Development of a novel fungicide, tolprocarb

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(Accepted June 15, 2019)

Tolprocarb developed by Mitsui Chemicals Agro, Inc. (Tokyo, Japan) was discovered as a new oomycete fungicide. However, its antifungal spectrum and action mechanisms against fungi are completely different from those of the original compound, iprovilacarb. Tolprocarb has a potent and highly controlled effect on a rice blast fungus Magnaporthe grisea, and its mode of action was revealed to be the inhibition of polyketide synthase in the melanin biosynthesis pathway. In addition, tolprocarb induced systemic acquired resistance in Arabidopsis thaliana and rice (Oryza sativa L.). Owing to these double modes of action, tolprocarb can effectively control not only rice blast but also bacterial diseases, and has a low risk of developing fungicide-resistant isolates. Tolprocarb also provides long-term residual activity. A meta-analysis was performed in order to demonstrate tolprocarb’s superior control against panic blast in paddy fields. In addition, tolprocarb did not show cross-resistance against the fungi that are resistant to dehydratase inhibitor in melanin biosynthesis (Melanin Biosynthesis Inhibitor-Dehydratase; MBI-D) or respiratory complex III: cytochrome bc1 at Quinone outside site inhibitor (Quinone outside Inhibitor; QoI). Owing to its stable effects, tolprocarb appears to be a suitable choice for practical use against fungi in the rice production field.

Keywords: Melanin biosynthesis inhibitor, MBI-P, Polyketide synthase, Systemic acquired resistance, Magnaporthe grisea, Xanthomonas oryzae pv. oryzae.

Introduction

Fungal or bacterial diseases in crops are important factor to decrease yields in crop productions. Fungicides are used for control such diseases to maintain yield and quality of harvest stably. Rice (Oryza sativa L.) is one of the most important crop in Asian countries including Japan. In spite of that the production still be threatened by many kinds of phytopathogenic microorganisms, therefore novel fungicides are required to control those stably.

Tolprocarb (Fig. 1) is a fungicide developed by Mitsui Chemicals Agro, Inc., Tokyo, Japan, to control rice blast (causal agent; Magnaporthe grisea) in paddy fields.10 Rice blast is an extremely harmful disease that affects the rice production; controlling this fungus poses is major challenge because fungicide-resistant strains can develop. Although there are many modes of action to control the disease, some fungicides become insensitive to disease. Fungicide resistant isolates of M. grisea, such as respiratory complex III: cytochrome bc1 at Quinone outside site inhibitors (Quinone outside Inhibitors; QoIs) or dehydratase inhibitors in melanin biosynthesis (Melanin Biosynthesis Inhibitors-Dehydratase; MBI-Ds), were developed.2–5) Studies were conducted on the two action mechanisms of tolprocarb: the inhibition of polyketide synthase in the melanin biosynthesis pathway (MBI-P) and the induction of systemic resistance in plants.6,7) Since tolprocarb has unique modes of action, it is presumed that the risk of developing resistance in the target fungi would be low. Tolprocarb has the potent biological activity not only on rice blast, but also on bacterial diseases, with only a small ef-

Fig. 1. Chemical structure of tolprocarb. A–C indicate the substituents that were modified for the structure–activity relationship studies.

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Published online #M## #D##, #Y##
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fect on mammals or the environment. Here, we describe the synthesis, biological activities, and the modes of action of tolprocarb.

1. Discovery

1.1. Discovery of lead compound
During the development of a new oomycete fungicide, "compound 1" was found to show disease controlling efficacy against *M. grisea* in rice. Compound 1 and iprovalicarb differed only in terms of the presence or absence of a methyl group and the position of the carbonyl group. The iprovalicarb, oomycete fungicide showed no activity against *M. grisea*. Interestingly, compound 1 showed no activity against oomycete plant pathogens such as *Phytophthora infestans* which causes blight in potatoes and tomatoes. A slight change in the structure of iprovalicarb greatly altered the spectrum of its fungicidal activities, as summarized in Fig. 2. As will be demonstrated, the mode of action of compound 1 is different from that of iprovalicarb.

