New Three- and Tetraoxygenated Coumarin Glucosides from the Mosses *Atrichum undulatum* and *Polytrichum formosum**

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Dedicated to Professor Robert Hegnauer, Leiden, on the occasion of his 75th birthday

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Atrichum undulatum as well as *Polytrichum formosum* are containing each six different coumarin glycosides of which three are common to both species. From the nine different glycosides eight are new natural compounds. The structures have been elucidated spectroscopically.

Introduction

In the course of our studies on phenolic constituents of mosses it was observed that species of the Polytrichaceae contained phenolic substances whose chromatographic properties (R_f values and spot appearance) differed markedly from other substances that have so far been detected in bryophytes. In the present communication the isolation and structures of these compounds from two representative species – *Atrichum undulatum* and *Polytrichum formosum* – are reported (Jung, 1993).

Results and Discussion

Atrichum undulatum (Fig. 1)

From the 80% methanol extract of *A. undulatum* six different pure compounds (**1, 2, 3, 4, 5** and **6**) have been isolated by a combination of various methods (see Experimental). Their structures were deduced as follows:

The ^{13}C NMR spectrum of the main compound (**1**) (see Table I) shows 15 signals. 6 of which are suggesting that **1** is a β-glucopyranoside (Markham *et al.*, 1982); the remaining 9 signals (7 quarternary and 2 tertiary) must be assigned to the aglycone. The ^{1}H NMR spectrum of **1** (Table II) shows in accordance with the ^{13}C data besides of the typical β-glucopyranoside signals (Markham and Geiger, 1993) only two olefinic protons as two 1,2-cis-coupled doublets (J = 9.6 Hz). The FAB mass spectrum (negative mode) gives rise to anion signals at m/e = 371 and 209. The difference of 162 amu between these two values points to the elimination of a glucose moiety. Thus M_R of the glucoside **1** is 372 and M_R of the corresponding aglycone must be 210. Together with the NMR

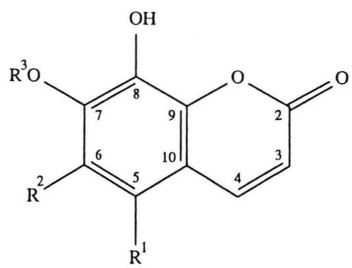

Fig. 1. Structure of the coumarin glycosides from *A. undulatum* (A) and *P. formosum* (P).

	R^1	R^2	R^3	Source
1	O-β-1-glc	OH	H	A + P
2	O-β-1-glc-6-acetylester	OH	H	A
3	O-β-1-glc-6-malonylester	OH	H	A
4	O-β-1-glc-6-malonylester	OH	CH_3	A
5	O-β-1-glc	H	H	A + P
6	O-β-1-glc	H	CH_3	A + P
7	O-β-1-glc-6 ← 1-β-glc	H	H	P
8	O-β-1-glc-6 ← 1-β-glc	H	CH_3	P
9	H	H	β-1-glc	P

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Table I. ^{13}C NMR data (in ppm) of the coumarin glucosides **1–9** from *Atrichum undulatum* and *Polytrichum formosum* (DMSO-d_6, 100 MHz, ambient temperature).

