Stereochemistry of Hydrogen Migration from C-24 to C-25 during Phytosterol Biomethylation

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(S)-Adenosyl-L-methionine: $\Delta^{24(25)}$-Sterol methyl transferase (SMT) enzymes catalyze the methylation of Δ^{24}-sterol acceptor molecules to 24(28)-methylene sterol products that serve as substrates for the synthesis of 24α- and β-alkyl sterol membrane inserts.[1] Methylation is the first committed step in phytosterol turnover and a critical slow step in the control of cycloartenol transformation to 24-alkyl Δ^5-sterols (e.g., sitosterol, (24*R*)(α)-ethyl cholest-5-en-3β-ol).[2,3] Rahier et al. examined sterol biomethylation inhibitors with the corn (*Zea mays*) SMT enzyme and reported that phytosterol methylation operated by a *Re*-face mechanism.[4] The stereochemistry of biomethylation in corn and other vascular plants is thought by some investigators to be different from that in fungi;[4−9] plants produce 24α-alkyl sterols by a *Re*-face mechanism of "methyl cation attack" on the 24,25-double bond, whereas fungi produce 24β-alkyl sterols (e.g., ergosterol, (24*R*)(β)-methyl cholesta-5,7,*E*22-trien-3β-ol) by a *Si*-face mechanism (Scheme 1). As such, the SMT enzyme from plants and fungi has emerged as an attractive target for inhibitor design.[4,10−14]

Alternatively, we considered that the stereochemistry of the methyl transfer reaction evolved by a similar *Si*-face mechanism in nature. In our model (termed the "steric electric plug model"),[15] the conformation of the sterol side chain in the ternary complex is postulated to regulate the steric course of biomethylation. We proposed that AdoMet juxtapositioned appropriately in the active site methylates from the backside (β-face) of the double bond, which, as shown in Scheme 1 (path a), gives rise to the 25*R*-stereochemistry from a [27-^{13}C]-isotopocally labeled sterol. In support of this hypothesis, we discovered that the SMT enzymes catalyzing $\Delta^{24(28)}$-methylenation from sunflower and yeast binds sterols with their side chain in a pseudocyclic conformation which serves to orient the side chain 24,25-double bond with its *Si*-face spatially

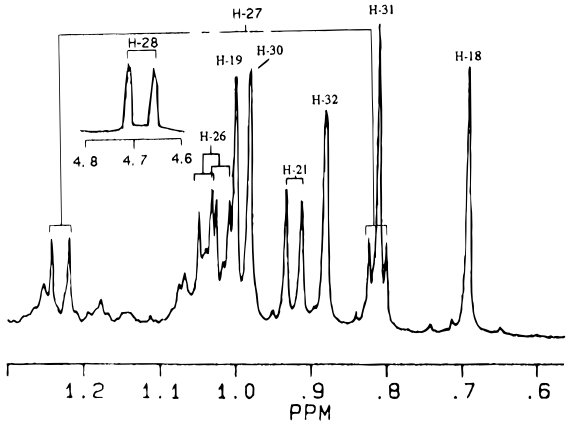

Figure 1.

complimentary to AdoMet[13−16] and reports that show 24β-methyl $\Delta^{25(27)}$-sterols may be formed by direct methylation from the *Si*-face of the 24,25-double bond.[17] The purpose of this communication is to report ^{1}H- and ^{13}C-NMR assignments of ^{13}C-isotopically labeled sterols synthesized by the SMT enzyme from corn . The spectral data indicate that the SMT enzyme from corn operates the same sterol methylation mechanism that operates in fungi and marine organisms.[16,17]

Sterol acceptor molecule, [27-^{13}C]lanosterol, prepared as described,[16] and coenzyme, AdoMet, were incubated in a microsome-bound SMT enzyme preparation from 4-day old *Zea mays* seedlings.[18] The resulting nonsaponifiable lipid fraction of the incubation product was fractionated by flash chromatography to give a fraction containing a mixture of 4,4-dimethyl sterols. These sterols were separated by RP-HPLC using Whatman and TSK gel C_{18}-columns.[19] The major 24(28)-methylene sterol recovered from the HPLC column (350 μg) was identified by chromatographic and spectral methods as [27-^{13}C]24(28)-methylene-24,25-dihydrolanosterol **1**(structure *1* in Scheme 1): GLC (RRTc on 3% SE-30 packed column operated at 245 °C, 1.88); MS (*m*/*z* 441 (M^+), 426, 408, 393, 365, 341, 323, 286, 259, 241); ^{1}H-NMR δ ppm, 0.688 (H-18, s), 0.806 (H-31, s), 0.877 (H-32, s), 0.918 (H-21, d J = 6.2 Hz), 0.977 (H-30,s), 0.996 (H-19,s), 1.017 (H-27,dd, J = 125.5 Hz, 6.8 Hz), 1.024 (H-26, d, J = 5.2 Hz, 6.8 Hz), 4.683 (H-28, d, J = 15.3 Hz), 3.225 (H-3, dd, J = 11.2 Hz, 11.6 Hz); ^{13}C-NMR (side chain carbons) δ ppm, C-20 (36.46), C-21 (18.68), C-22 (18.68), C-23 (31.25), C-24 (156.90), C-25 (33.78), C-26 (21.84), C-27 (21.97), C-28 (105.84).

