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Review

Enzyme mechanisms for sterol *C*-methylations

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Dedicated to the memory of Professor Jeffrey B. Harborne

Abstract

The mechanisms by which sterol methyl transferases (SMT) transform olefins into structurally different *C*-methylated products are complex, prompting over 50 years of intense research. Recent enzymological studies, together with the latest discoveries in the fossil record, functional analyses and gene cloning, establish new insights into the enzymatic mechanisms of sterol *C*-methylation and form a basis for understanding regulation and evolution of the sterol pathway. These studies suggest that SMTs, originated shortly after life appeared on planet earth. SMTs, including those which ultimately give rise to 24α- and 24β-alkyl sterols, align the *si*(β)-face π-electrons of the Δ^{24}-double bond with the *S*-methyl group of AdoMet relative to a set of deprotonation bases in the active site. From the orientation of the conformationally flexible side chain in the SMT Michaelis complex, it has been found that either a single product is formed or cationic intermediates are partitioned into multiple olefins. The product structure and stereochemistry of SMT action is phylogenetically distinct and physiologically significant. SMTs control phytosterol homeostasis and their activity is subject to feedback regulation by specific sterol inserts in the membrane. A unified conceptual framework has been formulated in the *steric-electric plug* model that posits SMT substrate acceptability on the generation of single or double 24-alkylated side chains, which is the basis for binding order, stereospecificity and product diversity in this class of AdoMet-dependent methyl transferase enzymes. The focus of this review is the mechanism of the *C*-methylation process which, as discussed, can be altered by point mutations in the enzyme to direct the shape of sterol structure to optimize function.

Keywords: Sterol methyltransferase; Stereochemical mechanisms; Phytosterols; Sitosterol; *C*-methylation; AdoMet-dependent methyltransferase

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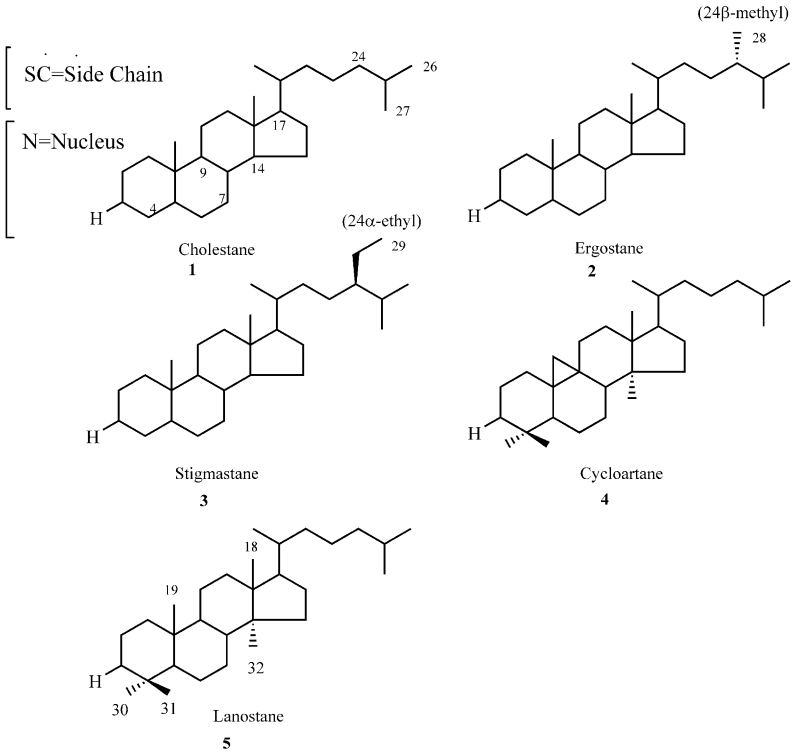
1. Introduction

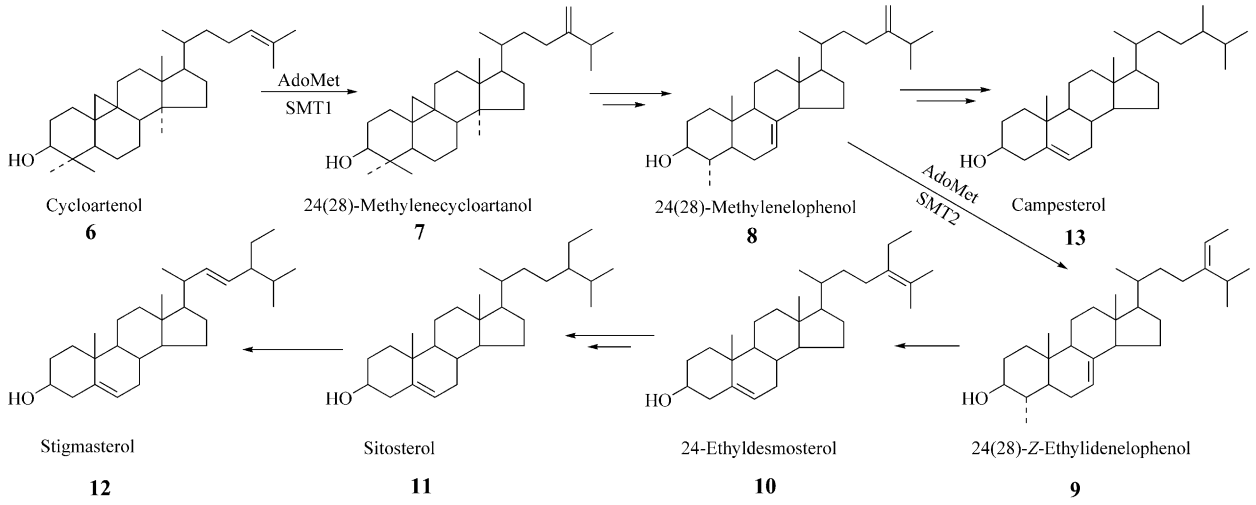
Phytosterols of plant and fungal origin are 28- to 32-carbon compounds containing a 24-alkyl group in the side chain derived by transmethylation from *S*-adenosyl-L-methionine (AdoMet = SAM) (Nes and Mckean, 1977; Goodwin, 1982). The *C*-methylation reactions which generate product diversity (Scheme 1) are catalyzed by enzymes collectively known as sterol methyl transferases (SMTs; EC 2.1.1.41). The bisubstrate reaction catalyzed by SMT includes a methyl donor (AdoMet = SAM) and a sterol acceptor and is characterized by a reorganization of at least three bonds: (a) cleavage of the C-S bond in the methyl donor, (b) formation of a C-C-bond on the sterol acceptor with attendant 1,2-hydride shift (potentially in a reversible reaction) on the opposite face of the substrate double bond, and (c) loss of a proton from the donor or acceptor. For a given SMT, modifications in the sterol acceptor molecule define enzyme specificity, while topological considerations of the active center, binding and allosteric domains delineate the essential features for kinetics and substrate channeling that control the level of *C*-methylation activity and establish the type and number of multiple products. All SMTs investigated to date are capable of catalyzing the coupled *C*-methylation-deprotonation reactions, with *C*-methylation of the sterol side chain being essentially irreversible in all cases.

Phytosterols derived from the blue-green algae (Nes and Nes, 1980; Brocks et al., 1999), cyanobacteria, are among the oldest biomolecules (membrane-associated lipids) recovered from sediments estimated to be 3.9 billion years (Brocks et al., 1999). Carbon flow through the phytosterol pathway from the cyanobacteria to tracheophytes shown in Scheme 2 accounts for approximately 90% of the products identified in edible plants (Nes and McKean, 1977; Akihisa et al., 1991). Product specificity at the level of 24-alkylation is species dependent and a feature of sterol-based chemosystematics (Nes et al., 1977). The size and direction of the 24-alkyl group is phylogenetically significant; 24β-methyl sterols occur predominantly in primitive systems and 24α-ethyl sterols are synthesized to a greater degree by advanced organisms (Scheme 1).

Sterol *C*-methylation is an energy expensive process costing the cell about 14 ATP per methyl group (Parker and Nes, 1994), suggesting an essential role for 24-alkyl sterols. As 24-alkylation is a committed step in the cycloartenol **6**- sitosterol **11** pathway (plant) and usually occurs early in the lanosterol–ergosterol pathway (fungi), such additions are emphasized to be critical slow steps (Chappell et al., 1995; Nes, 2000; Holmberg et al., 2002) and possibly branch points (Clouse, 2002) in these pathways and therefore serve as key targets for rational drug design and genetic engineering for value-added traits. SMTs generate intermediates that ultimately are converted to end products that control plant and fungal physiology. The natural balance of intermediates to end products consists of 4,4-dimethyl and 4-monomethyl sterols to 4,4-desmethyl sterols in a 1 to



Scheme 1. Structures and numbering of steranes.



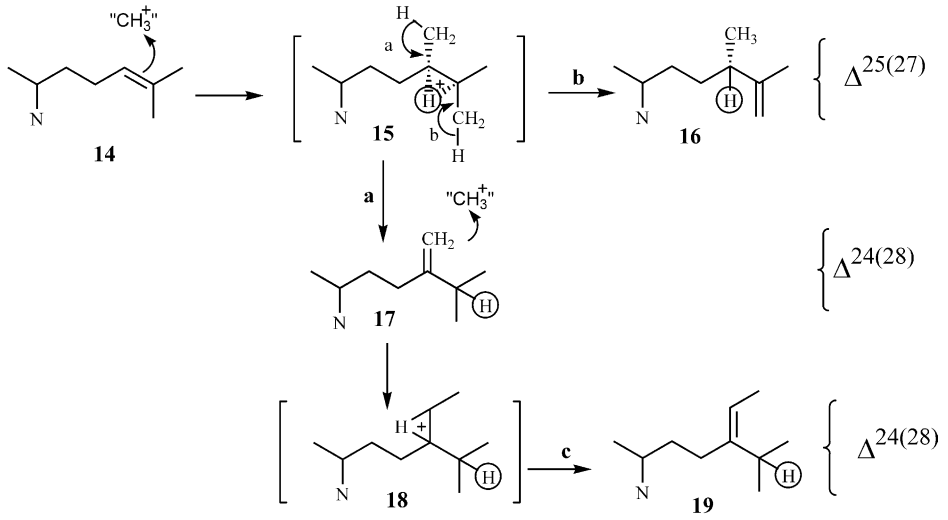
Scheme 2. Hypothetical cycloartenol-24-alkyl sterol pathway.

9 ratio in proliferating cells and actively growing tissues. Disturbance of this phytosterol homeostasis leads to microbial (Nes et al., 1978, 1986; Nes, 1987; Mangla and Nes, 2000) and cultured plant cell (Nes et al., 1991a) modifications in growth and development and to altered plant maturation (Diener et al., 2000; Schaeffer et al., 2001; Schrick et al., 2002). In the case of certain yeasts, such as *Saccharomyces cerevisiae*, transmethylation of the sterol acceptor molecule occurs after C-4 demethylation of lanosterol (Nes and McKean, 1977) with lanosterol failing to bind productively to the SMT (Venkatramesh et al., 1996a). Although a relatively small number of SMTs appear to determine the basic structural character of the phytosterols produced by a given species, the great variety of simple derivatives of each structural type encountered in plants and fungi suggests numerous SMTs are involved in *C*-methylation of the parent compounds. There can be as many as 60 sterols synthesized by a single plant with the occurrence of specific 24-alkyl sterols changing as a function of plant development (Guo et al., 1995). In some organisms, a single SMT appears to influence carbon flux, such as in the lanosterol–ergosterol yeast pathway, whereas in other organisms, multiple SMTs are required to control diversity of structural types, such as in the cycloartenol **6**-sitosterol **11** pathway (Schaeffer et al., 2001). The timing of synthesis of individual SMTs can be under developmental control leading to the suggestion that ontogeny recapitulates phylogeny of sterols in certain plant families such as the cucurbits (Garg and Nes, 1985). Rather surprisingly, given the similarity in structure, ergosterol has been shown to be a membrane reinforcer (Bloch, 1983) whereas stigmasterol **12** has not (Schuler et al., 1991; Krajewski-Bertrand et al., 1992; Marsan et al., 1996) and campesterol **13** is converted to brassinosteroids whereas 24-epicampesterol is not (Asami and Yoshida, 1999). From studies to establish the manner in which sitosterol, campesterol **13** and stigmasterol **12** serve as membrane inserts in artificial membranes, sitosterol **11** was found to be favored (Schuler et al., 1991; Krajewski-Bertrand et al., 1992; Marsan et al., 1996) consistent with intact plant observations that show carbon flux is directed to sitosterol as the first end product formed under conditions of active cell proliferation (Guo et al., 1995 and references cited therein).

