Pergamon

S0031-9422(96)00172-0

Phytochemistry, Vol. 42, No. 6, pp. 1517–1522, 1996

0031-9422/96 $15.00 + 0.00

CHARACTERIZATION OF TWO CYANO GLUCOSYLTRANSFERASES FROM CASSAVA LEAVES

HEIKE MEDERACKE, BÖLE BIEHL and DIRK SELMAR*

Botanical Institute, Technical University Braunschweig, Mendelssohnstr. 4, D-38092 Braunschweig, Germany

(*Received in revised form* 10 *February* 1996)

Key Word Index—*Manihot esculenta*; Euphorbiaceae; cyanogenesis; cyanohydrin; glucosyltransferase; hydroxynitrile.

Abstract—The terminal step in the biosynthesis of cyanogenic glucosides is the glucosylation of labile hydroxynitriles by UDP-glucose:hydroxynitrile-β-D-glucosyltransferases. These enzymes have not been characterized previously because this reaction is strongly affected by the instability of the hydroxynitriles, which dissociate to carbonyls and cyanide. Dissociation constants of cyanohydrins in aqueous solutions have been determined. Using these constants we are able to calculate the equilibrium concentrations of undissociated hydroxynitriles at a specific pH and thereby to determine the K_m for several cyanohydrins of glucosyltransferases that synthesize cyanogenic glucosides. Two soluble glucosyltransferases occurring in cassava leaves were separated by chromatographic methods and their kinetic properties determined. The two glucosyltransferases could be distinguished by different K_m-values, supporting the assumption that they represent distinct isoenzymes.

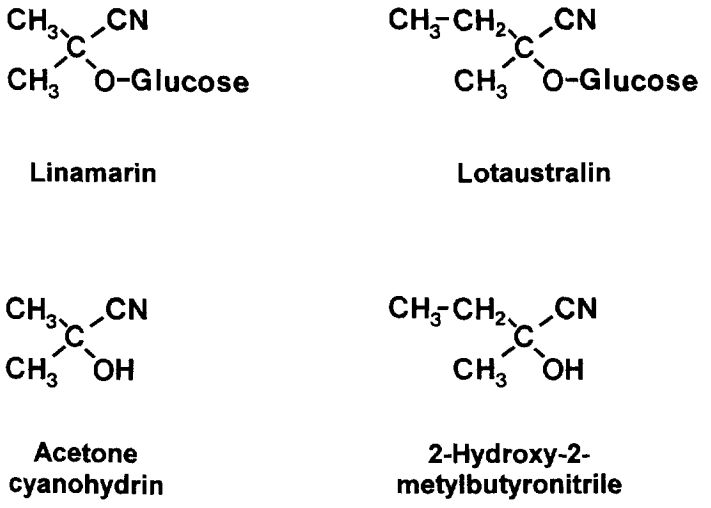
INTRODUCTION

Cyanogenic plants are characterized by the accumulation of cyanogenic glucosides. The biosynthesis of these compounds, which has been investigated intensively in recent decades (for review see ref. [1]), involves the conversion of certain amino acids into corresponding α-hydroxynitriles [2]. All steps of this channelled synthesis are catalysed by a membrane bound multienzyme complex, containing multifunctional cytochrome P-450 complexes [2–4]. The intermediates are not released from this complex until the α-hydroxynitriles are formed [2, 5]. In the terminal step these labile hydroxynitriles are glucosylated to yield cyanogenic glucosides. This glucosylation is catalysed by a soluble UDP-glucose:hydroxynitrile-β-D-glucosyltransferase [6–8]. It is not yet known how this glucosylation is linked to the membrane bound biosynthesis of hydroxynitriles. Moreover, nothing is known on interactions of various glucosylation reactions occurring in one cell, i.e. whether cyanogenic glucosides are synthesized by distinct enzymes or if those glucosyltransferases known to glucosylate other natural products, such as coumarins or flavonoids, are also responsible for glucosylation of hydroxynitriles. In cassava (*Manihot esculenta*) several soluble glucosyltransferases, capable of synthesizing the cyanogenic glucosides linamarin and lotaustralin (Formula 1) from the corresponding hydroxynitriles *in vitro* have been detected [9]. To understand, how glucosylation of hydroxynitriles is achieved *in vivo*, it is necessary to differentiate these linamarin producing enzymes. The kinetics are strongly affected by the instability of the cyanogenic substrates and thus, it is therefore necessary to study the dissociation of hydroxynitriles in detail. We were then able to calculate the actual concentrations of undissociated hydroxynitriles in equilibrium, providing valid kinetic data for the glucosyltransferases involved in the synthesis of cyanogenic glucosides.

