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Relationship Between Antifungal Activity and Inhibition of Sterol Biosynthesis in Miconazole, Clotrimazole, and 15-Azasterol†

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The availability of *Saccharomyces cerevisiae* mutants which are defective in sterol biosynthesis makes it possible to determine whether the ability of several antifungal agents to inhibit cell growth is due to their effect on sterol production. 15-Aza-24-methylene-8,14-cholestadien-3β-ol (15-azasterol) is known to block the reduction of the sterol Δ^{14} bond following C-14 demethylation. This agent inhibits the growth of wild-type *S. cerevisiae* but does not inhibit the growth of a strain that is defective in the removal of the C-14 methyl group of lanosterol and in the introduction of the 5,6 double bond. 15-Azasterol does not inhibit the growth of a sterol auxotrophic strain growing on an exogenous supply of sterol. Therefore, the effect of 15-azasterol on sterol biosynthesis is clearly the cause of its ability to inhibit growth. On the other hand, growth inhibition by two imidazole antifungal agents, clotrimazole and miconazole, cannot be ascribed to their ability to prevent the removal of the C-14 methyl group of lanosterol, because they inhibit the growth of the sterol auxotrophic strain as well as that of the demethylase mutant.

A series of antifungal agents have been developed (16, 22) that includes a group of imidazole-containing compounds, such as miconazole and clotrimazole (18, 34), which share the ability to block sterol C-14 demethylation. This results in an accumulation of the sterol intermediate, 14α-methyl-$\Delta^{8,24(28)}$-ergostadien-3β-ol (14-methylfecosterol), in fungal cells. Because the concentration dependence of growth inhibition by one of these compounds parallels the degree of inhibition of demethylation, it has been suggested that the presence of the unusual sterol biosynthetic intermediate is the cause of the antifungal properties (33). This conclusion may be questioned, since it has been found that some C-14 methyl sterols will support the growth of yeast cells (6, 21, 32).

We have presented studies on another compound, 15-aza-24-methylene-8,14-cholestadien-3β-ol (15-azasterol), with strong antifungal activity (13, 14, 23). This compound inhibits a step in C-14 demethylation (4) which leads to the accumulation of $\Delta^{8,14}$-ergostadien-3β-ol (ignosterol). Previous work from our laboratory documented a close correlation between ignosterol production and growth inhibition, but the cause-and-effect relationship has not been proven. Furthermore, there has been no other basis on which to judge whether a sterol with a $\Delta^{8,14}$-diene would be adequate to support cell growth.

In an attempt to clarify these issues, we tested the effect of the three antifungal agents on the growth of a *Saccharomyces cerevisiae* strain with blocks in lanosterol C-14 demethylation and Δ^5-desaturation and of another mutant strain blocked at the 2,3-oxidosqualene cyclization. The pattern of inhibition we have found suggests that the basis of growth inhibition by clotrimazole and miconazole is not due to their effects on sterol biosynthesis. In contrast, growth inhibition by low levels of 15-azasterol is clearly caused by inhibition of sterol biosynthesis.

A comprehensive review of the metabolism of sterols in yeast strains has been reported (20).

MATERIALS AND METHODS

Strains of *S. cerevisiae* were used exclusively. The sterol wild-type strain S288C (α *gal1*) was obtained from the yeast culture collection of the University of California, Berkeley, and strain A364a (α, *ade1 ura1 his7 tyr1 lys7 gal1*) was obtained from George Sprague at the University of Oregon, Eugene. Strain JR4 is a nystatin-resistant derivative of S288C (J. R. Ramp, honors thesis, Oregon State University, Corvallis, 1981); its sterol phenotype will be described in Results. JR1 (Ramp, honors thesis) and 3701b-N3 (31) are independently isolated, nystatin-resistant strains defective in the Δ^5-desaturase. Strain FY3 is blocked in sterol biosynthesis by both a heme mutation and a mutation in 2,3-oxidosqualene cyclase (29).

