Conversion of 6-Deoxocastasterone to Brassinolide in a Liverwort, *Marchantia polymorpha*

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We have recently demonstrated that a liverwort, *Marchantia polymorpha*, contained steroidal plant hormones, collectively named brassinosteroids (BRs), such as 6-deoxocastasterone (6-deoxoCS), castasterone (CS) and brassinolide (BL).[1,2] In addition, 24α-methylcholesterol (campesterol, CR) and 24α-methylcholestanol (campestanol, CN) as potent biosynthetic precursors of the endogenous BRs have been found in *M. polymorpha*.[3] These BRs and phytosterols are members of a biosynthetic pathway for BRs established in higher plants, namely the late C6-oxidation pathway (Fig. 1), proposing that BRs in the lower plant are also biosynthesized by a similar biosynthetic pathway to that in higher plants.[4-7] However, any concrete evidences for the presence of the BRs biosynthetic pathway in the lower plant have not been made yet. This led us to investigate the presence of a partial biosynthetic sequence of the late C6-oxidation pathway, 6-deoxoCS $\rightarrow$ CS $\rightarrow$ BL, in *M. polymorpha* by *in vivo* and *in vitro* conversions using deuterium-labeled ([$^2H_6$-C26, 28]) substrates in the study, which gives a clue for biogenesis of the BRs biosynthetic pathways in plant kingdom as well as how BRs are biosynthesized in lower plants.

30 μg of [$^2H_6$]-6-deoxoCS or 100 μg of [$^2H_6$]-CS was added to the media of suspension cultured cells of *M. polymorpha*.[8] After incubated for a week, the cells were harvested, and extracted with 80% methanol. The extracts were combined with the media, and purified by column chromatographies followed by a reversed phase HPLC. When [$^2H_6$]-6-deoxoCS was fed to the *Marchantia* cells, BRs activity appeared in HPLC fraction 12-14 eluted with 45% acetonitrile which were identical to that of [$^2H_6$]-CS, implying that [$^2H_6$]-CS was contained in the fraction as a metabolite of [$^2H_6$]-6-deoxoCS (Table 1). The active principle in the fraction was then derivatized to be a bismethaneboronate (BMB), and analyzed by GC-selected ion monitoring (SIM). As expected, selected ions at *m/z* 518, 441, 358, 287 and 161 to identify [$^2H_6$]-CS BMB were detected at the same GC retention time as those derived from authentic [$^2H_6$]-CS BMB (Table 1). Therefore, the active compound in the fraction was determined to be [$^2H_6$]-CS. When [$^2H_6$]-CS was fed to the cell, HPLC fraction 14, 15 eluted with 33% acetonitrile showed biological activity. The fractions were combined, and analyzed by GC-SIM after bismethane-boronation. Characteristic ions to identify [$^2H_6$]-BL BMB at *m/z* 534, 374, 343 and 161 were detected at the same GC retention time as those derived from authentic [$^2H_6$]-BL BMB, indicating that the metabolite of [$^2H_6$]-CS was [$^2H_6$]-BL (Table 1).

