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Biosynthesis of 2,3-epoxybrassinosteroids in seedlings of *Secale cereale*

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Abstract

Two new brassinosteroids, (22*R*,23*R*,24*S*)-22,23-dihydroxy-24-methyl-5α-cholest-2-en-6-one (secasterol) and (22*R*,23*R*,24*S*)-22,23-dihydroxy-2α,3α-epoxy-24-methyl-5α-cholest-6-one (2,3-diepisecasterone) have been identified together with a known 2,3-epoxybrassinosteroid, secasterone, in seedlings of *Secale cereale*. Deuterated secasterol, teasterone, and typhasterol, upon administration to rye seedlings, were incorporated into secasterone and 2,3-diepisecasterone, indicating a biosynthetic route via teasterone/typhasterol to secasterol to 2,3-epoxybrassinosteroids.

Keywords: *Secale cereale*; Gramineae; Biosynthesis; Brassinosteroids; Deuterium labeling; Epoxides; Secasterol; Secasterone

1. Introduction

Brassinosteroids represent plant hormones with essential effects on plant growth and development. Typical members of this class of steroidal compounds, such as brassinolide and castasterone, are characterised by vicinal 2α,3α- and 22,23-dihydroxy functionalities in ring A and in the side chain, respectively, and a 6-oxo or 7-oxalactone moiety in ring B. Brassinosteroids exhibiting these structural features and their biosynthetic precursors are ubiquitously distributed in the plant kingdom (Adam et al., 1999; Fujioka, 1999; Khripach et al., 1999; Bajguz and Tretyn, 2003). The biosynthetic functionalisation of the steroidal skeleton has been established by means of labeling experiments and mutant studies in *Arabidopsis thaliana* (Noguchi et al., 2000; Fujioka et al., 2002) and *Catharanthus roseus* (Suzuki et al., 1994; Choi et al., 1997). In addition to the typical substitution pattern, brassinosteroids possessing unusual functional groups have been found in several plants. 2,3-Epoxybrassinosteroids such as secasterone (**1**) from *Secale cereale* (Schmidt et al., 1995) and 24-episecasterone from *Lychnis viscaria* (Friebe et al., 1999) represent the first examples with an epoxide structure in ring A. The occurrence of such compounds in both mono- and dicotyledonous plants suggests a more general distribution in plants but the biosynthesis of 2,3-epoxybrassinosteroids has not been investigated. In this study, seedlings of *Secale cereale* were analysed for the occurrence of brassinosteroids with specific emphasis on 2,3-epoxybrassinosteroids and putative biosynthetic precursors. Feeding experiments using deuterated intermediates were performed in the same plant species to investigate biosynthetic incorporation into 2,3-epoxybrassinosteroids.

2. Results and discussion

2.1. Synthesis of deuterated precursors and analytical standards

The common occurrence of secasterone (**1**), teasterone (**3**), typhasterol (**4**), and further brassinosteroids in seeds of *Secale cereale* (Schmidt et al., 1995) suggested a biogenetic relationship between these sterols and prompted the study of the biosynthesis of 2,3-epoxybrassinosteroids in

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plants. As a prerequisite for these investigations, [26-$^2H_3$]secasterone (**1**) and [26-$^2H_3$]2,3-diepisecasterone (**2**) were synthesised as deuterated analytical standards, and deuterated teasterone (**3**), typhasterol (**4**), and (22*R*,23*R*,24*S*)-22,23-dihydroxy-24-methyl-5α-cholest-2-en-6-one (**5**) were synthesised as putative biosynthetic precursors of **1**. The synthesis of deuterated 2,3-epoxides **1** and **2** using Claisen rearrangement in the construction of the side chain and reductive introduction of deuterium from $LiAlD_4$ was published by Khripach et al. (2002). The [26-$^2H_3$]-olefin **5** (Zhou et al., 1990; Voigt et al., 1995) was prepared by alkaline deprotection of its 22,23-di-*O*-acetyl derivative, which is an intermediate in the synthesis of deuterated secasterone (**1**) and 2,3-diepisecasterone (**2**) (Khripach et al., 2002). The synthesis of [25,26-$^2H_6$]teasterone (**3**) will be published elsewhere.

