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A "flavone-polysaccharide" redefined as a mixture of 6-methoxyluteolin penta- and hexa-*O*-glycosides

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Abstract

The incomplete structure proposed in 1972 for a unique "flavone-polysaccharide" compound, MF-1, from the liverwort *Monoclea forsteri*, has been re-examined. Rather than the proposed 8-methoxyluteolin structure with polysaccharides attached to the 7- and 4′-hydroxyls, MF-1 has been shown to be primarily a mixture of penta- and hexa-*O*-glycosides of 6-methoxyluteolin, which are accompanied by their luteolin analogues. MS and NMR evidence is marshalled to define the structure of MF-la as 6-methoxyluteolin 7-*O*-[2-*O*-α-rhamnosyl-3-*O*-α-arabinosyl-β-glucuronide]-4′-*O*-[2-*O*-α-rhamnosyl-3-*O*-β-xylosyl-β-glucuronide], and MF-1b as 6-methoxyluteolin 7-*O*-[2-*O*-α-rhamnosyl-β-glucuronide]-4′-*O*-[2-*O*-α-rhamnosyl-3-*O*-β-xylosyl-β-glucuronide]. This report is the first to provide substantive evidence for the existence of flavone penta- and hexa-*O*-glycosides in nature. The newly defined structure(s) for MF-1 more closely align *M. forsteri* with the only other species in the order Monocleales, *M. gottschei*.

Keywords: *Monoclea forsteri*; Monocleales; Liverwort; Flavone-polysaccharide; 6-Methoxyluteolin 7, 4′-*O*-glycosides; Flavone penta- and hexa-*O*-glycosides

1. Introduction

In 1972 a unique "flavone-polysaccharide" compound was reported to have been isolated from the giant New Zealand liverwort, *Monoclea forsteri* (Markham, 1972). However, the size and complexity of this natural product defied attempts to fully define its structure using the then available physical techniques. The study cited mass and ^{1}H NMR spectral data to define the aglycone as 8-methoxyluteolin, and UV shift data to demonstrate that the "polysaccharide" comprised two "polysaccharide" moieties which were attached at the 7- and 4′-hydroxyls via their galacturonic acid units. The composition and size of the "polysaccharide" was deduced from sugar analyses following acid hydrolysis, and a molecular weight of ca. 3200 kD was estimated from a quantitative absorption spectrum using the Beer–Lambert law. Determination of a meaningful molecular weight by this means however, requires an accurate extinction coefficient and a truly pure compound for weighing. In the reported study, a literature derived extinction coefficient for a "related" aglycone was used, and the absence of contaminating polysaccharide material could not be assured. Such contamination could also invalidate the analytical results relating to the sugar composition of the attached "polysaccharides".

In view of the novelty of the reported compound, and the current availability of significantly improved chromatographic, NMR and MS techniques, a reinvestigation of this compound has been undertaken in order to fully define its structure. The structure information is also needed to support forthcoming studies of the possible involvement of this compound in the long-term survival of this most unusual liverwort.

2. Results and discussion

As reported previously (Markham, 1972) the water-methanol solubles from pulverized, fresh *M. forsteri* thallus material produced only one flavonoid spot ("MF-1″) on a 2D-PC. This spot runs at the solvent front in 15% HOAc but barely moves from the origin in TBA. However, it was found to separate into two, following multiple runs in TBA. Fractions A and B were

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separated by these means and cleared of saccharide contaminants using an RP-18 column. HPLC analysis of these fractions revealed that whilst the more mobile fraction B was comprised essentially of one component, MF-1b, (λ_{max} 253 sh, 273, 338 nm), fraction A contained four components, evident from two HPLC peaks, each with a shoulder. The front running peak/shoulder at RT 30.0 min contained two compounds, MF-1a (λ_{max} 253 sh, 273, 338 nm) and MF-1a′ (λ_{max} 253 sh, 270, 336 nm), and the second peak/shoulder at RT 30.6 min, contained another two, MF-1b and MF-1b′, which possessed absorption spectra identical to those of MF-1a and MF-1a′. Refining fraction A further by repeated paper and RP-18 chromatographic processes produced a fraction enriched in MF-1a and MF-1a′ which was used for subsequent NMR studies.

