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METABOLISM OF 24-*EPI*-CASTASTERONE AND 24-*EPI*-BRASSINOLIDE IN CELL SUSPENSION CULTURES OF *ORNITHOPUS SATIVUS*

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Key Word Index—*Ornithopus sativus*; Leguminosae; cell suspension cultures; metabolism; tritium-labelled brassinosteroids; 24-*epi*-brassinolide; 24-*epi*-castasterone; 20-keto-pregnane; hydroxylation; catabolism.

Abstract—Exogenously applied 24-*epi*-brassinolide and 24-*epi*-castasterone are transformed by cell suspension cultures of *Ornithopus sativus* in a multistep pathway to give 20-keto-pregnane derivatives. As intermediates of these degradations 20*R*-hydroxy-3,24-bis*epi*-brassinolide and 20*R*-hydroxy-3,24-bis*epi*-castasterone, respectively, as well as 2α,3β-dihydroxy-5α-pregnane-6,20-dione were isolated from the culture medium and their structures were elucidated by means of ^{1}H NMR and mass spectrometry. In addition to side-chain cleavage, 25-hydroxylation to give 25-hydroxy-3,24-bis*epi*-brassinolide was also observed.

INTRODUCTION

The brassinosteroids are a class of plant hormones that show high growth-promoting activity as well as other multiple effects on the development of plants [1,2]. There are only a few studies on the metabolism of this group of native growth regulators [3,4]. Recently, we have shown that cell suspension cultures of *Lycopersicon esculentum* are able to convert exogenously applied 24-*epi*-brassinolide to 25-β-D-glucopyranosyloxy-24-*epi*-brassinolide [5] and 26-β-D-glucopyranosyloxy-24-*epi*-brassinolide [6]. In cell suspension cultures of *Ornithopus sativus*, exogenously applied 24-*epi*-castasterone (**1**) and 24-*epi*-brassinolide (**2**), respectively, after epimerization at C-3 were conjugated to fatty acyl esters [7]. Following a hitherto hypothetical metabolic pathway, other portions of **1** and **2** were converted to pregnane-type compounds with a 20-keto function [8]. In this paper we report on the isolation and structural elucidation of several intermediates, completing the pathways from exogenously applied 24-*epi*-castasterone (**1**) and 24-*epi*-brassinolide (**2**) to metabolites derived from 20-keto-pregnane (Figs 1 and 2).

RESULTS

Tritium-labelled 24-*epi*-castasterone (**1**) and 24-*epi*-brassinolide (**2**) (3.3 μM), respectively, were applied to sterile cell suspension cultures of *O. sativus* (Brot.) at day 4 of the growth cycle as previously described [8]. After an incubation period of 24 hr, 36 hr and 4 days, respectively, the cells were separated from the medium and both fractions were individually studied. There was a clear compartmentalization of the different types of metabolites between the cells (about 75% of the radioactivity after application of **1** and 60% for **2**) and the medium (25% and 40%, respectively). While in the medium mainly nonconjugated metabolites were found, the cells contained a major fraction of lipophilic and a minor fraction of hydrophilic conjugates. The lipophilic conjugates recently were identified as fatty acyl esters of 3,24-bis*epi*-castasterone (**7–9**) and 3,24-bis*epi*-brassinolide (**13–15**), respectively [7]. The hydrophilic compounds, probably glucosides were not further investigated. The culture medium, both after application of **1** and **2**, respectively, contained several nonconjugated metabolites, which belong to metabolic sequences leading to 2α,3β-dihydroxy-B-homo-6a-oxa-5α-pregnane-6,20-dione (**12**) and 2α,3β,6β-trihydroxy-5α-pregnane-20-one (**6**), respectively [8] (Figs 1 and 2).

