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C-glycosylanthocyanidins synthesized from C-glycosylflavones

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ABSTRACT

Nine *C*-glycosyldeoxyanthocyanidins, 6-*C*-β-glucopyranosyl-7-*O*-methylapigeninidin, 6-*C*-β-glucopyranosyl-7-*O*-methylluteolinidin, 6-*C*-β-(2″-*O*-β-glucopyranosylglucopyranosyl)-7-*O*-methylapigeninidin, 6-*C*-β-(2″-*O*-β-glucopyranosylglucopyranosyl)-7,4′-di-*O*-methylapigeninidin, 8-*C*-β-glucopyranosylapigeninidin, 8-*C*-β-(2″-*O*-α-rhamnopyranosylglucopyranosyl)apigeninidin, 8-*C*-β-(2″-*O*-α-(4‴-*O*-acetylrhamnopyranosyl)glucopyranosyl)apigeninidin, 6,8-di-*C*-β-glucopyranosylapigeninidin (**8**), 6,8-di-*C*-β-glucopyranosyl-4′-*O*-methylluteolinidin (**9**), have been synthesized from their respective *C*-glycosylflavones (yields between 14% and 32%) by the Clemmensen reduction reaction using zinc-amalgam. The various precursors (*C*-glycosylflavones) of the *C*-glycosylanthocyanidins were isolated from either flowers of *Iris sibirica* L., leaves of Hawthorn 'Crataegi Folium Cum Flore', or lemons and oranges. This is the first time *C*-glycosylanthocyanidins have been synthesized. The structures of all flavonoids including the flavone rotamers were elucidated by 2D NMR techniques and high-resolution electrospray MS. The distribution of the various structural forms of **8** and **9** are different at pH 1.1, 4.5, and 7.0, however, the two pigments undergoes similar structural transformations at the various pH values. Pigments **8** and **9** with *C*–*C* linkages between the sugar moieties and the aglycone, were found to be far more stable towards acid hydrolysis than pelargonidin 3-*O*-glucoside, which has the typical anthocyanidin *C*–*O* linkage between the sugar and aglycone. This stability may extend the present use of anthocyanins as nutraceuticals, pharmaceuticals or colorants.

1. Introduction

The anthocyanins produce most red to blue colours in plants, and nearly 600 natural anthocyanins have hitherto been reported (Andersen and Jordheim, 2006). In recent decades there have been considerable interests in the development of new anthocyanin-based food colorants (Francis, 1989), including the use of deoxyanthocyanins (Awika et al., 2004). The motive of extended use is increasing with their potential positive health effects (Clifford and Brown, 2006), including for instance antioxidant activity (Zheng and Wang, 2003; Prior and Wu, 2006), anticarcinogenic effects (Hou, 2003; Dreiseitel et al., 2008) and anti-viral activity (Andersen et al., 1997; Knox et al., 2001). Their stabilities are affected by a number of factors including temperature, light, oxygen, enzymes, etc. (Francis, 1989; Matsufuji et al., 2007). A particular problem is the pH influence on the anthocyanin properties; the stability of most anthocyanins is particularly low under weakly acidic and neutral conditions (Brouillard and Dangles, 1994; Cabrita et al., 2000; Torskangerpoll and Andersen, 2004). In nearly all previously reported natural anthocyanins the sugar moieties are connected to the anthocyanidins through an *O*-linkage. When ingested the anthocyanidin *O*-glycoside may be hydrolysed to anthocyanidins (aglycones), which are significantly more unstable than the intact anthocyanins. Loss of sugar units may also limit *in vivo* transportation.

The conversion of flavonoids to the corresponding anthocyanins by the Clemmensen reduction using zinc-amalgam has previously been performed on a limited number of flavonols (Iacobucci and Sweeny, 1983). Elhabiri et al. have synthesized cyanidin and cyanidin 3-rutinoside by reduction of the corresponding flavonols, quercetin (1995a) and quercetin 3-rutinoside (1995b), respectively, and converted synthetic 7,3′,4′-tri-*O*-methylquercetin 3-*O*-β-D-glucoside and 5,3′-di-*O*-methylquercetin 3-*O*-β-D-glucoside to the corresponding anthocyanins (1995b). Recently, Oyama et al. (2007) synthesized pelargonidin 3-*O*-(6″-acetylglucoside) from the analogous synthetic kaempferol-glucoside. Some flavanones and flavone aglycones have been reduced with sodium-amalgam to their corresponding benzopyrylium compounds (Asahina et al., 1929). The flavone *O*-glucoside, apiin, and some flavanone *O*-glycosides likewise gave on reduction substances forming red to violet-red dyes. However these latter products could not be isolated in pure form (Asahina et al., 1929). It has also been reported that when the flavone *O*-glucoside, toringin, was reduced using magnesium, the acidic alcoholic solution turned pink (Shibata et al., 1919).

Although *C*-glycosylflavones are relatively common in nature (Jay et al., 2006), only two *C*-glycosylanthocyanidins, 8-*C*-β-glucosylcyanidin 3-*O*-β-(6″-*O*-malonylglucoside) and 8-*C*-β-(6‴-*O*-*trans*-

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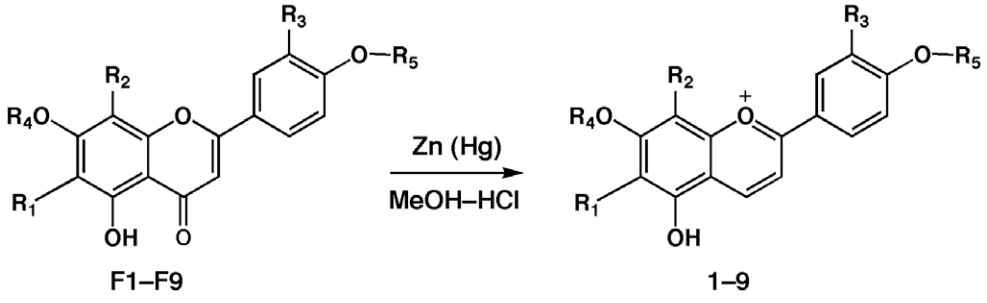
sinapoylglucosyl)cyanidin 3-*O*-β-(6″-*O*-malonylglucoside), have previously been reported (Saito et al., 2003; Tatsuzawa et al., 2004). These anthocyanins were together with four common cyanidin *O*-glycoside isolated from flowers of the toad lily (*Tricyrtis formosana*). No *C*-glycosylanthocyanidin has previously been synthesized, and *C*-glycosylflavones have as far as we know not been used before as starting material for production of anthocyanins in reductive synthesis.

In this paper we have extended the use of zinc-amalgam in the Clemmensen reduction reaction to include the synthesis of nine *C*-glycosylanthocyanidins (**1**–**9**) from their corresponding *C*-glycosylflavones (**F1**–**F9**), which were isolated from various natural sources. Flavones are lacking the 3-hydroxyl group, and pigments **1**–**9** synthesized from their precursors (**F1**–**F9**) are thus 3-deoxyanthocyanins. The reason for preparing the *C*-glycosyl-3-deoxyanthocyanidins is founded on two characteristics, which we wanted to combine in the new pigments; The relatively high colour stability of 3-deoxyanthocyanidins compared with their anthocyanidin analogues (Sweeny and Iacobucci, 1983), and the high stability of *C*-glycosylflavonoids towards acid hydrolysis, relative to flavonoid *O*-glycosides (Markham, 1982).

