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A BRASSINOLIDE BIOSYNTHETIC PATHWAY VIA 6-DEOXOCASTASTERONE

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Key Word Index—*Catharanthus roseus*; Apocynaceae; *Nicotiana tabacum*; Solanaceae; *Oryza sativa*; Gramineae; biosynthesis; cultured cells; seedlings; brassinolide; castasterone; 6-deoxocastasterone.

Abstract—The occurrence of 6-deoxocastasterone was demonstrated by GC–mass spectrometry in cultured cells of *Catharanthus roseus*. Its concentration was in the range 5.9–18.9 ng g^{-1} fr. wt. A feeding experiment with deuterium labelled 6-deoxocastasterone revealed that 6-deoxocastasterone was converted into castasterone and brassinolide in cultured cells of *C. roseus*. In addition, the conversion of 6-deoxocastasterone into castasterone was also demonstrated in seedlings of *C. roseus*, and both seedlings and cultured cells of *Oryza sativa* and *Nicotiana tabacum*. These results showed 6-deoxocastasterone being involved in the biosynthesis of castasterone and the presence of an alternative pathway of brassinolide biosynthesis other than that via teasterone and typhasterol.

INTRODUCTION

Brassinosteroids have been found to occur in a wide variety of higher plants and shown to have strong and unique biological activities on plant growth and development [1]. Although more than 30 natural brassinosteroids have been chemically characterized, most details of their biosynthesis remain unknown. During the course of our studies on the biosynthesis of brassinosteroids using cultured cells of *Catharanthus roseus*, the biosynthetic sequence of campesterol → campestanol → 6α-hydroxycampestanol → 6-oxocampestanol [2] and the sequence of cathasterone → teasterone → typhasterol → castasterone → brassinolide (**3**) [3–7] were established. Each step was demonstrated by feeding experiments with labelled substrates. All compounds in both sequences were found to be endogenous in cultured cells of *C. roseus* [2, 3, 6–8]. In addition, possible involvement of 3-dehydroteasterone in the conversion of teasterone into typhasterol was shown by a feeding experiment [9]. In other plant systems, the sequence of teasterone → typhasterol → castasterone (**2**) was shown in seedlings of *C. roseus*, *Nicotiana tabacum* and *Oryza sativa* [10], and possible involvement of 3-dehydroteasterone was also shown in several plant systems [11, 12].

The occurrence of 6-deoxocastasterone (**1**) was first demonstrated in *Phaseolus vulgaris* [13]. As the biological activity of **1** is weak [13], this brassinosteroid was considered to be a dead-end product in the biosynthesis of brassinosteroids [14]. Recently, **1** was identified in wheat grain [12], rice bran [15], seeds of *Secale cereale* [16], pollen of *Cupressue arizonica* [1] and pollen of *Robinia pseudo-acacia* l. [18]. Furthermore, 3-dehydro-6-deoxoteasterone and 6-deoxotyphasterol, possible precursors of **1**, were also identified in the pollen of *Cupressue arizonica* [17]. We therefore examined the possible involvement of **1** in the biosynthesis of **3** which is the most biologically active natural brassinosteroid.

In the present study, we investigated the natural occurrence of **1** in cultured cells of *C. roseus* and the conversion of **1** into **2** and/or **3** using both cultured cells and seedlings of *C. roseus*, *N. tabacum* and *O. sativa*.

RESULTS AND DISCUSSION

The cultured cells (V208) of *C. roseus* were extracted with methanol, and the extract was purified according to the methods described in Experimental. The HPLC-purified fraction corresponding to **1** was

