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Purple anthocyanin colouration on lower (abaxial) leaf surface of *Hemigraphis colorata* (Acanthaceae)

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ABSTRACT

The functional significance of anthocyanin colouration of lower (abaxial) leaf surfaces is not clear. Two anthocyanins, 5-*O*-methylcyanidin 3-*O*-(3″-(β-glucuronopyranosyl)-β-glucopyranoside) (**1**) and 5-*O*-methylcyanidin 3-*O*-β-glucopyranoside (**2**), were isolated from *Hemigraphis colorata* (Blume) (Acanthaceae) leaves with strong purple abaxial colouration (2.2 and 0.6 mg/g fr. wt., respectively). The glycosyl moiety of **1**, the disaccharide 3″-(β-glucuronopyranosyl)-β-glucopyranoside), has previously been reported to occur only in a triterpenoid saponin, lindernioside A. The structural assignment of the aglycone of **1** and **2** is the first complete characterisation of a natural 7-hydroxy-5-methoxyanthocyanidin. Compared to nearly all naturally occurring anthocyanidins, the 5-*O*-methylation of this anthocyanidin limits the type of possible quinoidal forms of **1** and **2** to be those forms with keto-function in only their 7- and 4′-positions.

1. Introduction

Hemigraphis colorata (Blume) (=*H. alternate* (Burm. f.) in family Acanthaceae is native to the eastern Malesia region. Various extracts of leaves of *H. colorata* has recently been shown to have anti-bacterial activity against some pathogens (Anitha et al., 2012), while leaf paste of *H. colorata* seems to promote wound healing in mice (Subramoniam et al., 2001). The leaves of *H. colorata* are green on the adaxial side and purple on the abaxial (lower) side. Permanent red to purple colouration of abaxial leaf surfaces is most often seen in deeply-shaded understory plants, especially in the tropics. However, the functional significance of abaxial anthocyanin colouration, including its role in photosynthetic adaptation, remains unclear (Hughes et al., 2008).

The major aim of this paper was to present isolation, structure elucidation and quantitative data for the two unusual anthocyanins causing purple colouration of the abaxial leaf surfaces of *H. colorata* and *H. colorata* var. 'Exotica'.

2. Results and discussion

HPLC analysis (detection at 520 ± 20 nm) of fresh leaf extracts of *H. colorata* and *H. colorata* 'Exotica' showed two anthocyanins, **1** and **2**. The quantitative amounts of **1** and **2** in *H. colorata* 'Exotica' leaves were found to be 2.17 and 0.64 mg/g fr. wt., respectively. When the acidified methanolic extract of *H. colorata* 'Exotica' was stored for 16 days at room temperature, a third anthocyanin (**3**) (20.1%) was observed, while the amount of **1** was reduced accordingly.

2.1. Structure elucidation

The UV–visible spectra of **1–3** recorded on-line during HPLC (Fig. 1) were quite similar showing $\lambda_{vis\text{-}max}$ at 518 nm (**1** and **2**) and 520 nm (**3**) in accordance with the same anthocyanidin having two oxygen-containing functional groups on the B-ring (Jordheim et al., 2011). Their $A_{440}/A_{vis\text{-}max}$ values of 23%, were interesting by being neither in accordance with analogous anthocyanidin 3, 5-di-*O*-glycosides (below 20%) nor with anthocyanidin 3-*O*-glycosides (around 31%) (Jordheim et al., 2011). The anthocyanins were isolated by various chromatographic techniques, and their structures (Fig. 2) were elucidated by UV–visible spectroscopy, high-resolution LC–MS (Table 1), and NMR (Table 2) data.

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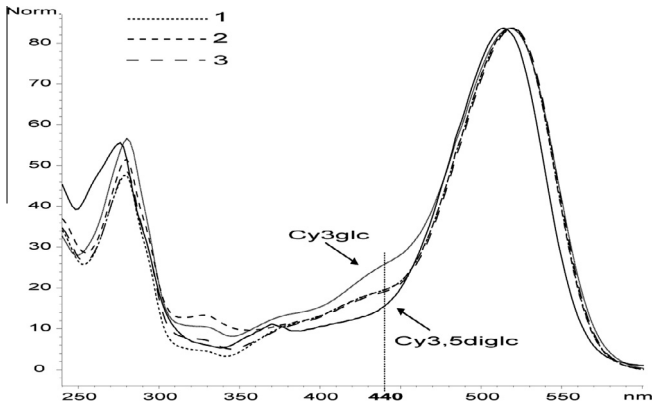

Fig. 1. UV–vis absorption spectra of compounds **1**–**3** on their flavylium cationic forms isolated from leaves of *Hemigraphis colorata* together with cyanidin 3-*O*-glucoside (Cy3glc) and cyanidin 3,5-di-*O*-glucoside (Cy3,5diglc) recorded on-line during HPLC analysis. The $A_{440}/A_{vis\text{-}max}$ ratio has diagnostic value for identification of 5-methoxyanthocyanidins.

