Pergamon

PII: S0031-9422(98)00037-5

Phytochemistry, Vol. 48, No. 3, pp. 467–470, 1998

Printed in Great Britain
0031 9422/98 $19.00 – 0.00

METABOLIC INVERSION OF THE 3-HYDROXY FUNCTION OF BRASSINOSTEROIDS

ADELHEID KOLBE, BERND SCHNEIDER,* ANDREA PORZEL and GÜNTER ADAM

Institut für Pflanzenbiochemie Halle, Weinberg 3, Postfach 110432, D-06018 Halle, Germany

(*Received* 26 *September* 1997)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; cell suspension cultures; biosynthesis; brassinosteroids; 3-dehydro-24-*epi*-teasterone; 24-*epi*-teasterone; 24-*epi*-typhasterol.

Abstract—Exogenously applied 3-dehydro-24-*epi*-teasterone is transformed by cell suspension cultures of *Lycopersicon esculentum* to give the metabolites 24-*epi*-teasterone and 24-*epi*-typhasterol in about equal but low quantities. The major portion of 24-*epi*-teasterone was found as carbohydrate conjugates while 24-*epi*-typhasterol occurred in free form, indicating significant influence of glycosidation on the equilibrium between both compounds. The importance of these conjugation processes for the regulation of the brassinosteroid biosynthesis is discussed. © 1998 Elsevier Science Ltd. All rights reserved

INTRODUCTION

The 3β-hydroxyl group of steroids originates from monooxygenase catalyzed oxidation of squalene followed by cyclization of the formed 2,3-oxidosqualene by squalene cyclase to afford cycloartenol as the primary steroidal compound in plants. Further oxidation of the 3β-hydroxyl group during biosynthesis of various steroids yields 3-keto type compounds. Steroids bearing a 3α-hydroxyl functionality, suggested to be formed by inversion of configuration via a redox mechanism, occur within various classes of plant and animal steroids, e.g. cardenolides [1] and ecdysteroids [2]. Such a mechanism has been proposed also in the biosynthesis of brassinosteroids [3] which recently have been established as a class of new phytohormones [4–6].

In fact, brassinosteroids bearing a 3α-hydroxyl group, e.g. 24-*epi*-castasterone and 24-*epi*-brassinolide, have been biotransformed to metabolites having 3β-hydroxy functionalities [7]. 3-Dehydro-24-*epi*-castasterone was identified as an intermediate in this metabolic reaction [8]. Furthermore, transformation of teasterone to typhasterol [9] via 3-dehydroteasterone, which is also a naturally occurring brassinosteroid [10], has been confirmed during brassinosteroid biosynthesis by labelling experiments.

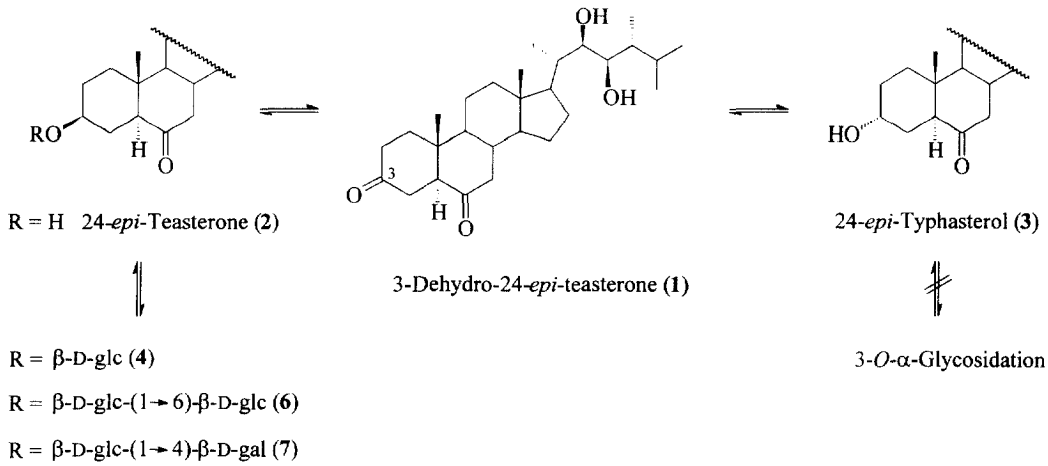
The epimerization of the hydroxyl functionality at C-3 might be a convenient pathway to regulate the cellular level of hormonal active brassinosteroids located in the biosynthetic sequence beyond that step. Therefore, in this paper the steps between 24-*epi*-teasterone and 24-*epi*-typhasterol have been studied by feeding experiments in cell cultures of *Lycopersicon esculentum*. Furthermore, the involvement of conjugation reactions in that process was demonstrated.

