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Leo W. Parks

The effects of 15-aza-24-methylene-8,14-homocholestadien-3β-ol on growth and sterol synthesis in S. cerevisiae were determined. Azasterol concentrations equal to 0.01% of the ergosterol content of the culture modified sterol synthesis. Biphasic growth curves were seen when the antimycotic was present in amounts equal to one percent of culture sterol. When equal to less than 20% of the ergosterol present, growth was inhibited.

The 15-azasterol was a very effective inhibitor of ergosterol synthesis, and caused accumulation of an unusual sterol, ergosta-8,14-dien-3β-ol, in amounts equal to the normal cellular sterol content. The inhibitor appears to affect primarily the yeast sterol 14-reductase enzyme. In vitro inhibition of the sterol 24-methyltransferase was
demonstrated with an apparent $K_i$ of 39 $\mu$m. In vivo accumulation of the 8,14-diene and the subsequent conversion to ergosterol was demonstrated.

Several isomers and analogs of the 15-azasterol were tested for in vitro inhibition of the sterol methyltransferase. The 14(15) carbon-nitrogen double bond was seen to be the most important characteristic of the inhibitors, followed by the 8(9) unsaturation. Reduction of the D-ring nitrogen eliminated the effectiveness of the compounds as inhibitors.
The Physiological Effects of an Antimycotic Azasterol in Saccharomyces cerevisiae

by

Phillip Roy Hays

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I would like to thank Drs. A. C. Oehlschlager and H. D. Pierce, Jr., for their Nuclear Magnetic Resonance studies of ergosta-8,14-dien-3β-ol, and Dr. G. W. Patterson for supplying samples of this compound for comparative analysis.

I appreciate the valuable assistance provided by the individuals and groups listed above, but it is to Professor Leo Parks that I owe my sincerest gratitude for his support and encouragement in this project.
# TABLE OF CONTENTS

I. Introduction .................................................. 1

II. Materials and Methods ........................................ 9
    Organism and culture conditions .......................... 9
    Growth inhibition studies ................................. 10
    Sterol biosynthetic studies *in vivo* ................... 11
    Sterol methyltransferase studies *in vitro* .......... 13
    Sterol extraction, purification and identification .... 16

III. Results and Discussion ...................................... 18
    Agar inhibition tests ...................................... 18
    Broth inhibition tests .................................... 20
    Azasterol effects on sterol synthesis ................... 25
    Azasterol minimum effective concentration ............. 28
    Ignosterol identification ................................. 29
    Conversion of ignosterol to ergosterol *in vivo* ...... 32
    Sterol methyltransferase *in vitro* assay ............... 35
    Azasterol analog studies .................................. 42

IV. Conclusions .................................................. 45

Bibliography .................................................... 54
**LIST OF ILLUSTRATIONS**

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>The structural similarity of the 15-azasterol and fecosterol</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>The numbering system in sterols</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>Sterol biosynthesis in <em>S. cerevisiae</em></td>
<td>5</td>
</tr>
<tr>
<td>4</td>
<td>Ergosta-8,14-dien-3β-ol</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>Azasterol effects on culture growth in broth with ethanol as the carbon source</td>
<td>21</td>
</tr>
<tr>
<td>6</td>
<td>Azasterol effects on culture growth in broth with dextrose as the carbon source</td>
<td>23</td>
</tr>
<tr>
<td>7</td>
<td>Azasterol inhibition of sterol synthesis in aerobically adapting cells</td>
<td>27</td>
</tr>
<tr>
<td>8</td>
<td>Ultraviolet absorption spectra of nonsaponifiable lipid extracts of cultures grown in the presence of varying amounts of the azasterol</td>
<td>30</td>
</tr>
<tr>
<td>9</td>
<td>Lineweaver-Burk plot of the azasterol's inhibition of the sterol methyltransferase in vitro</td>
<td>41</td>
</tr>
<tr>
<td>10</td>
<td>Structures of the azasterol A25822B and four analogs</td>
<td>44</td>
</tr>
<tr>
<td>11</td>
<td>Proposed pathway illustrating the biosynthesis of ergosta-8,14-dien-3β-ol as a result of azasterol inhibition of normal sterol biosynthetic enzymes</td>
<td>50</td>
</tr>
</tbody>
</table>
THE PHYSIOLOGICAL EFFECTS OF AN ANTIMYCOTIC
AZASTEROL IN SACCHAROMYCES CEREVISIAE

I. INTRODUCTION

Antimycotic compounds are chemicals which cause detrimental effects in fungi. The known medically important antimycotics are all sufficiently toxic to humans to restrict their use to potentially fatal infections only. Furthermore, the common antimycotics do not necessarily kill the fungal pathogen, but often merely prevent further growth as long as the antibiotic is present. Naturally occurring antifungal antibiotics are uncommon, and are of particular interest to medical mycologists. The research described in this thesis was concerned with the characterization of the antifungal activity of a new antimycotic.

Azasterols are compounds similar in structure to the common animal sterol, cholesterol, with the addition of one or more nitrogen atoms. Studies of the effects of azasterols on bacterial growth and yeast sterol synthesis have been reported (21,4). The particular azasterol used in this research was 15-aza-24-methylene-8,14-homocholestadien-3β-ol (11). The structure of this compound, referred to hereafter as azasterol or 15-azasterol, is shown in Figure 1 compared to a common fungal sterol,
Figure 1. The structural similarity of the 15-azasterol and fecosterol.
fecosterol. Details of the discovery, isolation and characterization, and general biological activity of the 15-azasterol have been reported (9,24,16). It was the similarity of structures of the azasterol and fecosterol that prompted the investigation of the effects of yeast sterol biosynthesis caused by the antibiotic.

The numbering system in sterols is illustrated in Figure 2, with the common sterols cholesterol and ergosterol shown as examples. Table 1 lists the trivial names and chemical names of the sterols mentioned in this thesis. The azasterol nitrogen in the D ring is at position 15, with the two carbons following numbered 16 and 17, respectively. The carbon to which the side chain attaches is numbered 17a, thereby preserving the numbering shown in Figure 2 in the remainder of the molecule.

The fungal biosynthesis of sterols proceeds directly from acetyl-coenzyme A through a series of condensations to the straight-chain unsaturated intermediate, squalene (I in Figure 3). This compound is then converted into the first cyclic intermediate, lanosterol (III), a 30-carbon sterol intermediate. Conversion of lanosterol into ergosterol (XII), the final product of fungal sterol biosynthesis, involves three demethylations of the cyclic portion of the molecule, a methylation in the side chain, and several rearrangements of the unsaturations in the
Figure 2. The numbering system in sterols.

