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Original Article

Resistance buster compounds for MBI-D insensitive rice blast fungus —Inquiry on effective compounds among derivatives of MBI-D fungicides—

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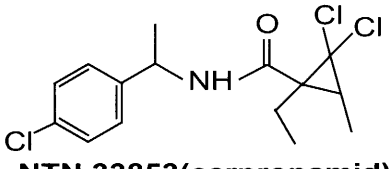
Effective molecules against resistant strains of *Pyricularia oyzae* to the melanin biosynthesis inhibitors (MBI-D) were designed by modifying the amine and acid parts of carpropamid {(1*RS*,3*SR*)-2,2-dichloro-*N*-[(*R*)-1-(4-chlorophenyl)ethyl]-1-ethyl-3-methylcyclopropanecarboxamide}. Substitution of the 1-phenylethylamine moiety with a 2-phenylethylamine increased the inhibitory activity against MBI-D-resistant strains. Reduction of the bulkiness of the amine part by replacing the benzene ring of 2-phenylethylamine with thiophene was effective to improve the activity. Among the derivatives, 5-chloro-3-ethylthiophene compounds showed the highest efficacy. Through the studies regarding structure–activity relationship of the compounds with five-membered heterocyclic rings, the discovery of new resistance buster compounds could be prospective. ©Pesticide Science Society of Japan

Keywords: resistant strains of MBI-D fungicides, resistance buster compounds, chemical modification of MBI-D compounds.

Introduction

Melanization in appressoria is essential for the virulence of *Pyricularia oryzae*.[1] Two types of enzyme, reductase and dehydratase, function in fungal melanin biosynthesis,[2] and are good targets for developing rice blast control agents. PCBA[3] and fthalide[4] were developed in the 1960's as the first melanin biosynthesis inhibitors followed by two more compounds, tricyclazole[5] and pyroquilon[6] in the 1970's. Although the functions of these compounds have been unknown for a while, they were widely used as potent fungicides for rice blast disease in Japan. All compounds developed in the 1960's and 1970's were ascertained to be inhibitors of reductase for melanin biosynthesis (MBI-R).[7]

A new class of melanin biosynthesis inhibitors acting on dehydratase (MBI-D) has been anticipated for some time. Carpropamid {(1*RS*,3*SR*)-2,2-dichloro-*N*-[(*R*)-1-(4-chlorophenyl)ethyl]-1-ethyl-3-methylcyclopropane-carboxamide}, the first MBI-D fungicide for rice blast disease was found in the 1980's. The compound was registered in 1997 and launched on the market as the first MBI-D fungicide.[8–10] Carpropamid has been widely used in Japan since 1998, because of its outstanding long-term control of rice blast disease. Subsequently, diclocymet[11] and fenoxanil,[12] compounds with the same mode of action as carpropamid, were developed in 2000 and 2001, respectively.



NTN 33853(carpropamid)

In 2001, carpropamid treatment by nursery box application, however, did not work well, and severe leaf and panicle blast infections appeared in a restricted area in the Matsuura River

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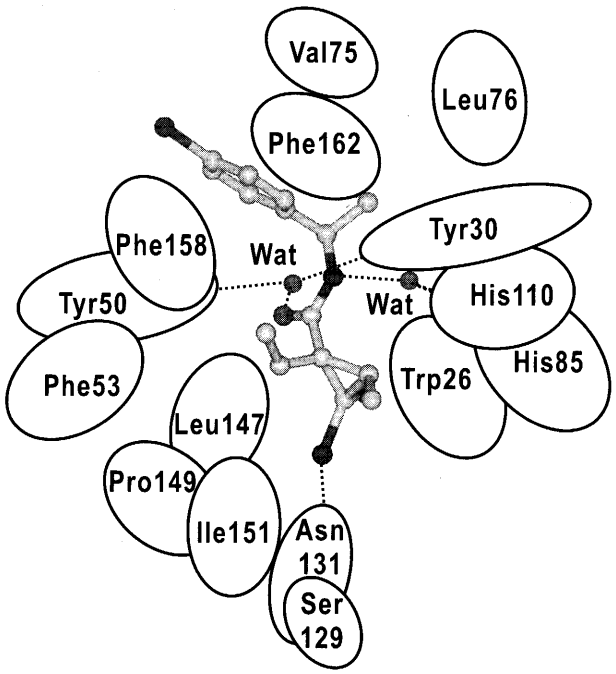


Fig. 1. The structure of carpropamid (ball-and-stick model) in a complex with wild-type scytalone dehydratase (the accession code of Protein Data Bank: 2STD).[16] The side chains of amino acid residues forming the active-site pocket are shown as stick models. The two small spheres are hydration water molecules. The dotted lines indicate possible hydrogen bonds between carpropamid and the side chains.

Basin in Saga Prefecture. Many strains of *Pyricularia oryzae* isolated from blast lesions in a carpropamid-treated field exhibited low sensitivity to the compound and showed cross-resistances to other MBI-D fungicides.[13]

The cause of the resistance was identified as a point mutation in the gene encoding scytalone dehydratase (SDH) determined through systematic genomic analyses. Namely, valine is mutated to methionine in the SDH of resistant strains (Val75Met).[14,15] In the crystal structure of the SDH–carpropamid complex, valine 75 at the tip of the C-helix forms the mouth of a binding pocket (Fig. 1).[16] MBI-D fungicides are tight-binding inhibitors, and their phenyl ring tightly bind with the valine residue.[17] Because methionine has a larger side chain than valine, it is inferred that the side chain of methionine protrudes toward an active pocket.[18] Therefore, the binding affinity of MBI-D fungicide molecules for an active cavity of mutant SDH is drastically reduced. However, because the enzymatic activity for dehydrating scytalone to 1,3,8-trihydroxynaphthalene is not severely reduced in mutant SDH,[18] the mutant is expected to have little influence on the enzyme's structure around the active center. Thus, the pathogenicity of the resistant strains is maintained.

