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Identification of New 2-Deoxy Type Brassinosteroids in Immature Seed of *Phaseolus vulgaris* by Gas Chromatography-Mass Spectrometry

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In immature seeds of *Phaseolus vulgaris*, the presence of three new 2-deoxy type brassinosteroids (trihydroxybrassinosteroids) have been demonstrated. These less polar brassinosteroids have been tentatively characterized to be (3ξ, 22ξ, 23ξ)-2-deoxy-25-methyldolicholide, (3ξ, 22ξ, 23ξ)-2-deoxy-dolichosterone and (3ξ, 22ξ, 23ξ)-2-deoxy-24-ethylbrassinone by analysis of gas chromatography-mass spectrometry. Their less biological activity and oxidation state than those of tetrahydroxybrassinosteroids suggest that they are potent biosynthetic precusors of tetrahydroxybrassinosteroids.

Keywords : *Phaseolus vulgaris*, immature seed, GC-MS, 2-deoxy type brassinosteroid, strutcure characterization

Brassinosteroids are steroidal plant hormones which promote plant growth. To date thirty one members of brassinosteroids have been identified from various plant sources (Kim, 1991).

We have previously reported that the seed of *Phaseolus vulgaris* contains over sixty members of brassinosteroids (Kim, 1988). Among them, nineteen brassinosteroids including thirteen new brassinosteroids have been fully characterized (Kim *et al.*, 1987; Yokota *et al.*, 1987; Kim, 1991). Because of continuing interest in the structure and biosynthesis of brassinosteroids in *Phaseolus* seed, we reinvestigated the endogenous brassinosteroids in the plant materials. Recently, three new 2-deoxy type brassinosteroids in the seed were successfully identified by the analysis of gas chromatography-mass spectrometry (GC-MS). Herein we report methods of purification and structural characterization by GC-MS of the 2-deoxy type brassinosteroids. In addition, the possible role of the 2-deoxy-brassinosteroids in biosynthetic pathway of brassinosteroids is discussed.

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MATERIALS AND METHODS

Bioassay

The rice lamina inclination assay (Wada *et al.*, 1984) was carried out using the cultivar Koshihikari as described by Arima *et al.* (1984).

Silica gel column chromatography

An ethyl acetate-soluble fraction (64.3 g) was dissolved in 300 mL of chloroform and charged on a column of silica (500 g). Mixture of methanol-chloroform were used for elution, and methanol contents were increased stepwise (0%, 4L; 2%, 4L; 3%, 3L; 4%, 3L; 5%, 3L; 6%, 3L; 7%, 3L; 8%, 3L; 13%, 5L; 20%, 5L; 30%, 5L; 50%, 5L; 100%, 5L).

Sephadex LH ; 20 column chromatography

As previously described (Yokota *et al.*, 1985), 1740 mL columns were used with a 4 : 1 mixture of methanol-chloroform as the elution solvent. Flow rate was 20 mL/h, and a fraction was 20 mL.

Activated charcoal column chromatography

Approximately 3.56 g of active fraction obtained from Sephadex LH-20 chromatography was purified by using 40 g of pre-activated charcoal. Samples were dissolved in 60% methanol and applied on the column. Elutions were undertaken by every 1 L of aqueous methanol (60, 80, 100%) and of mixture of methanol-chloroform (2 : 1, 1 : 1, 1 : 2, 1 : 4).

Reversed phase HPLC

A reversed phase column (20×300 mm) of Senshu Pak LRP-1 was eluted at a flow rate of 9.9 mL/min with acetonitrile-H_2O mobile phase containing 1% acetic acid, comprising 45% acetonitrile (0 to 40 min) and 80% acetonitril (40 to 65 min). Fractions were collected every min.

Derivertization of 2-deoxy type brassinosteroids for GC-MS

Samples were heated at 70℃ for 30 min in pyridine containing 2 mg/mL of methaneboronic acid (Sigma) (Takatsuto *et al.*, 1982). Prior to GC-MS, 50 uL of bis(trimethylsilyl)acetamide-trimethylchlorosilane (3 : 1) solution was added (Yokota *et al.*, 1983).

