Original Article

Mode of Action of (*E*)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol (S-3308) in *Ustilago maydis*

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The new fungicide, S-3308 [(*E*)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol] inhibited sporidial multiplication and caused morphological changes in *Ustilago maydis*. Sporidia were branched, swollen and multicellular in the presence of 2 µg/ml fungicide. Increase in dry weight was inhibited 66% during 8 hr incubation. Both endogenous and exogenous respirations were inhibited by S-3308 but a high concentration (100 µg/ml) was required for complete inhibition. Syntheses of protein, DNA and RNA were only slightly affected by the fungicide. A relative amount of ergosterol severely decreased in the treated sporidia and the concomitant accumulation of 24-methylenedihydrolanosterol, obtusifoliol and 14α-methyl- $\Delta^{8,24(28)}$ -ergostadienol was observed. Incorporation of ^{14}C -acetate into C-4-desmethyl sterols and sterol esters was inhibited more than 80%. On the contrary, the accumulation of radioactivities in the fractions of 4,4-dimethyl sterols and C-4-methyl sterols was observed. These results indicate that S-3308 particularly inhibits the demethylation at the C-14 position of 24-methylenedihydrolanosterol in the sterol biosynthetic pathway.

INTRODUCTION

S-3308 [(*E*)-1-(2,4-dichlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-yl)-1-penten-3-ol] (Fig. 1) is a new azole fungicide with a systemic activity and shows fungitoxicity to broad range of fungal species, especially to those belonging to Ascomycetes and Basidiomycetes. The fungicide has excellent preventive and curative activities on cereal diseases, e.g., powdery mildew (*Erysiphe graminis*), stripe rust (*Puccinia striiformis*), stem rust (*Puccinia graminis*) and brown rust (*Puccinia recondita*, *Puccinia hordei*). Seed treatment by the fungicide controls many kinds of seed-born diseases, e.g., loose smut of barley (*Ustilago nuda*) and wheat (*Ustilago tritici*), bunt of wheat (*Tilletia caries*) and stripe of barley (*Pyrenophora graminis*). Moreover, fruit crop diseases, e.g., scab of apple (*Venturia inaequalis*) and pear (*Venturia pirina*, *Venturia nashicola*), and

powdery mildew of apple (*Podosphaera leucotricha*) and vine (*Uncinula necator*) are also effectively controlled by S-3308.

During the last decade, a number of agricultural fungicides, which have diverse chemical structures, have been found to inhibit ergosterol biosynthesis in fungi. Among these, pyrimidines [fenarimol,^{1,2)} nuarimol^{1,2)} and triarimol^{3,4)}], pyridines [buthiobate^{5,6)}], imidazoles [imazalil^{2,9,10)} and prochloraz¹¹⁾], triazoles [triadimefon,¹²⁻¹⁴⁾ etaconazole¹⁵⁾ and diclobutrazol], piperazines [triforine^{7,8)}], and morpholines [tridemorph²⁶⁾] are included. Medical antifungal imidazole compounds, miconazole,^{16,17)} clotrimazole¹⁸⁾ and ketoconazole¹⁹⁾ are also known as inhibitors of ergosterol biosynthesis. Furthermore, recent investigations in our laboratory indicated that another antifungal compound, S-3308, strongly inhibited ergosterol biosynthesis rather than the other metabolic activities in fungi. This

Fig. 1 Chemical structure of S-3308.

investigation was undertaken to clarify the mode of action of S-3308.

MATERIALS AND METHODS

1. Culture Method

Sporidia of *Ustilago maydis* were grown in 200 ml Erlenmeyer flasks containing 40 ml of a nutrient solution.⁵⁾ The initial concentration of sporidia in the present experiments was adjusted to 4×10^6 sporidia/ml unless otherwise stated. Flasks were incubated at 27°C on a rotary shaker at 160 rpm.

