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REPORT

Identification of a New Brassinosteroid, 23-Dehydro-2-Epicastasterone, from Immature Seeds of *Phaseolus vulgaris*

Jung Yun Hwang, Chan Ho Park, Hojin Namgung, and Seong Ki Kim*
Department of Life Science, Chung-Ang University, Seoul 156-756, Korea

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Brassinosteroids (BRs), a new class of plant hormones, show a broad spectrum of physiological activities in regulating growth (Clouse et al., 1996; Li and Chory, 1997; Yokota, 1997; Clouse and Sasse, 1998; Oh and Clouse, 2003). To date, over 40 different steroids have been identified in plants (Kim, 1991; Fujioka, 1999). Among those species tested for their BR content, *Phaseolus vulgaris* is one of the richest. Its immature seeds are notable for the 11 BRs that have been fully characterized already, while the presence of over 60 additional, but still unknown, BRs have been demonstrated by capillary GC-MS analyses (Kim et al., 1987; Yokota et al., 1987; Kim, 1991; Fujioka, 1999; Park et al., 2000). Our interest in brassinosteroid structure and metabolism led us to investigate those unknown BRs by scaling up and using a large quantity of immature *P. vulgaris* seed. As a result, we have now identified a new BR -- 23-dehydro-2-epicastasterone (**1**; Fig. 1) from that tissue. Herein, we report its isolation and characterization.

The neutral ethyl acetate-soluble fraction obtained from 136 kg of immature seed was purified by silica gel, repeated Sephadex LH-20 separation, and charcoal column chromatography (Kim et al., 1987; Kim, 1991). The effective enrichment of the biological activity in less than 500 mg, as indicated by a rice lamina inclination assay, was purified by reverse-phase HPLC (Senshu pak 5251-D, Develosil ODS 5 μm, 20 × 200 mm; 9.9 mL min^{-1}, 45% acetonitrile for 0 to 40 min and 80% for 40 to 70 min; fractions collected every min), giving rise to multiple potentially biologically active fractions. Among these, the HPLC fraction that eluted at 14 to 15 min showed a BR-like blue-purplish spot at Rf 0.31 on the HPTLC (Merck) when developed with a 6:1 mixture of methanol:chloroform. This fraction was further purified by normal-phase HPLC (Senshu pak, Aquasil, 10 × 100 mm; 3 mL min^{-1}; chloroform:methanol:H_2O=95:5:0.1; fractions collected every min). As a final step, Compound **1** was isolated in a pure state from Aquasil fraction number 21 (Fig. 2).

As summarized in Table 1, the 400 MHz proton NMR ($CDCl_3$) of **1** showed the resonances derived from ring protons at δ 0.69 (3H, s), 0.97 (3H, s), 3.93 (H, br. s, $W_{1/2}$ =10.5 Hz), 3.98 (H, br. s, $W_{1/2}$=10.5 Hz), 2.73 (H, dd, *J*=4, 13 Hz), and 2.33 (H, dd, *J*=4, 13 Hz). These resonances are not equal but are quite similar to those in castasterone (**2**), implying that the ring structure of **1** is analogous to that of **2**. In the proton NMR of **1**, two broad singlets ($W_{1/2}$ =10.5 Hz) at δ 3.93 and 3.98 indicate the presence of two equatorial protons that are attached to two secondary hydroxyls, suggesting the existence of two axial hydroxyls at either C-1 and C-2, C-2 and C-3, or C-3 and C-4. A double doublet at δ 2.73, which is assignable for H-5, indicates the presence of a methylene at C-4. In the vicinal hydroxyls at C-1 and C-2, an absorption for H-1 should appear as a sharp doublet, but no doublet assignable for H-1 was detected. Therefore, it is clear that a hydroxyl group is not attached at C-1 or C-4, meaning that the two axial hydroxyls are indeed positioned at C-2 and C-3. Theoretically, these C-2 and C-3 biaxial hydroxyls should give broad singlets because of spin couplings with three neighboring protons (Silverstein and Webster, 1998), which is matched to the NMR data. Because the C-2 and C-3 biaxial hydroxyls are 1, 3-diaxial configurations against C-19 methyl and H-5, those down-shifted signals at δ 0.97 and 2.73 for C-19 methyl and C-5 proton can also be explained. Taken together, the ring structure of **1** is determined to be 2-epimeric of that of **2** carrying the 2β, 3α-vicinal hydroxyls, a 6-oxo group, and trans A/B rings.

