Pergamon

PII: S0031-9422(97)00390-7

Phytochemistry, Vol. 46, No. 6, pp. 1019–1022, 1997

Printed in Great Britain
0031-9422/97 $17.00+0.00

DIGLYCOSIDIC METABOLITES OF 24-*EPI*-TEASTERONE IN CELL SUSPENSION CULTURES OF *LYCOPERSICON ESCULENTUM* L.

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(*Received* 17 *March* 1997)

Key Word Index—*Lycopersicon esculentum* L.; Solanaceae; brassinosteroid diglycosides; cell suspension cultures; metabolism; glycosylation; tritium-labelled brassinosteroids; 24-*epi*-teasterone.

Abstract—Exogenously applied 24-*epi*-teasterone is converted to 24-*epi*-teasterone-3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside and 24-*epi*-teasterone-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside by cell suspension cultures of *Lycopersicon esculentum* L. Both structures were elucidated by LC-mass spectral and NMR analysis, representing the first brassinosteroid diglycosides.

INTRODUCTION

The brassinosteroids are a class of native plant growth regulators which recently have been established as new phytohormones [1, 2]. As discussed for other phytohormones, e.g. the gibberellins, brassinosteroid glycosides might be involved in regulation of biosynthesis, modulation of hormone activity as well as transport, storage and compartmentation processes [3]. As naturally occurring brassinosteroid glucosides hitherto only 25-methyl-dolichosterone and 2-*epi*-25-methyl-dolichosterone from *Phaseolus vulgaris* have been described, both glucosylated at position O-23 [4]. However, a number of glucosides has been found in metabolism experiments. Thus, brassinolide was converted to its 23-*O*-β-D-glucopyranoside by cell cultures of *Vigna radiata* [5]. In cell suspensions of *Lycopersicon esculentum*, metabolically introduced hydroxyl groups at C-25 and C-26, respectively of 24-*epi*-brassinolide and 24-*epi*-castasterone were readily glucosylated [6, 7]. 24-*epi*-Castasterone has been glucosylated also at 2α-OH while conjugation of the hydroxyl group at C-3 was found only after epimerization to the 3β-configuration. In this paper we report on the isolation and structural elucidation of two disaccharide conjugates of brassinosteroids formed from exogenously applied 24-*epi*-teasterone by cell suspension cultures of *Lycopersicon esculentum* L.

RESULTS AND DISCUSSION

24-*epi*-Teasterone (**1**) [8], a putative intermediate in the biosynthesis of 24-*epi*-brassinolide, has been labelled with tritium by the procedure previously described employing slight modifications [9]. Tritium labelled **1** was applied to sterile cell suspension cultures of *Lycopersicon esculentum* at day 4 of the growth cycle in 4×10^{-6} M concentration. Compared with untreated controls, normal cell growth was not influenced under these conditions. As indicated by radioactivity measurements, **1** was rapidly taken up by the suspended cells. In a characteristic experiment after 5 hr only 7.7% of the recovered radioactivity was still found in the medium. The remainder was absorbed by the cells (methanol extract 87.1%; insoluble material 5.2%). At this time, the cells were harvested and extracted with 80% aqueous methanol. After evaporation of the methanol the residue was partitioned between chloroform and water. The chloroform extract contained 83% of the applied radioactivity. Radio-TLC indicated three radioactive zones, centred at R_f 0.1 (45% of the radioactivity), R_f 0.2 (22%) and R_f 0.5 (33%), respectively. The latter zone co-chromatographed with an authentic sample of compound **1**. Identification of the TLC zone moving to R_f 0.2 was not possible because of the impurity of the sample.

