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Efficient Target-Site Assay of Chemicals for Melanin Biosynthesis Inhibition of *Magnaporthe grisea*

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A rapid and efficient assay to determine melanin biosynthesis inhibition of *Magnaporthe grisea*, a causal agent of the rice blast, by chemicals was developed. Wells in 24-well plates were loaded with spore suspension of the fungus and three known melanin biosynthesis inhibitors of KC10017, tricyclazole, and carpropamid. Subsequent color changes of mycelia and culture media in the wells were observed 7 days after incubation. The wells treated with KC10017 (an inhibitor of polyketide synthesis step and/or pentaketide cyclization step) became colorless, whereas tricyclazole (an inhibitor of 1,3,8-trihydroxynaphthalene reductase) or carpropamid (an inhibitor of scytalone dehydratase)-treated wells exhibited red color. They did not show any inhibitory effect on fungal growth. The inhibition of reaction steps prior to 1,3,6,8-tetrahydroxynaphthalene formation was easily determined by colorless medium and mycelia. However, it was impossible to distinguish between inhibition of reduction steps and inhibition of dehydration steps by colors of the cultures. It was accomplished through HPLC analysis of the melanin biosynthesis-involving pentaketide metabolites accumulated by the inhibitors. Through screening of a number of synthetic chemicals using the *in vitro* assay, we could find a novel chemical group of melanin biosynthesis inhibitor.

Keywords : melanin, *Magnaporthe grisea*, assay, carpropamid, tricyclazole, KC10017.

Specific penetration processes such as melanin biosynthesis and appressorium formation essential for fungal pathogenicity to plants may be unimportant or lacking in non-target organisms. Therefore, compounds which affect these would generally show less of an environmental hazard than conventional fungicides which act on processes, such as spore germination and mycelial growth, etc., common to many organisms. The most representative group of non-fungitoxic antipenetrant compounds used for practical control of plant disease is members of a group of compounds that specifically block melanin biosynthesis in the pathogen such as *Magnaporthe grisea*.

The compounds commonly referred to as melanin biosynthesis inhibitors (MBIs) include tricyclazole (5-methyl-1,2,4-triazolo[3,4-b]benzothiazole) (Okuno et al., 1983; Suzuki et al., 1982; Woloshuk and Sisler, 1982; Woloshuk et al., 1980; Yamaguchi et al., 1982), pyroquilon (1,2,5,6-tetrahydropyrrolo[3,2,1-*ij*]quinolin-4-one) (Woloshuk and Sisler, 1980; Yamaguchi et al., 1982; Woloshuk et al., 1981), fthalide (4,5,6,7-tetrachlorophthalide) (Chida and Sisler, 1987b; Inoue et al., 1984b; Yamaguchi et al., 1982), 2,3,4,5,6-pentachlorobenzylalcohol (PCBA) (Woloshuk and Sisler, 1980; Yamaguchi et al., 1982), chlorobenthiazone (4-chloro-3-methylbenzothiazol-2(3*H*)-one) (Inoue and Kato, 1983; Inoue et al., 1984a; Inoue et al., 1984b), pp389 (4,5-dihydro-4-methyltetrazolo[1,5-a]quinazolin-5-one) (Yamaguchi et al., 1982), carpropamid {(1R*,3S*)-2,2-dichloro-*N*-[1-(4-chlorophenyl)ethyl]-1-ethyl-3-methylcyclopropanecarboxamide} (Kurahashi et al., 1996; Kurahashi et al., 1997), and KC10017 (3-[4'-bromo-2',6'-dimethylphenoxy]methyl-4-[(3"-methylphenyl)aminocarbonyl]methyl-1,2,4-oxadiazol-5-one) (Kim et al., 1998). They commonly interfere fungal melanin biosynthesis without inhibitory effect on mycelial growth, spore germination, and appressorial formation of *M. grisea*. Within this group, only tricyclazole, pyroquilon, fthalide, and carpropamid are currently being used in practical applications, which are limited to the control of rice blast disease caused by *M. grisea*. The fungus produces 1,8-dihydroxynaphthalene (1,8-DHN) melanin, which is critical for the function of the appressoria formed from spores on rice leaves in penetration into host plants (Okuno et al., 1983; Woloshuk and Sisler, 1982; Woloshuk et al., 1980; Yamaguchi et al., 1982).

