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ANTIFUNGAL ACTIVITY OF BRASSINOSTEROID BIOSYNTHESIS INHIBITORS YUCAIZOL DERIVATIVES AGAINST *MAGNAPORTHE ORYZAE*

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ABSTRACT

Rice blast disease (RBD) is one of the most devastating diseases of cultivated rice caused by the pathogenic fungi *Magnaporthe oryzae*. To explore new compounds with antifungal activity against *Magnaporthe oryzae*, a select group of compounds synthesized in our laboratory targeting CYP90D1, a key enzyme in brassinosteroid biosynthesis, has been evaluated. The antifungal activity of the test compounds against *Magnaporthe oryzae* was determined by using *in vitro* mycelial growth inhibition tests. Structure-activity relationship analysis indicated that introducing a double bone to the alkyloxy substituent at the position 2 of phenoxy moiety of this synthetic series enhanced the antifungal activity. Moreover, a bulkyl substituent at the alkyloxy moiety exhibited a negative effect on promoting the antifungal activity. Among the 11 test compounds, we found that *2RS*, *4RS*-1-[4-chlorophenyl-(2-methylphenoxy)-ethyl]-1,3-dioxolan-2-yl-methyl]-1*H*-1,2,4-triazole (**7h**) displayed potent antifungal activity against *Magnaporthe oryzae* with an IC_{50} value approximately 23.3±0.5 μM.

Keywords: *Magnaporthe oryzae*, riazole derivatives, brassinsoteroid biosynthesis inhibitors, fungicide, rice blast disease.

INTRODUCTION

Diseases caused by plant pathogenic fungi is a leading constraint in world's crop production. One of the most devastating diseases of cultivated rice (*Oryza sativa* L) is the rice blast disease (RBD), which is caused by the pathogenic fungi *Magnaporthe oryzae* (Talbot, 2003; Clergeot *et al*., 2001; Bechinger *et al*., 1999). It is estimated that the amount of rice lost by this disease could feed over 60 million people annually worldwide. It affects all growth stages of the plant with some sever damage during the seedling stage. To control the RBD, tremendous efforts have been made and various management strategies like controlled use of nitrogen fertilizers, application of silica and flooding of paddy fields have been applied in use (Pageau *et al*., 2003). Even though, chemical fungicide is the most common solution effectively to minimize the severity of RBD and increase rice production. In the early of last century, the copper fungicides were first introduced to control BRD (Thurston, 1998). Subsequently, many systemic fungicides with different mode of action, like anti-mitotic compounds (Davidse, 1986), melanin inhibitors (Knight *et al*., 1997), ergo sterol biosynthesis inhibitor (EBI) and other organic compounds were discovered and used for RBD control (Baldwin and Rathmell, 1988).

Different from vertebrate, fungal membrane plays critical role of maintaining cell order and integrity. Hence, chemicals that directly or indirectly target fungal membranes or their components is a feasible method for fungal disease control. Some of these antifungal compounds affect the synthesis of specific membrane components (e.g., sterol biosynthesis inhibitors) are among the most effective fungicides in plant disease control. Currently, many types of fungicides have been developed and some of which targeting the biosynthesis of sterols thereby altering the structure and function of the cell membrane (Hartmann, 1998). 14α-demethylase (CYP51A1) is a well-known target for fungicides (Zarn *et al*., 2003). This class of P450 enzyme plays an essential role in mediating membrane permeability. Based on the action mechanism of monooxygenase of P450s (Koymans *et al*., 1993), it is believed that the essential steps are the binding of substrate and following the reduction of ferric, resting state of P450s to the ferrous state. The hydroxylation step is initialed by binding of molecular oxygen to give a ferrous P450-dioxygen complex. This event which is the key step in the action of P450s has been applied to design P450s inhibitors (Szklarz and

