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Flavonoids from flowers of two *Crocus chrysanthus-biflorus* cultivars: "Eye-catcher" and "Spring Pearl" (Iridaceae)

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Abstract

Eight flavonoids, of which five are new flavonols, were isolated from perianth segments of two *Crocus chrysanthus-biflorus* cultivars. The new flavonols were identified as 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranosides of kaempferol, quercetin and myricetin, and two were the corresponding modified kaempferol triglycosides acylated at OH-6 of the 7-glucoside with malonic acid or acetic acid. The flavonols coexist with the known 7-*O*-β-D-glucosides of dihydrokaempferol and apigenin and with isorhamnetin 3,4′-di-*O*-β-D-glucoside. Complete structural determination of all compounds was achieved using 1-D and 2-D NMR techniques and other spectral evidence.

Keywords: Crocus; Iridaceae; Flower pigments; Flavonoids; Malonic acid; Acetic acid; HPLC

1. Introduction

Several aglycones have been isolated from leaves or pollen of *Crocus* (Iridaceae) (Kuhn & Low, 1944; Bate-Smith, 1968; Harborne & Williams, 1983; Williams, Harborne, & Goldblatt, 1986). However, only kaempferol, quercetin and myricetin have been detected in perianth segments of the genus (Price, Robinson, & Robinson, 1939; Bate-Smith, 1968; Harborne & Williams, 1983). They were glycosylated but the carbohydrate moieties have only been identified by chromatographic studies (Harborne & Williams, 1983). Acylated flavonoids have not been detected earlier in *Crocus*, although they seem to occur regularly in Iridaceae (Pryakhina, Sheichenko, & Blinova, 1984; Kachroo et al., 1990; Abdul & Kumar, 1992).

More than 100 cultivars of *Crocus* are known today. They are derived from selection within and hybridization between relatively few species. Various characters including distribution pattern, habitat and morphological trait of the naturally occurring ca. 80 species have been described (Harborne, 1967; Mathew, 1982). However, about *Crocus chrysanthus-biflorus* cultivars, it is not possible to say to what extent they are hybrids between *C. chrysanthus* and *C. biflorus* (Jacobsen, Van Scheepen, & Ørgaard, 1997). We hope to elucidate the problems by using chemical markers in combination with cytological results (Ørgaard & Heslop-Harrison, 1994; Ørgaard, Jacobsen, & Heslop-Harrison, 1995aØrgaard, Jacobsen, & Heslop-Harrison, 1995b).

As a part of ongoing chemotaxonomic investigations of the genus, we report here on the identification of flavonoids from perianth segments of "Spring Pearl" and "Eye-catcher".

2. Results and discussion

Perianth segments of "Spring Pearl" and "Eye-catcher" were extracted with aqueous acetonitril con-

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Table 1
Analytical HPLC retention times (R_t) and FAB-MS of flavonoids found in two *Crocus chrysanthus-biflorus* cultivars. **1**: kaempferol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranoside, **2**: quercetin 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranoside, **3**: myricetin 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranoside, **4**: kaempferol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-(6-*O*-malonyl-β-D-glucopyranoside), **5**: kaempferol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-(6-*O*-acetyl-β-D-glucopyranoside), **6**: dihydrokaempferol 7-*O*-β-D-glucopyranoside, **7**: isorhamnetin 3,4′-di-*O*-β-D-glucopyranoside, **8**: apigenin 7-*O*-β-D-glucopyranoside

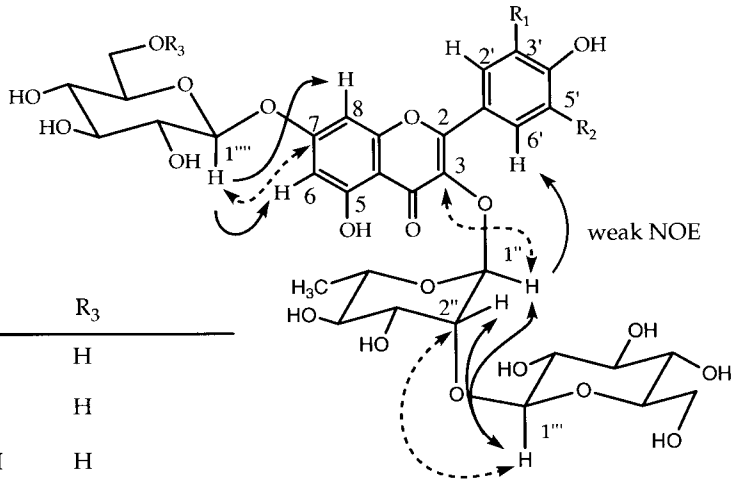
Flavonoid	Anal. HPLC R_t (min)	FAB-MS $[M]^+$, fragment ions
1	15.0	757 $[C_{33}H_{40}O_{20}]^+$, 595, 449, 287
2	12.2	773 $[C_{33}H_{40}O_{21}]^+$, 611, 465, 303
3	9.7	789 $[C_{33}H_{40}O_{22}]^+$, 627, 481, 319
4	27.7	843 $[C_{36}H_{42}O_{23}]^+$, 535, 287
5	30.2	799 $[C_{35}H_{42}O_{21}]^+$, 595, 491, 287
6	8.4	451 $[C_{21}H_{22}O_{11}]^+$, 289
7	21.6	641 $[C_{28}H_{32}O_{17}]^+$, 479, 317
8	30.7	433 $[C_{21}H_{20}O_{10}]^+$, 271

