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Phytosterol Biosynthesis: Isotope Effects Associated with Biomethylation Formation to 24-Alkene Sterol Isomers

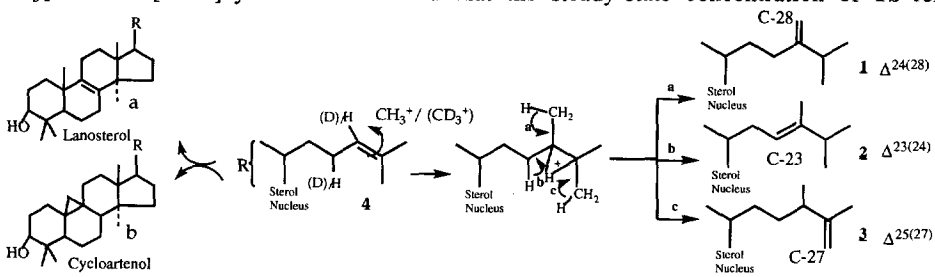
De-an Guo, Zhonghua Jia, and W. David Nes*
Department of Chemistry and Biochemistry
Texas Tech University, Lubbock, Texas 79409

Abstract: Changes in 24-alkene sterol product distribution resulting from deuterium substitution on the coenzyme methyl group of AdoMet and on the sterol acceptor molecule at C-23 and C-24 were used to determine kinetic isotope effects for the terminating deprotonations involved in sterol biomethylation catalyzed by (S)-adenosyl-L-methionine-Δ^{24}-sterol methyl transferase (SMT) enzyme. By this method 24(28)-methylene cycloartanol and cyclosadol were shown to be synthesized by two different SMT enzymes. Copyright © 1996 Published by Elsevier Science Ltd

The mechanism of biomethylation of phytosterols posits a series of stereochemically related events-electrophilic alkylations, rearrangements and deprotonations that may arise from a common set of enzymatic intermediates. A crude cell-free *Zea mays* (corn) SMT enzyme was shown to catalyze formation of three 24-alkene sterol isomers, 24(28)-methylene cycloartanol **1b,** cyclosadol **2b**, and cyclolaudenol **3b**, from the cycloartenol substrate **4b** (Scheme 1)[1,2]. A mechanistic approach that may be used to distinguish the number of SMT enzymes involved in sterol biomethylation consists of isotopic branching experiments. Branching experiments can only be observed if there is a common intermediate for both enzymatic products, one branch must be sensitive to the kinetic isotope effect (KIE)[3]. Mihailovic using this approach found that 24-alkene sterol isomers **1b** and **3b** were produced by an alga on the same (S)-adenosyl-L-methionine: Δ^{24}-sterol methyl transferase (SMT) enzyme surface[4]. Alternatively, KIE and mechanistic enzyme studies on the SMT enzyme from *Saccharomyces cerevisiae* indicated that the yeast enzyme was homogenous and capable of producing only one 24-alkene sterol product (viz. a 24(28)-methylene sterol) from suitable sterol acceptor molecules[5]. In this paper we report mechanistic information that shows for the first time that **1b** and **2b** are formed in corn by distinct isoforms of the SMT enzyme.

The product distributions and kinetics of multiple 24-alkene sterol products resulting from incubations with several pairs of deuteriated and nondeuteriated sterol acceptor molecules and coenzyme (AdoMet) in a soluble *Zea mays* SMT enzyme preparation from 8-day dark-grown seedlings are given in Table 1[6]. An induced KIE may result in these experiments from primary or secondary deuterium isotope effects that accelerate the rate of formation of one enzymatic product by suppressing the rate of formation of a second enzymatic product in a compensatory manner, supporting the role of a single SMT enzyme giving rise to **1b** and **2b**. Alternatively, induced KIEs may produce singular rate changes that lead to slowed formation of the only 24-alkene sterol isomer derived from deuterium elimination, or they may produce concurrent rate reductions in the formation of alternate 24-alkene sterol products, supporting two distinct SMT enzymes, operationally referred to as SMT I and SMT II, that catalyze the formation of **1b** and **2b**, respectively.

The change in product ratios of **1b** to **2b** can be used to calculate the isotope effect on the methyl deprotonation. Comparison of the radioactivity ratios for the pair AdoMet/[3-^{3}H]cycloartenol and [*methyl*-$^2H_3$]AdoMet/[3-^{3}H]cycloartenol showed that the steady-state concentration of **1b** relative to **2b**



