Externally Accumulated Flavonoids in Three Mediterranean Ononis Species

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The Mediterranean Ononis species, O. fruticosa, O. natrix subsp. ramosissima and O. tridentata, have been analyzed for their exudate flavonoids. More than 20 flavonoid aglycones were identified, some of which are rather rare natural compounds. One of them, namely hypolaeitin-8,3',4'-trimethyl ether, had been found only once before. The results are presented in a table along with literature data, and the chemotaxonomic impact of the flavonoid patterns is discussed.

Key words: Ononis, Epicuticular Flavonoids, Chemosystematics

Introduction

Ononis is a Fabaceae genus (tribe Trifolieae) that comprises some 75 species, occurring in the Canaries, the Mediterranean region, Europe to Central Asia (Willis, 1973). Many species bear glandular trichomes that produce a more or less viscid excretion, which in several cases has been reported to contain flavonoids. In the scope of our ongoing studies on the occurrence and distribution of externally accumulated flavonoid aglycones in higher plants we now studied three species: O. fruticosa, O. natrix subsp. ramosissima and O. tridentata.

Material and Methods

Plant material

Ononis tridentata L. was field collected in July, 2000 at Los Castaños (Sorbas, Almeria, Spain) by D. Rivera Nuñez (DRN) and C. Obón. Ononis fruticosa L. was collected in May, 1990 near Salida de Tíbi direcció a Jijona (Alicante, Spain). Ononis natrix ssp. ramosissima Desf. comes from a) Molino Rio Aguas (DRN, 04/1990) and b) Los Castaños (Sorbas, Almeria; DRN and C. Obón, 07/2000). Voucher specimens are deposited at the Herbarium of Murcia University.

Extraction and isolation

Fresh material was briefly rinsed with acetone to dissolve the lipophilic material accumulated on the plant surfaces and the solutions were evaporated to dryness. Amounts of air-dried plant material and resinous exudates were as follows: O. tridentata 97 g/0.85 g, O. fruticosa 54 g/1.33 g, O. natrix a) 57 g/3.26 g, and b) 170 g/12.3 g. The resinous residues were dissolved in a small volume of hot methanol, cooled to –10 °C, and the precipitating material was eliminated by centrifugation. The thus defatted solutions were passed over Sephadex LH-20 (Pharmacia), eluted with MeOH, to separate the flavonoids from the overwhelming terpenoids. In the cases of O. fruticosa and O. tridentata, the flavonoid aglycones were then identified directly by TLC comparisons with authentic samples. The flavonoid fractions of the two different collections of O. natrix subsp. ramosissima were essentially identical and were therefore combined. They were further chromatographed over acetylated polyamide (Macherey-Nagel), eluted with toluene and increasing quantities of methylketone and methanol.

Fractions were monitored and comparisons with markers were done by TLC on polyamide (DC 11, Macherey-Nagel) with the solvents i) PE100–140/toluene/MeCOEt/MeOH (12:6:1:1 v/v/v/v), ii) to-
luene/PE100–140, MeCOEt/MeOH (12:6:2:1 v/v/v/v), and iii) toluene/MeCOEt/MeOH (12:5:3 v/v/v/v), and on silica gel with solvent iv) toluene/MeCOEt (9:1 v/v). Chromatograms were viewed under UV light (366 nm) before and after spraying with “Naturstoffreagenz A” (1% of diphenyl-boric acid ethanolamine complex in MeOH). Authentic samples of flavonoids were available in E.W.’s lab.

Some flavonoids were isolated (purified) after a further passage over Sephadex LH-20, eluted with CHCl3/MeOH (99:1 v/v), followed by CTLC on the Chromatotron with benzene/2-propanol (95:5).

