# Identification of Brassinosteroids with 24*R*-Methyl in Immature Seeds of *Phaseolus vulgaris*

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Brassinosteroids are a new class of steroidal plant hormones which are requisite for normal growth and development.[1,2] To date, over 40 brassinosteroids have been characterized from a wide range of plant kingdom including higher and lower plants.[3] Among the plant materials in which endogenous brassinosteroids have been investigated hitherto, immature seeds of *Phaseolus vulgaris* have been most extensively examined. Thirteen brassinosteroids including two brassinosteroids conjugates have been successfully identified from the seeds.[3-7] In addition, HPLC and GC-MS analysis revealed that a number of unknown brassinosteroids were also contained in the seeds.[4-7] To understand more about the structure and biosynthesis of brassinosteroids, we attempted to characterize the unknown brassinosteroids in the seeds of *P. vulgaris* and succeeded to identify two additional endogenous brassinosteroids with 24*R*-methyl, 24*R*-epicastasterone (**9**) and 3$\beta$,24*R*-diepicastasterone (**10**). Herein, identification and biogenesis of these brassinosteroids with 24*R*-methyl in the seeds of *P. vulgaris* are reported.

The chloroform soluble extract obtained from immature seeds of *P. vulgaris* (Cultivar Kentucky Wonder, 136 Kg) was solvent-partitioned and purified by the methods reported previously.[4,7] After separation by a reversed phase HPLC, endogenous brassinosteroids in the HPLC fractions were analyzed by a capillary GC-MS.

As a bismethaneboronate (BMB), an active compound in HPLC fraction 31 showed prominent ions at m/z 512[$M^+$], 441, 399, 358, 328, 287 and 155, which were basically identical with those of castasterone (**5**) BMB (Table 1). However, retention times of the compound in the HPLC and GC were distinguished from those of castasterone (**5**) which has been already identified from the same plant material (Table 1), suggesting that the compound is a stereoisomer of castasterone (**5**). Among the stereoisomers of castasterone (**5**), 24*R*-epicastasterone (**9**) was eluted at the same HPLC fraction (Table 1). Furthermore, BMB of 24*R*-epicastasterone (**9**) gave the same mass spectrum and GC retention time as those of the active compound in HPLC fraction 31. Therefore, the active compound was determined to be 24*R*-epicastasterone (**9**).

GC-MS and HPLC analyses revealed that a stereoisomer of castasterone (**5**) was also contained in HPLC fraction 23 and 24 (Table 1). The active compound in the fractions was further purified by a normal phase HPLC, and analyzed by 400 MHz $^1$H-NMR. As summarized in Table 2, signals for four methyls at C21, 26, 27 and 28 were detected at $\delta$ 0.85 (3H, d), 0.87 (3H, d), 0.92 (3H, d), 0.98 (3H, d). Two proton signals at C22 and 23 were shown at $\delta$ 3.42 (H, t, $J$ = 6.3 Hz) and 3.70 (H, dd, $J$ = 1.8, 8.8 Hz), respectively. These side-chain proton signals were superimposable with those of authentic 24*R*-epicastasterone (**9**, Table 2), indicating that the side-chain structure of the compound is identical to that of 24*R*-epicastasterone (**9**). Signals due to C18, C19, C2 and C3 at the ring structure were detected at $\delta$ 0.68 (3H, s), 0.81 (3H, s), 3.38-3.43 (H, m) and 3.58-3.63 (H, m), respectively, which were identical to those derived from 3$\beta$-epicastasterone (**7**) identified from the same plant material (Isolation and structure determination will be reported elsewhere). This provided that the ring structure of the compound is equal to that of 3$\beta$-epicastasterone (**7**). Taken together, the active compound in the fraction 23 and 24 was characterized to be 3b, 24*R*-diepicastasterone (**10**), a new naturally-occurring brassinosteroid.

