ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1990, p. 989–993
0066-4804/90/060989-05$02.00/0

Vol. 34, No. 6

In Vivo Effects of Fenpropimorph on the Yeast *Saccharomyces cerevisiae* and Determination of the Molecular Basis of the Antifungal Property

CHRISTOPHE MARCIREAU,* MICHEL GUILLOTON, AND FRANCIS KARST

Laboratoire de Biochimie et Génétique des Microorganismes, Université de Poitiers, 40, avenue du Recteur Pineau, 86022 Poitiers Cedex, France

Received 9 November 1989/Accepted 20 March 1990

The effects of fenpropimorph on sterol biosynthesis and growth of *Saccharomyces cerevisiae* were examined to pinpoint the mode of action of fungicides that inhibit ergosterol biosynthesis. Taking advantage of sterol auxotrophy and sterol permeability in mutant strains, we show that growth inhibition is strongly correlated with inhibition of sterol biosynthesis. We confirm that in vivo and at low concentrations, fenpropimorph inhibits $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, and in addition, when it is used at higher concentrations, it inhibits Δ^{14}-sterol reductase. We show also that the fungistatic effect of fenpropimorph is not due to the accumulation of abnormal sterols in treated cells but is linked to the specific inhibition of ergosterol biosynthesis, leading to the arrest of cell proliferation in the unbudded G_1 phase of the cell cycle.

Fenpropimorph {*N*-[3-(*p-tert*-butylphenyl)-2-methylpropyl]-*cis*-2,6-dimethylmorpholine} belongs to the class of morpholine fungicides used in agriculture against powdery mildews of cereals and oleaginous and vine cultures. Treatment of fungal cultures with morpholines leads to the accumulation of sterol biosynthetic precursors such as ergosta-8,22-dienol and ergosta-8,22,24(28)-trienol in *Botrytis cinerea* and *Penicillium italicum* (8); ergosta-8,14,24(28)-trienol in *Penicillium italicum* (7); ignosterol and ergosta-8-enol in *Ustilago maydis* (10); and ergosta-8,22-dienol(8), 4,4-dimethyl-cholesta-8,14-dienol, 4,4-dimethyl-cholesta-8,24-dienol, and ignosterol in *Saccharomyces cerevisiae* (10).

These results strongly suggest that the $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and Δ^{14}-sterol reductase are the target enzymes for morpholines (2). This has been confirmed recently by in vitro assays of the two enzymes (9).

However, the basis of the antifungal property of fenpropimorph has not been demonstrated clearly. It has been suggested that the inhibition of cell growth could be the consequence of the modification of membrane properties caused by the abnormal sterols that accumulate in treated cells (16–19). However, mutants of *S. cerevisiae* blocked in late steps of ergosterol biosynthesis have been isolated (20) and characterized. For instance, strains carrying the *erg2* and *erg5* mutations (1), which affect $\Delta^8 \rightarrow \Delta^7$-sterol isomerase and $\Delta^{22,23}$ sterol dehydrogenase, respectively, are perfectly able to grow despite the accumulation of Δ^8 (*erg2*) or $\Delta^{8,14}$-sterols (*erg5*).

To investigate further the relationship between inhibition of sterol biosynthesis and inhibition of cell growth, we used the properties of sterol auxotrophic mutant strains. These mutants, which were isolated as temperature-sensitive clones, are blocked before lanosterol synthesis (6). We show first that growth inhibition is specifically the consequence of inhibition of the ergosterol pathway and is not due to the effect of fenpropimorph on another metabolic pathway. Then, we took advantage of a sterol-permeable (not requiring ergosterol) mutant strain that allowed us to discriminate between toxic sterol accumulation and starvation for a specific ergosterol as the causes of growth inhibition.

