JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

New Anthocyanidin and Anthocyanin Pigments from Blue Plumbago

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ABSTRACT: Phytochemical investigations of blue plumbago (*Plumbago auriculata* Poir. syn. *Plumbago capensis* Thunb.) flowers have led to the isolation of six new anthocyanins based on three new anthocyanidins with 5,7-dimethoxylated A-rings. Their structures were identified by 2D nuclear magnetic resonance and high-resolution mass spectrometry as the 3-*O*-*β*-galactopyranosides (**1**,**2**,**4**) and 3-*O*-*α*-rhamnopyranosides (**3**,**5**,**6**) of 5,7-dimethyldelphinidin, 5,7-dimethylpetunidin, and 5,7-dimethylmalvidin. Identification of **1**−**6** implies new structures for the previously reported anthocyanidins pulchellidin, europinidin, and capensinidin to be 5,7-dimethoxy-3,3′,4′,5′-tetrahydroxyflavylium, 5,7,3′-trimethoxy-3,4′,5′-trihydroxyflavylium, and 5,7,3′,5′-tetramethoxy-3,4′-dihydroxyflavylium cations, respectively. The anthocyanins (0.4 mg/g flowers) were accompanied by the dihydroflavonol taxifolin 3′-*O*-*β*-glucopyranoside (1.4 mg/g) and the flavonols 5-methylquercetin 3-*O*-*α*-rhamnopyranoside (8.8 mg/g) and 5-methylquercetin (0.4 mg/g). The anthocyanins **1**−**6** are the first reported natural anthocyanins with no free hydroxyl groups in their 5- and 7-positions on their A-rings. They have thus no possibility of forming the tautomeric quinonoidal bases (anhydrobases), which are related to the free hydroxyl groups in the 5- and 7-positions of previously reported anthocyanins. The genes behind the 5,7-dimethoxylated anthocyanins might be useful for making anthocyanins with special properties (colors, etc.).

KEYWORDS: *Plumbago auriculata syn. P. capensis, 5,7-dimethoxylated anthocyanidin A-rings, capensinidin, europinidin and pulchellidin*

INTRODUCTION

Plumbago auriculata Poir. (syn. *Plumbago capensis* Thunb.) is native to South Africa, and it is a popular ornamental plant in subtropical gardens across the world. Most of the 650 reported anthocyanins (>90%) found in plants are based on only 6 anthocyanidins.[1,2] In addition, three 6-hydroxyanthocyanidins, nine 3-deoxyanthocyanidins, some pyranoanthocyanidins, two riccionidins, three sphagnorubins, and seven anthocyanidins with monomethoxylation on their A-rings have previously been reported.[1] Anthocyanins are outstanding in the way each anthocyanidin may be involved in a series of equilibria giving rise to different forms (secondary structures), which exhibit their own properties including color expression and stability. All of the previously reported natural anthocyanins with anthocyanidins with C15 skeletons have one or two free hydroxyl groups in the 5- and/or 7-position on their A-rings, which is a necessity for forming the tautomeric quinonoidal bases (anhydrobases) involving these positions.[3]

The major aim of this paper is to present the isolation and structure elucidation of six anthocyanins and three flavonoids from the sky blue corollas of blue plumbago. The anthocyanins are based on three new anthocyanidins, which are the first reported anthocyanidins with the normal C15 skeleton, which have no free hydroxyl groups on their A-rings. New structures are suggested for the previously reported anthocyanidins capensinidin, europinidin, and pulchellidin.[4−6]

MATERIALS AND METHODS

Plant Material. The sky blue flowers of blue plumbago (leadwort or Cape plumbago) were collected from Kampala City council gardens on Bombo Road (Uganda) in March 2009. The identification of the plant was done at the Herbarium of the Botany Department of Makerere University, and a voucher specimen (voucher RB 23/2009) was deposited in the same place.

Extraction and Isolation. Flowers (1.1 kg) were extracted for 7 h in 2.5 L of methanol containing trifluoroacetic acid (TFA) (1%, v/v). The combined filtered extract was concentrated under reduced pressure at 27 °C, purified by partition (several times) against ethyl acetate, and applied to an Amberlite XAD-7 column. The flavonoids adsorbed to the column were washed with water and eluted from the column with methanol containing 0.5% TFA. For isolation of anthocyanins (**1**−**6**), 80% of the concentrated eluate was subjected to a Sephadex LH-20 column (80 × 2.6 cm) using H_2O/MeOH/TFA (79.5:20:0.5) as eluent. For isolation of flavonoids **7**−**9**, 20% of the concentrated eluate was subjected to a Toyopearl HW-40F column (79 × 2.6 cm) using H_2O/MeOH/TFA (89.5:10:0.5) as eluent. Eluted fractions were checked for homogeneity and purity by analytical HPLC, and compounds were further purified by preparative HPLC using a Gilson 321 pump equipped with an Ultimate 3000 variable-wavelength detector, a 25 × 2.2 cm (10 *μ*m) Econosphere C18 column (Grace), and the solvents A, water (0.5% TFA), and B, acetonitrile (0.5% TFA). The elution profile for preparative HPLC consisted of initial conditions with 90% A and 10% B followed by linear gradient elution for the next 10 min to 14% B, isocratic elution (10−14 min), and the subsequent linear gradient conditions; 14−18 min (to 16% B), 18−22 min (to 18% B), 22−26 min (to 23% B), 26−31 min (to 28% B), and 31−32 min (to 40% B), isocratic elution 32−40 min (40% B), and final linear gradient elution 43−46 min (to 10%

