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Short Communication

Cyanogenic Glycosides in Cassava, *Manihot esculenta* Crantz

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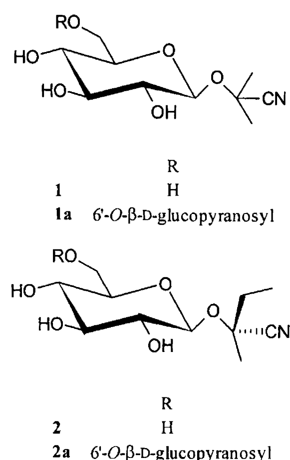
Cassava, *Manihot esculenta* Crantz, is extensively cultivated in the tropics for its large tuberous root, the starch of which forms a valuable foodstuff.[1–3] Cassava accumulates the cyanogenic glycosides linamarin (2-β-D-glucopyranosyloxy-2-methylpropiononitrile) **1** and lotaustralin [(2*R*)-2-β-D-glucopyranosyloxy-2-methylbutyronitrile] **2** in roots and leaves in ratios of about 93: 7.[4] Upon cell disruption they give rise to the release of hydrogen cyanide through the action of catabolic β-glucosidases (linamarases).[5–7] The linamarase-resistant cyanogenic disaccharides linustatin [2-(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyloxy)-2-methylpropiononitrile) **1a** and neolinustatin [(2*R*)-2-(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyloxy)-2-methylbutyronitrile] **2a** have previously been identified in *Hevea brasiliensis*,[8,9] *Linum usitatissimum*,[10] and in different species of *Passiflora*,[11] which, like cassava, contain **1** and **2**. In seedlings as well as in mature cassava plants, the glucosides **1** and **2** are synthesized in the leaves and transported to the roots.[12,13] From results obtained with *Hevea brasiliensis*,[9] Selmar *et al.* have concluded that the disaccharides **1a** and **2a** are transient transport forms of **1** and **2**, able circumvent their cleavage when exposed to linamarases present in the cell wall,[14] latex,[15] or the extracellular space.[16] Mkpong *et al.* recently suggested the operation of this so-called 'linustatin pathway' in cassava, but they did not substantiate their proposal by isolating **1a**.[14] In the present study we provide direct support for the operation of the 'linustatin pathway' in cassava by documenting the presence of trace amounts of **1a** as well as of **2a** in cassava shoots.



The production in cassava shoots of trace amounts of the two disaccharides **1a** and **2a** together with larger amounts of **1** and **2** was demonstrated in biosynthetic

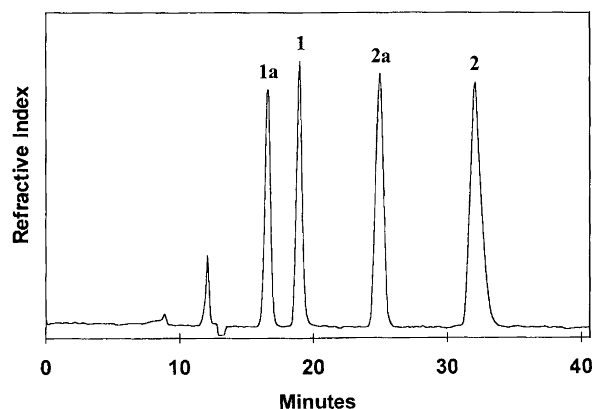


Fig. 1. Separation of **1**, **1a**, **2**, and **2a** by reversed phase HPLC. The data obtained from liquid scintillation counting showed specific co-elution of the radioactivity with the authentic standards of **1**, **1a**, **2** and **2a**.

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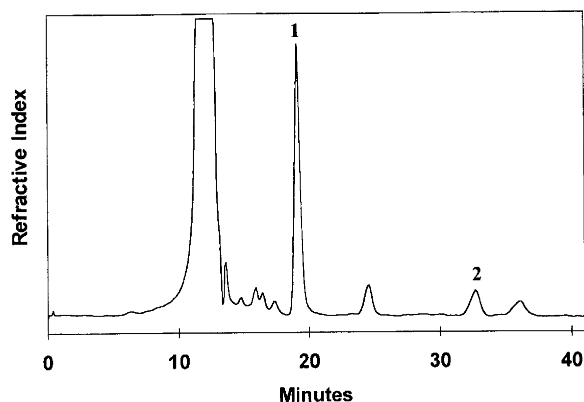

Fig. 2. HPLC profile of a cassava extract showing the presence of **1** and **2**. The detection method is not sensitive enough to reveal the presence of **1a** and **2a**.

experiments using [UL-^{14}C]valine and [UL-^{14}C]isoleucine as substrates, followed by TLC purification, HPLC separation, and analysis of the HPLC effluent. The identification of **1a** and **2a** was based on their co-migration with authentic standards on TLC as monitored by autoradiography and by subsequent co-elution of the extracted TLC bands with authentic standards on HPLC (Fig. 1) as detected by liquid scintillation counting. Furthermore, the radiolabelled bands corresponding to the disaccharides disappeared after pre-incubation with β-glucuronidase.

