Metabolic Transformation of the Brassinosteroid 24-*Epi*-castasterone by the Cockroach *Periplaneta americana*

Jürgen Schmidt[a,*], Klaus Richter[b], Brunhilde Voigt[a] and Günter Adam[a]

[a] Leibniz-Institut für Pflanzenbiochemie, Abteilung Naturstoffchemie, Weinberg 3, D-06120 Halle/S., Germany. Fax: 0345–5582166. E-mail: jschmidt@ipb.uni-halle.de
[b] Sächsische Akademie der Wissenschaften, Forschungsgruppe Jena, Neugasse 23, D-07743 Jena, Germany

* Author for correspondence and reprint request

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After feeding of 24-*epi*-castasterone to the cockroach *Periplaneta americana* an organ-specific epimerization of the brassinosteroid to 2,24-*diepi*-castasterone could be detected in female insects. The metabolite being observed only in the ovaries and not in the testes of the insect was identified by GC/MS in comparison with a synthesized authentic sample. Contrary, 24-*epi*-brassinolide is not metabolized in the sexual organs of *Periplaneta americana.* This is the first evidence of a metabolic transformation of a brassinosteroid in insects.

Introduction

Brassinosteroids represent a group of ubiquiteous occurring phytohormones with a high plant growth-promoting and anti-stress activity (Khripach *et al.,* 1999; Sakurai *et al.*, 1999). More than 40 naturally occurring members of this type of compounds have been found in a wide variety of higher plants (Adam *et al.,* 1999; Khripach *et al.,* 1999; Sakurai *et al.*, 1999). Brassinosteroids have striking structural similarity with insect hormones of the ecdysteroid type (Adler and Grebenok, 1995; Lafont, 1997). Ecdysteroids are the main biosynthetic products of moulting glands in arthropods. Members of this group of polyhydroxylated sterols act as moulting hormones.

The structural similarities between brassinosteroids and ecdysteroids gave rise to studies on the bioactivity of brassinosteroids on arthropods, whereas also antiecdysone activity of some members has been found (Richter and Koolman, 1991; Spindler *et al.,* 1992; Sobek *et al.,* 1993; Charrois *et al.,* 1996). Especially, upon feeding of nymphs of the cockroach *Periplaneta americana* with several brassinosteroids the duration of the last larval instar was increased leading to retardation of the moulting process (Richter *et al.,* 1987). Also a neurodepressing effect of brassinosteroids in the cockroach has been observed (Richter and Adam, 1991). However, whereas first metabolic transformations of brassinosteroids in microorganism have been reported (Voigt *et al.*, 1993a; 1993b), till now nothing is known about the resorption and metabolic transformation of brassinosteroids applied in insects. This paper describes the identification of a first brassinosteroid metabolite of the native phytohormone 24-*epi*-castasterone (**1**) (Scheme 1) formed in ovaries of *Periplaneta americana* after feeding.

Materials and Methods

Animals and feeding experiments

Nymphs and adult males and females of the cockroach *Periplaneta americana* were selected from mass culture and used for the experiments on the fifth day after imaginal moult and on the 23rd day in the last instar. Animals were kept in groups of 5 in piacryl containers with perforated lids (15 × 25 × 10 cm) under constant conditions (28 °C, 50–60% relative humidity, 12:12 light-dark cycle, water *ad libitum).* A methanolic solution of the brassinosteroid was mixed with pulverized rat standard food (16 mg brassinosteroid, 2.5 g food). During the feeding period 3.2 mg brassinosteroid in 0.5 g food was available statistically to each of the 5 animals. Four days before start of the feeding experiments, animals were reared without food. Consumption of the food preparations was different. Females consumed food completely already after one day, males and nymphs used in

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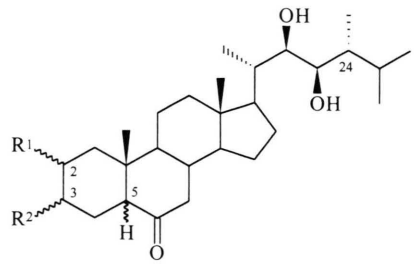


