Pergamon

PII: S0031-9422(97)00177-5

*Phytochemistry*, Vol. 45, No. 7, pp. 1325–1327, 1997

0031-9422/97 $17.00+0.00

# BRASSINOSTEROIDS FROM SEEDS OF *ARABIDOPSIS THALIANA*

JÜRGEN SCHMIDT,* THOMAS ALTMANN† and GÜNTER ADAM

Institute of Plant Biochemistry, Weinberg 3, D-06120 Halle/S., Germany; † Max-Planck-Institute of Molecular Plant Physiology, Karl-Liebknecht-Straße 25, D-14476 Golm, Germany

(*Received* 22 *November* 1996)

**Key Word Index**—*Arabidopsis thaliana*; Cruciferae; seeds; brassinosteroids; 24-*epi*-brassinolide; castasterone; gas chromatography-mass spectrometry.

**Abstract**—Extracts of seeds of *Arabidopsis thaliana* (ecotype 24) were analysed for the presence of free and conjugated brassinosteroids. 24-*epi*-Brassinolide (*ca* 220 ng $kg^{-1}$) and castasterone (*ca* 360 ng $kg^{-1}$) could be isolated and unambiguously identified as native brassinosteroids by GC/MS. © 1997 Elsevier Science Ltd. All rights reserved

## INTRODUCTION

Brassinosteroids (BRs) represent a new class of phytohormones [1] with structural similarities to animal steroidal hormones. They have been detected in many higher plants and therefore appear to be of ubiquitous occurrence [2–5]. A wide spectrum of physiological responses are elicited by exogenous application of low doses of these compounds to intact plants or to explants, whereas stimulation of cell growth is the most prominent effect [6]. Their high biological activity suggested an important role of BRs in the regulation of physiological processes in plants. Recent molecular genetic studies on dwarfed and de-etiolated *Arabidopsis thaliana* mutants provided strong evidence for an essential role of BRs in plant development processes such as cell elongation and etiolation. Feeding studies with BRs indicated a deficiency for BR biosynthesis in the *det*2, *dwf*1–6 (*cbb* 1), and the *cpd* (*cbb* 3) mutants [7–9]. On the other hand, the *cbb*2 and *bri*1 mutants appeared BR-insensitive indicating a defect in the BR-signal perception or transduction [8, 10].

However, no information was hitherto available about the occurrence of BRs in *Arabidopsis thaliana*. As seeds, together with pollen, are generally found to be the richest source of BRs in plants, we have investigated seed material of *Arabidopsis thaliana* wild type with respect to brassinosteroids.

## RESULTS AND DISCUSSION

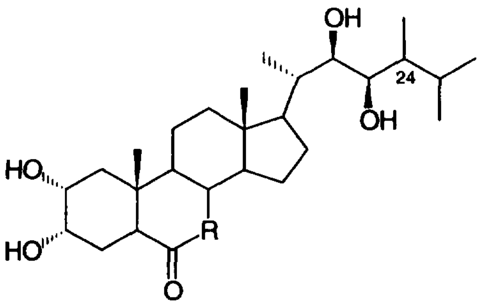
Dry seeds of *A. thaliana* were extracted with methanol and methanol–ethyl acetate. After concentration of the combined extracts *in vacuo* the residue was partitioned between water and chloroform. The water-soluble fraction was tested for the presence of conjugated BRs, while the chloroform extract was further partitioned between *n*-hexane and 80% methanol. Repeated silica gel column chromatography of the concentrated 80% methanol extract using several methanol–chloroform gradient systems led to two fractions eluted with 5 and 7% methanol which showed bioactivity in the rice lamina inclination test. The combined bioactive fractions were further purified by silica gel, DEA and RP-18 cartridges, respectively. The residue was further separated by preparative HPLC and the resulting biologically active fractions were derivatized and analyzed by GC-mass spectrometry.

Two BRs, 24-*epi*-brassinolide (**1**) and castasterone (**4**) were identified from the HPLC-fractions 16 and 20, respectively, by comparison with authentic samples through GC-mass spectrometry (Table 1). Whereas castasterone represents the most widely-distributed brassinosteroid [2–5, 11], 24-*epi*-brassinolide hitherto was only found in pollen of *Vicia faba* [12] and very recently in seeds of *Gypsophila perfoliata* [13]. Interestingly, 24-*epi*-castasterone (**3**) as the expected biosynthetic precursor of 24-*epi*-brassinolide could not be detected and, conversely, brassinolide (**2**) was not observed.

The water-soluble fraction was treated with cellulase to liberate BRs enzymatically from potential sugar conjugates. The aqueous layer was extracted with chloroform and the residue of the chloroform extract was chromatographed on a silica gel column (see Experimental). However, in the collected fractions no activity in the rice lamina inclination test could be detected.