taining 0.5% trifluoroacetic acid (TFA). Purification of eight flavonoids was achieved by column chromatography on Amberlite XAD-7 with subsequent preparative HPLC. A new series of flavonol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranosides (**1**–**5**) was isolated, together with dihydrokaempferol 7-*O*-β-D-glucopyranoside (**6**) (Slimestad, Andersen, & Francis, 1994), isorhamnetin 3,4′-di-*O*-β-D-glucopyranoside (**7**) (Tsushida & Suzuki, 1995; Krauze-Baranowska & Cisowski, 1996) and apigenin 7-*O*-β-D-glucopyranoside (**8**) (Hmamouchi, Es-safi, Lahrichi, Fruchier, & Essassi, 1996; Skaltsa, Lazari, Loukis, & Harvala, 1996).

UV spectra of all compounds except **6** and **8** showed the characteristics of flavonol glycosides. Positive FAB-MS suggested that **1**–**3** had identical triglycosyl moieties and **4**–**5** were the malonate and the acetate of **1**, respectively (Table 1). The observed *m*/*z* 287 of **1**, **4** and **5** corresponded to kaempferol, while $[aglycone]^+$ of **2** and **3** were 16 and 32 mass units larger than **1**, indicating that **2** and **3** are quercetin and myricetin derivatives, respectively. This suggestion was supported by the reversed-phase HPLC retention times (**3** > **2** > **1** in order, Table 1).

^{1}H NMR and/or ^{13}C NMR spectra assigned by HSQC and HMBC confirmed the identity of the aglycones of **1**, **2** and **3** as kaempferol, quercetin and myricetin, respectively (Tables 2 and 3). The aromatic signals of **4** and **5** were identical with those of **1**, indicating that **4** and **5** are kaempferol glycosides.

^{1}H NMR signals of the sugar parts of **1**–**5** were identical (Table 2) except for a lowfield-shift of a signal corresponding to methylene group in **4** and **5**. By assignment using 1-D-HOHAHA, ^{1}H–^{1}H-COSY and homo-decoupling spectra, **1**–**5** were found to contain three hexose units. The signals at δ_H 0.84 (d, J = 6.0 Hz, H-6″), 5.65 (br s, H-1″), 3.52 (dd, J = 3.6 and 9.0



	R_1	R_2	R_3
1	H	H	H
2	OH	H	H
3	OH	OH	H
4	H	H	malonyl
5	H	H	acetyl

Fig. 1. ← NOE (negative), ↔ HMBC.

Table 2
^{1}H NMR spectral data of five new flavonoids from two *Crocus chrysanthus-biflorus* cultivars. The assignment of the sugar protons were carried out by 1-D-HOHAHA. By irradiation of H-1″ of rhamnoside, a negative NOE was observed at H-6′ of the nucleus. A negative NOE was also observed between the same anomeric proton and H-1‴. A strong negative NOE observed at H-6 and H-8 of the nucleus appeared by irradiation of H-1⁗ of the glucoside. Coupling constants *J* (in Hz) in parentheses. Columns with two sets of ppm data show values measured in C_5D_5N-d_5 in parentheses. The remaining data are in DMSO-d_6–10% TFA-d

