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Dimeric anthocyanins from strawberry (*Fragaria ananassa*) consisting of pelargonidin 3-glucoside covalently linked to four flavan-3-ols

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Abstract

Flavanol–anthocyanin complexes were isolated by successive use of Amberlite XAD-7 chromatography, Sephadex LH-20 gel filtration and preparative HPLC from acidified, methanolic extract of strawberries (*Fragaria ananassa* Dutch.). These purple minor pigments were characterized by UV–Vis spectroscopy, 1D and 2D NMR techniques, and electrospray mass spectrometry to be catechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**1**), epicatechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**2**), afzelechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**3**) and epiafzelechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**4**). The stereochemistry at the 3- and 4-positions of the flavan-3-ol units was based on assumption of *R*-configuration at C-2. Each of the four pigments occurred in the NMR solvent as a pair of rotamers. Proved by cross-peaks in the ^{1}H–^{1}H NOESY NMR spectra of **1**, **2** and **4**, the two conformations within each rotameric pair were in equilibrium with each other. Even though **1** and **2** are based on a different aglycone, their structures may be similar to tentatively identified pigments, which have been assumed to contribute to the colour of red wines.

Keywords: Strawberry; *Fragaria ananassa*; Rosacea; Flavanol–anthocyanin; Catechin–pelargonidin 3-glucoside; Epicatechin–pelargonidin 3-glucoside; Afzelechin–pelargonidin 3-glucoside; Epiafzelechin–pelargonidin 3-glucoside; Rotamers; NOESY NMR exchange

1. Introduction

The major flavonoids in strawberry fruits have been described as the 3-glucosides of pelargonidin, cyanidin (anthocyanins), kaempferol and quercetin (flavonols), catechin (flavanol), and leucocyanidins of different degrees of polymerisation (Co and Markakis, 1968; and references herein), in addition to pelargonidin 3-(6-malonylglucoside) and the 3-rutinosides of cyanidin and pelargonidin (Tamura et al., 1995; Bridle and García-Viguera, 1997; Lopes da Silva et al., 2002). From the roots of *Fragaria* x *ananassa*, (+)-taxifolin 3-*O*-α-L-arabinofuranoside, a hydrolysable tannin (pedunculagin), (+)-catechin, and the condensed tannins, (+)-afzelechin-(4α-8)-(+)-catechin, procyanidin B-3 and procyanidin B-6, have been isolated (Ishimaru et al., 1995). Recently, 5-carboxypyranopelargonidin 3-glucoside has been isolated in small amounts from acidified, methanolic extract of strawberries (Andersen et al., 2004). Strawberries have been found to inhibit oesophageal cancer and to reverse the course of neuronal and behavioural aging in rats (Joseph et al., 2000; Torronen and Maatta, 2002). The antioxidant activity of strawberries in vitro oxidation assays has been correlated with anthocyanin content in the fruits (Heinonen et al., 1998; Wang and Lin, 2000). Hebert et al. (2002) have recently reported that strawberry proanthocyanidins may act both as antifungal compounds to extend shelf life and as antioxidants to enhance quality preservation.

The major objective of this study was to present structure identification of four novel dimeric flavonoids (**1**–**4**) isolated from strawberry extracts, comprising of an anthocyanin unit (pelargonidin 3-*O*-β-glucopyranoside) connected with a *C*–*C* binding to four different flavan-3-ols. These purple flavonoids may be formed

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along similar mechanisms as postulated for some of the pigments contributing to the colour of red wines (Jurd, 1967; Haslam, 1980; Remy et al., 2000; Salas et al., 2004), however, the complete structure of this type of dimeric flavonoids has previously not been elucidated. Some stereochemical considerations including chemical shifts (^{1}H and ^{13}C) of two rotamers of each pigment are also presented.

2. Results and discussion

The HPLC profile of the acidified methanolic extract of strawberries (*Fragaria ananassa* Dutch.) detected in the visible region showed one major, pelargonidin 3-*O*-β-glucopyranoside, **5** (Pedersen et al., 1993), and several minor anthocyanins. The retention times of some of the minor pigments were relatively short compared to the corresponding retention time of **5** (Table 1). The UV–Vis spectra of these pigments recorded on-line during HPLC analysis showed two visible absorption maxima at 516–520 and 432–438 nm (Fig. 1 and Table 1), indicating dimeric flavonoids containing one anthocyanidin unit linked to another flavonoid moiety. After purification of these dimeric flavonoids in the extract by partition against ethyl acetate followed by column chromatography (Amberlite XAD-7 and Sephadex LH-20), they were separated by preparative HPLC into two groups. Pigment **1** (major) and **2** (minor) were separated from **4** (major) and **3** (minor). The two groups were analysed separately by NMR. Thereafter, **1** and **2** were separated from each other by preparative HPLC, and analysed by ^{1}H NMR. Mass spectra of the individual pigments were achieved by LC–MS (Table 1).

