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HYDROXYCINNAMIC ACID ESTERS OF GLUCURONOSYLGLUCOSE FROM CELL SUSPENSION CULTURES OF *CHENOPODIUM RUBRUM*

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Key Word Index—*Chenopodium rubrum*; Chenopodiaceae; cell culture; hydroxycinnamic acid; glucuronic acid; glucose; sophorobiuronic acid; ester; phenylpropanoid metabolism.

Abstract—New hydroxycinnamic acid esters have been isolated from cell suspension cultures of *Chenopodium rubrum* and identified as 1-*O*-(*E*)-*p*-coumaroyl- and 1-*O*-(*E*)-feruloyl-(β-[1 → 2]-glucuronosyl)-β-glucose on the basis of TLC, HPLC, enzymatic degradation, FAB mass, ^{1}H NMR and UV spectroscopy.

INTRODUCTION

In the course of our recent work on secondary metabolism in cell suspension cultures of *Chenopodium rubrum* [1] we found some unknown hydroxycinnamic acid (HCA) conjugates besides glucose esters of *p*-coumaric and ferulic acids. In this paper we report on the structural elucidation of *p*-coumaric and ferulic acid esters of glucuronosylglucose from *Chenopodium rubrum*, which are new plant constituents.

RESULTS AND DISCUSSION

Cell suspension cultures of *Chenopodium rubrum* accumulate several red-coloured major pigments (2-3 μmol per g dry weight; not documented), identified as betacyanins amaranthin, betanin, and celosianin [2] and several hydroxycinnamic acid conjugates, *p*-coumaroyl- and feruloylglucoses [1], and the new *p*-coumaroylglucuronosylglucose and feruloylglucuronosylglucose (**1**). HPLC fractionation and (Fig. 1), quantification of extracts from 13-day-old cell cultures using *p*-coumaroyl- and feruloylglucoses as external standards gave values of 7.9 μmol *p*-coumaroylglucose, 10.4 μmol feruloylglucose, 1.3 μmol *p*-coumaroylglucuronosylglucose, and 8.2 μmol **1** per g dry weight.

For isolation and structure elucidation, **1** was isolated from the crude methanolic extract by ion exchange chromatography, TLC (cellulose) and column chromatography on Sephadex LH-20. Enzymatic hydrolysis with β-glucuronidase yielded 1-*O*-feruloylglucose. This latter method also allowed identification of a second analogous structure, *p*-coumaroylglucuronosylglucose which was isolated together with 1. The structure assignments are supported by the relative retentions (α values) on HPLC (Fig. 1) of *p*-coumaroylglucose/*p*-coumaroylglucuronosylglucose and feruloylglucose/**1** of 1.11 and 1.12, respectively.

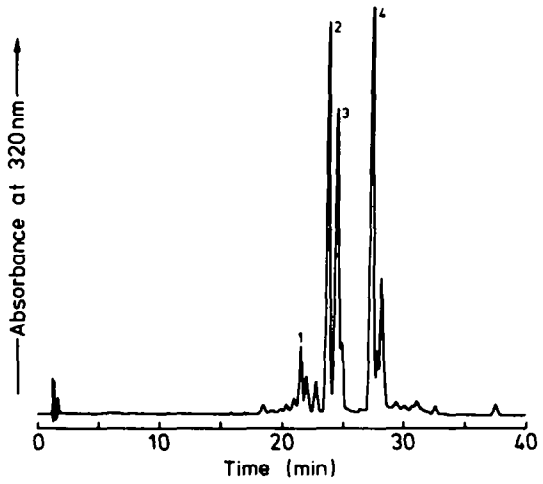

Fig. 1. HPLC analysis of a methanolic extract from a cell suspension culture of *Chenopodium rubrum* on a Nucleosil C_{18} column (5 μm, 250 × 4 mm i.d.; Macherey–Nagel, Düren, F.R.G.). Peak identification: 1, 1-*O*-*p*-coumaroylglucuronosylglucose; 2, 1-*O*-*p*-coumaroylglucose; 3, 1-*O*-feruloylglucuronosylglucose; 4, 1-*O*-feruloylglucose. Development: linear gradient elution within 40 min from solvent A (1.5% H_3PO_4 in H_2O) to 40% solvent B (1.5% H_3PO_4, 20% HOAc, and 25% MeCN in H_2O) in (A + B) at a flow rate of 1.5 ml/min. Detection was at 320 nm (0.128 absorbance full scale).

