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QUERCETIN AND PATULETIN 3,3′-DISULPHATES FROM *FLAVERIA CHLORAEFOLIA*

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Abstract—Quercetin 3,4′-disulphate and an equimolar mixture of two novel flavonol sulphates, quercetin 3,3′-disulphate and patuletin 3,3′-disulphate, were isolated from the butanolic extract of the leaves of *Flaveria chloraefolia*. Purification of these components was carried out by gel filtration, and their structures elucidated by UV, IR, ^{1}H and ^{13}C NMR spectroscopy, as well as FAB-MS. The effect of 3′- and 4′-sulphation on the ^{13}C NMR spectra of flavonols is discussed.

INTRODUCTION

The genus *Flaveria* is known to be a good source of flavonol sulphates, especially those having a high level of sulphation [1–6]. Apart from quercetin and isorhamnetin 3-sulphates [4], *Flaveria bidentis* accumulates a variety of flavonol sulphate esters, including isorhamnetin 3,7- and quercetin 3,4′-disulphates, quercetin 3,7,4′- and 3,7,3′-trisulphates, as well as quercetin 3-acetyl-7,3′,4′-trisulphate and quercetin 3,7,3′,4′-tetrasulphate [1–5]. Investigation of another species, *F. chloraefolia*, led to the isolation and characterization of an equimolar mixture of quercetin 3-sulphate and patuletin 3-sulphate (**1**) [6]. The present paper describes the identification of quercetin 3,4′-disulphate **2**, as well as two novel flavonol sulphates, quercetin and patuletin 3,3′-disulphates, which were isolated as an equimolar mixture **3**. Structural elucidation of **2** and **3** was carried out by UV, IR, ^{1}H and ^{13}C NMR spectroscopy, besides FAB-MS and atomic absorption.

RESULTS AND DISCUSSION

A butanolic extract of *F. chloraefolia*, after evaporation and solubilization in methanol [6], was concentrated to give a crystalline residue which after chromatography on a Sephadex G-10 column yielded **3**. Further chromatography of the supernatant on a Sephadex LH-20 column allowed the separation of quercetin 3,4′-disulphate (**2**) from the previously characterized quercetin and patuletin 3-sulphates (**1**) [6]. Compounds **2** and **3** were identified as sulphated derivatives on the basis of the following data. Their IR spectra showed a strong sulphate band at 1050 cm^{-1}. On cellulose TLC, they appeared as dark, arrow-shaped spots in UV and their R_f values (Table 1) were lower than those of quercetin and patuletin 3-sulphates. On the other hand, their R_t in HPLC after ion-pairing, as well as their R_f values on polyamide TLC, were higher than those of quercetin and patuletin 3-sulphates (Table 1). Such behaviour suggested that **2** and **3** were disulphate esters. This was supported by their rate of migration on electrophoresis which was similar to that of other flavonol disulphates. Furthermore, hydrolysis of **2** and **3** with aryl sulphatase afforded a product whose electrophoretic and chromatographic properties were similar to those of the 3-monosulphate ester **1**.

Acid hydrolysis of **2** at room temperature yielded quercetin. Negative FAB-MS gave a molecular ion at *m/z* 460, which corresponded with a disulphate ester of quercetin. The presence of a $[M+K]^-$ ion at *m/z* 499 demonstrated the occurrence of K^+ in the sample and atomic absorption analysis also indicated the presence of Na^+ and Ca^{2+}. Its dark UV absorbance indicated that **2** was substituted in the 3-position. Addition of HCl resulted in a bathochromic shift in the UV spectrum of 30 nm, indicating that **2** was a 3-sulphated flavonol [7]. A bathochromic shift of 10 nm was observed for band II after addition of sodium acetate, indicating that the 7 position was free, whereas the absence of a borate shift demonstrated that either the 3′ or 4′ positions were substituted. After analysis of the ^{13}C NMR spectrum of **2** (Table 3) and calculation of the sulphation shifts (Table 4), the 4′ position was unequivocally shown to be substituted with the second sulphate group. Unlike the shifts induced by 3-sulphation [6], those resulting from 4′-sulphation were characteristic of a phenol sulphate ester [9], i.e. upfield displacement for the carbon carrying the sulphate group (C-4′), while both *ortho* (C-3′ and C-5′) and *para* (C-1′) carbons underwent significant downfield displacements (Table 4). The shifts for carbons 2, 3 and 4 were, otherwise, in complete agreement with 3-sulphation [6]. From these results **2** is identified as quercetin 3,4′-disulphate in the form of a K^+, Na^+ and/or Ca^{2+} salt.

