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Synthesis and Biological Activity of Novel Pyrazol-5-yl-benzamide Derivatives as Potential Succinate Dehydrogenase Inhibitors

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ABSTRACT: To promote the discovery and development of new fungicides, a series of novel pyrazol-5-yl-benzamide derivatives were designed, synthesized by hopping and inversion of amide groups of pyrazole-4-carboxamides, and evaluated for their antifungal activities. The bioassay data revealed that compound **5IIc** exhibited an excellent in vitro activity against *Sclerotinia sclerotiorum* with an EC_{50} value of 0.20 mg/L, close to that of commercial fungicide **Fluxapyroxad** (EC_{50} = 0.12 mg/L) and **Boscalid** (EC_{50} = 0.11 mg/L). For *Valsa mali*, compound **5IIc** (EC_{50} = 3.68 mg/L) showed a significantly higher activity than **Fluxapyroxad** (EC_{50} = 12.67 mg/L) and **Boscalid** (EC_{50} = 14.83 mg/L). In addition, in vivo experiments proved that compound **5IIc** has an excellent protective fungicidal activity with an inhibitory rate of 97.1% against *S. sclerotiorum* at 50 mg/L, while the positive control **Fluxapyroxad** showed a 98.6% inhibitory effect. The molecular docking simulation revealed that compound **5IIc** interact with TRP173, SER39, and ARG43 of succinate dehydrogenase (SDH) through a hydrogen bond and p−π interaction, which could explain the probable mechanism of the action between compound **5IIc** and target protein. Also, the SDH enzymatic inhibition assay was carried out to further validate its mode of action. These results demonstrate that compound **5IIc** could be a promising fungicide candidate and provide a valuable reference for further investigation.

KEYWORDS: *succinate dehydrogenase inhibitors, pyrazolamide, antifungal activity, structure−activity relationships, molecular docking*

INTRODUCTION

Plant pathogenic fungi may cause crop yield losses,[1,2] which not only causes huge economic losses[3] but also threatens global food security.[4] Chemical fungicide protection is one of the most economical and effective measures to control fungal diseases at present.[5] Succinate dehydrogenase (SDH) inhibitors, which can inhibit the growth of pathogens through disrupting the mitochondrial tricarboxylic acid cycle and the respiration chain,[6,7] have been widely used for crop protection against plant diseases due to their broad-spectrum and excellent antifungal activities.[8,9] **Carboxin**, the first commercial SDH inhibitor, was invented by Uniroyal in 1969.[10] To date, there are 24 SDH inhibitors in the market, which are divided into 11 categories, including oxathilin carboxamides (**Carboxin**, 1969), furan carboxamides (**Fenfuram**, 1974), phenyl-benzamides (**Flutolanil**, 1986), thiazole-carboxamides (**Thifluzamide**, 1997), pyridinyl-ethyl benzamides (**Fluopyram**, 1997), pyridine carboxamides (**Boscalid**, 2003), *N*-methoxy-(penylethyl)-pyrazole-carboxamides (**Pydiflumetofen**, 2016), pyrazine carboxamides (**Pyrziflumid**, 2018), phenyl-oxo-ethyl-thiophene amides (**Isofetamid**, 2005), *N*-cyclopropyl-*N*-benzyl-pyrazole-carboxamides (**Isoflucypram**, 2020), and pyrazole-4-carboxamides (**Fluxapyroxad**, 2012)[11] (**Flubeneteram**, 2020).[12] Nevertheless, with the repeated use and misuse of SDH inhibitors, fungal resistance has become an increasingly serious problem. Also, due to a lack of effective alternative control options, plant pathogenic fungi are still challenging to control.[13] Therefore, it is an urgent and unmet need to develop novel SDH inhibitors with unique skeletons and improved fungicidal activity.[14]

Among the marketed SDH inhibitors, pyrazole-4-carboxamides occupies a great proportion.[15] For example, **Fluxapyroxad** is one of the widely used pyrazole-4-carboxamide fungicides with broad-spectrum antifungal activity,[16] high efficiency, and low toxicity.[17] As shown in Figure 1, all of these pyrazole-4-carboxamides SDH inhibitors structurally contain an amide function and a difluoromethyl or trifluoromethyl group as a pharmacophoric motif.

In a pesticide or a pharmaceutical drug development process, the design strategies by scaffold hopping[18,19] and bioisosterism[20] have been extensively used for lead compound optimization, which can provide compounds with novel skeletons, improved potency, reduced toxicity, enhanced physicochemical properties, more favorable absorption, metabolism, distribution, and excretion (ADME) profiles.[21−24] Other novel techniques, such as high-performance computation, structure biology, and artificial intelligence, have also been widely utilized.[25−27] For example, some high potent SDH inhibitors were discovered by Yang and Zhu using the pharmacophore-linked fragment virtual screening (PFVS) method.[7,28−30] In this work, to develop novel pyrazolamide SDH inhibitors, pyrazole-4-carboxamides were used as lead compounds, and we attempted to transfer the amide groups

