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Biosynthesis of cholestanol in higher plants

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Abstract

To understand the early steps of C_{27} brassinosteroid biosynthesis, metabolic experiments were performed with *Arabidopsis thaliana* and *Nicotiana tabacum* seedlings, and with cultured *Catharanthus roseus* cells. [26, 28-$^2H_6$]Campestanol, [26-$^2H_3$]cholesterol, and [26-$^2H_3$]cholestanol were administered to each plant, and the resulting metabolites were analyzed by gas chromatography–mass spectrometry. In all the species examined, [$^2H_3$]cholestanol was identified as a metabolite of [$^2H_6$]campestanol, and [$^2H_3$]cholest-4-en-3-one and [$^2H_3$]cholestanol were identified as metabolites of [$^2H_3$]cholesterol. This study revealed that cholestanol (C_{27} sterol) was biosynthesized from both cholesterol (C_{27} sterol) and campestanol (C_{28} sterol). It was also demonstrated that cholestanol was converted to 6-oxocholestanol, and campestanol was converted to 6-oxocampestanol.

Keywords: *Arabidopsis thaliana*; Cruciferae; *Catharanthus roseus*; Apocynaceae; *Nicotiana tabacum*; Solanaceae; Biosynthesis; Campestanol; Cholestanol; Cholest-4-en-3-one; Cholesterol; 6-Oxocampestanol; 6-Oxocholestanol

1. Introduction

The biosynthesis of brassinolide, the most active C_{28} brassinosteroid (BR), has been extensively studied using cultured cells of *Catharanthus roseus*. Brassinolide is biosynthesized from campesterol in two parallel pathways, namely the early and late C-6 oxidation pathways, which branch after the formation of campestanol (Fujioka and Sakurai, 1997a,b; Sakurai, 1999; Fujioka et al., 2000a). Recently, most of the steps in these pathways have been confirmed in seedlings of *Arabidopsis thaliana* (Noguchi et al., 2000), but some steps have yet to be demonstrated. Although many C_{27} BRs and C_{29} BRs occur naturally, their biosynthetic pathways have not yet been established. 28-Norcastasterone, the major C_{27} BR, may be biosynthesized from cholestanol using a pathway similar to the biosynthesis of castasterone from campestanol. Very recently, some possible precursors, such as 6-deoxo-28-norcastasterone and 6-deoxo-28-nortyphasterol, were identified in tomato (Yokota et al., 2001). On the other hand, it was reported that 28-norcastasterone was biosynthesized from castasterone in some plant species (Fujioka et al., 2000b). These studies suggest that the biosynthetic pathway of C_{27} BRs is not straightforward.

The biological activity of 28-norcastasterone is approximately 10% that of castasterone (Fujioka et al., 2000b). Therefore, BR activity might be partially regulated by the conversion of C_{28} BRs to C_{27} BRs. We have examined the early steps of BR biosynthesis in order to understand C_{27} BR biosynthesis and its importance in the regulation of BR activity.

In this paper, we demonstrate that both cholesterol (C_{27} sterol) and campestanol (C_{28} sterol) can be biosynthetic precursors of cholestanol (C_{27} sterol). We also provide evidence for the conversion of cholesterol to 6-oxocholestanol via cholest-4-en-3-one and cholestanol, and the conversion of campestanol to 6-oxocampestanol.

2. Results and discussion

2.1. Metabolism of [26, 28-$^2H_6$]campestanol in *A. thaliana*

Although the full biosynthetic sequence of the late C-6 oxidation pathway has been established in *A. thaliana*

