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UNIQUE BIFLAVONOID TYPES FROM THE MOSS *DICRANOLOMA ROBUSTUM*

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Key Word Index—*Dicranoloma robustum*; *D. billardieri*; Dicranaceae; moss; Bryales; biflavones; biluteolins; 2′,6″-biluteolin; dicranolomin; 2″,3″-dihydro-5′,6″-biluteolin; 2,3-dihydro-2′,6″-biluteolin.

Abstract—The flavonoids in *Dicranoloma robustum* are predominantly biflavones of the rare biluteolin type. The unique biflavones, 2′,6″-biluteolin (dicranolomin), 2,3-dihydro-2′,6″-biluteolin (2,3-dihydrodicranolomin) and 2″,3″-dihydro-5′,6″-biluteolin are reported for the first time and were characterized together with the known 5′,8″-and 5′,6″-biluteolins. A microscale dehydrogenation method is described for the quantitative conversion of dihydro-biluteolins to biluteolins.

INTRODUCTION

In comparison with other plant groups, only very limited data are available on the flavonoids of mosses [1]. Most data relate to the order Bryales and in particular, suborder Bryineae sensu Vitt. (= Eubryales or 'true mosses'). Species within the suborder Dicranineae have been the subject of several investigations by Swedish workers who have reported the isolation from *Dicranum scoparium* of the 7-*O*-(2″,4″-*O*-dirhamnosyl)glucosides of apigenin and diosmetin [2, 3], the 7-*O*-rhamnoglucoside of luteolin [4] and 5′,8″-biluteolin (**1**), [5]. The only other report of flavonoids in this suborder relates to the finding of luteolin in the sporophytes of *Ceratodon purpureus* [6].

The first isolation of a biflavone from a moss in 1974 has recently been followed by one further report [7] in which 5′,3″-dihydroxyrobustaflavone (5′,6″-biluteolin, **2**) and **1** are identified as components of *Hylocomium splendens* (Hypnineae : Hylocomiaceae). Recently published work [8] on *Bryum capillare* (Bryineae : Bryaceae) has established the presence of 'heterobryoflavone' (orobol linked 5′-8″ to luteolin) and bryoflavone (orobol linked 5′-6″ to luteolin) in this moss. Both mosses are in the order Bryales, which remains the only reported natural source of biluteolins.

A chemotaxonomic study of New Zealand *Dicranoloma* (Dicranineae : Dicranaceae) species has revealed that the major flavonoids in this genus are biflavonoids. The present paper describes the isolation and structure elucidation of a number of these biflavonoids, some of which are unique.

DISCUSSION

A 2D-PC survey of a large number of *Dicranoloma* species revealed that the predominant flavonoids in this genus are aglycones. The aglycones in *D. robustum* were isolated by DCCC followed by 1D-PC separation of individual components. Pure flavonoids were prepared for further study by HPLC. Seven aglycones were isolated, DR4, 4a, 5, 5a, 6, 7 and 8, some in milligram quantities (e.g. DR5) and others in only trace amounts (DR8).

The absorption spectra of DR4, 4a, 5, 7 and 8 all closely approximate to those of luteolin while those of DR6 and DR5a approximate to a superimposition of the spectra of luteolin and dihydroluteolin (eriodictyol, see Table 1). All compounds exhibited luteolin-like characteristics on chromatograms in that all appeared as dark UV-absorbing spots which turned the typical fluorescent, yellow-orange with NA spray reagent.