TABLE 1. YEAST STEROL NOMENCLATURE

<table>
<thead>
<tr>
<th>Trivial name</th>
<th>Chemical name</th>
<th>Figure number</th>
</tr>
</thead>
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<tr>
<td>Lanosterol</td>
<td>4,4,14-trimethyl-cholesta-8,24-dien-3β-ol</td>
<td>III</td>
</tr>
<tr>
<td>Zymosterol</td>
<td>cholesta-8,24-dien-3β-ol</td>
<td>VII</td>
</tr>
<tr>
<td>Fecosterol</td>
<td>ergosta-8,24(28)-dien-3β-ol or 24-methylene-cholesta-8-en-3β-ol</td>
<td>VIII</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>ergosta-5,7,22-trien-3β-ol</td>
<td>XII</td>
</tr>
<tr>
<td>Ignosterol</td>
<td>ergosta-8,14-dien-3β-ol</td>
<td></td>
</tr>
<tr>
<td>Azasterol</td>
<td>15-aza-24-methylene-8,14-homocholestadien-3β-ol</td>
<td>Figure 1</td>
</tr>
</tbody>
</table>
Figure 3. Sterol biosynthesis in *S. cerevisiae*. 
molecule. It should be noted that the cyclization of squalene requires molecular oxygen (27,35). In addition, oxygen is required for the demethylations at C-4 (2,32). These phenomena are commonly utilized to force the accumulation of squalene during anaerobic growth, with the resulting culture being depleted of sterol content (22). The synthesis of sterols in fungi and animals apparently proceeds by similar pathways up to the sterol intermediate lanosterol (III) (27). Animal sterol synthesis involves the conversion of 30-carbon lanosterol into 27-carbon cholesterol through demethylations at C-4 and C-14 and the rearrangement of the unsaturations in the molecule. Fungi convert lanosterol into 28-carbon ergosterol with the addition of a methyl group at position 28 in the side chain, demethylations at C-4 and C-14, and subsequent rearrangements of the unsaturations in the molecule. The order of the demethylations and the transmethylation varies in different fungi (27), and the pathway in S. cerevisiae is illustrated in Figure 3. Sterol synthesis in higher plants and algae is similar to fungal sterol synthesis up to the intermediate squalene (27). Beyond this point the pathways diverge, leading to different final sterol products. Of particular interest with respect to the work presented in this thesis are the fungal demethylation of lanosterol (III) at carbon 14 and the transmethylation from S-adenosyl-
methionine (SAM) to zymosterol (VII) to produce fecosterol (VIII).

The demethylation of 24-dihydrolanosterol at carbon 14 by rat liver homogenates has been reported to result in the "obligatory formation" of 4,4-dimethylcholesta-8,14-dien-3β-ol, and evidence was given that the product could not be the 7,14-diene (34). However, it has been demonstrated that this demethylation will occur in sterol substrates having an unsaturation at position 7(8) to produce 7,14-dienes (2). The accumulation of 8,14-diene sterols has been demonstrated in green algae as the result of treatment of the culture with a hypocholesterolemic drug (12). Conversion of synthetic ergosta-8,14-dien-3β-ol (Ignosterol, Figure 4) into ergosterol by the yeast Saccharomyces cerevisiae has been reported (1).

Transmethylation from the donor SAM to the sterol zymosterol to form fecosterol and S-adenosylhomocysteine has been studied in detail in yeast systems (6,25,26,29,33). All of the sterol methyltransferase studies published to date have utilized enzyme preparations from aerobically grown cultures.

In this thesis I will present data describing the physiological effects produced by the 15-azasterol in cultures of Saccharomyces cerevisiae, and demonstrate that the primary effect of the antimycotic appears to be the inhibition of ergosterol synthesis.
Figure 4. Ergosta-8,14-dien-3β-ol.

IGNOSTEROL
II. MATERIALS AND METHODS

Organism and culture conditions.

*Saccharomyces cerevisiae* strain 3701B, a haploid, of a mating type carrying a uracil auxotrophic marker, was used in these experiments. Cultures were grown in broth or 1.5% agar medium containing 1% tryptone, 0.5% yeast extract, and 2% carbon source (dextrose or ethanol), at 27-30°C, except as noted. Aerobic cultures were grown either in Erlenmeyer flasks with rotary shaking, in screw-cap culture tubes with reciprocating shaking, or in five-gallon carboys with rapid airflow bubbling through fritted-glass gas dispersion tubes into the liquid. The airflow was "sterilized" by passing through sterilized glass wool in a two-liter flask. The caps on screw-cap tubes were left untightened and taped into place to allow airflow into the tubes. Anaerobic cultures were incubated without shaking in flasks or carboys fitted with bunsen valves. The anaerobic broth medium was allowed to cool after autoclaving with argon bubbling through the liquid to reduce the amount of dissolved oxygen in the broth. The initial inoculum was maintained on agar slants at 4°C, and all subsequent cultures were started from this inoculum. One-liter aerobic starter cultures were used to inoculate the 18-liter carboy cultures.
The carbon source of the starter cultures was the same as in the larger cultures.

Growth inhibition studies.

The azasterol minimum inhibitory concentration (MIC) in agar was determined by spreading 0.1 ml of a 2000 cell/ml inoculum onto agar plates containing the appropriate azasterol dilutions in the agar. Azasterol was prepared as a solution in 95% ethanol and was added to precooled 50°C agar. The agar was immediately mixed and poured into sterile petri dishes. Ethanol concentration in the agar was 2% if no dextrose was added or 1% if 2% dextrose was in the agar.

Percent petite colony composition was determined by reduction of 2,3,5-triphenyltetrazolium chloride dye in soft agar overlays (28).

Culture cell density was determined using a Coulter model F cell counter (100µ aperture) and optical density was measured with a Klett-Summerson photoelectric colorimeter (filter wavelength 540 nm). An optical density of 20 Klett units was approximately equal to 2 x 10^6 cells/ml.

Azasterol MIC in broth was assayed by inoculating with logarithmic phase inocula to produce an increase in optical density of 20 Klett units, and optical density of the
cultures was recorded with time. The inoculum was cultivated with the same carbon source used in the subsequent growth studies. Azasterol was added to the broth in 95% ethanol, with the final ethanol concentration 2% if no dextrose was added and from 0.1% to 1% in dextrose broth. Cultures were incubated in 13x150mm screw cap tubes.

Sterol biosynthetic studies in vivo.

Aerobic adaptation experiments were conducted by growing anaerobic cultures in YMAF broth (0.1 M phosphate buffer (0.927% KH$_2$PO$_4$ and 0.556% K$_2$HPO$_4$) with 0.1% NH$_4$Cl and 2% dextrose). The stationary phase anaerobic cultures were allowed to settle overnight at 4°C and were then harvested by centrifugation. The cells were washed in ice water and again centrifuged 3000xg for one minute. The cell pack was resuspended in 0.1 M phosphate buffer with 1% dextrose, and azasterol in ethanol was added to the test culture to give 200 μg/ml and 1% ethanol. Ethanol only was added to the control culture. Culture samples were taken periodically and saponified to extract the sterols.

