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2,3-Epoxybrassinosteroids are intermediates in the biosynthesis of castasterone in seedlings of *Secale cereale*

Andrey Antonchick [a,b], Aleš Svatoš [a], Bernd Schneider [a,*], Olga V. Konstantinova [b], Vladimir N. Zhabinskii [b], Vladimir A. Khripach [b]

[a] *Max-Planck-Institute for Chemical Ecology, Beutenberg-Campus, Hans-Knöll-Str. 8, D-07745 Jena, Germany*
[b] *Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, Kuprevich Str., 5/2, 220141 Minsk, Belarus*

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Abstract

The involvement of the 2,3-epoxybrassinosteroids secasterone and 2,3-diepisecasterone in the biosynthesis of castasterone has been demonstrated in seedlings of *Secale cereale* by LC–ESI-MS. Deuterated secasterone, upon administration to rye seedlings, was incorporated into castasterone and its 2β- and 3β-epimers. Administration of deuterated 2,3-diepisecasterone resulted in castasterone and 2-epicastasterone. A biosynthetic subpathway from typhasterol/teasterone via 2,3-epoxybrassinosteroid intermediates to castasterone is discussed.

Keywords: *Secale cereale*; Gramineae; Biosynthesis; Brassinosteroids; Castasterone; Deuterium labelling; Epoxides; Epoxysteroids; Secasterone; Steroids

1. Introduction

Brassinosteroids are plant hormones of ubiquitous distribution in the plant kingdom (Adam et al., 1999; Fujioka, 1999; Khripach et al., 1999; Bajguz and Tretyn, 2003). Among steroidal natural products, castasterone and brassinolide are the most active regulators of plant growth and development (Brosa, 1999), and their biosynthesis has been a major subject of brassinosteroid research for several years (Sakurai, 1999; Yokota, 1999; Schneider, 2002). In general, the phytosterol skeleton is biosynthesized via the mevalonate pathway (Schwender et al., 1997), but recent results show that biosynthesis may originate from both the mevalonate and the deoxyxylulose pathways (De-Eknamkul and Potduang, 2003). Functionalization of the skeleton was primarily assumed to follow the sequence of the early C-6 oxidation pathway (C-6 oxidation → C-22α hydroxylation → C-23α hydroxylation → 3β-OH/3α-OH inversion → C-2α hydroxylation) (Suzuki et al., 1994). However, the discovery of the late C-6 oxidation (Choi et al., 1997) and early C-22 oxidation routes (Fujioka et al., 2002) indicated that the sequence of oxidation is interchangeable. In addition, some brassinosteroids have been found in plants which so far do not fit this biosynthetic network. 2,3-Epoxybrassinosteroids, such as secasterone (**2**) from *Secale cereale* (Schmidt et al., 1995) and 24-episecasterone from *Lychnis viscaria* (Friebe et al., 1999) are interesting examples. Recently we reported the formation of secasterone (**2**) and 2,3-diepisecasterone (**3**) from teasterone (**4**)/typhasterol (**5**) via secasterol (**1**) in rye (Antonchick et al., 2003).

In the present study, the conversion of deuterated 2,3-epoxybrassinosteroids to castasterone (**6**) has been

* Corresponding author. Tel.: +49 3641 571600; fax: +49 3641 571601.
E-mail address: schneider@ice.mpg.de (B. Schneider).

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investigated in seedlings of *Secale cereale* by means of labelling experiments using putative intermediates. An improved LC–ESI-MS method developed by us (Svatoš et al., 2004) was used in order to analyze brassinosteroids in plant extracts and to determine incorporation of deuterated precursors.

2. Results and discussion

2.1. Identification of naturally occurring brassinosteroids

Seeds and leaves of rye seedlings (*Secale cereale*, cv. "Sorom") were analyzed for naturally occurring brassinosteroids. Relevant fractions were obtained by the procedures described in the experimental section and converted to their dansyl-3-aminophenylboronates with an excess of dansyl-3-aminophenylboronic acid. Native brassinosteroids were identified by means of LC–ESI-MS according to the method developed by Svatoš et al. (2004) based on retention times of 22,23-dansyl-3-aminophenylboronates (DAPB), which are formed from bis-derivatives under HPLC conditions (Gamoh and Brooks, 1993; Konstantinova et al., 2001) and their molecular ions ($[DAPB + H]^+$). Endogenous castasterone (**6**) was found both in leaves and seeds. In addition, 2-epicastasterone (**7**) and 3-epicastasterone (**8**) were detected in seeds (Table 1). No substantial amounts of brassinolide (**BL**) were found in leaves and seeds of *Secale cereale*. The analytical data of native compounds were in full agreement with those of authentic references.

