Phytochemistry, Vol. 27, No. 7, pp. 2335–2338, 1988.
Printed in Great Britain.

0031-9422/88 $3.00 + 0.00

HYDROCHLORIC ACID AND ARYL-SULPHATASE AS REAGENTS FOR UV-SPECTRAL DETECTION OF 3- AND 4′-SULPHATED FLAVONOIDS

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(*Received* 30 *October* 1987)

Key Word Index—UV-spectroscopy; flavonoid sulphates.

Abstract—The UV spectra of 31 naturally occurring and synthetic flavonoid sulphates were analysed in the presence of hydrochloric acid and aryl-sulphatase reagents. After the addition of HCl, the spectra of sulphated flavones showed a bathochromic shift of about 8 nm when a 3′-sulphate group was present, while bathochromic shifts of 15 to 25 nm and 35 nm were indicative of 4′-mono- and 3′,4′-disulphation, respectively. Similarly, in the flavonol series, bathochromic shifts of 15 to 20 nm were indicative of sulphation in position 3; 25 nm, of 3,3′-; 30 to 40 nm, of 3,4′-; and 48 to 53 nm, of 3,3′,4′-sulphation. The aryl-sulphatase shift was found useful in the diagnosis of flavonol 3,3′-, 3,4′-disulphates and 3,3′,4′-trisulphates, which exhibited bathochromic shifts after treatment of 7 to 10, 17 to 22, and 30 to 65 nm, respectively.

INTRODUCTION

Until recently, it was believed that sulphated flavonoids exhibit the same UV-spectral characteristics as those of the corresponding flavonoid methyl ethers [1, 2]. However, it was later demonstrated [3] that introduction of a 3-sulphate group in gossypetin produced a hypsochromic shift of 12 nm. This observation, together with the fact that the sulphate group is unstable in acidic conditions [1, 2], led to the use of HCl as a spectral shift reagent for 3-sulphated flavonols [4]. Indeed, after the addition of HCl, Band I of 3-sulphated flavonols showed a bathochromic shift of 20 to 30 nm [4]. Although it has been applied to the identification of a variety of flavonol 3-sulphates [5–11], the HCl shift has yet to be widely used for this purpose, mostly because of the lack of general rules that can be derived from the available data. Very recently, however, we developed new chemical and enzymatic methods for the synthetis of flavonoids sulphated at specific positions [12, 13]. This allowed us to acquire, for the first time, a library of reference sulphated flavonoids which we used for ^{13}C NMR and FAB-MS spectroscopic studies [12, 13]. We wish to report here on the application of the HCl shift reagent, not only in the identification of 3-monosulphated conjugates, but also for the detection of flavonoid 4′- and 3′-sulphates. Furthermore, the distinction between 3,3′- and 3,4′-flavonol disulphates can be achieved by the use of the novel reagent, aryl-sulphatase.

RESULTS AND DISCUSSION

It is well known that substitution of the 3- or 4′-hydroxyl groups of flavonoids produces a hypsochromic shift of Band I of *ca* 10–20 nm and 2–10 nm, respectively [14], whereas substitution of the 7-hydroxyl does not have any effect on either Band I or II [14]. Examination of the UV absorption maxima of the methylated, glycosylated or sulphated flavonoids (Table 1), indicates that similar effects are induced by sulphation, except that the hypsochromic shifts obtained by 3- and 4′-sulphation of the flavonoid ring are appreciably higher (i.e. 20 to 30 and 8 to 14 nm, respectively) than the corresponding methylation or glycosylation shifts. After cleavage of the sulphate ester bond by HCl or aryl-sulphatase, the UV spectrum reverts to that of the corresponding aglycone and the resulting bathochromic shift of Band I indicates the presence of sulphate groups in positions 3 and 4′/3′, but not at position 7.

Spectra of sulphated flavones in presence of HCl

All 4′-sulphated flavones underwent a bathochromic shift of 15 to 25 nm after the addition of HCl, while this shift was only 8 nm in the case of luteolin 3′-monosulphate (Table 2). No shift was observed with flavone 7-monosulphates. On the other hand, the effects of 4′- and 3′- sulphation were additive in luteolin 7,3′,4′-trisulphate which gave a pronounced shift of 35 nm.

Spectra of sulphated flavonols in presence of HCl

Unlike the flavone analogues, flavonol 4′-monosulphates and 7,4′-disulphates did not exhibit any significant or reproducible shifts. Instead, when position 3 was sulphated, bathochromic shifts of 15 to 20 nm were observed in the case of flavonol 3-monosulphates and 3,7-disulphates, and of 25 nm with 3,3′-disulphates and 3,7,3′-trisulphates, and as much as 30 to 40 nm for 3,4′-disulphates and 3,7,4-trisulphates (Table 3). These results are in agreement with our previous report of similar shifts with various 6-methoxyflavonol 3-monosulphates [11]. In the

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Table 1. Comparison of UV absorption maxima (Band I) of a number of flavonoid glycosides, methyl esters and sulphate esters (λ_{max}^{MeOH})

