Epicuticular Flavonoids of Some Scrophulariaceae

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Twenty-two species of Scophulariaceae have been found to accumulate flavonoid aglycones externally on their leaves and stems. They belong to the genera *Anarrhinum, Antirrhinum, Asarina, Calceolaria, Mimulus,* and *Odontites.* Most of the flavonoids are methylated flavones and flavonols, some with 6-O and/or 8-O-substitution. One of them is the natural isobutyryl ester of a rare flavone.

Introduction

Scrophulariaceae have so far only rarely been reported to exhibit externally accumulated flavonoid aglycones. Wollenweber recently (1995) listed the genera *Diplacus, Mimulus* and *Odontites.* In addition, we have now studied one *Anarrhinum* species, six *Antirrhinum,* two *Asarina,* five *Calceolara*, and two *Mimulus* species for the occurrence of such products, because their aerial parts are more or less glandulous and sometimes sticky to the touch. We found a series of more or less trivial flavones and flavonols, several less abundant flavonoids, and a novel natural flavone ester.

Material and Methods

Anarrhinum forskalii (J. F. Gmel.) Cufod., *Antirrhinum barrelieri* Bor., *A. braun-blanquetii* Rothm., *A. graniticum* Rothm., *A. hispanicum* Chav., *A. majus* L. ssp. *cirrhigerum* (Ficalho) Franco, *A. nutallii* Benth., *Asarina barklaiana* (Lindl.) Pennell, *A. procumbens* Mill. [syn. *Antirrhinum asarina* L.], *Calceolaria arachnoidea* R. Grah., *C. chelidonioides* H. B. K., *C. irazuensis* J. D.Smith, *C. mexicana* Benth.; *C. pavonii* Benth., *C. scabiosaefolia* Sims, *C. tenella* Poepp. et Endl., *C. tripartita* Ruiz et Pav., *Mimulus moschatus* Dougl. ex Lindl., and *M. pilosus* (Benth.) S.Wats. were cultivated in the Botanischer Garten der TU Darmstadt. Aerial parts were collected from flowering plants, or just after the flowers had faded. They were briefly rinsed with acetone to dissolve the lipophilic exudate material accumulated on leaf and stem surfaces. The concentrated material was defatted (MeOH, −10°, centrifugation) and passed over Sephadex LH-20, eluted with MeOH, to separate the flavonoids from the predominant terpenoids. Comparative TLC of Sephadex fractions with authentic substances allowed the identification of most of the flavonoids present. Fractions were monitored and comparisons with markers were done by TLC on silica with toluene – MeCOEt 9:1 v/v and with toluene – dioxane – HOAc 18:5:1 v/v/v. For TLC on polyamide DC-11 we used toluene – petrol$_{100-140}$ – MeCOEt – MeOH 12:6:2:1 v/v/v/v, toluene – dioxane – MeOH 8:1:1 v/v/v and toluene – MeCOEt – MeOH 12:5:3 v/v/v. Chromatograms were viewed under UV before and after spraying with "Naturstoffreagenz A". Terpenoids were visualized by spraying silica plates with $MnCl_2$ reagent, followed by heating (Jork *et al.,* 1989). NMR spectra were recorded in DMSO-d_6 or acetone-d_6 at 400 MHz (^1H) and 100 MHz (^{13}C) on a Bruker ARX-400 instrument. Synthetic samples of 5,6,4′-trihydroxy-7,8-dimethoxyflavone and 5,8,4′-trihydroxy-6,7-dimethoxy flavone were obtained from T. Horie (Horie *et al.,* 1995).

In the case of *Asarina procumbens*, the flavonoid portion was subjected to column chromatography on polyamide SC-6. A minute amount of the major flavonoid was isolated by subsequent preparative TLC on silica. This compound (**1**) appears as a dark spot on TLC which remains dark

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on spraying with Naturstoffreagenz A. MS m/z (% rel. int.): 400 (20%, M^+), 330 (100, $M^+ - (CH_3)_2C{=}C{=}O$), 315 (45, $M^+ - (CH_3)_2C{=}C{=}O - CH_3$), 297 (17). ^1H NMR (d_6-acetone) δ 12.90, s (5-OH); 7.85, d, $J = 8$ Hz (H-2′ and H-6′); 7.00, d, $J = 8$ Hz (H-3′ and H-5′); 6.67, s (H-3); 4.01, s (7-OMe); 3.93, s (8-OMe).; 3.01, pentet, $J = 7$ Hz (H-2″); 1.37, d, J = 7 Hz (H-3″ and H-4″). ^{13}C NMR δ 165.5 (C-2); 104.1 (C-3); 183.5 (C-4); 152.4 (C-5); 124.2 (C-6); 152.7 (C-7); 137.4 (C-8); 145.3 (C-9); 107.2 (C-10); 123.0 (C-1′); 129.3 (C-2′ and C-6′); 116.9 (C-3′ and C-5′); 162.3 (C-4′); 61.5 (7-OMe); 60.7 (8-OMe); 174.4 (C-1″); 33.1 (C-2″); 18.9 (C-3″ and C-4″).

