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ACYL-CONJUGATED METABOLITES OF BRASSINOSTEROIDS IN CELL SUSPENSION CULTURES OF *ORNITHOPUS SATIVUS*

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Key Word Index—*Ornithopus sativus*; Leguminosae; cell suspension cultures; metabolism; tritium-labelled brassinosteroid; brassinosteroid fatty acyl esters; 24-epi-brassinolide; 24-epi-castasterone; 3,24-bisepi-brassinolide.

Abstract—Exogenously applied 24-epi-brassinolide and 24-epi-castasterone were transformed by a cell suspension culture of *Ornithopus sativus* to give fatty acid conjugates of 3,24-bisepi-brassinolide and 3,24-bisepi-castasterone, respectively, esterified at the 3β-position. The conjugates were identified by means of ^{1}H NMR and mass spectrometry. Lauric acid, myristic acid and palmitic acid were the acyl components for both brassinosteroid conjugate types. Non-conjugated 3,24-bisepi-brassinolide was also found for the first time in a plant system.

INTRODUCTION

The brassinosteroids are a class of plant hormone, showing growth promoting activity as well as other multiple effects on the growth and development of plants [1, 2]. Only few studies on the metabolism of this group of native growth regulators have been done until now [3]. In mung bean explants, castasterone was metabolized to unknown water soluble glycosidic and non-glycosidic compounds, whereas brassinolide was transformed mainly to 23-*O*-β-D-glucopyranosylbrassinolide [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]. Cell suspension cultures of *Ornithopus sativus* are able to transform exogenously applied 24-epi-brassinolide and 24-epi-castasterone in a multi-step metabolic process including epimerization at C-3 and side chain cleavage leading to pregnane-type metabolites [6].

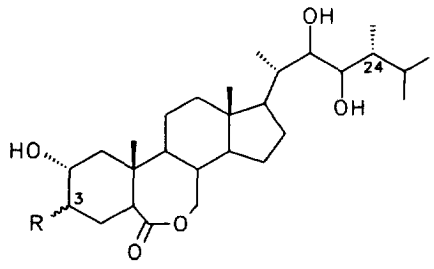
In this paper we report the formation of acyl conjugated brassinosteroids from exogenously applied 24-epi-brassinolide (**1**) and 24-epi-castasterone (**3**). As an intermediate of the metabolic sequence, also 3,24-bisepi-brassinolide (**2**) was isolated from the culture medium.

RESULTS AND DISCUSSION

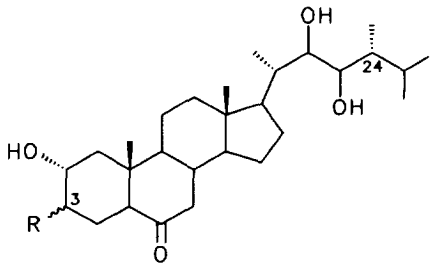
Tritium-labelled 24-epi-brassinolide (**1**) and 24-epi-castasterone (**3**) were applied to sterile cell suspension cultures of *Ornithopus sativus* (Brot.) at day 4 of the growth cycle. The concentrations of **1** and **3** were 3.3 μM. The distribution of the radioactivity between the medium and the cells did not change significantly during the whole incubation period of 7 days. About 40 and 25% of the radioactivity after application of **1** and **3**, respectively, were found in the medium and the remainder was associated with the cells. In experiment 1 the cells were harvested 24 hr after application of **1**. The culture medium was subjected to further investigations.

The chloroform extract contained 72% of the radioactivity of the culture medium. TLC indicated one major radioactive peak with R_f 0.49, which was purified using TLC followed by reversed phase HPLC (R_t 16.6 min). No starting compound (R_f 0.51) was detectable. The structure of the metabolite **2** was identified using EI-mass spectrometry and especially 2D NMR spectrometry, as 3,24-bisepi-brassinolide. The EI-mass spectrum showed the same peaks as the compound applied, thus indicating isomerization. The relevant ^{1}H NMR signals of **2** (Table 2) were assigned by comparison with the well known ^{1}H chemical shifts of **1** [7] and by the correlations found in the ^{1}H–^{1}H COSY 2D NMR spectrum. While the side chain signals of **2** were identical in comparison to **1**, the ring A signals were different. In particular H-3 exhibited coupling constants (J = 14.4, 9.5, 4.9 Hz) which indicated an axial position of this proton and hence epimerization at C-3.