1,8-DHN melanin is derived via a polyketide pathway through a series of steps involving pentaketide synthesis and/or cyclization to form 1,3,6,8-tetrahydroxynaphthalene (1,3,6,8-THN), subsequent reduction and dehydration of

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1,3,6,8-THN to form 1,8-dihydroxynaphthalene (1,8-DHN), and final oxidation and polymerization of 1,8-DHN. Except carpropamid and KC10017, the other MBIs block two reduction steps of the melanin biosynthesis pathway; the conversion of 1,3,6,8-THN to scytalone and 1,3,8-trihydroxynaphthalene (1,3,8-THN) to vermelone (Chida and Sisler, 1987a; Kubo et al., 1983; Woloshuk and Sisler, 1982; Yamaguchi et al., 1982), and thus cause accumulation of shunt products such as 3,4-dihydro-4,8-dihydroxy-1-(*2H*)-naphthalene (4,8-DDN), 3,4-dihydro-3,4,8-trihydroxy-1-(2*H*)-naphthalenone (3,4,8-DTN), 3,4-dihydro-4,6,8-trihydroxy-1-(2*H*)-naphthalenone (4,6,8-DTN), and 4-hydroxyscytalone [3,4-dihydro-3,4,6,8-tetrahydroxy-1-(2*H*)-naphthalenone] (4-HS). Carpropamid inhibits melanin biosynthesis by blocking the dehydration steps between scytalone and 1,3,8-THN, and between vermelone and 1,8-DHN, resulting in accumulation of a large amount of scytalone and a small amount of vermelone (Kurahashi et al., 1996; Kurahashi et al., 1997; Kurahashi et al., 1998). On the other hand, KC10017 inhibits the pentaketide synthesis and/or cyclization prior to 1,3,6,8-THN formation, and none of shunt products involved in melanin biosynthesis accumulate in the cultures (Kim et al., 1998). Thus, enzymes in the melanin biosynthetic pathway have been the prime targets for fungicide development. Many companies have been trying to develop new fungicides for rice blast with the same mode of action as MBIs.

In the past, lead generation for new fungicide development was usually conducted by *in vivo* screening of synthetic chemicals and natural products. *In vivo* assay requires large amounts of chemicals at levels of milligram or gram quantities. Recently, pesticide companies have changed technologies involved in lead generation, and so have been developing various *in vitro* methodologies that afford a step change in the ability to cope with diverse chemical formats, small quantities and high-throughput rates. Additionally, it is becoming increasingly important to find the action mode of a lead at an early stage of characterization process. We developed an efficient *in vitro* assay to rapidly determine melanin biosynthesis inhibition by chemicals using 24-well plate. In addition, the target site of MBIs has been elucidated by HPLC analysis.

Materials and Methods

Chemicals. Technical-grade tricyclazole was kindly supplied from Eli Lilly Co. (Greenfield, IN, USA). Carpropamid was obtained from Nihon Byer Agrochem Co. (Yuki, Japan). KC10017 was synthesized in Korea Research Institute of Chemical Technology (KRICT; Taejon, Korea). 3,4,8-DTN, 4,6,8-DTN and 4-HS were isolated in our laboratory (Kim et al., 1998). Scytalone was from Microbial Toxicology Laboratory at the Institute of Physical and Chemical Research (RIKEN; Wako, Japan). All organic solvents were purchased from Duksan Pure Chemicals (Ansan, Kyunggi-do, Korea) with the exception of HPLC solvents, which were obtained from Mallincrodt, Inc. (Paris, Kentucky, USA).

