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Discovery of Novel Pyrazole Carboxylate Derivatives Containing Thiazole as Potential Fungicides

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ABSTRACT: Inspired by commercially established fluxapyroxad as the lead compound of novel efficient antifungal ingredients, novel pyrazole carboxylate derivatives containing a flexible thiazole backbone were successfully designed, synthesized, and detected for their *in vitro* and *in vivo* biological activities against eight agricultural fungi. The antifungal bioassay results showed that compound **24** revealed excellent bioactivities against *Botrytis cinerea* and *Sclerotinia sclerotiorum*, with median effective concentrations (EC_{50}) of 0.40 and 3.54 mg/L, respectively. Compound **15** revealed remarkable antifungal activity against *Valsa mali*, with an EC_{50} value of 0.32 mg/L. For *in vivo* fungicide control against *B. cinerea* and *V. mali*, compounds **3** and **24** at 25 mg/L, respectively, displayed prominent efficacy on cherry tomatoes and apple branches. Molecular docking results demonstrated that compound **15** could form an interaction with several crucial residues of succinate dehydrogenase (SDH), and the *in vitro* enzyme assay indicated that the target compound **15** displayed an inhibitory effect toward SDH, with an IC_{50} value of 82.26 μM. The experimental results indicated that phenyl pyrazole carboxylate derivatives displayed a weak antifungal property and low activity compared to the other title substituent pyrazole carboxylate derivatives. Compounds **3**, **15**, and **24** are promising antifungal candidates worthy of further fungicide development due to their prominent effectiveness.

KEYWORDS: *pyrazole, thiazole, antifungal, Botrytis cinerea, Valsa mali, molecular docking*

INTRODUCTION

Pathogenic fungus has been regarded as a worldwide threat for plant growth and food security. *Botrytis cinerea* is a commercially necrotrophic pathogenic fungus, affecting devastating diseases and significant crop losses in a wide variety of plant species.[1,2] The complex interactions between this necrotrophic pathogenic fungus, the plants it infects, and significant crop losses caused by *B. cinerea* on over 1400 species of cultivated plants attract particular attention from the main global agrochemical researchers and companies.[3]

Pyrazole carboxamide derivatives showed a broad spectrum of antifungal activities as the succinate dehydrogenase inhibitor (SDHI),[4−7] and some of them reached the market in the past decade, such as penthiopyrad, penflufen, sedaxane, bixafen, fluxapyroxad, and pydiflumetofen (Scheme 1A).[8,9] Despite the remarkable effectiveness of commercial SDHI fungicides to agricultural production and food security in the past decade, invasive fungal diseases continue to cause global agricultural production reduction because these commercial SDHI fungicides possess similar structural skeletons, which may easily lead to drug resistance with continuous drug therapy.[10−12] Novel pharmacophores should be urgently explored to avoid SDH-based fungi cross-resistance.

It is well known that most SDHI fungicides consist of a common amide function, a structurally diverse "core" moiety attached to the carbonyl of the amide bond and a substituted amine moiety.[13−15] Fluxapyroxad as a classical SDHI fungicide is a commercially available agricultural pesticide against various necrotrophic pathogenic fungi for crop protection.[16,17] Encouraged by its remarkable antifungal activity, fluxapyroxad was selected as the lead compound and the substituted pyrazole "core" as the original pharmacophore fragment was reserved.[18] Inspired by the classical design strategy of bioisosterism,[19] the common amide bond was substituted by the ester linkage between the pyrazole fragment and pharmacophore moiety. Notably, pyrazole carboxylate derivatives are much less frequently found than pyrazole carboxamide derivatives in pesticide development, which may indicate that pyrazole carboxylate derivatives in agrochemicals have still not been explored to their full scope in crop protection lead discovery and optimization. Keeping in mind the tremendous contributions of thiazole as the privileged scaffold in medicinal and agrochemical exploration, as shown in Scheme 1B, the thiazole backbone was widely employed in antifungal,[20] antibacterial,[21] anticancer,[22] insecticidal,[23] and anti-inflammatory agents.[24] Thiazole derivatives with such broad spectrum biological activities have sparked enormous interest in the design and synthesis of compounds over the past few years.[25] The aromatic structure enables thiazole to be modified into compounds with various biological activities. The compounds obtained by substituting thiazole on their

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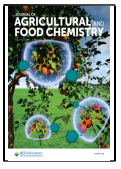

