

Structure-activity relationship study for fungicidal activity of 1-(4-phenoxyethyl-2-phenyl-[1,3]dioxolan-2-ylmethyl)-1H-1,2,4-triazole derivatives against rice blast

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Abstract: To explore new antifungal agents for rice blast control, the antifungal activity of a series of novel 1,2,4-triazole derivatives against *Magnaporthe oryzae* has been evaluated. The antifungal activity was determined by using *in vitro* mycelial growth inhibition tests. Among the 19 test compounds, we found that the compound 1-(4-phenoxyethyl-2-phenyl-[1,3]dioxolan-2-ylmethyl)-1H-1,2,4-triazole (Gj) displayed potent antifungal activity against *M. oryzae*. The IC₅₀ value was found approximately 3.8±0.5 µM and the IC₅₀ value of propiconazole was found to be approximately 3.7±0.2 µM, respectively. Structure-activity relationship studies on aromatic ring structures provided insight and information about the structural requirements for antifungal activity of this synthetic series against *M. oryzae*.

Key words: brassinosteroid biosynthesis inhibitors, fungicide, *Magnaporthe oryzae*, rice blast disease, triazole derivatives

Introduction

Rice (*Oryza sativa* L.) is a major food in the diets of a large part of the world's human population, especially in Asia. Rice blast disease (RBD) which is caused by the pathogenic fungi of *Magnaporthe oryzae*, is a leading constraint in world rice production (Thurston 1998; Bechinger *et al.* 1999; Clergeot *et al.* 2001; Talbot 2003). The high incidence of plant mortality and the lack of effective control methods cause billions of dollars in losses worldwide each year. Hence, great efforts and various management strategies have been made to control the RBD (Knight *et al.* 1997), including controlled use of nitrogen fertilisers, application of silica, and flooding the paddy fields (Solomon *et al.* 2003). Chemical fungicide is the most common solution to minimize the severity of RBD and increase rice production (Baldwin *et al.* 1988). With the use of fungicides, drug resistance among fungal pathogens has become a problem. It is necessary to develop some novel antifungal compounds with high efficacy, less toxicity and low resistance. Azoles are conventional and the most frequently used antifungal agents (Davidse *et al.* 1986), due to their broad spectrum of activity and more favorable safety profile (Jiang *et al.* 2013).

The mode of action of azole fungicides is the inhibition of 14 α -demethylase (CYP51A1), a well-known target for fungicides (Zarn *et al.* 2003). This class of P450 enzyme plays an essential role in mediating membrane permeability. Fungal membrane differs from vertebrate in that fungal membrane plays a critical role in the maintenance

of the cell order and integrity (Hartmann 1998). Consequently, chemicals that directly or indirectly target fungal membranes or their components are a feasible method for fungal disease control. Moreover, azole derivatives have been demonstrated to have a widespread ability as inhibitors of P450s (Koymans *et al.* 1993), due to the intrinsic affinity of the nitrogen electron pair in heterocyclic molecules for the prosthetic heme iron (Szklarz and Halpert 1998). The azoles bind not only to lipophilic regions of the protein but also simultaneously to the prosthetic heme iron (Testa and Jenner 1981).

Based on these observations, we conducted a systemic search for inhibitors targeting P450s in brassinosteroid (BR) biosynthesis, a plant growth-promoting hormone (Clouse and Sasse 1998). Using ketoconazole as a molecular scaffold (Fig. 1), we prepared a series of new triazoles and we found a new class of BR biosynthesis inhibitors YCZ (Oh *et al.* 2012). Structure-activity relationship studies revealed yucaizol (the structure is shown in figure 1), which is the most potent inhibitor of BR biosynthesis found to date (Yamada *et al.* 2012, 2013; Oh *et al.* 2013). Using YCZ-18, an analogue of yucaizol, we demonstrated that yucaizol is a specific inhibitor of BR biosynthesis (Oh *et al.* 2015). It has been reported that 1-[2-(2,4-dichlorophenyl)-4-alkoxymethyl-1,3-dioxolan-2-yl]methyl-1,2,4-triazoles display antifungal activity against *M. oryzae* (Lin *et al.* 2005). Considering the structural similarity of YCZ to the compounds that Lin *et al.* (2015) reported, it is possible that YCZ may exhibit antifungal activity against

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M. oryzae. In many cases, new drug development is a lengthy process and a slight change in the structure of a particular molecule results in a dramatic increase and/or decrease in biological activity. Thus, it is necessary to screen various molecules with similar structural features and study the structure-activity relationship.

