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AN ALTERNATIVE BRASSINOLIDE BIOSYNTHETIC PATHWAY VIA LATE C-6 OXIDATION

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Key Word Index—*Catharanthus roseus*; Apocynaceae; cultured cells; brassinosteroid biosynthesis; (22*R*, 23*R*, 24*S*)-3β,22,23-trihydroxy-24-methyl-5α-cholestane (6-deoxoteasterone); 3-dehydro-6-deoxoteasterone; 6-deoxotyphasterol; 6-deoxocastasterone; castasterone; brassinolide.

Abstract—6-Deoxoteasterone and 6-deoxotyphasterol were identified in cultured cells of *Catharanthus roseus*. Feeding experiments with deuterium-labelled substrates revealed the conversions of 6-deoxoteasterone to 6-deoxotyphasterol, and of 6-deoxotyphasterol to 6-deoxocastasterone in cultured cells of *C. roseus*. Furthermore, the conversion of 3-dehydro-6-deoxoteasterone to 6-deoxotyphasterol was also demonstrated in the cells. These results, together with our previously published data, demonstrate an alternative biosynthetic pathway of brassinolide production: 6-deoxoteasterone→3-dehydro-6-deoxoteasterone→6-deoxotyphasterol→6-deoxocastasterone→castasterone→brassinolide. This is termed the late C-6 oxidation pathway, and it is operated in cultured cells of *C. roseus* in addition to the early C-6 oxidation pathway. Copyright © 1997 Elsevier Science Ltd

INTRODUCTION

Brassinosteroids are steroidal plant hormones that are ubiquitously distributed in plants and stimulate growth and development [1]. Recently, dwarf mutants of *Arabidopsis* exhibiting de-etiolation, *cpd* [2], *det2* [3] and *dim* (N.-H. Chua and U. Klahre, personal communication) have been shown to benefit from external addition of brassinosteroids, suggesting that these mutants are deficient in biosynthesis of brassinosteroids. Moreover, the short *Pisum sativum* mutants *lka* and *lkb* have recently been found to be possible brassinosteroid-related mutants (T. Yokota and T. Nomura, unpublished results). These findings indicate that brassinosteroids play essential roles in growth and development of plants. Establishment of biosynthetic pathways of brassinosteroids is now crucial not only for characterizing the genes encoding enzymes of certain steps in brassinosteroid biosynthesis but also for elucidating the function of brassinosteroids in plant growth and development.

We have studied the biosynthesis of brassinosteroids using the cultured cells of *Catharanthus roseus*, which facilitated the investigation of metabolic conversion of the biosynthetic intermediates. Consequently, we have shown the biosynthetic sequence of campesterol → campestanol → 6α-hydroxycampestanol → 6-oxocampestanol, and the sequence cathasterone → teasterone **(7)** → 3-dehydroteasterone **(8)** → typhasterol **(9)** → castasterone **(5)** → brassinolide **(6)** [4–10]. We previously reported that 6-deoxocastasterone **(4)** was found in cultured cells of *C. roseus* and that it was converted to castasterone **(5)** in cultured cells and seedlings of *C. roseus* and also in *Oryza sativa* and *Nicotiana tabacum* [11]. In addition, the natural occurrence of 6-deoxocastasterone **(4)**, 6-deoxotyphasterol **(3)** and 3-dehydro-6-deoxoteasterone **(2)** was reported in various plant species [12–16]. These lines of evidence suggested the presence of two alternative pathways of brassinolide biosynthesis, namely, an early C-6 oxidation pathway, in which oxidation at C-6 occurs before the introduction of vicinal hydroxyls at C-22 and C-23 of the side chain, as we have shown already, and a late C-6 oxidation pathway, in which C-6 is oxidized after the introduction of hydroxyls at the side chain and C-2 of the A ring. This latter pathway remains unverified.

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In the present study, we first investigated the natural occurrence of 6-deoxo-type brassinosteroids such as 6-deoxoteasterone **(1)** and 6-deoxotyphasterol **(3)** in the cultured cells (V208) of *C. roseus*. We then carried out feeding experiments with deuterium-labelled 6-deoxo-type brassinosteroids using V208 cells to elucidate the existence of the late C-6 oxidation pathway.

RESULTS

Identification of 6-*deoxoteasterone* **(1)** *and* 6-*deoxotyphasterol* **(3)** *in cultured cells of* C. roseus

The cultured cells (V208) of *C. roseus* were extracted with methanol, and [$^2H_6$]6-deoxoteasterone and [$^2H_6$]6-deoxotyphasterol (100 pg g^{-1} fresh wt each) were added to the extract as internal standards, and then purified according to the methods described in the Experimental. In the final purification step, by C_{18} reversed phase HPLC, fractions were collected every 2 min (R_t 40–60 min). Each fraction was derivatized to give the methaneboronate-trimethylsilyl ether (MB-TMSi) and subjected to GC-selected ion monitoring or GC–mass spectrometry.

