Notes

2-Deoxy-25-methyldolichosterone and 3-*epi*-2-Deoxy-25-methyldolichosterone in Immature Seeds of *Phaseolus vulgaris*

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Received June 20, 2009, Accepted August 17, 2009

Key Words: Brassinosteroids, *Phaseolus vulgaris*, Biosynthesis, Biological activity, 24-Methylene-25-methyl-BRs

Steroidal plant hormones, collectively named Brassinosteroids (BRs), control various aspects of the growth and development of plants such as stem elongation, photomorphogenesis, leaf and flower development, stress modulation and sink/source relationship.[1-2] A number of naturally-occurring BRs have been identified in the plant kingdom from algae to higher plants.[3] The naturally-occurring BRs can be classified as C_{27}-, C_{28}- and C_{29}-BRs based on numbers of the carbon skeleton.[4] 25-Methyldolichosterone (**1**, Fig. 1) which has been identified from immature seeds of *P. vulgaris* is a unique C_{29}-BR because it carries a tertiary butyl moiety at the end of the side.[5] A comparison of the bioactivities of dolichosterone and **1** revealed that methylation at C-25 increases BRs activity, implying that **1** is a physiologically important BR.[6] Nevertheless, how **1** is biosynthesized in plants has not been established yet. This prompted us to search again for the presence of biosynthetic precursors of **1** in immature seeds of *P. vulgaris*. That search led to identification in seeds of two new BRs with a tertiary butyl at the end of the side chain, 2-deoxy-25-methyldolichosterone (**2**) and 3-*epi*-2-deoxy-25-methyldolichosterone (**3**). Here, structural elucidation, biological activity and biogenesis of the BRs are reported.

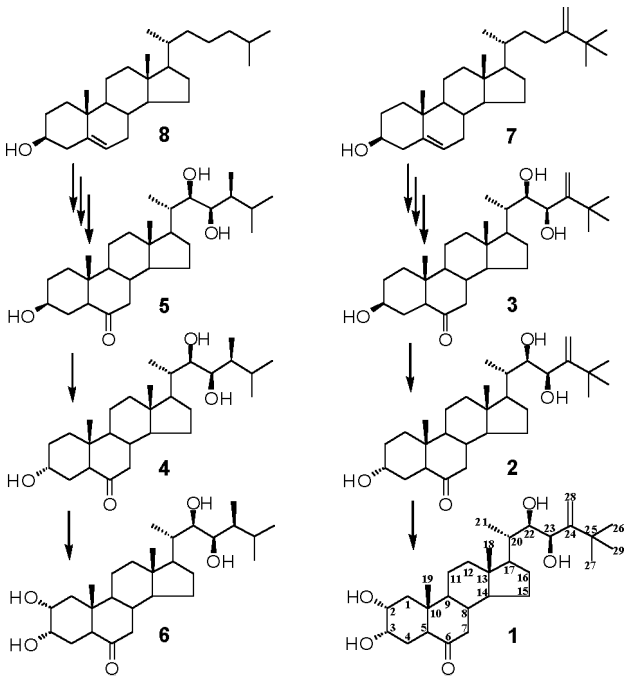

Figure 1. Biosynthetic pathway for 24-methyl-BRs and 24-methylene-25-methyl-BRs in *P. vulgaris*. The multiple arrows indicate multi-biosynthetic steps 1:25-methyldlichosterone, 2:2-deoxy-25-methyldlichosterone, 3:3-*epi*-2-deoxy-25-methyldlichosterone, 4:2-deoxycastasterone, 5:3-*epi*-2-deoxycastasterone, 6:castasterone, 7:24-methylene-25-methylcholesterol, 8:24-methylcholesterol.

Extraction, solvent partitioning and column chromatography for purification of BRs from *P. vulgaris* have been reported.[7,8] Using a large-scale reverse-phase HPLC method (Shenshu Pak. Develosil ODS 5 μm, 20 × 250 mm) with an elution flow rate of 20 mL min^{-1} with 45% acetonitrile as the mobile phase, a compound in fraction 45 showed a blue-purplish spot on HPTLC (Merck, F_{254}) with an R_f of 0.52 after heating followed by spraying with 70% sulfuric acid. The compound (**I**) was further purified by a small-scale, normal phase HPLC method (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 17 and 18 min showed biological activity in the rice lamina inclination. The fractions were combined and used for instrumental analyses. After the same large-scale reversed-phase HPLC, a compound (**II**) in fraction 50 also showed a blue-purplish spot on HPTLC with an R_f of 0.52 after heating followed by spraying with 70% sulfuric acid. **II** was further purified by the aforementioned small-scale normal phase HPLC, giving rise to biologically active fractions 10 and 11 in the rice lamina inclination assay. The fractions were combined and used for instrumental analyses.