Based on these facts, in the present work, we carried out a biological evaluation of yucaizol analogues on the antifungal activity against *M. oryzae*. Structure-activity relationship analysis provided insight and information about the structural requirements needed to enhance the antifungal activity of this synthetic series.

Materials and Methods

Chemicals

The brassinosteroid biosynthesis inhibitor compound library was synthesized by a method that was described previously (Oh *et al.* 2012; Yamada *et al.* 2012, 2013). Stock solutions of the test compound were dissolved in dimethyl sulfoxide (DMSO) at a concentration of 100 μM , and stocked at -30°C . The other reagents were of the highest grade and purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan).

Magnaporthe oryzae strain

Rice blast isolate designated as APU00-093A (race 007.0), was obtained by mono-spore isolation from diseased rice panicle on a paddy field from the Akita Prefecture, Japan, in 2000. This isolate was kept on Potato Dextrose Agar (PDA) at 15°C .

Fungicidal activity assay

Poisoned food technique was performed to investigate the antifungal effect of the test compounds against *M. oryzae*. Mycelial growth inhibition tests were carried out.

Each test-compound dissolved and diluted in DMSO, was added to Potato Sucrose Agar (PSA) medium (kept at 50°C after autoclaving) to the appropriate concentration. The final concentration of DMSO of each medium was 0.1%. Three mycelial pellets (1 mm in diameter) of *M. oryzae*, pre-cultured on PDA, were placed on the PSA medium containing the given concentrations of the test compound. The diameter of the mycelial mat of *M. oryzae* was measured when the diameter of each corresponding untreated control reached about 40–50 mm. The concentration for 50% inhibition (IC_{50} , μM) of mycelial growth was calculated by the linear regression formula obtained from the logarithm of the concentration and the inhibition rate at each concentration against the untreated control. All experiments were carried out in triplicate and the data in the report represents the average values.

Statistical analysis

All measurements were carried out at least in triplicate. Data analysis (t-test and analysis of variance) was applied to determine the significant difference with the use of significance throughout the manuscript being based on $p < 0.05$, unless stated otherwise.

Results and Discussion

Chemistry

The general structure of the YCZ is shown in figure 1. There are two aromatic ring structures in YCZ which allowed us to synthesis a series of analogues for structure-activity relationship studies. The method for the chemical modification of ring A was carried out as we previously described. The synthesis route was shown in scheme 1 (Yamada *et al.* 2012). To prepare compounds with a different substitution at ring B, we used a method as we reported previously, that was shown in scheme 2 (Oh *et al.* 2012). Briefly, preparation of analogues with different

