

Brief Report

## Discovery of novel pyridine carboxamides with antifungal activity as potential succinate dehydrogenase inhibitors

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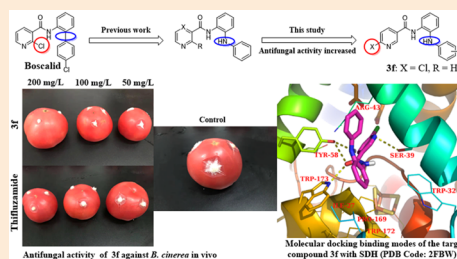
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### Supplementary material

Fifteen novel pyridine carboxamide derivatives bearing a diarylamine-modified scaffold were designed, synthesized, and their antifungal activity was evaluated. Preliminary bioassay results showed that some of the synthesized compounds exhibited moderate to good *in vitro* antifungal activity. Further, compound 6-chloro-*N*-(2-(phenylamino)phenyl)nicotinamide (**3f**) displayed good *in vivo* antifungal activity against *Botrytis cinerea*. The enzymatic test on *B. cinerea* succinate dehydrogenase (SDH) showed that the inhibitory activity possessed by compound **3f** equally matches that of thifluzamide. Molecular docking results demonstrated that compound **3f** could commendably dock with the active site of SDH *via* stable hydrogen bonds and hydrophobic interactions, suggesting the possible binding modes of the title compounds with SDH. The results above revealed that the target compounds would be the leading fungicide compound for further investigation.



**Keywords:** pyridine carboxamide, antifungal activity, succinate dehydrogenase, molecular docking.

### Introduction

Fungi have been known to be an extensive threat to plant species for a long time, accounting for more than 70% of plant diseases, and pathogenic fungi cause about 10% of the world's major crops to be reduced each year.<sup>1–3</sup> Moreover, fungi are easily spread through air and water flow, which increases the difficulty of their prevention.<sup>4</sup> Currently, people are mainly using chemical agents for the prevention and control of fungal diseases in agricultural production crops. As a matter of fact, the use of fungicides has been one of the most economical and effective

ways to reduce huge losses in crop productivity. However, the long-term and increasing application of chemical fungicides has caused ever-increasing fungicide resistance in plant pathogenic fungi. It is necessary to develop novel scaffolds or action mode fungicides to control these crop diseases and solve the problem of fungicide resistance.

Succinate dehydrogenase inhibitors (SDHIs) have been developed for more than 50 years, since the first commercial product, carboxin, was launched in 1966.<sup>5</sup> The mode of action of these fungicides is based on disruption of the mitochondrial tricarboxylic acid cycle and respiratory chain.<sup>6</sup> The SDHIs have common heterocyclic acid scaffolds, such as pyrazole, pyridine and thiazole rings. Recent studies have mainly focused on modification of the amine moiety and retaining the core moieties of commercial SDHIs, which may lead to structural similarity and better cross-resistance.<sup>7</sup> Pyridine carboxamide has attracted great attention since boscalid, the first pyridine carboxamide fungicide, was commercialized by the chemical company BASF.<sup>8</sup>

The diarylamine group represents an important structure in many agrochemicals,<sup>9</sup> and it may be a promising group to be

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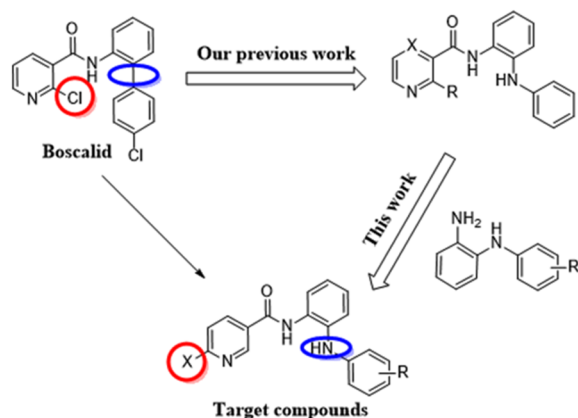


Fig. 1. Design strategy for the title compounds.

integrated with some pharmacophores.<sup>10</sup> In our previous work, six novel nicotinamide derivatives with a diarylamine-modified scaffold were synthesized and evaluated for their antifungal activity.<sup>11</sup> To continue our ongoing work on the discovery of novel nicotinamide fungicides, a series of novel nicotinamide derivatives were synthesized and widely investigated (Fig. 1).

## Materials and methods

### 1. General information

All reagents and solvents were commercially available and used directly without further purification unless specified otherwise. <sup>1</sup>H nuclear magnetic resonance (<sup>1</sup>H NMR) was obtained with chloroform-*d* as a solvent and tetramethylsilane as an internal standard by using a 400 MHz Bruker NMR spectrometer (Bruker Co., Switzerland). Electrospray ionization mass spectrometry (ESI-MS) were recorded on a Thermo Scientific ISQ™ EC spectrometer (Thermo Fisher Technology Co., Ltd., China). Thin-layer chromatography was performed on silica gel 60 F<sub>254</sub> (Qingdao Marine Chemical Co., Ltd., Qingdao, China). Column chromatography purification was conducted on silica gel (200–300 mesh, Qingdao Marine Chemical Co., Ltd., Qingdao, China).

