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Sterol C24-methyltransferase: Physio- and stereo-chemical features of the sterol C3 group required for catalytic competence ☆

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ABSTRACT

Sterol C24-methyltransferases (24-SMTs) catalyze the electrophilic alkylation of Δ^{24}-sterols to a variety of sterol side chain constructions, and the C3- moiety is the primary determinant for substrate binding by these enzymes. To determine what specific structural features of the C3-polar group ensure sterol catalysis, a series of structurally related C3-analogs of lanosterol that differed in stereochemistry, bulk and electronic properties were examined against the fungal 24-SMT from *Paracoccidioides brasiliensis* (*Pb*) which recognize lanosterol as the natural substrate. Analysis of the magnitude of sterol C24-methylation activity (based on the kinetic constants of V_{max}/K_m and product distributions determined by GC–MS) resulting from changes at the C3-position in which the 3β-OH was replaced by 3α-OH, 3β-acetyl, 3-oxo, 3-OMe, 3β-F, 3β-NH_2 (protonated species) or 3H group revealed that lanosterol and five substrate analogs were catalyzed and yielded identical side chain products whereas neither the 3H- or 3α-OH lanosterol derivatives were productively bound. Taken together, our results demonstrate a chemical complementarity involving hydrogen bonding formation of specific active site contacts to the nucleophilic C3-group of sterol is required for proper orientation of the substrate C-methyl intermediate in the activated complex.

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Introduction

So far as is known the isoprenoid biosynthesis pathway to 24-alkyl sterols is an ancient and ubiquitous property of eukaryotes with the different sterol side constructions arising from the product specificities of the sterol C24-methyltransferase enzymes (24-SMT) [1–3]. An illustrative example of this core pathway for ergosterol biosynthesis is provided by the concerted C24-methylation of lanosterol by the fungal *Paracoccidioides brasiliensis Pb*SMT yielding a single $\Delta^{24(28)}$-methylated sterol product, eburicol [4]. On the other hand, substrate-specific reaction channeling can occur during the C24-methylation reaction catalyzed by plant and protozoan 24-SMTs to form multiple carbocation intermediates en route to the biosynthesis of diverse 24β-methyl and 24α-ethyl sterols (Fig. 1) [1]. Recent metabolite profiling has uncovered complicated sterol patterns in a range of organisms [1,2,5,6] and helped to define aspects of the role of disordered sterol homeostasis in sterol biosynthesis in animals and plants [7–9].

Based on current concepts [1,11], a steric-electric plug model for the coupled methylation–deprotonation of Δ^{24}-sterols to C24-methyl(ene) products has been proposed [10]. This C24-methylation reaction can be viewed as a nucleophilic attack by the Δ^{24}-double bond of various sterols on the methyl group of the sulfonium group of S-adenosyl-L-methionine (SAM). A central feature of this stereochemical model is the enzyme recognition of sterol functional groups at either end of the molecule. Notably, a crucial difference in the anatomy of these flexible substrates targets the shape of the nucleus and the ring structure's influence on the tilt of sterol C3 hydroxyl group and 17(20)-bond [11,12] (Fig. 2).

Comparison of active site substrate requirements directed at the phyla-specific product specificities for sterol biosynthesis enzymes has been undertaken with limited success in part due to the absence of sterol-bound three-dimensional structures of these catalysts. Therefore, a non-structural approach that compares active sites of related enzymes on the basis of substrate specificities has proven useful. This specificity for substrates allows 24-SMTs to be studied for their discrimination between analogs of the natural substrate that differ by a single functional group. Yet, despite several structure–activity studies that focus on sterol ring A features [11,13–24], still unclear are what factors in substrate binding govern catalytic orientation of the reactants in the activated complex.

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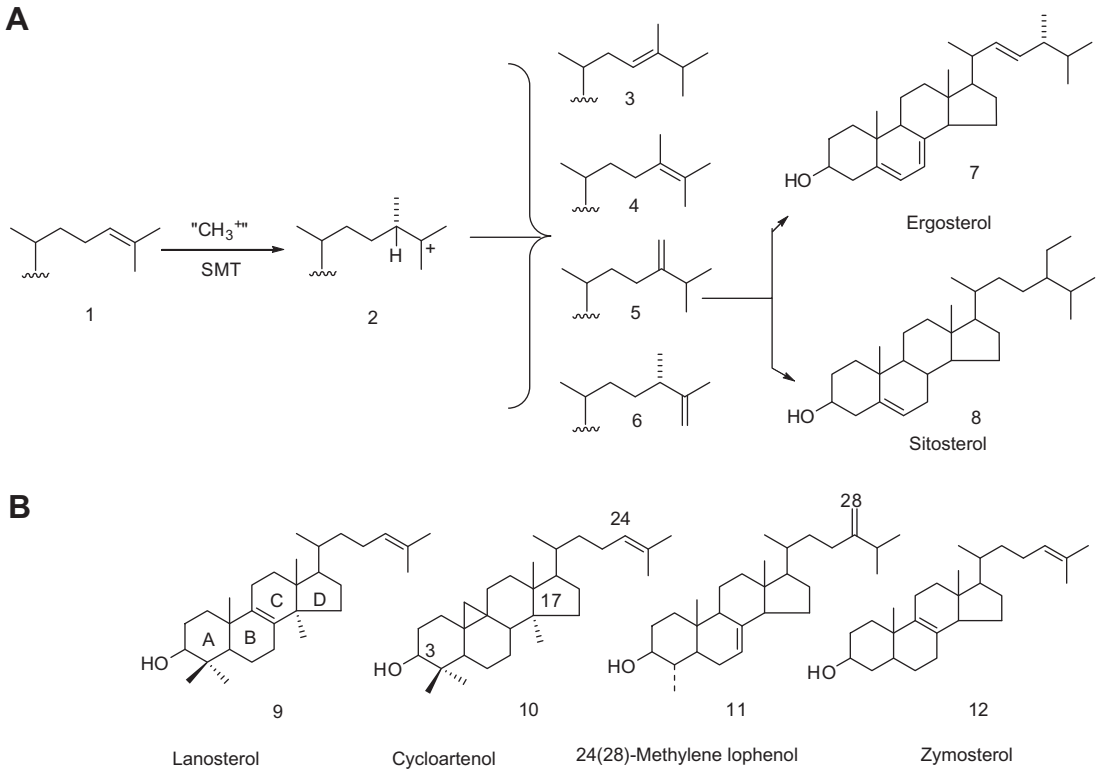

Fig. 1. Pathways for ergosterol and sitosterol side chain construction based on the C24-methylation of a Δ^{24}-sterol acceptor molecule **1** (Panel A) and structures of natural substrates recognized by sterol C24-methyltransferase enzymes from fungi **9** and **12** and plants **10** and **11** (Panel B).

