98	35.06 ± 1.15	**92.19** ± 0.90	59.38 ± 1.27	59.85 ± 0.76
7	51.01 ± 4.63	31.43 ± 1.43	47.06 ± 2.94	32.18 ± 1.52	88.02 ± 1.04	64.58 ± 1.80	37.12 ± 1.52
8	48.33 ± 1.65	26.11 ± 3.44	**100**	25.19 ± 3.92	**100**	50.00 ± 1.57	**95.5** ± 0.55
9	47.98 ± 0.87	40.48 ± 1.65	81.25 ± 0.64	<5	75.00 ± 0.26	70.31 ± 0.90	71.67 ± 1.45
10	51.52 ± 0.81	56.19 ± 4.12	**91.67** ± 0.28	<5	**92.22** ± 0.13	91.67 ± 0.12	88.33 ± 1.22
11	76.77 ± 1.51	50.95 ± 2.98	72.22 ± 0.33	31.48 ± 0.37	54.36 ± 2.25	31.77 ± 0.50	73.91 ± 2.13
12	35.86 ± 1.75	36.19 ± 0.83	42.08 ± 3.11	19.35 ± 4.52	53.33 ± 2.12	61.46 ± 1.21	71.11 ± 1.03
13	48.48 ± 1.52	33.33 ± 0.72	**100**	50.00 ± 2.63	**97.92** ± 0.22	63.02 ± 0.98	**96.97** ± 0.31
14	45.57 ± 3.2	15.31 ± 0.90	46.67 ± 0.59	30.30 ± 1.61	39.49 ± 0.51	27.02 ± 1.56	48.48 ± 0.62
15	66.16 ± 0.68	57.90 ± 1.32	**100**	48.85 ± 2.17	**93.75** ± 1.10	85.42 ± 0.89	**98.48** ± 0.25
16	59.60 ± 2.31	63.81 ± 0.83	75.00 ± 1.20	27.01 ± 3.59	**91.67** ± 0.65	90.63 ± 1.57	**100**
17	80.81 ± 0.88	67.62 ± 0.82	84.17 ± 0.85	43.68 ± 2.51	88.54 ± 0.75	75.00 ± 0.91	**91.67** ± 1.52
18	45.96 ± 2.31	11.09 ± 2.72	38.33 ± 3.52	7.29 ± 5.11	37.84 ± 2.53	41.33 ± 1.42	<5
19	36.36 ± 1.51	10.95 ± 0.83	22.00 ± 4.25	18.75 ± 2.53	39.19 ± 2.47	39.33 ± 2.58	26.34 ± 3.55
20	46.46 ± 0.89	28.10 ± 1.69	34.67 ± 3.10	28.65 ± 4.88	59.46 ± 1.28	52.00 ± 2.02	32.80 ± 2.38
21	51.52 ± 1.52	45.71 ± 2.47	75.00 ± 1.02	55.21 ± 2.62	**90.99** ± 0.42	75.33 ± 1.15	80.65 ± 0.66
22	46.46 ± 0.72	19.05 ± 3.86	41.67 ± 3.05	24.48 ± 4.55	55.41 ± 1.37	48.00 ± 1.65	23.66 ± 4.21
quercetin	33.33 ± 2.15	<5	100	8.62 ± 0.99	73.96 ± 1.80	<5	9.09 ± 4.32
C[i]	70.73 ± 1.45	76.19 ± 0.55	100	86.15 ± 0.64	71.67 ± 1.65	66.67 ± 2.08	91.6 ± 0.58
boscalid	97.62 ± 0.48	50.00 ± 0.78	100	66.67 ± 0.60	62.72 ± 0.52	44.59 ± 0.78	85.76 ± 0.61

[a]Data are given as the mean of triplicate experiments. [b]*C.o*: *Colletotrichum orbiculare*. [c]*F.o*: *Fusarium oxysporum*. [d]*S.s*: *Sclerotinia sclerotiorum*. [e]*P.p*: *Physalospora piricola*. [f]*V.m*: *Valsa mali*. [g]*A.a*: *Altenaria alternariae*. [h]*B.c*: *Botrytis cinerea*. [i]C: chlorothalonil.