2. Results and discussion

Nine *C*-glycosyl-3-deoxyanthocyanidins (**1**–**9**) (Fig. 1) were independently synthesized from their respective *C*-glycosylflavones, **F1**–**F9**, by the Clemmensen reduction reaction using zinc-amalgam. Analysis of the reaction products by HPLC proved that the reduction of **F1–F7** gave the corresponding *C*-glycosyldeoxyanthocyanidins, **1**–**7**, which individually during the purification steps provided a mixture of the 6-*C*-glycosyldeoxyanthocyanidin and the 8-*C*-glycosyldeoxyanthocyanidin. The mechanism for this isomerisation may be similar to that given by Jurd (1962), where 5,8-dihydroxyflavylium salts isomerised, probably via chalcone intermediates, to their corresponding 5,6-dihydroxyflavylium salts under mildly acidic conditions. As verified by the HPLC profiles detected at 475 nm (±20 nm), no significant 6/8 isomerisation (**1–7**), demethylation (**1–4**, **9**), or loss of any acetyl moiety (**7**) nor terminal *O*-glycoside (**3–4, 6–7**) occurred during the reduction reaction. However the HPLC profiles detected at 280 nm (±10 nm) revealed that the reduction of the *C*-glycosylflavones gave several unidentified aromatic compounds in addition to the *C*-glycosyldeoxyanthocyanidin pigments. The structures of **1**–**9** were elucidated by means of NMR (Tables 3 and 4), on-line UV–vis spectroscopy (Table 5), and high-resolution electrospray mass spectrometry (HR-ESMS) (Table 5), in a similar way as described in the next paragraph for **1**. *C*-glycosyl-3-deoxyanthocyanidins have previously not been made or isolated from any source.

The anthocyanin yield of the Clemmensen reduction of 6-*C*-glucosyl-7-*O*-methylapigenin (swertisin, **F1**) isolated from iris (Kawase, 1968; Asen et al., 1970) was 20% (Table 5). The aromatic region of the ^{1}H NMR spectrum of **1** revealed an AA′XX′ system at δ 8.48 (H-2′,6′) and δ 7.18 (H-3′,5′), and 3 protons at δ 9.26 (*dd*, 8.9 and 0.9 Hz, H-4), δ 8.30 (*d*, 8.9 Hz, H-3) and δ 7.47 (*d*, 0.9 Hz, H-8), respectively, corresponding to a 3-deoxyanthocyanidin with a symmetrical B-ring (Table 3). A 3H singlet at δ 4.21 (OMe) belonging to the aglycone was confirmed to be at the 7-position by the crosspeak at δ 4.21/169.7 (OMe/C-7) in the long-range ^{1}H-^{13}C HMBC spectrum, in accordance with the deoxyanthocyanidin 7-*O*-methyl-apigeninidin. All the sugar proton resonances were assigned by the 2D ^{1}H–^{1}H DQF-COSY experiment, and the corresponding ^{13}C resonances (Table 4) were then identified by the 2D ^{1}H–^{13}C HSQC and 1D ^{13}C CAPT experiments. The anomeric shift value δ 5.16 (H-1″) with a $^3J_{HH}$ = 9.8 Hz, together with the six ^{13}C resonances between 61 ppm and 83 ppm were in accordance with a β-glucopyranosyl attached to the aglycone by a *C*–*C* bond. The crosspeaks at δ 5.16/114.8 (H-1″/C-6), δ 5.16/169.7 (H-1″/C-7) and δ 5.16/156.7 (H-1″/C-5), in the HMBC spectrum of **1**, confirmed



	R_1	R_2	R_3	R_4	R_5
F1 and **1**:	Glc	H	H	Me	H
F2 and **2**:	Glc	H	OH	Me	H
F3 and **3**:	2GlcGlc	H	H	Me	H
F4 and **4**:	2GlcGlc	H	H	Me	Me
F5 and **5**:	H	Glc	H	H	H
F6 and **6**:	H	2RhaGlc	H	H	H
F7 and **7**:	H	2(4AcRha)Glc	H	H	H
F8 and **8**:	Glc	Glc	H	H	H
F9 and **9**:	Glc	Glc	OH	H	Me

Fig. 1. Structures of the *C*-glycosyl-3-deoxyanthocyanidins obtained by Clemmensen reduction of corresponding *C*-glycosylflavones. **1**: 6-*C*-β-glucopyranosyl-7-*O*-methylapigeninidin, **2**: 6-*C*-β-glucopyranosyl-7-*O*-methylluteolinidin, **3**: 6-*C*-β-(2″-*O*-β-glucopyranosylglucopyranosyl)-7-*O*-methylapigeninidin, **4**: 6-*C*-β-(2″-*O*-β-glucopyranosylglucopyranosyl)-7,4′-di-*O*-methylapigeninidin, **5**: 8-*C*-β-glucopyranosylapigeninidin, **6**: 8-*C*-β-(2″-*O*-α-rhamnopyranosylglucopyranosyl)apigeninidin, **7**: 8-*C*-β-(2″-*O*-α-(4‴-*O*-acetylrhamnopyranosyl)glucopyranosyl)apigeninidin, **8**: 6,8-di-*C*-β-glucopyranosylapigeninidin, **9**: 6,8-di-*C*-β-glucopyranosyl-4′-*O*-methylluteolinidin, **F1**: 6-*C*-glucosyl-7-*O*-methylapigenin (swertisin), **F2**: 6-*C*-glucosyl-7-*O*-methylluteolin (swertiajaponin), **F3**: 6-*C*-sophorosyl-7-*O*-methylapigenin (flavoayamenin, spinosin), **F4**: 6-*C*-sophorosyl-7,4′-di-*O*-methylapigenin (embinoidin), **F5**: 8-*C*-glucosylapigenin (vitexin), **F6**: 8-*C*-neohesperidosylapigenin (2″-*O*-rhamnosylvitexin), **F7**: 8-*C*-(4‴-acetylneohesperidosyl)apigenin (4‴-*O*-acetyl-2″-*O*-rhamnosylvitexin), **F8**: 6,8-di-*C*-glucosylapigenin (vicenin), **F9**: 6,8-di-*C*-β-glucopyranosyl-4′-*O*-methylluteolin (6,8-di-*C*-glucosyldiosmetin).

Table 1
^{1}H NMR data for the *C*-glycosylflavones, **F1–F9**, dissolved in either S1 (5% CF_3COOD in $(CD_3)_2SO$, v/v), S2 ($(CD_3)_2SO$) or S3 (CD_3OD), at 25 °C. See Fig. 1 for structures.

