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Stereochemistry of Yeast Δ^{24}-Sterol Methyl Transferase

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Abstract—*S*-Adenosyl-l-methionine:Δ^{24}-sterol methyl transferase (24-SMT) mediates introduction of the C-28 carbon of yeast sterols. It has been shown that sulfonium analogues of the presumptive cationic intermediates of the methylenation reaction are potent in vivo and in vitro inhibitors of this process. In the presence of these inhibitors, cultures of yeast produced increased proportions of zymosterol, the natural substrate of the enzyme, while proportions of ergosterol and ergostatetraenol were decreased. New C27-sterol metabolites were also found. The in vivo inhibitory power of the analogues [I_{50} (μM)] was determined from the proportion of C-24 methylated sterols to C-24 nonmethylated sterols in treated cultures to be in the following order: 25-thiacholesterol iodide (0.07) > 24(*S*)-methyl-25-thiacholesteryl iodide (0.14) > 24(*R*)-methyl-25-thiacholesteryl iodide (0.25). Kinetic inhibition as revealed by radiolabeled *S*-adenosyl-l-methionine (SAM), crude enzyme and 25-thiacholesteryl iodide revealed this inhibitor to be uncompetitive with respect to zymosterol and competitive with respect to SAM. The greater inhibitory power of 24(*S*)-methyl-25-thiacholesteryl iodide compared to 24(*R*)-methyl-25-thiacholesteryl iodide suggests that methyl donation to Δ^{24} occurs from the *si* face. When considered in conjunction with Arigoni's previous work, the present results infer the methylenation mediated by yeast 24-SMT proceeds by alkylation from the *si* face of Δ^{24} followed by migration of a hydrogen from C-24 to C-25 across the *re* face and final loss of a hydrogen from C-28 on the *re* face. © 1997 Elsevier Science Ltd.

Introduction

Most fungal and plant sterols possess side chains with a methyl group at C-24 that is added as a C-1 unit to Δ^{24} sterols by 24-SMTs.[1–3] These enzymes utilize SAM as the methyl donor and most often produce $\Delta^{24(28)}$-methylene sterols as initial products. The three steps in this methylenation process, alkylation at C-24, hydrogen migration from C-24 to C-25 and loss of a hydrogen from C-28 are considered to involve the formation of enzyme-stabilized cations at C-25 (A) and C-24 (B). Support of the intermediacy of A and B has been obtained through observation that mimics of these species are effective inhibitors of 24-SMTs. Several groups have shown that while sterols with heteroatoms substituted for C-24 or C-25 are effective inhibitors of 24-SMT in yeast (e.g., **1** and **2**)[4–8] and maize (e.g., **3**)[9,10] those sterols (e.g., **4–7**)[5–8,11–14] containing positively charged heteroatoms in place of C-24 or C-25 are the most potent.

Investigation of the relative stereochemistry of the steps in the conversion of Δ^{24} to $\Delta^{24(28)}$ methylene sterols is a challenging problem because three separate reactions occur prior to release of the product. Rahier et al.[12]

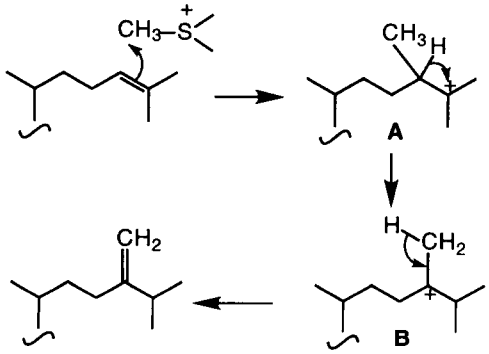
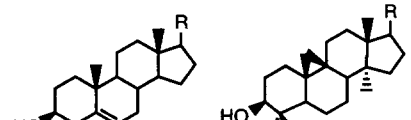


Diagram 1.



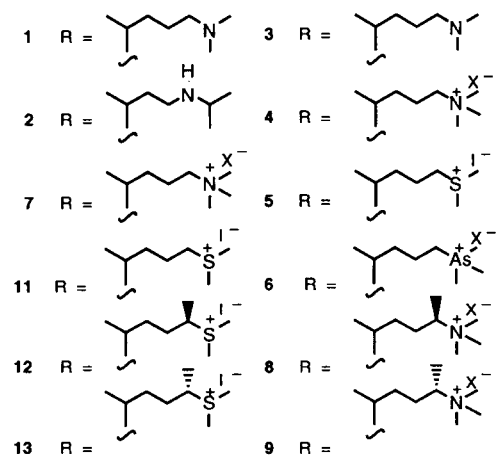


Diagram 2.

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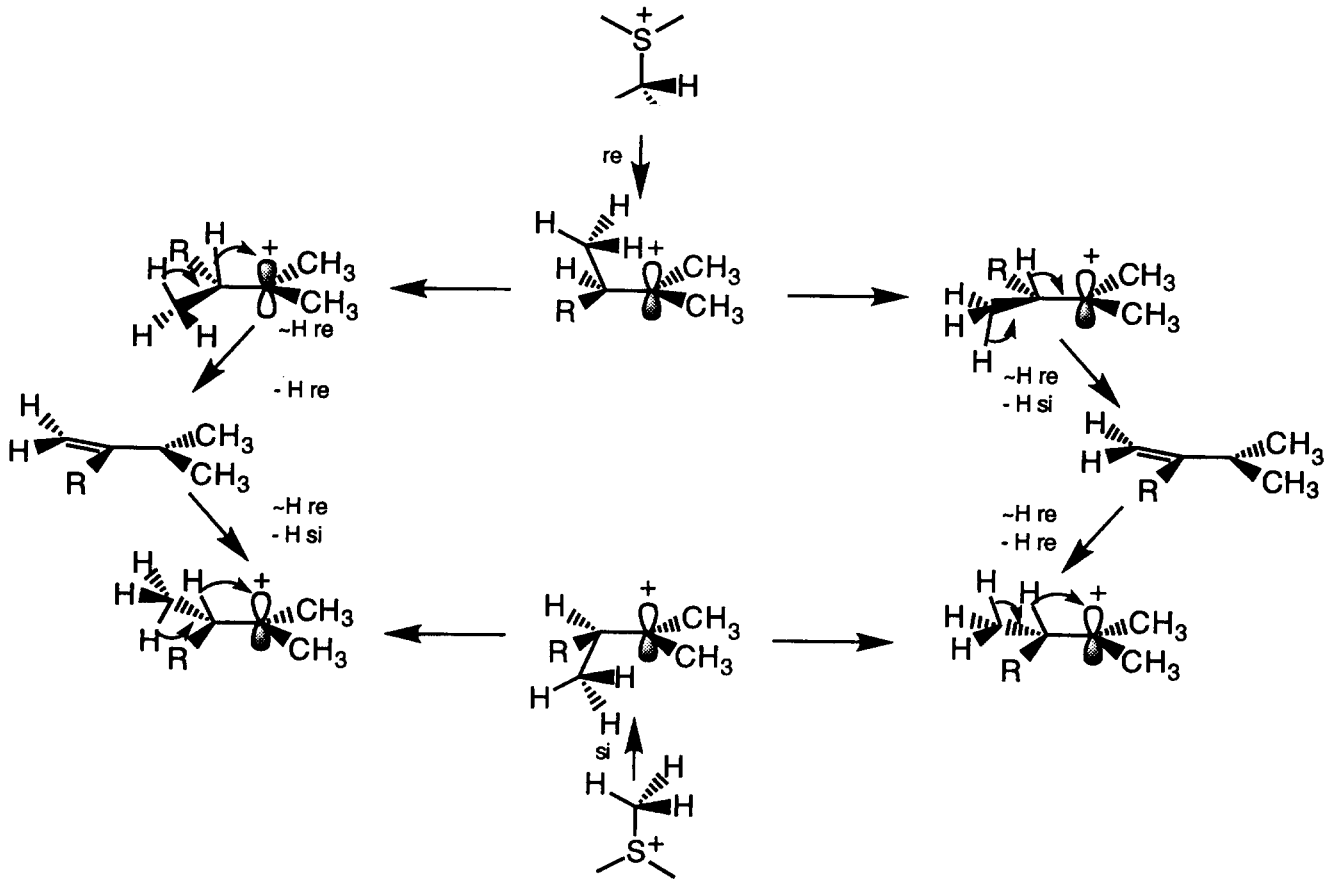


Figure 1. Stereochemical pathways possible for 24-SMT.

used the greater efficiency of inhibition of **8** compared to **9** to deduce that the initial C-24 alkylation of cycloartenol by maize 24-SMT occurred from the *re* face. Alkylation from the *si* face was deduced to occur in alga (*Trebouxia spp.*).[15] Hydrogen migration from C-24 to C-25 has been shown to occur across the *re* face in the yeast *Saccharomyces cerevisiae*,[1,3] the fungus *Claviceps paspali*[1] and alga[15] but across the *si* face in *Pinus pinea*.[16]

