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Chemical Studies on Bryophytes. 17. A New Luteolin Tetraglycoside from *Hedwigia ciliata*

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The moss *H. ciliata* contains twelve flavonoid compounds. One of the flavonoids has been assigned the structure luteolin-7,4′-di-*O*-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) (*1*) by a combination of spectroscopic methods and a sugar sequence analysis by GLC-MS of the methylated alditol acetates.

In this series of chemical studies of liverworts and mosses, the common moss *Hedwigia ciliata* was investigated. This communication describes the isolation and separation of the flavonoids from this moss, and the structural determination of one luteolin tetraglycoside. The structures of the other flavonoids will be reported later.

The ethanolic extract from 4.8 kg of the moss *H. ciliata* after gel filtration, paper and column chromatography, contains twelve flavonoid compounds (Table 1). Four of these flavonoids (*1*, *3*, *10*, and *11*) were obtained in larger quantities than the others. The yields of these main components were each larger than 0.5 g. One of the minor flavonoids (*12*) is probably identical with the biluteolin isolated from *Dicranum scoparium*.[1]

One of the main components (*1*) was purified by gel filtration. By R_F values and UV spectral studies of the products obtained after acid hydrolysis of *1* the aglycone was identified as luteolin and the sugars as glucose and rhamnose. The sugars are linked to luteolin at the 7- and the 4′-position according to UV spectral data of *1* using diagnostic shift reagents.[2]

Partial hydrolysis of *1* gave two intermediate luteolin glycosides, showing that *1* contains at least three monosaccaride molecules. The

Table 1. R_F values of the isolated flavonoids from *H. ciliata* on 0.1 mm pre-coated cellulose TLC plates.

Compound	Solvent		
	TBA	BAW	15 % HOAc
1	0.09	0.15	0.75
2	0.04	0.07	0.66
3	0.04	0.07	0.63
4	0.16	0.28	0.62
5	0.11	0.24	0.52
6	0.10	0.24	0.45
7	0.13	0.20	0.34
8	0.54	0.50	0.32
9	0.30	0.37	0.18
10	0.15	0.22	0.40
11	0.07	0.11	0.25
12	0.92	0.94	0.02

osmometrically determined molecular weight of *1* (mol. wt. 885) indicates that *1* is probably a tetraglycoside of luteolin. Only one luteolin glycoside with more than two monosaccaride molecules has been reported earlier.[3] The latter is a luteolin-triglycoside with sugars attached in the 7- and the 4′-positions.[4]

The ^{1}H NMR spectrum of the TMS ether of *1* (Fig. 1) shows four sugar C-1 proton signals confirming that *1* is a tetraglycoside. The two doublets at 5.14 and 5.45 must be due to two glucosyl C-1 protons, since the coupling constants of about 7 Hz agree with a diaxial coupling between the C-1 and the C-2 protons in a β-linked glucose.[2,5] The two signals at 4.76 and 4.86 correspond to two rhamnosyl C-1 protons.[2] Since the two C-1 proton signals at the lowest field are due to glucose, this

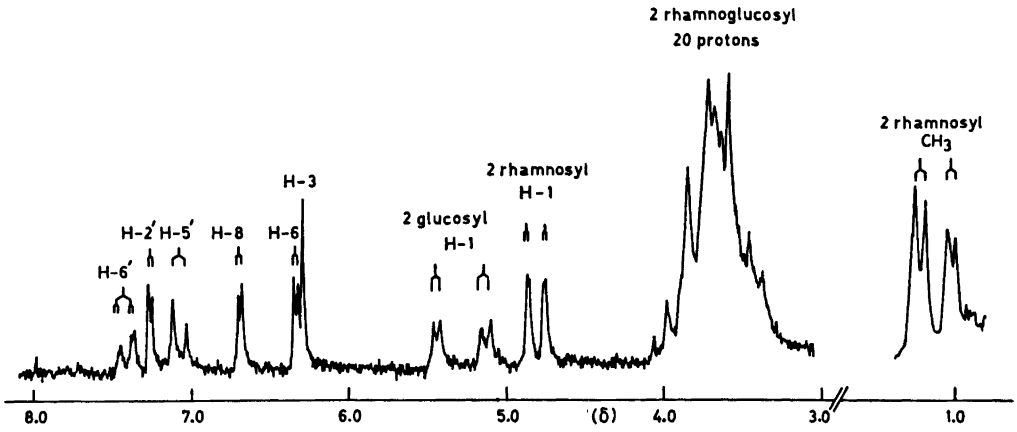


Fig. 1. ^{1}H NMR spectrum of the TMS ether of luteolin-7,4'-di-*O*-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside).

