A STUDY OF SIDE-CHAIN THIASTEROL INHIBITORS OF Δ^{24}-STEROL METHYLTRANSFERASE IN YEAST *SACCHAROMYCES CEREVISIAE*

by

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ABSTRACT

The carbon atom in the 28 position of ergosterol is derived from the methyl group of S-adenosylmethionine (SAM). S-Adenosyl-L-methionine:Δ24-sterol methyltransferase (24-SMT) is the enzyme responsible for the introduction of this carbon at C-24 of yeast sterols. Sulfonium analogs of cationic intermediates of the methylenation reaction were shown to be potent inhibitors of the conversion of zymosterol, the natural substrate, to its methylene derivative, fecosterol. Inhibitory power [I_{50} (μM)] was in the order: 25-thiacholesteryl iodide (0.07) > 24β-methyl-25-thiacholesteryl iodide (0.14) > 24α-methyl-25-thiacholesteryl iodide (0.25). In the presence of these inhibitors, cultures of yeast produced increased amounts of zymosterol, while amounts of ergosterol and ergostatetraenol were decreased. New C_{27}-sterol metabolites were also found. Kinetic inhibition studies using radiolabelled SAM, partially purified enzyme and 25-thiacholesteryl iodide, showed this inhibitor acted uncompetitively with respect to zymosterol (K_i'= 2.4 nM) and competitively with respect to SAM (K_i= 1.1 nM). Initial velocity studies on the reaction mechanism of the methyltransferase reaction yielded a series of parallel lines, regardless of which substrate was varied at fixed levels of a second substrate. Overall, these results constitute strong evidence supporting a Ping-Pong mechanism for yeast 24-SMT in which the essentially irreversible step in the process is the transfer of the S-methyl group of SAM

to the enzyme.

A HUGO Y BLANQUITA

A MIS PADRES

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ABBREVIATIONS

GLC	gas liquid chromatography
$K_{A\langle B\rangle}$	Michaelis constant for substrate A(B)
K_i	inhibition constant
K_i'	apparent inhibition constant
K_m	Michaelis constant (concentration of substrate when v= V/2)
K_m'	apparent Michaelis constant
24(28)-MSR	24-methylene-sterol-$\Delta^{24\langle 28\rangle}$-reductase
NSF	non-saponifiable fraction
SAH	S-adenosyl-L-homocysteine
SAM	S-adenosyl-L-methionine
24-SMT	Δ^{24}-sterol methyltransferase
TLC	thin layer chromatography
Tris	tris-(hydroxymethyl)-amino-ethane
V	maximum velocity (value of v at saturation)
$V_{A\langle B\rangle}$	maximum velocity for substrate A(B)
V'	apparent maximum velocity

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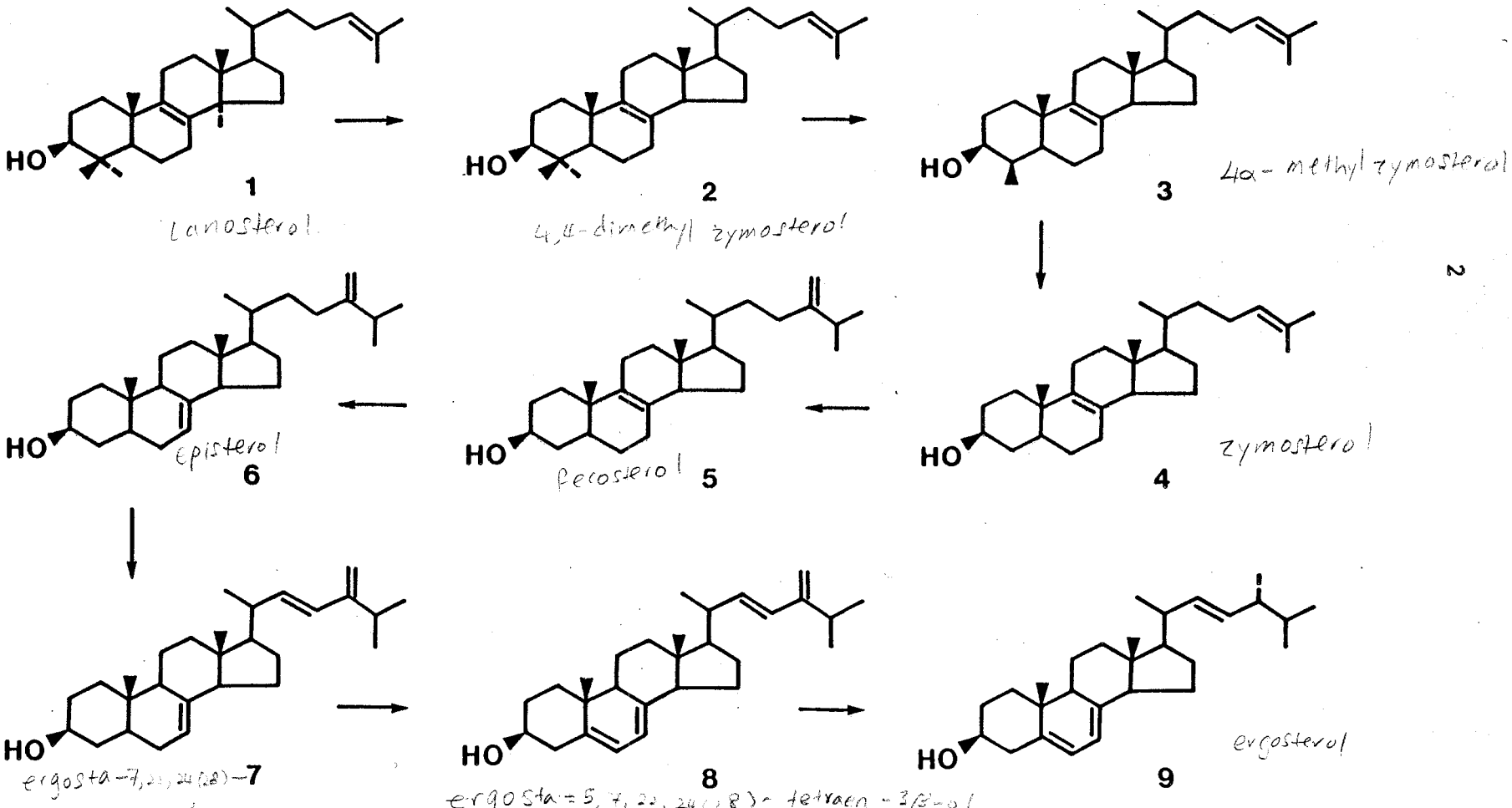
1. INTRODUCTION

The biosynthetic transformation of lanosterol,1, to ergosterol, 9, has been extensively studied in the yeast *Saccharomyces cerevisiae*. Several sterol intermediates have been recognized through the isolation and identification of endogenous sterols from both wild-type and mutant strains,[1-3] and by studying the metabolism of synthetic compounds in whole cells as well as in crude cell-free extracts of yeast.[4-8] From these studies simultaneous sequences have been proposed.[3,4,8] The major biosynthetic sequence is determined by the efficiency of specific enzymatic systems operating on available sterol substrates. An abbreviated sequence of the terminal events in yeast sterol synthesis is shown in Figure 1.

The majority of plant and yeast sterols are unsaturated in the side chain between C-22 and C-23, and are alkylated at C-24. Of special significance to this work is the C-24 alkylation reaction. It is well known that acetate serves as the major source of carbon for sterol biosynthesis, however, it is not a precursor of C-28 of 9.[9] In 1957, Alexander and co-workers[10a,b] conclusively demonstrated that the "extra carbon" atom in the 28 position of 9 was derived from the methyl group of methionine. Parks,[11] subsequently, proved that S-adenosyl-methionine (SAM) rather than methionine was the most effective methylating agent in yeast. The methylation is mediated by the enzyme S-adenosyl-L-methionine:Δ^{24}-sterol methyltranferase (E.C.2.1.1.41) (24-SMT).

Fig. 1. Sterol Biosynthesis in *S.cerevisiae*



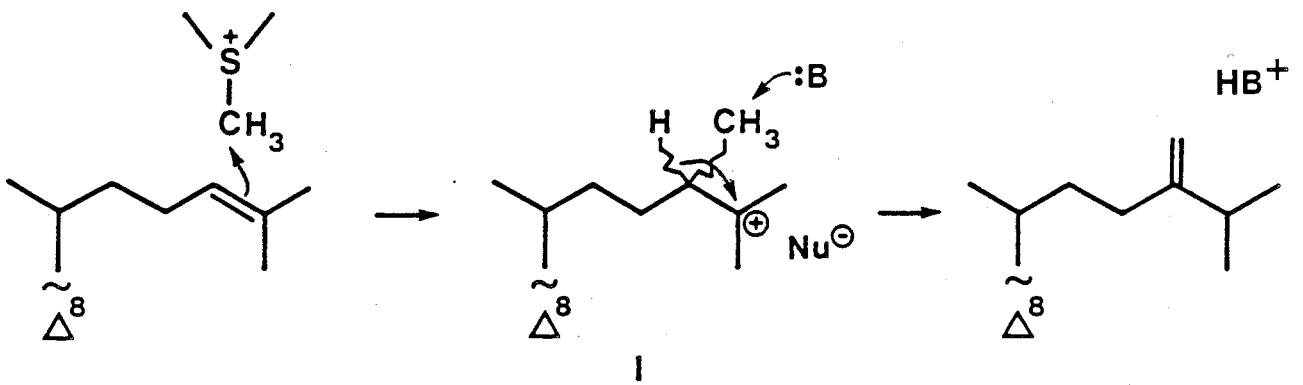
Moore and Gaylor[7] have isolated and highly purified this enzyme from yeast subcellular particles. The purified enzyme requires glutathione, Mg^{++} and a neutral pH for maximal activity.

A variety of studies, particularly those involving investigation of enzyme-substrate specificity, have provided evidence regarding the main sterol acceptor for the methylation. Using cell-free preparations of 24-SMT, Katsuki and Block[12] found that the methylated sterol intermediates formed from SAM contained an ergostane skeleton. These authors proposed a C_{27}-sterol as the methyl acceptor. Moore and Gaylor[13] found 5α-cholesta-8,24-dien-3β-ol, zymosterol, 4, to be the preferred substrate for methylation. Sterols methylated at C-4 were much poorer substrates while C_{27}-sterols containing no double bond between C-24 and C-25 were not methylated. Other groups, however, have suggested that methyl transfer to the sterol side-chain occurs before demethylation in positions 4α, 4β and 14α of the sterol ring has gone to completion.[14,15] Other C_{27}-sterols have also been reported as natural acceptors of the methyl group in ergosterol biosynthesis in yeast.[16,17] The immediate product of zymosterol methylation was found to be a methylene derivative, fecosterol, 5.[18] It has been suggested that the role of the 24-SMT is as a regulatory enzyme in the control of the biosynthesis of 9.[12,13]

Parks and co-workers[19] studied the sub-cellular location of yeast 24-SMT during aerobic adaptation and found it to be in the mitochondria and promitochondria. The same authors found the

enzyme to be present at low levels during anaerobic growth. They have also demonstrated the inhibition of this enzyme by high concentrations of cations such as Na^+ and K^+ in cell-free extracts of yeast[20,21] and have presented evidence for the presence of more than one enzyme capable of methylating sterols, one of which may be responsible for *in vivo* methylation of 4.[21]

Mechanistically, the methylation of the sterol side-chain is viewed as a nucleophilic attack of the Δ^{24}-π electrons on the S-methyl group of SAM generating a C-24 methylated sterol intermediate possessing a cationic site at C-25. Migration of a hydride from C-24 to C-25 and subsequent loss of a proton from C-28 leads to the formation of the $\Delta^{24(28)}$-methylene sterol in yeast and higher plants[22] (Scheme I). The carbonium ion in I could be stabilized by a nucleophilic residue of the active site of the enzyme leading to a transient interaction of I with the enzyme.[23-25]



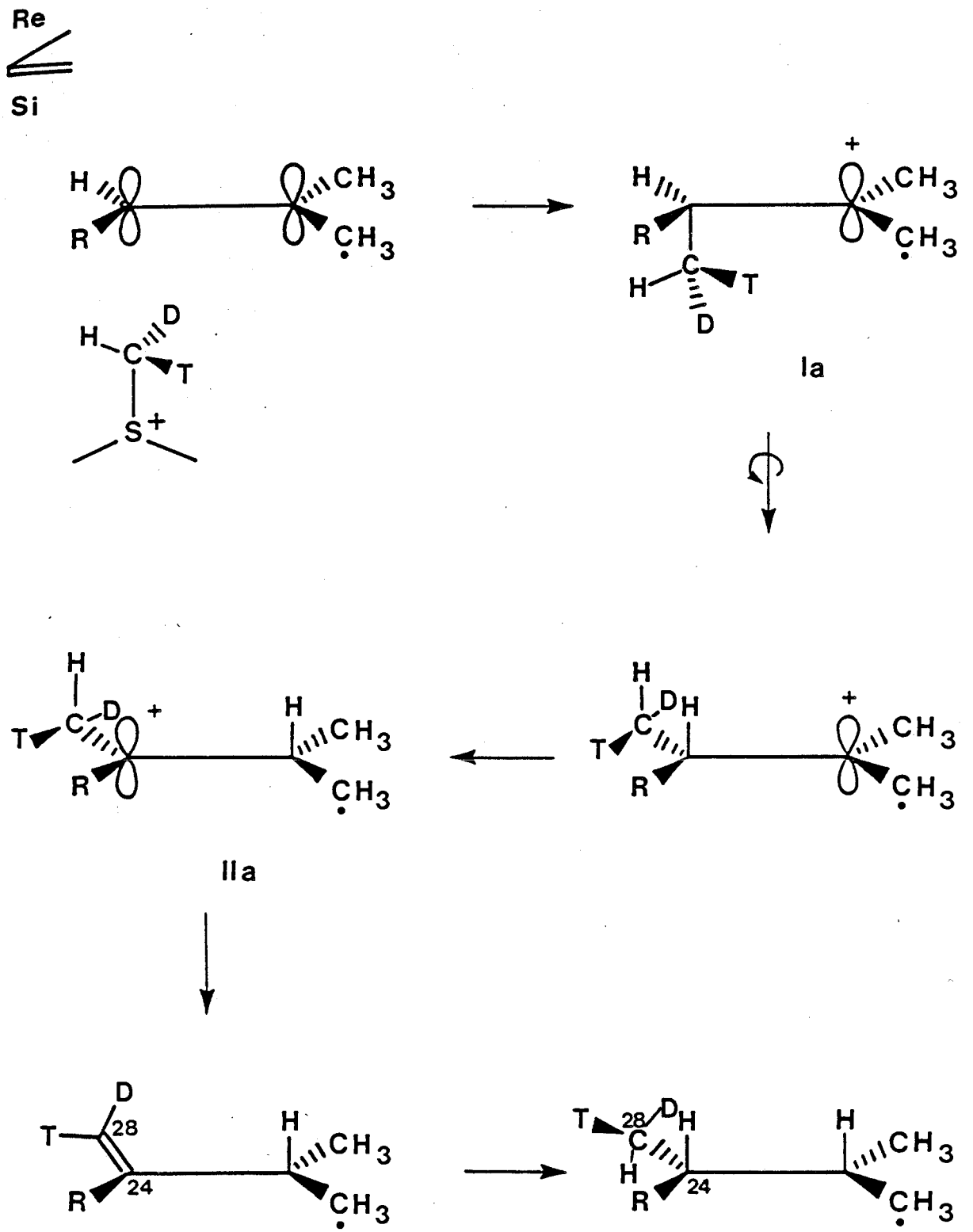
I

Scheme I

On the basis of this mechanism, one would expect 24-SMT could be inhibited by structural analogs of either the Δ^{24} substrate or the intermediate I.[26]

Stereochemical studies of this enzymic C-methylation, have been carried out using a C^{13} labelled sterol and, in separate experiments, methionine bearing a chiral methyl group (CHDT) of known configuration.[24] These investigations (Schemes II and III), revealed an S configuration at C-25 of the final sterol product. It was also shown that retention of configuration ocurred in conversion of the methionine methyl, to the (24S)-methyl sterol isolated from a whole cell incubation. Subsequent work (Arigoni, D., personal communication) showed that the $\Delta^{24(28)}$-methylene product of the 24-SMT reaction was reduced to the (24S)-methyl sterol in a trans fashion. These facts taken together impose restrictions on the stereochemical model for 24-SMT and, assuming direct transfer of the methyl from SAM to sterol, leave only two possibilities.

