Note

Evidence for Brassinosteroids in Strobilus of *Equisetum arvense* L.Suguru TAKATSUTO,[†] Hiroki ABE
and Keiji GAMOH*Department of Chemistry, Joetsu University of Education,
Joetsu-shi, Niigata 943, Japan* Faculty of Education, Kochi University,
Akebono-cho, Kochi-shi 780, Japan

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Since the discovery of brassinolide as the first plant growth hormonal steroid, a number of its related compounds, brassinosteroids, have been found to occur in substances from angiosperms (monocots and dicots) to gymnosperms, suggesting their wide distribution in phanerogams.^{1,2)} However, in cryptogams, only one report of the occurrence of brassinosteroids in green alga, *Hydrodictyon reticulatum*, has been published.³⁾ As part of our continuing work on brassinosteroids in the plant kingdom, and particularly on cryptogams, we have now investigated their presence in pteridophytes. In this paper, we report the evidence for brassinosteroids in strobilus of *Equisetum arvense* L., which was obtained by both a rice lamina inclination test⁴⁾ and by our newly developed HPLC microanalytical method.^{5,6)}

From the strobilus of *Equisetum arvense* L., a chloroform-soluble neutral fraction was obtained and chromatographed on silica gel. The eluates with 5-15% MeOH in CHCl₃, which were expected to contain brassi-

nosteroids,^{5,6)} showed slight inhibitory activity in the rice lamina inclination test, a highly sensitive and specific bioassay for brassinosteroids.⁴⁾ Thus, in order to examine the presence of the steroids, further purification of these fractions was carried out. The concentrated oil was fractionated by normal-phase preparative thin-layer chromatography (*p*-TLC) and then by reversed-phase *p*-TLC to give a partially purified bioactive fraction, the *R_f* values of which corresponded to those of typical brassinosteroids (see Experimental section). The biological activity of one-sixth of this fraction in the rice lamina inclination test was roughly estimated to be equivalent to that of a 1-10 ppb solution of brassinolide (Table I). The result strongly

Table I. EFFECT OF THE BIOACTIVE FRACTION FROM *Equisetum arvense* L. IN THE RICE LAMINA INCLINATION TEST

Test solution	Bending angle ^a
Control (H ₂ O)	37.4 ± 3.7 ^b
Bioactive fraction ^c	85.8 ± 3.5
Brassinolide 100 (ppb)	102.5 ± 5.2
10	90.7 ± 4.5
1	81.6 ± 6.2
0.1	63.5 ± 4.1

^a 180°—the internal angle between the lamina and sheath.

^b Mean ± standard error (*n* = 15).

^c 122 g fresh wt. equivalent in 5 ml of water solution.

[†] To whom correspondence should be addressed.

Fig. 1. Chromatogram of the Bis-9-phenanthreneboronates of Natural Brassinosteroids Obtained from *Equisetum arvense* L.

Peaks: 1, norbrassinolide; 2, unknown; 3, dolichosterone; 4, norcastasterone; 5, unknown; 6, castasterone.

suggests the presence of brassinosteroid-like substances in *Equisetum arvense* L.

An aliquot of the active fraction was derivatized with 9-phenanthreneboronic acid and then analyzed by reversed-phase HPLC with fluorimetric detection (*Ex.* 305 nm/*Em.* 375 nm) using CH₃CN-H₂O (90:10, v/v) as the mobile phase, according to our previous papers.^{5,6)} The chromatogram is presented in Fig. 1. The sharp peaks with retention times of 8.66, 11.06, 11.77 and 14.10 min were tentatively identified as the bis-9-phenanthreneboronates of norbrassinolide, dolichosterone, norcastasterone and castasterone, respectively, by co-chromatography using authentic boronates of the brassinosteroids.⁷⁾ The peaks with retention times of 9.93 and 12.95 min are at present unidentified. The amounts of the identified steroids were calculated by calibrating against authentic samples as 15.2, 74.6, 34.9 and 17.1 ng/100 g of fresh wt., respectively. These values are obviously underestimates because of losses of the steroids during the purification procedure. The extremely small content of the steroids in the fern precludes further rigorous identification by GC/MS at this stage.

