Identification of Brassinolide, Castasterone and Norcastasterone (Brassinone) in Sunflower (*Helianthus annuus* L.) Pollen

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From the pollen of sunflower (*Helianthus annuus* L.), the biologically active substances in the rice lamina inclination test were extracted and partially purified. They were converted to bis-9-phenanthreneboronates and bismethaneboronates, and analyzed by fluorescence HPLC and GC/MS analytical methods, respectively. The active principles were identified as brassinolide, castasterone and norcastasterone (brassinone).

Pollens have been found to be rich sources of the new plant growth promoter, brassinosteroid.¹⁾ Since the discovery of brassinolide from the pollen of rape (*Brassica napus* L.),²⁾ several pollens have been investigated and demonstrated to contain brassinosteroids: *Typha latifolia* L.,³⁾ *Pinus thunbergii* Parl.,⁴⁾ *Zea mays* L.,⁵⁾ *Alnus glutinosa* (L.) Gaertn.⁶⁾ and *Vicia faba* L.⁷⁾ We have developed a GC/MS microanalytical method for brassinosteroids as bismethaneboronate derivatives,⁸⁾ and more recently we have also developed a fluorescence HPLC microanalytical method for them as bis-9-phenanthreneboronate derivatives.⁹⁾ Further interest in the brassinosteroids contained in pollens prompted us to investigate the presence of the steroids in the pollen of sunflower (*Helianthus annuus* L.). In this paper, we report the identification of brassinosteroids in this pollen by the HPLC and GC/MS analytical methods.

The sunflower pollen (2.9 kg) was extracted with methanol and then with ethyl acetate-methanol (1:1, v/v). The combined extracts were concentrated *in vacuo* to give an aqueous residue, which was partitioned between chloroform and water.

The organic phase was concentrated and next partitioned between *n*-hexane and 90% methanol. The aqueous methanol phase was concentrated and further partitioned between chloroform and sat. sodium bicarbonate solution. The chloroform phase showed strong biological activity in a rice lamina inclination test,¹⁰⁾ which was used to guide the further fractionation. The concentrated chloroform fraction was subjected to chromatography on silica gel, eluting step-wise with increasing concentrations of methanol in chloroform. The eluates with 5~10% methanol in chloroform were found to be biologically active (Fig. 1). These fractions were combined, concentrated and further purified by preparative thin-layer chromatography (p-TLC), using chloroform-methanol (10:1, v/v) as the developing solvent. The biological activity appeared in the region of *R_f* 0.2~0.4, which corresponds to the *R_f* values of typical brassinosteroids. These fractions were combined and concentrated to give a partially purified active oil.

An aliquot of this active oil was derivatized with 9-phenanthreneboronic acid. The result-

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Fig. 1. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test after Silica Gel Adsorption Chromatography of the Extract from Sunflower Pollen.

Fig. 2. HPLC Chromatogram of the Bis-9-phenanthreneboronates of Natural Brassinosteroids Obtained from Sunflower Pollen. Peaks: 1=brassinolide; 2=norcastasterone; 3=castasterone.

ing boronates were analyzed by reversed-phase HPLC with acetonitrile-water as the mobile phase, and monitored by a fluorometric detector (Ex 305 nm/Em 375 nm).⁹⁾ The chromatogram is presented in Fig. 2. The peaks with retention times of 8.67, 10.07 and 12.15

Fig. 3. Distribution of Biological Activity Determined by the Rice Lamina Inclination Test after Sephadex LH-20 Column Chromatography of the Partially Purified Active Oil.

Fig. 4. Total Ion Current Trace in GC/MS of the Methaneboronated Highly Purified Fraction Using a DB-1 Fused Silica Capillary Column. Peaks: 1=norcastasterone; 2=castasterone; 3=brassinolide.

min were identical with those of the bis-9-phenanthreneboronates of authentic brassinolide, norcastasterone (brassinone) and castasterone, respectively. The amounts of these brassinosteroids were calculated by calibrating against authentic samples as 106, 65 and 21 $\mu\text{g}/\text{kg}$, respectively.

In order to more precisely identify these brassinosteroids, GC/MS analysis was carried out. The partially purified fraction was further

Fig. 5. Electron Impact Mass Spectra of the Bismethaneboronates of Norcastasterone (A), Castasterone (B) and Brassinolide (C) Obtained from the Highly Purified Fraction.

purified by Sephadex LH-20 column chromatography, using methanol-chloroform as the mobile phase. The biological activity was found to be in the elution volume $0.66 \sim 0.74 V_e/V_t$, corresponding to that of typical brassinosteroids (Fig. 3). These fractions were collected and concentrated to give a highly purified fraction. A small portion of this was converted to bismethaneboronate derivatives⁸⁾ and analyzed by GC/EI/MS, using a capillary column. A total ion chromatogram is presented in Fig. 4. The retention times of 12.13, 13.20 and 15.06 min, and mass spectra (Fig. 5) of the peaks were identical with those of the bismethaneboronates of authentic norcastasterone, castasterone and brassinolide, respectively. Thus, the biologically active brassinosteroids in the sunflower pollen were rigorously identified.

