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Sterol *C*-methyl Transferase from *Prototheca wickerhamii* Mechanism, Sterol Specificity and Inhibition†

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Abstract—The membrane-bound sterol methyl transferase (SMT) enzyme from *Prototheca wickerhamii*, a non-photosynthetic, yeast-like alga, was found to *C*-methylate appropriate $\Delta^{24(25)}$-sterol acceptor molecules to $\Delta^{25(27)}$-24β-methyl products stereoselectively. Incubation with pairs of substrates—[$^2H_3$-*methyl*]AdoMet and cycloartenol, and AdoMet and [27-^{13}C]lanosterol—followed by ^{1}H and ^{13}C NMR analysis of the isotopically labeled products demonstrated the *si*-face (β-face attack) mechanism of *C*-methylation and the regiospecificity of $\Delta^{25(27)}$-double bond formation from the pro-*Z* methyl group (C27) on lanosterol. The enzyme has a substrate preference for a sterol with a 3β-hydroxyl group, a planar nucleus and a side chain oriented into a 'right-handed' structure (20*R*-chirality)—characteristic of the native substrate, cycloartenol. The apparent native molecular weight of the SMT was determined to be approximately 154,000, as measured by Superose 6 FPLC. A series of sterol analogues which contain heteroatoms substituted for C24 and C25 or related structural modifications, including steroidal alkaloids, havs been used to probe further the active site and mechanism of action of the SMT enzyme. Sterol side chains containing isoelectronic modifications of a positively charged moiety in the form of an ammonium group substituted for carbon at C25, C24, C23 or C22 are particularly potent non-competitive inhibitors (K_i for the most potent inhibitor tested, 25-azacycloartanol, was ca. 2 nM, four orders of magnitude less than the K_m for cycloartenol of 28 μM), supporting the intermediacy of the 24-methyl C24(25)-carbenium ion intermediate. Ergosterol, but neither cholesterol nor sitosterol, was found to inhibit SMT activity ($K_i = 80$ μM). The combination of results suggests that the interrelationships of substrate functional groups within the active center of a $\Delta^{24(25)}$ to $\Delta^{25(27)}$ 24β-methyl-SMT could be approximated thereby allowing the rational design of *C*-methylation inhibitors to be formulated and tested.

Introduction

Ergosterol (24β-methyl cholesta-5,7,22*E*-trien-3β-ol) and related phytosterols are produced by all forms of microorganisms, including algae.[1,2] These natural products manifest many important biological functions and activities in the form of membrane inserts and bioregulators.[3,4] Ergosterol synthesis proceeds by the initial cyclization of squalene oxide to lanosterol in fungi or to cycloartenol in plants.[1,5] The sterol methyl transferase (SMT), a slow and perhaps rate-limiting enzyme in the extended biosynthetic sequence leading to ergosterol, exhibits unique types of catalytic activities and mechanisms and several of the responsible enzymes catalyzing $\Delta^{24(28)}$-formation have been isolated, cloned, purified and characterized.[6–10] The phytosterol pathway flows along different channels in yeast and algae to generate $\Delta^{24(28)}$- or $\Delta^{25(27)}$-*C*-methylated sterol side chains (Fig. 1).

The frequency of occurrence of human infections from yeast and related microorganisms have been increasing over the past decade in response to a combination of factors. For 30 years, drug companies have recognized that the lanosterol–ergosterol biosynthetic pathway can be a clinical target of powerful fungicides that impair a synthetic step thereby disrupting ergosterol homeostasis.[11,12] There is broad-application of these fungicides to microorganisms that are morphologically similar to yeast, as typified by the disease Protothecosis caused by the non-photosynthetic alga *Prototheca wickerhamii*.[13–15] Unfortunately, many of the antifungal agents used to treat infection block post-lanosterol steps common to pathogen and host or they are polyene antibiotics, nystatin or Amphotericin B, that can complex

Abbreviations: SMT, sterol methyl transferase; AdoMet, *S*-adenosyl-L-methionine; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; GLC, gas–liquid chromatography; HEI, high energy intermediate; FPLC, fast protein liquid chromatography; NSF, non-saponifiable lipid fraction.

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with sterol in the fungal or animal membranes.[16–19] Because these treatments may compromise the host there is a renewed search for alternative drugs.[20]

As part of an ongoing effort to develop new treatment strategies to inhibit sterol pathways that are synthesized by the pathogen, and are themselves not synthesized by the host,[21–25] we have investigated the biosynthesis of ergosterol in *P. wickerhamii*. Accordingly, we established a signature lipid for diagnostic purposes with respect to the operation of a cycloartenol–ergosterol pathway in *P. wickerhamii* and identified an enzyme that can be targeted for drug formulation and testing. A series of substrate analogues and high energy intermediate and product analogues suitably designed so as to prevent completion of the normal catalytic cycle of *C*-methylation and inhibit growth were tested with a cell-free SMT and intact cell cultures. 25-Azacycloartenol and related ammonium-containing sterol analogues, including the steroidal alkaloids solanidine and solasodine,[22,23] were found to be potential lead compounds in drug design to inhibit growth of this pathogenic alga. The *P. wickerhamii* SMT defines a unique family of $\Delta^{25(27)}$-SMTs that are highly conserved in action mechanism between algae and vascular plants but absent from yeast, which produce exclusively the family of $\Delta^{24(28)}$-SMTs[20,26] and SMTs are absent from animals that synthesize cholesterol.

Results

Sterol composition of *Prototheca wickerhamii* strain YB4330

In our earlier study we examined *P. wickerhamii* strain Y-6870.[2,27] In this study we examined strain YB-4330. The main difference between the strains is the high level of prototheca sterol (24β-methyl cholesta-5,7,22E, 25(27)-tetraen-3β-ol) and no significant ergost-7-enol synthesized by strain Y-6870 compared with the production of high levels of ergost-7-enol and no significant prototheca sterol by strain YB-4330. As found in preliminary studies using GC–MS, the major sterols of strain YB-4330 were ergosterol and ergost-7-enol in a ratio of about 2 to 1 (ca. greater than 95% of the total sterols). Trace amounts of the 4,4-dimethyl sterols cycloartenol, cyclolaudenol and 24(28)-methylenecycloartanol, were detected in a ratio of ca. 1:1:0.1. From a preparative scale incubation, five sterols with chromatographic mobilities corresponding to ergosterol, ergost-7-enol, cycloartenol, cyclolaudenol and 24(28)-methylene cycloartanol were isolated and purified to homogeneity. Structural determination was based on a comparison of the mass and ^{1}H NMR spectra of each compound with that of authentic specimens available to us. The configuration of the C24-methyl group in ergosterol, ergost-7-enol and cyclolaudenol was confirmed by ^{1}H and ^{13}C NMR to be β-oriented and in each case there was no evidence for contamination from the α-epimer.[28–30] By ^{1}H NMR analysis the minor 4,4-dimethyl sterol identified as 24(28)-methylene cycloartanol was found not to be contaminated by the olefin with a $\Delta^{23(24)}$-bond.[25,26] The results show that *P. wickerhamii* operates a novel cycloartenol-ergosterol pathway (Fig. 1).

