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BIOSYNTHESIS OF 24β-ALKYL-Δ^{25}-STEROLS IN HAIRY ROOTS OF *AJUGA REPTANS* VAR. *ATROPURPUREA*

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Key Word Index—*Ajuga reptans* var. *atropurpurea*; Labiatae; sterol biosynthesis; 24-alkylation; hairy root; clerosterol; 22-dehydroclerosterol; codisterol; desmosterol; 24-methylenecholesterol.

Abstract—A hairy root culture of *Ajuga reptans* var. *atropurpurea* contains clerosterol, 22-dehydroclerosterol and cholesterol as its sterol constituents. Feeding of [26-, 27-$^{13}C_2$]desmosterol to this culture and ^{13}C NMR analysis of the resulting biosynthesized sterols showed that the substrate was efficiently incorporated into clerosterol and codisterol. Feeding of [26-^{13}C] and [27-^{13}C]desmosterols revealed that the C-24 alkylation takes place in a highly specific manner wherein the 26- and 27-methyl groups of the substrate becomes C-26 (vinyl methyl) and C-27 (exomethylene carbon), respectively, of the two Δ^{25}-sterols. Further, feeding of [24-^{2}H]desmosterol and ^{2}H NMR analysis of the products showed that H-24 of clerosterol and codisterol is derived from H-24 of desmosterol. Finally, [28-^{13}C]ergosta-5,24(28)-dien-3β-ol was shown to be converted into clerosterol and 22-dehydroclerosterol, but not into codisterol. On the basis of these data, possible biosynthetic mechanism of 24β-alkyl-Δ^{25}-sterols in this plant is proposed.

INTRODUCTION

We have recently demonstrated that a hairy root clone of *Ajuga reptans* var. *atropurpurea* formed by infecting with *Agrobacterium rhizogens* is a suitable tool for studying ecdysteroid biosynthesis in plants [1]. In the feeding experiments with this transformed species, [2-^{13}C]acetate was incorporated into 20-hydroxyecdysone, cyasterone and 29-norcyasterone, whereas [26,27-$^{13}C_2$]cholesterol was incorporated only to the first ecdysteroid [2]. Further, 3β-hydroxy-5β-cholest-7-en-6-one was identified as an obligatory intermediate in the biosynthesis of ecdysteriods [3]. In order to get further insight into the mechanism of ecdysteroid biosynthesis, especially at the side chain portion, the knowledge of the sterol biosynthesis in *Ajuga* sp. seemed to be prerequisite. Here we describe the results of feeding experiments of variously labelled desmosterols and 24-methylenecholesterol and propose a mechanism of sterol biosynthesis in this species.

RESULTS AND DISCUSSION

HPLC profile of the sterol fraction of the cultured hairy roots of the transformed *Ajuga* sp. is shown in Fig. 1A. These sterols were separated by HPLC, and identified as clerosterol [4] (24β-ethylcholesta-5,25-dien-3β-ol) (**1**, 48%, corresponding to the second peak), 22-dehydroclerosterol [4] (24β-ethylcholesta-5,22,25-trien-3β-ol) (**2**, 44%, corresponding to the first peak) and cholesterol (**3**, 8%, corresponding to the third peak) on the basis of ^{1}H NMR and mass spectral data (the sterol % was expressed on the basis of GC analysis).

Since the ^{13}C-labelled cholesterol was efficiently incorporated into 20-hydroxyecdysone, it is expected that desmosterol would also taken-up with the root culture. We hoped that the alkylation might take place for a Δ^{24}-sterol with cholest-5-ene nuclei structure [5], although the optimal nuclei structure for the side chain modification (alkylation and reduction) is not known with this species. To examine this point, [26,27-$^{13}C_2$]desmosterol (**6**) was fed to *Ajuga* hairy root culture as described previously [2]. HPLC analysis of the sterol fraction (illustrated in Fig. 1B) showed an increased intensity of the first peak compared to that without exogenous substrate.

The ^{13}C NMR spectrum of the first peak in Fig. 1B exhibited four prominent ^{13}C-enriched signals. Among these, the signals of δ25.7 and 17.7 were apparently due to C-26 and C-27 methyl groups of desmosterol. However, the remaining two signals at δ18.6 and 109.4 (*ca.* 6 times enriched) could not be assigned to any carbon of 22-dehydroclesterol (**2**) and desmosterol (**5**), indicating the presence of another sterol in this fraction. The signals of C-26 (δ20.3) and C-27 (δ109.6) of 22-dehydroclerosterol were not enriched by ^{13}C, indicating that desmosterol was not incorporated into 22-dehydroclerosterol. The chemical shifts of the two unidentified signals were

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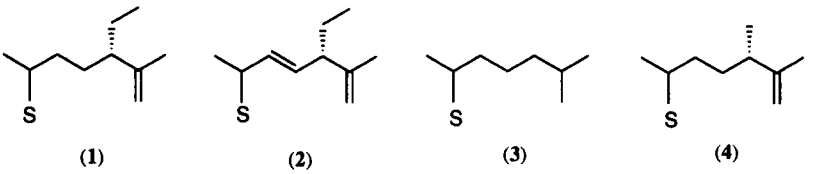

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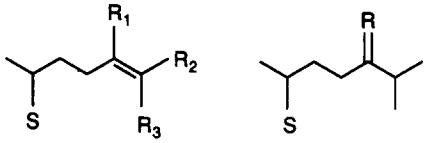
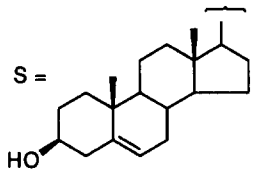