The chemical structure of carpropamid is divided into two parts, the amine part, 1-(4-chlorophenyl)ethylamine, and the acid part, 2,2-dichloro-1-ethyl-3-methyl-cyclopropanecarboxylic acid. In this study, chemical modifications of each part were carried out, and many new derivatives of MBI-D were synthesized and examined for their controlling effect on resistant and susceptible *Pyricularia oryzae* strains. In addition, the inhibitory activity of the new derivatives was examined *in vitro* for recombinant wild-type and Val75Met-mutated enzymes. In this paper, we present the structure–activity relationships of these derivatives and candidates for novel resistance buster fungicides.

Materials and Methods

1. Chemicals

1.1. Standard compounds

Tricylazole and carpropamid were used as standards. In the enzyme assays, the active isomer (KTU 3616b) of carpropamid was used as a reference. Carpropamid and its isomer were supplied by Bayer Crop Science. Tricyclazole was provided by Kumiai Chemical Co.

1.2. Derivatives of MBI-D blasticides

All the derivatives of MBI-D were synthesized at Gifu University. The derivatives were designed by inspecting the chemical structures of carpropamid and diclocymet.

1.2.1. Modification of amine moiety

Amides of 2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxylic acid, the acid form of carpropamid, were synthesized. The amides were classified into the following five groups according to structure.

a-1) alkyl-, cycloalkylmethyl- and trifluoromethylamines. (**1**–**7**).

a-2) 4-chloroaniline, 4-chlorobenzylamine, 1- or 2-(4-chlorophenyl)ethylamines and 1-, 2- or 3-(4-chlorophenyl)propylamines. (**9**–**15**)

a-3) 2-phenylethylamine derivatives having various substituents at different positions on the benzene ring. (**16**–**24**)

b-1) 1- or -2-thienylethylamines, in which the thiophene ring is substituted with one or two chlorine atoms or a bromine atom. (**25**–**35**)

b-2) 2-(chloro-substituted thiazolyl)ethylamines. (**36**–**37**)

1.2.2. Modification of carboxylic acid moiety

The amine part was fixed with 5-chloro-3-thienylethyl amine. The carboxylic acids are classified into two groups.

c-1) 2,2-dichlorocyclopropanecarboxylic acid, in which the ring is alkylated at its 1- and/or 3-position. (**38**–**42**)

c-2) 3,3-dimethylbutyric acid, in which the 2-position is substituted with a cyano, a chlorine or a bromine atom. (**43**–**45**)

1.2.3. Preparation of compounds

All melting points (mp) are uncorrected. NMR spectra were obtained with a Varian Gemini 2000 C/H (400 MHz). The chemical shifts were recorded in δ (ppm) and the coupling constants J in Hz. Mass spectra were recorded using a Joel JMS-700.

The final products were prepared from acid chlorides[11,19]

and the corresponding amines. The representative entry of new amines started with the reduction of arylacetic acid using boron-dimethylsulfide. The alcohol was then tosylated and the tosylate was substituted with sodium azide. The reduction of the azide with triphenylphosphine gave the amine.

N-[2-(5-Chloro-3-thienyl)ethyl]-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**26**). To an ice-cold solution of 5-chloro-3-thienylethylamine (161 mg, 1 mmol), triethylamine (152 mg, 1.5 mmol) in toluene (15 ml) was added a solution of 2,2-dichloro-1-ethyl-3-methylcyclopropanecarbonyl chloride (215 mg, 1 mmol) in toluene (5 ml) dropwise. Stirring was continued at ambient temperature for 10 hr, then at 80°C for 20 min. After cooling, the mixture was diluted with 15 ml of toluene, and washed with water (30 ml), 1% aq HCl (20 ml) and then brine, and dried over anhydrous magnesium sulfate. The toluene was evaporated and column chromatography of the residual liquid on SiO_2 with *n*-hexane/isopropyl ether 3 : 1 gave 278 mg (82% yield) of product. Mp: 106–107°C. IR (KBr) cm^{-1}: 1635. ^1H NMR δ ($CDCl_3$): 0.92 (3H, t, J=7.3, $CH_2C\underline{H}_3$), 1.20 (3H, d, J=6.6, 3-cyclopropyl-$C\underline{H}_3$), 1.52 (1H, m, $C\underline{H}_{2a}CH_3$), 1.87 (1H, m, $C\underline{H}_{2b}CH_3$), 2.19 (1H, q, J=6.6, 3-cyclopropyl-$\underline{H}$), 2.80 (2H, m, thiophene-$C\underline{H}_2$), 3.57 (2H, q, J=6.6, $CH_2C\underline{H}_2NH$), 5.90 (1H, bs, N$\underline{H}$), 6.80 (2H, s, 2,4-thienyl-$\underline{H}$). EI-MS *m/z* (relative intensity; %): 339 (M^+, 2), 144 (100).

The other products were prepared similarly from the corresponding amines and acid chlorides.

N-(1-Cyclopentylethyl)-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**5**). Mp: 84°C. IR (KBr) cm^{-1}: 1630. ^1H NMR δ ($CDCl_3$): 0.99 (3H, m, $CH_2C\underline{H}_3$,), 1.15–2.00 (17H, overlap, 3-cyclopropyl-$C\underline{H}_3$+cyclopentyl+NCHC$\underline{H}_3$, $C\underline{H}_{2a}CH_3$+$C\underline{H}_{2b}CH_3$), 2.20 (1H, q, J=6.6, 3-cyclopropyl-$\underline{H}$), 3.95 (1H, m, NHC$\underline{H}$), 5.60 (1H, bs, NH). EI-MS *m/z* (relative intensity; %): 291 (M^+, 5), 179 (64), 55 (100).