C	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Aglycone									
2	160.3	160.2	160.2	159.9	160.4	160.1	160.2	160.1	159.9
3	111.3 *tert*	111.1 *tert*	111.3 *tert*	114.0 *tert*	109.6 *tert*	110.9 *tert*	109.6 *tert*	110.9 *tert*	113.3* *tert*
4	140.6 *tert*	140.3 *tert*	140.3 *tert*	140.1 *tert*	140.0 *tert*	139.6 *tert*	139.8 *tert*	139.7 *tert*	144.5 *tert*
5	133.3	133.1	133.1	132.9*	147.0	146.6	146.7	146.6	118.2 *tert*
6	135.7*	135.6*	135.7*	135.6*	100.4 *tert*	97.3 *tert*	100.3 *tert*	97.2 *tert*	112.2* *tert*
7	139.5*	139.5*	139.5*	139.9*	150.4	151.3	150.1	151.4	148.1
8	130.3*	130.3*	130.4*	132.9*	127.3	128.4	127.2	128.5	133.9
9	136.7	136.6	136.6	135.9*	143.9	142.7	143.7	142.8	142.6
10	105.7	105.7	105.7	109.8	103.5	104.3	103.3	104.3	114.4
OMe	–	–	–	60.4 *pr*	–	56.1 *pr*	–	56.6 *pr*	–
Glucose									
1′	106.6 *tert*	106.2 *tert*	106.2 *tert*	106.2 *tert*	102.3 *tert*	102.3 *tert*	102.1 *tert*	102.1 *tert*	101.8 *tert*
2′	73.8 *tert*	73.7 *tert*	73.6 *tert*	73.7 *tert*	73.2 *tert*	73.2 *tert*	73.6 *tert*	73.5 *tert*	73.2 *tert*
3′	76.0 *tert*	75.6 *tert*	75.6 *tert*	75.6 *tert*	76.3 *tert*	76.4 *tert*	76.4 *tert*	76.8 *tert*	75.7 *tert*
4′	69.6 *tert*	69.9 *tert*	69.7 *tert*	69.7 *tert*	69.6 *tert*	70.1 *tert*	70.1 *tert*	70.0 *tert*	69.7 *tert*
5′	77.3 *tert*	74.1 *tert*	74.0 *tert*	74.0 *tert*	77.1 *tert*	77.3 *tert*	75.8 *tert*	75.7 *tert*	77.2 *tert*
6′	60.7 *sec*	63.4 *sec*	64.0 *sec*	64.1 *sec*	60.7 *sec*	60.9 *sec*	68.2 *sec*	69.2 *sec*	60.6 *sec*
1″	–	–	–	–	–	–	103.2 *tert*	103.7 *tert*	–
2″	–	–	–	–	–	–	73.1 *tert*	73.2 *tert*	–
3″	–	–	–	–	–	–	76.8 *tert*	76.8 *tert*	–
4″	–	–	–	–	–	–	69.5 *tert*	70.0 *tert*	–
5″	–	–	–	–	–	–	76.0 *tert*	76.1 *tert*	–
6″	–	–	–	–	–	–	60.7 *sec*	61.0 *sec*	–
Acyl									
COOR	–	170.1	166.9	166.8	–	–	–	–	–
CH_3	–	20.6 *pr*	–	–	–	–	–	–	–
CH_2	–	–	41.6 *sec*	41.4 *sec*	–	–	–	–	–
COOH	–	–	167.9	167.8	–	–	–	–	–

Aglycone signals that are marked by an asterisk (*) are assigned just by "best fit" and might be exchangeable. Sugar signals are assigned according to the literature (Markham and Geiger, 1993; Veit *et al.*, 1990; Aritomi *et al.*, 1986). *pr, sec, tert* = primary, secondary and tertiary carbon atoms as determined by the DEPT technique.

data this leads to the molecular formulae $C_{15}H_{16}O_{11}$ for the glucoside **1** and $C_9H_6O_6$ for its aglycone. Considering also the NMR data 5,6,7,8-tetrahydroxycoumarin is the only plausible structure for the aglycone. The site of glucosidation at OH-5 was proved by two independent methods: a NOE experiment and a long-range C–H correlation. With the NOE experiment irradiation of the anomeric proton of the glucose enhanced the H-4 signal, thus indicating the spatial vicinity of these two protons. The observed long-range C–H couplings are shown in Fig. 2, they are not only revealing the site of glucosidation but allow also, with the exception of C-6, C-7 and C-8, the unambiguous assignment of all other carbons of the aglycone. Thus **1** is 5,6,7,8-tetrahydroxycoumarin-5-β-glucopyranoside.

Compound **2** is the 6′-acetyl ester of **1.** This is evident from its ^{13}C NMR spectrum, which contains additional signals at 20.6 and 170.1 ppm. These are typical of an O-acetyl group (Markham *et al.*, 1982).

