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Anthocyanins acylated with gallic acid from chenille plant, *Acalypha hispida*

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Abstract

Three anthocyanins were isolated from the red flowers of chenille plant, *Acalypha hispida* Burm. (Euphorbiaceae) by a combination of chromatographic techniques. Their structures were elucidated mainly by homo- and heteronuclear nuclear magnetic resonance spectroscopy and electrospray mass spectrometry, and supported with complete assignments of ^{13}C NMR resonances. The novel pigment, cyanidin 3-*O*-(2″-galloyl-6″-*O*-α-rhamnopyranosyl-β-galactopyranoside) (5%), contains the disaccharide robinoside. The other anthocyanins were identified as cyanidin 3-*O*-(2″-galloyl-β-galactopyranoside) (85%), and cyanidin 3-*O*-β-galactopyranoside (5%). Anthocyanins acylated with gallic acid have previously been identified in species from the families Nymphaeaceae and Aceraceae, and tentatively in *Abrus precatorius* (Leguminosae).

Keywords: *Acalypha hispida*; Euphorbiaceae; Flowers; Anthocyanins; Robinoside; Cyanidin 3-*O*-(2″-galloyl-6″-*O*-α-rhamnopyranosyl-β-galactopyranoside); ^{13}C NMR; Chemotaxonomy

1. Introduction

The genus *Acalypha* (Euphorbiaceae) contains about 430 species of evergreen shrubs and trees, and annuals, from tropical and subtropical regions. Reports on anthocyanin identification from this genus have hitherto been restricted to the principal anthocyanin of *Acalypha hispida*, tentatively identified as cyanidin 3-arabinosylglucoside (Bailoni et al., 1998). Collectively from other genera in the family Euphorbiaceae the 3-rutinosides of cyanidin, delphinidin and pelargonidin, the 3-glucosides of cyanidin and pelargonidin and cyanidin 3-galactoside have been reported (Asen, 1958; Stewart et al., 1979; Del V. Galarza et al., 1983). Capsules containing anthocyanins from *Euphorbia splendens* for the treatment of blood circulation disorders have been patented (Fujii et al., 1987).

During our survey of the anthocyanin content of plants from tropical regions, we found that the major anthocyanin of flowers of the horticultural important chenille plant, *A. hispida* Burm., was cyanidin 3-*O*-(2″-galloyl-β-galactopyranoside), and not cyanidin 3-arabinosylglucoside as reported by Bailoni et al. (1998). In this paper we present the isolation and structure elucidation of three anthocyanins from flowers of *Acalypha hispida* including a novel anthocyanin containing the disaccharide robinose acylated with gallic acid.

2. Results and discussion

The HPLC chromatogram of the crude extract of *A. hispida* showed one major, **2**, and two minor anthocyanins, **1** and **3** (Table 1). The pigments in the extract were purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography, and isolated as three separated bands by Sephadex LH-20 chromatography. The isolated anthocyanins were checked for homogeneity by analytical HPLC.

The visible part of the UV–vis spectra of **1** and **2** (Table 1) were in accordance with cyanidin 3-/peonidin 3-glycosides (Andersen, 1985). The UV–vis spectrum of **2** taken on-line during HPLC showed a visible maximum at 523 nm with A_{440}/A_{523} and A_{280}/A_{523} of 28 and 99%,

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Table 1
Chromatographic (HPLC) and spectral (UV–vis and MS) data recorded for the anthocyanins, **1**–**3**, isolated from flowers of *Acalypha hispida*

