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Anthocyanins from red onion, *Allium cepa,* with novel aglycone

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Abstract

Four anthocyanins with the same novel 4-substituted aglycone, carboxypyranocyanidin, have been isolated from acidified, methanolic extracts of the edible scales as well as from the dry outer scales of red onion, *Allium cepa* L. The structures of **1** and **2** were identified as the 3-*O*-β-glucopyranoside and 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) of 5-carboxypyranocyanidin, respectively. This aglycone, 5-carboxy-2-(3,4-dihydroxyphenyl)-3,8-dihydroxy-pyrano[4,3,2-*de*]-1-benzopyrylium, is with exception of the substitution pattern on the phenyl ring similar to carboxypyranomalvidin (vitisidin A) recently isolated from red wines. In addition to **1** and **2**, two analogues of **2** methylated at the terminal carboxyl group of the acyl moiety (**3**) or at the aglycone carboxyl (**4**), respectively, were also identified. These latter compounds are most probably formed by esterification of **2** with the solvent (acidified methanol) during the isolation process. The structures were elucidated by 2D NMR spectroscopy and LC–MS.

Keywords: Red onion; *Allium cepa*; Alliaceae; Pigmented scales; Anthocyanins; 5-Carboxypyranocyanidin; Nomenclature; 2D NMR

1. Introduction

In recent years several colour stable 4-substituted anthocyanins have been discovered in small amounts in red wine and grape pomace (Bakker et al., 1997; Bakker and Timberlake, 1997; Fulcrand et al., 1998; Asenstorfer et al., 2001; Vivar-Quintana et al., 2002). Vitisin A and acetylvitisin A were identified as the 3-glucoside and the 3-acetylglucoside of malvidin containing an additional $C_3H_2O_2$ unit linking C-4 and the C-5 hydroxyl group. Vitisin B and acetylvitisin B were identified as analogous pigments having a CH=CH moiety instead of the $C_3H_2O_2$ unit (Bakker and Timberlake, 1997). The suggested structure for carboxypyranomalvidin (vitisidin A) was later slightly revised by Fulcrand et al. (1998), who proved that the $C_3H_2O_2$ unit was part of a pyran ring having a free acid group. They suggested that vitisin A was formed by cycloaddition of pyruvic acid involving both C-4 and the hydroxyl at C-5 of malvidin. In addition to the carboxypyranoanthocyanins based on malvidin, an analogous petunidin derivative has been suggested by its UV-Vis and MS spectra to occur in red wine (Vivar-Quintana et al., 2002). Four reported methylpyranoanthocyanins from blackcurrant seeds (Lu et al., 2000) were later shown to be the oxidative cycloaddition products of the extraction solvent (acetone) and the natural anthocyanins (Lu and Foo, 2001). Recently, Fukui et al. (2002) characterized the 4-substituted anthocyanin rosacyanin B isolated from petals of *Rosa hybrida* cv. 'M'me Violet. This anthocyanin contains, however, no sugar units.

The four main anthocyanins of red onion have previously been identified as the 3-(3″-glucosyl-6″-malonylglucoside), the 3-(6″-malonylglucoside), the 3-(3″-glucosylglucoside) and the 3-glucoside of cyanidin, respectively (Terahara et al., 1994; Fossen et al., 1996). In addition some minor anthocyanin pigments have been detected: the 3-(3″,6″-dimalonylglucoside), 3-(3″-malonylglucoside) and 3,5-diglucoside of cyanidin, the 3-glucoside, 3,5-diglucoside and 3-malonylglucoside of peonidin (Fossen et al., 1996; Donner and Mazza, 1997).

In this paper we report on isolation and characterisation of anthocyanins with the same 4-substituted anthocyanidin, 5-carboxypyranocyanidin, isolated from the pigmented scales of red onion.

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2. Results and discussion

The aqueous concentrate of the acidified methanolic extract of pigmented scales from red onion, *Allium cepa*, was purified by partition against ethyl acetate followed by Amberlite XAD-7 column chromatography. The anthocyanins in the purified extract were fractionated by Sephadex LH-20 column chromatography. The individual anthocyanins, **1**–**4**, in the Sephadex fraction shown in Fig. 1, were separated by preparative HPLC.

The UV-Vis spectrum of **2** taken on-line during HPLC showed a visible maximum at 507 nm with A_{440}/A_{507} of 35%, and a relatively low intensity of the UV absorption bands compared to the visible absorption maximum, indicating a 4-substituted anthocyanin (Bakker and Timberlake, 1997). The downfield part of the 1D ^{1}H NMR spectrum of **2** showed a 3H AMX system at δ 8.00 (*dd,* 8.7 Hz, 2.0 Hz; H-6′), δ 7.88 (*d,* 2.0 Hz; H-2′) and δ 6.92 (*d,* 8.7 Hz; H-5′), a 1H singlet at δ 7.92 (H-4), and an AX system at δ 7.25 (*d*, 1.8 Hz; H-9) and δ 7.16 (*d,* 1.8 Hz; H-7), revealing a 4-substituted anthocyanin having an asymmetrically substituted B-ring. The 18 ^{13}C resonances belonging to the aglycone were in accordance with carboxypyranocyanidin, 5-carboxy - 2 - (3,4 - dihydroxyphenyl)-3,8-dihydroxy-pyrano[4,3,2-*de*]-1-benzopyrylium.

