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Note

High-performance liquid chromatography of brassinosteroids in plants with derivatization using 9-phenanthreneboronic acid

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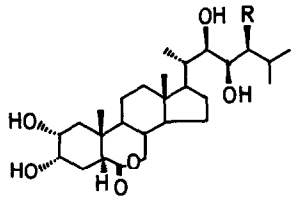
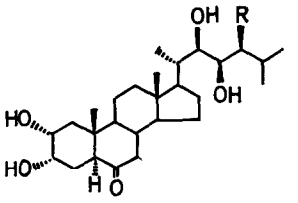
Since the discovery of brassinolide (**1**) as a new plant growth hormonal steroid[1], a number of related steroids (general term brassinosteroids) have been found to occur in a wide variety of higher plants[2]. The structures of some brassinosteroids are shown in Fig. 1. In a previous paper[3], we described the microanalysis of brassinosteroids using a labelling technique with 1-naphthaleneboronic acid. Although the boronic acid was very useful as a labelling reagent for brassinosteroids that have two vicinial diol groups in both the A ring and the side-chain, the resulting naphthaleneboronates were detected with ultraviolet absorption at 280 nm. As a continuation of our research on microanalytical methods for brassinosteroids, we have screened a number of derivatizing reagents in the hope of developing a more sensitive and selective analytical method.

In this paper, we report the determination of brassinosteroids as bis-9-phenanthreneboronate derivatives by high-performance liquid chromatography (HPLC) with fluorimetric detection and its application to the identification of brassinosteroids in plant extracts.



R = CH_3 : Brassinolide(1)
C_2H_5: Homobrassinolide(2)
2H : Norbrassinolide(3)



R = CH_3 : Castasterone(4)
C_2H_5: Homocastasterone(5)
2H : Norcastasterone(6)
$=CH_2$: Dolichosterone(7)

Fig. 1. Structure of brassinosteroids.

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EXPERIMENTAL

Plant material

The bee-collected pollen of broad bean (*Vicia faba* L.) was obtained from China and was kindly supplied by Nippon Kayaku (Tokyo, Japan). Identification of the pollen was carried out by microscopic examination.

Rice-lamina inclination test

The procedure for the purification of brassinosteroids was based on the rice-lamina inclination test. The bioassay was carried out according to our previous method[4] using etiolated seedlings of rice (*Oryza sativa* L. cv. Koshihikari).

Chemicals

Authentic brassinosteroids, brassinolide (**1**), 28-homobrassinolide (**2**), 28-norbrassinolide (**3**), castasterone (**4**), 28-homocastasterone (**5**), 28-norcastasterone (**6**) and dolichosterone (**7**) were synthesized in our laboratory[5–7]. 9-Phenanthreneboronic acid (PBA) was purchased from Kanto Chemical (Tokyo, Japan). All other reagents were of analytical-reagent grade.

Extraction of brassinosteroids

The broad bean pollen (25 g) was extracted with methanol (200 ml) for 1 week and then ethyl acetate–methanol (1:1, v/v) (200 ml) for 1 week. The combined extracts were concentrated below 30°C *in vacuo* and then partitioned between ethyl acetate (100 ml, twice) and water (100 ml). The organic layer was concentrated and further partitioned between *n*-hexane (100 ml) and methanol–water (9:1, v/v) (100 ml, twice). The aqueous methanol layer was concentrated and then partitioned between ethyl acetate (100 ml) and saturated sodium hydrogencarbonate solution (100 ml). The organic layer was dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to give a biologically active oil (240 mg).

Silica gel adsorption chromatography

The active oil (240 mg) was charged on to a column (200 mm × 10 mm I.D.) of silica gel (Merk Kieselgel 60, 70–230 mesh). Elution was carried out stepwise with chloroform (50 ml) and then chloroform–methanol (98:2, 50 ml; 95:5, 50 ml; 90:10, 50 ml; 85:15, 50 ml; 80:20, 50 ml). The eluates were collected in 25-ml fractions. One hundredth of each fraction was subjected to the bioassay. Biological activity appeared in the 5–10% methanol in chloroform eluates and these fractions were combined and concentrated *in vacuo* to give the active fraction (31.3 mg).