Negative ion electrospray MS of fraction B, i.e. MF-1b, produced a molecular ion of 1092.3 kD, while fraction A gave a major molecular ion of 1224.3 kD (MF-1a) accompanied by another of lesser intensity (MF-1a′) at 1194.3 kD. Lower level molecular ions of MF-1b (1092.3 kD) and MF-1b′ (1062.3 kD) were observed also in the MS of fraction A. The difference in molecular weight between both MF-1a and MF-1a′, and MF-1b and MF-1b′, is the same at 30 m.u. and suggests that they represent pairs of related compounds differing only by one methoxyl. MF-1a and 1a′ differ from MF-1b and 1b′ by 132 m.u. which equates to one pentose unit (150-H_2O).

The high resolution molecular weight of MF-1b at 1092.2541 defines the molecular composition as $C_{45}H_{54}O_{31}$ (1092.2638) which equates to the presence of two glycuronic acid units, two rhamnoses and one pentose, together with a methoxyluteolin aglycone. Similarly, the high resolution M^+ for MF-1a at 1224.3105 defines the molecular composition as $C_{50}H_{62}O_{35}$ (1224.3061) supporting the presence of two glycuronic acid units, two rhamnoses and two pentoses.

The close structural relationship between MF-1a and MF-1b was confirmed by HPLC, TLC and PC analyses of the products produced by controlled acid treatment of each. Both produced a glycuronic acid, rhamnose and xylose, and the higher molecular weight MF-1a also yielded arabinose. Both also produced the same aglycone (**1**) together with the same three partially hydrolysed glycosidic products.

The major of these products proved to be 6-methoxyluteolin 7,4′-diglucuronide (**2**). Its ^{1}H NMR spectrum (Table 1) contains a pattern of flavonoid signals similar to that reported for the ''MF-1'' aglycone (Markham, 1972), but revealed a previously obscured singlet at 7.07 ppm. This singlet exhibited long-range (HMBC)

Table 1
^{1}H NMR spectral data for MF-1 compounds in DMSO-d_6[a]

Proton No.	MF-1a (**3**)	MF-1a′ (**5**)[b]	MF-1b (**4**)	6OMeL-7,4′-diglur (**2**)
Aglycone				
H-3	6.77 *s*	6.72 *s*	6.72 *s*	6.89 *s*
H-6		6.41 *br.s*		
H-8	7.03 *s*	6.78 *br.s*	7.02 *s*	7.07 *s*
H-2′	7.45 *br.s*		7.45 *br.s*	
H-5′	7.21 *d* (8.4)		7.21 *d* (8.5)	7.21 *d* (8.1)
H-6′	7.45 *br.d* (8.4)		7.49 *br.d* (8.5)	7.56 *br.d* (8.1)
OMe	3.75		3.80	3.77
β-Glucuronic acid				
H-1	5,51 *br.d*, 5.35 d (6.5)[c]		5.35 *d* (7), 5.3 *br.d* (6)	5.32 *d*, 5.11 *d* (6.6)
H-2	3.78		3.83, 3.63	3.37, 3.35
H-3,4	ca 3.6		Obscured	3.3–3.43
H-5	3.98 *d*, 3.95 *d* (9)[c]		Obscured	4.05 *d*, 3.98 (9.4)
α-Rhamnose				
H-1	5.11 *s*, 5.09 *s*		5.18 *s*, 5.07 *s*	
H-2	3.85		3.88, 3.76	
H-6	0.99 *d* (6.5)		1.10 *d*, 1.00 *d* (6)	
β-Xylose				
H-1	4.41 *d* (7.6)		4.42 *d* (8.0)	
H-2	3.17		3.15	
α-Arabinose				
H-1	4.40 *d* (6.5)[d]			
H-2	3.45			

