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LUTEOLIN 7-GLUCURONIDE-3′-MONO(*TRANS*)FERULYLGLUCOSIDE AND OTHER UNUSUAL FLAVONOIDS IN THE AQUATIC LIVERWORT COMPLEX, *RICCIA FLUITANS*

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Key Word Index—*Riccia fluitans*; *Riccia duplex*; *Riccia crystallina*; Hepaticae; luteolin 7-glucuronide-3′-mono-(*trans*)ferulylglucoside; luteolin 7,4′-diglucuronide-3′-glucoside; luteolin 7,4′-diglucuronide-3′-mono(*trans*)-ferulylglucoside; luteolin and apigenin 4′-glucuronides; chemotaxonomy.

Abstract—Thirteen flavonoid glycosides, including eight which are new have been identified in *Riccia fluitans*; aquatic and terrestrial forms of this plant have the same pattern. Luteolin 7-*O*-glucuronide-3′-*O*-mono(*trans*)-ferulylglucoside is proposed as the type flavonoid for this species. Its absence from, and the presence of chrysoeriol in *R. duplex*, support the proposed separation of *R. duplex* from the *R. fluitans* complex. A micro-deacylation technique is described which can also be used for specific deglycosylation of luteolin glycosides at the 4′-hydroxyl.

INTRODUCTION

Riccia fluitans is generally considered to exist in two morphologically distinct forms, the common, ribbon-like, free floating 'aquatic' form and a terrestrial form which has a thicker, broader thallus. Because of the high variability of the aquatic form some doubt exists as to whether *R. fluitans* is a single taxon and it is regarded by some workers as an aggregation of the aquatic forms of a number of distinct terrestrial species of *Riccia* [1–3]. Based on the observations of Lorbeer [4], Müller [5] has suggested a division of the *R. fluitans* complex into four species: *R. fluitans* (amend. Lorbeer) *R. rhenana* Lorbeer, *R. canaliculata* Hoffm. and *R. duplex* Lorbeer. However, Berrie [6] has since claimed from culture studies that two of these species are in fact diploid forms of the other two taxa. Thus he found that colchicine-induced diploids of *R. fluitans* ($n = 8$) were identical with *R. rhenana* ($n = 16$) and that colchicine-induced diploids of *R. canaliculata* ($n = 8$) resembled *R. duplex* ($n = 16$).

We report here a study of the flavonoid chemistry of interconvertible aquatic and terrestrial forms of *R. fluitans* ($n = 8$) from two different localities in Germany, a 'stock' glasshouse grown type and a naturally occurring specimen from Wadern, Saarland. In addition, aquatic forms of this species from two different New Zealand localities [7] and an Australian sample of *R. duplex*, have been surveyed for flavonoids.

RESULTS

The aquatic form of *R. fluitans*, found in Wadern, West Germany, was used for the bulk of the work in the present study. Samples from other sources were examined by PC screening and compound identifications made by co-chromatography. The pattern of flavonoid glycosides found in an acetone:water extract of *R. fluitans* is presented in Fig. 1 together with compound identification and chromatographic characteristics.

Compounds **1**, **2**, **3**, **4** and **6** are all flavonoid glycosides that have been isolated previously from liverworts of the order Marchantiales [8] and accordingly these were identified by direct UV-visible spectrophotometric and PC (TBA, HOAc, H_2O) comparisons with authentic compounds from *Conocephalum conicum* [9]. The two intermittently occurring constituents, **1A** and **1B**, proved to be new natural products; luteolin 4′-*O*-glucuronide and apigenin 4′-*O*-glucuronide respectively. In each case the absorption spectrum indicates that the 4′-hydroxyl alone is substituted and β-glucuronidase treatment yielded glucuronic acid. Compound **1A** is chromatographically identical with luteolin 4′-*O*-glucuronide produced from luteolin 3′,4′-di-*O*-glucuronide [10], and compound **1B** cochromatographed with apigenin 4′-*O*-glucuronide isolated by partial hydrolysis of compound **6**.

Compound **8** gave luteolin on acid hydrolysis and showed spectrophotometric and chromatographic properties similar to those of luteolin 7,3′-di-*O*-glucuronide [11]. However it differs from this compound in its 50% lower PC mobility in H_2O. Treatment of **8** with β-glucosidase gave luteolin 7-*O*-glucuronide quantitatively as also did mild acid hydrolysis. The glucose liberated was identified by GLC, so defining compound **8** as luteolin 7-*O*-glucuronide-3′-*O*-glucoside.