Received: May 3, 2011
Revised: January 10, 2012
Accepted: January 19, 2012
Published: January 19, 2012

dx.doi.org/10.1021/jf2048004 | J. Agric. Food Chem. 2012, 60, 1510−1515

Table 1. Quantitative Amounts, Online HPLC, and High-Resolution Electrospray Ionization Mass Spectral Data Recorded for Anthocyanins 1−6[a] Isolated from Blue Plumbago

	quantitative amount (μg/g fresh wt flower)	t_R (min)	vis_{max} (nm)	UV_{max} (nm)	$A_{440}/A_{vis\text{-}max}$ (%)	$[F]^+$ obsd (m/z)	$[F]^+$ calcd (m/z)	$[M]^+$ obsd (m/z)	$[M]^+$ calcd (m/z)	molecular formula
1	10	24.2	524	275	18	331.0844	331.0818	493.1364	493.1346	$C_{23}H_{25}O_{12}^+$
2	6	26.5	526	275	*b*	345.0998	345.0974	507.1508	507.1503	$C_{24}H_{27}O_{12}^+$
3	12	32.1	524	275	18	331.0833	331.0818	477.1392	477.1397	$C_{23}H_{25}O_{11}^+$
4	14	32.8	527	277	16	359.1133	359.1131	521.1655	521.1659	$C_{25}H_{29}O_{12}^+$
5	50	35.5	526	277	17	345.0996	345.0974	491.1556	491.1553	$C_{24}H_{27}O_{11}^+$
6	348	37.0	527	277	16	359.1138	359.1131	505.1704	505.1709	$C_{25}H_{29}O_{11}^+$

[a]See Figure 2 for structures. [b]Value missing, influenced by impurity.

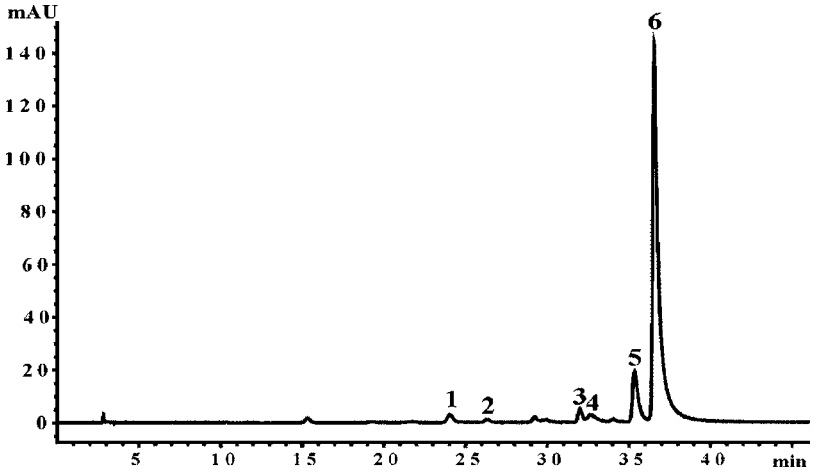

Figure 1. HPLC chromatogram of the six anthocyanins (**1**−**6**) isolated from blue flowers of *Plumbago auriculata* detected at 520 ± 20 nm.

B). The flow rate was 15 mL min^{-1}, and aliquots of 250 μL were injected.

Analytical HPLC. The Agilent 1100 HPLC analytical system was equipped with a HP 1050 diode array detector and a 200 × 4.6 mm i.d., 5 μm, ODS Hypersil column (Agilent). Two solvents, A, water (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 gradient elution for the next 10 min to 14% B, isocratic elution (10−14 min), and the subsequent linear gradient conditions: 14−22 min (to 18% B), 22−26 min (to 23% B), 26−31 min (to 28% B), and 31−32 min (to 40% B), isocratic elution 32−40 min (40% B), and final linear gradient elution 43−46 min (to 10% B). The flow rate was 1.0 mL min^{-1}, and aliquots of 15 μL were injected with an Agilent 1100 series Micro Autosampler. Prior to injection, all samples were filtered through a 0.45 μm Millipore membrane filter. Anthocyanins isolated from gooseberries (*Ribes grossularia*),[7] black beans (*Phaseolus vulgaris*),[8] and fuchsia flowers[9] were used as references. The UV−vis absorption spectra were recorded online during HPLC analysis over the wavelength range 200−600 nm in steps of 2 nm.

