PERGAMON

Phytochemistry 51 (1999) 237–242

PHYTOCHEMISTRY

Monitoring brassinosteroid biosynthetic enzymes by fluorescent tagging and HPLC analysis of their substrates and products

Jochen Winter[a, 1], Bernd Schneider[b,*], Sabine Meyenburg[a], Dieter Strack[a], Günter Adam[a]

[a]*Institut für Pflanzenbiochemie, Weinberg 3, D-06120 Halle, Germany*
[b]*Max-Planck-Institut für Chemische Ökologie, Tatzendpromenade 1a, D-07745 Jena, Germany*

Received 6 October 1998; received in revised form 24 November 1998

Abstract

Both the vicinal side chain hydroxyl groups and the 6-oxo function of brassinosteroids were modified by fluorescence tagging. Dansylaminophenylboronic acid was used as a derivatizing agent to form fluorescent esters of brassinosteroids containing a side-chain *cis*-diol structure. 6-Oxo type brassinosteroids were derivatized by means of dansylhydrazine. The modified brassinosteroids, as far as possible derivatized both at the diol and the oxo group, were separated by HPLC and the optimal emission wavelength was determined. By this approach almost all brassinosteroids, including biosynthetic precursors, were susceptible to highly sensitive analysis in the fmol range. This method has been verified as an analytical tool to determine brassinosteroids in cell culture extracts and to monitor brassinosteroid biosynthetic enzymes. 24-Epibrassinolide has been detected in tomato cell suspension cultures. Several steps of brassinosteroid biosynthesis, including the Baeyer–Villiger oxidation of 24-epicastasterone to give 24-epibrassinolide, were monitored in vitro with protein preparations of the same cell culture line.

Keywords: *Lycopersicon esculentum*; Solanaceae; Biosynthesis; Brassinosteroids; Dansylhydrazones; Dansylaminophenylboronates; Enzyme assay

1. Introduction

Brassinosteroids are a group of plant hormones which occur ubiquitously throughout the plant kingdom (Adam, Porzel, Schmidt, Schneider, & Voigt, 1996). The biosynthetic (Fujioka, & Sakurai, 1997) and metabolic (Adam et al., 1996) pathways and the molecular mode of action (Kauschmann, Jessop, Koncz, Szekeres, Willmitzer, & Altmann, 1996; Clouse, 1996; Szekerez and Koncz, 1998) have been subject of detailed investigations over recent years, while enzymatic studies have hitherto been limited to hydroxylations at the terminal part of the side chain (Hai, Schneider, & Adam, 1995; Winter, Schneider, Strack, & Adam, 1997). The analytical approaches to identify brassinosteroids depend on the type of the investigation carried out. Naturally occurring brassinosteroids are usually isolated by bioactivity guided fractionation, using the rice lamina inclination assay in combination with GC–MS analysis of methylboronates or trimethylsilyl ethers (TMS) (Takatsuto, Ying, Morisaki, & Ikekawa, 1982). However, the rice lamina inclination bioassay is unable to detect bioinactive brassinosteroids.

Biosynthetic intermediates and metabolites carrying stable or radioactive isotopes are generally detected either by means of gas chromatography combined with selected ion monitoring mass spectrometry (GC–MS–SIM) (Takatsuto, 1994) or by radioanalytical methods (Kolbe, Schneider, Voigt, & Adam, 1998). Due to the absence of a suitable chromophore in the steroidal skeleton, trace concentrations of these plant hormones

* Corresponding author.
E-mail address: schneider@ice.mpg.de (B. Schneider)
[1] Present address: MPI für Züchtungsforschung, Carl-von-Linné-Weg 10, D-50829 Köln, Germany.

0031-9422/99/$ - see front matter
PII: S0031-9422(98)00760-2

cannot be detected by UV. Therefore, derivatization with fluorescent (Gamoh, Okamoto, Takatsuto, & Tejima, 1990) and electrochemical (Gamoh, Sawamoto, Takatsuto, Watanabe, & Arimoto, 1990) precolumn labelling reagents has been used in brassinosteroid analysis. Dansylaminophenylboronic acid has proved to be the most effective reagent for this purpose (Gamoh, & Takatsuto, 1994). The dansylation procedure has also been used to determine ketosteroids by HPLC (Kawasaki, Maeda, & Tsuji, 1982; Weinberger, Koziol, & Millington, 1985). In addition, a small number of other examples of brassinosteroid analysis by HPLC, with fluorimetric detection, have been described (Taylor et al., 1993; Griffiths, Sasse, Yokota, & Cameron, 1995), including detection of biosynthetic intermediates (Kolbe, Schneider, Porzel, & Adam, 1998). This paper reports our efforts to monitor individual transformations in brassinosteroid biosynthesis by fluorescence tagging and HPLC analysis.