Table 2. *In Vitro* Fungicidal Activities (Inhibition Rate) of the Compounds 23−33 (50 mg/L)[a,b,c,d,e,f,g,h,i]

compd[a]	*C.o*[b]	*F.o*[c]	*S.c*[d]	*P.p*[e]	*V.m*[f]	*A.a*[g]	*B.c*[h]
23	56.67 ± 2.14	62.22 ± 2.04	**98.33** ± 0.13	57.04 ± 0.37	**97.95** ± 0.58	78.13 ± 0.90	**96.14** ± 0.30
24	58.33 ± 1.65	68.44 ± 1.03	**98.06** ± 0.20	52.22 ± 0.98	**95.38** ± 0.14	76.56 ± 0.90	**97.67** ± 0.13
25	65.00 ± 1.32	69.33 ± 0.28	**100**	63.33 ± 0.63	**100**	79.85 ± 0.14	**100**
26	54.30 ± 0.69	32.78 ± 2.57	72.22 ± 1.41	67.41 ± 0.97	82.05 ± 0.95	73.44 ± 1.82	88.56 ± 0.22
27	52.22 ± 1.25	41.11 ± 1.10	50.00 ± 2.44	41.11 ± 2.30	86.67 ± 0.83	75.00 ± 1.57	85.55 ± 0.55
28	65.00 ± 1.05	62.78 ± 1.42	84.44 ± 1.58	72.22 ± 0.63	87.69 ± 1.32	62.50 ± 0.14	**90.34** ± 0.30
29	60.00 ± 0.45	20.00 ± 3.23	**98.33** ± 0.22	3.33 ± 0.63	38.97 ± 2.75	34.90 ± 0.50	81.23 ± 0.79
30	45.00 ± 2.61	16.67 ± 3.57	42.78 ± 3.11	54.44 ± 0.42	47.18 ± 2.35	46.35 ± 0.53	86.71 ± 1.85
31	37.78 ± 2.78	40.00 ± 1.65	75.56 ± 2.02	42.59 ± 0.74	85.13 ± 0.88	59.90 ± 0.57	88.21 ± 0.52
32	40.00 ± 2.65	38.33 ± 2.41	46.67 ± 3.44	69.63 ± 0.98	63.59 ± 1.22	67.71 ± 0.53	**92.12** ± 0.79
33	53.33 ± 0.88	62.78 ± 1.58	30.56 ± 4.20	42.96 ± 0.37	49.74 ± 1.38	66.15 ± 0.53	**88.86 ± 0.60**
quercetin	33.33 ± 2.15	<5	100	8.62 ± 0.99	73.96 ± 1.80	<5	9.09 ± 4.32
C[i]	70.73 ± 1.45	76.19 ± 0.55	100	86.15 ± 0.64	71.67 ± 1.65	66.67 ± 2.08	91.6 ± 0.58
boscalid	97.62 ± 0.48	50.00 ± 0.78	100	66.67 ± 0.60	62.72 ± 0.52	44.59 ± 0.78	85.76 ± 0.61

[a]Data are given as the mean of triplicate experiments. [b]*C.o*: *Colletotrichum orbiculare*. [c]*F.o*: *Fusarium oxysporum*. [d]*S.s*: *Sclerotinia sclerotiorum*. [e]*P.p*: *Physalospora piricola*. [f]*V.m*: *Valsa mali*. [g]*A.a*: *Altenaria alternariae*. [h]*B.c*: *Botrytis cinerea*. [i]C: chlorothalonil.