In experiment 1 the cells were harvested 36 hr after application of 24-*epi*-castasterone (**1**). The chloroform extract of the filtrate contained 75% of the radioactivity of the culture medium. TLC indicated one radioactive peak with R_f 0.24. Further purification and separation were achieved using TLC followed by reversed-phase HPLC (gradient 2, R_t 17.9 min). The compound with R_t 17.9 min was found to be 20*R*-hydroxy-3,24-bis*epi*-castasterone (**4**). The EI-mass spectrum of **4** showed the parent peak, an intensive peak at *m/z* 349, indicating predominant fission between C-20/C-22 and peaks owing to a stepwise loss of three H_2O from this fragment

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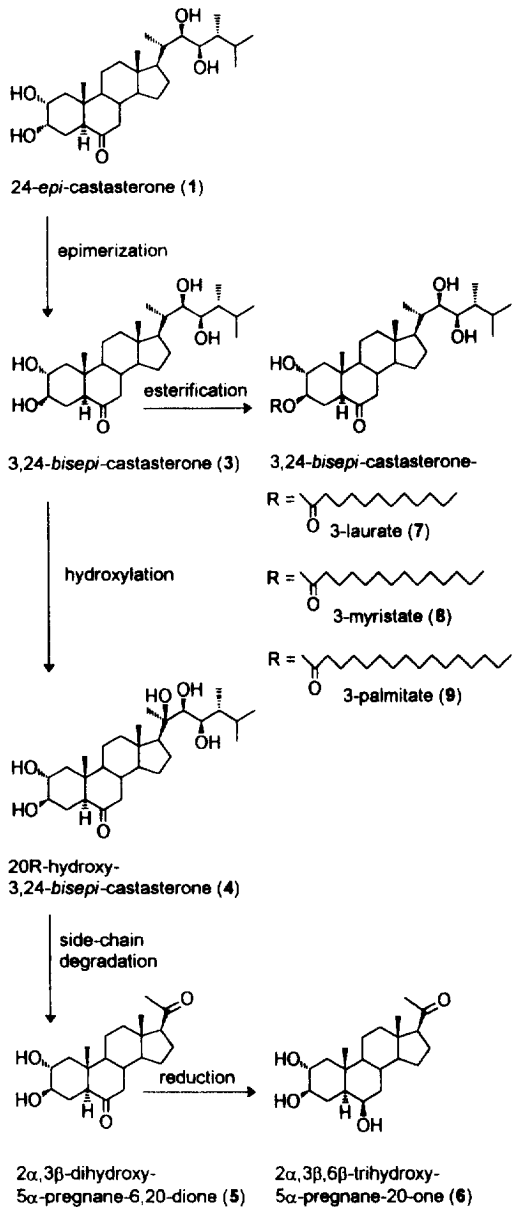


Fig. 1. The metabolism of 24-*epi*-castasterone in cell suspension cultures of *Ornithopus sativus*