Rice blast fungus. *M. grisea* strain P-2 maintained at Microbial Toxicology Laboratory at RIKEN was obtained and used throughout the experiments. The culture was kept on a rice bran agar (RBA) medium.

Culture conditions. RBA medium was also used for the spore production. As the inocula for RBA medium, mycelial suspension from 4-day-old potato dextrose broth (PDB) cultures was used and the final inoculum volume was less than 1% (v/v). After incubation at 25°C for 7 days, the mycelia formed on the agar medium were removed by scrapping with a paint brush and then the medium was further incubated at 25°C for 2 days under Black Light-Blue lamps. The spores were washed with PDB medium by using a paint brush and filtered through four layers of cheesecloth. They were adjusted to 1×10^5 spores/ml using a hemocytometer and used for target assay of chemicals for melanin biosynthesis inhibition.

Target assay for melanin biosynthesis inhibition. A 1-ml of spore suspension was added to each well of 24-well plates and then 10 μl of each chemical solution was mixed. KC10017, tricyclazole, and carpropamid were added as dimethyl sulfoxide (DMSO) solutions with appropriate controls. The plates were incubated at 25°C and 150 rpm. Color change of cultures and growth inhibition of the fungus were observed after incubation for 7 days. In the screening of a number of synthetic chemicals, they were dissolved in DMSO and then treated at two concentrations of 5 μg/ml and 50 μg/ml. The experiment was conducted with two replications per treatment. The chemicals that cause color change of cultures without inhibition of fungal growth were determined to act as MBIs.

HPLC analysis of pentaketide metabolites involved in melanin biosynthesis. When scytalone dehydratase and THN reductase were inhibited by chemicals, the mycelia and culture media of both cultures exhibited red color. Therefore, it is impossible to distinguish between scytalone dehydratase inhibition and THN reductase inhibition by color changes of the cultures. In order to do it, the pentaketide metabolites accumulated by the inhibitors were analysed by using HPLC (Waters; Milipore Co., Milford, Mass., USA). The cultures taken from wells were filtered through a 0.45 μm membrane filter and then injected directly into HPLC. The following equipment and conditions were used for the analysis: column, μ Bondapak C_{18} (3.9 by 300 mm); mobile phase, linear gradient of $CH_3OH : H_2O : AcOH$ (10 : 89 : 1, v/v/v) to $CH_3OH : H_2O : AcOH$ (64 : 35 : 1, v/v/v) for 40 min; flow rate, 1 ml/min; UV detector, 335 nm. The retention times of 4-HS, 3,4,8-DTN, scytalone, and 4,6,8-DTN were 12.1, 13.3, 18.0, and 19.3 min, respectively.

Spore germination, appressorium formation, and appressorial pigmentation. As the results of screening of a number of synthetic chemicals by using the target-site assay, a series of chemicals belonging to 2-imino-1,3-thiazoline group, containing a five-membered and two six-membered rings in the structure, showed to inhibit melanin biosynthesis. Among them, KSC35421

compound was chosen and used to examine its activity to spore germination, appressorium formation and appressorium pigmentation of *M. grisea*. Spores obtained from RBA medium were suspended in the deionized water to give a density of 1×10^5 spores/ml. KSC35421 was added to the spore suspension, adjusting the solvent not to exceed 1% in the final concentration. The treated spore suspension was loaded onto a cellophane piece of 20 mm square on a glass slide and incubated at 25°C. Spore germination, appressorium formation, and appressorium pigmentation were assessed 24 h after the spore suspension application. Approximately 100 spores were observed under a light microscope in each of four replicates to determine the percentages of spore germination and appressorium formation. Pigmentation of appressoria was expressed as black appressoria (+) and hyaline appressoria (–).