Secasterol (**1**), secasterone (**2**), and 2,3-diepisecasterone (**3**) were recently detected in seedlings of *Secale cereale* (Antonchick et al., 2003). Identification of compounds **2** and **3** was based on comparing their GC–MS data with those of side chain-deuterated (26-$^2H_3$)-standards. The molecular masses (*m*/*z* 470 $[M]^{\bullet+}$) of the nondeuterated compounds from rye samples and two characteristic fragments, *m*/*z* 245 (fragment of ring system) and *m*/*z* 155 (fragment of the nondeuterated side chain), were analogous to the molecular mass *m*/*z* 473 ($[M]^{\bullet+}$) and fragments of *m*/*z* 245 (ring system) and *m*/*z* 158 (deuterated side chain) of the deuterated standards. Due to the small number of three deuterium atoms relative to the total number of protons in the molecule, no significant differences in the retention times between nonlabelled and deuterated compounds **2** and **3** were observed. In order to avoid false-positive detection of native secasterone (**2**) and 2,3-diepisecasterone (**3**) caused by contamination with synthetic compounds, preparative synthesis and isolation from plant samples were carried out in separate institutes at Minsk and Jena. Only deuterated but no nondeuterated samples of synthetic compounds **2** and **3** were available at the Jena laboratory where analytical studies were performed.

Secasterone (**2**), teasterone (**4**), typhasterol (**5**), castasterone (**6**), and some other brassinosteroids were previously found in rye seeds (Schmidt et al., 1995). The data shown in Table 1, together with those obtained in a recent study using the same plant material (Antonchick et al., 2003), and information available from Schmidt et al. (1995) demonstrate the natural occurrence of the complete series of precursors, intermediates, and products involved in this study, in *Secale cereale*.

2.2. Synthesis of labelled precursors and analytical standards

In order to examine the conversion of 2,3-epoxybrassinosteroids *in planta*, putative intermediates were required in deuterated form and as standards. The synthesis of [26-$^2H_3$]secasterone (**2**), [26-$^2H_3$]2,3-diepisecasterone (**3**), [26-$^2H_3$]castasterone (**6**), and [26-$^2H_3$]3-epicastasterone (**8**) has been described recently (Khripach et al., 2002). HRMS data of synthetic deuterated compounds confirmed the occurrence of three deuterium atoms per molecule. The deuterium labels are located in a metabolically inert position at a side chain methyl group (C-26), which is not involved in metabolic conversion and not subject to spontaneous H–D-exchange. Thus, contamination of deuterated precursors with nondeuterated isotopomers was excluded, which is an important criterion for their suitability in biosynthetic investigations.

[26-$^2H_3$]2-Epicastasterone (**7**) was synthesized from [26-$^2H_3$]-**2** according to a procedure published for the synthesis of 2,24-diepicastasterone (Voigt et al., 2002). For analytical data, see Section 3.

Table 1
Detection of brassinosteroids in seeds and leaves of *Secale cereale*, retention times, and *m*/*z* data of their 22,23-dansyl-3-aminophenylboronates (DAPB)

	Seeds (pg g^{-1})	Leaves[a]	R_t (min)	*m*/*z* $[DAPB + H]^+$
Castasterone (**6**)	574	+	15.2	799
2-Epicastasterone (**7**)	201	–	13.0	799
3-Epicastasterone (**8**)	115	–	12.1	799

[a] Qualitative analysis without internal standard.

2.3. Biosynthesis

2.3.1. Opening the 2,3-epoxide

The biosynthetic formation of secasterol (**1**), secasterone (**2**), and 2,3-diepisecasterone (**3**) from teasterone (**4**) and/or typhasterol (**5**) in seedlings of *Secale cereale* (Antonchick et al., 2003) and the co-occurrence of **1**–**3** with a downstream brassinosteroid, castasterone (**6**) in *Secale cereale* raised a question: are the 2,3-epoxybrassinosteroids **2** and **3** final products of the biosynthesis or do they undergo further metabolism? Opening of the epoxide was anticipated as a possible conversion, one that would result in the formation of two different 2,3-dihydroxybrassinosteroids, 2-epicastasterone (**7**) and 3-epicastasterone (**8**).

In order to check for conversion to these hypothetical metabolites, feeding experiments using deuterated precursors were performed. In first biosynthetic experiments, [26-$^2H_3$]secasterone (**2**) was administered hydroponically to excised seedlings of the rye cultivar "Sorom" by immersing the leaves in the feeding solution. After an incubation time of 70 h, the leaves were extracted according to the procedure used to detect endogenous brassinosteroids in leaves. Relevant fractions were converted to their dansyl-3-aminophenylboronates and analyzed by LC–ESI-MS. Peaks with the same retention times as the standards were identified by selected ion monitoring (SIM, Fig. 1). SIM for the molecular ion of triply deuterated castasterone (**6**) (*m*/*z* 802, $[DAPB + H]^+$) resulted in the detection of three isomeric compounds: 3-epicastasterone (**8**) (R_t 12.1 min), 2-epicastasterone (**7**) (R_t 13.0 min), and castasterone (**6**) (R_t 15.2). Half-widths of peaks were used as a measure for resolution. Using this criterion, all peaks were clearly resolved and peak widths were significantly below 1 min. Castasterone (**6**) appears baseline-separated and did not interfere with any other peak. Integration of the LC peaks revealed a ratio of the three metabolites, **6**:**7**:**8**, resulting from conversion of the parent compound **2**, of approximately 3:15:2, indicating that 2-epicastasterone (**7**) is clearly the major product. However, the occurrence of significant amounts of castasterone (**6**) is the most remarkable finding of this feeding experiment.

Administering [26-$^2H_3$]2,3-diepisecasterone (**3**) to seedlings of *Secale cereale* under identical experimental conditions, including DAPB derivatization, resulted in the identification by LC–ESI-MS–SIM of triply labelled 2-epicastasterone (**7**) (*m*/*z* 802, $[DAPB + H]^+$, R_t 13.0 min) as the only product.

