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Anthocyanins from mauve flowers of *Erlangea tomentosa* (*Bothriocline longipes*) based on erlangidin – The first reported natural anthocyanidin with C-ring methoxylation

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ABSTRACT

Above 700 different anthocyanins have been isolated from plants. These flavonoid pigments are grouped after the number and position of hydroxy- and methoxy-groups on their anthocyanidin A- and B-rings, which influence their properties. In this study two new anthocyanins, erlangidin 5-*O*-(4″-(*E*-caffeoyl)-6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) and erlangidin 5-*O*-(6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) were isolated from purple flowers of *Erlangea tomentosa* (*Bothriocline longipes*) Oliv. & Hiern (Asteraceae) in amounts of ~1.6 and 0.6 mg/g fr. wt., respectively. They were found to contain the first reported natural anthocyanidin with a methoxy-group on the heterocyclic C-ring, which we have given the name erlangidin." During extraction, isolation and storage in acidified methanolic solvents the two anthocyanins were both partly converted to their demalonylated and methylmalonyl esterified forms. Spectroscopic and chromatographic characteristics for erlangidin in comparison with the common anthocyanidins, which it might be mistaken for, are included.

1. Introduction

The last two decades have witnessed renewed research activities on anthocyanins, mainly related to their potential health-promoting properties, their use as natural food colorants, and their appearance in cultivars and plant mutants with new colors and color patterns (Andersen and Jordheim, 2010; Davies, 2009; Zhao et al., 2017). Besides affecting the colors, various anthocyanin structures, including type of aglycone (anthocyanidin), have in many papers been reported to have different impact on the biological activity of these flavonoids. The position and number of hydroxy- and methoxy-groups on the various anthocyanidins have been considered as pivotal features for their stability and reactivity (Cabrita et al., 2000; Kamonpatana et al., 2012). The type of anthocyanidin have been shown to influence the bioavailability and degradation routes of anthocyanins, and their ability to form various phase II metabolites in metabolism (Andersen and Jordheim, 2013; Jakesevic et al., 2013; Sinela et al., 2017).

Around 92% of the reported anthocyanins (above 700 in 2018; Andersen, unpublished data) are based on the six anthocyanidins, cyanidin, delphinidin, pelargonidin, malvidin, peonidin and petunidin, referred to as the common anthocyanidins. These anthocyanidins have different number of hydroxyl- and methoxy-groups on their B-rings. Nearly all anthocyanins isolated from plants have an *O*-glycosyl moiety located at their anthocyanidin 3-positions. The exceptions are the 3-deoxyanthocyanins (which are lacking any substitution at their 3-positions) found in several sources, including mainly bryophytes, ferns, sorghum and some Gesneriacea (Andersen and Jordheim, 2010), and two anthocyanins found in the blue flowers of African water lily (*Nymphaéa caerulea*) (Fossen and Andersen, 1999) and two anthocyanins isolated from red onion (*Allium cepa*) (Fossen et al., 2003) having a free hydroxyl group in their anthocyanidin 3-positions.

In our continuing survey of the East-African flora looking for new nutraceutical or pigmentation sources, an investigation of *Erlangea tomentosa*(Oliv. & Hiern) S.Moore (*Bothriocline longipes* (Oliv. & Hiern) N.E.Br.) (family Compositae) a native tropical African plant widely distributed in South, Central and East Africa (Bailey, 1939), revealed that the relative complex anthocyanins isolated from its mauve flowers were based on an extraordinary anthocyanidin. In Uganda, *E. tomentosa* has been used to treat various health conditions including colic pains, stomachache, syphilis, fever, miscarriage, mental confusion,

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conjunctivitis, and convulsion in children (Asiimwe et al., 2014). With exception of the content of the seed oil (Phillips et al., 1969), the phytochemical composition of *E. tomentosa* is largely unknown.

The aim of this paper is to report anthocyanins from nature with methoxylation of the anthocyanidin C-ring for the first time, and to provide chromatographic and spectroscopic characteristics of this unusual anthocyanidin.

2. Results and discussion

2.1. Structural elucidation

HPLC analysis (detection at 520 ± 20 nm) of fresh flower extract of *E. tomentosa* revealed the presence of mainly two anthocyanins (**1** and **2**) in quantitative amounts found to be 1.55 and 0.60 mg/g fr. wt., respectively. The extract was purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography before individual anthocyanins were isolated using combinations of Sephadex LH-20 column chromatography and reversed phase preparative HPLC.

