# Synthesis of Flavonoid Sulfates. III. Synthesis of 3′,4′-*ortho* Disulfates Using Sulfur Trioxide-trimethylamine Complex, and of 3′-Sulfates Using Aryl Sulfatase*

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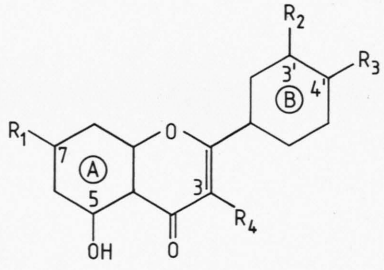
A number of flavonoid 3′,4′-disulfates were synthesized from the corresponding 4′-sulfate esters, using sulfur trioxide-trimethylamine complex. Desulfation of the sulfate esters using aryl sulfatase demonstrated that the rate of hydrolysis of the 3′-sulfate group was slower than either the 7- or 4′ groups, thus allowing the specific synthesis of flavonol 3,3′-disulfates. The effects of *ortho*-disulfation on the $^{13}C$ NMR spectra of flavonoids, and the negative FAB-MS spectra of di- and trisulfated flavonoids are discussed.

## Introduction

Although the first sulfated flavonoid, isorhamnetin 3-sulfate or persicarin was discovered as early as 1937 [2], this group of organic sulfur compounds was considered of rare occurrence until recently [3]. The development, during the seventies, of chromatographic methods such as paper electrophoresis for the rapid survey of sulfated flavonoids led to their characterization in a number of plant families [3–5] especially the Compositae [6–11] among which *Brickellia spp.* [12–18] and *Flaveria spp.* [19–29] are the most documented. Furthermore, important developments in the fields of enzymatic synthesis [30, 31] and organic synthesis [1, 32] of sulfated flavonoids have recently appeared and led us to review this subject [33]. The first published method of organic synthesis of sulfated flavonoids made use of sulfamic acid in boiling pyridine [34]. Using this procedure, sulfation of quercetin gave primarily the 3′-monosulfate ester, and very little of the disulfated products [35]. In contrast, sulfation of quercetin using the dicyclohexylcarbodiimide (DCC) plus tetrabutylammonium hydrogen sulfate (TBAHS) method provided good access to the 3,7,4′-trisulfate ester [32]. The latter method, however, yielded only trace amounts of the 3,7,3′,4′-tetrasulfate ester (**1**), the naturally occurring conjugate of highest sulfation level [5]. This low yield was attributed to the low reactivity of the 3′-hydroxyl group, as well as to steric hindrance created by the presence of the bulky 4′-sulfate group [32]. When we attempted to sulfate quercetin in presence of a large excess of sulfamic acid, quercetin 7,3′-disulfate and 7,3′,4′-trisulfate were produced, but none of the 3,7,3′,4′-tetrasulfate (Barron, unpublished results) due to the fact that chelation of the 3-hydroxyl with the neighboring carbonyl group precludes 3-sulfation. On the other hand, direct sulfation of quercetin with sulfur trioxide-trimethylamine complex in dimethylformamide




**1**: $R_1 = R_2 = R_3 = R_4 = OSO_3K$
**2**: $R_1 = R_3 = R_4 = OSO_3K$, $R_2 = OH$
**3**: $R_1 = OCH_3$, $R_2 = OH$, $R_3 = R_4 = OSO_3K$
**4**: $R_1 = OCH_3$, $R_2 = R_3 = R_4 = OSO_3K$
**5**: $R_1 = R_3 = OSO_3K$, $R_2 = OH$, $R_4 = H$
**6**: $R_1 = R_2 = R_3 = OSO_3K$, $R_4 = H$
**7**: $R_1 = R_2 = R_4 = OSO_3K$, $R_3 = OCH_3$
**8**: $R_1 = R_3 = R_4 = OSO_3K$, $R_2 = H$
**9**: $R_1 = OH$, $R_2 = R_4 = OSO_3K$, $R_3 = OCH_3$
**10**: $R_1 = OCH_3$, $R_2 = R_4 = OSO_3K$, $R_3 = OH$
**11**: $R_1 = R_3 = OH$, $R_2 = R_4 = OSO_3K$
**12**: $R_1 = R_3 = OH$, $R_2 = OSO_3K$, $R_4 = H$
**13**: $R_1 = R_3 = OCH_3$, $R_2 = R_4 = OSO_3K$

* For part II, see Ref. [1].

