ANALYSIS OF UNDERIVATIZED BRASSINOSTEROIDS BY HPLC/APCI-MS. OCCURRENCE OF 3-EPIBRASSINOLIDE IN *Arabidopsis thaliana*

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Received October 5, 2001
Accepted December 2, 2001

Dedicated to the memory of Dr Václav Černý.

In the course of investigations on brassinosteroid (BS) biosynthetic and metabolic pathways in plants, an approach using HPLC/APCI-MS analysis of underivatized BS for their identification in plant material has been developed. Its application to root-callus suspension cultures of *Arabidopsis thaliana* led to the first identification of 3-epibrassinolide as natural brassinosteroid.

Keywords: *Arabidopsis thaliana*; Steroids; Brassinosteroids; Isotopic labelling; HPLC/APCI-MS; Metabolism; Mass spectrometry.

The occurrence of brassinosteroids in plants is usually studied by means of gas chromatography/mass spectrometry (GC/MS) of their volatile cyclic methylboronates using selected ion monitoring[1] (SIM). However, only compounds bearing vicinal hydroxy groups are detectable by this technique. Brassinosteroids with single hydroxy groups in the side chain or ring A, such as cathasterone, teasterone and typhasterol, require trimethylsilylation instead of, or in addition to, boronation. Conformational rigidity of vicinal axial/equatorial hydroxy groups in ring A may also hinder complete boronate ring closure, thereby reducing analytical sensitivity. It was, therefore, desirable to develop microanalytical techniques that did not require derivatization to identify such special brassinosteroids. Owing to the rather polar character of brassinosteroids, direct GC/MS is not useful for this

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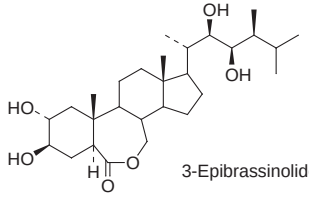
purpose. Gamoh and collaborators introduced reverse-phase liquid chromatography/mass spectrometry (LC/MS) as alternative method, which can be successfully applied to the detection of naphthaleneboronate derivatives of brassinosteroids[2]. In the present investigation, LC/MS coupling was used for the detection of underivatized brassinosteroids. Using this approach, 3-epibrassinolide (**1**) was identified as a natural product in plants for the first time.



3-Epibrassinolide (**1**)

RESULTS AND DISCUSSION

Initially, experiments on LC/MS of cyclic brassinosteroid boronates were carried out. Samples of authentic standards (brassinolide and [26-$^2H_3$]3-epibrassinolide[3]) were derivatized with phenylboronic acid. The phenylboronates were subjected to LC/MS under isocratic chromatographic conditions on a reverse-phase column using acetonitrile–water (90 : 10) as an eluent. Atmospheric pressure chemical ionization ($APCI^+$) was used for LC/MS-SIM detection. Monoboronate derivatives were found exclusively with retention times 12.6 min for 22,23-phenylboronate of [26-$^2H_3$]3-epibrassinolide and 14.4 min for brassinolide. The boronates of brassinolide and [26-$^2H_3$]3-epibrassinolide gave protonated molecular species ($[M + H]^+$) but, in addition, brassinolide gave an acetonitrile adduct ion ($[M + CH_3CN + H]^+$ = *m/z* 608), which was chosen for SIM analysis. Injection of 15 ng brassinolide phenylboronate led to a SIM spectrum with signal-to-noise ratio 22 : 1 (monitoring *m/z* 608); for the same amount of [26-$^2H_3$]3-epibrassinolide phenylboronate, this ratio was 7 : 1 (monitoring $[M + H]^+$ = *m/z* 570). Detection of monoboronates can be rationalized by hydrolysis of the initially formed bisboronate derivatives under aqueous conditions of HPLC analysis and is in accord with the corresponding data on the instability of bisboronates[4]. In contrast, 22,23-monoboronates have been observed to be remarkably stable, even in slightly acidic medium and hence phenylboronates are suitable derivatives in LC/MS analysis of some brassinosteroids. However, the different properties and reactivities for derivatization of individual brassinosteroids as well as variable hydrolytic stability are limiting factors in general use of this approach.

Therefore, we examined the application of LC/MS-SIM method to analyze underivatized brassinosteroids. Appropriate reverse-phase HPLC conditions for separation of brassinolide and 3-epibrassinolide (**1**) were established (see Experimental). As with the phenylboronates, $APCI^+$ was used for ionization. Detection limits were determined to be 12 ng for brassinolide and [26-$^2H_3$]3-epibrassinolide. During LC/MS analysis, the signal of standard brassinolide was detected at an average retention time of 14.3 min as the protonated molecular species (*m/z* 481 $[M + H]^+$) and molecular ion adducts (*m/z* 522 $[M + CH_3CN + H]^+$ and 544 $[M + CH_3CN + Na]^+$). [26-$^2H_3$]3-Epibrassinolide was found at an average retention time of 11.7 min with *m/z* 547 $[M + CH_3CN + Na]^+$ under the above conditions. Solvent adduct formation is commonly observed with APCI, and is well documented[5,6]. Owing to the ability of the procedure to detect such low concentrations, it was applied to the analysis of endogenous brassinosteroids in plant tissue extracts.

