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THE FIRST IDENTIFICATION OF ISOFLAVONES FROM A BRYOPHYTE

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Abstract—*Bryum capillare* is shown to accumulate the isoflavones orobol and pratensein as the 7-*O*-glucosides and predominantly, as the 7-(6″-malonylglucosides). This is the first finding of isoflavonoids in bryophytes. The phylogenetic relevance of this observation is briefly discussed.

INTRODUCTION

During the 1970s it was shown that flavonoids are more common in bryophytes than had earlier been assumed [1–3]. The most widespread flavonoids in mosses and liverworts are flavones [1, 2] and these occur mainly as *O*- and *C*-glycosides. Other flavonoid types have also been found, but often only in a few species [1]. In a recent review, Ingham [4] reported that over 90% of isoflavonoids are known to be produced by species belonging to the subfamily Papilionoideae of the Leguminosae. Isoflavonoids are also found in at least thirteen other angiosperm and two gymnosperm families [4], but have yet to be reported from a nonvascular plant. We now describe the first isolation and structure elucidation of isoflavones from a bryophyte, namely the moss *Bryum capillare*.

RESULTS AND DISCUSSION

Along with other flavonoids (details of which will be published later), two isoflavone glycosides and their aglycones were isolated from aqueous methanolic extracts of air dried *Bryum capillare*. Their structures were determined as 5,7,3′,4′-tetrahydroxyisoflavone (orobol) and 5,7,3′-tetrahydroxyisoflavone 4′-methyl ether (pratensein) and their respective 7-*O*-glucosides. These were detected in fresh and air dried plant material after extraction with 80% MeOH at room temperature and subsequent 2D-TLC (see Experimental). Extraction with iced acetone of fresh plant material followed by HPLC analysis (see Experimental) did not however reveal any free isoflavone aglycone. Thus the question whether pratensein and orobol occur as free aglycones in *Bryum* cannot be finally answered, though one can speculate that the aglycones arose by chemical and enzymic hydrolysis during extraction [6]. On the other hand the simultaneous occurrence of aglycones and *O*-glycosides in different plants has been reported by other authors [5, 6].

The absorption spectra (Table 1) and mass spectra of the aglycones are typical for 5,7-dihydroxyisoflavones [7]. The molecular ions also indicate that one aglycone is the monomethyl ether of the other; the methyl group is clearly sited on a B-ring hydroxyl as adjudged from the B_1 fragment ions at *m/z* 134 and 148. The isoflavone formulations are also supported by the ^{13}C NMR spectra of the two glycosides both of which show the typical C-2 resonance at δ154.4 together with patterns of signals consistent with 5,7,3′,4′-oxygenated isoflavone 7-*O*-glycosides [8]. Resonance signals in the sugar carbon region define the sugar as β-glucopyranose, and the rapid and complete hydrolysis of both glucosides with β-glucosidase establishes the glycosidic linkage as β. ^1H NMR spectra of both glycosides also confirm the results obtained from ^{13}C NMR spectra. The signal for H-2 at about δ8.4 is typical only of isoflavones [9] and the chemical shifts of H-6 and H-8 confirm 7-*O*-glycosylation. The anomeric glucose proton appears as a doublet with $J = 7$ Hz which proves the β-linkage of the sugar [9]. Consequently, the two glycosides are assigned the structures, orobol 7-*O*-β-D-glucopyranoside and pratensein 7-*O*-β-D-glucopyranoside. In confirmation, acid or enzymic hydrolysis of these glycosides gave the aglycones which were proven identical with authentic samples by TLC, HPLC and MS.

