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Mechanism-based active site modification of the soybean sterol methyltransferase by 26,27-dehydrocycloartenol

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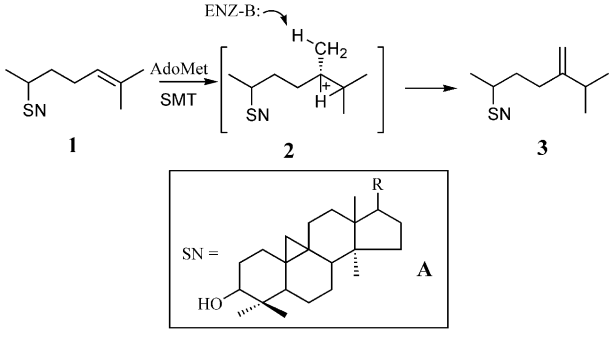
Abstract—26,27-Dehydrocycloartenol (26,27-DHC) was shown to be a substrate for the soybean sterol methyltransferase (SMT) as well as a mechanism-based inhibitor of enzyme action. The K_m and k_{cat} for 26,27-DHC was 10 μM and 0.018 min^{-1}, respectively. SMT catalyzed 26,27-DHC to two products tentatively identified as 26-homocholesta-9,19-cyclo-23(24)*E*,26(26′)-dienol and 26-homocholesta-9,19-cyclo-26(26′)-en-3β,24β-diol by GC–MS. Inhibitor treatment was concentration- and time-dependent (pseudo-first-order kinetics). A replot of the half-lives for inactivation versus the inverse of the inactivator concentrations gave an apparent K_i of 42 μM and a maximum rate of inactivation of 0.29 min^{-1}. A partition ratio (k_{cat}/k_{inact}) was calculated to be 0.06.

Sterol methyltransferase (SMT) from plants catalyzes the *C*-methylation of cycloartenol (**1A**), the ubiquitous C_{30} intermediate of the phytosterol pathway, to a wide variety of sterols containing Δ^{23}-, Δ^{24}- and Δ^{25}-side chains.[1] The crucial role of SMTs in the origin of the different olefins has stimulated considerable interest in the details of the *C*-methylation mechanisms and in determining the topography of the active-site structures for purposes of evaluating protein evolution and engineering new product compositions that lead to value-added traits. The first C_1-transfer pathway catalyzed by SMT1 proceeds in soybean plants as shown in Scheme 1.[1,2]

Three types of mechanistic probes have been used to provide evidence for the electrophilic *C*-methylation reactions catalyzed by SMTs. The known SMT inhibitors include substrate mimics (e.g., 26,27-dehydrozymosterol), product mimics that can act as dead-end inhibitors (e.g., cyclolaudenol with a side chain containing a 24β-methyl group and $\Delta^{25(27)}$-bond) and transition state analogues (25-azacycloartenol).[1] The last group includes a series of high energy intermediate analogues that possess structural and charge analogy to the presumptive C-24 (or C-25) cation generated during the reaction sequence and these inhibitors have been tested with a range of plant and fungal SMTs. On the other hand, substrate mimics of the mechanism-based inhibitor class have been developed only for the yeast and yeast-like microbes synthesizing SMT.[3]

Since there is as yet no three-dimensional structure of SMT and the topographical relations of SMT active site structures determined by enzymatic studies is limited, we considered an alternative route for obtaining comparative information of SMT structure and function by using the results of affinity labeled plant and fungal SMTs and homology sequencing data. Indeed, we have used such approach to identify a highly conserved region rich in aromatic amino acids, referred to as Region I, containing a signature motif Y83EYGWG88 not present in other AdoMet-dependent methyltransferases, and to



Scheme 1. Pathway for the *C*-methylation of cycloartenol, the preferred substrate of plant SMT1s.[1]

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characterize the sterol binding site of yeast SMT using 26,27-dehydrozymosterol (26,27-DHZ), the methylene cyclopropane analogue of the normal substrate, zymosterol.[4] Therefore to shed light on the bioactivity of a mechanism-based inhibitor designed to behave as 26,27-DHZ and to learn more about the active site structure of a plant SMT, we describe herein that 26,27-dehydrocycloartenol (26,27-DHC) can undergo *C*-methylation to side chain structures similar to those generated by yeast SMT action and that it also acts to covalently modify the soybean SMT.