Root-callus suspension cultures of *Arabidopsis thaliana*[7], which have been employed very recently to monitor 3β-brassinosteroid dehydrogenase activity[8], were used as the plant system in phytochemical studies on brassinosteroids. The extract from *Arabidopsis* cultures was treated as described in the Experimental, subjected to TLC on silica gel, and further separated twice by reverse-phase HPLC using acetonitrile–water as a mobile phase. Since brassinosteroids lack a suitable chromophore, the matrix material was visualized in UV light at 254 nm and TLC zones of brassinosteroids were scraped off according to the R_F values of standards determined on separate TLC plates. UV trace of HPLC (205 nm) was used to detect the peaks of matrix compounds coeluting with brassinsteroids. Thus, brassinosteroids were monitored indirectly, and HPLC fractions were collected corresponding to retention times of brassinosteroid standards detected at 205 nm. The fractions thus obtained were subjected to LC/MS with on-line UV detection (205 nm). Mass spectra were recorded in the SIM mode searching for pseudomolecular ion and its clusters, which were determined in preliminary LC/MS experiments using standard samples. Using this approach, fractions of root-callus suspension cultures of *A. thaliana* signals corresponding to brassinolide were detected at average retention time 14.5 min (*m/z* 481 $[M + H]^+$; *m/z* 522 $[M + CH_3CN + H]^+$). Based on comparison of signal intensity in obtained spectrum and spectra of standard brassinolide, the estimated concentration was 4 ng per g of fresh weight. An additional signal at 11.9 min in the same SIM experiment (*m/z* 522 $[M + CH_3CN + H]^+$) and another signal (Fig. 1) found by SIM (*m/z* 544 $[M + CH_3CN + Na]^+$) was assigned to 3-epibrassinolide (**1**) on the basis of the retention time 11.96 min,

which was determined before in separate experiments using [26-$^2H_3$]3-epibrassinolide (11.7 min; *m/z* 547 [M + CH_3CN + Na]$^+$). Replications confirmed the occurrence of endogenous 3-epibrassinolide in *A. thaliana*.

Brassinolide and 3-epibrassinolide (**1**) were identified in root-callus suspension cultures of *Arabidopsis thaliana*. The identification of 3-epibrassinolide represents the first isolation of the natural product from a plant, whereas brassinolide and other brassinosteroids have been already detected in *Arabidopsis*[9–11]. A related brassinosteroid, 3,24-bisepibrassinolide, different from 3-epibrassinolide in the configuration of C-24, has hitherto been found as a metabolite in plants only after feeding 24-epibrassinolide to *Ornithopus sativus*[12].

EXPERIMENTAL

HPLC. A Merck–Hitachi LiChrograph HPLC system (L-6200A gradient pump, UV-VIS detector L-4250) with Merck Nucleosil C18 (10 μm; 10 × 250 mm) and Waters Symmetry Shield™ (5 μm; 4.6 × 250 mm) reverse-phase columns were used.

LC/MS. Atmospheric-pressure-chemical-ionization (APCI) mass spectra were recorded in the positive ion mode on a Micromass Quattro II tandem quadrupole mass spectrometer (corona voltage 3.5 kV, cone voltage 30 V). An HP1100 HPLC system, equipped with a variable wavelength detector, was fitted to the mass spectrometer. A Waters Symmetry Shield™ reverse-phase column (5 μm; 4.6 × 250 mm) was used at the flow rate 0.5 ml min^{-1}; injection volume: 35 μl of fractions from plant extracts, 10 μl of standard samples.

Brassinosteroids and labelled standards. [26-$^2H_3$]3-Epibrassinolide was synthesized as described[3]. Brassinolide was purchased from CIDtech Research Inc., Mississauga (ON), Canada.

Derivatization. Phenylboronate derivatives were prepared from brassinolide and [26-$^2H_3$]3-epibrassinolide (each 320 μg, 0.667 μmol). The brassinosteroid was added to a solution of phenylboronic acid (240 μg, 1.995 μmol) in pyridine–acetonitrile (32 μl; 2 : 98 v/v) and heated to 70 °C for 20 min. Volumes of 10 μl of these solutions were injected to LC/MS.