The conversion of ergosta-8,14-dien-3β-ol to ergosterol in vivo was followed by first forcing the accumulation of the 8,14-diene by incubating the cultures with 75 nanograms/ml azasterol in dextrose broth. The sterols produced were
labeled by adding L-[methyl-\textsuperscript{14}C]\textsuperscript{m}ethionine (0.1 \mu Ci/ml, 2 \mu M) to the broth. The culture was harvested by centrifugation and washed twice with 4\degree C distilled water. A sample of this initial culture was withdrawn and the remaining cells were added to fresh broth lacking both the azasterol and the \textsuperscript{14}C label. Sterols were extracted from both the sample of the initial culture and the final culture. After separation of the sterols by thin-layer chromatography (TLC) (5), the radioactive bands on the plates were located with a radiochromatogram scanner. The free sterols were esterified overnight in pyridine-acetic anhydride (1:2 v/v) at room temperature, and the 4-demethyl steryl acetates were further purified on silica gel TLC plates impregnated with a 10\% solution of silver nitrate in methanol-water (3:1 v/v). The fractions were analyzed by gas chromatography and were counted in a liquid scintillation counter using PPO-POPOP toluene cocktail (3g 2,5-diphenyloxazole (PPO) and 0.1g 1,4-bis-[2-(5-phenyloxazolyl)]-benzene (POPOP) in 1L toluene). Specific procedures for extraction, purification, and identification of sterols are detailed later in this thesis.
Sterol methyltransferase studies in vitro

Although several yeast sterol methyltransferase preparation and assay procedures have been published (6, 26, 29) the procedures used in these experiments were sufficiently different to justify a detailed description. Both aerobic and anaerobic cultures were used for the isolation of the partially purified enzyme, and the preparation procedures differ only slightly for the two culture types. Ethanol was the carbon source for aerobic cultures and dextrose was used in the anaerobic experiments. Aerobic cultures were processed at room temperature, but anaerobic preparations were carried out at 4°C under argon to limit oxygen-dependent sterol synthesis in the cell fractions. Tris(hydroxymethyl)aminomethane-hydrochloride (Tris HCl) buffer (0.1 M, pH 7.5) was used throughout the enzyme preparations and assays. Eighteen-liter cultures were allowed to settle overnight at 4°C, and most of the supernatant was decanted. The cells were resuspended and harvested by centrifugation at 3000xg for one minute. The cell pack was resuspended in buffer to give 0.5 g/ml cells, and this suspension was treated for 30 seconds in a Braun homogenizer with liquid CO₂ cooling, using 0.25 mm glass beads. Unbroken cells and heavy cell debris were pelleted by two successive centrifugations at 3000xg for five
minutes. Mitochondria from aerobic cultures were collected by centrifugation at 8000xg for 20 min.; the promitochondria from anaerobic cultures were sedimented at 16000xg for 20 min. These pellets were washed in buffer and again were centrifuged, first at 3000xg, and this supernatant was centrifuged at 8000xg or 16000xg as was appropriate. The pellet was resuspended in buffer and sonicated in a model W140C Branson Ultrasonic cell disruptor three times (20 seconds each) at 80 watts output using an ice bath for cooling. The sonicated material was again centrifuged at 8000xg or 16000xg to remove unbroken particles, and this supernatant was centrifuged at 100,000xg for 60 min. The resulting pellets were resuspended in buffer and constituted the partially purified enzyme preparation. The aerobic preparations were resuspended to 15 mg protein/ml and used for the assays. The anaerobic preparations were deionized in Sephadex G75 coarse columns (bed volume approximately four to five times the protein suspension volume) at 4°C, and then diluted to 5 mg protein/ml. Protein assay was by the method of Lowry et al (23).

Assays of the aerobic enzyme preparations were essentially the same as have been previously reported except that 3 ml reaction volumes were used (6). The anaerobic preparations were assayed in a similar manner, but either S-adenosylmethionine or zymosterol was used as the variable
substrate in experiments to determine reaction kinetics. Sterols were added to the reaction mixtures in 95% ethanol except as otherwise noted. Aerobic preparation assays contained magnesium chloride (4 µM), but the MgCl₂ was omitted from the anaerobic preparation assays.

Anaerobic culture methyltransferase assay reaction mixtures were typically 6.7% ethanol with 1.7 mg protein/ml. SAM \( K_m \) determinations were made at the zymosterol concentration of 300 µM. Zymosterol \( K_m \) was determined at a SAM concentration of 500 µM. Azasterol was added to a final concentration of 40 µM. Azasterol inhibition was determined at a zymosterol concentration of 22 µM and SAM concentrations from 10 to 200 µM.

Reaction tubes (25 x 150 mm) were incubated with shaking at 27°C for 45 min. The assay was stopped by adding one-half the reaction volume of 5% trichloroacetic acid. The protein pellet was collected by centrifugation and the supernatant discarded. The reaction mixtures were dissolved in 0.5 ml dimethyl sulfoxide at 100°C for 60 min. Water (5 ml) was added to trap hydrophilic compounds, and the sterols were extracted by mixing three times with nine ml n-hexane. Methanol (0.5 ml) was added to each extraction to separate the resulting emulsion. The hexane was segregated and evaporated, and labeled sterols were detected by liquid scintillation methods.
Sterol extraction, purification and identification

Sterols were extracted from cultures by methanolic saponification (cell pellets were refluxed in 5% potassium hydroxide and 0.5% pyrogallol in 50% methanol for more than two hours, and the resulting mixture was shaken with n-hexane), and purification of the hexane extracts was accomplished with thin-layer chromatography (silica gel 60 F254) (5). Sterol identification and quantitation were determined by gas chromatography and the modified Lieberman-Burchard colorimetric assay (22). Gas chromatography was performed on a six foot by 2 mm inside diameter 1% OV-17 glass column with 100/120 mesh Chromosorb WHP support at 275°C in a Varian 2740 gas chromatograph equipped with a flame ionization detector. Carrier gas was prepurified nitrogen at a flow rate of 25 ml/min. Data accumulation and processing was performed by a Varian CDS 111 electronic data processor. Lieberman-Burchard assays were carried out in a Spectronic 20 colorimeter and ultraviolet absorption spectra were determined with a Cary model 11 recording spectrophotometer. Absorption maxima were determined in a Zeiss PMQ II spectrophotometer. Mass spectra were determined as described by Bailey and Parks (5).

Zymosterol was prepared by extraction from mutant S. cerevisiae strain 8R1, using a 30 minute steaming of the
cell pack in 0.1N HCl followed by methanolic saponification. After extracting with n-hexane and drying, the non-saponifiable lipid fraction was dissolved in acetone-absolute ethanol (1:1 v/v) and an equal volume of digitonin solution (4.8 mg/ml in ethanol-water 1:1 v/v) was added to precipitate the sterol digitonides. The digitonides were allowed to settle for 22 hours at 4°C and were then harvested by centrifugation (1000xg, 15 min.). The digitonides were washed twice with diethyl ether-acetone (1:1 v/v) and then twice with diethyl ether. The sterols were recovered from the digitonides by steaming (100°C) for three hours in dimethyl sulfoxide and extracting with repeated hexane washings (18). The sterols were purified on 2.0 mm silica-gel 60 F-254 plates using a filter-paper wick with cyclohexane:ethyl acetate (85:15) solvent running overnight. The zymosterol recovered was determined to be at least 98% pure by gas chromatographic analysis.