**NMR and MS**

Carbon and proton NMR spectra were recorded in DMSO-d6 on a Bruker AMX 400 spectrometer at 100 MHz and 400 MHz, respectively. Electron impact mass spectra were obtained on a Varian MAT 212 Spectrometer at 70 eV. The mass and NMR spectra confirmed the structures of the following flavonones: acerosin, agecorynin D, desmethylsudachitin, hymenoxin, nevadensin, pectolinarigenin, sideritiflavone, xanthomicrol and 5,7-dihydroxy-8,3′,4′-trimethoxyflavone. In the following we report the NMR data of the less common compounds [DMSO-d6, δ (ppm) downfield from tetramethylsilane].

**Sideritiflavone**: 13C NMR: δC = 164.3 (C-2), 102.5 (C-3), 182.3 (C-4), 148.4 (C-5), 135.7 (C-6), 152.3 (C-7), 132.6 (C-8), 145.1 (C-9), 106.1 (C-10), 121.3 (C-1′), 113.3 (C-2′), 145.7 (C-3′), 150.0 (C-4′). 116.1 (C-5′), 119.1 (C-6′) 60.5 (6-OMe), 61.9 (7-OMe), 61.4 (8-OMe). 1H NMR: δH = 6.77 (s, H-3), 7.47 (d, J = 2.4 Hz, H-2′), 6.92 (d, J = 8 Hz, H-5′), 7.46 (dd, J = 8, 2.4 Hz, H-6′), 12.79 (s, 5-OH), 3.82 (s, OMe), 3.93 (s, OMe), 4.02 (s, OMe).

**Acerosin**: 13C NMR: δC = 163.3 (C-2), 102.9 (C-3), 182.1 (C-4), 148.3 (C-5), 131.5 (C-6), 150.8 (C-7), 127.9 (C-8), 145.3 (C-9), 102.9 (C-10), 123.1 (C-1′), 112.8* (C-2′), 146.8 (C-3′), 151.2 (C-4′), 112.3* (C-5′), 118.5 (C-6′), 60.1 (6-OMe), 61.2 (8-OMe), 55.7 (4′-OMe). 1H NMR: δH = 6.77 (s, H-3), 7.12 (d, J = 8.4 Hz, H-5′), 7.55 (dd, J = 2.4, 8.4 Hz, H-6′), 12.77 (s, 5-OH), 9.52 (s, 7-OH), 10.37 (s, 3′-OH), 3.79 (s, OMe), 3.87 (s, OMe), 3.89 (s, OMe).

**Xanthomicrol**: 13C NMR: δC = 164.1 (C-2), 102.6 (C-3), 182.5 (C-4), 145.5* (C-5), 135.8 (C-6), 152.4 (C-7), 132.6 (C-8), 145.1* (C-9), 106.1 (C-10), 121.0 (C-1′), 128.4 (C-2′ and C-6′), 116.1 (C-3′ and C-5′), 161.4 (C-4′), 60.5 (6-OMe), 61.8 (7-OMe), 61.4 (8-OMe). 1H NMR: δH = 6.88 (s, H-3), 7.95 (d, J = 8.8 Hz, H-2′ and H-6′), 6.97 (d, J = 8.8 Hz, H-3′ and H-5′), 12.77 (s, 5-OH), 10.41 (s, 4′-OH), 3.82 (s, OMe), 3.92 (s, OMe), 4.02 (s, OMe).

**Agecorynin D**: 13C NMR: δC = 161.9 (C-2), 104.3 (C-3), 182.4 (C-4), 148.4 (C-5), 135.5 (C-6), 152.2 (C-7), 132.4 (C-8), 145 (C-9), 106.6 (C-10), 105.9 (C-1′), 153.3 (C-2′), 106.5 (C-3′), 151.9 (C-4′), 141.6 (C-5′), 110.6 (C-6′), 60.4 (6-OMe), 61.6 (7-OMe), 61.4 (8-OMe), 55.9 (5′-OMe). 1H NMR: δH = 6.58 (s, H-3), 7.12 (s, H-3′), 7.41 (s, H-6′), 12.85 (s, 5-OH), 10.50 (s, 2′-OH)*, 10.08 (s, 4′-OH)*, 3.80 (s, 5′-OMe), 3.82 (s, OMe), 3.93 (s, OMe), 4.02 (s, OMe).