Compound	On-line HPLC				ES-MS		
	$\lambda_{vis\text{-}max}$ (nm)	$\lambda_{UV\text{-}max}$ (nm)	$A_{UV\text{-}max}/A_{vis\text{-}max}$ (%)	$A_{440}/A_{vis\text{-}max}$ (%)	t_R (min)	M^+ m/z	A^+ m/z
1	520	280	73	30	13.24	449	287
2	523	280	99	28	14.45	601	287
3	523	280	98	28	14.80	747	287

respectively, indicating no 5-sugar substituents and the presence of an aromatic acyl group. The relative high mobility of **2** in the TLC system and the long retention time (HPLC) compared to pigment **1** (Table 1), confirmed aromatic acylation. Pigment **1** co-chromatographed (HPLC and TLC) with cyanidin 3-galactoside from *Vaccinium vites-idae* (Andersen, 1985). The structures of **1** and **2** were elucidated by one- and two-dimensional NMR results (Tables 2 and 3) to be cyanidin 3-*O*-β-galactopyranoside and cyanidin 3-*O*-(2″-galloyl-β-galactopyranoside), respectively. Their structures were confirmed by electrospray ESI-MS data (Table 1).

The UV–vis spectrum of **3** was very similar to the corresponding spectrum of **2**, indicating an anthocyanidin with two oxygen-functions on the B-ring (Table 1) (Andersen, 1985). This pattern was confirmed by the 3H AMX system at δ 8.10 (*dd*, 8.8 Hz, 2.2 Hz, H-6′), 7.90 (*d*, 2.2 Hz, H-2′) and 6.89 (*d*, 8.8 Hz, H-5′). In the downfield region additional signals were observed at δ 9.04 (H-4), and a 2H AX-system at δ 6.94 (H-8) and δ 6.90 (H-6) (Table 2). This latter system was influenced by a 5-bonding zig-zag coupling between H-4 and H-8 ($J = 0.9$ Hz). The chemical shifts for the corresponding

Table 2
^{1}H NMR spectral data for the anthocyanins, **1**–**3**, isolated from flowers of *Acalypha hispida* dissolved in CD_3OD:CF_3COOD (20:1) at 25 °C

	1 δ (ppm), *J* (Hz)	**2** δ (ppm), *J* (Hz)	**3** δ (ppm), *J* (Hz)
Aglycone			
4	9.11 *d* 0.9[a]	9.07 *s*	9.04 *d* 0.9[a]
6	6.74 *d* 2.0	6.71 *d* 2.0	6.75 *d* 2.0
8	6.98 *dd* 2.0, 0.9[a]	6.87 *s* br	6.94 *dd* 2.0, 0.9[a]
2′	8.17 *d* 2.4	7.84 *d* 1.8	7.90 *d* 2.2
5′	7.11 *d* 8.8	6.82 *d* 8.8	6.89 *d* 8.8
6′	8.34 *dd* 8.8, 2.4	8.02 *dd* 8.8, 1.8	8.10 *dd* 8.8, 2.2
3-O-β-Galactopyranoside			
1″	5.34 *d* 7.7	5.64 *d* 8.1	5.67 *d* 7.9
2″	4.08 *m*	5.76 *t* br	5.78 *dd* 9.9, 7.9
3″	3.76 *dd* 9.9, 3.3	4.05 *m*	4.07 *dd* 9.9, 3.3
4″	4.04 *t* 3.3	4.14 *m*	4.09 *dd* 3.3, 0.9
5″	3.89 *m*	4.03 *m*	4.23 *m*
6A″	3.87 *m*	3.99 *m*	4.00 *m*
6B″	3.87 *m*	3.94 *m*	3.84 *m*
6″-O-α-Rhamnopyranosyl			
1‴			4.78 *d* 1.8
2‴			3.93 *dd* 3.5, 1.8
3‴			3.76 *dd* 9.4, 3.5
4‴			3.46 *t* 9.4
5‴			3.71 *dd* 9.4, 6.4
6‴			1.36 *d* 6.4
2″-O-Galloyl			
2⁗/6⁗		7.10 s	7.08 *s*

See Fig. 1 for pigment identification.
[a] The coupling constant is determined by Gauss multiplication.