2. Results and discussion

2.1. Fluorescence tagging

Teasterone is the first intermediate in the biosynthetic sequence (Fujioka et al., 1997) of the (24*S*)-configured brassinosteroids, bearing a vicinal diol function in the side chain that is accessible to boronation. Although the biosynthesis of the (24*R*)-configured brassinosteroids has not been studied in detail, it is expected to proceed in an analogous manner. Consequently, for the enzymatic transformation of cathasterone to teasterone and of 24-epicathasterone (**1**) to 24-epiteasterone (**2**), respectively, the formation of both epimers from earlier precursors and all downstream transformation steps should be detectable by HPLC of dansylaminophenylboronate tagged brassinosteroids. Moreover, the transformation of 6α-hydroxy-campestanol to 6-oxo-campestanol, and further to cathasterone, in the early C-6-oxidation pathway (Fujioka et al., 1995), as well as the corresponding steps in the 24*R* series between 6-oxo-24-epi-campestanol and 24-epicathasterone (**1**), are expected to be detectable by precolumn derivatization of the keto function using dansylhydrazine and subsequent HPLC with fluorescence detection. Additionally, recently established early intermediates, (24*R*)-24-methylcholest-4-en-3-one and (24*R*)-24-methyl-5α-cholestan-3-one (Fujioka et al., 1997), are accessible by the same approach. Thus, the combination of both derivatization methods should allow the monitoring of most of the biosynthetic and metabolic reactions of brassinosteroids.

To check the sensitivity of the fluorescence tagging method, and to optimize the emission wavelength, dansylhydrazones of 24-epicathasterone (**1**), 24-epiteasterone (**2**), 3-dehydro-24-epiteasterone (**3**), 24-epityphasterol (**4**), and 24-epicastasterone (**5**) as well as dansylaminophenylboronates of **2**–**5** and 24-epibrassinolide (**6**) were prepared. The emission of the dansylaminophenylboronates was measured in steps of 5 nm between 400 and 600 nm (excitation 230 nm) and in most cases an optimum of 520 nm was found. A shift of the emission wave lengths of only 20–500 nm decreases the sensitivity by approximately 70 to 80% for compounds **2**, **5** and **6**, and by 20% for **4**. 3-Dehydro-24-epiteasterone (**3**) showed a slightly different behaviour. The emission maximum (100% at 510 nm) was broader (92% at 500 nm, 98% at 520 nm) than that of the other boronates. These findings are in agreement with published results (Gamoh et al., 1990). An amount of 100 pg (210 fmol) of 24-epibrassinolide was readily detectable, indicating the excellent sensitivity of this method (**6**).

The emission optimum of the dansylhydrazones was at 500–505 nm (excitation 230 nm). Again, a sharp decrease in sensitivity to about 60–80% occurred when the emission wavelength was changed to 520 nm. For common detection of all brassinosteroid hydrazones, a wavelength of 505 nm was used. The minimum amount of 1.5 ng (3.3 pmol) of the dansylhydrazone of 24-epicastasterone (**5**), for example, were required for detection. Thus, the sensitivity of the hydrazone method was in the pmol range corresponding to only about 10% that of the dansylaminophenylboronate method. Nevertheless, the advantage of using this method is that even brassinosteroids lacking a vicinal diol structure in the side chain, e.g. 24-epicathasterone (**1**), could be detected.

To extend the number of brassinosteroids accessible to fluorescence tagging in a single experiment, dansylation by the hydrazine and the boronate methods were combined. It is important to perform the conversion to the hydrazone first, before adding the dansylaminophenylboronic acid. The hydrazone is stable in the alkaline medium required to form the boronate ester, whereas the boronate ester is unstable under the acidic conditions required for synthesizing the hydrazone. As a compromise between the emission wavelengths of the dansylaminophenylboronates and the dansylhydrazones, a wavelengths of 515 nm was used in these experiments.