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and in presence of potassium carbonate, resulted in the formation of a complex mixture of quercetin mono- to trisulfate esters [32]. We wish to report in this paper the use of sulfur trioxide adducts in the sulfation of presulfated flavonoids and the preparation of 3′,4′-*ortho*-disulfate esters in excellent yield.

## Experimental

*Source of compounds, enzyme, and chemicals*

Luteolin 7,4 - and rhamnetin 3,4′-disulfates, as well as quercetin 3,7,4′- and tamarixetin 3,7,3′-trisulfates were chemically synthesized using the DCC + TBAHS method in the conditions described in [1] and [32]. Ombuin 3,3′-disulfate was synthesized by methylation of tamarixetin 3,3′-disulfate. Aryl sulfatase type H-1 from *Helix pomata* was purchased from Sigma. Sulfur trioxide-trimethylamine complex, tetrabutylammonium hydrogen sulfate, and tetramethylammonium chloride were obtained from Aldrich.

*General methods*

$^1$H NMR (400.13 MHz) and $^{13}$C NMR (100.1 MHz) were recorded using a Bruker spectrometer at the Montreal Regional High Field NMR Laboratory. $^{13}$C sulfation shifts were calculated according to [26]. Analytical HPLC was performed using the conditions and solvent compositions given in [1]; the initial solvent was 50% A + 50% B, increased to 40% A + 60% B in a 30 min period of time. Negative FAB-MS was carried out as in [1], except that the samples were dissolved in a glycerol-$H_2O$ matrix. The potassium salt of quercetin 3,3′-disulfate was converted to the tetrabutylammonium salt and the latter purified as described in [24]. The tetramethylammonium salt of quercetin 3,7,4′-trisulfate was prepared using similar procedure by addition of a saturated aqueous solution of tetramethylammonium chloride, except that the aqueous solution was diluted with an equal volume of MeOH and directly chromatographed on a column of Sephadex LH-20, using MeOH as solvent.

*Synthesis of quercetin 3,7,3′,4′-tetrasulfate (**1**), rhamnetin 3,3′,4′-trisulfate (**4**), and luteolin 7,3′,4′-trisulfate (**6**)*

0.19 mmol of quercetin 3,7,4′-trisulfate (**2**), rhamnetin 3,4′-disulfate (**3**), or luteolin 7,4′-disulfate (**5**) were dissolved in 20 ml of $H_2O$ containing 7.3 mmol of $K_2CO_3$. 5.7 mmol (30 equivalents) of sulfur trioxide-trimethylamine complex were added and the mixture was kept under stirring overnight. The reaction medium was adjusted to pH 7.0 with glacial acetic acid and the 3′,4′-*ortho*-disulfated products **1**, **4**, or **6** were converted to their tetrabutylammonium (TBA) salts by the addition of an aqueous solution of tetrabutylammonium hydrogen sulfate, until complete precipitation. The sulfated flavonol-TBA salts were extracted with ethyl acetate and evaporated to dryness. They were dissolved in MeOH and reconverted to their potassium salts according to [32]. Purification of the potassium salts was carried out by gel filtration on Sephadex G-10 using $H_2O$ as solvent.

