Site-Directed Mutagenesis of the Sterol Methyl Transferase Active Site from *Saccharomyces cerevisiae* Results in Formation of Novel 24-Ethyl Sterols

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$\Delta^{24(28)}$-Sterols are end products of a mono C-methylation pathway catalyzed by the native $\Delta^{24(25)}$- to $\Delta^{24(28)}$-sterol methyl transferase (SMT) enzyme from *Saccharomyces cerevisiae.* Using a Tyr^{81} to Phe mutant SMT enzyme of *S. cerevisiae*, generated by site-directed mutagenesis of a highly conserved residue in the sterol binding site, we found that several $\Delta^{24(25)}$- and $\Delta^{24(28)}$-sterols, which are not substrates for the native protein, were catalyzed to mono- and bis-C24-alkylated side chains. The mutant protein behaved similarly to the native protein in chromatography and in binding zymosterol, the preferred substrate. Zymosterol was converted to fecosterol by the Y81F mutant protein with similar turnover efficiency as the native protein (K_m = 12 μM and k_{cat} = 0.01 s^{-1}); trace 24-ethyl sterols were detected from these incubations. 4α-Methyl zymosterol, which is not a normal substrate for the wild-type SMT enzyme, was converted to 4α-methyl fecosterol in high yield. When fecosterol and 4α-methyl fecosterol were assayed individually at saturating concentrations only fecosterol served as an effective substrate for the second C-transfer step (K_m = 38 μM and k_{cat} = 0.002 s^{-1}), suggesting that successive C-methylation of $\Delta^{24(28)}$-substrates is limited by product release and that molecular recognition of sterol features involves hydrogen bond formation. Isomeric 24-ethyl sterol olefins generated from 24(28)-methylene cholesterol were characterized by chromatographic (GC and HPLC) and spectral methods (MS and ^{1}H NMR), viz., fucosterol, isofucosterol, and clerosterol. Changes in rate of C-methylation and product distributions resulting from deuterium substitution at C28 were used to establish the kinetic isotope effects (KIEs) for the various deprotonations leading to C24-methylene, C24-ethylidene, and C24-ethyl sterols. An isotope effect on C28 methyl deprotonation generated during the first C_1-transfer was detected with zymosterol and desmosterol paired with AdoMet and [$^2H_3$-*methyl*]AdoMet. A similar experiment to test for a KIE generated during the second C_1-transfer reaction with AdoMet paired with 24-(28)-methylenecholesterol and [28-$^2H_2$]24(28)-methylene cholesterol indicated an inverse isotope effect associated with C27 deprotonation. Alteration in the proportion of the C24 alkylated olefinic products generated by the pure Y81F mutant resulted from the suppression of the formation of $\Delta^{24(28)}$-ethylidene sterols (C28 deprotonation) by a primary deuterium isotope effect with a compensating stimulation of the formation of 24-ethyl sterols (C27 deprotonation). Kinetic study on the rate of product formation indicated a normal KIE of k_H/k_D = 2.62 for the first C_1-transfer. Alternatively, an inverse KIE was established with k_H/k_D = 0.9 for the second C_1-transfer resulting from conversion of the 24(28)-double bond (sp^2 hybridization) to a 24β-ethyl group (sp^3 hybridization). From the structures and stereochemical assignments of the C-ethyl olefin products, the stereochemistry of the attack of AdoMet in the second C_1-transfer was found to operate a *Si*-face (backside) attack at C24, analogous to the first C_1-transfer reaction.

Introduction

Sterol methyl transferases (SMT) are a family of *C*-methyl transfer enzymes that catalyze the conversion of olefinic precursors to a variety of 24-methyl and 24-ethyl sterol side chains.[1] Together these enzymes are responsible for the formation of more than 200 distinct sterol side chains in nature.[2] Fungi, like plants and marine organisms, are known to synthesize mono- and bis-C24-alkylated side chains, as shown in Figure 1.[3] Our interest in 24-alkyl sterol diversity and function stems from the SMT enzyme's regulatory properties to control 24-alkyl sterol production, which is necessary for cell growth and reproduction.[3] Earlier studies on the phylogenetic distribution of sterols were interpreted to imply that the evolution of the alkylation mechanism lead to 24β-methyl sterols (C_1-transfer) in primitive organisms, such as fungi and algae, and to 24α-ethyl sterols (C_2-transfer) in advanced organisms, such as vascular plants.[4,5] Mercer and Russell concluded after a review of the

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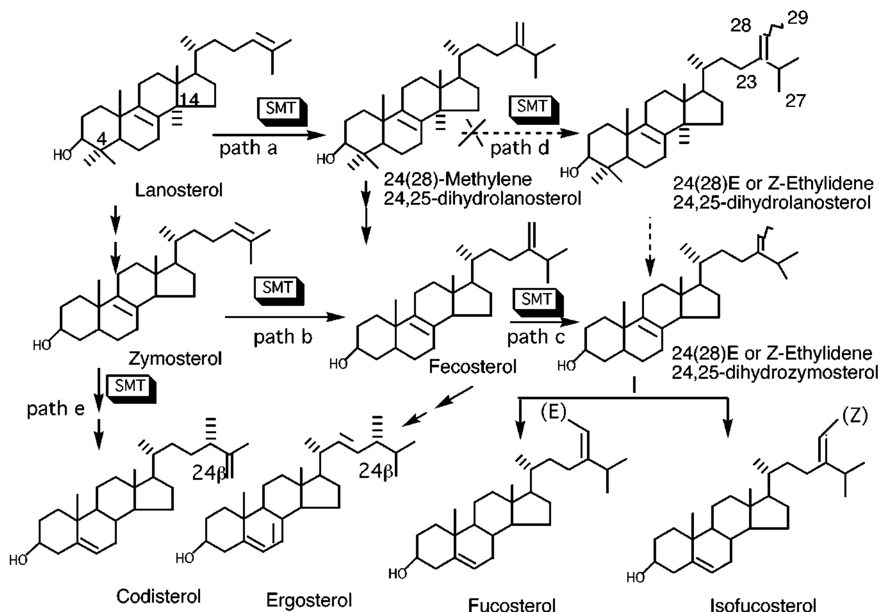

Figure 1. Hypothetical fungal sterol pathways from lanosterol to 24-methyl and 24-ethyl sterol end products.