The site of acetylation is evident from the fact, that apart from the acetyl signals the ^{13}C NMR spectra of **1** and **2** differ only in the chemical shift of C-5′ and C-6′. They have in the spectrum of **2** the typical values of 6-O-acylglucosides (Markham *et al.*, 1982). Therefore **2** is 5,6,7,8-tetrahydroxy-

Table II. ^{1}H NMR data (in ppm) of the coumarin glucosides **1–9** from *Atrichum undulatum* and *Polytrichum formosum* (DMSO-d_6, 400 MHz, ambient temperature, values in parentheses: coupling constants in Hz).

H	1	2	3*	4*	5	6	7	8	9
Aglycone									
3	6.15 d (9.6)	6.15 d (9.6)	6.17 d (9.6)	6.31 d (9.7)	6.11 d (9.7)	6.20 d (9.7)	6.13 d (9.7)	6.19 d (9.7)	6.30 d (9.4)
4	8.26 d (9.6)	8.10 d (9.6)	8.10 d (9.6)	8.13 d (9.7)	8.17 d (9.7)	8.22 d (9.7)	8.17 d (9.7)	8.21 d (9.7)	7.94 d (9.6)
5	–	–	–	–	–	–	–	–	7.11 d (8.7)
6	–	–	–	–	6.63 s	6.90 s	6.74 s	6.84 s	7.14 d (8.7)
7-OMe	–	–	–	3.81 s	–	3.85 s	–	3.87 s	–
Glucose**									
1′	4.49 d (7.8)	4.53 d (7.8)	4.54 d (7.8)	4.55 d (7.8)	4.71 d (6.6)	4.80 d (7.4)	4.70 d (7.2)	4.81 d (7.2)	4.85 d (7.4)
6′a	3.66 d (11.7)	4.26 d (11.7)	4.36 d (10.6)	4.38 d (10.5)	3.70 d (11.6)	–	–	–	–
6′b	3.49 dd (11.8/4.4)	4.11 dd (11.8/7.3)	4.13 dd (11.8/7.1)	4.13 dd (11.9/7.0)	3.51 dd (11.8/5.0)	–	–	–	–
1″	–	–	–	–	–	–	4.23 d (7.7)	4.17 d (7.8)	–
Acetyl									
CH_3	–	1.98 s	–	–	–	–	–	–	–

* The methylene signals of the malonic acid are obscured by the bulk of the glucose signals.
** Only the unambiguously assignable signals are presented.

coumarin-5-β-(6-O-acetylglucopyranoside). The ^{1}H NMR (Table II) and the FAB-MS data (M_R = 414) are also in accordance with this structure.

The ^{13}C NMR spectrum of **3** (Table I) differs from that of **2** only in the acyl part; instead of the acetyl signals there are signals at 41.6, 166.9 and 167.9 ppm typical for malonic acid monoesters (Veit *et al.*, 1990). Thus **3** is 5,6,7,8-tetrahydroxycoumarin-5-β-(6-O-malonylglucopyranoside). This is confirmed by the FAB mass spectrum (negative mode), which exhibits a *quasi*-molecular ion at 457 *m/e* and fragment ions at 413, 371 and 209 *m/e* corresponding to the loss of carbon dioxide, malonic acid and malonyl glucose respectively.

