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BETACYANINS FROM PLANTS AND CELL CULTURES OF *PHYTOLACCA AMERICANA*

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Key Word Index—*Phytolacca americana*; Phytolaccaceae; pokeweed; fruit; stem; cell culture; betalain; betacyanin; DAD–HPLC; LC–MS; electrospray MS; NMR.

Abstract—Betacyanins from cell cultures of *Phytolacca americana* were characterized and compared with those of the stems and ripening fruits of the plant. Whereas in fruits prebetanin (betanin 6′-*O*-sulphate) and its isoform predominate, in the stem and cell cultures feruloylated derivatives occur as the major components. These were rigorously identified by various spectroscopic techniques (DAD–HPLC, NMR, LC–MS and electrospray MS–MS) and carbohydrate analyses as betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside, a new betacyanin of higher plants, and betanidin 5-*O*-(6′-*O*-*E*-feruloyl)-β-D-glucopyranoside (lampranthin II), together with their isoforms.

INTRODUCTION

The red–violet pigments of the fruits of *Phytolacca americana* L. (*P. decandra* L., Phytolaccaceae) (Virginian pokeweed or pokeberry) have been used in the last century to dye wine and other foodstuffs [1, 2]. In early spectroscopic and chemical studies [1, 3, 4] it was suggested that the main pigment of pokeberries, phytolaccanin, was identical with betanin, the prevalent pigment from *Beta vulgaris* L., Chenopodiaceae. Whereas the main pigment from pokeberries was found to be chromatographically different from betanin [5], in later studies it was identified as betanin, and only a low level of prebetanin (betanin 6′-*O*-sulphate) was detected [6]. In a study of the distribution of betacyanins in plants of the order Caryophyllales (Centrospermae) the occurrence of betanin in fruits of *P. americana* was also mentioned [7].

We initiated cell cultures (callus and suspension) from stem explants of *P. americana*, which acquired the ability to produce betacyanins spontaneously during subculture [8]. This allowed us to study the effects of nutrients (sucrose, nitrogen source, and phosphate) and plant growth regulators (2,4-D and 6-benzylaminopurine) on betacyanin accumulation and growth in suspension cultures [9–14]. During these investigations, in addition to betanin and prebetanin known from fruits, other betacyanins have been detected. Their characterization and structural elucidation are reported here.

RESULTS AND DISCUSSION

Direct HPLC analysis of a freshly prepared methanolic extract from ripening fruits of *P. americana* confirmed the presence of the known betanin, prebetanin and their isoforms. In contrast to Wyler and Dreiding [6], who detected by electrophoresis only low levels (<1%) of prebetanin and its isoform together with the main components betanin/isobetanin, we found that prebetanin and its isoform are the predominant betacyanins (nearly 60%) detected at 540 nm (Fig. 1A). Presumably the ratio of prebetanin to betanin will depend on the ripening stage of the fruits, and also will be strongly influenced by the extraction conditions and purification steps prior to analysis. In analytical HPLC using 1% formic acid instead of 1.5% phosphoric acid in the solvent system, prebetanin (R_t 2.6 min) eluted considerably earlier than betanin (R_t 5.3 min) indicative of the high polarity of the ionized sulphuric acid half-ester (nor shown), whereas with 1.5% phosphoric acid prebetanin (R_t 7.4 min) eluted after betanin (R_t 5.6 min) (Fig. 1). In an extract from fruits of *P. acinosa* prebetanin/isoprebetanin were likewise the major betacyanins (not shown).

