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Sterol methyl transferase. Evidence for successive *C*-methyl transfer reactions generating $\Delta^{24(28)}$- and $\Delta^{25(27)}$-olefins by a single plant enzyme

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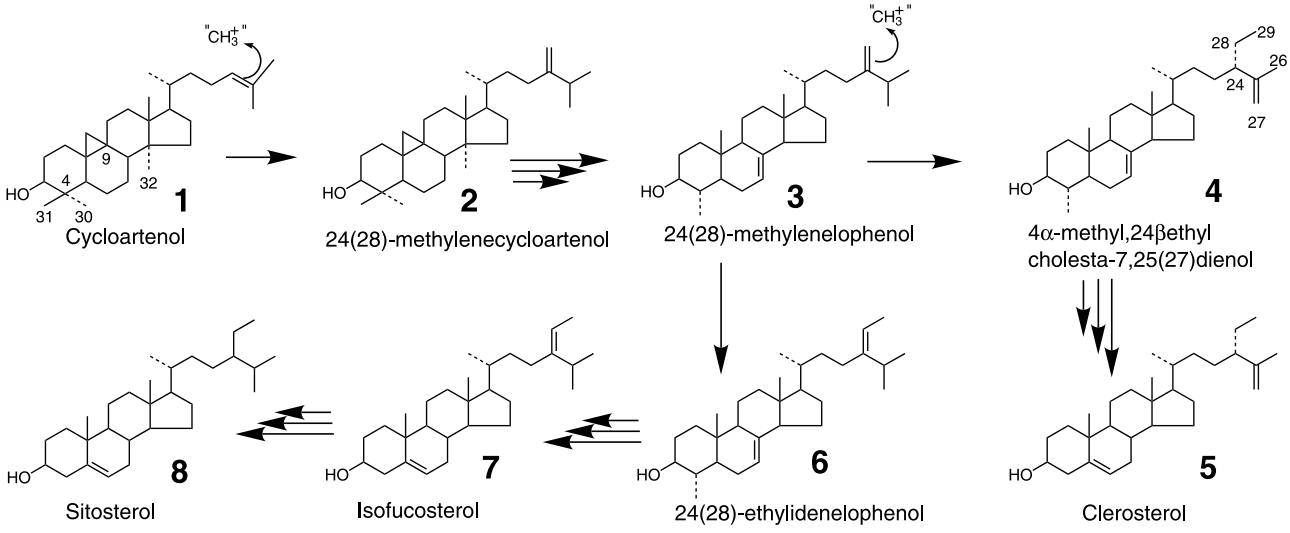
Abstract—The native SMT from *Glycine max* (soybean) expressed in *Escherichia coli* cells catalyzes the step-wise conversion of cycloartenol to 24(28)-methylene cycloartanol to a mixture of stereochemically related $\Delta^{24(28)}Z$-ethylidene-$\Delta^{24(28)}E$-ethylidene- and $\Delta^{25(27)}$-24β-ethylcyclosterols. [27-^{13}C]Cycloartenol was tested as a substrate to determine the C-25 configuration of the $\Delta^{24(28)}$-olefin formed during the first C_1-transfer reaction. This substrate yielded (25-*R*)-[27-^{13}C]24(28)-methylenecycloartanol. These results are entirely consistent with the observed stereochemistries of pathway specific 24-alkyl sterols found in plants and additionally revealed for the first time that a SMT can sequentially *C*-methylate the Δ^{24}-bond to generate multiple olefins.

Sterol methyl transferase (SMT) catalyzes the introduction of C-28 and C-29 at C-24 and formation of $\Delta^{24(28)}$- and $\Delta^{25(27)}$-olefin side chains giving rise to 24-alkyl sterol diversity.[1] The crucial role of these enzymes is to generate phytosterols that serve as membrane inserts and plant hormones.[2] The biosynthesis in more-advanced plants of sitosterol **8** (24α-ethyl) and in less-advanced plants of clerosterol **5** (24β-ethyl) is considered to proceed by pathway specific routes as a result of differences in plant evolution (Scheme 1).[1]

Differential inhibition studies and analyses of the structural requirements for substrate recognition by plant SMT[3] as well as a recent investigation of the cloning,



Scheme 1. Hypothetical pathway in phytosterol synthesis.