GC-MS with a capillary column

JEOL DX-303 mass spectrometer (70 eV) was used. A DB-1 column (0.258×15 m; film thickness 0.25 μm) purchased from J & W Scientific was used in a splitless injection mode with He gas. The injection temperature was 290℃. After maintaining oven temperature at 175℃ for 2 min, the temperature was increased by 32℃/min to 275℃. Finally the temperature was held at 275℃ (Yokota *et al.*, 1987).

RESULTS AND DISCUSSION

Immature seeds (136 kg) of *Phaseolus vulgaris* were extracted and solvent-partitioned as shown in Fig. 1. After examining of the biological activity of all collected fractions by the rice lamina inclination bioassay (Arima *et al.*, 1984), three active fractions were selected. Free brassinosteroids were considered

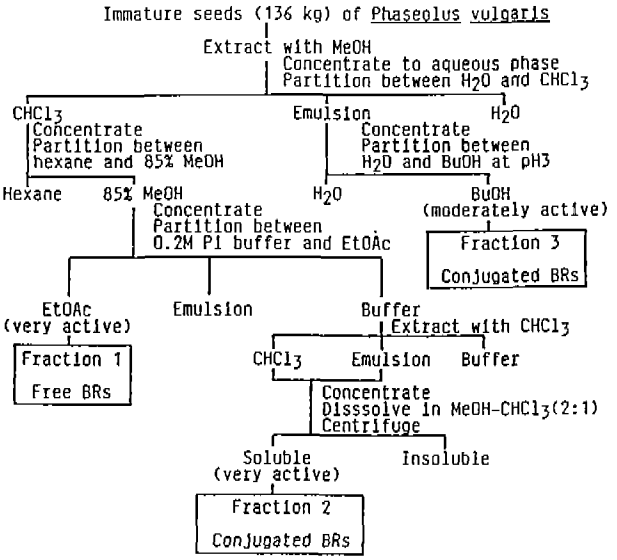

Fig. 1. Fractionation procedure of methanol extracts of *P. vulgaris* seed based on solvent partitioning.

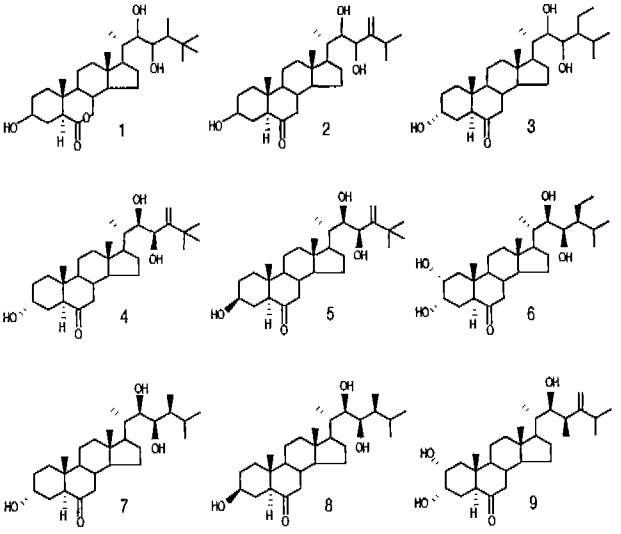

Fig. 2. Structures described in this paper.

to be partitioned in ethyl acetate soluble fraction.

A free brassinosteroid fraction obtained from solvent partitioning of methanol extract was subjected to silica gel chromatography. The fractions of 4, 5, 6, 7% methanol in chloroform were biologically active in the rice lamina inclination assay. The active fractions were collected and purified by repeated Sephadex LH-20 chromatography using elution solvent of methanol-chloroform. For further purification, charcoal chromatography (an elution solvent of mixtures of methanol-chloroform) was attempted,

