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Cyclobranol: A substrate for C25-methyl sterol side chains and potent mechanism-based inactivator of plant sterol methyltransferase

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ABSTRACT

Cyclobranol **8A**, an analog of the cycloartenol substrate **1A** for the plant sterol C24-methyltransferase (SMT), was shown to be an acceptor of the soybean SMT1 as well as an inhibitor of enzyme action. The K_m and k_{cat} for **8A** was 37 μM and 0.006 min^{-1}, respectively. The enzyme-generated product was identified by MS and ^1H NMR to be a C24, C25-doubly alkylated $\Delta^{24(28)}$-olefin **10A**. Inhibitor treatment was concentration and time-dependent affording an apparent K_i of 25 μM, a maximum rate of inactivation of 0.15 min^{-1} and a partition ratio (k_{cat}/k_{inact}) calculated to be 0.04.

Sterol C24-methyltransferases (SMT) are crucial enzymes in the biosynthesis of the phytosterol side-chain construction, and they are responsible for the formation of 24-alkyl sterols used as membrane inserts and phytohormones.[1] On the basis of several lines of evidence, it has recently been proposed that the conversion of cycloartenol **1A** to 24(28)-methylene cycloartanol **3A** by soybean SMT1 (E.C. 2.1.1.142) proceeds with the intermediacy of the discrete C25-cation **2** as shown in Scheme 1.[2] In this pathway, the intermediate **2** generated by a nucleophilic attack by the Δ^{24}-double bond of cycloartenol on the methyl group of the sulfonium group of AdoMet is catalyzed to **3** regio- and stereoselectivity. Typically, the C24(28)-methylene side chain of the first C_1-transfer reaction in plants provides the olefinic system required of the second C_1-transfer reaction giving rise to the C24-doubly alkylated side chains **5**, **6**, and **7** (Scheme 1).[3] However, in rare cases, as shown with the protozoan SMT1, intermediate **2** rather than converting to **3** exclusively can partition to **8** which then is C24 methylated to form the doubly alkylated sterol **12**.[4] On rare occasions, the side-chain structure **10** has been detected in plants,[5] although its enzymatic synthesis remains enigmatic.

One approach to developing mechanistic insight into the C-methyl transfer reaction involves determining the effects of substrate modifications on enzyme activity. Kinetic evaluation of a series of substrate analogs synthesized by soybean and related plants showed that none derail the soybean SMT1 activity in a time-dependent manner suggesting that the natural C24-methyl intermediate(s) **2** was safeguarded from entering into a covalent linkage with the enzyme.[2,6] Alternatively, the substrate mimic related to **1A** 26,27-dehydrocycloartenol was determined to undergo catalysis to a novel side chain elongated C26 sterol and to form an abortive complex.[6a] It was concluded that the soybean SMT1, like that of other SMTs, is highly stringent with regard to substrate recognition. We now report that the soybean SMT1 can C-methylate **8A**, and is specifically labeled by a C24-methylated cyclobranol intermediate, showing the enzyme has much greater versatility to accept natural substrate analogs than previously noted.[3b,6e] On the other hand, this acceptability of unnatural substrates can lead to negative consequences that inactivate the enzyme, and this may explain why the side-chain structure **8** is usually formed late in the phytosterol pathway by an isomerization-type reaction involving **9** and is attached to the cholesterol nucleus not readily accepted by the plant SMT1.[3b,6e,7]

Preliminary studies to evaluate the catalytic effectiveness of cyclobranol (**8A**) were carried out with crude microsome-bound sunflower SMT, which revealed that the enzyme binds the analog in an unproductive mode (no conversion to product), yet it can act as a competitive inhibitor generating a K_i value of approximately 100 μM.[8] To evaluate the potential for catalysis of **8A** directly using a cloned enzyme,[2] we synthesized fresh samples of cyclobranol from 24(28)-methylenecycloartanol C3-acetate by iodine isomerization followed by HPLC fractionation to generate the pure substrate.[8,9] In this case, the analog so tested generated similar catalytic competence to the C-methylation of cycloartenol by soybean SMT1.[10] The favorable kinetics for the conversion of substrate to product was determined for **1A** and **8A** to be K_m/k_{cat}

