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Flavonoid Glycosides from *Achillea roseo-alba*[x]

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In the first detailed study of the flavonoid pattern of *Achillea roseo-alba* rutin, apigenin-7-O-ß-glucopyranoside, luteolin-7-O-ß-glucopyranoside, isorhamnetin-3-O-rutinoside, schaftoside, luteolin-4'-O-ß-glucopyranoside and 6-OH-luteolin-7-O-ß-glucopyranoside were proven in the methanolic extract of aerial parts of the plant. Additionally quercetin-3-O-[ß-apiofuranosyl-(1‴→2″)-ß-glucopyranoside] was identified for the first time in the genus Achillea.

Keywords: Achillea roseo-alba; Asteraceae; flavonoids, quercetin-3-O-[ß-apiofuranosyl-(1‴→2″)-ß-glucopyranoside].

Introduction

Several species from the genus *Achillea* are used traditionally as aromatic bitters with adstringent, choleretic and anti-inflammatory activities [1]. Different studies [2, 3] showed that the anti-inflammatory effect is not only attributed to the presence of sesquiterpene lactones, but also to flavonoids. Within chemotaxonomic investigations of the *Achillea millefolium* aggregate the flavonoid complex of the tetraploid species *Achillea collina* BECKER [4] and the octoploid species *Achillea pannonica* SCHEELE [5] had been studied in detail. The object of this study was the flavonoid pattern of diploid *Achillea roseo-alba* EHREND. Until now only the flavonol glycoside rutin had been identified from this species [6]. The present communication describes the isolation of eight flavonoid glycosides from a methanolic extract of *Achillea roseo-alba*.

[x] Part of the Ph. D. thesis of D. Kasaj

Results and discussion

The fractionation of the purified methanolic extract of aerial parts of *Achillea roseo-alba* was performed by CC on polyamide and Sephadex® LH-20 using gradient elution with H_2O-MeOH mixtures. The resulting factions were further separated by DCCC with $CHCl_3$-MeOH-BuOH-H_2O mixtures, as well as by preparative HPLC. The occurrence of rutin (**1**) confirmed the results of an earlier investigation [6]. For the first time apigenin-7-O-ß-glucopyranoside (**2**), luteolin-7-O-ß-glucopyranoside (**3**), isorhamnetin-3-O-rutinoside (**4**), schaftoside (**5**), luteolin-4'-O-ß-glucopyranoside (**6**), 6-OH-luteolin-7-O-ß-glucopyranoside (**7**) and quercetin-3-O-[ß-apiofuranosyl-(1'''→2'')-ß-glucopyranoside] (**8**) were isolated from *Achillea roseo-alba* (Fig. 1).

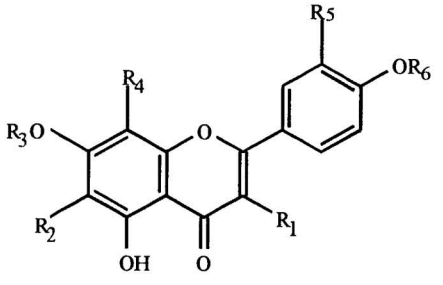
By comparison of the R*f*-TLC, R*t*-HPLC and CE-migration time as well as UV spectroscopic and ESI-MS data with those obtained from authentic substances the chemical structures of compounds **1-7** were proven. The structure of 7 was established additionally by NMR, 2D-NMR techniques. Compound **8** was identified by UV, ESI-MS, NMR and 2D-NMR-techniques. The sugars and their specific linkages in this compound were confirmed after permethylation, acid hydrolysis and trimethylsilylation by GC-MS [7].

Diagnostic UV shifts of compound **8** were comparable to rutin [8], thus suggesting quercetin as genin with 3-O-glycosidic linkage of the sugar residue. Negative ESI-MS showed a molecule ion at m/z = 595, which is 14 less than rutin with m/z = 609. The fragment ion at m/z = 301 [M-H-162-132 = aglycone]$^-$ confirmed an O-linked sugar chain and the one at m/z = 463 [M-H-132]$^-$ gave the first indication of a terminal pentose. The structure of 3-O-substituted quercetin was confirmed by the ^{1}H NMR spectral measurements and comparison with those of rutin [9]. A doublet at 6.90 ppm (5'-H), a double doublet at 7.65 ppm (6'-H) and a doublet at 7.63 ppm (2'-H) were according to the substitution pattern of ring B. The shifts of for 6-H and 8-H were 6.20 ppm and 6.40 ppm, respectively. The anomeric protons of a hexose and a pentose occurred at 5.58 ppm (1''-H) and 5.50 ppm (1'''-H). Correlation of the ^{1}H and ^{13}C NMR data by HSQC, HMBC and ^{1}H, ^{1}H-COSY experiments indicated the presence of ß-glucose and ß-apiose. Due to the good correlation of all ^{13}C NMR signals with quercetin-3-O-[ß-apiofuranosyl-(1'''→2'')-ß-glucopyranoside] [10], the attachment of the terminal apiose had to be via C_2-OH of the glucose.

