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A KAEMPFEROL 3-GLUCOSYLGALACTOSIDE AND FURTHER FLAVONOIDS FROM POLLEN OF *PETUNIA HYBRIDA*

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Key Word Index—*Petunia hybrida*; Solanaceae; pollen; flavonoids; flavonol glycosides; dihydroflavonol; kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside; quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside; taxifolin.

Abstract—The main flavonoids from pollen of *Petunia hybrida* were isolated and identified as taxifolin, and as quercetin and kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside on the basis of UV, ^{1}H NMR, ^{13}C NMR and FD mass spectral data and GC sugar analysis. The latter compound is a new natural product.

INTRODUCTION

Flavonoids occur in pollen of many angiosperm and gymnosperm species [1, 2]. Notably flavonol glycosides are frequently accumulated in pollen of higher plants [3–9]. With *Petunia hybrida*, often used in genetic experiments, only the flavonoid pattern of its flowers was known previously [10]. Here we report the isolation and identification of the main flavonoids of pollen of *Petunia hybrida* including a new kaempferol glycoside. Our recent experiments showed, that two of these flavonoids act as inducers of the *vir* region of the *Agrobacterium tumefaciens* Ti plasmid [11].

RESULTS AND DISCUSSION

A 2D TLC screening of the pollen extract of *Petunia hybrida* indicated a complex flavonoid pattern. In most cases the deep purple colour of the spots under UV light (366 nm) changed into a yellow or orange fluorescence after spraying with diphenyl boric acid aminoethyl ester suggesting the presence of kaempferol and quercetin compounds. One spot showed a dark brownish colour after application of this spray reagent.

The HPLC analysis of the pollen extract showed that the main flavonoids were found in two fractions. Fraction I was further separated by gel chromatography on a Sephadex LH 20 column and yielded taxifolin (**1**) and quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside (**2**); from fraction II kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside (**3**) was obtained.

Acid hydrolysis of **3** afforded kaempferol as aglycone, which was identified by its UV spectra [12]. The sugars were determined by quantitative GC as an equimolar mixture of glucose and galactose. (It is noteworthy, that like kaempferol 3-sophoroside [10] **3** resists enzymatic hydrolysis by sweet almond emulsin.) The FD mass spectrum shows a molecular ion at *m/z* 610 and fragment ions at *m/z* 448 and 286, thus identifying **3** as a diglycoside. The UV spectral analysis [12] suggested the presence of free hydroxyl groups at positions 5, 7 and 4′. Therefore the sugars must be linked to the aglycone at position 3. The site of glycosidation at position 3 is confirmed by the C-2 and C-3 signals in the ^{13}C NMR spectrum showing the characteristic shifts [13]. The magnitude of the vicinal proton couplings of the anomeric protons in the ^{1}H NMR spectrum indicates that both sugars are β-linked. The interglycosidic linkage and the sequential arrangement of the sugars were deduced from a comparison of the ^{13}C NMR spectral data of **3** with literature values. The C-2 signal of an unsubstituted β-D-galactopyranoside usually appears between δ71.2 and 71.8 [13–16]. In the ^{13}C NMR spectrum of **3**, however, there is no signal in this range, but at δ80.5. From this it follows, that the galactose is glucosylated at the OH-2 and must therefore be directly attached to the aglycone whereas the glucose is terminal. Therefore the glycoside **3** is kaempferol 3-*O*-(2″-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside. This compound has been recently prepared by partial hydrolysis of its caffeoyl ester from the fern *Brainea insignis* [17], but hitherto it has not been isolated from a natural source. Our spectral data are in good agreement with those of the synthetic material.