Determination of the face from which the hydrogen at C-28 is removed requires knowledge of the stereochemistry of C-24 alkylation, which is available by use of chirally labeled *S*-methyl methionine. Both *S. cerevisiae* and *C. paspali* convert chirally labeled *S*-[methyl-^{2}H,^{3}H]-methionine to ergosterol bearing a chiral C-24 methyl group of the same absolute configuration as the methionine precursor.[1] In *C. paspali* this conversion involves the *trans* addition of hydrogen to the intermediate $\Delta^{24(28)}$ sterol (Arigoni, D., personal communication). These results define the relative stereochemistry of the alkylation and C-28 hydrogen loss in this fungus as occurring from opposite faces if the methyl is transferred from methionine to C-24 of the sterol substrate with inversion of configuration. Algal 24-SMTs operate with net inversion of configuration at methyl[15] as do most transfers of methyl of *S*-methyl adenosyl methionine to oxygen, nitrogen, and sulfur.[17,18]

Early work by Moore and Gaylor[19] suggested that the 24-SMT of *S. cerevisiae* did not operate via a ping-pong process but that both sterol and SAM substrates became attached to the 24-SMT in a ternary complex. Recent work by the Nes group on 24-SMT from *S. cerevisiae* GL7 provided kinetic evidence most consistent with the operation a rapid-equilibrium random bi bi mechanism.[14] If the direct transfer of methyl occurs with inversion of configuration and reduction of the intermediate $\Delta^{24(28)}$ sterol product occurs *trans* as in *C. paspali* then complete stereochemical definition of the yeast methylenation requires knowledge of the stereochemistry of either the alkylation or C-28 hydrogen loss (Fig. 1). Although information concerning the absolute stereochemistry of both these steps is lost during the methylenation it is possible to query the facial specificity of the alkylation through the use of intermediate mimics. Accordingly, we prepared sulfonium mimics (**11**, **12**, and **13**) of presumptive intermediate A. Since all mimics were strong in vivo and in vitro inhibitors of yeast 24-SMT we were able to examine the stereochemistry of the alkylation through determination of the relative inhibitory power of **12** and **13**. Preliminary accounts of portions of this work have appeared.[20]

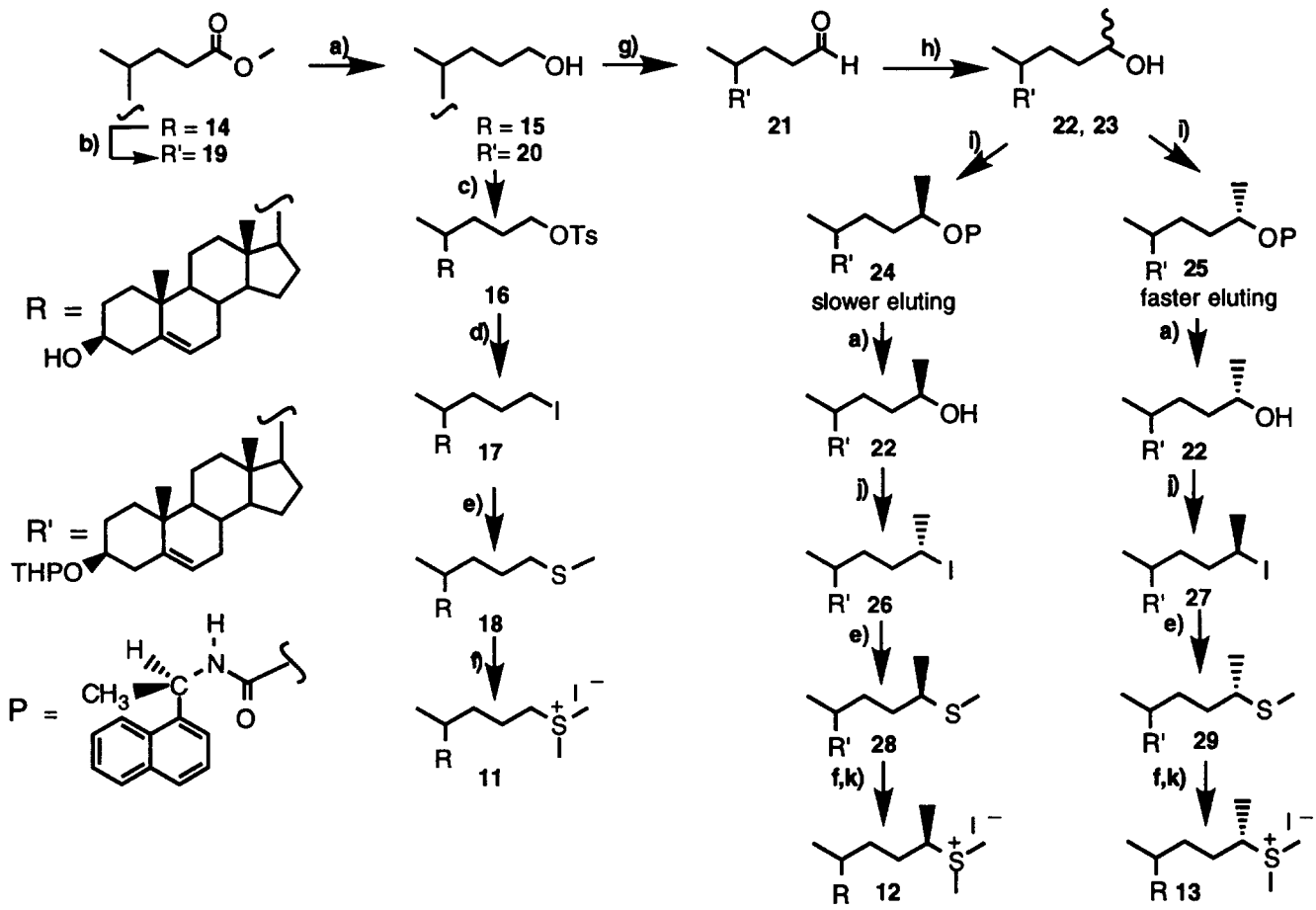


Figure 2. Synthesis of thiasterol inhibitors: (a) LAH, THF; (b) DHP, PTSA, CH_2CL_2; (c) Tos-Cl, Pyr; (d) Nal, acetone; (e) MeSLi, THF; (f) Mel; (g) PCC, CH_2Cl_2; (h) MeMgBr, Et_2O; (i) (*R*)-(-)-l-(1-naphthyl)ethyl isocyanate, Me_3N, toluene; (j) Ph_3P, diethyl diazodicarboxylate, Mel, benzene; (k) 1 N HCl, MeOH, THF.

Results and Discussion

Synthesis of thiasterol inhibitors

Synthesis of **11** commenced from diol **15** to give monotosylate **16** that was converted to iodide **17** with NaI in acetone (Fig. 2). Reaction of **17** with lithium thiomethylate in THF gave **18**, alkylation of which at sulfur with methyl iodide gave **11**.

The syntheses of thiasterols **12** and **13** bearing a C-24 methyl were designed to proceed via intermediates capable of chromatographic separation as diastereomeric carbamates.[21] Accordingly, the readily available 3-tetrahydropyranyl ether of **19** was reduced to alcohol **20** in 86% yield with lithium tetrahydridoaluminate. This sterol was oxidized to aldehyde **21** in 81% yield with chromic anhydride in pyridine. Reaction of **21** with methyl magnesium bromide gave a mixture of **22** and **23**, which were epimeric at C-24. Reaction of these with (*R*)-(−)-l-(1-naphthyl)-ethyl isocyanate gave diastereoisomeric carbamates **24** and **25** that were separated by repetitive chromatography. The arguments of Pirkle and Housks[21] lead to the assignment of the 24(*R*) configuration to the faster eluting carbamate. However, we determined by X-ray crystallographic analysis (A.C. Oehlschlager, E. Czyzewska and F.W.B. Einstein, unpublished work) that the diacetate of the diol generated by reduction of the faster eluting carbamate **25** had an *S* configuration at C-24. Mitsunobu reaction[22] of **22** [24(*R*)] and **23** [24(*S*)] separately with methyl iodide gave iodides **26** [24(*S*)] and **27** [24(*R*)], respectively. This reaction is known to proceed with inversion of configuration. When reacted with lithium thiomethylate each iodo sterol gave a thiomethyl sterol whose configuration at C-24 was assumed to be inverted. Thus, **26** [24(*S*)] gave **28** [24(*R*)] and **27** [24(*R*)] gave **29** [24(*S*)]. Conversion of **28** and **29** to **12** and **13**, respectively, was performed by reaction of each isomer with excess methyl iodide. The 24(*R*)-methyl-25-thiocholesterol iodide **12** was shown by NMR integration of the 26,27-methyl resonances to be contaminated with 4.8% of the 24(*S*) isomer **13**. Likewise, the 24(*S*) isomer **13** was shown to be contaminated with 10.5% of **12**.