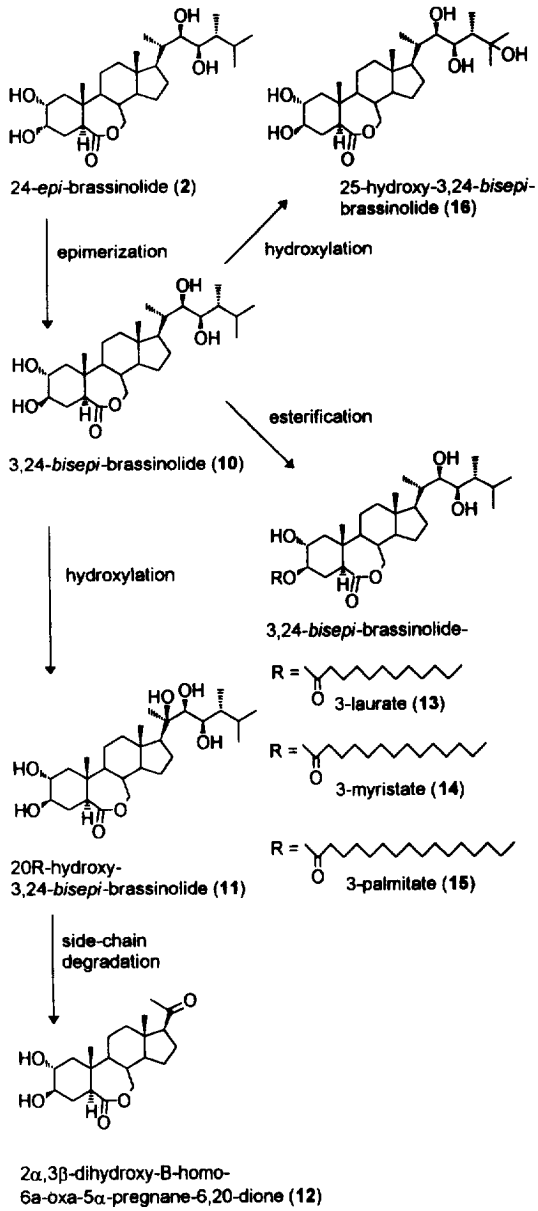
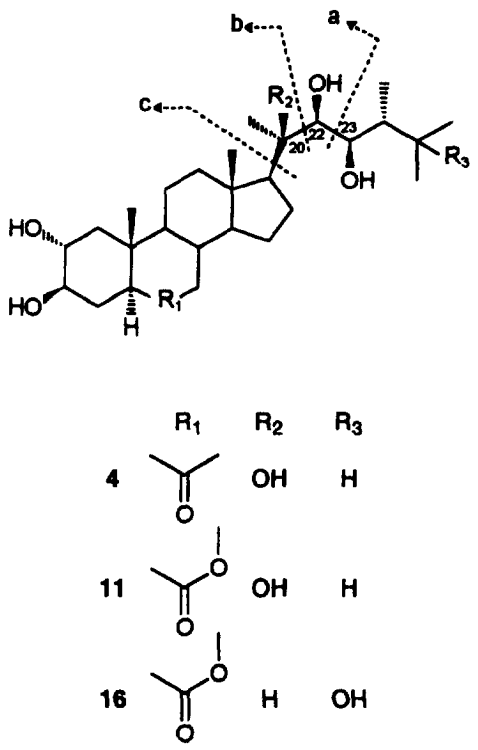


Fig. 2. The metabolism of 24-*epi*-brassinolide in cell suspension cultures of *Ornithopus sativus*

(Table 1). The ^{1}H NMR spectrum of **4** showed only three methyl doublets (δ 0.95, 0.93 and 0.87) but three methyl singlets (δ 1.34, 0.84, 0.83), indicating hydroxylation at C-20 or C-24. In the multiple-bond ^{1}H–^{13}C shift correlation experiment (HMBC) the carbon signal at δ 54.9 exhibits correlation peaks to both methyl singlets at δ 1.34 and δ 0.84. Therefore, these methyl signals had to be assigned to methyl-21 and methyl-18, respectively, because only these two methyl signals are expected to give a mutual correlation to one carbon signal (C-17). Methyl-21 gives two further correlations with the carbon signals at δ 77.8 and δ 75.4. One of these signals (δ 75.4) shows a correlation with a proton signal (δ 3.35) in the direct ^{1}H–^{13}C shift correlation experiment (HMQC), whereas no correlation for the ^{13}C signal at δ 77.8 were found. Thus, the latter signal was assigned to the quaternary carbon C-20. Further detailed analysis of the ^{1}H–^{1}H (COSY), direct ^{1}H–^{13}C (HMQC) and long-range ^{1}H–^{13}C (HMBC) shift-correlation two-dimensional NMR spectra yielded the assignments of almost all proton and carbon signals. The results of NOE difference suggests as most probable the 20*R*-configuration. The halfwidth of the ^{1}H multiplet of H-3 ($\Delta_{1/2}$ = 24 Hz) and the upfield shift of H-5α in comparison with **1** (δ 2.32 and 2.69, respectively) prove the epimerization at C-3.