	F1 (S1)	**F2** (S1)	**F3** (S2)	**F4** (S1)	**F5** (S2)*	**F6** (S3)*	**F7** (S3)*	**F8** (S1)	**F9** (S1)
3	6.81 *s* 6.83 *s*	6.70 *s* 6.72 *s*	6.84 *s* 6.86 *s*	6.93 *s* 6.92 *s*	6.77 *s* 6.79 *s*	6.66 *s* 6.67 *s*	6.68 *s* 6.72 *s*	6.81 *s* 6.80 *s*	6.77 *s* 6.79 *s*
6	–	–	–	–	6.26 *s* 6.24 *s*	6.36 *s* 6.35 *s*	6.39 *s* 6.34 *s*	–	–
8	6.81 *s* 6.80 *s*	6.76 *s* 6.75 *s*	6.80 *s* 6.77 *s*	6.79 *s* 6.83 *s*	–	–	–	–	–
2′	7.95 *′d′* 8.8 7.95 *′d′* 8.8	7.45 *d* 2.1 7.45 *d* 2.1	7.97 *′d′* 8.7 7.97 *′d′* 8.7	8.07 *′d′* 9.1 8.06 *′d′* 9.1	8.01 *′d′* 8.6 7.91 *′d′* 8.4	8.04 *′d′* 8.7 7.89 *′d′* 8.7	8.07 *′d′* 9.0 7.89 *′d′* 9.0	8.02 *′d′* 8.7 7.94 *′d′* 8.7	7.51 *d* 2.3 7.44 *d* 2.3
3′	6.92 *′d′* 8.9 6.92 *′d′* 8.9	–	6.93 *′d′* 8.7 6.93 *′d′* 8.7	7.12 *′d′* 9.1 7.11 *′d′* 9.1	6.88 *′d′* 8.6 6.94 *′d′* 8.4	7.01 *′d′* 8.7 7.02 *′d′* 8.7	7.02 *′d′* 9.0 7.00 *′d′* 9.0	6.89 *′d′* 8.7 6.93 *′d′* 8.7	–
5′	6.92 *′d′* 8.9 6.92 *′d′* 8.9	6.90 *d* 9.1 6.90 *d* 9.1	6.93 *′d′* 8.7 6.93 *′d′* 8.7	7.12 *′d′* 9.1 7.11 *′d′* 9.1	6.88 *′d′* 8.6 6.94 *′d′* 8.4	7.01 *′d′* 8.7 7.02 *′d′* 8.7	7.02 *′d′* 9.0 7.00 *′d′* 9.0	6.89 *′d′* 8.7 6.93 *′d′* 8.7	7.05 *d* 8.6 7.10 *d* 8.6
6′	7.95 *′d′* 8.8 7.95 *′d′* 8.8	7.44 *dd* 9.1, 2.1 7.44 *dd* 9.1, 2.1	7.97 *′d′* 8.7 7.97 *′d′* 8.7	8.07 *′d′* 9.1 8.06 *′d′* 9.1	8.01 *′d′* 8.6 7.91 *′d′* 8.4	8.04 *′d′* 8.7 7.89 *′d′* 8.7	8.07 *′d′* 9.0 7.89 *′d′* 9.0	8.02 *′d′* 8.7 7.94 *′d′* 8.7	7.66 *dd* 8.6, 2.3 7.58 *dd* 8.6, 2.3
7-MeO	3.88 *s* 3.85 *s*	3.88 *s* 3.86 *s*	3.89 *s* 3.89 *s*	3.91 *s* 3.90 *s*					
4′-MeO				3.85 *s* 3.85 *s*					3.88 *s* 3.86 *s*
	6-*C*-Glc	**6-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**
1″	4.58 *d* 9.8 4.60 *d* 9.7	4.59 *d* 9.9 4.61 *d* 9.8	4.69 *d* 9.9 4.67 *d* 9.9	4.70 *d* 10.1 4.68 *d* 10.1	4.67 *d* 9.8 4.82 *d* 9.8	5.12 *d* 9.9 5.21 *d* 9.9	5.13 *d* 9.9 5.24 *d* 9.8	4.80 *d* 9.7 4.66 *d* 9.8	4.79 *d* 9.8 4.66 *d* 9.8
2″	4.18 *dd* 9.8, 8.8 3.99 *dd* 9.7, 8.8	4.19 *t* br 9.2 4.00 *t* br 9.3	4.30 *d* 9.5 4.47 *d* 9.5	4.30 *dd* 10.1 8.9 4.47 *dd* 10.1 8.9	3.82 *t* 9.6 3.86 *m*	4.34 *dd* 9.9, 8.6 4.32 *t* 9.0	4.31 *dd* 9.9, 8.6 4.18 *dd* 9.8 8.9	3.49 *t* br 9.3 3.98	3.49 *t* br 9.2 3.48
3″	3.17 *t* 8.8 3.20 *t* 8.8	3.19 *m* 3.21 *m*	3.43 *m* 3.41 *m*	3.44 *t* 8.9 3.42 *t* 8.9	3.24 *m* 3.31 *m*	3.74 *m* 3.78 *m*	3.77 *dd* 9.2 8.6 3.84 *t* 8.9	3.30 *t* 9.1 3.21	3.29 *t* 9.0 3.21
4″	3.09 *m* 3.07 *m*	3.11 *m* 3.08 *m*	3.13 *m* 3.15 *m*	3.14 *m* 3.16 *m*	3.37 *t* 9.4 3.24 *m*	3.74 *m* 3.78 *m*	3.72 *t* 9.2 3.65 *m*	3.37 *t* 9.1 3.33	3.36 *t* 9.0
5″	3.14 *m* 3.16 *m*	3.14 *m* 3.16 *m*	3.17 *m* 3.16 *m*	3.16 *m* 3.18 *m*	3.21 *m* 3.34 *m*	3.55 *m* 3.64 *m*	3.57 *dd br* 9.2 5.9 3.67 *m*	3.33 *m* 3.45	3.32 *m* 3.43
6″A	3.69 *m* 3.69 *m*	3.69 *m* 3.69 *m*	3.68 *m* 3.68 *m*	3.69 *m* 3.69 *m*	3.75 *dd* 11.6, 1.4 3.68 *m*	4.06 *m* 4.03 *m*	4.09 *dd* 12.3 1.4 4.04 *dd* 12.3 1.4	3.63 *m* 3.68	3.62 *m* 3.69
6″B	3.36 *m* 3.36 *m*	3.37 *dd* 11.8 6.0 3.37 *dd* 11.8 6.0	3.37 *m* 3.37 *m*	3.38 *dd* 12.0 6.5 3.38 *dd* 12.0 6.5	3.51 *dd* 11.6 6.7 3.48 *m*	3.89 *m* 3.88 *m*	3.91 *m* 3.91 *m*	3.63 *m* 3.68	3.62 *m* 3.69
			2″-*O*-Glc	**2″-*O*-Glc**		**2″-*O*-Rha**	**2″-*O*-Rha**	**8-*C*-Glc**	**8-*C*-Glc**
1‴			4.17 *d* 8.0 4.15 *d* 8.0	4.19 *d* 7.9 4.16 *d* 7.9		5.19 *d* 1.8 5.29 *d* 1.8	5.39 *d* 1.8 5.50 *d* 1.7	4.75 *d* 9.9 5.00 *d* 9.6	4.74 *d* 9.8 4.98 *d* 10.0
2‴			2.84 *m* 2.84 *m*	2.85 *m* 2.84 *m*		3.94 *dd* 3.2, 1.8 3.88 *dd* 3.2, 1.8	3.91 *m* 3.80 *dd* 3.3 1.7	3.88 *t br* 9.3 3.56	3.86 *t br* 9.3 3.86
3‴			3.06 *m* 3.05 *m*	3.07 *t* 9.0 3.04 *t* 8.9		3.49 *dd* 9.5, 3.2 3.16 *m*	3.57 *dd* 9.8 3.3 3.10 *dd* 9.8 3.3	3.27 *t* 9.1 3.39	3.27 *m* 3.40
4‴			2.96 *m* 3.01 *m*	2.95 *t br* 9.3 3.00 *t br* 9.1		3.21 *t* 9.5 3.16 *m*	4.70 *t* 9.8 4.63 *t* 9.8	3.39 *t* 9.1 3.16	3.39 *t* 9.2 3.15
5‴			2.75 *m* 2.56 *m*	2.77 *dt* 9.6, 3.5 2.56 *ddd* 9.3 3.4, 2.7		2.53 *dd* 9.5, 6.3 2.40 *dd* 6.3, 9.5	2.41 *m* 2.17 *m*	3.23 *m* 3.18	3.25 *m* 3.17
6‴A			3.18 *m* 3.16 *m*	3.20 *d* 3.5 3.16 *m*		0.73 *d* 6.3 0.87 *d* 6.3	0.77 *d* 6.3 0.60 *d* 6.3	3.75 *dd* 12.0 1.8 3.69	3.79 *dd* 12.0, 1.8 3.69
6‴B			3.18 *m* 2.94 *m*	3.20 *d* 3.5 2.96 *m*				3.50 *dd* 12.0 5.8 3.42	3.54 *dd* 12.0, 6.5 3.42
							4‴-Ac		
2‴′							2.11 *s* 2.02 *s*		

Chemical shifts and coupling constants are in ppm and Hz, respectively. The two signals given for most positions correspond to two rotamers: major (top) and minor (bottom).
* Rayyan et al. (2005). *s* = Singlet, *d* = doublet, *dd* = double doublet, *t* = triplet, *q* = quartet, *m* = multiplet, br = broad, Glc = glucoside, Rha = rhamnoside, Ac = acetyl.

the *C*-*C* linkage between the sugar and the aglycone at the 6-position. The HR-ESMS spectrum of **1** exhibited a $[M]^+$ ion of *m*/*z* 431.1361, corresponding to the molecular formula $C_{22}H_{23}O_9$ (calc. 431.1342), confirming the identification of 6-*C*-β-glucopyranosyl-7-*O*-methylapigeninidin (6-*C*-β-glucopyranosyl-5,4′-dihydroxy-7-methoxy-2-phenylbenzopyrylium).

