Mechanism and Inhibition of Δ^{24}-Sterol Methyltransferase from *Candida albicans* and *Candida tropicalis*

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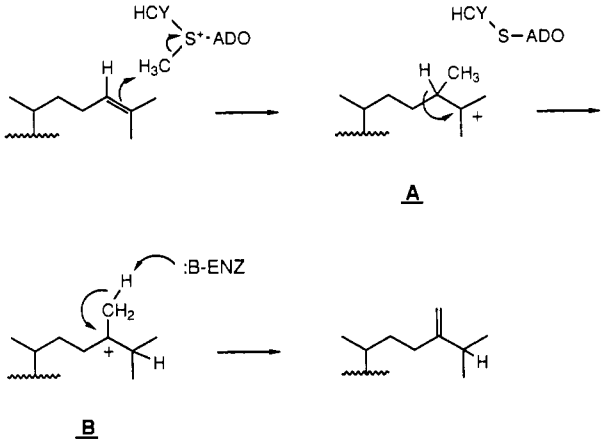
ABSTRACT: The *S*-adenosyl-L-methionine:Δ^{24}-sterol methyltransferase from *Candida albicans* has been solubilized with a mixture of octyl glucoside and sodium taurodeoxycholate. The enzyme has an apparent molecular weight of approximately 150 000 as measured by gel filtration chromatography. Zymosterol is the preferred substrate for the microsomal methyltransferase. Other nuclear double bond isomers support reduced rates of methenylation, while sterols which bear methyl groups at C-4 or C-14 are not substrates. Initial velocity and product inhibition studies are consistent with a rapid equilibrium ordered kinetic mechanism. A series of novel sterol analogues which contain heteroatoms substituted for C-24 or C-25 have been kinetically characterized as dead-end inhibitors of the methyltransferase, revealing three distinct mechanisms of interaction with the enzyme. Sterols which contain positively charged moieties in these positions are particularly potent inhibitors, supporting the proposed intermediacy of C-24 and C-25 carbocations. The methyltransferase is reversibly inhibited by low concentrations of 24-thiasterols, while behavior consistent with mechanism-based enzyme inactivation is apparent at higher concentrations. Possible mechanisms for this novel inactivation reaction are discussed.

The biosynthesis of ergosterol, an essential component of fungal cell membranes, requires the addition of a C_1 group to the sterol side chain (Mercer, 1984). This mechanistically intriguing transformation is catalyzed by *S*-adenosyl-L-methionine:Δ^{24}-sterol methyltransferase (EC 2.1.1.41), which is proposed to mediate the nucleophilic attack by the π electrons of the Δ^{24} double bond on the *S*-methyl group of *S*-adenosyl-L-methionine (SAM)[1] as depicted in Scheme I (Lederer, 1977; Arigoni, 1978). The resulting C-25 carbocation intermediate, A, undergoes a 24,25 hydride shift, generating a C-24 carbocation, B. Subsequent abstraction of a proton from the methyl group yields the $\Delta^{24(28)}$ product.

Support for this mechanism is derived from the observation that a series of sterol analogues in which C-25 is replaced by a positively charged functional group, such as a protonated tertiary amine, are potent inhibitors of the methyltransferase (Rahier et al., 1984; Oehlschlager et al., 1984). These compounds are isoelectronic with the postulated C-25 carbocation A (Scheme I) and are therefore suggested to be reaction intermediate analogues, which bind avidly to enzymes by exploiting the specific interactions involved in the binding of transition states and intermediates which are not present in the ground state (Wolfenden, 1972). Mimics of C-24 carbocation B (Scheme I) have been examined in less detail but are also highly effective inhibitors of the methyltransferase (Rahier et al., 1984; Oehlschlager et al., 1984). The mechanism of inhibition of the methyltransferase by these sterols is poorly defined, however, primarily because the enzyme has not been purified and is incompletely characterized.

The in vivo inhibition of the *Saccharomyces cerevisiae* Δ^{24}-sterol methyltransferase leads to a decrease in ergosterol synthesis, an accumulation of zymosterol (the substrate of the methyltransferase), and inhibition of growth (Oehlschlager et al., 1984), clearly demonstrating that the enzyme plays a vital role in the organism's metabolism. Encouraged by these observations, we have undertaken the rational design of inhibitors of the Δ^{24}-sterol methyltransferase from the opportunistic pathogens *Candida albicans* and *Candida tropicalis* as part of our effort to develop novel, effective antifungal therapy. In this paper we report the solubilization and characterization of the *Candida* methyltransferase and the elucidation of its kinetic mechanism. The mechanism of inhibition of the enzyme by sterol analogues **3**–**8** (Chart I) is presented, and the time-dependent inactivation of the methyltransferase by 24-thiasterols **3** and **4** is described.

Scheme I



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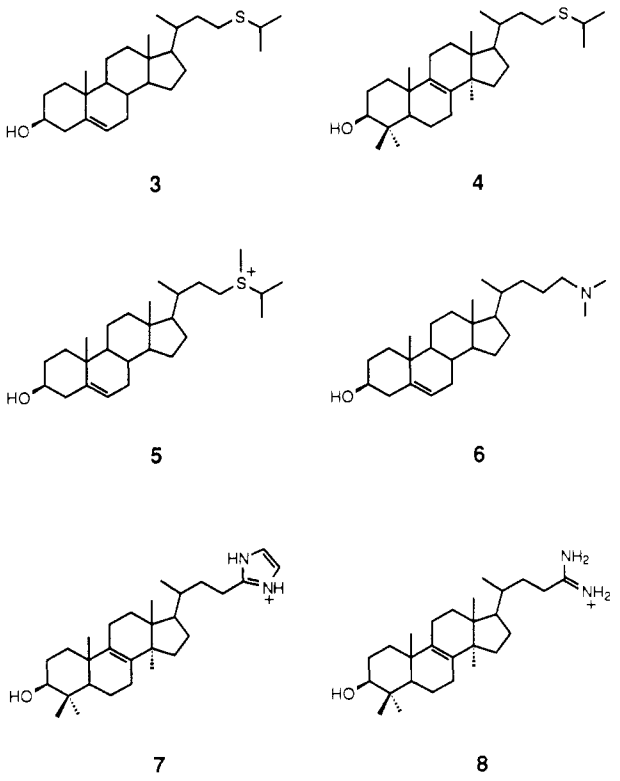
[1] Abbreviations and sterol common names: SAM, *S*-adenosyl-L-methionine; SAH, *S*-adenosyl-L-homocysteine; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; lanosterol, 4,4,14-trimethyl-5α-cholesta-8,24-dien-3β-ol; zymosterol, 5α-cholesta-8,24-dien-3β-ol; desmosterol, cholesta-5,24-dien-3β-ol; fecosterol, 5α-cholesta-8,24(28)-dien-3β-ol.

