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FERULOYLBETANIN FROM PETALS OF *LAMPRANTHUS* AND FERULOYLAMARANTHIN FROM CELL SUSPENSION CULTURES OF *CHENOPODIUM RUBRUM*

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Key Word Index—*Chenopodium rubrum*, Chenopodiaceae, *Lampranthus sociorum*, Aizoaceae, betalain, acylated betacyanin, celosianin, lampranthin, ferulic acid, hydroxycinnamic acid ester

Abstract—The structures of two acylated betacyanins, lampranthin II from petals of *Lampranthus peersii* and *L. sociorum* and celosianin II from cell suspension cultures of *Chenopodium rubrum*, were elucidated as betanidin 5-*O*-[6′-*O*-(*E*)-feruloyl-β-glucoside] and betanidin 5-*O*-[2″-*O*-(*E*)-feruloyl-β-(1″,2′)-glucuronosyl-β-glucoside].

INTRODUCTION

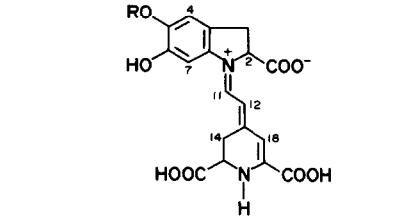
In a recent communication [1] we described the enzymic synthesis of hydroxycinnamic acid (HCA) esters of betacyanins, *p*-coumaroylbetacyanins (lampranthin I and celosianin I) and feruloylbetacyanins (lampranthin II and celosianin II) with protein preparations from petals of *Lampranthus sociorum* and cell suspension cultures of *Chenopodium rubrum*, respectively. Such esters occurring in members of the Caryophyllales [2, 3] are not yet fully characterized [4]. The structures of lampranthin [5] and celosianin [3] were proposed to be di- or tri-HCA-conjugates, which could not be verified in our previous study [1]. We obtained evidence for the presence of mono-HCA-conjugates, i.e. either the mono-(*p*-coumarate) or monoferulate ester of the betacyanins.

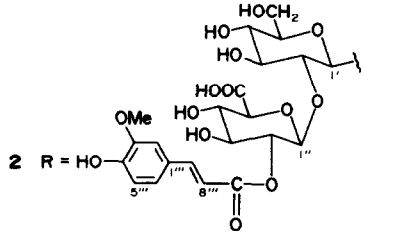
Two major HCA-acylated betacyanins, lampranthin II from *Lampranthus sociorum* and celosianin II from *Chenopodium rubrum*, have now been isolated and their structures conclusively elucidated. Lampranthin II is monoferuloylbetanin (**1**) and celosianin II monoferuloylamaranthin (**2**).

RESULTS AND DISCUSSION

HPLC analyses (Fig. 1) of methanolic extracts from petals of *Lampranthus sociorum* and cell suspension cultures from *Chenopodium rubrum* showed 10 distinct peaks at a detection wavelength of 535 nm. Four could readily be identified by cochromatography with reference extracts from various betacyanin-bearing plants, i.e. betanin and isobetanin from *Beta vulgaris* and amaranthin and isoamaranthin from *Amaranthus tricolor* [2]. The other unknown peaks were assumed to be celosianins (peaks 5, 6, 7) and lampranthins (peaks 8, 9, 10) according to Minale *et al.* [3] and Piattelli and Impellizzeri [5], respectively. This was supported by our previous enzymic study [1]. The major unknowns celosianin and lampranthin (peaks 6 and 9 in Fig. 1) were isolated by successive chromatography on ion exchanger (Dowex) eluted with aqueous formic acid and on Sephadex LH-20 eluted with water and aqueous methanol.