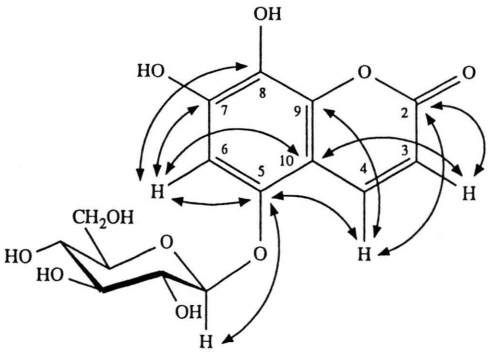

Fig. 2. Long-range ^{13}C–^{1}H couplings of **1.**

The FAB mass spectrum of compound **4** shows the [M−1] ion at 471 and fragments at 427, 385 and 223 *m/e, i.e.* compared with the spectrum of **3** all ions occur at 14 amu higher masses. This suggests that **4** is a malonylglucoside of a tetrahydroxycoumarin methylether. This is confirmed by the ^{13}C NMR spectrum (Table I) that shows 6-malonyl-β-glucopyranoside signals almost identical with those of **3** and a methoxyl signal at 60.4 ppm, which is typical of an aromatic methoxyl carbon situated between two oxygenated carbon atoms (Markham *et al.*, 1982). The ^{1}H NMR spectrum of **4** (Table II) shows the two olefinic protons (H-3 and H-4) as two doublets at 6.31 and 8.13 (J = 9.7 Hz) indicating an unsubstituted lactone ring, the methoxyl protons as a three proton singlet at 3.81 ppm and the 6-malonyl-β-glucopyranoside protons at the same positions as in the spectrum of **3.** The site of glucosidation was proved by a NOE experiment; irradiation of the anomeric proton enhances the H-4 signal, therefore these two protons must be in spatial vicinity. Thus **4** is like **3** a 5-glycoside. The UV spectra of **4** (Table III) are the same with and without added $AlCl_3$, this excludes an *ortho* position of the two free phenolic hydroxyl groups (Mabry *et al.*, 1970). Thus **4** must be 7-methoxy-5,6,8-trihydroxycoumarin-5-β-(6-O-malonylglucopyranoside).

Table III. UV and chromatographic data of the glycosides **1–9.**

	UV absorption maxima [nm]		TLC on cellulose sheets Spot appearance under UV		hR_f values		HPLC
	MeOH	MeOH + $AlCl_3$	untreated	NA	BAW	15% HOAc	t_R [min]
1	257 sh, 327	270 sh, 366	pg	t	31	55	10.9
2	258, 335	265 sh, 369	pg	t	51	58	28.4
3	256 sh, 334	266 sh, 369	pg	t	41	61	26.0
4	258, 311	258, 312	pg	pg	59	73	31.0
5	260, 326	281, 340, 380	pg	oy	37	58	8.0
6	265, 319	264, 319	pg	pg	40	67	14.6
7	268, 325	280, 336, 375	pg	oy	19	67	7.0
8	265, 320	265, 316	pg	pg	21	75	11.9
9	257, 310	255, 304	d	d	48	74	16.8

NA, sprayed with 0.1% diphenylboric acid-β-aminoethylester in MeOH; pg, pale-green; t, turquoise-green; oy, ochraceous yellow; d, dark (no fluorescence); BAW, *sec*-butanol/HOAc/H_2O (70:5:25); HPLC, column: 250×4 mm Nucleosil 100, C_{18}, 5 µm. Solvents: A, 1% aqu. H_3PO_4; B, CH_3CN. Elution profile: 0–8 min 8% B in A (isocratic), 8–35 min 8–25% B in A (linear gradient), 35–45 min 25% B in A (isocratic). Flow rate: 1 ml/min. Detection at 325 nm. Ambient temperature (*ca.* 20 °C).

The FAB mass spectrum of compound **5** ([M−1] at 355 *m/e* and an aglycone fragment at 193 *m/e*) gave a hint that this compound may be a trihydroxycoumarin hexoside. This assumption is corroborated by its ^{13}C NMR spectrum (Table I) which exhibits besides of six signals assignable to a β-glucopyranoside moiety nine aglycone signals of which, as expected for a trihydroxycoumarin three are tertiary and six quarternary. The ^1H NMR spectrum of **5** (Table II) shows in the aglycone range in addition to a singlet at 6.63 ppm the two doublets (J = 9.7 Hz) of H-3 and H-4 at 6.11 and 8.17 ppm respectively. Thus the lactone ring is unsubstituted. In the sugar region the resonances of H-1, H-6a and H-6b of a β-glucopyranoside moiety are readily identified. The exact substitution pattern of the carbocycle as well as the unambiguous assignment of all ^{13}C resonances can be deduced from the long-range $^{13}C-^1H$ correlation spectrum. The ^2J and ^3J $^{13}C-^1H$ couplings exhibited by this spectrum are shown in Fig. 3. The only structure that is compatible with all the spectroscopic data is 5,7,8-trihydroxycoumarin-5-β-glucopyranoside (**5**). The UV spectra of **5** (Table III) show in accordance with its structure a strong $AlCl_3$ shift indicating the *ortho*-dihydroxy grouping at the positions 7 and 8. Problems that were experienced with NOE experiments on **5** will be discussed later in connection with compound **8.**