Preparative thin-layer chromatography

The active fraction (31.3 mg) was applied to a precoated silica gel plate (Merck Kieselgel 60 F_{254} precoated plate, 20 × 20 cm^2, 0.25 mm film thickness). The plate was developed with chloroform–methanol (10:1). Silica gel was scraped off into ten bands and each was eluted with ethyl acetate. One hundredth of each fraction was subjected to the bioassay. The 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 (2.8 mg). This active fraction was estimated to contain several micrograms of

brassinosteroids by the rice-lamina inclination test using brassinolide as a reference compound.

Derivatization

The standard mixture of the biologically active brassinosteroid fraction was dissolved in a small amount of acetonitrile (100 μl). To the solution, 100 μl of 9-phenanthreneboronic acid[8] (1 mg/ml) in 1% (v/v) pyridine–acetonitrile was added. The mixture was heated at 70°C for 10 min. After cooling, several microlitres of the solution were injected directly into the analytical column (Fig. 2). The electron-impact (EI) mass spectrum of the bisphenanthreneboronate of brassinolide afforded a molecular ion at *m/z* 852 (Shimadzu GCMS-QP 1000A direct inlet system with EI ionization source; data not shown).

HPLC analysis

A Shimadzu Model LC-6A chromatograph equipped with a fluorimetric detector (Shimadzu Model RF-535) was employed (Excitation 305 nm, Emission 375 nm). A reversed-phase column of STR ODS-H (150 mm × 4.0 mm I.D.) (Shimadzu Techno Research, Kyoto, Japan) was used at 45°C. Samples were injected into the column using a Rheodyne rotary valve 7125 syringe-loading injector. The optimum mobile phase for the separation of the brassinosteroid bisphenanthreneboronates was acetonitrile–water (90:10) at a flow-rate of 0.8 ml/min.

RESULTS AND DISCUSSION

HPLC analysis

For the separation of the brassinosteroid bisphenanthreneboronates, an STR ODS-H column was found to afford better resolution than several conventional ODS columns tested. We examined the detection limit of these brassinosteroid derivatives using the ODS column and acetonitrile–water as the mobile phase.

As shown in Fig. 3, the bisphenanthreneboronates of norbrassinolide (**3**), brassinolide (**1**), dolichosterone (**7**), norcastasterone (**6**), homobrassinolide (**2**), castasterone (**4**) and homocastasterone (**5**) afforded sharp peaks at 7.2, 8.6, 9.1, 10.0, 10.6, 12.1 and 15.1 min, respectively. The amounts of these brassinosteroids were 200 pg for **5**, 150 pg for **3**, **6** and **4**, 120 pg for **1** and **7** and 75 pg for **2**. The method presented using 9-phenathreneboronic acid gave a detection limit for brassinolide 50 pg (*ca.* 500

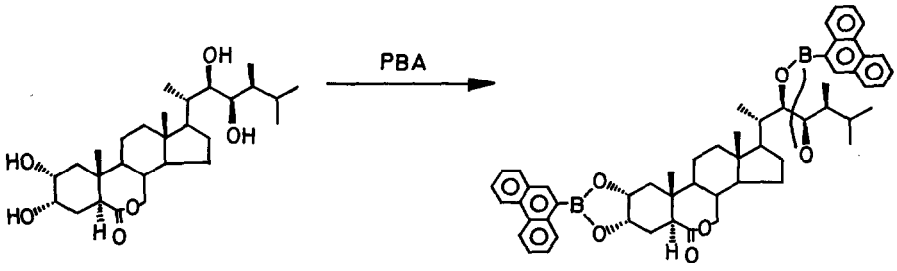

Fig. 2. Reaction of brassinolide with 9-phenanthreneboronic acid (PBA).