For the confirmation of the sugars and their linkages, GC-MS analysis of the TMS ethers of the methylglycosides was performed additionally [7] and resulted in 1-O-TMS-2,3,5-tri-O-

methylapiose arising from the terminal apiofuranose and 1,2-di-O-TMS-3,4,6-tri-O-methylglucose, which arose from 2-linked glucopyranose. The chemical and spectroscopic data proved the structure of quercetin-3-O-[ß-apiofuranosyl-(1‴→2″)-ß-glucopyranoside] for compound **8**. This is the first report of this compound in the genus *Achillea*.

The results of this investigation suggested rutin, apigenin-7-O-ß-glucoside and luteolin-7-O-ß-glucoside to be the leading compounds in the flavonoid complex of species of the *Achillea millefolium* group. The content of schaftoside and 6-OH-luteolin-7-O-ß-glucoside pointed to relations between *Achillea roseo-alba* and *Achillea collina*.



Compound	R1	R2	R3	R4	R5	R6
1	O-rhamnosyl-1‴→6″-glucose	H	H	H	OH	H
2	H	H	glucose	H	H	H
3	H	H	glucose	H	OH	H
4	O-rhamnosyl-1‴→6″-glucose	H	H	H	OCH_3	H
5	H	glucose	H	arabinose	H	H
6	H	H	H	H	OH	glucose
7	H	OH	glucose	H	OH	H
8	O-apiosyl-1‴→2″-glucose	H	H	H	OH	H

Fig. 1 Structures of flavonoids in *Achillea roseo-alba*

Experimental

General

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 x 20 cm, 0.5 mm. System C: n-BuOH-HOAc-H_2O (4:1:5) upper phase. Detection: 1% MeOH solution of Naturstoffreagens A and additionally with 5% EtOH solution of PEG 400. After drying the plates were controlled under UV_{366}.

Analytical HPLC was performed on a Perkin-Elmer Series 200 Liquid Chromatograph, with 600 LINK Controller, LC-235 Diode Array Detector and series 200 autosampler. Column: Nucleosil 100-5C 18 (250 x 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.

For UV- and NMR-spectra as well as ESI-MS see [4,5], for GC-MS identification and determination of the absolute configuration of the monosaccharide units see [7] and for capillary electrophoresis (CE) see [13].

DCCC was carried out on a Tokyo Rikakikai DCC-A apparatus (300 tubes; i.d. 2.0mm).

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

Reference flavonoids

Rutin (**1**), apigenin-7-O-ß-glucopyranoside (**2**), luteolin-7-O-ß-glucopyranoside (**3**) and isorhamnetin-3-O-rutinoside (**4**) were obtained from K. Roth, Germany. Schaftoside (**5**) and luteolin-4'-O-ß-glucopyranoside (**6**) were isolated during earlier works from *Passiflora incarnata* [11] and *Achillea nobilis* [12], respectively.

Plant material

The aerial parts of *Achillea roseo-alba* were collected at Rosental, Kärnten, Austria, in 1991. For the botanical authentication we are grateful to Prof. J. Saukel, Institute of Pharmacognosy, University of Vienna. A voucher specimen is deposited in the herbarium of the Institute.

Extraction and isolation of flavonoids

Air-dried, pulverized aerial parts (700 g) of *Achillea roseo-alba* were percolated with CH_2Cl_2 and EtOAc for the removal of apolar substances. The remaining drug was then successively extracted with 90% MeOH under reflux and the crude methanol extract (118.3 g) was partitioned between petrol/10% MeOH (v/v) (3x), CH_2Cl_2/10% MeOH (v/v) (3x) and EtOAc/10% MeOH (v/v) (6x), for the removal of accompanying substances. The aqueous and organic layers were evaporated to dryness. TLC screening of the organic layers showed the presence of flavonoids only in the EtOAc extract (7.2 g), which was further investigated. The residue of the aq.layer (78.4 g) was re-extracted with 500 ml MeOH (3 x 30 min) by sonification. After centrifugation and evaporation of the supernatant 20.1 g extract-A resulted. The remaining precipitate was re-extracted with 500 ml MeOH (3 x 30 min) under reflux. Centrifugation and evaporation of the supernatant yielded 14.1 g extract-B and 37.7 g precipitate.