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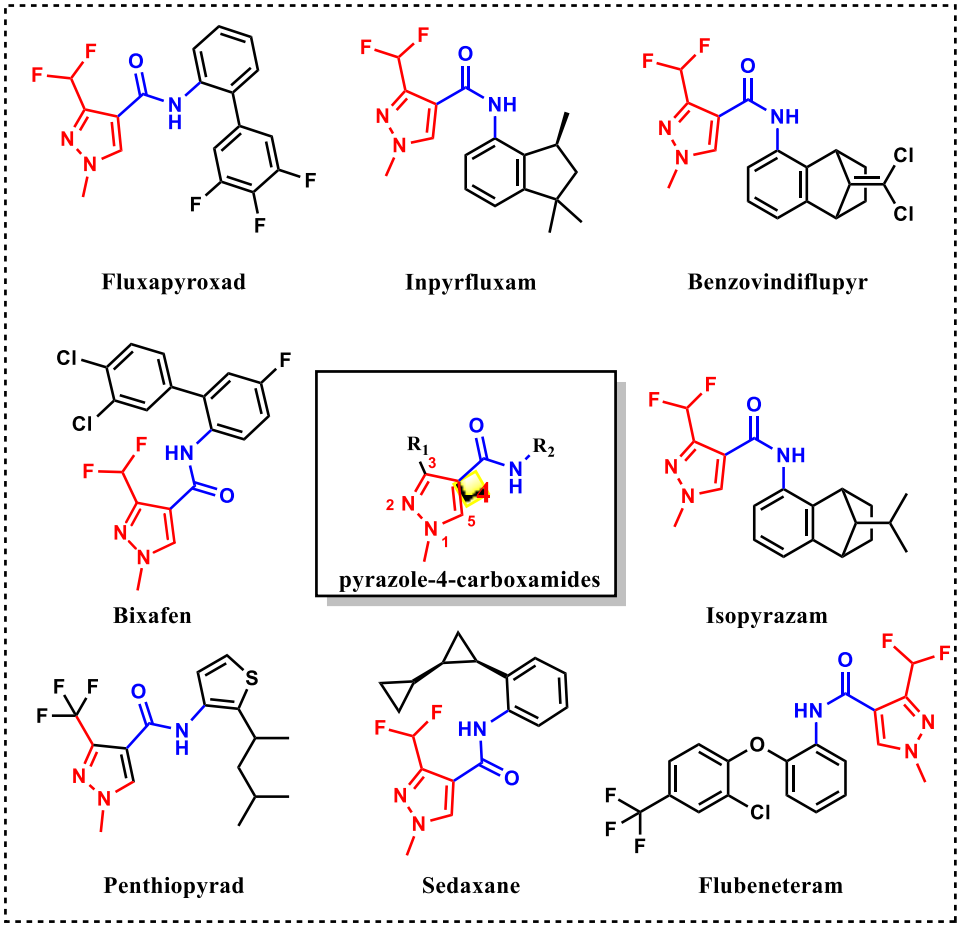

Figure 1. Representative chemical structures of pyrazole-containing SDHIs.

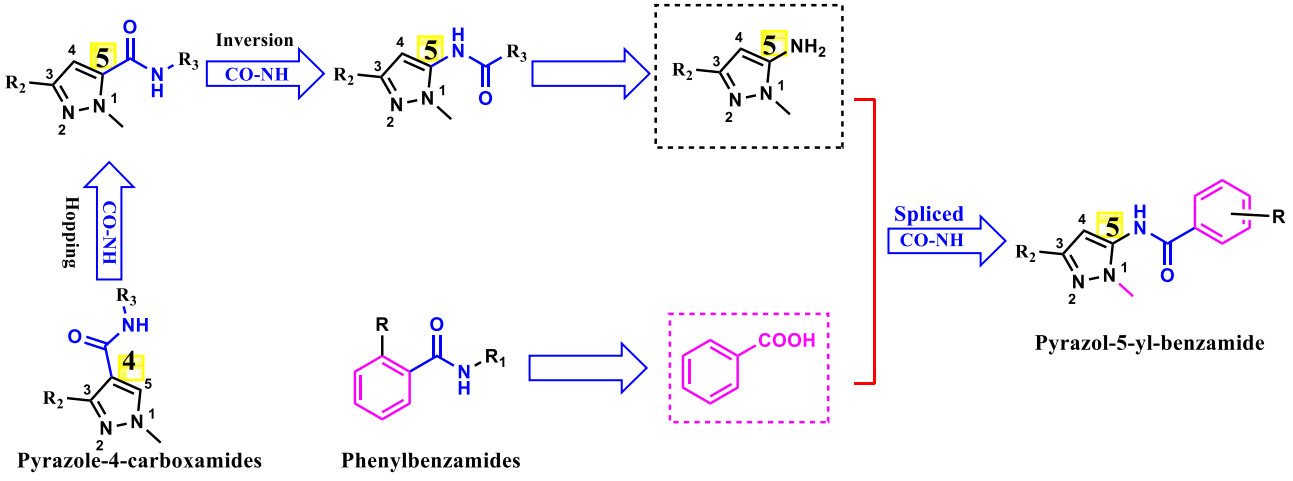

Figure 2. Design strategy for the target compounds.

from the 4th position to the 5th position of the pyrazole by scaffold hopping and inverse the amide groups based on the concept of bioisosterism. A series of novel *N*-pyrazol-5-yl-benzamide derivatives were designed and synthesized by a simple synthetic method (Figure 2). Structurally, pyrazol-5-amine and benzoic acid were spliced through an amide bond, and therefore the two types of SDHI phenylbenzamides and pyrazole-4-carboxamides were well combined. All of the target compounds were identified by ^{1}H NMR, ^{13}C NMR, and high-resolution mass spectrometry (HRMS). The in vitro fungicidal activities of these compounds were evaluated against five common plant pathogenic fungi, and in vivo fungicidal activities of compounds **5Ic** and **5IIc** with high antifungal activities were tested against *Sclerotinia sclerotiorum*. Furthermore, molecular docking and SDH enzymatic inhibition of compound **5IIc** were evaluated to validate its mode of action.

MATERIALS AND METHODS

Instruments and Chemicals. All chemicals (reagent grade) were purchased from commercial sources (Energy, Shanghai, China). All of

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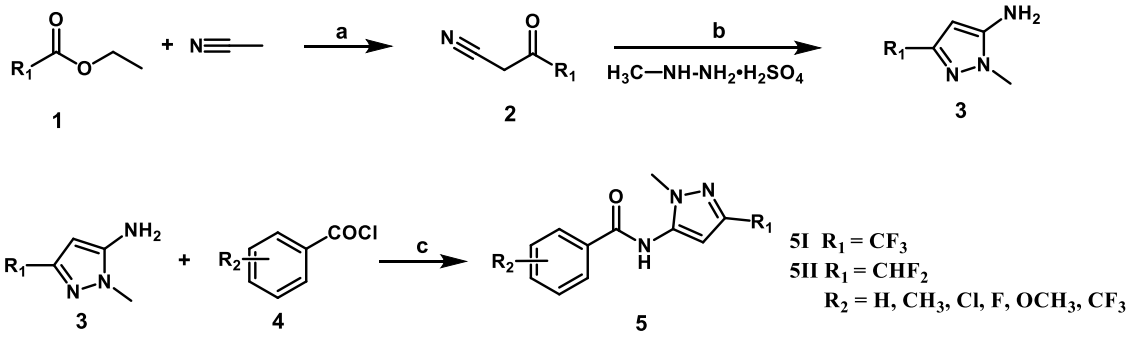

