Flavon- and Flavonolglycosides from *Achillea pannonica* Scheele[§]

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The detailed investigation of a methanolic extract of aerial parts of *Achillea pannonica* SCHEELE. within a chemotaxonomic study led to the isolation of 6 flavonoid glycosides. Besides rutin, apigenin-7-O-glucopyranoside, luteolin-7-O-glucopyranoside, apigenin-7-O-rutinoside and acacetin-7-O-rutinoside, an unusual flavondiglucoside was isolated. Its structure was established by UV, ^{1}H NMR and ^{13}C NMR spectroscopic methods including 2D-NMR techniques and ESI-MS as luteolin-7,4′-O-β-diglucoside. This substance is reported for the first time in the genus *Achillea*. Chemotaxonomic aspects are discussed briefly.

Introduction

Herbal teas from different species of the *Achillea millefolium* group are used in folk medicine against gastrointestinal disorders due to their antiphlogistic, spasmolytic and haemostyptic activities (Wichtl, 1997). It was shown that besides sesquiterpenes with proven antiphlogistic properties, flavonoids may contribute to the pharmacological activity of the drug (Della Loggia *et al.,* 1992). In a chemotaxonomical investigation among *Achillea* species the common occurrence of rutin and luteolin-7-O-glycosides as main flavonoids in the *A. millefolium* group was reported (Valant, 1978). Recently we studied the flavonoid pattern of *Achillea collina* BECKER. In this study we proved additional flavonoids, O-diglycosides as well as C-glycosides, in this tetraploid species of the *A. millefolium* group (Kasaj *et al.,* 2001). In the *A. millefolium* group the close relationship between the tetraploid *A. collina* and the octoploid *Achillea pannonica* SCHEELE was deduced from similarities in the flavonoid pattern (Valant-Vetschera, 1981). In a chemotaxonomic approach we investigated the flavonoid composition of this species and report the isolation and characterisation of six flavonoids from the aqueous methanolic extract.

Experimental

General

NMR-spectra were recorded on Varian Unity Inova 400 MHz (297 K) NMR-Spectrometer. 5 mm sample tubes, solvent resonance as internal standard. ^{1}H, ^{1}H-COSY: 90° pulse; ge-HSQC optimized to 140 Hz couplings, ge-HMBC optimized to 8 Hz couplings.

ESI-MS were recorded on a PE Sciex API 150 EX single quadrupole instrument, configurated for negative ionisation, the orifice plate voltage set at −20 and −80 V. Full scan spectra were acquired over the range 200 −700 mz. Scan time: 2 s.

GC-MS identification and determination of the absolute configuration of monosaccharide units were performed on a Shimadzu 5050A quadrupol mass spectrometer according to (De Bettignies-Dutz *et al.,* 1991).

Capillary electrophoresis (CE) was performed on SpectraPHORESIS 1000 according to (Marchart, 2001).

Analytical HPLC was performed on a Perkin-Elmer Series 200 Liquid Chromatograph, with 600 LINK Controller, LC-235 diode array detector

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and series 200 autosampler. Column: Nucleosil 100–5C 18 (250 × 4 mm) (Macherey&Nagel, Germany). Solvents: MeCN (A) and aq. H_3PO_4 pH 3 (B). Gradient elution: 0–20 min from 20 to 30% solvent A; 20–21 min from 30 to 100% A; 21–31 min 100% A; 31–32 min from 100 to 20% A; 32–42 min 20% A. Flow rate: 1.0 ml min^{-1}. Detection at 340 nm, room temperature.

Preparative HPLC was carried out on two ISCO 2350 HPLC pumps with a linear UVIS-205 absorbance detector.

UV spectra were recorded on Beckmann DU 640 Spectrophotometer using MeOH as blank. The preparation of shift-reagent solutions and analyses of the flavonoids after derivatisation were carried out by standard procedures (Mabry *et al.*, 1970).

Polyamide, Sephadex®-LH-20 and XAD-2 used for CC were obtained from ICN Pharmaceuticals (Eschwege, Germany), Pharmacia Biotech (Uppsala, Sweden) and Supelco (Bellefonte, USA), respectively.

TLC Silica gel plates (Merck, Germany), 0.25 mm. System A: EtOAc-HOAc-HCO_2H-H_2O (100:11:11:26). System B: EtOAc-butanone-HCO_2H-H_2O (50:30:10:10). TLC cellulose plates (Merck, Germany), 20 × 20 cm, 0.5 mm. System C: n-BuOH-HOAc-H_2O (4:1:5) upper phase. Detection: 1% MeOH solution of diphenyl-boric acid-ethanolamine complex (= Naturstoffreagens A) and additionally with 5% EtOH solution of PEG 400. After drying the plates were controlled under UV_{366}.

