Synthesis of Flavonoid Sulfates. II. The Use of Aryl Sulfatase in the Synthesis of Flavonol-3-sulfates*

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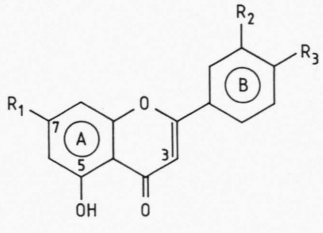
The rates of aryl sulfatase hydrolysis of several 7-, 4′- and 3-sulfated flavonoids were compared and found to follow the order 7 or 4′ >>> 3. The complete resistance of the 3-sulfate ester to enzyme hydrolysis provided a unique and convenient method for the synthesis of a number of naturally occurring flavonol-3-sulfates from the corresponding higher sulfated analogs in quantitative yield.

Introduction

There has been an increasing number of reports in recent years on the natural occurrence of flavonoid sulfates in the plant kingdom [2–21]. This considerable interest seems to be related to their suggested role in the detoxification of excess sulfate in response to high sulfur environment [2]. From structural point of view, the naturally occurring flavonoid sulfates are derivatives of common hydroxyflavones (Fig. 1) and hydroxyflavonols (Fig. 2) or their methyl ethers. The recent development of a novel sulfotransferase assay [22] and the design of an original method for the synthesis of specifically sulfated compounds [1] allowed, for the first time, to demonstrate the enzymatic synthesis of flavonoid sulfate esters [23]. The method used for their organic synthesis utilized N,N′-dicyclohexylcarbodiimide (DCC) plus tetrabutylammonium hydrogen sulfate (TBAHS) and performed stepwise sulfation of positions 7, 4′ and 3 of the flavonoid ring [1]. It allowed the synthesis of a number of naturally occurring flavone-7-sulfates, as well as polysulfated flavonol-3-sulfates. In addition, the DCC-mediated sulfation afforded one major product that was easily separated from by-products by gel filtration [1], and therefore, represents a significant improvement of the previously described sulfamic acid method [24] which gives rise to complex mixture of flavonoid sulfate isomers [25]. Although flavonol-3-sulfates represent one of the most common groups of naturally occurring sulfate esters [6, 7], however, no method is yet available for the specific sulfation of position 3, due to its chelation with the neighbouring carbonyl group. The fact that the 3-sulfate is the only group resistant to hydrolysis with aryl sulfatase [14] allows the specific synthesis of flavonol-3-sulfates from highly sulfated flavonoid-3-sulfates obtained by the DCC-TBAHS method. We wish to report here on the synthesis of a number of flavonol-3-sulfates and their identification by spectroscopic methods.

Fig. 1. Structures of the flavone sulfate esters.



Flavonoid compound	R_1	R_2	R_3
1 Apigenin	OH	H	OH
1a -7-sulfate	OSO_3K	H	OH
1b -4′-sulfate	OH	H	OSO_3K
1c -7,4′-disulfate	OSO_3K	H	OSO_3K
2 Luteolin	OH	OH	OH
2a -7-sulfate	OSO_3K	OH	OH
2b -4′-sulfate	OH	OH	OSO_3K
2c -7,4′-disulfate	OSO_3K	OH	OSO_3K

* For Part I, see Ref. [1].

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Abbreviations: DCC, NN′-dicyclohexylcarbodiimide; TBAHS, tetrabutylammonium hydrogen sulphate.

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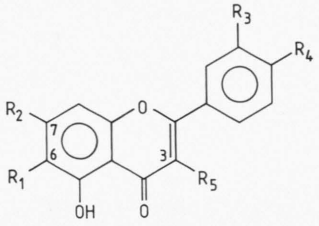
Fig. 2. Structures of the flavonol sulfate esters.



