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# Mechanism of the double bond isomerization from $\Delta^{24(28)}$ to $\Delta^{24(25)}$ in sitosterol biosynthesis in higher plants

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**Abstract**

Biosynthesis of sitosterol in higher plants involves the double bond isomerization of isofucosterol to 24-ethyldesmosterol followed by reduction of the latter. The mechanism of the double bond isomerization from $\Delta^{24(28)}$ to $\Delta^{24(25)}$ was investigated by feeding [28-$^{13}$C,28-$^{2}$H] doubly labeled isofucosterol to cell cultures of *Oryza sativa* and $^{2}$H-decoupled $^{13}$C, $^{1}$H shift correlation NMR analysis of the biosynthesized sitosterol. The $^{2}$H-decoupled HMQC spectrum revealed that the deuterium of isofucosterol stereospecifically becomes pro-*R*-$^{2}$H at C-28 of sitosterol, thus establishing that the double bond migration takes place in a *syn*-$S_E2'$ manner wherein the pro-*S*-hydrogen at C-28 of sitosterol is introduced from the 28-*Si* face of isofucosterol. 

*Keywords:* steroids; sterol; biosynthesis; NMR; stereochemistry.

In the biosynthesis of the side-chain of sitosterol (**3**), one of the most common sterols in higher plants, a 24(28)-olefinic sterol such as isofucosterol (**1**) is first isomerized to a 24(25)-olefinic sterol such as 24-ethyldesmosterol (**2**), which is then reduced to **3** (Scheme 1).[1] We have recently demonstrated that **2** is converted to **3** with cell cultures of *Oryza sativa*[2] and the reduction proceeds with *anti*-addition of hydrogen atoms which are introduced from the 24-*Si* and 25-*Re* faces.[3] More recently, it has been found that the pro-*S* and pro-*R* methyl groups on C-25 of **1** become pro-*S* and pro-*R* methyl groups, respectively, of **3** with the same cultures.[4] Taken together, the pro-*S*-methyl of **1**, the (*E*)-methyl of **2** and the pro-*S*-methyl of **3** should be derived from the same origin, i.e. C-2 of mevalonate (indicated by black circles in Scheme 1). The findings are in good agreement with the reported metabolic origins of C-26 and C-27 of isofucosterol and sitosterol in higher plants.[5] Under these circumstances, the step of the double bond isomerization from $\Delta^{24(28)}$ to $\Delta^{24(25)}$ would follow either one of two mechanisms, i.e. *anti*-$S_E2'$ or *syn*-$S_E2'$ wherein the newly introduced hydrogen (Hb) at C-28 of **2** and **3** comes from the same or opposite direction of the departing hydrogen (Hc). In theory, the two mechanisms would be distinguishable if there was a useful tool for differentiating the two hydrogens, Ha and Hb. However, it

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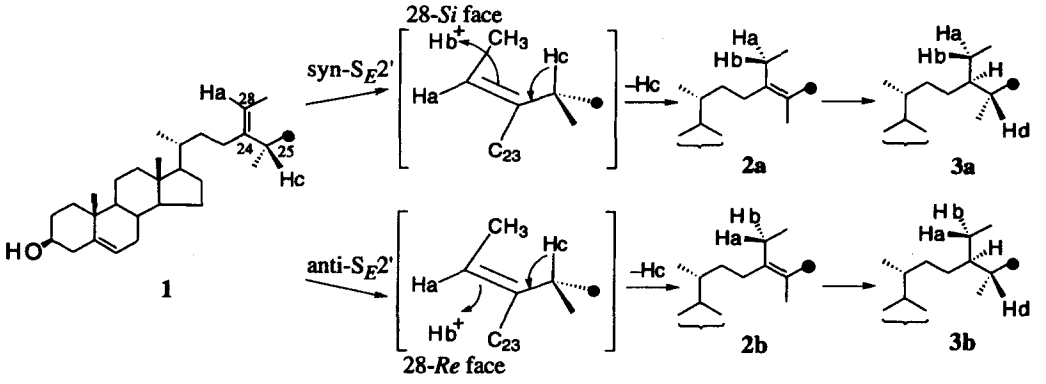
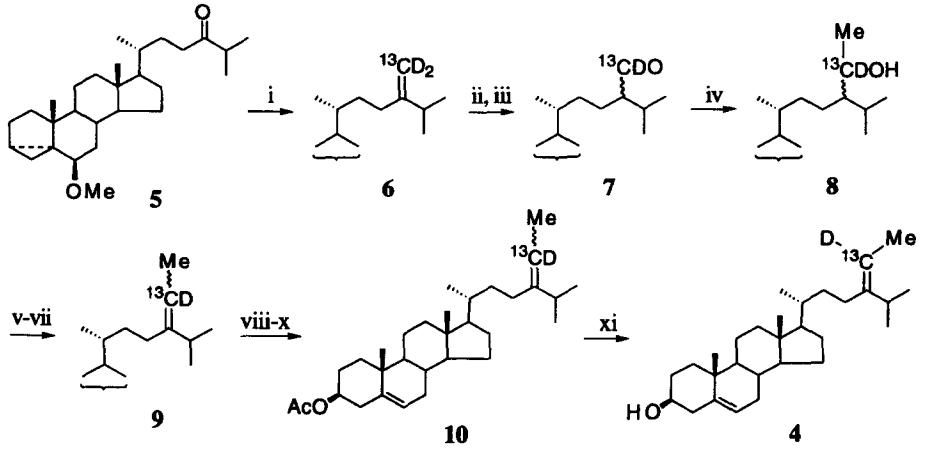
appeared difficult to differentiate the two hydrogens by routine approaches using a stable or radioisotope tracer method. For such a purpose it has been reported that $^{2}H$-decoupled $^{13}C$, $^{1}H$ shift correlation NMR spectroscopy is a powerful technique.[6] We decided to use this technique in combination with the feeding of a chemically synthesized $^{13}C$, $^{2}H$ doubly labeled substrate. The double label seemed essential for obtaining a reasonable S/N ratio in the NMR analysis of the resulting product.