One possible stereochemical model (Scheme II), involves transfer of the methionine methyl to the Si face of Δ^{24} of the sterol substrate with inversion of configuration. In order to obtain the required configuration at C-25 of Ia, migration of the C-24 hydrogen to C-25 must then occur across the Re face. This, requires a slight (60°) rotation to align the C-24 hydrogen to carbon bond and the proper empty p orbital at C-25. Removal of a C-28 hydrogen from the Re face of IIa generates the final $\Delta^{24(28)}$-methylene enzymatic product. This would be converted, by trans


Re
Si
H
R
CH3
CH3
D
H
T
S+
+
H
R
C
H
T
D
CH3
CH3
Ia
H
T
C
D
+
R
H
CH3
CH3
IIa
H
D
H
T
C
R
+
CH3
CH3
D
T
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R
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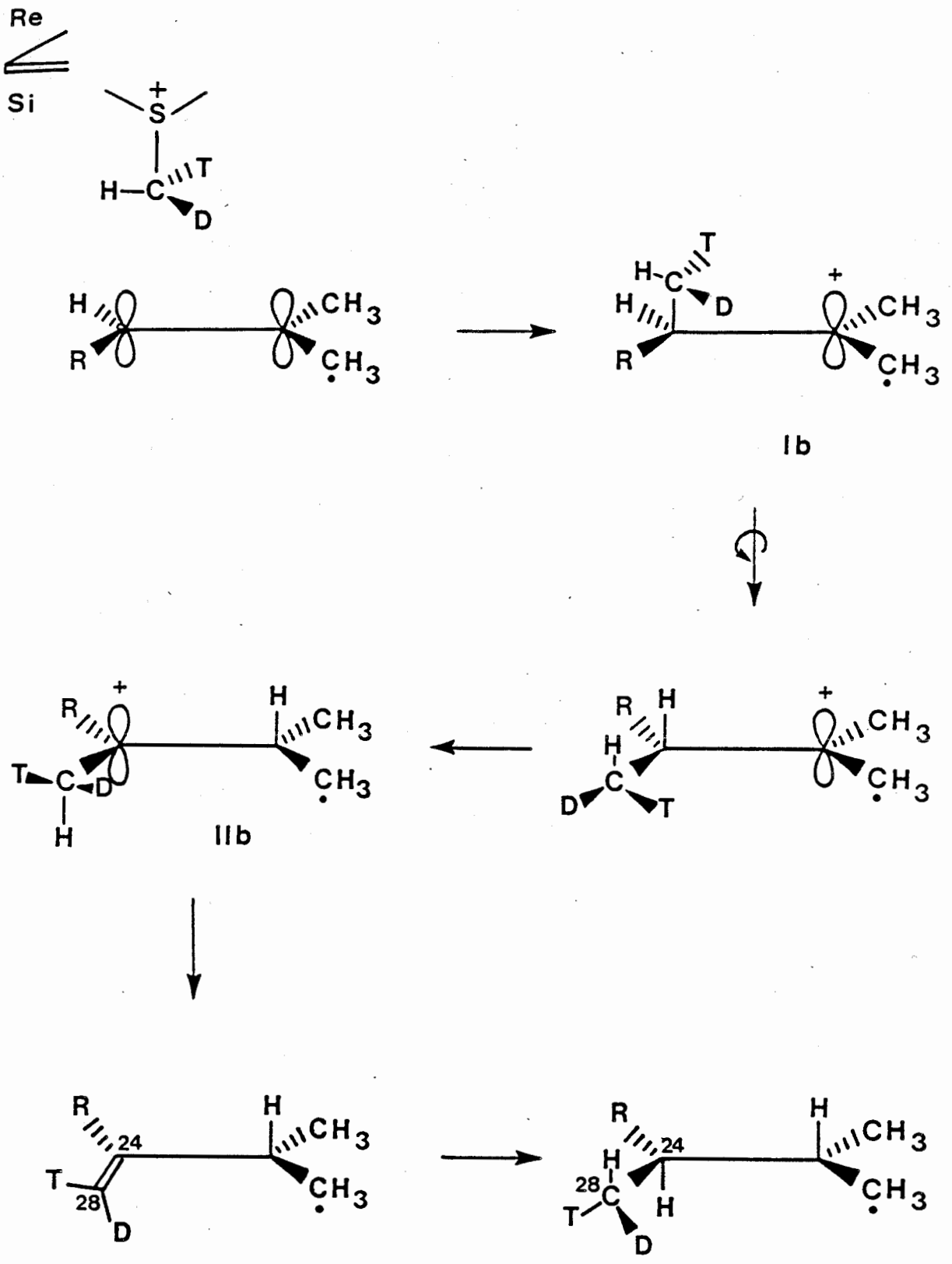
CH3
CH3

Scheme II



Scheme III

addition of hydrogen to $\Delta^{24(28)}$, to give a (24S)-methyl sterol with the same chirality at C-28 as the starting methionine.

A second possible stereochemical model (Scheme III), involves alkylation of the Re face of the Δ^{24}-sterol substrate, again with inversion of configuration of the transferred methyl. In order to achieve the required configuration at C-25, significant (120°) rotation must occur to align the C-24 hydrogen to carbon bond and the requisite face of the empty p orbital at C-25. Once this is achieved, C-24 to C-25 hydride migration can occur across the Re face to give Ib. Removal of a C-28 hydrogen from the Si face of IIb must, subsequently, occur to give the $\Delta^{24(28)}$-methylene product with the proper tritium/deuterium geometry to yield the same 24(S) C-28 chiral methyl sterol as before.

The stereochemical model (Scheme II) involving 24-Si alkylation requires the hydrogen removed at C-28, to be removed from the Re face of the substrate side chain, which is the same as that on which the C-24 $\longrightarrow$ C-25 hydride migration occurs. This is a suspect suggestion, since there is no *a priori* reason why the C-24 hydrogen should slide past an available base without being removed by it.

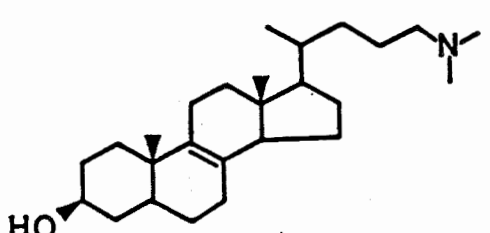
In the second stereochemical model (Scheme III), which is also consistent with the stereochemical probe experiments, alkylation occurs from the Re face of C-24. The questionable suggestion in this model is that rotation of nearly 120° about the C-24-C-25 bond, must occur before the C-24 hydrogen is aligned with an empty p orbital on C-25 to which it can migrate and give the observed

C-25 configuration.

Neither stereochemical model as proposed (Arigoni favored Scheme II), seems completely satisfactory. The major points of uncertainty are the stereochemistry of alkylation at C-24 (Re *vs* Si) and the chirality of transfer of the methionine methyl to the sterol substrate. Since 24-SMT mediates both the C-24 alkylation and C-28 deprotonation processes, the stereochemistry at C-24 of the initial C-24 alkylation product is not available for examination. It is therefore necessary to approach resolution of these questions by indirect methods.

One approach to the question of the C-24 stereochemistry and the configurational relationship between the methionine methyl and the C-28 methyl in the presumptive intermediate I, involves inhibition of 24-SMT with specifically designed mimics of I.

The two methods of enzyme inactivation employed most often are, genetic mutation[2,27,28] and chemical inhibition. Oehlschlager and co-workers[29] have shown that 25-aza-24,25-dihydrozymosterol, 10, a heteroatom substituted structural analog of the preferred sterol substrate[29-32] is a potent inhibitor of 24-SMT of yeast. 23-Azacholesterol, 11, was found to be a weaker inhibitor of this enzyme.[30] This inhibition has been attributed to the electronic resemblance at C-24 of the azasterol and the Δ^{24}-substrates.



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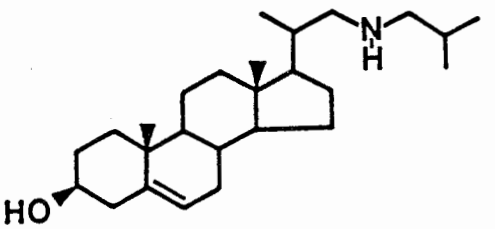

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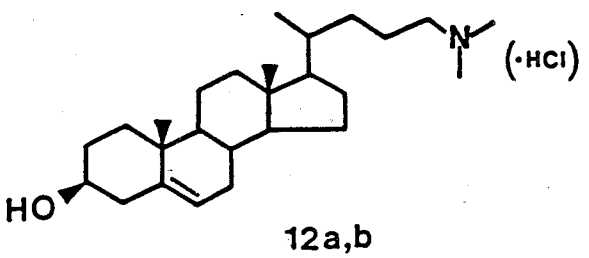
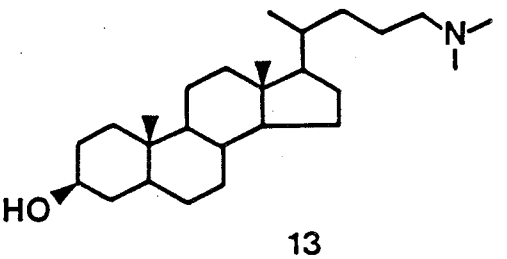
Since enzyme catalysis implies that the activated form of a substrate is bound more energetically by the active site than the substrate in its ground state,[33,34] molecules that bear a structural and electronic resemblance to metastable intermediates should be very strong inhibitors of the corresponding catalyzed reaction.[34-37]

A recent investigation in this laboratory[38] has shown that inhibition of 24-SMT of yeast by side-chain azasterols is related to their nuclear skeleton and side chain nitrogen position. Inhibitory power (I_{50}, μM) of these azasterols was found to be in the order of 25-azacholesterol hydrochloride salt, 12a, > 10, > 25-azacholesterol, 12b, ≈ 25-azacholestanol, 13, > (20R)- and (20S)22,25-diazacholesterol, 14, > 24-aza-cholesterol, 15, > 25-aza-24,25-dihydrolanosterol, 16, > 11.

Benveniste and co-workers using microsomes from maize seedlings[36,37] and cultures of bramble cells[39] have shown that derivatives of cycloartenol (the best substrate *in vivo* and *in vitro* of the C_{24}-methylation reaction in higher plants)



12a,b



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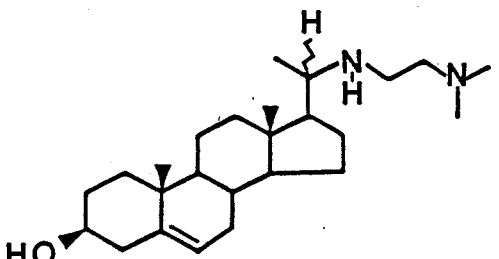

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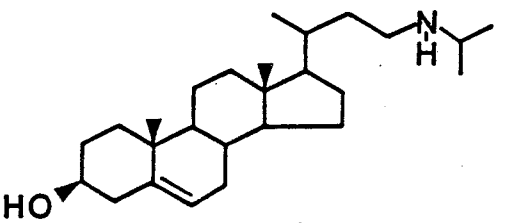

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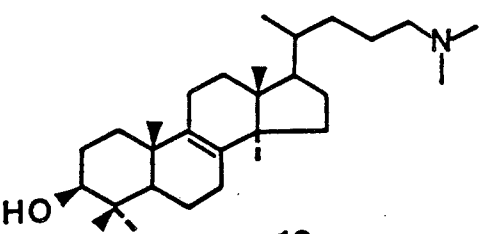

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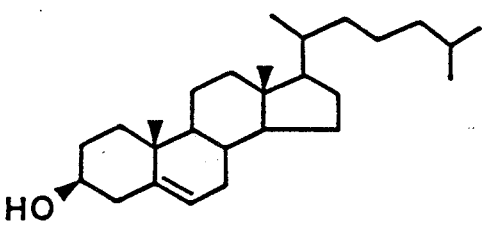
bearing a nitrogen atom at C-25 are potent inhibitors of S-adenosylmethionine:cycloartenol-Δ^{24}-methyltransferase (CMT). Very recently, the same authors[40] investigated structure-

activity relationship for C-25 heteroatom (N, As, S) substituted sterols. It was demonstrated that the presence of a positive charge at the 25 position is the major cause of the inhibition of CMT of maize due to an electronic resemblance of the sterol derivative to a "high energy intermediate", I. Similar concepts have been used to design inhibitors for other enzymes involved in sterol biosynthesis.[41-43]

Inhibition of sterol transmethylation in yeast by structural analogs of S-adenosylhomocysteine (SAH) has also been demonstrated.[44]

The mechanism of action of 24-SMT of *Saccharomyces cerevisiae* has been studied using steady-state kinetics. Preliminary results obtained with a highly purified enzyme preparation[7] showed that the enzyme exhibits saturation kinetics. The same authors studied inhibition of the yeast sterol methyltranferase reaction[13] and demonstrated that sterols possessing saturated side-chains such as cholesterol, 17, and 24,25-dihydrozymosterol, 18, are competitive inhibitors with respect to the natural substrate, zymosterol, 4. Furthermore, sterols possessing C-24 alkyl substituents such as 9, and stigmasterol, 19, enhanced the rate of the reaction at very low concentrations of 4, whereas at higher concentrations of 4 the maximal velocity was depressed. Later, Parks and co-workers[45] showed 9 acts non-competitively with respect to 4.

