Pergamon

0031-9422(95)00224-3

Phytochemistry, Vol. 40, No. 2, pp. 443 448, 1995

0031-9422/95 $9.50 + 0.00

METABOLIC CONVERSION OF 24-EPI-BRASSINOLIDE INTO PENTAHYDROXYLATED BRASSINOSTEROID GLUCOSIDES IN TOMATO CELL CULTURES

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(*Received* 4 *January* 1995)

Key Word Index—*Lycopersicon esculentum*; Solanaceae; brassinosteroids; cell suspension cultures; cytochrome P-450 inhibitors; glucosides; glucosyltransferase; hydroxylation; metabolism; rice lamina inclination test.

Abstract—Two isomeric metabolites, 25-β-D-glucopyranosyloxy-24-epi-brassinolide and 26-β-D-glucopyranosyloxy-24-epi-brassinolide, have been formed in cell suspension cultures of *Lycopersicon esculentum* from exogenously applied 24-epi-brassinolide. The two-step metabolic process involved hydroxylation of the side-chain at C-25 and C-26, respectively, followed by glucosidation of the newly formed hydroxyl groups. The ratio between both metabolites was significantly altered by *in vivo* treatment of the cell cultures with various cytochrome P-450-specific inhibitors, indicating the involvement of two different enzyme systems. Biosynthetically prepared 25-hydroxy-24-epi-brassinolide, reapplied to cell cultures, was exclusively glucosylated at the 25-hydroxyl group, strongly suggesting regiospecificity of the corresponding glucosyltransferase.

INTRODUCTION

Due to their multiple plant growth-regulating activities and widespread distribution in the plant kingdom, brassinosteroids are considered as a new group of plant hormones [1, 2]. Among the naturally occurring brassinosteroids, 24-epi-brassinolide (**1**) is one of the most important compounds used for biological experiments [3]. In cell suspension cultures of *Lycopersicon esculentum*, compound **1** was metabolized yielding 25-β-D-glucopyranosyloxy-24-epi-brassinolide (**3**) [4]. Now we have found a second metabolite formed under these conditions, which was identified as 26-β-D-glucopyranosyloxy-24-epi-brassinolide (**5**). The corresponding non-glucosidic intermediates, 25-hydroxy-24-epi-brassinolide (**2**) and 26-hydroxy-24-epi-brassinolide (**4**), respectively, were not detectable within the cell culture but were obtained by hydrolysis of the corresponding glucosides **3** and **5**. In this paper we report on the characterization of the metabolic reactions involved in the formation of these compounds, as well as on the bioactivity of both pentahydroxylated brassinosteroids **2** and **4**.

RESULTS

As shown by TLC of the cell extracts, 24-epi-brassinolide (**1**), after administration to cell suspension cultures of *Lycopersicon esculentum*, was converted into several hydrophilic metabolites. The TLC radioscan of an 80% aqueous methanol extract obtained at day 4 of a characteristic experiment exhibited two major peaks of compounds **3** (30%, R_f 0.46) and **5** (30%, R_f 0.32). The structure of compound **3** has recently been reported as 25-β-D-glucopyranosyloxy-24-epi-brassinolide[4]. Compound **5** is accompanied by several other glycosides. The glycosidic character of this product was shown by enzymatic hydrolysis using cellulase as well as β-glucosidase to give the aglycone **4** (R_f 0.32). The major compound of this fraction (60%), was purified as described for **3** [4] by chromatographic methods including reverse-phase HPLC (R_t 8.5 min) as the final step. The FAB-mass spectrum of **5** resembled that of compound **3** [4]. Both spectra exhibited identical fragmentation patterns and relative intensities with negligible differences between the corresponding peaks, suggesting that both compounds were isomers bearing an additional hydroxyl group in comparison with the parent compound **1**: (negative ionization) *m/z* 657 $[M - H]^-$ and 495 [aglycone − H] $^-$; (positive ionization) *m/z* 659 $[M + H]^+$ and 681 $[M + Na]^+$, aglycone fragments *m/z* 497 $[\text{aglycone} + H]^+$, 479 $[\text{aglycone} + H - H_2O]^+$, 461 $[\text{aglycone} + H - 2H_2O]^+$, 443 $[\text{aglycone} + H - 3H_2O]^+$ and 409 (bond fission between C-23 and C-24). These data indicated an additional fifth hydroxyl group within the aglycone, located in the terminal part of the side-chain. The NMR analysis of **5** (^1H ^1H COSY, HMBC, HMQC) confirmed an un

