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SYNTHESIS OF FLAVONOID SULFATES: I. STEPWISE SULFATION OF POSITIONS 3, 7, and 4' USING N,N'-DICYCLOHEXYLCARBODIIMIDE AND TETRABUTYLAMMONIUM HYDROGEN SULFATE

Denis Barron and Ragai K. Ibrahim*

Plant Biochemistry Laboratory, Department of Biology, Concordia University, 1455 de Maisonneuve Boulevard West, Montréal, Québec, Canada H3G 1M8

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Abstract: The dicyclohexylcarbodiimide-mediated esterification of flavones and flavonols with tetrabutylammonium hydrogen sulfate, resulted in the formation of mono-, di- and trisulfated products. Sulfation occured mainly at positions 7, 4' and 3 of the flavonoid skeleton and followed the order 7 > 4' > 3. Using this method, a number of mono- to trisulfate esters of various naturally occuring flavones and flavonols were synthesized, and their structures verified by spectroscopic methods. This method can be applied to the synthesis of other sulfated natural products, such as steroids and sugars.

Flavonoid sulfates are now considered of common occurrence in plants[1-9]. They belong to either the flavone (1,2) or flavonol (3-5) series (Scheme 1), that are commonly substituted at positions 7 and 3, respectively[3]. Flavonoid sulfates can be identified by electrophoretic, chromatographic and UV-spectroscopic methods[1,2] and, more recently, by ^{13}C NMR and FAB-MS spectroscopy[5,6,8,9]. The study of enzymatic sulfation of flavonoids[10] required an appropriate method for the synthesis of specifically substituted flavonoid sulfate esters to be used as enzyme substrates.

Among the methods used for the sulfation of phenolic compounds[11,12], the sulfamic acid procedure, applied to flavonoids[13,14], resulted in the sulfation of the 3'-position[13] and gave rise to a complex mixture of flavonoid mono- and disulfates, with trace amounts of 7- or 3-monosulfate esters[14]. Another common procedure involved sulfur trioxide adducts[11,12,15]. Attempts to sulfate quercetin 4 with sulfur trioxide trimethylamine complex and K_2CO_3[16], resulted in a mixture of quercetin mono- to trisulfate esters, while most of the quercetin remained unreacted (D. Barron, unpublished). The use of dicyclohexylcarbodiimide (DCC) and H_2SO_4 in DMF has been reported in the synthesis of sulfate esters of alcohols, phenols[17,18], carbohydrates[19], steroids[20], hydroxy amino acids and peptides[21]. Depending on reaction conditions, phenolic hydroxyl groups can be selectively sulfated in polyfunctional molecules[20]. However, attempts to sulfate quercetin by this method, resulted in the destruction of DCC before any sulfate ester was formed (D. Barron, unpublished).

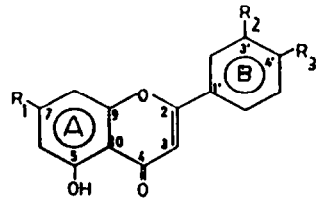
We wish to report here on the use of tetrabutylammonium hydrogen sulfate (TBAHS), instead of H_2SO_4, as a sulfating agent of flavonoid compounds. In contrast with previously used reagents, this method allowed the synthesis of various, specifically sulfated flavonoids in good yield (Scheme 1).

RESULTS

Characteristics of DCC+TBAHS Sulfation. TBAHS reacted with flavonoids in pyridine to give sulfate esters as TBA-salts, which were easily separated from minor byproducts by gel filtration. Conversion to the K-salt was performed by treatment with saturated methanolic K-acetate (Scheme 2). No dimerization of TBAHS took place in control reactions; whereas under the same conditions, H_2SO_4 rapidly reacted with DCC at first contact.