In experiment 2 (application of **1**) the cells were harvested after 7 days, and in experiment 3 (application of **3**) 36 hr, after application of the parent compounds. The cells were extracted with 80% aq. methanol. Radio-TLC indicated nearly complete metabolization. Several minor hydrophilic compounds were detected, but the major part of the radioactivity was in the less polar compounds. After purification of the lipophilic fraction by means of TLC (R_f 0.49) and separation by reversed phase HPLC, the structures of six new metabolites were elucidated from



1 R= ···· OH
2 R= — OH
5 R= — O-CO-$C_{11}H_{23}$
6 R= — O-CO-$C_{13}H_{27}$
7 R= — O-CO-$C_{15}H_{31}$



3 R= ···· OH
4 R= — OH
8 R= — O-CO-$C_{11}H_{23}$
9 R= — O-CO-$C_{13}H_{27}$
10 R= — O-CO-$C_{15}H_{31}$

the spectroscopic data. Three of them were derived from each compound applied, all in nearly the same quantity. Thus 3,24-bisepi-brassinolide-3β-laurate (**5**) (R_t 20.4 min), -3β-myristate (**6**) (R_t 30.6 min) and -3β-palmitate (**7**) (R_t 40.2 min) were found as metabolites of **1** in experiment 2, whereas 3,24-bisepi-castasterone-3β-laurate (**8**) (R_t 26.9 min), -3β-myristate (**9**) (R_t 36.7 min) and -3β-palmitate (**10**) (R_t 45.7 min) were metabolites of **3** in experiment 3.

The EI-mass spectrum of **5**–**10** produced molecular ion peaks at *m/z* 662, 690, 718, 646, 674 and 702 $[M]^+$, respectively, all of them of low relative intensity. Further typical peaks were a result of the loss of one and two H_2O, fission between C-22/C-23 (Table 1 fragment *a*), C-20/C-22 (*b*), C-17/C-20 (*c*), ester bonding (*d*) and C-3/O (*e*), respectively. The position of the fatty acid residue at the ring A position can be deduced from the ions, which appear after fission *a*, *b* or *c*. The basic peaks of the acyl-conjugated 3,24-bisepi-brassinolides appear at *m/z* 361 (*a*-RCO_2H) and of the acyl-conjugated 3,24-bisepi-castasterones at *m/z* 346 (*a* + H − RCO_2H), respectively.

The ^{1}H NMR spectra of **5**–**7** were very similar to each other (Table 2). In comparison to the spectrum of **2**, H-3β exhibited a downfield shift of about 1.2 ppm owing to an