0006-2960/89/0428-9633$01.50/0

Chart I



Experimental Procedures

Materials

Desmosterol, cholesterol, *S*-adenosyl-L-methionine (*p*-toluenesulfonate salt), *S*-adenosyl-L-homocysteine, octyl glucoside, sodium taurodeoxycholate, Triton WR-1339, dithiothreitol, pepstatin A, and phenylmethanesulfonyl fluoride were purchased from Sigma. [*methyl*-^{14}C]-*S*-Adenosyl-L-methionine (58.6 mCi/mmol) and [7-^{3}H(N)]cholesterol (11.4 Ci/mmol) were obtained from New England Nuclear. Ultrogel AcA 34 was from IBF Biotechnics, Inc. Bio-Beads SM-2 (20–50 mesh) were obtained from Bio-Rad and washed by the procedure of Holloway (1973) prior to use. Protein concentrations were estimated by use of the Pierce bicinchoninic acid reagent with bovine serum albumin as a standard (Smith et al., 1985). The Δ^{24}-sterols zymosterol, fecosterol, 5α-cholesta-8,14,24-trien-3β-ol (**1**), 5α-cholesta-8(14),24-dien-3β-ol (**2**), and 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol were prepared as described by Dolle et al. (1989). 25-Azacholesterol (**6**) was prepared by the method of Counsell et al. (1965) and had a melting point of 150–151.5 °C (lit. mp 147.5–149.5 °C). The synthesis of novel sterol analogues **3**–**5**, **7**, and **8** will be described separately.[2] Radioactivity was determined with a Beckman LS-3801 or LS-5801 scintillation counter which was calibrated for correction to decompositions per minute (dpm).

Aqueous sterol solutions were prepared as suspensions with the nonionic detergent Triton WR-1339 (tyloxapol) (Paik et al., 1984). Sterols were dissolved in acetone and added to sufficient Triton WR-1339 in acetone to give a detergent to sterol ratio of 75:1 (w/w). The solvent was evaporated and the residue dissolved in 0.1 M Tris-HCl (pH 7.5). The resulting solutions were clear and stable to storage at −70 °C. For assays of solubilized methyltransferase, desmosterol solutions which contained 25:1 tyloxapol/sterol were employed.

Methods

Fermentation of C. albicans and C. tropicalis. The growth medium used for *C. albicans* was CYGF, which contained the following (per liter): casamino acids (Difco Laboratories, Detroit, MI), 5.0 g; yeast extract (Difco), 5.0 g; glucose, 5.0 g; $FeSO_4 \cdot 7H_2O$, 0.15 g. The pH was adjusted to 7.0 prior to sterilization at 121 °C, 15 psig for 15–20 min. Stock cultures of *C. albicans* strain ATCC 28367 were stored by resuspending exponential-phase cells in 20% skim milk in 10% glycerol, freezing at a controlled rate, and storing under liquid nitrogen. Shake flasks of CYGF for use as seed cultures were inoculated from thawed stock cultures and incubated at 37 °C with shaking at 150 rpm. The shake-flask culture was used to inoculate 10 L of CYGF in a 15-L stirred tank fermentor (LSL Biolafitte Inc., Princeton, NJ) to an OD_{650} of 1.0. A direct digital control system (D/3 System, Texas Instruments, Hunt Valley, MD) was used to maintain the temperature, aeration rate, pH, and back pressure of the system at 37 °C, 5 slpm, 7.0, and 7 psig, respectively. The agitation rate was initially set at 300 rpm and was automatically increased as necessary to maintain the dissolved oxygen concentration at 20%. When the culture reached an OD_{650} of 10, it was chilled below 15 °C and concentrated 10-fold by hollow-fiber filtration (Model DC10L, Amicon, Inc., Danvers, MA). The cells were then pelleted by centrifugation and stored at −70 °C until use.

The medium used for growth of *C. tropicalis* was H1 (Hug et al., 1974) which was adjusted to pH 5.0 prior to sterilization. Stock cultures of exponential-phase cells of *C. tropicalis* strain ATCC 32113 were prepared in 0.9% NaCl in 25% glycerol, frozen, and stored in liquid nitrogen. The organism was fermented as described above, except the temperature was 28 °C and the pH was maintained at 5.0. When the culture reached an OD_{650} of 20–30, the cells were harvested as described above.

Preparation of Candida Δ^{24}-Sterol Methyltransferase. All enzyme preparation procedures were performed at 4 °C unless otherwise noted. Frozen *C. albicans* or *C. tropicalis* cells were thawed, suspended in two volumes of 0.1 M potassium phosphate (pH 7.4)/1 mM EDTA, and centrifuged at 5000*g* for 5 min. The washed cells were resuspended in 2.5 volumes of 0.1 M potassium phosphate (pH 7.4), 1 mM EDTA, 1 mM DTT, 0.25 mM PMSF, and 1 μM pepstatin A and broken by four passes through a Microfluidizer Model 110T (Microfluidics, Inc., Newton, MA) at an inlet air pressure of 50 psi. Cell debris was removed by centrifugation at 11000*g* for 20 min, and the supernatant was then centrifuged at 100000*g* for 1 h. The resulting microsomal pellet was resuspended in 0.1 M potassium phosphate (pH 7.4), 1 mM EDTA, and 20% glycerol (PEG buffer) at a protein concentration of 10–15 mg/mL, divided into aliquots, and stored at −70 °C. No loss of activity was detectable after 6 months of storage.

Solubilization of 24-SMT was achieved by dropwise addition of a 4% (w/v) solution of 3:1 octyl glucoside/sodium taurodeoxycholate in PEG buffer to a stirred microsomal suspension, yielding a final detergent to protein ratio of 2:1. The mixture was gently stirred for an additional 30 min and was then centrifuged at 100000*g* for 1 h. The pellet was discarded, and the solubilized microsomal protein was stored at −70 °C until use. Greater than 90% of the methyltransferase activity was retained after 1 month of storage.

The molecular weight of the solubilzed *C. albicans* 24-SMT was estimated by gel filtration chromatography on a 1.5 × 50 cm column of Ultrogel AcA 34 equilibrated with 50 mM

[2] S. J. Schmidt, J. L. Adams, R. E. Dolle, L. I. Kruse, and M. A. Ator, unpublished experiments. The spectroscopic and analytical data obtained for these compounds support the reported structures.