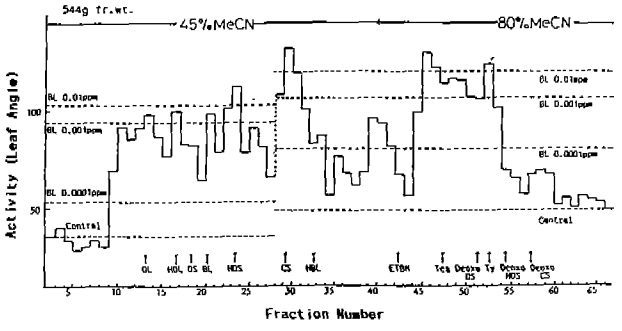

Fig. 3. Distribution of biological activity after reversed phase HPLC (Develosil ODS 5 μm, 20×250 mm). Arrow indicates the retention time of known brassinosteroids. DL, HDL, DS, BL, HDS, CS, HBL, ETBN, Tea, Deoxo-DS, Ty, Deoxo-HDS and Deoxo-CS represent dolicholide, homodolicholide, dolichosterone, brassinolide, homodolichosterone, castasterone, homobrassinolide, ethylbrassinone, teasterone, 6-deoxo-dolichosterone, typhasterol, 6-deoxo-homodolichosterone and 6-deoxo-castasterone, respectively.

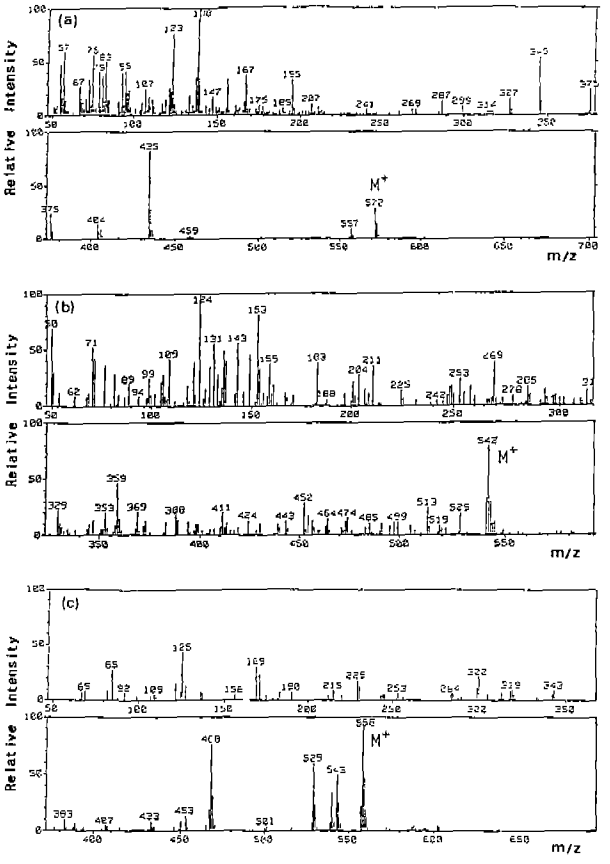

Fig. 4. Mass spectrum of methaneboronate-monoTMS ether of 2-deoxy type brassinosteroids in *P. vulgaris* seed. (a) 2-deoxy-25-methyldoli-cholide, (b) 2-deoxy-dolichosterone and (c) 2-deoxy-24-ethyl-brassinone.

resulting in the biological activity to be effectively enriched in one fraction (about a half gram) as indicated by the rice lamina inclination assay. Finally, active compounds in the purified fraction were distributed by reversed phase HPLC.

Fig. 3 shows the distribution of biological activities after reversed phase HPLC. Strong activities are detected in various fractions, indicating that a number of brassinosteroids are present in this species. Moreover retention times of many active fractions are not identical with those of known brassinosteroids, suggesting that numerous unidentified brassinosteroids are included.

