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Biosynthesis of brassinosteroids in cultured cells of *Catharanthus roseus*

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Abstract

Precursor administration experiments with ^{2}H-labeled 6-oxocampestanol, 6-deoxocastasterone and 6α-hydroxycastasterone in cultured cells of *Catharanthus roseus* were performed and the metabolites were analyzed by GC-MS. [$^2H_6$]Cathasterone was identified as a metabolite of [$^2H_6$]6-oxocampestanol, whereas [$^2H_6$]6α-hydroxycastasterone and [$^2H_6$]castasterone were identified as metabolites of [$^2H_6$]6-deoxocastasterone, and [$^2H_6$]castasterone was identified as a metabolite of [$^2H_6$]6α-hydroxycastasterone, indicating that 6-deoxocastasterone is converted to castasterone via 6α-hydroxycastasterone. In addition, 6-deoxocathasterone, a putative biosynthetic intermediate in the late C6-oxidation pathway, was identified as an endogenous brassinosteroid. These studies provide further evidence supporting our proposed biosynthetic pathways for brassinolide.

Keywords: *Catharanthus roseus*; Apocynaceae; Biosynthesis; Biosynthetic intermediates of brassinolide; Cathasterone; 6-Deoxocathasterone; 3-*epi*-6-Deoxocathasterone; 6-Deoxocastasterone; 6α-Hydroxycastasterone; Castasterone

1. Introduction

Brassinosteroids (BRs) are one of six classes of plant hormones that play an important role in plant growth and development. We have been studying their biosynthetic origin by administering labeled compounds of possible BR intermediates to cultured cells of *Catharanthus roseus* and analyzing the metabolites by GC-MS. The data suggests the presence of two parallel branched pathways, the early (see Fig. 1) and late C6-oxidation pathways (Fujioka & Sakurai, 1997a, 1997b). Genetic, molecular and biochemical studies with *Arabidopsis* (Li, Nagpal, Vitart, McMorris & Chory, 1996; Szekeres et al., 1996; Fujioka et al., 1997; Choe, Dilkes, Fujioka, Takatsuto, Sakurai & Feldmann, 1998; Klahre et al., 1998; Choe et al., 1999a, 1999b; Ephritikhine et al., 1999; Noguchi et al., 1999), pea (Nomura, Nakayama, Reid, Takeuchi & Yokota, 1997; Nomura, Kitasaka, Takatsuto, Reid, Fukami & Yokota, 1999) and tomato (Bishop et al., 1999; Koka et al., 2000) also support the presence of the proposed pathways. Some steps, however, have yet to be defined, e.g. the conversion of 6-deoxocastasterone (**1**) to castasterone (**3**). Although the conversion has been demonstrated not only in cultured cells of *C. roseus*, but also in cultured cells and intact seedlings of rice and tobacco (Choi, Fujioka, Harada, Yokota, Takatsuto & Sakurai, 1996), this conversion probably involves two steps, with 6α-hydroxycastasterone (**2**) as the biosynthetic intermediate in the conversion. These steps are analogous to the biosynthetic sequence cam-

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pestanol → 6α-hydroxycampestanol → 6-oxocampestanol, a sequence demonstrated in the earlier biosynthetic pathway (Suzuki et al., 1995). Another putative step is the conversion of 6-oxocampestanol (**4**) to cathasterone (**5**). Although the natural occurrence of 6-oxocampestanol (**4**) and cathasterone (**5**) has been demonstrated in cultured cells of *C. roseus* (Fujioka, Inoue, Takatsuto, Yanagisawa, Yokota & Sakurai, 1995; Suzuki et al., 1995), the metabolic conversion of 6-oxocampestanol (**4**) to cathasterone (**5**) has yet to be shown to occur. In addition, the natural occurrence of the biosynthetic precursor of 6-deoxoteasterone