MATERIALS AND METHODS

Strains and growth conditions. All strains used in this study were derived from *S. cerevisiae* FL100 (ATCC 28383; haploid, a mating type) and isogenic strain FL200 (ATCC 32119; haploid, α mating type). Sterol auxotrophic mutant strains FK*erg9* (squalene synthetase) and FK*erg7* (oxidosqualene-lanosterol cyclase) have been described elsewhere (6). Mutant strain FK*aux30* has been isolated from mutant strain FK*erg7* (6). The complete medium used consisted of the following (per liter): yeast extract (Biokar), 10 g; peptone (Biokar), 10 g; glucose, 20 g. Cultures were incubated at 30° with shaking.

Sterols were supplied by dilution of stock solutions in a mixture of tergitol Nonidet P-40–ethanol (1:1; vol/vol). Fenpropimorph (BASF) was dissolved in ethanol (the final solvent concentration in the culture medium did not exceed 1%). Ketoconazole (Janssen Pharmaceutica) was dissolved in sterile water.

Growth was assayed by dry weight determination or by measurement of the optical density of the culture at 700 nm. Cell numbers and budding cells were determined with a hemacytometer after vigorous agitation.

Sterol extraction and analysis. The cells were harvested by centrifugation and were washed twice with 0.1 M potassium phosphate buffer (pH 7.4) containing tergitol–ethanol 1% (vol/vol) and once with phosphate buffer.

The cells were saponified with methanolic KOH 40% (wt/vol) in the presence of pyrogallol at 90°C for 1 h. The sterols were extracted with *n*-heptane and analyzed by UV absorption and gas-liquid chromatography by using available standards. A gas chromatograph (GC 6000; Carlo-Erba) equipped with a flame-ionization detector and an on-column injector was used. The column was a capillary column (SE30). Cholesterol was used as the internal standard to calculate relative retention times, as follows: squalene, 0.72; cholesta-8,24-dienol (zymosterol), 1.06; ergosta-5,7,22-trienol (ergosterol), 1.09; ergosta-8,24(28)-dienol (fecosterol) and/or ergosta-8,14-dienol (ignosterol), 1.13; ergosta-8-enol,

* Corresponding author.

TABLE 1. Determination of MICs of fenpropimorph for wild-type, sterol auxotrophic, and FK*aux30* mutant strains[a]

Strain	MIC (μM) with the following supplement in complete medium[b]:			
	Control	Ergosterol	Lanosterol	Cholestanol
FL100	0.3	0.9	0.3	0.1
FK*erg7*		>60	0.1	0.1
FK*erg9*		>60	0.1	0.1
FK*aux30*	0.3	>60	0.1	0.1

[a] The concentration of fenpropimorph which prevented any appearance of growth after 48 h incubation was designated as the MIC.
[b] Cell suspensions were plated on complete solid medium with (4 μg/ml) or without sterol supplementation (control contained detergent mixture at 1% [vol/vol]).

1.14; ergosta-7,24(28)-dienol (episterol) and/or ergosta-5,7-dienol, 1.16; and 4,4,14-trimethyl-cholesta-8,24-dienol (lanosterol), 1.24. Sterols with 5,7- (ergosterol and ergosta-5,7-dienol) and 8,14- (ignosterol) diene systems were identified by their specific absorption spectra (4).

Ergosta-8-enol and ignosterol were authentified by gas chromatography-mass spectrometry analysis of the corresponding acetates.

RESULTS

Fenpropimorph-induced growth arrest may be the direct consequence of inhibition of the sterol pathway or may be due to the effect of the fungicide on another metabolic pathway. The latter hypothesis would be excluded if ergosterol supplementation relieved the inhibition of growth. However, yeast wild-type strains are impermeable to exogenous sterols (15), so we used two mutant strains that were auxotrophic for ergosterol and the FK*aux30* mutant strain, which is permeable to exogenous sterols, although it is not auxotrophic (15). In a preliminary experiment, we checked that all three mutant strains accumulated 10 to 15 times more [^{3}H]cholesterol than the wild-type strain after a 48-h culture period.