S-adenosyl-L-[methyl-14C]methionine (SAM) was a product of Amersham Searle Corp., and unlabeled SAM was obtained from Boehringer-Mannheim. Purity of the labeled SAM solution used in the assays was tested by paper chromatography (31). No S-adenosylhomocysteine was detected. The azasterols were products of the Lilly Research Laboratories. Synthetic ergosta-8,14-dien-3β-ol was a gift of G. W. Patterson.
III. RESULTS AND DISCUSSION

At the beginning of the investigations recounted in this thesis little was known about the antifungal activity of the 15-azasterol. A mixture of 15-azasterols was isolated from the mold *Geotrichum flavo-brunneum* at the Lilly Research Laboratories, and the principal active component, designated A25822B, was determined to be 15-aza-24-methylene-8,14-homocholestadien-3β-ol (11). The Minimal Inhibitory Concentration (MIC) of this compound for several pathogenic fungi had also been reported (16). It is this particular 15-azasterol which was used in the following experiments, unless otherwise stated.

Agar inhibition tests

The initial tests were to determine the MIC of the azasterol for the test organism, *Saccharomyces cerevisiae* strain 3701B. The results of these experiments, using azasterol concentrations from 0 to 18 ng/ml in agar, are shown in Table 2. The low concentration of 8 ng/ml (19.45 nM) was found to inhibit growth on agar having ethanol as the carbon source, and only approximately 7% of the colonies developed on glucose agar at this concentration.
### TABLE 2. AZASTEROL INHIBITION OF CULTURE GROWTH ON AGAR

<table>
<thead>
<tr>
<th>Azasterol concn (ng/ml)</th>
<th>Colonies/plate</th>
<th>Avg colony size (mm)&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2% Glucose</td>
<td>2% Ethanol</td>
</tr>
<tr>
<td></td>
<td>2% Glucose</td>
<td>2% Ethanol</td>
</tr>
<tr>
<td>0</td>
<td>175</td>
<td>104</td>
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<tr>
<td>2</td>
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<tr>
<td>18</td>
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<td>0</td>
</tr>
</tbody>
</table>

The numbers in parentheses indicate the percent petite colony types observed.

Concentrations of 4 ng/ml and less had no effect. At greater than 8 ng/ml a few colonies persisted on glucose agar. These resistant colonies persisted until concentrations as high as 200 ng/ml were used, above which no colonies grew from the stock 3701B culture. About 14% of the colonies on the glucose plates were found to be respiratory deficient petites incapable of reducing the dye 2,3,5-triphenyltetrazolium chloride in soft agar overlays. No petite colonies survived at concentrations of 8 ng/ml or greater.
Broth inhibition tests.

Liquid medium experiments were first conducted in ethanol broth. Four sets of tubes were prepared from the same large batch of broth, containing 52, 9, 0.032 and 0 \( \mu \text{g/ml} \) azasterol respectively. Serial dilutions from a log phase inoculum were made in each set to give cell concentrations of \( 10^6 \), \( 10^5 \) and \( 10^4 \) cells/ml at each azasterol concentration. Growth was followed by measuring culture optical density. The results of the \( 10^6 \) cells/ml inoculated cultures are shown in Figure 5. All control cultures obtained the same final optical density regardless of initial cell concentration. No growth was seen in any of the tubes containing 52 \( \mu \text{g/ml} \) azasterol. In the cultures with 9 \( \mu \text{g/ml} \) azasterol there was initial growth at a rate slightly slower than the control (G=430 min. and 400 min. respectively) in the two more heavily inoculated cultures, but growth ceased after about three generations. No growth was observed in the \( 10^4 \) cells/ml inoculated tube. All three cultures with 32 ng/ml azasterol obtained the same final optical density as the control cultures, but an intermediate slowdown of growth was observed in the \( 10^6 \) cells/ml inoculated tube. Similar biphasic growth curves were not observed in the cultures inoculated to lower starting optical densities, but these tubes did not
Figure 5. Azasterol effects on culture growth in broth with ethanol as the carbon source.
initially exhibit measurable density. Any modulation of the typical growth curve in these tubes may have happened before the cultures obtained measurable density. It was also observed that the more heavily inoculated tubes grew to a given optical density approximately one day before the same density was reached by tubes of the next lower inoculum concentration. This was observed in both the controls and in the 32 ng/ml tests. From these results it was apparent that the measured broth MIC was dependent upon the initial culture density, the initial azasterol/cell concentration and the time allowed for culture growth before "inhibition" was scored. The tubes containing 9 
\( \mu g/ml \) illustrate the problem; after 18 hours incubation, the culture inoculated to \( 10^4 \) cells/ml exhibited no growth while the \( 10^5 \) cells/ml inoculated culture showed slight growth (this tube was initially below measurable optical density) and the \( 10^6 \) cells/ml inoculated tube had the same optical density as the similarly inoculated control culture. In addition, growth in the 32 ng/ml tube initially inoculated to \( 10^6 \) cells/ml was obviously effected but this tube eventually obtained the final density of the control tubes.

The experiment was repeated using dextrose broth and azasterol concentrations of 0, 0.025, 0.05, 0.1, 0.25, 1.0, 10, and 50 \( \mu g/ml \). All tubes were inoculated with a log phase culture with sufficient cells to increase optical
Figure 6. Azasterol effects on culture growth in broth with dextrose as the carbon source.
density by 20 Klett units. This procedure ensured that approximately $2 \times 10^6$ cells/ml were added to each tube, and each tube was initially at an optical density which could be measured. Results similar to the ethanol tests were observed, as illustrated in Figure 6. Biphasic growth curves were observed at azasterol concentrations between 25 and 100 ng/ml. The 0.25 to 10 μg/ml curves were similar to the 9 μg/ml growth curve with ethanol carbon source, and 50 μg/ml again prevented all growth.

The results from these two experiments indicate that on either carbon source between $1.25 \times 10^{-14}$ gram azasterol per cell at inoculation adversely affects cell growth (biphasic growth curves), but the culture is able to overcome the inhibitory effect and achieve a final cell density equal to the control. Concentrations between $1.25 \times 10^{-13}$ and $9 \times 10^{-12}$ gram per cell result in premature cessation of growth at culture densities below that of the control, while 2.5 to $5 \times 10^{-11}$ g/cell prevents all growth. It has been found in subsequent experiments that 1 μg/ml azasterol will prevent growth of strain 3701B in dextrose or ethanol broth if the inoculum does not make the broth visibly turbid (about 10-20 Klett units). Concentrations as low as $1.25 \times 10^{-13}$ g/cell are sufficient to prevent continued growth of the test strain in broth.

One explanation for the biphasic growth curves could be that the more resistant organisms observed on the dextrose
agar plates were growing after the majority of the cells had ceased growth. Cells from the dextrose broth control and cells that had been cultured with 50 ng/ml of azasterol were reinoculated to the optical density of 20 Klett units into fresh dextrose broth with 0, 50, 100, and 250 ng/ml azasterol. Growth in the two sets of cultures was essentially the same, indicating that selection pressure for a more resistant population of cells, relative to the control, had not occurred in the original culture containing azasterol, even though these cells were "preconditioned" to the inhibitor.

Azasterol effects on sterol synthesis.

