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# TWO LUTEOLIN *O*-GLUCURONIDES FROM PRIMARY LEAVES OF *SECALE CEREALE*

MARGOT SCHULZ,* DIETER STRACK,* GOTTFRIED WEISSENBÖCK,* KENNETH R. MARKHAM,† GEORGETTE DELLAMONICA‡ and JEAN CHOPIN‡

*Botanisches Institut der Universität zu Köln, Gyrhofstr. 15, D-5000 Köln, West Germany; †DSIR, Chemistry Division, Petone, New Zealand; ‡Laboratoire de Chimie Biologique, Université de Lyon I, F-69622 Villeurbanne Cedex, France

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**Key Word Index**—*Secale cereale*; Poaceae; rye; luteolin glucuronides.

**Abstract**—Two luteolin *O*-glucuronides have been located exclusively in the photosynthetically active mesophyll of primary leaves of rye (*Secale cereale*). Their structures have been elucidated as luteolin 7-*O*-[*β*-D-glucuronosyl (1 → 2)*β*-D-glucuronide]-4′-*O*-*β*-D-glucuronide and luteolin 7-*O*-[*β*-D-glucuronosyl (1 → 2)*β*-D-glucuronide]. The former glycoside is a new natural compound.

## INTRODUCTION

As recently described, primary leaves of *Secale cereale* show a tissue specific distribution of their flavonoids [1]. Whereas the two *C*-glycosylflavone *O*-glycosides isovitexin 2″-*O*-arabinoside (**3**) and isovitexin 2″-*O*-galactoside (**4**) accumulate in the epidermal tissue [1, 2], two anthocyanins, cyanidin 3-*O*-glucoside (**5**) and cyanidin 3-*O*-diglycoside (**6**), together with the two luteolin derivatives, are exclusively located in the mesophyll [1]. In the present paper, we report on the structural elucidation of the two luteolin *O*-glucuronides.

## RESULTS AND DISCUSSION

During the first 5 days of seedling development, primary leaves of rye accumulate six flavonoid compounds belonging to three different classes. The *C*-glycosylflavone *O*-glycosides (**3**, **4**) and the anthocyanins (**5**, **6**) have recently been identified [1, 2]. The luteolin derivatives (**1** and **2**) were isolated as described previously [1] and their structures elucidated by means of hydrolysis, analytical thin-layer chromatography, high performance liquid chromatography, UV, mass and $^{13}$C NMR spectroscopy.

The thin-layer chromatographic mobilities of **1** and **2** indicated that they were glucuronides [3]. This was confirmed by enzymatic hydrolysis of **1** with *β*-glucuronidase (followed by HPLC) which yielded **2** as the major intermediate. Hydrolysis then proceeded further to produce luteolin as the end product (Fig. 1). Acidic hydrolysis with 50% formic acid gave identical results. The only other product present in the hydrolysate was identified by TLC (four solvents) as glucuronic acid. UV–visible absorption analysis [4] of **1** indicated that the 7- and 4′ (or 3′-)-hydroxyl groups are substituted, whereas for **2** only the 7-hydroxyl group is substituted [5].

Permethylation of **1** followed by TLC led to two main bands which gave undefined electron impact and chemical ionization ($NH_3$) mass spectra. However, the molecular weights of **1** and **2** could be determined by positive fast atom bombardment mass spectrometry. Thus, the mass spectrum of **1**, recorded using a glycerol matrix, showed the protonated peak $[MH]^+$ of a luteolin triglucuronide at *m/z* 815, confirmed by the presence of ions $[M + Na]^+$ and $[M + \text{glycerol}]^+$ at 837 and 906. In the mass spectrum of **2**, the pseudo-molecular ion $[MH]^+$ found at *m/z* 639 corresponded to a luteolin diglucuronide structure. From these results it followed that **2** was a luteolin 7-diglucuronide and **1** a luteolin 7-diglucuronide 4′-glucuronide.

$^{13}$C NMR spectra were measured for both **1** and **2** in order to confirm the presence of 4′-*O*-glycosylation (in **1**) and the nature and linkages of the sugars. The data are presented in Table 1 along with the spectral data

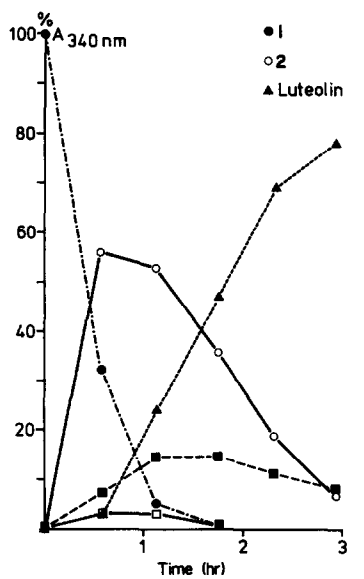


Fig. 1. Enzymatic hydrolysis of **1** (●—●), yielding **2** (○—○) as the major intermediate, and final accumulation of luteolin (▲—▲). The other two symbols represent minor, unidentified intermediates. HPLC analysis.