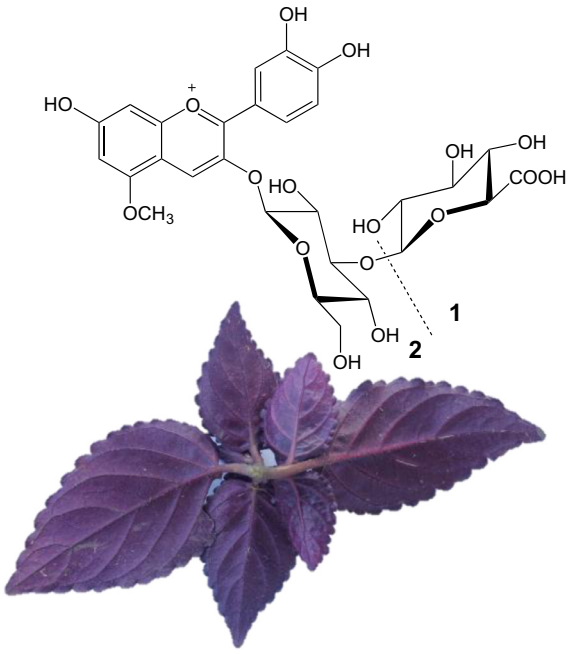

Fig. 2. Structures of 5-*O*-methylcyanidin 3-*O*-(3″-(β-glucuronopyranosyl)-β-glucopyranoside) (**1**) and 5-*O*-methylcyanidin 3-*O*-β-glucopyranoside (**2**) isolated from leaves of *Hemigraphis colorata* with purple abaxial surfaces.

The aromatic region of the 1D ^{1}H NMR spectrum of **2** with a small doublet at δ 9.13 (*d*, 0.7, H4), and a AMX system at δ 8.38 (*dd*, 2.4, 8.7, H6′), δ 8.15 (*d*, 2.4, H2′) and δ 7.12 (*d*, 8.8, H5′) (Table 2) was consistent with a B-ring similar to that of cyanidin occurring on its flavylium cationic form. However, the chemical shifts of H6 and H8 at δ 6.86 (*d*, 1.9) and δ 7.08 (*dd*, 0.8, 1.9), respectively, were shifted slightly downfield compared to analogous values of cyanidin (Andersen et al., 2010). Additionally, the 1D ^{1}H NMR spectrum of **2** contained a singlet integrated as 3H at δ 4.17, in accordance with a methoxy group. This group was found to be located at C-5 of the anthocyanidin by the HMBC NMR crosspeak at δ 4.17/159.8 (OCH_3/C-5) (Fig. 3). A fragment ion $[F]^+$ at *m/z* 301.0659 (Table 1) in the high-resolution ESI$^+$–MS spectrum of **2** was consistent with the molecular formula $C_{16}H_{13}O_6^+$ (calc. 301.0707 Da), in accordance with the anthocyanidin 5-methoxy-3,7,3′,4′-tetrahydroxyflavylium cation. The chemical shifts and coupling constants of the sugar moiety of **2** (Table 2) corresponded to a β-glucopyranosyl. A crosspeak at δ 5.38/146.2 (H1″/C3) in the HMBC NMR spectrum showed that this glucosyl was attached in the aglycone 3-position. The high-resolution ESI$^+$–MS spectrum of **2** exhibited a $[M]^+$ ion of *m/z* 463.1203, corresponding to the molecular formula $C_{22}H_{23}O_{11}^+$ (calc. 463.1235 Da), in agreement with 5-*O*-methylcyanidin 3-*O*-β-glucopyranoside (Fig. 2).

