Lipids (2007) 42:15–33
DOI 10.1007/s11745-006-3017-1

ORIGINAL ARTICLE

Sterol Biosynthesis Inhibitors: Potential for Transition State Analogs and Mechanism-Based Inactivators Targeted at Sterol Methyltransferase

Zhihong Song · W. David Nes

Received: 3 November 2006 / Accepted: 15 December 2006 / Published online: 14 February 2007

Abstract Sterol biosynthesis inhibitors (SBIs), discovered in the late 1960s and subsequently used commercially to treat ergosterol-dependent fungal diseases, represent a unique drug class targeted at an enzyme in a biosynthetic pathway. To date, few drugs have been commercialized as enzyme inhibitors; yet, prescription of SBIs has emerged as the gold standard for some cases of non-life-threatening antifungal chemotherapy and in crop protection. SBIs are not designed for their structural resemblance to the sterol molecule; they nonetheless can engender a curative effect by interfering with sterol production and homeostasis in the pathogenic organism. The increased use of SBIs in recent years, particularly the azole antifungals, has resulted in the development of resistance to those drugs, necessitating additional work to further our understanding of antifungal resistance and to explore opportunities to develop new enzyme inhibitors and uncover new enzyme targets that can regulate carbon flux in the post-lanosterol/cycloartenol pathway. This article reports general considerations for enzyme mechanism and active-site probes using inhibitors of the C-methylation reaction, including a potential new class of antifungal/antiparasitic agents of phytosterol synthesis tailored as mechanism-based inactivators. These steroid-based compounds prepared with different sterol side chain functionalities are designed to reversibly or irreversibly impair the sterol methyltransferase, an enzyme expressed in pathogenic microbes and plants but not in the human host. The salient aspects of these and related topics directed toward the enzyme recognition of sterol structure, and the inhibitory properties and catalytic competence of a series of specifically modified substrate analogs that affect sterol methyltransferase action are discussed.

Z. Song · W. D. Nes (✉)
Department of Chemistry and Biochemistry,
Texas Tech University, Lubbock, TX 79409, USA
e-mail: wdavid.nes@ttu.edu

Primarily due to their impact upon plant and human health, the similarities and differences in sterol content between microbes of eukaryotic origin and more advanced systems, the functions of the various sterols and biosynthetic intermediates, as well as the characterization of the enzymatic reactions involved have all received substantial investigation. An important goal of most of these studies is to provide basic information about sterol biochemistry from which drugs, herbicides, etc., can be developed to exploit a fundamental difference in sterol homeostasis (involving the type and amount of cellular sterol) between the pathogenic microbe and its host, whether human or plant. Excellent reviews that cover the topic of sterol biosynthesis inhibitors are available [1–4]; the last time this subject was reviewed in the journal *Lipids* was in 1986 by the Strasbourg group, who made seminal contributions to the field [5].

Ergosterol Homeostsasis

The major sterol of microbes, ergosterol accumulates in the cell at ca. 20–100 fg/cell; this contrasts with the sterols of advanced plant and animal systems, sitosterol and cholesterol respectively, that occur at ca. 3,000 fg/cell [6] (Fig. 1). Ergosterol and sitosterol are regarded as phytosterols (sterols that contain a 24-alkyl

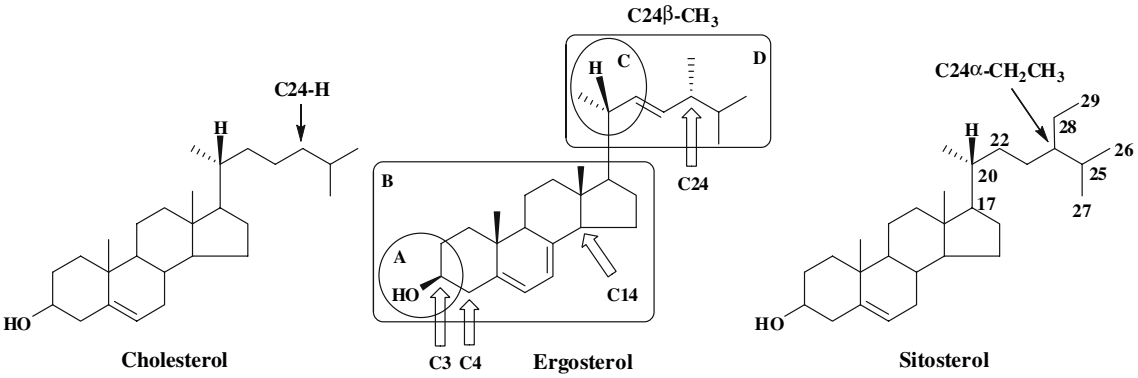


Fig. 1 Representative zoosterol and phytosterols (24-alkyl sterols). The structure of ergosterol is *boxed* to show different domains of the nucleus and a side chain of functional significance; *arrows* indicate structural features of key importance

group in the sterol side chain), whereas cholesterol is regarded as a zoosterol (sterols that lack a 24-alkyl group in the sterol side chain) [6]. The physiological importance of ergosterol to fungal–plant/human interactions provide a biochemical paradigm that ergosterol-dependent diseases can be cured or eradicated though the disruption of ergosterol homeostasis. The key element of this paradigm is that de novo sterol synthesis and the structural features of ergosterol are important to fungal growth and that loss of the native ergosterol structure, such as through blockage of the addition of the C24-methyl group to the intermediate structure, will harm cell physiology.

The first evidence that disruption of ergosterol homeostasis can lead to impaired fungal growth was the study by Nes and coworkers, who in the 1970s cultured yeast anaerobically to generate sterol auxotrophy. By supplementing the culture medium with different sterols, they established the functional significance of the structural features of yeast ergosterol and demonstrated that lanosterol and other intermediates were harmful to cell proliferation and morphology [7, 8]. From a functional perspective, the structural features can be divided into the ring and side chain requirements (cf. the ergosterol structure in Fig. 1). For the ring, the 3β-OH group is obligatory for growth, whereas the presence of C4 and C14 methyl groups in the nucleus did not allow growth. There appeared to be little advantage in the presence of double bonds compared to the fully saturated B-ring sterol. In the side chain, the 24β-methyl group was obligatory for growth, but better growth still was observed with the natural side chain containing a 24β-methyl group and Δ^{22}-double bond. By comparing the growth response to sterols with opposite configurations at C20 and C24, only sterols with the natural configuration at C20*R*- and C24β-methyl groups supported growth. This work highlighted, for the first time, the importance of sterol specificity that can manifest itself in ergosterol-dependent diseases, and permitted a rational manipulation of ergosterol homeostasis that will lead to cell death. The importance of ergosterol to other microbial systems and sitosterol to plants in growth has been reported and confirms the original observations. In further work on this issue, several groups were able to show that sterol played a dual function in microbial physiology [1, 6, 7, 9–11]: the first as a bulk membrane component and the second as a ''sparking'' compound to signal cell proliferation; this second function uses orders of magnitude less sterol than that required in the membrane. Clearly, if those features that are essential for growth could be exploited as antifungal targets, then it should be possible to obtain an effective inhibitor for therapeutic purposes and for crop protection.

The study of ergosterol biosynthesis in fungi and protozoa became of particular interest after it was discovered that specifically acting compounds, the ''azoles'' [4], a diverse group of compounds including the imidazoles, triazoles, pyrimidines and pyridines, interfere quite precisely in fungal metabolism, mainly in one or three steps of the lanosterol–ergosterol pathway [viz., with the Erg11p, Erg24p and Erg2p]. The mechanism of action by which many of the azoles interrupt ergosterol synthesis is based on their ability to bind to the 14α-demethylase enzyme [CYP51, Erg11p] with greater affinity in fungal cells than to the 14α-demethylase in animal cells. A common structural feature of these compounds that promote enzyme inhibition is an unsubstituted imidazole ring or triazol ring, bound via the *N*-3 atom of the heterocycle to a carbon atom in the remainder of the molecule.

From a compound intervention standpoint, impaired activity that disrupts carbon flux would be

expected when substrates are produced on which the enzymes fail to catalyze; consequently, these substrates should become deleterious to the cell because they are present in abnormally high concentration and contain structural features harmful to membrane structure and function. This proposal has been confirmed in studies which show that impaired Erg11p activity can lead to an accumulation of lanosterol and 24(28)-methylene lanosterol in inhibitor-treated cells, causing these cells to die [4]. A related set of experiments has been performed with anaerobic yeast and a no-growth result occurred when cells were supplemented with lanosterol, consistent with the observations of the inhibitor-treated fungi. Although the activity of the 14α-demethylase enzyme can be interrupted by azole treatment, thereby generating lanosterol in vivo, it is not necessarily the presence of the 14α-methyl group [C32] on the back face of the sterol molecule that prevents proper sterol–lipid interactions in the lipid leaflet; rather it is more likely the presence of the C4-geminal methyl group on the sterol molecule that can perturb the hydrogen bonding ability of the C3-hydroxyl group in the membrane [12]. Removal of the C14-methyl group is necessary to allow for the introduction of the Δ^5-bond in the nucleus, which can also have functional significance [10].

Ergosterol Biosynthesis

In the early stages of formation, the synthetic pathway to fungal ergosterol uses an isoprenoid pathway to squalene oxide similar to the one involved in cholesterol formation (viz, the acetate–mevalonate pathway); thereafter the pathway can diverge into routes that are phylogentically distinct based on whether the organism will synthesize lanosterol (animals and fungi) or cycloartenol (plants) (Fig. 2) [12, 13]. In most fungi and many protozoa, the following key transformations from lanosterol to ergosterol take place in the order: (1) methylation at C24; (2) demethylation at C4 followed by demethylation at C14; (3) double bond transformations in the nucleus from Δ^8 to Δ^5, and (4) double bond transformations in the side chain at C24(28) and C22(23). The yeast *Saccharomyces cerevisiae* is an important exception; methylation at C24 occurs after C4-demethylation. In *Prototheca wickerhamii* there is an additional enzyme system not present in fungi that is involved in catalyzing the opening of the 9β,19-cyclopropane ring in the cycloartenol structure characteristic of a plant pathway to ergosterol. Higher plants can also synthesize 24β-methyl sterols similar in structure to ergosterol but these are obtained through a different route (Path 3 in Fig. 2). They can also produce 24-ethyl sterols where the 24-ethyl group is stereochemically

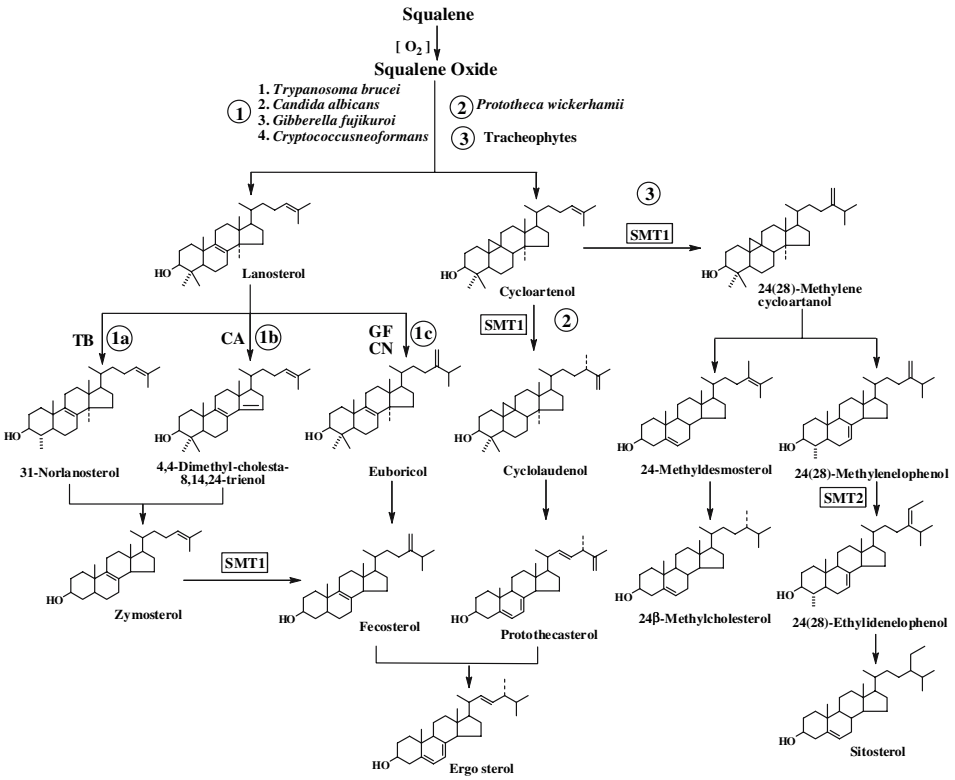


Fig. 2 Proposed general pathway for the biosynthesis of Δ^5-phytosterols from the first tetracyclic sterol formed by the cyclization of squalene oxide, lanosterol or cycloartenol

opposite to the C24-methyl group in fungal sterol ergosterol (Path 3 in Fig. 2).

