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Sterol C24-methyltransferase: Mechanistic studies of the C-methylation reaction with 24-fluorocycloartenol

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Abstract—The mechanism of the C-methylation reaction was studied with the allylic substrate analog 24-fluorocycloartenol **10** assayed with soybean sterol C24-methyltransferase (SMT). **10** is an effective competitive inhibitor (K_i = 32 μM) of the SMT, and the electron-withdrawing α-fluorine substituent was shown to suppress the rate of the C-methylation reaction by one order of magnitude relative to the natural cycloartenol substrate, k_{cat} = 0.02 min^{-1} versus 0.6 min^{-1}; alternately **10** can prevent the critical hydride shift of H24 to C25 to afford time-dependent inactivation of SMT (k_{inact} = 0.32 min^{-1}).

Sterol C-24 methyltransferases from plants differ from those from fungi and protozoa in that they consist of two sets of isoforms (SMT1; E.C. 2.1.1.142, cycloartenol substrate and SMT2; E.C. 2.1.1.143, 24(28)-methylene lophenol substrate); both are bifunctional and together they are responsible for the major phytosterol diversity.[1] These membrane-associated 160–172 kDa proteins cloned and purified in several cases from eukaryotes show 35–80% sequence identity and contain three conserved motifs that represent the sterol and AdoMet binding domains.[1] Extensive studies support the non-stop C-methylation mechanism illustrated in Scheme 1, in which a Δ^{24}-sterol acceptor is converted to a 24(28)-methylene product by way of the high energy intermediate (HEI) **2** and a second alkylation that proceeds similar to the first C_1-transfer reaction except the timing of the mechanism becomes step-wise utilizing HEI **4** with proton elimination from C28 or C27 generating multiple products.[2]

Fluorinated analogs of enzyme substrates have proven quite useful in mechanistic studies of the pre-squalene segments of the isoprenoid-sterol pathway. The unique aspect of fluoro derivatives is attributed to the slightly increased size and shape of the modified substrate so that binding is unaffected, yet at the same time the fluoro group exerts a strong influence on the electronic environment at the site of replacement to affect reactivity. These fluorinated compounds have served as inhibitors and substrates for which novel fluorinated terpenoids have been obtained from assay in cell-free systems.[3] Given the mechanistic findings of these analogs, an earlier study using the 24-vinyl halogen 24-bromolanosterol tested with the microsome-bound sunflower SMT1 and the proposed *steric-electric plug* model for sterol binding and methylation by the soybean SMT,[4] it occurred to us that fluorine-modified sterol side chains could be designed to examine the electrophilic nature of the common C-methylation reaction affecting C_1 and C_2-activities and might retard the C-methylation reaction to afford protein alkylation (Scheme 2). In this communication, we report the synthesis and kinetic analysis of 24-fluorocycloartenol treated with the cloned soybean SMT1, in which the critical 24H has been replaced by a fluorine atom; and as a chemically related inhibitor to test, 24-bromocycloartenol was prepared[5] and evaluated with the plant enzyme.[6]

24-Fluorocycloartenol was prepared from 24(28)-methylenecycloartenol, protected as the C3-acetate, in three steps (8% yield) as shown in Scheme 2.

The synthesis involved formation of the 24-difluoro intermediate **9** accomplished in a solution of hot diethyl amino sulfur trifluoride (DAST) as the key step. Dehydrohalogenation of one of the C24-gem-fluoride atoms and deprotection of the C3-hydroxyl group in the presence of 3 equiv potassium hydroxide dissolved in DMSO afforded 24-fluorocycloartenol. Pure **10** was obtained

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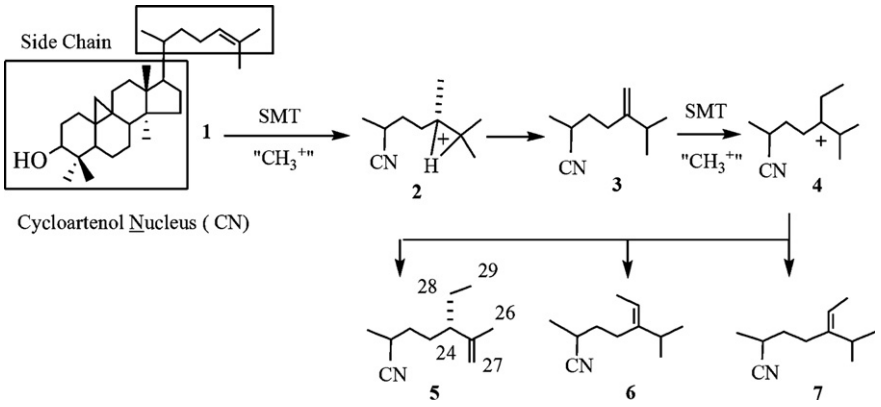
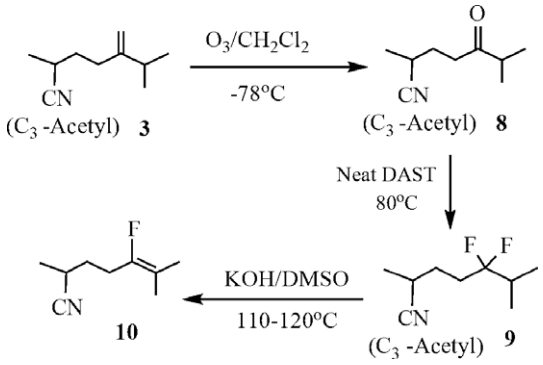