N-[2-(4-Chlorophenyl)ethyl]-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**11**). Mp: 118°C. IR (KBr) cm^{-1}: 1635. ^1H NMR δ ($CDCl_3$): 0.90 (3H, t, J=7.7, $CH_2C\underline{H}_3$), 1.19 (3H, d, J=6.6, 3-cyclopropyl-$C\underline{H}_3$), 1.52 (1H, m, $C\underline{H}_{2a}CH_3$), 1.85 (1H, m, $C\underline{H}_{2b}CH_3$), 2.18 (1H, q, J=6.6, 3-cyclopropyl-$\underline{H}$), 2.84 (2H, m, $NCH_2C\underline{H}_2$), 3.59 (2H, m, $NC\underline{H}_2CH_2$), 5.84 (1H, bs, N$\underline{H}$), 7.15 (2H, d, J=8.1, *ortho* $\underline{H}$), 7.28 (2H, d, J=8.1, *meta* $\underline{H}$). EI-MS *m/z* (relative intensity; %): 333 (M^+, 13), 138 (100).

N-[2-(2,4-Difluorophenyl)ethyl]-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**17**). Mp: 101°C. IR (KBr) cm^{-1}: 1650. 1H NMR δ ($CDCl_3$): 0.90 (3H, t, J=7.7, $CH_2C\underline{H}_3$), 1.19 (3H, d, J=6.2, 3-cyclopropyl-$C\underline{H}_3$), 1.53 (1H, m, $C\underline{H}_{2a}CH_3$), 1.89 (1H, m, $C\underline{H}_{2b}CH_3$), 2.18 (1H, q, J=6.2, 3-cyclopropyl-$\underline{H}$), 2.87 (2H, t, J=6.5, $NCH_2C\underline{H}_2$), 3.58 (2H, m, $NC\underline{H}_2CH_2$), 5.94 (1H, bs, N$\underline{H}$), 6.80 (2H, m), 7.20 (1H, dd, J=8.1_{H-F}/8.1_{H-F}, phenyl-3-$\underline{H}$). EI-MS *m/z* (relative intensity; %): 337 (M^+, 30), 141 (100).

N-[2-(4-Trifluoromethylphenyl)ethyl]-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**18**). Mp: 113°C. IR (KBr) cm^{-1}: 1645. ^1H NMR δ ($CDCl_3$): 0.89 (3H, t, J=7.5, $CH_2C\underline{H}_3$), 1.19 (3H, d, J=6.6, 3-cyclopropyl-$C\underline{H}_3$), 1.52 (1H, m, $C\underline{H}_{2a}CH_3$), 1.85 (1H, m, $C\underline{H}_{2b}CH_3$), 2.18 (1H, q, J=6.6, 3-cyclopropyl-$\underline{H}$), 2.93 (2H, m, $NCH_2C\underline{H}_2$), 3.63 (2H, m, $NC\underline{H}_2CH_2$), 5.86 (1H, bs, N$\underline{H}$), 7.34 (2H, d, J=8.0, *ortho* $\underline{H}$ to CH_2), 7.28 (2H, d, J=8.0, *meta* $\underline{H}$ to CH_2). EI-MS *m/z* (relative intensity; %): 367 (M^+, 94), 173 (100).

N-[2-(2,4-Dichlorophenyl)ethyl]-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**21**). Mp: 104°C. IR (KBr) cm^{-1}: 1625. ^1H NMR δ ($CDCl_3$): 0.93 (3H, t, J=7.3, $CH_2C\underline{H}_3$), 1.20 (3H, d, J=6.7, 3-cyclopropyl-$C\underline{H}_3$), 1.52 (1H, m, $C\underline{H}_{2a}CH_3$), 1.90 (1H, m, $C\underline{H}_{2b}CH_3$), 2.19 (1H, q, J=6.7, 3-cyclopropyl-$\underline{H}$), 2.98 (2H, m, $NCH_2C\underline{H}_2$), 3.60 (2H, m, $NC\underline{H}_2CH_2$), 5.82 (1H, bs, N$\underline{H}$), 7.21–7.26 (2H, m, aromatic), 7.39 (1H, m, aromatic). EI-MS *m/z* (relative intensity; %): 367 (M^+, 10), 179 (100).

N-[2-(4-Chloro-2-thienyl)ethyl]-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**27**). Mp: 102–105°C. IR (KBr) cm^{-1}: 1635. ^1H NMR δ ($CDCl_3$): 0.93 (3H, t, J=7.4, $CH_2C\underline{H}_3$), 1.20 (3H, d, J=6.6, 3-cyclopropyl-$C\underline{H}_3$), 1.52 (1H, m, $C\underline{H}_{2a}CH_3$), 1.87 (1H, m, $C\underline{H}_{2b}CH_3$), 2.19 (1H, q, J=6.6, 3-cyclopropyl-$\underline{H}$), 3.02 (2H, m, thiophene-$C\underline{H}_2$), 3.60 (2H, m, $CH_2C\underline{H}_2NH$), 5.93 (1H, bs, N$\underline{H}$), 6.74 (1H, d, J=1.5, 3-thienyl-$\underline{H}$), 6.95 (1H, d, J=1.5, 5-thienyl-$\underline{H}$). EI-MS *m/z* (relative intensity; %): 339 (M^+, 14), 144 (100).

N-[2-(4,5-Dichloro-2-thienyl)ethyl]-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**31**). Mp: 100°C. IR (KBr) cm^{-1}: 1635. ^1H NMR δ ($CDCl_3$): 0.92 (3H, t, J=7.3, $CH_2C\underline{H}_3$), 1.18 (3H, d, J=6.6, 3-cyclopropyl-$C\underline{H}_3$), 1.53 (1H, m, $C\underline{H}_{2a}CH_3$), 1.92 (1H, m, $C\underline{H}_{2b}CH_3$), 2.17 (1H, q, J=6.6, 3-cyclopropyl-$\underline{H}$), 2.94 (2H, m, thiophene-$C\underline{H}_2$), 3.54 (2H, m, $CH_2C\underline{H}_2NH$), 6.14 (1H, bs, N$\underline{H}$), 6.63 (1H, s, 3-thienyl-$\underline{H}$). EI-MS *m/z* (relative intensity; %): 375 (M^+, 10), 178 (100).