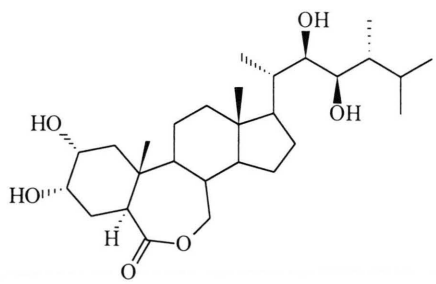
1: R^1=α-OH, R^2=α-OH, 5α-H (24-*epi*-castasterone)

2: R^1=β-OH, R^2=α-OH, 5α-H (2,24-*diepi*-castasterone)

3: R^1=α-OH, R^2=β-OH, 5α-H (3,24-*diepi*-castasterone)

4: R^1=β-OH, R^2=β-OH, 5α-H (2,3,24-*triepi*-castasterone)

5: R^1=β-OH, R^2=β-OH, 5β-H (2,3,5,24-*tetraepi*-castasterone)



24-*epi*-brassinolide (**6**)

Scheme 1. Structures of the brassinosteroids **1**–**6**.

the same time the half. Feeding deterrent effects, behavioral changes or other biological effects of the brassinosteroids could not be observed during the feeding period.

Two days after start of the feeding period three animals of each group were decapitated and the following organs were prepared: fatbody, malpighian tubules, midgut, hindgut and from adults additionally ovaries and testes. During the feeding period feces were collected.

Isolation of the brassinosteroids and metabolites

Organs of three animals were sonicated immediately after preparation in 150 µl ice-cold Ringers solution and pooled for extraction with methanol (500 µl, 3 ×). After filtration the extract was concentrated *in vacuo* (ovaries: 4.6 mg, testes: 7.1 mg). After partition of the residue between water and chloroform (3x) the chloroform extract (ovaries: 2 mg, testes: 1.4 mg) was further purified by DEA (Analytichem Bondesil, Preparative Grade 40 µm, 100 ms, 20fold amount) using 8 ml chloroform-methanol (1: 1 v/v). The obtained concentrate was then subjected to a silica gel cartridge (500 mg, Isolute™, ICT Bad Homburg) and eluted stepwise (3 ml fractions) with increasing concentrations of methanol in chloroform (0, 2, 10, 50% v/v). After evaporating the solvent of the fractions eluted with 10% methanol in chloroform (ovaries: 1 mg, testes: 0.3 mg) an aliquot of these fractions (1/50) was analysed by gas chromatography-mass spectrometry (GC-MS) after methylboronation and methylboronation/trimethylsilylation, respectively (see below).

Gas chromatography-mass spectrometry (GC-MS)

MD-800 (Fisons Instruments; EI (70 eV); source temp. 200 °C; column DB-5MS (J&W, 15 m × 0.32 mm, 0.25 μm film thickness), injection temp. 260 °C, interface temp. 300 °C, carrier gas He, flow rate 1 ml/min, splitless injection, column temperature program: 170 °C for 1 min, then raised to 290 °C at a rate of 30 grd/min and held on this temperature for 20 min. The relative retention times (RRT) of the derivatized brassinosteroids were calculated with respect to 5α-cholestane (RT = 5.35 min). The mass spectra were normalized to the most abundant peak above *m/z* 100.

24-*Epi*-castasteronebismethylboronate (**1-BMB**). RRT = 1.84, EIMS, *m/z* (rel. int.): 512 (M^+, 18), 441 (3), 399 (6), 358 (11), 329 (5), 287 (24), 228 (6), 155 (100), 109 (25).

2,24-*Diepi*-castasteronemethylboronate-ditrimethylsilylether (isolated **2-MB-TMS**). RRT= 1.91, EIMS, *m/z* (rel. int.): 632 (M^+, 2), 617 (19), 542 (11), 527 (3), 515 (100), 473 (4), 453 (4), 426 (31), 155 (8), 147 (16).

2,24-*Diepi*-castasteronemethylboronate-ditrimethylsilylether (authentic **2-MB-TMS**). RRT= 1.92, EIMS, *m/z* (rel. int.): 632 (M^+, 2), 617 (7), 542 (7), 527 (3), 515 (100), 473 (5), 453 (1), 426 (48), 155 (15), 147 (44).