LC-MS. High-resolution LC−electrospray mass spectrometry (ESI^+/TOF) spectra were recorded using a JEOL AccuTOF JMS-T100LC in combination with an Agilent Technologies 1200 series HPLC system. A Zorbax SB-C18 (50 mm × 2.1 mm, length × i.d., 1.8 μm) column was used for separation, and combinations of two solvents were used for elution: A, H_2O containing 0.5% TFA (v/v), and B, acetonitrile containing 0.5% TFA (v/v). The following solvent composition was used: 0−1.25 min, 10−22% B (linear gradient); 1.25−5 min, 22−30% B (linear gradient); 5−7 min, 30% B (isocratic); 7−8 min, 30−40% B (linear gradient); 8−14 min, 40% B (isocratic); and 14−15 min, 40−10% B (linear gradient). The flow rate was 0.4 mL min^{-1}.

NMR Spectroscopy. The NMR experiments (at 298 K) were obtained at 600.13 and 150.92 MHz for ^1H and ^{13}C, respectively, on a Bruker Biospin Ultrashield Plus AV-600 MHz instrument equipped with a TC1 $^1H-^{13}C/^{15}N$ cryoprobe head performing 1D ^1H NMR 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), total correlation spectroscopy ($^1H-^1H$ TOCSY), and compensated attached proton test (CAPT) experiments. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent, CD_3OD/CF_3COOD (95:5; v/v), were used as secondary references (δ 3.40 and δ 49.0 from TMS for ^1H and ^{13}C, respectively).

Quantitative Determination. A sample of blue plumbago flowers (12.38 g), collected in Les Issambres (France, July 2011), was extracted (7 h × 2) in methanol containing 0.5% TFA (v/v) in a refrigerator. The quantitative amounts of individual anthocyanins (**1**−**6**) (Figure 2) were determined from a HPLC calibration curve based on isolated **6** (purity > 98%, determined by HPLC-DAD, detected at 520 ± 20 nm) (Table 1), whereas the quantitative amounts of individual flavonoids (**7**−**9**) (Figure 2) were determined from a HPLC calibration curve based on isolated **8**. Each concentration in the two different calibration curves was based on average data from three parallel injections.

Determination of Molar Absorptivity (ε) Value. The molar absorptivity value of 5,7-dimethylmalvidin 3-*O*-α-rhamnopyranoside (**6**), ε (L cm^{-1} mol^{-1}), was calculated according to Lambert−Beer's law using the molecular mass including the mass of the counterion (CF_3COO^-, 113.16 g/mol).

Acid Hydrolysis. 5,7-Dimethylmalvidin 3-*O*-α-rhamnopyranoside (**6**; 2.2 mg) was taken into an ampule with 1 mL of superdistilled water and 1 mL of 6 M concentrated HCl, which was sealed and subjected to hydrolysis at 90 °C for 30 min. The reaction was monitored by TLC. TLC was carried out on microcrystalline cellulose (F5556, Merck) with the solvent FHW (HCO_2H/concentrated HCl/H_2O; 51.4:7.2:41.4, v/v).

dx.doi.org/10.1021/jf2048004 | J. Agric. Food Chem. 2012, 60, 1510−1515

RESULTS AND DISCUSSION

Identification of Anthocyanins 1−6. HPLC analysis (520 nm) of the crude extracts of blue plumbago flowers showed one major (**6**) and several minor peaks (Figure 1). The anthocyanins were isolated by various chromatographic techniques, and their structures (Figure 2) were elucidated

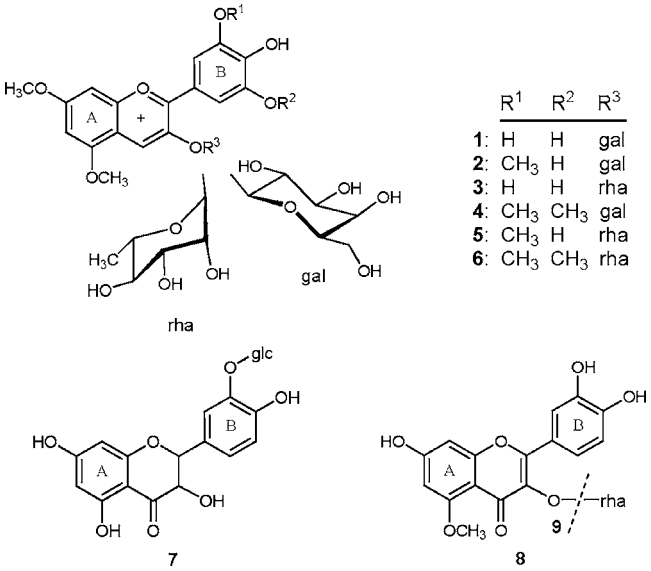

Figure 2. Structures of anthocyanins **1**−**6**, flavanonol **7**, and flavonols **8** and **9** isolated from blue plumbago. rha, α-rhamnopyranosyl; gal, β-galactopyranosyl; glc, β-glucopyranosyl.

mainly by high-resolution LC-MS (Table 1) and NMR (Tables 2 and 3) data. The absolute quantitative amount of **6**, which constituted 70% of the anthocyanins in the flowers, was 348 μg/g fresh weight. The amounts of the individual anthocyanins, **1**−**6**, are given in Table 1.