Table 1 shows that the growth of the wild-type strain was inhibited by a fenpropimorph concentration as low as 0.3 μM and that ergosterol supplementation relieved this inhibition only very slightly (MIC, 0.9 μM). On the contrary, the mutants that we tested showed a strong resistance (above 60 μM) if the culture medium was supplemented with ergosterol. The strong resistance of the sterol auxotrophic mutants showed that morpholine blocks cell proliferation by inhibiting specifically the sterol biosynthetic pathway. To confirm that growth arrest was correlated with the inhibition of late ergosterol biosynthetic steps, we supplemented with lanosterol the sterol auxotrophic mutants, which were blocked before lanosterol synthesis. As expected, they recovered a strong susceptibility (MIC, 0.1 μM) to fenpropimorph (Table 1). Therefore, the inhibited step is downstream of lanosterol synthesis, and growth inhibition might be the consequence of an accumulation of toxic sterol intermediates, as suggested above.

In order to search for the correlation between growth and inhibition of the sterol pathway, cell growth was measured in parallel with the sterol composition in wild-type cells treated with increasing fenpropimorph concentrations. At 15 and 60 nM, fenpropimorph concentrations which do not inhibit cell growth, the ergosterol content in cells was lowered by about 50 and 75%, respectively. The major sterols in treated cells were ergosta-8-enol and fecosterol (Table 2). At a concentration of 300 nM, cell growth was strongly inhibited. The ergosterol concentration was decreased 20-fold in comparison with the control, and ignosterol (34%), in addition to ergosta-8-enol (44%), accumulated. These results are consistent with the fact that in vivo low concentrations of fenpropimorph inhibit $\Delta^8 \rightarrow \Delta^7$-sterol isomerase, and higher concentrations also inhibit Δ^{14}-sterol reductase. They are in good agreement with results of the in vitro assays of the two enzymes, which showed that the inhibitor has a much stronger affinity for $\Delta^8 \rightarrow \Delta^7$-sterol isomerase than it does for Δ^{14}-sterol reductase (9).

Figure 1 shows that even when ergosta-8-enol reached its maximal level, no significant growth inhibition occurred. On the other hand, growth was already almost completely inhibited when the ignosterol level began to increase. Growth inhibition took place when the ergosterol content was under 15% of the total sterol content, which corresponds to about 0.20% of cell dry weight as ergosterol. In sterol auxotrophic mutant strains, we have observed that the minimal ergosterol level that can be obtained after starvation is very close to this value (15). Therefore, these results suggest that the toxic activity of fenpropimorph is due to the specific inhibition of ergosterol biosynthesis.

Studies on the role of sterols in *S. cerevisiae* have shown that ergosterol has at least two functions. Its role as a bulk membrane component can be fulfilled by numerous sterol molecules (even by the fully saturated cholestanol molecule). Another function is regulatory and is specific for ergosterol; very small amounts of ergosterol are required to

TABLE 2. Comparative sterol compositions of fenpropimorph-treated cells

Sterol	% of total sterols at the following fenpropimorph concn (nM)[a]:							
	Wild-type strain FL100				Mutant strain FK*aux30*			
	0	15	60	300	0	60	300	300 with ergosterol[b]
Zymosterol	12	12	15	5	9	7	4	5
Ergosterol	43	22	11	2	38	2	3	2
Fecosterol	7	22	20		3	26		
Ignosterol				34			33	37
Ergosta-8-enol		42	52	44		54	38	33
Episterol and ergosta-5,7-dienol	34				40			
Lanosterol	4	2	2	15	10	11	22	23

[a] The cells (inoculum, 10^6 per ml) were cultured for 15 h in the presence of different concentrations of fenpropimorph in complete medium. The sterols were quantified by the area method.
[b] The ergosterol supplement was 0.1 μg/ml.

