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ORIGINAL RESEARCH

Stereoisomers of Castasterone, 3-*Epi*castasterone and 2,3-*Diepi*castasterone, in Immature Seeds of *Phaseolus vulgaris*

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Abstract A capillary GC-MS analysis revealed that two stereoisomers of castasterone are contained in immature seeds of *Phaseolus vulgaris*. 400 MHz proton NMR analysis of the stereoisomers determined they are A ring epimers of castasterone, 3-*epi*castasterone and 2,3-*diepi*castasterone. In rice lamina inclination assay, 3-*epi*castasterone and 2,3-*diepi*castasterone showed reduced biological activity than that of castasterone. Together with our previous finding that 2-*epi*castasterone exhibits a less biological activity than CS, this result indicates that epimerization of hydroxyl at C-2 or/and C-3 is/are inactive processes to reduce biological activity of CS after exerting as a bioactive brassinosteroid in *P. vulgaris*.

Keywords Brassinosteroids · 3-*Epi*castasterone · 2,3-*Diepi*castasterone · Structure elucidation · Biological activity

Introduction

The steroidal chemical signals associated with the class of compounds known as the brassinosteroids (BRs) have been shown to be crucial for the normal growth and development of plants. Among more than 40 natural BRs, castasterone (CS, Fig. 1) is the most frequently and abundantly identified compound from plants (Sakurai and Fujioka 1997; Bishop and Yokota 2001; Fujioka and Yokota 2003; Bajguz and Tretyn 2003). The molecular genetic analysis of cytochrome P450 (CYP) 85A1 and A2 in *Arabidopsis* has revealed that CS, as well as brassinolide, functions as an active BR to regulate growth and development in plants, particularly vegetative growing plants (Castle et al. 2005; Kim et al. 2005; Kwon et al. 2005). It has been demonstrated previously that the immature seed of *Phaseolus vulgaris* contains CS and a number of uncharacterized stereoisomers of CS (Yokota et al. 1987; Fujioka and Yokota 2003). Among the stereoisomers of CS, 2-*epi*CS has been recently characterized from *Phaseolus* seeds (Park et al. 2009). Our continuous interest in the chemical structure of the CS isomers led us to determine two additional CS isomers—3-*epi*CS and 2,3-*diepi*CS—from the seeds. Herein, we report the characterization, biological activity, and physiological functions of 3-*epi*CS and 2,3-*diepi*CS.

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Results

A large quantity of immature seeds of *P. vulgaris* was extracted, solvent partitioned, and column chromatographed via previously described methods (Park et al. 2009). The purified fraction, which evidences enhanced BR activity, was subjected to a large-scale reversed-phase (RP) HPLC, thereby giving rise to a number of biological fractions that were evaluated via a rice lamina inclination bioassay. The RP HPLC fractions eluted in 20–21 and 24–25 min were further purified via normal-phase (NP) HPLC. The NP HPLC fractions eluted in 14–15 and 18–19 min, which generated a blue-purplish spot on HPLC at R_f 0.33 and 0.41, respectively, yielded pure samples of compounds I and II, respectively.

In GC-MS analysis, bismethaneboronate (BMB), in both compounds (I and II), yielded a molecular ion at *m/z* 512 and prominent ions at *m/z* 155, 287, 327, 357/358, and 441. These mass ions are identical to those of CS BMB (Table 1).