Since brassinosteroids are biosynthesized from phytosterols which have the same side chain carbon skeleton as that of brassinosteroids,[1,2,8-11] the identification of 24*R*-epicastasterone (**9**) and 3$\beta$,24*R*-diepicastasterone (**10**) strongly suggests that seeds of *P. vulgaris* should contain 24$\beta$-methylcholesterol (**8**) which is not always common in higher plants. In order to confirm that, 24-methylcholesterol in the seeds was analyzed as an acetate derivative by 400 MHz $^1$H-

**Table 1**. HPLC and GC-MS data for endogenous castasterone and 24*R*-epicastasterone in immature seeds of *P. vulgaris*

| Compound[a] | $R_t$[b] (min) on HPLC | $RR_t$[c] on GC | Prominent ions (m/z, relative intensity %) |
|---|---|---|---|
| Endogenous castasterone | 29-30 | 1.000 | 512($M^+$, 55) 441(5) 399(7) 358(24) 328(9) 287(26) 155(100) |
| Endogenous 24*R*-epicastasterone | 30-31 | 0.950 | 512($M^+$, 57) 441(6) 399(6) 358(23) 328(10) 287(20) 155(100) |
| Endogenous 3$\beta$, 24*R*-epicastasterone | 22-24 | 1.347 | 512($M^+$, 67) 441(5) 399(3) 358(7) 328(3) 287(13) 155(100) |
| Authentic castasterone | 29-30 | 1.000 | 512($M^+$, 61) 441(9) 399(11) 358(31) 328(10) 287(28) 155(100) |
| Authentic 24*R*-epicastasterone | 30-31 | 0.950 | 512($M^+$, 65) 441(8) 399(8) 358(26) 328(12) 287(21) 155(100) |

[a]The sample was analyzed as a derivative of bismethaneboronate. [b]$R_t$: Retention time. [c]$RR_t$: Relative retention time with respect to castasterone bismethaneboronate (14.21 min).

**Table 2**. $^1$H-NMR data (TMS internal standard) for castasterone isomers

| Compound | Ring protons | | | | Side chain protons | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | $H_3$-18 | $H_3$-19 | H-2 | H-3 | Me(1)* | Me(2)* | Me(3)* | Me(4)* | H-22 | H-23 |
| Endogenous 3$\beta$, 24*R*-diepicastasterone | 0.68s | 0.81s | 3.38-3.43m | 3.61 br.m | 0.85d | 0.87d | 0.92d | 0.98d | 3.42t ($J$ = 6.3 Hz) | 3.70dd ($J$ = 1.9, 8.8 Hz) |
| 24*R*-epicastasterone | 0.68s | 0.76s | 3.73 br.m | 4.05 br.s | 0.85d | 0.87d | 0.92d | 0.98d | 3.42t ($J$ = 6.3 Hz) | 3.70dd ($J$ = 1.9, 8.8 Hz) |
| 3$\beta$-epicastasterone | 0.68s | 0.81s | 3.38-3.43m | 3.61 br.m | 0.85d | 0.91d | 0.95d | 0.97d | 3.56d ($J$ = 8.8 Hz) | 3.73dd ($J$ = 1.9, 8.8 Hz) |

*Me(1), (2), (3), (4) indicate $CH_3$ at C21, 26, 27, and 28.

**Table 3**. $^1$H-NMR data (TMS internal standard) of 24$\alpha$- and 24$\beta$-methylcholesteryl acetate in immature seeds of *P. vulgaris*

| Compound | $H_3$-19 | $H_3$-18 | $H_3$-21 | $H_3$-26 | $H_3$-27 | $H_3$-28 | 3-OAc | H-3 | H-6 |
|---|---|---|---|---|---|---|---|---|---|
| 24$\alpha$-Methylcholesteryl acetate | 0.68s | 1.02s | 0.91d (6.3 Hz) | 0.85d (6.7 Hz) | 0.80d (6.6 Hz) | 0.78d (6.4 Hz) | 2.03s | 4.55-4.68m | 5.38br. d ($J$ = 3.2 Hz) |
| 24$\beta$-Methylcholesteryl acetate | 0.68s | 1.02s | 0.92d (6.3 Hz) | 0.85d (6.5 Hz) | 0.78d (6.6 Hz) | 0.77d (6.6 Hz) | 2.03s | 4.55-4.68m | 5.38br. d ($J$ = 3.2 Hz) |

NMR to determine the configuration of a methyl at C24. Signals at $\delta$ 0.68 (s), 1.02 (s), 2.03 (s), 4.55-4.68 (m) and 5.38 (br. d, $J$ = 3.2 Hz) were assigned for $H_3$-18, $H_3$-19, 3-OAc, H-3 and H-6, respectively (Table 3). However, four doublets due to the side chain methyls at C21, 26, 27 and 28 were divided into at $\delta$ 0.91, 0.85, 0.80 and 0.78 for 24$\alpha$-methylcholesteryl acetate, respectively, and $\delta$ 0.92, 0.85, 0.78 and 0.77 for 24$\beta$-methylcholesteryl acetate.[8,12] Thus, 24-methylcholesterol in *P. vulgaris* was found to be a mixture of the 24$\alpha$- (**1**) and 24$\beta$-isomer (**8**). By comparing the intensities of the doublets, the mixture was estimated to be composed of *ca* 57% of the $\alpha$-isomer and *ca* 43% of the $\beta$-isomer.