The *trans*-diaxial 2β,3α-diol **7**, which is the major metabolic product of secasterone (**2**), and 2,3-diepisecasterone (**3**) in rye may correspond to acid catalysis as a mechanism of epoxide hydrolysis because of its accordance with the Fürst–Plattner rule (Fürst and Plattner, 1949). In contrast, the diequatorial 2α,3β-diol **8** seems to be the result of a different process, with specificity of the putative enzyme for secasterone (**2**) rather than 2,3-diepisecasterone (**3**). Epoxide hydrolases (EHs) with different enantioselectivity (Summerer et al., 2002) have been identified from plants, microorganisms, and animals (Barth et al., 2004). They belong to the α/β hydrolase fold protein family (Ollis et al., 1992) and in plants are involved in, for example, xenobiotic metabolism, the biosynthesis of cuticular components (Blée and Schuber,

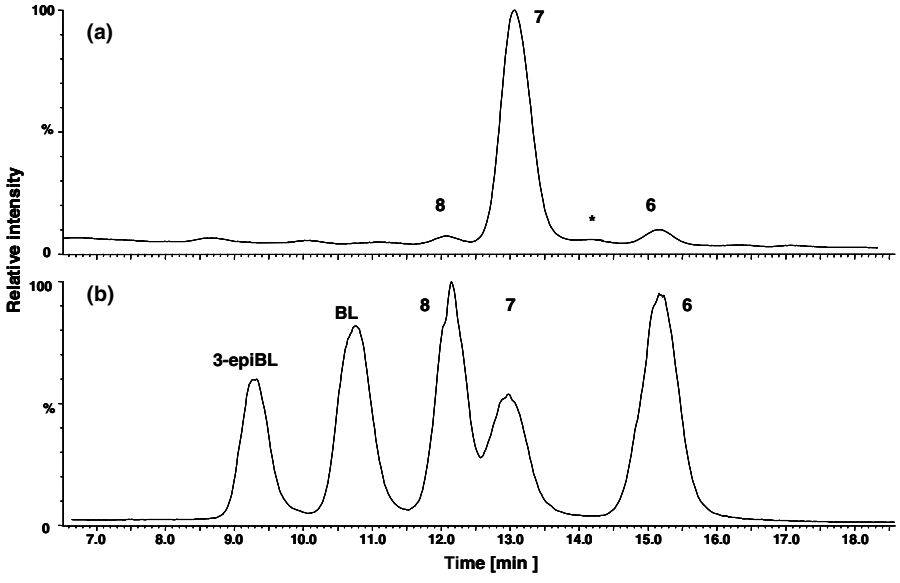

Fig. 1. Sections of mass chromatograms of brassinosteroid DAPB derivatives from ESI-LC-MS experiments in SIM mode. (a) Trace from administration of [26-$^2H_3$]secasterone (**2**) to seedlings of *Secale cereale* plotted at *m*/*z* 802 $[DAPB + H]^+$. The small peak denoted with an asterisk represents an unknown compound (see text). (b) Total ion chromatogram obtained from a mixture of authentic 3-epibrassinolide (**3-epiBL**), brassinolide (**BL**), 3-epicastasterone (**8**), 2-epicastasterone (**7**), and castasterone (**6**).

1993), and generation of lipid-derived defense-related signalling compounds. To our knowledge, a specific steroid EH has not yet been identified in plants. Thus, in terms of substrate specificity, mammalian microsomal cholesterol EHs (Fretland and Omiecinski, 2000) seems to be the closest known relative to epoxybrassinosteroid-hydrolyzing enzymes. In contrast to other microsomal EHs, cholesterol 5,6-oxide hydrolases do not covalently bind the substrate to the protein, indicating a uniquely different hydrolytic mechanism (Muller et al., 1997). On the other hand, evidence for the presence of members of the microsomal EH superfamily is missing in the plant genome; only cytosolic EHs were found (Barth et al., 2004). Thus, epoxide hydrolysis in plants might alternatively be catalyzed by glutathione-*S*-transferases (GSTs) (Eaton and Bammler, 1999). Characterization of the enzymes, whether EH or GST, involved in epoxybrassinosteroid metabolism and their regulation would be of considerable interest. In light of the putative involvement of EHs or GSTs, this especially is true with regard to the role of brassinosteroids in how plants respond to stress.

2.3.2. Inversion of configuration

The detection of castasterone (**6**) derived from [26-$^2H_3$]secasterone (**2**) in the feeding experiment described above suggested a biosynthetic route, which after ring opening presumably proceeds through an oxidation/reduction mechanism to **6**. Since 2-epicastasterone (**7**) and 3-epicastasterone (**8**) were both identified as metabolites of secasterone (**2**), two hypothetical possibilities exist for the formation of castasterone (**6**) from **2**: (a) secasterone (**2**) → 2-epicastasterone (2β-OH,3α-OH; **7**) → castasterone (**6**) or (b) **2** → 3-epicastasterone (2α-OH,3β-OH; **8**) → **6**. Compound **7** but not **8** was detected when plants were fed 2,3-diepisecasterone (**3**) and it accumulated at higher levels in comparison to **8** after being fed **2**. Formation of detectable levels of **7** from **2** and relatively high levels of **7** formed from **3** indicates that in rye conversion of 2-epicastasterone (**7**) to other products seems less efficient than conversion of 3-epicastasterone (**8**). Conformational changes of ring A hydroxyl groups are considered good candidates as metabolic reactions of compounds **7** and **8**. In the course of reversible inversion of configuration at C-3, conversion of 3α-OH to 3β-OH-brassinosteroids has been demonstrated several times in brassinosteroid metabolism (Kolbe et al., 1998; Noguchi et al., 2000). These reported data, in addition to different levels of **7** and **8** in the experiments above, support possibility (b) as well.