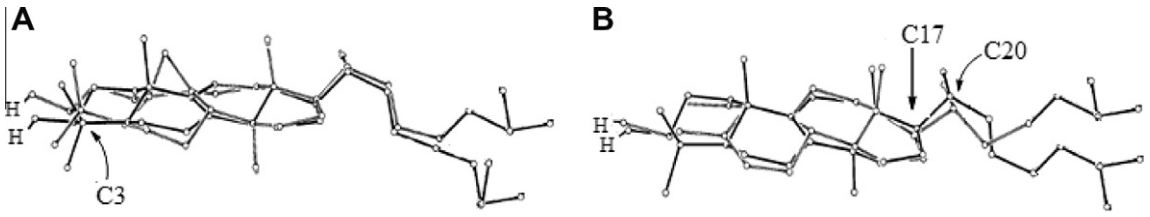

Fig. 2. Conformational perspectives of relevant sterols illustrating the flat structure and tilt of the C3– and C17(20)–bonds in the molecule. Panel A shows the X-ray structures of lanosterol overlapped on cycloartenol. Panel B shows the X-ray structures of lanosterol overlapped on sitosterol (Adapted from reference [11]).

Previously, we proposed directionality to be a relevant factor of interaction determined by the crucial non-reacting substrate group at the proximal end of the acceptor molecule [11]. This direction can be brought about by a stereospecific complexing of the substrate C3–OH to distinct active site contacts. Correct polarity and positioning between active site contacts and substrate would then give favorable entropy of activation leading to efficiency in sterol methylation. We now report the evaluation of a series of lanosterol analogs which systematically deviate from the C3β–OH group against the cloned *Pb*SMT in order to gain further insight into the nature of the enzyme–substrate complementarity utilized by 24-SMTs. Collectively, these and our earlier results [4,11,13–17] demonstrate the functional importance of the sterol ring A 3β-OH on formation of the C24-methyl(ene) intermediate and resulting product.

Materials and methods

Analytical methods

^{1}H Spectra were recorded at room temperature on a Varian Unity Inova 500 MHz spectrometer and chemical shifts reported in parts per million (ppm) downfield from tetramethyl silane of samples dissolved in $CDCl_3$ at ambient temperature. Sterols were analyzed by GC–MS using a Hewlett Packard GC 6890-MSD 5973 (70 eV EI, scan range 50–550 amu) equipped with 0.25 mm i.d. by 30 m fused silica columns coated with Zebron ZB5 (Phenomenex) with He as carrier at a flow rate of 1.2 mL/min. Cool, in column injection set for constant flow was used. The oven was set to be isothermal the first minute, and then ramp to 280 °C at 20 °C/min. The source temperature was 230 °C. Purification by a combination of flash chromatography on silica gel, Al_2O_3 and/or silver nitrate impregnated Al_2O_3 and TLC on 0.25 m or 1 mm glass plates and/or HPLC using Whatman or Phenomenex columns provided pure substrates as previously described [4,25]. Sterols were identified by their rates of movement in chromatography, expressed as R_F values (TLC) or retention times relative to cholesterol (RRT_c in GLC or α_c in HPLC) and by comparison of their mass and NMR spectra with authentic specimens available in our sterol collection.

Chemicals

SAM as the iodide or chloride salt was purchased from Sigma. [methyl-$3H_3$]AdoMet, diluted with non-radioactive SAM to a specific activity of 10 μCi/μmol, was purchased from Perkin-Elmer

(Waltham, MA). All other reagents and chemicals were purchased from Fisher, Aldrich or Sigma unless otherwise noted, and used without further purification.

Synthetic procedures

Lanosterol and eight C3-modified lanosterol analogs prepared from lanosterol are shown in Fig. 3. The starting material for the synthesis of the C3-modified lanosterol derivatives was lanosterol purified from commercial lanosterol (Sigma) containing lanosterol, 24-dihydrolanosterol, agnosterol and 24-dihydroagnosterol as described elsewhere [25]. For modification of the 3β-OH group, free lanosterol was converted to the C3-acetate using acetic anhydride in pyridine, to the C3–O methyl ether using methyl iodide and sodium hydride in anhydrous THF or to the 3-oxo derivative using pyridinium chlorochromate in chloroform by the standard procedures [24]. 3β-Amino lanosterol was prepared from 3-oxo lanosterol via the 3-oxime intermediate as previously described [26]. The 3-desoxy lanosterol was prepared from the 3-hydrazine intermediate in glycol/KOH under reflux followed by extraction using a water–diethyl ether mixture affording product in 88% yield.

Although we prepared 3α-lanosterol in past studies from the known route [27], the recovery has been low (0.5–1%). Therefore, we developed a new procedure as follows: To a stirred solution of ethylene glycol (20 mL) and KOH (200 mg) was added 3-oxolanosterol (50 mg) and the mixture refluxed for 5 h. After cooling to room temperature, the sample was poured into iced water and the precipitate filtered affording a 9:1 mixture of 3β- to 3α-lanosterol together with significant by-products. 3-Epilanosterol, generated in overall 5–10% yield, was easily separated from lanosterol and contaminants by a combination of TLC (developed in benzene/diethyl ether 85/15 (v/v)) and HPLC (reversed phase C_{18} Phenomenex semi-preparative column eluted with methanol at 2.5 m/min) (Table 1).