To verify the suitability of the HPLC method, a mixture of brassinosteroids was derivatized by both dansylhydrazine and dansylaminophenylboronic acid. This mixture, containing 25 pmol of each brassinosteroid derivative, was subjected to HPLC using a shallow gradient (method A) to achieve good resolution of the complete set of dansylated brassinosteroids, and separation from other nonbrassinosteroid plant matter and material derived from the derivatization procedure.

The following retention times were found: R_t 97.8 min, dansylhydrazone of 24-epicathasterone (**1**); R_t 101.0 min, dansylaminophenylboronate of 24-epibrassinolide (**6**); R_t 120.2 min, 'double derivative' of 24-epicastasterone (**5**); R_t 130.8 min, 'double derivative' of 24-epiteasterone (**2**); R_t 133.4 min, 'double derivative' of 3-dehydro-24-epiteasterone (**3**); R_t 137.4 min, 'double derivative' of 24-epityphasterol (**4**). Various gradient systems, adapted to detect dansyl derivatives of individual brassinosteroids, were used in further experiments.

2.2. Naturally occurring brassinosteroids

Cell suspension cultures of *Lycopersicon esculentum* had been used to study the metabolism and conjugation of exogenously applied brassinosteroids (Hai et al., 1995; Hai, Schneider, Porzel, & Adam, 1996; Kolbe et al., 1998) and the role of a cytochrome P450 in the brassinosteroid hydroxylation (Winter et al., 1997). By using the fluorescence detection method, this cell line was now investigated for its natural content of brassinosteroids. The crude methanolic cell extract (fr wt 15 kg) was treated as previously described (Schmidt, Altmann, & Adam, 1997) with slight modifications. An aliquot of the chloroform fraction was derivatized both by dansylaminophenylboronic acid and dansylhydrazine and subjected to HPLC (method B). The R_t of the major peak at 30.9 min was identical with that of the dansylaminophenylboronate of 24-epibrassinolide (**6**). Moreover, upon derivatization of another aliquot of this fraction by means of methaneboronic acid, compound **6** could be determined by GC–MS analysis as its methaneboronate derivative. Remarkably, a bioassay usually employed to detect trace amounts of native brassinosteroids in plant samples was not necessary, since the boronate method is suitable for detecting even nonactive brassinosteroids and biogenetic precursors.

2.3. Enzyme assay

The detection of 24-epibrassinolide (**6**) in tomato cell cultures by the fluorescence tagging method indirectly indicated the occurrence of biosynthetic precursors and the activity of the corresponding biosynthetic enzymes in that material. Consequently, these cultures could be of value for monitoring the enzyme activities in the biosynthetic sequence affording 24*R*-brassinosteroids. The fmol sensitivity of the fluorescence method should be sufficient to detect these plant hormones and their precursors at low concentration and therefore be used to monitor individual steps of brassinosteroid biosynthesis.

Since brassinosteroid biosynthesis involves several hydroxylation steps which are assumed to be cytochrome P450 dependent, an enzyme preparation was used which had been successfully employed to characterize and to purify P450 monooxygenases from various plants (Gabriac, Werck-Reichhart, Teutsch, & Durst, 1991; Stadler, & Zenk, 1993). Three fractions (15,000*g* pellet, 100,000*g* pellet, 100,000*g* supernatant) of the cell extract were incubated with intermediates **1**–**5** of the putative biosynthetic sequence (Fig. 1). Due to the inducibility of the 25- and 26-hydroxylases (Winter et al., 1997), it was assumed that the other enzymes might be also inducible. Therefore, 12 h before preparing the extract, the tomato cell cultures were induced by application of 100 nmol of the respective substrate (1 μM final concentration). Preparations from noninduced cells were used as controls. In fact, enzyme preparations obtained from induced cells metabolized substrates **3**, **4** and **5**, while the corresponding noninduced control experiments failed to do so. The presumed P450-dependent enzymes in the putative biosynthetic chain, namely the 23β-hydroxylase (*a*) and the 2α-hydroxylase (*f*), were not detectable. This could be due to a nonoptimal enzyme preparation or differences between the actual biosynthetic sequence and the expected pathway.