changed sterol ring system and the position of the new hydroxyl function at one of the terminal methyl groups [5]. As was assumed for similar side-chain hydroxylations in the ecdysteroid series [6], this newly functionalized methyl group may be C-26. The NMR data also indicated a β-D-glucosidic bond between 26-OH and the sugar moiety. Thus, the structure of **5** was shown to be 26-β-D-glucopyranosyloxy-24-epi-brassinolide.

Both glucosides **3** and **5** bear the glucose moiety at the new hydroxyl group. Thus, the metabolic conversion of **1** to **3** and **5**, respectively, is a two-step process including hydroxylation and subsequent glucosidation (Fig. 1). Different from this finding, the known 23-*O*-glucosides of brassinosteroids [7, 8] are formed without preceding hydroxylation. Obviously, the pentahydroxylated brassinosteroids **2** and **4** are much better substrates for glucosylation than **1**. This was supported by the observation that compounds **2** and **4** after an incubation period of four days, were not detectable in free form in the cultured cells but were completely glucosylated, while 10% of the parent compound **1** remained non-metabolized. 24-Epi-brassinolide (**1**) was directly glycosylated in small amounts to an unknown glucoside which was characterized by enzymatic hydrolysis. Furthermore, several highly hydrophilic minor glycosides of unknown structure were detected.

The metabolic reactions transforming **1** into **3** and **5**, respectively, as well as the bioactivity of these glucosides and aglycones **2** and **4** have been the subject of the following studies.

As well as in microbial and animal cells, cytochrome P-450 participates in the plant cell in different reactions, such as hydroxylation and mono-oxygenation of various natural and synthetic targets [9]. In animals, hydroxylation in the side chain at C-22 and C-25, respectively, of ecdysones, a group of steroids structurally closely related to brassinosteroids, was catalysed by typical P-450–mono-oxygenases [10]. For the formation of **2** and **4**, the hydroxylation of **1** in both cases occurs selectively in the terminal region of the side-chain. Positions other than C-25 and C-26, were not hydroxylated in significant amounts, if at all. It was suggested that the hydroxylation of **1** to give **2** and **4**, respectively, is also catalysed by cytochrome P-450-dependent mono-oxygenases. The question was now to find out whether both hydroxylations were catalysed by the same enzyme, or if two different, highly specific enzymes were involved. Cytochrome *c*, various other cytochrome P-450-specific inhibitors and carbon monoxide have been used as tools to characterize and manipulate different hydroxylation reactions *in vitro*. For instance, cytochrome *c*, plumbagine and menadione were used recently for inhibitory studies of a membrane-bound cytochrome P-450 enzyme from *Papaver somniferum* [11] and for glycyrrhetinic acid 24-hydroxylase from licorice cells (*Glycyrrhiza glabra*) [12].

