Physiological Effects of an Antimycotic Azasterol on Cultures of *Saccharomyces cerevisiae*¹

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The yeast *Saccharomyces cerevisiae* has been studied during cultivation with a naturally occurring antimycotic azasterol. At very low concentrations (1.0 to 10.0 ng/ml), where growth retardation is not observed, an unusual sterol, ergosta-8,14-dien-3 β -ol, accumulates in high concentrations. Upon removal of the azasterol from the culture, the 8,14-diene is converted to ergosterol. Much smaller amounts of another 8,14-sterol, but with an additional unsaturation, have also been observed. Total sterol accumulation is higher in cultures containing subinhibitory levels of the antimycotic agent than the amounts of normal sterol accumulation in control cultures. With between 10 and 100 ng of azasterol per ml a transitory cessation of growth is observed from which the culture is able to recover. At much higher concentrations growth inhibition and even cell lysis results. Competitive inhibition of sterol 24(28)methylene reductase is demonstrated.

Antibiotic agents specifically effective against fungi are rare in nature. A family of these agents has recently been isolated from *Geotrichum flavo-bruneum* (5), and the most abundant of the compounds was identified as 15-aza-24-methylene-D-homocholestadiene (6) (Fig. 1). These azasterols inhibit fungal growth at very low concentrations but are virtually without effect on bacteria. We became interested in these compounds when we recognized that they are structural analogues of yeast sterols, especially fecosterol. In a previous paper we showed in vitro inhibition of fecosterol formation by the antimycotic compound (3).

Inhibition of growth and lysis of fungi has been demonstrated with the sterol-binding polyene antibiotics. The polyenes rearrange existing sterols in membranes and modify membrane permeability (7). Since the control of sterols appears to be an effective way to control fungal growth, it was decided to investigate carefully the effects of the azasterol on yeast cell growth and sterol synthesis.

We report here that growth inhibition by the azasterol is probably a consequence of multiple effects on the organisms. The data presented further show that the inhibitor prevents ergosterol synthesis and is inhibitory to growth of test cultures at concentrations several orders of magnitude below polyene antibiotic inhibitory concentrations.

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MATERIALS AND METHODS

Saccharomyces cerevisiae strain 3701B was used in this study. Cultures were grown in broth containing 1% tryptone, 0.5% yeast extract, and 2% carbon source. All growth experiments were conducted at 28°C with shaking. Growth inhibition studies were conducted in broth with either 2% glucose or 2% ethanol. A logarithmic culture to be used as inoculum was harvested by centrifugation, and the cells were resuspended to 1/20 their original volume of broth. Tubes (15 by 150 mm) were prepared with 10 ml of broth and the appropriate azasterol concentration. The optical density of each culture tube was determined before inoculation, and sufficient cells were added to increase the density by 20 Klett units; changes in culture density were monitored with a Klett-Summerson photoelectric colorimeter.

Sterol extraction, thin-layer chromatographic separation and purification of sterols, and coupled gas chromatography-mass spectroscopy have been described previously (4). Gas chromatography was performed in a Varian 2740 gas chromatograph with an associated CDS 111 data processor. Ultraviolet absorption spectroscopy was performed on a Cary model 11 recording spectrophotometer.

Accumulation of labeled sterols was accomplished in broth containing L-[methyl-¹⁴C]methionine (0.1 μ Ci/ml, 2 μ M) and the azasterol (75 ng/ml, 0.18 μ M). Cultures (100 ml) were incubated until an optical density of about 50 Klett units was observed (log-phase growth). The azasterol and ¹⁴C label were then added, and the culture was allowed to grow to high turbidity (stationary phase). Cells were then harvested and saponified to extract the sterols.