3,24-*Diepi*-castasteronebismethylboronate (**3-BMB**). RRT = 2.04, EIMS, *m/z* (rel. int.): 512 (M^+, 7), 441 (2), 399 (5), 358 (8), 329 (7), 287 (10), 245 (3), 155 (100), 111 (23).

2,3,24-*Triepi*-castasteronebismethylboronate (**4-BMB**). RRT = 1.79, EIMS, *m/z* (rel. int.): 512 (M^+, 20), 441 (2), 399 (7), 358 (12), 329 (5), 287 (20), 179 (19), 155 (100), 95 (65).

2,3,5,24-*Tetraepi*-castasteronebismethylboronate (**5-BMB**). RRT = 1.70, EIMS, *m/z* (rel. int.): 512 (M^+, 11), 441 (2), 401 (31), 359 (9), 357 (9), 341 (6), 287 (11), 245 (30), 155 (51), 112 (100).

The ring A-epimers of 24-*epi*-castasterone (**2–5**) were synthesized by Voigt *et al.* (submitted for publication).

Derivatization of the brassinosteroids

The methylboronation of the brassinosteroids was carried out by treatment of the samples with pyridine containing methylboronic acid (2 mg/ml) at 70 °C for 30 min (Takatsuto *et al.*, 1982). Further trimethylsilylation for preparing the methylboronate-trimethylsilylethers was carried out by adding *N,O*-(bistrimethylsilyl)-acetamide to the foregoing reaction mixture at room temperature for 5 min.

Results

The fed brassinosteroids, 24-*epi*-castasterone (**1**) and 24-*epi*-brassinolide (**6**), could be detected in all investigated organs of *Periplaneta americana*, i.e. in fatbody, malpighian tubules, midgut, hindgut, and feces of larvae and adult males and females (Table). The fed brassinosteroids were found in ovaries and testes from adults in traces. Additionally, some not further characterized metabolites of 24-*epi*-castasterone (**1**), probably formed by hydroxylation at the steroid skeleton, appeared in feces of males, females and nymphs.

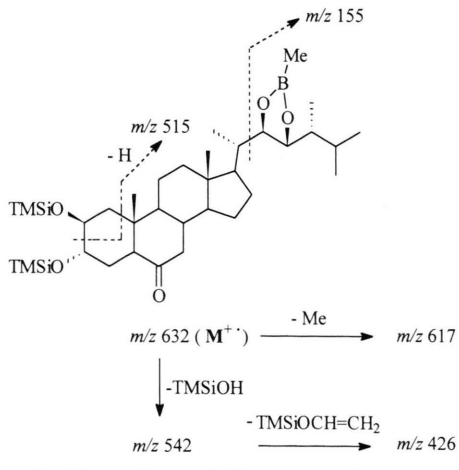
In ovaries and testes, 24-*epi*-brassinolide (**6**) was not metabolized by the cockroach. In testes of *Periplaneta americana* 24-*epi*-castasterone (**1**) was also not transformed. However, a specific metabolic transformation of 24-*epi*-castasterone (**1**) was found in the ovaries. After methylboronation and trimethylsilylation the EI mass spectrum of the isolated metabolite shows a molecular ion at *m/z* 632 corresponding to an isomer of 24-*epi*-castasterone (**1**) in which two hydroxy functions are methylboronated and two of them trimethylsilylated. The ion at *m/z* 155 indicates an untouched

Table. Brassinosteroids and their metabolites in organs of the cockroach *Periplaneta americana* after feeding of 24-*epi*-castasterone (**1**) and 24-*epi*-brassinolide (**6**).