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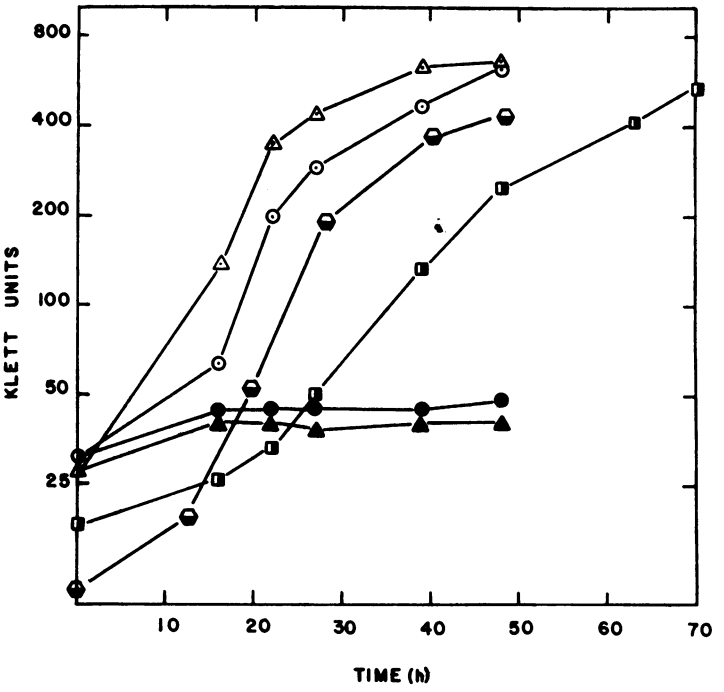

FIG. 1. Effect of 15-azasterol on wild-type and sterol mutants. Open and closed symbols indicate growth in the absence or presence (5 μg/ml) of 15-azasterol, respectively. Half-closed symbols indicate that growth was the same in the presence or absence of the antifungal agents. ○, Δ^5-Desaturase; □, C-14 demethylase–Δ^5-desaturase double mutant; △, sterol wild-type; ○, sterol auxotrophic strain FY3.

In most experiments the growth medium was 1% yeast extract–2% peptone–2% glucose; for solid medium, 2% agar was included. The sterol auxotrophic strain FY3 was cultured in a minimal medium, as previously described (30), which had 1% Tween 80 as a source of unsaturated fatty acid and 20 μg of ergosterol per ml as a source of sterol (FY medium). For growth measurement experiments, strains were cultured in tubes containing 5 ml of medium in a temperature gradient incubator (Scientific Industries) held isothermally at 30°C. Culture turbidity was monitored in a Klett-Summerson spectrophotometer equipped with a green filter. When the cell sterol content was analyzed, 20- to 50-ml cultures were grown in 250-ml flasks with shaking.

Stock solutions of (±)-miconazole nitrate and clotrimazole were dissolved in dimethyl sulfoxide; maximum concentrations were 0.33 and 0.17 M, respectively. The stock solution of 15-azasterol was dissolved in ethanol with a maximum concentration of 1 mg/ml. The final concentrations of dimethyl sulfoxide or ethanol in the growth medium never exceeded 2% and were routinely 0.5%. Appropriate controls were run to monitor the effects of these solvents on cell growth.

The techniques for genetic analysis were the same as those described by Sherman et al. (26).

Cultures for sterol analysis were grown to the stationary phase, centrifuged, and washed three times with 10 mM phosphate buffer (pH 6). Cellular sterols were isolated by an acid labilization procedure (12), followed by saponification and extraction with *n*-hexane. The lipids were then chromatographed on silica gel 60 F254 (0.25 mm thick; Merck & Co., Inc.) in the solvent system of Skipski et al. (27). The presence of the reference sterol (ergosterol) could be monitored by UV light. The sterol bands were then scraped into tubes and eluted with three washes of chloroform-methanol (4:1), and the free sterols were analyzed by both gas chromatography on a 3% SE30-packed column (30) and high-pressure liquid chromatography on a reverse-phase C-18 column (24). Gas chromatography-mass spectroscopy was performed on a mass spectrometer (Finigan model 4000) at 70 eV after separation of the sterols on 7% OV 101.