[a] Assignments based on ^{1}H,^{1}H-COSY and TOCSY correlations. Coupling (Hz) in parentheses.
[b] Signals distinguishable from those of MF-1a.
[c] Determined at 80° from a 500 MHz spectrum.
[d] Isomer, also evident at 4.35–4.37 (6.5) ppm.

coupling to the C-6, 7, 9 and 10 carbon signals and is accordingly assigned to H-8. The methoxyl is therefore sited at C-6 and the aglycone is 6-methoxyluteolin. This hydrolysis product is defined as a diglycoside containing two β-glucuronic acid residues from the ^{13}C NMR data (Table 2) and the two distinctively coupled (9.4 Hz) C-5 proton signals at ca. 4 ppm (Stochmal et al., 2001). These characteristic spectra clearly distinguish glucuronides from galacturonides (Flamini et al., 2002), and UV shift reagent studies position the glucuronic acid residues at the 7- and 4′-hydroxyls on the flavonoid nucleus.

The linkages of the remaining sugars to the 6-methoxyluteolin 7,4′-diglucuronide nucleus in MF-1a and MF-1b were deduced using NMR data from both compounds. TOCSY and COSY studies enabled the assignment of proton and carbon signals to each of the additional sugar moieties in both compounds (Tables 1 and 2) and comparisons of these data with literature values for α-rhamno-pyranosides (Markham et al., 1978), β-xylopyranosides (Webby, 1991) and α-arabino-pyranosides (Takemura et al., 2002), both confirmed their identities and established that all are terminally sited on the glucuronic acid residues.

Table 2
^{13}C NMR spectral data for MF-1 compounds in DMSO-d_6 (HMBC proton correlations in parentheses)

Carbon No.	MF-1a (**3**)	MF-1a′ (**5**)[a]	MF-1b (**4**)	6OMeL-7,4′-diglur (**2**)
Aglycone				
2	164.3		164.3	164.1
3	104.2		104.1	104.3
4	182.6	182.2	182.6	182.7
5	152.5a	161.0	152.4?	152.5a
6	133.4	99.7	133.1	133 (7.07)
7	155.8	162.3	156.0 (5.35)	156.4 (7.07)
8	94.7	ca 94	94.7	94.4
9	152.5b	157.4	152.4	152.9a (7.07)
10	106.4	106.0	106.2	106.4 (7.07)
1′	125.2		125.2	125.2
2′	114.2		114.2	114.2
3′	148.0b		147.9	147.4
4′	148.2b		148.3	147.4
5′	116.8		117.0	116.2
6′	118.8		118.9	118.9
OMe	60.7		60.7	60.7
β-Glucuronic acid				
1	98.4, 98.1		98.0, 98.6[b]	100.9, 99.9
2	78.0 (5.09,5.11)		77.0[b] (5.18), 78.1 (5.07)	73.3, 73.2
3	83.3 (4.40,4.41,4.42)		83.1	76.3, 75.9
4	69.9		ca 70.4	71.7, 71.6
5	75.1		75.2	75.8, 75.5
6	170.8		ca 171.2	170.3
α-Rhamnose				
0	101.5		101.5, 100.5[b]	
2	ca 70.5		ca 70.4	
3	ca 70.5		ca 70.4	
4	72.1		72.1	
5	69.2		ca 69.2	
6	17.8		17.7, 18.1	
β-Xylose				
1	103.3		103.1	
2	73.2		73.2	
3	76.4		76.3	
4	69.2		69.5	
5	65.9		65.9	
α-Arabinose				
1	103.3			
2	71.0c			
3	72.6c			
4	67.7			
5	65.9			

Assignments bearing the same letter may be interchanged.
[a] Shift data shown only where distinguishable from MF-1a.
[b] Signal associated with disaccharide.