The mycelia of *M. grisea* turned black owing to the formation of melanin on an ordinary potato dextrose agar plate, whereas the mycelia treated with compound 1 turned white without any inhibited mycelial elongation. The color of the mycelia treated with compound 1 was different from that treated with conventional MBIs, which suggests that the accumulated intermediates were different between the compound 1 and conventional MBIs treatments. In addition, compound 1 showed no cross-resistance to strains resistant to MBI-D. These results suggested that the compound 1 had a new target site in the melanin biosynthesis pathway. Therefore, we set compound 1 as a lead compound and optimized its structure.

1.2. Optimization of the lead compound and structure–activity relationship
The lead compound 1 was divided into three groups: A–C (Fig. 1), and optimization of each substructure was performed. A summary of the structure–activity relationships is shown in Table 1.

The carbamate or thiocarbamate bond in Group A exhibited higher activity compared to the amide or urea bond. The substitution of an isopropyl group by a 2,2,2-trifluoroethyl group at the end of Group A enhanced the activity. Methyl carbamate derivative in Group A was less active than the isopropyl carbamate derivative.

The S-enantiomers (Table 1) in Group B exhibited much higher activity compared to the R-enantiomers. The trifluoromethyl derivative exhibited the same level of activity as that of the isopropyl derivative. The n-propyl derivative was not effective as the i-propyl derivative. Introduction of the hydrophobic i-butyl group and the hydrophilic methysulfanyl methyl group was not favorable for the activity.

In Group C, the para-methyl group exhibited higher activity than that of the ortho- or meta-methyl group. The introduction of chlorine atom in the para-position exhibited less activity than that of the methyl group. Unsubstituted compound was more active than 2- and 3-methyl analog, but less potent than 4-methyl and 4-chloro analogs. Substitution at 4-position at C-ring seems to be favored.

Finally, after considering various factors, such as long residual activity in the field trial, tolprocarb was selected as the developed compound.

2. Synthesis
Tolprocarb was synthesized from *N*-tert-butoxycarbonyl-(N-Boc-) protected i-valine methyl ester (*a*), as shown in Fig. 3. The amine derivative (*d*) was prepared by hydrazinolysis after reduction using sodium borohydride and Mitsunobu coupling reactions. The amine derivative (*f*) was prepared by amide bond formation and *N*-Boc deprotection. Finally, tolprocarb was synthesized from the amine derivative (*f*) and chloroformate (*g*).

3. Melanin Biosynthesis Inhibitory Activity

3.1. Mode of action
In 1965, MBI and 2,3,4,5,6-pentachlorobenzyl alcohol were de-
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De velopment of a novel fungicide, tolprocarb developed by Sankyo Co., Ltd. (now called Mitsui Chemicals Agro, Inc., Tokyo, Japan). Reductase inhibitors in melanin biosynthesis (Melanin Biosynthesis Inhibitors-Reductase; MBI-Rs) were investigated in the 1970s, and MBI-Ds were investigated in 1990s. Before this, cerulenin was known as polyketide synthase (PKS) inhibitor in the melanin biosynthesis process, but no other compound that inhibits PKS before tolprocarb was entered for practical use; tolprocarb was the first practical PKS-inhibiting fungicide.11)

In the earlier stages of tolprocarb development, the inhibition of coloration of *M. grisea* was observed when the colony was grown with tolprocarb on agar media. The colony was less stained than those grown with conventional MBIs, such as MBI-R or MBI-D. A recovery test was conducted to estimate its action mechanism. *Magnaporthe grisea* was grown on agar media containing intermediary bodies of melanin and tolprocarb in order to determine the color of the mycelial colony. A less-colored colony on tolprocarb agar medium was recovered when grown on media containing 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN). Therefore, it was expected that tolprocarb inhibits colony coloration by inhibiting PKS upstream of 1,3,6,8-THN which regulates polyketide synthesis and pentaketide cyclization (Fig. 4). This was determined by quantifying the accumulation of 1,3,6,8-THN in transgenic *Aspergillus oryzae*, which has the PKS gene of *M. grisea*. Compared with some conventional MBIs, tolprocarb inhibited only PKS activity in vitro. These results indicated that tolprocarb acts on *M. grisea* PKS, which differentiates this fungicide from other conventional MBIs.6)