N-[2-(2-Chloro-4-thiazolyl)ethyl]-2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxamide (**36**). Mp: 55°C. IR (KBr) cm^{-1}: 1650. ^1H NMR δ ($CDCl_3$): 0.92 (3H, t, J=7.7, $CH_2C\underline{H}_3$), 1.21 (3H, d, J=6.6, 3-cyclopropyl-$C\underline{H}_3$), 1.54 (1H, m, $C\underline{H}_{2a}CH_3$), 1.97 (1H, m, $C\underline{H}_{2b}CH_3$), 2.21 (1H, q, J=6.6, 3-cyclopropyl-$\underline{H}$), 2.95 (2H, t, J=6.6, $NCH_2C\underline{H}_2$), 3.68 (2H, m, $NC\underline{H}_2CH_2$), 6.52 (1H, bs, N$\underline{H}$), 6.92 (1H, s, 5-thiazolyl-$\underline{H}$). EI-MS *m/z* (relative intensity; %): 340 (M^+, 28), 145 (100).

N-[2-(5-Chloro-3-thienyl)ethyl]-2,2-dichloro-1-isopropylcyclopropanecarboxamide (**41**). Mp: 108–109°C. IR (KBr) cm^{-1}: 1650. ^1H NMR δ ($CDCl_3$): 1.01 (3H, d, J=6.9, $CH(C\underline{H}_3)_2$), 1.20 (3H, d, J=6.9, $CH(C\underline{H}_3)_2$), 1.33 (1H, d, J=7.4, 3-cyclopropyl-$\underline{H}$), 1.66 (1H, m, $C\underline{H}(CH_3)_2$), 1.87 (1H, d, J=7.4, 3-cyclopropyl-$\underline{H}$), 2.78 (2H, m, thiophene-$C\underline{H}_2$ CH_2), 3.52 (1H, m, $CH_2C\underline{H}_{2a}NH$), 3.58 (1H, m, $CH_2C\underline{H}_{2b}NH$), 5.59 (1H, bs, N$\underline{H}$), 6.78 (2H, s, 2,4-thienyl-$\underline{H}$). EI-MS *m/z* (relative intensity; %): 339 (M^+, 4), 144 (100).

N-[2-(5-Chloro-3-thienyl)ethyl]-2-cyano-3,3-dimethylbutanamide (**43**). Mp: 80–82°C. IR (KBr) cm^{-1}: 2240, 1650. ^1H NMR δ ($CDCl_3$): 1.13 (9H, s, $C(C\underline{H}_3)_3$), 2.79 (2H, t, J=7.0, thiophene-$C\underline{H}_2CH_2$), 3.12 (1H, s, C$\underline{H}$CN), 3.53 (2H, m,

$CH_2C\underline{H}_2NH$), 6.06 (1H, bs, N<u>H</u>), 6.79 (1H, s, 2-thienyl-<u>H</u>), 6.80 (1H, s, 4-thienyl-<u>H</u>). EI-MS *m/z* (relative intensity; %): 284 (M^+, 3), 110 (100).

2. Pot tests

2.1. Rice plants, blast fungi and culture method

We used seedlings of *Oryza sativa* L. cv. Nihonbare throughout the pot test to evaluate the antiblast activity of the derivatives. Rice seedlings were grown in 7-cm plastic pots in a greenhouse kept at 22–30°C. A wild strain (Hoku-1) and a resistant strain SW (OYU-1) with Val75Met- SDH of the rice blast fungus were used for all pot tests. The Val75Met strain SW (OYU-1) was kindly supplied by Dr. So. All the strains were maintained on PDA slants, transplanted, and cultured for one week on oatmeal agar plates at 26°C. To induce sporulation, oatmeal agar plates were kept under a BLB lamp (Black Light Blue, near-ultraviolet radiation at 290–410 nm), after the scratching off of aerial hyphae on the plate. When many spores had formed on the plate, a blast spore suspension for pot tests was prepared by washing the surface of the plates with distilled water.

2.2. Procedure of pot tests

The efficacy of the test compounds against rice blast was evaluated through foliar spray and systemic application tests. Each derivative was dissolved to the extent of 1% (w/v) in DMF (*N*,*N*-dimethylformamide) solution containing 5% Tween 20®.

For the foliar application tests, every compound solution was diluted to 5 μg/ml with the blast spore suspension (30–40 spores/binocular ×100), and the resulting mixture was sprayed on rice seedlings grown in 7-cm plastic pots. The treated pots were incubated in a moisture chamber at 26°C. Then, the chemical treatment and rice blast inoculation were simultaneously advanced.

For the systemic application tests, hydroponic culture was carried out. The compound solution was diluted to a concentration of 5–10 μg/ml with a 500 μg/ml ammonium sulfate solution. Fifty milliliters of each diluted solution was poured into 50-ml black bottles. Rice seedlings at the 3 to 4-leaf stage grown in plastic pots were pulled out from the pots, and the roots were carefully washed with tap water to remove pot soil. Then, three rice seedlings were transplanted into each bottle. After the black bottles were kept in a greenhouse at 22–30°C for 3–4 days, the rice plants were inoculated with the blast spore suspension (30–40 spores/binocular ×100). The tests were carried out with 2–3 replicates.

Five to seven days after inoculation, the rice blast damage was evaluated by counting blast lesions formed on the 5 rice seedlings/pot in the spray tests and 3 rice seedlings/bottle in the systemic tests. A preventive value (PV) was calculated for each chemical using the following equation.

$$PV = \{1 - (\text{mean of lesions/treated stem})/(\text{mean of lesions/untreated stem})\} \times 100.$$

PV was calculated from the average of each test result. For simplicity, the efficacy of each chemical is represented by the following four ranks delineated on the basis of test results.

