Pergamon

Tetrahedron Letters, Vol. 38, No. 35, pp. 6115-6118, 1997

0040-4039/97 $17.00 + 0.00

PII: S0040-4039(97)01386-5

Stereochemical Features of C-methylations on the Path to $\Delta^{24(28)}$-Methylene and $\Delta^{24(28)}$-Ethylidene Sterols: Studies on the Recombinant Phytosterol Methyl Transferase from *Arabidopsis thaliana*

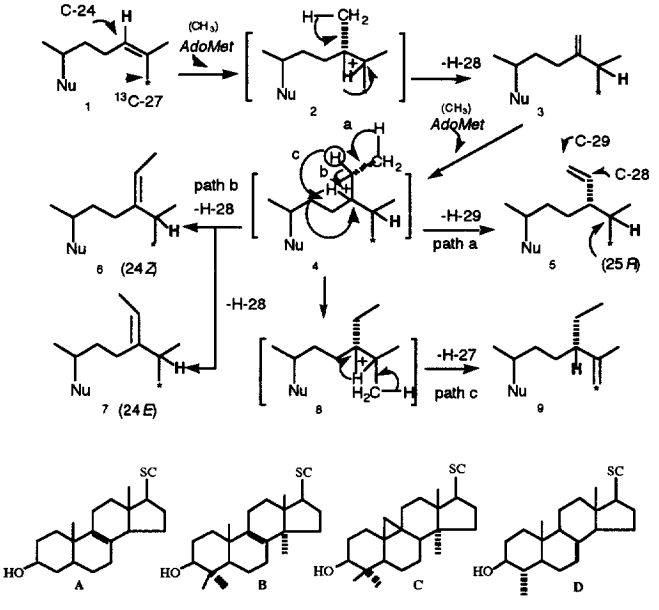
Yusen Tong, Brian S. McCourt, De-an Guo, Anil T. Mangla, Wen-Xu Zhou, Mark D. Jenkins, Wen Zhou, Monica Lopez and W. David Nes*
Department of Chemistry and Biochemistry
Texas Tech University
Lubbock, Texas 79409

Summary: Using a homogenate prepared from *Escherichia coli* cells that express the sterol methyl transferase (SMT) gene of *Arabidopsis thaliana*, migration of the hydrogen atom at C-24 to C-25 from the *Re*-face of the double bond was demonstrated in the biosynthesis of [27-^{13}C] 24(28)-methylenezymosterol (fecosterol) from [27-^{13}C]zymosterol and the chirality of the C-25 stereocenter (25*R*) was found to be retained after the stereospecific conversion of [27-^{13}C]24(28)-methylenezymosterol to [27-^{13}C](24(28)*Z*) -ethylidenecholest-8-en-3β-ol.

The (S)-adenosyl-L-methionine: Δ^{24}-sterol methyl transferase (SMT) enzyme is a regulatory enzyme synthesized by plants that catalyzes the committed step in cycloartenol transformation to Δ^{5}-24-alkyl sterols.[1] Vascular (advanced) plants are different from non-vascular (primitive) plants (including fungi), in that advanced plants always synthesize 24-ethyl sterols as the major sterol, whereas many primitive-plants synthesize 24-methyl sterols exclusively. On the basis of several lines of evidence, it has recently been proposed that the methylation of $\Delta^{24(25)}$-sterol acceptor molecules to C-28 phytosterols proceeds stereoselectively with the intermediacy of the discrete bridged carbenium ion (**2**) shown in scheme 1.[2] The formation of C-29 phytosterols has remained enigmatic. The possibility of a stereochemical inversion at C-25 resulting during the second methylation of the 24(28)-bond of intermediate **3** to form compound **6** has been considered.[3] Nonetheless, all C-29 phytosterols must proceed through a 24-methylene intermediate (**3**) and through a 24-ethyl sterol transition state (**4**), by double transmethylations from AdoMet (=SAM).