The *in vitro* application of inhibitors may cause complex variation of metabolic processes and it has been used therefore only in a few cases with cell suspension cultures [13]. The *in vivo* treatment of tomato cell cultures with various mono-oxygenase inhibitors simulta-

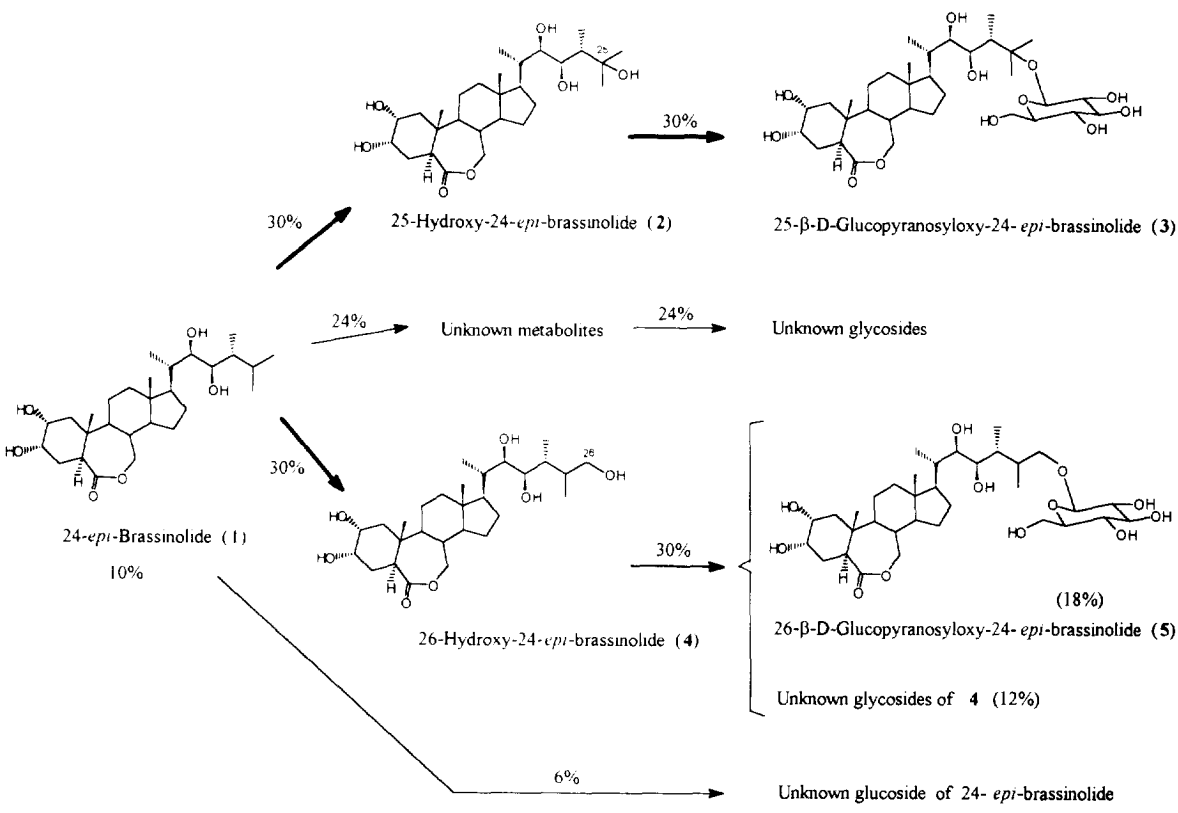

Fig. 1. Metabolism of 24-epi-brassinolide (**1**) in cultured cells of *Lycopersicon esculentum*.

neously with the administration of **1** was supposed to influence the pathway of hydroxylation. From this approach information on the specificity and character of the enzymes involved was expected. Prior to inhibitory studies, the influence of increasing doses of various exogenously applied P-450 inhibitors on the cell growth as well as the uptake of ^{3}H-**1** (1 μM) was determined *in vivo* to find the concentrations still tolerated by the cells. Concentrations of plumbagine and cytochrome *c*, for instance, which effect, as shown later, the hydroxylation processes, exhibited little or no influence on these parameters. Conversely, tetcyclasis and metyrapone significantly reduced both fresh weights and the portion of the radioactivity of the cell extracts. The influence of inhibitors on the position of hydroxylation of **1** was determined by ratio-TLC. The ratio of **3** and **5** (including accompanying minor glycosides of **4** in the latter case) within extracts of inhibitor non-treated cells was about 1:1. First, inhibitors competing with cytochrome P-450 for electron uptake were tested. As shown in Table 1, plumbagine, cytochrome *c* and 2,6-dichlorophenolindophenol (DCPIP) significantly reduced the formation of **5**, indicating inhibition of 26-hydroxylase activity. Cytochrome *c* (5 μM) showed the strongest effect of the inhibitors tested. Plumbagine and DCPIP, although applied in concentrations of 10 μM and 50 μM, respectively, showed less influence on the ratio of metabolites **3** and **5**. Menadione was inactive at the concentrations used.