Effect of thiasterols on yeast growth and sterol production

As shown in Table 1, **12** caused only a slight decline in cell production at 0.3 μM or below. Inhibitors **11** and **13** reduced the dry cell weight to approximately 60% of the control value when concentrations were increased to 0.5

Table 1. Effect of thiasterols on growth and sterol composition in *S. cerevisiae*

Inhibitor	25-thiacholesterol iodide (11)			24(*R*)-24-Me-25-thiacholesterol iodide (12)				24(*S*)-24-Me-25-thiacholesterol iodide (13)		
Inhibitor concentration (μM)	0	0.05	0.1	0.5	0.1	0.3	0.5	0.1	0.3	0.5
Dry cell wt (g l^{-1})	8.3	8.2	8.2	5.4	8.2	8.0	7.1	8.1	7.2	5.0
Sterols (% of dry cell wt)[a]	2.2	1.9	4.4	1.9	3.1	2.4	2.1	0.8	3.3	4.3
Δ5,7-dienes (% of dry cell wt)[b]	0.9	0.6	1.3	0.3	0.8	0.6	0.6	1.2	0.5	0.7
Sterol composition										
Lanosterol	5.2	5.3	1.8	2.4	7.3	2.9	1.8	3.4	3.5	4.0
4,4-Dimethylzymosterol	14.1	14.8	8.0	8.8	19.8	11.1	9.3	6.5	10.9	17.8
4 1-Methylzymosterol	6.2	5.5	1.7	4.4	6.6	6.7	6.5	2.8	4.9	8.9
4-Methylergosta-8,24(28)-dienol	2.0	1.7	nd[c]	0.2	nd	nd	nd	0.3	0.5	nd
Zymosterol	25.3	39.1	52.4	59.6	25.8	46.8	51.6	31.2	57.2	42.8
Fecosterol	5.4	3.9	0.6	nd	1.9	1.7	0.6	1.3	nd	nd
Episterol	2.4	1.9	1.4	nd	2.5	1.1	0.1	1.9	nd	nd
Ergosta-8,22,24(28)-trienol	1.9	nd	nd	nd	nd	nd	nd	nd	nd	nd
Ergosta-7,22,24(28)-trienol	0.6	nd	nd	nd	5.2	nd	nd	nd	nd	nd
Ergosta-5,7,22,24(28)-tetraenol	24.3	18.7	12.6	1.0	18.1	14.0	7.3	23.8	1.6	1.6
Ergosterol	12.6	6.3	2.0	nd	9.2	3.7	1.5	6.7	0.1	nd
Cholesta-7,24-dienol	nd	1.8	5.1	11.7	3.3	3.1	5.4	10.0	10.2	9.3
Cholesta-5,7,24-trienol	nd	nd	3.6	2.3	nd	3.1	4.3	3.4	3.3	2.9
Cholesta-5,7,22,24-tetraenol	nd	1.0	10.8	9.6	0.4	5.8	11.6	8.7	7.9	13.2
% inhibition: 24-SMT	0	34.7	64.8	97.9	21.8	56.6	80.0	28.6	96.4	96.6
24(28)-MSR[d]	0	29.4	56.4	100	8.8	39.5	48.5	31.9	82.8	100

[a]Determined by GLPC.
[b]Determined by UV.
[c]nd, < 0.1%.
[d]MSR = Methylene sterol reductase.

μM. The lower growth rate at high inhibitor concentrations may be a consequence of altered lipid composition of the yeast membrane.[24] Sterol content on a dry weight basis was usually increased when thiasterols were administered to growing yeast in the concentration range examined. Increased sterol production has also been observed in azasterol-inhibited cultures of yeast.[24]

Effect of thiasterols on sterol biosynthesis and sterol composition

The sterol composition of yeast grown in the presence of thiasterols differed from that in control cultures (Table 1). As inhibitor concentrations in cultures increased, ergosterol biosynthesis drastically declined and ceased at 0.5 μM in the case of experiments with **11** and **13**. The amount of 5,7,22,24(28)-ergostatetraenol also decreased whereas the relative proportion of zymosterol (**10**) increased. Three other C27-metabolites, cholesta-7,24-dienol (**30**), cholesta-5,7,24-trienol (**31**), and cholesta-5,7,22,24-tetraenol (**32**) also accumulated in treated cultures (Fig. 3). 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 (Δ8→Δ7 isomerase; 5,6-dehydrogenase, and 22,23-dehydrogenase) are not inhibited by these thiasterols. Accumulation of C27-sterols has been previously noted in azasterol-inhibited cultures of *S. cerevisiae*[4] in a double mutant blocked at both the 24-SMT and the 5,6-dehydrogenase,[25] in methionine-starved cultures[26] and in a *C. albicans* mutant.[27,28]

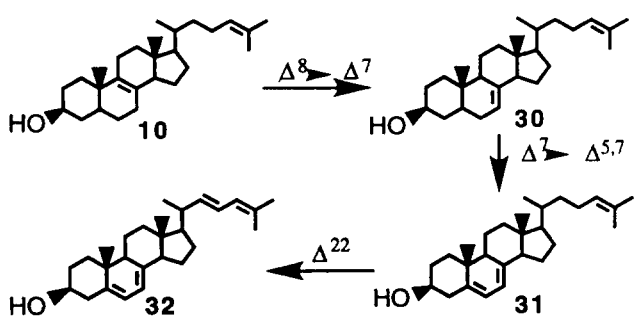


Figure 3. C27 Metabolites found in 24-SMT blocked yeast.

The per cent inhibition of 24-SMT (Table 1) was obtained by comparison of the proportion of C-24 alkylated sterols produced in the presence of the inhibitor (ΣC-24-alk_i) with that produced in control cultures (ΣC-24-alk_c) according to the expression:

$$\%\text{inhibition} = 100\left[1 - \frac{\text{C-24-alk}_i}{\text{C-24-alk}_c}\right]$$

The concentrations of **11**, **12**, and **13** required to reduce by 50% the amount of C-24 alkylated sterols is shown in Table 2. Both C-24 methylated thiasterols were less potent than thiasterol **11**.

The per cent inhibition of 24(28)-MSR was calculated by comparing the ratio of ergosterol to $\Delta^{24(28)}$-sterols in inhibited cultures ($\Sigma\Delta^{24(28)}$-$sterols_i$) with the ratio of ergosterol to $\Delta^{24(28)}$-sterols in the control cultures ($\Sigma\Delta^{24(28)}$-$sterols_c$) in the following expression

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

The 24(28)-MSR was inhibited to the greatest extent by thiasterol **11** (Tables 1 and 2).

Preparation 24-SMT for kinetic studies

Prior to carrying out inhibition and initial velocity studies, optimum conditions to assay 24-SMT in acetone powder extract were developed. Different protocols and varied cofactor requirements for maximal 24-SMT activity have been reported.[19,29–31] We found that the method of Moore and Gaylor[19,31] provided the most reproducible preparations of 24-SMT from *S. cerevisiae*. The most active methyl transferase fraction was found in the acetone powder extract. This extraction involved suspension of the acetone powder in buffer at pH 7.5 and centrifugation at 10,000 *g* for 20 min; the resulting pellet was found to be devoid of methyl transferase activity. The supernatant had a protein concentration of 3.5–7.0 mg mL^{-1} and specific transferase activity of 2.3 nmol h^{-1} per mg of protein. All stages of enzyme purification and incubation were conducted in 1 mM Mg^{2+}. In contrast to earlier reports,[19,29] neither glutathione nor bicarbonate were found to be requirements for maximal activity. Acetone powders prepared as described contain appreciable amounts of acetone-insoluble phospholipid[32] although endogenous sterol was removed. Methyl transferase activity was lost completely when the recommended ammonium sulfate precipitation was conducted. A study published after completion of this work yields soluble preparations of *S. cerevisiae* 24-SMT preparations.[14]

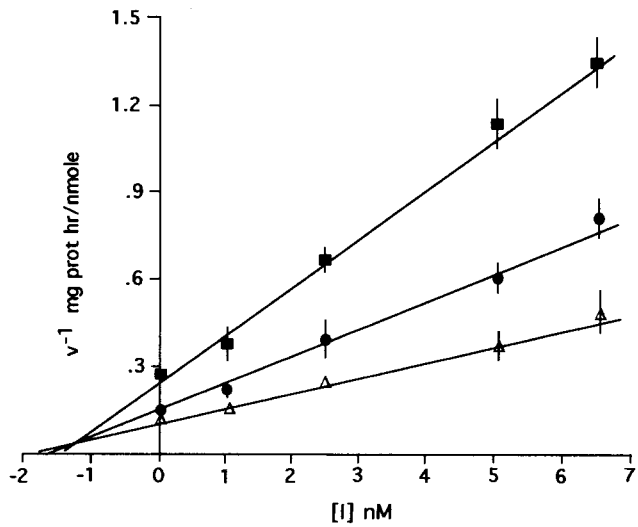


Figure 4. Dixon plot (1/*v* vs *I*) for inhibition of 24-SMT by 25-thiacholesterol iodide at fixed [zymosterol]. Acetone powder extract of methyl transferase (1.5 mL, ~4 mg of protein per mL) was incubated with 400 nmol of **10** and 100 nmol (■), 200 nmol (●), or 400 nmol (△) of *S*-methyl-[^{14}C]-SAM 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.