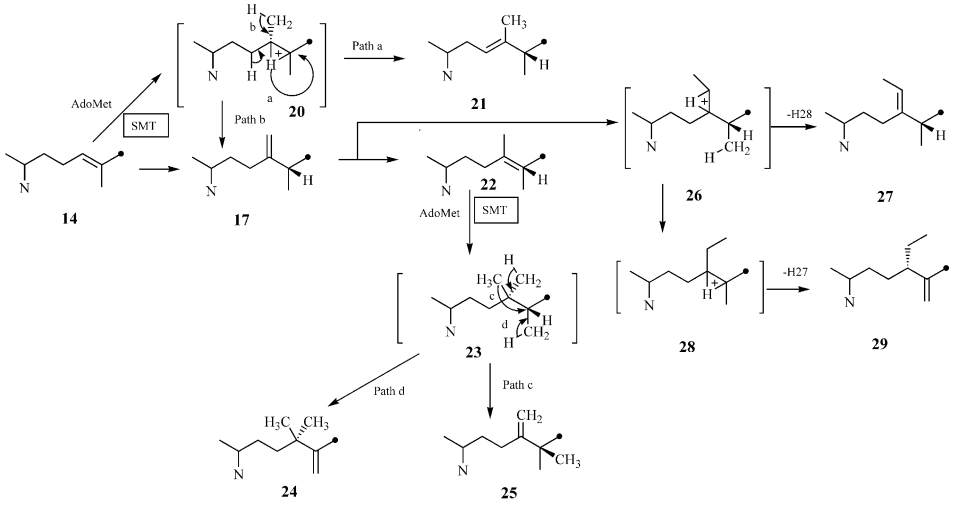
In this article we denote the regiochemistry of the attachment of the methyl group and deprotonation sites along the sterol side chain with the numerical convention recommended by Popják et al. (1977) and Parker and Nes (1994) as shown in Scheme 1.

2. X-group versus carbonium ion mechanisms

Biosynthetic tracer studies have been carried out on almost all classes of phytosterols, and in many instances cell-free preparations capable of converting Δ^{24}-sterol to various side chain skeletal types recorded (Nes and McKean, 1977; Seo et al., 1988; Zhou and Nes, 2000). The pattern of isomers formed, particularly structures dependent on a hydride shift, points to an ionic process. The blue-print for a cationic *C*-methylation pathway which generates these olefins, hypothesized by Castle et al. (1963), and extended by Goad and coworkers (1974), predicts the course of the enzymatic reaction operating in plants and fungi (Scheme 3). Djerassi and coworkers considered several novel *C*-methylation routes in marine invertebrates based on the original postulates to account for multi-branched 24-alkylated sterol side chains in these organisms (Giner and Djerassi, 1991a; Kerr et al., 1991). Several of the novel marine sterols also occur in plants generated as secondary 24-alkylations using 24-methyl $\Delta^{24(25)}$- sterol intermediates (Kalinowska et al., 1990; Giner and Djerassi, 1991b; Yano et al., 1992), as shown in Scheme 4. Interestingly,



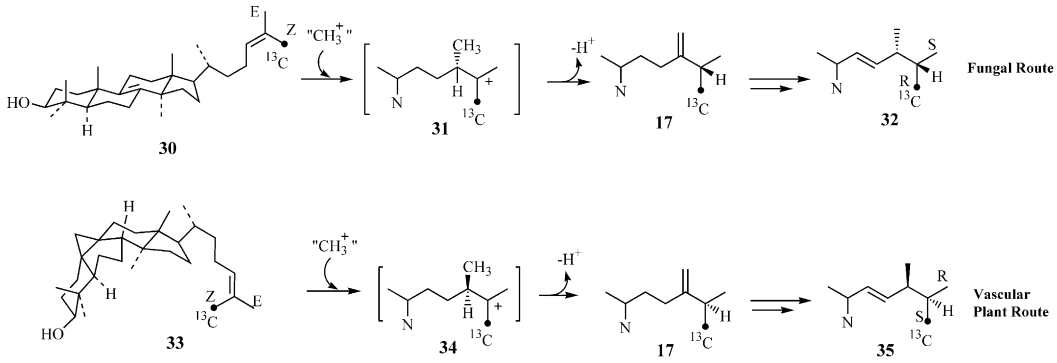
Scheme 3. Sterol *C*-methylation pathway to $\Delta^{24(28)}$- and $\Delta^{25(21)}$ -olefins.



Scheme 4. Hypothetical *C*-methylation pathway to unusual mono and doubly alkylated phytosterols. Dot represents ^{13}C-labelled C-26.

the work by Julia and Marazanao on the biomimetic methyl transfer to olefins (Julia and Marazano, 1985) reported the main compound of the *C*-methylation reaction would be a structure equivalent to a 24-methyl $\Delta^{24(25)}$-sterol which is never synthesized under physiological conditions by SMT action in fungi or plants (Nes and McKean, 1977). Arigoni hypothesized 24-methyl $\Delta^{24(25)}$-sterols will not be reaction products since the catalytic site of SMT contains a single negatively charged counter ion on the same side of the substrate double bond where AdoMet is located so as to facilitate its fixation at the active site (Arigoni, 1978). This counter ion is presumed to be identical with the basic group ultimately responsible for abstraction of the proton from C-28 (or C-27 in generating $\Delta^{25(27)}$-sterols). As a consequence, addition of the methyl group to C-24 and removal of the proton must necessarily occur from the face of the plane defined by the original 24,25- double bond of the substrate, thereby allowing H-24 to migrate easily to C-25 to eliminate the cation without necessarily being abstracted by the base acting as the deprotonating agent. For the cases shown in Scheme 5, the C-25 configuration of the final plant, sterol is determined at the stage of the reduction of $\Delta^{24(25)}$-intermediates.

Early considerations of the origin of the 24-alkyl stereochemistry in campesterol **13** (24α-methyl cholesterol) and sitosterol **11** (24α-ethyl cholesterol) related the successive transmethylation of a $\Delta^{24(25)}$-sterol to a 24-alkyl high energy intermediate possessing an initial α-orientation (Nicotra et al., 1981; Seo et al., 1983; Rahier et al., 1984). Substrate specificity was an additional factor considered to contribute to the stereochemistry of the enzyme-generated products (Nes and McKean, 1977; Rahier et al., 1986). In plants representing more-advanced (vascular plants) and less-advanced (algae) forms, it was found 24(28)-methylene



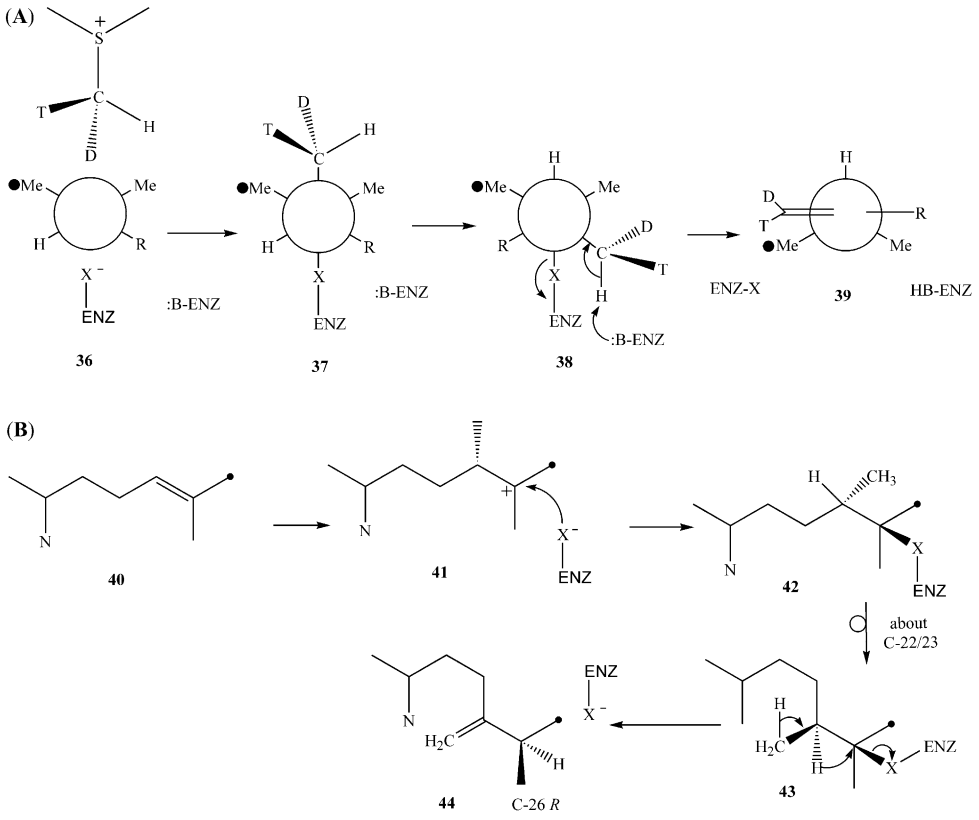
Scheme 5. Traditional view for *C*-methylation mechanism influenced by the conformation of the substrates - flat (**30**) or bent (**33**). C-27 (*Z*-methyl) is ^{13}C labeled.

cycloartanol (putative α-isomer pathway) gave rise to sitosterol and cyclolaudenol (putative β-isomer pathway) gave rise to ergosterol in a species-specific manner (Wojceichowski et al., 1973; Mercer and Russell, 1975; Nes and McKean, 1977; Goodwin, 1982). Ergosterol synthesis in fungi (putative β-isomer pathway) was found to proceed by the opposite steric course of transmethylation from campesterol **13** (putative α-isomer pathway) (Arigoni, 1978). For this reason, the evolution of two parallel pathways utilizing distinct SMTs for synthesis of the α- and β- 24-alkyl isomers was considered (Scheme 5) (Goodwin, 1982).

From a stereochemical point of view, the nucleophilic substitution can proceed with inversion or retention at the transferring chiral methyl center and the H-24 hydride migration can occur in two ways which lead to opposite configurations at C-25. In case (a) the *E* methyl group of **33** becomes the isopropyl *S* methyl group in the phytosterol side chain, whereas in case (b) the same methyl group assumes the *R* position (Scheme 5). There can be two conclusions concerning the timing of the individual steps: methylation of the substrate double bond with inversion of configuration at the methyl center proceeds with a concerted H-24–C-25 migration to form a new carbon–carbon bond, only in this way would there be no formation of a discrete C-25 cation; or formation of the carbon–carbon bond is not concerted with removal of a proton from C-28 of the 24-methylated structure since suprafacial (same side) reactions of that type were at the time thought to be stereoelectronically unfavorable. The latter view requires intervention of a nucleophilic moiety (X^--group) during the *C*-methylation event (Cornforth, 1968) to stabilize the high energy intermediate. A carbonium ion mechanism, based on the earlier paradigm (Arigoni, 1978; Nes, 2000), can be envisaged to account for the modern view of transmethylation. It differs from the X^--group mechanism to which side of the double bond *C*-methyl is attached and the proposed role of a covalent sterol-enzyme intermediate.

