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Two triacylated and tetraglucosylated anthocyanins from *Ipomoea asarifolia* flowers

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Abstract

Two triacylated and tetraglucosylated anthocyanins derived from cyanidin were isolated from the flowers of *Ipomoea asarifolia* and their structures elucidated using chemical, GC, MS and NMR methods (^{1}H and ^{13}C, TOCSY-1D, DQF-COSY, DIFFNOE and HMBC). These complex pigments were found to consist of cyanidin 3-*O*-[2-*O*-(6-*O*-*E*-caffeoyl-β-D-glucopyranosyl)]-{6-*O*-[4-*O*-(6-*O*-*E*-3,5-dihydroxycinnamoyl-β-D-glucopyranosyl)-*E*-caffeoyl]-β-D-glucopyranosyl}-5-*O*-β-D-glucopyranoside and cyanidin 3-*O*-[2-*O*-(6-*O*-*E*-*p*-coumaroyl-β-D-glucopyranosyl)]-{6-*O*-[4-*O*-(6-*O*-*E*-*p*-coumaroyl-β-D-glucopyranosyl)-*E*-caffeoyl]-β-D-glucopyranosyl}-5-*O*-β-D-glucopyranoside.

Keywords: *Ipomoea asarifolia*; Convolvulaceae; Acylated anthocyanins; Cyanidin; *p*-Coumaric acid; Caffeic acid; 3,5-Dihydroxycinnamic acid

1. Introduction

Ipomoea asarifolia [Desv.] Roem. and Schult. (Convolvulaceae) (Nacro and Millogo-Rasolodimbi, 1993) is a tropical creeping plant in west Africa. Our previous phytochemical investigation of the flowers led to the isolation of two diacylated and triglucosylated anthocyanins, cyanidin 3-*O*-[2-*O*-(6-*O*-*E*-caffeoyl-β-D-glucopyranosyl)]-{6-*O*-*E*-caffeoyl]-β-D-glucopyranosyl}-5-*O*-β-D-glucopyranoside and cyanidin 3-*O*-[2-*O*-(6-*O*-*E*-*p*-coumaroyl-β-D-glucopyranosyl)]-{6-*O*-*E*-caffeoyl]-β-D-glucopyranosyl}-5-*O*-β-D-glucopyranoside (Pale et al., 1997).

In the present study, the identification of the anthocyanin content of the flowers was completed by the structure elucidation of two new additional anthocyanins.

2. Results and discussion

Both anthocyanins **5** and **8** previously detected by HPLC in an extract of *I. asarifolia* flowers (Pale et al., 1997) (Fig. 1) were isolated as TFA salts after successive CC on Amberlite XAD-7, Sephadex LH-20, RP-18 silicagel, preparative TLC and finally again on RP-18 silicagel.

As observed for the earlier identified anthocyanins **1** and **3** from this plant, the UV–vis spectra of the pigments showed absorption at 530 nm, as well as two characteristic absorptions at 293 and 324 nm for **5**, and 295 and 312 for **8**, corresponding to phenolic acid substituents (Table 1).

The E_{acyl}/E_{vis} ratio (Table 1) confirmed the presence of three phenolic acid moieties for each compound. The E_{440}/E_{vis} ratio (Table 1) suggested that the anthocyanins were substituted at C-5 (Ribéreau-Gayon, 1968). The bathochromic shifts on the maximum visible wavelengths, observed after addition of $AlCl_3$, indicated the presence of a vicinal free hydroxyls in both anthocyanins. On acidic hydrolysis, **5** and **8** afforded only glucose which was identified by GC as TMS ether.