Tris-HCl (pH 8.0), 20% glycerol, 2 mM $MgCl_2$, 0.5 mM EDTA, 0.2% octyl glucoside, and 0.05% sodium taurodeoxycholate. Solubilized protein (8 mg) was applied to the column in a volume of 1 mL and eluted at a flow rate of 6 mL/h. The eluent was monitored at 280 nm, and fractions of 0.3 mL were collected and assayed for methyltransferase activity as described below. The column was calibrated with Bio-Rad gel filtration standards dissolved in the column buffer. The presence of detergent in the buffer had no effect on the elution volume of the standard proteins.

Assay of Δ^{24}-Sterol Methyltransferase. Enzyme activity was determined by a modification of published procedures (Thompson et al., 1974; Oehlschlager et al., 1984). A standard assay contained 0.1 M Tris-HCl (pH 7.5), 30 mM $KHCO_3$, 5 mM $MgCl_2$, 200 μM desmosterol, and 50 μM [*methyl*-^{14}C]SAM (3×10^6 dpm/μmol) in a final volume of 1.0 mL in a vial fitted with a Teflon-covered septum. Microsomal protein (0.25 mg) was added to initiate the reaction, which was maintained at 30 °C in a shaking water bath for 45 min. Assays were terminated by the addition of 1 mL of 20% ethanolic KOH and 6 nmol of [7-^{3}H(N)]cholesterol (2.5×10^6 dpm/μmol) in ethanol, which was included as an internal standard to allow quantitation of the efficiency of sterol extraction. The samples were heated to reflux under Ar for 15 min, cooled, and extracted twice with 2 mL of hexanes. The pooled organic layers were washed twice with 1 mL of water, dried over anhydrous Na_2SO_4, and filtered into scintillation vials. The solvent was evaporated, the residue was dissolved in 5 mL of Aquasol-2 (New England Nuclear) or Ready Safe (Beckman), and the amount of radioactivity present was quantitated by double-label scintillation counting. The recovery of the [^{3}H]cholesterol internal standard (typically 80 ± 5%) was calculated for each sample and was used to normalize the ^{14}C-labeled product dpm to 100% efficiency of extraction. With this procedure, the variation between duplicate samples was less than 5%.

Reversible Inhibition of Δ^{24}-Sterol Methyltransferase. Inhibition experiments in which desmosterol was the variable substrate were performed with 75–300 μM desmosterol at a constant SAM concentration of 50 μM. Triton WR-1339 was added as required to maintain equivalent detergent concentrations in all assays. Inhibitors were added at concentrations of approximately 0, 1, 2, and 3 times the apparent K_i as estimated by the method of Dixon (1953), assuming competitive inhibition versus sterol. When SAM was the variable substrate, its concentration ranged from 3 to 20 μM, while desmosterol was fixed at 200 μM. Triton WR-1339 was added to provide a detergent concentration equal to that present at 300 μM desmosterol.

Control experiments were performed to ensure that the methyltransferase reaction was linear with time in the presence of the highest concentrations of **3** and **4** examined in inhibition experiments. This eliminates the possibility that the results of inhibition experiments were influenced by time-dependent inactivation of the enzyme during the course of the reaction.

Time-Dependent Inactivation of Δ^{24}-Sterol Methyltransferase. A typical preincubation contained 0.1 M Tris-HCl (pH 7.5), 30 mM $KHCO_3$, 5 mM $MgCl_2$, inactivator, and 1.5 mg of solubilized *C. albicans* microsomal protein in a volume of 0.5 mL. The inactivation reaction was initiated by the addition of SAM to give a final concentration of 100 μM. At indicated times, 50-μL aliquots of the preincubation reaction were diluted into a standard assay mixture to yield a final volume of 1.0 mL. The amount of methyltransferase activity remaining was determined as described above.

Irreversibility of Inactivation of Δ^{24}-Sterol Methyltransferase by **3**. Two 1.5-mL preincubation mixtures were prepared as described above; one contained 2 μM **3** while the second lacked inactivator. SAM was added to each, and the reaction was allowed to proceed until 90% of the methyltransferase activity was lost. A 1-mL aliquot of each sample was chromatographed on a 1 × 25 cm column of Sephadex G-25 equilibrated in 0.1 M potassium phosphate (pH 7.5), 20% glycerol, 0.2% octyl glucoside, and 0.05% sodium taurodeoxycholate, and fractions of 0.7 mL were collected. The protein-containing fractions were pooled and dialyzed at 4 °C versus three 0.5-L changes of the same buffer which contained 5 g of washed Bio-Beads. Samples were taken after 0, 3, 6, and 22 h of dialysis for quantitation of methyltransferase activity and protein concentration. Enzyme from the control sample retained 83% of its activity after 6 h of dialysis and 51% after 22 h.

Data Analysis. Kinetic data from initial velocity, product inhibition, and dead-end inhibition experiments were analyzed with the FORTRAN programs of Cleland (1979). Data from experiments in which a single substrate was varied were fitted to eq 1 by the HYPERL program. The results of initial velocity experiments in which both substrates were varied were analyzed with eq 2 and the SEQUEN program. Data from inhibition experiments were fitted to linear competitive (COMP), noncompetitive (NONCOMP), and uncompetitive (UNCOMP) models (eq 3–5, respectively). The suggested statistical criteria of Cleland (1979) were used to determine which model provided the best fit to the data. If none of the linear inhibition models provided a satisfactory fit, data for each inhibitor concentration were analyzed separately with HYPERL. Slope and intercept replots were constructed and fitted to a straight line ($y = mx + b$) with LINE or to a parabolic function ($y = a + bx + cx^2$) with PARA. When parabolic replots were obtained, the entire data set was fit to eq 6 and 7, which describe parabolic competitive and noncompetitive inhibition, respectively (Segal, 1975). In these models, the binding of the first inhibitor molecule changes the intrinsic dissociation constant of the second binding site by the factor α. Data were fitted to eq 6 and 7 with Superfit, which is a numerical analysis system developed by Smith Kline & French Laboratories, using the SAS software package from SAS Institute, Inc. The method for solving the nonlinear equations is based upon the Marquardt algorithm (Marquardt, 1963). The results of double inhibition experiments were fitted to eq 8 (Yonetani & Theorell, 1964; Northrop & Cleland, 1974), where v_o and v_i are velocities observed in the absence and presence of two inhibitors, I and J, which have dissociation constants K_i and K_j. The degree of cooperativity in binding of I and J is described by the β term. Time-dependent inactivation data for each concentration of substrate analogue were expressed as

