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AZASTEROL INHIBITON OF Δ^{24}-STEROL METHYLTRANSFERASE IN YEAST AND ITS APPLICATIONS

by

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

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ABSTRACT

Previous work in this laboratory has shown that inhibition of Δ^{24}-sterol methyltransferase (24-SMT) in cultures of Saccharomyces cerevisiae by sterols containing nitrogen in key sidechain positions was efficient. The inhibition resulted in the increased production of C_{27} sterols such as zymosterol, cholesta-5,7,22,24-tetraen-3β-ol and cholesta-5,7,24-trien-3β-ol. The goal of this study was to achieve efficient production and synthetic utilization of these C_{27} sterol metabolites. In addition, this study was directed at determining the mode of enzymatic inhibition of these azasterols.

Production of zymosterol, free of its Δ^7 isomer, was achieved by growth of an erg 2 sterol mutant ($\Delta^7 \rightarrow \Delta^8$ isomerase blocked) of S. cerevisiae in the presence of 0.5 μM 25-azacholesterol (an inhibitor of 24-SMT).

The production of [^{13}C]-labelled zymosterol by S. cerevisiae cultured in the presence of [1-^{13}C]acetate and 25-azacholesterol was achieved. The conversion of zymosterol (obtained from inhibited yeast cultures, S. cerevisiae) to cholesterol in nine steps (6% overall yield) was effected.

A synthesis of 25-hydroxy provitamin D_3 (6% overall yield from yeast NSF) was executed from cholesta-5,7,24-trien-3β-ol, isolated from inhibited mutant cultures (erg 5, lacking Δ^{22}-desaturase).

The kinetics of the inhibition of 24-SMT by azasterols were studied using cell-free preparations of Δ^{24}-sterol methyltransferase. Attempts to develop a method of determining the rate of product formation or substrate disappearance by GLC were not successful because of endogenous sterol in the preparation. Inhibition kinetics were measured by the standard assay method using [^{14}C]-S-adenosyl-L-methionine (SAM).

At constant SAM and varying zymosterol concentrations, 25-azacholesterol (Ki' = 5.4 nM), 25-aza-24,25-dihydrozymosterol (Ki' = 5.1 nM), 23-azacholesterol (Ki' = 7.2 nM) and 25-methyl-25-azacholesterol (Ki' = 2.8 nM) exhibited uncompetitive inhibition with respect to zymosterol. At constant zymosterol and varying SAM concentrations 25-aza-24,25-dihydrozymosterol exhibited competitive inhibition (Ki = 1.25 nM). These results were interpreted in terms of a sequential binding of zymosterol and SAM to 24-SMT.

The same assay method showed that cycloartenol was not a substrate and that ergosterol was a non-competitive inhibitor. When zymosterol was kept at constant concentration and S-adenosyl-L-methionine concentrations were varied, the addition of S-adenosyl-L-homocysteine (2 μM) gave product inhibition for each SAM concentration.

In related work, it was shown that 24-methylene sterol $\Delta^{24(28)}$-reductase is inhibited only by 23- and

24-azacholesterol but not by 25-azasterols.

Candida utilis, grown in a culture containing 25-azacholesterol (1.0 μM), showed inhibition of that yeast's 24-sterol methyltransferase and production of C_{27} sterols. 25-Fluorocholesterol was not an inhibitor of any sidechain sterol-modifying enzymes.

To Ameera

and

Justine Marie

ACKNOWLEDGEMENTS

I would like to especially thank my research supervisor, Dr. A.C. Oehlschlager, for his enthusiasm, advice and direction during the course of this work.

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TABLE OF CONTENTS

ABBREVIATIONS

CI	chemical ionization
DPL	dipalmitoyl lecithin
EI	electron impact
EYL	egg-yolk lecithin
FID	flame ionization detector
GLC	gas liquid chromatography
K_M	Michaelis constant (concentration of substrate when $v = V_{max}/2$
MS	mass spectrum
24(28)-MSR	24-methylene-$\Delta^{24(28)}$-sterol reductase
NADPH	nicotinamide adenine dinucleotide phosphate (reduced)
NSF	non-saponifiable fraction
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
22,23-SD	22,23-sterol desaturase
24-SMT	Δ^{24}-sterol methyltransferase
Tris	Tris(hydroxymethyl)amino ethane
V_{max}	maximum velocity; i.e., value of v at saturation

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

A. Background

Several laboratories, including ours, have studied the latter stages of ergosterol biosynthesis in yeast (Fryberg et al., 1973; Barton et al., 1973; Fryberg et al., 1975). A general model has been developed for the conversion of lanosterol (1) to ergosterol (14) in Saccharomyces cerevisiae. The sequence of structural transformations involves: 1) loss of 14α-methyl and loss of the C_4 methyl groups; 2) methylation at C_{24} with reduction at C_{25} and generation of a $\Delta^{24(28)}$-methylene; 3) isomerization of Δ^8 to Δ^7; 5) introduction of Δ^{22} double bond; 5) introduction of Δ^5 double bond; and 6) reduction of $\Delta^{24(28)}$ double bond generating a C_{24}-methyl. Figure 1 illustrates many of the possible routes from lanosterol (1) to ergosterol (14), with a number of the possible metabolic intermediates shown.

This graphical representation was first put forward (Fryberg et al., 1973) as an aid in the design of experiments as well as to display the possible intermediates in an orderly fashion. Each structural transformation was treated as a single step, although most of these processes are multistep. In this particular scheme, a number of separate transformations can be dealt with and still have structural nearest neighbours adjacent. The six transformations, from lanosterol to ergosterol, when treated in this manner will yield sixty-four possible intermediates. Therefore, in order to represent the possible alternatives as in Figure 1, certain assumptions had to be

employed to simplify the graphical representation and give a more concentrated picture of the process.

The sequence from lanosterol (1) to zymosterol (4), wherein nuclear demethylation occurs prior to C_{24} alkylation, was suggested first by Gaylor's work with yeast Δ^{24}-sterol methyl transferase (24-SMT) which showed methylated sterols were poor substrates and zymosterol was the best substrate for this enzyme *in vitro* (Moore and Gaylor, 1970). Secondly, a study to clarify the routes operative from lanosterol (1) to fecosterol (6) has been reported. This study, through searches for metabolites such as 4,4-dimethylfecosterol and tracer time-course studies, using labelled lanosterol and zymosterol revealed that the principle pathway involved conversion of lanosterol (1) to 4,4-dimethylzymosterol (2) and then to 4α-methylzymosterol (3). At this point, the pathway to fecosterol diverges. In mature cells, fecosterol is produced equally from a pathway involving zymosterol and 4α-methyl fecosterol (5) (Fryberg *et al.*, 1975). 4α-Methyl fecosterol (5) and zymosterol were previously found as minor metabolites in commercial yeast mother liquors (Fryberg *et al.*, 1973) and in yeast sterol mixtures (Barton *et al.*, 1970). Fecosterol (6) has been shown by Gaylor to be the immediate product of the methyl transferase. All the other sterols in this particular scheme, except ergosta-8,22-dienol (8) and ergosta-5,7-dienol (15), were found in yeast (Fryberg *et al.*, 1973, Barton *et al.*, 1972; Barton *et al.*, 1970). Therefore, more than one unique pathway must be present. That is, the enzymes

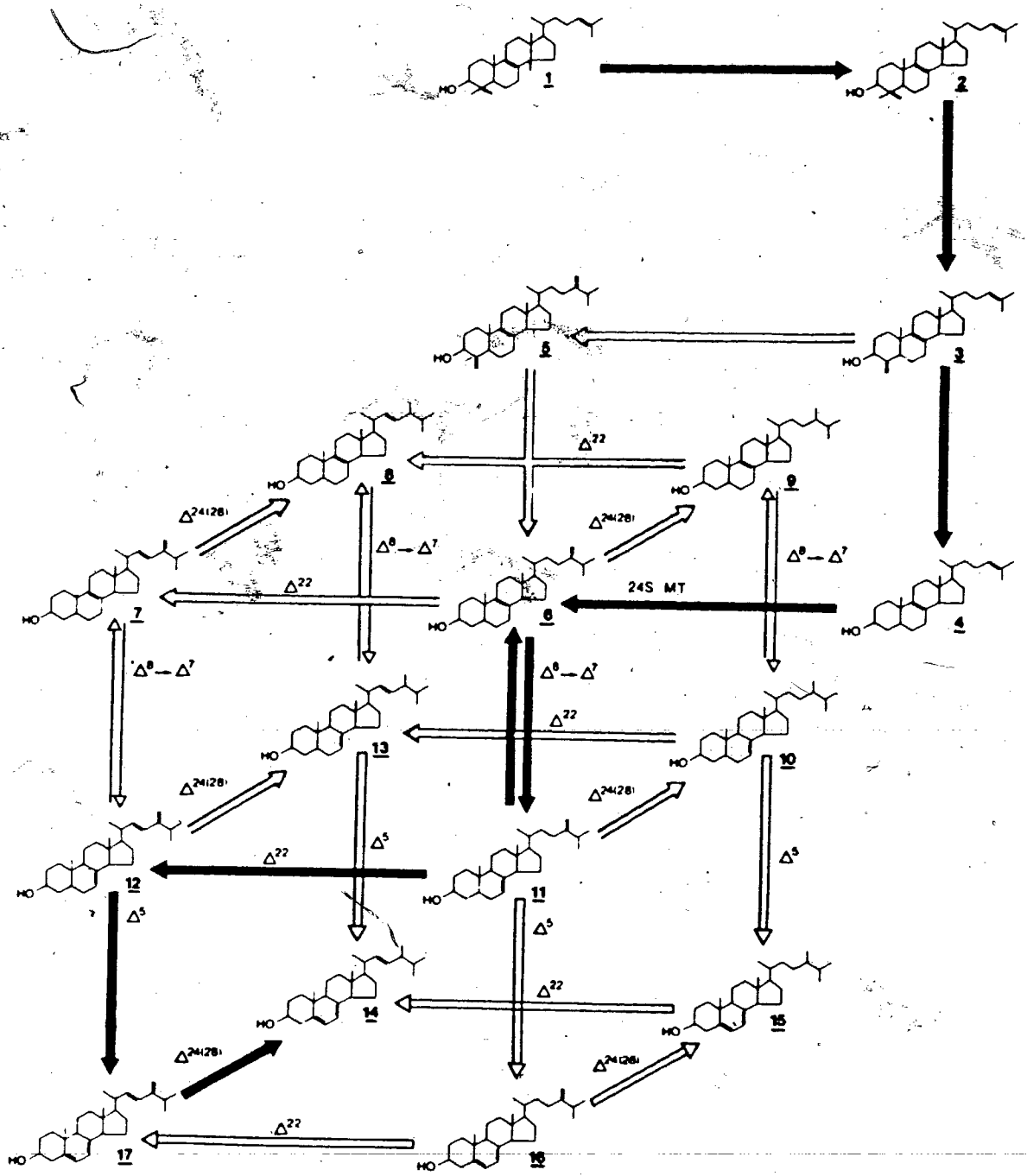
Figure 1

Sterol biosynthesis in S. cerevisiae.

major pathways ━━━

minor pathways ═══

Fig. 1



involved in each structural change possess sufficient flexibility to accept several substrates in the production of ergosterol.

The particular biosynthetic grid, represented in Figure 1, is a simplified biosynthetic scheme in that there is a bias against $\Delta^{5,8}$-sterols (which have been found in erg 2 sterol mutant of S. cerevisiae (Pierce et al., 1979)). It also does not show many of the possible sterols involved in demethylation at the 14 and 4 positions of the sterol. A more comprehensive grid (for several yeast) has been put forward by this lab which does not possess some of these biases (Pierce et al., 1978b). Finally, Figure 1 does not account for possible metabolites with $\Delta^{8,14}$ unsaturation (which have been found in erg 2 and 5 sterol mutants). However, the figure does show the major enzymatic transformations and the major metabolites of S. cerevisiae, wild type.

Tracer studies were carried out with labelled sterol metabolites present in yeast. Isolation of the labelled products of further metabolism, by trapping with unlabelled synthetic material (Fryberg et al., 1973; Barton et al., 1973), yielded information on major transformations. Studies in both Canadian and British laboratories concluded that the $\Delta^{8} \rightarrow \Delta^{7}$ isomerization was the only step that was reversible. Both studies found that the reduction at $\Delta^{24(28)}$ was irreversible. Fryberg et al. (1973) pointed out that when the tracer study was combined with time-course analysis, which gave information on sterol pool size, major pathways could be deduced by assuming that high

incorporation into a sterol metabolite coupled with a high pool size indicated an efficient transformation. This study concluded that the major sequence was fecosterol (6) → episterol (11) → ergosta-7,22,24(28)-trien-3β-ol (12) → ergosta-5,7,22,24(28)-tetraen-3β-ol (17) → ergosterol (14) as per Figure 1. Fryberg et al. (1973) concluded that the enzymes operating on the metabolites in lanosterol to ergosterol conversion can each operate on a variety of substrates, but there is a particular substrate which is preferred by each enzyme.

The realization that there was some latitude in substrate specificity for enzymes in this system led to the idea that manipulation of the sterol biosynthetic system, specifically to produce more desirable metabolites than ergosterol, would be possible. It was reasoned that if a specific sterol-modifying enzyme was inactivated, the remaining active enzymes would operate on available substrates to produce metabolites transformed by all enzymes except the one removed from action. Inactivation of specific enzymes in the yeast biosynthetic scheme may be accomplished by genetic mutation (Molzahn and Woods, 1972) or specific enzyme inhibitors.

Enzyme inhibitors are useful tools in determining substrate specificity, the type of functional groups at the active site, the mode of catalysis and conformational stability of enzymes. In biological systems, inhibition of certain enzymes by compounds in the cell can be a form of regulation of that system. The use and study of enzyme inhibition includes the fields of

enzymology, pharmacology, toxicology and pathology. Poisons, drugs and insecticides can all be considered enzyme inhibitors, if they act at the enzyme level. Two types of inhibition can take place: irreversible, which reduces enzyme activity by modifying the enzyme active site or structure, and reversible, where the inhibitor forms a complex with enzyme which modifies its activity without permanent damage. The activity can be regained by administering excess substrate or by using physical methods (e.g., dialysis). Substrate analogs are commonly used reversible inhibitors of enzymes. The strategy is to design the inhibitor such that it is structurally related to the normal substrate(s) of the enzyme, but is different chemically. One group of inhibitors, effective in blocking sterol modifying enzymes is sidechain azasterols.

Azasterols were first synthesized with the hope that they would function as hypocholesterolemic agents. When given to rats, blood serum cholesterol levels decreased and desmosterol in serum and liver increased (Counsell et al., 1962a,b). In these studies, 25-azacholesterol was the most potent azasterol investigated (Counsell et al., 1965). The accumulation of desmosterol (cholesta-5,24-dienol) led these workers to conclude that the Δ^{24}-sterol reductase was inhibited. A study with 20,25-diaza analogs of cholesterol showed that 20,25-diazacholesterol was also a potent inhibitor of cholesterol biosynthesis and that the dimethylamino end group and side chain length were important in determining inhibitory activity (Counsell et al.,

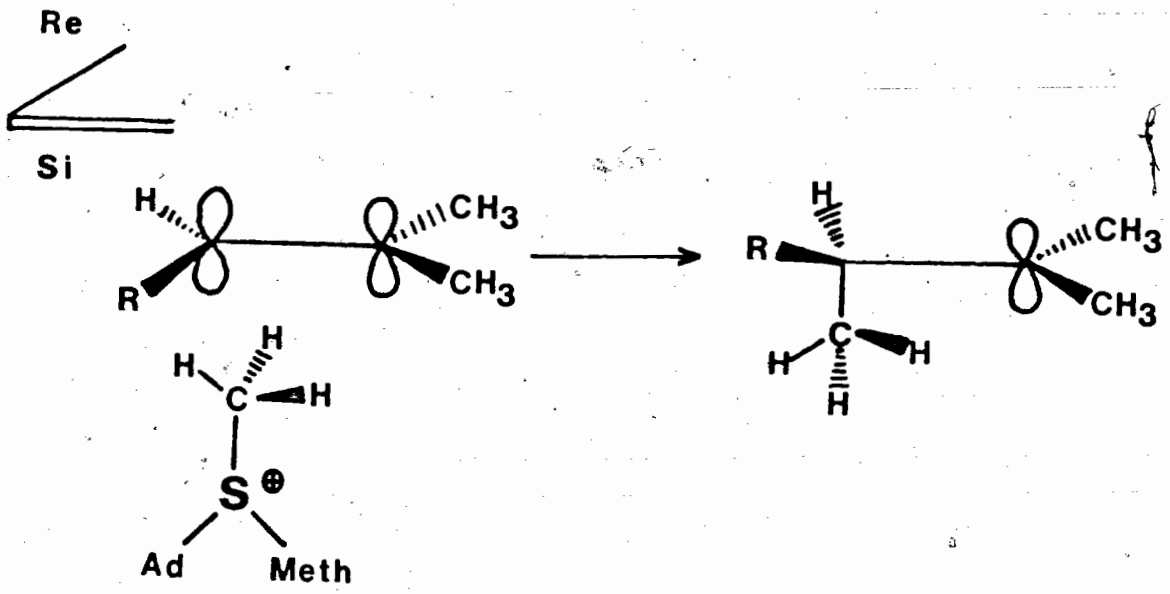
1962a,b). The implication was that the active site of the reductase was specific for a molecule with the dimensions of cholesterol.

Insect sterol metabolism was also effected by the administration of azasterols with nitrogen containing sidechains. These studies were aimed at blocking insect conversion of plant sterols (e.g., β-sitosterol) to ecdysones, which regulate molting and development. Studies with azasterols were carried out on the silkworm, Bombyx mori (Fujimoto et al., 1974) and the tobacco hornworm, Manduca sexta (Svoboda and Robbins, 1971). In the silkworm study, 24,28-iminofucosterol was fed to the insect along with β-sitosterol. Sterol analysis showed a marked decrease in cholesterol formation with an accumulation of β-sitosterol, indicating an inhibition of dealkylation activity in the insect. For Manduca sexta, several monoazasterols (20,22,23,24 and 25-azasterols) and 20,25-diazacholesterol were fed to the insects with dietary β-sitosterol, with the result that cholesterol levels decreased greatly while desmosterol levels increased slightly. 20,25-Diazacholesterol helped increase desmosterol levels in the insect even more when used with β-sitosterol. When the monoazasterols were administered to the hornworm with stigmasterol, cholesterol levels dropped significantly and the desmosterol levels increased as the nitrogen moved from the 20 to the 25 position in the sidechain . These results indicated an inhibition of the Δ^{24}-sterol reductase. When stigmasterol was given to the hornworm with azasterols,

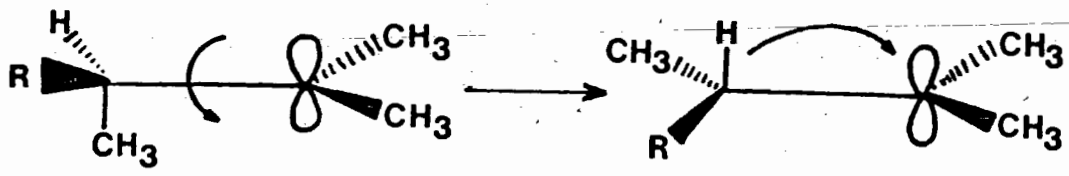
cholesta-5,22,24-trienol levels increased in the insect, especially when nitrogen was in the 20 and 22 positions indicating a possible block of the $\Delta^{22,24}$-reductase.

More recently, plant sterol biosynthesis has been examined using azasterols in in vitro as well as in vivo studies. 25-Azacycloartenol was administered to a cell-free extract of maize embryos (Zea mays) known to contain a microsomal C_{24}-sterol-methyltransferase. Inhibition of cycloartenol-C_{24}-methyltransferase in the presence of cycloartenol and ^{14}C-SAM as well as inhibition of the 24-methylene lophenol-C_{28}-methyltransferase was observed with increasing azasterol concentrations (Rahier et al., 1980). Bramble cells (Rubus fruticosus), incubated with 25-azacycloartenol (1.0 µM), showed large decreases in the amount of 24-ethyl sterols (sitosterol and isofucosterol) and an equally large increase in unalkylated sterols (cycloartenol, desmosterol, cholesterol) (Schmitt et al., 1981). This indicated inhibition of the cycloartenol-C_{24}-methyltransferase and the 24-methylene-lophenol-C_{28}-methyltransferase in vivo by the azasterol. Inhibition of the Δ^{24}-reductase was also postulated in the azasterol-treated bramble cells because of the accumulation of cycloartenol and desmosterol (Δ^{24}-sterols), as well as $\Delta^{24(28)}$-sterols (24-methylene cholesterol) . Recently, 20,24 and 25-azacholesterol inhibition of the methyl transferase in maize microsomes (in vitro) was investigated and C_{24}-methylation was inhibited most by 25-azacholesterol (Beneveniste, 1981, private communication).

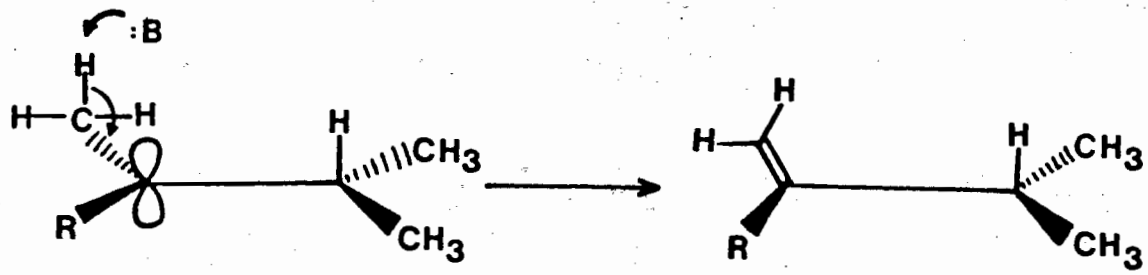
The use of azasterols to induce production of desired sterol metabolites in yeast was triggered by their successful use in rats and insects. The three sterol sidechain modifying enzymes active in yeast, Δ^{24}-sterol methyltransferase (24-SMT), 22,23-sterol desaturase (22,23-SD) and 24-methylene-$\Delta^{24(28)}$-sterol reductase (24,28-MSR), have been targeted for inactivation by azasterols (Avruch et al., 1976; Pierce et al., 1978a). The Δ^{24}-sterol methyltransferase (E.C. 2.1.1.41) has been the subject of the most detailed inhibition and substrate specificity studies (Moore and Gaylor, 1970; Bailey et al., 1974; Bailey et. al., 1976; McCammon and Parks, 1981). This enzyme system converts Δ^{24}-sterols to $\Delta^{24(28)}$-sterols (Moore and Gaylor, 1969) by mediating the transfer of the S-methyl group from S-adenosyl-L-methionine to Δ^{24}-sterol intermediates (Parks, 1958) with simultaneous $C_{24} \rightarrow C_{25}$ hydrogen migration (Goulston et al., 1967). In order to gain information about the active site, the stereochemistry of the methylation process, as well as the final reduction of ergost-5,7,22,24(28)-tetraenol to ergosterol, has been investigated by Arigoni and co-workers (Arigoni, 1978). The initial methyl transfer from methionine occurs on the Si face of the double bond with an inversion of the methyl group (Arigoni, 1978). The next step involves the



migration of the hydrogen at C_{24} to C_{25} which occurs on the opposite side of the double bond (Re face). At the same time

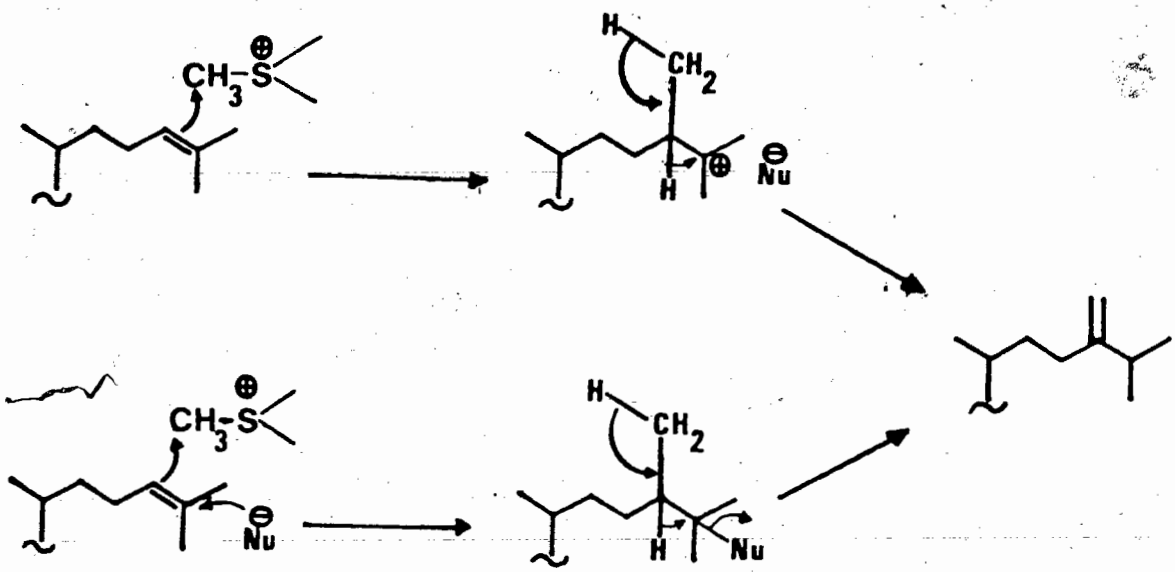


there is a loss of a hydrogen at C_{28} (also from the Re face) which produces the $\Delta^{24(28)}$-methylene group.