Initial studies revealed the stereochemistry of hydrogen migration from C-24 to C-25 during 24-alkylation takes place at the 24(28)-methylene sterol level and is such that the C-25 *E*- methyl group of the precursor takes up the *R* position in the methylene and ethylidene derivatives (Nicotra et al., 1981). Cornforth and Goad and coworkers (Cornforth, 1968; Wojceichowski et al., 1973; Goad et al., 1974) postulated a two-stage mechanism for the *C*-methylation of phytosterols in which an electron donor of unknown identity (designated X for a unspecific base in the active site) participates to stabilize the C-25 cation and this has become known as the "X-group" mechanism. The first stage of the reaction was envisioned as a *trans* addition of the methyl group to the *re*-face (α)- face of the substrate bond and concomitant addition of an X-group at C-25 to give a covalently bound intermediate. Rotation of C-24 by 120° would place the X-group and the migrating hydrogen in an antiperiplanar arrangement. Elimination of the X-group with concomitant hydride migration and deprotonation would give the 24(28)-methylene intermediate (Scheme 6, **A**). Intervention of an X-group bound intermediate obviates the stereoelectronic difficulties considered for a concerted addition-elimination reaction. It also allows for elimination of a cation that can inactivate the enzyme (Scheme 6). In a related X-group mechanism operating from the *si*-face of the substrate double bond, the side chain is rotated following *C*-methylation to generate the hypothetical C-25 stereochemistry for the $\Delta^{24(28)}$-methylene product (Scheme 6, **B**).

A problem with the X-group mechanism and stereochemistry of the 1,2-hydride shift proceeding on the *si*-face of the original substrate bond in 24(28)-methylene synthesis is that transmethylation would be expected to lead, after formation of cation **41**, not to the natural phytosterol 25-*S* configuration (Seo et al., 1988; Seo et al., 1990; Nes et al., 1992; Guo et al., 1996a; Zhou et al., 1996; Fujimoto et al., 1997; Tong et al., 1997), but to the unnatural 25-*R* arrangement **44**. Thus determination of the biosynthetic destination of [2-^{13}C]mevalonic acid



Scheme 6. Alternate X-group mechanisms for C-24 alkylation of sterols. Dot indicates ^{13}C-26 labeled carbon.

fed to cultured plants using ^{13}CNMR spectroscopic techniques confirmed the label was stereospecifically introduced into (25*S*)-[26-^{13}C]sitosterol **11** (Nes et al., 1992). In addition, assuming that SMT1 and SMT2 were acting on different substrates to give opposite C-25 stereochemistries, a stereochemical inversion might occur at C-25 during the second *C*-methylation of the $\Delta^{24(28)}$-double bond intermediate to form the 24(28)*Z*-ethylidene compound (Seo et al., 1990; Tong et al., 1997). The 24(28)-methylene structure is planar preventing a straightforward determination of the overall transmethylation process. It nonetheless has been possible to distinguish experimentally between the two catalytic mechanisms by determining the stereochemical fate of the ^{13}C-isotopically labeled methyl isopropyl group in the precursor using ^{1}H and ^{13}C-NMR techniques and relating this information to the configuration of the first formed intermediate by incubating samples of methionine carrying a chiral CHDT-group of known configuration and using ^{13}C-labeled intermediates (Arigoni, 1978; Seo et al., 1990; Nes et al., 1992; Guo et al., 1996a; Zhou et al., 1996; Fujimoto et al., 1997; Tong et al., 1997). In the yeast case, Arigoni showed that the SMT converts chiral labeled [*methyl*-^{2}H,^{3}H]AdoMet to ergosterol bearing a chiral C-24 methyl group of the same absolute configuration as the methionine precursor (Arigoni, 1978). Nes and coworkers subsequently demonstrated using a recombinant SMT that [27-^{13}C]zymosterol was converted to [27-^{13}C]24(28)-methylenezymosterol (fecosterol) (Nes et al., 1998a) and using the sterol yeast auxotroph that [27-^{13}C]lanosterol was converted to [27-^{13}C] ergosterol (Guo et al., 1996a; Zhou et al., 1996). These results demonstrated that the methylation step engages specifically the *si* (β)-face of the substrate double bond and proceeds by an inversion mechanism at the methyl center. It follows that the reduction of 24(28)-double bond takes place in an anti-manner to yield ergosterol (Arigoni, 1978). The side chain conformation recognized by SMT gives rise to the resulting stereochemistry at C-25 in both the first and second C_1-transfer reactions (Seo et al., 1990; Zhou et al., 1996; Tong et al., 1997). The *Z* methyl group on the sterol acceptor molecule at C-27 becomes the *R* methyl group of the enzyme-generated product as a result of migration of H-24 to C-25 along the original substrate *re*-face double bond. Further support of facial specificity for the 24-alkylation process in ergosterol synthesis was the kinetic study by Acuna-Johnson et al. (1997) where sulfonium mimics of the presumed high energy intermediate **15**, 24(*R*) and 24(S)-methyl-25-thiocholesterol iodides were assayed with a cell-free yeast SMT. The 24(*S*)- high energy intermediate analog tested against zymosterol was much more inhibitory to activity than the 24(*R*)-analog. Moore and Gaylor found that a covalent mechanism could not operate based on their kinetic observations using a crude yeast SMT prepara-

tion in which a ternary complex was formed involving sterol and AdoMet (Moore and Gaylor, 1970). Additional work by Nes and co-workers demonstrated the substrate binding order operated a rapid-equilibrium random bi bi mechanism, confirming the absence of a covalent intermediate during catalysis (Venkatramesh et al., 1996a).

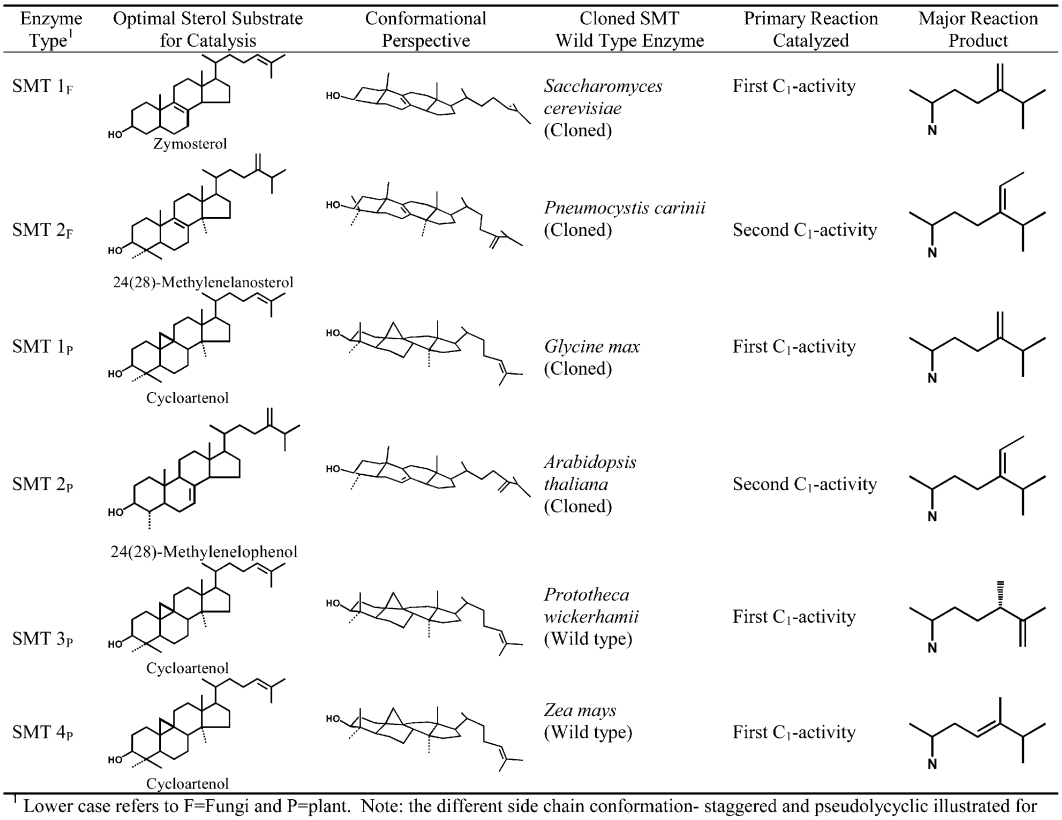
These mechanistic steps appear to be common to all SMT transformations, with sequential *C*-methylation or other types of product formation involving either termination of the reaction by remote deprotonation from C-28 to produce the $\Delta^{24(28)}$ **17**- $\Delta^{23(24)}$- **21** or $\Delta^{25(27)}$-**16** side chain or further electrophilic alkylation, hydride shifts and rearrangements before termination, as in the formation of the doubly alkylated clerosterol side chain **29** (Dennis and Nes, 2002). Consistent with the stereochemical model, the same results were obtained with corn and soybean SMT regarding the stereochemical fate of the 1,2-hydride shift in the formation of 24(28)-methylene sterol (Guo et al., 1996a; Guo et al., 1996b; Nes et al., 1999); kinetically, a ternary complex was demonstrated and the 24*S*-isomer (24β-methyl cycloartanol) inhibited 24-alkylation at ca. 25 μM when tested against cycloartenol **6** (K_m ca. 35 μM) whereas the *R*-isomer (24*a*-methyl cycloartanol) failed to inhibit activity at the highest concentration of inhibitor tested, 200 μM (Nes et al., 2003a; Zhou and Nes, 2003). In the case of *Prototheca wickerhamii* that synthesizes ergosterol by a cycloartenol-based pathway via cyclolaudenol, a net inversion of configuration at the methyl center was inferred based on the ^{1}H and ^{13}C- NMR structure determination of the 24β-methyl group in the cyclolaudenol enzyme-generated product (Mangla and Nes, 2000).

The general picture which emerges from these studies is one in which the *Z*-methyl group on cycloartenol corresponding to C-27 is transformed into the *R*- methyl group at C-25 on 24(28)-methylene cycloartanol by migration of the hydrogen atom at C-24 to C-25 from the *re*-face of the substrate double bond. For both products of cycloartenol *C*-methylation, 24(28)-methylene cycloartanol and cyclolaudenol, the methyl from AdoMet engages the *si*-face. In addition, the demonstration the 27-pro-*Z* methyl of cycloartenol **6** serves as substrate for deprotonation in cyclolaudenol formation whereas in 24(28)-methylene cycloartanol **6** the 28-methyl is deprotonated is consistent with the action of the yeast SMT where the coupled methylation-deprotonation reaction is regio- and stereospecific. The data now show channeling to a single $\Delta^{24(28)}$ olefin *C*-methylation proceeds via a non-covalent pathway whereby methyl addition to the *si* face of the Δ^{24}- and deprotonation of C-28 give rise to a nucleophilic rearrangement in which H-24 migrates to C-25 on the opposite face of the substrate double bond in concert with the initial ionization. For SMTs generating multiple olefins, viz., $\Delta^{25(27)}$- or $\Delta^{23(24)}$-products, some stabilization is received during catalysis by the consequent formation of corner-to-corner hydride migrations. Thus the high energy intermediate is sufficiently long-lived thereby uncoupling the concerted nature of proton transfer from C-24 to C-25 normally operating in $\Delta^{24(28)}$-product formation.