For GC-MS analysis (JEOL DX303, EI, 70 eV), **I** and **II** were derivatized with methaneboronic acid in pyridine followed by heating at 70 °C for 30 min. However, no ion peaks were found on a total ion chromatogram (TIC). Instead, methaneboronation followed by trimethylsilylation (MSTFA)

gave a sharp peak on TIC, implying that **I** and **II** have vicinal hydroxyls and an isolated hydroxyl in the structure. In methaneboronate-trimethylsilylic (MB-TMSi) ether, both **I** and **II** showed the same molecular ion at an *m/z* of 556 and prominent ions at *m/z* ratios of 541, 527, 443, 359, 329, 167, 138, and 123 (Table 1). Prominent ions at m/z 167, 138 and 123 are characteristic ions due to fission of C-20/C-22 and C-22/C-23 for 25-methyldolichosterone MB, suggesting that a vicinal hydroxyl is present in the side chain at C-22 and C-23.[9] Therefore, the location of an isolated hydroxyl in **I** and **II** is thought to be in the ring structure, most likely at C-2.

In a 400 NMR proton analysis, signals for protons derived from the side chain of **I** at δ 0.96 (3H, d, H_3-21), 1. 11 (9H, s, H_3-26, 27 and 29), 3.76 (H, d, H-22), 4.07 (H, d, H-23), 5. 09 (H, s, H-28) and 5.15 (H, s, H-28) were exactly the same as those for **1** (Table 2). Together with the same chemical shift at δ 0.61 for methyl at C-18, this shows that **I** has the identical side chain structure to that of **1**, C-21 methyl, C-22R and C-23R diols, C-24 exomethylene and a tertiary butyl at the end of the side chain. Absorptions for trans A/B ring protons of I were assignable to δ 0.73 (3H, s, H_3-19), 4.17(H, br.s, H-3), 2.73 (H, t, H-5) and 2.31 (H, dd, H-7). These chemical shifts are equal to those of 2-deoxycastasterone (**4**), indicating that **I** has the same C-3α hydroxyl and 6-ketone in its A/B ring structure as those of **4** (Table 2). Taken together, these findings suggested that **I** carried the same side chain structure as that of **1** and the same ring structure as that of **4**. Therefore, **I** was characterized and found to be **2**. (22*R*, 23*R*)-3α, 22, 23-trihydroxy-25-methyl-5α-ergost-24(28)-en-6-one (Fig. 1).

II also showed the same absorptions for protons derived from the side chain and C-18 methyl at δ 0.96 (3H, d, H_3-21), 1.11 (9H, s, H_3-26, 27, 29), 3.76 (H, d, H-22), 4.07 (H, d, H-23), 5.09 (H, s, H-28), 5.15 (H, s, H-28) and 0.61 (3H, s, H_3-18), indicating that the side chain of **II** is also identical to that of **1**. Proton signals for the A/B ring were assignable to δ 0.75 (3H, s, H_3-19), 3.58 (H, br, H-3), 2.22 (H, dd, H-5) and 2.31 (H, dd, H-7) which are superimposed on those of 3-*epi*-2-deoxycastasterone (**5**), showing that the A/B ring structure of **II** is identical to that **5**. Therefore, **II** was thought to have the same side chain and ring structure as that of **1** and **5**, respectively. Thus, **II** was determined to be **3**, (22*R*, 23*R*)-3β, 22, 23-trihydroxy-25-methyl-5α-ergost-24(28)-en-6-one (Fig. 1).

Biological activity of **2** and **3** was tested by the rice lamina inclination assay.[10] As shown in Figure 2, **3** showed almost no activity up to 0.002 ppm, and weak activity at 0.02 ppm. Similarly, **2** exhibited very weak activity up to 0.002 ppm, and moderate activity at 0.02 ppm. Compared to the biological activity of **1**, the activity was approximately 1/5 and 1/15 less

Table 1. GC-MS data for **1**, **I** and **II** indentified for immature seeds of *P. vulgaris*.

Compound	Rt[a] on GC	Prominent ion[b] (*m/z*, relative intensity)
1	27.25	524(M+, 18), 411(5), 387(17), 356(4), 327(21), 167(44), 138(100, 123(54)
I	16.48	556(M+, 59) 541(28), 527(21), 443(9), 359(23), 329(71), 167(42), 138(100), 123(45)
II	16.75	556(M+, 45) 541(21), 527(9), 443(11), 359(26), 329(83), 167(52), 138(100), 123(90)

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

Table 2. 400 MHz proton NMR (in $CDCl_3$) data for **1**, **4**, **5**, **I** and **II**. The chemical shifts are given in ppm from tetramethylsilane.

