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Azasterol Inhibition of Δ^{24}-Sterol Methyltransferase in *Saccharomyces cerevisiae*†

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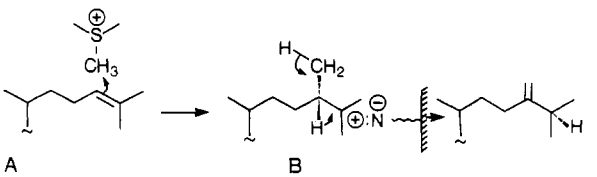
ABSTRACT: The inhibition of the Δ^{24}-sterol methyltransferase (24-SMT) of *Saccharomyces cerevisiae* by side-chain azasterols is related to their nuclear skeleton and side chain nitrogen position. Inhibitory power [I_{50} (μM)] was found to be in the order of 25-azacholesterol hydrochloride salt (0.05) > 25-aza-24,25-dihydrozymosterol (0.08) > 25-azacholesterol ≈ 25-azacholestanol (0.14) > (20*R*)- and (20*S*)-22,25-diazacholesterol (0.18) > 24-azacholesterol (0.22) > 25-aza-24,25-dihydrolanosterol (1.14) > 23-azacholesterol (4.8). In the presence of azasterols, *S. cerevisiae* produces increased amounts of zymosterol, decreased amounts of ergosterol and ergostatetraenol, and the new metabolites cholesta-7,24-dienol, cholesta-5,7,24-trienol, and cholesta-5,7,22,24-tetraenol. Kinetic inhibition studies with partially purified 24-SMT and several azasterols suggest the azasterols act uncompetitively with respect to zymosterol and are competitive inhibitors with respect to *S*-adenosyl-L-methionine (SAM). These results are consistent with at least two kinetic mechanisms. One excludes competition of azasterol and zymosterol for the same site, whereas a second could involve a ping-pong mechanism in which 24-SMT is methylated by SAM and the methylated enzyme reacts with sterol substrate.

The C-24 alkylation of Δ^{24}-sterols is viewed as a nucleophilic attack of the Δ^{24}-π electrons on the *S*-methyl group of SAM.[1] This generates an intermediate possessing a cationic site at C-25 in a C-24-methylated sterol. Migration of hydrogen from C-24 to C-25 and subsequent loss of a hydrogen from the C-24 methyl yields the 24-methylenesterol in yeast and higher plants (Arigoni, 1978):



The enzyme mediating this process might be inhibited by mimics of either the substrate A or the intermediate B. Recent work with the yeast *Saccharomyces cerevisiae* (Avruch et al., 1976) has shown that 25-aza-24,25-dihydrozymosterol (**1**) is

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[1] Abbreviations: 24-SMT, Δ^{24}-sterol methyltransferase; 24(28)-MSR, 24-methylenesterol $\Delta^{24(28)}$-reductase; SAM, *S*-adenosyl-L-methionine; MS, mass spectrum; TLC, thin-layer chromatography; THF, tetrahydrofuran; Tris, tris(hydroxymethyl)aminomethane; GLPC, gas–liquid partition chromatography.

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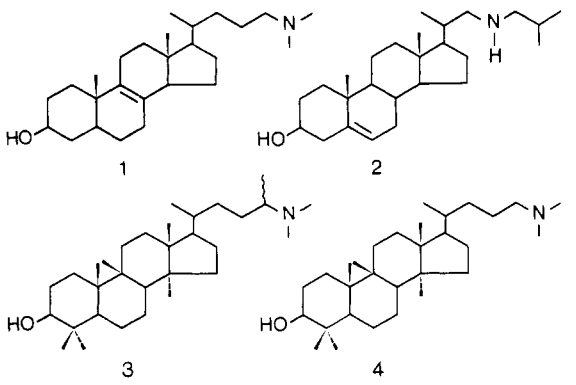
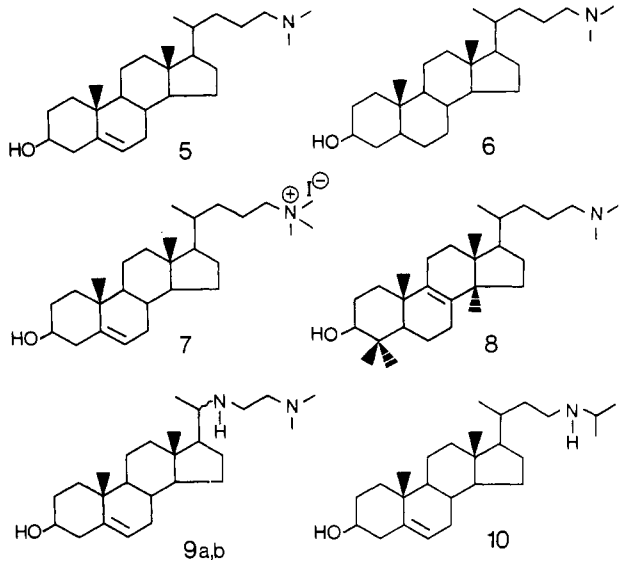


a potent inhibitor of the 24-SMT of this organism. This inhibition has been attributed (Oehlschlager et al., 1980) to the electronic resemblance at C-25 of the azasterol and the Δ^{24}-sterol substrates.

As expected by this rationale, 23-azacholesterol (**2**) is a weaker inhibitor of 24-SMT (Pierce et al., 1978). (24-*RS*)-24-Methyl-25-azacycloartanol (**3**) and 25-azacycloartanol (**4**) have been shown to be potent inhibitors of the *S*-adenosyl-L-methionine:cycloartanol C-24-methyltransferase of maize (Narula et al., 1981). The inhibition by these azasterols has been attributed to the electronic resemblance of the protonated form to intermediate B.