$$\log v = \log [V_m A/(A + K_a)] \quad (1)$$

$$v = V_m A/(K_{ia}K_b + K_a B + K_b A + AB) \quad (2)$$

$$v = V_m A/[K_a(1 + I/K_{is}) + A] \quad (3)$$

$$v = V_m A/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (4)$$

$$v = V_m A/[K_a + A(1 + I/K_{ii})] \quad (5)$$

$$v = V_m A/[K_m(1 + I/K_i + I^2/\alpha K_i^2) + A] \quad (6)$$

$$v = V_m A/[K_m(1 + I/K_{is} + I^2/\alpha K_{is}^2) + S(1 + I/K_{ii} + I^2/\alpha K_{ii}^2)] \quad (7)$$

$$v_i = v_o/[1 + I/K_i + J/K_j + IJ/(\beta K_i K_j)] \quad (8)$$

Table I: Substrate Specificity of *Candida* Δ^{24}-Sterol Methyltransferases[a]

substrate	*C. albicans*			*C. tropicalis*		
	$K_{m,app}$ (μM)	V (nmol h^{-1} mg^{-1})	V/K (nmol h^{-1} mg^{-1} μM^{-1})	$K_{m,app}$ (μM)	V (nmol h^{-1} mg^{-1})	V/K (nmol h^{-1} mg^{-1} μM^{-1})
zymosterol	71 ± 7	30 ± 2	0.424 ± 0.018	52 ± 3	43 ± 1	0.837 ± 0.027
desmosterol	281 ± 26	32 ± 2	0.113 ± 0.004	236 ± 22	36 ± 2	0.152 ± 0.006
1	48 ± 3	4.5 ± 0.1	0.093 ± 0.003	45 ± 6	5.6 ± 0.3	0.124 ± 0.006
2[b]	550 ± 70	11 ± 1	0.020 ± 0.001	650 ± 100	15 ± 2	0.023 ● 0.001

[a] Sterol (20–300 μM) was incubated with 50 μM [*methyl*-^{14}C]SAM and 0.25 mg of the indicated microsomal enzyme for 45 min at 30 °C, and product formation was quantitated as described under Experimental Procedures. The initial velocities were plotted in double-reciprocal form with the HYPERL program, as described in the text. [b] The K_m values obtained for this compound are the result of extrapolation from the concentration range which was examined and should be taken as estimates.

v_i/v_o values, fit to eq 9, and plotted in semilog form. Replots of $t_{1/2}$ versus $1/I$ were constructed to obtain the kinetic constants for the inactivation reaction, K_I and k_{inact} (Kitz & Wilson, 1962; Jung & Metcalf, 1975).

$$v_i/v_o = e^{-kt} \quad (9)$$

Results and Discussion

Solubilization of C. albicans Δ^{24}-Sterol Methyltransferase. The fungal Δ^{24}-sterol methyltransferase has been incompletely characterized, largely due to its microsomal nature. An early partial purification of the *S. cerevisiae* enzyme released the enzyme from the membrane by preparation of an acetone powder (Moore & Gaylor, 1969). This methyltransferase preparation allowed determination of the enzyme's substrate specificity and confirmation of the structure of the sterol product (Moore & Gaylor, 1969, 1970), but its lack of stability to storage limits it usefulness. Substantial advances have been made in the detergent solubilization of membrane-bound proteins since that time, justifying an attempt to purify the enzyme on the basis of that technology.

The *C. albicans* Δ^{24}-sterol methyltransferase was solubilized from the microsomal membrane under the conditions employed by Gaylor and co-workers to solubilize several enzymes of the cholesterol biosynthetic pathway (Grinstead & Gaylor, 1982; Paik et al., 1984, 1986). Addition of a 3:1 mixture of octyl glucoside and sodium taurodeoxycholate to microsomes at a protein to detergent ratio of 2:1 released an average of 75% of the total protein and 35% of the methyltransferase activity, resulting in an average specific activity 45% that of the membrane-bound enzyme. The yields of solubilized protein and methyltransferase activity were not enhanced by a number of modifications of the standard protocol, such as the inclusion of 0.15 M KCl or the protease inhibitors PMSF and pepstatin A, an increase in the detergent to protein ratio to 3:1, or utilization of different buffer mixtures. Substantially lower yields of enzyme activity were solubilized from *C. tropicalis* under all conditions examined. *C. albicans* was therefore routinely used as the source of solubilized methyltransferase.

The characteristics of the solubilized methyltransferase were virtually identical with those of its microsomal progenitor, indicating that the enzyme was not significantly modified by release from the membrane. Product formation was directly proportional to enzyme concentration in the range of 0.05–0.5 mg/assay, and the time course was linear for 60 min at 30 °C for both enzymes. The kinetic constants of the solubilized methyltransferase are in good agreement with those of the microsomal enzyme and will be discussed subsequently. The solubilized enzyme differs in its increased sensitivity to Triton WR-1339, the detergent used to prepare sterol suspensions. The detergent concentration in assays of solubilized methyltransferase was therefore minimized by use of sterol substrates prepared as 25:1 (w/w) detergent/sterol mixtures, in contrast to the 75:1 mixture used for the microsomal enzyme.

A number of approaches to the purification of the methyltransferase were pursued but were thwarted by the instability of the solubilized enzyme at 4 °C. Approximately 60% of the enzyme's activity remained after 1 day at 4 °C, and only 35% after 2 days. The addition of dithiothreitol, reduced glutathione, phosphatidylcholine, $MgCl_2$, or $CaCl_2$ failed to decrease the rate of loss of activity. The enzyme was, however, sufficiently stable to allow estimation of its molecular weight by gel filtration chromatography. Methyltransferase activity eluted from an Ultrogel AcA 34 column with an apparent molecular weight of approximately 150 000. While enzymes of this size are rarely single polypeptides, the quaternary structure of the methyltransferase remains unknown.

Substrate Specificity of Δ^{24}-Sterol Methyltransferase. The Δ^{24}-sterol methyltransferase of *S. cerevisiae* has been demonstrated to preferentially methenylate zymosterol, although desmosterol was also found to be an adequate substrate (Moore & Gaylor, 1970). Activity is dramatically diminished by the presence of methyl groups at C-4, and the 4,4,14-trimethyl sterol lanosterol is not metabolized. A similar specificity has been proposed for the methyltransferases of *C. albicans* and *Candida utilis* on the basis of sterol composition of these organisms (Fryberg et al., 1975). In contrast to *S. cerevisiae*, however, small amounts of 24-methylenelanosterol were isolated from the *Candida* species, suggesting that the *Candida* methyltransferase must also be able to utilize lanosterol as a substrate.