2.2. Identification of naturally occurring brassinosteroids

Two varieties of *Secale cereale*, cv. "Sorom" and cv. "Petka", were analysed in order to evaluate their suitability to study the biosynthesis of steroid 2,3-epoxides. In previous studies rye seeds (Schmidt et al., 1995) and *Lychnis viscaria* (Friebe et al., 1999) were used for isolation of steroid 2,3-epoxides. In our investigations seedlings were used because, in comparison with seeds, they are more suitable for feeding experiments. The leaves and roots of rye seedlings were extracted separately with MeOH and [26-$^2H_3$]secasterone (**1**) and [26-$^2H_3$]2,3-diepisecasterone (**2**) were added as internal standards before evaporation to a residue. After partitioning and chromatographic separation the fractions were converted to methaneboronates and subjected to GC–MS. Identification and quantification was based on retention time and relative intensities of three ions of deuterated compounds **1** and **2** (*m*/*z* 473, $[M]^{\cdot+}$, 245 [fragment containing ring system without D-ring], 158 [side chain fragment]) (Fig. 1) and corresponding fragments of unlabeled compounds (*m*/*z* 470, 245, 155) in the SIM mode. Both secasterone (**1**) (R_t 11.48 min) and 2,3-diepisecasterone (**2**) (R_t 11.20 min) were found in leaves (**1**: 52 pg g^{-1} fw; **2**: 20 pg g^{-1} fw) and roots (**1**: 107 pg g^{-1} fw; **2**: 32 pg g^{-1} fw) of the variety "Sorom". In the variety "Petka", only 2,3-diepisecasterone (**2**) was identified unambiguously (102 pg g^{-1} fw in the leaves and 22 pg g^{-1} fw in the roots) and no substantial amounts of **1** were detected. This is the first identification of 2,3-diepisecasterone (**2**) as a naturally occurring compound while secasterone (**1**) has been identified already earlier in seeds of *Secale cereale* (Schmidt et al., 1995).

The occurrence of the olefin **5**, (22*R*,23*R*,24*S*)-22,23-dihydroxy-24-methyl-5α-cholest-2-en-6-one, in leaves of the variety "Sorom" was determined separately using a procedure closely resembling that for the analysis of the 2,3-epoxides. The retention time on the GC column (R_t 8.92 min) of **5** from the plant extract was identical with that of the deuterated standard. Moreover, MS-SIM of three ions (*m*/*z* 458, 443, 430) (Fig. 1) of that GC peak clearly showed relative intensities consistent with synthetic compound **5**. (22*R*,23*R*,24*S*)-22,23-Dihydroxy-24-methyl-5α-cholest-2-en-6-one (**5**) is described here for the first time as a natural product for which the common name secasterol is proposed.

2.3. Biosynthetic hypothesis

Epoxides play a role as intermediates and products of a variety of biosynthetic pathways in plants. Examples are epoxidations involved in the biosynthesis and degradation of lipid-derived natural products such as absisic acid (Cutler and Krochko, 1999), the formation of acridone epoxide (Bohlmann and Eilert, 1994) and scopolamine from hyoscyamine (Hashimoto et al., 1993). Most important for steroid biosynthesis is the conversion of squalene to 2,3-epoxysqualene, an early steroid precursor. This process represents the only epoxidation of an olefin so far known that is catalyzed by a flavoprotein (Jandrositz et al., 1991). Steroid 5,6-epoxides have been discussed as intermediates in phytoecdysone biosynthesis (Fujimoto et al., 2000), and play a role in age-related processes in the plant cell (Meyer and Spiteller, 1997). Both secondary aliphatic alcohols and olefins can function as direct biosynthetic precursor of epoxides (Hashimoto et al., 1993). Since there is a constitutive hydroxyl group in position 3 of the steroid skeleton, this functionality was considered to

