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6-METHOXYFLAVONOL 3-MONOSULPHATES FROM *FLAVERIA CHLORAEFOLIA*

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Abstract—*Flaveria chloraefolia* leaves contain three novel 3-sulphates of 6-methoxykaempferol, spinacetin and eupalitin, together with the previously identified 3-sulphates of eupatolitin and eupatin. Purification of these compounds was carried out by gel filtration and chromatography of their tetrabutylammonium derivatives on polyamide and cellulose. Their structures were established by UV spectroscopy and negative FAB-MS of the tetrabutylammonium salts of the sulphate esters, as well as UV data and EI-MS of their aglycones.

INTRODUCTION

A significant number of flavonoid sulphates have been reported in the plant kingdom since 1975 [1–4]. It has been suggested that their occurrence may be more related to saline habitat, than to taxonomic considerations [2]; however, the Compositae seems to be particularly rich in sulphate ester conjugates [5–19] where species of *Brickellia* [5–10] and *Flaveria* [12–19] have received most attention. Very recently, we have shown that enzymatic sulphation in *Flaveria bidentis* is a later step in the biosynthesis of flavonol sulphate esters [19]. Furthermore, we have demonstrated the presence of several flavonol-specific, sulphotransferase activities in cell-free extracts of *Flaveria* species [20]. We wish to report here on the identification of three novel methylated flavonol 3-sulphates, as well as two known sulphate ester derivatives from the leaves of *F. chloraefolia*.

RESULTS AND DISCUSSION

After chromatography of the butanolic extract on Sephadex LH-20, the head fractions contained five methylated flavonol sulphates **1–5**, which were purified by preparative-layer chromatography on polyamide and cellulose plates. Of these, 6-methoxykaempferol 3-sulphate **1**, spinacetin 3-sulphate (6,3′-dimethylquercetagetin 3-sulphate) **2** and eupalitin 3-sulphate (6-methoxykaempferol 7-methyl ether 3-sulphate) **3**, are novel compounds; whereas eupatolitin 3-sulphate (6,7-dimethylquercetagetin 3-sulphate) **4** [6] and eupatin 3-sulphate (6,7,4′-trimethylquercetagetin 3-sulphate) **5** [5] are known. The chromatographic and spectral characteristics of the sulphated compounds **1–5** and their hydrolysis products **1a–5a** are summarized in Tables 1–3. Compounds **1–5** were characterized as flavonol 3-monosulphates on the basis of the following data: (i) they migrated at the level of monosulphates on electrophoresis; (ii) they were rapidly hydrolysed at room temperature in acid conditions; (iii) on cellulose-layer chromatography, they appeared as purple UV-absorbing arrow-shaped spots; (iv) their UV spectra in methanol + HCl underwent a bathochromic shift of 10–20 nm as compared with those in methanol [3]; (v) they were not readily hydrolysed in presence of aryl sulphatase [17].

6-*Methoxykaempferol* 3-*sulphate* 1

The UV spectrum (Table 2) of **1** exhibited a bathochromic shift of 17 nm after addition of aluminium trichloride + HCl, which demonstrated a 3-substitution and the presence of a 6-methoxy group [21, 22]. The bathochromic shift of 25 nm (band I) in presence of sodium acetate (NaOAc) showed that the hydroxyl group at position 4′ was free. The appearance of band III [23] at 325 nm in the sodium methoxide (NaOMe) spectrum (Table 2) indicated the presence of a 7-hydroxyl group; this was further supported by the fact that band I in the NaOAc spectrum appeared at a shorter wavelength than in the NaOMe spectrum [23]. On the other hand, the similarity between the spectra in presence of aluminium trichloride and $AlCl_3$ + HCl, together with the absence of a significant shift after addition of NaOAc + boric acid, indicated the absence of an *ortho* dihydroxy system on ring B. Acid hydrolysis at room temperature of compound **1** yielded an aglycone **1a** exhibiting a yellow spot on cellulose-layer chromatography and a pronounced bathochromic shift of 60 nm for its UV spectrum in presence of $AlCl_3$ + HCl (Table 2). This indicated that the hydroxyl group in position 3 became free. The EI mass spectrum of **1a** (Table 3) confirmed the 6-methoxy substitution by the presence of a M-18 peak larger than 10% and by a $[M]^+$ ion larger than the M − Me peak, the molecular ion being the base peak [24]. Furthermore, recording of the B_2 peak at *m/z* 121 demonstrated unequivocally a monohydroxy B-ring; thus identifying **1a** as 6-methoxykaempferol, $[M]^+$ 316, and **1** as 6-methoxykaempferol 3-sulphate, which is in agreement with a molecular ion at *m/z* 395 and a $[M-SO_3]^-$ ion at 315 in its negative FAB mass spectrum.