### 2. Synthesis

#### 2.1. The synthetic procedure for intermediates 1

Anhydrous potassium carbonate (20 mmol) was added to a mixture of 1-chloro-2-nitrobenzene (20 mmol) and substituted anilines (30 mmol) in macrogol 1000 (2 mmol), and the resulting mixture was heated at 180°C for 12 hr. The mixture was cooled and quenched with water at room temperature and then extracted with ethyl acetate (3×30 mL). The organic phase was washed with brine, dried with anhydrous sodium sulfate, and concentrated under reduced pressure. The crude product was subjected to flash column chromatography to produce intermediates 1.

#### 2.2. The synthetic procedure for intermediates 2

Intermediates 1 (15.0 mmol), reductive iron powder (15.0 mmol), ammonia chloride (45.0 mmol), and aqueous ethanol solution (75%, 30 mL) were added to a flask. The reaction

was refluxed at 90°C for 3 hr. When the reaction was finished, the mixture was cooled at room temperature, filtered, and extracted with ethyl acetate (3×20 mL). The organic phase was evaporated *in vacuo* to obtain intermediates 2.

#### 2.3. The synthetic procedure for title compounds 3

Intermediates 2 (1.1 mmol) were dissolved in 5 mL of dichloromethane solvent, and the mixture was cooled to 0°C. The mixture was initially supplemented with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.2 mmol) and 4-dimethylaminopyridine (0.2 mmol), subsequently supplemented with intermediate 2 (1.0 mmol), and reacted for 5 hr. When the reaction was completed, the mixture was quenched with water, and the water phase was extracted with dichloromethane (2×5 mL). Then the dichloromethane phase was combined, washed with brine (2×5 mL), dried with anhydrous sodium sulfate, and filtered. The solvent was removed *in vacuo*, and the crude product was purified through column chromatography to obtain pyridine carboxamides 3a–3o.

Data for *N*-(2-(phenylamino)phenyl)nicotinamide (3a): white solid; mp 122.2–125.0°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 9.15 (s, 1H), 9.04 (s, 1H), 8.63 (s, 1H), 8.27 (d, *J*=7.6 Hz, 1H), 8.08 (d, *J*=5.4 Hz, 1H), 7.51 (dd, *J*<sub>1</sub>=8.9 Hz, *J*<sub>2</sub>=3.6 Hz, 1H), 7.32–7.23 (m, 2H), 7.17 (m, 4H), 6.83 (m, 3H); Anal. Calcd. for C<sub>18</sub>H<sub>15</sub>N<sub>3</sub>O: C, 74.72; H, 5.23; N, 14.52; Found: C, 74.52; H, 5.27; N, 14.48; ESI-MS *m/z* 288.12 [M]<sup>-</sup>.

Data for *N*-(2-(*p*-tolylamino)phenyl)nicotinamide (3b): white solid; mp 136.1–138.2°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.91 (s, 1H), 8.70 (d, *J*=4.6 Hz, 1H), 8.61 (s, 1H), 8.19 (d, *J*=7.7 Hz, 1H), 8.05 (d, *J*=7.8 Hz, 1H), 7.44–7.32 (m, 1H), 7.25–7.11 (m, 4H), 7.03 (d, *J*=7.9 Hz, 2H), 6.73 (d, *J*=8.0 Hz, 2H), 2.26 (s, 3H); Anal. Calcd. for C<sub>19</sub>H<sub>17</sub>N<sub>3</sub>O: C, 75.23; H, 5.65; N, 13.85; Found: C, 75.31; H, 5.55; N, 13.83; ESI-MS *m/z* 302.13 [M]<sup>-</sup>.

Data for *N*-(2-((4-fluorophenyl)amino)phenyl)nicotinamide (3c): off-white solid; mp 149.5–149.9°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.96 (s, 1H), 8.73 (s, 1H), 8.53 (s, 1H), 8.10 (t, *J*=8.3 Hz, 2H), 7.41 (d, *J*=5.8 Hz, 1H), 7.24–7.08 (m, 4H), 6.93 (t, *J*=8.5 Hz, 2H), 6.78 (dd, *J*<sub>1</sub>=8.6 Hz, *J*<sub>2</sub>=4.4 Hz, 2H); Anal. Calcd. for C<sub>18</sub>H<sub>14</sub>FN<sub>3</sub>O: C, 70.35; H, 4.59; N, 13.67; Found: C, 70.30; H, 4.62; N, 13.63; ESI-MS *m/z* 307.18 [M]<sup>-</sup>.

Data for *N*-(2-((4-chlorophenyl)amino)phenyl)nicotinamide (3d): brown solid; mp 155.4–156.8°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 9.04 (s, 1H), 8.73 (s, 1H), 8.62 (s, 1H), 8.12 (m, 2H), 7.44 (d, *J*=2.6 Hz, 1H), 7.28 (d, *J*=8.0 Hz, 2H), 7.24–7.18 (m, 2H), 7.16 (d, *J*=8.0 Hz, 2H), 6.74 (d, *J*=8.2 Hz, 2H); Anal. Calcd. for C<sub>18</sub>H<sub>14</sub>ClN<sub>3</sub>O: C, 66.77; H, 4.36; N, 12.98; Found: C, 66.74; H, 4.32; N, 12.92; ESI-MS *m/z* 323.12 [M]<sup>-</sup>.