So-called pharmacological inhibitors, metyrapone and piperonyl butoxide, are normally used for animal cytochrome P-450 studies, e.g. for characterization of ecdysone metabolism in the invertebrate *Locusta migratoria* [14]. However, they have also been used to study cytochrome P-450 enzymes of plant origin [15]. In our experiments, both compounds, in concentrations of 1 mM, showed only negligible influence on the ratio of **3** to **5**. Tetcyclasis is a plant growth regulator strongly inhibiting the oxidation of *ent*-kaurene to *ent*-kaurenoic acid during gibberellin biosynthesis [16] and also affecting sterol biosynthesis, as well as many other mono-oxygenase enzymes [9]. In our studies, tetcyclasis at concentrations of 5 μM changed the ratio of **3** and **5** in favour of **5**. This finding is opposite to the effect of cytochrome *c*. These results strongly suggest that the hydroxylation of **1** at C-25 and C-26, respectively, is catalysed by two specific enzymes of different types. The glucosidation of both hydroxylated intermediates **2** and **4**, respectively, is not influenced by the inhibitors used.

Cytochrome P-450 enzymes are characterized by a haem iron in the active centre which is involved in the transfer of oxygen to the substrate. Carbon monoxide inhibits this reaction by competing with dioxygen for binding to the haem iron. This effect is reversible by light. The inhibition by carbon monoxide is a principal criterion for cytochrome P-450 involvement in an oxidation reaction [10].

To study the poisoning effect of carbon monoxide, cell suspensions of *L. esculentum* were flushed with a continuous gas stream composed of CO, O_2 and N_2 in different ratios (Table 2). Exposure to carbon monoxide (CO–O_2, 9:1) reduced the cell growth to 76% in the dark and 78% in the light. However, the uptake of ^{3}H-**1** (1 μM) was only slightly reduced (Table 2) in comparison with the gas-non-treated cells (Table 1). In the control experiments with air flushing, a portion of **5** is reduced to give a ratio with **3** of about 3:7, indicating the sensitivity of the *in vivo* cell culture system to the changed experimental conditions. After exposure to CO–O_2 (9:1) in the dark, the ratio of **5** to **3** was found to be 2:23. This carbon monoxide poisoning effect was reversible, in part, by light (Table 2). In contrast to the sensitivity of the C-26 hydroxylation to carbon monoxide, the C-25 hydroxylation, atypically for a cytochrome P-450 enzyme, was completely resistant to carbon monoxide. These results confirmed the different character of both the C-25 and C-26 hydroxylases from cell cultures of *L. esculentum*.

Hitherto, known naturally occurring [7] and metabolically formed glucosides [8] of brassinosteroids bear the carbohydrate moiety at the C-23 hydroxyl. Thus, the formation via the pentahydroxylated compounds **2** and **4** of 25-*O*- and 26-*O*-glucosidic compounds **3** and **5** as major metabolites of **1** was not expected and prompted

Table 1. Effect of *in vivo* applied mono-oxygenase inhibitors on the growth of cultured cells of *Lycopersicon esculentum*, uptake of exogenously applied [5,7,7-^{3}H]-24-epi-brassinolide (**1**) and ratio of hydroxylation at C-25 and C-26