Component **3**, after mild acid hydrolysis, gave two flavonoid aglycones, quercetin and patuletin. However, attempts to separate the two flavonol sulphate moieties of **3** were unsuccessful, either on TLC (cellulose or polyamide) or HPLC (Table 1). When **3** was hydrolysed with aryl sulphatase, it gave rise to **1**, identified on the basis of its chromatographic and electrophoretic properties as well as its UV and FAB-MS spectra. On the other hand, measurement of the ratios of protons at position 8 in the

Table 1. Comparison of the properties of components 1, 2 and 3

Component*	1	2	3
R_f values (TLC)			
Cellulose, solvent A†	0.30	0.14	0.14
Polyamide, solvent B†	0.08	0.43	0.41
Polyamide, solvent C†	0.06	0.24	0.17
Colour after exposure to NH_4OH (UV)	Yellow	Purple	Yellow-green
Colour after ADPB‡			
Visible	Orange	Yellow-green	Yellow-green
UV	Yellow	Green	Green
Electrophoretic mobility§	1	2	2
R_t (HPLC), min	38.8	60.0	60.0

* 1 = an equimolar mixture of quercetin and patuletin 3-sulphates; 2 = quercetin 3,4'-disulphate and 3 = an equimolar mixture of quercetin and patuletin 3,3'-disulphates.

†Solvent systems as described in the Experimental.

‡2-Aminoethyl diphenylborinate; on Avicel cellulose TLC plates.

§For conditions, see Experimental.

Table 2. ^{1}H NMR data of components of 2 and 3*

Protons	Components† 2	3
6 (Q)‡	6.20, 1 H, *d* J = 1.9 Hz	6.16, 1 H, *d* J = 2.0 Hz
8 (Q)	6.43, 1 H, *d* J = 1.9 Hz	6.38, 1 H, *d* J = 2.0 Hz
8 (P)‡	—	6.46, 1 H
2' (Q + P)	*ca* 7.6–7.8, *m*	7.87, 2 H, *d* J = 2.2 Hz
5' (Q + P)	7.31, 1 H, *d* J = 9.2 Hz	6.87, 2 H, *d* J = 8.6 Hz
6' (Q + P)	*ca* 7.6–7.8, *m*	8.03, 2 H, *dd* J = 2.2 and 8.6 Hz
OCH_3 (P)	—	3.74, 3 H

*At 80 MHz, DMSO-d_6, δ ppm/TMS.

†Compound 2 is quercetin 3,4'-disulphate; compound 3 is an equimolar mixture of quercetin and patuletin 3,3'-disulphates.

‡Proton assigned to: Q, quercetin moiety and P, patuletin moiety.

Table 3. ^{13}C NMR data of components 2, 3 and quercetin 3'-sulphate (100 MHz, DMSO-d_6, δ ppm/TMS)

Carbon	2*	Quercetin moiety in 3†	Patuletin moiety in 3†	Quercetin 3'-sulphate‡
2	155.8	155.7	155.7	146.1
3	133.1	132.6	132.2	135.9
4	177.8	177.6	177.8	175.9
5	161.2	161.2	152.3	160.7
6	98.6	98.6	131.4	98.2
7	164.1	164.3	156.2	163.9
8	93.5	93.5	93.8	93.4
9	156.2	156.1	151.4	156.1
10	104.2	104.0	104.1	103.1
1'	126.5	123.1	123.1	122.6
2'	117.3	121.6	121.6	122.2
3'	148.1	140.5	140.5	140.8
4'	143.3	152.0	152.0	151.2
5'	121.6	116.8	116.8	117.2
6'	120.6	126.9	126.9	124.9
OCH_3	—	—	59.8	—

*Assignments were made by comparison with the spectrum of quercetin 3-sulphate [6] and taking into account the reported sulphation shifts [9].