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sequencing and functional expression of cDNA encoding for SMT,[4] have led several investigators to suggest that a single enzyme catalyzes cycloartenol **1** to a monoalkylated $\Delta^{24(28)}$-sterol side chain.[4] Alternatively, the doubly alkylated sterol side chain with specifically the *Z*-ethylidene geometry **6** is thought to be generated by a different enzyme species that fails to accept 24(28)-methylenecycloartanol **2**.[5] However, since no plant SMT has been purified to date, we hypothesized a single plant SMT can catalyze both *C*-methylations by the same reaction mechanism.[3b] Indeed, a comparison of protein sequences deduced from the cDNAs for fungal and plant SMTs (Fig. 1)[4d] revealed a highly conserved region rich in aromatic amino acids, referred to as Region I, containing a signature motif YE*X*GW*X* not present in other AdoMet-dependent methyl transferases. As each of these plant enzymes uses cycloartenol in similar fashion to fungal SMT which catalyzes zymosterol, the aromatic-rich domain of Region I is proposed to be involved in substrate binding, and possibly product formation, by stabilizing intermediate carbenium ions generated during sterol *C*-methylation.[3]

Concerning the stereochemical features of these transformations, there is evidence that the direction of transfer of the *C*-methyl group from AdoMet proceeds by alkylation from the *re* (α)-face[6] or the *si* (β)-face[7] of Δ^{24}. To date, nothing has been reported about the ability of a native SMT enzyme to possess variant activities that include substrate channeling to form multiple olefins.[8] We now report investigations designed to identify enzyme-generated product stereochemistries and the stereochemistry of hydrogen migration from C-24 to C-25 in the biosynthesis of 24(28)-methylenecycloartanol using soluble recombinant soybean (*Glycine max*) SMT which allows the number of SMTs and the C-methylation mechanism involved with 24-ethyl sterol and multiple olefin formation to be established.

Shi et al.[4c] isolated the soybean SMT cDNA and provided us their plasmid pSERT carrying FLAG-epitope tagged SMT1 cDNA that generates a fusion protein. In the Shi et al. study, lanosterol was assayed using a cell-free homogenate of *Escherichia coli* expressing the recombinant SMT. Incubation with lanosterol generated 24(28)-methylene lanosterol. The investigators concluded the soybean SMT catalyzes only the first C_1-transfer reaction. We transferred the SMT cDNA from the pSERT to the T_7-based high-level expression system, pET23a plasmid, by introducing a restriction site *NdeI* at the 5′-end and a *BamHI* site at the 3′-end.[9] SMT cDNA localized in pET23a vector was transformed into BL21(DE3) cells at 37°C and expressed by addition of IPTG to the culture medium.[9] Cell were lysed using a sonifier, and the resulting native soybean SMT was resuspended in 50 mM Tris–HCl, 2 mM $MgCl_2$, 2 mM β-mercaptoethanol and 5% glycerol (v/v) at pH 7.5.[9] Activity assays using cell-free homogenates[7d] were performed overnight with saturating amounts of AdoMet and cycloartenol or 24(28)-methylene cycloartanol at 35°C and products of incubation were analyzed by GC–MS and ^{1}H NMR. Total sterols were recovered from the reactions essentially as described previously.[9] Reactions generated at least 300 μg of total product and the sterol mixture was purified to give individual compounds by HPLC using TSK gel C_{18}-columns.[10] GC–MS analysis of the enzyme-generated product from the cycloartenol incubation showed the product of *C*-methylation to correspond to an authentic specimen of 24(28)-methylenecycloartanol (Fig. 2; panel A).[10] By contrast, when 24(28)-methylenecycloartanol was incubated separately with the SMT enzyme three peaks were observed with base-line resolution in GC. The mass spectra of these compounds exhibited similar fragmentation patterns (M^+ 454 amu) corresponding to 24-ethyl sterol type lanostanoids, which we and others have isolated from fungi[11] (Fig. 2; panel B). HPLC chromatographic purification of the crude non-saponifiable lipid fraction of a preparative-scale incubation of 24(28)-methylenecycloartanol with SMT gave substrate and three novel products **9**, **10**, and **11**. The chromatographic and spectral data of the *C*-methylated cyclosterols generated by soybean SMT during the second C_1-transfer were as follows: **9**, GLC (retention time relative to cholesterol–RRT_c on DB5-capillary columns,[10] 2.39); MS (*m*/*z* 454 (M^+), 439, 421, 393, 355, 341, 323; 500 MHz ^{1}H NMR δ ppm, 0.538 (H-18, s),

