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ACYLATED FLAVONOL SOPHOROTRIOSIDES FROM PEA SHOOTS

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Key Word Index—*Pisum sativum*; Leguminosae; pea; flavonoids; triglycosides; acylated; *p*-coumaric, caffeic, ferulic, sinapic acids.

Abstract—Seven flavonols were isolated and identified from *Pisum sativum* (cv Solara) shoots. The 3-glucoside, and 3-sophorotrioside [β-D-glucopyranosyl(1 → 2)-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside] of quercetin, the 3-sophorotrioside of kaempferol, and the acylated derivatives of quercetin 3-sophorotrioside with *p*-coumaric, caffeic, ferulic and sinapic acids on the hydroxyl at the 6-position of the terminal sugar. The caffeic and sinapic acid esters are two new naturally occurring compounds. This is the first report in which the structures of the *p*-coumaryl- and feruloyl-sophorotriosides of quercetin, which were previously reported from pea leaves, have been completely established by means of ^{1}H NMR studies including COSY, NOESY and TOCSY experiments.

INTRODUCTION

Pea plants (*Pisum sativum*) have been classically used as a model system in physiological and biochemical studies [1–7]. In fact the biosynthesis of flavonols and anthocyanins [7], and composition changes with light and developmental events [1, 5], have been reported in the pea. In addition, the role of pea flavonoids as modulators of the IAA system [4], and their accumulation in the vacuoles of pea epidermal guard cells [6] have been reported. More recently, flavonoids released by seeds and roots of legumes have been reported to act as signal metabolites [8]. In the process of symbiotic nitrogen fixation, the host legume releases flavonoids that stimulate the coordinated expression of bacterial genes required for nodulation (nod genes), as a key step in biological nitrogen fixation [9, 10].

In spite of the number and relevance of these studies, the structures of flavonoids from pea plants have never been studied using the new analytical and spectroscopic techniques. In previous studies, the occurrence of the 3-sophorotriosides of quercetin and kaempferol and their acylated derivatives with *p*-coumaric and ferulic acids have been reported [1–8, 11]. However, their structures had never been studied in detail. As a first part of our study on the effect of mineral nutrition on flavonoid metabolism in the pea, the aim of the present work was the isolation and identification of pea leaf flavonoids.

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RESULTS AND DISCUSSION

From pea shoot extracts seven flavonoids (**1–7**) were separated and purified by a combination of column chromatography with Sephadex LH-20, reversed-phase LPLC and semipreparative HPLC. The purity of the compounds was tested by analytical HPLC, and capillary zone electrophoresis (CZE) which provided a better analytical resolution than HPLC.

The UV spectra of these compounds in methanol (recorded with the Diode Array Detector) clearly indicated that **4–7** were acylated with cinnamic acid derivatives [12], while the other three compounds (**1–3**) were flavonols non-acylated with aromatic acids. The UV study of **1– 3** in methanol and after the addition of the classical shift reagents, readily indicated that **1** and **3** were quercetin derivatives and that **2** was a kaempferol derivative, the hydroxyl at the 3-position being blocked in all instances [13]. All compounds were then submitted to alkaline hydrolysis. Compounds **1–3** remained unaltered, but the other four compounds (**4–7**) released **1** and the aromatic acids *p*-coumaric, caffeic, ferulic and sinapic, respectively, identified by HPLC comparisons with authentic markers. Acid hydrolysis of all compounds yielded glucose as the only sugar (TLC and PC) and quercetin with the exception of **2** which provided kaempferol (HPLC comparison with authentic markers).

The different flavonoids were then analysed by FAB-mass spectrometry (positive mode), and the spectra indicated that **1** is a triglucoside of quercetin (losses of three glucose residues were observed), **2** a triglucoside of kaempferol, **3** a monoglucoside of quercetin, and **4–7** the

caffeyl, *p*-coumaryl, ferulyl and sinapyl triglucosides of quercetin, respectively. The FAB-mass spectra showed clearly the peak for the quasimolecular ions [M + H], those of the aglycones and those of the intermediate monoglucosides. However, other expected fragments such as those of the loss of the acyl residue [M + H − acyl] or those of those of the loss of the acyl residue and the terminal glucose [M + H − acyl − glucose] were not detected in any of the spectra.