The ^{13}C NMR spectrum of **6** (Table I) is in the aromatic part very close to that of **5** and the glucose signals are almost the same, but it shows in addition an aromatic methoxyl at 56.1 ppm whose chemical shift indicates that it must be *ortho*-situated to an unsubstituted carbon (Markham *et al.*, 1982). The ^1H NMR spectrum is also similar to that of **5** except for an additional three-proton signal at 3.85 that is attributable to an aromatic methoxy group (Markham and Geiger, 1993). The relative molecular mass of **6** (370 amu, as determined by FAB-MS) also indicates that **6** is a methyl ether of **5.** As with **5** the structure can be deduced from a long-range $^{13}C-^1H$ correlation spectrum. This spectrum exhibits the same ^2J and ^3J C–H couplings as shown on Fig. 2 for compound **5** and in addition a ^3J coupling between the methoxyl protons and C-7. Thus the structure of **6** is 5,8-dihydroxy-7-methoxycoumarin-5-β-glucopyranoside.

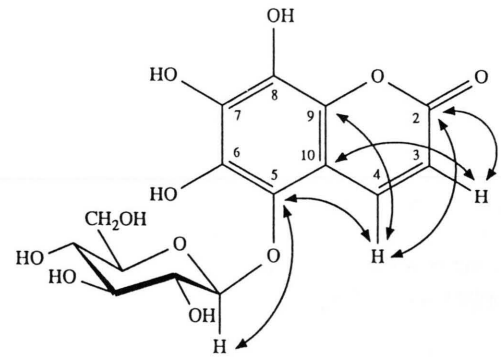

Fig. 3. Long-range $^{13}C-^1H$ couplings of **3.**

Polytrichum formosum (Fig. 1)

It yielded also six different coumarin glycosides. Three of them were identical (^{1}H and ^{13}C NMR, UV and FAB-MS) with the compounds **1, 5** and **6,** which are also occurring in *Atrichum undulatum.*

The ^{13}C NMR spectrum (Table I) of the first "unknown" from *P. formosum* – compound **7** – is in its aromatic region almost superimposable with that of compound **5.** The sugar region however does not show 6 but 12 carbon signals indicating that **7** is a dihexoside. A careful analysis of these signals and comparison with published data (Markham *et al.,* 1982) revealed, that **7** is 5,7,8-trihydroxycoumarin-5-β-gentiobioside. The FAB mass spectrum ([M−1] = 517 *m/e*) and the ^{1}H NMR data (Table II) are also in accordance with this structure of **7.**

Compound **8** yields ^{1}H and ^{13}C NMR spectra which are – within the limits of experimental error – in their aglycone parts identical with those of **6,** in their sugar part, however with those of **7.** The FAB mass spectrum of **8** shows a $[M-1]^-$ ion at 531 *m/e.* All together the spectroscopic evidence proves that **8** is 5,8-dihydroxy-7-methoxycoumarin-5-β-gentiobioside.

In the case of **8** it was also possible to demonstrate the arrangement of the substituents at the coumarin skeleton by a series of NOE experiments. In DMSO-d_6 – the best, but rather viscous solvent for these coumarin glycosides – irradiation of the anomeric proton at 4.81 leads only to an enhancement of the H-6, but not of the H-4 signal. This is also the case with compounds **5, 6** and **7.** But contrary to these compounds **8** is also soluble in a less viscous mixture of DMSO-d_6 and MeOH-d_4, and in this solution a NOE effect between the anomeric proton and H-6 as well as H-4 is observed. Thus **8** is a 5-glucoside, and in consequence a NOE interaction between the methoxyl protons and H-6 demonstrates, that the methoxyl group is located at C-7.

The eight glycosides discussed so far are all new natural compounds unique to mosses. Compound **9** from *P. formosum,* however, turned out to be the known daphnin, named after *Daphne alpina,* a seed plant (Karrer, 1958). It was identified by comparison of its ^{13}C NMR spectrum (Table I) with published data (Jewers and Zirivi, 1978), and the analysis of its ^{1}H NMR spectrum by several NOE experiments.