HPLC analysis of both glycosides revealed additional peaks (3–5% in relation to the main peaks) representing the 6″-malonyl esters. The structures of these esters were confirmed by HPLC comparison and cochromatography with enzymically synthesized 6″-malonyl esters of orobol and pratensein 7-*O*-glucosides. These esters were obtained with purified and isoflavone 7-*O*-glucoside-specific malonyltransferases from *Cicer arietinum* roots [10]. The amount of malonyl isoflavone glucosides in fresh plant material (harvested after a long desiccation period) was determined by extraction with iced acetone and subsequent analysis by HPLC. Using this extraction method the malonyl esters were shown to represent about 83% and 87% respectively of the total orobol 7-*O*-glucoside and pratensein 7-*O*-glucoside pools. Similar results were obtained for formononetin 7-*O*-(6″-malonylglucoside)

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Table 1. Chromatographic (hR_f values) and UV data of isoflavonoids from *Bryum capillare*

Solvents	Orobol 7-*O*-glucoside	Pratensein 7-*O*-glucoside	Orobol	Pratensein
15% HOAc	47	53	—	—
40% HOAc	71	80	58	63
TBA	46	58	75	93
BAW	60	68	—	—
PAW	55	64	—	—
BMM	—	—	41	70
BEM	—	—	30	63
80% MeOH	49	64	—	—
WEMA	40	51	—	—
CAF	—	—	30	49
Colour reactions				
UV (254, 350 nm)	P	P	P	P
UV/NH_3	P	P	P	P
UV/NA	P	ol	P	ol
UV data				
MeOH	261 290 sh 340 sh	261 292 sh 338 sh	261 290 sh 341 sh	261 288 sh 334 sh
NaOMe	267 344 (dec.)	261 306 sh 343 sh	270 339 (dec.)	270 319 sh
$AlCl_3$	269 298 sh 362	271 369	268 298 sh 364	272 313 sh 369
$AlCl_3$–HCl	271 363	271 369	272 365	272 314 sh 369
NaOAc	259 292 sh 332 sh	261 291 sh 336 sh	271 321 sh	271 322
NaOAc–H_3BO_3	259 267 sh 292 sh	261 291 sh 334 sh	266 292 sh	263 290 sh 338 sh

P = purple, ol = olive; for other abbreviations see Experimental.

and biochanin A 7-*O*-(6″-malonylglucoside) from *Cicer arietinum* [6].

The finding of isoflavones in a bryophyte is remarkable both in terms of the range of flavonoids previously found in bryophytes [1, 2] and in terms of the presently known distribution of isoflavones in the plant kingdom [4]. Of the major flavonoid groups, only the proanthocyanidins and the 3-hydroxylated anthocyanins remain to be found in bryophytes. The presence of isoflavones and even more the occurrence of malonyl esters of their glycosides adds further chemical support to the proposal [1] that bryophytes are not primitive but share a strong affinity with the vascular plants (cormophytes) and have developed parallel to them in a biochemical sense. It would appear therefore that the ability to develop enzyme systems for the biosynthesis of a range of secondary compounds similar to those of the vascular plants [3], was present before the bryophytes branched off from the main evolutionary line to higher plants. The moss *Bryum capillare* Hedw. is a cosmopolitan taxon belonging to the section *Capillaria* of the genus *Bryum* [11, 12]. Of the 14 samples investigated of this species, 12 showed essentially the same flavonoid pattern. Two samples from Switzerland and Luxemburg respectively contained only orobol glycosides in their isoflavone complement.

EXPERIMENTAL

Plant material. All specimens of Bryum capillare were identified by Prof. J.-P. Frahm, Duisburg. Samples were collected from 14 different sites (11 West Germany, 1 Switzerland, 1 Luxemburg, 1 Japan) for screening of flavonoid patterns. For isolation and structure determination plants were collected from three different sites:

1. Wall near Winkel, Rüdesheim/Rhein, W. Germany (14.4 g)
2. Wall at Grimburger Hof, Wadrill/Saarland, W. Germany (14.3 g)
3. Soil in a forest between Malsch and Freiolsheim, Baden-Württemberg, W. Germany (1.5 g).

As the 2D-TLC's of these samples proved to be identical in their flavonoid patterns they were extracted together. Voucher specimens are deposited in the Herbarium of the Fachrichtung Botanik, Universität des Saarlandes, Saarbrücken.

