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28-Norcastasterone is biosynthesized from castasterone

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Abstract

Metabolic experiments with deuterium-labeled castasterone in seedlings of *Arabidopsis thaliana*, *Oryza sativa* and *Lycopersicon esculentum*, and cultured cells of *Catharanthus roseus* were performed, and the metabolites were analyzed by GC–MS. In all the plant species examined, [$^2H_3$]28-norcastasterone was identified as a metabolite of [26,28-$^2H_6$]castasterone, indicating that castasterone is the biosynthetic origin of 28-norcastasterone. Moreover, the natural occurrence of 28-norcastasterone and 28-nortyphasterol in seedlings of *A. thaliana* has been demonstrated. This is the first report of the natural occurrence of 28-nortyphasterol in plants. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: *Arabidopsis thaliana*; *Catharanthus roseus*; Apocynaceae; Cruciferae; Biosynthesis; Cultured cells; Biosynthetic intermediates of brassinolide; Castasterone; 28-Norcastasterone; 28-Nortyphasterol

1. Introduction

Brassinosteroids (BRs) are now recognized as an important hormone controlling plant growth and development (Altmann, 1998; Clouse and Feldmann, 1999). Naturally occurring BRs so far identified are C_{27}-, C_{28}-, and C_{29}-steroids (Fujioka, 1999). The carbon skeletons of the side chain are common in plant sterols, suggesting that BRs are biosynthesized from the corresponding plant sterols. Brassinolide belonging to C_{28}-steroids is highly bioactive and is widely distributed in the plant kingdom. A biosynthetic pathway for brassinolide has been elucidated by feeding labeled compounds of possible brassinolide intermediates to cultured cells of *Catharanthus roseus*, followed by analyzing the metabolites produced by GC–MS. The present model includes parallel branched pathways, namely, early and late C-6 oxidation pathways (Fujioka and Sakurai, 1997a,b; Sakurai, 1999; Fujioka et al., 2000). By analogy with the biosynthesis of brassinolide, it is postulated that C_{27}-BRs and C_{29}-BRs could be biosynthesized from the corresponding C_{27}-sterols and C_{29}-sterols, respectively. Therefore, it is likely that 28-norcastasterone is synthesized from cholesterol, because 28-norcastasterone and cholesterol have the same structural skeleton. However, no experimental evidence has been obtained relating to biosynthesis of 28-norcastasterone. To date, 28-norcastasterone has been found in as many as twelve plant species (Fujioka, 1999). Thus, it would be important to clarify the biosynthesis of 28-norcastasterone. Unexpectedly, during our study of BR biosynthesis, we found that castasterone was converted to 28-norcastasterone. Here, we report the evidence for castasterone being the biosynthetic precursor of 28-norcastasterone. In addition, we describe the identification of 28-norcastasterone and 28-nortyphasterol in seedlings of *Arabidopsis thaliana*, and their biological activity.

2. Results and discussion

2.1. Metabolism of [$^2H_6$]castasterone in seedlings of *Arabidopsis thaliana*

[26,28-$^2H_6$]Castasterone was applied to seedlings of *A. thaliana* (wild type: Ws-2). After incubation, the plant was extracted, and the metabolites of interest purified using both a silica gel column and an ODS cartridge column, repeatedly followed an ODS-HPLC. HPLC-

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purified fractions were analyzed by gas chromatography–mass spectrometry (GC–MS) after conversion to the methaneboronate derivative. A prominent peak of a metabolite [GC retention time (Rt): 11.58 min] was found in the HPLC fraction (Rt: 6–8 min). Its mass spectral data was as follows: m/z 501 (M^+, 92), 486 (11), 459 (7), 399 (7), 358 (20), 287 (78), 144 (100). The mass spectral data was very similar to that of authentic 28-norcastasterone bismethaneboronate (28-norcastasterone bismethaneboronate; Rt: 11.60 min, m/z 498 (M^+, 94), 483 (13), 456 (8), 399 (9), 358 (24), 287 (82), 141 (100)). A clear difference in the mass spectra of the metabolite and authentic 28-norcastasterone, however, was observed at m/z 501 and 498, and 144 and 141. This difference must be derived from the side chain. As we used [26,28-$^2H_6$]castasterone as a substrate, we expected the corresponding [$^2H_6$]metabolite, if the C26 and C28 methyl groups were not involved in the metabolism. Unexpectedly, we observed a molecular ion of the metabolite at m/z 501. This suggested the removal of either the 26-methyl or 28-methyl group. From the similarity of the mass spectra between the metabolite and 28-norcastasterone, it was concluded that the 28-methyl group was removed during its metabolism. Another possible candidate for the metabolite, [28-$^2H_3$]26-norcastasterone was excluded, because the GC retention time (11.74 min) and the mass spectral data of 26-norcastasterone bismethaneboronate were different from those of the 28-norcastasterone bismethaneboronate. Thus, it was demonstrated that [26,28-$^2H_6$]castasterone was converted to [26-$^2H_3$]28-norcastasterone in the seedlings of *A. thaliana* (Fig. 1). In this administration experiment, the conversion ratio (the percentage of the detected amount of the metabolite versus the amount of added substrate) was ca. 5%.