literature documenting the number of deuterium atoms incorporated at C24 of organisms incubated with [d_3-*methyl*]methionine that different organisms introduce the *C*-methyl group by different mechanisms.[5] Goodwin subsequently suggested that migration of the hydrogen atom at C24 from either the *Re*-face or the *Si*-face of the $\Delta^{24,25}$-bond, following C-methylation of the Δ^{24}-bond, mediates the formation of the stereochemically opposite C24 stereochemistries.[6] On the basis of these and related experiments involving gene cloning and expression of SMTs, one group of investigators propose that differences in the degree of C-methylation at C24 results from distinct classes of SMT enzymes that catalyze either the first or second C_1-transfers,[7] whereas a second group of investigators propose that a single SMT enzyme can give rise to alternate and/or sequential enzyme activities.[2e,8]

In our search for an explanation for the operation of different SMT-catalyzed pathways in nature, we considered the possibility that a family of mechanistically similar SMT enzymes exists that operate C-methylation reactions by the same *Si*-face (β-face attack) mechanism. These enzymes can be distinguished from one another by the topology of the active site and alternate binding orientations of the sterol substrate, viz., the steric-electric plug model (Figure 2).[9] We proposed that plants and fungi synthesize three major classes of SMT enzymes; SMTI, SMTII, and SMTIII. SMTI generates $\Delta^{24(28)}$-sterols, SMTII generates $\Delta^{23(24)}$-sterols, although this enzyme species may also generate $\Delta^{24(28)}$-sterols from the same enzyme surface, thereby allowing for the second C-methylation to occur, and SMTIII generates $\Delta^{25(27)}$-sterols, although this enzyme species may also generate $\Delta^{24(28)}$-sterols from the same enzyme surface, thereby allowing for the second C-methylation to occur.[10] Each of these enzymes should bind and catalyze flat sterols with appropriate nucleophilic features at either pole of the substrate. We also considered that 24α-alkyl sterols are not synthesized by SMT enzymes directly; rather, they are formed by a reductase-type enzyme following C-methylation of the Δ^{24}-bond.[11] Therefore, it was not unexpected to find recently that SMT enzymes that give rise to $\Delta^{24(28)}$-sterol side chains by a *Si*-face mechanism

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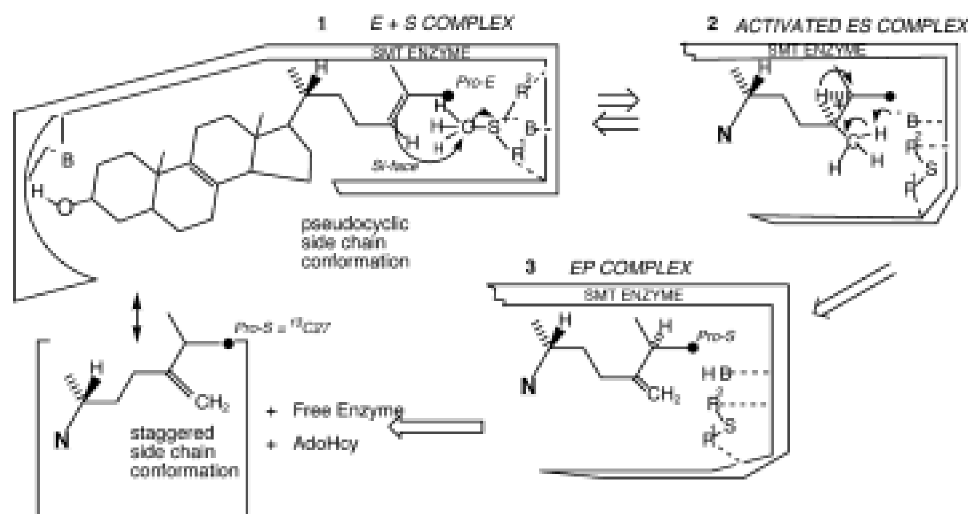

Figure 2. Hypothetical "steric-electric plug" model for the ternary complex of sterol-AdoMet-SMT and putative conformational changes in the enzyme and sterol side chain that occur during *Si*-face (β-face attack) C-methylation of the Δ^{24}-bond to give the $\Delta^{24(28)}$-structure. E = enzyme, S = substrate, P = product, B = base (enzymatic nucleophile).

show similarity in much of their primary structure and to contain four conserved regions; one region characteristic of SMT enzymes contains multiple aromatic amino acids that can give rise to a hydrophobic pocket for sterol binding and catalysis.[10] Still unexplained is the occurrence of isomeric 24-ethylidene sterols in fungi,[2a,c,d,h] the contribution of molecular recognition to stereoselective C-methylation activity, whether distinct enzymes are involved in successive C-methylation of the Δ^{24}-bond, and the nature of amino acids involved in substrate binding and catalysis.

In this paper, we define the last remaining stereochemical elements that have remained unexplained and determined the number of enzymes involved in double C-methylation of the Δ^{24}-bond by evaluating a mutant SMT enzyme of *S. cerevisiae*. Prior to studies on site-directed mutagenesis of the fungal SMT enzyme, we subcloned and overexpressed the native SMT enzyme from *S. cerevisiae* in *Escherichia coli.*[10] The resulting SMT enzyme was purified and found to be a homotetramer of 43 kD identical subunits. The pure SMT enzyme exhibited cooperativity among its subunits and was subject to down-regulation from ergosterol (24β-methyl cholesta-5,7,22(*E*)-trien-3β-ol) due to the C24 methyl group in the side chain.[12] The conserved aromatic-rich amino acid sequence unique to the yeast and related SMT enzymes has been referred to as Region I.[11b] The motif in the yeast SMT stretches between positions 79-D**F**Y**E**Y**G**W**G**SS**F**H**F**S-92. Region I has recently been found to be associated with the sterol binding site by chemical affinity labeling with [3-^{3}H]26,27-dehydrozymosterol.[13] In a preliminary examination of Region I by site-directed mutagenesis, a single active site residue Trp-85 was substituted with Ala and Phe.[11] The W85A mutant failed to bind zymosterol, consistent with Region I acting as the sterol binding site. Alternatively, the W85F mutant behaved similarly to the wild-type protein with respect to sterol specificity and C-methylation kinetics.[11,12] These results indicate that our hypothesis that W85 performs a dual function as a counterion and as deprotonating agent[12a] is incorrect. Nonetheless, the aromatic amino acid at position-85 may contribute to the energetically favorable binding orientation(s) of sterol in the ternary complex. A second aromatic amino acid residue has now been mutated at Tyr-81 to Ala and to Phe. The present study was undertaken to determine the resulting specificity in binding of a series of structurally related Δ^{24}-sterol substrates to characterize product distributions and to establish the degree of "isotopically sensitive branching"[14] catalyzed by these two new mutant SMT enzymes.