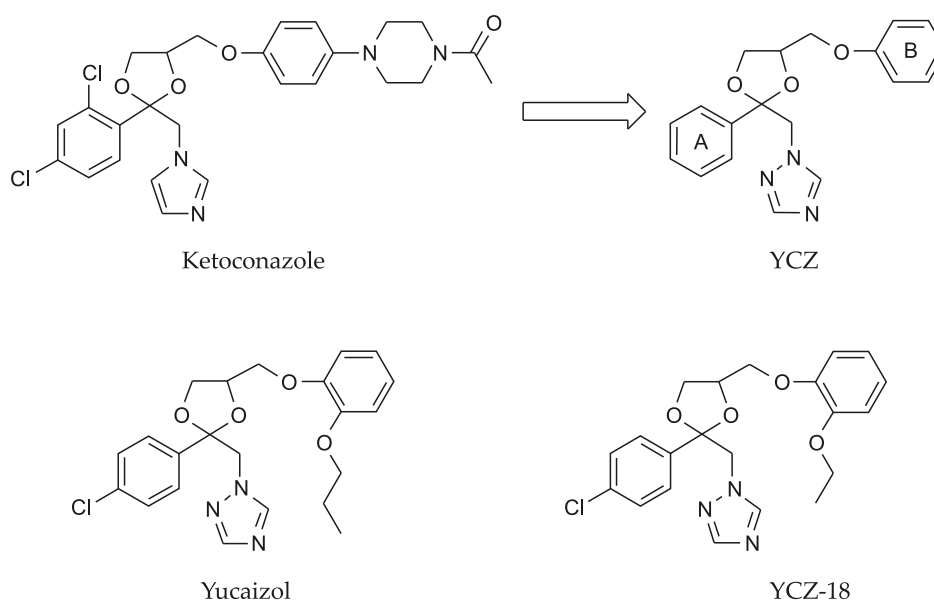
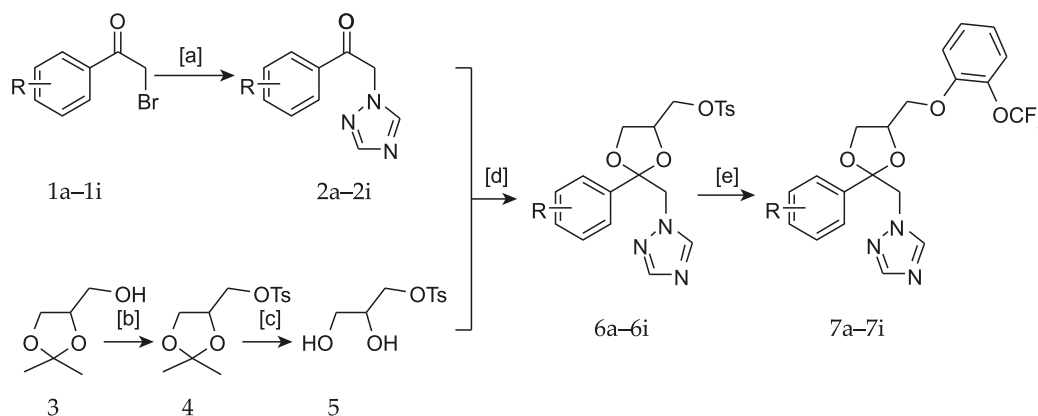
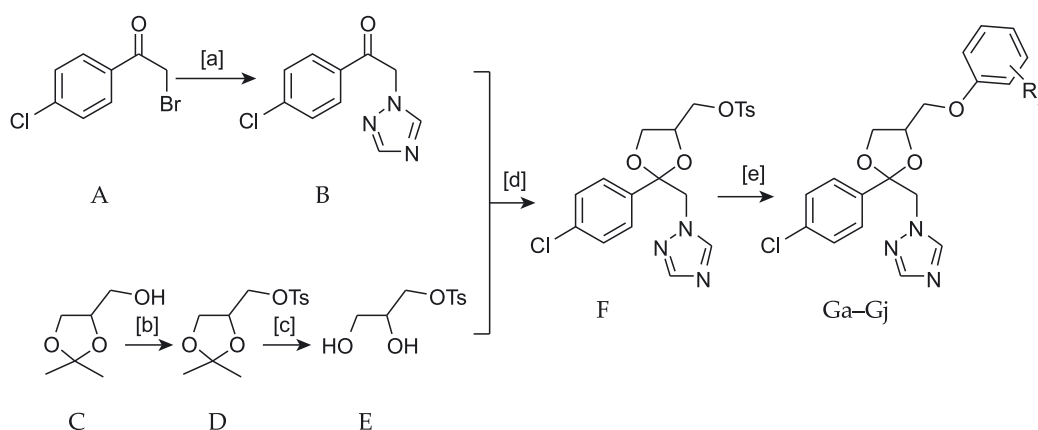


Fig. 1. The strategy of molecular scaffold (the arrow) of core structure of YCZ. The structures of lead compounds of brassinosteroid biosynthesis inhibitors: Yucaizol and YCZ-18 used in this study were shown