Flavonoid compound	R_1	R_2	R_3	R_4	R_5
3 Kaempferol	H	OH	H	OH	OH
3a -3-sulfate	H	OH	H	OH	OSO_3K
3b -3,7,4′-trisulfate	H	OSO_3K	H	OSO_3K	OSO_3K
4 Quercetin	H	OH	OH	OH	OH
4a -3-sulfate	H	OH	OH	OH	OSO_3K
4b -7-sulfate	H	OSO_3K	OH	OH	OH
4c -4′-sulfate	H	OH	OH	OSO_3K	OH
4d -3,4′-disulfate	H	OH	OH	OSO_3K	OSO_3K
4e -3,7-disulfate	H	OSO_3K	OH	OH	OSO_3K
4f -7,4′-disulfate	H	OSO_3K	OH	OSO_3K	OH
4g -3,7,4′-trisulfate	H	OSO_3K	OH	OSO_3K	OSO_3K
5 Tamarixetin	H	OH	OH	OCH_3	OH
5a -3-sulfate	H	OH	OH	OCH_3	OSO_3K
5b -3,7-disulfate	H	OSO_3K	OH	OCH_3	OSO_3K
6 Rhamnetin	H	OCH_3	OH	OH	OH
6a -3-sulfate	H	OCH_3	OH	OH	OSO_3K
6b -3,4′-disulfate	H	OCH_3	OH	OSO_3K	OSO_3K
7 Eupalitin	OCH_3	OCH_3	H	OH	OH
7a -3-sulfate	OCH_3	OCH_3	H	OH	OSO_3K
7b -3,4′-disulfate	OCH_3	OCH_3	H	OSO_3K	OSO_3K
8 Eupatolitin	OCH_3	OCH_3	OH	OH	OH
8a -3-sulfate	OCH_3	OCH_3	OH	OH	OSO_3K
8b -3,4′-disulfate	OCH_3	OCH_3	OH	OSO_3K	OSO_3K
9 Veronicafolin	OCH_3	OCH_3	OCH_3	OH	OH
9a -3-sulfate	OCH_3	OCH_3	OCH_3	OH	OSO_3K
9b -3,4′-disulfate	OCH_3	OCH_3	OCH_3	OSO_3K	OSO_3K

Experimental

Source of reference compounds and enzyme

Rhamnetin was obtained from Extrasynthese (Gernay, France). Tamarixetin-3,7-disulfate, apigenin-, luteolin-, and quercetin-7,4′-disulfates, as well as quercetin- and kaempferol-3,7,4′-trisulfates were synthesized according to [1]. Aryl sulfatase type H-1 from *Helix pomati* was purchased from Sigma (St. Louis, Mo.).

General methods

Analytical HPLC was carried out on m-Bondapack C18 column (300 × 3.9 mm) using Waters HPLC apparatus equipped with two pumps (Model 510), a Rheodyne injector (Model 7125), an automated gradient controller (Model 680) and a UV detector (Model 441) for detection at 340 nm. The following solvents were used for ion pairing chromatography: A, 0.1 M aqueous tetrabutylammonium dihydrogen phosphate; B, MeOH-H_2O-HOAc (90:5:5, v/v/v). ^1H

NMR (299.9 MHz) and ^{13}C NMR (75.4 MHz) spectra were recorded using a Bruker spectrometer at the Montreal Regional High Field NMR laboratory. For negative FAB-MS, a Kratos MS-50-TC-TA instrument (6.7 kV gun; Xe beam, 2 mA; 9 kV source) was used, after the sample had been dissolved in a glycerol matrix.

Synthesis of flavonoids

Eupalitin (**7**), eupatolitin (**8**) and veronicafolin (**9**) were synthesized according to the method of Wagner *et al.* [26, 27] except that the benzyl protecting groups were replaced with isopropyl groups. Isopropylation of *p*-hydroxybenzaldehyde, vanillin and protocatechualdehyde was carried out as described by Johnstone and Rose [28] and the isopropyloxyflavonols were obtained after transformation of their corresponding chalcones [29]. Deisopropylation and removal of the 5-methyl group were performed by refluxing the isopropyloxyflavonols in conc. HCl-glacial HOAc (1:1, v/v) for 2 h [26]. After dilution with ice, the precipitates of eupalitin, eupatolitin and veronicafolin were purified by column chromatography on cellulose using H_2O-Me_2CO-*n*-butanol-dioxane (80:10:5:5), v/v/v/v).

Rhamnetin (**6b**), eupalitin (**7b**), eupatolitin (**8b**) and veronicafolin (**9b**) 3,4′-disulfates were synthesized by sulfation of the corresponding aglycones using the DCC-TBAHS method [1]. The resulting TBA-salts were converted to their potassium salts using saturated K_2CO_3 in MeOH. They were separated by centrifugation, dissolved in water and further purified on Sephadex G-10 using water as solvent with **6b** and a gradient of 20–50% aqueous MeOH with **7b**, **8b** and **9b**. Yields ranged from 65 to 70%.

Synthesis of flavonoid-3-sulfates

Tamarixetin-3,7-disulfate (**5b**, 0.15 mmol) [1] was dissolved in 20 ml of citric acid-sodium citrate buffer [30] (25 mm, pH 4.5), to which were added 1100 units of aryl sulfatase and the mixture was incubated overnight at 30 °C. Each enzyme unit catalyzed the hydrolysis of 0.135 mmol of sulfated compound (*i.e.* hydrolyzable sulfate group). The incubation mixture was adjusted to pH 8.0 then chromatographed on Sephadex G-10 using a gradient of water and 50% aqueous MeOH to yield 0.14 mmol (93%) of tamarixetin-3-sulfate (**5a**). Under similar conditions, the 3-monosulfates of rhamnetin (**6a**), eupalitin (**7a**), eupatolitin (**8a**) and veronicafolin (**9a**) were prepared from their corresponding 3,4′-disulfate esters.

