PAPER I

New anthocyanins from stem bark of castor, *Ricinus communis*

New anthocyanins from stem bark of castor, *Ricinus communis*

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Abstract

Two new anthocyanins (**1**, **2**) were isolated from the stem bark of the castor plant, *Ricinus communis* L. by a combination of chromatographic techniques. Their structures were elucidated mainly by nuclear magnetic resonance spectroscopy and high-resolution electrospray mass spectrometry to be cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside, **1** (21 %) and cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-malonyl-*β*-glucopyranoside), **2** (79 %). In addition, cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-methylmalonate-*β*-glucopyranoside) (**3)** formed by methyl esterification of the malonyl unit of **2** during isolation and storage, was identified. Pigments **1–3** are among the few anthocyanins having a xylosyl moiety linked directly to the anthocyanidin.

Keywords: Castor; *Ricinus communis*; Euphorbiaceae; anthocyanins; cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside; cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-malonyl-*β*-glucopyranoside); cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-methylmalonate-*β*-glucopyranoside)

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1. Introduction

Castor *(Ricinus communis* L.) (Euphorbiaceae) is a soft-wooded small tree widespread throughout tropics and warm temperature regions of the world (Ivan, 1998). It is an important oilseed crop that produces an oil rich in ricinoleic acid, which confers unique properties to the oil (Velasco *et al*., 2005; Rojas-Barros *et al*., 2004; Zhang *et al*., 2005).

Bioactivities in mammalian cells of potential health benefit caused by anthocyanins and other flavonoids include effects on oxidative damage, detoxification enzymes, the immune system, blood pressure, platelet aggregation, and anti-inflammatory, antibacterial and anti-viral responses (Beattie *et al*., 2005; Ghosh, 2005). The structure of the anthocyanins in the castor plant has previously not been reported. However, it has recently been indicated that vacuolar anthocyanins in leaves of this plant may be an effective *in vivo* target for oxy-radicals, provided that the oxy-radical source and the anthocyanic detoxifying sink were in close vicinity (Kytridis and Manetas, 2006). It has been revealed that red and green stemmed castor varieties have differential impact on hemolymph biochemical and hemocyte count of the larvae of *Pericallia ricini* (Jeyakumar *et al.*, 1995), and aqueous leaf extract of the castor plant containing flavonoids has shown excellent insecticidal activity against *Callosobruchus chinensis* (Upasani *et al.*, 2003). Several flavanones and flavonols have previously been isolated from this plant (Khafagy *et al.*, 1985; Kang *et al.*, 1985; Aqil *et al.*, 1997; Bahroun *et al.*, 2002).

In this paper we report the isolation and identification of two new anthocyanidin 3,5-diglycosides (**1**, **2**) responsible for the bright red colour of the stem bark of the castor plant, and one anthocyanin (**3**) formed by methyl esterification of **2** during isolation and storage. The three anthocyanins, which all have a xylose unit linked directly to the anthocyanidin 3-position, were characterized by ^{1}H and ^{13}C NMR spectroscopy and high-resolution mass spectrometry.

2. Results and discussion

The HPLC chromatogram of the fresh acidified methanolic extract of the stem bark of *Ricinus communis* L. detected in the visible spectral region revealed two anthocyanins (**1** and **2)**. After storage in the extraction solvent the HPLC chromatogram ~~now~~ showed three anthocyanins (**1**–**3**) (Fig. 1, Table 1). The relatively amounts of **1** and **2** in the initial extract were 21 and 79 %, respectively. After storage in the extraction solvent for weeks the relative amounts of **1**–**3** were 51, 12 and 37% respectively. The UV–Vis spectra of the three anthocyanins recorded on-line during HPLC analysis showed visible maxima around 520 nm, and their $A_{440}/A_{\text{Vis-max}}$ were in the range of 15 to 20% (Table 1), indicating a 3,5-diglycoside based on cyanidin or peonidin aglycones. The pigments were purified by partition against ethyl acetate and Amberlite XAD-7 column chromatography, and separated by Toyopearl HW-40F column chromatography and preparative HPLC. Analysis of the NMR spectra (Tables 2 and 3), recorded in

CF_3COOD-CD_3OD (5:95; v/v), and MS spectra of **1**–**3** (Table 1) revealed that the aliphatic acylated anthocyanins **2** and **3** only differed with one methyl unit in **3**.