The anthocyanidin part of the NMR spectra of **1** was similar to the corresponding part of **2** (Table 2), in accordance with 5-*O*-methylcyanidin with a glycosyl moiety attached in the 3-position. However, the COSY and TOCSY NMR spectra of **1** showed resonances and coupling constants of 12 protons belonging to two monosaccharide units (Table 2). A crosspeak at δ 5.44/146.2 (H1″/C3) in the HMBC NMR spectrum of **1** showed that one of these monosaccharides, a β-glucopyranosyl, was attached in the aglycone 3-position. The downfield shift of C3″ (δ 88.0) indicated that this glucosyl was rather extraordinary substituted in its 3″-position. The five ^{1}H resonances in the 2D TOCSY spectrum of the second sugar unit, the large ^{1}H–^{1}H coupling constants showing di-axial couplings between these protons, their corresponding ^{13}C resonances assigned from the HSQC spectrum, and the crosspeak at δ 4.02/172.2 (H5‴/C=O) in the HMBC spectra of **1** were in accordance with a β-glucuronopyranosyl. The HMBC crosspeak at δ 4.81/88.0 (H1‴/C3″) confirmed the connection point between the two monosaccharides to be in the glucosyl 3″-position in accordance with the rare disaccharide 3″-(β-glucuronopyranosyl)-β-glucopyranoside. The high-resolution ESI$^+$–MS spectrum of **1** showed a molecular ion at *m/z* 639.1572 corresponding to the empirical formula $C_{28}H_{31}O_{17}^+$ (calc. 639.1556 Da) in agreement with the new compound 5-*O*-methylcyanidin 3-*O*-(3″-(β-glucuronopyranosyl)-β-glucopyranoside) (Fig. 2). The molar absorptivity value (ε) of **1** was found to be 16,500 L cm^{-1} mol^{-1} in MeOH containing 0.5% TFA (v/v), and it had a λ_{max}-value of 529 nm in the same solvent.

Compound **3** gave very similar NMR spectra compared to **1** (Table 2). However, **3** had longer HPLC retention time (1.3 min) (Table 1), and a molecular ion at *m/z* 653.1721 in the high-resolution ESI$^+$–MS spectrum, corresponding to $C_{29}O_{33}H_{17}^+$ (calc. 653.1712 Da) in accordance with 5-*O*-methylcyanidin 3-*O*-(3″-(methyl-*O*-β-glucuronopyranoate)-β-glucopyranoside) (Fig. 2). The additional mass of 14 Da (CH_2) of **3** compared to **1**, was observed as an extra methoxy signal at δ 3.88 in the ^{1}H spectrum, which had a crosspeak at δ 3.88/52.9 (CH_3) in the HSQC spectrum, and a crosspeak at δ 3.88/171.0 ($CH_3O/C=O$) in the HMBC spectrum. Compound **3** was not observed in fresh extracts of *H. colorata* and *H. colorata* 'Exotica'. This compound was made by methyl esterification of the carboxyl group of the glucuronic

Table 1
Chromatographic (HPLC) and spectral (UV–Vis and MS) data recorded for **1**–**3** found in leaves of *Hemigraphis colorata*.

Compound	On-line HPLC				ESI–MS	
	t_R (min)	Vis_{max} (nm)	Local UV_{max} (nm)	$A_{440}/A_{vis\text{-}max}$	M^+ *m/z* (observed)	F^+ *m/z*
1	29.7	518	280, 330	0.23	639.1572	301.0624
2	28.9	518	280, 329	0.23	463.1203	301.0659
3	31.0	520	280, 326	0.23	653.1721	301.0633

Table 2
^{1}H (600.13 MHz) and ^{13}C (150.92 MHz) NMR spectral data for pigments **1**–**3** from *Hemigraphis colorata* recorded in CF_3COOD–CD_3OD (5:95; v/v) at 25 °C. See Fig. 1 for structures.

