Biotransformation of 6-Deoxotyphasterol in a Liverwort, *Marchantia polymorpha*

Young-Soo Kim, Tae-Wuk Kim, and Seong-Ki Kim*

Department of Life Science, Chung-Ang University, Seoul 156-756, Korea
Received July 11, 2003

Key Words : Brassinosteroids, Biotransformation, 6-Deoxotyphasterol, Liverwort, *Marchantia polymorpha*

A series of our researches revealed that a Bryophyte, *Marchantia polymorpha*, contained steroidal plant hormones, collectively named brassinosteroids (BRs), such as 6-deoxocastasterone (**7**), castasterone (**8**) and brassinolide (**9**),[1,2] and potent biosynthetic precursors of the BRs, campesterol (**1**) and campestanol (**2**).[3] Together with demonstrating the presence of a biosynthetic sequence, **7** → **8** → **9**, the result strongly suggested that a biosynthetic pathway from **1** to **9** via **2**, **7** and **8**, so called the C6-oxidation pathway found in higher plants,[4-8] is operative to produce BRs in the lower plants (Fig. 1).[9] Nevertheless, biosynthetic intermediates between **2** and **7** in the C6-oxidation pathway have not been identified from the lower plant, so that the precise pathway to synthesize **7** from **2** in *M. polymorpha* still remains to be elucidated. This prompted us to investigate *in vivo* and *in vitro* conversion(s) of [26, 28-$^2H_6$]- and [$^2H_0$]-6-deoxotyphasterol (**6**), a potent biosynthetic precursor of **7**, in cultured cells of *M. polymorpha* in this study,[10,11] which gives information on biosynthetic processes for A-ring hydroxylation to produce the BRs identified from the lower plant.

First, [$^2H_6$]-**6** was fed to the media of suspension cultured cells of *M. polymorpha* to investigate *in vivo* conversion of **6** in the lower plant. After incubation for 7 days, the cells were harvested and extracted with 80% methanol followed by chloroform. The extract was reduced to aqueous phase, combined with the media, and purified by column chromatographies by the guidance of the rice lamina inclination assay.[12] The final purification for metabolites of [$^2H_6$]-**6** was carried out by a reversed phase HPLC. The obtained HPLC fractions were analyzed by a Preparative TLC, giving rise to BR-like purple-bluish spots in the HPLC fraction 42, 43, 50-52, 55 and 56. Among them, the fraction 55 and 56 was correspondent to the retention time of [$^2H_6$]-**6** added as a substrate, proposing that the active compounds in the fraction 42, 43 and 50-52 were metabolites of [$^2H_6$]-**6** in the *Marchantia* cells. The fractions were thus derivatized to be a methaneboronate (MB) or MB-trimethylsilylic ether (TMSi), and then analyzed by GC-MS/Selected Ion Monitoring (SIM).

As summarized in Table 1, a bis-MB (BMB) of the active compound in the fraction 42 and 43 showed characteristic ions for [$^2H_6$]-**7** BMB at *m/z* 504 (M^+, 39%), 489 (13), 273 (100) and 161 (34) at the same GC retention time as that of authentic [$^2H_6$]-**7** BMB. A MB of one of the active compounds in the fraction 50-52 gave a mass spectrum at *m/z* 462 (M^+, 47%), 447 (7), 385 (5), 301 (8), 246 (12), 231 (100) and 161 (45) whose mass spectrum and GC retention time were identical to those of authentic [$^2H_6$]-6-deoxo-3-dehydroteasterone ([$^2H_6$]-**5**) MB. In addition, a MB-TMSi ether of other active compound in the fraction appeared the same mass spectrum at *m/z* 536 (M^+, 45%), 521 (42), 479 (19), 446 (20), 431 (29), 305 (28), 215 (100) and 161 (41) at the equal