Clotrimazole, (±)-miconazole nitrate, Tween 80, and ergosterol were purchased from Sigma Chemical Co. Eli Lilly & Co. generously supplied the 15-azasterol. Growth media were from Difco Laboratories, gas chromatographic supplies were from Supelco, and liquid chromatographic equipment was from Beckman Instruments, Inc.

RESULTS

Through the use of a new procedure designed to obtain *S. cerevisiae* clones resistant to the polyene antibiotic nystatin, a strain which accumulated 14-methylfecosterol as its major sterol was isolated in this laboratory (Ramp, honors thesis). This sterol was identified by comparison of its mass spectrum with that published by

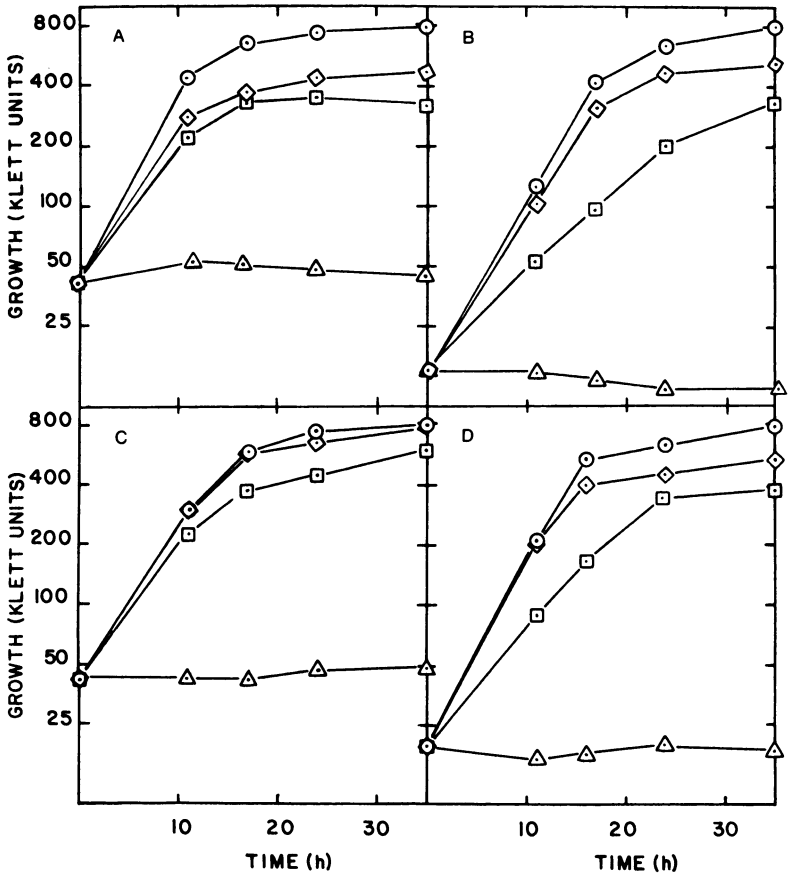

FIG. 2. Growth of wild-type and sterol mutants in the presence of (±)-miconazole nitrate. The growth medium was 1% yeast extract–2% peptone–2% glucose, and the culture conditions were as described in the text. (A) Sterol wild-type strain S288C; (B) C-14 demethylase–Δ^5-desaturase double-defective strain JR4; (C) Δ^5-desaturase-defective strain JR1; (D) Δ^5-desaturase-defective strain 3701b-N3. The miconazole concentrations were 100 μM (△), 10 μM (□), 1 μM (◇), and 0 μM (○).