Figure 3. General synthesis of compounds **5**. Reagents and conditions: (a) NaH, THF, 80 °C; (b) EtOH, 80 °C; and (c) Et_3N, DCM, 0 °C.

the ^1H and ^{13}C NMR spectra were measured on a Bruker AV 300, 400, 500, or 600 spectrometer or an Agilent DD2 600 Hz spectrometer with $CDCl_3$ or DMSO-d_6 as the solvent and tetramethylsilane as the internal standard. Chemical shifts were reported in ppm (δ). Electrospray ionization mass spectrometry (ESI-MS) spectra were carried out on a Mariner System 5304 mass spectrometer. Melting points (mp) were recorded on a WRS-1B melting point apparatus (Jingsong, Shanghai, China) and were uncorrected. The crystal structure was recorded on a Bruker APEX-II CCD diffraction meter. The microscopic morphology of the fungal hyphae was observed using a scanning electron microscope (Hitachi, S-3400 N, Tokyo, Japan). Molecular docking studies were performed with Discovery Studio 4.0.

Fungi. The plant pathogenic fungi (*S. sclerotiorum*, *Physalospora piricola*, *Valsa mali*, *FusaHum graminearum* sehw., and *Botrytis cinerea pers*) were provided by the College of Plant Protection, Northwest A&F University (Yangling, China).

Synthetic Procedures. The synthetic routes of the target compounds **5Ia−5IIp** are outlined in Figure 3.[31,32]

General Synthesis Procedure for Intermediate 2. To a 50 mL round-bottomed flask, acetonitrile (10 mmol), substituted ethyl acetate **1** (10 mmol), NaH (10 mmol), and tetrahydrofuran (THF) (30 mL) were added. The reaction flask was put into a preheated oil bath (80 °C) and monitored by TLC (thin layer chromatography) until the reaction was completed. Then, the reaction was cooled to room temperature, hydrochloric acid (10 mL, 12 mol/L) was added, and the resulting mixture was extracted with ethyl acetate (3 × 20 mL). The organic layers were combined and dried over anhydrous Na_2SO_4. The solvent was removed under reduced pressure to afford the residue, which was purified by flash column chromatography to give the intermediate **2** in a 81−92% yield.

General Synthesis Procedure for Intermediate 3. Intermediate **2** (10 mmol) was added to a solution of methylhydrazine sulfate in ethanol (15 mL). The mixture was heated at 80 °C for 4 h. The reaction was cooled to room temperature and concentrated in vacuo. The crude product was purified by silica gel flash chromatography (petroleum ether/ethyl acetate = 5:1) to afford the intermediate **3**.

*1-Methyl-3-(trifluoromethyl)-1H-pyrazol-5-amine (Intermediate **3a**).* White solid; yield: 95%; mp: 96.3−97.0 °C; ^1H NMR (500 MHz, chloroform-*d*) δ 5.76 (s, 1H, Ar−H), 3.67 (s, 3H, N−CH_3), 3.58 (s, 2H, NH_2); ^{13}C NMR (126 MHz, chloroform-*d*) δ 145.5, 140.7 (q, J = 37.8 Hz), 121.3 (q, J = 268.3 Hz), 89.5, 34.8; ^{19}F NMR (376 MHz, dimethyl sulfoxide (DMSO)-d_6) δ −61.1; HRMS (ESI) m/z: [M + H]$^+$ calculated for $C_5H_7F_3N_3$: 166.0587, found 166.0588.

*3-(Difluoromethyl)-1-methyl-1H-pyrazol-5-amine (Intermediate **3b**).* Yellow solid; yield: 31.6%; mp: 29.9−32.6 °C; ^1H NMR (500 MHz, DMSO-d_6) δ 6.66 (t, J = 55.1 Hz, 1H, CHF_2), 5.47 (s, 1H, Ar−H), 5.39 (s, 2H, NH_2), 3.54 (s, 3H, CH_3); ^{13}C NMR (126 MHz, DMSO-d_6) δ 148.6, 144.2 (t, J = 28.1 Hz), 112.6 (t, J = 230.7 Hz), 85.3, 34.9; ^{19}F NMR (376 MHz, DMSO-d_6) δ −110.3; HRMS (ESI) m/z: [M + H]$^+$ calculated for $C_5H_8F_2N_3$: 148.0681, found 148.0681.

General Synthetic Procedure for the Target Compounds 5Ia−5IIq. Intermediate **3** (1 mmol) and compound **4** (1 mmol) were dissolved in dichloromethane (3 mL), and triethylamine (0.55 mL) was added into the solution at 0 °C. Then, the mixture was stirred at room temperature for 3 h. Water was added and the resulting mixture was extracted with dichloromethane (3 × 5 mL). The combined organic layers were washed with brine (3 × 5 mL), dried over anhydrous $MgSO_4$, and concentrated in vacuo. The crude product was purified by silica gel flash chromatography (petroleum ether/ethyl acetate = 3:1) to afford compounds **5Ia−5IIq**. The physical data of **5Ia−5IIq** in detail are provided in the Supporting Information.

Crystal Structure Determination. Crystal structure determination of compound **5Ic** was carried out on a Bruker APEX-II CCD equipped with graphite monochromated MoKa (l 1/4 0.71073 Å) radiation (Figure 4). The structure was solved by direct methods and

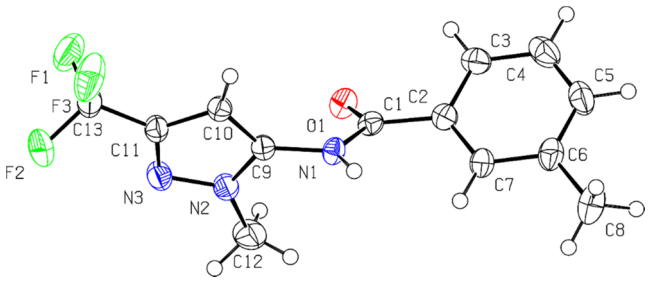

Figure 4. X-ray crystal structure of compound **5Ic** (CCDC number: 2005916, displacement ellipsoids are drawn at the 50% probability level).

refined on F^2 by full-matrix least-squares methods using SHELXTL. All nonhydrogen atoms were refined with anisotropic thermal parameters. All hydrogen atoms with the exception of those on nitrogen atoms were geometrically fixed and refined using a riding model. X-ray diffraction data and the refinement are shown in the Supporting Information.

