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Mechanism of clerosterol biosynthesis in *Ajuga* hairy roots: stereochemistry of C-28 methylation of 24-methylene sterol

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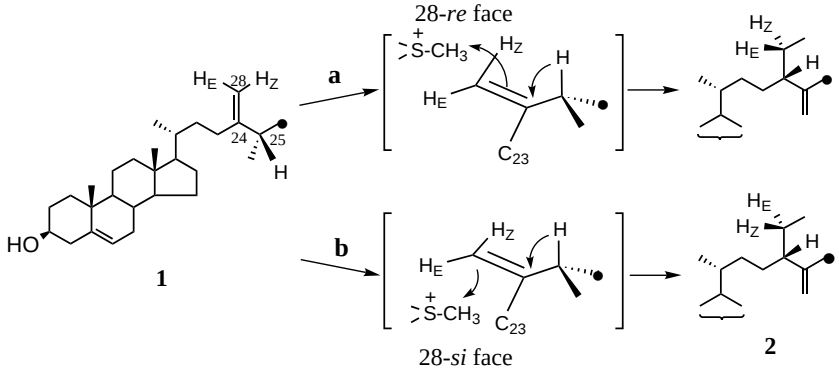
Abstract—Biosynthesis of clerosterol, (24*S*)-ethylcholesta-5,25-dien-3β-ol (**1**), involves transfer of the methyl group from *S*-adenosylmethionine to the C-28 position of a 24-methylene-sterol precursor. The resulting C-24 cationic species undergoes migration of hydrogen from C-25 to C-24, followed by deprotonation from C-26 to form **1**. We have now investigated the steric course of the methylation in hairy roots of *Ajuga reptans* var. *atropurpurea*. Feeding of [28*E*-^{2}H]- and [28*Z*-^{2}H]-24-methylenecholesterols and ^{2}H NMR analysis of clionasterol obtained by partial hydrogenation of the biosynthesized clerosterol have revealed that the methylation takes place from the 28-*si* face. © 2002 Published by Elsevier Science Ltd.

In plants, C_{29}-sterols are biosynthesized via methylation of C_{28}-sterols having a $\Delta^{24(28)}$-double bond. In most higher plants, a C-24 cationic intermediate arising from the methylation would be neutralized by elimination of C-28 hydrogen to form a C_{29}-$\Delta^{24(28)}$-sterol. We have recently reported that this methylation reaction proceeds in a '*trans*' mechanism, although the face of the transfer of the methyl group remains undefined.[1] In some families of plants and green algae, the C-24 cationic intermediate is neutralized by the migration of hydrogen from C-25 to C-24 with concomitant elimination of C-26 hydrogen to yield a 24β-ethyl-Δ^{25}-sterol such as clerosterol (**1**). The subsequent reduction of Δ^{25} yields a 24β-ethyl sterol such as 22-dihydrochondrillasterol in *Cucurbitaceae*[2] and clionasterol in algae.[3] *Ajuga* genus (*Laviatae*) is known to have clerosterol and 22-dehydroclerosterol.[4] We previously reported the mechanism of sterol side-chain biosynthesis in hairy roots of *Ajuga reptans* var. *atropurpurea*.[5] The C-24 hydrogen of desmosterol was shown to reside at the C-24 position of **1** in the hairy roots. Further, it was established that the isopropylidene (*E*) (derived from C-2 of mevalonate) and (*Z*) (derived from C-6 of mevalonate) methyl groups of desmosterol become stereospecifically olefinic methyl and exomethylene carbons, respectively, via (pro-*S*)- and (pro-*R*)-methyls at



Scheme 1. Two possible steric courses of the methylation of 24-methylene-sterol. ● designates carbon atoms derived from C-2 of mevalomate.

Keywords: steroids and sterols; clerosterol; clionasterol; biosynthesis; ^{2}H NMR; stereochemistry.

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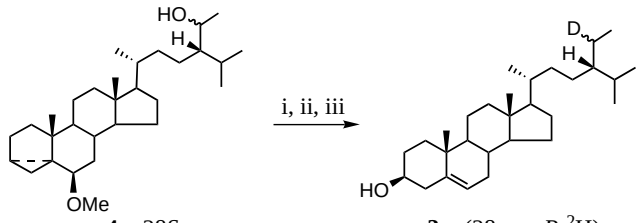
C-25 of 24-methylenecholesterol (**2**). This implies that migration of 25-H has to occur from the 24-*re*-face of the $\Delta^{24(28)}$-double bond, as depicted in Scheme 1.[6] We have now elucidated the steric course (path a or b in Scheme 1) of the transfer of the methyl group from *S*-adenosylmethionine.