Among the fractions obtained from the reversed phase HPLC, a less polar fraction 46 was analyzed by GC-MS using a fused silica capillary column. Methaneboronation (B-Me) followed by trimethylsilylation (TMS) of an active compound in the fraction gave a mass spectrum as shown in Fig. 4(a). Molecular ion peak at m/z 572 indicates that this compound has been derivatized as monomethaneboronate-monoTMS ether. Prominent ions at m/z 123, 138 and 167 are considered to have been due to the fission of the C22-C23 bond and C20-C22, respectively (Kim *et al.*, 1987), indicating that methaneboronate is carried in the side chain at C22, C23 vicinal hydroxyls. A fragment ion due to the elimination of side chain accompanied by the loss of two hydrogen atoms from the steroidal nucleus, which was known to be characteristic of C24- or C24(28)-unsaturated side chain (Ikekawa *et al.*, 1984), was also observed at m/z 375. These fragmentation patterns strongly suggest the presence of a 24-methylene group and a terminal tert-butyl group in the side chain (Kim *et al.* 1987). Fragment ions at m/z 345, 375, 404 and 435 show the presense of 7-oxa-lactone at B ring and an isolated hydroxyl at A ring. Because two 6-ketone congeners of this compound (2-deoxy-25-methyldolichosterone (Fig. 2-4), 3-epi-2-deoxy-25-methyldolichosterone (Fig. 2-5) have been identified from the same plant material (Kim *et al.*, 1988), it is considered that the compound also contains an isolated hydroxyl at C3 position. Because of low concentration, however, configuration of hydroxyls at C3, C22 and C23 still remains to be determined. Therefore, the compound is tentatively characterized to be (3ξ, 22ξ, 23ξ)-2-deoxy-25-methyldolicholide (Fig. 2-1). This fact provide the

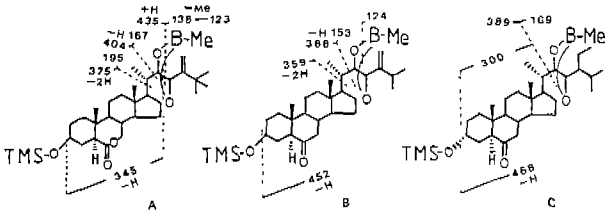

Fig. 5. Mass fragmentation pattern of methaneboronate-monoTMS ether of 2-deoxy type brassinosteroids in *P. vulgaris* seed. (A) 2-deoxy-25-methyldoli-cholide, (B) 2-deoxy-dolichosterone and (C) 2-deoxy-24-ethylbrassinone.

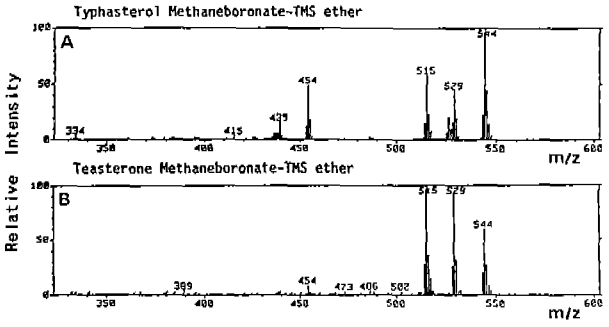

Fig. 6. Spectral comparison of methaneboronate-mono-TMS ether of typasterol (carrying 3α hydroxyl) (A) and teasterone (carrying 3β hydroxyl) (B).

first 2-deoxy type brassinosteroid containing 7-oxa-lactone at B ring.

Reversed phase HPLC fraction 48 is also considered to contain less polar brassinosteroids. GC-MS analysis of the active compound in fraction 48 after methaneboronation followed by TMS gave mass spectrum as shown in Fig. 4(b). Molecular ion peak at m/z 542 shows that the compound has been derivatized to be monomethaneboronate-monoTMS ether. Characteristic fragment ions at m/z 124 (base peak) and 153 are considered to have been due to fission of the C22-C23 bond and C20-C22 bond respectively. Therefore, it is thought that methaneboronate enter to C22, C23 vicinal hydroxlys in the side chain. Fragment ion at m/z 359 known to be characteristic of C24 unsaturated side chain was also observed. These fragmentation patterns are the same as those of dolichosterone (Fig. 2-9) bismethaneboronate (Yokota *et al.*, 1987). Therefore, the structure of side chain in this compound is identical with that of dolichosterone carrying C22, C23 vicinal hydroxlys and 24-exomethylene. Fragment ions at m/z 359, and 388 and 452 reveal the presence of 6-ketone at B ring and an isolated hydroxyl at A ring. Since all trihydroxybrassinosteroids have an isolated hydroxyl at C3, the isolated hydroxyl at A ring in the compound is probably present at C3. However, the configuration of hydroxyls at C3, C22 and C23 still remains to be determined. Thus, the active compound was tentatively characterized to be (3ξ, 22ξ, 23ξ)-2-deoxy-dolichosterone (Fig. 2-2).