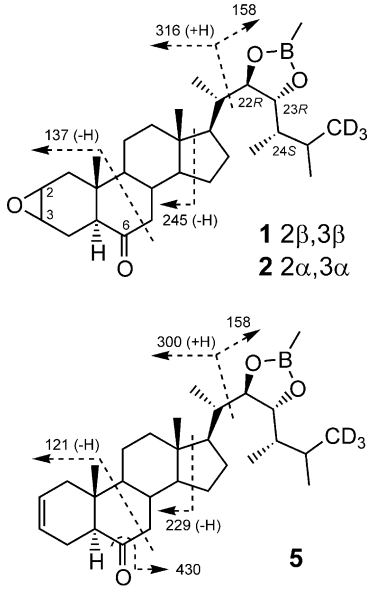

Fig. 1. MS fragmentation patterns of methaneboronate derivatives of deuterated standards [26-$^2H_3$]secasterone (**1**), [26-$^2H_3$]2,3-diepisecasterone (**2**), and [26-$^2H_3$]secasterol (**5**).

be a candidate to be stereospecifically converted to the 2,3-epoxide moiety, i.e. formation of 2α,3α-epoxides from 3α-precursors and 2β,3β-epoxides from 3β-precursors, respectively (Fig. 2). Alternatively, an olefinic steroid with a $\Delta^{2,3}$-double bond was taken into consideration as a putative precursor of both steroid 2α,3α- and 2β,3β-epoxides.

2.4. Biosynthesis

Excised seedlings of the rye cultivar "Sorom" were used for biosynthetic experiments. The putative precursors were administered by immersion of the seedlings into the feeding solution. Separate feeding experiments using precursors, which are hydroxylated at positions 3α ([26-$^2H_3$]typhasterol, **4**) or 3β ([26,27-$^2H_6$]teasterone, **3**) resulted in the conversion to both deuterated secasterone (**1**) and 2,3-diepisecasterone (**2**). Bi-directional inversion of configuration of a portion of each of the precursors **3** and **4** prior to epoxidation could explain the formation of 2,3-epoxysteroids **1** and **2** of both configurations. Alternatively, a $\Delta^{2,3}$-double bond could have been formed enzymatically, by dehydrogenase or reductase and desaturase activity, followed by formation of two stereoisomeric epoxides. Simultaneous administration of equal amounts of [26,27-$^2H_6$]teasterone (**3**) and [26-$^2H_3$]typhasterol (**4**) also resulted in [$^2H_3$]- and [$^2H_6$]-isotopomers of secasterone (**1**) and 2,3-diepoxysecasterone (**2**) but, in comparison with feeding of **5**, the extent of incorporation was significantly lower. In addition, both triply- and sixfold deuterated isotopomers of secasterol (**5**) were identified in the extracts obtained from this feeding experiment. Hence, compound **5** was suggested as an intermediate in the secasterone (**1**) biosynthesis, but it was not possible to determine whether inversion of configuration played a role in this pathway. However, since reversible interconversion between 3α-OH- and 3β-OH-brassinosteroids via the 3-keto derivative has been demonstrated several times (Kolbe et al., 1998; Noguchi et al., 2000), this possibility cannot be ruled out in these experiments with rye seedlings. Further feeding experiments using [26-$^2H_3$]secasterol (**5**) as a precursor proved its role as central intermediate in secasterone (**1**) and 2,3-diepisecasterone (2) biosynthesis (Fig. 3). Triply deuterated isotopomers of both stereoisomeric 2,3-epoxybrassinosteroids **1** and **2** were detected upon administration of deuterated compound **5**. Moreover, feeding [26,27-$^2H_6$]teasterone (**3**) in combination with equal amounts of [26-$^2H_3$]secasterol (**5**) to rye seedlings resulted in labeled **1** and **2** as well. Interestingly, the levels of the triply labeled isotopomers derived from **5** were significantly higher compared to the levels of the sixfold deuterated isotopomers coming from **3**, indicating a more distant precursor—product relation for teasterone (**3**) with both epoxides **1** and **2** than for secasterol (**5**) with the same products. From these findings, the biosynthetic sequence teasterone (**3**)/typhasterol (**4**)→secasterol (**5**)→secasterone (**1**)/2,3-diepisecasterone (**2**) (Fig. 3) has been demonstrated to be operative in *Secale cereale*.