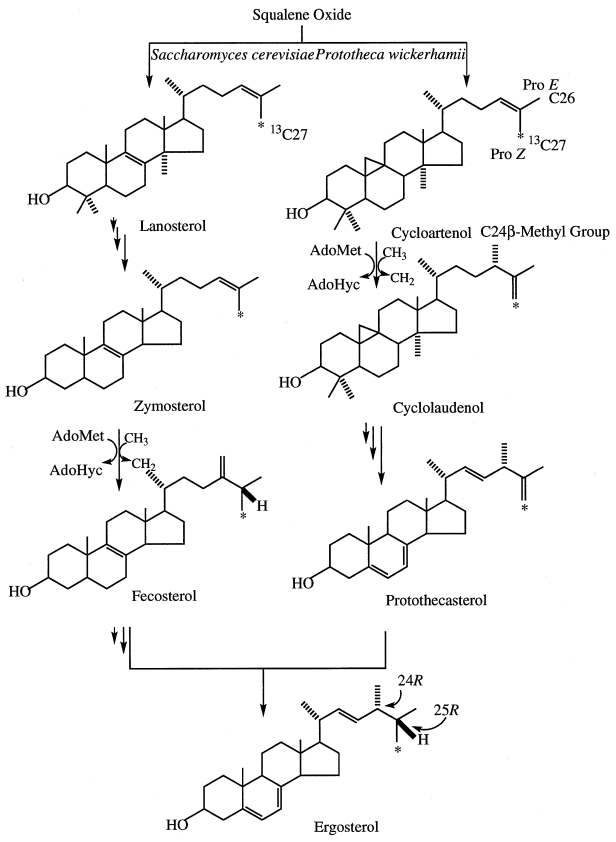

Figure 1. Hypothetical cycloartenol-lanosterol bifurcation to ergosterol in fungi and algae.

Feeding experiments with [2-^{3}H]acetate

The fate of the tritium label associated with [2-^{3}H]acetate was determined by incubating 5 μCi [2-^{3}H]acetate with *P. wickerhamii* cells in the presence and absence of 35 nM 25-azacycloartenol and analyzing the resulting sterol compositions for radioactivity. Approximately 20% of the radioactivity added to the culture medium was found in the non-saponifiable lipid fraction. From the TLC plate, three bands were found to possess ^{3}H-radioactivity corresponding to 4-desmethyl, 4-mono methyl and 4,4-dimethyl sterols. In the control, ca. 80% of the total radioactivity was associated with 4-desmethyl sterols whereas in the 25-azacycloartenol treated cells ca. 65% of the total radioactivity accumulated on the TLC plate matching the 4,4-dimethyl zone. An aliquot of the non-saponifiable lipid fraction from control incubation was injected into the HPLC[27] and radioactivity were monitored for each 30-s fraction. Mass corresponding to two HPLC peaks of radioactivity eluting between 15 and 18 min and representing 90% of the applied radioactivity was coincidental with HPLC peak retention times of ergosterol and ergost-7-enol. Alternatively, HPLC-radiocounting analysis of the non-saponifable lipid fraction from the 25-azacycloartenol-treated cells indicated that cycloartenol was the major sterol labeled (data not shown), consistent with the TLC data.

Properties of the SMT

The apparent molecular weight of the soluble SMT, estimated by chromatography on a calibrated (relative to BioRad Gel Filtration standards) Super 6 FPLC column eluted with 50 mM Tris HCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol, 0.2% emulphogen and 15% glycerol (pH 8.0) at 0.5 mL/min, was 154,000, suggesting a multimer. The pH optimum was about 7.5 (with one-half maximal activity at pHs 7.0 and 9.5). The apparent native molecular weight of the *P. wickerhamii* enzyme predicts a monomer of 38.5 kDa, which is in the range of monomeric units (38 to 43 kDa) of related SMTs.[6–10]

General features of sterol methyl transferase reaction

In a typical preparative experiment with *P. wickerhamii* SMT incubated with saturating levels of [$^3H_3$-*methyl*]AdoMet and cycloartenol, a total of 2.9×10^6 dpm was recovered from the non-saponfiable lipid fraction and a total of 2.4×10^6 dpm was recovered from the 4,4-dimethy TLC band. Two *C*-methylated products were detected by HPLC of the 4,4-dimethyl sterol fraction corresponding in chromatographic mobility to cyclolaudenol (6.9×10^5 dpm) and 24(28)-methylenecycloartanol (3.4×10^3 dpm), respectively. For routine assays involving the standard preparation, conversion of cycloartenol to 24(28)-methylenecycloartanol was considered negligible. We have assumed, therefore, that the *C*-methylation pathway involving 24(28)-methylenecycloartanol can be ignored in the analysis of the kinetics of cyclolaudenol formation.

From a preparative incubation with cycloartenol and AdoMet was isolated cyclolaudenol. The structure of the SMT enzyme generated C24β-methyl product was confirmed by ^{1}HNMR (δ ppm, 0.956 (s, H18), 0.555 (d, H_a endo), 0.330 (d, H_b exo), 0.859 (d, H21), 1.640 (br. s., H26), 4.655 (br. s., H27), 0.995 (d, H28), 0.967 (s, H30-α methyl group), 0.810 (s, H31-β-methyl group), and 0.885 (s, H32)). The ^{1}H NMR signals of the *P. wickerhamii* sample were in good agreement with that of an authentic specimen available to us.[29] These findings indicate that the facial stereochemistry of *C*-methylation proceeds stereospecifically from the β-face of the 24,25-double bond.

From a preparative scale incubation with [$^2H_3$-*methyl*] AdoMet paired with cycloartenol was isolated [28-$^2H_3$]cyclolaudenol. The structure was confirmed by the molecular weight of the product showing three deuterium atoms incorporated into the side chain (M^+443 and other diagnostic ions at the high mass end of 428, 225, 410, 382, 355, 341, 327, 315, 303, 297 amu) amd ^{1}H NMR spectrum indicating the incorporation of the deuterated methyl from AdoMet at C28 in the side chain (δ ppm, 0.956 (s, H18), 0.555 (d, H_a endo), 0.330 (d, H_b exo), 0.859 (d, H21), 1.640 (br. s., H26), 4.655 (br. s., H27), 0.995 (doublet for H28 missing), 0.967 (s, H30-α methyl group), 0.810 (s, H31-β-methyl group), and 0.885 (s, H32)). The deuterium introduced at C28 was established by the disappearance of the methyl signal corresponding to C28 in the spectrum. These findings rule out the initial formation of a 24(28)-methylene structure followed by isomerization of the exo methylene group to form a 24β-methyl $\Delta^{25(27)}$-side chain.

From a preparative scale incubation of AdoMet paired with [27-^{13}C]lanosterol was isolated [27-^{13}C]24β-methyl lanosta-8,25(27)-dien-3β-ol. The mass spectrum of [27-^{13}C]24β-methyl lanosta-8,25(27)-dien-3β-ol possessed a 1-mass unit increase (M^+ 441) in molecular weight compared with the molecular weight of the non-isotopically ^{13}C-labeled olefin (M^+ 440). For [27-^{13}C]24β-methyl lanosta-8,25(27)-dien-3β-ol, ions in the high mass region were found at M^+ 441, 426, 408, 400, 383, 300, 359, 353, 345, 317, 272 and 259 amu; ^{1}H NMR spectrum: δ 3.242 (H-3, 32;dd, $J=4.7/11.5$ Hz), 0.0.683 (H-18, s), 0.810 (H-31, s), 0.874 (H-32, s), 0.910 (H-21, d $J=6.0$ Hz), 0.980 (H-19, s), 1.000 (H-30, s), 1.044 (H-28, d, $J=9.5$ Hz), 1.620 (H-26, d, $J=5.9$ Hz), 4.662 (H-27, d, $J=154$ Hz). The regiospecific proton elimination at the *cis* terminal methyl (C27) group was established by the character and position of the chemical shifts in the ^{13}C NMR spectrum involving an enhanced signal at δ 109 ppm for C27 and in the ^{1}H NMR spectrum at δ