HPLC analysis of cassava extracts monitored by refractive index permits the detection only of **1** and **2** (Fig. 2). The effluent from the HPLC column was collected and the cyanide content of the individual fractions was determined after treatment with β-glucuronidase. Large amounts of HCN were released from fractions known to contain **1** and **2**. The lower detection limit of the cyanide assay is 1.0 nmol HCN[17] corresponding to a cyanogenic glycoside content of approximately 3 nmol g^{-1} plant material. The level of **1** and **2** in cassava seedlings is about 10 μmol g^{-1} and 0.7 μmol g^{-1}, respectively.[4] The failure to detect cyanide release from the fractions known to contain **1a** and **2a** demonstrates that the molar amounts of these two disaccharides are less than 0.03 % compared with **1**.

The co-occurrence of **1** and **2** with trace amounts of **1a** and **2a** strongly argues for the operation of the linustatin pathway in cassava when the cyanogenic glucosides **1** and **2** are transported from the leaves to the roots.

Experimental

Plant material. Cassava seeds, *M. esculenta* Crantz, (cross-code SM1234 derived from the female parent CM2967-8) were obtained from Dr. C.H. Hershey, *Centro Internacional de Agricultura Tropical*, Cali, Columbia. Cassava plants were cultivated in moss peat in a greenhouse at 30 °C.

Analysis. HPLC was performed at ambient temperature on a column of LiChrosorb RP18 (5 μm, 250 mm × 16 mm i.d.) operated at 3.0 ml 20 % MeOH per min. The column was coupled to a Gilson 131 Refractive Index Detector. TLC was carried out using Merck Kieselgel 60 F_{254}. Radioactive bands on TLC were visualized using autoradiography. Radioactivity of HPLC effluents were measured on an LKB 1217 Rackbeta scintillation counter. All solvents were of analytical grade. Linamarin was purchased from Sigma Chemical Company.

Isolation of cyanogenic glycosides. Excised shoots or seedlings were boiled in 80 % MeOH (approximately 10 ml g^{-1} fresh weight) for 5 min, homogenized using a mortar and pestle, and boiled for an additional 5 min. The suspension was filtered, and the combined extracts were concentrated *in vacuo*. The residue was dissolved in 20 % MeOH (20 ml) and subjected to HPLC analysis. A 500 μl aliquot of the concentrated extract was injected into the HPLC, and the effluent was collected in 6 ml fractions. The fractions were lyophilized and redissolved in 500 μl H_2O. An aliquot (100 μl) of each was added to test tubes containing 500 μl β-glucuronidase (Sigma, type H2, 6000 units ml^{-1}) and incubated at 37 °C for 24 h. Subsequent cyanide assays were as described by Halkier and Møller.[18]

Biosynthetic experiments. Incorporation experiments were carried out by administrating 1 μCi of either [UL-^{14}C]valine (Amersham, 266 mCi $mmol^{-1}$) or [UL-^{14}C]isoleucine (Amersham, 342 mCi $mmol^{-1}$) to single excised shoots. The plant material was allowed to absorb the tracer and kept for 24 h in continuous light with water added as needed. Each shoot was then extracted twice with boiling 90 % methanol for 5 min. The methanolic extracts were lyophilized, dissolved in 120 μl 100 mM sodium acetate buffer, pH 5.0, and divided into two equal portions. One portion was fractionated on TLC [EtOAc–$(CH_3)_2CO$–CH_2Cl_2–MeOH–H_2O, 20:15:6:5:4]. The areas corresponding to the authentic standards, **2** ($R_f = 0.48$), **1** ($R_f = 0.45$), **2a** ($R_f = 0.18$) and **1a** ($R_f = 0.15$), were extracted separately with methanol and injected into the HPLC. The HPLC effluent was collected in 3 ml fractions. Subsequent analysis of the fractions by liquid scintillation counting (1 ml sample to 5 ml scintillator, Ecoscint A, National Diagnostics, Manville, New Jersey) showed specific co-elution of the radioactivity with the authentic standards. The second portion was subjected to glucosidic cleavage by β-glucuronidase (37 °C, 24 h), after which the bands corresponding to **1**, **1a**, **2** and **2a** did not appear upon subsequent TLC analysis.

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