Lipophilic flavones of *Primula veris* L. from field cultivation and in vitro cultures

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Abstract

Ten lipophilic flavones were isolated from the leaves of *Primula veris* from field cultivation – the newly described 3'-hydroxy-4',5'-dimethoxyflavone and 3'-methoxy-4',5'-methylenedioxyflavone, the previously known from chemical synthesis 3',4'-dimethoxyflavone, 2',5'-dimethoxyflavone, and also flavone, 2'-hydroxyflavone, 2'-methoxyflavone, 3'-methoxyflavone, 3',4',5'-trimethoxyflavone and 5,6,2',6'-tetramethoxyflavone (zapotin) which were previously known from plants. The same flavones were found in the leaves of *P. veris* obtained by in vitro propagation. The structural assignments were derived from 1H NMR, 13C NMR, EIMS and UV spectral data and the influence of B-ring oxygen substituents on the C-2, C-3 and H-3 NMR resonances in flavones unsubstituted in the A ring is taken into consideration.

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Keywords: *Primula veris*; Primulaceae; Cowslip; Leaves; In vitro culture material; Flavonoids; Flavones; Methoxyflavones; Hydroxyflavone; Methylenedioxyflavone

1. Introduction

*Primula veris* L. (Primulaceae), one of more than 400 species from the genus, is distributed throughout Europe and Asia. It is a well-known medicinal plant, which provides rhizome and roots (*Primulae radix* (Schöpke, 1994). The flowers of *P. veris*, of similar medicinal use (*Primulae flos* (Schöpke, 1994), were found to contain a number of flavonol glycosides (Karl et al., 1981; Hegnauer, 1990) and several methoxyflavones (Huck et al., 1999, 2000; Stecher et al., 2003), the latter representing a group of lipophilic flavonoids found earlier in the leaf exudates of some *Primula* species (Hegnauer, 1969, 1990; Wollenweber et al., 1988, 1990; Wollenweber and Mann, 1986). A few flavonoids were also detected in the leaves of *P. veris* (Harborne, 1968; Hegnauer, 1990).

Here, we report the isolation and structural elucidation of 10 lipophilic flavones (1–10) from the leaves of cultivated *P. veris* plants and detection of these compounds in the leaves from the recently established in vitro culture of *P. veris* (Morozowska and Wesołowska, 2004). All flavones but one (1 – zapotin) are substituted only in the B ring and four of them (compounds 2, 4, 6, 7) are newly discovered in nature. The influence of the oxygen substituents in the B ring on the NMR shifts of C-2, C-3 and H-3 is also discussed.
2. Results and discussion

Classical two-dimensional thin-layer chromatography (2D TLC) on cellulose was performed on chloroform extracts from *P. veris* leaves, collected from field cultivation and those obtained by in vitro culture (Morozowska and Wesołowska, 2004). The results exhibited a cluster of overlapping spots with blue fluorescence under UV light and high Rf values in the organic mobile phase, presumably corresponding to the 5-deoxy or 5- O-substituted, lipophilic flavones (Harborne, 1991). A mixture of compounds was isolated, from the extract of the material from field cultivation, through column chromatography on cellulose and polyamide, and further separated by preparative thin-layer chromatography (prep. TLC) on silica gel and then on polyamide or cellulose to give 10 compounds 1-10.

Compound 2 exhibited a molecular ion [M]+ at m/z 298 and 298.08460 in the EIMS and HREIMS spectra, respectively, indicating a molecular formula of C17H14O5 which is consistent with the structure of mono-hydroxydimethoxyflavone. This was further supported by the presence of 17 carbon signals in the 13C NMR spectrum. The EIMS showed retro-Diels–Alder (RDA) ions at m/z 121 [A1 + H]+ and 181 [B3]+ indicative of an unsubstituted A ring, as well as the presence of one hydroxy group and two methoxyl groups in the B ring. The 1H NMR spectrum of 2 (Table 1), interpreted with the aid of an HH-COSY spectrum, showed a set of four coupled aromatic proton signals at δH 8.23, 7.43, 7.71 and 7.57 corresponding to the A ring H-5, H-6, H-7 and H-8, respectively (Freeman et al., 1981; Wollenweber et al., 1988). Moreover, the two meta-coupled protons present at δH 7.24 and 7.01 (each J = 2.4 Hz) were assigned to the B ring H-2' and H-6' on the basis of their 1J coupling to the C-2 signal at δC 163.1 in the HMBC spectrum. This pointed to the asymmetrical substitution of that ring with the hydroxyl group, resonating at δH 5.99, and two methoxyl groups present at δH 4.00 and 3.96, which therefore needed to be placed at positions 3', 4' and 5', respectively. The 13C NMR spectrum of 2, fully assigned with the help of HH-COSY, HSQC and HMBC spectra, is presented in Table 2. Hence, compound 2 is identified as 3'-hydroxy-4',5'-dimethoxyflavone.