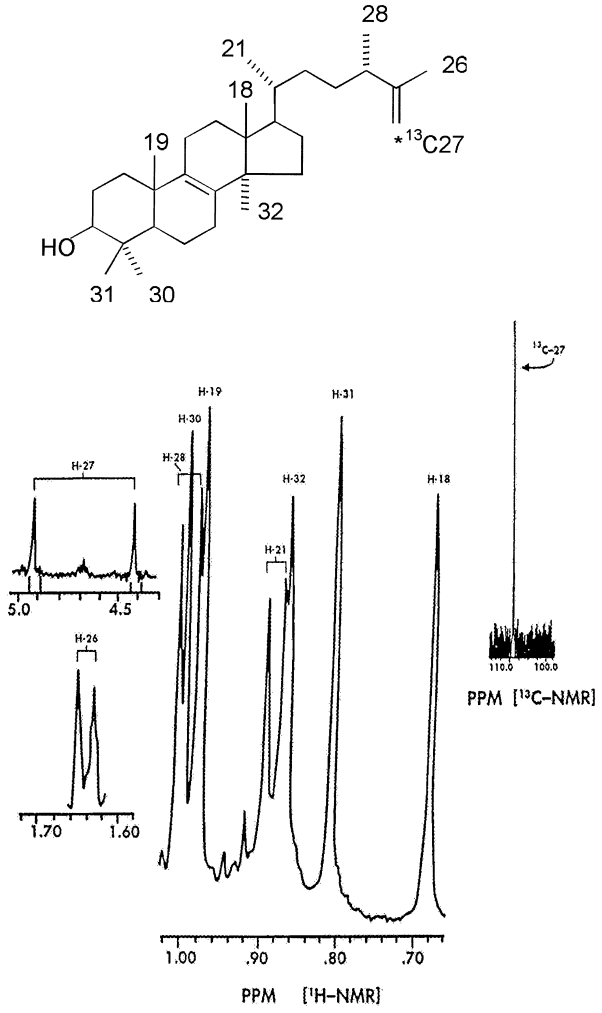

Figure 2. Partial ^{1}H and ^{13}C NMR (inset to right) spectra of 24β-methyl [27-^{13}C]lanosta-8,25(27)-dienol isolated from an incubation of the *P. wickerhamii* SMT and [27-^{13}C]lanosterol. The numbering system used is shown on the sterol structure at the top of the figure. See text for full details of the spectral analysis.

4.672 ppm (split signal) for the H27 olefinic proton and at δ 1.640 ppm (split signal) for the H26 methyl group (Fig. 2). The structure of the ^{13}C-labeled 24β-methyl lanosterol derivative is new. The stereochemistry at C24 of the new sterol is based on a comparison of the chemical shift for the H21 and H28 doublets associated with 24α- and 24 β-methyl sterols containing a $\Delta^{25(27)}$-sterols containing a $\Delta^{25(27)}$-sterol side chain.[28]

Classification of effects of structural modifications on SMT activity

Cycloartenol was assumed to be the preferred substrate based on the natural occurrence of cycloartenol in the cells of *P. wickerhamii*. A soluble SMT was used to assess structure–activity relationships. Dependence of *C*-methyl product formation on the sterol and AdoMet concentrations was found to obey Michaelis–Menten kinetics (data not shown) with apparent K_m and V_{max} values for cycloartenol and AdoMet of 28 μM and 36 pmol/min/mg and 10 μM and 13 pmol/min/mg protein, respectively. As shown in Table 1, no sterol was superior to cycloartenol in terms of substrate binding (K_m) or in catalytic competency (% transformation of substrate measured relative to the apparent V_{max}/K_m ratio of cycloartenol).

By examining the magnitude of the K_m values and the ratio of V_{max} to K_m of a series of sterols structurally related to cycloartenol, it has been possible to determine the relative structural requirements necessary for recognition and to measure the substrate acceptability for catalysis. Four domains of the cycloartenol structure were evaluated for their relevance to sterol binding (Fig. 3). The first structural domain examined involves the C3-hydroxyl group. Of the five sterols, cycloartenol, 3-desoxycycloartenol, 3-epicycloartenol, 3-ketocycloartenol or 3-OMe cycloartenol, only cycloartenol was catalyzed by the SMT under the assay conditions used here (Fig. 4, Table 1). These results indicate that the C3-hydroxyl group and its β-orientation are necessary features for recognition. Converting the hydroxyl group to the 3-keto or C3-methyl ether derivative impaired recognition thereby suggesting that added bulk at C3 affects the hydrogen-bonding abilities of the C3-hydroxyl group. Consistent with these observations was the finding that removal of an α-methyl group neighboring C3 at C4, as with 31-norcycloartenol, or complete removal of the C4-methyl groups, as with 30,31-dinorcycloartenol, produced substrates of less competency than the parent cycloartenol. Thus steric bulk from an angular C4 methyl group can also affect the recognition of the sterol.

The second domain examined was the nucleus (Table 1). To assess the importance of the 9β,19-cyclopropyl feature

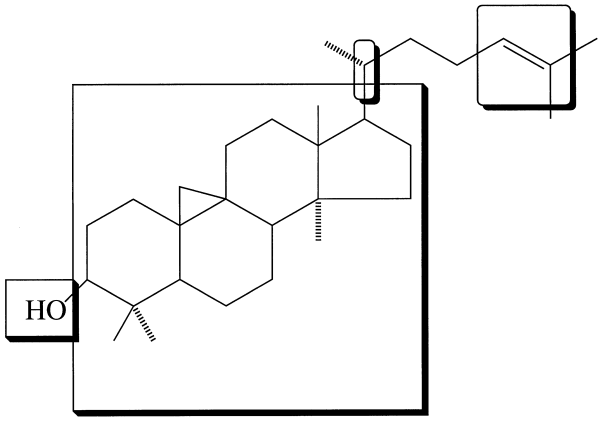

Figure 3. Domains on the cycloartenol substrate investigated for relevance to SMT recognition.

Table 1. Catalytic competence of sterols and substrate analogues to the SMT[a]

Substrate	K_m (μM)	V_{max} (pmol/min)	V_{max}/K_m	% Transformations of substrate compared to cycloartenol
Cycloartenol (**1**)	28	36	1.28	100
31-Norcycloartenol (**2**)	61	29	0.48	37
30,31-Dinorcycloartenol (**3**)	97	2	0.02	2
Parkeol (**4**)	80	62	0.78	60
Lanosterol (**5**)	176	35	0.20	16
Cholesta-5,20(22)*E*,24-trienol (**6**)	100	9	0.09	7
Desmosterol (**7**)	63	7	0.11	9
Cholesta-5,17(20)*E*,24-trienol (**8**)	276	2	0.01	1
Zymosterol (**9**)	38	7	0.18	14
14α-Methylzymosterol (**10**)	125	10	0.08	6
Cholesta-5,7,22*E*,24-tetraenol (**11**)	112	10	0.09	7
3-Epicycloartenol (**12**)	ND	ND	ND	ND
3-Desoxycycloartenol (**13**)	ND	ND	ND	ND
3-OMe Cycloartenol (**14**)	ND	ND	ND	ND
3-Keto-Cycloartenol (**15**)	ND	ND	ND	ND
Cholest-8-enol (**16**)	ND	ND	ND	ND
10α-Cucurbitacin (**17**)	ND	ND	ND	ND
Tirucallol (**18**)	ND	ND	ND	ND
20-Epidesmosterol (**19**)	ND	ND	ND	ND
Cholesta-5,17(20)*Z*,24-trienol (**20**)	ND	ND	ND	ND
Cholesta-5,25(27)-dienol (**21**)	ND	ND	ND	ND
Cycloartanol (**22**)	ND	ND	ND	ND
24-Methyldesmosterol (**23**)	ND	ND	ND	ND

[a]See text for details of enzymatic assay. Structures of the sterols are given in Figure 4. ND means not determined due to low or nonexistent of transmethylation.