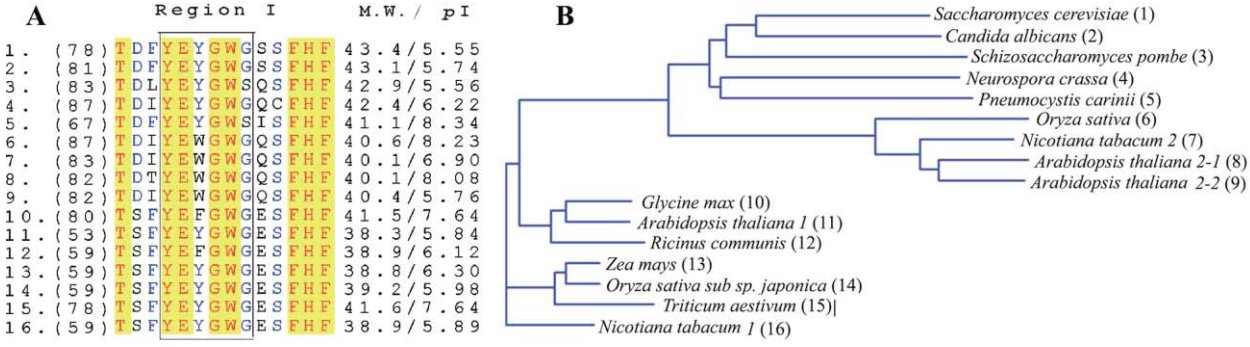


Figure 1. Partial sequence alignment and predicted Mr (kDa) and pI of 16 SMTs of plant and fungal origin (A).[4d] Yellow colored indicates a conserved residue and boxed area indicates signature moitf. Start residue in parentheses. Phylogenetic tree illustrating SMT relatedness was generated by Neighbor Joining Method embedded in the Vector NTI Suite 6.0 software package (Infomax) (B).

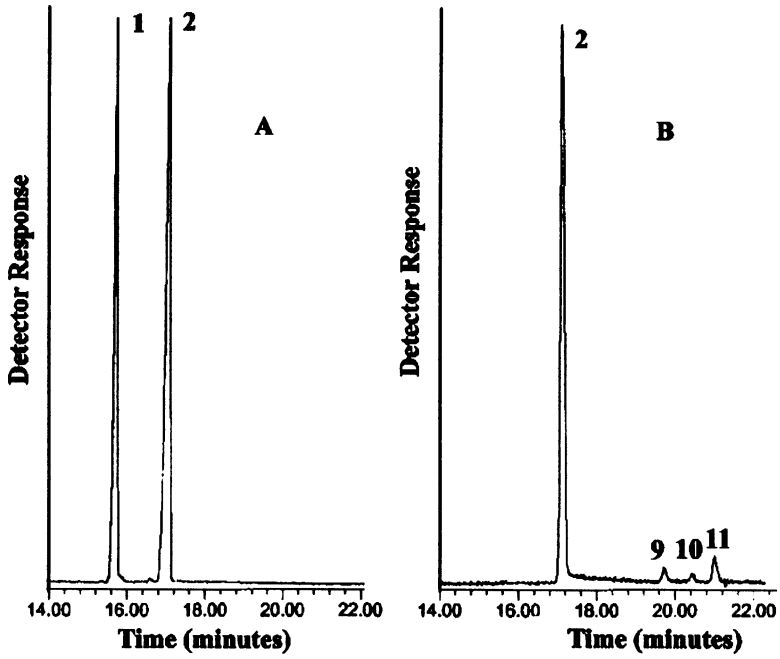


Figure 2. GC trace of (A) cycloartenol incubation with recombinant SMT and (B) 24(28)-methylenecycloartanol incubation with recombinant SMT. Peaks are as follow: 1, cycloartenol; 2, 24(28)-methylenecycloartanol; 9, 24β-ethyl-25(27)-dehydrocycloartanol; 10, 24(28)*E*-24-ethylidene cycloartanol and; 11, 24(28)*Z*-24-ethylidene cycloartanol.