The sugar region showed the presence of only one sugar unit. The anomeric coupling constant (7.7 Hz) and the six ^{13}C resonances in the sugar region of the ^{13}C CAPT spectrum of **2** were in accordance with β-glucopyranose (Fossen et al., 1996). All the ^{1}H sugar resonances were assigned by the DQF-COSY experiment, and the corresponding ^{13}C resonances were then assigned by the ^{1}H–^{13}C HSQC experiment. The acyl group was identified as malonic acid by the two carbonyl ^{13}C resonances at δ 167.03 (C-1‴) and 168.28 (C-3‴) in the ^{13}C CAPT spectrum (Fossen et al., 1996). The downfield shift of C-6″ (δ 64.30), H-6A″ (δ 4.04) and H-6B″ (δ 3.94) compared to the corresponding signals of an unsubstituted glucose unit of a similar pigment (Fulcrand et al., 1998; Fossen et al., 1996) indicated that the linkage between the acyl and the sugar moiety was at the 6″-hydroxyl. The weak crosspeaks at δ 4.04/167.0 (H-6A″/C-1‴) and δ 3.94/167.0 (H-6B″/C-1‴) in the HMBC spectrum confirmed the linkage between the acyl and the sugar unit to be at the 6″-hydroxyl. A molecular ion at *m*/*z* 603 in the ES-MS spectrum of **2** and the fragment ion at *m*/*z* 355 corresponding to the aglycone, confirmed the identity of **2** to be 5-carboxypyranocyanidin 3-*O*-(6″-*O*-malonyl-*β*-glucopyranoside); Fig. 2.

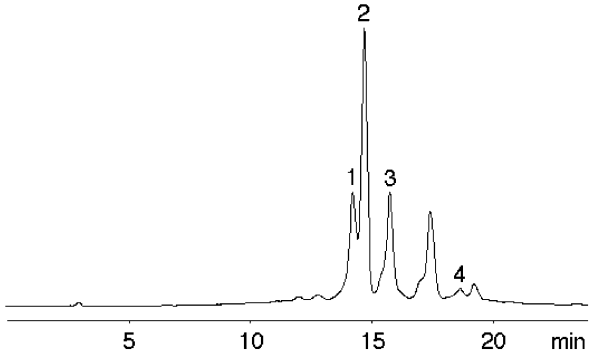

Fig. 1. The HPLC profile of the Sephadex LH-20 fraction containing the carboxypyranocyanidins **1**–**4**.

Pigment **1** exhibited a shorter retention time (HPLC) compared to **2**. However, the UV-Vis spectrum of **1** was nearly identical to that of **2** with a visible maximum at 507 nm with A_{440}/A_{507} of 35%, a local UV_{max} at 354 nm and a relatively low intensity of the UV absorption bands compared to the visible absorption maximum (Table 1). A fragment ion at *m*/*z* 355 in the ES–MS spectrum of **1** confirmed the identity of the aglycone to be carboxypyranocyanidin, and the molecular ion at *m*/*z* 517 was in agreement with the deacylated form of **2**, 5-carboxypyranocyanidin 3-*O*-*β*-glucopyranoside.

The UV-Vis and ^{1}H NMR spectra of pigment **3** were very similar to that of **2** (Tables 1 and 2). A molecular ion at *m*/*z* 617 in addition to a fragment ion at *m*/*z* 355 in the ES–MS spectrum of **3** were in accordance with 5-carboxypyranocyanidin 3-*O*-(6″-*O*-methylmalonyl-β-glucopyranoside. This compound is most probably formed during the isolation procedure by methyl esterification of the free acid function of the malonyl unit by the solvent (acidified methanol) (Fossen et al., 2001).

The retention time (HPLC) of pigment **4** was considerably longer than that of pigment **2** in a reverse

Table 1.
Chromatographic (HPLC) and spectral (UV-Vis and MS) data recorded for the 5-carboxypyranocyanidins **1–4**

Compound	On-line HPLC		$A_{440}/A_{vis\text{-}max}$ (%)	t_R (min)	ES-MS	
	Vis-max (nm)	Local UV-max (nm)			M^+ *m*/*z*	A^+ *m*/*z*
1	507	354	35	14.22	517	355
2	507	354	35	14.69	603	355
3	509	352	35	15.74	617	355
4	516		35	18.64	617	369

M^+ = molecular ion; A^+ = aglycone fragment ion.