The chromatographic behaviour and detection of these compounds and their liberated hydroxycinnamic acids on TLC under UV (350 nm) with and without treatment of ammonia vapour are consistent with *p*-coumaric and ferulic acids, changing from UV absorbing to blue fluorescence and blue to blue-green fluorescence, respectively. R_f values were: *p*-coumaric acid (0.5 in TAW), *p*-coumaroylglucose (0.4 in CAW; 0.84 in IEWA), *p*-coumaroylglucuronosylglucose (0.1 in CAW; 0.63 in

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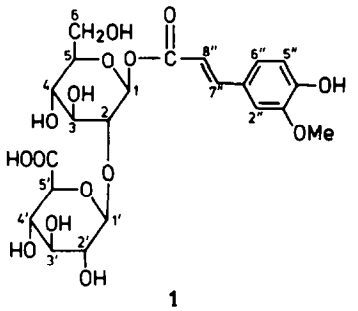

1

IEWA), ferulic acid (0.65 in TAW), feruloylglucose (0.5 in CAW; 0.8 in IEWA), and feruloylglucuronosylglucose (**1**) (0.17 in CAW; 0.59 in IEWA). The UV maximum was 328 nm with a bathochromic shift of 54 nm and an increase (2.5 fold) in absorbance intensity upon addition of alkali.

The identity of **1** as 1-*O*-(*E*)-feruloyl-(*β*-[1 → 2]-glucuronosyl)-*β*-glucose was based on the ^{1}H NMR and FABMS spectral data. The presence and nature of one aromatic and two sugar moieties were readily determined from the 2D-COSY ^{1}H spectrum, while their respective relative configurations were evident from the magnitude of the coupling constants in the 1D spectrum. The low-field shift of H-1 of the glucose moiety and comparison with 1-feruloyl-*β*-glucose indicated the aromatic moiety was attached at C-1 of glucose. The unusual low-field shift of H-2 was an indication of the point of attachment of the second sugar system. This was unambiguously demonstrated by the observation of a nuclear Overhauser enhancement of H-2 upon irradiation of H-1′ of the glucuronic acid moiety. This disaccharide moiety (sophorobiuronic acid) is identical with that from amaranthin (betanidin sophorobiuronic acid) [4] and its HCA derivative celosianin [5] present in the *Chenopodium rubrum* cell culture [1, 2].

High resolution-negative ion FAB mass spectroscopy of **1** confirmed the molecular formula as it showed a deprotonated molecular ion at *m/z* 531.1325 corresponding to $[M-H]^-$ of $C_{22}H_{27}O_{15}$ (calc. 531.1350). A fragmentation ion at *m/z* 355, $[M-C_{10}H_9O_3]^-$, corresponded to the loss of the intact feruloyl moiety, hence partially confirming the sequence found by NMR.

The present HCA esters of glucuronosylglucose are new plant constituents among a vast array of different HCA conjugates [6, 7]. They extend the number of pure HCA disaccharide esters so far described, i.e. gentiobiose [8–10], rutinose [9], galactosylglucose [11], and sucrose [12–17]. The possible biogenetic and regulatory relationship of the HCA-glucuronosylglucoses to the analogous structures of the *Chenopodium rubrum* amaranthin and celosianin [2, 5, 18], will be investigated with respect to activities of UDP-glucose-dependent- [e.g. 19] and UDP-glucuronic acid-dependent transferase activities [e.g. 20, 21].

EXPERIMENTAL

Plant material. Cultures of *Chenopodium rubrum* L. (cell line CH, red coloured, betalain accumulating) were established by Dr H. Harms (FAL, Braunschweig) from stem tissue of the intact plant. Suspension cultures were routinely maintained in 200 ml flasks, containing 70 ml MS-medium (+2 μM 2,4-D) [22] (2 g fr. wt inoculum, harvested after 14 days). The cell culture was incubated on a shaker operating at 120 rpm under fluorescent light (Osram 65 W Fluora and Phillips 65 W, 2200 lx).

Extraction and separation. Freeze-dried cells (7 g) were extracted with 150 ml 50% aq. MeOH (16 hr at 4°), the extract was filtered and the residue washed twice with 75 ml 50% aq. MeOH. The combined filtrates were evapd to dryness *in vacuo* and the residue suspended in 100 ml H_2O. The latter was transferred to an ion exchange column (Dowex 1X8, Cl^- form; Serva, Heidelberg, F.R.G.) using H_2O, 0.1 M, 0.5 M, 1 M, 2 M, and 7 M aq. HCO_2H (70 ml each). All fractions were evapd to dryness, redissolved in 1 ml 50% aq. MeOH and chromatographed (TLC) in IEWA. **1**, which was detected in the 0.5 M HCO_2H fraction was separated on 40 plates, scraped off and eluted with 50% aq. MeOH. **1** was finally purified on a Sephadex LH-20 column (90 × 2 cm i.d.; Pharmacia, Uppsala, Sweden) using H_2O as solvent.