In the general procedure for the fluorination of alcohols using DAST[5] [28], 50 mg of 3β-OH–lanosterol was added under nitrogen to a stirred solution of 50 mL dry heptane and dichloromethane (5 ml) to facilitate sterol to dissolve in solution, followed by addition of DAST (1 drop) at room temperature. The reaction mixture was stirred for 10 min and water (1 ml) added carefully to quench unreacted DAST, then the mixture washed again with water (100 ml × 2) and dried over magnesium sulfide. The solvent was removed in *vacuo*. The product was predominantly 3α-fluorolanosterol in poor yield (>1%). MS: 428.3 (M^+), 413.3 (M^+–Me), 408.4(M^+–HF), 393.3 (M^+ –Me–HF), 365.3329.0, 301.2, 283.1, 261.1, 69.1 (100%). ^{1}H NMR: 3.49 (3β-H, m, 1H). Apparently, synthesis of the desired 3β-fluoro derivative is not readily achieveable using the convententional procedure. Thus, we developed the following preparation of 3β-fluoro lanosterol 8 from lanosterol **1** in good yield outlined in Fig. 4. After the mono protection of **1** as the THP ether, **2** was oxidized to **3** followed by reduction using sodium hydride, then acetylation affording the C24-acetyl intermediate **4**. Preparations of **4** routinely gave a 1:1 mixture of C24-diastereoisomers in yields of 97–98% over the four steps. Oxidation of **4** with PCC led to **5**. This compound was fluorinated with DAST and the resulting Δ^2-olefin stereospecifically reduced in acidic solution using Adam's catalyst and hydrogen gas to provide a mixture of Δ^{23}/Δ^{24}-3β-fluorinated lanosterol (<99% β-isomer). Final purification of 3β fluoro-$\Delta^{24(25)}$-lanosterol was achieved by HPLC on TSK gel eluted with methanol. Relevant chromatographic and spectral properties of the lanosterol analogs are reported in Table 1.

[5] *Abbreviations used:* DAST, diethylaminosulfur trifluoride; 24-SMT, sterol C24-methyltransferase ; Pb, *Paracoccidiodes brasilienis*; SAM, S-adenosyl-L-methionine; GC-MS, Gas chromatography–mass spectroscopy.

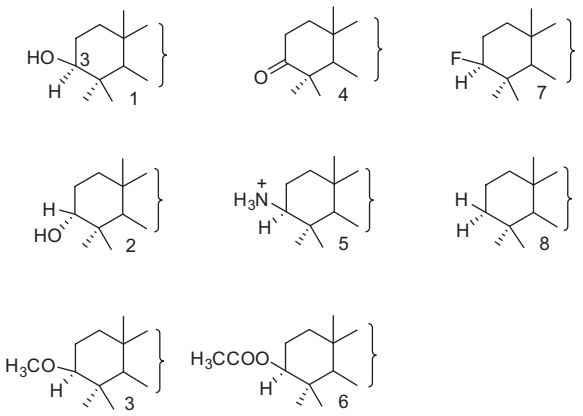

Fig. 3. Partial structure (only ring A) of lanosterol and modified lanosterol derivatives at C3 incubated with *Pb*SMT. In the case of 3-amino lanosterol **5,** the analog is protonated as shown at the buffer pH 8.

Table 1
Chromatographic and special properties for C3 modified analogs of lanosterol.

Substrate[a]	Structure[b]	RRT_c[c]	R_f[d]	MS (M^+)[e]	^{1}H NMR[f]
LA-3β-OH	1	1.33	0.70	426	3.28 dd, 1H
LA-3α-OH	2	1.30	0.88	426	3.43 d, 1H
LA-3β-OMe	3	1.18	0.97	440	3.36 m, 1H
LA-3-Oxo	4	1.25	0.94	424	ND
LA-3β-NH_2	5	1.25	0.03	425	2.67 m, 1H
LA-3β-OAc	6	1.49	0.98	468	4.12 m, 1H
LA-3β-F	7	1.05	1.00	428	4.15 m, 1H
LA-3-H	8	0.93	1.00	408	ND

[a] LA, Lanosterol frame.
[b] Structures are shown in Fig. 3.
[c] Retention time of sterol relative to the retention time of cholesterol in GC; ND, not determined.
[d] Retention factor (R_f) of sterol on silica gel TLC plate developed ×2 with benzene/ether (85:15, v/v).
[e] Molecular weight determined by electron-impact mass spectroscopy.
[f] ^{1}H NMR of C3–H signal; m, multiplet; d, doublet.

PbSMT assay

Procedures for the heterologous overexpression in *Escherichia coli* and isolation of the recombinant *Pb*SMT have been described [4]. The standard linear range assay procedure for *Pb*SMT involved incubation in triplicate in 10 mL test tubes containing 20 mM phosphate buffer (pH 8.0), 5% glycerol, lysate protein (1–2 mg), sterol (varied from 5 to 150 μM) and SAM (fixed at 100 μM) at 35 °C for 1 h in total volume of 600 μL. The incubation mixture was terminated by brief vortexing and the addition of a solution (600 μL) of 10% methanolic KOH. The C24-methylated sterol products were extracted in hexane and dried; an aliquot of the hexane extract was analyzed by GC–MS or in the case of radioactive samples analyzed by scintillation counting to determine the conversion rate. Control experiments (without sterol) were conducted with each enzyme preparation to determine solvolytic background, which generally afforded less than 500 dpm in experiments using [methyl-3H_3]SAM; for optimal sterol methylation the amount of radioactivity recovered from the organic extract was approximately 1×10^6 dpm per assay. Protein concentration was measured by the Bradford assay [29]. The initial velocity data was determined using SigmaPlot 2001 with the enzyme kinetics module software package. Data were fitted to the equation $v = V_{max}S/K_m + S$ using a non-linear least-squares approach. Kinetic constants ± standard errors estimated from the steady-state kinetic evaluation for each sterol were never greater than 10% of the experimental measurement, and R^2 values were between 0.90

