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Identification of brassinolide and castasterone in the pollen of orange (*Citrus sinensis* Osbeck) by high-performance liquid chromatography

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ABSTRACT

Brassinosteroids contained in the pollen of *Citrus sinensis* Osbeck were investigated. By using a rice lamina inclination test, a highly sensitive and specific bioassay for brassinosteroids, as a guide for purification, the pollen was extracted and the extract was subjected to solvent partitioning and subsequent purification by three chromatographic procedures. Two highly purified bioactive fractions were obtained and they were reacted with dansylaminophenylboronic acid. Each derivatized fraction was then analysed by reversed-phase high-performance liquid chromatography with fluorimetric detection. The bioactive compounds contained in the fraction were identified as brassinolide and castasterone by co-chromatography using authentic samples.

INTRODUCTION

Brassinosteroids (BRs) are a new class of plant growth hormone [1,2]. To date more than 30 natural BRs have been chemically characterized. It has been found that BRs occur in a wide variety of higher plants and also in some lower plants, and that pollens are the richest sources of BRs. In screening investigations, our microanalytical methods of analysing BRs by gas chromatography–mass spectrometry were very effective and they have contributed to a widening of our knowledge of the structural variation and distribution of BRs in the plant kingdom [3,4]. Recently, we have developed microanalytical methods of analysing BRs by high-performance liquid chromatography (HPLC) with fluorescence prelabelling reagents [5–8]. The HPLC method has been successfully applied to the identification of BRs in the pollens of broad bean [5], corn [8], sunflower [9] and buckwheat [10]. Of the prelabelling reagents developed for BRs, dansylaminophenylboronic acid is the most effective, because BR derivatives derived from this reagent can be detected at longer wavelength (excitation 345 nm/emission 515 nm) than those derived from the other fluorescence reagents. Therefore, the chromatogram obtained from the derivatives is subject to less interference from the matrix than those from the other derivatives. The HPLC method is very convenient, highly sensitive and specific for compounds with a vicinal diol function, such as BRs. As part of our research programme into the microanalysis of natural BRs and as an application of our HPLC microanalytical method to the identification of natural BRs, we have now

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investigated the pollen of *Citrus sinensis* Osbeck for the presence of BRs. The results are described in this paper.

EXPERIMENTAL

Plant material and chemicals

The bee-collected pollen of *Citrus sinensis* Osbeck was obtained from Spain and was kindly supplied by Tama Biochemical (Tokyo, Japan). Microscopic identification of the pollen was carried out by Dr. S. Ogawa of Joetsu University of Education. Authentic brassinolide and castasterone were synthesized as described previously [11].

Dansylaminophenylboronic acid was prepared as described in a previous paper [8]. All the other reagents were obtained from Wako (Osaka, Japan) and were of analytical grade.

Bioassay

Chromatographic purification of BRs was guided by the rice lamina inclination test. The bioassay was carried out according to the reported method [12], using etiolated seedlings of rice (*Oryza sativa* L. cv. Koshihikari).

Extraction of brassinosteroids

The pollen (100 g) of *Citrus sinensis* Osbeck was extracted with methanol (500 ml) for a week. The pollen residue was again extracted with methanol (300 ml) for another week. The combined extracts were concentrated *in vacuo* below 30°C to give an aqueous residue, to which was added ethyl acetate (250 ml) and water (250 ml). The mixture was partitioned and the organic phase was collected. The resulting water phase was then extracted twice with ethyl acetate (2 × 100 ml). The combined organic phases were concentrated to give an oil. The oil was dissolved in *n*-hexane (100 ml) and then partitioned with 90% methanol (100 ml). The separated aqueous methanol phase was collected. The remaining hexane phase was partitioned twice more with 90% methanol (2 × 50 ml). The combined aqueous methanol phases were concentrated and subsequently partitioned between chloroform (100 ml) and saturated sodium bicarbonate solution (100 ml). The aqueous phase was twice extracted with chloroform (2 × 50 ml). The combined chloroform phases were dried over anhydrous magnesium sulphate, filtered and concentrated *in vacuo* to give an oily material (680 mg), which showed significant biological response in the bioassay. No other fractions were found to be biologically active.

