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Sulphated flavonoid glycosides from leaves of *Atriplex hortensis*

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Abstract

Two flavonoid sulphates, i.e. quercetin 3-*O*-sulphate-7-*O*-α-arabinopyranoside and kaempferol 3-*O*-sulphate-7-*O*-α-arabinopyranoside, were isolated from leaves of *Atriplex hortensis* L. The structures of these compounds were established by UV, ^{1}H and ^{13}C NMR, 2D NMR and MS spectra. The compounds were isolated for the first time from plant material.

Introduction

Flavonoids are important in plant biochemistry and physiology, acting as antioxidants, enzyme inhibitors, pigments and light screens. These compounds are involved in photosensitization and energy transfer, growth regulation, respiration control, photosynthesis and defence against infection (Harborne 1994).

A variety of pharmacological effects were confirmed for flavonoids including antioxidative, superoxide scavenging, variety of enzymes inhibitory activity and antiinflamatory, antiviral, anticarcinogenic activities. One of the important limitations to the use of flavonoids for any therapeutic application is low solubility of most flavonoids in water. On the other hand, flavonoid sulphates belong to the group of compounds easily soluble in water. Studies on the biological activity of flavonoid sulphates included only few examples of kaempferol, quercetin, isorhamnetin and tamarixetin derivatives isolated from *Polygonum hydropiper*. The investigations showed that all of these compounds posses antioxidant properties stronger than α-tocopherol, a common natural antioxidant, and also act as lens aldose reductase inhibitors. (Yagi *et al.* 1994; Haraguchi *et al.* 1996).

Sulphate esters of flavonoids are relatively rare compounds. Up to now about 100 flavonoids substituted with the sulphate residue were isolated from natural sources, whereas more than 4000 common hydroxyflavones, hydroxyflavonols and their glycosylated derivatives are known (Harborne 1994). The flavonoid sulphates are found mainly in species occurring in coastal and swampy areas rich in mineral salts. The sulphates are also isolated from plants occurring in arid habitats. In plants binding of inorganic sulphate with polyphenolics, like flavonoids, is probably one of the mechanisms of its deactivation, and thereby is connected with

biochemical adaptation of species to environment (Barron *et al.* 1988b).

The species from the genus *Atriplex* (Chenopodiaceae) often occur on saline and arid soils, and are common weeds, like *Atriplex hortensis*.

The aerial parts of *A. hortensis* were used in folk medicine against diseases of respiratory tract, digestive and urinary systems, and due to their analgesic properties, in rheumatism (Hoppe 1975; Siddiqui *et al.* 1994; Nicol 1994). The plant was used as a vegetable, but it may be allergenic on prolonged intake, causing skin eruptions (Hoppe 1975).

Previous chemical investigations on the species of the genus *Atriplex* showed the presence of saponin glycosides, alkaloids, betains, proteins, amino acids, ascorbic acid, mineral salts (Hegnauer 1989; Siddiqui *et al.* 1994; Nicol 1994) and phytoecdysteroids (Dinan 1995; Dinan *et al.* 1998). Previous studies on flavonoids included only the analysis of aglycones resulting from acid hydrolysis of extracts obtained from leaves of eight species from the genus *Atriplex*, excluding *A. hortensis*. The following aglycones were found using chromatography: quercetin, kaempferol, isorhamnetin and sometimes patuletin, spinacetin and tricin. Isorhamnetin 3-*O*-glucoside and 3-*O*-rhamnosylglucoside as well as naringenin and its 3-*O*-glucoside were isolated from *A. farinosa* L. (Al-Jaber *et al.* 1991). Barron indicated the presence of flavonoid sulphates in three species from the genus *Atriplex*. However, the species names were not given and the compounds were not identified (Barron *et al.* 1988b).

The present paper deals with the identification of two sulphated flavonoids isolated from leaves of *Atriplex hortensis* L and never reported earlier in plant material.

Material and methods

Plant material

Leaves of *Atriplex hortensis* L. (50 g) were collected from plants cultivated in 1998 in the garden at the Department of Medicinal Plants, K. Marcinkowski University of Medical Sciences, and air-dried. A voucher specimen is deposited at the Department of Pharmacognosy, K. Marcinkowski University of Medical Sciences, Poznań, Poland.

