Identification of a Brassinosteroid, Castasterone from *Marchantia polymorpha*

Young-Soo Kim, Yun Hye Sup,† Tae-Wuk Kim, Se-Hwan Joo, and Seong-Ki Kim*

Department of Life Science, Chung-Ang University, Seoul 156-756, Korea
†Department of Biology, Yonsei University, Seoul 120-749, Korea
Received April 16, 2002

Keywords : Brassinosteroids, Castasterone, Liverwort, *Marchantia polymorpha*.

The presence of steroidal plant hormones, brassinosteroids (BRs), in lower plants has been demonstrated in a green alga and a fern.[1,2] Recently, we revealed that liverworts, which are phylogenically higher than algae, contain BRs based on the rice lamina inclination bioassay of the extract from cultured cells of a liverwort, *Marchantia polymorpha*.[3] However, the level was too low to identify BRs from the cultured cells of *M. polymorpha*. Nevertheless, the presence of enzymes responsible for the conversion of teasterone to castasterone, a partial biosynthetic sequence for BRs, was demonstrated in the *Marchantia* cells.[3,4] Furthermore, campesterol (24α-methylcholesteol) and campestanol (24α-cholestanol), which are known to be a biosynthetic precursors of BRs, were recently identified from *M. polymorpha*.[5] These events strongly suggested the presence of BRs in the lower plant, and led us to investigate the occurrence of BRs in naturally-grown *M. polymorpha*.

Whole bodies of naturally-grown *M. polymorpha* (4.3 Kg) were homogenized and extracted with 80% methanol (4 L × 3). The extracts were concentrated to aqueous phase *in vacuo* and re-extracted with chloroform (1 L × 4). After reducing to dryness *in vacuo*, the chloroform fraction was solvent-partitioned between *n*-hexane and 80% methanol (1 L × 3). The concentrated residue of the 80% methanol soluble fraction was partitioned again between phosphate buffer (pH 7.8) and ethyl acetate (1 L × 3). The concentrated ethyl acetate soluble fraction (23.1 g) was purified by silica gel column chromatography (150 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 fractions eluted with 4-8% methanol in chloroform showed activity based on the rice lamina inclination assay. The fractions were combined (3.9 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 the methanol content every 10% from 50% to 100% (100 mL each). The biologically active fractions eluted with 70-90% methanol were concentrated 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, dissolved in a small volume of methanol, and injected into ODS HPLC (Waters Novapak C_{18}, 4 μm, 8 × 100 mm) eluted at a flow rate of 1 mL min^{-1} with the solvent system programmed.[6]

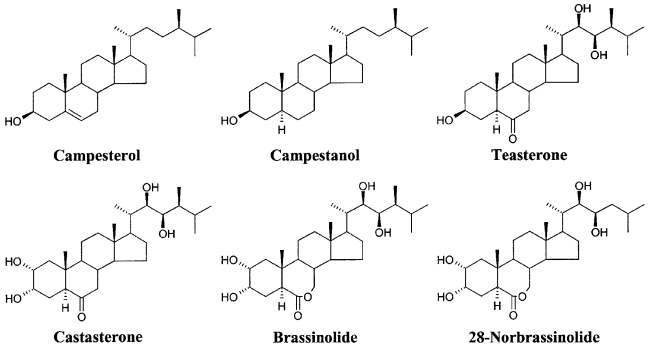

Figure 1. Structure of sterols and BRs mentioned in this study.

As shown in Figure 2, strong activity was detected in HPLC fraction 17-19, whose retention time was similar to that of castasterone (18.2 min), suggesting that castasterone was included in the fraction. In addition, moderate activity was observed in the fraction 12-13 whose retention time was almost equal to that of authentic brassinolide (12.8 min) or 28-norbrassinolide (12.8 min). To verify this, the compounds in the fractions were derivatized into a bismethaneboronate with methaneboronic acid (1 mg) in pyridine (2 mL) at 80 °C for 30 min, and analyzed by GC-MS (EI, 70 eV) using Hewlett-Packard 6890-5973 fitted with a capillary column (HP-5, 0.25 mm × 30 m, 0.25 μm film thickness).[7]

Bismethaneboronate of the active principle in fraction 17/18 gave a molecular ion at m/z 512 which was identical to that of authentic castasterone bismethaneboronate. In addition, characteristic ions at m/z 155 and 358, 327, and 287, which resulted from fission of C20-22, C17-20 and the breakdown of D ring, respectively, in authentic castasterone bismethaneboronate were also observed in the mass spectrum of the active principle bismethaneboronate. Moreover, GC retention time of the active principle bismethaneboronate was exactly the same as that of authentic castasterone bismethaneboronate, indicating that bismethaneboronate of the active compound in the fraction was castasterone bismethaneboronate.

Next, the presence of brassinolide and/or 28-norbrassinolide in the fraction 12-13 was examined by the identical GC-MS analysis. However, no trace of characteristic mass ions to determine bismethaneboronate of brassinolide or 28-norbrassinolide was detected in the fraction. Furthermore, the characteristic mass ions for brassinolide or 28-norbrassinolide were not observed even in GC-selected ion monitoring analysis, whose sensitivity for detection of BRs is approxi-