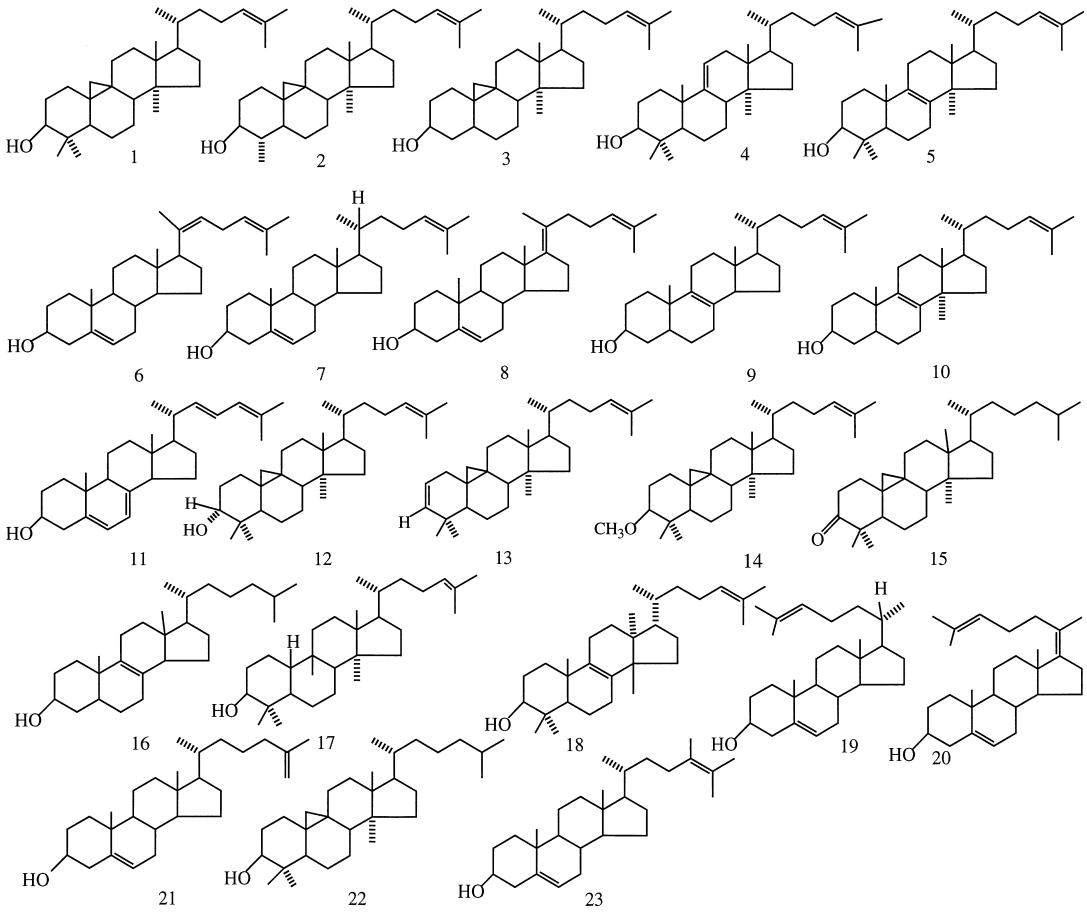

Figure 4. Substates tested and their corresponding catalytic competence ($V_{max}//K_m$) toward the *P. wickerhamii* SMT enzyme. **1**, cycloartenol; **2**, 31-norcycloartenol; **3**, 30,31-Dinorcycloartenol (24-dehydropollinstanol); **4**, parkeol; **5**, lanosterol; **6**, 20(22)*E*-dehydrodesmosterol; **7**, desmosterol; **8**, 17(20)*E*-dehydrodesmosterol; **9**, zymosterol; **10**, 14α-methylzymosterol; **11**, cholesta-5,7,22*E*,24-tetraenol; **12**, 3-epicycloartenol; **13**, 3-desoxy cycloartenol; **14**, 3-OMe cycloartenol. **15**, 3-ketocycloartenol, **16**, cholest-8-enol (24-dihydrozymosterol) **17**, 10α-cocurbitacin; **18**, tirucallol; **19**, 20-epidesmosterol; **21**, 17(20)*Z*-dehydrodesmosterol; **21**, cholesta-5, 25(27)-dienol; **22**, cycloartanol; **23**, 24-methyl desmosterol.

in the cycloartenol nucleus, we chose to compare cycloartenol with four related structural isomers of cycloartenol, parkeol, lanosterol, tirucallol and 10α-cucurbitacin. The ability of parkeol with a $\Delta^{9(11)}$-bond to bind to the SMT indicated that the 9β,19-cyclopropane ring was not essential to competency. However, placement of the double bond in the Δ^8-position generated a sterol that is less competent than parkeol. Inversion of the configuration of lanosterol at C13, C14 and C17, producing tirucallol,[31] or changing the nuclear conformation from flat (in cycloartenol)[23] to bent (10α-cucurbitacin,[32] led to a less competent structure than lanosterol. These results suggests that the position of the double bond and the three-dimensional shape of the nucleus affects recognition. It is possible that some non-substrates that were tested bind weakly to SMT but are not methylated, or methylated slowly. In support of this view, [3-^{3}H]lanosterol and [3-^{3}H]cholest-8-enol assayed with the pure recombinant yeast SMT failed to show productive binding (transformation),[29] but nevertheless, was found to bind weakly to the enzyme; K_d of lanosterol and cholest-8-enol was about two orders of magnitude less than the K_m of zymosterol (unpublished). The angular 14α-methyl group was found to minimally impair recognition based on a comparison of the activities of zymosterol and 14α-methyl zymosterol toward the *P. wickerhamii* SMT. A comparison of the activities of desmosterol (Δ^5) versus zymosterol (Δ^8) are consistent with the conclusion that the position of the nuclear bond is important for optimal substrate recognition. Thus the relative importance of nuclear features in binding measured as K_m values are: 9β,19-cyclo < Δ^9 < Δ^8 < Δ^5; 4,4-dimethyl < 4-monomethyl < 4, 4-desmethyl.

The third domain studied was the stereochemistry at C20 and the side chain conformation. To test the significance of the C20 chirality on sterol binding capabilities of five structurally similar sterols were compared desmosterol (20*R*-cholesta-5,24-dienol), 20-epidesmosterol (20*S*-cholesta-5,24-dienol), 17(20)*E*-dehydrodesmosterol (cholesta-5,17(20)*E*,24-trienol), 17 (20)*Z*-dehydrodesmosterol (cholesta-5,17(20)*Z*,24-trienol) and 20 (22)*E*-dehydrodesmosterol (cholesta-5,20(22)*E*,24-trienol). We chose to examine the desmosterol series of compounds since the relevant cycloartenol series of compounds was not available. Desmosterol was recognize by the SMT with an apparent K_m 63 μM in the same order of magnitude of cycloartenol.

Under the assay conditions used for these experiments, desmosterol, cholesta-5,20(22)*E*,24-trienol, and cholesta-5,17(20)*E*,24-trienol (K_m = 63 μM, 100 and 276 μM,

respectively) were catalyzed by the SMT whereas neither 20-epidesmosterol or cholesta-5,17(20)*Z*,24-trienol were effectively transformed by the SMT. The results indicate that the natural configuration of 20*R* is critical but not obligatory to sterol binding (as confirmed from the study of the binding constants (K_i) determined for the epimeric pair of inhibitors 20*R*- and 20*S*-22-azacholesterol (Table 2). The ability of 20(22)*E*-dehydrodes-mosterol and 17(20)*E*-dehydrodesmosterol to bind to the SMT proves that the C20 taetrahedral character, native to cycloartenol, is not essential for binding. Nonetheless, the side chain must orient into the right-handed conformation for optimal catalytic competency. Cholesta-5,7,22*E*,24-tetraenol was bound to the SMT similar to 20(22)*E*-dehydrodesmosterol, suggesting that the side chain conformation assumes a staggered conformation from C20 to C26 for transformation.