NMR experiments were then carried out to establish the interglycosidic linkage in the different flavonoids, and the position for the acylation with the aromatic acids. The ^{1}H NMR spectra confirmed the nature of the aglycones, the presence of three β-D-glucopyranoses in the molecules and the existence and nature of the acyl residues (Table 1). TOCSY experiments were used to extract the spectra of individual sugars from the spectrum of the flavonoid polyglycosides, as suggested previously for other natural compounds [14, 15]. By this means each of the three readily identifiable anomeric proton signals were related to the particular subset of sugar proton signals simplifying the further analysis of the spectrum. The interglycosidic linkage was unambiguously established by means of NOE experiments. Strong NOE correlations were observed between H-1 of the terminal sugar and H-2 of the second sugar, and H-1 of the second sugar and H-2 of the sugar linked directly of the flavonoid (Fig. 1). In addition, weak NOEs of the

Table 1. ^{1}H NMR spectral data of the isolated flavonoids (600 MHz, in DMSO-d_6, 2 mg flavonoid in 0.5 ml solvent)

H	1	4	5	6	7
Flavone					
6	6.17 *d* ($J_{6,8}$ 1.8)	6.17 *d* ($J_{6,8}$ 2.0)	6.18 *d* ($J_{6,8}$ 2.0)	6.18 *d* ($J_{6,8}$ 2.0)	6.17 *d* ($J_{6,8}$ 2.0)
8	6.38 *d*	6.37 *d*	6.37 *d*	6.37 *d*	6.38 *d*
2′	7.58 *d* ($J_{2',6'}$ 2.0)	7.56 *d* ($J_{2',6'}$ 2.1)	7.57 *d* ($J_{2',6'}$ 2.2)	7.57 *d* ($J_{2',6'}$ 2.1)	7.58 *d* ($J_{2',6'}$ 2.1)
5′	6.85 *d* ($J_{5',6'}$ 8.1)	6.85 *d* ($J_{5',6'}$ 8.4)	6.86 *d* ($J_{5',6'}$ 8.4)	6.86 *d* ($J_{5',6'}$ 8.4)	6.85 *d* ($J_{5',6'}$ 8.4)
6′	7.59 *dd*	7.58 *dd*	7.60 *dd*	7.58 *dd*	7.59 *dd*
Acyl					
α		6.31 *d* ($J_{\alpha\beta}$ 15.8)	6.42 *d* ($J_{\alpha\beta}$ 15.9)	6.48 *d* ($J_{\alpha\beta}$ 15.9)	6.55 *d* ($J_{\alpha\beta}$ 15.8)
β		7.47 *d*	7.56 *d*	7.58 *d*	7.60 *d*
2		7.03 *d* ($J_{2,6}$ 1.9)	7.54 *d* ($J_{2,3}$ 8.6)	7.28 *d* ($J_{2,6}$ 1.7)	6.98 *s*
3			6.72 *d*		
5		6.70 *d* ($J_{5,6}$ 8.7)	6.72 *d* ($J_{5,6}$ 8.6)	6.74 *d* ($J_{5,6}$ 8.5)	
6		7.02 *dd*	7.54 *d*	7.08 *dd*	6.98 *s*
OMe 3				3.76 *s*	3.73 *s*
OMe 5					3.73 *s*
Sugars					
A					
1	5.73 *d*	5.73 *d*	5.76 *d*	5.75 *d*	5.75 *d*
2	3.48	3.48	3.49	3.48	3.48
3	3.58	3.52	3.55	3.54	3.53
4	3.13	3.15	3.16	3.15	3.15
5	3.08	3.04	3.03	3.04	3.03
6a	3.52	3.45	3.41	3.44	3.43
6b	3.26	3.25	3.24	3.25	3.25
B					
1	4.71 *d*	4.75 *d*	4.76 *d*	4.75 *d*	4.75 *d*
2	3.27	3.28	3.29	3.29	3.29
3	3.39	3.40	3.40	3.40	3.40
4	3.20	3.19	3.18	3.18	3.18
5	3.05	3.03	3.05	3.04	3.04
6a	3.44	3.45	3.46	3.45	3.45
6b	3.40	3.40	3.40	3.40	3.40
C					
1	4.51 *d*	4.58 *d*	4.59 *d*	4.58 *d*	4.58 *d*
2	3.03	3.08	3.08	3.08	3.08
3	3.20	3.24	3.24	3.24	3.24
4	3.09	3.17	3.16	3.17	3.16
5	3.25	3.53	3.54	3.54	3.54
6a	3.74	4.42	4.46	4.44	4.44
6b	3.50	4.17	4.15	4.16	4.16