To determine the effect the azasterol had on total cellular sterol synthesis, aerobic adaptation experiments were conducted. First, anaerobic cultures were grown in YMAF broth until gas evolution ceased. These cells were then carefully harvested at 4°C to prevent sterol synthesis during the centrifugations and washings. These cells were then resuspended in 0.1M phosphate buffer with 1% dextrose and incubated with aeration. The test culture contained 200 μg/ml azasterol. The absence of a nitrogen source prevents significant growth under these conditions, but most of the squalene accumulated in the anaerobic cells is
converted into sterols upon aeration. The accumulation of sterols was followed using the modified Lieberman-Burchard colorimetric assay (22). Samples were taken at intervals after initiation of aerobic conditions and assayed for sterols. Sterol accumulation was prevented by the high concentration of azasterol (Figure 7). The experiment was repeated using 200 ng/ml and 200 \( \mu g/ml \) during aeration, and after three hours the cultures were harvested and the sterols extracted. After purification on thin-layer silica gel plates the sterol fractions were analyzed by gas chromatography. The major compounds recovered in all three cultures were 4-demethyl sterols: ergosterol from the control culture and a different compound from the cultures containing azasterol. The total amounts of these compounds were approximately the same in all three cultures.

An experiment was conducted to attempt to detect ergosterol in azasterol treated cultures and to follow sterol accumulation in these cultures. Dextrose broth containing 50 ng/ml azasterol was inoculated to 24 Klett units optical density from a log phase culture. This culture was then distributed to sterile culture tubes and incubated with shaking. Optical density was followed with time, and at intervals three tubes were removed, and growth was stopped with trichloroacetic acid. These samples were extracted and the sterol content determined. No ergosterol was detected, and accumulation of the unidentified sterol
Figure 7. Azasterol inhibition of sterol synthesis in aerobically adapting cells. Symbols: (O) control; (●) azasterol, 200 µg/ml.
was found to closely parallel growth. The culture did exhibit the biphasic growth seen earlier for 50 ng/ml cultures.

Cultures were grown aerobically in dextrose broth containing 75 ng/ml azasterol and L-[methyl-\(^{14}\)C]methionine (0.1 \(\mu\)Ci/ml, 2 \(\mu\)M). Under these conditions some of the labeled methionine will be converted to S-adenosylmethionine by the cells and used to transmethylate sterols by the addition of a methylene to the C-24 position in the sterol side chain (30). The compound recovered from the 4-demethyl sterol fraction on thin-layer chromatography plates was labeled, indicating that a 28 carbon sterol was being accumulated in these cultures.

Azasterol minimum effective concentration.

Ultraviolet absorption spectra of the sterols from the azasterol treated cultures revealed a single absorption peak at 250 nm. Ergosterol from untreated cultures produces two strong absorption peaks at 272 nm and 282 nm. Since the unidentified sterol from the azasterol treated cultures produced an ultraviolet absorption spectrum different from those of sterols normally accumulating in yeast this characteristic was utilized to determine the minimum detectable effective concentration in strain 3701B.
Cultures were grown in dextrose broth at azasterol concentrations of 0, 0.1, 0.25, 0.5, 0.75, 1.0, and 10 ng/ml. Sterols were extracted from these cultures and the UV absorption spectra of the whole-cell extracts were recorded from 210 to 310 nm as illustrated in Figure 8. The absorption spectrum of ergosterol is seen in the 0.1 ng/ml culture, which was identical to the control culture spectrum. The absorption spectrum of the unknown sterol is shown in the 10 ng/ml test. As the azasterol concentration was increased the absorption peak due to the unidentified sterol became evident at 0.5 nanograms azasterol/ml (1.2 nM), and changes appear to be seen in the 250 picogram/ml sample. The azasterol exhibits a UV absorption maximum at 238 nm, but is not seen in these spectra because of the very small amounts used in these tests. Quantitative gas chromatographic analysis of the 1.0 ng/ml sample showed the non-saponifiable lipid fraction to be 38% ergosterol and 51% unidentified sterol.

Ignosterol identification.

Identification of the unusual sterol, which was called ignosterol (for want of a better name), was accomplished by analyzing data from UV spectroscopy, $^{14}C$ labeling from methionine, mass spectroscopy and nuclear magnetic resonance
Figure 8. Ultraviolet absorption spectra of non-saponifiable lipid extracts of cultures grown in the presence of varying amounts of azasterol.
(NMR) spectroscopy. The UV absorption peak at 250 nm has been reported for sterols having the conjugated 8(9),14-diene system (12,13). Mass-spectral analysis of ignosterol gave a molecular ion peak at m/e 398 (100%) and strong peaks at 383 (70%), 365 (32%), and 271 (22%), which correspond to the empirical formula C_{28}H_{46}O and to loss of -CH$_3$, -CH$_3$+H$_2$O, and a saturated C$_9$H$_{19}$ side chain, respectively. These data indicated that the compound was an ergostadiene with a saturated side chain and a conjugated 8,14-diene system in the cyclic portion of the molecule. A sample of synthetic ergosta-8,14-dien-3β-ol was kindly provided by Prof. G. W. Patterson (12) and the mass spectrum of this compound was virtually identical to that of ignosterol. NMR spectroscopy was performed by H. D. Pierce, Jr. and A. C. Oehlschlager on both the synthetic 8,14-diene and ignosterol. The two spectra were virtually identical with C-18 and C-19 methyl resonances at δ 0.82 and 0.99, respectively. In addition, a one proton absorption at δ 5.33 agrees with a published δ 5.4 absorption for the olefinic hydrogen of the 8,14 conjugation. These values are close to those predicted for the 8,14-diene (8) and are similar to reported values for other 8,14-dienes (7). The NMR results support the 8,14-diene structure as opposed to the possible 7,14-diene, and the combined data indicate the structure of ignosterol is ergosta-8,14-diene-3β-ol.
Conversion of ignosterol to ergosterol in vivo.

Conversion of 8,14-dienes to other sterols has been reported in vitro with rat liver homogenates (34) and in vivo in S. cerevisiae (1). To determine whether strain 3701B could convert ignosterol into other sterols in vivo, cultures were first grown aerobically in dextrose broth with 75 ng/ml azasterol and L-[methyl-14C]methionine (0.1 μCi/ml, 2 μM) to cause accumulation of labeled ignosterol. These cells were harvested and washed to remove exogenous methionine and azasterol, one-fifth of the cells were saved for extraction and the remaining cells were resuspended in fresh broth and allowed to grow to stationary phase. Sterols were extracted and purified from the initial sample and the final culture and were identified by gas chromatography and UV spectroscopy. The incorporated 14C label was determined by liquid scintillation spectroscopy. Three labeled bands from the initial sample were revealed by radiochromatogram scans of the thin-layer chromatograms used for purification of the sterols. The major fraction was identified as ignosterol, and the minor labeled compound appeared to be ergosterol. The third band contained 29% of the label but only 7% of the mass of nonsaponifiable lipids from this sample. This compound exhibited UV absorption for an 8,14-diene, and had a mass
spectrum of an ergostatriene (acetate molecular ion m/e 438, doubly unsaturated acetate nucleus with loss of the side chain and one hydrogen m/e 312), but enough of this sterol has not been recovered to allow positive identification. It appears to be an ergosta-8,14,?,-triene with the third unsaturation in the side chain, possibly the 8,14,22-triene or the 8,14,24(28)-triene. Interestingly, both of the 8,14 unsaturated compounds appear to lose label in the final culture, while ergosterol gains the majority of the activity. The data are presented in Table 3. Initially, 71% of the recovered sterol label was located in the 8,14 unsaturated sterols and only 16% was in the ergosterol. In the final culture only 23% of the sterol label remained in the 8,14 compounds and 60% was in the ergosterol. Some caution is required when interpreting the data in Table 3, however. One-fifth of the initial culture was removed for analysis before the cells were transferred to the final culture. The label in the final culture, therefore, was seen to increase by about 33%. The label recovered in the ergosterol increased by 190%, however, or by 97613 cpm more than the total gain in the culture. In addition, the "other" fraction was also seen to accumulate 22% of the total label gain of the final culture. It seems, therefore, that at least 44% of the label gained by the ergosterol of the final culture must have originated in sterols of the
### TABLE 3. CONVERSION OF IGNOSTEROL TO ERGOSTEROL IN VIVO