indicates that *1* has two β-glucose molecules attached to luteolin and that the two rhamnose units are linked to the glucose. Treatment of *1* with β-glucosidase (emulsin) gave no hydrolysis, showing that there are no terminal β-glucose units.

Mass spectroscopy of the permethylated *1* gave no molecular peak and only a weak peak for the aglycone (*m/e* 314). A base peak at *m/e* 189 and peaks at *m/e* 157 (189 − CH_3OH) and 125 (157 − CH_3OH) indicated a terminal rhamnose unit. No fragments from a terminal glucose could be detected. The peak at *m/e* 393 indicated a rhamnoglucoside, and the peak at *m/e* 361 that the rhamnose unit is probably linked to the glucose at the 2-position.[6]

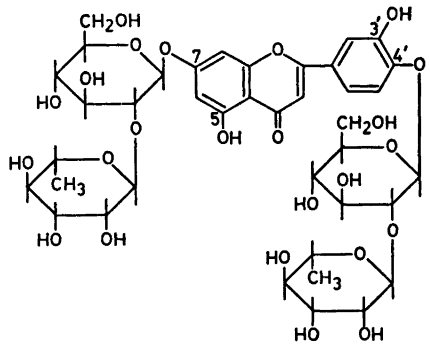
To establish the sugar sequence, a method used for the analysis of polysaccharides was applied.[7–10] This method has also been used for structural determinations of the sugar moieties in flavonoids.[11–13] The luteolin tetraglycoside was permethylated, hydrolyzed and the methylated sugars reduced and acetylated to give partially methylated alditol acetates. Results obtained by GLC-MS analysis of the alditol acetate mixture are given in Table 2. The ratio of the two GLC-peak areas was 1:1 proving that, after methylation and hydrolysis, there are only two different sugar units. The peak with a *T* value of 0.45 corresponds to 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol showing that the rhamnose units are terminal. The peak with a *T* value of 1.97 corresponds to both 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol and 1,3,5-tri-*O*-acetyl-2,4,6-tri-*O*-methyl-D-glucitol, but the MS data eliminate the second alternative.[10] This result shows that the two rhamnose units are linked to the two glucose units at the 2-position.

The aglycone isolated after permethylation

Table 2. GLC-MS analysis data of the methylated alditol acetates. *T* values are relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Alditol acetate	*T*	*T* [10]	Prominent fragments *m/e*
1,5-di-*O*-acetyl-2,3,4-tri-*O*-methyl-L-rhamnitol	0.45	0.46	175, 161, 131, 117, 115, 101, 89, 72, 45, 43
1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methyl-D-glucitol	1.97	1.98	205, 189, 161, 145, 129, 101, 99, 87, 71, 45, 43

and hydrolysis was identified as 5,3′-di-*O*-methylluteolin by means of MS and UV spectral data. Therefore *1* is a luteolin-7,4′-di-*O*-(2-*O*-rhamnopyranosyl-glucopyranoside). The optical rotation of synthetic luteolin-7-*O*-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside is $[\alpha]_D^{22}$ −97.55° (pyridine).[14] To explain the large negative rotation, $[\alpha]_D^{26}$ −192° (pyridine), of the isolated luteolin 7,4′-di-*O*-rhamnoglucoside, *1* must contain two units of β-D-glucose and two units of α-L-rhamnose. Taking these data together, the structure of *1* is proposed to be luteolin-7,4′-di-*O*-(2-*O*-α-L-rhamnopyranosyl-β-D-glucopyranoside) (*1*).



