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FLAVONOIDS IN THE GREEN ALGAE (CHLOROPHYTA)

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Abstract—A number of flavonoid C-glycosides have been isolated from the green alga *Nitella Hookeri* (F. Characeae). This extends the range of plants in which flavonoids are known to exist, to include the green algae.

INTRODUCTION

THE BIOSYNTHETIC pathway leading to flavonoids in plants involves C_6-C_3 cinnamic acid type precursors for the production of the B- and C-ring components.[1] These same precursors are also involved in the biogenesis of lignins[2] and, as a result, most plants which produce flavonoids are also ligniferous. Evidence is accumulating, however, which indicates that flavonoids may also be produced by some plant groups which are generally considered to be non-ligniferous.

Flavonoids to date have been found in a wide variety of plant taxa, including the lignin containing angiosperms, gymnosperms and ferns, and the doubtfully[3] ligniferous mosses.[4] Recently, reports have appeared which claim the existence of flavonoids in the slime moulds (Myxomycophyta)[5] and the liverworts (Bryophyta, Hepaticae),[6,7] both of which are considered to be non-ligniferous. These claims however have not all been fully substantiated. Although several reports of flavonoid-like compounds occurring in algae have been published,[8] it is currently considered[4] that these compounds are not flavonoids.

We wish to report here the first identification of flavonoid compounds in the green algae and so to extend the range of non-ligniferous plants in which flavonoids exist to include the Chlorophytes.

RESULTS AND DISCUSSION

In view of the key evolutionary position usually attributed to the green algae (Chlorophyta)[9–11] it was considered worthwhile to look for flavonoids in a relatively advanced

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[11] D. MARSLAND, *Principles of Modern Biology* (4th edition), pp. 566, 597, Holt, Rinehardt and Winston, New York (1964).

member of this group. *Nitella Hookeri* (F. Characeae, Cl. Charophyceae), a soft, branched, filamentous green algae, was chosen for this investigation because of its availability.

The plant material was extracted thoroughly with aqueous methanol and the ether-insoluble portion of the extract was examined by paper chromatography. The chromatograms showed ten dark-purple spots when viewed under u.v. (360 nm) light. Without exception these spots turned yellow or yellow-green in the presence of ammonia, typical of flavones or 3-substituted flavonols containing free 5- and 4′-hydroxyl groups.[12] The R_f values of these spots were also consistent with their being due to the mono- or diglycosides of these types of flavonoid;[12] as is evidenced by the comparisons made in Table 1 between the four more dominant spots (compounds 1, 1a, 2 and 3) and various known flavonoids.

TABLE 1. R_f VALUES OF *Nitella* COMPOUNDS AND KNOWN FLAVONOID C-GLYCOSIDES

	Solvent†	
	TBA	HOAc
Compound 1	0·14	0·45
Compound 1a	0·22	0·47
Compound 2	0·31	0·51
Compound 3	0·20	0·30
Lucenin-1	0·18	0·30
Orientin*	0·29	0·21
Isoorientin*	0·43	0·39
Vicenin-1	0·30	0·52
Vitexin	0·37	0·20
Isovitexin	0·51	0·30

* Values from Ref. 13.
† TBA; t-BuOH:HOAc:H_2O, 3:1:1. HOAc, 15% aqueous HOAc.

In order to isolate the flavonoids, the ether-insoluble portion of the original extract was chromatographed on a powdered cellulose column and by this method, together with repeated paper chromatography of the fractions so obtained, compounds 1, 1a, 2 and 3 were isolated in a pure condition in very small amounts.