RESULTS AND DISCUSSION

In two parallel experiments, 3-dehydro-24-*epi*-teasterone (**1**), a putative intermediate in the biosynthesis of 24-*epi*-brassinolide, has been administered to cell suspension cultures of *Lycopersicon esculentum*. An ethanolic solution (100 μl) of compound **1** (4.1 mg; final concentration 6×10^{-5} M), labelled with deuterium as previously described [11], was applied to the sterile cell suspension at day 5 of the growth cycle. The normal cell growth was not influenced under these conditions. After 19 h the cells were harvested and extracted with 80% aqueous methanol. Both extracts were evaporated to remove the methanol. The remaining aqueous phases were extracted with ethyl acetate.

One of these batches, prior to partition between water and ethyl acetate, was subjected to enzymatic hydrolysis by means of cellulase in citrate buffer in order to hydrolyze supposed glycosides. Preparative TLC was employed to remove both hydrophilic and lipophilic impurities from the ethyl acetate fraction. The combined TLC zones, co-chromatographing with 3-dehydro-24-*epi*-teasterone (**1**), 24-*epi*-teasterone (**2**) and 24-*epi*-typhasterol (**3**), were boronated with *m*-dansylaminophenylboronic acid and analysed by

* Author to whom correspondence should be addressed.
Present address: Max-Planck-Institut für Chemische Ökologie, Tatzendpromenade 1a, D-07745 Jena, Germany.



Scheme 1. Transformation of 3-dehydro-24-*epi*-teasterone (**1**) and 24-*epi*-teasterone (**2**) by cell suspension cultures of *Lycopersicon esculentum*.

reversed-phase HPLC (method 1). Integration of the fluorescence trace (515 nm/230 nm) indicated only residual quantities of the starting compound **1** (R_t 14.9 min) whereas the newly formed 24-*epi*-teasterone (**2**) (R_t 14.1 min) and 24-*epi*-typhasterol (**3**) (R_t 16.9 min) were found in a 23:1 ratio. The EI-mass spectra of both compounds exhibited approximately equal distribution of the deuterium as in compound **1**, indicating that no remarkable dilution by endogenous compounds had occurred.

In contrast, the second batch was left non-hydrolyzed. In that case, HPLC analysis (method 1) of the ethyl acetate fraction indicated a 1:1 ratio of 24-*epi*-teasterone (**2**) and 24-*epi*-typhasterol (**3**). This tremendous difference between both parallel experiments is due to the fact that 24-*epi*-teasterone (**2**) underwent extensive conjugation reactions at the equatorial 3β-OH while compound **3** with axial 3α-OH was not conjugated. This finding is in accordance with glucosidation and acylation, respectively, of the 3β-hydroxyl of brassinosteroids as found for native compounds as well as in metabolic processes [4]. In contrast, conjugation of the 3α-hydroxyl was not observed for brassinosteroids.

