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AN EQUIMOLAR MIXTURE OF QUERCETIN 3-SULPHATE AND PATULETIN 3-SULPHATE FROM *FLAVERIA CHLORAEFOLIA*

D. BARRON*, L. D. COLEBROOK* and R. K. IBRAHIM†

*Chemistry Graduate Faculty and †Department of Biology, Concordia University, 1455 De Maisonneuve Blvd. West, Montreal, Quebec, Canada H3G 1M8

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Key Word Index—*Flaveria chloraefolia*; Compositae; flavonol sulphates; ^{13}C NMR; FAB-MS.

Abstract—A fraction consisting of an equimolar mixture of quercetin 3-sulphate and patuletin 3-sulphate was isolated from a methanolic leaf extract of *Flaveria chloraefolia*. Structures were established by UV and NMR spectroscopy as well as FAB-MS. The effect of 3-sulphation on the ^{13}C NMR spectrum is discussed and the nature of a possible linkage of the two flavonol sulphate moieties is postulated.

INTRODUCTION

Flavonoid sulphates are known to occur in a large number of plant families [1–3]. However, their presence seems to be more related to aquatic or saline habitats, than to taxonomic considerations [2]. In the Compositae, flavonoid sulphates have been reported in 11 genera [1–4], among which *Brickellia* spp. [5–9] and *Flaveria bidentis* [10–14] are the most documented. The latter is known to accumulate isorhamnetin 3-sulphate and isorhamnetin 3,7-disulphate [10, 13] as well as a variety of mono- to tetrasulphate esters of quercetin [11–14].

This paper reports on the isolation and identification of a major flavonoid sulphate component from *Flaveria chloraefolia*, consisting of equimolar quercetin 3- and patuletin 3-sulphates. Their identification was carried out by UV, IR, ^{1}H and ^{13}C NMR spectroscopy, as well as FAB-MS and atomic absorption. NMR and FAB-MS data for both compounds are reported here for the first time.

RESULTS AND DISCUSSION

Extraction of *F. chloraefolia* leaves with 50% methanol and partition of the extract with solvents of increasing polarity afforded component 1, which was purified on Sephadex LH-20. On cellulose TLC, this component appeared as a dark UV-absorbing, arrow-shaped spot with low mobility in *n*-butanol–acetic acid–water (R_f 0.30). It did not migrate on polyamide TLC except after the addition of ammonia to the solvent system (R_f 0.12). Such chromatographic behaviour, together with a fairly high R_t (38.8 min) on HPLC after ion pairing, is characteristic of sulphated flavonoids [1–3, 15].

The dark UV-absorbing spot turned orange after spraying with 2-aminoethyl diphenylborinate, and its UV absorption spectrum exhibited a shift of only 10 nm in the presence of aluminium chloride + HCl, thus indicating substitution at position 3. The fact that sodium acetate caused a bathochromic shift of 17 nm (band II) and another of 25 nm (band I) after the addition of boric acid clearly indicated a free 7-position and the presence of an *o*-diphenol system on ring B, respectively. Furthermore, the addition of HCl resulted in a bathochromic shift of 20 nm (band I) which is characteristic of 3-sulphated flavonols [3]. On electrophoresis, 1 migrated at the level of a monosulphate. It exhibited a strong sulphate band in IR at 1050 cm^{-1}.

When acid hydrolysis of 1 was performed at room temperature, it yielded two flavonoid aglycones, quercetin and patuletin. However, 1 was not affected on hydrolysis with aryl sulphatase, which is commonly used for the characterization of sulphated flavonoids [2] including 3-sulphates [5, 8]. Comparison of the rate of enzyme-catalysed hydrolysis of quercetin 3-sulphate and quercetin 3′-sulphate indicated that, whereas the latter compound underwent complete hydrolysis, quercetin 3-sulphate was hardly affected. This may be ascribed to the partly phenolic nature of the 3-hydroxyl group and/or the steric hindrance associated with this position.

Examination of ^{1}H NMR ratios of the protons at position 8 of the quercetin and patuletin moieties in 1 indicated that they were present in equivalent amounts (Table 1). Furthermore, ^{1}H NMR of the tetrabutylammonium salt of the native compound indicated the presence of two sulphate groups (Table 1).

Negative FAB-MS of 1 revealed the occurrence of both quercetin monosulphate ($M_1 = 381$) and patuletin monosulphate ($M_2 = 411$) units. In addition, the presence of $[M_2 + K - H]^-$ and $[M_1 + K - H]^-$ ions, together with a few dimeric species, such as $[2M_2 + K]^-$ and $[M_1 + M_2 + K]^-$, gave evidence for the presence of K^+ in the sample. The latter was confirmed by atomic absorption which also demonstrated the occurrence of Na^+ and Ca^{2+} (Na^+: 4.7%; K^+: 2.1%; Ca^{2+}: 1.0%). The fact that ions involving Na^+ did not appear in the spectrum of 1 may be due to an exchange with K^+ [16].