Table 1. $^{13}$C NMR spectral data of luteolin glucuronides*

| Carbon No. | Luteolin 7-*O*-[β-D-glucuronosyl (1→2) β-D-glucuronide ex *Elodea* | 1 | 2 |
|---|---|---|---|
| C-4 | 181.9 | 182.1 | 182 |
| $CO_2H$ | 170.9, 169.8 | 174.1, 173.8, 173.4 | 172.5 |
| C-2 | 164.4 | 164.6 | ? |
| C-7 | 162.1 | 162.3 | 163 |
| C-5 | 161.1 | 161.3 | 161 |
| C-9 | 156.8 | 157.6 | 157 |
| C-4′ | 149.8 | 149.1 | 149 |
| C-3′ | 145.7 | | 145.5 |
| C-1′ | 121.4 | 125.1 | ? |
| C-6′ | 119.1 | 117.5† | 119.5 |
| C-5′ | 116.0 | 116.3† | 116 |
| C-2′ | 113.5 | 114.5 | 113.5 |
| C-10 | 105.4 | 106 | 105.6 |
| C-1‴ | 104.3 | 103.9 | 103.8‡ |
| C-3 | 103.1 | 102.3 | 103.1‡ |
| C-6 | 99.2 | 100.6 | 100.2 |
| C-1″ | 97.6 | 97.6 | 98.2 |
| C-8 | 94.5 | 94.5 | 95.8 |
| C-2″ | 82.3 | 79.8 | 80.9 |
| C-3″, 3‴, 5″, 5‴ | 75.7–75.0 | 75.7–75.1 | 75.8 |
| C-2‴ | 74.1 | 73.4§ | 73.9 |
| C-4″, 4‴ | 71.6, 70.7 | 71.6, 71.2 | 71.8 |
| C-1⁗ | — | 100.6 | — |
| C-3⁗, 5⁗ | — | 75.4 | — |
| C-2⁗ | — | 72.5§ | — |
| C-4⁗ | — | 71.6 | — |

*Solvent DMSO-$d_6$, 30°C; ″ and ‴ indicate sugars in disaccharide and ⁗ indicates sugar linked to 4′-hydroxyl.

†,‡,§Assignments bearing the same superscript may be interchanged.

of authentic luteolin 7-*O*-[β-D-glucuronosyl (1→2) β-D-glucuronide] from *Elodea* [6]. The small amount of **2** available (1.5 mg) was insufficient to produce a well-resolved spectrum, hence the incomplete data in Table 1. Signals that are clearly visible, however, are in good agreement with the spectrum of the *Elodea* compound, and in particular support the presence of glucuronic acid, the (1→2)-interglycosidic linkage and the absence of a glycosidic substituent on the B-ring. The HPL-chromatographic identity of **2** and the *Elodea* diglucuronide are thus confirmed by $^{13}$C NMR spectroscopy.

The spectrum of **1** also matches well, in part, the spectrum of the *Elodea* compound. There are important differences, however.

(i) The three carboxyl carbon resonances indicate the presence of three glycuronyl moieties in **1**.

(ii) The occurrence of both C-3′ and C-4′ at δ149.1 and the 4 ppm downfield shift of C-1′ is as expected for a 4′-*O*-glycosylated 3′,4′-dihydroxy-B-ring (cf. quercetin 4′-*O*-glucoside [7]).

(iii) The additional signals in the sugar carbon region (see Table 1) confirm the presence of one more glucuronyl moiety [3] which, with C-1⁗ at δ100.6, must be linked to a phenolic hydroxyl.

The $^{13}$C NMR data also require that the glucuronyl residues be in the pyranosyl form and β-linked.

On the basis of the above data, **2** is assigned the structure luteolin 7-*O*-[β-D-glucuronopyranosyl (1→2) glucuronopyranoside] and **1**, its 4′-*O*-β-D-glucuronopyranoside.

It is known that flavone 7-*O*-diglucuronides are relatively rare in the plant kingdom. Luteolin 7-*O*-[β-D-glucuronosyl (1→2)β-D-glucuronide] has recently been described in *Elodea* species for the first time [6] and an authentic sample of this compound was chromatographically (HPLC) identical with **2**. To our knowledge, luteolin 7-*O*-[β-D-glucuronosyl(1→2)-β-D-glucuronide]-4′-*O*-β-D-glucuronide, now isolated from *Secale cereale*, is a new natural compound.

## EXPERIMENTAL

*Plant material. Secale cereale* L. var. Kustro, the growing conditions and isolation of flavones have been described elsewhere [1].