Oehlschlager and co-workers[38] using partially purified yeast 24-SMT and several side-chain azasterols showed azasterols acted



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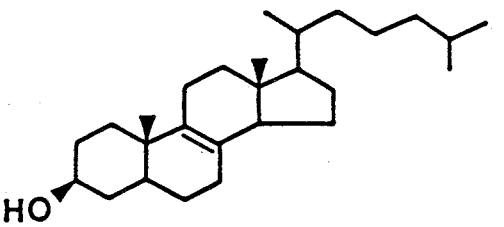

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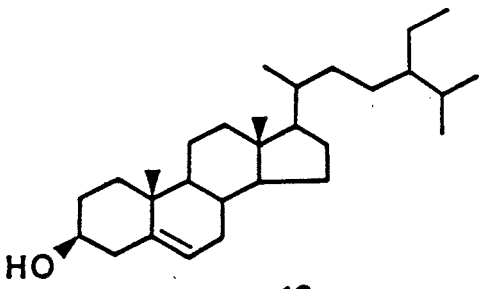

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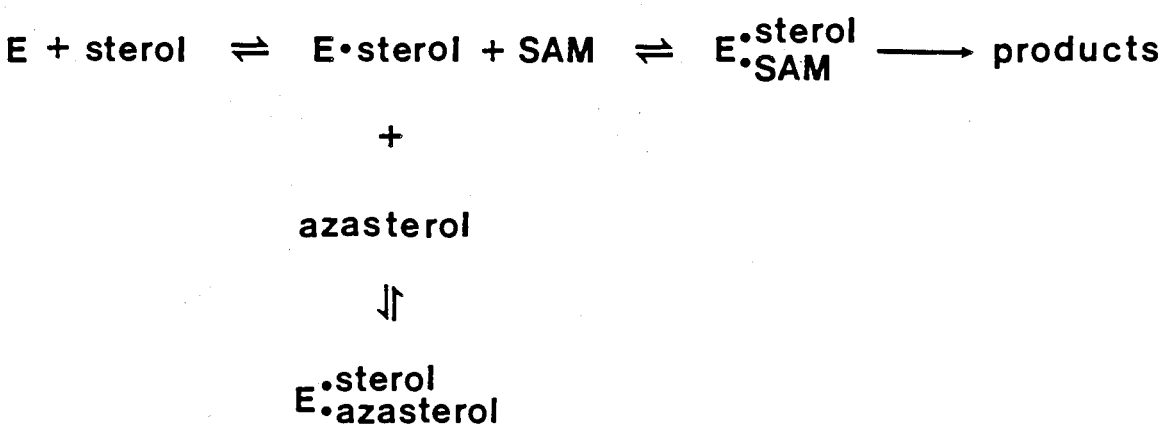
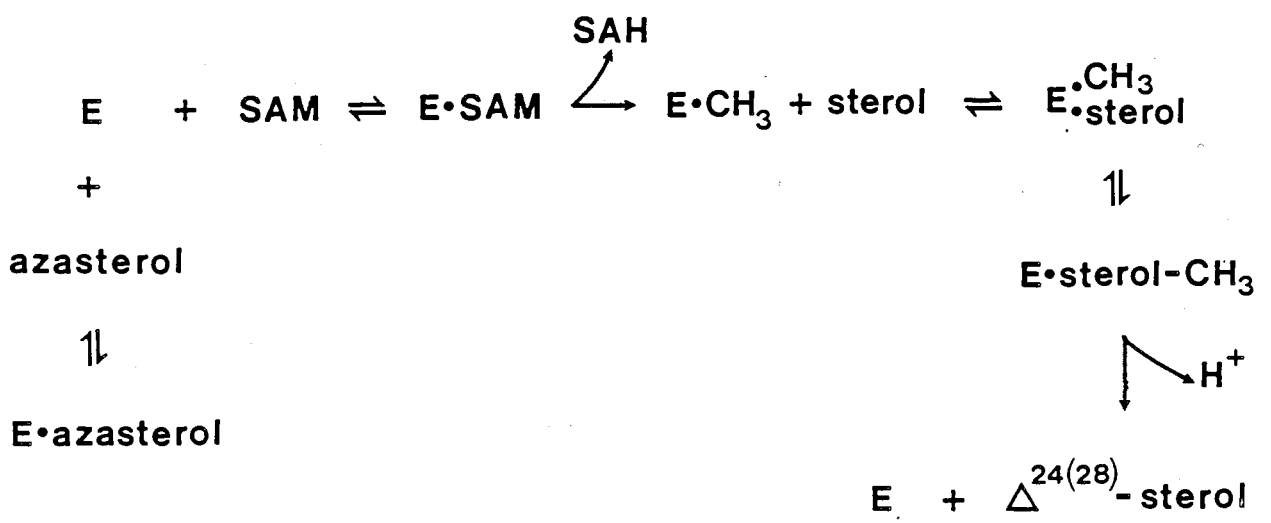
uncompetitively with respect to 4, and were competitive inhibitors with respect to SAM. Overall, these results, coupled with the observation that the efficiency of 25-azasterol inhibition changes with the same structural regularity as the specificity of the corresponding Δ^{24}-sterol substrates (4 > 17 » 1) led these authors to suggest two kinetic mechanisms for this reaction. The first, excludes the competition of azasterol and 4 for the same site on the 24-SMT. This would involve an ordered process where the sterol should be the first bound substrate (Scheme IV).



Scheme IV

The second, involves a Ping-Pong mechanism in which 24-SMT is methylated by SAM and the methylated enzyme reacts with the sterol substrate (Scheme V).



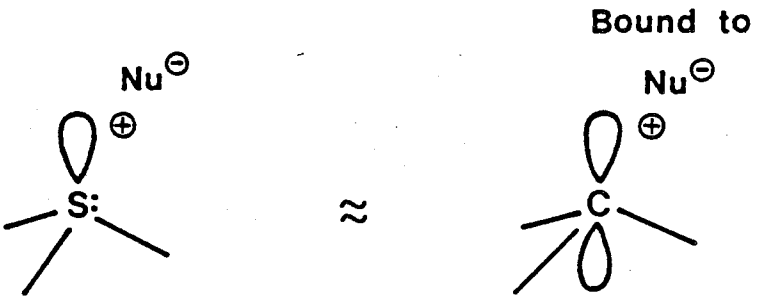
Scheme V

A Ping-Pong reaction mechanism has also been suggested for the enzyme SAM:Magnesium-Protoporphyrin-IX-Methyltransferase of wheat.[43,44]

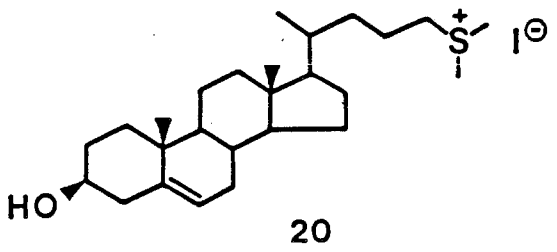
2. THESIS

At the time this research program was initiated, there was strong evidence that nuclear structure, side chain nitrogen position and nitrogen charge affected the enzymatic inhibitory power of azasterols. These effects were known, particularly, for yeast 24-SMT and 24(28)-MSR. Furthermore, kinetic studies of the inhibition of 24-SMT by these side-chain nitrogenated sterols carried out in our laboratory gave information on the mechanism of the methyl transfer and the nature of the inhibition for this particular system. These results, and the desire for a better understanding of the transmethylation process in yeast, stimulated the present work.

The main objective of this study was to investigate efficiency and kinetics of the inhibition of 24-SMT by sulfonium sterols which are considered to act as transition state analogs of this enzyme. Such analogs of enzyme bound intermediates should be tightly bound by target enzymes. We were especially interested in studying side-chain sulfonium containing sterols as possible inhibitors of 24-SMT in the yeast *S.cerevisiae*. We considered sulfonium ions to be reasonable analogs of enzyme bound tertiary carbonium ions (I and II, Schemes II and III). Thus, sterols bearing a sulfonium ion at C-25 such as 25-thiacholesteryl iodide, **20**, 24α-methyl-25-thia-cholesteryl iodide, **21a**,24β-methyl-25-thiacholesteryl iodide, **21b**, were expected to be good inhibitors of the enzyme. With reference to Schemes II and III, cultures of



yeast were to be grown with thiasterols at various concentrations, and their influence on yeast growth, sterol production, biosynthesis and composition studied.



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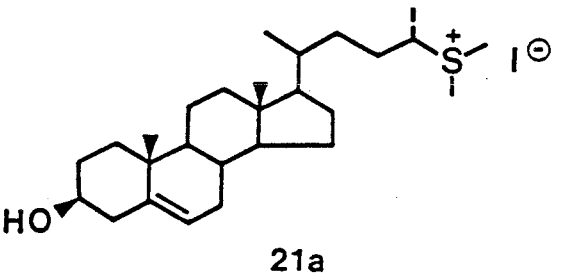
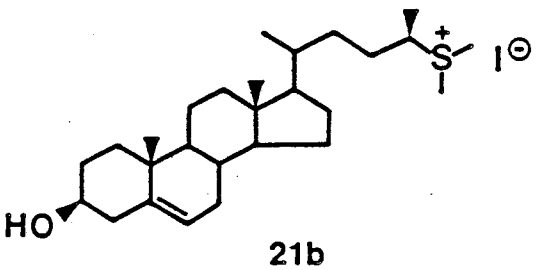


21a



21b

We planned to differentiate the two kinetic models proposed in our laboratory by studying the kinetics of the inhibition of 24-SMT by the thiasterols, using radiolabelled SAM and cell-free preparations of enzyme. The observation of kinetics consistent with a Ping-Pong mechanism, would then allow us to reinterpret Professor Arigoni's[24] elegant stereochemical work on this system outlined in Schemes II and III. A Ping-Pong process would implicate two inversions of the methyl group of SAM during transfer to C-28 of the sterol, and would require C-28 hydrogen removal from the face opposite to the C-24 $\longrightarrow$ C-25 hydride migration (Scheme II).

Depending on the kinetics observed, the relative inhibitory efficiency of thiasterols 20, 21a and 21b, would give an indication of the configuration at C-24 in intermediate I (Schemes II and III).

3. EXPERIMENTAL

3.1. Materials

3.1.1. Solvents and chemicals

Unless otherwise specified all solvents and chemicals were of the highest purity of commercially available reagents.

Sources of other synthetic substrates, inhibitors and chromatographic standards are indicated.

3.1.2. Biochemicals

Glucose oxidase (*Aspergillus niger* type II), and S-adenosyl-L-methionine (Grade II, chloride salt) were purchased from Sigma Chemical Co. Bacto-Yeast Extract and Bacto-Malt Extract were purchased from Difco Laboratories.

3.1.3. Radiochemicals

S-[Methyl-^{14}C]-SAM (lot No.1927-205, sp.act. 59.9 Ci/mol dil. sulfuric acid:ethanol, 9:1 pH 2) was purchased from New England Nuclear.

3.1.4. 25-Thiacholesteryl, 24α-Methyl-25-thiacholesteryl, and 24ß-Methyl-25-thiacholesteryl Iodide

25-Thiacholesteryl; 24α-methyl-25-thiacholesteryl and 24ß-methyl-25-thiacholesteryl iodide were synthesized in our laboratory by Dr. Eva Czyzewska. The melting points are 159-162° (with decomposition), 154-157°, and 145-147°, respectively.

3.1.5. Yeast Strains and Culture Medium

The wild type strain A184D (erg)$^{+}$ of *S.cerevisiae* and the sterol mutant *erg* 2 have been previously described.[27,48] Yeast strains were cultured in complete liquid medium.[49]

3.1.6. Isolation of Zymosterol

A sample of non-saponifiable fraction (NSF) obtained from several batch cultures of the *erg* 2 sterol mutant yeast of *S. cerevisiae* grown in the presence of 1.0 µM 25-azacholesterol was subjected to flash chromatography on Silica gel G60 (E. Merck No.9385).[50] Elution was with hexane-ethyl acetate, 75:25 (v/v). Separated sterols were detected by analytical TLC. Fractions containing zymosterol were acetylated with acetic anhydride-pyridine, 2:1 (v/v) and were analyzed by GLC on a SILAR-10C packed column (1.83 m x 2 mm i.d.) as the acetate derivatives. Zymosterol and ergosta-5,8,22-trienol (lichesterol) as well as small amounts of C_{28} and other $\Delta^{5,8}$-sterols eluted together. Zymosterol was separated by preparative TLC on Silica Gel GF254 impregnated with 25% silver nitrate, 0.5mm thick on 20 x 20 cm plates. The plates were developed twice with benzene-methylcyclohexane, 4:1 (v/v) and sprayed with Rhodamine 6G in acetone 0.1%, by weight. Bands were visualized under short wave UV light, removed and thoroughly extracted with ether. The presence of the individual sterols was confirmed by GLC (SILAR-10C packed column). Zymosteryl acetate was hydrolyzed and the free sterol was recrystallized from

hot methanol. This was analyzed as the TMS-derivative by GLC on a OV-101 capillary column (30 m x 0.25 mm i.d.).

3.2. Methods

3.2.1. 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 x 2 mm i.d. glass U-tube packed with 3% SILAR-10C on Gas Chrom Q (100-120 mesh) and OV-101 capillary, 30 m x 0.25 mm i.d. glass capillary coated with OV-101. Acetates were analyzed on both columns relative to cholestanyl acetate; OV-101 at 245°C, SILAR-10C at 220°C. Trimethylsilyl ethers were analyzed at 240°C relative to the trimethylsilyl derivative of cholestanol.[28] Ultraviolet spectra were recorded on a Cary 210 spectrophotometer. Fermentations were done on a Virtis fermenter, Model 40-300. Radioactivity was determined by a LKB Wallac liquid scintillation counter, Model 1217 Rack Beta using a Phase Combining System cocktail (Amersham). Centrifugations were carried out either on an IEC centrifuge or a Sorval RC-5 centrifuge. Color determinations for the Lowry protein assay were done on a Spectronic 20 spectrophotometer. Melting points were determined on a Fischer-Johns apparatus.

3.2.2. Inhibition Experiments and Isolation of NSF

The method was similar to that used by Oehlschlager and co-workers.[38] A 10 mL starter culture of wild-type *S.cerevisiae*

statically incubated at 30°C for 48-72 h was added to 100 mL of complete liquid medium containing the thiasteryl iodide of choice at a concentration of 0.1 µM unless otherwise specified. The culture was grown at 25°C with continuous stirring for 48-72 h. The inoculum was transferred to a 4L Virtis fermenter jar containing 1.5 L of medium to which the thiasteryl iodide had been added as a solution in ethanol (1-2 mL) to give a concentration of 0.1-0.5 µM. The culture was usually grown for 48 h at 30°C with 400 rev/min stirring and 1.9 L/min aeration. DOW antifoam spray was added to cultures to control foaming. The yeast cells were harvested by centrifugation (20 min at 2,500 g) washed three times with distilled water, weighed wet and either saponified immediately[29] or stored at -27°C until used. The non-saponifiable fractions were analyzed by GLC and UV spectroscopy.

This method was also employed when growing *erg* 2 sterol mutant yeast with 1.0 µM 25-thiacholesteryl iodide for zymosterol production, except that the starter culture was prepared by inoculating 2 x 10 mL medium.