Data for *N*-(2-((2,4-dimethylphenyl)amino)phenyl)nicotinamide (3e): gray solid; mp 142.4–143.1°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.97 (s, 1H), 8.69 (s, 2H), 8.18 (d, *J*=7.1 Hz, 1H), 8.09 (d, *J*=7.6 Hz, 1H), 7.48–7.09 (m, 4H), 6.98 (d, *J*=8.0 Hz, 1H), 6.63 (s, 1H), 6.57 (d, *J*=7.9 Hz, 1H), 2.17 (s, 6H); Anal. Calcd. for C<sub>20</sub>H<sub>19</sub>N<sub>3</sub>O: C, 75.69; H, 6.03; N, 13.24; Found: C, 75.65; H, 6.07; N, 13.19; ESI-MS *m/z* 317.17 [M+H]<sup>+</sup>.

Data for 6-chloro-*N*-(2-(phenylamino)phenyl)nicotinamide (**3f**): light red solid; mp 137.8–139.0°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.85 (s, 1H), 8.52 (d, *J*=2.2 Hz, 1H), 8.37 (s, 1H), 8.26 (d, *J*=7.6 Hz, 1H), 8.17 (d, *J*=6.0 Hz, 1H), 7.87 (dd, *J*<sub>1</sub>=8.3 Hz, *J*<sub>2</sub>=2.4 Hz, 1H), 7.62 (d, *J*=7.9 Hz, 1H), 7.46 (d, *J*=8.3 Hz, 1H), 7.40 (d, *J*=7.7 Hz, 1H), 7.37–7.31 (m, 1H), 7.21 (dd, *J*<sub>1</sub>=12.8 Hz, *J*<sub>2</sub>=6.8 Hz, 2H), 6.91 (t, *J*=7.3 Hz, 1H), 6.76 (d, *J*=7.7 Hz, 1H); Anal. Calcd. for C<sub>18</sub>H<sub>14</sub>ClN<sub>3</sub>O: C, 66.77; H, 4.36; N, 12.98; Found: C, 66.71; H, 4.34; N, 12.88; ESI-MS *m/z* 322.99 [M]<sup>-</sup>.

Data for 6-chloro-*N*-(2-(*p*-tolylamino)phenyl)nicotinamide (**3g**): light yellow solid; mp 138.1–139.2°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.89 (s, 1H), 8.61 (s, 1H), 8.46 (s, 1H), 8.28 (d, *J*=7.3 Hz, 1H), 7.94 (d, *J*=8.3 Hz, 1H), 7.52 (m, 1H), 7.40 (d, *J*=8.3 Hz, 1H), 7.29 (d, *J*=7.0 Hz, 1H), 7.28–7.22 (m, 1H), 7.08 (d, *J*=8.1 Hz, 2H), 6.73 (d, *J*=8.1 Hz, 2H), 2.31 (s, 3H); Anal. Calcd. for C<sub>19</sub>H<sub>16</sub>ClN<sub>3</sub>O: C, 67.56; H, 4.77; N, 12.44; Found: C, 67.52; H, 4.75; N, 12.24; ESI-MS *m/z* 337.07 [M]<sup>-</sup>.

Data for 6-chloro-*N*-(2-((4-fluorophenyl)amino)phenyl)nicotinamide (**3h**): light red solid; mp 182.3–183.1°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.62 (s, 1H), 8.37 (s, 1H), 8.15 (s, 1H), 7.99 (d, *J*=8.1 Hz, 1H), 7.40 (d, *J*=8.1 Hz, 1H), 7.25–7.11 (m, 4H), 6.95 (d, *J*=8.2 Hz, 1H), 6.75 (d, *J*=8.2 Hz, 1H); Anal. Calcd. for C<sub>18</sub>H<sub>13</sub>ClF<sub>3</sub>N<sub>3</sub>O: C, 63.26; H, 3.83; N, 12.30; Found: C, 63.22; H, 3.73; N, 12.26; ESI-MS *m/z* 341.12 [M]<sup>-</sup>.

Data for 6-chloro-*N*-(2-((4-chlorophenyl)amino)phenyl)nicotinamide (**3i**): light yellow solid; mp 128.4–130.1°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.56 (s, 1H), 8.40 (s, 1H), 8.25 (s, 1H), 7.90 (d, *J*=8.3 Hz, 1H), 7.36 (d, *J*=8.3 Hz, 1H), 7.25–7.11 (m, 4H), 7.04 (d, *J*=8.0 Hz, 1H), 6.68 (d, *J*=8.2 Hz, 1H); Anal. Calcd. for C<sub>18</sub>H<sub>13</sub>Cl<sub>2</sub>N<sub>3</sub>O: C, 60.35; H, 3.66; N, 11.73; Found: C, 60.30; H, 3.67; N, 11.68; ESI-MS *m/z* 357.09 [M]<sup>-</sup>.

Data for 6-chloro-*N*-(2-((2,4-dimethylphenyl)amino)phenyl)nicotinamide (**3j**): light brown solid; mp 150.2–150.6°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.64 (s, 1H), 8.48 (s, 1H), 8.28 (s, 1H), 7.95 (d, *J*=8.2 Hz, 1H), 7.40 (d, *J*=8.2 Hz, 1H), 7.30–7.13 (m, 1H), 7.03 (d, *J*=7.9 Hz, 1H), 6.64 (s, 1H), 6.56 (d, *J*=7.7 Hz, 1H), 2.22 (s, 6H); Anal. Calcd. for C<sub>20</sub>H<sub>18</sub>ClN<sub>3</sub>O: C, 68.28; H, 5.16; N, 11.94; Found: C, 68.25; H, 5.14; N, 11.88; ESI-MS *m/z* 351.09 [M]<sup>-</sup>.

