Identification of Flavone *C*-Glycosides Including a New Flavonoid Chromophore from Barley Leaves (*Hordeum vulgare* L.) by Improved NMR Techniques

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Six flavone *C*-glycosides were isolated from young leaves of barley. One of the *C*-glucosides has a new type of nucleus, a 2′,4′,5,5′,7-penta-OH-substituted flavone bearing a 6-*C*-β-D-glucoside, which has apparently never been isolated before. One mono- and two di-*C*-glycosyl flavones were isolated for the first time from barley and identified as isoscoparin 7-*O*-β-D-glucoside, carlinoside, and shaftoside, respectively. Other flavones were 7-*O*-β-D-glucosides of isoorientin and isovitexin. The known problematic NMR structure elucidation of *C*-glycosyl flavonoids has been solved by using both a temperature close to the freezing point of the solvent (22.5 °C in DMSO-d_6) and a high temperature (70, 90 °C) for comparison during NMR measurements. Structural determination of all the compounds was achieved by employing 1D and 2D NMR techniques.

Keywords: *Barley; Hordeum vulgare; C-glycosyl flavone; phenolic hydroxyl group; NMR, NOE*

INTRODUCTION

Several flavonoids, mostly flavone *C*-glycosides, have been isolated from leaves of barley (Bekele, 1983; Fröst et al., 1977; Liu et al., 1995; Osawa et al., 1992). However, generally ^{1}H NMR results of flavonoid *C*-glycosides are limited, most likely because of difficulties in finding the relevant measuring conditions (Besson et al., 1985; Chopin et al., 1974; Haribal and Renwick, 1998; Hörhammer et al., 1965; Krauze-Baranowska and Cisowski, 1995; Marston et al., 1976; Theodor et al., 1980, 1981). For example, for the flavonoid 6-*C*-glucoside-7-*O*-glucosides isolated from barley we observe two rotational isomers. Their frequency of rotation is close to the NMR frequency, resulting in double and broadening signals. However, in this study of flavonoids isolated from young barley leaves a new method has been developed resulting in clear ^{1}H NMR and difference NOE spectra.

In general, the fast exchange of phenolic protons on the NMR time scale gives an average chemical shift spectrum. By using a temperature very close to the freezing point of the solvent (22.5 °C in DMSO-d_6) during NMR measurements the exchange rate of the phenolic protons of the flavone slows considerably. This point attracted our attention. The phenolic proton signals are able to be observed individually because of a slow rotation of the molecules compared with the NMR frequency (600 MHz). Because of the slow correlation time, the rotational isomers can each be observed (Markham et al., 1987; Jay, 1994) and the negative NOE between the nearest protons can clearly be visualized.

Earlier identification of phenolic compounds in barley leaves has been connected to studies on UV-B effects (Liu et al., 1995), chemotaxonomy (Bekele, 1983; Fröst et al., 1975, 1977), and antioxidant activity (Osawa et al., 1992), respectively. In a previous study, the fingerprints from analytical HPLC analyses of barley plants infected and noninfected with powdery mildew (*Erysiphe graminis*) gave a general overview of changes in phenol composition, but it had been used only to establish whether differences were present among samples of plant material, not to compare specific effects on particular classes of compounds (Doll et al., 1994).

The purpose of the present study was to describe the complete assignment of the *C*-glycosyl flavonoid structures in barley by improved NMR techniques as a part of a project searching for a possible relation between particular classes of flavonoid structures and resistance against leaf diseases (Doll et al., 1994; Christensen et al., 1998). Furthermore the contents of phenolic acids in barley are of interest, probably influencing both nutritional qualities and disease resistance and will be discussed in a future study.

EXPERIMENTAL PROCEDURES

Collection of Plant Material. Field-grown young plants (7 months) of the winter barley cultivar Alexis were collected at the Danish Institute of Agricultural Sciences, Aarslev, in May 1998. No signs of leaf diseases could be observed.