After separation by preparative TLC, the most polar radioactive zone (R_f 0.1) was subjected to reversed-phase HPLC. This fraction contained two radiolabelled metabolites **2** (R_f 12.2 min) and **3** (R_f 14.5) as indicated by liquid scintillation counting of fractions collected in 1 min intervals. The structures of **2** and **3** which were found in the ratio 3.5:1 have been elucidated from the spectroscopic data. ESI-mass spectroscopy indicated the same molecular ion peaks of *m/z* 772 $[M]^+$ for both compounds **2** and **3** suggesting conjugation of 24-*epi*-teasterone (**1**) with

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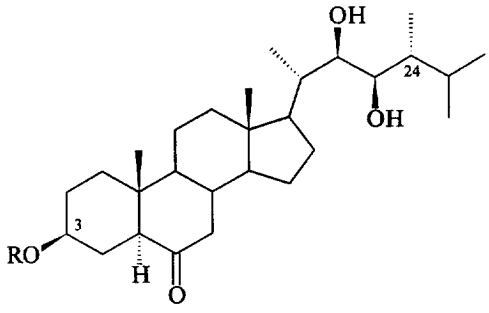
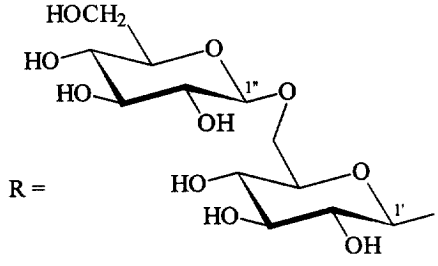
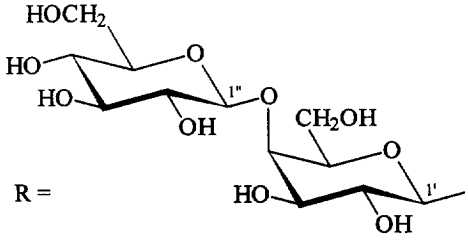


R = H 24-*epi*-Teasterone (**1**)



24-*epi*-Teasterone-3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**2**)



24-*epi*-Teasterone-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**3**)

two hexose units or one dihexose moiety. Fragment peaks with significant intensities did not occur in the mass spectra. In the NMR spectra the one-bond ^1H, ^{13}C shift correlation (HMQC) of both **2** and **3** showed nearly identical cross peaks for the genin signals as 24-*epi*-teasterone (**1**) except that in each case the H-3α signal was shifted upfield and the C-3 signal was shifted downfield in comparison with **1**, indicating glycosidation at position C-3. Furthermore, both HMQC spectra of **2** and **3** showed signals of two hexose units. From careful inspection of the ^1H NMR spectra two glucose units could be recognized in case of **2** whereas for **3** one glucose and one galactose were found (Table 1). ^1H, ^1H 2D COSY and long-range ^1H, ^{13}C shift correlation (HMBC) experiments allowed the complete and unambiguous assignment of all ^1H and ^{13}C resonances of **2** and most of **3** (Table 1 ^1H and ^{13}C; owing to the poor signal to noise ratio of the HMQC spectrum not all signals of **3** could be detected). The ^1H NMR signals of the two anomeric protons of **2** were nearly completely superimposed. However, from the intrasugar HMBC and COSY connectivities all sugar signals could be assigned unequivocally. The sugar chains of **2** and **3** were sequenced by observing interglycosidic connectivities through long-range ^1H, ^{13}C couplings. Thus, H-3α of **2** exhibited a HMBC correlation with C-1′ and H-6′A/H-6′B with C-1″. In a similar manner, H-1′ of **3** showed a HMBC correlation with C-3 of the genin, as well as H-1″ with C-4′. From these findings, the two metabolites were shown to be 24-*epi*-teasterone-3-*O*-β-D-glucopyranosyl-(1 → 6)-β-D-glucopyranoside (24-*epi*-teasterone-3-*O*-D-gentiobioside) (**2**) and 24-*epi*-teasterone-3-*O*-β-D-glucopyranosyl-(1 → 4)-β-D-galactopyranoside (24-*epi*-teasterone-3-*O*-β-D-lycobioside) (**3**), respectively, representing the first brassinosteroid diglycoside conjugates.