*Isolation and purification of* **1** *and* **2**. *Secale cereale* leaf material was extracted either with MeOH–$H_2O$ (4:1) or with hot distilled $H_2O$, and **1** and **2** isolated from the crude extracts by polyamide

CC. Initial elution with MeOH yielded *C*-glycosylflavones together with anthocyanins. Subsequent elution with 0.01% $NH_4OH$ in MeOH gave **1** and **2**, which were separated from one another by prep. TLC (solvents 1–3). Final purification was achieved on LH-20 using $H_2O$ as eluant. Pure **1** had the following properties: $R_f$ values (solvents 1, 2, 3 and 4), 0.0, 0.5, 0.03 and 0.6; $\lambda_{max}$ (MeOH) nm: 246 sh, 267, 332; + NaOMe 267, 295 sh, 398; + $AlCl_3$, 261 sh, 276, 289 sh, 339, 379 sh; + $AlCl_3$–HCl 256 sh, 276, 289 sh, 338, 378; + NaOAc 268, 296 sh, 323, 385 sh; + NaOAc–$H_3BO_3$ 267, 296 sh, 334. Pure **2** had the following properties: $R_f$ values (solvents 1, 2, 3 and 4), 0.02, 0.25, 0.24, and 0.15; $\lambda_{max}$ (MeOH) nm: 255 sh, 266, 344; + NaOMe 266, 299 sh, 397; + $AlCl_3$ 271, 294 sh, 335 sh, 419; + $AlCl_3$–HCl 260 sh, 271, 290 sh, 351, 383; + NaOAc 264, 406; + NaOAc–$H_3BO_3$ 260, 364.

*Solvents for TLC.* (a) For glycosides (on cellulose 'Avicel', Macherey & Nagel, Düren, F.R.G.); see refs. [3, 8, 9]: (1) $CHCl_3$–HOAc (3:2, $H_2O$ saturated), (2) 15% HOAc, (3) pyridine–EtOAc–HOAc–$H_2O$ (36:36:7:21), and (4) 15% propionic acid. (b) For the aglycone (on cellulose): (1) $CHCl_3$–HOAc (3:2, $H_2O$ saturated), (2) 15% HOAc, (5) BuOH–HOAc–$H_2O$ (5:2:3), (6) EtOAc–HCOOH–$H_2O$ (66:14:20), and (7) $C_6H_6$–HOAc–$H_2O$ (7:5:3, upper phase), Reagents: $NH_3$ vapour [5], Naturstoffreagenz [10], Benedict [11, 12]. (c) For glucuronic acid [8]: (3) pyridine–EtOAc–HOAc–$H_2O$ (36:36:7:21) on silica gel, saturated with aq. 0.1 M $H_3BO_3$, activated by heating at 100° for 1 hr; (8) *i*-PrOH–$Me_2CO$–0.1 M lactic acid (4:4:2) on silica gel, saturated with 0.2 M $NaH_2PO_4$, activated by heating at 100° for 1 hr [13]; (9) *n*-BuOH–pyridine–$H_2O$ (3:1:1) on silica gel and cellulose; and (10) MeOH–$CHCl_3$–MeCOMe–$NH_3$ (42:16.5:25:16.5) on silica gel, saturated with 0.1 M $H_3BO_3$, activated by heating at 100° for 1 hr. Spray reagents: (a) aniline–diphenylamine–$Me_2CO$ in 80% $H_3PO_4$, (b) *p*-anisidinephthalate in EtOH, and (c) 4-methoxybenzaldehyde–$H_2SO_4$ in HOAc [8].

*Acid hydrolysis.* For partial and complete hydrolysis: 50% HCOOH (aq.), 100° up to 3 hr. Extraction of the aglycone with EtOAc. The glucuronic acid was present in the $H_2O$ phase.

*Enzymatic hydrolysis* was performed with β-D-glucuronidase from *Helix pomatia*, type H-3AF (Sigma, F.R.G.), diluted 1:20 in 0.1 M NaOAc buffer, pH 5.0.

*HPLC conditions.* HPLC System 8800 Gradient Controller, chromatographic pump, flotation degasser. Dupont Instruments, Bad Nauheim, F.R.G. Absorbance detection: Spectroflow 773, absorbance detector, Kratos Analytical Instruments, Trappenkamp, F.R.G. Evaluation: Sp 4270 Integrator, Spectra-Physics, Santa Clara, CA, U.S.A.; detection wavelength 340 nm. The column was prepacked with Lichrosorb RP-8 (Standard 250-4, Cat. 15540, Merck, Darmstadt) and separation was accomplished within 20 min by elution with a linear gradient from 100% $H_2O$ (1% $H_3PO_4$) to 100% MeCN (HPLC grade, Baker Chemicals, Deventer, The Netherlands). Peaks (measured at 340 nm) were identified by co-chromatography with references. Retention times (min): **1** (9.4); **2** (10.21) and luteolin (11.19).

*Spectroscopy.* UV spectral analysis was according to Mabry *et al.* [4]. MS were run at the Centre de Spectrométrie de Masse de Lyon. $^{13}C$ NMR spectroscopy: see Table 1.

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