Phytosterol Diversity

It has become increasingly evident that considerable variability can exist during the post-squalene stages of ergosterol (phytosterol) synthesis among microbes, and this may partially explain the different responses to sterol biosynthesis inhibitors in pathogenic organisms. In some cases, the organism's sterol content and/or composition and hence its synthetic pathway can undergo a marked change as a function of host–parasite interactions or from morphological switching of the single cell to the hyphal phase of growth. In order for these changes to occur in the sterol synthetic pathway, the relevant enzymes that compose the pathway must undergo a change in activity and/or transcript level accompanied by a change in protein level. In the parasite *Trypanosoma brucei*, which causes sleeping sickness, the protozoan form cultured on a lipid-deficient medium contains significant levels of 24-methyl sterols, whereas the main sterol is cholesterol in the bloodstream form; there are no detectable 24-methyl sterols in these cells [14]. Presumably, the cholesterol is absorbed from the host, causing the set of ergosterol enzymes in the parasite to be down-regulated. In a similar fashion, the opportunistic pathogen *Pneumocystis carinii*, isolated from its animal host, contains 31 sterols but no ergosterol; the main sterol cholesterol is also absorbed from the animal host [15].

In the phytopathogen *Gibberella fujikuroi*, 38 different sterols have been identified at different stages of development with multiple 24β-methyl sterol end-products produced, including ergosterol during mycelial growth [16]. In the fungal-like pathogen *P. wickerhamii*, the microbe can synthesize ergosterol by a cycloartenol-based pathway [13], whereas in the zygomectous fungus *Mortierella alpina* [17], the major sterol synthesized by the mycelia is cholesta-5,24-dienol (desmosterol), with minor amounts of 24-alkyl sterols, suggesting that desmosterol is the membrane insert and the 24-alkyl sterol(s) is the ''sparking'' compound. In *M. alpina*, as reported for *P. carinii*, no ergosterol can be detected in the organism.

Several microbes have been found to utiliize an unconventional phytosterol synthetic pathway that includes the ability to use a mevalonate-independent route to squalene [18, 19]. In the case of unconventional phytosterol side chain construction, multiple 24-alkyl sterol products are formed by sterol methyltransferase (SMT) catalysis in *T. brucei* [20], rather than a single product as found in *S. cerevisise* [21]. Other examples of unconventional phytosterol C-methylation pathways include formation of the $\Delta^{25(27)}$-olefins with a 24β-methyl group in *P. wickerhammi* [22] and in *T. brucei*, the formation of a 24-dimethyl group in trypanosomoid phytosterols [20], the formation of multiple C24-alkylated sterol side chains in *P. caranii* (analogous to the product outcome that has recently been found to be catalyzed by plant SMTs [23]), and the synthesis of the 24β-methyl group in 22,23-dihydrobrassicasterol in *G. fujikuroi* by a route distinct from the route to ergosterol [24].

A distinguishing feature of phytosterols is the SMT-catalyzed introduction of the 24-alkyl group. This class of catalyst can be appealing targets for the design of inhibitors to inhibit microbe growth, since they lie on a pathway that is essential to phytosterol formation and are absent from the sterol pathway in animals. SMTs are unique since they not only catalyze the C-methylation reaction sterospecifically and with a high degree of substrate specificity, but they can also provide a critical slow step that is used to regulate carbon flux in post-squalene pathway transformations [6]. Interestingly, when the SMT is blocked in certain pathogens it will promote the accumulation of lanosterol or cycloartenol analogous to the situation in azole-treated fungal cells [1–6]. Having identified the SMT as a biochemical target, we can now address what is known about its C-methylation reaction, enzymatic properties and tests involving substrate analogs, concluding with future directions that incorporate recent findings about reversible and irreversible inhibitors affecting SMT action and their potential use in medicine and agriculture.

SMT: Steric–Electric Plug Model of Binding and Catalysis

The crucial C-methylation reactions that generate the parent phytosterol side chain skeletons are catalyzed by enzymes collectively known as sterol methyltransferases [25]. These enzymes bind a Δ^{24}-sterol acceptor molecule and a methyl donor AdoMet cofactor to produce a 24-methylated (ethylated) sterol product and AdoHcy. The SMTs can be divided into two families based on their genetics and substrate preference for a sterol with a side chain structure of $\Delta^{24(25)}$-(SMT1) or $\Delta^{24(28)}$-(SMT2) [26]. However, largely based on our recent work which showed that SMT isoforms from different sources exhibit a high degree of substrate specificity for either zymosterol, cycloartenol, lanosterol and 24(28)-methylene lophenol, there has been a refinement of the

Enzyme Commission's (E.C.) classification of the reactions they catalyze [21]. By also considering the genetics and substrate specificity, the SMTs can be classed as follows; SMT1 zymosterol 24-methyltransferase (E.C. 2.1.1.41); SMT1 cycloartenol 24-methyltransferase (E.C. 2.1.1.142); (no E.C. number assigned for a lanosterol-binding SMT) and SMT2 24-methylene lophenol C-methyltransferase (E.C. 2.1.1.143). Therefore, in the E.C. classification of enzymes, SMT activity can be classified according to the catalytic mechanism of their active site into one of four mechanistic sets that involve the generation of a C-methyl-$\Delta^{24(28)}$- and $\Delta^{25(27)}$-, $\Delta^{23(24)}$- and $\Delta^{24(25)}$-olefin. Although, as we will show below, SMT1 or SMT2 may be characterized by one of these catalytic mechanisms, this characteristic is not yet used in the E.C. nomenclature.

Since 1978, a series of investigations carried out using intact organisms and recombinant SMT by several research groups has greatly added to the present understanding of C-methyl transfer reactions catalyzed by SMT. In one case, the results support a nonstop (concerted) mechanism for the first C_1-transfer that produces a single product with a 24-methyl $\Delta^{24(28)}$-olefin, whereas the second series of studies provides the first evidence for a step-wise ionic mechanism for the second C_1-transfer, which generates multiple product sets such as those with a mixture of 24-methyl (or ethyl) $\Delta^{24(28)}$- and $\Delta^{25(27)}$-olefins. The stereochemical features on the path to the second C_1-transfer reaction were the same as those noted in the first C_1-transfer reaction [25]. Thus, the enzymatic transfer of a methyl group of AdoMet to a $\Delta^{24(28)}$-olefin acceptor sterol occurs on the *Si*-face of the original substrate double bond such that the C25 configuration obtained during the first C_1-transfer reaction is retained during the second C_1-transfer reaction. Based on the above considerations, related work with plant, fungal and protozoan SMT isoforms with unique sterol specificity, the results from other relevant biogenetic studies, and the conclusions drawn from model reactions [27], a general scheme for the first and second C_1-transfer methylation reactions has been proposed (Fig. 3) and embodied in the ''steric–electric plug'' model of sterol/AdoMet binding and catalysis [28] (Fig. 4).

This stereochemical model predicts the structural features of the sterol molecule needed to generate a productive Michaelis–Menten complex that includes a free 3β-OH group, a planar tetracyclic nucleus, a side chain that orients to the ''right'' (C20*R*-configuration) in a pseudocyclic conformation, and a double bond located at position C24. A related, central prediction of the model is that the Pro*E*-26 of the Δ^{24}-substrate derived from C2-MVA becomes the ProS-C26 of the 24-methyl(ene) product as a consequence of an S_N2 type mechanism that involves β-face nucleophilic attack by the π-electrons of the Δ^{24}-bond on the *S*-methyl group of AdoMet coupled to a stereospecific 1,2-hydride shift of H24 to C25 on the *Re*-face of the original substrate double bond (Fig. 4).

C-Methylation Reaction Progress

The observed regiochemistry and stereochemistry of any particular sterol C-methylation reaction depends upon the precise amino acid contacts resulting in the active center with specific nucleophilic groups on the sterol acceptor. One approach to designing potent inhibitors of the SMT that can disrupt these interactions is to unmask the probable structure of the transition state of the chemical reaction catalyzed by the enzyme. Kinetic constants involved with catalytic efficiency, k_{cat} and k_{cat}/K_m, are measures of the stabilization of the catalytic transition state of the enzyme, where the K_d, K_i and K_m values are measures of substrate/inhibitor binding affinity [29]; the term ''transition state stabilization'' is often used in the literature to refer to specific electrostatic interactions that are strengthened in the transition state. In the case of the yeast SMT-catalyzed reaction, one can envisage the involvement of a transient interaction in the activated complex between the C24 and C25 cationic intermediate(s) and the active site of the enzyme. Electrostatic interactions that occur in the SMT active site between enzyme and substrate can be disrupted by transition state analogs, such as 24(*R*,*S*)25-epiminocholest-8-enol, that possess a positively charged functional group at physiological pH. In our research laboratory we recently determined (for the first time for any enzyme that acts on sterol) the relevant kinetic constants to establish a free-energy diagram for an SMT enzyme-catalyzed reaction [6, 30]. As demonstrated schematically in Fig. 5, the reaction coordinate proceeds energetically uphill, from the binding interactions of the ground state (associating sterol and AdoMet) to the interactions of the activated complex (which involve stabilizing the making and breaking of chemical bonds associated with the methylation–deprotonation reaction, product formation, and then the release of the products from the enzyme)[6, 30].