Minimum effective inhibitor concentration (MIC) determinations were conducted in culture tubes (25

FIG. 1. Structure of the azasterol (15-aza-24-methylene-D-homocholesta-8,14-dien-3-ol) and ignosterol (ergosta-8,14-dien-3-ol).

by 150 mm) containing broth with 2% glucose and the appropriate azasterol concentrations. The cultures were incubated until high turbidity was achieved. After harvest and saponification, ultraviolet absorption spectra of the whole-cell non-saponifiable lipid fractions were determined.

The sterol 24(28)methylene reductase was followed by measuring the conversion of [28-methylene- ^{14}C]ergosta-5,7,22,24(28)-tetraen-3-ol to [28-methyl- ^{14}C]ergosterol. The 24(28)methylene reductase preparation was carried out as previously reported (13), with the following modifications. After cell homogenization, unbroken cells and other cellular fragments were removed by centrifugation twice at $2,500 \times g$ for 20 min. The supernatant was centrifuged at $13,000 \times g$ for 20 min. The pellet was collected and washed twice in 1.3 M sorbitol-0.1 M tris(hydroxymethyl)aminomethane-hydrochloride. The pellet was then resuspended in the same buffer and used as the enzyme fraction. [^{14}C]ergostatetraene [ergosta-5,7,22,24(28)-tetraen-3-ol] was isolated from *S. cerevisiae* (strain NCYC 366) grown in broth medium supplemented with L-[methyl- ^{14}C]methionine (0.02 $\mu\text{Ci}/\text{ml}$). The purity and specific activity of the [^{14}C]ergostatetraene have already been described (13).

The enzyme assay was performed as previously reported (13). For the inhibition studies, [^{14}C]ergostatetraene concentrations ranged from 5 to 100 μM . Inhibitory effects were checked at various azasterol concentrations (72, 97, and 122 μM). The azasterol was dissolved in 0.1 ml of 100% ethanol before being added to the reaction tubes. A 0.1-ml amount of 100% ethanol was added to the control tubes.

The azasterol, designated A25822B, used in this study was a generous gift of Eli Lilly & Co. L-[Methyl- ^{14}C]methionine was a product of International Chemical and Nuclear Co. Silica gel HF-254 plates were the product of E. M. Laboratories, Inc. Gas chromatographic supplies were from Supelco, Inc. Organic solvents were purchased from Mallinckrodt and redistilled before use.

RESULTS

To determine the effects of the azasterol on the growth of yeast, cultures containing various azasterol concentrations were monitored. Figure 2 shows the results of cultivation with increasing amounts of the inhibitor. Approximately equal cell numbers were added to each culture. Azasterol concentrations from 25 to 100 ng/ml produced biphasic growth curves show-

ing increasingly longer intermediate lag phases with increasing azasterol concentrations. At 250-ng/ml to 10- $\mu\text{g}/\text{ml}$ concentrations, increasing generation rates, earlier termination of growth, and lower final culture yields were observed. A concentration of 50 $\mu\text{g}/\text{ml}$ prevented any increase in culture density, and 100 $\mu\text{g}/\text{ml}$ resulted in a gradual loss of density. Figure 3 illustrates similar results when the organism is cultured with 2% ethanol as the carbon source.

On both substrates low levels of the azasterol (≤ 100 ng/ml) produced a temporary lag after about three generations. Subinhibitory concentrations are considered to be those that do not prevent the culture from eventually achieving the same stationary-phase culture density as a control culture without the inhibitor under otherwise identical growth conditions. At levels in excess of 250 ng/ml, increasing concentrations of the azasterol produced corresponding decreases in the number of generations before stationary phase occurred. We have found that this strain is more sensitive to the inhibitor on ethanol-containing agar than on agar with glucose as carbon source (3). The data presented here show a similar sensitivity in broth with either ethanol or glucose as the carbon source.

Since we have previously shown (3) the aza-

FIG. 2. Azasterol inhibition of growth of strain 3701B cultured with glucose as the energy source.