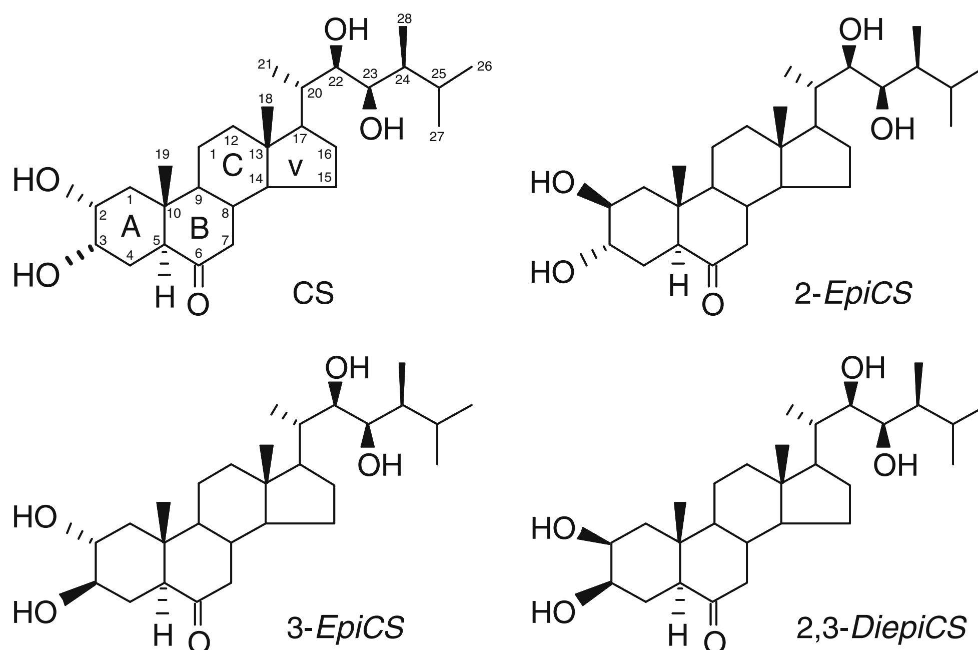

Fig. 1 Chemical structure of CS and its A ring epimers, 2-*epi*CS, 3-*epi*CS, and 2,3-*diepi*CS

However, the retention time on GC for the BMB of compound I and II was distinct from that of CS BMB, thereby implying that compounds I and II are stereoisomers of CS.

On 400 MHz proton NMR (Table 2), both compounds I and II yielded four methyl signals (C-21, C-26, C-27, and C-28) at δ 0.85 (d), 0.91 (d), 0.95 (d), and 0.97 (d). Two methine signals for H-22 and H-23 in the side chain were noted at δ 3.56 (d, J=9 Hz) and 3.73 (dd, J=2, 9 Hz), respectively. These side chain-derived signals were identical to those for CS, thereby indicating that the structure of the side chain in compounds I and II is identical to that of CS. Therefore, the structural variation of compounds I and II and CS appear to involve the ring structure. The signals for H_3-18 at δ 0.69 (s) and H_2-7 at δ 2.32 (dd, J=4, 14 Hz) of compound I and II are basically identical to those of CS, thus indicating that the structures of the B, C, and D rings in the CS isomers were also identical to those of CS.

Signals for two broad multiplet singlets ($W_{1/2}$=21 Hz) in compound I at δ 3.40 and 3.61 reflect the presence of two axial protons attached to secondary hydroxyls. Coupled with the fact that the isomer can be derivatized as BMB, these results demonstrate that a vicinal hydroxyl is located equatorially at C-2α and C-3β. In the presence of 2α, 3β vicinal hydroxyls, the absorption of H_3-19 and H-5 is down- and up-shifted at δ 0.81 and 2.32, respectively, relative to the results observed with CS (δ 0.76 and 2.69, respectively). According to this result, compound I is believed to be 3-epimeric CS. The quantity of 3-epimeric CS obtained herein (ca. 30 μg) was not sufficient for ^{13}C-NMR and/or 2D-NMR measurements that would corroborate the ^{1}H-NMR analysis results. However, the mass spectrum and GC retention time of the BMB of 3-epimeric CS was precisely identical to those of synthetic 3-*epi*CS BMB (Table 1). Therefore, compound I was characterized as 3-*epi*CS, (22*R*, 23*R*, 24*S*)-2α, 3β, 22, 23-tetrahydroxy-5α-cholestan-6-one.

