C-3 Epimerization of 6-Deoxocastasterone in *Phaseolus vulgaris*

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Brassinosteroids (BRs) are steroidal plant hormones, which exert a variety of regulatory effects relevant to plant growth and development.[1-5] Thus far, over 40 different BRs have been identified throughout the entirety of the plant kingdom.[6,7] Among the plants so far tested for their BR contents, the immature seeds of *Phaseolus vulgaris* represent one of the richest. 11 BRs have been fully characterized from this source, whereas the presence of more than 60 additional unknown BRs has been demonstrated *via* capillary GC-MS analyses. Our interest in the structure and metabolism of BRs compelled us to conduct a series of investigations into the unknown BRs contained in the seeds of *P. vulgaris*, by scaling up and employing a sizeable quantity of the plant materials. As a result, we have identified a new BR, designated 3-*epi*-6-deoxoCS. Herein, we report the isolation, characterization, and biogenesis of this BR in *Phaseolus* seeds.

The ethyl acetate soluble fraction acquired from the *Phaseolus* seeds (136 Kg) was purified with SiO_2, repeated Sephadex LH-20, and charcoal chromatography,[8] under the guidance of a rice lamina inclination bioassay.[9] Further purification was then conducted *via* reversed-phase HPLC (Senshupak Develosil, 20×250 mm),[8] and the resulting HPLC fractions were derivatized to bismethaneboronate (BMB), then analyzed using a capillary GC-MS: HP 5973 mass spectrometer (EI, 70 eV, Hewlett Packard) connected to a 6890 gas chromatograph equipped with a fused silica capillary column (HP-5, DB-5, 0.25×30 mm).[10]

In HPLC fractions 51 and 52, the BMB of a compound evidenced a mass spectrum almost identical to that of the BMB of 6-deoxocastasterone (6-deoxoCS), but the GC-retention time of the BMB compound was longer than that of 6-deoxoCS BMB (Table 1), thereby indicating that the compound was an epimeric 6-deoxoCS. In order to determine the chemical structure of the epimeric 6-deoxoCS, fractions 51 and 52 were purified further *via* normal-phase HPLC (Senshupak Aquasil 10×200 mm) and eluted with chloroform- methanol-H_2O (97:3:0.1) at a flow rate of 3 mL/min^{-1}. Fractions were collected every minute. Finally, the epimeric 6-deoxoCS was isolated in a pure state in HPLC fractions 16 and 17, and verified *via* 400 MHz proton NMR analysis (JEOL-FX400).

As summarized in Table 2, chemical shifts due to four methyls for C-21, C-24, C-26 or C-27 were observed at δ 0.85 (d), 0.89 (d), 0.95 (d) and 0.97 (d). Additionally, two proton signals assignable to H-22 and H-23 were detected as broad singlets at δ3.56 ($W_{1/2}$ = 9 Hz) and 3.73 ($W_{1/2}$ = 9 Hz), respectively. These signals for the side chain protons were completely identical to those derived from 6-deoxoCS, which indicates that the structure of the side chain in epimeric 6-deoxoCS was identical to that of 6-deoxoCS. However, absorptions for H-2 (δ3.76) and H-3 (δ3.97) in 6-deoxoCS were shifted up-field, at δ 3.39 and 3.59, respectively. Further, a broad H-3 singlet ($W_{1/2}$ = 10.5 Hz) in 6-deoxoCS was altered, appearing instead as a broad multiplet ($W_{1/2}$ = 21 Hz). These results suggest that the C-3 equatorial

Table 1. GC-MS/SIM data of endogenous BRs and their metabolites in *P. vulgaris*

Compound [a]	GC RRt [b]	Prominent ions (*m/z*, relative intensity %)
6-deoxoCS [c]	0.769	498 (M+, 75), 483 (22), 343 (20), 314 (10), 288 (21), 273 (100), 213 (16), 205 (36), 155 (26)
3-*epi*-6-deoxoCS [c]	1.036	498 (M+, 72), 483 (18), 343 (16), 314 (12), 288 (19), 273 (100), 205 (15), 155 (34)
CS [c]	1.000	512 (M+, 76), 441 (11), 399 (8), 358 (38), 327 (13), 287 (34), 155 (100)
Metabolite I [d]	1.000	512 (M+, 73), 441 (10), 399 (8), 358 (36), 327 (13), 287 (33), 155 (100)
Metabolite II [d]	1.036	498 (M+, 72), 483 (16), 343 (16), 314 (12), 288 (18), 273 (100), 205 (13), 155 (32)

[a]Samples were analyzed as bismethanboronate (BMB). [b]RRt : Relative Retetion time. [c]Samples were analyzed by GC-MS. [d]Samples were analyzed by GC-SIM.