FIG. 3. Azasterol inhibition of growth of strain 3701B cultured with ethanol as the energy source.

sterol to inhibit ergosterol accumulation in this yeast strain, we examined sterols accumulated by cultures treated with growth-subinhibitory levels (25 to 100 ng/ml) of the inhibitor. In treated cultures an unusual sterol accumulates as the major portion of the total cellular 4-demethyl sterol fraction. We have identified this novel sterol, ignosterol, as ergosta-8,14-dien-3 β -ol (P. R. Hays, L. W. Parks, H. D. Pierce, and A. C. Ochlschlager, *Lipids*, in press). A comparison of the structures of azasterol and ignosterol is shown in Fig. 1. Ignosterol may accumulate to greater than 90% of the total 4-demethyl sterols. The non-saponifiable lipid from these cultures also contained a compound(s) that did not migrate from the base line on AgNO₃-silica gel thin-layer chromatography plates. This fraction typically contained primarily (80 to 90%) a compound that exhibited an ultraviolet absorption $\lambda_{\max}$ of 250 nm, which is characteristic of the sterol-conjugated $\Delta^{8,14}$ -diene system also found in ignosterol. Mass spectral analysis of the acetate revealed a molecular ion peak at m/e 438 (97%), which corresponds to a C₂₈-steryl triene acetate. A peak at m/e 395 (42%) corresponds to an

ergostatriene with loss of the acetate. An ion peak at m/e 312 (58%) is equivalent to a doubly unsaturated steryl acetate nucleus after loss of the side chain and one hydrogen. Other major ion peaks were m/e 424 (35%), 423 (57%), 363 (33%), 340 (25%), 339 (100%), 313 (24%), and 312 (58%). The major ion of m/e 339 may result from cleavage between C-20 and C-22 in the side chain, with transfer of two hydrogens. This is similar to the fragmentation pattern of Δ^{22} -unsaturated sterols proposed by Wyllie and Djerassi (15). Since a hydrogen migration from C-14 or C-15 is postulated to occur in this fragmentation, the differences observed from published 14-15-saturated Δ^{22} -sterol spectra in the $\Delta^{8,14}$ mass spectrum are understandable. The compound seems, therefore, to be an ergosteryl triene with nuclear $\Delta^{8,14}$ -unsaturations and a singly unsaturated side chain. We have not yet recovered enough of this compound to allow a more precise analysis, and the sample we have may be a mixture of trienes, which we have thus far been unable to resolve. This fraction may be an 8,14,22-triene or an 8,14,24(28)-triene, or both. Neither 8,14-unsaturated trienes or ergosta-8,14-dien-3 β -ol (ignosterol) accumulate in untreated cultures.

Since a 24(28)methylene compound is an obligate precursor of the 28-methyl sterols, we tested the sensitivity of this reaction to azasterol in vitro. Figure 4 shows that this reduction is competitively inhibited by the antimycotic agent with a calculated apparent K_i of 17 μ M. The K_m of the control reaction was 10 μ M.

Accumulation of total non-saponifiable lipids is shown in Table 1. Cultivation with 75 ng of azasterol per ml resulted in approximately 20% greater accumulation of total sterol than was

FIG. 4. Lineweaver-Burk plots of data generated using two different 24(28)-methylene reductase enzyme preparations. Competitive inhibition of the 24(28)methylene reductase was observed after the addition of azasterol. V, [¹⁴C]ergosterol formed in counts per minute (10⁴). Symbols: (●) controls; (○) azasterol, 122 μ M.

seen in the control culture. The majority of sterol in the inhibitor-treated culture was ignosterol. Accumulation of ignosterol in the treated culture was 23% greater than ergosterol accumulation in a control culture of equal optical density.

