Phytochemistry, Vol. 28, No. 7, pp. 1901–1903, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00

8-METHOXYKAEMPFEROL 3-SOPHOROSIDE, A YELLOW PIGMENT FROM ALMOND POLLEN

FEDERICO FERRERES, FRANCISCO A. TOMÁS-BARBERÁN, FRANCISCO TOMÁS-LORENTE, JOSÉ L. NIETO,* ANGEL RUMBERO† and JOSÉ M. OLÍAS‡

Laboratorio de Fitoquímica, C.E.B.A.S., (C.S.I.C.) Apdo. 195, Murcia 30080, Spain; *Inst. Estructura de la Materia, Serrano 119, Madrid 28006, Spain; †Departamento de Química Orgánica, U.A.M., Canto Blanco, Madrid 28049, Spain; ‡Instituto de la Grasa, C.S.I.C., Apdo, 1078, Sevilla 41012. Spain

(*Received in revised form* 5 *December* 1988)

Key Word Index—*Prunus amigdalus*; Rosaceae; almond pollen; flavonols; 8-methoxykaempferol 3-*O*-(2″-β-D-glucopyranosyl-β-D-glucopyranoside); bee pollen.

Abstract—The novel flavonol 8-methoxykaempferol 3-*O*-(2″-β-D-glucopyranosyl-β-D-glucopyranoside) has been found as a yellow pigment in almond pollen. In addition, trace amounts of kaempferol and quercetin 3-diglucosides have been detected. These compounds have been isolated from almond bee pollen, which is a convenient source for the study of flavonoids from natural pollen. HPLC studies show no differences in the flavonoid patterns of pollen and bee pollen, and demonstrate that 8-methoxykaempferol 3-sophoroside is the main flavonoid in almond pollen, while other flower and leaf tissues are devoid of this pigment. This compound is also absent from other botanically related pollens (plum, apple, cherry, and pear).

INTRODUCTION

Plant pollens are well known as an interesting source of flavonoids, and glycosylated flavonols have been reported from *Corylus avellana* [1], and *Zea mays* [2] recently. These flavonol glycosides are located on the surface of the exine and are not covalently attached to the sporopollenin [3]. They have been found as pollen specific markers in the pollen of some trees [4]. The physiological, biochemical or ecological roles of these hydrophilic flavonoids in pollen remain unknown. The main problem in the study of the pollen flavonoids of most species is the difficulty in collecting sufficient pollen to achieve structural determinations or biological activity assays. In a recent study in which flavonoids were used as biochemical markers of the plant origin of bee pollens, no differences were found between the flavonoid patterns of bee pollen and natural plant pollen of the same species [5]. In the present study we have used bee pollen for the isolation and identification of the yellow pigment of almond pollen. In addition, an HPLC examination of the occurrence of this pigment in different flower tissues and in pollen from different botanically related species (Rosaceae) has been carried out.

RESULTS AND DISCUSSION

The yellow pigment (**1**) was isolated from almond bee pollen extracts by PC and LPLC on a reversed phase column and by CC on Sephadex LH-20. Its UV spectrum in methanol and the shapes and positions of bands I and II after addition of $AlCl_3$+HCl and alkaline reagents suggested that **1** was a flavone with free hydroxyls at the 5, 7 and 4′-positions, and substituted hydroxyls at the 3 and 8 positions [6, 7]. After acid hydrolysis, glucose and 8-methoxykaempferol (3,5,7,4′-tetrahydroxy-8-methoxyflavone) were detected. Upon controlled acid hydrolysis three fractions were visualized under UV light (360 nm) and identified as 8-methoxykaempferol 3-glucoside, the aglycone and the naturally occurring glycoside. Acid hydrolysis of the permethylated glycoside yielded an aglycone with a free hydroxyl at the 3-position as suggested by its UV analysis [7], and 2,3,4,6-tetramethylglucose and 3,4,6-trimethylglucose, tentatively identified by paper chromatography studies [8]. The EIMS spectrum of the permethylated derivative of **1** lacked fragments $[OS]^+$, $[S+60]^+$ and $[S]^+$ which are characteristic of a (1→6) linkage in flavonol disaccharides [9]. However, fragment $[OS-32]^+$, which was reported as characteristic of (1→2) linkages, was present in this spectrum with a relative abundance of 8%. The ^1H NMR data confirmed that this was a 8-methoxykaempferol 3-diglucoside and that the two glucoses were β-D-glucopyranoses [7]. The disaccharide was obtained by hydrogen peroxide treatment [10], and the R_g values obtained when analysed on PC, suggested that this could be sophorose or cellobiose. Chromatographic comparison with an authentic sample of cellobiose indicated that the disaccharide was sophorose. The structure of **1** was confirmed by ^{13}CNMR analysis in which the chemical shifts of the flavonoid nucleus carbons and the disaccharide carbons were in accordance to reported spectra [11–13]. All these data unambiguously indicate that the structure of the pigment of almond pollen is 8-methoxykaempferol 3-*O*-(2″-β-D-glucopyranosyl-β-D-glucopyranoside) (**1**), a new naturally occurring flavonoid glycoside [14].