From *Antirrhinum graniticum*, small amounts of two crystalline non-polar flavonoids were isolated by prep. TLC of relevant Sephadex fractions on silica. The molecular weights of both compounds were determined to be 388 by EIMS (M^+ with m/z 388), indicating flavones or flavonols each with 1 OH and 5 OMe groups. By direct comparisons with markers, one of them was readily identified to be 5-OH-6,7,8,3′,4′-pentamethoxy flavone (5-desmethyl-nobiletin). The second product (comp. **2**) was identified by its NMR spectrum as 5-OH-3,6,7,8,4′-pentamethoxy flavone (5-hydroxy-auranetin) (see Discussion).

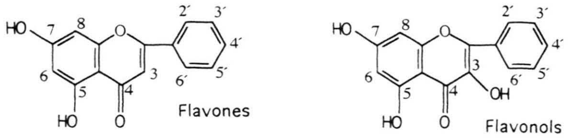
A series of further Scrophulariaceae have been checked for the presence of exudate flavonoids. Some of them exhibit a glandular exudate, but seem to be devoid of flavonoids, nonetheless. In *Alonsoa acutifolia, Antirrhinum orontium, Antirrhinum siculum, Asarina antirrhinifolia, Asarina lophospermum, Calceolaria cana, Collinsia heterophylla,* and *Paulownia tomentosa* no external flavonoid aglycones were detected. In *Antirrhinum sempervirens, Calceolaria glandulifera, C. pavoni, C. pinnata* and others there are only traces of flavonoids present.

Results and Discussion

Core structures of the flavones and flavonols found in this study are presented in Fig. 1. In the text, abbreviations OH = hydroxy-group, OMe = methoxy group, and Me = methyl ether are used throughout.

Structure elucidation

The base peak in the MS of compound **1**, from *Asarina procumbens*, is a fragment ion at $M^+ - 70$ (loss of dimethyl ketene), suggesting that **1** contains a four carbon acyl moiety. The proton NMR spectrum had signals corresponding to a 5-OH flavone with a fully substituted A ring, a *para* substituted B ring and a 2-propyl moiety (methyl doublet and methine pentet). The carbon spectrum displayed B and C ring signals virtually identical to those of other 4′-OH flavones, confirmed the presence of an isobutyryl moiety and two sterically compressed OMe's. The A ring of **1** therefore has a 5-OH, two OMe's and a second OH esterified with isobutyric acid. The carbon signals can be assigned to accommodate either of two isomeric flavones: 5,4′-diOH-6,7-diOMe-8-O-isobutyryl flavone or 5,4′-diOH-7,8-diOMe-6-isobutyryl flavone. Long range H-C coupling experiments revealed interactions between OH-5 and the C-3″/4″ methyl carbons of the isobutyl group, which indicates that the isobutyryl substituent is attached to C-6 and not to C-8. Compound **1** was therefore identified as 5,4′-dihydroxy-7,8-dimethoxy-6-O-isobutyryl flavone.



Flavones:
5,7-OH-flavone: chrysin
5,7,4′-OH-flavone: apigenin
5,6,7,4′-OH-flavone: scutellarein
5,7,8,4′-OH-flavone: isoscutellarein
5,7,3′,4′-tetraOH-flavone: luteolin
5,6,7,3′,4′-OH-flavone: 6-OH-luteolin
5-OH-6,7,8,3′,4′-OMe-flavone: 5-desmethylnobiletin
5,4′-OH-7,8-OMe-6-O-isobutyryl-flavone: comp. **1**

Flavonols:
3,5,7,4′-OH-flavone: kaempferol
3,5,7,8,4′-OH-flavone: herbacetin
3,5,7,3′-4′-OH-flavone: quercetin
5-OH-3,6,7,8,4′-OMe-flavone: 5-OH-auranetin
3,5,7,8,3′,4′-OH-flavone: gossypetin

A small sample of **1** was refluxed in acetic acid to which a few drops of HCl had been added; after 14 hrs a sample removed and analyzed by TLC showed that a considerable amount of unchanged **1** remained and reflux was continued for an additional 48 hrs. NMR spectra of the purified product (LH-20 in $CHCl_3$) showed that two isomeric flavones had been formed: 5,8,4′-triOH-6,7-diOMe flavone and 5,6,4′-triOH-7,8-diOMe flavone, presumably *via* acid-catalyzed opening and closing of the C ring. The latter product was, indeed, obtained by acid treatment of the former by Ferreres *et al.* (1985).