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(Noguchi et al., 2000), some early steps of this pathway have yet to be validated in this species. Conversion of campestanol to 6-oxocampestanol was demonstrated in cultured cells of *C. roseus* (Suzuki et al., 1995), but the conversion has not yet been shown in *A. thaliana*. To test whether this conversion occurs in *A. thaliana*, the metabolism of [26, 28-$^2H_6$]campestanol was examined using *A. thaliana* seedlings. After a 2-day incubation, metabolites were extracted and purified using a silica gel cartridge and ODS–HPLC. HPLC-purified fractions were analyzed by gas chromatography–mass spectrometry (GC–MS) after conversion to the trimethylsilyl (TMSi) derivatives. Most of the substrates remained unmetabolized; however, a small amount of [$^2H_6$]6-oxocampestanol was detected [GC retention time relative to cholesterol-TMSi (relative GC R_t): 1.142] as a metabolite of [$^2H_6$]campestanol in the HPLC fraction (R_t: 5.5–6.5 min), together with endogenous 6-oxocampestanol (relative GC R_t: 1.144). The mass spectral data were as follows: (*, metabolite; #, endogenous) *m*/*z* 494* [M^+, 2%], 488# [M^+, 11%], 479* [5%], 473# [22%], 465* [9%], 459# [37%], 159*# [30%]. Therefore, it was shown that campestanol was converted to 6-oxocampestanol in *A. thaliana*.

In addition, a major peak (relative GC R_t: 1.002) of a [$^2H_6$]campestanol metabolite was found in the HPLC fractions with R_t: 14.0–15.5 min. Its mass spectral data are shown in Fig. 1 (*m*/*z* 463 [M^+, 13%], 448 [20%], 406 [9%], 373 [15%], 358 [23%], 215 [100%]). The mass spectrum was very similar to that of authentic [26-$^2H_3$]cholestanol (relative GC R_t: 1.002, *m*/*z* 463 [M^+, 13%], 448 [20%], 406 [8%], 373 [15%], 358 [22%], 215 [100%]). Another possible candidate for the metabolite, [28-$^2H_3$]26-norcampestanol, was excluded because its GC retention time differed from that of the metabolite. Thus, [$^2H_3$]cholestanol was identified as a metabolite of [26, 28-$^2H_6$]campestanol. In this study, [$^2H_3$]cholestanol was detected together with endogenous cholestanol (relative GC R_t: 1.004, Fig. 1). To confirm this finding, we repeated the experiment several times using [$^2H_6$]campestanol. In all experiments, [$^2H_3$]cholestanol was detected as a metabolite of [$^2H_6$]campestanol, and the conversion ratio (the percentage of the detected amount of the metabolite versus the amount of added substrate) averaged 10% (minimum 4%, maximum 16%). Therefore, [$^2H_6$]campestanol is converted to [$^2H_3$]cholestanol in *A. thaliana* seedlings.

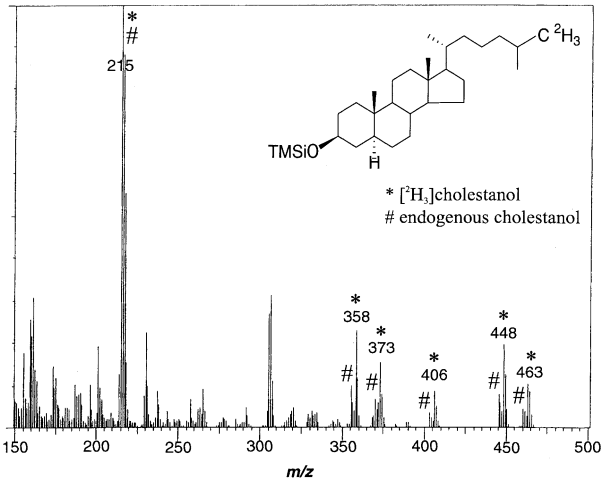

Fig. 1. Gas chromatography–mass spectrometry (GC–MS) analysis of a cholestanol fraction obtained from feeding [26,28-$^2H_6$]campestanol to seedlings of *Arabidopsis thaliana*. *, Metabolite; #, endogenous.