The u.v. spectra of the isolated compounds (Table 2) confirm the paper chromatographic assessments and indicate that compounds 1, 1a and 3 are of the luteolin (5,7,3′,4′-tetrahydroxyflavone) type and that compound 2 is of the apigenin (5,7,4′-trihydroxyflavone) type. The relationship between the R_f value of compound 2 and those of 1, 1a and 3 is also consistent with this difference in hydroxylation pattern.[13] Further information was gained from the u.v. spectra by the use of the standard diagnostic reagents,[13] NaOMe, NaOAc, NaOAc/H_3BO_3, $AlCl_3$ and $AlCl_3$/HCl. The spectra observed on addition of each of these reagents (Table 2) show spectral shifts which are not only entirely consistent with those observed for either apigenin or luteolin derivatives, but which also indicate that the oxygen functions at C-5,7 and 4′ in compound 2, and C-5,7,3′ and 4′ in compounds 1, 1a and 3, are all unsubstituted hydroxyl groups. Thus, since these compounds behave as mono- or di-glycosides on paper chromatography, they must be C-glycosides and not O-glycosides. This was con-

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TABLE 2. U.V. SPECTRA OF *Nitella* FLAVONOIDS (λ_{max}, nm)

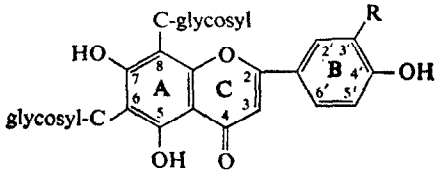
Flavonoid	MeOH	NaOMe	NaOAc	$NaOAc/H_3BO_3$	$AlCl_3$	$AlCl_3/HCl$
Compound 1	258, 271, 353	282, 417	271, 280, 410	266, 377, 430sh	277, 303sh, 330sh, 426	264sh, 277, 297, 359, 383
Compound 1a	259, 272, 348	282, 410	270–278, 399	267, 376	277, 303sh, 336sh, 426	263sh, 278, 300sh, 358, 388
Compound 3	258, 272, 351	276, 415	273, 278, 397	269, 380, 430sh	278, 305sh, 332sh, 429	262sh, 279, 300sh, 362, 389
Lucenin-1*†	256, 270, 347	267sh, 280 343sh, 408	272, 279, 326sh, 389	265, 378, 431sh,	277, 303sh, 332sh, 430	264sh, 277, 297, 364, 386
Orientin*†	255, 267, 346	268, 278, 334sh, 405	278, 325sh, 386	264, 375, 430sh	276, 302sh, 329, 429	265sh, 276, 296sh, 357, 384
Compound 2	271, 331	276, 330sh, 400	280, 305sh, 390	273, 325sh, 350sh	279, 306sh, 348, 390	279, 306sh, 348, 390
Vicenin-1	273, 333	281, 332, 398	281, 304sh, 388	274, 330sh, 348	281, 305, 350, 384	282, 306, 347, 383
Vitexin*†	270, 336	279, 329, 395	280, 300sh, 380	271, 329sh, 344	277, 305, 350, 386	278, 303, 343, 383

* Vitexin and orientin are the 8-C-glucosides of apigenin and luteolin respectively, and vicenin and lucenin are 6,8-di-C-glycosides of apigenin and luteolin respectively.

† Spectral data from Ref. 12.

firmed by prolonged acid hydrolyses of these compounds which failed to affect their chromatographic behaviour. The possibility of their being C-glycosyl-O-glycosides is thus also excluded.

Attempted acid hydrolyses were carried out under conditions which had previously been used to isomerize 8-C-monoglycosides, such as vitexin and orientin, to their 6-C-monoglycoside equivalents and vice versa.[14] Compounds 1, 1a, 2 and 3 however were recovered from this treatment unchanged, thus indicating that either the same C-glycosyl unit is present at both C-6 and C-8, as in lucenin-2 and vicenin-2[14] (I), or that the C-glycosyl unit or units are present only in the B-ring. This latter possibility is unlikely in view of the fact that all known flavonoid C-glycosides are A-ring derivatives. Thus, the flavonoids isolated from *Nitella* appear to be of the vicenin and lucenin types similar to those recently isolated from the wood of *Vitex lucens*.[14] Of interest in this respect is the close similarity between the R_f values of compound 2 and vicenin-1, and compound 3 and lucenin-1 (see Table 1).