As recently demonstrated, disaccharide conjugates are the predominating metabolites of 24-*epi*-teasterone (**2**) in tomato cell cultures [12]. In these experiments, 24-*epi*-teasterone-3-*O*-β-D-glucopyranosyl-(1→6)-β-D-glucopyranoside (**5**) and 24-*epi*-teasterone-3-*O*-β-D-glucopyranosyl-(1→4)-β-D-galactopyranoside (**6**) have been isolated as metabolites of ^{3}H-**2** from tomato cell suspension cultures by radioactivity guided fractionation. Additionally, 24-*epi*-teasterone-3-*O*-β-D-glucopyranoside (**4**) was formed from ^{3}H-**2** (final concentration 4×10^{-6} M; incubation time 5 h) in minor concentrations and has been isolated by TLC (R_f 0.2) and HPLC (R_t 7.6 min, method 2). The NMR data (Table 1), obtained by 1D TOCSY, HMQC, HMBC and NOE difference spectra, indicated a glucoside of **2**. After acetylation and another HPLC run (R_t 17.1 min, method 3), the structure of **4** was confirmed by the 1D TOCSY and HMQC spectra of the peracetylated derivative **5** (Table 1). In this experiment the same assignment strategy as previously described [12] has been employed. Remarkably, the formation of glycosides, **4**, **6**, and **7** was observed within 5 h after application of the parent compound.

Additionally, after feeding of ^{3}H-**2**, a more lipophilic fraction, co-chromatographing with compound **3** (R_f 0.5), was detected by radio-TLC. This radiolabelled fraction was subjected to derivatization with *m*-dansylaminophenylboronic acid. Reversed-phase HPLC (method 1) revealed 24-*epi*-teasterone (**2**) (R_t 14.1 min) and of 24-*epi*-typhasterol (**3**) (R_t 16.9 min) as demonstrated by comparison with authentic *m*-dansylaminophenylboronates of **2** and **3**. ESI-MS of both derivatives indicated the same molecular mass of *m/z* 783 $[M + H]^+$. As shown by integration of the HPLC peaks and by liquid scintillation counting (LSC), compounds **2** and **3** appeared in a 1:1 ratio. That ratio, both observed after application of ^{2}H-**1** and ^{3}H-**2**, suggested a dynamic equilibrium between **2** and **3**.

Our investigations confirmed that 3-dehydro-24-*epi*-teasterone (**1**) is a biogenetic intermediate in the formation of 24-*epi*-brassinosteroids. Since either epimerization and conjugation are reversible metabolic reactions, both processes are appropriate for regulation of brassinosteroid biosynthesis in *Lycopersicon esculentum*.

EXPERIMENTAL

Labelled compounds and measurements of radioactivity

[2,2,4,4,5,7,7-^{2}H]3-dehydro-24-*epi*-teasterone (^{2}H-**1**) was prepared by isotopic exchange [11]. Triethylamine (100 μl) and D_2O (400 μl) were added

Table 1. Selected NMR data (125.70/499.84 MHz) of **4** (MeOH-d_4) and its peracetylated derivative **5*** ($CDCl_3$)

	4		**5**	
	^{13}C†	^{1}H‡	^{13}C†	^{1}H‡
Aglycone				
3	78.5	*3.71*	78.4	3.57 *tt* (11.5/4.5)
18	12.1	0.72 *s*	11.6	0.67 *s*
19	13.2	0.76 *s*	13.0	0.73 *s*
20	41.7	n.d.	n.d.	n.d.
21	13.0	0.98 *d* (6.7)	13.3	0.93 *d* (6.7)
22	73.4	3.65 *m*	74.6	5.24 *d* (7.3)
23	77.3	3.33 *dd* (6.5/5.5)	77.4	5.07 *dd* (7.3/4.9)
24	42.7	*1.48*	n.d.	n.d.
25	28.0	*1.95*	n.d.	n.d.
26	17.5	0.86 *d* (6.8)	17.0	0.86 *d* (6.8)
27	22.5	0.91 *d* (6.9)	22.3	0.93 *d* (6.9)
28	11.1	0.83 *d* (7.0)	10.7	0.82 *d* (7.1)
Glucosyl				
1′	102.4	4.39 *d* (7.8)	99.2	4.61 *d* (7.9)
2′	75.2	3.14 *dd* (9.1/7.8)	71.3	4.96 *dd* (9.5/7.9)
3′	78.1	3.34 *t* (9.1)	72.8	5.18 *t* (9.5)
4′	71.4	*3.25*	68.4	5.08 *t* (9.5)
5′	77.9	*3.25*	71.7	3.65 *ddd* (9.5/4.6/2.5)
6′	62.8	3.85 *m*	61.9	4.26 *dd* (12.2/4.6)
		3.64 *m*		4.12 *dd* (12.2/2.5)