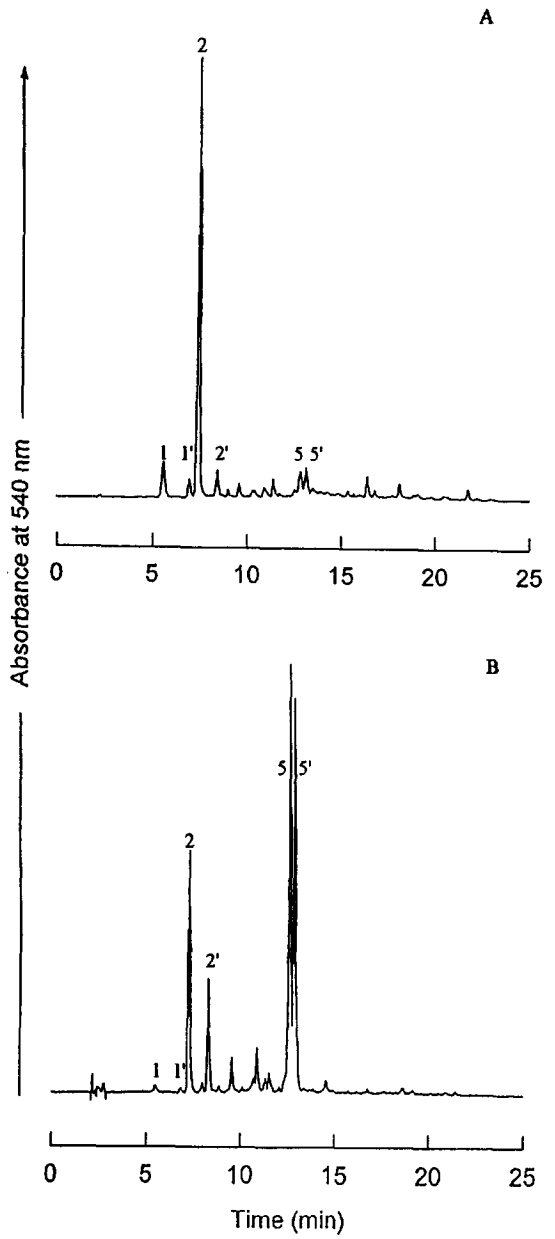


Fig. 1. HPLC elution profile of methanolic extracts from plants of *P. americana*. Peak identification: peak numbering corresponds to numbering in Table 1. Peak numbers with superior primes (1′, 2′, 5′) correspond to the isoforms (2*S*, 15*R*) of the respective normal betacyanins (2*S*,15*S*). (A) Extract from ripening fruits; (B) extract from epidermal layers of the stem of the same plant.

In addition to prebetanin/isoprebetanin and betanin/isobetanin, a pair of isomeric acyl betacyanins (**5**, **5′**) in low amounts was detected in fruit extracts of *P. americana* (Fig. 1A) and characterized as hydroxycinnamoyl (HCA) derivatives by DAD–HPLC. Interestingly, these HCA derivatives are the main betacyanins of the extract from epidermal layers of the stems (Fig. 1B), but their concentrations are not sufficient for isolation and characterization. It has been shown that feeding labelled tyrosine to cell cultures derived from stem explants of *P. americana* [13] causes label to be introduced not only into betanin/isobetanin, but also into other less polar betacyanins with retention characteristics of HCA-betacyanins. Hence, we have analysed extracts from cell suspension and callus cultures of *P. americana* by HPLC (Fig. 2; Table 1). The betacyanins **1–3** were readily identified by comparison (retention times, UV data) and co-chromatography with authentic **1** (betanin) from *B. vulgaris* suspension culture [15] and **3** (gomphrenin I) from fruits of *Basella rubra* [16],