The aromatic region of the 1D ^{1}H NMR spectrum of **6** showed a 1H singlet at δ 9.12 (H-4), a 2H singlet at δ 7.99 (H-2′/6′), and an AX system at δ 7.51 (d, 1.9 Hz; H-8) and δ 6.99 (d, 1.9 Hz; H-6), revealing an anthocyanidin having a symmetrically substituted B-ring. The chemical shifts of the corresponding carbons of the aglycone of **6** (Table 3) were assigned from the HSQC experiment, whereas the remaining quaternary C-atoms were assigned from the HMBC spectrum. The 6H singlet at δ 4.12 (2 × OCH_3) in the 1D ^{1}H NMR spectrum and the crosspeak at δ 4.12/149.6 (OCH_3/C-3′/5′) in the HMBC spectrum (Figure 3) showed that the aglycone was homologous to that of malvidin. However, two additional singlets at δ 4.21 (3H) and δ 4.19 (3H), respectively, were also revealed. These two latter signals might be mistaken as a

Table 3. ^{13}C NMR Spectral Data for Anthocyanins 1−6 Isolated from Blue Plumbago Recorded in CF_3COOD/CD_3OD (5:95, v/v) at 25 °C (δ in Parts per Million)

	1	2	3	4	5	6
2	165.2	165.3	165.7	164.9	165.1	164.6
3	147.0	146.9	146.2	146.9	146.0	146.4
4	134.9	133.5	133.4	135.2	133.5	133.5
5	158.5	158.3	158.7	158.8	158.3	158.5
5 (OCH_3)	57.7	57.8	57.8	57.9	57.8	57.9
6	100.9	101.1	101.1	101.2	101.3	100.9
7	170.3	169.8	170.2	170.4	170.1	170.4
7 (OCH_3)	57.7	57.7	57.7	57.7	57.7	57.7
8	93.4	93.5	93.4	93.7	93.5	93.6
9	157.3	157.8	157.4	157.6	157.9	157.4
10	113.3	113.2	113.7	113.8	113.7	113.9
1′	119.4	119.5	120.8	119.2	119.8	119.5
2′	113.1	108.7	112.6	111.2	108.7	110.5
3′	145.6	149.7	145.7	149.5	149.8	149.6
3′ (OCH_3)		57.3		57.3	57.3	57.3
4′	147.3	146.5	147.8	146.9	146.4	147.2
5′	145.6	147.4	145.7	149.5	147.3	149.6
5′ (OCH_3)				57.3		57.3
6′	113.1	114.3	112.6	111.2	114.5	110.5
1″	104.6	104.6	102.5	104.5	102.6	102.7
2″	71.9	72.2	71.4	72.2	71.5	71.6
3″	74.9	74.8	72.2	75.1	72.4	72.3
4″	70.2	70.8	73.1	70.2	73.1	73.2
5″	78.1	78.2	72.4	78.2	72.4	72.4
6″	62.5	62.6	17.9	62.6	18.1	17.4

Table 2. ^{1}H NMR Spectral Data for Anthocyanins 1−6 Isolated from Blue Plumbago Recorded in CF_3COOD/CD_3OD (5:95, v/v) at 25 °C (δ in Parts per Million, J in Hertz)[a]

	1	2	3	4	5	6
4	9.09 (d, 0.8)	9.09 (s)	9.03 (d, 0.8)	9.18 (d, 0.6)	9.08 (s)	9.12 (s)
5 (OCH_3)	4.18 (s, 3H)	4.182 (s, 3H)	4.20 (s, 3H)	4.19 (s, 3H)	4.20 (s, 3H)	4.21 (s, 3H)
6	6.99 (d, 2.2)	6.95 (d, 2.1)	6.99 (d, 2.1)	6.98 (d, 1.9)	6.98 (d, 1.9)	6.99 (d, 1.9)
7 (OCH_3)	4.18 (s, 3H)	4.179 (s, 3H)	4.18 (s, 3H)	4.20 (s, 3H)	4.18 (s, 3H)	4.19 (s, 3H)
8	7.33 (dd, 0.8, 2.2)	7.49 (d, 2.1)	7.33 (dd, 0.8, 2.1)	7.52 (d, 1.9)	7.42 (d, 1.9)	7.51 (d, 1.9)
2′	7.96 (s)	7.72 (d, 2.1)	7.75 (s)	8.22 (s)	7.86 (d, 2.2)	7.99 (s)
3′ (OCH_3)		4.11 (s, 3H)		4.13 (s, 3H)	4.11 (s, 3H)	4.12 (s, 3H)
5′ (OCH_3)				4.13 (s, 3H)		4.12 (s, 3H)
6′	7.96 (s)	7.74 (d, 2.1)	7.75 (s)	8.22 (s)	7.89 (d, 2.2)	7.99 (s)
1″	5.39 (d, 7.7)	5.39 (d, 7.80	5.91 (d, 1.7)	5.43 (d, 7.7)	5.94 (d, 1.7)	5.96 (d, 1.8)
2″	4.13 (dd, 7.7, 9.6)	4.13[B]	4.36 (dd, 1.7, 3.6)	4.10 (dd, 7.7, 9.6)	4.36 (dd, 1.8, 3.3)	4.36 (dd, 1.8, 3.6)
3″	3.78 (dd, 3.4, 9.6)	3.79 (dd, 3.4, 9.7)	4.02 (dd, 3.6, 9.1)	3.77 (dd, 3.4, 9.6)	4.00 (dd, 3.4, 9.1)	4.01 (dd, 3.6, 8.9)
4″	4.05 (dd, 0.6, 3.4)	4.06[B,S]	3.67 (dd, 9.1, 9.5)	4.02 (dd, 0.6, 3.4)	3.64 (dd, 9.0, 9.5)	3.67 (dd, 9.0, 9.4)
5″	3.90 (dd, 0.6, 7.1)	3.91 (dd, 0.6, 6.7)	3.70 (dd, 5.9, 9.5)	3.90 (m)	3.67 (dd, 5.8, 9.5)	3.70 (dd, 5.9, 9.4)
6A″	3.91 (m)	3.92 (m)	1.36 (d, 5.9)	3.91 (m)	1.36 (d, 5.8)	1.37 (d, 5.8)
6B″	3.85 (dd, 2.3, 7.1)	3.83 (dd, 2.8, 6.2)		3.83 (dd, 2.7, 9.7)		