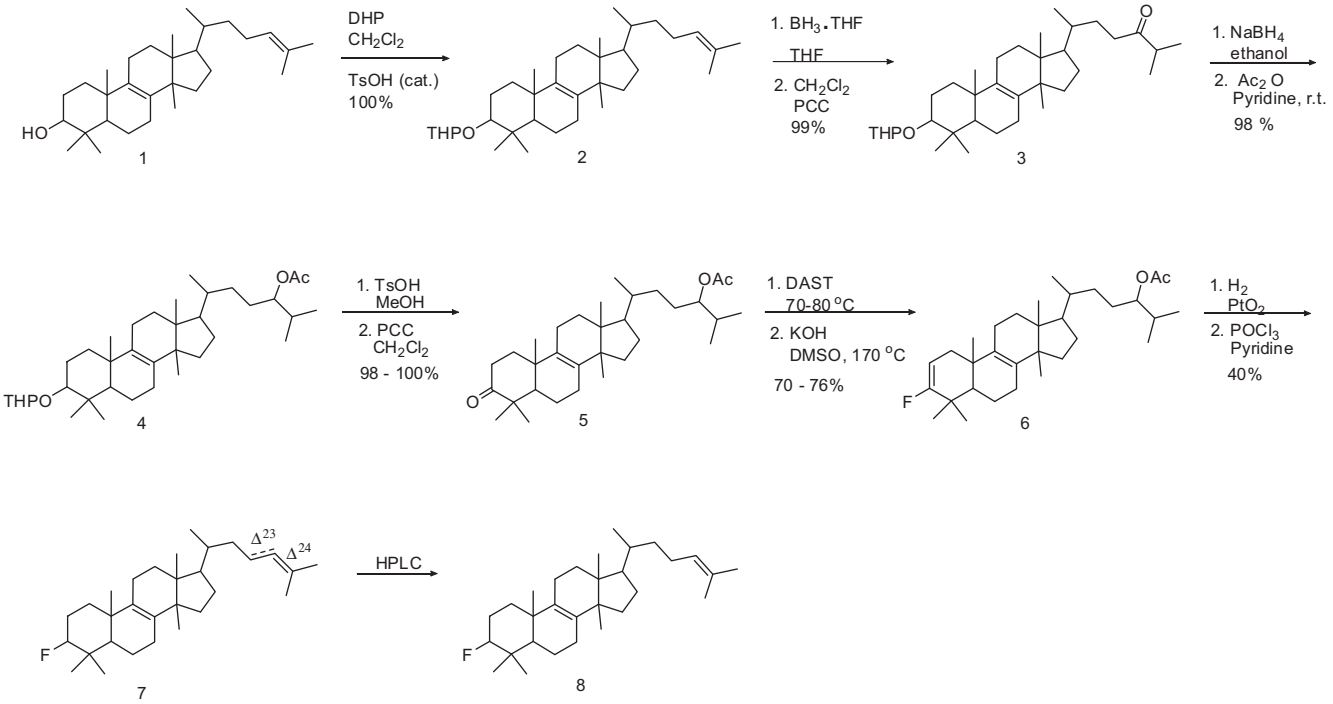

Fig. 4. Outline for the synthesis of 3β-fluorolanosterol **8** from lanosterol **1** as described in "Materials and methods".

and 0.99. K_{mapp} and V_{maxapp} values were initially determined by varying the substrate concentration in the presence of fixed cofactor at 100 μM. To test for non-productive binding of "inactive substrates", in the one case where sufficient analog was available for incubation with *Pb*SMT, increasing concentrations of 3-desoxylanosterol was varied (5–150 μM) against two concentrations of lanosterol at 25 and 100 μM of substrate and fixed concentrations of SAM (150 μM) and the kinetics evaluated as described previously [15].

Results and discussion

Catalytic competence of PbSMT in the presence of C3-modified lanosterol analogs

In attempt to dissect the retention mechanism for substrate bound to sterol catalysts, several strategies were devised for anchoring sterol in the active site. A limitation in many of these earlier studies was a chemical correlation between the substrates, predicted intermediate of the reaction and active site contacts due to few analogs tested. In our ongoing research we have evaluated several C3-variants in different systems and observed that sterol A-ring has several non-reacting features that can influence enzymatic activity [11,17,18]. As a first approximation, the natural substrates recognized by sterol biosynthesis enzymes can possess: (i) slightly different nuclear conformations affected by the number and position of double bonds in the molecule, (ii) varied C4-substitution and/or (iii) a distinct C3-polarity that is associated with either a 3β-OH or 3-oxo group. Currently, there are few reports that examine the accessibility of the 3β-oxygen in binding. Thus, to shed further light on the catalytic role of the polar group at C3, the nature and magnitude of C24-methylation of a series of synthetic lanosterol derivatives that differed in C3-electronics, bulk or stereochemistry were examined kinetically with *Pb*SMT and the specificity constants, $V_{max}/K_{m,}$ of the test substrates compared to the catalytic competence of lanosterol normalized to 100%.

Preliminary evaluation of the *Pb*SMT was to determine whether the C3–OH group is necessary in catalysis. Thus, we tested analogs in which the molecules' polarity at C3 was systematically decreased from that of C3 in lanosterol. Based on chemical reasonableness, polarity ranking of functional groups associated with biomolecules should follow the order: alcohol > ketone > amine > ester > ether > alkane or hydrogen atom. In agreement with this ranking, *Pb*SMT recognition of the analogs ranked in order: C3-hydroxyl (100%) > C3-oxo (47%) > C3-amino (21%) > C3-hydrogen (0%) (Table 2). The binding affinity of the acceptable analogs is comparable to that of the natural substrate (K_{mapp} = 21 μM). Alternatively, catalytic competence for these analogs differed kinetically in V_{maxapp} showing that not only the presence of the oxygen in the 3-position but also its polarity affects substrate transformation. In the case of the 3-amino analog, at pH 8 the nitrogen group is fully protonated, and therefore bears an acidic proton which can H-donor interact in the active site in similar fashion to the interactions of the C3-hydroxyl to contact residues. However, a surprising result in the present study is the observation that the C3-methyl ether derivative (18%), which like the C3-acetyl derivative (15%) are less reactive substrates, did not fall in the rankings below the 3-amino lanosterol activity to zero as might by expected [11,13].

Table 2
Catalytic competence of *Pb*SMT tested with substrate analogs.

Substrate	Structure[a]	V_{max}/K_m	Competence%[b]
LA-3β-OH	1	53/27 = 1.96	100
LA-3α-OH	2	0/0 = 0	0
LA-3β-OMe	3	9/25 = 0.36	18
LA-3-Oxo	4	22/23 = 0.95	48
LA-3β-NH_2	5	13/30 = 0.43	22
LA-3β-OAc	6	16/28 = 0.57	29
LA-3β-F	7	8/25 = 0.32	16
LA-3-H	8	0/0 = 0	0

[a] Structures are shown in Fig. 3.
[b] V_{max}(pmol/min/mg protein)/km(μM) ratio normalized to Lanosterol, LA, as 100%.