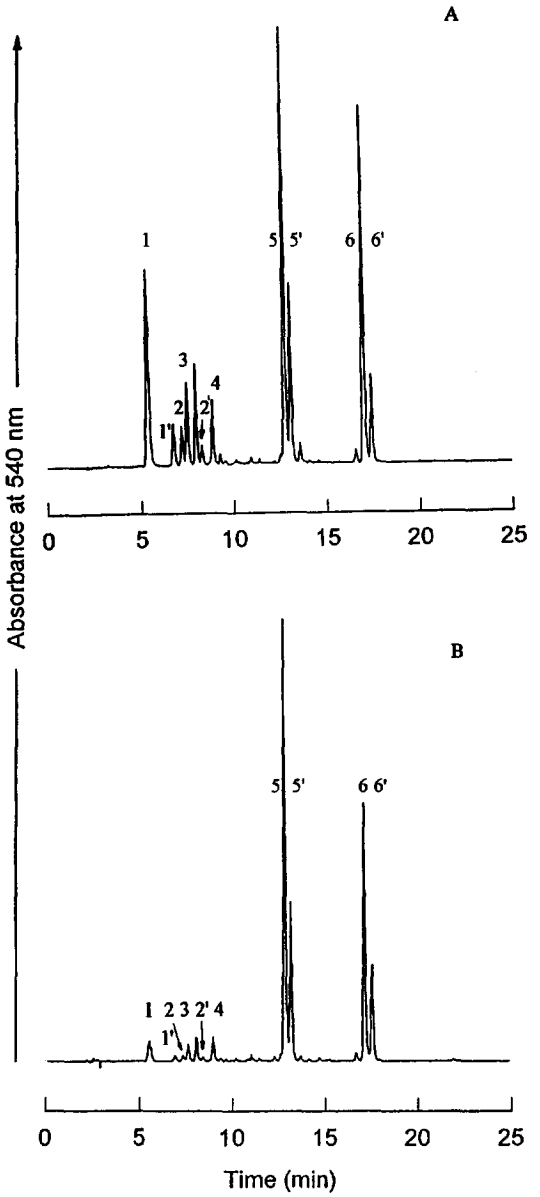


Fig. 2. HPLC elution profile of methanolic extracts from cultured cells of *P. americana*. Peak identification: peak numbering corresponds to numbering in Table 1. Peak numbers with superior primes (1′, 2′, 5′, 6′) correspond to the isoforms (2*S*,15*R*) of the respective normal betacyanins (2*S*, 15*S*). (A) Extract from suspension cultured cells; (B) extract from callus cells.

Table 1. DAD–HPLC and MS data for the betacyanins **1–6**

Peak	Compound	DAD–HPLC (λ_{max}, nm) I: HCA moiety	II: BC* moiety	Ratio *A*† II:I	MS $[M+H]^+$
1	Betanin	—	538	—‡	—
1′	Isobetanin	—	538	—‡	—
2	Prebetanin	—	538	—‡	—
2′	Isoprebetanin	—	538	—‡	—
3	Gomphrenin I	—	538	—‡	—
4	HCA-betacyanin§	326	553	1:0.39	—
5	HCA-betacyanin‖	331	548	1:0.37	[859]
5′	HCA-betacyanin	331	548	1:0.36	[859]
6	Lampranthin II	327	541	1:0.41	[727]
6′	Isolampranthin II	327	541	1:0.32	[727]

*BC = betacyanin glycoside moiety.
†*A* = absorbance.
‡Ratio of absorbance at λ_{max} and 320 nm is *ca* 1:0.08.
§Unknown HCA-betacyanin.
‖Betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside.

while **2** (prebetanin) is a known constituent of fruits of *P. americana* [6]. The betacyanins **4–6** (Table 1) showed UV spectra typical of HCA-substituted betacyanins [17]. Comparison of the DAD–HPLC data (Table 1), as well as co-chromatography, showed that **6** in both cell culture extracts was identical with lampranthin II [betanidin 5-*O*-(6′-*O*-*E*-feruloyl)-β-D-glucoside] from *B. vulgaris* suspension culture [15]. For further characterization the main HCA-betacyanins were isolated. Prior to preparative HPLC an attempt was made to purify the concentrated extract from suspension cultured cells by conventional anion exchange column chromatography on Dowex 1 × 8 [18] using a gradient of aqueous formic acid. Subsequent analytical HPLC of the concentrated fractions revealed that *ca* 95% of the compounds **5**, **5′** was lost by degradation to **1**, **1′**. Hence, this previously recommended step had to be omitted, and purification of the crude extracts was initiated with gel filtration on Sephadex LH-20. Three fractions were collected by visual monitoring, the latter two contained mainly compounds **5**, **5′** and **6**, **6′**. These were finally purified by preparative HPLC.

Liquid chromatography–mass spectrometry (LC–MS) of the extract from suspension cultured cells and the purified HCA-betacyanins showed the presence of two protonated molecular ions ($[M+H]^+$): *m/z* 859 for **5**, **5′** and *m/z* 727 for **6**, **6′**, the latter corresponding exactly with lampranthin II and its isoform [18]. Parent ion scans of *m/z* 389 [betanidin + H]$^+$ revealed that both $[M+H]^+$ ions were derived from the same aglycone betanidin/isobetanidin.

Alkaline hydrolysis of both purified HCA-betacyanin fractions yielded ferulic acid, which was identified by HPLC. These data, together with the more polar character of **5**, **5′** compared to **6**, **6′** and their mass difference (*m/z* 859–727) of 132, suggested the presence of an additional pentose moiety. As **5**, **5′** did not yield any trace of **6**, **6′** under acidic conditions (50% aqueous formic acid), the ferulic acid moiety is most likely attached to the pentose. Subsequently, the complete structure of this novel feruloyl-betacyanin derivative was elucidated using complementary data derived from various mass spectrometric and NMR techniques as described below.

Carbohydrate compositional analysis of the novel feruloyl-betacyanin derivative **5**, **5′** confirmed the presence of glucose and the rare pentose apiose in a ratio of about 1:1. The identity of the pentose residue was unambiguously established by comparison with an authentic sample of apiose obtained from apiin.