0.556 and 0.333 (H-19, d, 4.2=*endo*, d, 4.2=*exo*), 0.898 (H-21, d, 7.0), 1.568 (H-26, s), 0.804 (H-29, t), 0.984 (H-30, s), 3.126 (H-3, dd, 11.7, 4.4), 4.731 and 4.647 (H-27, s, s); **10**, GLC (RRT_c, 2.43); MS (*m*/*z* 454 (M^+), 439, 421, 393, 355, 341, 323); ^1H NMR, 0.538 (H-18, s), 0.556 and 0.333 (H-19, d, 4.2=*endo*, d, 4.2=*exo*), 0.896 (H-21, d, 6.4), 2.358 (H-25, sept, 7.0), 0.984 (6Hs, H-26, H-27, d, 6.6), 1.570 (H-29, d, 6.7), 0.984 (H-30, s), 0.811 (H-31, s), 0.901 (H-32, s), 3.126 (H-3, m), 5.185 (H-25, q, 6.7,), 5.181 (H-28, q, 6.8; **11**, GLC (RRT_c, 2.47); MS (*m*/*z* 454 (M^+), 439, 421, 393, 355, 341, 323); ^1H NMR, 0.538 (H-18, s), 0.556 and 0.333 (H-19, d, 4.2=*endo*, d, 4.2=*exo*), 896 (H-21, d, 6.4), 2.834 (H-25, sept, 7.0), 0.984 (6Hs, H-26, H-27, d, 6.6), 1.584 (H-29, d, 6.7), 0.984 (H-30, s), 0.811 (H-31, s), 0.901 (H-32, s), 3.126 (H-3, m); 5.110 (H-28, q, 6.8). The stereochemistry at C-24 as β-oriented in **9** is demonstrated by the signal for C-29 [7e,12] and the *E*/*Z*-geometry at C-24 for **10** and **11** is demonstrated by the signals for C-25, C-28 and C-29.[13] Full ^1H and ^{13}C NMR assignments of cycloartenol are reported.[12c] From these data the second C_1-transfer in soybean sterol *C*-methylation may be concluded to occur from the *si*-face of the Δ^{24}-bond.

The stereochemistry of hydrogen migration from C-24 of cycloartenol to C-25 in the biosynthesis of 24(28)-methylene cycloartanol is problematic since the product of *C*-methylation is an exomethylene structure. Therefore, to unambiguously establish the product 25*R*-stereochemistry in **2** soybean SMT was incubated with [27-^{13}C]**1**. The product possessed the following chromatographic and spectral cycloartenol characteristics: GLC (RRTc, 1.87); MS (*m*/*z* 441 (M^+)), 426, 408, 380, 354, 301 (cyclopropane ring fragment); ^1H NMR δ ppm, 0.968 (H-18, s), 0.556 and 0.333 (H-19, d, 4.2=*endo*, d, 4.2=*exo*), 0.899 (H-21, d, 6.4), 1.024 (H-26, dd, 5.2, 6.8), 1.017 (H-27, dd, 1.25.5, 6.8), 0.968 (H-30, s), 0.811 (H-31, s), 0.901 (H-32, s), 3.126 (H-3, m), 4.716, 4.665 (H-28, d, 1.1; d, 1.1); ^{13}C NMR (terminal side chain carbons) 125 MHz δ ppm C-24 (156.94), C-25 (33.65), C-26 (21.86), C-27 (21.99), C-28 (105.90). As shown in Fig. 3, the signal enhanced in the ^{13}C NMR spectrum of enzyme-generated ^{13}C-labeled **2** corresponds to C-27 and reveals this carbon atom contains the pro-*R* methyl group.[7d]

Based on steady-state kinetic analysis, 24(28)-methylenecycloartanol (K_{mapp}=12 μM/V_{maxapp}=14 pmol/min/mg) is a worse substrate to cycloartenol (K_{mapp}=20 μM/V_{maxapp}=243 pmol/min/mg) for soybean SMT catalysis. As much as 10 μM cycloartenol added to the incubation containing 24(28)-methylenecycloartanol prevents 24-ethyl sterol product formation,