Extraction and isolation. Plants were carefully cleaned with forceps and separated from other plant material. Fresh gametophytic plant material was extracted according to ref. [6] (HPLC) and 80% MeOH (TLC). Air-dried gametophytes (30.2 g) were ground and extracted several times, first with $CHCl_3$ and later with 80% MeOH; this extract was evaporated to the water phase and extracted first with $CHCl_3$ to separate the rest of the chlorophyll, and later with EtOAc. Free aglycones were isolated from the EtOAc fraction by repeated 1D-PC on Whatman 3 MM paper in *n*-BuOH–HOAc–H_2O (4:1:5, upper layer, BAW); 40% HOAc, the bands being eluted with MeOH. Glycosides were isolated from the water fraction by repeated 1D-PC on Whatman 3 MM paper with *n*-pentanol–HOAc–H_2O (2:1:1, PAW), the bands being eluted with 80% MeOH. Compounds were further purified by CC through a Sephadex LH-20 column with MeOH as solvent. Finally the glycosides were crystallized from aq. MeOH. Orobol 7-glucoside: 90 mg, mp 275–276°. Pratensein 7-glucoside: 70 mg, mp 145–147°.

Hydrolysis. (1) β-Glucosidase (Koch-Light): the sample in H_2O was left at room temp with the enzyme for 30 min. Under these conditions the 7-*O*-glucosides were completely hydrolysed (cf. ref. [7]). (2) TFA: the sample was heated with 1 N TFA under reflux for 2 hr. Aglycones were identified from the ether-soluble fraction, and sugars from the water.

Chromatography. TLC, solvent systems: (a) aglycones: TBA, 40% HOAc (cellulose); C_6H_6–EtOAc–MeOH (60:26:34, BEM),

C_6H_6–MeCOEt–MeOH (4:3:3, BMM) (polyamide); $CHCl_3$–Me_2CO–HCO_2H (9:2:1, CAF) (silica gel). (b) Glycosides: *n*-pentanol–HOAc–H_2O (2:1:1, PAW), TBA, BAW, 15% and 40% HOAc (cellulose); 80% MeOH, H_2O–MeCOEt–MeOH–3,5-pentanedione (13:3:3:1, WEMA) (polyamide). TLC on precoated sheets: cellulose F 1440 (Schleicher and Schüll), polyamide-6, Polygram (Macherey and Nagel), silica gel 60 (Merck). (c) Sugars: EtOAc–C_5H_5N–HOAc–H_2O (36:36:7:21, cochromatography with standards on cellulose). Spray reagents: Naturstoffreagenz A (NA) [13] 0.1% in MeOH (isoflavones), anilinphthalate, Merck (sugars). HPLC: Kontron chromatograph; Lichrosorb RP 18 column, 5 μ, 250 × 4 mm; flow of 0.8 ml/min; gradients: 20–60% acetonitrile in 3% HOAc, 35 min (orobol and pratensein), 10–60% acetonitrile in 3% HOAc, 45 min (orobol and pratensein 7-*O*-glucosides and their malonylesters).

Spectroscopic methods. UV-spectra: according to ref. [9]. Mass spectra: EI, 70 eV, ion source 150°, probe-temperature 160–230°. The terminology for the fragmentation pattern of the *O*-glycosides is used according to ref. [14]. ^{1}H NMR spectra: measured in DMSO-d_6 at 30°. ^{13}C NMR spectra: measured on a Varian FT-80 A; solvent, DMSO-d_6; temp. 30°C; acquisition time 0.8 sec.