---

* Author to whom correspondence should be addressed



| | R | configuration at C-24 |
|---|---|---|
| **1** | —O—CH₂— | 24*R* |
| **2** | —O—CH₂— | 24*S* |
| **3** | —CH₂— | 24*R* |
| **4** | —CH₂— | 24*S* |

The co-occurrence of 24-*epi*-brassinolide (24*R*-configuration) and castasterone (24*S*-configuration) in *Arabidopsis* seeds, originating probably from the two 24-epimeric sterols campesterol and 22,23-dihydrobrassicasterol, respectively, indicate a parallel BR-biosynthesis in *Arabidopsis* of both the 24*S*- and the 24*R*-series. These two pathways may be separately controlled because in the 24*R*-series the more active member 24-*epi*-brassinolide is formed, while in the 24*S*-series castasterone but not brassinolide is accumulated.

## EXPERIMENTAL

*Gas chromatography-mass spectrometry.* The GC-MS measurements were carried out under the following conditions: MD-800 (Fisons Instruments); EI (70 eV); source temp. 200°; column DB-5MS (J&W, 15 m × 0.32 mm, 0.25 μm film thickness) equipped with a precolumn FS-Phenyl-Sil (3 m × 0.32 mm), injection temp. 260°, column temp. programme for the biomethylboronates of brassinosteroids: 170° for 1 min, then raised to 290° at a rate of 30° $\text{min}^{-1}$ and held on this temperature for 20 min; interface temp. 300°, carrier gas He, flow rate 1 ml $\text{min}^{-1}$, splitless injection.

*Plant material.* The seeds of *Arabidopsis thaliana* L. (ecotype C24) were collected from soil grown plants cultivated under a 16 hr day (9000 lux fluorescent light, 20°)–8 hr night (17°) regime at a relative humidity of 70%. After drying at room temp. and ambient relative humidity, the seeds were stored at 4° for up to 1 year and at −20° for up to 6 years.

*Bioassay.* The rice lamina inclination test was carried out using the cultivar 'Koshihikari' as described previously [14].

*Extraction and purification.* The powdered seeds (541 g) were extracted × 4 with MeOH (1000 ml) and twice with MeOH–EtOAc (1:1, 1 l). The combined extracts were evapd to dryness *in vacuo*. The residue (87.7 g) was partitioned × 3 between $H_2O$ (300 ml) and $CHCl_3$ (300 ml). The residue after evapn of the $CHCl_3$ phase (61.4 g) was partitioned between *n*-hexane (500 ml) and 80% MeOH (500 ml). The *n*-hexane phase was partitioned a second time with 80% MeOH, and the combined 80% MeOH frs were concd (6.21 g). The residue resulting from the 80% MeOH fr. was chromatographed on a silica gel column (30 g). Elution was carried out with $CHCl_3$ (500 ml), $CHCl_3$–MeOH (4:1, 500 ml) and MeOH (500 ml). The eluate with 20% MeOH (972 mg) was subjected to a second silica gel column (10 g). Elution was carried out stepwise with 11 frs (100 ml) of MeOH in $CHCl_3$ (0, 2, 3, 4, 5, 7, 10, 15, 30, 50, 100%). The frs eluted with 5 and 7% MeOH were biologically active and were combined. The residue (108 mg) was further purified by a silica gel cartridge (1 g, VARIAN) eluting with 6 ml MeOH in $CHCl_3$ (2, 10, 50%). The fr. eluted with 10% MeOH (87 mg) was further purified by DEA ion exchange chromatography (1 g, Analyti-

Table 1. GC-MS data of the brassinosteriods from *A. thaliana* seeds

| Brassinosteroid | HPLC fraction | $RR_t$ (GC) | EI-MS (*m*/*z*, rel. int.) | Estimated content (ng $\text{kg}^{-1}$ plant material) |
|---|---|---|---|---|
| 24-*epi*-Brassinolide (**1**)* | 16 | 2.52† | 528 ($M^+$, 2), 457 (3), 415 (6), 374 (23), 345 (17), 332 (22), 177 (65), 155 (100), 85 (87) | *ca* 220 |
| Castasterone (**4**)* | 20 | 1.97† | 512 ($M^+$, 17), 441 (3), 399 (6), 358 (11), 329 (4), 287 (17), 155 (100), 85 (60) | *ca* 360 |

* As bismethylboronate.

† Brassinolide (**2**): 2.39.