Data for *N*-(2-(phenylamino)phenyl)-6-(trifluoromethyl)nicotinamide (**3k**): yellow solid; mp 139.4–139.8°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.91 (s, 1H), 8.61 (s, 1H), 8.30 (d, *J*=8.1 Hz, 1H), 8.08 (d, *J*=8.2 Hz, 1H), 7.71 (d, *J*=8.1 Hz, 1H), 7.34–7.15 (m, 6H), 6.95 (d, *J*=7.4 Hz, 1H), 6.80 (d, *J*=7.9 Hz, 2H); Anal. Calcd. for C<sub>19</sub>H<sub>14</sub>F<sub>3</sub>N<sub>3</sub>O: C, 63.86; H, 3.95; N, 11.76; Found: C, 68.88; H, 3.94; N, 11.74; ESI-MS *m/z* 357.13 [M]<sup>-</sup>.

Data for *N*-(2-(*p*-tolylamino)phenyl)-6-(trifluoromethyl)nicotinamide (**3l**): yellowish green solid; mp 113.6–114.7°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.94 (s, 1H), 8.60 (s, 1H), 8.29 (d, *J*=8.0 Hz, 1H), 8.12 (d, *J*=9.2 Hz, 1H), 7.73 (s, 1H), 7.45–7.14 (m, 4H), 7.04 (d, *J*=12.4 Hz, 2H), 6.70 (d, *J*=12.4 Hz, 2H), 2.28 (s, 3H); Anal. Calcd. for C<sub>20</sub>H<sub>16</sub>F<sub>3</sub>N<sub>3</sub>O: C, 64.69; H, 4.34; N, 11.32; Found: C, 64.74; H, 4.33; N, 11.35; ESI-MS *m/z* 371.11 [M]<sup>-</sup>.

Data for *N*-(2-((4-fluorophenyl)amino)phenyl)-6-(trifluoromethyl)nicotinamide (**3m**): gray white solid; mp 183.8–185.2°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.98 (s, 1H), 8.54 (s, 1H), 8.20 (s, *J*=5.2 Hz, 2H), 7.76 (d, *J*=8.4 Hz, 1H), 7.30–7.12 (m, 2H), 6.94 (d, *J*=10.4 Hz, 2H), 6.75 (d, *J*=10.0 Hz, 2H), 3.61 (m, 2H); Anal. Calcd. for C<sub>19</sub>H<sub>13</sub>F<sub>4</sub>N<sub>3</sub>O: C, 60.80; H, 3.49; N, 11.20; Found: C, 60.82; H, 3.46; N, 11.22; ESI-MS *m/z* 375.11 [M]<sup>-</sup>.

Data for *N*-(2-((4-chlorophenyl)amino)phenyl)-6-(trifluoromethyl)nicotinamide (**3n**): gray white solid; mp 190.7–191.2°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 9.01 (s, 1H), 8.50 (s, 1H), 8.26 (d, *J*=7.2 Hz, 1H), 8.22 (d, *J*=7.6 Hz, 1H), 7.79 (d, *J*=8.0 Hz, 1H), 7.36–7.24 (m, 4H), 7.21 (d, *J*=8.4 Hz, 2H), 6.73 (d, *J*=8.0 Hz, 2H); Anal. Calcd. for C<sub>19</sub>H<sub>13</sub>ClF<sub>3</sub>N<sub>3</sub>O: C, 58.25; H, 3.34; N, 10.73; Found: C, 58.21; H, 3.34; N, 10.75; ESI-MS *m/z* 392.02 [M]<sup>-</sup>.

Data for *N*-(2-((2,4-dimethylphenyl)amino)phenyl)-6-(trifluoromethyl)nicotinamide (**3o**): yellow solid; mp 119.7–121.5°C; <sup>1</sup>H NMR (400 MHz, Chloroform-*d*) δ 8.80 (s, 1H), 8.34 (s, 1H), 7.93 (d, *J*=7.8 Hz, 1H), 7.74–7.65 (m, 1H), 7.37 (m, 1H), 7.25–7.16 (m, 4H), 7.14 (m, 2H), 7.04 (d, *J*=7.5 Hz, 1H), 2.38 (s, 3H), 2.32 (s, 3H); Anal. Calcd. for C<sub>21</sub>H<sub>18</sub>F<sub>3</sub>N<sub>3</sub>O: C, 65.45; H, 4.71; N, 10.90; Found: C, 65.41; H, 4.72; N, 10.88; ESI-MS *m/z* 386.12 [M+H]<sup>+</sup>.