*Author to whom correspondence should be addressed.

RESULTS AND DISCUSSION

In aqueous solutions, α-hydroxynitriles dissociate to form the corresponding carbonyl compounds and HCN. Whereas this spontaneous dissociation is reduced in acid solutions, it occurs rapidly in neutral and alkaline media [10]. Nevertheless, glucosylation rates of α-hydroxynitriles are highest between pH 7 and 9 (see next section; Fig. 3). Therefore, when α-hydroxynitriles are used as glucose acceptors for glucosyltransferases, due to this dissociation, the actual substrate concentration declines rapidly after addition to the enzyme assays. In earlier glucosylation experiments, the initial concentrations of hydroxynitriles used were very large [6–8]. Therefore, the substrate concentration might still be saturating, even after most of the hydroxynitriles added initially have dissociated during the incubation. This was checked by simultaneously monitoring the time courses of the declining concentrations of acetone cyanohydrin and the increase of its glucosylated product linamarin, within the same



Formula 1.

incubation. Figure 1 shows that, as *ca* 90% of the hydroxynitriles added initially (11 mM) dissociates over a period of 25 min, the rate of glucosylation remains constant. This demonstrates that during incubation no substrate limitation occurs. Thus, by using very high hydroxynitrile concentrations, e.g. 30 mM in the standard assay, the rate of glucosylation is not influenced by the spontaneous dissociation of cyanohydrins.

In contrast to the standard assay, the determination of kinetic data (i.e. K_m values) requires much lower substrate concentrations where the rate of glucosylation