Scheme 1. Two possible steric courses of the double bond migration in sitosterol biosynthesis

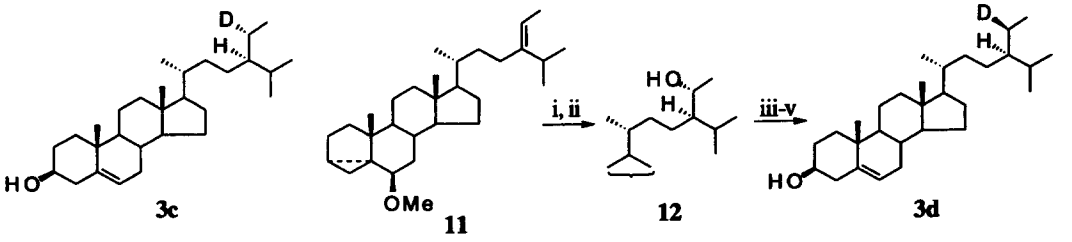
The requisite [28-$^{13}C$,28-$^{2}H$]isofucosterol **4** was synthesized according to Scheme 2. Wittig methylenation of the 6β-methoxy-3α,5-cyclo-5α-cholestan-24-one **5** with an ylide prepared from $^{13}CD_3I$ gave the exomethylene **6**. Hydroboration–oxidation of **6** and Dess–Martin oxidation of the resulting primary alcohol afforded the aldehyde **7**. Methyl Grignard addition to the aldehyde and dehydration of the diastereoisomeric C-28 alcohol **8** afforded a mixture of (*E*)- and (*Z*)-olefin **9** (*E*:*Z* 4:1). The ratio *Z*:*E* was increased to *Z*:*E* (7:3) by the subsequent hydroboration–oxidation and dehydration sequence. The (*Z*)-enriched olefin **9** was converted into the 3-acetate derivative via the 3-alcohol to facilitate the separation of the geometric isomer. Separation of the acetate by *p*-TLC using $AgNO_3$-impregnated silica gel afforded the pure (*Z*)-olefin **10**. Deacetylation of this olefin furnished the desired doubly labeled isofucosterol **4**.




Scheme 2. Synthesis of [28-$^{13}C$,28-$^{2}H$]isofucosterol **4**. Reagents: (i) $Ph_3P^{13}CD_3I$, *n*-BuLi (ii) $BH_3$–THF, then NaOH, $H_2O_2$ (iii) Dess–Martin ox. (iv) MeMgI (v) $POCl_3$, Py (vi) $BH_3$–THF, then NaOH, $H_2O_2$ (vii) $POCl_3$, Py (viii) *p*-TsOH, aq. dioxane (ix) $Ac_2O$, Py (x) separation from (*E*)-isomer on $AgNO_3$–silica gel (xi) $LiAlH_4$

In order to aid unequivocal assignment of the chemical shifts of the pro-*S* and pro-*R*-hydrogens at C-28 of **3**, stereochemically defined [28-pro-*R*-$^{2}H$]- (**3c**) and [28-pro-*S*-$^{2}H$]- (**3d**) sitosterols were

synthesized (Scheme 3). The synthesis of **3c** was carried out by modification of the published method,[7] using $NaBD_4$ in place of $NaBH_4$. Application of the same method to the [24(28)*Z*]-olefinic isomer **11** afforded (24*R*,28*R*)-28-alcohol **12** and its diastereoisomer (24*S*,28*S*)-28-alcohol, the stereochemistry of which was established by application of the advanced Mosher method.[8] The (24*R*,28*R*)-alcohol **12** was separated from the diastereoisomer by silica gel Lobar column and converted to **3d** in three steps.




Scheme 3. Structures of [28-pro-*R*-$^2$H]-sitosterol **3c** and [28-pro-*S*-$^2$H]-sitosterol **3d**, and synthesis of **3d**. Reagents: (i) $BH_3$–THF, then NaOH, $H_2O_2$ (ii) separation of the isomer (iii) MsCl, Py (iv) $NaBD_4$, HMPA (v) *p*-TsOH, aq. dioxane

The labeled isofucosterol **4** (90 mg) was fed to the cell cultures (six 500 ml flasks each containing 250 ml of N6 medium) of *O. sativa* as described previously,[2] and extraction and purification of the sterol fraction furnished 15 mg of sitosterol.