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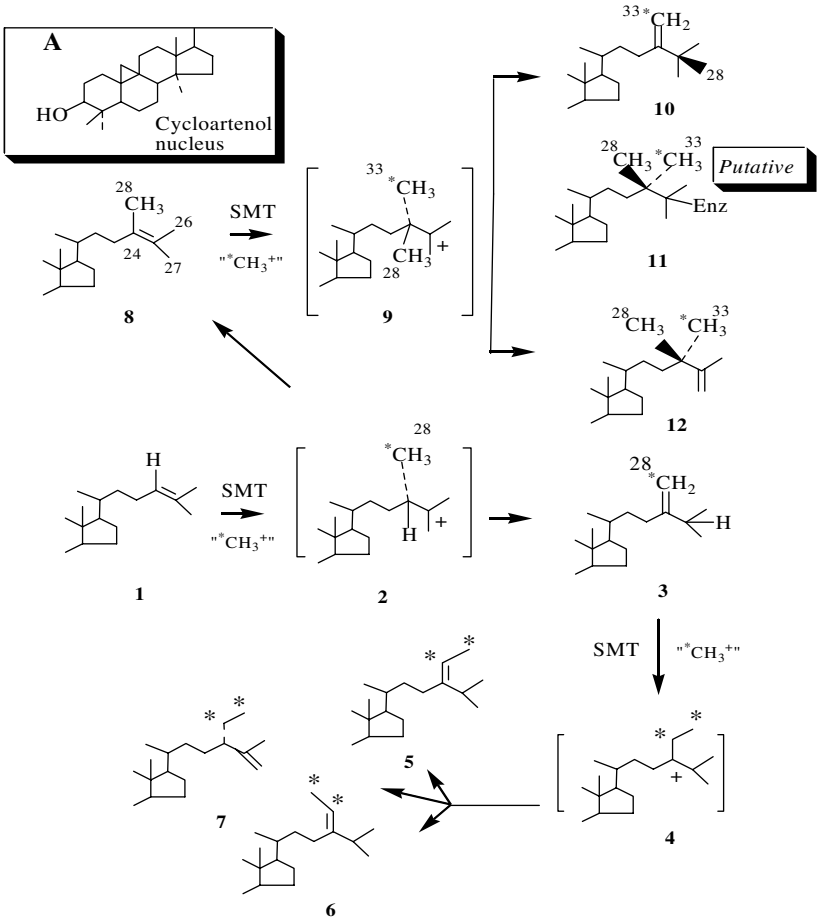

Scheme 1.

of 30 μM/0.06 min^{-1} and 37 μM/0.006 min^{-1}, respectively. These results indicate that the replacement of a methyl group for a hydrogen atom at C24 to the cycloartenol side chain has minimal effect on sterol methylation activity.

Analysis of the product profile of the incubation with cyclobranol by GC revealed a compound that eluted after the substrate peak, as would be expected for the chromatographic movement of a doubly alkylated sterol side chain, in approximately 20% yield (Fig. 1).[11]

The retention time in GC and mass spectrum (Fig. 1) of this late eluting material was similar to that of the three 24-ethyl(idene) sterols with a M^+ 454 amu formed by the soybean SMT1 incubated with 24(28)-methylene cycloartanol.[12]