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Halpert, 1998). Azole derivatives have been demonstrated to have widespread ability as inhibitors of P450s, due to the intrinsic affinity of the nitrogen electron pair in heterocyclic molecules for the prosthetic heme iron. 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 found a series of new BR biosynthesis inhibitors YCZ (Oh *et al*., 2012). Structure-activity relationship studies revealed yucaizol (the structure is shown in Fig. 1) which is the most potent inhibitor of BR biosynthesis found to date (Yamada *et al*., 2012, 2013; Oh *et al*., 2013). Use of YCZ-18, an analogue of yucaizol (the structure is shown in Fig. 1) we demonstrated that yucaizol is a specific inhibitor of BR biosynthesis. Quantitative analysis BRs levels in YCZ-18 treated *Arabidopsis* provided evidence that BRs contents were significantly reduced. Providing intermediates of BRs biosynthesis to YCZ-18-treated *Arabidopsis* seedlings indicated that YCZ-18 targeted the step responsible for the C-23 hydroxylation of cathasterone. Assessment of the binding affinity of YCZ-18 to purified recombinant CYP90D1 indicated that YCZ-18 induced a typical type II binding spectrum (Oh *et al*., 2015).

Based on these findings, we have started working on the application use of yucaizol as a new plant growth regulators. 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 *Magnaporthe oryzae* (Lin *et al*., 2005). Considering the structural similarity of the compounds to yucaizol, it is possible that yucaizol may exhibits antifungal activity against *Magnaporthe oryzae*. To verify this hypothesis, we report herein the biological evaluation the antifungal activity of yucaizol and its derivatives against *Magnaporthe oryzae.*

MATERIALS AND METHODS

Chemicals

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

Magnaporthe oryzae strain

Rice blast isolate designated APU00-093A (race 007.0) was obtained by mono-spore isolation from diseased rice panicle on the paddy field of Akita Prefecture Japan in 2000. This isolate was kept on potato dextrose agar at 15^{o}C.

Antifungal activity assay

Poisoned food technique was performed to investigate antifungal effect of test compounds against *Magnaporthe oryzae*. The 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^{o}C after autoclaving) to the appropriate concentration. The final concentration of DMSO of each medium was 0.1%. Three mycelial pellet (1mm in diameter) of *Magnaporthe oryzae* pre-cultured on potato dextrose agar (PDA) medium were placed on the PSA medium containing the given concentrations of the test compound. The diameter of the mycelial mat of *Magnaporthe oryzae* was measured when the diameter of each corresponding untreated control reached about 20-30mm. Concentration for 50% inhibition (IC_{50}, μM) of mycelial growth was calculated by the linear regression formula obtained from the logarithm of concentration and the inhibition rate at each concentration against untreated control. All experiments were carried out in triplicate and the report data represents 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 upon $P<0.05$ unless stated otherwise.

RESULTS AND DISCUSSION

Chemistry

The test compounds were synthesized by a method as we previously described. Briefly, preparation of target compound **7** is outlined in Scheme 1. The key transformation of **2** with **5** consisted of four steps: (1) formation of 1-(4-chlorophenyl)-2-(1*H*-1,2,4-triazol-1yl) ethanone **2**; (2) tosylation of isopropylidene-glycerol **3**; (3) deprotection of isopropylidene ketal **4**; and (4) ketal formation to generate **6**. Compound **2** was prepared by reacting α-bromoketone **1** with triazole in DMF using a method that we described previously (Oh *et al*., 2008). The tosylation of isopropylidene glycerol **3** was achieved using a standard protocol (tosyl chloride in pyridine at 0 ^{o}C), and hydrolysis with 1 M HCl in MeOH yielded glyceryl tosylate **5**. Ketal formation to generate **6** was carried out using 3 equivalent of trifluoromethanesulfonic acid (TfOH) in toluene at room temperature for 60 h, according to a method previously described (Tanoury *et al*., 2003). The target compound **7** was prepared by reacting **6** with corresponding phenols in a basic condition, as described previously (Tanoury *et al*., 1998).