Compound	**1**	**2**	**3**[a]	**4**	**5**
Aglycone					
6	(6.78) 6.51 d(1.2)	(6.78) 6.50 d(1.2)	6.75 d(1.2)	6.49 d(2.4)	6.43 d(2.4)
8	(6.98) 6.80 d(1.8)	(6.98) 6.78 d(1.8)	6.82 d(1.2)	6.78 d(2.4)	6.72 d(2.4)
2′	(7.97) 7.84 d(8.4)	(7.97) 7.58 br s	7.62 br s	7.82 d(8.4)	7.77 d(9.0)
3′	(7.22) 6.98 d(8.4)			6.97 d(8.4)	6.92 d(9.0)
5′	(7.22) 6.98 d(8.4)	(7.22) 6.95 d(8.4)		6.97 d(8.4)	6.92 d(9.0)
6′	(7.97) 7.84 d(8.4)	(7.97) 7.35 m	7.62 br s	7.82 d(8.4)	7.77 d(9.0)
Rhamnoside (H″)					
1	(6.28) 5.65 br s	(6.28) 5.56 br s	6.21 br s	5.65 br s	5.59 br s
2	(5.03) 4.16 m	(5.03) 4.18 dd(3.0;8.7)	4.99 m	4.15 m	4.09 m
3	(4.56) 3.52 dd (3.6; 9.0)	(4.58) 3.60 m	4.57 dd(2.4; 10)	3.58 dd(3.6; 10.2)	3.53 m
4	(4.40) 3.10 t(9.6)	(4.39) 3.14 t(9.0)	4.40 t(9.0)	3.17 t(9.6)	3.11 t(9.0)
5	(4.18) 3.30 m	(4.18) 3.53 m	4.15 m	3.34 m	3.31 m
6	(1.18) 0.84 d(6.0)	(1.18) 0.90 d(6.0)	1.18 d(6.0)	0.91 d(6.0)	0.86 d(6.0)
Glucoside (H‴)					
1	(5.28) 4.31 d(7.8)	(5.28) 4.28 d(7.8)	5.26 d(8.4)	4.30 d(7.2)	4.23 d(7.8)
2	(4.05) 3.22 t(9.0)	(4.05) 3.13 t(9.0)	4.03 t(9.0)	3.15 t(8.4)	3.16 t(9.0)
3	(4.22) 3.19 t(9.0)	(4.20) 3.12 t(9.0)	4.19 t(9.0)	3.12 t(9.0)	3.15 t(9.0)
4	(4.28) 3.05 t(9.6)	(4.28) 2.96 t(9.6)	4.30 t(9.0)	2.98 t(9.0)	3.01 t(9.0)
5	(3.93) 3.07 m	(3.94) 2.99 m	3.96 m	3.02 m	3.03 m
6	(4.44) 3.52 m	(4.39) 3.33 m	4.36 dd(5.4; 12.6)	3.46 m	3.48 m
	(4.55) 3.52 m	(4.43) 3.45 m	4.44 m	3.46 m	3.48 m
7-Glucoside (H⁗)					
1	(5.81) 5.10 d(7.2)	(5.81) 5.12 d(7.8)	5.75 d(7.2)	5.14 d(7.2)	5.07 d(7.2)
2	(4.33) 3.31 t(9.0)	(4.33) 3.30 t(8.4)	4.32 t(9.0)	3.30 t(9.0)	3.29 t(8.4)
3	(4.15) 3.27 t(9.0)	(4.18) 3.26 t(9.6)	4.15 t(9.0)	3.27 t(9.0)	3.27 t(8.4)
4	(4.38) 3.18 t(9.0)	(4.38) 3.18 t(9.6)	4.37 t(9.0)	3.18 t(9.0)	3.15 t(9.6)
5	(3.92) 3.43 m	(3.94) 3.43 m	3.95 m	3.72 m	3.69 m
6	(4.46) 3.47 dd(4.8; 11.7)	(4.42) 3.47 m	4.46 m	4.12 dd(6.6;12.0)	4.03 dd(6.6;11.4)
	(4.55) 3.70 m	(4.55) 3.70 m	4.51 dd(2.4; 12.6)	4.37 m	4.33 m
Acid				3.41–3.42	2.02 s

[a] Only measured in C_5D_5N-d_5.

Hz, H-3″) and 3.10 (t, $J=9.6$ Hz, H-4″) indicate the presence of a rhamnopyranosyl unit. The signals of two other sugars appeared at 4.31 (d, $J=7.8$ Hz, H-1‴) and 5.10 (d, $J=7.2$ Hz, H-1⁗) and 3.0–3.7 ($J_{2,3}=J_{3,4}=J_{4,5}=$ca. 9.0 Hz). Thus, the remaining two sugars must be β-D-glucopyranose.