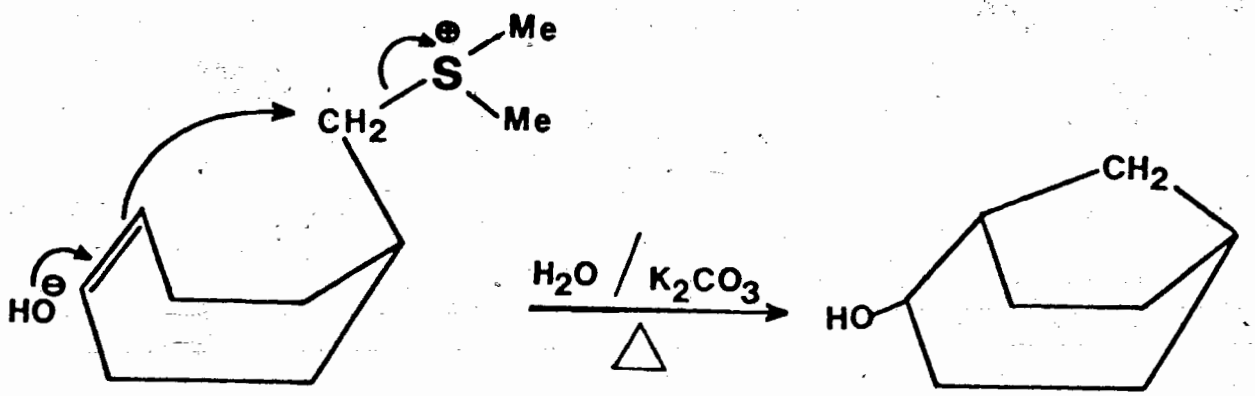


The migration of the hydrogen from C_{24} to C_{25}, however, would require the hydrogen to pass in the vicinity of the base which helps in the removal of the hydrogen at C_{28}. If we consider the possible mechanisms that have been described for this

process (Oehlschlager et al., 1980), which involve a nucleophilic group mediating the methylation process, an explanation of the migration of the hydrogen can be put forward. The first mechanism is a carbocation rearrangement (a), where C-25 is electron deficient providing the impetus. In the nucleophilic addition-rearrangement (b) C-24 and C-25 are electron-rich. A



neighbouring group, such as a nucleophile introduced during methylation, could participate in migration of the hydrogen from C_{24} to C_{25} by acting as a bridging group to induce the transfer. Such a group would be trans-antiparallel to the hydrogen lost from C_{28} during formation of the $C^{24(28)}$-methylene. There is one report of a non-enzymatic alkylation that provides an example of participation by a nucleophile in the alkylation of a double bond by a dimethylsulphonium group (Chiut and Felkin, 1967) as illustrated below:

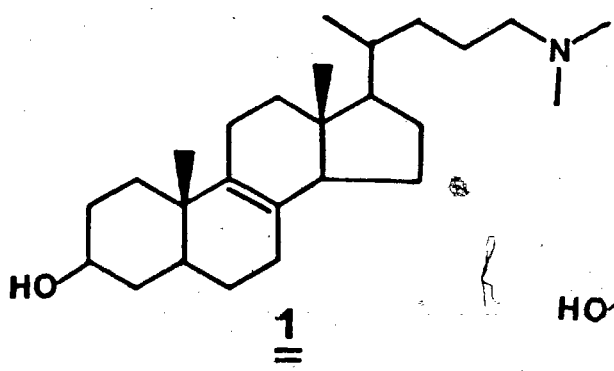


In this case, the entering nucleophile attacks the double bond undergoing alkylation in a trans fashion. The stereochemistry of the reduction of $\Delta^{24(28)}$ has been investigated. The observation was made that there was a net retention of configuration for the methyl group from methionine through the methylene intermediate to ergosterol. For this to happen, the final reduction must occur from the same side of the double bond as the methyl group originally approached. Since the stereochemistry at C_{24} involves attack of H (using NADPH, Neal and Parks, 1977) from the side of $\Delta^{24(28)}$ opposite to this, the reduction must proceed in a trans fashion.

Inhibition of the methylation process could result from blocking actual methyl donation, hydrogen transfer or removal of a C_{28} hydrogen.

An initial study (Avruch et al., 1976) showed the 24-SMT enzyme was efficiently inactivated by 25-aza-24,25-dihydrozymosterol (1) added to aerobically growing yeast. Its inhibitory action has been attributed to the nuclear identity of

25-aza-24,25-dihydrozymosterol (1) with zymosterol, the preferred substrate of 24-SMT (Moore and Gaylor, 1970), as well as its electronic resemblance at the 25 position to Δ^{24}-sterols (Oehlschlager et al., 1980). 23-Azacholesterol (2) was investigated to determine if it would inhibit the 22,23-sterol desaturase in cultures of S. cerevisiae (Pierce et al., 1978a). It failed to inhibit this enzyme, but was found to be an efficient inhibitor of the 24-methylene-$\Delta^{24(28)}$-sterol reductase. In this case, the nitrogen lone pair at C_{23} was cited as a reason



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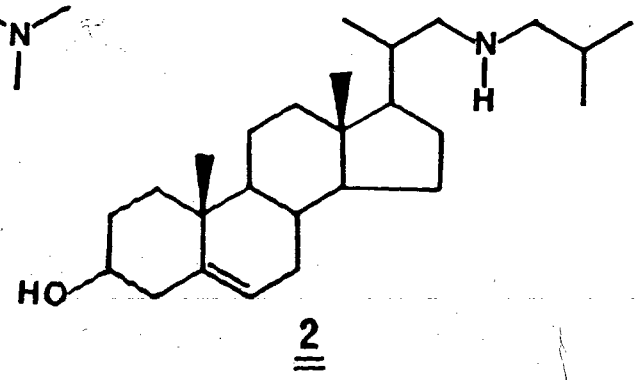

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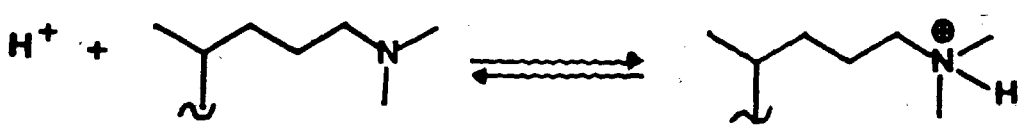
for the inhibition of the reductase. It was postulated that the lone pair of the nitrogen at position 23 in the side chain could mimic the Δ^{22} double bond in ergosta-5,7,22,24(28)-tetraen-3β-ol, which is the preferred substrate (Jarman et al., 1975) of the reductase. The azasterol could imitate an electrophilic or nucleophilic center depending on its state of protonation. As a result, the enzymes may bind the azasterol as well as or better



than the preferred substrate. In the study with 23-azacholesterol, the 22,23-sterol desaturase was not inhibited, presumably, because of the electronic resemblance of the inhibitor at the 23 position to the product of this enzyme (ergosta-7,22,24-(28)-trien-3β-ol) which is produced from episterol (11), the preferred substrate of 22,23-SD (Fryberg et al., 1973).

Genetic mutation has also been used to manipulate yeast sterol biosynthetic systems. This technique utilizes a number of isolated nystatin resistant mutants of S. cerevisiae (Molzahn and Woods, 1972). These mutants which were blocked at one particular enzyme in the conversion of zymosterol to ergosterol, were grown and their sterols were isolated and characterized (Barton et al., 1974), in order to determine which enzymes were affected for each mutant. In each case, the active enzymes, acting on available substrates, produced in large amounts the final sterol metabolite expected for each blocked system based on the sterol biosynthetic grid presented earlier. The sterol composition also showed an increase in the preferred substrate for each inactivated enzyme when compared to the amount found in the wild type yeast. Double mutants, yeast blocked at two enzymes, have also been grown and their sterols isolated (Barton et al., 1975; Bard et al., 1977). There was a significant drop in sterol production in double mutants and the sterols produced indicated that the unaffected enzymes were restricted in their operation on available substrates.

Finally, simultaneous chemical and genetic manipulation of the yeast sterol biosynthetic apparatus has been used to alter the sterols produced by yeasts (Pierce et al., 1978; Pierce et al., 1979). In one study, a mutant strain of S. cerevisiae was incubated with 25-aza-24,25-dihydrozymosterol resulting in the inhibition of the Δ^{24}-sterol methyltransferase and the loss of the 14-methyl group (Pierce et al., 1978b). In the other study (Pierce et al., 1979), sterol mutant yeasts (Molzahn and Woods, 1972; Bard et al., 1977) were grown with 23-azacholesterol, 25-azacholesterol or 25-azacholestanol and their sterols were analyzed. This study showed that specific sterols could be efficiently produced. One particular sterol, cholest-5,7,24-trien-3β-ol could be produced in larger amounts when 25-azacholestanol was used with erg 5 sterol mutant (lacking Δ^{22}-desaturase) than when double mutants (Barton et al., 1975), single mutants (Barton et al., 1974) or wild type S. cerevisiae inhibited by 25-aza-24,25-dihydrozymosterol, (Avruch et al., 1976) were grown. In all instances, the major transformation blocked was the Δ^{24}-sterol methyltransferase.

B. Aims

The first aim of the current study was the efficient production of C_{27} sterols in yeast with particular attention to obtaining cholesta-5,7,24-trien-3β-ol, because of its potential use as a synthetic precursor of 25-hydroxy provitamin D_3.

Vitamin D_3 is presently produced from cholesterol by introduction of the Δ^7-double bond and irradiation (Fieser and

Fieser, 1959). Poultry can only utilize vitamin D_3 (Sebrell and Harris, 1973) and are major consumers of vitamin D (Standen, 1970), also the conversion of cholesterol to vitamin D_3 accounts for a large part of the world production of D vitamins. 25-Hydroxy vitamin D_3 ($25(OH)D_3$) was the first metabolite of vitamin D isolated and chemically characterized (Blunt et al., 1968) and is the major circulating metabolite (DeLuca, 1979, p. 12; Norman, 1979, p. 254). Both vitamin D_2 and D_3 are hydroxylated in the liver (Jones et al., 1976) as the first step in their conversion to the more active metabolites, the 1,25-dihydroxy derivatives (DeLuca, 1979, p. 17; Norman, 1979, p. 242). The level of 25-hydroxy vitamin D_3 circulating in an organism is a general guide to its nutritional status (Preece et al., 1975; Arnaud et al., 1976). 25-Hydroxy vitamin D_3 has been found to be at low levels when intake or production of vitamin D is inadequate (Preece et al., 1975; Arnaud et al., 1976), vitamin D intestinal absorption is defective (Preece et al., 1973) and when steroid therapy (Klein et al., 1977) or anti-convulsive drugs are administered (Stamp et al., 1972). Currently, the Upjohn Company is developing 25-hydroxy D_3 for commercial distribution for administration in case of chronic renal failure (Norman, 1979, p. 413). Lesser amounts of this compound are required than vitamin D itself to treat rickets (Fraser et al., 1973) and the problem of vitamin D toxicity could be corrected for by administering the hydroxy compound, which has a shorter half-life than vitamin D in the body (DeLuca, 1979, p. 59). At

present, however, production of 25-OH-D_3 and its potential uses are limited by the cost and difficulty of its chemical synthesis.

A second aim of this study developed because of the initial results of the inhibition of the Δ^{24}-sterol methyltransferase. As pointed out earlier, studies in which wild type yeast and sterol mutants were treated with azasterols (Avruch et al., 1976; Pierce et al., 1979) produced several C_{27} sterols with a cholestane skeleton, the main one being zymosterol (4). This development, plus the fact that [^{14}C]-acetate (Hanahan and Al-Wakil, 1952; Fryberg et al., 1973) and [^{13}C]-enriched acetate [≈35%, Cushley and Filipenko, 1976] are efficiently incorporated into ergosterol by S. cerevisiae, encouraged pursuit of production of [^{13}C]-enriched sterols, specifically [^{13}C]-enriched cholesterol. Highly enriched [^{13}C]-cholesterol could be utilized for ^{13}C NMR studies in artificial membrane preparations, enzyme-substrate interactions as well as biosynthetic processes. [^{13}C]-cholesterol has already been produced from [^{13}C]-acetate and [^{13}C]-mevalonate fed to rats, but with [^{13}C]-enrichment only twice that of natural abundance at the enriched positions (Popjak et al., 1977). At present, membrane studies with [^{13}C]-enriched compounds have been conducted with [$^{13}C_4$]-cholesterol in model membrane preparations (DeKruijff, 1978) as well as [^{13}C]-enriched choline headgroups in biological membranes (DeKruijff et al., 1980). Enzyme-substrate interactions for the enzyme luciferase have been studied using [^{13}C]-enriched

substrates produced biosynthetically (Viswanathan et al., 1979). In a recent study, [^{13}C]-enriched peptides were used to study opiate receptors in biological membranes (Deslaurier et al., 1980). If it is possible to produce enriched sterols in large enough quantities, these sterols could be manipulated synthetically to produce a variety of enriched compounds of use in NMR studies of biological systems.

The final aim of this work was to study the kinetics of the inhibition of Δ^{24}-sterol methyltransferase by sidechain nitrogenated sterols. The type of inhibition caused by these azasterols should give information on the mechanism of the methyl transfer and the inhibition model.

EXPERIMENTAL

I. Materials

A. General

S-Adenosyl-L-methionine (Grade II, chloride salt) and S-adenosyl-L-homocysteine were purchased from Sigma Chemical Co. S-Adenosyl [Me-^{14}C]-L-methionine (Lot No. 850672, sp. act. 53.4 m Ci/mM, in dil. HOAc, pH 4) was purchased from ICN Pharmaceuticals, Inc. Sodium acetate-1-[^{13}C] (90 atom % C-13, Lot No. B-1100) was obtained from Merck Sharpe and Dohme Canada, Ltd. Ergosterol was obtained from ICN and recrystallized from methanol-chloroform. Cycloartenol acetate was a gift from Prof. P. Beneveniste. 25-Fluorocholesterol was obtained from Merck Sharp and Dohme Research Lab, Merck and Co., Inc., U.S.A.

B. 25-Azacholesterol, 23-Azacholesterol, 24-Azacholesterol and 25-Azacholestanol

25-Azacholesterol was prepared by the method of Counsell et al. (1965) and melted at 146.5-148.5° (reported 147.5-149.5 (Counsell et al., 1965)).

23-Azacholesterol was synthesized by Dr. H.D. Pierce using the method of Counsell et al. (1965) and melted at 128-129° (reported 129-131° (Counsell et al., 1965)).

24-Azacholesterol was synthesized by Mr. M. McGuire from 3β-acetoxy-22,23-bisnor-5-cholenic acid (Steraloids, Inc.) by way of an Arndt-Eistert chain extension (Sax and Bergmann, 1955). This product was then converted to the azasterol by the

method of Counsell et al. (1965) and melted at 159.5-162.5° (reported 164-166° (Counsell et al., 1965)).

25-Azacholestanol was prepared using a method similar to that of Bruce (1966) and gave a melting point of 144.5-145.5°. MS (calculated $C_{26}H_{47}NO$, 389) 389 (M^+, 45), 374 (M^+-CH_3, 25), 58 ($(CH_3)_2N^+$, 100).

C. 25-Aza-24,25-Dihydrozymosterol

25-Aza-24,25-dihydrozymosterol was synthesized by the method of Avruch et al. (1976) with the following modifications. The zymosterol was obtained from a culture of the erg 5 mutant of S. cerevisiae (Pierce et al., 1979) grown with 25-azacholesterol (.5 μM). The zymosterol was purified as the acetate by flash chromatography (Still et al., 1978) and by silver nitrate (25%) preparative thin layer chromatography (P-TLC) to 99+% purity (by GLC, OV-101, 10 m, 0.25 mm i.d., capillary column). Equimolar amounts of zymosterol acetate (.150 g, .35 mmoles) and N-bromosuccinimide (.060 g, .35 mmoles) (recrystallized from water) were added to THF-water (5:1) slowly over 10 min and then stirred for 2 h. The workup procedure was the same as one used previously (dilution with water, extraction with ether, drying, filtration, removal of solvent). The residue was dissolved in acetone:MeOH (3:1) and stirred with an excess of K_2CO_3 (.180 g) at 25° for 1.5 h. The mixture was again diluted with water and extracted with ether. The ethereal extract was washed with water and the extract dried. The residue (.140 g) was purified by preparative thin layer chromatography to yield .132 g of the

24,25-epoxy-zymosterol. The epoxide was immediately manipulated as previously described to the final product, 25-aza-24,25-dihydrozymosterol (overall yield 50%) which melted at 112-114° (lit. 110-116° (Avruch et al., 1976)). The azasterol was analyzed by GLC and found to be 96% pure. MS (calculated $C_{26}H_{45}NO$, 387) (CH_4-CI), 388.1 (P+1, 56), 387 (P, 54.4), 386 (P-1, 88.7), 370 (P+1-18 (H_2O), 100).

D. 25-Aza-24,25-Dihydrolanosterol

25-Aza-24,25-dihydrolanosterol was prepared from commercial grade lanosterol. Oxidation of the Δ^{24} linkage (Bernassau and Fetizon, 1975) gave the tri-noracid which was then converted to the methyl ester of 3β-acetoxy-25,26,27-trisnorlanost-8-en-24-oic acid. This was converted to the azasterol as follows. Dimethyl amine (0.945 g), dissolved in THF (8 mL), was added to a suspension of $LiAlH_4$ (0.235 g) in THF (50 mL) and allowed to stir at r.t. for 1 hour. The amine-$LiAlH_4$ product was cooled to 0° and methyl-3β-acetoxy-25,26,27-8-en-24-oate (0.15 g) dissolved in THF (25 mL) was added. The mixture was stirred for 2 h at 0° and 1.5 h at r.t. (Khanna et al., 1975). Water:THF (1:1, v/v, 10 mL), 3 N NaOH solution (10 mL) and water (20 mL) were added successively to the suspension. The mixture was filtered and the filtrate extracted with ether (3 × 50 mL). The extract was dried over anhydrous Na_2SO_4, filtered and concentrated in vacuo. The residue was recrystallized from acetone-dioxane to give 0.08 g, m.p. 163-165°C (reported 162-164° (Lu et

al., 1971)). NMR ($CDCl_3$) δ 0.70 (s, 3H, $C_{18}H$), 0.81 (s, 3H, 4βMe), 0.88 (s, 3H, $C_{14}Me$), 0.99 (s, 6H, 4αMe and $C_{19}H$), 2.22 (s, 6H, C_{26} and $C_{27}H$), 3.03-3.90 (m, 1H, C_3H). MS (calculated for $C_{29}H_{51}NO$, 429) 429 (M^+, 90) 414(M^+-CH_3, 40), 58 ($(CH_3)_2NCH_2^+$, 100).

E. Synthesis of (20R) and (20S) 22,25-Diazacholesterol

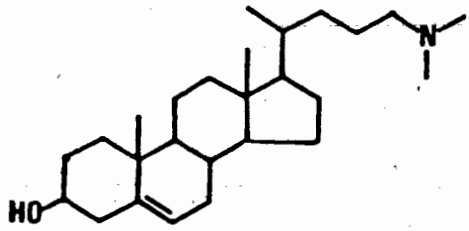
(20R) and (20S)-22,25-Diazacholesterols were synthesized by Dr. H.D. Pierce, Jr. as previously reported (Counsell et al., 1965). Separation and purification of the (20R) and (20S) 22,25-diazacholesterols (2 g) was achieved by column chromatography on 300 g of silica gel. Elution with 500 mL of $CHCl_3$:CH_3OH (1:1, v/v) taking 10 mL fractions gave 0.35 g of a 5-pregnendiol (M^+, 318). Elution with an additional 400 mL of this solvent system gave the (20R) isomer which was one spot on TLC (Silica Gel, GP 254, $CHCl_3$:CH_3OH, 1:1, v/v). The compound was crystallized from ethanol:water (3:7) to give 0.67 g, m.p. 129.5-130.5°C. NMR ($CDCl_3$) δ 0.75 (s, 3H, $C_{18}H$), 0.99 (d, J = 6 Hz, 3H, $C_{21}H$), 1.02 (s, 3H, $C_{19}H$), 2.24 (s, 6H, C_{26} and $C_{27}H$), 5.3 (m, 1H, C_5H). M.s. (calculated for $C_{24}H_{44}N_2O$, 388) 388 (M^+, 0.6), 330 ($M^+-CH_2N(CH_3)_2$, 66.3), 58 ($(CH_3)_2N^+-CH_2$, 100).

Elution with an additional liter of $CHCl_3$:CH_3OH (1:1) gave after crystallization from methanol:water, (20S) 22,25-diazacholesterol, 0.125 g, m.p. 112.5-113.5°C (reported 110-113°C (Counsell et al., 1965)). NMR ($CDCl_3$), δ 0.70 (s, 3H, $C_{18}H$),

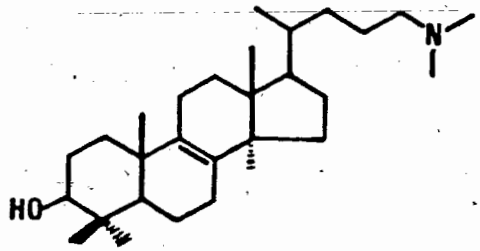
1.01 (s, 3H, $C_{19}H$), 1.08 (d, J = 6 Hz, 3H, $C_{21}H$), 2.22 (s, 6H, C_{26} and $C_{27}H$), 5.31 (m, 1H, C_5H). MS (calculated for $C_{24}H_{44}N_2O$, 388) 388 (M^+, 0.4), 330 ($M^+-CH_2N(CH_3)_2$, 51.5), 58 ($(CH_3)_2N^+-CH_2$, 100).

F. 25-Methyl-25-Azacholesterol Iodide

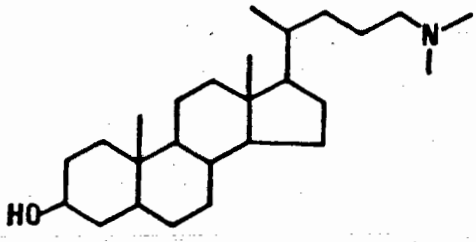
25-Azacholesterol (30 mg) dissolved in 5 mL of methanol was stirred with .1 g of K_2CO_3 and .1 mL of methyl iodide at r.t. (Chen and Benoiton, 1976). The reaction was monitored by TLC ($CHCl_3$:MeOH:NH_4OH, 60:40:.6) and stopped by the addition of water after 24 h. The reaction mixture was evaporated and the solids dissolved in hot acetone-water, filtered and crystallized (on cooling). The solid product was filtered and washed with ether to yield 12 mg (38%). NMR (CD_3OD) δ 0.69 (s, 3H, $C_{18}H$), 1.02 (s, 3H, $C_{19}H$), 3.20 (s, 9H, $-N(CH_3)_3$), 5.27 (m, 1H, C_5H). MS (calculated for $C_{27}H_{48}NO$, 402) 387 (M^+-15 (CH_3), 10), 360(20), 342 ($M-(CH_3)_3NH$, 40) (low resolution). M.p. 270° (turns brown), 280° (melts). TLC (60:40:.6, $CHCl_3$:MeOH:NH_4OH) R_f = .029 (R_f for 25-azacholesterol = .58).