Isolation of Flavonoids. Freeze-dried barley leaves (500 g) were extracted with 50% aqueous CH_3CN at room temperature for 1 h. The concentrated extract was adsorbed on an Amberlite XAD-7 column, washed with H_2O, and then eluted stepwise from 8 to 50% aqueous CH_3CN. The flavonoid fractions eluted from 18 to 20% aqueous CH_3CN were further purified by preparative ODS-HPLC (20 ∅ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) in the same solvent system with a flow rate of 7 mL min^{-1} and monitoring at 254 nm. The pure fractions were concentrated to dryness in vacuo and

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Table 1. HPLC Retention Time and UV λ_{max} Measured in MeOH

compound	retention time (t_R) (min)	UV λ_{max} (nm)
1[a]	36.2	262, 378
2	41.8	271, 345
3	25.5	270, 350
4	41.3	270, 348
5	22.3	290, 356
6	37.4	272, 332

[a] Additional spectral measurements of **1**: UV λ_{max}(nm) 262, 378; + $AlCl_3$, 273, 329, 425; + $AlCl_3$ + HCl, 274, 329, 434; +NaOAc, 263, 412; + NaOAc + H_3BO_3, 265, 406.

Table 2. ^1H and ^{13}C Chemical Shift of 1 Measured in DMSO-d_6 at 22.5 °C

no.	^1H	^{13}C	no.	^1H	^{13}C
2		161.3	1′		106.8
3	7.00 s	104.1	2′		151.6
4		181.8	3′	6.49 s	106.7
5		160.6	4′		150.2
6		108.5	5′		138.6
7		162.9	6′	7.25 s	113.3
8	6.40 s	93.2	1″	4.55 d (9.9)	73.0
9		156.1	2″	4.00 t (8.9)	70.1
10		103.2	3″	3.17 t (8.8)	78.8
			4″	3.09 t (9.2)	70.4
			5″	3.14 m	81.5
			6″	3.38 m	61.4
			6″	3.65 m	
OH[a]					
2′	10.18				
4′	9.87				
5′	8.75				
5	13.65				
7	10.50				

[a] All hydroxy protons were broadening singlet.

stored at − 80 °C. From the leaves **1** (15 mg), **2** (50 mg), **3** (15 mg), **4** (10 mg), **5** (50 mg), and **6** (15 mg) were isolated.

Analysis of Flavonoids. Analytical HPLC was conducted on an ODS-HPLC column (4.6 ∅ × 250 mm, Develosil ODS-HG-5, Nomura Chemicals) using an elution profile as follows: 0 min, 100% A; 20 min, 95% A; 30 min, 90% A; 45 min, 80% A; 50 min, 75% A; 60−79 min, 0% A; 90−95 min, 100% A; solvent A was CH_3CN/H_2O, 8:42, and solvent B was CH_3CN/H_2O, 1:1; flow rate was 1.0 mL min^{-1}.

Spectral Measurements. FAB-MS spectra were recorded in a positive mode using glycerol and NBA (*m*-nitrobenzyl alcohol) as a matrix.

Using a 600 MHz instrument (JNM alpha 600, JEOL), ^1H, $^1H-^1H$ COSY, TOCSY, 1D-HOHAHA, NOE difference, ^{13}C (only **1**, **2**, and **5**), HSQC, and HMBC (only **1** and **5**) spectra were measured in DMSO-d_6 with internal standard $CD_2\underline{H}OD$ (3.326 ppm) at 22.5 °C. For comparison some measurements were performed at 50, 70, 90, and 110 °C. 1D-HOHAHA, homodecoupling, and 2D spectra were obtained using a pulse sequence supplied from JEOL.

RESULTS AND DISCUSSION

When NMR measurements were performed very close to the freezing temperature of the solvent (22.5 °C in DMSO-d_6), the molecular rotation and exchange among the protons were highly hindered, so that the rotamers could be observed separately because of the slow correlation time (Table 3). However, spin diffusion has not been observed despite the high viscosity. Therefore, this method made it possible to observe clear NOE signals between spatially close protons. By preirradiation of a signal for longer time than the correlation time, but shorter than the chemical exchange time for difference NOE, the residue signals differed from each other. Also, NOE signals between the protons of the hydroxyl groups and the chromophore could be observed due to the slow proton exchange among phenolic hydroxyl groups in DMSO at low temperature (parts per million values only shown in Table 2). For comparison, the measurements of flavone 6-*C*-glucoside-7-*O*-glucosides (**2**, **5**, and **6**) were performed at relatively high temperatures (50, 70, or 90 °C) before the temperature was lowered to 22.5 °C. At high temperatures the increasing motion of the residues sharpened and unified the signals, but NOE between H-8 and H-2′ was not possible to observe. Likewise, when various high temperatures were used during NOE measurements of flavone 6,8-di-*C*-glycosides (**3** and **4**), no appreciable NOE signal between the proton of the sugar attaching at C-8 and the proton on the B-ring appeared. The ^1H NMR data of rotational isomers of **5** are presented in Table 3.

