Cytochrome \( P-450 \) catalysing the 14\( \alpha \)-demethylation of sterol is inhibited in fungi byazole antifungal compounds resulting in a fungistatic or afungicidal effect [1]. The azoles themselves represent a useful tool for investigating \( P-450 \) structure through the differential affinities with different \( P-450 \) forms. [2]. Of practical importance is the identification of the molecular features which result in the strong inhibition of a cytochrome \( P-450 \) and which may lead to the development of better antifungals, as well as other therapeutic agents and agrochemicals. The extensive use of azoles is also resulting in increasing reports of resistance in agriculture and in the clinic and we have been interested in the relationship of the resistance mechanisms to the target enzyme and the mode of action of azole antifungals [1,3].

The antifungal effect of azoles may be related to either the accumulation of C-14-methylated sterols or to depletion of ergosterol, and results in a variety of phenomena such as disruption of chitin and fatty acid metabolism, inhibition of membrane-bound enzymes and, at higher doses, direct membrane damage [4]. In \textit{Saccharomyces cerevisiae} grown on glucose we have observed considerable mitochondrial petite induction is induced at doses below the minimal inhibitory concentration. Should this mitochondrial sensitivity to sterol perturbation be found in other fungi which do not form petites then this would cause the initial arrest of growth [1].

We have been interested in theazole resistance of a number of polynuclear resistant sterol 14\( \alpha \)-demethylase mutants isolated both in \textit{S. cerevisiae} and \textit{Candida albicans}. These strains grow on 14\( \alpha \)-methylated sterols at a reduced rate in comparison with the wild types, but still contain trace levels of ergosterol. Investigation of their sensitivity to azoles has indicated them to be resistant and this has been associated with the \( P-450 \) mutations involved [5], and with uptake of azole by a \textit{C. albicans} mutant [6]. Investigation of two \textit{S. cerevisiae} \( P-450 \) mutant strains SG1 and NYS P-100 showed them to be resistant to azole treatment. However, the resistance was not associated with drastically reduced cellular levels of azole, or with the alteration in the target enzyme, but with a second sterol mutation in these strains in \( \Delta^{5,6} \) desaturase. The second mutations are required for the viability of these \( P-450 \) mutant strains [7]. In isolating azole-resistant mutants directly [3] we found that they were exclusively mutants in this latter sterol biosynthetic step. No evidence for resistance via increased levels of target enzyme or through alterations in the target enzyme were observed, and resistance of 14\( \alpha \)-demethylase defective strains can be ascribed to their second sterol mutation in \( \Delta^{5,6} \) desaturase. It would be of interest, however, to determine whether the leaky levels of ergosterol in these strains is sensitive to azole treatment.

The finding that mutations in sterol \( \Delta^{5,6} \) desaturation results in resistance led to the investigation of sterol composition of \textit{S. cerevisiae} both with and without azole treatment. In strains treated with the 14\( \alpha \)-demethylase inhibitor fluconazole the parent accumulated lanosterol and 14\( \alpha \)-methylergosta-8,24(28)-dien-3\( \beta \),6\( \alpha \)-diol (14-methyl-3,6-diol). The stringent desaturase mutant \textit{A2} accumulated 14\( \alpha \)-methyl-8,24(28)-dien-3\( \beta \)-ol (14-methyl fecosterol) and lanosterol as the major sterol components on fluconazole treatment. Resistant isolate \textit{A3} was only partially blocked in sterol \( \Delta^{5,6} \) desaturation and when fluconazole-treated accumulated 14-methyl-3,6-diol, 14-methyl fecosterol and lanosterol. These results indicated that sterol \( \Delta^{5,6} \) desaturation was required for the formation of 14-methyl-3,6-diol under conditions of azole inhibition, and led to the hypothesis that efficient conversion of 14-methyl fecosterol to 14-methyl-3,6-diol produced sterols unable to support growth. Growth can occur through utilization of 14-methyl fecosterol, and retention of a 14\( \alpha \)-methyl group is not in itself an obstruction to growth. Cell division can also occur when 14-methyl fecosterol is present as a mixture, as in the fluconazole-treated mutant \textit{A3}.

The cause of cell arrest following azole treatment appears therefore to depend not on the accumulation of C-14 methyl sterols, but on the type of such sterols. This may apply to all the roles of sterol in the cell as it has been found that strains of \textit{S. cerevisiae} containing a 14\( \alpha \)-demethylase gene disruption, and hence synthesizing no ergosterol, can also grow aerobically without an ergosterol supplement when compensated by a second mutation in \( \Delta^{5,6} \) desaturation [7a]. Also the partial dominance of
some sterol Δ^5,6 desaturase mutants indicates the relevance of this mechanism of resistance to diploid yeast (Fig. 1).