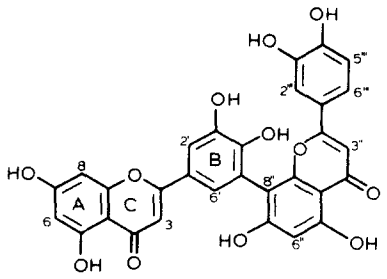
The FABMS of DR6 gave an $(M+H)^+$ of 573 consistent with the dihydro-biluteolin structure suggested by the absorption spectra. The ^1H NMR spectrum confirmed this (Table 2), a dihydroflavone moiety being evidenced by the characteristic [9] H-2 double doublet centred at 4.97 ppm and by the relatively high field H-6 and 8 signals [10]. Signals characteristic of luteolin but lacking an H-6 resonance are also present. The interflavonoid linkage must therefore bridge H-6 of the luteolin moiety with the B-ring of eriodictyol. Two *ortho*-coupled doublets at 6.96 and 6.84 ppm represent the B-ring protons in the eriodictyol moiety, and as such require that the interflavonoid linkage be 2′,6″. DR6 on this basis is assigned the structure, 2,3-dihydro-2′,6″-biluteolin (**3**).

DR7, a biluteolin from the absorption spectral data, gave an ^1H NMR spectrum with some similarities to that of DR6. It contained essentially the same proton resonances for the C-6 substituted luteolin moiety as did DR6, and while the spectrum of the other half of the molecule resembled that of luteolin rather than eriodictyol, the B-ring protons were again represented by a pair of ortho-coupled doublets. DR7 is, therefore, considered to be the dehydro-equivalent of DR6, 2′,6″-biluteolin (**4**).

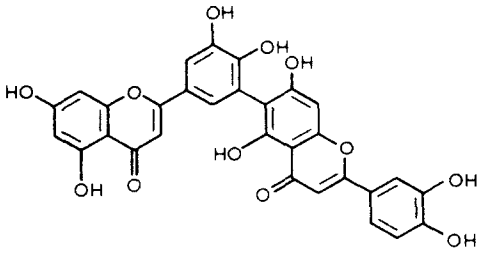
Dehydrogenation of DR6 to DR7 confirmed this relationship. Attempts to carry out this conversion using the technique of Fatma *et al.* [11] involving DMSO–iodine–sulphuric acid failed due to excessive

Table 1. Absorption spectra of *Dicranoloma*

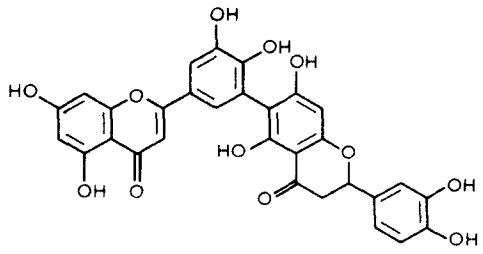
	MeOH	NaOMe	NaOAc
DR4	258, 268 sh 286 sh, 360	269 sh, 273 sh, 415	274, 325 sh, 382
DR4a (Luteolin)	253, 267 sh 291 sh, 350	266, 329 sh, 401	269, 326 sh, 384
DR5	248, 258 sh, 270 sh 291, 350	269, 274 sh 290 sh, 400	270, 294 sh, 380
DR5a	259, 267 sh, 288 358	266, 325, 410	270, 319, 375
DR6	276, 288, 346	280 sh, 324. 407	277, 286 sh 332, 365
Eriodictyol [9]	289, 324 sh	246 sh, 324	289 sh, 325
DR7	260 *br*, 349	266, 272 sh, 400	268 *br*, 373
DR8	~260, ~345	~270, 406	
HS5	248, 258, 270 sh 290, 350	270, 273 sh 293 sh, 396	272, 294 sh, 380
HS6	256, 270 sh, 288 sh 362	268 sh, 274 sh, 408	



1 5',8'' -Biluteolin (DR5)



2 5',6'' -Biluteolin (DR4)



5 2'',3'' -Dihydro-5',6''-biluteolin (DR5a)