Ragsdale (22) and Trocha et al. (32). In addition, radioactivity was incorporated into the sterol from [*methyl*-^{14}C]methionine, which demonstrated a side chain methylene and lacked UV absorbance above 210 nm. Therefore, strain JR4 appears to have a defect in the enzyme(s) catalyzing the removal of the C-14 methyl of lanosterol and would appear to be similar to the strains isolated by Trocha et al. (32) and Pierce et al. (21). In the course of a genetic analysis of JR4, it became clear that a mutation in the sterol Δ^5-desaturase reaction was also present in this strain. A more detailed report on the minor sterols of this strain and the apparently obligatory coupling of the C-14 demethylase defect with the desaturase mutation will be presented in a separate publication (F. R. Taylor, R. J. Rodriguez, and L. W. Parks, manuscript in preparation).

The characterization of this mutant offered a means for testing the mechanism of growth inhibition by a potent inhibitor of sterol biosynthesis, 15-azasterol. This compound inhibits the Δ^{14}-reductase reaction, which follows the sequence of reactions that result in the removal of the C-14 methyl group of lanosterol (4), forcing the accumulation of ignosterol. We reasoned that if the accumulation of ignosterol was responsible for growth inhibition, the C-14 demethylase–Δ^5-desaturase double mutant should not be inhibited by 15-azasterol; this strain would only accumulate 14-methylfecosterol even in the presence of 15-azasterol. Growth experiments (Fig. 1) proved this to be true; strain JR4 and six demethylase-desaturase double mutants, resulting from a segregation analysis of JR4 crossed with sterol wild-type strain A364a, were resistant to 5- to 10-fold higher levels of 15-azasterol than either the wild-type strain or segregants carrying only the Δ^5 desaturase mutation. We also tested the effect of 15-azasterol on an *S. cerevisiae* strain, FY3, that is

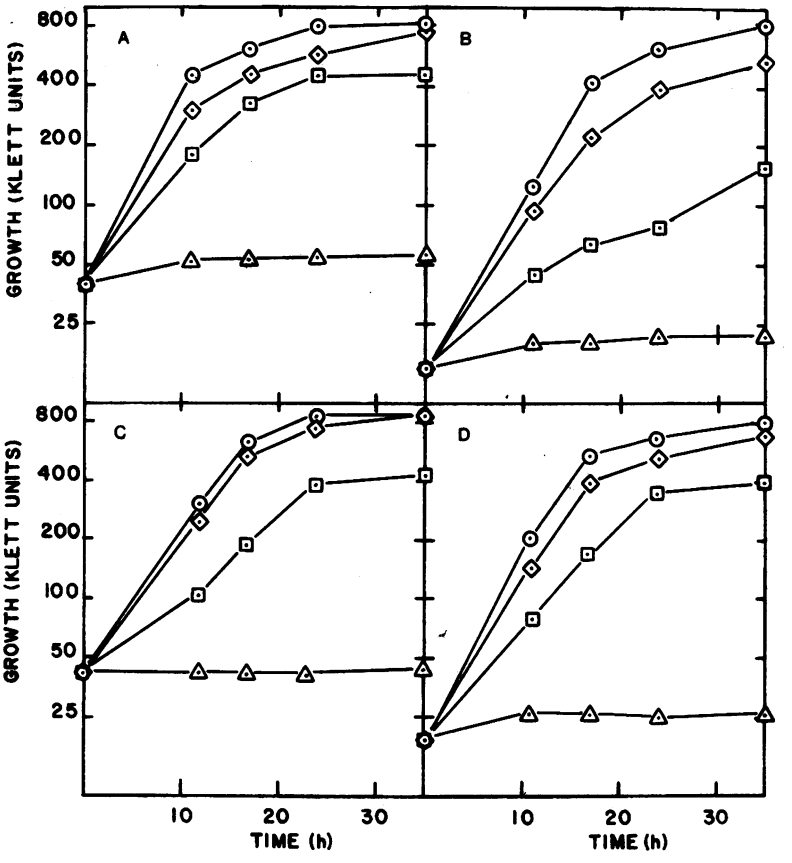

FIG. 3. Growth of wild-type and sterol mutants in the presence of clotrimazole. For explanation of symbols, see legend to Fig. 2.

defective in the cyclization of 2,3-oxidosqualene and in heme biosynthesis (29). These mutations make this strain an obligate sterol auxotroph, so that growth occurs only when an exogenous supply of sterol is provided. This strain was also resistant to 15-azasterol (Fig. 1).