	1		**2**		**3**	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
2		164.4		164.5		164.7
3		146.0		146.2		146.1
4	9.14 *d* 0.7	136.4	9.13 *d* 0.7	136.4	9.14 *d* 0.6	136.4
5		159.6		159.8		159.9
5 (OMe)	4.18 *s*	57.7	4.17 *s*	57.7	4.18 *s*	57.7
6	6.86 *d* 1.9	100.9	6.86 *d* 1.9	100.9	6.87 *d* 1.8	100.8
7		170.4		170.3		170.4
8	7.08 *dd* 0.7, 1.9	95.7	7.08 *dd* 0.8, 1.9	95.8	7.08 *dd* 0.7, 1.8	95.7
9		157.8		157.8		157.8
10		113.3		113.2		113.5
1′		121.0		121.0		121.3
2′	8.15 *d* 2.4	118.5	8.15 *d* 2.4	118.4	8.15 *d* 2.3	118.5
3′		147.5		147.4		147.8
4′		156.0		156.0		156.1
5′	7.11 *d* 8.8	117.3	7.12 *d* 8.8	117.4	7.11 *d* 8.8	117.3
6′	8.39 *dd* 2.4, 8.7	128.5	8.38 *dd* 2.4, 8.7	128.5	8.40 *dd* 2.3, 8.7	128.4
1″	5.44 *d* 7.9	103.6	5.38 *d* 7.8	103.8	5.44 *d* 7.8	103.6
2″	3.99 *dd* 7.9, 8.9	74.1	3.76 *dd* 7.8, 9.1	74.8	3.98 *dd* 7.9, 9.0	74.0
3″	3.82 *t* 8.8	88.0	3.62 *t* 9.1	78.2	3.81 *t* 8.9	88.0
4″	3.62 *dd* 8.7, 9.6	69.7	3.50 *dd* 9.0, 9.6	71.3	3.61 *dd* 8.9, 9.7	69.7
5″	3.71 *ddd* 2.2, 6.4, 9.6	78.5	3.66 *ddd* 2.2, 6.7, 9.7	79.0	3.70 *ddd* 2.2, 6.4, 9.7	78.4
6A″	4.04 *dd* 2.1, 12.2	62.4	4.03 *dd* 2.2, 12.1	62.5	4.03 *dd* 2.2, 12.3	62.4
6B″	3.80 *dd* 6.3, 12.2	62.4	3.77 *dd* 6.6, 12.1	62.5	3.79 *dd* 6.3, 12.3	62.4
1‴	4.81 *d* 7.8	105.1			4.82 *d* 7.8	105.1
2‴	3.48 *dd* 7.8, 9.2	75.0			3.47 *dd* 7.8, 9.2	75.1
3‴	3.55 *t* 9.1	77.2			3.54 *t* 9.1	77.2
4‴	3.64 *dd* 8.9, 9.7	73.0			3.64 *dd* 9.1, 9.8	73.0
5‴	4.02 *d* 9.7	76.2			4.05 *d* 9.8	76.5
C=O		172.2				171.0
6‴ (OMe)					3.88 *s*	52.9

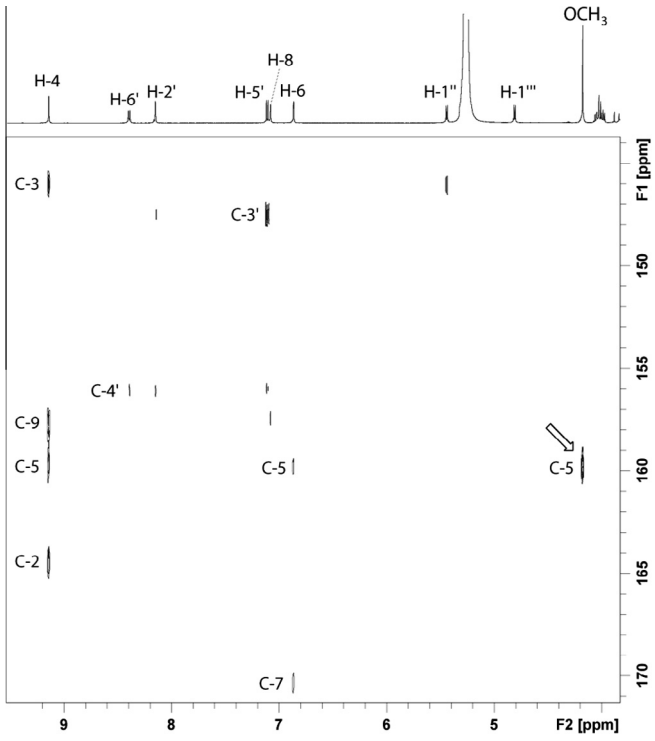

Fig. 3. Expanded region of the HMBC NMR spectrum of 5-*O*-methylcyanidin 3-*O*-(3″-(β-glucuronopyranosyl)-β-glucopyranoside) (**1**). The arrow points to the cross-speak showing the linkage of the OCH_3 group to the anthocyanidin 5-position.

acid of **1** occurs during extraction, isolation and storage of **1** in acidified methanolic solvents.

