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Inactivation of soybean sterol 24-C-methyltransferase by elongated sterol side chains at C26

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Abstract—The enzymatic C-methylation reaction catalyzed by the *Glycine max* sterol 24-C-methyltransferase was studied with substrate analogs containing a cycloartenol nucleus (CA) and a double bond (**8**) or triple bond (**14**) attached to C26. The production of the corresponding C24(28)-methylene olefin and time-dependent inhibition kinetics of k_{inact} 0.24 min^{-1} (CA-**8**) or 0.06 min^{-1} (CA-**14**) indicates an active-site directed process and partitioning to produce novel products.
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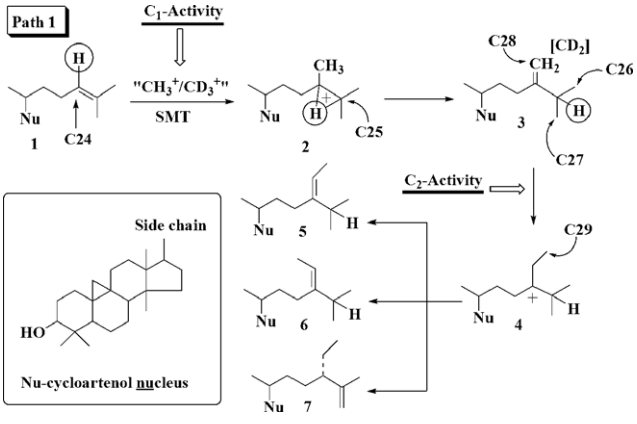
The pattern of 24-alkyl sterol diversity is determined by a family of sterol 24-C-methyltransferases (SMT) that perform distinct C_1-activities exemplified in Scheme 1.[1] In the case of the bifunctional SMT from soybean plants, the second alkylation leads to phytosterols with elongated sterol side chains at C28 that affect membrane fluidity.[2] The SMT-catalyzed reaction is a critical slow step in plant sterol metabolism as well as a potential target in chemotherapy for ergosterol-dependent diseases.[3] SMT of different origins are known and several of them have been cloned, purified to homogeneity, and characterized kinetically.[4] These enzymes are slow-acting (k_{cat} ca. 0.6 min^{-1}), membrane-associated 165–175 kDa tetrameric proteins and show ca. 40–80% sequence identity. Affinity labeling and site-directed mutagenesis experiments involving two conserved regions in the primary structure of the fungal SMT revealed the active center and that it contained subsites for a sterol and AdoMet binding cleft.[5]

Substrate analogs that include a double bond at C24 and an olefin appended to the sterol side chain at C26 were among the first mechanism-based inhibitors of sterol metabolism and of the SMT.[1] Inactivation was postulated to involve an initial C25-cation intermediate and a subsequent change in the side chain structure of the inhibitor to form a highly reactive carbon-centered charged species that when directed to a region of the protein that does not normally encounter reactive electrophilic centers can be alkylated by the SMT. Since there is as yet no three-dimensional structure of SMT, comparison of the enzymatic properties of phylogenetically different SMTs tested with rationally designed substrate analogs provides an alternative approach for obtaining comparative information on their active-site topographies and reaction mechanisms. We demonstrate here that the plant *Glycine max* SMT, in contrast to microbes tested with **8** and **14** which failed to generate detectable products from their activity assay,[6,7] both C-methylates and is specifically labeled by these substrate mimics and provide strong suggestive evidence for the intermediacy of the predicted cationic species involved with the mechanism-based inactivation, and



Scheme 1.

Keywords: Sterol methyltransferase; Mechanism-based inhibitor; Cycloartenol; Phytosterol; Sterol methylation.
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thus for the electrophilic nature and course of this reaction type.

The side chains **8** and **14** coupled to the cycloartenol (CA) nucleus (isolated from γ-Orazanol) were prepared by minor modification of the published routes.[6,7] Briefly, side chain extension and olefin introduction to C26 was accomplished by selective oxidation using selenium dioxide to afford the C26 aldehyde followed by Wittig olefination to generate CA-**8** (retention time relative to cholesterol of RRTc 1.69, M^+ 438) or using the Corey–Fuchs procedure to generate the corresponding enyne CA-**14** (RRTc 1.85, M^+ 436) via the dibromide intermediate.[8]

Recombinant soybean SMT expressed in *Escherichia coli*[9] was employed for the chemical affinity labeling experiments and product distribution determinations. Activity assay of 100 μM each of **8** or **14** in the presence of 100 μM [$^3H_3$-*methyl*]AdoMet (1 μCi per 600 μL assay) and 0.5 μM purified enzyme at pH 7.5 in 50 mM Tris/HCl buffer containing 20% glycerol, 2 mM $MgCl_2$, and 2 mM 2-mercaptoethanol (Buffer A) for 8 h specifically labeled the plant SMT (Fig. 1).