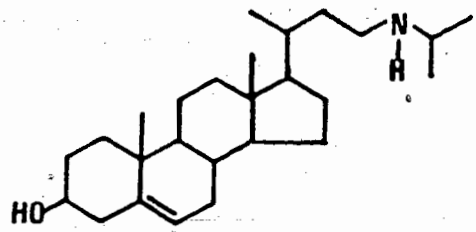
25- azacholesterol



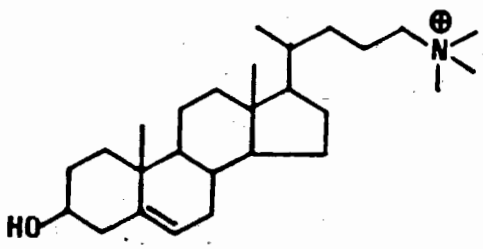
25-azalanosterol



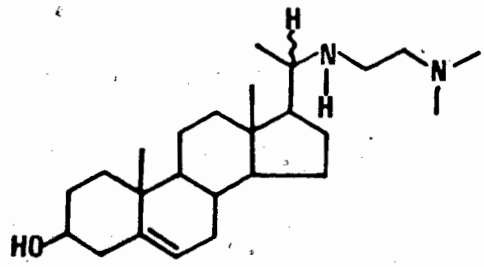
25-azacholestanol



24- azacholesterol



25-methyl-25-azacholesterol



22,25- diazacholesterol

G. Isolation of Zymosterol

A culture of wild type S. cerevisiae was grown in the presence of 1.0 μM 25-azacholesterol. The yeast was harvested by centrifugation after 24 h growth, washed and hydrolyzed in 10% KOH-ethanol (10 mL per g of yeast). The non-saponifiable fraction (NSF) was obtained by diluting the base hydrolysate with an equal volume of water and then extracting the mixture with an equal volume of hexane (divided into four equal portions). The hexane fraction was washed with water, dried with $MgSO_4$ and filtered. The solvent was removed and the NSF was kept at -25°

until used. One gram of the NSF was dissolved in hexane and chromatographed on alumina (act III, 100 g). Elution was performed with hexane (400 mL), hexane/ethyl acetate, 95:5 (v/v) and successively with 90:10, 85:15 and 80:20 (v/v) hexane/ethyl acetate (500 mL each). Fractions of 15 mL were taken and analyzed by GLC on an OV-101 capillary column (10 m/.25 mm i.d.) as the TMS derivatives. Zymosterol and cholesta-7,24-dien-3β-ol as well as small amounts of C_{28} and $\Delta^{5,7}$-sterols eluted together in the last two solvent systems. In order to remove these final impurities (C_{28} and $\Delta^{5,7}$-sterols) these fractions were acetylated with acetic anhydride/pyridine (2:1). The crude acetates (0.65 g) were then subjected to chromatography on alumina (act III, 65 g) impregnated with 25% $AgNO_3$. Elution was performed with 9:1 hexane/benzene (400 mL) then successively 8:2 and 7:3 hexane/benzene (400 mL each, v/v), while collecting 15 mL fractions and monitoring by the Liebermann-Burchard test (Liebermann, 1885). Zymosterol acetate eluted in the last solvent system. The zymosterol acetate was hydrolyzed and the free sterol was recrystallized from MeOH-$CHCl_3$. This was analyzed by GLC and found to be 96% pure (OV-101 capillary column).

H. Culture Medium and Yeast Strain

The culture medium and S. cerevisiae strain (wild type) used were as previously described (Avruch et al., 1976). The sterol mutants erg 2 and erg 5 were derived from the wild type strain A184D (erg^+) of S. cerevisiae and have been previously

described (Molzahn and Woods, 1972; Bard et al., 1977). Candida utilis (ATCC 8205) was the strain of yeast used.

II. Methods

A. Instrumentation

A Varian 2100 gas chromatograph equipped with a flame ionization detector was employed for sterol analysis using two types of columns: Silar 10C, 1.83 m × 2 mm glass U-tube packed with 3% Silar 10C on Gas Chrom Q (100/120); OV-101 capillary, 30 m × 0.25 mm glass capillary coated with OV-101. Acetates were analyzed on both columns relative to cholestanol acetate (OV-101 at 245°, Silar 10C at 220°). Trimethylsilyl ethers were analyzed at 240° relative to trimethylsilyl cholestanol (Pierce et al., 1979). Mass spectra of sterol acetates and free sterols were obtained on two machines. One was a Hitachi-Perkin Elmer RMU-6E mass spectrometer (ionization voltage of 80 eV and probe temperature 180°) coupled to a Varian 1400 gas chromatograph using a 1.83 m × 2.2 mm glass column packed with 3% SILAR 10C on Gas Chrom Q (100/120) at 230°. The other was a Hewlett-Packard 5985B GC/MS system mass spectrometer (ionization voltage 70 eV, CH_4 chemical ionization; direct insertion probe temperature, 200-280°). NMR spectra were recorded on three machines: Varian A-60, XL-100 or EM-360 spectrometers; samples were dissolved in $CDCl_3$ containing tetramethylsilane at 25°. In the case of 25-methyl-25-azacholesterol iodide, methanol-d_4 was used as the solvent. Ultraviolet spectra were recorded on a Unicam SP8000 spectrophotometer. Radioactivity was determined by Beckmann liquid scintillation counters, Model No. LS-8000 and LS-200B. Fermentations were done on a Virtis fermenter, Model 40-300.

Melting points were determined on a Fisher-Johns apparatus. Color determinations for the Lowry protein assay were done on a Coleman II Junior spectrophotometer. Centrifugations were carried out on either an IEC centrifuge, a Sorval RC-5 centrifuge or a Beckmann L5-75 preparative ultracentrifuge.

B. Inhibition Experiments and Isolation of the Non-Saponifiable Fraction

A starter culture was prepared by inoculating 10 mL of medium with a loop of S. cerevisiae (wild type) cells and incubating for 24 hours at 30°. This was added to 100 mL of medium containing azasterol at the same concentration as used in the 0.1-1 µM inhibition experiments. This inoculum culture was grown for 48 hours at 25°C. This method was also employed when growing erg 5 sterol mutant yeast, except that 25-azacholesterol was used at 1.0 µM. The C. utilis yeast cultures were also started as above, but only grown in the 100 mL inoculum stage for 24 h. For the erg 2 mutant (grown with .5 µM 25-azacholesterol) as well as for 5 and 10 µM inhibitor experiments, the starter culture and inoculum culture volumes were 20 mL and 200 mL, respectively. Each inoculum culture was transferred to a 4 L Virtis fermentor jar containing 1.5 L of stirred (400 rev/min) medium to which azasterol, as a solution in ethanol (1-2 mL), had been added before inoculation. The azasterol was added in .4 mL aliquots for the sterol mutants erg 2, erg 5 and C. utilis before inoculation. After stirring for 0.5 h, the cultures were aerated (1.9 L/min) for 24 h, or 48 h for the mutants, at 30° on

a Virtis fermenter. Dow Antifoam A spray or Dow Corning Antifoam "C" emulsion was added to cultures to control foaming.

The yeast cells were harvested by centrifugation (20 min at 2500 × g), washed twice with distilled water, weighed wet and either saponified immediately (Avruch et al., 1976) or stored at -27°C until later processing.

C. General Method for Separation and Analysis of Yeast Sterols

A chloroform solution containing about 90 mg of the non-saponifiable fraction of each culture was applied to three plates (20 × 20 cm), coated with a 0.5 mm layer of silica gel GF-254 impregnated with 25% silver nitrate and 0.2% rhodamine 6G, by weight. The plates were developed with methylcyclohexane/ethyl acetate (95:5, v/v) and several bands were visualized under shortwave ultraviolet light. The R_f values and composition of the bands on developed plates are as follows: 0.2-0.3, $\Delta^{5,7}$-dienes; 0.35-0.46, desmethyl sterols; 0.47-0.51, 4α-methyl sterols; 0.52-0.56, 4,4-dimethyl sterols; 0.60-0.67, squalene. The bands were removed and thoroughly extracted with ether. After evaporation of the ether, the fractions were processed to remove colored impurities as described previously (Fryberg et al., 1973). Each purified fraction was dried to constant weight, taken up in chloroform (1-2 mL) and aliquots of the solution transferred to microsample tubes (Clayton, 1962) (25-30 × 6 mm) containing 0.1 mg cholestanol. Solvent was removed under an N_2 stream. The tubes were stoppered with

sleeve-type rubber septa (4.5 mm) and the trimethylsilyl ether derivatives were prepared by injection of 50-100 µL Tri-Sil "TBT" and heating to 50°C for 5-10 minutes. Acetate derivatives were prepared in stoppered microsample tubes by overnight reaction with pyridine/acetic anhydride (1:1, v/v). Reagent was removed by evaporation under a nitrogen stream. The residue was dissolved in 50-100 µL benzene or heptane for analysis. Each derivatized fraction was analyzed in duplicate by gas chromatography. The presence of the individual sterols in the separated fractions was established by comparison of the relative retention times of the peaks on the chromatograms to standard values (Pierce et al., 1978a; Pierce et al., 1979).

D. Production of [^{13}C] Enriched Sterols and Conversion of Zymosterol to Cholesterol

1. Generation of [^{13}C] enriched sterols

A starter culture of S. cerevisiae was prepared by inoculating 10 mL of culture medium (Avruch et al., 1976) with a loop of cells and incubation for 24 h at 30°. Three such tubes were added to a 4-L Virtis fermenter jar containing 1 L of stirred (300 rpm) medium with 1-2 g of 1-[^{13}C]-sodium acetate (dissolved in 10 mL of distilled water and autoclaved prior to addition). After addition of the yeast, the culture was aerated (.5 L/min) for 72 h at 30° on the fermenter. Dow antifoam A or Dow Corning Antifoam "C" emulsion was added to the culture to control foaming.

Azasterol treatment involved generation of a 10 mL starter

culture which was transferred to 100 mL of medium containing .2-.4 μM azasterol and [^{13}C] acetate as above.

The yeast was harvested by centrifugation (20 min at 2500 × g), washed twice with distilled water, weighed and either saponified immediately or stored at -27° until processed.

2. Analysis of [^{13}C]-enrichment of the sterols

The NSF obtained from yeast (S. cerevisiae), grown as above, was separated into various fractions by preparative TLC as outlined earlier. In the case of zymosterol, the appropriate PTLC band was removed and extracted with ether. The sterol isolated was analyzed by mass spectrometry and ^{13}C NMR. Enrichment of the sterol with ^{13}C can be determined in two ways. By comparing the peak heights of enriched carbon signals to natural abundance carbon signals in the ^{13}C NMR spectra, one can determine the relative increase in the various resonances. Enrichment can also be calculated from the mass spectrum of the isolated sterol by assuming 100% enrichment was 100% of ^{13}C at each of twelve positions.

Enriched ergosterol was isolated as the acetate from preparative TLC plates, impregnated with silver nitrate, which were developed with benzene as eluent.

3. Hydrogenation of zymosteryl acetate (unenriched)

Following a procedure similar to Kircher (1974), zymosteryl acetate (.400 g), 10% Pd/C (.350 g), ethyl acetate (15 mL) and glacial acetic acid (5 mL) were placed in a 150 mL glass-lined Parr autoclave. Hydrogenation was conducted with stirring at

150° over 1.5 h using a H_2 pressure of 13.5 atm followed by 34 atm for 1 h at 150°. Removal of the catalyst and solvent gave an 85% yield of cholestanyl acetate, m.p. 109-111° (confirmed by MS and NMR).

4. Transformation of cholestanyl acetate to Cholest-4-en-3-one

Cholestanyl acetate obtained above (unenriched) was deacetylated with base (Fryberg et al., 1973) in a yield of 85%, giving a product of m.p. 141-142° (lit. 142-143° (Bruce, 1943)). The cholestanol was then oxidized to 5α-cholesta-3-one (Bruce, 1943) in 85% yield m.p. 128-129° (lit. 129-130° (Bruce, 1943)). Bromination (Wilds and Djerassi, 1946) gave 2α,4α-dibromo-cholestan-3-one in a 68% yield, m.p. 185-187° (lit. 187-188° (Wilds and Djerassi, 1946)). Conversion of the dibromo derivative to the Δ^4-ketone, via the iodo derivative (not isolated (Rosenkranz et al., 1950)), yielded cholest-4-ene-3-one in 78% yield, m.p. 81-82° (lit. 80-81° (Rosenkranz et al., 1950). Structures were confirmed by NMR and mass spectral data.

5. $\Delta^{3,5}$-Cholestadiene-3β-acetate

A solution of .40 g of cholesta-4-ene-3-one in 10 mL of isopropenyl acetate and .08 g of p-toluenesulphonic acid was refluxed for 2 h (Hagemeyer and Hull, 1948). At the end of the first hour, the condenser was removed and the solution heated gently for 15 min. The solution was then refluxed for an additional hour. At that time, .2 g of anhydrous sodium acetate was added and the mixture was concentrated in vacuo. The residue

was diluted with chloroform and filtered from the sodium acetate. Additional chloroform was added and this solution was added to boiling methanol. Crystallization from chloroform-methanol yielded .220 g (50% yield), m.p. 70-72° (lit. 76-78° (Hagemeyer and Hull, 1948)). NMR ($CDCl_3$) δ 2.11 (s, 3H, C-3βAcO), 5.41 (m, 1H, C_5H), 5.75 (d, 1H, C_3H). MS (calculated for $C_{29}H_{43}O_2$, 426) 426 (M^+, 18), 366 (M^+-CH_3COOH, 100).

6. Cholesterol

Reduction of the enol acetate was accomplished following procedures similar to Dauben and Eastham (1951) and Belleau and Gallagher (1951), with some modifications. A sample of .10 g of $\Delta^{3,5}$-cholestadiene-3β-acetate was dissolved in 95% ethanol (65 mL) and cooled to 5°. A sample of .20 g of $NaBH_4$ dissolved in 70% ethanol (7.5 mL) was added and kept at 5° for 2 h. The ethanol solution was then heated to reflux and 2.5 mL of 5% NaOH was added to the solution. This mixture was then cooled and the ethanol evaporated. The residue was dissolved in ether and washed with 2 N HCl solution (50 mL). The ether was evaporated and the residue dissolved in ethanol (25 mL). This was heated to reflux with .5 mL of conc. HCl for 1 h. The ethanol was removed and the residue dissolved again in ether. The ether was washed with water, dried and removed. Efforts to recrystallize the residue (.080 g) gave low yields (.035 g) of impure material m.p. 119-130° consisting of cholesterol and cholestanol in a 70:30 ratio, which was confirmed by mass spectral and gas chromatographic analysis. The total residue (.080 g) was

recovered for further purification.

7. Purification of Cholesterol

A sample of the sterol mixture (.055 g) from the reduction was acetylated asbefore (pyridine/Ac_2O) and the crude product (.060 g) was separated (.030 g per plate) on .5 mm thick, Silica Gel HF-366 and 254-20% $AgNO_3$ plates (by weight), doubly developed in hexane/benzene, 5:2 (V/V), in an unlined tank as described by Idler and Safe (1972). The band at R_f = .61, which matched the value of the cholestanyl acetate standard, was removed from the plate and eluted with ether. After the ether was removed (stream of N_2), the white crystalline solid left weighed .013 g, m.p. 109° (lit. 111° (Windholz, 1976)) (21% yield). The band at R_f = .50, which matched the value of the cholesteryl acetate standard, was removed from the plate and eluted with ether which after solvent removal, gave a white crystalline solid weighing .028 g, m.p. 113-114° (lit. 115-116° (Windholz, 1976)) (47% yield). After base hydrolysis, the residue was recrystallized from MeOH-H_2O giving .022 g (88% yield) of cholesterol m.p. 144-146° (lit. 148° (Windholz, 1976)).

8. ^{13}C NMR experiments with [^{13}C] enriched zymosterol

Zymosterol, unenriched, (.030 g) was dissolved in 1.0 mL of $CDCl_3$ and put into a 5 mm OD tube, and a ^{13}C NMR spectrum was taken (Fig. 4) (P. 59).

Zymosterol, (.015 g, 7% enriched) was dissolved in 1.0 mL of $CDCl_3$ and put into a 5 mm OD tube, and a ^{13}C NMR spectrum was taken (Fig. 4).

DL-α-phosphatidyl choline, dipalmitoyl (DPL) was dissolved in $CDCl_3$ (.5 M) and portions of this solution were added to the tube containing the enriched steroid. Spectra of the DPL steroid mixture were taken after every addition. The DPL concentrations were 40, 60, and 70 mole percent (Fig. 5).

Spectra were run on a Varian XL-100-15 in a pulse Fourier transform (FT) operation at 25.2 MHz with ^1H noise decoupling.

E. 25-Hydroxy Provitamin D_3 Synthesis

1. Erg 5 growth: Isolation and analysis of NSF

The growth of the erg 5 mutant in the presence of 1.0 μM 25-azacholesterol was as described above. The procedure for the isolation of the NSF was modified as follows. After extraction of crude NSF, hexane was removed and the residue dissolved in pyridine/acetic anhydride (1:2). The solution was left in a stoppered Erlenmeyer flask, wrapped in foil, in the dark. After 20 h, this solution was worked up by addition of the contents to twice their volume of ice. The mixture was extracted with ether and the ether extract was washed with .1 N HCl, 2% $NaHCO_3$ and saturated NaCl solution in succession. The ether was dried, filtered and the solvent removed. The crude acetates were analyzed by GLC on an OV-101 quartz capillary (10 m × .25 mm i.d.) and 3% Silar 10C packed column with cholestanyl acetate (.1 mg) as the internal standard. The TMS derivative of the crude NSF was also analyzed (a small sample taken before acetylation) in the standard fashion. The crude acetates were kept in the dark (aluminum foil wrap) at -27° until used.

2. 1,4-Cycloaddition of 4-Phenyl-1,2,4-triazoline-3,5-dione to $\Delta^{5,7}$-diene NSF acetates

A sample of crude NSF acetates (.900 g) was dissolved in dry acetone (60 mL) and cooled with dry ice acetone to -70° in a 250 mL, two-necked flask equipped with a drying tube (Drierite) and an pressure equalizing addition funnel. To this was added slowly, dropwise with stirring .128 g of 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (Barton et al., 1971) dissolved in 10 mL of acetone, until a red colour persisted. The mixture was maintained at -70° for 3 h. At that time, 2.5 g of neutral alumina, activity grade V (15% water) was added and the mixture allowed to warm to 25° over 1.5 h. The mixture was filtered and the solvent removed. TLC (95:5, benzene/ethyl acetate) of the crude product showed one spot (R_f = .2) which ran close to an authentic sample of the 1,4-cycloadduct of ergosteryl acetate (R_f = .23). The crude product (1.08 g) was used below without further purification.

3. Column chromatography of 1,4-cycloadducts of $\Delta^{5,7}$-dienes

The crude product from the 1,4-cycloaddition reaction was dissolved in benzene and added to a column of silica gel, 60-200 mesh (120 g) in benzene. The column was eluted with benzene (1500 mL) until no additional sterol was eluted. The column was then eluted with benzene/ethyl acetate, 70:30 (500 mL). Each fraction was concentrated and the residues analyzed first by TLC (95:5, benzene/ethyl acetate) and then by GLC. The initial

fraction (benzene) contained the NSF acetates lacking the $\Delta^{5,7}$ dienes (.689 g), while the second fraction (benzene/ethyl acetate, 70:30) contained the cycloadducts of $\Delta^{5,7}$-diene steryl acetates (.358 g).

4. Epoxidization of side chain double bonds and separation of products

A sample of .130 g of 1,4-cycloadduct of PTAD to $\Delta^{5,7}$ steryl acetates (from column chromatography) was dissolved in methylene chloride (7 mL). To this solution was added, with stirring at 0°, .040 g of m-chloroperbenzoic acid (≈ 95% pure). The mixture was stirred for 18 h (Morisaki et al., 1973) then shaken with aqueous solutions of potassium iodide, sodium thiosulphate, sodium bicarbonate and then water. The organic layer was dried with anhydrous sodium sulphate, filtered and concentrated. The residue was purified into two major components by PTLC (15:85, ethyl acetate/benzene) on Silica Gel GF. The bands were visualized by spraying with a solution of Rhodamine 6G in acetone. The sterols isolated from the bands were analyzed by NMR in $CDCl_3$ and MS. The upper band (.058 g, R_f = .62) was the 1,4-cycloadduct of PTAD to ergosta-5,7-dien-3β-acetate, m.p. 181-183°. NMR ($CDCl_3$) δ .72, .80, .90, .98 (15H, Me groups), 2.00 (3H, 3β-AcO), 3.1 (1H, C_3H), 6.16 (1H, d, J = 8 Hz), 6.4 (1H, d, J = 8 Hz), 7.33 (5H, m, Ph). MS (calculated for $C_{38}H_{53}O_4$, 615) (CH_4-CI) 614 (P-1,5), 381 (P+1-AcO-$C_8H_5N_3O_2$, 95), 379 (P-1-AcO-$C_8H_5N_3O_2$, 100).

The more polar band (.060 g, R_f = .34) was the 1,4-cycloadduct of PTAD to 24,25-epoxy-cholesta-5,7,24-trien-3β-acetate, m.p. 170-173°. NMR ($CDCl_3$) .80 (s, 3H, C_{18}-Me), .97 (6H, C_{21} and C_{19}Me), 1.23 and 1.28 (6H, C_{26} and C_{27}-Me), 2.00 (s, 3H, 3β-AcO), 6.17 (1H, d, J = 8 Hz), 6.37 (1H, d, J = 8 Hz), 7.33 (5H, m, Ph). MS (calculated for $C_{37}H_{47}N_3O_5$, 613) 440 ($M-C_8H_5N_3O_2$, .17), 380 ($M-C_8H_5N_3O_2$-AcO, 100), 365 ($M-C_8H_5N_3O_2$-AcO-CH_3, 11).

5. Production of cholesta-5,7-dien-3β,25-diol

A sample of the 1,4-cycloadduct of PTAD to 24,25-epoxy-cholesta-5,7,24-trien-3β-acetate (.035 g) was dissolved in dry THF (5 mL) and treated with lithium aluminum hydride (.060 g). The mixture was refluxed under nitrogen for 18 h. Excess hydride was destroyed with wet THF (2:1, THF/water) and then 2 N NaOH (2 mL) was added. After stirring for 15 min, water (5 mL) was added and the mixture was stirred for another 15 min. The mixture was extracted with ether/THF (1:1), 3 × 20 mL and the organic layer was washed with saturated NaCl solution. The organic layer was then dried over anhydrous sodium sulphate, filtered and concentrated. The residue was recrystallized from hot methanol-water to give after drying .013 g (57% yield) of platelet crystals, m.p. 187-190° (lit. 188.5-191.5° (ethanol-ethyl acetate) (Halkes and Van Liet, 1969). NMR ($CDCl_3$ + CD_3OD) δ 1.62 (s, 3H, C_{18}-Me), .97 (6H, C_{21} and C_{19}-Me), 1.19 (s, 6H, C_{26} and C_{27}-Me), 4.59 (b, 1H, C_3-H), 5.14 (m, 2H, $C_{6,7}$-H). MS ($C_{27}H_{44}O_2$, 400.6) 400.5 (M^+, 45), 367 (M^+-2H_2O, 52), 341

(M^+-$(CH_3)_2COH$, 42.5), 271 (M^+-side chain or $C_{19}H_{27}O$), 59 ($(CH_3)_2COH$, 100).

F. Preparation of Cell Free Δ^{24}-Sterol Methyltransferase

A starter culture of yeast was prepared by inoculating 4 × 10 mL of medium with a loop of S. cerevisiae, wild type, cells and incubating them for 24 h at 30°. These 10 mL inoculum were added to 2 × 1.5 L (two 10 mL inoculum to each 1.5 L of medium) of growth medium in 4 L Virtis fermenter jars. The cultures were stirred at 400 rev/min for 15 min and then aerated for 20 h at 30° on a Virtis fermenter with continuous stirring. The remaining steps were essentially the same as the method of Bailey et al. (1974). The yeast cells were harvested by centrifugation (20 min at 2500 × g), washed once with .1 M Tris-HCl buffer (pH 7.6, containing .1 mM Mg^{++} ($MgCl_2$)) and weighed wet. The yeast cells were suspended in .1 M Tris-HCl buffer to a final concentration of 1 g of cells/mL. The remaining steps were carried out at 4°. Twenty-five mL of yeast cells were added to a 75 mL Duran flask containing 40 g of .25 mm glass beads. The flask was stoppered and secured in a Braun MSK cell homogenizer. The cells were given a 45s burst and the homogenate was put on ice. The total homogenate was centrifuged at 25,000 × g for 20 min and the pellet was discarded. The supernatant was centrifuged at 105,000 × g for 1 h in a Beckmann L5-75 preparative ultracentrifuge. The supernatant was removed and the pellet was resuspended in .1 M Tris-HCl buffer, pH 7.6. The protein concentration was determined by the method of Lowry et

al. (1951) using bovine serum albumin (Sigma) as a standard. The suspension was divided into aliquots and stored at -25° until needed. The preparation was stable at this temperature for 3 weeks.

G. General Enzyme Assay Procedure

The method of assaying Δ^{24}-sterol methyltransferase activity was similar to that used by Thompson et al. (1974). Enzyme activity was assayed by measuring [^{14}C] incorporation from [^{14}C-S-methyl]-S-adenosyl-L-methionine into the non-saponifiable lipid fraction of enzyme preparations to which zymosterol had been added. All incubations were carried out in 25 mL Erlenmeyer flasks equipped with 14/20 ground glass joints. The assay flasks contained 3.2 mL of 0.1 M Tris-HCl buffer, pH 7.6, 10 µmoles of $MgCl_2 \cdot 5H_2O$, 60 µmoles of $KHCO_3$, 50 to 400 nmoles of sterol substrate (e.g., zymosterol), and .5 mL of enzyme preparation (protein concentration of enzyme preparation ≈15 mg/mL) (Lowry et al, 1951). Substrate and azasterol, dissolved in a total of .2 mL of ethanol, were then added, successively, to the reaction mixture. When S-adenosyl-L-homocysteine was used as an inhibitor it was dissolved in buffer (.1 M Tris-HCl, pH 7.6), rather than ethanol. The reaction was initiated by the addition of 0.1 mL of buffer containing .1 µCi of S-adenosyl-[Me-^{14}C]-L-methionine diluted in 400 nmoles of SAM. The flasks were stoppered and were incubated with gentle shaking at 30° in a water bath. The reaction was stopped by addition of 2 mL of 60% KOH solution. The flasks were removed and heated to reflux (boiling

chips added) for 1 h under N_2. Each sample solution was diluted with 1 mL of distilled water and extracted twice with 10 mL hexane. The combined hexane extracts were washed twice with water, dried over anhydrous Na_2SO_4 and filtered. The hexane was evaporated and the residue transferred into a liquid scintillation vial with 10 mL of Aquasol I (ICN) counter fluid and counted for 10 min. The amount of transmethylation was calculated from the count rates and the specific activity of the substrate. The transmethylation reaction by the cell extract was linear for up to 1.5 mL of enzyme preparation for 30 min.

