Identification of 6-Deoxocastasterone and Brassinolide from a Liverwort, *Marchantia polymorpha*

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Brassinosteroids (BRs) are steroidal plant hormones which play essential roles in plant growth and morphogenesis.[1-4] The presence of BRs in lower plants has been already demonstrated,[5-7] but biosynthesis of BRs in lower plants has not been well known yet. To get information on biosynthesis of BRs in lower plants, we have previously investigated endogenous BRs and their biosynthetic precursors in a liverwort, *Marchantia polymorpha*, resulting in identification of castasterone (CS) and its two biosynthetic precursors, campesterol (CR) and campestanol (CN) in the lower plant.[8,9] CS, CR and CN are members of the early and late C6-oxidation pathway (Fig. 1) in higher plants,[10~15] which suggested that the liverwort contained the early and/or late C6-oxidation pathway to produce BRs. To verify that, we attempted to identify other BRs included in the pathway with a large amount of *M. polymorpha* in the study.

Naturally-grown *M. polymorpha* (5.3 Kg) collected in early July, 2001, at Banpo area, Seoul, was extracted with 80% methanol. After concentrating to aqueous phase *in vacuo*, 200 ng of deuterium labeled ([26, 28-$^2H_6$]) teasterone (TE), typhasterol (TY), 6-deoxocastasterone (6-deoxoCS), CS and brassinolide (BL) were added to the extract as internal standards for quantitative analysis. By guidance of the rice lamina inclination assay,[16] the extract was re-extracted, solvent-partitioned and column-chromatographed using silica gel, Sephadex LH-20 and ODS (RP-18). The resulting active fractions were combined, and further purified by a reversed phase HPLC.

After HPLC, BR-like activities were detected in several fractions (Fig. 2). To get hint for the presence of BRs in the active fractions, authentic TE, TY, 6-deoxoCS, CS and BL were analyzed by the same HPLC condition, providing that the authentic BRs were eluted in the fraction 36, 41, 46, 20 and 14-15, respectively. Therefore, it was thought that fraction I, II, III, IV and V may contain [$^2H_6$]-BL, -CS, -TE, -TY and -6-deoxoCS added as internal standard possibly accompanied with deuterium unlabeled ([$^2H_0$]) endogenous BL, CS, TE, TY and 6-deoxoCS, respectively. To confirm that, the fractions were derivatized to a bismethaneboronates (BMBs) or monomethaneboronate (MB)-trimethylsilyl (TMSi) ethers, and analyzed by GC-MS and/or GC-selected ion monitoring (SIM).

In the GC-SIM, BMB of an active compound in fraction I gave mass ions at m/z 534 and 161 which are a molecular and a base peak for [$^2H_6$]-BL BMB at the same retention time as that of authentic sample (Table 1). In addition, the other active compound in the same fraction showed ions at m/z 528 and 155 which were 6 mass less than those derived from [$^2H_6$]-BL BMB, at identical GC retention time to that of [$^2H_0$]-BL BMB, indicating that the fraction I contained both [$^2H_6$]- and [$^2H_0$]-BL which were both an internal standard added and an endogenous BR, respectively. The amount of endogenous BL calculated by ratio of [$^2H_0$]-/[$^2H_6$]-BL added as internal standard was 0.04 ng g^{-1} fresh weight.

We have previously demonstrated that CS is contained in *M. polymorpha*. Thus, fraction II which showed identical retention time in HPLC as that of CS was thought to contain [$^2H_6$]- and [$^2H_0$]-CS. In fact, a full scan GC-MS analysis of fraction II after methaneboronation gave prominent ions for both [$^2H_0$]- and [$^2H_6$]-CS BMB at the same retention time as those of authentic samples (Table 1). The occurrence of CS in the lower plants was confirmed.[6,7] The endogenous level of CS in the plant was determined on the basis of [$^2H_6$]-CS added as internal standard being 0.34 ng g^{-1} fresh weight.