In conclusion, we have been able to present the biological and chromatographic evidence for the occurrence of endogenous brassinosteroids in strobilus of *Equisetum arvense* L., which suggests the possibility that brassinosteroids could play an important role in the phyto-physiological phenomena of the fern. The present work is the first report of brassinosteroids in pteridophytes and has widened our knowledge of brassinosteroids in the plant kingdom.

Experimental

Plant material and chemicals. The strobilus of *Equisetum arvense* L. was collected at Akakura in the Joetsu region of Niigata Prefecture during April 1989. The authentic brassinosteroids used in this study were synthesized in our laboratory according to the reported method.⁷⁾ 9-Phenanthreneboronic acid was obtained from Kanto Kagaku Co., Ltd. (Tokyo), all other reagents and solvents used being of reagent grade.

Bioassay. The rice lamina inclination test was carried out according to the reported method,⁴⁾ using etiolated seedlings of rice (*Oryza sativa* L. cv Koshihikari).

Extraction and solvent fractionation. The strobilus (733 g fresh wt.) of *Equisetum arvense* L. was extracted with MeOH (3 l) for one week and then with CHCl₃-MeOH (1:1, v/v, 3 l) for one more week. The combined extracts were concentrated *in vacuo* below 30°C to give an aqueous residue, to which a mixture (1:1, v/v, 800 ml) of hexane and 90% MeOH was added and then partitioned. The hexane phase was further partitioned with 90% MeOH (200 ml × 2). The combined aq. MeOH phases were concentrated and then dissolved in CHCl₃ (300 ml). The

solution was washed with sat. NaHCO₃ solution (200 ml × 2). The CHCl₃ soluble neutral fraction was dried over anhydrous MgSO₄, filtered and concentrated to give an oil (1.25 g).

Purification of the brassinosteroid fraction. The oil (1.25 g) was chromatographed on silica gel (2.0 cm i.d. × 34 cm, E. Merck, Kieselgel 60, 70–230 mesh, Art. 7734), eluting stepwise with increasing concentrations of MeOH in CHCl₃, and fractions were collected in 100 ml quantities as described in our previous papers.^{5,6)} One-hundredth of each fraction was submitted to the bioassay, but no positive response was found in any fraction. The eluates with 5–15% MeOH in CHCl₃ were collected and concentrated to give an oil (0.33 g), which was then purified by normal-phase *p*-TLC (20 × 20 cm, six plates, E. Merck, Kieselgel 60F₂₅₄, Art. 5744, 0.5 mm film thickness) using CHCl₃-MeOH (9:1, v/v) as the developing solvent. The silica gel was scrapped off into ten bands and each was eluted with CHCl₃-MeOH (15:1, v/v). The fractions with *R*_f 0.31–0.50, which correspond to the *R*_f values of typical brassinosteroids, were collected and concentrated to give an oil (37.2 mg). The oil was further purified by reversed-phase *p*-TLC (20 × 20 cm, two plates, E. Merck, Kieselgel 60F₂₅₄, Art. 5746, 0.25 mm film thickness) using MeOH-H₂O (3:1, v/v) as the developing solvent. The silica gel was scrapped off into ten bands and each was eluted with CHCl₃-MeOH (15:1, v/v). One-hundredth of each fraction was submitted to the bioassay, and biological activity appeared in the region of *R*_f 0.40–0.60, which corresponds to the *R*_f values of typical brassinosteroids. These fractions were combined and concentrated to give a partially purified bioactive fraction (6.3 mg).

HPLC analysis. One-sixth of the active fraction was derivatized with 100 µl of 9-phenanthreneboronic acid (1 mg/ml) in a 1% pyridine-CH₃CN solution at 70°C for 20 min. The resulting boronated samples were analyzed by reversed-phase HPLC with fluorimetric detection (*Ex.* 305 nm/*Em.* 375 nm) using an STC ODS-H column (4.6 mm i.d. × 15 cm, Shimadzu Techno Research) at 45°C with a flow rate of 0.8 ml/min of CH₃CN-H₂O (90:10, v/v) as described in our previous papers.^{5,6)}

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