From the genetic analysis of the demethylation-desaturation double mutations in JR4 (Taylor et al. manuscript in preparation), it appears that the inability to remove the sterol C-14 methyl is lethal in *S. cerevisiae* strains which are still able to introduce a Δ^5-bond into 14-methylfecosterol. It seems possible that this might have some bearing on the antifungal action of miconazole and clotrimazole. These compounds are known to block C-14 demethylation in fungi (18, 34), and their growth inhibitory action may result from blocking this step in wild-type *S. cerevisiae* strains capable of introducing the Δ^5-bond. If this were the case, these compounds would not be lethal to *S. cerevisiae* strains carrying a mutation in the Δ^5-desaturase. Growth experiments did not prove this to be the case. Growth of the strains carrying the Δ^5-desaturase mutation or the C-14 demethylase mutation were just as susceptible to these antifungal agents as were the parental strains (Fig. 2 and 3). In addition, an examination of the sterols produced by wild-type strain S288C in the presence of these agents revealed that the predominant sterol formed was 14-methylfecosterol. Small amounts of a sterol which has further desaturations (possibly a $\Delta^{5,8,22,24(28)}$-ergostatetraene) were also observed. This result agrees with that of Van den Bossche et al. (34) and suggests that miconazole and clotrimazole may inhibit the Δ^5-desaturase mutation in vivo.

The question then arose as to whether the antifungal activity of these imidazole compounds has any connection to sterol synthesis at all. This could be tested with *S. cerevisiae* FY3, which is completely blocked in sterol biosynthesis and therefore dependent on the utilization of sterol supplied in the medium. This strain was inhibited by even lower concentrations of miconazole and clotrimazole than were the wild-type controls, even though ergosterol is present in the medium as a source of sterol (Fig. 4). In