The major flavonoid glycoside in most *R. fluitans* samples, compound **5**, was converted to **8** by saponification. Liberated in this process is a blue fluorescent (UV) compound which gave a MS with major ions at *m/e* 194 (M^+), 179 ($M-CH_3$) and 150 ($M-CO_2$). Its identity as *trans*-ferulic acid was confirmed by spectrophotometry and by co-chromatography with an authentic marker. Ferulic acid and **8** were produced in a 1:1 molar ratio on saponification of **5**. Compound **5** is unaffected by β-glucosidase but with β-glucuronidase produces a glycoside which appears on paper as a dull green spot in NH_3 vapour (R_f 0.78 (TBA), 0.18 (HOAc), 0.06 (H_2O)) and

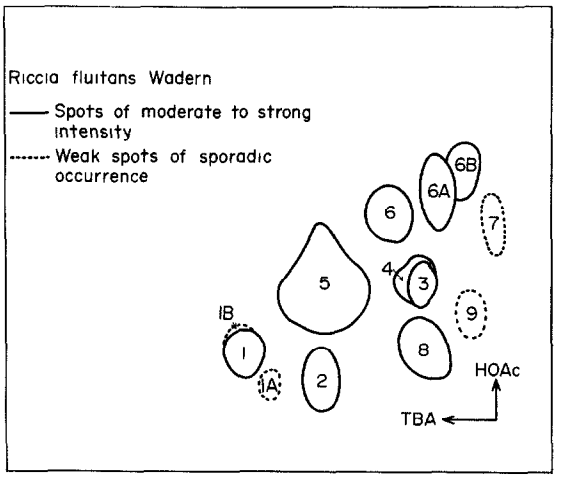

Fig. 1. Pattern of flavonoid glycosides in *Riccia fluitans*, aquatic form (Wadern).

Key

Spot no.	Flavonoid	Spot colour (NH_3/366 nm)	R_f* (TBA)	R_f* (HOAc)	R_f (H_2O)
1	Apigenin 7-glucuronide	lemon	0.50	0.23	0.45
1A	Luteolin 4'-glucuronide	purple	0.45	0.13	0.6
1B	Apigenin 4'-glucuronide	purple	0.51	0.24	-
2	Luteolin 7-glucuronide	yellow	0.35	0.14	0.6
3	Luteolin 7,4'-diglucuronide	brown	0.23	0.35	0.95
4	Lucenin-2	dull yellow	0.25	0.35	0.15
5	Luteolin 7-glucuronide-3'-ferulylglucoside	yellow-green	0.36	0.35	0.75
6	Apigenin 7,4'-di-glucuronide	purple	0.31	0.53	0.85
6A	Luteolin 7,4'-di-glucuronide-3'-ferulylglucoside	purple	0.18	0.60	0.85
6B	Luteolin 7,4'-di-glucuronide-3'-glucoside	purple	0.10	0.64	0.85
7	Luteolin 7-glucuronide-3'-(2-hydroxypropionyl)glucoside-4'-(2-hydroxypropionyl)-glucuronide	purple	0.04	0.50	0.9
8	Luteolin 7-glucuronide-3'-glucoside	lemon	0.18	0.20	0.7
9	Luteolin 7-glucuronide-3'-(2-hydroxypropionyl)-glucoside	dull yellow green	0.08	0.28	0.85

* R_f values all measured from 2DPCs of plant extract

which possesses absorption spectra consistent with it being a 3'-*O*-glycosylated luteolin. The low PC mobility in H_2O confirms that it is not a glucuronide. Ferulylation in **5** must therefore be on the 3'-*O*-glucose moiety and the structure follows as luteolin 7-*O*-glucuronide-3'-*O*-mono-(*trans*)ferulylglucoside.