The ^{13}C NMR spectrum of 1 (Table 2) was found to be similar to the spectra reported for quercetin and patuletin [17], except for the signals of carbons 2, 3, 4, 10 and 6′. Only the 3-carbons afforded upfield displacements, whereas the carbons α (C-2 and C-4) and β (C-10) to this position displayed downfield shifts (Table 3). These shifts

Table 1. ^{1}H NMR data of 1 and its tetrabutylammonium salt (80 MHz, δ ppm/TMS)

Proton	Compound 1*	Tetrabutylammonium salt†
$(C_4H_9)_4$-N		*ca* 0·8–1.1, 72H, *m*
6 (Q‡)	6.16, 1H, *d*	6.17, 1H, *d*
	J = 2 Hz	J = 1.9 Hz
8 (Q)	6.37, 1H, *d*	6.38, 1H, *d*
	J = 2 Hz	J = 1.9 Hz
8(P‡)	6.47, 1H	6.47, 1H
2′ (Q + P)	*ca* 7.56, 2H	7.70, 2H
5′ (Q + P)	6.80, 2H, *d*	6.86, 2H, *d*
	J = 8.4 Hz	J = 8.2 Hz
6′ (Q + P)	7.62, 2H, *dd*	7.65, 2H, *dd*
	J = 2 and 8.4 Hz	J = 2 and 8.2 Hz
OMe (P)	3.74, 3H	3.86, 3H

*DMSO-d_6.
†CD_3OD.
‡Q, Quercetin; P, patuletin.

Table 2. ^{13}C NMR data of 1 (100 MHz, DMSO-d_6, δ ppm/TMS)

Carbon	Quercetin moiety*	Patuletin moiety*
2	156.6	156.6
3	132.3	132.0
4	177.7	178.0
5	161.3	152.5
6	98.4	131.1
7	163.9	157.0
8	93.3	93.6
9	156.1	151.3
10	104.1	104.5
1′	121.6	121.6
2′	115.1	115.1
3′	144.7	144.7
4′	148.3	148.3
5′	115.9	115.9
6′	121.6	121.6
OMe	—	60.0

*Assignments were made by comparison with reference spectra [14, 17].

Table 3. ^{13}C NMR sulphation shifts induced by 3-sulphation in 1 and two reference compounds*

Carbon	Quercetin moiety in 1	Patuletin moiety in 1	Quercetin 3,7,3′-tri sulphate†	7,4′-DiMe-kaempferol-3-sulphate *
2	−9.7	−9.5	−8.0	−9.3
3	+3.3	+3.5	+4.3	+3.5
4	−2.0	−1.9	−1.9	−1.8
10	−1.1	−1.0	—	−1.1
2′	+0.2	+0.1	—	−1.5
6′	−1.6	−1.6	—	−1.5

*Refers to $\delta_{aglycone} - \delta_{sulphate\ ester}$. Values for quercetin and patuletin were from ref. [17]; those for quercetin 3,7,3′-trisulphate from ref. [14]; those for 7,4′-dimethylkaempferol and 7,4′-dimethylkaempferol 3-sulphate from ref. [20].
†Carbons 10, 2′ and 6′ are not considered here since they were affected by 7- and 3′-sulphation.

are indicative of sulphation at position 3, being in agreement with the previously observed upfield displacements for carbons carrying sulphate groups and the downfield shifts for the α carbons [18]. However, the magnitude of the shifts induced by 3-sulphation differed from those reported for true phenolic compounds [18]. No downfield shifts were observed for the *para* carbons (C-9), whereas shifts for the *ortho* carbons (C-2) were quite pronounced (Table 3). Similar unusual shifts have been observed with 3-glycosylation [19] and have been attributed to an incomplete overlap of the π-orbital system due to steric hindrance created by the 3-glycosyl residue. It appears, therefore, that 3-sulphation induces similar effects which become evident after examination of the sulphation shifts (Table 3) in quercetin 3,7,3′-trisulphate [14] and 7,4′-dimethylkaempferol 3-sulphate [20]. The 1.6 ppm shift for C-6′ in compound 1 is unexpected; however, it can be attributed to the proximity of the 6′-position to the 3-sulphate group. In 7,4′-dimethylkaempferol 3-sulphate similar shifts affect both C-2′ and C-6′ (Table 3), which may be due to free rotation of ring B in the absence of the 3′-hydroxyl group.

Attempts to separate the two flavonol sulphates in 1 on cellulose TLC, polyamide TLC or HPLC were unsuccessful. However, chromatography of the fully methylated derivative of 1 on silica gel in ethyl acetate–acetone (1:1) gave two compounds with blue fluorescence, presumably the quercetin and patuletin derivatives. These results indicate that 1 is an equimolar mixture of quercetin 3-sulphate and patuletin 3-sulphate, containing Na^+, K^+ and/or Ca^{2+}. The fact that calcium sulphate is often precipitated during the isolation of 1, as well as the existence of the component flavonol sulphate moieties in equivalent amounts, suggest that Ca^{2+} may be involved in

the linkage of the two flavonol sulphates; i.e. 1 may be mixed salt, possibly involving additional sulphate anions.

