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

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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 posesses 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 N_2 and ground in a pestle and mortar prior to cold extraction with EtOH–H_2O(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–H_2O, 3:1:1 (TBA) and 15% HOAc, J. T. Baker, Baker-flex polyamide 6-F using MeOH–HOAc–H_2O, 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.

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