2.2. Metabolism of [26-$^2H_3$]cholesterol in A. thaliana

The conversion of campesterol to campestanol via (24*R*)-24-methylcholest-4-en-3-one has been demonstrated in cultured cells of *C. roseus* and seedlings of *A. thaliana* (Fujioka et al., 1997; Noguchi et al., 1999), and the conversion of campestanol to 6-oxocampestanol has also been demonstrated in cultured cells of *C. roseus* (Suzuki et al., 1995). Therefore, the conversion of cholesterol to 6-oxocholestanol via cholest-4-en-3-one and cholestanol may be possible. To verify this hypothesis, we examined the metabolism of [26-$^2H_3$]cholesterol in seedlings of *A. thaliana*. [$^2H_3$]Cholesterol metabolites were identified by GC–MS. [$^2H_3$]Cholest-4-en-3-one (relative GC R_t: 1.032; HPLC fraction, R_t: 12.5–13.0 min), [$^2H_3$]cholestanol (relative GC R_t: 1.002; HPLC fraction, R_t: 13.5–14.5 min), and [$^2H_3$]6-oxocholestanol (relative GC R_t: 1.090; HPLC fraction, R_t: 5.0–5.5 min) were identified as metabolites of [$^2H_3$]cholesterol, together with endogenous compounds (Table 1). Therefore, cholesterol is converted to cholest-4-en-3-one, cholestanol, and 6-oxocholestanol in *A. thaliana*. Together with the metabolic study of [$^2H_6$]campestanol, this study clearly showed that cholestanol can be biosynthesized from both campestanol (C_{28} sterol) and cholesterol (C_{27} sterol). Metabolic experiments with [$^2H_3$]cholesterol in *A. thaliana* confirmed these conversions. The average conversion ratio of cholesterol to cholestanol was approximately 2% (ranging from 1 to 4%). Since the conversion ratio of campestanol to cholestanol (10%) was five times higher than that of cholesterol to cholestanol (2%), campestanol might be a better biosynthetic source of cholestanol than cholesterol, at least in *A. thaliana*.

2.3. Metabolism of [26-$^2H_3$]cholestanol in A. thaliana

To confirm the conversion of cholestanol to 6-oxocholestanol, seedlings of *A. thaliana* were incubated with [26-$^2H_3$]cholestanol, and the resulting metabolites were analyzed by GC–MS. Although most of the substrate was found to be unmetabolized, a small amount of [$^2H_3$]6-oxocholestanol was identified (relative GC R_t:

Table 1
GC–MS data for the metabolites of [26-$^2H_3$]cholesterol and their endogenous compounds detected in seedlings of *Arabidopsis thaliana*

Identified compounds	Relative GC R_t[a]	Prominent ions m/z [relative intensity %]	Conversion ratio (%)
		(*, metabolite; #, endogenous)	
Cholestanol	1.002* (1.004#)	463* [M^+, 8%], 460# [12%], 448* [13%], 445# [18%], 406* [5%], 403# [6%], 373* [9%], 370# [14%], 358* [13%], 355# [17%], 215*# [100%]	4
Cholest-4-en-3-one	1.032* (1.033#)	387* [40%], 384# [5%], 372* [13%], 369# [5%], 345* [23%], 342# [5%], 302* [12%], 299# [4%], 264* [45%], 261# [9%], 229*# [100%]	23
6-Oxocholestanol	1.090* (1.092#)	477* [M^+, 17%], 474# [7%], 462* [49%], 459# [18%], 448* [100%], 445# [41%], 159*# [41%]	0.3

[a] R_t: retention time relative to cholesterol-TMSi on GC.