#### 2.4. Antifungal activity assay in vitro

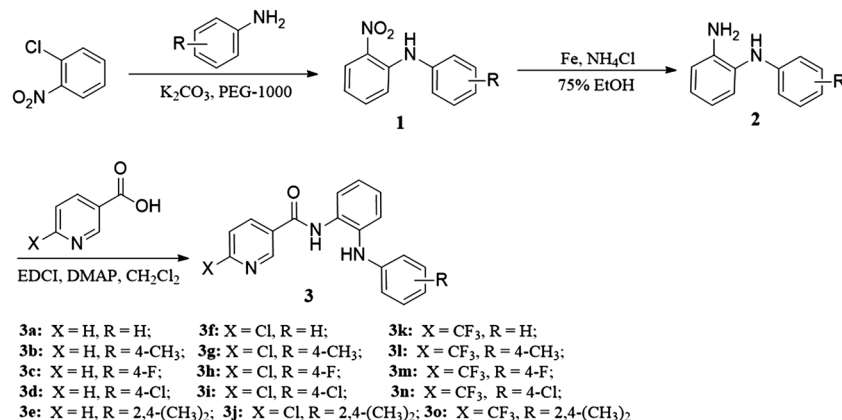
The antifungal activities of target compounds **3a–3o** against eight plant pathogens, *Fusarium solani*, *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Phytophthora capsici*, *Fusarium oxysporum*, *Cytospora ambiens*, *Gibberella zeae*, and *Alternaria alternata*, were tested *in vitro* according to the mycelium growth rate method. The test pathogens grown on PDA medium slants were subcultured for 48 hr in petri dishes prior to testing and used for inoculation of fungal strains on PDA plates. The commercially available fungicides boscalid and thifluzamide were used as the positive control, whereas acetone was set as the negative control. The compounds were dissolved in acetone to prepare a 1000 mg/L stock solution for the following antifungal test. The diameter of each strain was measured after the mycelia were incubated at 25°C for a certain duration. The percentage of inhibition was calculated as follows:

$$I = (B - A) / (B - 5.0) \times 100\%$$

where *I* is the inhibition percentage, *A* is the average mycelial diameter (mm) with the compounds in petri dishes, and *B* is the average mycelial diameter with the compounds in blank petri dishes. The inhibition percentage of the compounds was determined at 50 mg/L, and these compounds with an inhibition rate of more than 70% against *B. cinerea* were further determined for their median effective concentration (EC<sub>50</sub>) values according to the method described above. The stock solution was mixed with the autoclaved PDA medium to prepare a set of media containing 1.5625, 3.125, 6.25, 12.5, 25, and 50 μg/mL of the test compounds.

#### 2.5. Antifungal activity assay in vivo

Tomato fruits were collected from the Sanhe sub-district of



Scheme 1. Synthetic route of title compounds 3a–3o.

Huainan City. Healthy tomatoes were washed and treated with water and 75% aqueous ethyl alcohol in advance and rinsed with water, which was then evaporated at room temperature. The fruits were wounded ( $d=5$  mm) using an inoculating needle and then inoculated with each pathogen. The test compound **3f** was dissolved in 200  $\mu$ L of dimethyl sulfoxide; then 0.2% Tween-80 aqueous water was added at concentrations of 50, 100, and 200 mg/L. The fruits treated with aqueous dimethyl sulfoxide (2%) containing Tween-80 (0.5%) were used as the control. The commercial fungicide boscalid was used as the positive control. All of the treated fruits were then placed into an illumination incubator ( $25\pm 2^\circ\text{C}$  and 95% relative humidity) for 4 days. These

experiments were repeated three times. The protective activity rate (%) was calculated using the following formula: (diameter of lesion in the negative control – diameter of lesion in the treatment)/diameter of lesion in the negative control  $\times 100$ .

#### 2.6. SDH inhibition assay in vitro

The *in vitro* inhibitory effects of title compound **3f** and thifluzamide against *B. cinerea* SDH were determined using a succinate dehydrogenase assay kit that was purchased from the Nanjing Jiancheng Bioengineering Institute. Cultures were inoculated at 0.05 OD<sub>600</sub> nm and grown on a reciprocal shaker (180 rpm,  $28^\circ\text{C}$ ) for 7 days in Sabouraud Maltose Broth (SMB), and the mycelia of *B. cinerea* was collected for preparing the tested mito-