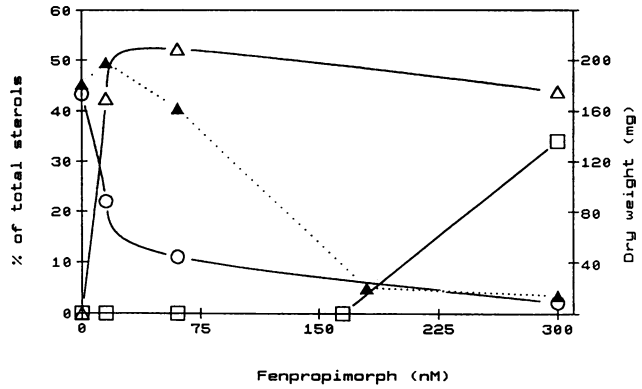

FIG. 1. Growth and sterol composition of the wild-type strain as a function of fenpropimorph concentration. The inoculum was 10^6 cells per ml. Cells were harvested after 15 h. Cell yield was determined and the sterols were analyzed as described in the text. Symbols: ○, ergosterol; △, ergosta-8-enol; □, ergosta-8,14-dienol; ▲, growth.

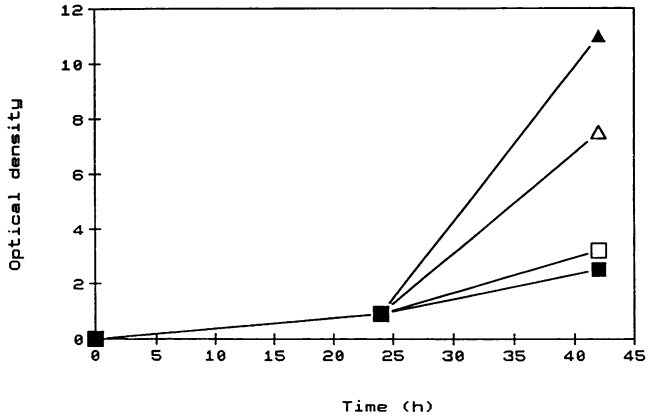

FIG. 3. Effect of sterol addition on growth of the FK*aux30* mutant strain blocked with fenpropimorph (0.6 μM) for 24 h. The inoculum was 10^5 cells per ml. Growth yield was measured after 18 h. Symbols: △, ergosterol, 0.1 μg/ml; ▲, ergosterol, 1 or 10 μg/ml; □, cholestanol, 4 μg/ml; ■, tergitol.

ensure this physiological function, which has been described as a "sparking" effect (12–14).

Because Fig. 1 suggests that the inhibition is correlated with ergosterol starvation rather than the accumulation of unusual sterols, specific ergosterol feeding should relieve inhibition. As the wild-type strain was not permeable to exogenous sterols, we used the FK*aux30* mutant strain, permeable when grown under aerobic conditions, as are the strains that carry *hem* mutations (11). The FK*aux30* mutant strain has a sterol composition similar to that of the parental wild-type strain in the presence or absence of inhibitor (Table 2). The results (Table 1) show that only ergosterol, but not cholestanol, obviates growth inhibition in sterol auxotrophic and FK*aux30* mutant strains. An ergosterol concentration as low as 0.1 μg/ml relieved growth inhibition of the FK*aux30* mutant strain (Fig. 2). As expected, growth inhibition of the wild-type strain was very poorly relieved by ergosterol, whatever its concentration. Moreover, when grown in the presence of ergosterol, the FK*aux30* mutant strain accumulated amounts of ignosterol similar to those accumulated in nonsupplemented inhibited cultures (Table 2). Taken together, these results confirm that ignosterol, which accumulated as a consequence of fenpropimorph treatment, is not toxic for yeast cells and that inhibition is the consequence of a specific ergosterol deprivation.