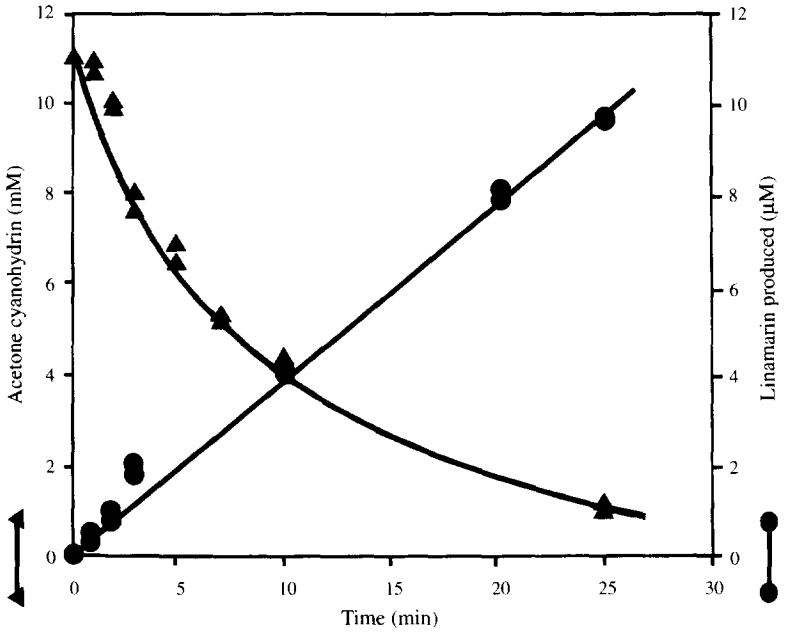


Fig. 1. Dissociation of acetone cyanohydrin during glucosylation assay. Incubation was performed according to the standard protocol using GT 3 (partially purified enzyme fraction according to ref. [9], which contains exclusively glucosyltransferase GT 3). In contrast to the standard assay, where 30 mM cyanohydrins are used, the initial concentration of acetone cyanohydrin was 11 mM, and incubation time was varied from 1 to 25 min. Glucosylation was monitored by the incorporation of radioactivity from [^{14}C]-UDP-glucose into linamarin. The concentration of undissociated acetone cyanohydrin was estimated as the difference between the initial concentration (11 mM) and concentration of free cyanide (CN^- plus HCN), determined by a standard cyanide test.

is limited. Unfortunately, the degree of dissociation of cyanohydrins is much higher in such diluted solutions than in concentrated ones [11]. Moreover, under the required substrate-limiting conditions, any major change in substrate concentrations leads to significant changes in reaction velocity and thus to erroneous data. Due to their dissociation, the concentration of hydroxynitriles will not stay constant, unless the reaction is in equilibrium. Therefore, corresponding glucosyltransferase assays should only be performed after the dissociation of hydroxynitriles has reached equilibrium, i.e. by starting the enzymic reaction after appropriate preincubations of the cyanohydrins. Thus, it becomes necessary to calculate the equilibrium concentrations of undissociated hydroxynitriles for various initial concentrations. Indeed, some dissociation constants for cyanohydrins are already available, but only for ethanol as solvent [12, 13]. Unfortunately, the dissociation constants of hydroxynitriles in aqueous solutions have not yet been published. A major problem in the calculation results from the further dissociation of HCN into cyanide and protons, which strongly depends on the pH. Nevertheless, by combining the equations for dissociation of acetone cyanohydrin and HCN [equations (1) and (2) in Formula 2] and determining the 'total cyanide' concentration (x) experimentally, we were able to calculate the following dissociation constants (equation 5) for acetone cyanohydrin (K_{AC}) and 2-hydroxy-2-methylbutyronitrile (K_{HMBN}), since K_{HCN} ($10^{-9.21} = 0.6$ nM) is known: $K_{AC} = 53$ kmM; $K_{HMBN} = 29$ mM. These values are very similar to those determined for ethanolic solutions ($K_{AC\ eth.} = 70$ mM [13]) and are in accordance with the dissociation curves mentioned in ref. [11], which are based on unpublished data of A. Bauer. Using the dissociation constants and knowing the initial concentration of hydroxynitrile added, we are able to calculate the equilibrium concentration for any amount of hydroxynitrile added initially at specific pH values (equation 6).

Formula 2. Dissociation of acetone cyanohydrin:

$$\text{Acetone cyanohydrin} \rightleftharpoons \text{Acetone} + \text{HCN}$$

$$\text{HCN} \rightleftharpoons \text{H}^+ + \text{CN}^-$$

(a) Calculation of K_{AC}

$$K_{AC} = \frac{[\text{HCN}] \cdot [\text{Acetone}]}{[\text{AC}]} \quad (1)$$

$$K_{HCN} = \frac{[\text{H}^+] \cdot [\text{CN}^-]}{[\text{HCN}]} \quad (2)$$

$$[\text{Acetone}] = [\text{HCN}] + [\text{CN}^-] = x \quad (3)$$

$$[\text{AC}] = [\text{AC}_0] - x \quad (4)$$

$$K_{AC} = \frac{[\text{H}]^+ \cdot x^2}{(K_{HCN} + [\text{H}^+]) \cdot [\text{AC}]} \quad (5)$$