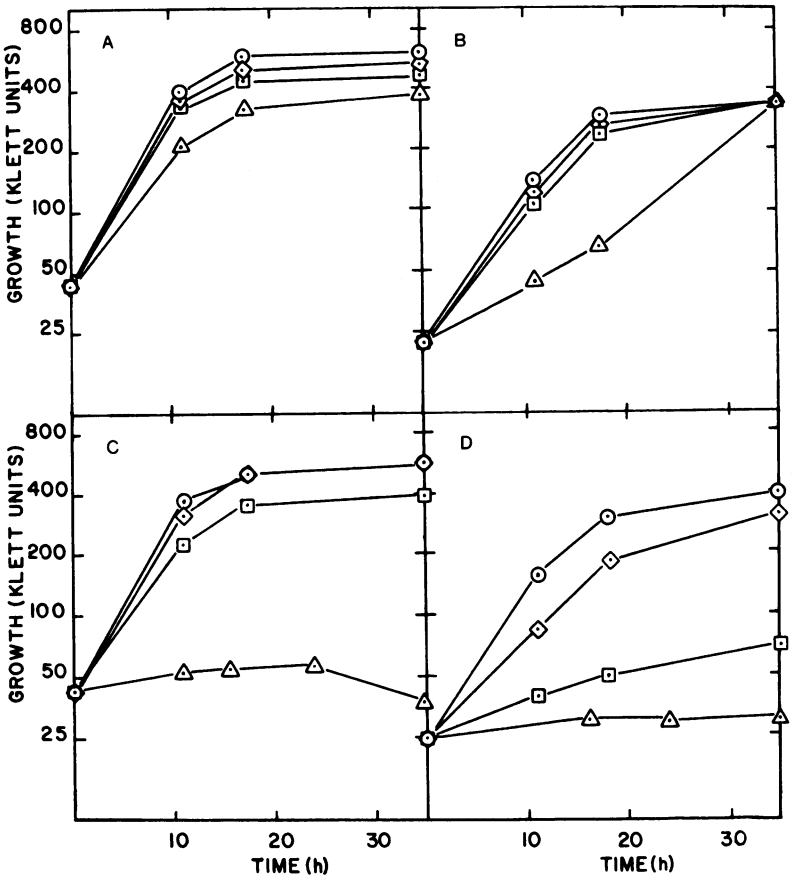

FIG. 4. Growth of wild-type and sterol auxotrophic strains in the presence of clotrimazole and (±)-miconazole nitrate. The strains were cultured in FY medium as described in the text. (A) and (C) Sterol wild-type strain S288C, (B) and (D) sterol auxotrophic strain FY3. The concentrations of clotrimazole (A and B) were 1 mM (△), 100 μM (□), 10 μM (◇), and 0 μM (○). The concentrations of (±)-miconazole nitrate (C and D) were 6.7 mM (△), 1 mM (□), 100 μM (◇), and 0 μM (○). The concentrations required for growth inhibition were higher than those in the experiments shown in Fig. 2 and 3. This is presumably because the detergent in the FY medium lowered the effective concentrations of the antifungal agents.

addition, the presence or absence of ergosterol in the medium had no effect on the inhibition of growth of the sterol wild-type strains, thus eliminating the possibility that sterol biosynthesis is the site of growth inhibition in these strains.

DISCUSSION

Current models of sterol-phospholipid interaction stress the importance of the planarity of the sterol α-face for biologically effective packing with the fatty acyl groups of phospholipids (1, 2, 15, 19). From this point of view, removal of the C-14 methyl of lanosterol would be an essential reaction in sterol biosynthesis because this methyl group projects into, and therefore disrupts, the α-face planarity. This hypothesis has been supported by experiments on the effectiveness of 14-methyl sterols in model membranes (7, 8, 17, 35) and as growth supplements for *Mycoplasma capricola* (7, 8, 9).

Results with *S. cerevisiae* have been at variance with this theory, however. Nystatin-resistant *S. cerevisiae* mutants have been isolated which are defective in the removal of the C-14 methyl and yet are viable with 14-methylfecosterol as their major sterol (21, 32; Ramp, honors thesis). This finding was reinforced by a report (6) that some C-14 methyl sterols are an effective supplement in a sterol auxotrophic strain of *S. cerevisiae*, although lanosterol itself will not support aerobic growth of *S. cerevisiae* (5, 29).

This issue has practical significance in that several antifungal agents, including a group of imidazole-containing compounds such as clotrimazole and miconazole (18, 34), block sterol C-14 demethylation (16, 22). Our demonstration here that the ability of miconazole and clotrima-

zole to inhibit growth is not related to the inhibition of sterol C-14 demethylation is consistent with results obtained in other studies of *S. cerevisiae*. It has been reported that, in addition to their effects on sterol synthesis, the imidazole antifungal agents affect the plasma membrane ATPase of *S. cerevisiae* (11) as well as other membrane enzymes, membrane transport, and fatty acid metabolism (see Borgers and Van den Bossche [3] for review). Imidazole antifungal agents have also been found to cause physical membrane damage (28). It is perhaps these or other activities which give these agents their antifungal properties.

If the results presented here are valid for other fungal species as well as for *S. cerevisiae*, it is puzzling why a large group of antifungal agents should all be effective inhibitors of sterol C-14 demethylation if this is not the cause for growth inhibition. It is possible that other types of fungi do require the removal of the sterol C-14 methyl group for a biologically effective sterol and that growth inhibition by these compounds in *S. cerevisiae* is due to their effect on some other reaction. On the other hand, perhaps the sensitive metabolic step is in some way related to C-14 demethylation by sharing a common component or by taking place in the same intracellular organelle.

