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DHURRIN-6′-GLUCOSIDE, A CYANOGENIC DIGLUCOSIDE FROM *SORGHUM BICOLOR*

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Key Word Index—*Sorghum bicolor*; Gramineae; cyanogenesis; cyanogenic glucosides; dhurrin.

Abstract—A novel cyanogenic diglucoside has been isolated from methanolic extracts of young seedlings of *Sorghum bicolor*. Its structure was established as dhurrin-6-glucoside from NMR, mass spectrometry and enzymatic hydrolysis data. Compared with dhurrin, which is the major cyanogenic glucoside in *Sorghum* leaves, dhurrin-6-glucoside occurs only in low concentrations. In contrast, however, the diglucoside is present in significant amounts in guttation droplets of young *Sorghum* seedlings. The presence of the diglucoside and its occurrence in apoplasmic exudates supports the hypothesis that diglucosides represent metabolites of cyanogenic monoglucosides which can be translocated within the plant. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Cyanogenic plants are characterized by the liberation of HCN in the course of tissue injury. This cyanogenesis is caused by the hydrolysis of cyanogenic glucosides [1]. In addition to such post mortem processes, cyanogenic glucosides are also involved in various metabolic processes within the living plant. Depending on the plant's physiological status, cyanogens can be translocated or converted to noncyanogenic compounds [2–4]. According to the 'linustatin pathway' hypothesis, cyanogenic (mono)-glucosides must be converted into the corresponding diglucosides before they are translocated and/or metabolized [2, 5]. To date, such diglucosidic metabolites have been demonstrated in several cyanogenic plants, e.g. linustatin and neolinustatin in species containing linamarin and lotaustralin (*Hevea brasiliensis*, *Manihot esculenta* or *Linum usitatissimum*), and amygdalin in various species containing prunasin (e.g. *Prunus* spp.) (for review see [6]).

Most of our knowledge of cyanogenicity is based on investigations of *Sorghum bicolor*, which contains large quantities of the cyanogenic glucoside, dhurrin (**1**). Conn and coworkers provided comprehensive information on the compartmentation and biosynthesis of cyanogenic glucosides from analysing young *Sorghum* plants (for reviews see [7, 8]). If the hypothesis involving diglucosidic metabolites of cyanogens represents a general feature of the metabolism of cyanogenic glucosides, then diglucosidic cyanogens must also be present in this model cyanogenic plant.

In the present paper, we report the extraction and purification of a new cyanogenic diglucoside from *Sorghum* seedlings. This new cyanogen represents a dhurrin-glucoside (**2**) and, thus adds further evidence for the general occurrence of diglucosidic metabolites of cyanogenic monoglucosides.

RESULTS AND DISCUSSION

Fractionation of methanolic leaf extracts from *Sorghum* by HPLC yielded at least three cyanogenic compounds. The major component was dhurrin, which is the main cyanogenic (mono)-glucoside occurring in *Sorghum* [1]; this eluted in tetrahydrofuran (5%) after *ca* 10 min. A second substance was present in the void volume and this was shown to be *p*-hydroxy-mandelonitrile, the aglycone of dhurrin. The presence of the hydroxynitrile could be demonstrated by a positive cyanide test, even when the assay for hydrolysis was performed without β-glucosidase. The presence of cyanohydrin is obviously due to some hydrolysis of cyanogenic glucosides during the extraction procedure. The third unknown component eluted after *ca* 5 min. Using methanol (10%) as eluant, the retention of dhurrin and the unknown substance were reversed, dhurrin eluted at 12 min and the new cyanogen at 14 min.

Enzymatic hydrolysis of the new cyanogenic glucoside gave a molar ratio of glucose to cyanide of $2.08:1 \pm 0.15$ ($n = 6$), indicating a cyanogenic diglucoside. The M_r of the compound from the FAB-mass

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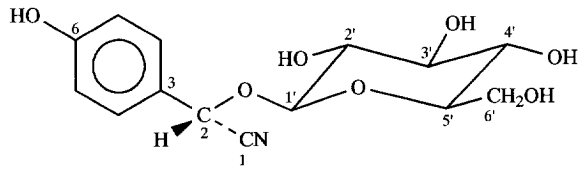
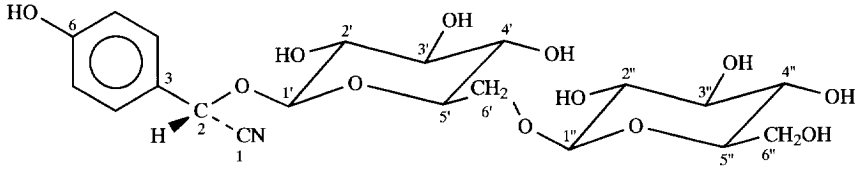


Dhurrin (1)