The HPLC retention times for two moderate active fractions III and IV implied the presence of [$^2H_6$]-TE and -TY added as internal standard. To examine the co-existence of endogenous [$^2H_0$]-TE and -TY, respectively, in the fractions, the fraction III and IV were derivatized to be MB-TMSi ethers and analyzed by GC-SIM. As expected, selected ions at m/z 550 (M^+), 535 (M-15), 521 (M-29) and 161 (base peak) for [$^2H_6$]-TE and -TY MB-TMSi ether were detected at the same GC-retention times as those of authentic [$^2H_6$]-TE and -TY MB-TMSi (Table 1). However, ion peaks due to endogenous [$^2H_0$]-TE and -TY MB-TMSi ether at m/z 544 (M^+), 529 (M-15), 515 (M-29) and 155 (base peak) were not detected, indicating that TE and TY do not exist in *M. polymorpha*.

To examine whether endogenous [$^2H_0$]-6-deoxoCS is also present in the fraction, fraction V was derivatized, and analyzed by GC-SIM. Mass ions at m/z 504 (M^+), 489 (M-15) and 161 (base peak) due to [$^2H_6$]-6-deoxoCS BMB were successfully detected at the same GC-retention time as that of authentic sample. Selected ions for endogenous [$^2H_0$]-6-deoxoCS BMB at m/z 498 (M^+), 483 and 155 (base peak)