Table 1. *In vitro* antifungal activities of title compounds 3a–3o at 50 mg/L

Compd.	Inhibition rate <sup>a)</sup> (%)							
	<i>F.s.</i> <sup>b)</sup>	<i>S.s.</i>	<i>B.c.</i>	<i>P.c.</i>	<i>F.o.</i>	<i>C.a.</i>	<i>G.z.</i>	<i>A.a.</i>
<b>3a</b>	2.1 $\pm$ 0.1	8.5 $\pm$ 1.3	22.4 $\pm$ 0.5	23.7 $\pm$ 0.2	14.6 $\pm$ 1.7	35.9 $\pm$ 1.8	31.1 $\pm$ 0.7	9.6 $\pm$ 0.7
<b>3b</b>	1.1 $\pm$ 0.2	5.9 $\pm$ 0.8	13.1 $\pm$ 1.2	11.2 $\pm$ 0.3	1.8 $\pm$ 0.1	48.1 $\pm$ 1.9	26.4 $\pm$ 1.1	3.1 $\pm$ 0.4
<b>3c</b>	0	0	19.4 $\pm$ 0.8	18.1 $\pm$ 1.1	2.8 $\pm$ 0.1	38.7 $\pm$ 1.6	11.3 $\pm$ 1.2	0
<b>3d</b>	2.7 $\pm$ 0.2	1.6 $\pm$ 0.2	13.8 $\pm$ 0.7	5.6 $\pm$ 0.3	0	26.6 $\pm$ 0.8	0	0
<b>3e</b>	1.8 $\pm$ 0.2	0	22.7 $\pm$ 1.9	7.2 $\pm$ 1.0	9.9 $\pm$ 0.3	49.1 $\pm$ 0.2	27.1 $\pm$ 0.4	7.8 $\pm$ 0.9
<b>3f</b>	16.4 $\pm$ 0.4	57.5 $\pm$ 2.2	76.9 $\pm$ 0.9	10.8 $\pm$ 0.5	20.9 $\pm$ 0.4	29.8 $\pm$ 0.4	7.4 $\pm$ 0.1	31.7 $\pm$ 1.2
<b>3g</b>	2.7 $\pm$ 0.3	14.9 $\pm$ 0.5	24.9 $\pm$ 1.7	10.6 $\pm$ 1.0	20.1 $\pm$ 1.2	84.1 $\pm$ 1.5	22.3 $\pm$ 1.2	17.6 $\pm$ 0.7
<b>3h</b>	3.3 $\pm$ 0.2	0	28.5 $\pm$ 1.0	8.8 $\pm$ 0.6	10.0 $\pm$ 1.1	9.2 $\pm$ 0.3	23.7 $\pm$ 0.9	11.7 $\pm$ 0.6
<b>3i</b>	0	0	29.4 $\pm$ 0.3	5.0 $\pm$ 0.6	8.9 $\pm$ 0.5	42.1 $\pm$ 1.2	24.4 $\pm$ 0.9	13.2 $\pm$ 1.1
<b>3j</b>	1.8 $\pm$ 0.2	0	33.6 $\pm$ 3.0	11.8 $\pm$ 2.6	12.9 $\pm$ 1.0	9.0 $\pm$ 0.4	15.2 $\pm$ 0.5	8.3 $\pm$ 0.7
<b>3k</b>	12.1 $\pm$ 1.6	27.4 $\pm$ 1.7	53.9 $\pm$ 2.3	11.5 $\pm$ 0.7	4.3 $\pm$ 2.4	16.7 $\pm$ 1.9	46.4 $\pm$ 0.9	18.9 $\pm$ 1.3
<b>3l</b>	10.5 $\pm$ 1.1	28.7 $\pm$ 1.7	48.9 $\pm$ 4.4	23.4 $\pm$ 0.6	5.3 $\pm$ 1.5	27.7 $\pm$ 2.2	44.7 $\pm$ 2.0	19.4 $\pm$ 1.2
<b>3m</b>	3.0 $\pm$ 0.4	13.5 $\pm$ 0.7	19.8 $\pm$ 1.5	0	0	13.8 $\pm$ 2.2	33.7 $\pm$ 5.5	0
<b>3n</b>	12.4 $\pm$ 0.4	22.7 $\pm$ 0.7	28.9 $\pm$ 2.3	6.3 $\pm$ 0.5	0	8.2 $\pm$ 0.3	27.5 $\pm$ 1.1	0
<b>3o</b>	13.0 $\pm$ 1.2	25.8 $\pm$ 1.0	40.0 $\pm$ 0.1	25.5 $\pm$ 0.5	6.5 $\pm$ 1.7	15.1 $\pm$ 1.3	38.1 $\pm$ 2.7	11.7 $\pm$ 0.8
Thifluzamide	1.1 $\pm$ 0.2	100	75.5 $\pm$ 1.1	11.1 $\pm$ 0.5	6.9 $\pm$ 0.8	11.0 $\pm$ 0.5	10.8 $\pm$ 0.8	81.1 $\pm$ 2.1
Boscalid	7.9 $\pm$ 0.7	100	100	15.0 $\pm$ 1.3	10.5 $\pm$ 1.4	63.4 $\pm$ 1.5	21.3 $\pm$ 1.3	100

<sup>a)</sup> Values were the mean  $\pm$  standard deviation (SD) of three replicates.

<sup>b)</sup> Abbreviations: *F.s.*, *Fusarium solani*; *S.s.*, *Sclerotonia sclerotiorum*; *B.c.*, *Botrytis cinerea*; *P.c.*, *Phytophthora capsici*; *F.o.*, *Fusarium oxysporum*; *C.a.*, *Cytospora ambiens*; *G.z.*, *Gibberella zeae*; *A.a.*, *Alternaria alternata*.



**Table 2.** EC<sub>50</sub> value of compound **3f** against *B. cinerea*

Compd.	Regression equation	EC <sub>50</sub> (mg/L)	95% Confidence interval (mg/L)	R <sup>2</sup>
<b>3f</b>	$y = -3.12 + 2.34x$	21.72	18.01–27.03	0.952
Thiﬂuzamide	$y = -1.80 + 1.56x$	14.71	11.36–19.86	0.948
Boscalid	$y = -1.33 + 2.42x$	3.52	2.78–4.29	0.989

chondrial suspension. The tested compounds were added to the working solution according to the operating instructions for the purchased SDH assay kit. After adding mitochondrial suspensions containing *B. cinerea* SDH to the above-mentioned solution, the absorbance values of the obtained mixtures were monitored at 600 nm to calculate the median inhibitory concentration (IC<sub>50</sub>) against fungal SDH.<sup>12)</sup>

### 2.7. Molecular docking

Molecular docking studies were performed to investigate the binding modes of compound **3f** and thiﬂuzamide to SDH (PDB Code: 2FBW<sup>13,14)</sup>) using the molecular docking software Ledock (<http://www.lephar.com>), which was chosen for its high speed and accuracy.<sup>15,16)</sup> The 3D structures of compound **3f** and thiﬂuzamide were drawn using ChemBio3D Ultra 19.0 software, and the docking results were analyzed and visualized using the PyMOL Molecular Graphics System, Version 1.8.0.0, from Schrödinger, LLC (<http://www.pymol.org/pymol>). The dimension of the binding box was set as [Xmin=36.5, Xmax=53.3, Ymin=-23.0, Ymax=-8.5, Zmin=-2.7, Zmax=13.5]. The conformations of the test compounds were generated by energy minimization using the MM2 force field.