*2.1 Identification of pigment **1***

The downfield part of the 1D ^{1}H NMR spectrum of **1** showed a singlet at 9.04 ppm (H-4), a 3H AMX system at 8.42 ppm (*dd*, 8.8 Hz, 2.3 Hz; H-6'), 8.15 ppm (*d*, 2.3 Hz; H-2') and 7.12 ppm (*d*, 8.8 Hz; H-5') and an unresolved 2H AB system at 7.17 ppm (H-8) and 7.13 ppm (H-6), respectively (Table 2), in accordance with the anthocyanin, cyanidin. After the chemical shifts of the protons of the aglycone of **1** were assigned, the chemical shifts of the corresponding carbons (Table 3) were assigned from the HSQC experiment. The remaining quaternary C-atoms were assigned using the HMBC spectrum, which was optimized for $^2J_{CH}$ and $^3J_{CH}$ couplings (Table 3).

The sugar region of the 1D ^{1}H NMR of **1** showed the presence of two sugar units revealed by two anomeric protons with a *β*-configuration (H-1": $^3J_{HH}$ = 7.0 Hz, H-1"': $^3J_{HH}$ = 7.9 Hz). The DQF-COSY and TOCSY spectra were in accordance with 13 protons, which indicated that one of the sugar units was a pentose, and another a hexose. Starting from H-1" at δ 5.49 (J =7.0 Hz), the observed cross-peak at 5.49/3.81 ppm in the DQF-COSY spectrum supported by corresponding crosspeaks in the HSQC spectrum (Table 2), permitted the assignment of H-2". The chain of coupled protons H-2", H-3", H-4", H-5A" and H-5B" were there after assigned similarly (Table 2). In cases where several sugar protons showed similar chemical shifts, the assignment was assisted by the TOCSY experiment. The chemical shifts and the coupling constants (Table 2) of this glycosyl unit

were in accordance with a *β*-xylopyranosyl. A crosspeak at δ 5.49/145.3 in the HMBC spectrum between H-1" and C-3 of the aglycone showed that the xylosyl unit was connected to the 3-position of the aglycone. By using the doublet at δ 5.28 (*J* = 7.9 Hz) as the starting point in the DQF-COSY spectrum together with the crosspeaks in the TOCSY spectrum, it was likewise possible to assign all the chemical shifts for the second monosaccharide moiety, *β*-glucopyranosyl (Table 2). A cross peak at δ 5.28/157.0 in the HMBC spectrum between H-1"" and C-5 of the aglycone showed that this sugar unit was connected to the 5-position of the aglycone. All the ^{13}C of the two sugar units were assigned from the HSQC experiment (Table 3). The molecular mass (m/z 581.1495) corresponding to $C_{26}H_{29}O_{15}^{+}$ in the ESI+ high resolution mass spectrum of **1** confirmed the structure to be cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside (Fig. 1), which is a new anthocyanin in plants.

*2.2 Identification of pigment **2** and **3***

The NMR resonances of pigment **2** shared many similarities with the corresponding resonances of **1** (Tables 2 and 3) in accordance with cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside. However, the chemical shift values of H-6A"' (*δ* 4.62), H-6B"' (*δ* 4.42), H-5"' (*δ* 3.89) (Table 2) and C-6"' (*δ* 65.3) (Table 3), indicated the presence of acylation at the 6"'-hydroxyl. The crosspeaks at *δ* 4.62/168.7 (H-6A"'/C-M^{I}), and 4.42/168.7 (H-6B"'/C-M^{I}) in the HMBC spectrum confirmed that the acid moiety, was linked to the hydroxyl at the C-6"' of the 5-*O*-*β*-glucopyranoside. The molecular mass (m/z 667.1478) corresponding to $C_{29}H_{31}O_{18}^{+}$ in the ESI+ high resolution mass

spectrum of **2** was in accordance with cyanidin 3-xylopyranoside-5-glucopyranoside with an additional malonyl unit. Thus the identity of **2** was confirmed as the new anthocyanin cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-malonyl-*β*-glucopyranoside).