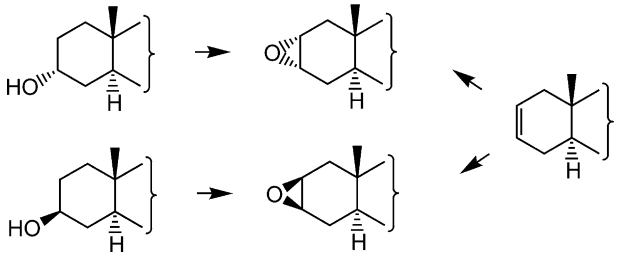

Fig. 2. Hypothetical alternative biosynthetic formation of 2,3-epoxysteroids from 3-hydroxy- or 2,3-olefinic precursors.

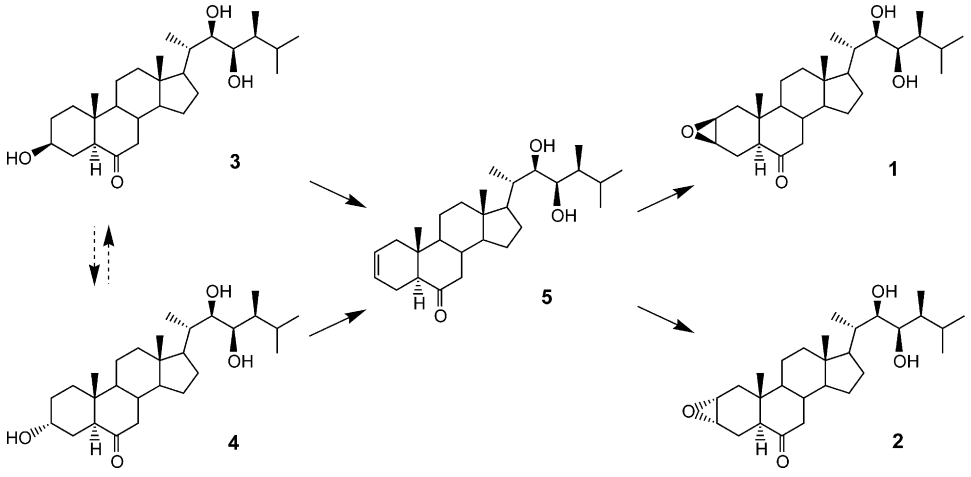

Fig. 3. Biosynthesis in secasterone (**1**) and 2,3-diepisecasterone (**2**) from teasterone (**3**) and/or typhasterol (**4**) via secasterol (**5**) in seedlings of *Secale cereale*. Dashed arrows: hypothetical steps.

3. Experimental

3.1. Plant materials

Seeds of *Secale cereale* cv. "Sorom" and "Petka" were purchased from Lochow-Petkus GmbH, Bergen-Wohlde, Germany. Prior to germination, seeds were stratificized and then grown in the green house 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 and a temperature of 22–24 °C.

3.2. Spectroscopic methods

GC–MS–SIM analysis of brassinosteroid 22,23-monomethaneboronates was carried out on ThermoQuest CE Instruments TraceGC-2000 series (Finnigan) at 70 eV with a capillary DB-5 column (0.25 mm×15 m, 0.25 μm film thickness, J&W Scientific). The carrier gas was He at a flow rate of 1 ml min^{-1} (constant flow), the injector temperature was 280 °C, and the samples were injected by splitless injection. The column oven temperature was programmed at 170 °C for 2 min, before being elevated to 280 °C at 40 °C min^{-1}, and then to 320 °C at 1.5 °C min^{-1}. ^{1}H, ^{13}C NMR and ^{1}H–^{1}H COSY spectra of synthetic brassinosteroids were measured on a Bruker Avance DRX 500 NMR spectrometer, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. Chemical shifts are given in δ values relative to TMS as internal standard.