In order to further determine the fungicidal potency and probe the SARs of these novel 3-(iso)quinolinyl-4-chromenone derivatives, several compounds with superior fungicidal activities were selected for further study. Their EC_{50} values against *S. sclerotiorum*, *V. mali*, and *B. cinerea* are shown in Table 3. In general, the compounds bearing methoxy groups showed better fungicidal activities than that with other substituents. For example, compound **8** (R_2 = 5-OCH_3, EC_{50} = 3.79 mg/L) was more active than compound **5** (R_2 = 5-F, EC_{50} = 4.60 mg/L) and compound **13** (R_2 = 6-OCH_3, EC_{50} = 3.65 mg/L) was more active than compound **10** (R_2 = 6-F, EC_{50} = 7.50 mg/L) against *S. sclerotiorum*. Compound **13** (R_2 = 6-OCH_3, EC_{50} = 2.61, 2.32 mg/L) was more active than compound **10** (R_2 = 6-F, EC_{50} = 3.47, 4.72 mg/L) against *V. mali* and *B. cinerea*, respectively. Meanwhile, the compounds with substitution at the 5/6-position of the quinoline ring showed better fungicidal activities than that with substitution at other positions. For instance, compounds **8** (R_2 = 5-OCH_3, EC_{50} = 3.79, 2.37 mg/L) and **13** (R_2 = 6-OCH_3, EC_{50} = 3.65, 2.32 mg/L) were more active than compounds **15** (R_2 = 7-OCH_3, EC_{50} = 5.27, 2.47 mg/L), **16** (R_2 = 8-OCH_3, EC_{50} = 8.55, 6.54 mg/L), and **17** (R_2 = 3-OCH_3, EC_{50} = 18.18, 13.54 mg/L), against *S. sclerotiorum* and *B. cinerea*. For *V. mali*, compound **8** (EC_{50} = 1.65 mg/L) was more active than compounds **13** (EC_{50} = 2.61 mg/L), **17** (EC_{50} = 4.97 mg/L), **15** (EC_{50} = 3.33 g/L), and **16** (EC_{50} = 3.43 mg/L).

The above SARs of the 3-quinolinyl-4-chromenones **3**−**22** against *S. sclerotiorum*, *V. mali*, and *B. cinerea* could be

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Table 3. Fungicidal Activities of the Target Compounds with EC_{50} Values against *S. sclerotiorum*, *V. mali*, and *B. cinerea*[a]

