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IDENTIFICATION OF THE GLYCOSYLFLAVONES OF *EPHEDRA* AND *BRIZA* BY MASS SPECTROMETRY OF THEIR PERMETHYL ETHERS

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Abstract—The application of permethylation and mass spectrometry to several glycoflavone samples homogenous by paper chromatography showed them in most cases to be mixtures of closely related isomers. A sample isolated from *Ephedra andina* leaves was identified by these means as a mixture of vicenin-1 and -2. Application of the procedure to a sample from *Briza media* thought to be vitexin showed it to be 8-*C*-galactosylapigenin and this was confirmed by co-chromatography with a synthetic specimen; an acylated 8-*C*-galactosylapigenin was also detected in the same source.

INTRODUCTION

Identification of the carbon-linked sugar moieties in glycosylflavones is difficult because there is no micromethod available for releasing the carbon-bonded sugar intact in reasonable yield. Indirect methods have therefore been used up to now and identifications have usually been based on spectral and R_f comparisons with synthetic specimens or natural compounds of known structure. Recently, a more powerful indirect method has been developed involving the mass spectrometry of their permethyl ethers [1].

We have applied this new procedure to a number of glycosylflavones available to us and have found that several samples, apparently homogenous by paper chromatography, were clearly mixtures of closely related structures. Our results suggest that some of the literature data, particularly on the disubstituted vicenins and lucenins, will need reassessment in the light of this new development. We report here the first identification of two vicenin derivatives in *Ephedra* and the re-examination of two vitexin derivatives in *Briza*.

RESULTS

Preliminary studies were carried out on samples of vicenins (di-*C*-substituted apigenins) isolated by PC in apparently pure form variously from *Commelina*, *Dioscoreophyllum*, *Ephedra* and *Triticum* (flavonoid B, see ref. [2]). Permethylation with MeI and NaH [1] gave mixtures of permethyl ethers which separated on TLC on silica gel in chloroform-acetone (4:1). In all cases, it was clear that at least two major components were present in the original samples and this was confirmed by the pairs of permethyl ethers having different MS fragmentations; parent ions peaks indicated that interference by products of incomplete methylation or of *C*-methylation could be ruled out.

More detailed studies were then carried out on the glycosylflavone sample of *Ephedra andina*. This appeared to be homogenous by PC and from its UV and R_f data and stability to acid treatment it was provisionally identified as a 6,8-di-*C*-hexosylapigenin. On permethylation, it gave two main permethylated flavones of molecular masses 748 and 704 with MS fragmentation patterns characteristic of 6-substituted PM-*C*-hexosyl- and PM-*C*-pentosylapigenin, with an 8-substituted PM-*C*-hexosyl in each case. The compound with the pentose sugar was found to be chromatographically distinct from samples of PM-schaftoside and PM-isoschaftoside, two derivatives with glucose and arabinose in the 8,6- and 6,8-positions respectively [1], which indicated that it probably contained the only other common pentose, xylose. Comparison with the fragmentation pattern of the same two compounds showed that the pentose was attached to the 6-position and the hexose to the 8-. Its identification as 6-*C*-xylosyl,8-*C*-glucosylapigenin was confirmed by direct chromatographic comparison with synthetic material. This compound is also known as 6-xylosylvitexin or as vicenin-1, and has previously been isolated from *Vitex lucens* and several other sources [3].

The original glycoflavone material from *Ephedra*, when submitted to TLC on activated silica gel using the system of Chopin and Bouillant [3], separated into its two components, the one of higher R_f being vicenin-1 and the one of lower R_f the 6,8-di-*C*-hexosylapigenin. Comparison with the literature data indicated that this second compound is 6,8-di-*C*-glucosylapigenin (vicenin-2), although the possibility of galactose units being present has not been completely ruled out. Vicenin-1 and -2 have been found together in several plant sources, including *Vitex lucens* [3], so that their co-occurrence is expectable. Their discovery in *Ephedra*, however, is the first report of them in the Gymnospermae. Indeed, although glycoflavones are probably present in a range of conifers, the only other complete report of these compounds in gymnosperms appears to be that of Niemann [4], who has isolated several vitexin derivatives from needles of *Larix* species.

Table 1. R_f Data for Glycosylflavones of *Briza* and *Ephedra*

Support	Solvent	R_f (×100) *Briza* Vitexin	Apigenin 8-*C*-Gal	Acyl. Ap. 8-*C*-Gal	*Ephedra* Vicenin-1	Vicenin-2
Silica gel	APWM	69	61	77	19	08
Paper	15% HOAc	23*	22*	40	45	45
Paper	BAW (4:1:5)	42	42	60	31	29
Microcrystalline cellulose	15% HOAc	15	15	31	—	—
Microcrystalline cellulose	BAW (6:1:2)	28	30	60	—	—
Silica gel	$CHCl_3$–Me_2CO (4:1)	—	24†	24†	39†	87†

*Separation could be achieved by over-development for 15 hr in this solvent. †Of the permethyl ether.