The positions of the glycosidic linkages of **1**–**5** were determined by NOE difference spectra. By irradiation of H-1″, strong negative NOE was observed on H-1‴ and weaker on H-6′, but not on H-3″ and H-5″, indicating that rhamnose is directly linked to OH-3 of the flavonol and in α-configuration. By irradiation of H-1‴ glucosyl unit, strong negative NOEs appeared to H-2″ and H-1″ indicating that a glucosyl unit is linked to OH-2″ by the (β1–2) linkage. The (1–2) linkage was also confirmed by the lowfield-shift of H-1″ by ca. 0.4 ppm more than for other glucorhamnosyl units (Table 2) (Markham, Geiger, & Jaggy, 1992). Strong negative NOEs to H-6 and H-8 on A-ring of the chromophore were also observed by irradiation of H-1⁗, indicating that the other glucosyl unit is attached on OH-7. Finally, the glycosidic linkages were confirmed by HMBC (Table 3). Thus, **1**–**3** were identified as 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-glucopyranosides of kaempferol, quercetin and myricetin, respectively. The glucosyl(β1-2)rhamnosyl moiety has been found in a few other flavonoids (Oshima, Okamoto, & Hikino, 1987; Fukai & Nomura, 1988; Mizuno et al., 1990; Markham et al., 1992).

Comparison of H-6⁗ (-CH_2-) signals of **4** and **5** with that of **1**, showed a lowfield-shift of ca. 0.65 and 0.56 ppm, respectively (Liu, Shi-Lin, Roberts, &

Table 3
^{13}C NMR spectral data of flavonoids. Assignments of carbons having almost the same chemical shifts may be reversed

Flavonol	C	**1**[a,b]	**2**[a]	**3**[a]	**5**[b]	**6**[b]	**7**[b]
	2	156.9	156.9	156.9	158.2	83.4	156.1
	3	136.0	135.9	138.9	135.3	71.9	133.9
	4	178.7	178.4	178.9	178.3	198.9	177.8
	5	161.7	162.2	162.3	161.1	162.8	161.5
	6	100.3	100.3	100.2	99.7	97.1	99.0
	7	163.9	163.9	163.8	163.1	165.7	164.5
	8	95.0	95.1	95.1	95.0	95.7	94.1
	9	156.9	156.9	156.9	156.5	158.6	156.9
	10	107.6	107.7	107.9	106.2	102.4	104.6
	1′	121.2	122.1	120.9	120.6	127.7	124.1
	2′	131.2	117.0	109.4	130.9	129.8	113.9
	3′	116.2	147.1	162.1	115.6	115.2	148.5
	4′	158.3	158.8	158.8	160.5	158.6	148.9
	5′	116.2	116.5	162.1	115.6	115.2	115.0
	6′	131.2	121.7	109.4	130.9	129.8	121.8
	OMe						56.0
Sugar at C-3	1″	102.4	102.6	102.8	101.2		101.2
	2″	82.5	82.7	82.9	81.6		74.6
	3″	72.1	72.2	72.2	70.0		76.6
	4″	71.6	71.7	71.7	71.9		70.1
	5″	73.4	73.7	73.7	70.7		77.8
	6″	18.1	18.1	18.2	17.6		60.9
	1‴	106.8	106.9	106.8	106.5		
	2‴	75.6	75.7	75.7	70.4		
	3‴	79.0	78.4	78.3	76.4		
	4‴	70.9	71.2	70.9	74.3		
	5‴	78.4	79.0	79.1	77.0		
	6‴	62.2	62.3	62.3	60.7		
Sugar at C-7	1⁗	101.5	101.6	101.6	100.0	100.0	
	2⁗	74.6	74.7	74.6	73.2	73.2	
	3⁗	78.4	78.3	78.2	76.5	76.5	
	4⁗	70.9	70.9	70.7	69.5	69.8	
	5⁗	78.2	78.5	78.4	74.1	77.4	
	6⁗	62.1	62.2	62.2	63.7	60.8	
Sugar at C-4′	1⁗						100.0
	2⁗						73.4
	3⁗						77.0
	4⁗						69.8
	5⁗						77.4
	6⁗						60.9
OCOMe					170.5		
OCO*Me*					20.7		

[a] Compounds measured in pyridine-d_5. Remaining compounds measured in DMSO-d_6–10% TFA-d.
[b] Assignments have been confirmed by 2-D techniques (^{1}H–^{1}H COSY, HSQC or HMBC). In the HMBC spectra of **1** and **5**, appreciable shift correlations were observed; correlations of **1** in pyridine-d_5 were C-7(δ_c 163.9)-H-1⁗(δ_H 5.81), C-3(δ_c 136.0)-H-1″(δ_H 6.28), and C-2″(δ_c 82.5)-H-1‴(δ_H 5.28); these of **5** in DMSO-d_6-10% TFA-d., C-7(δ_c 163.1)-H-1⁗(δ_H 5.07), C-3(δ_c 135.3)-H-1″(δ_H 5.59) and C-2″(δ_c 81.6)-H-1‴(δ_H 4.23).