These analogs, together with the C3-oxo derivative, have contrasting polarity to the protonated amine and possess a lone pair of electrons on the oxygen which are available for nucleophilic interactions such that the oxygen group may H-atom accept in H-bonding interactions.

It is thus apparent for *Pb*SMT that the hydrogen atom from the 3-hydroxyl group of lanosterol may not engage in hydrogen bonding solely as a H-donor leading to fixation to the enzyme.

Anchoring mechanism for sterol-PbSMT interactions

As posed above, although of the analogs incubated with *Pb*SMT thus far reveals that the 3β-OH group of lanosterol to be the optimal feature for recognition by *Pb*SMT, several of them were unexpectedly effective substrates, including those that contained C3-structures represented by the 3-amino and C3-acetate derivatives never recognized by a sterol catalyst. Analogs that showed that the C3-oxygen can act as a H-acceptor in H-bonding interactions leads to the possibility that some active site contact other than histidine observed in homology modeling of 24-SMT [31] is involved in anchoring sterol. For this reason, we prepared the halogenated substrate 3β-flurolanosterol. This additional substrate was chosen for two reasons: it was not expected to perturb the conformation of the enzyme's active site since the steric size of a fluorine and hydroxyl group are similar, and the isosteric replacement of the sterol hydroxyl group with a fluorine atom can hydrogen bond accept in a manner similar to the oxygen of a hydroxyl group and never function as a hydrogen-bond donor [31]. Steady-state kinetic measurements of lanosterol compared to its 3-fluoro analog shows that *Pb*SMT maintains the same binding efficiencies (K_m) for both substrates (Supplementary Fig. S1), but 3β-fluoro lanosterol is transformed much less (16%) efficiently than 3β-OH lanosterol (100%) (Table 2). These data suggest that burying of the fluorine atom in the hydrophobic pocket, normally occupied by a hydroxyl group, angles the C3-F to an acidic hydrogen (active site donor) to provide the optimal situation for F–H bonding. Thus, the 3β-OH group in lanosterol might engage in hydrogen bonding with active site contacts as a H-bond acceptor, which agrees and contrasts with other sterol structure–activity investigations of 24-SMT [11,13]. The experimental data for productive substrates, 3β-OH and 3β-F, as well as for 3β-NH_3^+, 3β-OMe, 3-oxo, and 3β-OAc, showing similar K_m values but differences in V_{max} further suggests that substrate specificity is determined mainly by the transition state structure, rather than by that of the ground state structure.

Product characterization

Typically, the methyl–methylene eliminations that form the exocyclic double bond of the enzyme-bound acceptor molecule takes place at C28 in the sterol side chain. However, catalysis of the Δ^{24}-sterol substrate from several 24-SMTs of plant, fungal or protozoan origin can lead to channeling to generate alternate C24 methyl(ene) products possessing the $\Delta^{23(24)}$-, $\Delta^{24(25)}$-, $\Delta^{24(28)}$- and $\Delta^{25(27)}$-olefin structure. The different C24-methylation reaction products are readily distinguished by their chromatographic behaviors in GC [8,21]. Thus, it seemed prudent to consider whether any of the test substrates can generate products other than a $\Delta^{24(28)}$-sterol. To assure that our enzyme preparation was generating the same high yields of 24(28)-methylenelanosterol (eburicol) from lanosterol as reported previously [4], sterol C24-methylation of the various lanosterol derivatives was initially evaluated using lanosterol (100 μM); in agreement with our earlier findings we detected eburicol as the sole product by GC–MS analysis in approximate 40% yield (Fig. 5, Panel A).

The elution of eburicol relative to lanosterol in GC moves with a retention factor of 1.07 corresponding to the change in side chain structure from $\Delta^{24(25)}$-substrate to $\Delta^{24(28)}$-product [13]. This retention factor in product formation was observed routinely in substrate transformation typified in the incubations of lanosterol and 3β-fluorolanosterol with *Pb*SMT (Fig. 5). The mass spectra of enzyme-generated products are shown in Fig. 6, except for the 3-amino lanosterol product which was anomalous (M^+ 465 peak). Activity assay of 3-acetyl lanosterol led to 3-acetyl 24(28)-methylene lanosterol (M^+ 482). The acetylated methylene sterol was a minor product in GC analysis and detected only in cases of incomplete saponification of the enzyme preparation. Consequently, lanosterol and eburicol were the main sterols in GC–MS analyses of 3-acetyl lanosterol transformation. Incubations of 3-desoxy (3-H) or 3α-lanosterol sterol derivatives with the recombinant enzyme did not lead to any hexane extractable products as judged by GC–MS analysis, even after prolonged incubation or increasing the protein to 5 mg per assay tube or increasing cofactor concentrations to 200 μM in the incubation system. Steady state kinetic analysis of the inactive substrate 3-desoxy lanosterol against lanosterol showed competitive-type inhibition ($K_{i3\text{-desoxylanosterol}}$ approximately 67 μM (Supplementary Fig. S2), consistent with non-productive binding of the analog in the active site. In none of the GC–MS analyses of the enzyme-generated 24(28)-methylene sterol was a byproduct detected or hydroxylated sterol intermediate formed as reported for incubation of suicide substrates with 24-SMTs [1], showing the analogs are stable and that the reaction progress proceeded to completion. These data suggest that when the chemical reactivities of the C3 group were altered, the strict regio- and stereo-specificities of the C-24 methylation reaction were