In work carried out by the Strasbourg group [5], it was found that the high-energy intermediate [carbocation (**2** in Fig. 4)] generated at C25 during the methylation–deprotonation reaction could be replaced by a positively charged nitrogen atom (e.g., protonated form) introduced at C25 to mimic the structure of the

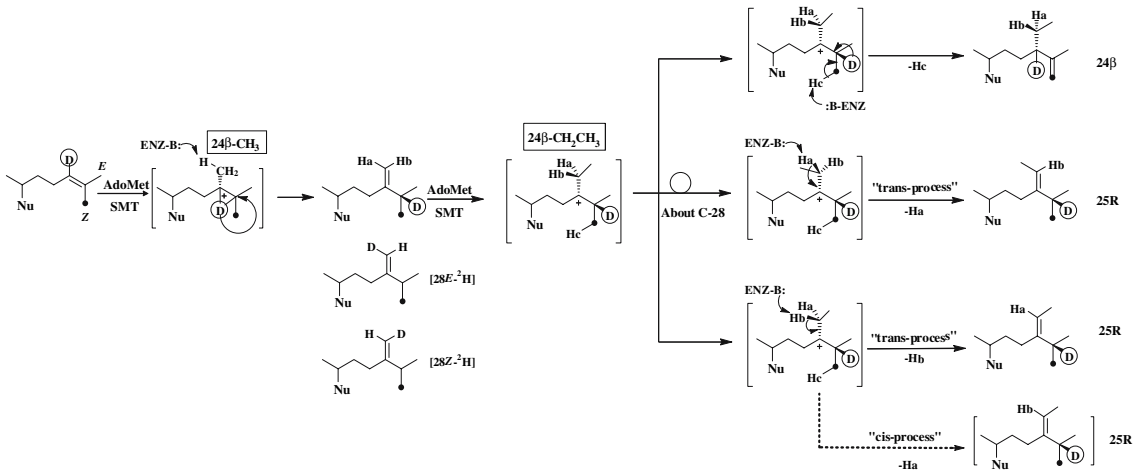


Fig. 3 Proposed mechanism for the biosynthesis of phytosterols that generates successive mono and double C24-alkylation in the side chain from a Δ^{24}-acceptor molecule. Deuterium-labeled sterol side chains were studied to reveal the mechanism (adapted from [23, 32])

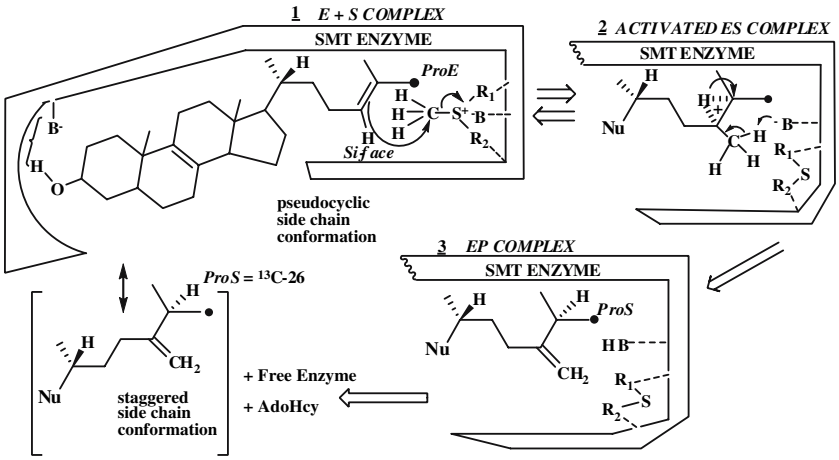


Fig. 4 Steric–electric plug model of the sterol methyltransferase binding and catalysis of sterol and S-adenosyl-L-methionine (AdoMet). *SMT* sterol methyltransferase enzyme; *E* enzyme; *S* substrate or acceptor molecule; *P* C24-methylated product; *B* unidentified enzymic base; S-adenosyl-L-homocysteine (AdoHcy) (adapted from Parker and Nes in [28])

putative ''charged'' intermediate formed during catalysis. If the transition state stabilization is equated to the binding energy, a stable analog that mimics the structure of the transition state should bind to an enzyme more tightly than the native substrate, i.e., it should bind with a strength at least three orders of magnitude higher than that of the best substrate for the enzyme [5]. In support of this view, utilizing the yeast SMT we found that the K_m for zymosterol is 30 μM, whereas a transition state analog and potent noncompetitive inhibitor of the C-methylation reaction, 24(*R,S*),25-epiminocholest-8-enol, generated a K_i value of 3 nM [31]. From the K_i/K_i values for the isostere cholest-8-enol and its ''charged'' counterpart 24(*R,S*),25-epiminocholest-8-enol [30], we calculate a $\Delta(\Delta G)$ of 5.6 kcal/mol; this amount of binding energy reflects the contribution of electrostatic association to the total energy of formation of the inhibitor–enzyme complex in wild-type enzyme. In related work using a plant SMT, Rahier et al. [5] reported a similar value for the K_m of cycloartenol and K_i values for 25-azacycloartenol in the micromolar and nanomolar range, respectively, as well as a value of 5.1 kcal/mol when comparing a similar set of inhibitors. However, the mechanistic work fails to show whether SMT actually lowers the activation energy for the making and breaking of bonds in the activated complex, or whether it simply forces the selection of a single reaction channel through the precise control of substrate conformation and the positions of counter ions that affect product outcome.

SMT1 and SMT2 Isoforms

Over the past several years there have been remarkable advances in the study of SMT enzymes, because it has been possible to generate recombinant species of

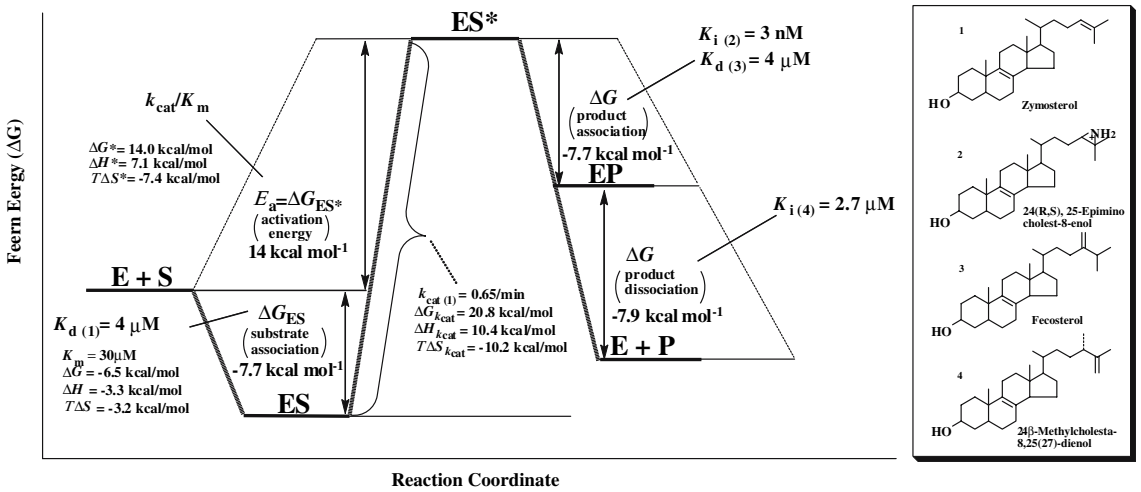


Fig. 5 A hypothetical free energy profile for the sterol methyltransferase-catalyzed activity representing the C-methylation reaction that proceeds through the formation of a high-energy chemical intermediate (activated complex) by way of a C24-methyl C25-cationic [or that of a C24(25)-bridged carbenium ion] intermediate. Compounds 1–4 assayed with the enzyme and the experimental values provided in the energy diagram are taken from [6, 30]

the native enzyme. Until now, the type and number of SMTs involved in catalyzing the product diversity observed in nature were not known. These enzymes had remained incompletely characterized until recently because of their low concentration in tissues, their membrane-bound nature and their lack of stability during purification efforts. Using cloned enzyme, it is now known that the pure SMT has unique properties that contribute to sterol specificity, kinetic and reaction properties, and to slow turnover, which serves to control carbon flux under physiological conditions. The cloned SMT from plant, fungi and protozoa possesses a pH that ranges from 6 to 8 with an optimum pH of 7.5, it is tetrameric with four identical subunits, and it has theoretical pI values that range from 5.4 to 7.5. The predicted molecular masses of the monomeric SMTs range from 38 to 43 kDa. The amino acid sequences deduced are reasonably similar to each other (21–78% identity), with 130–197 amino acids conserved in the primary structure [10, 21, 25, 27].

Fungi and protozoa have the genetics to express a single SMT (SMT1), whereas plants have a genome for SMT families and can express up to three SMT isoforms—one of the SMT1 type (C_1-methylation activity) and two of the SMT2-type (C_2-methylation activity) [21]; the different SMT activities possess different substrate properties and can produce various C24-methylated products. Both SMT1 and SMT2 from plants are currently known to be capable of performing either of the C_1-transfer methylation activities, whereas the yeast SMT1 only performs the first C_1-transfer methylation activity. The protozoa SMT1 can perform two C_1-methyl transfers, but the doubly C24-alkylated product is different from that formed in plants [21]. There appear to be at least five sterol molecules that can serve as optimal substrate for the SMT, as shown in Fig. 6. In the case of fungi and protozoa, either lanosterol, 24(28)-methylene lanosterol (euboricol) or zymosterol are the preferred substrates of the enzyme. In the case of plants, cycloartenol or 24(28)-methylene lophenol are the preferred substrates for the enzyme. Equilibrium dialysis and time-dependent inhibition experiments indicate that the enzyme characteristically contains one active center with subsites for sterol and AdoMet. The turnover numbers for SMT1 and SMT2 are similarly slow, about 0.01 s^{-1}, and the K_m values for the optimal substrates for these enzymes are about the same, around 30 μM [20, 25, 27].

Biosynthetic pathways often involve enzymes that catalyze reversible bond formation, as determined for the Δ^8- to Δ^7-sterol isomerase. Alternatively, the plant SMT was found to act in an irreversible manner, with AdoMet binding predominantly in the forward direction. The enzyme activity responds to allosterism; in soybean, the SMT1 is subject to down-regulation by the final product (K_i of 65 μM), sitosterol (it is ergosterol in the case of yeast SMT1), but not by either cholesterol or ergosterol, and to up-regulation by physiological concentrations of ATP (400 μM) [25]. The position of SMT1 in, for instance, the soybean phytosterol pathway (in the first step), the relatively irreversible nature of the binding isotherm involving the cosubstrates, and the allosteric nature of the

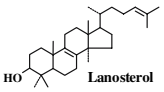
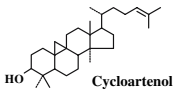
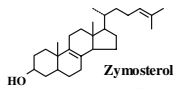
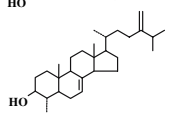
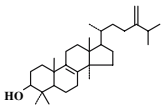
SMT Classification	Substrate	Primary Catalytic Activity	Gene Family (Example)
SMT1	 Lanosterol	C_1-transfer to 24,25-double bond	SMTc-fungal (Gibberella) (Cryptococcus)
SMT1	 Cycloartenol	C_1-transfer to 24,25-double bond	SMTa-plant (Glycine)
SMT1	 Zymosterol	C_1-transfer to 24,25-double bond	SMTb-fungal (Saccharomyces) SMTe-protozoan (Trypanosoma)
SMT2	 24(28)-Methylenelophenol	C_1-transfer to 24(28)-double bond	SMTd-plant (Araba dopsis) (Glycine)
SMT2	 24(28)-Methylenelanosterol	C_1-transfer to 24(28)-double bond	SMTc-fungal (Pneumocystis) (Glycine)

Fig. 6 Functional characteristics of the sterol methyltransferase in relation to sequence relatedness. SMT1 and SMT2 are distinguished by substrate specificity toward the $\Delta^{24(25)}$- and $\Delta^{24(28)}$-olefin side chain structure. The gene families are grouped into five distinct subfamilies based on sequence relatedness and substrate preference, as indicated in the figure (adapted from Nes et al. in [6, 21, 25])

enzyme, together with the fact the SMT gives rise to the product diversity are all consistent with the SMT acting as the rate-limiting enzyme of phytosterol synthesis.

In cases where there is credible steady-state kinetic data for enzyme-catalyzed bisubstrate reactions, the yeast SMT1 is shown to proceed by a different reaction pathway than the one that operates for the plant SMT1 [23, 31]. In both cases, the cosubstrates, sterol and AdoMet, are bound to the enzyme at the same time at some point during the course of the reaction, forming a ternary complex. The possibility of the intervention of an X-group bound intermediate was eliminated experimentally; rather, for yeast a concerted addition–elimination C-methylation reaction was demonstrated kinetically [25]. In the yeast SMT1 case, a random ordered *bi bi* mechanism operates, where either substrate can bind first to the enzyme, and either product can leave first. For the plant SMT1, it was found that the AdoMet binds first to the SMT. Interestingly, the plant SMT1 can catalyze the second C_1-transfer reaction in a mechanistically different way to the first C_1-transfer, using a *bi ter* reaction to catalyze the $\Delta^{24(28)}$-substrate [32].