Extraction

The leaves were extracted subsequently with MeOH and 60 % MeOH. The combined extracts were concentrated under reduced pressure, treated with hot distilled water and the resulted precipitate was filtered off. The filtrate was extracted with $CHCl_3$.

Isolation procedure

Flavonoids present in the water layer were separated by preparative TLC using plates coated with microcrystalline cellulose Avicel (Merck) and *n*-BuOH-HOAc-H_2O (4:1:1) as a solvent system (Harborne 1989). The plates were viewed under a UV light (λ=366 nm), the bands were scraped off the plates and extracted subsequently with MeOH and 80 % MeOH at room temperature. The extract was filtrated, concentrated under reduced pressure and dissolved in 80 % MeOH. Compound (1) 15 mg crystallized from the corresponding fractions. The fractions containing compound (2) were purified on a Sephadex LH-20 column eluted with MeOH. The yield of compound (2) was *ca* 2 mg.

General

^{1}H NMR (300 MHz) and ^{13}C NMR (75,5 MHz) spectra were recorded in DMSO-d_6 on a Varian 300 MHz spectrometer, using TMS as internal standard.

Mass spectra were performed on an AMD 604 two sector mass spectrometer (AMD Intectra, Germany) of B/E geometry, liquid secondary ion mass spectrometry (LSIMS) was used as an ionization method. Low and high resolution spectra and B/E linked scan mass spectra were registered. The ion gun supplied primary Cs^+ ion beam at energy 10 keV. The secondary ion beam was accelerated to 8 kV. Triethanolamine was used as matrix in the negative mode and glycerol in the positive mode.

Gas chromatography-mass spectrometric analyses were performed on a Hewlett-Packard Gas Chromatograph model 5890/II equipped with Mass Se-

lective Detector model 5971A. The instrument was equipped with XTI-5 (Restek Co., USA) fused silica capillary column (0.25mm x 30m). The carrier gas was helium at flow rate 1ml/min. The column temperature was programmed from 140 °C (held for 2 min), at 5 °C/min to 300 °C. The latter temperature was maintained for 5 min. The injector temperature was 250 °C.

Standards of kaempferol, quercetin and arabinopyranoside were obtained from Sigma Chemical (St Louis, USA).

TLC was performed on cellulose plates (Merck, Darmstadt, Germany) with *n*-BuOH-HOAc-H_2O (4:1:1); H_2O; HOAc-H_2O (15:85) as a solvent systems, and spots were observed in UV light (λ = 366 nm) before and after spraying with 1% solution Naturstoffreagenz A (ß-aminoethyl ester of diphenylboric acid) from Roth (Karlsruhe, Germany) in MeOH and 1% $AlCl_3$ in MeOH.

Chemical modifications

Acid hydrolysis

Total hydrolysis: flavonoid (ca 1 mg) was refluxed in a mixture of MeOH (2 ml) and 2% HCl (2 ml) for 2 hours. After evaporation of MeOH, aglycones were extracted with EtOAc and analysed by TLC (cellulose C_6H_6 – HOAc – H_2O 125:72:3). The water layer was analysed for sugars by TLC on silica gel, *n*-PrOH – EtOAc – H_2O 7:2:1; sprayed with aniline phtalate (Harborne 1989).

Hydrolysis in trifluoroacetic acid (TFA): 2M TFA in H_2O (250 µl) was added to a dry sample and heated at 120 °C for 1h. The samples were cooled, evaporated to dryness, treated with isopropanol (300 µl) and again evaporated. The samples were dried overnight in a desiccator.

Methanolysis of glycosides (1) and (2) was performed in screw capped vials in 0,5 ml of 1 M HCl in MeOH for 2 h at 100 °C. Next the solvent was evaporated in a stream of dry nitrogen.

Reduction

Reduction of sugars to alditols was performed with sodium borohydride, according to the known procedure (Manzi and Varki 1993).