The standard error depicted by the error bars in the figures was calculated from the variation between duplicate assays. The counting efficiency of the scintillation counter was 95 ± 1.0%. The counting error was typically between .5 and 1.5%. The counts for the samples varied between 1500 cpm ± 1.5% (background) to 2.4×10^4 cpm ± .5% (100 μM zymosterol, uninhibited). The error in work-up from sample to sample as expressed in cpm varied between 5 and 15% for typical samples in a kinetic experiment.

Dixon plots (Dixon, 1953) were used to determine type of inhibition caused by the administration of the azasterols. The data was plotted using a linear regression analysis which yielded correlation coefficients of .94 or better for the lines drawn (Hewlett-Packard 33E Scientific Calculator).

RESULTS

A. In Vivo Inhibition Studies on S. cerevisiae

1. Effect of azasterols on yeast growth and sterol production

The presence of sidechain azasterols did not appreciably affect yeast growth at inhibitor concentrations below 1.0 μM, except for 25-aza-24,25-dihydrozymosterol (25 AZ). At .5 μM 25-AZ, dry cell weight was approximately 50% of that for other inhibitors and sterol yield decreased as the concentration of 25-AZ increased. Other inhibitors caused a slight decline in cell yield at above 5 μM concentrations.

2. Effect of azasterol inhibitors on sterol biosynthesis and composition

The 25-azasterols influenced yeast sterol composition greatly as seen from the relative amounts of zymosterol (4), ergosterol (14) and ergostatetraenol (17) in the various cultures (Table I). The amount of ergosterol and ergostatetraenol decreased while zymosterol increased in relative proportion. There was also an increase in the amount of C_{27} sterols, cholesta-7,24-dienol (18), cholest-5,7,24-trienol (19) and cholesta-5,7,22,24-tetraenol (20), when the yeast was treated with 25-azasterols, because of the inhibition of 24-SMT (Figure 2). An estimate of the effectiveness of each inhibitor in blocking this enzyme was obtained by comparison of the proportion of C^{24}-alkylated sterols produced in the presence of the inhibitor ($\sum C^{24}\text{-alk}_i$) with that produced in control cultures

Table I. Effects of Azasterols on Growth and Sterol Biosynthesis of S. cerevisiae[a]

	Concentration of Azasterols in Culture (μM)								
	25-aza-24,25-dihydro-zymosterol				25-aza cholesterol				
	0	.1	.2	.5	0.1	0.2	0.5	1.0	5.0
Dry cell wt (g/L)	11.2	6.4	6.2	4.4	10.7	9.9	9.1	9.9	6.7
Sterols (% of dry cell wt)	4.3	2.6	.47	.23	5.5	6.2	5.7	5.2	4.1
$\Delta^{5,7}$-Dienes (% of dry cell wt)[b]	2.1	1.22	.21	.11	2.2	1.8	1.8	1.6	1.1
Sterol composition (%)[c]									
Lanosterol (1)	4.8	2.0	5.0	3.7	3.2	3.8	3.1	3.6	3.0
4,4-Dimethylzymosterol (2)	4.5	6.1	12.8	13.5	5.1	6.2	4.8	7.5	8.4
4α-Methylzymosterol (3)	2.8	3.5	5.6	4.4	3.4	3.9	3.1	4.2	3.9
Zymosterol (4)	19.7	39.7	46.6	39.8	38.5	48.4	53.6	55.2	59.5
Cholesta-7,24-dienol (18)	nd	4.6	5.1	12	2.5	3.0	3.6	4.3	3.8
Cholesta-5,7,24-trienol (19)	nd	3.6	4.5	6.8	1.3	3.0	5.8	5.3	5.4
Cholesta-5,7,22,24-tetraenol (20)	nd	11.8	12.0	15.0	4.3	11.4	16.1	14.6	16.0
Fecosterol (6)	4.8	5.2	1.4	.3	1.8	0.6	nd	nd	nd
Episterol (11)	5.0	.5	.4	.1	2.0	1.2	nd	nd	nd
Ergosta-7,22,24(28)-trienol (12)	5.1	nd	nd	nd	2.9	1.3	nd	nd	nd
Ergosta-5,7,22,24(28)-tetraenol (17)	21.5	5.0	2.5	2.1	15.0	7.6	3.6	2.2	nd
Ergosterol (14)	30.0	18.0	4.2	2.0	20.0	9.6	6.3	3.1	nd
% Inhibition 24-SMT	0	58	87	94	39	70	85	92	100
% Inhibition 24(28)-MSR	0	nil	nil	nil	nil	nil	nil	nil	nil

Table I (Cont'd)

	Concentration of Azasterols in Culture (μM)							
	25-aza cholestanol	25-aza lanosterol	22,25-diazacholesterol 20R			22,25-diazacholesterol 20S		
	1.0	1.0	0.2	0.5	1.0	0.2	0.5	1.0
Dry cell wt (g/L)	10.4	10.9	12.0	9.9	9.9	12.3	10.9	14.1
Sterols (% of dry cell wt)	5	6	3.4	4.1	5.2	3.9	3.8	3.9
$\Delta^{5,7}$-Dienes (% of dry cell wt)[b]	1.5	2.0	1.1	1.1	1.6	1.2	1.1	0.9
Sterol composition (%)[c]								
Lanosterol (1)	3.5	6.5	7.2	5.5	3.8	5.6	6.2	5.1
4,4-Dimethylzymosterol (2)	6.5	9.1	6.8	7.4	6.0	6.5	7.1	6.2
4α-Methylzymosterol (3)	3.4	4.4	3.1	2.7	3.6	4.0	3.1	4.2
Zymosterol (4)	52.2	37	43.5	49.8	51	45.2	50.3	56.9
Cholesta-7,24-dienol (18)	4.3	2.1	3.4	4.1	4.3	3.3	4.7	4.2
Cholesta-5,7,24-trienol (19)	7.5	nd	1.6	2.8	3.8	3.4	2.4	3.4
Cholesta-5,7,22,24-tetraenol (20)	nd	2.5	3.8	9.7	14.7	2.4	9.8	10.8
Fecosterol (6)	nd	2.6	nd	nd	nd	nd	nd	nd
Episterol (11)	nd	1.7	1.5	nd	nd	2.1	nd	nd
Ergosta-7,22,24(28)-trienol (12)	nd	1.2	2.8	1.5	nd	1.7	1.1	nd
Ergosta-5,7,22,24(28)-tetraenol (17)	1.5	15.2	9.1	5.7	5.6	8.9	6.6	3.7
Ergosterol (14)	4	17.7	17.2	10.8	7.2	16.9	8.7	5.5
% Inhibition 24-SMT	92	44	55	74	81	57	76	86
% Inhibition 24-(28)-MSR	nil	nil	nil	nil	nil	nil	nil	nil

Table I (Cont'd)

	Concentration of Azasterols in Culture (μM)									
	24-aza Cholesterol					23-aza Cholesterol				
	0.2	0.5	1.0	5.0	10.0	0.2	0.5	1	5	10
Dry cell wt (g/L)	10.9	10.4	9.6	10.1	9.1	11.2	10.3	8.6	7.7	2.7
Sterols (% of dry cell wt)	4.3	3.2	4.1	4.7	4.0	4.8	3.9	4.3	5.0	2.0
$\Delta^{5,7}$-Dienes (% of dry cell wt)[b]	1.4	0.8	1.2	1.0	0.6	2.9	2.3	1.7	1.2	0.2
Sterol composition (%)[c]										
Lanosterol (1)	5.8	6.5	3.7	5.8	5.0	5.4	5.4	3.0	2.0	4.0
4,4-Dimethylzymosterol (2)	3.8	4.4	5.1	9.0	9.4	3.5	5.3	2.9	3.7	7.5
4α-Methylzymosterol (3)	2.5	3.0	3.1	4.5	4.6	2.3	3.4	2.1	1.3	4.9
Zymosterol (4)	45.4	56.5	61.8	61.1	61.7	23.8	24.4	36.6	58.4	61.1
Cholesta-7,24-dienol (18)	3.1	2.5	3.1	4.8	4.8	nd	nd	1.6	1.8	2.2
Cholesta-5,7,24-trienol (19)	nd	2.0	1.9	2.2	2.8	nd	nd	nd	nd	nd
Cholesta-5,7,22,24-tetraenol (20)	2.1	3.2	6.4	5.2	5.5	nd	nd	nd	nd	nd
Fecosterol (6)	0.8	nd	nd	nd	nd	5.1	7.5	7.8	9.6	53
Episterol (11)	2.3	1.2	0.2	nd	nd	3.8	3.1	1.6	0.4	0.9
Ergosta-7,22,24(28)-trienol (12)	3.4	2.1	0.2	nd	nd	3.9	2.5	3.3	1.0	0.8
Ergosta-5,7,22,24(28)-Tetraenol (17)	16.8	12.2	10.7	5.7	4.2	35.6	37.3	36.5	20.7	8.6
Ergosterol (14)	14.0	6.4	4.2	1.7	2.0	14.9	9.8	3.6	0.6	0.6
% Inhibition 24-SMT	45	68	78	89	91	5	10	21	52	74
% Inhibition 24(28)-MSR	24	45	52	54	39	62	76	91	97.6	95.6

Table I (Cont'd)

a In addition, the sterol mixtures were analyzed by gas-liquid partition chromatography for the sterols (Fryberg et al., 1973).

b Determined by ultraviolet spectroscopy.

c Determined by g.l.p.c., ergosta-8,22,24(28)trienol was present (1.8%) in the control.

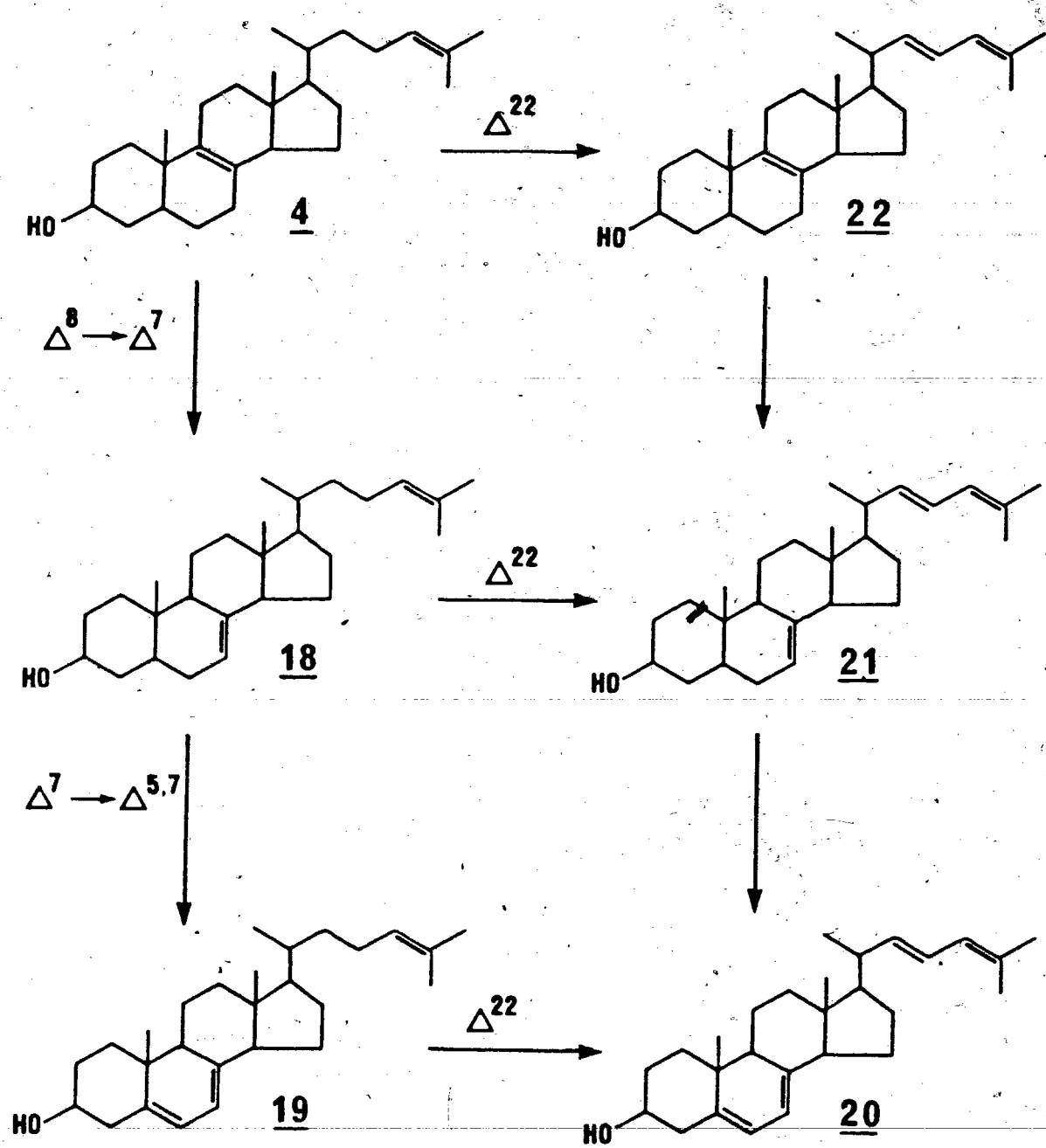
d Values from Pierce et al. (1978a).

nd = not detected (<0.1%).

Figure 2

Expected Sterols from Δ^{24}-Sterol Methyltransferase Block.

Fig. 2



($\sum C^{24}\text{-alk}_c$) according to the expression:

$$\text{percent inhibition} = 100\left[1-\left(\frac{\sum C^{24}\text{-alk}_i}{\sum C^{24}\text{-alk}_c}\right)\right]$$

The % inhibition for each concentration of inhibitor is given in Table I.

Of the 25-azasterols, 25-aza-24,25-dihydrozymosterol was the most effective inhibitor of 24-SMT while 25-aza-24,25-dihydrolanosterol was the least effective. The inhibition of 24-SMT decreases slightly when the nitrogen is moved from 25 to 24, but greatly when moved from 24 to 23 (Oehlschlager et al., 1980).

The 22,25-diazacholesterols inhibited 24-SMT less than the other 25-azasterols. There was little difference in the inhibition of 24-SMT between the 20S and 20R isomers.

The relative effectiveness of each azasterol tested as an inhibitor of the 24-SMT can be seen most clearly, when comparing the concentration of each azasterol required to effect 50% inhibition of the 24-SMT enzyme (Table II).

Comparing the relative amounts of sterols of S. cerevisiae cultures grown with and without 23 and 24-azacholesterol and 25-azasterols revealed 24-methylene $\Delta^{24(28)}$-sterol reductase (24(28)-MSR) is inhibited by 23 and 24-azacholesterol, but not by 25-azasterols. This inhibition was calculated by comparing the ratio of ergosterol to $\Delta^{24(28)}$ sterols in inhibited cultures ($\sum \Delta^{24(28)}$-sterols i) with the ratio of ergosterol to

Table II. Efficiency of Azasterol Inhibition of Δ^{24}-Sterol Methyltransferase and 24-Methylene $\Delta^{24(28)}$-Sterol-reductase in S. cerevisiae[a]

Azasterol[b]	Concentration (μM) Required for 50% Inhibition		Relative Effectiveness as Inhibitor	Activity of Corresponding Δ^{24}-Sterol as Substrate for 24-SMT (Moore and Gaylor, 1970)
	24-SMT	24(28)MSR	24-SMT/24(28)MSR	
25-aza-24,25-dihydro zymosterol	0.08	NI		100
25-azacholesterol	0.14	NI		45
25-azacholestanol	0.14	NI		-
20(R) 22,25-diazacholesterol	0.18	NI		45
20(S) 22,25-diazacholesterol	0.18	NI		45
25-aza-24,25-dihydro lanosterol	1.14	NI		0
24-azacholesterol	0.22	0.7	3	45
23-azacholesterol	4.8	0.12	0.025	45

a Oehlschlager et al., 1980.

b Azasterol administered to culture of S. cerevisiae (Fryberg et al., 1973) and using previously described growth conditions and sterol analysis procedures (Pierce et al., 1979). Growth period for the present cultures was 24 hrs.

$\Delta^{24(28)}$ sterols in the control cultures ($\sum \Delta^{24(28)}$-sterols c), in the following expression:

$$\text{percent inhibition} = 100\left[1 - \frac{\dfrac{\text{ergosterol i}}{\sum \Delta^{24(28)}\text{-sterol i)}}}{\dfrac{\text{ergosterol c}}{\sum \Delta^{24(28)}\text{-sterols c)}}}\right]$$

The % inhibition, for each concentration of inhibitor, of 24(28)-MSR is given in Table I. The inhibition of 24(28)-MSR by 23-aza-cholesterol is higher than by 24-azacholesterol. The rather constant ergosterol:ergostatetraenol ratio in control and 25-azasterol inhibition experiments suggests none of the 25-aza-sterols inhibit 24-methylene sterol-24(28)-reductase.

No inhibition of the 22,23-desaturase was detected in any of the experiments by comparison of Δ^{22} metabolites with C_{22} and C_{23} saturated compounds.

B. Effect of 25-Fluorocholesterol on S. cerevisiae and 25-Azacholesterol on C. utilis

When S. cerevisiae was grown with 1.0 µM 25-fluorocholesterol, cell production was lower but sterol production increased. Little difference was found in the sterol composition of control and fluorosterol cultures and no modified sterols, such as cholesta-5,7,24-trienol or cholesta-5,7,22,24-tetraenol, were detected.

When a culture of C. utilis was grown with 1.0 µM

concentration of 25-azacholesterol, there was inhibition of the methyl transferase that was calculated to be 33% by the formula stated previously. There was a decrease in the percentage of ergosterol in the NSF from 69.3% for the control, to 44.5% as well as an increase in zymosterol to 38.1% from 8.3% for the control. C_{27}-Sterols were also produced in these cultures. Cholesta-5,7,24-trienol contributed 3.3% and cholesta-5,7,22,24-tetraenol 1.3% of the sterol. The cell yield was the same for the inhibited and non-inhibited cultures, however, the sterol production decreased.

C. Production of Pure Zymosterol for Synthetic Manipulation

The erg 2 sterol mutant of S. cerevisiae (Bard et al., 1977), grown with .5 μM 25-azacholesterol, produced zymosterol which represented 50% of the sterols. The major impurity was ergosta-5,8,22-trienol with smaller amounts of cholesta-5,8,24-trienol, cholesta-5,8,22,24-tetraenol and fecosterol. These impurities were removed by a combination of flash chromatography (Still et al., 1978) and preparative argentation ($AgNO_3$, 20%)-TLC to produce zymosterol, 99+% pure by gas chromatography. The elimination of cholesta-7,24-dienol, which is a major impurity in azasterol inhibited wild type yeast (Avruch et al., 1976) and baker's yeast (Taylor et al., 1981), from the zymosterol by the use of the erg 2 (blocked at $\Delta^8 \rightarrow \Delta^7$-isomerase) sterol mutant yeast is most effective. The yeast produces 7.5 g/L dry cell weight with 1% of the dry weight being sterols.

D. Production of [^{13}C] Enriched Sterols and Conversion of Zymosterol to Cholesterol

1. Enrichment levels versus sterol production

When S. cerevisiae was grown in the presence of 2.0 g/L, 1-[^{13}C]-sodium acetate, 749 mg/L of NSF were obtained from which was isolated 80 mg of ergosterol acetate enriched by 17.2% over the natural abundance by MS and 5 to 20-fold by comparison of peak heights in the ^{13}C NMR.

This compares favorably with the same yeast grown in the presence of .2 μM 25-azacholesterol in medium containing 1.0 g/L 1-[^{13}C]-sodium acetate which produced 1.23 g/L NSF. The isolated zymosterol (410 mg/L) was enriched by 7.2% over the natural abundance by MS and 4 to 7-fold by ^{13}C NMR (Figure 4).

In an effort to increase enrichment, yeast was grown anaerobically in the presence of .4 μM 25-azacholesterol in medium containing 1.0 g/L 1-[^{13}C]-sodium acetate. The overall yield of zymosterol (8 mg) from 65 mg of NSF was very low although enrichment (29%) was high by MS.

As sodium acetate concentrations were increased in yeast controls to 2 g/L, the amount of yeast and NSF decreased slightly. It was thus decided to limit the maximum acetate concentration to 2.0 g/L.

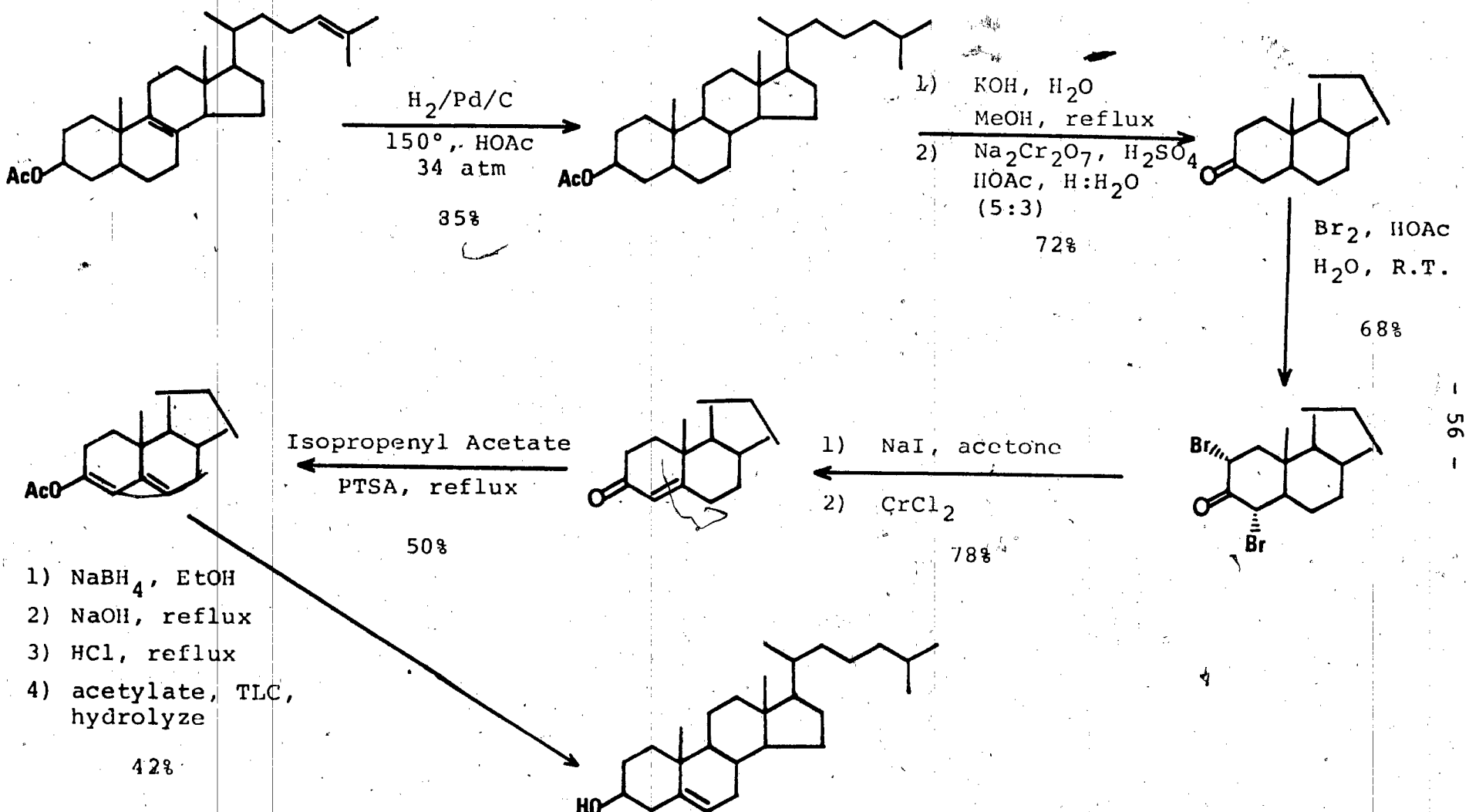
2. Cholesterol production from zymosterol

Figure 3 shows the steps and yields in the synthesis of cholesterol from zymosterol. The initial step was developed

Figure 3

Synthesis of Cholesterol from Zymosterol.