Isolation by HPLC[9] and characterization of the biomethyl product by high field (500 MHz) ^1H NMR revealed the presence of distinct signals in the spectrum corresponding to the side-chain methyl groups at δ C21 (d, 0.968, J= 5 Hz), C26 (s, 1.061), C27 (s, 10.61), C28 (s, 4.835 and s, 4.667 corresponding to 1H each), and C33 (s, 1.061) and other relevant signals of methyl groups in the nucleus at δ C18 (s, 0.967), 9β,19-cyclopropane signals at C19 (endo/exo- 0.553, J = 4.5 Hz/d, 0.335 J = 4.5 Hz) and nuclear methyl groups at C30 (s, 0.967), C31 (s, 0.881), and C32 (0.892). These data unambiguously establish that the side chain of the cyclobranol metabolite is different from the previously synthesized 24-ethyl(idene) sterols from 24(28)-methylene cycloartanol incubated with the soybean SMT1; this new metabolite is a doubly alkylated biomethyl sterol constructed with an exocyclic methylene group at C28 and a quaternary methyl group at C25 to give **10** (Fig. 2).[5c]

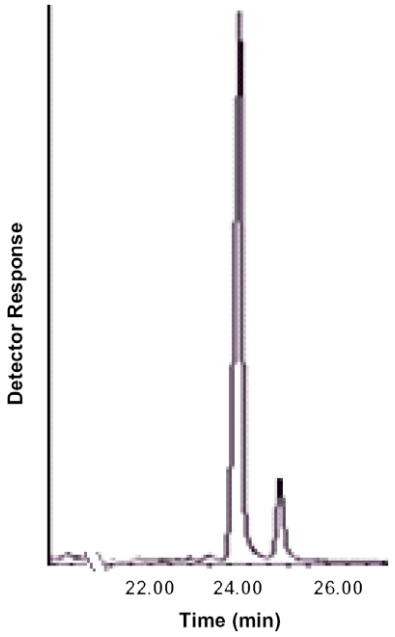

Figure 1. Partial gas-liquid chromatographic separation of the non-saponifiable lipid fraction derived from a preparative incubation (combined 12 independent assays) of soybean SMT1 with saturating amounts of cyclobranol and AdoMet extended for 12 h.

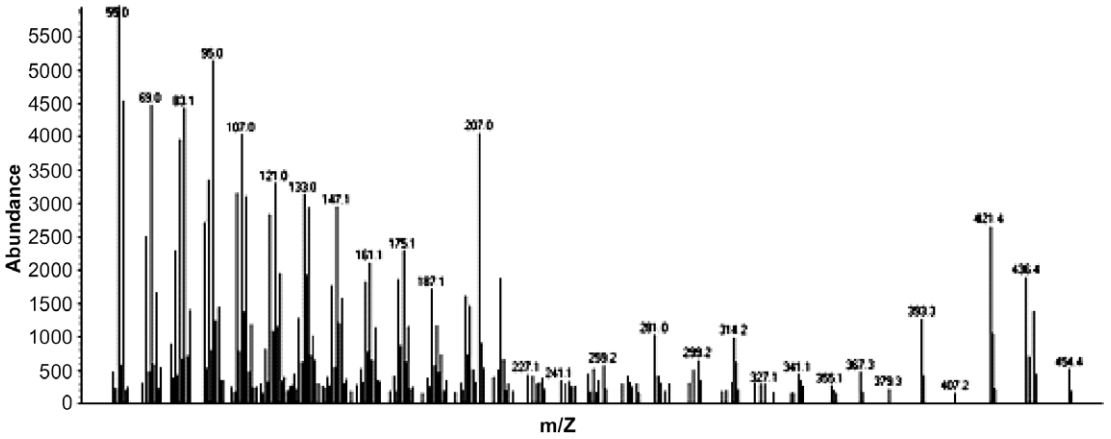

Figure 2. Mass spectrum of the soybean SMT1-generated cyclobranol.

Initial inhibition kinetics revealed that cyclobranol was a competitive-type inhibitor (K_i = 25 μM) (Fig. 3), as shown previously for the sunflower SMT. However, it is not a dead-end analog as we considered before in view of our current findings that cyclobranol is converted to a biomethyl product. The new finding of **10A** formation by the soybean SMT1 can likely arise from the greater abundance of the cloned enzyme and amount of substrate used in these assays than was used with the crude microsome-bound sunflower enzyme preparation.