2. Toxicity Studies

S-3308 was dissolved in methanol and added to the culture. The final concentration of the solvent in the medium was 1.0%. Sporidial number and dry weight were investigated every 2 hr-incubation period. After observation of sporidial number and morphology, sporidia were collected by centrifugation and washed with distilled water. Pellets of sporidia were transferred to weighed glass beakers, kept at 80°C for 18 hr and weighed after cooling.

3. RNA, DNA and Protein Analysis

RNA, DNA and protein were extracted from sporidia, which were incubated with or without the fungicide, by the method of Ragsdale and Sisler.⁴⁾ Amounts of RNA, DNA and protein were determined by the ribose-orcinol method,²⁰⁾ the diphenylamine method,²¹⁾ and the method of Lowry *et al.*,²²⁾ respectively.

4. Respiratory Measurement

The effect of various concentrations of S-3308 on oxygen uptake was determined with a Gilson differential respirometer. Sporidia preincubated for 1.5 hr were collected by centrifugation, washed twice with 0.2 M phos-

phate buffer (pH 6.0) and resuspended in the phosphate buffer. Starvation was performed by incubating for 3 hr on a rotary shaker. Aliquots (1.5 ml) of sporidial suspension were transferred to respective reaction vessels. Glucose or sodium succinate was added as an exogenous substrate at a final concentration of 0.1 M. Various concentrations of S-3308 in methanol were added to respective vessels and 2 N NaOH solution was put in the internal wells of vessels to absorb CO₂. The samples were incubated at 30°C and after 1 hr incubation, measurements of oxygen uptake were initiated and continued at 15 min intervals during the following 1.5 hr-period.

5. Sterol Analysis

Sporidia of *U. maydis* grown for 6 hr in a nutrient medium with or without the fungicide collected by centrifugation and washed twice with distilled water. The packed sporidia were freeze-dried and then the dried cells (350 mg) were saponified for 1 hr with 10 ml of 10% KOH in 60% ethanol at 80°C. Unsaponifiable lipids were extracted twice with *n*-hexane. The solvent containing extracts was evaporated to dryness under reduced pressure at 40°C. The resulting residue was dissolved in 1 ml of chloroform and sterols were analyzed by a Hitachi Model 163 gas chromatograph equipped with a flame ionizing detector. Aliquots of the sterol solution were injected into a 1 m × 3 mm glass column packed with Gas-chrom Q (100–120 mesh) coated with 3% SE-30. The temperature of column was adjusted to 250°C. Authentic cholesterol was used as a standard to determine the relative retention time and the relative amount of sterols in the extracts. Each sterol was identified by gas chromatograph-mass spectrometric (GC-MS) analysis at 70 eV.

6. Incorporation of ¹⁴C-Acetate into Lipid Fractions

Sporidial suspension (1.5 ml) at 10⁷ cells/ml was transferred to a 25 ml glass flask, incubated for 3 hr at 27°C on a reciprocal shaker and then S-3308 was added to the suspension. Just after the addition of the fungicide, 1 μCi of sodium acetate-2-¹⁴C was added to respective samples. After 3 hr incubation, the

reaction was stopped by adding 4.5 ml of a solvent mixture (chloroform : methanol, 1 : 2, v/v), and total lipid was extracted by the method of Bligh and Dyer modified by Yung and Mudd.²³⁾ The solvents were evaporated, and the residue was redissolved in 100 μ l of chloroform. Lipid extracts were applied to a thin-layer plate (Silica gel 6G; 0.25 mm thick) and developed by using a solvent mixture, heptane-isopropyl ether-acetic acid (60 : 40 : 4, v/v). The thin-layer plate was autoradiographed for 10 days. The 14 C-labeled lipid fractions were scraped out and transfer-

red into counting vials. The radioactivity was counted with a liquid scintillation counter.