Related to this compound is **6A**, the 4'-*O*-glucuronide of **5**. Compound **6A** gave luteolin on complete acid hydrolysis and its absorption spectra indicate that the 7,3' and 4'-hydroxyls are derivatized. On partial acid hydrolysis **6A** produced luteolin 7,4'-di-*O*-glucuronide (**3**), luteolin 7-*O*-glucuronide (**2**) and luteolin 4'-*O*-glucuronide (**1A**) together with ferulic acid and a trace of luteolin 7-*O*-glucuronide-3'-*O*-ferulylglucoside (**5**). In contrast, β-glucuronidase treatment of **6A** gave small amounts of **5** and only one major product, luteolin 3'-*O*-ferulylglucoside, which was chromatographically identical with that produced from **5**. Compound **6A** is therefore assigned the structure luteolin 7,4'-di-*O*-glucuronide-3'-*O*-mono-(*trans*)ferulylglucoside. Compound **6B** was hydrolysed completely to luteolin with a mixture of β-glucuronidase and β-glucosidase and was derived quantitatively from **6A** by saponification with the liberation of ferulic acid. It follows that the structure of **6B** is luteolin 7,4'-di-*O*-glucuronide-3'-*O*-glucoside.

The two remaining compounds, **9** and **7**, could be isolated in only very small amounts. Compound **9** gave luteolin and luteolin 7-*O*-glucuronide on acid hydrolysis and its absorption spectra closely resemble those of luteolin 7-*O*-glucuronide-3'-*O*-glucoside (**8**), the compound into which it is converted on saponification. Treatment of **9** with β-glucuronidase gave a luteolin 3'-*O*-glucoside derivative of low PC mobility (R_f 0.26 (TBA), 0.06 (HOAc)) which was not hydrolysed by β-glucosidase. The acyl group in **9** must therefore be attached to the 3'-*O*-glucosyl function. It must also be considerably more polar than ferulyl (or acetyl) since unlike ferulyl its presence causes a reduction in the PC mobility of compound **8**. TLC of the ether soluble saponification product of **9** under recommended conditions for the identification of organic acids [12–15] revealed the presence of only one major acid, 2-hydroxypropionic acid. Due to reagent impurities blank saponifications did give low levels of the same acid however and for this reason compound **9** can be only tentatively assigned the structure, luteolin 7-*O*-glucuronide-3'-*O*-(2-hydroxypropionyl)glucoside.

Compound **7** was generally the weakest flavonoid spot on the *R. fluitans* 2DPC. Its absorption spectra indicate that it is a 7,3',4'-tri-*O*-glycosylated luteolin. Acid hydrolysis yields luteolin 7-, 4'- and 7,4'-*O*-glucuronides, together with a derivative of 7,4'-di-*O*-glucuronide with lower PC mobility. This derivative gives a 4'-substituted luteolin on treatment with β-glucuronidase suggesting that in **7**, the 4'-*O*-glucuronide is derivatized. As with compounds **5**, **6A**, **6B**, **8** and **9**, no 3'-derivatized luteolin was produced on acid hydrolysis of **7**, thus the sugar on the 3'-hydroxyl is not glucuronic acid. It was shown to be glucose by alkaline treatment of **7** (at 40°) which gives luteolin 7-*O*-glucuronide-3'-*O*-glucoside (**8**) in good yield. Minor flavonoid products of the acid hydrolysis were luteolin 7,4'-di-*O*-glucuronide and a lower mobility (2DPC) 7,4'-di-*O*-glucuronide derivative. Compound **7** is therefore a derivative of luteolin 7,4'-di-*O*-glucuronide-3'-*O*-glucoside (**6B**) and further, since treatment with β-glucosidase plus β-glucuronidase removes only the 7-linked sugar, it is considered that both the 3'- and 4'-linked sugars are derivatized. Traces of only one acid, 2-hydroxypropionic acid, were detected following 40° saponification. Compound **7** is therefore tentatively assigned the structure luteolin 7-*O*-glucuronide-3'-*O*-(2-hydroxypropionyl)glucoside-4'-*O*-(2-hydroxypropionyl)glucuronide.

The apigenin, luteolin and chrysoeriol 7-*O*-glucuronides from *R. duplex* were identified by co-chromatography (TBA, H_2O) with authentic material and additionally, chrysoeriol 7-*O*-glucuronide was hydrolysed with β-glucuronidase to produce chrysoeriol which in turn was identified by co-chromatography.

DISCUSSION

As part of a continuing study of the taxonomic and phylogenetic significance of the flavonoid chemistry of the order Marchantiales, *Riccia fluitans* has been investigated as one of the few examples of aquatic liverworts.