The present investigation was undertaken to probe the effects of nuclear structure, side chain nitrogen position, and nitrogen charge on inhibitory power of azasterols on the 24-SMT of *S. cerevisiae*. We also sought to determine the kinetics of azasterol inhibition of this enzyme.

Inhibitors used were 25-aza-24,25-dihydrozymosterol (**1**), 25-azacholesterol (**5**), 25-azacholesterol hydrochloride, 25-



azacholestanol (**6**), 25-azacholesterol methiodide (**7**), 25-aza-24,25-dihydrolanosterol (**8**), the 20*R* and 20*S* isomers of 22,25-diazacholesterol (**9a,b**), 24-azacholesterol (**10**), and 23-azacholesterol (**2**; Pierce et al., 1978).

Experimental Procedures

Instrumentation. A Varian 2100 gas chromatograph equipped with a flame-ionization detector was employed for sterol analysis (Pierce et al., 1978, 1979). Mass spectra of sterol acetates were obtained on a Hitachi Perkin-Elmer RMU-6E mass spectrometer (ionization voltage of 80 eV and probe temperature of 180 °C) coupled to a Varian 1400 gas chromatograph having a 1.83 m × 2.2 mm glass column packed with 3% SILAR-10C on Gas Chrom Q (100/120) at 230 °C. NMR spectra were recorded on a Varian XL-100 spectrometer in $CDCl_3$ containing tetramethylsilane at 25 °C. Ultraviolet spectra were recorded on a Unicam SP8000 spectrophotometer. Melting points were determined on a calibrated Fisher-Johns apparatus.

Azasterol Preparation. The synthesis of 25-aza-24,25-dihydrozymosterol (**1**) was as previously reported (Avruch et al., 1976). 25-Azacholesterol (**5**) was prepared by the method of Counsell et al. (1965) and melted at 146.5–148.5 °C [lit. mp 147.5–149.5 °C (Counsell et al., 1965)].

The preparation of small amounts (50–100 mg) of the hydrochloride salt of 25-azacholesterol was as follows. Azasterol was placed in a tapered, screw-cap centrifuge tube and dissolved in a minimum amount of isopropyl alcohol. The solution was clarified by centrifugation to remove small amounts of insoluble matter and debris, and the clear supernatant was transferred by capillary pipet to a clean, dust-free tube. A few drops of 6 M HCl was added, and the mixture was stirred with a glass rod until precipitation of the salt was complete. The salt was collected by centrifugation and, after removal of the supernatant by capillary pipet, washed 3 times with anhydrous diethyl ether. After being partially dried under a stream of nitrogen, the damp salt was transferred to a vial or small crystallizing dish and dried under reduced pressure in a desiccator.

25-Azacholestanol (**6**) was prepared by a method similar to that of Bruce (1943) and gave a melting point of 144.5–145.5 °C: MS, *m/z* (relative intensity) 389 (45, M^+), 374 (25, $M^+ - CH_3$), 58 [100, $(CH_3)_2N^+CH_2$].

25-Azacholesterol methiodide (**7**) was prepared from 25-azacholesterol (**5**) by reaction of the latter (30 mg) in 5 mL of methanol with 0.1 g of K_2CO_3 and 0.1 mL of methyl iodide at room temperature (Chen & Benoiton, 1976). The reaction was monitored by TLC ($CHCl_3$–MeOH–NH_4OH, 60:40:0.6) and stopped by the addition of water after 24 h. The reaction mixture was evaporated, and the solids were dissolved in hot acetone–water, filtered, and crystallized (on cooling). The solid product was filtered and washed with ether to yield 12 mg (38%): NMR (CD_3OD) δ 0.69 (s, 3 H, C-18 H), 1.02 (s, 3 H, C-19 H), 3.20 [s, 9 H, $-N(CH_3)_3$], 5.27 (m, 1 H, C-5 H); MS, *m/z* (relative intensity) 387 (10, $M^+ - CH_3$), 360 (20), 342 [40, $M^+ - (CH_3)_3NH$]; mp 270 °C (turns brown), 280 °C (melts).

25-Aza-24,25-dihydrolanosterol (**8**) was prepared from commercial-grade lanosterol. Oxidation of the Δ^{24} linkage of lanosteryl acetate (Bernassau & Fetizon, 1975) gave the trinor acid, which was then converted to the methyl ester of 3β-acetoxy-25,26,27-trinorlanost-8-en-24-oic acid. This was converted to the azasterol as follows. Dimethylamine (0.945 g), dissolved in THF (8 mL), was added to a suspension of $LiAlH_4$ (0.235 g) in THF (50 mL) and allowed to stir at room temperature for 1 h. The amine–$LiAlH_4$ product was cooled to 0 °C, and methyl 3β-acetoxy-25,26,27-trinorlanost-8-en-24-oate (0.15 g) dissolved in THF (25 mL) was added. The mixture was stirred for 2 h at 0 °C and 1.5 h at room temperature (Khanna et al., 1975). Water–THF (1:1 v/v, 10 mL), 3 N NaOH solution (10 mL), and water (20 mL) were added successively to the suspension. The mixture was filtered and the filtrate extracted with ether (3 times 50 mL). The extract was dried over anhydrous Na_2SO_4, filtered, and concentrated in vacuo. The residue was recrystallized from acetone–dioxane to give 80 mg of product: mp 163–165 °C [lit. mp 162–164 °C (Lu et al., 1971)]; NMR ($CDCl_3$) δ 0.70 (s, 3 H, C-18 H), 0.81 (s, 3 H, 4β-Me), 0.88 (s, 3 H, C-14 Me), 0.99 (s, 6 H, 4α-Me and C-19 H), 2.22 (s, 6 H, C-26 and C-27 H),

3.03–3.90 (m, 1 H, C-3 H); MS, *m/z* (relative intensity) 429 (90, M^+), 414 (40, $M^+ - CH_3$), 58 [100, $(CH_3)_2N^+CH_2$].