Rank:	A	B	C	D
PV:	≧90	<90~≧65	<65~≧40	<40

3. Measurement of inhibitory activities of new compounds

To evaluate the inhibitory activities of the compounds for wild-type and Val75Met SDHs, we carried out an *in vitro* enzyme assay to measure the reaction rate catalyzing the conversion of scytalone to 1,3,8-trihydroxynaphalene in the presence of the compounds.

Wild-type and Val75Met SDHs were prepared using the overexpression system of *E. coli*, as described previously.[17,18] Scytalone was purchased from Extrasynthese (France). Because carpropamid has three asymmetric carbon atoms, eight stereoisomers are possible. In this assay, we used the purified isomer KTU3616b which acts as a tight-binding inhibitor of wild-type SDH.[17]

Enzymatic activity at 30°C in the presence of the compounds was measured by monitoring the changes in UV absorption at 282 and 352 nm caused by the conversion of scytalone to 1,3,8-trihydroxynaphthalene using the spectrophotometer U-2001 (HITACHI, Tokyo, Japan). The sample solutions contained 0.6 nM SDH, 50 μM scytalone, 75 nM compound and 0.5% of dimethylsulfoxide, for dissolving the compounds and scytalone in 50 mM PIPES buffer (pH 7.0). The reaction rate was calculated from the tangent of the progress curves at the initial stage of the reaction, assuming differences in the molar extinction coefficient $\Delta\varepsilon_{282}$ of $-8.0\,mM^{-1}\,cm^{-1}$ and $\Delta\varepsilon_{352}$ of $5.9\,mM^{-1}\,cm^{-1}$ at the two wavelengths.

Results

1. Modification of amine moiety

1.1. Amides of alkylamine

In the trials to reduce the bulkiness of the amine moiety, the benzene ring was replaced with a less bulky linear-chain, branched-chain or cycloalkyl group. The results for the alkyl amino compounds in this test are summarized in Table 1. In the pot tests, almost all the alkyl derivatives were ineffective against rice blast infection. Only the compound with cyclopentyl (**5**) exhibited any efficacy in the hydroponic tests. In the enzyme assay, however, none of the compounds including compound **5** showed any inhibitory activity against the metabolism of scytalone. In the first step of the trials, we looked for a signal to direct our next molecular design in the induction of weak activity of the cyclopentyl derivative.

1.2. Modification of the alkyl side chain

The test results for the phenyl-alkyl amino compounds are

Table 1. Antifungal and enzyme inhibitory activity of derivation compounds—Conversion of amine moiety

Compd. No.	R		Effectiveness of pot test (A–D) R-strain Foliar spray	R-strain Systemic	S-strain Foliar spray	S-strain Systemic	SDH Enzyme Assay[a] Metabolism ratio of scytalone (1.00–0.00) R-strain	S-strain
	R–I	Alkyl						
1		*i*-Pr	D	D	D	D	1.05	0.98
2		*s*-Bu-	D	D	D	D	1.04	0.93
	R-II	Haloalkyl						
3		CF_3–CH_2–CH(–CH_3)–	D	D	D	D	1.08	1.05
	R-III	Cycloalkyl *n*: 4–7						
4		*n*=4	D	D	D	D	1.08	0.98
5		*n*=5	C/D	D	D	C	1.08	1.03
6		*n*=6	D	D	D	D	1.04	0.98
7		*n*=7	D	D	D	D	0.99	0.75
8a		Carpropamid	C	C	A	A		
8b		KTU 3616b (Active isomer)					0.60	0.00
Blank							1.00	1.00

[a] Conditions of reaction: inhibitors, 75 nM; enzyme, 0.6 nM; substitute, 50 μM; PIPES, 50 mM; pH 7.0; Temp., 30°C.

shown in Table 2. Carpropamid maintained moderate efficacy against the resistant rice blast strain Val75Met in the pot tests. Of the various (substituted-phenyl)alkyl groups, 2-(4-chlorophenyl)ethyl (**11**) was as effective as carpropamid in controlling MBI-D-resistant rice blast disease. As the stereo structure of the inhibitor-binding cavity in the enzyme is changed by the mutation, the inhibitors tested appeared to combine little with either the wild-type or mutated enzyme. However, 2-(4-chlorophenyl) ethyl (**11**) was effective against both the wild-type and Val75Met-mutated strains. This feature was observed more clearly in the enzyme assay; **11** and **14** {2-methyl-2-(4-chlorophenyl)ethyl} showed inhibitory activities for both wild and Val75Met enzymes.

Benzyl (**10**), 1-(4-chlorophenyl)ethyl (KTU 3616b, **8a**), 3-(4-chlorophenyl)propyl (**12**), 1-(4-chlorophenyl)propyl (**13**) and 1-methyl-2-(4-chlorophenyl)ethyl (**15**) were active against the wild-type enzyme, but were clearly less active against Val75Met. The benzene derivative with no alkyl side chain (**9**) did not show any activity.

1.3. Substitution at the phenyl ring

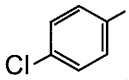
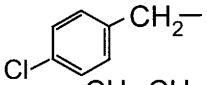
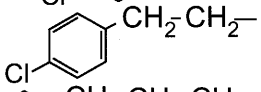
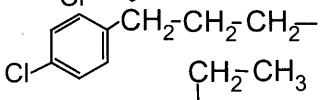
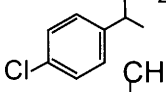
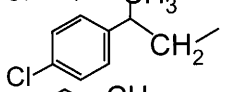
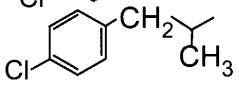
In the modification of the alkyl side chain, we selected the 2-(4-chlorophenyl)ethyl (**11**) as the most suitable lead compound for the next stage. To improve the inhibitory efficacy against the wild-type and MBI-D-resistant strains, halogens, trifluoromethyl and methyl-substituted derivatives were synthesized and tested (Table 3). 2-Phenylethylamine derivatives with various substituents, except 4-methyl (**24**), were somewhat effective against both strains. Of the derivatives, 4-trifluoromethyl (**18**) was found to be the most effective against both strains through the spray and systemic treatments. The 2,4-dichloro (**21**) and 3,4-dichloro (**22**) derivatives were inferior to the 4-trifluoromethyl derivative (**18**) in terms of the efficacy in the systemic treatment. The 4-fluoro (**16**) and 2,4-difluoro (**17**) derivatives were less effective than the 4-trifluoromethyl derivatives against the resistant strain. The substitution by 2-, 3- or 4-chloro (**19**, **20** and **11**) resulted in almost the same efficacies.