26,27-Dehydrocycloartenol (**4A**) was prepared from the C3-acetate of cycloartenol **1A** (20 mg) using the Wittig reaction and aldehyde produced by ozonolysis.[5] **4A** was purified to homogeneity by HPLC using a semi-preparative Phenomenex column to give a 30% overall yield. The relevant chromatographic and spectral characteristics of **4A** are: GC on the capillary column,[4] $RRT_c = 1.50$; IR (neat) V_{max} cm^{-1} 3436, 2931, 2860, 1649, 1625, 1460, 1372, 1096, 1049; MS, (EI-MS) *m*/*z* (relative intensity) M^+ 424 (5), 409 (15), 391 (32), 363 (12), 337 (8), 315 (7), 284 (24, fragment corresponding to 9,19-cyclopropane cleavage), 259 (10); ^1H NMR (300 MHz, $CDCl_3$) δ 0.965 (s, 3H, 18-H), 0.551 and 0. 333 (d, 2H, 19-H, corresponding to the cyclopropane *exo*/*endo* ring hydrogens), 0.905 (d, 3H, 21-H), 5.742 (m, 1H, 24-H), 1.010 (bs, 4H, 26-H and 27-H), 0.984 (s, 3H, 30-H), 0.811 (s, 3H, 31-H), 0.892 (s, 3H, 32-H); ^{13}C NMR (75 MHz, $CDCl_3$) δ (side-chain carbons) 35.76 (C-20), 18.12 (C-21), 35.86 (C-22), 28.79 (C-23), 118.86 (C-24), 120.59 (C-25), 2.19 (C-26), 1.80 (C-27).

Incubation of purified soybean SMT with 26,27-DHC led to a pseudo-first-order time- and concentration-dependent loss of enzyme activity (Fig. 1).[6] From a replot of the half-lives for inactivation ($t_{1/2}$) versus the inverse of the inactivator concentrations (1/[I]) were obtained K_i and k_{inact} values of 42 μM and 0.29 min^{-1}, respectively. These values compare to a K_m of 30 μM and k_{cat} of 0.60 min^{-1} for soybean SMT with cycloartenol.[2] In a separate experiment, the kinetic pattern for 26,27-DHC was established to be of the competitive-type relative to varied cycloartenol (5–100 μM) and saturating concentrations of AdoMet (data not shown).[2,6] Although the kinetic constants for 26,27-DHC are approximate, the results indicate that the analogue binds well but reacts more slowly than the natural substrate. Cycloartenol assayed at 50 and 100 μM concentrations together with saturated concentrations of [*methyl*-$^3H_3$]AdoMet against 100 μM 26,27-DHC for 45 min was shown to protect the enzyme from inactivation, generating 33 and 48% *C*-methylation activity, respectively, relative to the *C*-methylation activity of a control incubation. These results indicate that inactivation is active site directed.

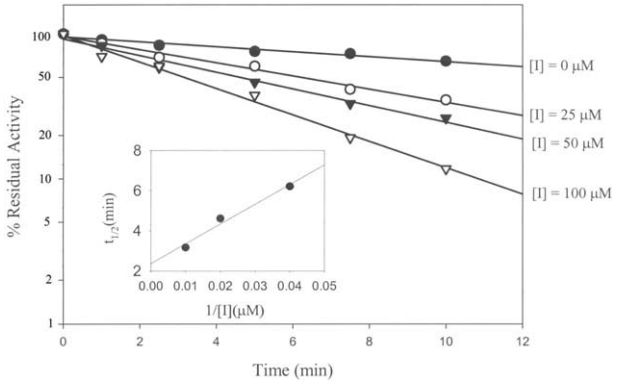


Figure 1. Time dependent inactivation of soybean SMT with 26,27-DHC. Semilog plots of residual activity versus time at 0 (●), 25 (○), 50 (▼) and 100 (▽) μM concentrations of inhibitor. The time indicated in the figure was 1, 2.5, 5, 7.5 and 10 min. (Inset) Replot of enzyme half lives ($t_{1/2}$) for inactivation versus 1/[I].

