Investigation of the Sterol Composition and Azole Resistance in Field Isolates of *Septoria tritici*

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We report here a biochemical study of resistance to azole antifungal agents in a field isolate (S-27) of a fungal phytopathogen. Isolates of *Septoria tritici* were compared in vitro, and their responses reflected that observed in the field, with S-27 exhibiting resistance relative to RL2. In untreated cultures, both RL2 and S-27 contained isomers of ergosterol and ergosta-5,7-dienol, although in differing concentrations. Under azole treatment, this phytopathogen exhibited a response similar to that of other pathogenic fungi, with a reduction in desmethyl sterols and an accumulation of 14 α -methyl sterols, indicative of inhibition of the P450-mediating sterol 14 α -demethylase. Growth arrest was attributed to the reduction of ergosterol combined with an accumulation of nonutilizable sterols. Strain S-27 exhibited an azole-resistant phenotype which was correlated with decreased cellular content of azole.

The target for azole antifungal agents has been shown to be the sterol P450 14 α -demethylase (P450_{14 α -dm}; CYP51A1) (15), which catalyzes three sequential hydroxylation reactions resulting in the C-14 demethylation of the sterol molecule (1). Accumulation of 14 α -methyl sterols, predominately eburicol, obtusifolol, and 14 α -methylfecosterol, and, in *Saccharomyces cerevisiae*, 14 α -methyl-3,6-diol, at the expense of ergosterol (the most abundant desmethyl sterol of most fungi [for a review, see reference 25]) (Fig. 1), has been attributed as the cause of growth arrest associated with azole treatment (for example, see reference 4).

The increasing incidence of treatment failures involving azole antifungal agents in the medical (3, 7) and agricultural fields (9) has provoked increased interest in the molecular and biochemical cause(s) of resistance. Mechanisms of azole resistance may be characterized into two main groups. The most diverse group involves alteration in the enzymes associated with ergosterol biosynthesis, while the second group involves the altered ability of azole to permeate (Fig. 1). Studies of resistance to azoles in *S. cerevisiae* have indicated several sites within the pathway which may give rise to azole resistance. Mutants of *S. cerevisiae* (29) and *Ustilago maydis* (10, 13) containing lesions in sterol $\Delta^{5(6)}$ -desaturase have been observed to accumulate, on azole treatment, 14 α -methyl fecosterol, which supports fungal growth. This occurred instead of accumulation of the attempted $\Delta^{5(6)}$ desaturation product of this sterol, 14 α -methyl-3,6-diol, which cannot fulfill requirements for growth (29).

Mutations inactivating P450_{14 α -dm} are lethal and result in a sterol phenotype identical to that observed with azole treatment. However, a second suppressor mutation in the $\Delta^{5(6)}$ -desaturase will result in cell viability (for example, see reference 29). Such mutants are cross resistant to polyene antifungal agents, such as amphotericin B, since the cells pro-

duce only 14 α -methyl sterols and not ergosterol, to which polyene antifungal agents bind (22).

An alteration in the target P450_{14 α -dm} giving rise to reduced azole binding, while retaining partial activity for demethylation of eburicol, has also been reported in *U. maydis* (11) as a mechanism of resistance to azole antifungal agents. Such mutants do not appear to require the second suppressor mutation in the $\Delta^{5(6)}$ -desaturase for viability, since they retain leaky sterol 14 α -demethylase activity and are not lethal.

An alteration in the cellular content of azole resulting in resistance has been reported for various pathogenic strains, such as *Candida albicans* (7), *Cryptococcus neoformans* (12), and *Penicillium italicum* (6). These mutants show cross-resistance to other azoles, and the *C. neoformans* mutants also show high levels of amphotericin B resistance. The mechanisms of the decreased uptake or increased antifungal efflux remain unclear in these strains but may involve multidrug resistance mechanisms similar to those identified in mammalian and other eukaryotic organisms (for example, see reference 26).