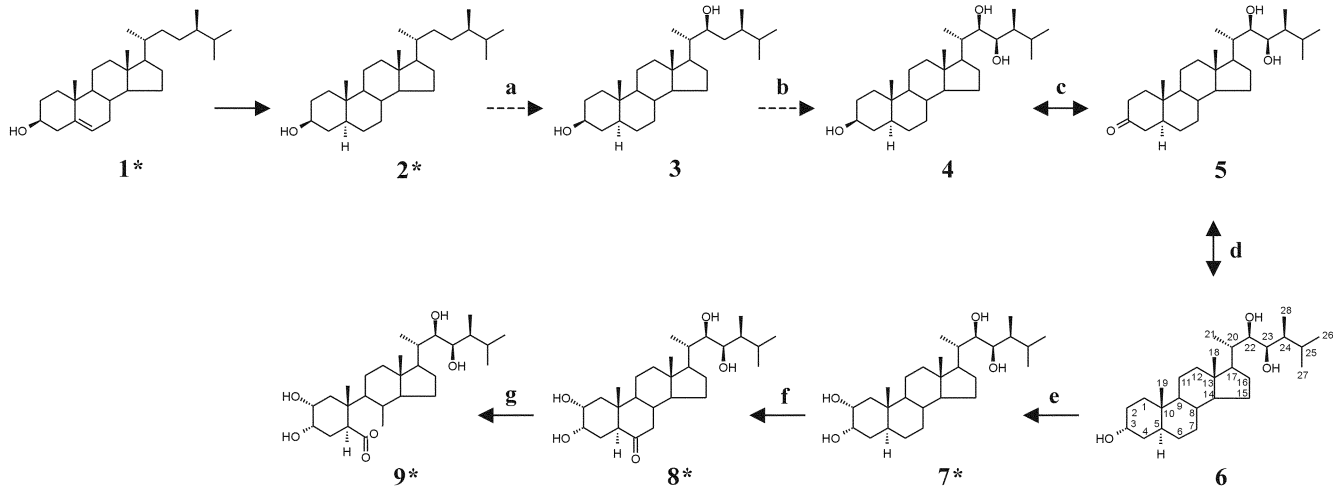

Figure 1. The late C6-oxidation pathway to produce BRs possibly involved in *M. polymorpha*. Asterisks indicate steroids identified from *M. polymorpha*. Dashed arrows indicate steps which are not verified yet. **1**, campesterol; **2**, campestanol; **3**, 6-deoxocathasterone; **4**, 6-deoxoteasterone; **5**, 6-deoxo-3-dehydroteasterone; **6**, 6-deoxotyphasterol; **7**, 6-deoxocastasterone; **8**, castasterone; **9**, brassinolide; **a**, **2** 22*R*-hydroxylase; **b**, **3** 23*R*-hydroxylase; **c**, **4** dehydrogenase; **d**, **5** reductase; **e**, **6** 2a-hydroxylase; **f**, **7** oxidase; **g**, **8** oxidase.

Table 1. HPLC and GC-MS data for identified BRs as *in vivo* and *in vitro* metabolites in *M. polymorpha*

Compound	Rt[1] on HPLC	Rt[1] on GC	Prominent ions (*m/z*, relative intensity %)
Authentic **4** [2]	50-52	14.02	530 (M^+, 49), 515 (44), 473 (17), 440 (17), 425 (29), 305 (28), 215 (100), 155 (41)
Authentic [$^2H_6$]-**4**[2]	50-52	13.78	536 (M^+, 50), 521 (46), 479 (17), 446 (18), 431 (29), 305 (28), 215 (100), 161 (42)
Authentic **5** [3]	50-52	14.18	456 (M^+, 51), 441 (6), 385 (4), 301 (7), 246 (12), 231 (100), 155 (47)
Authentic [$^2H_6$]-**5**[3]	50-52	13.91	462 (M^+, 46), 447 (6), 385 (4), 301 (8), 246 (12), 231 (100), 161 (44)
Authentic **6** [2]	55, 56	12.22	530 (M^+, 23), 515 (4), 479 (3), 440 (48), 425 (49), 305 (12), 215 (100), 155 (29)
Authentic [$^2H_6$]-**6**[2]	55, 56	12.01	536 (M^+, 23), 521 (3), 479 (2), 446 (47), 431 (48), 305 (12), 215 (100), 161 (29)
Authentic **7** [4]	42, 43	14.47	498 (M^+, 39), 483 (15), 273 (100), 155 (34)
Authentic [$^2H_6$]-**7**[4]	42, 43	14.22	504 (M^+, 40), 489 (14), 273 (100), 161 (34)
Identified **4** [2]	50-52	14.02	530 (M^+, 44), 515 (42), 473 (23), 440 (23), 425 (33), 305 (31), 215 (100), 155 (38)
Identified [$^2H_6$]-**4**[2]	50-52	13.78	536 (M^+, 45), 521 (42), 479 (19), 446 (20), 431 (29), 305 (28), 215 (100), 161 (41)
Identified **5** [3]	50-52	14.18	456 (M^+, 52), 441 (3), 385 (5), 301 (8), 246 (10), 231 (100), 155 (37)
Identified [$^2H_6$]-**5**[3]	50-52	13.91	462 (M^+, 47), 447 (7), 385 (5), 301 (8), 246 (12), 231 (100), 161 (45)
Identified **6** [2]	55, 56	12.22	530 (M^+, 23), 515 (5), 479 (4), 440 (47), 425 (48), 305 (12), 215 (100), 155 (30)
Identified [$^2H_6$]-**6**[2]	55, 56	12.01	536 (M^+, 24), 521 (4), 479 (3), 446 (46), 431 (47), 305 (12), 215 (100), 161 (29)
Identified **7** [4]	42, 43	14.47	498 (M^+, 36), 483 (14), 273 (100), 155 (35)
Identified [$^2H_6$]-**7**[4]	42, 43	14.22	504 (M^+, 39), 489 (13), 273 (100), 161 (34)