We found that zymosterol was readily methylated by 24-SMT and exhibited saturation kinetics. Exogenously supplied zymosterol (**10**) has been shown to be the most efficient substrate for fungal 24-SMTs.[6,13,14,19,29]

Kinetic studies of 24-SMT

The rates of transmethylation of zymosterol (**10**) at several concentrations at a single nonsaturating concentration of SAM yielded linear double reciprocal plots that allowed determination of a K_m for **10** of 60.6 μM, which is close to reported values.[13,19,31] The K_m for zymosterol for recently reported solubilized 24-SMT is nearly identical to the K_m for this substrate determined using the insoluble microsomal preparations.[14] Double reciprocal plots of the rate of transmethylation at several SAM concentrations and a single nonsaturating level of **10** were linear and yielded a K_m for SAM of 55.6 μM. The K_m for SAM for solubilized 24-SMT of *S. cerevisiae* is 29 μM.[14] The linear behavior of the double reciprocal plots obtained in these experiments is indicative of sequential substrate binding.

Because of efficient in vivo inhibition of 24-SMT by thiasterol **11**, it was chosen for initial in vitro studies. When the SAM concentration was varied and [**10**] was kept constant, Dixon plots of the reciprocal of the initial velocity vs [**11**] gave a family of lines intersecting in the fourth quadrant that yielded the K_i for **11** vs SAM (Fig. 4). The kinetic pattern observed is indicative of competitive inhibition with respect to SAM and occurs when the inhibitor **11** and varied substrate (SAM) are competing for the same enzymatic species, or for two different species that are in equilibrium.[33–36]

Administering **11** to 24-SMT under conditions in which [**10**] was varied and SAM concentration was constant yielded a Dixon plot with a set of parallel lines which is

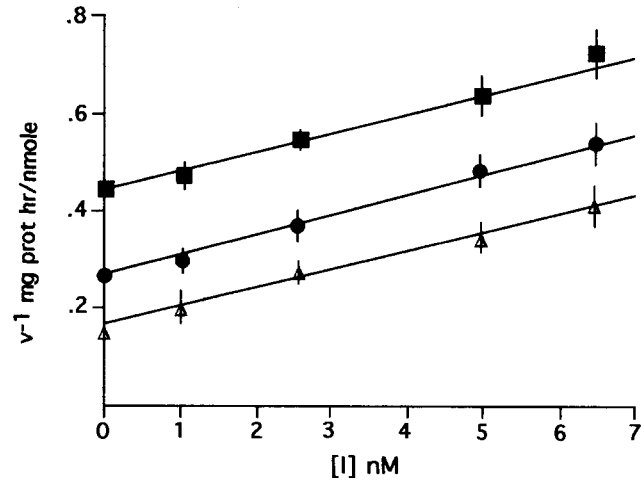


Figure 5. Dixon plot (1/*v* vs *I*) for inhibition of 24-SMT by 25-thiacholesterol iodide at fixed [SAM]. Acetone powder extract of methyl transferase (1.5 mL, ~4 mg of protein per mL) was incubated with 400 nmol of *S*-methyl-[^{14}C]-SAM and 50 nmol (■), 100 nmol (●), or 200 nmol (△) of **10** at various 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.

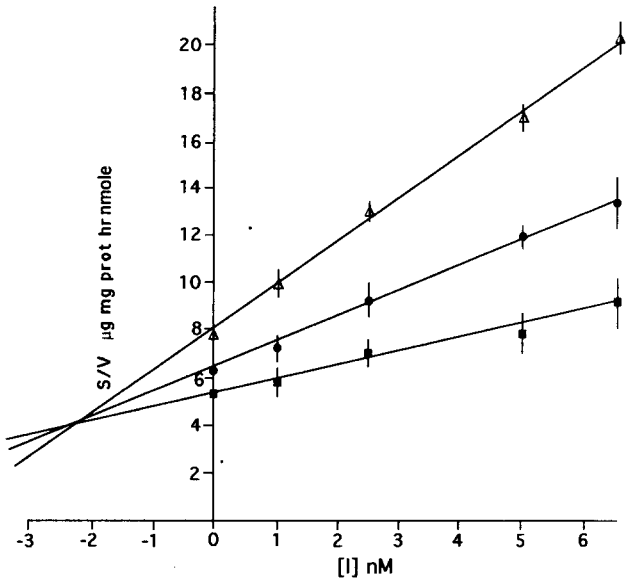
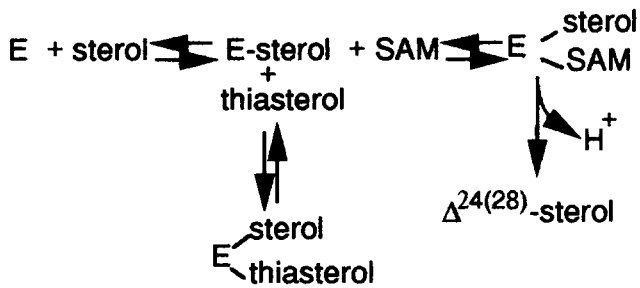


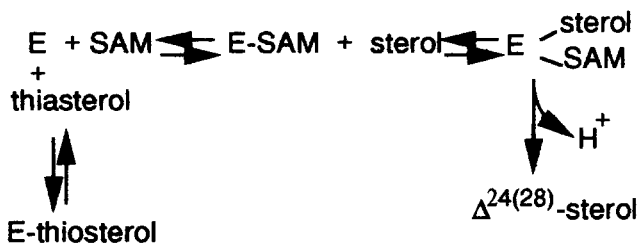
Figure 6. Plot (s/v vs I) for inhibition of 24-SMT by 25-thiacholesterol iodide at fixed [SAM]. Acetone powder extract of methyl transferase (1.5 mL, ~4 mg of protein per mL) was incubated as described in Figure 5.

consistent only with uncompetitive inhibition (Fig. 5). In this case the K_i for **11** vs **10** was obtained from the plots of [**10**]/velocity vs [**11**] (Fig. 6). The uncompetitive kinetic pattern observed for **11** with respect to **10** is expected if the enzyme species to which **11** and **10** bind are different and separated by an essentially irreversible step. This pattern is consistent with the binding of **11** before **10**.[33-36] These kinetic results parallel those obtained in studies of inhibition of yeast 24-SMT by 25-azasterols[13] and suggest that the inhibitor binds to sites normally occupied by sterol but does not compete with it for the same enzymatic species.

These kinetic results can be accommodated by two mechanisms. In one mechanism zymosterol binds first



Scheme 1.



Scheme 2.

to 24-SMT. This is followed by binding of **11** to the 24-SMT-zymosterol complex to form a dead-end ternary complex (Scheme 1). This mechanism suggests thiasterol binds to an allosteric site normally occupied by zymosterol. Evidence against this interpretation comes from the observation that 24-SMT does not exhibit sigmoidal kinetics with respect to zymosterol expected of this kinetic model.[33-36] In the second mechanism SAM binds first (Scheme 2). In this scenario thiasterol competes with SAM for 24-SMT but is uncompetitive with respect to zymosterol because the latter binds after SAM.