Dhurrin-6'-glucoside (2)

spectrometric data was 473 and was compatible with a molecular formula of $C_{20}H_{27}NO_{12}$. The one dimensional ^1H NMR spectrum showed characteristic signals of an aromatic AA'BB' spin system and a singlet at δ 5.96 that corresponded closely to those in dhurrin, and two anomeric protons which, from the positions of the remaining sugar protons in the two-dimensional COSY spectrum, belonged to two β-glucopyranose systems. The lowfield shift of one pair of H6 signals suggested the two systems had a 1-6 linkage. This was confirmed from the GC-mass spectral analysis of the partially methylated alditol acetates, which showed characteristic retention times and fragmentation patterns of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylglucitol and 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylglucitol [9], indicative of the presence of terminal glucose and 6-substituted glucose moieties, respectively. These data unambiguously identified the new cyanogen as dhurrin 6'-β-D-glucopyranoside (**2**).

According to the linustatin pathway, dhurrin 6'-glucoside should be produced by glucosylation of dhurrin and then translocated within the plant. The ratio of dhurrin to dhurrin 6'-glucoside was determined from the complete hydrolysis of the cyanogens in the corresponding HPLC fractions. In extracts from young *Sorghum* leaves the dhurrin concentration was *ca* 20 to over 100 times higher than that of dhurrin 6'-glucoside. These small amounts of dhurrin 6'-glucoside do not contradict the assumption that it represents a transport metabolite of dhurrin because of the nonhomogenous distributions of transport molecules within the plant. Such distinct spatial distribution would result if dhurrin 6'-glucoside is produced in source-tissues just before the diglucoside is exported, and (as is known for other transport metabolites) after its translocation within the plant, dhurrin 6'-glucoside immediately is hydrolysed when it enters the sink-tissue. Thus, high concentrations of the diglucosidic metabolite should be present only in the vascular tissue, whereas in other tissues its concentration should be negligible. Therefore, when calculated on a whole-leaf basis, the concentration of transport metabolites might be very low.

Translocation can be performed via either the phloem or xylem vessels. In both cases, transport metabolites occur in the apoplastic space and thus they should be present in the apoplastic liquids, such as guttation droplets. For apoplastic transport via the xylem, the guttation liquid corresponds directly to the xylem sap. Also, in the case of symplastic translocation *via* phloem, transport metabolites have to pass the apoplasm in the course of phloem loading. When vascular tissues are rinsed intensively, e.g. in the course of the massive water fluxes during guttation, substances present in the vascular apoplastic space should be present in the guttation exudates. In the present work, we were able to collect about 100 ml of guttation liquid from 1000 young *Sorghum* seedlings, which corresponded to *ca* 7000 single droplets. Analysis of the exudates from very young seedlings (three to six days after germination) revealed the presence of *ca* 10–20 nmol dhurrin 6'-glucoside ml^{-1} exudate, indicating an apoplasmic occurrence of this diglucoside. In contrast, in guttation droplets of seedlings older than two weeks, only traces of dhurrin 6'-glucoside (less than 0.5 nmol ml^{-1}) were detectable. The finding that dhurrin 6'-glucoside is present in guttation droplets exactly in that developmental stage when biosynthesis of dhurrin is at a maximum [10], supports the hypothesis that the metabolism of dhurrin and dhurrin 6'-glucoside are linked together.

Surprisingly, in various guttation samples, dhurrin, which is reported to be located in the epidermal vacuoles [11], was also present in the guttation liquid. From this, it is deduced that, in the case of *Sorghum* seedlings, the guttation liquid does not represent pure apoplasmic fluids. The presence of symplasmic components, such as dhurrin, may result from the 'bleeding' of cells after minor injuries to the tissue, e.g. when the

coleoptile is damaged by the growing primary leaves. This is supported by the finding that, in contrast to dhurrin 6′-glucoside, dhurrin is hydrolysed by β-glucosidases, which also are present in guttation exudates, causing cyanogenesis. Thus, the presence of dhurrin 6′-glucoside in the guttation droplets and its resistance against cleavage by β-glucosidases is not unequivocal proof, but suggestive of its apoplasmic occurrence and its putative function as transport metabolite.

EXPERIMENTAL

Plant material. Sorghum seedlings were cultivated in a greenhouse at *ca* 24° with day-night periods of 14 hr light and 10 hr dark. The r.h. was 60–70%. Extracts were taken from 4-week-old seedlings. To induce guttation, *Sorghum* plants were cultivated in containers with a 3-cm layer of moist vermiculite. In the evening, the plants were watered intensively and the containers were closed with glass panes. The next morning, almost every young plant revealed one to three guttation droplets of *ca* 2–50 μl.