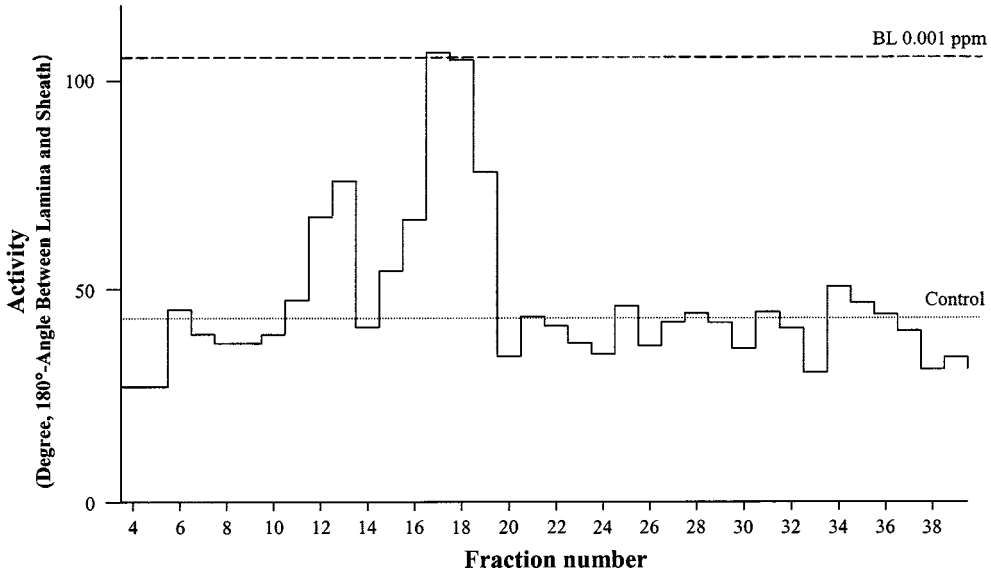

Figure 2. Distribution of BRs activity after a reversed phase HPLC. The activity was measured by the rice lamina inclination assay.

Table 1. GC-MS data for authentic and endogenous castasterone in *M. polymorpha*

Compound	Rt[a] (min) on GC	Prominent ions (m/z, relative intensity %)
Endogenous CS	16.52	512 (M^+, 92), 441 (14), 358 (38), 287 (48), 155 (100)
Authentic CS	16.52	512 (M^+, 84), 441 (16), 358 (24), 287 (39), 155 (100)

Rt[a]; Retention time

mately more than 10 times higher than that of GC-MS analysis, suggesting that amount of the active compound in the fraction is very low. In general, while brassinolide shows very strong activity, 26-norbrassinolide exhibits weak activity in the rice inclination assay.[8] Thus, the high activity against low concentration shown in fraction 12-13 suggested that the active compound in the fraction may be brassinolide. However, the conclusion still remains to be established.

The present study, for the first time, demonstrated the occurrence of a BR, castasterone in a liverwort, *Marchantia polymorpha*. In addition, the presence of brassinolide which is biosynthesized from castasterone, was suggested in the lower plant. Although physiological roles of BRs in lower plants have not been established yet, the result implies that BRs are probably involved in regulation of some events in growth and differentiation of lower plants.

In higher plants, castasterone and brassinolide are biosynthesized from campesterol via the early and late C6-oxidation pathway.[9-12] Together with the presence of campesterol and campestanol in *M. polymorpha*,[5] identification of castasterone in the present strongly suggests that the same biosynthetic pathway(s) that produce castasterone and brassinolide, the early or/and late C6-oxidation pathway in higher plants, is also active in lower plants. To confirm this, identification of other intermediates involved in the biosynthetic pathways of BRs and enzymatic conversions of the intermediates is underway with a large amount of *M. polymorpha*.

References

1. Fujioka, S. *Brassinosteroids: Steroidal Plant Hormones;* Sakurai, A., Yokota, T., Clouse, S. D., Eds.; Springer-Verlag: Tokyo, 1999; p 21.
2. Yokota, T.; Kim, S.-K.; Fukui, Y.; Takahashi, N.; Takeuchi, Y.; Takematsu, T. *Phytochemistry* **1987**, *26*, 503.
3. 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.
4. Kim, T.-W.; Park, S.-H.; Joo, S.-H.; Kim, Y.-S.; Choo, J.-K.; Kim, S.-K. *Bull. Korean Chem. Soc*. **2001**, *22*(6), 651.
5. Kim, S.-K.; Kim, Y.-S.; Kim, T.-W.; Joo, S.-H.; Shim, J.-K. *Bull. Korean Chem. Soc.* **2001**, *22*(12), 1299.
6. HPLC analysis: Mobile phase, 0 to 25 min, 50% acetonitrile; 25 to 35 min, 50% to 100% acetonitrile; 35 to 45 min, 100% acetonitrile. Fractions were collected every min, and BR activity in the fractions were examined by the rice lamina inclination assay.
7. GC condition: 1 mL min^{-1} He, on-column injection mode, temperature program: 175 °C for 2 min, thermal gradient 40 °C min^{-1} to 280 °C, and then maintained at 280 °C.
8. Watanabe, T.; Noguchi, T.; Yokota, T.; Shibata, K.; Koshino, H.; Seto, H.; Kim, S.; Takatsuto, S. *Phytochemistry* **2001**, *58*, 343.
9. Choi, Y. H.; Fujioka, S.; Nomura, T.; Harada, A.; Yokota, T.; Takatsuto, S.; Sakurai, A. *Phytochemistry* **1987**, *26*, 503.
10. Sakurai, A. *Brassinosteroids: Steroidal Plant Hormones;* Sakurai, A., Yokota, T., Clouse, S. D., Eds.; Springer-Verlag: Tokyo, 1999; p 91.
11. Clouse, S. D.; Sasse, J. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **1998**, *49*, 427.
12. Kim, S.-k.; Akihisa, T.; Tamura, T.; Matsumoto, T.; Yokota, T.; Takahashi, N. *Phytochemistry* **1988**, *27*, 629.