The ^{1}H-NMR spectrum of specimen **1** shows that C-26 and C-27 in a 24(28)-methylenesterol may be resolved for the first time. The signal corresponding to C-27 resonates upfield from C-26 in the ^{1}H-NMR spectrum, whereas the signal for C-27

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(19) In ref 3 we describe methods for the separation of 24-alkene phytosterol isomers by HPLC. NMR measurements were perfomed in $CDCL_3$ with a Bruker 300 (300 MHz). Chemical shifts were referenced to TMS as internal standard.

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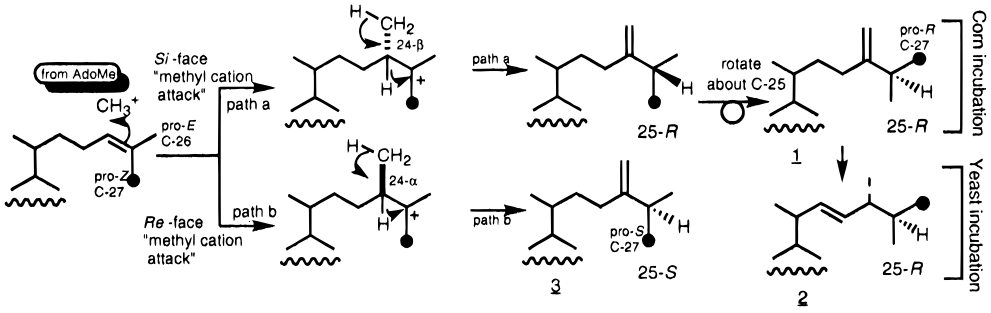
Scheme 1



resonates downfield relative to C-26 in the ^{13}C-NMR spectrum (Figure 1). There are no model compounds to correlate the labeling patterns of C-26 and C-27 in 24(28)-methylene sterols in ^{1}H- and ^{13}C-NMR. Horibe et al. prepared the pair of epimeric 24-alkyl sterols deuteriated stereospecifically at one of the two methyl groups at C-25 and found a correlation between the chirality at C-24 and the chemical shift assignment.[20] However, the spectral data of Horibe et al. was not helpful in this study.

To pursue further our NMR analysis, sample **1** was isotopically diluted with 4.0 mg of freshly prepared nonlabeled **1** and incubated at 5 mg/L with a strain (GL7) of *Saccharomyces cerevisiae* that is auxotrophic for sterols and transforms dietary supplements of lanosterol to ergosterol in 40% yield.[16] Figure 2b shows the partial ^{13}C-NMR spectrum of sample **1** mixed with nonlabeled **1**[21] in a ca. 1:10 ratio. Figure 2a shows the partial ^{13}C-NMR spectrum of [27-^{13}C]ergosterol produced by GL7 from incubation with **1** isotopically diluted with nonlabeled **1**. The position of the C-27 signal in Figure 2a matched that of an authentic specimen of [27-^{13}C]ergosterol, indicating that C-27 was the pro-*R*-methyl group[16] (structure **2** shown in Scheme 1). In the transformation of [27-^{13}C]**1** to [27-^{13}C]-ergosterol by GL7, the stereochemistry at C-25 is not affected and the ^{13}C-labeled ergosterol is not diluted by endogenously formed nonlabeled ergosterol.[16] Thus the known configuration of C-25*R* in the product [27-^{13}C]ergosterol may now safely be assigned to the fungal substrate **1** derived from seedling material. The results indicate that under physiological conditions the pro-*Z*-methyl group on cycloartenol corresponding to C-27[22] is transformed into the pro-*R*-methyl group at C-25 on 24(28)-methylene cycloartanol by migration of the hydrogen atom at C-24 to C-25 from the *Re*-face of the 24,25-double bond of the intermediate, cycloartenol.

In view of our recent demonstration that [2-^{13}C]mevalonic acid is converted to (25*S*)-[26-^{13}C]sitosterol[22] and the current finding that [27-^{13}C]lanosterol is converted to (25*R*)-[27^{13}C]**1** (IUPAC nomenclature: (25*R*)-[26-^{13}C]**1**)[22] suggests a general process: the biosynthesis of 24α-ethyl phytosterols passes through three steps—(i) successive methylation at C-24, (ii) isomerization of the 24(28)-bond to the 24,25-bond, and (iii) reduction of the 24,25-bond—with a net retention in configuration at C-25 in the final chiral product. Interestingly, the stereochemical course of [26-^{13}C]desmosterol reduction in the biosynthesis of cholesterol in mammals and insects leads to (25*R*)-[26-^{13}C]cholesterol, which is opposite stereochemically to the product of the reduction step from iii above.[23] Hence phylogenetic differences in the evolution of the sterol side chain pathway in plants and animals exist in reduction stereochemistry.

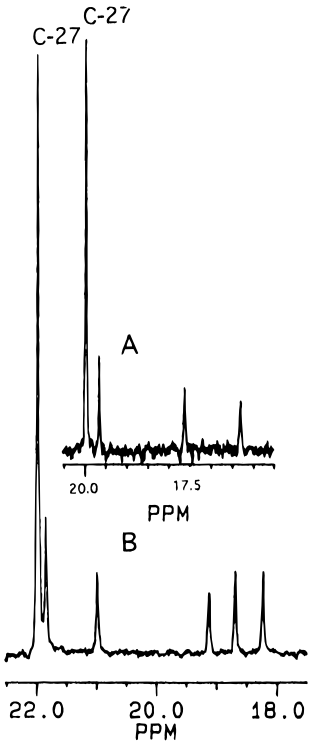


Figure 2.

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