Silica gel adsorption chromatography

One-half of the active oily material was charged onto a column (30 cm × 3.0 cm I.D.) of silica gel (E. Merck, Kieselgel 60, 70–230 mesh, Art. 7734). Elution was carried out stepwise with chloroform (200 ml) and then with chloroform–methanol (98:2, v/v, 200 ml; 95:5, 200 ml; 90:10, 200 ml; 85:15, 200 ml; 80:20, 200 ml; 70:30, 200 ml). The fractions were collected in 100-ml quantities, and an aliquot (1.0 ml) of each fraction was subjected to the bioassay. Biological activity appeared in the 10–15% methanol in chloroform eluates, and these fractions were combined and concentrated *in vacuo* to give an active fraction (50 mg).

Preparative thin-layer chromatography

The active fraction (50 mg) was applied to two precoated plates of reversed-phase silica gel (E. Merck, Kieselgel $60F_{254}$, 20 cm × 20 cm, 0.25-mm film thickness, Art. 5746). The plate was developed with methanol–water (3:1, v/v) as the developing solvent. The plate was dried and silica gel was scraped off into seven bands, and each was eluted with chloroform–methanol (15:1, v/v). Each eluate was adjusted to 50-ml quantities and one-hundredth of each fraction was subjected to the bioassay. Activity appeared in the regions of $R_F = 0.49$–0.62, this R_F value being in good agreement with those of authentic brassinolide and castasterone by co-chromatography. These fractions were combined and concentrated to give an active fraction (1.8 mg). This was then applied to a precoated plate of normal-phase silica gel (E. Merck, Kieselgel $60F_{254}$, 20 cm × 10 cm, 0.25-mm film thickness, Art. 5744). The plate was developed with chloroform–methanol (15:1, v/v) as the developing solvent. The plate was dried and silica gel was scraped off into ten bands, and each was eluted with chloroform–methanol (15:1, v/v). Each

eluate was adjusted to 50-ml quantities and one-hundredth of each fraction was subjected to the bioassay. Strong and weak activities appeared in the regions of $R_F = 0.20$–0.28 and $R_F = 0.28$–0.35, respectively, these R_F values being in good agreement with those of authentic brassinolide and castasterone, respectively, by co-chromatography. Each fraction was concentrated to give two highly purified bioactive fractions, fractions I (0.7 mg) and II (0.8 mg).

Derivatization procedure

As described previously [8], one-fifth of the active fractions I and II was derivatized with 100 μl of a solution of dansylaminophenylboronic acid (1.0 mg/ml) in pyridine–acetonitrile (1:99, v/v) at 70°C for 20 min. After cooling, an aliquot of the resulting solution was injected into the analytical column.

HPLC analysis

Our previous method [8] was slightly modified in terms of analytical column and the flow-rate of a mobile phase. A Shimadzu Model LC-6A chromatograph equipped with a fluorimetric detector (Shimadzu Model RF-530) was employed (excitation 345 nm, emission 515 nm). A reversed-phase column of STR ODS-H (150 mm × 4.6 mm I.D.) (Shimadzu Techno Research, Kyoto, Japan) was used at 45°C. Samples were injected into the column using a Rheodyne 7125 rotary valve syringe-loading injector. The mobile phase was acetonitrile–water (80:20, v/v) at a flow-rate of 0.8 ml/min.

RESULTS AND DISCUSSION

The pollen of *Citrus sinensis* Osbeck was extracted with methanol and the extract was concentrated *in vacuo* below 30°C to give an aqueous residue. The residue was extracted with chloroform and concentrated to give an oil. The oil was partitioned between *n*-hexane and 90% methanol. The aqueous methanol phase was concentrated and further partitioned between chloroform and a saturated sodium bicarbonate solution. In a rice lamina inclination test, which is highly sensitive and specific to BRs, biological activity appeared only in the chloroform phase. The bioassay was used to guide the subsequent fractionation at each purification step. The bioactive phase was concentrated and then chromatographed on silica gel, eluting stepwise with increasing concentrations of methanol in chloroform. The eluates with 10–15% methanol in chloroform were found to be biologically active. These fractions were combined and concentrated, and further purified by reversed-phase preparative thin-layer chromatography (p-TLC), using methanol–water (3:1, v/v) as the developing solvent. Biological activity appeared in the region of $R_F = 0.49$–0.62, which corresponded to the R_F values of typical brassinosteroids (brassinolide and castasterone). These fractions were combined and concentrated to give a bioactive fraction. This fraction was then purified by normal-phase p-TLC, using chloroform–methanol (15:1, v/v) as the developing solvent. Strong and weak biological activities were found to be in the regions of $R_F = 0.20$–0.28 and $R_F = 0.28$–0.35, respectively, and these R_F values were in good agreement with those of authentic brassinolide and castasterone, respectively. Each fraction was concentrated to give two bioactive fractions, fractions I and II.

