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7- AND 8-*O*-METHYLHERBACETIN-3-*O*-SOPHOROSIDES FROM BEE POLLENS AND SOME STRUCTURE/ACTIVITY OBSERVATIONS

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Key Word Index—*Ranunculus sardous*; *R. raphanistrum*; Ranuculaceae; *Ulex europaeus*; Leguminosae; bee pollen; 7-*O*-methylherbacetin 3-*O*-sophoroside; 8-*O*-methylherbacetin 3-*O*-sophoroside; 8-*O*-methylherbacetin-3-glucoside; structure/activity.

Abstract—A new flavonol glycoside, 7-*O*-methylherbacetin-3-*O*-sophoroside, has been characterized from *Ranunculus sardous* pollen, 8-*O*-methylherbacetin-3-*O*-sophoroside from *Ulex europaeus* pollen and 8-*O*-methylherbacetin 8-*O*-glucoside from *Raphanus raphanistrum* pollen; all pollens were obtained from bee pollen. It is demonstrated that the unusual UV–visible absorption spectra of 7- and 8-*O*-methylherbacetin 3-*O*-glycosides can be used conveniently to distinguish these and their aglycones. The distribution of various flavonol glycosides in pollen is discussed in terms of its significance to the previously established role of flavonols in pollen germination. No evidence for taxa specificity of individual flavonol glycosides was found; however, a case is made for elevating the significance of flavonol glycosides, particularly the 2″-*O*-glycosides of flavonol-3-*O*-glycosides in stimulating pollen tube growth. Copyright © 1996 Elsevier Science Ltd

INTRODUCTION

Bee pollen is collected from selected flower species by the honey bee *Apis mellifera*, to feed their larvae in the early stages of development, and normally consists of pollens from a mixture of species. These pollens have been shown to be unaffected, in terms of their flavonoid constituents, by the bees collection and pelletization procedures, and thus can provide a worthwhile source of species-specific flower pollen [1]. Flower pollen has been shown to contain on its surface a wide range of flavonoids, both glycosides and aglycones together with cinnamic acid derivatives [1–10]. Flavonols are commonly encountered in the pollen of flowering plants and these have recently been shown to perform an essential physiological function in pollen germination and pollen tube growth [11–13]. While structure–activity studies carried out with *Petunia* define important structural features necessary in the aglycone for effective function, there remains doubt about the role and significance of the widely encountered glycosidic forms [12, 13].

In the course of an HPLC survey of the flavonoids in a range of pollens sourced from New Zealand and Portuguese bee pollens, a number of novel and rare herbacetin-based glycosides were encountered. This paper describes the structure elucidation of these compounds and uses these data, together with related published data, to speculate on structure–activity relationships in the glycosides of pollen flavonoids.

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RESULTS

HPLC analyses of a range of bee pollens from New Zealand and Portugal revealed a number of components with unusual absorption spectra. These were accompanied by other components clearly identifiable as flavonoids and cinnamic acid derivatives from their absorption spectra. The unusual spectra of compounds **1**, **2** and **3** and their aglycones (Fig. 1) were not immediately recognized as flavonoid spectra (e.g. see ref. [14]) although subsequent NMR studies confirmed that these compounds were indeed flavonoids.

Compound **1** (RT 30.94) was isolated from pollen of *Ranunculus sardous* Crantz. and appeared on paper as a dark UV-absorbing spot that was largely unaffected by NH_3. It hydrolysed readily under normal hydrolysis conditions to give an aglycone, **1a**, which appeared yellowish–olive in UV. These observations are suggestive of a flavonol-3-*O*-glycoside formulation for **1**. Compound **1** was shown to possess free 5- and 4′-hydroxyl groups and to lack an *ortho*-dihydroxyl group, and possibly a 7-hydroxyl group, by the effects of shift reagents [14] on the absorption spectrum. ^{1}H NMR specroscopy (Table 1) revealed the presence of 2′, 6′ and 3′, 5′ protons on the B-ring and a single isolated