The EtOAc extract (7.2 g) was fractionated by CC (polyamide, 50 x 3 cm) starting with 10% MeOH as eluent and increasing the MeOH concentration stepwise to 100%, yielding the fractions Ia (1.07 g), IIa (0.16 g), IIIa (1.64 g) and IVa (1.38 g). Extract-B (14.1 g) was separated in the same way into four fractions: Ib (8.28 g), IIb (0.25 g), IIIb (0.57 g) and IVb (0.64 g). The fractionation of extract-A (20.1 g) by CC (polyamide, 63 x 3 cm) like above yielded Ic (13.05 g), IIc (0.17 g), IIIc (0.14 g), IVc (1.02 g), Vc (0.45 g) and VIc (0.13 g). Similar CC of the precipitate (37.7 g) (polyamide, 50 x 3 cm) gave Id (21.97 g), IId (0.36 g), IIId (0.23 g), IVd (0.34 g) and Vd (0.75 g). The combined fraction of IIb, IIc and IId was separated by CC (Sephadex® LH-20, 50 x 3 cm, mobile phase 20% MeOH), to obtain four subfractions. Two of the subfractions were successively purified by DCCC using $CHCl_3$-MeOH-BuOH-H_2O (10:10:2:6) as solvent system in the ascending mode to give 11 mg and 21 mg of compounds **5** and **4**, respectively. The fractionation of the combined fractions of IIIa, IVb, Vc and IVd by CC over Sephadex® LH-20 (60 x 3 cm) eluted with H_2O and gradually increasing portions of MeOH up to 60%, afforded thirteen subfractions. Compounds **2** and **3** were identified in the subfractions 5, 6, 7, 8 and 9. Further purification of subfraction 6 was achieved by preparative RP-HPLC on Nucleosil 100-7 C 18 (250 x 21mm, Macherey & Nagel, Germany) by isocratic elution with 50% MeOH (pH adjusted to 3 using TFA) at a flow rate of 12 ml min^{-1}, detection at 340 nm. Thus 7 mg of compound **7** were obtained. The combined fractions of IIa, IIIb, IVc and IIId were separated by CC (Sephadex® LH-20, 50 x 3 cm) eluted with H_2O with gradually increasing amounts of MeOH to afford eight subfractions. Compound **1** was detected in subfraction 6, 7 and 8. The isolation of compounds **1** and **8** (3 mg)

from subfraction 8 was achieved by preparative RP-HPLC with 50% MeOH under the same conditions like substance 7. Purification of the combined fractions of IVa, VIc and Vd afforded compound **6** after gel chromatography on Sephadex® LH-20 (50 x 3 cm) eluted with 10%MeOH with gradually increasing the MeOH concentration up to 70%.

Physico-chemical properties of **1** – **3** and **5** see [4].

Isorhamnetin-3-O-rutinoside (**4**). TLC Rf: 0.42 (system A); 0.39 (system B); 0.51 (system C).*Rt*-HPLC: 11.43 min. CE migration time: 8.75 min. Negative ESI-MS ($C_{28}H_{32}O_{16}$) m/z: 623 [M-H]$^-$, 477 [M-H-146]$^-$, 315 [M-H-146-162]$^-$= [aglycone-H]$^-$.

Luteolin-4'-O-ß-glucopyranoside (**6**). TLC Rf: 0.64 (system A); 0.65 (system B); 0.53 (system C). *Rt*-HPLC: 13.67 min. CE migration time: 9.66 min. UV λ_{max} MeOH nm: 269, 336; +NaOAc: 273, 369; +NaOAc+H_3BO_3: 270, 339; +$AlCl_3$: 259sh, 278, 295sh, 351, 387sh; +$AlCl_3$ +HCl: 256sh, 280, 293sh, 345, 387sh; +NaOH: 269, 302sh, 379. Negative ESI-MS ($C_{21}H_{20}O_{11}$) m/z: 447 [M-H]$^-$; 285 [M-H-162]$^-$= [aglycone-H]$^-$.

