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Brief Report

Synthesis and fungicidal activities of positional isomers of the *N*-thienylcarboxamide

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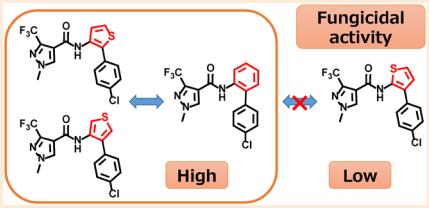
Supplementary material

To investigate the effects of bioisosteric replacement of the phenyl group with the thienyl group, *N*-phenylcarboxamide and three regioisomers of *N*-(substituted-thienyl)carboxamide were synthesized. The inhibitory activity on the succinate dehydrogenase prepared from the gray mold *Botrytis cinerea* as well as the fungicidal activity against *B. cinerea* were evaluated. Two isomers, *N*-(2-substituted-3-thienyl)carboxamide and *N*-(4-substituted-3-thienyl) carboxamide exhibited the same level of activity as the phenyl derivative, whereas *N*-(3-substituted-2-thienyl)carboxamide exhibited lower activity than the phenyl derivative, suggesting that the 2-substituted-3-thienyl and 4-substituted-3-thienyl groups functioned as bioisosteres of the phenyl group in *N*-phenylcarboxamide, but the other did not.



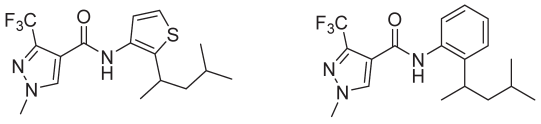
Keywords: penthiopyrad, thiophene, positional isomers, bioisostere, succinate dehydrogenase inhibitor (SDHI).

Introduction

Penthiopyrad (**1**; Fig. 1) is a heterocyclic carboxamide fungicide that was developed by Mitsui Chemicals Agro, Inc., and it exhibits high activity against various plant pathogens that belong to the Ascomycota and Basidiomycota phyla.[1,2] Penthiopyrad is classified as a succinate dehydrogenase inhibitor (SDHI), which inhibits mitochondrial respiration in plant pathogenic fungi by binding to succinate dehydrogenase (SDH).[3,4] Penthiopyrad consists of a pyrazole carbonyl moiety and a thiophene amine moiety. With regard to the pyrazole carbonyl moiety, there is a large difference in the activity against gray mold between positional isomers, and this difference has been attributed to the difference in binding affinities to SDH in a docking study.[5]

With regard to the thiophene amine moiety, penthiopyrad (**1**) and the corresponding *N*-phenyl compound, *N*-[2-(1,3-dimethylbutyl)]phenyl-1-methyl-3-(trifluoromethyl)-pyrazole-4-carboxamide (**2**), exhibit the same level of activity against various diseases, suggesting that the 2-substituted-3-thienyl group can function as a bioisostere of the 2-substituted-phenyl group.[1] As a thienyl group with a substituent in the *ortho* position of the amino group, three possible regioisomers are permitted (*i.e.*, the 2-substituted-3-thienyl, 4-substituted-3-thienyl, and 3-substituted-2-thienyl groups; Fig. 2). Thus, it is an interesting question whether these isomers can be bioisosteres of the phenyl group similar to the 2-substituted-3-thienyl group.

Several studies that compared the biological activities of the two positional isomers of thiophene have been reported.[6–9] However, few studies have compared the biological activities of the three positional isomers of thiophene with two or more sub-



1: penthiopyrad　　**2**

Fig. 1. Penthiopyrad and relevant compound.