3.2.3. Separation and Analysis of Yeast Sterols

The method of separation and analysis of yeast sterols was similar to that used by Oehlschlager and coworkers.[38]

3.2.4. Preparation of Acetone Powder of S-Adenosyl-L-methionine: Δ^{24}-Sterol methyltransferase

Cells of *S.cerevisiae* were grown with slight modification to the method described by Oehlschlager and coworkers.[38] Starter cultures of yeast were prepared by inoculating 4 x 10 mL of medium with a loop of cells followed by static incubation for 48 h at 30°C. These inocula were added to 2 x 1.5 L of medium (2 x 10 mL inocula to each 1.5 L of medium) in 4L Virtis fermenter jars. The cultures were stirred (400 rev/min) and aerated (1.9 L/min) for 24 h at 30°C. All subsequent steps were carried out at 4°C according to the method of Parks and coworkers.[20] Cells were harvested by centrifugation (10 min at 5,000 g), washed twice with 0.1 M Tris-HCl buffer (pH 7.5, containing 1 mM Mg^{++} ($MgCl_2$)), and weighed wet. Cells were suspended in 0.1 M Tris-HCl buffer to a final concentration of 1 g/mL. A total of 25 mL of cell suspension was added to a 75-mL Duran flask containing 40 g of 0.25 mm glass beads. The cells were given a 45 second burst in a Braun MSK cell homogenizer, and the homogenate was put on ice. Unbroken cells and cell debris were removed by centrifugation at 3,000 g for 10 min. The supernatant was centrifuged at 25,000 g for 30 min and the resulting pellet discarded. A layer of lipid floating on the surface of the supernatant was carefully removed with a Pasteur pipette.

The acetone powder was prepared as described by Moore and Gaylor.[7] The supernatant fraction was dialyzed for 9 h against chilled distilled water. A 100-fold volume of water was used and

changed three times. The dialyzed suspension was added dropwise to rapidly stirred acetone at -15°C. The volume of acetone was five times the volume of the supernatant fraction. After all the supernatant fraction had been added to the acetone, stirring was continued for an additional 30 min. The suspension was centrifuged at 10,000 g for 30 min at -15°C. The precipitate was dispersed in five volumes of fresh, chilled acetone stirring at a rapid rate for 20 min, and centrifuged. After the third treatment with acetone, the sedimented material was taken up in three volumes of anhydrous ether (-15°C) from a newly open can, and rapidly centrifuged as described above. This procedure was repeated three times. Ether vapors were removed under vacuum from the resulting powder at -15°C. The acetone powder was stored under vacuum at -20°C.

Protein was determined by the method of Lowry[51] using bovine serum albumin as a standard.

3.2.5. Assay of Enzyme Activity

The assay of 24-SMT activity was done using a procedure similar to that described by Moore and Gaylor.[7] All incubations were carried out in 25-mL Erlenmeyer flasks equipped with 14/20 glass joints. Unless otherwise specified reaction mixtures contained a final volume of 4.0 mL: 4.0 µmoles of $MgCl_2x6H_2O$; 222 µmoles of glucose; 3.5 mg of glucose oxidase; 400 µmoles of Tris-HCl; 20 to 400 nmoles of sterol substrate (e.g. zymosterol) dissolved in ethanol. Finally, 20 µL of a solution containing 20 nmoles of

S-[methyl-^{14}C]-SAM per µL (0.01 µCi/µL) were added. Just prior to use, the S-[methyl-^{14}C]-SAM solution was thawed. A portion was removed and diluted with a solution of S-adenosyl-L-methionine chloride in 0.1 M Tris-HCl buffer (pH 7.5) to provide a solution of S-[methyl-^{14}C]-SAM with a specific activity of 0.5 µCi/µmol. Assays of methyltransferase activity with the enzyme inhibitor 25-thiacholesteryl iodide ,20, were carried out as described above using an inhibitor concentration of 1.0 to 6.5 nM.

The order of addition of components to the reaction was as follows. Buffer containing the magnesium salt and the enzyme preparations (in 0.1 M Tris-HCl, pH 7.5, containing 1 mM Mg^{++}) were added first to prechilled Erlenmeyer flasks. The flasks were flushed with nitrogen for 20 seconds and then sealed with rubber septum stoppers. Glucose oxidase in buffer was injected into each of the flasks, and the contents were incubated for 5 min at 4°C to remove dissolved oxygen.[92] Simultaneously, anaerobic sterol substrate dissolved in ethanol (enough ethanol was added to make the final volume of ethanol 0.2 mL) and inhibitor as a solution in ethanol were injected. The reaction was initiated by injection of S-[methyl-^{14}C]-SAM solution. Incubations were carried out immediately at 37°C in a water bath with gentle shaking. Reactions were stopped by the injection of 5 mL of a 10% solution of KOH in 95% ethanol; saponification under nitrogen was then carried out for 1 h. The non-saponifiable fractions were extracted three times with 10 mL of hexane. The combined hexane extracts were washed with water until the wash was neutral. The hexane was

evaporated, the residue taken up in anhydrous ether (1-2 mL) and transferred into a liquid scintillation counting vial. The solvent was removed with a stream of nitrogen and 10 mL of PCS (Amersham) scintillation cocktail was added. The samples were counted for 5 min. Transmethylation was calculated from the count rates and specific activities of the substrate.

3.2.6. Analysis of Kinetic Data

Kinetic studies were examined by standard graphical procedures. Dixon plots[53] were used to analyse inhibition patterns. The data was plotted by least-squares fits according to a Polynomial Least-Squares Program (200 Poly, APL Public Files, Simon Fraser University). Correlation coefficients for the lines drawn were > 0.98. The error in work-up from sample to sample varied between 5 and 10% expressed as rate for typical samples in a kinetic experiment.

4. Results

4.1. *In vivo* Inhibition Studies on *S.cerevisiae*

4.1.1. Effect of Thiasterols on Yeast Growth and Sterol Production

Thiasterols,24α-methyl-25-thiacholesteryl iodide, 21a, did not appreciably affect the yeast growth and caused only a slight decline in cell production at 0.3 μM or below. Thiasterols, 25-thiacholesteryl iodide, 20, and 24β-methyl-25-thiacholesteryl iodide, 21b, reduced the dry cell weight to approximately 60% of the control value when its concentration increased to 0.3.

Sterol content on a dry weight basis was highly affected throughout the thiasterol concentrations examined. All three of the different thiasterols tested reduced to some degree production of ergosterol, 9.

4.1.2. Effect of Thiasterols on Sterol Biosynthesis and Sterol Composition

As inhibitor concentrations in cultures increased, ergosterol biosynthesis drastically declined (Table I). For 20 and 21b ergosterol production ceased at the 0.5 μM level. The amount of ergostatetraenol, 8, also decreased whereas zymosterol, 4, increased in relative proportion. Three other C_{27}-metabolites, cholesta-7,24-dienol, 22, cholesta-5,7,24-trienol, 23, and cholesta-5,7,22,24-tetraenol, 24, also accumulated in treated cultures (Fig.2).

Table I. Effect of Thiasterols on Growth and Sterol Composition in Yeast *S.cerevisiae*.

	25-thiacholesteryl iodide			
Inhibitor concentration (μM)	0	0.05	0.1	0.5
Dry cell wt. (g/l)	8.3	8.2	8.2	5.4
Sterols (% of dry cell wt)[b]	2.2	1.9	4.4	1.9
$\Delta^{5,7}$-dienes (% of dry cell wt)[a,b]	0.9	0.6	1.3	0.3
	0.8	0.5	1.4	0.2
Sterol composition (%)[b]				
Lanosterol	5.2	5.3	1.8	2.4
4,4-dimethylzymosterol	14.1	14.8	8.0	8.8
4α-methylzymosterol	6.2	5.5	1.7	4.4
4α-methylergosta-8,24(28)-dienol	2.0	1.7	nd	0.2
Zymosterol	25.3	39.1	52.4	59.6
Fecosterol	5.4	3.9	0.6	nd
Episterol	2.4	1.9	1.4	nd
Ergosta-8,22,24(28)-trienol	1.9	nd	nd	nd
Ergosta-7,22,24(28)-trienol	0.6	nd	nd	nd
Ergosta-5,7,22,24(28)-tetraenol	24.3	18.7	12.6	1.0
Ergosterol	12.6	6.3	2.0	nd
Cholesta-7,24-dienol	nd	1.8	5.1	11.7
Cholesta-5,7,24-trienol	nd	nd	3.6	2.3
Cholesta-5,7,22,24-tetraenol	nd	1.0	10.8	9.6
% Inhibition: 24-SMT	0	34.7	64.8	97.9
24(28)-MSR	0	29.4	56.4	100

[a]: determined by UV

[b]: determined by GLPC

Table I. Cont'.

	24α-methyl-25-thiacholesteryl iodide		
Inhibitor concentration (μM)	0.1	0.3	0.5
Dry cell wt. (g/l)	8.2	8.0	7.1
Sterols (% of dry cell wt)[b]	3.1	2.4	2.1
$\Delta^{5,7}$-dienes (% of dry cell wt)[a,b]	0.8	0.6	0.6
	0.9	0.6	0.4
Sterol composition (%)[b]			
Lanosterol	7.3	2.9	1.8
4,4-dimethylzymosterol	19.8	11.1	9.3
4α-methylzymosterol	6.6	6.7	6.5
4α-methylergosta-8,24(28)-dienol	nd	nd	nd
Zymosterol	25.8	46.8	51.6
Fecosterol	1.9	1.7	0.6
Episterol	2.5	1.1	0.1
Ergosta-8,22,24(28)-trienol	nd	nd	nd
Ergosta-7,22,24(28)-trienol	5.2	nd	nd
Ergosta-5,7,22,24(28)-tetraenol	18.1	14.0	7.3
Ergosterol	9.2	3.7	1.5
Cholesta-7,24-dienol	3.3	3.1	5.4
Cholesta-5,7,24-trienol	nd	3.1	4.3
Cholesta-5,7,22,24-tetraenol	0.4	5.8	11.6
% Inhibition: 24-SMT	21.8	56.6	80.0
24(28)-MSR	8.8	39.5	48.5

[a]: determined by UV

[b]: determined by GLPC

Table I. Cont'.

	24β-methyl-25-thiacholesteryl iodide		
Inhibitor concentration (μM)	0.1	0.3	0.5
Dry cell wt. (g/l)	8.1	7.2	5.0
Sterols (% of dry cell wt)[b]	2.8	3.3	4.3
$\Delta^{5,7}$-dienes (% of dry cell wt)[a,b]	1.2	0.5	0.7
	1.2	0.4	0.8
Sterol composition (%)[b]			
Lanosterol	3.4	3.5	4.0
4,4-dimethylzymosterol	6.5	10.9	17.8
4α-methylzymosterol	2.8	4.9	8.9
4α-methylergosta-8,24(28)-dienol	0.3	0.5	nd
Zymosterol	31.2	57.2	42.3
Fecosterol	1.3	nd	nd
Episterol	1.9	nd	nd
Ergosta-8,22,24(28)-trienol	nd	nd	nd
Ergosta-7,22,24(28)-trienol	nd	nd	nd
Ergosta-5,7,22,24(28)-tetraenol	23.8	1.6	1.6
Ergosterol	6.7	0.1	nd
Cholesta-7,24-dienol	10.0	10.2	9.3
Cholesta-5,7,24-trienol	3.4	3.2	2.9
Cholesta-5,7,22,24-tetraenol	8.7	7.9	13.2
% Inhibition: 24-SMT	28.6	96.4	96.6
24(28)-MSR	31.9	82.8	100

[a]: determined by UV

[b]: determined by GLPC

The percent inhibition of 24-SMT was obtained by comparison of the proportion of C-24 alkylated sterols produced in the presence of the inhibitor ($\Sigma C_{24}\text{-alk}_i$) with that produced in control cultures ($\Sigma C_{24}\text{-alk}_c$) according to the expression:

$$\% \text{ inhibition} = 100 \left[1 - \left(\Sigma C_{24}\text{-alk}_i / \Sigma C_{24}\text{-alk}_c \right) \right]$$

The concentration of 20, 21a, and 21b required to reduce by 50% the amount of C-24 alkylated sterols is shown in Table II. Thiasterol 20 was found to be the most potent inhibitor of 24-SMT, while 21a was the least potent. Both C-24 methylated thiasterols were less potent than thiasterol 20.

When the ratio of ergosterol to $\Delta^{24(28)}$-sterols in inhibited cultures ($\Sigma\Delta^{24(28)}\text{-sterols}_i$) was compared with the ratio of ergosterol to $\Delta^{24(28)}$-sterols in the control cultures ($\Sigma\Delta^{24(28)}\text{-sterols}_c$):

$$\% \text{ inhibition} = 100 \left\{ 1 - \left[\frac{(\text{ergosterol}_i / \Sigma\Delta^{24(28)}\text{-sterol}_i)}{(\text{ergosterol}_c / \Sigma\Delta^{24(28)}\text{-sterol}_c)} \right] \right\}$$

it was found that the enzyme system 24-methylene sterol-$\Delta^{24(28)}$-reductase (24(28)-MSR) was greatly inhibited by these sulfur containing sterols (Table I). Thiasterol 20 appeared to be a very powerful inhibitor of the 24(28)-MSR system.

Fig.2. Expected C_{27}-metabolites from 24-SMT Block

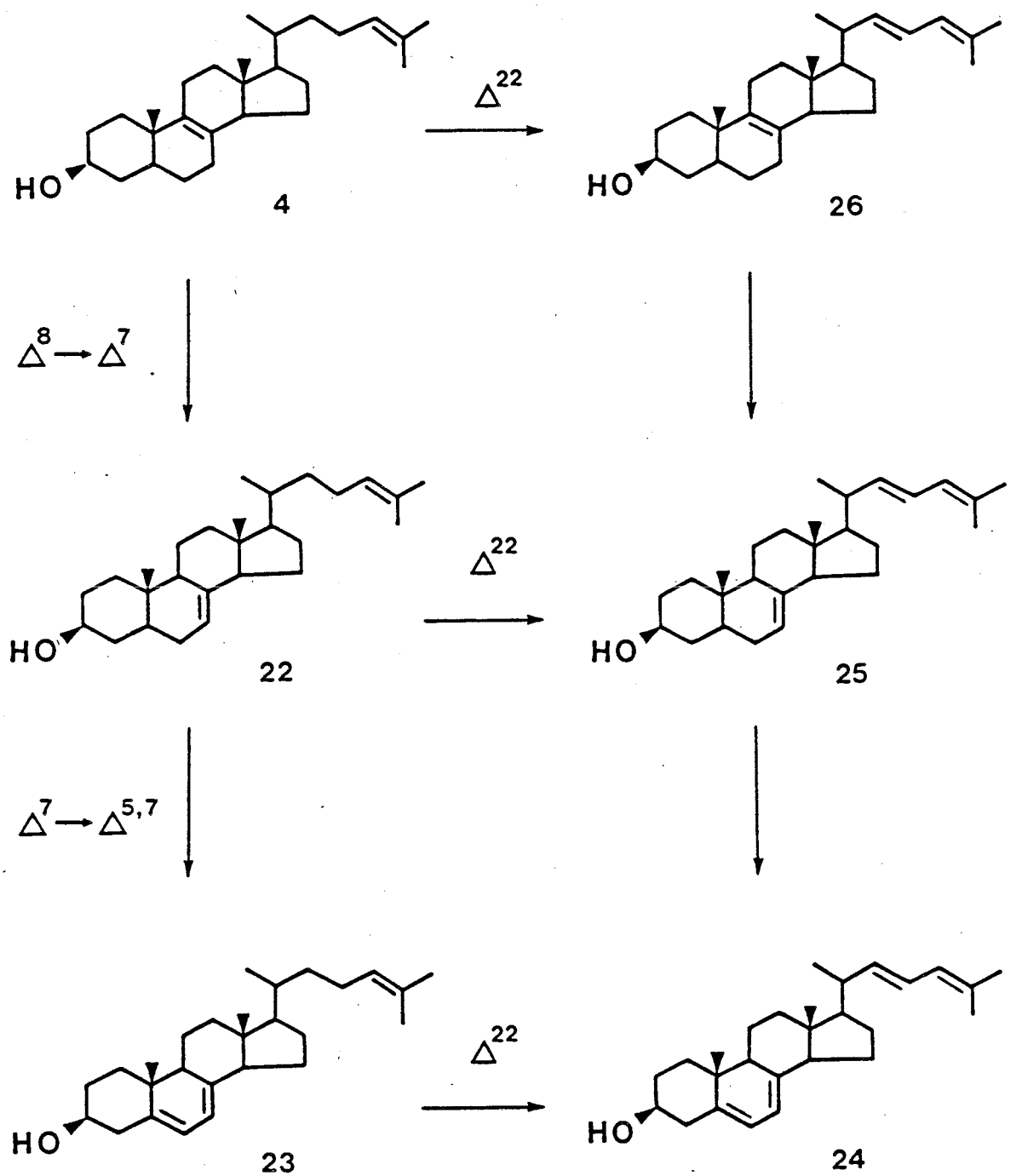


Table II. Efficiency of Thiasterol Inhibition of 24-SMT and 24(28)-MSR in Yeast *S.cerevisiae*.