The sterols present at the point of cell arrest were also of interest to us rather than those present at sub-inhibitory doses. Fig. 2 shows the sterols and growth of the wild-type S. cerevisiae strain XY 529-5a during treatment. The growing cells were treated with ketoconazole and with time the 14-methyl sterols accumulated and ergosterol decreased. The cells arrested at about 5 h after initial treatment giving approximately two residual divisions. Lanosterol reached a peak at about 2 h with 14α-methyl-3,6-diol accumulating later, presumably through conversion from lanosterol. At cell arrest the amount of 14α-methyl-3,6-diol had reached a maximum and ergosterol remained at a detectable level. Hence the combination of sterols present were apparently unable to sustain growth and points to the inability of 14α-methyl-3,6-diol and lanosterol to support growth even in the presence of ergosterol.

The analysis of nystatin resistance in the diploid yeast C. albicans has revealed P-450 mutants accumulating 14-methylfecosterol [8] as well as mutants accumulating 14-methyl-3,6-diol [9]. The latter have demonstrated spontaneous secondary mutants carrying an additional block in sterol Δ^5,6 desaturation. Further evidence that C. albicans is subject to this interaction between sterol Δ^5,6 desaturase and 14α-desaturase blocks may be gained through gene disruption of the C. albicans sterol 14α-demethylase. Similar experiments using the RIP phenomenon in Neurospora crassa [10] or the molecular genetic system of Ustilago maydis will extend these findings to other species. In some fungi treatment with azole does not result in the sterol 14α-methyl-3,6-diol accumulating at a high level even with prolonged treatment, and sterols such as 24-methylenedihydrolanosterol, obtusifoliol

![Fig. 1](image1)

**Fluconazole sensitivity of diploids produced by crossing parent strain XY729-5A and resistant mutants A1, A2, and A3, with isogenic mata strain SQI-2C**

Key to symbols: (●), XY729-5A:SQI-2C; (▲), A1/SQI-2C; (○), A3/SQI-2C; (★), A2/SQI-2C.

![Fig. 2](image2)

**The effect of an inhibitory dose of ketoconazole on sterol biosynthesis and growth rate in an exponential phase culture of parent strain XY729-5A**

The relative g.c. peak areas of the major sterol components: (●), ergosterol; (▲), lanosterol; and (○), 14-methyl-3,6-diol, are shown, and the culture density as measured by absorbance, (●). Ketoconazole (2 × 10^{-4} M) was added at t = 0 h.

![Fig. 3](image3)

**The fluconazole sensitivity of XY729-5A::pEMS and the control strain XY729-5A::pEMBLYe30**

Key to symbols: (●), pEMBLYe30; (○), pEMS.
and obtusifolione are observed. It may be that in some fungi C-4 demethylase enzymes are either inhibited by the retention of the 14α-methyl group or are not active through physiological control. Reduced demethylation of the C-4-methyl group would limit the formation of 14-methyl-stericosterol, the precursor of 14α-methyl-3,6-diol. The other sterols mentioned above may also be unsuitable for growth.

Despite the potential for further elucidation of the mechanisms by which sterol C-14 demethylase inhibition results in growth arrest the target for the antifungal azoles remains the cytochrome P-450 responsible for the reaction. Azoles bind through the N-3 of the imidazole ring or the N-4 of the triazole ring to the haem moiety as a sixth ligand. Relative affinity is determined by interaction between the N-1 substituent group and the apoprotein of cytochrome P-450. It might be feasible to produce resistant enzymes through mutagenesis in vitro, which have a lower affinity for azole and which will indicate important domains and residues for azole/P-450 interaction. Target enzymes of pathogens may be expressed in S. cerevisiae, such as the 14α-demethylase of C. albicans. The resistance level of transformants containing the episomal plasmid pEMBL Ye30 and the same plasmid with the C. albicans 14α-demethylase gene inserted are shown in Fig. 3. The C. albicans protein is overexpressed from this multicopy vector and can be placed in strains with undetectable or no levels of the corresponding S. cerevisiae protein. Expression of 18 pmol mg⁻¹ microsomal protein of the C. albicans protein have been detected using the C. albicans promoter while use of the PGK promoter directly adjacent to the coding sequence raised this to only 89 pmol mg⁻¹ microsomal protein. A combination of rational alteration complementary to molecular modelling studies [2] and biological selection for altered resistance in transformants allow an approach to a refined picture of fungal sterol 14α-demethylase structure.

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