Inversion of the 3-hydroxy function of brassinosteroids proceeds via a redox mechanism. This has been elucidated by identification of naturally occurring 3-dehydro type brassinosteroids (Yokota, Nakayama, Wakisaka, Schmidt, & Adam, 1994) and by feeding experiments (Suzuki et al., 1994; Hai et al., 1996). Very recently, the involvement of carbohydrate conjugation in the equilibrium between **2** and **4** has been described (Kolbe et al., 1998). Incubation of 3-dehydro-24-epiteasterone (**3**) and HPLC analysis of the dansylaminophenylboronates (R_t 14.6 min, method C) afforded both epimeric reduction products **2** (R_t 16.5 min) and **4** (R_t 18.1 min, method C). The 3β-reductase reaction (*c*) required with the 100,000*g* pellet and NADPH as coenzyme. In contrast, the 3α-reductase (*d*) was catalyzed only by a soluble protein occurring in the 100,000*g* supernatant (Table 1) with NADH as a coenzyme. The reverse reaction (*e*) was found in the same enzyme fraction, indicating that both reactions of the equilibrium between **3** and **4** were probably catalyzed by the same soluble enzyme. These two oxidoreductase reactions were obviously catalyzed by a typical dehydrogenase. Reaction (*b*) did not operate in vitro with the preparations and NAD or NADP as cofactors. The finding of (*c*) and (*d*/*e*) in different fractions clearly indicated the involvement of different enzymes in the redox process.

The Baeyer–Villiger transformation (*g*) of 24-epicastasterone (**5**) (R_t 20.2 min, method D) to 24-epibrassinolide (**6**) (R_t 13.7 min) was detected in the 100,000*g* pellet Table 1. Interestingly, the correspond-

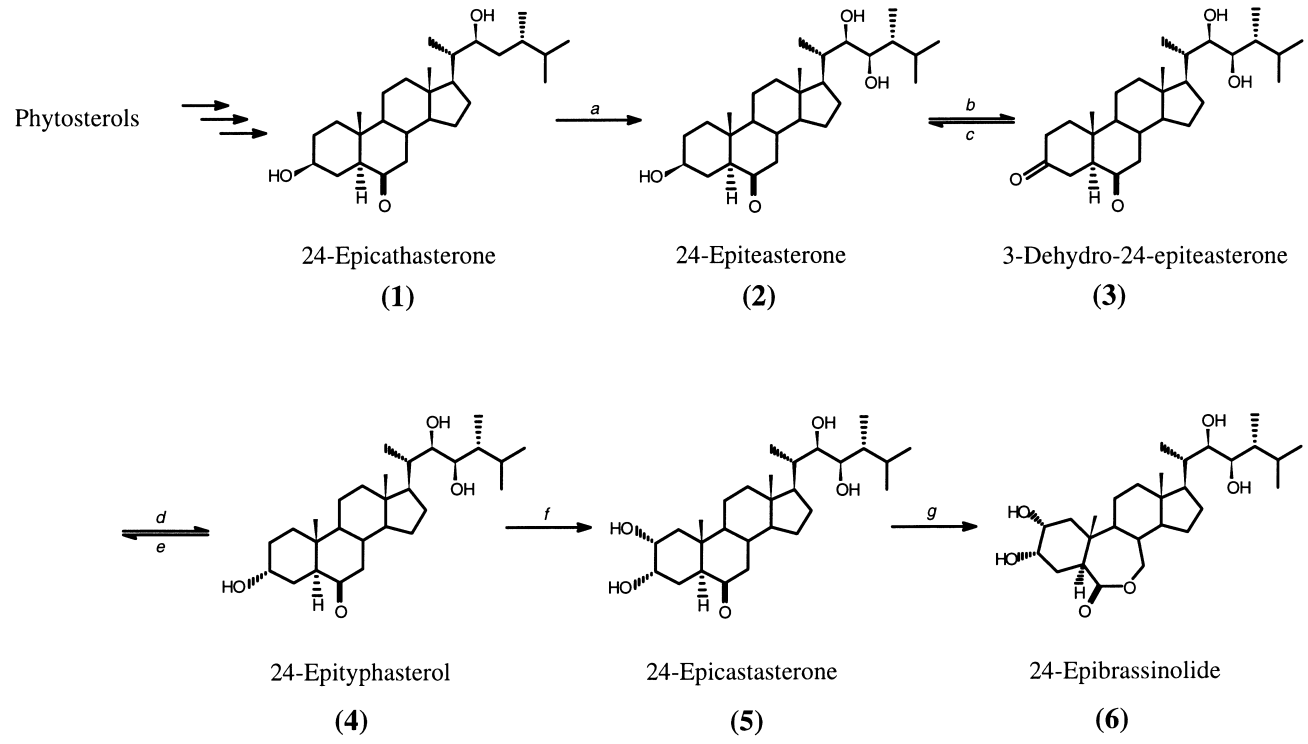

Fig. 1. Putative biosynthetic pathway of 24*R*-brassinosteroids.