Scheme 1.



Scheme 2.

after HPLC on TSK Gel: *RRT*c, 1.99 compared to the *RRT*c for cycloartenol of 1.58, α_c of **10** = 1.14 versus the α_c for cycloartenol = 1.04; MS M^+, 444, 429, 411, 383, 357, 339, 304 (fragment corresponding to the 9,19-cyclopropane ring), 262; ^{1}H NMR δ, 0.330 (1H, δ, J = 4, H-19), 0.550 (1H, δ J = 4, H-19), 0.810 (3H, s, H-31), 0.891 (3H, s, H-32), 0.91 (1H, δ, J = 6.5 Hz, H-21), 0.967 (6H, s, H-18/H-30), 1.56 (6H, s, H-26/H-27), 3.28 (1H, dd, J = 4.5 Hz, H-3).

As in the case of 24-bromolanosterol treated with a microsome-bound SMT1 from sunflower (K_i = 25 μM relative to cycloartenol),[4b] 24-bromocycloartenol was a competitive inhibitor (K_i = 36 μM relative to cycloartenol) of the soybean SMT1 and in similar fashion **10** was a competitive inhibitor (K_i = 32 μM relative to cycloartenol) of the enzymatic C-methylation reaction as well (Fig. 1).

In the earlier study with the crude sunflower preparation no effort was made to evaluate whether the 24-vinyl bromide structure could undergo catalysis or serve to inactivate the enzyme.[4b] In this study, the 24-bromocycloartenol was tested as substrate using [^{3}H$_3$-methyl]AdoMet and no radioactive product was detected in the quenched enzyme lipid extract, nor was there evidence by fluorography of covalent protein binding (Fig. 2). It would appear that the large bulk in the sterol side chain of 24-bromocycloartenol, like the steric

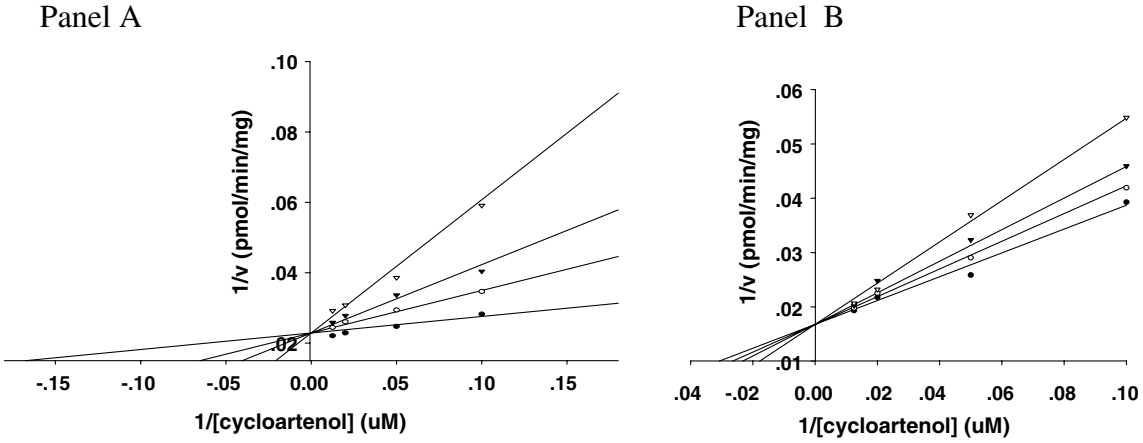


Figure 1. Double-reciprocal plots illustrating the inhibition of C-methylation of cycloartenol to 24(28)methylenecycloartenol by 24-bromo cycloartenol (A) and 24-fluorocycloartenol (B). Incubations were performed with 1 mg total protein of a 100,000 g supernatant fraction from *Escherichia coli* containing 0.4 μM SMT in a final volume of 600 μL of the standard assay. The reactions were performed at 35 °C for 45 min. The constant substrate [^{3}H$_3$-methyl]AdoMet was held at saturation (100 μM) and the varied substrate cycloartenol was changed at increasing fixed concentrations from 10 to 100 μM. The concentration of the substrate analogs was varied at 5, 15, 25, and 50 μM. Results are the average of samples from three separate experiments with variation amongst the trials at <10%.