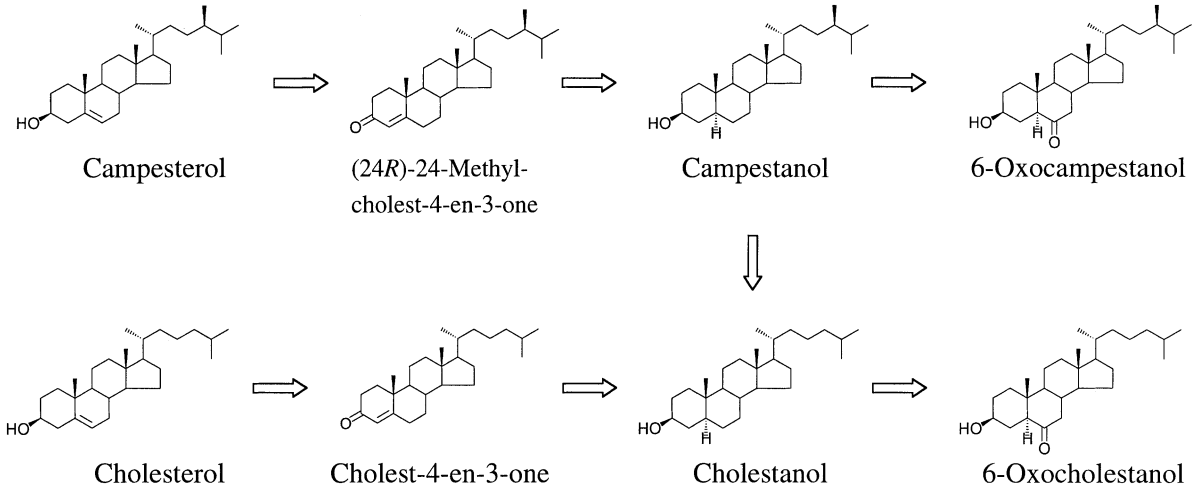

Fig. 2. The proposed biosynthetic pathway of 6-oxocampestanol and 6-oxocholestanol.

1.090) as a metabolite of [$^2H_3$]cholestanol in the HPLC fraction (R_t: 5.0–6.0 min), together with endogenous 6-oxocholestanol (relative GC R_t: 1.092). The prominent ions in the MS of the metabolites were as follows: (*, metabolite; #, endogenous) m/z 477* [M^+, 11%], 474# [M^+, 26%], 462* [21%], 459# [55%], 448* [40%], 445# [100%], 159*# [27%]. Therefore, cholestanol is converted to 6-oxocholestanol in *A. thaliana*. It was concluded that cholestanol is biosynthesized from cholesterol via cholest-4-en-3-one and then converted to 6-oxocholestanol.

2.4. Metabolism of [26, 28-$^2H_6$]campestanol and [26-$^2H_3$]cholesterol in C. roseus and N. tabacum

To test whether campestanol is converted to cholestanol in other higher plants besides *A. thaliana*, the metabolism of [$^2H_6$]campestanol was examined in cultured cells of *C. roseus* and seedlings of *N. tabacum*. After administering [26, 28-$^2H_6$]campestanol, the resulting metabolites were analyzed by GC–MS. [$^2H_3$]Cholestanol was identified as a metabolite of [$^2H_6$]campestanol in both *C. roseus* (conversion ratio: *ca.* 9%), and *N. tabacum* (conversion ratio: *ca.* 3%). Therefore, campestanol is converted to cholestanol in both *C. roseus* and *N. tabacum*.

We also examined whether the conversion of [$^2H_3$]cholesterol to [$^2H_3$]cholestanol occurred in *C. roseus* and *N. tabacum*. GC–MS analysis revealed the presence of [$^2H_3$]cholest-4-en-3-one, [$^2H_3$]cholestanol, and [$^2H_3$]6-oxocholestanol as metabolites of [$^2H_3$]cholesterol in cultured *C. roseus* cells. The average conversion ratios were 48, 24, and 0.4%, respectively. Therefore, cholesterol is converted to cholest-4-en-3-one, cholestanol, and 6-oxocholestanol in *C. roseus*. In *N. tabacum* seedlings, [$^2H_3$]cholest-4-en-3-one and [$^2H_3$]cholestanol were identified as metabolites of [$^2H_3$]cholesterol, but their conversion ratios were less than 1%.