Thiasterol[a]	Concentration Required for 50% Inhibition (μM)		Relative Efectiveness as Inhibitor
	24-SMT	24(28)-MSR	24-SMT/24(28)-MSR
20	0.07	0.08	0.875
21a	0.25	0.50	0.500
21b	0.14	0.15	0.933

[a] Thiasterol administered to culture of *S.cerevisiae* and using previously described growth conditions and sterol analysis procedures.[28] Growth period for the cultures was 48 h.

4.2. Production of Pure Zymosterol for Enzyme Assays

The erg 2 sterol mutant yeast of *S.cerevisiae* treated with 1.0 µM 25-azacholesterol produced zymosterol, 4, which represented 50+% of the sterol content. The major impurity was ergosta-5,8,22-trienol while smaller amounts of cholesta-5,8,24-trienol, cholesta-5,8,22,24-tetraenol and fecosterol were also present. Impurities were removed by flash chromatography and preparative argentation ($AgNO_3$, 25%) TLC. Zymosterol isolated by this procedure was found to be 99% pure by GLC (m.p.108°C). This yeast produced 8.0 g/L dry cell weight which was 1.5% by weight sterols.

4.3. *In vitro* Studies on *S.cerevisiae*

4.3.1. 24-SMT Extract for Kinetic Studies

Partial purification of 24-SMT from *S.cerevisiae* following the method of Moore and Gaylor[7] was attempted. The most active methyltransferase fraction was found in the acetone powder extract, while the methyltransferase activity was lost completely when the recommended ammonium sulfate precipitation was conducted. We thus used the acetone powder extract in our kinetic experiments.

The acetone powder as prepared was suspended in 0.1 M Tris-HCl buffer (pH 7.5 and containing 1mM Mg^{++}). The suspension was centrifuged at 10,000 g for 20 min and the resulting pellet found to be devoid of methyltransferase activity. The supernatant had a protein concentration of 3.5-7.0 mg per mL, and specific transferase activity of 2.31 nmole per hour per mg of protein.

All stages of enzyme purification and incubation were conducted in 1mM Mg^{++}. In contrast with earlier reports,[7,21] neither glutathione nor bicarbonate were found to be requirements for maximal enzymic activity.

4.3.2. Inhibition Studies with 25-Thiacholesteryl iodide on 24-SMT

Due to the results obtained in the *in vivo* inhibition studies with thiasterols, 25-thiacholesteryl iodide, 20, was chosen for *in vitro* studies.

When SAM concentration was varied and zymosterol concentration was kept constant, the Dixon plot of the reciprocal of the initial velocity *vs* inhibitor concentration gave a family of lines, all intersecting at the same point in the fourth quadrant (Fig.5) This pattern is indicative of competitive inhibition. However, when zymosterol concentration was varied and SAM concentration was held constant, the Dixon plot yielded a set of parallel lines which are observed for uncompetitive inhibition (Fig.6). In this case, K_i' values were obtained from the plots of substrate concentration/initial velocity *vs* inhibitor concentration (Fig.7).

4.3.3. Kinetics of the Transmethylation Reaction

The initial rate of the reaction was measured, and a regular rectangular hyperbole plot for zymosterol was obtained (Fig.3). Reciprocal expressions of velocity and substrate concentration gave a Lineweaver-Burk plot (Fig.4). As shown in Fig.8, double reciprocal plots of SAM concentration at three fixed concentrations of zymosterol gave a set of parallel lines. No apparent convergence of the lines at a common point was evident. The plot of the reciprocal of the zymosterol concentration at three fixed concentrations of SAM (Fig.10) also yielded a family of parallel lines. The intercepts on the ordinate are the reciprocals of the apparent maximal velocities, $1/V'$, and the intercepts on the abscissae are negative reciprocals of apparent Michaelis constants, $1/K_m'$. The Michaelis constants of the enzyme for SAM (K_A) and zymosterol (K_B) were obtained graphically from secondary plots of the values of $1/V'$ obtained in Figs.9 and 11.

Figure 3

Dependence of Velocity (v) on Substrate Concentration ([zymosterol]). (v *vs* S)

Acetone powder extract of methyltransferase (1.0 mL, ~6 mg of protein per mL) was incubated with 20 to 400 nmoles of zymosterol and 400 nmoles of S-[methyl-^{14}C]-SAM in a final volume of 4.0 mL at 37°C for 30 min. Results are the average of samples from four separate experiments.

Fig.3.

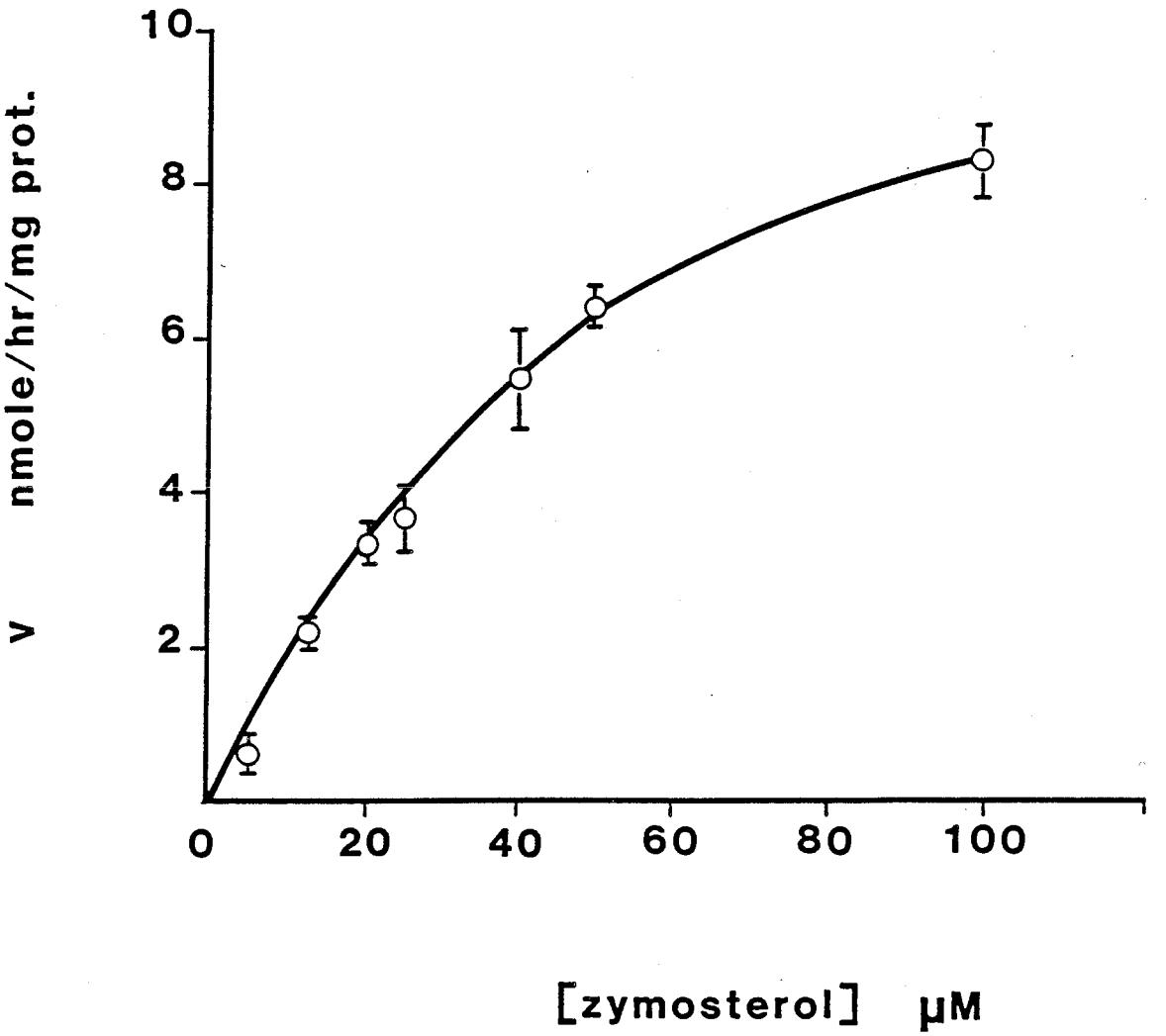


Figure 4

Lineweaver-Burk Plot for Zymosterol. (1/v vs 1/S)

Acetone powder extract of methyltransferase (1.0 mL, ~6 mg of protein per mL) was incubated as described in Fig.3. Results are the average of samples from four separate experiments.

$K_m' = 58.8\ \mu M$

$V' = 13.3$ nmole x hr/mg prot.

Fig.4.

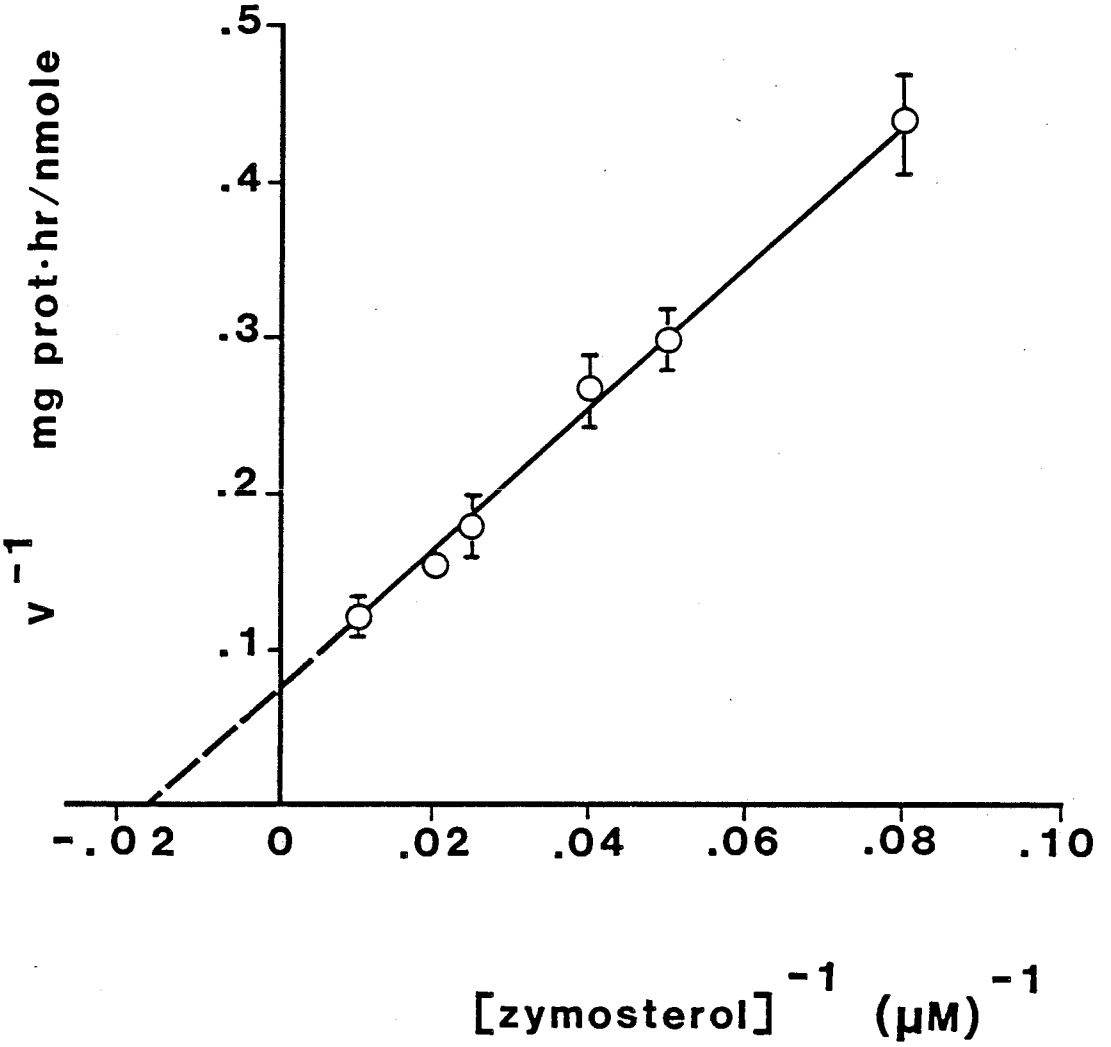


Figure 5

Dixon Plot (1/v *vs* I) for the Inhibition of 24-SMT Transmethylation by 25-Thiacholesteryl Iodide at fixed Zymosterol Concentration

Acetone powder extract of methyltransferase (1.5 mL, ~4 mg of protein per mL) was incubated with 400 nmoles of zymosterol and 100 nmoles (■), 200 nmoles (●) or 400 nmoles (△) of S-[methyl-^{14}C]-SAM at various inhibitor concentrations in a final volume of 4.0 mL at 37°C for 30 min. Results are the average of samples from four separate experiments.

K_i = 1.1 nM

Fig.5.