A major new achievement of the present work is thus the demonstration of a method to measure NOE signals of *C*-glycoside flavonoids clearly by lowering the temperature to near the freezing point of DMSO. This strategy may also be applicable to other types of compounds for the determination of $^1H-^1H$ relations by the idea of using the phenolic exchangeable proton for NOE structural analysis.

Compound **1** was identified as 2′-hydroxyisoorientin (Figure 1), revealing the existence of a flavone chromophore not reported before. The FAB mass spectrum showed an $[M + H]^+$ at 465, in good agreement with the mass calculated for $C_{21}O_{12}H_{20}$. Compound **1** showed λ_{max} at 262 and 378 nm (UV measurements using shift reagents are shown in Table 1).

The ^1H NMR at 22.5 °C revealed a 2′,4′,5,5′,7-penta-OH-substituted flavone from the aromatic spin systems: δ_H 7.25 (s) and 6.49 (s), corresponding to H-6′ and H-3′, respectively, and δ_H 7.00 (s) and 6.40 (s) corresponding to H-3 and H-8, respectively (Table 2). The hexoside was assigned as a *C*-β-linked glucoside because all vicinal coupling constants were 8.8−9.9 Hz, including the anomeric proton at δ_H 4.55 (*d*, J = 9.9 Hz) and H-2″ at δ_H 4.00 (*t*, J = 8.9 Hz) (Table 2). The position of the glucosidic linkage was determined by an NOE difference spectrum. A negative NOE by irradiation of H-1″ was observed at HO-5 (δ 13.65) and HO-7 (δ 10.50). Irradiation at H-8 (δ_H 6.40) confirmed that the sugar moiety is linked to C-6 of the aglycon because no NOE was observed to the anomeric proton. By irradiation of H-3 of the chromophore, negative NOE appeared to H-6′ and HO-2′ (δ 10.18). The H-3′ was assigned to the para position to H-6′ because the $J_{3',6'}$ value was 0 Hz. The NOE network indicated the existence of a 2′,4′,5,5′,7-penta-OH-substituted flavone (Figure 1). The HMBC measured in DMSO-d_6 at 22.5 °C (Table 2) confirmed this substituent position. Thus, **1** is 6-*C*-β-D-glucopyranosyl-2′,4′,5,5′,7-pentahydroxyflavone (Figure 1).

The FAB-MS spectrum of **2** showed a molecular ion at *m*/*z* 625 [M + H], in good agreement with the mass calculated for $C_{28}O_{16}H_{32}^+$, and the UV λ_{max} was identical with data previously introduced for isoscoparin-7-*O*-β-D-glucoside (Marston et al., 1976) (Table 1). Analysis of the ^1H NMR spectrum of **2** revealed the presence of an aglycon containing −OMe (δ_H 3.90) consistent with chrysoeriol, containing one *O*-β-linked and one *C*-β-linked glucoside residue. The assignment of the two glucoside units was carried out by 1D-HOHAHA and $^1H-^1H$ COSY, but some signals from the sugar moieties

Table 3. ^{1}H NMR Spectral Data Measured in DMSO-d_6 at 22.5 °C of Five Flavone Glycosides from Young Barley Leaves[a]