The diglycoside lycobiose has been found earlier also as a component of steroid alkaloid glycosides of tomato plants and some other Solanaceae [10]. Since conjugation of plant hormones is a reversible process, one might speculate that the disaccharides **2** and **3** are involved in the regulation of the brassinosteroid biosynthesis, for instance, to regulate the level of the intermediate 24-*epi*-teasterone (**1**). Such a process must be relatively fast because 5 hr after application 45% of the applied compound was already conjugated with disaccharides.

EXPERIMENTAL

Synthesis of [5,7,7-^3H]24-epi-*teasterone*. Triethylamine (100 μl) and 120 μl ^3H_2O (specific radioactivity 525 MBq mmol^{-1}) were added successively to a soln. of 91.4 mg 22,23-isopropylidene dioxy-24-*epi*-teasterone in 200 μl dry DMF under N_2. The mixt. was heated to 80° for 64 hr in a sealed glass ampoule. The reaction mixt. was then evapd under N_2 and exchangeable tritium was removed with MeOH. The product was deprotected by 1.5 ml 4 N HCl in 15 ml MeOH (50°, 3 hr) and purified by silica gel CC. Elution with *n*-hexane–EtOAc (3:7 and 1:4) afforded 41.5 mg [5,7,7-^3H]24-*epi*-teasterone (specific radioactivity 57 MBq mmol^{-1}) [9].

Measurement of radioactivity. [5,7,7-^3H]24-*epi*-Teasterone was adjusted to a specific radioactivity of 10.4 MBq mmol^{-1} by addition of non-labelled **1** before application. 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 by means of an automatic TLC linear analyser. For quantification of metabolites the ratios of the peak areas from TLC radioscans were used.

Cell cultures and application of sample. Cell cultures of *Lycopersicon esculentum* L. were grown in a

Table 1. ^1H and ^{13}C NMR data of **2** and **3** (125.70/499.84 MHz, $CDCl_3$; values in italic faces are chemical shifts of HMQC cross peaks)

Position	**2**		**3**	
	^1H	^{13}C	^1H	^{13}C
1	*1.33/1.78*	37.7	n.d.	37.7
2	*1.91/1.49*	29.9	*1.90/1.51*	30.0
3	3.72 *tt* (11.3/4.7)	78.6	3.65 *m*	78.7
4	*1.98/1.42*	27.3	*2.00/1.42*	27.4
5	2.37 *dd* (12.4/2.5)	57.5	2.37/*dd* (12.6/2.6)	57.4
6	—	213.8	—	213.8
7	*2.11/2.22*	47.4	*2.11/2.22*	47.2
8	*1.78*	39.4	*1.78*	39.5
9	*1.36*	54.8	*1.37*	54.9
10	—	42.2	—	42.2
11	*1.66/1.37*	22.6	n.d.	22.6
12	*1.30/2.04*	40.9	n.d.	40.9
13	—	43.9	—	44.0
14	*1.35*	57.9	*1.33*	57.9
15	*1.59/1.22*	24.9	n.d.	25.0
16	*2.01/1.32*	28.7	n.d.	28.8
17	*1.59*	54.2	*1.60*	54.2
18	0.72 *s*	12.2	0.72	12.3
19	0.76 *s*	13.3	0.75	13.4
20	*1.48*	41.7	n.d.	41.7
21	0.98 *d* (6.7)	13.1	0.978 *d* (6.7)	13.2
22	3.65 *dd* (4.7/1.6)	73.4	3.65 *dd* (4.8/1.6)	73.4
23	3.33 *dd* (6.2/4.7)	77.6	3.33 *dd* (6.2/4.8)	77.3
24	*1.47*	42.7	*1.48*	42.7
25	*1.94*	28.0	n.d.	28.0
26	0.86 *d* (6.8)	17.5	0.86 *d* (6.7)	17.6
27	0.91 *d* (6.9)	22.6	0.91 *d* (6.7)	22.7
28	0.83 *d* (7.0)	11.1	0.83 *d* (7.0)	11.2
1′	4.40 *d* (7.8)	102.4	4.35 *d* (7.6)	102.9
2′	3.15 *dd* (8.7/7.9)	75.1	3.49 *dd* (9.8/7.6)	73.2
3′	3.34 *dd* (8.8/8.8)	78.0	3.56 *dd* (9.8/3.3)	75.2
4′	*3.28*	71.6	4.04 *dd* (3.3/1.0)	79.2
5′	3.45 *ddd* (9.4/6.1/2.0)	77.1	3.50 *ddd* (7.7/5.8/1.0)	75.5
6′	4.11 *dd* (11.8/2.0)	69.8	3.86 *dd* (11.1/7.7)	61.3
	3.77 *dd* (11.8/6.1)		3.60 *dd* (11.1/5.8)	
1″	4.40 *d* (7.8)	104.9	4.51 *d* (7.8)	106.1
2″	3.20 *dd* (8.9/7.8)	75.1	3.27 *dd* (9.1/7.8)	75.7
3″	3.34 *dd* (8.8/8.9)	78.0	3.36 *dd* (9.1/8.8)	78.3
4″	*3.28*	71.6	3.20 *dd* (9.7/8.8)	72.0
5″	*3.26*	78.0	3.30 *ddd* (9.7/7.3/2.4)	78.0
6″	3.86 *dd* (11.8/2.0)	62.8	3.89 *dd* (11.5/2.4)	63.1
	3.66 *dd* (11.8/5.5)		3.58 *dd* (11.5/7.3)	