†Assignments were made by comparison with the spectra of quercetin 3-sulphate [6], patuletin 3-sulphate [6] and quercetin 3'-sulphate.

‡Assignments were made by comparison with the spectrum of quercetin [8] and taking into account the reported sulphation shifts [9].

^{1}H NMR spectrum of 3 (Table 2) showed that the quercetin and patuletin moieties were present in an equivalent amount. The UV data of 3 were similar to those of 2, and demonstrated sulphation at the 3 position (+ 28 nm after addition of 3 N HCl), as well as a free hydroxyl group at position 7 (+ 8 nm for band II in presence of NaOAc). However, the second sulphate group must be attached to the 3'-position since the spectrum in aluminium chloride did not change after the addition of HCl, while sodium acetate induced a pronounced bathochromic shift of 32 nm. In the ^{13}C NMR spectrum of 3 (Table 3), the chemical shifts of the carbons assigned to ring B (C-1'–C-6') were found to be similar to those of quercetin 3'-sulphate (Table 3). Additional evidence for the 3'-sulphation came from calculation of the sulphation shifts (Table 5). The 3' carbons which carry the sulphate groups exhibited an upfield displacement of 4–5 ppm. *Ortho* (C-2' and C-4') and *para* (C-6') carbons were shifted downfield by 5.5–6.9, 3.6–4.6 and 6.8 ppm, respectively. These data demonstrate that 3 is an equimolar mixture of quercetin

Table 4. ^{13}C NMR sulphation shifts* induced by 3- and 4'-sulphations in quercetin 3,4'-disulphate (2)

Carbon	Sulphation shift
2	−8.9
3	+2.5
4	−2.1
10	−1.2
1'	−4.5
2'	−2.0
3'	−3.2
4'	+4.3
5'	−6.0
6'	−1.6

*Refers to $\delta_{aglycone} - \delta_{sulphate\ ester}$.

Table 5. ^{13}C NMR sulphation shifts* induced by 3- and 3'-sulphations in quercetin 3'-sulphate, 3, and quercetin 3,7,3'-trisulphate

Carbon	Quercetin 3'-sulphate	Quercetin moiety in 3	Patuletin moiety in 3	Quercetin 3,7,3'-trisulphate†
2	—	−8.8	−8.6	−8.0
3	—	+3.0	+3.3	+3.1
4	—	−1.9	−1.7	−1.9
10	—	−1.0	−0.6	−3.0
1'	−0.6	−1.1	−1.0	−1.0
2'	−6.9	−6.3	−6.4	−5.5
3'	+4.2	+4.5	+4.6	+4.9
4'	−3.6	−4.4	−4.2	−4.6
5'	−1.6	−1.2	−1.1	−1.0
6'	−4.9	−6.9	−6.8	−6.8

*Refers to $\delta_{aglycone} - \delta_{sulphate\ ester}$.
†Values for quercetin 3,7,3'-trisulphate were calculated from [5].

3,3'-disulphate and patuletin 3,3'-disulphate. The fact that the quercetin sulphate moiety could not be separated from its patuletin analogue is not surprising since a similar situation was encountered during the separation of quercetin 3-sulphate and patuletin 3-sulphate, the two flavonol sulphate moieties of 1 [6]. More intriguing is the fact that the two flavonol sulphate moieties co-exist in an equimolar ratio, suggesting the involvement of a divalent cation in their linkage. Although the calcium content of 3 was found to be 1.47%, calcium sulphate constituted a major constituent of the butanolic extract that did not dissolve in methanol. This suggests the possible participation of $CaSO_4$ in the formation of the equimolar mixture, as was previously hypothesized [6].

Quercetin 3,4'-disulphate was previously characterized in the leaves of *F. bidentis* [4]; however, this is the first report of the quercetin and patuletin 3,3'-disulphates in nature.