In the NMR spectra, the ring proton signals of the cyanidin for **5** and **8** appeared at approximately the same chemical shifts observed for **1** and **3** (Pale et al., 1997). In addition, three pairs of doublets at δ 5.8 and 7.1, δ 6.6 and 6.9 and δ 6.1 and 7.3 with large coupling constants ($J = 16$ Hz) were observed for **5** and **8**, indi-

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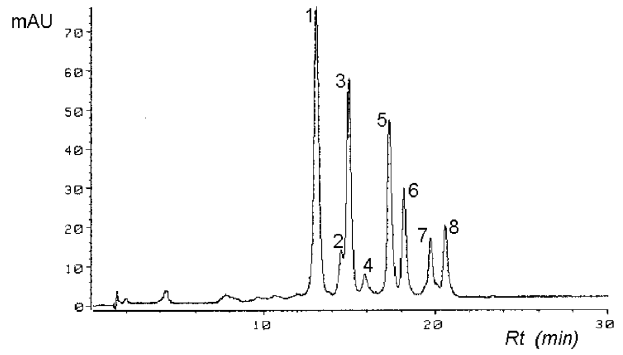


Fig. 1. HPLC chromatogram of a crude methanol extract of *Ipomoea asarifolia* flowers.

cating the *E*-configuration of the olefinic protons of the phenolic acid substituents.

Four characteristic doublets corresponding to the anomeric protons of GlcA, B, C and D were clearly separated at respectively δ 5.4, 4.8, 5.1 and 4.9 for both **5** and **8**. Their coupling constants (J = 7–8 Hz) confirmed the β-D-pyranoside configurations of each.

The downfield shift of ^{1}H NMR signal of H-6 protons of GlcA, B and D in both anthocyanins suggested that these position was subtituated. An NOE oberved between protons H-1 of glucosyl D and H-5 of acyl I confirmed the presence of an additional acylglucoside moiety linked to I. Each additional aromatic acyl group could be placed on the C-6 of GlcD due to the appearance of long range C–H coupling between this position sugar protons and the acyl carbonyl carbon. The H-6 protons of GlcA, B and D showed long range correlation (HMBC) to the organic acid carbonyl signals. Finally GlcD was linked at the C-4 of the caffeoyl group (the anomeric proton of this sugar demonstrated a long range correlation with the characteristic ^{13}C NMR signal of the caffeoyl C-4 at 149.5 and 147.2 ppm) in both anthocyanins.

The ESI-MS of **5** showed a molecular ion at 1421 (100%) M^+ consistent with a $C_{66}H_{69}O_{35}$ molecular formula, 1259 (M–3,5-dihydroxycinnamoyl)$^+$, 1097 (M–3,5-dihydroxycinnamoyl–caffeoyl)$^+$, 773 (M–3,5-dihydroxycinnamoyl–caffeoyl–2 glucosyl)$^+$, 449 (M–3,5-dihydroxycinnamoyl - 2 caffeoyl - glucosyl- sophorosyl)$^+$, 287 (M–3,5-dihydroxycinnamoyl–2 caffeoyl–sophorosyl–2 glucosyl)$^+$. Alkaline hydrolysis afforded caffeic acid, *p*-coumaric acid and an additional non common phenolic acid as confirmed by TLC. The later was isolated by preparative TLC and unambigously identified by ^{1}H NMR and MS. Its ^{1}H NMR spectrum showed indeed two characteristic doublets at δ 6.53 and 7.59 with a large coupling constant (J = 16 Hz) correspondind to the (*E*) olefinic protons (Table 2). Two triplets appeared at δ 7.42 (H-2 and H-6) and at 7.69 (H-4) with short coupling constants (J = 3 Hz). Two characteristic singlets were also observed at δ 8. 3 and 12.3 corresponding respectively to the phenolic and the carboxylic group protons. This phenolic acid was therefore identified as *E*-3,5-dihydroxycinnamic acid. Extensive NMR experiments (^{1}H, ^{13}C, TOCSY 1D, DFQ-COSY, DIFFNOE and HMBC; Table 2) confirmed that the structural features of **5** were identical to those of **1** with an additional acylglucoside. Anthocyanin **5** was therefore identified as cyanidin 3-*O*-[2-*O*-(6-*O*-*E*-caffeoyl-β-D-glucopyranosyl)]-{6-*O*-[4-*O*-(6-*O*-*E*-3,5-dihydroxycinnamoyl-β-D-glucopyranosyl)-*E*-caffeoyl]-β-D-glucopyranosyl}-5-*O*-β-D-glucopyranoside.