Compound 6 gave EIMS and HREIMS spectra with molecular ion peak [M]+ at m/z 296 and m/z 296.06911, respectively, indicating the formula C17H15O5 and corresponding to a flavone with oxygen substituents. The EIMS fragmentation suggested presence of an unsubstituted A ring owing to the RDA ions at m/z 120 [A1]+ and 121 [A1+H]+ and the attachment of oxygen functions to the B ring from the RDA ions at m/z 162 [B1]+ and 165 [B2]+. The 1H NMR spectrum (Table 1) exhibited signals with splitting patterns similar to those of compound 2, i.e., four protons of ring A and two meta-positioned protons at δH 7.11 and 7.14 (each J = 1.6 Hz) ascribable to the B ring, besides a singlet at δH 4.00, which integrated with three protons – typical for the methoxyl group and a singlet at δH 6.09, which integrated with two protons and thus was assigned to a methylenedioxy group. The 13C NMR spectrum of 6, assigned with the aid of 2D NMR spectra, showed methoxyl and methylenedioxy group signals at δC 56.9 and 102.3, respectively, besides the expected fifteen carbon signals of the flavone nucleus (Table 2). From these data the B ring protons can only be H-2' and H-6' and hence compound 6 is identified as 3'-methoxy-4',5'-methylenedioxyflavone.

The remaining compounds were identified by interpretation of their 1H NMR, 13C NMR, EIMS and UV spectral data and by comparisons to those available in the literature, as the known flavones – 5,6,2',6'-tetramethoxyflavone (zapotin) (1) (Dreyer and Bertelli, 1967; Ito et al., 1998), 2'-hydroxyflavone (3) (Blaskó et al., 1988; Wollenweber et al., 1988), 3',4'-dimethoxyflavone (4) (Mabry et al., 1970; Miyake et al., 2003; Ahmed et al., 2003), 3',4',5'-trimethoxyflavone (5) (Gaydou and Bianchini, 1978; Obrecht, 1989; Inumia et al., 1992), 2',5'-dimethoxyflavone (7) (Gallagher et al., 1953; Inumia et al., 1980; Tanaka et al., 1986), 2'-methoxyflavone (8) (Freeman et al., 1981; Blaskó et al., 1988), 3'-methoxyflavone (9) (Inumia et al., 1980; Freeman et al., 1981), and flavone (10) (Mabry et al., 1970; Blaskó et al., 1988).

In the literature cited above, the NMR data for some known compounds are missing (compound 7), the signal assignment is absent (4), incomplete (5, 9) or partly incorrect (1). There exist the possibility to observe the influence of B ring oxygen substitution on the shifts of flavones unsubstituted in the A ring. We wish to present the 1H and 13C NMR data of the isolated compounds in...
Table 1

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*In CDCl₃.*

**Table 2.** Our HSQC and HMBC data for zapotin (1) indicate that previously reported ¹³C NMR shifts for C-9 (δc 119.4) and C-10 (δc 149.6) (Ito et al., 1998) need to be interchanged. 2'-Hydroxyflavone (3) exhibited B ring protons signals at somewhat different shifts than reported earlier (Blaskó et al., 1988), probably owing to the low concentration of our sample, and exhibited a 2'-hydroxyl signal at δH 10.79, not previously reported. 2',5'-Dimethoxyflavone (5) could be discriminated from the alternative 2',4'- and 3',4'-dimethoxy isomers (Inunaka et al., 1980; Gaydou and Bianchini, 1978) on the basis of its ¹³C NMR spectrum but as far as ¹H NMR data are considered only an H-3 shift has been reported (Tanaka et al., 1986). In the case of 3'-methoxyflavone...
Among the compounds identified in the present work, 3',4',5'-trimethoxyflavone (5), 3'-methoxyflavone (9) and flavone (10) were reported from the flowers of *P. veris* (Huck et al., 1999, 2000; Stecher et al., 2003) and the latter compound (10) also from other species of the genus *Primula* and the family Primulaceae (Hegnauer, 1969); 2'-methoxyflavone (8) was found in *Primula kwensensis* (Wollenweber and Mann, 1986) and *Pimelea simplex* (Thymelaeaceae) (Freeman et al., 1981), and 2'-hydroxyflavone (3) in *Primula florindae* (Bouillant et al., 1971) and *Daphnopsis sellowiana* (Thymelaeaceae) (Blaskó et al., 1988). The remaining compounds (1, 2, 4, 6, 7) are new for the species *P. veris* and the genus *Primula*. Zapotin (1) has been previously isolated from *Casimiroa edulis* (Rutaceae) (Kincl et al., 1956) (structure elucidation by Dreyer and Bertelli, 1967; Garratt et al., 1967) and Sargentia greggi (Rutaceae) (Meyer et al., 1985). 3',4'-Dimethoxyflavone (4) and 2',5'-dimethoxyflavone (7) are new from the natural source, although they have been obtained by chemical synthesis (Gaydou and Bianchini, 1978; Gallagher et al., 1953; Inuma et al., 1980), while 3'-hydroxy-4',5'-dimethoxyflavone (2) and 3'-methoxy-4',5'-methylenedioxyflavone (6) are found for the first time in nature and have not yet been reported by chemical synthesis.