Despite the moderate efficacy against both strains in the pot tests, the *in vitro* assay demonstrated that both the 2,4- and the 3,4-dichloro-substituted derivatives of 2-phenylethylamine (**21** and **22**) were most active against the wild-type and resistant strains. Subsequently, 4-trifluoromethyl (**18**) and 2,4-difluoro (**17**) showed relatively stronger activity against the mutated enzyme than the 4-chloro-substituted derivatives. In the *in vitro* and *in vivo* tests, the 2-chloro, 3-chloro and 4-fluoro derivatives (**19**, **20** and **16**) were comparable to the 4-chloro derivative (**11**).

1.4. Thiophene analogues

Because only the cyclopentyl derivative (**5**) among the alkyl derivatives showed any efficacy, five members of the hetero-

Table 2. Antifungal and enzyme inhibitory activity of derivation compounds—Change of alkyl chain bound with phenyl ring

Compd. No.	R	Effectiveness of pot test (A–D)				SDH Enzyme Assay[a]	
		R-strain		S-strain		Metabolism ratio of scytalone (1.00–0.00)	
		Foliar spray	Systemic	Foliar spray	Systemic	R-strain	S-strain
9		D	D	D	D	1.06	1.03
10		D	C/D	C/D	C	1.02	0.30
11		C	C	C	C	0.69	0.52
12		D	C/D	C	C	1.01	0.31
13		D	D	C/D	D	0.85	0.20
14		C	C/D	C	C	0.65	0.73
15		D	C/D	D	D	0.97	0.75
Blank						1.00	1.00

[a] Conditions of reaction: inhibitors, 75 nM; enzyme, 0.6 nM; substitute, 50 μM; PIPES, 50 mM; pH 7.0; Temp., 30°C.

cyclic ring were introduced as less bulky compounds.

In the beginning, a thiophene ring was introduced instead of a cyclopentyl or phenyl ring. In all the derivatives of this group, the side chain was fixed by an ethyl. The thiophene ring was substituted with one or two chlorine atoms.

Among the thiophene derivatives, 5-chloro-3-ethylthiophene (**26**) was the most effective against both strains. 4-Chloro-2-ethylthiophene (**27**) was comparable or slightly inferior to **26** in efficacy in the spray and systemic tests. The effects of 5-chloro-2-ethylthiophene and 2,5-dichloro-3-ethylthiophene (**28** and **30**) were comparable to that of **26** on spray application, but inferior on systemic application.

The effects of the thiophene derivatives with a branched ethyl side chain (**32**–**35**), particularly against R-strain, were clearly inferior to those of the derivatives with a linear ethyl side chain. In the enzyme assay, derivatives with linear ethyl side chains showed some inhibitory activity against both enzymes. Meanwhile, the derivative with a branched ethyl side chain was obviously inferior to that with a linear ethyl side chain regarding the activity against Val75Met-SDH, despite has strong activity against the wild-type enzyme.

The derivatives with linear alkyl groups, 5-chloro-3-ethyl (**26**), 3-ethyl-5-bromo (**29**) and 2,5-dichloro-3-ethyl (**30**), showed marked inhibitory activity against both SDH enzymes. However, the results did not correlate with those in the pot tests. In particular, **30** and **31** having strong activity in the enzyme assay were not very effective in the pot tests.

1.5. Other heterocyclic derivatives

In the modification of the five-membered heterocyclic ring, 1,3-thiazole derivatives (**36** and **37**) were synthesized. Despite the weak activity in the enzyme assay, the derivatives showed relatively high efficacy but were inferior to thiophene derivatives with the same substitutions in the pot test.

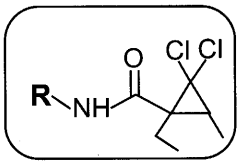
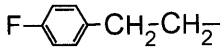
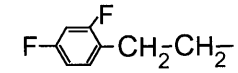
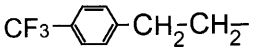
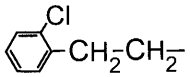
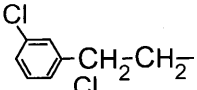
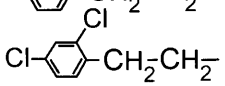
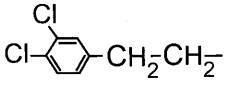
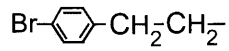
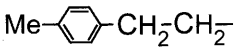
2. Modification of the acid moiety

In modifying the acid moiety, the amine part of the derivatives was fixed with 3-ethyl-5-chlorothiophene.

2.1. Acid moiety mimicking carpropamid

Alkyl substituents at the cyclopropane ring in the acid moiety of carpropamid were changed. The derivative of 1-isopropyl (**41**) showed relatively high efficacy in the pot tests, followed by the 1,3,3-trimethyl derivative (**40**). However, the compound with the 2,2-dichloro-1-ethyl-3-methyl substitution (**26**), which was the acid moiety of carpropamid, was most effective, and no other compounds with the substituents surpassed **26** in efficacy.