	2	**3**[b]	**4**[b]	**5**[c]	**6**[d]
aglycon					
3	6.58 s	6.63s	6.69 s	6.86 s, 6.82 s	6.86 s
8	6.47 s			6.77 s, 6.73 s	6.93 s
2′	7.56 br s	7.45 s	7.91 d (8.5)	7.45 d (2.0), 7.44 d (2.0)	7.94 d (8.5)
3′			6.91 d (8.5)		6.94 d 8.5)
5′	7.38 br s	6.90 d (8.0)	6.91 d (8.5)	6.92 d (8.0), 6.90 d (8.0)	6.94 d (8.5)
6′	6.94 m	7.56 br s	7.91 d (8.5)	7.45 d (2.0), 7.44 d (2.0)	7.94 d (8.5)
OMe	3.90 s				
6-*C*-glucoside (H″)					
1	4.64 d (9.5)	4.71 d (9.5)	4.72 d (9.5)	4.64 d (9.5), 4.70 d (9.5)	4.64 d (9.5)
2	4.00 t (9.5)	3.93 m	3.87 br	3.91 t (9.5)	3.91 t (9.5)
3	3.20 m	~3.25 m	~3.3 m	3.20 t (9.0)	3.20 t (9.0)
4	3.10 m	~3.25 m	~3.3 m	3.18 m	3.19 t (9.0)
5	3.20 m	~3.25 m	~3.3 m	3.14 m	3.15 m
6	3.30 m	3.51 m	3.52 m	3.26 m	3.26 m
	3.50 m	3.66 d (12.0)	3.66 d(12.0)	3.57m	3.58 m
7-*O*-glucoside (H‴)					
1	4.96 d (7.5)			4.96 d (7.5), 5.03 d (7.5)	4.95 d (7.5)
2	3.43 t (8.8)			3.31 m	3.48 m
3	3.35 t (9.0)			3.28 m	3.33 t (9.0)
4	3.45 m			3.33 m	3.30 t (9.0)
5	3.22 m			3.28 m	3.21 m
6	3.47 m			3.49 m	3.48m
	3.90 m			3.76 m	3.77m
8-*C*-arabinoside (H‴)					
1		4.81 d (9.5)	4.77 d (9.5)		
2		4.04 br t	4.06 br s		
3		3.54 dd (9; 2.5)	3.56 dd (9; 3.5)		
4		3.87 br s	3.85 br s		
5		3.67 d (12.0)	3.69 d (12.0)		
5		3.93 dd (12.0; 1.5)	3.91 dd (12; 1.5)		

[a] Assignments of the sugar protons carried out by 1D-HOHAHA. Coupling constants *J* (in hertz) in parentheses. [b] Measured at 70 °C. In ^{1}H NMR signals of **5** and **6** rotamers were observed, so that each signal showed two peaks corresponding to the isomers. [c] Signals of both rotational isomers of **5** are shown. [d] The minor signal of **6**; H″-1 and H‴-1 appeared at 5.01 d (7.5) and 4.70 d (9.5), respectively.

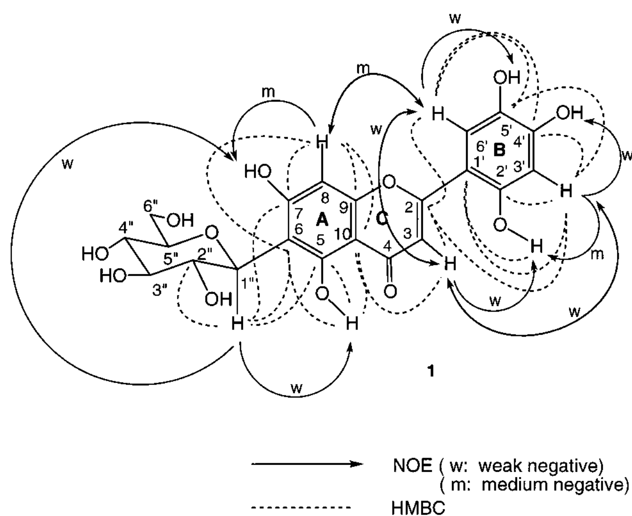

Figure 1. Appreciable information of the new natural product 2′-hydroxyisoorientin (**1**) for the structure determination by ^{1}H NMR in DMSO at 22.5 °C. The signal of HO-5 (δ 13.65) appeared at characteristic low field, arising from a strong intramolecular hydrogen bonding. A negative NOE by irradiation of H-1″ was observed at HO-5 and HO-7. Furthermore, HO-7 also showed clear NOE to H-8, indicating that the sugar attaches to C-6 but not to C-8. By irradiation of H-6′ a NOE appeared at H-8, weaker at H-3 (δ 7.00) and HO-5′ (δ 8.75). Therefore, the B and C rings were directly connected; also it was confirmed by correlation between C-2 and H-6′ by HMBC. The 1′,2′,4′,5′-substitution on the B ring ($J_{3',6'} = 0$ Hz indicates para-substitution) was elucidated by NOE observation on HO-4′ and HO-2′ by irradiation of H-3′, and that on HO-5′ by irradiation of H-6′. The structure was confirmed by HMBC.