The data of standard UV spectral analysis of **2** are consistent with those of a quercetin derivative without a free 3-OH group [12]. Acid hydrolysis of **2** gave quercetin, identified by its UV spectroscopic properties [12]. The FD mass spectrum shows signals at *m/z* 626, 464 and 302 corresponding to a diglycoside molecular ion and a sequential loss of two hexose moieties. The ^{13}C NMR spectral data confirm the linkage of the sugars to the aglycone at position 3. Besides, the number and characteristic shifts of the ^{13}C glycosidic signals indicate the presence of two hexose moieties. Both sugars have a β-configuration because there can be found diaxial couplings between the anomeric sugar protons and the C-2

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sugar protons. A comparison of the ^1H and ^{13}C NMR spectra of **2** with those of **3** shows, that the same glycosidation pattern exists in both flavonol glycosides. The structure of **2** was therefore established as quercetin 3-*O*-(2″-*O*-β-D-glucopyranosyl)-β-D-galactopyranoside, which has been previously found in pollen of *Corylus avellana* [8] and of many other species of Fagales and some further taxa [9].

The identity of compound **1** with taxifolin was proved by co-chromatography (TLC and HPLC) with an authentic sample and by comparison of the UV spectra and the EIMS with reported data [12, 18].

Related to the dry weight of *Petunia hybrida* pollen, kaempferol 3-glucosylgalactoside makes up 3.4%, quercetin 3-glucosylgalactoside 0.6% and taxifolin 2.6%. Other flavonoids occur only in minor quantities. Because of their chromatographic behavior these could be the aglycones kaempferol and quercetin, corresponding monoglycosides and further di- and triglycosides.

EXPERIMENTAL

General. TLC was performed on cellulose in TBA (*t*-BuOH–HOAc–H_2O [3:1:1]) and in 15% HOAc. Flavonoids were visualized by UV light (366 nm) and by spraying with 1% diphenyl boric acid aminoethyl ester (Naturstoffreagenz A) in MeOH. The HPLC equipment and the chromatographic conditions have been described elsewhere [11]. GC analysis was carried out with a FID and a 3 m × 3 mm column packed with 5% OV-101 (N_2 at 75 ml/min; temp. program: 180–280° at 4°/min). For the GC analysis the sugars were dissolved in 30 μl of dry pyridine and the TMSi ethers were prepared by the successive addition of 100 μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide and of 30 μl of trimethylchlorosilane. The injection volume was 3 μl. ^1H and ^{13}C NMR spectra were recorded at 24° and at 250 and 62.89 MHz, respectively. The solvent (DMSO-d_6) signals at δ2.49 (^1H) and δ39.5 (^{13}C) were used as an internal standard. EIMS were measured at 190°.

Plant material. Ripe pollen was collected from *Petunia hybrida* Cyanidin-type [19] grown under greenhouse conditions and subsequently stored at −20°. The pure line Cyanidine-type of *Petunia hybrida* has been inbred for about 20 years at the Institut für Pflanzenphysiologie, Universität Hohenheim, and is kept there in cultivation.

Extraction and isolation. Ten 3.5 g portions of pollen were extracted by 20 ml of 5% SDS for 1 hr and after adding 80 ml of EtOH for another hr on a steam bath at 95°. The pollen was removed by filtration through a fluted filter and by subsequent centrifugation. The supernatant was filter-sterilized and then lyophilized. Each portion was redissolved in 5 ml of 20% MeOH. These crude extracts were submitted to repeated prep. RP HPLC. Two fractions I and II were collected. Chromatography of fraction I on a Sephadex LH 20 column gave pure compounds **1** (65 mg) and **2** (15 mg). Fraction II, containing only one compound, was purified by repeated prep. HPLC to yield **3** (85 mg).

Acid hydrolysis. 2 mg of **3** were hydrolysed in 10 ml of 2 M HCl for 1 hr at 95°. The aglycone was extracted with 20 ml of EtOAc, evapd to dryness, redissolved in MeOH and identified by its spectra in MeOH and with the usual shift reagents [12]. The aq. layer was lyophilized and the sugars were determined by GC. 2 mg of **2** were hydrolysed in 1 ml of 2 M HCl for 2 hr at 70°. The reaction mixture was passed through a polyamide column. The column was washed with 5 ml of H_2O. Elution of the column with MeOH afforded the aglycone, which was identified by its spectra in MeOH and after addition of shift reagents [12].