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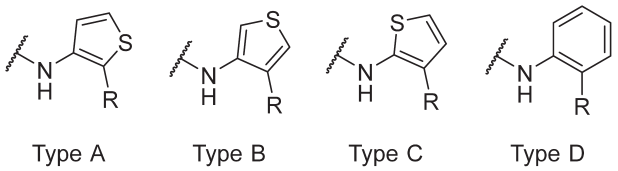


Fig. 2. Three thiophene regioisomers and corresponding phenyl derivative.

stituents at the thiophene ring,[10] and it has been reported that one of the three isomers could not be synthesized due to the instability of its intermediate.[11] Because bioisosterism has the potential to play an important role in the rational modification of lead compounds into safer and more effective agents, it is scientifically significant to investigate the biological activities of the three thiophene regioisomers in detail.

In this study, we used *N*-[2-(substituted)phenyl-3-thienyl]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamides, which have the same level of activity as penthiopyrad against gray mold,[1,2] as model compounds, and two other thiophene regioisomers were synthesized to evaluate the differences in their fungicidal activities. Additionally, to determine the factors that cause the difference in fungicidal activity, *in vivo* and *in vitro* antifungal activities against *Botrytis cinerea* (which is a pathogen of gray mold), SDH inhibitory activity, and the stability of each regioisomer in water were examined.

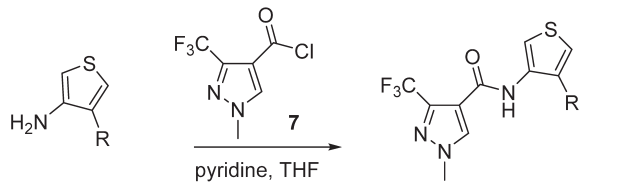
Materials and methods

1. Preparation of compounds

Melting points were measured using a Mettler FP62 melting-point apparatus (Mettler-Toledo, Greifensee, Switzerland). IR spectra were obtained using a JASCO FT-IR-7300 spectrometer (JASCO, Tokyo, Japan). ^{1}H-NMR spectra were obtained using a JEOL JNM-Λ400 FT-NMR system at 400 MHz (JEOL Ltd., Tokyo, Japan) with tetramethylsilane as the internal standard. Melting-point, IR spectral, and ^{1}H-NMR spectral data of each compound are provided in Supplemental Table S1.

1.1. 3-Amino-2-substituted thiophene derivatives

Penthiopyrad (**1**), *N*-(2-phenyl-3-thienyl)-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide (**3**), and *N*-[2-(4-chlorophenyl)-3-thienyl]-1-methyl-3-(trifluoromethyl)-pyrazole-4-carboxamide (**4**) were synthesized according to the method in the literature.[1]



5a (R=phenyl)
6a (R=4-chlorophenyl)

5 (R=phenyl)
6 (R=4-chlorophenyl)

Scheme 1.

1.2. 4-Amino-3-substituted thiophene derivatives (*Scheme 1*)

1.2.1. N-*[3-(4-Chlorophenyl-4-thienyl)]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide (**6**)*

1-Methyl-3-(trifluoromethyl)pyrazole-4-carbonyl chloride (**7**), which was prepared from 1-methyl-3-(trifluoromethyl)pyrazole-4-carboxylic acid (0.38 g, 1.96 mmol) and thionyl chloride (1 mL), was added to a solution of 3-amino-4-(4-chlorophenyl)-thiophene[12] (**6a**; 0.41 g, 1.96 mmol) in pyridine (3 mL) at room temperature. The reaction mixture was stirred for 1 hr at room temperature and then poured into a 5% hydrochloric acid solution (20 mL) and extracted with ethyl acetate (20 mL). The organic layer was washed successively with saturated sodium hydrogen carbonate solution and brine, then dried over anhydrous sodium sulfate. The solvent was distilled off under reduced pressure, and finally, compound **6** (0.53 g, 70%) was obtained as a white solid *via* silica gel purification (hexane : ethyl acetate=7 : 3).

1.2.2. N-*(3-Phenyl-4-thienyl)-1-methyl-3-(trifluoromethyl) pyrazole-4-carboxamide (**5**)*

Compound **5** was synthesized from 3-amino-4-phenylthiophene (**5a**)[12] using a method similar to that described in Section 1.2.1.