Inhibitor	Concentration (μM)	Cell growth (% fr. wt)	Uptake of **1** (% RA*)	Ratio **5** to **3**
Plumbagine	10	90	84	2:3
Cytochrome *c*	5	105	88	31:69
DCPIP	50	80	84	2:3
Menadione	100	80	90	1:1
Metyrapone	100	70	78	12:13
Piperonyl butoxide	100	64	85	11:9
Tetcyclasis	5	78	62	63:37
Control	0	100	100	1:1

*RA = Radioactivity found 4 days after application of [5,7,7-^{3}H]-**1** in the methanol extract of the cells.

Table 2. Effect of carbon monoxide on the growth of cultured cells of *Lycopersicon esculentum* uptake of exogenously applied [5,7,7-^{3}H]24-epi-brassinolide (**1**), and ratio of hydroxylation at C-25 and C-26

Gas mixture	Illumination	Cell growth (% fr. wt)	Uptake of **1** (% RA*)	Ratio **5** to **3**
CO–O_2 9:1	–	76	60	2:3
CO–O_2 9:1	+	78	70	1:1
N_2–O_2 9:1	–	97	71	2:3
N_2–O_2 9:1	+	94	70	31:69
Air	–	90	76	12:13
Air	+	100	75	1:1

*RA = Radioactivity found 4 days after application of [5,7,7-^{3}H]-**1** in the methanol extract of the cells.

us to examine the regiospecificity of the glucosyltransferases involved. Tritium-labelled compounds **2** and **4** obtained by enzymatic hydrolysis of biosynthetically prepared **3** and **5**, respectively, were applied to cell cultures of *L. esculentum* at day 3 of the growth cycle. After four days of incubation, over 90% of the applied radioactivity was recovered in the methanol extract of the cells. Radio-TLC (R_f 0.46) and reverse-phase HPLC (R_f 6.5 min) indicated that one individual polar compound was formed from **2** which was identical with **3**. Conversely, after enzymatic hydrolysis **2** and glucose (PC, R_f 0.28) were released from **3**. FAB-mass spectrometry of **3**, obtained from reapplication experiments (*m/z* 409 [4]) confirmed the position of the glucose moiety at the terminal part of the side-chain. These results strongly suggested that **2** underwent neither conversion to compounds other than **3** nor noticeable catabolism. In contrast, from ^{3}H-labelled **4** under the same conditions at least four glucosyl conjugates were formed. Among them the major compound (18% of total radioactivity, Fig. 1) was identical with metabolite **5**. The glucosidic fraction, obtained after reapplication of **4** to tomato cell cultures after hydrolysis, released only a single radioactively labelled compound identical with **4**, as well as glucose. Thus, it was concluded that **4** may be glucosylated at various hydroxyl groups but the most preferred position is 26-OH. Comparing the behaviours of **2** and **4** in reapplication experiments it has to be stated that the glucosidation of the 25-hydroxy compound **2** proceeds with high regiospecificity in this position, while the glucosidation of the 26-hydroxy compound **4** was less regiospecific. This implies the involvement of the aglycone **2** and its glucoside **3** in the mode of action of 24-epi-brassinolide (**1**) in plants.

The rice lamina inclination test (RLIT) was used to evaluate the activity of the new pentahydroxylated brassinosteroids **2** and **4** and their glucosides **3** and **5**. As a reference, the parent compound 24-epi-brassinolide (**1**) was used. The results obtained with *Oryza sativa* L., cv. Nep IR-415, a Vietnamese rice cultivar [17], are shown in Fig. 2. Compound **2** at a 2 nM concentration demonstrated nearly the same inclination angles as 20 nM of **1**, and 20 nM of **2** induced a greater inclination angle then 200 nM of **1**. Thus, **2** is about ten times more active than **1** indicating that the hydroxylation at C-25 is an activation step in brassinosteroid metabolism. Following these results, **2** next to brassinolide, is one of the most active brassinosteroids known until now. In comparison with compound **2**, the 26-hydroxylated metabolite **4** was clearly less active. As in other groups of steroidal hormones, for instance vitamin D metabolites, hydroxylation at C-25 seems to be essential for high activity. In comparison with the aglycones, the glucosides **3** and **5** exhibited less but significant activity in the RLIT, which may be due to hydrolysis within the test system.