The 1D ^{1}H NMR spectrum of **1** showed in the area downfield for the sugar region signals corresponding to sixteen protons. Three of these at δ 8.42 (*d*,*J* = 2.1 Hz, H-2′), δ 8.39 (*dd*, *J* = 2.1, 8.8 Hz, H-6′) and δ 7.27 (*d*,*J* = 8.8 Hz, H-5′) were caused by the AMX-system of the anthocyanidin B-ring, while δ 8.64 (s, H-4), δ 7.09 (*d*, *J* = 2.0 Hz, H-6) and δ 7.02 (*d* (broad), H-8) belonged to the A- and C-rings, respectively (Table 1). The chemical shifts of the corresponding carbons of the aglycone of **1** were assigned from the HSQC NMR experiment, whereas the remaining quaternary carbon atoms of the aglycone were assigned from the HMBC spectrum (Table 1) indicating an anthocyanidin with a similar substitution pattern as cyanidin. However, a strong cross-peak at δ 4.25/149.19 (C-3) was observed in the HMBC spectrum of **1** (Fig. 2). This proton signal at δ 4.25 was integrated to three protons in the ^{1}H NMR spectrum, in accordance with a methoxy group extraordinary located at the 3-position of the anthocyanidin. This location of a methoxy group on an anthocyanidin was also supported by the cross-peak in the NOESY spectrum at δ 8.64/4.25 between H-4 and the methoxy group (Fig. 2). A fragment ion $[F]^+$ at *m/z* 301.0853 in the HR-ESI-MS spectrum of **1** was in accordance with a 3-methoxy-5,7,3′,4′-tetrahydroxyflavylium cation (3-methoxycyanidin) (calcd 301.0707 Da, $C_{16}H_{13}O_6^+$). No anthocyanidin with a methoxy group on its C-ring has previously been isolated from nature, and we suggest to name this anthocyanidin erlangidin.

Five of the protons in the downfield ^{1}H NMR region were due to coupled aromatic proton resonances at δ 6.96 (*d*, *J* = 1.8 Hz, H-2^{II}), δ 6.71 (*d*, *J* = 8.2 Hz, H-5^{II}) and δ 6.63 (*dd*, *J* = 8.2, 1.9 Hz, H-6^{II}), and the coupled olefinic resonances at δ 7.22 (*d*, *J* = 15.9 Hz, β^{II}) and δ 6.10 (*d*, *J* = 15.9 Hz, α^{II}) (Table 1). These signals together with resonances for the three quaternary carbons at δ 150.72 (C-4^{II}), δ 146.89 (C-3^{II}) δ 126.96 (C-1^{II}) and the ester carbonyl carbon at δ 168.06 showed the presence of one *p*-caffeoyl moiety. Similarly, the remaining five protons in the downfield region were found to belong to the second *p*-caffeoyl moiety (Table 1). The *E*-configuration of the double bond of both of the caffeoyl moieties were established by the coupling constant (15.9 Hz) of the olefinic protons.

The sugar region of the ^{1}H and TOCSY spectra of **1** indicated the presence of three sugar units. Starting from the anomeric proton at δ 5.35 (*d*, *J* = 7.8 Hz, H-1″), the observed cross-peak at δ 5.35/3.92 in the DQF−COSY spectrum supported by the cross-peak in the HSQC spectrum at δ 5.35/102.47, permitted the assignment of H-2″. Similarly, the chain of coupled protons H-2″, H-3″, H-4″, H-5″, H-6A″/H-6B″ and their coupling constants were assigned in accordance with a β-glucopyranosyl (Table 1). A cross-peak at δ 5.35/156.00 in the HMBC spectrum between H-1″ and C-5 of the aglycone and the cross-peak at δ 5.35/7.09 in the NOESY spectrum between H-1″ and H-6 (Fig. 2) showed that this sugar was connected to the aglycone 5-position. By using the doublet at δ 5.16 (*J* = 7.6 Hz) as the starting point in the DQF−COSY spectrum it

Table 1
^{1}H and ^{13}C NMR spectral data for the anthocyanins erlangidin 5-*O*-(4″-(*E*-caffeoyl)-6″-(malonyl)-β-glucoside)-3′-*O*-(6‴-(3^{II}-(β-glucosyl)-*E*-caffeoyl)-β-glucoside) (**1**) and erlangidin 5-*O*-(6″-(methylmalonyl)-β-glucoside)-3′-*O*-(6‴-(3^{II}-(β-glucosyl)-*E*-caffeoyl)-β-glucoside) (**2b**) isolated from mauve flowers of *Erlangea tomentosa* (*Bothriocline longipes*) recorded in CF_3COOD-CD_3OD (5:95, v/v) at 25 °C.