The 3-sulfates of kaempferol (**3a**) and quercetin (**4a**) were synthesized from their respective 3,7,4′-trisulfate esters [1] (**3b**) and **4g** as described for tamarixetin-3-sulfate, except that double the amount of aryl sulfatase was used.

The 4′-sulfates of apigenin (**1b**), luteolin (**2b**) and quercetin (**4c**) were obtained after aryl sulfatase hydrolysis of their corresponding 7,4′-disulfates **1c**, **2c** and **4f** using the same conditions as described for tamarixetin-3,7-disulfate. Separation of the isomeric 4′- and 7-sulfate esters of apigenin, luteolin and quercetin was performed on Sephadex G-10 using a gradient of water and 20% aqueous MeOH, which was followed by column chromatography on cellulose using water in the case of quercetin isomers.

Results and Discussion

Tamarixetin-3,7-disulfate (**5b**) [1], after hydrolysis with aryl sulfatase, yielded tamarixetin-3-sulfate (**5a**) [31] as the only product. Similarly, using double the amount of enzyme, quercetin (**4g**) and kaempferol (**3b**) 3,7,4′-trisulfates [1] gave quercetin-3-sulfate (**4a**) [32] and kaempferol-3-sulfate (**3a**) [33], respectively. Rhamnetin (**6**), eupalitin (**7**), eupatolitin (**8**) and veronicafolin (**9**), on sulfation with DCC + TBAHS gave their respective 3,4′-disulfate esters **6b**, **7b**, **8b** and **9b**, and upon aryl sulfatase hydrolysis gave their corresponding 3-monosulfates **6a** [32], **7a** [17], **8a** [34] and **9a** [34]. The rates of enzymic hydrolysis of the 7- and 4′-sulfate groups were compared by subjecting a number of flavonoid 7,4′-disulfates to the same hydrolytic conditions as the respective monosulfates (*i.e.* 7.4 units of aryl sulfatase per mmol of compound). Thus, apigenin-7,4′-disulfate (**1c**) [1] gave apigenin-7-sulfate (**1a**) [1] (19%), 4′-sulfate (**1b**) (16%) apigenin (**1**) (6%) and 59% of the unhydrolyzed substrate (based on HPLC analysis). Similarly, luteolin-7,4′-disulfate (**2c**) [1] yielded luteolin-7-sulfate (**2a**) [1], 7,4′-disulfate (**2c**) [1] (total 53%), 4′-sulfate (**2b**) [32] (18%) as well as luteolin (**2**) (29%); and quercetin-7,4′-disulfate (**4f**) gave quercetin-7-sulfate (**4b**) [1] (8%), 4′-sulfate (**4c**) (32%), 7,4′-disulfate (**4f**) (33%) as well as

quercetin (**4**) (27%). In most cases, hydrolysis was difficult to control, and changes in the amount of enzyme resulted in either incomplete hydrolysis or totally desulfated flavonoid aglycone. Similar results were obtained with quercetin-3,7,4′-trisulfate (**4g**) [1] which afforded a mixture of quercetin-3,4′-disulfate (**4d**) [16] (14%), 3,7-disulfate (**4e**) [15] (4%), 3,7,4′-trisulfate (**4g**) (80%) and 3-sulfate (**4a**) (2%).

Table I. ^{13}C NMR data for the synthesized flavonoid sulfates* (75.43 MHz, DMSO-d_6, δppm/TMS).

Compound	**1b**	**3a**	**5a**	**6b**
C-2	162.8	156.4	154.7	155.5
C-3	102.8	132.2	131.1	133.5
C-4	181.3	177.5	178.6	177.6
C-5	161.5	161.3	160.7	163.2
C-6	99.7	99.2	97.8	98.2
C-7	167.3	166.6	164.7	165.0
C-8	94.5	93.9	95.6	90.8
C-9	157.7	156.0	157.7	156.7
C-10	104.0	103.3	102.6	106.1
C-1′	125.1	121.2	124.8	126.6
C-2′	127.5	130.7	116.8	117.5
C-3′	102.2	115.2	146.0	148.6
C-4′	156.8	160.1	152.0	143.3
C-5′	120.2	115.2	110.4	121.5
C-6′	127.5	130.7	123.5	120.4
OMe	–	–	55.0	55.9

* The ^{13}C NMR spectrum of quercetin-3-sulfate (**4a**) was similar to that published [14] and is not reported in this Table.