2.2. Anthocyanidins with 5-methoxylation

The anthocyanins **1**–**3** are built on the anthocyanidin, 5-*O*-methylcyanidin. This anthocyanidin has previously, without complete structural assignments based on NMR or MS data, been reported to occur naturally only in detached leaves of *Egeria densa* immersed in 0.1 M sucrose solution and incubated (24–26 °C) for 4–5 days under continuous illumination (*ca* 4000 lx), and in small amounts in reddish spring shoots of the same plant and its relative *Elodea nuttallii* (Planch.) St. John, (Momose et al., 1977) – both are aquatic plants. The previously reported 7-hydroxy-5-methoxyanthocyanidin structures of pulchellidin (5-methoxy-3,7,3′,4′,5′-pentahydroxyflavylium), europinidin (5,3′-dimethoxy-3,7,4′,5′-tetrahydroxyflavylium) and capensinidin (5,3′,5′-trimethoxy-3,7,4′-trihydroxyflavylium), have recently been revised to be the corresponding 5,7-dimethoxyanthocyanidins; 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, respectively (Skaar et al., 2012). The structural assignment of the aglycone of **1**–**3**, 5-*O*-methylcyanidin (Table 2), is thus the first complete characterisation of a 7-hydroxy-5-methoxyanthocyanidin isolated from a natural source. The UV–vis spectrum of 5-*O*-methylcyanidin with a visible absorption maximum at 529 nm shows a bathochromic shift of 11 nm compared to the corresponding spectra of the 5-*O*-methylcyanidin glycosides (**1**–**3**) in the applied HPLC solvent.

For diagnostic purposes in identification of 5-*O*-methylanthocyanidins, we want to highlight three points: (A) The ratio of the absorption at 440 nm to the absorption at the visible absorption maximum of all the three 5-*O*-methylcyanidin derivatives, **1–3**, on their flavylium cationic forms is found to be 23% in the applied HPLC solvent. For cyanidin 3-*O*-glucoside and cyanidin 3,5-di-*O*-glucoside the corresponding values are 30% and 15%, respectively (Fig. 1). The presence of a 5-*O*-methyl substituent on cyanidin on

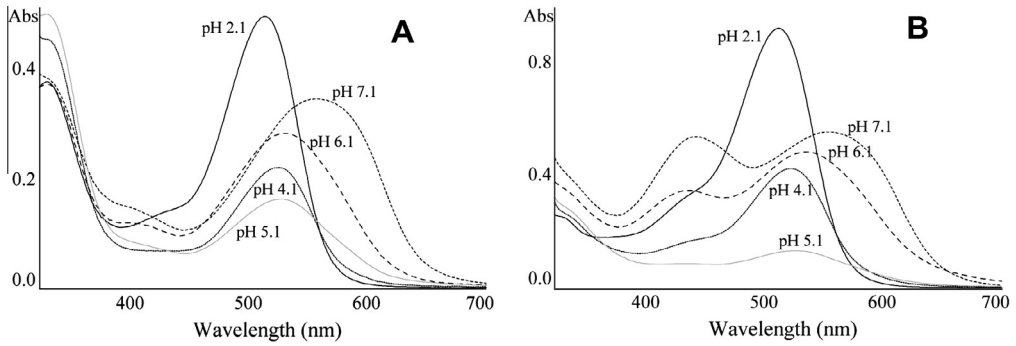

Fig. 4. UV–vis absorption spectra of **1** (A) and cyanidin 3-*O*-glucoside (B) recorded immediately after dissolution between 320 and 700 nm in solutions with pH at 2.1, 4.1, 5.1, 6.1 and 7.1, respectively.