Measurements of inhibition of the SMT-catalyzed conversion of cycloartenol to 24(28)-methylene cycloartanol by the substrate analogs CA-**8** and CA-**14** were carried out using semi-purified recombinant soybean SMT.[9] The enzyme solution was preincubated with 100 μM of AdoMet and different concentrations of inhibitor from zero to a concentration of 100 μM for 1 h at 35 °C, then the co-substrates, cycloartenol and [$^3H_3$-*methyl*]AdoMet were added at saturating concentrations of 100 μM each, and the conversion to 24(28)-methylene cycloartanol was determined at 2.5 min intervals (assay by radiometric determination of the non-saponifiable lipid fraction). Consistent with the findings drawn in Figure 1, incubation of increasing concentrations of CA-**8** with recombinant soybean SMT resulted in pseudo-first-order time-dependent inactivation of the SMT, as evidenced by the linear dependence of the log residual activity against time (Fig. 2).

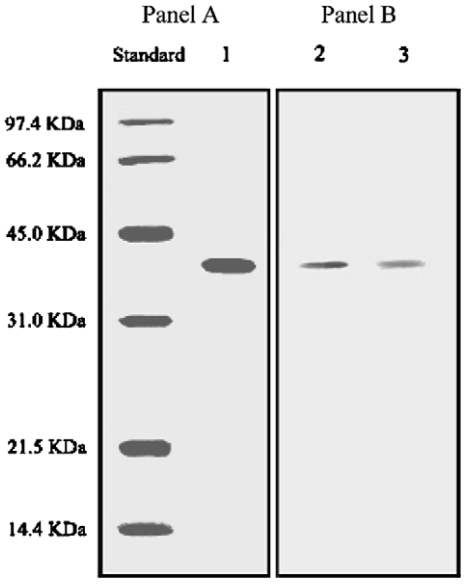


Figure 1. Affinity labeling of *Glycine max* SMT expressed in *E. coli*: (Panel A) SDS–PAGE gel (12%) stained with Coomassie blue. (Lane 1) Purified soybean SMT; (Panel B) Corresponding fluorogram. (Lane 2) purified enzyme affinity labeled with CA-**8**. (Lane 3) Purified enzyme affinity labeled with CA-**14**.

The rate of inactivation by CA-8 was saturable, with a maximum rate of inactivation, k_{inact} of 0.24 ± 0.01 min^{-1} and a K_i 47 μM. These values compare favorably with the steady state kinetic parameters for the normal substrate cycloartenol (k_{cat} = 0.6 min^{-1} and K_m = 30 μM).[9] Co-incubation of CA-**8** with the normal substrate at 50 μM or 100 μM cycloartenol afforded protection against inactivation generating 25% and 45% C-methylation, relative to the C-methylation activity of a control incubation containing saturating amounts of substrate and AdoMet only.

In addition, inhibitor CA-**8** was found to be catalyzed by soybean SMT to a product detected by GC–MS analysis, CA-**11** (Scheme 2). Kinetic study of CA-**8** catalysis, as described for the initial velocity rate measurements of the normal substrate, revealed kinetic constants of turn-

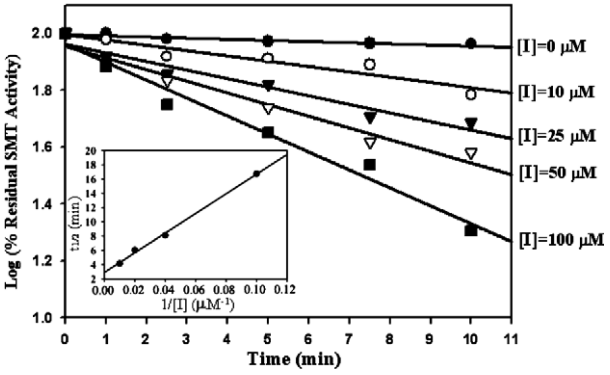
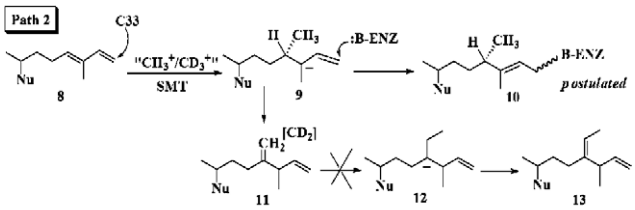