The other antifungal agent tested, 15-azasterol, does not inhibit the growth of *S. cerevisiae* strains in which it cannot force the accumulation of the $\Delta^{8,14}$-diene sterol. This makes a strong case for the argument that the production of ignosterol is the cause of growth inhibition and further suggests that differing arrangements of double bonds in the sterol ring system can profoundly affect sterol biological activity. Obviously, these changes affect sterol conformation and flexibility, but it is difficult to predict the effect on membrane properties on the basis of what is currently known about sterol-membrane interactions. It is also possible that ignosterol cannot fulfill some other indispensable function of sterols in cells which may not be related to bulk membrane properties (10, 25), or its presence may result in problems in the cellular distribution of sterol. More information on the metabolism of these sterols by yeast strains and their effects on model systems and yeast physiology will be necessary to reach an understanding of the biological properties of these and other sterols.

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LITERATURE CITED

1. **Bloch, K.** 1976. On the evolution of a biosynthetic pathway, p. 143–150. *In* A. Kornberg, B. L. Horecker, L. Cornudella, and J. Oro, Jr. (ed.), Reflections in biochemistry. Pergamon Press, Inc., Oxford, England.
2. **Bloch, K.** 1981. Sterol structure and membrane function. Curr. Top. Cell. Regul. **18**:289–299.
3. **Borgers, M., and H. Van den Bossche.** 1982. The mode of action of antifungal drugs, p. 25–47. *In* H. B. Levine (ed.), Ketoconazole in the management of fungal diseases. Adis Press, New York.
4. **Bottema, C. K., and L. W. Parks.** 1978. Δ^{14}-Reductase in *Saccharomyces cerevisiae*. Biochim. Biophys. Acta **531**:301–307.
5. **Buttke, T. M., and K. Bloch.** 1980. Comparative responses of the yeast mutant strain GL7 to lanosterol, cycloartenol, and cyclolaudenol. Biochem. Biophys. Res. Commun. **92**:229–236.
6. **Buttke, T. M., and K. Bloch.** 1981. Utilization and metabolism of methyl-sterol derivatives in the yeast mutant strain GL7. Biochemistry **20**:3267–3272.
7. **Dahl, C. E., J. S. Dahl, and K. Bloch.** 1980. Effect of alkyl-substituted precursors of cholesterol on artificial and natural membranes and on the viability of *Mycoplasma capricolum*. Biochemistry **19**:1462–1467.
8. **Dahl, C. E., J. S. Dahl, and K. Bloch.** 1980. Effects of cycloartenol and lanosterol on artificial and natural membranes. Biochem. Biophys. Res. Commun. **92**:221–228.
9. **Dahl, J., C. Dahl, and K. Bloch.** 1980. Sterols in membranes: growth characteristics and membrane properties of *Mycoplasma capricolum* cultured on cholesterol and lanosterol. Biochemistry **19**:1467–1472.
10. **Dahl, J. S., C. E. Dahl, and K. Bloch.** 1981. Effect of cholesterol on macromolecular synthesis and fatty acid uptake by *Mycoplasma capricolum*. J. Biol. Chem. **256**:87–91.
11. **Dufour, J. P., M. Boutry, and A. Goffeau.** 1980. Plasma membrane ATPase of yeast. J. Biol. Chem. **255**:5735–5741.
12. **Gonzales, R. A., and L. W. Parks.** 1977. Acid-labilization of sterols for extraction from yeast. Biochim. Biophys. Acta **489**:507–509.
13. **Hays, P. R., W. D. Neal, and L. W. Parks.** 1977. Physiological effects of an antimycotic azasterol on cultures of *Saccharomyces cerevisiae*. Antimicrob. Agents Chemother. **12**:185–191.
14. **Hays, P. R., L. W. Parks, H. D. Pierce, and A. C. Oehlschlager.** 1977. Accumulation of ergosta-8,14-dien-3β-ol by *Saccharomyces cerevisiae* cultured with an azasterol antimycotic agent. Lipids **12**:666–668.
15. **Huang, C.** 1977. Configurations of fatty acyl chains in egg phosphatidylcholine-cholesterol mixed bilayers. Chem. Phys. Lipids **19**:150–158.
16. **Kato, T., and Y. Kausse.** 1976. Selective inhibition of the demethylation of C-14 in ergosterol biosynthesis by the fungicide, Denmert (S-1358). Agric. Biol. Chem. **40**:2379–2388.
17. **Lala, A. K., H. K. Lin, and K. Bloch.** 1978. The effect of some alkyl derivatives of cholesterol on the permeability properties and microviscosities of model membranes. Bioorg. Chem. **7**:437–445.
18. **Marriott, M. S.** 1980. Inhibition of sterol biosynthesis in *Candida albicans* by imidazole-containing antifungals. J. Gen. Microbiol. **117**:253–255.
19. **Nes, W. R., and M. L. McKean.** 1977. Biochemistry of steroids and other isopentanoids, p. 536–557. University Park Press, Baltimore, MD.
20. **Parks, L. W.** 1978. Metabolism of sterols in yeast. CRC Crit. Rev. Microbiol. **6**:301–341.
21. **Pierce, A. M., R. B. Mueller, A. M. Unrau, and A. C. Oehlschlager.** 1978. Metabolism of Δ^{24}-sterols by yeast