Acetylation

Acetylation of alditol was performed in a mixture of pyridine – acetic anhydride (1:1) v/v, for 20 min. at 120 °C. The toluene was added to the reaction mixture and this was followed by evaporation in stream of N_2. The evaporation was carried out twice. Residue was dissolved in water and acetylated alditols were extracted twice with CH_2Cl_2. After concentration samples were injected on GC/MS.

Methylation

Methylation reactions of intact flavonoid glycosides or products of their methanolysis were performed according to the modified Hakomori procedure in dimethylsulfoxide (DMSO) with methyl iodide (Ciucanu and Kerek 1984). The sample in a tightly screwed capped vial (about 100 µg) was dissolved in 0.5 ml of DMSO and 30 mg of fine powdered dry KOH was added. The reaction was started with the injection of 50 µl of methyl iodide and performed with agitation for five minutes at room temperature. After this time 1 ml of water was added and the methylated products extracted with $CHCl_3$.

Kaempferol 3-O-sulphate-7-O-α-arabinopyranoside (1)

Colourless plates, mp>290 °C.

Rf (TLC cellulose): 0.66 (15% HOAc); 0.30 (*n*-BuOH-HOAc- H_2O 4:1:1); 0.65 (H_2O).

UV_{366} nm – brown; + $AlCl_3$ – yellow; + NA – yellow olive.

$UV_{\lambda\ max}$ nm: MeOH 270,350; +NaOAc 270,355; +NaOAc-H_3BO_3 270,354; +NaOMe 273,400; $AlCl_3$ 280,306sh,355,408; $AlCl_3$-HCl 240,280,305sh, 360, 408; +25%HCl in MeOH 270,370.

^1H NMR (DMSO-d_6) δ: 8.14 (d, J=8.8 Hz, H-2',6'); 6.88 (d, J=9.0 Hz, H-3',5'); 6.77 (d, J=2.1 Hz, H-8); 6.40 (d, J=2.2 Hz, H-6); 5.05 (d, J=6.8 Hz, H-1''); 3.61 (m, H-2''); 3.78-3.66 (m, H-3'',4'',5'').

^{13}C NMR (DMSO-d_6) δ: 177.7 (C-4); 162.5 (C-7); 160.8 (C-5); 160.0 (C-4'); 156.8 (C-9); 155.6 (C-2); 132.5 (C-3); 130.7 (C-2',6'); 120.9 (C-1'); 115.0 (C-3',5'); 105.5 (C-10); 98.9 (C-6); 94.1 (C-8); 100.1 (C-1''); 72.3 (C-2''); 70.0 (C-3''); 67.3

(C-4''); 65.7 (C-5'').
HR MS (LSI) $[M-H]^-$ $[C_{20}H_{17}O_{13}S]^-$ calculated = obtained = 497.0411

Quercetin 3-O-sulphate 7-O-α-arabinopyranoside (2)

Pale yellow needles, mp. 228-230 °C.

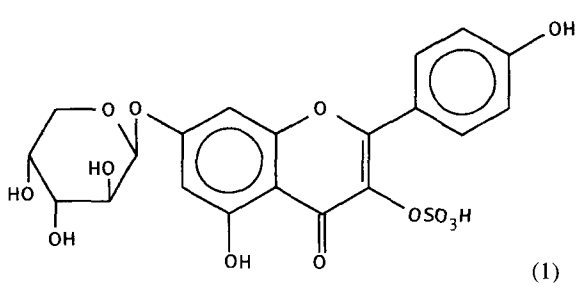
Rf (TLC cellulose): 0.51 (15% HOAc); 0.14 (*n*-BuOH-HOAc- H_2O 4:1:1); 0.52 (H_2O).

UV_{366nm} – brown; + $AlCl_3$ – yellow; + NA – orange.