The 2α, 3β-, and 2α,3β-vicinal hydroxyls of BRs can be derivatized into a methaneboronate by treatment with methaneboronic acid in pyridine under heating conditions (Takatsuto et al., 1982; Ikekawa et al., 1984). However, 2β, 3α-vicinal hydroxyls are difficult to methaneboronate because of their biaxial configurations. Further, the produced methaneboronate of 2β, 3α-vicinal hydroxyls can be easily disrupted by treatment with trimethylsilylic (TMS) reagent, yielding a di-TMS ether derivative (Fig. 3). Indeed, as shown in Figure 4, methaneboronation followed by trimethylsilylation of **1** yields a molecular ion at *m/z* 630. Moreover, characteristic ions appear at *m/z* 540 $[M\text{-}TMSOH]^+$, 513 $[M\text{-}TMSOCH{=}CH_2]^+$, and 424 $[M\text{-}TMSOCH{=}CH_2\text{-}TMSOH]^+$, indicating that the neighboring hydroxyls at C-2 and C-3 were not derivatized into a methaneboronate but rather to a di-TMS ether. Therefore, the absolute configurations of the vicinal hydroxyls at C-2 and C-3 are now re-verified as β and α, respectively.

In the GC-MS application, a characteristic ion at *m/z* 153 suggests that the side chain of **1** is unsaturated, just like that of dolichosterone (**3**) (Baba et al., 1983). By proton NMR, however, the two proton absorptions for the C-28 exomethylene of **3** are not observed in that of **1**. Four methyl signals assignable for C-21, C-26, C-27, or C-28 are shown at δ 1.03, 1.07, 1.08, and 1.13; their split patterns are all doublets, indicating that, as with **2**, the methyl moieties are

*Corresponding author; fax +82-2-820-5206
e-mail skkimbio@cau.ac.kr

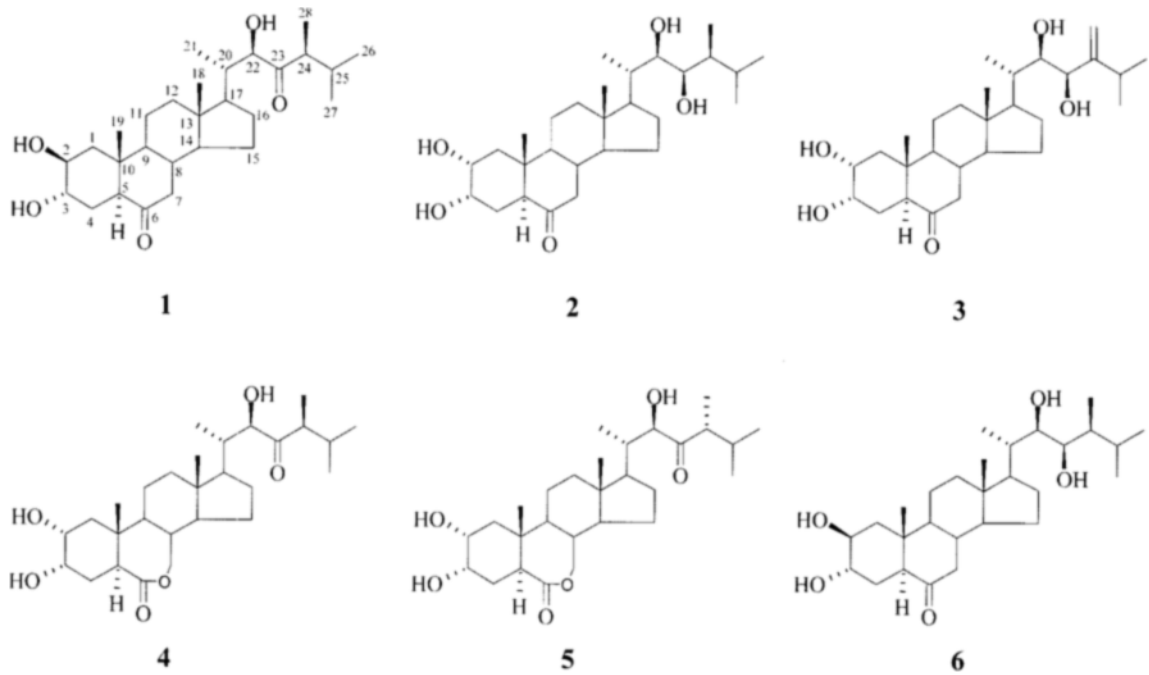


Figure 1. Chemical structure of 23-dehydro-2-epicastasterone (**1**), castasterone (**2**), dolichosterone (**3**), 23-dehydrobrassinolide (**4**), 23-dehydro-24-epibrassinolide (**5**), and 2-epicastasterone (**6**).