n.d., Not detected because of poor signal-to-noise ratio.

*^{1}H chemical shifts of side chain acetyl groups: δ 2.04, 2.03, and of the acetyl groups of glucose: δ 2.08, 2.07, 2.02, 2.00.

†Chemical shifts of ^{1}H, ^{13}C correlation cross peaks.

‡Values in *italic* are chemical shifts of ^{1}H, ^{13}C correlation cross peaks.

successively to a soln of 3-dehydro-24-*epi*-teasterone (25 mg) in MeOH-d_4 (300 μl) under nitrogen. The mixture was heated to 70° for 50 h in a sealed glass ampoule. The reaction mixture was then evaporated under a stream of N_2 gas. Exchangeable deuterium was removed with MeOH. The reaction product was purified by silica gel column chromatography by stepwise elution with *n*-hexane–EtOAc from 9:1 to 1:1. The fraction eluting with *n*-hexane–EtOAc (1:1) afforded the deuterated product (21 mg). The labelling procedure of [5,7,7-^{3}H]24-*epi*-teasterone (**2**) has been previously described [12]. The spec. radioactivity was adjusted to 10.4 MBq mmol^{-1} by addition of non-labelled **2** before application. Radioactivity of all frs was measured by LSC. The radioactive zones of the TLC plates were analyzed by means of an automatic TLC linear analyzer.

Cell cultures and application

Cell cultures of *Lycopersicon esculentum* L. were grown in a Linsmaier-Skoog medium [13] at 22° on a gyratory shaker (100 rpm) under constant diffuse light (4.4 μmol m^{-2}s^{-1}) in conical flasks. Subculturing was performed every 7 days. Labelled compounds were administered at day 4 (^{3}H-**2**) and 5 (^{2}H-**1**), respectively, of the growth cycle. The cell suspensions were held under identical conditions during the entire incubation period.

Extraction and chromatography

For isolation of **2** and **3**, 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 evaporated *in vacuo* at less than 40°. The residue was extracted with EtOAc (2 × 50 ml). The combined EtOAc extracts were separated after concentration *in vacuo* by TLC (Merck silica gel 60; 0.25 mm layer thickness; developed twice in $CHCl_3$–MeOH 9:1) and reversed-phased HPLC (method 1: LiChrospher 100 RP18; 5 μm; 250 × 4 mm; 45°; 1 ml min^{-1}; MeCN–H_2O 17:3, isocratic mode; fluorescence detection 230 nm / 515 nm). Prior to EtOAc extraction, one of the extracts was incubated with 25 mg cellulase in 25 ml citrate buffer (pH 4.0) at 37° on a gyratory shaker (185 rpm) for 21 h. The isolation of **6** and **7** has been described in Ref. [12]. Purification of compound **4** was carried out by reversed-phase HPLC (method 2: LiChrospher 100 RP18; 10 μm; 250 × 10 mm; 5 ml min^{-1}; MeCN–H_2O 11:9; isocratic mode;

detection 204 nm and LSC of aliquots). After acetylation of **4**, the peracetyl derivative **5** was again purified by reversed-phase HPLC (method 3: LiChrospher 100 RP8; 5 μm; 250 × 4 mm; 0.8 ml min^{-1}; gradient from MeCN–H_2O 3:2 to MeCN in 40 min; UV detection 204 nm and LSC of aliquots).