Owing to such a new action mechanism, tolprocarb has been classified as MBI-PKS (MBI-P) by the Fungicide Resistance Action Committee (FRAC).12)

3.2. Effect on the life cycle of *M. grisea*

The effect of conventional MBIs, such as MBI-D and MBI-R, on the life cycle of *M. grisea* has already been identified. Those fungicides inhibit the melanization of *M. grisea* appressoria and hinder the dispersal of conidia from the conidiophores.13–17) The effects of tolprocarb against these cells and spores were also identified. Most likely, MBI-D/MBI-R and tolprocarb inhibit the melanization of *M. grisea* appressoria and hinder conidia dispersal.18) It is unlikely that conventional MBIs and tolprocarb inhibit the melanization of conidia more strongly than either MBI-D or MBI-R. The decolorized conidia resulting from exposure to tolprocarb were more sensitive to sunlight (ultraviolet [UV] light) than colorized conidia. The germination rate of the decolorized conidia after treatment with tolprocarb was lower than that after treatment with MBI-D or MBI-R fungicides, even on cloudy or rainy days (UV intensity: 500–2,000 µW/cm²).19) Melanization inhibition in appressoria or conidia was observed using transmission electron microscopy (TEM).20) Melanization of *M. grisea* appressoria is essential for creating turgor pre-
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sure for the infection peg to penetrate into the host cell. In contrast, the function of melanin in conidia has not been much studied. As mentioned, MBIs inhibit conidial dispersal from the conidiophore, but the mechanism remains unclear; however, melanization is related to the ability to separate the conidium from the conidiophore.

4. Systemic Acquired Resistance (SAR)

4.1. SAR in Arabidopsis thaliana

SAR is a nonspecific systemic immune response in plants, which is induced by the signal compound salicylic acid (SA). NPR1 and WRKY45 regulate various pathogenesis-related (PR) genes in the SA signaling pathway, such as PR-1, PR-2, PR-5, and PBZ1, which encode the PR proteins, such as glucanase and chitinase, in addition to regulating the phytoalexin synthesis pathways.

The SAR activity of tolprocarb in A. thaliana was revealed by determining that the transgenic A. thaliana harbors the tobacco PR-1a promoter-Fluc (firefly luciferase) reporter gene fragment (PR-1a::Fluc). PR-1a promoter activities were observed in two different growth stages of A. thaliana: the newly germinated seedling and the three-week-old adult stage. In the A. thaliana seedling, PR-1a promoter activities were detected 96 hr after treatment. In the adult plant, the activities were observed at 96 and 120 hr after treatment by irrigation. In the adult plant, the potential for controlling Pseudomonas syringae pv. maculicola (Psm) was also evaluated at 3 days after inoculation. Arabidopsis thaliana leaves treated with tolprocarb when 10 days before inoculation were reduced the amount of Psm. The results indicated that SAR activity is induced in response to tolprocarb treatment.

4.2. SAR activity in rice

Tolprocarb has been identified as a potent inducer of SAR activity in A. thaliana; therefore, we assessed whether tolprocarb also induces SAR activity in rice.

The activities of the PR genes in rice (Oryza sativa L. cv. Nipponbare) after treatment with tolprocarb were observed using quantitative reverse transcription polymerase chain reaction. Tolprocarb accelerated SA-mediated signaling pathway-related genes, such as PBZ1, β-1,3-glucanase, and chitinase 1 24 or 72 hr after treatment. Conversely, it did not accelerate the jasmonic acid (JA-) mediated signaling pathway-related genes. It was then suggested that tolprocarb induced SAR activity by accelerating the SA signaling pathway.

SA and JA are known as signaling compounds that induce plant immunity. In particular, the SA signaling pathway is closely related to the function of protecting the plant from pathogenic fungi or bacteria. Therefore, the potential to control the activity against bacterial diseases in rice was expected, and the activity against Xanthomonas oryzae pv. oryzae was observed. Tolprocarb prevented the expansion of disease symptoms in rice leaves.