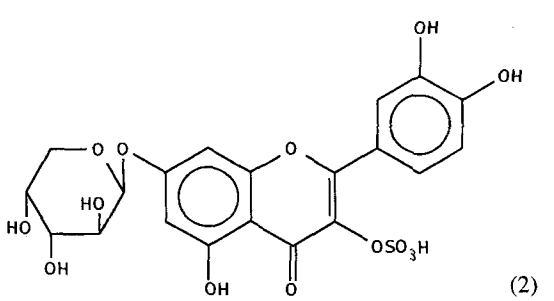
$UV_{\lambda\ max}$ nm: MeOH 258,358; +NaOAc 258,362; +NaOAc-H_3BO_3 263,385; +NaOMe 268,413; +$AlCl_3$ 278,457; +$AlCl_3$-HCl 270,370,398; +25 % HCl in MeOH 258,380.
HR MS (LSI) $[M-H]^-$ $[C_{20}H_{17}O_{14}S]^-$ calculated = obtained = 513.0378

Results and discussion

The leaves of *A. hortensis* L. were extracted exhaustively with MeOH and MeOH–H_2O. The combined extracts were concentrated, treated with hot water and the insoluble part was filtered off. The filtrate was extracted with $CHCl_3$. Compounds in the water layer were separated by preparative TLC using microcrystalline cellulose plates (Avicel) to yield two predominant flavonoids (1) and (2), see Fig. 1.



(1)



(2)

The UV spectra of compounds (1) and (2), registered in a methanolic solution, without and after addition of specific reagents, were characteristic for flavonoids with free hydroxyls at C-5,4' in compound (1) and C-5,3',4' in compound (2), and with substituted hydroxyls at C-3 and C-7 in both compounds (Mabry *et al.* 1970). The bathochromic shift 20–22 nm after addition of HCl indicated the presence of a sulphate residue at C-3 in both compounds (Barron and Ibrahim 1988a).

The low resolution mass spectra of compounds (1) and (2) were recorded in negative and positive ion modes. In the negative ion mode $[M-H]^-$ ions were observed at m/z=497 for (1) and m/z=513 for (2), whereas in the positive ion mode only $[M+Na]^+$ ions at m/z 520 and 536 were formed, respectively. The accurate masses obtained from the high resolution LSIMS spectra for the $[M-H]^-$ ions of compounds (1) and (2) were 497.0411 (Ä= -2.1 mmu) and 513.0378 (Ä= -3.9 mmu), respectively. The molecular formulas $C_{20}H_{18}O_{13}S$ for (1) and $C_{20}H_{18}O_{14}S$ for (2) were estimated. In the linked scan mass spectra (B/E=const) of $[M-H]^-$ ions for compounds (1) and (2), daughter ions created after elimination of sugar ring $[M-H-132]^-$ and SO_3 molecule $[M-H-80]^-$ were observed. Further fragmentation of the above mentioned ions resulted in the formation of aglycone $[A-H]^-$ fragment ions at m/z 285 and 301 for compound (1) and (2) respectively. From these data it was possible to estimate placement of the sulphonyl group on the aglycone moiety.

Normal and linked scan (B/E = const) mass spectra of quercetin 3-*O*- sulphate -7-*O*-α-arabinopyranoside are presented on Fig. 2.

The structures of the sugars and aglycones in glycosides (1) and (2) were established after GC/MS analyses of products obtained after hydrolysis. Compound (1) was hydrolized in 2 M TFA and than methylated. The obtained products had the same retention times and mass spectra like standards of 1,2,2,4-tera-O-methylarabinose and 3,4',5,7-tetra-O-methylkaempferol. The presence of arabinose in compound (2) was confirmed after identification of

Fig. 1. The structures of kaempferol 3-*O*-sulphate 7-*O*-α-arabinopyranoside (1) and quercetin 3-*O*-sulphate 7-*O*-α-arabinopyranoside (2).