The first and second methylation mechanisms should operate in a similar manner such that the enzymatic transfer of the methyl group of AdoMet to carbon as acceptor occurs via *Si*-face attack (β-face attack as shown in **2**, **4** and **8**) with inversion of configuration. This suggests a similar S_N2 transition state is obtained during successive transmethylations.[4] These observations indicate that the second C-methylation may proceed to give a 24-vinyl sterol, e.g., **5**, although the natural occurrence of 24-ethylated sterols shows otherwise, e.g., occurrence of side chain types **6**, **7** and **9** .[5] Since SMT enzymes from plants distinguish between cycloartenol (**C1**) and 24(28)-methylenelophenol (**D3**) as substrates, and 24(28)-methylenecycloartanol (**C2**) fails to replace 24(28)-methylenelophenol as a suitable substrate for the second C-methylation,[6] different SMT enzymes in plants have been considered to catalyze the first and second C-methylation of phytosterols.[6] Alternatively, it is known that generation of a $\Delta^{24(28)}$-

methylene sterol involves addition of the methyl group and removal of the proton from C-28 from the *same* face of the plane defined by the double bond on the $\Delta^{24(28)}$-substrate, whereas generation of the 24(28)-ethylidene sterol involves addition of the methyl group and removal of the proton from C-28 on the *opposite* face from the AdoMet attack.[4a,b,e] Thus, the spatial relation of the nucleophile in the active site relative to the sterol C=C bond and its identity may be different for the two methylation reactions. For this reason, we hypothesize that methylation of a $\Delta^{24(25)}$- and $\Delta^{24(28)}$-sterol may occur from different binding sites on the SMT enyzme. This paper reports the first direct evidence showing that a single SMT enzyme species may give rise to mono and double alkylations and provides confirmation that the *pro*-Z methyl group on the $\Delta^{24(25)}$-substrate becomes the isopropyl *pro-R* methyl group on the 24-methylene and 24-ethylidene products.



Scheme 1

Sterol methylation reactions were performed on sonicated suspensions of recombinant SMT enzyme of *A. thaliana* expressed in *E. coli* cells under the control of T7 promoter.[7] We chose to study the pair of substrates [27-^{13}C]zymosterol[8a] (**A 1**) and [27-^{13}C]fecosterol (**A 3**) rather than the pair of substrates [27-^{13}C]cycloartenol and [27-^{13}C]24(28)-methylenecycloartanol, after we discovered that neither 24(28)-methylenecycloartanol (**C 3**) nor 24(28)-methylenelanosterol (**B 3**) were methylated by the SMT enzyme. GLC and TLC showed clean conversion of each of the ^{13}C-isotopically labeled sterol substrates to a single methylated product (ca. 25 % yield of each), which was isolated by chromatography on HPLC.[8a] The NMR spectra of these two products (Fig. 1) were compared with that of structurally

related compounds where the ^{1}H and ^{13}C NMR signals of the *pro-R-* and *pro-S-* methyl groups on C-25 in the side chain were assigned.[9] The ^{1}H- and ^{13}C-chemical shifts and chromatographic data of **A3** confirm its identity and stereochemistry.[10] The chemical shift of H-25 in the ^{1}H-NMR and signal for C-29 in the ^{13}C-NMR of **A6** confirm that the second C-methylation proceeds stereoselectively to produce the *Z*-geometry of the 24-ethylidene group.[8b,11] The results indicate that *A. thaliana* synthesizes a SMT enzyme which catalyzes *Re*-face hydrogen migration from C-24 to C-25 and the resulting C-25 (*25R*) configuration is retained during the second C-methylation to produce a 24(28)Z-ethylidene structure (**A6**).[12]