3. Conclusion

This study demonstrated that cholestanol is biosynthesized from both campestanol and cholesterol in

A. thaliana, *C. roseus*, and *N. tabacum* (Fig. 2). Moreover, we showed that cholesterol is converted to cholest-4-en-3-one, cholestanol, and 6-oxocholestanol in *A. thaliana* and *C. roseus*, and cholesterol is converted to cholest-4-en-3-one and cholestanol in *N. tabacum*. The conversion of campestanol to 6-oxocampestanol was demonstrated for the first time in *A. thaliana*, although this conversion had already been shown in *C. roseus*. Thus, this study provides evidence to support a biosynthetic sequence cholesterol→cholest-4-en-3-one→cholestanol→6-oxocholestanol, and cross-linked paths of campestanol to cholestanol synthesis in higher plants (Fig. 2). Although we looked for the conversion of campesterol to cholesterol, and the conversion of (24*R*)-24-methyl-5α-cholest-4-en-3-one to cholest-4-en-3-one, such conversions were not found. Only the conversion of campestanol to cholestanol was detected. Perhaps the conversion of C_{28} sterols to C_{27} sterols occurs only at particular points in the pathway, and this substrate specificity may be important for understanding the physiological significance of sterol metabolism.

4. Experimental

4.1. General

GC–MS analysis was carried out on a JEOL Automass JMS-AM 150 mass spectrometer connected to a Hewlett-Packard 5890-A-II gas chromatograph with a capillary DB-5 column (0.25 mm×15 m, 0.25 μm film thickness). The analytical conditions were the same as previously described (Noguchi et al., 1999).

4.2. Synthesis of [26-$^2H_3$]cholesterol and [26-$^2H_3$]cholestanol

According to the published method (Takatsuto et al., 1981), [26-$^2H_3$]cholesta-5,22*E*-dien-3β-ol (86.1 mg), mp 131–132 °C (MeOH) [non-labeled form, mp 130–132 °C (Takatsuto et al., 1981)], was prepared from a known 3β-tetrahydropyranyloxycholesta-5,22*E*-dien-26-oic acid ethyl ester (163.2 mg; Eguchi et al., 1982) using $LiAlD_4$ in place of $LiAlH_4$.

[26-$^2H_3$]Cholesta-5,22*E*-dien-3β-ol (16 mg) was hydrogenated (H_2/10% Pd-C, ethyl acetate, room temp., overnight) and then purified by preparative thin layer chromatography (TLC; Merck Kiesel gel 60, 0.5 mm thickness; R_f 0.09–0.19; developing solvent, *n*-hexane/ethyl acetate, 5/1, v/v) to give [26-$^2H_3$]cholestanol (5.5 mg): mp 138–139 °C (MeOH), ^{1}H NMR spectral data (400 MHz, $CDCl_3$) δ: 0.647 (3H, *s*, H-18), 0.802 (3H, *s*, H-19), 0.856 (1H, *d*, *J*=6.83 Hz, H-26), 0.860 (2H, *d*, *J*=6.34 Hz, H-27), 0.897 (3H, *d*, *J*=6.84 Hz, H-21), 3.586 (1H, *m*, H-3α); EIMS *m*/*z*: 391 (M^+, 100), 376 (21), 358 (8), 265 (7), 248 (13), 233 (60), 215 (43), 165 (21), 121 (11), 107 (15); HR–EIMS $[M]^+$ *m*/*z*: 391.3889 (calc. 391.3896) for $C_{27}H_{45}D_3O$.