ing reaction in the 24*S* series to give brassinolide hitherto could be demonstrated only in *Catharanthus roseus* cell cultures (Suzuki et al., 1993), while attempts to show this reaction in vivo in other systems failed (Suzuki et al., 1995).

In summary, the present studies demonstrated that fluorescence tagging of brassinosteroids combined with HPLC analysis is a suitable tool with which to monitor enzymatic activities which are involved in the biosynthesis of these plant hormones. Moreover, the preliminary results obtained by this method represent the first demonstration of enzyme activities of brassinosteroid biosynthesis in vitro. Further biochemical investigation with an extended number of putative substrates are required to characterize these enzymes in more detail and to monitor those transformations for which enzyme activities have not been detected so far.

Table 1
Transformation of brassinosteroid biosynthetic intermediates by enzyme preparations from cell suspension cultures of *L. esculentum*. Synthetic reference compounds were derivatized with dansylaminophenylboronic acid and used as HPLC standards to identify the enzyme products

Substrate[a]	Cofactors	Reaction[a]	Product[a]	Pellet 15,000 *g* (fkat mg^{-1} protein)	Pellet 100,000 *g* (fkat mg^{-1} protein)	Supernatant 100,000 *g* (fkat mg^{-1} protein)
24-Epicathasterone (**1**)	NADH, NADPH	*a*	–[b]	–	–	–
24-Epiteasterone (**2**)	NAD, NADP	*b*	–[c]	–	–	–
3-Dehydro-24-epiteasterone (**3**)	NADPH	*c*	24-Epiteasterone (**2**)	–	361	–
	NADH	*d*	24-Epityphasterol (**4**)	–	–	82
24-Epityphasterol (**4**)	NAD	*e*	3-Dehydro-24-epiteasterone (**3**)	–	–	21
	NADH, NADPH	*f*	–[d]	–	–	–
24-Epicastasterone (**5**)	NADPH	*g*	24-Epibrassinolide (**6**)	–	230	–

[a] See Fig. 1.
[b] The expected product **2** was not observed.
[c] The expected product **3** was not observed.
[d] The expected product **5** was not observed.

3. Experimental

3.1. Cell cultures

Cell cultures of *L. esculentum* L. were grown in Linsmaier–Skoog medium (Linsmaier, & Skoog, 1965) at 22°C on a gyratory shaker (100 rpm) under constant diffuse light (4.4 μmol m^{-2} s^{-1}) in 1-l conical flasks containing 440 ml cell suspension. Subculturing was performed every 7 days using an inoculum of ca. 140 ml.

3.2. Isolation of 24-epibrassinolide (**6**)

The cells (15 kg fr. wt) 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 a temperature not exceeding 40°C. The residue was partitioned between $CHCl_3$–H_2O. The residue, after evapn of the $CHCl_3$ phase, was partitioned between *n*-hexane and 80% MeOH (1:1). The *n*-hexane phase was partitioned a second time with 80% MeOH, and the combined 80% MeOH frs were concd. The residue (59 mg) obtained from the 80% MeOH fr. was chromatographed on silica gel (0.063–0.200 mm) (2.7 g) by stepwise elution with 10 frs (17 ml each) of MeOH in $CHCl_3$ (0, 2, 3, 4, 5, 7, 10, 15, 20, 50%) (Schmidt et al., 1997). The fraction eluted with 10% MeOH was subjected, after evaporation, to diethylaminopropyl ion exchange chromatography and again rechromatographed on silica gel using the same conditions. The fraction eluted with 10% MeOH was chromatographed by reversed-phase HPLC (RP-18, column 8×250 mm, flow rate 2.0 ml min^{-1}, gradient: MeCN–H_2O (9:11) for 40 min, then raised to (4:1) over 5 min, followed by isocratic elution at (4:1) for 25 min). Seventy 2 ml-frs were collected. Aliquots (10%) of five fractions each were pooled, evaporated in vacuo, derivatized with dansylhydrazone and dansylaminophenylboronic acid and analyzed by fluorescence detection for brassinosteroid contents. The brassinosteroid containing HPLC fractions 16–20 were then analyzed by GC–MS of methylboronates (Takatsuto et al., 1982).