It is noteworthy that flavones, which are widespread constituents of bryophytes (Markham, 1990) could not be detected in *Polytrichum* and *Atrichum.* As a possible role of flavonoids it is discussed that they may act as a screen against UV-B (*l.c.* Markham, 1990, p. 155). Since the coumarins **1–9** are strongly absorbing in the same spectral range as flavones (Table III and Mabry *et al.,* 1970), they may compensate in this respect the lack of flavones in *Polytrichum* and *Atrichum.*

Experimental

Plant material

Atrichum undulatum (Hedw.) P. Beauv. was collected in September 1990 in a forest near Herrstein, Rheinland-Pfalz, Germany.

Polytrichum formosum Hedw. was gathered at Lautzkirchen, Saarland, Germany in November 1992. The material was identified by R. Mues. Voucher specimen (3889 *A. undulatum,* 3890 *P. formosum*) are deposited in the herbarium SAAR.

Extraction and isolation

Air-dried gametophytic material was ground and pre-extracted with CH_2Cl_2 to remove lipophilic constituents. Thereafter it was exhaustively extracted with MeOH/H_2O (4:1). The combined aqueous MeOH extracts were reduced *in vacuo* to an aqueous solution, which was extracted with $CHCl_3$ to remove chlorophyll. Isolation of the individual glycosides was achieved by repeated MPLC on Lichroprep RP-18, 40–63 μm (E. Merck, Darmstadt) with gradients or isocratic mixtures of MeOH or CH_3CN with 2% formic acid in H_2O. The sequence of elution is in both solvent combinations almost the same as with the analytical HPLC system (see Table III). The choice of the system was often dictated by the solubility of the mixture to be separated (especially **1, 5, 6** and **9** are sparingly soluble in most solvent combinations). The final clean-up of almost pure compounds was performed by CC on Sephadex LH-20 with H_2O as eluent, to which if necessary MeOH up to 30% was added.

By these methods 400 g (dry weight) *A. undulatum* yielded 1200 mg **1,** 25 mg **2,** 187 mg **3,** 11 mg **4,** 57 mg **5** and 15 mg **6.** The yields from 750 g (dry weight) *P. formosum* were 35 mg **1,** 26 mg **5,** 58 mg **6,** 7 mg **7,** 44 mg **8** and 47 mg **9.**

FAB mass spectra were run on a Finnigan MAT 90, with 5–8 keV Xe and glycerol as matrix.

NMR and UV spectra see Tables I–III.

Acknowledgements

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Aritomi M., Komori T. and Kawasaki T. (1986), Flavonol glycosides in leaves of *Spinacia oleracea.* Phytochemistry **25,** 231.

Jewers K. and Zirvi K. A. (1978), The coumarin glycosides of *Daphne acuminata:* use of ^{13}C NMR spectroscopy for their identification. Planta Med. **33,** 403.

Jung M. (1993), Dissertation, Saarbrücken.

Karrer W. (1958), Konstitution und Vorkommen der organischen Pflanzenstoffe. Birkhäuser Verlag, Basel, p. 539.

Mabry T. J., Markham K. R. and Thomas M. B. (1970), The Systematic Identification of Flavonoids. Springer Verlag, Berlin, Heidelberg, New York.

Markham K. R. (1990), Bryophyte flavonoids, their structures, distribution and evolutionary significance. Chapter 10. In: Bryophytes, their Chemistry and Chemical Taxonomy (H. D. Zinsmeister and R. Mues, eds.). Proceedings of the Phytochemical Society of Europe **29.** Clarendon Press, Oxford, p. 143.

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

Markham K. R. and Geiger H. (1993), ^{1}H NMR spectroscopy of flavonoids and their glycosides in hexadeuterodimethylsulphoxide. In: The Flavonoids – Advances in Research since 1986 (J. B. Harborne, ed.). Chapman and Hall, London.

Veit M., Geiger H., Czygan F.-C. and Markham K. R. (1990), Malonylated flavone 5-O-glucosides in the barren sprouts of *Equisetum arvense.* Phytochemistry **29,** 2555.