[a]s, singlet; d, doublet; dd, double doublet; m, multiplet. [B], overlapped by another signal. [S], chemical shift value from HSQC spectrum.

dx.doi.org/10.1021/jf2048004 | J. Agric. Food Chem. 2012, 60, 1510−1515

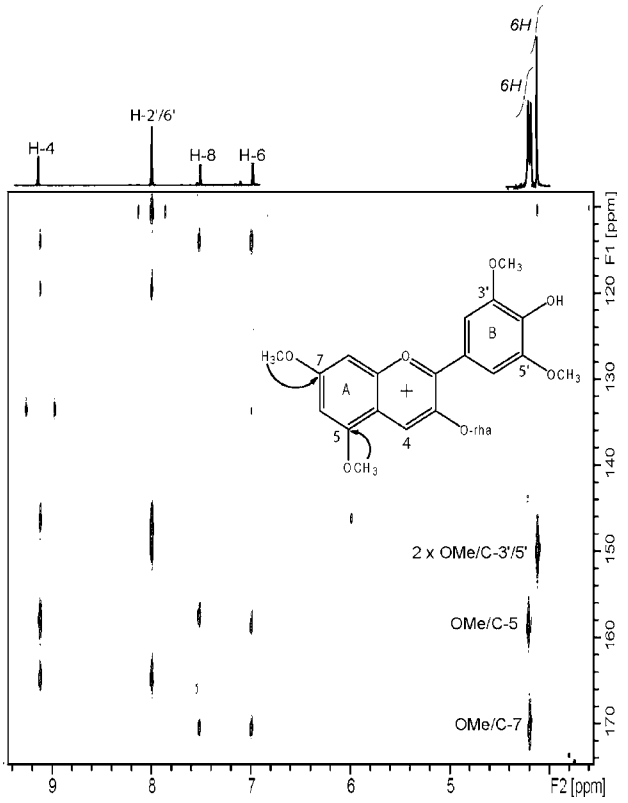

Figure 3. Expanded region of the HMBC spectrum (600.13 MHz) of 5,7-dimethylmalvidin 3-*O*-α-rhamnopyranoside (**6**) highlighting the linkages of the different OCH_3 groups to the aglycone.

doublet, but the HSQC spectrum revealed individual ^{13}C values at δ 4.21/57.9 and 4.19/57.7. The HMBC spectrum showed that these two methoxyl units were linked to the A-ring of the anthocyanidin through the crosspeaks at δ 4.21/158.5 (OCH_3/C-5) and δ 4.19/170.4 (OCH_3/C-7), respectively (Figure 3). A fragment ion $[F]^+$ at m/z 359.1138 in the HR-ESI-MS spectrum of **6** was in accordance with a 5,7,3′,5′-tetramethoxy-3,4′-dihydroxyflavylium cation (calcd 359.1131, $C_{19}H_{19}O_7^+$) (Table 1). The anomeric coupling constant (1.8 Hz), the chemical shifts of the six ^{13}C resonances in the sugar region of the 2D $^1H-^{13}C$ HSQC spectrum, and the crosspeaks in the $^1H-^1H$ DQF-COSY and $^1H-^1H$ TOCSY spectra of **6** were in agreement with a rhamnopyranoside (Tables 2 and 3).[10] The anomeric configuration of the sugar was confirmed to be α by the large $^1J_{CH}$ coupling constant in the HMBC spectrum (174.7 Hz), showing that the anomeric proton is in the equatorial position.[11] A crosspeak at δ 5.96/146.4 in the HMBC spectrum between H-1″ and C-3 of the aglycone showed that the rhamnosyl unit was connected to the 3-position of the aglycone. The HR-ESMS spectrum of **6** exhibited a $[M]^+$ ion of m/z 505.1704, corresponding to the molecular formula $C_{25}H_{29}O_{11}^+$ (calcd 505.1709), confirming the identity of pigment **6** to be 5,7-dimethylmalvidin 3-*O*-α-rhamnopyranoside (Figure 2).