Eight of the 13 flavonoid glycosides isolated in the

present work are new natural products and all but the 4′-glucuronides, **1A** and **1B**, are derivatives of luteolin 7-*O*-glucuronide-3′-*O*-glucoside (**8**). The plant shows a remarkable ability to modify this starting material including ferulylation of the glucose (**5**), probable hydroxypropionylation of the glucose (**9**), glycosylation of the 4′-hydroxyl with glucuronic acid (**6B**) and subsequent ferulylation of this sugar (**6A**) and possible hydroxypropionylation of both this sugar and the 3′-linked glucose (**7**). Ferulic acid has been found as an acyl function on flavone glycosides previously but this appears to be a new role for 2-hydroxypropionic acid [16].

The high level of acylated flavonoids in this liverwort is of interest because acylation of flavonoids has been reported only once previously in the Hepaticae, in the primitive New Zealand liverwort, *Haplomitrium gibbsiae* [17]. However, reinvestigation of the flavonoids of the only other previously studied *Riccia* species, *R. crystallina* [18], has shown that two of the previously unidentified constituents (RC-3 and RC-4b) are acylated derivatives of apigenin 7-*O*-glucuronide.

The two German aquatic samples of *R. fluitans* gave qualitatively similar flavonoid patterns but differed with respect to the relative levels of different glycosides. High levels of **1**, **2**, **3** and **4** characterized the glasshouse sample, which contained only low levels of other flavonoids, whereas the Wadern sample was rich in **5** and contained moderate levels of all other flavonoids but **3**, **4**, **7** and **9**. The influence of the aquatic habitat on flavonoid biosynthesis appears to be variable since conversion of both of these aquatic forms into terrestrial forms in the laboratory failed to produce consistent changes. Flavonoid production in the Wadern sample was unchanged by this transformation apart from a reduction in the levels of **7** and **9**, whereas the pattern of flavonoids in the glasshouse sample was enriched with the acylated and glucosylated flavones **5**, **6A**, **6B**, **7**, **8** and **9**. However, it is clear from their flavonoid patterns that both aquatic and terrestrial forms are recognizably the same species.

The complex patterns of flavonoids observed in the German samples contrast markedly with the single spot pattern (compound **5**) associated with both of the indigenous New Zealand samples. This may be a reflection of the considerable morphological variation observed within this species (see Introduction) and may thus justify subdivision of the *R. fluitans* complex. It could also indicate a continuation of the 'reduction' process which is thought [19] to have occurred in this genus. The flavonoid glycoside common to all samples, luteolin 7-*O*-glucuronide-3′-*O*-mono(*trans*)ferulylglucoside (**5**) is a new natural product and embodies most of the unusual structural features found in flavonoids of this species. In this respect it may be considered the type flavonoid for the species. Significantly, this compound was not found in the closely related Australian species, *R. duplex*, which contains only apigenin and luteolin 7-*O*-glucuronides in common with the German *R. fluitans*. *R. duplex* also produces chrysoeriol 7-*O*-glucuronide, a compound which has not previously been isolated from any species in the family Ricciaceae. These findings support the proposed [4–6] separation of *R. duplex* from the rest of the *R. fluitans* liverwort complex.

In the course of this work a new micro-deacylation technique has been developed in which alkali treatment is carried out in a microlitre syringe. This permits saponifications to be carried out on 1 μl of solution or less, the plunger action being used both to mix the sample with alkali and to exclude all air. The conditions suitable for the removal of acyl functions from most glycosides (1.5 N NaOH, 20°, 1 hr) were ineffective with **7**, for which overnight treatment at 40° was necessary, resulting not only in complete deacylation but also in almost specific loss of the 4′-linked sugar (some loss of the 3′-linked sugar was also observed). A similar pattern of sugar loss was obtained when this treatment was applied to two compounds of known constitution, luteolin 7,4′-di-*O*-glucuronide-3′-*O*-glucoside and luteolin 7-*O*-glucuronide-3′,4′-di-*O*-rhamnoside, suggesting that this technique may have general application in structural investigations with this class of compound. The present observation extends those of Litvinenko *et al.*, [20] on the alkaline hydrolysis of 3- and 7-*O*-glycosides of flavonoids and suggests possible refinements in terms of technique and conditions.