Phillipson, 1989; Nørbæk & Kondo, 1998) (Table 2). From MS data, **4** must be malonated, and the methylene of malonate appeared at δ_H 3.41–3.42. Thus, **4** is kaempferol 3-*O*-α-L-(2-*O*-β-D-glucopyranosyl)rhamnopyranoside-7-*O*-β-D-(6-*O*-malonyl)glucopyranoside. In **5** additional CH_3CO-signals (δ_H 2.02 and δ_C 170.5, 20.7 ppm) was observed Liu et al., 1989; Allais et al., 1991; Allais, Chulia, Kaouadji, Simon, & Delage, 1995). The lowfield-shift of C-6⁗ by ca. 3 ppm and the smaller highfield-shift of the neighboring carbon (C-5⁗) indicates that the acetyl group was attached to the 6-OH (Liu et al., 1989) (Table 3). This result was con-

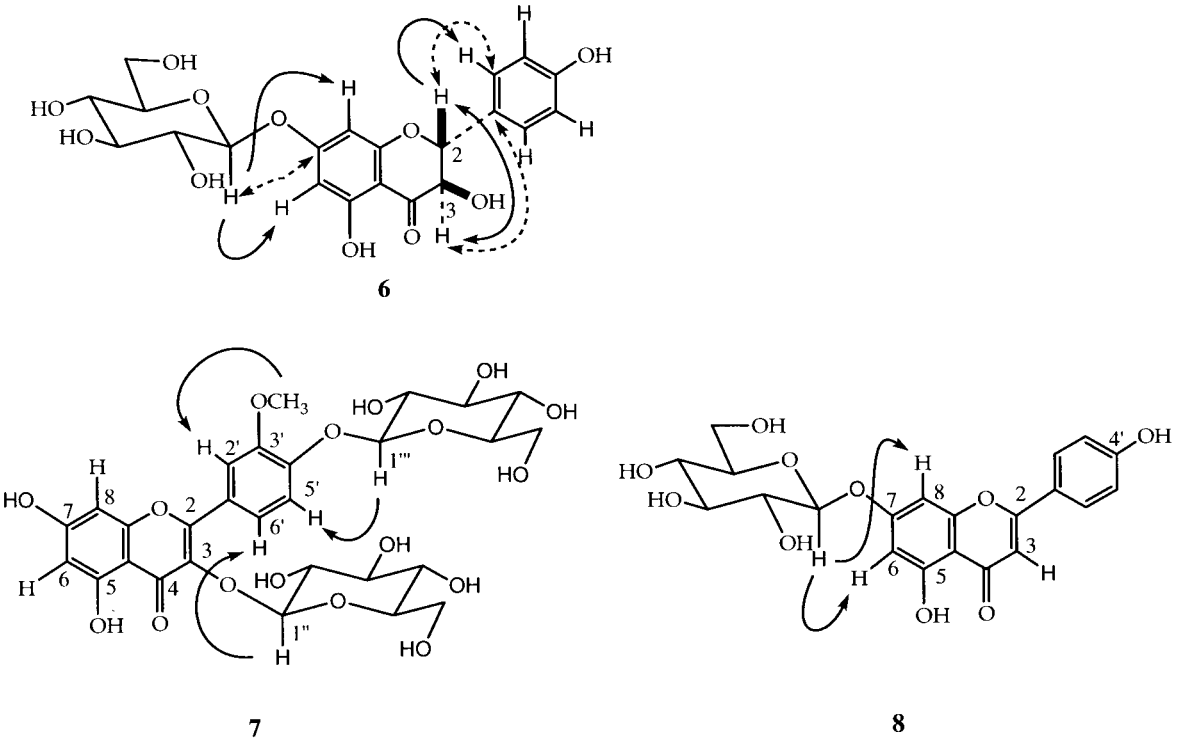


Fig. 2. ← NOE, ↔ HMBC. Only important correlation was described.

sistent with the HMBC spectrum, showing shift-correlation between ^{13}C of carbonyl and H-6‴. Thus, **5** is OH-6‴-*O*-acetate of **1** (Fig. 1).

From "Spring Pearl" **6–8** also were isolated. Compound **6** was eluted earlier and its UV spectrum showed similarity with dihydrokaempferol (λ 284 and 335 sh) (Markham, 1982) and the molecular ion *m/z* 451 (Table 1) suggested a dihydroflavonoid monoglucoside (Slimestad et al., 1994). ^1H and ^{13}C NMR signals were assigned by using 1-D-HOHAHA and ^1H–^1H-COSY. Compound **6** had two aromatic systems linked through -CH–CH–CO-, and one β-D-glucopyranosyl unit. Connection of the components was directly determined by NOE difference, HSQC and HMBC (as shown in Fig. 2). All data were consistent with those previously reported (Slimestad et al., 1994) (Tables 3 and 4). Thus, **6** was determined to be dihydrokaempferol 7-*O*-β-D-glucopyranoside (Fig. 2).