The ESI-MS of **8** showed a molecular ion at 1389 (100%) M^+ consistent with a $C_{66}H_{69}O_{33}$ molecular formula, 1227 (M–glucosyl)$^+$, 1097 (M–2 coumaroyl)$^+$, 935 (M–2 coumaroyl–glucosyl)$^+$, 773 (M–2 coumaroyl–cafeoyl–glucosyl)$^+$, 611 (M–2 coumaroyl–cafeoyl–2 glucosyl)$^+$, 449 (M–2 coumaroyl–caffeoyl–3 glucosyl)$^+$, 287 (M–2 coumaroyl–caffeoyl–4 glucosyl)$^+$. The TLC analysis of the products obtained by alkaline hydrolysis allowed the identification of caffeic and *p*-coumaric acids. The results of the NMR experiments (Table 2) confirmed that the structural features of **8** were identical to those of **3**, with an additional *p*-coumaroylglucoside

Table 1
Chromatographic and UV–vis data of the anthocyanins **5** and **8** from *Ipomoea asarifolia* flowers

	TLC (R_f×100)		HPLC[a]	UV–vis			
Anthocyanins	BAW[b]	EAFW[c]	R_t (min)	λ_{max} (nm) (log ε)	$E_{max\ acyl}/E_{max\ vis}$ (%)	$E_{440l}/E_{max\ vis}$	+$AlCl_3$ [d] Δ (nm)
5	18	28	17.39	293 (4.57) 324 (4.52) 531 (4.41)	136	11	+ 20
8	24	33	20.20	295 (4.60) 312 (4.57) 532 (4.36)	160	12	+ 20

[a] HPLC conditions: the Nova Pak C_{18} column Waters (160×4 mm, 4 μ m) was eluted at a flow rate of 0.8 ml min^{-1} with a linear gradient solvent system for 20 min from 40 to 75% (MeOH–H_2O–HCO_2H, 75:24.5:0.5) in A (H_2O–HCO_2H, 60:1); an isocratic elution was then maintained for 10 min. The monitoring was achieved with a photodiode array detector operating at 520 nm [2].

[b] TLC on microcrystalline cellulose F_{254} (0.1 mm;Merck) with 1-BuOH–HOAc–H_2O, 4:1:5 upper phase (BAW) as mobile phase.

[c] TLC on silicagel 6O (Merck) with EtOAc–AcOH–HCO_2H–H_2O, 100:11:11:26 (EAFW) as mobile phase.

[d] 2–3 drops 5% $AlCl_3$ in MeOH were added.