The NMR spectra revealed rotameric pairs of the 8-*C*-glycosylanthocyanidins, **5–9**, and their parent *C*-glycosylflavones, **F5–F9**,

Table 2
^{13}C NMR data (in ppm) for the *C*-glycosylflavones, **F1–F9**, dissolved in either S1 (5% CF_3COOD in $(CD_3)_2SO$, v/v), S2 ($(CD_3)_2SO$) or S3 (CD_3OD), at 25 °C. See Fig. 1 for structures.

	F1 (S1)	**F2** (S1)	**F3** (S2)	**F4** (S1)	**F5** (S2)*	**F6** (S3)*	**F7** (S3)*	**F8** (S1)	**F9** (S1)
2	163.9	164.1	163.89	163.2	164.02	166.53	166.66	163.9	163.6
	163.9	164.1	164.04	163.3	163.81	166.74	165.95	163.6	163.2
3	103.1	103.2	103.20	103.6	102.52	103.53	103.58	102.5	103.1
	103.1	103.2	103.30	103.6	102.72	103.46	104.21	102.6	103.0
4	182.3	182.3	182.51	181.9	182.18	183.99	184.11	182.2	182.1
	182.1	182.0	182.19	182.2	181.53			182.2	182.1
5	160.4	160.4	160.75	159.6	160.46	162.56	162.82	158.6	158.4
	159.6	160.1	159.88	160.4	160.79	162.47	163.20		159.9
6	109.8	109.8	108.79	108.5	98.20	99.81	99.77	107.3	107.3
	109.7	109.8	108.74	108.5	99.46	101.06	101.32		108.9
7	163.9	163.8	164.04	165.1	162.63	164.04	164.12	160.7	160.6
	165.0	165.0	165.27	163.8	162.96	164.35	164.34		161.6
8	90.2	90.2	90.50	90.7	104.68	105.52	105.75	105.2	105.2
	91.0	91.0	90.96	90.2	104.57	105.41	105.61		102.9
9	156.8	156.9	157.16	157.0	156.06	157.77	157.80	154.9	154.8
	157.0	156.9	157.28	156.9	156.03	156.60	156.34		153.7
10	104.8	104.8	104.63	104.1	104.12	105.89	105.98	103.8	103.8
	104.2	104.3	104.38	104.4	103.89	105.86	104.74	103.2	103.1
1′	121.1	121.5	121.27	122.6	121.68	123.42	123.36	121.4	123.1
	121.1	121.5	121.23	122.6	121.62	123.35	123.32	121.3	122.8
2′	128.5	113.6	128.76	128.3	129.05	129.99	130.13	129.0	113.4
	128.5	113.6	128.74	128.3	128.57		129.52	128.7	113.0
3′	116.0	145.7	116.19	114.4	115.87	116.91	117.01	115.7	146.6
	116.0	145.7	116.19	114.4	116.10		117.15	115.9	146.6
4′	161.4	149.9	161.48	162.3	161.22	162.56	162.79	161.2	151.0
	161.4	149.9	161.48	162.3	161.35	162.47		161.2	151.0
5′	116.0	116.1	116.19	114.4	115.87	116.91	117.01	115.7	111.5
	116.0	116.1	116.19	114.4	116.10		117.15	115.9	111.9
6′	128.5	119.1	128.76	128.3	129.05	129.99	130.13	129.0	119.0
	128.5	119.1	128.74	128.3	128.57		129.52	128.7	118.7
7-MeO	56.5	56.5	56.73	56.1					
	56.2	56.3	56.32	56.4					
4′-MeO				55.5					55.7
				55.5					55.5
	6-*C*-Glc	**6-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**
1″	72.9	73.0	71.24	70.6	73.45	73.59	73.77	74.0	73.8
	72.6	72.7	70.91	70.9	74.35	74.86	75.30	73.1	73.0
2″	69.6	69.7	81.45	81.1	70.89	78.04	76.09	71.9	71.7
	70.3	70.4	80.99	80.6	71.09	77.88	76.29	70.2	71.6
3″	79.1	79.1	78.89	78.6	78.70	81.48	81.80	77.7	77.6
	79.1	79.1	78.49	78.3	78.72	81.17	81.45	78.8	78.7
4″	70.9	70.8	70.65	70.3	70.25	72.12	72.51	68.9	68.8
	70.9	71.0	70.65	70.3	70.60	71.56	71.50	68.9	69.1
5″	81.7	81.7	82.15	81.6	81.74	82.68	82.99	80.8	80.7
	81.9	81.9	81.85	81.8	81.93	82.75	83.00	81.2	81.2
6″	61.7	61.8	61.66	61.3	61.27	63.01	63.13	59.7	59.6
	61.7	61.8	61.66	61.3	61.35	62.55	62.45	59.9	59.9
			2″-*O*-Glc	**2″-*O*-Glc**		**2″-*O*-Rha**	**2″-*O*-Rha**	**8-*C*-Glc**	**8-*C*-Glc**
1‴			105.66	105.2		102.37	101.15	73.2	73.1
			105.48	105.3			101.19	74.9	74.8
2‴			74.92	74.5		72.37	72.09	70.8	70.6
			74.75	74.5		72.02	71.80	71.8	70.6
3‴			76.58	76.2		71.84	70.02	78.7	77.8
			76.54	76.2		71.88		78.0	78.8
4‴			69.65	69.3		73.43	75.16	70.4	70.5
			69.33	69.0		73.14		70.3	70.2
5‴			76.88	76.6		69.84	67.27	81.8	82.0
			76.62	76.3		69.91	67.03	81.7	81.4
6‴			60.79	60.5		17.97	17.84	61.1	61.4
			60.24	59.9		17.90	17.95	61.1	61.0
							4‴-Ac		
1⁗							172.54		
2⁗							21.00		
							21.03		

Signals with two and one significant decimal are recorded from the ^{13}C CAPT and the heteronuclear experiments, respectively. The two signals given for most positions correspond to two rotamers: major (top) and minor (bottom).
* Rayyan et al. (2005). Glc = glucoside, Rha = rhamnoside, Ac = acetyl.

caused by restricted rotation around the linkage between the anomeric carbon (1″-*C*; sp^3) and the aglycone (8-*C* and possibly 6-*C* in **8**, **9**, **F8** and **F9**; sp^2) (Tables 1–4). The equilibrium between the two rotamers of each of **5**–**9** was confirmed by observation of strong exchange peaks between equivalent protons of each rotameric pairs in the NOESY and ROESY spectra. Rotamers were not observed for the 6-*C*-glycosylanthocyanidins, **1–4**, dissolved in acidified deuterated methanol, however rotameric pairs were observed for their parent *C*-glycosylflavones, **F1**–**F4**, dissolved in deuterated DMSO. In this context one should keep in mind that distinct NMR

Table 3
^{1}H NMR data for the C-glycosylanthocyanidins, **1**–**9**, dissolved in either S4 (5% CF_3COOD in CD_3OD, v/v) or S5 (20% CF_3COOD in $(CD_3)_2SO$, v/v) at 25 °C. See Fig. 1 for structures.