Table 2. Blockage of SMT activity by substrate analogues and rationally designed intermediate inhibitors[a]

Inhibitor	K_i	K_i/K_m	Kinetic pattern
25-Azacycloartenol (**24**)	3 nM	1.1×10^{-4}	NC
25-Azalanosterol (**25**)	7 nM	2.5×10^{-4}	NC
25-Azacholesterol (**26**)	10 nM	3.6×10^{-4}	NC
24-Azazymosterol (**27**)	15 nM	5.6×10^{-4}	NC
23-Azacholesterol (**28**)	20 nM	7.1×10^{-4}	NC
20*S*,22-Azacholesterol (**29**)	25 nM	8.9×10^{-4}	NC
24(*R*,*S*),28-Epiminocholesterol (**30**)	85 nM	3.0×10^{-3}	NC
24(*R*,*S*),25-Epiminolanosterol (**31**)	13 nM	4.6×10^{-4}	NC
20*R*,22-Azacholesterol (**32**)	50 μM	1.78×10^{0}	C
Solanidine (**33**)	2 μM	0.7×10^{0}	C
Solasodine (**34**)	75 μM	2.7×10^{0}	C
Cyclolaudenol (**35**)	23 μM	0.8×10^{0}	C
24(28)-Methylenecycloartanol (**36**)	85 μM	3.6×10^{0}	C
Ergosterol (**37**)	80 μM	2.9×10^{0}	C
Cholesterol (**38**)	ND	ND	ND
Sitosterol (**39**)	ND	ND	ND

[a]See text for details of enzymatic assay. Structures of the sterols are given in Figure 5. K_m is that of cycloartenol; C-competitive; N.C., non-competitive; N.D., no activity determined.

The fourth domain examined was the $\Delta^{24(25)}$-bond. The importance of a $\Delta^{24(25)}$-bond in the sterol side chain was demonstrated by comparing the activities of cycloartenol with cycloartenol and zymosterol and cholest-8-enol. No transformation was evident with either cycloartenol or cholest-8-enol as substrate. Regiospecificty for the $\Delta^{24(25)}$-bond was demonstrated by comparing the activities of desmosterol and cholesta-5,25(27)-dienol. No transformation was evident with cholesta-5,25(27)-dienol as substrate. Bulk at C24 interferes with *C*-methylation activity as shown by the effective transformation of desmosterol (24H-atom) and the lack of transformation of 24-methyl desmosterol (24-methyl group). 24(28)-Methylene cycloartenol (Table 2, Fig. 5) was not transformed under the assay conditions tested, suggesting that the algal SMT most likely will not perform the consecutive *C*-methylations of cycloartenol to 24-ethylidene cycloartanol during active cell proliferation.

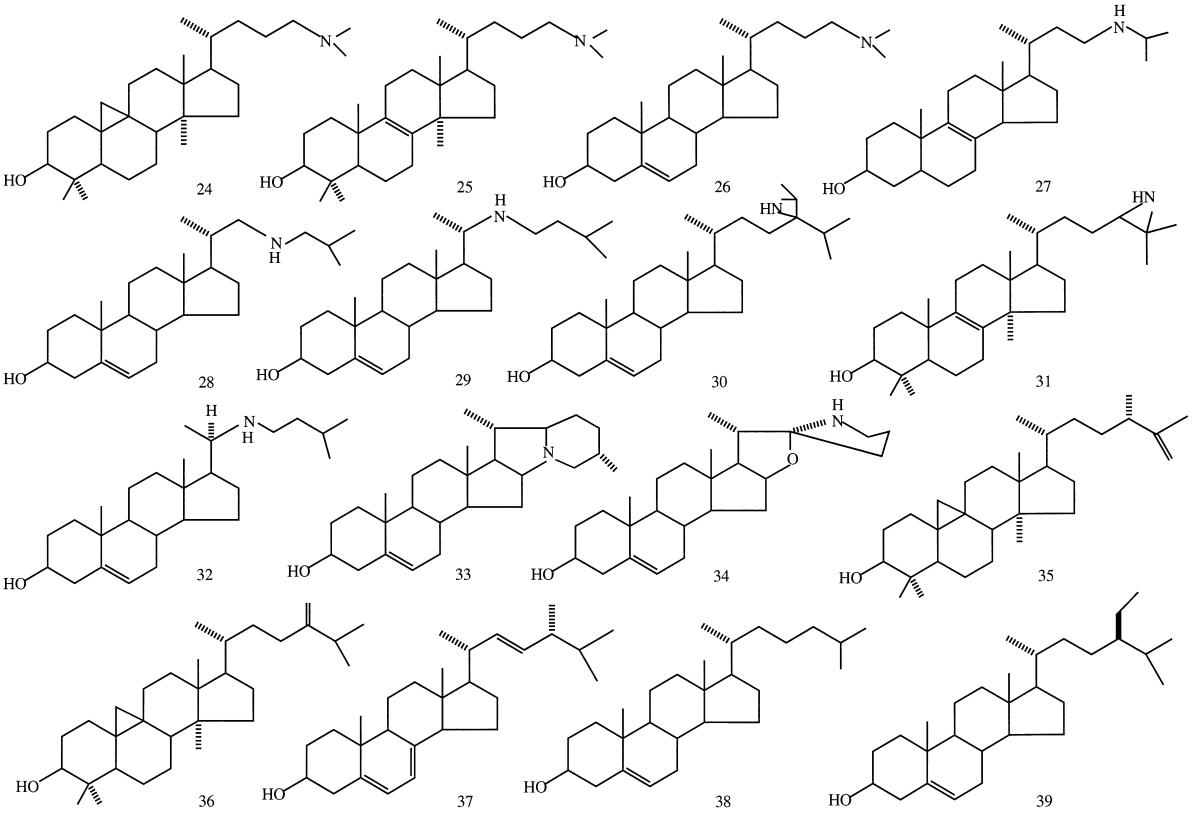

Figure 5. Inhibitors tested (range from 1 nM to 100 μM) with the SMT from *P. wickerhamii*. Cycloartenol and AdoMet were added at a fixed concentration of 50 μM. Compounds shown are: **24**, 25-azacycloartenol; **25**, 25-azalanosterol; **26**, 25-azacholesterol; **27**, 24-azazymosterol; **28**, 23-azazymosterol; **29**, 20*S*,22-azacholesterol; **30**, 24(*R*,*S*),28-epiminofucosterol; **31**, 24(*R*,*S*),25-epiminolanosterol; **32**, 20*R*,22-azacholesterol; **33**, solanidine; **34**, solasodine; **35**, cyclolaudenol; **36**, 24(28)-methylenecyclartanol; **37**, ergosterol; **38**, cholesterol; **39**, sitosterol.

High energy intermediate analogues

To help define the structural requirements of the SMT active site, high energy intermediate analogues with a nitrogen substitutent were examined for their ability to inhibit the SMT-catalyzed reaction in the direction of cyclolaudenol formation. A list of potential inhibitors (tested to a concentration of 100 μM) and a summary of inhibition characteristics are reported in Table 2 (cf. accompanying structures in Figure 5). For mechanistic reasons, we examined a series of ammonium derivatives in which an azridine group or aza group was introduced in the lateral chain between carbon atoms C22 and C25. Most of the sterol analogues were potent inhibitors of the SMT activity exhibiting non-competitive type kinetic patterns with low K_i values in the nM range.

The effectiveness of the sterol analogues on inhibition of the SMT activity was found to be related to the nuclear structure of the inhibitor and on the structure of the side chain. The sterol analogues were found to group into three groups related to their kinetic patterns and degree of inhibitory power. Group I (Table 2: **24**–**31**) contains compounds that exhibited non-competitive type kinetics with respect to cycloartenol whereas Groups II (**32**–**35**) and III (**36**–**39**) contained compounds that exhibited competitive-type kinetics. Group I inhibited in the nanomolar range whereas Groups II and III inhibited in the micromolar range, similar to the K_m of cycloartenol. None of the inhibitors were found to exhibit time-dependent inactivation type kinetics, suggesting they were all reversible inhibitors. The expectation from a non-competitive-type kinetic pattern is that binding of the inhibitor occurs at a site on the enzyme other than the site for the normal substrate. The high potency is considered to result from tight-binding and from the ammonium-containing side chain to interact at a subsite in the active center that differs from the location normally occupied by the $\Delta^{24(25)}$-bond at initial binding.