	1 (^{1}H)		**2b** (^{1}H)		**1** (^{13}C)	**2b** (^{13}C)
Erlangidin						
2					162.66	162.4
3					149.19	149.0
4	8.64	*s*	8.65	*d* 2.0	129.86	129.8
5					156.00	155.9
6	7.09	*d* 2.	7.04	*d* 2.0	106.65	106.3
7					168.32	168.3
8	7.02	(*d*)	7.01	*dd* 2.0, 0.8	97.60	97.4
9					155.68	155.4
10					113.76	113.7
1’					121.24	121.3
2’	8.42	*d* 2.1	8.43	*d* 2.2	120.23	120.3
3’					146.89	146.6
4’					157.40	157.1
5’	7.27	*d* 8.8	7.27	*d* 8.9	118.90	118.7
6’	8.39	*dd* 8.8, 2.1	8.39	*dd* 8.8, 2.2	130.68	130.4
3-OMe	4.25	*s*	4.25	*s*	58.35	58.3
5-*O*-glucoside						
1”	5.35	*d* 7.8	5.27	*d* 7.7	102.47	102.5
2”	3.92	*dd* 9.2, 7.8	3.79	*m*	74.69	74.5
3”	4.00	*t* 9.2	3.59	*m*	75.32	77.5
4”	5.16	*dd* 9.9, 9.3	3.58	*dd* 9.8, 9.1	71.94	71.1
5”	4.22	*ddd* 9.9, 6.6, 3.3	3.94	m	73.90	75.9
6A”	4.44	*m*	4.70	*dd* 12.0, 2.0	64.78	65.4
6B”	4.44	*m*	4.50	*dd* 12.0, 7.0		
3’-*O*-glucoside						
1”’	5.16	*d* 7.6	5.15	*d* 7.7	102.15	102.0
2”’	3.74	*dd* 9.1, 7.7	3.72	*dd* 9.4, 7.7	74.47	74.4
3”’	3.68	*t* 9.0	3.69	*t* 8.6	77.56	78.6
4”’	3.49	*dd* 9.3, 8.9	3.49	*m*	72.68	72.7
5”’	3.93	*m*	3.93	*ddd* 9.6, 6.3, 2.0	76.07	75.9
6A”’	4.94	*dd* 11.7, 2.3	4.91	*dd* 11.9, 2.1	64.96	64.9
6B”’	4.32	*dd* 11.7, 9.6	4.34	*dd* 12.0, 7.0		
3^{II}-*O*-glucosyl						
1””	4.69	*d* 7.6	4.63	*d* 7.5	103.86	103.8
2””	3.64	*dd* 9.2, 7.6	3.61	*m*	74.75	74.9
3””	3.63	*t* 9.2	3.60	*m*	77.61	77.4
4””	3.54	*dd* 9.3, 8.7	3.52	*m*	71.35	71.3
5””	3.39	*m*	3.36	*m*	78.63	78.3
6A””	3.98	*dd* 11.6, 2.1	4.00	*dd* 11.9, 2.2	62.27	62.4
6B””	3.81	*dd* 11.6, 5.6	3.80	*m*		
4”-*O*-caffeoyl						
1^{I}					127.66	
2^{I}	7.18	*d* 2.1			115.31	
3^{I}					146.85	
4^{I}					149.88	
5^{I}	6.89	*d* 8.1			116.53	
6^{I}	7.08	*dd* 8.1, 2.1			123.21	
α^{I}	6.45	*d* 15.9			114.38	
β^{I}	7.75	*d* 15.8			148.13	
COO^{I}					168.18	
6”’-*O*-caffeoyl						
1^{II}					126.96	126.8
2^{II}	6.96	*d* 1.8	6.95	*d* 2.0	116.73	116.6
3^{II}					146.89	146.6
4^{II}					150.72	150.3
5^{II}	6.71	*d* 8.2	6.70	*d* 9.3	117.49	117.6
6^{II}	6.63	*dd* 8.2, 1.9	6.63	*m*	125.11	125.1
α^{II}	6.10	*d* 15.9	6.10	*d* 15.9	115.99	115.9
β^{II}	7.22	*d* 15.9	7.21	*d* 15.9	145.75	145.7
COO^{II}					168.06	168.2
6”-*O*-malonyl						
1^{III}					168.40	168.9
2^{III}	3.58		3.61		41.65	41.4
3^{III}					170.25	170.3
1^{III}-OMe			3.79	*s*		52.9

s = singlet, *d* = doublet, *dd* = double doublet, *m* = multiplet. See Fig. 1 for structures.