Next, *in vitro* enzymatic conversion of 6-deoxoCS to BL

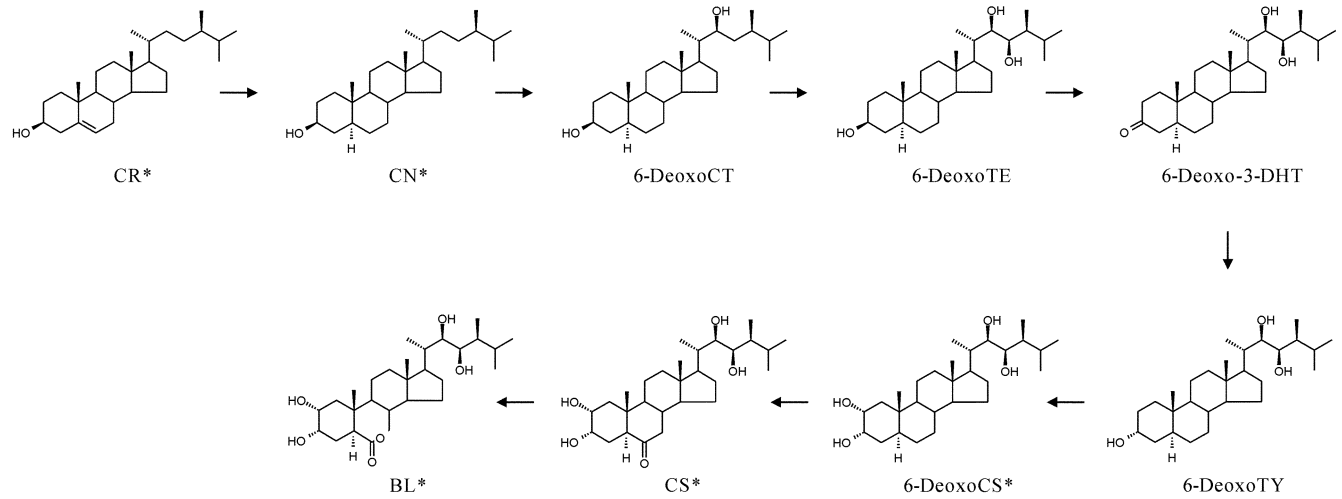

Figure 1. The late C6-oxidation pathway to produce BRs possibly involved in *M. polymorpha*. Asterisks indicate steroids identified from *M. polymorpha*. 6-DeoxoCT, 6-deoxoTE, 6-deoxo-3-DHT and 6-deoxoTY is 6-deoxocathasterone, 6-deoxoteasterone, 6-deoxo-3-dehydroteasterone and 6-deoxotyphasterol, respectively.

Table 1. GC-MS data for authentic [$^2H_6$]-BRs and metabolite of [$^2H_6$]-6-deoxoCS and CS in *M. polymorpha*

Compound*	Rt** (min) on HPLC	Rt** (min) on GC	Prominent ions (*m/z*, relative intensity %)
[$^2H_6$]-CS	12-14***	15.58	518 (M^+, 84), 441 (16), 358 (24), 287 (39), 161 (100)
[$^2H_6$]-BL	14, 15****	19.10	534 (M^+, 2), 374 (33), 343 (15), 161 (100)
Metabolite of [$^2H_6$]-6-deoxoCS	12-14***	15.58	518 (M^+, 87), 441 (17), 358 (24), 287 (42), 161 (100)
Metabolite of [$^2H_6$]-CS	14, 15****	19.10	534 (M^+, 2), 374 (32), 343 (15), 161 (100)

*Compound was analyzed as a bismethaneboronate derivative. **Rt: Retention time. ***HPLC was carried out with 45% acetonitrile. ****HPLC was carried out with 33% acetonitrile.

Table 2. Enzyme activities in subcellular enzyme solutions prepared from *M. polymorpha*

Subcellular fraction	Enzyme activity		
	6-DeoxoCS oxidase*	CS 6-oxidase*	NADPH-Cyt c reductase**
Cytosolic	ND	ND	0.09
Microsomal	5.90	0.62	2.80
+ Cyt c	1.79	ND	NE

*The enzyme activity was expressed as a specific activity, ng of the product mg protein^{-1} min^{-1}. **The enzyme activity was expressed as a specific activity, μmol of the product mg protein^{-1} min^{-1}. ND: Not Detected. NE: Not Determined.

via CS was examined by the use of crude enzyme solutions prepared from *Marchantia* cells. To the end, the cultured cells were homogenized, and a microsomal and cytosolic enzyme solution were separately collected by ultra-centrifugation. The activity of NADPH-Cytochrome (Cyt) c reductase, a marker enzyme for microsomal enzymes, in the cytosolic solution was only one thirtieth of that in the microsomal solution, indicating that both microsomal and cytosolic enzymes were well divided in the subcellular solutions with almost no contamination (Table 2).

Because endogenous amount of BRs in the culture cells (15 g) was negligible,[9] unlabeled ([$^2H_0$]) 6-deoxoCS was added as a substrate to the microsomal or cytosolic enzyme solution. After the enzyme assay, 50 ng of [$^2H_6$]-CS was added to the assay mixtures as an internal standard for quantitative analysis, and the product was analyzed by GC-SIM after methaneboronation. Only in the microsomal solution, selected ions at *m/z* 512 [M^+] and 155 (base peak) were observed at the same GC retention time as those of authentic CS BMB (Fig. 2A and Table 2). The enzyme activity was clearly decreased by addition of Cyt c, a Cyt P450 inhibitor (Table 2). Taken together, the enzyme mediating the conversion of 6-deoxoCS to CS, namely 6-deoxoCS oxidase, was thought to be a Cyt P450 mono-oxygenase which is bound to the membrane of cells, most likely endoplasmic reticulum. The specific activity of 6-deoxoCS oxidase calculated by ratio of [$^2H_0$]-/[$^2H_6$]-CS in the assay solution was 5.90 ng mg protein^{-1} min^{-1}.