(20*R*)- and (20*S*)-22,25-diazacholesterols (**9a,b**) were synthesized as previously reported (Counsell et al., 1965). Separation and purification of the (20*R*)- and (20*S*)-22,25-diazacholesterols (2 g) were achieved by column chromatography on 300 g of silica gel. Elution with 500 mL of $CHCl_3$–CH_3OH (1:1 v/v) for 10-mL fractions gave 0.35 g of a 5-pregnendiol (M^+, 318). Elution with an additional 400 mL of solvent gave the 20*R* isomer, which was one spot on TLC (silica gel GF-254; $CHCl_3$–CH_3OH, 1:1 v/v). The compound was crystallized from ethanol–water (3:7) to give 0.67 g: mp 129.5–130.5 °C; NMR ($CDCl_3$) δ 0.75 (s, 3 H, C-18 H), 0.99 (d, J = 6 Hz, 3 H, C-21 H), 1.02 (s, 3 H, C-19 H), 2.24 (s, 6 H, C-26 and C-27 H), 5.3 (m, 1 H, C-5 H); MS, *m/z* (relative intensity) 388 (0.6, M^+), 330 [66.3, $M^+ - CH_2N(CH_3)_2$], 58 [100, $(CH_3)_2N^+CH_2$].

Elution with an additional liter of solvent gave, after crystallization from methanol–water, (20*S*)-22,25-diazacholesterol: 0.125 g; mp 112.5–113.5 °C [lit. mp 110–113 °C (Counsell et al., 1962)]; NMR ($CDCl_3$) δ 0.70 (s, 3 H, C-18 H), 1.01 (s, 3 H, C-19 H), 1.08 (d, J = 6 Hz, 3 H, C-21 H), 2.22 (s, 6 H, C-26 and C-27 H), 5.31 (m, 1 H, C-5 H); MS, *m/z* (relative intensity) 388 (0.4, M^+), 330 [51.5, $M^+ - CH_2N^+(CH_3)_2$], 58 [100, $(CH_3)_2N^+CH_2$].

24-Azacholesterol (**10**) was synthesized from 3β-acetoxy-22,23-dinor-5-cholenic acid (Steraloids, Inc.) by way of an Arndt–Eistert chain extension (Sax & Bergmann, 1955). This product was then converted to the azasterol by the method of Counsell et al. (1965) and melted at 159.5–162.5 °C [lit. mp 164–166 °C (Counsell et al., 1965)].

Culture Medium and Yeast Strain. The culture medium and *S. cerevisiae* strain were as previously described (Avruch et al., 1976).

Inhibition Experiments and Isolation of the Nonsaponifiable Fraction. A starter culture was prepared by inoculating 10 mL of medium with a loop of cells and statically incubated for 24 h at 30 °C. This was added to 100 mL of medium containing azasterol at the same concentration as used in the 0.1–1 μM inhibition experiments. The inoculum culture was grown for 48 h at 25 °C with stirring. For 5 and 10 μM inhibitor experiments, the starter culture and inoculum culture volumes were 20 and 200 mL, respectively, and the inoculum contained 2 μM azasterol. The inoculum was transferred to a 4-L Virtis fermenter jar containing 1.5 L of stirred (400 rev/min) medium to which azasterol as a solution in ethanol (1–2 mL) or 50% aqueous ethanol (2–3 mL) (25-azacholesterol hydrochloride experiments) had been added before inoculation. After being stirred for 0.5 h, the cultures were aerated (1.9 L/min) for 24 h at 30 °C on a Virtis fermenter. Dow Antifoam A spray was added to the cultures to control foaming. Cells were harvested by centrifugation (20 min at 2500*g*), washed twice with distilled water, weighed wet, and either saponified immediately (Avruch et al., 1976) or stored at −27 °C until later processing.

Separation and Analysis of Yeast Sterols. A chloroform solution containing about 90 mg of the nonsaponifiable fraction of each culture was applied to three plates (20 × 20 cm), coated with a 0.5-mm layer of silica gel GF-254 impregnated with 25% silver nitrate, by weight. The plates were developed with methylcyclohexane–ethyl acetate (9:5 v/v). After these were sprayed with an acetone solution containing 0.2% rhodamine 6G by weight, the bands were visualized under short-wave ultraviolet light. The R_f values and composition of the bands on a developed plate are as follows: 0.2–0.3, $\Delta^{5,7}$-dienes; 0.35–0.46, desmethylsterols; 0.47–0.51, 4α-methylsterols; 0.52–0.56, 4,4-dimethylsterols; 0.60–0.67, squalene. The bands were removed and thoroughly extracted with ether. Each fraction was dried to constant weight and taken up in chloroform (1–2 mL), and aliquots of the solution were transferred to microsample tubes (Clayton, 1962) [(25–30) × 6 mm] containing 0.1 mg of cholestanol. Solvent was removed under a N_2 stream. The tubes were stoppered with sleeve-type rubber septa (4.5 mm), and the trimethylsilyl ether derivatives were prepared by injection of 50–100 μL of Tri-Sil "TBT" into the stoppered tubes and by heating at 50 °C for 5–10 min. Acetate derivatives were prepared in stoppered microsample tubes by overnight reaction with pyridine–acetic anhydride (1:1 v/v). Reagent was removed by evaporation under a N_2 stream. The residue was dissolved in 50–100 μL of benzene or heptane for analysis. Each derivatized fraction was analyzed in duplicate by gas chromatography. The presence of the individual sterols in the separated fractions was established by comparison of the relative retention times of the peaks on the chromatograms to standard values (Pierce et al., 1979, 1978). The results of the sterol analyses of the nonsaponifiable fractions isolated from the control and inhibited cultures are given in Table I. Sterols present in the nonsaponifiable fractions of the control and inhibited cultures as trace levels (less than 0.1%) have been excluded from the list in Table I.