	1	**4**	**5**	**I**	**II**
Ring protons					
H_3-18	0.61 s	0.61 s	0.61 s	0.61 s	0.61 s
H_3-19	0.75 s	0.73 s	0.75 s	0.73 s	0.75 s
H-2	3.77 br	-	-	-	-
H-3	4.05 br.s	4.17 br.s	3.58 br.s	4.17 br.s	3.58 br.s
H-5	2.69 dd (J = 4, 13 Hz)	2.73 t (J = 8, 16 Hz)	2.22 dd (J = 5, 14 Hz)	2.73 t (J = 8, 16 Hz)	2.22 dd (J = 5, 14 Hz)
H-7	2.29 dd (J = 5, 13 Hz)	2.31 dd (J = 5, 14 Hz)	2.31 dd (J = 5, 14 Hz)	2.31 dd (J = 5, 14 Hz)	2.31 dd (J = 5, 14 Hz)
Side chain protons					
H_3-21	0.96 d	0.85 d	0.85 d	0.96 d	0.96 d
H_3-26	-	0.91 d	0.91 d	-	-
H_3-27	-	0.95 d	0.95 d	-	-
H_3-28	-	0.97 d	0.97 d	-	-
H_9-26,27,29	1.11 s	-	-	1.11 s	1.11 s
H-22	3.76 d (J = 8 Hz)	3.56 d (J = 9 Hz)	3.56 d (J = 9 Hz)	3.76 d (J = 9 Hz)	3.76 d (J = 9 Hz)
H-23	4.05 d (J = 8 Hz)	3.72 d (J = 9 Hz)	3.72 d (J = 9 Hz)	4.07 d (J = 9 Hz)	4.07 d (J = 9 Hz)
H_2-28	5.09 s 5.15 s	-	-	5.09 s 5.15 s	5.09 s 5.16 s

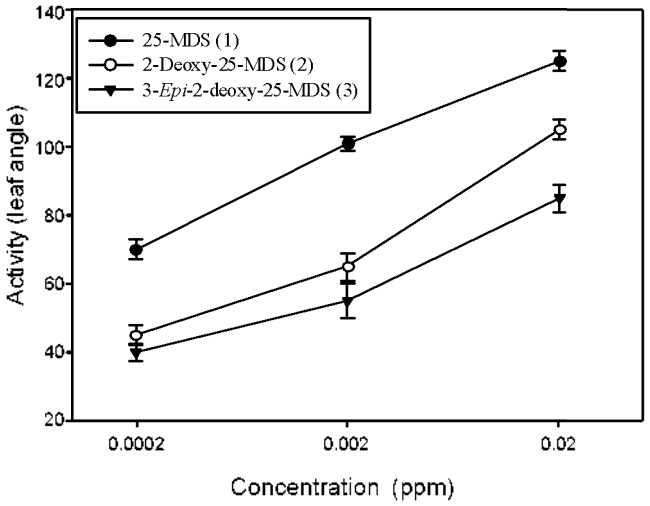

Figure 2. Biological activity for **1**, **2** and **3** in the rice lamina inclination assay.

active for **2** and **3**, respectively.

Among the naturally-occurring BRs, castasterone (**6**) is the most frequently identified BR in the plant kingdom.[3] Coupled with strong biological activity and a high endogenous level of **6**, biosynthesis of BRs has been mainly focused on how **6** is biosynthesized in plants. As a result, the biosynthetic pathways from campesterol to **6**, namely the early and late C-6 oxidation pathway for C_{28}-BRs, have been established (Fig 1). It has been previously reported that *P. vulgaris* contains **6** as a major BRs.[11] Additionally, the presence of biosynthetic precursors such as 6-deoxoCS, **4**, **5** for biosynthesis of **6** were demonstrated in *p. vulgaris*.[12] Further, a crude enzyme solution prepared from *p. vulgaris* successfully catalyzed almost all biosynthetic reactions involved in the early and late C-6 oxidation pathway to generate **6**, showing that the biosynthetic pathways for C_{28}-BRs are operating in these plants.

In *P. vulgaris*, **1** is also a major BR whose endogenous level is comparable to that of **6**. The presence of 24-methylene-25-methyl-cholesterol (**7**) in the *Phaseolus* plant implies that biosynthetic pathways for synthesizing **1** from **7** are also functional in the plant. In this study, we identified two new BRs, **2** and **3** from immature seeds of *P. vulgaris*. **2** and **3** are considered to be counterparts of **4** and **5** in the early C-6 oxidation pathway for **6**. Coupled with the presence of **7** and **1**, this strongly suggests that a biosynthetic pathway from **7** to **1** *via* **3** and **2**, possibly named the early C-6 oxidation pathway for 24-methylene-25-methyl-BRs, is also operating in *Phaseolus* plants (Fig. 1). It is thus thought that a steady-state level of endogenous BRs is maintained by multiple biosynthetic pathways, at least two, in *Phaseolus vulgaris*.

Experimental Section

Bioasay. The rice lamina inclination assay was carried out using Cultivar Koshihikari as described Arima *et al.*[12]

Instrumental Analysis. GC-MS analysis was carried out with JEOL DX303 (EI: 70 eV) fitted with a capillary column (DB-1, J & W Co., -.254 mm × 15 m, 0.25 μm film thickness). GC condition; 1 mL min^{-1} He : splitless injection mode: 175 °C for 2 min, thermal gradient 32 °C min^{-1} to 275 °C, and then maintained at 275 °C.

400 MHz proton NMR analysis was performed by JEOL FX-400 using TMS as an internal standard.

Acknowledgments. This work is supported by the grants from the Plant Diversity Research Center of the 21st Century Frontier Research Program (PF06304-03) and KOSEF/MEST (R01-2007-000-20074-0).

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