	1 (S4)	**2** (S4)	**3** (S4)	**4** (S4)	**5** (S4)	**6** (S4)	**7** (S4)	**8** (S5)	**9** (S5)
3	8.30 *d* 8.9	8.23 *d* 8.9	8.23 *d* 8.9	8.34 *d* 8.7	8.13 *d* br 8.7	8.12 *d* 8.8 8.12 *d* 8.8	8.18 *d* 8.7 8.25 *d* 8.7	8.28 *d* 8.8 8.28	8.26 *d* 8.7 8.32 *d* 8.7[a]
4	9.26 *dd* 8.9, 0.9	9.20 *dd* 8.9, 0.9	9.24 *d* 8.9	9.34 *dd* 8.7, 0.8	9.20 *d* 8.7	9.16 *d* 8.8 9.16 *d* 8.8	9.22 *d* 8.7 9.29 *d* 8.7	9.20 *d* 8.8 9.20	9.19 *d* 8.7 9.21 br
6	–	–	–		6.86 *s*	6.85 *s* 6.84 *s*	6.90 *s* 6.85 *s*	–	–
8	7.47 *d* 0.9	7.40 *d* 0.7	7.37 *s*	7.45 *d* 0.8	–	–	–	–	–
2′	8.48 *′d′* 9.1	7.89 *d* 2.4	8.34 *′d′* 8.9	8.56 *′d′* br 9.1	8.49 *′d′* br 8.0 8.43	8.48 *′d′* 9.1 8.37 *′d′* 9.0	8.54 *′d′* 8.9 8.42 *′d′* 8.9	8.45 *′d′* 8.8 8.40 *′d′* br	7.86 *d* 1.7[a] 7.80 br
3′	7.18 *′d′* 9.1	–	7.18 *′d′* 8.9	7.37 *′d′* br 9.1	7.18 *′d′* 8.9	7.17 *′d′* 8.9 7.14 *′d′* 9.0	7.19 *′d′* 8.9 7.22 *′d′* 8.9	7.06 *′d′* 8.8 7.10 *′d′* br	–
5′	7.18 *′d′* 9.1	7.14 *d* 8.6	7.18 *′d′* 8.9	7.37 *′d′* br 9.1	7.18 *′d′* 8.9	7.17 *′d′* 8.9 7.14 *′d′* 9.0	7.19 *′d′* 8.9 7.22 *′d′* 8.9	7.06 *′d′* 8.8 7.10 *′d′* br	7.25 *d* 8.5 7.28 br
6′	8.48 *′d′* 9.1	8.04 *dd* 8.6, 2.4	8.34 *′d′* 8.9	8.56 *′d′* br 9.1	8.49 *′d′* br 8.0 8.43	8.48 *′d′* 9.1 8.37 *′d′* 9.0	8.54 *′d′* 8.9 8.42 *′d′* 8.9	8.45 *′d′* 8.8 8.40 *′d′* br	8.11 *dd* 8.5, 1.7[a] 8.09 br
7-MeO	4.21 *s*	4.20 *s*	4.18 *s*	4.22 *s*					
4′-MeO				4.10 *s*					3.98 *s*
	6-*C*-Glc	**6-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**
1″	5.16 *d* 9.8	5.15 *d* 9.8	5.24 *d* 10.0	5.26 *d* 9.9	5.15 *d* br 5.30	5.21 *d* 10.1 5.36 *d* 9.6	5.23 *d* 10.1 5.44 *m*	5.12 *d* 9.8 4.94 *d* br	5.12 *d* 9.7 4.93 *d* 9.7[a]
2″	3.75 *m*	3.75	4.3 *m*	4.34 *m*	4.14 *t* br	4.26 *dd* 10.1, 8.7 4.39 *t* br 9.6	4.27 *dd* 10.1, 8.6 4.29 *dd* 9.9, 8.8	3.46 *m* 3.70	3.46 *m* 3.70
3″	3.64 *t* 8.9	3.64	3.9 *m*	3.83 *t* 9.0	3.69	3.79 *t* 8.7 3.83 *m*	3.81 *t* 8.8 3.90 *t* 8.8	3.38 *m* 3.42	3.38 *m* 3.33
4″	3.73 *m*	3.72 *dd* 9.8, 8.9	3.8 *m*	3.77 *m*	3.86	3.86 *t* br 9.4 3.70 *m*	3.87 *t* br 9.3 3.64 *dd* 9.8, 9.0	3.46 *m*	3.46 *m* 3.37
5″	3.60 *ddd* 9.8, 3.7, 2.5	3.62	3.6 *m*	3.61 *m*	3.61	3.60 *m* 3.68 *m*	3.58 *m* 3.70 *ddd* 9.8, 5.4, 2.3	3.41 *m*	3.43 *m* 3.37
6″A	3.98 *d* 3.7	3.97	4.0 *m*	3.98 *m*	4.04 *dd* br	4.05 *m* 4.04 *m*	4.03 *dd* 12.2, 2.2 4.02 *dd* 11.9, 2.3	3.69 *m*	3.70 *m* 3.7
6″B	3.96 *d* 2.5	3.97	3.9 *m*	3.78 *m*	3.94	3.94 *m* 3.87 *m*	3.93 *dd* 12.2, 4.9 3.88	3.66 *m*	3.66 *m* 3.61
			2″-*O*-Glc	**2″-*O*-Glc**		**2″-*O*-Rha**	**2″-*O*-Rha**	**8-*C*-Glc**	**8-*C*-Glc**
1‴			4.38 *d* 7.8	4.39 *d* 7.8		5.16 *d* 1.9 5.26 *d* 1.9	5.33 *d* 1.8 5.45 *m*	4.88 *d* 10.0 5.30 *d* br	4.88 d 9.8 5.25 *d* 9.1[a]
2‴			3.1 *m*	3.06 *dd* 8.9, 7.8		3.96 *m* 3.85 *m*	3.96 *dd* 3.3, 1.8 3.80 *m*	3.83 *t* br 9.3 3.52	3.83 *m* 3.55
3‴			3.3 *m*	3.29 *t* 9.1		3.47 *dd* 9.6, 3.2 2.93 *dd* 9.6, 3.2	3.58 *dd* 9.8, 3.3 2.89 *dd* 9.8, 3.3	3.35 *m* 3.35	3.36 *m* 3.56
4‴			3.0 *m*	3.02 *t* 9.1		3.20 *t* 9.6 3.12 *t* 9.6	4.72 *t* 9.8 4.61 *t* 9.8	3.52 *m*	3.51 *t* 9.1 3.50
5‴			2.9 *m*	2.93 *m*		2.49 *m* 2.16 *m*	2.48 *′dq′* 9.8, 6.3 2.14 *′dq′* 9.8, 6.3	3.33 *m*	3.38 *m* 3.57
6‴A			3.3 *m*	3.26 *m*		0.58 *d* 6.3 0.82 *d* 6.3	0.47 *d* 6.3 0.75 *d* 6.3	3.74 *m*	3.82 *m* 3.79
6‴B			3.1 *m*	3.07 *m*				3.59 *m*	3.63 *m* 3.70
							4‴-Ac		
2‴′							2.10 *s* 2.02 *s*		

Chemical shifts and coupling constants are in ppm and Hz, respectively. The two signals given for **5**–**9** correspond to two rotamers: major (top) and minor (bottom). *s* = Singlet, *d* = doublet, *dd* = double doublet, *t* = triplet, *q* = quartet, *m* = multiplet, br = broad, *′dq′* = double quartet, Glc = glucoside, Rha = rhamnoside, Ac = acetyl.

[a] Coupling constant was determined at 292 K.

signals of rotameric conformers of *C*-glycosylflavones seem to be more observable in DMSO than in methanolic solutions (Rayyan et al., 2005).

On the basis of examination of the UV–visible spectra of **8** and **9** in aqueous buffer solutions at various pH values it seems that both pigments undergoes similar structural transformations (Fig. 2). At pH 1.1 it is assumed that both pigments occur mainly on their respective flavylium forms with visible λ_{max} at 472 nm and 485 nm. At pH 4.5 the pigments have a local absorption maximum around 400 nm and a broad absorption band around 500 nm with

Table 4
^{13}C NMR data (in ppm) for the *C*-glycosylanthocyanidins, **1**–**9**, dissolved in either S4 (5% CF_3COOD in CD_3OD, v/v) or S5 (20% CF_3COOD in $(CD_3)_2SO$, v/v) at 25 °C. See Fig. 1 for structures.