The presence of the isobetacyanins in Fig. 1 (peaks 2, 4, 7, 10) was confirmed by treatment of the extracts with an aqueous citric acid solution [6] according to Piattelli and Minale [2]. The major (feruloyl) and minor (*p*-coumar-





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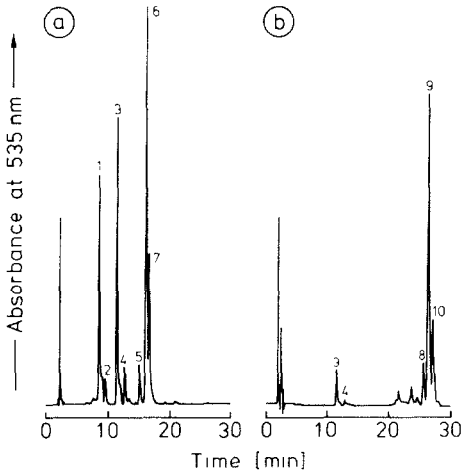

Fig 1 High-performance liquid chromatographic analyses of betacyanins from crude methanolic extracts of cell suspension cultures of *Chenopodium rubrum* (a, 0 32 absorbance unit full scale) and petals of *Lampranthus sociorum* (b, 0 08 absorbance unit full scale) Peak identification 1, amaranthin, 2, isoamaranthin, 3, betanin, 4, isobetanin, 5, celosianin I (*p*-coumaroylamaranthin); 6, celosianin II (feruloylamaranthin), 7, isocelosianin II (feruloylisoamaranthin), 8, lampranthin I (*p*-coumaroylbetanin), 9, lampranthin II (feruloylbetanin), 10, isolampranthin II (feruloylisobetanin) Chromatographic column (250 × 4 mm i d) prepacked with Nucleosil 120-5 C_{18} (Macherey-Nagel, Duren) Development linear gradient elution within 30 min from 10 to 55% solvent B (1% H_3PO_4, 20% HOAc, 25% MeCN in H_2O) in solvent A (1 5% H_3PO_4 in H_2O) at a flow rate of 1 ml/min

oyl) celosianins and lampranthins have been tentatively identified by cochromatography with the enzymically formed HCA-acylated betacyanins [1]. Protein preparations from petals of *L. sociorum* catalyse, with betanin (betanidin 5-*O*-glucoside) as acceptor molecule, the formation of lampranthin I when incubated with *p*-coumaroylglucose and lampranthin II when incubated with feruloylglucose. An analogous reaction occurred on incubation of the protein from *Ch. rubrum* cell suspension cultures with amaranthin (betanidin 5-*O*-glucuronosylglucoside) and *p*-coumaroylglucose with the formation of celosianin I and feruloylglucose with the formation of celosianin II That these compounds are mono-HCA-conjugates was confirmed by the HPLC analyses at different wavelengths (535 and 320 nm) which allowed the calculation of the respective molar ratios of the betanidinglycoside to the HCA-moiety They were 1 . 1 for both the lampranthins and celosianins [1]

The spectral maxima in methanol were 298, 316, and 534 nm for **1** and 298, 324, and 538 nm for **2** TLC of the *L. sociorum* and *Ch rubrum* crude extracts in three solvents showed the following R_f values 0.22 (S1), 0.15 (S2), and 0 13 (S3) for betanin; 0.12 (S1), 0 07 (S2), and 0.04 (S3) for amaranthin; 0 28 (S1), 0 28 (S2), and 0 34 (S3) for **1**, and 0 35 (S1), 0 23 (S2), and 0 22 (S3) for **2**

The definitive structures of **1** and **2** were determined as betanidin 5-*O*-[6′-*O*-(*E*)-feruloyl-*β*-glucoside] and betanidin 5-*O*-[2″-*O*-(*E*)-feruloyl-*β*-(1″,2′)-glucuronosyl-*β*-glucoside] by ^{1}H NMR in acidic DMSO-d_6 Both compounds showed characteristic shifts for the betacyanin residues which could be assigned by comparison with literature data [7] From the integrated spectra one feruloyl moiety (*trans* form) per molecule, and one or two sugar moieties were evident in **1** and **2**, respectively This was confirmed by observations of the appropriate protonated molecular ions in the positive ion FAB mass spectra of the two compounds. From the cross peaks in the 2D COSY ^{1}H NMR spectra the connectivity of the individual protons in the sugar residues of both molecules could be unambiguously assigned