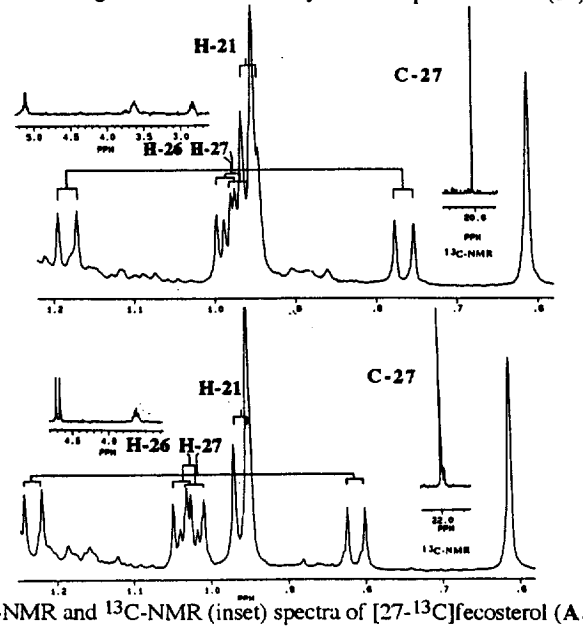


Figure 1. Partial ^{1}H-NMR and ^{13}C-NMR (inset) spectra of [27-^{13}C]fecosterol (**A3**) (bottom) and [27-^{13}C](24(28)*Z*)-ethylidenecholest-8-en-3β-ol (**A6**) (top) generated by the *A. thaliana* SMT enzyme.

References and Notes

1. Nes, W.D.; Venkatramesh, M. in *Biochemistry and Function of Sterols.* (Parish, E. J.; Nes, W. D., eds.): CRC Press, Boca Raton, **1997**, pp. 111-122.

2. Nes, W. D.; Guo, D.; Zhou, W. *Arch. Biochem. Biophys.* **1997**, *342*, 68-81.

3. Seo, S.; Uomori, A.; Yoshimura, Y.; Seto, H.; Ebizuka, Y.; Noguchi, H.; Sankawa, U.; Takeda, K. *J. Chem. Soc. PERKIN TRANS 1* **1990**, 105-109, have reported on the stereochemistry of the hydride shift from C-24 to C-25 in isofucosterol synthesis using different ^{13}C-acetate species fed to plant cell cultures. Their mechanistic observations differ from that of Nicotra, F.; Ronchetti, F.; Russo, G.; Lugaro, G.; Casellato, M. *J.. Chem. Soc. PERKIN TRANS 1* **1981**, 498-501.

4. (a) Arigoni, D, *Ciba Found. Symp.* **1978**, *60*, 243-261; (b) Guo, D; Jia, Z; Nes, W.D. *J. Amer. Chem. Soc.* **1996**, *118*, 8507-8508; (c) Zhou, W.; Guo, D.; Nes, W.D. *Tetrahedron Lett.* **1996**, *37*, 139-1342; (d) Fujimoto, Y; Ohyama, K; Sato, N; Yamada, J.; Morisaki, M. *Chem.*

Pharm.. Bull. **1997**, *45*, 224-226; (e) Zimmerman, M.P.; Djerassi, D. *J. Amer. Chem. Soc.* **1991**, *113*, 3530-3533.

5. For a review see Akihisa, T.; Kokke, W.C.M.C.; Tamura, T. in *Physiology and Biochemistry of Sterols.* (Patterson, G.W.; Nes, W. D., eds): American Oil Chemists' Society Press, Champaign, **1992**, pp. 172-228.

6. Rahier, A; Taton; M; Bouvier-Navé, P.; Schmitt, P.; Benveniste, P; Schuber, F.; Narula, A.S.; Cattel, L; Anding, C.; Place, P. *Lipids* **1986**, *21*, 52-62.

