Flavonoid Diversity in the Liverwort Genus *Monoclea* Hooker*

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Many samples of *Monoclea gottschei* from Central and South America and the Caribbean Islands have been compared for their flavonoids. The species exhibits the most diverse flavonoid pattern ever observed for a liverwort. From different chemotypes 28 flavones were isolated: 8 aglycones, 3 monoglycosides, 9 diglycosides, 6 triglycosides and 2 oligo- or polyglycosides. The chromatographic and spectroscopic data of previously unidentified compounds are presented. Samples of *M. forsteri* from New Zealand have been analyzed for flavonoids too, but their flavone patterns are much less diverse: 1 aglycone, 1 polyglycoside and 2 further mono-or-diglycosides were isolated. The differences in flavonoid production of both species are an additional feature for the distinction of the two *Monoclea* species.

Introduction

The taxonomy of the liverwort genus *Monoclea* Hook. has been discussed by various authors [1–6]. The essential point was whether *M. forsteri* Hooker and *M. gottschei* Lindberg are distinct species or whether *M. gottschei* is only a subspecies or a variety of *M. forsteri.* In a recent paper we presented new morphological and phytochemical results from the investigation of a larger number of samples from both taxa [7]. We came to the conclusion that both should not only be maintained as distinct species, but because of the clearly different antheridial receptacles, *M. gottschei* should be subdivided into two subspecies: subsp. *gottschei* with orbicular antheridial receptacles and subsp. *elongata* Gradstein and Mues with mostly elongated antheridial receptacles. In that paper we reported the accumulation of no less than 31 flavonoids in different chemotypes by the various *Monoclea* samples investigated, and presented the isolated compounds in a table without details on their structure elucidation and specific chromatographic and spectral data. These are now reported here for all previously unidentified compounds.

Abbreviations: Bd I, long wave length absorption; BEW, BuOH(2)–HOAc–H_2O = 14:1:5; BR, Benedict's reagent; NA, Naturstoffreagenz A.

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Results and Discussion

A total of 49 samples of *M. gottschei* from 12 countries of Central and South America and the Caribbean Islands (for details see [7]) were analyzed for their flavonoids. Twenty-eight flavonoids including aglycones, mono-, di-, tri- and oligo-glycosides (Table I) were isolated. In 6 samples from 4 different countries, flavonoids were not detected. This could be explained by the age, the quantity and the condition of some herbarium samples, but also by the fact that plants from these regions may have lost the ability to synthesize flavonoids.

Compared to the diversity of the *M. gottschei* flavonoid patterns, the samples of *M. forsteri* from New Zealand contained only a few flavonoids. Eight samples were analyzed and only one free aglycone was isolated, luteolin 3′,4′-dimethylether (**4**), and two types of flavone glycosides: onopordin 7,4′-di-O-polyglycoside (**31**), which was previously isolated from this species [8] and two hispidulin-type di- or mono-glycosides (**15, 16**) (Table I). Dr. K. R. Markham (pers. commun.) screened 18 further samples originating from Stewart Island in the south, to the region around Auckland of New Zealand and found no additional flavonoid types.

The flavonoid aglycones were isolated from $CHCl_3$- or CH_2Cl_2-extracts and glycosides from aq. MeOH-extracts. The aglycones and glycosides were separated by combined prep. CC-, PC- or TLC-techniques, and their structures were elucidated by chromatographic comparisons (TLC, HPLC) and spectroscopy (UV-, NMR- and mass-

Table I. Flavonoids of *Monoclea gottschei* and *M. forsteri*.