The signals of the glucuronic acid H-1 protons in both MF-1a and MF-1b are shifted downfield from their positions in 6-methoxyluteolin 7,4-diglucuronide (Table 1), which indicates that in both compounds there is a glycosidic linkage to the 2-hydroxyl in each of the glucuronosyl groups (Markham and Geiger, 1994). The concomitant downfield shift of the glucuronosyl H-2 signals identified from ^{1}H, ^{1}H-COSY experiments confirms this (Markham and Geiger, 1994). That the 2-linked sugars are rhamnoses was confirmed from HMBC studies (see Table 2). By this means, with MF-1a, the rhamnose H-1 signals at 5.09 and 5.11 ppm were found to be long-range coupled to the carbon signal at 78.0 ppm which was demonstrated to represent the glucuronosyl C-2 through its correlation with H-2 at 3.78 ppm by ^{1}H, ^{13}C-COSY. In a like manner for MF-1b, the rhamnose H-1 signals at 5.07 and 5.18 were long-range coupled to the 77.0 and 78.1 ppm C-2 signals which in turn correlated with the glucuronosyl H-2s at 3.83 and 3.63 ppm.

Using a similar approach, the xylose and arabinose sugars were shown to be attached to the glucuronosyl 3-hydroxyls. The identified xylose (MF-1a and 1b) and arabinose (MF-1a) H-1 signals were found to be long-range coupled (see Table 2) to a glucuronosyl carbon at 83.3 ppm, and since such a glycosylation would cause a downfield substitution shift of ca. 8 ppm (Markham and Chari, 1982), this signal must be the glucuronosyl C-3 which is shifted from around 76 ppm (cf. 6-methoxyluteolin 7,4′-diglucuronide, Table 2). The C-4 signal is excluded as a possibility since an excessively large downfield shift of 13 ppm would be required to account for the signal at 83.3 ppm.

The above data defines the oligosaccharides in the MF-1a structure as [2-*O*-α-rhamnopyranosyl-3-*O*-β-xylopyranosyl]-β-glucuronopyranoside and [2-*O*-α-rhamnopyranosyl-3-*O*-α-arabinopyranosyl]-β-glucuronopyranoside, and in MF-1b as [2-*O*-α-rhamnopyranosyl-3-*O*-β-xylopyranosyl]-β-glucuronopyranoside and 2-*O*-α-rhamnopyranosyl-β-glucuronopyranoside. The only remaining question is which oligosaccharide is linked to the 7-hydroxyl of the aglycone and which to the 4′-hydroxyl.

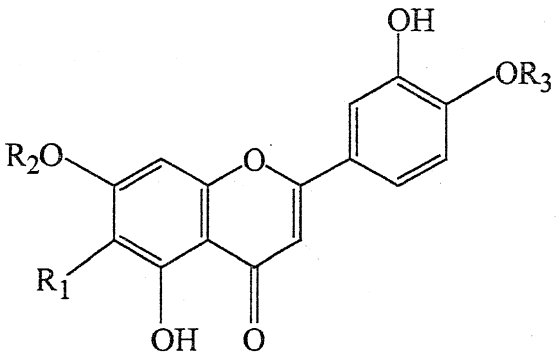
In the HMBC spectrum of MF-1b, the glucuronic acid (No. 1) H-1 at 5.35 ppm is long-range coupled to a carbon resonating at 156.0 ppm. This carbon is identified as C-7 rather than C-4′ from the same spectrum, since it is one of four (including C-6, 9 and 10) which long-range couple to H-8 (7.07 ppm) in the hydrolysis product (**2**)—see Table 2. Glucuronic acid (No. 1) is therefore glycosidically linked to the 7-hydroxyl. The H-2 signal associated with this glucuronic acid is defined from the ^{1}H, ^{1}H-COSY spectrum as that at 3.63 ppm. This is at higher field than the H-2 signal correlated with the glucuronic acid (No. 2) H-1 at 3.83 ppm, due to the lack of glycosylation at C-3 [which would bring about a downfield shift of 1-4 ppm in the neighbouring H-2 (Markham and Geiger, 1994; Stochmal et al., 2001) as seen in the H-2 of glucuronic acid (No. 2)]. In contrast in MF-1a, where the 2- and 3-positions of the glucuronic acid residues are *both* glycosylated at C-2 and C-3, both H-2 signals are found at 3.78 ppm. Confirmation of this interpretation was obtained from the ^{1}H^{13}C-TOCSY spectra which reveals that the glucuronic acid (No. 1) H-1 signal at 5.35 ppm originates from the same sugar as does the carbon signal at 77 ppm, whereas the glucuronic acid (No. 2) H-1 signal at 5.30 ppm originates from the same sugar that produces the carbon signals at 78.1 and 83.1 ppm which result from 3-glycosylation. It follows therefore that the oligosaccharide attached to the 7-hydroxyl of the aglycone in MF-1b is lacking the 3-linked arabinose, whereas in MF-1a the 7-hydroxyl-linked oligosaccharide is the one containing a 3-linked arabinose.