The linkage between the two sugar moieties was established by methylation analysis [19]. The detection of 1,4-di-*O*-acetyl-2,3,5-tri-*O*-methylapitol by GC–mass spectrometry, identified by its characteristic fragmentation pattern [20], clearly showed the terminal position of this pentose (all acyl-linked organic acids are lost under the basic conditions of the derivatization procedure). This pentose residue is bound to position 2 of the glucose as indicated by the detection of 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylglucitol. Traces of 1,5-di-*O*-acetyl-2,3,4,6-tetramethylglucitol, characteristic of terminal glucose, can be explained by partial degradation of the compound during the permethylation procedure or contamination of the sample.

Mass spectrometric analysis of betacyanins **5**, **5′** was performed by electrospray ionization mass spectrometry (ESI-MS). In the positive mode a protonated molecular ion $[M+H]^+$ was observed at *m/z* 859 confirming the LC-MS data. Collision induced dissociation (CID) of the parent at *m/z* 859 yielded a rather uninformative daughter ion spectrum (not shown) dominated by a fragment ion at *m/z* 389, characteristic of betanidin/isobetanidin system. An additional weak signal at *m/z* 551 [betanidin + (hexose − H_2O) + H]$^+$ suggests that the feruloyl residue is not bound to this part of the molecule.

In the negative mode, the respective deprotonated

molecular ion at m/z 857 $[M-H]^-$ was obtained, which yielded a complex daughter ion spectrum, depicted in Fig. 3. The fragmentation pathway is explained in the affixed scheme. Unfortunately, the position of the acyl-linked feruloyl residue could not be unequivocally determined due to ambiguities in the assignment of the fragment ions: The elimination of three CO_2 molecules leads to a fragment with the same nominal mass as the loss of a pentose residue and, therefore, these two fragmentation pathways cannot be distinguished by the low resolution daughter ion spectrum produced by the triple quadrupole mass analyser. Furthermore, the loss of one CO_2 molecule plus a pentose residue is equivalent to the elimination of a feruloyl fragment. However, CID of the ion at m/z 664 (cf. Fig. 3), generated by nozzle/skimmer fragmentation, which obviously cannot eliminate more than one CO_2 molecule and therefore should give clearer results, did not yield a fragment attributable to elimination of a pentose residue. Only the loss of a pentose plus ferulic acid was observed, strongly suggesting the linkage of the feruloyl moiety to the apiose residue. Confirmation of this linkage pattern was obtained by positive ion tandem mass spectrometry (MS–MS) experiments of the partially methylated compound obtained by addition of diazomethane to a methanolic solution at room temperature. Formerly, under these conditions a 14,15-dehydrogenation was observed, which results in a neobetanin derivative [21]. A molecular ion at m/z 927 [neobetanidin + (hexose + pentose + ferulic acid –