I Aglycones	**1** Apigenin 7,4′-di-OMe
	2 Scutellarein 6,7,4′-tri-OMe (Salvigenin)
	3 Luteolin 7,3′-di-OMe (Velutin)
	4 Luteolin 3′,4′-di-OMe
	5 6-Hydroxyluteolin 7-OMe (Pedalitin)
	6 6-Hydroxyluteolin 6,7-di-OMe (Cirsiliol)
	7 6-Hydroxyluteolin 7,3′-di-OMe
	8 6-Hydroxyluteolin 6,3′,4′-tri-OMe (Eupatilin)
II Monoglycosides	**9** Pectolinarigenin 7-O-glucuronide*
	10 Pectolinarigenin 7-O-β-galacturonide**
	11 Luteolin 7-O-glucuronide
III Diglycosides	**12** Apigenin 7,4′-di-O-glucuronide
	13 Acacetin 7-O-diglucuronide
	14 Acacetin 7-O-digalacturonide*
	15, 16 Hispidulin 7-O-glycosides*
	17 Scutellarein 4′-OMe-7-O-glucuronorhamnoside*
	18, 19 Scutellarein 4′-OMe-7-O-di-or triglycosides*
	20 Luteolin 4′-O[-α-L-rhamnosyl-β-D-galacturonide]*
	21 Chrysoeriol 7,4′-di-O-glucuronide
	22 Nodifloretin 7-O-rhamnoside-4′-O-glucuronide*
IV Triglycosides	**23** Apigenin 7-O-rhamnoglucuronide-4′-O-glucuronide or Apigenin 7-O-glucuronide-4′-O-rhamnoglucuronide*
	24, 25 Mixture of Acacetin 7-O-rhamnoarabinosylgalacturonide and Acacetin 7-O-rhamnoxylosylgalacturonide*
	26 Acacetin 7-O-rhamnoglucosylglucuronide*
	27, 28 Mixture of Luteolin 3′,4′-di-OMe-7-O-rhamnoarabinosylgalacturonide and Luteolin 3′,4′-di-OMe-7-O-rhamnoxylosylgalacturonide*
V Oligo-(Poly?)-glycosides	**29** Luteolin – Oligoglycosides*
	30 Nepetin – Oligoglycosides*
	31 Onopordin 7,4′-di-O-polyglycoside***

* Structures only tentative. ** New compound. *** For Reference see [8].

spectrometry). Additionally EI-MS of most free aglycones (except **6**) were recorded.

Aglycones

The aglycones (**1–8**) belong to four different types of flavone aglycones: apigenin, scutellarein, luteolin and 6-hydroxyluteolin. We present here the ^{13}C NMR data for pedalitin (**5**) (see Experimental), which are not published in the recent ^{13}C NMR review [9]. Although the methoxylated aglycones **2, 3, 6–8** are known from tracheophytes [10, 11], they are reported here for the first time from a bryophyte.

Glycosides

Because of the limited amount of plant material available for analysis (for details see [7]), most glycosides were isolated in trace quantities which did not permit determination of the interglycosidic linkages of di-, tri- and oligo-glycosides. The chromatographic and UV/visible data of previously unpublished compounds are presented in Table II. UV/visible data for **17–19** and **24–26** are almost identical, thus only one set of data is shown in Table II for each group of compounds. The NaOMe-spectra of all glycosides in Table II are stable and show a decreased intensity in Bd I, indicating a 4′-OR-group [12, 13]. The ^1H NMR data for **10** and **20** are given in the Experimental.

Monoglycosides

Among the three isolated monoglycosides (Table I; **9–11**) only pectolinarigenin 7-O-β-D-galacturonide (**10**) is new. Acid and enzymatic hydrolysis with β-glucuronidase of **10** gave the aglycone pectolinarigenin (= 6,4′-diOMe-apigenin) and galacturonic acid, both of which were identified by TLC and HPLC comparisons with standard sam-

Table II. Chromatographic and UV/visible data of compounds **10, 14, 17–20, 22–28***.

Compound	**10**	**14**	**17**	**18/19**	**20**
Spot appearance					
UV (366 nm)	purple	purple	purple	purple	purple
UV/NH_3	purple	purple	purple	purple	purple
UV/NA	purple	olive	purple	purple	olive
UV/BR	brown-olive	olive	olive	olive	olive
TLC hR_f values					
Adsorbent: Cellulose F 1440					
H_2O	64	78	75	73	83
15% HOAc	31	41	36	48	41
BAW	75	53	32	38	64
BEW	62	64	20	37	63
Adsorbent: Polyamide-6-WEMA = H_2O–MeOH–MeCOEt–Acetylaceton (13:3:3:1)	4	18	16	8	7
Adsorbent: Silica gel 60 F_{254} EtOAc–MeCOEt–HCOOH–H_2O (5:3:1:1)	47	44	22	21	16
UV/visible data					
MeOH	276, 327	268, 326	287, 327		248, 268, 285 sh, 336
NaOMe	296, 377	290, 375	276 sh, 323		268, 294 sh, 376
$AlCl_3$	286 sh, 300, 355	275, 298, 340, 373 sh	300, 357		257 sh, 276, 292 sh, 351, 384 sh
$AlCl_3$/HCl	286 sh, 300, 348	273, 295, 335, 373 sh	300, 357		254 sh, 280, 292 sh, 343, 384 sh
NaOAc	276, 328	268, 324	292, 325 sh		275, 317, 366
$NaOAc/H_3BO_3$	276, 328	268, 325	287, 323 sh		269, 334