The low field shifts of H-6′A and H-6′B in **1** caused by acylation indicated attachment of the feruloyl moiety at C-6′ of *β*-glucose which was attached to C-5 of the betanidin moiety from NOE difference spectra For **2**, NOE difference spectra indicated at the *β*-glucuronic acid moiety was bound to C-2′ of the *β*-glucose moiety which in turn was attached to C-5 of the betanidin moiety The presence of the feruloyl moiety at C-2″ of *β*-glucuronic acid was evident from the low field shift of H-2″ These structures were confirmed by the FAB mass spectra which are compatible with a M_r of 727 $[M+H^+]$ for **1** and a M_r of 903 $[M+H^+]$ for **2**

The low field portion of the ^{1}H NMR spectra of both compounds clearly shows the presence of two major isomers From the NMR evidence alone it is not possible to distinguish between the isomers arising from the characteristic epimerization at C-15 or by hindered rotation about the C-12/C-13 bond of the betanidin moiety Since our previous study on the enzymic synthesis of these compounds [1] suggests that both betanidin and isobetanidin forms are present in the material used for the structure elucidation, we conclude that this is the cause of the doubling of signals observed here

EXPERIMENTAL

Plant material Flowering plants of *Lampranthus sociorum* (L Bol.)N.E.Br. and *L. peersii* (L. Bol.)N.E.Br. were grown in the botanic garden of the University of Cologne Cell suspension 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 In the present study a new high betacyanin-producing cell line (celosianin II as major component) was used, selected by Dr J Berlin (GBF, Braunschweig) from the callus culture Suspension cultures were routinely maintained in 200 ml flasks, containing 70 ml MS medium [8] (+2 μM 2,4-D) [9] (2 g fresh weight inoculum, harvested after 14 days) The culture was incubated on a shaker operating at 120 rpm under fluorescent light (Osram 65 W Fuora and Phillips 65 W, 6000 lx)

Isolation of **1** *and* **2** (i) Freshly harvested petals of *Lampranthus sociorum* and *L. peersii* and freeze-dried cell suspension material of *Chenopodium rubrum*, respectively, were homogenized (4 min) in 50% aq MeOH The homogenates were allowed to stand for 1 hr with continuous stirring (ii) The filtrates were taken to complete dryness (under vacuum at 30) and the residues redissolved in 100 ml H_2O which was (iii) transferred to a Dowex column (20 × 2 cm i d , 1 × 8, Cl-, 100- 200 mesh, Serva, Heidelberg) washed with H_2O followed by increasing concn of HCO_2H, 0 5, 1 0, 2 0, 3 5, and 7 M aq HCO_2H (iv) The latter fraction was concd under vacuum to a few ml, diluted with H_2O and concd again This was repeated × 3 (v) Final purification was achieved by repeated chromatography on a Sephadex LH-20 column (90 × 2 5 cm i d , Pharmacia, Uppsala, Sweden) using H_2O (first run) and 50% aq MeOH (second run) as eluants Elutions of **1** and **2** were visually controlled and purity checked by HPLC and TLC

TLC On microcrystalline cellulose ('Avicel', Macherey-Nagel, Duren) development in S1, *iso*-PrOH–EtOH–H_2O–HOAc (6 7 6 1) [10], in S2, *n*-BuOH–HOAc–H_2O (12 3:5), and in S3, EtOAc–HCO_2H–H_2O (33 7 10)

HPLC The liquid chromatograph (LKB) and the data processor have been described [11] For further details see Fig 1

NMR and MS ^{1}H NMR spectra were recorded at ambient temp on a Bruker AM 300 NMR spectrometer, operating at 300 MHz, locked to the deuterium resonance of the solvent, DMSO-d_6 containing a trace of DCl The 2D COSY ^{1}H NMR spectra were recorded with a $90°$–t_1 – $90°$–FID (t_2) pulse sequence In all cases the data were multiplied by sine-bell functions and one level of zero-filling was used for both t_1 and t_2 All 1D (normal and NOE difference) and 2D spectra were recorded using the standard Bruker software package Chemical shifts are given in ppm relative to TMS and coupling constants in Hz Chemical shifts given to two decimal places were derived from the COSY spectrum Two isomers are present in both compounds in the ratio of 11 9. Where two signals are found for a particular proton (indicated by '× 2') the most intense signal is given first