Orobol 7-O-glucoside. R_t (HPLC): 12, 78 min.; 16, 77 min.; (malonylester) MS* (pertrimethylsilylated derivative): $[M]^+$ 952 (3), $[M-15]^+$ 937 (2), $[M-72]^+$ 880 (7), $[A+H+72]^+$ 574 (1), $[A+H+72-15]^+$ 559 (3), $[A+H]^+$ 502 (9), $[A+H-15]^+$ 487 (12), $[A+H-72]^+$ 430 (5), $[A+H-88]^+$ 414 (3), $[T-H]$ 450 (5), $[T-H-89]$ 361 (67), $[T-H-179]$ 271 (10). ^{1}H NMR [δ-values (ppm) downfield from TMS in DMSO-d_6]: 10.79 (H-5, *s*, OH), 8.38 (H-2, *s*), 7.03 (H-2′, *s*), 6.82 (H-5′, H-6′, *s*), 6.72 (H-8, *d*, J = 2 Hz), 6.48 (H-6, *d*, J = 2 Hz), 5.07 (glucose H-1, *d*, J = 7 Hz), 3–4 (multiplet, sugar protons + H_2O). ^{13}C NMR: 180 (C-4), 162.9 (C-5), 161.6 (C-7), 157.1 (C-9), 154.4 (C-2), 145.6 (C-4′), 144.9 (C-3′), 122.7 (C-3), 121.4 (C-1′), 119.9 (C-6′), 116.5 (C-2′), 115.4 (C-5′), 106.0 (C-10), 99.8 (C-1″), 99.5 (C-6), 94.5 (C-8), 77.1, 76.3 (C-3″, C-5″), 73.0 (C-2″), 69.6 (C-4″), 60.6 (C-6″).

Orobol (both free and ex TFA treatment). R_t (HPLC): 15.5 min (both isolated and standard aglycone). MS: $[M]^+$ 286 (100), $[M-29]^+$ 257 (6), $[M-57]^+$ 229 (10), $[A_1+H]$ 153 (93), $[B_1]$ 134 (48).

Pratensein 7-O-glucoside. R_t (HPLC): 18.0 min, 21.37 min (malonylester). MS: $[M]^+$ 462 (0.2), $[M-17]^+$ 445 (0.8), $[M-35]^+$ 427 (0.6), $[M-53]^+$ 409 (2.3), $[A+H]^+$ 301 (100), $[A+H-15]^+$ 286 (27), $[A+H-29]^+$ 272 (8), $[A+H-43]^+$ 258 (21), $[A+H-72]^+$ 229 (23), $[A_1]$ 153 (30), $[B_1]$ 148 (7), $[B_1-15]$ 133 (19), $[T]$ 163 (0.9), $[T-18]$ 145 (3), $[T-36]$ 127 (3). ^{1}H NMR [δ-values (ppm) downfield from TMS in DMSO-d_6]: 10.7 (H-5, *s*, OH), 8.42 (H-2, *s*), 7.05 (H-2′, *s*), 6.98 (H-5′, H-6′, *s*), 6.72 (H-8, *d*, J = 2 Hz), 6.48 (H-6, *d*, J = 2 Hz), 5.07 (glucose H-1, *d*, J = 7 Hz), 3.80 (*s*, OMe), 3–3.7 (multiplet, sugar protons + H_2O). ^{13}C NMR: 180.4 (C-4), 162.9 (C-5), 161.6 (C-7), 157.1 (C-9), 154.7 (C-2), 147.8 (C-4′), 146.1 (C-3′), 123.1 (C-3), 122.4 (C-1′), 119.8 (C-6′), 116.3 (C-2′), 112.0 (C-5′), 106.1 (C-10), 99.9 (C-1″), 99.6 (C-6), 94.5 (C-8), 77.1, 76.4 (C-3″, C-5″), 73.0 (C-2″), 69.6 (C-4″), 60.6 (C-6″), 55.6 (OMe).

Pratensein. R_t (HPLC): 21.9 min (both isolated and standard aglycone) (conditions as for orobol). MS: $[M]^+$ 300 (100), $[M-15]^+$ 285 (24), $[M-29]^+$ 271 (4), $[M-43]^+$ 257 (18), $[M-71]^+$ 229 (19), $[A_1+H]$ 153 (38), $[B_1]$ 148 (7), $[B_1-15]$ 133 (22).

*Relative intensities in parentheses.

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