The in vitro substrate specificity of the microsomal *C. albicans* and *C. tropicalis* Δ^{24}-sterol methyltransferases was examined with a series of naturally occurring and synthetic Δ^{24}-sterols. The substrate profiles for the two enzymes are indistinguishable, as shown in Table I, and correlate well with the specificity of the *S. cerevisiae* congener. The data demonstrate that the methyltransferase is exquisitely sensitive to the location and number of double bonds in the sterol nucleus. The $\Delta^{8,24}$-sterol, zymosterol, is the preferred substrate and displays optimal values for both K_m and V_m. Desmosterol, which contains a Δ^5 nuclear double bond instead of Δ^8, has a significantly elevated K_m but a V_m comparable to that of zymosterol. Introduction of an additional double bond into the sterol D ring to yield 5α-cholesta-8,14,24-trien-3β-ol (**1**) decreases V_m without altering K_m. The $\Delta^{8(14)}$-sterol, 5α-cholesta-8(14),24-dien-3β-ol (**2**), binds very poorly but clearly is a substrate. While trends are difficult to discern from such a limited series of compounds, it appears that the hybridization states of C-9 and C-14 have substantial influence on the values of K_m and V_m, respectively. When C-9 is changed from sp^2 to sp^3, the conformations of the B and C rings of the molecule are altered, and an increase in the apparent K_m values results. Decreases in V_m are observed when C-14 is sp^2 as opposed to sp^3. This structural modification would change the conformation of the D ring and likely affect the position of the sterol side chain.

Table II: Initial Velocity Patterns for Δ^{24}-Sterol Methyltransferase[a]

enzyme	sterol	sterol K_m (μM)	sterol K_i (μM)	SAM K_m (μM)	SAM K_i (μM)
microsomal *C. tropicalis*	desmosterol	286 ± 37	449 ± 96	5.6 ± 1.2	8.9 ± 1.0
microsomal *C. tropicalis*	zymosterol	57 ± 8	22 ± 5	11.7 ± 1.3	4.5 ± 1.1
microsomal *C. albicans*	desmosterol	201 ± 20	489 ± 106	7.9 ± 1.6	19.3 ± 2.0
solubilized *C. albicans*	desmosterol	27 ± 5	170 ± 21	16.4 ± 1.8	100 ± 21

[a] Initial velocity data were fit to the SEQUEN program as described in the text.

Table III: Dead-End Inhibition of Δ^{24}-Sterol Methyltransferase

inhibitor	variable substrate	inhibition pattern[a]	apparent inhibition constant (nM) K_{is}	apparent inhibition constant (nM) K_{ii}	α[b]
3	desmosterol	C	1 100 ± 100		
	SAM	UC		1 700 ± 100	
4	desmosterol	C	198 000 ± 12 000		
	SAM	UC		192 000 ± 10 000	
5	desmosterol	PC	34 ± 9		0.02 ± 0.01
	SAM	PNC	52 ± 30	81 ± 47	0.01 ± 0.01
6	desmosterol	PC	63 ± 22		0.01 ± 0.01
	SAM	PNC	42 ± 16	66 ± 24	0.03 ± 0.03
7	desmosterol	NC	20 ± 3	26 ± 8	
	SAM	NC	17 ± 2	15 ± 1	
8	desmosterol	NC	40 ± 5	83 ± 24	
	SAM	NC	39 ± 7	35 ± 4	

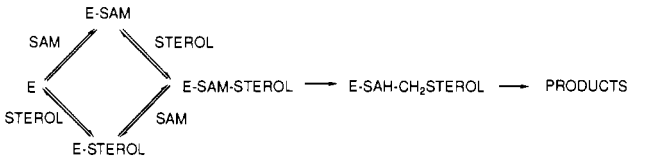
[a] Data were fit to the following models according to the procedures described under Experimental Procedures: C, linear competitive; UC, linear uncompetitive; NC, linear noncompetitive; PC, parabolic competitive; PNC, parabolic noncompetitive. [b] α is a factor which describes the cooperativity of binding of two identical inhibitor molecules, as described in the text.

No activity is observed with lanosterol or 4,4-dimethyl-5α-cholesta-8,14,24-trien-3β-ol, demonstrating the deleterious effect of methyl groups at C-4 or C-14 on the ability of a sterol to serve as a methyltransferase substrate. The presence of lanosterol at concentrations up to 300 μM has no effect on the rate of desmosterol methenylation, indicating that the methylated sterol does not bind to the enzyme.

Kinetic Mechanism of Δ^{24}-Sterol Methyltransferase. Initial velocity and product inhibition experiments were performed to define the kinetic order of substrate binding to the methyltransferase. Initial velocity data for Δ^{24}-sterol methyltransferase were obtained under several sets of conditions, as described in Table II. In all cases, a sequential mechanism provided the best fit to the data. The kinetic constants obtained for the microsomal enzymes (Table II) were in accord with the apparent K_m values described above, while the K_m of desmosterol for the solubilized *C. albicans* methyltransferase is nearly an order of magnitude lower. At least part of this effect is due to the decreased detergent concentrations employed in solubilized enzyme reactions, since the K_m for sterol also decreases with decreasing detergent in initial velocity patterns conducted with the microsomal enzyme (data not shown).

Fecosterol and *S*-adenosyl-L-homocysteine (SAH) were examined as product inhibitors of the microsomal *C. tropicalis* Δ^{24}-sterol methyltransferase in order to differentiate between random and ordered sequential mechanisms. Fecosterol did not inhibit the enzyme at concentrations of 20–300 μM. Competitive inhibition by SAH versus both desmosterol and SAM was observed with K_{is} values of 14.0 ± 0.6 and 4.3 ± 0.2 μM, respectively. This result uniquely defines a rapid equilibrium random kinetic mechanism (Scheme II) in which the E·sterol·SAH ternary complex does not form (Rudolph, 1979). Since fecosterol does not inhibit the enzyme, no conclusions can be drawn regarding the existence of an enzyme·SAM·fecosterol ternary complex. While it is unusual for a random system not to form a dead-end complex containing the substrate and product which lack the transferred group (Segal, 1975), this behavior has been reported previously

Scheme II



(Krenitsky, 1976). Due to the presumedly irreversible nature of the methyltransferase reaction, no attempt was made to discern the order of product release.

Dead-End Inhibition of Δ^{24}-Sterol Methyltransferase by Sterol Analogues. Previous studies of the inhibition of sterol methyltransferases have focused on the synthesis of appropriate analogues of the carbocationic intermediates A and B which are proposed to occur in the normal catalytic process, as depicted in Scheme I. Compounds which are isoelectronic and isosteric with these intermediates would be expected to bind tightly to the enzyme through the same interactions which the enzyme utilizes to bind its intermediates. A variety of functionalities which are positively charged, including ammonium, sulfonium, and arsonium groups, or which require protonation to yield a positive charge, such as secondary or tertiary amines, have been incorporated into the sterol side chain in place of C-24 or C-25 and demonstrated to be effective methyltransferase inhibitors (Oehlschlager et al., 1984; Rahier et al., 1984).