Nevertheless, in order to check for the conversion of 2-epicastasterone (**7**) and 3-epicastasterone (**8**) to castasterone (**6**), both deuterated intermediates **7** and **8** were administered to rye seedlings in separate experiments. Screening the extract prepared from the feeding of [26-$^2H_3$]3-epicastasterone (**8**) for the mass of triply deuterated **6** and its isomers (*m/z* 802, $[DAPB + H]^+$) showed a large peak corresponding to derivatized labelled precursor **8** (R_t 12.1 min) and a small peak corresponding to labelled DAPB derivative of castasterone **6**

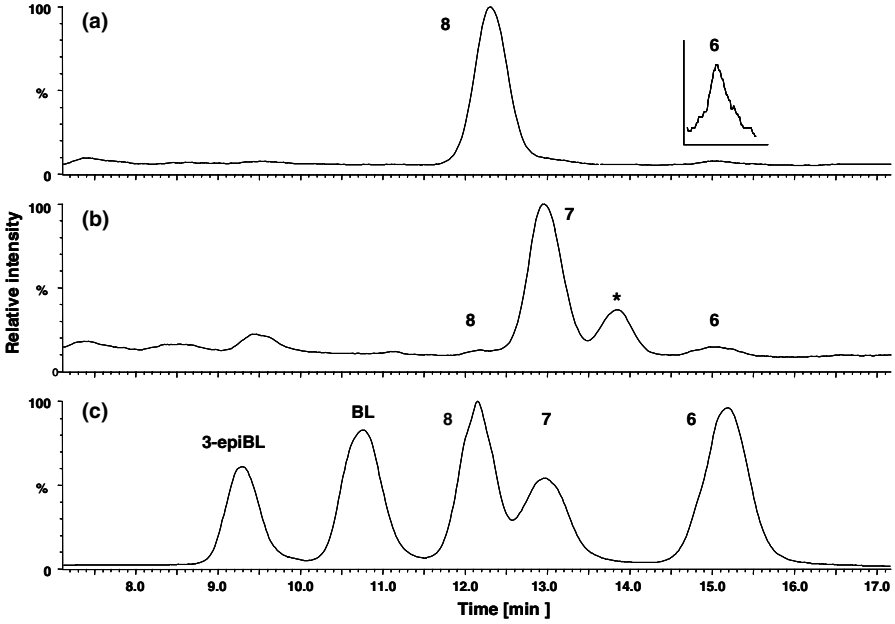

Fig. 2. Sections of mass chromatograms of brassinosteroid DAPB derivatives from ESI-LC-MS experiments in SIM mode. (a) Trace from administering [26-$^2H_3$]3-epicastasterone (**8**) to seedlings of *Secale cereale* plotted at *m/z* 802 $[DAPB + H]^+$. The inset represents the 40 times magnified region of 14.5–16 min. (b) Trace from administering [26-$^2H_3$]2-epicastasterone (**7**) to seedlings of *Secale cereale* plotted at *m/z* 802 $[DAPB + H]^+$. The peak denoted with an asterisk represents an unknown compound (see text). (c) Total ion chromatogram obtained from a mixture of 3-epibrassinolide (**3-epiBL**), brassinolide (**BL**), 3-epicastasterone (**8**), 2-epicastasterone (**7**), and castasterone (**6**).

(R_t 15.2 min). From this result, we can assume that 3-epicastasterone (**8**) has been biosynthetically converted to castasterone (**6**). The precursor:product ratio was 97:3 (Fig. 2(a)), which is due to the large excess of the administered **8**.

A similar result was obtained when plants were fed [26-$^2H_3$]2-epicastasterone (**7**), Fig. 2(b). Again, the SIM chromatogram (*m/z* 802, $[DAPB + H]^+$) exhibited a large peak corresponding to derivatized labelled precursor, [26-$^2H_3$]2-epicastasterone (**7**) (R_t 13.0 min) and a small peak corresponding to that of DAPB derivative of labelled castasterone (R_t 15.2 min). In this case the ratio was 95:5, which indicated an even higher conversion rate of **7** to castasterone (**6**), compared to the conversion of **8** to **6**. Resolution for [26-$^2H_3$]2-epicastasterone (**7**), the peak marked with an asterix, and [26-$^2H_3$]castasterone (**6**) in Fig. 2(b) exemplarily were calculated to be $R_{s\ (7,*)} = 1.23$ and the $R_{s\ (7,6)} = 2.56$, respectively. The number of theoretical plates for peak **7** in Fig. 2(b) is $N = 39333$.