Results and Discussion

Melanin biosynthesis inhibition of KC10017, tricyclazole, and carpropamid in 24-well plates. *M. grisea* mycelia started to pigment in the liquid cultures untreated from 5 days after the inoculation and the pigmentation level reached a maximum in 7 days. After 7-days incubation, melanization of *M. grisea* P-2 in liquid cultures treated with KC10017, tricyclazole, and carpropamid was shown in Fig. 1. As for KC10017, dark melanin pigments were accumulated in the cultures treated with 0.1 μg/ml, but not in the cultures treated with 1.0 and 10 μg/ml. Because KC10017 blocks pentaketide synthesis and/or pentaketide cyclization steps prior to 1,3,6,8-THN formation in the melanin biosynthesis, none of shunt products exhibiting color are accumulated in the cultures. So, the cultures treated with KC10017 became colorless. The cultures treated with 0.1 μg/ml of tricyclazole or carpropamid exhibited to be black, but those with 1.0 and 10 μg/ml turned red. This is due to inhibition of reductase and dehydratse reactions by tricyclazole and carpropamid, respectively, and then accumulation of shunt products in the cultures.

The cultures cotreated with both KC10017 and tricycalzole or both KC10017 and carpropamid at a concentration of 5 μg/ml each became colorless like as those treated with KC10017 alone. In contrast, the cultures cotreated with tricyclazole and carpropamid exhibited red color like as those treated with tricyclazole or carpropamid alone. The above results indicate that it is easy to distinguish between inhibition of reaction steps prior to 1,3,6,8-THN formation and inhibition of other two reduction and dehydration steps by color changes of the cultures, but not between inhibition of reduction steps and inhibition of dehydration steps. In order to do it, the shunt products and intermediates involved in 1,8-DHN melanin biosynthesis were examined and compared in the cultures treated with KC10017, tricyclazole, and carpropamid by HPLC analyses.

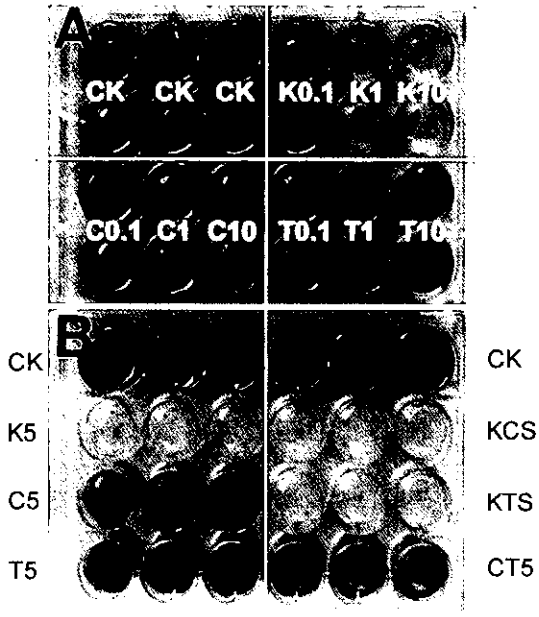

Fig. 1. Melanization of *Magnaporthe grisea* P-2 in liquid cultures using 24-well plates treated with KC10017, tricyclazole, or carpropamid alone (A), and cotreated with two of the three melanin biosynthesis inhibitors (B). CK, no treatment; K0.1, K1, and K10, treated with 0.1, 1, and 10 μg/ml of KC10017, respectively; C0.1, C1, and C10, treated with 0.1, 1, and 10 μg/ml of carpropamid, respectively; T0.1, 1, and 10, treated with 0.1, 1, and 10 μg/ml of tricyclazole, respectively; K5, C5, and T5, treated with 5 μg/ml of KC10017, carpropamid, and tricyclazole, respectively; KC5, treated with KC10017 (5 μg/ml) and carpropamid (5 μg/ml); KT5, treated with both KC10017 (5 μg/ml) and tricyclazole (5 μg/ml); CT5, treated with both carpropamid (5 μg/ml) and tricyclazole (5 μg/ml).