Table 3
^{13}C NMR spectral data for the anthocyanins, **1**–**3**, isolated from flowers of *Acalypha hispida* dissolved in CD_3OD:CF_3COOD (20:1) at 25 °C

	1 δ (ppm)	**2** δ (ppm)	**3** δ (ppm)
Aglycone			
2	164.82	163.93	164.32
3	145.92	145.11	145.34
4	137.00	136.29	135.77
5	159.35	159.20	158.85
6	103.39	103.30	103.40
7	170.44	170.41	170.37
8	95.09	95.08	95.12
9	157.87	157.46	157.90
10	113.50	113.08	113.09
1′	121.47	120.77	121.09
2′	118.48	117.67	117.80
3′	147.50	147.31	147.49
4′	155.91	155.72	156.17
5′	117.45	117.30	117.39
6′	128.07	128.47	128.68
3-O-β-Galactopyranoside			
1″	104.41	102.14	101.98
2″	71.94	73.07	72.98
3″	74.98	73.04	72.92
4″	70.16	70.30	70.65
5″	77.73	78.00	76.46
6″	62.37	62.31	68.01
6″-O-α-Rhamnopyranosyl			
1‴			99.38
2‴			71.80
3‴			72.52
4‴			73.98
5‴			69.88
6‴			17.80
2″-O-Galloyl			
1⁗		120.96	121.21
2⁗/6⁗		110.48	110.48
3⁗/5⁗		146.29	146.36
4⁗		140.00	140.03
C=O		167.76	167.97

See Fig. 1 for pigment identification.

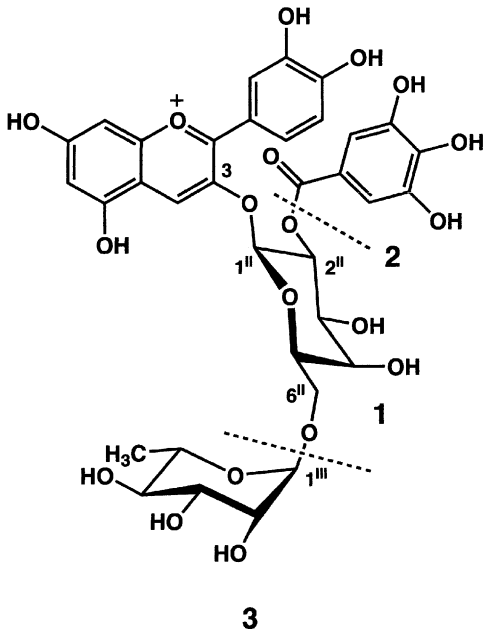

Fig. 1. The structures of the anthocyanins in flower extract of *Acalypha hispida*. **1** = cyanidin 3-*O*-β-galactopyranoside, **2** = cyanidin 3-*O*-(2″-*O*-galloyl-β-galactopyranoside), and **3** = cyanidin 3-*O*-(2″-*O*-galloyl-6″-*O*-α-rhamnopyranosyl-β-galactopyranoside).

aglycone carbons and the quarternary carbons were assigned by the HSQC and HMBC NMR spectra, respectively, in accordance with the anthocyanidin cyanidin (Table 3). The procedure used for assignments of the ^{13}C NMR signals of cyanidin has previously been reported by Andersen et al. (1991).

The two-dimensional TOCSY NMR spectrum of **3** was in agreement with two sugar units. Starting from the anomeric proton at δ 5.67 and the two 6″-sugar protons we could, through the crosspeaks in the DQF-COSY spectrum supported by cross-peaks in the HSQC spectrum assign all the seven sugar protons (Table 2). The corresponding sugar carbons (Table 3) were assigned by their cross-peaks in the HSQC spectrum. The five non-anomeric carbons had chemical shift values from δ 68.0 to δ 76.5 (Table 3), indicating a hexose with a pyranose form (Markham and Chari, 1982). The chemical shifts and the coupling constants (Tables 2 and 3) were in accord with a substituted β-galactopyranoside. A cross-peak at δ 5.67/145.44 in the HMBC spectrum showed that this sugar unit was connected to the 3-position of the aglycone.