The covalent nature of binding was established by incubation of 26,27-DHC and [*methyl*-$^3H_3$]AdoMet with pure soybean SMT. After activity assay of the incubation mixture and chromatographic separation on SDS-PAGE, stained with Coomassie blue confirmed the presence of a single protein of the expected molecular weight, while treatment of the gel with fluoroenhancer and radio-fluorography revealed the presence of a single radioactive component with mobility identical to that of soybean SMT (Fig. 2).

In control experiments, saturated concentrations of the inhibitor with [*methyl*-$^3H_3$] AdoMet was separately incubated with heat-treated SMT. Analysis of the corresponding SDS-PAGE gels by radiofluorography failed to indicate any comigration of tritium with the protein, thereby confirming the specificity of the covalent modification of the SMT by 26,27-DHC.

The kinetics and metabolic fate of 26,27-DHC assayed with soybean SMT were as follows.[6] The inhibitor **4A** can act as a substrate for soybean SMT, having a K_m of 10 μM and k_{cat} of 0.018 min^{-1}. The partition ratio, a measure of the production of product per inactivation

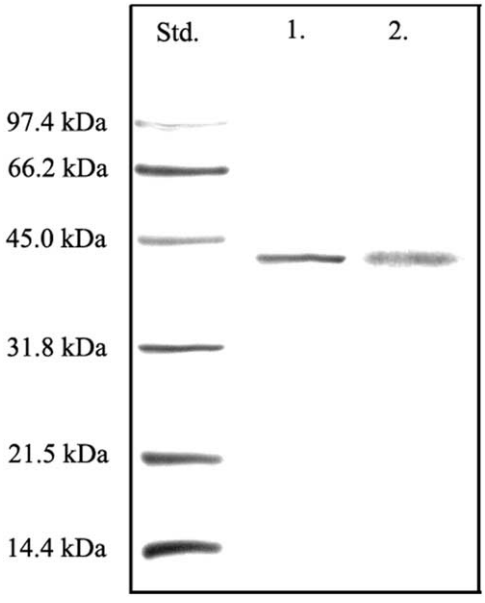


Figure 2. SDS-PAGE of different samples of purified soybean SMT. Migration of standards is indicated. Lane 1, soybean SMT and lane 2 is a radiofluorogram of soybean SMT affinity labeled by 26,27-DHC reacted with [*methyl*-$^3H_3$]AdoMet.

