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Mechanism of the second methylation in sitosterol side-chain biosynthesis in higher plants: metabolic fate of 28-hydrogens of 24-methylenecholesterol in *Morus alba* cell cultures

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Abstract

Biosynthesis of the side-chain of sitosterol in higher plants involves two methylation steps by attack of *S*-adenosylmethionine. The stereochemical features of the second methylation, namely, of the conversion from 24-methylenecholesterol to isofucosterol in higher plants has been investigated. Feeding studies of synthesized [28*E*-^{2}H]- and [28*Z*-^{2}H]-24-methylenecholesterols to cultured cells of *Morus alba* followed by ^{2}H NMR analysis of the resulting isofucosterol established that the second methylation proceeded in such a manner that addition of the methyl group and proton loss occur on opposite faces of the original $\Delta^{24(28)}$-double bond.

Keywords: steroids and sterols; biosynthesis; NMR; stereochemistry.

In the biosynthesis of the side-chain of sitosterol (**4**), one of the most popular sterols in higher plants, 24-methylenecholesterol (**1**) which is produced by the first methylation of 24(25)-olefinic sterol, is subjected to a second methylation leading to isofucosterol (**2**). Isofucosterol is isomerized to 24-ethyldesmosterol (**3**) which is then reduced to yield **4** (Scheme 1).[1] Concerning the stereochemical features of this transformation, we have recently reported that the reduction of **3** to **4** takes place with an *anti*-addition of hydrogen atoms from the 24-*Si* and 25-*Re* face in cell cultures of *Oryza sativa*.[2] It has also been demonstrated that isomerization of **2** to **3** proceeds in a *syn*-S_E2' fashion in which the hydrogen newly introduced at C-28 of **2** comes from the 28-*Si* face in the same culture.[3] However, the mechanism of the second methylation in higher plants remains to be established, although Djerassi's group have reported the mechanism of the second methylation in a marine sponge which contains **2** and its $\Delta^{24(28)E}$-isomer, fucosterol (vide infra).[4]

Although the direction of transfer of the methyl group from *S*-adenosylmethionine (SAM) in higher plants has not been fully established, the methyl transfer is suggested to occur from the 28-*Si* face of

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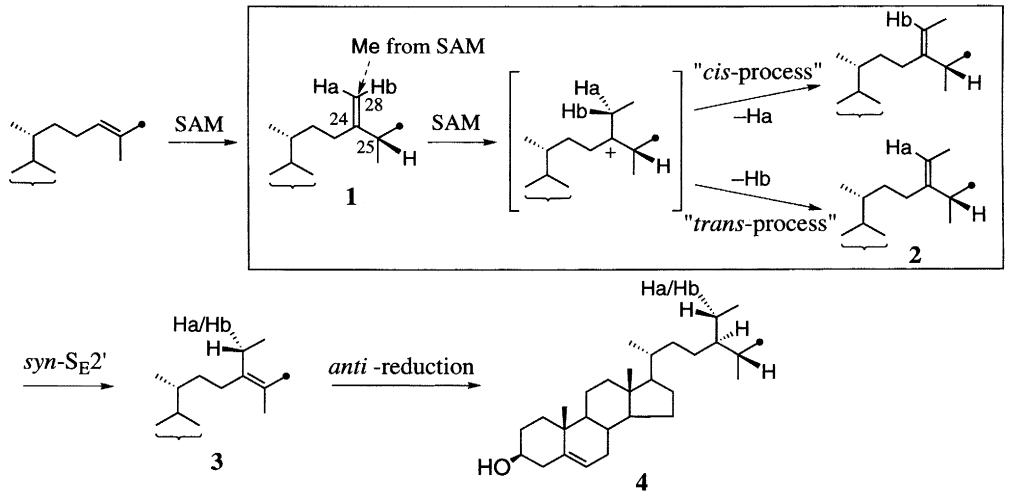
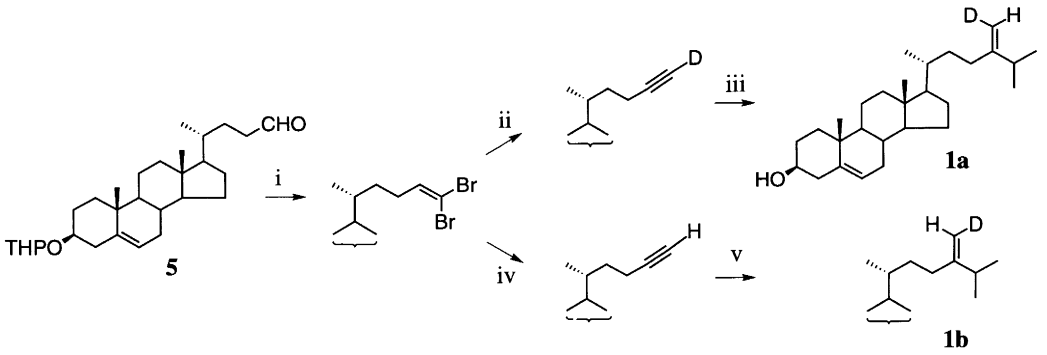