On the basis of the above evidence MF-1b is defined as 6-methoxyluteolin 7-*O*-[2-*O*-α-rhamnopyranosyl-β-glucuronopyranoside]-4′-*O*-[2-*O*-α-rhamnopyranosyl-3-*O*-β-xylopyranosyl-β-glucuronopyranoside] (**4**), and MF-1a is defined as 6-methoxyluteolin 7-*O*-[2-*O*-α-rhamnopyranosyl-3-*O*-α-arabinopyranosyl-β-glucuronopyranoside]-4′-*O*-[2-*O*-α-rhamnopyranosyl-3-*O*-β-xylopyranosyl-β-glucuronopyranoside] (**3**).

The additional presence of the MF-1a and MF-1b analogues, MF-1a′ and MF-1b′, was revealed via HPLC (before and after acid treatment) and MS studies of fraction A from the original work-up (see above). These analogues differ from MF-1a and MF-1b respectively, in that they lack the equivalent of one methoxyl. This suggests that MF-1a′ and MF-1b′ might be the luteolin analogues of MF-1a and MF-1b, **5** and **6**. Indeed, the on-line measured absorption spectra are identical with one another and with authentic luteolin 7,4′-diglucuronide (Campos and Markham, 2003), and evidence distinctive of luteolin was seen in the ^{1}H and ^{13}C NMR spectra of fraction A (see Tables 1 and 2).

The reinvestigation of the structure of the "flavone-polysaccharide", MF-1, from the liverwort *Monoclea forsteri* has thus revealed that MF-1 is in fact a mixture of penta- and hexa-glycosides of 6-methoxyluteolin, and to a lesser extent, luteolin. Although flavonol tetraglycosides (e.g. Nielsen et al., 1998; Semmar et al., 2002) and a flavonol pentaglycoside (Asada et al., 1988) have been found previously in plants they are rarely encountered and the current report is the first to provide substantive evidence for the existence of flavone penta- and hexa-*O*-glycosides in nature (Williams and Harborne, 1994).

Until recently there has been a tendency to consider *M. forsteri* to be the sole species in the order Monocleales (e.g. Campbell, 1987), an order aligned with, but isolated from, the Marchantiales in the subclass Marchantiidae (Gradstein et al., 1992). The order is



	R_1	R_2	R_3
1	OCH_3	H	H
2	OCH_3	β-glucuronosyl	β-glucuronosyl
3	OCH_3	[2-O-α-rhamnosyl-3-O-α-arabinosyl]-β-O-glucuronosyl	[2-O-α-rhamnosyl-3-O-β-xylosyl]-β-O-glucuronosyl
4	OCH_3	2-O-α-rhamnosyl-β-glucuronosyl	[2-O-α-rhamnosyl-3-O-β-xylosyl]-β-O-glucuronosyl
5	H	[2-O-α-rhamnosyl-3-O-α-arabinosyl]-β-O-glucuronosyl	[2-O-α-rhamnosyl-3-O-β-xylosyl]-β-O-glucuronosyl
6	H	2-O-α-rhamnosyl-β-glucuronosyl	[2-O-α-rhamnosyl-3-O-β-xylosyl]-β-O-glucuronosyl