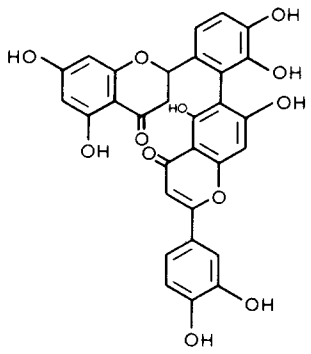
degradation occurring during the reaction process. Varying the reaction time was likewise unsuccessful and it is apparent that this method, which was successfully used with underivatized biapigenins [11], is unsuited to the more readily oxidized biluteolins. The conversion was finally accomplished in high yield using the peracetate of DR6, which after dehydrogenation and deacetylation yielded DR7 as the only product (TLC, HPLC, ^{1}H NMR). DR6 is the first example of a dihydro-biluteolin to be reported as a natural product, and DR6 and 7 are representatives of a hitherto unique biluteolin series involving the 2',6'' inter-flavone linkage. Accordingly, the trivial names dicranolomin and 2,3-dihydrodicranolomin are proposed for DR7 and DR6 respectively.

It is thought likely that DR8, which was isolated in only trace amounts, is the 2',8''-isomer of DR7 based on its absorption spectra, colour reactions and its chromatographic properties. On cellulose TLC, DR8 bears the same relationship to DR7 as does DR5 to its equivalent isomer (see below) DR4, and on HPLC DR7 and DR8 cochromatograph as also do DR4 and 5.

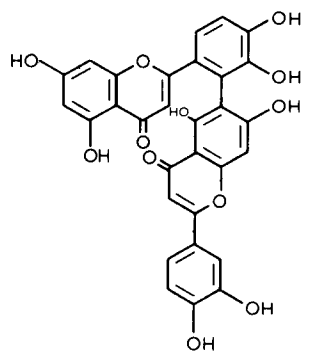
The 2',6''-interflavonoid linkage in biluteolins creates a doubly *ortho*-disubstituted biphenyl moiety involving the B-ring of one flavone and the A-ring of the other. Steric hinderance in such a system would be expected to prevent free rotation about the biphenyl linkage and to give rise to distinguishable rotamers in certain circumstances [12]. This is thought to be the case with DR6, in the ^{1}H NMR spectrum of which H-8'' (and to a lesser extent H-3'') appears as two, half proton singlets. An ^{1}H NMR spectrum was run at 70° in an attempt to confirm the presence of rotamers but the amount of DR6 available did not permit the measurement of a sufficiently noise-free spectrum. That DR6 did indeed comprise two similar species, however, was confirmed by their HPLC resolution (after many unsuccessful attempts at TLC separation) in the approximate ratio of 1:1. Steric hinderance is also in evidence in the ^{1}H NMR spectrum of DR7. The abnormally high field resonance of H-3 at 6.04 ppm is more akin to that of a chromone (e.g. leptaric acid,

and reference biflavones

$NaOAc-H_3BO_3$	$AlCl_3$	$AlCl_3$–HCl
264, 377	274, 303 sh, 424	274 *br*, 300 sh 364, 392 sh
260, 372	274, 300 sh, 328 sh 426	266 sh, 275, 295 sh, 355, 385
262, 374	274, 305 sh, 416	264, 278 sh, 300 sh 360, 390 sh
260 sh, 291, 376	275, 294 sh, 416	264 sh, 279, 297 sh 361, 388
267, 274 sh 290 sh, 373	280, 300 sh, 410	264 sh, 274, 298 sh 362, 390 sh
289, 325 sh	309, 378	309, 373
263,372	272, 303 sh, 406	264, 274 sh, 301 sh 360, 390 sh
	~273, ~310 sh, ~410	~270 sh, ~310 sh, ~355
264, 374	274, 305 sh, 418	265, 279 sh, 300 sh, 360, 390 sh



3 2,3-Dihydro – 2',6'' -biluteolin (DR6 ; 2,3-dihydrodicranolomin)



4 2',6''-Biluteolin (DR7; dicranolomin)

6.06 ppm [13]) than a flavone. This suggests either (i) that π-electron overlap between the relevant B- and C-rings is limited, as is known to be the case with 2'-hydroxyflavones [14] or (ii) that the A-ring of the attached luteolin is twisted out of plane such that H-3 is influenced by the shielding zone of this ring.