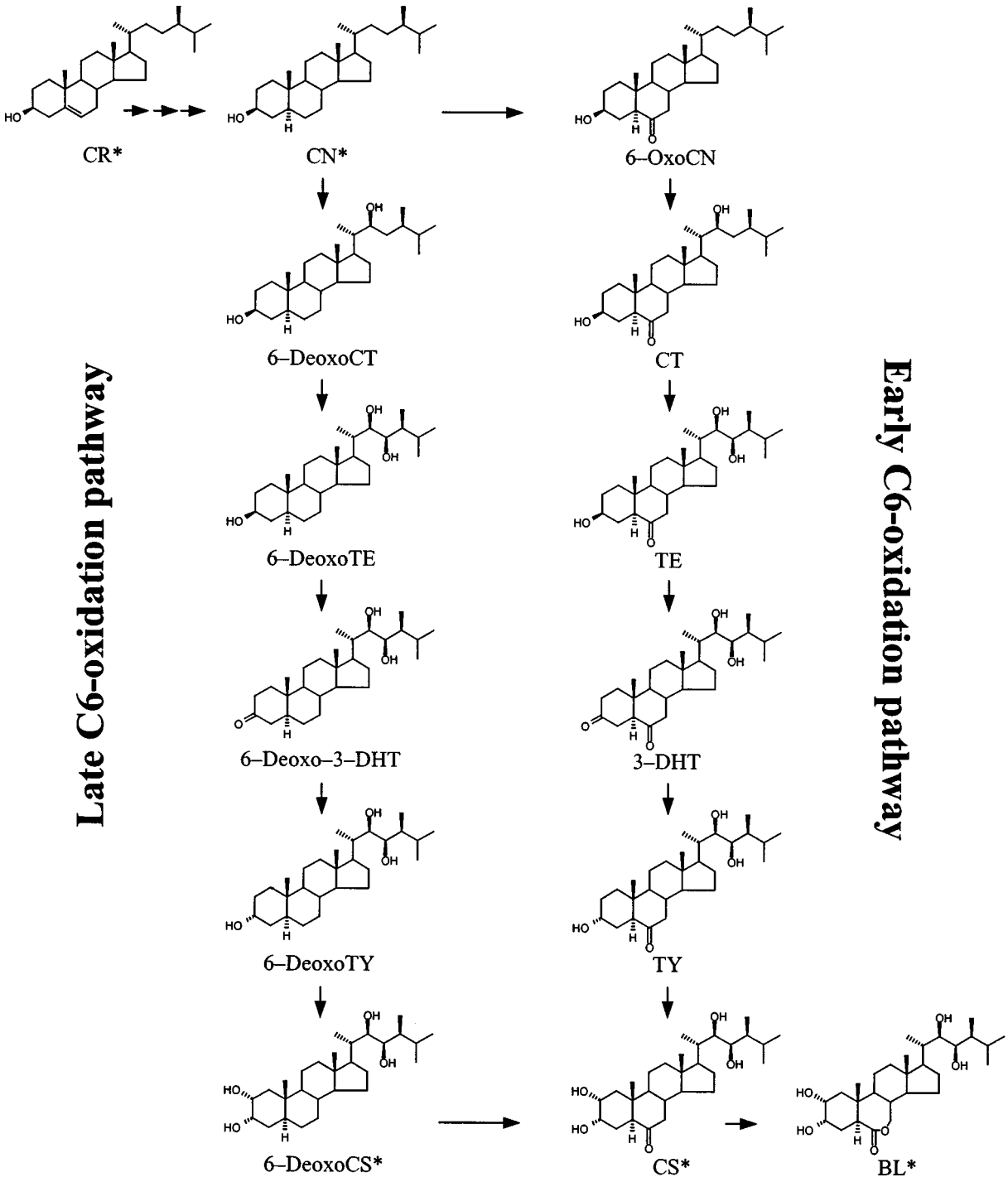

Figure 1. The early C6-oxidation pathway and late C6-oxidation pathway for BRs biosynthesis. Asterisks indicate BRs/sterols identified from *M. polymorpha*. CT: cathasterone, 3-DHT: 3-dehydroteasterone.

were also shown at identical GC-retention time to those of authentic [$^2H_0$]-6-deoxoCS BMB. This demonstrated that both [$^2H_6$]-deoxoCS and [$^2H_0$]-6-deoxoCS were contained in the fraction. The endogenous level of 6-deoxoCS in *M. polymorpha* was determined as 0.14 ng g^{-1} fresh weight.

Among the biosynthetic intermediates shown in Figure 1, 6-deoxoCS, TE and TY are frequently and abundantly identified BRs together with CS and BL from the entire plant kingdom.[12,17] In a plant which contains BL or /and CS, therefore, co-existence and endogenous level of 6-deoxoCS or TE and/or TY may confer a clue which pathway(s) is present and prominently operative to synthesis of BRs in a plant. For the reason, we tried to identify and quantify 6-deoxoCS, TE, TY, CS and BL in *M. polymorpha* by use of [$^2H_6$]-labeled BRs as internal standards, which revealed that 6-deoxoCS, CS and BL were contained but not TE and TY in the lower plants. Together with the presence of campesterol and campestanol, the result indicated that BRs in *M.*

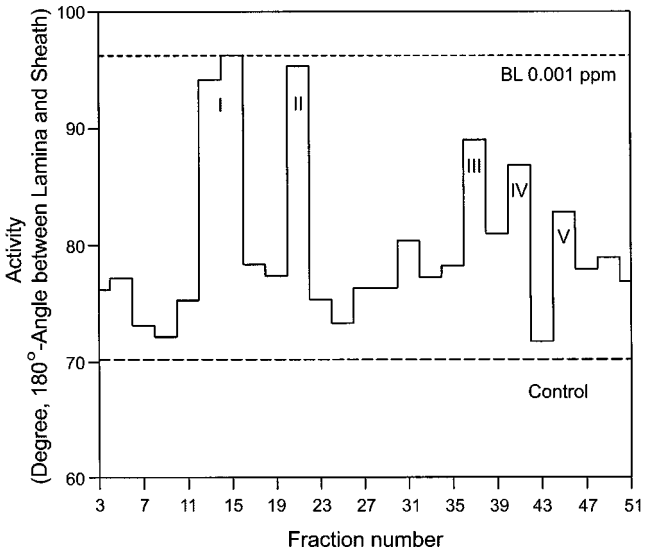

Figure 2. Distribution of biological activity determined by the lice lamina inclination assay after a reversed phase HPLC.

polymorpha is biosynthesized from campesterol to BL *via* the late C6-oxidation pathway, suggesting that a lower plant contains the same biosynthetic pathway to synthesize BRs as that in higher plants. Therefore, it is thought that BRs biosynthetic pathways in higher plants are biogenetically evolved from those in lower plants.