Other quercetin and kaempferol 3-diglucosides were also detected, but in too small amount to allow their full

characterization. Their structures are suggested on the basis of UV studies of the natural glycosides and aglycones, chromatographic comparisons of the aglycones and sugars obtained by acid hydrolysis against markers, and by study of the chromatographic behaviour (TLC and HPLC) of the naturally occurring glycosides and the monoglucosides obtained by mild acid hydrolysis.

Other tissues of *Prunus amigdalus* were also examined for the presence of the yellow pigment (**1**) by means of HPLC coupled with a photodiode array detector. Initial screening showed that **1** was restricted to flower tissues and a more detailed examination of the different reproductive organs (Table 1), showed that it was the major flavonoid in pollen. Other tissues such as anthers and filaments were characterized by hydroxycinnamic derivatives (R_ts 1.71 and 1.05, respectively). Another quercetin 3-glycoside which is absent from pollen and is the major flavonoid in filaments and ovarids.

8-Methoxykaempferol 3-sophoroside was not found in an HPLC examination of the pollen of the related rosaceous fruit trees: cherry, plum, apple and pear. But was present in pollen of all the almond cultivar varieties tested. However, 8-methoxykaempferol 3-glucoside was previously identified within this family in the inflorescences of *Sorbus aucuparia* [14]. In addition, the 3-galactoside was detected in *Dryas octopetala* [15], the 3-rutinoside in *Fagonia arabica* [14], the 3-glucoside 7-rhamnoside in *Gossypium hirsutum* [16], the 3-glucoside-7-rutinoside in *Ephedra alata* [12] and the 3-rutinoside-7-rhamnoside in *Sedum sexangulare* [17].

The presence of flavonoid diglycosides in almond pollen confirms that such hydrophilic flavonoids are accumulated in pollen from different species, and that monoglycosides and triglycosides are much less frequent in these tissues. In addition, these results support the occurrence of pollen specific flavonoid patterns.

EXPERIMENTAL

Plant material. Almond bee pollen was obtained from the market and natural almond pollen and almond (different cvs), cherry, plum, apple and pear flowers were collected from cultivated trees. All plant material was identified and deposited in the Department of Botany, Murcia University.

Extraction and isolation. Almond bee pollen (*ca* 50 g) was defatted with *n*-hexane and extracted with EtOH–H_2O (24:1). The concd extract was redissolved in H_2O, partitioned with *n*-BuOH and the butanol extract chromatographed on Whatman No 3 paper with 2% HOAc and the flavonoid fractions purified by a combination of CC on Sephadex LH-20 with MeOH and LPLC (Lobar Lichroprep RP-8) with MeOH–H_2O (3:7) flow = 5 ml/min. 200 mg of the purified yellow pigment were obtained.