6-OH-luteolin-7-O-ß-glucopyranoside (**7**). TLC Rf: 0.41 (system A); 0.44 (system B); 0.22 (system C). *Rt*-HPLC: 6.58 min. CE migration time: 9.86 min. UV λ_{max} MeOH nm: 253sh, 285, 346; +NaOAc: 270, 288 sh, 353sh, 394; +NaOAc+H_3BO_3: 262, 286 sh, 359; +$AlCl_3$: 273, 300sh, 391 sh, 428 ; +$AlCl_3$+HCl: 261, 295, 370; +NaOH: 266, 304sh, 389. ^{1}H NMR (400 MHz, MeOH): δ ppm 3.42 (1H, *m*, H-4''), 3.53 (1H, *m*, H-3''), 3.58 (1H, *m*, H-5''), 3.61 (1H, *m*, H-2''), 3.74 (1H, *dd*, H-6a''), 3.95 (1H, *dd*, H-6b''), δ 5.05 (1H, *d*, *J* 8Hz, H-1''), 6.57 (1H, br *s*, H-3), 6.88 (1H, *d*, *J* 8 Hz, H-5'), 6.98 (1H, br *s*, H-8), 7.40 (1H, *d*, *J* 2 Hz, H-2'), 7.42 (1H, *dd*, *J* 8 Hz and 2 Hz, H-6'). ^{13}C NMR: δ ppm, 62.6 (C-6''), 71.5 (C-4''), 74.8 (C-2''), 75.7 (C-3''), 78.6 (C-5''), 96.1 (C-8), 103.2 (C-1''), 103.8 (C-3), 107.5 (C-10), 114.5 (C-2'), 117.3 (C-5'), 121.0 (C-6'), 124.0 (C-1'), 132.0 (C-6), 147.8 (C-3'), 148.1 (C-5), 151.9 (C-4'), 153.8 (C-9), 167.8 (C-2), 184.8 (C-4). Negative ESI-MS ($C_{21}H_{20}O_{12}$) m/z: 463 [M-H]$^-$; 301 [M-H-162]$^-$= [aglycone-H]$^-$.

Quercetin-3-O-[ß-apiofuranosyl-(1'''→2'')-ß-glucopyranoside] (**8**). TLC Rf: 0.41 (system A); 0.40 (system B); 0.50 (system C). *Rt*-HPLC: 7.66 min. CE migration time: 12.87 min. UV λ_{max} nm MeOH: 260, 357; +NaOAc: 269, 325sh, 373; +NaOAc+H_3BO_3: 262, 307sh, 377; +$AlCl_3$: 275, 307sh, 431; +$AlCl_3$+HCl: 273, 300sh, 353, 402; +NaOH: 272, 328sh, 401. ^{1}H NMR(400MHz, MeOH): δ 3.18 (1H, *m*, H-2''), 3.31 (1H, *m*, H-4''), 3.52 (1H, *dd*, H-6a''), 3.55 (1H, *m*, H-3''), 3.66 (1H, H-5a'''), 3.67 (1H, *m*, H-5''), 3.69 (1H, H-4a'''), 3.69 (1H, *dd*, H-6b''), 3.76 (1H, H-

5b'''), 3.72 (1H, H-4b'''), 4.06 (1H, *m*, H-2'''), 5.50 (1H, H-1'''), 5.58 (1H, *d*, *J* 8 Hz, H-1''), 6.20 (1H, br *s*, H-6), 6.40 (1H, br *s*, H-8), 6.90 (1H, *d*, *J* 8 Hz, H-5'), 7.63 (1H, *d*, *J* 2 Hz, H-2'), 7.65 (1H, *dd*, *J* 8 Hz and 2 Hz H-6'). ^{13}C NMR: δ ppm, 62.7 (C-6''), 66.4 (C-5'''), 71.7 (C-4''), 75.7 (C-4'''), 78.1 (C-2'''), 78.2 (C-3''), 78.6 (C-2''), 79.3 (C-5''), 81.1 (C-3'''), 94.8 (C-8), 99.2 (C-6), 101.0 (C-1''), 105.7 (C-10), 110.8 (C-1'''), 116.2 (C-5'), 117.2 (C-2'), 123.8 (C-6'), 123.8 (C-1'), 135.5 (C-3), 146.4 (C-3'), 149.9 (C-4'), 158.3 (C-9), 158.5 (C-2), 163.4 (C-5), 166.1 (C-7), 179.2 (C-4). Negative ESI-MS ($C_{26}H_{28}O_{16}$) m/z: 595 $[M-H]^-$; 463 $[M-H-132]^-$, 301 $[M-H-132-162]^-$= [aglycone-H]$^-$.

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