Characterization of 2-*Epi*castasterone from Immature Seeds of *Phaseolus vulgaris*

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Brassinosteroids (BRs), steroidal plant hormones, regulate diverse phenomenon in the growth and differentiation of plants.[1-2] Among naturally-occurring BRs, castasterone (CS) has been identified as the most abundant BR in the plant kingdom, one which strongly regulates the overall development of plants, especially vegetatively growing plants.[3] We have previously reported that immature seeds of *Phaseolus vulgaris* contain CS and a number of unknown BRs showing the same MS spectrum as that of CS with different retention times on GC and HPLC, suggesting that the unknown BRs are isomers of CS (Fig. 1).[4] This prompted us to investigate the chemical structure of the unknown CS isomers, and lead to our identification of a new naturally-occurring BR, 2-*epi*-castasterone (2-*epi*CS) from immature seeds of *P. vulgaris*. Herein, we report elucidation of the structure and biological activity of 2-*epi*CS.

Extraction, solvent partitioning and column chromatography for purification of BRs from *P. vulgaris* have been reported.[5] In a large-scale reverse-phase HPLC (Shenshu Pak, Develosil ODS 5 μm, 20 × 250 mm) eluted at a flow rate of 20 mL min^{-1} with 45% acetonitrile as the mobile phase, a compound in fraction 24 and showed a blue-purplish spot on HPTLC (Merck, F_{254}) with an R_f of 0.30 after heating followed by spraying of 70% sulfuric acid. The compound was further purified by a small-scale, normal phase HPLC (Shensu Pak, Aquasil, 10 × 200 mm). It eluted at a flow rate of 3 mL min^{-1} with a gradient of increasing iso-propanol in *n*-hexane (0-20 min: 25%, 20 - 40 min: gradient to 60% iso-propanol in *n*-hexane). The fraction eluting between 31 and 32 min gave a pure solid-state substance which we used for instrumental analyses.

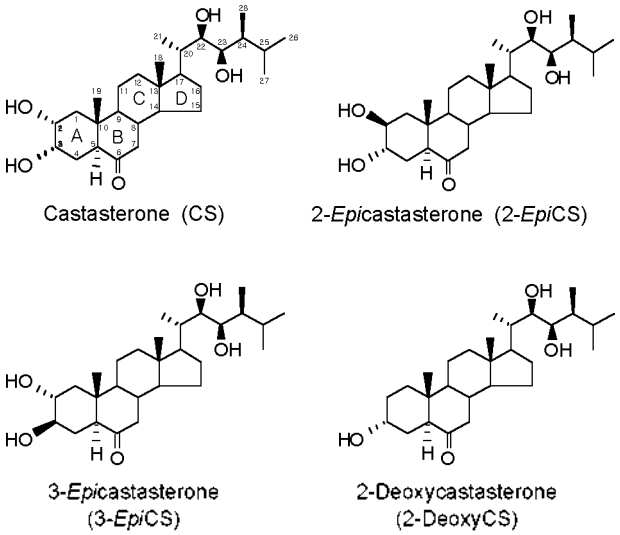

Figure 1. Structure of castasterone (CS), 2-*epi*castasterone (2-*epi*CS), 3-*epi*castasterone (3-*epi*CS) and 2-deoxycastasterone (2-deoxyCS).

The compound was derivatized with methaneboronic acid in pyridine followed by heating at 70 °C for 30 min, and analyzed by capillary GC-MS (JEOL DX303, EI, 70 eV).[6] As a bismethaneboronate (BMB), the compound showed a molecular ion at *m/z* 512 and prominent ions at *m/z* 470, 357/358, 327, 287 and 155 (Table 1). These molecular and prominent ions, except the one at *m/z* 470, are the same as those of the BMB derivative of CS. High resolution MS showed the molecular formula of the characteristic ion at *m/z* 470. The ion peak at 470.3508 was $C_{29}H_{47}O_4B$ (calculated 470.3567), which indicates that the characteristic ion is derived from the loss of CH_3OB from the molecular formula of BMB of the compound ($C_{30}H_{50}O_5B_2$), most likely from an A ring methaneboronate. Coupled with a distinct retention time on GC from that of CS BMB, our findings suggest that the compound is an isomer of CS.