Table 1. EI-mass spectroscopy data of 20*R*-hydroxy-3,24-bis*epi*-castasterone (**4**), 20*R*-hydroxy-3,24-bis*epi*-brassinolide (**11**) and 25-hydroxy-3,24-bis*epi*-brassinolide (**16**)

Ion	*m/z* (rel. int.)		
	4	**11**	**16**
$[M + H]^+$	481 (0.8)	497 (0.5)	497 (0.9)
a		395 (0.9)	379 (100)
a-H_2O			361 (13)
a-$2H_2O$			343 (16)
a-$3H_2O$			325 (11)
(b + H)			350 (41)
b	349 (100)	365 (79)	
b-H_2O	331 (88)	347 (57)	
b-$2H_2O$	313 (23)	329 (26)	
b-$3H_2O$	295 (14)	311 (20)	
c	305 (18)	321 (11)	321 (8)



The cells obtained in experiment 1 (cell harvest 36 hr after application of **1**) were extracted with 80% aqueous methanol. After purification of the chloroform fraction of this extract by means of TLC (R_f 0.25) and reversed-phase HPLC (gradient 3) the fraction with R_t 25.4 min was found to be 3,24-bis*epi*-castasterone (**3**). The ^{1}H NMR signals of **3** were assigned by comparison with the ^{1}H chemical shifts of **1** [7]. While the side-chain signals of **3** are identical to those of **1**, the A-ring signals are different. In particular the upfield shift of H-3 (δ 3.39, *ddd*) and the ^{1}H–^{1}H coupling constants (11.7/9.0/4.9 Hz) indicate an axial position of this proton and hence epimerization at C-3.

The chloroform extract of the cell culture medium obtained 24 hr after administration of 24-*epi*-brassinolide (experiment 2) was subjected to TLC. The radioscan indicated a major labelled zone with R_f 0.49 and a minor one with R_f 0.38. The latter radioactive fraction was twice purified by reversed-phase HPLC (gradient 1, R_t 10–13 min; gradient 2, R_t 15 min; see Experimental). Structure elucidation of the obtained compound by ^{1}H–^{1}H-COSY and EIMS revealed 20*R*-hydroxy-3,24-bis-*epi*-brassinolide (**11**). The EI mass spectrum showed the parent peak and the peak due to the fission between C-22/C-23 both of very lower intensity. As was found for compound **4**, the fission of compound **11** takes place predominantly between C-20/C-22 followed by stepwise loss of H_2O (Table 1). Considering chemical shifts and coupling patterns, the ^{1}H NMR spectrum of **11** shows side-chain methyl signals nearly identical to those of **4**, indicating a 20-β-hydroxylation for this metabolite also. Again, H-3 exhibits a large linewidth and H-5α (δ 2.90) a high-field shift of about 0.2 ppm in comparison with **2**. Thus, H-3 has to be axial and therefore in the β-position.

As previously described, 3,24-bis*epi*-brassinolide (**10**) (R_f 0.49; gradient 1, R_t 16.6 min) has been isolated from the chloroform extract of the medium [7]. From the same TLC zone another metabolite was purified by reversed-phase HPLC (gradient 1, R_t 12.4 min). Structure elucidation by EI-mass spectrometry and ^{1}H NMR revealed 25-hydroxy-3,24-bis*epi*-brassinolide (**16**). The EI-mass spectrum showed the parent peak and peaks resulting from the fission between C-22/C-23 and stepwise loss of three H_2O (Table 1). In contrast to the 20-hydroxy compounds **4** and **11**, preferred fragmentation of **16** occurred between C-22 and C-23. Thus, the fragments *m/z* 379 and *m/z* 365, respectively, represent diagnostic ions of pentahydroxylated brassinosteroids, derived from 24-bis*epi*-brassinolide (**2**), for 25-hydroxylation in the first case and for 20-hydroxylation in the second. The ^{1}H NMR methyl signals of **16** show the typical chemical shifts and coupling patterns for an 24-*epi*-brassinolide side-chain with 25-hydroxylation (two doublets at δ 1.00 and 0.82; four singlets at δ 1.30, 1.28, 0.98 and 0.71). The chemical shift of H-5α and the large halfwidth of the H-3 signal indicate epimerization at C-3.