To elucidate the steric course in question, it is essential to differentiate the C-28 methylene hydrogens of **1**. In our previous work, the differentiation of the C-28 methylene hydrogens of sitosterol (C-24 epimer of clionasterol) was uniquely achieved.[7] Thus, we initially focused on assigning the C-28 methylene hydrogens of clionasterol (**3**) instead of **2**, since **2** could be readily converted into **3** by selective hydrogenation.[8] The methylene protons of **3** were found to be much more diagnostic from each other in the ^{1}H NMR spectrum than those (δ 1.24 for pro-*R*, δ 1.28 for pro-*S*) of sitosterol, as evidenced by the HMQC spectrum of **3** (ca. δ 1.11 and 1.31). In order to assign the ^{1}H chemical shifts of the methylene hydrogens, [28-pro-*R*-^{2}H]- and [28-pro-*S*-^{2}H]-clionasterols were prepared as shown in Scheme 2.

The known (24*S*,28*S*)-6β-methoxy-3,5-*cyclo*-5α-stigmastan-28-ol (**4a**)[7,9] was converted into mesylate which was then reduced by Super-deuteride to give [28-pro-*R*-^{2}H]-cycloether. The use of Super-deuteride was found to be superior to $NaBD_4$, since the formation of olefinic byproducts was much reduced.[7] The ether was converted to [28-pro-*R*-^{2}H]-clionasterol (**3a**)[10] in a standard acidic treatment. Starting from the epimeric (28*R*)-28-ol (**4b**),[7,11] there obtained [28-pro-*S*-^{2}H]-clionasterol (**3b**).[12] The ^{2}H NMR spectra of these reference samples showed signals at δ 1.32 for **3a** and 1.14 for **3b**, as illustrated in Fig. 1.



4a: 28*S* **4b**: 28*R* **3a**: (28-pro-*R*-^{2}H) **3b**: (28-pro-*S*-^{2}H)

Scheme 2. Synthesis of [28-pro-*R*-^{2}H]- and [28-pro-*S*-^{2}H]-clionasterols (**3a** and **3b**). *Reagents and conditions*: (i) MsCl, Py; (ii) Super-deuteride, THF; *m*CPBA (separation of olefinic byproducts as epoxides), 65–70% for two steps; (iii) *p*-TsOH, aq. dioxane, 85%.

Hence, pro-*R* and pro-*S* hydrogens at C-28 of **3** were unequivocally assigned to the signals of δ 1.31 and 1.11, respectively.

The requisite [28*E*-^{2}H]- (**1a**) and [28*Z*-^{2}H]- (**1b**) 24-methylenecholesterols were synthesized as described previously.[1] Compound **1a** (32 mg) was fed to *Ajuga* hairy roots (four 500 ml flasks each containing 250 ml of MS medium) as described previously.[5] Extraction and purification of the sterol fraction furnished 1.6 mg of clerosterol. This was hydrogenated in the presence of Pt/C to give clionasterol in good yield. Compound **1b** was similarly fed to *Ajuga* hairy roots and the biosynthesized clerosterol was converted to clionasterol. Fig. 2 illustrates the ^{2}H NMR spectra of the clionasterol samples. The spectra A and B are essentially identical to those of [28-pro-*R*-^{2}H]- and [28-pro-*S*-^{2}H]-clionasterols, respectively. This implies that 28*E*- and 28*Z*-hydrogens of **1**