The downfield region of the ^{1}H NMR spectrum of **1** showed a 1H singlet at δ 9.14 (H-4A); a 4H AA′XX′ system at δ 8.30 (*d*, 9.1 Hz; H-2′A, 6′A) and δ 7.06 (*d*, 9.1 Hz; H-3′A,5′A); and a 1H singlet at δ 6.90 (H-6A) (Table 2). This singlet was assigned to H-6A by the cross-peak at δ 6.90/102.7 in the ^{1}H–^{13}C HSQC spectrum of **1** after comparison with the chemical shifts of C-6A and C-8A of **5** (Table 3). The one-bond correlation at δ 9.14/138.3 in the ^{1}H–^{13}C HMBC spectrum of **1** was assigned to C-4A. In the same spectrum H-4A revealed long-range correlations to C-2A, C-3A, C-5A, C-9A and C-10A (Fig. 3). The assignments of C-2A, C-5A and C-10A were confirmed by the cross-peaks at δ 8.30/163.9 (H-2′A,6′A/C-2A), δ 6.90/157.9 (H-6A/C-5A) and 6.90/

Table 1
Chromatographic (HPLC) and spectral (UV–Vis spectroscopy and electrospray mass spectrometry) data of catechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**1**), epicatechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**2**), afzelechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**3**), epiafzelechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**4**) and pelargonidin 3-*O*-β-glucopyranoside (**5**) isolated from strawberry extract

Compound	ES-MS[a]		On-line HPLC (diode array detection)		
	$[A]^+$ (*m*/*z*)	$[M]^+$ (*m*/*z*)	$\lambda_{Vis-max}$ (nm)	$A_{440}/A_{vis-max}$ (%)	t_R (min)
1	559	721	518, 438	81	10.40
2	559	721	518, 433	70	10.59
3			516, 434	69	11.73
4	543	705	520, 432	68	12.28
5	271	433	504	43	17.65

[a] $[A]^+$, aglycone fragment ion; $[M]^+$, molecular ion.

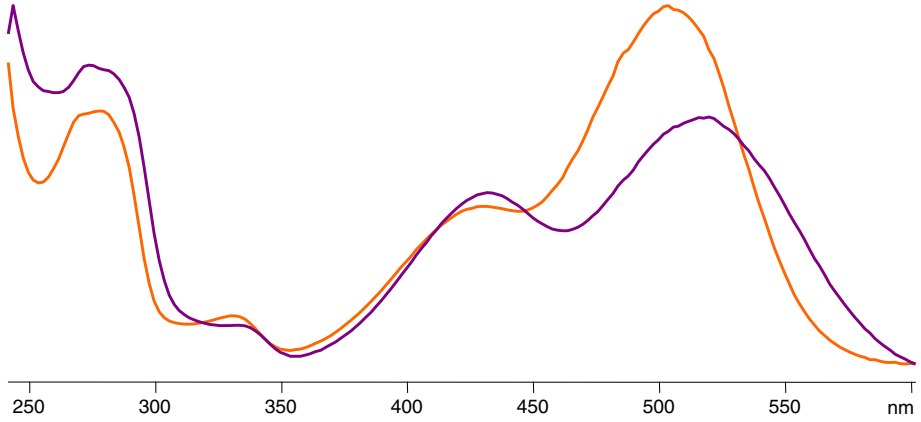

Fig. 1. The UV–Vis spectra of epiafzelechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**4**) (purple) and pelargonidin 3-glucoside (**5**) (orange) isolated from strawberry extract.

Table 2
^{1}H NMR chemical shifts and coupling constants of the anthocyanin-flavanol complexes (**1**–**4**), and pelargonidin 3-glucoside (**5**) from strawberries in CD_3OD-CF_3COOD (95:5; v/v) at 25 °C