It has been already demonstrated that brassinolide (**6**),

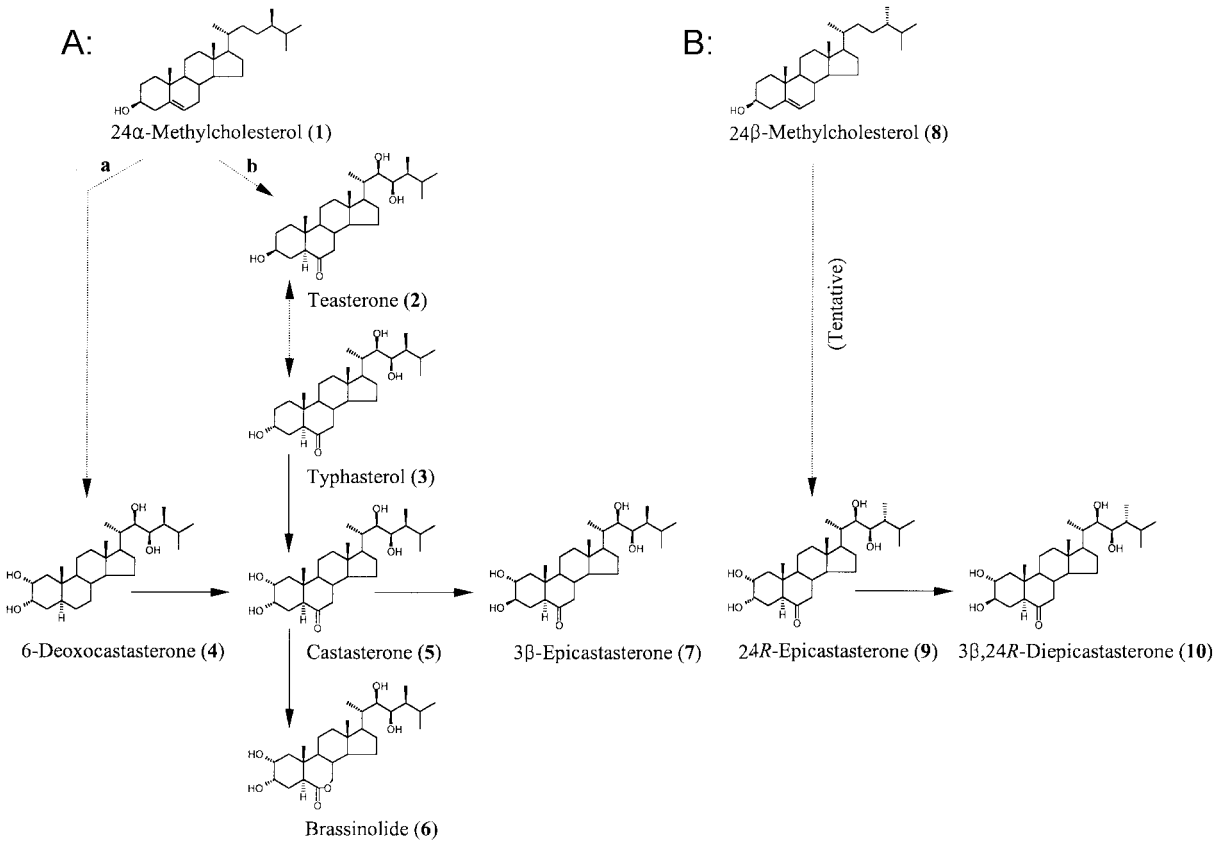


**Figure 1**. Two possible pathways (A and B) for brassinosteroids biosynthesis and catabolism included in *P. vulgaris* seeds. The early- and late-C6-oxidation pathway in A are represented as 'a' and 'b', respectively.

castasterone (**5**), typhasterol (**3**), teasterone (**2**) and 6-deoxocastasterone (**4**) exist in immature seeds of *P. vulgaris*.[3-7] These brassinosteroids are all 24*S*-methylated and members of the early- and/or late-C6 oxidation pathway. Together with the presence of 24$\alpha$-methylcholesterol (**1**), this indicates that the two pathways for biosynthesis of castasterone (**5**) and brassinolide (**6**) from 24$\alpha$-methylcholesterol (**1**) are operative in the seeds of *P. vulgaris* (Figure 1).