(5): R_1=H, R_2=R_3=CH_3
(6): R_1=H, R_2=R_3=$^{13}CH_3$
(7): R_1=H, R_2=$^{13}CH_3$, R_3=CH_3
(8): R_1=H, R_2=CH_3, R_3=$^{13}CH_3$
(9): R_1=^{2}H, R_2=R_3=CH_3

(10): R=CH_3
(11): R=$^{13}CH_2$



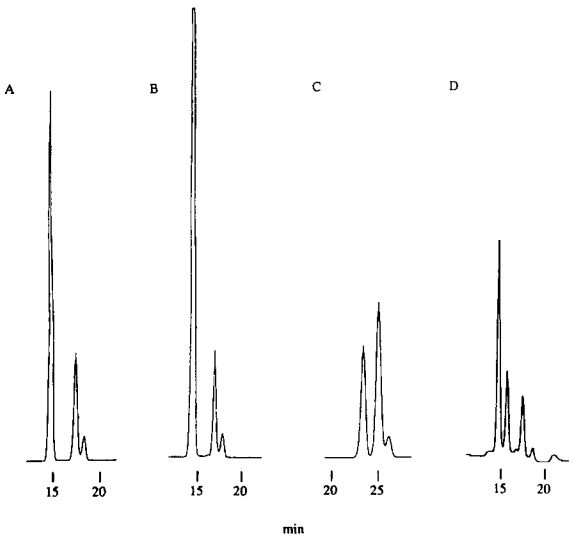


Fig. 1. HPLC analysis of the sterol fractions biosynthesized with transformed *Ajuga reptans* var. *atropurpurea*. A: without exogenous sterol (the peaks at 14.7, 17.4 and 18.3 min correspond to 22-dehydroclerosterol, clerosterol and cholesterol, respectively), B: fed with [26,27-$^{13}C_2$]desmosterol (**6**), C: the first peak (14.7 min) of B was rechromatographed with CH_3CN–MeOH, (2:1) as an HPLC solvent (the peaks at 23.2, 25.0 and 26.4 min correspond to desmosterol, 22-dehydroclerosterol and codisterol, respectively], D: fed with [28'-^{13}C]ergosta-5,24(28)-dien-3β-ol (**11**) (the peaks at 15.6 and 21.5 min correspond to 24-methylenecholesterol and campesterol, respectively). For HPLC conditions, see Experimental, unless otherwise stated.

found to coincide with those reported for C-26 and C-27 of 31-norcyclolaudenol [6]. Comparison with the reported retention times of HPLC and GC [7] suggested this sterol to be codisterol (**4**) [8]. Further, HPLC analysis of the first peak of Fig. 1B using CH_3CN–MeOH (2:1) as eluent revealed three peaks (Fig. 1C) due to desmosterol, 22-dehydroclerosterol and codisterol. Definite assignment of the last peak to codisterol rest on direct comparison with the synthetic sample (Experimental) in respect to GC, mass spectral, HPLC and ^{13}C NMR data. Importantly, the mass spectrum of codisterol showed the molecular ion peak at *m/z* 400, not at *m/z* 398 (M^+ of non-labelled **4**). These observations clearly indicated that codisterol was not endogenous in *Ajuga*, but efficiently biosynthesized from [$^{13}C_2$]desmosterol. It should be noted that the sterol metabolism was modulated by feeding desmosterol, and now codisterol which was not observed in the original *Ajuga* hairy roots was accumulated.

^{13}CNMR analysis of the second peak in Fig. 1B showed a pair of signals at δ17.8 and 111.3 (assignable to C-26 and C-27 of **1**), with much higher intensity (*ca.* 5 times) than the non-labelled background signals. This clearly indicated that desmosterol was efficiently incorporated into clerosterol. This feeding experiment has demonstrated that desmosterol is a good substrate for the study on C-24 alkylation mechanism in *Ajuga* hairy roots. Further, [26,27-$^{13}C_2$]desmosterol was incorporated also into cholesterol as indicated by ^{13}C-enriched signals of δ22.5 and 22.8 (C-26 and C-27).

The aforementioned negligible conversion of [^{13}C] desmosterol into 22-dehydroclerosterol is rather unexpected (*vide infra*), and this might be due to a limited availability of a C_{29} and/or C_{28}-sterol precursor or a pool of such a sterol. It has been reported that sitosterol is incorporated into stigmasterol in *Sorghum bicolor* [9], and it has also been suggested that sterol pools might be responsible for the different rates of sitosterol and stigmasterol biosynthesis in *Zea mays* [10].

With transformations of desmosterol into clerosterol and codisterol demonstrated, we then incubated the regioselectively labelled [26-^{13}C]desmosterol (**7**) [11] and [27-^{13}C]desmosterol (**8**) [12]. As can be seen in Fig. 2 and Fig. 3, **7** was converted into [26-^{13}C]clerosterol and [26-^{13}C]codisterol, whereas **8** produced [27-^{13}C]clerosterol and [27-^{13}C]codisterol. These results indicate that C-24 alkylation of desmosterol proceeded in a highly