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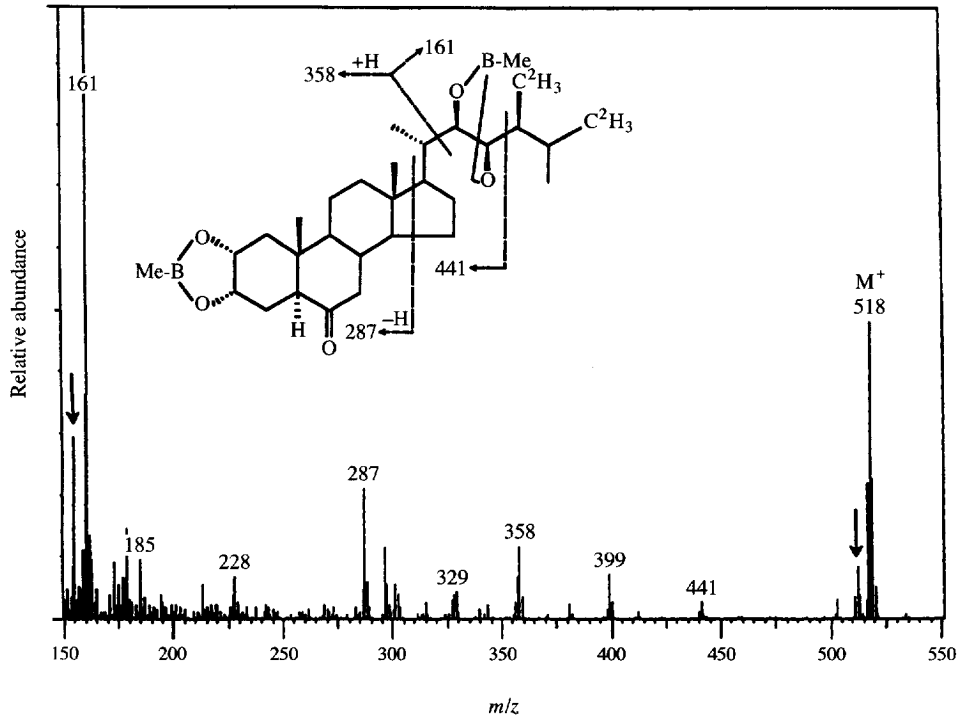
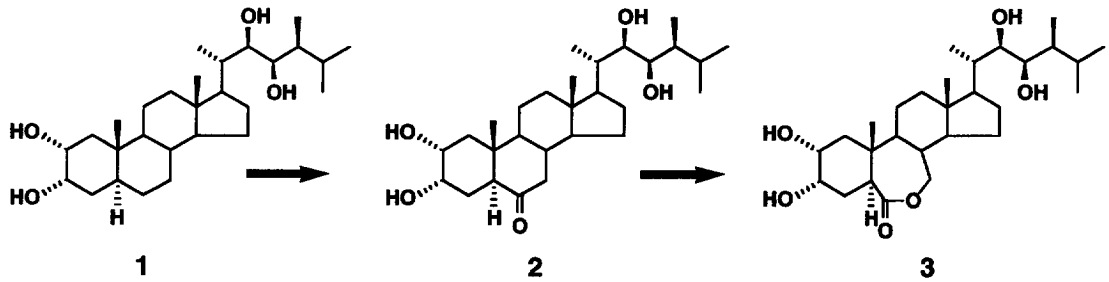


Fig. 1. Mass spectrum of the bismethaneboronate of [$^2H_6$]castasterone converted from [$^2H_6$]6-deoxocastasterone in cultured cells of *Catharanthus roseus*. Arrows indicate the ions of endogenous castasterone (**2**).

analysed by GC-mass spectrometry after methaneboronation. The R_t on GC and the full-scan mass spectrum of the fraction were in agreement with those of the authentic bismethaneboronate of **1**. The mass spectral data were as follows: *m/z* (ret. int.) 498 (62) $[M]^+$, 483 (10), 273 (100), 205 (30) and 155 (34). Thus, the occurrence of **1** in the cultured cells of *C. roseus* was established.

During growth of V208 cells, the concentrations of endogenous **1** were measured by GC–selected ion monitoring using deuterium labelled **1** as an internal standard. The concentrations of endogenous **1** in the growth period day 5, 7, 9 and 11 were 18.9, 12.2, 5.9 and 17.5 $ng^{-1}g$ fr. wt, respectively. The concentration of **1** rapidly decreased in the logarithmic phase (days 5–9) and increased again in the stationary phase (day 11). It is interesting to note that the concentrations were the highest among the endogenous brassinosteroids in V208 cells [19]. Its fluctuation pattern showed that the decrease of **1** corresponded to the increase of **2** [19], suggesting that **1** might be a precursor of **2**. We carried out feeding experiments with deuterium labelled substrate to investigate whether **1** could be a biosynthetic precursor of **2** in cultured cells of *C. roseus*. V208 cells (eight days old) were fed with [$^2H_6$]**1** and incubated for two days. The partially purified extract of the cells was derivatized and analysed by GC–mass spectrometry. From the fraction corresponding to **2**, ions (*m/z* 518 $[M]^+$, 399, 358, 287 and 161) of [$^2H_6$]**2**-bismethaneboronate were detected along with ions (*m/z* 512 and 155) of endogenous **2**-bismethaneboronate (Fig. 1). Furthermore, from the fraction corresponding to **3**, [$^2H_6$]**3** was detected by GC–selected ion monitoring. These results indicated that **1** was converted into **3** via **2** (Scheme 1) in cultured cells of *C. roseus*.