SMT Sequence Relatedness

Over the past ten years, SMTs have been cloned, sequenced and purified from each of the major phylogenetic groups, including plant, fungi and protozoa. Including the four families which represent enzymes of the SMT1 and SMT2 isoforms, sequences of another 60 SMTs are now available. Of the 383 amino acids in the *S. cerevisiae* SMT, 78 residues were completely conserved (21%) in those that we studied, including the SMT1-types *Glycine max*, *Crytpoccocus neoformans*, and *Trypanosoma brucie* and the SMT2-type *Arabidopsis thaliana*. Overall, yeast SMT contains a distinct number of aromatic amino acids that are completely conserved (16 out of 47 with 34% identity), and two notable conserved regions of about ten amino acids each, referred to as Regions 1 and 3. In the yeast SMT, Region 1, which is highly hydrophobic with about 50% aromatic residues, spans Tyr81 to Phe91, and Region 3 spans Tyr192 to Pro201. Regions 1 and 3 are found only in the SMT, suggesting that they might have functional importance with respect to sterol biosynthesis. A third region that also contains ten amino acids, referred to as Region 2, spans Leu124 to Pro133. This signature motif is present in other AdoMet-dependent methyl transferases, where it is known to be part of the AdoMet binding site and therefore likely functions in the SMT structure in a similar fashion.

The possibility that several of the electron-rich aromatic residues with π-systems of indoles and phenyl groups might be part of the active center that directs the folding of the sterol side chain close to the AdoMet and contributes negative point charges that allow ion pairs to form, which could stabilize the developing cationic center(s) on the methylating substrate, was considered [25, 27]. The high degree of sequence

conservation in Regions 1–3 led us to perform leucine screening by site-directed mutagenesis of the aromatic residues in these regions [29–31]. We discovered that none of the conserved aromatic residues were essential to activity, but in the case of the *S. cerevisiae* SMT Tyr81 (Region 1) and Tyr192 (Region 3), substitution with a related aromatic amino acid can lead to altered substrate specificity, product formation and differential control of the first versus the second C_1-transfer reaction, consistent with the hypothesis that aromatic amino acid residues at the active site play a crucial role in the C-methylation reactions. On the other hand, loss of activity was associated specifically with His-90 and Glu-195.

Chemical and photoaffinity labeling studies provided direct proof that Regions 1 and 3 are part of the sterol binding subsite and Region 2 is part of the AdoMet binding subsite. Supporting data, using site-directed mutagenesis, revealed that the aromatic amino acids of Regions 1 and 3 play a functional role in catalysis by managing reaction channeling that contributes to product diversity, whereas the histidine-90 residue in Region 1 and the glutamic acid-195 residue in Region 3 may act as the deprotonating agent and counter ion, respectively. According to Kyte–Doolittle hydropathy plot analysis, the SMTs are moderately hydrophilic proteins without apparent membrane-spanning domains [25]. Thus, microsomal SMTs appear to associate weakly with membranes; this may explain the ease of solubilization from wild-type sources under mild conditions and their association with lipid droplets upon cell disruption.

Substrate Analogs and Topological Considerations

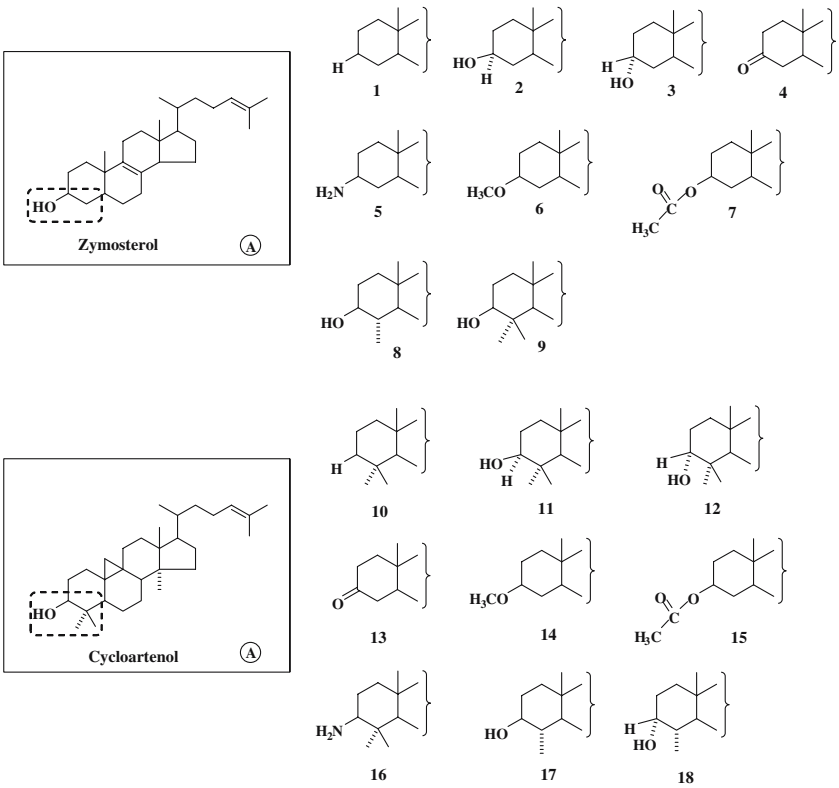
Substrate, transition state and dead-end analogs tested with SMTs have long been useful for mapping the substrate specificity of the active site with regard to stereochemistry, three-dimensional shape, charge significance of functional groups or for determining the binding order of substrates and the molecular parameters of inhibition. In addition, we recently found that tight-binding SMT inhibitors can be used in an experimental strategy to determine the active enzyme concentration in wild-type cultured cells. The substrate specificity of SMT has been explored with a variety of analogs of zymosterol, cycloartenol and lanosterol. In a manner reminiscent of the forces involved in binding sterol to phospholipids in the membrane, the affinity of SMT for its substrates depends on polar interactions with the C3-hydroxyl group and C-24 double bond and nonpolar interactions with the nucleus and side chain. The molecule must be flat and the sterol side chain oriented to the ''right-handed'' conformation for optimal catalysis. The most extensive investigations of analogs used to test a specific region of the SMT1 and SMT2 were performed in this laboratory using compounds that differed in a single structural modification associated with a specific domain of the putative native substrate, referred to as Domain A, B, C or D (cf. Fig. 1), as summarized in Structures 1–5. The SMT1 can accept either a 4-demethyl sterol, such as zymosterol, or a 4,4-dimethyl sterol, such as cycloartenol, depending on the source of the enzyme (fungus or plant, respectively).

Structure–Activity Studies: Substrates

When using V_{max}/K_m determinations as the measure of catalytic competence for substrates assayed with the SMT, the SMT1 from a fungus and plant was found to recognize the stereochemistry, bulk and polarity at C3 (and at C4) [25]. Structure 1 shows substrates with modifications in Domain A. The yeast SMT1 prefers compound 2 with a 3β-hydroxyl group that is unhindered by a neighboring methyl group [3] at C4. It also accepts compounds **4** and **6** in which the 3-OH group has been replaced with a related oxygen-containing group. Alternatively, the plant SMT1 prefers a substrate that possesses the 3β-hydroxyl group with C4 substituted by a geminal methyl group, compound **10**. The plant SMT1 will also accept compounds **13** and **15**, but not the related methyl ether (compound **14**), which is the case for the yeast SMT1. The results imply that the active sites of these SMT1 enzymes differ with respect to specific contact amino acids that are involved in hydrogen bonding to Domain A of the sterol molecule. The plant SMT2, which is not as extensively studied as the SMT1 isoforms because it is difficult to generate test sterols with a Δ^7-nucleus, has been shown to prefer C4-monomethyl sterols with a 3β-hydroxyl group (e.g., compounds **17** vs. **18**) [32].

In Structure 2, modifications of the sterol nucleus that refer to Domain B are explored. Sterols with a $\Delta^{8(9)}$, Δ^7 or Δ^5 nucleus (monoene or diene) are good substrates for the plant or fungal enzyme, with $\Delta^{8(9)}$-sterols being the optimal substrates. Alternatively, sterols with a $\Delta^{8(14)}$-nucleus are poor substrates. Dreiding models reveal that $\Delta^{8(9)}$-sterols are flatter than the other sterols tested with the SMT. An unexpected finding was that the yeast SMT1 utilized both of the 14α-methyl sterols tested (compounds **25** and **26**), converting them to C24(28)-methylene product (as judged by HPCL radiocounting against appropriate reference

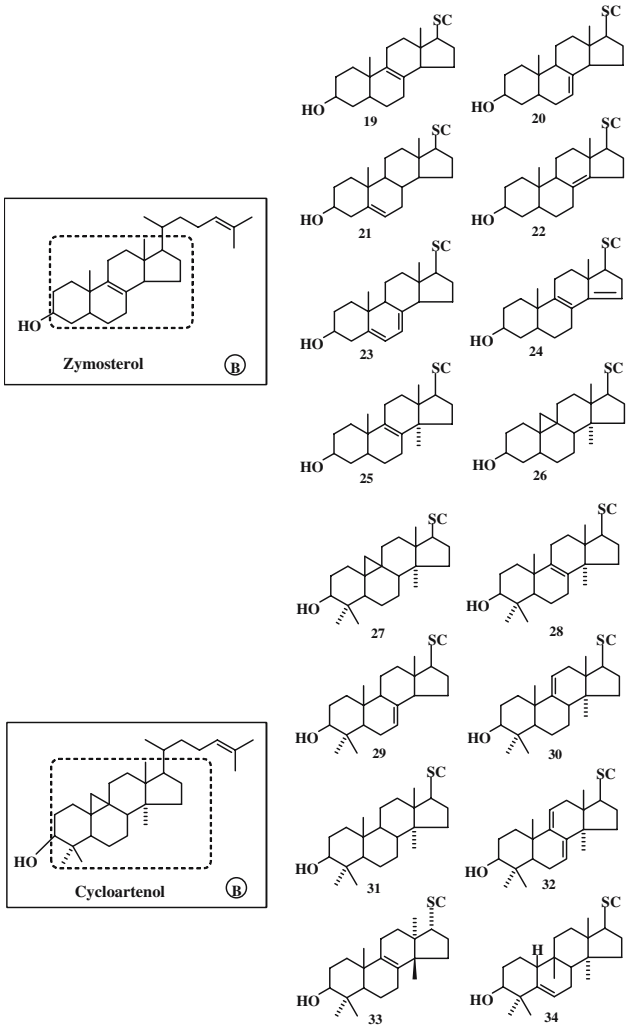
Structure 1



specimens and GC–MS). Earlier speculations [5] that 9β,19-cyclopropane plant sterols are bent in shape, that they produce 24α-methyl sterols as a direct consequence of C-methylation, but that they cannot be converted to a 24β-methyl sterol product because of their three-dimensional shape (Fig. 7), indicated that the ring structures in these substrates were similar [33]. Since plant sterols that contain a 9β,19-cyclopropane ring structure have been found to be ''flat'' in solution and the solid state [39], the enzymatic results obtained with the two substrates (compounds **25** and **26**) are consistent with one another. In a second set of activity assays using plant SMT1 from soybean and sunflower [23, 34], we discovered that by assaying the structural isomers with a Δ^{24}-bond and a 9β,19-cyclopropane group, Δ^{9}–Δ^{8}–Δ^{7} (compounds **27** to **30** and **32**) or no double bond in the nucleus (compound **31**), the optimal substrate for the SMT contained a 9β,19-cyclopropyl group whereas the corresponding saturated sterol (lanostanol) that can flex somewhat into various physiological conformations failed to bind productively, suggesting that double bond character is important for locking the nucleus into a preferred conformation for binding. Further modification of the ring by changing the structure of the A/B ring system from flat to bent (compound **34**) or inverting the centers at C13, C14 and C17 to rearrange the methyl groups in C/D ring junctions and the stereochemistry of the 17(20) bond (compound **33**) eliminated productive binding of the substrate. These results are keeping with the proposal that the substrate must possess a specific flat shape to be catalyzed by the SMT [25].