In GC-selected ion monitoring analysis, ions at *m/z* 530 $[M]^+$ and 515 $[M-15]^+$ for non-labelled 6-deoxoteasterone **(1)** MB-TMSi and ions at *m/z* 536 $[M]^+$ and 521 $[M-15]^+$ for [$^2H_6$]6-deoxoteasterone MB-TMSi were monitored. From the HPLC fraction 4 (R_t 46–48 min), ions derived from non-labelled, endogenous 6-deoxoteasterone **(1)** were clearly detected along with ions derived from [$^2H_6$]6-deoxoteasterone. Relative intensities of ions at *m/z* 530 and 515 were similar to those of ions at *m/z* 536 and 521. The R_t of the MB-TMSi derivative of endogenous 6-deoxoteasterone **(1)** on GC was *ca* 1 sec later than that of [$^2H_6$]6-deoxoteasterone due to the isotope effect.

In GC-mass spectral analysis of the HPLC fraction 9 (R_t 56–58 min), ions characteristic of the non-labelled, endogenous 6-deoxotyphasterol **(3)** MB-TMSi were observed at *m/z* 530 ($[M^+]$, 28), 515 (6), 440 (33), 425 (46), 215 (100), and 155 (24). Low abundance ions (at *m/z* 536, 521 and 161) characteristic of the [$^2H_6$]6-deoxotyphasterol MB-TMSi were observed in the mass spectrum. Thus, the above evidence demonstrated that 6-deoxoteasterone **(1)** and 6-deoxotyphasterol **(3)** are endogenous in cultured cells of *C. roseus*. The contents of 6-deoxoteasterone **(1)** and 6-deoxotyphasterol **(3)** in the cells were 47 and 758 pg g^{-1} fresh wt, respectively.

The metabolism of 6-*deoxoteasterone* **(1)**

The cultures in the stationary phase were incubated with 2 μg of [$^2H_6$]6-deoxoteasterone. After incubation for 24 h, the metabolites were extracted and separated into 3-dehydro-6-deoxoteasterone **(2)** and 6-deoxotyphasterol **(3)** fractions. Each fraction was derivatized and subjected to GC–mass spectral analysis. From the fraction corresponding to 6-deoxotyphasterol **(3)**, [$^2H_6$]6-deoxotyphasterol was identified as its MB-TMSi derivative [536 (M^+, 61), 521 (8), 446 (17), 431 (17), 215 (100), 161 (54)], indicating the conversion of 6-deoxoteasterone **(1)** to 6-deoxotyphasterol **(3)**. However, neither the endogenous 3-dehydro-6-deoxoteasterone **(2)** nor [$^2H_6$]3-dehydro-6-deoxoteasterone was detected in this feeding experiment.

The metabolism of 3-*dehydro*-6-*deoxoteasterone* **(2)**

The metabolites obtained from the cultures incubated with 2 μg of [$^2H_6$]3-dehydro-6-deoxoteasterone for 24 hr, were separated into 6-deoxoteasterone **(1)** and 6-deoxotyphasterol **(3)** fractions. From the fraction corresponding to 6-deoxotyphasterol **(3)**, [$^2H_6$]6-deoxotyphasterol was identified after conversion to the MB-TMSi derivative [536 ($[M]^+$, 61), 521 (8), 446 (18), 431 (17), 215 (100), 161 (74)]. Thus, the conversion of 3-dehydro-6-deoxoteasterone **(2)** to 6-deoxotyphasterol **(3)** was demonstrated in cultured cells of *C. roseus*. Unlike the conversion of 3-dehydroteasterone **(8)** to teasterone **(7)** [7], the conversion of [$^2H_6$]3-dehydro-6-deoxoteasterone to [$^2H_6$]6-deoxoteasterone was not detected.