## Results and discussion

Scheme 1 provides the details of the synthesis route of compounds **3a–3o**. The diarylamine intermediate **2** was obtained through the reduction of intermediate **1**, which can be easily prepared with high yields. The title compounds were prepared by reacting pyridine carboxylic acid with intermediate **2** via a classic synthetic approach called condensation reaction. The structure of **3a–3o** was confirmed through <sup>1</sup>H NMR, ESI-MS, and elemental analysis. The <sup>1</sup>H NMR and ESI-MS spectra of representative compounds are given in the supplemental material.

**Table 3.** *In vivo* protective effects of compound **3f** on tomatoes infected by *B. cinerea*

Compd.	Treatment (mg/L)	Protective activity <sup>a)</sup> (%)
<b>3f</b>	200	53.9±5.9
	100	49.0±2.2
	50	27.1±1.5
Thiﬂuzamide	200	55.2±1.5
	100	41.2±1.3
	50	33.8±3.1
Negative Control	—	—

<sup>a)</sup> Values were the mean±standard deviation (SD) of three replicates; “—”, not tested.

### 1. Antifungal activity in vitro

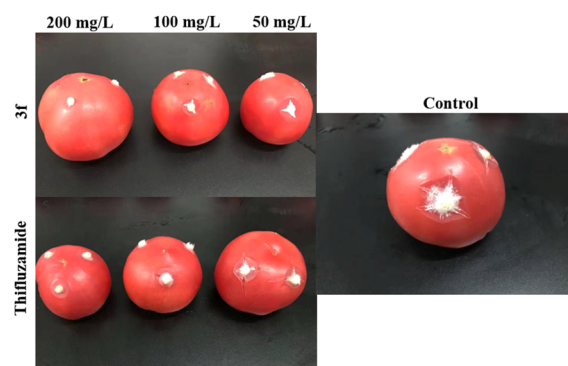
The preliminary *in vitro* screening results for the antifungal activities of compounds **3a–3o** against eight plant pathogens are listed in Table 1. The bioassay results indicated that these synthesized compounds have different degrees of activity against the eight tested plant pathogens at 50 mg/L. Among them, compound **3f** showed a 76.9% inhibition rate against *B. cinerea*, and compound **3g** had an inhibition rate of 84.1% against *C. ambiens*. Although most compounds showed low to moderate antifungal activity, with the inhibition ranging from 0 to 50%, compound **3f** impressed me most for its similar *in vitro* antifungal activity against *B. cinerea* to thiﬂuzamide (Table 2), and compound **3g** also had better antifungal activity against *C. ambiens* than the positive control.

### 2. In vivo antifungal activity assay

Compound **3f** demonstrated the best antifungal activity against *B. cinerea* during the *in vitro* tests, and the efficacy was compared with that of the commercial fungicide thiﬂuzamide. Table 3 presents the results of *in vivo* tests. The preventative efficacies of compound **3f** were 53.9%, 49%, and 27.1% at concentrations of 200 mg/L, 100 mg/L, and 50 mg/L, respectively, whereas those of thiﬂuzamide were 55.2%, 41.2%, and 33.8%. The above results confirmed that compound **3f** had antifungal activity against *B. cinerea* similar to that of thiﬂuzamide, both *in vivo* and *in vitro*. Further, compound **3f** showed preventative efficacy against *B. cinerea* on tomato fruits, and its effects exhibited concentration-dependent properties (Fig. 2). In fact, we could conclude that compound **3f** has potential applications as a fungicide.

### 3. SDH enzymatic inhibition activity

To further prove the possible antifungal mechanism of the novel

**Fig. 2.** *In vivo* protective effects of compound **3f** on tomatoes infected by *B. cinerea*.

**Table 4.** Inhibitory effect of **3f** against *B. cinerea* SDH

Compd.	IC <sub>50</sub>	
	mg/L	μM
<b>3f</b>	5.60±0.49	17.3±1.5
Thifluzamide	7.61±0.58	14.4±1.1

pyridine carboxamide derivatives, compound **3f**, with promising fungicidal activity against *B. cinerea*, was selected and evaluated for SDH enzymatic inhibition determination for its target site validation. As shown in Table 4, compound **3f** exhibited preferable SDH inhibition with an IC<sub>50</sub> value of 5.6 mg/L (17.3 μM), which is the same level of inhibitory activity as thifluzamide (IC<sub>50</sub>=7.61 mg/L, 14.4 μM). This proved that the pyridine carboxamide derivatives designed in this paper displayed as good inhibitory effects against *B. cinerea* SDH as other successfully developed carboxamide fungicides.

