

Biosynthetic Connection of 24-Methylene- and 24-Methyl-brassinosteroids in *Phaseolus vulgaris*

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Plant steroidal hormones, brassinosteroids (BRs), show a broad spectrum of regulatory activities in plant growth and differentiation.^{1,2} Over forty members of naturally occurring BRs can be classified as C₂₇-, C₂₈-, or C₂₉-BRs based on the number of carbon atoms in the alkyl group side chain, especially at C-24.^{3,4} These congeners are biosynthesized from phytosterols carrying the same carbon skeletons as the BRs. This suggests that plants use multiple pathways to generate BRs, but the reasons are not well-known.

It has been shown that *Phaseolus vulgaris* (*P. vulgaris*) contains 6-deoxodolichosterone (6-deoxoDS), DS and dolicholide (DL) as 24-methylene-BRs. *P. vulgaris* also contains 6-deoxocastasterone (6-deoxoCS), CS and brassinolide (BL) as 24S-methyl-BRs.^{5,6} Together with the presence of 24-methylene-cholesterol and 24S-methyl-cholesterol as biosynthetic precursors for 24-methylene-BRs and 24S-methyl-BRs, respectively, it is implied that two biosynthetic pathways to produce 24-methylene- and 24S-methyl-BRs exist in plants (Fig. 1). This prompted us to investigate the possibility that these pathways in *P. vulgaris* are biosynthetically connected.

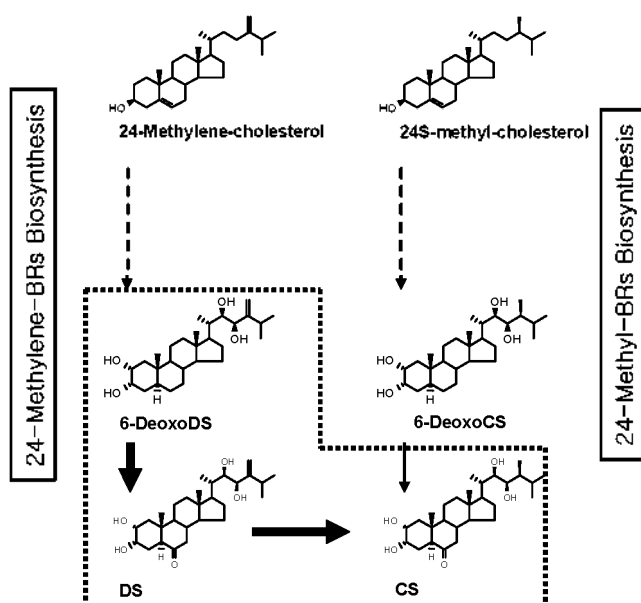


Figure 1. The simplified biosynthetic pathway for 24-methylene-BRs and 24-methyl-BRs. The dotted box indicates the connection of two BRs biosynthesis established in this study. The dotted arrow indicates multiple biosynthetic steps. The solid arrow shows a single biosynthetic step.

Results and Discussion

When 6-deoxoDS was used as a substrate (Fig. 2a), the BMB derivative of an active compound (metabolite 1) in I showed a molecular ion at m/z ratio of 510 and prominent ions at m/z ratios of 495, 411, 387, 356/355, 327, 287, 153 and 124. The mass spectrum and GC retention time (Rt, 27.46 min) were identical to those of DS BMB (Table 1), demonstrating that the metabolite 1 is DS. The BMB derivative of an active principle (metabolite 2) in II gave a molecular ion at an m/z ratio of 512 and prominent ions at m/z ratios of 497, 441, 399, 358, 327, 287 and 155 at the same GC Rt (29.20 min) as that of CS BMB, proving that the metabolite 2 is CS. When DS was added as a substrate (Fig. 2b), BMB of the metabolite 3 in III gave the same mass spectrum and GC retention time as those of authentic CS BMB. This demonstrated that the metabolite 3 is CS. Taken together, the above evidence clearly indicates that 6-deoxoDS is converted into a CS intermediate by DS.