3.3. Derivatization

Brassinosteroids bearing a vicinal side chain diol group were converted to boronates by treatment with 10 molar equivalents of dansylaminophenylboronic acid (1 mg in 1 ml MeCN–pyridine 99:1) for 20 min at 75° (Gamoh et al., 1990). Aliquots from cell culture extracts and enzyme assays were treated with 100 μl dansylaminphenylboronic acid as described above. 6-Oxo-brassinosteroids were incubated overnight at room temperature with a 10-fold molar excess of dansylhydrazine in MeOH/10 mM HCl (Anderson, 1986). For combined derivatization, the brassinosteroids were first converted to their hydrazones. The acid methanolic samples were basified by adding 0.1 volume pyridine followed by immediate boronation as described above. Methylboronation was carried out by treating the samples with methylboronic acid (Takatsuto et al., 1982).

3.4. Enzyme preparation

Freshly harvested cells (20 g fr. wt) were frozen with liquid N_2 and homogenized in a mortar and pestle. The homogenate was suspended in buffer (10 mM Na–K-phosphate; 250 mM sucrose; 400 mM sodium ascorbate; pH 7.0) and centrifuged twice for 10 min at 5000*g*. The supernatant was centrifuged for 15 min at 15,000*g*. The 15,000*g* pellet was resuspended in buffer (10 mM Na–K-phosphate; 250 mM sucrose; pH 7.0) and centrifuged for 2 h at 100,000*g*. The 100,000*g* pellet was resuspended in buffer (10 mM Na–K-phosphate; 15 vol% glycerol; pH 7.0). The 15,000*g* pellet, the 100,000*g* pellet, and the 100,000*g* supernatant were used for the enzyme assays.

3.5. Enzyme assay

All assays were carried out in a total volume of 0.5 ml and incubated for 4 h at 30°C. The assays contained 50 μg protein, 2 μM brassinosteroid, 1 mM electron donor (NADPH, NADH) or electron acceptor (NADP, NAD) and the appropriate buffer used for cell fractionation. The enzyme assays were extracted three times with 0.5 ml $CHCl_3$. The organic phase was separated by centrifugation, removed from the upper phase and evaporated with a SpeedVac. Derivatization was carried out as described above. 50% aliquots of the samples were analyzed by HPLC with fluorescence detection. The enzyme activity was measured as the relative peak area of substrate and product.

3.6. HPLC of dansyl derivatives

Chromatographic separation of dansyl derivatives was carried out on LiChrospher 100 RP18; 5μm; 250×4 mm; 0.8 ml min^{-1}. An excitation wavelength of at 230 nm was used in all cases. Dansylaminophenylboronates were detected at an emisssion wavelength of 520 nm, dansylhydrazones at 505 nm and double-derivatized brassinosteroids at 515 nm. Separations were carried out using MeCN–H_2O linear gradients: Method A: MeCN–H_2O (2:3) for 25 min, then raised to (9:1) over 125 min, followed by isocratic elution at (9:1) for 15 min. Method B: MeCN–H_2O (2:3) for 11 min, then raised to (9:1) over 30 min.

Method C: MeCN–H_2O (9:1), isocratic elution. Method D: MeCN–H_2O (4:1), isocratic elution.

Acknowledgements

The authors wish to thank Dr Jürgen Schmidt for GC–MS analysis and Dr Brunhilde Voigt for generous gifts of the brassinosteroids. We thank Dr Neil Oldham for improving the English of the manuscript. This investigation was supported by the Deutsche Forschungsgemeinschaft, Bonn and the Fonds der Chemischen Industrie, Frankfurt/Main.

References

Adam, G., Porzel, A., Schmidt, J., Schneider, B., & Voigt, B. (1996). In: Atta-ur-Rahman. Studies in natural products chemistry, Vol. 18 (pp. 495). Elsevier.