Table 1. The EI-mass spectra of compounds **5**–**10**

Ion	**5**	**6**	**7** *m/z*	**8** (rel. int.)	**9**	**10**
$[M + H]^+$	663(2)	691(0.4)				
$[M]^+$	662(1)	690(0.3)	718(2)	646(0.7)	674(0.8)	702(0.7)
$[M - H_2O]^+$	644(3)	672(0.8)	700(3)	628(0.9)	656(0.6)	684(0.9)
$[M - 2H_2O]^+$	626(1)	654(0.6)	682(1)	610(0.5)	638(0.3)	
(*a* + H)				546(10)	574(13)	602(13)
a	561(46)	589(10)	617(28)	545(11)	573(12)	
(*a* − H_2O)	543(46)	571(11)	599(21)	527(7)	555(8)	583(9)
(*b* + H)	532(18)	560(6)	588(8)	516(3)	544(3)	
b						571(2)
(*b* + H − H_2O)	514(7)	542(3)	570(8)			
(*c* − 2H)	501(6)	529(5)	557(5)	485(4)	513(4)	541(4)
(*d* + 2H)	481(6)	481(5)				
e	463(8)	463(6)		447(4)	447(28)	447(27)
(*e* − H_2O)	445(4)	445(4)		429(2)	429(5)	429(7)
(*e* − $2H_2O$)	427(4)	427(5)	427(15)			
(*a* + H − RCO_2H)	362(87)	362(80)	362(70)	346(100)	346(100)	346(100)
(*a* − RCO_2H)	361(100)	361(100)	361(100)	345(84)	345(43)	345(99)
(*a* − RCO_2H − H_2O)	343(63)	343(77)	343(73)	327(35)	327(18)	327(40)
(*b* + H − RCO_2H)	332(27)	332(41)	332(60)	316(17)	316(15)	316(19)
(*a* − RCO_2H − $2H_2O$)	325(14)	325(32)	325(25)	309(13)	309(11)	309(12)
(*c* − RCO_2H)	303(23)	303(56)	303(80)	287(15)	287(7)	287(15)
(*c* − RCO_2H − H_2O)	285(21)	285(47)	285(70)	269(15)	269(6)	269(13)

Table 2. ^{1}H NMR spectral data of compounds **1**, **2**, **5**–**7** (500 MHz, $CDCl_3$)*

H	1	2†	5	6	7
2β	3.72	3.46	3.72	3.72	3.68
3α	4.03‡	3.34	4.56	4.55	4.55
5α	3.12	2.91	2.97	2.97	2.97
7β	*ca*4.1	4.13	4.13	4.12	4.13
7α	*ca*4.1	4.03	4.02	4.02	4.02
H_3-18	0.70	0.71	0.71	0.71	0.71
H_3-19	0.92	0.96	0.97	0.97	0.97
H_3-21	0.97	0.96	0.97	0.97	0.97
22	3.69	3.66	3.68	3.68	3.68
23	3.41	3.38	3.41	3.41	3.41
H_3-26[a]	0.92	0.92	0.92	0.92	0.92
H_3-27[a]	0.87	0.87	0.87	0.87	0.87
H_3-28	0.85	0.84	0.85	0.85	0.85
$(-CH_2-)_n$	—	—	1.26	1.26	1.25
Me	—	—	0.88	0.88	0.88

*Typical multiplicities and coupling constants (Hz): H-2β: *br m* ($\Delta_{1/2}$ = 23 Hz); H-3β: *br s*; H-3α: *ddd* (*J* = 14.4, 9.5, 4.9 Hz); H-5α: *dd* (*J* = 11.9, 4.9 Hz); H-7β: *br d* (*J* = 12.2 Hz); H-7α: *dd* (*J* = 12.2, 9.1 Hz); H_3-18: *s*; H_3-19: *s*; H_3-21: *d* (*J* = 6.7 Hz); H-2: *br dd* (*J* = 4.5, 4.5 Hz); H-23: *ddd* (*J* = 5.0, 4.5, 4.5 Hz); H_3-26: *d* (*J* = 7.0 Hz); H_3-27: *d* (*J* = 7.0 Hz); H_3-28: *d* (*J* = 7.0 Hz); $(-CH_2-)_n$: *br s*; Me: *t* (*J* = 6.7 Hz).

†Solvent: 95% $CDCl_3$ + 5% CD_3OD.

‡H-3β.

[a]May be reversed.

Table 3. ^{1}H NMR spectral data of compounds **3**, **8**–**10** (500 MHz, $CDCl_3$)*

H	3	8	9	10
2β	3.77	3.80	3.79	3.80
3α	4.05†	4.58	4.57	4.58
5α	2.69	2.38	2.38	2.38
H_3-18	0.68	0.68	0.67	0.68
H_3-19	0.76	0.81	0.81	0.81
H_3-21	0.98	0.98	0.98	0.98
22	3.7	3.69	3.69	3.70
23	3.41	3.41	3.41	3.41
H_3-26[a]	0.92	0.92	0.92	0.92
H_3-27[a]	0.87	0.87	0.87	0.87
H_3-28	0.85	0.85	0.85	0.85
$(-CH_2-)_n$	—	1.26	1.25	1.25
Me	—	0.88	0.88	0.88