Inhibition by 12 and 13

These thiasterols were designed to mimic the first presumptive intermediate (**A**) in the alkylation process. We expected that the sulfonium ion mimic possessing the same stereochemistry at C-24 as A would be the most efficient inhibitor of 24-SMT. As can be seen by the I_{50} values in Tables 2 and 3, **13** is a more potent inhibitor of both 24-SMT and 24(28)-MSR in vivo and of 24-SMT in vitro. These results indicate that the most probable stereochemistry at C-24 for the presumptive intermediate is 24(*S*). The small difference ~2 × in the inhibitory power of **13** compared to **12** could be due to a poor fit of both mimics in the active site due to the presence of the large sulfur. Indeed, both **12** and **13** are less potent inhibitors than **11** which, although it contains a common sulfur, does not have the added bulk of a C-24 methyl. The binding site for these intermediate mimics is expected to be small if direct methyl transfer is involved since, as has been elegantly reasoned such transfers occur through compressed transition states.[37]

Conclusions

Our view of the methylenation process in yeast is summarized in Figure 7. The kinetic analysis suggests SAM is the first to bind. This induces a conformational change in the 24-SMT that allows zymosterol to be simultaneously bound with SAM. As deduced from the greater potency of **13** compared to **12**, the methyl is

Table 2. Efficiency of thiasterol inhibition of 24-SMT and 24(28)-MSR in *S. cerevisiae*

Thiasterol[a]	Concentration required for 50% inhibition, µM 24-SMT	24(28)-MSR[b]	Relative effectiveness as inhibitor I_{50} (24-SMT) I_{50} [24(28)-MSR]
11	0.07	0.08	0.87
12	0.27[c]	0.55[c]	0.49[c]
13	0.13[c]	0.13[c]	1.00[c]

[a]Thiasterol administered to culture of *S. cerevisiae* and using previously described growth conditions and sterol analysis procedure.[9] Growth period was 48 h.
[b]MSR: methylene sterol reductase.
[c]Corrected for cross-contamination of each isomer.

Table 3. Efficiency of thiasterol inhibition of 24-SMT in vitro

Thiasterol[a]	$K_{i'}$ vs Zymosterol (nM ± SEM)	K_i vs SAM (nM ± SEM)
11	2.38 ± 0.05	1.08 ± 0.05
12	4.61 ± 0.04[b]	2.30 ± 0.06[b]
13	2.98 ± 0.05[b]	1.70 ± 0.06[b]

[a]Thiasterols **12** and **13** administered to partially purified 24-SMT from *S. cerevisiae* using replications and conditions described for **11** (Figs 4 and 5). Both **12** and **13** were competitive inhibitors with respect to SAM and noncompetitive inhibitors with respect to zymosterol.
[b]Corrected for cross-contamination of each isomer.

transferred to the *si* face of the Δ^{24}-bond with a concomitant change in conformation that favors binding of A. A rotation of approximately 60° around the C-24(25)-bond is required at this point to bring the proper face of the presumptive empty *p* orbital on C-25 into alignment with the C-24 H bond. Migration of hydrogen from C-24 to C-25 then occurs across the *re* face to generate **B**. Mimics of **B** (**2**) that have been examined in yeast are very efficient in vivo inhibitors of 24-SMT.[6,7,12–14] The last step in the methylenation process is loss of a hydrogen from the *re* face of C-28. As shown in Figure 7 it is possible that the same center can act as a stabilizing nucleophile for both A and B. A remaining enigma is the apparent requirement for C-24 to C-25 hydrogen migration and C-28 hydrogen removal to occur from the same face.

Experimental

Instrumentation and general methodology

Unit resolution mass spectra were obtained on a Hewlett–Packard 5985B coupled gas chromatograph–mass spectrometer using electron-impact ionization at 70 eV or chemical ionization (CI) with isobutane as the ionizing gas. High-resolution mass spectra were obtained on a Kratos MS80 RFA mass spectrometer. IR spectra were measured on a Perkin–Elmer 599B spectrophotometer. Ultraviolet spectra were recorded on a Cary 210 spectrophotometer. The ^{1}H NMR spectra were recorded in $CDCl_3$ on a Bruker 400 WM spectrometer. A Varian 2100 gas chromatograph with flame-ionization detection was employed for sterol analysis using two types of columns: SILAR-10C, 1.83 m × 2 mm id glass U-tube packed with 3% SILAR-10C on Gas Chrom Q (100–120 mesh) and OV-101 capillary, 30 m × 0.25 mm id glass capillary coated with OV-101. Acetates were analyzed on both columns relative to cholestanyl acetate, OV-101 at 245 °C and SILAR-1OC at 220 °C. Trimethylsilyl ethers were analyzed on the OV-101 column at 240 °C relative to the trimethylsilyl derivative of cholestanol.[27,28] Fermentations were conducted on a Virtis fermenter, Model 40-300. Radioactivity was determined by an 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 Lowery protein assay were done on a Spectronic 20 spectrophotometer. Melting points were determined on a Fischer–Johns apparatus.

All reactions requiring anhydrous conditions and (or) oxygen-free conditions were run in flame-dried glassware under a positive pressure of argon. Tetrahydrofuran (THF) was freshly distilled from lithium tetrahydridoaluminate. Column chromatography was performed by flash chromatography[38] on Silica Gel (Kieselgel 60, 40–63 mm, Merck, Darmstadt). Solvents were distilled before use.

Preparation of 25,26,27-trisnorcholest-5-en-3β,24-diol (15). A solution of methyl 3β-hydroxy-5-cholenoate (**14**) (Steraloids, Wilton NH, 0.5 g, 1.3 mmol) in 20 mL THF was added dropwise to a suspension of lithium

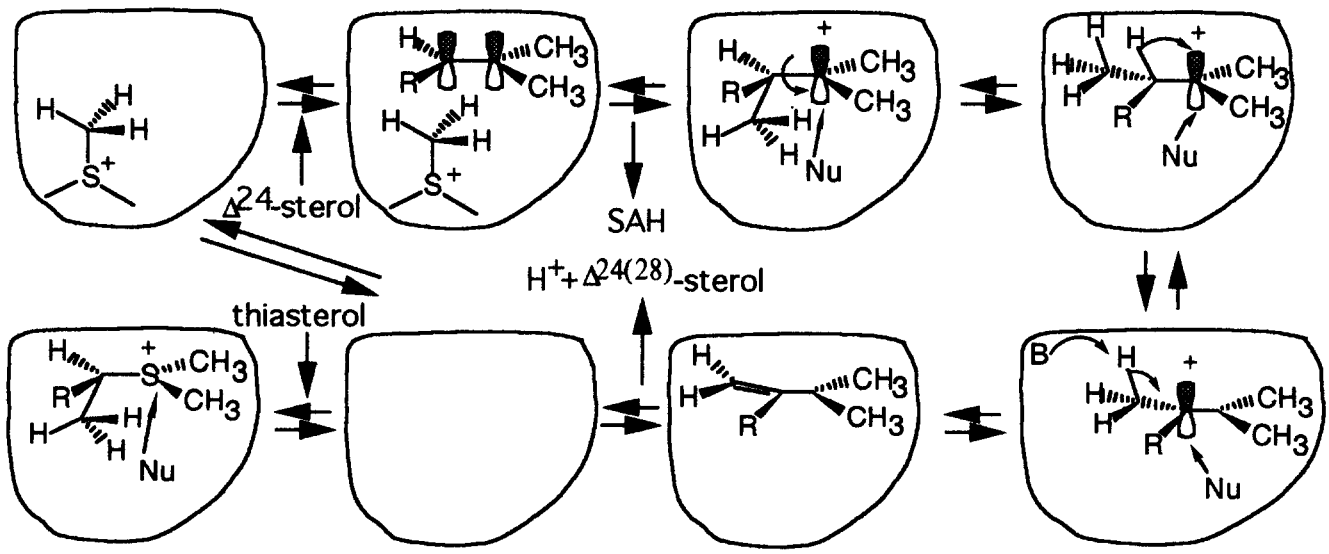


Figure 7. Proposed mechanism and stereochemistry of *S. cerevisiae* sterol methylenation.

tetrahydridoaluminate (0.05 g, 1.3 mmol) in 100 mL of dry THF. The mixture was refluxed for 2 h and the reaction quenched by careful addition of 10 mL of 10% aq NH_4Cl. The product was extracted with ether (4 × 15 mL), washed with H_2O, dried ($MgSO_4$), and concentrated in vacuo to yield 0.45 g (96%) of 3β,24-diol **15**: mp (methanol) 193–195 °C; ^1H NMR ($CDCl_3$) δ 0.69 (s, 3 H, C-18 H), 0.96 (d, J = 6.7 Hz, 3 H, C-21 H), 1.02 (s, 3 H, C-l9 H), 3.53 (m, 1 H, C-3 H), 3.62 (m, 2 H, C-24 H), 5.35 (br, d, 1-H, C-6 H); MS, *m/z* (relative intensity) 360 (96, M^+), 345 (30), 342 (60), 327 (48), 309 (5), 275 (70), 273 (33), 255 (42), 249 (40), 231 (35), 213 (80), 161 (52), 145 (88). Anal. calcd for $C_{24}H_{40}O_2$: C:79.93, H:11.20; found C:79.68, H:11.27.