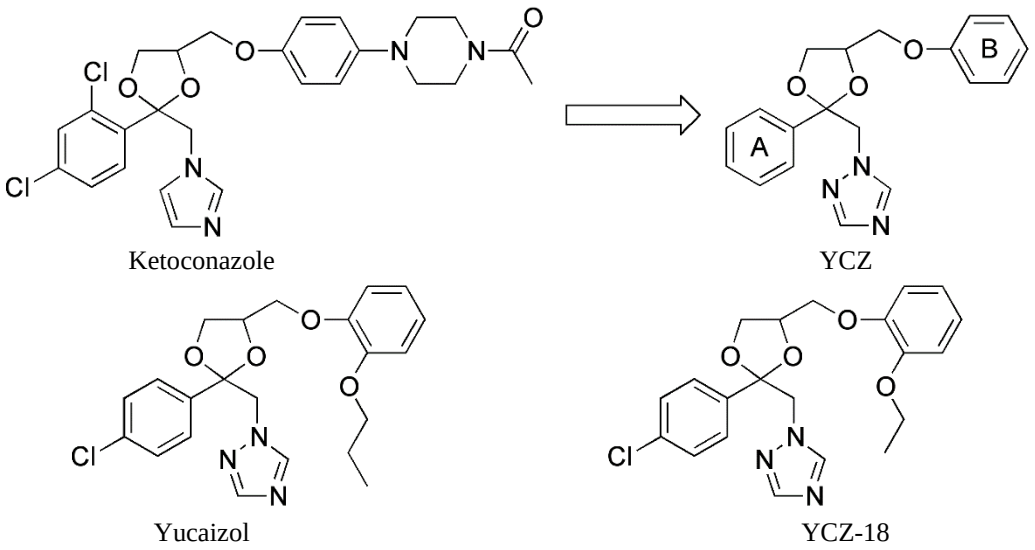
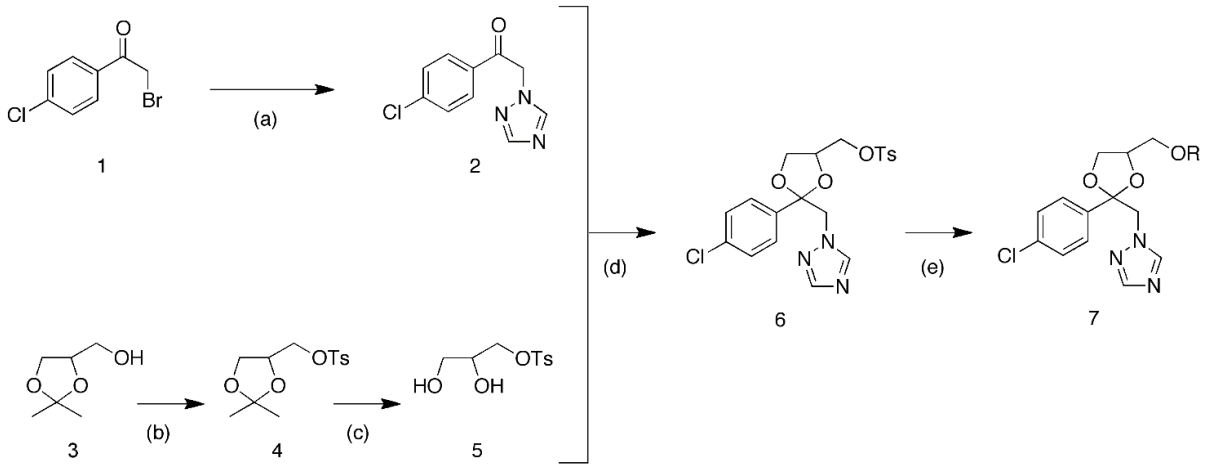


Fig. 1. Chemical structures of brassinosteroid biosynthesis inhibitors and structures of chemicals in this study.



Scheme 1. Reagents and conditions: (a) 1,2,4-triazole, triethylamine, DMF, -10 ^{o}C, 1 h, rt, 3 h; (b) TsCl, pyridine, 0^{o}C, acetone; (c) HCl, Reflux, 6 h; (d) 3 equiv TfOH, toluene, rt, 60 h; and (e) phenol, KOH, DMF, 50^{o}C, 12 h.

Data for characterization the test compounds using NMR and HR-MS were achieved and have been shown in our previous report (Yamada *et al*., 2013). All the compounds synthesized in this work consist of four stereoisomers, and they were subjected to biological studies without further purification.