* For solvent systems see Experimental.

ples. In the FAB-MS the $(M-H)^-$ at *m/z* 489 is indicative of a pectolinarigenin mono-hexuronide. The aglycone ion $(A-H)^-$, *m/z* 313, is the base peak. EI-MS of the isolated aglycone revealed the M^+ at *m/z* 314, accompanied by RDA-fragment-peaks characteristic of pectolinarigenin [14]. The structure was confirmed by ^1H NMR spectroscopy (see Experimental).

Di-, Tri- and Oligo-glycosides

The proposed structures of these flavone glycosides (Table I; **12–31**) were determined by chromatographic and UV/visible data. Aglycones, monoglycosides (isolated as products of partial acid hydrolysis) and sugars were identified by comparison with standard samples (apart from the sugars of **15/16**). For **20, 24/25** and **27/28** FAB-mass spectra (neg. mode) were recorded. The $(M-H)^-$ signal at *m/z* 607 for **20** is indicative of a luteolin hexuronide-deoxyhexoside and a fragment peak at *m/z* 461 is in accordance with the loss of the deoxyhexosyl-moiety. As expected from results of total acid hydrolysis the base peak at *m/z* 285 relates to the $(A-H)^-$ ion of a tetrahydroxyflavone, luteolin. Despite the absence of a shoulder between 320 and 335 nm [13] in the UV/vis-spectrum of this glycoside it is still probable that a free 7-OH is present. The evidence for this is as follows. Firstly, in the UV/vis-spectrum NaOAc induces a 7 nm bathochromic shift in band II absorption [13]. Secondly, the FAB-MS shows only one monoglycoside fragment peak. This is support for a flavone disaccharide formulation, since if the sugars were attached to separate hydroxyls two monoglycoside

Table II (Continued).

Compound	**22**	**23**	**24/25**	**26**	**27/28**
Spot appearance					
UV (366 nm)	purple	purple	purple	purple	purple
UV/NH_3	purple	purple	purple	purple	purple
UV/NA	purple	olive	olive	olive	olive
UV/BR	olive	olive	olive	olive	olive
TLC hR_f values					
Adsorbent: Cellulose F 1440					
H_2O	71	91	79	82	70
15% HOAc	15	44	43	45	31
BAW	28	40	64	57	51
BEW	16	17	43	33	39
Adsorbent: Polyamide-6-WEMA = H_2O–MeOH–MeCOEt–Acetylaceton (13:3:3:1)	20	2	11	11	18
Adsorbent: Silica gel 60 F_{254} EtOAc–MeCOEt–HCOOH–H_2O (5:3:1:1)	21	7	8/14 or 14/18	7	10/17 or 17/10
UV/visible data					
MeOH	254 sh, 281, 340	269, 316	267, 322		250, 269, 337
NaOMe	268, 310, 370	291, 380	287, 364		287, 313 sh, 385
$AlCl_3$	295, 361	278, 296, 335, 375	276, 299, 344, 377		262 sh, 274, 294 sh, 360, 380
$AlCl_3$/HCl	295 sh, 360	277, 297, 329, 375	277, 298, 336, 376		259, 274, 292 sh, 350, 382 sh
NaOAc	281, 336	269, 316	267, 323		249, 268, 339
NaOAc/H_3BO_3	281, 336	269, 316	268, 326		249, 268, 339

fragments would be expected. Finally, no hydrolysis of **20** with β-glucuronidase was observed within 24 h. This supports a rhamnogalacturonyl structure, as an underivatized galacturonide at the 7 or 4′-position should be completely cleaved by this enzyme in less than 2 h [13]. This evidence supports the structure of luteolin 4′-O[-α-L-rhamnosyl-β-D-galacturonide] for **20.**

