



## Isolation and Characterization of Brassinolide and Castasterone In the Pollen of Pumpkin

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### ABSTRACT

The two brassinosteroids contained in the pollen of pumpkin (*Cucurbita moschata* Duch.) were investigated. The bioactive compounds from the purified extract were identified by GC-MS as brassinolide and castasterone. The content of brassinolide and castasterone were estimated to be 36  $\mu\text{g kg}^{-1}$  and 112  $\mu\text{g kg}^{-1}$  (dry weight) respectively. This study provides the first evidence for the occurrence of brassinolide and castasterone in the pollen of pumpkin.

**Keywords:** brassinolide, brassinosteroids, castasterone, *Cucurbita moschata*, pumpkin, rice lamina inclination

### 1. INTRODUCTION

Brassinosteroids (BRs) are steroidal plant hormones, which are essential for plant growth and development [1-4]. Since brassinolide (BL) was isolated from rape pollen (*Brassica napus*) [5, 6] as the first steroidal plant growth regulator, the intensive research on the isolation and characterization of active substances related to BL has been continued [7-10]. The BRs have been detected in many higher plants and some lower plants [11-14]. Therefore, the BRs appear to be ubiquitous in the plant kingdom. At present more than 50 compounds of natural occurrence of BRs have been characterized from various plant sources [15-17].

In this present work, we investigated the natural BRs in the pollen of pumpkin. It is a by-product collected from the stamina of pumpkin flowers during the time of pumpkin harvesting. The approximate harvest of the

pollen was about 15-17 kg per acre (fresh weight). In this study, we have isolated and characterized brassinolide and castasterone (CS) in the pollen of pumpkin, Mun-Seok Jang *et al.*[18] have isolated and characterized brassinolide, castasterone and their biosynthetic precursors from the seeds of pumpkin, but the occurrence of brassinolide and castasterone in the pollen has never been previously reported.

### 2. EXPERIMENTAL

#### 2.1 General Data

The GC-MS analysis was carried out with the following condition: Automass (JMS-AM150) mass spectrometer connected with a Hewlett-packard 6890-5973 gas chromatograph, EI (70 eV), source temperature 280 °C, column HP-1MS (HP19091S-933, 30 m x 250  $\mu\text{m}$ , 0.25  $\mu\text{m}$  film thickness), injection temperature 275 °C column temperature

programmed 200 °C for 2 min, then raised to 280 °C at rate of 20 °C min<sup>-1</sup> and held on this temperature for 25 min; interphase temperature 275 °C, carrier gas He, flow rate 1.0 mL min<sup>-1</sup> split injection. Column chromatography was carried out on silica gel (Merck; 70-230 mesh) and Sephadex LH-20 (Amersham Biosciences) and the RP-18 cartridge (1 g, Merck; Licholut RP-18) for cleaning up the bioactive residues. The authentic brassinolide and castasterone were purchased from Kento Chemical Co., Ltd. (Japan). The other chemicals were analytical grade and all the commercial solvents used for the extraction were freshly distilled before using.

## 2.2 Plant Material

The pollen of pumpkin was collected from Mae-Ai district, Chiang Mai province Thailand. A voucher specimen of this plant (Pachthong No.24) is deposited at CMU Herbarium, Department of Biology, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand. The stamina of pumpkin (37.8 kg, fresh weight) were collected from flowers of pumpkin, and drying at room temperature to give dried stamina (9.52 kg), which was sieved through a 100 mesh screen, whereby the dried pumpkin pollen (2.76 kg) was obtained.

## 2.3 Bioassay

The rice lamina inclination bioassay was carried out to guide the isolation and purification of the active compounds from the crude extract, using the rice (*Oryza sativa* L.) cultivar of "RD-7" as described previously [19]. The rice seeds were obtained from the Multiple Cropping Center of Chiang Mai University, Chiang Mai, Thailand.