3. Substrate structure and conformation

Whether the side chain *C*-methylations of sterol acceptor molecules are catalyzed by commonly occurring enzymes of low specificity, or by unique, highly specific enzymes, has been a matter of considerable speculation. The natural occurrence of structurally similar 24-alkyl derivatives in plants and fungi suggests a high degree of positional specificity in the *C*-methylation of Δ^{24}-sterols. Few sterol *C*-methylations have been studied thus far, and most of these enzyme preparations exhibit a significant degree of substrate specificity for select sterol features. SMTs can be grouped into enzymes that recognize positional-specific Δ^{24}-substrates with either the $\Delta^{24(25)}$- or $\Delta^{24(28)}$-structures (Fig. 1). Nuclear features contribute to substrate acceptability but only in so far as they influence the side chain orientation in the active center. To date, two major classes of SMTs have been identified based on cloning and substrate preference for microsome-bound or partially pure wild-type enzymes (Bansal and Knoche, 1981; Shi et al., 1996; Venkatramesh et al., 1996b; Fonteneau et al., 1977; Bouvier-Navé et al., 1997; Bouvier-Navé et al., 1998; Kaneshiro et al., 2002; Nes et al., 2003a; Zhou and Nes, 2003). The first C_1-transfer reaction is catalyzed by SMT1 to produce a $\Delta^{24(28)}$-exomethylene structure and the second C_1-transfer reaction is catalyzed by SMT2 to produce a $\Delta^{24(28)}$*Z*-olefin structure. There is apparent stereospecificity in the second C_1-transfer reaction to generate the *Z*-geometry (Tong et al., 1997). Substrate preferences for fungal SMT1 (*Saccharomyces cerevisiae*) are zymosterol **76** or lanosterol (*Uromyces phaseoli*) (Moore and Gaylor, 1970; Bansal and Knoche, 1981; Venkatramesh et al., 1996a; Venkatramesh et al., 1996b), fungal SMT2 (*Pneumocystis carinii*) is 24(28)-methylene lanosterol (Kaneshiro et al., 2002), plant SMT1 (*Arabidopsis thaliana*, *Prototheca wickerhamii*, *Glycine max, Helianthus annus, Rubus fruticous*) is cycloartenol (Nes et al., 1991a; Diener et al., 2000; Mangla and Nes, 2000; Nes et al., 2003a; Zhou and Nes, 2003) and plant SMT2 (*Arabidopsis thaliana*) is 24(28)-methylene lophenol-(Bouvier-Navé et al., 1997; Zhou and Nes, 2003) (Fig. 1).

24-Methyl desmosterol and cyclobranol have also been tested as substrates with several fungal and plant SMTs catalyzing the first and second C_1-transfer reac-



[1] Lower case refers to F=Fungi and P=plant. Note: the different side chain conformation- staggered and pseudolycyclic illustrated for zymosterol, 24(28)-methylenelophenol and cycloartenol , 24(28)-methylenelanosterol, respectively.

Fig. 1. Preferred sterol substrates recognized by SMTs from fungi and plants.

tions and in no case were these compounds acceptable substrates (Venkatramesh et al., 1996a; Mangla and Nes, 2000; Nes et al., 2003a; Zhou and Nes, 2003), suggesting that either a novel isoform exists in plants to generate the unusual olefins or the catalytic action is slow and beyond our limits of detection during the assay condition employed. However, the $\Delta^{24(25)}$-24-methyl sterols can inhibit SMT action of either the plant or fungal SMTs at comparable concentrations similar to other inhibitors such as ergosterol and sitosterol **11** e.g., 100 μM (Janssen and Nes, 1992; Nes et al., 1997), indicating these olefins bind to SMT non-productively. Cycloartenol-type substrates which were predicted not to bind productively to the yeast SMT such as 4,4-dinor cycloartenol have been found to be transformed to 24(28)-methylene pollinastanol by the yeast SMT (Venkatramesh et al., 1996a), suggesting it is not the conformation of cycloartenol **6** that prevents productive binding rather it is the presence of the 4,4-dimethyl group. Zymosterol **7** was found to be an acceptable substrate for the SMT1 and SMT2 from plants (Nes et al., 2003a; Zhou and Nes, 2003), suggesting sterols preferred by fungal SMTs can compete for native structures in phytosterol synthesis. These observations become relevant when transgenic plants can be engineered to possess foreign yeast SMTs, as we recently accomplished (unpublished). In addition to SMT1 and SMT2, two related isoforms have been identified based on isotopically-sensitive branching experiments, and substrate specificity studies to generate the $\Delta^{25(27)}$- and $\Delta^{23(24)}$-olefins and these catalysts we now operationally refer to as SMT3 and SMT4, respectively (Guo et al., 1996b; Mangla and Nes, 2000).

In the case of plant SMT1, the initial hypothesis was the substrate preference was for a "bent" cycloartenol **33** whereas the substrate preference for the fungal SMT1 was for a "flat" lanosterol **30** or zymosterol (Nes and Mckean, 1977; Goodwin, 1982; Bloch 1983; Rahier et al., 1984). In order for sitosterol **11** to be synthesized, the cycloartenol **6** substrate would have to undergo a change structurally to accommodate the active site of SMT2 which recognizes the "flat" 24(28)-methylene lophenol **8** (Rahier et al., 1984; Rahier et al., 1986). The rationale for cycloartenol **6** to be bent is based on the *syn-cis* configuration at the A/B and B/C ring junctions resulting in an unfavorable interaction between the 9,10-bridgehead and the 8β-hydrogen atom at C_8. Furthermore, on the basis of manipulation of ball-and-stick models, it was proposed that ring B in 9β,19-cyclosterols becomes a boat and the A/B/C rings orient in the chair–boat–chair conformation. However, recent analyses of cycloartenol **6** and other 9β,19-cyclosterols by Nes and co-workers clearly show that the solution and solid state conformations are essentially the same and the B and C

rings possess a 6β,7α-half-chair B-ring conformation and an 13β,14α-half chair (twist boat) C ring conformation forming a flat molecule similar to lanosterol, zymosterol **76** and sitosterol (Nes et al., 1998b). Most interesting is that, when the molecular structure of lanosterol is compared to that of cycloartenol **6** and sitosterol **11**, lanosterol is the more bent and crowded molecule (Fig. 1).

By examining the magnitude of the K_m values and the catalytic competence (ratio of V_{max} to K_m) of a series of sterol analogs considered to be possible substrates for the SMT, it has been possible to determine the minimum substrate requirements necessary for generating the Michaelis complex. The new data indicate the requirements for productive binding of substrate are strict and differ amongst the SMTs in regards to subtle differences in the topology of the active center (Nes et al., 1991b; Venkatramesh et al., 1996a; Mangla and Nes, 2000; Nes et al., 2003a; Zhou and Nes, 2003). Specificity is largely determined by differences in rate phenomena (k_{cat}/V_{max}) rather than to differences in affinity for the compounds tested since the K_m for acceptable substrates are relatively the same. A rule for substrate recognition can now be developed based on the set of sterol features found to determine fitness (Fig. 2). Thus, the SMT recognizes four sterol domains: Domain 1 consists of a free 3-hydroxyl group in an equatorial orientation. The 3-hydroxy group can be replaced by a 3-ketone group but activity is greatly reduced; Domain 2 consists of a tetracyclic ring system in a planar orientation. Nuclear methyl groups, a cyclopropane ring and double bonds in the B- or C-ring may be acceptable for binding to specific SMTs. However, steric hindrance from the neighboring C-4 methyl groups may contribute to effectiveness of the 3-hydroxyl group in binding and double bonds located at different positions in the rings can permit some flexing of the nucleus that can perturb tight binding; Domain 3 consists of a 20*R*-configuration which orients the side chain in a ''right-handed'' conformation (Nes et al., 1978; Nes et al., 1998b). The side chain can extend in a staggered or pseudocyclic conformation from C-20 to C-26(27) (Fig. 1); Domain 4 consists of a C_8 or C_9- side chain containing a Δ^{24}-bond. As the position of the double bond is moved from C-24 or the number of carbon atoms at C-24 is increased or the side chain length at C-26(27) is increased beyond two atoms or branching at C-25 is altered, activity decreases dramatically. Domains 1 and 4 possess nucleophilic character to assist substrate anchoring to the sterol binding site. All four domains interact to produce an amphipathic and flat structure. Differences from conformational transmission effects that extend through the substrate molecule resulting from the different numbers and locations of double bonds in the nucleus affect the tilt of the 17(20)-bond and hence the side chain orientation as well as the tilt of the 3β-hydroxyl group (Nes et al., 1991b). These modifications in SMT structure can limit the type of olefins formed and scope of the reaction; for instance, a 24-methyl $\Delta^{24(25)}$- sterol is never a product of *C*-methylation (Scheme 4) which attests to tight catalytic control. The latter olefins, produced by isomerization of the 24(28)-methylene intermediate are the substrates which give rise to 24α-stereochemistry in the terminal campesterol **13** and sitosterol **11** structures (Nes and McKean, 1977). Collectively, these studies show the size and shape of the substrate and its nucleophilic character establish sterol specificities, with the $\Delta^{24(28)}$-product of the first methylation expected to be more nucleophilic than the starting $\Delta^{24(25)}$-olefin and therefore to compete with it for the methylating agent in cases involving the second C_1-transfer reaction.

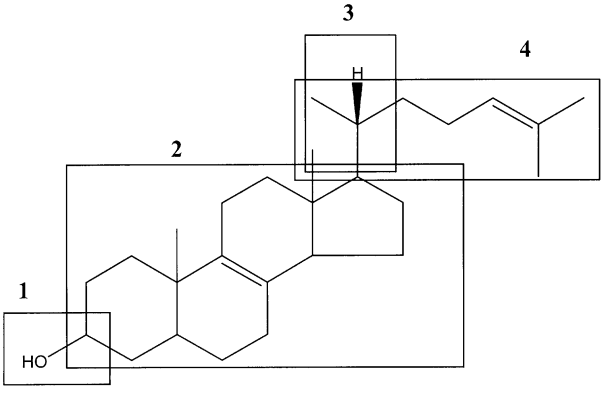


Fig. 2. Sterol domains recognized by SMT. Zymosterol is used as an example.

In many instances, the absence of detecting the second C_1-transfer activity can be due to the absence of testing the appropriate substrate (e.g., 24(28)-methylene lophenol **8**) (Nes, 2000). In the case of vascular-plant SMT2 catalysis, the reaction product 24(28)-*Z*-ethylidene lophenol is an intermediate on the path to sitosterol **11** (Scheme 2). The general failure to detect 24-ethyl(ethylidene) sterols with the β-stereochemistries and *E*-geometries (e.g., **27**) in advanced-plants which operate the cycloartenol-sitosterol pathway (Kalinowska et al., 1990; Yagi et al., 1996) suggest additional SMT2s will be identified. The inability for several SMT1s to perform the second C_1-transfer reaction using $\Delta^{24(28)}$- substrates has been interpreted to imply these SMTs catalyze formation of a single product. However, using cloned SMTs purified to homogeneity from a bacterial host has allowed Nes and coworkers to reinvestigate the plant SMT action previously examined by others (Nes et al., 1999; Dennis and Nes, 2002; Nes et al., 2003a). Thus either SMT1 from *Glycine max*—or a yeast SMT1 mutated to perform the second C-methylation were found to utilize a range of Δ^{24}-substrates and to generate multiple products. The conversion rates to generate the novel products is trace and may be one reason they have been missed in a crude

enzyme system where the abundance of SMT is low and provide an explanation for why they may not occur in any appreciable levels in intact organisms or tissues.