The major flavonoids in *D. robustum*, and in fact all *Dicranoloma* species studied, are DR4 and DR5. The FABMS of DR5 gave an $[M+H]^+$ ion of *m/z* 571 confirming the biluteolin formulation suspected from its absorption spectra. The ^1H NMR spectrum was consistent with a 5',8''-biluteolin (**1**) structure and indeed was closely similar to that determined on a small sample of **1** (see Table 2) supplied from the original source [5; Oesterdahl, B.-G., personal communication]. Furthermore, DR5 was shown to be spectroscopically (Table 1) and chromatographically (HPLC, TLC) indistinguishable both from this authentic sample and HS-5 (ex *Hylocomium splendens*) which was assigned the same structure [7]. DR4 is thought to be the 5',6''-isomer (**2**) of DR5. In accordance with this, DR4 has the same distinctive absorption spectra as does HS-6 to which this structure has previously been assigned [7]. Additionally, it appeared to isomerize in low yield to DR5 on treatment with hot HBr, and gave an ^1H NMR spectrum which exhibits a pattern of aromatic proton signals consistent with this formulation. Although some chemical shift values in this spectrum differed somewhat from those recently reported for HS-6[8], DR4 was found never-theless to be chromatographically indistinguishable from authentic HS-6 on reversed phase HPLC, and silica, cellulose and polyamide TLC.

Biflavonoid DR5a is considered to be a dihydro-derivative of DR4 and, although found in *D. robustum*, it was isolated in higher yield from *D. billardieri*. The dihydro-flavone nature of DR5a is evident from the

Table 2. ^{1}H NMR spectra of *Dicranoloma* biflavones (DMSO-d_6, 200 MHz, 20°)

Flavonoid nuclear protons	DR4 (2)*	Luteolin [8]	DR5 (5′,8″-biluteolin†, 1)		DR5a (5)	DR6 (3)	DR7 (4)
2 (Flavanone)	—	—	—	—	5.31 *dd*	4.97 *dd*	—
3	6.58 *s*	6.69 *s*	6.67 *s*	(6.69)	6.52 *s*	obscured	6.04 s
3″	6.53 *s*		6.64 *s*	(6.65)	obscured	6.66 *s*	6.69 *s*
						6.65 *s*	
6	6.16 d^m	6.22 d^m	6.15 d^m	(6.17)	6.16 d^m	5.79 s	5.97 d^m
6″	—	—	6.27 *s*	(6.34)	—	—	—
8	6.44 d^m	6.47 d^m	6.36 d^m	(6.39)	6.43 d^m	5.79 *s*	6.08 d^m
8″	6.26 *s*	—	—	(—)	5.66 *s*	6.47 *s*	6.54 *s*
						6.54 *s*	
2′	7.20 d^m	7.43 d^m	7.43 d^m	(7.46)	7.17 d^m	—	—
2‴	7.39 (*m*)	—	7.12 (*m*)	(7.09)	6.76 (*s*)	7.38 (*m*)	7.41 (*m*)
5′	—	6.92 d^o	—	—	—	6.85 d^o	6.94 d^o
5‴	6.89 d^o	—	6.60 d^o	(6.58)	6.76 d^o	6.87 d^o	6.89 d^o
6′	7.82 d^m	7.44 (*m*)	7.59 d^m	(7.54)	7.75 d^m	6.96 d^o	7.16 d^o
6‴	7.39 (*m*)	—	7.10 (*m*)	(7.07)	6.91 (d^o)	7.40 (*m*)	7.43 (*m*)

*Assignments confirmed by decoupling experiments.