*Synthesis of tamarixetin 3,3′-disulfate (**9**) and rhamnetin 3,3′-disulfate (**10**)*

0.015 mmol of tamarixetin 3,7,3′-trisulfate (**7**), or rhamnetin 3,3′,4′-trisulfate (**4**) was dissolved in 5 ml of citric acid-sodium citrate buffer [36] (25 mm, pH 4.5). 110 units (7.4 units/mmol sulfated compound/hydrolyzable sulfate group) of aryl sulfatase were added and the mixture incubated overnight at 30 °C. The incubation medium was adjusted to pH 8.0 with aqueous $K_2CO_3$ and chromatographed on a column of Sephadex G-10, using a gradient of $H_2O$ to 50% aqueous MeOH, yielding pure **9** or **10**, respectively.

*Synthesis of quercetin 3,3′-disulfate (**11**) and luteolin 3′-sulfate (**12**)*

The same procedure as for the synthesis of **9** and **10** was used, except that double the amount of aryl sulfatase was added to the hydrolysis medium.

## Results and Discussion

Sulfation of quercetin 3,7,4′-trisulfate (**2**) [32] in aqueous potassium carbonate using sulfur trioxide-trimethylamine complex yielded its 3,7,3′,4′-tetrasulfate ester (**1**) as the only product. Similarly, rhamnetin 3,4′-disulfate (**3**) [1] and luteolin 7,4′-disulfate (**5**) [32] yielded their 3,3′,4′-trisulfate (**4**) and 7,3′,4′-trisulfate (**6**) [35] esters as the only products, respectively. These products were identified by $^{13}$C NMR (Table I), $^1$H NMR (Table II), UV spectroscopic (Table III) and negative FAB-MS (Table IV)

Table I. $^{13}C$ NMR data for the synthesized flavonoid sulfates (100.1 MHz, DMSO-$d_6$, δppm/TMS).

| Carbon | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 1′ | 2′ | 3′ | 4′ | 5′ | 6′ | OMe |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **1** | 155.3 | 133.3 | 178.1 | 160.4 | 101.9 | 159.5 | 97.4 | 156.7 | 106.3 | 125.0 | 120.7 | 142.9 | 146.9 | 119.1 | 124.0 | – |
| **6** | 163.7 | 102.7 | 181.8 | 162.5 | 104.4 | 159.6 | 97.4 | 156.6 | 106.2 | 124.2 | 118.3 | 143.8 | 147.7 | 120.1 | 121.5 | – |
| **9** | 154.2 | 133.1 | 177.6 | 160.9 | 99.1 | 164.6 | 95.9 | 157.6 | 102.6 | 123.3 | 121.1 | 141.6 | 152.4 | 111.8 | 125.7 | 55.7 |

Table II. $^1H$ NMR data for the synthesized sulfated flavonoids (400.13 MHz, DMSO-$d_6$, δppm/TMS).

| Proton | 3 | 6 | 8 | 2′ | 5′ | 6′ | OMe |
|---|---|---|---|---|---|---|---|
| **1** | – | 6.56 d<br>2.0 Hz | 6.89 d<br>2.0 Hz | 8.12 d<br>2.3 Hz | 7.61 d<br>8.9 Hz | 8.04 dd<br>8.9 & 2.3 Hz | – |
| **6** | 6.74 s | 6.52 | 6.96 | 8.13 | 7.72 | 7.72 | – |
| **9** | – | 5.50 | 5.59 | 7.93 d<br>2.3 Hz | 6.99 d<br>8.8 Hz | 8.02 dd<br>8.8 & 2.3 Hz | 3.80 s |