‡ 24-*epi*-Castasterone (**3**): 2.06.

chem Bondesil). The residue (50 mg) was charged onto a RP-18-cartridge (1 g, Varian) and eluted with 10 ml 80% MeCN–$H_2O$. The residue (36 mg) was subjected to HPLC (Eurospher 80-C18, column 8 × 250 mm); flow rate, 2 ml $min^{-1}$, mobile phase, MeCN–$H_2O$ (45% MeCN for 40 min, then raised to 80% MeCN within 5 min and hold on 80% MeCN for 25 min, two run), 70 2 ml-frs. The frs with activity ($R_t$ 16 and 20 min) were concd, derivatized and examined by GC-MS.

The bismethylboronation was carried out by treatment of the bioactive frs with 20 $\mu$l pyridine containing methylboronic acid (2 mg $ml^{-1}$) at 70° for 30 min [15]; 1 $\mu$l was subjected to the GC-MS analysis. The relative retention times ($RR_t$ values) were calculated with respect to 5α-cholestane ($R_t = 5.78$ min). The quantitative analysis was performed by GC-SIM comparing the peak areas at $m/z$ 512 in fr. 20 (castasterone) with the peak area at $m/z$ 512 of a 5 ng-injection of 24-*epi*-castasterone as well as $m/z$ 528 in fr. 16 (24-*epi*-brassinolide) with a 10 ng-injection of authentic 24-*epi*-brassinolide.

*Enzymatic cleavage of the water-soluble fraction.* To the $H_2O$-soluble fr. (23.5 g) 150 ml buffer soln (pH 4, 0.1 M citric acid and 0.5 M $Na_2HPO_4 \times 2H_2O$) and 1 g cellulase (Onozuka R10, Japan) were added. After stirring the mix. for 24 hr at 37° the layer was partitioned × 4 with $CHCl_3$. The combined $CHCl_3$-extracts were dried with $Na_2SO_4$, filtered off and dried *in vacuo*. The residue (680 mg) was chromatographed on a silica gel column (7 g) with 100 ml $CHCl_3$ and each 50 ml using a $CHCl_3$–MeOH gradient system (2, 10, 20, 50% MeOH). An aliquot of these frs (5%) was investigated by the rice-lamina-inclination bioassay.

*Acknowledgements*—The authors gratefully acknowledge Mrs Helga Mernzky for technical assistance and Mrs Christine Kuhnt for the GC-MS measurements as well as the Deutsche Forschungsgemeinschaft (DFG) for financial support.

## REFERENCES

1. *Brassinosteroids—Chemistry, Bioactivity, Application*, ed. H. G. Cutler, T. Yokota and G. Adam, ACS Symposium Series 474. American Chemical Society, Washington DC, 1991.
2. Adam, G. and Marquardt, V., *Phytochemistry*, 1986, **25**, 1787.
3. Marquardt, V. and Adam, G., in *Chemistry of Plant Protection*, ed. W. Ebing. Springer, Heidelberg, 1991, p. 103.
4. Sakurai, A. and Fujioka, S., *Plant Growth Regulation*, 1993, **13**, 147.
5. Adam, G., Porzel, A., Schmidt, J., Schneider, B. and Voigt, B., in *Studies in Natural Products Chemistry*, Vol. 18, ed. Atta-ur-Rahman. Elsevier, Amsterdam, 1996, p. 495.
6. Mandava, N. B., *Annual Review of Plant Physiology and Plant Molecular Biology*, 1988, **39**, 23.
7. Li, J., Nagpal, P., Vitart, V., McMorris, T. C. and Chory, J., *Science*, 1996, **272**, 398.
8. Kauschmann, A., Jessop, A., Koncz, C., Szekeres, M., Willmitzer, L. and Altmann, T., *Plant Journal*, 1996, **9**, 701.
9. Szekeres, M., Nemeth, K., Koncz-Kalman, Z., Mathur, J., Kauschmann, A., Altmann, T., Redei, G. P., Nagy, F., Schell, J. and Koncz, C., *Cell*, 1996, **85**, 171.
10. Clouse, S. D., Langford, M. and McMorris, T. C., *Plant Physiology*, 1996, **111**, 671.
11. Yokota, T., Arima, M. and Takahashi, N., *Tetrahedron Letters*, 1982, **23**, 1275.
12. Ikekawa, N., Nishiyama F. and Fujimoto, Y., *Chemical and Pharmaceutical Bulletin*, 1988, **36**, 405.
13. Schmidt, J., Böhme, F. and Adam, G., *Zeitschrift für Naturforschung*, 1996, **57c**, 897.
14. Arima, M., Yokota, T. and Takahashi, N., *Phytochemistry*, 1984, **23**, 1587.
15. Takatsuto, S., Ying, B., Morisaki, M. and Ikekawa, N., *Journal of Chromatography*, 1982, **239**, 233.