Enzymatic conversion of CS to BL was also carried out with the same subcellular solutions by the addition of [$^2H_0$]-CS and [$^2H_6$]-BL as a substrate and an internal standard, respectively. In GC-SIM analysis, as shown in Fig. 2B, characteristic ions to determine BL as a product at *m/z* 528 [M^+] and 155 (base peak) were detected in the microsomal solution but not in cytosolic solution, indicating that the enzyme, namely CS 6-oxidase (BL synthase), was a mono-oxygenase bound to membrane of endoplasmic reticulum.

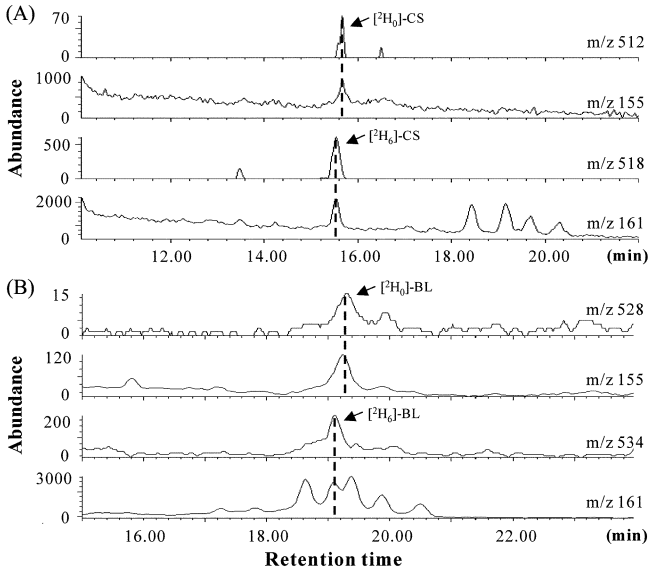

Figure 2. GC-SIM profile for the product of 6-deoxoCS oxidase (A) or CS 6-oxidase (B) in *M. polymorpha.*

Inhibitory effect by Cyt c on *Marchantia* CS 6-oxidase was examined, but a reliable result was not obtained due to a low activity (0.62 ng mg protein^{-1} min^{-1}) of CS 6-oxidase in *Marchantia* cells.

In summary, we the first demonstrated *in vivo* and *in vitro* conversion of 6-deoxoCS to CS in a liverwort, *M. polymorpha.* This provides that *Marchantia* cells contain enzyme systems mediating a BRs biosynthetic sequence, 6-deoxoCS $\rightarrow$ CS $\rightarrow$ BL, which is the last two steps in the late C6-oxidation pathway to produce BL found in higher plants.[4-7] Coupled with the presence of CR and CN, this strongly suggested that BRs in *M. polymorpha* can be biosynthesized by the same pathway as that in higher plants, most likely the late C6-oxidation pathway. It can thus be hypothesized that BRs biosynthesis in higher plants is sustained from that in

lower plants during evolutional processes.

Conversion of 6-deoxoCS to CS is a reaction in which an oxygen atom is incorporated. In many biological systems, the reaction is catalyzed by Cyt P450 enzymes. In fact, the C-6 oxidation has been demonstrated to be mediated by a Cyt P450 by analyzing the tomato *Dwarf (D)* gene which had high homology to mammalian steroid hydroxylase genes.[10] In addition, the enzyme responsible for the C6-oxidation, 6-deoxoCS oxidase, was characterized to be a P450 from *Phaseolus vulgaris*, where the enzyme was located in the membrane of endoplasmic reticulum, required NADPH and molecular oxygen for activity, and inhibited by several P450 inhibitors. Especially, the inhibition by a specific inhibitor of P450, carbon monoxide, was reversibly recovered to control value by illumination of a blue light (will be published elsewhere). In this study, 6-deoxoCS in *Marchantia* was also found in the membrane fraction, and showed its activity under the aerobic condition in the presence of NADPH. Furthermore, the enzyme activity was inhibited by a P450 inhibitor, Cyt c. Therefore, it is thought that the *Marchantia* 6-deoxoCS oxidase is also a P450 enzyme.