Δ^{24}-Sterol Methyltransferase Activity Assay Procedures. Starter cultures of yeast were prepared by inoculating 4 times 10 mL of medium each with a loop of *S. cerevisiae* cells and statically incubating for 24 h at 30 °C. These 10-mL inocula were added to 2 times 1.5 L of medium (two 10-mL inocula to each 1.5 L of medium) in 4-L Virtis fermenter jars. The cultures were stirred at 400 rev/min for 15 min and then aerated for 20 h at 30 °C on a Virtis fermenter with continuous stirring. The remaining steps were according to the method of Bailey et al. (1974). Cells were harvested by centrifugation (20 min at 2500*g*), washed once with 0.1 M Tris-HCl buffer [pH 7.6, containing 0.1 mM Mg^{2+} ($MgCl_2$)], and weighed wet. Cells were suspended in 0.1 M Tris-HCl buffer to a final concentration of 1 g/mL. Remaining steps were carried out at 4 °C. A total of 25 mL of cells was added to a 75-mL Duran flask containing 40 g of 0.25-mm glass beads. The flask was stoppered and secured in a Braun MSK cell homogenizer. The cells were given a 45-s burst, and the homogenate was put on ice. Total homogenate was centrifuged at 25000g for 20 min, and the pellet was discarded. The supernatant was centrifuged at 105000*g* for 1 h in a Beckmann L5-75 preparative ultracentrifuge. The supernatant was removed, and the pellet was resuspended in 0.1 M Tris-HCl buffer, pH 7.6. Protein concentration was determined by the method of Lowry et al. (1951), with bovine serum albumin (Sigma) as a standard. The suspension was divided into aliquots and stored at −25 °C until needed. The preparation was stable at this temperature for 3 weeks.

The method of assaying 24-SMT activity was similar to that used by Thompson et al. (1974). Enzyme activity was assayed by measuring ^{14}C incorporation from [*methyl*-^{14}C]SAM into the nonsaponifiable lipid fraction of enzyme preparations to which zymosterol had been added. All incubations were carried out in 25-mL Erlenmeyer flasks equipped with 14/20 ground glass joints. The assay flasks contained 3.2 mL of 0.1 M Tris-HCl buffer, pH 7.6, 10 μmol of $MgCl_2 \cdot 5H_2O$, 60 μmol of $KHCO_3$, 50–400 nmol of sterol substrate (e.g., zymosterol), and 0.5 mL of enzyme preparation (protein concentration of enzyme preparation ≈15 mg/mL) (Lowry et al., 1951).

Table I: Effects of Azasterols on Growth and Sterol Biosynthesis of *S. cerevisiae*

	Concentration of Azasterols in Culture (μM)	Dry Cell Weight (g/L)	Sterols (% of dry cell wt)[a]	$\Delta^{5,7}$-Dienes (% of dry cell wt)[b]	Sterol Composition[c]												
					Lanosterol (17)	4,4-Dimethylzymosterol (18)	4α-Methylzymosterol (19)	Zymosterol (13)	Cholesta-7,24-dienol (14)	Cholesta-5,7,24-trienol (15)	Cholesta-5,7,22,24-tetraenol (16)	Fecosterol (20)	Episterol (21)	Ergosta-7,22-24(28)-trienol (22)	Ergosta-5,7,22,24(28)-tetraenol (12)	Ergosterol (11)	% Inhibition 24-SMT	% Inhibition 24(28)-MSR
25-aza-24,25-dihydro zymosterol	0	11.2	4.3	2.1	4.8	4.5	2.8	19.7	nd	nd	nd	4.8	5.0	5.1	21.5	30.0	0	0
	0.1	6.4	2.6	1.2	2.0	6.1	3.5	39.7	4.6	3.6	11.8	5.2	0.5	nd	5.0	18.0	58	0
	0.2	6.2	0.5	0.2	5.0	12.8	5.6	46.6	5.1	4.5	12.0	1.4	0.4	nd	2.5	4.2	87	0
	0.5	4.4	0.2	0.1	3.7	13.5	4.4	39.8	12.0	6.8	15.0	0.3	0.1	nd	2.1	2.0	94	0
25-azacholesterol	0.1	10.7	5.5	2.2	3.2	5.1	3.4	38.5	2.5	1.3	4.3	1.8	2.0	2.9	15.0	20.0	39	0
	0.2	9.9	6.2	1.8	3.8	6.2	3.9	48.4	3.0	3.0	11.4	0.6	1.2	1.3	7.6	9.6	70	0
	0.5	9.1	5.7	1.8	3.1	4.8	3.1	53.6	3.6	5.8	16.1	nd	nd	nd	3.6	6.3	85	0
	1.0	9.9	5.2	1.6	3.6	7.5	4.2	55.2	4.3	5.3	14.6	nd	nd	nd	2.2	3.1	92	0
	5.0	6.7	4.1	1.1	3.0	8.4	3.9	59.5	3.8	5.4	16.0	nd	nd	nd	nd	nd	100	0
25-azacholesterol hydrochloride	0.1	8.0	4.7	1.4	1.6	6.3	3.1	56.2	3.2	3.5	11.5	0.3	0.4	0.4	3.6	9.9	78	0
	0.2	6.8	3.7	1.2	1.6	7.0	2.6	59.4	3.4	3.9	14.5	0.2	0.3	nd	2.1	5.0	88	0
	0.5	7.0	3.6	1.1	1.9	7.6	4.2	59.9	4.6	3.3	13.9	0.1	0.3	nd	0.8	3.4	93	0
	1.0	3.0	3.5	1.0	1.6	6.4	3.7	60.4	6.8	4.1	15.3	nd	nd	nd	0.3	1.4	97	0
25-azacholestanol	1.0	10.4	5.0	1.5	3.5	6.5	3.4	52.2	4.3	7.5	nd	nd	nd	nd	1.5	4.0	92	0
25-aza-24,25-dihydrolanosterol	1.0	10.9	6.0	2.0	6.5	9.1	4.4	37.0	2.1	nd	2.5	2.6	1.7	1.2	15.2	17.7	44	0
22,25-diazacholesterol (20R)	0.2	12.0	3.4	1.1	7.2	6.8	3.1	43.5	3.4	1.6	3.8	nd	1.5	2.8	9.1	17.2	55	0
	0.5	9.9	4.1	1.1	5.5	7.4	2.7	49.8	4.1	2.8	9.7	nd	nd	1.5	5.7	10.8	74	0
	1.0	9.9	5.2	1.6	3.8	6.0	3.6	51.0	4.3	3.8	14.7	nd	nd	nd	5.6	7.2	81	0
(20S)	0.2	12.3	3.9	1.2	5.6	6.5	4.0	45.2	3.3	3.4	2.4	nd	2.1	1.7	8.9	16.9	57	0
	0.5	10.9	3.8	1.1	6.2	7.1	3.1	50.3	4.7	2.4	9.8	nd	nd	1.1	6.6	8.7	76	0
	1.0	14.1	3.9	0.9	5.1	6.2	4.2	56.9	4.2	3.4	10.8	nd	nd	nd	3.7	5.5	86	0
24-azacholesterol	0.2	10.9	4.3	1.4	5.8	3.8	2.5	45.4	3.1	nd	2.1	0.8	2.3	3.4	16.8	14.0	45	24
	0.5	10.4	3.2	0.8	6.5	4.4	3.0	56.5	2.5	2.0	3.2	nd	1.2	2.1	12.2	6.4	68	45
	1.0	9.6	4.1	1.2	3.7	5.1	3.1	61.8	3.1	1.9	6.4	nd	0.2	0.2	10.7	4.2	78	52
	10.0	9.1	4.0	0.6	5.0	9.4	4.6	61.7	4.8	2.8	5.5	nd	nd	nd	4.2	2.0	91	39
23-azacholesterol	0.2																5	62
	0.5																10	76
	1.0						(Pierce et al., 1978)										21	91
	5.0																52	98
	10.0																74	96