Scheme 1. Two possible steric courses of the second methylation in sitosterol side-chain biosynthesis. The present study has established that the Ha hydrogen is retained at the C-28 positions of compound **2**, **3** and **4**. The carbons derived from C-2 of mevalonate are indicated by black circles

$\Delta^{24(28)}$-double bond of **1** from the analogy of the first methylation[5] as well as from stereochemical consideration of clerosterol [(24*S*)-stigmasta-5,25-dien-3β-ol] biosynthesis.[6] For simplicity, this text including Scheme 1 is written on the assumption that this is correct. Thus, the purpose of this paper is to elucidate whether Ha (designated as a '*cis*-process', since addition of the methyl group and proton loss occur on the same face of the $\Delta^{24(28)}$-double bond) or Hb (designated as a '*trans*-process', since the two events occur on opposite faces) is eliminated. To differentiate the two mechanisms, [28*E*-^{2}H]- (**1a**) and [28*Z*-^{2}H]- (**1b**) 24-methylenecholesterols were synthesized and their feeding studies were carried out using cell cultures of *Morus alba*,[7] which was selected from several plant cell cultures since our preliminary studies indicated a labeled-**1** was most efficiently incorporated into **2**.



Scheme 2. Synthesis of [28*E*-^{2}H]- (**1a**) and [28*Z*-^{2}H]- (**1b**) 24-methylenecholesterols. Reagents: (i) Ph_3P, CBr_4, py, 80%; (ii) *n*-BuLi, then D_2O, 99%; (iii) *i*-$Pr_2CuMgCl$, then HCl, 66%; (iv) *n*-BuLi, then H_2O, 95%; (v) *i*-$Pr_2CuMgCl$, then DCl, D_2O, 73%

28*E*-^{2}H compound **1a** was synthesized from the known aldehyde **5** via deuterated acetylene in three steps (dibromoolefination,[8] elimination–deuteration, and carbocupration–deprotection) according to the published method (Scheme 2).[9] The synthesis of 28*Z*-^{2}H compound **1b** was also achieved in a similar three-step sequence, but introducing the deuterium atom by quenching the carbocupration reaction with D_2O/DCl. The ^{1}H NMR spectra (Fig. 1) of **1a** (δ 4.70 for 28*Z*-H), **1b** (δ 4.64 for 28*E*-H), and non-

labeled **1** (δ 4.71 and 4.65 for 28*Z*-H and 28*E*-H, respectively) clearly indicated that the deuterium label was regiospecifically introduced. The (*E*)-geometry for the $\Delta^{24(28)}$ of **1a** was confirmed by NOE studies in which irradiation of the C-26 or C-27 methyl signal caused enhancement of the signal intensity of 28-H, whereas analogous NOE studies of **1b** did not show such signal enhancement.

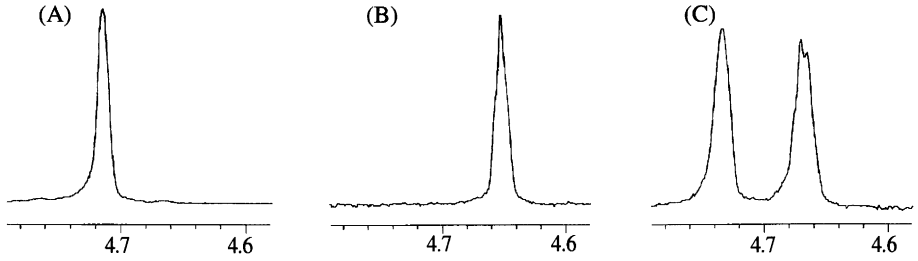


Fig. 1. Partial ^{1}H NMR spectra (300 MHz, in $CDCl_3$) of **1a** (A), **1b** (B), and non-labeled **1** (C)

Compounds **1a** and **1b** (50 mg each) were individually fed to cell cultures of *M. alba* (three 500 ml flasks containing 250 ml of Murashige–Skoog medium) which had been preincubated for 2 weeks, and the cultures were incubated for another 2 weeks. The sterol fraction was obtained by silica gel chromatography of the $CHCl_3$–MeOH extract of the cells. This was further separated by RP-HPLC to give a mixture of isofucosterol (**2**) and cholesterol.† The ^{2}H NMR spectrum (Fig. 2A) of the mixture