EXPERIMENTAL

NMR spectra were recorded on a Varian A 60-D instrument, UV-visible spectra on a Bausch & Lomb Spectronic 505 spectrophotometer and on a Zeiss spektralphotometer DMR 10. MS were recorded on a LKB 9000 and an AEI MS 30 instrument. GLC were performed on a Perkin Elmer 990 Gas Chromatograph as described earlier.[11] Gel filtrations were performed on Sephadex G-25 columns with EtOH−H_2O (1:1) as eluent. Molecular weight determination was made with a Knauer vapour pressure osmometer. Solvent systems: BuOH−HOAc−H_2O, 6:1:2 (BAW), *t*-BuOH−HOAc−H_2O, 3:1:1 (TBA).

Isolation. The moss *H. ciliata* was collected in the surroundings of Enköping and Uppsala. Foreign materials were carefully removed and the air-dried moss (4.8 kg) was pulverized and first extracted with $CHCl_3$, air-dried and then extracted with 80 % aqueous EtOH at room temperature. The ethanolic extract was evaporated in vacuum at room temperature and the residue suspended in H_2O and continually extracted with ether. The water phase was concentrated to a small volume and, after gel filtration, gave four flavonoid fractions. The first fraction consisted of nine flavone glycosides (*1*−*9*), the second and third fractions gave two flavone C-glycosides (*10* and *11*) and the fourth fraction gave one biflavone (*12*). PC on Whatman 3 MM paper with BAW and CC on cellulose with 5 % HOAc of the first fraction led to complete separation of the nine flavone glycosides. R_F values, see Table 1.

*Luteolin-7,4′-di-*O*-rhamnoglucoside* (*1*). The crude fraction *1* was further purified by gel filtration. Drying at 100 °C/0.1 mmHg over P_2O_5 gave 0.5 g of a pale yellow compound, m.p. > 360 °C, $[\alpha]_D^{26}$ −192° (*c* 0.27, pyridine). Found: C 48.24; H 5.95. Calc. for $C_{39}H_{58}O_{28}$: C 48.05; H 6.00. Mol. weight, obs. 885, calc. for $C_{39}H_{50}O_{24}$: 902.8. UV (99.9 % CH_3OH): 272, 340; (+$AlCl_3$): 279, 295sh, 354, 380sh; (+$AlCl_3$/HCl): 281, 293sh, 352, 379sh; (+MeONa): 270, 371; (+NaOAc): 271, 338; (+NaOAc/H_3BO_3): 271, 342 nm.

Acid hydrolysis of *1* with 6 % HCl at 100 °C gave luteolin, glucose and rhamnose. Luteolin was identified by MS data, R_F values and UV data. Glucose and rhamnose were identified by PC and TLC by comparison with authentic samples.

Partial hydrolysis of *1* with 10 % HCl at room temperature for 140 h gave two intermediates; *1a* and *1b*. *1a* gave on complete hydrolysis luteolin, glucose and rhamnose, partial hydrolysis with 10 % HCl at room temperature gave *1b*. *1b* gave on complete hydrolysis luteolin, rhamnose and glucose. *1a*: R_F values 0.48 (15 % HOAc) and 0.15 (TBA). UV (99.9 % CH_3OH): 269, 336; (+$AlCl_3$): 279, 294sh, 351, 392sh; (+$AlCl_3$/HCl): 279, 294sh, 349, 392sh; (+MeONa): 276, 380; (+NaOAc): 269, 313sh, 330; (+NaOAc/H_3BO_3): 271, 340 nm. This indicated a luteolin-7,4′-tri-*O*-glycoside. *1b*: R_F values 0.21 (15 % HOAc) and 0.38 (TBA). UV (99.9 % CH_3OH): 266, 348; (+$AlCl_3$): 277, 292sh, 430; (+$AlCl_3$/HCl): 277, 292sh, 358, 381sh; (+MeONa): 276, 409; (+NaOAc): 260, 407; (+NaOAc/H_3BO_3): 260, 370 nm. This indicated a luteolin-7-*O*-glycoside.