	1 (S4)	**2** (S4)	**3** (S4)	**4** (S4)	**5** (S4)	**6** (S4)	**7** (S4)	**8** (S5)	**9** (S5)
2	173.62	173.4	173.20	172.7	172.6	173.07 173.0	173.41 173.9	170.72	170.22
3	112.36	112.6	112.00	111.9	110.0	110.38 110.89	110.57 110.98	110.67 110.7	110.89 111.32
4	149.58	149.0	149.88	150.0	149.3	149.53 150.03	149.68 149.9	149.03 149.0	148.97 149.1
5	156.68	156.5	156.7	156.7	159.5	160.05 160.00	160.14 160.0	155.06	155.12
6	114.82	114.7	114.6	114.8	102.0 103.5	102.73 102.56	102.37 103.93	113.25	113.43
7	169.72	169.3	170.1	170.4	170.3	170.39 170.3	170.24 170.7	166.74	167.38
8	93.40	93.2	93.4	93.1	106.4	107.22 106.17	107.41 106.36	107.43	107.38
9	159.25	159.1	159.7	159.8	157.8	157.82 157.21	157.90 157.0	155.53	155.75
10	114.19	113.9	113.66	115.0	113.8	114.05 113.46	113.95 113.4	112.92	113.5
1′	120.97	121.3	121.29	122.4	121.2	121.53 121.00	121.46 121.2	120.19	121.99
2′	133.92	116.4	133.66	132.9	133.5	133.94 133.58	134.19 133.56	133.26 133.2	115.58 115.0
3′	118.66	148.3	118.53	116.8	118.2	118.46 118.56	118.55 118.80	117.54 117.6	147.91
4′	168.19	157.3	167.8	168.0	167.3	167.51 167.58	167.84 168.0	165.98	155.51
5′	118.66	118.1	118.53	116.8	118.2	118.46 118.56	118.55 118.80	117.54 117.6	113.12 113.2
6′	133.92	126.1	133.66	132.9	133.5	133.94 133.58	134.19 133.56	133.26 133.2	124.21 124.5
7-MeO	58.27	58.2	58.14	58,0					
4′-MeO				56.6					56.66
	6-*C*-Glc	**6-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**8-*C*-Glc**	**6-*C*-Glc**	**6-*C*-Glc**
1″	76.51	76.5	75.11	74.8	74.7	73.36 74.19	73.39 74.32	75.38 74.4	75.29 74.58
2″	74.07	74.1	79.0	81.2	72.7	78.65 77.85	77.36 76.94	73.01 71.4	73.02 71.8
3″	79.14	79.1	80.2	78.8	79.8	81.31 81.05	81.46 81.13	77.64 77.5	77.66 78.2
4″	70.84	70.8	70.3	70.2	71.8	72.06 72.1	72.08 71.55	68.90	68.92 69.67
5″	82.74	82.9	82.7	82.4	82.7	82.98 83.0	83.10 83.02	81.38	81.39 81.7
6″	61.58	61.5	61.3	62.9	62.3	62.48 62.70	62.41 62.49	59.68	59.69 59.94
			2″-*O*-Glc	**2″-*O*-Glc**		**2″-*O*-Rha**	**2″-*O*-Rha**	**8-*C*-Glc**	**8-*C*-Glc**
1‴			105.2	104.9		102.85 102.5	101.95 101.7	73.71 75.0	73.71 75.2
2‴			75.8	75.6		72.12 72.04	71.99 71.6	71.17 72.4	71.05 72.6
3‴			77.7	77.6		71.92 72.27	70.05 70.0	78.52 77.1	78.60 77.51
4‴			71.0	70.9		73.29 73.05	75.12 75.02	70.46	70.94 70.8
5‴			77.2	77.4		70.18 70.11	67.68 67.38	82.20	82.52 81.6
6‴			62.2	62.0		18.12 17.97	17.91 17.91	61.04	61.71 61.7
							4‴-Ac		
1⁗							172.17 172.0		
2⁗							20.95 20.9		

Signals with two and one significant decimal are recorded from the ^{13}C CAPT and the heteronuclear experiments, respectively. The two signals given for **5**–**9** correspond to two rotamers: major (top) and minor (bottom). Glc = glucoside, Rha = rhamnoside, Ac = acetyl.

shoulders on both sides. Based on these absorption characteristics, it is assumed that at pH 4.5 each pigment occur mainly as a mixture of chalcone form(s) (λ_{max} around 400 nm), flavylium cation (shoulder in absorption spectrum with λ_{max} around 470 nm) and quinonoidal form(s) (shoulder in absorption spectrum with λ_{max} around 530 nm) similar to the distribution of the transformed forms of apigeninidin (Brouillard et al., 1982). At pH 7 the picture is comparable to that at pH 4.5 for both **8** and **9**, however, the proportion(s) of the chalcone form(s) seem to increase on the expense of the other forms. Possible colourless hemiketal forms are not considered.

To compare the stability of the *C*–*C* linkage of *C*-glycosylanthocyanidins with the *C*–*O* linkage of anthocyanidin *O*-glycoside towards acid, mixtures of nearly equal parts of 6,8-di-*C*-β-glucosylapigeninidin (**8**), and pelargonidin 3-*O*-β-glucoside (P1), and 6,8-di-*C*-β-glucopyranosyl-4′-*O*-methylluteolinidin (**9**) and P1 were prepared. Aliquots of these solutions were subjected to acid hydrolysis at 110 °C at different time intervals (15, 30, 60, 90, 120, 180, 240 and 500 min, respectively). The HPLC profiles (Fig. 3) revealed that after 30 min hydrolysis more than half of the amount of P1 was converted to the aglycone pelargonidin (P2), after 60 min P1 was not detected at all, while most of P2 was degraded further after 500 min. Similar acid hydrolysis products were not observed for the *C*-glycosylanthocyanidins, **8** and **9**, during the hydrolysis period. Thus, contrary to the *3*-*O* linkage of P1, the corresponding 8-*C* and 6-*C* linkages of **8** and **9**, which are not found in normal anthocyanidin *O*-glycosides, seemed to be far more stable towards acid hydrolysis. This may encourage extended use of anthocyanins like the *C*-glycosylanthocyanidins, **1**–**9**, as nutraceuticals, pharmaceuticals or colorants in the future.

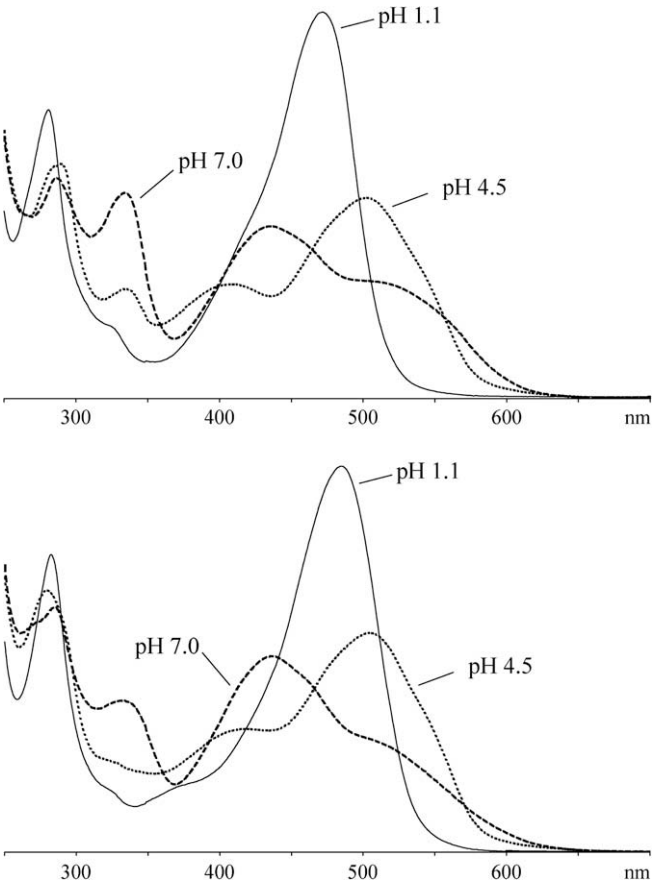


Fig. 2. Effect of pH on the UV–visible absorption spectra of 6,8-di-*C*-β-glucopyranosylapigeninidin (0.17 mM), **8** (top), and 6,8-di-*C*-β-glucopyranosyl-4′-*O*-methylluteolinidin (0.21 mM), **9** (bottom) in aqueous buffer solutions measured minutes after dissolution.