1.3. 2-Amino-3-substituted thiophene derivatives (*Scheme 2*)

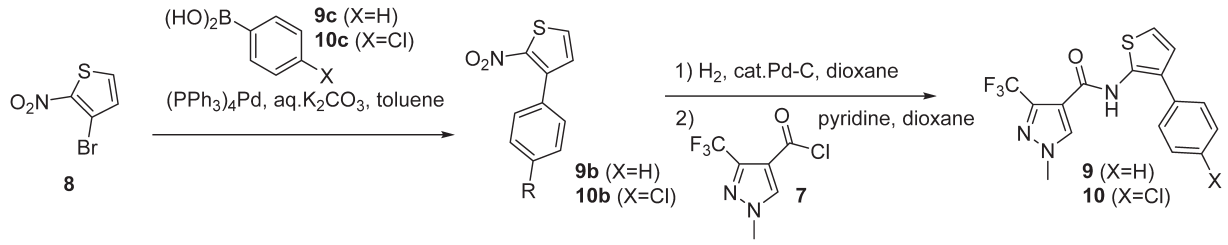
1.3.1. N-*[3-(4-Chlorophenyl)-2-thienyl]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide (**10**)*

*1.3.1.1. 2-Nitro-3-(4-chlorophenyl) thiophene (**10b**)*

Tetrakis(triphenylphosphine)palladium (0.64 g, 0.56 mmol) was added under nitrogen to a solution of 3-bromo-2-nitorothiophene (**8**; 2.0 g, 9.61 mmol), 4-chlorophenylboronic acid (**10c**, 1.50 g, 9.61 mmol), ethanol (5 mL), and 2 M potassium carbonate solution (10 mL) in toluene (60 mL). The mixture was stirred under reflux for 7 hr under nitrogen. The cooled mixture was washed with water (40 mL), and the organic layer was dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was purified using silica gel (hexane : ethyl acetate=10 : 1) to produce the compound **10b** (2.1 g, 91%) as a yellow solid.

*1.3.1.2. N-[3-(4-Chlorophenyl)-2-thienyl]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide (**10**)*

To convert 2-nitro-3-(4-chlorophenyl)thiophene (**10b**) to 2-amino-3-(4-chlorophenyl)phenylthiophene, we first tried nitro reduction with iron under acidic and heating conditions (Bechamp reduction) used for synthesizing 3-amino-2-phenylthiophene in high yield.[1] However, only a complicated reaction mixture was obtained as determined *via* TLC analysis, and the desired amino compound could not be obtained under those conditions. This fact suggests that 2-amino-3-(4-chlorophenyl)-thiophene is unstable upon Bechamp reduction. Thus, we performed a catalytic hydrogenation reaction using Pd/C, which is a milder reaction condition as compared to Bechamp reduction, and carried out the next amidation without isolating the obtained 2-amino-3-(4-chlorophenyl)thiophene. We describe the detailed procedure below. 2-Nitro-3-(4-chlorophenyl)-thiophene (**10b**; 0.5 g, 2.09 mmol) and 1.0 g of 5% Pd/C (50% wet) were added to 1,4-dioxane (20 mL) and stirred under a hy-



Scheme 2.

drogen atmosphere at room temperature for 11 hr, and the reaction mixture was filtrated to remove the Pd/C catalyst. Pyridine (1.65 g, 20.9 mmol) and 1-methyl-3-(trifluoromethyl)pyrazole-4-carbonyl chloride (**7**), which was prepared from 1-methyl-3-(trifluoromethyl)pyrazole-4-carboxylic acid (0.49 g, 2.53 mmol) and thionyl chloride (2 mL), were added to the filtrate at room temperature, and the resulting mixture was stirred at room temperature for 4 hr. Ethyl acetate (50 mL) was added to the reaction mixture and washed successively with 5% hydrochloric acid solution (30 mL), saturated with sodium hydrogen carbonate solution (30 mL) and brine (30 mL), and then dried over anhydrous sodium sulfate. The solvent was removed *in vacuo*, and the residue was purified using silica gel (hexane : ethyl acetate=7 : 3) to produce the compound **10** (0.36 g, 45%) as a white solid.