n.d.: not detected because of poor signal-to-noise ratio of the HMQC spectrum.

Linsmaier–Skoog medium [11] at 22° on a gyratory shaker (100 rpm) under constant diffuse light (4.4 μmol m^{-2} s^{-1}) in 1 l. Erlenmeyer-flasks containing 440 ml cell suspension. Subculturing was performed every 7 days using an inoculum of *ca* 140 ml. A soln. of [5,7,7-^{3}H]24-*epi*-teasterone (8.6 mg in 9 ml DMSO) was administered to 9 Erlenmeyer-flasks (1 ml soln. per flask) at day 4 of the growth cycle.

Isolation and purification of metabolites. The cells 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 filtrate was evapd *in vacuo* at less than 40°. The residue was extracted with $CHCl_3$ and sepd after concn *in vacuo* by TLC (Merck silica gel 60; 0.25 mm layer for prep. mode; silica gel sheets for analyt. mode; developed ×2 in $CHCl_3$–MeOH, 9:1) and reversed-phase HPLC (Nucleosil C18; 10 μm; 250 × 10 mm; flow rate 5 ml min^{-1}; detection UV 204 nm and LSC of aliquots; gradient H_2O–MeCN from 7:3 to 1:1 in 20 min).

Spectrometric methods. LC-MS: The ESI MS measurements were carried out on a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV, sheath gas and auxillary gas was N_2) using a const-Metric 4100 HPLC instrument equipped with a LiCh-

rospher 100 RP18-column (5 μm, 2 × 100 mm). The following HPLC conditions were used: eluent MeCN–H_2O (containing 0.2% HOAc) 9:11; flow rate 0.2 ml min^{-1}; injection vol. 1 μl; injected amount *ca* 100 ng. NMR analysis was 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.

Acknowledgements—The authors are grateful to Dr B. Voigt (Halle) for a gift of 24-*epi*-teasterone, to Prof. F. Führ and Dr M. Roeb (Jülich) for providing the possibility for tritium labelling, to Dr J. Schmidt (Halle) for MS analysis, and to Prof. M. H. Zenk (München) for providing tomato cell cultures. This investigation was supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt/Main.

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