Fig. 3


AcO
H_2/Pd/C
150°, HOAc
34 atm
85%
AcO
1) KOH, H_2O
MeOH, reflux
2) $Na_2Cr_2O_7$, H_2SO_4
HOAc, H:H_2O
(5:3)
72%
Br_2, HOAc
H_2O, R.T.
68%
Br
Br
1) NaI, acetone
2) $CrCl_2$
78%
Isopropenyl Acetate
PTSA, reflux
50%
AcO
1) $NaBH_4$, EtOH
2) NaOH, reflux
3) HCl, reflux
4) acetylate, TLC, hydrolyze
42%
HO

from a procedure by Kircher (1974), wherein zymosterol was successfully converted to cholestanol (85%). The steps up to and including the production of the enol acetate were the same as in the references cited. The reduction of the enol acetate gave a mixture of cholesterol and cholestanol (70:30) confirmed by GLC and mass spectral data, which was different in composition from the published results (Belleau and Gallagher, 1951; Dauben and Eastham, 1951). The use of low temperature reactions (-20°) did not increase the yield of cholesterol. Final purification, using preparative thin-layer chromatography ($AgNO_3$, 20%) (Idler and Safe, 1972), gave cholesterol of high purity in an overall yield of 6%.

3. ^{13}C NMR experiments

The ^{13}C NMR spectrum of enriched (7%) zymosterol (Figure 4) shows a significant increase in the signal of the enriched carbons over those that were not enriched.

On addition of increasing amounts of dipalmitoyl lecithin (DPL) to the sample of enriched zymosterol (7%), the resonances of enriched carbons become less and less distinguishable from those of DPL-carbons (Figure 5). At 70 mole percent DPL, the resonances of vinyl carbons of zymosterol (tail and nuclear) were still recognizable.

Figure 4

^{13}C NMR, Zymosterol

a) Zymosterol (.030 g), natural abundance was dissolved in 1.0 mL of $CDCl_3$ and put into a 5 mm tube, 1000 scans.

b) Zymosterol (.015 g), enriched (≈ 7%) was dissolved in 1.0 mL of $CDCl_3$ and put into a 5 mm OD tube, 1000 scans.

Scale in parts per million (ppm).

Fig. 4

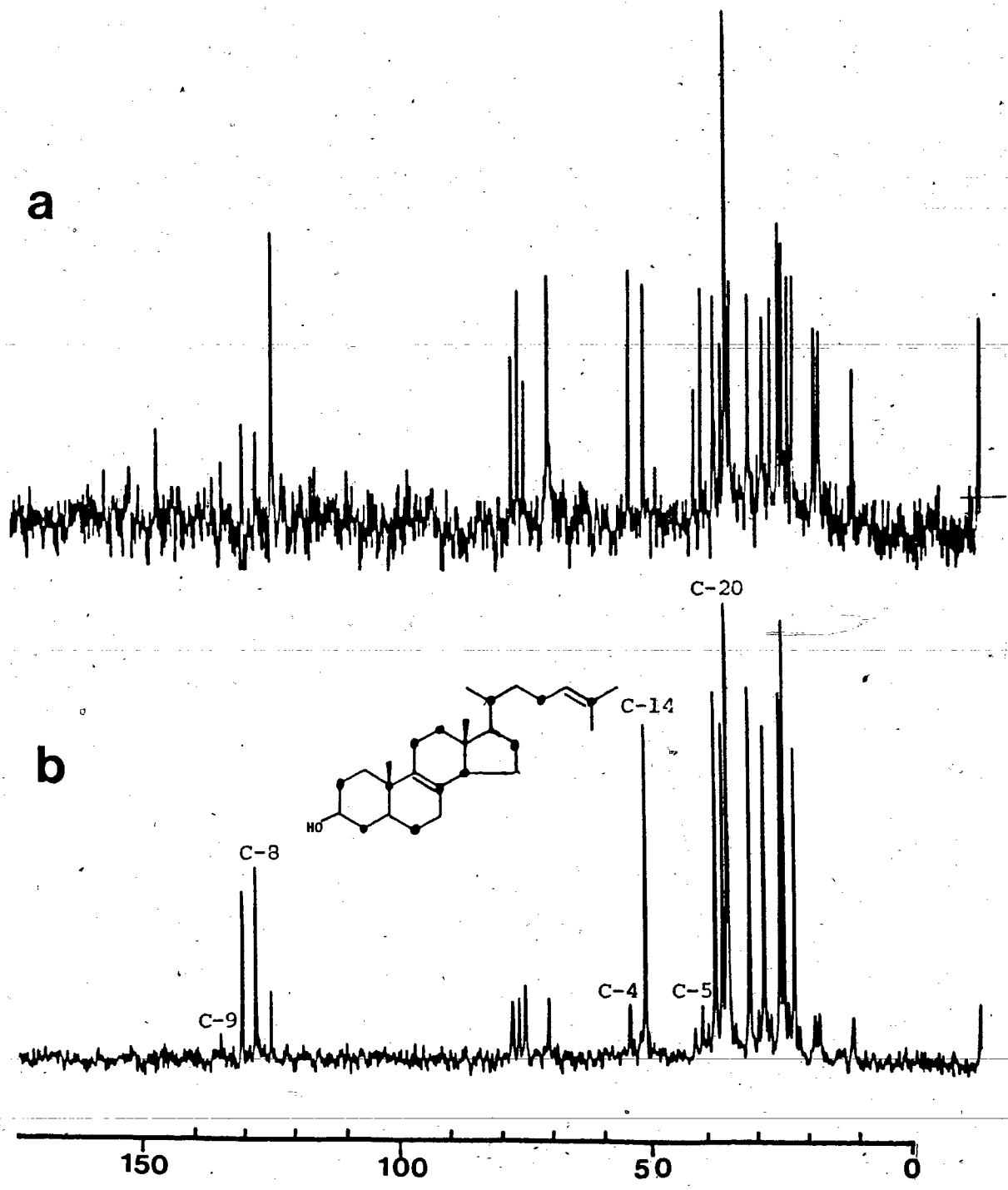


Figure 5

^{13}C NMR, Zymosterol and Dipalmitoyl Lecithin (DPL).

a) 70 Mole percent DPL, 30 mole percent enriched zymosterol.

b) 60 Mole percent DPL, 40 mole percent enriched zymosterol.

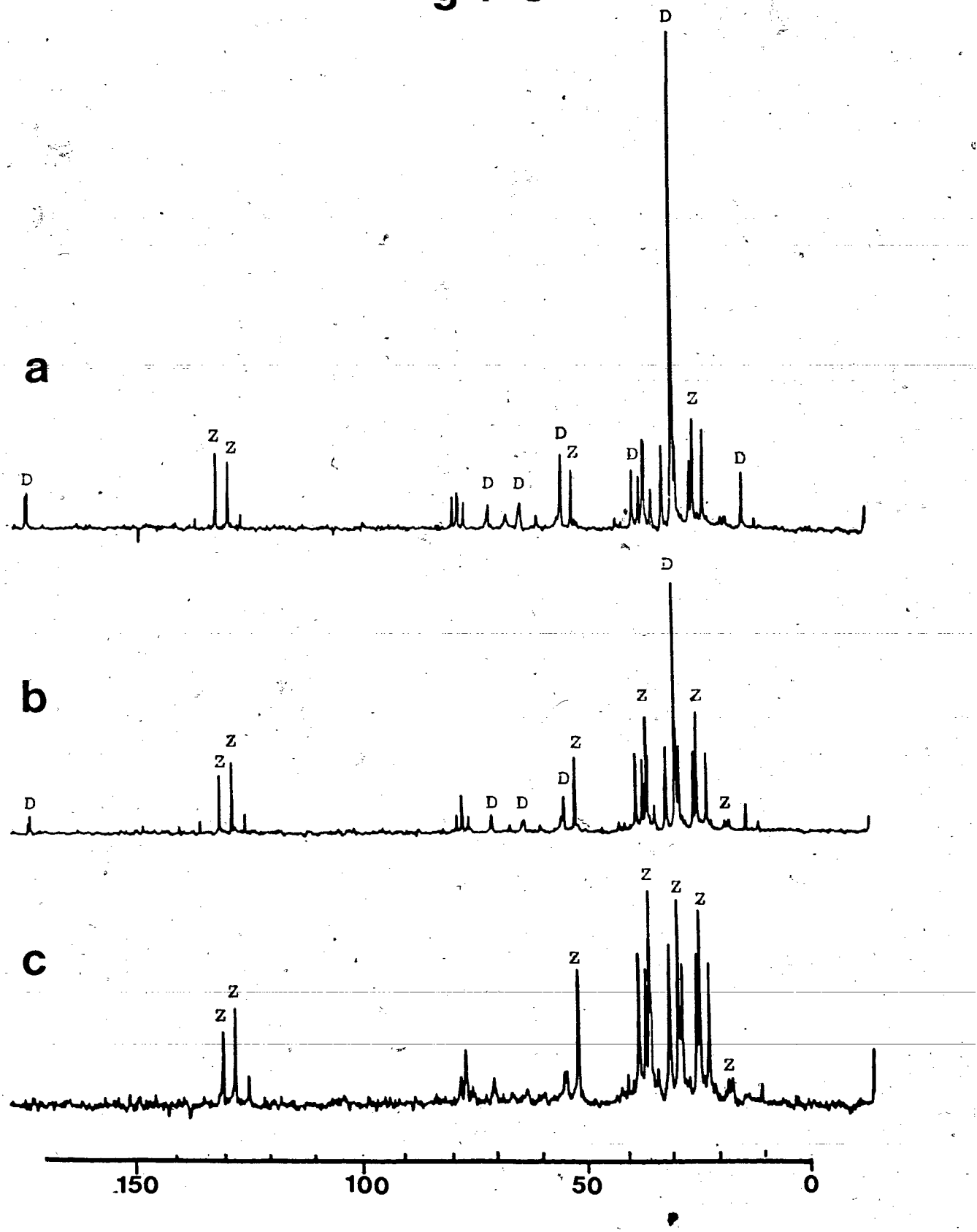
c) 40 Mole percent DPL, 60 mole percent enriched zymosterol.

Scale in parts per million (ppm).

DPL peaks (D)

Zymosterol peaks (Z)

Fig. 5



E. Production of 25-Hydroxy Provitamin D_3 from Erg 5 Mutant Yeast and 25-Azacholesterol Inhibition

1. Sterol composition and production of NSF from erg 5 yeast culture inhibition by 25-azacholesterol

Growth of two 3 L cultures of erg 5, each containing 1.0 μM 25-azacholesterol, gave 230 g (wet weight) of yeast. The combined cultures yielded 1.18 g of crude steryl acetates. GLC analysis showed cholesta-5,7,24-trienol (15 ± 2%) and ergosta-5,7-dienol (12 ± 2%) were the two major $\Delta^{5,7}$-diene containing sterols in the non-saponifiable fraction.

2. Isolation of $\Delta^{5,7}$-diene compounds

Cycloaddition of 4-phenyl-1,2,4-triazoline-3,5-dione to the $\Delta^{5,7}$ steryl acetates was most efficient at -70°, as judged by TLC.

Column chromatography on silica gel proved to be the most efficient method to obtain the $\Delta^{5,7}$-steryl acetate cycloadducts. Non-ring B derivatized steryl acetates (including zymosteryl acetate and methylated steryl acetates) were eluted rapidly with benzene while the derivatized steryl acetates eluted with benzene/ethyl acetate. The efficiency of the 1,4-cycloaddition reaction, as determined by GLC analysis of the benzene eluate for $\Delta^{5,7}$-steryl acetates, revealed that 1% of the $\Delta^{5,7}$-steryl acetates remained in the NSF. This corresponds to a reaction efficiency of 94%. Analysis of the 1,4-cycloadduct fraction by GLC was futile as the compounds decomposed upon injection. The GLC recording showed peaks (broad and not well

defined) with retention times corresponding to the steryl acetates of cholesta-5,7,24-trienol and ergosta-5,7-dienol.

3. Isolation of 1,4-cycloadduct of cholesta-5,7,24-trienyl-3β-acetate

The 1,4-cycloadduct fraction was treated with m-chloroperbenzoic acid to epoxidize the Δ^{24}-double bond of the cholesta-5,7,24-trienyl acetate adduct, thereby facilitating the separation of the 1,4-cycloadduct of ergosta-5,7-dienyl acetate from the cholesta-5,7,24-trienyl acetate cycloadduct. Separation was accomplished by preparative TLC and confirmed by NMR. NMR spectra of each isolated cycloadduct showed that the more polar band was the 1,4-cycloadduct of 24,25-epoxy-cholesta-5,7,24-trien-3β-acetate and the upper band to be the 1,4-cycloadduct of ergosta-5,7-dien-3β-acetate. GLC and molecular ion mass spectral evidence were difficult to obtain as the 1,4-cycloadducts were unstable to heat.

4. Production of 25-hydroxy provitamin D_3

Reaction of lithium tetrahydroaluminate with the 24,25-epoxy-1,4-cycloadduct of cholesta-5,7-dien-3β-yl acetate accomplished reduction of the epoxide, cleavage of the acetyl group at the 3β-position and the conversion of the ring B, 1,4-cycloadduct moiety to a $\Delta^{5,7}$-diene in 57% yield. The overall yield of cholesta-5,7-dien-3β,25-diol from yeast NSF was 6%.

F. In vitro Studies of Δ^{24}-Sterol Methyltransferase with Azasterols

1. Obtaining an active 24-SMT enzyme preparation

Attempts at purification of the 24-SMT by the method of Moore and Gaylor (1969) gave an enzyme preparation with inconsistent activity. Therefore, the method of Bailey et al. (1974) was used to produce a cell-free preparation possessing 24-SMT activity. Yeast were grown as in the inhibited culture experiments with 24 h aeration and rapid stirring. After being processed, the preparation was frozen at -25° until required.

Initially, attempts were made to determine enzyme activity, either by following zymosterol disappearance and/or fecosterol (the product of the enzymic reaction) appearance by gas chromatography. However, endogenous sterols present in the preparation (even in the pellet from ultracentrifugation at 105,000 × g) prevented the detection of fecosterol by gas chromatography. Zymosterol content after the reaction showed no detectable decrease compared to a control sample. Therefore, the standard assay of using [^{14}C-Me]-S-adenosyl-L-methionine as the indicator for transmethylation was employed (Moore and Gaylor, 1969; Thompson et al., 1974).

Figure 6 represents the effect of increasing zymosterol concentrations, [S], on the initial velocity, v, of the enzyme's transmethylation. Figure 7 is the standard Lineweaver-Burk transformation of the data which gives a K_M of 57.1 μM and a V_{max} of 11.7 nmoles/hr/mg protein.

Figure 6

Regular Rectangular Hyperbola Plot for Zymosterol.

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of zymosterol indicated (●). A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl methionine (.1 μ Ci per 400 nmoles). The points are an average of five separate experiments (duplicate samples).

[S] = zymosterol

v = nmoles of [^{14}C-Me] of SAM incorporated into NSF/hr per mg protein.

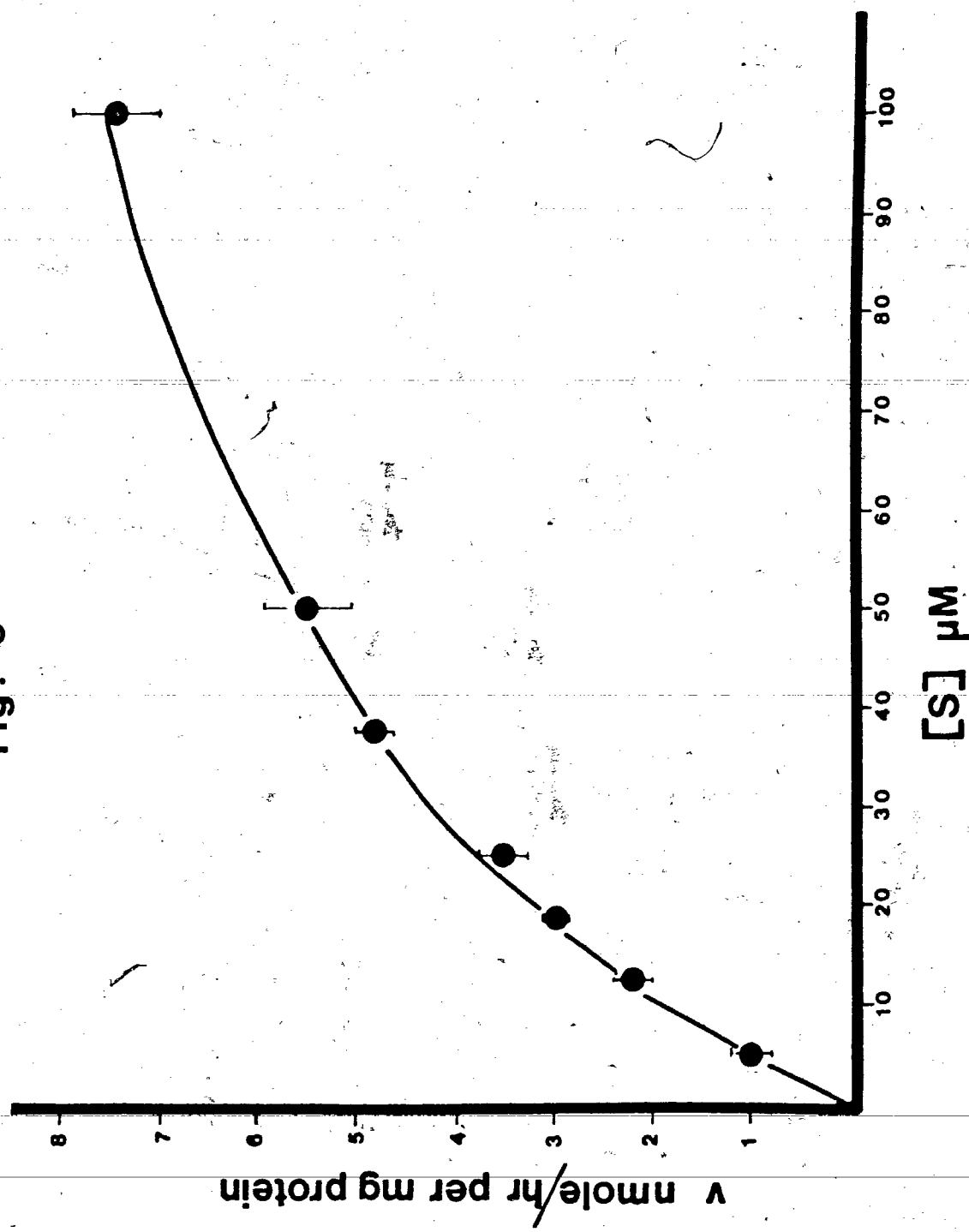

v nmole/hr per mg protein
1
2
3
4
5
6
7
8
[S] µM
10
20
30
40
50
60
70
80
90
100

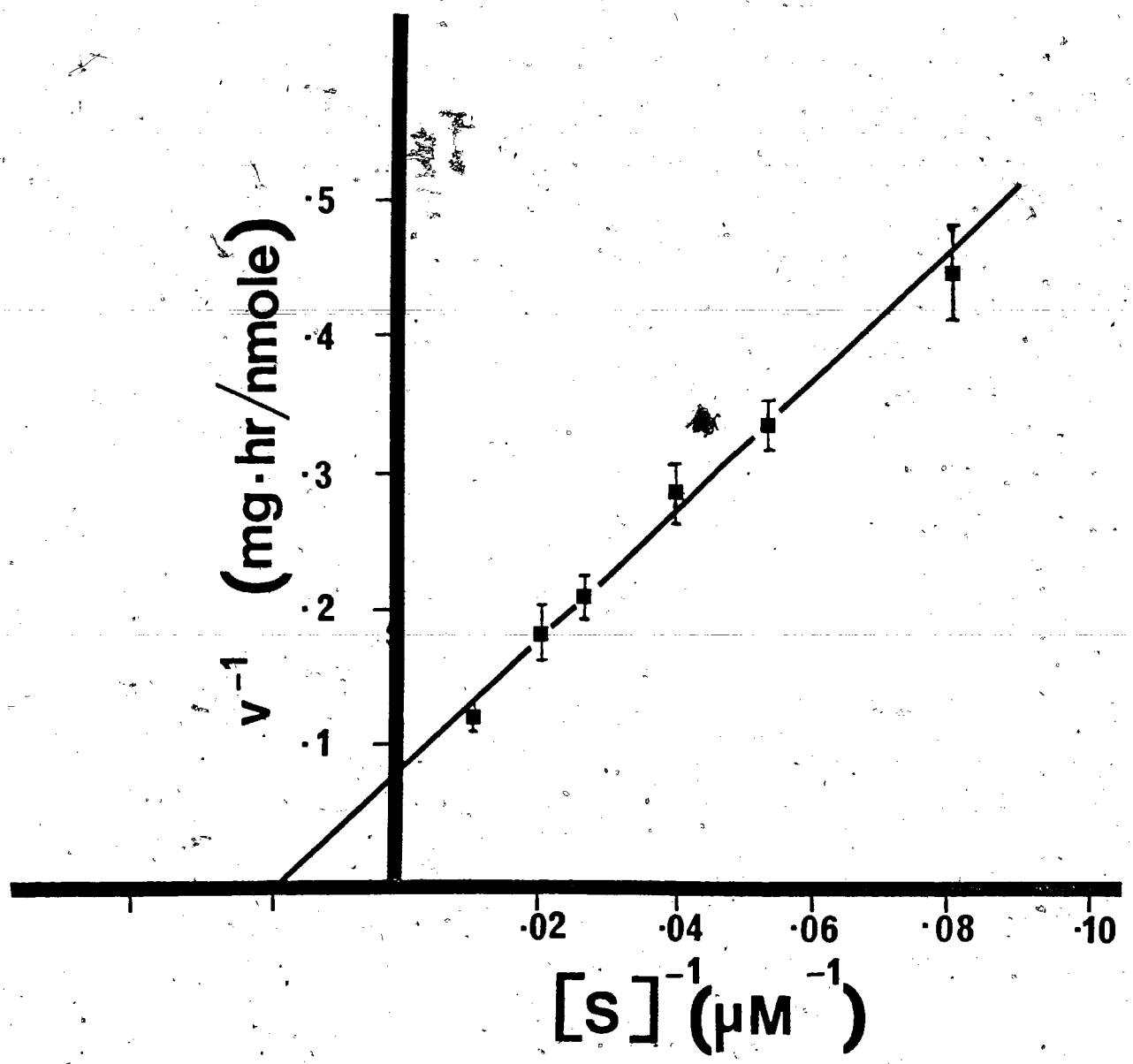
Fig. 6

Figure 7

Lineweaver-Burk Plot for Zymosterol.

The standard assay procedure was used (P. 41). Each flask contained .1 M tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of zymosterol indicated (■). A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 μ Ci per 400 nmoles). The points are an average of five separate experiments (duplicate samples.

Fig. 7



In the standard experiment we showed cycloartenol is not a substrate for this yeast methyltransferase enzyme.

2. Inhibition studies with azasterols on Δ^{24}-sterol methyltransferase

In the initial experiments with 25-azacholesterol, a concentration was used that gave 50% inhibition in the *in vivo* experiments (100 nM). However, this concentration proved to be completely inhibitory *in vitro* so the concentration of the inhibitor was decreased by a factor of ten (to 10 nM). Figures 8 to 11 are Dixon plots (Dixon, 1953) showing the effect of azasterols on the activity of the Δ^{24}-sterol methyltransferase preparation. When SAM concentrations are constant, the plot of 1/v *vs.* [I] (reciprocal of initial velocity *vs.* inhibitor concentration) gives parallel lines which is indicative of uncompetitive inhibition (Cornish-Bowden, 1974). This can be confirmed by examining the plots of s/v *vs.* [I] (substrate concentration/initial velocity *vs.* inhibitor concentration), which show intersection of the lines giving a K_I' (Figures 12 to 15). The K_I' values (see figures) are similar in magnitude indicating nearly equal potency of the inhibitors *in vitro*.

When the zymosterol concentrations are constant (100 µM), the Dixon plot for the effect of 25-aza-24,25-dihydrozymosterol gives intersecting lines, which is indicative of competitive inhibition (Figure 16). For this type of inhibition, a K_I (inhibitor constant) is obtained which gives a value of 1.25 nM.

Figure 8

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 25-Azacholesterol (1/v *vs.* I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol, either 12.5 µM (▲), 25 µM (■) or 50 µM (●), was added, then the inhibitor. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). The points are an average of 4 determinations in two separate experiments.