considered to be primitive and phylogenetically isolated, and the occurrence of *Monoclea* in only New Zealand and South and Central America suggests that it is "geologically very old and Gondwana-derived" (Gradstein et al., 1992). The species name, *M. gottschei*, has been reinstated recently by Gradstein et al. (1992) to describe the New World *Monoclea* population which they found to differ from *M. forsteri* both morphologically and chemically. In terms of their flavonoid profiles, *M. gottschei* populations were shown to accumulate a much more diverse range of structural types than *M. forsteri*, to produce 6-oxygenated but not 8-oxygenated flavones, and to di- and tri-glycosylate the flavone aglycones rather than attach "polysaccharides". However, the structures for the MF-1 group of compounds as defined in the present paper narrow the distinction between these two species in that the originally proposed 8-methoxyluteolin has been redefined as 6-methoxyluteolin, luteolin glycosides have been found, and the polysaccharides have been characterized as di- or tri-saccharides. Furthermore, the structures of the di- and trisaccharides bear a striking resemblance to the glycosidic moieties in several of the partly characterized *M. gottschei* flavone glycosides, e.g. the 7-*O*-rhamnoarabino "galacturonides", 7-*O*-rhamnoxylosyl "galacturonides", 4′-*O*-rhamnosylglucuronides, 7-*O*-rhamnoglucuronides, etc. The structural data defined in the present paper thus provide biosynthetic evidence which strengthens the link between the two species and in so doing, enhances the homogeneity and distinctiveness of this primitive and isolated order of liverworts, the Monocleales.

3. Experimental

3.1. General

High resolution mass spectrometry was carried out using the electrospray method in negative ion mode, and the data was processed using a MARINER Biospectrometry Workstation, system 5158. NMR spectroscopy was carried out on Brucker-Avance 300 (300 MHz) and Varian Unity 500 (500 MHz) machines using 5 mm probes and DMSO-d_6 or DMSO-d_6/D_2O as solvents. Spectra were measured at 30° (300 MHz and 500 MHz) and 80° (500 MHz). The 2D homonuclear double quantum filtered (dqf) ^1H, ^1H-COSY, heteronuclear inverse one bond HSQC and long-range HMBC were

all standard Bruker PFG versions. TOCSY experiments were carried out in heteronuclear mode, and all 2D spectra incorporated pulsed field gradients.

3.2. Plant material and extraction/purification procedures

Fresh *M. forsteri* thallus material (ca. 1 kg) which was identified by Dr. John Braggins, bryologist, School of Biological Sciences, University of Auckland (NZ), was collected from a 50 m long population in Huapai, West Auckland. The thallus material was washed clean of adhering soil with water, and then ground in a Waring Blendor with 1.5 l of MeOH:H_2O (20:80). After standing overnight the macerate was filtered through cotton wool. The residue was re-extracted and the filtrates combined and evaporated to dryness in vacuo. The solid extract was dissolved in H_2O and clarified by centrifugation (5000 rpm, 5 min). After concentration, a portion of the clarified soln. was applied to 20 sheets of Whatmans 3MM (57 × 46 cm) for 1D separation in *t*-BuOH:HOAc:H_2O, 3:1:1 (TBA). These sheets were run twice in this solvent, being dried completely between runs. The partially separated bands, still close to the origin, were cut out and eluted with H_2O to yield fractions B (front running) and A, which were analysed by HPLC (see below, and detail in Results and Discussion). Fraction A was re-applied to 3MM sheets and these were run four times to remove most of the remaining MF-1b (=Fraction B). This "purified" Fraction A and the original Fraction B were cleaned of unattached carbohydrate by applying them to "Extract Clean" RC C-18 (Alltech), 500 mg columns in 0.03 M trifluoroacetic acid (TFA), washing the column thoroughly with TFA, and then eluting the flavonoids with MeOH containing ca. 5% H_2O. HPLC analysis of purified Fraction B revealed one major peak (MF-1b, 30.6 min) with a slight shoulder at ca. 30.7 min (MF-1b′), whilst HPLC analysis of "purified" Fraction A gave one major peak (MF-1a, 30.0 min) with a shoulder at ca. 29.9 min (MF-1a′), plus a smaller peak containing MF-1b and MF-1b′. These fractions were used for subsequent MS and NMR studies.