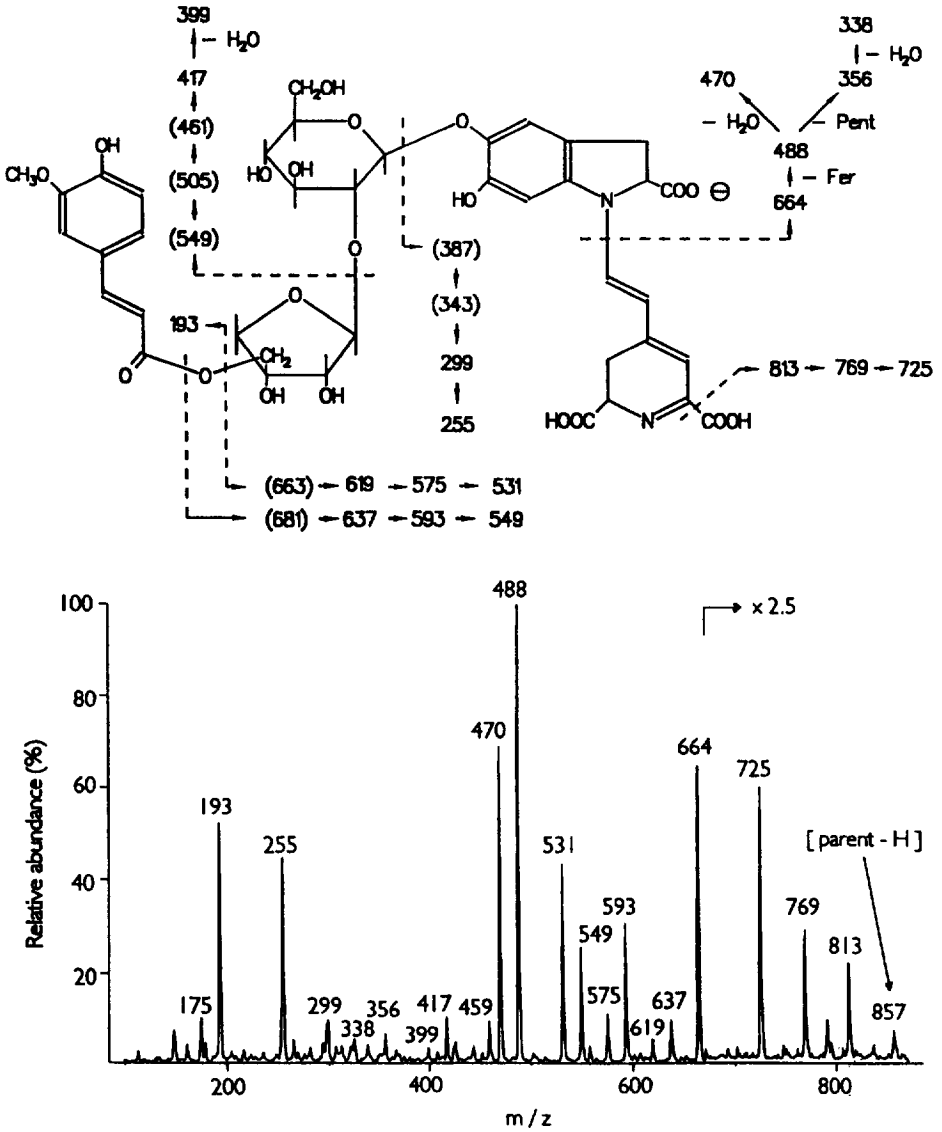


Fig. 3. Negative daughter ion spectrum of the deprotonated molecular ion of the betacyanins **5,5'**, isolated from suspension cultured cells of *P. americana*. The fragmentation pathway is explained in the affixed scheme (for enhanced clarity, the exact structure of the compound, obviously not deducable from these MS data alone, is given; cf. text). Fragmentation arrows not marked are all due to loss of CO_2. The fragmentation pathway of the ion at m/z 664 was confirmed by CID of the respective nozzle/skimmer fragment. (Fer = ferulic acid–H_2O; Pent = pentose–H_2O.)

$3H_2O$) + 5Me + H]$^+$, compatible with the methylation of the three carboxyl groups, the free phenolic hydroxyl groups of the betanidin system and the feruloyl moiety, as well as the aromatization of the dihydropyridyl ring, were observed. The daughter ion spectrum displayed a fragmentation pathway similar to the positive ion ESI-MS–MS of the underivatized compound. An intense fragment at *m/z* 443 [neobetanidin + 4Me + H]$^+$ confirmed the addition of four methyl groups to the betanidin system. Methylation of the acidic proton of the feruloyl residue, however, led to the detection of clear signals at *m/z* 191 [ferulic acid + Me − H_2O + H]$^+$, *m/z* 323 [ferulic acid + Me + pentose − $2H_2O$ + H]$^+$, *m/z* 305 [ferulic acid + Me + pentose − $3H_2O$ + H]$^+$ and a rather weak peak at *m/z* 485 [ferulic acid + Me + pentose + hexose − $3H_2O$ + H]$^+$, unequivocally confirming the linkage position of the feruloyl moiety at the pentose part of the molecule. A relatively intense peak at *m/z* 605 [neobetanidin + 4Me + hexose − H_2O + H]$^+$ agrees with the deduced structure.