mutants blocked in removal of C-14 methyl group. Can. J. Biochem. **56**:794–800.

22. **Ragsdale, N. N.** 1975. Specific effects of triarimol on sterol biosynthesis in *Ustilago maydis*. Biochim. Biophys. Acta **380**:81–96.
23. **Rodriguez, R. J., and L. W. Parks.** 1981. Physiological response of *Saccharomyces cerevisiae* to 15-azasterol-mediated growth inhibition. Antimicrob. Agents Chemother. **20**:184–189.
24. **Rodriguez, R. J., and L. W. Parks.** 1982. Application of high-performance liquid chromatographic separation of free sterols to the screening of yeast sterol mutants. Anal. Biochem. **119**:200–204.
25. **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**:435–441.
26. **Sherman, F., G. F. Fink, and C. W. Lawrence.** 1974. Methods in yeast genetics: laboratory manual. Cold Spring Harbor Press, Cold Spring Harbor, N.Y.
27. **Skipski, V. P., A. F. Smolowe, R. C. Sullivan, and M. Barclay.** 1965. Separation of lipid classes by thin-layer chromatography. Biochim. Biophys. Acta **106**:386–396.
28. **Sud, I. J., and D. S. Feingold.** 1981. Heterogeneity of action mechanisms among antimycotic imidazoles. Antimicrob. Agents Chemother. **20**:71–74.
29. **Taylor, F. R., and L. W. Parks.** 1980. Adaptation of *Saccharomyces cerevisiae* to growth on cholesterol: selection of mutants defective in the formation of lanosterol. Biochem. Biophys. Res. Commun. **95**:1437–1445.
30. **Taylor, F. R., and L. W. Parks.** 1981. An assessment of the specificity of sterol uptake and esterification in *Saccharomyces cerevisiae*. J. Biol. Chem. **256**:13048–13057.
31. **Thompson, E. D., and L. W. Parks.** 1974. The effect of altered sterol composition on cytochrome oxidase and S-adenosylmethionine: Δ^{24} sterol methyltransferase enzymes of yeast mitochondria. Biochem. Biophys. Res. Commun. **57**:1207–1213.
32. **Trocha, P. J., S. J. Jasne, and D. B. Sprinson.** 1977. Yeast mutants blocked in removing the methyl group of lanosterol at C-14. Separation of sterols by high-pressure liquid chromatography. Biochemistry **16**:4721–4726.
33. **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.
34. **Van den Bossche, H., G. Willemsens, W. Cools, W. F. J. Lauwers, and L. Le Jeune.** 1978. Biochemical effect of miconazole on fungi. II. Inhibition of ergosterol biosynthesis in *Candida albicans*. Chem. Biol. Interact. **21**:59–78.
35. **Yeagle, P. L., R. B. Martin, A. K. Lala, H. K. Lin, and K. Bloch.** 1977. Differential effects of cholesterol and lanosterol on artificial membranes. Proc. Natl. Acad. Sci. U.S.A. **74**:4924–4926.