The NMR data of **5** revealed the same sugar moiety (α-rhamnopyranosyl) and 5,7-dimethoxylation of the A-ring as found for **6** (Tables 2 and 3), but the aromatic region of the ^1H NMR spectrum showed a 1H singlet at δ 9.08 (s; H-4), two 1H resonances at δ 7.86 (d, 2.2 Hz; H-2′) and δ 7.89 (d 2.2 Hz; H-6′), and an AX system at δ 7.42 (d, 1.9 Hz; H-8) and δ 6.98 (d, 1.9 Hz; H-6), revealing an anthocyanidin having an asymmetrically substituted B-ring. A third methoxyl group located at C-3′ by the HMBC NMR crosspeak at δ 4.11/149.8 (OCH_3/C-3′) was in accordance with a 5,7,3′-trimethoxy-3,4′,5′-trihydroxyflavylium cation ($[F]^+$ obsd at m/z 345.0996, $C_{18}H_{17}O_7^+$). The HR-ESI-MS spectrum of **5** showed a $[M]^+$ ion at m/z 491.1556 (Table 1), confirming the identity to be 5,7-dimethylpetunidin 3-*O*-α-rhamnopyranoside (Figure 2).

The aromatic region of the 1D ^1H NMR spectrum of **3** contained a 1H doublet at δ 9.03 (d 0.8 Hz), a 2H singlet at δ 7.75 (H-2′/6′), and an AX system at δ 7.33 (dd, 0.8, 2.1 Hz; H-8) and δ 6.99 (d, 2.1 Hz; H-6), revealing an anthocyanidin having a symmetrically substituted B-ring homologous to delphinidin. In a similar manner as for **5** and **6**, two methoxyl signals were found to be located at the A-ring of **3** by the HMBC NMR crosspeaks at δ 4.20/158.7 (OCH_3/C-5) and δ 4.18/170.2 (OCH_3/C-7). A fragment ion $[F]^+$ at m/z 331.0833 (Table 1) in the HR-ESMS spectrum of **3** was in accordance with a 5,7-dimethoxy-3,3′,4′,5′-tetrahydroxyflavylium cation. The sugar moiety connected to the 3-position was identified to be α-rhamnopyranosyl (Tables 2 and 3), and the identity of **3** was confirmed by the $[M]^+$ ion at m/z 477.1392 (Table 1) in the HR-ESI-MS spectrum to be 5,7-dimethyldelphinidin 3-*O*-α-rhamnopyranoside.

The NMR resonances of pigments **4**, **2**, and **1** were similar to those of the aglycones of **6**, **5**, and **3**, respectively (Tables 2 and 3). However, the chemical shifts, coupling constants, and crosspeaks of the sugar moieties were in accordance with a β-galactopyranosyl linked to the 3-position of each anthocyanidin.[10,11] The molecular ions $[M]^+$ observed in the respective HR-ESI-MS spectra (Table 1) confirmed their identities to be 5,7-dimethylmalvidin 3-*O*-β-galactopyranoside (**4**), 5,7-dimethylpetunidin 3-*O*-β-galactopyranoside (**2**), and 5,7-dimethyldelphinidin 3-*O*-β-galactopyranoside (**1**), respectively.

All of the anthocyanins **1**−**6** have the same A-ring with 5,7-dimethoxylation. These are the first anthocyanins with C15 anthocyanidin skeletons without any free hydroxyl groups on their A-rings. For diagnostic purposes in the identification of this type of anthocyanidin A-ring we want to highlight three points: (1) The ratio of the absorption at 440 nm to that at the visible absorption maximum of 5,7-dimethylmalvidin 3-galactoside (**4**) and 5,7-dimethylmalvidin 3-rhamnoside (**6**) is 16%. For malvidin 3,5-diglucoside and malvidin 3-glucoside the corresponding values are 14 and 23%, respectively. The presence of an 5-*O*-methyl substituent thus seems to have the same lowering effect on the 440 nm/visible absorption maximum as an 5-*O*-glycosyl substituent. Analogous spectral values are reported for **1**−**3**, and **5** (Table 1). (2) The chemical carbon shifts of the A-ring methoxyl groups of **1**−**6** are observed at 57.7−57.9 ppm (Table 3). Similar values for the carbons of the B-ring methoxyl groups are observed at 57.3 ppm. An A-ring methoxyl carbon may thus be identified by a downfield chemical shift effect of ca. 0.5 ppm compared to similar values for B-ring methoxyl carbons (Figure 2). (3) The chemical proton shifts of the A-ring methoxyl groups of **1**−**6** are observed at 4.18−4.21 ppm (Table 2). Similar values observed for the protons of the methoxyl groups of the B-rings are at 4.11−4.13 ppm. Methoxyl protons located at the A-ring may thus be identified by a small downfield chemical shift effect compared to similar values for B-ring methoxyl protons (Figure 2). The molar absorptivity value of 5,7-dimethylmalvidin 3-*O*-α-rhamnopyranoside (**6**), ε, was found to be 18400 L cm^{-1} mol^{-1} in TFA/MeOH (0.5:99.5, v/v). For comparison, the ε value for malvidin 3-glucoside in concentrated HCl/MeOH