5,4′-Dihydroxy-6,7,8,3′-tetramethoxyflavone: 13C NMR: δC = 164.0 (C-2), 102.9 (C-3), 182.5 (C-4), 148.4 (C-5), 135.8 (C-6), 152.4 (C-7), 132.5 (C-8), 145.1 (C-9), 106.1 (C-10), 121.3 (C-1′), 121.3 (C-2′), 148.0 (C-3′), 151.0 (C-4′), 115.9 (C-5′), 112.3 (C-6′), 60.5 (6-OMe), 61.8 (7-OMe), 61.4 (8-OMe), 55.8 (3′-OMe). 1H NMR: δH = 7.00 (s, H-3), 6.98 (d, J = 8.8 Hz, H-5′), 7.59 (m, H-2′ and H-6′), 12.76 (s, 5-OH), 10.02 (s, 4′-OH), 3.82 (s, OMe), 3.90 (s, OMe), 3.93 (s, OMe), 4.02 (s, OMe).

**Results and Discussion**

**Structure elucidation**

A good number of flavonoid aglycones were found in the lipophilic material accumulated on aerial parts of Ononis fruticosa, O. natrix, and O. tridentata. The following compounds from O. natrix were identified by their MS, NMR, and UV spectra: scutellarein-6,4′-di-O-methyl ether (pectolinarigenin), 5,7,4′-trihydroxy-6,8-dimethoxy flavone (desmethylsudachitin), 5,4′-dihydroxy-6,7,8-trimethoxy flavone (xanthomicrol), 5,7-dihydroxy-6,8,4′-trimethoxy flavone (nevadensin), 5-hydroxy-6,7,8,4′-tetramethoxy flavone (gardenin B), 5,7-dihydroxy-8,3′,4′-trimethoxy flavone (hypo-laetin-8,3′,4′-tri-O-methyl ether), 5,3′,4′-trihydroxy-6,7,8-trimethoxy flavone (sideritiflavone), 5,7,3′-trihydroxy-6,8,4′-trimethoxy flavone (acerosin), 5,4′-dihydroxy-6,7,8,3′-tetramethoxy flavone, 5,7-dihydroxy-6,8,3′,4′-tetramethoxy flavone (hymenoxin) and 5,2′,4′-trihydroxy-6,7,8,5′-tetrame-
Table I. Exudate Flavonoids of *Ononis* species (Me, methyl ether; OMe, methoxy substituent).

<table>
<thead>
<tr>
<th>Flavone</th>
<th><em>Ononis fruticosa</em></th>
<th><em>Ononis matrix</em> sp. <em>hispanica</em></th>
<th><em>Ononis matrix</em> sp. <em>natrix</em></th>
<th><em>Ononis matrix</em> sp. <em>ramossissima</em></th>
<th><em>Ononis matrix</em> sp. <em>ramossissima</em> (2 coll)</th>
<th><em>Ononis parviceps</em></th>
<th><em>Ononis psilostachya</em></th>
<th><em>Ononis pratensis</em></th>
<th><em>Ononis vaginalis</em></th>
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<td>5,7,4′′-triOH-3,6,8′′-′′-tetraOMe</td>
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<td>Flavanones, chalcones, dihydrochalcones</td>
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thoxy flavone (agecorynin D). The structures of these products (except for 5,4’-dihydroxy-6,7,8,3’-tetramethoxy flavone) and the remaining compounds were confirmed by co-TLC with authentic markers.

In Table I, the flavonoids herein identified are listed together with those reported earlier for various collections of *O. natrix* and for further species found to exhibit flavonoid aglycones.