*Typical multiplicities and coupling constants (Hz): H-2β: *br m* ($\Delta_{1/2}$ = 23 Hz); H-3β: *br s*; H-3α: *ddd* (*J* = 11.9, 9.5, 5.2 Hz); H-5α: *dd* (*J* = 12.5, 2.7 Hz); H_3-18: *s*; H_3-19: *s*; H_3-21: *d* (*J* = 6.7 Hz); H-2: *br s*; H-23: *ddd* (*J* = 5.0, 5.0, 5.0 Hz); H_3-26: *d* (*J* = 7.0 Hz); H_3-27: *d* (*J* = 7.0 Hz); H_3-28: *d* (*J* = 7.0 Hz); $(-CH_2-)_n$: *br s*; Me: *t* (*J* = 7.0 Hz).

†H-3β.

[a]May be reversed.

ester bonding at this position. In addition to the signals of the 3,24-bisepi-brassinolide, the ^{1}H NMR spectra of **5**–**7** showed the presence of fatty acid methylene protons (δ1.25, *br s*) and the terminal methyl group (δ0.88, *t*).

The ^{1}H NMR spectra of **8**–**10** showed no significant differences. The low-field shift of H-3 and the found ^{1}H–^{1}H coupling constants (Tables 3) proved the esterification of the 3-hydroxyl as well as the epimerization at C-3. A strong signal at δ1.25 and the methyl triplet at δ0.88 were from the fatty acid residue.

Whereas phytosteryl esters are quite common in the plant kingdom [8], for brassinosteroids there is only one recent publication on teasterone-3-myristate, isolated from anthers of *Lilium longiflorum* [9]. Our results represent the first report on fatty acid conjugates as metabolites of exogenously applied brassinosteroids. In all identified metabolites **5**–**7** and **8**–**10**, respectively, the epimerization at C-3 to the equatorial hydroxyl function is indicated as a prerequisite for the enzymatic acyl transfer. The non-conjugated compound 3,24-bisepi-brassinolide (**2**), found for the first time in a plant system, was isolated from the medium, whereas the fatty acyl esters were present within the cells.

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 [10]. [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. 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 ratios of peak areas from TLC radioscans were used.

Cell cultures and application. The suspended cells of *Ornithopus sativus* cultures were grown in a Linsmaier–Skoog-medium [11] at 26° on a gyratory shaker (125 rpm) in the dark in 300 ml conical flasks containing 150 ml cell suspension. Subculturing was performed every 7 days using an inoculum of *ca* 40 ml.

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

Isolation and purification of metabolite **2**. 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 MeOH–$CHCl_3$ 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 H_2O–MeCN from 3:1 to 1:1 in 15 min, then 5 min H_2O–MeCN 1:1).

Isolation and purification of metabolites **5**–**10**. The cells obtained after 7 days and 36 hr after application of **1** and

3, respectively, were harvested by suction filtration through a nylon mesh, 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. This Me_2CO extract was purified after concn *in vacuo* by TLC (Merck silica gel 60; 0.25 mm layer for prep. mode; developed successively with C_6H_6, n-hexane–Et_2O, 3:7, $CHCl_3$–Me_2CO, 4:1) and reversed phase HPLC (Nucleosil C8; 10 μm; 250 × 10 mm; flow rate 4 ml min^{-1}; detection UV 204 nm and LSC of aliquots; MeCN–H_2O, 4:1, for 20 min, gradient MeCN–H_2O from 4:1 to 9:1 in 20 min, to 1:0 in 2 min then 1:0 for 13 min).

Spectrometric methods. EI-MS (70 eV) was performed with an AMD 402 mass spectrometer. NMR analyses were carried out on a Varian Unity 500 spectrometer at 499.84 MHz (^1H). 2D ^1H–^1H COSY-90 spectra were recorded according to standard pulse programmes.

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