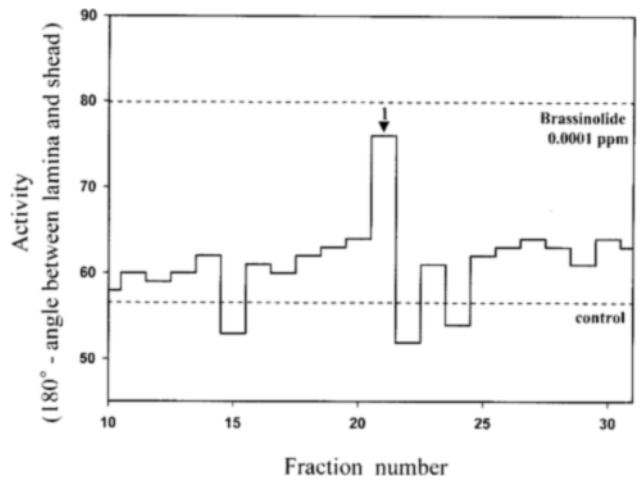


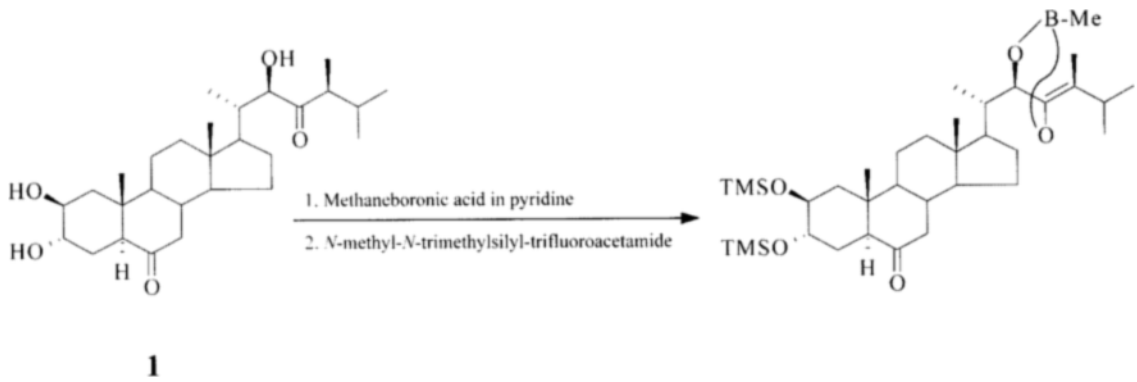
Figure 2. Distribution of biological activity for **1** after normal-phase HPLC (Senshu pak 5251-D, Develosil ODS 5 μm, 20 × 200 mm; 9.9 mL min^{-1}, 45% acetonitrile for 0 to 40 min and 80% for 40 to 70 min; fractions collected every min).

attached to C-20, C-24, and C-25. Therefore, the location of a double bond in the side chain of **1** appears to be between C-22 and C-23, or C=O at C-22 or C-23. A doublet at δ 3.88 (J=5 Hz) that arises as a side-chain proton attached to a secondary alcohol can exclude the possible location of the double bond between C-22 and C-23, thereby providing the presence of a keto group at C-22 or C-23. Because only C-23 keto BRs are natural, the doublet for that side-chain proton can be assigned to H-22. Therefore, the presence of tetramethyl at C-21, -26, -27, and -28; a C-22 hydroxyl; and C-23 oxo on the side-chain carbon skeleton of **1** has been demonstrated.

Although it does not carry vicinal hydroxyls in the side chain, **1** is derivatized to be a methaneboronate, suggesting that a double bond of C=O at C-23 is migrated to between C-22 and C-23 or C-23 and C-24 by methaneboronation. Yokota et al. (1998) have reported that 23-dehydrobrassinolide (**4**), which has a side-chain structure identical to that of **1**, can be derivatized by methaneboronation to be Δ^{22} or Δ^{23} dehydrobrassinolide bismethaneboronate. This can be distinguished by the presence of ions at m/z 153 or 182 in their MS spectra. In Δ^{22} dehydrobrassinolide bismethaneboronate, the ion at m/z 181 is shown, but the one at m/z 153 is not; the result is opposite in Δ^{23} dehydrobrassinolide bismethaneboronate (Yokota et al., 1998). In view of the methaneboronate

Table 1. ^{1}H-NMR data (400 MHz, $CDCl_3$) of BR compounds **1**, **2** and **3** from immature seeds of *P. vulgaris*. The chemical shifts are given in ppm from tetramethylsilane.