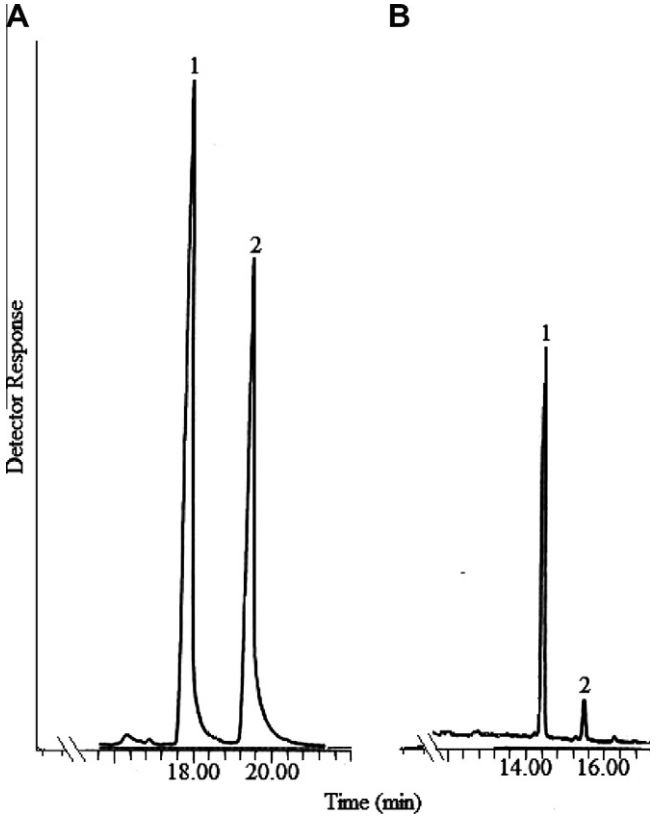

Fig. 5. GC analysis (reported as a partial total ion current chromatogram) of the C24-methylated sterol products, peak 2, generated from *Pb*SMT incubated with lanosterol peak 1 (Panel A) or 3β-fluorolanosterol peak 1 (Panel B). The retention time in GC for cholesterol is approximately 13.4 min.

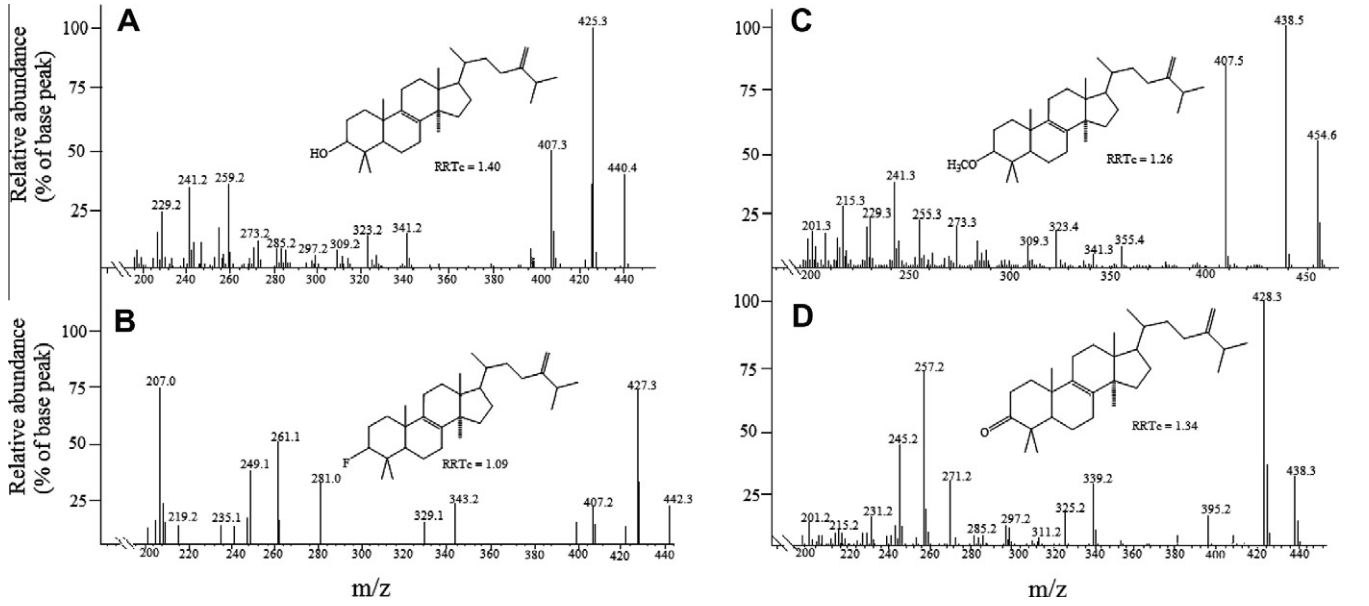

Fig. 6. Mass spectra (EI, 70 eV) of the C24-methylated products generated by *Pb*SMT incubated with lanosterol (Panel A), 3β-fluorolanosterol (Panel B), 3-methyl ether lanosterol (Panel C) or 3-oxo lanosterol (Panel D).

still retained consistent with correct anchoring of the test acceptor molecule in the activated complex. In this model, a non-productive substrate lacks the necessary structural feature at C3 to induce the conformational change required for catalytic activity, and thus does not undergo a reaction.

General considerations

The results of this investigation have established the sterol C3–OH group and its 3β-stereochemistry are absolute substrate requirements to be catalytically functional in *Pb*SMT catalysis. Although this binding feature is not predictable from the comparative sequence alignments or reaction mechanisms of sterol biosynthesis enzymes that form the core pathway, typically these non-homologous enzymes show narrow substrate selectivity for the hydrophilic part of the sterol molecule. Several reports indicate that one or more active site amino acids can interact to form a hydrogen bond at the 3β-hydroxyl group, including serine, methionine, threonine or aspartate in sterol 14-demethylase [32–35], histidine in sterol C24-methyltransferase (24-SMT) [31], serine in sterol C24-reductase (24-SR) [36], threonine in sterol 8,7-isomerase (8-SI) [37] and tyrosine or serine in 3β-hydrosteroid-dehydrogenase/C4-decarboxylase [3βHSD/4-SD] [38], as suggested by bioinformatic analyses, homology modeling studies and/or site-directed mutagenesis.