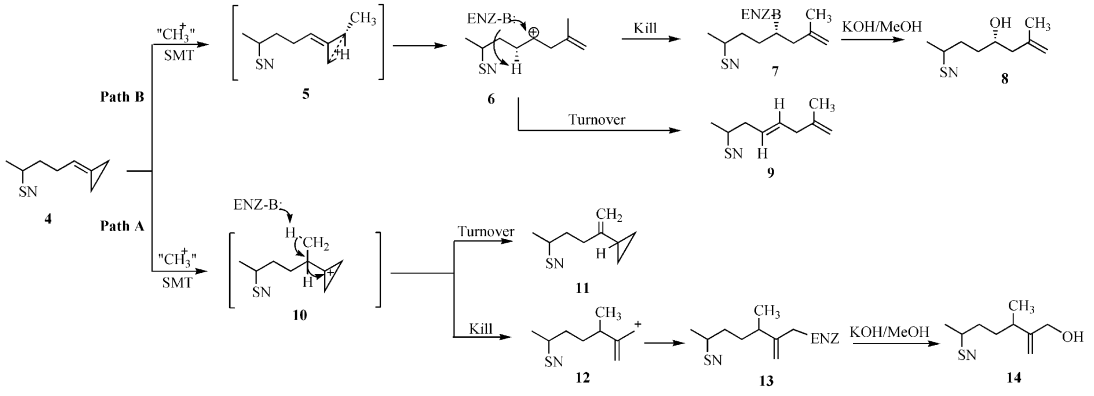
event, can be calculated from the ratio k_{cat}/k_{inact} to be 0.06, which is similar to the partition ratio of 0.03 established from activity assay of the corresponding 26,27-DHZ with the yeast SMT.[4] The different catalytic competence exhibited by sterol substrates possessing a Δ^{24}-bond catalyzed by the soybean SMT are revealed in k_{cat}/K_m values for cycloartenol **1A**, 24(28)-methylenecycloartanol **3A** (established in our earlier study[2]) and 26,27-DHC **4A**; catalytic competence for **1A** is 0.01/30 = 0.0003, for **3A** is 0.001/30 = 0.00003 and for **4A** is 0.0003/10 = 0.00003. These results show the rate at which SMT affects *C*-methylation of the substrate correlates with the regiospecificity and nucleophilicity of the $\Delta^{24(25)}$-bond in the sterol side chain and strongly indicates a preference of the soybean SMT to catalyze the $\Delta^{24(25)}$ -bond in comparison to other olefinic systems.

Reaction channeling of SMT promoting 26,27-DHC catalysis was followed by GC–MS. As expected from the results of 26,27-DHZ treated with the yeast SMT[4] and on retention factors of sterol features calculated from the relevant sterol mobility in GLC relative to the cholesterol retention time, two products were identified in the chromatogram at RRT_c of 1.52 and 2.03, in a ratio of ca. 3 to 1, along with the substrate [M^+ 424] at RRT_c 1.50, corresponding to a <1% yield of a tentatively assigned *C*-methylated monol (putative turnover product) and *C*-methylated diol (putative ligand-bound intermediate). By comparison, the RRT_c of cycloartenol and its methylated product 24(28)-methylenecycloartanol chromatographed on the same capillary column as the substrate and products of the incubation with the inhibitor was 1.39 and 1.49, respectively. The mass spectral fragmentation pattern for the *C*-methylated monol contained the relevant ions at: M^+ (438), $M^+ - CH_3$ (423), $M^+ - H_2O$ (420), $M^+ - CH_3–H_2O$ (405) and a fragment at M^+ 298 corresponding to the retention of the cyclopropane ring. The *C*-methylated monol eluted slightly after the substrate on GC whereas the *C*-methylated diol eluted later consistent with the polar nature of the compound. The mass spectral fragmentation pattern for the *C*-methylated diol contained the relevant ions at: M^+ (456), $M^+ - CH_3$ (441), $M^+ - H_2O$ (438), $M^+ - CH_3–H_2O$ (423) and a fragment at M^+ 316 corresponding to the retention of the cyclopropane ring. Due to a paucity of substrate and enzyme yields using 26,27-DHC NMR analysis was not pursued. Incubation with saturated concentrations of 26,27-DHC and [*methyl*-$^2H_3$]AdoMet led to two products that co-migrated in GC with a retention time similar to the retention time of the pair of compounds produced from control assays.

The monol and diol produced from [*methyl*-$^2H_3$]AdoMet contained three mass unit increases in their atomic mass (M^+ 441 and M^+ 459 for **8A** and **9A**, respectively) which is to be expected assuming that methyl from AdoMet is added to C-26 rather to C-24 (e.g., expected M^+ 440 for $^2H_2$-**11**) of the substrate undergoing *C*-methylation.[4] These chromatographic and spectral data, along with the elution profiles and fragmentation patterns of the enzyme-generated products established elsewhere for 26,27-DHZ assayed with the yeast SMT,[4] suggested that the two compounds were 26-homocholesta-9,19-cyclo-26(26′)-en-3β,24β-diol **8A** and 26-homocholesta-9,19-cyclo-23(24)*E*,26(26′)-dienol **9A**, respectively (Scheme 2).

