Reversible Conversion in the Brassinosteroid Quartet Castasterone, Brassinolide and their 3β-Epimers

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Z. Naturforsch. 61b, 1039 – 1044 (2006); received January 31, 2006

The metabolism of deuterated brassinosteroids has been studied in excised leaves of Secale cereale, and in vitro in seedlings of Arabidopsis thaliana and cell suspension cultures of Lycopersicon esculentum. In addition to the known biosynthetic conversion of castasterone to brassinolide and epimerization to 3-epicastasterone, inversion of the 3α-configured hydroxyl group of brassinolide to a 3β-configured one in 3-epibrassinolide has been observed using liquid chromatography (HPLC) with electrospray ionization (ESI) and selected ion-monitoring mass-spectrometry (SIM-MS). Administration of deuterated 3-epicastasterone and 3-epibrassinolide to Arabidopsis and Secale seedlings resulted in the formation of castasterone and brassinolide, respectively, indicating conversion of configuration at C-3 of brassinosteroids is reversible.

Key words: Biosynthesis, Brassinosteroids, Epimerization

Introduction

The biosynthesis and metabolism of brassinosteroids in plants has been studied by means of labelling experiments and mutant studies, and on the enzymatic level [1 – 5]. Clearly brassinosteroids are a part of a complex regulatory system controlling plant homeostasis [6, 7]. An important precondition of its correct functioning is maintenance of a proper level of this hormone, which can be achieved by combining the corresponding biosynthetic (activation) and metabolic (inactivation) reactions. Recent studies have provided increasing evidence for a cross-linked biosynthetic grid of interchangeable steps rather than a distinct route to brassinolide [8 – 10] as well as strong evidence for deactivation of brassinosteroids in plants. The last may be the reason why results of experiments on the practical use of brassinolide (the most potent phytohormone among brassinosteroids) have been disappointing [11]. Evidently, the level of this endogenous hormone is strictly regulated in plants, whereas inactivation mechanisms of its analogs may be less efficient. This could be the reason why brassinolide analogs are more effective in field trials [12]. An excess of brassinolide and other brassinosteroids can be removed (or stored) in various ways including hydroxylation/degradation of the side chain and conjugation with fatty acids, sugars or sulfate [12 – 16] or inactivated by unknown mechanisms [17]. The present work deals with the possibility that transformations in ring A are involved in brassinolide-level control.

Inversion of configuration of the hydroxy group at C-3 represents one of the reactions, which can occur at different steps in the pathway. Inversion from 3β to 3α configuration has been demonstrated in the course of early and late C-6 oxidation [18 – 19], and 3-epi-6-deoxocathasterone (3α-configuration) was identified as a metabolite of 6-deoxocathasterone (3β-configuration) [9]. Formation of teasterone (3β-epimer) from typhasterol (3α-epimer) through the 3-dehydro intermediate showed the reversibility of this reaction [20]. Brassinosteroids of the 24β series have also been used to show that inversion of configuration at C-3 is influenced by regio- and stereospecific formation of carbohydrate conjugates of the 3β-epimer [21]. Compared to 3β-isomers, 3α-isomers generally are more active in the rice lamina inclination bioassay. The conversion of 3β to 3α is supposed to represent an activation step, and the opposite, 3α to 3β, e.g. castasterone to 3-epicastasterone [22], is con-
sidered an inactivation step. Using 24-epicastasterone and 24-epibrassinolide, transformation to \(3\beta\)-isomers was shown by cell suspension cultures of *Ornithopus sativus* [23]. Thus, inversion of configuration at C-3 is one of the putative possibilities to inactivate brassinolide, the most active brassinosteroid, but this specific reaction has not yet been experimentally demonstrated. The reverse conversion of 3-epicastasterone and 2-epicastasterone to castasterone was detected recently in seedlings of *Secale cereale* [10]. The conversion of brassinolide to 3-epibrassinolide, a naturally occurring brassinosteroid in *Arabidopsis* [24], and the reverse conversion are reported here for the first time and, together with the corresponding conversions between castasterone and 3-epicastasterone, are discussed in the context of hormone homeostasis in plants.

**Results and Discussion**

**Brassinosteroids in *Arabidopsis thaliana* and *Secale cereale***

The occurrence of the brassinosteroids castasterone (1), brassinolide (2), 3-epicastasterone (3) and 3-epibrassinolide (4), which are subject to the present metabolic study, was searched in the literature and investigated in *Arabidopsis thaliana* and *Secale cereale*. In *A. thaliana*, brassinosteroids [25–26] have been reported several times [27–29]. Castasterone (1) and brassinolide (2) are native also to *Arabidopsis* shoots and seeds, and 3-epibrassinolide (4) was recently found in root-callus suspension cultures of eco-type C24 [24]. Schmidt *et al.* [30] reported castasterone (1) together with other brassinosteroids from seeds of *S. cereale*. Recently we demonstrated the presence of castasterone (1) and 3-epicastasterone (3) in seeds of this plant [10].