overlapped with each other (Table 3). However, clear signals appeared from H-1‴ of the *O*-β-linked glucoside unit at δ_H 4.96 (d, $J = 7.5$ Hz), H-1″ of the *C*-β-linked glucoside unit at δ_H 4.64 (d, $J = 9.5$ Hz), and H-2″ at δ_H 4.00 (t, $J = 9.5$ Hz). The positions of the glucosidic linkages were determined by NOE difference spectra. A strong negative NOE was observed at H-8 (δ_H 6.47) on the chromophore by irradiation of H-1‴, indicating that this sugar moiety is linked to 7-OH of the aglycon. A weaker NOE was also observed at H-8 by irradiation of H-2′ (δ_H 7.56). Furthermore, all ^{13}C sugar signals were assigned from comparison with previous results (Krauze-Baranowska and Cisowski, 1995) (Table 4), and **2** is identified as isoscoparin-7-*O*-β-D-glucoside (Figure 2). This compound has been isolated before from *Gentiana* but has not been previously found in the Gramineae (Marston et al., 1976).

The molecular ion at *m*/*z* 581 [M + H]$^+$ of **3** corresponded to $C_{26}O_{15}H_{28}$, and UV data were identical with results from carlinoside (Besson et al., 1985; Proliac and Raynaud, 1977) (Table 1).

^{1}H NMR signals of the aglycon at 70 °C revealed a 5,7,3′,4′-tetra-OH-substituted flavone, a luteolin, whereas the ^{1}H signals of the sugar moieties corresponded to *C*-β-glucoside and *C*-α-arabinoside. By ^{1}H−^{1}H COSY the anomeric proton signals, δ_H 4.71 and 4.81, could be assigned to H-1″ of D-glucoside and H-1‴ of the arabinoside, respectively. Their coupling constants ($J_{1,2}$) were 9.5 Hz (relation between H-1 and H-2 is antiperiplanar), and the signals of H-2″ and H-2‴ were δ_H 3.93 and 4.04, respectively, indicating that their sugar attachments are of *C*-β and *C*-α configuration linkages, respectively (Table 3). From difference NOE spectra at 22.5 °C weak NOE was observed between the anomeric proton (H-1‴) and H-2′ (δ_H 7.45) of the chromophore, indicating C-8 attachment of arabinoside. By irradiation of H-1″

Table 4. ^{13}C NMR Spectral Data Measured in DMSO-d_6 at 22.5 °C of *C*-Glycosyl Flavones 2 and 5[a]

	C	2	5[b]		C	2	5[b]
flavone	2	164.3	164.2	glucoside at C-6	1″	73.1	73.3
	3	104.7	101.1		2″	72.7	72.6
	4	181.7	181.7		3″	78.6	78.3
	5	159.2	160.1		4″	69.5	69.5
	6	110.2	110.2		5″	80.9	80.5
	7	162.2	162.2		6″	60.5	60.6
	8	93.4	93.8				
	9	156.2	156.2	glucoside at C-7	1‴	101.1	101.1
	10	102.9	104.7		2‴	73.5	73.5
	1′	121.2	121.1		3‴	75.5	74.9
	2′	121.2	113.0		4‴	69.4	69.4
	3′	149.3	149.3		5‴	73.1	73.3
	4′	160.1	159.2		6‴	60.2	60.2
	5′	115.3	115.8				
	6′	118.8	118.6				
	OMe	60.2					

[a] Assignments of carbons having almost the same chemical shifts may be reversed. [b] Assignments have been confirmed by 2D techniques (^{1}H−^{1}H COSY, HSQC, or HMBC). Additional measurements of remaining compound were not performed because of limited amounts.