Two mechanistic variants can be hypothesized for the mode of *C*-methylation of 26,27-DHC (Scheme 2) based in part on studies using a similarly designed inhibitor with a terpene cyclase.[7] In path A, the enzymatic methylation of C-24 generates the cyclopropyl cation **10**. The latter can undergo facile electrocyclic rearrangement to the more stable cation **12** in which the positive charge should be in the immediate vicinity of the enzyme base involved in the normal *cis*-methyl deprotonation step which can generate the $\Delta^{25(27)}$-sterol side chain. Since the allylic cation **12** cannot be eliminated directly by deprotonation, alkylation of the protein at the active site base is strongly favored. In path B, backside nucleophilic attack of a methyl group from AdoMet on the methylenecyclopropane of the substrate can lead to ring opening and ring expansion to generate the elongated sterol side chain of **9**.

In retrospect, path A is highly unlikely since it requires a $\Delta^{25(27)}$-type olefin formed during the first C_1-transfer activity and such a structure has not been detected in soybean plants.[8] However, path B is a consideration, since it has been demonstrated recently for yeast SMT action and invokes a unique covalent catalysis considered novel in cyclopropane chemistry. As illustrated in Scheme 2, if the catalysis advanced by mechanism B,



Scheme 2. Alternative mechanisms for inhibition of SMT catalyzed by 26,27-DHC.

as predicted, reaction with 26,27-DHC would lead to irreversible inactivation, accompanied by possible formation of compounds **8** and **9** as turnover products. While the outcomes of these two modes of attack can lead to monol structures with the same molecular weight, they can be distinguished by assaying [*methyl*-$^2H_3$]AdoMet paired with 26,27-DHC which should produce either a 2 or 3 mass unit increase in the product based on the mechanism (**8** and **9**). The deuterium content of the methylated monol and diol species indicated a 3 mass unit increase for each compound, consistent with the methylation of a cyclopropane carbon atom to produce the cation (**6**) which can be eliminated by C-23 deprotonation or it could be trapped by an active-site nucleophile to give a covalently modified enzyme. It is worth noting, that Glu68 (*ERG*6 nomenclature) was identified as the amino acid residue to which 26,27-DHZ became attached in the yeast SMT study, and the related acidic amino acid, aspartate positioned at residue 70 (*ERG*6 nomenclature, position-68)[1] is similarly conserved in the primary sequence of the soybean SMT.[2] In addition, the product set after activity assay of the inhibitor with soybean SMT resembles that previously observed upon incubation of 26,27-DHZ with the native yeast SMT.

The similar behavior of the yeast and soybean SMT towards the substrate analogues, as well as the considerable similarity in their molecular sizes and substrate preference for a position-specific olefin containing a $\Delta^{24(25)}$-bond, suggests common features in the structures of the active site as well as global similarities. However, the intrinsic capability of these SMTs to catalyze sterol analogues composed of methylene cyclopropane-containing side chains may be unusual for AdoMet-dependent methyltransferases and suggests that with sufficient comparative information about the active site structure–function relationships, it may be possible to develop enzyme-specific inhibitors in which an olefinic system in the sterol side chain can be designed to undergo covalent attachment to a highly conserved amino acid residue specific to SMTs as a result of catalysis. The finding that 26,27-DHC is converted to **8A** and **9A** supports the proposed step-wise mechanism (Scheme 2) and is distinctly different from the concerted pathway pursued in the first C_1-transfer reaction. Studies are in progress to identify the amino acid residue attached to 26,27-DHC.

Acknowledgements

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