†Authentic (ex Oesterdahl).

dd = Double doublet, $J = ca$ 12.5, 2.5 Hz, d^m = doublet, *meta*-coupled, $J = ca$ 2 Hz, d^o = doublet, *ortho*-coupled, $J = ca$ 8 Hz, *s* = singlet, *m* = multiplet, ( ) = multiplicities bracketed are ill-defined.

absorption data (Table 1) and in particular the absorption at 286 nm which undergoes a characteristic [9] bathochromic shift to 324 nm in NaOMe. This, and the shift of the other band II absorption from 259 to 270 nm with NaOAc, indicates that both the 7- and 7″-hydroxyl groups are unsubstituted. Nine aromatic proton signals are visible in the ^{1}H NMR spectrum of DR5a, and the additional one proton double doublet at 5.31 ppm confirms the presence of a single dihydroflavone moiety. The presence of a one proton singlet at 5.66 ppm requires that the interflavonoid linkage be in the A-ring of this moiety, and the signals at 6.91 and 6.76 ppm are indicative of a 3′,4′-dioxygenated B-ring [10]. The linkage to the luteolin moiety must be at C-5′ to account for the two pairs of *meta*-coupled signals at 7.75/7.17 and 6.43/6.16 ppm, the former of which represent the B-ring protons. DR5a is, therefore, defined as the dihydro-derivative of either 5′,6″-or 5′,8″-biluteolin. Dehydrogenation using the peracetate (as for DR6) produced DR4 quantitatively (HPLC, TLC) and not 5′,8″-biluteolin (DR5), therefore, DR5a is assigned the structure 2″,3″-dihydro-5′,6″-biluteolin (**5**). Its relationship with DR4 is confirmed not only by its conversion by dehydrogenation, but also by the near identity of the chemical shift pattern of the signals associated with the luteolin moiety.

The only remaining flavonoid isolated in this study is DR4a. This compound was chromatographically similar to DR4, but was distinguished clearly on TLC by its yellow (rather than orange) colouration with the NA spray. DR4a proved to be spectroscopically (Table 1) and chromatographically (HPLC, TLC) identical with luteolin.

In summary, the current investigation of *D. robustum* has led not only to the finding of the rare 5′,6″- and 5′,8″-biluteolins in a further moss genus, but also to the first isolation and identification of two dihydro-biluteolins and the recognition of a new series of biluteolins with a 2′,6″-interflavonoid linkage. A survey of a wide variety of species is currently under way and it is clear that biluteolins are a common characteristic of most, if not all, *Dicranoloma* species. The finding of such compounds in this genus is in accord with the classification (by Vitt [15]) of *Dicranoloma* in the order Bryales, suborder Dicraninneae.

EXPERIMENTAL

Plant material. Dicranoloma robustum and *D. billardieri* samples supplied and identified by Dr A. Fife (Botany Division, DSIR, New Zealand) were collected at Broad Stream, Canterbury (Nov. 1985) and Lake Thomson, Fiordland Natl Park (May 1986) respectively. Voucher specimens are deposited in the Botany Division herbarium, reference: AJF 7278 (CHR 405867) and AJF 7755 (CHR 406932) respectively.

Sample extraction and work-up. In a typical work-up, plant material (20 g) was dried in a microwave oven and powdered with a pestle and mortar. The sample was extracted twice at room temp. with 10% aqueous MeOH and once with 10% aqueous acetone and the dried, combined extract redissolved in a mixture (20 ml) of the stationary and mobile phases used for the DCCC. DCCC (using 250–300 columns in a Tokyo Rikakikai Eyela model DCC-300 linked to a Cecil CE212A monitor and a fraction collector) was carried out in the descending mode using the solvent, $CHCl_3$–*n*–BuOH–MeOH–H_2O (10:1:10:6) and monitored at 340 nm. A flow rate of *ca* 40 ml/hr was maintained throughout. With *D. robustum*, eight obvious peaks (Nos 1–8) were evident and each was collected and worked up as a separate fraction. Large scale 1DPC (Whatmans 3 MM paper) using 50% HOAc led to the isolation of no flavonoids from fractions 1-3, DR-4 and 4a from 4, DR-4 and 5 from 5, DR5 and 6 from 6, DR6 and 7 from 7 and DR8 from 8. With *D. billardieri*, DR5a accompanied DR4 in significant quantities.