3. Experimental

3.1. Isolation of the C-glycosylflavones

Flowers of *Iris sibirica* L. (550 g) were extracted twice with MeOH containing 0.5% trifluoroacetic acid (v/v), TFA, for 16 h at 4 °C. The combined extracts were concentrated under reduced pressure, and purified by partition against equal portions of ethyl acetate (EtOAc) (five times). The concentrated aqueous layer was applied on an Amberlite XAD-7 column. The flavonoids adsorbed on the column were washed with H_2O before they were eluted with MeOH containing 0.2% TFA. The flavonoids were further purified by Sephadex LH-20 column chromatography using MeOH:H_2O (containing 0.5% TFA) (1:4, v/v) as eluent. After concentration of the Sephadex LH-20 fractions containing the various flavones, individual flavones, **F1–F4** (Kawase, 1968; Asen et al., 1970; Hirose et al., 1981; Hilsenbeck and Mabry, 1983, 1990), were precipitated after storage at 4 °C for 24 h. **F2** and **F4** were further purified by preparative HPLC. Embinoidin (**F4**), 6-*C*-sophorosyl-7,4′-di-*O*-methylapigenin, has previously been reported to occur only in the genus *Siphonoglossa* (Hilsenbeck and Mabry, 1983, 1990).

The herbal drug "Crataegi folium cum flore" (dried hawthorn leaves and flowers) was purchased from Norsk Medisinaldepot ASA (Bergen, Norway). This drug has been specified for example in the German, Swiss and French pharmacopeias (DAB 10, Ph. Helv. VII, Ph Franc. X) (Sticher and Meier, 1998). Plant material (200 g) was extracted three times with 20% aqueous MeOH at 4 °C. The extracts were after filtration concentrated under reduced pressure, purified by partition against diethyl ether, and subjected to Amberlite XAD-7 column chromatography. The columns were washed with distilled water before the flavonoids were eluted with methanol. The individual flavonoids, including **F5**–**F7** (Nikolov et al., 1982; Rayyan et al., 2005), were separated by Sephadex LH-20 column chromatography using MeOH–H_2O (40:60; v/v) to MeOH–H_2O (70:30; v/v) (stepwise gradient) as eluents.

Orange juice (20 l) was filtered prior to purification by partition against EtOAc. The aqueous layer was then concentrated under reduced pressure, and applied to an Amberlite XAD-7 column. The column was washed with H_2O before the adsorbed flavonoids were eluted with MeOH containing 0.2% TFA (v/v). The MeOH fraction was concentrated to a dark brown syrup, and subjected to Sephadex LH-20 chomatography. The flavones were separated using isocratic elution with MeOH–H_2O (1:4, v/v) containing 0.2% TFA. Flavonoids from lemon juice (20 l) were treated according to a similar procedure, before the Sephadex LH-20 fractions containing **F8** and **F9** from both juices were separately combined. **F8** and **F9** (Tomas et al., 1978; Kumamoto et al., 1985) were then further purified by subjecting them individually to a second Sephadex LH-20 column, using the same separation conditions as the first Sephadex column, followed by preparative HPLC. HPLC-fractions containing **F8** were combined and evaporated to dryness, and the solid was dissolved in a minimum amount of H_2O containing 0.2% TFA (v/v). **F8** was precipitated after 3 days storage at 4 °C. HPLC-fractions containing **F9** were also combined and evaporated to dryness, and the solid was suspended into cold MeOH containing 0.2% TFA (v/v) before the suspension was filtered giving **F9**.

The structures of **F1**–**F9** including their rotamers were elucidated by means of one- and two-dimensional NMR spectroscopic techniques (Tables 1 and 2), UV–vis spectroscopy and high-resolution electrospray mass spectrometry.

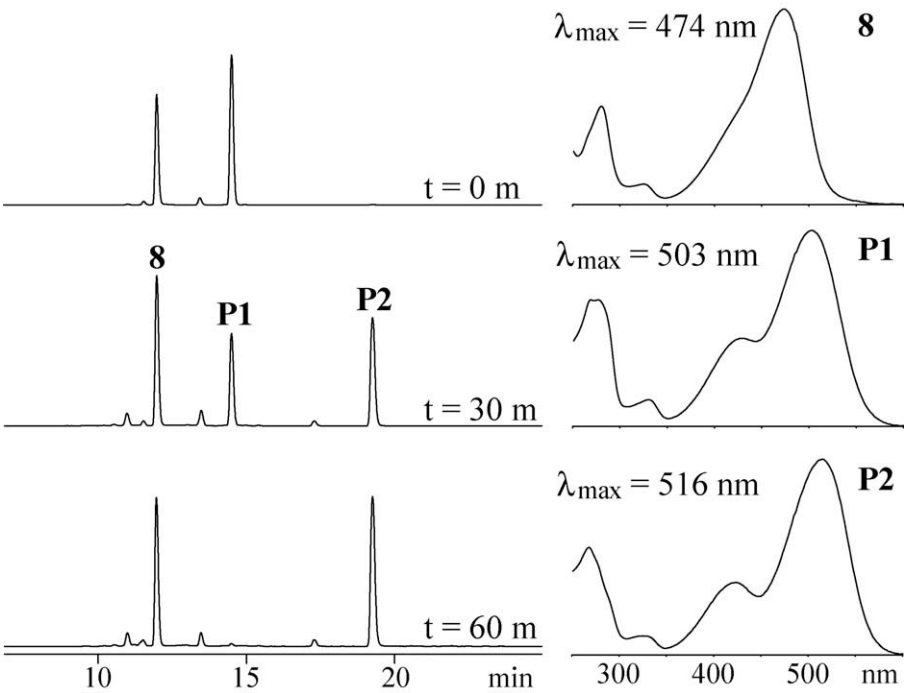


Fig. 3. Left: Various HPLC profiles detected at 485 ± 20 nm of the same mixture of 6,8-di-*C*-β-glucopyranosylapigeninidin (**8**) and pelargonidin 3-*O*-β-glucopyranoside (P1) dissolved in MeOH–HCl (2 M) (1:1, v/v) subjected to 110 °C at different time intervals. Each chromatogram is scaled to its highest peak. Right: UV–visible spectra of **8**, P1 and P2. P2 = pelargonidin.

3.2. Synthesis of anthocyanidin C-glycosides

General procedure: Zinc-amalgam was prepared as described by Elphimoff-Felkin and Sarda (1977). **F1**–**F9** (70–200 mg) were individually dissolved in 20 ml dry methanol containing 3% HCl gas by weight (Vogel et al., 1989). The solutions were, if necessary, gently heated to 40 °C to dissolve the compounds before being chilled to room temperature on ice. Zinc-amalgam (3 g) was thereafter added, and after thorough stirring for 30 min the liquid phases containing the products were separated from amalgam residues by filtration through glass wool. The reaction time was notably influenced by the quality of the zinc-amalgam being as short as 12 min using fresh zinc. By using an alternative procedure, $HgCl_2$ (1 g) and Zn (12.5 g) were mixed with 200 ml H_2O–conc. HCl (96:4, v/v) with stirring for 30 min. The liquid phase was removed and the zinc-amalgam washed with ethanol followed by diethyl ether. **F9** (200 mg) dissolved in 250 ml MeOH–conc. HCl (97:3, v/v) was then added to the zinc-amalgam. This latter procedure appeared not to influence the yield of the product (**9**), however, the use of HCl gas and dried anhydrous MeOH was positively prevented. The increased reaction volume was also beneficial for dissolution of **F9**. The various crude products were first analysed by analytical HPLC, and thereafter the *C*-glycosylanthocyanidins, **1**–**9**, were purified by preparative HPLC, or by a small column (30 × 1 cm) packed with Sephadex LH-20 material. The yields obtained during the various reductive syntheses are presented in Table 5.

Prior to the main synthesis of the various *C*-glycosylanthocyanidins, a test synthesis with 20 mg **F3** (spinosin) was performed. The reaction proceeded for 30 min. Samples from the reaction mixture were collected after every 5th min, respectively, and analysed by analytical HPLC. After 5 min, most of the spinosin sample was still intact (spinosin:*C*-glycosylanthocyanidin, ~50:1). After 15 min, a significant amount of the flavone was still present (spinosin:*C*-gly-

Table 5
Chromatographic (HPLC), UV–vis* and high-resolution electrospray MS data recorded for the *C*-glycosylanthocyanidins, **1**–**9**. The yields give the quantitative amounts of the various pigments formed after reductive synthesis of their respective *C*-glycosylflavones. See Fig. 1 for structures.

