Original Article

Structure-activity relationships of positional isomers in aromatic heterocyclic carboxamide fungicides

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Positional isomers of various aromatic heterocyclic carboxamides were synthesized and their fungicidal activity was examined. Among them, N-(biphenyl-2-yl) and N-[2-(1,3-dimethylbutyl)-3-thienyl]-1-methyl-3-CF3-pyrazole-4-carboxamides showed high activity against gray mold, while their corresponding 5-CF3 carboxamide isomers showed no fungicidal activity. Similar results were observed for carboxamide isomers containing CF3-thiazole as well as methyl-substituted thiophene and furan derivatives. Docking studies were performed on these carboxamides with a fungus succinate dehydrogenase homology model. Based on our docking study, we proposed a model for the interaction between carboxamides and a conserved histidine residue in succinate dehydrogenase. © Pesticide Science Society of Japan

Keywords: penthiopyrad, carboxamide, positional isomer, structure-activity relationships, succinate dehydrogenase, docking study.

Electronic supplementary materials: The online version of this article contains supplementary materials (Supplemental Table S1), which is available at http://www.jstage.jst.go.jp/browse/jpestics/.

Introduction

Carboxin and related carboxamides inhibit succinate dehydrogenase (SDH) and play an important role in plant protection against many phytopathogenic fungi.1,2) These molecules bind specifically to the ubiquinone-binding site of mitochondrial complex II and inhibit fungal respiration. Because of their specific mode of action, they generally show no cross-resistance with other chemical classes (strobilurins, benzimidazoles, and dicarboximides).3,4) Furthermore, they show high activity against various diseases. Therefore, they are excellent candidates for resistance management of fungicides and were first used in agriculture in the late 1960s. The development of carboxin-related fungicides is described in Fig. 1.5) Early SDH inhibitors (SDHIs), such as carboxin and flutolanil, are shown in the upper part of Fig. 1. They have fungicidal activity only against diseases caused by Basidiomycetes, such as rusts, and Rhizoctonia diseases. Newer SDHIs, such as boscalid and penthiopyrad, are shown in the lower part of Fig. 1. In contrast to early SDHIs, they are characterized by broad-spectrum antifungal activity and can control diseases caused by Ascomycetes and Basidiomycetes. Previously, we reported high fungicidal activity of penthiopyrad (1) against various pathogens.6,7) During our study of this compound, positional isomers of various aromatic heterocyclic carboxamides showed large differences in their activity against gray mold. A similar difference was observed in fungicidal activity among positional isomers of N-(1,1,3-trimethylindan-4-yl) heta-
erocyclic carboxamide derivatives. In this study, we describe fungicidal properties of positional isomers in various aromatic heterocyclic carboxamides and show that the difference in activity can be accounted for by the interaction between carboxamides and the highly conserved histidine residue of complex II, which is involved in binding carboxamides.

**Materials and Methods**

1. **Preparation of compounds**
Melting points (mp) were measured with a Mettler FP62 melting point apparatus. IR spectra were obtained using the JASCO FT-IR-7300 spectrometer. $^1$H-NMR spectra were obtained using the JEOL JNM-A400 FT-NMR system at 400 MHz using tetramethylsilane as the internal standard.

Preparation, mp, IR spectral data, and $^1$H-NMR spectral data of compounds 1, 2, 5, 6, 8, 9, 12, 13, 16, and 17 have been described previously. Other carboxamide derivatives were prepared by the reaction of corresponding carboxylic acids and amines. Table S1 shows their mps, IR spectral data, and $^1$H-NMR spectral data.

2. **Biological evaluation for protection against kidney bean gray mold**
Each compound was dissolved in acetone, diluted with water, and sprayed on kidney beans (Phaseolus vulgaris, cv. Green Top, Tokita Seed Co., Ltd., Japan) at the cotyledonal stage until runoff of the test solution was observed. After the dried leaves were cut and placed in plastic cups, they were covered with wet paper discs to maintain humidity. Spores of gray mold (B. cinerea, MCAG stock culture No. 40212) were collected in a potato sucrose broth medium, and the paper discs were inoculated by soaking them in the spore suspension ($1 \times 10^5$ spores/mL). The plastic cups were kept in dark conditions at 20°C. Four days after inoculation, the size of each gray mold lesion was measured, and the protective value was calculated by the following formula:

$$\text{Protective value} (%) = \left[ \frac{(\text{diameter of lesion on untreated leaf} - \text{diameter of lesion on treated leaf})}{(\text{diameter of lesion on untreated leaf})} \right] \times 100$$

0: Protective value is less than 95% at 500 ppm.
1: Protective value is more than 95% at 500 ppm and less than 50% at 62.5 ppm.
2: Protective value is more than 50% and less than 95% at 62.5 ppm.
3: Protective value is more than 95% at 62.5 ppm.