	1	**2**	**3**	**4**	**5**[a]
Pelargonidin					
4A	9.14 *s*	9.15 *s*	9.14 *s*	9.16 *s*	9.10 *s*
	9.21 *s*	9.20 *s*	9.21 *s*	9.21 *s*	
6A	6.90 *s*	7.00 *s*	6.89 *s*	6.99 *s*	6.74 *d*, 1.2 Hz
	6.79 *s*	6.76 *s*		6.75 *s*	
8A					6.96 '*d*', 1.2 Hz
2′A,6′A	8.30 '*d*', 9.1 Hz	7.79 '*d*', 9.1 Hz	8.31 '*d*', 9.2 Hz	7.79 '*d*', 9.2 Hz	8.61 '*d*', 9.1 Hz
	8.77 '*d*', 9.2 Hz	8.64 '*d*', 9.2 Hz	8.77 '*d*', 9.2 Hz	8.66 '*d*', 9.2 Hz	
3′A,5′A	7.06 '*d*', 9.1 Hz	6.97 '*d*', 9.1 Hz	7.06 '*d*', 9.2 Hz	6.97 '*d*', 9.2 Hz	7.10 '*d*', 9.1 Hz
	7.17 '*d*', 9.2 Hz	7.09 '*d*', 9.2 Hz	7.17 '*d*' 9.2 Hz	7.08 '*d*', 9.2 Hz	
3-Glucoside					
1″	5.26 *d*, 7.8 Hz	5.35 *d*, 7.7 Hz	5.26 *d*, 7.9 Hz	5.35 *d*, 7.7 Hz	5.37 *d*, 8.0 Hz
	5.39 *d*, 7.7 Hz	5.41 *d*, 7.8 Hz	5.27 *d*, 7.9 Hz	5.41 *d*, 7.7 Hz	
2″	3.69 *dd*, 7.7, 9.1 Hz	3.66 *dd*, 7.7, 9.2 Hz	3.70 *m*	3.66 *dd*, 7.7, 9.2 Hz	3.76 *dd*, 8.0, 9.1 Hz
	3.74 *dd*, 7.7, 9.2 Hz	3.75 *m*		3.74 *dd*, 7.7, 9.2 Hz	
3″	3.58 *t*, 9.1 Hz	3.63 *t*, 9.2 Hz	3.57 *m*	3.59 *t*, 9.2 Hz	3.69 *dd*, 9.1, 8.9 Hz
	3.65 *m*	3.65 *m*	3.58 *m*	3.65 *m*	
4″	3.52 *dd*, 9.8, 9.1 Hz	3.48 *dd*, 9.8, 9.2 Hz	3.51 *m*	3.48 *t*, 9.2 Hz	3.58 *dd*, 8.9, 9.9 Hz
	3.55 *dd*, 9.8, 9.2 Hz	3.53 *m*	3.53 *m*	3.52 *m*	
5″	3.60 *ddd*, 9.8, 5.9, 2.4 Hz	3.61 *m*	3.59 *m*	3.63 *m*	3.22 *ddd*, 9.9, 6.0, 2.3 Hz
	3.65 *m*	3.65 *m*		3.66 *m*	
6A″	4.00 *dd*, 12.1, 2.4 Hz	3.99 *dd*, 12.1, 2.4 Hz	3.99 *m*	3.98 *dd*, 12.1, 2.2 Hz	4.05 *dd*, 12.3, 2.3 Hz
	4.01 *dd*, 12.1, 2.3 Hz	4.00 *m*		4.01 *dd*, 12.1, 2.2 Hz	
6B″	3.80 *dd*, 12.1, 5.9 Hz	3.79 *dd*, 12.1, 5.9 Hz	3.80 *m*	3.77 *dd*, 12.1, 5.9 Hz	3.82 *dd*, 12.3, 6.0 Hz
	3.83 *dd*, 12.1, 5.9 Hz	3.78 *dd*, 12.1, 5.9 Hz		3.82 *dd*, 12.1, 5.9 Hz	
Flavan-3-ol					
2F	4.81 *d*, 9.2 Hz	4.95 *m*	4.88 *d*, 9.4 Hz	5.01 *d*, 1.5 Hz	
	4.62 *d*, 9.3 Hz	5.34 *d*, 2.4 Hz	4.97 *d*, 9.4 Hz	5.39 *d*, 2.2 Hz	
3F	4.35 *t*, 9.2 Hz	4.19 *dd*, 2.9, 1.7 Hz	4.38 *t*, 9.4 Hz	4.18 *dd*, 2.9, 1.5 Hz	
	4.61 *m*	4.46 *m*	4.38	4.43 *dd*, 4.4, 2.2 Hz[b]	
4F	5.02 *d*, 9.2 Hz	4.84 *d*, 2.9 Hz	5.02 *d*, 9.4 Hz	4.82 *d*, 2.9 Hz	
	5.01	4.93 *d*, 4.6 Hz	5.02	4.94 *d*, 4.4 Hz	
6F	6.89 *d*, 2.0 Hz	6.82 *s*(b)	nd[c]	nd[c]	
8F	nd[c]	6.82 *s*(b)	nd[c]	nd[c]	
2′F	6.92 *d*, 2.0 Hz	6.97 *d*, 2.2 Hz	7.29	7.28 '*d*', 8.8 Hz	
	7.07 *d*, 2.0 Hz	7.05 *m*	7.45 '*d*', 8.8 Hz	7.36 '*d*', 8.8 Hz	
3′F			6.77 '*d*', 8.8 Hz	6.80 '*d*', 8.8 Hz	
			6.92 '*d*', 8.8 Hz	6.86 '*d*', 8.8 Hz	
5′F	6.76 *d*, 8.3 Hz	6.76 *d*, 8.3 Hz	6.77 '*d*', 8.8 Hz	6.80 '*d*', 8.8 Hz	
			6.92 '*d*', 8.8 Hz	6.86 '*d*', 8.8 Hz	
6′F	6.82 *dd*, 8.3, 2.0 Hz	6.73 *dd*, 2.2, 8.3 Hz	7.29	7.28 '*d*', 8.8 Hz	
	6.95 *dd*, 8.3, 2.0 Hz		7.45 '*d*', 8.8 Hz	7.36 '*d*', 8.8 Hz	