In the present paper, we describe an in-depth biochemical examination of azole resistance in field isolates of a phytopathogen. These isolates were purified as single cell lines before use. Other studies of phytopathogens have concentrated on laboratory isolates (for examples, see references 6 and 28). Strain S-27 (of *Septoria tritici*) was observed to have an azole-resistant phenotype when compared with the *S. tritici* strain RL2 both in vitro and in response to fungicide treatment of plants (9a). A detailed analysis indicated that the underlying mechanism of azole resistance was associated with decreased accumulation of azole within the fungal cell. We also observed the presence of an isomer of ergosterol, probably containing a 3 α -OH group (epi-ergosterol), which may alter hydrogen bonding with phospholipids in the membrane. Its significance is discussed.

MATERIALS AND METHODS

Chemicals. Unless otherwise indicated, all chemical reagents were supplied by Sigma, Poole, United Kingdom.

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FIG. 1. Sterol biosynthetic pathway. (A) Normal route of ergosterol biosynthesis in filamentous fungi; (B) alternative sterol biosynthetic pathway present as a result of azole antifungal inhibition.

Culture conditions. Two field isolates of *S. tritici*, RL2 and S-27, were used. RL2 was yeast-like in growth characteristics until the late log phase, when mycelial growth was observed. S-27 was similar in morphology to RL2, although cells were clumped and tended to appear longer in shape. Cells were grown in liquid medium containing 1% (wt/vol) Difco yeast extract, 2% (wt/vol) Difco peptone, and 2% (wt/vol) glucose (YEPD medium) at 18°C and 150 rpm or on solid YEPD plates containing 2% (wt/vol) Difco Bacto Agar.

Antifungal studies. Unless otherwise specified, all azoles were supplied by IACR-Long Ashton Research Station. Prochloraz, tebuconazole, and the pyrimidine fenarimol were dissolved in methanol to a stock concentration of 10^{-2} M, while triadimenol and dichlobutrazol (Zeneca Agrochemicals) were dissolved at the same concentration in dimethyl sulfoxide. Amphotericin B (Sigma) was dissolved in dimethyl sulfoxide to a final stock concentration of 1.08×10^{-3} M. Minimum inhibitory concentrations (MICs) were determined for sporidia harvested in the late log phase by centrifugation at 4,000 rpm and inoculated into 2 ml of YEPD medium to give 10^6 cells ml^{-1} . Triplicate treatments with azole and pyrimidine doses between 1×10^{-4} and 5×10^{-8} M and amphotericin B doses between 1.08×10^{-8} and 5.4×10^{-5} M were used. Test MIC cultures were incubated for 72 h at 18°C and 150 rpm, with growth assessed by cell counts (hemocytometer) and number of CFU milliliter^{-1} .

RL2 growth study. Experiments to determine the effect of treatment with time at the MIC concentration (5×10^{-7} M) of tebuconazole were conducted under conditions identical to those described above, except that 1-liter cultures in 2-liter flasks were employed, with 100 ml of culture harvested for sterol analysis, dry cell weight, and cell count determinations.

Sterol isolation and analyses. Extraction of nonsaponifiable sterols followed the method outlined by Woods (32), with gas chromatography-mass spectrometry analyses conducted as described by Loeffler and Hayes (18). Peak analysis and identification were undertaken with reference to current literature (18, 19, 24) for relative retention time (RRT) values and fragmentation patterns.