Table 2
^{1}H and ^{13}C NMR data of anthocyanins **5** and **8**

		5		**8**	
Assignment[a]		δ_H (ppm) *J* (Hz) [b]	δ_C (ppm)[b]	δ_H (ppm) J (Hz)[b]	δ_C (ppm)[c]
Aglycone	2		165.2		162.3
	3		145.5		144.1
	4	8.77, *s*	129.2	8.83, *s*	133.1
	5		156.9		155.2
	6	6.77, *d* (1.9)	105.4	6.82, *d* (2.0)	104.5
	7		170.1		167.6
	8	6.41, *d* (1.9)	97.5	6.41, *d* (2.0)	96.3
	9		156.7		155.1
	10		113.0		111.6
	1′		120.7		119.3
	2′	7.76, *d* (1.9)	118.6	7.77, *d* (2.3)	117.5
	3′		147.3		146.6
	4′		156.5		155.0
	5′	6.92, *d* (8.6)	117.7	6.94, *d* (8.6)	116.9
	6′	8.08, *dd* (8.6, 1.9)	129.2	8.15, *dd* (2.3, 8.6)	127.5
Glucose A	1	5.36, *d* (7.8)	102.6	5.41, *d* (7.2)	101.3
	2	3.98, *dd* (7.8, 9.0)	84.1	4.00, *dd* (7.2, 9.0)	81.7
	3	3.85, *dd* (9.0, 8.4)	76.1	3.83, *t* (9.0)	74.7
	4	3.58, *dd* (8.4, 9.6)	71.0	3.59, *dd* (9.0, 9.6)	69.3
	5	3.90, *dd* (9.6, 7.2)	77.7	3.93, m	75.9
	6a	4.50, *dd* (7.2, 10.6)	64.6	4.56, *dd* (8.6, 12.0)	63.3
	6b	4.38, *d* (10.6)	64.6	4.42, *dd* (2.3, 12.0)	63.3
Glucose B	1	4.80, *d* (7.8)	106.1	4.81, *d* (7.5)	104.9
	2	3.50- 3.53, m	75.4	3.45- 3.6, m	73.2
	3	3.50- 3.53, m	77.3	3.45- 3.6, m	75.7
	4	3.48, *dd* (9.6, 9.0)	71.6	3.48, *dd* (9.0, 9.6)	69.7
	5	3.5.- 3.53, m	78.6	3.54, m	77.7
	6a	4.34, *d* (12.0)	64.0	3.32, *dd* (2.0, 12.0)	62.4
	6b	4.23, *d* (12.0)	64.0	3.27, *d* (12.0	62.4
Glucose C	1	5.14, *d* (7.8)	102.3	5.17, *d* (7.8)	99.4
	2	3.76, *dd* (7.8, 9.0)	74.5	3.77, *dd* (7.8, 9.0)	73.2
	3	3.64, *dd* (9.0, 8.4)	75.9	3.64, *dd* (9.0, 9.6)	74.0
	4	3.54, *dd* (8.4, 9.6)	71.4	3.54, *t* (9.0)	69.7
	5	3.61, *dd* (9.6, 3.5)	77.6	3.62, *dd* (2.3, 9.0)	75.8
	6a	3.84, *dd* (3.5, 11.4)	62.4	4.05, *dd* (2.3, 12.0)	60.8
	6b	4.05, *d* (11.4)	62.4	3.84, *d* (12.0)	60.8
Glucose D	1	4.94, *d* (7.8)	104.1	4.99, *d* (7.8)	102.2
	2	3.64, *dd* (7.8, 9.0)	74.7	3.67, *dd* (7.8, 9.0)	73.2
	3	3.63, *t* (9.0)	75.7	3.62, *dd* (9.0, 9.6)	73.9
	4	3.45, *dd* (9.0, 9.6)	72.2	3.47, *t* (9.0)	70.1
	5	3.80, m (9.6, 7.2)	78.0	3.82, m (2.0, 5.4, 9.0)	76.1
	6a	4.26, *dd* (7.2, 10.6)	65.0	4.78, *dd* (2.0, 12.0)	63.5
	6b	4.77, *d* (10.6)	65.0	4.4.28, *dd* (5.4, 9.0)	63.5
Acyl I		*E*-Caffeoyl		*E*-Caffeoyl	
	1		130.4		127.5
	2	6.90, *d* (1.9)	115.4	6.95, *d* (2.0)	115.6
	3		148.4		144.6
	4		149.5		147.2
	5	7.07, *d* (8.6)	116.5	7.12, *d* (8.6)	115.9
	6	6.64, *dd* (8.6, 1.9)	122.8	6.71, *dd* (2.0, 8.6)	120.6
	α	5.85, *d* (16.0)	114.8	5.94, *d* (15.7)	114.2
	β	7.11, *d* (16.0)	146.6	7.18, *d* (15.7)	146.2
	C=O		168.6		166.3
Acyl II		*E*-Caffeoyl		*E-p*-Coumaroyl	
	1		127.0		124.9
	2	6.55, *d* (1.9	115.1	6.97, *d* (8.6)	130.1