Biology

The chemical structures of the test compounds were listed in table 1. The concentration of all the test compounds were adjusted to a final concentration of 100 μM in potato sucrose agar (PSA) medium while propiconazole (10 μM), a commercially available fungicide, was used as a positive control. Yucaizol was used as a baseline for structure-activity relationship discussion. As shown in table 1, at a concentration of 100 μM, yucaizol display the growth inhibition of the fungal at approximately 19.7±5.5%. When the propyl moiety was changed into ethyl (**7j**) or trifluoromethoxy (**7g**) the inhibition activity of the analogues were increased from 19.7±5.5% to 60.6±1.5 and 53.0±1.5%, respectively. This result indicated that a short side chain at this position have a positive effect on promoting the inhibitory activity of this synthetic series. Likewise, the antifungal potency for compound **7h** and **7i** are higher than that of yucaizol. When change the propyl moiety into 3-methylbutyl substituent (**7e**), the antifungal potency was decreased from19.7±5.5 to 15.2±4.0%. This result indicated that a bulkyl substituent at the side chain displays a negative effect on promoting the antifungal activity of this

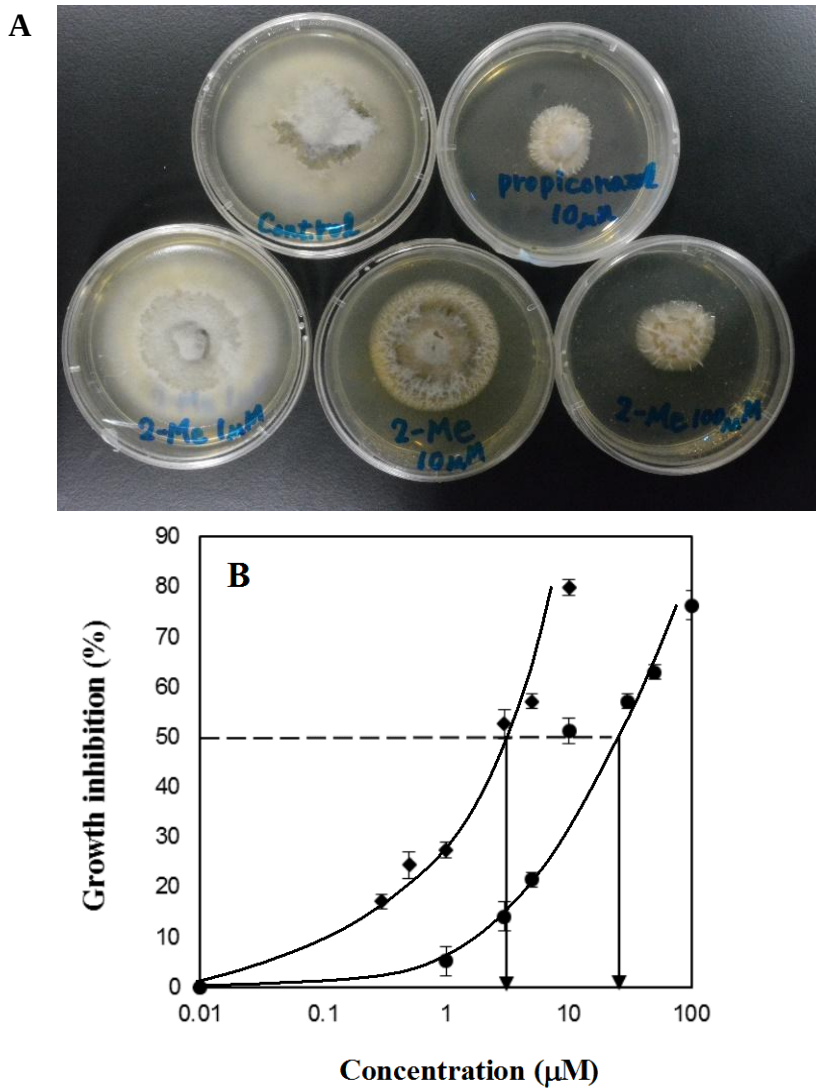


Fig. 2. Antifungal activity of compound **7h** against *Magnaporthe oryzae.* (A) Effect of compound **7h** on growth of *Magnaporthe oryzae*, upper left, control, upper right 10 μM propiconazole*;* lower left. 1 μM **7h**, lower middle, 10 μM **7h**, lower right, 100 μM **7h**. (B) Does dependent effect of **7h** on growth of *Magnaporthe oryzae,* filled cycle: compound **7h**; filled diamond: propiconazole. All the experiments were taken three times to establish the repeatability.