A series of novel sterol analogues which contain heteroatomic substitutions in place of C-24 or C-25 of the side chain have been evaluated as inhibitors of the microsomal *C. tropicalis* Δ^{24}-sterol methyltransferase. The results summarized in Table III provide additional support for the intermediacy of C-24 and C-25 carbocations. 24-Thiasterols **3** and **4** are analogues of the ground state of the methyltransferase reaction and bind to the enzyme with K_i values which approximate the K_m values of sterol substrates. Methylation of the side chain sulfur of **3** to produce 24-(methylsulfonium) analogue **5** yields a gain in inhibitory potency, in accordance with the expected increase in binding affinity of a reaction intermediate analogue over a mimic of the ground state. Because both diastereomers

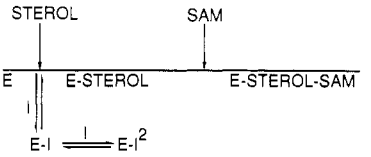
of the methylsulfonium group were present, the inhibition constants for the single diastereomer which correctly reflects the stereochemistry at C-24 of carbocation intermediate B would likely be even lower than those reported in Table III. The known methyltransferase inhibitor 25-azacholesterol (**6**) as well as the novel imidazole (**7**) and amidine (**8**) derivatives of lanosterol also prove to be suitable mimics of the C-25 carbocation, as evidenced by their effective inhibition of the methyltransferase (Table III). It is particularly notable that lanosterol analogues **7** and **8** are powerful inhibitors of the methyltransferase in spite of the inability of the parent sterol to bind to the enzyme. The strong interactions between the charged side chain and the enzyme must overwhelm the negative attributes of the methylated sterol nucleus.

Characterization of the mechanism of combination of inhibitors with the Δ^{24}-sterol methyltransferase has rarely appeared in the literature, and in no case has the result been set within the context of the enzyme's kinetic order. Examination of the kinetic patterns described in Table III for inhibition of the methyltransferase by compounds **3**–**8** reveals a surprisingly diverse and complex set of interactions, as evidenced by three distinct mechanisms of inhibition. 24-Thiasterols **3** and **4** display the most straightforward mode of combination with the enzyme. These compounds are competitive inhibitors versus desmosterol and uncompetitive inhibitors versus SAM, consistent with the operation of a single branch of the random mechanism: binding of the sterol analogue to the enzyme·SAM binary complex to yield a dead-end ternate (Fromm, 1979).

A second class of inhibitors is comprised of compounds **5** and **6**. Inhibition patterns for compounds similar to 25-azacholesterol (**6**) have been determined previously for related sterol methyltransferases. Oehlschlager et al. (1984) reported that inhibition of the *S. cerevisiae* methyltransferase by 24-aza-24,25-dihydrozymosterol is competitive versus SAM and uncompetitive versus zymosterol, while Rahier et al. (1984) presented noncompetitive kinetic patterns versus both substrates for inhibition of the cycloartenol methyltransferase from maize by 25-azacycloartenol. Data for the inhibition of the *C. tropicalis* methyltransferase by sterol analogues **5** and **6** did not conform to linear competitive, noncompetitive, or uncompetitive kinetic models. Parabolic slope replots were obtained when desmosterol was varied, while both slope and intercept replots were parabolic when SAM was varied. Such results are indicative of a combination of a second inhibitor molecule with the E·I complex (Cleland, 1970). Attempts to fit the data to an equation which describes parabolic inhibition with equal dissociation constants for each of the two inhibitor molecules were also unsatisfactory. However, a kinetic model containing the term α which describes the effect of binding of the first inhibitor molecule on the dissociation constant of the second binding site provided an excellent fit to the data. The magnitude of α is very small for inhibition by **5** and **6** (Table III), demonstrating that the binding of the two inhibitor molecules is highly cooperative.

Sterols **5** and **6** were parabolic competitive inhibitors versus desmosterol and parabolic noncompetitive inhibitors versus SAM, which is typical of inhibitor binding in a random mechanism but is also consistent with association of the compound exclusively with free enzyme in an ordered process (Fromm, 1979). One reasonable interpretation of these results is prompted by the observation that the sulfonium group of **5** and the protonated tertiary amine of **6** not only resemble carbocation intermediates A and B (Scheme I) but are also similar to the methylsulfonium group of SAM. The observed

Scheme III



inhibition patterns may result from combination of the substrate analogue with free enzyme in the sterol binding site, followed by binding of a second inhibitor molecule in the SAM site to yield an E·I^2 complex (Scheme III).

The third class of inhibitor behavior is exemplified by cationic lanosterol analogues **7** and **8** (Table III). Noncompetitive inhibition was observed when either desmosterol or SAM was the variable substrate. Binding of the sterol analogue to an allosteric site would provide a trivial explanation for these results. These patterns could also arise from the binding of the inhibitors to two different enzyme forms. For example, if the inhibitor bound competitively with desmosterol, yielding a slope effect, and also bound to an enzyme·SAH binary complex, producing an intercept effect, the two results would combine additively to give a noncompetitive pattern versus sterol (Cleland, 1970).

The hypothesis that **7** and **8** bind to the enzyme·SAH complex was tested in a series of double-inhibition experiments. This technique involves the variation of two inhibitors, I and J, at fixed, subsaturating substrate concentrations. The factor β which describes the degree of cooperativity in the binding of the two inhibitors is determined (Yonetani & Theorell, 1964; Northrop & Cleland, 1974). Values of β less than 1 indicate the synergistic binding of I and J, while values between 1 and ∞ are obtained when there is negative cooperativity in the binding of the inhibitors. Mutually exclusive binding is represented by an infinite value of β. The double inhibition of Δ^{24}-sterol methyltransferase by SAH and **7** is characterized by a β value of 0.13, consistent with the cooperative binding of the two compounds. Sterol inhibitor **8** and SAH also bind synergistically with a β value of 0.28. It is interesting that these positively charged inhibitors will form a ternary complex with SAH, while uncharged substrates will not. The presence of a positive charge, supplied either by SAM or a cationic sterol, is apparently required for formation of a ternary complex.