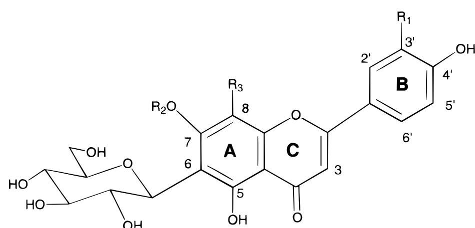

Figure 2. Structures of flavone *C*-glycosides: **2**, R_1 = OCH_3, R_2 = β-glucoside, R_3 = H; **3**, R_1 = OH, R_2 = H, R_3 = α-arabinoside; **4**, R_1 = H, R_2 = H, R_3 = α-arabinoside; **5**, R_1 = OH, R_2 = β-glucoside, R_3 = H; **6**, R_1 = H, R_2 = β-glucoside, R_3 = H.

a weak NOE was observed at H-3 (δ_H 6.63), confirming C-6 attachment of glucoside (H-1″). Thus, **3** is the known carlinoside (Figure 2) found earlier in Gramineae but not until now in barley (Besson et al., 1985; Proliac et al., 1973; Proliac and Raynaud, 1977; Raynaud and Rasolojaona, 1976).

Positive FAB-MS of **4** showed a molecular ion at *m*/*z* 565 [M + H]$^+$ corresponding to $C_{26}O_{14}H_{28}$, and the compound was spectrally (UV−vis) identical with schaftoside (Besson et al., 1985; Chopin et al., 1974) (Table 1). A ^{1}H NMR spectrum confirmed the aglycon as apigenin. By assignment using ^{1}H−^{1}H HOHAHA and ^{1}H−^{1}H COSY, **4** was shown to have one hexoside and one pentoside moiety. The signal at δ_H 4.77 (d, J = 9.5 Hz) corresponded to H-1‴ of a *C*-α-linked arabinoside. A clear signal appeared at δ_H 4.72 (d, J = 9.5 Hz), corresponding to H-1″ of a *C*-β-linked glucoside. The remaining arabinoside signals were identical to the corresponding signals of **3**, although the glucoside signals overlapped (Table 3).

The positions of the glycosidic linkages of **4** were determined by a ^{1}H−^{1}H NOESY experiment at 22.5 °C. The same NOE pattern of signals as for **3** was found, indicating that **4** is schaftoside (Figure 2), which has been isolated from many sources including Gramineae and Caryophyllaceae (Besson et al., 1985; Chopin et al., 1974), but not from barley. Another related compound, isovitexin-x″-*O*-β-D-arabinoglucoside, has been reported from barley leaves (Fröst et al., 1977). This compound was not found in the present investigation.

From MS data, **5** and **6** showed a difference of 16 mass units: *m*/*z* 611 corresponding to $C_{27}O_{16}H_{30}$ (**5**) and *m*/*z* 595 [M + H]$^+$ corresponding to $C_{27}O_{15}H_{30}$ (**6**) and gave UV−visible absorption data identical to those of isoorientin 7-*O*-β-D-glucoside (Fröst et al., 1977; Krauze-Baranowska and Cisowski, 1995) and isovitexin 7-*O*-β-D-glucoside (Fröst et al., 1977), respectively. This assignment was supported by the reversed-phase HPLC retention times, **5** being eluted earlier than **6** (Table 1). In the downfield region of the 600 MHz ^{1}H NMR, the pattern of aromatic proton signals corresponded to luteolin and apigenin, respectively (Table 3). However, the sugar proton regions of **5** and **6** were mutually identical and identical to the sugar part of **2** (Table 3). These observations were supported by ^{13}C NMR and HSQC spectra of **5**, and the ^{13}C assignment of the sugar part was almost identical to that of **2** (Table 4). ^{1}H NMR data showing each rotational isomer of **5** are shown in Table 3.

The positions of the glucosidic linkages of **5** and **6** were determined by NOE difference spectra. Appreciable NOE signals were observed at H-8 by irradiation of H-1‴, indicating that the *O*-glucosides are linked to HO-7 of the aglycon. Likewise, NOE signals were observed at HO-5 and H-3 by irradiation of H-1″, suggesting that the *C*-glucoside moieties are linked to C-6. Thus, **5** and **6** were identified as 7-*O*-β-D-glucosides of isoorientin and isovitexin, respectively (Figure 2). These compounds are common also in barley (Fröst et al., 1977; Krauze-Baranowska and Cisowski, 1995; Hörhammer et al., 1965).

The isolated six flavonoids have a common structural part, 6-*C*-glucoside, indicating the existence of a *C*-glucoside step in the flavone biosynthesis system in barley. Further analyses of the occurrence of these compounds may give information of biological interactions of barley with outside stresses, infections, and damages.

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