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Stereochemistry of Hydrogen Migration from C-24 to C-25 during Biomethylation in Ergosterol Biosynthesis

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Abstract: [*methyl-*$^{2}H_3$]Methionine and zymosterol, [27-^{13}C]lanosterol, [24-^{2}H]lanosterol and lanosterol were separately incubated with the sterol auxotroph *Saccharomyces cerevisiae* strain GL7. Spectral evidence (^{1}H and ^{13}C-NMR) obtained on three different isotopically labeled ergosterol samples indicated that C-28 was derived from AdoMet, H-24 migrated to C-25 and the C-25 hydrogen on (27-)-methyl ^{13}C-labeled ergosterol was introduced from the *Re* -face to produce the 25R-stereochemistry.

The (S)-adenosyl-L-methionine:$\Delta^{24(25)}$-sterol methyl transferase (SMT: EC: 2.1.1.41) synthesized by *Saccharomyces cerevisiae* catalyzes the transfer of the methyl group from AdoMet to the 24,25-double bond of the sterol side chain (Fig.1). [1,2] The mechanism of sterol biomethylation involves a 1,2-hydride shift of H-24 to C-25. The steric course of hydrogen migration may proceed in two ways which lead to opposite configurations at C-25: in case (A) the pro-R methyl group on lanosterol becomes the isopropyl pro-R methyl group in the ergosterol side chain, whereas in case (B) the same methyl group assumes the pro-S position. Arigoni[2] and Seo et al.,[3] investigated the stereochemistry of the migration of the hydrogen atom from C-24 to C-25 by examining the ^{13}C-NMR spectra of ^{13}C-isotopically labeled ergosterol samples formed in *Claviceps paspali* and *Saccharomyces cerevisiae*, respectively. Arigoni prepared an authentic specimen of (27-) methyl ^{13}C-labeled ergosterol and used the NMR spectra on this sample (which was not published) to reference and locate the chemical shift for C-26 and C-27 in the biosynthetically formed (26-) methyl ^{13}C-labeled ergosterol. The ^{13}C-ergosterol was biosynthesized from fungal conversion of [2-^{13}C]mevalolactone to lanosterol, which hypothetically possessed the C-26 (E-methyl group) isotopically labeled with ^{13}C. On the basis of NMR analysis, Arigoni concluded the pro-E methyl group on lanosterol becomes the pro-S methyl group on ergosterol, which results in the C-25 S-configuration in ergosterol (Fig. 1).

When Seo et al.,[3] repeated the mechanistic studies in *S. cerevisiae* using [$^{13}C^{2}H_3$] acetate as substrate for ergosterol production, they reported the ^{13}C-NMR signals for isotopically C-26 and C-27 labeled ergosterol were located in positions δ 19.89 and 19.61 ppm, respectively, which agreed with the NMR interpretation of others,[4,5] that C-26 resonated downfield from C-27 in 24-alkyl sterols. The ^{13}C-enhanced signals in the reported spectrum of ergosterol were not obvious, nevertheless, Seo et al, claimed to confirm the Arigoni proposal regarding the mechanism of fungal biomethylation. Following this seminal work, the characterization of several new ^{13}C-isotopically labeled sterols caused the assignments for C-26 and C-27 signals of 24-alkyl sterols to be revised.[6]

The new information clearly demonstrated that in 24-alkyl sterol epimers the signals for C-26 and C-27 should be ***reversed*** from their original location to- in nonlabeled ergosterol:^{1}H-NMR, δ 0.821 (d) and 0.836(d) ppm, and ^{13}C-NMR, δ 19.69 (s) and 20.02 (s) ppm, respectively. In view of the conflicting data and the possibility that *C. paspali* and *S. cerevisiae* might operate different biomethylation pathways, we re-examined the ability of *S. cerevisiae* to produce stereospecifically ^{13}C-labeled ergosterol. Our results unambiguously show that the pro-Z methyl (C-27) on lanosterol becomes the pro-R methyl (C-27) on ergosterol, thereby producing the 25R-stereochemistry. This result clearly demonstrates that the 1,2-hydrogen migration in ergosterol biosynthesis operates via a *Re*-face mechanism and is the same as the mechanism operating in *C. paspali.*