1: Retention time (min). 2: The sample was analyzed as a methanboronate (MB)-trimethylsilylic ether (TMSi). 3: The sample was analyzed as a methanboronate (MB). 4: The sample was analyzed as a bismethanboronate (BMB).

GC retention time to that of authentic [$^2H_6$]-6-deoxoteasterone ([$^2H_6$]-**4**) MB-TMSi ether. In consequence, [$^2H_6$]-**4**, -**5** and -**7** were identified as metabolites of [$^2H_6$]- **6**, which indicated that **6** could be transformed into **4**, **5** and **7** in the *Marchantia* cells.

Next, *in vitro* conversion of **6** was investigated by the use of a crude enzyme solution prepared from the cultured cells of *M. polymorpha*. Because endogenous amount of BRs in the cells (15 g) was negligible,[11] non-deuterium labeled ([$^2H_0$]) **6** was used as a substrate, and deuterium labeled expected products, [$^2H_6$]-**4**, -**5** and -**7**, were added for quantitative analyses after finishing enzyme assays. The enzyme products were extracted and purified by the methods described in Experimental Section, and the HPLC fractions 42, 43 and 50-52 from which *in vivo* metabolites of [$^2H_6$]-**6**, [$^2H_6$]-**4**, -**5** and -**7** were detected, were analyzed by a capillary GC-MS/SIM. In the HPLC fraction 42 and 43, BMB of the enzyme product showed mass ions at *m/z* 498 (M^+, 36), 483 (14), 273 (100) and 155 (35) that were detected at the same GC retention time as that of authentic **7** BMB (Table 1). In the HPLC fraction 50-52, a MB-TMSi and a MB of the product gave the identical mass spectrum and GC retention time to those of authentic **4** MB-TMSi and **5** MB, respectively (Table 1). Coupled with the result obtained from *in vivo* feeding experiment, these revealed that **6** was converted into **7** by 2α-hydroxylation, and **4** *via* **5** by C6-oxidation and C3-epimerization. Therefore, the presence of a biosynthetic sequence, **4** ← **5** ← **6** → **7**, was demonstrated in the cells (Table 2).

In the late C6-oxidation pathway, 3-epimerization of **4** to **6** *via* **5** is known to be a reversible reaction.[11,13] Thus, the presence of **6** → **5** → **4** strongly suggested that a reversed reaction of the sequence can be also functional in the *Marchantia* cells. To confirm that, enzymatic conversions of **4** and **5** in the cells were subsequently performed. When **4** was added to the enzyme solution as a substrate, **5** and **6** were identified as the products. When **5** was used, **4** and **6** were characterized to be the products (Table 2). These indicated that conversions of **4** → **5** → **6** and **4** ← **5** → **6** also occurred in the cells, proofing the presence of a reversible 3-epimerization of **4** to **6** *via* **5**, **4** ↔ **5** ↔ **6**, in the *Marchantia* cells.