fungi	compd	R_1	R_2	EC_{50}	95% CI[a]	regression equation	R^2
S. sclerotiorum	**5**	H	5-F	4.60	2.34−4.87	$y = -0.673 + 1.205x$	0.992
	8	H	5-OCH_3	3.79	1.03−6.35	$y = -0.908 + 1.571x$	0.985
	9	H	6-CH_3	23.49	14.63−51.40	$y = -2.528 + 1.844x$	0.992
	10	H	6-F	7.50	5.94−9.14	$y = -1.237 + 1.414x$	0.970
	13	H	6-OCH_3	3.65	2.74−4.51	$y = -1.001 + 1.781x$	0.986
	15	H	7-OCH_3	5.27	4.45−6.22	$y = -1.066 + 1.477x$	0.952
	16	H	8-OCH_3	8.55	6.55−10.75	$y = -1.088 + 1.167x$	0.990
	17	H	3-OCH_3	18.18	15.72−21.29	$y = -2.507 + 1.989x$	0.991
	21	CH_3	6-OCH_3	7.82	5.73−10.01	$y = -0.946 + 1.059x$	0.948
	23	4-CH_3	H	4.20	3.54−4.96	$y = -0.928 + 1.489x$	0.941
	24	4-F	H	4.42	2.18−7.15	$y = -0.892 + 1.382x$	0.993
	25	4-Cl	H	1.94	1.61−2.29	$y = -0.487 + 1.692x$	0.990
	26	4-OCH_3	H	2.17	1.72−2.64	$y = -0.449 + 1.338x$	0.980
	28	3-CH_3	H	4.37	3.58−5.28	$y = -0.796 + 1.243x$	0.973
	29	Ph	H	3.56	2.35−4.74	$y = -0.696 + 1.262x$	0.997
	chlorothalonil			1.57	0.95−2.22	$y = -0.241 + 0.954x$	0.960
	boscalid			0.67	0.47−0.89	$y = 0.183 + 1.042x$	0.979
V. mali	**4**	H	5-CH_3	3.55	0.03−8.13	$y = -0.184 + 0.334x$	0.993
	6	H	5-Cl	2.97	1.83−4.09	$y = -0.583 + 1.231x$	0.989
	7	H	5-Br	5.79	4.10−7.50	$y = -0.859 + 1.126x$	0.977
	8	H	5-OCH_3	1.65	1.36−1.95	$y = -0.386 + 1.769x$	0.987
	9	H	6-CH_3	3.38	2.54−4.32	$y = -0.493 + 0.934x$	0.987
	10	H	6-F	3.47	2.77−4.20	$y = -0.809 + 1.498x$	0.968
	13	H	6-OCH_3	2.61	2.16−3.10	$y = -0.630 + 1.511x$	0.977
	15	H	7-OCH_3	3.33	2.47−4.24	$y = -0.610 + 1.167x$	0.976
	16	H	8-OCH_3	3.43	2.72−4.21	$y = -0.619 + 1.156x$	0.959
	17	H	3-OCH_3	4.97	3.87−6.18	$y = -0.810 + 1.163x$	0.958
	21	CH_3	6-OCH_3	6.81	5.43−8.41	$y = -0.971 + 1.165x$	0.983
	23	4-CH_3	H	3.52	2.83−4.23	$y = -0.842 + 1.543x$	0.982
	24	4-F	H	3.18	2.48−3.92	$y = -0.705 + 1.401x$	0.954
	25	4-Cl	H	1.56	1.23−1.89	$y = -0.286 + 1.490x$	0.968
	26	4-OCH_3	H	3.02	2.37−3.69	$y = -0.711 + 1.481x$	0.985
	27	4-CF_3	H	3.57	2.52−4.58	$y = -0.820 + 1.486x$	0.994
	28	3-CH_3	H	3.98	3.21−4.80	$y = -0.883 + 1.471x$	0.950
	chlorothalonil			11.24	8.20−15.12	$y = -0.941 + 0.895x$	0.991
	tebuconazole			0.27	0.16−0.40	$y = 0.528 + 0.937x$	0.962
B. cinerea	**3**	H	H	8.20	6.69−9.84	$y = -1.396 + 1.528x$	0.977
	8	H	5-OCH_3	2.37	1.64−3.17	$y = -0.558 + 1.493x$	0.953
	9	H	6-CH_3	25.87	19.42−38.46	$y = -1.388 + 0.982x$	0.951
	10	H	6-F	4.72	3.59−5.83	$y = -1.049 + 1.558x$	0.987
	13	H	6-OCH_3	2.32	1.38−3.31	$y = -0.311 + 0.852x$	0.988
	15	H	7-OCH_3	2.47	1.92−3.03	$y = -0.619 + 1.575x$	0.986
	16	H	8-OCH_3	6.54	5.53−7.58	$y = -1.693 + 2.076x$	0.999
	17	H	3-OCH_3	13.54	9.52−19.66	$y = -0.862 + 0.761x$	0.978
	21	CH_3	6-OCH_3	4.78	3.70−5.82	$y = -1.121 + 1.651x$	0.986
	23	4-CH_3	H	3.57	2.80−4.38	$y = -0.754 + 1.364x$	0.958
	24	4-F	H	4.52	3.66−5.44	$y = -0.933 + 1.425x$	0.959
	25	4-Cl	H	1.54	1.12−1.98	$y = -0.209 + 1.125x$	0.988
	26	4-OCH_3	H	7.65	6.24−9.13	$y = -1.410 + 1.597x$	0.990
	27	4-CF_3	H	6.45	4.69−8.24	$y = -0.928 + 1.147x$	0.988
	28	3-CH_3	H	5.28	3.68−6.86	$y = -0.829 + 1.147x$	0.974
	29	Ph	H	38.48	25.11−82.53	$y = -1.160 + 0.732x$	0.988
	32	H	6-CH_3	4.90	3.50−6.27	$y = -0.874 + 1.268x$	0.982
	chlorothalonil			2.92	1.61−4.22	$y = -0.485 + 1.043x$	0.986
	boscalid			1.26	0.61−2.04	$y = -0.059 + 0.581x$	0.967
	procymidone			0.10	0.07−0.14	$y = 0.891 + 0.905x$	0.970