The relative binding efficiency of 25-azacycloartenol, 25-azalanosterol and 25-azacholesterol follows the order of acceptability of the corresponding Δ^{24}-sterols as substrates for *P. wickerhamii*. This trend suggests that 25-azasterols behave as sterol mimics of the SMT enzyme. Regardless of whether the nitrogen atom was introduced at C25, or at C24, C23 or C22, the inhibitory power of the azasterol was about the same. 24(*R,S*),28-Epiminofucosterol (K_i 85 nM) was much less effective at binding than 24(*R,S*),25-epiminolanosterol ($K_i = 13$ nM), confirming the observation with 24-methyldesmosterol, that increased bulk at C24 inhibits *C*-methylation activity. The NSF fraction from select azridine- or aza-inhibitor treated cycloartenol incubations was examined by HPLC.

There was no evidence of formation of 25-hydroxy sterols by HPLC from the incubation with 25-azacycloartenol, suggesting that a discrete C25 cation had been formed during the reaction cycle involving cycloartenol as substrate either the 25-hydroxy sterol was not released (covalent attachment to the enzyme) or there was not sufficient time to react with a water molecule. Another possibility is that carbenium ion character is developed in a concerted reaction such that *C*-24-alkylation and C-27 proton elimination are simultaneous thereby eliminating the possibility of the formation of a C25 cation during *C*-methylation.

The steroidal alkaloids solasodine and solanidine with the ammonium group caged in a ring system were tested and found to inhibit SMT activity in the micromolar range. The 22-azacholesterol sample with the unnatural configuration at C20*R* (20α-hydrogen atom) was also found to bind to the SMT but much less efficiently than 22*S*-azacholesterol with the natural C20-configuration (20β-hydrogen atom). The molecular parameters of the inhibition indicate that the sterol side chain may bind to the SMT with different structural parameters (open chain versus caged) or in different orientations (C20*R* or C20*S*-alternate configurations that affect the side chain conformation). The nature of these interactions suggest that an anionic center exists in the active site in the vicinity of C24/C25 of the native substrate corresponding to the 24-methyl 24(25)-carbenium ion intermediate formed during *C*-methylation of the 24,25-double bond. In order to interact with the anionic center (deprotonating base) optimally the side chain must orient to the "right-handed" conformation.[31]

Product analogues and related inhibitors

To determine the inhibition from product analogues (Fig. 5), 24(28)-methylene cycloartanol was compared to cyclolaudenol, and ergosterol was tested relative to sitosterol and cholesterol. 24(28)-Methylenecycloartanol was an effective competitive inhibitor of SMT enzyme activity with a K_i of 85 μM, whereas the K_i of the competitive inhibitor cyclolaudenol was 23 μM. The greater efficacy of binding of cyclolaudenol compared to 24(28)-methylene cycloartanol is consistent with the *C*-methyl mechanism to generate a 24β-methyl sterol. Ergosterol was also a competitive inhibitor of SMT activity with a K_i of 80 μM whereas neither cholesterol nor sitosterol were found to inhibit SMT activity, even at the highest concentration of inhibitor tested of 200 μM. The specificity for ergosterol to down-regulate SMT activity indicates the binding pocket of the SMT distinguishes the size of the group at C24.

Inhibition of growth and sterol analysis

25-Azacycloartenol, solasodine and ergosterol were tested as inhibitors of growth. Only the nitrogen-containing compounds inhibited growth, presumably because only they were actively absorbed by the cells. Inhibition of cell growth was found to lead to cell death as determined by cell fragmentation microsopically of 25-azacycloartenol treated cells. The IC_{50} value of cells treated with 25-azacycloartenol was 35 nM and the IC_{50} value for solasodine was 2 μM. At the IC_{50} concentration of inhibitor, the sterol compositions resulting from the two treatments were similar with cycloartenol predominating the sterol mixture. Ergosterol was the major sterol observed in untreated *P. wickerhamii,* total cellular sterol = 19 fg/cell, whereas in 25-azacycloartanol- and

solasodine- treated cells, the total cellular sterol at IC_{50} was significantly increased to 104 fg/cell and 372 fg/cell, respectively. The IC_{50} value for 25-azacycloartanol correlated well with the K_i value for inhibition of SMT enzyme activity. The specific accumulation of cycloartenol in the 25-azacycloartenol and solasodine-treated cells confirms the mode of action of these compounds is to target the inhibition of the SMT enzyme activity and that cycloartenol cannot replace ergosterol as a membrane insert, similar to our earlier observations with yeast.[33,34]

Discussion

Carbocations typical of those generated during biomimetic *C*-methyl transfer reactions of an olefin are notoriously unselective in their reactions.[35] Nonetheless, the fine-tuning of SMT enzymes under the pressure of natural selection has resulted in efficient catalysts that is reflected in the regio- and stereoselectivity to the reactions they promote and in the high degree of sterol specificity for binding.[36–43] Two mechanisms have been proposed for the *C*-methylation of a sterol acceptor molecule, a covalent (X-group)[36–39,44] and a noncovalent (steric-electric-plug)[40–42] mechanism. Crucial to the structure and stereochemistry of the 24-methylated products generated by these different mechanisms is the orientation of the sterol side chain at initial substrate binding; in the covalent mechanism two side chain conformations are employed to generate the $\Delta^{24(28)}$- or $\Delta^{25(25)}$-olefins whereas in the noncovalent mechanism a single side chain orientation is employed to generate the product distribution (Fig. 6). Additional features of the steric-electric plug model of sterol *C*-methylation involve a reaction mechanism whereby the facial disposition of the Δ^{24}-bond relative to AdoMet and its counter ion promotes alkylation from the *si* face of Δ^{24} followed by migration of a hydrogen atom from C24 to C25 across the *re* face and a kinetic mechanism whereby specific molecular interactions develop in the ternary complex of the active centre between key nucleophilc groups associated with the sterol (C3 hydroxyl group and Δ^{24}-bond) and a polar amino acid(s). Although many aspects of this model have been verified experimentally, including studies on fungal and vascular plant SMTs involving substrate and HEI analogues[50–60] and structural and kinetic analyses involving site-directed mutagenesis of the SMT sterol binding site from *S. cerevisiae*,[42] little is known about SMT structure and function from primitive organisms.