Table 2. Enzymatic conversions of BRs in the cells of *M. polymorpha*

Substrate	Product(s)	Enzyme		Biosynthetic Sequence(s)
		Name	Activity[a]	
6	**7**	**e**	23.5	**6** → **7**
	5	**d**	ND[b]	**5** ← **6**
	4	**d, c**	ND[b]	**4** ← **5** ← **6**
5	**4**	**c**	ND[b]	**4** ← **5**
	6	**d**	57.9	**5** → **6**
4	**5**	**c**	100.0	**4** → **5**
	6	**c, d**	ND[b]	**4** → **5** → **6**
				Sum : **4** ↔ **5** ↔ **6** → **7**

[a]Enzyme activity is expressed as ng product mg protein^{-1} min^{-1}. [b]ND: Not determined.

The activity of enzymes mediating the conversion of **4** to **6** *via* **5** and **6** to **7**, namely **4** dehydrogenase (**c**), **5** reductase (**d**) and **6** 2α-hydroxylase (**e**), respectively, in the *Marchantia* cells calculated by the ratio of the products to the internal standards ([$^2H_6$]-**4**, -**5** and -**7**) added was 100.0, 57.9 and 23.5 ng mg protein^{-1} min^{-1}, respectively, which are much higher than that of **7** oxidase (**f**, 5.9 ng mg protein^{-1} min^{-1}) and **8** 6-oxidase (**g**, 0.6 ng mg protein^{-1} min^{-1}) previously determined in the cells.[9] This probably means that **4** is quickly converted into **7** *via* **5** and **6**, but the next conversion of **7** to **9** *via* **8** slowly occurs. For the reason, the endogenous level of **7**, **8** and **9** seems to be kept higher than those of **4**, **5** and **6** in the *Marchantia* cells, which probably allowed us to identify **7**, **8** and **9** but not **4**, **5** and **6** from *M. polymorpha*.[1,2]

Although **4**, **5** and **6** has not been identified from *M. polymorpha*, the presence of a biosynthetic sequence of BRs, **4** ↔ **5** ↔ **6** → **7**, in the *Marchantia* cells was

demonstrated by *in vivo* and *vitro* conversions in this study. Because the presence of biosynthetic sequences, **1** → **2** and **7** → **8** → **9**, was already verified in the cells,[1-3] the result indicates that *M. polymorpha* contains biosynthetic sequences, **1** → **2** and **4** ↔ **5** ↔ **6** → **7** → **8** → **9**, to produce BRs. These biosynthetic sequences are included in the C6-oxidation pathway established in higher plants, which suggests that the same biosynthetic pathway in higher plants is working to produce BRs in a liverwort, *M. polymorpha*. Therefore, it is thought that the biosynthetic pathway to BRs in higher plants, at least the C6-oxidation pathway, is evolved from that in lower plants. To complete the genesis of BRs biosynthesis in plant kingdom, demonstration of the presence of the skipped biosynthetic pathway from **2** to **4** *via* **3** in *Marchantia* cells is underway now.

Experimental Section

Purification of *in vivo* metabolites of [$^2H_6$]-6. [$^2H_6$]-**6** (50 μg) was added to suspension-cultured cells (15 g) of *M. polymorpha*.[10] After 1 week, the cells were harvested and extracted with 80% methanol (100 mL × 3). The extract was reduced to aqueous phase, combined with the culture media (30 mL), and re-extracted with chloroform (100 mL × 3). The chloroform soluble extract was concentrated and partitioned between *n*-hexane and 80% methanol. The biological active 80% methanol fraction was evaporated, and partitioned again between phosphate buffer (0.1 M, pH 7.8) and ethyl acetate. The ethyl acetate soluble fraction was then loaded on a silica gel (20 g, Merck Co.) column. Elution was carried out with every 100 mL of chloroform, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 50% methanol in chloroform. The fraction 4-6% methanol in chloroform shown BR-activity was combined and subjected to C18 bulk chromatography (100 mL, Waters, Preparative C18) that was eluted with 50, 60, 70, 80, 90 and 100% methanol. The fraction eluted 70-90% methanol was further purified by a reversed phase HPLC (Senshu Pak Pegasil-B, 10 × 150 mm) at a flow rate 2.5 mL min^{-1} using aqueous acetonitrile as a mobile phase (0-20 min: 45%, 20-40 min: gradient to 100%, and then 100%). The HPLC fractions were collected every min, and analyzed by a Preparative F254 TLC (Merck. Co) developed with a mixture of methanol-chloroform (1 : 6). After treatment of 70% sulfuric acid followed by heating, BR-like purple-bluish spots were detected in the HPLC fraction 42-43, 50-52 and 55-56. The fractions were analyzed by GC-MS/SIM.