Results and Discussion

Expression of Mutant SMT in *E. coli* Cells and Protein Purification. Using the expression and protein purification systems for mutant SMTs that we developed earlier for generation of the recombinant native SMT, we observed that the mutant and wild-type SMT enzymes were expressed to similar levels in *E. coli*,[10] to approximately 50% of the cellular protein as estimated from SDS−PAGE of the crude homogenates. Starting from 9.0 g of BL21(DE3) cells, 15 mg of relatively pure recombinant protein is recovered after two chromatographic steps. Purification of SMT involves a series of fractionations: crude homogenate, 100000*g* supernatant, ammonium sulfate precipitation, Q-Sepharose, and ω-aminodecyl agarose (hydrophobic interaction chromatography: HIC). In two cases, the native construct and Y81F mutant, the purified protein showed a single band on SDS−PAGE with a molecular weight of 43 kDa as predicted by the complementary DNA sequence. However, the Y81A mutant behaved differently during purification, which interfered with our ability to purify the mutant protein to homogeneity. Nonetheless, Y81A was overexpressed as determined by SDS−PAGE analysis of the crude homogenates. To test for activity, incubations with Y81A were performed using crude homogenates. Incubation with [^{3}H$_3$-*methyl*]AdoMet paired with zymo-

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sterol (**A1**), desmosterol (**B1**), 24(28)-methylene cholesterol (**B3**), and fecosterol (**A3**) failed to generate any active C-methylated sterol products, even at the highest concentration of sterol, AdoMet, and protein tested, ca. 1 mg of each in the reaction mixture. Using similar assay conditions, the native SMT from *E. coli* routinely generates 6.8 μmol of fecosterol per milligram of protein. Therefore, the Y81A mutant was not studied further.

Using the same chromatographic steps and amount of bacterial cells to generate the native protein for Y81F, the mutant was found to purify to homogeneity identically to the wild-type protein. Incubations were performed with labeled and nonlabeled substrates with pure Y81F protein in a manner similar to that described for the native SMT. Incubation of pure Y81F mutant with zymosterol paired with AdoMet generated a [^{3}H]C-methylated product in the nonsaponifiable lipid fraction of the quenched reaction mixture, tentatively considered to be [28-$^3H_2$]fecosterol. When fecosterol was paired with AdoMet, low levels of C-methylation were apparent from the radioactivity present in the nonsaponifiable lipid fraction. Therefore, additional study of the Y81F mutant was undertaken to establish the identity of the C-methylated products and scope of the C-methylation reaction using $\Delta^{24(25)}$- and $\Delta^{24(28)}$-sterol substrates.

Initial experiments to identify the reaction products were performed with saturating amounts of desmosterol (**B1**) and zymosterol (**A1**) incubated with a total of 500 μg of pure SMT protein for 15 h. From these incubations ca. 100 μg of total sterol was recovered, as determined by GC–MS. From analysis of the total ion current chromatograms and mass spectral fragmentation patterns, ca. 99% of the total 24-alkyl sterol product was identified as 24(28)-methylene sterol, and 1% of the total 24-alkyl sterol product was identified as a mixture of three 24-ethyl sterols (cf. Supporting Information). The absence of significant formation of 24-ethylated sterols from incubation with the $\Delta^{24(25)}$-sterol substrate indicates that the $\Delta^{24(28)}$-methylene sterol product is not an enzymic intermediate in the reaction sequence leading to successive double methylation of a Δ^{24}-substrate.

Structure–Function Analysis. To gain greater insight into the structural features of sterols required for catalysis by Y81F, we examined the reactivity of a series of sterol acceptor molecules, all of which have been tested earlier with the native SMT enzyme from yeast. Ten sterols[12] were assayed with the Y81F mutant to test for catalytic competence (Scheme 1, Table 1). Zymosterol (**A1**), the preferred substrate for the wild-type enzyme, was tested relative to 3-desoxy zymosterol (**G1**), 4α-methyl zymosterol (**C1**), lanosterol (**E1**), and cycloartenol (**F1**) in order to determine the essential nature of the C3-hydroxyl group in binding and to assess recognition of substrate methyl groups at C4 that affect the hydrogen-bonding abilities of the C3-hydroxyl group. Zymosterol was bound by the enzyme, whereas lanosterol, cycloartenol, and 3-desoxy zymosterol were bound nonproductively, as to be expected. However, uncharacteristic of the yeast SMT enzyme[12] was the observation that 4α-methyl zymosterol (**C1**), with an equatorial C4 methyl group, was converted to 4α-methyl fecosterol (**C3**) in high yield (ca. 50% conversion): Preparative scale incubation of Y81F with AdoMet and 4α-methyl zymosterol gave a single C-methylated product, 4α-methyl fecosterol (**C3**): GLC, RRT$_c$ 1.33; TLC, R_f 0.46; MS, M$^+$ 412, 397, 394, 379, 357, 313, 285, 267, 245, 241, 227); ^{1}H NMR, H18, 0.611 (s), H19, 0.975 (s), H21, 0.958 (d, J = 6.5 Hz), H26, 1.030 (d, J = 7.5 Hz), H27, 1.020 (d, J = 6.9 Hz), H28 δ at 4.714 (s) and 4.661 (s) representing two olefinic protons resonating at distinct δ values, H31, 0.994 (d, J = 6. 4 Hz). These structural assignments are consistent with that of 4α-methyl zymosterol isolated from yeast by Barton et al. 30 years ago.[15] The consensus of opinion has been that lanosterol conversion to ergosterol in yeast proceeds by path b, rather than path a, which can generate 4α-methyl fecosterol as an intermediate in the biosynthesis of fecosterol (Figure 1).[15,16] Neither 24(28)-methylenelophenol (**D3**) (the preferred substrate for the second C$_1$-transfer catalyzed by plant SMTs) nor 4α-methyl fecosterol (**C2**) were substrates for the second C-methylation reaction. Desmosterol K_m 15 μM (**B1**) was recognized about as effectively as zymosterol 10 μM (**A1**) by Y81F, consistent with the recognition of this pair of sterols by the native protein.[12b] Alternatively, higher affinity for 24(28)-methylene cholesterol (**B3**) and fecosterol (**A3**) was observed in the mutant compared to the wild-type SMT. The latter observation is based on the K_i values of 24(28)-methylene cholesterol (**B3**) and fecosterol (**A3**), 200 and 54 μM, respectively, tested as inhibitors with the native SMT,[12a] compared with the K_m values of **B3**, 100 μM, and **A3**, 38 μM, tested as substrates with the mutant enzyme. Clearly, modifications in product distribution observed uniquely in Y81F are related to the