As a control experiment, a double-inhibition experiment was performed with SAH and **3**. Since **3** is a competitive inhibitor versus desmosterol, it must bind to the enzyme·SAH complex with a low affinity, if at all, and it would not be expected to bind synergistically with SAH. A series of parallel lines was obtained, consistent with a β value of ∞, indicating that the binding of SAH and **3** is mutually exclusive.

While these results demonstrate that **7** and **8** can bind to the enzyme·SAH complex, it is not clear that this binary complex exists under initial velocity conditions. The maximum amount of SAH formed in uninhibited reactions is approximately 2.5 μM, which is still below its K_i concentration, so a limited amount of enzyme·SAH complex would be expected to result from reassociation of SAH with the methyltransferase. If binding of inhibitor to an enzyme·SAH complex is the correct explanation for the observed kinetic patterns, it is likely that SAH is the second product released from the enzyme and that the binary complex is trapped by inhibitor prior to dissociation of the product.

Time-Dependent Inactivation of Δ^{24}-Sterol Methyltransferase by 24-Thiasterols. 24-Thiasterols **3** and **4** display duplicitous behavior toward the Δ^{24}-sterol methyltransferase:

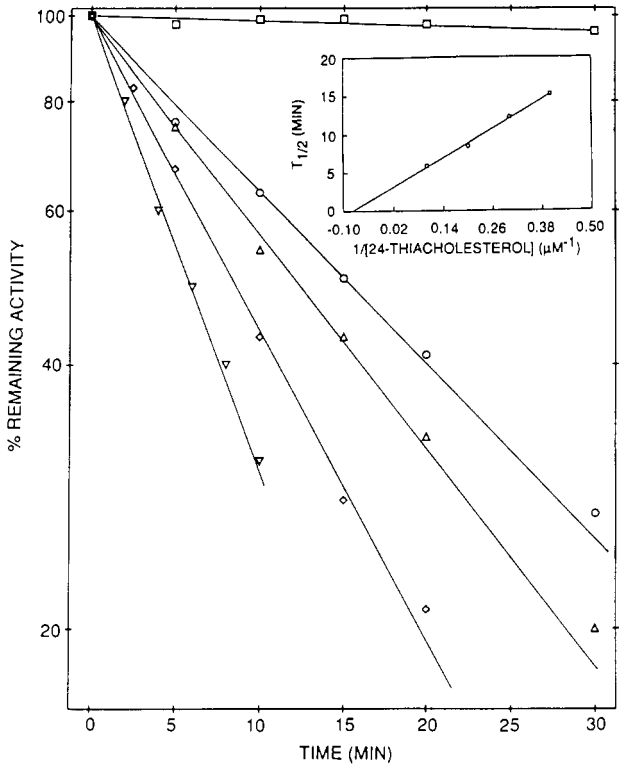
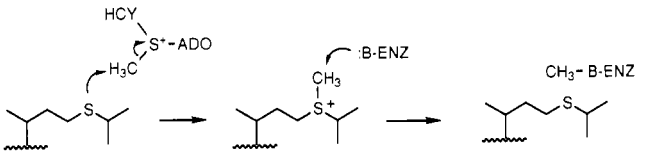


FIGURE 1: Time-dependent inactivation of Δ^{24}-sterol methyltransferase by **3**. The methyltransferase was preincubated with 0 (□), 2.5 (○), 3.3 (△), 5 (◇), and 10 μM (▽) **3**, and aliquots were withdrawn at the indicated times and assayed for remaining activity. A replot of the half-life of the enzyme at each inhibitor concentration versus the reciprocal inhibitor concentration is shown in the inset.

while low concentrations of the substrate analogue cause reversible inhibition, time-dependent inactivation ensues in a higher concentration range. All inactivation studies were performed with solubilized *C. albicans* methyltransferase, since nonlinear inactivation kinetics were obtained in pilot experiments with the microsomal enzyme. The kinetic constants for the inactivation process were determined from the concentration dependence of the reaction, as shown in Figure 1 (Kitz & Wilson, 1962; Jung & Metcalf, 1975). Pseudo-first-order inactivation kinetics were maintained through at least three half-lives. Inactivation of the methyltransferase by the desmosterol analogue, **3**, occurs with a rate constant for inactivation, k_{inact}, of 0.28 ± 0.05 min^{-1} and a K_I of 13 ± 3 μM. The lanosterol analogue, **4**, inactivates the enzyme with a comparable k_{inact} of 0.13 ± 0.03 min^{-1} but binds much less tightly than the desmosterol derivative, with a K_I of 290 ± 90 μM. The values of K_I for the two analogues reflect the substrate specificity of the methyltransferase, while the values of k_{inact} are quite comparable, consistent with common chemistry in the rate-determining step of the inactivation process independent of differences in the structure of the sterol nucleus.

The inactivation of the methyltransferase by 24-thiasterols meets the criteria for consideration as an example of mechanism-based enzyme inactivation (Silverman, 1988). Loss of activity is a time-dependent, pseudo-first-order process which displays saturation kinetics (Figure 1), consistent with the formation of an E·I complex analogous to a Michaelis complex. The half-life for inactivation by 2 μM **3** was increased from 21 to 40 min in the presence of 300 μM zymosterol, demonstrating association of the thiasterol with the substrate binding site. Methyltransferase which had been inactivated by **3** was chromatographed on Sephadex G-25 and dialyzed for 24 h without measurable recovery of catalytic activity, indicative of an irreversible reaction. The necessity of SAM for the

Scheme IV



inactivation reaction supports the hypothesis that the catalytic action of the enzyme is required to methylate the substrate analogue and produce the activated species which causes loss of catalytic competence. If the thiasterol is methylated, it would be the first functional group other than a Δ^{24}-olefin reported to undergo a methyltransferase-catalyzed reaction.

At least two distinct chemical mechanisms can be envisioned for inactivation of the methyltransferase by 24-thiasterols. Enzyme-catalyzed methylation of the side-chain sulfur of **3** at the expense of SAM would yield 24-methyl-24-thia derivative **5**, which has been established as a potent reversible inhibitor of the methyltransferase. Formation of this compound during the catalytic cycle might lock the enzyme into a conformation corresponding to that which binds intermediate A in the normal catalytic reaction (Scheme I). The rate of dissociation of the intermediate analogue from the active site might be very slow, resulting in a functionally irreversible inactivation of the enzyme. The suppression of growth of *Crithidia fasciculata* by 10-thiasteric acid is proposed to result from the inhibition of the mechanistically related cyclopropane synthetase by a similar process (Pascal et al., 1986).