[a] In addition, the sterol mixtures were analyzed by gas–liquid partition chromatography for the sterols (Fryberg et al., 1973). [b] Determined by ultraviolet spectroscopy. [c] Determined by GLPC; ergosta-8,22,24(28)-trienol was present (1.8%) in the control. nd = not detected (<0.1%).

Substrate and azasterol, dissolved in a total of 0.2 mL of ethanol, were then added, successively, to the reaction mixture. The reaction was initiated by the addition of 0.1 mL of buffer containing 0.1 μCi of [*methyl*-^{14}C]SAM diluted in 400 nmol of SAM. The flasks were stoppered and were incubated with gentle shaking at 30 °C in a water bath. The reaction was stopped by addition of 2 mL of 60% KOH solution. The flasks were removed and heated to reflux (boiling chips added) for 1 h under N_2. Each sample solution was diluted with 1 mL of distilled water and extracted twice with 10 mL of hexane. The combined hexane extracts were washed twice with water, dried over anhydrous Na_2SO_4, and filtered. The hexane was evaporated and the residue transferred into a liquid scintillation vial with 10 mL of Aquasol I (ICN) counter fluid and counted for 10 min. The amount of transmethylation was calculated from the count rates and the specific activity of the substrate. The transmethylation reaction by the cell extract was linear for up to 1.5 mL of enzyme preparation for 30 min.

The standard error depicted by the error bars in the figures was calculated from the variation between duplicate assays. The counting efficiency of the scintillation counter was 95 ± 1.0%. The counting error was typically between 0.5 and 1.5%. The counts for the samples varied between 1500 cpm (±1.5%) (background) to 2.5 × 10^4 cpm (±0.5%) (100 μM zymosterol, uninhibited). The error in workup from sample to sample as expressed in cpm varied between 5 and 15% for typical samples in a kinetic experiment.

Dixon plots (Dixon, 1953) were used to determine the type of inhibition caused by the administration of the azasterols. The data were plotted by a linear regression analysis that yielded correlation coefficients of 0.94 or better for the lines drawn (Hewlett-Packard 33E scientific calculator).

Results and Discussion

Yeast Growth and Sterol Production. Growth was not appreciably affected by the presence of azasterols containing nitrogen in the side chain at inhibitor concentrations below 1.0 μM, except for 25-aza-24,25-dihydrozymosterol (Table I). At 0.5 μM, this azasterol caused dry cell weight to be approximately 50% of that for other inhibitors at the same concentration. At concentrations above 5 μM, all azasterols caused a decline in cell yield.

The decline in cell production could be related to a decrease in total sterol or a decrease in ergosterol ($\Delta^{5,7}$-sterols) at higher inhibitor concentrations. Ergosterol has been reported to be important in the respiratory growth of yeast (Adams & Parks,