In view of the fact that a single enzyme can perform successive transmethylations from the same active center, it would appear the same stereochemical restrictions apply for *C*-methylation of the vinylic $\Delta^{24(28)}$-substrate as those reported for the trisubstituted $\Delta^{24(25)}$-substrate. However, due to the planar nature of the 24(28)-methylene and 24(28)-ethylidene intermediates in phytosterol synthesis, it has so far been hard to establish the orientation of proton removal relative to AdoMet attack in the formation of 24(28)-ethylidene sterols. Biosynthetic experiments with isotopically labeled compounds have provided preliminary evidence for the course of *C*-methylation leading to the formation of 24(28)*Z*-ethylidene sterols by *Morus alba* plant cell cultures (Okuzumi et al., 2000). By examining the stereochemical product distributions by mass spectrometry and ^{1}H- and ^{2}H-nuclear magnetic resonance spectroscopy that result from incubation with C-28*E*- and *Z*- deuterium-labeled 24(28)-methylene sterols it has been possible to test which of two 24-alkyation pathways is favored under physiological conditions. The stereochemistry catalyzed by the second C_1-transfer activity can be established by whether Ha (designated as a "*cis*-process" [*syn*-mechanism], since addition of the methyl group and proton loss occur on the face of the $\Delta^{24(28)}$-bond) or Hb (designated as a "trans-process" [anti-mechanism], since the two events occur on opposite faces) is eliminated from C-28 establishes the mechanism (Zimmerman and Djerassi, 1991; Okuzumi et al., 2000). Based on the appearance and position of deuterium signals due to 28-H(^{2}H) in cell cultures that synthesize isofucosterol [24(28)Z-ethylidene cholesterol] following individual assay of the deuterated species established unambiguously that the 28*E*-hydrogen (Ha) of 24(28)-methylene sterol is retained while the 28*Z*-hydrogen (Hb) is eliminated during the conversion of substrate to product **54**. These results suggest the second C_1-transfer reaction proceeds by the "*trans*-process", as similarly demonstrated in a sponge (Zimmerman and Djerassi, 1991).

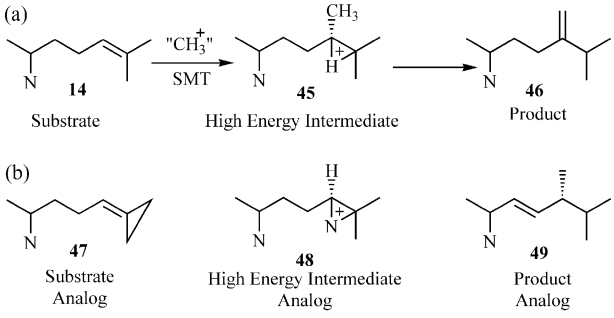
Using cloned soybean SMT to catalyze [28*E*-^{2}H] and [28*Z*-^{2}H]-24(28)-methylenelanosterols to 24(28)*Z*- and *E*-ethylidene sterols as well as 24β-ethyl sterols from a common active site, we similarly established directly the antimechanism operates for formation of the pair of geometric isomers (Nes et al., 2003a). Thus when [28*E*-^{2}H]sterol was assayed, sterols with clerosterol **52** and isofucosterol **54** side chains were labeled with two and one ^{2}H-atoms, respectively. Alternatively, when [28*Z*-^{2}H]sterol was assayed, the clerosterol **52** and fuscosterol **54a** side chains contained two and one ^{2}H-atoms, respectively. The 24β-ethyl sterol **52** was found to possess deuterium at C-28 from either progenitor. This result suggests a lack of stereochemical control resulting from the evolution of position-specific high energy intermediates during the reaction progress. The configuration at C-24 in **52** is determined by the hydride shift of H-25 in the substrate to eliminate the C-24 cation generated during the reaction progress in analogous manner to the formation of 24β-methyl sterols generated in the first C_1-transfer reaction (Yagi et al., 1996; Nes et al., 2003a). Taken together, the biosynthetic data for *C*-methylation of the $\Delta^{24(28)}$-bond to give the product set **52**, **54**, **54a** (Scheme 8) indicate the C-24 double bond is formed via processing of the isofucosterol cation **51** by proton removal on the opposite face from AdoMet attack. These predicted results serve to confirm the step-wise sequence illustrated in Scheme 8 for the second C_1-transfer reaction and provide presumptive evidence that the isofucosterol **54** and fucosterol **54a** side chains are generated by the same active site base.

In addition to the chemical studies, we performed isotopically sensitive branching experiments with the soybean SMT (Nes et al., 2003a). A kinetic isotope effect (KIE: k_H/k_D observed) related to the C-28 deprotonation step was observed on the overall catalyzed reaction with [28*E*-^{2}H]- and [28*Z*-^{2}H]sterol paired with AdoMet of 0.92 and 1.23, respectively. The inverse value established by these experiments is strong evidence that the proton transfer from the substrate to the active site base has come to equilibrium prior to the rate-determining transition state and supports the proposal of a discrete carbocation intermediate. The chemical mechanisms associated with the first and second C_1-transfer activities therefore must differ in the order the two bonds are cleaved in the $\Delta^{24(25)}$- and $\Delta^{24(28)}$-substrates. In the carbocation mechanism, the bond leaving group is broken first while in the concerted mechanism both bonds are cleaved simultaneously without intervention of an intermediate. There can be much variability in the timing of steps in the nucleophilic rearrangements. Thus, it is possible that the first C_1-transfer reaction can operate a non-stop concerted mechanism while the second C_1-transfer reaction can operate a step-wise mechanism. The change in reaction mechanisms with the binding of distinct position-specific olefins suggest the reactants occupy different subsites in the active center.

4. Substrate-analogs: topological considerations

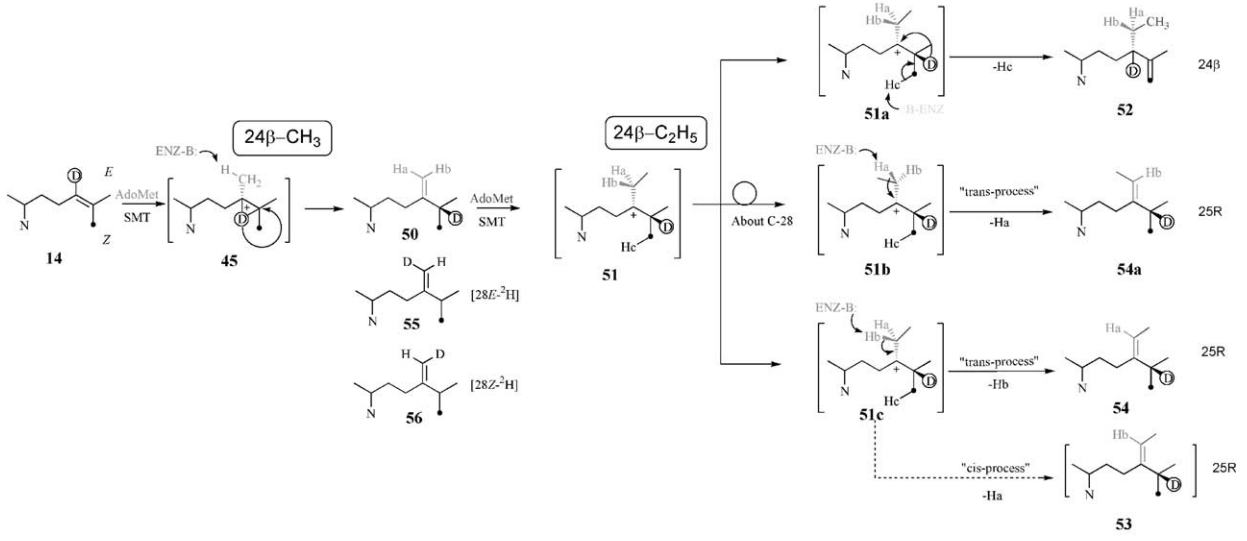
Most of the work in this area can be subdivided into one of four categories: (i) confirming the molecular parameters for interaction with the active site with regard to geometry and electronic features of the substrate, (ii) distinguishing which sterols in the native mixture down-regulate activity, (iii) determining the

number and location of the sterol binding site(s) and (iv) determining the kinetic mechanism. The rational design and isolation of these compounds from nature is related to the carbenium ion mechanism and three sets of inhibitors have been prepared; (i) substrate analogs, (ii) high energy intermediate (HEI) analogs and (iii) product analogs (Scheme 7). The SMTs share many common properties (Nes, 2000; Nes et al., 2003a; Zhou and Nes, 2003). All possess molecular weights between 40,000-43,000 Daltons and exhibit pH optima in the 7–8 range, p*I* in the range 5.5–8.5. None of them require any cation(s) or oxygen for activity. They are tetrameric with one binding site for sterol and AdoMet and subject to allostery from ATP and feed-back effects from select 24-alkyl sterols. Their K_m values for the preferred substrate ranges from 15 to 40 μM, whereas k_{cat} value for the preferred substrate regardless of whether it is a SMT1 or SMT2-type enzyme is about 0.01 sec^{-1}, K_d for sterol and AdoMet is about 4 μM and the enzyme is inhibited by sterol analogs with varied hetereoatoms and other functionalities added to the side chain. The turnover number for these SMTs is very slow and slower than other AdoMet-dependent methyl transferase enzymes. The k_{cat} for the yeast SMT is in the same range as the purified yeast 14α-demethylase (Lamb et al., 1999), suggesting the activities of the fungal sterol enzymes are coordinately regulated and intimately associated with cell growth and membrane genesis. Typically, the plant synthesizes ca. 4 μg/day/shoot of total sterol in actively growing seedlings (Guo et al., 1995). As shown by studies on the transcriptional regulation of sterol enzymes, sterol biosynthesis is localized in apical meristems, with much lower levels observed in leaves and roots (Devarenne et al., 2002). Light/dark conditions affect C_1- and C_2-activities, such that the introduction of light supplied to germinating seeds modulates SMT1 and SMT2 activity (Sauvaire et al., 1987). The C_1- and C_2-activities are differentially regulated by ATP which may relate to the role of these SMTs as branch point enzymes (Nes, 2000). When the ATP concentration is high (high energy charge), phytosterol synthesis might be expected to be favored (compared to cholesterol synthesis which can be significant in some plant families) thereby directing carbon flux in the phytosterol pathway away from sitosterol **11** and into campesterol **13** synthesis (Sauvaire et al., 1987). When the enzyme interacts with the sterol substrate cooperatively, there can be a large change in reaction velocity over a very narrow range of substrate concentrations. The change produced by subsequent inhibition is much more dramatic, particularly at low substrate concentrations.



Scheme 7. Inhibitors tested with the SMT: A. Native *C*-methylation reaction progress; B. Rationally designed inhibitors.