DISCUSSION

Starting from phytosterol skeletons the brassinosteroids are biosynthesized by introduction of structural characteristics increasing their phytohormone-like activity in each step [7, 18]. The bioactivity reached a temporary maximum when two couples of vicinal hydroxyl groups with 2α,3α- and 22*R*,23*R*-structure, respectively, are present in ring A and in the side-chain. Furthermore, structure/activity relationships have indicated the importance of the terminal region of the side-chain, whereas small structural differences may cause dramatic changes in bioactivity [3, 19]. Details of the side-chain structure are also important for the activity in other classes of steroidal effectors. Thus, 25-hydroxyvitamin D_3 demonstrates much higher activity than analogues non-hydroxylated in this position [20]. From this point of view the formation of brassinosteroids bearing a fifth hydroxyl group at C-26 and C-25, respectively, in cell cultures of *L. esculentum*, as well as the extraordinary high activity of compound **2** in the rice lamina inclination test, was not unexpected.

The superior bioactivity of 25-hydroxy-24-epi-brassinolide (**2**), and the regiospecificity of both the C-25 hydroxylase and 25-*O*-glucosyltransferase, suggested that metabolite **2** and its 25-*O*-glucoside **3** are not detoxification products of exogenously applied 24-epi-brassinolide (**1**) but could be regarded as final members of the biosynthetic chain of brassinosteroids. As demonstrated by inhibitor experiments presented in this paper, the hydroxylases catalysing the formation of **2** and **4**, respectively, belong to separate types of mono-oxygenases. The

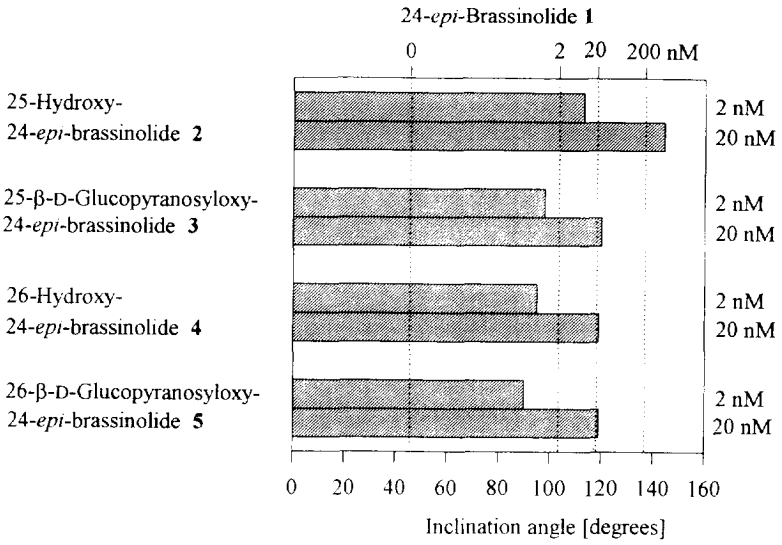

Fig. 2. Activity of metabolites of 24-epi-brassinolide (**1**) in the rice lamina inclination test using *Oryza sativa* L., cv. Nep IR-415.

C-26-hydroxylase is a typical cytochrome P-450-dependent enzyme. In response to specific cytochrome P-450 inhibitors, e.g. cytochrome *c*, DCPIP and carbon monoxide, the formation of **4** is significantly decreased. In contrast, the C-25 hydroxylase is a non-typical mono-oxygenase enzyme. This system was not sensitive to the above-mentioned inhibitors. In particular, it was completely insensitive to carbon monoxide poisoning. Such a phenomenon was previously shown for the C-25-hydroxycalciferol hydroxylase classified as a carbon monoxide-insensitive cytochrome P-450-dependent oxygenase [21].