Cell-free sterol biosynthesis. The assay of azole inhibition of ergosterol biosynthesis used the cell-free system described elsewhere (2, 27). Flasks (500 ml) containing 200 ml of YEPD medium were inoculated with strains RL2 and S-27 to give a concentration of 10^6 sporidia per ml and grown at 18°C at 150 rpm. Cells were harvested in the late log phase by centrifugation (4,000 rpm for 10 min) and resuspended in 25 ml of 0.2 M phosphate buffer (pH 6.8) containing 140 ml of 0.2 M KH_2PO_4 , 360 ml of 0.2 M K_2HPO_4 , 20% (vol/vol) glycerol, and 1 mM EDTA. Cell disruption was achieved with a Braun MSK homogenizer with three 30-s disruptions. Cell extracts were harvested by centrifugation at 4,000 rpm for 10 min. Cofactor mix (0.5 ml; 20 mg of NADPH, 18 mg of NAD, 17 mg of NADP, 55 mg of ATP, 20 mg of glutathione, 30 mg of glucose-6-phosphate dissolved in 1 ml of phosphate buffer) was added to 9.5 ml of supernatant with 20 μl of 1 M magnesium chloride and 10 μl of 1 M manganese chloride. To 1 ml of this mix, tebuconazole was added at various concentrations. [^{14}C]mevalonic acid (10 μl ; specific activity, 53 mCi/mmol) was added to each sample, and the samples were incubated at 18°C for 2 h. Sterol extraction of nonsaponifiable sterols was conducted as described above.

Sterol class identification was achieved through thin-layer chromatography (TLC) on TLC plastic-coated Silica Gel 60 F_{254} sheets (20 by 20 cm; Merck) with a solvent system of cyclohexane-diethyl ether (4:1) with ergosterol and lanosterol

TABLE 1. Sensitivities of *S. tritici* RL2 and S-27 to azole antifungal agents, the pyrimidine fenarimol, and the polyene antibiotic amphotericin B, expressed as MICs

Strain	MIC (μ M)				
	Triadimenol	Tebuconazole	Prochloraz	Fenarimol	Amphotericin B
RL2	10	0.5	1	0.5	1.1
S-27	50	10	5	10	1.1

as co-run standards. Autoradiograms were developed over 7 days at -80°C , and the radioactivity incorporated into ergosterol was quantified with a Beckman LS 1801 scintillation counter.

Accumulation of triadimenol. Cells were inoculated into 100 ml of YEPD medium in 250-ml flasks at 10^6 cells ml^{-1} and incubated at 18°C and 150 rpm until growth reached the stationary phase. The cells were collected by centrifugation at 4,000 rpm and washed in phosphate buffer (pH 7.0). [^{14}C]triadimenol was added to give a concentration of 10^{-5} M from a $3.5\text{-}\mu\text{Ci ml}^{-1}$ stock, and the cells were resuspended in 1 ml of phosphate buffer (pH 7.0) at a final concentration of 2×10^9 cells ml^{-1} . [^{14}C]triadimenol was added to give a concentration of 10^{-5} M from a $3.5\text{-}\mu\text{Ci ml}^{-1}$ stock and incubated for 2 h at 18°C and 150 rpm. The cells were harvested at this time, because accumulation was shown to have reached a maximum, and washed in 5 ml of nonradioactive triadimenol (5×10^{-5} M). They were collected by vacuum filtration on Whatman no. 1 filter paper and washed quickly three times with 5.0 ml of phosphate buffer. Cells and filters were dried at 80°C , and dry cell weights were determined for each sample. Radioactivity was measured with a Beckman LS 1801 liquid scintillation counter. Cellular content was calculated in triplicate as micromoles of triadimenol milligram dry cell weight $^{-1}$.

Metabolism of triadimenol. Cells were inoculated into 200 ml of YEPD medium in 500-ml flasks to a density of 10^6 cells ml^{-1} and incubated at 25°C (150 rpm) until growth reached the late log phase. The cells were harvested by centrifugation at 4,000 rpm and resuspended in 10 ml of YEPD medium in 50-ml flasks at an inoculum density of 5×10^7 cells ml^{-1} . [^{14}C]triadimenol was added to give a concentration of 10^{-5} M from a $3.5\text{-}\mu\text{Ci ml}^{-1}$ stock, and cultures were incubated at 25°C (150 rpm) for 12 h. Extraction of triadimenol from the culture and detection of possible metabolites were conducted on the basis of the method described by Woestenborghs et al. (31) with a heptane-isoamyl alcohol (98.5:1.5 [vol/vol]; 5 ml) solvent system. The organic phase was back extracted with 50 mM sulfuric acid (3 ml). Following centrifugation at 1,000 rpm, the organic phase was made alkaline with concentrated ammonia (pH 9.0) and reextracted with the heptane-isoamyl alcohol mix (2 ml) and dried under N_2 (g) at 55°C .