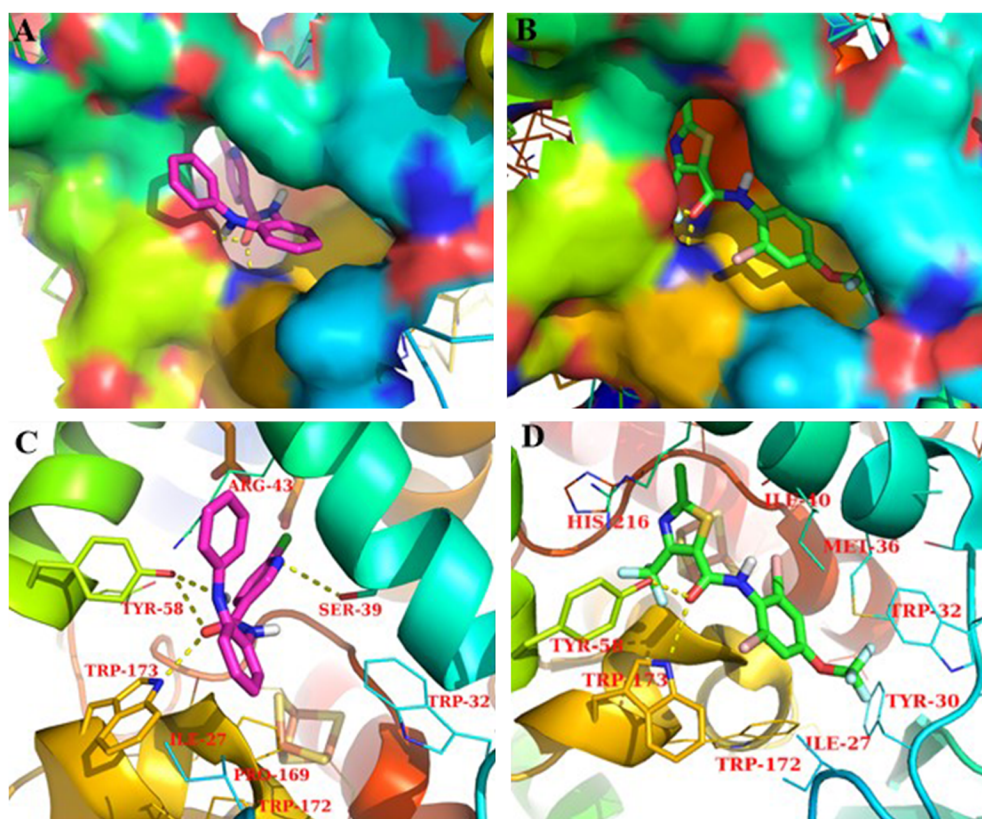
#### 4. Molecular docking

Since compound **3f** and thifluzamide had similar inhibitory effects on *B. cinerea* SDH, they were selected for molecular docking analysis. The three-dimensional schematic diagrams clearly explained the possible optimal combination between the ligands and the receptor protein. As shown in Fig. 3A and B, compound

**3f** and thifluzamide could be embedded well into the protein cavity. The docking total scores for compound **3f** and thifluzamide were  $-6.87$  and  $-7.65$  kcal/mol, respectively. Compound **3f** was well bound to the receptor protein by forming four hydrogen bonds with TYR-58, TRP-173, and SER-39 (Fig. 3C), and the pyridine ring and diphenylamine structure played a key role in the bond formation. In addition, the hydrophobic cavities formed by amino acid residues such as ILE-27, PRO-169, TRP-172, and TRP-32 could create a good hydrophobic interaction with the phenyl fragment of compound **3f**. In fact, thifluzamide's better docking results were related not only to its ability to form two hydrogen bonds with TYR-58 and TRP-173 but, more importantly, to the good hydrophobic interaction between thifluzamide and its surrounding amino acid residues (Fig. 3D). Docking analysis revealed that to design target compounds with a higher docking score, hydrophobic substituents should be introduced into the structure of compound **3f** to increase the hydrophobic interaction between compound **3f** and its surrounding amino acid residues so that target compounds can be better embedded into the protein cavity.

#### Conclusion

Fifteen novel pyridine carboxamides bearing the substituted diarylamine-modified scaffold were designed, synthesized, and



**Fig. 3.** (A) Solid surface of complex II with compound **3f** shown as a stick model. (B) Solid surface of complex II with thifluzamide shown as a stick model. (C) Main interaction of compound **3f** and amino acid residues near the ligands. (D) Main interaction of thifluzamide and amino acid residues near the ligands.

evaluated for their antifungal activity based on our previous study. The results of preliminary fungicidal bioassays revealed that some of the target compounds exhibited a certain degree of antifungal activity against the tested pathogens. Among them, compound **3g** had an inhibition rate of 84.1% against *C. ambiens*, and **3f** showed 76.9% inhibition rate against *B. cinerea* *in vitro*. Meanwhile, it showed a good *in vivo* control effect against *B. cinerea* that was comparable to that of thifluzamide. The test of inhibitory activity against *B. cinerea* SDH showed that compound **3f** had enzyme inhibitory activity equal to that of thifluzamide. The molecular docking results demonstrated that compound **3f** could dock well into the active site of SDH *via* stable hydrogen bonds and hydrophobic interactions, suggesting the possible binding modes of the title compounds with SDH. Compared with the structure–activity relationship reported in our previous work, it was found that the activity of these compounds was slightly better than that of 2-substituted compounds when 6-substituted on the pyridine ring. In addition, the absence of substitution on the terminal benzene ring may be beneficial to the activity. These results suggested that individual compounds should be the lead fungicide compounds for further investigation.

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#### Electronic supplementary materials

The online version of this article contains supplementary materials, which are available at <https://www.jstage.jst.go.jp/browse/jpestics/>.

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