When two numbers are given for the same proton, they represent a pair of rotamers. The chemical shift values of the minor rotamer are at the bottom.

s, singlet; *d*, doublet; *dd*, double doublet; *ddd*, doublet of a double doublet; *m*, multiplet or overlapping signals in the 1D H NMR spectrum.

[a] See Pedersen et al. (1993)

[b] Coupling constant derived from the 2D ^{1}H–^{1}H COSY spectrum

[c] Not detected

113.9 (H-6A/C-10A), respectively. The carbons belonging to the pelargonidin B-ring were assigned by the long-range couplings in the HMBC spectrum (Fig. 3), in addition to the one-bond correlation at δ 7.06/117.5 (H-3′A,5′A/C-3′A,5′A) in the ^{1}H–^{13}C HSQC spectrum. These assignments are in accordance with the anthocyanidin pelargonidin linked to another moiety in its 8-position.

Additionally, a 3H ABX system at δ 6.92 (*d*, 2.0 Hz; H-2′F), δ 6.82 (*dd*, 2.0 and 8.3 Hz; H-6′F) and δ 6.76 (*d*, 8.3 Hz; H-5′F); a 3H ABX system at δ 4.81 (*d*, 9.2 Hz; H-2F), δ 4.35 (*t*, 9.2 Hz; H-3F) and δ 5.02 (*d*, 9.2 Hz; H-4F) were observed in the downfield region of the ^{1}H NMR spectrum of **1**. C-2F was assigned by the cross-peaks in the HMBC spectrum (Fig. 3). H-2F was then confirmed by the one-bond correlation at δ 4.81/83.5 (H-2F/C-2F) in the HSQC spectrum. The remaining protons of the catechin C-ring (H-3F and H-4F) could then be confirmed by the ^{1}H–^{1}H coupling constants in the 1D ^{1}H spectrum and the cross-peaks in the 2D ^{1}H–^{1}H

Table 3
^{13}C NMR chemical shifts and coupling constants of dimeric flavanol–anthocyanin complexes (**1**, **2** and **4**), and pelargonidin 3-glucoside (**5**) from strawberries in CD_3OD-CF_3COOD (95:5; v/v) at 25 °C

	1	**2**	**4**	**5**[a]
Pelargonidin				
2A	163.9 (163.6)	165.2 (163.5)	165.4 (164.2)	163.9
3A	144.5 (144.6)	144.8 (144.4)	144.6 (144.6)	145.3
4A	138.3	nd	137.3	137.1
5A	157.9 (158.2)	157.3 (158.5)	157.9 (157.3)	157.5
6A	102.7 (103.4)	103.0 (103.6)	102.8 (103.4)	103.5
7A	170.1 (169.9)	nd	nd	170.6
8A	111.4	110.4	110.8	95.3
9A	154.5 (155.5)	154.6 (154.8)	155.9 (155.3)	157.4
10A	113.9 (114.1)	113.0 (113.6)	nd	113.4
1′A	121.0 (121.1)	121.3 (121.2)	120.5 (121.5)	120.6
2′A,6′A	135.7 (135.3)	136.2 (135.6)	136.1 (135.5)	135.7
3′A,5′A	117.5 (117.9)	115.8 (116.2)	117.1 (117.5)	117.9
4′A	165.8 (165.8)	165.6 (166.1)	166.2 (166.2)	166.6
3-Glucoside				
1″	103.9 (103.8)	103.1 (103.9)	103.2 (103.8)	103.7
2″	74.7 (74.8)	74.6 (75.0)	74.8 (74.8)	74.8
3″	78.2 (78.5)	78.3 (78.5)	78.1 (78.3)	78.1
4″	71.0 (70.7)	71.0 (70.9)	71.0 (71.1)	71.1
5″	78.4 (78.5)	78.3 (78.5)	78.3 (78.7)	78.7
6″	62.3	62.5 (62.3)	62.3 (62.5)	62.5
Flavan-3-ol				
2F	83.5 (84.0)	77.9 (78.4)	78.2 (77.9)	
3F	72.8 (72.6)	73.0 (71.2)	72.8 (71.7)	
4F	39.0 (39.2)	37.9	37.9	
5F	157.7	nd	nd	
6F	nd	nd	nd	
7F	nd	nd	nd	
8F	nd	nd	nd	
9F	154.0 (154.1)	nd	nd	
10F	104.3	101.6	101.4 (101.8)	
1′F	131.6	131.5	131.0 (131.5)	
2′F	115.5 (116.2)	115.8 (115.0)	129.5 (129.4)	
3′F	146.0 (146.1)	145.4	115.5 (115.7)	
4′F	146.3 (146.1)	145.7 (145.7)	157.8 (158.0)	
5′F	115.5 (115.5)	115.5	115.5 (115.7)	
6′F	120.3 (121.1)	119.1 (120.9)	129.5 (129.4)	