(b) Calculation of acetone cyanohydrin concentrations

$$[\text{AC}] = \frac{p}{2} - \sqrt{\frac{p^2}{4} - q} \quad (6)$$

with

$$p = \frac{2[\text{H}^+] \cdot [\text{AC}_0] + K_{AC}(K_{HCN} + [\text{H}^+])}{[\text{H}^+]}$$

$$= 2[\text{AC}_0] + K_{AC} + \frac{K_{AC} \cdot K_{HCN}}{[\text{H}^+]}$$

and

$$q = [\text{AC}_0]^2$$

[HCN], [H^+], [CN^-] and [Acetone] = concentrations of HCN, protons, cyanide and acetone; [AC] = concentration of undissociated acetone cyanohydrin at equilibrium; [AC_0] = concentration of acetone cyanohydrin initially added, corresponding to [AC] + [Acetone].

When the acetone cyanohydrin equilibrium concentration does not result from the addition of cyanohydrins, but is due to the addition of cyanide and acetone, similar activities of glucosyltransferase are determined (Fig. 2). By varying the concentrations of both components, several equilibrium concentrations of acetone cyanohydrin were obtained. The corresponding incubations result in the same saturation pattern for glucosyltransferase activities (Fig. 2) as was observed when acetone cyanohydrin was added as substrate, e.g. for determining the K_m-values. This demonstrates that the relationship defined in Formula 2 describes the complex dissociation of acetone cyanohydrin correctly.

The concentration of acetone cyanohydrin initially added does not show a linear relation to the concentration of the undissociated hydroxynitrile in the equilibrium (Table 1). Whereas in highly concentrated solutions more than 20% of the acetone cyanohydrin remains undissociated, more than 99% is dissociated when the initial concentrations are less than 1 mM. This clearly points out that corresponding kinetic data cannot be determined without the consideration of the dissociation of hydroxynitriles. Nevertheless, in the standard incubation assay [9], the substrate concentration is saturating: when 30 mM acetone cyanohydrin is added at pH 9.0, the corresponding equilibrium concentration is 6.5 mM, being more than 20 times higher than the K_m values determined (see below).

From cassava leaves, four glucosyltransferases (GT 1–4) were separated and purified by gel filtration, ion exchange chromatography on DEAE-sephacel and affinity chromatography on Reactive Red agarose according to ref. [9]. One enzyme (GT 4) accepts only coumarin and flavonoid aglycones. Each of the other three (GT 1–3) glucosylate hydroxynitriles *in vitro* [9]. While transferase GT 1 and GT 3 exhibit high activity, GT 2 is less active on hydroxynitriles. Thus, it was assumed that *in vivo* either GT 1 or GT 3 might be