Table 3. Antifungal and enzyme inhibitory activity of derivation compounds—Change of substitution at phenethyl ring

Compd. No.	 R	Effectiveness of pot test (A–D)				SDH Enzyme Assay[a]	
		R-strain		S-strain		Metabolism ratio of scytalone (1.00–0.00)	
		Foliar spray	Systemic	Foliar spray	Systemic	R-strain	S-strain
16		C	C	B/C	B/C	0.66	0.88
17		C	C	B/C	B/C	0.51	0.67
18		B/C	B	B/C	B/C	0.40	0.31
19		C	C	C	C	0.62	0.72
20		C/D	C	C	C	0.67	0.77
21		B/C	C	B/C	C	0.27	0.17
22		B/C	C	C	C	0.22	0.25
23		C	D	C/D	D	0.68	0.71
24		D	D	D	D	0.81	0.79

[a] Conditions of reaction: inhibitors, 75 nM; enzyme, 0.6 nM; substitute, 50 μM; PIPES, 50 mM; pH 7.0; Temp., 30°C.

In the enzyme assay, a clear difference was not observed in the activity of any of the derivatives between the wild-type and Val75Met-SDH. The compound with the 1-ethyl-3-methyl substitution (**26**) showed strong activity and none of the other compounds were better than **26** in the enzyme assay (Table 6).

2.2. Acid moiety mimicking diclocymet

In modifying the acid moiety of diclocymet (2-cyano-3,3-dimethyl-butanoic acid), the cyano group was replaced with a bromine or chlorine atom. The cyano derivative was very effective but inferior to compound **26**. In the enzyme assay, all the derivatives with the acid of diclocymet type showed strong activity against the wild-type enzyme but weakly inhibited Val75Met (Table 6).

Discussion

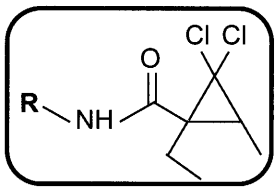
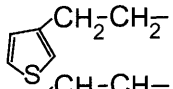
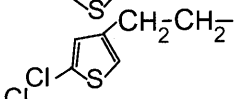
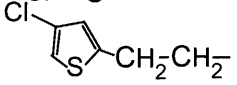
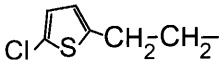
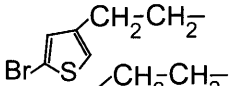
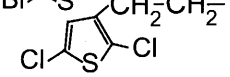
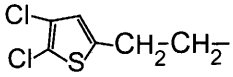
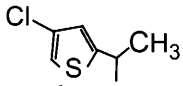
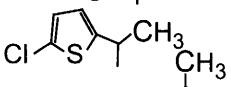
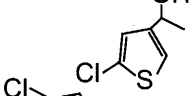
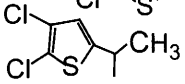
In this study, several compounds effective against the MBI-D-resistant strain of rice blast were found by modifying the chemical structures of known MBI-D fungicides. Here, we discuss the role of the chemical groups introduced in the derivatives and the novel fungicides effective against the wild-type and MBI-D-resistant strains.

On the basis of the crystal structure of the SDH-carpropamid complex, the Val75Met substitution is expected to cause a small structural change in the active-site pocket as well as the entire structure of SDH. The Val75Met substitution may result in a small protrusion of the side chain into the mouth of the pocket. MBI-D fungicides are classified as tight-binding inhibitors,[17] and their amine moieties bind to the mouth of the pocket.[18] Thus, even small changes in ligand structure may reduce their affinity for the active pocket as discussed previously.[18] To recover the affinity of inhibitors for the cavity, we attempted to reduce the bulkiness of the amine moiety in the chemical structure.

Of the derivatives in which 1-phenylethylamine groups in the amine moiety are substituted by several linear, branching alkyl chains or cycloalkyl groups, only the planar cyclopentyl derivative showed any efficacy (Table 1). From this result, an aromatic ring seemed to be an important factor in acquiring anti-blast activity, and the introduction of a five-membered heterocyclic ring into the amine moiety is being planned.

When inspecting the structure of carpropamid complexed

Table 4. Antifungal and enzyme inhibitory activity of derivatives having heterocyclic ring—Introduction of thienyl ring

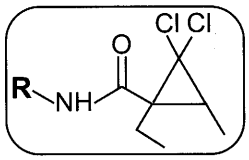
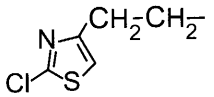
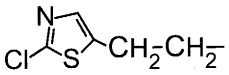
Compd. No.	 R	Effectiveness of pot test (A–D)				SDH Enzyme Assay[a]	
		R-strain		S-strain		Metabolism ratio of scytalone (1.00–0.00)	
		Foliar spray	Systemic	Foliar spray	Systemic	R-strain	S-strain
25		D	D	C	C/D	0.88	0.93
26		B	B	B	B	0.45	0.50
27		B	B	B/C	B/C	0.54	0.56
28		B/C	B	B/C	C	0.69	0.76
29		C	C	B/C	D	0.43	0.44
30		B	C/D	B/C	D	0.29	0.36
31		C	C/D	C/D	C	0.32	0.32
32		D	C/D	C/D	C/D	0.92	0.19
33		D	D	C/D	C/D	0.96	0.48
34		D	C/D	C/D	C	0.97	0.23
35		D	C/D	C	C/D	0.68	0.01

[a] Conditions of reaction: inhibitors, 75 nM; enzyme, 0.6 nM; substitute, 50 μM; PIPES, 50 mM; pH 7.0; Temp., 30°C.

with wild-type SDH,[14] the methyl substitution at the α-position of the benzyl side chain seems to protrude from the facet of other parts of the compound such as the phenyl and cyclopropyl rings. The protrusion may cause steric hindrance for the inhibitor when binding to the active-site pocket of Val75Met-SDH.[16] Then, the side chain of the benzyl group is modified to the other alkyl chain. The 2-phenylethylamine derivatives with linear ethyl groups (**11**, **16**, **17**, **18** and **21**) show an improved efficacy, but 1-(4-chloro-phenyl)propyl and 1-methyl-2-(4-chlorophenyl)ethyl derivatives with branched side chains (**13** and **15**) had neither efficacy in the pot tests nor activity in the enzyme assay against Val75Met-SDH. Among the branched-side-chain derivatives, **14** {2-methyl-2-(4-chlorophenyl)ethyl} showed relatively strong inhibitory activity against Val75Met-SDH and was comparable to **11** with a linear ethyl side chain in efficacy against the wild-type and mutant strains in the pot tests. From these results, it is suggested that an ethyl side chain is one of the key structures for improving the binding affinity of inhibitors for Val75Met-SDH. According to the structure–activity relationships examined, an aromatic ring and an ethyl side chain were incorporated for further derivation of the compounds (Table 2).