Pigment **3** showed higher mobility in the FHW-TLC system and longer retention time than pigment **2** (Table 1). The NMR resonances of **3** showed, however, many similarities with the corresponding resonances of **2** (Tables 2 and 3). The molecular ion at *m*/*z* 681.1647 in the positive ion ESI was in accordance with cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-malonyl-*β*-glucopyranoside) with an additional mass of 14 amu. The crosspeak at *δ* 3.76/168.6 (H-M^{IV}/C-M^{III}), and the crosspeaks at *δ* 4.66/167.9 (H-6A'''/C-M^{I}) and 4.42/167.9 (H-6B'''/C-M^{I}) in the HMBC confirmed the identity of pigment **3** to be cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-methylmalonate-*β*-glucopyranoside). Pigment **3** was identified as the esterified form of pigment **2**. Methyl esterification of the terminal carboxyl group of malonyl units occur easily in the acidified methanolic solvents normally used for extraction and isolation (Fossen et al., 2001; Bloor and Abrahams, 2002).

Altogether sixty-four among the properly identified anthocyanins in the previous reported literature contain a xylosyl moiety (Andersen and Jordheim, 2006; Andersen, 2007). However, only four of these (pelargonidin 3-xyloside, cyanidin 3-xyloside, malvidin 3-xyloside-5-glucoside and malvidin 3-(6''-*p*-coumaryl-glucoside)-5-(2'''-acetyl-xyloside) in addition to pigments **1** and **2** have this monosaccharide linked directly to the anthocyanidin.

3. Experimental

3.1. Plant material

Red stems of *Ricinus communis* were collected on Entebbe Road in Kampala (Uganda) in February 2006. The identification of the plant was carried out in the Botany Department at Makerere University, and voucher specimen has been deposited in the herbarium of that Department, voucher No. RB37/2006. The stems (cut into short pieces) were kept in a freezer before extraction.

3.2 Isolation of anthocyanins

The red stem bark was peeled from the frozen stems, weighed (400 g) and extracted with for 5h in a 750 ml of methanol containing TFA (1% v/v). The filtered extract was concentrated under reduced pressure at 27ºC, purified by partition (several times) against ethyl acetate and applied to an Amberlite XAD-7 column. The anthocyanins adsorbed to the column were washed with water, and eluted from the column with methanol containing 1 % TFA. The concentrated anthocyanin extract was purified by Sephadex LH-20 chromatography using 50% aqueous methanol containing 1% TFA as eluent. The anthocyanins were separated by Toyopearl HW-40F (TOSOH) into three bands using H_2O-MeOH-TFA (89.5:10:0.5) solvent system; first band contained pigment **1**, second band contained pigment **3** and third band pigments **3** and **2**. The individual anthocyanins were further separated using preparative HPLC (Gilson

305/306 pump equipped with a UV 6000LP detector) equipped with an ODS Hypersil column (25 × 2.2 cm; i.d.; 5 μm). Two solvents were used for elution: A = HCO_2H-H_2O (1:9; v/v) and B = HCO_2H-H_2O-MeOH; (1:4:5; v/v). The elution profile consisted of a linear gradient from 10 % to 100 % B for 30 min, isocratic elution (100 % B) for the next 10 min, followed by a linear gradient from 100 % to 10 % B for 1 min. The flow rate was 14 ml/min for 41 min, and aliquots of 250 μl were injected.

3.3 Analytical HPLC and TLC

Chromatography included TLC and on-line HPLC. TLC was carried out on microcrystalline cellulose (F 5556, Merck) with the solvent FHW (HCO_2H-conc HCl-H_2O; 1:1:2 v/v). The HPLC instrument (HP-1050 module, system, Hewlett-Packard) was equipped with an ODS Hypersil column (25 × 0.46 cm, 5 μm). Two solvents; A, water with 0.5 % TFA and B, acetonitrile (0.5 % TFA) were used for elution. The elution profile for HPLC consisted of initial conditions with 90 % A and 10 % B followed by linear elution for 10 min (14 % B), isocratic elution 10-14 min, and the subsequent linear conditions; 18 min (16 % B), 22 min (18 % B), 26 min (23 % B), 31 min (28 % B) and 32 min (40 % B), isocratic elution 32-40 min, and final linear elution 40-41 min (10 % B). Aliquots of 15 μL were injected and the flow rate was 1 ml/min. Prior to injection, all samples were filtered through a 0.45 μm Millipore membrane filter. All the UV–Visible absorption spectra were recorded on-line during HPLC analysis, and the spectral measurements were made over the wavelength range 200–600 nm in steps of 2 nm.