Scheme 1

increased from 2. 54/1 to 4.76/1, from which the equilibrium isotope effect ($K_{eq\ H}/K_{eq\ D}$) can be calculated[3a] to be 0.53, suggesting an apparent inverse kinetic isotope effect. However, the rate of product formation of **1b** and **2b** decreased concurrently from 2.7 to 2.4 pmol/min and 0.9 to 0.5 pmol/min, respectively. The apparent change in product ratios resulted from the greater rate reduction of **2b** relative to the rate reduction in **1b**, suggesting that the branching reactions are generated by competing formation of products by distinct SMT enzymes, with one of the SMT enzymes (SMT II) more sensitive to terminating deprotonations than the other (SMT I). Similar relative results were observed using lanosterol as the sterol acceptor molecule. When the rate constants are considered from initial velocity experiments the isotope effect may be calculated for **1b** and **1a**, in which the proton lost is from C-28, k_H/k_D = 2.7/ 2.4 =1.1 and 1.2/1.0 = 1.2, respectively, and for **2b** and **2a**, in which the proton lost is from C-23, 0.9/0.5 = 1.8 and 0.6/0.3 = 2.0, respectively. The absence of an induced KIE for 24(28)-methylene sterol production in corn was similarly observed in sterol biomethylation in ergosterol biosynthesis[5], whereas an apparent KIE was observed in the formation of $\Delta^{23(24)}$-24-alkene sterols for the first time. Observation of an apparent inverse isotope effect when [*methyl*-$^2H_3$]AdoMet is used must mean that the formation of the 23/24 product is significantly slowed. The only step in the reaction course that would be consistent with this rate decrease would be the first step in the mechanism common to both pathways, which is formation of a dative bond by an S_N2 mechanism[7].

When the pair of sterol acceptor molecules was lanosterol/[23-$^2H_2$]lanosterol and the coenzyme [*methyl*-$^3H_3$]AdoMet, the resulting k_H/k_D was calculated for **1a** to be 1.0 and for **2a** to be 2.0[8]. The isotope effect could again be related to the slowed formation of the 23(24)-product. If the first step was rate-limiting, then an inverse isotope effect (measured from the product distributions at saturating concentrations of substrates) could only be explained by more than one enzyme. Since saturating concentrations of sterol acceptor molecule and coenzyme were employed and rates of net product formation from deuteriated and nondeuteriated substrates were compared, the observed rate suppressions represent effects on V_{max} rather than V_{max}/K_m [3b].

Different K_m values[2] were found for the sterol specificities toward SMT I and SMT II, which was further reason to believe that corn synthesized SMT isoforms. The apparent K_m for cycloartenol and lanosterol conversion to biomethylated product with side chain **1** was 30 μM ± 5 μM and 19 μM ± 2 μM, respectively, whereas the apparent K_m for side chain **2** production was 10 μM ± 2 μM and 7 μM ± 2 μM, respectively. The

K_m for cycloartenol and lanosterol was unaffected by the addition of saturating levels of AdoMet or [*methyl-*$^2H_3$]AdoMet. The K_m for AdoMet and [*methyl-*$^2H_3$]AdoMet was similar and was not affected by the type of sterol added to the assay mixture, 5 μM ± 2μM, respectively.

To determine whether intramolecular isotope effects were associated with methylation of the sterol 24,25-double bond, [24-^{2}H]cycloartenol and [24-^{2}H]lanosterol were assayed with the SMT enzyme preparation (Table 1). Neither substrate induced a KIE on product distributions or effected the rates of product formation. The lack of an isotope effect resulting from 1,2-hydride transfer of H-24 to C-25 during biomethylation is consistent with our proposal that the rate-determining step is early in the mechanism and that side chain **1** and **2** arise naturally from different SMT enzymes[9].

Table 1. Product Distribution and Rates of C-24 Sterol Alkene Formation Catalyzed by SMT Enzyme from Corn Sheaths with Deuterium Labeled Sterol and AdoMet Substrates

Substrate	Product Distribution[a]	*Vapp* (pmol/min/mg)[b]	
1/2	$\Delta^{24(28)}$-sterol/$\Delta^{23(24)}$-sterol	$\Delta^{24(28)}$-sterol	$\Delta^{23(24)}$sterol
Cycloartenol and [*methyl-*$^3H_3$]AdoMet	2.28/1	2.6	0.9
[24-^{2}H]Cycloartenol and [*methyl*$^3H_3$]AdoMet	2.40/1	2.5	0.9
[3-^{3}H]Cycloartenol and AdoMet	2.54/1	2.7	0.9
[3-^{3}H]Cycloartenol and [*methyl-*$^2H_3$]AdoMet	4.76/1	2.4	0.5
Lanosterol and [*methyl-*$^3H_3$]AdoMet	1.56/1	1.3	0.6
[24-^{2}H]Lanosterol and [*methyl-*$^3H_3$]AdoMet	1.53/1	1.3	0.6
[3-^{3}H]Lanosterol and AdoMet	1.63/1	1.2	0.6
[3-^{3}H]Lanosterol and [*methyl-*$^2H_3$]AdoMet	3.51/1	1.0	0.3
[23-$^2H_2$]Lanosterol and [*methyl-*$^3H_3$]AdoMet	3.31/1	1.3	0.3

[a]24-Alkene sterol production was determined by standard assay of aliquots of the same soluble enzyme preparation at saturating substrate concentration of 75 μM (n = 4); Distribution of 24-alkene sterol products was determined by radio reversed-phase HPLC analysis.
[b]Variation in apparent velocity *(Vapp)* was calculated from Lineweaver-Burk plots of sterol substrate concentration varied from 5 to 100 μM, AdoMet concentration was fixed at 50 μM.