Homology modeling of 24-SMT has shown the sterol aligns in the active site of 24-SMTs with the A-ring pointed toward a basic residue, neutral histidine [30]. Thus, one might conclude that the sterol OH– group functions as the H donor in a H-bond to the lone pair of electrons in the nitrogen atom which corresponds to the active site amino acid in the steric-electric plug model [10]. In contrast, from the recently obtained X-ray structure of a suicide substrate (14α-methylencyclopropyl-Δ^7-24,25-dihydrolanosterol) complexed to a sterol 14α-demethylase from *Trypanosoma brucei* which shows a thermodynamically favorable conformation and orientation that clearly reflects the catalytically relevant binding mode to the heme, do any of these amino acids interact directly with the sterol C3-hydroxyl group; rather, the C3β–OH group is positioned close to the channel entrance of the active site, 3.5A from a backbone oxygen associated with Met358 residue [39]. These new structural findings together with prior structure–activity studies of the cycloeucalenol-obtusifoliol isomerase [24] are particularly noteworthy because in both cases bonding to sterol C3–OH is proposed to proceed from the main chain interactions. Since the putative contact 24-SMT histidine residue that interacts with the sterol C3-hydroxyl group can be replaced with leucine in the *Sc*SMT (=Erg6p) without abolishing activity [40] and C3-methyl ether and C3-oxo derivatives are suitable substrates for *Sc*SMT, it is reasonable to extrapolate to the *Pb*SMT that these substrate–enzyme interactions are likely more wired than previously considered. Thus, the anchoring mechanism involved with sterol C24-methylation in *Pb*SMT catalysis may consist of a topologically constrained side carbonyl and a polar amino acid side chain which can surround the substrate C3–OH and firmly attach it in the active site cleft by H-bonds directly or by way of a water bridge. In this tightly coupled H-bonding ternary complex, the binding and reaction of the intermediate generated at the active site are greatly favored over dissociation. This new model, which envisages the 24-SMT interacts with the sterol C3-hydroxyl group from two directions (Fig. 7), enables the dynamic conformational changes that accompany C-24-methylation of acceptor molecule to play a role in chaperoning product outcome. Since structural differences between substrates undergoing C24-methylation are small and local, the difference in specificity amongst this family of enzymes is likely caused by only a few amino acid differences in the substrate-recognition site and/or in subtle differences in the main frame organization relative to the sterol ring A functional groups. Indeed, a conserved aromatic residue-phenylalanine, has been shown to act as a key discriminator between C4α-methylated and C4α/β-dimethylated substrates of the sterol 14-demethylase enzymes [33,41].

A key feature in sterol recognition, and therefore specificity, to distinguish a plant 24-SMT from a fungal 24-SMT is the degree of C4-substituion in the acceptor molecule. The contribution of one or two methyl groups at C4 and associated Δ^7 or Δ^8-bond in rings B or C for substrate binding can have a positive or negative effective on 24-SMT catalysis. On the other hand, sterols that contain a monoene Δ^5-bond system and lack C4 methyl groups, important features for sterols to function as membrane components [42–44], are generally poor substrates for 24-SMTs. It seems to be reasonable to

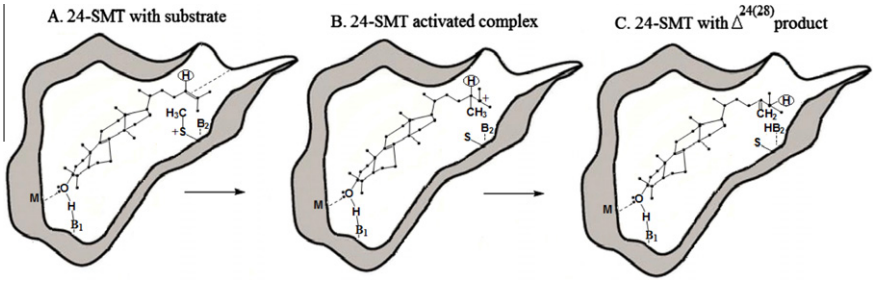

Fig. 7. Proposed aspects of substrate (A) intermediate (B) and product (C) binding to the *Pb*SMT active site. For lanosterol binding in A, the enzyme presents a binding site that is sterically and electronically complementary, to which the substrate becomes anchored at its C3-hydrophilic group. The sterol C3–OH group interacts in a pre-organized active site with contacts that form hydrogen bonds against the 3-oxygen atom (from a main frame moiety, M) and hydrogen atom of the 3-oxygen atom (from a basic amino acid, B_1) forming a hydrogen bonded network to stabilize the ground state structure at the proximal end of the acceptor molecule and the side chain assumes a pseudocyclic conformation. Productive orientation of the substrate side chain affords backside (S_N2) addition of "methyl cation" from S-adenosyl-L-methionine (represented by the catalytic sulfur atom, S) to the Δ^{24}-bond generating the C24β-methyl C25 cation shown in B. Deprotonation of the C28 methyl group from a basic amino acid (B_2) can lead to the C24(28)-methylene product shown in C, followed by disassociation of the methylated sterol from the enzyme (*cf.* steric-electric model discussed in references [10,11]).

suppose that for 24-SMTs precise positional control of the substrate C3-group requires a Δ^8- over Δ^5-substrate for effective catalysis and that the homoallylic effect from the sterol Δ^5-bond on the C–O bond at C3 can degrade this control, either by holding the C3–OH group fixed by donation of electrons through an inductive effect which should strengthen the H–O bond and therefore weaken H-bonding of the sterol to the active site contacts, or by allowing multiple alternative orientations which are non-productive.

It is notable that the active site cavity of *Pb*SMT is sufficiently tight that changes to the active site volume resulting from incubation of the different analogs failed to generate multiple products which have been detected in related 24-SMT enzymes catalyzing the first C_1-transfer reaction [1]. Tight conformational control by the enzyme also implies proximity of the cationic center with the Δ^{24}-bond of the substrate undergoing C24-methylation, a charge-stabilizing mechanism. The weaker binding of the 3β-fluorinated analog did not alter the product outcome, consistent with a directional component to sterol catalysis independent of the mechanistic type. Thus, we suggest efficient anchoring of the C3-hydroxyl group which generates the productive C24-methylation intermediate in the *Pb*SMT active site affords an element of kinetic control over competition between "slow" deprotonation and "fast" methylation affecting product specificity. Although a more precise formulation of ternary enzyme–sterol–SAM interactions must await the three-dimensional structure of the 24-SMT, the present studies in conjunction with previous work [11,13,15], provide a foundation for substrate binding selectivity amongst this class of enzyme. Additionally, evaluation of the results, and those relevant to substrate binding by related catalysts [17–24], allows effective rational design of mechanism-based inactivators targeted to inhibit acute enzymes of sterol biosynthesis synthesized in opportunistic pathogens, efforts currently in progress.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.abb.2012.03.002.