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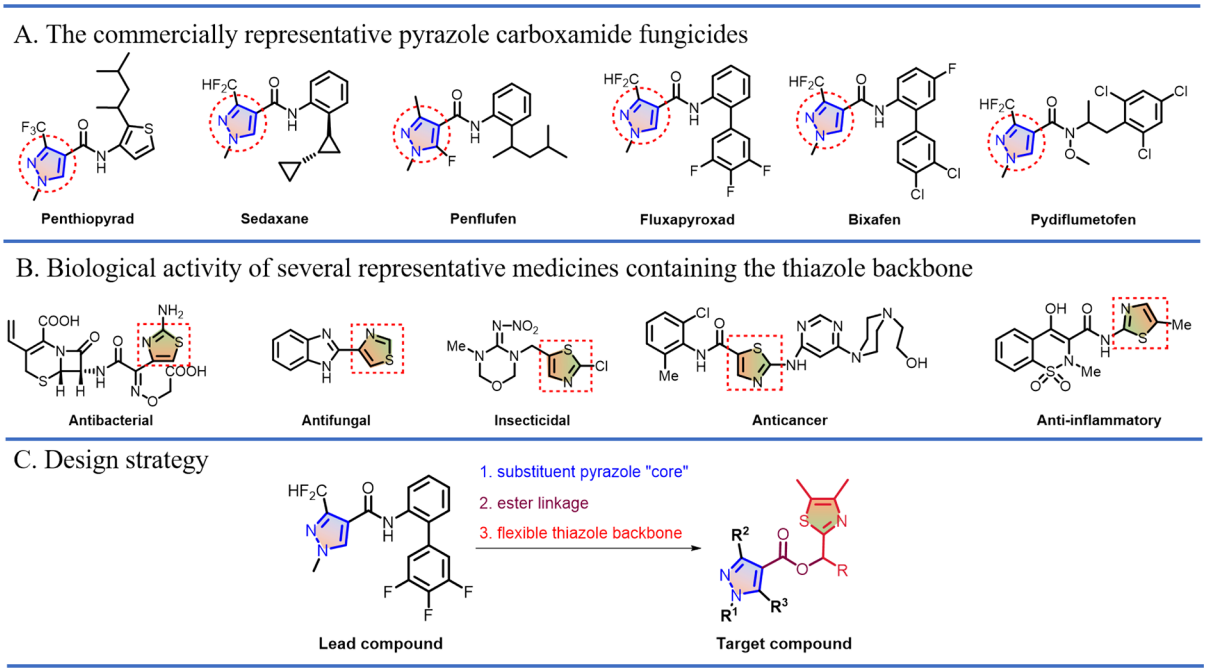
Scheme 1. (A−C) Design Strategy of the Target Compounds



rings have great worth in the pharmaceutical and agrochemical industries. Therefore, we envisaged that the rigid biphenyl group was modified by attachment of flexible substituents containing the thiazole backbone, which may improve the bioactivity of the designed compounds. The benzene ring is significant in drug design, and different substituted phenyl groups improve a variety of the target molecules. Additionally, naphthyl or isopropyl also was introduced to the target compounds. Three series of novel pyrazole carboxylate derivatives containing thiazole were designed, synthesized, and detected for their *in vitro* and *in vivo* biological activities against eight agricultural fungi.

MATERIALS AND METHODS

Chemicals and Instruments. Unless otherwise stated, all the solvents and reagents were purchased from Energy, Meryer, and Aladdin Chemicals and were analytically or chemically pure. Using tetramethylsilane as the internal standard, ^{1}H NMR and ^{13}C NMR spectra were carried out by using an Agilent DD2 600 Hz spectrometer with $CDCl_3$ as the solvent. Electrospray ionization−mass spectroscopy (ESI-MS) spectra were carried out on a Mariner System 5304 mass spectrometer.

Chemical Synthesis. The synthetic route of the target compounds **1**−**12**, **13**−**24**, and **25**−**36** is shown in Scheme 2.

General Procedures for Preparing Intermediates ***a***−***l***.[26,27] 4,5-Dimethylthiazole (10 mmol) was added in a flame-dried round-bottom flask under nitrogen conditions, then tetrahydrofuran (THF, 45 mL) was added, and the solution was cooled to −78 °C. Whereafter, *n*-butyl lithium (1.9 M, 12 mmol) was added dropwise to the cold reaction mixture. The resulting dark red solution was stirred for 45 min at −78 °C. The appropriate aldehyde (30 mmol) was then added, causing the solution to turn yellow, and the reaction was stirred for 1 h at −78 °C. The reaction was warmed to room temperature and stirred for 5 h. After completion of the reaction step, water was added to quench the reaction. The reaction mixture was diluted with ethyl acetate and washed with saturated salt water. The aqueous layer was extracted two additional times with ethyl acetate. The combined organic extracts were dried over sodium sulfate, filtered, and concentrated *in vacuo*. The resulting residue was purified by recrystallization (ethyl acetate/hexanes).

General Procedures for Preparing the Target Compounds ***1***−***12***. To a flame-dried round-bottom flask were successively added intermediates **a**−**l** (1 mmol),1-methyl-3-(difluoromethyl)-1*H*-pyrazole-4-carboxylic acid (1.5 mmol),1-ethyl-3(3-dimethylpropylamine)-carbodiimide (1.5 mmol),4-dimethylaminopyridine (1.5 mmol),and moderate triethylamine followed by dichloromethane (10 mL). The resulting solution was then stirred overnight at ambient temperature. The reaction mixture was diluted with dichloromethane and successively washed three times with diluted hydrochloric acid and sodium bicarbonate solution. The combined aqueous layers were washed with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude products were recrystallized from ethanol or purified by column chromatography (ethyl acetate/hexanes) to obtain the pure target compounds **1**−**12**.

General Procedures for Preparing the Target Compounds ***13***−***24***. To a flame-dried round bottom flask were added intermediates **a**−**l** (1 mmol), 1-methyl-3-(trifluoromethyl)-1*H*-pyrazole-4-carboxylic acid (1.5 mmol), 1-ethyl-3(3-dimethylpropylamine)carbodiimide (1.5 mmol), 4-dimethylaminopyridine (1.5 mmol), and moderate triethylamine followed by dichloromethane (10 mL). The reaction mixture was diluted with dichloromethane and successively washed three times with diluted hydrochloric acid and sodium bicarbonate solution. The combined aqueous layers were washed with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude products were recrystallized from ethanol or purified by column chromatography (ethyl acetate/hexanes) to obtain the pure target compounds **13**−**24**.