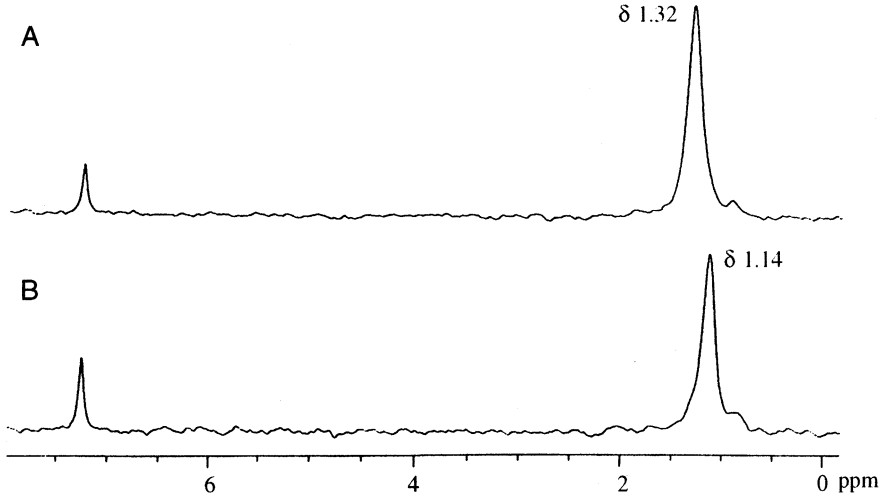
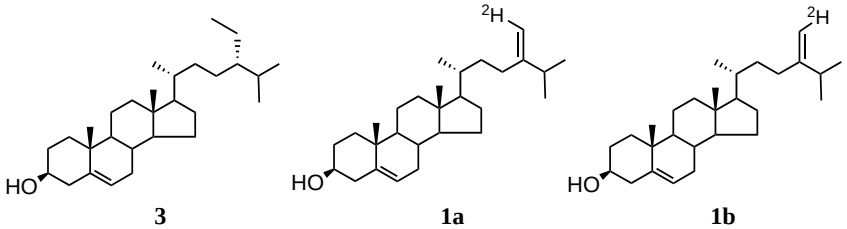


Figure 1. ^{2}H NMR spectra (61 MHz, $CHCl_3$) of [28-pro-*R*-^{2}H]-clionasterol **3a** (A) and [28-pro-*S*-^{2}H]-clionasterol **3b** (B).



3 **1a** **1b**

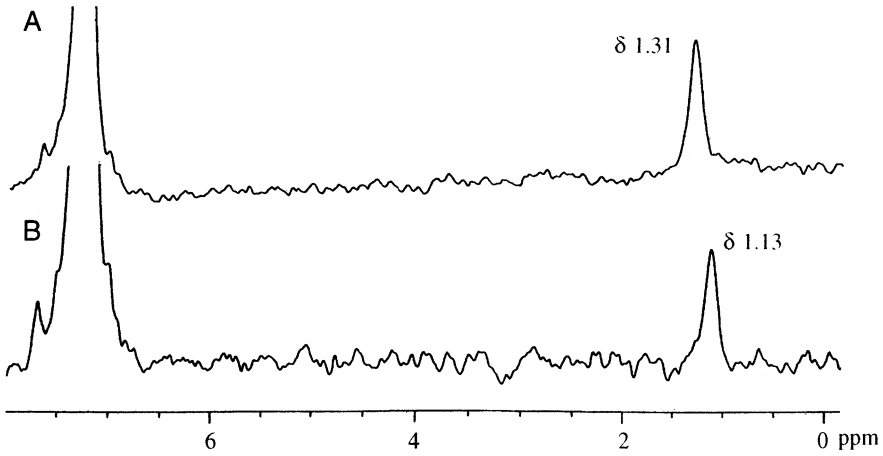


Figure 2. ^{2}H NMR spectra (61 MHz, $CHCl_3$) of clionasterol. (A) **3** derived from clerosterol biosynthesized from **1a**. (B) **3** derived from clerosterol biosynthesized from **1b**.

become C-28 pro-*R*- and pro-*S*-hydrogens, respectively, of **2**.

In conclusion, it has been established that the transfer of the methyl group from *S*-adenosylmethionine to C_{28}-$\Delta^{24(28)}$ sterol takes place from the 28-*si* face (path b in Scheme 1) in *Ajuga* hairy roots. This work is the first to determine the steric course of the methyl transfer in the conversion of C_{28}- to C_{29}-sterol. In *Ajuga* hairy roots, the transfer of the methyl group in the conversion of C_{27}- to C_{28}-sterol, e.g. from desmosterol to codisterol (24β-methyl-Δ^{25}-cholesterol), proceeds from the 24-*si* face of $\Delta^{24(25)}$ double bond.[5] Therefore, it appears that the methyl group of *S*-adenosylmethionine may be held in the backside of the $\Delta^{24(25)}$ and $\Delta^{24(28)}$ double bond in both methyl transfer reactions.