Three major families of SMT proteins are speculated to exist according to the nature of the predominant product formed from catalysis:[6] SMTI ($\Delta^{24(28)}$-olefin), SMTII ($\Delta^{23(24)}$-olefin) or SMTIII ($\Delta^{25(27)}$-olefin), all of which should pass through a 24β-methyl high energy intermediate. SMTIII can be considered a more primitive SMT enzyme based on the product, 24β-methyl $\Delta^{25(27)}$, which characterizes the sterol composition of primitive plants.[48] SMTIII can generate a $\Delta^{24(28)}$ olefin, but the $\Delta^{24(28)}$-product is expected to be a minor

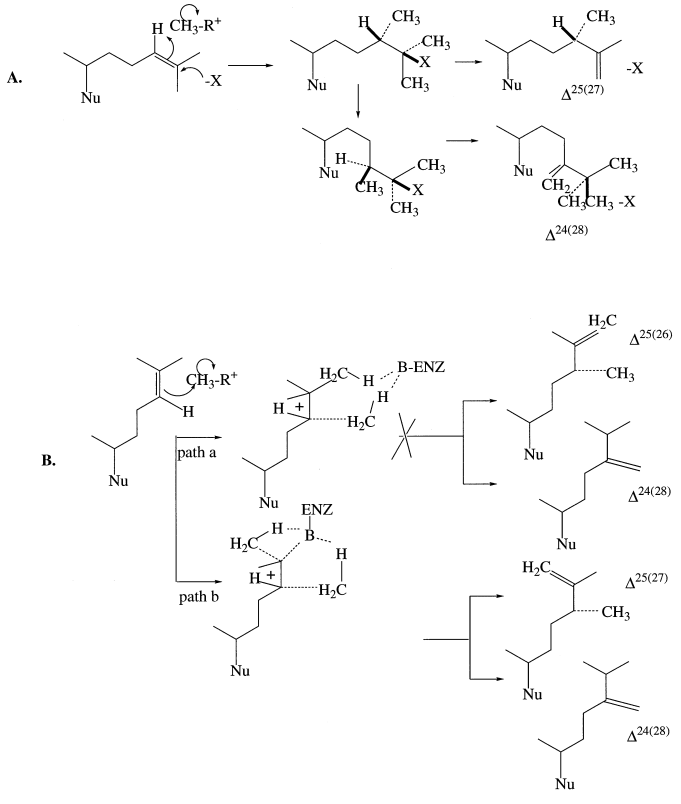

Figure 6. Two oppposing views of the $\Delta^{24(28)}$- and $\Delta^{25(27)}$-*C*-methylation pathway(s) operating in alga phytosterol synthesis: (A) The X-group mechanism involves a step-wise mechanism of two different sterol side chain conformations to achieve the final product distribution catalyzed by the SMT; (B) the steric–electric plug model involves a concerted mechanism of a single phytosterol side chain conformation to achieve the final product distribution catalyzed by the SMT; path b is the expected route to olefin formation. See text for further details.

component of the *C*-methylated sterol mixture. In view of the recent demonstration that as many as three SMTs (isoenzymes) from the same plant (tobacco and Arabidopsis) are formed to catalyze a $\Delta^{24(28)}$-structure with either a C_1- or C_2-group attached to C24,[10,45,46] and the observation herein that *P. wickerhamii* synthesizes trace levels of 24-methylene sterols in situ, is reason that SMTs may exist in select organisms or tissues singly or in multiple molecular forms. One of the enzyme forms can be bifunctional to permit the consecutive *C*-methylations of the Δ^{24}-bond. Assuming that SMTs exist as isoenzymes, they can be expected to be differentiated by sterol specificity, allosteric modulators, such as ergosterol, and/or electrophoretically based on different pI values for each isoenzyme. Thus, the observation that the *P. wickerhmaii* SMT prefers cycloartenol to zymosterol, indicates a 'plant-derived' SMT. Alternatively, the observation that the activity of the *P. wickerhamii* SMT is inhibited by ergosterol and not by sitosterol, suggests that the *P. wickerhamii* SMT is a primitive SMT.

The results establish conclusively that sterol competency of the algal SMT catayzed reactions involves recognition by both poles of the sterol molecule, similar to the action from fungal and vascular plant SMTs.[28,29] Structure–activity studies of substrate analogues tested with the *P. wickerhamii* SMT indicated four molecular domains on cycloartenol, the native substrate, were essential to binding: (i) 3β-OH group, (ii) planar nucleus, (iii) 20*R*-stereochemistry in which the side chain is oriented to the right (C22 *transoid* to C18 in the usual view of the molecule) and (iv) C_8-side chain with an intact terminal isopropyl group and an unhindered 24,25-double bond. Of less importance to sterol competency was substitution at C4 or the degree of unsaturation in the nucleus.

The coupled methylation-deprotonation catalyzed by the *P. wickerhamii* SMT was found to be stereoselective and regiospecific. Based on the ^{1}H NMR analysis of the enzyme-generated cyclolaudenol, the *C*-methylation of the $\Delta^{24(25)}$-bond associated with cycloartenol the methyl group introduced at C24 was β-oriented. The retention of three deuterium atoms at C28 from incubation with [$^2H_3$-*methyl*]AdoMet indicates a $\Delta^{24(28)}$-intermediate is not involved with the reaction pathway. Thus, the C24 methyl group must be delivered from the β-face of the 24,25-double bond, similar to the *si* face reaction mechanism catalyzed by SMTs from yeast and vascular plants.[6,7,49] Based on the ^{1}H and ^{13}C NMR analysis of the enzyme-generated [27-^{13}C]25(27)-24β-methyl 24,25-dihydrolanosterol, the regiospecific proton elimination occurred at the *cis* terminal (*Z*)-methyl group to become C27 (an exo-methylene group). The observed regiospecific CH_3 to CH_2 eliminations generated during *C*-methylations of the 24,25-double bond to produce related 24β-methyl or ethyl-Δ^{25}-sterols investigated thus far occur consistently at the *cis*-methyl group.[44,47] We conclude that the formation of the $\Delta^{25(27)}$-bond is concerted with C-24 methylation and that the C24 configuration of cyclolaudenol should directly reflect the direction (i.e., *si*-face or backside attack) of methylation of the $\Delta^{24(25)}$-bond of the corresponding sterol substrate, cycloartenol, consistent with the steric–electric plug model.[40]

Conclusion

The demonstration that select HEI analogues can inhibit the *C*-methylation step and inhibit cell proliferation of *P. wickherhamii* appears to result from the initial positioning of the *C*-methylation step in the cycloartenol–ergosterol pathway compared to its positioning the lanosterol–ergosterol pathway in yeast and from the inability of cycloartenol to replace ergosterol in sterol-controlled membrane competence thereby having a greater disruptive influence on ergosterol homeostasis in the alga. These results contrast with a yeast or protozoan system where the *C*-methylation step occurs late in the lanosterol–ergosterol pathway, so that upon cellular treatment with 25-azasteroids, zymosterol (a membrane competent sterol) accumulates leading to reduced growth rates.[51–58] The different sensitivities of microorganisms toward 24-amino and 24-epimino steroids may also be from the positioning of the SMT in the post-lanosterol pathway. For these reasons, rational drug design targeted for the *C*-methylation reaction, occurring as a first step in the post-lanosterol–cycloartenol pathway may offer a new approach to treat disease, including Protothecosis. The search for natural products such as plant alkaloids solasodine, solanidine and the marine alkaloid plakinamine and related compounds[57] to block SMT activity of microbes sensitive to these compounds may also warrant a renewed interest.

The combination of results suggest that SMTIII from *P. wickerhamii* may have a different complement of active-site amino acid residues involved in substrate binding and catalysis than those of SMTs from yeast and higher plants operating as SMTI- type enzymes. Further investigations to define the active-site structure of the *P. wickerhamii* SMT should reveal the specific catalytic features that are responsible for the observed differences in properties and reaction pathways between the alga SMT and related SMT enzymes.

Experimental

Algal material, substrates and reagents

P. wickerhamii strain YB-4330 was obtained from Dr. C. P. Kurtzman (US Department of Agriculture/ Northern Regional Research Center, Peoria, IL). The cultures were grown at 29 °C on a medium of 1% Bacto-yeast extract/2% Bacto peptone/2% dextrose. The cultures (300 mL medium per 1-L Erlenmyer Flask) were inoculated with 0.5×10^5 cells/mL and shaken continuously for 48 h to growth arrest, ca. 1.0×10^8 cells/mL, yielding per flask ca. 5.7 g fresh weight of packed cells. The cells were cultured in the dark on a New Brunswick Scientific Shaker at 180 rpm. Cells were harvested by centrifugation (10,000×*g* for 10 min) using a J2-HS Beckman centrifuge and the resulting packed cells stored at −20 °C. Cell counting methods were followed as described.[21] All sterol substrates and inhibitors were purchased commercially, isolated from natural sources or synthesized as described in our earlier papers,[22–25] except for the synthesis of 20-epidesmosterol and 3-des-

oxycycloartenol which will be described elsewhere. [27-^{13}C]Lanosterol was prepared as described.[26] AdoMet iodide salt was purchased from Sigma, [$^3H_3$-*methyl*]AdoMet (10–15 Ci/mmol) and [2-^{3}H]acetate (75–150 mCi/mmol) were purchased from NEN DuPont and [$^2H_3$-*methyl*]AdoMet (99.3 at % deuterium) as the *p*-toluenesulfonate salt was purchased from MSD Isotopes (Canada). All other biochemicals and reagents were purchased from Sigma or Aldrich, unless otherwise noted.