The metabolism of 6-*deoxotyphasterol* **(3)**

A culture in the log phase was incubated with 5 μg of [$^2H_6$]6-deoxotyphasterol. After incubation for 12 hr, the metabolite was purified as described in the Experimental section. The fraction corresponding to 6-deoxocastasterone **(4)** was analysed by GC-mass spectrometry. Although ions derived from endogenous 6-deoxocastasterone **(4)** were predominant in the mass spectrum, minor but distinct ions (at *m/z* 504 and 161) corresponding to [$^2H_6$]6-deoxocastasterone MB-TMSi were detected (*ca* 8% of the endogenous 6-deoxocastasterone **(4)**. GC-selected ion monitoring analysis with *m/z* 504, 498, 489, 483, 161 and 155 ions confirmed the presence of [$^2H_6$]6-deoxocastasterone and also endogenous 6-deoxocastasterone **(4)** (R_t of [$^2H_6$]6-deoxocastasterone MB-TMSi was *ca* 1 sec earlier than that of 6-deoxocastasterone, **(4)**. The results showed the conversion of 6-deoxotyphasterol **(3)** to 6-deoxocastasterone **(4)** in cultured cells of *C. roseus*.

DISCUSSION

Previously, we have identified 6-deoxocastasterone **(4)** [11] as well as cathasterone, teasterone **(7)**, typhasterol **(9)**, castasterone **(5)** and brassinolide **(6)** [6, 9, 17] as endogenous brassinosteroids of cultured cells of *C. roseus*. The present study revealed the occurrence of 6-deoxoteasterone **(1)** and 6-deoxotyphasterol **(3)** in the cultured cells of *C. roseus*. This is the first report on the occurrence of 6-deoxoteasterone **(1)** in the plant kingdom, while the occurrence of 6-deoxotyphasterol **(3)** has already been reported in the pollen of *Cupressus arizonica* [15]. The concentration of 6-deoxo-

teasterone **(1)** was lower than 50 pg g^{-1} fresh wt, being about the same as the concentration of teasterone **(7)** and typhasterol **(9)** [6], but the amount of 6-deoxotyphasterol **(3)** was 15-fold higher.

As with the early C-6 oxidation pathway, 6-deoxoteasterone **(1)** could be converted to 6-deoxotyphasterol **(3)** via 3-dehydro-6-deoxoteasterone **(2)**, because the conversions of 6-deoxoteasterone **(1)** and 3-dehydro-6-deoxoteasterone **(2)** to 6-deoxotyphasterol **(3)** were conclusively demonstrated. Further, the conversion of 6-deoxotyphasterol **(3)** to 6-deoxocastasterone **(4)** was established. We have previously shown the conversion of 6-deoxocastasterone **(4)** to brassinolide **(6)** [11]. Therefore, the sequential steps of the late C-6 oxidation pathway, 6-deoxoteasterone **(1)** → 3-dehydro-6-deoxoteasterone **(2)** → 6-deoxotyphasterol **(3)** → 6-deoxocastasterone **(4)** → castasterone **(5)** → brassinolide **(6)**, were verified (Scheme 1). Since 6-deoxoteasterone **(1)** and 6-deoxotyphasterol **(3)** were not converted to teasterone **(7)** and typhasterol **(9)**, respectively, it is most likely that in the C-6 oxidation pathway only 6-deoxocastasterone **(4)** suffers oxidation at C-6 to give castasterone **(5)**.

Thus, we have shown that both an early C-6 oxidation pathway and a late C-6 oxidation pathway are operating in the cultured cells of *C. roseus*, suggesting the presence of multiple pathways of brassinosteroid biosynthesis in plants similar to the other plant hormones. Campesterol or campestanol should also be the precursor of 6-deoxoteasterone **(1)**. However, the metabolic pathway from these plant sterols has not been elucidated.

EXPERIMENTAL

Plant material. The cultured cells (V208) of *C. roseus* was used. They were cultured in a manner described in ref. [5].

GC-MS analysis. GC-MS analysis was carried out on a JEOL Automass JMS-AM 150 mass spectrometer connected with a Hewlett-Packard 5890A-II gas chromatograph with capillary column DB-5 (0.25 mm × 15 m, 0.25 μm film thickness). The temp. of GC and analytical conditions were the same as ref. [6]. The sample corresponding to 6-deoxocastasterone **(4)** was treated with pyridine containing methaneboronic acid (2 mg ml^{-1}). The samples corresponding to 6-deoxoteasterone **(1)**, 3-dehydro-6-deoxoteasterone **(2)** and 6-deoxotyphasterol **(3)** were treated with pyridine containing methaneboronic acid (2 mg ml^{-1}), then with *N*-methyl-*N*-trimethylsilyltrifluoroacetamide. Typical R_t values of 6-deoxocastasterone, 6-deoxotyphasterol and 6-deoxoteasterone derivatives were 10.55, 10.05 and 10.45 min, respectively.