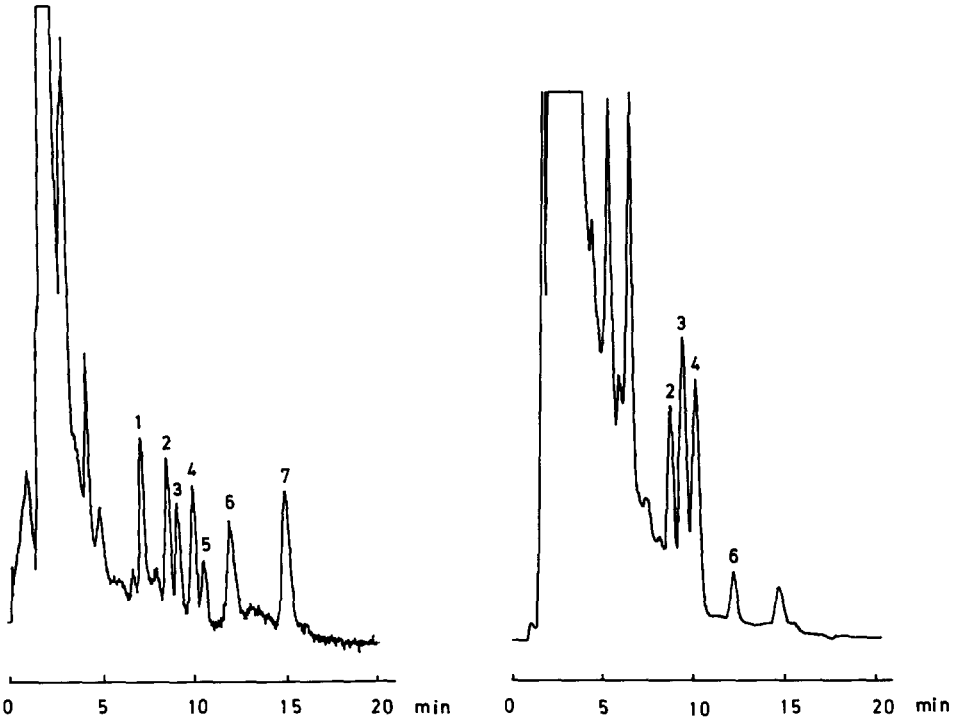

Fig. 3. Chromatogram of bisphenanthreneboronates of authentic brassinosteroids. Peaks: 1 = norbrassinolide (**3**); 2 = brassinolide (**1**); 3 = dolichosterone (**7**); 4 = norcastasterone (**6**); 5 = homobrassinolide (**2**); 6 = castasterone (**4**); 7 = homocastasterone (**5**). Conditions: STR ODS-H (5 μm) column, 15 cm × 4.0 mm I.D.; mobile phase, acetonitrile–water (9:1); flow-rate, 0.8 ml/min; temperature, 45°C.

Fig. 4. Chromatogram of bisphenanthreneboronates of natural brassinosteroids extracted from the pollen of broad bean. Peaks: 2 = brassinolide (**1**); 3 = dolichosterone (**7**); 4 = norcastasterone (**6**); 6 = castasterone (**4**). Conditions as in Fig. 3.

fmol) per injection with a signal-to-noise ratio of 3. A five-fold increase in detectability was observed when the fluorescence of the phenanthreneboronates was compared with UV absorption detection of the naphthaleneboronates.

Application

The method was applied to the identification and determination of brassinosteroids in the bee-collected pollen of the broad bean (*Vicia faba* L.). Although brassinosteroids have previously been reported in the pollen[9], we examined independently the same pollen in this present study. The biologically active fraction (280 μg) obtained from the pollen was derivatized with 9-phenanthreneboronic acid as described above and analysed by HPLC. As shown in Fig. 4, brassinolide (**1**), dolichosterone (**7**), norcastasterone (**6**) and castasterone (**4**) were determined. The amounts of these brassinosteroids were calculated by use of authentic samples for calibration and the results were summarized in Table I. The amount of brassinolide calculated was in good agreement with that reported using gas chromatography–mass spectrometry[9].

TABLE I

AMOUNTS OF BRASSINOSTEROIDS IN THE POLLEN OF BROAD BEEN DETERMINED BY HPLC

Brassionosteroid	*Amount (ng/g)*[a]
Brassinolide (**1**)	180.8 ± 3.1
Dolichosterone (**7**)	536.5 ± 4.5
Norcastasterone (**6**)	628.4 ± 3.6
Castasterone (**4**)	134.4 ± 2.2

[a] Mean ± S.D. ($n = 4$).

Hence it is possible to determine the other brassinosteroids in the pollen by the proposed HPLC method.

A recovery test was carried out by adding a mixture of 4 ng of brassinolide and 6 ng of castasterone to the divided extraction fraction. The samples were derivatized as described above and analysed by HPLC. The added compounds were recovered at a rate of more than 92% ($n = 4$, coefficient of variation = 2.2%).

In conclusion, we have developed an HPLC method with fluorescence detection for the determination of brassinosteroids and demonstrated its usefulness in the identification of several brassinosteroids in broad bean pollen. As the bisphenanthreneboronate was found to be a sensitive and suitable derivative, simultaneous identification and determination of brassinosteroids in plant extracts could be performed by this HPLC method.

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