The subfractions obtained by the preparative separation of chloroform extracts of the leaves from *P. veris* were chromatographically indistinguishable from the relevant subfractions of material (9), the first-order spin system of B ring proton signals appeared in the NMR spectrum recorded at 600 MHz.

It has already been stated that, in 2'-oxygenated (but not 2',6'-dioxygenated) flavones, the H-3 signal occurs at low field, around δH 7.0, in contrast to the high field value, δH 6.8–6.7, observed for the flavone itself and flavones oxygenated at other positions of the B ring (Tannaka et al., 1986). This appears to be valid for compounds obtained in our work. From the NMR data (measured in CDCl3) presented in Tables 1 and 2, it may be observed that 2'-oxygenation of the flavones, compounds 7 and 8, causes an upfield shift of the C-2 signal by ca. −2.7 ppm (δC around 160.6) and the downfield shift of the C-3 signal by ca. +5.2 ppm (δC around 113), relative to the resonances of flavone (10) at δC 163.4 (C-2) and δC 107.6 (C-3). Oxygen substituents at the 3',4' or 5' positions, as in compounds 2, 4, 5, 6, 9, have negligible influence on C-ring NMR shifts. This is in accordance with NMR shifts (measured also in DMSO-d6) reported earlier for the other flavones bearing oxygen substituents in the B ring (Gaydou and Bianchini, 1978; Inuma et al., 1980). An exception to the above is 2',4'-dimethoxyflavone where assignments for C-2 at δC 162.7 and C-4' at δC 160.9 (Inuma et al., 1980) should be reversed, especially as the shift value for C-4' in 4'-oxygenated flavones, lacking substituents at both adjacent carbons (C-3', C-5'), were reportedly previously to be around δC 162 (Gaydou and Bianchini, 1978).

### Table 2

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a Assignments aided with HSQC and HMBC at 600 MHz.
b Signal did not emerge from the background.
from field cultivation, indicating that biosynthesis of flavones 1–10 is maintained under in vitro culture conditions (Morozowska and Wesołowska, 2004).

It is noteworthy that, apart from the possible role of the detected flavones 1–10 as UV-protectants for the plant (Harborne and Williams, 2000) and chemotaxonomic markers for the genus Primula, some flavones have been found to possess interesting biological properties. 2′-Hydroxyflavone shows cytostatic activity (Tokalov et al., 2004), 2′-methoxyflavone and zapotin exhibit some antimutagenic activity (Edenharder and Tang, 1997), while 3′,4′-dimethoxyflavone and zapotin have been reported to possess anti-cancer properties (Lee and Safe, 2000; Mata-Greenwood et al., 2001). These properties may be expected for the lipophilic flavone fractions from P. veris, which can be obtained not only from field cultivated plants, but also by means of biotechnological methods, i.e., in vitro culture.

3. Experimental

3.1. General

Spectroscopy. UV spectra were recorded in MeOH using a Specord M-40 spectrophotometer (Zeiss, Jena) equipped with M-40 computer software according to procedures described earlier (Mabry et al., 1970). NMR spectra were taken on the Varian Unity 300 (1H: 300 MHz, 13C: 75 MHz) and Bruker MDX-600 (1H: 600 MHz, 13C: 150 MHz) spectrometers in CDCl3 or Me2CO-d6 with spectra referenced to residual 1H in the deuterated NMR solvents. EIMS spectra were recorded on an AMD 402 spectrometer (Intectra) at 70 eV (probe).