EXPERIMENTAL

General methods. TLC was carried out on polyamide 6 (Macherey-Nagel) pre-coated plates, or on cellulose (Avicel) plates. The use of pre-coated cellulose sheets (Macherey-Nagel) is not recommended since it leads to the degradation of the flavonoid sulphates. TLC plates were developed in the following solvent systems: A, *n*-BuOH–HOAc-H_2O (3:1:1); B, MeOH–H_2O–29% NH_4OH (10:9:1); C, MeOH–H_2O–29% NH_4OH (15:4:1). The plates were sprayed with 2-aminoethyl diphenylborinate (0.1% in MeOH) and examined in UV light. Analytical HPLC was performed on two Waters Model 510 pumps, equipped with a Rheodyne injector (Model 7125), an automated gradient controller (Waters Model 680) and a UV detector (Waters Model 441, detection at 340 nm), in the conditions reported in [6]. Electrophoresis, acid hydrolysis, hydrolysis with aryl sulphatase and atomic absorption analysis were carried out as in [6]. For component 3, calcium analysis by atomic absorption was performed before the gel filtration on Sephadex G-10, whereas cation analysis was carried out on the purified compound 2. ^{1}H NMR spectra (80.1 MHz) were recorded in DMSO-d_6 using a Bruker WP-80SY spectrometer. ^{13}C NMR spectra (100.1 MHz) were recorded in DMSO-d_6 using a Bruker WH-400 spectrometer at the Montréal Regional High Field NMR Laboratory. The FAB-MS spectra were recorded after dissolution of the sample in a glycerol–H_2O (2:1) matrix, and using a Kratos MS 50-TC-TA instrument (Xe beam, 2 mA; 6–7 kV gun; 8 kV source). UV spectra in acidic conditions were recorded after addition of 5 drops of 3 N HCl in the cuvette and 30 min wait.

Chemicals. Quercetin 3'-sulphate was synthesized according to [10] and purified as in [6].

Plant material. Seeds of *Flaveria chloraefolia* A. Gray (Compositae) were obtained from Professor A. M. Powell, Sul Ross State University, Alpine, TX, and were raised to fully grown plants under greenhouse conditions.

Extraction and purification of the flavonoid sulphates. Extraction and liquid–liquid partition of the extract were carried out according to [6]. The BuOH extract was evaporated and the residue extracted with MeOH. The insoluble fraction contained mostly $CaSO_4$ (IR ν_{max}^{KBr} cm^{-1}: 1620, *ca* 1100–1170, 665 and 600; similar to that of reference $CaSO_4$), while the soluble fraction was left to concentrate under the fume hood for 1 week to give crystals which, after gel filtration on Sephadex G-10 (H_2O), afforded 90 mg of 3. The supernatant was chromatographed on a Sephadex LH-20 column using MeOH. An equimolar mixture of quercetin and patuletin 3-sulphates (1), which was previously characterized [6], eluted first, followed by quercetin 3,4'-disulphate 2 (100 mg).

Quercetin 3,4'-disulphate (2). Yellow crystals; UV λ_{max}^{MeOH} nm: 268, 335; + NaOAc: 278, 320 *sh*, 360; + NaOAc + H_3BO_3: 268, 335; + $AlCl_3$: 278, 415; + $AlCl_3$ + HCl: 278, 358 *sh*, 407; + NaOMe: 278, 380; + 3 N HCl: 255, 260 *sh*, 365; IR ν_{max}^{KBr} cm^{-1}: 3440, 2950, 2920, 1655, 1605, 1500, 1360, 1280, 1245 (S=O), 1195, 1165, 1115, 1050 (C–O–S), 990, 935, 860, 820, 760 and 710; neg FAB-MS, *m/z* 499 [M + K], 460 [M].

Component 3 (quercetin plus patuletin 3,3'-disulphates). Yellow crystals; UV λ_{max}^{MeOH} nm: 267, 340; + NaOAc: 275, 315 *sh*, 372; + NaOAc + H_3BO_3: 267, 350; + $AlCl_3$: 277, 315 *sh*, 347, 390 *sh*; + $AlCl_3$ + HCl: 277, 315 *sh*, 347, 390 *sh*; + NaOMe: 275, 330 *sh*, 398; + 3 N HCl: 260, 368; IR ν_{max}^{KBr} cm^{-1}: 3440, 2920, 1650, 1600, 1500, 1470, 1430, 1335, 1250 (S=O), 1190, 1050 (C–O–S), 990, 860, 805, 760 and 715; hydrolysis of 3 with aryl sulphatase yielded a product whose chromatographic and electrophoretic properties, as well as UV, IR and neg FAB-MS spectra, were identical to those of 1.

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