When two numbers are given for the same carbon, they represent a pair of rotamers.
The chemical shift values for the minor rotamer are given in brackets.
nd, not detected
[a] See Pedersen et al. (1993).

DQF-COSY (double quantum filtered correlation spectroscopy) experiment (Table 2). The corresponding carbons were then assigned by the one-bond correlations at δ 4.35/72.8 (H-3F/C-3F) and δ 5.02/39.0 (H-4F/C-4F) in the HSQC spectrum. The carbon shifts corresponding to the catechin B-ring were identified by the long-range correlations in the HMBC spectrum (Fig. 3). Due to the rapid exchange of H-6F and H-8F with deuterium in the acidified deuterated solvent, these signals and their corresponding ^{13}C resonances (C-6F and C-8F), as well as their long-range correlation to C-7F, were absent in the HSQC and HMBC spectra, respectively. C-9F and C-10F were, however, assigned by the cross-peaks in the HMBC spectrum at δ 5.02/154.0 (H-4F/C-9F) and δ 5.02/104.3 (H-4F/C-10F), respectively. The large coupling constants between H-2F, H-3F and H-4F (9.2 Hz) confirmed *trans*-configurations. Assuming the usual *R*-configuration at C-2F, these coupling constants were in accordance with (+)-catechin ((2*R*,3*S*,4*R*)-3,5,7,3′,4′-pentahydroxyflavan) lacking one H-4 proton.

The cross-peaks at δ 5.02/170.1 (H-4F/C-7A), δ 5.02/154.5 (H-4F/C-9A) and δ 5.02/111.4 (H-4F/C-8A) were in accordance with a covalent *C–C* linkage between the catechin 4-position and the pelargonidin 8-position. The anomeric coupling constant (7.9 Hz) and the 6 ^{13}C resonances belonging to the sugar in the CAPT spectrum were in accordance with a β-glucopyranose unit (Pedersen et al., 1993). All the ^{1}H sugar resonances were assigned by the DQF-COSY experiment (Table 2), and the corresponding ^{13}C resonances were then assigned by

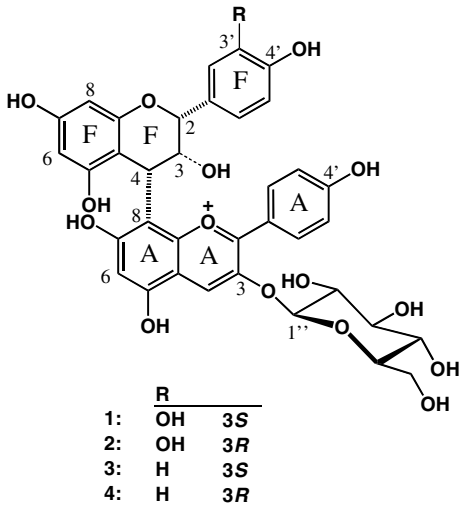

Fig. 2. The structures of the flavan-3-ol(4α → 8)pelargonidin 3-*O*-β-glucopyranosides (**1**–**4**) isolated from strawberry extract. The letter A denotes the aglycone ring systems belonging to the anthocyanidin substructure, whereas the letter F denotes the aglycone ring systems belonging to the flavanol substructure.