Since 8,14-unsaturated sterols do not normally accumulate in yeast cultures, an experiment was carried out to determine if such sterols will serve as substrates for ergosterol synthesis. Accumulation of ^{14}C -labeled ignosterol was forced by cultivating the yeast in the presence of L-[methyl- ^{14}C]methionine and 75 ng of azasterol per ml. When the culture had grown to high cell density, a sample was taken for analysis and the remaining cells were harvested, washed, and suspended in 10 times the original volume of the same medium without azasterol or ^{14}C label. This culture was allowed to grow into stationary phase, and then the cells were recovered. Both the initial culture sample and the final culture were assayed for sterol content. Table 2 shows the changes in mass and ^{14}C label in the major sterol fractions

TABLE 1. Effect of the azasterol on sterol accumulation in strain 3701B grown with glucose as the energy source

Sterol	Control		Azasterol (75 ng/ml)	
	μg	% Total	μg	% Total
Total non-saponifiable lipids	245	100	282	100
Ergosterol	221	90 ^a	0	0
$\Delta^8,^{14}$ Sterols ^b	0	0	273	97

^a The minor sterols were not quantitated in this experiment. Previous studies with this organism have shown them to be principally zymosterol, fecosterol, and lanosterol.

^b Approximately 90% ignosterol.

after transfer to fresh medium. Ergosterol increased from 10% of the total 4-demethyl sterols to 26% in the final culture and ignosterol decreased from 59 to 17%. The unidentified triene fraction was 7% of the initial demethyl sterol. A fraction with the same relative carbon number (gas-liquid chromatography analysis) was recovered from the final culture in a mixture of compounds which were not separable, so a valid mass determination could not be made.

The relative amount of ^{14}C label in the ergosterol increased from 16% of the total 4-demethyl sterol activity of the initial culture to 60%. The radioactivity of the ignosterol band decreased from 42 to 15%. The unidentified triene contained 29% of the initial culture label, and the mixture recovered from the final culture contained 8% of the total 4-demethyl sterol label. These data show that 71% of the recovered radioactivity from the initial culture was associated with $\Delta^8,^{14}$ -unsaturated sterols, and after growth in the absence of inhibitor, 60% of the recovered activity was found in the ergosterol of the final culture. No significant change in total label incorporated in the 4-demethyl sterols was observed after transfer to fresh medium, and no radioactivity was recovered in 4,4-dimethyl sterols or 4 α -methyl sterols from either culture. Although the transfer of label from the $\Delta^8,^{14}$ -sterols to ergosterol has been repeatedly demonstrated, the relative amounts of ignosterol, ergosterol, and the unidentified triene in the initial cultures are somewhat variable with the specific experiment.

Since the azasterol was effective at very low concentrations, we sought to determine the minimum concentration that would produce a measurable effect on sterol synthesis. Because the azasterol causes ignosterol accumulation and severely reduces ergosterol formation, we looked for an increase of ignosterol as a func-

TABLE 2. Conversion of ignosterol to ergosterol *in vivo*^a

Fraction (acetates) ^b	Relative carbon no.	Mass (μg)	Activity (cpm)	% Mass	% Activity
Initial sample total		262	93,811		
Ignosterol	3,584	155	39,743	59	42
Ergosterol	3,559	30	15,338	10	16
Unidentified triene	3,596	19	27,218	7	29
Final culture total		4,212	499,871		
Ignosterol	3,590	718	74,418	17	15
Ergosterol	3,558	1,093	298,930	27	60
Unidentified triene (mixture)	3,598	227	41,562		8

^a Accumulation of ^{14}C -labeled sterols was forced in the initial culture by incubation with 75 ng of azasterol per ml. After a sample of the initial culture was removed, the remaining cells were harvested, washed, and suspended in fresh medium. Sterols were recovered from the initial culture sample and from the final culture, and relative amounts of each fraction and the respective label incorporation were determined.