Extraction of cyanogenic glucosides. Sorghum leaves (60 g) were frozen, crushed in liquid N_2 and subsequently freeze-dried. The freeze-dried powder was defatted with CH_2Cl_2 in a Soxhlet apparatus. To extract the cyanogenic glucosides, the lipid-free residue was suspended in MeOH (1 ml g^{-1} fresh wt) and homogenized in an Ultra Turrax homogenizer 93 × 60 sec). After filtration, the MeOH extract was evapd. The residue was dissolved in 5 ml aq. eluant (5% THF), filtered through a 0.45 μm filter (Spartam 30B, Schleicher & Schüll) and fractionated by HPLC. Directly after collection of guttation droplets the liquid was frozen. After freeze-drying, the residue was extracted with MeOH, the solvent evapd and samples prepd for HPLC as described above.

Purification of dhurrin-6′-glucoside. Extracts were fractionated by HPLC using a prep. RP-18 column (16 mm i.d.; 100 mm long; 15 μm particle size). Sepn was performed isocratically with THF–H_2O (1:19), flow rate 5 ml min^{-1}. After evapn of THF, cyanogenic glucosides were detected by cyanide liberation after hydrolysis with β-glucosidase. The frs containing cyanogenic glucosides were freeze-dried and rechromatographed using the same system. A third run was applied on an analytical column (RP-18; 4 mm i.d.; 250 mm long; 5 μm). Again, sepn was performed isocratically with THF–H_2O (1:19), flow rate 1 ml min^{-1} and monitored at 230 nm. Finally, the fr. containing the new cyanogenic glucoside was rechromatographed on an analytical RP-18 column using MeOH–H_2O (1:19).

Hydrolysis of dhurrin-6-glucoside. Portions of each fr. were combined with β-glucosidase (emulsin, Serva, 2 mg ml^{-1} in Pi buffer, pH 5.5) and incubated for 1 hr. In order to prevent loss of HCN, the assay was performed in Thunberg vessels. After incubation, 1 ml of 0.1 N NaOH, initially added into the side bulb of the vessels, was mixed with the assay to ensure complete dissociation of the hydroxynitriles produced. After neutralization, the cyanide concn was determined with the Spectroquant kit for cyanide (Merck), an assay based on the method of ref. [12]. Frs containing the new cyanogenic glucoside were also analysed for glucose, which was liberated during incubation, with the Merck GOD-PAP (oxidation of glucose by glucose oxidase) test for glucose.

Carbohydrate methylation analysis. Durrin-6′-glucoside was methylated according to ref. [13]. After hydrolysis, reduction and peracetylation, the partially methylated alditol acetates were identified by GC–MS [14] on a Finnigan GCQ ion-trap GC–mass spectrometer.

Mass spectrometry and ^{1}H *NMR spectroscopy.* Positive and negative ion mode FAB-MS were recorded with glycerol as matrix. ^{1}H 1D and 2D COSY NMR spectra were recorded at 300 K on a Brucker ARX 400 NMR spectrometer locked to the major deuterium signal of the solvent, CD_3OD. Chemical shifts are reported in δ relative to TMS.

Dhurrin. ^{1}H NMR (CD_3OD): δ 7.39 [*'d'*, H4/H8, *J*(4-5) + (4-7) 8.6 Hz], 6.82 [*'d'*, H5/H7), 5.88 [*s*, H2], 4.63 [*d*, H1′, *J*(1′-2′) 7.7 Hz], 3.93 [*dd*, H6′A, *J*(6′A-6′B) 11.9, *J*(6′A-5′) 2.0 Hz], 3.69 [*dd*, H6′B, *J*(6′B-5′) 6.1 Hz], 3.43 [*dd*, H3′, *J*(3′-2′) 9.0, *J*(3′-4′) 9.0 Hz], 3.38 [*ddd*, H5′, *J*(5′-4′) 9.7 Hz], 3.30 [*dd*, H4]′, 3.24 [*dd*, H2′].

Dhurrin-6-glucoside. ^{1}H (CD_3OD): δ 7.45 [*'d'*, H4/H8, *J*(4-5) + (4-7) 8.6 Hz], 6.87 [*'d'*, H5/H7], 5.96 [*s*, H2], 4.65 [*d*, H1′, *J*(1′-2′) 7.7 Hz], 4.45 [*d*, H1″, *J*(1″-2″) 7.7 Hz], 4.26 [*dd*, H6′A, *J*(6′A-6′B) 11.8, *J*(6′A-5′) 2.0 Hz], 3.92 [*dd*, H6″A, *J*(6″A-6″B) ~ 12, *J*(6″A-5″) ~ 2 Hz], 3.82 [*dd*, H6′B, *J*(6′B-5′) 7.1 Hz], 3.71 [*m*, H6″B], 3.59 [*m*, H5′], 3.72–3.31 [*m*, H3′, H4′, H3″ to H5″], 3.28 [*m*, H2′, H2″].

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