A similar experiment was performed using the seedlings of BR-insensitive mutants (*bri1-5*) of *A. thaliana*, where [$^2H_3$]28-norcastasterone was identified as a metabolite of [26,28-$^2H_6$]castasterone by full-scan GC–MS analysis, confirming that castasterone is the biosynthetic origin of 28-norcastasterone in *A. thaliana*.

2.2. Metabolism of [26,28-$^2H_6$]castasterone in cultured cells of C. roseus

To test whether or not our finding in *A. thaliana* occurs in other higher plants, we examined the metabolism of [26,28-$^2H_6$]castasterone in cultured cells of *Catharanthus roseus*.

[26,28-$^2H_6$]castasterone was administered to cultured cells of *C. roseus*, and incubated for 2 days. The culture was extracted with methanol, and the extract was purified. The purified HPLC fractions were converted to methaneboronate derivatives and analyzed by GC–MS. A prominent peak, co-migrating with authentic 28-norcastasterone, was found in the HPLC fraction (Rt: 6–8 min).

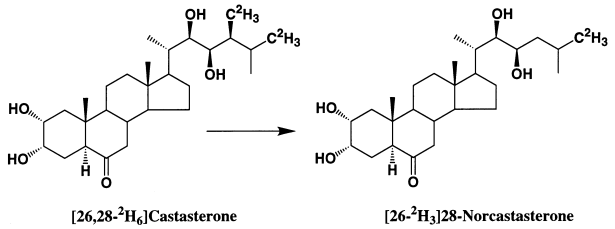

Fig. 1. Conversion of castasterone to 28-norcastasterone. In this study, this conversion was demonstrated in seedlings of *Arabidopsis thaliana*, rice and tomato, and cultured cells of *Catharanthus roseus*.

Mass spectral data of the metabolite was as follows: m/z 501 (M^+, 90), 486 (12), 459 (6), 399 (7), 358 (22), 287 (76), 144 (100). Molecular and fragment ions such as m/z 501 and 144 corresponding to m/z 498 and 141 of non-labeled 28-norcastasterone-bismethaneboronate derivative was detected, along with characteristic ions of 28-norcastasterone bismethaneboronate (such as m/z 399, 358 and 287). Thus, [$^2H_3$]28-norcastasterone was identified as a metabolite of [26,28-$^2H_6$]castasterone, demonstrating that castasterone is converted to 28-norcastasterone in cultured cells of *C. roseus*. In the same HPLC fraction, [$^2H_6$]brassinolide was identified as a major metabolite of [$^2H_6$]castasterone by full-scan GC–MS analysis, confirming our previous work (Suzuki et al., 1993). Conversion ratios in this feeding were ca. 12% ([$^2H_6$]brassinolide) and ca. 4% ([$^2H_3$]28-norcastasterone).

2.3. Metabolism of [$^2H_6$]castasterone in seedlings of Oryza sativa and Lycopersicon esculentum

The metabolism of [$^2H_6$]castasterone in seedlings of rice (*Oryza sativa*) and tomato (*Lycopersicon esculentum*) was also investigated. In both plant species, [$^2H_3$]28-norcastasterone was identified as a metabolite of [26,28-$^2H_6$]-castasterone by full-scan GC–MS analysis. In tomato, ca. 4% of the substrate was converted to the metabolite following 2 day incubation, while lower conversion was found in rice (ca. 1%).