From the TOCSY spectrum it was observed that one of the proton signals of the second sugar unit was at high-field (δ 1.36). This 3H-doublet (J = 6.4 Hz) is typical for a rhamnose moiety. By using the DQF-COSY and the HSQC spectra, it was possible to assign all the chemical shifts and the ^1H–^1H coupling constants for the rhamnopyranosyl moiety in accordance with a α-rhamnopyranosyl (Tables 2 and 3). Confirmed by the downfield shift of C-6″ (δ 68.01) and the cross-peak at δ 68.01/4.78 between C-6″ and H-l‴ in the HMBC-spectrum, the rhamnosyl moiety was found to be connected to the galactosyl 6-position. Thus the sugar moiety is determined as the disaccharide robinose.

The UV–vis spectrum of **3** showed higher absorbances around 280 nm ($A_{280}/A_{\text{vis-max}}$ = 98%) than those of **1**, cyanidin 3-galactoside, ($A_{280}/A_{\text{vis-max}}$ = 73%), indicating the presence of an aromatic acyl group. The CAPT spectrum of **3** showed in addition to the corresponding signals of **1,** four positive and one negative carbon signals in the aromatic region (Table 3). Two of the signals (δ 146.36 and 110.48) represented each two carbon atoms. The latter negative CAPT signal (C2‴″/C6‴″) was correlated (revealed by the HSQC spectrum) with a 2H singlet at δ 7.08. In the HMBC spectrum this 2H singlet showed ^3J(CH) responses to C4‴″ (δ 7.08/140.03) and COO (δ 7.08/167.97) of a galloyl (3,4,5-trihydroxybenzoyl) moiety. C1‴″ and C3‴″/C5‴″ were thereafter assigned by the weaker ^2J(CH) responses to the same singlet (δ 7.08/121.21 and (δ 7.08/146.36, respectively). The cross-peak at δ 5.78/167.97 in the HMBC spectrum between H-2″ and the carboxylic carbon showed that the galloyl moiety was connected to C-2″ on the galactose ring. This linkage was also confirmed by the pronounced downfield shift of H-2″ (1.7 ppm), and that H-1″ and H-3″ of **3** were ca 0.3 ppm more downfield than the corresponding signals of **1** (Table 2). Similarly, C-2″ was deshielded (1 ppm) and C-1″ and C-3″ were shielded (3.4 and 2.1 ppm, respectively) compared to the corresponding signals of **1** (Table 3). A molecular ion at m/z 747, and a fragment ion at m/z 287 in the ESI-MS spectrum of **3** corresponding to cyanidin, confirmed the identity of **3** to be cyanidin 3-*O*-(2″-*O*-galloyl-6″-*O*-α-rhamnopyranosyl-β-galactopyranoside).

The relative proportions of **1**–**3** are 5, 85 and 5%, respectively. Thus, galloylated anthocyanins constitute 90% of the total anthocyanin content of *A. hispida*. Even though acylation of anthocyanins with aromatic acids of the cinnamoyl type has widespread occurrence, similar acylation with gallic acid is much more restricted; it has been identified in Aceraceae as cyanidin 3-(6″-galloylglucoside) in *Dipteronia sinensis* and several *Acer* taxa, as cyanidin 3-(″-galloylrutinoside) in some *Acer* taxa (Ji et al., 1992a,b; Fossen and Andersen, 1999a), and as cyanidin 3-(2″,3″-digalloylglucoside) from red leaves of *Acer platanoides* (Fossen and Andersen, 1999a). In Nymphaeaceae the 3-(2″-galloylgalactosides) and 3-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranosides) of delphinidin and cyanidin have been identified (Strack et al., 1992; Fossen and Andersen, 1997, 2001; Fossen et al., 1998), as well as the 3′-*O*-(2″-*O*-galloyl-β-galactopyranoside) and 3′-*O*-(2″-*O*-galloyl-6″-*O*-acetyl-β-galactopyranoside) of delphinidin in blue flowers of *Nymphaéa caerulea* (Fossen and Andersen, 1999b). In addition, delphinidin (coumarylgalloyl)-glucoside has been tentatively identified in the seed coat of *Abrus pre-*

catorius (Legumiosae) (Karawya et al., 1981). Thus, the major pigment of *A. hispida*, cyanidin 3-*O*-(2″-galloyl-β-galactopyranoside) (**2**) which has previously been isolated from *Victoria amazonica* leaves (Strack et al., 1992), and the novel cyanidin 3-*O*-(2″-*O*-galloyl-6″-*O*-α-rhamnopyranosyl-β-galactopyranoside) (**3**) may have chemotaxonomic significance.