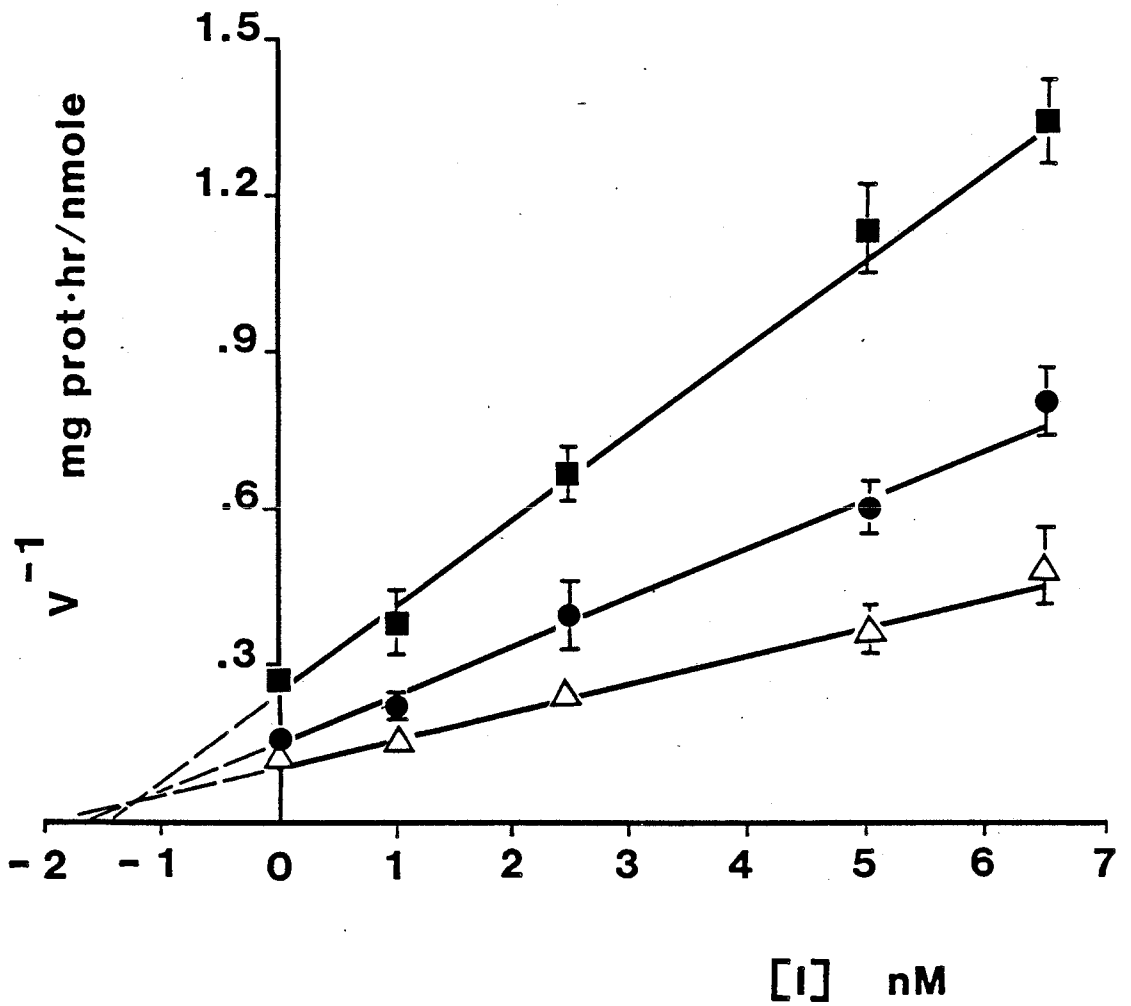


Figure 6

Dixon Plot (1/v *vs* I) for the Inhibition of 24-SMT Transmethylation by 25-Thiacholesteryl Iodide at fixed SAM Concentrations

Acetone powder extract of methyltransferase (1.5 mL, ~4 mg of protein per mL) was incubated with 400 nmoles of S-[methyl-^{14}C]-SAM and 50 nmoles (□), 100 nmoles (o) or 200 nmoles (▲) of zymosterol at various inhibitor concentrations in a final volume of 4.0 mL at 37°C for 30 min. Results are the average of samples from four separate experiments.

Fig.6.

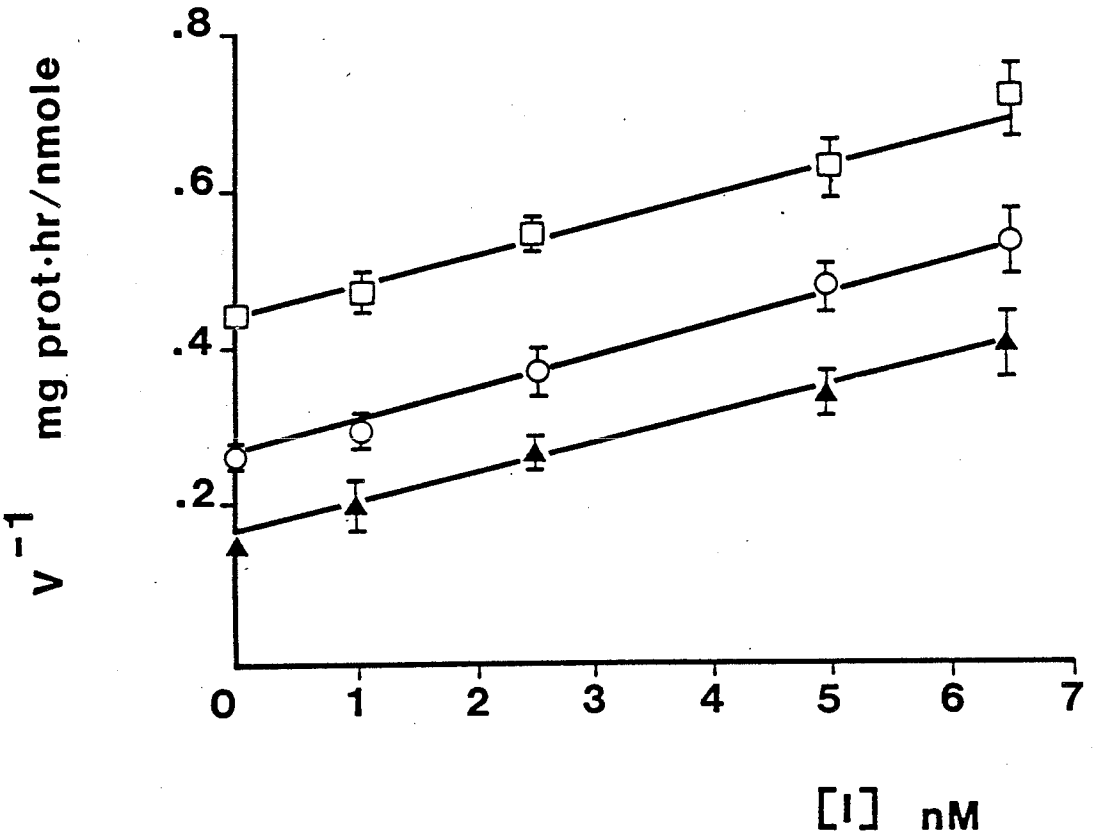


Figure 7

Plot (s/v vs I) for the Inhibition of 24-SMT Transmethylation by 25-Thiacholesteryl Iodide at fixed SAM Concentration

Acetone powder extract of methyltransferase (1.5 mL, ~4 mg of protein per mL) was incubated as described in Fig.6.

$K_i' = 2.4$ nM

Fig.7.

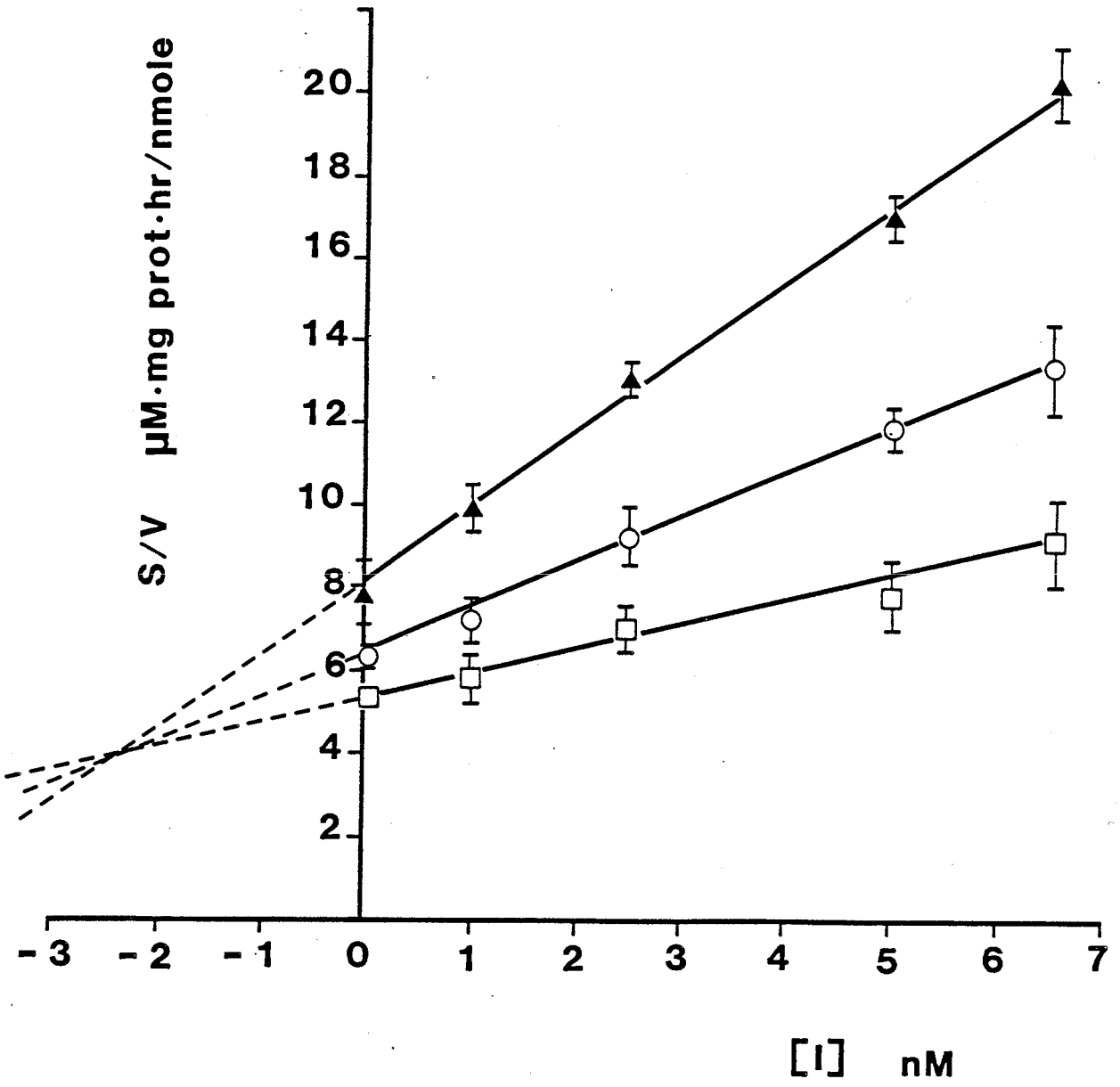


Figure 8

Double-Reciprocal Plots ($1/v$ *vs* $1/S_A$) of the Initial Velocity *vs* SAM Concentration at fixed Zymosterol Concentrations

Acetone powder extract of methyltransferase (1.5 mL, ~4 mg of protein per mL) was incubated with 50 to 800 nmoles of S-[methyl-^{14}C]-SAM (S_A, the varied substrate, 0.5 µCi/µmole) and 50 nmoles (o), 100 nmoles (●) or 200 nmoles (□) of zymosterol (S_B, fixed substrate) in a final volume of 4.0 mL at 37°C for 30 min. The results are the average of samples from at least five separate experiments.

Fig.8.

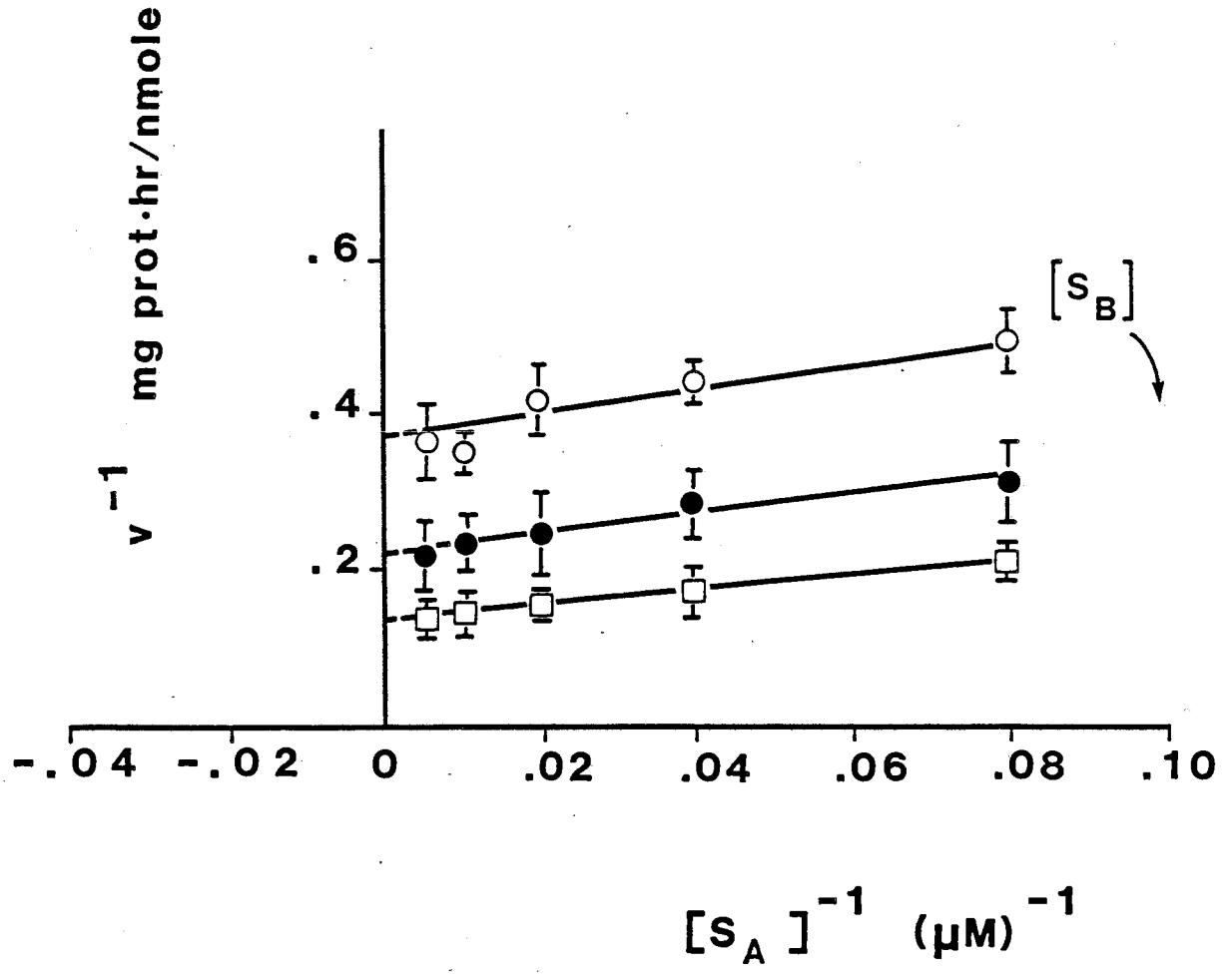


Figure 9

Secondary Plot from the Intercepts on the Abscissa of Fig.8.