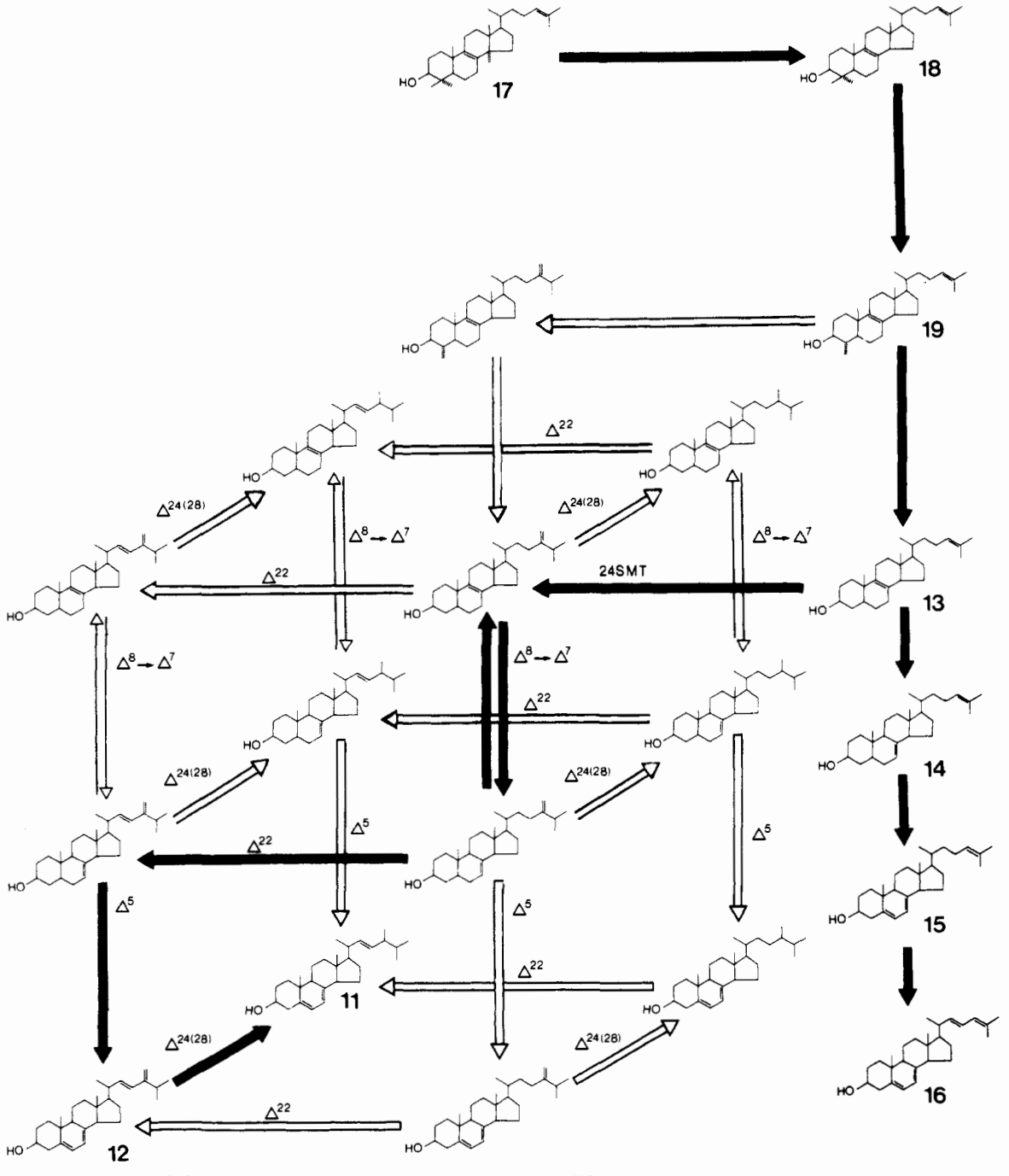


FIGURE 1: Sterol biosynthesis in *S. cerevisiae*: major pathways operative (solid arrows); minor pathways operative (open arrows).

1969; Parks et al., 1979), and the 24β-methyl-Δ^{22} moiety was reported to be important to the growth of *S. cerevisiae* (Nes et al., 1978). Another possible explanation is that azasterols behave as regulators of sterol biosynthesis as do hydroxysterols in mammalian systems (Parish et al., 1979; Parish & Schroepfer, 1979; Schroepfer, 1981).

Effect of Azasterol Inhibition on Sterol Composition. The effects of azasterols on yeast sterol composition can be most easily interpreted in terms of the simplified model for sterol biosynthesis previously published (Figure 1; Fryberg et al., 1973).

Comparing the sterol composition (Table I) of *S. cerevisiae* cultures grown with and without 23- and 24-azacholesterol and 25-azasterols revealed 24(28)-MSR is inhibited by 23- and 24-azacholesterol but not by 25-azasterols. This inhibition was calculated by comparing the ratio of ergosterol to $\Delta^{24(28)}$-sterols in inhibited cultures ($\sum\Delta^{24(28)}$-sterols$_i$) with the ratio of ergosterol to $\Delta^{24(28)}$-sterols in the control cultures ($\sum\Delta^{24(28)}$-sterols$_c$) in the following expression:

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

The percent inhibition of 24(28)-MSR for each concentration of inhibitor is given in Table I. The inhibition of 24(28)-MSR by 23-azacholesterol is higher than that by 24-azacholesterol (Tables I and II). The rather constant ergosterol to ergostatetraenol ratio in control and 25-azasterol inhibition experiments suggests none of the 25-azasterols inhibit 24(28)-MSR. If azasterols are acting as substrate mimics for the 24(28)-MSR, the nitrogens at C-23 and C-24 could be viewed as mimics (Pierce et al., 1978; Oehlschlager et al., 1980) for the Δ^{22} and $\Delta^{24(28)}$ linkages of the best substrate of the reductase, ergosta-5,7,22,24(28)-tetraen-3β-ol (**12**) (Jar-

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

azasterol[b]	concn (μM) required for 50% inhibition		relative effectiveness as inhibitor [24-SMT/24(28)-MSR]	act. of corresponding Δ^{24}-sterol as substrate for 24-SMT[c]
	24-SMT	24(28)-MSF		
25-aza-24,25-dihydrozymosterol	0.08	NI[d]		100
25-azacholesterol	0.14	NI		45
25-azacholesterol hydrochloride	0.052	NI		45
25-azacholestanol	0.14	NI		
(20*R*)-22,25-diazacholesterol	0.18	NI		45
(20*S*)-22,25-diazacholesterol	0.18	NI		45
25-aza-24,25-dihydrolanosterol	1.14	NI		0
24-azacholesterol	0.22	0.7	3	45
23-azacholesterol	4.8	0.12	0.025	45