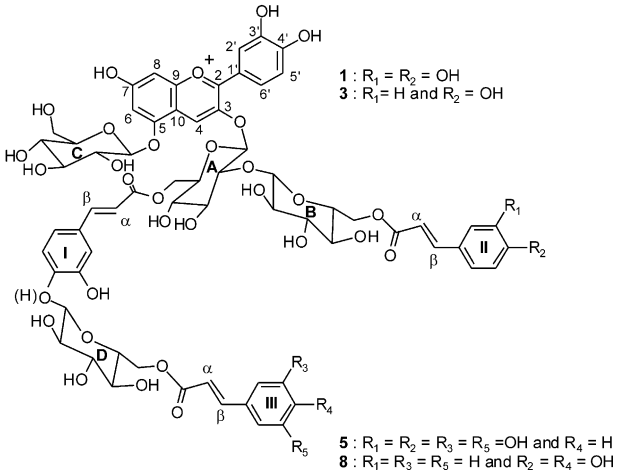
(continued on next page)

Table 2 (*continued*)

Assignment[a]		5		8	
		δ_H (ppm) *J* (Hz) [b]	δ_C (ppm)[b]	δ_H (ppm) J (Hz)[b]	δ_C (ppm)[c]
	3		148.0	6.55, *d* (8.6)	115.8
	4		149.1		159.7
	5	6.60, *d* (8.2	116.3	6.55, *d* (8.6)	115.8
	6	6.44, *dd* (8.2, 1.9)	121.0	6.97, *d* (8.6)	130.1
	α	5.63, *d* (16.0	114.2	5.70, *d* (15.7)	113.7
	β	6.93, *d* (16.0	146.0	7.03, *d* (15.7)	144.5
	C=O		168.5		166.2
Acyl III		*E*-3.5 dihydroxycinnamoyl		Coumaroyl	
	1		127.6		125.0
	2	6.51, *s*	122.8	7.10, *d* (8.6)	130.1
	3		146.6	6.65, *d* (8.6)	115.8
	4	6.81, *s*	139.0		159.8
	5		146.6	6.65, *d* (8.6)	115.8
	6	6.51, *s*	122.8	7.10, *d* (8.6)	130.1
	α	6.16, *d* (16.0	115.1	6.23, *d* (15.7)	115.5
	β	7.39, *d* (16.0	146.8	7.48, *d* (15.7)	144.8
	C=O		168.9		166.5

[a] Assignments based upon COSY, HMBC and TOCSY 1D experiments. Coupling constants (in Hz) are given in parentheses.
[b] In [CD_3OD-TFA-d_1 (5:1)].
[c] [DMSO-d_6-TFA-d_1 (5:1)].

substitution. Finally, anthocyanin **8** was identified as cyanidin 3-*O*-[2-*O*-(6-*O*-*E*-coumaroyl-β-D-glucopyranosyl)]-{6-*O*-[4-*O*-(6-*O*-*E*-coumaroyl-β-D-glucopyranosyl)-*E*-caffeoyl]-β-D-glucopyranosyl}-5-*O*- β-D-glucopyranoside.



3. Experimental

3.1. General procedures

Similar procedures to those previously published (Pale et al., 1997) were used.

3.2. Isolation of anthocyanins

The flowers of *Ipomoea asarifolia* [Desv.] Roem. and Schult. (Convolvulaceae) (Nacro and Millogo-Rasolodimbi, 1993) were freshly harvested in October 2001 at the station of the University of Ouagadougou where a voucher specimen has been deposited. The material was immediately freeze-dried, grounded and stored in the dark and kept in a dry place. Freeze-dried sample (100 g) was extracted at 5 °C by maceration successively by 1000 ml and then twice by 200 ml of a mixture of TFA–MeOH (1:99). The filtrates were pooled and concentrated under vacuum to dryness at 30 °C. 50 ml of (TFA–H_2O, 0.5:99)/MeOH (7:3 ml) was added. The solution was filtered and concentrated to 25 ml. The mixture was transferred to an Amberlite XAD-7 column that was first washed with TFA–H_2O (0.5:99). Anthocyanins were eluted with (TFA–H_2O, 0.5:99)/MeOH (3:7). The resulting solution was concentrated and fractionated by Sephadex LH-20 CC using [(TFA–H_2O, 0.5:99)/MeOH (7:3)]. Each fraction was purified twice by a LiChroPrep RP-18 column (40-60 μm, Merck) using [TFA–H_2O, 0.5:99)/MeOH (6:4)] at a flow rate of 3 ml min^{-1}. Fractions (approximately 10 ml) were collected and controlled by TLC on cellulose with BAW as solvent (Table 1). The fractions corresponding to **5** and **8** were concentrated and their purification was achieved by preparative TLC using EtOAc–AcOH–HCO_2H–H_2O as mobile phase. The isolated bands were eluted with TFA–MeOH (0.5:99), concentrated and finally