According to published methods (Fujimoto and Ikekawa, 1979; Hirano et al., 1984), [26-$^2H_3$]cholesta-5,22*E*-dien-3β-ol (64.9 mg) was converted by sulfonation, methanolysis, hydrogenation as mentioned earlier, and acid treatment to [26-$^2H_3$]cholesterol (23.5 mg); mp 146–147 °C (MeOH), ^{1}H NMR spectral data (400 MHz, $CDCl_3$) δ: 0.679 (3H, *s*, H-18), 0.859 (1H, *d*, *J*=6.34 Hz, H-26), 0.863 (2H, *d*, *J*=6.83 Hz, H-27), 0.915 (3H, *d*, *J*=6.35 Hz, H-21), 1.009 (3H, *s*, H-19), 3.518 (1H, *m*, H-3α), 5.352 (1H, *m*, H-6); EIMS *m*/*z*: 389 (M^+, 100), 371 (33), 356 (19), 304 (22), 278 (32), 255 (14), 231 (11), 213 (15), 145 (13), 107 (12); HR–EIMS $[M]^+$ *m*/*z*: 389.3739 (calc. 389.3739) for $C_{27}H_{43}D_3O$.

4.3. Metabolism of [26, 28-$^2H_6$]campestanol, [26-$^2H_3$]cholesterol, and [26-$^2H_3$]cholestanol in seedlings of *A. thaliana*

Before the precursor-administration experiments, 7-day-old *A. thaliana* (wild type: Columbia: 15 seedlings) seedlings were transferred to 200-ml flasks containing 30 ml of half-strength MS medium supplemented with 1% sucrose. The plants were grown at 22 °C under continuous light. Seven days after transfer, [26, 28-$^2H_6$]campestanol (10 μg) dissolved in MeOH solution (10 μl) was added to each flask. The seedlings were incubated for 2 days at 22 °C in the light, on a shaker (120 rpm), and then extracted with MeOH. The MeOH extract was partitioned between $CHCl_3$ and H_2O and the $CHCl_3$-soluble fraction was purified with a silica gel cartridge (Sep-Pak Vac 2 g; Waters, Milford, MA), which was eluted with 40 ml $CHCl_3$. This fraction was purified by HPLC on a 150×4.6-mm Senshu Pak ODS-1151-D column (Senshu Scientific Co., Ltd., Tokyo) using MeOH as the mobile phase at a flow rate of 1.0 ml/min. Fractions were collected at 0.5-min intervals (Rt of 2–20 min). Each fraction was subjected to GC–MS analysis after derivatization with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide at 80 °C for 30 min. Experiments involving administration of a MeOH solution (5 μl) of [26-$^2H_3$]cholesterol (5 μg) and an acetone solution (20 μl) of [26-$^2H_3$]cholestanol (50 μg) were carried out similarly.

4.4. Metabolism of [26, 28-$^2H_6$]campestanol and [26-$^2H_3$]cholesterol in cultured cells of *C. roseus*

Cultured cells of *C. roseus* (V208) were grown in MS media supplemented with 3% sucrose at 27 °C by shaking at 100 rpm in the dark. A MeOH solution (10 μl) of [$^2H_6$]campestanol (10 μg) was added to a 100-ml flask containing cultured cells, which were grown for 7 days in 30 ml MS medium. After a 2-day incubation, cultures were extracted with MeOH, and the extract was

purified and analyzed by the same method as described for *A. thaliana*. Similar experiments were carried out in which a MeOH solution (5 μl) of [$^2H_3$]cholesterol (5 μg) was added to 200-ml flasks containing cultured cells, which were grown for about 7 days in 60-ml MS medium.

4.5. Metabolism of [26, 28-$^2H_6$]campestanol and [26-$^2H_3$]cholesterol in seedlings of N. tabacum

Seedlings of *N. tabacum* were grown in pots containing soil for 4 weeks at 22 °C under continuous light. Before the administration experiment, the plants were transferred to water culture in 30-ml conical flasks containing 20 ml H_2O and allowed to grow for 3 days. The seedlings were then ready to be used for metabolism experiments. Through all growth stages, the plants were grown at 22 °C under continuous light. A MeOH solution (10 μl) of [$^2H_6$]campestanol (10 μg) was added to each 30-ml flask containing a seedling. After a 2-day incubation, seedlings were extracted with MeOH, and the extract was purified and analyzed using the method described earlier. The experiments using a MeOH solution (10 μl) of [$^2H_3$]cholesterol (10 μg) were carried out by the same method.

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