## 2.4 GC-MS Analysis

The bismethaneboronation was carried out by treatment of the bioactive compounds and authentic samples with 20 µL of pyridine containing of methylboronic acid (2 mgL<sup>-1</sup>) at 70 °C for 30 min; 1 µL was subjected to

GC-MS analysis [20].

## 2.5 Extraction and Purification

The dried pollen (2.76 kg) was extracted with methanol (3 x 4 L), by stirring with mechanical stirrer at room temperature for three days. The methanol extract was evaporated to dryness in *vacuo* below 30 °C. The residue (648.63 g) was partitioned between H<sub>2</sub>O (1.5 L) and CHCl<sub>3</sub> (1.5 L), and the separated aqueous phase was partitioned twice with CHCl<sub>3</sub> (2 x 800 mL). The combined CHCl<sub>3</sub> phase was dried over anh.Na<sub>2</sub>SO<sub>4</sub> and evaporated to dryness. The residue (274.32 g) was divided into portions for qualitative and quantitative analysis. The first portion (137.16 g) was partitioned between n-hexane (1 L) and 80% MeOH (1 L). The n-hexane phase was partitioned twice with 80% MeOH (2x400 mL), and the combined 80% MeOH fractions were concentrated to expel methanol. The resulting aqueous phase from 80% MeOH was added with sat.NaHCO<sub>3</sub> solution (100 mL), then extracted with CHCl<sub>3</sub> (500 mL), and the aqueous phase after separation of CHCl<sub>3</sub> was partitioned twice with CHCl<sub>3</sub> (2x200 mL), and the combined CHCl<sub>3</sub> phase after drying with anh.Na<sub>2</sub>SO<sub>4</sub> was evaporated to dryness. The residue (32.31 g) from the CHCl<sub>3</sub> phase was chromatographed on silica gel column (400 g, Merck). The elution was carried out stepwise with (1 L) 10 fractions of MeOH in CHCl<sub>3</sub> gradients (0, 3, 5, 10, 15, 20, 25, 30, 40 and 50%). The resulting residue (3.09 g) from 10 and 15% MeOH fractions was chromatographed on silica gel column (100 g, Merck). The elution was carried out stepwise with (300 mL) 11 fractions of MeOH in CHCl<sub>3</sub> gradients (0, 2, 3, 4, 5, 7, 10, 15, 20, 30 and 50%). The resulting residue from 5 and 7% MeOH fractions (1.52 g) was subjected to RP-18 column chromatography (bed volume 100mL). The elution was carried out with MeOH in H<sub>2</sub>O to give 11 fractions (0, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100%). The combined bioactive residue (241.4mg) from 80 and 90% MeOH was subjected to

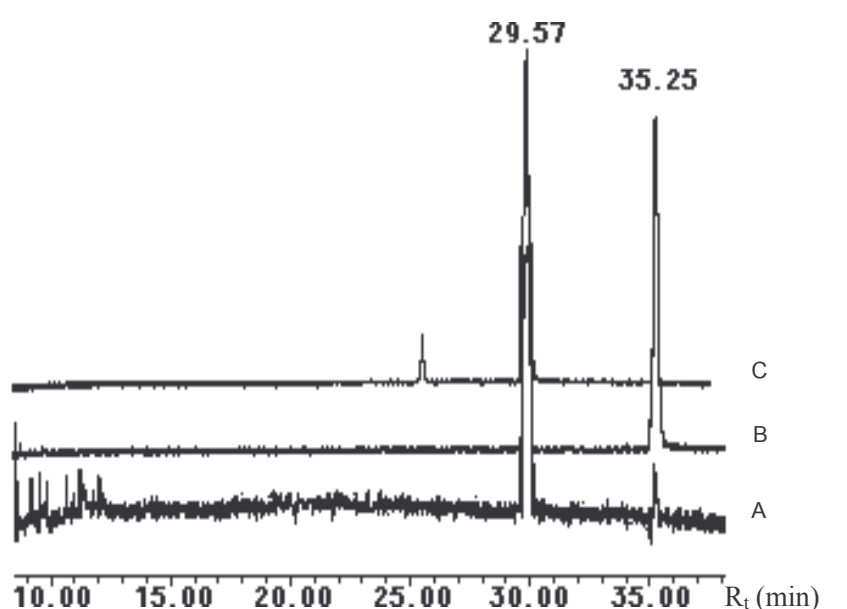
Sephadex LH-20 column (bed volume 200 mL). The elution was carried out with 25% MeOH in  $\text{CHCl}_3$  with flow rate  $0.5 \text{ mL min}^{-1}$  to give a biologically active fraction ( $V_e/V_t$  0.65-0.75). After evaporation, the residue (13.52 mg) was charged onto RP-18 cartridges (1 g) and eluted with 80% MeOH- $\text{H}_2\text{O}$ . The bioactive residue (2.61 mg) was obtained, the aliquot of which was derivatized with methylboronic acid, by treatment with 20  $\mu\text{L}$  pyridine containing methylboronic acid ( $2 \text{ mg mL}^{-1}$ ) at  $70^\circ\text{C}$  for 30 min; 1  $\mu\text{L}$  was subjected to GC-MS analysis.