Determination of target sites of MBIs by HPLC analyses. None of shunt products and intermediates such as 4-HS, 3,4,8-DTN, 4,6,8-DTN, and scytalone were detected in KC10017-treated and untreated cultures (Fig. 2). However, three shunt products of 4-HS, 3,4,8-DTN, and 4,6,8-DTN were found at relatively low levels in the cultures treated with tricyclazole, which blocks reductase reactions in melanin biosynthetic pathway. Compared to this, one shunt product (4-HS) and one intermediate (scytalone) of melanin biosynthesis were detected at much higher levels in the cultures treated with carpropamid, which blocks dehydratase reactions.

Kurahashi et al. (1998) reported that a large amount of scytalone and a small amount of vermelone accumulated in the culture treated with 10 μg/ml of carpropamid. However, vermelone was not detected in the culture broth by HPLC in our study and in stead, 4-HS was found. This may be due to complete inhibition of a dehydration step between scytalone and 1,3,8-THN, and an irreversible reaction between 1,3,6,8-THN and scytalone; the accumulated scytalone is

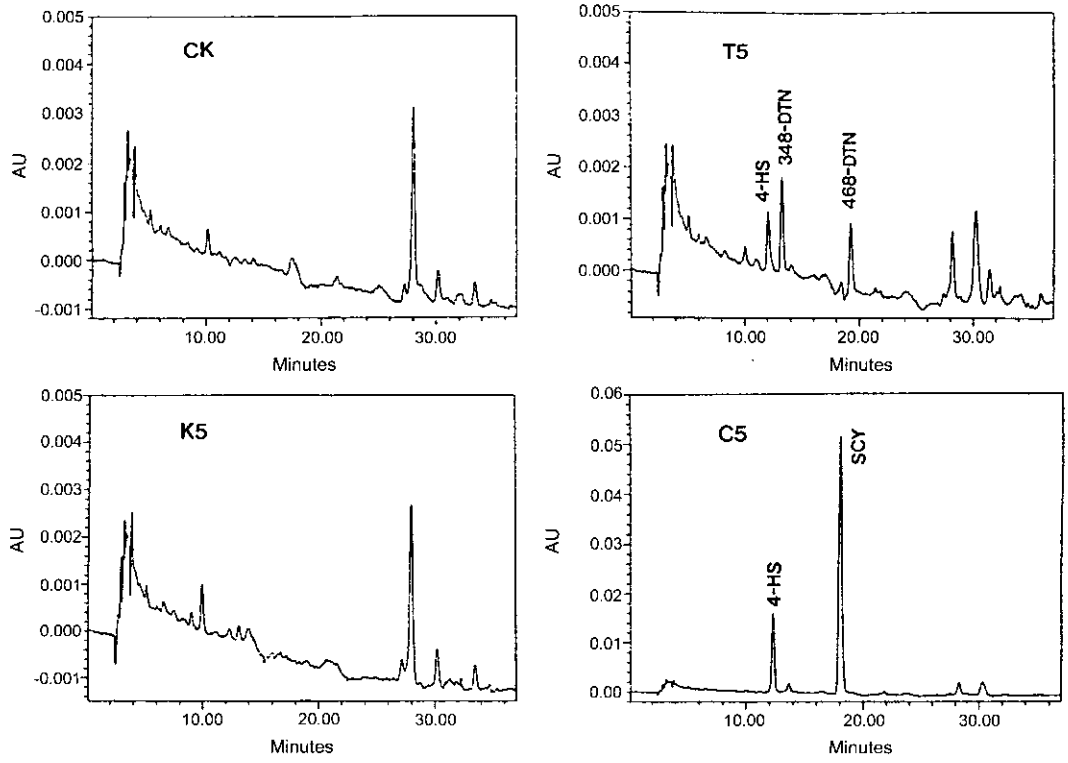

Fig. 2. HPLC separations of pentaketide metabolites involved in 1,8-DHN melanin biosynthesis in the culture broths untreated (CK), treated with 5 μg/ml of tricyclazole (T5), treated with 5 μg/ml of KC10017 (K5), and treated with 5 μg/ml carpropamid (C5).

converted to 1,3,6,8-THN through irreversible reaction step, and then the latter substance is transformed into 4-HS via flaviolin. The above results reveal that HPLC analyses of the culture broths enable to easily distinguish between inhibition of reduction steps and inhibition of dehydratation steps.