5,6,4′-triOH-7,8-diOMe-flavone has been known previously as "thymusin" (see e.g. Hernández *et al.*, 1987). Its 6-O-isobutyrate **1**, however, is a novel natural flavone. Besides chrysin-7-O-benzoate from *Baccharis bigelovii* (Arriaga-Giner *et al.,* 1986) and the 5′-O-acetate of 2′,5′.dihydroxyflavone from *Primula pulverulenta* (Wollenweber *et al.*, 1988), compound **1** is the only other naturally occurring ester of a flavone that has thus far been found.

The molecular weight of compound **2** from *Antirrhinum graniticum* (M^+ 388 by APCI-MS) was consistent with a flavone bearing one hydroxy and five methoxy substituents. MS-MS fragmentation of the $[MH]^+$ ion with *m/z* 389 yielded daughter ion fragments with *m/z* 373 $[MH\text{-}CH_4]^+$ (9%), 359 $[MH\text{-}2CH_3]^+$ (100%), 341 $[359\text{-}H_2O]^+$ (37%), 331 $[359\text{-}CO]^+$ (25%), 316 $[331\text{-}CH_3]^+$ (22%), and *m/z* 135 $[\text{B-ring}]^+$ (23%). The latter fragment ion pointed to a B-ring with one methoxy substituent, which was confirmed by ^1H NMR analysis. The B-ring protons gave rise to two doublets (δ_H 8.07 and δ_H 7.20 *d* , both $J = 8.8$ Hz, H-2′/6′ and H-3′/5′) which were shifted downfield compared to the B-ring resonances of kaempferol (δ_H 8.06 *d* and δ_H 6.94 *d*), indicating O-methylation of the *para* hydroxyl. The low-field resonance at δ_H 12.43 was taken as evidence for a free hydroxyl at C-5. These assignments left the five methoxy groups (δ_H 4.03, 3.90, 3.88, 3.83, and δ_H 3.82) to be placed at carbons 3, 6, 7, 8, and 4′. The compound was therefore identified as 5-hydroxy-3,6,7,8,4′-pentamethoxy flavone, trivially known as 5-hydroxy auranetin. Its identity was confirmed by direct comparison with an authentic sample. This flavonol has previously been known from several taxonomically independent plants, including e.g. *Polanisia trachysperma* (Wollenweber *et al.*, 1989a) and *Rosa centifolia* (Wollenweber *et al.*, 1993).

Chemotaxonomy

The exudate flavonoid patterns of the Scrophulariaceae we studied are presented in Table I. In addition to the results of the present study, we included earlier published results with three *Calceolaria* sepcies (Wollenweber *et al.,* 1989b) , one *Mimulus* and two *Odontites* species (Wollenweber, 1990). In the literature, the occurrence of flavone and flavonol aglycones has also been reported for species belonging to the genera *Adenosma, Digitalis, Hebe, Kickxia, Limnophila, Pedicularis, Rhinanthus, Scoparia, Striga, Verbascum,* and *Veronica*. However, since in these reports no mention is made of the flavonoid localization, they must not be considered here. Plant material was "percolated with ethanol" (Imre *et al.,* 1984: *Digitalis*), or "cut and extracted with methanol under reflux" (Nakanishi *et al.*, 1985: *Striga*). In our lab, *Digitalis ferruginea, D. luta* and *D. purpurea* were found devoid of external flavonoids. Phuong *et al.* (1997) found methoxylated flavones in the hexane (!) extract of dried *Adenosma capitatum*; these are likely, therefore, to be exudate constituents. Bohm (1992) reported the external occurrence of some flavonol aglycones in the leaf exudate of *Mimulus lewisii*. Only once has a trivial flavanone been found in Scrophulariaceae, namely sakuranetin (naringenin-7-Me) in *Hebe cupressoides* (Perry and Foster, 1994). For *Diplacus aurantiacus*, the existence of C_{10}-substituted flavanones and dihidroflavonols in leaf resin has been reported (Lincoln and Walla, 1986; Wollenweber *et al.*, 1989c), while for *Mimulus clevelandii* it can only be assumed that the geranyl flavanones and geranyl dihydroflavonols found (Phillips *et al.*, 1996: "whole plant") are exudate constituents.