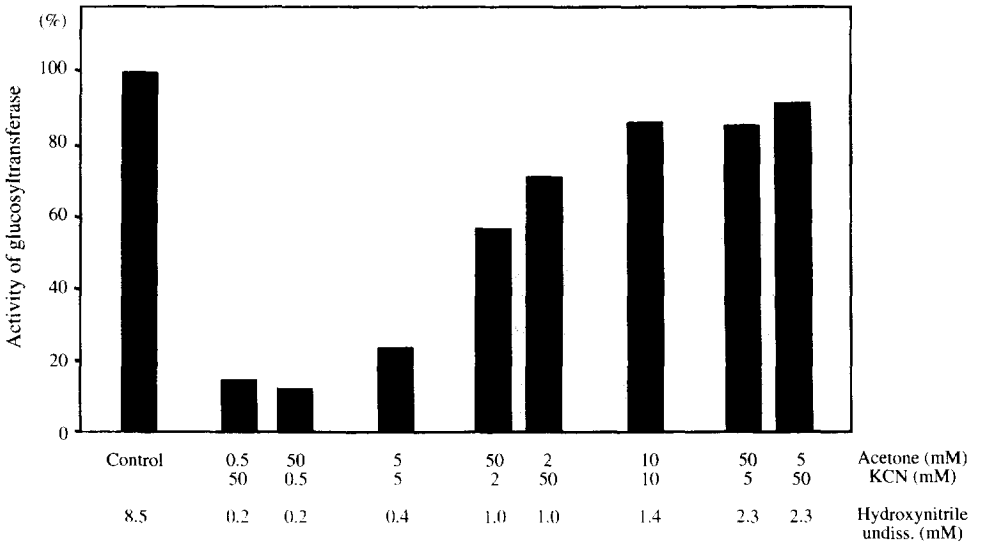


Fig. 2. Acetone and HCN form the substrate for the glucosyltransferase. Incubation was performed by a modified assay using GT 3. Instead of adding acetone cyanohydrin (control), various concentrations of acetone and KCN were added. The concentration of undissociated acetone cyanohydrin was calculated according to equations (5) and (6), respectively.

responsible for the biosynthesis of cassava cyanogens. In contrast to GT 1, which is able to glucosylate various flavonoids and phenols as well as hydroxynitriles, GT 3 accepts only hydroxynitriles as substrates [9]. Due to the different substrate preferences of GT 1 and GT 3, it was assumed that these fractions correspond to different enzymes (isoenzymes). Despite the high purification achieved, the possibility that two distinct glucosyltransferases co-chromatographed and are present in the GT 1 fraction could not be eliminated. In order to determine whether the activity of GT 1 towards hydroxynitriles is due to a distinct isoenzyme or is due to a multiple form of GT 3, further characterization of GT 1 and GT 3 was needed. Therefore, the kinetic data for both enzymes were determined.

The pH dependence of these two glucosyltransferases shows similar patterns with no well defined pH optimum (Fig. 3). In both cases, two maxima are obtained. The highest activity for GT 1 is found at pH 8.5, for GT 3 at pH 7.0 and in both cases the transferase activity declines abruptly above pH 10. This decrease in activity could be due to limiting substrate because of the extensive dissociation of acetone cyanohydrin in alkaline media. Indeed, when 30 mM acetone cyanohydrin is added at pH 7 or 8.5, the concentration in the assay of undissociated acetone cyanohydrin is 8.6 and 7.8 mM, respectively, whereas at pH 11 only 265 μM remains undissociated. Yet this concentration approximates the K_m values for both enzymes (Table 1) and should result in 50% of their activity. Thus, the strong decrease in activity to less than 10% at pH 11 could be caused mainly by limiting substrate. Nevertheless, the similar pH dependency of both transferases does not allow valid conclusions about their homology.

Table 1. Concentration of acetone cyanohydrin in equilibrium at pH 7.5

Acetone cyanohydrin initially added (mM)	Acetone cyanohydrin (undissociated) in equilibrium (mM)	(%)
30	8.53	28.4
20	4.47	22.3
10	1.38	13.8
5	0.39	7.9
2.5	0.11	4.2
1	0.018	1.8
0.5	0.0045	0.9
0.1	0.00018	0.2

Data for the amount of undissociated acetone cyanohydrin were calculated according to equation (5).

In comparison to other cyanogenic glucosyltransferases, the two enzyme fractions from cassava show quite similar pH dependencies. Like GT 1 and GT 2, all transferases described in the literature exhibit their highest activity in slightly alkaline solutions: *Linum usitatissimum* (pH 8–9 [14]), *Sorghum bicolor* (pH 8.2–8.5 [7]), *Prunus serotina* (pH 7–8 [8]) and *Triglochin maritima* (pH 6.5–8.5 [15]). Yet the pH optima of these enzymes are much more narrow than those of GT 1 or GT 3, which are active over a very wide pH range.