General Procedures for Preparing Intermediate ***q***. Compound **o** is commercially available. Using compound **2** as the starting material, the reaction mixtures were added to a solution of DMF and $POCl_3$ and cooled to 0 °C with an ice bath to obtain intermediate **p**. To a 100 mL round-bottom flask, intermediate **p** and $KMnO_4$ dissolved in H_2O were mixed and reacted for 4 h at room temperature. A solution of 75% aqueous HCl was added slowly and dropwise with an addition funnel under vigorous stirring. After completion of 75% aqueous HCl, the white solids precipitated out from the reaction mixtures. The organic extracts were filtered from the reaction mixtures, dried to obtain intermediate **q**, and used for the next step without further purification.

General Procedures for Preparing the Target Compounds ***25***−***36***. To a flame-dried round bottom flask were added intermediates **a**−

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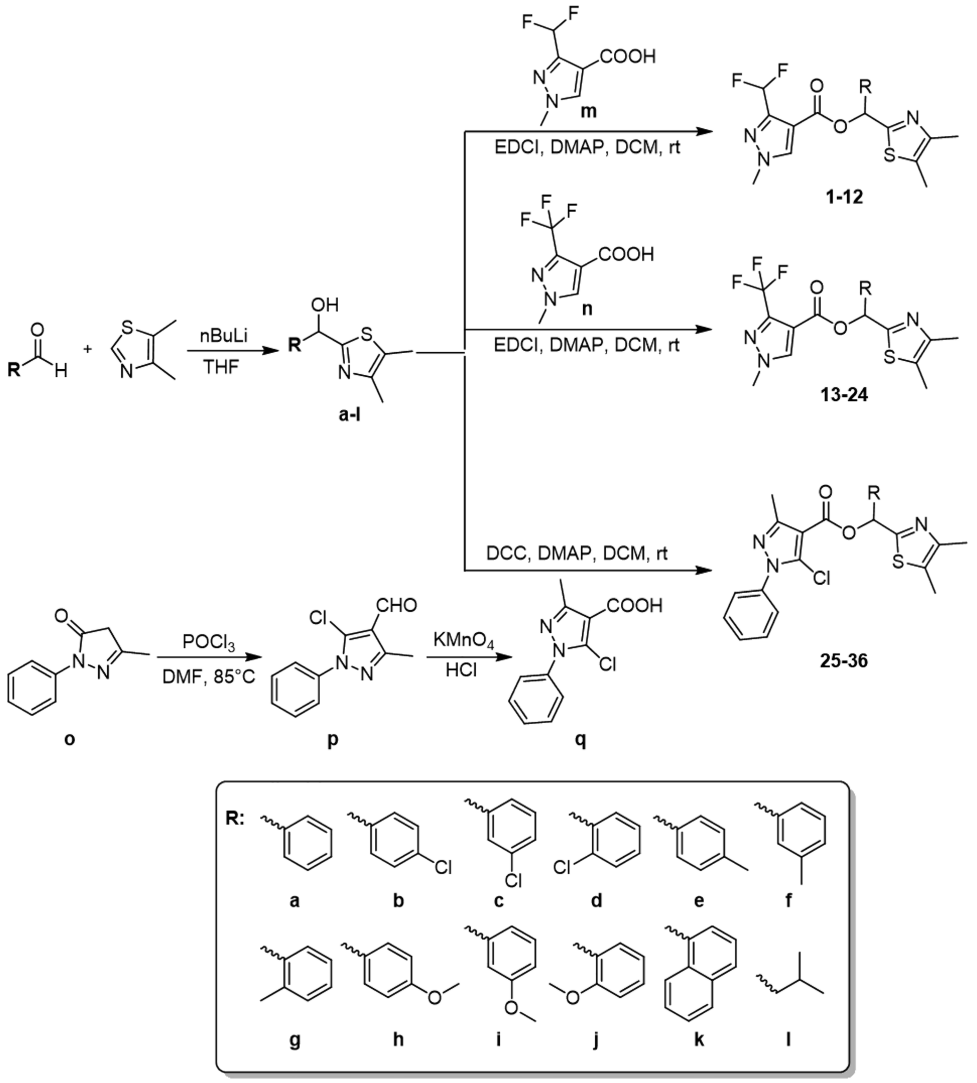
Scheme 2. Synthetic Route of the Target Compounds



l (1 mmol), intermediate **q** (1.5 mmol), 1,3-dicyclohexylcarbodiimide (2 mmol), and 4-dimethylaminopyridine (0.1 mmol) followed by dichloromethane (10 mL). The resulting solution was then stirred overnight at ambient temperature. After filtration of the reaction mixture, the filtrate was diluted with dichloromethane and washed three times with saturated salt solution. The combined aqueous layers were washed with dichloromethane. The combined organic layers were dried over Na_2SO_4, filtered, and concentrated *in vacuo*. The crude products were recrystallized from ethanol or purified by column chromatography (ethyl acetate/hexanes) to obtain the pure target compounds **25**−**36**.