As shown in Fig. 4(c), monomethaneboronate-mono TMS ether of an active compound in reversed phase HPLC fraction 58 showed a molecular ion peak at m/z 558 in GC-MS analysis. A characteristic fragment ion (base peak) for 24-ethylbrassinone (Fig. 2-6) bismethaneboronate at m/z 169 (Ikekawa *et al.*, 1984) is found in the mass spectrum of the compound, indicating that the compound possesses C22, C23 vicinal hydroxyls and 24-ethyl group in side chain. Fragment ions at m/z 300 and 468 show the presence of 6-ketone at B ring and an isolated hydroxyl at A ring. Mass fragmentation pattern at high mass charge (relative intensity of fragment ions of $[M]^+$, $[M-15]^+$, $[M-18]^+$, $[M-29]^+$ and $[M-90]^+$) of the compound is not identical with that of teasterone (Fig. 2-8) (3-epi-2-deoxycastasterone carrying 3β hydroxyl) but identical with that of typhasterol (Fig. 2-7) (2-deoxycastasterone carrying 3α hydroxyl), suggesting that the C3 hydroxyl in the compound is α oriented (Fig. 6). Therefore the active compound in the fraction was tentatively determined to be (3α, 22ξ, 23ξ)-2-deoxy-24-ethyl-brassinone (Fig. 2-3), although the confuguration of vicinal hydroxyls at C22 and C23 still remains to be determined.

We already reported that *P. vulgaris* seed contains 2-deoxycastasterone (typhasterol), 3-epi-2-deoxycastasterone (teasterone), 2-deoxy-25-methyl-dolichosterone and 3-epi-2-deoxy-25-methyldolichosterone. Now we demonstrate the presence of three new 2-deoxy-brassinosteroids in the seed.

Because these 2-deoxy type brassinosteroids have a less activity in rice inclination assay (unpublished data) and a less oxidation state than those of tetrahydroxybrassinosteroids (carrying vicinal hydroxyls at C2 and C3), they are considered to be potent biosynthetic precusors of tetrahydroxy-brassinosteroids in the seed. Also the presence of various 2-deoxy-brassinosteroids could suggest that hydroxylation of 5-cholestan skeleton for brassinosteroids biosynthe-

sis primarily occurs in the side chain rather than in A ring.

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Gas Chromatography-Mass Spectrometry 分析에 의한 강낭콩 未熟種子에 含有되어 있는 새로운 2-Deoxy型 Brassinosteroid의 同定

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적 요

강낭콩 미숙종자내에 3개의 새로운 구조의 2-deoxy형의 brassinosteroid가 존재함을 처음으로 증명하였다. 이들 비교적 비극성의 brassinosteroid(trihydroxybrassino-steroid)는 gas chromatography-mass spectrometry 분석을 통하여 잠정적으로 (3ξ, 22ξ, 23ξ)-2-deoxy-25-methyldolicholide, (3ξ, 22ξ, 23ξ)-2-deoxy-dolichosterone, 그리고 (3ξ, 22ξ, 23ξ)-2-deoxy-24-ethylbrassinone로 구조결정하였다. 이들 화합물은 tetrahydroxybrassinosteroid와 비교하여 낮은 생리활성과 산화상태를 나타내고 있어 tetrahydroxybrassinosteroid의 생합성 과정의 전구물질로 사료된다.

주요어 : 강낭콩, 미숙종자, GC-MS, 2-deoxy형 brassinosteroid, 구조결정

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