dx.doi.org/10.1021/jf2048004 | J. Agric. Food Chem. 2012, 60, 1510−1515

Table 4. ^{1}H and ^{13}C NMR Data for Flavanonol 7 and Flavonols 8 and 9 Isolated from Blue Plumbago Recorded in CF_3COOD/CD_3OD (5:95, v/v) at 25 °C[a]

	^{1}H δ, J (Hz)			^{13}C δ		
	7	8	9	7	8	9
2	5.07 (d, 11.7)			84.9	156.7	144.9
3	4.66 (d, 11.7)			73.5	138.2	138.5
4				198.3	175.4	173.7
5				164.5	162.7	162.2
5 (OCH_3)		3.99 (s)	4.01 (s)		56.4	55.1
6	5.98 (d, 2.0)	6.50 (d, 1.6)	6.46 (d, 1.5)	96.3	97.1	96.8
7				168.7	164.6	164.7
8	6.02 (d, 1.9)	6.56 (d, 1.6)	6.60 (d, 1.5)	97.3	96.0	95.8
9				165.4	160.3	160.2
10				101.8	108.7	106.3
1′				129.9	123.0	124.2
2′	7.47 (d, 1.8)	7.40 (d, 2.0)	7.81 (d, 1.8)	118.2	116.9	115.6
3′				146.5	146.1	146.2
4′				148.8	149.3	148.3
5′	6.99 (d, 8.2)	6.99 (d, 8.3)	6.97 (d, 8.5)	116.8	116.3	116.3
6′	7.19 (dd 1.7, 8.3)	7.37 (dd 2.1, 8.3)	7.71 (dd 1.8, 8.5)	124.6	122.6	121.2
1″	4.92 (dd 7.7)	5.39 (d, 1.3)		104.0	103.3	
2″	3.61 (dd 7.7, 9.2)	4.35 (dd 1.3, 3.2)		74.9	72.0	
3″	3.56 (t 9.3)	3.84 (dd 3.3, 8.8)		77.6	72.2	
4″	3.47 (t 9.1)	3.41[C] (m)		71.5	73.4	
5″	3.53 (m)	3.43[C] (m)		78.3	72.0	
6(A)″	3.98 (dd 2.0, 12.0)	0.99 (d, 5.8)		62.6	17.6	
6(B)″	3.76 (dd 6.0, 12.0)					

[a][C]Chemical shift value attributed from COSY spectrum. s, singlet; d, doublet; dd, double doublet; m, multiplet.

(0.01:99.99, v/v) has been found to be 25100 L cm^{-1} mol^{-1}.[12] Pigment **6** was conducted to acid-mediated hydrolysis, which gave 5,7-dimethylmalvidin with a 9 nm bathochromic shift of its visible λ_{max} compared to the visible λ_{max} of the unhydrolyzed compound.

Revision of the Structures of Capensinidin, Pulchellidin, and Europinidin. The anthocyanidin capensinidin has previously been reported to occur in sky blue corollas of *P. capensis*, where it occurred as the 3-rhamnoside.[4] Its structure was tentatively identified to be the 5,3′,5′-trimethoxy-3,7,4′-trihydroxyflavylium cation on the basis of the fact that on controlled demethylation it yielded malvidin, petunidin, and delphinidin. The presence of a 5-*O*-methyl group in the aglycone was indicated by its vivid fluorescence in UV light and its low 440 nm/visible maximum ratio in the absorption spectrum. No experimental data supporting the nature of the 5-*O*-substituent was presented. On the basis of similar experimental data as presented above, the anthocyanidin pulchellidin, isolated from petals of *P. pulchella*, has been suggested to be 5-methoxy-3,7,3′,4′,5′-pentahydroxyflavylium,[5,6] whereas europinidin, isolated from petals of *P. europea*, has been suggested to be 5,3′-dimethoxy-3,7,4′,5′-tetrahydroxyflavylium or a 5-deoxyanthocyanidin having the 3,7,8,3′,4′,5′-hexahydroxyflavylium skeleton.[5,6] On the basis of the identification of the anthocyanidins of **1**−**6**, the present paper suggests revision of the structures of capensinidin, pulchellidin, and europinidin to be 5,7,3′,5′-tetramethoxy-3,4′-dihydroxyflavylium, 5,7-dimethoxy-3,3′,4′,5′-tetrahydroxyflavylium, and 5,7,3′-trimethoxy-3,4′,5′-trihydroxyflavylium cations, respectively.

Identification of Flavonoids 7−**9.** HPLC analysis of the crude extracts of plumbago flowers revealed, in addition to the anthocyanins detected at 520 nm, three other flavonoids (**7**−**9**) detected at 280 nm. Compounds **7**−**9** were individually isolated by various chromatographic techniques, and their structures were elucidated by NMR data (Table 4).