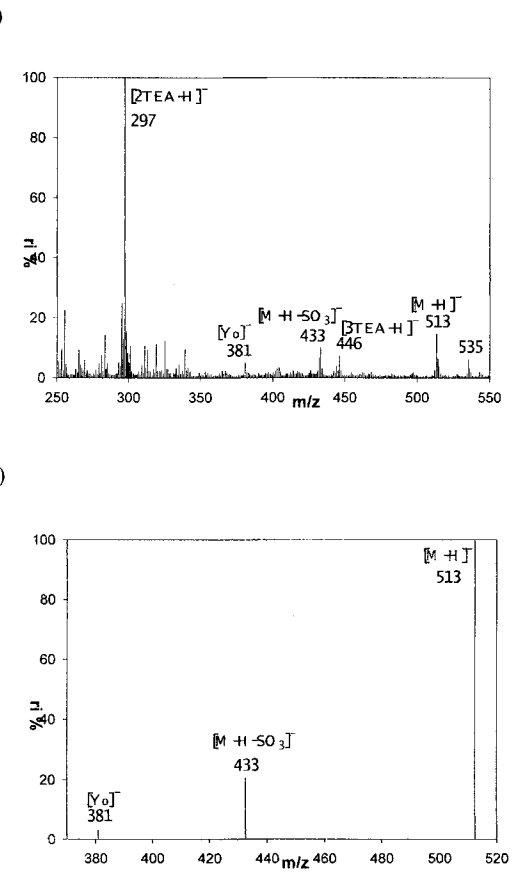


Fig. 2. LSI mass spectra of quercetin 3-*O*-sulphate 7-*O*-α-arabinopyranoside (2) registered in triethanolamine (TEA) as matrix:
a) normal LSI mass spectrum
b) linked scan (B/E=const) mass spectrum of product ions for deprotonated molecule $[M-H]^-$

products obtained after hydrolysis in 2 M TFA, reduction and acetylation of the obtained reaction mixture, the retention time and mass spectrum corresponded to an arabinitol standard. Quercetin was identified in the glycoside (2) after methanolysis of the sample in 1 M HCl in MeOH and methylation of products. In the total ion chromatogram after GC/MS analysis of the reaction mixture presence of permethylated quercetin was confirmed. The GC/MS analysis supported earlier information about aglycones structures obtained from TLC after total acid hydrolysis of glycosides. The data obtained after different mass spectrometric analyses combined with information from UV spectra permitted to propose the structure of compound (2).

The ^1H and ^{13}C NMR spectra were recorded for compound (1). The signals in the ^1H NMR spectrum were in accordance with earlier obtained UV data about 3,7-substitution of kaempferol (Harborne 1994). The signals matching the *meta* protons appeared at 6.77 ppm (1H, d, J=2.1 Hz, H-8) and 6.40 ppm (1H, d, J=2.2 Hz, H-6), doublets at 8.14 (J=8.8 Hz) and 6.88 ppm (J=9.0 Hz) corresponded to the H-2',6' and H-3',5' protons, respectively. The signal for the anomeric proton of the sugar (H-1) appeared at 5.05 ppm with coupling constant J=6.8 Hz and was characteristic for α-arabinopyranose. The ^1H-^1H COSY experiment enabled assignment of all sugar protons. In the ^{13}C NMR spectrum the C-1" arabinose signal was observed at 100.1 ppm, and the consecutive signals for the carbon atoms C-2", C-3", C-4" and C-5" appeared at 72.3, 70.0, 67.3 and 65.7 ppm, respectively. Such an arrangement of the signals is in accordance with literature data for arabinopyranose (Harborne and Mabry 1982). Fifteen signals between 94.1 and 177.7 ppm corresponded to the aglycone and their arrangement is in agreement with literature data for a 3,7-substituted kaempferol (Harborne and Mabry 1982). Glycosylation of the C-7 hydroxyl group was confirmed by an upfield shift of 1.4 ppm of the C-7 signal in conjunction with insignificant downfield shifts (0.7 and 0.8 ppm) of the *ortho* carbon atoms C-6 and C-8 and a downfield shift of 2.4 ppm of the *para* carbon atom C-10.

An upfield shift of 3.1 ppm of the C-3 carbon atom signal together with a significant downfield shift of 9.0 ppm of the *ortho* carbon atom C-2 as well as downfield shifts of the C-4 atom of 1.8 ppm proved, that the C-3 position was substituted (Barron *et al.* 1988b).

Combined information obtained after physicochemical analyses of sulphated flavonoid glycosides permitted to elucidate structure of compound (1) and (2) as: kaempferol 3-*O*-sulphate 7-*O*-α-arabinopyranoside and quercetin 3-*O*-sulphate 7-*O*-α-arabinopyranoside, respectively. These compounds, isolated from *Atriplex hortensis*, were found in plant material for the first time.

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