Biological Assay. *In Vitro Fungicidal Activities.* All synthesized compounds were screened for their in vitro antifungal activities against *S. sclerotiorum* (*Lib.*) *de bary*, *P. piricola*, *V. mali*, *F. graminearum* sehw., and *Botrytis cinereal* pers at 50 mg/L for the preliminary screening according to a mycelial growth inhibition method.[33,34]

In Vivo Fungicidal Activities against S. sclerotiorum. The *Brassica napus L.* leaves of rape were collected from the Key Laboratory of Botanical Pesticide R&D of Northwest A&F University. For protective activity assay, healthy leaves of *B. napus L.* were sprayed with the target compounds (50 mg/L), respectively, and then cultivated at 25 °C for 24 h before inoculation with *S. sclerotiorum*. For curative activity assay, healthy leaves of *B. napus L.* were sprayed with the target compounds (50 mg/L), respectively, and then cultivated at 25 °C for 24 h after inoculation with *S. sclerotiorum*.

Effect of 5IIc on the Mycelial Morphology of S. sclerotiorum. *S. sclerotiorum* grew in Fries medium for 3 days. Fresh fungus dishes (5 mm in diameter) were made from the edge of the colonies. The mycelia were inoculated on PDA medium plates containing no compound (negative control) and **5IIc** with a concentration of 1 mg/L. Then, the mycelia were cultured at 25 °C for 2 days. The mycelial

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Table 1. In Vitro Fungicidal Activities (Inhibition Rate %) of All Compounds at 50 mg/L[a]

compd.	R_1	R_2	*S. s.*[b]	*P. p.*[c]	*V. m.*[d]	*F. g.*[e]	*B. c.*[f]
5Ia	CF_3	H	13.33 ± 1.6	55.91 ± 1.0	55.00 ± 1.4	29.43 ± 4.1	17.78 ± 3.6
5Ib	CF_3	2-CH_3	45.71 ± 2.8	22.81 ± 0.2	61.67 ± 3.6	23.69 ± 0.9	27.78 ± 1.2
5Ic	CF_3	3-CH_3	**100**	58.06 ± 1.8	77.50 ± 2.5	55.48 ± 2.4	65.55 ± 1.1
5Id	CF_3	4-CH_3	55.24 ± 1.3	24.73 ± 1.9	52.12 ± 0.9	32.87 ± 2.1	36.67 ± 1.9
5Ie	CF_3	2-Cl	22.86 ± 1.4	25.81 ± 2.1	45.00 ± 1.4	22.96 ± 4.3	21.11 ± 0.9
5If	CF_3	3-Cl	87.14 ± 3.2	40.43 ± 1.0	72.50 ± 3.3	53.76 ± 2.4	62.56 ± 1.1
5Ig	CF_3	4-Cl	71.33 ± 2.1	29.35 ± 1.9	65.15 ± 3.0	27.12 ± 1.0	46.67 ± 1.9
5Ih	CF_3	2-F	<5.0	24.73 ± 1.1	52.50 ± 1.4	<5.0	20.10 ± 2.0
5Ii	CF_3	3-F	77.14 ± 2.1	30.11 ± 1.0	62.50 ± 0.1	55.48 ± 2.4	58.89 ± 1.1
5Ij	CF_3	4-F	47.14 ± 0.8	39.78 ± 2.4	12.50 ± 3.3	13.91 ± 3.5	40.00 ± 0.3
5Ik	CF_3	2-OCH_3	23.16 ± 3.7	24.73 ± 3.3	46.67 ± 1.9	18.27 ± 1.6	16.77 ± 2.6
5Il	CF_3	3-OCH_3	65.71 ± 1.1	39.78 ± 1.0	61.66 ± 2.2	54.83 ± 1.1	53.33 ± 3.3
5Im	CF_3	4-OCH_3	37.14 ± 3.3	38.71 ± 1.8	53.33 ± 3.6	33.73 ± 2.3	47.78 ± 1.1
5In	CF_3	2-CF_3	48.10 ± 1.6	12.90 ± 1.9	50.00 ± 2.4	15.45 ± 1.9	11.12 ± 3.0
5Io	CF_3	3-CF_3	83.33 ± 2.2	35.48 ± 1.9	61.67 ± 1.9	58.49 ± 1.1	36.67 ± 0.4
5Ip	CF_3	4-CF_3	77.10 ± 0.8	25.81 ± 1.8	53.33 ± 0.4	36.95 ± 0.9	22.35 ± 1.5
5IIa	CHF_2	H	21.42 ± 1.2	61.2 ± 0.7	70.00 ± 0.8	22.83 ± 1.9	32.22 ± 1.1
5IIb	CHF_2	2-CH_3	25.41 ± 1.3	39.78 ± 1.9	70.83 ± 1.4	33.72 ± 1.5	23.33 ± 1.8
5IIc	CHF_2	3-CH_3	**100**	78.71 ± 1.9	95.00 ± 0.4	43.06 ± 1.3	89.33 ± 1.9
5IId	CHF_2	4-CH_3	63.33 ± 2.2	35.48 ± 1.8	74.16 ± 1.7	29.31 ± 1.6	40.00 ± 2.0
5IIe	CHF_2	2-Cl	26.10 ± 0.7	12.90 ± 1.9	65.00 ± 1.4	42.58 ± 1.4	51.11 ± 1.1
5IIf	CHF_2	3-Cl	98.67 ± 2.1	31.20 ± 1.0	80.20 ± 0.5	51.22 ± 1.1	71.28 ± 0.2
5IIg	CHF_2	4-Cl	85.71 ± 2.8	25.81 ± 1.9	78.33 ± 0.7	44.40±1.9	62.22 ± 2.1
5IIh	CHF_2	2-F	37.14 ± 1.4	17.20 ± 3.8	51.67 ± 0.8	22.83 ± 1.8	34.44 ± 1.1
5IIi	CHF_2	3-F	94.76 ± 0.8	43.24 ± 2.1	77.40 ± 1.4	32.62 ± 1.7	55.55 ± 0.7
5IIj	CHF_2	4-F	47.62 ± 2.2	34.41 ± 1.9	65.00 ± 2.8	33.66 ± 2.6	40.00 ± 0.2
5IIk	CHF_2	2-OCH_3	40.00 ± 1.3	17.20 ± 1.1	32.55 ± 3.8	<5.0	13.47 ± 3.5
5IIl	CHF_2	3-OCH_3	88.57 ± 1.4	33.33 ± 1.0	61.21 ± 1.5	31.25 ± 1.7	51.14 ± 1.8
5IIm	CHF_2	4-OCH_3	47.14 ± 6.5	24.73 ± 2.1	56.67 ± 0.8	26.12 ± 2.1	32.22 ± 2.2
5IIn	CHF_2	2-CF_3	61.00 ± 2.8	35.21 ± 4.3	44.16 ± 2.2	43.48 ± 0.9	34.44 ± 1.1
5IIo	CHF_2	3-CF_3	96.19 ± 0.8	48.38 ± 3.3	88.83 ± 0.8	50.03 ± 1.6	77.78 ± 0.4
5IIp	CHF_2	4-CF_3	90.95 ± 0.7	38.48 ± 1.3	76.28 ± 2.2	41.76 ± 1.2	53.33 ± 2.0
Boscalid			100	45.16 ± 2.3	65.83 ± 1.5	37.86 ± 2.5	86.91 ± 1.1
Fluxapyroxad			100	59.39 ± 0.6	78.67 ± 0.7	38.33 ± 1.0	82.67 ± 0.7