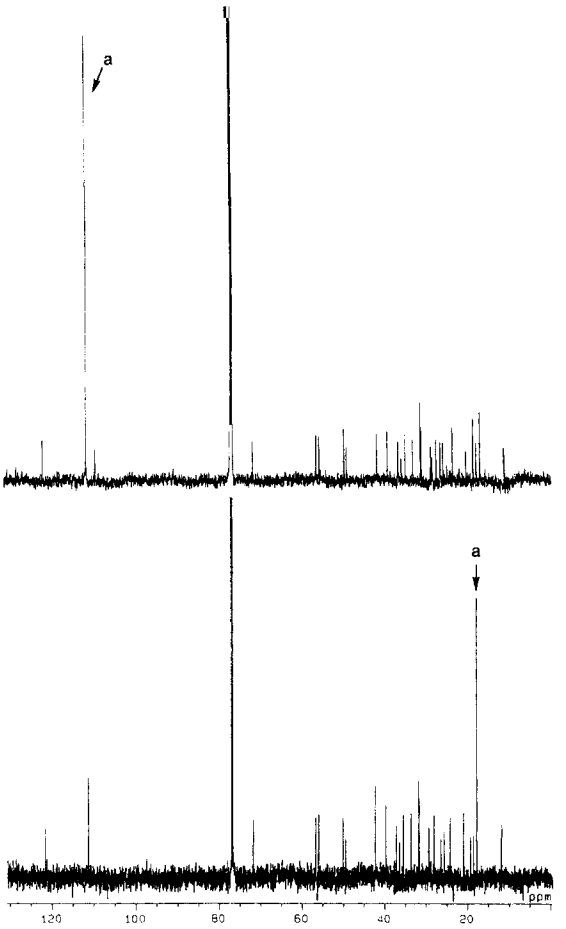


Fig. 2. ^{13}C NMR spectra (100 MHz, $CDCl_3$) of clerosterol produced from [26-^{13}C]desmosterol (**7**) (Lower) and from [27-^{13}C]desmosterol (**8**) (Upper). Signals at δ17.8 and 111.3 (arrow a) are due to C-26 and C-27 of clerosterol, respectively.

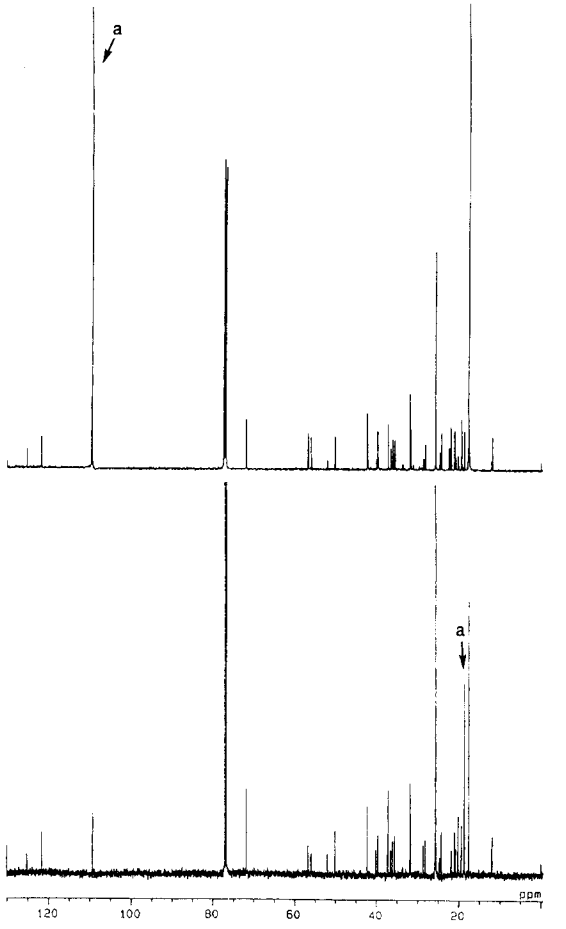


Fig. 3. ^{13}C NMR spectra (100 MHz, $CDCl_3$) of 22-dehydroclerosterol fraction (accompanied with desmosterol and codisterol) of Fig. 1B, produced from [26-^{13}C]desmosterol (**7**) (Lower) and with [27-^{13}C]desmosterol (**8**) (Upper). Signals at δ18.6 and 109.3 (arrow a) were assigned to C-26 and C-27 of codisterol. The signals of δ25.7 and 17.6 are due to C-26 and C-27 of desmosterol.

specific manner, wherein C-26, the (*E*)-methyl derived from C-2 of mevalonate [13] of desmosterol, becomes the C-26 (vinyl methyl) of clerosterol and codisterol, whereas C-27, the (*Z*)-methyl derived from C-6 of mevalonate [13], becomes C-27 (exo-methylene carbon). The observed metabolic fate is in agreement with those reported for the biosynthesis of 24β-ethyl-Δ^{25}-sterols, 22-dehydroclerosterol in *Clerodendrum campbellii* [14] and 22-dihydro-25-dehydrochondrillasterol in *Trichosanthes kirilowii* [15], as well as a 24β-methyl-Δ^{25}-triterpene, cyclolaudenol in *Polypodium vulgare* [16].

Additional information on the mechanism of C-24 alkylation was obtained by the feeding experiment of [24-^{2}H]desmosterol (**9**). The ^{2}H NMR spectrum of the resulting codisterol showed a signal at δ2.05 (Fig. 4). The chemical shift corresponds to that of H-24 (δ2.08, *m*) of codisterol (determined by decoupling and H–H COSY experiments). Similarly, clerosterol derived from **9** exhibited a ^{2}H signal at δ1.8 (Fig. 5) which is assignable to ^{2}H at C-24 (H-24 of clerosterol: δ1.82, *m*). These results clearly demonstrate that H-24 of clerosterol and codisterol is derived from H-24 of desmosterol. The retention of H-24 during the conversion of desmosterol into codisterol is consistent with a concerted dehydrogenation to introduce Δ^{25}-bond and suggests that neither a $\Delta^{24(25)}$- nor a $\Delta^{24(28)}$-sterol is involved during transformation. To our knowledge the present paper is the first to establish the fate of 24-H of Δ^{24}-sterol substrate during the conversion into 24β-methyl-Δ^{25}-sterol [16]. On the other hand, the retention of H-24 during the conversion of desmosterol into clerosterol agrees with the involvement of a $\Delta^{24(28)}$-sterol, e.g., ergosta-5,24(28)-dien-3β-ol (**10**), in which H-24 of desmosterol migrates to the C-25 position and back to C-24, as suggested for the biosynthesis of 22-dihydro-25-dehydrochondrillasterol [15].