Reference flavonoids

Rutin (**1**), luteolin-7-O-glucoside (**2**) and apigenin-7-O-glucoside **(3)** were obtained from K. Roth, Germany. Apigenin-7-O-β-rutinoside was isolated from *Achillea collina* (Kasaj *et al.*, 2001).

Plant material

The aerial parts of *A. pannonica* were collected in Falkenstein, Austria, in 1991. The material was identified by J. Saukel, Institute of Pharmacognosy, Univ. Vienna, a voucher specimen is deposited in the herbarium of the institute.

Extraction and isolation

Dried, pulverised aerial parts (840 g) of *A. pannonica* were percolated with CH_2Cl_2 for the removal of unpolar substances. The purified drug was extracted exhaustively with 40% MeOH under reflux. The aq. methanolic extract (100 g) was separated in six portions by CC on polyamide (50 × 3 cm) using H_2O-MeOH mixtures as solvent to yield ten fractions (fr. 1a-10a) from the first portion, nine fr. (fr. 1b-9b) from the second, eight fr. (fr. 1c-8c) from the third, nine fr. (fr. 1d-9d) from the fourth, ten fr. (fr.1e-10e) from the fifth and nine fr. (fr.1f-9f) from the sixth one. Compounds **2** and **3** were identified after purification of the fractions 4c, 4d and 4e (3 g) on CC Sephadex® LH-20 (65 × 4 cm) eluted with 45%-100% MeOH. The combined fraction of 5c, 5d and 6c (1.49 g) was separated by CC on XAD-2 (65 × 4 cm) with H_2O-MeOH mixtures to obtain four subfractions (Ia-IVa). The fractionation of 2b and 2c (0.37 g) by CC over Sephadex® LH-20 (2 × 30 cm) eluted with 50% EtOH, afforded also four subfractions (Ib-IVb). The separation of the subfractions IIa and IVb (0.1 g) by the use of preparative RP-HPLC on Nucleosil 100–7C 18 (250 × 21 mm, Macherey&Nagel, Germany) and isocratic elution with 15% AcCN at a flow rate of 12 ml min^{-1}, detection at 340 nm, afforded 20 mg compound **1** and 4 mg compound **4**. The fractionation of the combined fractions of 3b, 3c and 3d (2.63 g) by CC on Sephadex® LH-20 (2 × 65 cm) eluted with 20% MeOH with gradually increasing amounts of MeOH afforded subfractions Ic-Vc. Similar CC of the fractions 3e and 2f (1.42 g) gave subfractions Id-IXd. Further purification of subfractions Ic and VId (0.44 g) after gel chromatography on Sephadex® LH-20 (1 × 20 cm) with 25% MeOH afforded 5 mg of compound **6**. 3 mg of compound **5** were isolated by preparative HPLC of the subfraction IXd (0.045 g) with 20% AcCN (pH adjusted to 3 using TFA) under the same conditions like substances **1** and **4**.

Acacetin-7-O-rutinoside (**5**). TLC *Rf*: 0.47 (system A); 0.46 (system B); 0.49 (system C). *Rt*-HPLC: 20.30 min. CE migr.time: 5.98 min. UV λmax MeOH nm: 269, 324; +NaOAc: 269, 326; +NaOAc+H_3BO_3: 269, 326; +$AlCl_3$: 276, 300sh, 342, 385sh; +$AlCl_3$+HCl: 277, 300sh, 338, 385sh; +NaOMe: 285, 362. ^{1}H NMR (400 MHz, MeOH):

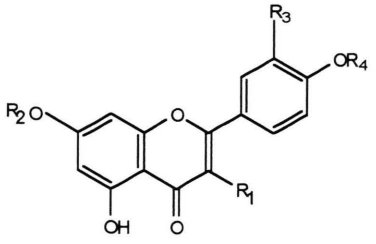
δ ppm 1.22 (3H, d, H-6‴), 3.34 (1H, m, H-4″), δ 3.39 (1H, m, H-4‴), δ 3.50 (1H, d, H-2″), 3.57 (1H, m, H-3″), 3.68 (1H, m, H-5″), 3.71 (1H, m, H-5‴), 3.72 (1H, dd, H-6a″), 3.80 (1H, m, H-3‴), 3.93 (3H, s, O-CH_3), 4.04 (1H, d, H-2‴), 4.08 (1H, dd, H-6b″), 4.75 (1H, d, H-1‴), 5.09 (1H, d, H-1″), 6.57 (1H, d, *J* = 2Hz, H-6), 6.74 (1H, s, H-3), 6.82 (1H, d, *J* = 2Hz, H-8), 7.15 (1H, d, *J* = 8.8Hz, H-3′ and H-5′), 8.02 (1H, d, *J* = 8.8Hz, H-2′and H-6′). Multiplicities of most sugar resonances not determined because of signal overlap.^{13}C NMR: δ ppm 17.0 (C-6‴), 56.0 (O-CH_3), 67.8 (C-6″), 69.8 (C-5‴), 71.1 (C-2‴) 71.8 (C-4″), 72.3 (C-3‴), 74.0 (C-4‴), 74.7 (C-2″), 78.0 (C-5″), 78.1 (C-3″), 96.2 (C-8), 101.6 (C-1″), 101.7 (C-6), 102.6 (C-1‴), 104.9 (C-3), 107.0 (C-10), 115.6 (C-3′ and C-5′), 124.0 (C-1′), 129.2 (C-2′ and C-6′), 158.5 (C-5), 162.5 (C-9), 163.0 (C-4′), 164.3 (C-2), 165.5 (C-7), 183.1 (C-4). Negative ESI-MS ($C_{28}H_{32}O_{14}$) *m/z*: 591 [M-H]$^-$, 445 [M-H-146]$^-$, 283 [M-H-146–162]$^-$= [agycone-H]$^-$.