To determine whether cyclobranol can act as an irreversible inhibitor of the soybean SMT1, we examined the time-dependent inhibition kinetics of increasing concentrations of **8A** in the standard assay with cycloartenol paired with [$^3H_3$-*methyl*]AdoMet (Fig. 4).[2] A replot of the inactivation data as log % residual activity against time for the four inhibitor concentrations gave a series of straight lines (Fig. 4), showing that the inactivation followed pseudo first-order kinetics under these assay conditions. A plot of the reciprocal of k_{inact}, determined from the half-lives of inactivation, versus the reciprocal of analog concentration (Fig. 4, inset) gave for the intercept a maximum rate of inactivation for soybean SMT1 with cyclobranol 0.15 min^{-1}. Interestingly, in contrast to earlier reports of inhibitor action for the natural substrate analogs tested with variant SMTs, **8A** was found to permanently immobilize the enzyme in this model.

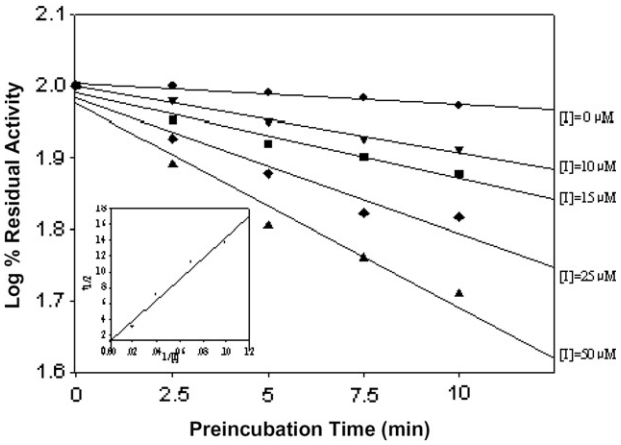

Figure 4. Time-dependent inactivation of soybean SMT1 with cyclobranol. Log plot of residual activity versus time: incubations were carried out with pure recombinant enzyme diluted to 0.8 μM as described in Ref. 2; concentrations of cyclobranol (I) were varied at 0, 10, 15, 25 and 50 μM for the pre-incubation time that ranged from 0, 2.5, 5, 7.5–10 min; inset plot of enzyme half-lives ($t_{1/2}$) for inactivation versus 1/[I].

Consistent with an active site directed inhibition of cyclobranol, co-incubation with cycloartenol at 50 and 100 μM afforded protec-

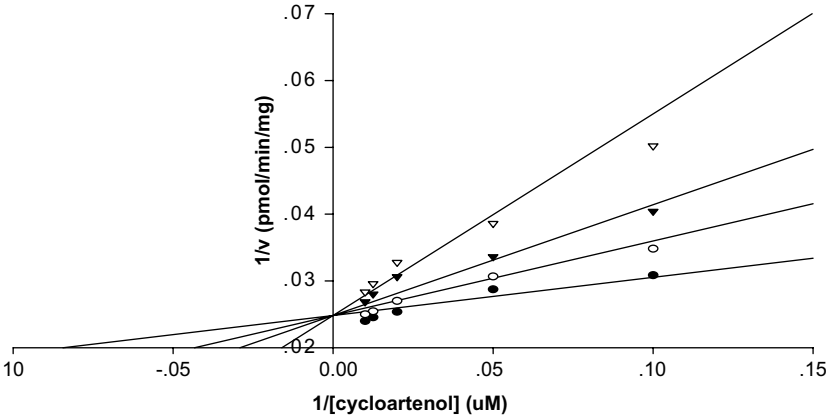

Figure 3. Double reciprocal plot of initial rates versus the concentration of substrate for soybean SMT1 catalyzed turnover of cycloartenol in the presence of cyclobranol for increasing concentrations of inhibitor at 10, 15, 25, and 50 μM. Incubations were performed with 1 mg of total protein of a 100,000 g supernatant fraction derived from *Escherichia coli* harboring the soybean SMT gene (0.4 μM SMT, concentration of SMT was estimated from the SDS–PAGE gel) in a final volume of 600 μL of the standard assay. The reactions were performed at 35 °C for 45 min. The soybean SMT1 was assayed with 10, 25, 50, and 100 μM cycloartenol with AdoMet fixed at 100 μM.