Following the seminal paper by Malhotra and Nes (1971) showing that triparanol can inhibit SMT activity, a series of sterol analogs with a heteroatom- bromine, nitrogen, sulfur or arsenic- positioned along the lateral side chain were prepared and tested as mechanistic probes of the fungal and plant SMTs (Narula et al., 1981; Oehlschlager et al., 1984; Rahier et al., 1984; Ator

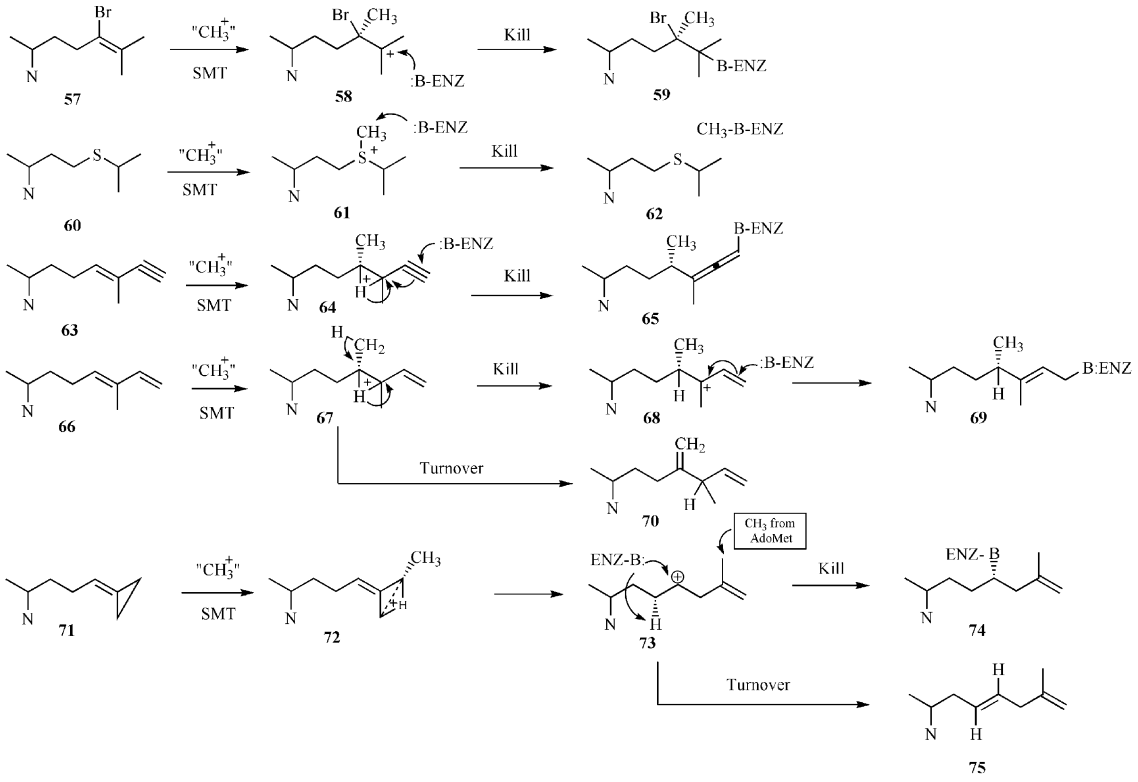


Scheme 8. Stereochemical model for the formation of phytosterol olefins by C_1- and C_2- activities performed by soybean SMT; N = sterol nucleus.

et al., 1989; Janssen and Nes, 1992; Venkatramesh et al., 1996a; Venkatramesh et al., 1996b; Acuna-Johnson et al., 1997; Nes et al., 1997; Mangla and Nes, 2000). The results of chemical structure-inhibitory activity relationship studies with these inhibitors indicated an anionic center on the active site in the vicinity of C-24-C-25 in the native substrate exists. They also provided strong evidence for the intermediacy of the predicted cationic species which the HEI analog inhibitors were intended to mimic (Narula et al., 1981), and thus the electrophilic nature and course of this reaction type. The formation of a bridged carbenium ion species in the HEI was highlighted by the ability for the 24,25-aziridine (24(*R,S*),25-epimino-) containing sterols to be the most highly effective inhibitor of SMT activity (Janssen and Nes, 1992; Venkatramesh et al., 1996a; Nes et al., 1997; Mangla and Nes, 2000). However, other aspects of substrate features for catalysis—the presence of a 3β-hydroxyl group, the 20*R*-configuration and a specific length, bulk and branching in the lateral side chain were determined not to be essential. The kinetic patterns for many of the ammonium-containing inhibitors were of the reversible, non-competitive type against the native acceptor molecule consistent with the proposal that the molecular features which determine the affinity of HEI analogs, assuming they are protonated at physiological pH, should be different from those affecting the recognition of the substrate in the ground state (Rahier et al., 1986). The ammonium-containing inhibitors were also found to be competitive with AdoMet, suggesting the inhibitors bind or crossover in a subsite of the active center which is not suited for sterol substrate such as binding to the AdoMet subsite which has the electrons to accept positively charged moieties (Nes et al., 2003a). The ammonium-containing 25-aza and 24(*R,S*)-epimino sterols have been tested against substrates for the first and second C_1-transfer activities using a recombinant SMT from soybean and Arabidiopsis SMT (Nes et al., 2003a; Zhou and Nes, 2003) and in every case, the K_i values for the inhibitors against both activities were about the same, ca. 30 nM. These results indicate the first and second *C*-methylations occur at the same binding site on the enzyme.

A set of sterol substrates viewed as end products of sterol synthesis have been tested with plant and fungal SMTs. For the yeast and algal systems which synthesize ergosterol, ergosterol but neither sitosterol **11** nor cholesterol down-regulated SMT activity with a K_i ca. 50 μM which is around the K_m for the respective native substrate, zymosterol or cycloartenol, respectively (Venkatramesh et al., 1996a; Venkatramesh et al., 1996b; Nes et al., 1997; Mangla and Nes, 2000). For the plant systems, Helianas (sunflower) Arabidopsis or Glycine (soybean) which synthesize sitosterol **11**, only sitosterol **11** was an effective inhibitor of SMT activity with a K_i about 50 μM which is similar to the K_m of the native substrate, cycloartenol **6**, of ca. 40 μM. The inhibitors exhibited competitive-type kinetic patterns. The apparent species-specific down-regulation of SMT activity by ergosterol for ergosterol-synthesizing microbes and sitosterol **11** synthesized by vascular plants and the fact HEI analogs can inhibit growth of fungi and cultured plant cells by blocking the *C*-methylation reaction is suggestive that carbon flux is controlled by the availability of specific membrane inserts to bind SMT and down-regulate its activity.

It was anticipated, based on the structures of compounds of the native phytosterol pathway, that substrate analogs of the type shown in Scheme 9 can, following *C*-methylation of the Δ^{24}-bond, undergo rearrangement or delocalization to place a positive charge in a region of the active site that does normally encounter electrophilic centers and therefore under influence of the SMT enzyme would be susceptible to alkylation. Several methylenecyclopropane analogs of the lanosterol, cycloartenol **6**, zymosterol **76** and desmosterol nucleus were prepared as an inhibitor of the fungal and plant SMTs (Scheme 9) (Nes et al., 1997; Nes et al., 2003a, Zhou and Nes, 2003). Only analogs with structures that closely mimicked the three-dimensional structure of the native substrate were potent inhibitors of SMT action, showing competitive-type kinetics and dissociation constants (ca. 2 to 20 μM) on the order of the K_m values for the substrate (Nes, 2000). The similar recognition of substrate analogs tested as inhibitors as observed with sterol analogs tested as substrates is consistent with the requirements for substrate acceptability for SMT1- and SMT2- type enzymes. These compounds also displayed substrate protection and time-dependent inactivation kinetics, consistent with properties of a mechanism-based inhibitor with K_{inact} from 0.2 to 2.0 min^{1} (Ator et al., 1989; Nes et al., 1998c; Nes et al., 1998d; Nes, 2000; Nes et al., 2002; Nes et al., 2003a; Zhou and Nes, 2003). The turnover to kill aspects of SMT action were established unambiguously for the yeast enzyme and a partition ratio of 0.03 determined for 26,27-dehydrozymosterol (Nes et al., 2002). The covalent interaction of 26,27-dehydrozymosterol was demonstrated by monitoring a tryptic digestion fragment which indicated Glu68 bound the sterol analog. Saponification of the enzyme-ligand adduct demonstrated the sterol was bound to the acidic residue in an ester linkage. The enzyme was nearly quantitatively inactivated with a stoichiometry of labeling of 1 mole of inhibitor incorporated per catalytic site. Equilibrium dialysis showed 26,27-dehydrozymosterol **71** behaved in a similar manner to the native substrate zymosterol **76** with a K_d of ca. 2 μM for SMT binding. In related work, Scatchard analysis from equilibrium dialysis studies with pure yeast enzyme indicated there is one sterol binding site per enzyme (Nes et al., 2002). The fact that a single point mutation of the yeast SMT in the sterol binding site can change both its sterol speci-

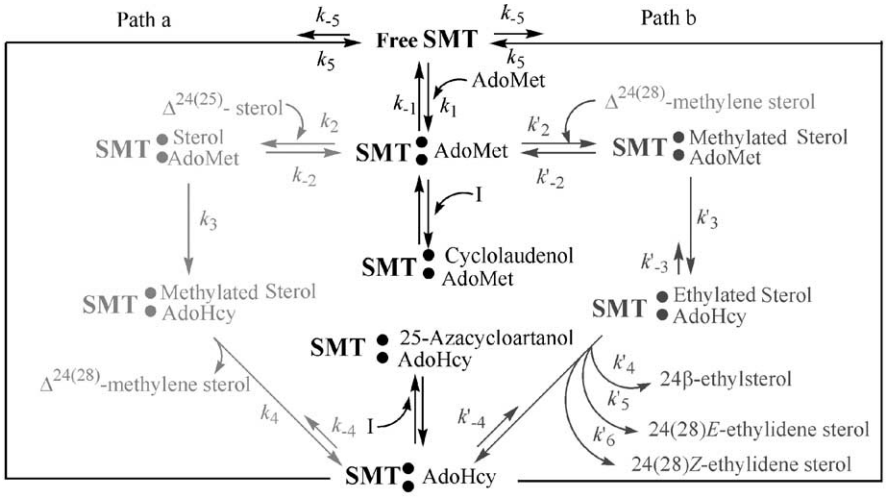


Scheme 9. Proposed pathways for mechanism-based inactivation of SMT. "kill" implies covalent attachment of inhibitor to enzyme and "turnover implies the substrate is transformed to a *C*-methyl product and ultimately is released from the enzyme.

ficity and ability to carry out the second C_1-transfer activity allowing the enzyme to mimic a SMT2 catalytically (Nes et al., 1999), is further support that only one sterol binding site exists for both *C*-methylation activities.

Chemical analysis of the neutral and polar structures generated from the enzyme after saponification suggested the methylenecyclopropane was the substrate from *C*-methylation rather than the Δ^{24}-bond. The *C*-methylation of 26,27-dehydrozymosterol **71** was directional, that is, it was initiated through a backside nucleophilic attack of a methyl group on the methylenecyclopropane leading to ring opening and ring expansion. The termination step gives rise to either olefinic (elimination) or hydroxyl (water addition) products. Ring strain and the rich π-electron density associated with the overlapping sp^2 systems of the monosubstituted cyclopropane ring make the cyclopropanoid susceptible to C-methylation from AdoMet and nucleophilic rupture leading to selectivity with respect to the direction of bond cleavage (Scheme 9). We have proposed (Nes et al., 2002) that the C-methylation-elimination reaction involving 26,27-dehydrozymosterol proceeds by the C-methylation of C-26 to give intermediate **72**, which can undergo corner to corner nucleophilic rearrangement and cyclopropane ring fission to generate chain extension to produce intermediate **73** which can either be deprotonated to give **75** or be trapped by an active site nucleophile, probably by a nonconcerted ionization thereby rendering the protein inactive, to give **74**. In a related study, we recently characterized the neutral product **70** from conversion of analog **66** assayed with the yeast SMT (unpublished). In addition, the proposed operation for SMT inactivation by sulfur and bromine tagged inhibitors also point to novel routes of inactivation (Scheme 9). Thus assuming the yeast SMT can *C*-methylate each of these inhibitors producing a cation at different strategic locations in the side chain, it may be possible to locate different bases in the active center that control substrate channeling. The different behavior of the Prototheca and Saccharomyces SMTs to methylenecyclopropane containing inhibitors (Nes et al., 1998c; Nes et al., 2002), as well as their difference in molecular size (Nes, 2000) and reaction products generated ($\Delta^{25(27)}$- vs $^{\Delta 24(28)}$-), suggests species differences in the structures of the active site as well as global differences.