Quercetin 3-sulphate was first reported in *Ammi visnaga* and *Oenanthe crocata* [21] and later in *F. bidentis* [13]. Until now, patuletin 3-sulphate has been known to be present only in the genus *Brickellia* [5, 6, 8, 9]. This is the first reported incidence of patuletin 3-sulphate in *Flaveria*.

EXPERIMENTAL

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

Source of reference compounds. Quercetin 3-sulphate was a generous gift from Dr. H. R. Juliani, Cordoba, Argentina. Quercetin 3′-sulphate was synthesized according to ref. [22], and the product was purified by recrystallization and chromatography on Sephadex LH-20, using MeOH as solvent.

Extraction and isolation. Fresh leaves (1.4 kg) were frozen in liquid N_2 and extracted with 50% aq. MeOH. The extract was concd under red. pres. and the resulting aq. layer was partitioned against hexane, $CHCl_3$, EtOAc and BuOH. The BuOH extract was evaporated and the residue was extracted with MeOH. The extract was chromatographed on a Sephadex LH-20 column using MeOH, affording 80 mg of 1.

General methods. Cellulose TLC was performed in *n*-BuOH–HOAc–H_2O (3:1:1); polyamide TLC, in MeOH–H_2O–29% NH_4OH (10:9:1). TLC plates were sprayed with 2-aminoethyl diphenylborinate (0.1% in MeOH) and examined in UV light. Analytical HPLC was carried out on a μ-Bondapack C_{18} column (300 × 3.9 mm) using an UV-detector (340 nm), and a flow rate of 1 ml/min. The following solvents [15] were used for ion pairing chromatography: A, 0.01 M aq. tetrabutylammonium phosphate; B, MeOH–H_2O–HOAc (90:5:5). The initial solvent was 60% A + 40% B over 10 min, increased to 50% A + 50% B for 40 min, and finally to 40% A + 60% B for 10 min. Electrophoresis was carried out for 3 hr on Whatman No. 3 paper in HCO_2H–HOAc–H_2O buffer (33:147:1820), pH 2.2 at 250 V. Atomic absorption analysis was performed after ashing of 2 mg of 1 in 50 μl conc. HNO_3 at 150° for 12 hr. Acid hydrolysis was conducted at room temp. in 3 N HCl for 30 min. After extraction with EtOAc, the hydrolysis products were identified by co-chromatography with reference compounds on polyamide TLC plates using C_6H_6–MeCOEt–MeOH (3:1:1). Hydrolysis with aryl sulphatase (type V from limpets, Sigma) was performed at 30° (pH 5.0) for 12 hr. The assay mixture was chromatographed on cellulose plates for the identification of the hydrolysis products. ^{1}H NMR spectra (80.13 MHz) were recorded using a Bruker WP-80 SY spectrometer. ^{13}C NMR spectra (100 MHz) were recorded using a Bruker WH-400 spectrometer at the Montreal Regional High Field NMR Laboratory. For FAB-MS a Kratos MS 50-TC-TA instrument (6–7 kV gun; Xe beam, 2 mA; 8 kV source) has been used, and the sample was dissolved in a thioglycerol matrix.

Component 1. Yellow crystals; UV λ_{max}^{MeOH} nm: 255, 267 sh, 350; + NaOMe: 273, 330 sh, 410; + $AlCl_3$: 275, 303, 437; + $AlCl_3$ + HCl: 270, 360; + NaOAc: 272, 320 sh, 375; + NaOAc + H_3BO_3: 262, 375; + 3 N HCl: 255, 370; IR ν_{max}^{KBr} cm^{-1}: 3340, 1660, 1600, 1550, *ca* 1470–1500, 1445, 1360, 1290, 1245 (S=O), 1195, 1115, 1085, 1050 (C–O–S), 1025, 990, 957, 935, 870, 815, 785, 755, 670, 590 and 580; Neg FAB-MS, *m/z*: 898 [M_2 + 2K − 2H], 868 [M_1 + M_2 + 2K − 2H], 861 [2M_2 + K], 831 [M_1 + M_2 + K], 801 [2M_1 + K], 449 [M_2 + K − H], 419 [M_1 + K − H], 411 [M_2], 381 [M_1], 331 [M_2 − SO_3] and 301 [M_1 − SO_3].

Tetrabutylammonium salt. Compound 1 was dissolved in H_2O and an aq. soln of tetrabutylammonium-HSO_4 was added dropwise until no more pptn occured. The ppt was isolated by centrifugation, washed with H_2O, dissolved in MeOH and purified by chromatography on Sephadex LH-20, using MeOH as solvent.

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