***In Vitro* Antifungal Activity Test.** The selected plant pathogenic fungi, *B. cinerea*, *Fusarium verticillioide*, *Fusarium oxysporum*, *Valsa mali*, *Sclerotinia sclerotiorum*, *Alternaria alternata*, *Fusarium graminearum*, and *Physalospora piricola*, were supplied by the Laboratory of Plant Disease Control, Anhui Agricultural University (Hefei, China). *In vitro* antifungal activities of novel pyrazole carboxylate derivatives containing thiazole against *B. cinerea*, *F. verticillioide*, *F. oxysporum*, *V. mali*, *S. sclerotiorum*, *A. alternata*, *F. graminearum*, and *P. piricola* were detected at 10 mg/L according to the reported mycelial growth inhibition methods by using the commercial fungicides boscalid and carbendazim as positive controls.[28,29] Each compound was dissolved in DMSO to prepare the 10,000 mg/L stock solution. Percentage inhibition was calculated by the following equation: percentage inhibition (%) = (1 − PT/CK) × 100, where PT is the mean colony diameter with compounds and CK is the mean colony diameter without tested compounds. For the potent compounds with an average inhibitory rate >70% at 10 mg/L, their median effective concentrations (EC_{50}) against *B. cinerea*, *V. mali*, and *S. sclerotiorum* were further measured at concentrations of 16, 8, 4, 2, 1, and 0.5 mg/L. All experiments were replicated three times, and the statistical analyses of the antifungal bioassay were performed using SPSS software version 26.0.

In Planta Fungicidal Activities. *For the Protective Activity against B. cinerea In Vivo.* Based on the preceding test of *in vitro* antifungal activity, the potent compounds **3**, **24**, and **27** were further carried out *in vivo* on cherry tomatoes, according to reported common methods.[30,31] The selected compounds and positive controls boscalid and carbendazim in 0.1 mL of DMSO were dissolved in 10 mL of deionized water at 25 mg/L. Boscalid and carbendazim were used as positive controls because they are specific commercial fungicides for controlling *B. cinerea*. Each sample measured in triplicate was sprayed evenly onto the cherry tomatoes, which had been already washed and treated with water and 75% aqueous ethyl alcohol. After 24 h, pathogen was inoculated on cherry tomatoes. DMSO (1%) in 10 mL of water was set up as the blank control. All the treated samples were then placed into an illumination incubator in 25 °C and 100% relative humidity for 7 days. Each treatment was performed three times as technical replicates.

For the Protective Activity against V. mali In Vivo. Based on the preceding test of *in vitro* antifungal activity, compounds **3**, **15**, and **24** were further measured *in vivo* on apple branches, according to a

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Table 1. *In Vitro* Fungicidal Activities (Inhibition Rate) of the Target Compounds at 10 mg/L[j]

Compd.	Inhibitory Rate[a] at 10 mg/L (%)							
	B. c[b]	*F. v*[c]	*F. o*[d]	*V. m*[e]	*S. s*[f]	*A. a*[g]	*F. g*[h]	*P. p*[i]
1	47.3±1.2	-	-	-	24.4±1.5	20.3±1.6	-	-
2	63.2±0.7	-	-	-	56.3±1.3	40.4±0.8	-	-
3	85.3±0.9	64.1±0.9	48.4±1.3	80.2±1.9	60.1±0.8	60.2±1.3	-	48.2±1.7
4	80.2±1.3	68.3±1.2	44.1±0.6	72.3±1.1	64.2±1.2	60.3±1.1	-	40.3±1.1
5	21.3±1.1	-	-	-	28.1±0.9	-	-	-
6	26.1±0.6	-	-	-	27.4±1.1	-	-	-
7	20.1±1.1	-	-	-	35.2±1.7	-	-	-
8	63.2±0.7	-	-	-	36.3±1.3	-	-	-
9	74.2±1.3	-	-	-	49.1±0.6	-	-	-
10	21.4±1.7	-	-	-	24.2±1.9	-	-	-
11	84.2±1.3	60.1±1.5	36.2±0.9	48.1±1.3	56.1±1.2	56.2±1.6	-	40.3±1.4
12	32.2±0.9	-	-	-	28.3±1.4	28.1±0.7	-	-
13	32.3±1.6	20.1±1.3	-	-	-	-	-	-
14	74.3±1.2	68.4±0.8	48.2±1.1	72.4±0.8	64.2±0.7	56.3±1.9	-	-
15	72.4±1.4	26.2±1.1	35.4±1.1	95.1±1.1	62.4±0.9	58.4±0.6	28.2±0.8	60.2±1.3
16	12.1±0.8	12.3±1.2	-	-	-	-	20.1±1.4	40.3±0.6
17	17.4±1.1	-	14.1±1.2	64.4±1.8	30.4±1.3	33.3±1.4	-	50.2±1.1
18	75.1±0.8	31.2±1.7	40.3±1.8	52.2±1.2	67.2±1.5	37.3±1.8	38.3±0.8	50.4±1.7
19	72.3±1.4	31.3±0.9	33.2±1.3	34.2±1.3	65.1±1.1	22.1±1.3	38.3±1.9	50.4±0.9
20	-	-	12.2±0.7	50.1±1.8	-	18.2±0.6	-	60.2±1.3
21	72.3±1.8	33.1±0.8	37.2±0.8	60.4±0.8	64.1±1.0	56.4±1.1	42.2±0.7	56.1±1.1
22	25.2±1.4	-	11.4±1.8	-	50.3±1.1	11.2±0.7	34.1±1.1	30.1±1.5
23	50.3±0.9	17.2±1.7	16.1±1.3	-	40.2±1.2	33.1±0.8	16.2±1.7	40.2±0.7
24	80.3±0.6	48.1±1.3	60.2±1.1	92.2±1.7	70.2±1.8	55.2±1.8	45.2±1.4	54.3±0.8
25	30.4±1.1	34.1±1.3	-	12.3±1.3	15.4±0.8	-	-	-
26	27.1±1.7	-	-	-	26.1±0.9	-	-	-
27	73.1±0.8	50.1±0.7	-	26.1±0.7	65.2±1.7	12.2±1.4	-	31.4±1.1
28	32.1±1.9	13.2±1.6	-	-	-	-	-	-
29	18.3±1.4	-	-	-	-	-	-	-
30	40.1±1.5	-	-	-	-	-	-	-
31	34.2±1.3	-	-	-	-	-	-	-
32	-	21.4±0.7	-	-	-	-	-	-
33	23.2±0.8	-	-	-	-	-	-	-
34	24.2±0.6	-	-	-	-	-	-	-
35	15.4±1.7	35.2±1.1	-	-	25.2±1.5	-	-	11.2±1.4
36	14.1±1.4	-	-	-	-	-	-	-
Boscalid	80.2±1.1	24.3±1.6	35.4±1.2	40.3±1.1	100	84.3±0.7	88.2±1.3	70.4±0.8
Carbendazim	84.9±1.5	89.1±2.3	100	77.7±1.4	100	94.3±2.0	100	91.4±1.6