Proton assignation was achieved by TOCSY (60 mecs).
Coupling constants (J in Hz) in parentheses.
$J_{1,2}$ was around 8.0 Hz in all sugars.

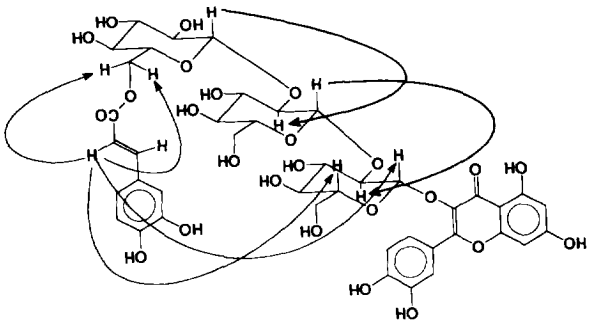

Fig. 1. Compound **4**, observed NOEs are indicated by arrows.

anomeric protons with H-1 and H-3 of the glucose at which they were linked were also observed confirming the 1 → 2 linkage between glucoses in all compounds. The NOE experiment was equivocal for the unambiguous determination of acylation since the proton α to the acyl residue showed NOEs with protons H-6a and H-6b of the terminal glucose and protons H-1 and H-5 of the glucose linked directly to the flavonol nucleus (Fig. 1). This suggests that the linkage of the acyl residue could be to the hydroxyl at the 6-position of the terminal sugar, and that the acyl residue is stereospatially close to the sugar directly linked to the aglycone. The acylation site was elucidated by comparison of the spectra of the acylated flavonoids (**4**–**7**) with that of the unacylated triglucoside (**1**). This was readily established since the responses of the δ values for H-6a and H-6b in the terminal sugars were shifted to lower fields in the acylated derivatives when compared with those values for the unacylated triglycoside. Therefore, for **5**, the $\Delta\delta = \delta_5 - \delta_1$ was 0.72 ppm for H-6a of the terminal sugar, 0.65 ppm for H-6b of the terminal sugar and 0.29 ppm for H-5 of the same sugar, while the $\Delta\delta$ for the rest of the protons was smaller than 0.1 ppm (Table 1). The same values of $\Delta\delta$ were observed for **4**, **6** and **7** showing clearly the same acylation position as for **5**.

Thus **1** is quercetin 3-sophorotrioside, **2** is kaempferol 3-sophorotrioside, **3** is quercetin 3-glucoside, **4** is quercetin 3-[6-caffeyl-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside], **5** is quercetin 3-[6-*p*-coumaryl-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside], **6** is quercetin 3-[6-feruloyl-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside], and **7** is quercetin 3-[6-sinapyl-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranosyl(1 → 2)-β-D-glucopyranoside]. As recently reported [16], acylation of flavonoids is a rare feature in the tribe Vicieae of the Leguminosae, since aromatic acylation is still only known in *Pisum*, although aliphatic acylation has been detected in *Vicia faba* [17].

EXPERIMENTAL

Plant material and flavonoid extraction. Pea shoots (2 kg fresh weight), without flowers or pods were macerated at room temp. in 3 l MeOH–H_2O (7:3) for two days. The extract was concd to dryness under red press (40°) and redissolved in MeOH.