I (Lucenin R = OH, Vicenin R = H)

Due to the extremely small yield of the flavonoids from *Nitella* and to the difficulty of obtaining plant material clear of contaminating debris, insufficient of the natural products have so far been isolated for confirmatory NMR studies. The evidence presented above, however, is difficult to interpret in any way other than that *N. Hookeri* contains a number of flavonoid glycosides with apigenin and luteolin hydroxylation patterns, which are probably present as 6,8-di-C-glycosides.

Flavonoid C-glycosides have previously been found in a number of the less-advanced plant groups including the mosses[4,15] and the liverworts.[7] This present finding extends for the first time the range of plant taxa in which flavonoids have been found to the Chlorophytes, and would seem to provide another biochemical relationship between the Chlorophytes (Charophyta) and the higher plants (cf. Ref. 16). If flavonoids are not subsequently found in other members of the Chlorophyta, the isolation of flavonoids from *Nitella*, a member of the class Charophyceae, could well have some bearing on the taxonomy and the evolutionary position of the Charophytes as distinct from other members of the Chlorophyta.

EXPERIMENTAL

Nitella Hookeri plant material (Voucher specimen No. CHR 185330, Botany Division, D.S.I.R., Christchurch) was collected near Lower Hutt, New Zealand. Paper chromatograms were run on Whatman 3 MM paper (46 × 57 cm) using t-BuOH:HOAc:H_2O, 3:1:1 (TBA), and 15% HOAc (HOAc) as solvents. The u.v. spectra were measured in AR MeOH and diagnostic reagents were made up and used as described in Ref. 13.

[14] M. K. SEIKEL, J. H. S. CHOW and L. FELDMAN, *Phytochem.* **5**, 439 (1966).
[15] R. E. ALSTON and T. E. MELCHERT, *Science* **150**, 1170 (1965).
[16] P. POHL and H. WAGNER, *Phytochem.* **7**, 1565 (1968).

Extraction Procedure

Wet *Nitella* plant material was thoroughly washed and then hand-picked clear of debris. It was dried in air at 40° for 1–2 hr to yield 22 g of dry plant material, which was then mixed with 20% aqueous MeOH (400 ml) in a Waring blendor. The resultant "pulp" was heated at 80° on a water-bath for 4 hr and the solubles were removed by filtration. This extraction process was carried out three times and the extracts were combined and evaporated to dryness *in vacuo*. The chlorophylls were removed from the dry extract by washing it several times with hot diethyl ether and the remaining solid material was used for the paper chromatography.

Isolation of Flavonoids

The ether-insoluble extract was dissolved in a minimum of hot water, filtered, and applied to the top of a 15 cm column (5 cm dia.) of powdered cellulose. The column was eluted with water, and 250-ml fractions were taken. All fractions were analysed by 2D paper chromatography in TBA/HOAc. The first three fractions were a bright yellow colour but contained none of the compounds of interest. Fraction (4) contained compounds 1 and 2; fraction (5), 1, 2 and 3; fractions (6–9), 1, 1a and 3; and fractions (10) and (11), 1 and 3. The individual compounds were separated by paper chromatography in TBA and/or HOAc and the purity of each was checked by polyamide TLC. Quantitative u.v. spectroscopy indicated a yield of about 0·5 mg (0·002 per cent of dry wt.) for the major compound, compound 1. U.v. spectra and R_f values for each compound are presented in Tables 1 and 2.

Hydrolysis Conditions

Each compound isolated was hydrolysed for 5–6 hr in MeOH : 2 N HCl (50 : 50) at 100° and then co-chromatographed in TBA and/or HOAc with unhydrolysed material. In no case could the hydrolysed compound be distinguished from the unhydrolysed. (Compounds 1 and 3 did show additional streaking on the paper chromatogram after 7 hr hydrolysis.) In a parallel hydrolysis under the same conditions vitexin isomerized to produce a mixture of vitexin and isovitexin.

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