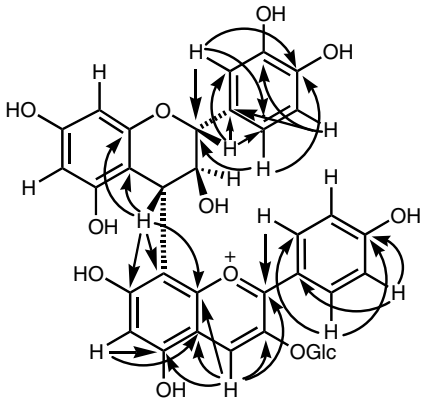

Fig. 3. Most of the long-range heteronuclear couplings of the aglycone in the ^{1}H–^{13}C HMBC spectrum of catechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**1**).

the HSQC spectrum and the 1D ^{13}C CAPT spectrum (Table 3). The cross-peak at δ 5.26/144.5 (H-1″/C-3A) in the HMBC spectrum confirmed that the glucose unit was attached to the pelargonidin 3-position. A molecular ion in the electrospray MS spectrum of **1** at *m*/*z* 721 corresponded to a covalent catechin-pelargonidin dimer with one hexose. A fragment ion at *m*/*z* 559 showed the same flavonoid dimer without the sugar moiety. Thus, pigment **1** was identified as catechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside.

The NMR resonances of pigment **2** shared many similarities with the corresponding resonances of **1** (Tables 2 and 3), in accordance with a flavonoid dimer consisting of pelargonidin 3-glucoside covalently linked to one 3,5,7,3′,4′-pentahydroxyflavan unit. However, the chemical shift values and the coupling constants of H-2F (δ 4.95 *s*(b)), H-3F (δ 4.19 *dd*, 2.9 Hz, 1.7 Hz) and H-4F (4.84 *d*, 2.9 Hz) were different from the corresponding values of **1** (Table 2). These small coupling constants were in accordance with (2*R*,3*R*,4*R*)-3,5,7,3′,4′-pentahydroxyflavan ((−)-epicatechin) lacking one proton in the 4-position. The strong cross-peak at δ 4.95/4.84 (H-2F/H-4F) in the ^{1}H–^{1}H NOESY spectrum of **2** established the all-*cis*-stereochemistry for this compound. A molecular ion and fragment ion at *m*/*z* 721 and 559, respectively, in the electrospray MS spectrum, corresponded to epicatechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**2**). The signal corresponding to H-2′A,6′A of the pelargonidin unit in the ^{1}H NMR spectrum of pigment **2** was observed considerably upfield (δ 7.79) compared to the corresponding value of pelargonidin 3-glucoside (δ 8.61) (Table 2), most probably because of the steric influence of the flavanol unit (Fig. 2).

The 1D ^{1}H and the 2D DQF-COSY, HSQC and HMBC NMR spectra of pigment **4** showed many similarities with the corresponding spectra of **1** and **2**. However, the substitution pattern of the flavanol B-ring revealed a AA′XX′ system at δ 7.28 (*d*, 8.8 Hz; H-2′F,6′F) and δ 6.80 (*d*, 8.8 Hz; H-3′F,5′F). After assignments of the proton and carbon resonances (Tables 2 and 3), it was shown that **4** contained pelargonidin 3-glucoside linked to a 3,5,7,4′-*cis*-tetrahydroxyflavan (epiafzelechin) unit. The signal corresponding to H-2′A,6′A of the pelargonidin unit in the ^{1}H NMR spectrum of pigment **4** showed the same upfield shift (δ 7.79) as observed for **2** (Table 2). A fragment ion and molecular ion in the electrospray MS spectrum of **4** at *m*/*z* 543 and 705, respectively, were thus in accordance with epiafzelechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside.

After assignments of the ^{1}H resonances of pigment **3** (Tables 2 and 3), based on similar arguments as presented for **4**, pigment **3** was identified to be a dimeric flavonoid consisting of pelargonidin 3-glucoside covalently linked to a 3,5,7,4′-tetrahydroxyflavan unit. The large coupling constants of the flavanol C-ring protons (9.4 Hz) indicated that these protons were in *trans*-configuration to each other, in accordance with afzelechin(4α → 8)pelargonidin 3-*O*-β-glucopyranoside (**3**).