Fig. 1 illustrates the $^2$H-decoupled HMQC spectra of the sitosterol samples. In the spectrum (D) of non-labeled sitosterol, a broad cross-peak appeared between the C-28 (δ 23.11) and 28-methylene protons (δ ca. 1.2–1.3). In the $^2$H-decoupled HMQC spectrum (B) of [28-pro-*R*-$^2$H]-sitosterol **3c**, a cross-peak appeared between deuterium isotope shifted C-28 (22.72 ppm, $^1\Delta\delta_C$=–0.39) and β-deuterium isotope shifted pro-*S* hydrogen at C-28 (δ 1.26), while [28-pro-*S*-$^2$H]-sitosterol **3d** displayed a cross-peak between deuterium isotope shifted C-28 (22.71 ppm, $^1\Delta\delta_C$=–0.40) and β-deuterium isotope shifted

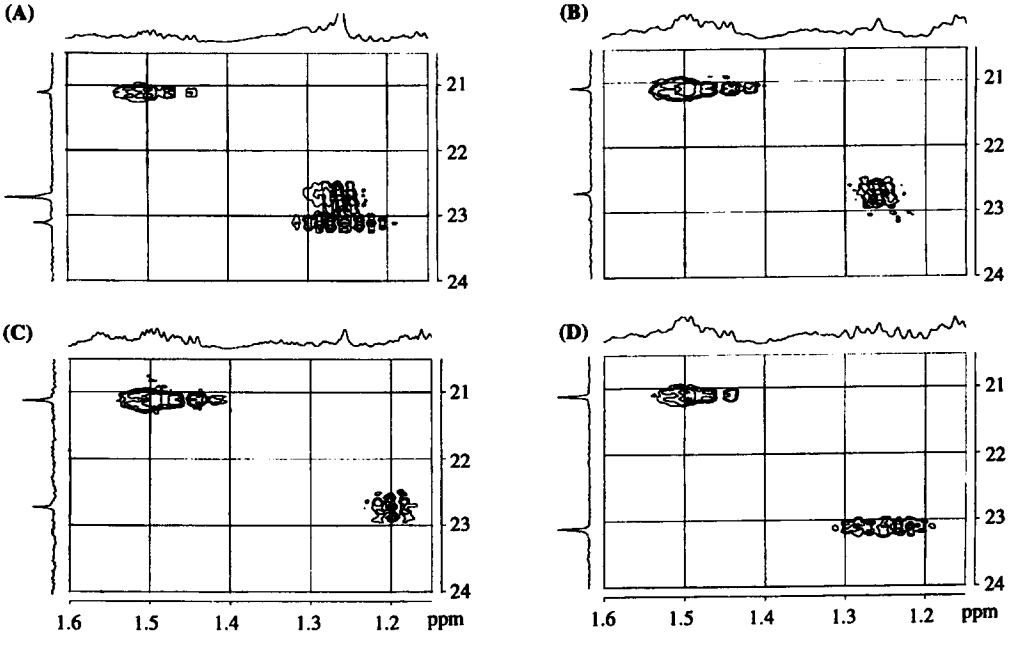



Figure 1. $^2$H-decoupled HMQC spectra (500 MHz for $^1$H/125 MHz for $^{13}$C, in $CDCl_3$) of sitosterol biosynthesized from **4** (A), [28-pro-*R*-$^2$H]-sitosterol **3c** (B), [28-pro-*S*-$^2$H]-sitosterol **3d** (C), non-labeled sitosterol (D)

pro-*R* hydrogen (δ 1.20). The spectra of **3c** and **3d** allowed definitive stereochemical assignment that the pro-*R* and pro-*S*-hydrogens at C-28 are upfield and downfield, respectively. The spectrum (A) of the biosynthesized sitosterol displayed a cross-peak between deuterium isotope shifted C-28 and hydrogen at δ 1.26, accompanied with a cross-peak due to the non-labeled sample. The position δ 1.26 is superimposable over that of the spectrum (B). This indicated that the deuterium atom arising from the labeled substrate occupies the pro-*R* position at C-28 and the newly introduced hydrogen (Hb in Scheme 1) is located at the pro-*S* position. Thus, it is concluded that the double bond isomerization reaction proceeds in a *syn*-$S_E2'$ mechanism wherein a hydrogen is introduced from the 28-*Si* face. The spectrum (A) also indicated that the isomerization reaction is highly stereospecific.

The present study has established the mechanism of the double bond isomerization of isofucosterol into 24-ethyldesmosterol in the cell cultures of *O. sativa* by the successful monitoring of a small difference in the chemical shifts between the pro-*R* and pro-*S* hydrogens at C-28 of **3** using $^2$H-decoupling HMQC NMR technique combined with the feeding of synthetic $^2$H, $^{13}$C doubly labeled substrate.

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