In experiment 3 the cells were harvested 4 days after application of 24-*epi*-castasterone (**1**). In this experiment the chloroform extract of the medium contained 70% of the radioactivity of the culture medium. TLC indicated two radioactive peaks with R_f 0.22 and R_f 0.38 in the ratio 2:1. Purification was achieved using TLC followed by reversed-phase HPLC (R_t 10.2 and R_t 11.4 min, isocratic mode). As previously reported, the compound with R_t 10.2 min was 2α,3β,6β-trihydroxy-5α-pregnane-20-one (**6**) [8]. On the basis of EI-mass spectrometry and ^{1}H NMR analysis, the structure of the compound with R_t 11.4 min was proved to be 2α,3β-dihydroxy-5α-pregnane-6,20-dione (**5**). The EI-mass spectrum showed *m/z* 348 (38) $[M]^+$ and *m/z* 330 (24) $[M-H_2O]^+$. The ^{1}H NMR spectrum of **5** exhibited only two methyl singlets in the high-field methyl region (δ 0.81 and 0.62),

indicating degradation of the side chain. The methyl singlet at δ 2.13 is assigned to methyl-21 in a 20-keto-pregnane side-chain moiety. The halfwidth of the H-3 proton signal (25 Hz) as well as the ^{1}H chemical shift of H-5α (δ 2.34, as derived from the ^{1}H–^{1}H COSY spectrum) prove the epimerization at C-3. Only two proton signals appear in the low-field region (δ 3.61 H-2β; δ 3.40 H-3α). In addition, no significant shift of the proton signals of H-5α and methyl-19 were observed compared with **4**, indicating that the 6-keto group in **5** was unchanged.

DISCUSSION

In this report four new metabolites of exogenously applied brassinosteroids in cell suspension cultures of *Ornithopus sativus* were described. 20-Hydroxy-3,24-bis*epi*-castasterone (**4**) and 2α,3β-dihydroxy-5α-pregnane-6,20-dione (**5**), isolated after application of 24-*epi*-castasterone, are intermediates of the degradation pathway to 2α,3β,6β-trihydroxy-5α-pregnane-20-one (**6**). These metabolites complete the metabolic scheme of **1** in *Ornithopus sativus* cell suspension cultures (Fig. 1). First, 24-*epi*-castasterone (**1**) is epimerized at C-3 to 3,24-bis*epi*-castasterone (**3**). This epimerization probably follows a redox mechanism via the corresponding 3-ketone. In the following step hydroxylation at C-20 takes place. This 20-hydroxy compound (**4**) is split between C-20/C-22 to give 2α,3β-dihydroxy-5α-pregnane-6,20-dione (**5**). Reduction of the latter compound at the 6-keto-group in the final step revealed 2α,3β,6β-trihydroxy-5α-pregnane-20-one (**6**).

The metabolism of 24-*epi*-brassinolide (**2**) follows an analogous pathway except the reduction of the 6-keto group in the last step (Fig. 2), which is not operating with the lactone structure.

Assuming an oxidative mechanism of this side-chain cleavage, 2-hydroxy-3,4-dimethylpentanoic acid should be expected as a side-chain fragment. An analogous compound, 4-hydroxy-4-methylpentanoic acid was found as a metabolite of 20-hydroxyecdysone in insects [9] and in crustaceous organisms [10]. However, in our experiments no pentanoic acid fragment could be detected because of lack of label in the side chain. To our knowledge, this is the first report on the metabolic sequence revealing pregnane-type compounds from brassinosteroids in plant systems. The hydroxylation at C-20 is especially remarkable, yielding a hitherto unknown type of pentahydroxylated brassinosteroid.

Both 3,24-bis*epi*-castasterone (**3**) and 3,24-bis*epi*-brassinolide (**10**) are branching points in the metabolism of **1** and **2**. In addition to the pregnane pathway, conjugation with fatty acids to acyl conjugates **7**–**9** and **13**–**15**, respectively, was observed [7]. Furthermore, in the experiments with 24-*epi*-brassinolide (**2**), hydroxylation at C-25 occurred. This reaction is well known for other steroid compounds, e.g. for ecdysones [11], and was recently observed by us also for **1** and **2** in tomato cell cultures [5, 6]. In contrast to tomato, 25-hydroxylation in *O. sativus* occurred not with the parent compounds **1** and **2**, but only after epimerization at C-3. Unlike tomato, the 25-hydroxylated compound **16** was found in the nonglucosylated state.