In ^{1}H-NMR of compound II, absorptions of an axial proton at δ 3.64 ($W_{1/2}$=21 Hz) and an equatorial proton at δ 4.03 ($W_{1/2}$=10.5 Hz) were observed. Coupled to the finding that compound II can be derivatized to a BMB, this appears to reflect the presence of the C-2β and C-3β hydroxyls at the A ring. Relative to CS, which harbors the C-2α and C-3α hydroxyl, the signal for H_3-19 was down-shifted at δ

Table 1 GC-MS data for CS, 3-*epi*CS, 2,3-*diepi*CS, compound I, and compound II in *P. vulgaris*

Compound[a]	RRt	Prominent ions (relative intensity, %)
CS	1.00	512 (M+, 60), 441 (20), 357/358 (10), 327 (8), 287 (60), 155 (100)
3-*Epi*CS	1.11	512 (M+, 49), 441 (19), 357/358 (18), 327 (6), 287 (52), 155 (100)
2,3-*Diepi*CS	1.02	512 (M+, 51), 441 (19), 357/358 (15), 327 (7), 287 (48), 155 (100)
Compound I	1.11	512 (M+, 48), 441 (17), 357/358 (18), 327 (6), 287 (49), 155 (100)
Compound II	1.02	512 (M+, 50), 441 (18), 357/358 (19), 327 (8), 287 (46), 155 (100)

RRt relative retention time

[a] Compounds were analyzed as BMB derivatives

Table 2 Proton NMR (400 MHz, in $CHCl_3$) data for CS, compound I, and compound II

	CS	Compound I	Compound II
Ring protons			
H_3-18	0.69 s	0.69 s	0.69 s
H_3-19	0.76 s	0.81 s	0.97 s
H-2	3.77 br.m ($W_{1/2}$=21 Hz)	3.40 br.m ($W_{1/2}$=10.5 Hz)	4.03 br.s ($W_{1/2}$=21 Hz)
H-3	4.06 br.s ($W_{1/2}$=10.5 Hz)	3.61 br.m ($W_{1/2}$=10.5 Hz)	3.64 br.m ($W_{1/2}$=10.5 Hz)
H-5	2.69 dd (J=4, 14 Hz)	2.32 d[a] (J=4, 14 Hz)	2.23 dd (J=4, 13 Hz)
H_2-7	2.30 dd (J=4, 14 Hz)	2.32 d[b] (J=4, 14 Hz)	2.32 dd (J=4, 14 Hz)
Side chain protons			
H_3-21	0.85 d	0.85 d	0.85 d
H_3-26	0.91 d	0.91 d	0.91 d
H_3-27	0.95 d	0.95 d	0.95 d
H_3-28	0.97 d	0.97 d	0.97 d
H-22	3.56 d (J=9 Hz)	3.56 d (J=9 Hz)	3.56 d (J=9 Hz)
H-23	3.73 dd (J=2, 9 Hz)	3.73 dd (J=2, 9 Hz)	3.73 dd (J=2, 9 Hz)

The chemical shifts are given in ppm from tetramethylsilane

[a] Overlapped with H-5

[b] Overlapped with H-7

0.97. Additionally, the signal for H-5 was up-shifted at δ 2.32. The observed A ring proton signals clearly implicate compound II as 2,3-diepimeric CS. Although ^{13}C-NMR and/or 2D-NMR were/was not performed to confirm the structural identification, owing to the small amount (ca. 25 μg) of compound II obtained, the direct comparison of the BMB of compound II to synthetic 2,3-*diepi*CS BMB conclusively confirmed that compound II is 2, 3-*diepi*CS (22*R*, 23*R*, 24*S*)-2β, 3β, 22, 23-tetrahydroxy-5α-cholestan-6-one (Table 1).

The biological activity of CS, 2-*epi*CS, 3-*epi*CS, and 2,3-*diepi*CS was evaluated via a rice lamina inclination assay. As shown in Fig. 2, the biological activity of 3-*epi*CS was approximately one fifth that of CS. 2-*Epi*CS evidenced only a 1/50th reduction in activity relative to CS. 2,3-*Diepi*CS exhibited extremely weak activity up to a concentration of 0.02 ppm, which was approximately 1/500th that of CS.