Subsequently, the possibility for reversible conversions from CS to 6-deoxoDS via DS was examined using the enzyme solution. When DS was used as a substrate, the BMB derivative

Table 1. GC-MS data for authentic BRs (DS, CS and 26-norCS) and metabolites (1, 2, 3 and 4) in *P. vulgaris*

Compound	Rt ^a on GC (min)	Prominent ions (m/z , relative intensity)								
Metabolite 1	27.46	510 (M ⁺ , 27),	495 (14),	411 (15),	387 (16),	355/356 (18),	327 (98),	287 (9),	153 (72),	124 (100)
Metabolite 2	29.20	512 (M ⁺ , 92),	497 (8),	441 (14),	399 (23),	358 (37),	327 (11),	287 (45),	155 (100)	
Metabolite 3	29.20	512 (M ⁺ , 85),	497 (9),	441 (15),	399 (20),	358 (40),	327 (9),	287 (41),	155 (100)	
Metabolite 4	26.28	498 (M ⁺ , 100),	483 (8),	399 (13),	358 (28),	328 (10),	287 (42),	141 (94)		
Authentic DS	27.46	510 (M ⁺ , 25),	495 (16),	411 (13),	387 (18),	355/356 (18),	327 (96),	287 (9),	153 (70),	124 (100)
Authentic CS	29.20	512 (M ⁺ , 84),	497 (9),	441 (16),	399 (21),	358 (24),	327 (12),	287 (40),	155 (100)	
Authentic 26-norCS	26.28	498 (M ⁺ , 100),	483 (10),	399 (15),	358 (25),	328 (10),	287 (44),	141 (92)		

The samples were analyzed as a derivative of bismethaneboronate. ^aRetention time

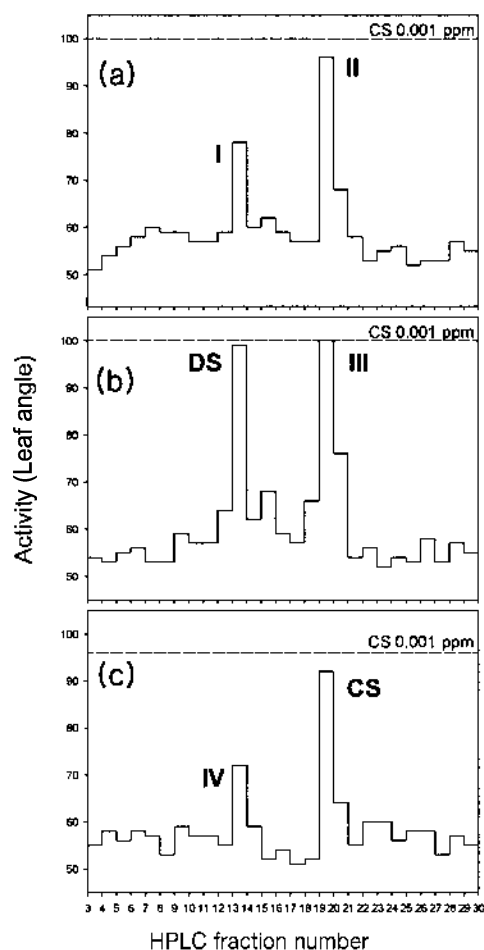


Figure 2. Distribution of biological activity for the metabolites in I, II, III and IV derived from 6-deoxoDS (a), DS (b) and CS (c) after a reversed phase HPLC. The activity was evaluated by the rice lamina inclination assay.

of the compound in the fractions corresponding to 6-deoxoDS in reversed phase HPLC gave no mass spectrum for 6-deoxoDS BMB, indicating that 6-deoxoDS is not generated from DS in the enzyme assay. When CS was used, the BMB derivative of the HPLC fraction corresponding to DS did not show a mass spectrum for DS BMB. Instead, the BMB derivative of an active compound (metabolite 4) in IV (Fig. 2C) gave the same mass spectrum and GC retention time as that of 26-norCS BMB, demonstrating that the compound is 26-norCS which is known to be a CS catabolite. Therefore, we confirmed that a biosynthetic sequence, 6-deoxoDS \rightarrow DS \rightarrow CS is not reversible, and CS is biodegraded to 26-norCS in *P. vulgaris* similar to what occurs in other plants.

In the rice lamina inclination assay (Fig. 3), 6-deoxoDS showed almost no activity from 0.01 to 0.00001 ppm. DS exhibited approximately 1/2-1/3 the activity compared to CS over the same concentration range. 28-NorCS has only 1/10 the activity that CS has. These findings also indicate that the conversion of 6-deoxoCS to CS *via* DS is a biosynthetic route to synthesize CS, and the conversion of CS to 26-norCS is a biodegradative pathway.