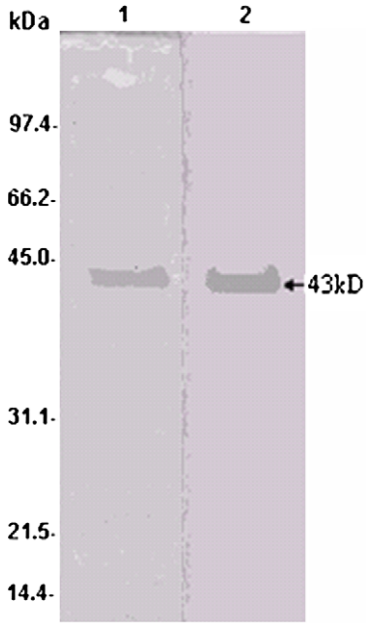

Figure 5. Radiofluorogram of soybean SMT1 treated with cyclobranol. Lane 1 is a radiofluorogram of soybean SMT1 labeled by cyclobranol reacted with [$^3H_3$-*methyl*]AdoMet and lane 2 is the migration of pure soybean SMT1 in SDS–PAGE gel electrophoresis under similar chromatographic conditions as shown for lane 1. The labeling reaction was carried out with 100 μM [$^3H_3$-*methyl*]AdoMet (0.6 μCi), 100 μM cyclobranol, and 0.8 μM pure SMT at 35 °C for 8 h.

tion against inactivation, generating 37% and 49% C-methylation, relative to the C-methylation activity of a control incubation containing saturating amounts of cycloartenol and AdoMet only.

Incubation of cyclobranol paired with [$^3H_3$-*methyl*]AdoMet resulted in specific covalent modification of the soybean SMT1, as detected by fluorography of the SDS–PAGE separated proteins (Fig. 5).

The results support the following mechanism whereby the biomethyl product C24-methylene C25-methyl cycloartanol **10A** arises from the corresponding C24-dimethyl cation **2**, followed by 1,2-methyl migration of C28 on the *Re*-face of the original substrate double bond generating alkylation at C25 and proton elimination at C33 to form an exocyclic methylene group (Scheme 1). Covalent modification of soybean SMT1 requires partial C-methylation of **8A** at the active site of the enzyme with trapping of the cationic intermediate (presumably **2**) by an active site nucleophile. It is noteworthy that the C-methylation of cyclobranol by soybean SMT1 was directional so that side chain **12** was not a product of the C-methylation reaction. This observation leads to the conclusion that formation of the transient carbocationic intermediates from the "product-like" analog **8A** orients in an active site pocket more similar to the intermediate of cycloartenol catalysis than to the intermediate of 24(28)methylene cycloartanol catalysis; as a result proton abstraction can occur at C28 to yield a single turnover product. However, the added bulk at C24 of **8A** appears to contribute to the bound sterol side chain in the activated complex thereby slowing the timing of sterol C24-methylation to afford alkylation of the enzyme. Further characterization of the labeled protein will certainly provide valuable information about the active site of this important class of catalyst whose crystal structure is still lacking.