In a 400 MHz proton NMR study (Table 2), absorptions for four methyls (C-21, C-26, C-27 and C-28) and two protons at C-22 and C-23 in the side chain of the isomer of CS were exactly the same as those of CS, indicating that the side chain structure of the isomer is identical to that of CS. In addition, signals for H_3-18 at δ0.69 (s) and H_2-7 at δ 2.33 (dd, J = 4, 14 Hz) are basically equal to those of CS, indicating that the structures of the B, C and D rings in the isomer of CS is also

Table 1. Movement of CS and its isomer in *P. vulgaris* on HPTLC, HPLC and GC-MS.

Compound	R_f on HPTLC	Rt[a] on HPLC	RRt[b] on GC	Prominent ion[c] (*m/z*, relative intensity)
CS	0.43	30-31	1.000	512(M+, 60), 357/358(10), 327(8), 287(60), 155(100)
CS isomer	0.30	23-24	1.347	512(M+, 49), 470(25), 357/358(18), 287(52), 155(100)

[a]Rt: Retention time (min). [b]RRt: Relative Retention time. [c]The samples are analyzed by a capillary GC-MS as BMB derivatives.

Table 2. 400 MHz proton NMR data for CS, 2-*epi*CS and an isomer of CS in *P. vulgaris*. The chemical shifts are given in ppm from tetramethylsilane.

	CS	CS isomer	2-*Epi*CS
Ring protons			
H_3-18	0.69s	0.69s	0.69s
H_3-19	0.76s	0.97s br.s	0.97s br.s
H-2	3.77 br.m ($W_{1/2}$ = 21 Hz)	3.93 br.s ($W_{1/2}$ = 10.5 Hz)	3.93 br.s ($W_{1/2}$ = 10.5 Hz)
H-3	4.06 br.s ($W_{1/2}$ = 10.5 Hz)	3.98 br.s ($W_{1/2}$ = 10.5 Hz)	3.98 br.s ($W_{1/2}$ = 10.5 Hz)
H-5	2.69 (J = 4, 14 Hz)	2.73 (J = 3, 13 Hz)	2.73 (J = 3, 13 Hz)
H_2-7	2.30 (J = 4, 14 Hz)	2.33 (J = 4, 14 Hz)	2.33 (J = 4, 14 Hz)
Side chain protons			
Me(1)	0.85d	0.85d	0.85d
Me(2)	0.91d	0.91d	0.91d
Me(3)	0.95d	0.95d	0.95d
Me(4)	0.97d	0.97d	0.97d
H-22	3.56d (J = 9 Hz)	3.56d (J = 9 Hz)	3.56d (J = 9 Hz)
H-23	3.73dd (J = 2, 9 Hz)	3.73dd (J = 2, 9 Hz)	3.73dd (J = 2, 9 Hz)

the same as that of CS. Signals for two broad singlets ($W_{1/2}$ = 10.5 Hz) at δ 3.93 and 3.98 represent the presence of two equatorial protons attached with secondary hydroxyls. Coupled with the fact that the isomer can be derivatized as BMB, these results indicate that a vicinal hydroxyl is axially located at C-1 and C-2, C-2 and C-3, or C-3 and C-4. However, absorption at δ 2.73 as a double doublet (J = 3, 14 Hz) was assigned to H-5 to verify the presence of the proton at C-4, excluding the possibility of 3,4-diaxial hydroxyls. If the diaxial hydroxyls are located at C-1 and C-2, H-1 must give a sharp doublet; but the observed signals for both protons attaching to the hydroxyl at δ 3.93 and 3.98 were broad singlets, also denying the possibility of 1,2-diaxial hydroxyls. In 2,3-diaxial hydroxyls, two equatorial protons at C-2 and C-3 couple with at least three protons, giving rise to broad singlets. Therefore, the location of the vicinal hydroxyls in the A ring is at C-2 and C-3. Because the obtained amount of the isomer of CS was small (*ca.* 30 μg), ^{13}C-NMR and/or 2D NMR to confirm the position and configuration of vicinal hydroxyls at C-2 and C-3 could not carried out. However, proton NMR data for the isomer of CS was exactly the same as that of synthetic 2-*epi*CS (Table 2). Therefore, the structure of the isomer of CS is conclusively characterized to be 2-*epi*CS, (22R,23R,24S)-2β,3α,22,23-tetrahydroxy-5α-cholestan-6-one.

