

EXTENDED FLAVONOID BIOSYNTHETIC CAPABILITY IN THE AQUATIC FERN GENUS *PILULARIA*

KENNETH R. MARKHAM and ENZO S. VIOTTO

Chemistry Division, DSIR, Petone, New Zealand

(Received 14 May 1987)

Key Word Index—*Pilularia globulifera*; *P. americana*; Marsileaceae; fern; flavonol glycosides; 7-*O*-glycosylation.

Abstract—The marsileaceous fern, *Pilularia globulifera* is shown to accumulate the following quercetin glycosides: 3-glucoside, 3-rhamnoside, 3-xyloside, 3-arabinopyranoside, 3-arabinofuranoside and 3,7-di-rhamnoside. Kaempferol 3-glucoside is also present and kaempferol 3,7-dirhamnoside is the major component. 7-*O*-Glycosylation has not been observed previously in the marsileaceae. Amendments are proposed to structure assignments in previous work on *P. americana*.

INTRODUCTION

The only family of 'rooted' aquatic ferns, the Marsileaceae, comprise three genera, the widespread *Marsilea* and the more restricted *Regnellidium* and *Pilularia*. *Pilularia*, the pillwort, is a small genus of about six species and may be the most primitive [1]. Only one incomplete study of the flavonoids of a *Pilularia* species has appeared to date [1]. In this, *P. americana* was shown to accumulate kaempferol-3-*O*-glucoside and the 3-*O*-glucoside, 3-*O*-rhamnoside and 3-*O*-arabinoside of quercetin together with other flavonoids not fully characterized. The present paper extends the range of flavonoids known to be synthesized by *Pilularia*, and suggests amendments to structure proposals made in the earlier paper.

RESULTS

A 2D-PC of the ethanol–water extract of *P. globulifera* revealed a pattern of seven dominant flavonoids together with two very weak components. All but two of the dominant flavonoids possessed the chromatographic mobilities of monoglycosides. A series of standard procedures [2] involving purification, absorption spectroscopy, hydrolysis, followed by sugar and aglycone analyses and finally TLC co-chromatography with authentic samples (except for the xyloside), revealed these glycosides to be quercetin 3-*O*- α -L-rhamnoside, 3-*O*- β -D-glucoside, 3-*O*-xyloside, 3-*O*- α -L-arabinopyranoside and 3-*O*- α -L-arabinofuranoside, and kaempferol 3-*O*- β -D-glucoside. One very weak monoglycoside possessed relative R_f values and colour reactions approximately equivalent to those previously attributed to 'quercetin 3-*O*-gentiobioside' [1]. The major flavonoid possessed the absorption spectra of a flavonol 3,7-diglycoside, gave kaempferol and rhamnose on hydrolysis, kaempferol 7-*O*-rhamnoside on partial hydrolysis and co-chromatographed with authentic kaempferol 3,7-di-*O*-rhamnoside. This was accompanied by smaller amounts of quercetin 3,7-di-*O*-rhamnoside which behaved in an equivalent manner.

The finding of both the arabinofuranoside and the arabinopyranoside of quercetin in *P. globulifera* (confirmed by cochromatography with authentic samples from *Arctostaphylos uva-ursi* [3]) is very unusual, and unique in ferns [4]. It may however have been observed in the earlier work on *P. americana* [1]. The flavonol described in this work as quercetin 3-*O*-arabinoside possesses the relative (to quercetin 3-*O*-glucoside) R_f values of quercetin 3-*O*- α -L-arabinofuranoside. Additionally, the compound described as (possibly) quercetin 3-*O*-lyxoside is almost certainly quercetin 3-*O*- α -L-arabinopyranoside, since it possesses the same relative R_f values (see Experimental) and as expected, gives a GC sugar pattern which is qualitatively the same as that of arabinose [1]. In the present work the arabinose from quercetin 3-*O*-arabinopyranoside, although not distinguished from lyxose by GC, was clearly distinguished by PC using BBPW [2], a technique not used by the earlier workers. Xylose from the chromatographically similar quercetin 3-*O*-xyloside was distinguished from lyxose by GC of the TMS-ethers on OV-1.

P. globulifera significantly, differs from *P. americana* in its accumulation of substantial quantities of kaempferol, and is unique (to date) in the family Marsileaceae in its ability to glycosylate flavonoids at the 7-hydroxyl.

EXPERIMENTAL

Pilularia globulifera was collected by J. M. Camus and A. M. Paul from Hatchets Pond, Hampshire, Southern England on 3 July 1986 and supplied and identified by Dr Clive Jermy of the British Museum, London. A voucher specimen is deposited at the British Museum.