Sulfation of Flavones. Reaction of apigenin 1 with 2 eq. TBAHS and 10 eq. DCC at 4° for 3 days, yielded apigenin-7-sulfate 1a as major product (70%) and 12% apigenin-7,4'-disulfate 1b. The reaction proceeded faster at room temperature (2 days) or 80° (15 min.), but with lower yield. Increasing the TBAHS and DCC to 8 and 20 eq., respectively resulted in 86% yield of 1b. Similarly, luteolin 2 when subjected to sulfation with 2 eq. TBAHS and 10 eq. DCC, yielded 30% of luteolin-7-sulfate 2a. Using 8 and 20 eq. of the respective reagents, luteolin-7,4'-disulfate 2b was formed in 90% yield. No 7,3',4'-trisulfate ester was formed, even when an excess of both reagents was used at room temperature. Therefore, depending on the amounts of reagents used,

Scheme 1



1: Apigenin
$R_1=R_3=OH$, $R_2=H$

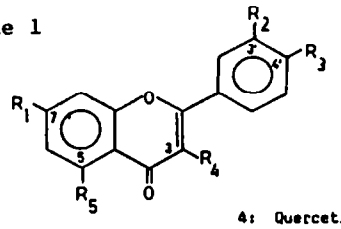
1a: $R_1=OSO_3K$, $R_2=H$, $R_3=OH$

1b: $R_1=R_3=OSO_3K$, $R_2=H$

2: Luteolin
$R_1=R_2=R_3=OH$

2a: $R_1=OSO_3K$, $R_2=R_3=OH$

2b: $R_1=R_3=OSO_3K$, $R_2=OH$



3: Kaempferol
$R_1=R_3=R_4=R_5=OH$, $R_2=H$

3a: $R_1=OSO_3K$, $R_2=H$, $R_3=R_4=R_5=OH$

3b: $R_1=R_3=OSO_3K$, $R_2=H$, $R_4=R_5=OH$

3c: $R_1=R_3=R_4=OSO_3K$, $R_2=H$, $R_5=OH$

5: Tamarixetin
$R_1=R_2=R_4=R_5=OH$, $R_3=OCH_3$

5a: $R_1=R_4=OSO_3K$, $R_2=R_5=OH$, $R_3=OCH_3$

5b: $R_1=R_2=R_4=OSO_3K$, $R_3=OCH_3$, $R_5=OH$

4: Quercetin
$R_1=R_2=R_3=R_4=R_5=OH$

4a: $R_1=OSO_3K$, $R_2=R_3=R_4=R_5=OH$

4b: $R_1=R_3=OSO_3K$, $R_2=R_4=R_5=OH$

4c: $R_1=R_3=R_4=OSO_3K$, $R_2=R_5=OH$

4d: $R_1=R_2=R_3=R_4=OSO_3K$, $R_5=OH$

4e: $R_1=R_3=R_4=R_5=OSO_3K$, $R_2=OH$

Table 1. ^{13}C NMR data for sulfated flavones 1a-2b.
(100 MHz, DMSO-d_6, δ ppm/TMS)

Carbon number	1a	1b	2a	2b
2	165.7	164.6	165.8	164.0
3	103.5	103.2	101.2	102.3
4	182.9	182.8	181.7	181.9
5	161.1	161.3	160.8	160.6
6	103.6	105.1	102.6	104.3
7	159.4	160.0	159.2	159.6
8	99.3	99.0	98.2	97.6
9	157.1	160.0	156.5	156.4
10	106.9	106.8	106.0	105.9
1'	121.1	125.3	120.6	125.8
2'	129.4	129.5	110.9	114.6
3'	117.2	121.1	147.5	149.9
4'	162.9	157.0	151.8	145.0
5'	117.2	121.1	116.1	121.7
6'	129.4	129.5	120.6	117.0

Table 2. ^{13}C NMR data for sulfated flavonols 3a-5b.
(100 MHz, DMSO-d_6, δ ppm/TMS)

