

Characterization of C₂₉-Brassinosteroids and Their Biosynthetic Precursors in Immature Seeds of *Phaseolus vulgaris*

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Brassinosteroids (BRs) are unique steroids which show hormonal actions in plant growth and development.^{1,2} The BRs can be classified into C₂₇, C₂₈ and C₂₉ based on their carbon skeleton.^{3,4} Metabolic and molecular genetic studies revealed that the C₂₈-BRs are generated from campesterol (CR).^{5,6} Recently, we demonstrated that a C₂₇-BR, 28-norcastasterone (28-norCS) is biosynthesized from cholesterol (CHR) that carries an identical carbon skeleton to that of 28-norCS.⁷ These results suggested that the C₂₉-BRs are also biosynthesized from a phytosterol carrying the same skeleton as that of the C₂₉-BRs. Solid evidence to support this notion, however, is not yet to be obtained. This deficiency has prompted us to investigate the presence of BRs with a C-24 ethyl group and their potent biosynthetic precursors in the immature seeds of *Phaseolus vulgaris* wherein 24 α -ethylcholesterol, sitosterol (STR), is a major sterol, which provides a clue for possible C₂₉-BRs biosynthesis in plants.

As summarized in Table 1, the strongest peak on the total ion chromatogram (TIC) at 17.29 min gave a mass spectrum at *m/z* 486 (M⁺), 471, 396, 381, 357, 255, 213, 145 and 129 whose mass spectrum and retention times on GC are identical to those of authentic 24*S*-ethylcholesterol (STR) trimethylsilyl (TMSi) ether, demonstrating that the compound is STR. On TIC, a peak appeared at 17.57 min showing a

molecular ion at *m/z* 488 and prominent ions at *m/z* 473, 398, 383, 359, 257, 215, 147 and 131. The molecular ion and characteristic ions in the TMSi compound are 2 mass increased than those of sitosterol TMSi, suggesting that the compound is dihydro-sitosterol, most likely sitostanol (STN).

As shown in Table 2, 300 MHz proton NMR of the acetylated STN compound gave the key proton resonances at δ 0.64 (Me -18, s), 0.81 (Me -19, s), 4.68 (H-3, m), 2.02 (3-OAc, s), 0.90 (Me -21, d, *J* = 6.6 Hz), 0.85 (Me -26, d, *J* = 7.2 Hz), 0.83 (Me -27, d, *J* = 7.2 Hz) and 0.80 (Me -29, d, *J* = 5.7 Hz). The signals for H-4 (δ 2.32, d) and H-6 (δ 5.38, m) in STR-acetate were not observed in the compound, however, indicating that a double bond between C-5 and C-6 in STR acetate is not present in the compound. By saturating the ring structure, most of the aforementioned proton signals from the compound are upfield shifted compared to those from STR-acetate. The downfield shifted signal for H-3 is also understandable because the absorption of H-3 (δ 4.60 m) derived from CHR-acetate is downfield shifted to δ 4.69 (m) by diminishing the double bond at C-5. Taken together, the compound is undoubtedly STN-acetate, determining that the natural structure of the compound is STN.

The ethyl acetate soluble fraction (64 g) obtained from immature seeds of *P. vulgaris* was purified by several

Table 1. GC-MS data for authentic and endogenous sterols in immature seeds of *P. vulgaris*

Compound	Rt ^a on GC	Prominent ions (<i>m/z</i> , relative intensity)
Authentic STR	17.29	486 (M ⁺ , 52), 471 (15), 396 (91), 381 (40), 357 (100), 255 (15), 213 (10), 145 (26), 129 (96)
Authentic STN	17.57	488 (M ⁺ , 75), 473 (16), 398 (32), 383 (48), 359 (9), 257 (10), 215 (100), 147 (25), 131 (10)
Endogenous STR	17.29	486 (M ⁺ , 50), 471 (15), 396 (93), 381 (40), 357 (100), 255 (17), 213 (10), 145 (25), 129 (95)
Endogenous STN	17.57	488 (M ⁺ , 77), 473 (16), 398 (34), 383 (49), 359 (9), 257 (10), 215 (100), 147 (26), 131 (11)

The samples were analyzed as a derivative of trimethylsilylic (TMSi) ether. ^a: Retention time

Table 2. ¹H-NMR data (300 MHz, CDCl₃) of CHR-, CHN-, STR- and STN-acetate

Compound	Ring protons						Side chain protons			
	Me-18	Me-19	H-3	H-4	H-6	3-OAc	Me-21	Me-26	Me-27	Me-29
CHR-acetate	0.677 s	1.019 s	4.602 m	2.318 d (<i>J</i> = 7.6 Hz)	5.376 d (<i>J</i> = 4.8 Hz)	2.033 s	0.915 d (<i>J</i> = 6.6 Hz)	0.863 d (<i>J</i> = 6.9 Hz)	0.867 d (<i>J</i> = 6.6 Hz)	
CHN-acetate	0.645 s	0.816 s	4.685 m			2.019 s	0.896 d (<i>J</i> = 6.6 Hz)	0.859 d (<i>J</i> = 6.6 Hz)	0.863 d (<i>J</i> = 6.6 Hz)	
STR-acetate	0.677 s	1.018 s	4.602 m	2.318 d (<i>J</i> = 7.6 Hz)	5.380 m	2.031 s	0.920 d (<i>J</i> = 6.4 Hz)	0.854 d (<i>J</i> = 7.2 Hz)	0.835 d (<i>J</i> = 7.2 Hz)	0.813 d (<i>J</i> = 5.7 Hz)
STN-acetate	0.640 s	0.814 s	4.675 m			2.015 s	0.897 d (<i>J</i> = 6.6 Hz)	0.848 d (<i>J</i> = 7.2 Hz)	0.825 d (<i>J</i> = 7.2 Hz)	0.802 d (<i>J</i> = 5.7 Hz)