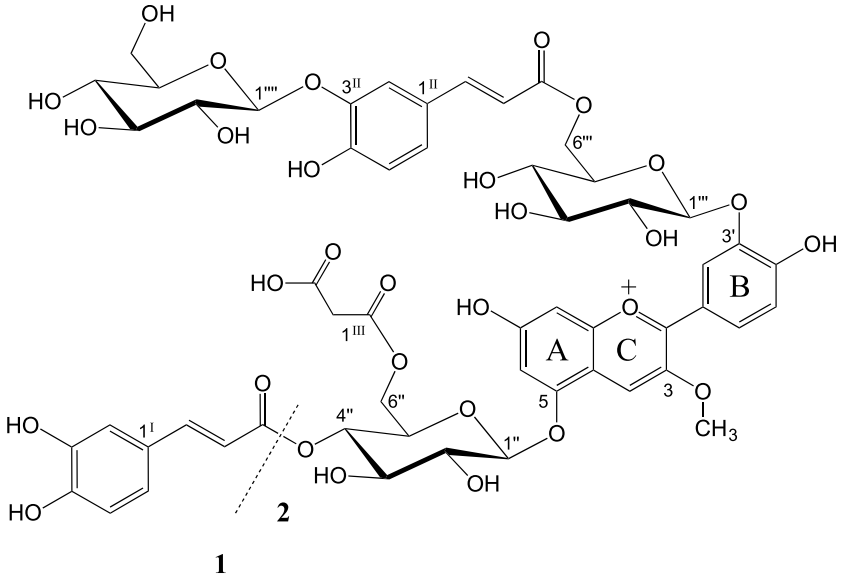

Fig. 1. Structures of erlangidin 5-*O*-(4″-(*E*-caffeoyl)-6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) (**1**) and erlangidin 5-*O*-(6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) (**2**) isolated from mauve flowers of *Erlangea tomentosa* (*Bothriocline longipes*). Compounds **1a** and **2a** are the demalonylated derivatives of **1** and **2**, while **1b** and **2b** are the 6″-(methymalonyl)-derivatives of **1** and **2**, respectively.

was likewise possible to assign all the chemical shifts for the second monosaccharide moiety, β-glucopyranosyl (Table 1). In cases where sugar protons had overlapping chemical shifts, the assignment was assisted by the TOCSY experiment. A cross-peak at δ 5.16/146.89 (H-1‴/C-3′) in the HMBC spectrum and the cross-peak at δ 5.16/8.42 in the NOESY spectrum between H-1‴ and H-2′ (Fig. 2) showed that this sugar unit was connected to the 3′-position of the aglycone. The third and last anomeric doublet at δ 4.69 (J = 7.6 Hz) was also identified to be part of a β-glucopyranosyl moiety (Table 1). The chair conformation of the three β-glucopyranosyls were confirmed by cross-peaks in the NOESY spectrum between H-1, H-3 and H-5 of all three moieties (Fig. 2).

A cross-peak at δ 4.69/146.89 (H-1″/C-3^{II}) in the HMBC spectrum and the cross-peak at δ 4.69/6.96 in the NOESY spectrum between H-1″ and H-2^{II} (Fig. 2) showed that this sugar unit was connected to the 3^{II}-position of the second caffeoyl moiety. This aromatic acyl group was itself found to be linked at the glucosyl 6‴-position by the crosspeaks between H-6A‴ and the C^{II}-carbonyl (δ 4.94/168.06) and between H-6B‴ and the C^{II}-carbonyl (δ 4.32/168.06) in the HMBC spectrum, while the other caffeoyl moiety was confirmed to be linked at the glucosyl 4″-position by the crosspeak between H-4″ and the C^{I}-carbonyl (δ 5.16/168.18) in the HMBC spectrum (Fig. 2). Furthermore, an aliphatic acyl moiety was found to be connected in the glucosyl 6″-position by the crosspeak at δ 4.44/168.40 between H-6A″/6B″ and the C-1^{III}-carbonyl (Fig. 2). This group was identified as malonyl by the crosspeaks at δ 3.58/168.40 (H-2^{III}/ C-1^{III}-carbonyl) and δ 3.58/170.25 (H-2^{III}/ C-3^{III}-carbonyl) in the HMBC spectrum and at δ 3.58/41.65 (H-2^{III}/ C-2^{III}) in the HSQC spectrum. The HR-ESI-MS spectrum of **1** showed a molecular ion at m/z 1197.2903 corresponding to the empirical formula $C_{55}H_{57}O_{30}^{+}$ (calc. 1197.2929 Da) in agreement with the novel compound erlangidin 5-*O*-(4″-(*E*-caffeoyl)-6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) (Fig. 1).

During extraction, isolation and storage in acidified methanolic solvents, **1** and **2** were partly converted to **1a**/**1b** and **2a**/**2b**, respectively (Fig. 3), and the NMR assignments in the structure elucidation of **2** were mainly based on NMR spectra recorded for **2b** (Table 1). The compounds (**1**, **1a**, **1b**, **2**, **2a** and **2b**) showed UV–vis spectra with $\lambda_{Vis\text{-}max}$ values around 530 nm (Table 2) for all compounds. However, while **1, 1a** and **1b** showed strong local λ-values around 325 nm, indicating anthocyanins acylated with two aromatic acyl groups, the lower intensity of the local absorption maxima around 322 nm for **2, 2a** and **2b** indicated that these compounds were acylated with only one aromatic acyl group (Table 2). Based on analogous NMR assignments as reported for **1**, the identity of **2** was confirmed to be **1** lacking one caffeoyl moiety. The HR-ESI-MS spectrum of **2** showed a molecular ion at m/z 1035.2709 corresponding to the empirical formula $C_{46}H_{51}O_{27}^{+}$ (calc. 1035.2612 Da) in agreement with the novel compound erlangidin 5-*O*-(6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) (Fig. 1).