2.4. Identification of 28-norcastasterone and a novel brassinosteroid, 28-nortyphasterol in seedlings of Arabidopsis thaliana

In *A. thaliana*, the natural occurrence of 28-norcastasterone has not yet been demonstrated. Accordingly we have re-examined the natural occurrence of BRs in the seedlings of *A. thaliana*. Thus, *bri1-5* seedlings were grown and used for the identification of BRs, because *bri1-5* was shown to accumulate very high levels of endogenous BRs (Noguchi et al., 1999). The final HPLC-purified fractions were derivatized and analyzed by GC–MS. From the ODS-HPLC fraction (Rt: 8–10 min), 28-norcastasterone and brassinolide were identified by full-scan GC-MS analysis. Mass spectra and retention

Table 1
GC–MS identification of Brassinosteroids in *Arabidopsis thaliana*

Brassinosteroid	Retention time (min)	Characteristic ions *m/z* (relative intensity)
28-Norcastasterone		
standard	11.60	498 [M$^+$] (80), 483 (6), 399 (4), 358 (12), 329 (11), 287 (51), 228 (19), 141 (100)
endogenous	11.60	498 [M$^+$] (88), 483 (7), 399 (4), 358 (13), 329 (11), 287 (48), 228 (19), 141 (100)
Brassinolide		
standard	13.20	528 [M$^+$] (4), 457 (2), 397 (7), 374 (21), 345 (11), 332 (22), 177 (82), 155 (100)
endogenous	13.22	582 [M$^+$] (4), 457 (2), 397 (7), 374 (24), 345 (12), 332 (24), 177 (90), 155 (100)
Castasterone		
standard	12.23	512 [M$^+$] (53), 497 (5), 441 (4), 399 (8), 358 (20), 329 (11), 287 (28), 155 (100)
endogenous	12.22	512 [M$^+$] (56), 497 (6), 441 (4), 399 (10), 358 (22), 329 (13), 287 (31), 155 (100)
28-Nortyphasterol		
standard	11.05	530 [M$^+$] (91), 515 (58), 501 (100), 440 (48), 425 (16), 229 (33), 141 (51)
endogenous	11.05	530 [M$^+$] (88), 515 (56), 501 (100), 440 (42), 425 (15), 229 (30), 141 (50)

times on GC were identical to those of authentic specimens (Table 1). This is the first full-scan GC–MS identification of 28-norcastasterone and brassinolide in *A. thaliana*, although the presence of brassinolide has been already shown by GC–SIM analysis (Fujioka et al., 1998b; Noguchi et al., 1999). From the HPLC fractions (Rt: 10–12 min, 12–14 min), castasterone was identified by full-scan GC-MS analysis (Table 1). The natural co-occurrence of castasterone and 28-norcastasterone, and the conversion of castasterone to 28-norcastasterone support our assumption that castasterone is the biosynthetic origin of 28-norcastasterone.

In this series of experiments, a novel BR was identified. From the HPLC fraction (Rt: 20–23 min), the following mass spectral data was obtained as a methaneboronate-trimethylsilyl derivative; prominent ions at *m/z* 530 [M$^+$], 515, 501 (base peak) and 440. This mass spectral data is very similar to that of a typhasterol-methaneboronate-trimethylsilyl derivative (prominent ions at *m/z* 544 [M$^+$], 529, 515 (base peak) and 454), suggesting that this BR is a typhasterol analog lacking a methyl group. By direct comparison with an authentic specimen, this BR was shown to be 28-nortyphasterol (Table 1). This is the first report of the natural occurrence of 28-nortyphasterol in the plant kingdom.

2.5. Biological activity of 28-norcastasterone and castasterone, and 28-nortyphasterol and typhasterol

Biological activity was evaluated using the rice lamina inclination test (Fujioka et al., 1998a). Two assay methods were employed: single application of the sample (Fig. 2), and co-application with 5 μg of indole-3-acetic acid (Fig. 3). In both assay methods, the activity of 28-norcastasterone was shown to be ca. 10% of that of castasterone (Figs. 2 and 3). Activity of 28-nortyphasterol was also shown to be ca. 10% of that of typhasterol (Figs. 2 and 3). Relative activities were as follows: castasterone (100), 28-norcastasterone (10), typhasterol (10), 28-nortyphasterol (1).