<table>
<thead>
<tr>
<th>Fraction (acetates)(^a)</th>
<th>Relative mass carbon no.</th>
<th>Activity (cpm)</th>
<th>Mass %</th>
<th>Activity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial culture total(^b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ignosterol</td>
<td>3,584</td>
<td>775</td>
<td>198,715</td>
<td>59</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>3,559</td>
<td>150</td>
<td>76,690</td>
<td>11</td>
</tr>
<tr>
<td>Unidentified triene</td>
<td>3,596</td>
<td>95</td>
<td>136,090</td>
<td>7</td>
</tr>
<tr>
<td>Other</td>
<td>290</td>
<td></td>
<td>57,560</td>
<td>22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1,310</td>
<td>469,055</td>
<td></td>
</tr>
<tr>
<td>Final culture total</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ignosterol</td>
<td>3,590</td>
<td>718</td>
<td>74,418</td>
<td>17</td>
</tr>
<tr>
<td>Ergosterol</td>
<td>3,558</td>
<td>1,093</td>
<td>298,930</td>
<td>26</td>
</tr>
<tr>
<td>Unidentified triene (mixture)</td>
<td>3,598</td>
<td>227</td>
<td>41,562</td>
<td>--</td>
</tr>
<tr>
<td>Other</td>
<td>2,174</td>
<td></td>
<td>84,961</td>
<td>52</td>
</tr>
</tbody>
</table>

\(^a\)Initial and final culture 4-demethyl sterol acetates.

\(^b\)Initial culture values were determined by multiplying the initial sample values by five.
initial culture. Actually, the activity lost from the 8,14-unsaturated sterols was only equal to 78% of the label gain of the ergosterol, so about 20% of the activity in the ergosterol of the final culture must represent de novo synthesis. It was not possible to separate the remaining 8,14,?-triene of the final culture from the other compounds in the same TLC fraction, so accurate mass determinations could not be made. The activity reported for this compound in the final culture represents the label in the corresponding band from the thin-layer plate. In both the initial culture and the final culture numerous other minor compounds were detected, some carrying 14C label, but none were present in sufficient quantity to allow identification. The conversion of ignosterol into ergosterol demonstrated here provides more evidence that S. cerevisiae possesses an enzyme capable of reducing the 14-15 unsaturation, and a 22-desaturase which functions in vivo on a saturated-side-chain substrate.

Sterol methyltransferase in vitro assay.

The S. cerevisiae sterol methyltransferase enzyme has been carefully investigated (6,33), and the sterol product of the methyltransfer is fecosterol (VIII) (25) (Figure 1) which is very similar in structure to the 15-azasterol.
Zymosterol (VII), cholesta-8,24-dien-3β-ol, has been found to be the best methyl group acceptor in this reaction (26). The methyl donor was determined to be S-adenosylmethionine (29), which is converted into S-adenosylhomocysteine. Several problems exist in the reported assay systems, however, which limit investigations of azasterol inhibition of the methyltransferase reaction. Preparations of the mitochondrial insoluble protein fraction (100,000xg pellet) were made as previously described (6). This enzyme preparation was found to have an activity (cpm/mg protein) half-life of about two-and-one-half days when stored at 4°C, and less than one percent of initial activity remained after two weeks. Dialysis of the deionized anaerobic culture methyltransferase in 50% glycerol-Tris HCl buffer, and subsequent storage at 4°C resulted in an activity half-life of about 20-30 days, with 21% of initial activity (after dialysis) remaining after 10 weeks. The dialysis procedure actually increased the activity of the preparation somewhat. All attempts to purify the enzyme further with ammonium sulfate precipitation resulted in almost complete loss of activity. Freezing was found to reduce activity by about 60% with each freezing.

The greatest problem with the methyltransferase inhibition assay results from sterols endogenous to the enzyme preparation itself. Assays were conducted using only endogenous sterol substrates and with exogenously
supplied zymosterol (300 μM), and only two percent increase in reaction rate was noted as a result of the exogenous zymosterol. Sterols were extracted from the mitochondrial protein preparation and purified. A fraction with gas chromatographic retention properties similar to zymosterol was present in the preparation at a concentration of approximately 23 μM, which is several times the zymosterol $K_m$ for this enzyme, as demonstrated in this thesis. In these reactions, zymosterol was added as a solution in ethanol. In subsequent assays zymosterol was added in solution in lecithin micelles or with Triton x-100 with no significant enhancement in activity noted over the original method.

Attempts to remove sterols from the enzyme preparations using digitonin precipitation of the sterol digitonide resulted in 90% reduction in activity due to endogenous sterols, but only one-fourth of the total initial enzyme activity remained. The hydrophobic materials Amberlite XAD-4 and Sephadex LH-20 were used in chromatographic columns in attempts to remove sterols by exchange or gel filtration, but no reduction in endogenous sterols could be detected. The endogenous sterol substrate does not significantly hamper methyltransferase assays if excess zymosterol is added to the reaction mixture and S-adenosylmethionine is used as the variable substrate. However, it is not possible to measure directly the zymosterol $K_m$ in these
preparations, and the most abundant sterol in the preparations, ergosterol, is demonstrated in this thesis to be an effective inhibitor of the reaction. Attempts to measure azasterol effects on the methyltransferase demonstrated definite inhibition of the reaction, but kinetic calculations were not repeatable, and mixed inhibition was often indicated by the results.