To examine the intermediary role of 24-methylenecholesterol in the formation of codisterol, clerosterol and 22-dehydroclerosterol, [28-^{13}C]ergosta-5,24(28)-dien-3β-ol (**11**) was fed to the root culture. The HPLC profile of the sterol fraction is shown in Fig. 1D. ^{13}C NMR analysis of the first HPLC peak showed two

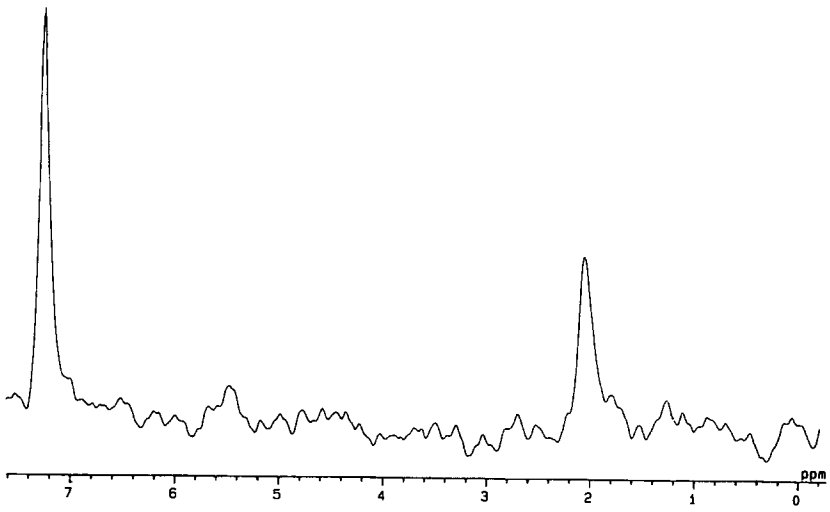


Fig. 4. ^{2}H NMR spectrum (61 MHz, $CHCl_3$) of codisterol produced from [^{2}H]desmosterol (**9**). A signal at δ2.05 is assignable to H-24 of codisterol.

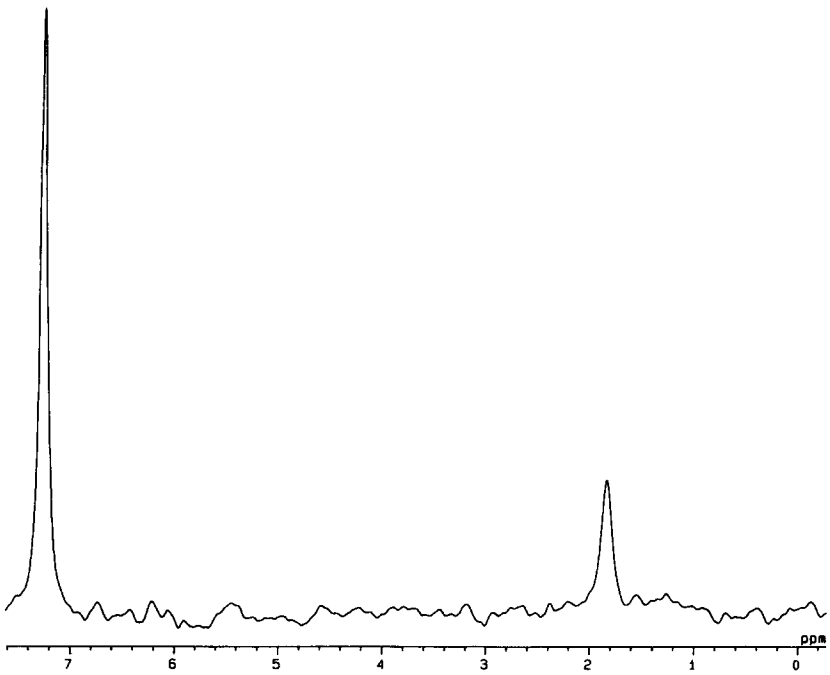


Fig. 5. ^{2}H NMR spectrum (61 MHz, $CHCl_3$) of clerosterol produced from [^{2}H]desmosterol (**9**). A signal at δ1.8 is assignable to H-24 of clerosterol.

^{13}C-enriched signals (shown in Fig. 6, Upper). The signal at δ105.9 is due to C-28 of the recovered substrate, whereas the signal at δ25.7 (*ca.* 3 times enriched) was assigned to C-28 of 22-dehydroclerosterol, indicating that 24-methylenecholesterol was transformed into 22-dehydroclerosterol. GC and ^{1}H and ^{13}C NMR analysis of the first peak showed that codisterol was absent in this fraction.

^{13}C NMR analysis (Fig. 6, Lower) of clerosterol fraction (the third peak in Fig 1D) showed an enriched (*ca.* 6 times) signal at δ26.5 (C-28 of **1**). Thus, it is now evident that 24-methylenecholesterol is a precursor of both clerosterol and 22-dehydroclerosterol, although the incorporation yield into the latter sterol was approximately half of the former.