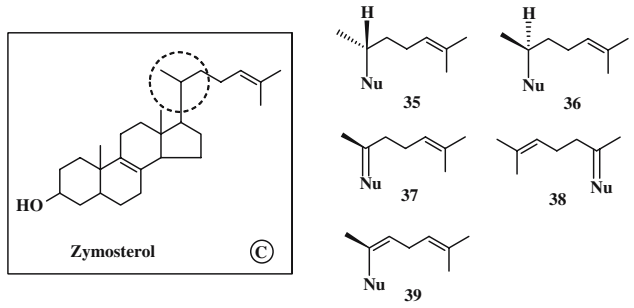
In Structure 3, the structure and stereochemistry at C20 were tested using SMTs from *S. cerevisiae* and *P. wickerhamii* [22, 35]. First, compounds **35** and **37** (which place the sterol side chain into the ''right-handed'' conformation) were converted to methylated product, whereas compounds **36** and **38** (which orient the sterol side chain into a ''left-handed'' conformation) were not accepted as substrates. These results suggest that the natural 20*R* configuration in the sterol side chain is crucial to SMT-catalyzed reactions. The SMT enzyme can also catalyze a reaction with the planar $\Delta^{20(22)E,\ 24}$-dienol substrate (compound **39**), which proves that tetrahedral character at C20 is not essential for sterol binding to the SMT.

In Structure 4, the sterol side chain structure has been modified in terms of double bond position, double bond character, length and heteroatom substitution [22, 35, 36]. The presence of a Δ^{24}-bond is crucial to catalysis. However, as found by studying the binding abilities of the substrate cholest-8-enol (compound **48**, K_d of 10 μM), it binds somewhat less effectively than zymosterol (cholesta-8,24-dienol; compound **40**, K_d of



Structure 2

4 μM) and it can impair catalytic activity since it has a K_i of ca. 100 μM, suggesting that sterol can compete in the active site with native substrate. Activity is lost when the double bond in the side chain is moved to the



Structure 3

Δ^{22}- (compound **41**) or Δ^{25}- (compound **42**) position, but the $\Delta^{24(25)}$-sterol can undergo methylation to product (compound **44**) [20]. Steric hindrance produced by the addition of a methyl group at C24 (compounds **44** and **51**) can prevent methylation of the Δ^{24}-substrate [33]. However, compounds **44** and **51** can both inhibit the yeast SMT, suggesting that the yeast SMT active site has limited freedom to accept added bulk to the side chain. Moving the substrate double bond from $\Delta^{24(25)}$- to generate a $\Delta^{24(28)}$-acceptor prevents the substrate from undergoing methylation by the wild-type yeast SMT, but in the case of the native SMT1 from plants, the $\Delta^{24(28)}$-acceptor is recognized in the second C_1-transfer reaction; both of the methylation activities can occur at the same active center in plant SMT1 and SMT2 isoforms, suggesting distinct subsites for binding the $\Delta^{24(25)}$- and $\Delta^{24(28)}$-olefins. Conjugated double bonds in the side chain at C22 and C24 or at C24 and C26 are not harmful to activity. Upon testing compound **45** with a truncated side chain, a requirement for the terminal branch in the side chain becomes apparent. Upon testing a homolog, compound **46**, a modest lengthening of the chain by one carbon was found to be tolerated by the SMT. The latter observation is worth noting, since in the case of the methylene cyclopropane (compound **47** [37]) the

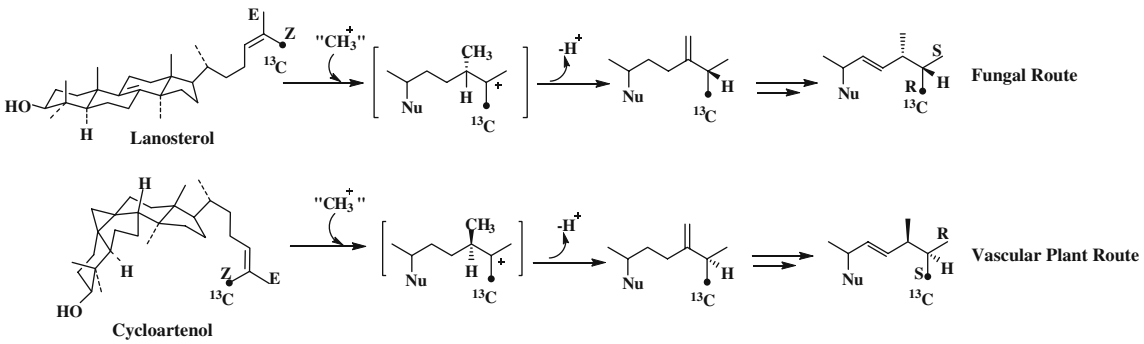
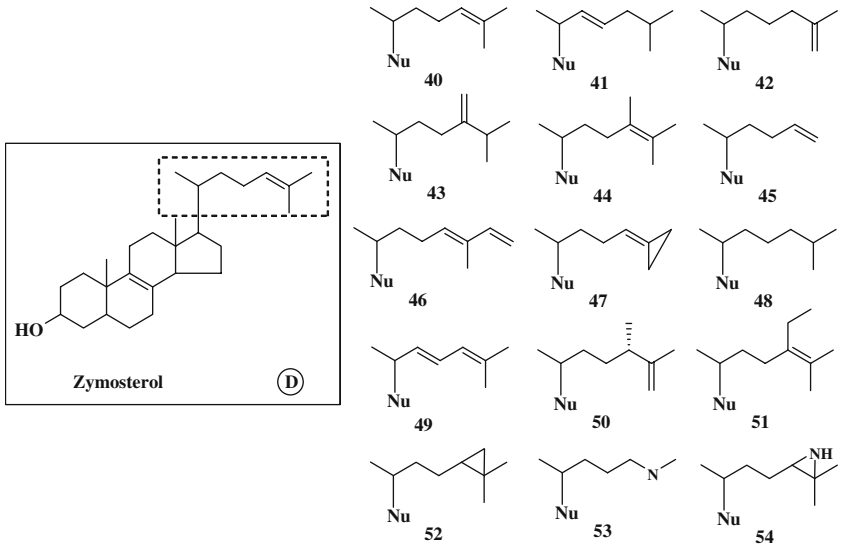


Fig. 7 Alternative C-methylation reaction pathways that form 24α-methyl and 24β-methyl sterols from substrates of possible distinct conformations: flat (lanosterol) or bent (cycloartenol); work by the Nes research group discovered that the plant pathway illustrated does not operate in nature (adapted from Nes [25])

Structure 4



substrate was methylated on the cyclopropane bridgehead C26 rather at C24, which is typical of all other Δ^{24}-acceptor sterols. The Δ^{24}-acceptor sterols catalyzed by the SMT were incorporated into the methylated sterol product regiospecifically; the methylene cyclopropane gave rise to a diene with double bonds at Δ^{23} and Δ^{25}, whereas all other Δ^{24}-acceptors produced a $\Delta^{24(28)}$-olefin product.

The question of whether the 24,25-double bond in the sterol side chain could be replaced with an analog containing a fixed-locus nitrogen atom to mimic the developing olefin nucleophile of zymosterol by heteroatomic substitution of C24 or C25 has been examined with SMT from yeast, soybean and sunflower. Neither the azirdine-containing compound **54** nor its cyclopropane-containing surrogate (compound **52**) nor the 25-aza-containing side chain (compound **53**) were methylated by SMT, consistent with kinetic data that showed they were reversible inhibitors [35, 39].

The first studies directed at defining the structural parameters necessary for substrate recognition and subsequent initiation of the C-methylation reaction catalyzed by different SMT isoforms were confirmed and extended by examining the natures and magnitudes of inhibition (measured via experimentally determined K_i/K_m values) of a series of structurally related substrate analogs with nitrogen or sulfur introduced into the nucleus and/or side chain [35–40]. Using those nitrogen-containing analogs, which differ in a single structural feature but are otherwise similar to the natural substrate, and assuming that the recognition of an analog and natural substrate arises from a common set of enzyme interactions, it has been possible to identify and evaluate at least four critical structural elements of recognition involving the same four domains reported before for the sterol structure. The principal determinant during binding appears to be associated with Domain A and the C3-hydroxyl group, since removal of the C3-hydroxyl group in the analog by replacement with hydrogen or by making a derivative of the C3-acetate alone is capable of noncompetitively inhibiting the SMT at concentrations at least an order of magnitude lower than the inhibitor with the C3-hydroxyl group. The order of effectiveness of 25-azasteroids at inhibiting yeast SMT is 25-azazymosterol > 25-azalanosterol, and for plant SMT it is 25-azacycloartenol > 25-azacholesterol or 25-azalanosterol, consistent with the recognition of the sterol nucleus by the fungal and plant SMT1.

The stereochemistries at C20 and Domain C are also considered to be a major contributor to enzyme–substrate interactions for SMT isoforms. Consistent with earlier observations obtained using stereochemically varied substrates that show that the enzyme prefers 20*R*-sterols with the 20*β*-hydrogen atom (Structure 3), testing the diastereoisomeric pair of C20*R* and C20*S*-22-azacholesterols with side chain 20*α*- or 20*β*-hydrogens, respectively (thereby placing the sterol side chain in a ''left-handed'' or ''right-handed'' conformation), revealed that the analog with the natural C20-configuration was a relatively potent noncompetitive inhibitor of SMT (K_i 250 nM), whereas the analog with the unnatural C20 configuration was a weak competitive inhibitor of SMT (K_i 1.2 μM). The change in kinetic pattern suggests that the two inhibitors bind to different subsites in the active center of the enzyme.

The Δ^{24}-bond associated with Domain D appears to be recognized electronically and specific to the natural side chain length, with a stringency paralleling that for the binding of the hydrophobic portion of the substrate

side chain. This follows from the observations that transition state analogs with a C25-nitrogen (in the salt form) inhibit the reaction three orders of magnitude more than the K_m for the natural substrate, as compared to the related inhibitor with an oxygen function (compound **64**) which failed to inhibit the reaction, and the 25-azasteroid with the terminal branch of the side chain was eliminated from the structure, producing a K_i/K_m value that was an order magnitude less than that of the corresponding natural 25-azasteroid.