Compounds **24** and **25** were isolated as a 1:1 (approximately) mixture, which was chromatographically difficult to separate. The single $(M-H)^-$ peak for **24/25** at *m/z* 737 suggests that both compounds are acacetin triglycosides with a pentose (xylose or arabinose), a hexuronic acid (galacturonic acid) and a deoxyhexose (rhamnose). In confirmation, partial acid hydrolysis of this mixture gave the following products, each of which was separated and analyzed for aglycone and sugars: a single aglycone (acacetin), a single monoglycoside (acacetin 7-O-galacturonide), and two diglycosides as a mixture (acacetin 7-O-xylogalacturonide and 7-O-arabinogalacturonide). This evidence suggests that the mixture **24/25** consists of acacetin 7-O-rhamnoarabinosylgalacturonide and acacetin 7-O-rhamnoxylosylgalacturonide.

In the same manner, the analogous mixture, **27/28,** which gave $(M-H)^-$ at *m/z* 767, was shown to consist of luteolin 3′,4′-di-OMe-7-O-rhamnoarabinosylgalacturonide and 7-O-rhamnoxylosylgalacturonide.

All structures in Table I with an asterisk must be regarded as tentative only as the amount isolated was insufficient for complete structure determination.

Experimental

Plant material

Details regarding the origin and the quantities of the investigated plant material and location of voucher specimens are given in [7].

Extraction and isolation

For 2D-TLCs between 35 and 200 mg air-dried thalli (gametophytes) were ground and either pre-extracted with CH_2Cl_2, followed by extraction with 80% aq. MeOH or immediately extracted with 80% aq. MeOH. Comparative TLC-analysis was accomplished according to [15]. The plant material of those samples, sufficient for isolation of flavonoids [7], was also ground and in general extracted similarly as described above, lipophilic extracts mainly with $CHCl_3$. From lipophilic extracts the free flavone aglycones were isolated 1. by CC on Sephadex LH-20 with CH_2Cl_2–MeOH = 1:1, on silica gel (Merck) with toluene–MeOH–HOAc = 45:3:2 and on polyamide (Macherey and Nagel) with toluene–EtOAc–MeOH = 30:13:17; 2. by prep. PC on Whatman 3MM with the following solvents: AEW = *n*-pentanol–HOAc–H_2O = 2:1:1; BAW = *n*-BuOH–HOAc–H_2O = 4:1:5, upper layer; 40% HOAc; 3. by prep. TLC on silica gel (Merck), self made plates, solvent: toluene–ethylformate–HCOOH = 5:4:1. The isolated aglycones were finally purified (before spectroscopic investigation) by CC on Sephadex LH-20 with 90% aq. MeOH as solvent.

The aq. MeOH-extracts were reduced to a small volume and partitioned between $CHCl_3$ and the H_2O-phase. The $CHCl_3$-layer was combined with the other lipophilic extracts. The crude H_2O-phase was first passed through a cellulose column (Avicel, Merck, for CC) with 3–20% HOAc as solvent to get crude flavonoid fractions. These were further purified by repeated prep. PC on Whatman 3MM with BAW and AEW as solvents. Final purification was achieved by CC on Sephadex LH-20 with 70 or 80% aq. MeOH as solvent. Yields: 1. aglycones: < 1 mg, except for pedalitin (**5;** 3.2 mg, crystallized from hot MeOH); 2. glycosides: traces of < 1 mg except for **10:** 3 mg; **20:** 3.3 mg; mixture of **25/26:** 6 mg; mixture of **27/28:** 3.2 mg, all crystallized from aq. MeOH.

TLC all compounds was performed on precoated sheets: cellulose F 1440 (Schleicher and Schüll); polyamide-6, Polygram (Macherey and Nagel); silica gel 60 F 254 (Merck); solvent systems: see above and Table II; spray reagents: Naturstoffreagenz A (NA, 0.1% in MeOH, Roth, Karlsruhe, F.R.G.), Benedicts Reagenz (BR) and anilinephthalate (Merck, for sugars) according to [15]; naphthoresorcin of Aldrich 0.2 g in 100 ml EtOAc + 2 ml conc. H_2SO_4 (for sugars) according to [16]. HPLC: Waters, two M-45 pumps with "automated gradient controller", variable wavelength detector 450, Rheodyne 7125 injector; Nucleosil 5 C_{18}-column 250 × 4 mm (Macherey and Nagel); flow rate 1 ml/min, isocratic, 35–80% MeOH in 5% HOAc or 1% H_3PO_4; detection at 280 or 330 nm.