The present study revealed that castasterone is converted to 28-norcastasterone at least in four plant species, that is, *A. thaliana*, *C. roseus*, rice and tomato, indicating that castasterone is the biosynthetic origin of 28-norcastasterone. This was a totally unexpected result, because we originally thought that 28-norcastasterone would be biosynthesized from 28-nor-6-deoxocastasterone by analogy of the established step of 6-deoxocastasterone to castasterone. The present study clearly shows that 28-norcastasterone is biosynthesized from castasterone. This result explains the fact that when 28-norcastasterone was identified in a plant, castasterone was always found to co-occur in the same plant (Fujioka, 1999). However, castasterone may not be the only biosynthetic origin of 28-norcastasterone. As the natural occurrence of 28-nor-6-deoxocastasterone has been found in several plant species (Fujioka, 1999), this BR may be another biosynthetic precursor of 28-norcastasterone. In any case, the natural occurrence of 28-nortyphasterol is intriguing:

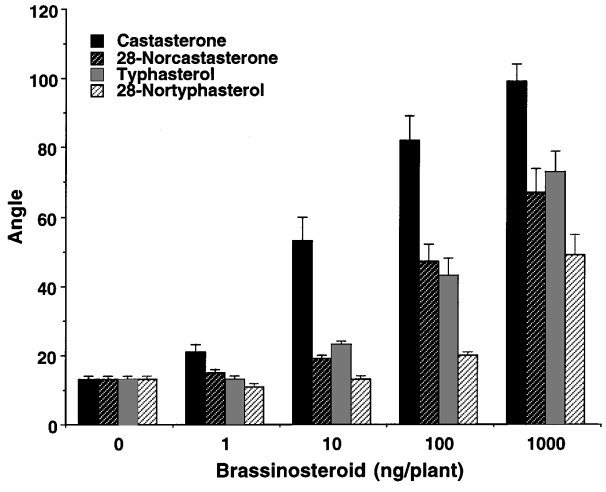

Fig. 2. Rice lamina inclination test of castasterone, 28-norcastasterone, typhasterol and 28-nortyphasterol by single application of the test sample. Each data point represents the mean of 30 replicates ± SE.

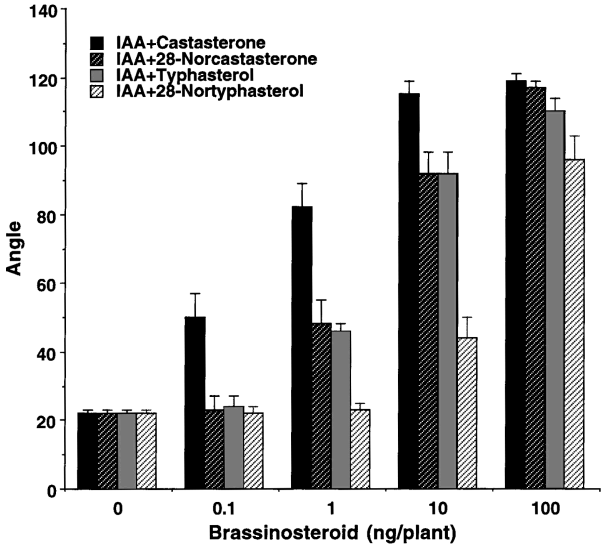

Fig. 3. Rice lamina inclination test of castasterone, 28-norcastasterone, typhasterol and 28-nortyphasterol by co-application of the test sample with IAA (5 μg/plant). Each data point represents the mean of 30 replicates ± SE.

the co-occurrence of 28-nortyphasterol and 28-norcastasterone suggests that 28-norcastasterone may be biosynthesized from 28-nortyphasterol. Furthermore, typhasterol may be predicted to be a precursor of 28-nortyphasterol by analogy of the findings in the present study. Furthermore, 28-nortyphasterol may be another precursor of 28-norcastasterone; detailed metabolic studies of these BRs will clarify these possibilities.

The biological activity of 28-norcastasterone was shown to be ca. 10% of that of castasterone. This means that the level of castasterone could be regulated by this metabolism. Thus, the conversion of castasterone to 28-norcastasterone may, in effect, have some regulatory role in maintaining the proper level of castasterone, an immediate precursor of brassinolide. This speculation must be also clarified by further studies.