Table 3. GC-MS data for authentic and endogenous HCS and HTY in immature seeds of *P. vulgaris*

Compound	Rt ^a on GC	Prominent ions (<i>m/z</i> , relative intensity)
Authentic HCS ^b	38.08	526 (M ⁺ , 38), 441 (13), 399 (12), 358 (36), 343 (6), 287 (20), 169 (100)
Authentic HTY ^c	29.34	558 (M ⁺ , 100), 543 (48), 540 (31), 529 (60), 468 (75), 169 (30)
Endogenous HCS ^b	38.08	526 (M ⁺ , 39), 441 (13), 399 (13), 358 (36), 343 (7), 287 (19), 169 (100)
Endogenous HTY ^c	29.34	558 (M ⁺ , 100), 543 (50), 540 (33), 529 (60), 468 (77), 169 (30)

^a: Retention time. ^b: The samples were analyzed as a derivative of bismethanboronate. ^c: The samples were analyzed as a derivative of methanboronate (MB)-TMSi ether.

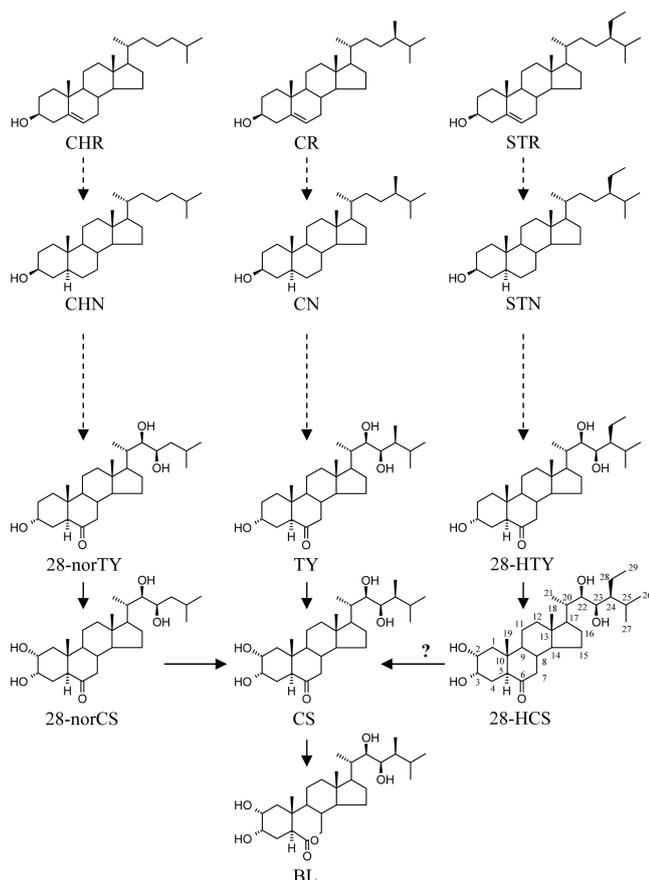


Figure 1. Biosynthetic pathway for C₂₇-, C₂₈-, C₂₉-BRs and their connections in plants. The dotted and solid arrow indicates multi- and single-biosynthetic step, respectively.

column chromatographies followed by a reversed phase HPLC (Senshu Pak Develosil, 20 × 250 mm) with elution using aqueous acetonitrile (0-40 min: 55%, 40-80: 80%) at a flow rate 9.9 mL min⁻¹. The fraction 43, whose retention time is equal to that of authentic 28-homocasterone (HCS), was derivatized to bismethanboronate (BMB) and analyzed by capillary GC-MS.⁷ The BMB of an active compound in fraction 43 showed a molecular ion at *m/z* 526 and characteristic ions at *m/z* 441, 399, 358, 343, 287 and 169 whose retention times on GC were identical to that of authentic HCS BMB, demonstrating that the active compound is HCS (Table 3).

Methaneboronation followed by trimethylsilylation indicated that the active principle in HPLC fraction 58 gave an

MS spectrum at *m/z* 558[M⁺], 543, 540, 529, 468 and 169 which was identical to that shown in 28-homotyphasterol (28-HTY) methaneboronate (MB)-TMSi ether (Table 3). Further, the GC-retention time of the active compound MB-TMSi ether was the same as that of authentic 28-HTY MB-TMSi, verifying that the compound is 28-HTY.

This study is the first to demonstrate that immature seeds of *P. vulgaris* contain STN, 28-HTY and 28-HCS as well as STR. All these steroids carry the 24*S*-oriented ethyl group, and are counterparts of CR, cholestanol (CN), typhasterol (TY) and CS in C₂₈-BRs biosynthesis and CHR, cholestanol (CHN), 28-norTY and 28-norCS in C₂₇-BRs biosynthesis, indicating that a similar biosynthetic pathway to C₂₈- and C₂₇-BRs biosynthesis, most likely the early C-6 oxidation pathway for C₂₉-BRs, is operative in the seeds (Fig. 1).

The conversion of 28-norCS to CS has been demonstrated in the tomato plant.⁷ Additionally, we found that the same conversion occurs in *Arabidopsis* as in *P. vulgaris* (data published elsewhere), providing that the C₂₇-BRs biosynthesis is an alternative pathway to generate an active C₂₈-BR, CS. To examine whether the C₂₉-BRs biosynthesis is an alternative route for the C₂₈-BRs, a metabolic study of C₂₉-BRs, especially the C-29 demethylation of 28-HCS to CS is underway in our laboratory.

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