Experimental Section

Purification of endogenous BRs in *M. polymorpha*. *M. polymorpha* (5.3 Kg) were homogenized and extracted with 80% methanol (5 L × 3). The extract was concentrated to aqueous phase in vacuo and re-extracted with chloroform (1 L × 4). The chloroform soluble fraction was concentrated and solvent-partitioned between *n*-hexane and 90% methanol (1 L × 3). The bioactive 90% methanol soluble fraction was partitioned again between phosphate buffer (pH 7.8) and ethyl acetate (1 L × 3). The ethyl acetate soluble fraction (14.2 g) was then purified by silica gel column chromatography (100 g, Merck) eluted stepwise with chloroform containing 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 50 and 100% methanol (100 mL each). The biologically active fractions eluted with 4-5% methanol in chloroform were combined (1.13 g) and loaded on Sephadex LH-20 column (bed volume 340 mL, 22 × 900 mm) eluted with a mixture of methanol-chloroform (4 : 1) at a flow rate of 0.5 mL min^{-1}. The bioactive fractions with 0.65-0.75 of elution volume/ total volume were combined (0.175 g), dissolved in 50% methanol (100 mL), and subjected to ODS column (100 mL, Merck LiChroprep RP-18) chromatography. The elution was carried out with aqueous methanol increasing methanol content every 10% from 50 to 100% (100 mL each). The bioactive fractions eluted with 80-90% were concentrated (0.051 g), dissolved in small volume of methanol, and injected into a reversed phase HPLC (Pegasil C_{18}, 10 × 150 mm, Senshu Chemical Co.) eluted with 50% for 25 min, gradient from 50% to 100% for 15 min, and then 100% acetonitrile for 10 min at a flow rate of 2 mL min^{-1}. Based on polarity, the active fractions were referred as I, II, III, IV, and V and analyzed by GC-MS/SIM.

Bioassay. The rice lamina inclination assay using a cultivar Koshihikari was carried out to examine BRs activity.[16]

GC-MS/SIM analysis. GC-MS/SIM analyses were carried out with a 5973 mass spectrometer (70 eV, Hewlett-Packard) connected to 6890 gas chromatography fitted with a fused silica capillary column (HP-5, 0.25 × 30 m, 0.25 μm film thickness). GC conditions in the analyses were as follow: on-column injection mode; He 1 mL min^{-1}; oven temperature, 175 °C for 2 min, thermal gradient from 175 °C to 280 °C at 40 °C min^{-1}, and then 280 °C. The sample was prior to injection, treated with pyridine containing methanboronic acid (2 mg mL^{-1}) or pyridine containing methanboronic acid (2 mg mL^{-1}), then with *N*-methyl-*N*-trimethylsilytrifluoroacetamide at 80 °C for 30 min for bismethaneboronate (BMB) or methaneboronate-trimethylsilylation, respectively.