The difference of 85.9890 Da in the high-resolution ESI-MS spectra of **1** and **1a** (Table 2) was in accordance with the lack of an aliphatic acyl moiety (malonyl) in **1a**. Compound **1a** was thus made by demalonylation of the malonyl unit of **1**, and the HR-ESI-MS spectrum of **1a** showed a molecular ion at m/z 1111.3013 (Table 2) in agreement with erlangidin 5-*O*-(4″-(*E*-caffeoyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside). The difference of 14.0164 Da between of **1** and **1b** (Table 2) was confirmed by an extra methoxy signal at δ 3.77 in the ^{11}H spectrum, a crosspeak at δ 3.77/53.3 (CH_3O) in the HSQC spectrum, and a crosspeak at δ 3.77/170.16 (CH_3O/C-3^{III}-carbonyl) in the HMBC spectrum of **1b**. Compound **1b** was thus made by methyl esterification of the free carboxyl group of the malonyl unit of **1**, and the HR-ESI-MS spectrum of **1b** showed a molecular ion at m/z 1211.3067 (Table 2) in agreement with erlangidin 5-*O*-(4″-(*E*-caffeoyl)-6″-(methylmalonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside). In a similar way **2a** and **2b** were identified as erlangidin 5-*O*-β-glucopyranoside-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) and erlangidin 5-*O*-(6″-(methylmalonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside), respectively.

Numerous studies have confirmed that aromatic acyl groups situated on the glycosyl moieties increase the *in vitro* and *in vivo* stability of anthocyanins (Dangles et al., 1993; Yoshida et al., 2009; Andersen and Jordheim, 2010; Trouillas et al., 2016; Zhao et al., 2017). Anthocyanins without aromatic acylation are assumed to be more quickly transformed into non-cyanic forms by hydration at the 2-position of the anthocyanidin nucleus. The aromatic acyl moieties seem to protect against nucleophilic attack by water at this position. The shift to more bluish colors of in particular acylated anthocyanins based on delphinidin (Yoshida et al., 2009; Andersen and Jordheim, 2010), have in some cases been indicated to be achieved by intramolecular copigmentation involving stacking between the anthocyanidin and one or two of the aromatic acyl groups, which stabilize the cyanic forms of the anthocyanidin. It has also been shown for 3-deoxyanthocyanidins how *O*-glycosidation can perturb the acid-base properties of the chromophore and with a large impact on the distribution of colored and colorless forms in aqueous solution (Basílio et al., 2016; Sigurdson et al.,

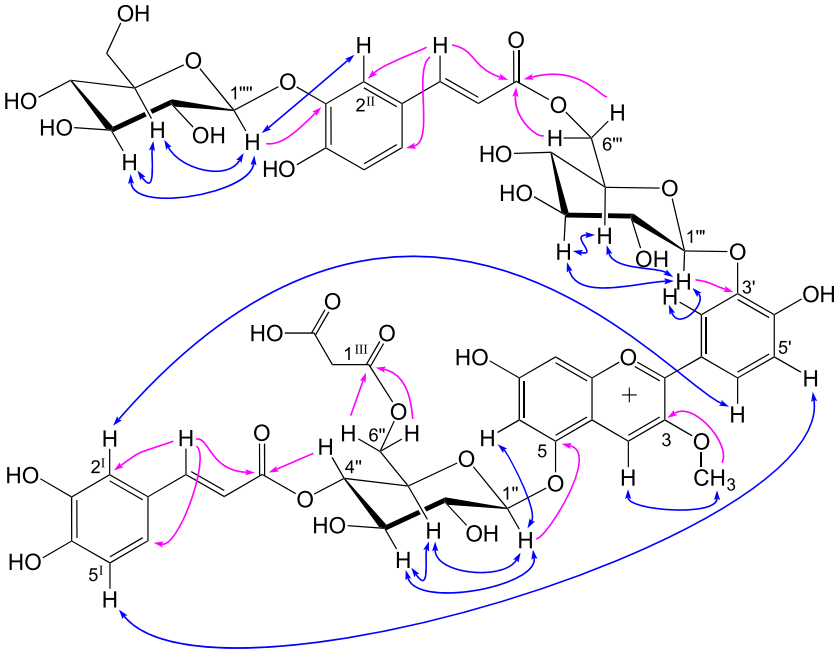

Fig. 2. Highlighted NMR correlations observed as cross-peaks in ^{1}H-^{1}H NOESY (two-sided blue arrows from ^{1}H to ^{1}H) and ^{1}H-^{13}C HMBC (one-way pink arrows from ^{1}H to ^{13}C) spectra of erlangidin 5-*O*-(4″-(*E*-caffeoyl)-6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) (**1**) isolated from mauve flowers of *Erlangea tomentosa* (*Bothriocline longipes*).