TLC was performed on microcrystalline cellulose ('Avicel', Macherey-Nagel, Düren, F.R.G.): IEWA = *iso*-PrOH–EtOH–H_2O–HOAc (6:7:6:1), CAW = $CHCl_3$–HOAc (3:2, H_2O std), TAW = toluene–HOAc (2:1, H_2O std).

Alkaline hydrolysis was performed at room temp. 1 M aq. NaOH for 30 min. The solution was acidified with HCl and chromatographed in TAW.

HPLC. The HPLC apparatus (LKB) and the data processor (Shimadzu) are described elsewhere [23]. *p*-Coumaroyl- and feruloylglucose as external standards were from petals of *Antirrhinum majus* [10] and were isolated as described [3]. For the chromatographic conditions for analysis of the *Chenopodium rubrum* extract see Fig. 1. The enzymatic assays (*β*-glucuronidase hydrolysis) were analysed isocratically with 30% solvent B in (A + B) at a flow rate of 1 ml/min (*p*-coumaroylglucuronosylglucose R_t 4.2 min; feruloylglucuronosylglucose R_t 5.3 min; *p*-coumaroylglucose R_t 6.3 min; feruloylglucose R_t 8.6 min).

Enzymatic hydrolysis was performed with *β*-glucuronidase from *Helix pomatia* (40 U/ml at pH 3.8; Merck, Darmstadt, F.R.G.), diluted 1:40 in 0.1 M NaOAc buffer (pH 3.5) with a mixt. of 0.5 mM 1 and 0.2 mM *p*-coumaroylglucuronosylglucose (80% conversion in 20 min at 30°).

NMR and MS. 1D- and 2D-COSY ^{1}H NMR spectra were recorded at ambient temp., at 400 MHz, on a Bruker WM-400 NMR spectrometer locked to the major deuterium resonance of the solvent, CD_3OD. The standard Bruker software package was used throughout. Chemical shifts are reported in ppm relative to TMS and coupling constants in Hz. Negative ion fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS-50 mass spectrometer equipped with a Kratos FAB source. Glycerol was used as matrix.

1-O-(E)-*Feruloyl-(β-[1 → 2]-glucuronosyl)-β-glucose* (**1**). ^{1}H NMR (CD_3OD): δ = 7.754 [*d*, H-7″ *J*(7″–8″) 15.9], 7.295 [*d*, H-2″, *J*(2″–6″) 1.9], 7.164 [*dd*, H-6″, *J* (6″–5″) 8.2], 6.844 [*d*, H-5″], 6.473 [*d*, H-8″], 5.750 [*d*, H-1, *J*(1–2) 8.2], 4,743 [*d*, H-1′, *J*(1′–2′) 7.8], 3.963 [*s*, OMe], 3.894 [*dd*, H-6A. *J*(6A–6B) 11.7, *J*(6A–5) 1.1], 3.860 [*dd*, H-2, *J*(2–3) 9.3], 3.729 [*dd*, H-6B, *J*(6B-5) 4.7], 3.721 [*dd*, H-3, *J*(3–4) ≃ 9], 3.614 [*d*(*m*), H-5′, *J*(5′–4′) 9.5], 3.48–3.45 [*m*, H-4, H-5], 3.47–3.43 [*m*, H-4′], 3.278 [*dd* (*m*), H-2′, *J*(2′–3′)9.3]. Irradiation of H-1′ gave nuclear Overhauser enhancements of H-2 and H-5′. Negative ion FABMS: *m/z*: 531 $[M-H]^-$, 355 $[M-C_{10}H_9O_3]^-$; high resolution *m/z*: 531.1325, $C_{22}H_{27}O_{15}$ (calc. 531.1350).

1-O-(E)-*Feruloyl-β-glucose* (see scheme of structure **1**): ^{1}H NMR (CD_3OD): δ = 7.769 [*d*, H-7″, *J*(7″–8″) 15.9], 7, 245 [*d*, H-6″, *J*(2″–6″) 1.9], 7.140 [*dd*, H-6″, *J*(6″–5″) 8.3], 6.857 [*d*, H-5″], 6.442 [*d*, H-8″], 5.617 [*d*, H-1, *J*(1–2) 7.9], 3.935 [*s*, OMe], 3.897 [*dd*, H-6A, *J*(6A–6B) 12, *J*(6A–5) ≃ 2], 3.734 [*dd*, H-6B, *J*(6B–5) 4.7], 3.53–3.34 [*m*, H-2, H-3, H-4, H-5].

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