References

1. Yokota, T. *Trends Plant Sci.* **1997**, *2*, 137.

Table 1. GC-MS/SIM data for authentic and endogenous BRs in *M. polymorpha*

Compound	Rt[a] (min) on GC	Prominent ions (m/z, relative intensity %)
Endogenous BL[*, 1]	23.01	528 (M^+, 5), 155 (100)
Authentic BL[*, 1]	23.01	528 (M^+, 6), 155 (100)
Authentic[$^2H_6$]BL[*, 1]	22.81	534 (M^+, 6), 161 (100)
Endogenous CS[*, 2]	19.43	512 (M^+, 92), 441 (14), 358 (38), 287 (48), 155 (100)
Authentic CS[*, 2]	19.43	512 (M^+, 84), 441 (16), 358 (24), 287 (48), 155 (100)
Authentic[$^2H_6$]CS[*, 2]	19.26	518 (M^+, 84), 441 (16), 358 (24), 287 (48), 161 (100)
Endogenous 6-DeoxoCS[*, 1]	14.94	498 (M^+, 24), 483 (19), 155 (100)
Authentic 6-DeoxoCS[*, 1]	14.94	498 (M^+, 29), 483 (20), 155 (100)
Authentic[$^2H_6$]6-DeoxoCS[*, 1]	14.77	504 (M^+, 29), 489 (20), 161 (100)
Authentic[$^2H_6$]TE[**, 1]	19.18	550 (M^+, 23), 535 (64), 521 (100), 161 (8)
Authentic[$^2H_6$]TY[**, 1]	16.34	550 (M^+, 63), 535 (44), 521 (100), 161 (24)

Rt[a]: Retention time. [*]: BMB derivative. [**]: MB-TMSi derivative. [1]: Analyzed by GC-SIM. [2]: Analyzed by GC-MS (full scan)

2. Clouse, S. D.; Sasse, J. M. *Annu. Rev. Plant Physiol. Plant Mol. Biol*. **1998**, *49*, 427.
3. Clouse, S. D. *The Plant Journal* **1996**, *10*, 1.
4. Li, J. M.; Chory, J. *Cell* **1997**, *90*, 929.
5. Fujioka, S. *Brassinosteroids: Steroidal Plant Hormones*; Sakurai, A., Yokota, T., Clouse, S. D., Eds.; Springer-Verlag: Tokyo, 1999; p 21.
6. Yokota, T.; Kim, S.-K.; Fukui, Y.; Takahashi, N.; Takeuchi, Y.; Takematsu, T. *Phytochemistry* **1987**, *26*, 503.
7. Takatsuto, S.; Abe, H.; Gamoh, K. *Biol. Chem.* **1990**, *54*, 1057.
8. Kim, S.-K.; Kim, Y.-S.; Kim, T.-W.; Joo, S.-H.; Shim, J.-K. *Bull. Korean Chem. Soc*. **2001**, *22*, 1299.
9. Park, S.-H.; Han, K.-S.; Kim, T.-W.; Shim, J.-K.; Takatsuto, S.; Yokota, T.; Kim, S.-K. *Plant Cell Physiol*. **1999**, *40*, 955.
10. Sakurai, A. *Brassinosteroids: Steroidal Plant Hormones*; Sakurai, A., Yokota, T., Clouse, S. D., Eds.; Springer-Verlag: Tokyo, 1999; p 91.
11. Bishop, G. J.; Yokota, T. *Plant Cell Physiol*. **2001**, *42*, 114.
12. Suzuki, H.; Fujioka, S.; Takatsuto, S.; Yokota, T.; Murofushi, N.; Sakurai, A. *Plant Growth Regul*. **1994**, *13*, 21.
13. Suzuki, H.; Inoue, T.; Fujioka, S.; Saito, S.; Takatsuto, S.; Yokota, T.; Murofushi, N.; Yanagisawa, T.; Sakurai, A. *Phytochemistry* **1995**, *40*, 1391.
14. Suzuki, H.; Fujioka, S.; Takatsuto, S.; Yokota, T.; Murofushi, N.; Sakurai, A. *Biosci. Biotech. Biochem*. **1995**, *59*, 168.
15. Yokota, T.; Ogino, Y.; Takahashi, N.; Saimoto, H.; Fujioka, S.; Sakurai, A. *Agric. Biol. Chem.* **1990**, *54*, 1107.
16. Arima, M.; Yokota, T.; Takahashi, N. *Phytochemistry* **1984**, *23*, 1587.
17. Yokota, T.; Morita, M.; Takahashi, N. *Agric. Biol. Chem*. **1983**, *47*, 2149.