Here we investigate the occurrence of brassinosteroids in *Arabidopsis thaliana* seedlings grown under *in vitro* conditions. The seedlings were extracted according to the procedure described in Materials and Methods. Triply deuterated brassinosteroids, \([26-^2H_3]3\)-epicastasterone, and \([26-^2H_3]3\)-epibrassinolide [31] (0.4 \(\mu\)g in 10 \(\mu\)l of 75% EtOH each), were added as internal standards. Several solvent partitioning and chromatographic steps resulted in brassinosteroid-containing fractions, which after derivatization with an excess of dansyl-3-aminophenylboronic acid were analyzed by liquid chromatography (HPLC) with electrospray ionization (ESI) and using an established ion-monitoring mass-spectrometric (SIM-MS) detection (HPLC-ESI-SIM-MS) method [10, 32]. Identification of brassinosteroids based on the relative retention time of 22,23-dansyl-3-aminophenylboronates (Table 1). Using this approach, castasterone (1), brassinolide (2), 3-epicastasterone (3) and 3-epibrassinolide (4) were detected in *Arabidopsis* seedlings. The relative retention times of authentic standards and mass spectral data (Table 1) also revealed the presence of castasterone (1) and 3-epicastasterone (3) in seedlings of *S. cereale*.

**Metabolism of castasterone and brassinolide**

\([26,28,^2H_6]3\)-Castasterone (1) and \([26,28,^2H_6]3\)-brassinolide (2) [33] were administered in a series of separate experiments in different plant materials, seedlings of *A. thaliana S. cereale*, and cell suspension cultures of *L. esculentum*. After a two-day incubation, the plant material was extracted according to the procedure used to detect endogenous brassinosteroids. Brassinosteroid-containing fractions were converted to their dansyl-3-aminophenylboronates and analyzed by HPLC-ESI-SIM-MS [10, 32].

Administering \([26,28,^2H_6]3\)-castasterone (1) to *A. thaliana* and *L. esculentum* and analysing the extracts by HPLC-ESI-SIM-MS, we identified sixfold deuterated brassinolide (2) \((m/z 821; [M+H]^+; RR_1 = 1.000)\) in both plants. The conversion of castasterone (1) to brassinolide (2) (Fig. 1) confirmed that this well-established final step of brassinolide biosynthesis is operating in our experimental system. SIM analysis for the molecular mass of sixfold deuterated castasterone (1) \(m/z 805 (\text{[M+H]}^+)\) allowed us to identify the parent compound 1 at \(RR_1 1.419\) and 3-epicastasterone (3) at \(RR_1 1.100\) of the same molecular mass. The natural abundance isotopomer of 3, the \(3\beta\)-epimer
of 1 (m/z 799; [M+H]+; R_R 1.101), was detected in seedlings of A. thaliana as well. Furthermore, in seedlings of S. cereale [26,28-2H6]castasterone (1) was converted to labelled 3-epicastasterone (3) (m/z 805; [M+H]+) as the only product (Fig. 1). The discovery of compound 3 as a metabolite of 1 resembles previous results [22], where in the seedlings of Catharanthus roseus the conversion of 1 to 2 and 3 was observed, while the conversion of 1 to only 3 was found in the seedlings of Nicotiana tabacum and Oryza sativa.

Administering [26,28-2H6]brassinolide (2) followed by an analogous separation procedure resulted in identification of sixfold labelled 3-epibrassinolide (4) (m/z 821; [M+H]+; R_R 0.857) in A. thaliana, S. cereale seedlings, and tomato cell suspension (Fig. 1). The metabolic conversion of brassinolide, the most active plant steroid hormone, to its 3β-epimer is considered an inactivation process. This result is the first experimental evidence for metabolic inactivation of brassinolide (1) in plants by stereochemical alteration. However, inversion of configuration at C-3 has already been observed for 24-epibrassinolide in cell cultures of Ornithopus sativus [34].

Metabolism of 3-epicastasterone and 3-epibrassinolide

The previously reported reversibility of the epimerization of the 3-hydroxyl group of brassinosteroids [20,21,35,36] encouraged us to investigate the fate of 3-epicastasterone (3) and 3-epibrassinolide (4) in seedlings of A. thaliana and S. cereale.

In order to check for the conversion of [26-2H3]3-epicastasterone (3) and 3-epibrassinolide (4) to 3α-hydroxybrassinosteroids, both deuterated compounds 3 and 4 were administered in separate experiments. Screening the analogously prepared extract from feeding [26-2H3]3-epicastasterone (3) for the mass of triply deuterated 1 and its isomers (m/z 802; [M+H]+) showed a peak corresponding to labelled precursor 3 (R_R 1.000) and a peak corresponding to labelled castasterone 1 (R_R 1.419) in the seedlings of A. thaliana and S. cereale. In addition a peak corresponding to triply deuterated 3-epibrassinolide (4) (m/z 818; [M+H]+; R_R 0.856) in the seedlings of S. cereale has been detected (Fig. 1). This conversion is the first evidence that 3-epicastasterone (3) is an alternative biosynthetic precursor of 3-epibrassinolide (4). However, additional studies with substrates specifically deuterated at C-2 and C-3 are necessary to check for the course 3 → 1 → 2 → 4 including so far undetected direct lactonization of 1 to 2 and hypothetical conversion of 3-dehydrocastasterone to 3-dehydrobrassinolide in this plant.