Taxifolin (**1**). TLC: (Cellulose, TBA) R_f 0.78; (Cellulose, 15% HOAc) R_f 0.58; UV λ_{max} nm: (MeOH) 290, 327sh; (NaOMe) 247, 326; ($AlCl_3$) 315, 373; ($AlCl_3$ + HCl) 292sh, 313, 373; (NaOAc) 291 sh, 323; (NaOAc + H_3BO_3) 293, 325 sh. EIMS, 70 eV, *m/z* (rel. int.): 304 $[M]^+$ (49), 275 $[M-CHO]^+$ (57), 165 $[M-C_7H_7O_3]^+$ (30), 153 $[M-C_8H_7O_3]^+$ (100), 152 $[M-C_8H_8O_3]^+$ or $[M-C_7H_4O_4]^+$ (24), 137 $[M-C_8H_7O_4]^+$ (7), 123 $[M-C_8H_5O_5]^+$ (53).

Quercetin 3-O-(2″-O-β-D-glucopyranosyl)-β-D-galactopyranoside (**2**). TLC: (Cellulose, TBA) R_f 0.62; (Cellulose, 15% HOAc) R_f 0.69; UV λ_{max} nm: (MeOH) 257, 266sh, 304sh, 358; (NaOMe) 272, 329, 405; ($AlCl_3$) 275, 302sh, 335, 434; ($AlCl_3$ + HCl) 269, 299sh, 363sh, 403; (NaOAc) 274, 326, 384; (NaOAc + H_3BO_3) 263, 300sh, 379. ^1H NMR (250 MHz, DMSO-d_6): δ7.67 (1H, *dd*, J = 2.0 Hz and 8.5 Hz, H-6′); 7.51 (1H, *d*, J = 2.0 Hz, H-2′); 6.83 (1H, *d*, J = 8.5 Hz, H-5′); 6.37 (1H, *d*, J = 1.8 Hz, H-8); 6.16 (1H, *d*, J = 1.8 Hz, H-6); 5.65 (1H, *d*, J = 7.5 Hz, H-1‴); 4.56 (1H, *d*, J = 7.6 Hz, H-1″). ^{13}C NMR (62.89 MHz, DMSO-d_6): δ176.9 (C-4), 163.5 (C-7), 160.6 (C-5), 155.7 (C-9), 154.9 (C-2), 147.9 (C-4′), 144.2 (C-3′), 132.6 (C-3), 121.7 (C-6′), 120.6 (C-1′), 115.4 (C-5′), 114.8 (C-2′), 103.8 (C-1‴), 103.3 (C-10), 98.1, 97.9 (C-6, C-1″), 92.9 (C-8), 80.2 (C-2″), 76.2, 75.9 (C-3‴, C-5‴), 75.3 (C-5″), 73.8 (C-2‴), 72.8 (C-3″), 69.0 (C-4‴), 67.0 (C-4″), 60.1 (C-6‴), 59.3 (C-6″). FDMS, *m/z*: 626 $[M]^+$, 464 $[M-C_6H_{10}O_5]^+$, 302 $[M-2C_6H_{10}O_5]^+$.