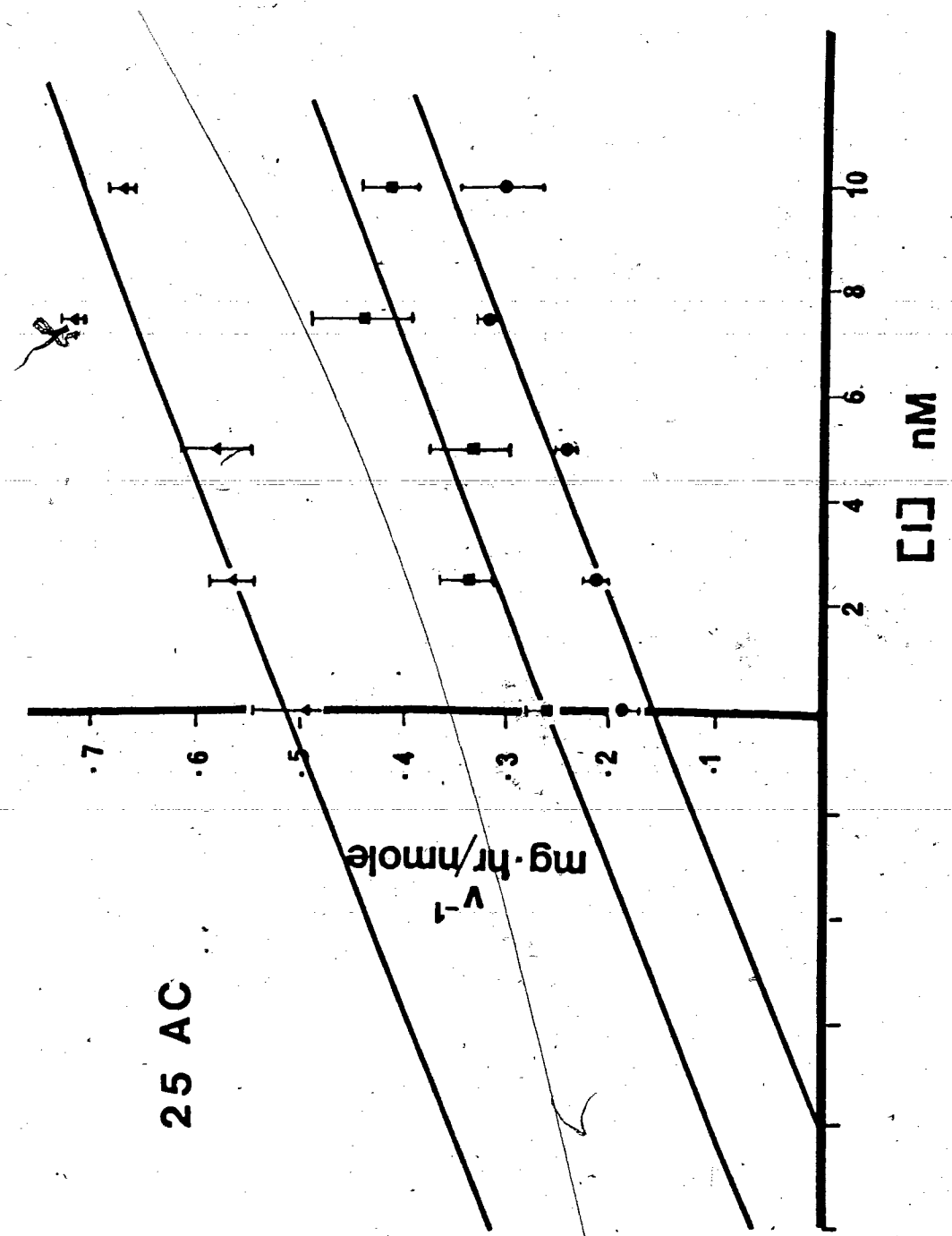


Fig. 8

Figure 9

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 25-Aza-24,25-dihydrozymosterol (1/v *vs.* I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol, either 12.5 µM (▲), 25 µM (■) or 50 µM (●), was added, then the inhibitor. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). The points are an average of 4 determinations in two separate experiments.

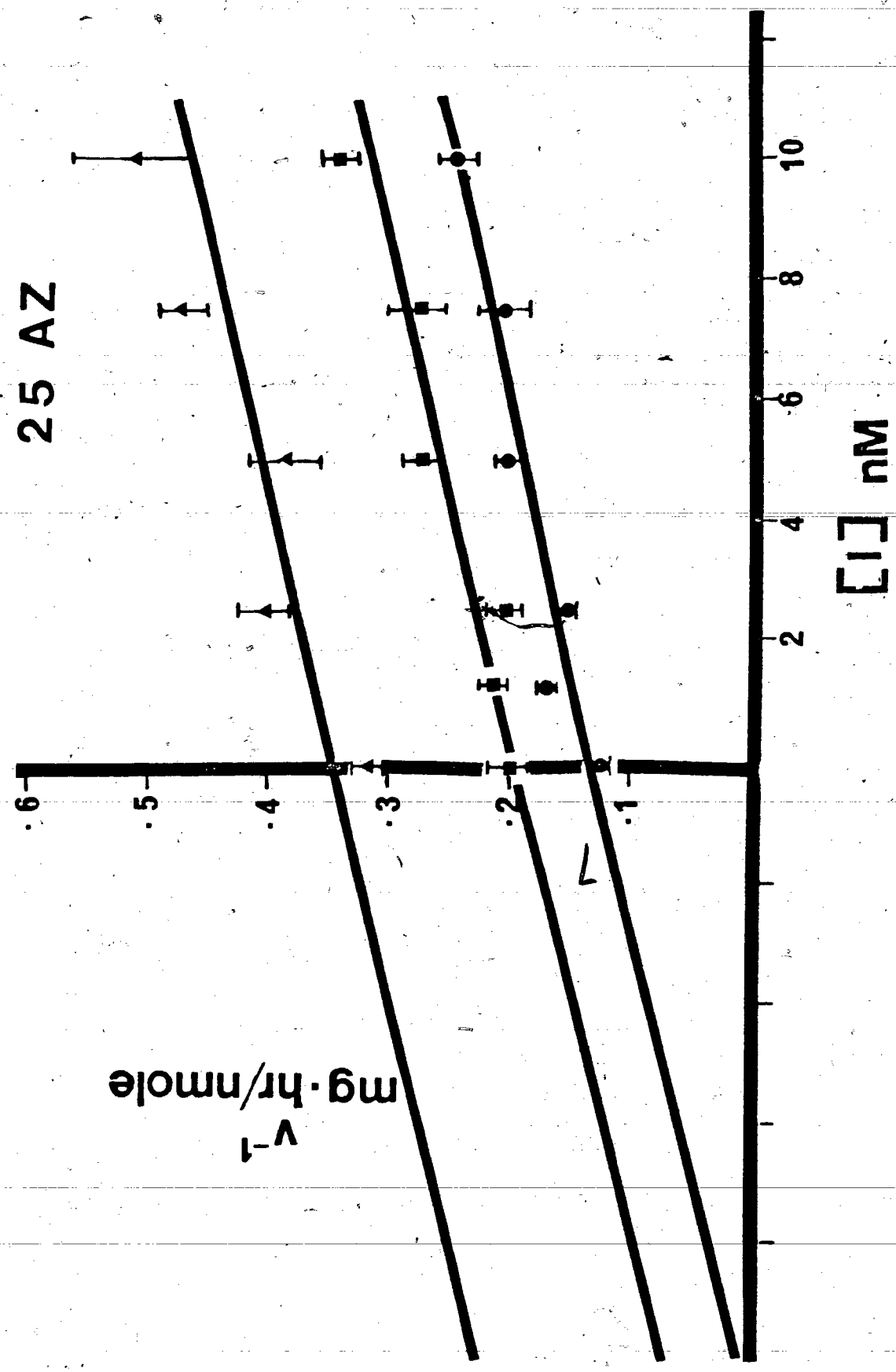


Fig. 9

Figure 10

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 23-Azacholesterol (1/v vs. I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol, either 25 µM (▲) or 50 µM (■), was added, then the inhibitor. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). The points are an average of 4 determinations in two separate experiments.

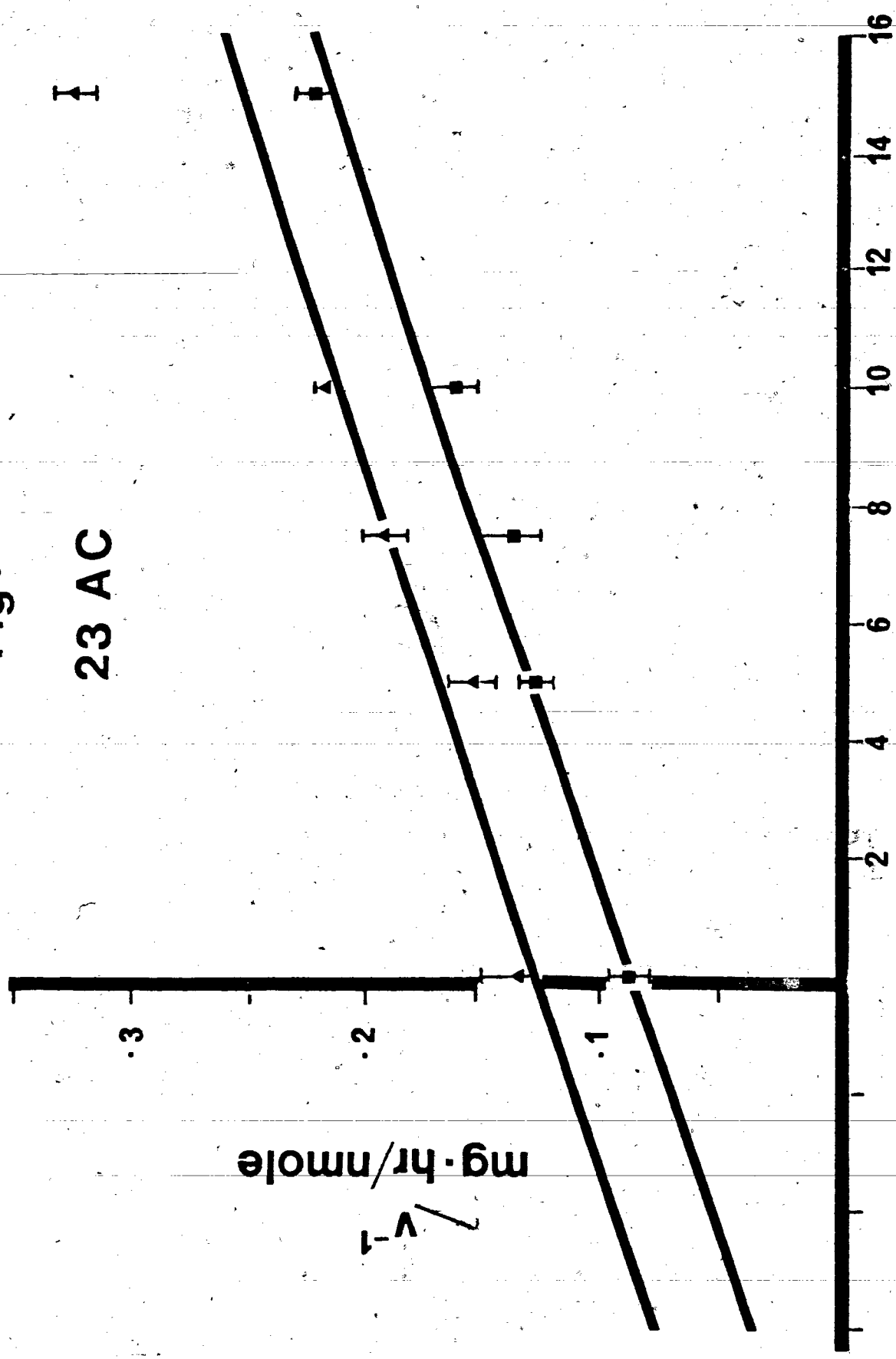


Fig. 10
23 AC
I
$\sqrt{v^{-1}}$
mg·hr/nmole
.3
.2
.1
[I] nM
2
4
6
8
10
12
14
16

Figure 11

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 25-Methyl-25-azacholesterol (1/v vs. I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol, either 25 µM (■) or 50 µM (●), was added, then the inhibitor. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). The points are an average of 4 determinations in two separate experiments.

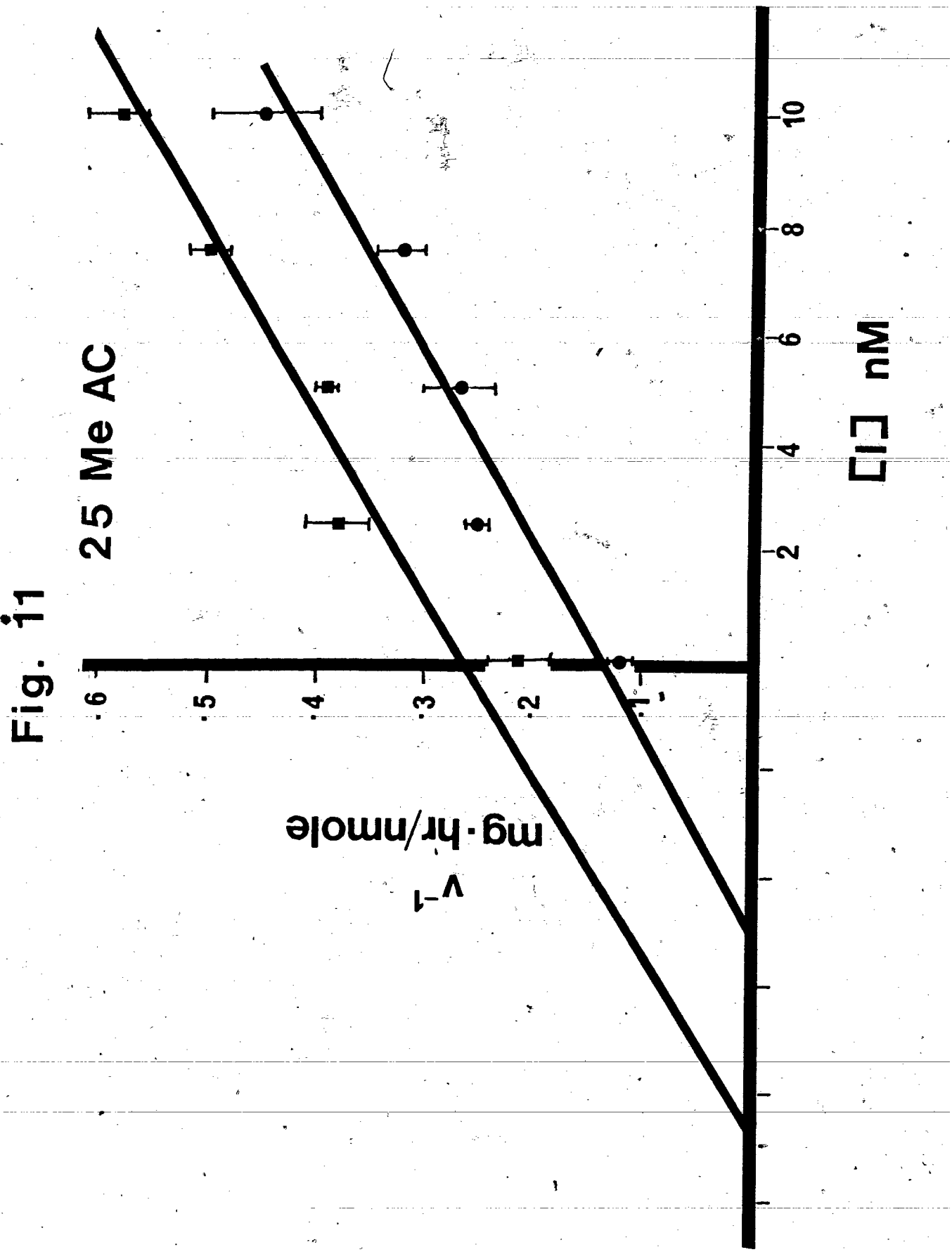


Fig. 11

Figure 12

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 25-azacholesterol (s/v vs. I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol, either 12.5 µM (●), 25 µM (■) or 50 µM (▲), was added, then the inhibitor. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). The points are an average of 4 determinations in two separate experiments.

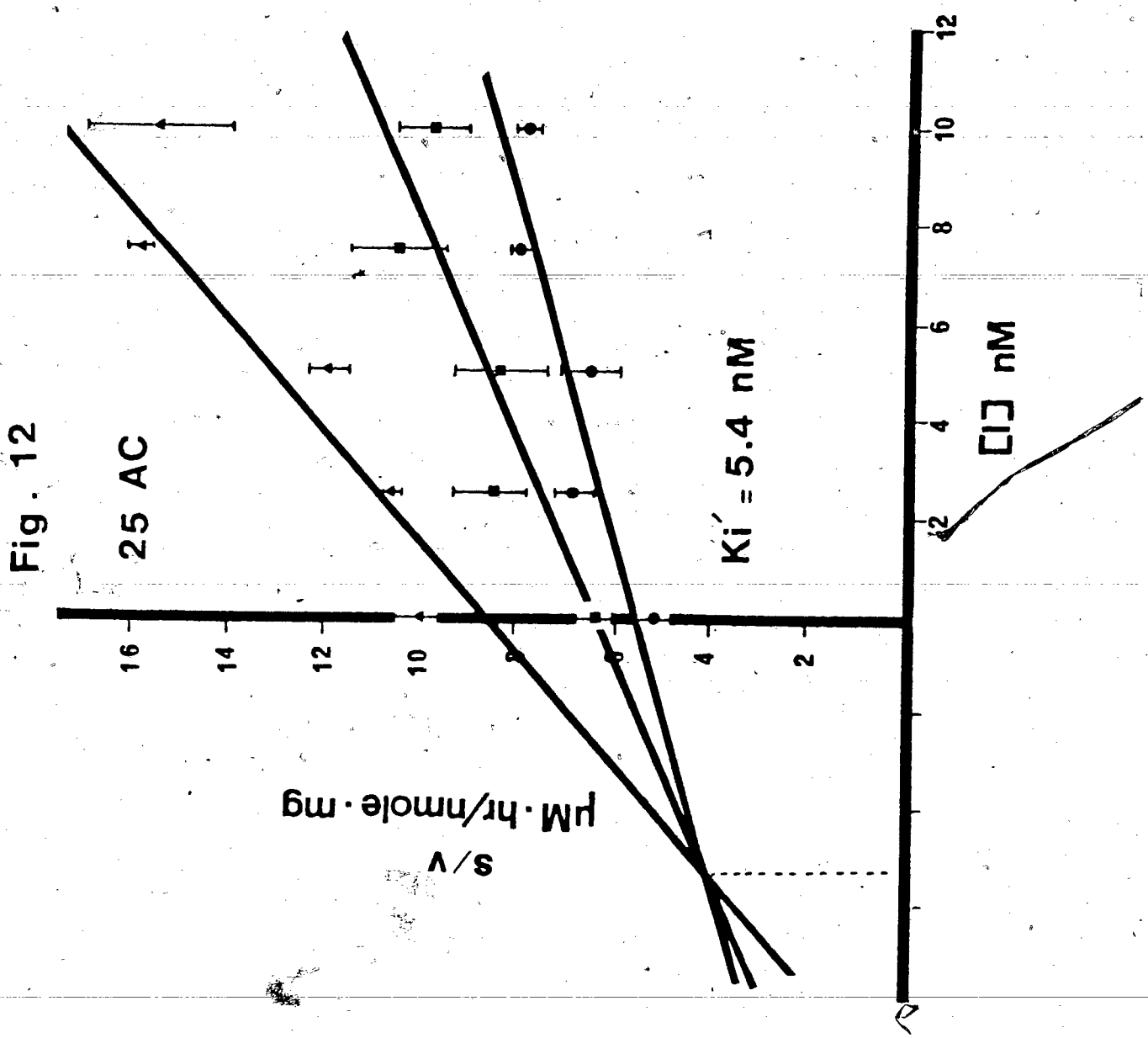


Fig. 12
25 AC
Ki' = 5.4 nM
[I] nM
s/v
μM · hr/nmole · mg
16
14
12
10
8
6
4
2
2
4
6
8
10
12

Figure 13

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 25-Aza-24,25-dihydrozymosterol (s/v vs. I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol, either 12.5 µM (▲), 25 µM (●) or 50 µM (■), was added, then the inhibitor. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). The points are an average of 4 determinations in two separate experiments.

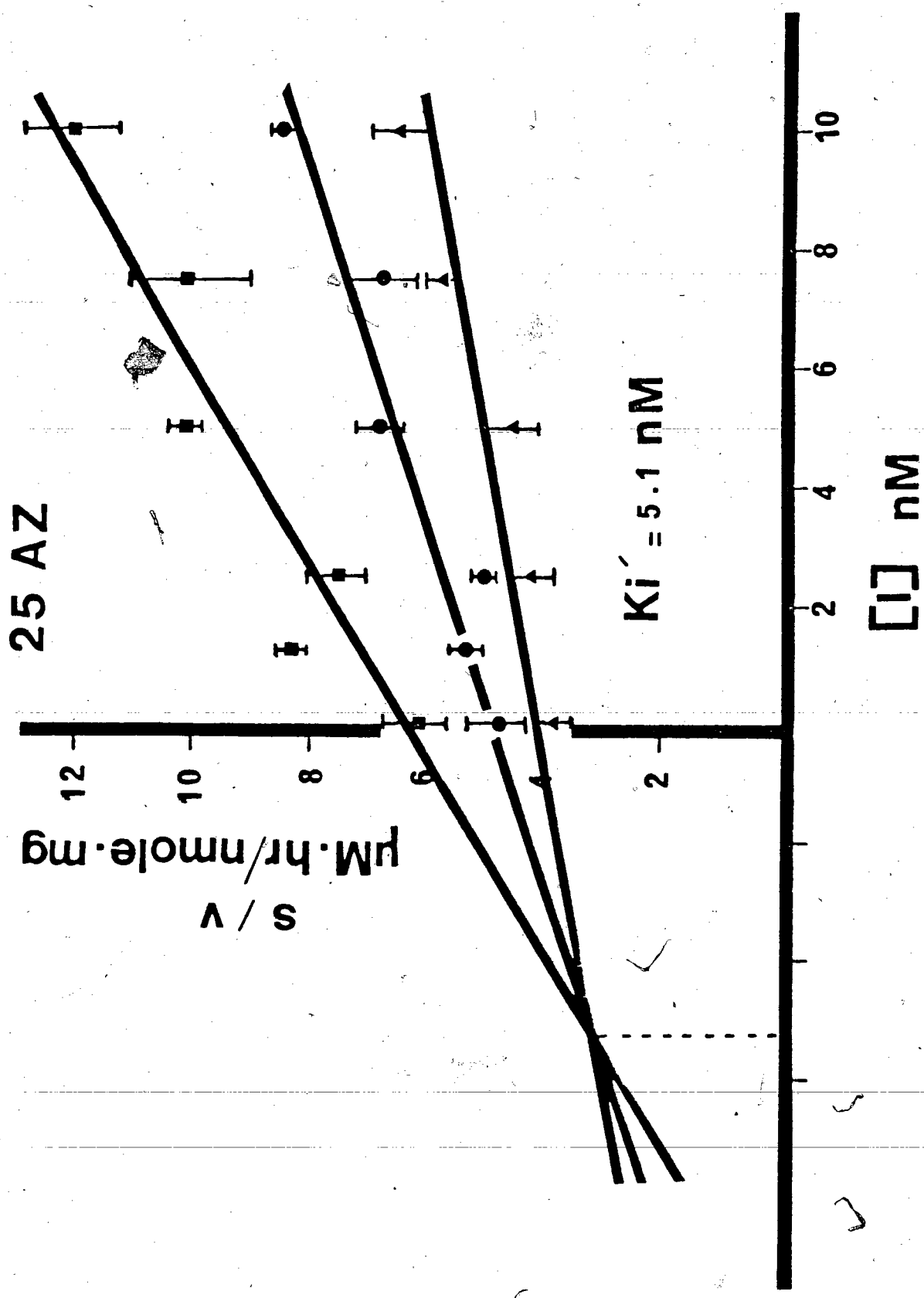


Fig. 13

Figure 14

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 23-azacholesterol (s/v vs. I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol, either 25 µM (▲) or 50 µM (■), was added, then the inhibitor. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). The points are an average of 4 determinations in two separate experiments.