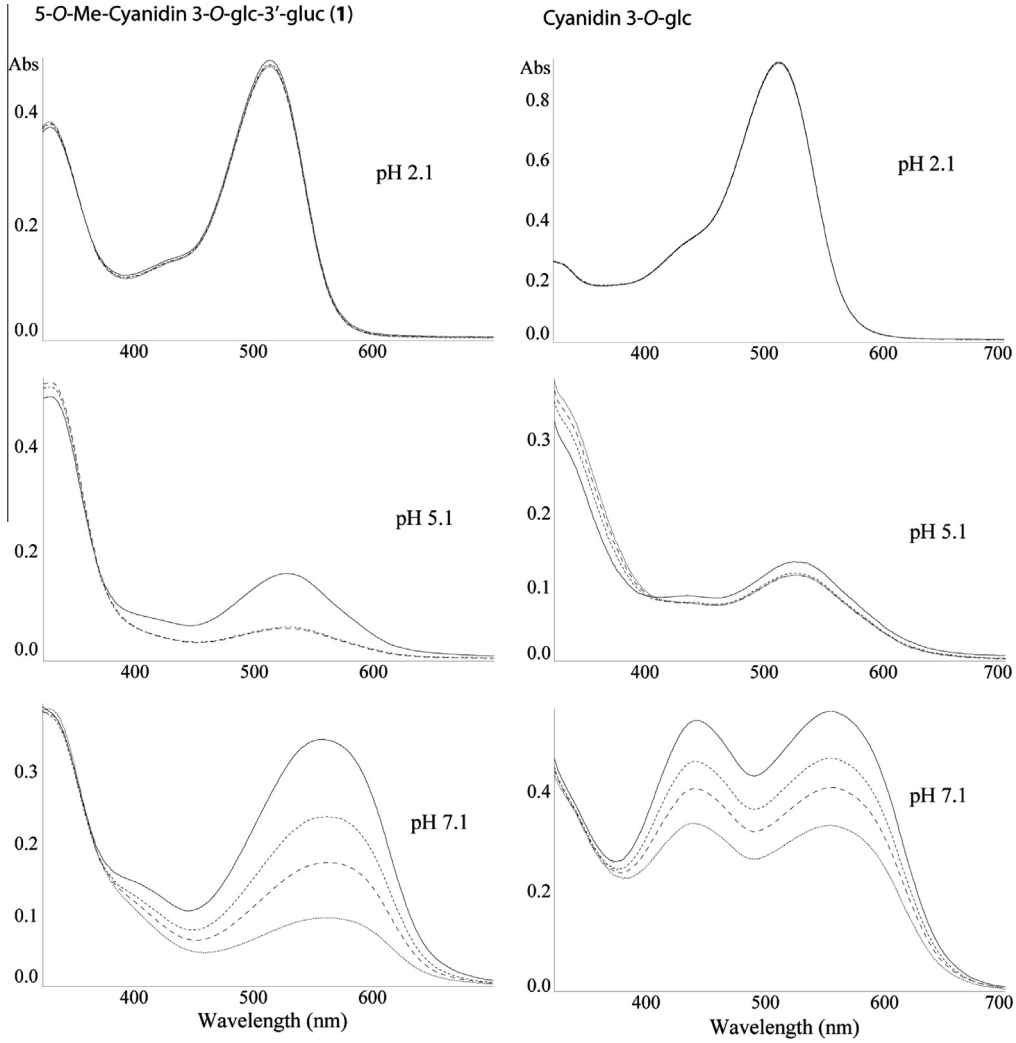

Fig. 5. UV–vis absorption spectra (between 320 and 700 nm) recorded after 0 min, 30 min, 60 min and 120 min for **1** and cyanidin 3-*O*-glucoside at pH 2.1, pH 5.1 and pH 7.1. When differences between absorption spectra of an anthocyanin are observed at different times at a specific pH value, a decrease in absorption is observed with time in all cases.

its flavylium cationic form thus seem to have a lowering effect (7%) on the $A_{440}/A_{vis\text{-}max}$ ratio compared to the corresponding value of cyanidin 3-*O*-glucoside, and this lowering effect is not as large as found for the 5-*O*-glycosyl substituent of cyanidin 3,5-di-*O*-glucoside (15%). As seen in Fig. 4 this effect is even more profound when the pH is increasing and these anthocyanins occur on various other forms than their flavylium cationic forms. In the various solutions of **1** the $A_{440}/A_{vis\text{-}max}$ ratios are around 30% for all pH values, while similar values for cyanidin 3-*O*-glucoside is increasing from 38% at pH 2.1 to 97% at pH 7.1. This increase in $A_{440}/A_{vis\text{-}max}$ ratios for cyanidin 3-*O*-glucoside reflects most probably the impact of a keto-function in the 5-position, which is lacking for anthocyanins like **1** and **2**, which have 5-methoxy-substitution of their aglycones. (B) The chemical carbon shifts of the A-ring 5-methoxyl

group of **1**–**3** are for all three anthocyanins observed at 57.7 ppm (Table 2). Similar value for the carbon of the B-ring methoxyl group of peonidin 3-*O*-glucoside obtained in the same solvent is observed at 56.9 ppm (unpublished). (C) The chemical proton shifts of the A-ring 5-methoxyl groups of **1**–**3** are observed at 4.17–4.18 ppm. Similar value observed for the protons of the methoxyl groups of the B-ring of peonidin 3-*O*-glucoside is observed at 4.06 ppm (unpublished). Both methoxyl protons and carbon located at the A-ring 5-position may thus be identified by downfield chemical shift effect compared to similar values for the B-ring methoxyl group of peonidin 3-*O*-glucoside. Similar diagnostic points have recently been observed for anthocyanins based on 5,7-dimethoxylated anthocyanidins (Skaar et al., 2012).