8-Methoxykaempferol 3-sophoroside. TLC (cellulose) H_2O, R_f 0.57; 2% HOAc, R_f 0.57; 15% HOAc, R_f 0.78; BAW, R_f 0.56. HPLC [Lichrospher 100 RP 18 (5 μm), MeOH–H_2O (2:3) isocratic 1 ml/min, detection at 340 nm], R_t 3.31 min. UV λ_{max}^{MeOH} nm: 355 sh (0.51), 322 (0.55), 296sh (0.53), 272 (1.00), 220 i (0.95); + NaOMe: 408 (inc.), 331, 282; + $AlCl_3$: 408, 352, 311, 280, 235 i; + $AlCl_3$ + HCl: 408, 349, 309, 280, 235 i; + NaOAc: 398, 307sh, 281; + NaOAc + H_3BO_3: 356sh, 325, 272. EIMS permethylated derivative, *m/z* (rel. int.) (70 eV) probe: 780 $[M]^+$ (1), 578 $[S+H+32]^+$ (3), 546 $[S+H]^+$ (2), 391 $[OS-32]^+$ (8), 385 (9), 358 $[A+H]^+$ (89), 329 $[A-28]^+$ (8), 218 $[T_1]^+$ (8), 187 $[T_2]^+$ (73), 155 $[T_3]^+$ (29), 111 (100). ^1H NMR (360 MHz, DMSO-d_6): δ 3.0–3.6 (sugar protons), 3.79 (3H, *s*, OMe), 4.62 (1H, *d*, *J* = 8 Hz, H-1‴), 5.65 (1H, *d*, *J* = 7 Hz, H-1″), 6.31 (1H, *s*, H-6), 6.96 (2H, *d*, *J* = 8.5 Hz, H-3′ and H-5′), 8.05 (2H, *d*, *J* = 8.5 Hz, H-2′ and H-6′). ^{13}C NMR (300 MHz, DMSO-d_6): 175.8 (C-4), 169.2 (C-4′), 160.2 (C-7), 156.6 (C-2), 153.1 (C-5), 148.4 (C-9), 131.9 (C-3), 130.4 (C-2′, 6′), 129.5 (C-8), 121.4 (C-1′), 115.4 (C-3′, 5′), 103.9 (C-1‴), 102.8 (C-10), 98.6 (C-1‴[a]), 98.2 (C-6[a]), 82.4 (C-2″), 77.3 (C-3‴[b]), 77.0 (C-5‴[b]), 76.6 (C-3″, 5‴[b]), 74.4 (C-2‴), 69.7 (C-4‴[c]), 69.5 (C-4‴[c]), 60.8 (C-6‴[d]), 60.5 (6‴[d]), 59.8 (MeO-) (assignments bearing the same superscript may be reversed).

Acid hydrolysis. The glycoside (**1**) was hydrolysed by means of aq. 2 M HCl (1 hr, 90°) in a sealed tube yielding 3,5,7,4′-tetrahydroxy-8-methoxyflavone identified by its UV study [7] and glucose (PC comparison with authentic samples). Mild acid hydrolysis was carried out on a Whatman No 3 paper on which a MeOH solution of the pigment was deposited as a narrow strip, and 2 M HCl was then deposited onto the dried flavonoid strip and heated for 1 min with a hair-drier. This was chromatographed with 15% HOAc and three fractions were observed: R_f 0.14 identified as 8-methoxykaempferol, R_f 0.70 identified as 8-methoxykaempferol 3-glucoside (UV comparision with naturally occurring glycoside). TLC (cellulose): H_2O, R_f 0.23; 2% HOAc, R_f 0.22; 15% HOAc, R_f 0.49; BAW, R_f 0.70. HPLC (see above) R_t 7.22 min), and R_f 0.86 coincident with the unhydrolysed glycoside.

Hydrogen peroxide hydrolysis. The disaccharide obtained by H_2O_2 treatment of an alkaline solution **1** [10], run on Whatman No 1 paper developed for 89 hours in *n*-BuOH–HOAc–H_2O (4:1:5, upper phase) against an authentic sample of cellobiose (cellobiose R_g 0.51; sophorose R_g 0.57; gentiobiose R_g 0.41; laminaribiose R_g 0.69).