The purification of the second portion (137.16g) was carried out similarly as described. The resulting purified sample was subjected to quantitative analysis performed by GC-MS compared with authentic brassinolide and castasterone (as bismethaneboronate derivatives).

### 3. RESULTS AND DISCUSSION

The dried pollen of pumpkin was extracted with methanol. After evaporation to dryness, the residue was partitioned between water and chloroform. Only the residual chloroform phase showed biological

activity by rice lamina inclination test after evaporation under reduced pressure to dryness. The residue resulting from the chloroform phase was partitioned between n-hexane and 80% methanol. The n-hexane phase did not show biological activity and 80% methanol fraction was evaporated to concentrate. The remaining aqueous phase after adding saturated sodium hydrogen carbonate solution was extracted with chloroform. The active residue from the chloroform phase was chromatographed on silica gel column, eluting with methanol in chloroform gradients. The combined highly bioactive residue from the elution with 5 and 7% methanol in chloroform was purified by silica gel column, RP-18 column, Sephadex LH-20 column chromatographies and finally with RP-18 cartridges. The bioactive residue was derivatized with methylboronic acid and analyzed by GC-MS. Based on the GC retention time (Figure 1) and mass spectra (Table 1. and Figures 2, 4) of the bismethaneboronate (BMB) of the bioactive compound I ( $R_t=29.57 \text{ min}$ ) it was shown to be identical to authentic castasterone bismethaneboronate (BMB-CS) under the