In the reaction that converts CS to BL, an oxygen atom is inserted into C-C bond. Such reactions have been classified into the Baeyer-Villiger reaction. In bacteria and fungi, enzymes responsible for the biological Baeyer-Villiger are all monooxygenases belong to a group of flavoprotein with FAD as a prosthetic group, requiring NADPH as an electron donor.[11-13] In a plant, *P. vulagris*, the enzyme mediating the conversion of CS to BL showed all enzyme properties of a P450, especially inhibitory effect by carbon monoxide which do not exhibit any inhibitory effect on flavoprotein activity.[14] As far as the biological Baeyer-Villiger oxidation, plants seem to be different from bacteria and fungi because lactonization from CS to BL *in planta* is elaborated by a P450 rather than flavoprotein. *M. polymorpha* is a lower plant which has the same BRs biosynthetic pathway as that in higher plants, suggesting that the *Marchantia* CS 6-oxidase is also a P450. Properties for the *Marchantia* CS 6-oxidase such as intracellular localization and requirement of oxygen and NADPH for the activity obtained in this study are included in general characteristics for P450s, but they are overlapped for those for flavoprotein. To confirm the possibility for CS 6-oxidase as a P450, more physical evidences, most likely inhibitory effect of P450 and flavoprotein inhibitors on the CS 6-oxidase should be addressed, which will be helpful to understand evolution of monooxygenation, at least the Baeyer-Villiger oxidation in biological systems.

Experimental Section

Isolation of metabolite. The 95% ethanolic solution (30 μL) of [$^2H_6$]-6-deoxoCS (30 μg) or [$^2H_6$]-CS (100 μg) was added to the medium of the cell suspension culture at the 14th d of growth. After incubation for 7 d, the cells were collected and extracted with 80% methanol (200 mL × 3). The extracts were concentrated to aqueous phase in vacuo, combined with the culture medium (350 mL), and subjected to ODS (bed volume 100 mL, Merck LiChroprep RP-18) chromatography. Elution was carried out with the aqueous methanol increasing methanol content every 200 mL (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100%). The biologically active 70 and 80% methanol fraction were combined and chromatographed on Sephadex LH-20 column (bed volume 340 mL; 22 × 900 mm) using a 4 : 1 mixture of methanol-chloroform at a flow rate of 0.5 mL min^{-1}. The bioactive fractions with 0.65-0.75 of Ve/Vt were combined, dissolved in small volume of methanol, and subjected to a reversed phase HPLC (8 × 100 mm, 4 μm Novapak C18 column) at a flow rate of 1 mL min^{-1} with 45% or 33% acetonitrile for the metabolite of [$^2H_6$]-6-deoxoCS or CS, respectively. The active fractons 12-14 were analyzed by GC-MS/SIM.

Enzyme preparation and assays. During all enzyme preparation steps, the temperature was maintained at 0 to 4 ^oC. The harvested cells (15 g) were ground in 80 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 250 mM sucrose, 15 mM 2-mercaptoethanol, 1 mM EDTA, 40 mM ascorbate, 1 mM dithiothreitol, 0.1 mM phenylmethyl-sulfonyl fluoride, 15% (v/v) glycerol and 1% (w/v) insoluble polyvinylpyrrolidone. The homogenate was filtered and centrifuged at 15,000 × g for 20 min. The resulting super-natant was re-centrifuged at 190,000 g for 120 min (Kontron Centrikon T-1180). The obtained pellet was re-suspended in 4 mL of 0.1 M sodium phosphate buffer (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% (v/v) glycerol and used as a microsomal enzyme solution. Cytosolic enzymes were precipitated from the supernatant by addition of cold acetone to 40% (v/v) final concentration. The supernatant-acetone mixture was kept at −25^oC for 20 min and centrifuged at 13,000 × g for 10 min. The resulting precipitate was dissolved in 8 mL of 0.1 M sodium phosphate (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% (v/v) glycerol and used as a cytosolic enzyme solution. Protein concentrations of the microsomal and cytosolic solution were estimated with a microassay from Bio-Rad using BSA as a standard.