1.3.2. N-*(3-Phenyl-2-thienyl)-1-methyl-3-(trifluoromethyl) pyrazole-4-carboxamide (**9**)*

Compound **9** was synthesized from 3-bromo-2-nitorothiophene (**8**) and phenylboronic acid (**9c**) by methods similar to those described in Sections 1.3.1.1. and 1.3.1.2.

1.4. 2-Substituted aniline derivatives

Compound **2**, *N*-(2-phenylphenyl)-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide (**11**), and *N*-[2-(4-chlorophenyl)phenyl]-1-methyl-3-(trifluoromethyl)pyrazole-4-carboxamide (**12**) were synthesized according to the method in the literature.[1,13]

2. Biological evaluation of kidney bean gray mold (in vivo *assay*)

Each compound was dissolved in acetone, diluted with water, and sprayed onto kidney beans (*Phaseolus vulgaris*, variety: Green Top from Tokita Seed Co. Ltd., Japan) at the cotyledonous stage until a runoff of the test solution was observed. Then, after the dried leaves were cut and placed in plastic cups, they were covered with wet paper discs to maintain the humidity. Spores of gray mold (*Botrytis cinerea*, MCAG stock culture No. 40212) were collected in potato sucrose broth medium, and paper discs were inoculated by soaking them in the spore suspension (1×10^5 spores/mL). The plastic cups were kept in the dark at 20°C. Four days after inoculation, the gray mold lesions were measured, and the protective value was calculated using the following formula:

$$\text{Protective value (\%)} = \frac{(\text{Diameter of lesion on untreated leaf} - \text{Diameter of lesion on treated leaf})}{\text{Diameter of lesion on untreated leaf}} \times 100.$$

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

3. Inhibition of mycelium growth (in vitro *assay*)

Botrytis cinerea (MCAG stock culture No. 40226) was precultured on PDA (potato dextrose agar: Difco, Detroit, MI) medium at 24°C for 5 days under dark conditions. A 5 mm^2 disk from precultured mycelia was transferred to a PDA plate containing a test compound at 0.01, 0.1, 1, and 10 ppm, and mycelia were grown at 24°C for 6 days under dark conditions. Each colony was measured, and the inhibition rate of each test compound was calculated and compared with an untreated control.

4. Inhibition of SDH in the cell-free system

4.1. Mitochondria preparation

Mitochondria were extracted from the *B. cinerea* protoplast, which was prepared *via* the following procedure. *Botrytis cinerea* (MCAG stock culture No. 40226) was grown on PDA (Difco) medium under BLB light, and conidial suspensions were collected. The conidial suspension of *B. cinerea* was incubated with 150 mL of modified liquid medium (KH_2PO_4, 2 g; $MgSO_4\cdot7H_2O$, 0.5 g; glucose, 10 g; starch, 10 g; K_2HPO_4, 1.5 g; $(NH_4)_2SO_4$, 1 g; yeast extract (Difco), 2 g; distilled water, 1,000 mL) at 22°C for 23 hr while shaking (115 rpm min^{-1}).[14] The resulting mycelia were harvested by filtration through a nylon mesh (0.63 mm), rinsed with distilled water, and then suspended in 10 mL/g of an enzyme solution (2% Driselase 20 (ASKA Animal Health Co., Ltd.), 0.5% Cellulase Onozuka RS (Yakult HONSYA Co., Ltd.,), 0.25% Zymolyase 20-T (NACALAI TESQUE, Inc.), 2% Meicelase (SERVICETEC JAPAN Corp.), and 0.6 M KCl). After incubation at 37°C for 1 hr, the resulting protoplasts were obtained by centrifugation at 1,500×*g* for 10 min.[15] The protoplast pellet was resuspended in 3 mL of extraction buffer (0.5 M sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% bovine serum albumin, 5 mM cysteine, and 1/15 M sodium phosphate buffer; pH 7.4) and disrupted using a sonicator at 0–4°C.