In light of the above discussion, this finding was rather surprising, but seems to indicate how highly flexible plants' production of regulatory active compounds can be. The peak with *m/z* 802 at R_t 13.8 min, which in smaller amounts was also noticed in native samples and after administering [26-$^2H_3$]secasterone (**2**) (Fig. 1(a)), is probably 2,3-diepicastasterone. However, since no authentic standard was available, the identity of this compound remains uncertain.

Inversion of configuration at C-3 of brassinosteroids has been demonstrated to proceed via 3-dehydro intermediates (Kolbe et al., 1998; Noguchi et al., 2000). Thus, although so far unknown as natural products, 2-dehydrocastasterone and 3-dehydrocastasterone may be anticipated as intermediates of inversion of configuration of C-2 and C-3, respectively. Unfortunately these compounds were not available in labelled form for testing this hypothesis by feeding experiments. Therefore, although direct epimerization of **7** to **6** and **8** to **6** is less likely, the present study does not rule it out.

In summary, together with recent results on secasterone (**1**) biosynthesis from teasterone (**4**) and/or typhasterol (**5**) (Antonchick et al., 2003), this study provides evidence for a new biosynthetic networking in the pathway to castasterone in the studied plant, *Secale cereale*. These findings demonstrate that the biosynthetic sequence teasterone (**4**)/typhasterol (**5**) → secasterol

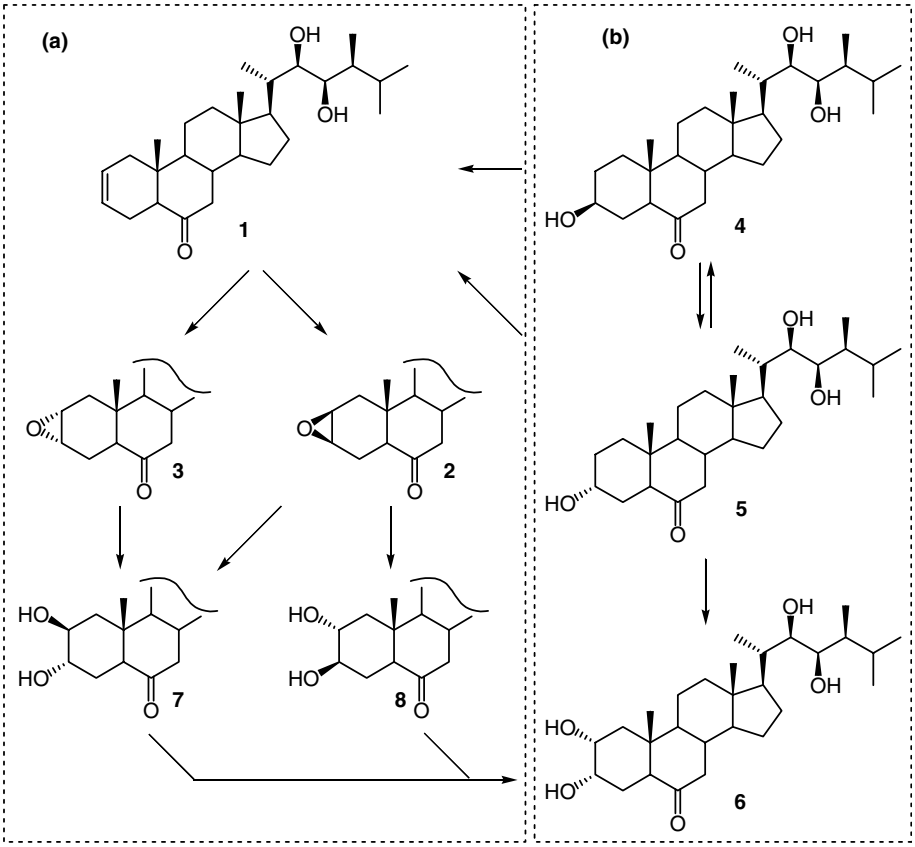

Fig. 3. Hypothetical network of brassinosteroid biosynthesis in seedlings of *Secale cereale*. (a) Biosynthetic steps established in the present study and by Antonchick et al. (2003). (b) Part of the known early C-6 oxidation pathway (Suzuki et al., 1994; Noguchi et al., 2000), which was not investigated in this study.

(**1**) → secasterone (**2**) → 2-epicastasterone (**7**)/3-epicastasterone (**8**) → castasterone (**6**) (Fig. 3) is operative in *Secale cereale*. This route represents an alternative to the direct C-2α-hydroxylation of typhasterol (**5**) to castasterone (**6**) in the early C-6 oxidation pathway (Suzuki et al., 1994). The C-2α-hydroxylation step is generally accepted also in the late C-6 oxidation pathway in dicotyledons between 6-deoxotyphasterol and 6-deoxocastasterone (Choi et al., 1997). Whether or not, and under which physiological conditions, the new epoxide pathway or direct C-2α-hydroxylation is operating, alternatively or in parallel, remains to be investigated.

3. Experimental

3.1. Plant materials

Seeds of *Secale cereale* cv. "Sorom" were purchased from Lochow–Petkus GmbH, Bergen-Wohlde, Germany. Prior to germination, seeds were stratificized and then grown in the greenhouse at 22–24 °C using a mixture of Vermiculite and sand (ratio 3:1) as a substrate. The natural photoperiod was supplemented with 16 h illumination from Philips Sun-T Agro 400 Na lights. Biosynthetic experiments were performed using 14-day-old excised seedlings of *Secale cereale* cv. "Sorom".