Although the limited amount of material and the inherent instability of this type of compound in solution makes a rigorous structural elucidation by NMR improbable, the 1D and 2D ^{1}H NMR spectra at 600 MHz afforded sufficient data to complement and confirm the mass spectrometric findings that completed the structural elucidation of the compound. Not unexpectedly the 1D ^{1}H spectrum in CD_3OD was complex as betalains are known to display configurational isomerism at C-15 and to exist as *E/Z* isomers at the partial C-12/C-13 double bond [18]. Two main components were observed in a ratio of 13:7, and the 2D ^{1}H COSY and TOCSY spectra showed that both had the same constituents of a betanidin system, i.e. two sugar moieties and a ferulic acid moiety. The NMR data shown in Table 2, in particular the similarity of the shifts of H-12 in both components, and the HPLC data, indicated that these two components correspond to the 15*S* and 15*R* isomers. The TOCSY cross peaks associated with H-11 and H-12 of the main components each showed exchange cross peaks to a further small component (in each case ≤22% of the main components). From comparison with literature data [18] the upfield shift observed for H-12 and downfield shift for H-18 for these minor components, and their exchange behaviour, support the conclusion that these are the 12*Z*-isomers, while the two main components are the corresponding 12*E* isomers. The small chemical shift difference between H-4 and H-7 of ≤0.1 ppm is characteristic of substitution at the hydroxyl group at C-5 of betanidin, as opposed to that at C-6 where differences of *ca* 0.8 ppm are to be expected [22].

The NMR data for the sugar moieties are compatible with the presence of glucose and apiose units, found from the mass spectral data, in the β-glucopyranose and apiofuranose forms. Although in general the magnitude of $^3J(1-2)$ of the apiose system is insufficient to define conclusively the relative configuration of the anomeric proton, the similarity of the coupling found here (2.7 Hz) and that of the apiose moiety, e.g. in xeranthin (2.8 Hz) [23], together with similarities of their chemical shifts, is suggestive of a β-configuration of the glycosidic linkage. Significantly the chemical shifts of H-5A and H-5B of this same moiety are

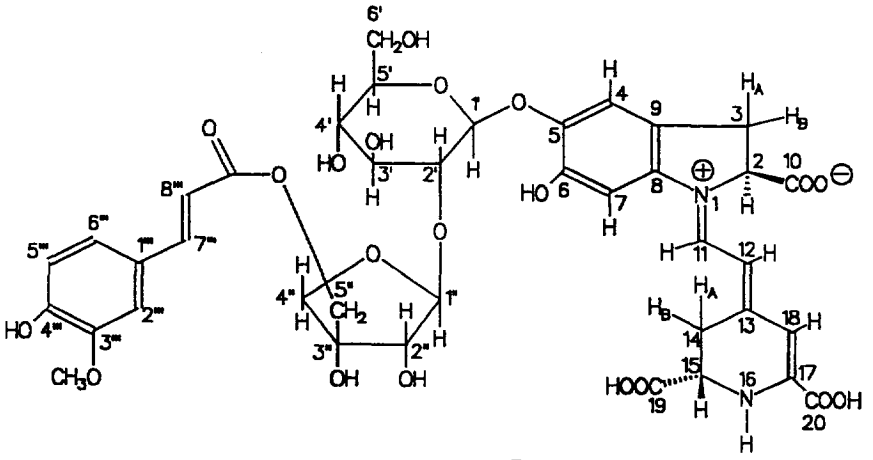
Table 2. ^{1}H NMR data for betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside in CD_3OD/DCl

Chemical shifts*	Aglycone										
	H-2	H-3A	H-3B	H-4	H-7	H-11	H-12	H-14A	H-14B	H-15	H-18
First component† (65%)	5.27	3.65	3.35	7.23	7.20	8.43	6.09	3.47	3.30	4.75	6.44
Second component† (35%)	5.26	3.75	3.34	7.18	7.18	8.23	5.96	3.47	3.00	4.75	6.39
	β-Glucopyranose moiety					Apiose moiety‡					
	H-1′	H-2′	H-3′—H-6′B		H-6′A	H-1″	H-2″	H-4″A	H-4″B	H-5″A	H-5″B
First component	5.01	3.72	3.77 – 3.40		3.93	5.46	4.03	4.27	3.88	4.31	4.20
Second component	5.12	3.75	3.77 – 3.40		3.90	5.47	3.92	4.30	3.85	4.31	4.20
	Feruloyl moiety										
	H-2‴	H-5‴	H-6‴	H-7‴	H-8‴	3‴-OMe					
First component	7.08	6.79	7.05	7.54	6.28	3.89					
Second component	7.12	6.80	7.05	7.44	6.21	3.91					

*Through-bond couplings were detected using 2D ^{1}H COSY and TOCSY spectra. Their magnitudes were taken from the 1D ^{1}H spectra and were comparable in both components and their minor isomers (see footnote †). The following values were measured for the first component: (2–3) *ca* 2, *ca* 10, (11–12) 12.3, (1′–2′) 7.7, (6′A–5′) *ca* 2, (6′A–6′B) *ca* 12, (1″–2″) 2.7, (4″A–4″B) 9.7, (5″A–5″B) 11.3, (2″–6″) 1.6, (5″–6″) 8.1, (7″–8″) 15.9 Hz. A number of long-range couplings were unambiguously identified in the 2D TOCSY spectrum in both components between H-4 and H-3A, H-4 and H-3B, H-12 and H-14A, H-12 and H-14B, H-2‴ and 3‴-OMe.