Scheme 1

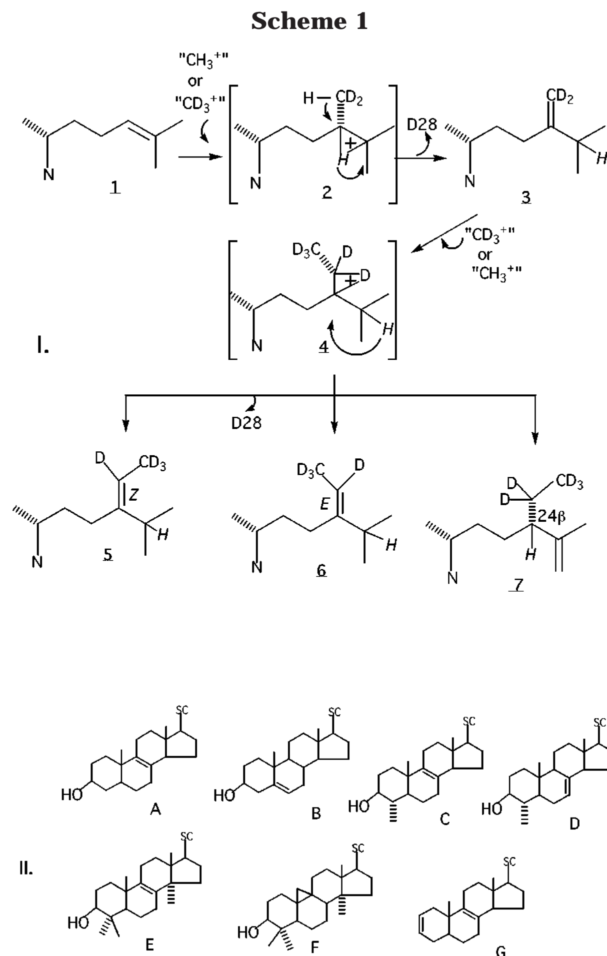

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Table 1. Kinetic Parameters of Mutant Y81F Sterol Methyl Transferase from *Saccharomyces cerevisiae*

substrate		K_m[a] (μM)	k_{cat}[b] (s^{-1})	k_{cat}/K_m ($s^{-1}\ \mu M^{-1}$)	catalytic competence (%) Y81F[c]	native protein[d]
zymosterol	**A1**	12	10.9×10^{-3}	9.08×10^{-4}	100	100
fecosterol	**A3**	38	2.67×10^{-3}	0.70×10^{-4}	8	NA
desmosterol	**B1**	15	2.05×10^{-3}	1.37×10^{-4}	15	29
desmosterol and [$^2H_3$-*methyl*]AdoMet	**B1**	10	0.79×10^{-3}	0.79×10^{-4}	8	
24(28)-methylene cholesterol	**B3**	100	9.37×10^{-3}	0.94×10^{-4}	10	NA
[28-$^2H_2$]24(28)-methylene cholesterol	**B3**	105	10.4×10^{-3}	0.99×10^{-4}	11	NA
4α-methyl zymosterol	**C1**	14	5.26×10^{-3}	3.76×10^{-4}	41	NA
4α-methyl fecosterol	**C3**	NA				
24(28)-methylene lophenol	**D3**	NA				
lanosterol	**E1**	NA				
cycloartenol	**F1**	NA				
[3-^{3}H]zymosterol and [$^2H_3$-*methyl*]AdoMet	**A1**	10	4.10×10^{-3}	3.42×10^{-4}	38	90
[3-^{3}H]zymosterol and AdoMet	**A1**	12	10.8×10^{-3}	9.08×10^{-4}	100	100
3-desoxy zymosterol	**G1**	NA				

[a] Michaelis–Menten constants were measured at 29 °C as described in the Experimental Section. NA, not active due to lack of productive binding; unless otherwise noted sterols were paired with AdoMet isotopically diluted with catalytic amounts of [$^3H_3$-*methyl*]AdoMet. [b] Per molecule of homotetramer; each identical subunit has a molecular weight of 43 kDa. [c] This study. [d] Data from ref 12.

point mutation of Tyr81 to Phe that affects the substrate binding (specificity) and the relationship of binding energy to catalysis (approximation effects).

