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

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Received September 28, 2006

Key Words: Brassinosteroids, 6-Deoxocastasterone, 3-Epi-6-deoxocastasterone, *Phaseolus vulgaris*

Brassinosteroids (BRs) are steroidal plant hormones, which exert a variety of regulatory effects relevant to plant growth and development.\(^1\) Thus far, over 40 different BRs have been identified throughout the entirety of the plant kingdom.\(^2,3\) Among the plants so far tested for their BR contents, the immature seeds of *Phaseolus vulgaris* represent one of the richest.\(^4\) Over 40 different unknown BRs have been demonstrated via capillary GC-MS analyses. Our interest in the structure and biogenesis of this BR, designated 3-epi-6-deoxocastasterone, in *Phaseolus vulgaris* seeds.

The ethyl acetate soluble fraction acquired from the *Phaseolus* seeds (136 Kg) was purified with SiO2, 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 bismethanoborane (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-H2O (97:3:0.1) at a flow rate of 3 mL/min. 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 δ 3.56 (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 (δ 3.76) and H-3 (δ 3.97) 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...
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, (22R, 23R, 24S)-2α,3β,22,23-tetrahydroxy-24-methyl-5α-cholestan-3β-ol (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). BR detected in the bioassay, the aforementioned results strongly suggest that 3-epi-6-deoxoCS may be a catabolite of 6-deoxoCS in Phaseolus vulgaris seeds.

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. 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 x 3). The obtained ethyl acetate soluble fractions were then purified by a reversed phase HPLC and analyzed via GC-MS/SIM following methanethoranization 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-epiCS, has also been demonstrated (data will be published elsewhere). In Oryza sativa, Nicotiana tabacum and Catharanthus roseus seedlings, the C-3 epimerization of CS to 3-epiCS has been demonstrated, in feeding experiments utilizing deuterium-labeled CS.12 In rice lamina inclination assays, 3-epiCS evidences approximately one-fifth less biological activity than is detected with CS,12 thereby indicating that 3-epiCS 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 vulgaris seeds.

Acknowledgement. This research was supported by a grant (PF06304-03) from Plant Diversity Research Center of 21st Century Frontier Research Program funded by Ministry of Science and Technology of Korean government.

References