Our studies provide for the first time information on enzymes of the brassinosteroid metabolism. *In vivo* treatment of cell cultures with various inhibitors has proved to be a useful approach.

EXPERIMENTAL

Radiochemicals and measurement of radioactivity. The synthesis of [5,7,7-^{3}H]24-epi-brassinolide (**1**) was recently described [22]. Compound **1** with sp. act. of 22.2 MBq mmol^{-1} was used. Radioactivity of all frs was measured by liquid scintillation counting (LSC). TLC plates were analysed for radioactive zones with an automatic TLC linear analyser. For quantification of metabolites the ratios of peak areas from TLC radioscans were used.

Cell culture. Plant cell cultures of *Lycopersicon esculentum* were obtained from the cell culture laboratory of Prof. Zenk (München). The suspended cells were grown in Linsmaier–Skoog medium [23] at 20° on a gyratory shaker (100 rpm) under constant diffuse light (600 lux) in 300-ml Erlenmeyer flasks containing 150 ml cell suspension. Subculturing was performed every 7 days using an inoculum of *ca* 70 ml.

Administration of labelled precursors and inhibitors. The filter-sterilized ethanolic solns (< 1 ml) of [5,7,7-^{3}H]24-epi-brassinolide (**1**) (final concn of cell suspension 10^{-5} M), [5,7,7-^{3}H]25-hydroxy-24-epi-brassinolide (**2**) (10^{-5} M), and [5,7,7,-^{3}H]26-hydroxy-24-epi-brassinolide (**4**) (10^{-5} M) were administered to the cell cultures at day 3 of growth. The cell suspensions were maintained under identical conditions for another 7 (following administration of **1**) and 4 (**2**, **4**) days. Inhibitors were applied simultaneously with the brassinosteroids as filter-sterilized ethanolic or aq. solns (100 μl) in concs given in Table 1. The air and gas stream composed of CO and O_2, or N_2, respectively, were sterilized by filtration and bubbled through the cell suspensions with a flow rate of 4 ml min^{-1}.

Isolation and purification of metabolites. The isolation of compound **3** (TLC, $CHCl_3$–MeOH, 9:1 and 4:1) has been described [4]. Compound **5** was isolated and purified as described for **3** with the following changes: reverse phase HPLC (Nucleosil C18; 10 μm, 250 × 10 mm; flow rate 4 ml min^{-1}; detection UV 204 nm and LSC of aliquots: gradient MeCN–H_2O from 1:3 to 9:11 in 15 min). Following enzymatic hydrolysis [4], compounds **2** and **4**, respectively, were subjected to prep. TLC (CH_2Cl_2–MeOH, 9:1) and reverse 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 2:3 for **2** (R_t 8.9 min); MeCN for **3** (R_t 7 min).

Rice lamina inclination test. The rice seeds (*Oryza sativa* L., cv. Nep IR-415) were washed thoroughly before germinating at 25°–26° under illumination by an incandescent lamp (60 W). After germination, the seedlings were placed into 1% solid agar medium and maintained for 5 days at 27° in the dark. Then leaf segments were excised and floated on water for 24 hr. For RLIT the segments were transferred to solutions of the compounds to be tested (27° in darkness, concn in Table 2) and

incubated for 2 days before the inclination angles between second leaf and sheath were determined.

Spectrometric methods. FAB-MS was performed with an AMD 402 mass spectrometer; neg. ionization 4 kV; pos. ionization 9 kV. NMR spectra were recorded on a Varian Unity 500 spectrometer at 499.84 MHz (^{1}H) and 125.7 MHz (^{13}C) in CD_3OD, with TMS as int. standard.