Inhibitory Effect: low → high

[a]Values are the mean ± standard deviation (SD) of three replicates. [b]*B. c*: *B. cinerea*. [c]*F. v*: *F. verticillioide*. [d]*F. o*: *F. oxysporum*. [e]*V. m*: *V. mali*. [f]*S. s*: *S. sclerotiorum*. [g]*A. a*: *A. alternata*. [h]*F. g*: *F. graminearum*. [i]*P. p*: *P. piricola*. [j]-: No activity.

reported common approach.[29] Fresh and clean apple branches were prepared by artificial removal of the epidermis after disinfection. The target compound (25 mg/L) was then applied to the wound, and after drying, *V. mali* was inoculated at the wound and cultured for 7 days in an incubator at 25 °C under moisturizing conditions (100% humidity) before evaluation of the symptoms. Boscalid and carbendazim were employed as the positive control. Each treatment was performed three times as technical replicates.

Scanning Electron Microscopy (SEM) Observations. SEM observations on the hyphae of *V. mali* were carried out according to the reported methods.[32,33] Mycelial disks were cut from the periphery of the colony grown on potato dextrose agar (PDA) containing EC_{50} (0.32 mg/L) of compound **15** and 0.1% DMSO (blank control). The samples were fixed in a 4 °C stationary liquid (5% glutaraldehyde, 0.1 M phosphate buffer) at room temperature for 12 h, were washed with 0.1 M phosphate buffer for 20 min, and were dehydrated successively with 30, 50, 70, 80, 95, and 100% ethanol and 100% acetone at 4 °C temperature. After drying at critical points by CO_2 and gold coating, the mycelial cell wall morphology was carried out under SEM (HITACHI, S-4800, Japan) at an accelerating voltage of 3.0 kV.

***In Vitro* Enzyme Inhibition Assay.** The *in vitro* inhibitory effects of title compounds **3**, **15**, and **24** and boscalid against fungal succinate dehydrogenase (SDH) were determined by a succinate dehydrogenase assay kit that was purchased from the Nanjing Jiancheng Bioengineering Institute. After being incubated in PDA for 72 h, the mycelia of *B. cinerea* and *V. mali* were collected for preparing the tested mitochondrial suspension. The tested compounds were added into working solution according to the operating instruction of the purchased SDH assay kit. After adding mitochondrial suspensions containing fungal SDH into the mentioned-above mixture solution, the absorbance values of the obtained mixtures were monitored at 600 nm to calculate median inhibitory concentration (IC_{50}) against fungal SDH.[34]

Molecular Docking. Molecular docking of compound **15** and boscalid to SDH was performed with the software Discovery Studio 3.5. The crystal structure of SDH (PDB entry: 2fbw) was downloaded from the RCSB Protein Data Bank. After preparing protein to get protein that can be used for docking, defining the active site of the receptor protein from current selection. Compound **15** and boscalid were imported into Discovery Studio for ligand preparation. The molecular docking procedures were performed by using the CDOCKER protocol for the receptor−ligand interaction section of Discovery Studio 3.5.

RESULTS AND DISCUSSION

Chemistry. To develop novel fungicides by integrating the substituent pyrazole "core" and the active scaffold of thiazole into one molecule, three series of novel pyrazole carboxylate compounds were successfully designed, synthesized, and well characterized. General procedures for preparing the target compounds **1**−**36** are depicted in Scheme 2. According to the reported procedure,[26] using different substituted benzaldehyde and 4,5-dimethylthiazole as the starting materials, intermediates **a**−**l** were prepared in a yield of 75−90% through reduction reaction. Target compounds **1**−**36** were obtained through the classical esterification reactions of the corresponding intermediates **a**−**l**. The chemical structures of all the synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and MS. It should be noted in this context that, to the best of our knowledge, all of the target compounds are first reported.