[a]The data are the mean values of triplicate experiments. [b]*S. s. Sclerotinia sclerotiorum*. [c]*P. p. Physalospora piricola*. [d]*V. m. Valsa mali*. [e]*F. g. FusaHum graminearum* sehw. [f]*B. c. Botrytis cinerea pers.*

cell wall structures 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).

Molecular Docking. The crystal structures of succinate dehydrogenase (PDB entry: 2FBW) were retrieved from the Protein Data Bank. The molecular docking procedure was performed using the CDOCKER protocol for receptor−ligand interaction section of DS 4.0 (Discovery Studio 4.0, Accelrys, Inc., San Diego, CA).[35]

SDH Enzyme Assay In Vitro. The SDH enzyme activity of **5IIc** and **Fluxapyroxad** were determined using a succinate dehydrogenase assay kit (Mlbio, ml076573). *S. sclerotiorum* was grown in the sterilized Fries medium for 3 days and then treated with different concentrations of **5IIc** and **Fluxapyroxad**, separately. The SDH enzyme activity was measured after 24 h of treatment with the selected compounds, and the absorbance value was recorded at 600 nm using a microplate reader. Compounds were tested at five different concentrations with three replicates, and the IC_{50} value was calculated by SPSS 24.0.

RESULTS AND DISCUSSION

Synthesis of Target Compounds. A series of novel pyrazol benzamide derivatives were synthesized, and the general pathway is outlined in Figure 3. The chemical structures of all synthesized compounds were confirmed by ^{1}H NMR, ^{13}C NMR, and HRMS (ESI) studies, and characterization data are provided in the Supporting Information.

Fungicidal Activities and SAR Discussion. The fungicidal activities of all synthesized compounds were evaluated against five fungi including *S. sclerotiorum*, *P. piricola*, *V. mali*, *F. graminearum* sehw., and *B. cinereal*, where **Fluxapyroxad** and **Boscalid** were used as positive controls. The results are presented in Table 1, and the EC_{50} values of the selected compounds are shown in Table 2.

As shown in Table 1, most of the compounds showed antifungal activity against five kinds of phytopathogenic fungi at 50 mg/L, and some of them displayed good fungicidal activity against specific fungi. Among them, nine target compounds displayed good antifungal activities against *S. sclerotiorum* (inhibition rate > 80%) at 50 mg/L. Compounds **5If**, **5Io**, **5IIf**, **5IIi**, **5IIl**, **5IIo**, and **5IIp** showed fungicidal activities of 87.14, 83.33, 98.67, 94.76, 88.57, 96.19, and 90.95%, respectively. In addition, compounds **5Ic** and **5IIc** showed excellent fungicidal activity toward *S. sclerotiorum* with a 100% inhibition rate, which was same as those of **Fluxapyroxad** and **Boscalid** (100%). As for *P. piricola*, the two compounds **5IIa** and **5IIc** have higher antifungal activities than **Fluxapyroxad** (59.36%). For *V. mali*, three target

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Table 2. Fungicidal Activities with EC_{50} against *S. sclerotiorum* and *V. mail* of the Selected Compounds