We have previously shown in tomato plants that CS, an active BR in 24-methyl-BR biosynthesis, can be generated from

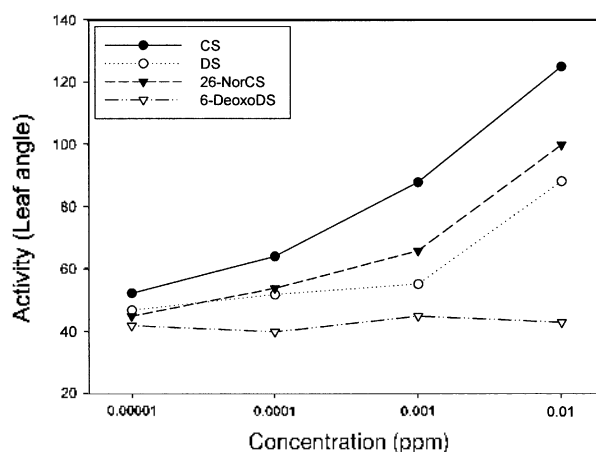


Figure 3. Biological activity for CS, DS, 6-deoxoDS and 26-norCS in the rice lamina inclination assay.

28-norCS, an end product of C_{27} -BR biosynthesis.⁵ Recently, we also found that ethylbrassinone, the end product of a C_{29} -BR biosynthesis, is converted to CS in *P. vulgaris*, rice and *A. thaliana* (data will be published elsewhere). This study is the first to demonstrate that CS can be generated from 24-methylene-BR biosynthesis. This strongly suggests that CS, one of the most important BRs in BR physiology, can be biosynthesized through multiple biosynthetic pathways starting from different phyosterols. Plants use at least three BR biosynthetic pathways, which funnel the end product to CS to maintain a steady-state level of the active BR, CS. For this reason, the endogenous concentration of CS is generally much higher than those of other naturally-occurring BRs in plants.

Experimental Section

After confirming that there was no detectable amount of CS, the most abundant BR in *P. vulgaris*, in the enzyme solution (data not shown), 6-deoxoDS or DS was added to the solution as a substrate in the presence of NADPH as a cofactor. The enzyme assay was carried out at 37 °C for 30 min. The metabolites produced from 6-deoxoDS or DS were extracted, purified and analyzed by GC-MS.

Preparation of crude enzyme solution from *P. vulgaris*. Seedlings of *P. vulgaris* (5 g) were homogenized with 0.1 M sodium-phosphate buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 15 mM 2-mercaptoethanol, 15% glycerol, 250 mM sucrose, 40 mM ascorbate and 1% insoluble polyvinylpyrrolidone and centrifuged at 8,000 \times g for 10 minutes. The resulting supernatants were re-centrifuged at 20,000 \times g for 30 min. After adding cold acetone to the supernatants (final volume 40%), the precipitates were suspended in 0.1 M sodium-phosphate buffer (pH 7.4) containing 1.5 mM 2-mercaptoethanol and 30% glycerol to create a crude enzyme solution.

Purification and characterization of metabolites. Three times with ethyl acetate (1.2 mL each) and loaded on a Sep Pak C18 column and eluted with 50 and 100% MeOH. The biologically active 100% MeOH fraction was evaporated and further purified by a reversed phase HPLC (Senshu Pak C18,

10 × 150mm) with a flow rate of 2.5 mL/min using 45% MeCN. Fractions were collected every minute. Biologically active fractions in the rice lamina inclination assay were combined, derivatized to the bismethaneboronates (BMB) by heating in pyridine-containing methaneboronic acid (2 mg/mL) at 70 °C for 30 min. and analyzed by a capillary GC-MS: using a Hewlett-Packard 5973 mass spectrometer (EI, 70 eV) coupled to a 6890 gas chromatograph fitted with a fused silica capillary column (HP-5, 0.25 mm × 30 m, 0.25 μm film thickness). The oven temperature was maintained at 175 °C for 2 min, elevated to 280 °C at a rate of 40 °C/min and then maintained at 280 °C. Helium was used as the carrier gas at a flow rate of 1 mL/min and samples were introduced using an on-column injection mode.

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