†Each component shows exchange signals in the 2D ^{1}H TOCSY spectrum with a further minor isomer that is present in a ratio of *ca* 2:9. The shifts of H-11, H-12 and H-18 in these isomers are 8.47, 5.55 and 6.74 ppm, respectively, for the first component, and 8.23, 5.70 and 6.86 ppm for the second component.

‡The corresponding non-acylated apiose in apigenin 7-*O*-(2′-*O*-β-D-apiofuranosyl)-β-D-glucopyranoside (apiin) in CD_3OD had the following ^{1}H chemical shifts and coupling constants: H-1 5.50, H-2 4.00, H-4A 4.08, H-4B 3.86, H-5AB 3.59 ppm and (1–2) 1.8, (4A–4B) 9.6 Hz.



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comparable with those in xeranthin, but *ca* 0.6 to 0.7 ppm to low field of the same unit in apiin (see footnote ‡ to Table 2). This is definitive evidence that the *E*-feruloyl system is bound to C-5 of the apiose moiety. The identity of this acyl system is readily established from the vicinal coupling of H-7‴ and H-8‴ (15.9 Hz) and the long-range coupling between H-2‴ and the methoxyl group at C-3‴. The present NMR data are not sufficient to determine the position of the apiose linkage to glucose, but this has been unambiguously established from the mass spectral data.

Thus, despite the complex nature of the NMR spectra, they can be readily interpreted and, in combination with the substantial amount of information from the various mass spectral and chromatographic techniques, unambiguously established the structure of the major and minor components of the mixture **5**, **5′** as the (2*S*,12*E*,15*S*)- and (2*S*,12*E*,15*R*)-betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranosides, respectively.

In accordance with previous results from UV–Vis spectroscopy [17, 22], the bathochromic shift in λ_{max} of the betanidin moiety of **5**, **5′** compared to those in unacylated compounds (Table 1) arise most likely from intramolecular association, a phenomenon of co-pigmentation which is also corroborated by significant chemical shift differences in NMR.

In summary, the results obtained showed that fruit extracts of *P. americana* contain prebetanin as a major betacyanin and low amounts of betanin and a new HCA-betacyanin, which in contrast predominates in extracts from epidermal layers of stems and cell cultures. From cultured cells this compound was isolated and structurally elucidated as betanidin 5-*O*-[(5″-*O*-*E*-feruloyl)-2′-*O*-β-D-apiofuranosyl]-β-D-glucopyranoside, a new betacyanin of higher plants. Additionally, betanidin 5-*O*-(6′-*O*-*E*-feruloyl)-β-D-glucopyranoside (lampranthin II) was identified from this material for the first time, known to be a minor betacyanin constituent of cell cultures of *B. vulgaris* [15].

EXPERIMENTAL

Plant material and plant cell culture. Plants of *P. americana* L. (pokeweed) and *P. acinosa* Roxb. with ripening fruits were obtained from the botanical garden in Halle. Cells were maintained in Schenk–Hildebrandt medium [24] with 4×10^{-6} M 2,4-D. Suspensions were subcultured every 7 days in 40 ml medium on a shaker (120 rpm) in darkness at 25°. Calluses were subcultured every 3 weeks.

Isolation of betacyanins. Freshly harvested fruits were frozen in liquid N_2, homogenized in a mortar and extracted with 50% aq. MeOH. The reddish-coloured epidermal layers of stems of *P. americana* were peeled off, processed similarly, centrifuged (10 min, 15 000 *g*) and analysed immediately by analyt. HPLC. Cells from suspension cultures of *P. americana* were harvested 1 week after transfer of cells to fresh medium and sepd from culture media by suction through a glass sinter funnel. 17 g cells (fr. wt) were frozen in liquid N_2, homogenized in a mortar and extracted with 34 ml 50% aq. MeOH. After centrifugation (10 min, 15 000 *g*) the supernatant was taken to near dryness in SpeedVac. The concd extract was transferred on to a Sephadex LH-20 column (500 × 15 mm i.d.) and the betacyanins eluted with H_2O. Elution of 3 distinct betacyanin frs were detected visually.