By applying the calculations mentioned above, K_m values for GT 1 and GT 3 were determined. Unfortunately, the corresponding K_m values are in the same order of magnitude, and thus no valid statistical based differentiation could be presented. Nevertheless, the K_m values for both fractions differ clearly (Table 2), pointing to the presence of two distinct isoenzymes. For a final proof, purification to homogeneity of both enzymes will be necessary. To decide which of these putative different enzymes is involved in biosynthesis of cyanogenic glucosides *in vivo*, further studies are

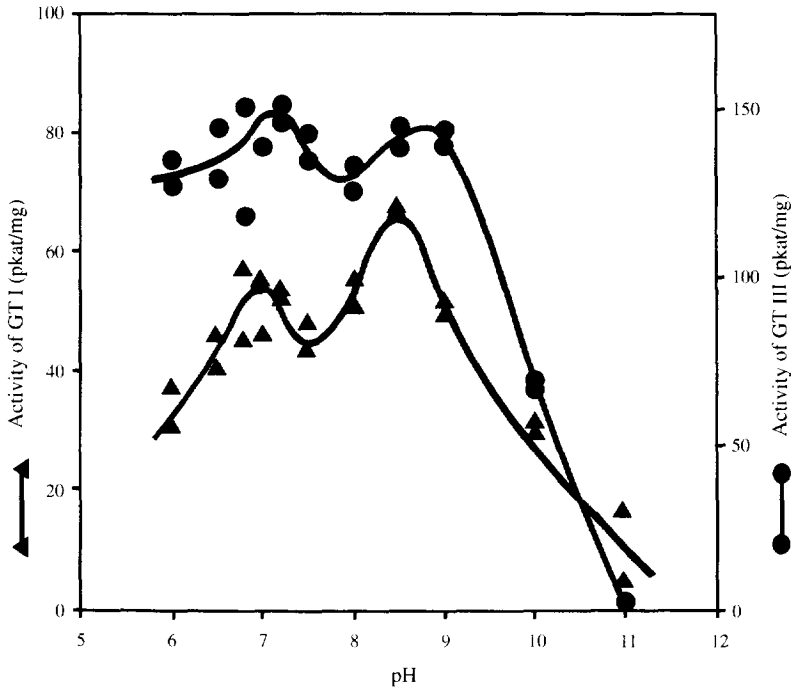


Fig. 3. pH dependence of GT 1 and GT 3. Activities of glucosyltransferases were determined by the standard assay, modified by varying the pH values.

Table 2. Kinetic data for GT 1 and GT 3

Substrate	GT 1	GT 3
	K_m (μM)	
UDP-glucose	11	36
Acetone cyanohydrin	305	205
2-Hydroxy-2-methylbutyronitrile	344	184

Kinetic data were determined by varying the corresponding substrate concentration in a modified standard assay at pH 7.0. When K_m values for hydroxynitriles were determined, concentration of UDP-glucose was maintained at 100 μM; when K_m values for UDP-glucose were estimated, concentrations of undissociated hydroxynitriles were *ca* 8.5 mM. $N = 3$, deviation was always <30%.

required, especially those which focus on their localization and on the interaction with the biosynthesis of cyanohydrins. The K_m values for UDP-glucose of GT 1 (11 μM) and GT 3 (36 μM) are much lower than those of the glucosyltransferases from other cyanogenic plants: *P. serotina* (320 μM [8]), *T. maritima* (500 μM [15]), and *L. usitatissimum* (1000 μM [14]. Only the enzyme from *S. bicolor* reveals a similar low value (29 μM [7], pointing to the fact that various cyanogenic glucosyltransferases have evolved with quite different properties.

EXPERIMENTAL

Plant material. Cassava plants were cultivated in a greenhouse at a day/night cycle of 14 hr light and 10 hr dark. The temp. varied between 25 and 40° and the r.h. was 60–90%.