Figure 2. Kinetics of inactivation of *Glycine max* by inhibitor (*I*) CA-**8**. Enzyme (0.5 μM) was incubated at 35 °C with 100 μM of AdoMet and the concentrations of inhibitor shown above. Total incubation volume was 0.2 mL in Buffer A. At the indicated time intervals, 30 μL aliquots were withdrawn, diluted to a total volume of 0.6 mL in Buffer A containing 100 μM each CA and [$^3H_3$-*methyl*]AdoMet (0.6 μCi), and assayed for SMT activity for 45 min as previously described. The logarithmic percentage of remaining enzyme activity was plotted against incubation time of enzyme-inhibitor mixture to determine the half-life of inactivation. Inset is a Kitz and Wilson plot of 1/[*I*] versus $t_{1/2}$ (min) from which the apparent k_{inact} and IC_{50} values were estimated.



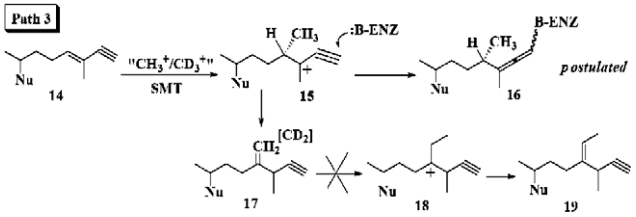
Scheme 2.

over $k_{cat} = 0.0003\ min^{-1}$ and $K_m = 5\ \mu M$. The movement in GC and mass spectrum (M^+ 452 amu, consistent with a 14 mass unit increase for the addition of a methylene unit to C24) of the new compound indicated it was a C24-methylated sterol (Fig. 3). Activity assay of [$^2H_3$-*methyl*]AdoMet paired with CA-**8** generated a methylated product with a two mass unit increase compared to the unlabeled product (Fig. 3) which confirms the methyl unit from AdoMet is added to C24 (Scheme 2).

Interestingly, no other monol-type sterols were evident in the HPLC 'sterol region' (eluting between 20 and 60 min) or in the GC profile of CA-**8** catalysis,[10] suggesting compromised C_2-activity such that CA-**11** is not further methylated at C28 as can be the case in the conversion of some $\Delta^{24(28)-}$ sterols by this SMT (Scheme 1).[1]

Incubation of CA-**14** paired with AdoMet (Scheme 3) together with recombinant SMT as described above for assay of CA-**8** generated inhibition kinetics that were non-competitive and time-dependent ($IC_{50} = 67\ \mu M$, $k_{inact} = 0.06\ min^{-1}$) (Fig. 4). Compound CA-**14** can also serve as a poor substrate for the plant SMT, being converted in low yield ca. 1% incorporation and having a $K_m = 2\ \mu M$ and $k_{cat} = 0.00005\ min^{-1}$. The potency of inhibition of CA-**8** and CA-**14** toward soybean SMT was significantly less than related mechanism-based inactivators tested with the soybean SMT, 24-thiacycloartanol $K_i = 2.0\ \mu M$, $k_{inact} = 0.30\ min^{-1}$ or 24-dehydrocycloartenol $K_i = 42\ \mu M$, $k_{inact} = 0.29\ min^{-1}$.[11]

The chromatographic mobility of the product eluting before the substrate and its mass spectrum



Scheme 3.