Mixtures of compounds **24/25** and **27/28** were difficult to separate. A separation of both mixtures was possible by TLC only on silica gel with EtOAc–MeCOEt–HCOOH–H_2O (5:3:1:1) and EtOAc–HOAc–HCOOH–H_2O (100:11:11:27) as solvents. HPLC-separation was achieved only after a long retention time of 50 min with the solvent 38% MeOH in 1% H_3PO_4.

Glucuronic and galacturonic acid moieties were easily separated after acid hydrolysis of the glycosides for 6 h with 1 N TFA. Under these conditions glucuronic acid (GA) undergoes at least partly lactonization to the glucuronolactone (GL), clearly separating from GA in the solvent: *n*-BuOH–C_5H_5N–H_2O (50:33:17) on silica gel 60 F_{254}, Merck, plastic sheets. In this solvent GA and galacturonic acid (GaA) are also clearly separated; R_f-values: GA = 0.15; GL = 0.58; GaA = 0.8.

Hydrolysis methods

1. Total acid hydrolysis: 1 N TFA, 6 h, reflux; for **29** and **30:** 1 N HCl, 6 h, reflux.

2. Partial acid hydrolysis: 0.1 N or 1 N TFA. 0.5–1 h, reflux.

3. Enzymatic hydrolysis: β-glucuronidase ("β-Glucuronidase pulv.", Fluka, Switzerland. No. 49320), 4 h, deion. H_2O; room temp.; β-glucuronidase + pectinase ("pectinase fungal", Koch-Light, No. 4443-01), 48 h, deion. H_2O; room temp.

Spectroscopic methods

UV/visible spectroscopy: according to [12, 13]; NMR spectroscopy: Bruker AM 400, DMSO-d_6, ambient temperature, 400 MHz (^{1}H), 100 MHz

(^{13}C); mass spectrometry: EI, Varian MAT 311, 70 eV, ion source 150 °C, probe 150–250 °C; FAB, Finnigan MAT 90, xenon, 7–8 keV, glycerol as matrix.

^1H and ^{13}C NMR data for pedalitin (**5**): ^1H NMR: 6.69 (s, H-3), 6.86 (s, H-8), 7.44 (d, $J = 7$ Hz, H-2′, 6′), 6.89 (d, $J = 8.9$ Hz, H-5′), 3.91 (s, 7-OMe); ^{13}C NMR: 163.9 (C2), 102.4 (C3), 181.9 (C4), 149.5 (C5)*, 129.5 (C6), 154.2 (C7), 90.9 (C8), 146.2 (C9), 104.9 (C10), 121.5 (C1′), 113.3 (C2′), 145.7 (C3′), 149.7 (C4′)*, 115.8 (C5′), 118.8 (C6′), 56.2 (7-OMe).

^1H NMR data for glycosides:

1. Pectolinarigenin 7-O-β-D-galacturonide (**10**): 6.96 (s, H-3), 7.05 (s, H-8), 8.05 (d, $J = 8.9$ Hz, H-2′, 6′), 7.13 (d, $J = 8.8$ Hz, H-3′, 5′), 3.86 (s, 6-OMe)*, 3.80 (s, 4′-OMe)*, 5.16 (d, $J = 7.7$ Hz, H-1″).

* Assignments exchangeable.

2. Luteolin 4′-O[-α-L-rhamnosyl-β-D-galacturonide] (**20**): 6.76 (s, H-3), 6.20 (d, $J = 2$ Hz, H-6), 6.46 (d, $J = 2$ Hz, H-6), 6.46 (d, $J = 2$ Hz, H-8), 7.48 (d, $J = 8.2$ Hz, H-2′, 6′), 7.17 (d, $J = 8.4$ Hz, H-5′), 5.21 (d, $J = 7.5$ Hz, H-1 gal), 5.11 (s, H-1 rham), 1.08 (d, $J = 6$ Hz, CH_3).

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