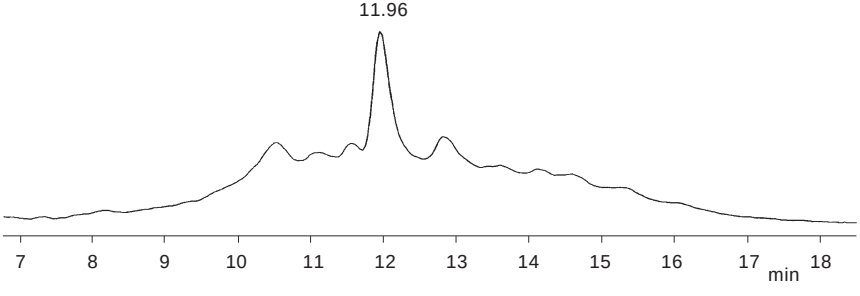


FIG. 1
Detection of 3-epibrassinolide (**1**) (RT 11.96, *m/z* 544 [M + CH_3CN + Na]$^+$) in root-callus suspension cultures of *Arabidopsis thaliana* by means of HPLC/APCI-MS-SIM

Plant material and culture conditions. Sterile seeds of *Arabidopsis thaliana* cv. C24 were germinated on auxin- and cytokinin-free B5 medium[13] containing sucrose (20 g l^{-1}) at 23 °C on a rotary shaker at 90 rpm. After growing for two weeks, the roots were separated from the seedlings, minced under sterile conditions, and placed into RM 28 medium[7] (100 ml) in 250-ml Erlenmeyer flasks. The cultures were maintained for three weeks under the same conditions, with weekly replacement of the nutrient medium. During that time, root-callus suspension cultures developed which were used for further investigations.

Extraction and separation. The root-callus material of *Arabidopsis thaliana* was harvested by suction filtration, disrupted with an Ultra-Turrax grinder at room temperature in MeOH (3 × 60 ml). The combined extracts were evaporated, taken up with water, and extracted with $CHCl_3$ (3 × 60 ml). The residue remaining after evaporation of the $CHCl_3$ extract was partitioned between hexane (60 ml) and 80% aqueous MeOH (60 ml). The crude material (approximately 50 mg) remaining after evaporation of aqueous MeOH was subjected to TLC (Merck silica gel $60F_{254}$, 20 × 20 cm, 0.5 mm layer thickness; $CHCl_3$–MeOH 88 : 12). The TLC zone corresponding to standards (brassinolide and [26-$^2H_3$]3-epibrassinolide) was scraped off and eluted with $CHCl_3$–MeOH 50 : 50. Brassinosteroid standards were chromatographed on separate TLC plates to avoid contamination of extracts. The material remaining after elution (approximately 5 mg) was further purified twice by reverse-phase HPLC. Initial separation was carried out on a Merck Nucleosil C18 column (10 μm, 250 × 10 mm, CH_3CN–H_2O 50 : 50 under isocratic conditions, flow rate 2 ml min^{-1}, UV 205 nm). Retention times of standards: [26-$^2H_3$]3-epibrassinolide 15.0 min, brassinolide 18.7 min. The 13–21 min fraction was collected and rechromatographed on Waters Symmetry Shield™ reverse-phase column (5 μm, 4.6 × 250 mm, CH_3CN–H_2O 50 : 50 under isocratic conditions, flow rate 0.5 ml min^{-1}, UV 205 nm). Retention times of standards: [26-$^2H_3$]3-epibrassinolide 11.2 min and brassinolide 13.4 min. The 10–15 min fraction was collected and further subjected to LC/MS analysis using the same Waters Symmetry Shield™ reverse-phase column.

Dr D. J. Fowler (Jena, Germany) is gratefully acknowledged for linguistic support in the preparation of this manuscript. This investigation was financially supported by the Deutsche Forschungsgemeinschaft (Bonn, Germany) and the Fonds der Chemischen Industrie (Frankfurt/Main, Germany).

REFERENCES

1. Takatsuto S.: *J. Chromatogr., A* **1994**, *658*, 3.
2. Gamoh K., Abe H., Shimada K., Takatsuto S.: *Rapid Commun. Mass Spectrom.* **1996**, *10*, 903.
3. Khripach V. A., Zhabinskii V. N., Konstantinova O. V., Antonchick A. P., Schneider B.: *Steroids*, in press.
4. Gamoh K., Brooks C. J. W.: *Anal. Sci.* **1993**, *9*, 549.
5. Warrack B. M., Didonato G. C.: *Biol. Mass Spectrom.* **1993**, *22*, 101.
6. Honig M., Barcelo D., van Baar B. L. M., Brinkman U. A. T.: *J. Mass Spectrom.* **1996**, *31*, 527.
7. Ecker J.: *Methods (San Diego)* **1990**, *1*, 186; *Chem. Abstr.* **1992**, *116*, 100219.
8. Stuendl U., Schneider B.: *Phytochemistry* **2001**, *58*, 989.
9. Fujioka S., Choi Y.-H., Takatsuto S., Yokota T., Li J., Chory J., Sakurai A.: *Plant Cell Phys.* **1996**, *37*, 1201.

10. Schmidt J., Altmann T., Adam G., Schmidt J.: *Phytochemistry* **1997**, *45*, 1325.
11. Nomura T., Sato T., Bishop G. J., Kamiya Y., Takatsuto S., Yokota T.: *Phytochemistry* **2001**, *57*, 171.
12. Kolbe A., Schneider B., Porzel A., Adam G.: *Phytochemistry* **1996**, *41*, 163.
13. Gamborg O. L., Miller R. A., Ojima K.: *Exp. Cell Res.* **1968**, *50*, 151.