References

[1] W.D. Nes, Chem. Rev. 111 (2011) 6423–6451.
[2] L.J. Goad, T. Akihisa, Analysis of Sterols, Blackie Academic & Professional, New York, 1997. pp. 1–40.
[3] R.B. Kolner, R.E. Summons, A. Pearson, N. King, A.H. Knoll, Proc. Natl. Acad. Sci. U.S.A. 105 (2008) 9897–9902.
[4] M. Pereira, Z. Song, L.K. Santos-Silva, M.H. Richards, T.T.M. Nguyen, J. Liu, C.M.A. de Almeida Soares, A.H. da Silva Cruz, K. Ganapathy, W.D. Nes, Biochim. Biophys. Acta 1801 (2010) 1163–1174.
[5] W. Zhou, G.A.M. Cross, W.D. Nes, J. Lipid Res. 48 (2007) 665–673.
[6] K. Schrick, C. Cordova, G. Li, L. Murray, S. Fujioka, Phytochemistry 72 (2011) 465–475.
[7] J.X. He, S. Fujioka, T.C. Li, S.G. Kang, H. Seto, S. Takatsuto, S. Yoshida, J.C. Jang, Plant Physiol. 131 (2003) 1258–1269.
[8] F.D. Porter, G.E. Herman, J. Lipid Res. 52 (2011) 6–34.
[9] P. Benveniste, Annu. Rev. Plant Biol. 55 (2004) 429–457.
[10] W.D. Nes, Biochim. Biophys. Acta 1529 (2000) 63–88.
[11] W.D. Nes, G.G. Janssen, A. Bergenstrahle, J. Biol. Chem. 266 (1991) 15202–15212.
[12] W.D. Nes, K. Koike, Z. Jia, Y. Sakamoto, T. Satou, T. Nikaido, J.F. Griffin, J. Am. Chem. Soc. 120 (1998) 5970–5980.
[13] M. Venkatramesh, D. Guo, Z. Jia, W.D. Nes, Biochim. Biophys. Acta 1299 (1996) 313–324.
[14] A.T. Mangla, W.D. Nes, Med. Chem. 8 (2000) 925–936.
[15] W.D. Nes, A. Song, A.L. Dennis, W. Zhou, J. Nam, M.B. Miller, J. Biol. Chem. 278 (2003) 34505–34516.
[16] W. Zhou, G.I. Lepesheva, J. Biol. Chem. 281 (2006) 6290–6296.
[17] W.D. Nes, W. Zhou, A.L. Dennis, H. Li, Z. Jia, R.A. Keith, T.M. Piser, S.T. Furlong, Biochem. J. 387 (2002) 587–599.
[18] A. Bellamine, A.T. Mangla, A.L. Dennis, W.D. Nes, M.R. Waterman, J. Lipid Res. 42 (2001) 128–136.
[19] M. Taton, A. Rahier, Biochem. J. 277 (1991) 483–492.
[20] Y. Aoyama, Y. Yoshida, Y. Sonoda, Y. Sato, Biochim. Biophys. Acta 1006 (1989) 209–213.
[21] Y. Aoyama, Y. Yoshida, Biochem. Biophys. Res. Commun. 183 (1992) 1266–1272.
[22] G.I. Lepesheva, N.G. Zaitseva, W.D. Nes, W. Zhou, M. Arase, J. Liu, G.C. Hill, M.R. Waterman, J. Biol. Chem. 281 (2006) 3577–3585.
[23] S. Darnet, A. Rahier, Biochim. Biophys. Acta 1633 (2003) 106–117.
[24] A. Rahier, M. Taton, P. Benveniste, Eur. J. Biochem. 181 (1989) 615–626.
[25] S. Xu, R.A. Norton, F.G. Crumley, W.D. Nes, J. Chromatogr. 452 (1988) 377–398.
[26] D. Guo, J. Hu, J-H. Zheng, S.A. Ross, W.D. Nes, J. Chin. Pharm. Sci. 6 (1997) 133–137.
[27] J.W. Huffman, J. Org. Chem. 24 (1959) 447–451.
[28] W.J. Middleton, J. Org. Chem. 40 (1975) 574–578.
[29] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[30] B.K. Park, N.R. Kitteringham, P.M. O'Neill, Annu. Rev. Pharmacol. Toxicol. 41 (2001) 443–470.
[31] J. Liu, Biochem. J. 439 (2011) 413–422.
[32] D.C. Lamb, D.E. Kelly, J. Biol. Chem. 272 (1997) 5682–5688.
[33] L.M. Podust, L.V. Yermalitskaya, G.I. Lepesheva, V.N. Podust, E.A. Dalmasso, M.R. Waterman, Structure 12 (2004) 1937–1945.
[34] C. Sheng, Z. Miao, H. Ji, J. Yao, W. Wang, Z. Che, G. Dong, J. Lu, W. Guo, W. Zhang, Antimicrob. Agents Chemother. 53 (2009) 3487–3495.
[35] N. Strushkevich, S.A. Usanov, J. Mol. Biol. 397 (2010) 1067–1078.
[36] A. Pedretti, E. Bocci, R. Maggi, G. Vistoli, Steroids 73 (2008) 708–719.
[37] A. Rahier, S. Pierre, G. Riveill, F. Karst, Biochem. J. 414 (2008) 247–259.
[38] A. Rahier, M. Bergdoll, G. Genot, F. Bouvier, B. Camara, Plant Physiol. 149 (2009) 1872–1886.

[39] T.Y. Hargrove, Z. Wawrzak, J. Liu, M.R. Waterman, W.D. Nes, G.I. Lepesheva, J. Lipid Res. 53 (2012) 311–320.
[40] W.D. Nes, P. Jayasimha, W. Zhou, K. Ragu, C. Jin, T.T. Jaradat, R.W. Shaw, J.M. Bujinicki, Biochemistry 43 (2004) 569–576.
[41] G.I. Lepesheva, W.D. Nes, W. Zhou, G.C. Hill, M.R. Waterman, Biochemistry 43 (2004) 10789–10799.
[42] W.D. Nes, G.G. Janssen, F.G. Crumley, M. Kalinowska, M. Akihisha, T. Akihisha, Arch. Biochem. Biophys. 300 (1993) 724–733.
[43] K.E. Bloch, Crit. Rev. Biochem. Mol. Biol. 14 (1983) 47–92.
[44] R. Bitmann, Subcell. Biochem. 28 (1997) 145–171.