Acetone powder extract of methyltransferase (1.5 mL, ~4 mg of protein per mL) was incubated as described in Fig.8.

K_m= 60.6 µM

V_m= 15.4 nmole x hr/mg prot.

Fig.9.

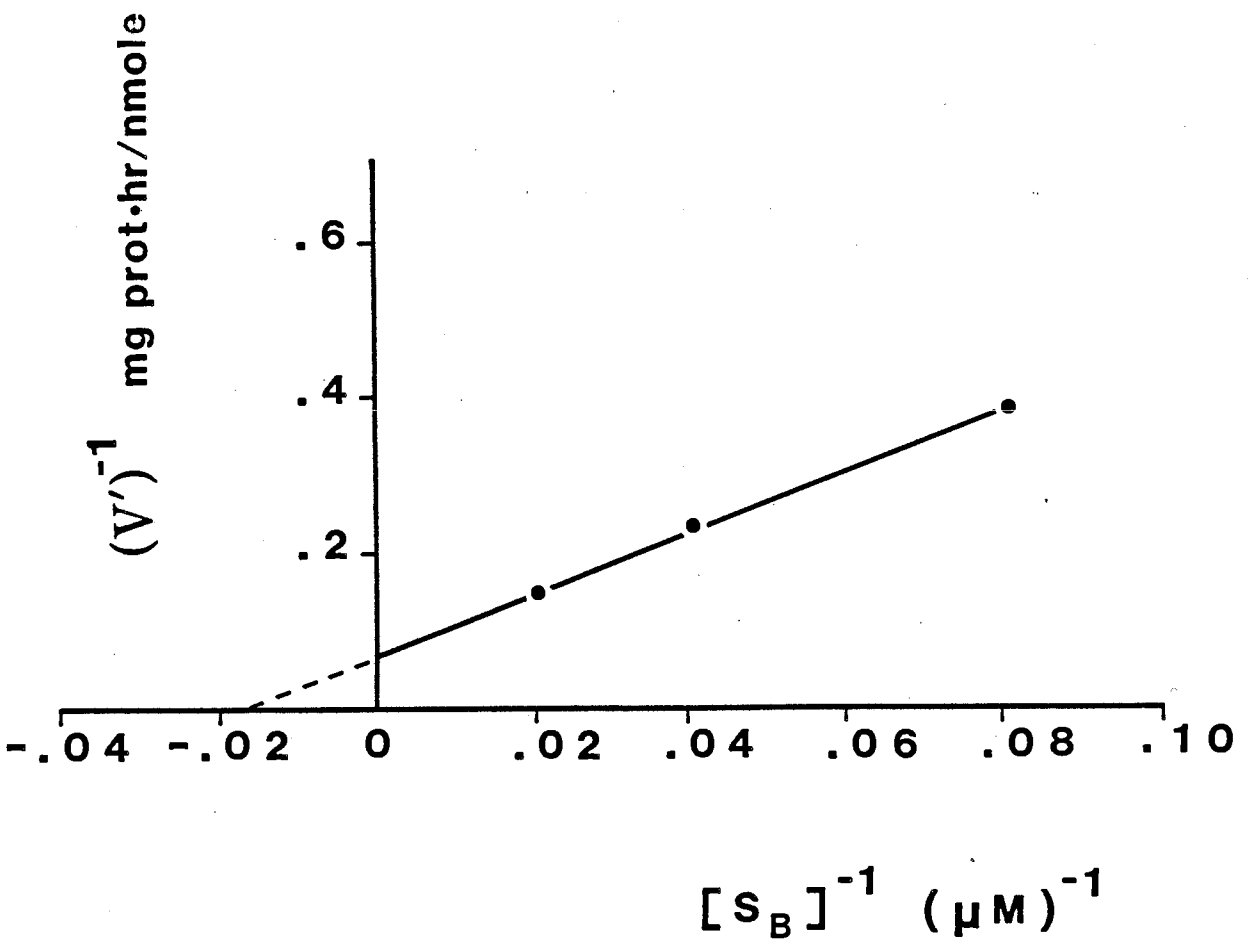


Figure 10

Double-Reciprocal Plots (1/v *vs* 1/S_B) of the Initial Velocity *vs* Zymosterol Concentration at fixed SAM Concentrations

Acetone powder extract of methyltransferase (1.5 mL, ~4 mg of protein per mL) was incubated as described in Fig.8., with 50 to 200 nmoles of zymosterol (S_B, the varied substrate) and 50 nmoles (▲), 100 nmoles (△) or 800 nmoles (■) of S-[methyl-^{14}C]-SAM (S_A, fixed substrate). The results are the average of samples from at least five separate experiments.

Fig. 10.

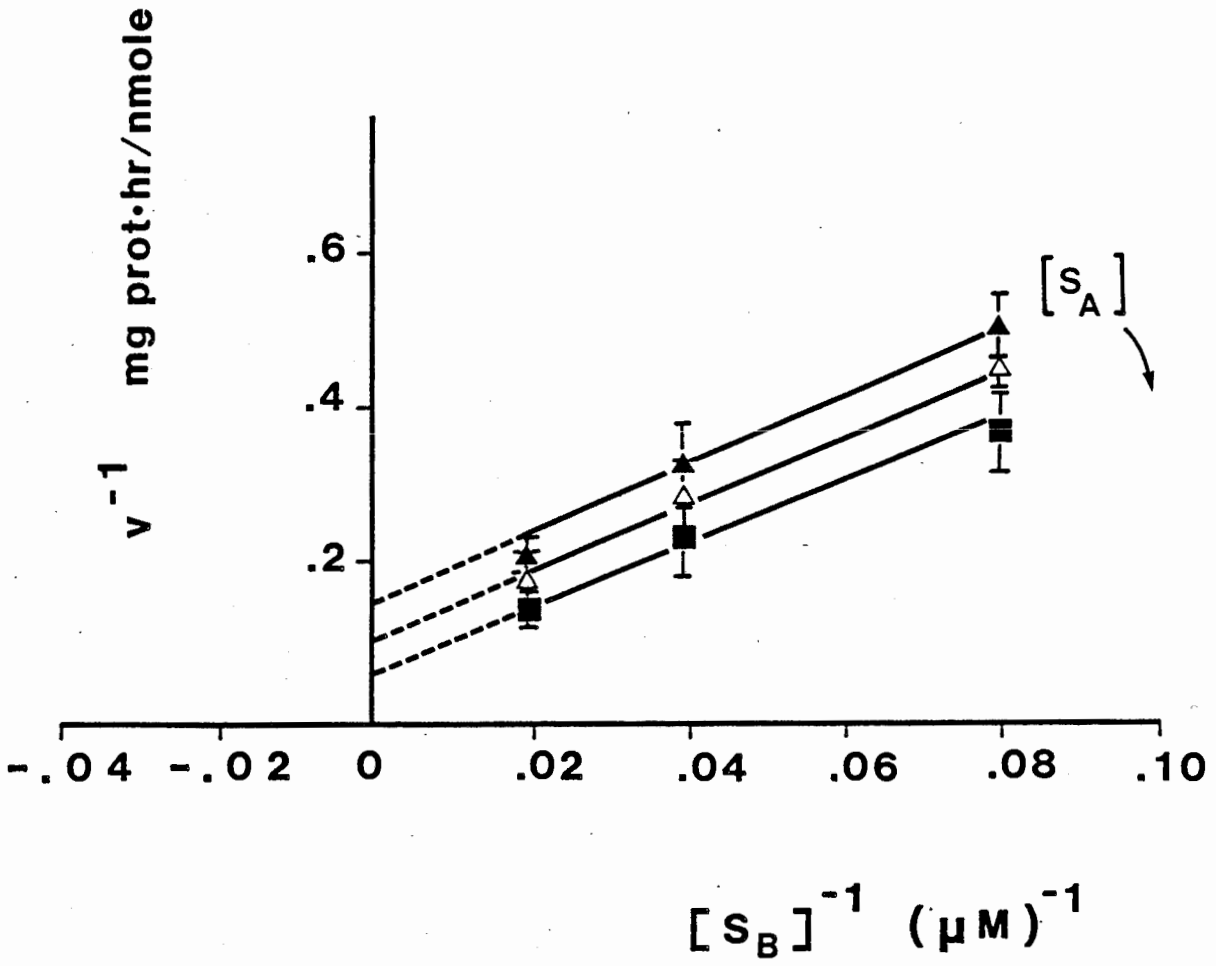


Figure 11

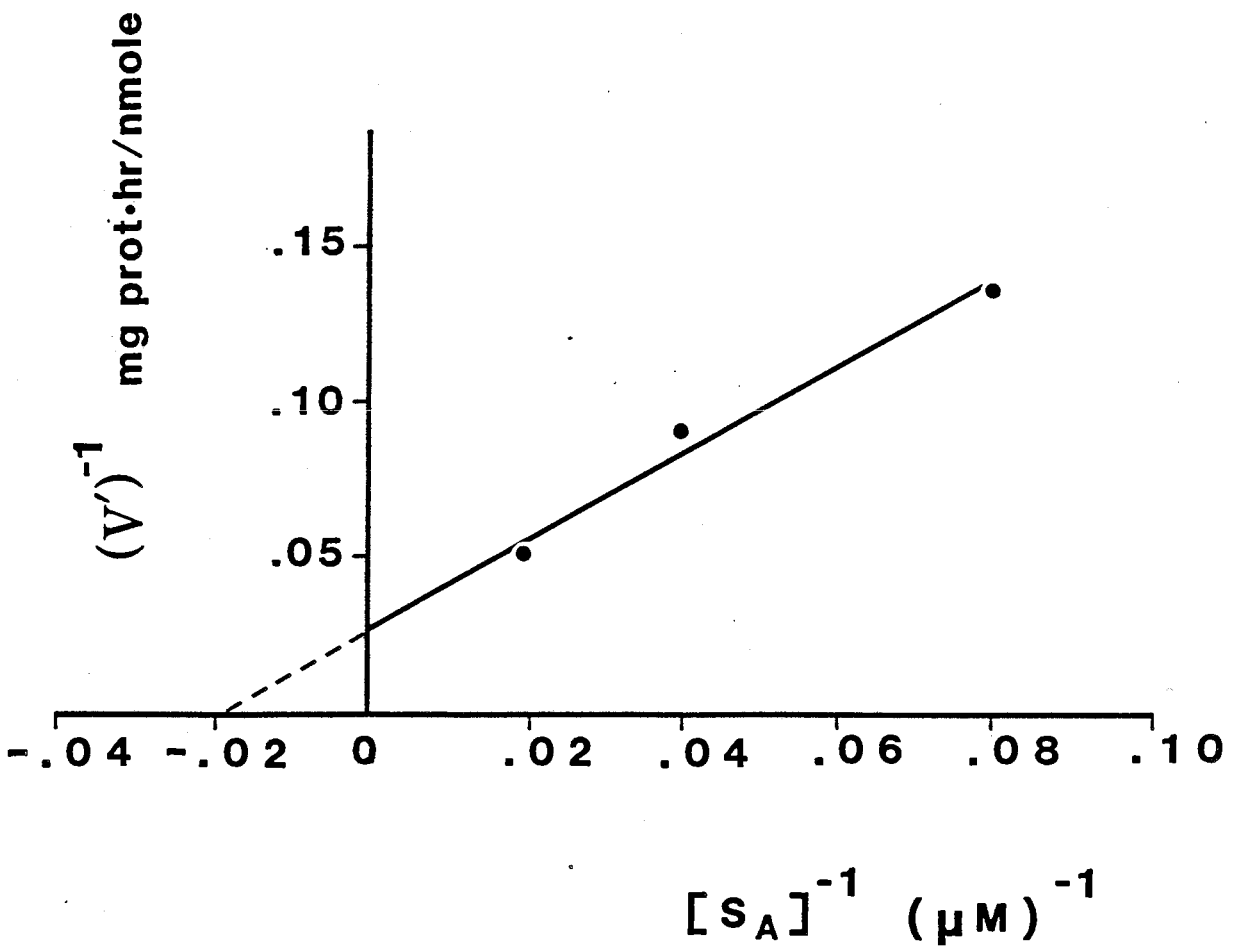
Secondary Plot from the Intercepts on the Abscissa of Fig.10.

Acetone powder extract of methyltransferase (1.5 mL, ~4 mg of protein per mL) was incubated as described in Fig.8.

K_A= 55.6 µM

V_A= 40 nmole x hr/mg prot.

Fig. 11.



5. DISCUSSION

The addition of selected thiasterols, at several concentrations, to cultures of the yeast *S.cerevisiae*, results in a significant decrease in growth (Table I). The poor growth may be a consequence of altered lipid composition of the yeast membrane.[54] Electron microscopy studies on the ultrastructural organization of the cell wall of *C.utilis* have provided evidence that the structure of the cell wall changes when cultures are grown with sulfur-containing compounds.[55]

The decreased cell growth is coupled, in most cases, with increased sterol content. Increased sterol production has also been observed in azasterol-inhibited cultures of yeast.[28] The sterol composition of yeast grown in the presence of thiasterols is different from that observed in control cultures (Table I). In treated cultures, zymosterol, 4, accumulates, while both ergosterol, 9, and ergostatetraenol, 8, decrease in the same proportion as 4 increased. Thiasterol-treated cultures contain measurable levels of C_{27}-sterol metabolites (Fig.2). These results are consistent with blockage of 24-SMT and another side-chain modifying enzyme, 24(28)-MSR, and imply that the other three sterol modifying enzyme systems in yeast ($\Delta^8 \longrightarrow \Delta^7$-isomerase; 5,6-dehydrogenase, and 22,23-dehydrogenase) are not inhibited by the thiasterols tested. The most abundant C_{27}-sterol, cholesta-7,24-dienol, is the product of $\Delta^8 \longrightarrow \Delta^7$-isomerase action on 4. Accumulation of C_{27}-sterols has been previously noted in

azasterol-inhibited cultures of *S.cerevisiae*,[29] in a double mutant blocked at both the 24-SMT and the 5,6-dehydrogenase,[56] in methionine starved-cultures,[16] and in a *C.albicans* mutant.[57]

Prior to carrying out kinetic inhibition and initial velocity studies on the methyltransferase reaction, optimum conditions to assay 24-SMT in acetone powder extract were evaluated.