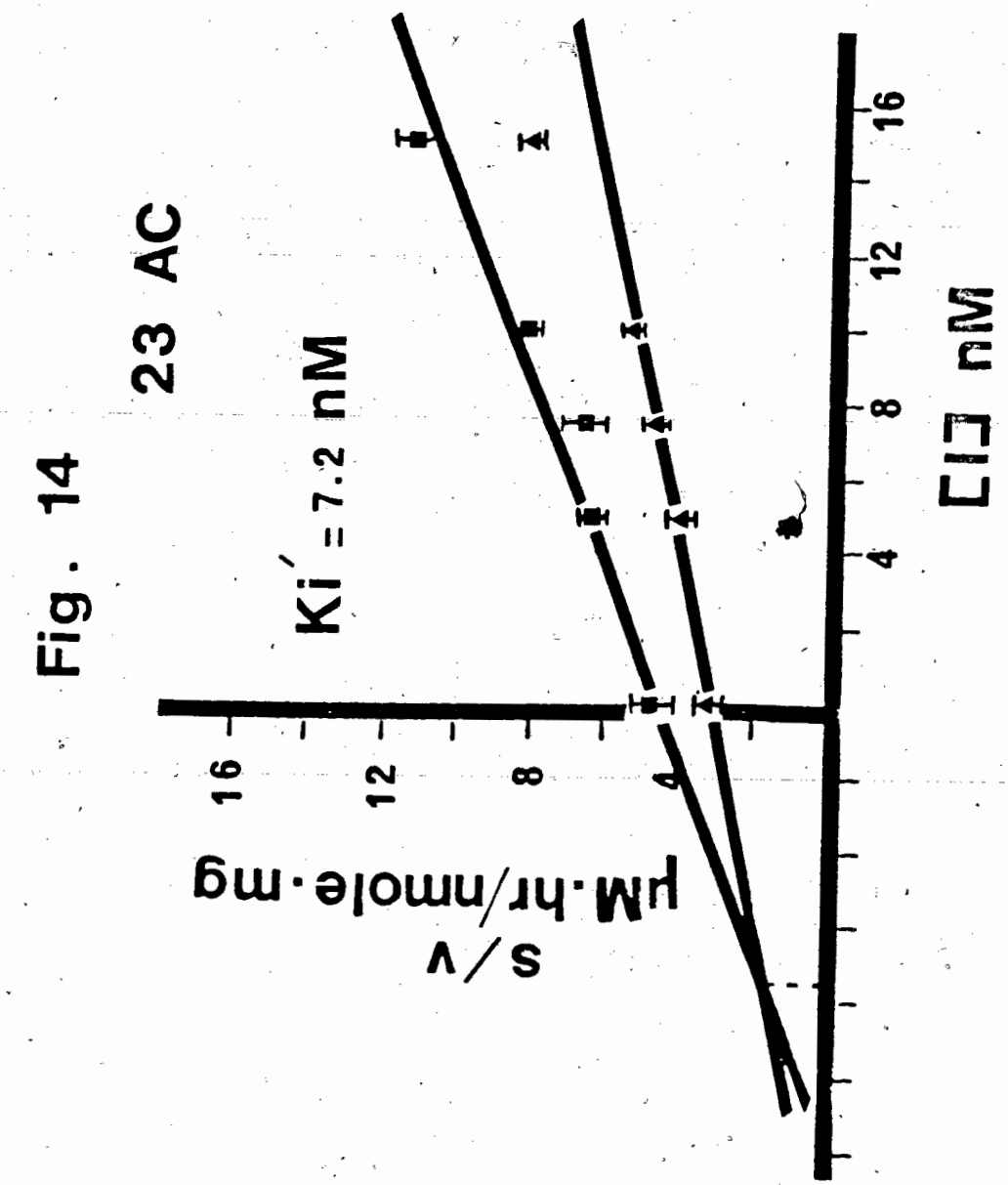


Fig. 14
23 AC
Ki′ = 7.2 nM
S/V
μM·hr/nmole·mg
16
12
8
4
4
8
12
16
[I] nM

Figure 15

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 25-Methyl-25-Azacholesterol (s/v vs. I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol, either 25 µM (■) or 50 µM (▲), was added, then the inhibitor. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by [^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). The points are an average of 4 determinations in two separate experiments.

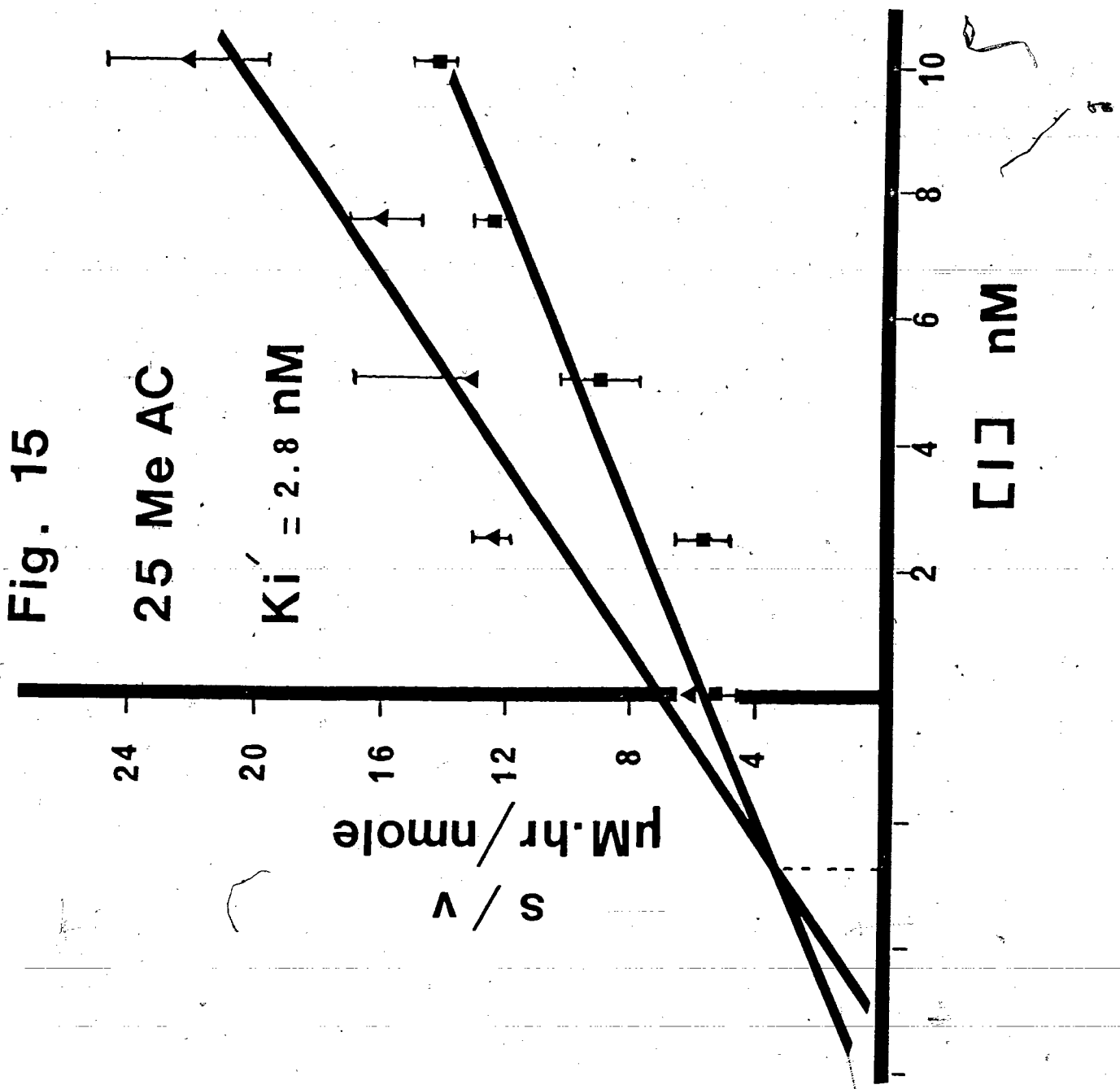


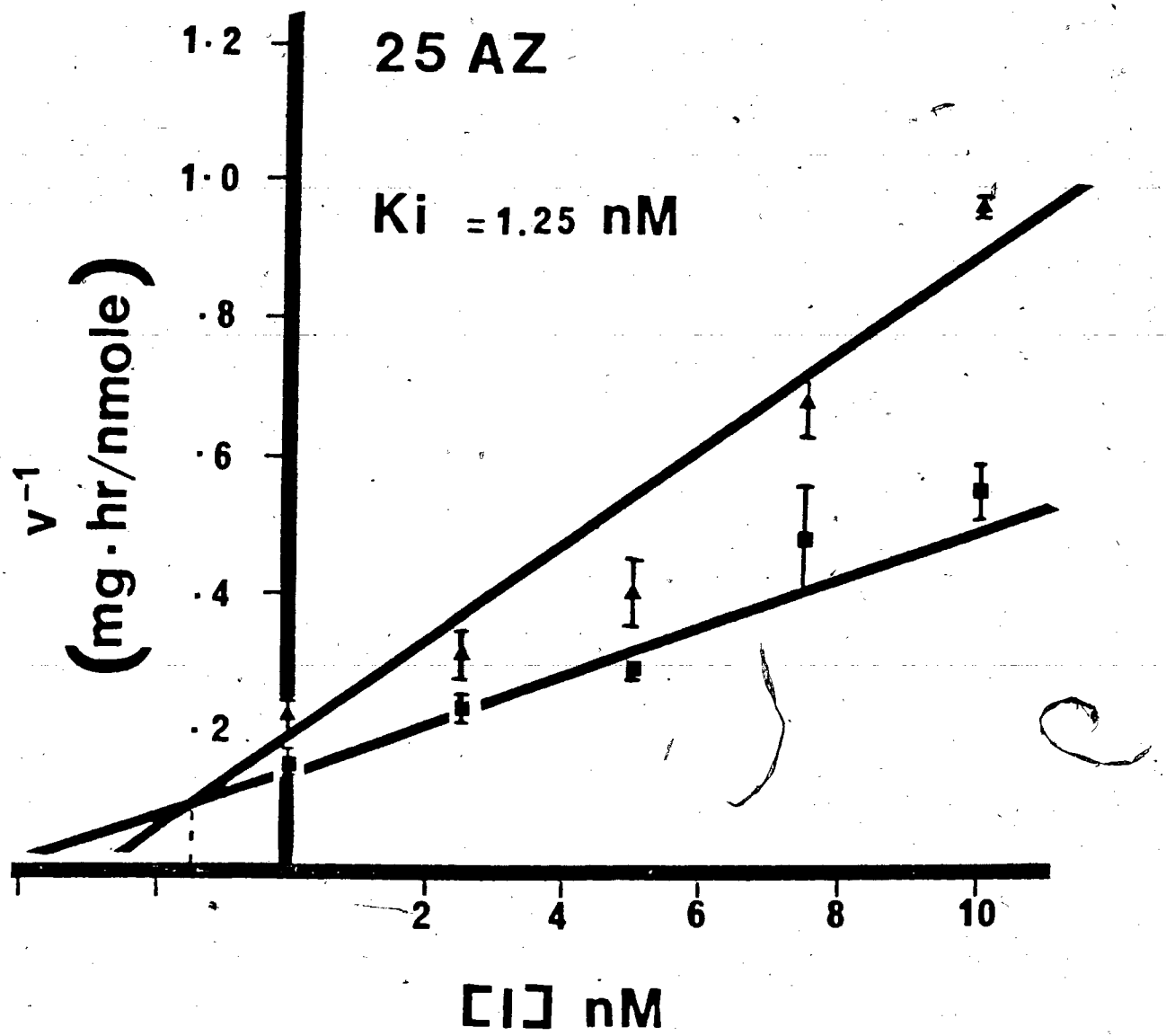
Fig. 15
25 Me AC
Ki' = 2.8 nM
S / V
μM.hr/nmole
24
20
16
12
8
4
2
4
6
8
10
[I] nM

Figure 16

Dixon Plot for the Inhibition of 24-SMT Transmethylation by 25-Aza-24,25-Dihydrozymosterol (1/v vs. I)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of inhibitor indicated. Zymosterol added was at a concentration of 100 µM. A volume of .5 mL of enzyme preparation was added. S-adenosyl-L-methionine was either 25 µM (▲) or 50 µM (■). Points are an average of 4 determinations in two separate experiments.

Fig. 16



3. Inhibition of 24-SMT by Ergosterol and S-Adenosyl-L-Homocysteine (SAH)

A Lineweaver-Burk plot of ergosterol inhibition of Δ^{24}-sterol methyltransferase methylation of zymosterol (Figure 17) indicates non-competitive inhibition by ergosterol, K_I (calculated from the graph) is 120 µM.

The inhibition of transmethylation of zymosterol by S-adenosyl-L-homocysteine (dissolved in Tris-HCl buffer, pH 7.6, .1 mM Mg^{++}) is given in Figure 18. When the SAH concentration is at 2 µM, there is a definite inhibition of the rate of reaction of the enzyme even when the concentration of S-adenosyl-L-methionine is increased (zymosterol concentration is constant, 100 µM). The complex nature of the [S] *vs.* v curve indicates that product inhibition is occurring with an effect on the ability of SAM to bind the ~~en~~zyme resulting in a decreased rate.

Figure 17

Lineweaver-Burk Plot for the Inhibition of 24-SMT Transmethylation by Ergosterol

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of zymosterol indicated (■). Ergosterol, 50 µM (⧈) was added to each substrate concentration. A volume of .5 mL of enzyme preparation was added and the reaction was initiated by[^{14}C]-S-adenosyl-L-methionine (.1 µ Ci per 400 nmoles). Points are an average of 2 determinations.

Fig. 17

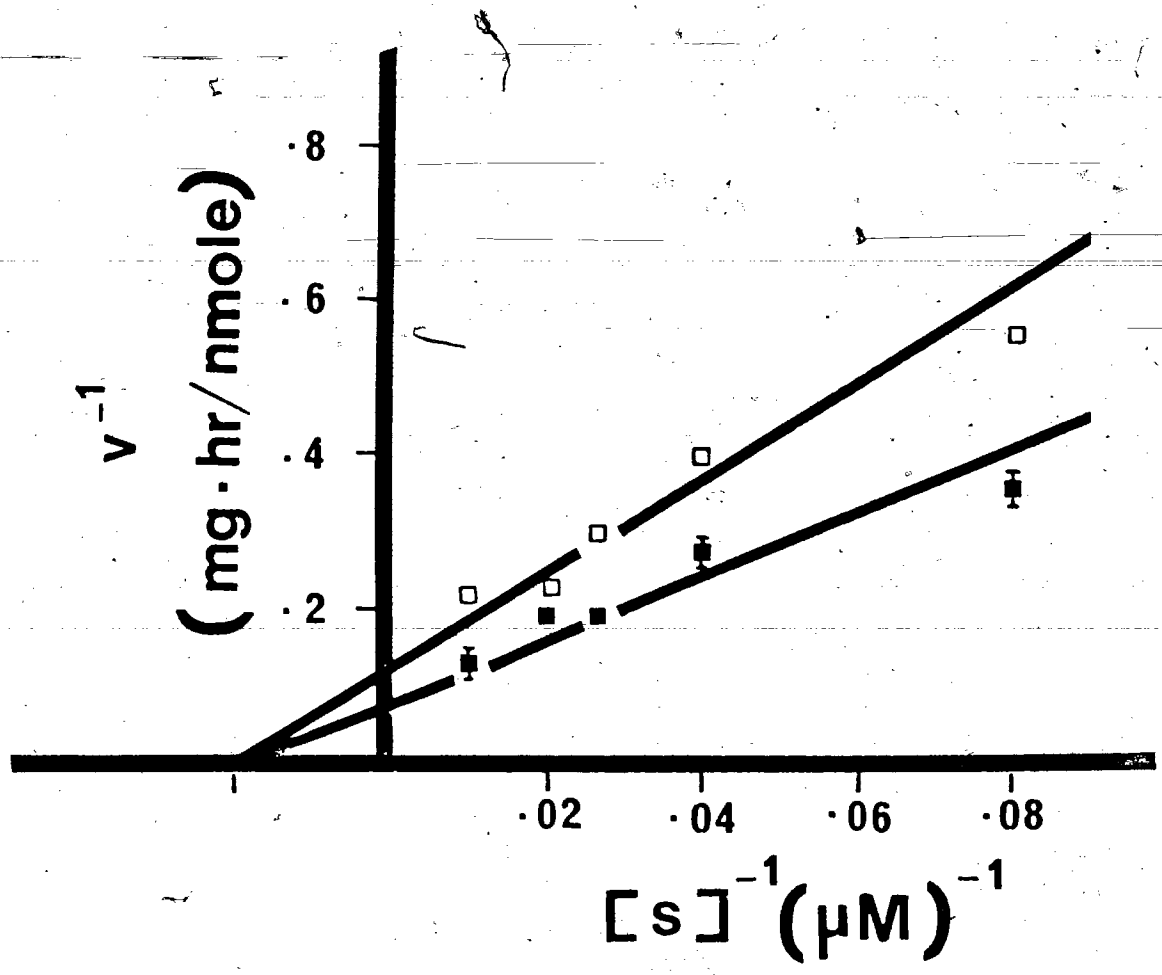
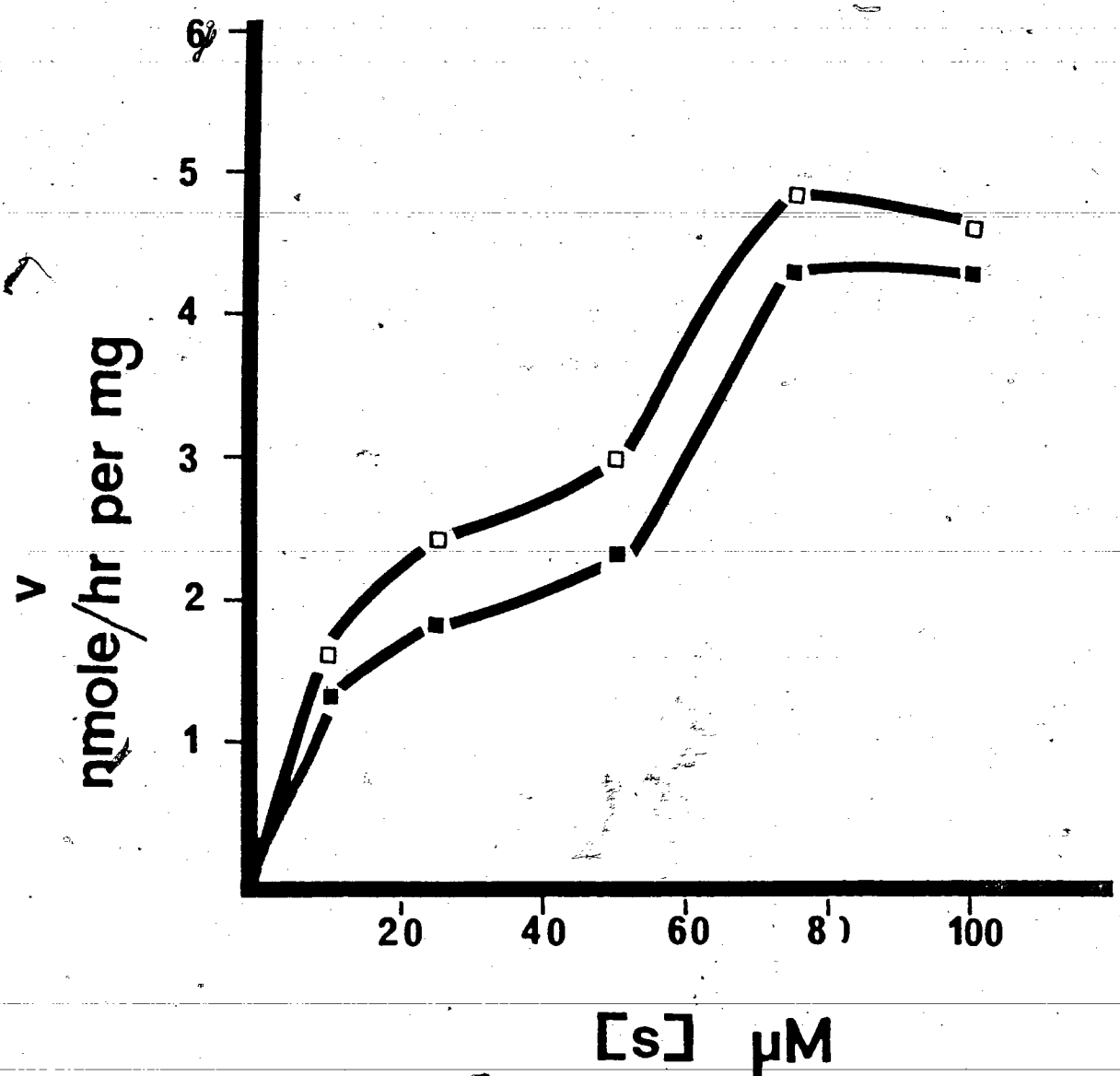


Figure 18

Plots of v vs. [S] for S-Adenosyl-L-Methionine and SAM + 2 μM S-Adenosyl-L-Homocysteine (SAH)

The standard assay procedure was used (P. 41). Each flask contained .1 M Tris-HCl buffer, pH 7.6, .1 mM Mg^{++} and the concentration of S-adenosyl-L-methionine indicated (□). Zymosterol is at a concentration of 100 μM. SAH, 2 μM (■) was added to each SAM concentration. A volume of .5 mL of enzyme preparation was added and the reaction is initiated by [^{14}C]-S-adenosyl-L-methionine (.1 μ Ci per 400 nmoles). Points are an average of 3 determinations.

Fig. 18

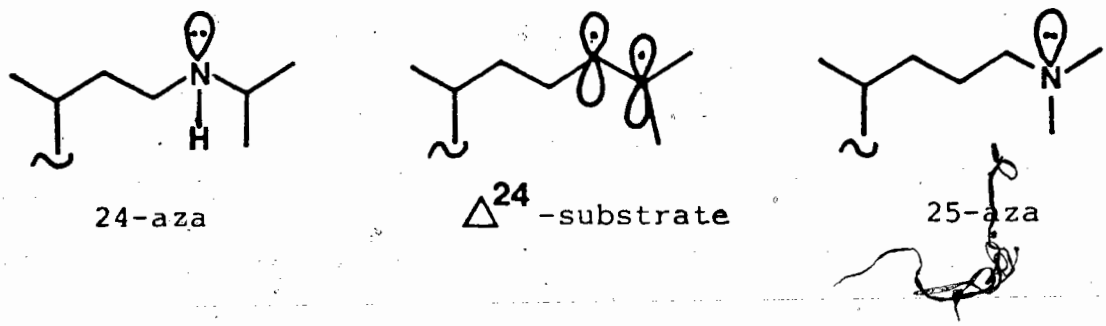


DISCUSSION

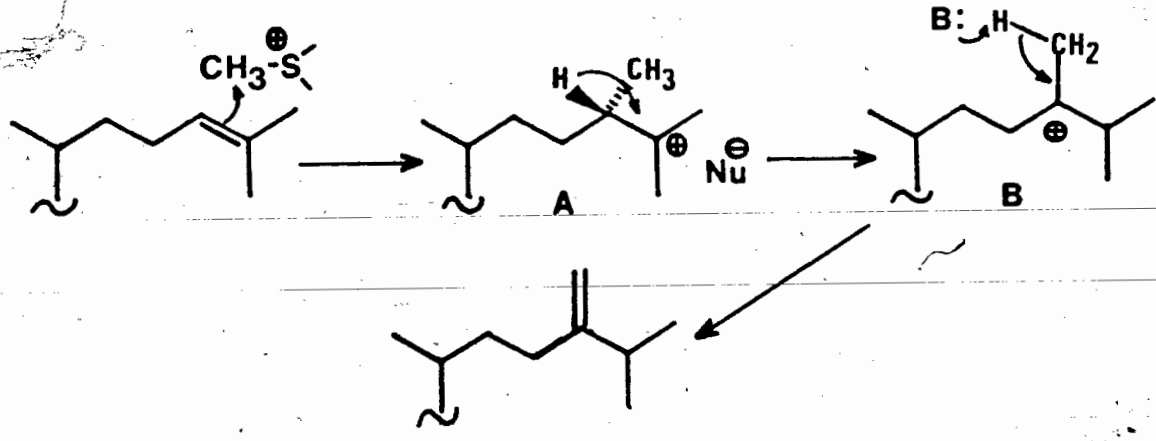
Growth of S. cerevisiae, in the presence of several side chain nitrogen-containing sterols, resulted in decreased cell growth and sterol production at azasterol concentrations of 5 μM or greater (Table I). Decreased cell production could be related to a decrease in total sterol or a decrease in ergosterol ($\Delta^{5,7}$-sterols) at higher inhibitor concentrations. Ergosterol has been reported to be important in the respiratory growth of yeast (Adams and Parks, 1969; Parks et al., 1979) and the 24β-methyl-Δ^{22} moiety was reported to be important to the growth of S. cerevisiae (Nes et al., 1978). Another possible explanation is that these azasterols could behave as regulators of sterol biosynthesis as do hydroxy sterols in mammalian systems (Parish et al., 1979; Parish and Schroepfer, 1979). Hydroxy-sterols are believed to have an effect on enzymes involved in sterol biosynthesis, specifically hydroxy-methylglutaryl-CoA reductase (HMG-CoA reductase) (Schroepfer, 1981).

The sterol composition of yeast cultures grown in the presence of various azasterols was different from that of control cultures (Table I). The alteration of sterol composition of yeast cultures, when grown in the presence of azasterols, indicated an inhibitory effect of azasterols on the Δ^{24}-sterol methyltransferase (24-SMT) and other sidechain modifying enzymes ($\Delta^{24(28)}$-methylene sterol reductase). For example, the appearance of new sterol metabolites, such as

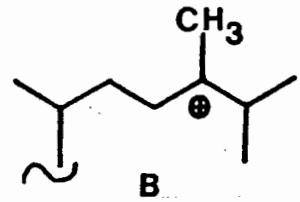
cholesta-5,7,22,24-tetraen-3β-ol and cholesta-5,7,24-trien-3β-ol, and the decrease in ergosterol production observed when 24- and 25-azasterols were administered to S. cerevisiae indicates inhibition of 24-SMT (Table I). This inhibition exhibited saturation kinetics indicating azasterol enzyme complexation. It has been postulated (Avruch et al., 1976; Oehlschlager, 1980) that 24-SMT inhibition by 24 and 25-azasterols is due to the close electronic relationship between the nitrogen lone pair and the Δ^{24}-system of normal 24-SMT substrates.