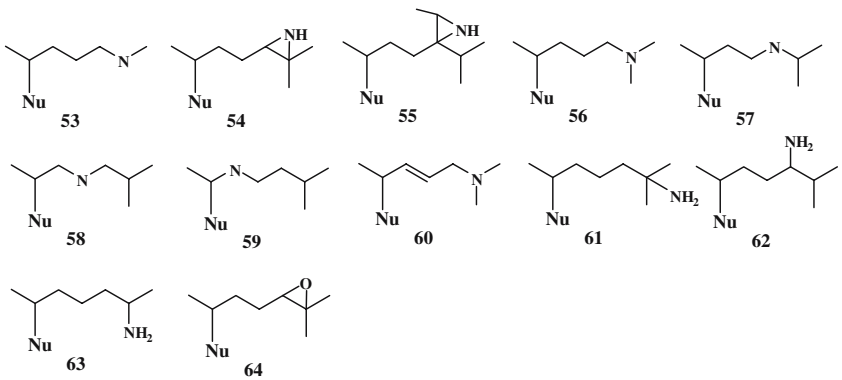
Structure–Activity Studies: Inhibitors

Structure 5 shows a set of inhibitors where modifications have been made to the structure and the location of the nitrogen atom along the lateral side chain. The ''charged'' aziridine compound **54** is more potent against the yeast SMT than any of the other inhibitors of similar structure for which compound **54** has been tested. The 24(28)-ethyl-containing aziridine, compound **55**, is less effective against the SMT than the 24(25)-aziridine inhibitor due to the added steric bulk at C24, which interferes with tight binding of the compound in the active site of the yeast SMT. The structure of the nitrogen—modified from a secondary amine (compound **54**) to a primary amine (e.g., compound **62**) to a tertiary amine (e.g., compound **61**)—had a significant effect on the inhibitor potency; the primary and tertiary amines were less potent by about an order magnitude than the secondary amine. Restricting rotation of the side chain by including a Δ^{22}-bond (compound **60**) or by modifying the terminal branching in the side chain (e.g., compounds **61** and **63**) affected the binding such that these compounds were about an order magnitude less potent than the best inhibitor tested with the yeast SMT 24,(*R,S*),25-epiminocholest-8-enol. These results are consistent with the substrate preference for a Δ^{24}-sterol and a distinct C-methylation mechanism performed by the yeast SMT; in this case, the high-energy intermediate involves a bridged 24,25-carbenium ion, and the mechanism is short-lived and nonstop. On the other hand, in cases where the ionic C-methylation mechanism is rather long-lived and occurs step-wise to allow for a reversible hydride shift of H24 to C25, the C24,25-aziridine-containing inhibitor is less potent against the plant SMT than the 25-aza-containing compounds (e.g., compound 56). When the C25-sulfur derivatives are prepared in the salt form [5, 35], they act in a similar fashion to C25-azasteroids and inhibit the SMT with equal effectiveness. The results described here provide strong suggestive evidence for the intermediacy of the predicted cationic species which the sulfonium and ammonium analogs were intended to mimic, and thus for the electrophilic nature and course of this reaction type.

The nitrogen atom can be moved toward the distal or proximal end of the sterol side chain, and the inhibitor will continue to bind quite well to the SMT. Inhibitors that contain a nitrogen function around C24–C25 usually impair catalysis between 1 and 50 nM. However, as the nitrogen atom is moved closer to C20, the inhibitor becomes less potent. When the inhibitor potency rises from the low nanomolar range to the low micromolar range, we observed that the kinetic pattern of inhibition changes from a noncompetitive-type to a competitive-type inhibition with respect to zymosterol. In a similar fashion the steroidal alkaloids solanidine and solasodine, which have the sterol side chain cyclized into a fifth ring and contain a nitrogen atom at about the equivalent of the C22–C23 position in the open extended side chain of cholesterol (Fig. 8), also generate a competitive-type kinetic pattern and will inhibit the enzyme in the low micromolar range [22, 36, 39]. The different K_i values and kinetic patterns of the various inhibitors tested with the SMT indicate that they possess different modes of binding in the active center.

It appeared possible that ''charged'' nitrogen-containing compounds (synthetically prepared or natural

Structure 5



products) might serve as inhibitors of SMT until it was appreciated that these compounds lack specificity in terms of inhibiting enzymes of sterol biosynthesis, particularly during cholesterol synthesis [41–44]. For instance, the animal Δ^{24}-reductase enzyme operates in a mechanistically similar way to the C-methylation reaction, so high-energy intermediate analogs with side chains shown in Structure 5 that inhibit SMT action should, and indeed have been found to, inhibit the animal Δ^{24}-reductase. For instance, testing triparinol (a structural mimic of the natural products berberine and palmatine that inhibit SMT [45]) which, like 24(*R,S*),25-epiminolanosterol, can inhibit both the SMT and Δ^{24}-reductase enzyme (Fig. 9) [33, 42], as an antihypercholesterolemic agent led to a range of side effects of sufficient severity to require withdrawal of the drug from the market [46]. The steroidal alkaloids from Veratrum (e.g., verazine) and Solanum steroidal alkaloids (e.g., solasodine) have good antifungal properties with IC_{50} values in the range 35–50 μM [22, 47–49]. However, they can induce teratogenic effects in cows, causing malformations during embryonic development [50]. A further lack of specificity of these inhibitors is indicated by 25-azacholesterol binding to the human Δ^8- to Δ^7-isomerase with a K_i of 21 μM [43], which might interrupt carbon flux in humans.

Mechanism-Based Inhibition of SMT Catalysis

A final series of analogs related to the native Δ^{24}-sterol acceptor molecule have been prepared for use as possible mechanism-based inactivators to probe the topography of the active site, to serve as leader compounds for a new generation of antifungal/antiparasitic agents, and for use as compounds to quantify active SMT concentrations in microbes and tissues. Figures 10 and 11 show hypothetical mechanisms for the inactivation of SMT by sterols with side chain modifications (including heteroatomic substitution with fluorine and sulfur or chemical alteration with novel olefin combinations to render them more electron-withdrawing), thereby allowing for the production of a unique reactive intermediate capable of trapping an enzyme nucleophile. Each of the side chain structures shown in Figs. 10 and 11 were prepared with a cycloartenol, lanosterol, desmosterol or zymosterol nucleus and the relevant inhibitor was tested with SMT from plant, fungus or protozoan [20, 32, 38, 40, 51–53, Song Z, Nes WD, unpublished data]. Our hypothesis is that mechanism-based inactivation of SMT requires ternary complex formation of the inhibitor followed by substrate methylation that undergoes rearrangement or delocalization to place a positive charge in a region of the active site that does not normally encounter electrophilic species. One or more of the resulting allylic cation species generated in the activated complex can serve as substrate to react with an active site base or nearby nucleophilic amino acid side chain, generating a covalent adduct with the enzyme. Alternatively, as in the case of the sulfur derivative (**A**, Fig. 11), the methylated intermediate may serve as a methyl donor to methylate an amino acid in the active site.

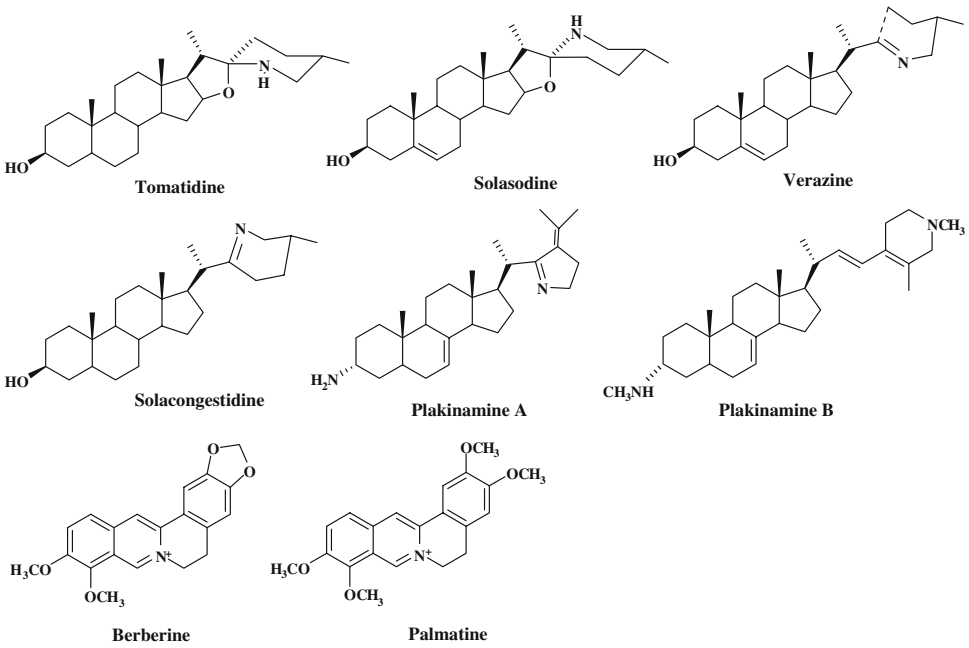


Fig. 8 Representative natural products with a nitrogen in the structure that are found to inhibit fungal growth and to inhibit the sterol methyltransferase

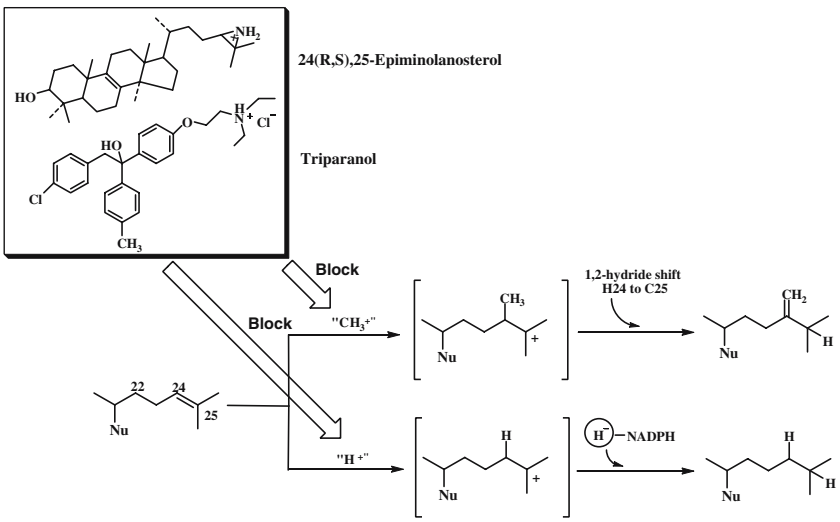


Fig. 9 Postulated similarity between the C-24 alkylation and reduction of the sterol side chain Δ^{24}-bond and inhibition by a hypothetical transition state analog of the reaction mechanism; the substrate mimic 24(*R,S*),25-epiminolanosterol and the related compound triparanol with structural features that can mimic the hydrophobic part of the sterol nucleus and a charged nitrogen at the tail portion of the molecule that can mimic the protonated nitrogen group in the tail portion of the 24(*R,S*),25-epiminolanosterol side chain

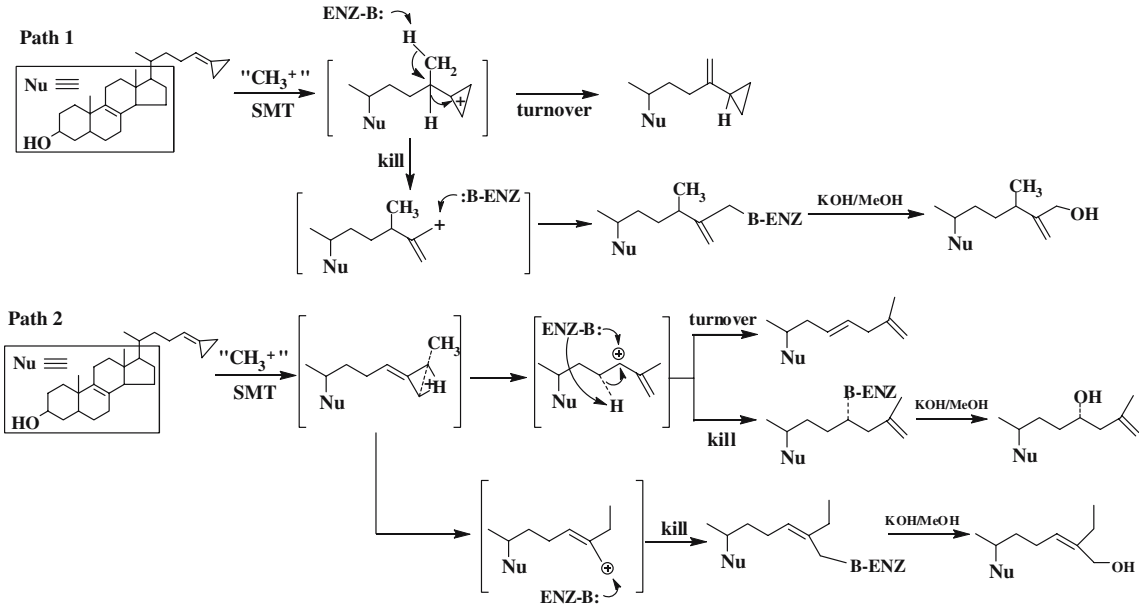


Fig. 10 Two possible mechanisms for the conversion of 26,27-dehydrozymosterol (DHZ) to methylated product and potential modes of inactivation by DHZ; of the two alternatives, only Path 2 is catalyzed by the SMT (adapted from Nes et al. in [38, 51])