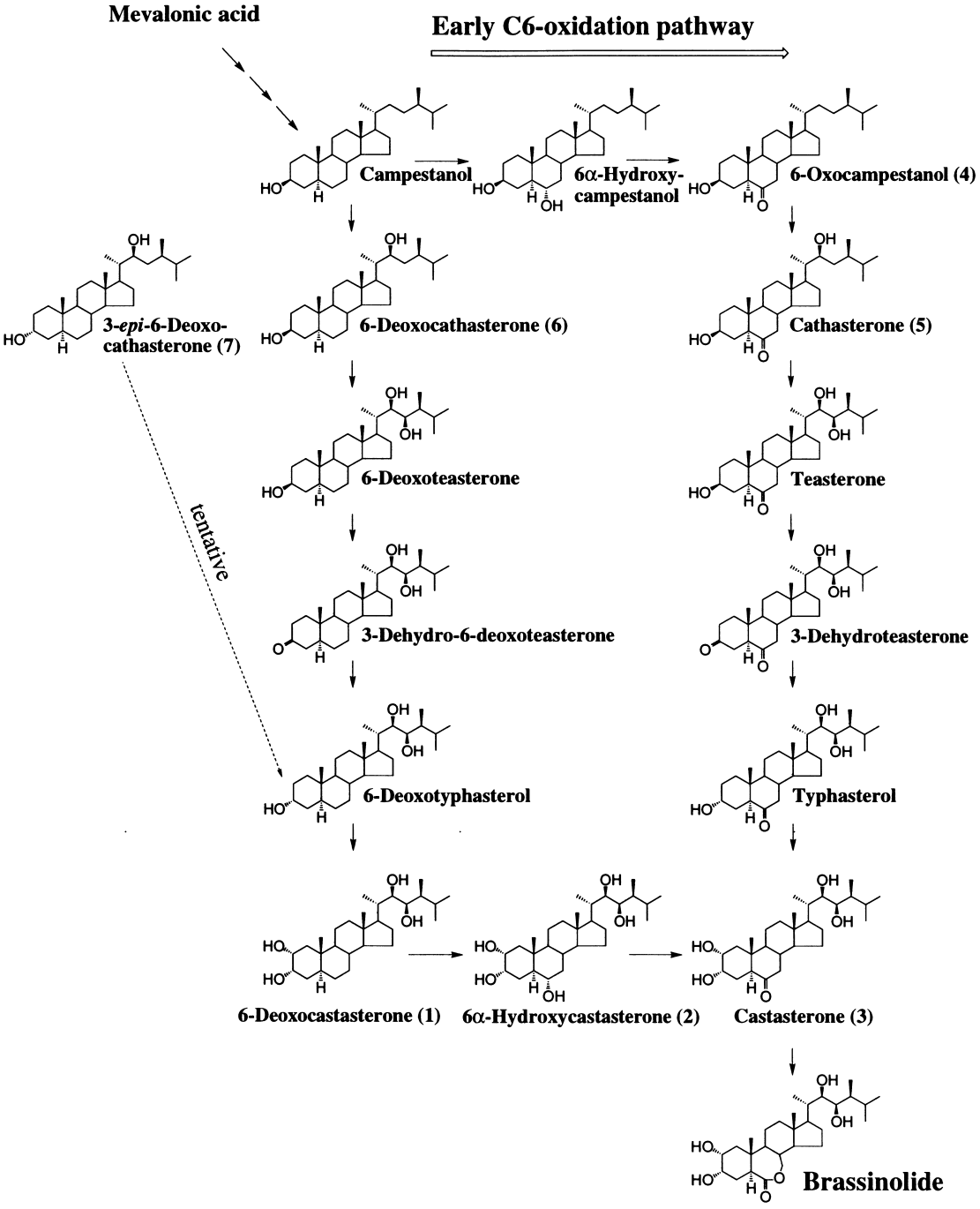

Fig. 1. Proposed biosynthetic pathways for brassinolide. In this study, the conversion of 6-oxocampestanol (**4**) to cathasterone (**5**), and the conversion of 6-deoxocastasterone (**1**) to castasterone (**3**) via 6α-hydroxycastasterone (**2**) have been demonstrated in cultured cells of *Catharanthus roseus*. In addition, the occurrence of 6-deoxocathasterone (**6**) and 3-*epi*-6-deoxocathasterone (**7**) in *C. roseus* has been established. The conversion of 3-*epi*-6-deoxocathasterone (**7**) to 6-deoxotyphasterol has not yet been demonstrated.

remains unknown in cultured cells of *C. roseus*, although recently the presumed intermediate has been identified from tomato (Bishop et al., 1999).

In the present study, we report metabolic experiments to provide conclusive evidence for the conversion of 6-oxocampestanol (**4**) to cathasterone (**5**), and the conversion of 6-deoxocastasterone (**1**) to castasterone (**3**) via 6α-hydroxycastasterone (**2**). In addition, we have examined the natural occurrence of 6-deoxocathasterone (**6**).

2. Results and discussion

To test the involvement of 6α-hydroxycastasterone (**2**) in the conversion of 6-deoxocastasterone (**1**) to castasterone (**3**), the metabolism of [$^2H_6$]**1** and [$^2H_6$]**2** was examined in the cultured cells of *C. roseus*.