Table 2
^{1}H and ^{13}C spectral data for 5-carboxypyranocyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside), 2, in CF_3COOD-DMSO-d_6 (1:4; v/v) at 25 °C

	^{1}H δ (ppm) J (Hz)	^{13}C δ (ppm)
Aglycone		
2		165.01
3		135.25
3a		109.79[a]
4	7.92 *s*	106.8
5		154.46
COOH		160.52
6a		153.07
7	7.16 *d*, 1.8 Hz	101.30
8		168.40
9	7.25 *d*, 1.8 Hz	101.05
9a		153.20
9b		109.67[a]
1′		120.33
2′	7.88 *d*, 2.0 Hz	118.5
3′		146.14
4′		153.89
5′	6.92 *d*, 8.7 Hz	116.89
6′	8.00 *dd*, 2.0 Hz, 8.7 Hz	126.52
3-*O*-β-glucopyranoside		
1″	4.59 *d*, 7.7 Hz	105.58
2″	3.49 *t*, 8.7 Hz	74.13
3″	3.21 *m*	76.52
4″	3.17 *m*	70.05
5″	3.23 *m*	74.78
6A″	4.04 *d (b)*, 11.8 Hz	64.30
6B″	3.94 *dd*, 11.8 Hz, 6.1 Hz	
6″-*O*-malonyl		
1‴		167.03
3‴		168.28

[a] May be interchanged.

phase system, indicating that the former pigment was less polar than the latter. The molecular ion m/z 617 in the ES-MS spectrum of **4** corresponded to a methylated form of pigment **2**. The fragment ion at m/z 369 was in agreement with methylesterfication of the carboxyl group of the aglycone 5-carboxypyranocyanidin. This esterification, which seems to produce a bathochromic shift in the visible absorption maximum (Table 1), may also be caused by the solvent (acidified methanol).

The carboxypyranoanthocyanins **1** and **2** were recognized by their characteristic UV-Vis spectra recorded during HPLC with diode array detector (Table 1). However, due to the fact that these anthocyanins occur as minor pigments in red onion, they were difficult to detect in HPLC profiles of the crude extract. They were identified in enriched fractions after size exclusion chromatography (Sephadex LH-20). There were no qualitative differences between the carboxypyranoanthocyanin content of the dry outer scales and the rest of the pigmented scales. Discovery of pyranocyanidins in *Rosa* petals (Fukui et al., 2002) and red onions suggest that carboxypyranoanthocyanins may have a more widespread occurrence than in grape marc and wines. It has been shown that anthocyanins with 4-substituted aglycones have favourable properties such as higher resistance to bleaching by sulphur dioxide and higher colour intensity at pH values up to 7, compared for instance to malvidin 3-glucoside (Bakker and Timberlake, 1997; Romero and Bakker, 2000).

We suggest to use carboxypyranocyanidin as trival name for the aglycone of **1** and **2**. Similarly we suggest to use carboxypyranopetunidin and carboxypyranomalvidin for the petunidin and malvidin analogous, respectively. The sugar derivative of this latter compound has been given the name vitisin A. However, the name vitisin A has for many years been used for a stilbene tetramer.

3. Experimental

3.1. Isolation of pigments

The pigmented scales of 2.37 kg red onion bought on the local food market were cut into pieces and extracted (two times) with 0.5% TFA in MeOH at 4 °C. The filtered extract was concentrated under reduced pressure, purified by partition against EtOAc (equal volume) four times before application to an Amberlite XAD-7 column (Andersen, 1988). The anthocyanins were further purified on a Sephadex LH-20 column (100×5 cm) using MeOH–H_2O–TFA (39.6:60:0.4; v/v) as an eluent. The flow rate was 2.5 ml min^{-1}. Prior to elution of pigment **1**–**4** (175 ml), 2515 ml of the mobile phase was eluted. Pure anthocyanins were then isolated by preparative HPLC.

From another portion of red onions (2.87 kg), the dry outer scales were separated from the rest of the pigmented scales. Thereafter the pigments were isolated "simultaneously" from both portions using the same procedure as described above including extraction, purification by ethyl acetate and Amberlite XAD-7 chromatography, and Sephadex LH-20 chromatography.

Preparative HPLC (Gilson 305/306 pump equipped with an HP-1040A detector) was performed with an ODS-Hypersil column (25×2.2 cm, 5 μm) using the solvents HCOOH–H_2O (1:19, v/v) (A) and HCOOH–H_2O–MeOH (1:4:5, v/v) (B). The elution profile consisted of a linear gradient from 10% B to 100% B for 45 min, isocratic elution (100% B) for the next 13 min, followed by linear gradient from 100% B to 10% B for 1 min. The flow rate was 14 ml min^{-1}, and aliquots of 300 μl were injected.