All compounds were identified on the basis of their ^{13}C NMR (Table I), ^{1}H NMR (Table II), UV spectroscopic (Table III) and negative FAB-MS (Table IV) data. Their retention times on HPLC are summarized in Table V.

These data demonstrate that the rates of enzymic hydrolysis of the 7- and 4′-sulfate groups differ appreciably from one compound to another. However, the common resistance of the 3-sulfate group to hydrolysis with aryl sulfatase provides a unique method for the specific synthesis of flavonol-3-sulfates. Our results seem to be in contrast with previous studies [34–36] which claimed the enzymatic hydrolysis of various flavonol-3-sulfates without reporting the yields of hydrolysis products. We observed that when large amounts of enzyme were used traces of aglycones were liberated from their corresponding 3-sulfate esters, suggesting auto-degradation of the original substrate under such 'forced' assay conditions.

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Table II. ^{1}H NMR data for the synthesized sulfated flavonoids (299.9 MHz, DMSO-d_6, δppm/TMS).

Compound	**1b**	**3a**	**5a**	**6b**
H-3	6.79, s	–	–	–
H-6	6.12	6.10	5.34, d J=1.9 Hz	6.25
H-8	6.40	6.32	5.45, d J=1.9 Hz	6.56
H-2′	7.94, d J=8.7 Hz	8.06, d J=8.7 Hz	*ca* 6.95– 6.98, m	*ca* 7.64– 7.69, m
H-3′	7.34, d J=8.7 Hz	6.83, d J=8.7 Hz	–	–
H-5′	7.34, d J=8.7 Hz	6.83, d J=8.7 Hz	6.56, d J=8.2 Hz	7.30, d J=9.1 Hz
H-6′	7.94, d J=8.7 Hz	8.06, d J=8.7 Hz	*ca* 6.95– 6.98, m	*ca* 7.64– 7.69, m
OMe	–	–	3.78, s	3.83, s

Table III. UV spectral data for the synthesized flavonoid sulfates.

Compound*	MeOH	NaOMe	$AlCl_3$	$AlCl_3$ + HCl	NaOAc	NaOAc + H_3BO_3
1b	315 265	360 275	375 327 280	375 327 280	355 274	320 267
2b	325 267	380 272	380 *sh* 343 272	380 *sh* 343 272	360 272	355 *sh* 265
3a	337 265	385 272	385 343 300 *sh* 275	385 343 300 *sh* 274	370 272	345 265
4c	365 295 *sh* 252	402 320 270	427 350 *sh* 300 *sh* 265	420 345 300 *sh* 262	383 315 270	375 255
5a	340 265 *sh* 250	360 270	390 *sh* 350 265	390 *sh* 352 265	360 272	345 265 250
6b	330 265	335 *sh* 265	390 *sh* 335 275	390 *sh* 335 275	330 265	330 263
7b	320 270	320 *sh* 270	345 277	348 275	320 270	320 270
8b	320 268	320 *sh* 268	350 280	350 280	320 *sh* 269	320 267
9b	325 270	325 273	350 282 255	352 280 253	325 270	325 265

* The UV spectra for other compounds were found similar to published data [14, 15, 17, 32, 34] and are not included in this Table.

sh, shoulder.

Table IV. Negative FAB-MS data for the synthesized flavonoid sulfates (glycerol matrix)*.

Compound	**1b**	**3a**	**4c**	**5a**	**6a**	**6b**	**7b**	**8b**	**9a**	**9b**
M + 2K − H						551				
M + K						*513*	*527*	*543*		*557*
M + K − H				433						
M + H						475	489			
M	*349*	*365*	*381*	*395*	*395*				*439*	
M − SO_3 + H						395	409	425		439
M − SO_3	*269*		301	315	315				359	
M − 2 SO_3 + H						315	329	345		359

* M represents the negatively charged sulfate conjugate, in absence of counter-ion. Fragments in italics correspond to the molecular or pseudo-molecular ions.

Table V. HPLC analysis of flavonoid sulfate esters.

Flavonoid compound	R_t* [min]	Flavonoid compound	R_t* [min]
1	31.4	**4**	16.9
1a	25.4	**4a**	16.0
1b	23.4	**4b**	13.8
1c	18.5	**4c**	20.0
		4d	24.6
2	20.4	**4e**	19.8
2a	16.2	**4f**	14.9
2b	21.7	**4g**	28.7
2c	20.4		
		5a	13.1
		5b	9.5

* The initial solvent was 50% A + 50% B over 10 min, increased to 40% A + 60% B in a 40 min period of time, except for compounds **4a**, **4d**, **4e** and **4g** where the gradient was completed in a 20 min period of time.

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