Separation of triadimenol and its possible metabolites was conducted on TLC plastic-coated Silica Gel 60 F₂₅₄ sheets (20 by 20 cm; Merck) with two sequential solvent systems of butanol-acetic acid-water (60:15:25 [vol/vol]) and then chlo-

roform-methanol-ammonia (80:19:1 [vol/vol]). [^{14}C]triadimenol was co-run as a standard. Autoradiograms were developed over 7 days at -80°C .

RESULTS

The response of *S. tritici* to azole treatment at the minimum inhibitory dose was examined to identify sterol profiles leading up to and including the growth arrest rather than with sub-MIC treatments. Similar experiments have been conducted with various organisms, including *S. cerevisiae* (24), *U. maydis* (13), and *C. neoformans* (12), but not in field isolates. The role of sterols in this process of azole-induced growth arrest has indicated that depletion of sterols which support growth, such as ergosterol, ergosta-7,22-dienol, and 14α -methyl fecosterol, to a combined level below 20% of the total relative sterol concentrations within the cell is responsible for the observed arrest rather than accumulation of possible toxic sterol intermediates (13). An azole-sensitive field isolate of *S. tritici* (strain RL2) (Table 1) was examined to characterize the response of this pathogen to azole treatment.

Investigation of antifungal resistance in S-27. Two field isolates of *S. tritici* were examined for resistance to azoles, the pyrimidine fenarimol, and amphotericin B (Table 1). S-27, when compared with RL2, exhibited a five-fold greater level of resistance to the azoles triadimenol and prochloraz and 20-fold greater resistance to tebuconazole. This strain was also observed to have a 20-fold greater resistance to fenarimol compared with RL2. Both strains showed equal sensitivities to amphotericin B.

Inhibition of RL2 sterol biosynthesis with the MIC of tebuconazole. Treatment of RL2 with the MIC of tebuconazole resulted in an accumulation of 14α -methyl sterols and a decrease in desmethyl sterols (Table 2). This general response was similar to that in previous studies involving other fungal species (for example, see reference 13) and indicated that the sterol P450_{14 α -dm} of *S. tritici* was inhibited by tebuconazole. Growth arrest, determined as the point when both dry cell weight and cell number reached a static level, occurred between 24 and 36 h after inoculation. Growth prior to this was

TABLE 2. Alteration of sterol profile in RL2 following tebuconazole treatment at the MIC^a

Sterol	Relative sterol content (%) at:									
	0 h	3.0 h	6.0 h	12.0 h	18.0 h	24.0 h	36.0 h	48.0 h	60.0 h	60.0 CONT ^g
Epiergosterol ^b	27.2	11.8	11.8	11.4	8.4	2.6	3.2	3.0	2.6	24.9
Ergosterol ^c	47.2	42.7	41.4	34.0	23.8	8.4	8.1	6.7	3.8	56.9
14α -Methyl fecosterol	— ^d	—	—	—	—	—	3.5	4.7	5.7	—
Ergosta-7,22-dienol	7.6	2.4	2.1	2.7	1.3	0.5	0.8	0.9	Trace	3.2
Ergosta-5,7-dienol ^e	—	1.4	1.4	0.4	0.6	—	0.9	1.2	1.0	Trace
Ergosta-5,7-dienol ^f	—	1.5	2.1	1.5	1.2	Trace	0.9	Trace	—	Trace
Episterol	6.1	2.4	0.8	Trace	Trace	—	—	—	—	7.6
Ergosta-7-enol	6.1	1.2	0.6	1.5	Trace	—	—	—	—	4.1
14α -Methyl fecosterone	—	1.1	—	—	—	—	—	—	—	—
Obtusifoliol	—	—	0.8	4.6	5.5	7.5	12.4	11.7	9.9	—
4-Methyl ergosta-7-enol	—	4.1	2.2	3.1	4.2	—	—	—	—	—
Eburicone + obtusifolione	—	—	—	—	—	—	3.3	1.6	0.6	—
Eburicol	—	31.0	37.1	40.1	54.9	81.1	70.1	70.2	76.5	—
Lanosterol	5.7	—	—	—	—	—	—	—	—	3.3
Cell counts (10^6)	1.2	2.0	2.1	2.4	2.9	3.5	3.8	3.8	3.8	30

^a MIC, 5×10^{-7} M.