Synthesis of [$^2H_6$]*6-deoxoteasterone.* [$^2H_6$]Teasterone (2.5 mg) was subjected to the Huang–Minlon reduction according to ref. [18] followed by silica gel CC (elution with $CHCl_3$) and successive crystallization from MeCN to give [$^2H_6$]6-deoxoteasterone (1.5 mg), mp 227°. ^1H NMR (400 MHz, $CDCl_3$); δ 0.68 (3H, *s*), 0.81 (3H, *s*), 0.90 (3H, *d*, J = 5 Hz), 0.97 (3H, *d*, J = 7 Hz), 3.56 (1H, *d*, J = 8.5 Hz), 3.60 (1H, *m*), 3.71 (1H, *d*, J = 8.5 Hz). GC–MS (as MB-TMSi) 70 eV, *m/z* (rel. int.): 536 $[M]^+$ (92), 215 (100).

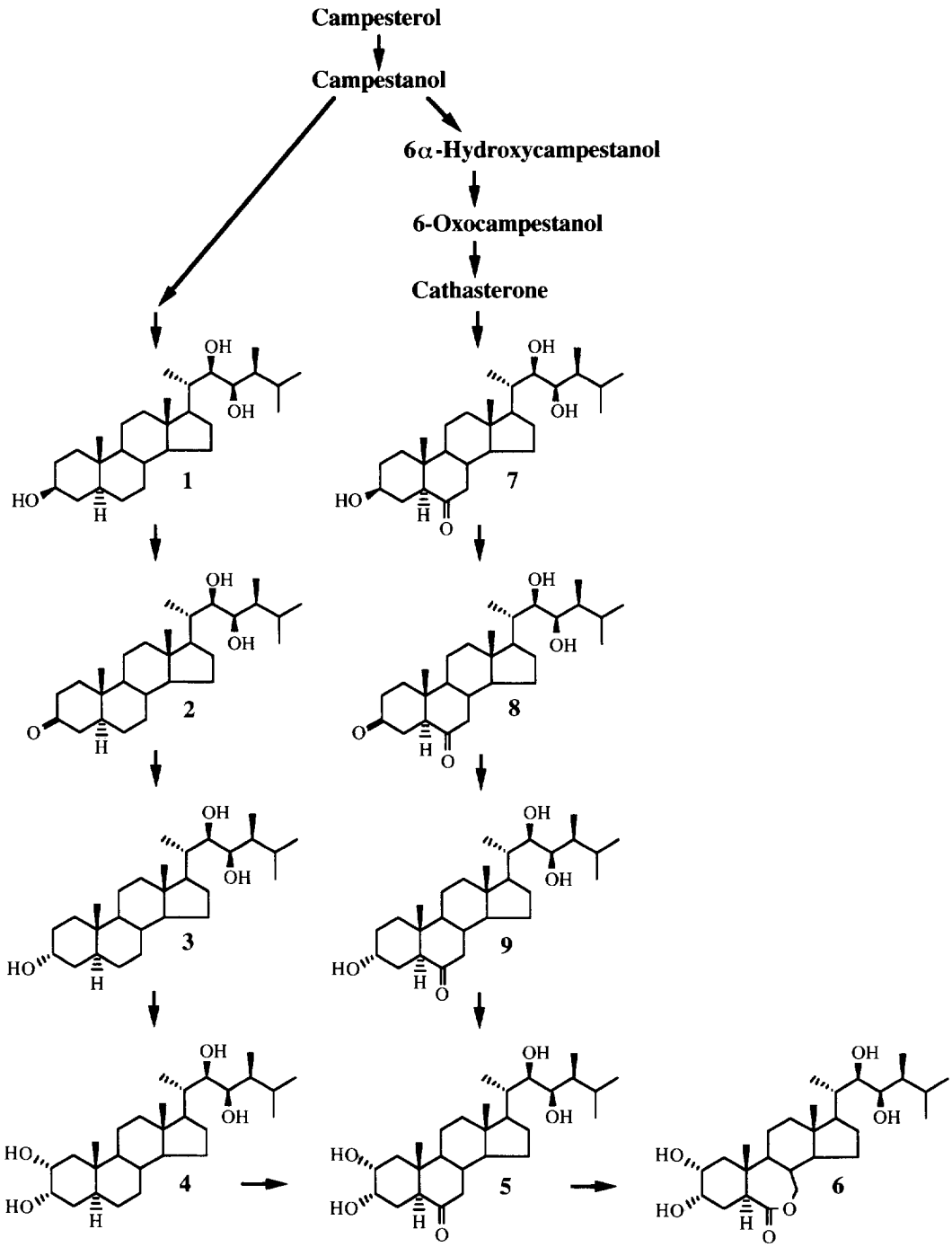
Synthesis of [$^2H_6$]*6-deoxotyphasterol.* [$^2H_6$]Typhasterol (5.5 mg) was subjected to the Huang–Minlon reduction according to ref. [18]. The reaction mixt. was purified by silica gel CC (elution with 0.5% MeOH in $CHCl_3$) and then by C_{18} RP-HPLC (gradient from 45% MeCN to 100% MeCN) to yield fast-moving [$^2H_6$]6-deoxoteasterone (0.9 mg) and slow-moving [$^2H_6$]6-deoxotyphasterol (1 mg), mp 224°. ^1H NMR of the latter (400 MHz, $CDCl_3$); δ 0.66 (3H, *s*), 0.78 (3H, *s*), 0.89 (3H, *d*, J = 6 Hz), 0.96 (3H, *d*, J = 6 Hz), 3.56 (1H, *d*, J = 8 Hz); 3.72 (1H, *d*, J = 8 Hz), 4.04 (1H, *br*). GC–MS (as MB-TMSi) 70 eV, *m/z* (rel. int.): 536 $[M]^+$ (47), 215 (100).