Carbon number	3a	3b	3c	4a	4b	4c	5a	5b
2	147.6	146.3	155.6	148.2	146.2	155.6	155.4	155.6
3[a]	136.2	--	133.3	136.9	--	133.5	133.1	133.1
4	176.3	178.2	177.7	176.5	177.8	178.2	178.0	177.7
5[b]	159.7	159.9	161.4	160.0	159.8	160.7	160.9	161.5
6	101.3	100.9	102.3	101.4	100.9	102.3	101.9	102.4
7[b]	159.2	158.7	159.3	159.0	158.9	159.6	159.5	159.4
8	97.5	97.5	96.6	97.7	97.4	97.6	96.9	96.4
9	155.2	154.2[c]	155.9[c]	155.2	155.0	156.7	156.6	156.2
10	105.0	105.4	106.7	105.4	105.4	106.7	106.4	106.9
1'	121.6	126.6	124.7	121.3	127.8	126.3	122.9	122.2
2'	129.6	127.8	129.8	114.9	115.9	117.3	115.3	121.3
3'	115.5	119.8	119.2	145.8	148.7	148.4	146.1	141.9
4'	159.4	155.0[c]	155.6[c]	149.5	142.1	143.6	150.2	153.2
5'	115.5	119.8	119.2	116.1	121.8	121.6	111.5	112.0
6'	129.6	127.8	129.8	120.5	118.2	120.9	121.7	126.3
OMe	--	--	--	--	--	--	55.6	55.8

[a] Carbon 3 could not be unequivocally assigned for compounds 3b and 4b.

[b] Assignments for carbons 5 and 7 may be reversed.

[c] Assignments for carbons 9 and 4' can be reversed.

stepwise sulfation of flavones followed the sequence 7 > 4'. ^{13}C NMR data of synthesized flavone sulfates is summarized in Table 1.

Sulfation of Flavonols. The sequence of sulfation of the 7- and 4'-positions was found to be similar to that of flavones. Therefore, under the same conditions described for flavones, kaempferol **3** and quercetin **4**, gave rise to their 7-monosulfate esters **3a** and **4a**, as well as their 7,4'-disulfate esters **3b** and **4b**, respectively. However, using 20 eq. TBAHS and 30 eq. DCC for 12 days, the 3-hydroxyl was sulfated as well, giving rise to the 3,7,4'-trisulfate esters **3c** and **4c**, respectively. A trace amount of quercetin-3,7,3'4'-tetrasulfate **4d** was detected as well. The sulfation of tamarixetin **5** with 20 eq. TBAHS and 30 eq. DCC gave rise to its 3,7-disulfate **5a** and 3,7,3'-trisulfate **5b**, esters in 1:3 ratio, respectively. This indicates that when the 4'-hydroxyl is methylated, sulfation can be directed to the 3'-position. Furthermore, it appears that sulfation at position 3 occurs prior to that at 3', since the second major reaction product was the 3,7-disulfate ester, and not the 7,3'-analog. Therefore, the sequence of sulfation in flavonols was established as 7 > 4' > 3 > 3'. ^{13}C NMR data of sulfated flavonoids is summarized in Table 2. Further attempts to sulfate flavonoids with three vicinal phenolic groups, such as quercetagetin (6-hydroxyquercetin) or myricetin (5'-hydroxyquercetin), resulted in a number of degradation products, possibly due to the instability of these aglycones in pyridine.

DISCUSSION

In the TBAHS+DCC mediated synthesis of sulfated flavonoids, the reaction temperature seems to control both the sequence and yield of sulfation. A low temperature (ca 4°) favors the synthesis of 7-monosulfates in 30-70% yield, whereas 7,4'-disulfate esters can be synthesized in better yield (84-99%) at room temperature. It should be noted, however, that the 3-position of flavonols was resistant to sulfation due to its chelation with the adjacent carbonyl group. The latter position was sulfated only in presence of a large excess of TBAHS+DCC, to yield the flavonol-3,7,4'-trisulfate ester. The 3'-hydroxyl group exhibited low reactivity towards the TBAHS+DCC mediated sulfation, probably due to its relatively weak acidic nature. It could be sulfated when the 4'-hydroxyl was substituted with a methyl but not sulfate group, possibly because of steric hindrance of the bulky 4'-sulfate group. The purified yields of flavonoid sulfates depended on the efficiency of conversion of TBA-salts to their K-salts, i.e. yields increased with increasing insolubility of the K-salts in methanol (see Exptl. Section).