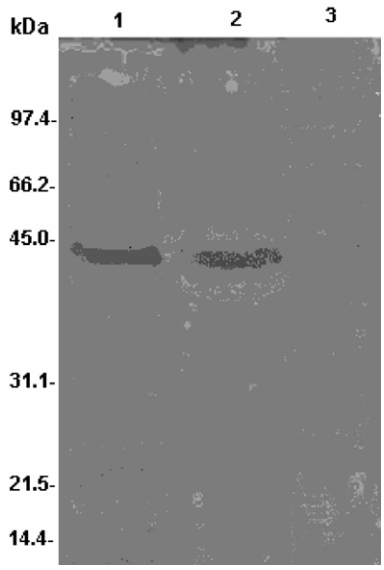


Figure 2. Fluorogram showing affinity labeling of partially purified soybean SMT (lane 1 is the migration of pure recombinant soybean enzyme on SDS–PAGE) treated with 24-fluorocycloartenol (lane 2) or 24-bromocycloartenol (lane 3). Each labeling reaction was carried out with 100 μM [$^3H_3$-methyl]AdoMet, 100 μM analog, and 0.8 μM SMT at 35 °C for 8 h.

features of 24-alkyl groups in dead-end analogs,[6] is sufficient to interfere with SMT catalysis in the direction of C24 methylene formation.

In contrast, using identical activity assay conditions to study 24-fluorocycloartenol as assayed with the 24-bromocycloartenol the reaction involving recombinant soybean SMT1 resulted in specific covalent binding as detected by fluorography of SDS–PAGE separated proteins (Fig. 2). In addition, the inhibition kinetics of **10** followed a time-dependent process (Fig. 3), consistent with the action of a suicide substrate that irreversibly inactivates the SMT. The time-dependence of inhibition

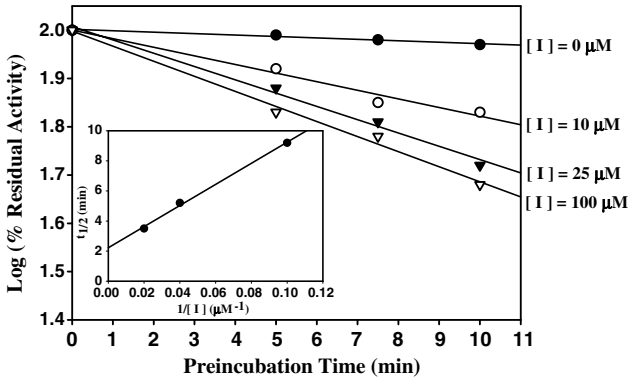
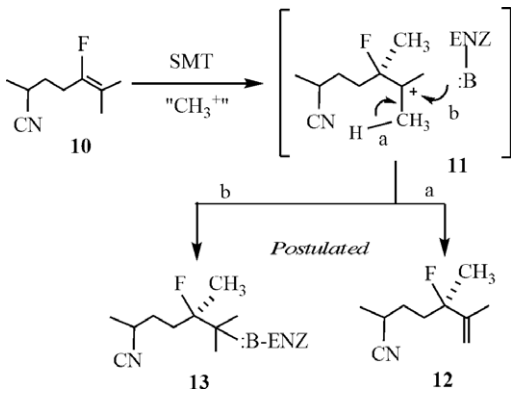


Figure 3. Time-dependent inactivation of soybean SMT1 assayed with 24-fluorocycloartenol. Log plot of residual activity versus time: incubations were performed with a pure enzyme diluted to 0.8 μM as described in Ref. 7; concentrations of 24-fluorocycloartenol (I) were varied at 0, 10, 25, and 50 μM for the pre-incubation time that ranged from 0, 5, 7.5 to 10 min; inset plot of enzyme half-lives ($t_{1/2}$ min) for inactivation versus 1/[I].