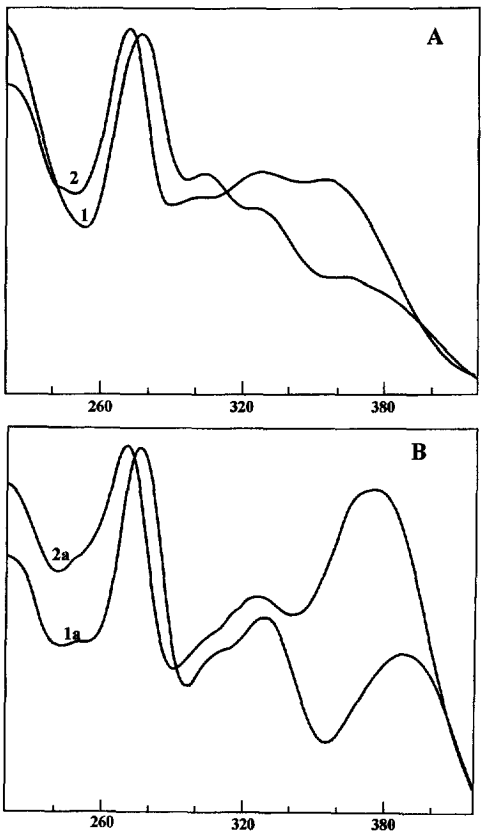

Fig. 1. UV–visible absorption spectra of (A) 7-*O*-methylherbacetin 3-*O*-sophoroside (**1**) and 8-*O*-methylherbacetin 3-*O*-sophoroside (**2**) and (B), their aglycones **1a** and **2a**, as recorded during HPLC analysis.

proton (δ 6.55) on the A-(or C-) ring. One methoxyl was also evident, and the two doublets at δ 5.69 and δ 4.63 showing *ca* 7 Hz coupling indicated the presence of two sugars, each β-linked through C-1. The ^{13}C NMR spectrum (Table 1) confirmed the above and also defined the disaccharide as a 3-*O*-linked sophorose [15]. The methoxyl signal at 56.6 ppm is representative of a noncrowded methoxyl [15].

On the basis of the above, the structure of compound **1** is indicated to be the 3-*O*-sophoroside of an A-ring *O*-methylated 5,6,7,4′- or 5,7,8,4′-oxygenated flavonol. HMQC (^{13}C, ^{1}H-COSY) and HMBC (inverse long-range ^{13}C–^{1}H COSY) measurements were used to define the remaining structural features. The proton singlet at δ 6.55 correlated strongly with the carbon signal at 95.7 ppm which in tri-oxygenated A-ring flavonols equates to C-6 if the 7-hydroxyl is methylated [15, 16]. That this singlet represents H-6 was confirmed by the HMBC connectivities to C-5, 7, 8 and 10. Connectivity between the methoxyl signal (δ 3.90) and C-7 (154.1 ppm) in the HMBC established the site of methylation as the 7-hydroxyl group. The structure of compound **1** is therefore established as 7-*O*-methylherbacetin 3-*O*-sophoroside. Although the aglycone has been found twice before [17], it has not been found in pollen. This is the first recorded example of a glycoside of 7-*O*-methylherbacetin [17].

Compound **2**, with similar HPLC retention time (31.54 min), similar appearance on paper to **1** and which hydrolysed readily to a similar but different (HPLC, TLC) aglycone, **2a**, was isolated from *Ulex europaeus* L. pollen and detected in *Lotus* (*corniculatus*?) pollen. Like compound **1** it possessed an unusual absorption spectrum (Fig. 1). The NMR spectra differed from those of compound **1**, only in that the A-ring proton singlet appeared at δ 6.29, a more usual position for a C-6 proton, and in that the ^{13}C signal for the methoxyl appeared at 60.6 ppm, indicating an *ortho*-disubstituted site for the methoxyl [15]. The NMR spectra confirmed the presence of a 3-*O*-linked sophorosyl moiety (Table 1). As for compound **1**, HMQC measurements confirmed the siting of the A-ring proton at C-6, but in contrast to compound **1**, HMBC correlated the methoxyl with C-8 (127.5 ppm). These data together with the absorption spectra which identified free hydroxyls at positions 5, 7 and 4′, define the structure of compound **2** as 8-*O*-methylherbacetin 3-*O*-sophoroside. This flavonoid has been found only once before [1], in the pollen of *Prunus amygdalus* (almond), although an unidentified 8-*O*-methylherbacetin glycoside has been detected in the pollen of *Nothofagus antarctica* (Fagaceae) [18].