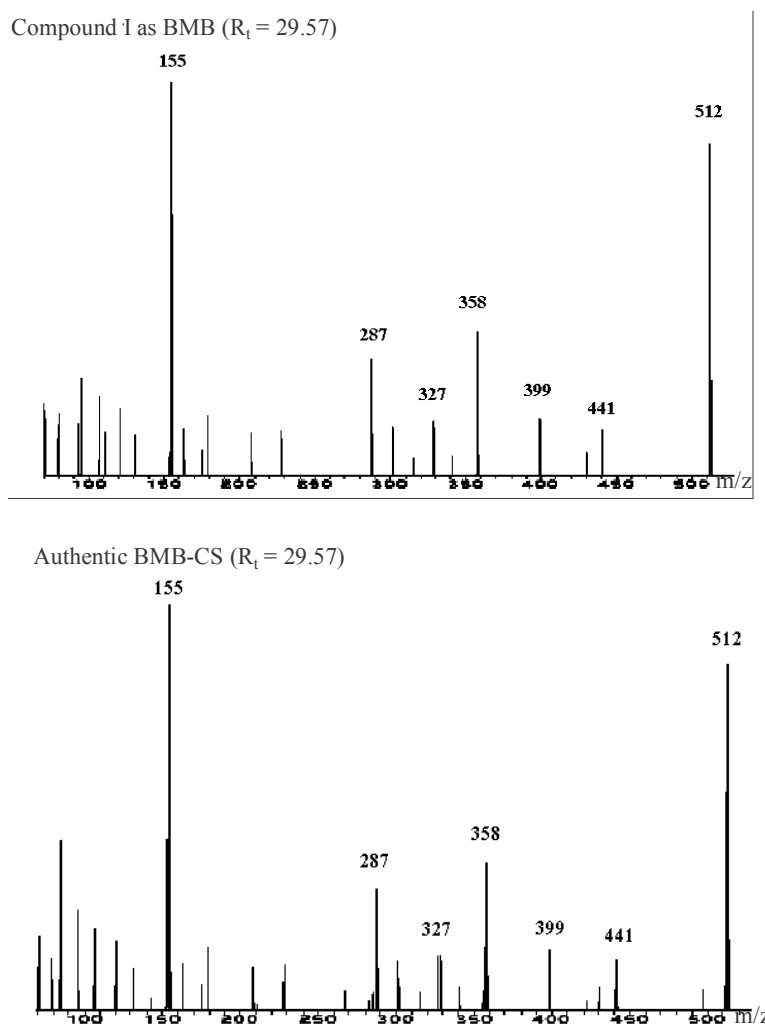


**Figure 1.** Chromatogram of the bioactive compounds from the extract of pollen of pumpkin as BMB (A), authentic BMB-BL (B) and authentic BMB-CS (C) analyzed by GC-MS.

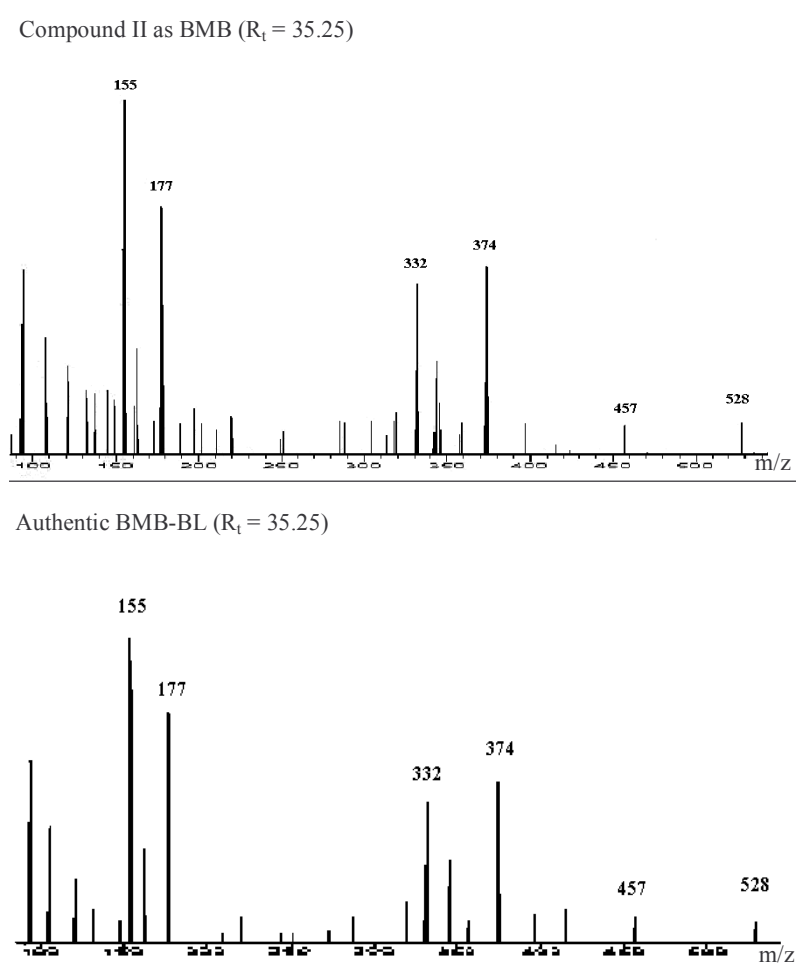
**Table 1.** Characteristic ions of brassinosteroids analyzed by GC-MS