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Table 1. Chromatographic properties for flavonol sulphates 1–5

Compound	R_f values (× 100)				
	Cellulose			Colour reactions‡	
	Solvent A*	Solvent D*	Polyamide†	NH_4OH	NA§
6-Methoxykaempferol 3-Sulphate 1	26	51	23	YG	GB
Spinacetin 3-sulphate 2	17	47	54	YG	GB
Eupalitin 3-sulphate 3	30	78	75	YG	GB
Eupatolitin 3-sulphate 4	16	65	65	Y	Or
Eupatin 3-sulphate 5	20	59	84	P	P

*For solvent composition, see Experimental section.
†After two migrations each in solvents B and C.
‡YG: yellow-green; GB: green-brown; Y: yellow; Or: orange; P: purple.
§Naturstoffreagenz A (diphenylboric acid-2-amino ethyl ester).

Table 2. UV data for flavonol sulphates 1–5 and their hydrolysis product 1a–5a

Compound	(λ_{max}, nm)						
	MeOH	HCl	NaOMe	$AlCl_3$	$AlCl_3$ + HCl	NaOAc	NaOAc + H_3BO_3
6-Methoxy-kaempferol 3-sulphate 1	330 262	340	390 325 270	355 300 sh 275	347 300 sh 275	355 295 sh 265	335 262
Spinacetin 3-sulphate 2	345 270 253	365	410 330 272	372 300 sh 275	366 300 sh 275 sh 267	367 320 sh 274	350 270
Eupalitin 3-sulphate 3	335 270	355	390 275	362 300 sh 280	355 300 sh 280	405 sh 345 270	340 270
Eupatolitin 3-sulphate 4	345 270 sh 255	362	405 272	415 300 sh 275	365 300 sh 280 sh 265	425 sh 352 270 sh 255	370 262
Eupatin 3-sulphate 5	335 270 sh 253	355	375 265	370 300 sh 280 sh 265	360 300 sh 280 sh 260	340 270 sh 253	340 270 sh 253
6-Methoxy-kaempferol 1a	360 295 sh 253	—	410 320 273	425 365 sh 305 sh 270	420 360 sh 305 sh 270	370 295 sh 268	360 395 sh 263
Spinacetin 2a	365 252	—	395 275	422 305 sh 262	422 365 sh 300 sh 260	380 255	365 252
Eupalitin 3a	355 265 250	—	405 264	415 360 sh 300 sh 263	413 355 sh 300 sh 260	365 265 sh 250	355 265 sh 250
Eupatolitin 4a	363 255	—	435 275	440 330 sh 300 sh 270	420 370 sh 300 sh 265	370 255	380 260
Eupatin 5a	360 253	—	412 267	418 365 sh 300 sh 263	416 365 sh 300 sh 260	420 sh 365 253	360 253

Table 3. EIMS data for hydrolysis products 1a–5a*

Compound	m/z (rel. int.)							
	$[M]^+$	$[M-Me]^+$	$[M-18]^+$	$[M-COMe]^+$	$[A_1+H]^+$	$[A_1-Me]^+$	$[B_2]^+$	$[B_2-CO]^+$
6-Methoxykaempferol **1a**	316 (100)	301 (12)	298 (30)	273 (82)	183 (5)	167 (4)	121 (28)	93 (11)
Spinacetin **2a**	346 (100)	331 (19)	328 (32)	303 (80)	—	167 (3)	151 (13)	123 (7)
Eupalitin **3a**	330 (100)	315 (8)	312 (31)	287 (76)	—	181 (3)	121 (22)	93 (6)
Eupatolitin **4a**	346 (100)	331 (11)	328 (33)	303 (71)	197 (15)	181 (9)	137 (70)	109 (34)
Eupatin **5a**	360 (100)	345 (8)	342 (36)	317 (98)	—	—	151 (18)	123 (11)

*The terminology for fragments A_1 and B_2 is as given in [28]. For conditions, see Experimental.