at [**10**] = 10, 25, and 50 μM allowed determination of the k_{inact} value of 0.32 min^{-1} for soybean SMT1 (Fig. 3), which is of the same magnitude as that reported for the k_{inact} of the substrate analogs 24-thiacycloartenol and 26,27-dehydrocycloartenol (0.3 min^{-1} and 0.29 min^{-1}, respectively) compared to the reported K_m = 30 μM and k_{cat} of 0.6 min^{-1} of cycloartenol.[7]

Co-incubation of **10** with cycloartenol at 50 and 100 μM afforded protection against inactivation, generating 34% and 56% C-methylation, relative to the C-methylation activity of a control incubation containing saturating amounts of substrate and AdoMet only. Compound **10** was tested as a substrate with [$^3H_3$-methyl]AdoMet (the pooled organic extracts of 8 incubations) and shown to be converted to a single methyl product eluting in the sterol region of the HPLC chromatogram somewhat after the substrate at α_c = 1.19 (suggesting a methyl group is added to the side chain) in ca. 0.5% yield.[6]

When 24-fluorocycloartenol was tested as substrate with the soybean SMT1, the kinetic constants were found to be K_m = 33 μM and k_{cat} of 0.02 min^{-1}. A partition ratio (k_{cat}/k_{inact}) of 0.07 calculated for 24-fluorocycloartenol is very similar to that generated by incubation with 26,27-dehydrocycloartenol (0.06). A paucity of analog and low conversion rate contributed to the failure to detect a methylated product by GC–MS. However, on the basis of the regiospecificity of methyl addition and proton elimination reactions and assuming that fluorine was not lost during partitioning the radioactive sterol in the HPLC is postulated to be 25(27)-dehydro-24-methyl-24-fluorocycloartenol as shown Scheme 3. It appears further that the inductive electron-withdrawing effect of the fluoro substituent can affect the timing of the C-methylation reaction to prevent the 24-fluorine transfer, normally occupied by H24, to C25 and thereby block the C-methylation reaction after formation of the C25 cation high energy intermediate **11**. The substantial rate-retarding effect on formation of methylated product measured as catalytic competence (K_m/k_{cat}) by **10** is greater than 10-fold different from that of the native substrate, suggesting that steric perturbations by the fluorine atom may alter the optimally oriented sterol side chain in the active



Scheme 3.

site of the enzyme to favor a different partitioning directed to the formation of species **13**.

Yet, because the enzyme typically deprotonates the introduced methyl to eliminate a C24 carbocation, the 'sessile' C25 carbocation produced upon methylation of 24-fluorocycloartenol is not readily quenched by the catalytic general base; thus a kinetic competition between 'slow' deprotonation and 'fast' alkylation can develop setting up the fluoro substituent to be predominantly electronic in nature. An exacting analysis of the contribution of steric versus electronic effects of the 24-fluoro substituent to the C24-methylation mechanism awaits further study.

Efforts underway to obtain an X-ray crystallographic structure of this unique enzyme and peptide mapping of **10** bound to the plant SMT may provide details of the topography of the active center and its role in the reaction progress.

Acknowledgments

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5. The modified substrate 24-bromocycloartenol was prepared with slight modification of the procedure of Boar, R.B.; Lewis, D.A.; McGhie, J.F. *J. Chem. Soc. Perkin Trans. I*, **1973**, 1583, in 70% overall yield (35 mg) from cycloartenol in two steps: bromination of the C3-acetate protected cycloartenol followed by saponification in methanolic KOH. The resulting vinyl bromide was purified by C_{18}-HPLC on a TSK Gel column developed isocratically in methanol (ambient temperature) at 2.5 mL/min to give a retention time relative to cholesterol of $\alpha_c = 0.84$; on GLC with the packed 3% SE-30 column operated isothermally at 245 °C the retention time relative to cholesterol is $RRT_c = 4.41$; mass spectrum (*m*/*z*) (M^+ and other diagnostic ions in the high mass region) M^+ 506/504, 489/491, 471/473, 443/445, 425, 364/366 (fragment illustrating retention of 9,19-cyclopropane ring), 339, 315, 285; ^{1}H NMR (in ppm relative to TMS at 500 MHz in $CDCL_3$): δ 0.330 (1H, δ, $J = 4$, H-19), 0.550 (1H, d, $J = 4$, H-19), 0.810 (3H, s, H-31), 0.895 (3H, s, H-32), 0.910 (1H, δ, $J = 6.5$, H-21), 0.967 (6H, s, H-18/H-30), 1.755 (3H, s, H-26), 1.853 (3H, s, H-27), 3.28 (1H, dd, $J = 4.5$ Hz, H-3); relevant C24 with the 24-bromo atom attached (referenced to TMS) appears at $\delta = 122.633$ in the ^{13}C NMR spectrum.
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