***In Vitro* Antifungal Activities.** *In vitro* antifungal activities of novel pyrazole carboxylate derivatives containing thiazole against *B. cinerea*, *F. verticillioide*, *F. oxysporum*, *V. mali*, *S. sclerotiorum*, *A. alternata*, *F. graminearum*, and *P. piricola* were detected at 10 mg/L according to a reported mycelial growth inhibition method by using boscalid and carbendazim as

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positive controls. As listed in Table 1, the results indicated that majority of the newly synthesized compounds displayed remarkable inhibitory effects toward *B. cinerea* and *V. mali*, and even several of these were superior to the commonly used fungicides boscalid and carbendazim. Among all the synthesized compounds, compounds **3**, **4**, **14**, **15**, **21**, and **24** showed remarkable fungicide activity against more than half of the tested fungi, with an inhibitory rate of over 50% at 10 mg/L.

For the outstanding compounds with an average inhibition >70% at 10 mg/L, their median effective concentrations (EC_{50}) against *B. cinerea*, *V. mali*, and *S. sclerotiorum* were measured according to the reference standard by using boscalid and carbendazim as positive controls. As listed in Table 2, compounds **3**, **24**, and **27** revealed excellent antifungal effects toward *B. cinerea*, with EC_{50} values of 0.77, 0.40, and 0.87 mg/L, respectively. Compounds **3**, **15**, and **24** displayed prominent antifungal effects toward *V. mali*, with EC_{50} values of 0.67, 0.32, and 0.57 mg/L, respectively. To evaluate inhibitory effects of the outstanding compounds mentioned above, these compounds were further tested for their *in vivo* antifungal activities.

Table 2. *In Vitro* Antifungal EC_{50} Value against *B. cinerea*, *V. mali*, and *S. sclerotiorum* of the Target Compounds

fungi	compd	EC_{50} (mg/L)	95% confidence interval	regression equation	r
B. c	**3**	0.77	0.40−1.13	$y = 1.805x + 0.209$	0.991
	4	1.75	1.19−2.30	$y = 1.739x - 0.421$	0.985
	9	2.25	1.52−3.01	$y = 1.488x - 0.523$	0.998
	11	1.18	0.75−1.59	$y = 1.896x - 0.132$	0.985
	14	3.50	2.79−4.35	$y = 2.061x - 1.122$	0.989
	15	4.38	3.53−5.48	$y = 2.058x - 1.320$	0.990
	18	2.15	1.42−2.92	$y = 1.445x - 0.481$	0.998
	19	2.17	1.44−2.93	$y = 1.461x - 0.490$	0.985
	21	3.17	2.38−4.09	$y = 1.690x - 0.846$	0.993
	24	0.40	0.12−0.74	$y = 1.542x + 0.608$	0.977
	27	0.87	0.30−1.45	$y = 1.139x + 0.072$	0.985
	boscalid	0.79	0.32−1.28	$y = 1.327x + 0.134$	0.971
	carbendazim	0.91	0.29−1.51	$y = 1.658x + 0.068$	0.972
V. m	**3**	0.67	0.31−1.05	$y = 1.648x + 0.285$	0.976
	4	2.86	2.20−3.59	$y = 1.913x - 0.872$	0.991
	14	3.83	3.07−4.77	$y = 2.055x - 1.200$	0.977
	15	0.32	0.02−0.78	$y = 0.928x + 0.461$	0.960
	24	0.57	0.22−0.93	$y = 1.578x + 0.389$	0.980
	boscalid	13.91	10.13−22.63	$y = 1.795x - 2.052$	0.964
	carbendazim	4.22	3.55−5.14	$y = 1.674x - 1.047$	0.980
S. s	**24**	3.54	2.90−4.30	$y = 2.343x - 1.286$	0.977
	boscalid	0.96	0.68−1.25	$y = 1.789x + 0.029$	0.994
	carbendazim	0.50	0.02−1.01	$y = 2.253x + 0.668$	0.995

In Planta Fungicidal Activities. Based on the preceding test results of *in vitro* antifungal activity, compounds **3**, **24**, and **27** at 25 mg/L were further tested for *in vivo* protective activity against *B. cinerea* on cherry tomatoes. As shown in Figure 1A,

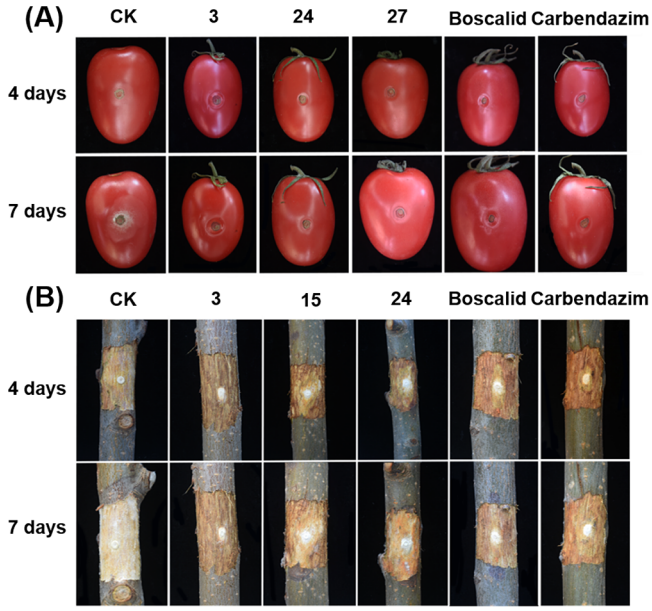

Figure 1. In planta fungicidal activities. (A) *In vivo* fungicidal activities against *B. cinerea* on cherry tomatoes. (B) *In vivo* fungicidal activities against *V. mali* on apple branches.

cherry tomatoes of control showed that the mycelia developed white filamentous sectors on the surface of cherry tomatoes without pesticide application. The tested compounds exhibited a significant biocontrol effect toward *B. cinerea* and were almost equal to positive controls, where merely the inoculated position was slightly sunken.