^b α -Hydroxy ergosterol.

^c β -Hydroxy ergosterol.

^d —, none detected.

^e α -Hydroxy ergosta-5,7-dienol.

^f β -Hydroxy ergosta-5,7-dienol.

^g CONT, untreated control sample.

TABLE 3. Mass spectral data of sterol isomers and 4-methylergosta-7-enol

Ion ^a	Relative abundance (% of base peak) of sterol isomer ^b :				
	A (468) ^c	B (468)	C (470)	D (470)	E (486)
[M] ⁺	13	13	11	11	65
[M-CH ₃] ⁺	4	3			23
[M-TMSiOH] ⁺	9	18	6	15	23
[M-CH ₃ -TMSiOH] ⁺	100	100	96	100	32
[M-(TMSiO=CH-CH ₂ -CH ₃)] ⁺	70	70	100	99	14
[M-CH ₃ -TMSiOH-SC-C ₂ H ₂ -H] ⁺			30	36	
[M-SC] ⁺					7
[M-(SC+H)] ⁺					7
[M-(SC+2H)] ⁺					20
[M-SC-TMSiOH] ⁺	33	43	26	22	100
[M-(SC+2H)-TMSiOH] ⁺	3	4			25
[M-SC-TMSiOH-C ₂ H ₂] ⁺	10	10			27
[M-(SC+H)-TMSiOH-C ₂ H ₂] ⁺	2	3			6
[M-(SC+2H)-TMSiOH-C ₂ H ₂] ⁺	2	2			
[M-SC-TMSiOH-42] ⁺	36	39	32	35	58

^a SC, side chain.^b A, α -hydroxy ergosterol; B, β -hydroxy ergosterol; C, α -hydroxy ergosta-5,7-dienol; D, β -hydroxy ergosta-5,7-dienol; E, 4-methyl ergosta-7-enol.^c Values in parentheses are [M⁺].

probably supported by endogenous ergosterol levels, enabling the strain to undergo approximately three successive cell divisions, but with an observed slowdown in division after 3 h. The predominant sterol at the point of growth arrest was eburicol (81%), with the combined levels of ergosterols at 11.0%.

Sterol analysis. Identification of sterols was achieved from their RRTs and mass spectral fragmentation patterns (Table 3). Sterols 1 and 2 had RRTs of 32.904 and 34.114 min, respectively, with the latter corresponding to the RRT of ergosterol. However, the mass ion [M⁺] m/z ratio was 468 for both of these sterols, and each fragmentation ion pattern corresponded with that reported for ergosterol.

Sterols 1 and 2 were ergosterol isomers, with the differences probably occurring at either C-3 (3 α -OH and 3 β -OH isomers) or at C-24 in the sterol molecule, with a $\beta \rightarrow \alpha$ isomerization of the methyl moiety occurring during the double-bond reduction in the latter, but absolute identification was not possible by the available techniques. Of the two possible sites, the C-3-OH site was considered the more likely because of the presence of 4-methylated intermediates in the untreated samples and the identification of ketosteroids (obtusifolione and eburicone) in the treated samples, indicating altered activity and/or specificity of C-3 ketoreductase. The RRT difference between both isomers was approximately 1.1 min, which suggests C-3 as the point of isomerization since it is the site of TMSi derivatization. Alteration of the orientation at C-3 would be expected to give rise to alterations in column interaction and, therefore, in RRTs. However, in the absence of authentic standards of these possible isomers, it is not possible to assay for this. By analogy to the synthetic 3 α -hydroxy cholesterol isomer, we called sterol 2 epiergosterol (5).