Organ	Animals	**1**	Metabolites	**6**	Metabolites
Feces	males	+	+	+	–
Feces	females	+	+	+	–
Feces	nymphs	+	+	+	–
Fatbody	males	+	–	+	–
Fatbody	females	+	–	–	–
Fatbody	nymphs	+	–	(+)	–
Malp.tub.	males	+	–	(+)	–
Malp.tub.	females	+	–	(+)	–
Malp.tub.	nymphs	+	–	+	–
Midgut	males	+	(+)	+	–
Midgut	females	+	(+)	+	–
Midgut	nymphs	+	(+)	+	–
Hindgut	males	+	(+)	+	–
Hindgut	females	+	(+)	+	–
Hindgut	nymphs	+	(+)	+	–
Ovaries		(+)	+	+	–
Testes		(+)	–	+	–

+ : present;
(+) : weakly present;
– : not present.

side chain moiety (Ikekawa and Takatsuto, 1984). Accordingly, the two hydroxy groups at ring A have to be trimethylsilylated indicating that an epimerization at ring A has taken place. After derivatization the metabolite was compared with the synthetic reference compounds **2–5** by GC-MS. Whereas both the diols **3** (2α,3β-OH), **4** (2β,3β-OH) and **5** (2β,3β-OH; 5β-H) formed in accordance to 24-*epi*-castasterone (**1**, 2α,3α-OH) a bismethylboronate (**BMB**), the *trans*-diaxial diol 2,24-*diepi*-castasterone **(2,** 2β,3α-OH) afforded a methylboronate-ditrimethylsilylether (see Materials and Methods). The EI-MS of **2-MB-TMS** displays key ions at *m/z* 632 (M^+), 617 ($[M\text{-}Me]^+$), 542 ($[M\text{-}TMSiOH]^+$), 515 (base peak), 426 and 155 (side chain fragment). The ion at *m/z* 515 is originated by cleavages in ring A (C-1/C-10 and C-2/C-3) (Scheme 2). Both the mass spectral fragmentation and the relative retention time (RRT) of derivatized 2,24-*diepi*-castasterone (**2-MB-TMS**) were in good agreement with the corresponding data obtained from the isolated metabolite. Therefore, we conclude that female cockroaches are able to epimerize in the ovaries 24-*epi*-castasterone (**1**) to 2,24-*diepi*-castasterone (**2**). Contrary to 24-*epi*-castasterone (**1**) with a 6-keto function, after feeding the lactone-type brassinosteroid 24-*epi*-brassinolide (**6**) was detected both in the ovaries and testes without any metabolization.



Scheme 2. Mass Spectral Fragmentation of 2,24-*Di-epi*-castasteronemethylboronate-ditrimethylsilylether (**2-MB-TMS**).

Discussion

It is well known, that large amounts of ingested ecdysteroids are excreted by insects and can be found in the feces as unchanged hormones and a mixture of several metabolites such as 3α-epimers and a variety of conjugates (Lafont and Koolman, 1984). In our experiments, besides not further characterized minor metabolites of 24-*epi*-castasterone, both ingested brassinosteroids could be detected unchanged in feces. Therefore, one can exclude that brassinosteroids are significantly metabolized by the intestinal flora.

The fact that traces of the ingested brassinosteroids are found in ovaries as well as in testes from adults suggests an identic intestinal pathway and resorption of the ingested compounds in both sexes. The only difference between males and females is the presence of the newly formed 2,24-*diepi*-castasterone (**2**) in ovaries. In our experiments only traces of both fed brassinosteroids but no metabolites could be detected in the testes. Also Briers *et al.* (1983) could not find male-specific metabolites of ecdysteroids in testes of the fly *Sarcophaga bullata*. Metabolic transformation after resorption into the hemolymph can be excluded, because no other organs show metabolized compounds besides the ovaries. Therefore, a metabolic transformation of 24-*epi*-castasterone (**1**) specifically in the ovaries is obviously.

The presence of substantial amounts of ecdysteroids in different organs besides moulting glands was documented in many arthropod species. In larval and adult ovaries such as the cockroach *Nauphoeta cinerea* (Imboden *et al.*, 1978), the spider crab *Acanthonyx lunulatus* (Chaix and De Reggi, 1982), and the locust *Locusta migratoria* (Goltzené *et al.*, 1978, Lagueux *et al.*, 1984) e.g. ecdysteroids are taken up by and stored in the maturing eggs. Ecdysteroids in the ovaries may serve for hormonal regulation of embryonic moult during embryogenesis. In locusts more than 95% of all body ecdysteroids in the reproducing females stored in the ovaries consist of ecdysone conjugates (Tawfik *et al.*, 1999). In *Periplaneta* mature ovaries can esterify ecdysone to at least six apolar compounds which are stored in the eggs to an amount of 53 ± 10 ng/g (Slinger and Isaac, 1988).