Table 1. Fungicidal activity of *N*-thienyl carboxamide against gray mold[a)]

R	Fungicidal activity against gray mold			
	Type A	Type B	Type C	Type D
	3 (**1**)	ND[b)]	ND[b)]	3 (**2**)
	3 (**3**)	3 (**5**)	1 (**9**)	3 (**11**)
	3 (**4**)	3 (**6**)	0 (**10**)	3 (**12**)

a) Numbers in parentheses represent compound No. *b*) ND: No Data.

The total mitochondria pellet was collected by centrifugation at 3,000×*g* for 10 min and resuspended in 5 mL of wash buffer (0.5 M sucrose, 1 mM EDTA, and 1/15 M sodium phosphate buffer ; pH 7.4).[16)]

4.2. SDH activity in the cell-free system

The mitochondrial suspension used for the SDH assay was adjusted to 1 μg/mL by measuring the amount of protein using the Bradford reagent (595 nm). SDH inhibitory activity was measured using the MitoCheck Complex II Activity Assay Kit as per the manufacturer's protocol (Cayman Chemical Company, Ann Arbor, MI, USA).

5. Stability in water

Each test compound was dissolved in acetonitrile to prepare a 2,500 ppm solution, and the solution (1 μL) was added to 0.5 mL of buffer solution in a glass vial. The buffer solution (pH 4, 7) was prepared following OECD TG111 (ANNEX3)[17)] except for the pH 11 solution, which was prepared by mixing 0.1 N NaOH solution and 0.2 M borax solution.[18)] The glass vial was sealed with a vial cap and left to stand in a thermostatic chamber at 50°C under dark conditions. After 14 days, the vial was collected from the chamber, and 0.5 mL of acetonitrile was added to the vial to obtain a sample for measurement. The sample (20 μL) was injected into a high-performance liquid chromatography column (Chemicals Evaluation and Research Institute, L-column ODS 5 μm, 4.6×250 mm) maintained at 40°C under isocratic conditions (water : acetonitrile=45 : 55, v/v) with a combined flow rate of 1.0 mL/min. The area of the peak was determined, and the attenuation rate was calculated with the value of day 0 as 100%.

6. Data analysis

Statistical analysis was performed using the GraphPad Prism 6.07 software (GraphPad Prism, San Diego, CA, USA). The IC_{50} and IC_{90} values were determined using a four-parameter logistic Hill equation with an upper constraint of 100% extension and a lower constraint of 0% extension. Data from at least three independent experiments were used with two technical repeats for each condition. One-way analysis of variance followed by Tukey's *post hoc* test was used to assess the significant differences ($p<0.05$) between inhibitors.

Results and discussion

1. Difference in fungicidal activity among the positional isomers of N-(*substituted-thienyl*)*carboxamides determined by* in vivo *assay*

The *in vivo* fungicidal activities of various positional isomers against gray mold with regard to *N*-(substituted-thienyl)-carboxamides and the corresponding *N*-(2-substituted-phenyl)-carboxamides are shown in Table 1. We selected the phenyl or 4-chlorophenyl group as the substituent because these substituents are expected to show high activity against gray mold.[1,2)]