Spinacetin 3-sulphate **2**

Negative FAB-MS of **2** gave a molecular ion at *m/z* 425 and an $[M-SO_3]^-$ ion at 345, the molecular ion showing a difference of 30 units as compared to **1**, due to an additional methoxyl substituent. Apart from the presence of a 3-sulphate group (HCl: +20 nm), the UV spectra of **2** (Table 2) demonstrated: (i) a methoxy group at position 6 ($AlCl_3$ + HCl: +21 nm only); (ii) the absence of an *ortho* dihydroxy system on ring B (no significant shift with either NaOAc + boric acid or $AlCl_3$, as compared with their corresponding spectra in MeOH and $AlCl_3$ + HCl); (iii) a free hydroxyl group at position 7 (appearance of band III at 330 nm on the NaOMe spectrum; Band I in NaOAc: 367 nm positioned at a shorter wavelength than Band I in NaOMe: 410 nm); (iv) a free hydroxyl group at position 4′ (Band I in NaOAc: +22 nm). After exposure to ammonia vapours, compound **2** turned yellow-green (Table 1) which confirmed the presence of the free 4′-hydroxyl group [25]. These data suggest that the additional methoxyl group is situated at position 3′. Acid hydrolysis of **2** gave an aglycone **2a** whose chromatographic properties, UV spectra and EIMS (Tables 1–3) were identical to those of reference spinacetin. Therefore, **2** is identified as the 3-sulphate ester of spinacetin.

Eupalitin 3-sulphate **3**

This compound was identified as a 6-methoxy derivative on the basis of a shift of 20 nm only on its UV spectrum after addition of $AlCl_3$ + HCl (Table 2), the presence of a M − 18 peak higher than 10% and a molecular ion higher than the M − Me peak on the EIMS of its aglycone **3a** (Table 3). Compound **3** turned yellow-green after exposure to ammonia vapours (Table 1), therefore suggesting a free hydroxyl group at 4′. This was confirmed by a shift of 10 nm on its UV spectrum after addition of NaOAc (Table 2). On the other hand, **3** turned green-brown after spraying with 2-aminoethyl diphenylborinate (Table 1), and did not show any significant shift in UV (Table 2) between $AlCl_3$ and $AlCl_3$ + HCl, as well as after the addition of NaOAc + boric acid. This demonstrated the absence of an *ortho* dihydroxy system on ring B. Finally, the absence of band III on the NaOMe spectrum (Table 2) suggested that position 7 was blocked. The EI mass spectrum of the hydrolysis product **3a** (Table 3) showed the molecular ion at *m/z* 330, corresponding to a flavonol with three hydroxyl and two methoxyl groups. Presence of the B_2 and B_2-CO peaks at *m/z* 121 and 93, respectively, indicated that ring B was bearing one hydroxyl group only. These data identified **3a** as eupalitin, while the FAB mass spectrum of **3** $[M]^-$ 407, $[M-SO_3]^-$ 327 demonstrated that the latter was a monosulphate ester of **3a**. A shift of 20 nm in UV after the addition of HCl assigned the sulphate group at position 3, therefore compound **3** is eupalitin 3-sulphate.