Ecdysteroids are also made in the follicular sheath of the testes as was first shown in the blow-

fly *Calliphora vicina* (Koolman *et al.,* 1979) and in the budworm *Heliothis virescens* (Loeb *et al.,* 1982). Obviously, they play a role in spermiogenesis (Delbecque *et al.,* 1990).

Whereas the metabolism of ecdysteroids in insects is quite well investigated, till now nothing is known about the metabolic pathway of brassinosteroids in insects. In regard to ring A in the metabolism of ecdysone and 20-hydroxyecdysone the two enzymes ecdysone oxydase and 3-oxo-ecdysteroid 3α-reductase are involved cooperating to facilitate the 3-epimerization of ecdysteroids. This epimerization of ecdysone and 20-hydroxyecdysone at C-3 is irreversible (Weirich, 1989) and was mainly found in larval midguts (Weirich and Bell, 1997). Ecdysone 3-epimerase was purified from meconium and midgut of the tobacco hornworm *Manduca sexta (Mayer et al.,* 1979), from midguts of the gypsy moth *Lymantria dispar* (Weirich, 1989) and from the cotton leafworm *Spodoptera littoralis* (Chen *et al.,* 1996). This enzyme also transforms the 3β-hydroxyl of ecdysone and 20-hydroxyecdysone to the corresponding 3α-epimers, which are 10 to 15 times less biologically active than ecdysone in puff inducing abilities on *Drosophila* salivary gland giant chromosomes as well as in the *Calliphora* bioassay (Spindler *et al.,* 1977). Thus, this epimerization is a step of inactivation of moulting the hormones (Kaplanis *et al.,* 1979; Lafont *et al.,* 1980).

In our investigations, epimerization of the fed 24-*epi*-castasterone (**1**) and 24-*epi*-brassinolide (**6**) at C-3 could not be observed. However, in plant systems, especially cell cultures of *Lycopersicon esculentum* and *Ornithopus sativus* both brassinosteroids **1** and **6** interestingly undergo a comparable epimerization leading via 3-oxo intermediates to corresponding metabolites with 3β-hydroxy function (Adam and Schneider, 1999). The enzymology of this transformation remains still open. Thus, in both series the enzymatic formation of an equatorial 3-hydroxy group can be observed (ecdysteroids with 5β-configuration: 3β–OH→α-OH; brassinosteroids with 5α-configuration: 3α–OH→β-OH) representing in the brassinosteroid series a preferred position for a subsequent conjugation (Adam and Schneider, 1999).

In insects and crustaceans stereospecific 2β-hydroxylation is one of the key reactions during the biosynthesis of ecdysone from cholesterol in the ovaries (Hetru *et al.,* 1982; Greenwood *et al.,* 1984; Fujimoto *et al.,* 1989; Lafont, 1997), whereas epimerization at this position has not been hitherto observed. The equatorial 2β-hydroxy group was shown to be the preferred position for conjugation reactions during ecdysone metabolism (Lafont and Koolman, 1984). Furthermore, such conjugation at C-2 are main terminal steps in the biosynthesis of 20-hydroxyecdysone in ovaries of the locust *Schistocerca gregaria* (Rees and Isaac, 1984).

The herewith described epimerization of 24-*epi*-castasterone (**1**) to 2,24-diepi-castasterone (**2**) in ovaries of *Periplaneta americana* represents the first example of a metabolic transformation of a brassinosteroid in an insect. It is of special interest that the formed metabolite **2** exhibits an unusual trans-diaxial 2β,3α-position of both ring A hydroxyls hitherto found only in the scarce brassinosteroids 2-*epi*-castasterone and 2-*epi*-25-methyldolichosterone (Fujioka, 1999).

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