fungi	compd.	R_1	R_2	EC_{50}	95% CI[a]	regression equation	R^2
S. sclerotiorum	**5Ic**	CF_3	3-CH_3	1.19	0.92−1.48	$y = -0.0862 + 1.1564x$	0.978
	5If	CF_3	3-Cl	3.02	1.47−4.58	$y = -0.4204 + 0.8772x$	0.953
	5Ig	CF_3	4-Cl	21.22	17.40−26.85	$y = -1.8741 + 1.4125x$	0.981
	5Ii	CF_3	3-F	5.63	3.90−7.88	$y = -0.5908 + 0.7864x$	0.998
	5Io	CF_3	3-CF_3	7.78	5.53−10.51	$y = -0.7776 + 0.8729x$	0.994
	5Ip	CF_3	4-CF_3	9.24	7.76−10.87	$y = -1.6679 + 1.7273x$	0.972
	5IIc	CHF_2	3-CH_3	0.20	0.16−0.25	$y = 0.9094 + 1.3097x$	0.991
	5IIf	CHF_2	3-Cl	2.62	2.08−3.20	$y = -0.7511 + 1.3582x$	0.955
	5IIg	CHF_2	4-Cl	7.36	5.93−9.06	$y = -1.0354 + 1.1944x$	0.996
	5IIi	CHF_2	3-F	2.68	1.95−3.42	$y = -0.5298 + 1.2383x$	0.996
	5IIo	CHF_2	3-CF_3	4.64	3.83−5.51	$y = -1.0478 + 1.5713x$	0.963
	5IIp	CHF_2	4-CF_3	5.07	4.06−6.19	$y = -0.9140 + 1.2958x$	0.992
	Boscalid			0.11	0.07−1.13	$y = 1.0464 + 1.0523x$	0.990
	Fluxapyroxad			0.12	0.07−0.17	$y = 0.6599 + 0.7285x$	0.962
V. mail	**5Ic**	CF_3	3-CH_3	8.30	5.67−11.22	$y = -0.7939 + 0.8639x$	0.956
	5If	CF_3	3-Cl	6.46	0.91−9.50	$y = -0.9509 + 1.1737x$	0.964
	5Ig	CF_3	4-Cl	10.08	7.63−12.97	$y = -1.0499 + 1.0462x$	0.986
	5Ii	CF_3	3-F	13.51	8.44−24.57	$y = -0.7060 + 0.6244x$	0.976
	5Io	CF_3	3-CF_3	9.65	5.57−14.88	$y = -0.5928 + 0.6022x$	0.993
	5Ip	CF_3	4-CF_3	21.19	13.16−47.01	$y = -0.7125 + 0.5373x$	0.956
	5IIc	CHF_2	3-CH_3	3.68	2.37−4.98	$y = -0.6659 + 1.1747x$	0.968
	5IIf	CHF_2	3-Cl	4.11	2.48−5.72	$y = -0.6079 + 0.9898x$	0.965
	5IIg	CHF_2	4-Cl	6.07	3.40−8.81	$y = -0.5732 + 0.7316x$	0.954
	5IIi	CHF_2	3-F	6.12	4.40−8.38	$y = -0.6689 + 0.8497x$	0.998
	5IIo	CHF_2	3-CF_3	5.01	3.12−6.18	$y = -0.6830 + 0.9763x$	0.965
	5IIp	CHF_2	4-CF_3	8.08	6.51−9.77	$y = -1.2658 + 1.4136x$	0.951
	Boscalid			14.83	8.33−30.32	$y = -0.5625 + 0.4802x$	0.968
	Fluxapyroxad			12.67	9.20−20.12	$y = -0.9514 + 0.8626x$	0.998

[a]Confidence interval.

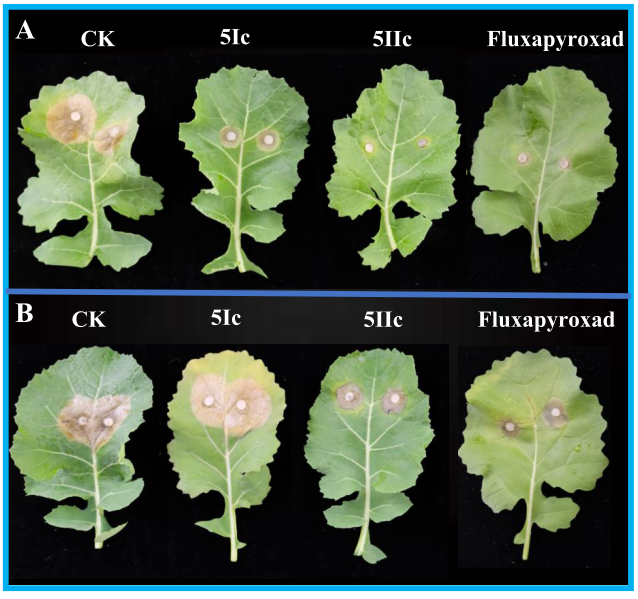

Figure 5. In vivo activity of the target compounds against *S. sclerotiorum*-infected *B. napus L.* leaves. (A) Protective activity and (B) curative activity.

compounds exhibited higher activities than **Fluxapyroxad** (78.67%), and compound **5IIc** displayed the best fungicidal activity of 95.00%. However, for *B. cinerea*, only compound **5IIc** (89.33%) displayed higher activity than **Fluxapyroxad** (82.67%). Unfortunately, for *F. graminearum* sehw., most

Table 3. In Vivo Activity of the Target Compounds against *S. sclerotiorum*-Infected *B. napus L.* Leaves at 50 mg/L[a]

	protective activity		curative activity	
compd.	diameter of lesions (cm)	control efficacy (%)	diameter of lesions (cm)	control efficacy (%)
5Ic	1.1 ± 0.2	82.8	3.7 ± 0.4	15.8
5IIc	0.6 ± 0.1	97.1	1.1 ± 0.1	84.2
Fluxapyroxad	0.5 ± 0.1	98.6	0.8 ± 0.1	92.1
CK	4.0 ± 0.3		4.3 ± 0.2	

[a]Data are given as the mean of three experiments.

compounds did not show high inhibition activities (less than 60%), but some of them were more active than **Fluxapyroxad** (38.33%). Notably, compounds **5Ic** and **5IIc** exhibited a broader fungicidal spectrum than other compounds, in which the 3-methyl phenyl moiety seems to be an important functional group for high activity.

To further determine the fungicidal potency and probe the SAR of these novel derivatives, several compounds with superior fungicidal activities were selected for further study. Their EC_{50} values against *S. sclerotiorum* and *V. mail* are shown in Table 2. In general, the compounds bearing the 3-difluoromethyl group of a pyrazole ring showed better fungicidal activities than that with the 3-trifluoromethyl group. For example, **5IIc** (EC_{50} = 0.20, 3.68 mg/L) was more active than **5Ic** (EC_{50} = 1.19, 8.30 mg/L) and **5IIo** (EC_{50} = 4.64, 5.01 mg/L) was more active than **5Io** (EC_{50} = 7.78, 9.65 mg/L) against *S. sclerotiorum* and *V.mali*, respectively.

