Identification of 24α-Methylsterols from *Marchantia polymorpha*

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Brassinosteroids (BRs) are steroidal hormones to regulate growth and differentiation of plants.[1-3] Among over forty members of BRs, castasterone (CS, **1**) and brassinolide (BL, **2**) are considered to be the most important BRs because of their strong activities and wide distributions in plant kingdom.[4,5] The feeding experiment using isotope-labeled intermediates and molecular genetic analyses of BR-deficient mutants revealed that CS (**1**) and BL (**2**) are biosynthesized from 24α-methylcholesterol (campesterol, **3a**) carrying the same carbon skeleton as those of the BRs *via* early- and/or late-C6 oxidation pathway.[6,7] Recently, we identified CS (**1**) and BL (**2**) from a liverwort, *Marchantia polymorpha* (the result will be published elsewhere), suggesting that the early- and/or late-C6 oxidation pathway are/is contained to synthesize BRs in the lower plant. In view that lower plants generally contain 24β-alkylated sterols, however, the result was unusual. This led us to investigate the presence of 24α-methylsterols as precursors of BRs in *M. polymorpha* in the study.

The dried residue of *n*-hexane soluble fraction obtained from naturally grown *M. Polymorpha* was saponified with 80% ethanol (200 mL) containing 5% KOH at 70 °C for 90 min and extracted with *n*-hexane. The unsaponified lipids were charged on silica gel column (22 × 220 mm) using a mixture (1 : 1) of *n*-hexane and methylene chloride as an elution solvent. Based on the movement on a F254 preparative TLC (developing solvent: ethanol free chloroform), the 4-demethylsterols were separated from non-polar lipids, 4,4-dimethylsterols and 4-methylsterols. The 4-demethylsterols were acetylated with acetic anhydride (1 mL) and pyridine (2 mL) at room temperature for 18 h. Then, the mixtures were adjusted to pH 7 with HCl solution (pH 3) and extracted with *n*-hexane (200 mL × 3). After removing *n*-hexane, 4-demethylsteryl acetates (420 mg) were collected and subjected to a reversed phase HPLC (Novapak C_{18}, 8 × 100 mm) using a mixture (97 : 3) of methanol and *n*-hexane as a mobile phase at a flow rate of 5 mL min^{-1}. Fractions were collected every min and analyzed by GC-MS using Hewlett-Packard 5973 mass spectrometer (Electron Impact ionization, 70 electron voltage) connected to 6890 gas chromatograph (GC condition: 1.5 mL He min^{-1}, on-column injection mode, oven temperature: 150 °C for 2 min, thermal gradient 40 °C min^{-1} to 280 °C, and then 280 °C) fitted with a fused silica capillary column (HP-5, 0.25 × 30 m, 0.25 μm film thickness).

In GC-MS, HPLC fraction 16 showed a single peak at 15.1 min on total ion chromatogram which gave prominent ions at m/z 382 $[M\text{-}AcOH]^+$, 367 $[M\text{-}(AcOH+Me)]^+$, 274 $[M\text{-}(AcOH + \text{side chain}) + Me + 2H]^+$, 260, 255$[M\text{-}(AcOH + \text{side chain})\text{-}2H]^+$, 213 [M-(AcOH + side chain + C_3H_5 due to fissions of C13-17 and C14-15)] (Table 1) , providing that a pure state of 24-methylcholesteryl acetate was contained in the fraction. Because 24α- and 24β- isomer of 24-methylcholesteryl acetate could not be separated in the GC condition, determination of configuration of methyl at C24 was carried out by 300 MHz ^{1}H-NMR ($CDCl_3$, Varian Gemini 2000). As summarized in Table 2, Signals at δ 0.68 (s), 1.02 (s), 2.03 (s), 4.55-4.68 (m) and 5.38 (br.d) were assigned for H_3-18, H_3-19, 3-OAc, H-3 and H-6, respectively. However, for doublets due to the side chain methyls at C21, 26, 27 and 28 were divided into at δ 0.91, 0.85, 0.78 and 0.80, respec-

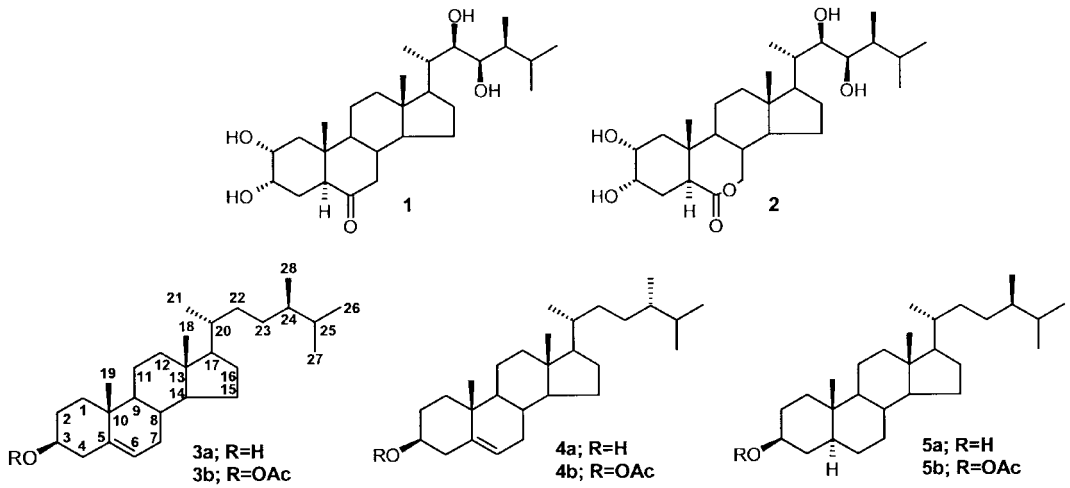
Table 1. GC-MS data for 24-methylcholesterol and 24α-methylcholestanol in *M. polymorpha*