Compound	Ring protons						Side-chain protons			
	H_3-18	H_3-19	H-2	H-3	H-5	H-7	Me (1)Me (2)Me (3)Me (4)	H-22	H-23	H_2-28
1	0.69 s	0.97 s	3.93 br. s ($W_{1/2}$=10.5 Hz)	3.98 br. s ($W_{1/2}$=10.5 Hz)	2.73 dd (J=4, 13 Hz)	2.33 dd (J=4, 13 Hz)	1.03 d1.07 d1.08 d1.13 d	-	3.88 d (J=5.0 Hz)	-
2	0.69 s	0.76 s	3.77 br. m ($W_{1/2}$=21.0 Hz)	4.06 br. s ($W_{1/2}$=10.5 Hz)	2.69 dd (J=4, 13 Hz)	2.30 dd (J=4, 13 Hz)	0.85 d0.91 d0.95 d0.97 d	3.56 d (J=9.0 Hz)	3.72 d (J=9.0 Hz)	-
3	0.62 s	0.75 s	3.76 br. m ($W_{1/2}$=21.0 Hz)	4.05 br. s ($W_{1/2}$=10.5 Hz)	2.68 dd (J=4, 13 Hz)	2.30 dd (J=4, 13 Hz)	0.95 d1.08 d1.11 d	- 3.63 d (J=8.0 Hz)	4.03 d (J=8.0 Hz)	5.30 s 5.06 s



1

Figure 3. Methaneboronation followed by trimethylsilylation of **1**.

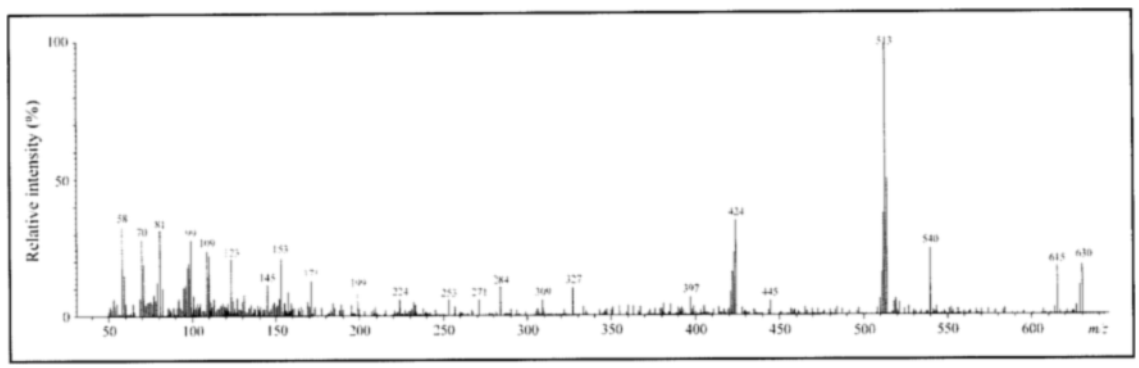


Figure 4. MS spectrum of 1 methaneboronate-trimethylsilylic ether.

of **1** to show an ion at *m/z* 153, we speculate that a double bond at the C-23 keto group has migrated between C-23 and C-24 to produce the methaneboronic derivative (Fig. 2).

Based on the aforementioned MS and NMR data, we can characterize **1** as 23-dehydro-2-epicastasterone (2β, 3α, 22-trihydoxy-24-methy-5α-cholestan-5, 23-dione). Compared with the many previously characterized BRs in the plant kingdom, this steroid is quite unusual because of the β-oriented hydroxyl at C-2 and the oxo group at C-23. In a rice lamina inclination assay, the 23-dehydro-24-epibrassinolide (**5**) shows four- to ten-fold less biological activity than the 24-epibrassinolide (Yokota et al., 1998). Likewise, 2-epicastasterone (**6**) is approximately twenty-fold less potent than **2** (data not shown). These results suggest that both the 23-dehydrogenation and the 2-epimerization are deactivation processes in the metabolism of BRs in plants. Therefore, we conclude that **1** is a catabolite of **2**, the most abundant and potent bioactive BR in *P. vulgaris*.

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