Analytical HPLC was performed with an ODS-Hypersil column (25×0.3 cm, 5 μm) using the solvents HCOOH–H_2O (1:9) (A) and HCOOH–H_2O–MeOH (1:4:5) (B). The elution profile consisted of a linear gradient from 10% B to 100% B for 17 min, isocratic elution, 100% B, for the next 6 min, followed by linear gradient from 100% B to 10% B for 1 min. The flow rate was 0.5 ml min^{-1}, and aliquots of 10 μl were injected.

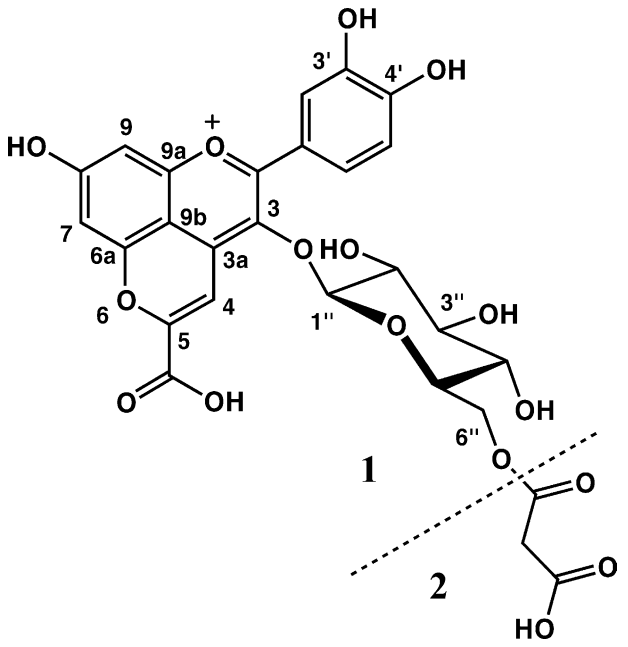

Fig. 2. The structures of 5-carboxypyranocyanidin 3-*O*-β-glucopyranoside (**1**) and 5-carboxypyranocyanidin 3-*O*-(6″-*O*-malonyl-β-glucopyranoside) (**2**) isolated from red onions.

3.2. Spectroscopy

UV-Vis absorption spectra were recorded on-line during HPLC analysis over the wavelength range 240–600 nm in steps of 2 nm. The NMR experiments were obtained at 600.13 MHz and 150.90 MHz for ^{1}H and ^{13}C respectively, on a Bruker DRX-600 instrument equipped with a multinuclear inverse probe for the 1D ^{1}H and the 2D Heteronuclear Single Quantum Coherence (HSQC), Heteronuclear Multiple Bond Correlations (HMBC) and Double Quantum Filtered Correlation Spectroscopy (DQF-COSY) experiments. The ^{13}C 1D CAPT experiment was performed on a $^{1}H/^{13}C$ BBO probe. Sample temperatures were stabilised at 25 °C. The deuteriomethyl ^{13}C signal and the residual ^{1}H signal of the solvent (CF_3CO_2D–DMSO-d_6; 1:4, v/v) were used as secondary references (δ 39.6 and δ 2.49 from TMS, respectively). See Fossen et al. (2001) for more experimental details.

5-Carboxypyranocyanidin 3-*O*-(6″-*O*-methylmalonyl-β-glucopyranoside), **3**. ^{1}H NMR: 7.92 (1H, *s*, H-4), 7.16 (1H, *d*, $J=1.9$ Hz, H-7), 7.25 (1H, *d*, $J=1.9$ Hz, H-9), 7.88 (1H, *d*, $J=2.2$ Hz, H-2′), 6.92 (1H, *d*, $J=8.9$ Hz, H-5′), 7.99 (1H, *dd*, $J=2.2$, 8.9 Hz, H-6′), 4.60 (1H, *d*, $J=7.8$ Hz, H-1″), 3.50 (1H, *m*, H-2″), 3.20 (1H, *m*, H-3″), 3.14 (1H, *m*, H-4″), 3.22 (1H, *m*, H-5″), 4.02 (1H, *dd*, $J=11.8$, 1.9 Hz, H-6A″), 3.99 (1H, *dd*, $J=11.8$, 5.7 Hz, H-6B″).

Mass spectral data on **1**–**4** were achieved by a LC–MS system (Waters 2690 HPLC-system connected to Micromass LCZ mass spectrometer) with electrospray ionization in positive mode (ESP+). The following ion optics were used: Capillary 3 kV, cone 30 V and 60 V, and extractor 7 V. The source block temperature was 120 °C and the desolvation temperature was 150 °C. The electrospray probe-flow was adjusted to 100 μl/min. Continuous mass spectra were recorded over the range m/z 150-800 with scan time 1 s and interscan delay 0.1 s.

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