Acknowledgment. We thank the Asgrow Seed Company for financial support.

References and Notes

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2. In Guo, D; Venkatramesh, M.; Nes, W.D. *Lipids* **1995**, *30*, 203-219, we confirmed that corn synthesizes multiple sterol side chain isomers **1**, **2** and **3** in intact tissue using NMR.^{14}C-acetate and ^2H_2O incubations with growing seedlings indicated that isomers **1** and **2**, but not **3**, were biosynthesized by young plants, an observation that was confirmed from subsequent in vitro enzyme assays performed on maturing roots and shoots (unpublished). SMT enzyme assays were performed in quadruplicate (n = 4),

with minimal variation among the trials as discussed in the text.

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6. Reaction rates for the bisubstrate biomethylation reaction catalyzed by corn sheath SMT enzymes were established using a soluble enzyme prepared from microsomes as described in ref. 5, except the assays were performed at 35°C. Initial velocity data was obtained by isolation of the 4,4-dimethyl sterol fraction (generated under linear conditions) followed by radio-HPLC analysis of this material to determine the product distribution. Catalytic amounts of [*methyl-*$^3H_3$]AdoMet used in select incubations (^{3}H-methyl AdoMet was diluted with non-radioactive AdoMet to produce a sp. act. 20 μCi/mmol) did not affect biomethylation rates significantly. In calculating radioactivity ratios a factor of 1.5 was used to account for the loss of one tritium atom from the methyl on the coenzyme producing side chain **1** compared to no loss of ^{3}H in formation of side chain **2**. Endogenous levels of sterol was trace in soluble enzyme preparations (in microsomes the ratio of cycloartenol /24(28)- methyleneycloartanol/cyclosadol is 62/27/11, or for the biomethylated sterols **1b** and **2b** in a ratio of 2.45/1). The deuteriated and tritiated sterols were prepared according to Le, P.H.; Nes, W.D. *Chem. Phys. Lipids* **1986**, *40*, 57-69; [3-^{3}H]cycloartenol, sp. act. 5.4 x 10^8 dpm/mg and [3-^{3}H] lanosterol, sp. act. 4.3 x 10^8 dpm/mg. [*methyl-*$^2H_3$]AdoMet (98% atom enrichment) was purchased from MSD Isotopes, Canada. [24-^{2}H]- and [23-$^2H_2$]-Sterol was prepared from the 24-keto sterol.
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8. The deuterium content of deuteriated **1b** and **2b** and the products from biomethylation of [23-$^2H_2$] lanosterol and [24-^{2}H]lanosterol was obtained from analysis of the 4,4-dimethyl sterol fraction of 300 pooled assays by GC-MS as described in reference 5. Compared with the mass spectrum of authentic nondeuteriated specimens available to us, dideuteriated **1b** possessed a two amu increase (m/z, M^+ 442, 427, 424, 409, 381, 355, 302) and trideuteriated **2b** possessed a three amu increase (m/z, M^+ 443, 428, 425, 382, 356, 303), similar to the observations of Misso and Goad in reference 1b.Catalysis of [23-$^2H_2$] lanosterol gave rise to loss of no deuterium atoms in production of [23-$^2H_2$]**1a** (m/z, M^+ 442, 427, 409, 393, 365, 339,259) and loss of one deuterium atom in the production of [23-^{2}H]**2a** (m/z, M^+ 441, 426, 401, 393, 375, 259) whereas [24-^{2}H]lanosterol gave rise to [25-^{2}H]**1a** (m/z, M^+ 441, 426, 408, 385, 259).
9. The facility of 1,2-hydride transfer in formation of side chain **2** indicates that H-23 might be axially directed in the ternary complex to the same face of the double bond which was methylated by AdoMet, otherwise H-24 should be similarly captured by the base involved in H-23 removal. These observations provide a rationale for Arigoni's proposal (Arigoni, D.*Ciba Found. Symp.* **1978**, 243-261) that $\Delta^{24(25)}$-24-methyl sterols are never formed from direct alkylation of the 24,25-bond and is further reason to believe that the conformation of the sterol side chain in the ternary complex mediates the steric course of biomethylation of sterols cf. Zhou, W.; Guo, D.; Nes, W.D. *Tetrahedron Lett.* **1996,** 37, 1339-1342.

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