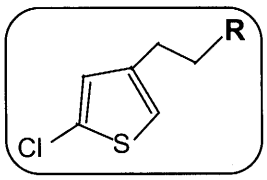
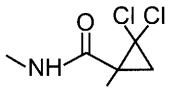
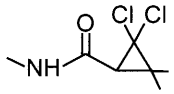
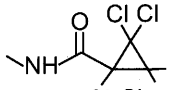
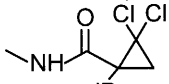
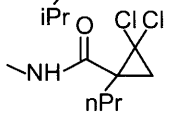
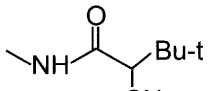
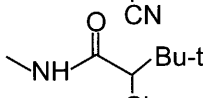
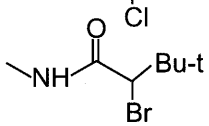
Among the derivatives with a phenethyl ring substituted by various halogens, **18** with 2-(4-trifluoromethylphenyl)ethyl is most favorable, but **24** with a 2-(4-methylphenyl)ethyl has little efficacy. In the enzyme assay, all the compounds except **24** in this group showed some level of activity against both enzymes. These results suggest that electron-withdrawing substituents are required for designing fungicides effective against both strains. The two dichloro-substituted derivatives (**21** and **22**) showed the greatest activity in this group in the enzyme assay, but were not sufficiently effective in the pot

Table 5. Antifungal and enzyme inhibitory activity of derivatives having heterocyclic ring—Introduction of thiazole ring

Compd. No.	 R	Effectiveness of pot test (A–D)				SDH Enzyme Assay[a]	
		R-strain		S-strain		Metabolism ratio of scytalone (1.00–0.00)	
		Foliar spray	Systemic	Foliar spray	Systemic	R-strain	S-strain
36		B/C	B/C	B/C	B/C	0.89	0.96
37		B/C	C	B/C	C	0.90	1.03

[a] Conditions of reaction: inhibitors, 75 nM; enzyme, 0.6 nM; substitute, 50 μM; PIPES, 50 mM; pH 7.0; Temp., 30°C.

Table 6. Antifungal and enzyme inhibitory activity of derivatives modified at acid moiety

Compd. No.	 R	Effectiveness of pot test (A–D)				SDH Enzyme Assay[a]	
		R-strain		S-strain		Metabolism ratio of scytalone (1.00–0.00)	
		Foliar spray	Systemic	Foliar spray	Systemic	R-strain	S-strain
	1) Acid moiety carpropamid type						
38		D	D	C	C	1.00	0.97
39		D	D	D	D	1.00	0.87
40		C	C	C	C	0.91	0.90
41		B/C	B/C	B/C	B/C	0.71	0.82
42		D	D	D	D	0.89	0.83
	2) Acid moiety diclocymet type						
43		B/C	B/C	B/C	B	0.86	0.64
44		C/D	C/D	B/C	C	0.99	0.56
45		B/C	C	B/C	C	0.79	0.34

[a] Conditions of reaction: inhibitors, 75 nM; enzyme, 0.6 nM; substitute, 50 μM; PIPES, 50 mM; pH 7.0; Temp., 30°C.

tests. In particular, they are less effective with systemic application. These compounds may have a low mobility to the fungal acting site through rice plants on systemic application (Table 3).

On the basis of the results described above, five-membered heteroaromatic rings were incorporated into the subsequent derivatives. As expected, the effect and activity of **26** (5-chloro-3-ethyl-thiophene) are good in the pot tests and enzyme assay, and the compound is superior to **11**, 2-(4-chlorophenyl)ethyl. Thiophene compounds **32–35** with branched alkyl side chains show outstanding inhibitory activities against the wild-type enzyme. On the other hand, their inhibitory activities against Val75Met-SDH are clearly weak. Thus, branched alkyl side chains tend to reduce the inhibitory activity of Val75Met-SDH in the case of the thiophene as well as phenyl rings (Tables 2 and 4). Compounds **36** and **37** having thiazole rings were somewhat inferior to **26** having thiophene rings in efficacy in pot tests and activity in the enzyme assay (Table 5).

When modifying the acid moiety in the amido derivatives, the amine moiety was fixed as 5-chloro-3-ethylthiophene. The first group is of the carpropamid type, and the derivatives of various alkyl-substituted cyclopropanes at 1,3,3-positions were synthesized. The structure–activity relationships of the substituted cyclopropane derivatives are similar to those of the carpropamid derivatives in the former study[9] (Table 6). The second is of the diclocymet type, and 2-halogen-substituted-3,3-dimethylbutanic acid was synthesized. In comparison with the carpropamid type, there are no compounds superior to the compounds with 2,2-dichloro-1-ethyl-3-methylcyclopropanecarboxylic acid in the *in vitro* and *in vivo* tests. The chloro- and bromo-substituted derivatives (**44** and **45**) of diclocymet acid are not better than the cyano-substituted derivative (**43**) in efficacy. In the enzyme assay, the diclocymet-type compounds have considerably strong activity for the wild-type enzyme but weak activity for Val75Met-SDH (Table 6).

This study is still in progress. Summarizing the results so far, an ethyl side chain linked with an aromatic ring was proved to be important in improving the inhibitory activity of new derivatives against the wild-type and mutant enzymes. A thiophene or thiazole ring substituted with an electron-withdrawing group is superior to the derivatives of the similarly substituted benzene ring in efficacy. The introduction of the other heterocyclic aromatic rings is expected to further improve the activity.

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