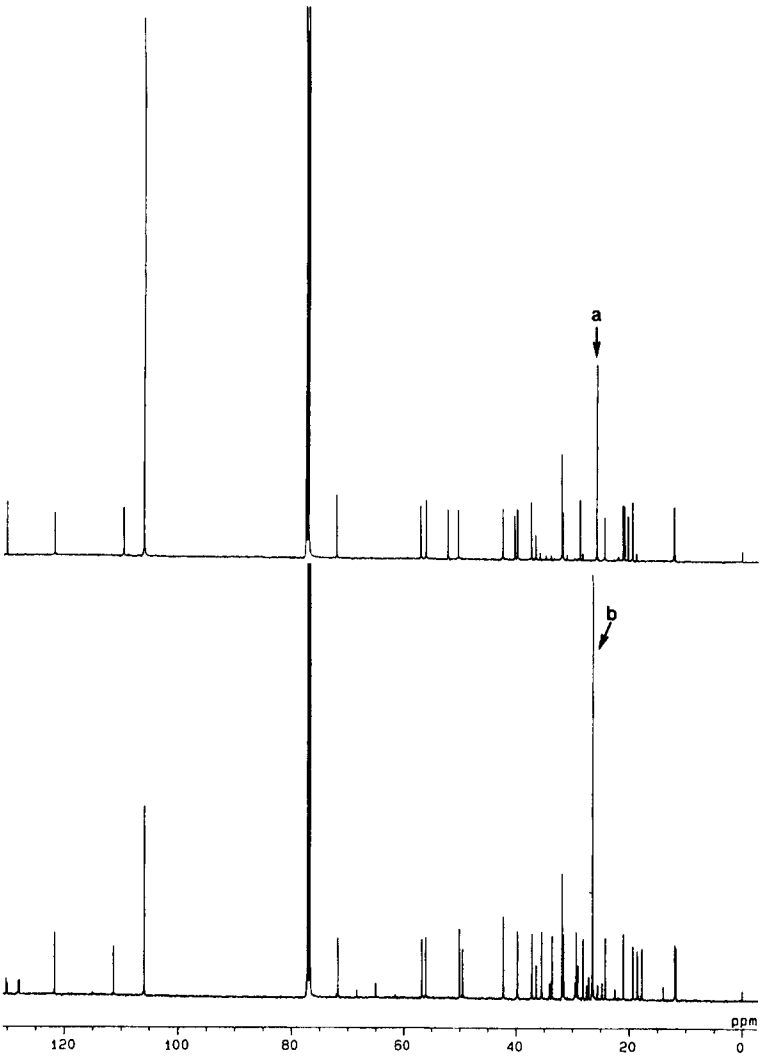


Fig. 6. ^{13}C NMR spectrum (100 MHz, $CHCl_3$) of 22-dehydroclerosterol (Upper) and clerosterol (Lower) produced from [28-^{13}C] ergosta-5,24(28)-dien-3β-ol (**11**). Signals at δ 25.7 (arrow a), 26.5 (arrow b) and 105.9 are due to C-28 of 22-dehydroclerosterol, clerosterol and ergosta-5,24(28)-dien-3β-ol, respectively.

In this feeding experiment, campesterol (*ca.* 5% of total sterol) was identified by spectral evidence and direct HPLC comparison with the authentic sample. The ^{13}CNMR spectrum of the campesterol, separated by HPLC, showed a ^{13}C-enriched signal at δ15.4 (C-28). Further, in the ^1H NMR spectrum the signal of 28-H_3 was observed as a double doublet (J = 124.7 and 6.6 Hz) at δ0.77, and in the mass spectrum a molecular ion peak appeared at m/z 401 instead of m/z 400 for the non-labelled sample. These spectral data showed that the conversion of 24-methylenecholesterol into campesterol occurred without significant dilution. This constitutes another example of the formation of a sterol which is absent in the original *Ajuga* roots.

A possible pathway of the C-24 alkylation consistent with these experimental data is depicted in Fig. 7. The C-25 carbonium ion intermediate generated by transfer of the methyl group from (S)-adenosylmethionine (SAM) to the C-24 position of desmosterol would be deprotonated by two distinct pathways *a* and *b*. Pathway *a* directly leads to codisterol by deprotonation at one of the *gem*-dimethyl groups. It is rigorously controlled that the methyl group which loses hydrogen is that derived from (*Z*)-methyl of desmosterol. The formation of Δ^{25}-bond concerted with C-24 methylation was strongly supported by the experimental evidence that H-24 of desmosterol turned out to be H-24 of codisterol and 24-methylenecholesterol was not incorported into codisterol. Thus, the C-24 configuration of codisterol should directly reflect the direction (i.e., *si*-face) of methylation of Δ^{24}-bond.

Pathway *b* leads to 24-methylenecholesterol *via* the migration of hydrogen at C-24 to C-25 followed by

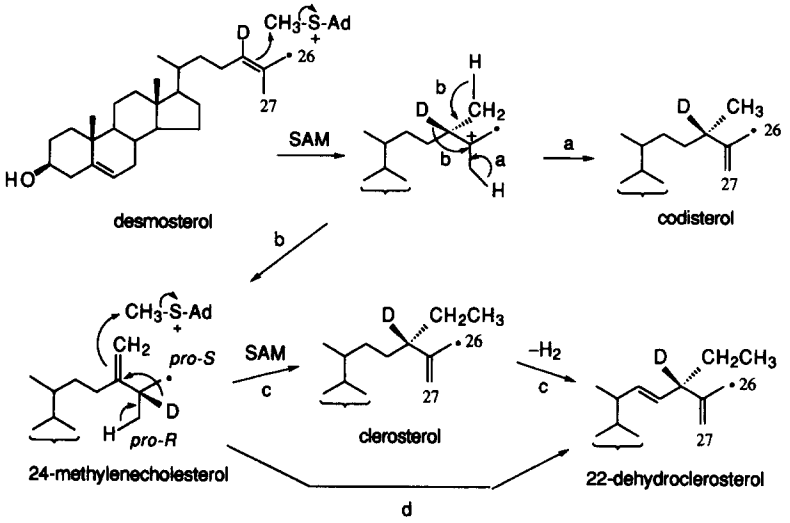


Fig. 7. Proposed biosynthetic pathway of codisterol, clerosterol and 22-dehydroclerosterol, from desmosterol. •, Indicates the metabolic fate of (*E*)-methyl group (C-26) of desmosterol. SAM refers to (S)-adenosylmethionine.