The second mechanistic alternative also requires conversion of the 24-thiasterol to the methylsulfonium analogue, which is then proposed to undergo further reaction with the enzyme (Scheme IV). In the mechanistic hypothesis for the normal catalytic reaction, an active site base is suggested to abstract a proton from carbocation B to yield product (Scheme I). Rather than serving as a base, this residue might be poised to act as a nucleophile toward the methylsulfonium moiety, which should be comparable to SAM in its strength as a methyl donor. Inactivation of the enzyme would therefore result from covalent methylation of the active site residue. While the methyl group of the sulfonium salt would presumably be oriented properly to react with the hypothetical active site base, transfer of either of the other two alkyl groups might also occur with the same outcome. Peptidyl sulfonium salts have been designed as affinity labels of cysteine proteases on the basis of the reactivity of trialkylsulfonium groups as alkylating agents, although their efficacy appears to be derived from transfer of the peptidyl group to the enzyme rather than a methyl group (Shaw, 1988).

Both mechanistic hypotheses invoke the intermediacy of a 24-methyl-24-thiasterol, so inactivation of the methyltransferase by that compound would therefore support the veracity of these proposals. However, no inactivation is observed on preincubation of **5** with the methyltransferase in the presence of either SAM or SAH. A number of reasonable explanations for this result can be envisioned. The $E \cdot I^2$ complex resulting from the addition of **5** to the methyltransferase is, in fact, probably not the same complex which is obtained from catalytic methylation of the thiasterol. It is also possible that the enzyme conformation which is required for covalent modification or slowly dissociable binding of the reaction intermediate is achieved only during the catalytic process and cannot be attained on addition of exogenous **5**. Alternatively, the inability of **5** to inactivate the methyltransferase may simply be due to the narrow range of concentrations which can be explored. The potent reversible inhibition by **5** limits the concentrations which can be employed in preincubations

to 50 nM, so if inactivation occurs in a higher concentration range than inhibition, it would not be observed.

Additional experiments to test these mechanistic proposals can readily be designed. Unfortunately, attempts to demonstrate radiolabeling of the methyltransferase by [*methyl*-^{3}H]SAM in the presence of **3** and to isolate **5** as a product of the inactivation reaction have failed due to the lack of a homogeneous methyltransferase preparation. Purification of the enzyme and further study of the inactivation reaction will provide valuable insight into the nature of the methyltransferase's active site and the means by which it catalyzes its fascinating reaction.

ACKNOWLEDGMENTS

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Registry No. **1**, 64284-65-7; **2**, 117556-83-9; **3**, 123333-28-8; **4**, 123333-29-9; **5** (diastereomer 1), 123333-30-2; **5** (diastereomer 2), 123333-33-5; **6**, 1973-61-1; **7**, 123333-31-3; **8**, 123333-32-4; SAM, 29908-03-0; SAH, 979-92-0; zymosterol, 128-33-6; desmosterol, 313-04-2; Δ^{24}-sterol methyltransferase, 37257-07-1.

REFERENCES

Arigoni, D. (1978) *Ciba Found. Symp. 60*, 243–261.
Cleland, W. W. (1970) *Enzymes (3rd Ed.) 2*, 1–65.
Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–138.
Counsell, R. E., Klimstra, P. D., Nysted, L. N., & Ranney, R. E. (1965) *J. Med. Chem. 8*, 45–48.
Dixon, M. (1953) *Biochem. J. 55*, 170–171.
Dolle, R. E., Schmidt, S. J., Erhard, K. F., & Kruse, L. I. (1989) *J. Am. Chem. Soc. 111*, 278–284.
Fromm, H. J. (1979) *Methods Enzymol. 63*, 467–486.
Fryberg, M., Oehlschlager, A. C., & Unrau, A. M. (1975) *Arch. Biochem. Biophys. 173*, 171–177.
Grinstead, G. F., & Gaylor, J. L. (1982) *J. Biol. Chem. 257*, 13937–13944.
Holloway, P. W. (1973) *Anal. Biochem. 53*, 304–308.
Hug, H., Blanch, H. W., & Fiechter, A. (1974) *Biotechnol. Bioeng. 16*, 965–985.
Jung, M. J., & Metcalf, B. W. (1975) *Biochem. Biophys. Res. Commun. 67*, 301–306.
Kitz, R., & Wilson, I. B. (1962) *J. Biol. Chem. 237*, 3245–3249.
Krenitsky, T. A. (1976) *Biochim. Biophys. Acta 429*, 352–358.
Lederer, E. (1977) in *The Biochemistry of Adenosylmethionine* (Salvatore, F., Borek, E., Zappia, V., Williams-Ashman, H. G., & Schlenk, F., Eds.) pp 89–126, Columbia University Press, New York.
Marquardt, D. W. (1963) *J. Soc. Ind. Appl. Math. 11*, 431.
Mercer, E. I. (1984) *Pestic. Sci. 15*, 133–155.
Moore, J. T., Jr., & Gaylor, J. L. (1969) *J. Biol. Chem. 244*, 6334–6340.
Moore, J. T., Jr., & Gaylor, J. L. (1970) *J. Biol. Chem. 245*, 4684–4688.
Northrop, D. B., & Cleland, W. W. (1974) *J. Biol. Chem. 249*, 2928–2931.
Oehlschlager, A. C., Angus, R. H., Pierce, A. M., Pierce, H. D., Jr., & Srinivasan, R. (1984) *Biochemistry 23*, 3582–3589.
Paik, Y.-K., Trzaskos, J. M., Shafiee, A., & Gaylor, J. L. (1984) *J. Biol. Chem. 259*, 13413–13423.
Paik, Y.-K., Billheimer, J. T., Magolda, R. L., & Gaylor, J. L. (1986) *J. Biol. Chem. 261*, 6470–6477.
Pascal, R. A., Jr., Mannarelli, S. J., & Ziering, D. L. (1986) *J. Biol. Chem. 261*, 12441–12443.
Rahier, A., Genot, J.-C., Schuber, F., Benveniste, P., & Narula, A. S. (1984) *J. Biol. Chem. 259*, 15215–15223.
Rudolph, F. B. (1979) *Methods Enzymol. 63*, 411–436.
Segal, I. H. (1975) *Enzyme Kinetics*, Wiley, New York.
Shaw, E. (1988) *J. Biol. Chem. 263*, 2768–2772.
Silverman, R. B. (1988) *Mechanism-Based Enzyme Inactivation: Chemistry and Enzymology*, CRC Press, Boca Raton, FL.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76–85.
Thompson, E. D., Bailey, R. B., & Parks, L. W. (1974) *Biochim. Biophys. Acta 334*, 116–126.
Wolfenden, R. (1972) *Acc. Chem. Res. 5*, 10–18.
Yonetani, T., & Theorell, H. (1964) *Arch. Biochem. Biophys. 106*, 243–251.