Calculation of ^{13}C NMR sulfation shifts for positions 3,3' & 4' of flavonoid sulfates (Table 3) were in accordance with those reported for naturally occurring compounds[6,8]. Flavonoids with 3,4'-dihydroxy grouping exhibited downfield shifts induced on the ortho carbons by sulfation at position 4' or 3' which were more pronounced (Table 3) for the alpha carbon lacking a hydroxyl group (C-2' or C-5'), than for that bearing a hydroxyl group (C-3' or C-4'). On the other hand, the shifts induced by 7-sulfation were characteristic of a phenol sulfate ester[22] (Table 3), i.e. upfield displacement for the carbon carrying the sulfate group (C-7) and downfield displacement for ortho (C-6 and C-8) and para (C-10) carbons. In ^{1}H NMR (Table 4), introduction of a sulfate group at positions 7 or 4' induced downfield shift of the protons attached to the carbon alpha to the sulfate group. Thus, H-5' (and H-3' in 4'-monosubstituted flavonoids) was shifted downfield by 0.37 to 0.48 ppm when a 4'-sulfate was present. On the other hand, if a sulfate group was attached to position 7, H-6 and H-8 were shifted downfield by 0.30 to 0.37 and 0.53 to 0.55 ppm, respectively.

To our knowledge, the TBAHS+DCC mediated sulfation represents the first reported method for the introduction of sulfate groups at specific positions on flavonoid rings. It is particularly useful for the synthesis of naturally occurring 7-sulfated flavones, and 3-sulfated flavonols. The use of DCC allows the synthesis of trisulfated, flavonol-3-sulfates from which lower 3-sulfated intermediates can be obtained by enzymatic desulfation (Barron & Ibrahim, in preparation). This method has the following advantages: (a) allows the synthesis of substrates and reaction products for enzyme studies[10], (b) permits sulfation of selected positions, (c) eliminates dimerization of the sulfating agent[21] and/or formation of pyrosulfate esters[17] and (d) can be applied to the synthesis of other natural products such as steroid and sugar sulfates.

Table 3. ^{13}C NMR sulfation shifts[a] observed with the synthesized flavonoid sulfates **1a-5b**.

Carbon	Position of sulfation			
	3	7	3'	4'
Ipso	+2.1 to +3.1	+4.1 to +5.5	+4.3	+3.6 to +5.5
Ortho(C-OH)	--	--	-3.7(C-4')	-3.4 to -5.1(C-3')
Ortho(C-H)[b]	-8.1 to -8.8(C-2)	-2.7 to -6.3(C-6)	-6.1(C-2')	-3.8 to -6.2(C-5')
	-1.7 to -2.5(C-4)	-2.5 to -5.0(C-8)	--	--
Meta	--	+1.5 to -0.7(C-5)	+1.2(C-1')	+1.7 to -1.1(C-2')
	--	+1.2 to -0.5(C-9)	+0.4(C-5')	+1.7 to -1.1(C-6')
Para	--	-1.7 to -3.6(C-10)	-6.5(C-6')	-3.0 to -5.8(C-1')

[a] In ppm (DMSO-d_6) refers to $\delta_{aglycone} - \delta_{sulfate\ ester}$.
[b] Including C-2 and C-4.

Table 4. ^{1}H NMR data for sulfated flavonoids **1a-5b**. (400 MHz, DMSO-d_6, δ ppm/TMS)