^b Initial and final culture 4-demethyl sterol acetates.

tion of inhibitor concentration. Both ergosterol and ignosterol produce different characteristic ultraviolet absorption spectra which are readily distinguished. Cultures containing various concentrations of the inhibitor were grown, harvested, and saponified. Absorption characteristics of the non-saponifiable lipid extracts between 210 and 310 nm were recorded. The resulting spectra are illustrated in Fig. 5. A typical spectrum for ergosterol from strain 3701B is seen in the 0.1-ng/ml figure, which is identical to the control. The major peaks due to absorption of the $\Delta^{5,7}$ -conjugated diene system of ergosterol are at 272 and 282 nm. A spectrum typical of cultures treated with relatively high concentrations of azasterol is seen in the 10-ng/ml figure, with an absorption at 250 nm. The azasterol absorbs at 238 nm and can contribute to background absorption, but it is not detectable in the non-saponifiable lipid fractions because of the low inhibitor concentrations used in these experiments. Since the extinction coefficient of ignosterol is significantly higher than that of ergosterol (ϵ 18,000, $\lambda_{\max}$ 250 nm: ignos-

terol; ϵ 11,900, $\lambda_{\max}$ 282 nm: ergosterol) (7, 8), relatively small amounts of ignosterol modify the normal spectrum. This effect can be seen as the appearance of a shoulder at 250 nm on the ergosterol absorption spectrum. The change is clearly evident at 0.5 ng/ml and is discernible in the 0.25-ng/ml spectrum. Gas-liquid chromatography quantitative analysis of the 1.0-ng/ml sample showed the non-saponifiable lipid fraction to be 38% ergosterol and 51% ignosterol.

DISCUSSION

The biphasic growth curves obtained by culturing *S. cerevisiae* with subinhibitory levels of the azasterol indicate that the compound produces an effect that the cells are able to overcome with time. Although we have shown in earlier work (3) that ergosterol synthesis is halted abruptly by addition of the azasterol, this effect does not result in immediate cessation of growth. The addition of azasterol causes accumulation of an unnatural sterol, ignosterol, which has been identified as ergosta-8,14-dien-3 β -ol (Hays et al., *Lipids*, in press), as well as at least one other $\Delta^{8,14}$ -sterol that appears to be an ergostatriene. The conjugated 8,14-diene system is not normally found in yeast sterols. Because extremely small quantities of the inhibitor are used, we have not been able to determine if the azasterol is modified by the culture.

We have observed that under various growth conditions in the presence of 25 nM (10 ng/ml) or higher concentrations of azasterol, ignosterol accumulates to equal or greater amounts than does ergosterol in control cultures. It is also interesting that other naturally occurring sterols (zymosterol and fecosterol) were not observed in the azasterol-treated yeast cultures. Concentrations of the drug as low as 1.2 nM (0.5 ng/ml) have been found to alter the normal pattern of sterol synthesis. Terminal reactions in sterol biosynthesis are shown in Fig. 6. Our data indicate that ignosterol can be converted to ergosterol in vivo when azasterol is removed from the culture. It is apparent that the wild-type yeast possesses enzymes to reduce the Δ^8 - and Δ^{14} -unsaturations. Similar conclusions have been reached previously by others (1, 9, 14). Our results suggest that the azasterol blocks the natural reduction of the Δ^{14} -unsaturation, which has been proposed to result from the C-14 demethylation of lanosterol (14). Failure to carry out this reduction prevents maturation of the B ring from an 8(9)-monounsaturated ring to a 5,7-conjugated diene. However, the sterol methyltransferase and 24(28)methylene reductase (13) are capable of converting

FIG. 5. Ultraviolet absorption spectra of non-saponifiable lipid extracts of cultures grown in the presence of varying amounts of the azasterol. Ergosterol, the normal yeast sterol, absorbs at 272 and 282 nm, as illustrated in the 0.1-ng/ml spectrum. Ignosterol absorbs at 250 nm, as shown in the 10-ng/ml spectrum.