2.3. Colours and equilibrium forms of *1*

Anthocyanins are outstanding compounds in the way each anthocyanidin (aglycone) may be involved in a series of equilibria giving rise to different forms, which exhibit their own properties including colour expression and stability. Although a number of factors will influence the final anthocyanin colour of a plant (Andersen and Jordheim, 2010), when anthocyanin colouration is expressed as nuances of red, the anthocyanin(s) occur on their flavylium cationic form. When purple to blue nuances are revealed under more neutral and slightly alkaline conditions, the anthocyanidins are reckoned to occur on their quinoidal forms (Brouillard and Dangles, 1994). Most anthocyanidins have free hydroxyl groups in their anthocyanidin 5-, 7- and 4′-positions, which is a necessity for forming the quinoidal forms having keto-functions in either the 5-, 7- or 4′-positions. Structural transformations of cyanidin 3-*O*-glucoside from the flavylium cation through the colourless hemiketal and chalcone forms to the mixture of quinoidal forms, have been characterised on the basis of thermochemical parameters calculated by functional theory B3LYP method (Borkowski et al., 2005). The quinoidal form formed by deprotonation of the C4′–OH was found to be most stable when it was stabilized by H-bonding between the C5–OH and the sugar moiety. However, the 5-*O*-methylation of the anthocyanidin of **1** excludes the possibility of forming the keto-function in the 5-position of quinoidal forms of this pigment, and generates also a different situation with respect to H-binding involving the substituent in the 5-position.

Cyanidin 3-*O*-glucoside has the same anthocyanidin B-ring as 5-*O*-methylcyanidin, however, an additional free hydroxyl group in the 5-position representative for the anthocyanidin A-ring pattern of most anthocyanins. When **1** was dissolved in alkaline methanol (CD_3OD with 2% 2,2,6,6-tetramethylpiperidine), a bluish solution was observed, however, within seconds this colouration vanished and precipitation started. When a sample of cyanidin 3-*O*-glucoside was dissolved in the same alkaline solvent, a similar bluish solution was observed, which stayed without any precipitation for several days. In accordance with the absorption spectra shown in Fig. 5 the colour of both **1** and cyanidin 3-*O*-glucoside, when they occur on their flavylium cation forms, are approximately the same at pH 2.1 during a period of two hours. However, at pH 5.1 and pH 7.1 both anthocyanins show decrease in their visible absorptions during the same two hours period. This is caused by formation of colourless hemiketal forms and potential irreversible degradation at the expense of colourful quinoidal forms. This conversion from quinoidal forms to colourless hemiketal forms is clearly faster for **1** compared to cyanidin 3-*O*-glucoside at both pH 5.1 and 7.1.

Glucuronic acid moieties of anthocyanins have been suggested to imply increased colour stability compared to more common monosaccharides of anthocyanins like glucosyl(s) in response to at least light (Osmani et al., 2009). The carboxylate group might contribute to anthocyanin stability facilitated by H-bonding between the carboxylate group and the aglycone, as previously suggested for the acid group of malonic acid (Dangles, 1997; Osmani et al., 2009). Potential stabilisation might also be caused by the fact that the acid function of the glucuronic acid moiety of the anthocyanins can imply higher occurrence of the more stable flavylium cationic form of these anthocyanins at the expense of their quinoidal forms, compared to other sugar moieties of anthocyanins, which are lacking this acid function. When **1** was dissolved in methanolic solutions it was more reddish than **2** in the same solvent showing higher proportions of the flavylium cationic form compared to **2**. During storage in methanol the glucuronic acid moiety of **1** was esterified with methanol making **3** from **1**, with the consequence that the pH of the solvent increased and the more purple quinoidal forms of **1** appeared at the expense of the flavylium form. However, under *in vivo* conditions the impact of the acidity function of the glucuronic acid of **1** on pH is more likely overcome by the natural buffers of the vacuoles.

2.4. Concluding remarks

The disaccharide moiety of the major anthocyanin (**1**), 3″-(β-glucuronosyl)-β-glucoside, has previously not been found in any flavonoid. This disaccharide has been identified only in a triterpenoid saponin, lindernioside A isolated from *Lindernia pyxidaria* (Linderniaceae) (Miyase et al., 1995). It has been reported to constitute part of a trisaccharide moiety of a flavone from seeds of *Oroxylum indicum* (Bignoniaceae) (Yan et al., 2011) and a triterpenoid saponin from roots of *Aralia spinifolia* (Araliaceae) (Yu et al., 1994). *H. colorata* belong to the family Acanthaceae. From a chemotaxonomic point of view, the families Acanthaceae, Linderniaceae and Bignoniaceae belong all to the order Lamiales. The laminariobioside (3″-(β-glucosyl)-β-glucoside) moiety, found in anthocyanins from *Allium* spp. (Terahara et al., 1994; Fossen and Andersen, 2003) and the fern *Blechnum novae-zelandiae* (Swinny, 2001), is the only other disaccharide type of anthocyanins reported to have a 3″-linkage between the two monosaccharide units.