Tolprocarb was effective against M. grisea mainly through MBI-P activity, but it was not clear whether the second mode of action, SAR activity, participated its activity. It has been difficult to find whether the activity was caused by MBI-P or SAR. However, we conducted two recovery tests using a melanin intermediate to distinguish them. First, an M. oryzae infection trial was conducted on rice leaf cells with a melanin intermediate, 1,8-dihydroxynaphthalene. An M. grisea infection trial was conducted on surface-cross-linked (SCL) agar media, in which M. grisea infection could be observed through the appressoria on artificial media. In the first trial, the melanin-recovered M. grisea appressoria on the rice cells were prevented from penetrating the rice after tolprocarb treatment. The penetration by those on SCL media was not inhibited after treatment. These two contrasting results indicated that living plant cells are necessary to prevent melanin-recovered M. grisea infection after tolprocarb treatment. Therefore, it is suggested that tolprocarb in-
hibits *M. grisea* infection through two action mechanisms: MBI activity and inducing the host defense response.33)

5. Activity against Rice Blast

5.1. Activity against panicle blast in the field

Tolprocarb is a systemic fungicide with granule formulations containing 9% or 4% tolprocarb, which are spread around the plant roots in nursery boxes and another granule formulation containing 3% tolprocarb, which are spread on paddy water. Tolprocarb exhibited potent control activity against both leaf blast and panicle blast, but especially superior control activity against panicle blast. Its special feature was confirmed by a meta-analysis using the restricted maximum likelihood method and the efficacy data from field trials after treatment with 9% or 4% tolprocarb granules in nursery boxes from 2007 to 2015. Statistical analyses using the data from treatment with 3% granules in paddy water were also conducted and showed superior control efficiency against panicle blast when treated from 5 to 29 days before heading.34)

Tolprocarb is stable regardless of the soil type. Tolprocarb performed lesser adsorption in 36 soil types, including 25 an- dosols in Japan, regardless of the organic carbon content, cation exchange capacity, phosphoric acid absorption, and pH. Soil adsorption rates of tested soils were 7–42%, and 20–100% of ad sorbed tolprocarb was desorbed by water.35)

5.2. Activity against pesticide-resistant strains

The emergence of pesticide-resistant isolates is a particular problem because they hinder stable rice production. In Japan, resistant isolates against MBI-Ds or QoIs emerged, which became problematic in the production of rice tolprocarb. It was then confirmed to have stable control against MBI-D- or QoI-resis tant isolates. Owing to its effect, tolprocarb appears to be a suitable choice for practical use against fungi in rice crops.

Concluding Remarks

Tolprocarb was invented by the derivatization of iprovalicarb anlogs, but the mode of action was completely different from that of MBI-P. Tolptoared also has an ability to induce SAR activity in *A. thaliana* and rice, and exhibited potent activity against bacterial diseases and rice blast by inducing SAR activity. Owing to these two action mechanisms and tolprocarb’s potency, fewer resistant isolates against this fungicide would develop. In paddy fields, granules containing tolprocarb were observed to have a higher efficacy against race blast, with especially superior efficacy against panicle blast, including isolates resistant to MBI-D or QoI fungicides. According to these characteristics, tolprocarb showed potent stable control efficacy against rice diseases, comprising not only blast but also bacterial diseases. This is a good prospect for its use in stable crop production.

Acknowledgements

Observations of *M. grisea* by TEM were conducted by collaborating with Dr. Tomoko Suzuki, Japan Women’s University. Studies of inducing SAR activity of tolprocarb in *A. thaliana* or rice were conducted by collaborating with Prof. Kazuyuki Hiratsuka, Yokohama National University, and Dr. Rieko Ogrura, Yokohama Biotechnology Company, Ltd. The technique of preparing the SCL media was instructed by Assoc. Prof. Eiji Tanaka, Ishikawa Prefectural University. The authors would like to thank all of these contributors for their significant collaboration.

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