Enzyme preparation and assay. Suspension cultured cells (15 g) of *M. polymorpha* were homogenized with Na-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 phenylmethylsulfonyl 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 supernatant was collected and used as a crude enzyme solution to examine enzymatic transformations in the study.

In vitro enzymatic conversions were undertaken by addition of **6** (5 μg × 3) to the crude enzyme solution (500 μL × 3) The assay mixture was incubated at 37 °C from 30 min, and enzyme products were extracted with ethyl acetate after adding [$^2H_6$]-**4**, -**5** and -**7** as internal standards for quantitative analysis. The obtained ethyl acetate soluble fraction was concentrated, re-dissolved in 50% methanol (10 mL), and loaded on Sep-Pak C18 (Waters Co.) cartridge eluted with aqueous methanols (10 mL each, 50, 60 and 100%). The fraction eluted with 100% methanol that showed a BR-activity was subjected to a reversed phase HPLC (Senshu Pak Pegasil-B, 10 × 150 mm) at a flow rate 2.5 mL min^{-1} using aqueous acetonitrile as a mobile phase (0-20 min: 45%, 20-40 min: gradient to 100%, and then 100%). The HPLC fractions 42-43 and 50-52 were collected and analyzed by GC-MS/SIM.

GC-MS/SIM analysis. Metabolites of [$^2H_0$]- and [$^2H_6$]-**6** were analyzed by 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. Prior to injection, samples were derivatized to be a methaneboronate (MB) or MB-trimethylsilylic ether (TMSi).[11]

Acknowledgement. This work was supported by Korea Research Foundation Grant (KRF-2001-015-DP0483).

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. Yokota, T.; Kim, S.-K.; Fukui, Y.; Takahashi, N.; Takeuchi, Y.; Takematsu, T. *Phytochemistry* **1987**, *26*, 503.
5. Yokota, T.; Ogino, Y.; Takahashi, N.; Saimoto, H.; Fujioka, S.; Sakurai, A. *Agric. Biol. Chem.* **1990**, *54*, 1107.
6. Choi, Y. H.; Fujioka, S.; Nomura, T.; Harada, A.; Yokota, T.; Takatsuto, S.; Sakurai, A. *Phytochemistry* **1997**, *44*, 609.
7. Sakurai, A. *Brassinosteroids: Steroidal Plant Hormones*; Sakurai, A., Yokota, T., Clouse, S. D., Eds.; Springer-Verlag: Tokyo, **1999**; p 91.
8. Suzuki, H.; Fujioka, S.; Takatsuto, S.; Yokota, T.; Murofushi, N.; Sakurai, A. *Plant Growth Regul.* **1994**, *13*, 21.
9. Kim, Y.-S.; Kim, T.-W.; Kim, S.-K. *Bull. Korean Chem. Soc.* **2003** (in press).
10. Ohta, Y.; Katoh, K.; Miyaki, K. *Planta* **1977**, *136*, 229.
11. Park, S.-H.; Han, K.-S.; Kim, T.-W.; Shim, J.-K.; Takatsuto, S.; Yokota, T.; Kim, S.-K. *Plant Cell Physiol* **1999**, *40*(9), 955.
12. Arima, M.; Yokota, T.; Takahashi, N. *Phytochemistry* **1984**, *208*, 1.
13. Kim, T.-W.; Park, S.-H.; Joo, S.-H.; Kim, Y.-S.; Choo, J.; Kim, S.-K. *Bull. Korean Chem. Soc.* **2001**, *22*(6), 651.