3.2. Synthesis of [26-$^2H_3$]2-epicastasterone (7)

[26-$^2H_3$]Secasterone (**2**) (5.3 mg) (Khripach et al., 2002) was converted to [26-$^2H_3$]2-epicastasterone (**7**) (4.1 mg) by an acid-catalyzed epoxide opening, according to a procedure published for the synthesis of 2,24-diepicastasterone (Voigt et al., 2002). ^{1}H NMR, ^{1}H–^{1}H COSY, HMBC and HMQC spectra were measured at 500 MHz (Bruker Avance DRX 500 spectrometer) and ^{13}C spectra at 100 MHz (Bruker Avance 400 spectrometer). Chemical shifts are given in δ values relative to TMS as internal standard. ^{1}H NMR ($CDCl_3$): δ 0.69 (3H, *s*, CH_3-18), 0.85 (3H, *d*, J = 6.9 Hz, CH_3-28), 0.91 (3H, *d*, J = 6.6 Hz, CH_3-21), 0.95 (3H, *d*, J = 6.9 Hz, CH_3-27 or CH_3-26), 0.96 (3H, *d*, J = 6.5 Hz, CH_3-26 or CH_3-27), 0.97 (3H, *s*, CH_3-19), 1.10 and 1.57 (H_2-15), 1.22 (H-24), 1.26 and 1.97 (H_2-16), 1.28 and 1.76 (H_2-12), 1.31 (H-14), 1.33 (H-9), 1.34 and 1.64 (H_2-11), 1.50 (H-20), 1.58 (H-17), 1.61 (H-25), 1.70 and 2.07 (H_2-1), 1.76 and 2.02 (H_2-4), 1.81 (H-8), 2.00 and 2.31 (H_2-7), 2.74 (1H, *dd*, J = 2.3, 12.9 Hz, H-5), 3.56 (1H, *br d*, J = 8.5 Hz, H-22), 3.72 (1H, *br d*, J = 8.5 Hz, H-23), 3.93 (1H, *m*, H-2), 3.98 (1H, *m*, H-3). ^{13}C NMR ($CDCl_3$): δ 10.1 (C-28), 11.9 (C-21), 12.0 (C-18), 15.3 (C-19), 20.7 and 20.8 (C-26 and C-27), 21.2 (C-11), 23.3 (C-1), 23.8 (C-15), 27.7 (C-16), 30.5 (C-25), 36.8 (C-20), 37.4 (C-8), 39.4 and 39.6 (C-4 and C-12), 40.0 (C-24), 41.0 (C-10), 42.8 (C-13), 46.6 (C-7), 51.5 (C-5), 52.3 (C-17), 54.5 (C-9), 56.6 (C-14), 69.7 (C-3), 70.5 (C-2), 73.5 (C-23), 74.7 (C-22), 212.5 (C-6). For mass spectral analysis, [26-$^2H_3$]-**7** was converted to the dansyl-3-aminophenylboronate and subjected to HPLC–ESI-MS as described below (R_t 13.0 min, *m*/*z* 802).

3.3. Isolation of endogenous brassinosteroids

Leaves (80 g) of 14-day-old rye seedlings of *Secale cereale*, cv. "Sorom" were homogenized using an Ultra–Turrax and extracted with MeOH (3 × 300 ml). The extract was evaporated to a residue and partitioned between equal volumes of EtOAc and 0.5 M K_2HPO_4 (3 × 100 ml). The EtOAc phase was evaporated and partitioned between *n*-hexane and 80% MeOH (3 × 100 ml). The 80% MeOH extract was evaporated to dryness and partitioned between $CHCl_3$ and water (3 × 100 ml). After evaporation the $CHCl_3$ phase was partitioned between *n*-hexane and 80% MeCN (3 × 100 ml). The MeCN extract was evaporated and subjected to TLC (silica gel 60 F_{254} 0.5 mm thickness, 200 × 200 mm; $CHCl_3$–MeOH 88:12). Based on the retention times of authentic deuterated standards, which were determined on a separate plate (compound **6**: R_f 0.41; **7**: R_f 0.31; **8**: R_f 0.34; BL: R_f 0.31; 3-epiBL: R_f 0.25), the brassinosteroid-containing zone (R_f 0.20–0.45) was collected and eluted sequentially with a mixture of $CHCl_3$–MeOH 1:1 (20 ml) and MeOH (30 ml). The combined solution was evaporated and the residue separated by reversed-phase HPLC (LiChrosphere® 100 RP-18; 10 μm; 250 × 10 mm) using a linear gradient MeCN–H_2O (0.01% trifluoro acetic acid) from 30% to 100% MeCN in 30 min and held at 100% MeCN for a further 15 min (flow rate 2 ml min^{-1} and UV detection of matrix compounds at 205 nm). Based on the retention times of authentic deuterated standards (compound **6**: R_t 24.6 min; **7**: R_t 22.5 min; **8**: R_t 23.7 min; BL: R_t 20.9 min; 3-epiBL: R_t 19.1 min), the brassinosteroid-containing fraction (R_t 19.0–27.0 min) was collected and evaporated. The residue was converted to 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 MeCN (1:19) and used for analysis by HPLC–ESI-MS.