Compound **7** gave the characteristic UV spectrum of isorhamnetin and a free 7-OH group (Markham, 1982) and the FAB-MS fragmentation pattern *m/z* 479 (M-162), 317 (M-324 M- 2 × hexose, isorhamnetin + 1) (Table 1). The ^1H NMR confirmed the presence of one isorhamnetin nucleus since methoxy (δ_H 3.89) and two aromatic spin systems, δ_H 7.58 (J = 1.8, 9.0), 7.26 (J = 9.0) and 8.01 (J = 2.4 Hz) corresponded to H-6′, H-5′ and H-2′, respectively, and δ_H 6.51 (J = 2.4) and δ 6.27 (J = 1.8) to H-8 and H-6, respectively (Table 4). *J*-values of all vicinal couplings in the sugar regions were 7.8–9.6 Hz, indicating that the two sugars must be β-D-glucopyranose. Thus, **7** is isorhamnetin 3,4′-di-*O*-β-D-glucopyranoside (Tsushida & Suzuki, 1995; Krauze-Baranowska & Cisowski, 1996) (Fig. 2).

UV of **8** was identical with a flavone monoglucoside (Markham, 1982) which was supported by the FAB-MS, showing peaks of *m/z* 433 $[M+H]^+$ and 271 [M-hexose, apigenin] (Table 1). The structure was identified by ^1H NMR. A characteristic signal appeared at δ_H 7.28 (s), corresponding to H-3, indicating the chromophore is a flavone, apigenin (Table 4). The hexose was a β-linked glucopyranoside because all vicinal coupling constants were 7.2–9.6 Hz. From the glycosidic linkage determination by NOE difference, **8** is apigenin 7-*O*-β-D-glucopyranoside, previously isolated from other plants (Hmamouchi et al., 1996; Skaltsa et al., 1996) (Fig. 2).

3. Experimental

3.1. Plant material

Field grown flowers of the *Crocus chrysanthus-biflorus* cultivars "Eye-catcher" and "Spring Pearl" were collected in Noordwijk, Holland, in March 1996. The identity was verified by Professor N. Jacobsen,

Table 4
^{1}H NMR spectral data of three known flavonoids from perianth segments of *Crocus chrysanthus-biflorus* "Spring Pearl" in DMSO-d_6–10% TFA-d. Coupling constants *J* (in Hz) in parentheses

	H No.	Aglycone (ppm)	H No.	3-Glucoside (H″) (ppm)	4′-Glucoside (H‴) (ppm)	7-Glucoside (H⁗) (ppm)
6	2	5.15 d(12.0)	1			5.00 d(7.8)
	3	4.67 d(10.8)	2			3.30 t(9.0)
	6	6.18 br s	3			3.25 t(9.0)
	8	6.21 br s	4			3.19 t(9.0)
	2′	7.36 d(9.0)	5			3.42 m
	3′	6.84 d(7.8)	6			3.47 dd(5.4,11.7)
	5′	6.84 d(7.8)				3.69 m
	6′	7.36 dd(9.0)				
7[a]	6	6.27 d(1.8)	1	5.62 d(8.4)	5.10 d(7.8)	
	8	6.51 d(2.4)	2	3.31 t(9.0)	3.36 t(9.6)	
	2′	8.01 d(2.4)	3	3.27 t(9.6)	3.34 t(8.4)	
	5′	7.26 d(9.0)	4	3.16 t(9.6)	3.25 t(9.6)	
	6′	7.58 dd(1.8, 9.0)	5	3.17 m	3.41 m	
	OMe	3.89 s	6	3.44 m	3.51 dd(4.8;11.7)	
				3.63 m	3.72 m	
8	3	7.28 br s	1			5.10 d(7.2)
	6	6.82 d(1.2)	2			3.30 d(8.4)
	8	6.46 d(1.8)	3			3.25 t(8.4)
	2′	8.10 d(8.4)	4			3.18 t(9.6)
	3′	6.97 d(8.4)	5			3.43 m
	5′	6.97 d(8.4)	6			3.46 m
	6′	8.10 d(8.4)				3.73 m

[a] Negative difference NOE measured at 10°.

Royal Veterinary and Agricultural University, Copenhagen.