Luteolin-7,4′-O-β-diglucoside (**6**). TLC R*f*: 0.20 (system A); 0.17 (system B); 0.24 (system C). *Rt*-HPLC: 4.42 min. CE migr. time: 7.74 min. UV λmax MeOH nm: 270, 336; +NaOAc: 267, 338; +NaOAc+H_3BO_3: 269, 337; +$AlCl_3$: 277, 296sh, 348, 388; +$AlCl_3$+HCl: 272, 297sh, 348, 398; + NaOMe: 267, 299sh, 369. ^{1}H NMR (400 MHz, MeOH): δ ppm 3.40–3.60 (H-2″- H-5″ and H-2‴-H-5‴), 3.75 (1H, dd, H-6a″ and H-6a‴), 3.97 (1H, dd, H-6b″ and H-6b‴), 4.96 (1H, d, *J* = 7.5Hz, H-1‴), 5.12 (1H, d, *J* = 7.5Hz, H-1″), 6.55 (1H, d, *J* = 2.1Hz, H-6), 6.72 (1H, s, H-3), 6.87 (1H, d, *J* = 2.1Hz, H-8), 7.35 (1H, d, *J* = 11.0Hz, H-5′), 7.49 (1H, dd, $J_{5',6}$= 11.0Hz, $J_{2',6'}$ = 2.0Hz, H-6′), 7.51 (1H, d, *J* = 2.0Hz, H-2′). Multiplicities of most sugar resonances not determined because of signal overlap. ^{13}C NMR: δ ppm 62.6 (C-6″ and C-6‴), 71.6 (C-4″ and C-4‴), 75.1 (C-2″), 75.5 (C-2‴), 78.5 (C-3″ and C-3‴), 79.1 (C-5″ and C-5‴), 96.8 (C-8), 102.0 (C-6), 102.2 (C-1″), 104.0 (C-1‴), 105.9 (C-3), 108.2 (C-10), 115.9 (C-2′), 118.5 (C-5′), 119.8 (C-6′), 129.5 (C-1′), 149.8 (C-3′), 151.9 (C-4′), 159.0 (C-9), 161.0 (C-5), 166.3 (C-7), 168.0 (C-2), 185.8 (C-4). Negative ESI-MS ($C_{27}H_{30}O_{16}$) *m/z*: 609 [M-H]$^-$, 447 [M-H-162]$^-$, m/z 285 [M-H-162–162]$^-$= [agycone-H]$^-$.

Results and Discussion

From a 40% methanolic extract of aerial parts of *A. pannonica* 6 flavonoids were isolated by CC on polyamide and Sephadex® LH-20 by gradient elution with H_2O-MeOH and H_2O-EtOH mixtures. Further purification by CC on XAD-2 and by preparative HPLC on C 18 yielded rutin (**1**), luteolin-7-O-glucopyranoside (**2**) and acacetin-7-O-rutinoside (**5**), which had been described before in *A. pannonica* (Valant 1978; Valant-Vetschera 1981). In addition apigenin-7-O-glucopyranoside (**3**), apigenin-7-O-rutinoside (**4**) and luteolin-7,4′-O-diglucoside (**6**) were isolated for the first time from this species of the *A. millefolium* group (Fig. 1).