As shown in Fig. 10, two possible mechanisms for the inactivation of yeast SMT by activity assay with 26,27-dehydrozymosterol can be envisioned. In Path 1, the analog is expected to bind normally to the SMT and undergo C-methylation at C24, as with the natural substrate zymosterol. The resulting high-energy intermediate containing a cyclopropyl cation should undergo facile electrocyclic rearrangement to the more stable allylic cation, in which a positive charge is generated at C26. Presumably, the C26 cation can be placed in the immediate vicinity of the enzymic base involved with the normal C28-deprotonation step, which generates fecosterol [cholesta-8,24(28)-dienol]. Since the allylic cation cannot be discharged directly by deprotonation, and it is presumably shielded from capture by water, alkylation of the SMT can proceed to inactivate the enzyme. The mechanism illustrated in Path 2 involves the nucleophilic attack of a methyl group from AdoMet on the C26 methylene cyclopropane, leading to a cyclopropyl cation followed by ring opening and ring expansion. In this case, an alternative set of high-energy intermediates can be predicted, including (1) the secondary carbonium ion at C24, which can eliminate a proton directly from C23 to produce a $\Delta^{23(24)}$-bond, or the positive charge can be eliminated through covalent attachment with an enzyme base, or (2) during the

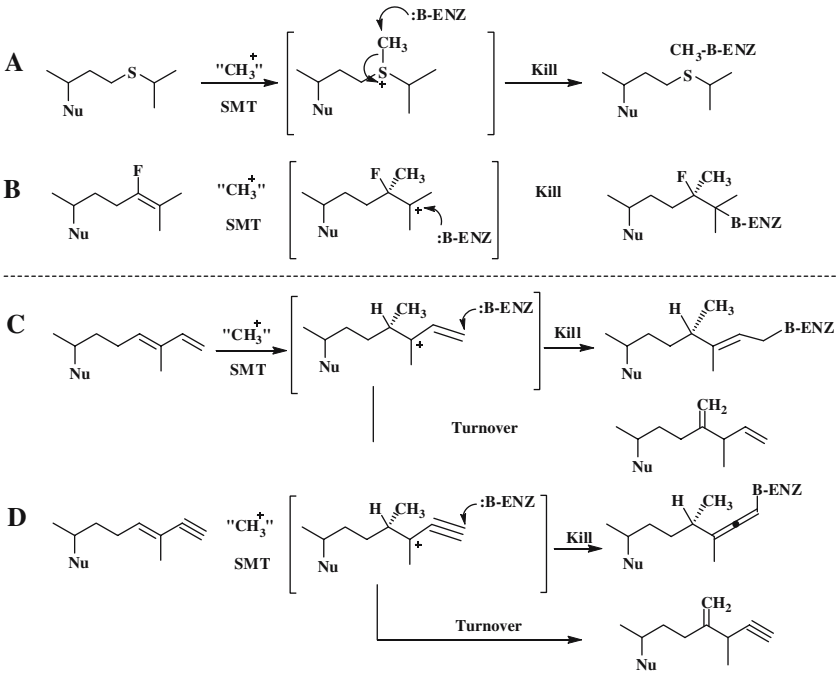


Fig. 11 Proposed modes of inactivation of the SMT by substrate analogs designed to be mechanism-based inactivators

initial rearrangement a second high-energy intermediate is formed with C26 methylated by AdoMet and C27 generating a cation, which can then interact with a base from the enzyme. Ring strain and the rich π-electron density associated with the overlapping sp^2 system make the cyclopropanoid susceptible to C-methylation from AdoMet and nucleophilic rupture, leading to selectivity with respect to the direction of bond cleavage shown in Path 2. Recent studies performed using wild-type and a Tyr81Trp mutant of the yeast SMT indicate that the mechanism-based inactivation process for 26,27-dehydrozymosterol-treated SMT follows Path 2 in Fig. 11 [30, 38]. The wild-type yeast SMT does not appear to produce significant amounts of the C25(26)-methyl, C25-OH product. However, by using the Tyr81Trp yeast SMT mutant it has been possible to show that the alternate high-energy intermediate that leads to the C25(26)-methyl, C25-OH product together with the other high-energy intermediate that leads to the C25(27)–C24-OH product will associate with Regions 3 and 1, respectively, to covalently modify the enzyme [30]. The methylene cyclopropane-containing substrate analog has been attached to the zymosterol and cycloartenol nucleus and the resulting mechanism-based inactivator tested with SMT1 and SMT2 from fungi, plant and protozoa. The SMT from each group was shown to catalyze the inhibitor to a similar methylated product. In addition, the SMT1 from soybean, yeast and *Trypanosoma* was strongly inactivated, with k_{inact} values of 0.3, 1.5 and 0.3 min^{-1} observed, respectively [20, 38, 51], whereas the SMT2 from *Arabidopsis* was inactivated (k_{inact} of 0.60 min^{-1}), and the yeast-like *Prototheca* SMT1 was not inactivated by the inhibitor [51, 52]. The variation in the recognition of the inhibitor by the yeast and yeast-like SMTs suggests that subtle differences exist in the topographies of the active sites of these enzymes. The results reported here provide, for the first time, a route towards active site labeling involving a substrate mimic that is complementary to the previously described use of 24-sulfur-containing steroids [35, 40, 54]; it is mechanistically relevant that the location of the cations generated during the catalysis of 26,27-dehydrozymosterol become spatially distributed in a segment of inhibitor that is different from the region on the natural substrate that forms a C25 cation during catalysis. Thus, this class of inhibitor should not necessarily be harmful to a Δ^{24}-reductase reaction (and hence, we discovered the long sought after differential specificity in enzyme mechanism that can be used in the design of second-generation mechanism-based inhibitors containing multiple sites in the sterol molecule modified either via heteroatomic substitution or with another site developed to inhibit additional enzyme targets in sterol synthesis, which can serve as dual action drugs). Finally, the generation of a covalent adduct using 26,27-dehydrozymosterol is entirely consistent with the postulated carbonium ion mechanism for the C_1-(wild-type yeast SMT) and C_2-transfer activities (Tyr81Phe yeast SMT mutant).

The inhibitory properties of a set of rationally designed analogs were tested recently with plant and yeast SMTs [40, 51–53, Song Z, Nes WD, unpublished data]. A shown in Fig. 11, two inhibitors were constructed with side chains of heteroatomic substitution at C24 (**A** and **B**), and two inhibitors were constructed with side chains of multiple olefin character involving a Δ^{24}-bond conjugated to a double or triple bond attached to a C26 appendage (**C** and **D**). In each case, the test compound inhibited one or more SMTs. The inhibitors exhibited an irreversible-type and time-dependent inactivation kinetic pattern against the SMTs tested. For the soybean SMT, the sulfur and fluorine-derivatives generated k_{inact} values of 0.3 and 0.1 min^{-1}, respectively, and the 24,26-diene compound and 24,26-triple bond-containing compounds generated k_{inact} values of 0.2 and 0.1 min^{-1}, respectively. Marked specificity toward these inhibitors was observed between the fungal and plant SMTs. For instance, the inhibitor with the 24,26-dienol side chain attached to either the zymosterol or the cycloartenol nucleus to ensure optimal binding generated a k_{inact} value of 0.2 min^{-1} for soybean SMT and 0.3 min^{-1} for *P. wickerhamii* SMT, whereas no detectable enzyme inactivation was observed following the assay of the 24,26-dienol-containing inhibitor with the yeast SMT, although in the latter case the inhibitor was turned over to 24-methylated product, as determined by MS and NMR characterization. These results suggest that the SMT-active sites of the different enzymes are subtly different. The proposed mechanism for inhibiting the sulfur derivative is different from that of the other analogs; the sulfur derivative is considered to undergo methylation and then serve as a substrate to methylate the enzyme active site (**A**, Fig. 11) [53]. Alternatively, the other inhibitors are considered to form an adduct with the enzyme during catalysis (**B**, **C** and **D**, Fig. 11). The results indicate that, as with assays using the other substrate and transition state analogs, interactions between the enzyme and the Δ^{24}-bond in the sterol side chain appear to be the major binding forces that affect product formation, and this therefore provides a key site in the sterol structure for chemical modification in rational drug design.

An exciting new analytical method of quantifying proteins crucial to phytosterol synthesis has emerged from our work, which uses the knowledge that the mechanism-based inhibitors bind to the SMT in stoichiometric fashion. Thus, it has become possible to use these inhibitors, especially sterols constructed with the methylene cyclopropane-containing side chain, to determine the concentration of *active* SMT in organisms/tissues and to use this information to establish the turnover number (k_{cat} values) for a specific enzyme system in an intact system. In *S. cerevisiae* cultured to the onset of growth arrest, the active SMT concentration was measured at 0.7 fg/cell, which contrasts with the total SMT concentration measured from the same preparations by Western blotting at ca. 5.0 fg/cell, and the cellular ergosterol content, determined to be 40 fg/cell by GC–MS (Song Z, Nes WD, unpublished data). In separate work, the turnover numbers of the yeast and soybean SMTs from microsome preparations of wild-type material were found to be in excellent agreement with that shown for the corresponding pure enzyme, at ca. 0.01 s^{-1} (Song Z, Nes WD, unpublished data).

Summary

The past few years have seen major advances in the design and testing of SBIs. The first generation of SBIs that target ergosterol-dependent diseases are a defined class of highly effective antifungal agents, whose common structural features are the presence of a nitrogen atom in the molecule and a greasy nature, possessing limited volume to fit the active site. The structures and mechanisms of action of those drugs are unrelated to the class of lead compounds under review here, many of which contain a nitrogen atom in the steroid frame; however, several of the azasteroids tested so far, which show good specific activities in the low nanomolar range in vivo and in vitro toward the SMT, are not suitable for commercial use because cholesterol synthesis can be affected in animals, causing side effects. In order to avoid the biological and mechanistic concerns related to azasteroid-treatment of enzymes of the cholesterol pathway, a second generation of sterol biosynthesis inhibitors—mechanism-based inactivators of SMT—are being developed as new alternatives for plant protection and for use as therapeutics. Moreover, these compounds could potentially be used in biochemical diagnostics to quantify active enzyme isoforms, since they can be prepared for specific enzymes of the phytosterol pathway and they can be used to probe the uniformity and differences in active site topography amongst SMTs of different origins. With the arsenal of sterol analogs available to us now, we can begin to address the many challenging mechanistic and evolutionary questions about sterol enzymes that still remain:

– What are the substrate–enzyme interactions involved in the initiation and termination of C-methylation?