Cultures in the log phase of growth were incubated with [$^2H_6$]6-deoxocastasterone. After incubation (for 2 and 4 days), the metabolites were extracted and separated into **2** and **3** fractions, respectively. Each fraction was derivatized and subjected to GC-MS. Both incubations gave similar results. From the fraction corresponding to **2**, [$^2H_6$]6α-hydroxycastasterone was identified by full-scan GC-MS ([$^2H_6$]**2**-bismethaneboronate-trimethylsilyl ether (BMB-TMSi); *m*/*z* (rel. int.) 592 [M^+] (5), 577 (29), 502 (100), 443 (10), 271 (97) and 161 (90)). In addition, from the fraction corresponding to **3**, [$^2H_6$]castasterone was identified by full-scan GC-MS ([$^2H_6$]**3**-bismethaneboronate (BMB); *m*/*z* (rel. int.) 518 [M^+] (48), 441 (3), 399 (9), 358 (16), 287 (32), 161 (100)). Ions (*m*/*z* 512 and 155) of endogenous castasterone-bismethaneboronate were also detected. These results demonstrated that 6-deoxocastasterone (**1**) is converted to 6α-hydroxycastasterone (**2**) and castasterone (**3**).

The metabolism of [$^2H_6$]6α-hydroxycastasterone was also investigated. Cultures in log phase were incubated with [$^2H_6$]6α-hydroxycastasterone. After incubation (for 2, 4 and 6 days), the metabolites were extracted and purified. Each castasterone (**3**) fraction was derivatized and analyzed by GC-MS; [$^2H_6$]castasterone was identified, along with a small amount of endogenous castasterone (**3**), in all precursor administration experiments examined. The mass spectra and retention time were identical to those of an authentic specimen. Thus, the conversion of 6α-hydroxycastasterone (**2**) to castasterone (**3**) was demonstrated. From both experiments, it was shown that 6-deoxocastasterone (**1**) is converted to castasterone (**3**) via 6α-hydroxycastasterone (**2**) (see Fig. 1).

In a previous study, cathasterone (**5**) was identified as a novel BR in cultured cells of *C. roseus* (Fujioka et al., 1995). However, the biosynthetic origin of cathasterone (**5**) remained unknown. To determine the immediate biosynthetic precursor of cathasterone (**5**), the metabolism of [$^2H_6$]6-oxocampestanol was investigated. Cultures in the early log phase of growth were incubated with [$^2H_6$]6-oxocampestanol. After incubation for 10 days, the metabolites were extracted and purified. The fraction corresponding to cathasterone (**5**) was trimethylsilylated and analyzed by GC-MS. A mass spectrum derived from a mixture of endogenous cathasterone (**5**) and [$^2H_6$]**5** (a metabolite of [$^2H_6$]6-oxocampestanol) was obtained. Prominent ion peaks were as follows (∗: metabolite, #: endogenous): *m*/*z* (rel. int.) 567∗ [M^+-15] (1), 561# (4), 462∗# (48), 193∗ (18), 187# (100)). Therefore, the biosynthetic origin of cathasterone (**5**) was shown to be 6-oxocampestanol (**4**). This result, together with our previous studies (Fujioka & Sakurai, 1997b), completes the elucidation of the early C6-oxidation pathway (see Fig. 1).

Recently, 6-deoxocathasterone (**6**), a possible biosynthetic precursor of 6-deoxoteasterone, was identified in tomato (Bishop et al., 1999). The natural occurrence of 6-deoxocathasterone (**6**) in cultured cells of *C. roseus* was examined in this study. V208 cells in the stationary phase were extracted and purified by silica gel and ODS cartridges, and by ODS-HPLC. The HPLC-purified fraction corresponding to 6-deoxocathasterone (**6**) was trimethylsilylated and analyzed by GC-MS. The retention time on GC and the mass spectrum of the fraction were identical to those of the trimethylsilyl drivative of authentic 6-deoxocathasterone (**6**). The mass spectral data were as follows: *m*/*z* (rel. int.) 547 (1) [M^+-15], 297 (3), 187 (85), 97 (100)). Thus, the occurrence of 6-deoxocathasterone (**6**) in the cultured cells of *C. roseus* was established. The endogenous level of 6-deoxocathasterone (**6**) was measured by GC-MS using ^{2}H-labeled 6-deoxocathasterone as an internal standard. The concentration of endogenous 6-deoxocathasterone (**6**) in the stationary phase cell culture was estimated to be 30 ng g^{-1} fr. wt. From the later eluted HPLC fraction, the same mass spectrum as 6-deoxocathasterone (**6**) was obtained, although the retention time of this compound on GC was ca. 30 s shorter than that of 6-deoxocathasterone (**6**), suggesting the occurrence of an epimer of 6-deoxocathasterone (**6**). As a possible candidate, we next chemically synthesized 3-*epi*-6-deoxocathasterone (**7**). By direct comparison with an authentic specimen, the compound was found to be 3-*epi*-6-deoxocathasterone (**7**); this is the first report of its occurrence in plants. The presence of 3-*epi*-6-deoxocathasterone (**7**) suggests that there might be a branched pathway of brassinolide biosynthesis. For instance, 3-*epi*-6-deoxocathasterone (**7**) might be another biosynthetic precursor of 6-deoxotyphasterol (see Fig. 1). Further studies should clarify this possibility.