deprotonation at the methyl group donated from SAM. Although this $\Delta^{24(28)}$-sterol was not detected in *Ajuga* hairly roots, it seems highly reasonable to presume the $\Delta^{24(28)}$-sterol as a precursor of clerosterol, since its positive conversion into clerosterol and 22-dehydroclerosterol was proved in the present study. The C-25 prochirality of 24-methylenecholesterol, in which the *pro-S* methyl group is derived from the (*E*)-methyl of desmosterol, was tentatively assigned from analogy of the reported examples [17, 18]. *Si*-face (back-side) attack at C-24 of the methylation leading to 24-methylenecholesterol in higher plants is speculated without direct evidence [19, 20]. This paper presents evidence supporting the *si*-face attack, since the initially formed C-25 carbonium ion intermediate should be a common precursor in pathways *a* and *b*.

The second methylation from SAM at C-28 of $\Delta^{24(28)}$bond would produce the C-24 carbonium ion intermediate. This will lead to clerosterol by the migration of hydrogen at C-25 to C-24 followed by deprotonation again from the methyl group (*pro-R* methyl of **10**) arising from the (*Z*)-methyl of desmosterol. This mechanism (pathway *c*) agrees with the fact that H-24 of desmosterol finally resides at the C-24 position of clerosterol and 22-dehydroclerosterol. The metabolic fate of the hydrogen excludes the possibility of a C_{28}- or a C_{29}-Δ^{24}-sterol as an intermediate. If the second transfer of the methyl group is a concerted process (i.e., *anti*-S_N2' process), the direction of the methyl transfer at C-28 of $\Delta^{24(28)}$-bond would take place preferably from *si*-face (back side).

An alternative pathway leading to 22-dehydroclerosterol from 24-methylenecholesterol, i.e. 22-desaturation of 24-methylenecholesterol followed by the second methylation (pathway *d*), cannot be ruled out with the presently available information. Feeding experiments of ^{13}C-labelled clerosterol and 22-dehydro-24-methylenecholesterol would provide information on this point.

In summary, the present study has successfully utilized the transformed *Ajuga* hairy root culture in combination with NMR spectroscopy and provided several lines of clear-cut evidence for the biosynthetic mechanism of 24β-alkyl-Δ^{25}-sterols in higher plants.

EXPERIMENTAL

Incubation procedure and sterol analysis. Hairy root clone of *Ajuga reptans* var. *atropurpurea*, maintained in solid MS medium, was inoculated into three 500 ml flasks, each containing 250 ml of MS liquid medium supplemented with 3% of sucrose, as described previously [1]. The inoculum was cultured for 4 weeks on a rotary shaker (120 rpm) at 25° in the dark. The root culture (90 g, wet wt) was ground in the presence of sea sand of $CHCl_3$–MeOH (1:1) in a mortar with a pestle. The mixture was centrifuged and the supernatant soln was separated. Removal of the solvent from the organic layer gave the residue, which was dissolved in *n*-BuOH and washed with brine. The separated *n*-BuOH layer was concentrated and the residue was taken up in $CHCl_3$–MeOH (1:1). The separated soln was concentrated and submitted to silica gel column chromatography eluted with a $CHCl_3$–MeOH gradient system. The fraction containing phytosterols were combined and further chromatographed on silica gel eluting with hexane–EtOAc (4:1), to give a purified phytosterol fraction (5 mg). An aliquot of the sample was analyzed by reverse phase HPLC (column, Shimadzu Shimpack CLC-ODS, 15 cm × 6 mm i.d.; solvent, methanol; flow rate, 1.0 ml min^{-1}; UV detector at 210 nm). The HPLC profile was shown in Fig. 1A.

Separation of these sterols by HPLC gave 22-dehydroclerosterol (**2**) (3.1 mg, R_t 14.7 min), clerosterol (**1**) (1.2 mg, R_t 17.4 min) and cholesterol (**3**) (0.27 mg, R_t 18.3

min). Clerosterol, ^{1}H NMR ($CDCl_3$) δ: 0.67 (*s*, 18-H_3), 0.80 (*t*, J = 7.7 Hz, 29-H_3), 0.91 (*d*, J = 6.7 Hz, 21-H_3), 1.01 (*s*, 19-H_3), 1.56 (*d*, J = 4.7 Hz, 26-H_3), 1.82 (*m*, 24-H), 3.52 (*m*, 3-H), 4.64 (*d*, J = 2.7 Hz, 27-H), 4.73 (*dt*, J = 4.0, 1.3 Hz, 27-H), 5.35 (*m*, 6-H), EI-MS (in the form of trimethylsilyl ether) *m/z* (rel. int.): 484 $[M]^+$ (11), 469 (3), 394 (13), 379 (6), 355 (9), 129 (68), 55 (100). 22-Dehydroclerosterol, ^{1}H NMR ($CDCl_3$) δ: 0.69 (*s*, 18-H_3), 0.83 (*t*, J = 7.4 Hz, 29-H_3), 1.01 (*s*, 19-H_3), 1.01 (*d*, J = 6.7 Hz, 21-H_3), 2.42 (*q*-like, J = 7.0 Hz, 24-H), 3.52 (*m*, 3-H), 4.70 (*m*, 27-H_2), 5.18 (*dd*, J = 15.4, 7.4 Hz, 22 or 23-H), 5.24 (*dd*, J = 15.4, 8.1 Hz, 23 or 22-H), 5.35 (*m*, 6-H); EI-MS (in the form of trimethylsilyl ether) *m/z* (rel. int.): 484 $[M]^+$ (7), 467 (2), 392 (10), 353 (5), 137 (95), 129 (70), 95 (98), 81 (96), 73 (100).