Positive ion fast atom bombardment (FAB) mass spectra were recorded on a Kratos MS 50 mass spectrometer equipped with a Kratos FAB source 3-Nitrobenzylalcohol was used as matrix

Betanidin 5-O-[6′-O-(E)-*feruloyl-β-glucoside*] [*lampranthin II* (**1**)] ^{1}H NMR· δ = 8.720, 8 685 [*d* × 2, H-11, *J* (11–12) 11 9, 12 6], 7 623, 7 642 [*s* × 2, H-7], 7 575 [*d*, H-7″, *J* (7″–8″) 15 9], 7 320 [*br s*, H-2″], 7.091 [*br d*, H-6″, *J* (6″–5″) 8.1], 7 104 [*s*, H-4], 6 826 [*d*, H-5″], 6.504 [*d*, H-8″], 6.300 [*s*, H-18], 6.235, 6 216 [*d* × 2, H-12], 5.35 [*m*, H-2], 4 776 [*br d*, H-1′, *J* (1′–2′) ≈6–7], 4.49 [*m*, H-15], 4 434 [*br d*, H-6′A, *J* (6′A–6′B) 11 9], 4.237 [*d, d*, H-6′B, *J* (6′B-5′) 5 7], 3 808 [*s*, 3″–OMe], 3 74 (*m*, H–14A], 3 74–3.00 [*m*, H-14B], 3 68 [*m*, H-5′], 3 54 [*m*, H-3A], 3 4–3.05 [*m*, H-3′], 3.33 [*m*, H-2′], 3 27 [*m*, H-4′], 3.20 [*m*, H-3B] Irradiation of H-1′ gave a negative NOE at H-4, of H-8″ at H-2″, of H-6″ + H-4 at H-5″, H-7″ and H-1′, and of H-7 at H-11 FABMS 727 $[M+H]^+$

Betanidin 5-O-[2″-O-(E)-*feruloyl-β*-(1″,2′)-*glucuronosyl-β-glucoside*] [*celosianin II* (**2**)]. ^{1}H NMR· δ = 8 74, 8 68 [*d* × 2, H-11, *J* (11–12) 12, 12], 7 659, 7 670 [*s* × 2, H-7], 7 515, 7 505 [*d* × 2, H-7‴, *J* (7‴–8‴) 15 8, 15 8], 7 284 [*br s*, H-2″], 7 130 [*s*, H-4], 7 106 [*br d*, H-6‴, *J* (6‴–5‴) 8 3], 6 809 [*d*, H-5‴], 6.444, 6.431 [*d* × 2, H-8‴], 6 338 [*s*, H-18], 6.273, 6.243 [*br d* × 2, H-12], 5 42–5.36 [*br d* × 2, H-2], 5.044 [*d*, H-1″, *J* (1″–2″) 7 8], 4 846 [*d*, H-1′, *J* (1′–2′) 7 6], 4 673 [*d, d*, H-2″, *J*(2″–3″) 9 1], 4 55–4 49 [*br*, H-15], 3 90–3 73 [*m*, H-6′, H-5″], 3 819 [*s*, 3‴–OMe], 3 78 [*m*, H-14A], 3 71–3.40 [*m*, H-4″], 3 62 [*m*, H-3A], 3.62 [*m*, H-2′], 3 53–3 40 [*m*, H-5′, H-3″], 3 38–3 25 [*m*, H-3′, H-4′], 3 28 [*m*, H-3B], 3 12 [*m*, H-14B] Irradiation of H-1′ gave a negative NOE at H-4, H-3′ and H-5′, of H-1″ at H-2′, H-3″ and H-5″, and of H-8‴ at H-2‴ and H-6‴ FABMS 903 $[M+H]^+$

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