Anthocyanins isolated from blackcurrant (*Ribes nigrum*) (Frøytlog *et al*., 1998) and black bean (*Phaseolus vulgaris* L.) (Takeoka *et al*., 1997) were used as references.

3.4. Spectroscopy

UV–Vis absorption spectra were recorded on-line during HPLC analysis, and the spectral measurements were made over the wavelength range 200–600nm in steps of 2 nm.

The NMR experiments were obtained at 600.13 MHz and 150.92 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DRX–600 instrument at 298 K. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent, CF_3COOD–CD_3OD (95:5; v/v), were used as secondary references (δ 49.0 and δ 3.40 ppm from TMS for ^{1}H and ^{13}C, respectively) (Fossen and Andersen, 2006). The 1D ^{1}H NMR and the 2D Heteronuclear Single Quantum Coherence (^{1}H-^{13}C HSQC), Heteronuclear Multiple Bond Correlations (^{1}H-^{13}C HMBC), Double Quantum Filtered Correlation Spectroscopy (^{1}H-^{1}H DQF-COSY) and Total Correlation Spectroscopy (^{1}H-^{1}H TOCSY) experiments were obtained with the 5 mm TB1 probe.

High-resolution LC-MS (ESI^{+}/TOF) spectra of **1**–**3** were recorded using a JMS-T100LC with an AccuTOF LP mass separator. The gradient used was equal to the one described for the analytical HPLC system. A Develosil C18 (100 mm × 2.0 mm, 3.0 μm; particle size) column was used for separation. The retention times of **1**–**3** were 15.4, 25.9 and 30.3 mins, respectively.

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Figure legends:

Fig. 1. Structures of cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside, **1**, cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-malonyl-*β*-glucopyranoside), **2**, and cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-methylmalonate-*β*-glucopyranoside), **3**.

Table 1

Chromatographic (HPLC and TLC) and spectral (UV-Vis and MS) data recorded for cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside (**1**) and cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-malonyl-*β*-glucopyranoside) (**2**) isolated from the stem bark of *Ricinus communis,* and cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-methylmalonate-*β*-glucopyranoside) (**3**) formed from **2** during isolation and storage. See Fig.1 for structures.

Compound	On-line HPLC				TLC R_f (100×)	ESI calculated	ESI observed	Molecular formula
	R_t (min)	$\lambda_{Vis\text{-}max}$ (nm)	$\lambda_{UV\text{-}max}$ (nm)	$A_{440}/A_{Vis\text{-}max}$ (%)	FHW	M^+ *m*/*z*	M^+ *m*/*z*	
1	19.2	516	277	15	50	581.1506	581.1495	$C_{26}H_{29}O_{15}^+$
2	27.4	516	277	20	55	667.1510	667.1478	$C_{29}H_{31}O_{18}^+$
3	30.6	520	278	20	67	681.1667	681.1647	$C_{30}H_{33}O_{18}^+$

M^+, molecular ion

Table 2
^{1}H NMR spectral data for cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside (**1)** and cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-malonyl-*β*-glucopyranoside) (**2)** isolated from stem bark of *Ricinus communis*, and cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-methylmalonate-*β*-glucopyranoside) (**3)** formed from **2** during isolation and storage. All anthocyanins are dissolved in CD_3OD:CF_3COOD (95:5, v/v) at 25°C.