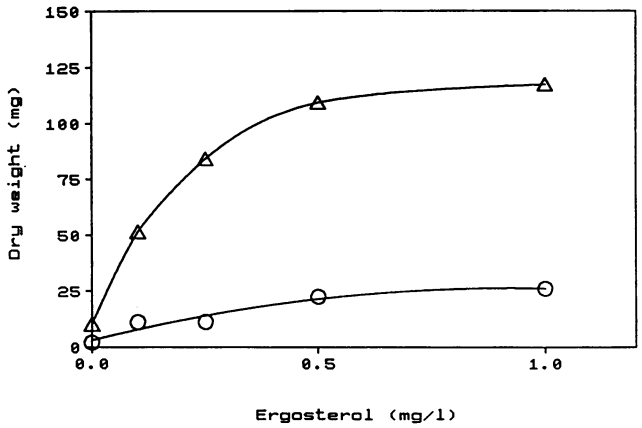

FIG. 2. Cell yield of wild-type and FK*aux30* mutant strains treated with 0.6 μM fenpropimorph as a function of ergosterol supplementation. The inoculum was 10^5 cells per ml. Cells were harvested after 48 h. Symbols: ○, wild-type; △, mutant strain FK*aux30*.

We wondered whether ergosterol might also relieve growth inhibition induced by azole compounds such as ketoconazole. Indeed, the FK*aux30* mutant strain was inhibited by 56 μM ketoconazole in medium containing detergent mixture alone (2%; vol/vol). In contrast, in ergosterol-supplemented medium, the MIC was greater than 376 μM.

We observed that the cells remained viable in extended cultures blocked by fenpropimorph (24 h). When the sterol-permeable FK*aux30* mutant strain was blocked with fenpropimorph for 24 h, the addition of ergosterol resulted in a sharp recovery of cell proliferation (Fig. 3). Figure 3 also shows that cholestanol could not replace ergosterol in relieving the inhibition. Since the inhibited cells remained viable, one could suppose that they stay blocked during the cell cycle. In fact, microscopic observation of treated cells showed that a majority of them appeared without buds, which suggests that they were blocked in the G_1 phase of the cell cycle. When inhibitory concentrations of fenpropimorph (>0.6 μM) were added to exponentially growing cultures of the FK*aux30* mutant strain, the inhibition of cell growth (Fig. 4A) was correlated with the increase in number of unbudded cells (Fig. 4B); after 5 h of treatment, the percentage of budded cells dropped from 62 to 23%. The addition of ergosterol stimulated budding and the recovery of cell proliferation. One can therefore conclude that the fungistatic property of fenpropimorph is due to ergosterol deprivation, which leads to the arrest of cell proliferation in the G_1 phase of the cell cycle. The observation that ergosterol deprivation blocks yeast cells in the G_1 phase has been already reported by Dahl et al. (3), who showed that ergosterol starvation blocks sterol auxotrophic mutant strain GL7 in the G_1 phase and that only a specific ergosterol addition triggers cell growth.

DISCUSSION

Several groups of investigators have described the effect of fungicides that inhibit ergosterol biosynthesis in yeast or filamentous fungi, but the basis of growth inhibition was not established. One possibility was that the abnormal sterols

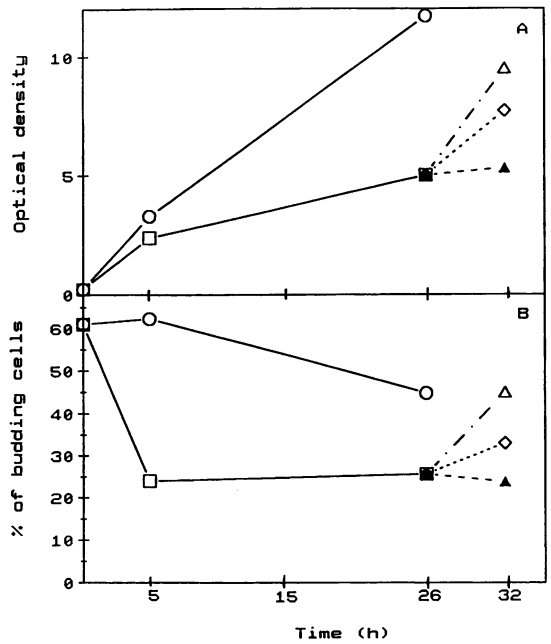

FIG. 4. Effect of fenpropimorph on cell proliferation (A) and budding (B) of the FK*aux30* mutant strain. Fenpropimorph was added in exponentially growing cultures at an optical density (700 nm) of 0.2. Symbols: □, fenpropimorph at 0.6 μM; ○, control without fenpropimorph. After incubation for 26 h, the sterol-free medium was supplemented with ergosterol at 0.1 (◇) and 1 (△) μg/ml. The control without ergosterol is also shown (▲).