RESULTS

1. Effect on Increase in Number of Sporidia and in Dry Weight of *U. maydis*

Increase in number of sporidia was inhibited strongly after the first 2 hr of incubation (Fig. 2A). Dry weight increase was also inhibited but the rate of inhibition was less than that in sporidial number (Fig. 2B). During the 8 hr-incubation period, increase in number and dry weight of sporidia were inhibited 87% and

Fig. 2 Effect of S-3308 on increase in number of sporidia (A) and in dry weight (B) of *U. maydis*.

○: control, ●: 2 μ g/ml S-3308-treated.

Fig. 3 Effect of S-3308 on sporidial morphology in *U. maydis* ($\times 400$).

A: control, B: 2 μ g/ml S-3308-treated.

Fig. 4 Effect of S-3308 on DNA (A), RNA (B) and protein (C) syntheses.

○: control, ●: 2 µg/ml S-3308-treated.

66%, respectively. Morphological changes were observed in the treated sporidia, which were branched, swollen and multicellular, after the incubation period of 6 hr (Fig. 3).

2. Effect on Nucleic Acid and Protein Syntheses

RNA synthesis was only slightly affected by the fungicide (2 µg/ml) (Fig. 4B). During the incubation period of 8 hr, increase in RNA content in the treated culture was inhibited by 10%. DNA synthesis was inhibited also slightly by the treatment of S-3308 (2 µg/ml) (Fig. 4A). The amount of DNA in the treated culture was 20% less than that in the untreated culture after 8 hr incubation. Protein synthesis was only moderately inhibited by the fungicide (2 µg/ml) (Fig. 4C). The protein amount in the treated culture was 73% of the control after the incubation period of 6 hr. These results indicate that there is no specific inhibition on RNA, DNA and protein syntheses by S-3308.

3. Effect on Respiration

The effect on respiration varied depending on the concentration of the fungicide (Table 1). Endogenous and exogenous respiration in the absence or presence of glucose were not affected at a concentration of 1 µg/ml, but exogenous respiration in the presence of succinate was slightly inhibited at the same concentration. However at 10 µg/ml, endogenous respiration was stimulated to 170% of the control, whereas

Table 1 Effect of S-3308 on respiration of *U. maydis*.

Treatment	Oxygen uptake (µl/mg dry weight/hr)		
	Endo- genous	Exogenous	
		0.1 M glucose	0.1 M succinate
Control	13	130	71
S-3308 1 µg/ml	14	126	61
10 µg/ml	22	126	30
100 µg/ml	0	5	0

exogenous respiration with succinate was inhibited 60% and exogenous respiration with glucose was not affected by the fungicide. Furthermore, at a concentration of 100 µg/ml, S-3308 almost completely inhibited both endogenous and exogenous respirations.

4. Effect on Sterol Synthesis

Gas-liquid chromatographic (glc) analysis of sterols revealed that three sterol components were present in the untreated cells (Fig. 5A). The sterol having a relative retention time (t_R) of 1.19 was identified as ergosterol by GC-MS analysis. Ergosterol was found as a major sterol component in *U. maydis* as in other fungi.^{24,25)} The two other sterols having t_R 1.39 and t_R 1.78 in the untreated cells were tentatively identified as Δ^7 -ergosterol and 24-methylenedihydrolanosterol by GC-MS. Presence of both sterols in control culture of *U.*

Fig. 5 Gas liquid chromatographic (*glc*) recordings of sterols in sporidia of *U. maydis* after the incubation period of 6 hr.