FIG. 6. Proposed pathway illustrating the biosynthesis of ergosta-8,14-dien-3 β -ol as a result of azasterol inhibition of normal sterol biosynthetic enzymes. The normal pathway from lanosterol through zymosterol to ergosterol is mediated by at least four enzymes that are inhibited by the azasterol: ① Δ^{14} reductase; ② Δ^{24} transmethylase; ③ Δ^{22} desaturase; and ④ $\Delta^{24(28)}$ reductase. Transmethylation involves the transfer of a methyl group from S-adenosylmethionine (SAM) to a Δ^{24} sterol substrate to produce S-adenosyl homocysteine (SAH) and a $\Delta^{24(28)}$ sterol product.

the $\Delta^{8,14}$ -intermediate into ignosterol through modification of the side chain. The sterol 22(23)desaturase is also probably very sensitive to azasterol, but we have been unable to develop a satisfactory assay for this enzyme to test this possibility.

At relatively high concentrations, azasterol inhibits the sterol methyltransferase enzyme *in vitro* (3). We also have shown here inhibition of the sterol 24(28) reductase. Since ignosterol contains the C-28 methyl group, the low levels of azasterol used to cause accumulation of ignosterol do not block transmethylation or methylene reduction *in vivo*. Based on our *in vitro* experiments, it seems logical that the 24(28)methylene reductase would be more sensitive to azasterol than the methyl transferase.

Kabara et al. (12) have tested a variety of synthetic azasterols for activity against several organisms, including *S. cerevisiae*. Of the compounds tested, the lowest MIC was 2.5 μ g/ml for their 23-azacholesterol. However, their procedure required observation of an initial 5×10^5 -cells/ml culture for an increase in culture turbidity after only 18 h, which, based on our experiments, may be insufficient time to allow recovery from a temporary drug-induced lag in growth of the yeast. We observed that 9 μ g of the 15-azasterol per ml, in broth with ethanol as the energy source, prevents observable

growth if the broth is inoculated to 10^4 cells/ml, but culture density increases sixfold during the first 15 to 20 h at a rate similar to that of the control if inoculated to 10^6 cells/ml. Inoculation to 10^5 cells/ml produced a slight increase in density, which was observable only after 24 h. However, in no case does this concentration allow the wild-type culture to attain a final density equal to that of the control. These data indicate that the relative amount of drug per cell in the test medium and the initial cell concentration, especially in turbidimetric measurements, may bias the observable MIC. We have based our MIC determinations upon observations taken after several days of incubation with an initial inoculation sufficient to allow monitoring of changes in culture turbidity. This procedure allows us to determine the minimal concentration of a drug that will prevent culture growth over an extended period, as well as concentrations producing only temporary inhibitions.

The 23-azacholesterol MIC (12) is similar to the reported MIC of 25-azazymosterol (5 to 10 μ M; between 1.9 and 3.8 μ g/ml) (2). Significantly, we have found that the 15-azasterol used here produces long-term inhibition at the much lower concentration of 250 ng/ml (0.61 μ M) and short-term inhibition at concentrations as low as 50 ng/ml (120 nM). This azasterol may be

10 to 50 times as effective as other known azasterols and is a more effective fungal inhibitor than the polyene antimycotics.

We have found that the 15-azasterol studied here has at least three specific inhibitory effects upon yeast sterol synthesis. The most sensitive effect appears to be a block of the reduction of the Δ^{14} -unsaturation, which results from demethylation at the C-14 position. At low drug concentrations, C_{28} sterols containing the $\Delta^{8,14}$ -conjugated diene system accumulate. The azasterol is a competitive inhibitor in vitro of the Δ^{24} -sterol transmethylase enzyme (K_i , 43 μ M) and prevents transmethylation in vivo (3). We have also demonstrated here inhibition of the 24(28)methylene reductase. As little as 5×10^{-10} g/ml of broth (1.2 nM) of the azasterol causes accumulation of the unusual $\Delta^{8,14}$ -sterols. We feel that the growth-inhibitory effects of this azasterol result from the cumulative effects of several modifications of the sterol synthetic pathway, which result in the production of an unacceptable sterol for the cells.

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