7. The coding region of SMT cDNA was isolated from an *A. thaliana* cDNA library by PCR amplification. DNA primers used in the PCR chain reactions were prepared based on the known sequence published by Husselstein, T.; Gachotte, D.; Desprez, T.; Bard, M; Benveniste, P. *FEBS Letts.* 1996, 381, 87-92. A 1086 bp fragment was subcloned into a PCR cloning vector (PCR II), sequenced, and engineered into expression vector pET15b. The resulting SMT enzyme possessed a His-tag (which unfortunately did not help in the purification of the enzyme). The recombinant protein was isolated from BL21(DE3) *E. coli* cells by cellular sonication, followed by low speed centrifugation (8,000 x g) to produce a supernatant fraction that served as the crude homogenate. Incubations were performed for 45 min. and 16 hrs.(product isolations) at 33^{0}C and assayed as described in Nes, W.D.; Janssen, G.G.; Bergenstrahle, A. *J. Biol. Chem.* **1991**, *266*, 15202-15212.

8 (a) [27-^{13}C]Zymosterol was prepared from zymosterol as described in 4c. HPLC methods to separate the geometric pair of *E/Z*-24-ethylidene sterols and related methods for sterol analysis were reported in Guo, D.; Venkatramesh, M.; Nes, W.D. *Lipids* **1995**, *30*, 203-219; (b) Patterson, G.W.; Hugly, S, Harrison, D. *Phytochemistry* **1993**, *33*, 1381-1383, reported that *A. thaliana* plants synthesize sitosterol (principal sterol in plant) and lesser amounts of 24*Z*- and 24*E*-ethylidene cholesterol, isofucosterol and fucosterol, respectively.

9. Synthetic standards of sterols have been prepared and the chemical shifts identified for the *pro-R* and *pro-S*-methyl groups on C-25 as described in ref. 4 and references cited therein.

10. Physical data for **A3**: R_f = 0.3 (tlc on sg plate developed in 85/15 benzene-diethyl ether); RRT_c = 1.31 (GLC: 3% SE-30 operated isothermally at 245°C); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.613 (s, H-18), 0.951 (s, H-19), 0.960 (d, *J* = 5.4 Hz, H-21), 1.019 (dd, *J* = 125.5 Hz, 6. 9 Hz, H-27), 1.028 (dd, *J* = 6.8 Hz each, H-26), 3.620 (m , H-3), 4.659 / 4.714 (2s, H_2-28); ^{13}C-NMR (75 MHz, $CDCl_3$): δ 31.050 (C-23), 156.877 (C-24), 33.526 (C-25), 21.98 (C26), 21.84 (C-27 = enhanced peak), 105.879 (C-28); EIMS (amu): M^+ 399, and other diagnostic ions in high mass region at 384, 366, 342, 314, 299, 271 (base peak), 245, 229, 213.

11. Physical data for **A6**: R_f = 0.3 (tlc performed as above); RRTc = 1.81 (GLC performed as above); ^{1}H-NMR (300 MHz, $CDCl_3$): δ 0.613 (s, H-18), 0.951 (s, H-19), 0. 960 (d, *J* = 5.4 Hz), 0.973 (dd, *J* = 125.2 Hz, 6.8 Hz, H-27) 0. 972 (dd, *J* = 6.70 each, H-26), 2.831 (sept, *J* = 7.2 Hz, H-25), 3. 619 (m, H-3), 5.107 (q, *J* = 7. 5. 107 Hz, H-28); ^{13}C-NMR (75 MHz, $CDCL_3$): δ 27.963 (C–23), 40.729 (C-24), 28.334 (C–25), 21.00 (C-26), 21.071 (C-27=enhanced peak), 116.398 (C-28), 12.753 (C-29); EIMS (amu): M^+ 413, and other diganostic ions in the high mass end at 398, 380, 356, 341, 327, 299, 285, 271, 246, 227, 213.

12. This work was supported by the Asgrow Seed Company and Welch Foundation (# D-1276).

(*Received in USA* 9 *May* 1997; *revised* 1 *July* 1997; *accepted* 2 *July* 1997)