Compounds*	R <sub>t</sub> on GC (min)	Characteristic ions (m/z , relative intensity)
I	29.57	155(100), 287(29), 327(13), 358(36), 399(14), 441(11), 512(84)
II	35.25	155(100), 163(38), 177(70), 332(49), 374(53), 457(8), 528(9)
Brassinolide	35.25	155(100), 163(33), 177(76), 332(46), 374(52), 457(8), 528(7)
Castasterone	29.57	155(100), 287(34), 327(10), 358(35), 399(13), 441(9), 512(78)

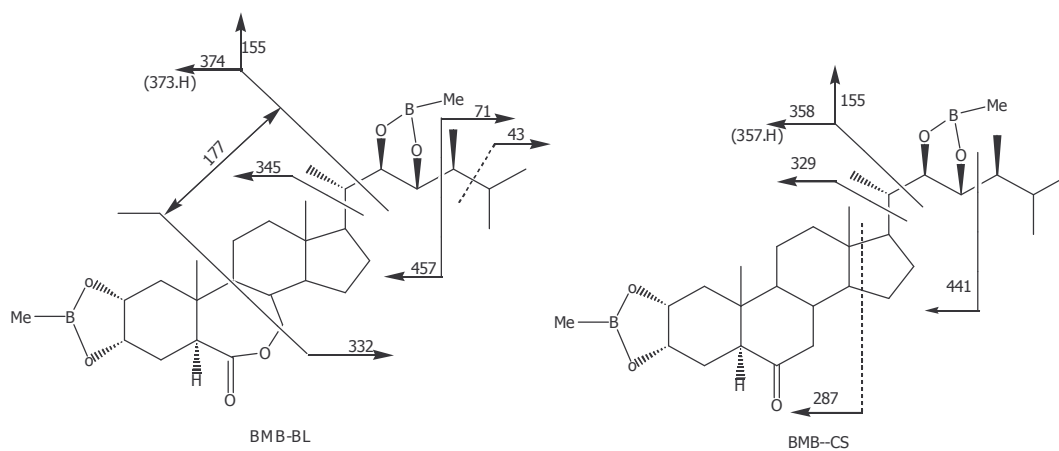
\* The compounds as BMB were analyzed by full scan GC-MS



**Figure 2.** Mass spectra of the bioactive compound I from the extract of pollen of pumpkin as BMB and authentic BMB-CS.



**Figure 3.** Mass spectra of the bioactive compound II from the extract of pollen of pumpkin as BMB and authentic BMB-BL.



**Figure 4.** The MS fragmentation pattern of BMB-BL and BMB-CS [20].

same condition. Thus, the active compound I was established to be CS. The BMB of active compound II ( $R_t=35.25$  min) (Figure1) showed the characteristic ions for brassinolide bismethaneboronate (BMB-BL) at  $m/z$  528( $M^+$ ), 457, 374, 332, 177, 163 and 155. This is consistent with the retention time and mass spectra fingerprint (Table1 and Figures 3, 4) shown by BL. Their approximate amounts were estimated based on the GC-MS data to be 112 and 36  $\mu\text{g kg}^{-1}$  (dried weight) for CS and BL respectively. Thus, to conclude, the partially purified extract from pollen of pumpkin was found to have a high content of BRs and it showed high biological activity, so it could possibly be used as an inexpensive source of these natural plant hormones for agriculture.