The acetate was prepared with Ac_2O in pyridine. M.P. 144−146 °C, Found: C 52.13; H 5.53. Calc. for $C_{67}H_{84}O_{41}$: C 52.07; H 5.48. ^1H NMR (100 MHz, $CDCl_3$): δ 7.73 (H6′), 7.64 (H2′), 7.31 (H5′), 7.03 (H8), 6.72 (H6), 6.58 (H3), 5.4−4.9 (sugar H), 4.3−3.7 (sugar H), 2.41 (aromatic acetyl H), 2.25−1.85 (sugar acetyl H), 1.22 and 1.12 (rhamnose CH_3).

The TMS ether was prepared according to standard procedures.[2] ^1H NMR (100 MHz, CCl_4): δ 7.42 (H6′), 7.26 (H2′), 7.08 (H5′), 6.70 (H8), 6.35 (H6), 6.30 (H3), 5.45 (glucose H1, *J* 6 Hz), 5.14 (glucose H1, *J* 7 Hz), 4.86 (rhamnose H1, *J* 2 Hz), 4.76 (rhamnose H1, *J* 2 Hz), 4.10−3.30 (sugar H), 1.22 and 1.02 (rhamnose CH_3).

The permethyl ether was prepared with NaH, DMSO and CH_3I using Hakomori's procedure.[15] The permethyl ether was purified by CC on silica gel with acetone as eluent. ^1H NMR

(100 MHz, $CDCl_3$): δ 7.44 (H6'), 7.32 (H2'), 7.10 (H5'), 6.70 (H8), 6.60 (H3), 6.53 (H6), 5.42 (glucose H1), 5.33 (glucose H1), 5.00 (rhamnose H1), 4.93 (rhamnose H1), 4.0 – 3.0 (sugar H and OCH_3), 1.33 and 1.25 (rhamnose CH_3). MS [70 eV; *m/e* (% rel. int.)]: 394 (0.5), 393 (1), 391 (0.6), 390 (0.4), 379 (0.4), 363 (0.3), 362 (1), 361 (5), 360 (4), 347 (0.7), 346 (0.4), 332 (0.4), 331 (0.3), 329 (0.3), 328 (0.4), 314 (0.3), 204 (13), 190 (11), 189 (100), 188 (23), 187 (7), 175 (8), 173 (5), 157 (33), 145 (13), 143 (5), 131 (6), 129 (10), 125 (8), 113 (7), 101 (35), 99 (33), 89 (10), 88 (22), 75 (18), 71 (10), 59 (20). Only peaks larger than 5 % (0.3 % *m/e* 300 – 500) of the base peak are given.

Sequence analysis of sugar. The permethylated glycoside was hydrolyzed with 8 % H_2SO_4 at 100 °C for 3 h. 5,3'-Di-*O*-methylluteolin deposited on cooling. The acid solution was neutralized with the anion exchanger IRA-400 in carbonate form and concentrated to 4 ml in vacuum at room temperature. Reduction with $NaBH_4$ and acetylation with Ac_2O in pyridine was performed as described earlier.[7] Retention times and prominent fragments in GLC-MS analysis are given in Table 2.

5,3'-Di-O-methylluteolin. M.p. 273 – 276 °C. UV (99.9 % CH_3OH): 241, 265, 337; (+$AlCl_3$): 241, 265, 337, 412; (+$AlCl_3$/HCl): 241, 265, 301, 340, 414; (+MeONa): 261, 270sh, 317sh, 392; (+NaOAc): 263sh, 270, 313, 384; (+NaOAc/H_3BO_3): 264, 337 nm. MS [70 eV; *m/e*; (% rel. int.)]: 315 (21), 314 (100), 313 (50), 300 (16), 298 (10), 297 (14), 286 (6), 285 (31), 284 (26), 283 (14), 270 (11), 269 (9), 268 (36), 256 (6), 255 (6), 253 (6), 242 (5), 241 (6), 238 (5), 213 (6), 167 (4), 151 (4), 149 (4), 148 (19), 143 (9), 138 (4), 137 (21), 136 (5), 133 (14), 128 (6), 123 (4), 118 (4), 108 (5), 105 (10). Only peaks larger than 4 % of the base peak are given.

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