Preparation of 3β-hydroxy-25,26,27-trisnorcholest-5-en-24-yl-*p*-toluene-sulfonate (16). To a solution of diol **15** (0.1 g, 0.28 mmol, 1 equiv) in 20 mL dry pyridine was added *p*-toluenesulfonyl chloride (0.055 g, 0.28 mmol, 1 equiv). The mixture was left for three days at 4 °C, then the reaction was quenched with water (30 mL). The product was extracted with Et_2O (3 ×, 10 mL), dried ($MgSO_4$), and concentrated in vacuo. Monotosylate **16** was purified by chromatography on silica gel using hexane:ethyl acetate (2:1 v/v) as eluant to give 0.065 g (46%) of crystalline product: mp (methanol) 76–78 °C; ^1H NMR ($CDCl_3$) δ 0.64 (s, 3 H, C-18 H), 0.87 (d, J = 6.7 Hz, 3 H, C-21 H), 1.01 (s, 3 H, C-19 H), 2.46 (s, 3 H, *p*-C_6H_4-CH_3), 3.53 (m, 1 H, 3αH), 4.00 (m, 2 H, C-21 H), 5.35 (m, 1 H, C-6 H), 7.34 (d, J = 6.7 Hz, 2 H, C_6H_4), 7.78 (d, J = 6.7 Hz, 2 H, C_6H_4); MS, *m/z* (relative intensity) 514 (4, M^+), 496 (23, M^+-H_2O), 481 (6, M^+-Me-H_2O), 429 (4), 375 (4), 342 (M^+-$C_7H_7SO_3H$, 2), 324 (342-H_2O, 3), 309 (324-Me, 10), 273 (5), 255 (25), 213 (53), 172 (43, $C_7H_7SO_3H$), 161 (30), 91 (100).

Preparation of 3β-hydroxy-25,26,27-trisnorcholest-5-en-24-yl iodide (17). To tosylate **16** (0.2 g, 0.39 mmol, 1 equiv) in 2 mL dry acetone was added a solution of NaI (0.2 g 1.3 mmol, 3 equiv) in 5 mL of dry acetone. The yellow mixture was left overnight at room temperature. The mixture was concentrated in vacuo and extracted with 15 mL $CHCl_3$ to give a crystalline material that was removed by filtration and the filtrate was concentrated in vacuo. The product was purified on silica gel using hexane:ethyl acetate (2:1.5 v/v) as the eluant to give 0.18 g (96%) of **17**: mp (methanol) 121–122 °C; ^1H NMR ($CDCl_3$) δ 0.68 (s, 3 H, C-18 H), 0.93 (d, J = 6.7 Hz, 3 H, C-21 H), 1.01 (s, 3 H, C-19 H), 3.16 (m, 2 H, C-24 H), 3.52 (m, 1 H, C-3 H), 5.35 (m, 1 H, C-6); MS, *m/z* (relative intensity) 470 (57, M^+), 455 (20), 452 (25), 437 (24), 385 (50), 359 (45), 331 (18), 273 (20), 255 (23), 231 (15), 213 (40), 161 (48).

Preparation of 3β-hydroxy-25,26,27-trisnorcholes-5-en-24-yl methyl thioether (18). Methanethiol was bubbled through a solution of *n*-BuLi (0.8 mL, 1.6 M, 1.3 mmol, 2.8 equiv) in 5 mL THF at −78 °C for 10 min. To this a solution of iodosterol 17 (0.21 g, 0.45 mmol, 1 equiv) in 5 mL THF was added. The mixture was stirred at −78 °C for 5 min and then stirred until it warmed to room temperature. The reaction was then quenched with 10 mL water, extracted with Et_2O (3 × 10 mL), dried ($MgSO_4$), and concentrated in vacuo to yield 0.15 g (86%) of **18** which, after chromatography on silica gel in hexane:ethyl acetate (2:1.5), gave 0.145 g, 83%): mp (hexane) 119–120 °C; ^1H NMR ($CDCl_3$) δ 0.66 (s, 3 H. C-18 H), 0.92 (d, J = 6.7 Hz, 3 H, C-21 H), 1.00 (s, 3 H, C-19 H), 2.08 (s, 3 H, C-26 H), 2.43 (m, 2 H, C-24 H), 3.51 (m, 1 H, C-3 H), 5.33 (br. d, 1 H, C-6 H); MS, *m/z* (relative intensity) 390 (95, M^+), 375 (52), 372 (100), 357 (62), 330 (15), 305 (55), 279 (52), 273 (35), 255 (55), 231 (35), 213 (70), 161 (38); exact mass (M^+) calcd for $C_{25}H_{42}OS$: 390.2956; found 390.2963.

Preparation of 25-thiacholesterol iodide (11). Thiasterol **18** (0.095 g, 0.24 mmol) was stirred overnight at room temperature in 1 mL of CH_3I. To the precipitate a few drops of methanol were added and the product removed by filtration. Recrystalization from methanol yielded **11**, 0.075 g (58%): mp (methanol) 159–162 °C (decomp); ^1H NMR (CD_3OD) δ 0.78 (s, 3 H, C-18 H), 1.05 (d, J = 6.7 Hz, 3 H, C-21 H), 1.06 (s, 3 H, C-19 H), 2.95 (s, 6 H, C-26 H and C-27 H), 5.38 (m, 1 H, C-6 H). Anal. calcd for $C_{26}H_{45}OSI$: C:58.65, H:8.46; found C:58.65, H:8.62.

Preparation of methyl-3β-tetrahydropyranyloxy-5-cholenoate (19). To a solution of **14** (2.06 g, 5.3 mmol) in 80 mL of CH_2Cl_2, dihydropyran (0.6 mL, 0.55 g, 6.6 mmol, 1.2 equiv) was added with a catalytic amount of *p*-toluenesulfonic acid. The mixture was stirred at room temperature for 1.5 h, then quenched by addition of 20 mL of 5% aq $NaHCO_3$. The product was extracted with ether (3 × 20 mL), washed with H_2O, dried ($MgSO_4$), and concentrated in vacuo to yield 2.1 g (84%) of **19**: mp 148–149 °C; ^1H NMR ($CDCl_3$) δ 0.68 (s, 3 H, C-18 H), 0.93 (d, J = 6.7 Hz, 3 H, C-21 H), 1.01 (s, 3 H, C-19 H), 3.51 (m, 2 H, THP), 3.68 (s, 3 H, CH_3O), 3.92 (m, 1 H, C-3 H), 4.72 (br sh, 1 H, THP), 5.35 (br t, 1 H, C-6 H); HRMS (no molecular ion observed), exact mass calcd for $C_{27}H_{38}O_2$ (M^+-8517): 370.2832, found 370.2852.

Preparation of 3β-tetrahydropyranyloxy-25,26,27-trisnorcholest-5-en-24-ol (20). To a stirred mixture of lithium tetrahydridoaluminate (0.170 g, 4.5 mmol) in 50 mL of dry THF a solution of **19** (2.1 g, 4.45 mmol) in 10 mL THF was added dropwise at room temperature. The mixture was refluxed for 2 h and the reaction quenched by careful addition of 10 mL of 10% aq NH_4Cl. The product was extracted with ether (4 ×, 15 mL), washed with H_2O, dried ($MgSO_4$), and concentrated in vacuo to yield 1.7 g (86%) of **20**: mp 137–138 °C; ^1H NMR ($CDCl_3$) δ 0.67 (s, 3 H, C18 H), 0.91 (d, J = 6.7 Hz, 3 H, C-21 H), 0.99 (s, 3 H, C-l9 H), 3.47 (m, 2 H, THP), 3.58 (m, 2 H, C-24 H), 3.88 (m, 1 H, C-3 H), 4.69 (br sh, 1 H, THP), 5.33 (br t, 1 H, C-6 H); exact mass calcd for $C_{29}H_{48}O_3$: 444.3587, found 444.3595.

Preparation of 3β-tetrahydropyranyloxy-25,26,27-trisnorcholest-5-en-24-al (21). To a stirred, cold solution of pyridine (8.6 g, 8.8 mL, 109 mmol, 19 equiv) in 100 mL CH_2Cl_2, CrO_3 (5.5 g, 55 mmol, 9.5 equiv) was added

over 10 min. The resulting mixture was stirred for 30 min. A solution of 20 (2.55 g, 5.7 mmol, 1 equiv) in 5 mL of CH_2Cl_2 was added and the mixture stirred for 30 min at 4 °C, followed by 1 h at room temperature. The reaction was quenched by addition of 100 mL of water. The product was extracted with Et_2O and CH_2Cl_2 several times, washed with water, and dried ($MgSO_4$). Purification on a silica gel column using hexane:ethyl acetate (4:1 v/v) as an eluant gave **21** (2.05 g, 81%), which was used in the next reaction.