One further related compound, **3**, was encountered in *Raphanus raphanistrum* L. pollen as a minor component. With an HPLC retention time of 35.3 min and an absorption spectrum identical to that of compound **2**, it appeared to be a monoglycoside of 8-*O*-methylherbacetin. Indeed, 8-*O*-methylherbacetin and not the 7-*O*-methyl isomer was produced, together with glucose, on acid treatment which liberated a 3-hydroxyl group. ^{1}H NMR of compound **3** confirmed the presence of this oxygenation pattern (Table 1) and of the single methoxyl. Only one sugar H-1 was evident (at δ 5.41), which at higher field than in compound **2**, is in the range expected for a 3-linked glucose lacking 2″-*O*-glycosylation [19]; furthermore, ^{1}H–^{1}H COSY revealed that H-2 and other sugar protons resonate in the δ 3–3.8 range expected for an unsubstituted glucosyl moiety [19]. Accordingly, compound **3** is assigned the structure, 8-*O*-methylherbacetin 3-*O*-β-D-glucoside. This is a known compound, but like **2** appears to be rare in nature.

DISCUSSION

The data above assign the structures of compounds **1** and **2** and their aglycones unequivocally, but distinguishing between the two aglycones or the two glycosides without reference standards, would require a detailed NMR study were it not for the highly distinctive absorption spectra (Fig. 1). Such spectra, now related to the respective structures, permit immediate recognition/distinction of such compounds during HPLC analysis.

Flavonols and/or their 3-glycosides in pollen have

Table 1. ^{1}H- and ^{13}C-NMR spectral data for compounds **1**, **2** and **3***

Position	**1**		**2**		**3**
	^{13}C (ppm)	^{1}H (δ)	^{13} (ppm)	^{1}H (δ)	^{1}H (δ)
2	156.0		155.5		
3	(132.8)		130.6		
4	178.8		177.7		
5	153.0	12.2 *s* (OH)	156.8[a]	12.30 *s* (OH)	12.23 *s* (OH)
6	95.7	6.55 *s*	98.9	6.29 *s*	6.23 *s*
7	154.1		155.9[a]		
8	126.2		127.5		
9	(149)		148.5		
10	104.4		103.8		
1′	121.3		121.0		
2′	131.3	8.13d, 8.8 Hz	130.8	8.07d, 8.5 Hz	8.05d, 9.0 Hz
3′	115.5	6.93d, 8.8 Hz	115.4	6.95d, 8.5 Hz	6.92d, 9.0 Hz
4′	160.3		160.0		
5′	115.5	6.93d, 8.8 Hz	115.4	6.95d, 8.5 Hz	6.92d, 9.0 Hz
6′	131.3	8.13d, 8.8 Hz	130.8	8.07d, 8.5 Hz	8.05d, 9.0 Hz
1″	98.2	5.69d, 7.0 Hz	98.0	5.72d, 7.2 Hz	5.41d, 7.0 Hz
2″	82.6	3.5	82.4	3.5	3.2
3″	76.0[b]	3.1–3.5	76.6[b]	3.1–3.5	3–3.8
4″	69.9[c]	3.1–3.5	69.6[c]	3.1–3.5	3–3.8
5″	77.7[b]	3.1–3.5	77.6[b]	3.1–3.5	3–3.8
6″	60.8[a]	3.1–3.7	61.0[d]	3.2–3.8	3–3.8
1‴	104.3	4.63d, 7.7 Hz	104.1	4.62d, 7.5 Hz	
2‴	74.6	3.1	74.4	3.1	
3‴	76.0[b]	3.1–3.5	76.6[b]	3.1–3.5	
4‴	69.8[c]	3.1–3.5	69.7[c]	3.1–3.5	
5‴	77.2[b]	3.1–3.5	77.0[b]	3.1–3.5	
6‴	60.1[a]	3.1–3.7	60.8[a]	3.2–3.8	
OMe	56.6	3.90 *s*	60.6	3.82 *s*	