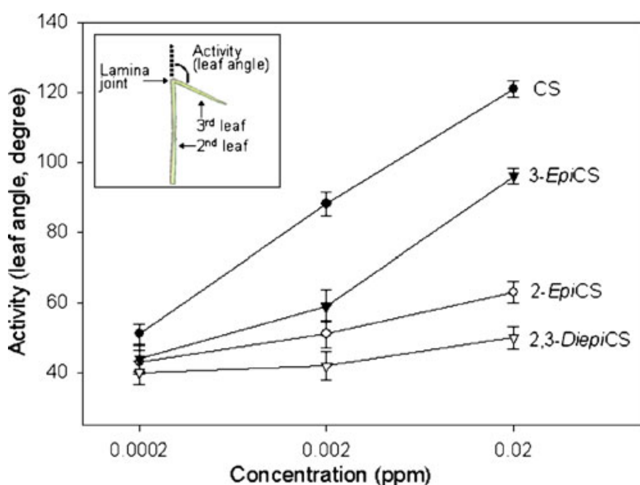

Fig. 2 Biological activity for CS, 2-*epi*CS, 3-*epi*CS, and 2,3-*diepi*CS in the rice lamina inclination assay

Discussion

In this study, two stereoisomers of CS, 3-*epi*CS and 2, 3-*diepi*CS, were identified from immature seeds of *P. vulgaris*; this is the first evidence of the presence of 3-*epi*CS and 2,3-*diepi*CS in the plant kingdom. Along with the observed presence of 2-*epi*CS, the identification of 3-*epi*CS and 2,3-*diepi*CS demonstrates that *Phaseolus* seeds harbor all possible configurations of vicinal hydroxyls at the C-2 and C-3 of CS, including 2α, 3α (CS), 2α, 3β (2-*epi*CS), 2β, 3α (3-*epi*CS), and 2β, 3β (2,3-*diepi*CS).

The rice inclination assay showed less biological activity, in the following order: CS>3-*epi*CS>2-*epi*CS>2,3-*diepi*CS. This indicates that the introduction of a β-configured hydroxyl at C-2 or/and C-3 reduced the biological activity observed for CS, thereby implying that the epimerization of C-2 or/and C-3 is an inactive process for an active BR, CS in *P. vulgaris*. Put in another way, 2α- and 3α-configured vicinal hydroxyls are required for the strong activity of CS.

2-*Epi*CS, 3-*epi*CS, and 2,3-*diepi*CS can be synthesized via the hydroxylation of 2-deoxy-analogs of CS, 2-deoxyCS (typhasterol), and 2-deoxy-3-*epi*CS (teasterone). Another possible biosynthetic route for CS epimers is the isomerization of CS at C-2 or/and C-3 of CS. In fact, exogenously applied CS is a metabolite of 3-*epi*CS in *Arabidopsis* (Fujioka 1999). The isomerization (epimerization) of CS at C-2 or/and C-3 may result in the generation of 2-*epi*CS, 3-*epi*CS, and 2,3-*diepi*CS in *P. vulgaris.*

In addition to biosynthesis, the inactivation of BRs is an important factor in maintaining a steady-state endogenous level of BRs in plants. We have demonstrated in *Marchantia polymorpha*, *Phaseolus vulgaris*, and the tomato plant that CS is metabolized into 26-norCS by C-26 demethylation in the side chain, via C-26 hydroxyl, C-26 aldehyde or/and C-26 carboxylic acid (Fig. 3; Kim et al. 2000; Kim et al. 2004a; Kim et al. 2004b). In the bioassay, 26-norCS evidenced 10% less activity than that of CS, thereby indicating that C-26 demethylation is a common inactivation process that reduces CS activity in planta. In this study, we also showed that the 2- and 3-epimerization of CS are inactivation reactions. The results of studies of the relevant metabolic and structure-activity relationships demonstrate that BR signaling receptors such as BRI1 and BAK1 can be used to determine the length of the side chain and the configuration of hydroxyls at C-2 and C-3. This is expected to prove a valuable insight in efforts in the synthetic chemistry field to develop commercially valuable compounds exhibiting BR activity.