Compound[a]	Rt[b] (min) on GC	Prominent ions (m/z, relative intensity %)
24-methylcholesterol (**3a** and **4a**)	15.1	382 (M^+-60, 100), 367 (30), 274 (15), 260 (12), 255 (15), 213 (14)
24-methylcholestanol (**5a**)	15.3	444 (22), 384 (40), 369 (31), 276 (34), 257 (10), 215 (100)
Authentic 24α-methylcholesterol (**3a**)	15.1	382 (M^+-60, 100), 367 (31), 274 (13), 260 (12), 255 (18), 213 (14)
Authetic 24α-methylcholestanol (**5a**)	15.3	444 (23), 384 (46), 369 (31), 276 (32), 257 (11), 215 (100)

[a]The compound was analyzed as a derivative of acetate. [b]Rt; Retention time

Table 2. ^{1}H-NMR data (TMS internal standard) of 24α- (**3a**) and 24β- (**4a**) isomer of 24-methylcholesterol in *M. polymorpha*

Compound	H_3-18	H_3-19	H_3-21	H_3-26	H_3-27	H_3-28	3-OAc	H-3	H-6
24α- isomer (**3a**)	0.68s	1.02s	0.91d (6.3Hz)	0.85d (6.7Hz)	0.78d (6.4Hz)	0.80d (6.6Hz)	2.03s	4.55-4.68m	5.38br.d (3.2Hz)
24β- isomer (**4a**)	0.68s	1.02s	0.92d (6.3Hz)	0.85d (6.5Hz)	0.77d (6.6Hz)	0.78d (6.6Hz)	2.03s	4.55-4.68m	5.38br.d (3.2Hz)



tively, for 24α-methylcholesteryl acetate (**3b**), and δ 0.92, 0.85, 0.77 and 0.78, respectively, for 24β-methylcholesteryl acetate (**4b**).[8,9] Therefore, 24-methylcholesterol in *M. polymorpha* was found to be a mixture of 24α- and 24β-isomer. Based on the intensities of the doublets (C21, 26, 27 and 28) in 24α- and 24β-methylcholesteryl acetate, ratio of the 24α- and 24β-isomer was calculated to be 5 : 3.

In the early- and late-C6 oxidation pathway for CS (**1**) and BL (**2**) biosynthesis, 24α-methylcholestanol (campestanol, **5a**) is also a common biosynthetic precursor which is synthesized from 24α-methylcholesterol (**3a**) by 5α-reduction.[10] Thus, co-existence of 24α-methylcholestanol (**5a**) with 24α-methylcholesterol (**3a**) in *M. polymorpha* was proposed. To confirm that, GC-MS analysis to identify 24α-methylcholestanyl acetate (**5b**) in the *Marchantia* 4-demethylsteryl acetates was subsequently undertaken. As expected, a weak peak at 15.3 min on GC gave characteristic ions at m/z 444 $[M]^+$, 384 $[M\text{-AcOH}]^+$, 369 $[M\text{-(AcOH + Me)}]^+$, 276 [M-(AcOH + side chain) + Me + 2H]$^+$, 257 [M-(AcOH + side chain)-2H]$^+$, 215 [M-(AcOH + side chain + C_3H_5 due to fissions of C13-17 and C14-15)]$^+$, whose retention time on GC and mass fragment ions were identical to those of authentic 24α-methylcholestanyl acetate (**5b**). Therefore, the presence of 24α-methylcholestanol (**5a**) in *M. polymorpha* was also demonstrated.

We have previously reported that *M. polymorpha* contained cholesterol, 24-methylenecholesterol, 24-methylcholesterol, stigmasterol, 24-ethylcholesterol and isofucosterol as principal 4-demethylsterols.[11,12] Among them, 24-methylcholesterol was found to be the most abundant 4-demethylsterol, which accounted for 50% of the total amount of 4-demethylsterols in the lower plant. This study attempted to determine the configuration of 24-methyl in the *Marchantia* 24-methylcholesterol, providing that 24α-methylcholesterol (**3a**) is prominently contained in the lower plant than 24β-methylcholesterol (**4a**). In addition, the presence of 24α-methylcholestanol (**5a**), a biosynthetically related 24α-methylsterol, was demonstrated in the same plant material. These are striking from phylogenetic and taxonomical aspects that the configuration at C24 of the 24-methylsterols in *M. polymorpha* is quite analogous to those for higher plants rather than lower plants.[8,13,14]

Since it was found that *M. polymorpha* contains 24α-methylsterols which have the same C24 configuration as the endogenous BRs (**1** and **2**), it is rational that both the BRs and sterols in *M. polymorpha* may be biosynthesized in a way analogous to the biosynthesis of BRs and sterols in higher plants. This is the first evidence that the biosynthetic pathways for BRs in higher plants are evolved from those in lower plants.

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