24*R*-Epicastasterone (**9**) has been initially identified as a 24*R*-epimeric brassinosteroid from a green alga, *Hydrodictyon reticulatum*.[8] This has been thought to be theoretical because lower plants generally contain 24$\beta$-alkylated sterols as main components. Then, the occurrence of 24*R*-epimeric brassinosteroids such as 6-deoxo-24*R*-epicastasterone, 24*R*-epicastasterone, 24*R*-epibrassinolide have been demonstrated in several higher plants.[9,10,14-16] However, biogenesis of the 24*R*-epimeric brassinosteroids in higher plant has not been established yet. In this study, we provided the first evidence that 24*R*-epimeric brassinosteroids, 24*R*-epicastasterone (**9**) and 3$\beta$,24*R*-diepicastasterone (**10**), co-existed with 24$\beta$-methylchosterol (**8**) in immature seeds of *P. vulgaris*. The result provides the fact that 24*R*-methylated brassinosteroids are biosynthesized from 24$\beta$-methylcholesterol (**8**) as 24*S*-methylated brassinosteroids are biosynthesized from 24$\alpha$-methylcholesterol (**1**) in the seeds (Figure 1). For the conclusion, however, the occurrence of possible intermediates involved in the pathway(s) such as 3$\beta$-epi-2-deoxy-24*R*-epicastaterone, 2-deoxy-24*R*-epicastasterone, 6-deoxo-24*R*-epicastasterone should be demonstrated in the same plant materials.

3-Epicastasterone (**7**) showed five times less biological activity than that of castasterone. Because castasterone (**5**) is biosynthesized from typhasterol (**3**) by 2$\alpha$-hydroxylation and exogenous [$^2H_6$]-castasterone was converted into [$^2H_6$]-3-epicastasterone in seedlings of *Catharansus roseus*, tabacco and rice, 3-epimerization of castasterone was thought to be a catabolic process of castasterone.[17] In this viewpoint, 3$\beta$, 24*R*-diepicastasterone (**10**) may be produced from 24*R*-epicastasterone (**9**) as a deactivation process in the *P. vulgaris* seeds (Figure 1). The fact that the conversion of teasterone (**2**) to typhasterol (**3**) *via* 3-dehydroteasterone is reversible implies that the conversion of 24*R*-epicastasterone (**9**) to 3$\beta$, 24*R*-diepicastasterone (**10**) is also intermediated by 3-oxo compound.

## Experimental Section

**Purification of brassinosteroids in immature seeds of *P. vulgaris***. Endogenous brassinosteroids obtained after solvent partitionings and column chromatographies[5-7] were purified by a reversed phase HPLC (Senshu Pak Develosil ODS, 20 $\times$ 250 mm) at a flow rate of 9.9 mL min$^{-1}$ with aqueous acetonitrile as an elution solvent (45% acetonitrile for 0-40 min and 80% for 40-70 min). The fractions eluted 22-24 min (fraction 23 and 24) were further purified by a normal phase HPLC using Aquasil column (Senshu Pak, 10 $\times$ 200 mm) at a flow rate of 3 mL min$^{-1}$ with a mixture of chroloform-methanol-water (150 : 25 : 3 for 0-20 min and gradient to 12 : 8 : 1 for 20-40 min). Fraction was collected every min and fraction 35 gave a pure state of 3$\beta$, 24*R*-diepicastasterone.

**Instrumental analysis**. GC-MS analysis was carried out with JEOL DX303 (EI; 70 eV) fitted with a capillary column (DB-1, J & W Co., 0.254 mm $\times$ 15 m, 0.25 $\mu$m film thickness). GC condition; 1 mL min$^{-1}$ He: splitless injection mode: 175 $^o$C for 2 min, thermal gradient 32 $^o$C min$^{-1}$ to 275 $^o$C, and then maintained at 275 $^o$C. Prior to injection, sample was treated with methaneboronic acid in pyridine (1 mg/2 mL) to produce bismethaneboronate.

400 MHz $^1$H-NMR analysis was performed by JEOL FX400 using TMS as an internal standard.

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