Feeding experiments with [$^2H_6$]**1** were also carried



Scheme 1.

out in seedlings of *C. roseus*, and in both seedlings and cultured cells of *N. tabacum* and *O. sativa*. In consequence, the conversion of **1** into **2** was demonstrated by GC–selected ion monitoring in all plant systems examined.

The present study provided the first evidence of **1** being a biosynthetic precursor of **2**. As the biosynthetic sequence of teasterone → typhasterol → **2** → **3** is already established, this study demonstrates that there are at least two different biosynthetic pathways leading to **3**.

EXPERIMENTAL

Plant materials. The cultured cells of *C. roseus* (V208), *N. tabacum* (BY-2) and *O. sativa* (Oc) were cultured as described before [20]. Seedlings of *C. roseus* cv. Downcarpet, *N. tabacum* cv. Wisconsin 38 and *O. sativa* cv. Nipponbare were grown in pots containing vermiculite at 28° under continuous light for 10 weeks, 7 weeks, and 9 days, respectively. The seedlings of *C. roseus* (2 plants/flask), *N. tabacum* (2 plants/flask) and *O. sativa* (10 plants/flask) were transferred to a 10-ml flask containing 5 ml 0.1% Hyponex soln and adapted to the medium for 2 days prior to feeding experiments.

GC-MS analysis. 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 analyt. conditions were the same as previously described [7]. Samples were dried and treated with pyridine containing methaneboronic acid (2 mg ml^{-1}). In quantitative analysis of **1**, the endogenous concn was calculated from the ratio of the peak areas of the ions that corresponded to the endogenous and deuterium labelled **1**.

Synthesis of authentic [$^2H_6$]*6-deoxocastasterone.* [$^2H_6$]**1** (mp 238°) was synthesized from [$^2H_6$]**2** according to the method of ref. [21]. Its structure was rigorously determined by NMR and GC-MS.

Qualitative analysis of endogenous 6-deoxocastasterone (**1**). V208 cells (533 g fr. wt) of *C. roseus* were extracted with 2× MeOH. The extract was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$-soluble fr. was subjected to CC on silica gel and Sephadex LH-20, and finally purified by ODS-HPLC (10 × 50 mm + 20 × 250 mm) according to the method previously reported [7]. The fr. (R_t 40–45 min) corresponding to **1** was subjected to GC-MS analysis after derivatization.

Quantitative analysis of the endogenous 6-deoxocastasterone (**1**). V208 cells were cultured in a 200-ml conical flask. Cells were harvested every 2 days for measurement of fr. wt. (day 5, 10.4 g; day 7, 12.7 g; day 9, 20.5 g; day 11, 20.6 g). Cells (10 g fr. wt. eq.) harvested from day 5 to day 11 were used for analysis of endogenous **1**. After extraction with MeOH, 100 ng [$^2H_6$]**1** was added to the extract as int. standard. The extract was purified on silica gel and ODS cartridges, and subjected to ODS-HPLC analysis. The fr. corresponding to **1** in ODS-HPLC analysis was collected from 30 to 35 min. The analysis was performed under the same conditions previously described [3].

Feeding of [$^2H_6$]**1**. A MeOH soln (4 μl) of [$^2H_6$] **1** (2 μg) was added into a 200-ml flask containing cultured cells, which were grown for 8 days (log phase) in 60 ml MS medium. The cells were allowed to grow for 2 days (V208), 0.5 day (BY-2) or 1 day (Oc). For seedlings, [$^2H_6$]**1** (5 μg) was added to the culture medium. The incubation time was 2 days for *C. roseus*, and 1 day for *N. tabacum* and *O. sativa*.

Purification of metabolites. After incubation, cells and seedlings were extracted with MeOH, and the extract was partitioned between $CHCl_3$ and H_2O. Purification of the extract was performed under the same conditions described in the section on quantitative analysis of endogenous **1**. Each fr. corresponding to **2** was subjected to GC-MS analysis after derivatization.

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