Sterols 3 and 4, with RRTs of 34.994 and 36.369 min and [M⁺] m/z of 470, were identified on a similar basis as 3 α - and 3 β -isomers of ergosta-5,7-dienol on the basis of their RRTs and fragmentation patterns.

Sterol 5, with an RRT of 41.083 min and [M⁺] m/z of 486, did not correspond to any published data. However, the fragmentation pattern contained high levels of similarity to ergosta-7,22-dienol, particularly with the high intensities of the mass ion and fragments at m/z 357, 269, and 227 corresponding to [M-SC-2H], [M-SC-TMSiOH], and [M-SC-TMSiOH-42] fragments, respectively, indicating the presence of a hydroxyl

moiety and a fully unsaturated side chain (SC; C₉H₁₉). This compound displayed stronger similarities to the fragmentation pattern of ergosta-7-enol than to that of ergosta-8-enol. The [M⁺] m/z of 486 for compound 5 was probably an indication of retention of a methyl moiety in the ergosta-7-enol-like molecule; the intensity of the [M-CH₃] ion (23%) was lower than would be expected if the methylation were at C-14 but similar to that observed for the loss of a C-4 methyl. This was further supported by the strong retention of the sterol on the column, which is characteristic of methylated sterols. Therefore, compound 5 was identified as 4-methyl ergosta-7-enol (Fig. 2).

Biochemical investigation on the basis of resistance. Examination of the sterol profiles of RL2 and S-27 in untreated cultures (Table 4) indicated that both strains were similar, and therefore, the resistance observed was not considered to be related to an alteration in this pathway. Tebuconazole treatment of RL2 at the dose identified as its MIC resulted in the accumulation of 14 α -methyl sterols and a decrease in the levels of the ergosterols to 2.6 and 8.4%, respectively. At this azole dose, weaker inhibition of P450_{14 α -dm} was observed in S-27 than in RL2 as indicated by the smaller reduction in levels of desmethyl sterols and the lower accumulation of eburicol (up to 33% of total sterols). The level of ergosterols was also still high when S-27 was treated with this dose of tebuconazole and amounted to 37.1 and 29.7% of total sterol for epiergosterol and ergosterol, respectively. Increasing the azole dose to the

FIG. 2. Proposed structure of sterol 5, i.e., 4-methyl ergosta-7-enol.

TABLE 4. Relative sterol compositions of RL2 and S-27 at various tebuconazole concentrations

Sterol	Relative sterol content (%)				
	Untreated		Treated with tebuconazole		
	RL2	S-27	5 × 10 ⁻⁷ M		S-27 at 1 × 10 ⁻⁵ M
Epiergosterol	27.2	43.1	2.6	37.1	15.5
Ergosterol	47.2	34.5	8.4	29.7	13.5
Ergosta-7,22-dienol	7.6	7.0	0.5	Trace	Trace
Episterol	6.1	5.4	— ^a	Trace	Trace
Ergosta-7-enol	6.1	5.0	—	—	—
Obtusifoliol	—	—	7.5	—	11.1
Eburicol	5.7	5.0	77.5	33.3	59.9

^a —, none detected.

MIC of tebuconazole in S-27 resulted in a further increase in the levels of 14 α -methylated sterols and a decrease in the levels of desmethyl sterols consistent with P450_{14 α -dm} inhibition.

Inhibition of sterol P450_{14 α -dm} activity in vitro. Alteration in the target site has been shown to be one mechanism for azole resistance (25). Examination of the in vitro cell-free system indicated that tebuconazole inhibition of the P450_{14 α -dm} from each strain (as indicated by the disappearance of the band corresponding to 14 α -, 4 α -, and 4 β -desmethyl C-14-incorporated sterol on the autoradiogram and an increase in the intensity of the 14 α -methyl sterol-containing bands) occurred at a similar dose for each strain (Table 5). This indicated that P450_{14 α -dm}s from both strains were equally inhibited at the same azole dose, with 50% inhibitory concentration (IC₅₀) values (concentration required to inhibit ergosterol synthesis by 50% of total ergosterol produced in the assay) of 0.07 ± 0.01 μ M for each.