(A) control, (B) 2 µg/ml S-3308-treated. Cholesterol (t_R : 1.00) was employed as an internal standard.

maydis has been reported by Ragsdale.²⁷⁾ When the sporidia were treated with S-3308 (2 µg/ml), a relative amount of ergosterol was reduced and three other sterols were accumulated (Fig. 5B). The sterols having t_R 1.27 and t_R 1.44, which were not observed in control samples, were identified as 14 α -methyl- Δ^8 ,²⁴⁽²⁸⁾-ergostadienol and obtusifolol by GC-MS. The

fragmentation patterns of both sterols were identical with those reported previously.²⁷⁾ The sterol t_R 1.39 present in the untreated cells was not observed in the treated cells. Although the sterol having t_R 1.78 was detected in the untreated cells, its relative amount was much greater than that in control sample. This sterol was identified as 24-methylenedihydrolanosterol by GC-MS and its fragmentation peaks were identical with those reported.²⁷⁾ These results revealed that the sterol composition in *U. maydis* were quantitatively and qualitatively changed by the addition of S-3308.

5. Incorporation of ¹⁴C-Acetate into Lipid Fractions

S-3308 affected the incorporation of ¹⁴C-acetate into some lipid fractions during a 3 hr-incubation period (Table 2). Incorporation of ¹⁴C-acetate into the fraction of C-4-desmethyl sterols was almost completely inhibited by S-3308 (2 µg/ml). On the contrary, radioactivities in the fractions of C-4-methyl sterols and 4,4-dimethyl sterols of treated sample were 5 times and 20 times as much as those in the control sample, respectively. The result coincided with that of *glc*-analysis and indicated that S-3308 affected sterol metabolism.

DISCUSSION

Multiplication of sporidia in *U. maydis* was almost completely inhibited by 2 µg/ml S-3308 after the first 2 hr-incubation period, whereas increase in dry weight of sporidia was less affected and biosyntheses of protein, DNA and

Table 2 Effect of S-3308 on the incorporation of ¹⁴C-acetate into lipids.

Lipid fraction	Radioactivity (10 ³ dpm/1.5 ml culture)		Ratio (T/C)
	Treated (2 µg/ml)	Control	
Phospholipids	104.13	106.26	0.98
Diglycerides	2.11	1.81	1.17
C-4-Desmethyl sterols	1.49	31.87	0.05
C-4-Methyl sterols	5.82	1.15	5.06
4,4-Dimethyl sterols	24.17	1.15	21.02
Free fatty acids	6.09	4.79	1.27
Triglycerides	31.15	35.86	0.87
Sterol esters	1.05	5.39	0.20
Total lipids	176.02	188.28	0.94

RNA were only slightly inhibited. S-3308 induced some typical changes in sporidial morphology; the treated sporidia were swollen, branched and multicellular after 6 hr incubation. These morphological abnormalities were quite similar to those treated with triarimol.⁴⁾ In the respiration studies, the effect of S-3308 was observed to vary depending on the substrate and the concentration of the fungicide. Succinate oxidation was suppressed more strongly than endogenous respiration and glucose oxidation. Surprisingly, at 10 $\mu\text{g}/\text{ml}$ of S-3308, endogenous respiration was enhanced about 70% over that of control sample. Kato *et al.* reported that oxygen uptake by the starved mycelium of *Botrytis cinerea* was stimulated by 100 μM tridemorph, which is known to inhibit sterol biosynthesis.²⁶⁾ Furthermore, at a concentration of 100 $\mu\text{g}/\text{ml}$, both endogenous and exogenous respirations were completely inhibited. However, the

toxicity of S-3308 is not explained only by the inhibition of respiration, because the antifungal action is already recognized at lower concentrations, at which there was no particular inhibition of the respiration. Gas-liquid chromatographic analysis revealed that quality and quantity of sterols in *U. maydis* were changed by S-3308. A relative amount of ergosterol was drastically reduced in the presence of the fungicide and three other sterols, 14 α -methyl- $\Delta^8,24(28)$ -ergostadienol, obtusifoliol and 24-methylenedihydrolanosterol, were accumulated in the treated sporidia. All of the accumulated sterols possess a methyl group at C-14, which must be eliminated during ergosterol biosynthetic process. A similar change has been recognized when triarimol,⁴⁾ triadimefon¹⁸⁾ and buthiobate⁶⁾ were applied against *U. maydis*, *U. avenae* and *Monilinia fructigena*, respectively. On the other hand, it was shown that the incorpora-

Fig. 6 Ergosterol biosynthetic pathway in *U. maydis*, indicating the site of inhibition by S-3308.