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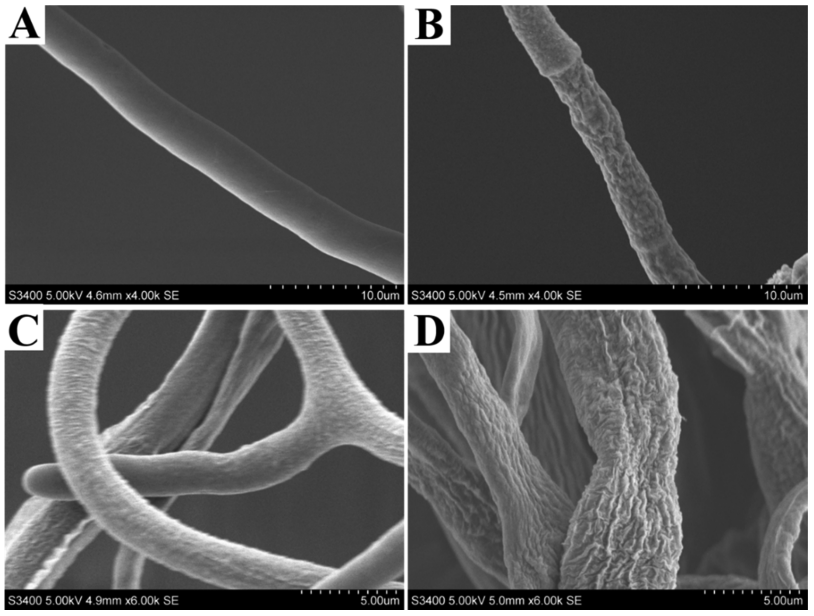

Figure 6. Scanning electron micrographs of the hyphae from the colony of *S. sclerotiorum*. (A) Partial enlarged view of the untreated hypha, (B) partial enlarged view of the hypha treated with **5IIc** (1 mg/L), (C) overall image of the untreated hypha, and (D) overall image of the hypha treated with **5IIc** (1 mg/L).

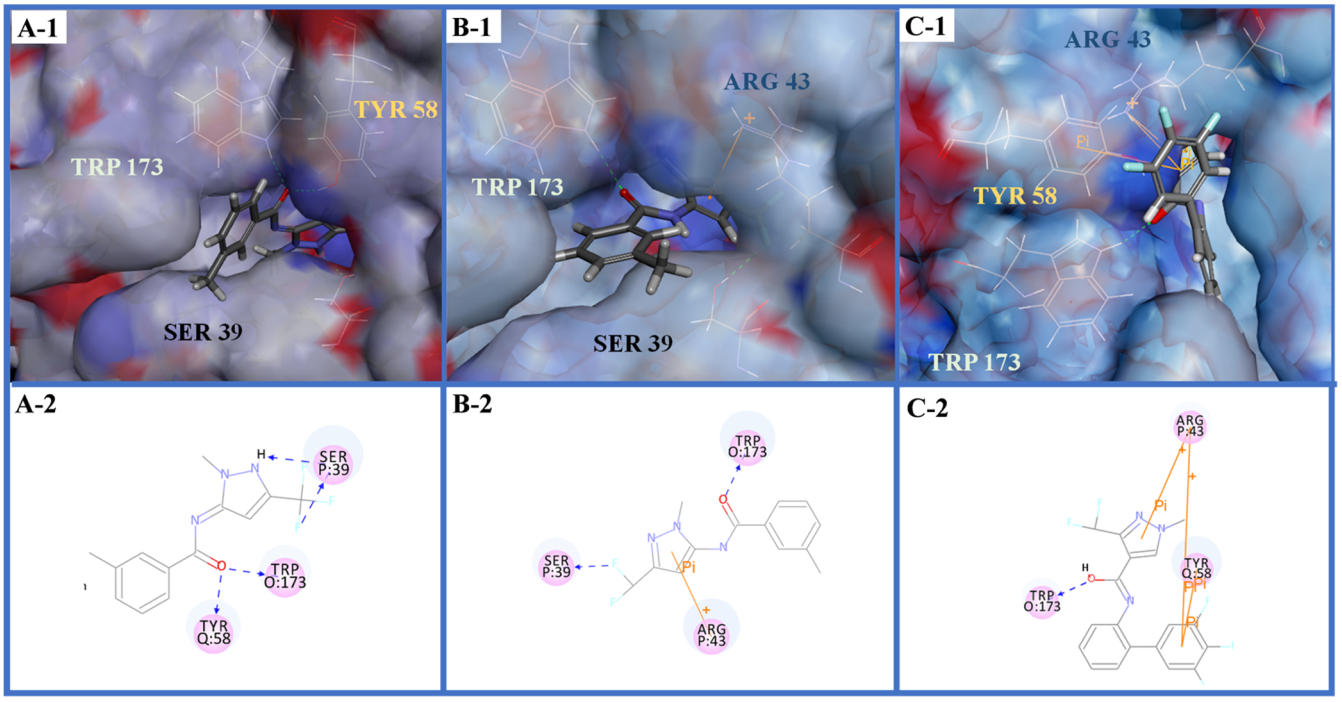

Figure 7. Binding model of **5Ic** (**A**), **5IIc** (**B**), and **Fluxapyroxad** (**C**) in the active site of SDH (PDB entry: 2FBW). The H bond is displayed as a line and π interaction is displayed as a solid yellow line.

Table 4. CDOCKER_INTERACTION_ENERGY (kcal/mol) Obtained from the Docking Study of All Synthesized Compounds by the CDOCKER Protocol

compd.	R_1	R_2	CDOCKER_INTERACTION_ENERGY (kcal/mol)
5Ic	CF_3	3-CH_3	−35.8619
5IIc	CHF_2	3-CH_3	−37.1253
Fluxapyroxad			−39.1853

Meanwhile, the compounds with meso-substitution of the phenyl ring showed better fungicidal activities than that with para-substitution. For instance, **5If** (EC_{50} = 3.02, 6.46 mg/L) was more active than **5Ig** (EC_{50} = 21.22, 10.08 mg/L) and

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Table 5. SDH Enzymatic Inhibition Activity (IC_{50})

compd.	IC_{50} (mg/L)	95% CI[a]	regression equation	R^2
5IIc	0.39	0.19−1.18	$y = -1.0902 + 0.4202x$	0.963
Fluxapyroxad	0.23	0.10−0.74	$y = -0.8291 + 0.3519x$	0.969

[a]Confidence interval.