Several authors have studied the enzymatic methyl transfer process in yeast with conflicting results.[7,12,13,21] Conflicting and varied cofactor requirements also have been reported.[7,21] While zymosterol, 4, has been shown to be the most efficient substrate for 24-SMT,[7,13] some authors[12] observed reaction only with an endogenous substrate; exogenously added zymosterol was not altered and even inhibited a crude cell-free methyltransferase preparation. To the contrary, other investigators[21] found that addition of zymosterol to a cell-free extract greatly stimulated the transmethylation reaction. In general, the presence of membrane lipids in enzyme preparations has been predicted to have a profound effect on the apparent ability of a particular enzyme to bind substrate.[58] Partially purified 24-SMT from *S.cerevisiae* as prepared by Moore and Gaylor[7] was used in this work. This preparation was reasonably pure with respect to liberation of the methyltransferase from microsomes and removal of endogenous substrate. Cofactor requirements for maximal enzymatic activity were also examined. We found the highest methyltransferase activity in the acetone powder extract of microsomes. Ammonium sulfate precipitation of

the acetone powder extract resulted in a total loss of transmethylase activity. A pH gradient (not measured) created *in situ* while adding the solid salt (ultrapure, special enzyme grade) may be responsible for the loss of methyltransferase activity in this fractionation step. Extraction of 24-SMT from the acetone powder proved to be quite reliable. Considerable enzyme purification was achieved in the extraction step and a reproducible enzyme activity was obtainable. Presumably, endogenous sterol was removed during treatment with acetone. Acetone powders prepared as described contain appreciable amounts of acetone-insoluble phospholipid.[59]

Inhibition studies of 24-SMT with 25-thiacholesteryl iodide, 20, yielded in contrast to *in vivo* results (TableII) nanomolar K_i values (Figs.5 and 7). A possible reason for this observation is that 24(28)-MSR, which presumably competes for thiasterols, might have been removed or denatured during the preparation of the acetone powder of 24-SMT. The competitive kinetic pattern obtained with respect to SAM occurs only when the inhibitor and varied substrate (i.e. SAM) are competing for the same enzymatic species, or for two different species that are in thermodynamic equilibrium.[60] In contrast, the uncompetitive kinetic pattern (parallel lines) (Fig.6) observed for 20 with respect to the sterol substrate is expected when inhibitor binds after substrate. However, if the two enzyme species to which the inhibitor and the varied substrate bind are separated by an irreversible step, this pattern is consistent with inhibitor

binding before the varied substrate.[60]

These kinetic results suggest that the inhibitor is binding to sites normally occupied by the sterol substrate (i.e. zymosterol). Thus, the kinetics suggest SAM and the inhibitor compete for the same enzymatic species, although not necessarily for the same site.[61] Furthermore, the kinetics suggest sterol and inhibitor could compete for different enzymatic species which are separated by an irreversible step (Scheme V).

Initial velocity studies provide additional evidence regarding this assumption. Double reciprocal plots yield a set of parallel lines, regardless of which substrate is varied at fixed levels of the second substrate. The observed kinetic pattern suggests that the two substrates, SAM and zymosterol, are not present simultaneously on the enzyme, since the lines do not converge at a common point. Instead, this type of kinetic behaviour is consistent with a Ping-Pong mechanism.[62] This kinetic model is generally observed for enzymes whose reactions involve covalent enzyme-substrate intermediates and therefore, it is often used as evidence for such an intermediate. This kinetic behaviour also suggests that an irreversible step lies between the addition of the two substrates. The irreversible step could be release of a product, such as is seen with the amino acid transaminases.[63]

On the basis of these, and previous[38] experimental results, we propose a Ping-Pong mechanism for the transmethylation reaction in yeast *S.cerevisiae* in which the irreversible process is the transfer of the S-methyl group of SAM to the enzyme. Since

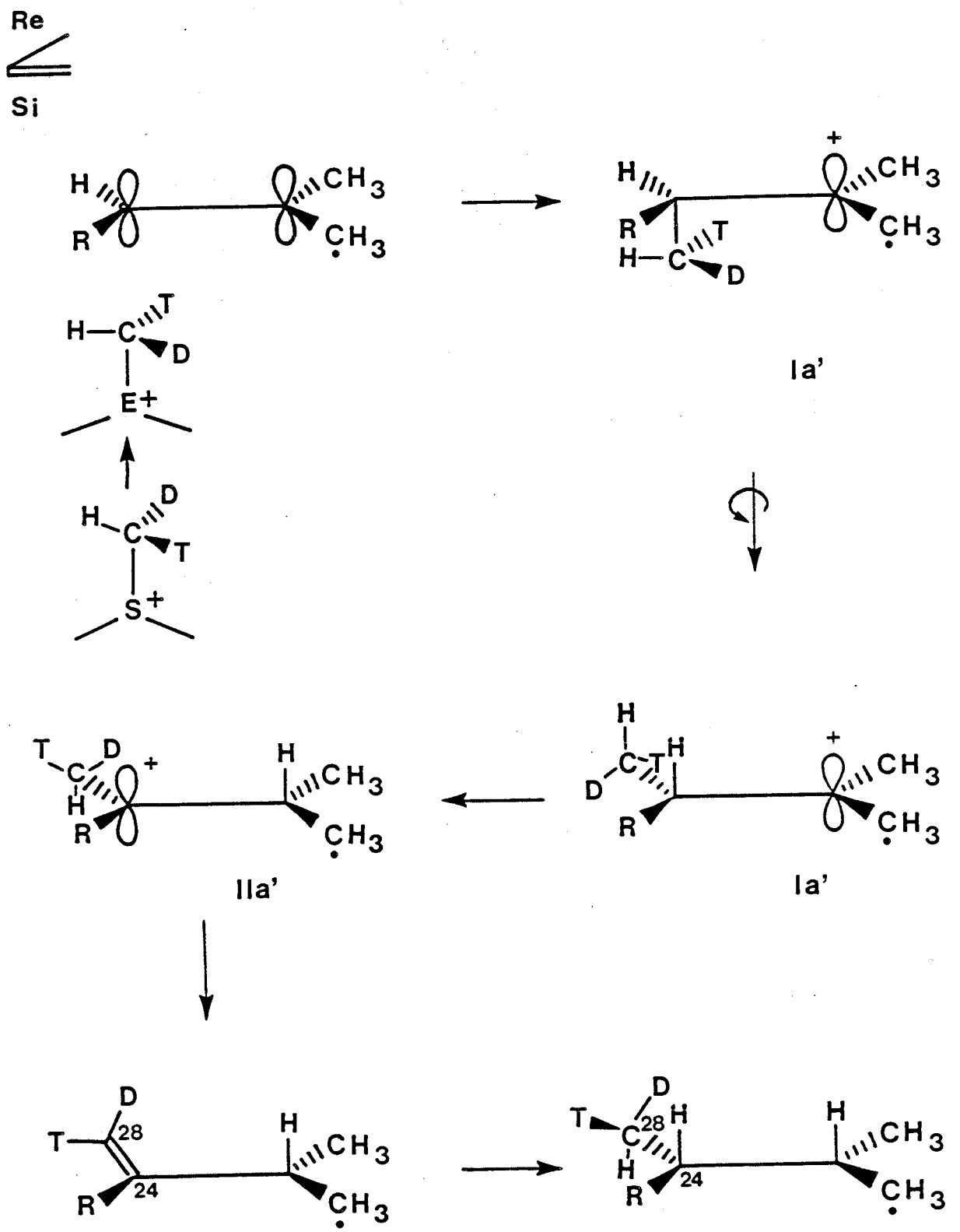
steady-state kinetics only gives information about the order of combination of the enzyme with substrates and products, but does not reveal the sequence of covalent bond cleavage and formation, it cannot be used as a sole test of a mechanism.[64]

The Ping-Pong mechanism may be used as a basis for analysis of the stereochemical experiments on yeast 24-SMT. It is clear from Schemes II and III, that if a Ping-Pong mechanism operates, the stereochemical arguments pertaining to the removal of the C-28 hydrogen change, and now become those represented in Schemes VI and VII, respectively. Scheme VI, traces the stereochemical requirements of reactions of the presumptive intermediates if, alkylation occurs on the Si face of Δ^{24} of the sterol substrate.

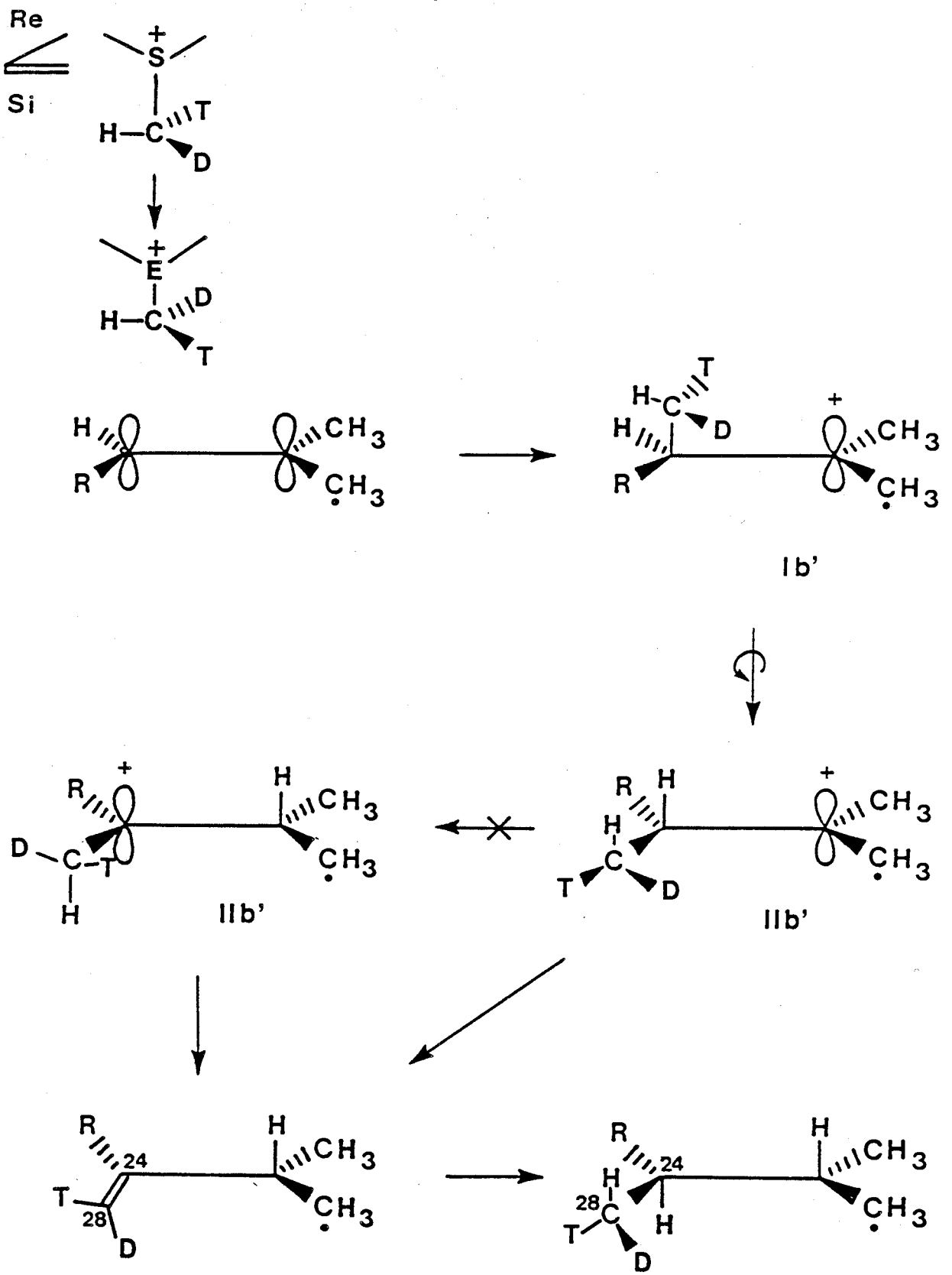
One important feature of Scheme VI compared to Scheme II, is that C-28 hydrogen removal must occur from the Si face of the presumptive intermediate IIa', which is <u>opposite</u> to that across which C-24 to C-25 hydrogen migration is required to occur. A second important feature in Scheme VI, is that in intermediate Ia', only a slight rotation (60°) about the C-24 to C-25 bond is required to align the C-24 hydrogen to carbon bond with the proper face of the empty p orbital at C-25.*

These two features lead one to favor Scheme VI as representing the most probable stereochemical course of the reaction.

* This alignment allows C-24 to C-25 hydrogen migration to occur to give the required configuration at C-25.



Scheme VI



Scheme VII

Thiasterols 20, 21a and 21b were designed as mimics of the first presumptive intermediate I(a,b) in the alkylation process. Our view was that during the alkylation there was, in all probability, a nucleophilic site near the developing C-25 carbocation, and that C-25 sulfonium sterols should bind to this site.

As can be seen by the I_{50} values (Table II), the stereochemistry at C-24 of the 24-methyl-25-thiacholesteryl iodides plays an important role in determining the relative potency of these C-24 substituted thiasterols as inhibitors of the 24-SMT (and 24(28)-MSR) enzyme in yeast. The relative inhibitory power of the sulfonium inhibitors of 24-SMT was in the order 20 > 21b > 21a. It is difficult to interpret the relative potency of 20, 21a and 21b as inhibitors of 24-SMT because the I_{50} values vary slightly compared to the difference observed for the K_i of 20 vs I_{50} for 20 (x70). In order to determine the efficiency of inhibition of 21a and 21b as inhibitors for 24-SMT, K_is for these inhibitors must be determined *in vitro*.

In terms of the Ping-Pong mechanism suggested by the kinetic analysis, the enzyme only binds sterol substrate <u>after</u> it has been methylated. Therefore, the methyl is the first to arrive in the active site and according to our stereochemical arguments (Scheme VI) is located near the Si face of Δ^{24} when the sterol binds. The kinetic analysis also shows that the inhibitor binds <u>before</u> the enzyme has been methylated. These observations are consistent with the assumption that the methyl transfer from the methylated enzyme

to the sterol substrate induces a conformational change in the enzyme causing the sterol intermediate (I) to bind more tightly to the unmethylated enzyme. Thus, before the conformational change takes place the methylated enzyme only binds sterol substrate. The kinetic results show that carbocation mimic, 20, of the presumptive intermediate (I) binds many orders of magnitude (10^4) more tightly to the unmethylated enzyme than that of substrate, 4, binding to the methylated enzyme. This is as expected for a transition state analog.

APPENDIX

IUPAC Nomenclature of Common Names Used

COMMON NAMES	IUPAC
Lanosterol, 1	4,4,14α-Trimethyl-5α-cholesta-8,24-dien-3β-ol
4,4-dimethylzymosterol, 2	4,4-Dimethyl-5α-cholesta-8,24-dien-3β-ol
4α-methylzymosterol, 3	4α-Methyl-5α-cholesta-8,24-dien-3β-ol
Zymosterol, 4	5α-Cholesta-8,24-dien-3β-ol
Fecosterol,5	5α-Ergosta-8,24(28)-dien-3β-ol
Episterol, 6	5α-Ergosta-7,24(28)-dien-3β-ol
Ergosterol, 9	Ergosta-5,7,22-trien-3β-ol
Cholesterol, 17	Cholest-5-en-3β-ol
24,25-dihydrozymosterol, 18	5α-Cholest-8-en-3β-ol
Stigmasterol, 19	24-Ethylcholest-5-en-3β-ol
Cycloartenol	4,4,14α-Trimethyl-9β,19-cyclo-5α-cholesta-24-en-3β-ol

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