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#### REFERENCES

- [1] Clouse S.D., and Sasse J.M., Brassinosteroids: essential regulations of plant growth and development, *Ann. Rev. Plant Physiol., Plant Mol. Biol.*, 1988; **49**:427-451.
- [2] Mandava N.B., Plant growth-promoting brassinosteroids, *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 1988; **39**:23-52.
- [3] Mussig C., and Altmann T., Brassinosteroid signaling in plants, *Trends Endocrinol. Metab.*, 2001; **12**:398-402.
- [4] Yokota T., Chemical structure of plant hormones, *Chem. Regul. Plants*, 1995; **30**:185-196.
- [5] Grove M.D., Spencer G.F., Rohwedder W.K., Mandava N., Worley J.F., Warthen Jr. J.D., Steffens G.L., and Flippen-Anderson J.L., Cook Jr. J.C., Brassinolide, a plant growth-promoting steroids isolated from *Brassica napus* pollen, *Nature*, 1979; **281**:216-217.
- [6] Mitchell J.W., Mandava N.B., Worley J.F., Plimmer J.R., and Smith M.V., Brassins: a new family of plant hormones from rape pollen, *Nature*, 1970; **225**:1065-1066.
- [7] Friebe A., Volz A., Schmidt J., Voigt B., Adam G., and Schnabl H., 4-Epi-secasterone and 24-epi-castasterone from *Lycchnis viscaria* seeds, *Phytochemistry*, 1999; **52**: 1607-1610.
- [8] Fujioka S., Noguchi T., Yokota T., Takatsuto S., and Yoshida S., Brassinosteroids in *Arabidopsis thaliana*, *Phytochemistry*, 1998; **48**:595-599.
- [9] Yokota T., Arima M., and Takahashi N., Castasterone, a new phytosterol with plant-hormone potency, from chestnut insect gall, *Tetrahedron Lett.*, 1982; **23**: 1275-1278.
- [10] Yokota T., Baba J., and Takahashi N., Brassinolide-related bioactive sterols in *Dolichos lablab*; brassinolide, castasterone and a new analog, homodolicholide, *Agric. Biol. Chem.*, 1983; **47**:1409-1411.
- [11] Abe H., Morishita T., Uchiyama M., Takatsuto S., Ikekawa N., Ikeda M., Sassa T., Kitsuwa T., and Marumo S., Occurrence of three new brassinosteroids: brassinone, 24(S)-24-ethylbrassinone and 28-norbrassinolide in higher plants, *Experientia*, 1983; **39**:351-353.
- [12] Kim Y.S., Sup Y.H., Kim T.W., Joo S.H., and Kim S.K., Identification of a brassinosteroid, castasterone from *Marchantia polymorpha*, *Bull. Korean Chem. Soc.*, 2002; **23**:941-942.
- [13] Takatsuto S., Abe H., and Gamoh K., Evidence for brassinosteroids in strobilus of *Equisetum arvense* L., *Agric. Biol. Chem.*, 1990; **54**:1057-1059.
- [14] Yokota T., Kim S.K., Fukui Y., Takahashi N., Takeuchi Y., and Takematsu T., Brassinosteroids and sterols from a green alga, *Hydrodictyon reticulatum*. configuration at C-24, *Phytochemistry*, 1987; **26**:503-506.
- [15] Bajguz A., and Tretyn A., The chemical

- characteristic and distribution of brassinosteroids in plants, *Phytochemistry*, 2002; **62**:1027-1046.
- [16] Khripach V., Zhabinskii V., and Groot A.D., Twenty Years of Brassinosteroids: Steroidal plant hormones warrant better crops for the XXI century, *Annals of Botany*, 2000; **86**:441-447.
- [17] Schmidt J., Almann T., and Adam G., Brassinosteroids from seeds of *Arabidopsis thaliana*, *Phytochemistry*, 1997; **45**:1325-1327.
- [18] Jang M.S., Han K.S., and Kim S.K., Identification of brassinosteroids and their biosynthetic precursors from seeds of pumpkin, *Bull. Korean Chem. Soc.*, 2000; **21**:161-164.
- [19] Fujii S., and Saka H., The promotive effect of brassinolide on lamina joint-cell elongation., germination and seedling growth under low-temperature stress in rice (*Oryza sativa* L.), *Plant Production Science*, 2001; **4**:209-214.
- [20] Takatsuto S., Brassinosteroids: distribution in plant, bioassays and microanalysis by gas chromatography-mass spectrometry, *J.Chromatogr. A*, 1994; **658**:3-15.