EXPERIMENTAL

Type specimens of *R. fluitans* aquatic (1 g) and terrestrial forms (10 g) were sampled from the University of Saarland greenhouse collection in February 1976, and a bulk supply of aquatic form (6 g) in May 1976. The terrestrial form had been derived from the aquatic form by 'planting out' on soil in November 1975. Voucher specimens of the aquatic form are held at Massey University, N.Z. (MPN 17020) and in the herbarium of the University of Saarland. The aquatic form of *R. fluitans* (6 g) was also collected from Wadern, Saarland in October 1976 and a voucher specimen deposited in the herbarium of the University of Saarland. After 'planting out' of this sample a small quantity (0.6 g) of the terrestrial form was harvested in January 1977. New Zealand specimens were collected near Waikouaiti in March 1975 and at Lake Rotorua in May 1977 (MPN 17010). *R. duplex* (MPN 17050) was collected in Victoria, Australia in September 1977 near the Grampians.

Isolation procedure. Plant specimens were dried at 100°, ground to a powder and extracted several times with Me_2CO–H_2O (1:1). Extracts were then applied to Whatman 3 MM sheets (at a loading level equivalent to 100 mg dry wt of plant material) and chromatographed in 2D using *t*-BuOH–HOAc H_2O, 3:1:1 (TBA) and 15% HOAc (HOAc). Overrunning the chromatograms in TBA was necessary to achieve good separation of all components. PC using H_2O as solvent was employed to distinguish monoglucosides (immobile) from monoglucuronides (R_f *ca* 0.4) and diglucuronides (R_f *ca* 0.9). Individual compounds were isolated by extraction from the paper chromatograms.

Hydrolysis procedures. (i) *Partial acid hydrolysis* routinely involved treatment of the flavonoid with 1.5 N HCl for 30 min at 100°. Aglycones produced in these hydrolyses were identified by PC or TLC comparison with authentic material using TBA and C_6H_6–HOAc–H_2O (125:72:3). (ii) *Alkaline hydrolyses* involved R.T. treatment of the acylated glycoside (2 μl soln) with 2N NaOH (5 μl) in a 10 μl syringe for 2 hr. With compound **7** (and subsequently model flavonoid glycosides) the alkaline treatment was carried out at *ca* 40° for 16 hr. In all cases blank hydrolyses were carried out. The product after acidification, was evaporated to dryness and extracted first with Et_2O and then EtOAc. These extracts were analysed for acids (see below) and the insoluble flavonoid products were dissolved in Me_2CO–H_2O for PC and TLC analysis. (iii) *Enzyme hydrolyses* were carried out for 20 min in dist. H_2O at 20° using β-glucosidase (*ex* sweet almonds, Koch-Light) and β-glucuronidase (*ex* bovine liver, Sigma, type B-3). Under these conditions authentic luteolin 7-*O*-glucoside was completely hydrolysed by both enzymes, while authentic luteolin 7-*O*-glucuronide was completely hydrolysed by the latter and only partially (10–20%) by the former. Sugars produced in the above hydrolyses were analysed when required by either PC or GC according to procedures detailed previously [21] and flavonoid glycosides by 2DPC

(using $AlCl_3$ as spray reagent to detect low level spots). Aglycones were analysed by TLC (cellulose) using TBA and C_6H_6–HOAc–H_2O (125:72:3).

Identification of acids. Both Et_2O and Me_2CO extracts of the saponification products were analysed by TLC and compared directly with authentic samples. Preliminary identifications were made on cellulose (Schleicher and Schüll, F1440) using the two solvents, *n*-BuOH–HCOOH–H_2O (10:1·5) [12] and MeOH–NH_4OH(0.91)–H_2O (16:1:3) [15]. In the case of 2-hydroxypropionic acid these identifications were confirmed (for compound **9** but not **7**) by TLC on Si gel (Schleicher and Schüll, F1500) using EtOH–$CHCl_3$–NH_4OH–H_2O (53:30:15:1.5) [14] and on polyamide (G1600) using CH_3CN–EtOAc–HCOOH (2:1:1) [14]. Acid spots, were located by UV (360 nm) light and spraying with a mixture of $KMnO_4$/Na_2CO_3/bromocresol green/bromothymol blue [12]. Additionally, ferulic acid was identified by MS and as the *trans*-isomer by spectrophotometry immediately following saponification. Quantification of both ferulic acid and luteolin 7-*O*-glucuronide produced from **5** (the latter by saponification followed by mild acid hydrolysis) was achieved by spectrophotometry.

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