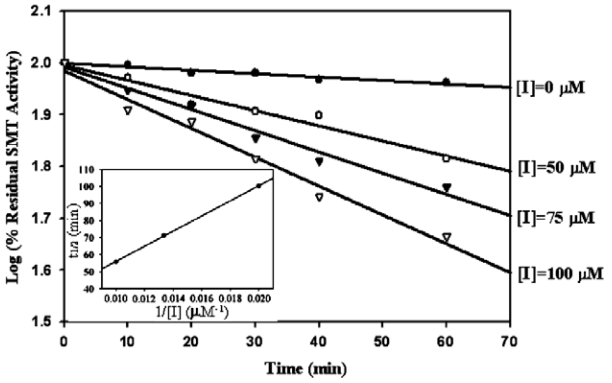


Figure 4. Kinetics of inactivation of *Glycine max* by inhibitor (*I*) CA-**14**. Enzyme (0.5 μM) was incubated at 35 °C with 100 μM of AdoMet and the concentrations of inhibitor shown above. Total incubation volume was 0.2 mL in Buffer A. At the indicated time intervals, 30 μL aliquots were withdrawn, diluted to a total volume of 0.6 mL in Buffer A containing 100 μM each CA and [$^3H_3$-*methyl*]AdoMet (0.6 μCi) and assayed for SMT activity for 45 min as previously described. The logarithmic percentage of remaining enzyme activity was plotted against incubation time of enzyme-inhibitor mixture to determine the half-life of inactivation. Inset is a Kitz and Wilson plot of 1/[*I*] versus $t_{1/2}$ (min) from which the apparent k_{inact} and IC_{50} values were estimated.

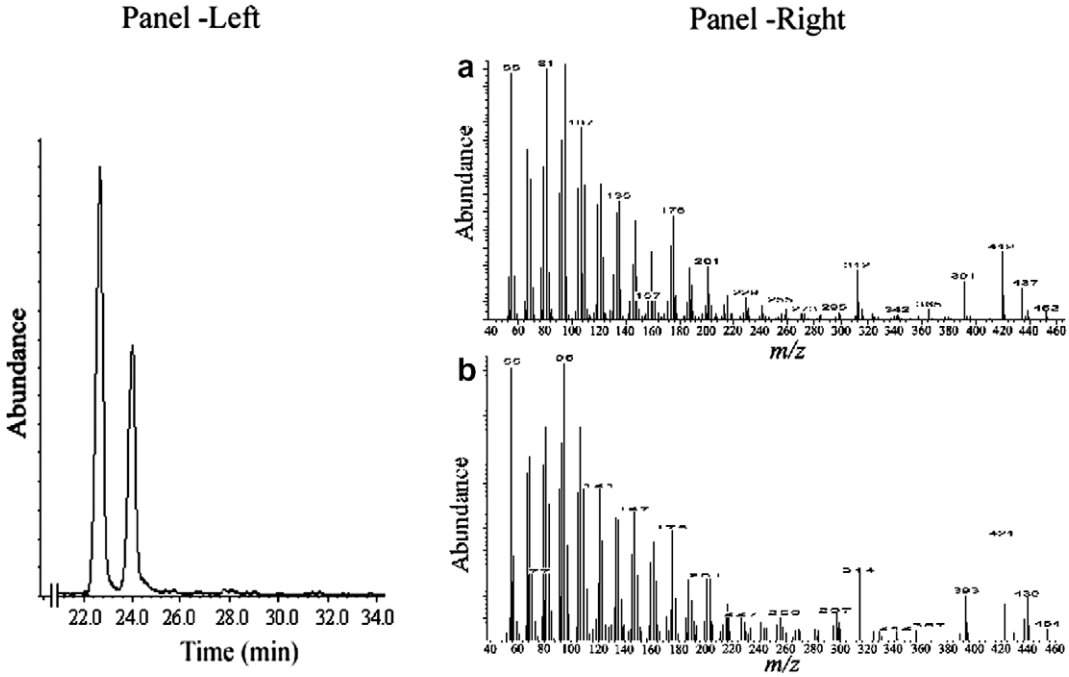


Figure 3. Gas–liquid chromatographic separation of the HPLC fraction eluting between 42 and 47 min derived from study of CA-**8** assayed overnight in preparative scale with soybean SMT; less polar eluting peak is the substrate (rt = 23 min) and more polar eluting peak is the product (Left). Mass spectrum of the enzyme-generated product of incubation with CA-**8** paired with AdoMet (a) or CA-**8** paired with [$^2H_3$-*methyl*]AdoMet (b) (Right).[10]