Compound	Proton Number							
	3	6	8	2'	3'	5'	6'	OMe
1a	6.70 s	6.55 d 2.0 Hz	6.96 d 2.0 Hz	7.84 d 8.8 Hz	6.87 d 8.8 Hz	6.87 d 8.8 Hz	7.84 d 8.8 Hz	-
1b	6.90 s	6.60 d 2.1 Hz	7.02 d 2.1 Hz	8.02 d 8.9 Hz	7.35 d 8.9 Hz	7.35 d 8.9 Hz	8.02 d 8.9 Hz	-
2a	6.42 s	6.48 d 2.1 Hz	6.87 d 2.1 Hz	7.14 d 2.5 Hz	-	6.35 d 8.5 Hz	7.34 dd 8.5 & 2.5 Hz	-
2b	6.80 s	6.56 d 2.0 Hz	7.00 d 2.0 Hz	7.59 d 1.8 Hz	-	ca 7.44- 7.48,m	ca 7.44- 7.48,m	-
3a	-	6.55 d 2.0 Hz	6.98 d 2.0 Hz	8.08 d 8.9 Hz	6.93 d 8.9 Hz	6.93 d 8.9 Hz	8.08 d 8.9 Hz	-
3b	-	6.50 d 2.0 Hz	6.95 d 2.0 Hz	8.20 d 8.9 Hz	7.30 d 8.9 Hz	7.30 d 8.9 Hz	8.20 d 8.9 Hz	-
3c	-	6.52 d 2.0 Hz	6.88 d 2.0 Hz	8.16 d 8.9 Hz	7.27 d 8.9 Hz	7.27 d 8.9 Hz	8.16 d 8.9 Hz	-
4a	-	6.50 d 1.9 Hz	6.95 d 1.9 Hz	7.67 d 1.9 Hz	-	6.84 d 8.5 Hz	7.61 dd 8.5 & 1.9 Hz	-
4b	-	6.47 d 2.0 Hz	6.93 d 2.0 Hz	7.90 d 2.0 Hz	-	7.31 d 8.6 Hz	7.70 dd 8.6 & 2.0 Hz	-
4c	-	6.54 d 1.9 Hz	6.94 d 1.9 Hz	7.64 d 2.2 Hz	-	7.33 d 8.4 Hz	7.69 dd 8.4 & 2.2 Hz	-
5a	-	6.50 d 2.0 Hz	6.94 d 2.0 Hz	7.60 d 2.2 Hz	-	7.01 d 8.9 Hz	7.84 dd 8.9 & 2.2 Hz	3.85 s
5b	-	6.48 d 1.9 Hz	6.82 d 1.9 Hz	8.06 d 2.3 Hz	-	7.07 d 8.9 Hz	8.12 dd 8.9 & 2.3 Hz	3.83 s

Scheme 2

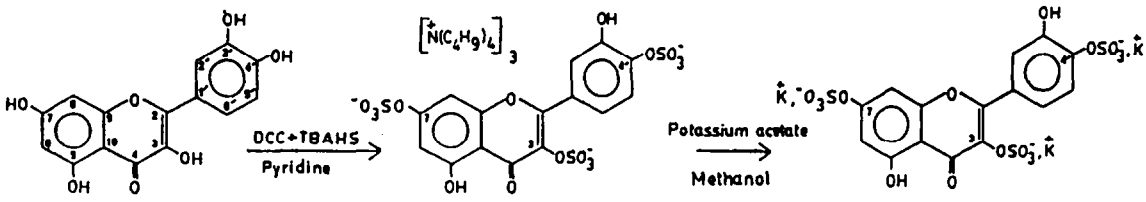


Table 5 UV data for the flavonoid sulfates 1a-5b.

	MeOH	NaOMe	$AlCl_3$	$AlCl_3$ + HCl	NaOAc	NaOAc + H_3BO_3
1a	330, 265	385, 263	380, 340 290s, 273	380, 340 290s, 273	390s, 340 265	330, 265
1b	300, 265	300s, 265	375, 320 290s, 275	375, 320 290s, 273	300, 265	300, 265
2a	340, 262s 250	382, 260	380, 342s 290s, 265	375s, 337 290s, 265	343, 262s 250	362, 252
2b	320, 265	315, 265	380, 325 287s, 275	375, 325 287s, 275	310, 265	320, 265
3a	360, 320s 262, 240	400, 250	415, 345 300s, 275s 255	415, 345 300s, 275s 255	430, 350 255	360, 262s
3b	358, 310 265, 245	410, 350s 265s, 250	415, 335 300s, 275s 250	415, 335 300s, 265	415s, 360 307, 260 245	358, 310 262
3c	335s, 305 265	340s, 300s 265	385, 327 295s, 227	385, 335 295s, 275	340s, 305 265	340s, 305 265
4a	365, 250	415, 357	425, 355s 262	415, 350 260	375, 250	380, 255
4b	360, 300s 265s, 250	415, 350s 250	422, 345 300s, 262	420, 345 300s, 260	420s, 365 265s, 250	360, 305s 265s, 250
4c	335, 265	410s, 315 265	390, 330 275	390, 330 275	337, 265	335, 265
5a	345, 265s 250	342, 265s 250	395, 355s 297s, 265	390, 350s 297s, 265	350, 265s 250	350, 265s 250
5b	340, 265	335, 265	390, 342 295s, 275	390, 345 295s, 270	340, 265	338, 265

