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

Soon Cheol Park,* Tae-Wuk Kim, and Seong-Ki Kim

Department of Life Science, Chung-Ang University, Seoul 156-756, Korea
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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 3β,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 δ 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 δ 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 δ 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 3β-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 3β-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 3β,24*R*-diepicastasterone (**10**) strongly suggests that seeds of *P. vulgaris* should contain 24β-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 3β, 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 3β, 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)
3β-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 24α- and 24β-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
24α-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)
24β-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 δ 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 δ 0.91, 0.85, 0.80 and 0.78 for 24α-methylcholesteryl acetate, respectively, and δ 0.92, 0.85, 0.78 and 0.77 for 24β-methylcholesteryl acetate.[8,12] Thus, 24-methylcholesterol in *P. vulgaris* was found to be a mixture of the 24α- (**1**) and 24β-isomer (**8**). By comparing the intensities of the doublets, the mixture was estimated to be composed of *ca* 57% of the α-isomer and *ca* 43% of the β-isomer.

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

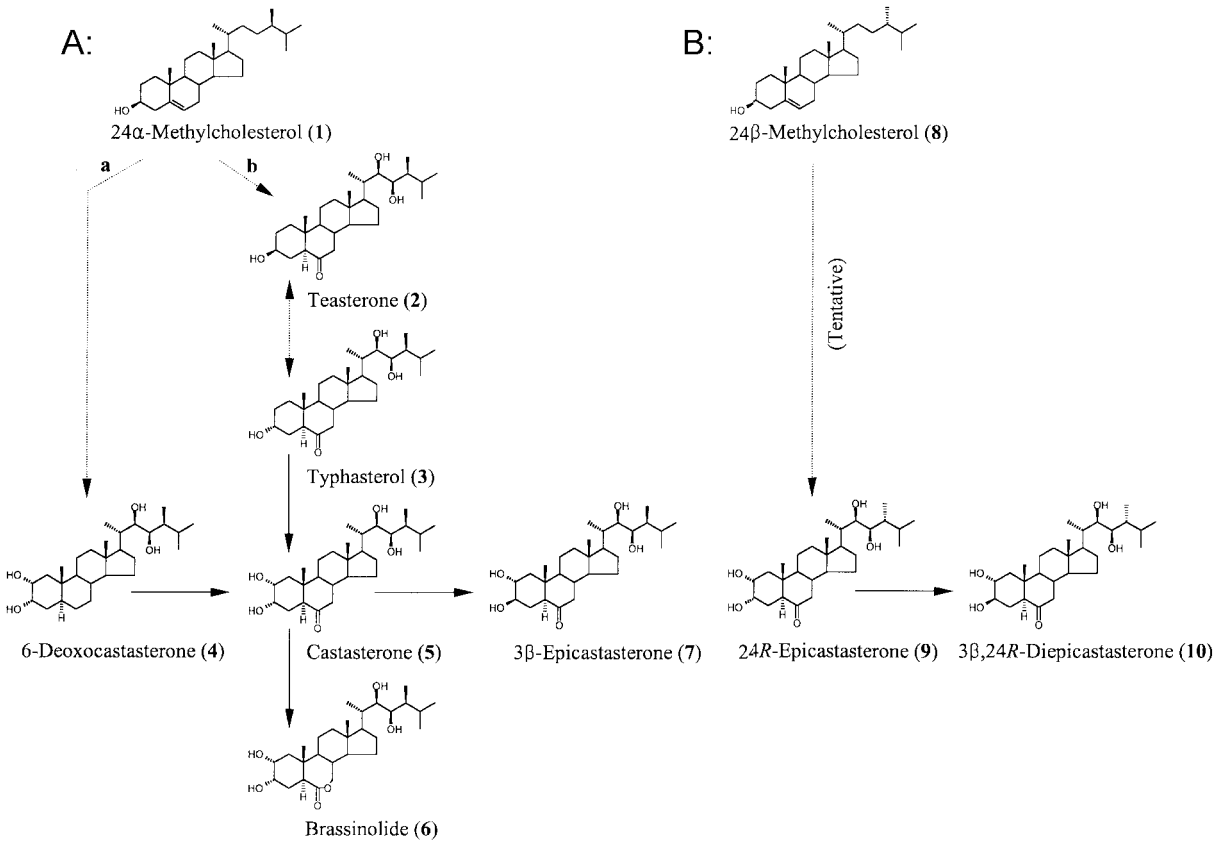

Figure 1. Two possible pathways (A and B) for brassinosteroids biosynthesis and catabolism included in *P. vulgaris* seeds. The early- and late-C6-oxidation pathway in A are represented as 'a' and 'b', respectively.

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 24α-methylcholesterol (**1**), this indicates that the two pathways for biosynthesis of castasterone (**5**) and brassinolide (**6**) from 24α-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 24β-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 3β,24*R*-diepicastasterone (**10**), co-existed with 24β-methylchosterol (**8**) in immature seeds of *P. vulgaris*. The result provides the fact that 24*R*-methylated brassinosteroids are biosynthesized from 24β-methylcholesterol (**8**) as 24*S*-methylated brassinosteroids are biosynthesized from 24α-methylcholesterol (**1**) in the seeds (Figure 1). For the conclusion, however, the occurrence of possible intermediates involved in the pathway(s) such as 3β-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 2α-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, 3β, 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 3β, 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 3β, 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 μm 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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