Enzyme purification. Enzymes were isolated from shoot tips and young leaves (with petioles) of mature plants. Extraction and purification were performed as described in ref. [9]. According to this protocol, plant material was used to prepare acetone dry powder, which was then extracted with Tris buffer (50 mM Tris–HCl, pH 7.5, containing 200 mM sucrose, 10 mM NaCl, 5 mM DTT and 5 mM Na ascorbate). After centrifugation (1 hr, 30 000 *g*) the supernatant of the crude homogenate was concd by $(NH_4)_2SO_4$ pptn and further purified by gel filtration on Sephacryl S-300 (Pharmacia), affinity chromatography with Reactive Red agarose and ion exchange chromatography on DEAE-Sephacel gel (Pharmacia).

Enzyme assays for glucosyltransferase. The enzyme was assayed by measuring the incorporation of [^{14}C]-labelled uridine-diphosphoglucose (UDPG) into linamarin or lotaustralin according to ref. [6]. This radioassay was modified as follows: 1.1 nmol UDPG, containing 62.5 nCi [^{14}C]-UDPG (glucose moiety uniformly labelled) and 3.3 μmol acetone cyanohydrin or 2-HMBN, respectively, were incubated in Tris–HCl buffer (0.2 M, pH 9) with 5–25 μl enzyme soln in a total vol. of 100 μl for 30 min at 30°. The reaction was stopped by adding 20 μl 80% TCA. The pptd protein was pelleted by centrifugation (16 000 *g*, 10 min), and the supernatant was passed through a HPLC system using a reversed phase column (C-18, 4.5 × 240 mm) and 3% MeCN in H_2O as mobile phase at 1 ml min^{-1}. Linamarin and lotaustralin were identified by co-chromatography with authentic substrates and by liberation of cyanide after treatment with β-glucosidase. For

quantification, HPLC frs were collected and their radioactivity estimated by liquid scintillation spectroscopy, using Quicksafe A (Zinsser) scintillant.

Cyanide determination. Total cyanide (HCN and CN^-) was determined with the Merck Spectroquant kit for cyanide (data sheet 130 259 8 Do dt/5, Fa. Merck). This assay is based on the method of ref. [16].

REFERENCES

1. Halkier, B. A., Scheller, H. V. and Møller, B. L. (1988) *Cyanide Compounds in Biology*, pp. 49–66. John Wiley, Chichester, U.K.
2. Møller, B. L. and Conn, E. E. (1979) *J. Biol. Chem.* **254**, 8575.
3. Koch, B., Nielsen, V., Halkier, B. A., Olsen, C., and Møller, B. L. (1992) *Arch. Biochem. Biophys.* **292**, 141.
4. Sibbesen, O., Koch, B., Halkier, B. A. and Møller, B. L. (1994) *Proc. Natl Acad. Sci. U.S.A.* **91**, 9740.
5. Halkier, B. A. and Møller, B. L. (1989) *Plant Physiol.* **90**, 1552.
6. Hahlbrock, K. and Conn, E. E. (1970) *J. Biol. Chem.* **245**, 917.
7. Reay, P. and Conn, E. E. (1974) *J. Plant. Physiol.* **249**, 5826.
8. Poulton, J. E. and Shin, S.-I. (1983) *Z. Naturforsch.* **38c**, 493.
9. Mederacke, H, Selmar, D. and Biehl, B. (1995) *J. Appl. Botany* **69**, 119.
10. Cooke, R. D. (1978) *J. Sci. Food Agric.* **29**, 345.
11. Conn, E. E. and Butler, G. W. (1969) in *Perspectives in Phytochemistry* (Harborne, J. B. and Swain, T., eds), Academic Press, London.
12. Lapworth, A. and Manske, R. H. F., (1928) *J. Chem. Soc.* 2533.
13. Evans, P. D. and Young, J. R. (1954) *J. Chem. Soc.* 1310.
14. Hahlbrock, K. and Conn, E. E. (1971) *Phytochemistry* **10**, 1019.
15. Hösel, W. and Schiel, O. (1984) *Arch. Biochem. Biophys.* **229**, 177.
16. Aldridge, W. N. (1944) *Analyst* **69**, 262.