Flavonoid isolation. The extract dissolved in MeOH was chromatographed through a Sephadex LH-20 column (Pharmacia) with MeOH and the flavonoid fractions followed under UV light (360 nm) and separated from chlorophylls and other compounds. The flavonoid fraction was then submitted to fractionation by LPLC on a Lobar C-18 column eluting with MeOH–H_2O mixtures starting with MeOH–H_2O (3:7) and increasing the MeOH proportion to end with MeOH–H_2O (1:1), and the elution of flavonoids was followed by UV light (360 nm). The different fractions obtained were analysed by HPLC to test their composition. The fractions needing further purification were re-chromatographed on the same column in order to recover pure fractions and then again by Sephadex LH-20 now eluting with MeOH–H_2O (9:1) as a previous step to the final purification by semiprepd HPLC. This was achieved on a Spherisorb ODS-2 column (5 μm particle size, 25 × 0.7 cm), using as solvent isocratic hydroalcoholic mixtures between 30 and 35% MeOH depending on the polarity of substance to be isolated, and the chromatographic behaviour observed on analytical HPLC.

Alkaline hydrolysis. The pure flavonoids (*ca* 1 mg) were dissolved in 1 ml of NaOH 3 M and left for 24 hr at room temp. in an stoppered test-tube under N_2 atmosphere. The solution was taken to pH 4 with conc HCl, and extracted with Et_2O (to recover free phenolic acids) and *n*-BuOH (to recover flavonol glycosides in which the acyl residues had been removed). The hydrolysis products were analysed by HPLC against authentic markers under the same conditions described below.

Acid hydrolysis. The isolated flavonoids were dissolved in 1 ml 2 M HCl and heated at 90° for 1 hr. The hydrolysate was extracted with EtOAc to recover flavonol aglycones and the remaining water contained the sugars. Flavonoid aglycones were identified by HPLC against authentic markers, and sugars by PC with PhOH–H_2O (4:1) and *n*-BuOH–HOAc–H_2O (4:1:5, upper phase), and using *p*-anisidine as spray reagent.

HPLC analysis. All extracts, fractions and isolated compounds were HPLC analysed with a LiChrospher 100 RP-18 column (5 μm particle size, 12.5 × 0.4 cm) using as solvents MeOH (solvent B) and H_2O–HCO_2H (19:1) (solvent A). Elution was performed with a gradient starting with 15% solvent B to reach 35% B at 40 min, and with a solvent flow rate of 1 ml min^{-1}, and column thermostated at 40°. Detection was achieved with a diode array detector (DAD).

CE analysis. Capillary zone electrophoresis was achieved on a fused silica capillary (50 cm effective length, 75 μm internal diameter), using as buffer 0.1 M boric acid taken to pH 9.5 with NaOH pellets, injected hydrodynamically (9 nl) 3 sec and detection at 280 nm (Beckman Pace-5000).

FAB-MS analyses. These were achieved in the positive mode, using *m*-NBA as matrix. **1**: UV λ_{max}^{MeOH} nm: 257, 267sh, 303sh, 356; + NaOMe, 272, 328, 404; + $AlCl_3$, 275, 303sh, 432; + $AlCl_3$ + HCl, 270, 298sh, 360, 390sh;