Structure and Stereochemistry of *C*-Methyl Products from First and Second C_1-Transfer Reactions. Product structure and stereochemistry were determined following preparative-scale incubations with nonisotopically labeled and deuterium labeled substrates and HPLC isolations of each olefin. Desmosterol and zymosterol were incubated with Y81F, and both substrates generated a single C-methylated product with the $\Delta^{24(28)}$-structure, similar in structure to the products generated by the native protein. No alternate isomeric C-methylated products were detected by GC–MS analysis from the reaction mixture containing either desmosterol or zymosterol. Confirmation of the exomethylene structure of 24(28)-methylene cholesterol and of fecosterol synthesized by Y81F was by co-injection on GLC and HPLC with authentic specimens and by MS and ^{1}H NMR analysis.[12a,b] Because of a paucity of zymosterol substrate, we turned our attention to study the metabolic fate of 24(28)-methylene cholesterol, which was available to us in significant amounts. Chromatographic purification on reversed-phase HPLC of the crude nonsaponifiable lipid fraction of a preparative scale incubation with 24(28)-methylene cholesterol paired with AdoMet yielded four sterols: **B3** (6.90 mg), **B7** (0.56 mg), **B6** (2.24 mg), and **B5** (1.12 mg). **B3** was determined by GC–MS and ^{1}H NMR to be substrate and to be uncontaminated with other sterols. The RRT_c and MS fragmentation patterns of **B6**, **B5**, and **B7** were identical to that of authentic fucosterol, isofucosterol, and clerosterol specimens.[17] Further confirmation of the 24-ethylated sterol stereochemistry was established by ^{1}H NMR spectroscopy (300 MHz). The ^{1}H NMR spectrum of fucosterol **B6** showed the presence of two methyl singlets for H18 and H19 (δ 0.687 and 1.011), four methyl doublets for H26/H27, H21, and H29 (δ 0.980 6H, 0.989 3H, 1.572 3H), two olefinic protons for H6 and H28 (δ 5.35 br d, 1H and 5.18 m, 1H), a proton geminal to a 3β-hydroxyl group (δ 3.51 m, 1H), and a signal for H25 (δ 2.21 sept., 1H J = 7.0 Hz), which is diagnostic for the *E*-geometry of the 24-ethylidene group.[8c,18] The ^{1}H NMR spectrum of isofucosterol **B5** showed the presence of two methyl singlets for H18 and H19 (δ 0.682 and 1.010), four methyl doublets for H26/H27, H21, and H29 (δ 0.980 6H, 0. 0.946 3H, 1.59 3H), two olefinic protons for H6 and H28 (δ 5.35 br d, 1H and 5.11 m, 1H), a proton geminal to a 3β-hydroxyl group (δ 3.51 m, 1H), and a signal for H25 (δ 2.831 sept, 1H, J = 7.2 Hz), which characterizes the *Z*-geometry of the 24-ethylidene group. The ^{1}H NMR spectrum of clerosterol **B7** showed three methyl singlets for H18, H19, and H26 (δ 0.671, 1.007, and 1.566), one doublet for H21 (δ 0.905), three olefinic protons for H6 (d 5.36 br d, 1H) and H27 (4.643 and 4.724 2H, br s), one triplet for H29 (δ 0.800 t, J = 7.3 Hz), and a proton geminal to a 3β-hydroxyl group (δ 3.51 m, 1H). No evidence was found of signals for H21 deshielded to δ 0.914 and for H29 shielded to δ 0.796, which are observed in the ^{1}H NMR spectrum of 24-epiclerosterol,[19] thereby proving that our sample of **B7** was epimerically pure at C-24. There was no evidence for synthesis of $\Delta^{25(27)}$-sterols following the first C_1-transfer step in the Y81F mutant enzyme, suggesting that the side chain with the $\Delta^{24(25)}$-bond is positioned differently in the active site from the orientation of the side chain with the $\Delta^{24(28)}$-bond bound to the enzyme.

To pursue these studies further, we developed a hypothesis involving isotopically sensitive branching for the enzyme-catalyzed C-methylation of a $\Delta^{24(25)}$-sterol to a $\Delta^{24(28)}$- or $\Delta^{25(27)}$-sterol (first C_1-transfer), which is applicable with minor modification to the second C_1-transfer reaction. Isotopically sensitive branching is defined as a rate enhancement in the formation of one product of a multiple-product enzyme caused by a reduction of the rate constant for the formation of a second product due to isotopic substitution in the substrate. The rate enhancement is defined as an induced kinetic isotope effect, and such an observation indicates that the resulting C-methylated olefins arise from a common intermediate formed at the same active site and, by implication, from the same enzyme. For the sterol methyl transferase-catalyzed reaction, a simplified kinetic model for the generation of two products can be described as shown in Scheme 1.

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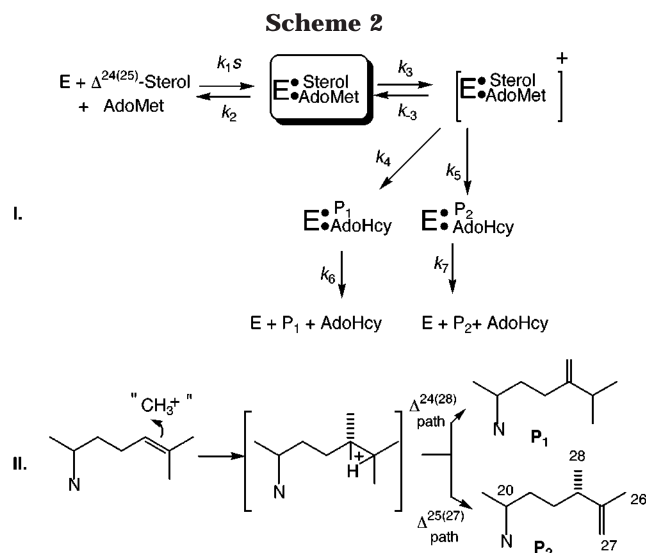
Scheme 2



$E^{\bullet Sterol}_{\bullet AdoMet}$ represents the ternary complex in the ground state, k_1 and k_2 are the binding constants, k_3 is taken as the rate constant for the irreversible dative bond formation step that leads to the branch point, high energy intermediate $[E^{\bullet Sterol}_{\bullet AdoMet}]^+$, and k_4 and k_5 are the rate constants for the competing deprotonations at C27 and C28 (and the dissociation steps) leading to the isomeric olefin products **P1** ($\Delta^{24(28)}$ and **P2** ($\Delta^{25(27)}$). The step represented by k_3 also involves conformational changes in the enzyme that modify the structure of the ground-state ternary complex to a catalytically competent one that recognizes the deuterium-sensitive step.

The formation of side chains of **B5**, **B6**, and **B7** can be explained in each case by a change in sterol specificity and binding orientation in the ternary complex that allows for electrophilic alkylation of a 24(28)-methylene sterol (Scheme 2). The steric course of C-methylation depends on the nature of the methylation−deprotonation step and the juxtaposition of the methyl donor and proton abstracting base relative to the C=C bond of the intermediate sterol acceptor molecule. Assuming a protein conformation change is accompanied by a methyl-addition reaction at C24 of the sterol acceptor molecule and that regiospecific proton abstraction from C28 and C27 to generate the $\Delta^{24(28)}$ and $\Delta^{25(27)}$ bond structures is directed from the same side of the enzyme-bound side chain, the possibility is raised that the same base controls deprotonation of the high energy intermediates **2** and **4** (Scheme 2). To further examine our hypothesis, pairs of deuterium-labeled and nonisotopically labeled 24(28)-methylene cholesterol with AdoMet were tested with Y81F.