Our results demonstrate close similarity to the metabolism of ecdysones in insects [11]. Like *O. sativus* cell suspension cultures, insects are able to split the bond between C-20 and C-22. Insects are also able to epimerize the hydroxyl-group of ecdysone at position C-3 and to hydroxylate the molecule at position C-20. In contrast to the cell suspension cultures of *Ornithopus sativus*, insects do not form conjugates with fatty acids at position C-3, but at C-22.

EXPERIMENTAL

Radiochemicals and measurement of radioactivity. The syntheses of [5,7,7-^{3}H]24-*epi*-brassinolide and [5,7,7-^{3}H]24-*epi*-castasterone were recently described [13]. [5,7,7-^{3}H]24-*epi*-brassinolide and [5,7,7-^{3}H]24-*epi*-castasterone with sp. act. of 10.3 and 4.8 MBq mmol^{-1}, respectively, were used in the experiments described in this paper. Radioactivity of all frs was measured by liquid scintillation counting (LSC). Before LSC the solid samples were incinerated. The radioactive zones of the TLC plates were analysed with an automatic TLC linear analyser. For quantification of metabolites the ratio of peak areas from TLC radioscans were used.

Cell cultures and application. The suspended cells of *O. sativus* cultures were grown in a Linsmaier–Skoog medium [13] at 26° on a gyratory shaker (125 r.p.m.) in the dark in 300 ml Erlenmeyer flasks containing 150 ml cell suspension. Subculturing was performed every 7 days using an inoculum of *c.* 40 ml.

The ethanolic solution (< 1 ml) of [5,7,7-^{3}H]24-*epi*-castasterone and [5,7,7-^{3}H]24-*epi*-brassinolide, respectively, (final concn 3.3 μM) were added to the cell suspension cultures at the day 4 of the growth cycle. The cell suspensions were held under identical conditions for another 24 hr, 36 hr or 4 days, respectively.

Isolation and purification of compounds **4**, **5**, **11**, **16**. The cells were harvested by suction filtration through a nylon mesh. The filtrate (culture medium) was extracted with $CHCl_3$. The $CHCl_3$ extract was purified after concn *in vacuo* by TLC (Merck silica gel 60; 0.25 mm layer for prep. mode; silica gel sheets for analytical mode; developed in $CHCl_3$:MeOH, 9:1) and reversed-phase HPLC (Nucleosil C18; 10 μm; 250 × 10 mm; flow rate 4 ml min^{-1}; detection UV 204 nm and LSC of aliquots; gradient 1, H_2O: MeCN from 3:1 to 1:1 in 15 min, then 1:1 for 5 min; gradient 2, H_2O: MeCN 7:3 for 10 min, then in 10 min to H_2O: MeCN 1:1; isocratic mode: H_2O: MeCN 7:3).

Isolation and purification of compound **3**. The cells were homogenized with an Ultra-turrax grinder at room temp. in 80% aq. MeOH, filtered and washed with MeOH. The aq. soln remaining after concn of the combined filtrates *in vacuo* at less than 40° was extracted with $CHCl_3$. The $CHCl_3$ was removed *in vacuo* and the residue was

extracted with Me_2CO. The Me_2CO fraction contained fatty acyl esters [7] and compound **3**, which was separated by TLC (Merck silicagel 60; 0.25 mm layer; developed with $CHCl_3$:MeOH 9:1) and reversed-phase HPLC (gradient 3, H_2O–MeCN 7:3 for 10 min, then in 10 min to H_2O:MeCN 1:1, then H_2O:MeCN 1:1 for 10 min.

Spectrometric methods. EI-MS (70 eV) was performed with an AMD 402 mass spectrometer. NMR experiments were carried out on a VARIAN UNITY 500 spectrometer at 499.84 MHz (^1H) using a NALORAC 3 mm microsample inverse-detection probe. $CDCl_3$ was used as solvent. Two-dimensional COSY, HMQC and HMBC spectra were recorded according to standard VARIAN pulse programs.

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