Acknowledgments

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References and notes

1. (a) Schaller, H. *Plant Physiol. Biochem.* **2004**, *42*, 465; (b) Hartmann, M.-A. *Trends Plant Sci.* **1998**, *3*, 170; (c) Asami, T.; Yoshida, S. *Trends Plant Sci.* **1999**, *4*, 348.
2. Nes, W. D.; Song, Z.; Dennis, A. L.; Zhou, W.; Nam, J.; Miller, M. B. *J. Biol. Chem.* **2003**, *278*, 34505.
3. (a) Bouvier, F.; Rahier, A.; Camara, B. *Prog. Lipid Res.* **2005**, *44*, 357; (b) Benveniste, P. *Annu. Rev. Plant Biol.* **2004**, *55*, 429.
4. Zhou, W.; Lepesheva, G. I.; Waterman, M. R.; Nes, W. D. *J. Biol. Chem.* **2006**, *281*, 6290.
5. (a) Akihisa, T.; Tamura, T.; Matsumoto, T. *Phytochemistry* **1987**, *26*, 575; (b) Kalinowska, M.; Nes, W. R.; Crumley, W. D. *Phytochemistry* **1990**, *29*, 3427; (c) Goad, L. J.; Akihisa, T. *Analysis of Sterols*; Blackie Academic & Professional: London, 1997. pp. 197–234.
6. (a) Song, Z.; Zhou, W.; Liu, J.; Nes, W. D. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 33; (b) Zhou, W.; Song, Z.; Liu, J.; Miller, M. B.; Nes, W. D. *Tetrahedron Lett.* **2004**, *45*, 875; (c) Song, Z.; Nes, W. D. *Bioorg. Med. Chem. Lett.* **2007**, *17*, 5902; (d) Wang, J.; Liu, J.; Song, Z.; Nes, W. D. *Bioorg. Med. Chem. Lett.* **2008**, *18*, 232; (e) Nes, W. D. *Biochim. Biophys. Acta* **2000**, *1529*, 63.
7. (a) Lenton, J. R.; Goad, L. J.; Goodwin, T. W. *Phytochemistry* **1975**, *14*, 1523; (b) Seo, S.; Uomori, A.; Yoshimura, Y.; Takeda, K.; Seto, H.; Ebizuka, Y.; Noguchi, H.; Sankawa, U. *J. Chem. Soc., Perkin Trans. I* **1988**, 2407.
8. Janssen, G. G.; Nes, W. D. *J. Biol. Chem.* **1992**, *267*, 25856.
9. Cyclobranol was prepared as previously described in Ref. 8 and (a) Nes, W. D.; Le, P. H. *Biochim. Biophys. Acta* **1990**, *1042*, 119. with the ^1H NMR signals for this compound in agreement with that shown in; Guo, D.; Venkatramesh, M.; Nes, W. D. *Lipids* **1995**, *30*, 203. In reviewing the results of the cyclobranol tested previously with the soybean SMT, we reported that it fails as a substrate (Ref. 2). Reexamination of the sample tested by GC–MS indicated that it was not in the free form, it was the C3-acetate (to protect the C3-group) which explains its inactivity.
10. The standard assays for generating kinetic constants and for product determination were as described in Ref. 2.
11. No other GC peak was evident in the chromatogram to 40 min. However, an anomalous GC peak routinely appeared in several different incubations at approximately Rt 21.8 min with a M^+ 440 amu in 10% yield. A compound that matches the Rt for a C24-methyl sterol of M^+ 440 amu was not evident in HPLC from incubation (12 pooled assays) with cyclobranol and [$^3H_3$-*methyl*]AdoMet (0.6 μCi). In this experiment, a single peak of radioactivity appeared in the sterol region of the chromatogram (which occurs from α_c 0.5–2.0) with a retention time relative to cholesterol (α_c) (Rt = 36 min; HPLC was performed with a TSK gel C_{18}-reversed phase semi-preparative column eluted with MeOH at a flow rate of 1 mL/min at ambient temperature) of α_c 1.36 (total radioactivity associated with this HPLC peak was ca. 160,000 dpm representing 100% of radioactivity recovered from the sterol region of the chromatogram). The HPLC peak possessing the radioactivity migrates in similar fashion as the material **10A** isolated from the HPLC for ^1H NMR analysis.
12. In Dennis, A.; Nes, W. D. *Tetrahedron Lett.* **2002**, 437017. we report the detailed characterization (GC, MS, ^1H NMR, and ^{13}C NMR spectra with assignment) of the product set of 24-ethyl(idene) sterols **5A**, **6A**, and **7A** generated from 24(28)-methylene cycloartenol assayed with the soybean SMT1.