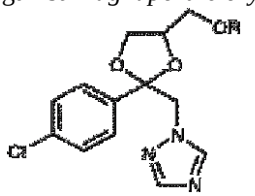
synthetic series against *Magnaporthe oryzae*. Another line of evidence indicated that a double bone structure may have a positive effect on promoting the antifungal activity. As shown in table 1, the antifungal potency for analogues with a double bond structure (**7a** and **7d**) are higher than those analogues have a same chemical structures except the double bond was replaced by a single bond (yucaizol and **7e**). The inhibitory activity was found 56.1±4.0% versus 19.7±5.5% for **7a** versus yucaizol; 31.8±2.6 versus 15.2±1.5% for **7d** versus **7e**. Interestingly, analogue without oxygen atom at position 2 of the phenoxy ring (**7h**) display the most potent inhibitory activity against *Magnaporthe oryzae.*

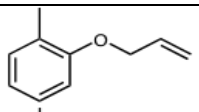
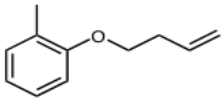
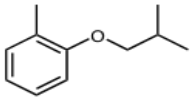
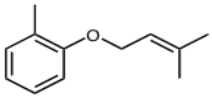
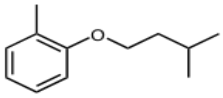
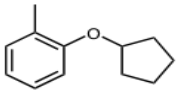
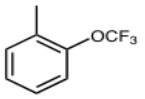
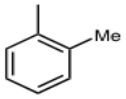
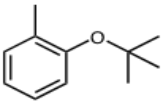
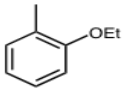
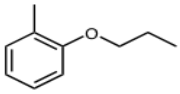
Next, we used compound **7h** to determine the dose-dependent effect of antifungal activity against *Magnaporthe oryzae* of this synthetic series. As shown in figure 2, compound **7h** inhibits *Magnaporthe oryzae* growth in a dose dependent manner. The IC_{50} was found approximately 23.3±0.5 μM. while the IC_{50} of the positive control of propiconazole was found approximately 3.7±0.2 μM in our assay system.

CONCLUSION

In the present work, we conducted a biological evaluation the antifungal activity of yucaizol and its derivatives

Table 1. Antifungal activity of test compounds against *Magnaporthe oryzae*.



Compound No.	R	Chemical name	Inhibition (%)*
7a		2-Allyloxyphenyl	56.1±4.0
7b		2-But-3-enyloxyphenyl	27.3±2.6
7c		2-Isobutoxyphenyl	15.2±4.0
7d		2-(3-Methyl-but-2-enyloxy)phenyl	31.8±2.6
7e		2-(3-Methylbutoxy)phenyl	15.2±1.5
7f		2-Cyclopentyloxyphenyl	21.2±5.5
7g		2-Trifluoromethoxyphenyl	53.0±1.5
7h		2-methylphenyl	72.7±2.6
7i		2-tert-butoxyphenyl	37.9±4.0
7j		2-Ethoxyphenyl	60.6±1.5
yucaizol		2-propoxyphenyl	19.7±5.5
Propiconazole (10 μM)			79.3 ±1.5

*The concentration of all the test compounds were 100 μM. All the experiments were done three times to establish the repeatability.

against *Magnaporthe oryzae*. Data obtained from present work provided evidences that the test compounds exhibited antifungal activity against *Magnaporthe oryzae*. Structure-activity relationship analysis indicated that introducing a long chain structure to the alkyoxy moiety at the position 2 of the phenoxy moiety significantly reduced the antifungal activity. In addition, introducing a double bond to the alkyloxy substituent to this synthetic series enhanced the antifungal activity. We found that compound **7h** is the most potent antifungal compound with an IC_{50} approximately 23.3±0.5 μM which is approximately 6.3 times to that of propiconazole in our

assay system. We expect further structure-activity relationship studies may lead to found new compounds with potent antifungal activity against *Magnaporthe oryzae.*

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