[a] Oehlschlager et al., 1980. [b] Azasterol administered to culture of *S. cerevisiae* (Fryberg et al., 1973) and with previously described growth conditions and sterol analysis procedures (Pierce et al., 1979). Growth period for the present cultures was 24 h. [c] Moore & Gaylor, 1970. [d] NI, no inhibition.

man et al., 1975). It is also possible that 24-azacholesterol binds 24-SMT, reducing effective inhibition of the 24(28)-MSR. The $\Delta^{22,23}$-sterol desaturase is not inhibited by any of the azasterols. 23-Azacholesterol resembles the product of the desaturase more than the substrate, episterol (Pierce et al., 1978; Fryberg et al., 1973).

The most significant change upon addition of side chain nitrogen containing sterols to *S. cerevisiae* can be attributed to their inhibition of the 24-SMT. The addition of azasterols caused the proportion of ergosterol (**11**) and ergostatetraenol (**12**) to decrease while zymosterol (**13**) increased in relative proportion along with other C_{27} sterols (**14–16**).

An estimate of the effectiveness of each inhibitor in blocking the 24-SMT is obtained by comparison of the proportion of C-24-alkylated sterols produced in the presence of the inhibitor ($\sum$C-24-alk$_i$) with that produced in control cultures ($\sum$C-24-alk$_c$) according to the expression:

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

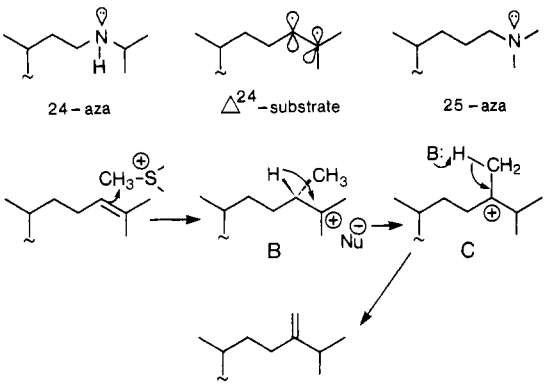
The percent inhibition for each concentration of inhibitor is given in Table I. The effectiveness of azasterols as inhibitors of yeast 24-SMT is most clearly seen by a comparison of the concentration of each azasterol required to effect 50% inhibition of 24-SMT (Table II).

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

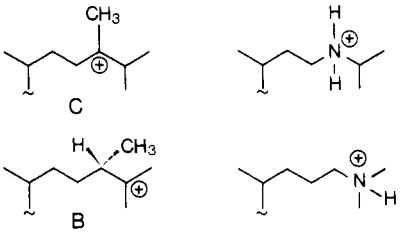
As expected from the hypothesis that side-chain azasterols mimic natural substrates, 23-azacholesterol is a weak 24-SMT inhibitor, and 24-azacholesterol is a somewhat less effective inhibitor of 24-SMT compared to 25-azacholesterol (Tables I and II). Since 24-azacholesterol also inhibits 24(28)-MSR, competition between this enzyme and the 24-SMT for this azasterol may reduce the effective concentration of the azasterol available to inhibit the 24-SMT.

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

25-Azacholesterol hydrochloride was found to be the most efficient inhibitor of the 24-SMT. The use of the hydrochloride salt ensures that the azasterol is protonated under physiological conditions (pH 6.5). If one assumes a carbocation rearrangement mechanism for the methylation process (Oehlschlager et al., 1980), then molecules that resemble intermediates in the sequence could be inhibitors of the reactions (Jencks, 1975; Wolfenden, 1976):



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



This explanation has been put forward to explain inhibition of cycloartanol C-24-methyltransferase in microsomes of maize seedlings (Narula et al., 1981) by 24-methyl-25-azacycloartanol and 25-azacycloartanol and in cultures of bramble cells (Schmitt et al., 1981) by 25-azacycloartanol.

Enzyme Inhibition Studies. To examine the mode of action of azasterols on 24-SMT, we studied its in vitro inhibition kinetics with partially purified enzyme. We adopted the method of Dixon (1953) to plot inhibition kinetics for 25-azacholesterol, 25-aza-24,25-dihydrozymosterol, 23-azacholesterol, and 25-azacholesterol methiodide. With this method, several inhibitor concentrations could be used in one experiment, and difficulties in determination of absolute concentrations of substrate were minimized. When zymosterol concentration was varied and SAM concentration held con-

Table III: Uncompetitive Inhibition of Δ^{24}-Sterol Methyltransferase by Azasterols When Zymosterol Concentration Is Varied and SAM Concentration Is Held Constant

azasterol	inhibition constant [K_I' (nM)]
25-azacholesterol	5.4
25-aza-24,25-dihydrozymosterol	5.1
23-azacholesterol	7.2
25-azacholesterol methiodide	2.8

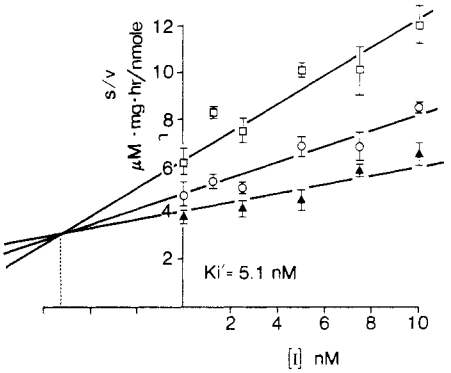


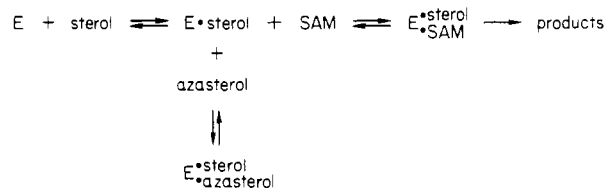
FIGURE 2: Plot for the inhibition of 24-SMT transmethylation by 25-aza-24,25-dihydrozymosterol (s/v vs. [I]). Zymosterol concentration was 12.5 (▲), 25 (○), or 50 μM (□).

stant, inhibition was uncompetitive (Table III and Figure 2).