2018). Pigment **1** isolated from *E. tomentosa* flowers contains two caffeoyl moieties, situated at the glucosyl moieties at the 5- and 3′-position, respectively, on the anthocyanidin (erlangidin). Based on the cross-peaks between H-5′ and H-5^{I} (δ 7.27/6.89) and between H-6′ and H-2^{I} (δ 8.39/7.18) in the NOESY NMR spectrum of **1** (Fig. 2), it is obvious that there is proximity between erlangidin and the caffeoyl moiety connected to the 5-glucosyl. This proximity might be achieved by intramolecular folding of the 5-glycosyl unit placing the aromatic ring of the coumaroyl moiety on top or bottom of the B-ring of erlangidin. However, the flowers of *E. tomentosa* might be described as mauve – not bluish, and the visible part of the absorption spectra of **1** and **2** are nearly identical (Table 2), even though pigment **2** is lacking the caffeoyl moiety connected to the 5-glucosyl of pigment **1**. Thus, the aromatic acylated anthocyanins **1** and **2**, which are based on erlangidin, do not achieve the bluing effect typical for aromatic acylated anthocyanins based on delphinidin.

2.2. Diagnostic features of erlangidin

For diagnostic purposes in the identification of anthocyanins based on anthocyanidins with a 3-methoxyl group on their C-ring, we want to highlight three points after comparison with own results on a variety of anthocyanins: a) Erlangidin (made by acid hydrolysis of **1** and **2**) was compared with common anthocyanidins with respect to retention times (TLC and HPLC) and spectral data (UV–vis) (Table 3). The most characteristic feature was the relatively high R_f value of erlangidin in an acidified aqueous TLC system – even when compared with peonidin having the same number of hydroxyl and methoxyl substituents as erlangidin.

b) The chemical carbon shifts of the B-ring methoxyl groups of anthocyanins are observed at 56.7–57.3 ppm in acidified deuterated methanolic solvents, while the carbons of the A-ring methoxyl groups are observed at 57.7–57.9 ppm. The chemical carbon shift of the C-ring 3-methoxyl carbon (58.3 ppm in the erlangidin-derivatives **1** and **2b**)

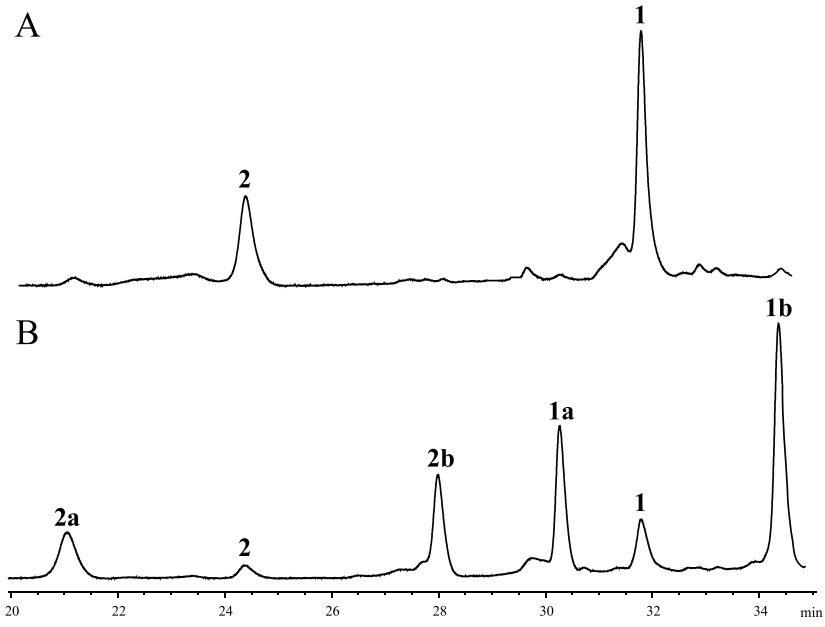

Fig. 3. HPLC profiles of raw extracts (methanol containing 0.5% TFA) of *Erlangea tomentosa* (*Bothriocline longipes*) detected at 520 ± 20 nm. **A**: After 24 h extraction at 4 °C. **B**: After 1-month storage at 4 °C showing how the two anthocyanins (**1** and **2**) are converted partly to their respective demalonylated (**1a** and **2a**) and methylmalonylated (**1b** and **2b**) derivatives. See Fig. 1 for structures.