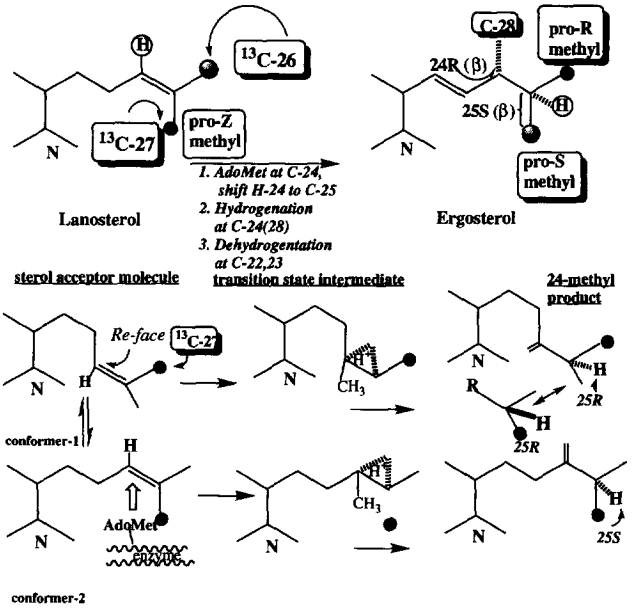

Figure 1. Sterol biomethylation reaction

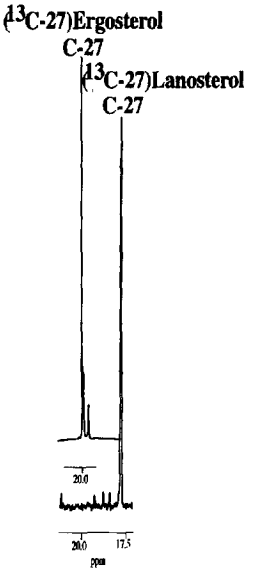

Figure 2. Partial ^{13}C-NMR of sterols

The compounds were prepared and purified by HPLC as described,[7] and incubated with *S. cerevisiae* strain GL7 (a sterol auxotroph) according to established culturing methods.[8] Four separate incubations were performed with each test sterol distributed into ca. 1 liter of medium: (i) 5 mg/L lanosterol, (ii) 5 mg/L zymosterol and 625 mg/L [$^{2}H_{3}$-*methyl*]methionine (which is converted to [*methyl*-$^{2}H_{3}$] AdoMet by GL7), (iii) 5 mg/L [24-^{2}H]lanosterol; and (iv) 5 mg/L of [27-^{13}C]-lanosterol. The major sterol (ca. 1 mg, representing 90% of the cellular sterol) recovered from the cells was isotopically-labeled ergosterol. Figure 3 shows the 300 MHz ^{1}H-NMR spectra of the four ergosterol samples recovered from HPLC: spectrum A represents native ergosterol (control spectrum) and its spectrum is an identical match with that of other spectra we obtained on ergosterol isolated from related fungi and plants.[9] The intensity of the doublet for C-28 at δ 0.912 originating from the methyl on [*methyl*-$^{2}H_{3}$]AdoMet (derived from [*methyl*- $^{2}H_{3}$]methionine) is reduced by addition of the two deuterium atoms (B, Fig. 3) , and the doublets for C-26 and C-27 resonating at δ 0. 82 and 0.84 collapsed into singlets after the hydrogen at C-25 is replaced with a deuterium (C, Fig.3). Additionally, the ^{13}C-NMR of the

[25-^{2}H]ergosterol was similar to the control sprectrum,[3,4] except the resonance for C-25 was absent (data not shown). The latter results confirm that AdoMet attacks C-24 and the hydrogen originally at C-24 migrates to C-25. The signal for C-27 in [27-^{13}C]ergosterol (D, Fig. 3) was characterized by the pair of doublets with a coupling constant J=250 Hz (^{1}H/^{13}C). This result shows the doublet resonating at δ 0.84 in the control ^{1}H-NMR spectrum may be assigned C-27 and the upfield doublet resonating at δ 0.82 may be assigned C-26. It can be seen from Figure 2 that the non-equivalence of the diastereotopic isopropyl group at C-27 in the two sterol samples was apparent in the proton decoupled ^{13}C-NMR; [27-^{13}C]ergosterol derived from incubation with [27-^{13}C]lanosterol possessed an intense signal at δ 19.925, which was shifted downfield from its position at δ 17.629 in the lanosterol spectrum.[10] By correlation of the NMR assignments for the absolute configuration of compounds in reference 6 with the positions of the resonances in the C-25 chiral isotopically-labeled ergosterol, we assigned the following absolute stereochemistry, (25-R)-[27-^{13}C]]ergosterol (IUPAC nomenclature: (25R)-[26-^{13}C]ergosterol)[11]. The spectroscopic results presented here suggest that conformer 1 (Fig. 1) plays a role in catalysis by orienting the side chain into a conformation which promotes *Re*-face migration of H-24 to C-25 following biomethylation of the 24,25-double bond at C-24.