Regarding the two substituents of the thiophene ring, *N*-(2-substituted-3-thienyl)carboxamides (Type A) and *N*-(3-substituted-4-thienyl)carboxamides (Type B) showed high activity against gray mold at a level similar to that of *N*-(2-substitituted-phenyl)carboxamides (Type D). Their activities are on the same level as penthiopyrad (**1**) and the corresponding phenyl compound (**2**). These results indicated that Types A and B can be bioisosteres of the corresponding phenyl compound (Type D). However, Type C compounds (**9** and **10**) exhibited lower activity than the other compounds (Types A, B, and D). The low fungicidal activity observed for Type C compounds was

Table 2. Antifungal activity of *N*-(4-chlorophenyl-thienyl)carboxamide against *B. cinerea*

No.	Type	IC_{50} (ppm)[a)]	IC_{90} (ppm)[a)]
4	A	0.271±0.0907[a]	1.53±0.602[a]
6	B	0.514±0.122[a]	1.62±0.402[a]
10	C	8.63±0.131[b]	84.6±78.6[b]
12	D	0.418±0.100[a]	1.63±0.501[a]

a): Letters (a, b) devote homogenous subsets at $p<0.05$. Data are expressed as mean±S.D.

Table 3. SDH inhibitory activity of *N*-(4-chlorophenyl-thienyl)carboxamide

No.	Type	IC_{50} (ppm)[a)]
4	A	2.57±0.851[a,b]
6	B	1.01±0.715[a]
10	C	7.41±2.36[b]
12	D	0.978±0.180[a]

a): Letters (a, b) devote homogenous subsets at $p<0.05$. Data are expressed as mean±S.D.

Table 4. Stability of *N*-(4-chlorophenyl-thienyl)carboxamide in various pH solution

No.	Type	Recovery rate (%)[a)]		
		pH 4	pH 7	pH 11
4	A	101.8±2.6	101.8±3.7	94.8±6.2
6	B	102.3±3.1	100.5±0.4	101.9±3.2
10	C	98.7±1.2	99.4±0.1	56.0±2.1
12	D	103.4±9.4	102.4±2.9	100.1±8.5

a): Incubation temperature; 50°C, Incubation period; 14days. Data are expressed as mean±S.D. from two experiments, each done in duplicate.

very interesting because the three-dimensional (3D) structure of Type C was presumed to be almost the same as those of Types A and B. Therefore, we conducted a series of studies to find the cause of the lower *in vivo* fungicidal activity of Type C compounds.

2. Difference in fungicidal activity in vitro *against* B. cinerea

The *in vitro* antifungal activity of each isomer was examined to confirm the direct effect of the compounds against the pathogen without the influence of the plants. The IC_{50} and IC_{90} values of each isomer against *B. cinerea*, which causes gray mold disease, are listed in Table 2.

Compounds **4** (Type A), **6** (Type B), and **12** (Type D), which exhibited high fungicidal activity *in vivo*, had almost the same *in vitro* IC_{50} and IC_{90} values, and no significant differences in the IC_{50} and IC_{90} values ($P<0.05$) in Tukey's *post hoc* test were observed. However, compound **10** (Type C), which exhibited lower activity *in vivo*, also exhibited low activity *in vitro*; *i.e.*, its IC_{50} and IC_{90} values were approximately 21-fold and 52-fold higher than those of the phenyl derivative (Type D), respectively. These results indicated that one of the reasons for the difference in activity against gray mold *in vivo* is the difference in direct antifungal activity against *B. cinerea*.

3. Difference in SDH inhibitory activity

We examined whether the difference in antifungal activity against *B. cinerea* was due to the difference in inhibitory activity against SDH, which is the action site of the test compounds. The inhibitory activities of the isomers on SDH are listed in Table 3.