Because of rotational hindrance at the C(sp^2)–C(sp^3) linkage of the C-8A–C-4F bond, conformational isomers of **1**–**4**, respectively, leading to duplication of the NMR signals, were identified in the NMR spectra (Tables 2 and 3). One major and one minor rotamer was identified for each of the pigments, respectively. Strong cross-peaks due to rotational equilibrium between equivalent protons within each pair were observed in the NOESY spectra of **1**, **2** and **4**. The strongest exchange peaks were observed for **2** and **4**, which have the protons of the flavanol *C*-ring in *cis*-configuration to each other. The strong exchange peaks in the NOESY spectrum between the protons of **4a** (major rotamer) and the

corresponding protons of **4b** (minor rotamer) at δ 9.16/9.21 (**4a**H-4A/**4b**H-4A), δ 6.99/6.75 (**4a**H-6A/**4b**H-6A), δ 7.79/8.66 (**4a**H-2′A,6′A/**4b**H-2′A,6′A), δ 6.97/7.08 (**4a**H-3′A,5′A/**4b**H-3′A,5′A), δ 5.35/5.41 (**4a**H-1″/**4b**H-1″), δ 3.66/3.74 (**4a**H-2″/**4b**H-2″), δ 5.01/5.39 (**4a**H-2F/**4b**H-2F), δ 4.18/4.43 (**4a**H-3F/**4b**H-3F), δ 4.82/4.94 (**4a**H-4F/**4b**H-4F), δ 7.28/7.36 (**4a**H-2′F,6′F/**4b**H-2′F,6′F) and δ 6.80/6.86 (**4a**H-3′F,5′F/**4b**H-3′F,5′F), respectively, revealed rotational equilibrium. Similar exchange peaks were observed in the NOESY spectrum of pigment **1** and **2**, respectively.

In accordance with previous studies on rotameric conformers of flavone C-glycosides (Zhang et al., 2003), the rotameric equilibrium is temperature-dependent. Based on integration data from the 1D ^{1}H experiments performed at different temperatures, the relative proportions of the major and minor rotamer of pigment **4** were 1.00:0.64, 1.00:0.75 and 1.00:0.80 at 277, 298 and 310 K, respectively. Rotameric isomers have previously been identified for several C-glycosylated flavones (Markham et al., 1987; Jay, 1993; Lewis et al., 2000; Nørbæk et al., 2000; Kumazawa et al., 2001) and dimeric flavonoids (procyanidins) (Weinges et al., 1970). However, no duplication of the NMR signals caused by restricted rotation was observed for the dimeric flavanol 3-glucosides recently identified in young leaves of *Inga umbellifera* (Lokvam et al., 2004). In this latter case it was indicated that the glycoside units prevent subunit rotation around the interflavanyl bond. According to our results (Tables 2 and 3), this is not the case for **1**–**4**, when the glucosyl moiety is linked to the 3-position of the lower anthocyanin moiety (Fig. 2).

Although pigment **1**–**4** are observed in the HPLC profile of the crude extract, we cannot totally exclude that these pigments are formed in vitro by similar processes that occur during wine conservation and maturation. A steady decline in the concentration of the original anthocyanins in wine is related to formation of more complex and stable pigments. Some of these pigments have been suggested formed by the condensation of anthocyanins with other grape-derived polyphenols such as flavan-3-ols/tannins either directly or mediated through acetaldehyde (Pissarra et al., 2003; Remy-Tanneau et al., 2003; and references herein). Two related mechanisms involving nucleophilic addition have been proposed for direct reaction of anthocyanins with flavanols (Remy-Tanneau et al., 2003). According to the first mechanism a flavylium ion is expected to react with nucleophiles owing to the partial positive charges born by the flavylium nucleus at the 2- and 4-positions. Indeed, recent analysis of reaction products after incubation of malvidin 3-glucoside and epicatechin in ethanol have shown the formation of colorless dimers (Remy-Tanneau et al., 2003), and the major pigment was identified as malvidin 3-glucoside(C2–O–C7,C4–C8)epicatechin. In the second type of reactions a carbocation formed from procyanidin reacts with the nucleophilic C-6 and C-8 positions of an anthocyanin in its hydrated hemiketal form (AOH) (Jurd, 1967; Haslam, 1980) producing a colourless dimer, which dehydrates to the flavylium form of the anthocyanin moiety. Such pigments have been tentatively identified in wine by liquid chromatography/mass spectrometry (Remy et al., 2000; Salas et al., 2004). Even though **1** and **2** are based on a different aglycone, their structures may be analogous to the tentatively identified pigments, which have been assumed to contribute to some of the colour of red wines.

3. Experimental

3.1. Isolation of pigments

Fresh strawberries (1.25 kg) were extracted three times with 1.5 litre MeOH containing 0.5% trifluoracetic acid (TFA) at 3 °C. The extract was filtered through glass wool, concentrated under reduced pressure, purified by partition against EtOAc (equal volume) and applied to an Amberlite XAD-7 column (Andersen, 1988).