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

| Compound | **1** | **4** | **6** | **9** | **10** | **11** | **12** |
|---|---|---|---|---|---|---|---|
| MeOH | 335 *s*, 310, 267 | 315, 265 | 305, 265 | 340, 265 | 340, 265 | 340, 265 | 330, 267 |
| NaOMe | 380 *s*, 280 | 345 *s*, 280 *s*, 268 | 305 *s*, 267 | 360, 300 *s*, 273 | 395, 263 | 390, 270 | 390, 273 |
| $AlCl_3$ | 393, 330, 300 *s*, 278 | 390 *s*, 325, 275 | 375 *s*, 320, 280 | 385 *s*, 340, 300 *s*, 275 | 390 *s*, 345, 300 *s*, 272 | 395 *s*, 345, 300 *s*, 272 | 375 *s*, 337, 300 *s*, 272 |
| $AlCl_3$ + HCl | 390, 332, 300 *s*, 278 | 390 *s*, 330, 275 | 375 *s*, 320, 275 | 385 *s*, 340, 300 *s*, 272 | 387 *s*, 345, 300 *s*, 275 | 395 *s*, 350, 300 *s*, 270 | 375 *s*, 340, 300 *s*, 270 |
| NaOAc | 345 *s*, 310, 267 | 345 *s*, 265 | 305 *s*, 267 | 360, 300 *s*, 273 | 397 *s*, 355, 265 | 385, 270 | 385, 273 |
| NaOAc + $H_3BO_3$ | 345 s, 310, 267 | 320, 265 | 305, 265 | 340, 265 | 340, 265 | 345, 265 | 340, 267 |

s, shoulder

Table IV. Negative FAB-MS data for the synthesized flavonoid sulfates (glycerol-$H_2O$ matrix)[a].

| Compound | **2** | **4** | **6** | **7** | **8** | **9** | **11** |
|---|---|---|---|---|---|---|---|
| M+2K | *617* | *631* | *601* | *631* | *601* | | |
| M+2K−H | | | 600 | | | 551 | |
| M+K+H | | | | 593 | | | |
| M+K | | | | | | *513* | *499* |
| M−H | | | | | | | 459 |
| M−$SO_3$+2K | 537 | 551 | | 551 | | | |
| M−$SO_3$+H+K | 499 | 513 | 483 | 513 | | | |
| N−$SO_3$+H | | | | | | | 381 |
| M−$SO_3$ | 459 | | | | 443 | | |
| M−2$SO_3$+2H | | | 365 | | | | |
| M−2$SO_3$+H | | | | | | | 301 |
| M−3$SO_3$ | | 313 | | | | | |

[a] M is the negatively charged sulfated conjugate, in absence of counter-ion. Fragments in italics correspond to the pseudo-molecular ions.

techniques [24, 26, 32]. Calculation of the carbon sulfation shifts for quercetin 3,7,3′,4′-tetrasulfate **1** indicated that individual effects of 3′- and 4′-sulfation [26] were not cumulative in the case of 3′,4′-*ortho*-disulfated compounds (Table V). In fact, while the upfield shifts undergone by the carbons carrying the sulfate groups (*i.e.* C-3′ and C-4′) tend to be higher than expected, the downfield shifts of carbons *ortho* (C-2′ and C-5′) and *para* (C-1′ and C-6′) to those bearing the sulfates were less pronounced (Table V). Similar *ortho*-disulfation shifts were observed in the case of luteolin 7,3′,4′-trisulfate (**6**) (Table V). When rhamnetin 3,3′,4′-trisulfate (**4**) and luteolin 7,3′,4′-trisulfate (**6**) were subjected to negative FAB-MS analysis [M+2K] pseudo-molecular ions were recorded at $m/z$ = 631 and 601, respective-

Table V. Sulfation shifts[a] induced on ring B carbons by sulfation in positions 3′ and/or 4′.

| | 3′ (Q) | 4′ (Q) | Calculated 3′ + 4′ (Q) | Observed 3′ + 4′ (Q) | Observed 3′ + 4′ (L) |
|---|---|---|---|---|---|
| C-1′ | −0.6 | −4.5 (*p*) | −5.1 (*p*-4′) | −3.0 (*p*-4′) | −2.1 (*p*-4′) |
| C-2′ | −6.9 (*o*) | −2.0 | −8.9 (*o*-3′) | −5.4 (*o*-3′) | −4.5 (*o*-3′) |
| C-3′ | +4.2 | −3.2 (*o*) | +1.0 | +2.1 | +2.4 |
| C-4′ | −3.6 (*o*) | +4.3 | +0.7 | +0.7 | +2.4 |
| C-5′ | −1.6 | −6.0 (*p*) | −7.6 (*o*-4′) | −3.5 (*o*-4′) | −3.7 (*o*-4′) |
| C-6′ | −4.9 (*p*) | −1.6 | −6.5 (*p*-3′) | −4.0 (*p*-3′) | −2.2 (*p*-3′) |