Cellular content of [¹⁴C]triadimenol. The possible involvement of resistance due to either reduced uptake or increased efflux of triadimenol in strains RL2 and S-27 was examined. Following a 2-h incubation with [¹⁴C]triadimenol, the cellular content of RL2 was 0.15 ± 0.005 μ mol/mg dry cell weight compared with that of S-27, i.e., 0.04 ± 0.005 μ mol/mg dry cell weight, indicating a fourfold difference in accumulation of triadimenol between the two strains (Table 5). Triadimenol was employed for this assay because [¹⁴C]tebuconazole was unavailable.

DISCUSSION

Our results demonstrate the presence of a novel ergosterol isomer in *S. tritici* and also point to reduced accumulation of the azole antifungal agent as the mechanism of resistance in the field isolate S-27.

Sterol analysis of *S. tritici*. The untreated sterol profile for each strain indicated an alteration in the biochemistry of sterol formation not previously observed for other fungi, with isomers of ergosta-5,7-dienol and ergosterol also present. The *cis*-to-*trans* isomerization of the C-3 hydroxyl group of the sterol probably occurred at the C-4 demethylation step and may follow the scheme described in Fig. 3. This may reflect a unique property of the 3-ketoreductase of *S. tritici* compared with that of other fungal species. Evidence that the isomers were formed at this step is provided by the absence of 3 α -OH isomers of sterols earlier in the pathway, particularly eburicol, but further studies are warranted to confirm this and whether isomerization occurred at C-24 rather than at the C-3-OH. The presence of the ketosteroid obtusifolione under azole treat-

ment and the presence of 4-methyl ergosta-7-enol in the untreated cultures indicated interference in the C-4 demethylation step at the C-3 ketoreductase step and may further support the involvement of this in the formation of the 3 α - and 3 β -hydroxy isomers of ergosta-5,7-dienol and ergosterol. However, involvement of epi-isomerases cannot be ruled out and similarly warrant further investigation, although why such enzymes would be active against sterols of *S. tritici* is unclear.

The postulated involvement of a single enzyme in the formation of the 3-keto group associated with sterol biosynthesis (20) may be questionable because eburicone was also detected under azole treatment of RL2. The ketosteroid could not have been formed by the mechanism described in Fig. 3 for sterol C-4 demethylation, since eburicone retains both methyl groups at C-4. It is possible that either the ketoreductase catalyzes the opposite reaction or other enzymes are involved in the production of 3-keto compounds in the fungus.

The response to azole treatment in both *S. tritici* strains was similar and consistent with the established mode of action of this group of antifungal agents, i.e., inhibition of P450_{14 α -dm}. Tebuconazole treatment resulted in a characteristic accumulation of 14 α -methylated sterols, with the predominant sterol being eburicol at the point of growth arrest (70.1% of total sterols) and with other 14 α -methyl sterols present in lower concentrations, such as eburicone, obtusifoliol, obtusifolione, and 14 α -methyl fecosterol (Table 2). Unlike with azole-treated cultures of *S. cerevisiae* (16), no 14 α -methyl-3,6-diol was detected. This indicates that formation of abnormal sterols through metabolism of eburicol, without removal of the 14 α -methyl group, does not occur efficiently, perhaps because of the substrate specificities of these enzymes in *S. tritici*, unlike those in *S. cerevisiae*.

Following growth arrest, the relative levels of the two ergosterol isomers continued to decline, indicating the continued tebuconazole inhibition of P450_{14 α -dm}. Eburicol was also observed to decline in level after the point of growth arrest which represents the further metabolism of this sterol, as indicated by the increase in levels of obtusifoliol and 14 α -methyl fecosterol.