Bi-substrate kinetic analysis using cycloartenol and AdoMet indicated both substrates must bind to form a ternary complex before catalysis of soybean SMT (Nes et al., 2003a). Product inhibition and dead-end inhibitor patterns using 24(28)-methylene cycloartanol and cyclolaudenol **8** and 25-azacycloartanol respectively, strongly support a steady-state sequential ternary complex (ordered bi–bi) kinetic mechanism, where AdoMet (=SAM) binds first before cycloartenol **6** and methylated sterol is released first before AdoHcy (=SAH) from the SMT (Scheme 10). These observations are dis-



Scheme 10. Kinetic scheme for soybean SMT catalyzed *C*-metylation of Δ^{24}-bond. Path a and path b refer to first and second C_1-transfer activities, respectively.

tinctly different from the kinetic mechanism for the yeast SMT which involves a random mechanism and a single catalytic activity in the temperature range 25–35 °C, which is similar to the temperature range for growth. Although not determined thus far, the large size and rigidity of the *C*-methylation products together with the presumed hydrophobicity of the active site raises the interesting possibility that the rate-determining step involves a conformational change of the enzyme active site to enforce a spatial restructuring of the AdoMet-sterol molecules to facilitate the *C*-methylation reaction or that the enzyme undergoes a conformational isomerization after formation of the Michaelis complex to eliminate the methylated sterol thereby promoting product release necessary for the second C_1-transfer activity to proceed. Substrate preferences based on chemotaxonomy per se cannot explain the operation of the different catalytic mechanisms since zymosterol **76**, the preferred substrate for the yeast SMT also binds to the soybean SMT, but with an effectiveness of ca. 40% compared to cycloartenol **6** (Nes et al., 2003a). The recent studies strongly argue against a ping-pong (covalent intermediate) type mechanism for SMT catalysis and therefore argues further against the X-group mechanism.

5. The *steric-electric plug* model and evolution

The structure–function studies of this class of enzymes have benefited from the combination of genetic and biochemical approaches. For instance, hydropathy analysis of these proteins (Fig. 3) show they are moderately hydrophobic (Venkatramesh et al., 1996b), and cellular location and solubilization characteristics suggest they are membrane-associated in plants and fungi (Hartmann and Benveniste, 1987; Zisner et al., 1993). No kinetic mechanism for binding order characterizes AdoMet-dependent methyl transferases. There is nonetheless a characteristic action in which the methyl transfer reaction either proceeds through a nucleophilic catalysis of a cysteine residue with a covalent intermediate (for example, cytosine-C5-DNA methyltransferase) or a simple S_N2 reaction (the vast majority of AdoMet-dependent methyltransferases). In *O*-methylations, where stereochemical studies were performed, a bimolecular transfer from the methyl sulfonium of the AdoMet to the oxygen of the substrate bearing a hydroxyl group was found to proceed through an S_N2-like transition state in similar fashion to sterol C-methylations (Woodward et al., 1980), suggesting a structurally similar AdoMet-binding domain amongst these proteins.

The putative active-site cavity of SMTs consists of a central section that contains conserved aromatic and hydrophobic amino acids that face the β-face of the sterol nucleus and surround the sterol side chain (Nes et al., 2002). Affinity labeling and mutational analyses provide information about the size and shape of the enzyme binding pocket and the identity of amino acids involved with catalysis. Studying the SMT from *Sac-*

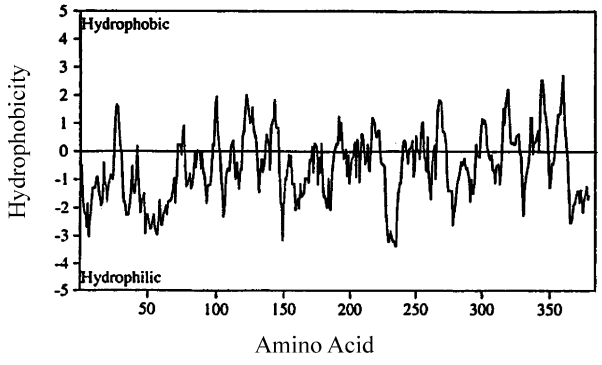


Fig. 3. Hydropathy plot for the *S. cerevisiae* sterol methyl transferase enzyme.

charomyces cerevisiae, we recently discovered a Region I for the sterol binding site through the chemical affinity attachment of 26,27-dehydrozymosterol to the enzyme (Nes et al., 2002). The AdoMet- binding site or Region II has been defined through photoaffinity labeling using radiolabeled AdoMet (Nes et al., 2003b). These affinity-labeling experiments together with directed mutagenesis and sequence homology have identified signature motifs for the sterol (Y81EYGW)- AdoMet (L124D*X*GCGVGGP)- binding sites of the yeast SMT. Whereas Regions I and II are absolutely conserved in all SMT sequences studied to date (Fig. 4), Region I is unique to the SMT and Region II is present generally in AdoMet-dependent methyl transferase enzymes. Regions I and II are always found in the same order on the polypeptide chain and are separated by comparable intervals. Comparing the deduced amino acid sequences of 16 SMT genes from plants and fungi reveals a fair degree of homology among them, ranging from 32 to 80% identity. Predictive secondary structure determi-

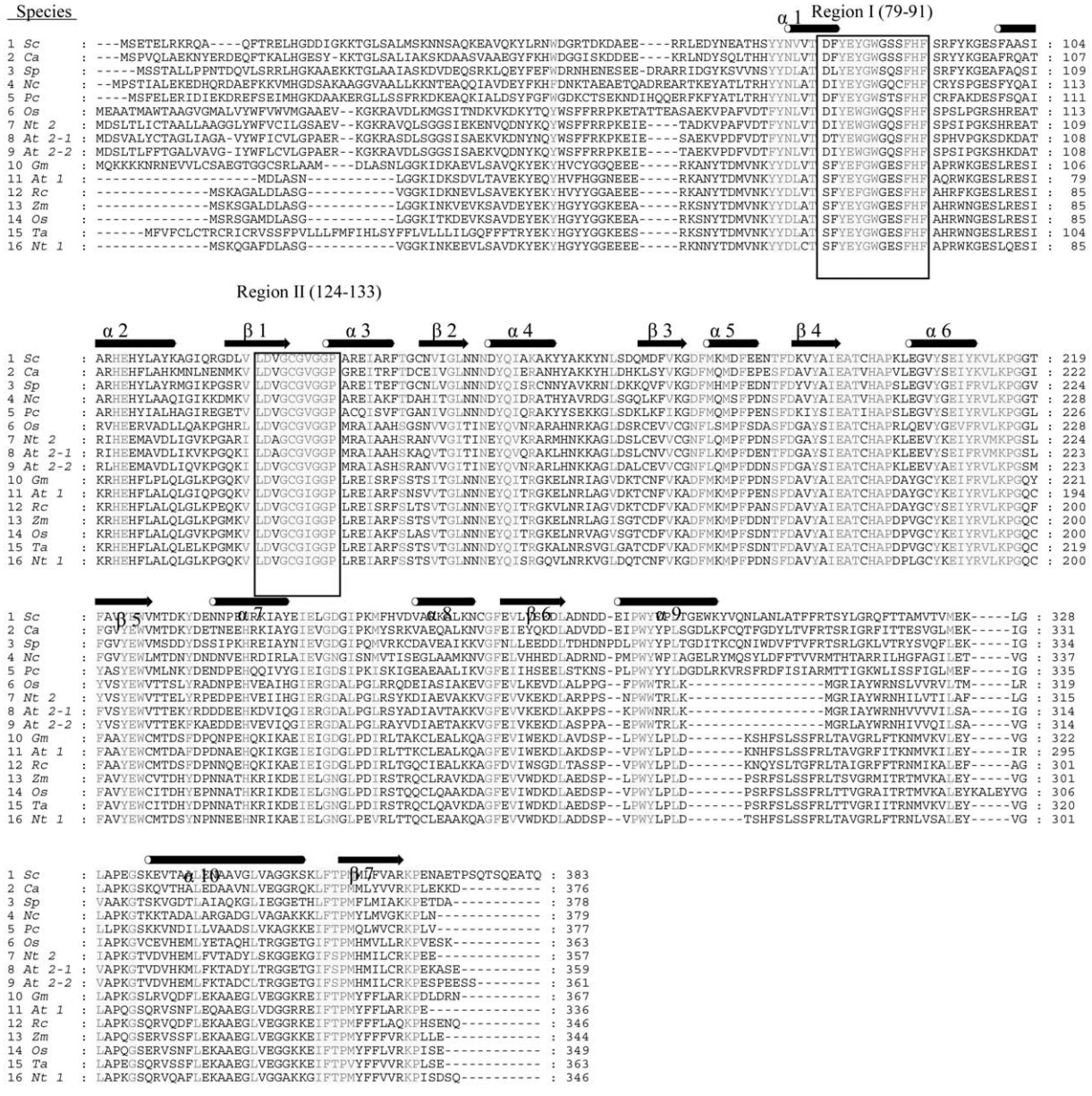


Fig. 4. Alignment of *ERG6* SMT family proteins. Key catalytic signature motifs are boxed; Region I is sterol binding site and Region II is the AdoMet binding site. Secondary structure elements reported as α-helices and β-sheets were determined from homology building with related AdoMet-dependent proteins. The 16 SMTs, indicated by the number on left side are from: 1, *Saccharomyces cerevisiae*, CAA52308; 2, *Candida albicans*, AAC26626; 3, *Schizosaccharomyces pombe*, CAB16897; 4, *Neurospora crassa*, CAB97289; 5, *Pneumocystis carinii*, AAK54439; 6, *Oryza sativa,* AAC34989; 7, *Nicotiana tabacum* 2, T03848; 8, *Arabidopsis thaliana 2-1*, CAA61966; 9, *Arabidopsis thaliana 2-2*, AAB62809; 10, *Glycine max*, AAB04057; 11, *Arabidopsis. thaliana 1*, AAG28462; 12, *Ricinus communis*, AAB62812; 13, *Zea mays*, AAC04265; 14, *Oryza sativa sub sp. Japonica*, AAC34988; 15, *Triticum aestivum*, AAB37769; and 16, *Nicotiana tabacum 1*, AAC34951.

nations, circular dichroism spectral analysis of soybean and yeast SMTs and hydropathy profiles were utilized to search for possible similarities in the conformational features of SMTs. The structures were found to be predominated by α-helices and to have a sequence of alternating α- helices and β-strands that surrounds the putative active center involving Regions I and II, stretching from Y79 to P133 (*ERG*6 nomenclature) (Fig. 5).