Table 2
Online HPLC, and high-resolution electrospray ionization mass spectral data recorded for anthocyanins **1** and **2** isolated from *Erlangea tomentosa* (*Bothriocline longipes*) and their respective demalonylated (**1a** and **2a)** and methylmalonylated (**1b** and **2b)** derivatives. See Fig. 1 for structures.

	t_R (min)	Vis-max (nm)	Local UV-max (nm)	$A_{440}/A_{vis\text{-}max}$ (%)	$A_{UV\text{-}max}/A_{vis\text{-}max}$ (%)	$[M]^+$ (obsd) (m/z)	$[M]^+$ (calcd) (m/z)	molecular formula
1	31.78	530	282 (sh), 294, 326	18	95	1197.2903	1197.2929	$C_{55}H_{57}O_{30}^+$
1a	30.25	530	280 (sh), 296, 324	19	106	1111.3013	1111.2925	$C_{52}H_{55}O_{27}^+$
1b	34.30	530	282 (sh), 296, 326	18	87	1211.3067	1211.3086	$C_{56}H_{59}O_{30}^+$
2	24.32	530	278, 294 (sh), 322	19	65	1035.2709	1035.2612	$C_{46}H_{51}O_{27}^+$
2a	21.11	528	278, 294 (sh), 320	20	63	949.2653	949.2608	$C_{43}H_{49}O_{24}^+$
2b	27.95	530	278, 294 (sh), 322	19	61	1049.2837	1049.2769	$C_{47}H_{53}O_{27}^+$

Table 3
Retention times (TLC and HPLC) and spectral data (UV–vis) recorded for erlangidin compared to five common anthocyanidins.

Aglycone	R_f TLC (FHW)	t_R HPLC (min)	UV-Vis_{max} (nm)	$A_{440}/A_{vis\text{-}max}$ (%)
Delphinidin	0.13	18.2	532	25
Cyanidin	0.23	23.7	525	25
Petunidin	0.20	24.9	534	20
Erlangidin	0.42	28.5	525	27
Peonidin	0.34	33.2	526	26
Malvidin	0.30	33.8	533	24

may thus be indicated by their downfield chemical shift effect.

c) The chemical proton shifts of the B-ring methoxyl groups are observed at 4.06–4.13 ppm in acidified deuterated methanolic solvents, while the protons of the A-ring methoxyl groups are observed at 4.17–4.21 ppm. 3-Methoxy protons located at the C-ring (4.25 ppm in the erlangidin-derivatives **1** and **2b** may thus be identified by their small downfield chemical shift effect compared to similar values for A- and B-ring methoxyl protons.

2.3. Concluding remarks

In this paper a novel natural anthocyanidin (erlangidin) with methoxylation of the heterocyclic C-ring is identified for the first time. Two anthocyanins having erlangidin as aglycone, erlangidin 5-*O*-(4″-(*E*-caffeoyl)-6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) (**1**) and erlangidin 5-*O*-(6″-(malonyl)-β-glucopyranoside)-3′-*O*-(6‴-(3^{II}-(β-glucopyranosyl)-*E*-caffeoyl)-β-glucopyranoside) (**2**) are isolated from mauve flowers of *E. tomentosa* either intact, as 6″-(methymalonyl)-derivatives (**1b** and **2b**), or as demalonylated derivatives (**1a** and **2a**). Most of the more than 700 reported anthocyanins (> 90%) found in plants are based on only common 6 anthocyanidins. In addition, three 6-hydroxyanthocyanidins, nine 3-deoxyanthocyanidins, ten anthocyanidins with mono- or dimethoxylation on their A-rings and some anthocyanidins with extended C15-skeleton (pyranoanthocyanidins, sphagnorubins) have previously been reported. The finding of erlangidin-glycosides with methoxylated C-rings will thus expand the diversity of known anthocyanidin structures and have chemotaxonomic significance.

Nearly all previously reported anthocyanins occur as anthocyanidin 3-glycoside derivatives. Further examinations of erlangidin 3-methoxy derivatives may contribute in the understanding of anthocyanidin equilibrium forms and thus anthocyanin chemistry (color, stability *etc.*) and the potential nutraceutical value of anthocyanins.

3. Experimental

3.1. General experimental procedures

The analytical HPLC system used for the anthocyanins (**1** and **2**) and anthocyanidins was the Agilent 1100 series equipped with a HP 1050 diode array detector and a 200 × 4.6 mm i.d., 5 µm ODS Hypersil column (Supelco, Bellefonte, PA). Two solvents, A) H_2O (+ 0.5% TFA, v/v) and B) acetonitrile (+ 0.5% TFA, v/v), were used for elution. The elution profile started 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), with isocratic elution at 32–40 min (40% B). and linear gradient elution at 40–43 min (back to 10% B). The gradient used for separation of anthocyanidins started with 90% A and 10% B followed by linear gradient conditions: 0–10 min (to 18% B), 10–20 min (to 22% B), 20–35 min (to 25% B), 35–36 min (to 40% B), with isocratic elution at 36–41 min (40% B) and linear gradient elution at 41–43 min (back to 10% B). The flow rate was 1.0 ml/min, and aliquots of 15 µl were injected with an Agilent 1100 series microautosampler. Prior to injection, all samples were filtered through a 0.45 µm Millipore membrane filter. The UV–vis absorption spectra were recorded online during HPLC analysis over the wavelength range of 240–600 nm in steps of 2 nm.