3.3. Synthesis of secasterol (*5*)

A mixture of (22*R*,23*R*,24*S*)-[26-$^{2}H_3$]22,23-diacetoxy-24-methyl-5α-cholest-2-en-6-one (1.53 mg, 2.96 μmol, Khripach et al., 2002) and MeONa [138 μl, 48.7 μmol, prepared from Na (73 mg) and MeOH (9 ml)] was stirred at room temperature for 7 days after which the reaction mixture was chromatographed over silica to give (22*R*,23*R*,24*S*)-[26-$^{2}H_3$]22,23-dihydroxy-24-methyl-5α-cholest-2-en-6-one (secasterol, **5**) (1.15 mg, 2.65 μmol, 89.5%). ^{1}H NMR (500 MHz, $CDCl_3$, assigned proton signals only) δ 0.70 (3H, *s*, CH_3-19 or CH_3-18), 0.72 (3H, *s*, CH_3-18 or CH_3-19), 0.85 (3H, *dd*, J =6.9, 1.0 Hz, CH_3-28), 0.92 (3H, *d*, J =6.8, CH_3-21), 0.95 (3H, *d*, J =6.8, CH_3-26 or CH_3-27), 0.97 (3H, *d*, J =6.8, CH_3-27 or CH_3-26), 1.23 (1H, *m*, H-24), 1.51 (1H, *m*, H-20), 2.00 (2H, *m*, H-1 and H-4), 3.56 (1H, *dd*, J=1.5, 8.4 Hz, H-22), 3.71 (1H, *dm*, J=8.4 Hz, H-23), 5.56 (1H, *m*, H-2 or H-3), 5.68 (1H, *m*, H-2 or H-3). ^{13}C NMR (125 MHz, $CDCl_3$) δ 10.10, 10.12, 11.86, 11.92, 13.53, 20.68, 21.15, 21.71, 23.82, 27.64, 30.54, 36.79, 37.77, 39.33, 39.51, 39.98, 40.06, 42.66, 46.96, 52.32, 53.31, 53.82, 56.63, 73.55, 74.76, 124.51, 124.95, 212.05. GC–MS–EI (monomethaneboronate, 70 eV): *m*/*z* (rel. int.) 107 (52), 121 (38), 158 (33), 211 (14), 229 (22), 253 (6), 283 (10), 430 (30) $[M–CO]^{+}$, 440 (19), 443 (100) $[M–CH_3]^{+}$, 458 (61) $M^{\cdot +}$.

3.4. Identification of secasterone (*1*) and 2,3-diepisecasterone (*2*)

The 18-day-old rye seedlings of varieties "Petka" and "Sorom" were separated and the roots and leaves used separately. Leaves were homogenised using an Ultra-Turrax and extracted with MeOH (2×500 ml). [26-$^{2}H_3$]Secasterone (**1**) (500 ng) and [26-$^{2}H_3$]2,3-diepisecasterone (**2**) (500 ng) (Khripach et al., 2002) were added to each of the MeOH extracts (obtained from 145 g leaves of "Petka", 134 g leaves of "Sorom", 90 g roots of "Petka", and 95 g roots of "Sorom", respectively) as internal standards before evaporation. The residue was partitioned between equal volumes of EtOAc and 0.5 M K_2HPO_4 (3×100 ml). The EtOAc phase obtained from leaves was evaporated and partitioned between *n*-hexane and 80% MeOH (3×100 ml). The 80% MeOH extract from leaves and the EtOAc extract from roots, respectively, were evaporated to dryness and partitioned between *n*-hexane and MeOH (100 ml). The MeOH phase was evaporated, re-dissolved in MeOH (10 ml), sonified for 15 min and centrifuged for 15 min at 4000 min^{-1} at 15 °C. The supernatant was evaporated and subjected to TLC (silica gel 60 F_{254} 0.5 mm thickness, 200×200 mm, Merck; $CHCl_3$–MeOH 88:12). Based on the retention times of authentic standards, which were determined on a separate plate (compound **1**: R_f 0.66; **2**: R_f 0.69), the brassinosteroid-containing zone (R_f 0.63–0.72) 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 (LiChrospher® 100 RP-18; 10 μm; 250×10 mm) using isocratic conditions (MeCN–H_2O 80:20, 0.01% trifluoro acetic acid; 2 ml min^{-1}) and UV detection of matrix compounds at 205 nm. Based on the retention times of authentic standards (compound **1**: R_t 25.1 min; **2**: R_t 26.2 min), the brassinosteroid-containing fraction (R_t 24–28 min) was collected and evaporated. The residue was converted to monomethaneboronates by heating at 62 °C for 30 min with a solution of methaneboronic acid (2 mg ml^{-1}) in a mixture of pyridine and acetonitrile (1:19) and used for analysis by GC–MS–SIM. Identification of secasterone (**1**) and 2,3-diepisecasterone (**2**) was based on retention time (compound **1**: R_t 11.48 min; **2**: R_t 11.20 min) and relative intensities of three ions, which were consistent with those of corresponding deuterated standards {[26-$^{2}H_3$]secasterone (**1**): R_t 11.48 min, *m*/*z* (rel. int.) 158 (100), 245 (38), 473 (35); [26-$^{2}H_3$]2,3-diepisecasterone (**2**): R_t 11.20 min, *m*/*z* (rel. int.) 158 (100), 245 (47), 473 (30)}. The content of compounds **1** and **2** was calculated from the peak area ratios of $^{2}H_3$ $M^{\cdot +}$ and $^{2}H_0$ $M^{\cdot +}$ ions.