	^{1}H *δ* (ppm) *J* (Hz)		
	1	**2**	**3**
Aglycone			
4	9.04 *s*	9.06 *s*	9.05 *s*
6	7.13 *s (br)*	7.10 *s (br)*	7.08 *d* 1.8
8	7.17 *s (br)*	7.18 *s(br)*	7.17 *d* 1.6
2'	8.15 *d* 2.3	8.15 *d* 2.0	8.15 *d* 2.2
5'	7.12 *d* 8.8	7.11 *d* 8.9	7.11 *d* 8.8
6'	8.42 *dd* 2.3, 8.8	8.44 *dd* 2.0, 8.9	8.42 *dd* 2.3, 8.7
3-β-Xylopyranoside			
1''	5.49 *d* 7.0	5.48 *d* 6.9	5.49 *d* 6.9
2''	3.81 *dd* 7.0, 9.4	3.80 *dd* 7.0, 9.3	3.80 *dd* 9.3, 7.0
3''	3.66 *m*	3.65 *t* 9.4	3.65 *t* 9.3
4''	3.75 *ddd* 12.3, 9.4, 4.5	3.74 *m*	3.75 *m*
5A''	4.11 *dd* 4.9, 11.6	4.10 *dd* 11.3, 5.0	4.11 *dd* 4.9, 11.5
5B''	3.60 *dd* 9.4, 11.5	3.59 *dd* 9.3, 11.5	3.60 *dd* 9.3, 11.5
5-β-Glucopyranoside			
1'''	5.28 *d* 7.9	5.29 d 7.7	5.29 *d* 7.7
2'''	3.74 *m*	3.73 *m**	3.74 *m*
3'''	3.69 *m*	3.65 *t* 9.3	3.66 *t* 9.0
4'''	3.55 *t* 9.4	3.54 *t* 9.3	3.56 *m*
5'''	3.65 *m**	3.89 *ddd* 9.2,6.9, 1.9	3.88 *ddd* 9.5, 6.8, 1.9
6A'''	4.04 *dd* 2.0, 12.1	4.62 *dd* 2.0, 12.0	4.63 *dd* 1.9, 11.9
6B'''	3.84 *dd* 12.1, 5.7	4.42 *dd* 12.0, 6.9	4.42 *dd* 11.9, 6.8
6'''-O-Malonyl			
M^{II}		#	3.44
M^{IV}			3.76

*Overlap; *br*, broad. See Fig. 1 for pigment identification. # Not detected adequately.

Table 3
^{13}C NMR spectral data for cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-*β*-glucopyranoside (**1)** and cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-malonyl-*β*-glucopyranoside) (**2)** isolated from stem bark of *Ricinus communis*, and cyanidin 3-*O*-*β*-xylopyranoside-5-*O*-(6'''-*O*-methylmalonate-*β*-glucopyranoside) (**3)** formed from **2** during isolation and storage. All anthocyanins are dissolved in CD_3OD:CF_3COOD (95:5, v/v) at 25°C.

	^{13}C δ (ppm)		
	1	**2**	**3**
Aglycone			
2	164.8	165.1	165.1
3	145.3	146.2	146.0
4	135.0	135.0	134.6
5	157.0	156.4	156.0
6	105.6	105.8	105.6
7	169.1	169.3	168.9
8	97.5	97.3	97.3
9	155.5	157.1	157.2
10	113.3	113.2	113.1
1'	120.5	121.0	120.7
2'	118.9	118.5	118.4
3'	147.3	147.6	147.3
4'	157.0	156.7	156.4
5'	117.9	117.6	117.6
6'	129.4	129.1	129.1
3-β-Xylopyranoside			
1''	104.1	103.9	103.9
2''	74.4	74.1	74.1
3''	78.9	77.4	77.0
4''	71.0	70.7	70.7
5''	67.4	67.0	66.9
5-β-Glucopyranoside			
1'''	102.8	102.4	102.3
2'''	74.9	74.6	74.6
3'''	78.1	77.4	77.7
4'''	71.5	71.2	71.3
5'''	77.7	75.9	75.8
6'''	62.5	65.3	65.2
6'''-O-Malonyl			
M^{I}		168.7	167.9
M^{II}		#	#
M^{III}		#	168.6
OCH_3 (M^{IV})			52.9

See Fig. 1 for pigment identification. # Not detected adequately