[a]Confidence interval.

summarized as follows: (1) The compounds bearing methoxy groups in the quinoline moiety show better fungicidal activities than that with other groups (H, CH_3, F, Cl, Br, and OCF_3). (2) The compounds with a 5/6-methoxy group in the

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quinoline part exhibit similar antifungal activities, which are relatively higher than that with other groups. (3) The compounds including 6-methoxy quinoline were chosen for further lead optimization due to the availability of the starting material and cost considerations. When R_2 is fixed as a 6-methoxy group, the compound **13** with R_1 = H at the 4-position of the phenyl ring displays the best antifungal activity.

As for the 3-isoquinolinyl-4-chromenones series, we initially explored the effect of the substituents on the phenyl ring. The results indicated that introduction of a chlorine atom on the phenyl ring (compound **25**) was favorable for increased fungicidal activities (Figure 5). Also, compound **25** displayed the most potent fungicidal activities (EC_{50} = 1.94, 1.56, 1.54 mg/L) against *S. sclerotiorum*, *V. mali*, and *B. cinerea*, respectively.

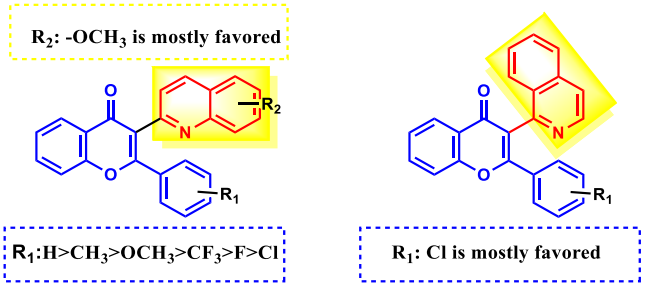

Figure 5. Summarized SARs of the target compounds against *S. sclerotiorum*, *V. mali*, and *B. cinerea*.

***In Vivo* Fungicidal Activities.** *In vivo* fungicidal activity of the target compounds against *B. cinerea* was carried out on *Cucumis sativus Linn.* leaves. The efficacy of the antifungal protection is shown in Figure 6A. Compounds **13** and **25** showed promising protective activities of 88.24 and 94.12%, respectively. Notably, compounds **13** and **25** showed better antifungal effects than chlorothalonil (76.47%) but slightly worse than boscalid (97.06%) at the concentration of 50 mg/L. (Table 4).

Table 4. *In Vivo* Activity of the Target Compounds 13 and 25 against *B. cinerea* on *Cucumis sativus Linn.* Leaves (50 mg/L)

	protective activity	
compd	diameter of lesions (cm)	control efficacy (%)
13	0.7 ± 0.1	88.24%
25	0.6 ± 0.1	94.12%
chlorothalonil	0.9 ± 0.2	76.47%
boscalid	0.5 ± 0.1	97.06%
CK	2.2 ± 0.2	

Effects of Compounds 13 and 25 on Cell Membrane Permeability of *S. sclerotiorum*, *V. mali*, and *B. cinerea*. The change of relative conductivity indicates the variation of cell membrane permeability. According to literature,[34,36] the cell membrane of fungi is an important target for fungicides, which can inhibit the synthesis of a phospholipid bilayer or protein and cause the leakage of internal electrolytes.[37,38] As shown in Figure 6B, after treatment with compounds **13** and **25**, the mycelial relative conductivities of *S. sclerotiorum*, *V. mali*, and *B. cinerea* increased with time, and the increase extent is much greater than that with the control, which indicates that