purified by chromatography again on a RP-18 silica gel column using (TFA–H_2O, 0.5:99)/MeOH (6:4). The eluates were concentrated and freeze-dried to yield 30 mg of **5** and 10 mg of **8** as TFA salts.

Hydrolysis procedures and identification of the acyl and the sugar moieties

Anthocyanins **5** (20 mg) and **8** (4 mg) were hydrolyzed in 0.5 ml of 1 N NaOH under nitrogen for 30 min at room temperature (Terahara et al., 1990). 1 N TFA (0.5 ml) was immediately added to the mixture and the acyl moieties were extracted twice with 1 ml of ethyl acetate. The organic phases were pooled, dried on Na_2SO_4, filtered and evaporated avoiding the exposure to light and oxygen. Preparative TLC on silica gel F_{254} using toluene–EtOAc–HCO_2H (40:10:5) as mobile phase was carried out to purify the organic acids. Three compounds were isolated and finally identified by analytical TLC in comparison with standards. Two of them were found to be caffeic acid (R_f = 0.40) and *p*-coumaric acid (R_f = 0.50). The third one (R_f = 0.44) was further unambigously identified by ^{1}H NMR and MS as *E*-3,5-dihydroxycinnamic acid.

Acidic hydrolysis was performed by dissolving 2 mg of each compound in 4 ml of 2 N TFA in a sealed vial and heating at 110 °C for 45 min. Cyanidin was extracted twice by 0.5 ml of 3-methylbutan-2-ol. The resulting aqueous fraction was evaporated to dryness. The residue was silylated in 0.5 ml pyridine/*N*-(trimethylsilyl) imidazole (2:1) and heated at 60 °C for 15 min. The TMS derivative of the sugar moietie was identified by GC on a CP-Sil-8-CB Chromapack column (25 m×0.32 mm I.D.) in comparison with authentic sugar devivatives.

3.3. *E-3,5-dihydroxycinnamic acid*

Chromatographic data: TLC on silicagel F_{254} with Toluene–EtOAc–HCO_2H (40:10:5) afforded at R_f = 0.44 a purple fluorescent spot under UV at 366 nm. UV $\lambda_{max,\ nm}$ 295, 312. Mass and NMR data: see reference (Heindl et al, 2002).

3.4. *Cyanidin 3-O-[2-O-(6-O-E-caffeoyl-β-D-glucopyranosyl)]-{6-O-[4-O-(6-O-E-3,5-dihydroxycinnamoyl-β-D-glucopyranosyl)-E-caffeoyl]- β-D-glucopyranosyl}-5-O-[β-D-glucopyranoside],* ***5***

Chromatographic and UV-vis data: see Table 1. HRESI-MS *m*/*z* 1421.369 (M^+, $C_{66}H_{69}O_{35}$ calc. 1421.362). ^{1}H and ^{13}C NMR data: see Table 2.

3.5. *Cyanidin 3-O-[2-O-(6-O-E-coumaroyl-β-D-glucopyranosyl)]-{6-O-[4-O-(6-O-E-coumaroyl-β-D-glucopyranosyl)-E-caffeoyl]-β-D-glucopyranosyl}-5-O-[β-D-glucopyranoside],* ***8***

Chromatographic and UV-vis data: see Table 1. HRESI-MS *m*/*z* 1389.370 (M^+, $C_{66}H_{69}O_{33}$ calc. 1389.372). ^{1}H and ^{13}C NMR data: see Table 2.

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