[a] In DMSO-$d_6$, calculated according to references 24, 26 and 32. Q, quercetin; L, luteolin.

ly (Table IV). The presence of these fragments is in agreement with a previous report of a [M+2Na] peak in the negative FAB-MS spectrum of quercetin 3,7,4′-trisulfate sodium salt [11]. In addition, the spectra of **4** and **6** showed [M−$SO_3$+2K] and [M−$SO_3$+H+K] peaks as well. The validity of these assignments was further demonstrated by the appearance of similar species in the spectra of tamarixetin 3,7,3′- (**7**), quercetin 3,7,4′- (**2**) and kaempferol 3,7,4′- (**8**) trisulfates [32] (Table IV). However, it is interesting to note that when potassium was replaced with a tetramethylammonium counter-ion, the pseudo-molecular ion of quercetin 3,7,4′-trisulfate was shifted to $m/z$ = 687, corresponding to a [M+2 tetramethylammonium] species.

The synthesized 3′-sulfated conjugates were subsequently used for comparative study of the rates of aryl sulfatase hydrolysis of the 3′-, 7-, and 4′-sulfate groups. Tamarixetin 3,7,3′-trisulfate (**7**) [32] and rhamnetin 3,3′,4′-trisulfate (**4**), when subjected to aryl sulfatase hydrolysis under the conditions used for removal of one sulfate group [1], yielded tamarixetin 3,3′-disulfate (**9**), and rhamnetin 3,3′-disulfate (**10**), respectively. Using the conditions for hydrolysis of two sulfate groups, quercetin 3,7,3′,4′-tetrasulfate (**1**) and luteolin 7,3′,4′-trisulfate (**6**), afforded quercetin 3,3′-disulfate (**11**) [26] and luteolin 3′-sulfate (**12**) [37], respectively. All hydrolysis products were obtained in more than 90% yield (HPLC analysis). However, in the presence of an excess of enzyme, quercetin 3,7,3′,4′-tetrasulfate (**1**) and rhamnetin 3,3′-disulfate (**10**) gave their corresponding flavonol 3-monosulfates, since the 3-sulfate group has previously been shown to be resistant to aryl sulfatase hydrolysis [1]. On the other hand, tamarixetin 3,3′-disulfate (**9**) was not hydrolyzed, even in presence of excess aryl sulfatase. Similar results were obtained with ombium 3,3′-disulfate (**13**) which failed to produce any ombuin 3-sulfate under the same enzymatic hydrolysis conditions. These results are in agreement with the fact that tamarixetin 3,3′-disulfate (**9**) did not exhibit any UV spectral shift in presence of aryl sulfatase reagent [38], and suggest that the 3′-sulfate group is resistant to enzymatic hydrolysis when a 4′-methoxy substituent is present. When subjected to negative FAB-MS, tamarixetin 3,3′-disulfate (**9**) and quercetin 3,3′-disulfate (**11**) gave [M+K] pseudo molecular-ions at $m/z$ = 513 and 499, respectively. After replacement of potassium with tetrabutylammonium in quercetin 3,3′-disulfate, a similar [M + tetrabutylammonium] pseudomolecular ion was observed at $m/z$ = 702. Additional [M−$SO_3$+H] and [M−2$SO_3$+H] fragments were recorded in the spectrum of **11**. Similar ions have been previously shown to occur in the spectra of rhamnetin-, eupalitin-, eupatolitin, and veronicafolin 3,4′-disulfates [1], as well as nodifloretin-6,7-disulfate [39].

These results clearly indicate that, in addition to the synthesis of flavonol-3-monosulfates, aryl sulfatase hydrolysis is useful in the specific synthesis of flavonol 3,3′-disulfates as well.

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