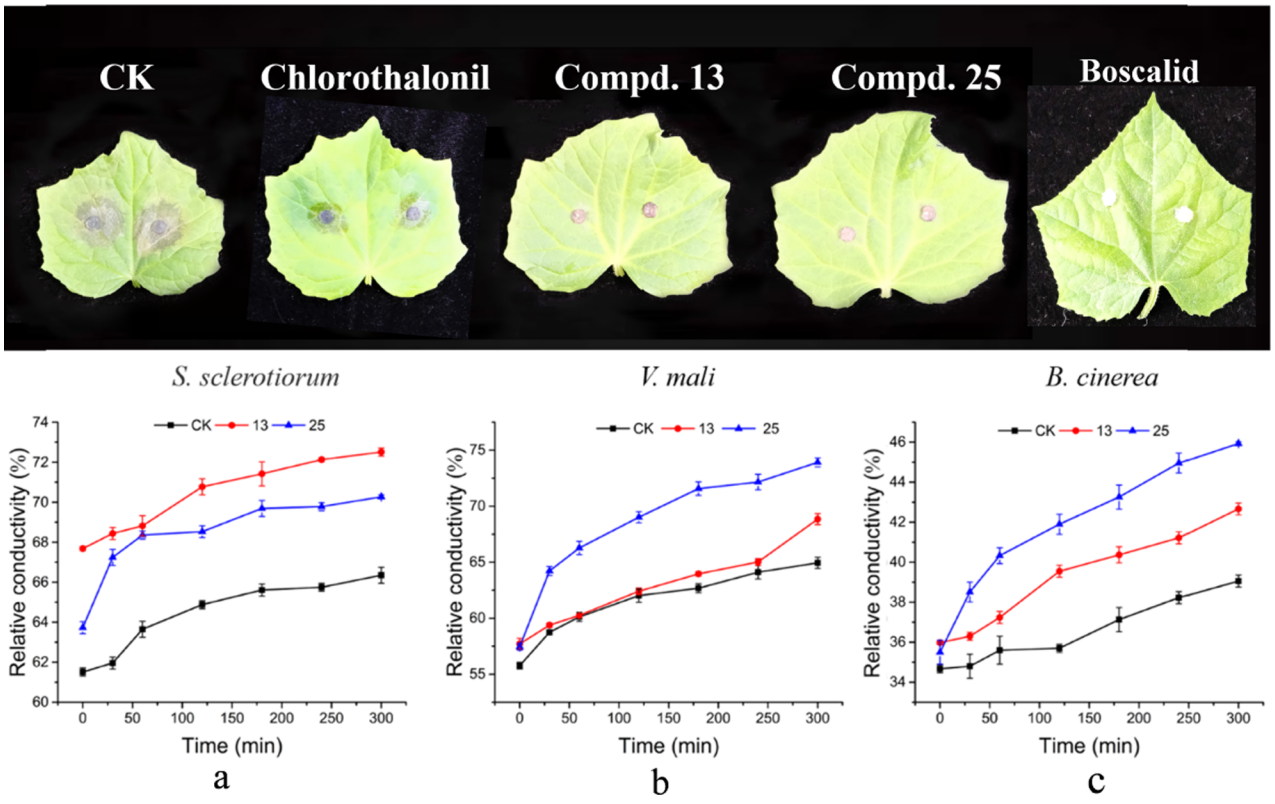

Figure 6. (A) *In vivo* fungicidal activity of the target compounds against *B. cinerea* was carried out on *Cucumis sativus Linn.* leaves. (B) Mycelial relative conductivity of *S. sclerotiorum*, *V. mali*, and *B. cinerea* in the presence or absence of compounds **13** and **25**.

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compounds **13** and **25** could enhance the mycelial cell membrane permeability of *S. sclerotiorum*, *V. mali*, and *B. cinerea*. Combined with the change of mycelial morphology after treatment with compounds **13** and **25**, it can also be inferred that compounds **13** and **25** could manifest antifungal properties by destroying the cell membrane structure. The above reported findings revealed that the action target site of compounds **13** and **25** on *S. sclerotiorum*, *V. mali*, and *B. cinerea* could be the cell membrane.