In conclusion, we were able to identify brassinolide, castasterone and norcastasterone

(brassinone) in the pollen of sunflower by fluorescence HPLC and GC/MS analyses, thereby indicating the usefulness of our fluorescence HPLC microanalytical method for brassinosteroids.

Experimental

Plant material. The bee-collected pollen of sunflower (*Helianthus annuus* L.) was obtained from China, and was kindly supplied to us by Nippon Kayaku Co. Ltd. (Tokyo). Identification of the pollen was carried out by a microscopic examination.

Brassinosteroids. Authentic brassinolide, castasterone and norcastasterone (brassinone) were synthesized in our laboratory, according to the reported method.¹¹⁾

Bioassay. The rice lamina inclination test was carried out according to the reported method,¹⁰⁾ using etiolated seedlings of rice (*Oryza sativa* L. cv Koshihikari).

Extraction. The sunflower pollen (2.9 kg) was extracted with MeOH (15 l) for a week and then with AcOEt-

MeOH (1:1, v/v, 10 l) for another week. The combined extracts were concentrated below 30°C *in vacuo* to give an aqueous residue, which was partitioned between chloroform (1 l × 2) and water (1 l). The CHCl₃ phase was concentrated and then partitioned between *n*-hexane (2 l) and 90% MeOH (2 l × 2). The aqueous MeOH phase was concentrated and further partitioned between CHCl₃ (1 l) and sat. NaHCO₃ solution (300 ml × 2). The CHCl₃ phase was dried over anhydrous MgSO₄, filtrated and concentrated to give a biologically active oil (42.5 g). The hexane phase was concentrated to give an oily material (108.7 g).

Silica gel adsorption chromatography. The active oil (42.5 g) was charged onto a column (3.5 cm i.d. × 35 cm) of SiO₂ (E. Merck, Kieselgel 60, 70~230 mesh). Elution was carried out step-wise with CHCl₃ (600 ml) and then with CHCl₃-MeOH (98:2, 600 ml; 95:5, 600 ml; 90:10, 600 ml; 85:15, 600 ml; 80:20, 600 ml). The fractions were collected in 300 ml quantities, and an aliquot (2 ml) of each fraction was subjected to the bioassay. The biological activity appeared in the 5~10% MeOH in CHCl₃ eluates (Fig. 1), and these fractions were combined and concentrated *in vacuo* to give an active oil (1.57 g).

Preparative thin-layer chromatography. The active oil (1.57 g) was applied to precoated silica gel plates (E. Merck, Kieselgel 60 F₂₅₄, 20 × 20 cm², 0.5 mm thickness), which were developed with CHCl₃-MeOH (10:1, v/v). The silica gel plates were scrapped off into ten bands, each being eluted with CHCl₃-MeOH (15:1, v/v), and the eluates were adjusted to 100 ml each fraction. An aliquot (0.1 ml) of each fraction was subjected to the bioassay. Strong activity appeared in the region of *R_f* 0.2~0.4, and these fractions were combined and concentrated *in vacuo* to give a partially purified fraction (210 mg), which was analyzed by HPLC.

HPLC analysis. To a small portion (1.05 mg) of the partially purified fraction was added 100 µl of a solution of 9-phenanthreneboronic acid (1 mg/ml) in 1% (v/v) pyridine/CH₃CN. The mixture was heated at 70°C for 20 min, and after cooling to room temperature, the solution was diluted with CH₃CN to 1 ml. One microliter of the solution was directly injected into the analytical column.

A Shimadzu Model LC-6A chromatograph equipped with a fluorometric detector (RF-530, Ex 305 nm/Em 375 nm) and a CR-3A data system was employed. An STR ODS-H reversed phase column (4.0 mm i.d. × 15 cm, Shimadzu Techno Research) was used at 45°C. Samples were injected into the column using a Rheodyne rotary valve 7125 syringe-loading injector. The mobile phase was a mixture of CH₃CN-H₂O (90:10, v/v) with a flow rate of 0.8 ml/min.

Sephadex LH-20 column chromatography. The partially

purified fraction (54 mg) obtained from p-TLC was charged onto a Sephadex LH-20 column (26.6 mm i.d. × 90 cm) and eluted with a mixture of CHCl₃-MeOH (1:4, v/v) at a flow rate of 25 ml/hr. Successive 10 ml fractions were collected, and an aliquot (20 µl) of each fraction was subjected to the bioassay. The biological activity was detected in the elution volume of 0.66~0.74 *Ve/Vt* (Fig. 3), which was combined and concentrated to give a highly purified fraction (9.0 mg) for analysis by GC/MS.

GC/MS analysis. Bismethaneboronation was carried out by treating a sample (0.9 mg) with 50 µl of a solution of methaneboronic acid in pyridine (2 mg/ml) at 70°C for 30 min.⁸⁾ One microliter of the solution was injected into the capillary column. The GC/MS analysis was carried out on a JEOL DX-303 gas chromatograph-mass spectrometer under the following conditions: ionization, EI (70 eV); ionization chamber temp., 180°C; column, DB-1 fused silica capillary (0.25 mm × 15 m, 0.25 µm film thickness); column temp., 170°C for 2 min, elevated to 275°C at 32°C/min and then elevated to 290°C at 2°C/min; carrier gas, He at 1.1 kg/cm²; splitless mode.

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