Permethylated derivative of **1**. This was obtained with MeI and NaH by standard methods [18]. The PM derivative showed dull

Table 1. Distribution of phenolic compounds in almond reproductive organs

R_t(min)	Anthers	Filaments	Stamens	Pollen	Bee-pollen	Ovary
1.05	+	+ + + + +	+ +	+	+	+
1.71	+ + + + +	+	+ + +	t	t	t
3.31	+ + +	+ +	+ +	+ + + + +	+ + + + +	+ +
4.58	+	t	+	t	t	t
6.86	+ +	+ + + +	+ + +	—	—	+ + + + +

(1.05) hydroxycinnamic acid derivative; (1.71) hydroxycinnamic acid derivative; (3.31) 8-methoxykaempferol 3-sophoroside; (4.58) kaempferol 3-diglucoside; (6.86) quercetin 3-glycoside. In some chromatograms, there is a small shoulder at R_t 3.15 coresponding to quercetin 3-diglucoside

brown fluorescence under UV light (360 nm) and was TLC purified on silica gel with EtOAc (R_f 0.12), $CHCl_3$–EtOAc–Me_2CO, 5:1:4 (R_f 0.36). HPLC (RP-18, MeOH–H_2O 7:3, 1 ml/min) R_t 9.8 min. UV λ_{max}^{MeOH} nm: 345, 307, 267. Acid hydrolysis of the PM derivative yielded 3-hydroxy-5,7,8,4′-tetramethoxyflavone UV λ_{max}^{MeOH} nm: 369, 305, 260, 242; +NaOMe: 407, 264, 218i; +$AlCl_3$: 434, 350, 300i, 265, 225i; +$AlCl_3$+HCl: 432, 350, 300i, 263, 225i) and 2,3,4,6-tetra-*O*-methyl-D-glucose and 3,4,6-tri-*O*-methyl-D-glucose (tentatively identified by PC in *n*-BuOH–EtOH–H_2O 5:1:4 upper layer [R_g 0.84] by comparison with an authentic marker of 2,3,4,6-tetra-*O*-methyl-D-glucose [R_g 1.00]) [8].

REFERENCES

1. Meurer, B., Wray, V., Grotjahn, L., Wiermann, R. and Strack, D. (1986) *Phytochemistry* **25**, 433.
2. Ceska, O. and Styles, E. D. (1984) *Phytochemistry* **23**, 1822.
3. Meurer, B., Wiermann, R. and Strack, D. (1988) *Phytochemistry* **27**, 823.
4. Pratviel-Sosa, F. and Percheron, F. (1972) *Phytochemistry* **11**, 1809.
5. Tomás-Barberán, F. A., Tomás-Lorente, F., Ferreres, F. and García-Viguera, C. (1989) *J. Sci. Food Agric.* (in press).
6. Voirin, B. (1983) *Phytochemistry* **22**, 2107.
7. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*. Springer, Heidelberg.
8. Lederer, E. and Lederer, M. (1957) *Chromatography*. Elsevier, Amsterdam.
9. Schmid, R. D. (1972) *Tetrahedron* **28**, 3259.
10. Harborne, J. B. (1973) *Phytochemical Methods*, pp. 222–225. Chapman & Hall, London.
11. Wu, T. S. and Furukawa, H. (1983) *Phytochemistry* **22**, 1061.
12. Nawwar, M. A. M., El-Sissi, H. I. and Barakat, H. H. (1984) *Phytochemistry* **23**, 2937.
13. Markham, K. R. and Chari, V. M. (1982) in *The Flavonoids; Advances in Research* (Harborne, J. B. and Mabry, T. J., eds). Chapman & Hall, London.
14. Harborne, J. B. and Williams, C. A. (1988) in *The Flavonoids: Advances in Research since 1980* (Harborne, J. B., ed.). Chapman & Hall, London.
15. Servettaz, O., Colombo, M. L., De Bernardi, M., Uberti, E., Vidari, G. and Vita-Finzi, P. (1984) *J. Nat. Prod.* **47**, 809.
16. Elliger, C. A. (1984) *Phytochemistry* **23**, 1199.
17. Mnajed, H., Raynaud, J. and Combier, H. (1972) *Compt. Rend.* **274**, 445.
18. Markham, K. R. (1982) *Techniques of Flavonoid Identification*. Academic Press, London.