In summary, we have shown that 6-deoxocastasterone (**1**) was converted to castasterone (**3**) via 6α-

hydroxycastasterone (**2**) in cultured cells of *C. roseus* (see Fig. 1). Previously the tomato *DWARF* gene was cloned, and shown to encode a cytochrome P450 (Bishop, Harrison & Jones, 1996) and recently, the tomato dwarf mutant has been shown to be defective in the conversion of 6-deoxocastasterone (**1**) to castasterone (**3**) (Bishop et al., 1999). Thus, the C6-oxidation of 6-deoxocastasterone (**1**) to castasterone (**3**) is presumed to be catalyzed by a cytochrome P450 enzyme. Functional expression of DWARF in yeast has revealed that DWARF catalyses two steps, namely, the conversion of 6-deoxocastasterone (**1**) to 6α-hydroxycastasterone (**2**), and the conversion of 6α-hydroxycastasterone (**2**) to castasterone (**3**) in BR biosynthesis (Bishop et al., 1999). The present study provides further evidence for 6α-hydroxycastasterone (**2**) being involved in the conversion of 6-deoxocastasterone (**1**) to castasterone (**3**). In addition, the biosynthetic origin of cathasterone (**5**) was clarified by metabolic studies using ^{2}H-labeled 6-oxocampestanol. Furthermore, 6-deoxocathasterone (**6**), a possible biosynthetic intermediate, was identified in cultured cells of *C. roseus*. Although the conversion of campestanol to 6-deoxocathasterone (**6**), and the conversion of 6-deoxocathasterone (**6**) to 6-deoxoteasterone have been examined in this cell system, the conversions have not yet been demonstrated. However, recently both conversions have been demonstrated in *Arabidopsis thaliana* (Fujioka et al., unpublished data). Therefore, both proposed biosynthetic pathways of early and late C6-oxidation pathways, have been substantiated.

3. Experimental

3.1. General

Melting points were determined under a hot-stage microscope (Yazawa micro melting point apparatus) and are uncorrected. ^{1}H NMR spectra were recorded on a JEOL JMN-ECP500 (500 MHz) spectrometer in $CDCl_3$ using TMS as an internal standard. EIMS, HR-EIMS and FABMS, HR-FABMS were measured with JEOL JMS-SX 102 and JEOL JMS-HX 110 mass spectrometers, respectively. GC-MS analysis was carried out on a JEOL Automass JMS-AM 150 mass spectrometer connected to a Hewlett–Packard 5890A-II gas chromatograph with a capillary column DB-5 (0.25 mm × 15 m, 0.25 μm film thickness). The analytical conditions were as previously described (Noguchi et al., 1999).

3.2. Cell cultures

The cultured cells of *Catharanthus roseus* (V208) were grown in Murashige-Skoog (MS) medium supplemented with 3% sucrose at 27°C with shaking at 100 rpm in the dark.

3.3. Synthesis of [$^2H_6$]6α-hydroxycastasterone 2

A solution of [26,28-$^2H_6$]castasterone **3** (Takatsuto & Ikekawa, 1986) (13.0 mg, 27.6 mmol) in THF (3 ml) and EtOH (1 ml) was added to a solution of lithium (19.3 mg, 2.76 mmol) in liquid ammonia (5 ml) at −78°C and the mixture was stirred at −78°C under Ar for 1 h. Dry solid NH_4Cl (200 mg) was added and ammonia was allowed to evaporate at room temperature. Work-up (EtOAc) and chromatography on silica gel (0.7 cm i.d. × 30 cm) with $CHCl_3$–EtOH (15:1, v/v) afforded [26,28-$^2H_6$]**2** (10.5 mg, 80.4%), mp 246–248°C (from MeOH), ^{1}H NMR spectral data (500 MHz, pyridine-d_5) δ:0.79 (3H, *s*), 0.94 (3H, *s*), 1.24 (3H, *d*, J = 6.5 Hz), 2.28 (1H, *dd*, J = 15.0 and 4.5 Hz), 2.98 (1H, *dd*, J = 11.0 and 3.3 Hz), 3.71 (1H, *dd*, J = 10.7 and 4.2 Hz), 3.97 (1H, *d*, J = 7.2 Hz), 4.14 (2H, *m*), 4.48 (1H, *d*, J = 3.0 Hz), HR-FABMS [M-H]$^-$ m/z: 471.3955 (calcd. 471.3957) for $C_{28}H_{43}O_5D_6$.