Biflavonoid purification and analysis. Before spectral analysis, all compounds were purified (from carotenoids and other contaminants) by HPLC on an Alltech-Applied Science Econosphere C-18, 5 μ column (25 cm × 4.6 mm i.d.) fitted with an RP-

18 precolumn. With most components 1–3 mg were purified using a programmed mix of MeOH (A) and 3% HCO_2H (B) beginning with 35% A and changing linearly to 80%A over a period of 25 min. The detector was set at 356 nm. Absorption spectra (Table 1, conditions, reagents etc. as in ref. [9]), ^1H NMR spectra (Table 2; Varian XL-200, DMSO-d_6, room temp. and MS (VG ZAB 2HF mass spectrometer, FAB probe, Ar gas, glycerol sample matrix) were all measured on HPLC purified material.

Chromatographic comparisons. Spray reagent: 2% tetraphenyl-diboroxide ethanolamine complex (K and K) in MeOH (NA). Approximate relative R_f values on TLC (Schleicher and Schull F1440 cellulose, 20 cm) using 60% HOAc: DR4a 0.41, DR4 0.53, DR5a 0.60, DR5 0.66, DR7 0.73, DR6 0.83, DR8 *ca* 0.8. Routine cochromatography was carried out using this system (1D and 2D) and using polyamide (Bakerflex 6-F) TLC, solvent; MeOH–HOAc–H_2O (18:1:1) and multiple runs. Co-chromatography of DR4 with HS6 involved the use of polyamide (EtOAc–MeCOEt–HCO_2H–H_2O, 5:3:1:1), cellulose (40% HOAc) and silica (toluene–HCO_2Et–H_2O, 5:4:1). Approximate relative HPLC retention times (conditions as above)R_t': DR4a 11.8, DR4 and 5 16.5, DR5a 14.4, DR6 12.5, DR7 and 8 10.1, 5′,8″-biluteolin ex *Hylocomium* and synthesis 16.5, 5′,6″-biluteolin ex *Hylocomium* 16.5. Special conditions (i) for the separation of DR6 into two components: initial ratio of MeOH–3% HCO_2H 9:11 programmed to reach 11:9 in 20 min via a linear gradient; (ii) to separate DR4 and 5: initial ratio of 10% aqueous MeOH–3% HCO_2H 30:70 programmed to reach 17:3 in 25 min via a linear gradient, R_t': DR4 21.8, DR5 22.3.

Dehydrogenation of DR5a and DR6. In a typical experiment the starting material (0.5 mg) in pyridine (0.25 ml) was mixed with Ac_2O (0.25 ml) and stood overnight in a stoppered flask. The mixture was then evapd to dryness and redissolved in (CaH_2 dried) DMSO (0.1 ml) and transferred to a Reacti-vial (0.3 ml, Pierce). To this was added one small crystal of I_2 and a trace of conc H_2SO_4, and the sealed vial was placed in an oven at 100° for 30 min. The reaction mixture was then added to *ca* 5 ml H_2O, $Na_2S_2O_3$ added to remove the I_2, and the solution extracted with EtOAc. The EtOAc solubles were recovered by evapn, and deacetylation was achieved by dissolving this material in 1M NaOH (10 μl), and leaving it at room temp. in the syringe for 2 hr. The product was obtained by ejecting the syringe contents into a vial containing 1M HCl (12 μl) and evapg the mixture to dryness. TLC monitoring was used throughout and confirmed that DR5a and DR6 produced DR4 and DR7 respectively, in essentially quantitative yield.

Isomerization of DR4 to DR5. DR4 (0.25–0.5 mg) in 48% HBr (0.5 ml) was heated at 145° under N_2 (and positive N_2 pressure) for 30 min and then taken to dryness by passing a stream of N_2 over the heated solution. TLC of the MeOH solubles (2D-cellulose, polyamide—see section on chromatography) revealed, after spraying, low level amounts of a product which cochromatographed with DR5. Most of the DR4 remained unchanged. Using the above technique, amentoflavone was converted substantially to robustaflavone in 1.5 hr. This period of reaction with DR4 led to extensive degradation.

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