Deuterium Incorporation Studies. The results of incubation of 24(28)-methylenecholesterol and [28-$^2H_2$]-24(28)-methylenecholesterol paired with AdoMet or [$^2H_3$-*methyl*]AdoMet are summarized in Table 2 (cf. Supporting Information). Following HPLC purification, the MS of [28-$^2H_2$]24(28)-methylenecholesterol indicated the incorporation of two deuterium atoms (Table 2). ^{1}H NMR analysis confirmed the deuterium atoms were located at C28 by the diminished intensity of signals for H28. Mass spectral analysis of the deuterium content in the 24-ethyl ^{2}H-labeled compounds clearly demonstrated that each of the products retained a minimum of 89−98% of the original deuterium present in the substrate. The MS of deuterated fucosterol, isofucosterol, and clerosterol showed the incorporation of three deuterium atoms each into the products when the deuterated substrate is [$^2H_3$-*methyl*]-AdoMet, one deuterium atom each into 28-^{2}H fucosterol and isofucosterol, and two deuterium atoms into 28-^{2}H clerosterol when the deuterated substrate is [28-$^2H_2$]-24(28)-methylenecholesterol. Ion clusters containing the side chain moiety [M − 33] ($M^+ - 33$, amu = apparent molecular ion minus methyl minus water) also gave peaks indicating incorporation of the predicted number of deuterium atoms introduced into the sterol side chain. On the basis of these findings, the second C_1-transfer cannot proceed by a stepwise C-methylation reaction in which fucosterol and isofucosterol are intermediates to clerosterol; rather, each of the three 24-ethyl sterol olefins is a direct product of C-methylation.

From the incubation of 24(28)-methylenecholesterol paired with [$^2H_3$-*methyl*]AdoMet was isolated [29-$^2H_3$]-fucosterol (ca. 200 μg) by HPLC. Mass spectral analysis of the doubly alkylated sterol showed it to possess M^+ 415 amu, indicating incorporation of three deuterium atoms at C29 (Table 2). The ^{1}H NMR analysis of the trideuterated fucosterol was similar to unlabeled fucosterol, except that the 28H had shifted from δ 5.18 to 5.17 (α-deuterium shift) and C29 at δ 5.17 had disappeared consistent with complete substitution of C29 with three deuterium atoms. The stereochemistry of the 1,2-hydride shift of H25 to C24 (a reversal from that which occurs during the first C_1-transfer) attending clerosterol formation indicates that corner to corner migration in the symmetrical bridged intermediate **B4** proceeds stereoselectively at the same active site that generates fucosterol and isofucosterol. Since the stereochemistry of the ethyl group at C24 of clerosterol has been established to be β-oriented and fucosterol and isofucosterol are generated from the same high energy intermediate as clerosterol, for mechanistic reasons, the three 24-ethyl sterol products must be generated by a common C-methylation pathway that involves *Si*-face attack.

Kinetic Isotope Effects. The effects of deuterium substitution at C28 on the total rate of conversion of $\Delta^{24(25)}$-sterols to products were evaluated with [3-^{3}H]-zymosterol paired with [$^2H_3$-*methyl*]AdoMet and desmosterol paired with [$^2H_3$-*methyl*]AdoMet (which was isotopically diluted with catalytic amounts of [$^3H_3$-*methyl*]AdoMet) at saturation to avoid any significant intermolecular V_{max}/K_m isotope effect discriminating against C-methylation of the deuterated substrates (Table 1). The intramolecular KIEs for the two pairs of compounds were estimated to be $k_{CH2}/k_{CD2} = 2.50$ and $k_{CH2}/k_{CD2} = 2.61$, respectively. The similar KIEs from the two incubations indicate that the observed KIEs were the intrinsic isotope effects on C28 deprotonation and confirm that the primary deuterium KIEs on C-methylation of sterols was dependent on the nature of the resulting high energy intermediate formed (Scheme 2) and relatively independent of the double bond condition in the nucleus, Δ^5 or Δ^8. The KIE on the C-methylation pathway catalyzed by the native protein was found to be ca. 1.00,[12c] suggesting the Y81F mutation altered the C-methylation pathway, either at the level of product release or in a step of the chemical reaction.

When the Y81F mutant was incubated with [28-^{2}H]-24(28)-methylene cholesterol or 24(28)-methylene sterol (either as a Δ^5- or Δ^8-sterol substrate) paired with AdoMet and [$^2H_3$-*methyl*]AdoMet, GC−MS analysis in-

Table 2. GC–MS Analysis and Deuterium Content of 24-Alkylsterols Biosynthesized by Y81F Enzyme-Catalyzed C-Methylation of Different Paired Substrates[a]

substrate pair	labeled products	GC–MS		isotopic composition (%)		
		M^+	$M^+ - CH_3 - H_2O$	d_1	d_2	d_3
desmosterol and [$^2H_3$-*methyl*]AdoMet	[28-$^2H_2$]-24(28)-methylenecholesterol	400	367	7.1	92.3	
	[29-$^2H_3$]clerosterol	415	382	1.54	0.59	97.7
24(28)-methylenecholesterol and [$^2H_3$-*methyl*]AdoMet	[29-$^2H_3$]fucosterol	415	382	3.1	7.1	89.8
	[29-$^2H_3$]isofucosterol	415	382	2.2	10.2	87.6
	[28-$^2H_2$]clerosterol	414	381	4.4	95.6	
[28-$^2H_2$]-24(28)-methylenecholesterol and AdoMet	[28-^{2}H]fucosterol	413	380	85.8		
	[28-^{2}H]isofucosterol	413	380	88.8		

[a] [28-^{2}H]-24(28)-methylenecholesterol > 92 atom % deuterium, [$^2H_3$-*methyl*]AdoMet > 98 atom % deuterium. Isotopic abundances were calculated from the *m*/*z* 400–418 peak intensities and corrected for contributions of natural isotopic abundances as described in the Experimental Section. No correction was made for the ^{12}C content of the deuterated substrate.