*In DMSO-d_6 solvent relative to TMS; assignments confirmed where possible by HMBC and HMQC measurements; assignments bearing the same superscript may be reversed; figures in parentheses are uncertain.

been shown to act as essential stimulants of pollen tube growth in some flowering plants [11–13] and as such appear to play a vital role in the plant fertilization process. Species-, genus-, and family-specific flavonoid patterns have been observed in a number of surveys of pollen flavonoids (e.g. refs [1] and [7]) and it is therefore tempting to speculate that the flavonoid constituents might act to provide a mechanism for restricting cross-pollination outside of a particular taxonomic grouping. In this report, the finding of the rare 8-*O*-methylherbacetin 3-*O*-sophoroside as the major flavonoid in *U. europaeus* is of interest. This compound has been encountered previously only in the pollen of *P. amygdalus* (Rosaceae) although its aglycone occurs as the 3-glucoside and 3-neohesperidoside in the pollen of hawthorn (*Crataegus monogyna*), another member of the Rosaceae [20]. Thus, although there is a familial relationship between some of the pollens containing this rare aglycone, its finding as the 3-*O*-sophoroside in the Leguminosae (*Lotus corniculatus*?) and as the 3-*O*-glucoside in *R. raphanistrum* L. (*Cruciferae*), suggests that the flavonol glycoside content is at best only one of a number of factors involved in the restriction of cross-pollination.

For the function of flavonols in stimulating pollen tube growth, it remains to be established unequivocally whether the germination stimulating form is the aglycone or the glycoside [12, 13]. Of interest in this respect is the observation both from our own findings and from the literature, that flavonol glycosides found in the pollens studied to date, from a wide range of families, almost invariably include a 2″-*O*-glycosylated flavonol-3-*O*-glycoside. For example: quercetin 3-*O*-glucosyl(1,2)galactoside from *Corylus avellana* (Betulaceae) [8] and from *Petunia hybrida* (Solanaceae) [21]; 8-*O*-methylherbacetin 3-*O*-sophoroside from *Prunus amygdalus* (Rosaceae) [1]; 8-*O*-methylherbacetin 3-*O*-neohesperidoside from *Crataegus monogyna* (Rosaceae) [20]; isorhamnetin 3-*O*-neohesperidoside from *Zea mays* (Gramineae) [17]; kaempferol and quercetin 3-*O*-sophorosides from trees in the families Juglandaceae, Betulaceae, Fagaceae and Oleaceae [5]; kaempferol 3-*O*-[2-rhamnosyllaminaribioside] from *Populus yunnanensis* (Salicaceae) [9]; and kaempferol 3-*O*-[2^{G}-rhamnosylrutinoside], and isorhamnetin and quercetin 3-*O*-neohesperidosides from *Typha augustifolia* (Typhaceae) [10]. This preponderance of flavonol 3-*O*-di- and tri-glycosides containing a 1,2 interglycosidic linkage is unlikely to be coincidence, especially as it encompasses such a wide range of taxa. Indeed, it strongly suggests that the presence and nature of these

glycosidic flavonol derivatives have significance in addition to that of the aglycone hydroxylation pattern parameters previously established [12, 13]. Interestingly, the only 3-linked sugars encountered in the polyglycosides, glucose and galactose, both have the same configuration at C-2. Thus, there is also a strong conformational relationship linking the reported flavonol polyglycosides in pollens.

EXPERIMENTAL

Plant material. Commercially marketed New Zealand bee pollen, representing a blended 1994 early summer collection, was provided by Comvita (NZ) Ltd, and Portuguese bee pollens were provided by Portuguese bee-keepers near Coimbra who collected pollen expressly for this research (see ref. [22] for details).