The most serious problems encountered in the assay of azasterol inhibition of the methyltransferase were due to the endogenous sterols in the preparations, especially zymosterol and ergosterol. These problems were circumvented by growing the cultures anaerobically. Under anaerobic conditions yeast cannot produce sterols, but accumulate the precursor squalene in large quantity (35). During fermentative growth yeast produce promitochondria rather than mitochondria, and the total insoluble promitochondrial protein recovered from the normal methyltransferase preparation procedure is much less than that from an equivalent aerobic culture. Since the methyltransferase is located in the mitochondria in aerobic cultures (33) there is some question whether or not promitochondria carry the same enzyme. However, a sterol methyltransferase is easily prepared from anaerobic cultures using a modified preparation procedure similar to that for the aerobic enzyme. The activity of this preparation is equivalent to that of
the aerobic enzyme preparations, and sterol assays of the anaerobic cultures reveal that 91% of the non-saponifiable lipid extract is squalene, 2.9% is ergosterol, and less than 1% is lanosterol. Insufficient quantities of the sterols were recovered to allow actual determination of concentrations. The methyltransferase activity in the assays was proportional to the exogenous zymosterol concentration, and could be as much as 23 times greater than endogenous activity. As has been demonstrated, sterol synthesis occurs at a high rate in aerobically adapting cultures, and it was necessary to prepare the anaerobic enzyme preparations with extra care to prevent aeration. The cultures were allowed to settle overnight at 4°C and all subsequent work was done on ice or in refrigerated equipment. Argon was layered over all preparations to prevent direct contact with air. Even with these precautions the cultures and preparations were not absolutely anaerobic, as evidenced by the small amounts of sterols recovered. If these precautions were not observed the endogenous substrates could produce activity of 30% or more of that due to exogenous zymosterol. The labeling of these endogenous substrates was subject to inhibition by the azasterol in a manner similar to the inhibition of labeling of the exogenously supplied zymosterol.

The $^{14}$C label recovered from the anaerobic culture enzyme assays was found to increase linearly with time for
75 minutes (not shown). To ensure that substrates did not become limiting during the assays, the reactions were terminated after 45 minutes incubation. The activity of the preparations was doubled by deionization in a Sephadex G-75 (coarse) column. Magnesium chloride has been reported to enhance the activity of the sterol methyltransferase (25), but addition of MgCl$_2$ (4 µM) to the anaerobic enzyme assay, either with or without preceding deionization of the enzyme preparation, resulted in the loss of about 50% of the enzyme activity. Inhibition of the methyltransferase by inorganic cations has been previously demonstrated (6).

The $K_m$ of both zymosterol and S-adenosylmethionine were determined from Lineweaver-Burk plots (Figure 9, zymosterol not shown). Zymosterol $K_m$ was determined to be 6.5 µM (+2 -2 µM) from the average of values from two separate enzyme preparations and assays. The $K_m$ of SAM was found to be 72 µM (+5 -8 µM) from the average of three independent assays.

The apparent $K_i$ for azasterol was also determined in a similar manner. The kinetics of inhibition of the sterol methyltransferase by azasterol is presented in Figure 9. The apparent $K_i$ is 39 µM (+3 -3 µM) as determined in two assays, and the inhibitor exhibited noncompetitive inhibition.
Figure 9. Lineweaver-Burk plot of the inhibition of the sterol methyltransferase in vitro by the azasterol. Symbols: (●) control; (▲) azasterol, 40 μM.
Azasterol analog studies.

Several analogs of the azasterol used in the preceding experiments were produced in the Lilly Research Laboratories, and the structures were determined there (19,20). Four of these azasterols, all 15-azasterols, were compared to the original compound in their abilities to inhibit the sterol methyltransferase reaction. The structures of these azasterols and their relative inhibitions of the reaction are shown in Figure 10. All were tested at approximately 60 μM in a reaction using the aerobic enzyme preparation. None of the analogs were as effective as the original azasterol, A25822B, which produced 48% inhibition. Compound 109038, which contained no cyclic unsaturations and had a reduced nitrogen, produced no inhibition. Similar to the original azasterol but lacking only the 8(9) unsaturation, 83833 was the most effective inhibitor of the four, producing 22% inhibition - about half that of the original azasterol. Compound 83834 differed from the original azasterol by having a reduced nitrogen and no 14(15) unsaturation. This azasterol produced only 8% inhibition. Total saturated 109039 was also not very effective, giving 13% inhibition. Also, a mixture (106701) of A25822B (4%) and two isomers (8,14,24(25) (61%) and 8,14,23 (35%) trienes) was tested and found to cause 25% inhibition. These data
indicate that the most effective inhibitors carry the 14(15) carbon-nitrogen unsaturation. Reduction of the nitrogen reduces the effectiveness of the compound as an inhibitor. For comparative purposes, ergosterol (84 μM), the most abundant yeast sterol, was found to produce 36% inhibition of the anaerobic culture methyltransferase.
Figure 10. Structures of the azasterol A25822B and four analogs. Percent inhibition of the in vitro methyltransferase assay caused by each azasterol (60 μM) are shown in parentheses.
IV. CONCLUSIONS

The purpose of this research was to characterize the effects produced by the 15-azasterol in the yeast *Saccharomyces cerevisiae*. Based upon the results presented, several interesting conclusions can be formulated relating to the inhibitory effect produced by the compound.

The azasterol is a potent inhibitor of yeast growth, with as little as 20 ng/ml producing measurable effects on culture growth in broth. There is, however, a more interesting way to look at this effect. The size of normal *Saccharomyces* cells varies from one to ten μm in diameter, with average cells about 5.8 μm diameter. Therefore, the volume of the typical cell is about $1.02 \times 10^{-10}$ cc, and the mass is about $1.02 \times 10^{-10}$ gram (assuming cell density at $20^\circ C$ to be approximately that of water, which is a little low). If about 80% of cell mass is water, the dry weight of a cell is about $2.04 \times 10^{-11}$ gram. The ergosterol content of *S. cerevisiae* has been found to vary typically from two to five percent of dry weight (14), or, therefore, to be approximately $7.14 \times 10^{-13}$ g/cell. This value is reasonably close to actual measurements and will serve as a reference for comparisons. Consideration will now be given to the amount of azasterol per cell in the culture medium that is required to produce the observed effects, and how
this relates to the approximate amount of ergosterol found in normal cells. The effects to be considered were observed to obtain over wide azasterol concentration ranges.

The transient cessation of growth (biphasic growth curves) was observed when the initial culture contained about $1.25 \text{ to } 5 \times 10^{-14} \text{ g/cell azasterol}$, or an amount of the compound equal to about one to seven percent of the ergosterol in the cells. Under these conditions ergosterol synthesis stopped, ignosterol accumulated, the culture grew at a rate slower than the control, and the culture eventually reached the same turbidity as control cultures. Azasterol represented less than 0.1% of the final total culture sterol, yet no ergosterol accumulation was detected.

Inhibition of culture growth at optical densities less than the control resulted when the initial amount of azasterol per cell was $1.25 \times 10^{-13}$ to $9 \times 10^{-12} \text{ g/cell}$, or about one-fifth to twelve times the ergosterol content, but the final sterol content of the cultures may have been one to fifty times greater than the azasterol alone. No growth occurred if the azasterol was present at concentrations thirty times greater than the cellular ergosterol, and a gradual lysis of the cultures was observed (this may have been the normal autolysis found in old yeast cultures).

If the azasterol content of the medium was as low as 0.05% of the ergosterol content of the inoculum (250 pg/ml
with $2 \times 10^6$ cells/ml some 8,14-conjugated sterol accumulation was observed in UV analyses, but ergosterol was the major sterol recovered.