Scheme 1. Reagents and conditions: [a] 1,2,4-triazole, triethylamine, DMF, -10°C , 1 h, then the reaction was continued at rt, 3 h; [b] TsCl, pyridine, 0°C , acetone; [c] HCl, Reflux, 6 h; [d] 3 equiv TfOH, toluene, rt, 60 h; [e] 2-trifluoromethoxyphenol, KOH, DMF, 50°C , 12 h. The variation of the substituents of "R" on phenyl ring which is shown in "a" to "i" is according to the chemical structure displayed in table 1



Scheme 2. Reagents and conditions: [a] 1,2,4-triazole, triethylamine, DMF, -10°C , 1 h, then the reaction was continued at rt, 3 h; [b] TsCl, pyridine, 0°C , acetone; [c] HCl, Reflux, 6 h; [d] 3 equiv TfOH, toluene, rt, 60 h; [e] corresponding phenol, KOH, DMF, 50°C , 12 h. The variation of the substituents of "R₂" on phenoxy ring which is shown in "Ga" to "Gj" is according to the chemical structure displayed in table 2

structures in ring A was carried out by using different α -bromoketone **1a–1i** as a starting material. Compound **2a–2i** was prepared by reacting compound **1a–1i** with triazole in dimethylformamide (DMF) using the previously described method (Oh *et al.* 2008). The tosylation of isopropylidene glycerol **3** was achieved using standard protocol (tosyl chloride in pyridine at 0°C), and hydrolysis with 1 M HCl in MeOH yielded glyceryl tosylate **5**. Ketal formation to generate **6a–6i** was carried out using 3 equiv of trifluoromethanesulfonic acid (TfOH) in toluene at room temperature for 60 h, according to the previously described method (Tanoury *et al.* 2003). The target compound **7a–7i** was prepared by reacting **6a–6i** with 2-trifluoromethoxyphenols in a basic condition, as described previously (Tanoury *et al.* 1998).

Chemical modification of ring B was achieved by the method shown in scheme 2. We used 4-chlorophenacyl bromide **A** as a starting material. The methods for preparing the target compound **Ga–Gj** are similar to those for preparing compound **7a** as outlined in scheme 2, except for the final step: variant structures at ring B were intro-

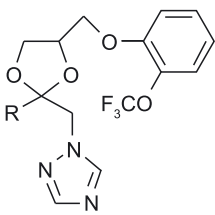
duced by using different phenols. Data for the characterization of the test compounds using Nuclear Magnetic Resonance (NMR) and High Resolution Mass Spectrometry (HR-MS), were achieved and were shown in our previous reports (Oh *et al.* 2012; Yamada *et al.* 2012).


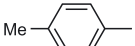
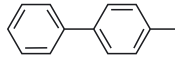
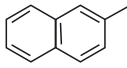
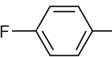
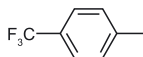
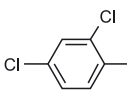
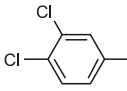
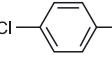
Biology

All the compounds used in this work consist of four stereoisomers, and they were subjected to biological studies without further purification. The concentration of all the test compounds were adjusted to a final concentration of 100 μM in PSA medium, while propiconazole (10 μM) was used as a positive control.

Effect of the chemical structure of ring A on antifungal activity

The chemical structures and antifungal activity of compounds with different structures, on ring A, and those that shared the same structure as ring B with 2-trifluorophenoxy moiety, were shown in table 1. Compound **7a** which

Table 1. Antifungal activity of compounds with variant structure of ring A


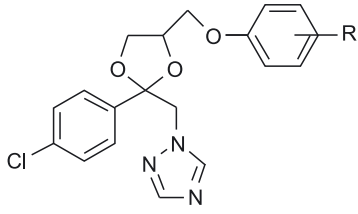
Compound No.	R	Chemical name	Inhibition [%]
7a		phenyl	65.2±3.0
7b		4-methylphenyl	51.5±4.0
7c		4-phenylphenyl	39.4±3.1
7d		naphhtatyl-2-yl	33.3±4.1
7e		4-fluorophenyl	69.7±1.5
7f		4-trifluoromethylphenyl	53.0±4.0
7g		2,4-dichlorophenyl	66.7±1.5
7h		3,4-dichlorophenyl	39.4±1.6
7i		4-chlorophenyl	53.0±1.52
Propiconazole (100 µM)	-	-	96.7±2.8