High-Resolution LC-Electrospray mass spectrometry (ESI^+/TOF), spectra were recorded using a JEOL AccuTOF JMS-T100LC instrument in combination with an Agilent Technologies 1200 Series HPLC system. A Zorbax SB-C18 (50 × 2.1 mm, length × i.d., 1.8 µm) column was used for separation. Two solvents, A) H_2O (+ 0.5% TFA, v/v) and B) acetonitrile (+ 0.5% TFA, v/v), were used for elution. The following solvent compositions were used: 0–1.25 min (10–22% B, linear gradient), 1.25–5 min (to 30% B, linear gradient), 5–7 min (30% B, isocratic), 7–8 min (to 40% B, linear gradient), 8–14 min (40% B, isocratic) and 14–15 min (back to 10% B, linear gradient). The flow rate was 0.4 ml/min.

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).

One-dimensional ^1H, compensated attached proton test (CAPT), 2D heteronuclear single quantum coherence (^1H–^{13}C HSQC), heteronuclear multiple bond correlation (^1H–^{13}C HMBC), 2D correlation spectroscopy (^1H–^1H COSY), and 2D total correlation spectroscopy (^1H–^1H TOCSY) were obtained on a Bruker Biospin Ultrashield Plus AV–600 MHz instrument equipped with a TCI ^1H–^{13}C/^{15}N CryoProbehead at 298 K. Sample temperatures were stabilized at 298 K. The deuteriomethyl ^{13}C signal and the residual ^1H signal of the solvent (CF_3COOD-CD_3OD; 5:95, v/v) were used as secondary references (δ 49.0 and 3.40 from TMS, respectively).

3.2. Plant material

Fresh flowers of *E. tomentosa* were collected from the margins of Zika forest in Entebbe, Uganda in May 2014. The plant was identified by the staff at the Herbarium of Botany Department of Makerere University, where a voucher specimen, Adaku No. 4, was deposited.

3.3. Extraction and isolation

The fresh mauve corollas of the flowers (300 g) were extracted with 0.8 l methanol containing trifluoroacetic acid (0.5%, v/v) (MeOH–TFA) for 12 h. The filtered extract was concentrated under reduced pressure at 28 °C, purified by partition against ethyl acetate (three times) and applied to an Amberlite XAD–7 column. The anthocyanins adsorbed to the column were washed with water to remove polar non-phenolic impurities, and eluted from the column with MeOH–TFA. The concentrated anthocyanin eluate was applied to a Sephadex LH-20 column and separated by gradient elution using H_2O–MeOH–TFA (from 79.5:20:0.5 to 69.5:30:0.5, v/v/v) solvent. The separated anthocyanins were purified by a preparative HPLC system equipped with a Gilson 321 pump, an Ultimate 3000 Variable Wavelength Detector, a 25 × 2.2 cm (10 µm) Econosphere C18 column (Grace, USA), and the solvents, A) H_2O (0.5% TFA, v/v) and B) acetonitrile (0.5% TFA, v/v). 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 linear gradient elution 43–46 min (back to 10% B). The flow rate was 15 ml/min, and aliquots of 250 µl were injected.

3.4. Preparation of anthocyanidins

Pigments from blueberries (*Vaccinium myrtillus*) (Prior et al. (1998)), *Fuchsia* spp. flowers (Jordheim et al. (2011)) and *E. tomentosa* flowers were subjected to acid hydrolysis by dissolving them in 1 ml water and 1 ml 6 M HCl at 90 °C and incubated for 120 min in sealed ampules. The reactions were monitored by TLC (every 30 min) and HPLC (every 60 min).

3.5. Quantitative determinations

Four samples (4–9 g) of *E. tomentosa* flowers were extracted with methanol containing 0.5% TFA (11–21 mL) at 3 °C for 24 h. The supernatants were concentrated under reduced pressure, dissolved in methanol containing 0.5% TFA (2.5–4.5 mL), filtered through a 0.45 µm Millipore membrane filter, before the quantitative amounts of anthocyanins **1** and **2** in the four samples (three HPLC replicates of each) were determined from a HPLC standard curve based on cyanidin 3-glucoside isolated from black rice (Lee, 2010). The purity of cyanidin 3-glucoside was > 99% based on integration of the signals in the aromatic region of its ^{1}H NMR spectrum. Each of the three concentration points of the standard curve was based on averaged data calculated from three HPLC replicates. The averaged quantitative results are presented as milligrams per cyanidin 3-glucoside equivalents per gram of fresh flower weight.

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