dicated an alteration in proportions of the olefinic products. The modified 24-alkyl sterol composition was associated with a suppression in the formation of fucosterol and isofucosterol (C28 deprotonation) by a primary deuterium isotope effect accompanied by a compensating stimulation in formation of clerosterol (C27 deprotonation). Using similar enzyme preparations and incubation conditions that generated similar amounts of 24-ethyl sterol products after 15 h, we found that incubation with 24(28)-methylene cholesterol paired with AdoMet exhibited on GC a ratio of clerosterol to fucosterol and isofucosterol of 8:92 and from incubation with [28-^{2}H]-24(28)-methylene cholesterol paired with AdoMet, a ratio of 25:75. The significant increase in clerosterol accompanied by a reduction in fucosterol and isofucosterol in the total sterol mixture indicates isotopically sensitive branching from a common intermediate **B4**. Isotopically sensitive branching in Y81F appears to be unique to the second C_1-transfer step. Rate changes in clerosterol formation from deuterium substitution resulted from the change in sp^2 to sp^3 hybridization that follows transformation of the trigonal substrate (**B3**) to the tetrahedral product (**B7**). The change in hybridization in the bridged carbenium ion intermediate lead to the rate enhancement. Detection of the inverse effect indicates that the KIE on C28 deprotonation in the formation of fucosterol and isofucosterol must have approached unity (k_H/k_D = 1. 00), similar to the observation for the side chain methyl-deprotonation leading to the generation of the $\Delta^{24(28)}$-structure by the native enzyme[8d,12b] and corresponding KIEs of k^H/k^D = 1.0 and 2.0 for $\Delta^{24(28)}$- and $\Delta^{23(24)}$-methyl-deprotonation leading to the generation of 24(28)-methylene cycloartanol and cyclosadol, respectively, in phytosterol synthesis.[8g]

Conclusions. It is clear from our studies using a set of genetically related SMTs, viz., as native forms and mutant forms derived from the native construct, that changes in sterol specificity and overall rates of *C*-methyl olefin biosynthesis can affect product distributions and regulate the number of C_1-transfer reactions catalyzed by a SMT enzyme. The kinetics of C-methylation activity and alteration in product ratios resulting from deuterium substitution permitted corroboration of isotopically sensitive branching in sterol methylation[8] and confirmed our suggestion[8c,g] that a single SMT enzyme can give rise to the first and second C_1-transfer reactions. With one enzyme catalyzing the first and second C_1-transfer reactions to give rise to one or multiple products, the observed specificity and change in the product ratio can be explained by the enzyme's molecular recognition of specific subsites on the sterol molecule (the proximal end containing the C3-hydroxyl group and the terminal end containing the Δ^{24}-bond) and a competition between the rates of two proton removal steps, with the isotope effect slowing down the removal of deuterium and therefore favoring the formation of the other product. Had two enzymes been involved (which is not the case for studies with the Y81F mutant), the change in the product ratio can be accounted for by a lack of sterol specificity or the proton removal being the rate-limiting step which leads to the slower formation of the only product derived by deuterium elimination. The kinetic data obtained on Y81F indicate that turnover during the second C_1-transfer of the $\Delta^{24(28)}$-substrate may be limited by a step following the initial reaction of the $\Delta^{24(25)}$-substrate (zymosterol), which may explain the confusion in the field in realizing the number of SMTs involved in catalysis and the role of sterol specificity in pathway sequencing in phytosterol synthesis.

The general picture that emerges from these and related studies is one in which the coupled methylation–deprotonation of the steroid Δ^{24}-bond is stereospecific. The spatial disposition of the $\Delta^{24(25)}$- and $\Delta^{24(28)}$-methylene bonds to AdoMet and the enzymic nucleophile (base) in the ternary complex holds the key to the generation of the β-face C-methyl attack, as evidenced clearly in the C_2-specific C24β-chirality of clerosterol. Thus, although the structure of the first C_1-transfer reaction is different from the structure of the products generated from the second C_1-transfer reaction, catalysis to achieve an overall stereochemical outcome that is similar in the two C-methylation cases must result from different side chain conformations of these olefins obtained at initial binding. That the $\Delta^{24(25)}$-sterol substrates do not produce a product set identical in composition to that produced by the $\Delta^{24(28)}$-sterol substrates with the Y81F mutant is further reason to believe that $\Delta^{24(25)}$- and $\Delta^{24(28)}$-sterol substrates are bound to the SMT with different side chain conformations.

The ability to tailor genetically the native yeast SMT enzyme to perform a second C-methylation step from the same enzyme surface that catalyzes the first C_1-transfer indicates that the stereochemically ordered C-methylation–deprotonation sequence to form multiple products arises by alternate deprotonations of a common high energy C-methylated intermediate. The second C_1-transfer follows the same steric course of C-methylation as found during the first C_1-transfer (i.e., β-face attack), suggesting that deprotonation from C28 and C27 methyl groups leading to 24(28)-methylene structure is mediated

by the same enzyme base that is used in deprotonation of the C28 methyl group during the first C_1-transfer. This base is most likely a residue of the native protein, but inaccessible to participate in catalysis of 24(28)-methylenecholesterol under normal physiological conditions. Plausible candidates for the immediate proton acceptor (B) would be a charged amino acid, such as Asp79 or Glu82 in Region 1, whereas the aromatic residues in Region 1 may serve to maintain a hydrophobic pocket and possibly to engage in hydrogen bonding activities with the C3-hydroxyl group. Thus, we propose that minor changes in the topography of the active site can lead to (evolve) differences in sterol specificity and product distributions, the basis for structural and functional diversification of SMT enzymes in nature.