3.5. Identification of (22R,23R,24S)-22,23-dihydroxy-24-methyl-5α-cholest-2-en-6-one (secasterol, 5)

Leaves (80 g) of 14-day-old rye plants, cv. "Sorom", were homogenized using an Ultra-Turrax, extracted with MeOH (3×300 ml) and evaporated. The residue was 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). The $CHCl_3$ extract was evaporated and partitioned between *n*-hexane and 80% MeCN (3×100 ml). The MeCN extract was evaporated to dryness and the residue subjected to TLC as described above. Based on the retention times of authentic standard compound **5** (R_f 0.71), which was determined on a separate plate, the brassinosteroid-containing zone (R_f 0.68–0.74) 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 (LiChrospher® 100 RP-18; 10 μm; 250×10 mm) using MeCN–H_2O containing 0.01% trifluoro acetic acid as a mobile phase (0 min: 80% MeCN, 30 min: 80% MeCN: 31 min: 100% MeCN; 50 min: 100% MeCN; flow rate 2 ml min^{-1}) and UV detection of matrix compounds at 205 nm. Based on the retention time of authentic standard **5** (R_t 44.8 min), the brassinosteroid-containing fraction (R_t 43–47 min) was collected, evaporated, converted to the monomethaneboronate, and identified by GC–MS–SIM as described above. Retention time (R_t 8.92 min) and relative intensities of three ions [*m*/*z* (rel. int.) 430 (30), 443 (100), 458 (61)] of compound **5** was consistent with the data of the deuterated standard, (22*R*,23*R*,24*S*)-[26-$^2H_3$]22,23-dihydroxy-24-methyl-5α-cholest-2-en-6-one.

3.6. Administration of deuterated precursors

Biosynthetic experiments were performed using excised seedlings of *Secale cereale* cv. "Sorom". In typical experiments, the respective deuterated precursor (10 μg) was dissolved in 75% EtOH (20 μl) and added to a flasks containing freshly harvested 13- to 14-day-old leaves (30–110 g) immersed into 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. Extraction with MeOH (3×200 ml), separation, and the analytical procedure as described above for the identification of compounds **1**, **2**, and **5**, has been employed, respectively. Each of the deuterated precursors available, i.e. [26,27-$^2H_6$]teasterone (**3**), [26-$^2H_3$]typhasterol (**4**), and [26-$^2H_3$]secasterol (**5**) was administered individually. In addition, feeding experiments were carried out using $^2H_3$- and $^2H_6$-labelled precursors in combination, i.e. compound **3** together with **4**, and **3** with **5**, respectively.

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