The anthocyanin extracts was further purified by Sephadex LH-20 chromatography (column: 100 × 5 cm, Pharmacia) using MeOH:TFA:H_2O (19.8:0.2:80.0, v/v) as mobile phase. The flow rate was 2.5 ml/min. Initially, 1500 ml mobile phase was eluted prior to the first fraction containing anthocyanins. Then, 1400 ml mobile phase containing the monomeric anthocyanins was collected before the mobile phase composition was changed to 49.5:0.5:50.0 (v/v). After collection of further 2430 ml mobile phase containing monomeric anthocyanins followed by elution of 2050 ml mobile phase, the fraction (appearing as a purple band) containing the flavanol–anthocyanin complexes (500 ml) was collected.

Individual anthocyanins in the Sephadex LH-20 fraction containing the flavanol–anthocyanin complexes were separated by preparative HPLC using the solvents HCOOH:H_2O (0.5:9, v/v) (A) and MeOH:HCOOH:H_2O (5:0.5:4, v/v) (B). The elution profile consisted of a linear gradient from 10% B to 100% B in 45 min, isocratic elution (100% B) for the next 13 min, followed by linear gradient from 100% to 10% B for 1 min. The flow rate was 14 ml/min and aliquots of 300 ml were injected.

3.2. Analytical HPLC

Analytical HPLC was performed with an ODS-Hypersil column (20 × 0.5 cm, 5 μm) using the solvents HCOOH:H_2O (0.5:9, v/v) (A) and MeOH:HCOOH:H_2O (5:0.5:4, v/v) (B). The elution profile consisted of a linear gradient from 10% B to 100% B in 23 min, isocratic elution (100% B) for the next 5 min, followed

by linear gradient from 100% to 10% B for 1 min. The flow rate was 0.75 ml/min and aliquots of 15 μl were injected.

3.3. Spectroscopy

The 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 recorded at 600.13 and 150.92 MHz for ^{1}H and ^{13}C, respectively, on a Bruker DRX-600 instrument equipped with a multinuclear inverse probe for all experiments but the Compensated Attached Proton Test (CAPT) experiment, which was performed on a $^{1}H/^{13}C$ BBO probe. The temperature was stabilized at 25 °C. Sample 1 (3 mg), which contained **1** and **2** in a 1.4:1.0 ratio, and sample 2 (2.5 mg), which contained **4** and **3** in a 3.7:1.0 ratio, were dissolved in CF_3COOD:CD_3OD (5:95; v/v). The residual solvent signal at 3.4 ppm for ^{1}H and solvent signal at 49.0 for ^{13}C was used as secondary references. The proton–carbon shift correlations by long-range coupling were established using the ^{1}H–^{13}C Heteronuclear Multiple Bond Correlation (HMBC) experiment. This experiment was optimized for $^{n}J_{CH}$ coupling constant of 8 Hz. The spectral widths were 27,165 Hz in f_1 and 4960 Hz in f_2. Seventy FIDs were recorded in t_1 and $2k$ points were recorded in t_2, and 2400 transients were collected for each t_1 increment. The one-bond proton–carbon shift correlations were established by heteronuclear single quantum coherence (HSQC) experiment. This experiment was optimized for a one-bond proton–carbon coupling constant of 145 Hz. One hundred FIDs were recorded in t_1 and $2k$ data points were recorded in t_2, and 700 transients were collected for each t_1 increment. The spectral widths were 21,128 Hz in f_1 and 4921 Hz in f_2. The one-bond proton–proton shift correlations were established by the DQF-COSY experiment. The DQF-COSY experiment was optimized for a one-bond proton–proton coupling constant of 7.5 Hz. One hundred and twenty FIDs were recorded in t_1 and $4k$ data points were recorded in t_2, and 140 transients were collected for each t_1 increment. The spectral width was 2201 Hz. Total proton–proton shift correlations were established by the total correlation spectroscopy (TOCSY) experiment. While 140 FIDs were recorded in t_1 and $2k$ data points were recorded in t_2, 160 transients were collected for each t_1 increment. The spectral width was 1395 Hz. Proton–proton couplings through space were established by the nuclear Overhauser effect spectroscopy (NOESY) experiment. While 142 FIDs were recorded in t_1 and $2k$ data points were recorded in t_2, 232 transients were collected for each t_1 increment. The spectral width was 4960 Hz. The CAPT experiment was performed using 31,240 transients and the spectral width was 24,038 Hz.

Mass spectral data were achieved by an LC–MS system (Waters 2690 HPLC-system connected to Micromass LCZ mass spectrometer) with electrospray ionisation in positive mode (ESP+). The following ion optics were used: capillary, 3 kV; cone, 30 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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