Synthesis of [$^2H_6$]*3-dehydro-6-deoxoteasterone.* [$^2H_6$]6-deoxoteasterone (1.64 mg) was treated according to Yokota *et al.* [19], purified on HPLC as described above and crystallized from MeCN to yield [$^2H_6$]3-dehydro-6-deoxoteasterone (750 μg), mp 167°. ^1H NMR (400 MHz, $CDCl_3$); δ 0.70 (3H, *s*), 0.90 (3H, *d*, J = 6), 0.96 (3H, *d*, J = 6.8 Hz), 1.01 (3H, *s*), 3.56 (1H, *d*, J = 8 Hz), 3.71 (1H, *d*, J = 8 Hz). GC–MS (as methaneboronate) 70 eV, *m/z* (rel. int.): 462 $[M]^+$ (87), 231 (100).

Identification of endogenous 6-deoxoteasterone **(1)** *and 6-deoxotyphasterol* **(3)**. The cells (400 g fresh wt) of *C. roseus* harvested at day-11 and day-12 were extracted 2 × with MeOH (1.5 l.). The extract was supplemented with [$^2H_6$]6-deoxoteasterone (40 ng) and [$^2H_6$]6-deoxotyphasterol (40 ng) as internal standards, and partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fr. was subjected to *CC* on silica gel and Sephadex LH-20, according to the method in ref. [6]. Finally, the fr. was purified by C_{18} RP-HPLC (10 × 50 mm + 20 × 250 mm), with 80% MeCN at a flow rate of 8 ml min^{-1}. Ten frs were collected every 2 min (R_t 40–60 min). All frs were subjected to GC–MS analysis after derivatization. The main frs of 6-deoxoteasterone **(1)** and 6-deoxotyphasterol **(3)** were fr. 4 (R_t 46–48 min) and fr. 9 (R_t 56–58 min), respectively. The levels of endogenous 6-deoxoteasterone **(1)** and 6-deoxotyphasterol **(3)** were determined as the ratio of the peak areas of molecular ions for the endogenous brassinosteroid and for the internal standard.

Feeding of [$^2H_6$]*6-deoxoteasterone,* [$^2H_6$]*3-dehydro-6-deoxoteasterone and* [$^2H_6$]*6-deoxotyphasterol.* Each deuterated compound, [$^2H_6$]6-deoxoteasterone (2 μg), [$^2H_6$]3-dehydro-6-deoxoteasterone (2 μg) and [$^2H_6$]6-deoxotyphasterol (5 μg), was dissolved in MeOH and added to a 200 ml flask containing V208 cells grown in 60 ml of Murashige–Skoog medium. The cells were incubated for 24 hr for the [$^2H_6$]6-deoxoteasterone and [$^2H_6$]3-dehydro-6-deoxoteasterone feeds, and for 12 hr for the [$^2H_6$]6-deoxotyphasterol feed.

Analysis of labelled metabolites. After incubation,



Scheme 1. Proposed biosynthetic pathways of brassinolide formation. **1**: 6-deoxoteasterone; **2:** 3-dehydro-6-deoxoteasterone; **3:** 6-deoxotyphasterol; **4:** 6-deoxocastasterone; **5:** castasterone; **6:** brassinolide; **7:** teasterone; **8:** 3-dehydroteasterone; and **9:** typhasterol.

the cultures were extracted with MeOH, and the extract was partitioned between $CHCl_3$ and H_2O. Purification was performed under the conditions as previously described [11]. Each fraction of the metabolites was subjected to GC-MS analysis after derivatization.

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