The aromatic region of the 1D ^{1}H NMR spectrum of **7** showed five protons indicating a tetrasubstituted aromatic system of the aglycone. An AMX spin system at δ 7.19, 6.99, and 7.47 was attributed to H-6′, H-5′, and H-2′, whereas the two proton AM spin systems at δ 5.07 amd 4.66 were assigned to H-2 and H-3, respectively (Table 4). These two protons showed a vicinal coupling interaction (11.7 Hz) and unsaturation of the C_2-C_3 bond. Together with the identification of the far most shielded carbon signal at 198.3 ppm in the HMBC as a C-4 keto group, interacting with H-2 and H-3, the aglycone of **7** was identified as taxifolin.[13] The ^{1}H and ^{13}C chemical shifts and coupling constants of the resonances in the sugar region of the HSQC, COSY, and TOCSY spectra of **7** were in agreement with a glucopyranosyl.[7] The sugar linkage between C-3′ and H-1″ in the HMBC spectrum confirmed the structure of **7** as the dihydroflavonol taxifolin 3′-*O*-β-glucopyranoside.[14] This compound has previously been reported to occur in a handful of plant species, in particular in the family Pinaceae.

Compounds **8** and **9** showed similar 1D ^{1}H NMR spectra including two spin system in the aromatic region, AMX (H-6′, H-5′, H-2′) and AX (H-8, H-6) (Table 4), for both compounds. The assignments of the ^{1}H and ^{13}C data obtained by the HSQC and HMBC spectra were in accordance with 5-methylquercetin (**9**). The 1D ^{1}H NMR spectrum of compound **8** showed an anomeric proton signal with a coupling constant of 1.3 Hz. The chemical shifts of the six ^{13}C resonances in the sugar region of the HSQC spectrum and the crosspeaks in the COSY and TOCSY spectra of **8** were in agreement with an α-rhamnopyranoside (Table 4).[10] The crosspeak at 5.39/138.2

ppm (H-1″/C-3) in the HMBC spectrum confirmed the identity of **8** to be in accordance with 5-methylquercetin 3-*O*-α-rhamnopyranoside. This compound has previously been reported to occur in *Plumbago* and *Rhododendron* species.[4]

Blue Plant Colors. When previously reported anthocyanins are considered, it is noticeable that nearly all blue anthocyanin-colored plants are based on just one anthocyanidin, delphinidin.[2] Among the 28 reported species with blue colors, only *Ophiopogon jaburan* and *Ipomoea tricolor* are exemptions,[15,16] having anthocyanins based on petunidin and peonidin, respectively. The finding of 5,7-dimethylmalvidin 3-*O*-α-rhamnoside (**6**) as the major anthocyanin of the flowers of blue plumbago will add 5,7-dimethylmalvidin to this exemption list. The blue colors of some flowers are explained by metal ion coordination to the 3′,4′-dihydroxyl groups of the B-ring of the anthocyanidins in so-called metalloanthocyanins.[17] However, 5,7-dimethylmalvidin, which is the major anthocyanidin of blue plumbago flowers, is without *o*-dihydroxylation on its anthocyanidin B-ring. In general, the majority of anthocyanins found in blue flowers also contain aromatic acyl group(s), or they are found together with copigments. None of the anthocyanins found in blue plumbago are acylated; however, they were accompanied by relatively high amounts of a dihydroflavonol, taxifolin 3′-*O*-β-glucopyranoside (**7**) (1.4 mg), and two flavonols, 5-methylquercetin 3-*O*-α-rhamnopyranoside (**8**) (8.8 mg) and 5-methylquercetin (**9**) (0.4 mg), per gram flowers.

5,7-Dimethoxylated Anthocyanins. In general, when anthocyanins are dissolved in water, secondary structures are formed according to different acid−base, hydration, and tautomeric reactions.[3] This kind of secondary structure, which is formed under various conditions, has an impact on anthocyanin properties (e.g., colors, stability) and most probably their pharmaceutical assets. The anthocyanins reported in this study, **1**−**6**, have no free hydroxyl groups in their 5- and 7-positions on their A-rings. They have thus no possibility of forming the tautomeric quinonoidal bases (anhydrobases), which are related to the free hydroxyl groups in the 5- and 7-positions of previously reported anthocyanins. The genes behind the 5,7-dimethoxylated anthocyanins of plumbago might be useful in genetic engineering with the purpose of coding for enzymes making anthocyanins with special properties (colors, etc.).

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Funding

This study was part of the EU project called MUTHI (Multidisciplinary University Traditional Health Initiative, FP7-AFRICA 2010, Grant Agreement 266005). The team at Makerere University gratefully acknowledges Carnegie Cooperation of New York for financial support.

ACKNOWLEDGMENTS

We are grateful to George Ogweng (Makerere University) for collection of plant material and to Sectional Engineer Terje Lygre (University of Bergen) for technical support during recording of the LC-MS spectra.

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