3.2. Isolation of flavonoids

Freeze-dried perianth segments of "Eye-catcher" (40 g) and "Spring Pearl" (50 g) were extracted with 50% aq. CH_3CN containing 0.5% TFA at room temp. for 1 h. The conc extracts were adsorbed on an Amberlite XAD-7 column, washed with 0.5% TFA aq. soln and then eluted stepwise from 4 to 20% aq. CH_3CN containing 0.5% TFA. The flavonoids were further purified by prep. ODS-HPLC (20ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) in the same solvent system; flow rate of 7 ml min^{-1}; monitoring at 280 nm. The pure fractions were conc to dryness in vacuo and stored at −80°. From "Eye-catcher" **1** (50 mg) and **2** (15 mg), from "Spring Pearl" **1** (30 mg), **2** (10 mg), **3** (10 mg), **4** (6 mg), **5** (17 mg), **6** (15 mg), **7** (8 mg) and **8** (10 mg) were isolated.

3.3. Analysis of flavonoids

Analytical HPLC was carried out on a ODS-HPLC column (4.6ϕ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) using an elution profile as follows: 0 min 16% B, 3 min 38% B, 10 min 44% B, 20 min 50% B, 25 min 67% B, 40–50 min 100% B; solvent A (H_2O–TFA, 99:1) and solvent B (CH_3CN–H_2O–TFA, 60:140:1); flow rate 1.5 ml min^{-1}.

3.4. Spectral measurements

3.4.1. Kaempferol 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-glucopyranoside (*1*)

UV λ_{max}(nm): 265, 310sh, 345; +NaOH: 269, 305sh, 335sh, 379; +$AlCl_3$: 269, 302sh, 348, 395; +$AlCl_3$+HCl: 269, 302sh, 348, 395; +NaOAc: 267, 385; +NaOAc+H_3BO_3: 267, 347.

3.4.2. Quercetin 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-glucopyranoside (*2*)

UV λ_{max} (nm): 255, 268sh, 301sh, 352; +NaOH: 272, 326, 399 (dec.); +$AlCl_3$: 272, 299sh, 325sh, 434; +$AlCl_3$+HCl: 270, 299sh, 362, 398; +NaOAc: 262, 296sh, 374, 410sh; +NaOAc+H_3BO_3: 261, 295sh, 370.

*3.4.3. Myricetin 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-glucopyranoside (**3**)*

UV λ_{max} (nm): 295, 358; +NaOH: 295, 398 (dec.); +$AlCl_3$: 280, 310, 425; +$AlCl_3$+HCl: 308, 365, 402; +NaOAc: 300, 408; +NaOAc+H_3BO_3: 378.

*3.4.4. Kaempferol 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-(6-O-malonyl)glucopyranoside (**4**)*

UV λ_{max} (nm): 266, 301sh, 315sh, 345; + NaOH: 265, 295, 340sh, 384; +$AlCl_3$: 267, 299, 346, 398; +$AlCl_3$+HCl: 269, 297, 342, 397; +NaOAc: 267, 290, 362, 398sh; +NaOAc+H_3BO_3: 267, 292, 315sh, 346.

*3.4.5. Kaempferol 3-O-α-L-(2-O-β-D-glucopyranosyl)rhamnopyranoside-7-O-β-D-(6-O-acetyl)glucopyranoside (**5**)*

UV λ_{max} (nm): 265, 290sh, 315sh, 345; +NaOH: 265, 295, 340sh, 381; +$AlCl_3$: 267, 299, 346, 398; +$AlCl_3$+HCl: 267, 297, 342, 397; +NaOAc: 267, 290, 362, 400sh; +NaOAc+H_3BO_3: 267, 292, 315sh, 346.

*3.4.6. Dihydrokaempferol 7-O-β-D-glucopyranoside (**6**)*

UV λ_{max} (nm): 284, 335sh; +NaOH: 244, 289, 360; +$AlCl_3$: 315, 367; +$AlCl_3$+HCl: 284, 315, 362; +NaOAc: 283, 330sh; +NaOAc+H_3BO_3: 283, 330sh.

*3.4.7. Isorhamnetin 3,4′-di-O-β-D-glucopyranoside (**7**)*

UV λ_{max} (nm): 268, 300sh, 351; +NaOH: 260, 301, 403; +$AlCl_3$: 266, 304sh, 352sh, 402; +$AlCl_3$+HCl: 267, 309sh, 351sh, 402; +NaOAc: 280, 319, 375; +NaOAc+H_3BO_3: 280, 350.