Experimental Section

Instrumentation and General Methods. General instrumentation, sources of reagents, sterol substrates, AdoMet, [$^3H_3$-*methyl*], AdoMet (10−15 Ci-mmol), [$^2H_3$-*methyl*]AdoMet, [3-^{3}H-zymosterol] (55 μCi/μmol), recombinant SMT from *E. coli* BL21(DE3) cells and chromatographic materials were as described in our earlier papers.[8c,f,10−12] [28-$^2H_2$]24(28)-Methylene sterol substrates were generated by incubating the native recombinant yeast SMT enzyme with zymosterol and desmosterol paired with [$^2H_3$-*methyl*]AdoMet and purification of the resulting C-methylated sterols by reversed-phase HPLC. The purity of the HPLC fractions was determined by capillary GC−MS as previously described. Protein concentration assays were performed initially according to the method of Bradford[20] with commercial reagents (Bio-Rad) using bovine γ-globulin as standard and stained for total protein using Coomassie blue (Bradford, 1976).[20] For more precise determination of protein concentration, the A_{280} (wavelength scan between 240 and 370 nm) was measured using an extinction coefficient of 58 370 M^{-1} cm^{-1}. The latter value was obtained by direct UV measurement of a sonicated (30 s burst) sample of homogeneous SMT enzyme derived from hydrophobic interaction chromatography or Mono Q chromatography in deionized water. Aliquots of this solution were loaded on SDS−PAGE (12% separating gel), and the concentration of the protein in the gel was estimated from known concentrations of standard markers. From this sample, comparative analysis was made in four ways: densitometry from SDS−PAGE, mass determination of lyophilized samples by gravimetric determination, UV scan, and Bradford determination. Comparison of the protein concentrations using the first three determinations varied by $<$5%, and when these assays were compared with the results of the Bio-Rad assay the indication was that the latter method overestimated protein concentrations by a factor of ca. 1.5. The accuracy of this comparison becomes less secure using impure protein fractions, e.g., derived from the supernatant, homogenate, etc., and fractions that contain salts and detergent.

Site-Directed Mutagenesis, Protein Overexpression, and Purification. The SMT gene, subcloned into the previously described T_7-based high-level expression system,[10] was used as template for all mutagenesis reactions. The plasmid construct, pSMTy,[10,12c] consists of a 1239 bp *Nde*I/*Nhe*I fragment that contains the entire open reading frame for the yeast *ERG*6 gene encoding SMT enzyme. Mutants were generated using site-directed mutagenesis with the GENE Editor in vitro site-directed mutagenesis system (Promega). The mutation converting Tyr81 to alanine (Y81A) was obtained with the 45 mer primer (corresponding to codons 74−88): 5′ TATAACGTCGTTACAGATTTC**GCT**GAATATGGTTGGGGTTCCTCT 3′. The mutation converting Tyr81 to phenylalanine (Y81F) was obtained with the 24 mer primer (corresponding to codons 78 to 85): 5′ ACAGATTTCT**T**TGAATATGGTTGG 3′. The codon corresponding to the introduced mutation is underlined. Plasmids containing the desired mutations were identified by sequencing using a model 310 Genetic Analyzer (Perkin-Elmer Applied Biosystems). Each of the mutant constructs were transformed to *E. coli* strain BL21 (DE3) and the resulting SMT enzyme purified to homogeneity by a combination of ammonium sulfate precipitation, anion exchange, and hydrophobic interaction chromatography.[10]

Steady-State Kinetics. Kinetic constants for each enzyme−substrate−product combination were determined by computer assisted Lineweaver−Burk plotting. The enzyme concentration used in kinetic and overnight experiments for GC−MS determination (ca. 15 h) was 50 μg/0.6 mL. The coenzyme concentration was held constant at 100 μM, and the substrate concentration varied in the range 5−150 μM. Duplicate assays were performed at each variable substrate concentration with excellent agreement between the assays.

Product Isolation and Characterization. Individual olefins were isolated from the nonsaponifiable lipid fraction of quenched reactions treated with KOH by HPLC using analytical reversed-phase C_{18}-Zorbax or TSK gel columns or a semipreparative C_{18}-Whatman column, as necessary. 24-Alkyl sterol fractions found to be a mixture eluting from one column were injected into a second column to resolve the sterol mixture, as described.[8f] Preparative-scale incubations involved incubating 15 mg of substrate in the usual manner at 100 μM with 100 μM of AdoMet (or of [$^2H_3$-*methyl*]AdoMet) and 50 μg of protein per assay, and the reaction vessels were incubated overnight (15 h) at 32 °C. ^{1}H NMR spectra were recorded in deuteriochloroform at 300 MHz using a Bruker AF300 spectrometer.[21] Signals in ^{1}H NMR of Y81F mutant generated sterols were referenced to authentic specimens characterized previously in this laboratory.[12,17] Chemical shifts (δ, ppm) were referenced to TMS (δ, 0). For combined GLC−MS analysis, a Hewlett-Packard 6890 coupled to a 5973 mass selector detector system was employed with on-column injection using He as carrier. Retention times in GC are relative to cholesterol (RRTc).[8f]

Calculations of Isotopic Abundance. Spectra of olefins generated from unlabeled desmosterol and 24(28)-methylenecholesterol paired with AdoMet, [$^2H_3$-*methyl*]AdoMet, and [28-$^2H_2$]24(28)-methylenecholesterol were obtained under conditions as nearly identical as possible, and the isotopic percent composition of the olefins derived from the labeled precursor was calculated by correcting for natural abundance as described in ref 22. In these calculations, contributions from the $[M - 1]^+$ ion, assumed to be entirely due to $[M - H]^+$ ion−molecule reaction product, were first subtracted from the ion abundance normalized areas of the unlabeled Δ^{24}-sterol acceptor molecule derived olefins. Diagnostic molecular ion clusters of peaks in the MS of the biosynthetically derived olefins incubated with deuterium labeled substrates were at the high mass end of the spectrum and were composed of ions at *m*/*z* values of M^+, $M^+ - CH_3$, $M^+ - H_2O$, $M^+ - CH_3 - H_2O$; only the M^+ and $M^+ - CH_3 - H_2O$ peaks are reported in the paper. In each of the labeled olefins the compound possessed the expected number of deuterium atoms added to the side chain.

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Supporting Information Available: Figures showing ^{1}H NMR spectra of B5, B6, B7, [29-$^2H_3$]B6, C3 and Figures showing MS spectra of A1, B1, A3, D3, C3, B7, B5, B6, A5, A6, A7, [28-$^2H_2$]B3, [29-$^2H_3$]B7, [28-^{2}H]B5, [28-$^2H_2$]B7, [28-^{2}H]-B5, [28-$^2H_2$, 29-$^2H_3$]B7, [29-$^2H_3$]B5, [29-$^2H_3$]B6, [28-^{2}H, 29-$^2H_3$]-B6, and [28-^{2}H, 29-$^2H_3$]B5. This material is available free of charge via the Internet at http://pubs.acs.org.

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