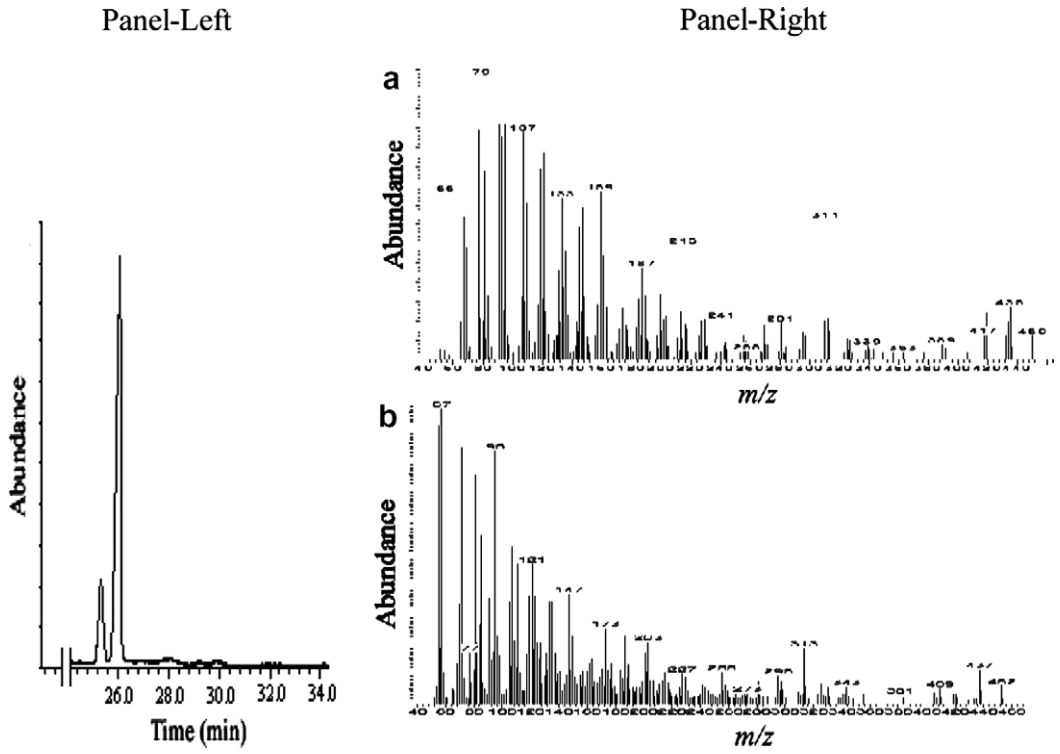


Figure 5. Gas–liquid chromatographic separation of the HPLC fraction eluting between 25 and 27 min derived from study of CA-**14** assayed overnight in preparative scale with soybean SMT; more polar eluting peak is the substrate (rt = 26 min) and less polar eluting peak is the product (Left). Mass spectrum of the enzyme-generated product of incubation with CA-**14** paired with AdoMet (a) or CA-**14** paired with [$^2H_3$-*methyl*]AdoMet (b) (Right).[10]

(M^+ 452 amu) indicated that the new compound was methylated at C24 (Fig. 5).

In similar fashion to the catalysis of CA-**8** by soybean SMT, no evidence was found for the successive transmethylation at C24 of CA-**14** (Scheme 3). It appears that sterol side chains elongated at C26 can, compared to the native substrate, be utilized as acceptor molecules for the first C_1-activity. Alternatively, the ability for side chain expansion at C28 is impaired likely due to the limited space in the active center of the soybean SMT to accommodate the longer sterol catalytically.

The C24-methylated compounds CA-**11** and CA-**17** most likely arise from the corresponding C26-olefin associated C25-cation, followed by proton elimination from C28. Reaction channeling involving the C25-cation leads to turn-over or enzyme inactivation. The partition ratio, a measure of the production of product per inactivation event, can be calculated from the ratio of k_{cat}/k_{inact} for each of the inhibitors with elongated sterol side chains tested with the plant SMT; for CA-**8** and C-**14** the partition ratio is about the same, approximately 0.001. Correlation of k_{inact} with product formation requires that C24 methylation occurs with formation of a C25-cationic intermediate that is stabilized by olefin rearrangement of the neighboring double bond or triple bond attached at C26 of CA-**8** or CA-**14**, respectively. Such a view is consistent with the postulated intermediacy of a C33-cationic intermediate trapped by nucleophilic attack of an enzyme base in the active-site (Schemes 2 and 3).

For the plant SMT assayed with substrate analogs possessing side chains **8** and **11**, it is most likely that the labeling site of the SMT bound by these inhibitors compared with 26,27-dehydrocycloartenol or 24-thiacycloartanol paired with AdoMet[5a,11b] would be different since different channeling outcomes are detected in their catalysis. Identification of the peptides in the plant SMT resulting from covalent binding by the mechanism-based inactivators is now in progress.

Acknowledgments

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