Derivatization

For boronation, a soln of *m*-dansylaminophenylboronic acid (0.1 mg) in MeCN–pyridine 99:1 (100 μl) was added to the dry sample. The soln was heated to 70° for 30 min. After cooling the soln was diluted with 1 ml MeCN. Then an aliquot was subjected to reversed-phase HPLC. Acetylation was done by Ac_2O in pyridine with 1% dimethylaminopyridine at 20° for 24 h.

Spectrometric methods

LC-MS: The ESI mass spectra measurements were carried out on a Finnigan MAT TSQ 7000 instrument (electrospray voltage 4.5 kV, sheath gas and auxiliary gas was nitrogen) using a const Metric 4100 HPLC instrument equipped with a LiChrospher 100 RP18-column (5 μm, 2 × 100 mm). The following HPLC conditions were used: eluent MeCN–H_2O (containing 0.2% HOAc) 45:55; flow rate 0.2 ml min^{-1}; injection volume 1 μl; injected amount ca 100 ng. NMR analysis was carried out on a VARIAN UNITY 500 spectrometer at 499.84 MHz (^{1}H) using a NALORAC 3 mm microsample inverse detection probe. MeOH-d_4 was used as solvent for compound **4** and $CDCl_3$ for the acetyl derivative **5**.

Acknowledgements—The authors are grateful to Dr B. Voigt (Halle) for a gift of 3-dehydro-24-*epi*-teasterone and 24-*epi*-teasterone and to Dr J. Schmidt (Halle) for MS analysis. These investigations were supported by the Deutsche Forschungsgemeinschaft, Bonn, and the Fonds der Chemischen Industrie, Frankfurt.

REFERENCES

1. Kawaguchi, K., Hirotani M. and Furuya, T. In *Biotechnology in Agriculture and Forestry*, Vol. 21, Y. P. S. Bajaj (ed.), Springer, 1993, p. 371.
2. Milner, N. P. and Rees, H. H., *Biochemical Journal*, 1985, **231**, 369.
3. Griffiths, P. G., Sasse, J. M., Yokota, T. and Cameron, D. W., *Bioscience, Biotechnology and Biochemistry*, 1995, **59**, 956.
4. Adam, G., Porzel, A., Schmidt, J., Schneider, B. and Voigt, B. In *Studies in Natural Products Chemistry*, Vol. 18, ed. Atta-ur-Rahman, Elsevier, 1996, p. 495.
5. Clouse, S. D., *The Plant Journal*, 1996, **10**, 1.
6. Fujioka, S. and Sakurai, A., *Natural Product Reports*, 1997, **14**, 1.
7. Kolbe A., Schneider, B., Porzel A. and Adam, G., *Phytochemistry*, 1996, **41**, 163.
8. Hai, T., Schneider, B., Porzel, A. and Adam, G., *Phytochemistry*, 1996, **41**, 197.
9. Suzuki, H., Inoue, T., Fujioka, S., Takatsuto, S., Yanagisawa, T., Yokota, T. Murofushi, N., Sakurai, A., *Bioscience, Biotechnology and Biochemistry*, 1994, **58**, 1186.
10. Yokota, T., Nakayama, M., Wakisaka, T., Schmidt, J. and Adam, G., *Bioscience, Biotechnology and Biochemistry*, 1994, **58**, 1183.
11. Kolbe, A., Schneider, B., Voigt, B. and Adam, G., *Journal of Labelled Compounds and Radiopharmaceuticals*, 1998, **41**, 131.
12. Kolbe, A., Porzel, A., Schneider, B. and Adam, G., *Phytochemistry*, 1997, **46**, 1019.
13. Linsmaier, E. M. and Skoog, F., *Physiologia Plantarum*, 1965, **18**, 100.