One-fifth of each bioactive fraction I and II was derivatized with dansylaminophenyl boronic acid and the resulting boronate derivatives were analysed by reversed-phase HPLC with a fluorimetric detection of excitation 345 nm/emission 515 nm, as previously described [8]. Under our HPLC conditions, the relationships between the peak areas and the amounts of the boronate derivatives of authentic brassinolide and castasterone were linear from 50 pg to 50 ng. The precision was established by repeated determinations ($n = 8$) and the relative standard deviations (R.S.D.) were 2.2 and 2.7%, respectively. As shown in Fig. 1a and b, peaks 1 and 2 were identified as those of the brassinolide and castasterone derivatives, respectively, by co-chromatography using the corresponding authentic dansylaminophenylboronate derivatives. The amounts of brassinolide and castasterone in the bioactive fractions were calculated by use of authentic samples for calibration and they were 36.2 ± 1.0 and 29.4 ± 0.7 μg per kg of pollen

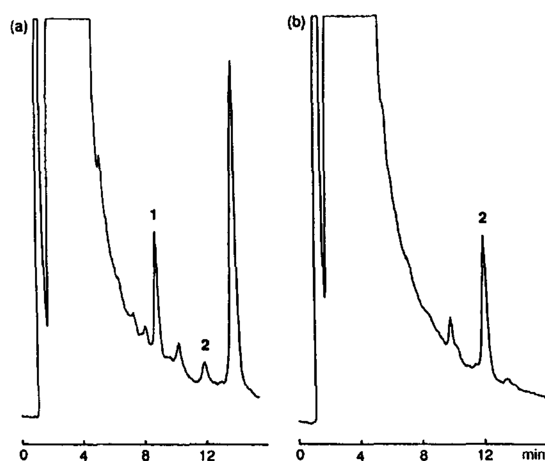

Fig. 1. Chromatograms of dansylaminophenylboronates of brassinosteroids, (a) bioactive fraction I; (b) bioactive fraction II, which were obtained from the pollen of *Citrus sinensis* Osbeck. Peaks: 1 = brassinolide, 2 = castasterone.

($n = 4$, mean ± S.D.), respectively. A recovery test was carried out by adding a mixture of 4 ng of brassinolide and 5 ng of castasterone to the divided bioactive fraction. The samples were derivatized as described above and analysed by HPLC. The recovery of the added steroids was more than 94% ($n = 4$; R.S.D. = 2.9%). It is evident from these data that the HPLC method is satisfactory in both accuracy and precision and that brassinolide and castasterone (Fig. 2) are unequivocally identified in the pollen of *Citrus sinensis* Osbeck.

With respect to the clean-up of the bioactive fraction, it was found that purification by reversed-phase p-TLC of bioactive fraction obtained from column chromatography on silica gel was very effective. The fraction obtained from

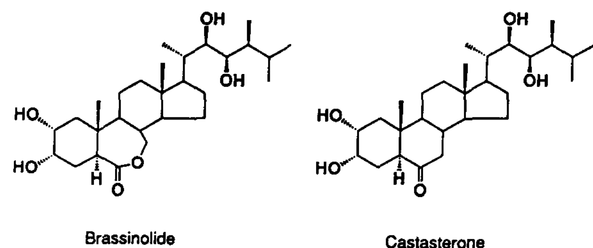

Fig. 2. Structures of brassinosteroids identified in the pollen of *Citrus sinensis* Osbeck.

the reversed-phase p-TLC seems to be sufficiently highly purified to be subjected to HPLC analysis, because it was found that an efficient purification was not attained by the subsequent purification by normal-phase p-TLC. Thus, for the small scale purification of BRs from natural sources, we recommend the use of the reversed-phase p-TLC, subsequent to column chromatography on silica gel.

In conclusion, we were able to identify brassinolide and castasterone in the pollen of *Citrus sinensis* Osbeck by an HPLC analytical method using dansylaminophenylboronic acid as a prelabelling reagent. From the chemotaxonomical points of view, the present work is the first report of identification of brassinosteroids in *Rutaceae*. In addition to our previous works, this report describes a further demonstration that our HPLC method is convenient, highly sensitive, specific and effective for determining trace amounts of naturally occurring BRs.

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