Feeding of the labelled desmosterols. The inoculum was cultured for 2 weeks as described above. [26,27-^{13}C] desmosterol (**6**) (50 mg) dissolved in a mixture of EtOH (2 ml), Tween 80 (1 ml) and H_2O (1 ml), were added to the three incubation flasks through a membrane filter. After another 2 weeks of incubation, the root culture (50 g wet wt) was treated as described above to give a purified phytosterol fraction (6 mg). HPLC profile of this fraction is shown in Fig. 1B. The three major peaks were separated by HPLC to give 22-dehydroclerosterol (2.1 mg, R_t 14.7 min, accomanied with desmosterol and codisterol), clerosterol (1.3 mg, R_t 17.4 min) and cholesterol (0.2 mg, R_t 18.3 min). The 22-dehydroclerosterol fraction was further analyzed by HPLC using CH_3CN–MeOH (2:1) as a solvent system (Fig. 1C). GC-MS data for codisterol biosynthesized from **6**: EI-MS *m/z* (rel. int.): 400 (65), 385 (13), 384 (14), 367 (15), 299 (40), 271 (60), 213 (47), 81 (100).

The feeding experiments of [26-^{13}C], [27-^{13}C] and [24-^{2}H]desmosterols were carried out in the same way. The 22-dehydroclerosterol fraction obtained from the incubation of [24-^{2}H]desmosterol was further separated by HPLC using CH_3CN–MeOH system. The codisterol and clerosterol thus obtained were separately analyzed by ^{2}H NMR spectroscopy.

The feeding experiment of [28-^{13}C] ergosta-5,24(28)-dien-3β-ol was carried out in the same manner as described above, and 22-dehydroclerosterol (accompanied with 24-methylenecholesterol), clerosterol and campesterol were separated by preparative HPLC. The spectral data for the campesterol: ^{1}H NMR ($CDCl_3$) δ: 0.68 (*s*, 18-H_3), 0.77 (*dd*, J = 124.7, 6.6 Hz, 28-H_3), 0.80 (*d*, J = 7.3 Hz, 26-H_3), 0.91 (*d*, J = 6.6 Hz, 21-H_3), 1.01 (*s*, 19-H_3), 3.53 (*m*, 3-H), 5.36 (*m*, 6-H); EI-MS *m/z*: (rel. int.): 401 $[M]^+$ (29), 386 (10), 383 (15), 368 (9), 316 (19), 290 (25), 273 (18), 255 (24), 213 (51), 105 (100), 231 (25), 105 (100).

The ^{13}C-labelled desmosterols. [26,27-$^{13}C_2$]Desmosterol (**6**) was prepared from $^{13}CH_3I$ (99.8% ^{13}C) as described previously [2]. [26-^{13}C] Desmosterol (**7**) and [27-^{13}C] desmosterol (**8**) were synthesized as described in our previous papers [11, 12]. The ^{13}C-lable of **7** is located 89% at C-26 (*E*-Me) and 11% at C-27 (*Z*-Me). The ^{13}C-label of **8** is located 4% at C-26 and 96% at C-27.

Synthesis of [24-^{2}H]desmosterol (**9**). To a soln of methyl 3β-tetrahydropyranyloxychol-5-en-24-oate [21] (2.0 g, 4.2 mmol) in dry THF (3 ml) was added $LiAlD_4$ (167 mg, 4.2 mmol) and the mixture was stirred at room temp. for 2.5 hr. Extractive (EtOAc) work-up gave a crude product, which was chromatographed on silica gel using hexane–EtOAc as an eluent to afford [24-$^2H_2$]-3β-tetrahydropyranyloxychol-5-en-24-ol (1.87 g, 99%), ^{1}H NMR ($CDCl_3$) δ: 0.68 (*s*, 18-H_3), 0.94 (*d*, J = 6.4 Hz, 21-H_3), 1.01 (*s*, 19-H_3), 3.50-3.91 (*m*, 3-H and 6'-H_2 of THP), 4.72 (*m*, 2'-H of THP), 5.35 (*m*, 6-H).