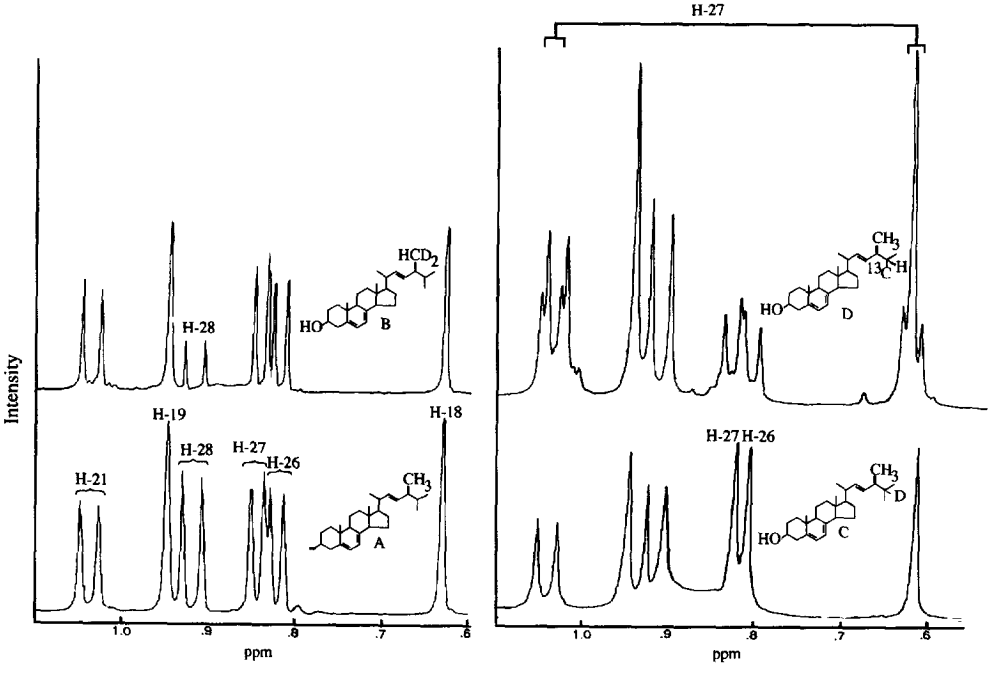

Figure 3. 300-MHz ^{1}H NMR spectra of:(A)ergosterol;(B)[28-^{2}H$_{2}$]ergosterol; (C) [25-^{2}H]ergosterol; (D) [27-^{13}C]ergosterol.

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References and Notes

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7. Isotopically labeled sterols were prepared by the methods of Raab, K.H.; DeSouza, N.J.; Nes, W.R. *Biochim. Biophys. Acta* **1968**, *152*, 742-748; Yagi, T.; Kobayashi, N.; Morisaki, M.; Hara, N.; Fujimoto, Y. *Chem. Pharm. Bull.* (Japan) **1994** , *42*, 680-682. Sterols were purified by TLC and HPLC as described by Guo, D.; Venkatramesh, M.; Nes, W.D. *Lipids* **1995**, *30*, 203-219. The chromatographic (TLC, GLC and HPLC) and spectral (^{1}H and ^{13}C-NMR) properties of [24-^{2}H]lanosterol and [27-^{13}C]lanosterol were consistent with the assigned structures. [*methyl-$^2H_3$*] Methionine was purchased from MSD Isotopes.The sample of [27-^{13}C]lanosterol contained ca. 8% of [26-^{13}C]lanosterol, as evidenced in the [27-^{13}C]lanosterol ^{13}C-NMR spectrum at δ 25.719 (data not shown), and in the [27-^{13}C]ergosterol ^{13}C-NMR spectrum shown in Figure 2, resonating at δ 19.616.
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10. The ergosterol samples possessed similar UV, TLC, GLC, and HPLC properties, cf. ref. 9 above. The partial mass spectrum of the ergosterol showed diagnostic ions of M^+ 396, 363, 337, 271 amu ; [25-^{2}H]ergosterol of M^+ 397,365,339, 271; [28-$^2H_2$]ergosterol of M^+ 398,364,338 amu and [27-^{13}C]ergosterol of M^+ 397, 364, 338, 271 amu.
11. We prefer to use the side chain nomenclature introduced by Popják and Nes (6a and 1e) for designating carbon positions, rather than using the revised 1989 IUPAC system. Hence, C-27 in lanosterol is derived from C-6 mevalonic acid and C-26 is derived from C-2 mevalonic acid. The numbering of the carbon atoms will not change following metabolic-induced saturation of the 24,25-double bond. When using the IUPAC rules, the apparent inversion of configuration at C-25 in [26/27-^{13}C]ergosterol produced from [26-^{13}C]lanosterol and [27-^{13}C]lanosterol is not real and is derived from conventions in nomenclature.

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