Chromatography. Analytical TLC was carried out on pre-coated silica gel F-254 or cellulose plastic-backed sheets, polyamide 11 aluminium-backed sheets (Merck) and polyamide DC6 (Macherey-Nagel) plates produced in-house. Preparative TLC was performed on pre-coated, glass-backed silica gel F-254 plates (0.25 mm, Macherey-Nagel) plates made in-house. Each extract or fraction (0.1 g), dissolved in an appropriate solvent (1.0 ml) – CHCl3 (CHCl3 fr.), MeOH (BuOH frs.) or EtOH–H2O 1:1 (H2O fr.), was analysed by 2D TLC on cellulose in 1-BuOH–HOAc–H2O 4:1:5 (first direction) and AcOH–H2O 3:7 (second direction). CHCl3 fractions were also chromatographed by 1D TLC on silica gel F-254 in n-hexane–AcOEt 7:3 (Huck et al., 2000), on polyamide DC6 or 11 in n-hexane–toluene–MeCOEt–MeOH 12:6:1:1; 25:6:1:1 and on cellulose in AcOH–H2O 3:17. For detection, plates were viewed under UV 365 and 254 nm light, before and after spraying with 1% AlCl3 or 0.1% 2-aminoethanol diphenylborate solutions in EtOH.

3.3. Extraction and fractionation

Samples of leaves (5.0 g each) were extracted under reflux with CHCl3 (7 × 150 ml) and MeOH (3 × 150 ml). The dried MeOH extracts were fractionated by partitioning between 1-BuOH (3 × 100 ml) and H2O (100 ml). The bulk sample (20 g) of the leaves of plants from field cultivation was extracted with CHCl3 (300 ml) and MeOH (400 ml) in a Soxhlet apparatus. Each extract and fraction was concentrated to dryness.

3.4. Chromatographic analyses

Each extract or fraction (0.1 g), dissolved in an appropriate solvent (1.0 ml) – CHCl3 (CHCl3 fr.), MeOH (BuOH frs.) or EtOH–H2O 1:1 (H2O fr.), was analysed by 2D TLC on cellulose in 1-BuOH–HOAc–H2O 4:1:5 (first direction) and AcOH–H2O 3:7 (second direction). CHCl3 fractions were also chromatographed by 1D TLC on silica gel F-254 in n-hexane–AcOEt 7:3 (Huck et al., 2000), on polyamide DC6 or 11 in n-hexane–toluene–MeCOEt–MeOH 12:6:1:1; 25:6:1:1 and on cellulose in AcOH–H2O 3:17. For detection, plates were viewed under UV 365 and 254 nm light, before and after spraying with 1% AlCl3 or 0.1% 2-aminoethanol diphenylborate solutions in EtOH.

3.5. Isolation

The CHCl3 fraction (0.85 g), obtained from the bulk sample of leaves from field cultivation, was separated by CC on cellulose with MeOH–H2O 7:3. The blue-fluorescent eluate obtained (0.4 g) was rechromatographed over a polyamide column eluted with MeOH to give a new eluate (0.35 g) which was further separated by prep. TLC on silica gel in n-hexane–AcOEt 7:3 into 10 bands – 1–10, in order of increasing Rf value. Band 2 gave compound 1 (14.2 mg) after prep. TLC on Avicel in HOAc–H2O 3:17 followed by polyamide and Sephadex LH20 CC in MeOH. Band 4 was separated by prep. TLC on polyamide in toluene–MeCOEt 9:1 (run x 3) to give compound 2 (4.8 mg). Band 5 yielded compounds 3 (1.0 mg) and 4 (1.8 mg) after separation by prep. TLC on polyamide in toluene–EtOH 19:1 and final clean-up by Sephadex CC in MeOH. Band 7 was separated by prep. TLC on polyamide in n-hexane–toluene–MeCOEt–MeOH 25:6:1:1 to afford compounds 6 (5.4 mg) and 7 (4.6 mg), each of which was further purified by Sephadex LH20 CC in MeOH. From bands 6, 8, 9 and 10 compounds 5 (3.2 mg), 8 (30.9 mg), 9 (12.5 mg) and 10 (1.8 mg) were obtained by CC on polyamide and Sephadex LH20 in MeOH, respectively.
Part (0.4 g) of the total CHCl₃ fraction (0.5 g) obtained from in vitro culture material, was separated by prep. TLC on silica gel as described above into 10 bands (1–10), which were compared by TLC on silica gel, polyamide and cellulose (see Section 3.4) with the relevant bands (1–10) obtained from the extract of the material from field cultivation.