A mixture of the alcohol (1.87 g, 4.2 mmol), pyridinium chlorochromate (2.5 g, 11.6 mmol) and NaOAc (140 mg) in dry CH_2Cl_2 was stirred at room temp. for 3 hr, and then filtered through a short column of Florisil with ether as eluent to afford [24-^{2}H]-3β-tetrahydropyranyloxychol-5-en-24-al (1.7 g, 91%), mp 179–180° (from MeOH–CH_2Cl_2).

n-BuLi (1.6M-hexane solution, 4.62 ml, 7.44 mmol) was added to a suspension of isopropyltriphenylphosphonium bromide (2.88 g, 7.44 mmol) in dry THF (10 ml) at room temp. under nitrogen, and the mixture was stirred for 45 min. A soln of the aldehyde (1.1 g, 2.48 mmol) in dry THF (1 ml) was added and the mixture was stirred at room temp. overnight. Extractive (EtOAc) work-up gave a crude product, which was chromatographed on silica gel using hexane–EtOAc as an eluent to afford [24-^{2}H]-3β-tetrahydropyranyloxycholesta-5,24-diene (650 mg, 56%), ^{1}H NMR ($CDCl_3$) δ: 0.67 (*s*, 18-H_3), 0.94 (*d*, J = 6.6 Hz, 21-H_3), 1.01 (*s*, 19-H_3), 1.59 (*s*, 27-H_3), 1.68 (*s*, 26-H_3), 3.49–3.89 (*m*, 3-H and 6'-H_2 of THP), 4.71 (*m*, 2'-H of THP), 5.34 (*m*, 6-H).

A mixture of the THP ether (650 mg, 1.38 mmol), THF (20 ml), MeOH (20 ml) and 6M HCl (0.3 ml) was stirred at room temp. for 1.5 hr. Extractive (EtOAc) work-up followed by purification on a silica gel column using hexane–EtOAc as an eluent afforded **9** (392 mg, 74%), mp 121–122°, ^{1}H-NMR ($CDCl_3$) δ: 0.67 (*s*, 18-H_3), 0.94 (*d*, J = 6.6 Hz, 21-H_3), 1.01 (*s*, 19-H_3), 1.60 (*s*, 27-H_3), 1.68 (*s*, 26-H_3), 3.50 (*m*, 3-H), 5.36 (*m*, 6-H).

Synthesis of codisterol (**4**). *n*-Bu_3P (18 μl, 0.072 mmol) was added to a mixture of (24*S*)-6β-methoxy-3α,5-cyclo-5α-ergostan-26-ol [22] (5.2 mg, 0.012 mmol) and 2-nitrophenylselenocyanate (16.4 mg, 0.072 mmol) in dry THF (100 μl) under nitrogen and the mixture was stirred at room temp. for 3 hr. The reaction mixture was cooled down to 0°, and 30% H_2O_2 (3 drops) was added. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel using hexane–EtOAc as an eluent to afford (24*S*)-6β-methoxy-3α,5-cyclo-5α-ergost-25-ene (3.6 mg, 66%).

A soln of the 25-ene in dioxane (0.6 ml), H_2O and a catalytic amount of *p*-TsOH was heated at reflux for 45 min. Extractive (Et_2O) work-up followed by HPLC purification using MeOH as an eluent afforded **4** (1 mg, 31%), mp 144–145°, ^{1}H NMR ($CDCl_3$) δ: 0.67 (*s*, 18-H_3), 0.93 (3H, *d*, J = 6.1 Hz, 21-H_3), 1.00 (*d*, J = 6.9 Hz, 28-H_3), 1.01 (*s*, 19-H_3), 1.64 (*s*, 26-H_3), 2.08 (*m*, 24-H), 3.53 (*m*, 3-H), 4.66 (*s*, 27-H_2), 5.36 (*m*, 6-H), ^{13}C NMR ($CDCl_3$): δ11.84 (C-18), 18.68 (C-26), 18.73 (C-21), 19.39 (C-19), 20.11 (C-28), 21.08 (C-11), 24.29 (C-15), 28.17 (C-16), 31.18 (C-23), 31.69 (C-2), 31.92 (C-7 and C-8), 33.68 (C-

22), 35.67 (C-20), 36.51 (C-10), 37.27 (C-1), 39.79 (C-12), 41.58 (C-24), 42.32 (C-4 and C-13), 50.15 (C-9), 56.01 (C-17), 56.78 (C-14), 71.82 (C-3), 109.31 (C-27), 121.71 (C-6), 140.78 (C-5), 150.24 (C-25).

Synthesis of [28-^{13}C]*ergosta*-5,24(28)-*dien*-3β-*ol* (**11**). *n*-BuLi (1.6 M-hexane soln, 1.24 ml, 1.98 mmol) was added to a suspension of [^{13}C] methyltriphenylphosphonium iodide (800 mg, 2.0 mmol, prepared from [^{13}C]methyl iodide with 99% ^{13}C) in dry THF (0.5 ml) at 0° under nitrogen, and the mixture was stirred at room temp. for 15 min. A soln of 3β-tetrahydropyranyloxycholest-5-en-24-one [23] (400 mg, 0.82 mmol) in dry THF (1 ml) was added and stirred for 4 hr. Extractive (Et_2O) work-up gave a crude product, which was chromatographed on silica gel using hexane–EtOAc as an eluent to afford [24′-^{13}C]-24-methylene-cholesterol tetrahydropyranyl ether (355 mg, 89%).

A mixture of the THP ether (355 mg, 0.73 mmol), THF (3 ml), MeOH (1 ml) and 2M HCl (0.1 ml) was stirred at room temperature for 5 hr. Extractive (EtOAc) work-up followed by purification on a silica gel column using hexane–EtOAc as an eluent afforded **11** (275 mg, 93%), mp 143–144°, ^{1}H NMR ($CDCl_3$): δ 0.68 (3H, *s*, 18-H_3), 0.95 (3H, *d*, *J* = 6.7 Hz, 21-H_3), 1.01 (3H, *s*, 19-H_3), 1.03 (6H, *d*, 26 or 27-H_3), 3.52 (1H, *m*, 3-H), 4.71 (2H, *dd*, *J* = 154, 28.7 Hz, 28-H_2), 5.36 (1H, *m*, 6-H). ^{13}C NMR ($CDCl_3$): δ105.9 (C-28).

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