3.4. Synthesis of 3-epi-6-deoxocathasterone (7)

According to the reported method (Takatsuto, Watanabe, Fujioka & Sakurai, 1998), the 3β-hydroxyl group of a known (22*S*,24*R*)-22-acetoxy-24-methyl-5α-cholestan-3β-ol (Takatsuto, Watanabe, Gotoh, Kuriyama, Noguchi & Fujioka, 1998) (22 mg) was inverted to provide a corresponding 3α-hydroxyl compound (13 mg). A solution of the product in THF (5 ml) was treated with lithium aluminum hydride (10 mg) at room temperature overnight. Extraction with EtOAc ultimately gave a crude product (8 mg), which was recrystallized from EtOAc to give **7** (4.5 mg): mp 237–239°C, ^{1}H NMR, spectral data (500 MHz, $CDCl_3$) δ:0.671 (3H, *s*), 0.781 (3H, *s*), 0.809 (3H, *d*, J = 6.93 Hz), 0.829 (3H, *d*, J = 6.60 Hz), 0.874 (3H, *d*, J = 6.93 Hz), 0.880 (3H, *d*, J = 6.27 Hz), 3.771 (1H, *m*), 4.041 (1H, *m*); EIMS m/z: 418 (M^+, 6), 400 (27), 385 (7), 382 (5), 367 (3), 333 (3), 315 (9), 304 (100), 302 (11), 297 (5), 289 (17), 286 (45), 273 (8), 271 (18), 258 (11), 256 (5), 249 (16), 234 (67), 217 (16), 215 (27), 203 (7); HR-EIMS [M]$^+$ m/z: 418.3811 (calcd. 418.3811) for $C_{28}H_{50}O_2$.

3.5. Precursor administration experiments

A MeOH solution (5 μl) of [$^2H_6$]**1** (5 μg) or [$^2H_6$]**2** (5 μg) was added to a 200-ml flask containing cultured cells (7-day-old) in 60 ml MS medium. The cells were allowed to grow for 2, 4 or 6 days. A Me_2CO solution (40 μl) of [$^2H_6$]**4** (100 μg) was added into 200-ml flask containing cultures cells (3-day-old) in 60 ml MS med-

ium. The cells were allowed to grow for 10 additional days.

3.6. Purification of metabolites

After incubation, the cultures were extracted with MeOH, and the extracts were partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fraction was subjected to silica gel and ODS cartridges, and finally purified by ODS-HPLC (Senshu Pak, ODS-1151-D, 4.6 × 150 mm; flow rate, 1.0 ml min^{-1}; mobile phase, 45% MeCN (for the purification of metabolites of $[^2H_6]$**1** and $[^2H_6]$**2**), 65% MeCN (for the purification of metabolites of $[^2H_6]$**4**). The main frs. of **2** and **3** were R_t 8–9 min and 10.5–11.5 min, respectively (45% MeCN) whereas that of the Main fr. of **5** R_t 14–16 min (65% MeCN).

3.7. Identification of 6-deoxocathasterone (**6**) and 3-epi-6-deoxocathasterone (**7**)

V208 cells (12-day-old, 16 g fr. wt.) were extracted with MeOH (200 ml, two times). The extract was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$-soluble fr. was subjected to silica gel and ODS chromatographic steps, and finally purified by ODS-HPLC (Senshu Pak, ODS-4150-N, 10 × 150 mm; flow rate, 2.0 ml min^{-1}; mobile phase, MeOH). Fractions were collected every 0.5 min (R_t, 3–8 min). Main frs. of **6** and **7** were as follows: **6** (R_t, 6.5–7 min) and **7** (R_t, 7–8 min).

For quantitative analysis, V208 cells (12-day-old, 18 g fr. wt.) were extracted with MeOH (250 ml, two times). One hundred ng of $[^2H_6]$**6** was added to the extract as an internal standard. The extract was purified according to the methods described above. Fr. corresponding to **6** was subjected to GC-MS analysis after derivatization. The endogenous level of **6** was determined as the ratio of the peak areas of fragment ions of m/z 187 (endogenous) and m/z 193 (int. standard).

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