Fungicidal activity of each compound is symbolized with ratings of 0, 1, 2, or 3 based on the determined protective value of each concentration.

3. **Homology modeling of SDH**
All calculations were carried out in the Discovery Studio 3.1 (DS) environment using CHARMM force field with the Momany-Rone partial charge estimation method except for the partial charges of iron–sulfur clusters. The partial charges of iron–sulfur cluster atoms were determined from electrostatic potential fit charges in a single point QM/MM calculation with only iron–sulfur cluster atoms as the quantum calculation part (PBE/DND level) using the Calculate Energy (QM-MM) protocol in DS. SDH consists of four subunits: SDHA, SDHB, SDHC, and SDHD. A homology model of SDH of B. cinerea has been generated using the standard settings of the Build Homology Model protocol in DS with carboxin-bound SDH crystal structure (PDB ID 2WQY) as a template. The amino acid sequence alignments between the template and the targets (ACL50597.1 for SDHA, AAW52508.1 for SDHB, ACT83440.1 for SDHC, and ACT83438.1 for SDHD) were performed using the BLOSUM62 substitution matrix. Twenty homology models with co-crystallized carboxin were constructed and the model with the lowest PDF total energy was selected for next refinement in the Prepare Protein protocol in DS.

The imidazole ring may be rotated by 180 degrees with respect to the PDB file's coordinates because of the inability to distinguish the oxygen and nitrogen atoms in X-ray crystallography at common resolutions. Therefore, two conformations were considered in the side chain of histidine residue (H272) at the SDHB subunit, which directly interacted with carboxin in the crystal structure. One conformation (Model-A) is the same as it is in the X-ray structure, and the other conformation (Model-B) shows a 180-degree flip at the imidazole ring of H272. To define the stable tautomeric form for H272, the free-energy differences among histidine tautomers were estimated using the Calculate Mutation Energy protocol in DS. From these calculations, the imidazole ring of H272 with Model-A conformation was assigned to the neutral Nε2-H tautomeric form, and the imidazole ring of H272 with Model-B conformation was assigned to the neutral Nơ1-H tautomeric form. Finally, carboxin-bound SDH homology models were energy minimized with the condition that only the side chain atoms of H272 in each conformation were allowed to move with a distance-dependent dielectric constant ($\varepsilon = R$, where $R$ is distance). After energy minimization in the Model-B structure, a hydrogen bond was formed between the oxygen atom of the oxathiin ring in carboxin and the hydrogen atom (Nơ1-H) of the imidazole ring in H272.

4. **Docking study**
Carboxamides were flexibly docked into the rigid SDH homology models by employing the CDOCKER module in DS. The top ten resulting poses ranked by CDOCKER score were visually inspected, and the most plausible pose was determined. The selected pose for each carboxamide was energy minimized using the In situ Ligand Minimization protocol in DS in a rigid SDH homology model. For evaluation of the binding affinity, the piecewise linear potential (PLP1) scoring method implemented in DS was applied.
Results and Discussion

1. Difference in fungicidal activity between two positional isomers of aromatic heterocyclic carboxylic acids

Fungicidal activity of various N-(biphenyl-2-yl) heterocyclic carboxamides (Type A) and N-[2-(1,3-dimethylbutyl)-3-thienyl] heterocyclic carboxamides (Type B) was evaluated (Table 1). With regard to Type A compounds, 1-methyl-3-CF₃-pyrazole-4-carboxamide (2) exhibited high activity against gray mold, while its 1-methyl-5-CF₃ derivative (3) had no activity. Moreover, the thiazole derivative (5) exhibited high activity while its isomer (7) exhibited no activity. Methyl-substituted thiophene (8) and furan (12) derivatives also exhibited high activity against gray mold, while their corresponding isomers (10, 14) exhibited low or no activity. A similar structure-activity relationship was observed with Type B compounds as shown in Table 1.

2. Interaction between carboxamide isomers and fungal SDH

Structures of carboxin-inhibited SDH were determined previously. In these structures, the methyl-oxathiin ring in carboxin lies close to the histidine residue (H272) of the SDH iron–sulfur protein (SDHB). This histidine residue is conserved among many species, and replacement of H272 by tyrosine has been reported to confer resistance to carboxin and boscalid.