Kaempferol 3-O-(2″-O-β-D-glucopyranosyl)-β-D-galactopyranoside (**3**). TLC: (cellulose, TBA) R_f 0.76; (cellulose, 15% HOAc) R_f 0.76; UV λ_{max} nm: (MeOH) 267, 303sh, 328sh, 350; (NaOMe) 276, 327, 397; ($AlCl_3$) 258sh, 275, 305, 352, 400; ($AlCl_3$ + HCl) 261sh, 278, 304, 349, 400; (NaOAc) 276, 306, 378; (NaOAc + H_3BO_3) 269, 303sh, 353. ^1H NMR (250 MHz, DMSO-d_6): δ8.07 (2H, *d*, J = 8.9 Hz, H-2′ and H-6′); 6.88 (2H, *d*, J = 8.9 Hz, H-3′ and H-5′); 6.43 (1H, *d*, J = 2.0 Hz, H-8); 6.19 (1H, *d*, J = 1.9 Hz, H-6); 5.67 (1H, *d*, J = 7.5 Hz, H-1‴); 4.56 (1H, *d*, J = 7.6 Hz, H-1″). ^{13}C NMR (62.89 MHz, DMSO-d_6): δ177.5 (C-4), 164.0 (C-7), 161.2 (C-5), 160.0 (C-4′), 156.3 (C-9), 155.5 (C-2), 132.9 (C-3), 131.0 (C-2′ and C-6′), 120.9 (C-1′), 115.3 (C-3′ and C-5′), 104.2 (C-1‴), 103.9 (C-10), 98.7, 98.3 (C-6, C-1″), 93.6 (C-8), 80.5 (C-2″), 77.0, 76.6 (C-3‴, C-5‴), 75.8 (C-5″), 74.4 (C-2‴), 73.4 (C-3″), 69.7 (C-4‴), 67.6 (C-4″), 60.8 (C-6‴), 59.9 (C-6″). FDMS, *m/z*: 610 $[M]^+$, 448 $[M-C_6H_{10}O_5]^+$, 286 $[M-2C_6H_{10}O_5]^+$.

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REFERENCES

1. Wiermann, R. (1968) *Ber. Dtsch. Bot. Ges.* **81**, 3
2. Stanley, R. G. and Linskens, H. F. (1974) *Pollen–Biology, Biochemistry, Management*. Springer, Berlin
3. Sosa, F. and Percheron, F. (1970) *Phytochemistry* **9**, 441
4. Pratviel-Sosa, F. and Percheron, F. (1972) *Phytochemistry* **11**, 1809
5. Strack, D., Sachs, G. and Wiermann, R. (1981) *Z. Pflanzenphysiol.* **103**, 291
6. Wiermann, R. and Vieth, K. (1983) *Protoplasma* **118**, 230
7. Ceska, O. and Styles, E. D. (1984) *Phytochemistry* **23**, 1822

8. Strack, D., Meurer, B., Wray, V., Grotjahn, L., Austenfeld, F. A. and Wiermann, R. (1984) *Phytochemistry* **23**, 2970
9. Meurer, B., Wiermann, R. and Strack, D. (1988) *Phytochemistry* **27**, 823
10. Birkofer, L. and Kaiser, C. (1962) *Z. Naturforsch.* **17b**, 359
11. Zerback, R., Dressler, K. and Hess, D. (1988) (submitted).
12. Mabry, T. J., Markham, K. R. and Thomas, M. B. (1970) *The Systematic Identification of Flavonoids*. Springer, Berlin
13. Markham, K. R., Ternai, B., Stanley, R., Geiger, H. and Mabry, T. J. (1978) *Tetrahedron* **34**, 1389
14. Markham, K. R. (1982) *Techniques of Flavonoid Identification*. p. 81. Academic Press, London
15. Barberá, O., Sanz, J. F., Sánchez-Parareda, J. and Marco, J. A. (1986) *Phytochemistry* **25**, 2361
16. Yasukawa, K. and Takido, M. (1987) *Phytochemistry* **26**, 1224
17. Murakami, T., Wada, H., Tanaka, N., Kido, T., Iida, H., Saiki, Y. and Chen, C.-M. (1986) *Yakugaku Zasshi* **106**, 982
18. Mabry, T. J. and Markham, K. R. (1975) in *The Flavonoids* (Harborne, J. B., Mabry, T. J. and Mabry, H., eds), pp. 103–105. Chapman & Hall, London
19. Hess, D. (1963) *Planta* **59**, 567