that accumulated in the membrane of treated cells would be toxic. We showed here that fenpropimorph inhibits cell growth by leading to a specific ergosterol starvation. In fact, it has been demonstrated (12–14) that bulk membrane function in yeast cells can be fulfilled by numerous different sterol molecules but that ergosterol is needed specifically for a regulatory function. This sparking effect was described only in yeast mutant strains harboring a complex genotype as RD5-R (*hem1 erg3 erg6* [13]), GL7 (*hem3 erg12* [12]), and FY3 (*hem1 erg7 met ura* [14]). These genotypes actually lead to sterol permeability and to sterol and unsatured fatty acid auxotrophy caused by *hem* mutations. In the present study we observed a similar effect of ergosterol using a mutant strain bearing an *aux30* mutation that led to sterol permeability but not to ergosterol or unsaturated fatty acid auxotrophy.

The question that arises concerns the normal growth of the sterol mutants that are blocked in the late steps of ergosterol biosynthesis. The simplest explanation is that they bear leaky mutations that allow weak but sufficient ergosterol synthesis. Another possibility could be that they have acquired additional mutations that suppress the specific need for ergosterol.

The rapid inhibition of growth observed in the presence of fenpropimorph (Fig. 4A) strongly suggests that membrane ergosterol cannot undergo an exchange with ergosta-8-enol or ignosterol, which would make it available for the regulatory function.

Ergosterol deprivation of a sterol auxotrophic mutant strain (3) as well as fenpropimorph treatment of a wild-type yeast strain (this study) block both cell cultures in the G_1 phase of the cell cycle, as do numerous nutritional deprivations (3). The isolation of many temperature-sensitive cell division cycle (*cdc*) mutants has led to a better understanding of cell proliferation. Genes implicated in the "start" event have been characterized, and several of them correspond to proteins related to cyclic AMP formation or encode proteins which show homology to mammalian protein kinases (5). Dahl et al. (3) showed that a protein kinase different from that encoded by *CDC28* is stimulated by very low levels of ergosterol and is involved in the exit from the G_1 phase of sterol-depleted yeast cells. The isolation and characterization of fenpropimorph-resistant mutants might thus permit identification of the proteins involved in ergosterol-linked cell division control, as well as in the regulation of ergosterol biosynthesis and uptake.

Finally, the results presented here are probably not specific for fenpropimorph. The fact that ergosterol efficiently relieves the growth inhibition induced by ketoconazole strongly suggests that they may be extended to the azole fungicides as well.

ACKNOWLEDGMENTS

We are grateful to Pierre Benveniste for stimulating discussions during the course of this work. We thank André Amblès for the mass spectrum analyses and F. Nau, M. Servouse, and R. Defaye for help in editing the manuscript.