According to the preceding screening results, compounds **3**, **15**, and **24** at 25 mg/L were further tested for *in vivo* protective activity against *V. mali* on apple branches. As shown in Figure 1B, under the fungal infection conditions, the experimental results demonstrated that the mycelia developed white filamentous sectors over the whole wound on the apple branch for CK. Among all the tested compounds, compound **3** could significantly inhibit *V. mali* to expand from the inoculation site to the surroundings of the strains at 25 mg/L, even slightly superior to boscalid and carbendazim. In addition, the other tested compounds showed prominent inhibitory potency toward *V. mali* on apple branches. The results demonstrated that compound **3** at 25 mg/L was a highly effective antifungal candidate toward *V. mali* in planta.

Scanning Electron Microscopy (SEM) Analysis. The mycelia morphology changes of each treated *V. mali* were observed in the appropriate size by scanning electron microscopy (SEM). As shown in Figure 2, normal mycelial morphological characteristics of *V. mali* in the untreated groups (Figure 2A,B) were relatively smooth and regular, whereas *V. mali* mycelia treated with compound **15** (Figure 2C,D) at 0.32 mg/L significantly shrunk. These SEM

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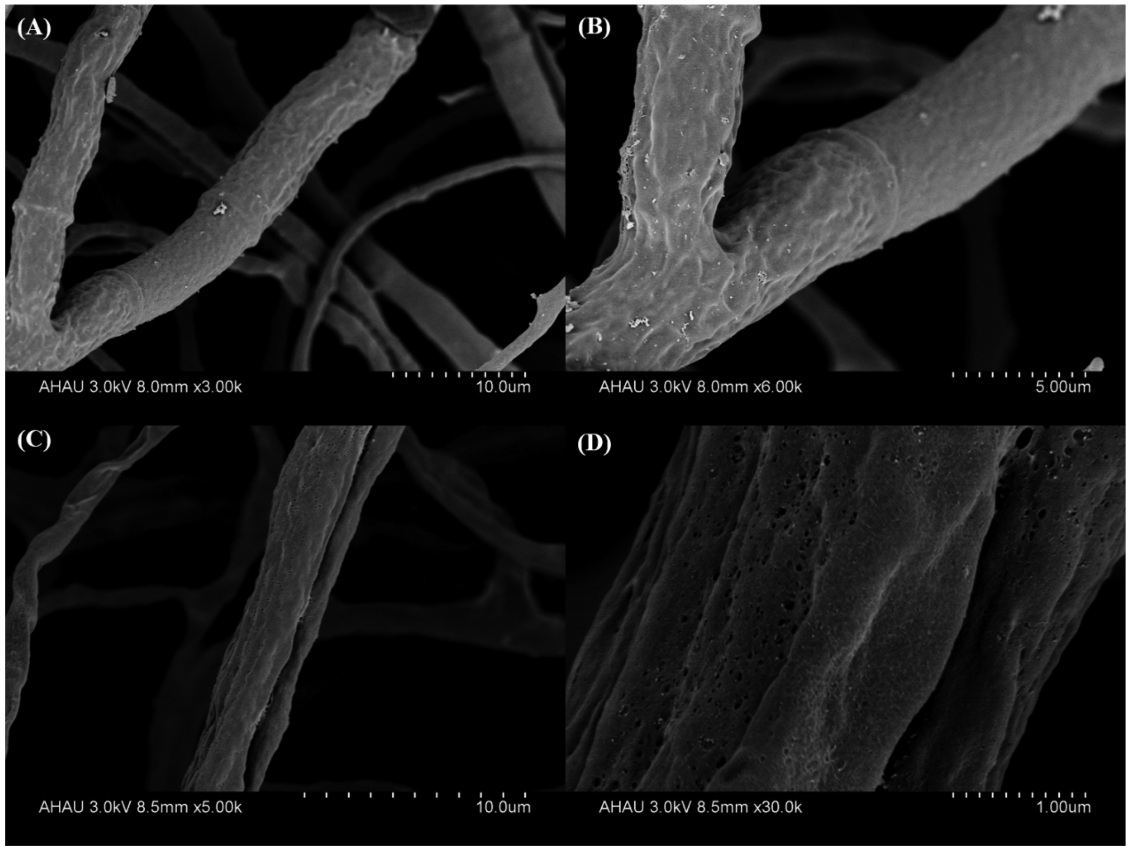

Figure 2. Scanning electron micrographs of the hyphae of *V. mali* grown on PDA with DMSO or compound **15** at 25 °C. (A, B) Untreated control; (C, D) after 72 h of compound **15** at 0.32 mg/L (EC_{50}) treatment.

observations revealed that compound **15** caused a certain degree of damage to the cell wall of *V. mali*.