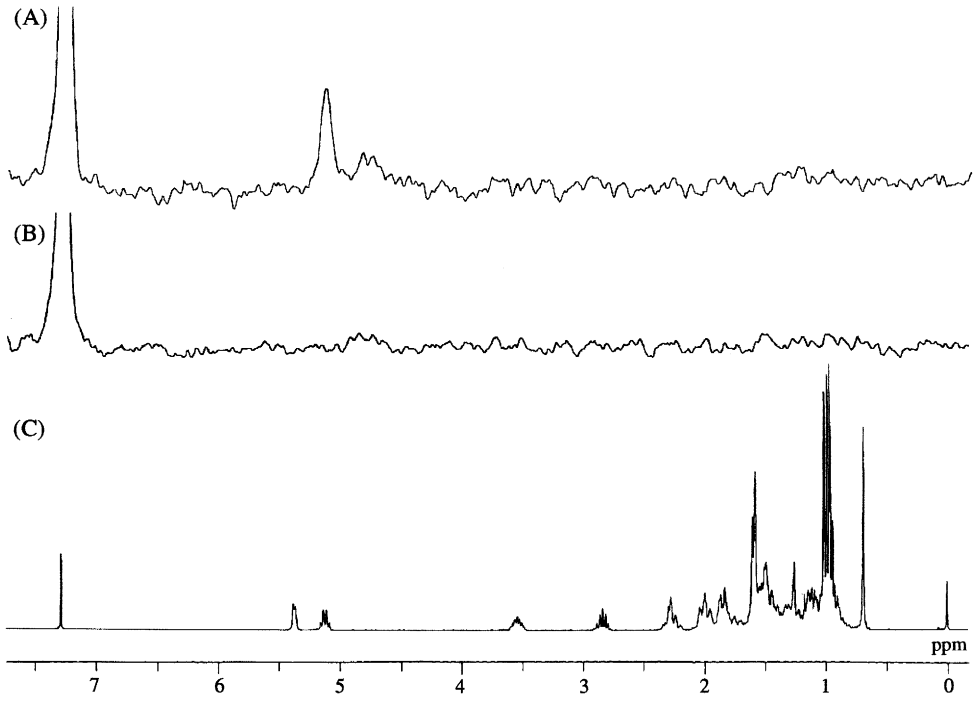


Fig. 2. ^{2}H NMR spectra (61 MHz, in $CHCl_3$) of **2** derived from **1a** (A), and from **1b** (B), and ^{1}H NMR spectrum (300 MHz, in $CHCl_3$) of authentic **2** (C). A weak signal around δ 4.70 is apparently due to 28*E*-^{2}H of the slightly contaminated substrate **1a**

† The cells grown without feeding external sterol contain sitosterol (81%), campesterol (4%), isofucosterol (8%) and cholesterol (7%).

derived from **1a** exhibited a deuterium signal at δ 5.13 due to 28-H of **2**. Complementary results were obtained from the incubation of **1b**, as shown in Fig. 1B in which no signal was observed at δ 5.13. The results unambiguously established that 28*E*-hydrogen (Ha) of 24-methylenecholesterol is retained while 28*Z*-hydrogen (Hb) is eliminated during the conversion of **1** to **2** in the cultured cells of *M. alba*. Thus, it can be concluded that the second methylation of **1** proceeded in the '*trans*-process'.

Furthermore, sitosterol **4** derived from **1a**, but not derived from **1b**, exhibited a signal at δ 1.23 which corresponds to a 28-hydrogen in the ^{2}H NMR spectrum (the pro-*R* and pro-*S* hydrogens of **4** resonate at δ 1.24 and 1.28, respectively[3]). This is compatible with the above findings that the Ha hydrogen is retained in the second methylation process. The observed ^{2}H signal in **4** is most likely to be assigned to 28-pro-*R* hydrogen in view of our finding that isomerization of **2** to **3** occurs in the S_N2' mechanism.[3]

It is interesting to compare the present results with those reported in the sponge, *Xestospongia testudinaria*.[4] In the case of the sponge, it was reported that the label of 28*E*-^{3}H-**1** was incorporated into isofucosterol, but not fucosterol, whereas the label of 28*Z*-^{3}H-**1** was incorporated into isofucosterol as well as fucosterol. The authors deduced from these results that a '*trans*-process' operates in a sponge and the tritium isotope effect seemed to be responsible for the anomalous behavior. In contrast to the complicated observations, our results in cell cultures of *M. alba* are very simple: feeding of **1a** and **1b** furnished isofucosterol, but not fucosterol, as evidenced by ^{1}H NMR analysis, and the elimination of Hb takes place in a highly stereospecific manner even when Hb is replaced with a deuterium atom.

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