Inhibition of Growth of *P. wickerhamii*

Duplicate flasks of growth medium containing a variety of concentrations of the chosen inhibitor dissolved in 100 μL ethanol (usually four concentrations, plus a control and none) were inoculated with equal numbers of cells and grown for 48 h. The approximate concentration of inhibitor supplied to medium for 50% inhibition of growth was reported as the IC_{50} value. Ethanol at the same concentration used to solubilize the inhibitors had no apparent effect on growth.

Enzyme isolation, solubilization and assay

P. wickerhamii cells cultured to the onset of growth arrest were used as the enzyme source for determination of constitutive sterol methyl transferase activity. Frozen cell pellets were added to a chilled mortar and allowed to thaw. The mass was refrozen by adding liquid nitrogen. An equal amount of sea sand to the weight of the frozen pellet was added to the mortar and the cells broken by grinding vigorously for 15 min. The resulting paste was washed with homogenizing buffer (buffer A: 0.1 M Tris–HCl (pH 7.5 to 8.0), 4 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5 M sucrose and 300 mg/100 mL bovine serum albumin) and filtered through four layers of cheesecloth. The filtrate was clarified by centrifugation at 7000×*g* for 20 min and further centrifuged at 100,000×*g* for 90 min to sediment the microsomes. From 53 g fr weight of cells, eight microsomal pellets were generated. Solubilization of the SMT enzyme was achieved by adding the combined microsomal suspension of eight pellets to a resuspension buffer containing 20% glycerol and the sample homogenized until the pellets were evenly distributed in buffer. The sample was poured into a beaker on ice and emulphogen (polyoxoethylene 10 tridecyl ether, Sigma; 10% (v/v)) was added to a final concentration of 0.3% (volume of detergent/volume of buffer containing 20% glycerol). The solubilized material was allowed to stand for 30 min on ice with periodic shaking. The sample was centrifuged at 100,000×*g* for 1 h. The resulting supernatant was used to assay the SMT. Protein determinations were as described with bovine albumin as the standard.[61]

The standard assay of the microsomal or solubilized SMT activity was performed in 0.5 mL of the respective suspension dissolved in buffer B (50 mM Tris–HCl, pH 7.5–8.0, 2 mM $MgCl_2$ and 2 mM β-mercaptoethanol) for 45 min at 35 °C in the presence of 50 μL of [$^3H_3$-*methyl*] AdoMet (0.5–1 μCi/mL), substrate or substrate analogue dissolved in 50 μL buffer A and Tween 80 (final concentration of 0.1% (w/v)) to give a total volume in the assay mixture of 0.6 mL and final concentration of each substrate of 50 μM, unless otherwise noted. The protein concentration in the microsomal suspension was maintained between 2.5 and 3.0 mg/mL. The production of radiolabeled *C*-methylated sterols using the soluble enzyme preparation was linear with respect to time for up to 3 h at a protein concentration of 1.5 mg/mL, and all subsequent experiments were carried out well within these linear assay conditions. The rates of *C*-methylation as a function of substrate concentration with cycloartenol gave rise to typical hyperbolic saturation curves. The double reciprocal plots were linear and the measured K_m values for cycloartenol were about 28 ± 3 μM where the number of different trials equaled 10. These assay conditions were found to be optimal and similar to those reported for related SMT enzymes from fungi and vascular plants.[50,51] The reaction was quenched by the addition of 10% KOH in ethanol (w/v). To increase the recovery of sterol from the quenched reaction, we found that adding 1 mL of DMSO to the quenched reaction and heating at 95 °C for 10 min followed by extraction with skelly solve F (mixed hexanes) (3×1 mL) led to an increase of sterol recovered from the incubation mixture by about 3-fold compared to experiments where the dimethyl sulfoxide (DMSO) treatment was not followed (65,000 dpm per assay versus 23,000 dpm per assay). Generally, the radioactivity associated with the DMSO-treated non-saponifiable lipid fraction served to establish product conversion. For some experiments, the combined hexane extracts from DMSO-treated incubations were analyzed by TLC to separate sterols by the degree of substitution at C4. The desired sterol class (4-desmethyl, 4-monomethyl or 4,4-dimethyl sterol) was eluted from the TLC plate, injected into HPLC and an aliquot of each fraction taken for liquid scintillation counting to determine the conversion rate or product distribution. Radioactivity was measured in a Beckman LS #6500. Liquid scintillation counting was performed in 5 mL of Scintiverse (Fisher) at a ^{3}H counting efficiency of 56%.

For preparative incubations, the number of assays was increased to 90–100, generating about 100 to 300 μg product. Each assay contained the standard protein concentration and saturating levels of AdoMet and sterol substrates, and the incubation time was extended to 15 h. The assays were pooled, saponified and extracted in the usual manner. The resulting non-saponifiable lipid fraction was chromatographed by TLC and HPLC to isolate sterols to homogeneity. For the isolation of sterols from whole cells, the combined cell pellets were ground to a paste and saponified by treatment with a refluxing solution of 10% KOH, 10% H_2O and 80% aqueous methanolic KOH (w/v/v) for 30 min. The resulting non-saponifiable lipid fraction was chromatographed on TLC and HPLC to obtain pure sterols.

Inhibitor assay

Boiled controls were included in each experiment, and in all cases non-enzymatic product formation was negligible. Preliminary studies indicated that the proportion

of $\Delta^{25(27)}$-24β-methyl product formed in the non-saponifiable lipid fraction was not influenced by the degree of inhibition with any of the analogues, thus permitting routine determination of sterol methylation as a measure of SMT enzyme activity. The sterol to protein ratio in the microsomes were found to be 20 μg/mg, and after solubilzation of the SMT to be 4 μg/mg. The only sterol detected in the soluble SMT fraction was ergosterol. Inhibition by each analogue, and velocity comparisons between the different sterol substrates were determined using single enzyme preparations thereby avoiding any preparation to preparation variation. All inhibition studies were performed using variable analogue concentrations in the presence of fixed concentrations of sterol substrate (usually cycloartenol) and AdoMet. Inhibition type and K_i values were determined by standard graphical procedures,[58] for which computer assisted linear regression analysis afforded correlation coefficients greater that 0.85 in all cases. For velocity determination with the substrate analogues, the production of methylated sterol was monitored as outlined above. The sample to sample variation (in dpm) was less than 10% in all cases as judged by direct comparison of duplicate experiments.

Identification of metabolites

The chromatographic and spectral properties of the metabolite(s) from selected incubations were determined by their rates of movement in TLC (Silica gel G plates developed in benzene:diethyl ether (85:15), GLC (3% SE-30 packed columns operated isothermally at 245 °C) and HPLC (two HPLC columns, Whatman C_{18} or Zorbax C_{18}, were employed to separate structurally similar compounds), by GC–MS (Hewlett-Packard 5973 mass detector) or by ^1H and ^{13}C NMR analysis (Bruker AF300-MHz for ^1H and 75 MHz for ^{13}C; samples referenced to tetramethyl silane or chloroform) as described in earlier publications.[27,62] Relative retention times in GLC (RRt_c) and HPLC (α_c) are relative to the mobility of cholesterol eluting from the column.

Acknowledgements

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