LITERATURE CITED

1. **Barton, D. H. R., J. E. T. Corrie, D. A. Widdowson, M. Bard, and R. A. Woods.** 1974. Biosynthesis of terpenes and steroids. Part IX. The sterols of some mutant yeasts and their relationship to the biosynthesis of ergosterol. Chem. Soc. Perk. Trans. **I**:1326–1333.
2. **Benveniste, P.** 1986. Sterol biosynthesis. Annu. Rev. Plant. Physiol. **37**:275–308.
3. **Dahl, C., H. P. Biemann, and J. Dahl.** 1987. A protein kinase antigenically related to pp60v-src possibly involved in yeast cell cycle control: positive *in vivo* regulation by sterol. Proc. Natl. Acad Sci. USA **89**:4012–4016.
4. **Dorfman, L.** 1953. Ultraviolet absorption of steroids. Chem. Rev. **53**:47–144.
5. **Jacquet, M., and J. Camonis.** 1985. Contrôle du cycle de division cellulaire et de la sporulation chez *Saccharomyces cerevisiae* par le système de l'AMP cyclique. Biochimie **67**: 35–43.
6. **Karst, F., and F. Lacroute.** 1977. Ergosterol biosynthesis in *Saccharomyces cerevisiae* mutant deficient in the early steps on the pathway. Mol. Gen. Genet. **154**:269–277.
7. **Kerkenaar, A., J. M. Van Rossum, G. G. Werluis, and J. W. Marsmamn.** 1984. Effect of fenpropimorph and imazalil on sterol biosynthesis in *Penicillium italicum*. Pestic. Sci. **15**: 177–187.
8. **Leroux, P., and M. Gredt.** 1983. Etudes sur les inhibiteurs de la biosynthèse des stérols fongiques. I. Fongicides provoquant l'accumulation de desméthylstérols. Agronomie **2**:123–130.
9. **Mercer, I., and R. Baloch.** 1987. Inhibition of sterol Δ^8-Δ^7 isomerase and Δ^{14} reductase by fenpropimorph, tridemorph and fenpropidin in cell free enzyme systems from *Saccharomyces cerevisiae*. Phytochemistry **23**:663–668.
10. **Mercer, I., R. Baloch, T. Wiggins, and B. Baldwin.** 1984. Inhibition of ergosterol biosynthesis in *Saccharomyces cerevisiae* and *Ustilago maydis* by tridemorph, fenpropimorph, and fenpropidin. Phytochemistry **23**:2219–2226.
11. **Parks, L. W., C. D. K. Bottema, R. J. Rodriguez, and T. A. Lewis.** 1985. Yeast sterols: yeast mutants as tools for the study of sterol metabolism. Methods Enzymol. **111**:333–345.
12. **Ramgopal, M., and K. Bloch.** 1983. Sterol synergism in yeast. Proc. Natl. Acad. Sci. USA **80**:712–715.
13. **Rodriguez, R. J., C. Law, C. D. K. Bottema, and L. W. Parks.** 1985. Multiple functions for sterols in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **837**:336–343.
14. **Rodriguez, R. J., F. R. Taylor, and L. W. Parks.** 1982. A

requirement for ergosterol to permit growth of yeast sterol auxotrophs on cholestanol. Biochem. Biophys. Res. Commun. **106**:435–438.
15. **Servouse, M., and F. Karst.** 1986. Regulation of early enzymes of ergosterol biosynthesis in *Saccharomyces cerevisiae*. Biochem. J. **240**:541–547.
16. **Stell, C. C., R. I. Baloch, E. I. Mercer, and B. C. Baldwin.** 1989. The intracellular and physiological effects of abnormal sterols in fungi grown in the presence of morpholine and functionally related fungicides. Pestic. Biochem. Physiol. **33**:101–111.
17. **Taylor, F. R., J. R. Rodriguez, and L. W. Parks.** 1983. Relationship between antifungal activity and inhibition of sterol biosynthesis in miconazole, clotrimazole, and 15-azasterol. Antimicrob. Agents Chemother. **23**:515–521.
18. **Van den Bossche, H., G. Willemsens, W. Cools, F. Cornelissen, W. F. Lauwers, and J. M. Van Cutsem.** 1980. In vitro and in vivo effects of the antimycotic drug ketoconazole on sterol synthesis. Antimicrob. Agents Chemother. **17**:922–928.
19. **Wholoshuk, C., H. Sisler, and S. Dutky.** 1979. Mode of action of the azasteroid antibiotic 15aza-24-methylene-D-homocholesta 8-14 dien-3-β-ol in *Ustilago maydis*. Antimicrob. Agents Chemother. **16**:98–103.
20. **Woods, R. A.** 1971. Nystatin-resistant mutants of yeast: alterations in sterol content. J. Bacteriol. **108**:69–72.