*3.4.8. Apigenin 7-O-β-D-glucopyranoside (**8**)*

UV λ_{max} (nm): 270, 323sh, 425; +NaOH: 269, 335, 421; +$AlCl_3$: 276, 304, 354, 423; +$AlCl_3$+HCl: 276, 304, 354, 423; +NaOAc: 268, 320sh, 398; +NaOAc+H_3BO_3: 268, 365.

FAB-MS spectra were obtained in a positive mode using glycerol (1 drop of HCl aq. was added) as a matrix.

Using a 600-MHz instrument (JNM alpha 600, JEOL) (^{1}H, ^{1}H–^{1}H-COSY, 1-D-HOHAHA, homodecoupling, NOE difference, ^{13}C, HSQC and HMBC), spectra were measured in DMSO-d_6 containing 10% TFA-d with internal standard CD_2HOD (3.326 ppm). Exceptionally pyridine-d_5 was used as solvent. 1-D HOHAHA, homodecoupling and 2-D spectra were obtained using a pulse sequence supplied from JEOL.

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References

Abdul, S. S., & Kumar, T. (1992). *Phytochemistry*, *31*, 1399.
Allais, D. P., Simon, A., Bennini, B., Chulia, A. J., Kaouadji, M., & Delage, C. (1991). *Phytochemistry*, *30*, 3099.
Allais, D. P., Chulia, A. J., Kaouadji, M., Simon, A., & Delage, C. (1995). *Phytochemistry*, *39*, 427.
Bate-Smith, E. C. (1968). *Botanical Journal of the Linnean Society*, *60*, 325.
Fukai, T., & Nomura, T. (1988). *Phytochemistry*, *27*, 259.
Harborne, J. B. (1967). *The comparative biochemistry of the flavonoids*. London: Academic Press.
Harborne, J. B., & Williams, C. A. (1983). *Zeitschrift fuer Naturforschung*., *39C*, 18.
Hmamouchi, M., Es-safi, N., Lahrichi, M., Fruchier, A., & Essassi, E. M. (1996). *American Journal of Enology and Viticulture*, *47*, 186.
Jacobsen, N., Van Scheepen, J., & Ørgaard, M. (1997). *The New Plantsman*, *4*, 6.
Kachroo, P. K., Razdan, T. K., Qurishi, M. A., Khuroo, M. A., Koul, S., & Dhar, K. L. (1990). *Phytochemistry*, *29*, 1014.
Krauze-Baranowska, M., & Cisowski, W. (1996). *Polish Journal of Chemistry*, *70*, 430.
Kuhn, R., & Low, I. (1944). *Chemische Berichte*, *77B*, 196.
Liu, K. C. S., Shi-Lin, Y., Roberts, M. F., & Phillipson, J. D. (1989). *Phytochemistry*, *28*, 2813.
Markham, K. R. (1982). *Techniques of flavonoid identification*. Academic Press.
Markham, K. R., Geiger, H., & Jaggy, H. (1992). *Phytochemistry*, *31*, 1009.
Mathew, B. (1982). *The Crocus*. London: B.T. Batsford Ltd.
Mizuno, M., Iinuma, M., Tanak, T., Sakakibara, N., Murata, J., & Lang, F. A. (1990). *Phytochemistry*, *29*, 1277.
Nørbæk, R., & Kondo, T. (1998). *Phytochemistry*, *47*, 861.
Ørgaard, M., & Heslop-Harrison, J. S. (1994). *Annals of Botany*, *73*, 195.
Ørgaard, M., Jacobsen, N., & Heslop-Harrison, J. S. (1995a). *Annals of Botany*, *76*, 253.
Ørgaard, M., Jacobsen, N., & Heslop-Harrison, J.S. (1995b). In: Brandham, P.E., & Bennett, M.D. (Editors). Kew Chromosome Conference IV, 291. Royal Botanic Gardens, Kew.
Oshima, Y., Okamoto, M., & Hikino, H. (1987). *Heterocycles*, *26*, 935.

Price, J. R., Robinson, G. M., & Robinson, R. (1939). *Chem. Soc.*, *1938*, 281.

Pryakhina, N. I., Sheichenko, V. I., & Blinova, K. F. (1984). *Khim. Prir. Soedin.*, 589.

Skaltsa, H., Lazari, D., Loukis, A., & Harvala, C. (1996). *Sciences Pharmaceutiques*, *64*, 103.

Slimestad, R., Andersen, Ø. M., & Francis, G. W. (1994). *Phytochemistry*, *35*, 550.

Tsushida, T., & Suzuki, M. (1995). *Nippon Skokuhin Kagaku Kogaku Kaishi*, *42*, 100.

Williams, C. A., Harborne, J. B., & Goldblatt, P. (1986). *Phytochemistry*, *25*, 2135.