has a phenyl substituent was used as a baseline reference for the structure-activity relationship studies. We found that compound **7a** displayed antifungal activity against *M. oryzae*. Compound **7a** inhibited fungi growth by approximately 65.2±3.0%. When a methyl was introduced to the phenyl ring (**7b**), we found that antifungal activity decreased from 65.2±3.0 to 51.5±4.0%. When introducing a phenyl moiety at position 4 of the phenyl moiety of compound **7a** (the compound **7c**), the antifungal activity significantly decreased from 65.2±3.0 to 39.4±3.1%. This result indicated that a bulky substituent may have a negative effect on enhancing the antifungal activity of this synthetic series. Likewise, the analogue of naphhtatyl-2-yl (**7d**) weakened antifungal activity with a growth inhibition of approximately 33.3±3.0%. When an electron-withdrawing fluorine atom was introduced into this position (**7e**), the antifungal activity was slightly enhanced from 65.2±3.0 to 69.7±1.5%. When comparing the antifungal activity of **7b** with **7f**, we found that an electron-with-

drawing group (trifluoromethyl group) had a positive effect on enhancing the antifungal activity of this synthetic series. Introducing two chlorine atoms to the phenyl moiety, had a different effect on the enhancement of the antifungal activity. As shown in table 1, the 2,4-dichlorophenyl analogue (**7g**) slightly enhanced antifungal activity, with a growth inhibition of approximately 66.7±1.5%, while the 3,4-dichlorophenyl analogue (**7h**) significantly reduced the antifungal activity, with a growth inhibition of approximately 39.4±1.6%. The mono chlorine substitution at position 4 of the phenyl ring (**7i**) displayed a negative effect on enhancing the antifungal activity of this synthetic series. When these results were taken together, we found that compound **7e** displayed the most potent antifungal activity in this synthetic series.

Effect of the chemical structure of ring B on antifungal activity

To further explore the structure-activity relationship of this synthetic series, we next determined the effect of the

Table 2. Antifungal activity of compounds with variant structure of ring B


Compound No.	R	Inhibition [%]
7i	2-OCF ₃	53.0±1.5
Ga	2-Cl	71.2±3.0
Gb	3-Cl	80.3±1.5
Gc	4-Cl	75.8±3.1
Gd	2,3-Cl ₂	80.4±3.0
Ge	2,4-Cl ₂	74.3±4.0
Gf	2,5-Cl ₂	25.8±6.1
Gg	2,6-Cl ₂	74.2±3.2
Gh	3,4-Cl ₂	74.0±3.0
Gi	3,5-Cl ₂	65.2±4.1
Gj	H	94±3.8
Propiconazole (100 µM)	-	96.7±2.8

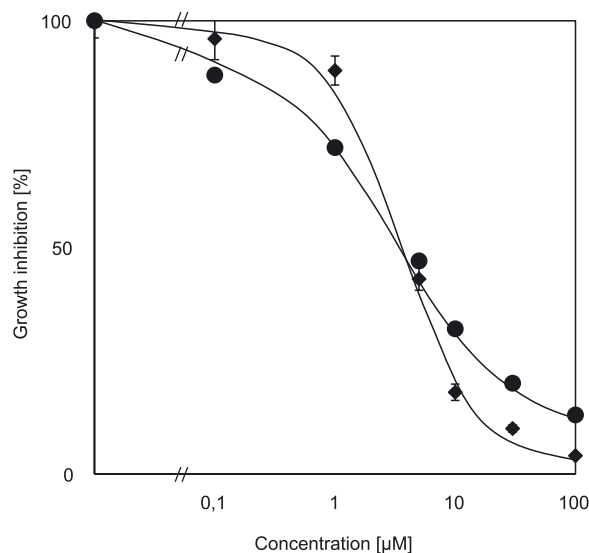


Fig. 2. Dose dependent effect of compound **Gj** on *Magnaporthe oryzae* growth. Filled circle is compound **Gj** and filled diamond is the positive control of propiconazole. Experiments were performed three times under the conditions described in the text