Comparison of the R*f*-TLC, R*t*-HPLC and CE-migration time as well as UV spectroscopic and



Compound	R_1	R_2	R_3	R_4
1	O-rhamnosyl-1‴→6″-glucose	H	OH	H
2	H	glucose	OH	H
3	H	glucose	H	H
4	H	O-rhamnosyl-1‴→6″-glucose	H	H
5	H	O-rhamnosyl-1‴→6″-glucose	H	CH_3
6	H	glucose	OH	glucose

Fig. 1. Flavonoids in *Achillea pannonica.*

ESI-MS data with those from authentic substances revealed the structures of compounds **1** – **4**. The structure of **5** was established additionally by NMR, 2D-NMR techniques and the sugars and their specific linkages were confirmed after permethylation, acid hydrolysis and trimethylsilylation by GC-MS. Structure elucidation of **6** was performed by UV, ESI-MS, NMR and 2D-NMR-techniques.

Negative ESI-MS of **6** showed a peak at *m/z* 609 $[M-H]^-$ suggesting the molecular formula ($C_{27}H_{30}O_{16}$). The fragment ions at *m/z* 447 $[M-H-162]^-$ and *m/z* 285 $[M-H-162-162]^-$=[agycone-H]$^-$ gave the indication of two hexose units and their O-glycosidic linkage to the aglycone. The UV spectrum in MeOH gave maxima at 270 and 336 nm. The diagnostic UV shifts, by comparison with those of luteolin-7-O-glucoside (Mabry *et al.*, 1970) and luteolin-4′-O-glucoside (Pieroni *et al.*, 1996; Williams *et al.*, 1993) suggested the attachment of the sugars at C_7-OH and $C_{4'}$-OH. Decreasing intensity of the signal of band I, observed in presence of NaOMe indicated a substituted 4′-OH. Moreover, the absence of a bathmochromic shift for band I when comparing spectra recorded in MeOH/$AlCl_3$ and in MeOH/$AlCl_3$+HCl confirmed the absence of a dihydroxylated B-ring. In the ^{1}H NMR spectrum the chemical shifts and the coupling constants of the protons indicated a 5,7-dihydroxylated pattern for ring A (two meta-coupled doublets at 6.55 ppm and 6.87 ppm, J = 2.1 Hz), a 3′,4′-dihydroxylation for ring B (a doublet at 7.51 ppm, *J = 2.0 Hz, for 2′-H, a doublet at 7.35 ppm,* J = 11.0 Hz, for 5-H′ and a double doublet at 7.49 ppm, $J_{5',6'}$ = 11.0 Hz, $J_{2',6'}$ = 2.0 Hz, for 6′-H) and a singlet at 6.72 ppm for 3-H, permitted to determine the aglycon luteolin. Two anomeric protons resonated at 5.12 ppm (J = 7.5 Hz) and 4.96 ppm (J = 7.5 Hz) in the ^{1}H NMR spectrum and correlated with 102.2 ppm and 104.0 ppm, respectively, in the HSQC spectrum. The subspectrum of the sugars with high digital resolution, obtained by irradiating the anomeric proton signals at 5.12 ppm and at 4.96 ppm (1D-TOCSY), the results of HMBC and ^{1}H,^{1}H-COSY experiments and the absolute values of the coupling constants indicated the presence of two glucopyranosyl moieties with β-configuration at the anomeric carbon. By comparison of the ^{1}H NMR data for ring A and B to those published for luteolin-7-O-glucoside (Harborne, 1993) and luteolin-4′-O-glucoside (Yoshizaki, 1987), the positions of the glucose units had to be at C-7 and C-4′. The correlations between 7-H and 1″-H as well as 4′-H and 1‴-H observed in the NOE and HMBC spectra confirmed the linkage of the glucoses. Thus compound **6** was assigned as luteolin-7,4′-O-β-diglucoside. This substance is quite rare in plant kingdom and it is its first prove in the genus *Achillea*.

In accordance with an earlier investigation we proved luteolin-7-O-glucoside as main and acacetin-7-O-rutinoside and rutin as minor flavonoids in *A. pannonica,* chrysoeriol- and diosmetin-glycosides, which had been described, were not detected (Valant-Vetschera, 1981).

The occurrence of luteolin- and apigenin-7-O-glucoside as major compounds and rutin underlined the importance of these substances for the chemotaxonomic classification of the species of the *A. millefolium* group (Hoffmann, 1993; Krenn, 1998a; Smolnig *et al.*, 2000) as well as the close relation of *A. collina* and *A. pannonica* (Valant-Vetschera, 1981). This relationship was additionally confirmed by the content of apigenin-7-O-rutinoside as minor flavonoid in both species. On the other hand *A. collina* showed similarities in the flavonoid pattern with *Achillea nobilis* L. due the minor amounts of isoschaftoside and quercetin-3-O-arabinosyl-glucoside (Kasaj *et al.*, 2001; Krenn *et al.*, 1998), while from *A. pannonica* luteolin-7,4′-O-β-diglucoside was isolated as outstanding minor compound.

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