Seeds (30 g) of *Secale cereale*, cv. "Sorom" were soaked in water (50 ml) at room temperature for 12 h, then homogenized and extracted with MeOH (3 × 100 ml). A solution of [26-$^2H_3$]3-epicastasterone (10 ng) in EtOH (10 μl) was added to the extract, which was evaporated and partitioned between equal volumes of AcOEt and 0.5 M K_2HPO_4 (3 × 100 ml). The AcOEt phase was evaporated and partitioned between *n*-hexane and 80% MeOH (3 × 100 ml). The 80% MeOH extract was evaporated to dryness and

subjected to TLC followed by reversed-phase HPLC, conversion to dansyl-3-aminophenylboronates using the conditions described above for isolating brassinosteroids from leaves. HPLC–ESI-MS (Svatoš et al., 2004) was used for analysis.

3.4. Mass spectrometry

HPLC–MS analyses were carried out using the electrospray ionization technique (ESI) in the single ion monitoring (SIM) mode. Dansyl-3-aminophenylboronates were injected into an Agilent 1100 LC system coupled to a Micromass Quattro II tandem quadrupole mass spectrometer operating in the positive ion mode. A Macherey-Nagel Nucleodur 100-3 C18 column (100×1 mm, 3 μm) and a gradient MeCN-0.1% HCOOH in H_2O (0–10 min: 75% MeCN; 10–15 min: 75–100% MeCN; 15–25 min: 100% MeCN; flow rate 0.05 ml min^{-1}) were used for separation. Identification of brassinosteroid mono-dansyl-3-aminophenylboronate derivatives was based on retention times of corresponding dansyl-3-aminophenylboronates of authentic deuterated standards (Table 1) and the corresponding deuterated compounds (Figs. 1(b) and 2(c)). Each sample run was followed by injecting blank sample (HLPC pure methanol) to limit sample carryover and to effectively clean the RP columns and the injector needle. After injecting 3 samples a mixture of standards was analyzed to check for R_t stability.

3.5. Administration of deuterated precursors

Biosynthetic experiments were performed using freshly harvested leaves of 14-day-old seedlings of *Secale cereale* cv. "Sorom". [26-$^2H_3$]Secasterone (**2**), [26-$^2H_3$]2,3-diepisecasterone (**3**), [26-$^2H_3$]2-epicastasterone (**7**), and [26-$^2H_3$]3-epicastasterone (**8**) were administered separately to plant material. In typical experiments, the respective deuterated precursor (10 μg) was dissolved in 75% EtOH (10 μl) and added to a flask containing freshly harvested leaves (35 g) immersed in water (30 ml). The precursor was absorbed hydroponically with the transpiration stream and incubated for 70 h, during which time the feeding solution was periodically complemented with water. The plant material was homogenized using an Ultra–Turrax and extracted with MeOH (3×300 ml). The analytical procedure described above was employed to identify endogenous brassinosteroids.

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References

Adam, G., Schmidt, J., Schneider, B., 1999. Brassinosteroids. In: Herz, W., Falk, H., Kirby, G.W., Moore, R.E., Tamm, C. (Eds.), Progress in the Chemistry of Organic Natural Products, vol. 78. Springer, Vienna, pp. 1–46.

Antonchick, A.P., Schneider, B., Zhabinskii, V.N., Konstantinova, O.V., Khripach, V.A., 2003. Biosynthesis of 2,3-epoxybrassinosteroids in seedlings of *Secale cereale*. Phytochemistry 63, 771–776.

Bajguz, A., Tretyn, A., 2003. The chemical characteristic and distribution of brassinosteroids in plants. Phytochemistry 62, 1027–1046.

Barth, S., Fischer, M., Schmid, R.D., Pleiss, J., 2004. Sequence and structure of epoxide hydrolases: a systematic analysis. Proteins 55, 846–855.

Blée, E., Schuber, F., 1993. Biosynthesis of cutin monomers – involvement of a lipoxygenase/peroxygenase pathway. Plant J. 4, 113–123.

Brosa, C., 1999. Structure-activity relationship. In: Sakurai, A., Yokota, T., Clouse, S.D. (Eds.), Brassinosteroids – Steroidal Plant Hormones. Springer, Tokyo, pp. 191–222.

Choi, Y.H., Fujioka, S., Nomura, T., Harada, A., Yokota, T., Takatsuto, S., Sakurai, A., 1997. An alternative brassinolide biosynthetic pathway via late C-6 oxidation. Phytochemistry 44, 609–613.

De-Eknamkul, W., Potduang, B., 2003. Biosynthesis of β-sitosterol and stigmasterol in *Croton sublyratus* proceeds via a mixed origin of isoprene units. Phytochemistry 62, 389–398.

Eaton, D.L., Bammler, T.K., 1999. Concise review of the glutathione S-transferase and their significance to toxicology. Toxicol. Sci. 49, 156–164.

Fretland, A.J., Omiecinski, C.J., 2000. Epoxide hydrolases: biochemistry and molecular biology. Chem.–Biol. Interact. 129, 41–59.

Friebe, A., Volz, A., Schmidt, J., Voigt, B., Adam, G., Schnabl, H., 1999. 24-*Epi*-secasterone and 24-*epi*-castasterone from *Lychnis viscaria* seeds. Phytochemistry 52, 1607–1610.