Anderson, J. M. (1986). *Analytical Biochemistry*, *152,* 146.

Clouse, S. D. (1996). *Plant Journal*, *10,* 1.

Fujioka, S., & Sakurai, A. (1997). *Natural Product Reports*, *14,* 1.

Fujioka, S., Inoue, T., Takatsuto, S., Yanagisawa, T., Yokota, T., & Sakurai, A. (1995). *Bioscience, Biotechnology and Biochemistry*, *59,* 1543.

Fujioka, S., Li, J., Choi, Y. -H., Seto, H., Takatsuto, S., Noguchi, T., Watanabe, T., Kuriyama, H., Yokota, T., Chory, J., & Sakurai, A. (1997). *Plant Cell*, *9,* 1951.

Gabriac, B., Werck-Reichhart, D., Teutsch, H., & Durst, F. (1991). *Archives of Biochemistry and Biophysics*, *288,* 302.

Gamoh, K., & Takatsuto, S. (1994). *Journal of Chromatography A*, *658,* 17.

Gamoh, K., Okamoto, N., Takatsuto, S., & Tejima, I. (1990). *Analytica Chimica Acta*, *228,* 101.

Gamoh, K., Sawamoto, H., Takatsuto, S., Watanabe, Y., & Arimoto, H. (1990). *Journal of Chromatography*, *515,* 227.

Griffiths, P. G., Sasse, J. M., Yokota, T., & Cameron, D. W. (1995). *Bioscience, Biotechnology and Biochemistry*, *59,* 956.

Hai, T., Schneider, B., & Adam, G. (1995). *Phytochemistry*, *40,* 443.

Hai, T., Schneider, B., Porzel, A., & Adam, G. (1996). *Phytochemistry*, *41,* 197.

Kawasaki, T., Maeda, M., & Tsuji, A. (1982). *Journal of Chromatography*, *232,* 1.

Kauschmann, A., Jessop, A., Koncz, C., Szekeres, M., Willmitzer, L., & Altmann, T. (1996). *Plant Journal*, *9,* 701.

Kolbe, A., Schneider, B., Voigt, B., & Adam, G. (1998). *Journal of Labelled Compounds and Radiopharmaceuticals*, *41,* 131.

Kolbe, A., Schneider, B., Porzel, A., & Adam, G. (1998). *Phytochemistry*, *48,* 467.

Linsmaier, E. M., & Skoog, F. (1965). *Physiologia Plantarum*, *18,* 100.

Schmidt, J., Altmann, T., & Adam, G. (1997). *Phytochemistry*, *45,* 1325.

Stadler, R., & Zenk, M. (1993). *Journal of Biological Chemistry*, *268,* 823.

Suzuki, H., Fujioka, S., Takatsuto, S., Yokota, T., Murofushi, N., & Sakurai, A. (1993). *Journal of Plant Growth Regulation*, *12,* 101.

Suzuki, H., Fujioka, S., Takatsuto, S., Yokota, T., Murofushi, N., & Sakurai, A. (1995). *Bioscience, Biotechnology and Biochemistry*, *59,* 168.

Suzuki, H., Inoue, T., Fujioka, S., Takatsuto, S., Yanagisawa, T., Yokota, T., Murofushi, N., & Sakurai, A. (1994). *Bioscience, Biotechnology and Biochemistry*, *58,* 1186.

Szekerez, M., & Koncz, C. (1998). *Plant Physiology and Biochemistry*, *36,* 145.

Takatsuto, S. (1994). *Journal of Chromatography A*, *658,* 3.

Takatsuto, S., Ying, B., Morisaki, M., & Ikekawa, N. (1982). *Journal of Chromatography*, *239,* 233.

Taylor, P. E., Spuck, K., Smith, P. M., Sasse, J. M., Yokota, T., Griffiths, P. G., & Cameron, D. W. (1993). *Planta*, *189,* 91.

Yokota, T., Nakayama, M., Wakisaka, T., Schmidt, J., & Adam, G. (1994). *Bioscience, Biotechnology and Biochemistry*, *58,* 1183.

Weinberger, R., Koziol, T., & Millington, G. (1985). *Chromatographia*, *19,* 452.

Winter, J., Schneider, B., Strack, D., & Adam, G. (1997). *Phytochemistry*, *45,* 233.