3.3. HPLC analyses

Crude and purified extracts, and products from acid hydrolyses (see below), were analysed by HPLC using the following conditions. Analyses were carried out on a Merck Superspher 100 end capped RP-18 column (12.5 × 0.4 cm I.D.: 4 μm) using a Waters (Milford, M.A. USA) 600E solvent controller, a Waters 996 photodiode array detector, a Jasco (Tokyo, Japan) 851-AS intelligent sampler and Millenium^{32} (Milford, MA, USA) Version 3.05.01 software. Injection volume was 20 μl and elution was performed using a flow rate of 0.8 ml min^{-1} at 24 °C. The solvents, water adjusted to pH 2.5 with orthophosphoric acid (A) and acetonitrile (B), were mixed using a linear gradient starting with 100% A, decreasing to 91% over 12 min, to 87% over the next 8 min and to 67% over the next 10 min. After holding at this composition for 2 min, A was decreased to 57% over 10 min and then held at this level until the end of the 60 min analysis. Spectral data for all peaks were accumulated in the range 220–400 nm, and standard chromatograms were plotted at 352 nm.

3.4. Controlled acid treatment of MF-1 compounds

Fraction B and "purified" Fraction A solids, ca. 3 mg, were each dissolved in 1 M TFA (1 ml) and heated to 100° for 30 min. Both were evaporated to dryness at ca. 50° in vacuo, redissolved in H_2O and applied to "Extra Clean" RC C-18 (Alltech) 500 mg columns. Washing with 2% aq. HOAc gave a sugar containing fraction, and washing with MeOH gave a flavonoid fraction. The sugar fraction was chromatographed on paper in 1D, alongside authentic sugar markers, in *n*-BuOH:pyridine:H_2O, 15:10:7.5, and the dried chromatograms were sprayed with 3% aniline phthalate in EtOH before heating at 100° to reveal sugar spots. Spots equivalent to rhamnose, xylose and a glycuronic acid (weak) were evident for both samples, and arabinose was also present in the 1D-PC representing "purified" Fraction A. The MeOH eluted flavonoids from both samples gave the same patterns of spots on 2D-TLC. The TLCs were run in TBA and 15% HOAc and sprayed with 1% diphenylboric acid 2-amino-ethyl ester in MeOH (Markham, 1982) for viewing under UV light (366 nm). The major spot in both cases was a diglycoside which was dark in UV, and this was accompanied by lower levels of monoglycosides and aglycone (cf. Markham, 1972). HPLC analyses of these same products produced chromatograms which were qualitatively the same. The flavonoid products, identified from their TLC properties and their RTs (min.) and on-line measured absorption spectra, include the following: MF-1b? (27.3), luteolin-7,4′-diglucuronide (30.1), 6-methoxyluteolin-7,4′-diglucuronide (31.2), luteolin-7-glucuronide (34.1), 6-methoxyluteolin-7-glucuronide (34.9), 6-methoxyluteolin-4′-glucuronide (36.7), 6-methoxyluteolin (42.3). The major product of acid treatment was 6-methoxyluteolin-7,4′-diglucuronide (**2**), and this was isolated from a larger scale hydrolysis of the more pure, MF-1b. The acid hydrolysis product was cleared of carbohydrate on an "Extra Clean" column (see above) for NMR study. Absorption spectra, with added shift reagents (Markham, 1982), for compound **2** further purified by 1D-PC (15% HOAc) are as follows: λ_{max} (H_2O) 250 *sh*, 274, 332 nm; (NaOMe) 270 *sh*, 286, 351 nm; (NaOAc) 274, 331 nm; (NaOAc/H_3BO_3) 250 sh, 274, 325 nm.