Fujioka, S., 1999. Natural occurrence of brassinosteroids in the plant kingdom. In: Sakurai, A., Yokota, T., Clouse, S.D. (Eds.), Brassinosteroids – Steroidal Plant Hormones. Springer, Tokyo, pp. 21–45.

Fujioka, S., Takatsuto, S., Yoshida, S., 2002. An early C-22 oxidation branch in the brassinosteroid biosynthetic pathway. Plant Physiol. 130, 930–939.

Fürst, A., Plattner, P.A., 1949. Über Steroide und Sexualhormone. 160. 2-Alpha,3-alpha- und 2-beta,3-beta-Oxido-cholestane – Konfiguration der 2-Oxy-cholestane. Helv. Chim. Acta 32, 275–283.

Gamoh, K., Brooks, C.J.W., 1993. Stability and reversed-phase liquid chromatographic studies of cyclic boronates. Anal. Sci., 549–552.

Khripach, V.A., Zhabinskii, V.N., de Groot, A.E., 1999. Brassinosteroids – a New Class of Plant Hormones. Academic Press, San Diego.

Khripach, V.A., Zhabinskii, V.N., Konstantinova, O.V., Antonchick, A.P., Schneider, B., 2002. Synthesis of [26-$^2H_3$]brassinosteroids. Steroids 67, 587–595.

Kolbe, A., Schneider, B., Porzel, A., Adam, G., 1998. Metabolic inversion of the 3-hydoxy function of brassinosteroids. Phytochemistry 48, 467–470.

Konstantinova, O.V., Antonchick, A.P., Oldham, N.J., Zhabinskii, V.N., Khripach, V.A., Schneider, B., 2001. Analysis of underivatized brassinosteroids by HPLC/APCI-MS. Occurrence of 3-epibrassinolide in *Arabidopsis thaliana*. Collect. Czech. Chem. Commun 66, 1729–1734.

Muller, F., Arand, M., Frank, H., Seidel, A., Hinz, W., Winkler, L., Hanel, K., Beetham, J.K., Hammock, B.D., Oesch, F., 1997. Visualization of a covalent intermediate between microsomal

epoxide hydrolase, but not cholesterol epoxide hydrolase, and their substrates. Eur. J. Biochem. 245, 490–496.

Noguchi, T., Fujioka, S., Choe, S., Takatsuto, S., Tax, F.E., Yosihida, S., Feldman, K.A., 2000. Biosynthetic pathway of brassinolide in *Arabidopsis*. Plant Physiol. 124, 201–209.

Ollis, D.L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S.M., Harel, M., Remington, S.J., Silman, I., Schrag, J., Sussmann, J.L., Verschueren, K.H.G., Goldmann, A., 1992. The α/β hydrolase fold. Protein Eng. 5, 197–211.

Sakurai, A., 1999. Biosynthesis. In: Sakurai, A., Yokota, T., Clouse, S.D. (Eds.), Brassinosteroids – Steroidal Plant Hormones. Springer, Tokyo, pp. 91–111.

Schmidt, J., Spengler, B., Yokota, T., Nakayama, M., Takatsuto, S., Voigt, B., Adam, G., 1995. Secasterone, the first naturally occurring 2,3-epoxybrassinosteroid from *Secale cereale*. Phytochemistry 38, 1095–1097.

Schneider, B., 2002. Pathways and enzymes of brassinosteroid biosynthesis. In: Esser, K., Lüttge, U., Beyschlag, W., Hellwig, F. (Eds.), Progress in Botany, vol. 63. Springer, Berlin, pp. 286–306.

Schwender, J., Zeidler, J., Groner, R., Muller, C., Focke, M., Braun, S., Lichtenthaler, F.W., Lichtenthaler, H.K., 1997. Incorporation of 1-deoxy-D-xylulose into isoprene and phytol by higher plants and algae. FEBS Lett. 414, 129–134.

Summerer, S., Hanano, A., Utsumi, S., Arand, M., Schuber, F., Blée, E., 2002. Stereochemical features of the hydrolysis of 9,10-epoxystearic acid catalysed by plant and mammalian epoxide hydrolases. Biochem. J. 366, 471–480.

Suzuki, H., Fujioka, S., Takatsuto, S., Yokota, T., Murofushi, N., Sakurai, A., 1994. Biosynthesis of brassinolide from teasterone via typhasterol and castasterone in cultured cells of *Catharanthus roseus*. J. Plant Growth Regul. 13, 21–26.

Svatoš, A., Antonchick, A.P., Schneider, B., 2004. Determination of brassinosteroids in the sub-femtomolar range using dansyl-3-aminophenylboronate derivatization and electrospray mass spectrometry. Rapid Commun. Mass Sp. 18, 816–821.

Voigt, B., Porzel, A., Adam, G., Golsch, D., Adam, W., Wagner, C., Merzweiler, K., 2002. Synthesis of 2,24-diepicastasterone and 3,24-diepicastasterone as potential brassinosteroid metabolites of the cockroach *Periplaneta americana*. Collect. Czech. Chem. Commun 67, 91–102.

Yokota, T., 1999. Brassinosteroids. In: Hooykaas, P.J.J., Hall, M.A., Libbenga, K.R. (Eds.), Biochemistry and Molecular Biology of Plant Hormones. Elsevier, Amsterdam, pp. 277–293.