3. Experimental

3.1. General

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 the same as previously described (Fujioka et al., 1997).

3.2. Metabolism of [$^2H_6$]castasterone in seedlings of A. thaliana

[26,28-^{2}H6]Castasterone (Takatsuto and Ikekawa, 1986), 28-norcastasterone (Takatsuto et al., 1984), and 26-norcastasterone (Watanabe, Seto, Yokota and Takatsuto, unpublished work) were chemically synthesized.

Before precursor administration experiments, seven-day-old seedlings of *A. thaliana* (Ws-2: 20 seedlings, *bri1-5*: 50 seedlings) were transferred to a 200-ml flask containing 30 ml of half-strength MS medium supplemented with 1% sucrose. Seven days after transfer, a MeOH solution (10 μl) of [26,28-$^2H_6$]castasterone (10 μg) was added. The seedlings were incubated for 2 days at 22°C in the light on a shaker (120 rpm), then extracted with MeOH. The MeOH extract was partitioned between $CHCl_3$ and H_2O. The $CHCl_3$-soluble fraction was purified with a cartridge of silica gel (Sep-Pak Vac 2 g; Waters, Milford, MA), which was eluted with 30 ml of chloroform, 2% MeOH in chloroform, and 7% MeOH in chloroform. The last fraction was purified with HPLC on a 150 × 4.6-mm Senshu Pak ODS-1151-D column (Senshu Scientific Co., Ltd., Tokyo) by using 45% acetonitrile at a flow rate of 1.0 ml/min. The fractions were collected at two-minute intervals (Rt of 4 to 30 min). Each fraction was subjected to GC–MS analysis after derivatization.

3.3. Metabolism of [$^2H_6$]castasterone in cultured cells of C. roseus

Cultured cells of *C. roseus* (V208) were grown in a MS medium supplemented with 3% sucrose at 27°C by shaking at 100 rpm in the dark. A MeOH solution (10 μl) of [$^2H_6$]castasterone (10 μg) was added to a 200-ml flask containing cultured cells, which were grown for 10 days (log phase) in 60 ml MS medium. The cells were allowed to grow for 2 days. After incubation, cultures were extracted with MeOH, and the extract was purified by the same method described above.

3.4. Metabolism of [$^2H_6$]castasterone in seedlings of O. sativa and L. esculentum

Seedlings (*O. sativa* cv. Koshihikari and *L. esculentum* cv. Alisa Craig) were grown in pots containing vermiculite at 25°C under continuous light. Prior to precursor administration, they were transferred to a water culture in a 10-ml conical flask containing 10 ml of water. A MeOH solution (10 μl) of [$^2H_6$]castasterone (10 μg) was added into the water and allowed to grow for additional 2 days. Extraction and purification were performed by the same method described above.

3.5. Identification of 28-norcastasterone and 28-nortyphasterol in A. thaliana

Thirty-two grams of five-week-old plants (*bri1-5*) were collected and extracted with MeOH. The MeOH extract was partitioned between $CHCl_3$ and H_2O. The

$CHCl_3$-soluble fraction was purified with a cartridge of silica gel (Sep-Pak Vac 10 g, Waters, Milford, MA), which was eluted with 100 ml of chloroform, 2% MeOH in chloroform, and 7% MeOH in chloroform. The last fraction was purified by ODS cartridge column (Sep-Pak Plus C18, Waters, Milford, MA), which was eluted with 20 ml of MeOH, and subjected to ODS-HPLC (Senshu Pak Pegasil ODS, 10×30 mm + Senshu Pak Pegasil ODS, 20×250 mm; Senshu Scientific Co., Ltd., Tokyo) by using 70% acetonitrile at a flow rate of 8 ml/min. The fractions were collected at 2-min intervals (Rt of 6–14 min) and 3-min intervals (Rt of 14 to 35 min). Each fraction was subjected to GC–MS analysis after derivatization. Authentic 28-nortyphasterol was chemically synthesized (Takatsuto and Ikekawa, 1987).

3.6. Rice lamina inclination test

Rice lamina inclination test was carried out according to the methods reported by Fujioka et al. (1998a).

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