Pigment	t_R (min)	$\lambda_{UV\text{-}max}$ (nm)	$\lambda_{VIS\text{-}max}$ (nm)	$A_{440}/A_{VIS\text{-}max}$ (%)	$[M]^+$ observed (*m*/*z*)	$[M]^+$ calculated (*m*/*z*)	Molecular formula	Yield[a]		
								mg	mmol	%
1	5.35	279, 326	469	63	431.1361	431.1342	$C_{22}H_{23}O_9$	24[b]	4.4×10^{-2}	20
2	4.74	281, 325s[A]	486	42	447.1294	447.1291	$C_{22}H_{23}O_{10}$	25[b]	4.5×10^{-2}	21
3	5.70	280, 327	472	61	593.1865	593.1870	$C_{28}H_{33}O_{14}$	35[b]	5.5×10^{-2}	25
4	12.13	280, 328	470	67	607.1998	607.2027	$C_{29}H_{35}O_{14}$	11[b]	1.5×10^{-2}	14
5	4.05	279, 323	424s, 476	60	417.1212	417.1186	$C_{21}H_{21}O_9$	13[b]	2.4×10^{-2}	15
6	3.86	279, 325	424s, 481	56	563.1749	563.1765	$C_{27}H_{31}O_{13}$	14[b]	2.1×10^{-2}	15
7	5.15	280, 326	426s, 482	52	605.1848	605.1870	$C_{29}H_{33}O_{14}$	36[b]	4.9×10^{-2}	32
8	3.57	281, 326	474	66	579.1700	579.1714	$C_{27}H_{31}O_{14}$	41	5.9×10^{-2}	18
9	3.63	283, 325s[A]	488	46	609.1790	609.1819	$C_{28}H_{33}O_{15}$	51	7.0×10^{-2}	22

* In MeCN–H_2O (1:4) containing 0.5% TFA (v/v/v);
[a] As trifluoroacetic acid salt.
[b] Total anthocyanin yield, which includes the 6-*C*- and 8-*C*-glycosylanthocyanidin isomers gathered (see Section 2 for further details).
[A] Weak shoulder.

cosylanthocyanidin, ~5:1). However, after 30 min spinosin had reacted completely, and no trace of the starting material could be detected in the HPLC profile.

3.3. Acid hydrolysis of anthocyanins

6,8-Di-*C*-β-glucosylapigeninidin, **8**, and pelargonidin 3-*O*-β-glucoside (P1) (Nerdal et al, 1992) dissolved in MeOH were mixed with aqueous HCl (2 M) (1:1, v/v) (Gao and Mazza, 1994). The mixture was distributed into nine equal portions and subjected to heating at 110 °C. After different time intervals: 0, 15, 30, 60, 90, 120, 180, 240 and 500 min, respectively, each sample was cooled in an ice bath and monitored by HPLC (Fig. 3). In a similar way a mixture of **9** and P1 were subjected to the same hydrolysis procedure. The identity of pelargonidin (P2) was confirmed by its molecular ion at *m*/*z* at 271.06 in the LC–MS spectra.

3.4. High performance liquid chromatography

Preparative HPLC (Gilson 305/306 pump equipped with an HP-1040A detector) was performed using an Econosil C18 column (250 mm × 22 mm; length × I.D., 10.0 μm), and combinations of two solvents were used for elution: A, H_2O–HCOOH (9:0.5, v/v) and B, H_2O–MeOH–HCOOH (4:5:0.5, v/v). See Rayyan et al. (2005) for more experimental details.

Analytical HPLC was performed with an ODS-Hypersil column (20 × 0.5 cm, length × i.d., 5 μm) using the solvents A, H_2O containing 0.5% TFA (v/v) and B, acetonitrile containing 0.5% TFA (v/v). The following gradient was used: 10% B (isocratic) in 0–4 min, 10–40% B (linear gradient) from 4 to 21 min, 40% B (isocratic) from 21 to 28 min. The flow rate was 1.0 ml min^{-1}.

3.5. Spectroscopy

UV–vis absorption spectra were recorded on-line during HPLC analysis using a photodiode array detector (HP 1050, Hewlett-Packard) (Table 5). All samples were dissolved in the same solvent as used for isocratic HPLC analysis, namely MeCN–H_2O (1:4) containing 0.5% TFA (v/v/v). Spectral measurements were made over the wavelength range 240–600 nm in steps of 2 nm. UV–vis absorption spectra of **8** (0.17 mM) and **9** (0.21 mM) in the following three buffer solutions (Fig. 2) were recorded between 250 and 800 nm, in steps of 1 nm, using a Varian Cary3 UV–vis Spectrophotometer. The spectra were recorded within 1 min after sample preparation, and care was taken to prevent the samples from being exposed to daylight. UV–vis absorption spectra recorded for the samples stored at +6 °C for 24 h showed no differences from those recorded immediately after sample preparation. The phosphate buffer was prepared from $K_2HPO_4 \cdot 3H_2O$ (660 mg, 2.89 mmol) and KH_2PO_4 (288 mg, 2.12 mmol) dissolved in H_2O. The total volume was extended with H_2O to 100 ml, and then the pH was adjusted to 7.0 by dropwise addition of a 0.2 M NaOH solution. The acetate buffer was prepared from $NaO(O)CCH_3$ (148 mg, 1.80 mmol) and CH_3COOH (192 mg, 3.20 mmol) dissolved in H_2O. The total volume was adjusted to 100 ml by adding H_2O, and thereafter the final pH of 4.5 was obtained by dropwise addition of aqueous acetic acid. The hydrochloride buffer with pH 1.1 was prepared by mixing an aqueous solution of KCl (405 mg, 5.43 mmol) and 72.8 ml 0.2 M HCl to a total volume of 100 ml. Accurate pH values were measured with a Hanna HI 9224 pH-meter equipped with a Hanna HI 1330B pH electrode.

The NMR experiments (^{1}H, ^{1}H–^{13}C HMBC, ^{1}H–^{13}C HSQC, ^{1}H–^{1}H COSY, ^{1}H–^{1}H TOCSY, ^{1}H–^{1}H ROESY, ^{1}H–^{1}H NOESY and CAPT were obtained at 600.13/500.13 and 150.90/125.76 MHz for ^{1}H and ^{13}C, respectively, on a Bruker Biospin AV-600 MHz instrument equipped with a TCI ^{1}H–^{13}C/^{15}N CryoProbe and a Bruker Ultrashield Plus AV-500 MHz instrument (Tables 1–4). All experiments were recorded at 298 K unless otherwise noted. Chemical shift values were set relative to the deuterio-methyl ^{13}C signal and the residual ^{1}H signal of the solvent; at δ 49.0 and δ 3.4 for CD_3OD (containing CF_3COOD), and at δ 39.6 and δ 2.49 for $(CD_3)_2SO$. NMR experiments of dissolved *C*-glycosylflavones were recorded using S1 (5% CF_3COOD in $(CD_3)_2SO$, v/v), S2 ($(CD_3)_2SO$) or S3 (CD_3OD), while *C*-glycosylanthocyanidins were dissolved in S4 (5% CF_3COOD in CD_3OD, v/v), or S5 (20% CF_3COOD in $(CD_3)_2SO$, v/v).

High-resolution LC–electrospray mass spectrometry (ESI^+/TOF), spectra were recorded using a JEOL AccuTOF JMS-T100LC in combination with an Agilent Technologies 1200 Series HPLC system. A Zorbax SB-C18 (50 mm × 2.1 mm, length × i.d., 1.8 μm) column was used for separation, and combinations of two solvents were used for elution: A, H_2O containing 0.5% TFA (v/v) and B, acetonitrile containing 0.5% TFA (v/v) (Table 5). The following solvent composition was used: 0–1 min 5% B (isocratic), 1–3 min 5 to 13% B (linear gradient), 3–6 min 13% B (isocratic), 6–8 min 13 to 30% B (linear gradient), 8–14 min 30 to 40% B (linear gradient). The flow rate was 0.4 ml min^{-1}.

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