Table 2. 400 MHz ^{1}H-NMR data of 6-deoxoCS and 3-*epi*-6-deoxoCS ($CDCl_3$, TMS as internal standard)

Compound	Ring protons				Side chain protons					
	Me-18	Me-19	H-2	H-3	Me (1)	Me (2)	Me (3)	Me (4)	H-22	H-23
6-deoxoCS	0.67s	0.80s	3.76br.m ($w^1/_2$ = 21 Hz)	3.97br.s ($w^1/_2$ = 10.5 Hz)	0.85d	0.89d	0.95d	0.97d	3.56br.s (J = 9 Hz)	3.73br.s (J = 9 Hz)
3-*epi*-6-deoxoCS	0.67s	0.86s	3.39br.s ($w^1/_2$ = 21 Hz)	3.59br.m ($w^1/_2$ = 21 Hz)	0.85d	0.89d	0.95d	0.97d	3.56br.s (J = 9 Hz)	3.73br.s (J = 9 Hz)

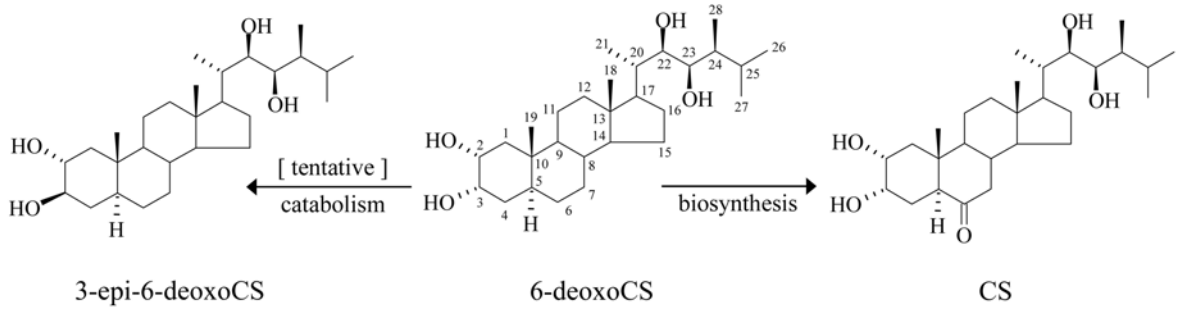

Figure 1. Enzymatic conversions of 6-deoxoCS in *Phaseolus vulgaris* cells.

proton co-attaching with the 3α-hydroxyl in 6-deoxoCS was altered to an axial proton which co-attached with the 3β-hydroxyl in the epimeric 6-deoxoCS. For this reason, the signal for CH3-19 (δ0.80, s) in 6-deoxoCS was downshifted to δ0.86 (s) in the epimeric 6-deoxoCS. Taken together, the epimer was shown to be 3-*epi*-6-deoxoCS, (22*R*, 23*R*, 24*S*)-2α,3β,22,23-tetrahydroxy-24-methyl-5α-cholestane.

The biogenesis of 3-*epi*-6-deoxoCS was determined via cell-free enzyme conversion(s), using a crude enzyme solution prepared from cultured *Phaseolus vulgaris* cells.[10] Prior to the enzyme assays, the presence of CS, the most abundant BR detected in the *Phaseolus* cells, was assessed in the crude enzyme solutions. GC-MS/selected ion monitoring (SIM) analyses revealed no CS in the solutions (data not shown), thereby indicating that the solutions harbored no detectable amounts of BR. Therefore, non-labeled 6-deoxoCS and NADPH (4.8 mM) were added to the enzyme solutions as a substrate and a cofactor, respectively, in order to characterize the metabolism of 6-deoxoCS occurring in the plants. After 30 minutes of incubation at 37 °C, the assay mixtures were extracted with ethyl acetate (1.2 mL × 3). The obtained ethyl acetate soluble fractions were then purified by a reversed phase HPLC and analyzed *via* GC-MS/-SIM following methaneboronation as described previously.[10]

On the full scan GC-MS, an active compound (metabolite I) in HPLC fractions 19 and 20 generated a mass spectrum and GC-retention time identical with those of authentic CS BMB (Table 1), thereby verifying that 6-deoxoCS had been converted into CS, as had been previously reported.[11] In the GC-SIM analysis, an active principle in HPLC fractions 40 and 41 (metabolite II) evidenced prominent ions at 498, 483, 343, 332, 314, 288, 273, 213, 205 and 155, at the same GC-retention time as that of the BMB of 3-*epi*-6-deoxoCS identified in the *P. vulgaris* seeds as mentioned above. This result indicated that 6-deoxoCS was metabolized to 3-*epi*-6-deoxoCS in the *Phaseolus* cells. Therefore, the biogenesis of 3-*epi*-6-deoxoCS from 6-deoxoCS was confirmed in the *Phaseolus* cells.

This study is the first, to the best of our knowledge, to identify the new BR, 3-*epi*-6-deoxoCS, in the immature seeds of *P. vulgaris*. Additionally, we demonstrated, *via* enzymatic conversion, that 3-*epi*-6-deoxoCS is generated from 6-deoxoCS within the seeds. Using the same plant material, the co-existence of CS and its 3-epimer, 3-*epi*CS, has also been demonstrated (data will be published elsewhere). In *Oryza sativa*, *Nicotiana tabacum* and *Catharansus roseus* seedlings, the C-3 epimerization of CS to 3-*epi*CS has been demonstrated, in feeding experiments utilizing deuterium-labeled CS.[12] In rice lamina inclination assays, 3-*epi*CS evidences approximately one-fifth less biological activity than is detected with CS,[12] thereby indicating that 3-*epi*CS must be considered a product for deactivation, rather than a biosynthetic product, of CS. Although the lower biological activity of 3-*epi*-6-deoxoCS as compared to 6-deoxoCS has yet to be verified, as both BRS are biologically inactive in the bioassay, the aforementioned results strongly suggest that 3-*epi*-6-deoxoCS may be a catabolite of 6-deoxoCS in *Phaseolus* seeds.

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