Both the glucuronosyl moiety and the aglycone of **1**, 5-*O*-methylcyanidin, seem to have different impact on anthocyanin properties like colours and colour stability compared to typical structural moieties of a common anthocyanin like cyanidin 3-*O*-glucoside.

3. Experimental

3.1. Isolation of anthocyanins for structure determination

Leaves of *H. colorata* collected from St. Mary's College Kisubi lawns in Entebbe (Uganda) in November 2011 were used for preparative isolation of individual anthocyanins. The identification of the plant was done at the Herbarium of the Botany Department of Makerere University, and a voucher specimen, Adaku No. 01, was deposited in the same place. The leaves (0.8 kg) were extracted for 16 h in 1.5 L of methanol containing trifluoroacetic acid (TFA) (0.5% v/v). The 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 anthocyanins adsorbed to the column were washed with water, and eluted from the column with methanol containing 0.5% TFA. The concentrated anthocyanin coloured eluate was separated by Sephadex LH-20 chromatography using H_2O-MeOH-TFA (79.5:20:0.5) as eluent.

Individual anthocyanins were purified by preparative HPLC using a Gilson 321 pump equipped with an Ultimate 3000 Variable Wavelength Detector, a 25 × 2.2 cm (10 μm) Econosphere C18 column (Grace, USA), and the solvents; A, water (0.5% TFA), and

B, acetonitrile (0.5% TFA). The elution profile 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% B). The flow rate was 15 ml min^{-1}, and aliquots of 250 μL were injected.

3.2. Analytical HPLC, spectroscopy and acid hydrolysis

See Andersen et al. (2010) for experimental procedures for obtaining analytical HPLC chromatograms, UV–vis absorption spectra, high-resolution LC-electrospray mass spectra, and the various NMR experiments. The molar absorptivity value ε (L cm^{-1} mol^{-1}) of 5-*O*-methylcyanidin 3-*O*-(3″-(β-glucuronopyranosyl)-β-glucopyranoside), **1**, was calculated using Lambert–Beer's law, including the mass of the counterion (CF_3COO^-, 113.16 g mol^{-1}) in the molecular mass. Solutions with five different pH values were prepared in the following manner: pH 2.1: 25 mL 0.2 M KCl and 67 mL 0.02 M HCl (v/v), pH 4.1: 50 mL 0.1 M potassium hydrogen phthalate (KHP) and 0.1 mL 0.1 M HCl (v/v), pH 5.1: 50 mL 0.1 M KHP and 22.6 mL 0.1 M NaOH (v/v), pH 6.1: 50 mL 0.1 M KH_2PO_4 and 5.6 mL 0.1 M NaOH (v/v), pH 7.1: 50 mL 0.1 M KH_2PO_4 and 29.1 mL 0.1 M NaOH (v/v). The accurate pH in each solution was measured with a Metrohm 827 pH lab pH meter equipped with a Metrohm combination electrode. Anthocyanins isolated from blackcurrant (*Ribes nigrum*) (Frøytlog et al., 1998), and *Fuchsia* spp. (Jordheim et al., 2011) were used as reference compounds in the HPLC and UV–vis analysis. Acid hydrolysis was performed by placing a 4 mg mixture of **1** and **2** into an ampule with 1 mL of super distiled water and 1 mL of 6 M HCl. The ampule was sealed and subjected to hydrolysis at 90 °C for 1 h. The completeness of the acid hydrolysis reaction was confirmed by using analytical HPLC with UV–vis detection.

3.3. Quantitative determinations

A fresh sample of *H. colorata* 'Exotica' leaves (0.65 g) from Green Park Tropical Fishfarm Pte Ltd., Singapore was extracted (3 h × 2) in 7 mL of methanol containing 0.5% TFA (v/v) in a refrigerator. The quantitative amounts of anthocyanins **1** and **2** in this sample were determined from a HPLC calibration curve based on compound **1**. Each of the concentration points of the calibration curve was based on average data from three parallel injections. The molar absorptivity value of **2** was assumed to be similar to the corresponding value of **1**.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2014.05.016.

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