The inhibitory activity of SDH was high in the order of compounds **12**, **6**, **4**, and **10**. Among them, compounds **4**, **6**, and **12** showed no significant difference in IC_{50} values ($p<0.05$) in Tukey's *post hoc* test. The SDH inhibitory activity of compound **10**, which exhibited lower activity in both the *in vivo* and *in vitro* tests, was significantly lower than that of compounds **6** and **12** in Tukey's *post hoc* test at the 95% confidence level. These results agree with the *in vivo* and *in vitro* test results. However, the difference in the IC_{50} of SDH between compounds **10** and **4** is relatively small (approximately 3 times) and not significant in Tukey's *post hoc* test at the 95% confidence level, whereas the difference in *in vitro* activity between compounds **10** and **4** is approximately 32 times. Thus, the SDH inhibitory activity seems insufficient to explain the difference in fungicidal activity between the two compounds.

4. Difference in chemical stability

The difference in activity between compound **10** and compounds **4**, **6**, and **12** in the *in vivo* and *in vitro* tests was likely influenced by differences in metabolic detoxification or chemical stability of each compound as well as the difference in activity at the action site. Therefore, the differences in chemical stability, *i.e.*, the stabilities of compounds **4**, **6**, **10**, and **12** in water, were compared. Because the *in vivo* test was conducted under dark conditions, this stability experiment was conducted under dark conditions as well. The results are provided in Table 4.

Compounds **4**, **6**, and **12**, which exhibited high activity both *in vivo* and *in vitro*, showed a high recovery rate at each pH, indicating that the chemical stability of these compounds was relatively high. However, compound **10**, whose *in vivo* and *in vitro* activities were considerably lower, showed a lower recovery rate under alkaline conditions of pH 11, though a high water stability at pH 4 and 7 was observed. These results indicated that the chemical stability of compound **10** was relatively low. It is uncertain whether this instability under alkaline conditions is directly related to the lower activity *in vivo*, but compound **10** may be more susceptible to some chemical reactions, including metabolic detoxification, than the other isomers. Further information could be obtained by examining the metabolites and degradation products of each isomer.

We described in Section 1.3.1.2 that 2-amino-3-(4-chlorophenyl)thiophene, an intermediate of compound **10**, is unstable under Bechamp reduction conditions. Although compound **10** seems to have been stabilized to some extent by covalent binding of an amine to the pyrazolyl carbonyl group, which is an electron-withdrawing group, the low stability of 2-amino-3-phenylthiophene as a partial structure might be one factor that lowers the stability of the entire compound.

Conclusion

In this study, we synthesized three types of positional isomers of the thiophene ring that were expected to be the bioisosteres of the phenyl ring and investigated their biological activities in detail. The *in vivo* fungicidal activity of each positional isomer was evaluated. *N*-(2-substitututed-3-thienyl)carboxamides (Type A) and *N*-(4-substituted-3-thienyl)carboxamides (Type

B) exhibited the same activity levels as the corresponding *N*-(2-substituted)-phenylcarboxamides (Type D). However, the *N*-(3-substituted-2-thienyl)carboxamides (Type C) showed significantly lower activity as compared to the other isomers. Therefore, the *in vitro* fungicidal activity of each positional isomer was evaluated. The Type C isomer, which showed lower activity *in vivo*, exhibited a lower activity *in vitro* than the other isomers, which is consistent with the *in vivo* fungicidal activity. The Type C isomer showed significantly lower inhibitory activity on SDH as compared to the other isomers. These results were qualitatively consistent with the *in vivo* and *in vitro* data.

The Type C isomer was significantly less stable in water than the other isomers under alkaline conditions. The Type C isomer exhibited lower fungicidal activity both *in vivo* and *in vitro* than other isomers, likely owing to multiple factors, such as inhibitory activity at the action site and compound stability.

In summary, we investigated whether a thiophene ring can become a bioisostere for a benzene ring for fungicidal activity against plant pathogens *in vivo* and *in vitro*. We also evaluated the inhibitory activity at the action site. Two of the thiophene regioisomers could act as bioisosteres for the benzene ring, whereas one isomer could not. Future studies, such as examining the metabolites of each isomer in detail, may clarify the factors that cause the difference in fungicidal activity between each regioisomer.

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Electronic supplementary materials

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

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