In analogous experiments, [26-2H3]3-epibrassinolide (4) was administered to the seedlings of A. thaliana and S. cereale. HPLC-ESI-SIM-MS analysis showed triply labelled brassinolide (2) (m/z 818; [M+H]+; R_R 0.856) in extracts of plants treated with the 3β-epimer 4 (Fig. 1). These results showed that the conversion of 3β- to 3α-epimers is operating in A. thaliana and S. cereale not only for 6-oxo but 7-homolactonetype brassinosteroids also. As demonstrated by the identifica-
Seeds were germinated and grown in 100 ml conical flasks established from surface-sterilized seeds for each experiment. The greenhouse at 22 – 24 °C was used for germination, seeds were stratificized and then grown in cultures of *L. esculentum* L. cv. “Tamina” were grown in Linsmaier-Skoog medium [41] in 300 ml conical flasks containing 100 ml cell suspension at 23 °C on a rotary shaker at 85 rpm under constant diffuse light (4.4 µmol m⁻² s⁻¹). Subculturing was performed every 14 d using an inoculum of approximately 40 ml. In typical experiments, a filter-sterilized solution of the respective deuterated precursor (1 to 20 µg) in 75% ethanol (20 µl) was added to flasks with plant material (55 to 60 g) and incubated for three days. Each of the deuterated precursors 1 and 2 was administered individually.

**Identification of endogenous brassinosteroids in Arabidopsis**

The seedlings of *A. thaliana* cv. C24 were separated from the medium and extracted with MeOH (3 × 80 ml) using an Ultra-Turrax homogenizer. [26-H₃]-Epicastasterone (3), and [26-H₂]-3-epibrassinolide (4) [31] (0.4 µg in 10 µl of 75% EtOH each) were added as internal standards before evaporation followed by the procedure described below. Identification of endogenous brassinosteroids 1 to 4 was based on a comparison of retention times and relative intensities of molecular ions.

**Isolation and analysis of brassinosteroid metabolites**

After incubation with labelled precursors, the plant materials were separated from the medium and extracted with MeOH (3 × 80 ml) using an Ultra-Turrax homogenizer. The aqueous residue remaining from evaporation of the methanolic extract was partitioned between equal volumes of EtOAc and 0.5 M K₂HPO₄ (3 × 100 ml). The culture medium was extracted with EtOAc (100 ml) and the EtOAc phase was combined with the corresponding extract obtained from the plant material. The combined EtOAc phase was evaporated; MeOH (10 ml) was added, sonified for 15 min, and centrifuged for 15 min at 4000 rpm at 15 °C. The supernatant was evaporated and subjected to TLC (silica gel 60 F₂₅₄, 0.5 mm thickness, 200 × 200 mm, Merck; CHCl₃-MeOH 88:12). Based on the retention times of authentic standards, which were determined on a separate plate (compound 1: *R*ₜ 0.41; 2 and 3: *R*ₜ 0.31; 4: *R*ₜ 0.25), the
brassinosteroid-containing fraction (Rf 0.21–0.45) was collected and eluted sequentially with a mixture of CHCl3-MeOH 1:1 (20 ml) and MeOH (30 ml). The combined solution was evaporated to dryness and subjected to reversed-phase HPLC (LiChrospher® 100 RP-18; 10 μm; 250 × 10 mm) using a linear gradient MeCN-0.01% trifluoroacetic acid in H2O from 30 to 100% MeCN in 30 min and held at 100% MeCN for a further 15 min (flow rate 2 ml min⁻¹; UV detection of matrix compounds at 205 nm). Based on the retention times of authentic standards (compound 1: t 23.0 min; 2: Rf 20.9 min; 3: Rf 21.3 min; 4: Rf 19.1 min), the brassinosteroid-containing fraction (Rf 17.5–24.5 min) was collected and evaporated. The residue was converted to bis-dansyl-3-aminophenylboronates by being heated to 62°C for 30 min with a solution of dansyl-3-aminophenylboronic acid (3 mg ml⁻¹) in a mixture of pyridine and acetonitrile (1:19) and used for analysis by HPLC-ESI-SIM-MS as previously described [10, 32].

Acknowledgements

We thank Dr. A. Kolbe and Prof. G. Adam (Halle) for providing [26,28-²H₆]castasterone and [26,28-²H₆]brassinolide and Emily Wheeler (Jena) for linguistic help in the preparation of this manuscript.