The biological activity of 2-*epi*CS was examined using the rice lamina inclination assay.[7] As shown in Fig. 2, 2-*epi*CS exhibited almost no biological activity up to 0.002 ppm. At 0.02 ppm, 2-*epi*CS showed weak activity that was approximately one fiftieth that of CS, indicating that replacement of the 2α-hydroxyl with a 2β-hydroxyl in CS greatly reduces biological activity. Another BR, 2-deoxycastasterone (2-deoxyCS), which has no hydroxyl at the C-2 position of CS, showed one tenth the activity of CS. Compared to 2-deoxyCS, 2-*epi*CS showed one fifth the activity, confirming that introduction of a β-configured hydroxyl at C-2 hinders expression of BR activity.

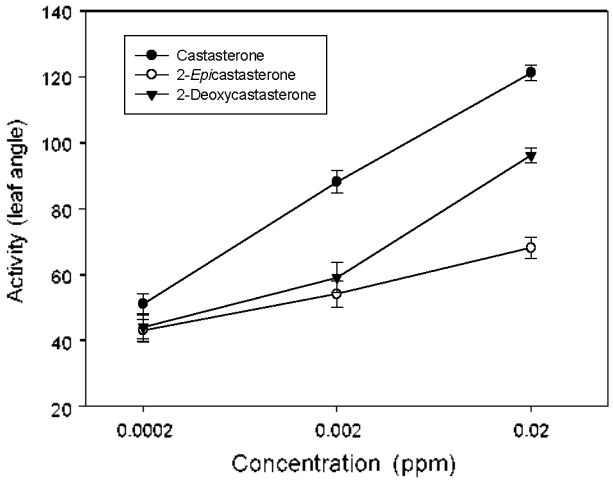

Figure 2. Biological activity for castasterone (CS), 2-*epi*castasterone (2-*epi*CS) and 2-deoxycastasterone (2-deoxyCS) in the rice lamina inclination assay.

This study is the first to demonstrate the presence of 2-*epi*CS in immature seeds of *P. vulgaris*. This novel compound showed less activity than 2-deoxyCS and CS, which indicates that introduction and configuration of the hydroxyl at C-2 are important for BR activity. In other words, the presence of an α-configured hydroxyl at C-2 is essential for the strong BR activity of CS. It has been previously reported that CS is inactivated by conversion to a less biologically active stereoisomer, 3-*epi*castasterone (3-*epi*CS).[8] A lower activity for 2-*epi*CS than for CS implies that 2-epimerization is also an inactivation reaction of CS by substitution of an α-configured to a β-configured hydroxyl at C-2.

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Reference

1. Bishop, G. J.; Yokota, T. *Plant Cell Physiol.* **2001**, *42*, 114.
2. Bajguz, A.; Tretyn, A. *Phytochemistry* **2003**, *62*, 1027.
3. Kim, T.-W.; Hwang, J.-Y.; Kim, Y.-S.; Joo, S.-H.; Chang, S. C.; Lee, J. S.; Takatsuto, S.; Kim, S.-K. *The Plant Cell* **2005**, *17*, 2397.
4. Yokota, T.; Kim, S.-K. *Agric Biol Chem.* **1987**, *51*, 1625.
5. Joo, S.-H.; Hwang, J.-Y.; Park, C. H.; Lee, S. C.; Kim, S.-K. *Bull. Korean Chem. Soc.* **2009**, *30*, 502.
6. Park, S. C.; Kim, T.-W.; Kim, S.-K. *Bull. Korean Chem. Soc.* **2000**, *21*, 1274.
7. Arima, M.; Yokota, T.; Takahashi, N. *Phytochemistry* **1984**, *23*, 1587.
8. Fujioka, S. In *Brassinosteroids: Steroidal Plant Hormones*; Sakurai, A.; Yokota, T.; Clouse, S. D., Eds.; Springer-Verlag: Tokyo, 1999; p 21.