Uncompetitive inhibition occurs when the inhibitor binds after the varied substrate. If the two enzyme species to which the inhibitor and varied substrate bind are separated by an irreversible step, however, the inhibitor binds before the varied substrate. The K_I' values of the azasterols studied are extremely low (lower than the in vivo concentrations) and are of the same order of magnitude (nanomolar). Even the K_I' value for 23-azacholesterol is similar to the K_I' values for other azasterols, which contrasts with the in vivo results (Table II). A possible reason for this observation is that the 24(28)-MSR was removed in the cell-free preparation of the 24-SMT at the initial centrifugation of 25000g. Maximum reductase activity was found in the 25000g pellet (Neal & Parks, 1977), which was discarded in 24-SMT preparations.

When SAM concentration was varied and zymosterol concentration kept constant, a competitive pattern was observed with 25-aza-24,25-dihydrozymosterol (Figure 3). Competitive inhibition occurs only when the inhibitor binds before the varied substrate and when the inhibitor and varied substrate are competing for the same enzymatic species or two different species that are in thermodynamic equilibrium.

One interpretation of the kinetic results is that the enzymatic mechanism involves an ordered process in which sterol is the first bound substrate. To be uncompetitive with respect to sterol substrate but competitive with respect to SAM, the azasterols would be required (Cleland, 1963) to bind after sterol as



Such an outcome is expected only if azasterols are binding to regulatory (allosteric) sites on the 24-SMT. Variable inhibition patterns with respect to zymosterol have been observed with sterols that are not substrates for this enzyme (Moore & Gaylor, 1970). Thus, cholesterol and 24,25-dihydrozymosterol exhibit competitive inhibition. Ergosterol and stigmasterol (which possess C-24-alkyl substituents) enhanced activity of 24-SMT at low concentrations of zymosterol and suppressed activity at higher concentrations of zymosterol. Of these two sterols, at least ergosterol is a noncompetitive inhibitor with respect to zymosterol (Parks et al., 1979). These latter observations are consistent with the view that regulatory sterol binding sites may be present on the 24-SMT.

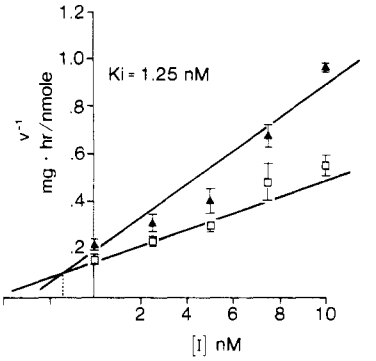
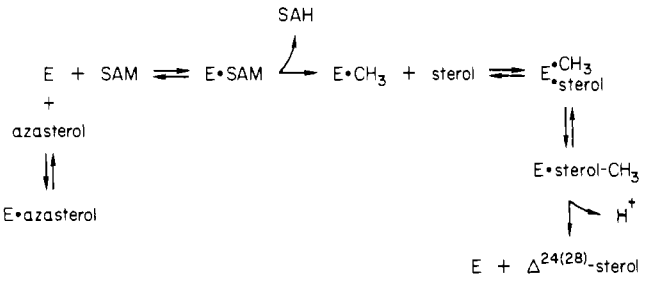


FIGURE 3: Dixon plot for the inhibition of 24-SMT transmethylation by 25-aza-24,25-dihydrozymosterol ($1/v$ vs. [I]). Zymosterol concentration was 100 μM. S-Adenosyl-L-methionine concentration was 25 (▲) or 50 μM (□).

Alternatively, the kinetic studies can be interpreted by assuming that the azasterols bind to the sites normally occupied by the sterol substrate. This view is strengthened by the observation (Table II) that the efficiency of 25-azasterol inhibition changes (25-aza-24,25-dihydrozymosterol > 25-azacholesterol > 25-aza-24,25-dihydrolanosterol) with the same structural regularity as the specificity of the corresponding Δ^{24}-sterol substrates (zymosterol > cholesterol ≫ lanosterol). If azasterols are binding to the sites normally occupied by the sterol substrates, then the kinetic results require SAM and inhibitor to compete for the same enzymatic species and sterol and inhibitor to compete for different enzymatic species that are separated by an irreversible process. We propose the irreversible process to be the transfer of the S-methyl of SAM to the enzyme; thus



Such a ping-pong reaction mechanism has been proposed for the SAM:magnesium-protoporphyrin IX methyltransferase of wheat (Ellsworth et al., 1974; Hinchigeri et al., 1981). Confirmation of this suggestion requires further kinetic work.

Registry No. **1**, 60189-90-4; **2**, 29588-39-4; **5**, 1973-61-1; **5**·HCl, 10454-60-1; **6**, 7727-87-9; **7**, 90531-18-3; **8**, 30093-16-4; **9a**, 40521-35-5; **9b**, 24887-57-8; **10**, 1249-82-7; **12**, 29560-24-5; **14**, 651-54-7; **15**, 1715-86-2; **16**, 34298-92-5; 24-SMT, 37257-07-1; ergosterol, 57-87-4; methyl 3β-acetoxy-25,26,27-trinorlanost-8-en-24-oate, 30655-16-4; 3β-acetoxy-22,23-dinor-5-cholenic acid, 1474-14-2.

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