HPLC. The liquid chromatograph and data processors (Millenium software, Baseline 810 software) from Waters™ Millipore (Eschborn, Germany) were used. Conditions for analyt. HPLC: column prepacked with Nucleosil C_{18} (Macherey-Nagel; 5 μm, 250 × 4 mm i.d.); linear gradient within 25 min from 13% solvent B (80% aq. MeCN) in solvent A (1.5% aq. H_3PO_4) to 45% B in A + B at a flow rate of 1 ml min^{-1}; 20 μl injection vol.; detection at 540 nm. Conditions for prep. HPLC: column prepacked with silica gel C-18 (Cartridge with two Waters Prep Nova-Pak HR C18, 6 μm, 100 × 25 mm i.d.); linear gradient within 60 min from 20% solvent B (80% aq. MeOH) in solvent A (1% aq. HCO_2H) to 40% B in A + B at a

flow rate of 5 ml min^{-1}; 0.1 ml injection vol.; detection at 540 nm.

NMR. 1D and 2D ^1H (COSY and TOCSY with a mixing time of 70 msec) NMR spectra were recorded at 300 K on a Bruker AVANCE DMX 600 NMR spectrometer locked to the major deuterium resonance of the solvent, CD_3OD containing a trace of DCl. All chemical shifts are given in ppm relative to TMS and coupling constants in Hz.

Sugar composition analysis. Monosaccharides were analysed as the corresponding Me glycosides after methanolysis and trimethylsilylation on a Carlo Erba Mega Series gas chromatograph incorporating a 30-m DB1 capillary column [25]. Apiin [apigenin 7-*O*-(2′-*O*-β-D-apiofuranosyl)-β-D-glucopyranoside] from Roth (Karlsruhe, Germany) was used as standard.

Carbohydrate methylation analysis. For methylation analysis the glycoconjugate was permethylated according to ref. [26]. Purification of the permethylated sample, hydrolysis, reduction, peracetylation and analyses were performed as described [22].

ESI-MS

(1) LC–ESI-MS. Ion spray mass spectra were recorded on a Sciex API III triple–quadrupole mass spectrometer equipped with an ion spray source; column prepacked with Nucleosil C-18 (Macherey–Nagel; 5 μm, 100 × 2 mm i.d.); linear gradient within 25 min from 15% solvent B (0.1% TFA in MeOH) in solvent A (0.1% TFA) to 70% B in A + B at a flow rate of 200 μl min^{-1} with 110:90 split; 7 μl injection vol.; detection at 540 nm. For direct injection into ion spray source, betacyanin samples were dissolved in MeOH–0.1 M ammonium acetate (1:1) and introduced at 5 μl min^{-1}.

(2) MS–MS. A Finnigan MAT TSQ 700 triple–quadrupole mass spectrometer equipped with a Finnigan electrospray ion source (Finnigan MAT, San Jose, U.S.A.) was used for ESI-MS-MS. The samples were dissolved in MeOH (concns *ca* 20 pmol μl^{-1}) and injected at 0.5–1.0 μl min^{-1} into the electrospray chamber. In the positive ion mode, a voltage of +5.5 kV was applied to the electrospray needle. For CID experiments, parent ions were selectively transmitted by the first mass analyser and directed into the collisions cell (Ar used as collision gas) with a kinetic energy set *ca* −30 eV. For detection of negative ions, all voltages were reversed, i.e. −4.5 kV was applied to the needle and the collision energy set at *ca* +30 eV for MS–MS experiments.

Alkaline HCA-betacyanin hydrolysis for HCA analysis. For HCA analysis, HCA-betacyanin frs containing **5**, **5′** and **6**, **6′** were hydrolysed with 0.1 N NaOH (10 min, 60°) and the liberated HCA identified by analyt. HPLC (linear gradient within 20 min from 15% B (80% aq. MeCN) in solvent A (1.5% aq. H_3PO_4) to 40% B in A + B at a flow rate of 1 ml min^{-1}; 20 μl injection vol.; detection at 320 nm. Ferulic acid from Sigma was used as standard.

Acidic HCA-betacyanin hydrolysis. To an aliquot (50 μl) of purified HCA-betacyanin fr. containing **5**, **5′**, 50 μl HCO_2H (98%) was added and the kinetics of hydrolysis was followed with the analyt. HPLC system; 10 μl injection vol.; detection at 540 and 320 nm.

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