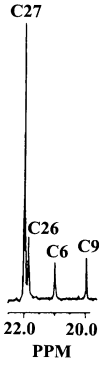
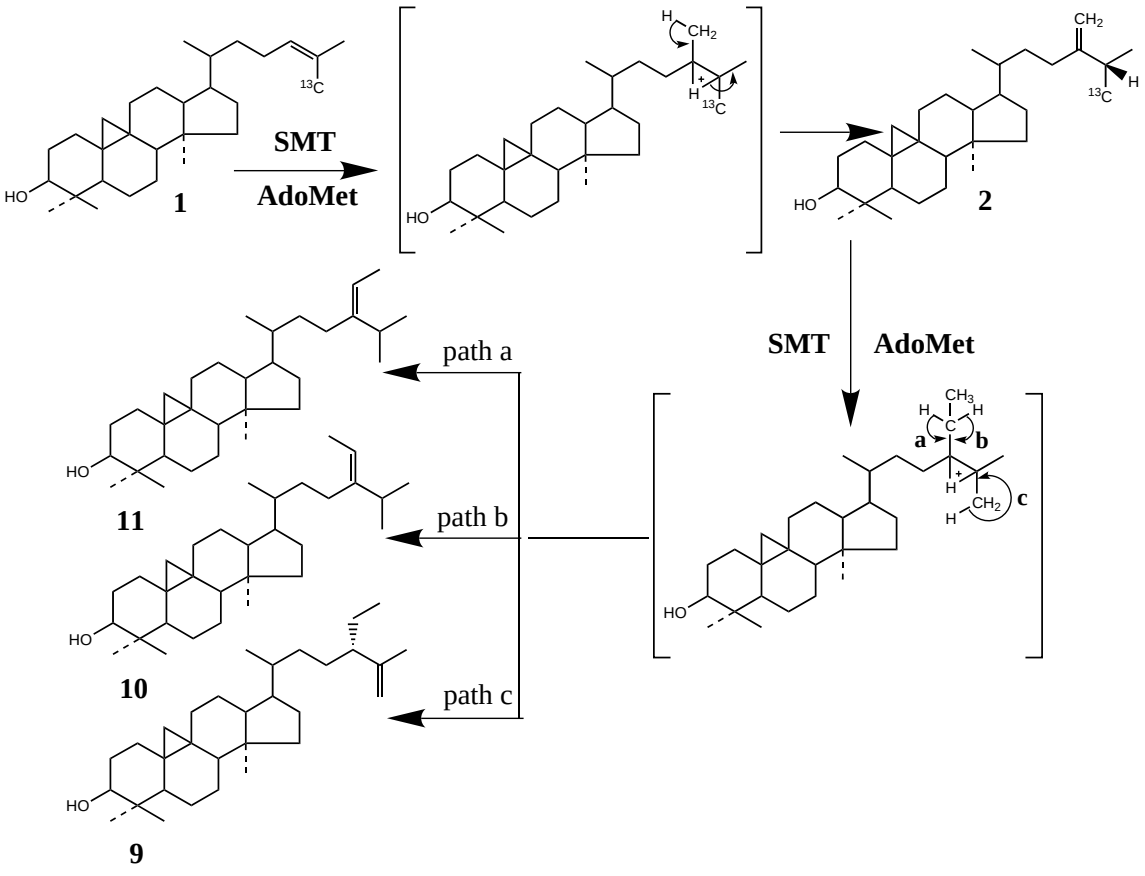


Figure 3. Partial ^{13}C NMR spectrum of (25*R*)-[27-^{13}C] 24(28)methylenecycloartanol recovered from soybean SMT incubated with [27-^{13}C].



Scheme 2. *C*-Methylation pathway in phytosterol synthesis.

suggesting that under normal physiological conditions the second C_1-transfer is limited. Recently, we characterized the kinetic parameters of a soybean SMT obtained from shoots which binds 24(28)-methylenelophenol preferentially to cycloartenol and 24(28)-methylenecycloartanol was not a substrate for the enzyme.[3a] In addition, soybean plants apparently do not normally synthesize $\Delta^{24(28)}(E)$ or 24β-ethyl $\Delta^{25(27)}$-sterol side chains.[14] These findings suggest binding order of substrate can contribute to product diversity and that at least two SMT isoforms exist in soybean to control substrate flux to sitosterol (24α-ethyl cholesterol).

The general picture which emerges from these and related studies is one in which the pro-*Z* methyl group on cycloartenol corresponding to C-27 is transformed into the pro-*R*-methyl group at C-25 on 24(28)-methylenecycloartanol by migration of the hydrogen atom at C-24 to C-25 from the *re*-face of the substrate double bond of the acceptor molecule (Scheme 2). The first C_1-transfer reaction is proposed to proceed via a non-covalent pathway whereby methyl addition to the *si* face of Δ^{24}-and deprotonation of C-28 gives rise to a nucleophilic rearrangement in which H-24 migrates to C-25 on the opposite face of the substrate double bond in concert with the initial ionization, consistent with yeast SMT action.[3a,7a] In the case of the second C_1-transfer reaction, the *C*-methylation reaction can also proceed from the *si*-face of the Δ^{24}-bond. The formation of three olefins during the second C_1-transfer reaction is presumably made possible by the relaxed control over substrate and intermediate conformations in the active site as a result of the increased steric bulk derived from the 24-ethyl sterol intermediate. Notably, the deprotonations leading to the formation of **9**, **10**, and **11** involve substrate channeling along different reaction coordinates (Scheme 2) suggesting the second C_1-transfer reaction is not necessarily concerted.

Acknowledgements

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