Evidence for a shared three-dimensional structure among these proteins is strongly supported by homology building of SMT with related AdoMet-dependent methyl transferase of known structure (Bujnicki et al., 2001; Nes and Bujnicki, unpublished). A key feature of the tertiary structure of AdoMet-dependent methyl transferases is the structural arrangement of their active center. In the three-dimensional perspective of several AdoMet-dependent methyltransferase crystal structures, a similar folding pattern with the central parallel β-sheet surrounded by α-helices is observed (Kagan and Clark, 1994; Niewmierzycka and Clark, 1999; Zubieta et al., 2001; Huang et al., 2002 and references cited therein). This domain is built around a Rossmann fold [β strands that form a four- or five-stranded parallel β sheet with two or three α helices residing on either side] and it appears from sequence alignment that the SMT contains this domain (Region II). It is noteworthy that the bulk of the conserved residues of Regions I and II are primarily hydrophobic and that Region I contains a high percentage of aromatic amino acids which are in close proximity to Region II. This feature, the cationic nature of the *C*-methylation/rearrangement reactions, and literature precedents (Kagan and Clark, 1994; Niewmierzycka and Clark, 1999 and references cited therein) have led us to consider an evolving *steric-electric plug model* (Fig. 6)—whereby a critically positioned deprotonating base, perhaps histidine based on pH profiling (Nes et al., 2003b), and a set of electron-rich aromatic sidechains from tryptophan, tyrosine and phenylalanine

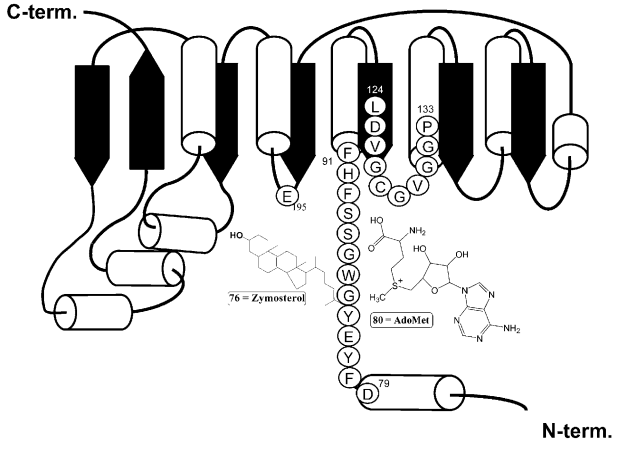


Fig. 5. Schematic secondary structure prediction showing sterol and AdoMet docked into the putative active center lined by residues corresponding to Regions I and II.

based on the cation-π hypothesis of Dougherty (Dougherty, 1996) are essential to proton shuttling and catalysis. Leucine screening (substitution of each all relevant conserved amino acid residues in the enzyme with leucine) of the yeast SMT provide presumptive evidence that one or both Asp276 and Glu195 may be interacting with the C-3 hydroxyl group and or the π system of the Δ^{24}-bond of the sterol directly or by way of a water bridge (Nes et al., 2003b). Within the topography of the active center, aromatic residues can direct folding of the sterol side chain and stabilize positively charged high energy intermediates generated during the reaction progress. Here, the π-systems of indole, phenyl and phenol sidechains from tryptophan, phenylalanine and tyrosine, respectively constitute ''negative point charges'' that can stabilize the side chain motion during the *C*-methylation reaction progress which generates a positively charged high energy intermediate. Aromatic residues can also serve as a counterion that interacts with the positively charged sulfonium on AdoMet via cation-π interactions to assist in AdoMet binding to the SMT (Dougherty, 1996). Relative to true anions such as those in the side chains of glutamic and aspartic acids, we expect that electron-rich π-systems would be less likely to be alkylated by carbenium ion intermediates formed during the coupled methylation–deprotonation reaction. Moreover, if an aromatic amino acid was found to be the counter-ion to AdoMet this would be a fundamental departure from the classic view that an acidic amino acid is spatially disposed to play two roles-as a counter ion to AdoMet and as the deprotonating agent involved in CH_3–CH_2 formation (Rahier et al., 1984). Site-directed mutagenesis experiments have shown that Asp79, Glu82 and Tyr81 serve as a wall in the sterol binding site and interact with the side chain (Nes et al., 1999). Although no crystal structure for a SMT has been determined, the activity and chemical profiles of different suicide substrates treated with SMTs from plant and fungal origin showing highly varied sensitivities in the kill to turnover ratios (Nes et al., 1998c; Nes et al., 1998d; Nes et al., 2002; unpublished), suggest variation in the

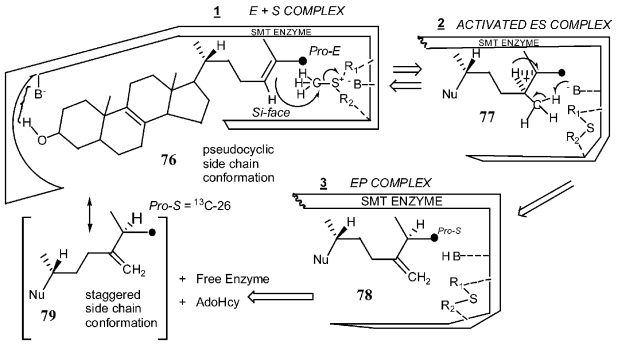


Fig. 6. *Steric-electric plug* model of sterol binding and catalysis by sterol methyl transferase.

identity or position of key amino acids exist amongst the active-site topography of these SMTs.

To compare the phylogenetic relationships among these proteins, a dendogram based on amino acid similarity was produced, as shown in Fig. 7. Taken strictly at face value, the phylogenies of the currently available sequence samples would suggest that the SMT1 and SMT2 groups can be assembled into different categories with the plant SMT1 preceding the plant SMT2 and the plant SMT2 being closely related to fungal SMT1. At present, using amino acid sequence there is no justification for SMT1 and SMT2 enzymes to be organized as they are in the evolution of life. For example, *P. carinii* is identified as an SMT1 according to sequence analyses rather than in a separate SMT2 class. Alternatively, the intrinsic substrate for plant SMTs, cycloartenol **6**, is the most primitive sterol produced by the cyclization of squalene-oxide in organisms with a photosynthetic lineage. Therefore, SMT1 with a preference for cycloartenol **6**, must precede the appearance of SMT2 that accepts 24(28)-methylene lophenol **8**, a metabolite of the cycloartenol **6** *C*-methylation pathway. This observation is in accord with the dendogram for SMT relationships amongst the plant SMTs (Fig. 7). The SMTs can be traced at the level of sequence similarity to a larger super family of AdoMet-dependent methyl transferases with similar catalytic properties, suggesting that several features of these proteins share common ancestral genes that underwent diversification and gene duplication prior to the existence of a completed sterol pathway. That SMTs are so similar in much of their primary sequence to other AdoMet-dependent methyl transferases in spite of taxonomic differences from which they were derived, that they most likely arose by divergent evolution from a common ancestral AdoMet-dependent methyl transferase early in evolution. After inclusion of the sterol binding site into a AdoMet-dependent methyl transferase, the primitive SMT was incorporated into the sterol biosynthetic pathway. At what point the SMT was introduced into the lineage that ultimately gave rise to phytosterols is unclear. In view of the hypothesis that the earliest microbes were non-photosynthetic organisms synthesizing lanosterol (Cavalier-Smith, 1987; Nes et al., 1990) and non-photsynthetic microbes can *C*-methylate lanosterol, the appearance of SMTs may have preceded the origin of photosynthesizing blue–green algae. Indeed, through gene duplication of an ancestral SMT gene at least two lines may have been created—one line gave rise to fungi and a second line introduced the "green" plants. The regulatory strictures by which these variant genes are expressed and their gene product activities controlled will not necessarily operate in a similar manner in the two lines. The different ordering of intermediates, number of SMTs synthesized and feedback responses from membrane inserts that affect SMT activity in plant and fungal systems is consistent with the latter hypothesis.

Kinetic analysis has allowed us to estimate the activation energy for the yeast SMT at 60 kJ/mol, measured over the temperature range 15–45 °C (unpublished). A determination of the reaction course and catalytic competence from steady–state analyses has allowed us to make conclusions as to the transient structural changes that accompany *C*-methylation of the substrate double bond, as envisaged in the *steric-electric plug* model (Fig. 6) (Parker and Nes, 1994). In this model, the enzyme active center is considered to be conformationally fluid to allow for changes in substrate structure and to promote enzyme catalysis using positional-specific olefins. A good substrate would be one that has nucleophilic features at C-3 and C-24, each one being complementary to a specific subsite within the active center. The side chain orientation as well as the location of the double bond in the side chain contribute to catalytic competence. It is generally understood, that the most catalytically favored conformation of the active site is one that does not optimally fit the ground state substrate conformation, but instead is complementary to the transition state of the reaction. For the ground substrate to bind, the enzyme must undergo a conformational deformation that is energetically unfavorable. The sulfonium and ammonium analogs e.g., **48** were designed with the expectation that the

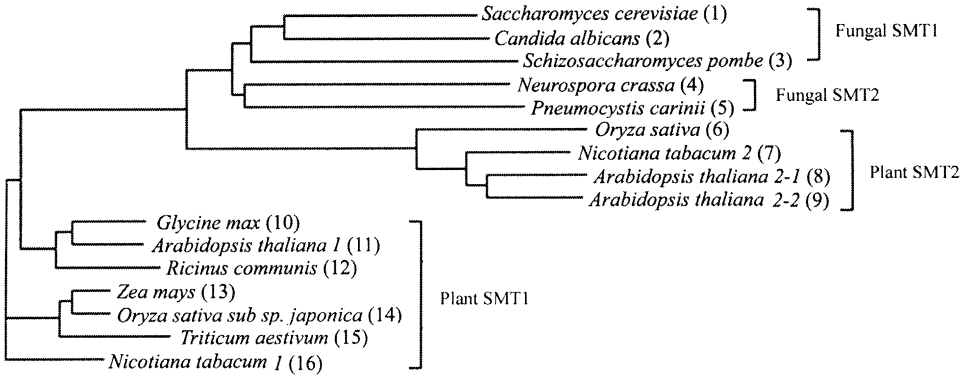


Fig. 7. Dendogram illustrating relationships among SMT family proteins based upon amino acid alignment of *ERG6* yeast family.

SMT in the ground state would recognize the stable "reaction intermediate" mimic and bind it many orders of magnitude more tightly than the substrate which binds in the 10–50 μM range. Based on the K_i values for the 24-alkylsterol analogs active in the nanomolar range, there is a greater affinity for the 24β-methyl olefin than the 24α-methyl olefin and based on product inhibition studies there is a greater affinity for the 24 β-$\Delta^{25(27)}$-structure than the $\Delta^{24(28)}$-structure (Janssen and Nes, 1992; Acuna-Johnson et al., 1997; Mangla and Nes, 2000), suggesting that 24β-sterols are more tightly bound to the enzyme and more energy is required to release these compounds. The SMT does not appear to lower the activation energy to generate the variant olefin bond formations; rather it selects for a single reaction channel by the precise control of substrate conformation and the positioning of counter ions. Although limited information is available for SMTs of prokaryotic origin (cf. accession number for a putative SMT type containing cyanobacterium Synechocystis- S76226-7469487), the operation of the sterol methylation pathway is known in these photosynthetic bacteria (Nes and Nes, 1980; Brocks et al., 1999).

The catalytic specificity and efficiency of SMTs, key features of the *C*-methylation process, are revealed in thermodynamic and kinetic characteristics optimized to accelerate the transformation of substrate to product. More efficient enzymes, and hence more evolutionarily-advanced, (i) can mediate a higher flux of substrate to product than less efficient enzymes, or (ii) can select for substrates that ultimately produce end products of biosynthesis that contribute to the survival and reproductive fitness of the organism. The addition of methyl groups at C-24 affects the physical properties of the enzyme-generated product which is transformed into a membrane insert (Krajewski-Bertrand et al., 1992; Marsan et al., 1996; Schuler et al., 1991). While energy expensive, each addition of methyl generates a 24-alkyl sterol that can affect the fluidity of the plant membrane bilayer in a fashion superior to cholesterol thereby influencing plant physiology where temperature variations occur during the growing season. In the case of fungi, the structure of ergosterol and the genes that generate sterol methylation in these microbes are essential to the manner in which these organisms grow in the wild (Nes et al., 1978; Palermo et al., 1997), suggesting that the evolution of the active center of the SMT is mated to the architectural function sterols play in physiology. We propose that pressure from the environment to improve catalytic effectiveness caused SMT function to evolve early, whereas specificity evolved slowly over time. Once substrate specificity evolves, relaxed substrate and product specificities of "mutant" enzymes were tolerated by a step-wise gain in function—through control of channeling and product diversity to produce new product shapes that optimized cell physiology. Hence, the $\Delta^{24(28)}$-route may be more primitive than the $\Delta^{25(27)}$-pathway with key amino acids in the SMT active center to have perhaps coevolved coincidentally with the $\Delta^{24(28)}$-channel that ultimately gives rise to 24-ethyl sterols. Since structural differences among these substrates are small and local, the observed differences in substrate recognition can be caused by a few amino acid substitutions occurring in the sterol binding domain. Work in progress in several laboratories will shed further light on the functional genomic and evolution of the phytosterol pathway.

Acknowledgements

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