3. Experimental

3.1. Isolation of pigments

Whole flowers of *A. hispida* (= *A. sanderi*, *A. sanderiana*, chenille plant, red-hot cat tail) were collected in Kampala (Uganda) in February 2002. Voucher specimens are deposited at Department of Chemistry, Makerere University (Byamukama No. 10). The frozen flowers (ca 77 g) were extracted for 2 h in 450 ml methanol containing H_2O (10%, v/v) and CF_3COOH, TFA (1%, v/v). After filtration and repeated extraction (250 ml), the combined extracts were concentrated and purified by partition against ethyl acetate before application on an Amberlite XAD-7 column. After washing the column with H_2O, the anthocyanins were eluted by methanol containing 1% TFA. The anthocyanins were isolated into three bands on a Sephadex LH-20 column (100 × 5.0 cm, Pharmacia) using H_2O–MeOH–TFA (first 80:19:1 and then 70:29:1, v/v) as eluent. Analytical HPLC was performed on an HP-1050 module system (Hewlett-Packard) using an ODS Hypersil column (20 × 0.5 cm, 5 μm). The elution consisted of a linear gradient from 10% B to 100% B during the first 17 min, isocratic elution using 100% B for the next 4 min followed by a linear gradient back to 10% B during 1 min. The flow rate was 0.75 ml min^{-1}, and aliquots of 10 μl were injected. TLC was carried out on microcrystalline cellulose (Art. 5565, DC-Plastikfolien, Cellulose F, Merck) using the solvent HCO_2H–conc. HCl–H_2O; 1:1:2 v/v. The R_F-values for **1**–**3** are 0.50, 0.56 and 0.68, respectively.

3.2. Spectroscopy

UV–vis absorption spectra were recorded on-line during HPLC over the wavelength range 240–600 nm in steps of 2 nm. Relative amounts of each anthocyanin are reported as percentages of total peak area in HPLC chromatograms based on absorptions recorded for every second nm between 500 and 540 nm.

The NMR experiments on **1**–**3** were obtained at 600.13 MHz and 150.90 MHz for ^1H and ^{13}C respectively, on a Bruker DRX-600 instrument equipped with a multinuclear inverse probe for the 1D ^1H and the 2D Heteronuclear Single Quantum Coherence (^1H–^{13}C HSQC), Heteronuclear Multiple Bond Correlations (^1H–^{13}C HMBC), Double Quantum Filtered Correlation Spectroscopy (^1H–^1H DQF-COSY) and Total Correlation Spectroscopy (^1H–^1H TOCSY) experiments. The ^{13}C 1D CAPT experiment, which was performed only on pigment **3**, was executed on a $^1H/^{13}C$ BBO probe. Sample temperatures were stabilised at 25 °C. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CD_3OD:CF_3CO_2D; 20:1, v/v) were used as secondary references (δ 39.0 and δ 3.4 from TMS, respectively). Mass spectral data on **1**–**3** were achieved by a LCMS system (Waters 2690 HPLC-system connected to Micromass LCZ mass spectrometer) with electrospray ionization in positive mode (ESP+). The following ion optics were used: Capillary 3 kV, cone 30 and 60 V, and extractor 7 V. The source block temperature was 120 °C and the desolvation temperature was 150 C. The electrospray probe-flow was adjusted to 100 μl/min. Continuous mass spectra were recorded over the range m/z 150–800 with scan time 1 s and interscan delay 0.1 s.

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