In our docking study, carboxamides with proton acceptors at the γ position (compounds 2, 5, 12, and 16) formed hydrogen bonds with the Nδ atom of H272 and had better scores with Model-B than they did with Model-A. This hydrogen bond
Table 2. PLP1 scores obtained by docking of carboxamides into a homology model of succinate dehydrogenases from B. cinerea

<table>
<thead>
<tr>
<th>Compound No.</th>
<th>PLP1 Score&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fungicidal Activity against gray mold</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Model-A</td>
<td>Model-B</td>
</tr>
<tr>
<td>2</td>
<td>−77.05</td>
<td>−82.82</td>
</tr>
<tr>
<td>3</td>
<td>−52.53</td>
<td>−52.31</td>
</tr>
<tr>
<td>5</td>
<td>−78.86</td>
<td>−85.93</td>
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<td>7</td>
<td>−69.78</td>
<td>−67.07</td>
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<tr>
<td>8</td>
<td>−75.35</td>
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<tr>
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<td>−69.24</td>
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<tr>
<td>12</td>
<td>−74.59</td>
<td>−77.04</td>
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<tr>
<td>14</td>
<td>−73.27</td>
<td>−68.28</td>
</tr>
<tr>
<td>16</td>
<td>−79.24</td>
<td>−82.01</td>
</tr>
<tr>
<td>18</td>
<td>−72.39</td>
<td>−67.78</td>
</tr>
</tbody>
</table>

<sup>a</sup> Lower PLP1 scores indicate stronger receptor–ligand binding.

Fig. 4. Schematic diagrams illustrating the proposed interaction of carboxamides with H272 of SDH. The side chain of H272 of SDH is shown. The groups attached to the nitrogen atom of the carbamoyl moiety are abbreviated for clarity (right part in each figure). The hydrogen bond and CH–π interaction are represented by dotted lines and a dotted arrow respectively. The solid arrow indicates the interaction between π electrons from the imidazole ring and lone-pair electrons from the sulfur atom. (A) H272 forms a hydrogen bond with carboxamides having proton acceptors at the γ position. (B) H272 sterically collides with carboxamides having substituents at the γ position. (C) H272 forms CH–π interactions with carboxamides having CH at the γ position. (D) H272 forms an unfavorable hydrogen bond with the sulfur atom in carboxamides. (E) H272 has an electrostatic repulsive interaction with the sulfur atom in carboxamides.

may contribute to the strong binding of carboxamides to SDH, which resulted in their high fungicidal activity (see Fig. 4A).

The carboxamide with an N-methyl substituent at the γ position (compound 3) showed the worst score among the calculated compounds (Table 2). Compound 3 slightly preferred docking into the Model-B structure due to avoiding collision between the γ substituent and the side chain of H272 (see Fig. 4B). However, rotation of the imidazole ring may not be sufficient to avoid this collision; hence, this insufficiency leads to poor binding with SDH and abolished fungicidal activity.

Carboxamides with CH at the γ position (compounds 8 and 14) got better scores with the Model-A structure where a CH–π interaction was formed between the imidazole ring of H272 and the heterocyclic ring of the carboxamides (see Figs. 2A and 4C). This CH–π interaction is weak but favorable<sup>26</sup> and is expected to lead to moderate fungicidal activity against gray mold.

Table 2 continued...

shown in Table 1, these carboxamides with CH at the γ position, such as 8 and 14, show large differences in activity against gray mold. We speculate that this favorable, albeit weak, interaction at the binding site reveals the presence of other factors (e.g., absorption, distribution, metabolism, and excretion) that may affect activity against gray mold.

Carboxamides with a sulfur atom at the γ position (compounds 7, 10, and 18) resulted in worse scores with both Model-A and Model-B than carboxamides with proton acceptors at the γ position (Table 2). These carboxamides were unable to form hydrogen bonds with the Nε atom of SDH H272. This may be due to poor hydrogen bonding of the sulfur atom in thiophene relative to the oxygen atom in furan.<sup>26</sup> Furthermore, the van der Waals volume of the sulfur atom in thiophene is too large to form a stable hydrogen bond with the imidazole ring of H272 (Fig. 4D). Moreover, when the imidazole ring of H272 adopts the orthogonal conformation and orients to the thiophene ring, the lone pair of electrons from the sulfur atom directly repels the π-electrons of the imidazole ring (Fig. 4E). Therefore, when the sulfur atom is located at the γ position, carboxamides may not interact favorably with H272, resulting in low fungicidal activity.

In summary, we synthesized positional isomers of aromatic heterocyclic carboxamides and examined differences in activity related to heterocyclic rings. Based on our docking study, we elucidated the difference in fungicidal activity between positional isomers of heterocyclic rings. Our proposed interaction model will help to understand the structure–activity relationships and the underlying resistance mechanisms of this class of fungicides.

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References