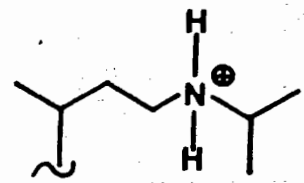


If one assumes a carbocation rearrangement mechanism for the methylation process (Oehlschlager et al., 1980), then molecules which resemble intermediates in the sequence could be inhibitors of the reaction (Jencks, 1975; Wolfenden, 1976).

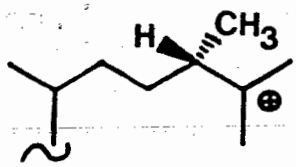


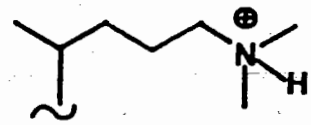
Thus, the protonated form of a 24-azasterol could mimic intermediate B while protonated 25-azacholesterol could mimic intermediate A.





$pK_a = 11$





$pK_a = 10-11$

This explanation has been put forward to explain inhibition of Δ^{24}-sterol methyltransferase in microsomes of maize seedlings (Narula et al., 1981) by 24-methyl-25-azacycloartenol and 25-azacycloartenol and in cultures of bramble cells (Schmitt et al., 1981) by 25-azacycloartenol.

In yeast, the effectiveness of 25-azasterols in inhibition of 24-SMT is related to their nuclear structure (25-azazymosterol > 25-azacholestanol > 25-azacholesterol > 25-azalanosterol). This is in the order of the acceptability of the corresponding Δ^{24}-sterols as substrates for yeast 24-SMT (Moore and Gaylor, 1970). This trend has been considered to indicate that 25-azasterols behave as sterol substrate mimics (Avruch et al., 1976; Pierce et al., 1978a; Oehlschlager et al., 1980).

As expected from the hypothesis, sidechain nitrogen- containing azasterols mimic natural substrates, 23-azacholesterol

is a weak 24-SMT inhibitor and 24-azacholesterol is comparable to 25-azacholesterol in inhibition of 24-SMT (Table I). Since 24-azacholesterol also inhibits the 24-methylene-$\Delta^{24(28)}$-sterol reductase, competition between this enzyme and the 24-SMT for this azasterol may reduce the effective concentration of the azasterol available to inhibit the latter enzyme.

Diazasterols, (20R) and (20S)-22,25-diazacholesterol, were less inhibitory than the other 25-azasterols investigated probably because the nitrogen at C_{22} decreases the effectiveness of the azasterol due to steric effects. 22,25-Diazasterols were also less effective at inhibiting sterol biosynthetic processes, in insects (Svoboda and Robbins, 1971) and rats (Counsell et al., 1965), than analogous 25-monoazasterols.

Inhibition of 24-methylene-$\Delta^{24(28)}$-sterol reductase by 23-azacholesterol was greater than by 24-azacholesterol which, in turn, was greater than by any of the other azasterols. If azasterols are acting as substrate mimics for the 24(28)-MSR, the nitrogens at C_{23} and C_{24} could be viewed as mimics (Pierce et al., 1978a; Oehlschlager et al., 1980) for the Δ^{22} and $\Delta^{24(28)}$ linkages of the best substrate of the reductase, ergosta-5,7,22,24(28)-tetraen-3β-ol (17) (Jarman et al., 1975). It is also possible that 24-azacholesterol binds 24-SMT reducing its effective inhibition of the 24-methylene-$\Delta^{24(28)}$-sterol reductase. The lack of inhibition of 24(28)-MSR by 25-azasterols is probably due to non-interaction of the nitrogen at C_{25} with any part of the enzyme.

The 22,23-sterol desaturase is not inhibited by any of the azasterols. 23-Azacholesterol resembles the product of the desaturase more than the substrate, episterol (Pierce et al., 1978a; Fryberg et al., 1973). 24-Azacholesterol and the 25-azasterols had no effect on the desaturase, presumably because nitrogens in these azasterols are too distant to affect the active site. The diazasterols, 20(R) and 20(S) 22,25-diazacholesterol, are also thought to be similar to the product of the desaturase and therefore ineffective (Oehlschlager et al., 1980).

The inhibition of Δ^{24}-sterol methyltransferase in C. utilis by 25-azacholesterol (1.0 μM) is not as pronounced as in S. cerevisiae (inhibition of 33% as opposed to 92% at identical concentration). This may be due to differences in the biosynthetic pathway used by the two yeasts. Since methylated sterols are substrates of the methyltransferase in C. utilis, desmethyl azasterols may not be as effective in this yeast.

25-Fluorocholesterol had no effect on any of the sidechain modifying enzymes indicating that the site involved in methylation is not affected by the size or electronegativity of the fluorine atom.

Isolation of specific yeast sterols, relatively pure and in large quantities, had previously been tedious and time consuming. In many instances, the target sterol was present in small quantities as a minor component in sterol mixtures. The use of azasterols with yeasts (wild type and mutant) to produce specific sterols has reduced some of these problems (Oehlschlager et

al., 1980). For example, growth of erg 2 sterol mutant yeast with 25-azacholesterol is the most effective way to produce large quantities of pure zymosterol completely free of cholesta-7,24-dienol. The latter sterol can be removed only by a long and involved chromatographic procedure at present (Taylor et al., 1981). The two practical applications that evolved from this work were the production of 25-hydroxy provitamin D_3 and [^{13}C]-enriched cholesterol.

When wild type S. cerevisiae is grown in the presence of small concentrations of 25-azacholesterol, the sterol fraction contains 40-60% zymosterol. This factor coupled with successful incorporation of 1-[^{14}C]-acetate (Hanahan and Al-Wakil, 1952; Fryberg et al., 1973) and 1-[^{13}C]-acetate (Filipenko and Cushley, 1976) into ergosterol suggested a route to significantly enriched [^{13}C]-zymosterol. Thus, S. cerevisiae grown in the presence of [^{13}C]-enriched acetate (90 atom %) and 25-azacholesterol produce [^{13}C]-enriched zymosterol. It was found that by reducing both aeration (by 80%) and stirring of the yeast (see P. 31) along with an increase in the [^{13}C]-acetate to 2.0 g/L in the medium, produced the best incorporation. High aeration increased sterol production, but decreased incorporation. Higher acetate concentrations decreased the yield of yeast and sterols. Therefore, the optimum conditions are a compromise between high incorporation and reasonable sterol production. Because of the requirement for [^{13}C]-enriched cholesterol for biological work, we developed a route for synthesis of

cholesterol from zymosterol. The important steps were efficient isolation of zymosterol and its hydrogenation to cholestanol. The isolation required two chromatographic steps, while the hydrogenation was fast and efficient. Subsequent reactions to the reduction with borohydride were done as described in the references (see P. 33). The reduction was accomplished as previously described (Belleau and Gallagher, 1951; Dauben and Eastham, 1951).

Spectral studies carried out with enriched zymosterol (7%) showed the increased ^{13}C-content of the sterol giving improved signal-to-noise (Figure 4). In order to increase resolution and sensitivity natural abundance ^{13}C NMR studies were recently carried out with cholesterol in egg yolk lecithin vesicles at high magnetic field (Brainard and Cordes, 1981). Large samples were used and accumulations were over long periods of time. The spectra obtained were sufficiently clear to detect carbon nuclei of cholesterol in the bilayer system. Only one carbon (C_6) resonance was unobscured by phospholipid resonance . Other sterol carbon resonances which should have been visible were not. It is probable, that if [^{13}C]-enriched sterols were used in these bilayer experiments further carbon resonances of cholesterol could be located.

Several difficulties have plagued production of 25-hydroxy provitamin D_3 (cholesta-5,7-dien-3β,25-diol) from wild type yeast. The greatest problem was that the ideal starting material, cholesta-5,7,24-trien-3β-ol, was only a minor component

(25%) in a mixture with cholesta-5,7,22,24-tetraen-3β-ol, from which it was separable only after multiple crystallization (Avruch et al., 1976). Efforts to reduce the unwanted tetraenol by inhibiting the 22,23-desaturase with 23-azacholesterol were unsuccessful (Pierce et al., 1978a). Growth of erg 5 sterol mutant with 25-azacholestanol did however provide an optimal route to cholesta-5,7,24-trienol (Pierce et al., 1979). In the present investigation, it was found that 25-azacholesterol gave inferior results. Mutant cultures grown with 1.0 μM 25-azacholesterol in this work compared to the cultures, grown in the previous study (Pierce et al., 1979), produced more sterols (1.55 vs. 1.30% of dry cell weight), but only a little more than half of the cholesta-5,7,24-trien-3β-ol predicted (15% vs. 26.6%) and more ergosta-5,7-dien-3β-ol than expected (13% as opposed to 1.8%). However, 24-SMT inhibition was only 85% compared with 92% for the earlier study. Thus, the best source of cholesta-5,7,24-trien-3β-ol is still from the mutant yeast erg 5. The synthesis of cholest-5,7-dien-3β,25-diol was accomplished in four steps. The sterols in the NSF were acetylated and then reacted with 4-phenyl-1,2,4-triazoline-3,5-dione in order to derivatize all of the $\Delta^{5,7}$-diene sterols. Chromatographic separation of the 1,4-cycloadducts of the $\Delta^{5,7}$-dienyl sterols from the unreacted NSF sterols gave the 1,4-cycloadducts of the target metabolite, cholesta-5,7,24-trien-3β-ol, and a small amount of ergosta-5,7-dien-3β-ol. The cycloadducts were

treated with m-chloroperbenzoic acid at low temperature to epoxidize the Δ^{24}-double bond of the trienol cycloadduct. The increased polarity of the epoxide of the C_{27}-sterol adduct facilitated its separation from the C_{28}-sterol adduct. Reduction by lithium aluminum hydride gave the provitamin in 57% yield. At present, use of the erg 5 mutant inhibited by 25-azacholestanol or 25-azacholesterol is an efficient source of precursor for the production of 25-hydroxy provitamin D_3 from yeast.

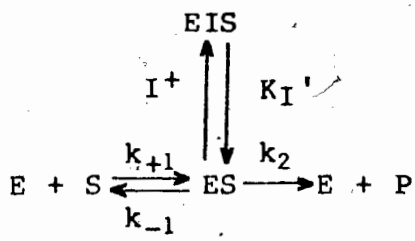
The Δ^{24}-sterol methyltransferase of yeast has been extensively studied in terms of its substrate specificity and inhibition (Moore and Gaylor, 1970; Bailey et al., 1974; Bailey et al., 1976; McCammon and Parks, 1981). The in vivo effects of azasterols on S. cerevisiae (and mutants) led us to consider their mode of action. These azasterols were extremely powerful inhibitors in yeast cultures over long periods of time (up to 72 h). Increases in the concentrations of the substrates zymosterol and ergostatetraenol did not seem to reverse the effects of the inhibitors of the methyltransferase and reductase enzymes. For 24-SMT, the observed inhibition could be due to a feedback mechanism because of the zymosterol buildup as was considered previously (Moore and Gaylor, 1970; Katsuki and Bloch, 1967). However, Parks found the addition of zymosterol increased 24-SMT activity greatly over the activity observed with endogenous sterols (Bailey et al., 1974). These conflicting results

for zymosterol, plus the desire to examine the validity of the sterol substrate-mimic model for the mode of action of azasterols on 24-SMT, led us to study the *in vitro* enzyme kinetics of the methylation using partially purified 24-SMT.

Gaylor's preparation of the 24-SMT proved long and gave inconsistent results, while Parks' cell-free preparation, although yielding 24-SMT of lower purity, gave more consistent kinetic results, was rapid and facile. An attempt to follow the enzyme reaction by gas chromatography failed, because of the extremely small changes in substrate and product concentration, with respect to the endogenous sterols, found in the cell-free preparations.

The amount of work involved in processing the enzyme assays led to the use of the Dixon plot (Dixon, 1953) to determine trends in inhibition. Using this plot, several inhibitor concentrations could be used in one experiment and difficulties with determination of absolute concentrations of substrate minimized. The data indicated that, when zymosterol was varied at constant S-adenosyl-L-methionine concentration, inhibition was uncompetitive. In this type of inhibition, the slopes of the lines for a Dixon plot are constant but possess different intercepts (Figures 8-11). For this type of inhibition, when the substrate concentration is divided by initial velocity (s/v), the lines converge in the second quadrant (Figures 12-15). A line drawn from this intersection, perpendicular to the inhibitor axis, gives the K_I' directly (Cornish Bowden, 1974). The

K_I' term is a dissociation constant for the binding of the inhibitor I with the ES complex. The K_I's of the azasterols studied are extremely low (lower than the *in vivo*

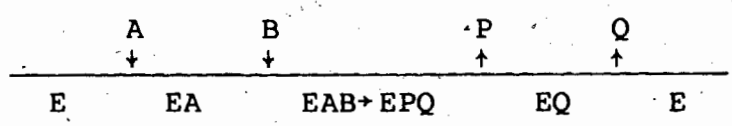


concentrations) and are of the same order of magnitude (nanomolar). Even the K_I' value for 23-azacholesterol is similar to the K_I' values for other azasterols which contrasts with the *in vivo* results (Table II). A possible reason for this observations is that the 24-methylene-$\Delta^{24(28)}$-sterol reductase is removed in the cell-free preparation of the 24-SMT at the initial centrifugation of 25,000 × g. Maximum reductase activity is found in the 25,000 × g pellet (Neal and Parks, 1977) which is discarded in 24-SMT preparations.

Uncompetitive inhibition occurs when an inhibitor binds with an ES complex meaning that there is no binding site available for the inhibitor until substrate binds with the enzyme. This type of inhibition is similar to ordered sequential reactions, where the second substrate can bind only when enzyme and the first substrate are complexed. Similarly, more complex multi-substrate cases are more likely to give results which fit the uncompetitive pattern. In order to analyze for this type of inhibition in a multi-reactant situation, the concentration of

one substrate must be varied while the concentration of the other is kept constant, when inhibitor is present. When S-adenosyl-L-methionine concentration is kept constant and zymosterol concentration is varied, one obtains an uncompetitive pattern (parallel lines).

Varying SAM concentration and keeping a constant zymosterol concentration gives a Dixon plot in which the lines converge (Figure 16), a competitive pattern. The inhibition patterns of multi-substrate enzyme reactions have been set out by Cleland (1963). In his study, a set of rules were derived, which give by inspection, a qualitative picture of the type of inhibition occurring. In this way, complex inhibition caused by either product or dead-end inhibition can be interpreted. The particular patterns given by the methyltransferase enzyme, when inhibited by the azasterols, indicated that they are dead-end inhibitors which bind to an EA complex. In this type of mechanism, A is the first substrate bound in an ordered bireactant mechanism as expressed below:



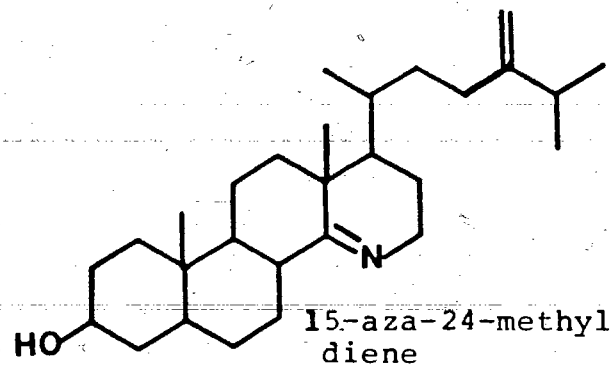
According to the rules set out by Cleland, dead-end inhibitors which resemble normal reactants, can bind to open parts of the enzyme or active site to give complexes which are unable to participate in the normal enzyme reaction. In such an instance,

the inhibition of the methyltransferase cannot be reversed by zymosterol if that is what binds first to the enzyme. The other part of the pattern would give competitive inhibition when the zymosterol concentration is kept constant and S-adenosyl-L-methionine concentration is varied. This result is observed with 25-aza-24,25-dihydrozymosterol as the inhibitor (Figure 16).

These arguments suggest that azasterols bind a form of the methyltransferase different from that which binds the zymosterol substrate lowering the total amount of enzyme available for substrate complexation (EA), methylation (EAB) and product release (EPQ, EQ). The increase in zymosterol concentration that occurs in mature cells would then not reverse this process. The competitive inhibition pattern observed for variable SAM concentrations indicates that a saturating SAM concentration would reverse the effects of the inhibitor. In vivo, when inhibitor is present and if the physiological concentration of SAM is lower than that required to reverse inhibition, the dead-end complex will persist. The in vitro concentration of SAM used was on the order of 100 μM.

The results indicate that azasterols bind to an enzyme-zymosterol complex to yield a ternary complex that does not bind S-adenosyl-L-methionine. The azasterols, thus, do not act strictly as mimics for Δ^{24}-sterols in terms of binding better to the enzyme than the preferred Δ^{24}-sterol substrates. On the other hand, 15-aza-24-methylene-D-homocholestadiene, a

metabolite from the mold Geotrichum flavo-brunneum, which contains a nitrogen in the D ring, is a competitive inhibitor of the Δ^{24}-sterol methyltransferase with respect to SAM (Bailey et al., 1976). Interestingly, cholesterol and dihydrozymosterol



15-aza-24-methylene-D-homocholesta-diene

have been shown to be competitive inhibitors of 24-SMT with respect to zymosterol at levels comparable to the K_M of zymosterol (Moore and Gaylor, 1970). The results of the in vivo study indicated that the nuclear structure of the inhibitor is important (25-aza-24,25-dihydro-zymosterol > 25-azacholesterol > 25-aza-24,25-dihydrolanosterol) possibly in the initial binding of the inhibitor or transport of the inhibitor in the yeast. However, the in vivo results also showed the placement of the nitrogen in the side chain is crucial to the potency of the inhibitor (25 > 24 > 23-azacholesterol). The ability of the nitrogen to become protonated might be the key to the inhibitory power of the azasterols studied. It is conceivable that in the microenvironment of the active site the nitrogen is charged and could compete for the S-adenosyl-L-methionine site. This would fit the kinetic pattern observed. Additional evidence for this line of argument comes from the in vitro inhibition of

Δ^{24}-sterol methyltransferase by the quarternary ammonium ion of 25-methyl-25-azacholesterol.

Kinetic experiments carried out to probe the inhibition of 24-SMT by ergosterol and S-adenosyl-L-homocysteine were done to compare the present enzyme preparation with previous preparations (Parks et al., 1979; McCammon and Parks, 1981). Ergosterol (Figure 17) is a non-competitive inhibitor with respect to zymosterol (Parks et al., 1979). This indicates that it binds to the 24-SMT at a site not involved with the binding of the sterol substrate. In this case, ergosterol, as has been pointed out (Moore and Gaylor, 1970), could be acting as a regulator of the methyltransferase by changing the enzyme conformation so that there is an effective decrease in the rate of methylation of zymosterol. The value of K_I calculated (120 μM) is higher than found in previous work (28 μM, Parks et al., 1979), however, zymosterol concentrations used in the present experiments were ten times higher and ergosterol concentration were twice that used previously (50 μM versus 25 μM). Use of a high concentration of zymosterol could reduce the concentration of the form of the enzyme that binds ergosterol, requiring a higher ergosterol concentration to affect inhibition.

The effect on 24-SMT enzyme activity of varying S-adenosyl-L-methionine concentrations has been extensively studied (Thompson et al., 1974). The atypical triphasic Michaelis-Menten results previously observed is also observed with our preparation (Figure 18). The explanation given

previously was that three enzymes were present in the preparation and were subject to changes in pH, zymosterol and SAM concentrations. Another way of rationalizing the result is to postulate that changes in S-adenosyl-L-methionine concentrations alter the efficiency of the enzyme. Three separate enzyme forms with separate affinities for SAM may be present in equilibrium, so, at low concentrations as well as high concentrations of SAM the enzyme methylates zymosterol but at different rates. If we relate this to an ordered bi-reactant enzyme system, we would expect various forms of the enzyme to be in equilibrium with each other depending on the concentrations of the reactants, zymosterol and SAM. Zymosterol, in the present case, is at a concentration which is saturating so that all the enzyme forms that bind S-adenosyl-L-methionine could be present. As the concentration of SAM changes, the efficiency of the enzyme could be regulated depending on the equilibrium between various forms of the enzyme. Then, as the concentration of SAM increases, one form of enzyme may be favored over another effectively shifting the equilibrium and increasing the rate of methylation of zymosterol. The 24-SMT enzyme may be more affected by changes of SAM concentration *in vitro* than it would be in the cell *in vivo*.

Competitive inhibition of the Δ^{24}-sterol methyltransferase with respect to SAM has been previously shown with S-adenosyl-L-homocysteine (Parks *et al.*, 1979). In the present work, there is a slight reversal of the inhibition as the SAM concentration increases (Figure 18), so, a competitive type inhibition could

be deduced. Since S-adenosyl-L-homocysteine is a product of the methylation reaction, the inhibition by this compound may be simply due to this product.

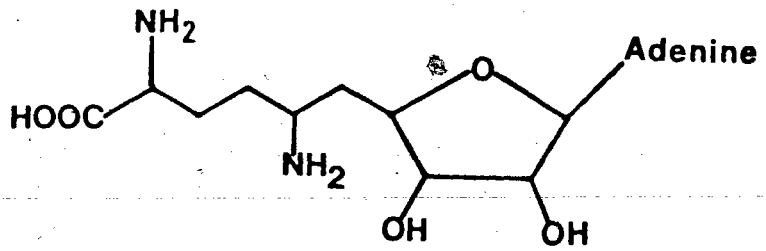
SUMMARY AND PROPOSED RESEARCH

Azasterol inhibition of Δ^{24}-sterol methyltransferase in yeasts results in reasonable production of $\Delta^{5,7}$-C_{27} sterols. Inhibition of 24-SMT of erg 5 by 25-azacholestanol or 25-azacholesterol provides acceptable amounts of cholesta-5,7,24-trien-3β-ol for synthesis of 25-hydroxy provitamin D_3. Inhibition of wild type S. cerevisiae with 25-azacholesterol, coupled with feeding [^{13}C]-acetate, produces [^{13}C]-enriched zymosterol. If erg 2 yeast is used, the Δ^7 impurity in zymosterol encountered in the wild type yeast work is eliminated but production of the sterol is decreased (5.2% versus 1.3% sterols of dry cell weight for erg 2).

The in vivo growth studies indicate that the Δ^{24}-sterol methyltransferase discriminates between the azasterol inhibitors possessing different nuclear structures just as it does for Δ^{24}-sterol substrates in vitro (Moore and Gaylor, 1970). The position of the nitrogen in the side chain is more crucial to the potency of the inhibitor in vivo than in vitro, possibly, because of in vivo competition for the azasterol inhibitor between different cellular enzymes (24-SMT versus 24(28)-MSR).

The in vitro inhibition results indicate that azasterol inhibitors have significant effects on the binding and utilization of S-adenosyl-L-methionine by the enzyme. It is conceivable that the side-chain nitrogen of the azasterol, if positively charged, could mimic the sulphonium species of the SAM molecule. The kinetic study shows that the azasterol inhibition of

24-SMT is due to its competition with SAM. This could be due to the charged nitrogen on the inhibitor binding with the SAM binding site. The antifungal agent sinefungin, a SAM analog and a competitive inhibitor of 24-SMT with respect to SAM (McCammon and Parks, 1981), also has a strategically placed nitrogen (a primary amino group in the δ-position) which is in the same position as the sulphonium-methyl moiety of SAM. The mode of action of sinefungin could also be similar in that it could compete for a negatively-charged site that binds S-adenosyl-L-methionine.



SINEFUNGIN

Study of azasterol inhibition of 24-SMT at various pH values, above and below the pH of the enzyme, could provide information concerning the state of protonation of the azasterol in its inhibitory form.

It has been suggested that a sulphonium group in the side chain could possibly be a better inhibitor of 24-SMT than the nitrogen in the azasterols are at present, as it would resemble the sulphonium group of SAM (Pierce, 1981, private communication). It is felt that the sulphur of the sterol could compete

with the sulphur group of SAM directly. However, if the size of the nitrogen atom in the azasterol is a factor in its effective inhibition of 24-SMT, then a sulphur atom may not inhibit as well as it could be displaced easily. If the environment around a SAM binding site is such that a nitrogen atom of the azasterol fits "tighter" than a sulphur atom of either SAM or the sulphonium group of a sterol sidechain, then the former may not be displaced as easily. A sulphonium sterol could be easily tested with the cell-free preparation of Δ^{24}-sterol methyltransferase. Presently, cultures of yeast are grown with inhibitors of 24-SMT at various concentrations, and the effectiveness of each inhibitor is determined by changes it causes in the sterol composition of the culture. The enzyme assay, when done on a smaller scale, would yield information about a potential inhibitor much more quickly. Although the *in vivo* results could still be different from the *in vitro* results as seen with 23-azacholesterol, preliminary testing could be carried out this way.

Product inhibition studies with fecosterol and S-adenosyl-L-homocysteine (the immediate products of the enzymatic reaction) could be expanded to determine the order of binding of substrates. The fecosterol could be obtained from *erg* 2 or 23-azacholesterol (1.0 µM) inhibited *erg* 2.

Finally, the effect of specific nucleophilic groups could be studied using cell-free 24-SMT enzyme preparation. For example, 24-SMT inhibition by a mercury(II) compound, such as mercury(II) p-hydroxy benzoate could be used to determine if a sulfhydryl group is important to its activity.

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