***In Vitro* Enzyme Assay.** For antifungal activity of the target compounds *in vitro*, compounds **3**, **15**, and **24** exhibited the highest activities against *B. cinerea* and *V. mali*, respectively. To further investigate the antifungal mechanism of the title compounds, compounds **3**, **15**, and **24** were detected for *in vitro* enzyme inhibition experiments. As shown in Table 3, the

Table 3. IC_{50} Values of Fungal SDH Inhibition Activity

	IC_{50} (μM)	
compd	*B. c*	*V. m*
3	>100	>100
15	>100	82.26 ± 2.55
24	>100	>100
boscalid	18.56 ± 1.30	25.22 ± 1.51

experimental results indicated that compound **15** showed inhibitory activity against SDH enzymes, with an IC_{50} value of 82.26 μM, and was inferior to boscalid. In addition, compounds **3** and **24** showed no inhibitory effect toward SDH enzymes of *B. cinerea* and *V. mali*, and these compounds may show *in vitro* inhibitory activities against *B. cinerea* and *V. mali* by other antifungal mechanisms.

Molecular Docking Analysis. The molecular docking model of the representative compound **15** and boscalid with SDH is shown in Figure 3. The binding mode of the positive drug boscalid with SDH indicated that boscalid could form an interaction with several crucial residues including TYR58, ARG43, and TRP173 in the semi-open ellipsoid region on succinate dehydrogenase, and the pyridine fragment of boscalid occupies the cavity of the SDH enzyme inside. Compound **15** formed a hydrogen bond interaction with residues TYR58 and SER39, and the benzene ring of compound **15** formed a $\pi-\pi$ interaction with the residue ARG43.

In summary, this current accomplished study highlighted pyrazole carboxylate derivatives containing thiazole assisted by the lead compound fluxapyroxad for the discovery of promising agrochemicals. From the *in vitro* bioassay results, majority of novel pyrazole carboxylate derivatives containing thiazole displayed prominent efficacy against the tested phytopathogenic fungi and structure−activity relationships were investigated. As shown in Table 1, the experimental results indicated that the introduction of the phenyl group at the pyrazole ring displayed a weak antifungal property and low activity compared to the other title substituent pyrazole carboxylate derivatives. This work demonstrated that the design strategy of new fungicides was a rational approach by optimized substituent group at the R position of the thiazole backbone to improve efficacy and specificity; especially, the introduction of the *m*-chlorophenyl group at the R site remarkably improved effectiveness and selectivity for treatment of phytopathogenic fungi, exemplified by compounds **3**, **15**, and **27**. Among all the pyrazole carboxylate derivatives with the trifluoromethyl group, compound **24** demonstrated that the profitably conglomeration of the isobutyl group and thiazole backbone exhibited the most excellent inhibitory effect against *B. cinerea* and *V. mali*. Furthermore, the *in vitro* enzyme assay indicated that the target

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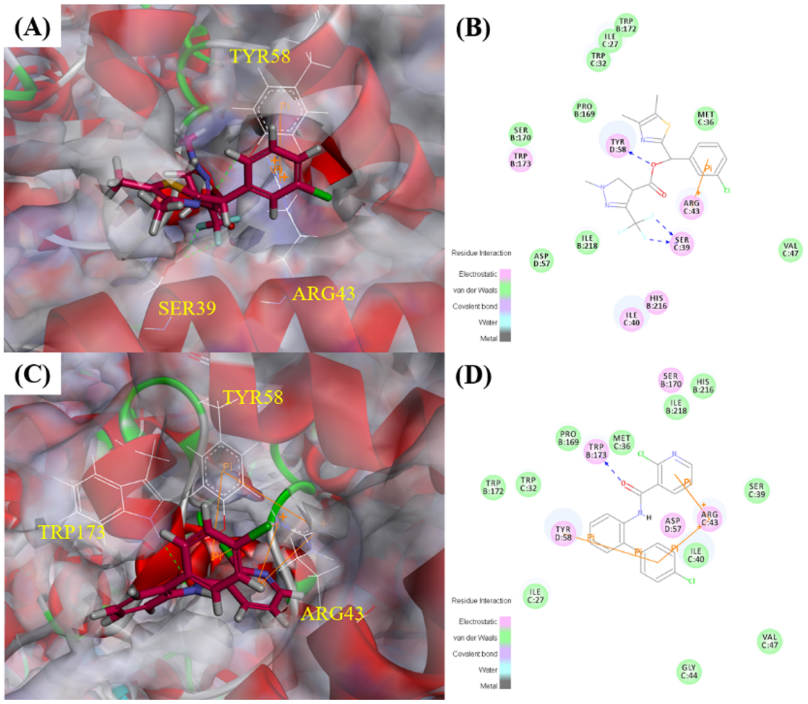

Figure 3. Binding modes of compound **15** (A, B) and boscalid (C, D) with SDH (3D and 2D diagram).

compound **15** displayed an inhibitory effect toward SDH, with an IC_{50} value of 82.26 μM, and molecular docking results demonstrated that compound **15** could form an interaction with several crucial residues of SDH. Compounds **3** and **24** showed no inhibitory activity against SDH despite their *in vitro* remarkable antifungal activity, which was worthy of further investigation to explore about their mechanism of inhibiting growth of phytopathogenic fungi. Considering prominent fungicidal effectiveness against phytopathogenic fungi, compounds **3**, **15**, and **24** are potential antifungal candidates worthy of further fungicide development.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.1c01189.

Chemical structures of all the synthesized compounds as confirmed by ^{1}H NMR, ^{13}C NMR, and MS, report of all of the target compounds, and more details of general procedures, yields, and physical and characterization data of all the synthesized compounds (PDF)

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Notes

The authors declare no competing financial interest.

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