Eupatolitin 3-sulphate **4**

On cellulose thin-layer chromatography, this compound turned orange after spraying with 2-aminoethyl diphenylborinate, indicating a 3′,4′-dihydroxy system. This was confirmed by the UV spectral data (Table 2) which showed bathochromic shifts of 50 nm in presence of $AlCl_3$ (as compared to $AlCl_3$ + HCl) and 25 nm after addition of NaOAc + boric acid. On the other hand, a shift of +20 nm in presence of $AlCl_3$ + HCl not only demonstrated the 3-substitution, but also the presence of a 6-methoxyl group. Finally, the absence of band III in the NaOMe spectrum suggested that position 7 was blocked. Acid hydrolysis of **4** gave an aglycone **4a** whose UV spectra and EIMS (Tables 2 and 3) were similar to those reported for eupatolitin (5). The negative FAB mass spectrum of **4** confirmed a monosulphate ester of eupatolitin (M: *m/z* 423 and $[M-SO_3]^-$ *m/z* 343), with the sulphate group attached to position 3, as shown by the UV spectrum in presence of HCl (Table 2).

Eupatin 3-sulphate **5**

The presence of M and $M-SO_3$ peaks at *m/z* 439 and 359, respectively on the negative FAB spectrum, demonstrated that **5** was the monosulphate ester of a flavonol having three hydroxyl and three methoxyl groups. In addition, the chromatographic properties, UV- and MS data for **5** and its hydrolysis product **5a** (Tables 1–3) were identical to those of reference eupatin 3-sulphate and eupatin, respectively.

Whereas eupatin 3-sulphate [5] and eupatolitin 3-sulphate [6] have been previously reported in *Brickellia* [5–9], this is the first report of their occurrence in *Flaveria*. On the other hand, 6-methoxykaempferol 3-sulphate, spinacetin 3-sulphate and eupalitin 3-sulphate are identified for the first time in the plant kingdom.

EXPERIMENTAL

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

Source of reference compounds. Spinacetin was from our laboratory collection. Eupatin and its 3-sulphate were kindly supplied by Dr B. Timmermann (University of Arizona, Tucson).

General methods. TLC plates were developed in the following solvent systems: A, *n*-BuOH–HOAc–H_2O(8:1:1); B, 0.1% (w/v) aq. tetrabutylammonium hydrogen sulphate (TBAHS)–C_5H_5N (8:2); C, 0.1% aq. TBAHS–C_5H_5N (7:3); and D, 0.1% aq. TBAHS. The plates were then sprayed with 0.1% (w/v) diphenylboric acid-2-amino ethyl ester (Sigma) in MeOH and examined in UV light (366 nm). Electrophoresis, acid hydrolysis, and enzymatic hydrolysis with aryl sulphatase were performed as in [17]. UV spectra were obtained following standard procedures [26, 27] except for the spectrum in presence of HCl which was recorded 30 min after the addition of 5 drops of 3 M HCl. FAB-MS was carried out as in [17] except that the flavonoid sulphates were analysed in the form of their tetrabutylammonium salt derivatives and after dissolution in a glycerol matrix. EIMS was performed using a VG Analytical 7070 E instrument and the following conditions: electron energy 70 eV, acceleration voltage 6 kV, probe temperature 30°, and source temperature 220°. All flavonoid aglycones were purified on prep. cellulose TLC plates (migration solvent: 40% aq. HOAc) prior to EIMS analysis.

Extraction and isolation of the flavonoid sulphates. Extraction, liquid–liquid partition of the extracts and chromatography of the BuOH extract on a column of Sephadex LH-20 were carried out according to [17]. The head fractions of the column contained a series of non-sulphated flavonoids, followed by a mixture of methylated flavonol sulphates. The fractions containing the methylated flavonol sulphates were pooled; an aq. solution of TBAHS was added, and the resulting tetrabutylammonium salts were extracted with EtOAc. The EtOAc extract was chromatographed on Polyamide-DC 6.6 (Macherey Nagel) preparative plates using solvent B (2 migrations), followed by solvent C (2 migrations). This resulted in the separation of four major bands 1–4 (top to bottom) which were rechromatographed on cellulose (Avicel) preparative plates using solvent D. Band 4 afforded 6-methoxykaempferol 3-sulphate **1**; band 3, spinacetin 3-sulphate **2**; band 2, eupalitin 3-sulphate **3** and eupatolitin 3-sulphate **4**; and band 1, eupatin 3-sulphate **5**.

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