Both *U. europaeus* and *R. raphanistrum* pollens were identified by microscopy and direct HPLC comparison with manually gathered pollen (Eastern Hutt hills, NZ, and Coastal, Central Portugal, respectively). *Ranunculus sardous* Crantz. pollen was identified by HPLC comparison with pollen from authentic *R. sardous* (Herbarium specimen: WELTU 16841) collected from the property of Dr Keith Hammett, Massey, Auckland, NZ.

Extraction and isolation. Bee pollen samples were sorted into species-specific pollens on the basis of colour, microscopy and HPLC patterns using a systematic method described in detail in ref. [22]. Pellets of species-specific pollen were fragmented in EtOH–H_2O (1:1) with ultrasound, and the dispersed pollen grains left to extract overnight. The extracts were sepd by centrifugation before application to sheets of Whatman 3 MM chromatography paper for 1D separation. Compound **1** was sepd in 10% HOAc (R_f 0.70) from an extract of *R. sardous* pollen, **2** in 15% HOAc (R_f 0.80) from *U. europaeus* pollen, and **3** in 15% HOAc (R_f 0.50) from *R. raphanistrum* pollen. Each was prepared for further study by chromatography on a C-8 reversed-phase column using MeOH–H_2O [23] and checked for purity by HPLC (see below).

7-O-*Methylherbacetin* 3-O-*sophoroside* (**1**). HPLC *Rt* = 30.94 min UV (λ_{max}^{MeOH}) nm: 278, 307, 330, 370 sh; +NaOMe: 252 sh, 278 sh, 305 sh, 377 (inc); +NaOAc: 278, 307, 330, 386; +NaOAc + H_3BO_3: as for MeOH; +$AlCl_3$: 282, 312, 355, 420 sh. NMR spectra: Table 1.

8-O-Methylherbacetin 3-O-sophoroside (**2**). HPLC *Rt* = 31.54 min. UV (λ_{max}^{MeOH}): 274, 300 sh, 324, 355 sh; +NaOMe: 284, 334, 407 (inc); +NaOAc: 284, 333, 406; +NaOAc + H_3BO_3: as for MeOH; $AlCl_3$: 235 sh, 282, 312, 354, 405. NMR spectra: Table 1.

8-O-*Methylherbacetin* 3-O-*glucoside* (**3**). HPLC *Rt* = 35.3 min. UV (MeOH, and +NaOMe) as for **2**. ^{1}H NMR spectrum: Table 1.

Hydrolysis conditions. Compounds **1**, **2** and **3** were each treated with 3 N HCl–MeOH (1:1) for 15 min at 100°. The resulting aglycones, **1a**, **2a** and **3a**, were removed at room temperature by extraction with EtOAc and compared by TLC on cellulose (50% HOAc). R_f values before (and after) hydrolysis: **1**, 0.90, **1a**, 0.50; **2**, 0.90, **2a**, 0.57; **3**, 0.81, **3a**, 0.57. Spot colours in UV (366 nm) before hydrolysis: dk → deep olive (NH_3); after hydrolysis: yellowish–olive → dk (NA spray). HPLC *Rts*: see below. Sugars from the aqueous layer were identified by PC in *n*-BuOH–pyridine–H_2O (6:4:3).

HPLC analysis. HPLC analyses were carried out at 24° on an end-capped 100 RP18, 5μ column with a flow rate of 0.8 ml min^{-1}. A linear solvent gradient was used starting with 100% of solvent A (H_2O adjusted to pH 2.5 with orthophosphoric acid). The level of solvent B (CH_3CN) was increased to 9% by 12 min, to 13% by 20 min, and to 33% by 40 min. This level was maintained for 2 min before being increased to 43% by 60 min. Absorption spectra for all significant peaks were recorded over the range 220–400 nm using diode-array detection. *Rt* values in min: **1**, 30.94; **1a**, 45.60; **2**, 31.54; **2a**, 46.04; **3**, 35.30; **3a**, 46.04. Absorption spectra: Fig. 1.

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