chemical structure of ring B (Fig. 1) on antifungal activity. We used analogues with 4-chlorophenyl moiety in common at ring A and with different phenoxy moiety at ring B (Table 2). Compound **7i** was used as a baseline reference for structure-activity relationship studies. We found that compound **7i** inhibits *M. oryzae* growth approximately $53.0 \pm 1.5\%$, at a concentration of $100 \mu\text{M}$, while the antifungal activity of the positive control of propiconazole ($10 \mu\text{M}$) was found to be approximately $70.4 \pm 2.8\%$. Next, we introduced chlorine atom(s) to the different position of the phenoxy moiety (ring B) to evaluate the effects on antifungal activity of this synthetic series. When one chlorine atom was introduced to the ring B (**Ga**, **Gb**, **Gc**), the antifungal activity of these compounds were enhanced from $53.0 \pm 1.5\%$ to 71.2 ± 3.0 , 80.3 ± 1.5 and $75.8 \pm 3.1\%$, respectively. This result indicated that a chlorine atom at position 3 of ring B displays a positive effect on promoting the antifungal activity of this synthetic series. The introduction of two chlorine atoms to ring B had different effects on the antifungal activity. The 2,3-dichlorophenoxy analogue (**Gd**) enhanced the antifungal activity. We found a growth inhibition approximately $80.4 \pm 3.0\%$. In contrast, the 2,5-dichlorophenoxy analogue (**Gf**) significantly reduced the antifungal activity of this synthetic series. Other analogues (**Ge**, **Gg**, **Gh**, **Gi**) displayed different antifungal activity against *M. oryzae* with an inhibition of fungal growth from 65.2 ± 4.1 to $74.3 \pm 4.0\%$. This result indicated that the position of the chlorine atoms among the double-chlorine-substituted-analogues, did not show significant effects on the antifungal activity of this synthetic series, except for compound **Gf**. Finally, we determined the antifungal activity of the analogue without a chlorine substituent at ring B (**Gj**). We found that compound **Gj** exhibited the most potent antifungal activity among the compounds listed in table 2 with a growth inhibition at $100 \mu\text{M}$; approximately $94.0 \pm 3.8\%$. Data obtained in the present work provided insight and information about the structure requirement for antifungal activity of this synthetic series against *M. oryzae*. Among all the

test compounds, we found that compound **Gj** displayed the most potent antifungal activity against *M. oryzae*.

Next, we used compound **Gj** to determine the dose-dependent effect of antifungal activity against *M. oryzae*. As shown in figure 2, compound **Gj** inhibits *M. oryzae* growth in a dose dependent manner. The IC_{50} was found to be approximately $3.8 \pm 0.5 \mu\text{M}$ and the IC_{50} of the positive control of propiconazole was found to be approximately $3.7 \pm 0.2 \mu\text{M}$ in our assay system, respectively.

In the present work, the antifungal activity of a series of new 1,2,4-triazole derivatives against *M. oryzae* was evaluated. Structure-activity relationship studies revealed that 1-(4-phenoxy-methyl-2-phenyl-[1,3]dioxolan-2-ylmethyl)-1H-1,2,4-triazole (**Gj**) displayed potent antifungal activity against *M. oryzae*. We found the IC_{50} value was approximately $3.8 \pm 0.5 \mu\text{M}$. Although the test compounds were synthesized based on the design of brassinosteroid biosynthesis inhibitors, the data obtained from the present work indicated that these compounds also displayed potent antifungal activity against *M. oryzae*.

The chemical structure of the compounds used in the present work are partially similar to that of ketoconazole, which is a well-known P450 inhibitor. Currently, ketoconazole is widely used experimentally and clinically (Scheinfeld 2008). Thus, yucaizol derivatives may inhibit P450 enzymes involved in fungi sterol biosynthesis.

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