3.6. 5,6,2',6'-Tetramethoxyflavone (zapotin) (1)

White crystalline solid (CHCl₃); EIMS: m/z (rel. int.): 238 [M⁺] (100); UV and MS data are comparable to published values (Dreyer and Bertelli, 1967); ¹H NMR: see Table 1; ¹³C NMR: see Table 2.

3.7. 2-(3-Hydroxy-4,5-dimethoxyphenyl)-4H-1-benzopyran-4-one or 3'-hydroxy-4',5'-dimethoxyflavone (2)

White crystalline solid (CHCl₃); UV (MeOH) λmax nm (log ε): 220 (4.11), 238 (sh.) (3.91), 311 (3.97), 335 (sh.) (3.82), +NaOAc 238, 311, 333, 365 (sh.), no shift with NaOAc, NaOAc/H₃BO₃, AlCl₃ and AlCl₃/HCl; ¹H NMR: see Table 1; ¹³C NMR: see Table 2; EIMS m/z (rel. int.): 298 [M⁺] (100), 255 [M – Me – CO]⁺ (18), 227 (19), 212 (9), 283 [M – Me]⁺ (54), 181 [B₁]⁺ (13), 117 [B₁]⁺ (2), 163 [B₁ – Me]⁺ (6), 121 [A₁ + H]⁺ (5), 120 [A₁]⁺ (9), 92 [B₂ – B₂]⁺ (13); HR–EI–MS m/z: 298.0846 [M⁺] (Calc. for C₁₇H₁₂O₅, 298.08414).

3.8. 2'-Hydroxyflavone (3)

White solid (needles) (CHCl₃); EIMS m/z (rel. int.): 238 [M⁺] (100); UV and MS data are comparable to published values (Blasko et al., 1988); ¹H NMR: see Table 1.

3.9. 3',4'-Dimethoxyflavone (4)

White crystalline solid (CHCl₃); EIMS: m/z (rel. int.): 282 [M⁺] (100); UV and MS data are comparable to published values (Mabry et al., 1970; Mabry et al., 1976; Ahmed et al., 2003); ¹H NMR: see Table 1; ¹³C NMR: see Table 2.

3.10. 3',4',5'-Trimethoxyflavone (5)

White solid (CHCl₃); EIMS: m/z (rel. int.): 312 [M⁺] (100); UV and MS data are comparable to published values (Gaydou and Bianchini, 1978; Obrecht, 1989); ¹H NMR: see Table 1; ¹³C NMR: see Table 2.

3.11. 2-(3-Methoxy-4,5-methylenedioxyphenyl)-4H-1-benzopyran-4-one or 3'-methoxy-4',5'-methylenedioxyflavone (6)

White crystalline solid (CHCl₃); UV (MeOH) λmax nm (log ε): 220 (4.01), 242 (sh.) (3.89), 310 (sh.) (3.76), 338 (3.85), no shift with NaOMe; ¹H NMR: see Table 1; ¹³C NMR: see Table 2; EIMS m/z (rel. int.): 296 [M⁺] (100), 281 [M – Me]⁺ (3), 268 [M – CO]⁺ (10), 176 [B₁]⁺ (51), 148 [B₁ – Me]⁺ (4), 121 [A₁ + H]⁺ (5), 120 [A₁]⁺ (4); HR–EI–MS m/z: 296.06911 [M⁺] (Calc. for C₁₇H₁₂O₅, 296.06848).

3.12. 2',5'-Dimethoxyflavone (7)

Light yellow solid (CHCl₃); EIMS: m/z (rel. int.): 282 [M⁺] (100); UV and MS data are comparable to published values (Gallagher et al., 1953; Tanaka et al., 1986); ¹H NMR: see Table 1; ¹³C NMR: see Table 2.

3.13. 2'-Methoxyflavone (8)

White crystalline solid (CHCl₃); EIMS: m/z (rel. int.): 252 [M⁺] (100); UV and MS data are comparable to published values (Freeman et al., 1981; Blasko et al., 1988); ¹H NMR: see Table 1; ¹³C NMR: see Table 2.

3.14. 3'-Methoxyflavone (9)

White crystalline solid (CHCl₃); EIMS: m/z (rel. int.): 252 [M⁺] (100); UV and MS data are comparable to published values (Freeman et al., 1981); ¹H NMR: see Table 1; ¹³C NMR: see Table 2.

3.15. Flavone (10)

White solid (CHCl₃); EIMS: m/z (rel. int.): 222 [M⁺] (100); UV and MS data are comparable to published values (Mabry et al., 1970; Blasko et al., 1988); ¹H NMR: see Table 1; ¹³C NMR: see Table 2.

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References