Analysis of azole resistance in the isolate S-27. Tebuconazole inhibition of in vitro ergosterol biosynthesis from [¹⁴C]mevalonic acid in RL2 and S-27 was shown to occur at similar doses. This indicated that no alteration of target site affecting binding by inhibitor could be attributed to the increased azole dose required to cause growth arrest in S-27 compared with that in RL2. Similarly, metabolism of triadimenol was not evident in either strain, which is consistent both with previous studies on azole antifungal metabolism in fungal species (14) and with the observation that triadimenol is a recalcitrant pollutant.

Resistance of S-27 to azole antifungal agents was, therefore, attributed to a decreased cellular content of azole, as shown by the reduced accumulation of radiolabelled triadimenol when compared with that of the sensitive strain RL2 (Table 5). This may be caused by decreased ability of azole to permeate or

TABLE 5. Comparison of cellular contents of [¹⁴C]triadimenol, IC₅₀ values, and growth rates for RL2 and S-27

Strain	Accumulation of [¹⁴ C]triadimenol ^{a,b}	Tebuconazole IC ₅₀ (μ mol) ^a	Doubling time (h)
RL2	1.5 ± 0.05	0.07 ± 0.01	4.0
S-27	0.4 ± 0.05	0.07 ± 0.01	7.5

^a Values are means ± standard deviations.^b Expressed in moles (10⁻⁷) per milligram dry cell weight.

FIG. 3. Proposed pathway for C-4 demethylation, indicating possible point of $\alpha \rightarrow \beta$ hydroxyl isomerization within the sterol molecule. (A) Mixed function oxidase; (B) C-3 ketoreductase.

enhanced efflux of the antifungal agent, as has been observed for *P. italicum* (6). Alteration of the sterol/phospholipid ratio within the lipid bilayer has also been attributed to azole resistance in some mutants of *C. albicans* (8). Although the combined levels of ergosterol isomers within the two isolates were similar (74.4 and 78.6% of total sterol for RL2 and S-27, respectively), the relative levels differed between the two strains, with RL2 and S-27 containing 47.2 and 34.5% of ergosterol, respectively. Studies have indicated that the physical and chemical properties of sterols for correct membrane incorporation are highly stringent (21). Many intermediate sterols in ergosterol biosynthesis, for example, eburicol, are unable to completely satisfy the bulk membrane functions (21). Our results indicate that orientation of the 3C-hydroxyl moiety of fungal sterols may affect membrane characteristics and lead to alterations in the integrity and fluidity of the membrane by

direct destabilization. This effect would presumably be similar to the bilayer-to-hexagonal H_{II} phase preference of epicholesterol-containing synthetic phosphatidylethanolamine membranes which, upon comparison with cholesterol-containing membranes, promotes a 10°C decrease in the temperature required for such a shift (5). This effect has also been linked to membrane instability and altered permeability (5) and may play a role in the azole resistance of S-27.

The effect of epiergosterol on growth and membrane function will require additional investigation. Studies on epicholesterol using artificial membranes may not be relevant to this organism, which may have adapted its phospholipid content and type to compensate for the presence of epiergosterol. Certainly, the presence of a mixture of ergosterol and epiergosterol may make such adjustments difficult. Purification of epiergosterol to allow sterol feeding experiments with *S. cer-*

evisiae sterol auxotrophs may allow a better understanding of its acceptability for supporting normal growth. Why this fungus should produce such sterols is difficult to understand at present.

The lower growth rate of S-27 compared with that of RL2 may result from the greater proportion of epiergosterol in the former. Alternatively, it may result from changes which conferred azole resistance. If the altered cellular content of azole was caused by a mechanism similar to that responsible for multidrug resistance in eukaryotes, i.e., by alteration of proteins involved in transport across the membrane, supply of important nutrients may have been affected. Recently, a gene capable of causing miconazole resistance has been characterized in laboratory studies with *C. albicans* (23). Examples similar to the data presented here can be anticipated from studies of the field isolates of other phytopathogens, warranting investigation of multidrug resistance proteins.

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