Materials and Methods

Purification of 3-*Epi*CS and 2,3-*Diepi*CS in Immature Seeds of *P. vulgaris*

Immature seeds of *P. vulgaris* (136 kg) were extracted, solvent partitioned, and column chromatographed as the methods described previously. The obtained active fraction was purified by a large-scale RP HPLC (Shenshu Pak, Develosil ODS 5 μm, 20×250 mm) eluted at a flow rate of 20 mL min^{-1} with 45% acetonitrile as the mobile phase. Fraction was collected every minute. The fraction 21 and 25 were further purified by a small-scale NP HPLC (Shensu Pak, Aquasil, 10×200 mm). It eluted at a flow rate of 1 mL min^{-1} with a mixture of $CHCl_3$–MeOH–H_2O (96:4:0.1). Fraction was collected every minute. Compound I in fraction 15 and compound II in fraction 19 showed a blue-purplish spot on HPTLC (Merck, F_{254}) with an R_f of 0.33 and 0.41, respectively, after heating followed by spraying of 70% sulfuric acid. A pure solid-state substance for compound I and II were used for instrumental analyses.

Bioassay

The rice lamina inclination assay using a cultivar Koshihikari was performed to examine BRs activity (Arima et al. 1984).

Instrumental Analyses

GC-MS was carried out with JEOL DX-303 mass spectrometer (70 eV). A DB-1 column (J & W Scientific, 0.258×15 m; film thickness 0.25 μm) was used in a splitless injection mode with He gas. The injection temperature was at 290°C. After maintaining at 175°C for 2 min, the temperature was increased by 32°C min^{-1} to 275°C, and kept at 275°C for 30 min. Prior to injection,

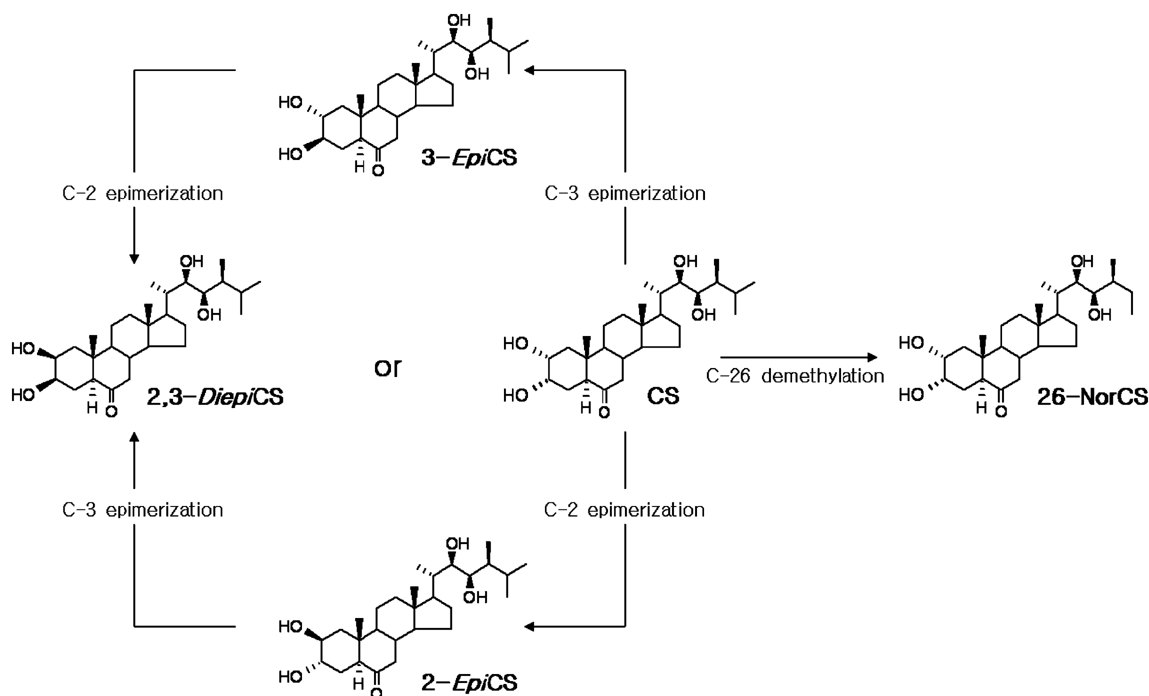

Fig. 3 Inactivation processes of CS in *P. vulgaris*

samples were heated at 70°C for 30 min in pyridine containing 2 mg/mL of methaneboronic acid (Sigma) to be BMB derivatives. Using tetramethylsilane (TMS) as an internal standard, 400 MHz proton NMR was measured by JEOL-FX400 NMR spectrometer.

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