(1) lanosterol, (2) 24-methylenedihydrolanosterol, (3) 4,4-dimethyl-fecosterol, (4) fecosterol, (5) episterol, (6) ergosterol, (7) obtusifoliol, (8) 14 α -methyl- $\Delta^8,24(28)$ -ergostadienol.

tion of ^{14}C -acetate into C-4-desmethyl sterols was strongly inhibited by S-3308 and the accumulation of radioactivities occurred in the fractions of C-4-methyl sterols and 4,4-dimethyl sterols. This result was similar to that obtained by using triadimefon.¹³⁾ The major components in C-4-methyl sterols and 4,4-dimethyl sterols were considered to be obtusifoliol and 24-methylenedihydrolanosterol, respectively, from the GC-MS analysis described above. All of the present results, therefore, demonstrate that S-3308 inhibits the conversion from 24-methylenedihydrolanosterol to 4,4-dimethylfecosterol; i.e., demethylation at the position of C-14 in the ergosterol biosynthetic pathway (Fig. 6). This inhibition was considered to result in rapid accumulation of 24-methylenedihydrolanosterol. Obtusifoliol and 14α -methyl- $\Delta^{8,24(28)}$ -ergostadienol were thought to accumulate, since the biosynthetic pathway was altered by S-3308. It is suggested that since these methyl sterols do not operate fully as the substitution of ergosterol, the lack of ergosterol causes abnormality in cell membrane function and finally fungal growth is arrested. The present experiments indicate that S-3308 has the same mode of action as triarimol,^{3,4)} triadimefon¹³⁾ and buthiobate^{5,6)} which are known to be the inhibitors of C-14 demethylation. Although these inhibitors are not known to affect fungal respiration, S-3308 was found to inhibit respiration completely at a high concentration. Recently, it was reported that another sterol-biosynthesis inhibitor, ketoconazole, blocked transport of electrons in the respiratory chain of *Candida albicans* under aerobic condition.²⁸⁾ Both S-3308 and ketoconazole may have the possibility of inhibiting the same electron transport system in the respiration.

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要 約

アゾール系新規殺菌剤 S-3308 の *Ustilago maydis* への作用機構

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S-3308 の抗菌作用機構について, トウモロコシ 裸黒

穂病菌 (*Ustilago maydis*) を用いて検討した。本菌を液体培地で振とう培養すると小生子の状態で増殖するが、S-3308 (2 ppm) 含有培地で培養すると小生子数および菌体乾重の増加は強く抑制され、分岐やくびれなどの特有の形態変化が認められた。これらの形態異常は、本菌にトリアリモールを処理したときのものと酷似していた。ところで本剤は、タンパク質および核酸の生合成にはほとんど影響を与えなかったが、100 ppm 処理によって呼吸阻害を引き起こした。しかし本剤の抗菌活性は、呼吸阻害が認められない低濃度区においても認められたことから、呼吸阻害作用のみで本剤の抗菌性を説明すること

は困難と考えられた。一方本剤は、2 ppm で本菌のエルゴステロール生合成を強く阻害した。すなわち、本剤処理によってエルゴステロールの相対量が低下し、その前駆体で 14 位にメチル基を有する 24-メチレンジヒドロラノステロール、14 α -メチル- $\Delta^{8,24(28)}$ -エルゴスタジエノールおよびオプツシホリオールの蓄積が認められた。また、 ^{14}C -アセテートの脂質画分への取込み実験においても、同様の結果が得られたことから、本剤の抗菌活性は、エルゴステロール生合成系における 14 位の脱メチル化反応の阻害に起因するものと考えられた。