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References

Asada, Y., Hirayama, Y., Furuya, T., 1988. Acylated flavonols from *Crocosmia crocosmiiflora*. Phytochemistry 27, 1497–1501.

Campbell, E.O., 1987. *Monoclea* (*Hepaticae*), distribution and number of species. Bryologist 90, 371–373.

Campos, M., Markham, K.R., 2003. Structure Information from HPLC and On-line Measured Absorption Spectra—Flavones, Flavonols and Phenolic Acids. Coimbra University Press, Coimbra, Portugal. (spectrum 48).

Flamini, G., Pardini, M., Morelli, I., Ertugrul, K., Dural, H., Bagci, Y., Kargioglu, M., 2002. Flavonoid glycosides from *Centaurea pseudoscabiosa* subsp. *pseudoscabiosa* from Turkey. Phytochemistry 61, 433–437.

Gradstein, S.R., Klein, R., Kraut, L., Mues, R., Sporle, J., Becker, H., 1992. Phytochemical and morphological support for the existence of two species in *Monoclea* (*Hepaticae*). Plant Syst. Evol. 180, 115–135.

Markham, K.R., 1972. A novel flavone-polysaccharide compound from *Monoclea forsteri*. Phytochemistry 11, 2047–2053.

Markham, K.R., 1982. Techniques of Flavonoid Identification. Academic Press, London, pp.15-51.

Markham, K.R., Ternai, B., Stanley, R., Geiger, H., Mabry, T.J., 1978. Carbon-13 NMR studies of flavonoids-III. Naturally occurring flavonoid glycosides and their acylated derivatives. Tetrahedron 34, 1389–1392.

Markham, K.R., Geiger, H., 1994. ^{1}H-NMR spectroscopy of flavonoids and their glycosides in hexadeuterodimethylsulphoxide. In: Harborne, J.B. (Ed.), The Flavonoids-Advances in Research Since 1986. Chapman and Hall, London, pp. 441–473.

Markham, K.R., Chari, V.M., 1982. Carbon-13 NMR spectroscopy of flavonoids. In: Harborne, J.B., Mabry, T.J. (Eds.), The Flavonoids: Advances in Research. Chapman and Hall, London, pp. 19–132.

Nielsen, J.K., Norbaek, R., Olsen, C.E., 1998. Kaempferol tetraglucosides from cabbage leaves. Phytochemistry 49, 2171–2176.

Semmar, N., Fenet, B., Gluchoff-Fiasson, K., Compte, G., Jay, M., 2002. New flavonol tetraglycosides from *Astragalus caprinus*. Chem. Pharm. Bull. 50, 981–984.

Stochmal, A., Simonet, A.M., Macias, F.A., Oleszek, W., 2001. Alfalfa (*Medicago sativa* L.) flavonoids. 2. Tricin and chrysoeriol glycosides from aerial parts. J. Agric. Food Chem. 49, 5310–5314.

Takemura, M., Nishida, R., Mori, N., Kuwahara, Y., 2002. Acylated flavonol glycosides as probing stimulants of a bean aphid, *Megoura crassicauda*, from *Vicia augustifolia*. Phytochemistry 61, 135–140.

Webby, R.F., 1991. A flavonol triglycoside from *Actinidia arguta* var. Giraldii. Phytochemistry 30, 2443–2444.

Williams, C.A., Harborne, J.B., 1994. Flavone and flavonol glycosides. In: Harborne, J.B. (Ed.), The Flavonoids-Advances in Research Since 1986. Chapman and Hall, London, pp. 337–370.