Acknowledgements

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6. Although nothing is known about whether the process is concerted or stepwise, the stereochemical discussion in the text is applicable even in a non-concerted process.
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9. Compound **4a**: oil, ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.43 (1H, dd, J=7.9, 5.3 Hz, 4eq-H), 0.64 (1H, t, J=5.3 Hz, 4ax-H), 0.72 (3H, s, 18-H_3), 0.90 (3H, d, J=6.6 Hz, 26-H_3), 0.94 (3H, d, J=6.8 Hz, 27-H_3), 0.95 (3H, d, J=6.3 Hz, 21-H_3), 1.02 (3H, s, 19-H_3), 1.18 (3H, d, J=6.3 Hz, 29-H_3), 2.77 (1H, m, 6-H), 3.32 (3H, s, OMe), 3.94 (1H, m, 28-H).
10. Compound **3a**: mp 136–138°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.680 (3H, s, 18-H_3), 0.811 (3H, d, J=6.8 Hz, 26-H_3), 0.832 (3H, d, J=6.8 Hz, 27-H_3), 0.848 (3H, d, J=6.4 Hz, 29-H_3), 0.927 (3H, d, J=6.8 Hz, 21-H_3), 1.010 (3H, s, 19-H_3), 3.53 (1H, m, 3-H), 5.35 (1H, brd, J=5.6 Hz, 6-H); ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.86 (C-18), 12.21 (C-29), 18.83 (C-21), 18.96 (C-27), 19.39 (C-19), 19.63 (C-26), 21.08 (C-11), 24.31 (C-15), 26.37 (C-23), 28.24 (C-16), 28.95 (C-25), 31.67 (C-2), 31.91 (C-7), 31.91 (C-8), 33.90 (C-22), 36.27 (C-20), 36.51 (C-10), 37.26 (C-1), 39.78 (C-12), 42.31 (C-4), 42.31 (C-13), 45.97 (C-24), 50.14 (C-9), 56.03 (C-17), 56.77 (C-14), 71.82 (C-3), 121.72 (C-6), 140.77 (C-5). A signal of C-28 (δ 23.1 for non-labeled compound) was negligible.
11. Compound **4b**: oil, ^{1}H NMR (300 MHz, $CDCl_3$) δ: 0.43 (1H, dd, J=8.0, 5.3 Hz, 4eq-H), 0.65 (1H, t, J=5.3 Hz, 4ax-H), 0.71 (3H, s, 18-H_3), 0.89 (3H, d, J=6.8 Hz, 26-H_3), 0.91 (3H, d, J=7.1 Hz, 27-H_3), 0.94 (3H, d, J=6.6 Hz, 21-H_3), 1.02 (3H, s, 19-H_3), 1.18 (3H, d, J=6.3 Hz, 29-H_3), 2.77 (1H, m, 6-H), 3.33 (3H, s, OMe), 3.80 (1H, m, 28-H).
12. Compound **3b**: mp 137–139°C; ^{1}H NMR (400 MHz, $CDCl_3$) δ: 0.680 (3H, s, 18-H_3), 0.812 (3H, d, J=6.8 Hz, 26-H_3), 0.830 (3H, d, J=7.6 Hz, 27-H_3), 0.847 (3H, d, J=6.0 Hz, 29-H_3), 0.926 (3H, d, J=6.4 Hz, 21-H_3), 1.010 (3H, s, 19-H_3), 3.53 (1H, m, 3-H), 5.35 (1H, brd, J=5.6 Hz, 6-H). ^{13}C NMR (100 MHz, $CDCl_3$) δ: 11.85 (C-18), 12.19 (C-29), 18.83 (C-21), 18.99 (C-27), 19.39 (C-19), 19.58 (C-26), 21.08 (C-11), 24.31 (C-15), 26.35 (C-23), 28.23 (C-16), 28.94 (C-25), 31.67 (C-2), 31.91 (C-7), 31.91 (C-8), 33.93 (C-22), 36.27 (C-20), 36.51 (C-10), 37.26 (C-1), 39.78 (C-12), 42.31 (C-4), 42.31 (C-13), 45.97 (C-24), 50.14 (C-9), 56.03 (C-17), 56.77 (C-14), 71.82 (C-3), 121.72 (C-6), 140.76 (C-5). A signal of C-28 was negligible.