Scanning Electron Microscopy (SEM) Analysis. SEM of *S. sclerotiorum* and *B. cinerea* revealed morphological changes in the fungal cell surface. As shown in Figure 7, the surfaces of *S. sclerotiorum* and *B. cinerea* cells in the untreated groups (A, E, I, and M) were relatively smooth and regular, whereas when treated with compounds **13** (B, F, J, and N) and **25** (C, G, K, and O), and boscalid (D, H, L, and P) at 5 mg/L, there were significant shrinkage. Of note, for the *B. cinerea*, the extent of mycelial shrinkage with compound **25** (Figure 7, K and O) is greater than that with compound **13** (Figure 7, J and N). This showed that compounds **13** and **25** have a certain degree of damage to the cell wall of *B. cinerea*, and the damage intensity with compound **25** is greater.

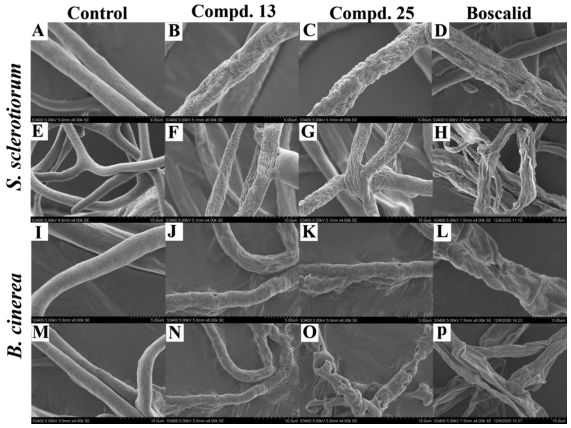

Figure 7. Surfaces of *S. sclerotiorum* (A, E) and *B. cinerea* (I, M) cells in the untreated groups were relatively smooth and regular, whereas when treated with compounds **13** (B, F, J, N), **25** (C, G, K, O), and **Boscalid** (D, H, L, P) at 5 mg/L there were significant shrinkage.

Effects of Compounds 13 and 25 on the Respiratory Metabolism of *B. cinerea*. The respiratory inhibition effects of compounds **13** and **25**, and boscalid on *B.cinerea* mycelia are shown in Table 5. Compounds **13** and **25** have inhibitory effects on the respiration rate of *B. cinerea* mycelia, and their inhibition rates were 32.16 and 19.06%, respectively. The inhibition rate of compound **13** is close to that of boscalid (33.02%). We speculate that the antifungal mechanism of compound **13** may be similar to that of boscalid, which involves the inhibition of fungal respiration.

Table 5. Respiratory Inhibition Rate of Compounds 13 and 25 and Boscalid on *B. cinerea* Mycelia

compd	R_0 (O_2, μmol/g min)	R_1 (O_2, μmol/g min)	IR (%)
13	3.83 ± 0.02	2.63 ± 0.03	32.16 ± 0.58
25	3.67 ± 0.03	2.87 ± 0.09	19.06 ± 1.43
boscalid	3.73 ± 0.03	2.53 ± 0.02	33.02 ± 0.59

In summary, series of 3-(iso)quinolinyl-4-chromenone derivatives were designed and synthesized by the active substructure splicing principle and evaluated for their antifungal activities. The lead optimization was guided by bioactivity. The antifungal bioassay discovered the highly active compounds **13** and **25** with a broad spectrum of excellent *in vitro* fungicidal activities and better *in vivo* efficacy against *B. cinerea*. Physiological and biochemical studies showed that the primary action of mechanism of compounds **13** and **25** on *S. sclerotiorum* and *B. cinerea* may involve changing mycelial morphology and increasing cell membrane permeability. In addition, compound **13** may also affect the respiratory metabolism of *B. cinerea*. This study reveals that compounds **13** and **25** could be promising antifungal candidates and provide a valuable reference for further development of 3-(iso)quinolinyl-4-chromenones in crop protection.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c06700.

The physical and spectroscopic data of **1a−h**, **3−33** (PDF)

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Notes

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