Preparation of 3β-tetrahydropyranyloxy-26,27-bis-norcholest-5-en-24-ols (22 and 23). Methyl magnesium bromide (1.9 mL, 2.98 M, 5.7 mmol, 1.2 equiv) was added under argon to a stirred solution of **21** (2.05 g, 4.6 mmol) in 50 mL of dry ether. After stirring at room temperature for 1 h the reaction was quenched by addition of water. The product was extracted with ether (3 ×, 20 mL), dried ($MgSO_4$), and concentrated in vacuo to yield 1.77 g (84%) of crude **22** and **23** that were used in the subsequent step without further purification: ^{1}H NMR ($CDCl_3$) δ 0.68 mmol) in 50 mL of dry THF a solution of **19** (2.1 g, 4.45 mmol) in 10 mL THF was added dropwise at room temperature. The mixture was refluxed for 2 h and the reaction quenched by slow addition of 10 mL of 10% aq NH_4Cl. The product was extracted with ether (4 ×, 15 mL), washed with H_2O, dried ($MgSO_4$), and concentrated in vacuo to yield 1.7 g (86%) of **20**: mp 137–138 °C; ^{1}H NMR ($CDCl_3$) δ 0.67 (s, 3 H, C-18 H), 0.91 (d, J = 6.7 Hz, 3 H, C-21 H), 0.99 (s, 3 H, C-19 H), 3.47 (m, 2 H, THP), 3.58 (m, 2 H, C-24 H), 3.88 (m, 1 H, C-3 H), 4.69 (br. sh., 1 H, THP), 5.33 (br. t, 1 H, C-6 H); exact mass calcd for $C_{29}H_{48}O_3$: 444.3587, found 444.3595.

Preparation of 3β-tetrahydropyranyloxy-25,26,27-tris-norcholest-5-en-24-al (21). To a stirred, cold solution of pyridine (8.6 g, 8.8 mL, 109 mmol, 19 equiv) in 100 mL CH_2Cl_2, CrO_3 (5.5 g, 9.5 equiv, 55 mmol) was added over 10 min. The resulting mixture was stirred for 30 min. A solution of **20** (2.55 g, 5.7 mmol, 1 equiv) in 5 mL of CH_2Cl_2 was added and the mixture stirred for 30 min at 4 °C, followed by 1 h at room temperature. The reaction was quenched by addition of 100 mL of water. The product was extracted with Et_2O and CH_2Cl_2 several times, washed with water, and dried ($MgSO_4$). Purification on a silica gel column using hexane:ethyl acetate (4:1 v/v) as an eluant gave **21** (2.05 g, 81%) that was used without further purification in the next reaction.

Preparation of carbamates of 24 and 25. A solution of **22** and **23** (0.14 g, 0.3 mmol, 1 equiv) and (*R*)-(–)-l-(1-naphthyl)ethyl isocyanate (0.063 g, 0.32 mmol, 1.07 equiv) in 2 mL of toluene containing triethylamine (0.016 g, 0.15 mmol, 0.5 equiv) was heated overnight at 120 °C in a sealed tube. The products (**24** and **25**) were concentrated in vacuo and purified by column chromatography on silica gel using hexane:ethyl acetate (4:1 v/v) as the eluant to yield 0.160 g (82%) of oily material. The diastereoisomeric carbamates **24** and **25** were separated by repetitive flash column chromatography on silica gel using hexane:ethyl acetate (95:5 v/v) as eluant. The individual diastereoisomers were isolated as oils and subjected to reduction without further purification.

The slow eluting carbamate **24** gave a ^{1}H NMR ($CDCl_3$) δ 0.69 (s, 3 H, C-18 H), 0.94 (d, J = 6.7 Hz, 3 H, C-21 H), 1.02 (s, 3 H, C-19 H), 1.18 (d, J = 6.7 Hz, 3 H, C-28 H), 1.66 (d, J = 6.5 Hz, 3 H, CH_3C-Ar), 3.51 (m, 2 H, THP), 3.92 (m, 1 H, C-3 H), 4.73 (m, 1 H, THP), 4.77 (m, 1 H, C-24 H), 4.94 (br. d, 1 H, NH), 5.35 (m, 1 H, C-6 H), 5.64 (m, 1 H, CHAr), 6.7–8.2 (m, 7 H, Ar).

The fast eluting carbamate **25** gave a ^{1}H NMR ($CDCl_3$) δ 0.65 (s, 3 H, C-18 H), 0.88 (d, J = 6.5 Hz, 3 H, C-21 H), 1.02 (s, 3 H, C-19 H), 1.23 (m, 3 H, C-28 H), 1.66 (d, J = 6.5 Hz, 3 H, CH_3C-Ar), 3.52 (m, 2 H, THP), 3.92 (m, 1 H, C-3 H), 4.73 (m, 1 H, THP), 4.80 (m, 1 H, C-24 H), 4.96 (br. d, 1 H, NH), 5.34 (m, 1 H, C-6 H), 5.64 (m, 1 H, CHAr), 6.7–8.2 (m, 7 H, Ar).

Regeneration of 3β-tetrahydropyranyloxy-26,27-bis-norcholest-5-en-24-ols (22 and 23). To a suspension of lithium tetrahydridoaluminate (0.05 g, 1.3 mmol, 9 equiv) in 10 mL of dry THF at room temperature was added a solution of 24 (0.37 g, 0.56 mmol, 1 equiv) in 2 mL THF. The mixture was refluxed for 2 h and worked up in the usual manner. Purification on silica gel using hexane:ethyl acetate 4:1 (v/v) as the eluant gave 0.23 g (90%) of **22**: ^{1}H NMR ($CDCl_3$) δ 0.675 (s, 3 H, C-18 H), 0.93 (d, J = 6.7 Hz, 3 H, C-21 H), 1.01 (s, 3 H, C-19 H), 1.18 (d, J = 6.7 Hz, 3 H, C-28 H), 3.50 (m, 2 H, THP), 3.73 (sextet, J = 6.7 Hz, 1 H, C-24 H), 3.92 (m, 1 H, C-3 H), 4.72 (m, 1 H, THP), 5.36 (m, 1 H, C-6 H).

Similarly, **25** was converted to **23** in 89% yield: mp (methanol) 145–147.5 °C: ^{1}H NMR ($CDCl_3$) δ 0.68 (s, 3 H, C-18 H), 0.92 (d, J = 6.7 Hz, 3 H, C-21 H), 1.00 (s, 3 H, C-19 H), 1.17 (d, J = 6.7 Hz, 3 H, C-28 H), 3.48 (m, 2 H, THP), 3.73 (sextet, J = 6.7 Hz, 1 H, C-24 H), 3.88 (m, 1 H, C-3 H), 4.71 (m, 1 H, THP), 5.33 (m, 1 H, C-6 H); HRMS (no molecular ion observed), exact mass calcd for $C_{25}H_{41}O_2$ (M^+-85): 373.3024, found 373.3065.

Preparation of 3β-tetrahydropyranyloxy-26,27-bis-norcholest-5-en-24-yl iodides (26 and 27). To a benzene solution (10 mL) containing triphenylphosphine (0.129 g, 0.49 mmol, 1.5 equiv) was added at room temperature 23 (0.15 g, 0.33 mmol, 1 equiv), methyl iodide (0.07 g, 0.49 mmol, 1.5 equiv), and diethyl azodicarboxylate (0.086 g, 0.49 mmol, 1.5 equiv). The resulting yellow solution was stirred overnight and the product concentrated in vacuo and purified by column chromatography on silica gel using hexane:ethyl acetate (95:5 v/v) as the eluant to yield 0.04 g (218) of **27**: mp (methanol) 133–135 °C; ^{1}H NMR ($CDCl_3$) δ 0.66 (s, 3 H, C-18 H), 0.925 (d, J = 6.5 Hz, 3 H, C-21 H) , 1.00 (s, 3 H, C-19 H), 1.90 (d, 3 H, J = 6.5 Hz, C-28 H), 3.49 (m, 2 H, THP), 3.91 (m, 1 H, C-3 H), 4.15 (sextet, J = 6.5 Hz, 1 H, C-24), 4.71 (m, 1 H, THP), 5.35 (m, 1 H, C-6 H).

In a similar fashion **22** was converted to **26** in 18% yield: ^{1}H NMR ($CDCl_3$) δ 0.68 (s, 3 H, C-18 H), 0.925 (d, J = 6.5 Hz, 3 H, C-21 H), 1.01 (s, 3 H, C-19 H), 1.92 (d, J = 6.5 Hz, 3 H, C-28 H), 3.50 (m, 2 H, THP), 3.92 (m, 1 H, C-3 H), 4.14 (sextet, J = 6.5 Hz, 1 H, C-24 H), 4.71 (m, 1 H, THP), 5.35 (m, 1 H, C-6 H).