Dried plant material (2.5 g) was frozen with liquid N₂ and ground in a pestle and mortar prior to cold extraction with EtOH–H₂O(4:1). 2D-PC using TBA and HOAc solvents (see below) was used to isolate components and these were further purified by repeat 1D-PC and finally by passing through an LH-20 column (MeOH solvent). Details of experimental procedures are generally as described in ref. [2], but partial hydrolyses of kaempferol and quercetin 3,7-dirhamnosides to the 7-*O*-

rhamnosides involved treatment with 1 N TFA at 100° for 5 min. Sugar analyses were carried out by PC using BBPW solvent and by GC on OV-1 (see [2]).

TLC characteristics of purified flavonoids. Plates: Schleicher and Schuell, F1440, Avicel (cellulose) using *t*-BuOH–HOAc–H₂O, 3:1:1 (TBA) and 15% HOAc; J. T. Baker, Baker-flex polyamide 6-F using MeOH–HOAc–H₂O, 18:1:1 (MAW). *R_f* values of purified components (TBA, HOAc, MAW): kaempferol 3,7-di-*O*-rhamnoside (0.70, 0.71, 0.76), quercetin 3,7-di-*O*-rhamnoside (0.58, 0.65, 0.72), quercetin 3-*O*-rhamnoside (0.75, 0.40, 0.48), quercetin 3-*O*-glucoside (0.56, 0.26, 0.41), quercetin 3-*O*-xyloside (0.60, 0.20, 0.39), quercetin 3-*O*-arabinopyranoside (0.54, 0.16, 0.34), quercetin 3-*O*-arabinofuranoside (0.74, 0.22, 0.34), kaempferol 3-*O*-glucoside (0.76, 0.40, 0.49). All co-chromatography with authentic flavonoids was carried out using these chromatographic systems, with 50% HOAc/cellulose being used where appropriate (e.g. with aglycones). Spray reagent: 1.0% diphenylboric acid 2-aminoethyl ester (Fluka) in MeOH (NA).

GC Retention times of key sugars. 3% OV-1 on silanized Chromosorb W; sugars acid treated; retention times relative to β -glucose: arabinose (both fur. and pyr.) 0.22, 0.27; lyxose 0.23, 0.27; xylose 0.36, 0.44.

2D-PC characteristics of flavonoids detected. (Structure, *R_f* values from 2D-PCs in TBA, 15% HOAc, spot intensity, colour in UV, colour with NA): kaempferol 3,7-di-*O*-rhamnoside, 0.59, 0.77, strong, dark, yellow-green; quercetin 3,7-di-*O*-rhamnoside, 0.47, 0.72, med, dark, yellow-orange; quercetin 3-*O*-rhamnoside, 0.59, 0.50, strong, dark, orange; quercetin 3-*O*-glucoside, 0.47, 0.45, med., dark, orange; unidentified flavonoid, 0.45, 0.45, v. weak, dark, orange; quercetin 3-*O*-xyloside, 0.50, 0.37, weak, dark, orange; quercetin 3-*O*-arabinofuranoside, 0.58, 0.33,

med-strong, dark, orange; quercetin 3-*O*-arabinopyranoside, 0.45, 0.30, med-strong, dark, orange; unidentified (flavonoid?), 0.33, 0.12, v. weak, yellow, fl., orange.

Comparison of relative (2D-PC) *R_f* values with those of *P. americana* flavonoids [1]. *R* (quercetin 3-*O*-glucoside) values: (TBA, 15% HOAc); 'quercetin 3-*O*-lyxoside' (0.98, 0.76) cf quercetin 3-*O*-arabinopyranoside (1.0, 0.72); 'quercetin 3-*O*-arabinoside' (1.4, 0.79) cf quercetin 3-*O*-arabinofuranoside (1.3, 0.79); 'quercetin 3-*O*-gentiobioside' (0.96, 1.02) cf unidentified flavonoid (1.0, 1.07).

Absorption Spectra. As detailed in ref. [5] for quercetin 3-*O*-glycosides, and kaempferol and quercetin 3,7-di-*O*-glycosides.

Acknowledgements—The authors are grateful to Dr Clive Jermy of the British Museum for the supply and identification of plant material.

REFERENCES

1. Wallace, J. W., Chapman, M., Sullivan, J. E. and Bhardwaja, T. N. (1984) *Am. J. Botany* **71**, 660.
2. Markham, K. R. (1982), *Techniques of Flavonoid Identification*. Academic Press, London.
3. Markham, K. R., Ternai, B., Stanley, R., Geiger, H. and Mabry, T. J. (1978) *Tetrahedron* **34**, 1389.
4. Markham, K. R. (1988) in *The Flavonoids: Advances in Research, Vol. 2*. (J. B. Harborne, ed.). Chapman & Hall (in press).
5. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970). *The Systematic Identification of Flavonoids*. Springer, New York.