Among the flavones identified in the present study, hypolaetin-8,3’,4’-tri-O-methyl ether is an extremely rare compound. It has thus far been reported only from the resin of *Gardenia gummi-fera* (dikamali gum; Krishnamurti et al., 1972) and from the leaf of *Gardenia lucida* (another source of dikamali gum; Kumari, 1989). 5,2’,4’-trihydroxy-6,7,8,5’-tetramethoxy flavone (agecorynin D) is also a rare compound, previously reported from *Ageratum corymbosum* (Quijano et al., 1980) and from *Gutierrezia microcephala* (Fang et al., 1986), and 5,7,3’-trihydroxy-6,8,4’-trimethoxy flavone (acerosin) is an uncommon flavone, too, that has been found only five or six times, thus far.

**Flavonoid distribution**

Wollenweber (1990) found a series of methylated flavones to be exudate constituents of *O. natrix*, *O. rotundifolia* and *O. spinosa*, and so they are in the species now studied. It is hence assumed that the flavonoid aglycones reported for *O. natrix* ssp. *hispanica* (Barrero et al., 1990), ssp. *natrix* (Al-Khalil et al., 1995) and ssp. *ramosissima* (Barrero et al., 1997) are also accumulated externally, although they were analysed from the extracts of aerial parts. It may be mentioned in this context that, for *O. natrix*, dihydroisocoumarins have also been reported as lipophilic constituents (San Feliciano et al., 1990).

*Ononis natrix* is a polytypic taxon for which 11 subspecies have been described (Sirjaev, 1932). It has recently been split into different species (Devesa, 2000). Barrero et al. (1997) pointed out that the flavonoid profiles of the three *O. natrix* subspecies they studied were clearly different. In subspecies *hispanica* they found only one flavone, but several resorcinol derivatives (Barrero et al., 1990), and Al-Khalil et al. (1995) found only three flavones in subspecies *natrix*. [Note that for Devesa (2000), subspecies *hispanica* does not exist and is either *O. natrix* ssp. *ramosissima* or *O. natrix* sensu stricto.] However Barrero et al. (1997) detected two flavanones, four chalcones and six dihydrochalcones in *O. natrix* ssp. *ramosissima*, along with alkylresorcinol. Flavanones, chalcones and dihydrochalcones have not been reported for any other *Ononis* species than *natrix*; however our examination of the exudate from *O. natrix* ssp. *ramosissima* revealed none of these types of compounds, a discrepancy for which we presently have no explanation. It is striking that a study of the glandulose *O. viscosa* did not reveal any flavonoid aglycones. Alkylresorcinols and pterocarpanes seem to be predominant in its exudate (Barrero et al., 1991, 1998). Unfortunately, material of *O. viscosa* was not available for our present studies.

With the exception of those flavanones, chalcones and dihydrochalcones previously reported for *O. natrix* ssp. *ramosissima* (Barrero et al., 1997), the majority of the flavonoid aglycones found on aerial parts of *Ononis* species are flavones; flavonols occur only occasionally. Furthermore the presence of 6- and 6,8-O-substitution seems to be a characteristic feature of these *Ononis* flavones and flavonoids.

While more or less lipophilic flavonoid aglycones have been reported from root, stem, bark, and seed of a number of genera (*Dalbergia, Derris, Distemonanthus, Flemingia, Glycyrrhiza, Lonchocarpus, Millettia, Pongamia, Prosopis, Sophora, Tephrosia*, to give some prominent examples), the occurrence of externally accumulated flavonoid aglycones is a rather rare phenomenon within the Fabaceae. It was reported from *Acacia neovernica* (Wollenweber and Seigler, 1982), from *Zuccagna punctata* (Pedriva and Giordano, 1984) and from the above cited *Ononis* species. In some further cases, where leaves or aerial parts were reported as the source of flavonoid aglycones, it is well possible that they also occurred externally: so e.g. in *Anthyllis* (Pistelli et al., 1996), *Crotalaria* (Krohn et al., 2002), *Dalea* (Domínguez et al., 1982), *Lonchocarpus* (Rousis et al., 1987), *Lupinus* (Nicholls and Bohm, 1983), *Millettia* (Ganapathy et al., 1998), and *Parkia* (Lemmich et al., 1996).