+ NaOAc, 270, 325sh, 380; + NaOAc + H_3BO_3, 263, 298sh, 379. FAB-MS (positive mode): 789 [M + H], 627 [M + H − Glc], 465 [M + H − 2Glucose], 303 [M + H − 3Glc]. Alkaline hydrolysis: no modification. Acid hydrolysis: quercetin and glucose. TLC cellulose (Merck) H_2O R_f 0.34; 15% HOAc R_f 0.70. HPLC R_t 11.3 min. CZE M_t 7.63 min. **2**: UV λ_{max}^{MeOH} nm: 255sh, 266, 296sh, 348; + NaOMe, 274, 318, 395; + $AlCl_3$, 275, 303, 350, 385sh; + $AlCl_3$ + HCl, 274, 300sh, 348, 383sh; + NaOAc, 274, 298, 372; + NaOAc + H_3BO_3, 266, 295, 355. FAB-MS (positive mode) : 773 [M + H], 611 [M + H − Glc], 449 [M + H − 2Glc], 287 [M + H − 3Glc]. Alkaline hydrolysis: no modification. Acid hydrolysis: kaempferol and glucose. TLC cellulose (Merck) H_2O R_f 0.39; 15% HOAc R_f 0.75. HPLC R_t 15.9 min. CZE M_t 7.50 min. **3**: UV λ_{max}^{MeOH} (nm): 256, 265sh, 297, 355; + NaOMe, 270, 330, 409; + $AlCl_3$, 248sh, 275, 305, 435; + $AlCl_3$ + HCl, 269, 302, 363, 386; + NaOAc, 269, 298sh, 330, 387; + NaOAc + H_3BO_3, 264, 295, 378. FAB-MS (positive mode): 465 [M + H], 303 [M + H − glucose]. Alkaline hydrolysis: no modification. Acid hydrolysis: quercetin and glucose. TLC cellulose (Merck) H_2O R_f 0.06; 15% HOAc R_f 0.32. HPLC R_t 30.0 min. CZE M_t 8.74 min. **4**: UV λ_{max}^{MeOH} nm: 256, 267sh, 300sh, 337; + NaOMe, 272, 320sh, 388; + $AlCl_3$, 274, 300sh, 355, 425; + $AlCl_3$ + HCl, 272, 298sh, 335, 390; + NaOAc, 269, 359; + NaOAc + H_3BO_3, 262, 298, 363. FAB-MS (positive mode): 951 [M + H], 465 [M + H − caffeyl − 2Glc], 303 [M + H − caffeyl − 3Glc]. Alkaline hydrolysis yielded: caffeic acid and **1**. Acid hydrolysis yielded: quercetin and glucose. TLC cellulose (Merck) H_2O R_f 0.16; 15% HOAc R_f 0.50. HPLC R_t 18.2 min. CZE M_t 8.95 min. **5**: UV λ_{max}^{MeOH} nm: 258, 268sh, 317, 362sh; + NaOMe, 272, 315sh, 373; + $AlCl_3$, 276, 310, 431; + $AlCl_3$ + HCl, 275, 314, 357sh, 390sh; + NaOAc, 272, 318, 381; + NaOAc + H_3BO_3, 264, 298sh, 316, 378. FAB-MS (positive mode): 935 [M + H], 465 [M + H − *p*-coumaroyl − 2Glc], 303 [M + H − *p*-coumaroyl − 3Glc]. Alkaline hydrolysis yielded: *p*-coumaric acid and **1**. Acid hydrolysis yielded: quercetin and glucose. TLC cellulose (Merck) H_2O R_f 0,23; 15% HOAc R_f 0.60. HPLC R_t 24.1 min. CZE M_t 7.98 min. **6**: UV λ_{max}^{MeOH} nm: 256, 268sh, 295sh, 331; + NaOMe, 270, 386; + $AlCl_3$, 276, 327, 435; + $AlCl_3$ + HCl, 274, 326, 390sh; + NaOAc, 272, 326, 387; + NaOAc + H_3BO_3. 263, 300sh, 326, 370sh. FAB-MS (positive mode): 965 [M + H], 465 [M + H − ferulyl − 2Glc], 303 [M + H − ferulyl − 3Glc]. Alkaline hydrolysis: ferulic acid and **1**. Acid hydrolysis: quercetin and glucose. TLC cellulose (Merck) H_2O R_f 0.21; 15% HOAc R_f 0.60. HPLC R_t 25.1 min. CZE M_t 8.19 min. **7**: UV λ_{max}^{MeOH} (nm): 256, 268sh, 332; + NaOMe, 270, 388; + $AlCl_3$, 276, 325, 434; + $AlCl_3$ + HCl, 274, 324, 390sh; + NaOAc, 272, 325, 388; + NaOAc + H_3BO_3, 263, 300sh, 325, 370sh. FAB–MS (positive mode): 995 [M + H], 465 [M + H − sinapyl − 2Glc], 303 [M + H − sinapyl − 3Glc]. Alkaline hydrolysis: sinapic acid and **1**. Acid hydrolysis: quercetin and glucose. TLC cellulose (Merck) H_2O R_f = 0.21; 15% HOAc R_f = 0.60. HPLC R_t = 25.7 min. CZE M_t = 8.31 min.

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