In cultures with high azasterol concentrations (greater than 5 µg/ml) some of the inhibitor could be recovered from the cell-free medium after 24 hours incubation, but similar determinations could not be made for cultures with lesser concentrations. No data are available to indicate what fraction of the azasterol in the medium actually enters the cells, and at what rate the uptake occurs. However, if the initial azasterol concentration was less than 1% of the cellular ergosterol, some change in sterol synthesis was seen. At amounts equal to one to ten percent of cellular ergosterol, ignosterol was seen to accumulate and a transitory inhibition of growth also occurred. Interestingly, the final azasterol concentration was 1% or less of the sterol content in these cultures but no ergosterol synthesis was detected. These results indicate that the effect of the azasterol on ergosterol synthesis was not likely due to overall gross alterations of membrane properties due to intercalation of the inhibitor into the normal sterol sites in the membranes, but was primarily due to inhibition of specific steps in sterol synthesis. This conclusion was supported by the accumulation of the abnormal sterol ignosterol, which contains the unusual conjugated
8,14-diene system. In the absence of the inhibitor this sterol was converted into ergosterol. The accumulation of ignosterol, and the absence of ergosterol, was not the cause of the antifungal activity of the inhibitor, however. Cultures have been repeatedly transferred for several years now on agar containing sufficient azasterol to prevent ergosterol synthesis.

Inhibition of cellular growth occurred when the amount of azasterol available to each cell was initially equal to 10% or more of the ergosterol in the cells. This may have been enough azasterol to alter membrane properties significantly. It has been suggested that the primary effect of azasterols in general, either directly or indirectly, is "most probably on membrane- or enzyme-bound systems" resulting in decreased levels of adenosine triphosphate, and other effects (21). If all of the azasterol in the medium was incorporated into cellular membranes it may well have affected membrane properties, but it must be noted that a very similar sterol, ignosterol, does not produce detrimental effects, even when it is present in the cells in amounts equal to the normal cellular ergosterol content. Since cellular sterol synthesis continues in the presence of moderate amounts of the azasterol, dilution of the azasterol in membranes would occur as more ignosterol was synthesized, and the azasterol-related membrane effects
would diminish with time. Additionally, some selectivity seems to be exhibited by the cell as to which sterols are placed into the membranes and which are esterified to long-chain fatty acids (F. R. Taylor, personal communication, and 5).

An alternative mode of inhibition of cellular growth can be derived from the analysis of the effect of the azasterol on sterol synthesis. Sterol-free mutants of yeast are exceedingly rare, and sterols have been reported to be essential for the growth of yeast under anaerobic conditions (3). If sterols are a requirement for normal cellular growth, total inhibition of sterol synthesis may prevent growth. The proposed pathway of accumulation of ignosterol and the normal ergosterol synthetic pathway from lanosterol is presented in Figure 11. For the accumulation of ignosterol to occur, four enzymatic steps in the ergosterol synthetic pathway must be blocked. Inhibition of three of these enzymes has now been demonstrated in vitro. The noncompetitive inhibition of the sterol methyltransferase (#2 in Figure 11) is reported here (K_i 39 μM), and the yeast sterol 24(28)-methylene reductase (#4 in Figure 11) is competitively inhibited (K_i 17 μM) (17). Inhibition of the 14-reductase (#1 in Figure 11) was indicated from the accumulation of ignosterol, and has been demonstrated in cell-free extracts (K_i 2 nM) (10). The sterol 22-desaturase (#3 in Figure 11) enzyme kinetics have not yet
Figure 11. The proposed pathway of accumulation of ergosta-8,14-dien-3β-ol in *S. cerevisiae* in the presence of the 15-azasterol.
been determined.

The very low reported $K_i$ for the 14-reductase is in agreement with the observed effects of the azasterol in vivo. This enzyme appears to be the most sensitive cellular component yet observed. The other two enzymes investigated were not remarkably inhibited by the compound in vivo, since both must function in the synthesis of ignosterol. However, these enzymes were inhibited in vitro by azasterol concentrations a thousand-fold or more than necessary for the inhibition of the 14-reductase. Coincidently, azasterol concentrations a thousand times greater than that required to cause ignosterol accumulation in vivo prevented cellular growth. Although not detailed here, RNA and DNA accumulation in *S. cerevisiae* in the presence of the azasterol were unchanged, even at azasterol concentrations as high as 200 µg/ml in the cultures (fifteen times the amount of ergosterol in the cultures), and both accumulations continued until culture growth slowed (RNA) or stopped (DNA). The inhibitor was demonstrated to enter the cells rapidly (Figure 7). Cellular growth may be used as a crude measure of protein synthesis, and growth did not cease immediately at any but the very highest azasterol concentrations. It seems, therefore, that the syntheses of protein, RNA and DNA were not especially sensitive to the inhibitor, although it must be assumed that a very
large number of membrane associated processes are involved in the synthesis of these three major cellular components. Perhaps the growth inhibition produced at the lower azasterol concentrations was the result of the blockage of several steps in ergosterol synthesis, causing either accumulation of a sterol which did not satisfy cellular requirements, or possibly no sterol synthesis at all. Growth continued temporarily after addition of moderate amounts of the inhibitor (1 µg/ml) for two generations, perhaps until all useable sterols had been diluted into new cells, although changes in sterol synthesis were brought about rapidly. Growth inhibition at very high concentrations of the azasterol (thirty times the cellular ergosterol content) may well have resulted from more generalized effects in membranes, or as a result of the combination of effects. However, at the lowest concentrations of the azasterol that inhibit cellular growth, interference with sterol synthesis may be a key factor in the action of the inhibitor.

Several conclusions may be drawn concerning sterol synthesis in yeast. The accumulation of ignosterol proves the existence of an 8,14-conjugated intermediate in yeast, and its subsequent conversion to ergosterol demonstrates the presence of a 14-reductase enzyme and a 22-desaturase enzyme capable of modifying sterol substrates with saturated
side chains. The 14-reductase functions with C-28 sterol substrates. In addition, demethylations from the C-4 position occur regardless of the 14(15) unsaturation. No 8,14-conjugated 4'-dimethyl sterols were detected from any of the azasterol cultures, although a thorough search was made and the 8,14 conjugation is easily detected by its strong UV absorption. Likewise, transmethylation at C-24 and the subsequent reduction of the 24(28) unsaturation also came about in the presence of the 8,14 conjugation. It is not possible to draw conclusions about the normal sterol synthetic steps which did not happen in ignosterol synthesis, because both the 8,14 conjugation in the newly synthesized sterol and the 15-azasterol were present, and both may have effects upon these processes.

The four analogs of the azasterol primarily used in this work provide clues about the properties of 15-azasterols which bring about inhibition in at least one enzyme reaction. The oxidized nitrogen, with a 14(15) unsaturation, seems to be the most effective portion of the molecule. Reduction of this nitrogen results in reduced effectiveness as an inhibitor. Of secondary importance is the 8(9) unsaturation. This double bond gives the azasterols a ring structure more nearly like that of normally occurring sterols.