Acknowledgements—The authors thank Dr A. Porzel and Dr J. Schmidt for NMR and MS analysis, respectively. Dr A. Kolbe is acknowledged for a gift of tritium labelled 24-epi-brassinolide and Prof. M. H. Zenk for providing cell suspension cultures. This investigation was supported by Fonds der Chemischen Industrie, Frankfurt.

REFERENCES

1. Adam, G. and Petzold, U. (1994) *Naturwissenschaften* **81**, 210.
2. Sakurai, A. and Fujioka, S. (1993) *J. Plant Growth. Regul.* **13**, 147.
3. Marquardt, V. and Adam, G. (1991) in *Chemistry of Plant Protection* (Ebing, W., ed.), Vol. 7, p. 103. Springer, Berlin.
4. Schneider, B., Kolbe, A., Porzel, A. and Adam, G. (1994) *Phytochemistry* **36**, 319.
5. Porzel, A., Hai, T., Kolbe, A., Schneider, B. and Adam, G. (in preparation).
6. Imai, S., Fujioka, S., Murata, E., Sasakawa, Y. and Nakanishi, K. (1968) *Tetrahedron Letters* 3887.
7. Suzuki, H., Kim, S.-K., Takahashi, N. and Yokota, T. (1993) *Phytochemistry* **33**, 1361.
8. Yokota, T., Ogino, Y., Suzuki, H., Takahashi, N., Saimoto, H., Fujioka, S. and Sakurai, A. (1991) in *Brassinosteroids—Chemistry, Bioactivity, and Applications* (Cutler, H. C., Yokota., T. and Adam, G., eds), p. 86. ACS Symp. Ser. 474, Am. Chem. Soc., Washington.
9. Durst, F. (1991) in *Frontiers in Biotransformation*, Vol. 4, *Microbial and Plant Cytochrome P-450: Biochemical Characteristics, Genetic Engineering and Practical Implications* (Ruckpaul, K. and Hein, H., eds), p. 191. Akademie-Verlag, Berlin.
10. Kappler, C., Hetru, C., Durst, F. and Hoffmann, J. (1989) in *Ecdysone—From Chemistry to Mode of Action* (Koolman, J., ed.), p. 161. Thieme, Stuttgart.
11. Gerardy, R. and Zenk, M. H. (1993) *Phytochemistry* **32**, 79.
12. Hayashi, H., Hanaoka, S., Tanaka, S., Fukui, H. and Tabata, M. (1993) *Phytochemistry* **43**, 1302.
13. Cole, J. D. and Owen, W. I. (1987) *Plant Sci.* **50**, 13.
14. Kappler, C., Kabbouh, M., Durst, F. and Hoffmann, J. (1988) *J. Steroid Biochem.* **31**, 891.
15. Tanahashi, T. and Zenk, M. H. (1990) *Phytochemistry* **29**, 1113.
16. Graebe, J. E. (1984) *Ber. Dt. Bot. Ges.* **97**, 67.
17. Hai, T., Schneider, B. and Adam, G. (in preparation).
18. Suzuki, H., Fujioka, S., Takatsuto, S., Yokota, T., Murofushi, N. and Sakurai, A. (1994) *J. Plant Growth. Regul.* **13**, 21.
19. Yokota, T. and Mori, K. (1992) in *Molecular Structure and Biological Activity of Steroids* (Bohl, M. and Duax, W. L., eds), p. 317. CRC, Boca Raton.
20. Ikekawa, N. (1993) *Bioorg. Med. Chem. Letters* **3**, 1789.
21. Warner, M. (1983) *J. Biol. Chem.* **258**, 11590.
22. Kolbe, A., Marquardt, V. and Adam, G. (1992) *J. Lab. Comp. Radiopharm.* **31**, 801.
23. Linsmaier, E. M. and Skoog, F. (1965) *Physiol. Plant.* **18**, 100.