– How is the active center organized to recognize acceptor molecule and inhibitor?
– Is there a critical amino acid in the active center that can be targeted for blockage via ligand binding to provide a species-specific drug?
– How and in what way has nature modified structural motifs in the SMT during the evolution of this class of catalyst to make them more or less susceptible to a mechanism-based inactivator?

Studies in progress in this and other research laboratories around the world will provide rationales to develop new lead inhibitors of SMT action.

Acknowledgments One of us (WDN) thanks his students and post-doctoral fellows that participated in the synthesis and testing of the compounds described here and cited in the references. The work was supported by a Welch Foundation Grant (-D–1276), a National Science Foundation Grant (MCB-0417436) and a National Institutes of Health Grant (GM63477).

References

1. Roberts CW, McLeod R, Rice DW, Ginger M, Chance ML, Goad JJ (2003) Fatty acid and sterol metabolism: potential antimicrobial targets in apicomplexan and trypansomatid parasitic protozoa. Mol Biochem Parasitol 126:129–142
2. Burbiel J, Bracher F (2003) Azasteroids as antifungals. Steroids 68:587–594
3. Rahier A, Taton M (1997) Fungicides as tools in studying postsqualene sterol synthesis in plants. Pest Biochem Physiol 57:1–27
4. Berg D, Plempel M (1988) Sterol biosynthesis inhibitors: pharmaceutical and agrochemical aspects. Ellis Horwood, Chichester, UK, p 583
5. Rahier A, Taton M, Bouvier-Nave P, Schmitt P, Benveniste P, Schuber F, Narula AS, Cattel L, Anding C, Place P (1986) Design of high energy intermediate analogues to study sterol biosynthesis in higher plants. Lipids 21:52–62
6. Jayasimha J, Bowman CB, Pedroza JM, Nes WD (2006) Engineering pathway enzymes to understand the function and evolution of sterol structure and activity. Rec Adv Phytochem 40:211–251
7. Nes WR, Sekula BC, Nes WD, Adler JH (1978) The functional significance of structural features of ergosterol. J Biol Chem 253:6218–6225
8. Pinto WJ, Nes WR (1983) Stereochemical specificity for sterols in *Saccharomyces cerevisiae.* J Biol Chem 258:4472–4476
9. Nes WD (1987) Biosynthesis and requirement for sterols in the growth and reproduction of oomycetes. Am Chem Soc Symp Ser 325:304–328
10. Rodriquez RJ, Low C, Bottema CDK, Parks LW (1985) Multiple functions for sterols in *Saccharomyces cerevisiae.* Biochim Biophys Acta 837:336–343
11. Bloch KE (1983) Sterol structure and membrane function. CRC Crit Rev Biochem 14:47–82
12. Nes WR, Nes WD (1980) Lipids in evolution. Plenum, New York, p 244
13. Nes WD, Norton RA, Crumley FG, Madigan SJ, Katz ER (1990) Sterol phylogenesis and algal evolution. Proc Natl Acad Sci USA 87:7565–7569
14. Zhou W, Cross GAM, Nes WD (2006) Cholesterol import fails to prevent catalyst-based inhibition of ergosterol synthesis and cell proliferation of *Trypanosoma brucei*. J Lipid Res (in press)
15. Zhou W, Nguyen TTM, Collins MS, Cushion MT, Nes WD (2002) Evidence for multiple sterol methyltransferase pathways in *Pneumocystis carinii*. Lipids 37:1177–1186
16. Nes WD, Xu X, Haddon WF (1988) Evidence for similarities and differences in the biosynthesis of fungal sterols. Steroids 53:533–558
17. Nes WD, Nichols SD (2006) Phytosterol biosynthesis pathway in *Mortierella alpine*. Phytochemistry 67:1716–1721
18. Zhou W, Nes WD (2000) Stereochemistry of hydrogen introduction at C-25 in ergosterol synthesized by the mevalonate-independent pathway. Tetrahedron Lett 41:2791–2795
19. Lichtenthaler HK (1999) The 1-deoxy-D-xylulose-5-phosphate pathway of isoprenoid biosynthesis in plants. Annu Rev Plant Physiol Mol Biol 50:47–65
20. Zhou W, Lepesheva GI, Waterman MR, Nes WD (2006) Mechanistic analysis of multiple product sterol methyltransferase implicated in ergosterol biosynthesis in *Trypanosoma brucei*. J Biol Chem 281:6290–6296
21. Nes WD (2005) Enzyme redesign and interactions of substrate analogues with sterol methyltransferase to understand phytosterol diversity, reaction mechanism and the nature of the active site. Biochem Soc Trans 33:1189–1196
22. Mangla A, Nes WD (2000) Sterol C-methyl transferase from *Prototheca wickerhamii*: mechanism, sterol specificity, and inhibition. Bioorg Med Chem 8:925–936
23. Nes WD, Song Z, Dennis AL, Zhou W, Nam J, Miller MB (2003) Biosynthesis of phytosterols: kinetic mechanism for the enzymatic C-methylation of sterols. J Biol Chem 278:34505–34516
24. Nes WD, Le PH (1990) Evidence for separate intermediates in the biosynthesis of 24β-methylsterol end products by *Gibberella fujikuroi*. Biochim Biophys Acta 1042:119–125
25. Nes WD (2003) Enzyme mechanisms for C-methylations. Phytochemistry 64:75–95
26. Bouvier-Nave P, Husselstein T, Benveniste P (1998) Two families of sterol methyltransferases are involved in the first and the second methylation steps of plant sterol biosynthesis. Eur J Biochem 256:88–96
27. Nes WD (2000) Sterol methyltransferase: enzymology and inhibition. Biochim Biophys Acta 1529:63–88
28. Parker SR, Nes WD (1992) Regulation of sterol biosynthesis and phylogenetic implications. Am Chem Soc Symp Ser 497:110–145
29. Nes WD, Jayasimha P, Zhou W, Kanagasabai R, Jin C, Jaradat TT, Shaw RW, Bujnicki JM (2004) Sterol methyltransferase: functional analysis of highly conserved residues by site-directed mutagenesis. Biochemistry 43:569–576
30. Jayasimha P (2006) Sterol methyltransferase: protein engineering, molecular mapping of adomet-binding site. Thermodynamic analysis and its phylogenetic implications. Ph.D. dissertation, Texas Tech University, p 193
31. Nes WD, Sinha A, Jayasimha P, Zhou W, Song Z, Dennis AL (2006) Probing the sterol binding site of soybean sterol methyltransferase by site-directed mutagenesis: functional analysis of conserved aromatic amino acids in region 1. Arch Biochem Biophys 448:23–30
32. Zhou W, Nes WD (2003) Sterol methyltransferase 2: purification, properties and inhibition. Arch Biochem Biophys 420:18–34
33. Venkatramesh M, Guo D, Jia Z, Nes WD (1996) Mechanism and structural requirements for transformation of substrates

by the (*S*)-adenosyl-L methionine: $\Delta^{24(25)}$-sterol methyl transferase from *Saccharomyces cerevisiae*. Biochim Biophys Acta 1299:313–324
34. Nes WD, Janssen GG, Bergenstrahle A (1991) Structural requirements for transformation of substrates by the (*S*)-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyl transferase. J Biol Chem 266:15202–15212
35. Kanagasabai R, Zhou W, Liu J, Nguyen TTM, Veeramachaneni P, Nes WD (2004) Disruption of ergosterol biosynthesis, growth and the morphological transition in *Candida albicans* by sterol methyltransferase inhibitors containing sulfur at C-25 in the sterol side chain. Lipids 39:737–746
36. Nes WD, Guo D, Zhou W (1997) Substrate-based inhibitors if the (*S*)-adenosyl-L-methionine $\Delta^{24(25)}$- to $\Delta^{24(28)}$-sterol methyltransferase from Saccharomyces cerevisiae. Arch Biochem Biophys 342:68–81
37. Venkatramesh M, Guo D, Harman JG, Nes WD (1996) Sterol specificity of the *Saccharomyces cerevisiae ERG*6 gene product expressed in *Escherchia coli*. Lipids 31:373–377
38. Nes WD, Marshall JA, Jia Z, Jaradat TT, Song Z, Jayasimha P (2002) Active site mapping and substrate channeling in the sterol methyltransferase pathway. J Biol Chem 277:42549–42556
39. Janssen GG, Nes WD (1992) Structural requirements for transformation of substrates by the S-adenosyl-L-methionine: $\Delta^{24(25)}$-sterol methyltransferase: inhibition of analogs of the transition state coordinate. J Biol Chem 267:25856–25863
40. Zhou W, Song Z, Liu J, Miller MB, Nes WD (2004) 24-Thiacycloartanol, a potent mechanism-based inactivator of plant sterol methyltransferase. Tetrahedron Lett 45:875–878
41. Malhotra HC, Nes WR (1971) The mechanism of introduction of alkyl groups at C-24 of sterols, IV. Inhibition by triparanol. J Biol Chem 246:4934–4937
42. Popják G, Meenan A, Parish EJ, Nes WD (1989) Inhibition of cholesterol synthesis and cell growth by 24(*R,S*),25-epiminolanosterol and triparanol in cultured rat hepatoma cells. J Biol Chem 264:6230–6238
43. Nes WD, Zhou W, Dennis AL, Li H, Jia Z, Keith RA, Piser TM, Furlong ST (2002) Purification, characterization, and catalytic properties of human sterol 8-isomerase. Biochem J 367:587–599
44. Ator MA, Schmidt SJ, Adams JL, Dolle RE, Kruse LI, Frey CI, Barone JM (1992) Synthesis, specificity, and antifungal activity of inhibitors of the *Candida albicans* Δ^{24}-sterol methyltransferase. J Med Chem 35:100–106
45. Park KS, Kang KC, Kim JH, Adams DJ, Johng TN, Paik YK (1999) Differential inhibitory effects of protoberberines on sterol and chitin biosyntheses in *Candida albicans*. J Antimicrob Chemother 43:667–674
46. Kirby TJ, Achor RWP, Perry HO, Winkelmann RK (1962) Cataract formation after triapranol therapy. Arch Opthalmol 68:84–87
47. Kusano G, Takahashi A, Sugiyama K, Nozoe S (1987) Antifungal properties of solanum alkaloids. Chem Pharm Bull 35:4862–4867
48. Barrett-Bee K, Ryder N (1992) Biochemical apsects of ergosterol biosynthesis inhibition. In: Sutcliffe J, Georgopapadakou NH (eds) Emerging targets and antifungal therapy. Routledge, Chapman and Hall, New York, pp 410–436
49. Roddick JG (1987) Antifungal activity of plant steroids. Am Chem Soc Symp Ser 325:286–303
50. Keeler RF (1984) Mammalian teratogenicity of steroidal alkaloids. In: Nes WD, Fuller G, Tsai L (eds) Isopentenoids in plants: biochemistry and function. Marcel Dekker, New York, pp 531–562
51. Song Z, Zhou W, Liu J, Nes WD (2004) Mechanism-based active site modification of the soybean sterol methyltransferase by 26,27-dehydrocycloartenol. Bioorg Med Chem Lett 14:33–36
52. Zhou W, Song Z, Kanagasabai R, Liu J, Jayasimha P, Sinha A, Veeramachanemi P, Miller MB, Nes WD (2004) Mechanism-based enzyme inactivators of phytosterol biosynthesis. Molecules 9:185–203
53. Nes WD, Marshall JA, Zhou W, He L, Dennis AL (1998) Mechanism-based site modification of sterol methyltransferase by tritium-labeled 26-homocholesta-8,14,26-yn-3β-ol. Tetrahedron Lett 39:8575–8578
54. Ator MA, Schmidt SJ, Adams JL, Dolle RE (1989) Mechanism and inhibition of delta 24-sterol methyltransferase from *Candida albicans* and *Candida tropicalis*. Biochemistry 28:9633–9640