EXPERIMENTAL

General methods. ^1H NMR, ^{13}C NMR, FAB-MS, cellulose TLC and paper electrophoresis (as K-salts) were performed as in[8]. TLC scanning of the Sephadex LH-20 fractions (see the "Isolation of flavonoid sulfate esters" Section) first required conversion of TBA-salts to K-salts. This was performed by addition of 100 μl of satd. K_2CO_3 in MeOH, to an equal volume of eluate. The alkaline solution was directly used for TLC when no precipitation occured (monosulfates), or after centrifugation and dissolution of the K-salt ppt. in water (di-and trisulfates). Analytical HPLC was carried out as in[8] using the following solvents: A, 0.01M aq. TBA dihydrogen phosphate; B, MeOH-H_2O-AcOH (90:5:5); isocratic 40% A + 60% B. Identification of sulfated reaction products was based on results of acid and aryl-sulfatase hydrolysis, electrophoresis and analysis of spectroscopic data including UV (Table 5), IR and FAB-MS, according to[6,8].

Isolation of flavonoid sulfate esters. The reaction medium was diluted with MeOH, the dicyclohexylurea ppt. was removed by centrifugation. The supernatant was chromatographed on Sephadex LH-20 using MeOH. The pooled eluates were converted to their K-salts with satd. K_2CO_3 in MeOH. The precipitates of flavonoid-7-monosulfates K-salts were dissolved in H_2O, the insoluble material eliminated by centifugation, and further purified by chromatography on Sephadex G-10 using 20-50% gradient of aq. MeOH. Since the MeOH supernatants of flavonoid monosulfates still contained appreciable amounts of K-salts, they were evaporated to dryness, taken in 20% aq. MeOH, the insoluble removed by centrifugation, and chromatographed on Sephadex G-10 in the same conditions. The precipitates of flavonoid di- and trisulfates K-salts were washed with MeOH, dissolved in H_2O and after centrifugation of the insoluble, chromatographed on Sephadex G-10 using 20% aq. MeOH (disulfates) or H_2O (trisulfates).

Conditions* for synthesis of sulfated flavonoids, yields and FAB-MS data

Compound	1a	1b	2a	2b	3a	3b	3c	4a	4b	4c	5a+5b
mmoles DCC in ml pyridine	3.70 2.00	7.70 3.00	7.00 5.00	14.00 7.00	7.00 5.00	7.00 3.00	21.00 8.00	2.90 2.50	5.80 3.00	8.70 3.00	18.90 8.00
mmoles flavonoid	0.37	0.37	0.70	0.70	0.70	0.35	0.70	0.29	0.29	0.29	0.63
mmoles TBAHS in ml pyridine	0.76 0.30	2.94 1.00	1.40 0.60	5.60 2.00	1.40 0.60	2.80 1.00	14.00 5.00	0.59 0.20	2.30 1.00	5.90 2.00	12.60 5.00
Temperature (°)	4	4	4	4	4	25	25	4	25	25	25
Time (days)	3	3	3	3	3	2	12	5	3	12	30
Products and % yield (HPLC)	1a (70) 1b (12)	1b (86) 1a (14)	2a (30)	2b (90) 2a (8)	3a (65)	3b (84) 3c (12)	3c (95)	4a (42) 4b (20)	4b (99)	4c (97)	5a 5b
Purified % yield	28	81	25	78	49	69	94	48	84	89	-
FAB-MS: $(M)^-$	349		365		365			301			
$(M+K)^-$		467		483		483	-		499	-	513 (5a)

* Using the following steps: i) dissolution of DCC+flavonoid in pyridine, ii) 15 min. latter, addition of TBAHS solution.

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