NADPH-Cyt c reductase activity was determined by the changes with time in the absorbance at 550 nm (Cyt c reduction) using a Spectra Max 340 spectrophotometer (Molecular devices Co.). The assay mixture contained 200 μL enzyme solution, 100 mM sodium phosphate (pH 7.4), 5 μM FMN, 10 mM $MgCl_2$, and 60 μM Cyt c in a total volume of 1 mL. The assay was initiated by addition of NADPH to a final concentration of 0.4 mM, and the data were collected every 9 s for 1 min. An extinction coefficient used to calculate enzyme activity was 29.5 mM^{-1} cm^{-1} (Cyt c reduction).

The assay for 6-deoxoCS oxidase and CS 6-oxidase were performed in duplicate and the standard assay mixture consisted of 5 mg protein mL^{-1} from either cytosolic or microsomal enzymes in the re-suspension buffer, 0.8 mM NADPH and 5 μg substrate (6-deoxoCS or CS) in a total volume of 1.2 mL. The reaction was initiated by addition of

NADPH. The reaction mixture was thoroughly mixed and incubated at 37°C for 30 min with shaking. The reaction was terminated by addition of 1.2 mL of ethyl acetate and C26, 28-$^{2}H_{6}$-labeled BR (CS or BL) 50 ng was added to the reaction mixtures as an internal standard for quantitative analysis. The reaction mixture was then centrifuged at 2,000 rpm for 10 min and ethyl acetate soluble fraction was obtained (three times in 1.2 mL). The ethyl acetate soluble fraction was concentrated *in vacuo*, dissolved in 50% methanol (5 mL), and charged on an octadecylsilane cartridge column (Sep-Pak Plus C18, Waters). The fraction eluted with 100% methanol (5 mL) was concentrated in vacuo and subjected to a reversed phase HPLC (Novapak C18, 8 × 100 mm) at a flow rate 1 mL min^{-1} with 45% and 33% acetonitrile for elution of the product for 6-deoxoCS oxidase and CS 6-oxidase, respectively.

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 min^{-1}, and then 280°C. The sample was prior to injection, treated with pyridine containing methanboronic acid (2 mg mL^{-1}) at 80°C for 30 min for bismethaneboronate.

References

1. Kim, Y.-S.; Yun, H. S.; Kim, T.-W.; Joo, S.-H.; Kim, S.-K. *Bull. Korean Chem. Soc.* **2002**, *23*(7), 941.
2. Kim, Y.-S.; Kim, S. Y.; Kim, S.-K.; Shim, J. K. *Bull. Korean Chem. Soc.* **2002**, *23*(10), 1473.
3. Kim, S.-K.; Kim, Y.-S.; Kim, T.-W.; Joo, S.-H.; Shim, J. K. *Bull. Korean Chem. Soc.* **2001**, *22*(12), 1299.
4. Choi, Y. H.; Fujioka, S.; Nomura, T.; Harada, A.; Yokota, T.; Takatsuto, S.; Sakurai, A. *Phytochemistry* **1997**, *44*, 609.
5. Sakurai, A. *Brassinosteroids: Steroidal Plant Hormones*; Sakurai, A., Yokota, T., Clouse, S. D., Eds.; Springer-Verlag: Tokyo, 1999; p 91.
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. *Agric. Biol. Chem.* **1990**, *54*, 1057.
8. Ohta, Y.; Katoh, K.; Miyaki, K. *Planta* **1977**, *136*, 229.
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. Bishop, G. J.; Nomura, T.; Yokota, T.; Harrison, K.; Noguchi, T.; Fujioka, S.; Takatsuto, S.; Jones, J. D.; Kamiya, Y. *PNAS* **1999**, *96*(4), 1761.
11. Donoghue, N. A.; Norris, D. B.; Trudgill, P. W. *NCIB 9871*. *Eur. J. Biochem*. **1976**, *63*, 175.
12. Walton, A. Z.; Stewart, J. D. *Biotechnol. Prog.* **2002**, *18*, 262.
13. Fraaije, M. W.; Kamerbeek, N. M.; Van Berkel, W. J. H.; Janssen, D. B. *FEBS. Letters* **2002**, *518*, 43.
14. El-Alfy, A.; Schlenk, D. *Toxicol. Appl. Pharmacol.* **1998**, *152*, 175.