5IIo (EC_{50} = 4.64, 5.01 mg/L) was more active than **5IIp** (EC_{50} = 5.07, 8.08 mg/L) against *S. sclerotiorum* and *V. mali*, respectively. Notably, the fungicidal activity of compound **5IIc** (EC_{50} = 0.20 mg/L) was close to those of **Fluxapyroxad** (EC_{50} = 0.12 mg/L) and **Boscalid** (EC_{50} = 0.11 mg/L) against *S. sclerotiorum*. Meanwhile, **5IIc** (EC_{50} = 3.68 mg/L) showed significantly higher activity than **Fluxapyroxad** (EC_{50} = 12.67 mg/L) and **Boscalid** (EC_{50} = 14.83 mg/L) against *V. mali*. When R_1 is the difluoromethyl group and R_2 is fixed at the meso-position of the phenyl ring, the influence of R_2 substituents was compared, and the results showed that the meso-methyl group increases the inhibition activity on *S. sclerotiorum* and *V. mali*, and the compound **5IIc** (EC_{50} = 0.20, 3.68 mg/L) showed the best antifungal activity. An electronegative group (Cl or CF_3) at the meso-position of the phenyl ring could decrease the inhibition property. For the compound **5IIf** (EC_{50} = 2.62, 4.11 mg/L) that contains a Cl substituent, the activities are higher than that of **5IIo** (EC_{50} = 4.64, 5.01 mg/L) with a trifluoromethyl group. The same effects were also shown for compounds **5Ia−p**, in which R_1 is the trifluoromethyl group.

The SAR of the selected compounds can be summarized as follows: (1) the compounds bearing a difluoromethyl group at the 3 position of pyrazole showed better fungicidal activities than those with a trifluoromethyl group and (2) the compounds with the meso-substitution of the phenyl ring may be essential for enhancing antifungal activity.

In Vivo Fungicidal Activities. In vivo fungicidal activity of the target compounds against *S. sclerotiorum* was carried out on *B. napus L.* leaves. The efficacy of the protective and curative activity is shown in Figure 5. The compounds **5Ic** and **5IIc** showed promising protective activities of 82.8% and 97.1%, respectively. Notably, compound **5IIc** showed apparent antifungal effects comparable to **Fluxapyroxad** (98.6%) at a concentration of 50 mg/L. In curative activity assay, unlike **5Ic** with low curative activity (15.8%), compounds **5IIc** and **Fluxapyroxad** displayed significant efficacies of 84.2 and 92.1%, respectively. Thus, remarkable differences existed between the treated and untreated groups for disease control experiments in the greenhouse cole leaves. In vivo fungicidal assay exhibited the same tendency as the data acquired using the mycelial growth inhibition assay in vitro (Table 3).

Scanning electron micrographs (SEM) of *S. sclerotiorum* revealed morphological changes in the fungal cell surface. As shown in Figure 6, the surface of *S. sclerotiorum* cells in the untreated groups (A) and (C) was relatively smooth, regular, and even the pores could be seen, whereas when treated with **5IIc** (B) and (D) at 1 mg/L, there was an obvious shrinkage. This showed that **5IIc** had a certain degree of damage to the cell wall of *S. sclerotiorum*.

Molecular Docking. The binding modes of compounds **5Ic**, **5IIc**, and **Fluxapyroxad** with SDH(PDB: 2FBW) are shown in Figure 7. Both the pyrazole rings of **5Ic** and **5IIc** were embedded into the binding pocket, and the oxygen of their amide bonds formed H bonds with TRP173 (**5Ic**, angle = 145.1°, distance = 2.0 Å; **5IIc**, angle = 128.7°, distance = 2.0 Å), similar to the binding mode of **Fluxapyroxad** in 2FBW (angle = 154.6°, distance = 1.91 Å). In addition, the fluorine atom of compounds **5Ic** and **5IIc** formed H bonds with SER39 (**5Ic**, angle = 109.4°, distance = 2.40 Å and angle = 145.1°, distance = 2.00 Å; **5IIc** angle = 110.6°, distance = 2.27 Å). As presented in Figure 7 **A/B/C-2**, the phenyl tail of **Fluxapyroxad** formed the $\pi-\pi$ interaction and $p-\pi$ conjugation with TYR58 and ARG43, respectively. Also, the pyrazole rings of **5IIc** and **Fluxapyroxad** formed the $p-\pi$ conjugation with ARG43. In addition, the CDOCKER_INTERACTION_ENERGY for docking was in the following order: **Fluxapyroxad** (−39.1853 kcal/mol) > **5IIc** (−37.1253 kcal/mol) > **5Ic** (−35.8619 kcal/mol) (Table 4), which could explain the observation that **5Ic** ($R_1 = CF_3$) has lower activity than **5IIc** ($R_1 = CHF_2$).

SDH Enzymatic Inhibition Activity. To further prove the possible antifungal mechanism of the novel pyrazol-5-yl-benzamide derivatives, compound **5IIc** with promising fungicidal activity was selected and evaluated for SDH enzymatic inhibition determination for its target site validation. As shown in Table 5, **5IIc** exhibited significant SDH inhibition with an IC_{50} value of 0.39 mg/L, which is the same level of inhibitory activity as **Fluxapyroxad** (IC_{50} = 0.23 mg/L). These IC_{50} values are consistent with the order of their CDOCKER_INTERACTION_ENERGY, which may be related to the fact that **5IIc** does not form the $\pi-\pi$ interaction with TYR58. The results indicate that SDH is a possible target enzyme of the synthesized compound **5IIc**, and its binding mode is not exactly the same as that of the marketed SDHI **Fluxapyroxad**, which may provide some valuable clues for further optimizing the SDHIs and solving the drug-resistance problem.

In summary, a series of novel pyrazol-5-yl-benzamide derivatives were designed, synthesized, and evaluated for their antifungal activities against five fungi (*S. sclerotiorum*, *P. piricola*, *V. mali*, *F. graminearum* sehw., and *B. cinereal*). The antifungal bioassay discovered a highly active compound **5IIc** with a broad spectrum of excellent in vitro fungicidal activity and better in vivo efficacy against *S. sclerotiorum*. The molecular docking for SDH target validation indicated that compound **5IIc** exhibited similar properties as that of the SDHI positive control **Fluxapyroxad**. These results demonstrate that compound **5IIc** could be a promising fungicide candidate and provide a valuable reference for further investigation.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jafc.0c08094.

The X-ray crystallography data of **5Ic** and physical and spectroscopic data of compounds **5Ia−5IIp** (PDF)

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Notes

The authors declare no competing financial interest.

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