Preparation of 3β-tetrahydropyranyloxy-27-norcholest-5-en-24-yl-methyl thioethers (28 and 29). An ether solution (10 mL) of *n*-BuLi (0.3 mL, 2.3 M, 0.69 mmol, 17 equiv) was saturated with methanethiol under nitrogen at −78 °C. Iodosterol 27 (0.025 g, 0.04 mmol, 1 equiv) in 2 mL of ether was added and the mixture stirred for 30 min at −78 °C, followed by stirring at room temperature. After formation of a very fine white ppt, the reaction was quenched by addition of 10 mL water. The product was extracted with ether (3 ×, 10 mL), dried ($MgSO_4$), and concentrated in vacuo to yield 0.018 g (83.6%) of **29**: mp 135–136 °C; ^{1}H NMR ($CDCl_3$) δ 0.68 (s, 3 H, C-18 H), 0.925 (d, J = 6.5 Hz, 3 H, C-21 H), 1.00 (s, 3 H, C-19 H), 1.26 (d, J = 6.5 Hz, 3 H, C-28 H), 2.07 (s, 3 H, C-26 H), 2.60 (sextet, J = 6.5 Hz, 1 H, C-24 H), 3.50 (m, 2 H, THP), 3.92 (m, 1 H, C-3 H), 4.72 (br sh, 1 H, THP), 5.35 (m, 1 H, C-6 H). In a similar fashion **26** was converted to 28 in 80% yield, mp (methanol) 133–134 °C.

Deprotection of the 3β-alcohol of **29** was carried out in 5 mL MeOH:THF (3:1 v/v) in the presence of 0.5 mL 1 N aq HCl at room temperature. Extraction with Et_2O (3 ×, 10 mL) gave 0.01 g (67%) of the 3β-alcohol of **29**: mp (methanol) 116–118.5 °C; ^{1}H NMR ($CDCl_3$) δ 0.68 (s, 3 H, C-18 H), 0.925 (d, J = 6.5 Hz, 3 H, C-21 H), 1.02 (s, 3 H, C-19), 1.27 (d, J = 6.5 Hz, 3 H, C-28 H), 2.07 (s, 3 H, C-26 H), 3.52 (m, 1 H, C-3 H), 5.35 (m, 1 H, C-6 H); exact mass calcd for $C_{26}H_{44}OS$: 404.3161, found 404.3137.

In a similar fashion the 3β-alcohol of **28** was deprotected to give the corresponding alcohol: ^{1}H NMR ($CDCl_3$) δ 0.69 (s, 3 H, C-18), 0.94 (d, J = 6.5 Hz, 3 H, C-21 H), 1.02 (s, 3 H, C-19 H), 1.26 (d, J = 6.5 Hz, 3 H, C-28 H), 2.08 (s, 3 H, C-26 H), 2.60 (m, 1 H, C-24 H), 3.53 (m, 1 H, C-3 H), 5.34 (m, 1 H, C-6 H).

Preparation of 24(*R*) and 24(*S*)-methyl-25-thiocholesterol iodides (12 and 13). The 3β-alcohol of thioether 28 (0.01 g, 0.025 mmol) was dissolved in 2 mL methyl iodide at room temperature. The yellow solution was stirred overnight and then refluxed for 2 h. The resulting ppt was isolated by filtration and crystallized from methanol to yield 0.007 g (52%) of **13**: mp (methanol) 154–157 °C; ^{1}H NMR (CD_3OD) δ 0.73 (s, 3 H, C-18 H), 1.01 (d, J = 6.7 Hz, 3 H, C-21 H), 1.02 (s, 3 H, C-19 H), 1.48 (d, J = 6.75 Hz, 3 H, C-28 H), 2.83 (s, 3 H, C-25 H), 2.89 (s, 3 H C-27 H), 4.8% contamination with **12**; HRMS (no molecular ion observed), exact mass calcd for $C_{26}H_{44}OS$ (M^+-142): 404.311, found 404.3116. In a similar manner **28** was converted to **12** in 45% yield: mp 145–147 °C (methanol); ^{1}H NMR (CD_3OD) δ 0.77 (s, 3 H, C-18 H), 1.01 (d, J = 6.5 Hz, 3 H, C-21 H), 1.02 (s, 3 H, C-19 H), 1.41 (d, J = 6.75 Hz, 3 H, C-28 H), 2.86 (s, 3 H, C-27 H), 2.90 (s, 3H, C-26), 10.5% contamination with 13; HRMS (no molecular ion observed), exact mass calcd for $C_{26}H_{44}OS$ (M^+-142): 404.3143, found 404.3128.

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.

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.

Yeast strains and culture medium

The wild-type strain Al84D (erg^+) of *S. cerevisiae* and the sterol mutant erg 2 derived from this stock have been previously described.[39,40] Yeast strains were cultured in complete liquid medium.[27,28]

Isolation of zymosterol

The NSFs (nonsaponifiable fractions) obtained from several batch cultures of the erg 2 mutant grown in the presence of 1.0 pM 25-azacholesterol (8.0 g L^{-1} dry cell weight which was 1.5% by weight sterols) was subjected to chromatography on silica gel using hexane:ethyl acetate (75:25 v/v) as the eluant. Fractions (TLC) containing zymosterol were combined and acetylated with acetic anhydride:pyridine (2:1 v/v). Zymosteryl acetate was further purified by preparative TLC on silica gel GF254 impregnated with 25% silver nitrate, by weight (0.5 mm thick on 20 × 20 cm plates). Plates were developed twice with benzene:methylcyclohexane (4:1 v/v) and sprayed with Rhodamine 6G in acetone (0.1% w/w). 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). Zymosteryl acetate was hydrolyzed and the free sterol was recrystallized from hot methanol. Analysis as the TMS-derivative by GLC (OV101) revealed that zymosterol (mp 108 °C) purified by this procedure was 99% pure.

Inhibition experiments

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 thiasterol 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 4 L Virtis fermenter jar containing

1.5 L of medium to which the thiasterol 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 grown for 48 h at 30 °C with 400 rev min^{-1} stirring and 1.9 L min^{-1} aeration. DOW antifoam spray was added to the culture to control foaming. Cells were harvested by centrifugation (20 min at 2500 *g*), washed three times with distilled water, weighed wet, and either saponified immediately[4] or stored at −27 °C until used. The nonsaponifiable fractions were analyzed by GLC and UV spectroscopy. This method was also employed when growing erg 2 sterol mutants with 1 μM 25-thiacholesterol iodide for zymosterol production, except that the starter culture was prepared by inoculation of a 2 × 10 mL medium. The nonsaponifiable fractions were analyzed for sterol content and composition by methods reported elsewhere.[13]

Preparation of acetone powder of *S*-adenosyl-L-methionine. Δ^{24}-Sterol methyl transferase *S. cerevisiae* was grown with slight modification of a previously described method.[13] Starter cultures (4) of yeast were prepared by inoculation of 10 mL of medium with a loop of cells followed by static incubation for 48 h at 30 °C. Two starter cultures were added to each 1.5 L of medium of medium in 4 L Virtis fermenter jars. The cultures were stirred (400 rev min^{-1}) and aerated (1.9 L min^{-1}) for 24 h at 30 °C. All subsequent steps were carried out at 4 °C.[41] 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 $MgCl_2$), and weighed wet. Cells were suspended in 0.1 M Tris-HCl buffer to a final concentration of 1 g mL^{-1}. 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 s burst in a Braun MSK cell homogenizer, and the homogenate was put on ice. Unbroken cells and cell debris were removed by centrifugation at 3000 *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 supernatant fraction was dialyzed three times for 9 h against 100 vol of chilled distilled water. 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 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 with 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 opened 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[42] using bovine serum albumin as a standard.

Assay of enzyme activity

The assay of 24-SMT activity was conducted using a procedure similar to that described by Moore and Gaylor.[19] 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 μmol of $MgCl_2 \cdot 6H_2O$; 222 μmol of glucose; 3.5 mg of glucose oxidase; 400 μmol of Tris-HCl; 20–400 nmol of sterol substrate (e.g., zymosterol) dissolved in ethanol. Finally, 20 μL of a solution containing 20 μmol of *S*-[methyl-^{14}C]-SAM per μL (0.01 mCi pL^{-1}) was 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^{-1}. Assays of methyl transferase activity with the enzyme inhibitor 25-thiacholesterol iodide[12] were carried out as described above using an inhibitor concentration of 1.0–6.5 nM. The order of addition of components to the reaction was as follows. Buffers containing the magnesium salt and the enzyme preparations (in 0.1 M Tris-HCl, pH 7.5, containing 1 mM Mg^{2+}) were added first to prechilled Erlenmeyer flasks. The flasks were flushed with nitrogen for 20 s 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.[43] Sterol substrate (e.g., zymosterol) 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 simultaneously. 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 nonsaponifiable 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.

Analysis of kinetic data

Kinetic studies were examined by standard graphical procedures. Dixon plots[44] were used to analyze inhibition patterns. The data were 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.

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