Screening of synthetic chemicals and effect of KSC-35421 on infection behaviors of *M. grisea*. A total of 2114 chemicals (1757 from KRICT and 357 from Korea Institute of Science and Technology, Seoul, Korea) of a large diverse chemical structures were screened by using the target-site assay for melanin biosynthesis pathway of *M. grisea*. As the results, a series of chemicals belonging to 2-imino-1,3-thiazoline group were found to inhibit melanin biosynthesis with no or low inhibitory effects to fungal growth at concentrations up to 50 μg/ml. The mycelia and culture media of cultures treated with the chemicals became colorless like those treated with KC10017. In addition, none of pentaketide metabolites involved in melanin biosynthesis were detected in HPLC analyses of the culture broths. These results indicate that the chemicals of 2-imino-1,3-thiazoline group inhibit one of reaction steps prior to 1,3,6,8-THN formation.

Among the chemicals, KSC35421 was selected and used to examine further its activity on spore germination, appressorium formation, and appressorium pigmentation. The chemical inhibited pigmentation in the appressoria formed on cellophane membranes treated with the concentrations of more than 0.1 μg/ml (Table 1). It had no or low effect on spore germination and appressorium formation of *M. grisea* P-2 at concentrations lower than 10 μg/ml, whereas it almost inhibited both spore germination and appressorium

Table 1. Effect of KSC35421 on spore germination, appressorium formation, and appressorium pigmentation of *M. grisea* P-2[a]

KSC35421 (μg/ml)	Spore germination (%)[b]	Appressorium formation (%)[c]	Appressorium pigmentation[d]
0	94 ± 5.2	74 ± 4.3	+
0.1	94 ± 3.4	84 ± 5.2	–
1	89 ± 2.3	79 ± 2.4	–
10	79 ± 1.3	57 ± 1.4	–
100	17 ± 2.0	9.0 ± 1.2	–

[a] One milliliter of spore suspension (10^5 spores/ml) of *M. grisea* P-2 was applied on a cellophane piece (20 × 20 mm) with or without KSC35421. Spore germination, appressorium formation and appressorium pigmentation were determined 24 h after the application.

[b] Spore germination was represented as the percentage of germinated spores. Each value is the average of four replications with standard deviation.

[c] Appressorium formation was represented as the percentage of germ tube tips with appressoria. Each value is the average of four replications with standard deviation.

[d] +, black appressoria; –, hyaline appressoria.

formation at a concentration of 100 μg/ml. However, since much higher concentrations of the chemical are required to inhibit fungal growth than to block melanin biosynthesis, it was thought to act as an antipenetrant toward *M. grisea* like other MBIs. Also, it showed potent controlling activity against only rice blast disease in *in vivo* assay; it completely controlled the development of rice blast disease when applied as foliar treatments at a concentration of 100 μg/ml, whereas it did not show any controlling activity against other plant diseases such as rice sheath blight, tomato gray mold, tomato late blight, wheat leaf rust, and barley powdery mildew at rates lower than 5 %.

In conclusion, a rapid assay using 24-well plate to specifically determine inhibition of melanin biosynthesis of *M. grisea* by chemicals has been developed. It is labor- and time-saving, and inexpensive. We could find new chemicals in the form of 2-imino-1,3-thiazoline derivatives inhibiting fungal melanin biosynthesis of *M. grisea* through the assay. It has proved useful to the fungicide industries for rapid screening of chemical samples.

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