Table I comprises 9 species from 3 genera in the Scrophularioideae-Antirrhinae (*Anarrhinum, Antirrhinum* and *Asarina*), 8 species of the genus *Calceolaria* in the Scrophularioideae-Calceolariae, 3 species of *Mimulus* in the Scrophularioideae-Gratioleae, and 2 species of *Odontites* in the Rhinanthoideae-Rhinantheae. *Anarrhinum, Asarinum* and *Antirrhinum* show a rather uniform pattern of flavones, with the exception of *Antirrhinum graniticum* which exhibits a higly methylated flavone (5-desmethyl-nobiletin) plus a highly methylated flavonol (5-hydroxyauranetin), and *Asarina procumbens,* which produces a flavone ester (comp. **1**). Flavonols are predominant in *Calceolaria*; however *C. irazuensis* shows three flavones. Several 8-O-substituted products are found in this genus, two of them being flavones. In *Mimulus*, the flavonoid profiles are more diverse: three species exhibit only trivial flavones, but one also produces

Table I. Flavonoid aglycone distribution in selected Scrophulariaceae.

	Species																					
Flavonoid	*Anarrhinum forskalii*	*Antirrhinum barrelieri*	*Antirrhinum braun-blanquetti*	*Antirrhinum graniticum*	*Antirrhinum hispanicum*	*Antirrhinum majus ssp. cirrhigerum*	*Antirrhinum nutalli*	*Asarina barkleyana*	*Asarina procumbens*	*Calceolaria arachnoidea*	*Calceolaria chelidonioides*	*Calceolaria irazuensis*	*Calceolaria mexixana*	*Calceolaria pavonii*	*Calceolaria scabiosifolia*	*Calceolaria tenella*	*Calceolaria tripartita*	*Mimulus cardinalis*	*Mimulus moschata*	*Mimulus pilosus*	*Odontites lutea*	*Odontites viscosa*
Flavones																						
Chrysin																			x			
Apigenin		x	x		x	x			x									x		x	x	x
Ap-7-Me		x		x	x	x																x
Ap-4′-Me												x								x		x
Ap-7,4′-diMe	x	x	x			x	x															x
Scutellarein-6,7-diMe					x				x													
Scut-6,4′-diMe																					x	
Isoscut-8,4′-diMe												x										
Isoscut-7,8,4′-triMe												x										
Luteolin		x			x	x		x										x				
Lut-7-Me		x			x	x																
Luteolin-3′-Me			x	x		x		x										x		x		x
Lut-7,3′-diMe		x			x		x															x
Lut-7,4′-diMe			x	x																		
Lut-3,4′-diMe								x														
Lut-7,3′,4′-triMe	x		x	x	x	x	x															
6-OH-luteolin-6,7-diMe				x																		
Di-OH-di-OMe-flavone-ester									x													
5-OH-6,7,8,3′,4′-OMe-flavon				x																		
Flavonols																						
Kaempferol-3-Me										x	x				x			x				x
Kae-7-Me										(x)		x						x				
Kae-4′-Me												x										
Kae-3,7-diMe										x								x				
Kae-3,4′-diMe										(x)												
Kae-7,4′-diMe												x										x
Kae-3,7,4′-triMe										x								x				
Herbacetin-3,8-diMe										x												
Herb-7,8-diMe										(x)												
Herb-8,4′-diMe												x										
Herb-3,7,8-triMe										x						x						
Herb-3,8,4′-triMe										x												
Herb-7,8,4′-triMe												x										
Quercetin-3-Me											x		x	x	x		x					
Qu-3,7-diMe										x			x				x	x				
Qu-3,3′-diMe											x		x		x		x	x				
Qu-3,7,3′-triMe																		x				
Qu-3,7,3′,4′-tetraMe																		x				
5-OH-3,6,7,8,4′-pentaOMe-fl.				x																		
Gossypetin-3,7,8-triMe																x						
Goss-3,8,3′-triMe											(?)				x							
Goss-7,8,3′-triMe												x										
Goss-3,7,8,3′-tetraMe																x						
Myricetin-3,x′-Me													x	x			x					

a series of flavonols. The flavonoid chemistry of *Odontites* (Rhinanthoideae-Rhinantheae) is similar to that of *Mimulus* (Scrophularioideae-Gratioleae). Both genera are capable of accumulating flavones and flavonols, and they seem to hold an intermediate position between the Scrophularioideae-Antirrhineae (flavones) and the Scrophularioideae-Calceolarieae (flavonols). The flavonoid patterns clearly support the recognition of the tribes Antirrhineae and Calceolarieae (Tahtajan, 1997).

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