The only hexose so far reported as a *C*-sugar in natural glycosylflavones is glucose, but there is clearly the possibility of galactose occurring in this context, although there are difficulties in distinguishing between such closely similar isomeric compounds. The discovery of such an isomer arose completely by chance when a sample of what was thought to be vitexin isolated in our laboratory by Williams and Murray from *Briza media* [5] was permethylated and its MS examined. Although the fragmentation pattern was the same as vitexin, there were some small differences in ion intensities, sufficient to indicate the possibility of it having a different structure. Chopin and Bouillant [3] have devised a TLC system for separating synthetic 8-*C*-galactosylapigenin from the 8-*C*-glucosyl isomer and when this system was applied to the *Briza* compound, it had a different mobility from a specimen of vitexin isolated from *Vitex*. The identity of the *Briza* glycosylflavone with 8-*C*-galactosylapigenin was then confirmed by direct comparison with a synthetic sample. Although TLC gave the best separations (Table 1), it was also possible to distinguish the slower running *C*-galactoside from vitexin on paper chromatograms, by over-developing (for 15 hr) in 15% HOAc.

This discovery of 8-*C*-galactosylapigenin in *Briza* represents the first report of a galactose-containing glycosylflavone in plants. Its discovery suggested immediately that other glycosylflavones of *Briza* reported as *C*-glucosides [5] should be re-investigated. One of these, reported as isovitexin was found not to be the 6-*C*-galactoside but to be an acylated derivative of 8-*C*-galactosylapigenin, since it gave this both on alkaline and mild acid hydrolysis. An IR spectrum of the original sample showed free carbonyl stretching absorption, confirming the presence of an acyl group, but it proved impossible to identify the acyl moiety since it lacked any UV absorption.

Compounds previously reported in *Briza* as orientin and iso-orientin derivatives have also been reinvestigated and the structures proposed earlier have had to be modified (C. A. Williams and B. Murray, unpublished); the results obtained will be reported elsewhere. Finally, there is the possibility that 8-*C*-galactosylflavones may occur in other grasses besides *Briza* and taxonomic aspects are also being actively investigated.

EXPERIMENTAL

Isolation. Glycoflavones were extracted and purified by standard PC methods [6]: that from *Briza* was kindly supplied by Drs. C. A. Williams and B. G. Murray.

Identification. *C*-Glycoapigenins were examined initially by TLC (Si gel, activated 1 hr 105°) using APWM (EtOAc–Py–H_2O-MeOH, 80:20:5:10) and by TLC on cellulose using *n*-BuOH–HOAc–H_2O (6:1:2) and 15% HOAc. UV spectral measurements were applied with the usual reagents. Spectra and shifts corresponded closely to those of the parent aglycone, apigenin. *Ephedra* *C*-glycoapigenins, λ_{max} (EtOH) 275, 337; 285, 405 (NaOEt); 282, 306, 345 ($AlCl_3$, unchanged by HCl). IR spectra were run using KBr discs.

Permethylation. The method described for carbohydrates by Brimacombe [7] was used except that initial drying of DMF was by 4Å molecular sieve before distillation under reduced pressure onto CaH_2, and that partition between $CHCl_3$ and H_2O was omitted. The reaction mixture was repeatedly diluted with EtOH and evaporated *in vacuo* to remove all the DMF. The alcoholic soln was streaked on pre-coated TLC Si gel plates (Merck) and developed in $CHCl_3$–EtOAc–Me_2CO (5:4:1). Main bands were eluted with aq EtOH and further purified on pre-washed plates using $CHCl_3$–Me_2CO (4:1). These systems were also used for comparison of final products with PM-glycoflavones prepared by Bouillant and Chopin (Table 1).

Mass spectra of permethylethers were obtained on an AEI MS 12-DS 30 (HDIS) and results were compared with those of Bouillant *et al.* [1]. ST 178°, PT *ca* 200°, EI 70 eV. Natural isotope peaks omitted, *m/e* (rel. int.): *PM*-6-C-*xylosyl*-8-C-*glucosylapigenin*, (from *Ephedra*) M^+ 704 (17), M-Me 689 (22), M-OMe 673 (100), 657 (10), 645 (2), 585 (13), 573 (8), 559 (5), 543 (2), 541 (3), 529 (8), 312 (6). *PM*-6,8-*di*-C-*hexosylapigenin*, (from *Ephedra*) M^+ 748 (16), M-Me 733 (25), M-OMe 717 (100), 703 (22), 689 (11), 645 (9), 585 (17), 573 (32), 559 (5), 543 (3), 383 (6), 353 (2). *PM*-8-C-*galactosylapigenin*, (from *Briza*) M^+ 530 (56), 369 (12), 355 (100), 341 (20), 325 (4).

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REFERENCES

1. Bouillant, M-L., Favré-Bonvin, J. and Chopin, J. (1975) *Phytochemistry* **14**, 2267.
2. King, H. G. C. (1962) *J. Sci. Food Agr.* **27**, 446.
3. Chopin, J. and Bouillant, M-L. (1975) In *The Flavonoids* (eds. Harborne, J. B., Mabry, T. J. and Mabry, H.) pp. 632–691. Chapman & Hall, London.
4. Niemann, G. J. and Bekooy, R. (1971) *Phytochemistry* **10**, 893; Niemann, G. J. *Phytochemistry* **12**, 2056; **14**, 1436.
5. Williams, C. A. and Murray, B. G. (1972) *Phytochemistry* **11**, 2705.
6. Harborne, J. B. (1967) *Comparative Biochemistry of the Flavonoids* Academic Press, London.
7. Brimacombe, J. S., Jones, B. D., Stacey, M. and Willard, J. J. (1966) *Carbohyd. Res.* **2**, 167.