Substitution	Kaempferol	Quercetin	Isorhamnetin	Eupatin	Eupatolitin	Luteolin	Jaceidin
Aglycone	367 [15]	370 [15]	370 [15]	360 [11]	363 [11]	349 [15]	351 [21]
3-OMe	350 [16]	358 [15]		347 [17]	354 [6]		
4′-OMe						344 [15]	348 [4]
3-Glucoside	345 [9]				353 [18]		
3-Rhamnoglucoside		359 [15]					
3-Galactoside			357 [15]				
3-Sulphate	337 [13]	350 [8]	350 [9]	335 [11]	345 [11]		
4′-Sulphate						335 [20]	343 [21]
3,7-Di-	345 [19]	350 [9]	350 [9]				
3,3′-Di-		340 [10]					
3,4′-Di-		335 [10]	333 [7]				
3,7,4′-Tri-		335 [12]	337 [7]				

Table 2. UV spectra of sulphated flavones in presence of HCl

Sulphated conjugate	Source and reference	λ_{max} Band I (nm) MeOH	HCl	HCl shift
Apigenin				
7-Mono-	CS [12]	330	330	0
4′-Mono-	CS [13]	315	332	+17
7,4′-Di-	CS [12]	300	325	+25
Luteolin				
7-Mono-	CS [12]	340	340	0
4′-Mono-	CS [13]	325	343	+18
3′-Mono-	CS [13]	330	338	+8
7,4′-Di-	CS [12]	320	335	+15
7,3′,4′-Tri-	CS [13]	305	340	+35

CS, chemical synthesis.

same conditions, no bathochromic shift was observed with patuletin 3-glucoside (MeOH: 350 nm; HCl: 325 nm); thus demonstrating the specificity of the HCl reagent for the detection of sulphate ester conjugates. It is also evident that the effect of 3′- and 4′-sulphation is cumulative in 3,3′,4′-trisulphated flavonols which gave shifts of 48 to 53 nm. However, there was no difference observed in the shifts exhibited by quercetin 3,7,4′- and 3,7,3′-trisulphates, considering the magnitude of the shift given by the 3′-sulphated isomer (Table 3). Because of the lack of reference compounds, we could not evaluate the effect of B-ring sulphation on 3-methylated or 3-glycosylated flavonoids. It is likely, however, that HCl may provide indication of 4′-sulphation, since a 5 nm bathochromic shift has previously been observed in the spectrum of jaceidin (3,6,3′-trimethyl quercetagetin) 4′-sulphate, after addition of HCl [21].

Spectra of sulphated flavonols in presence of aryl-sulphatase

Since 4′- or 3′-sulphation in flavonols can only be detected in the presence of a 3-substitution, the use of aryl-sulphatase (sulphohydrolase, EC 3.1.5-) as spectral shift reagent allows the specific characterization of 4′- or 3′-sulphate groups in polysulphated flavonol 3-sulphates. This enzyme is known to selectively hydrolyse all the sulphate groups on the flavonoid ring, except that at position 3 [8, 10, 13]. Such specific cleavage of the 4′- and 3′-sulphate groups, leaving the 3-ester bond intact, is impossible to perform using the HCl reagent. Better distinction between 3,4′- and 3,3′-sulphation can also be achieved using the aryl-sulphatase reagent, since enzymatic cleavage of the 4′-sulphate group produced shifts of +17 to 22 nm, as compared to +7 to 10 nm only with the 3′-sulphate (Table 3). Thus, quercetin 3,7,3′- and 3,7,4′-trisulphates, which exhibited comparable HCl shifts, gave clearly distinguishable shifts with aryl-sulphatase (Table 3). On the other hand, no shift was observed with either tamarixetin 3,3′- or 3,7,3′-conjugates tested. Therefore, more studies will be needed to investigate the effect of 4′-methylation on the enzyme-catalysed 3′-sulphation shift. Furthermore, it is interesting to note that 3′,4′-disulphated esters gave cumulative shifts of +30 to 35 nm.

It should be noted, however, that kaempferol 3,7,4′-trisulphate gave unexpectively high 4′-shifts (Table 3), with both HCl and aryl-sulphatase reagents (+60 and +35 nm, respectively). This case seems to be exceptional in the appearance of two maxima (335 and 300 nm) in Band I (Table 3).

The interpretation of the effects of both HCl and aryl-sulphatase on the UV-spectral shifts of flavone and flavonol sulphates is summarized in Table 4. The use of both reagents allows the identification of sulphated positions in small amounts of flavonoid compounds.

EXPERIMENTAL

Determination of HCl *spectra.* The normal MeOH spectrum was recorded after dissolving the flavonoid sulphate in H_2O and addition of a few drops of the aq. soln to MeOH. The HCl spectra were obtained 30 min. after addition of 3 drops of a 25% HCl in MeOH to the aq. methanolic solution. Hydrolysis of the sulphate groups was usually complete after 15 min.

Preparation of the aryl-sulphatase reagent. 220 Units of aryl-sulphatase (Type H-1 from *Helix*; Sigma) were disssolved in 25 mM citrate buffer pH 4.5 [22], containing 10% glycerol. The enzymatic solution was divided in 50 μl aliquots (eppendorff tubes) and stored at −15°. Under such conditions the activity was stable for several weeks.

Table 3. UV spectra of sulphated flavonols in presence of HCl and aryl-sulphatase (λ_{max} Band I, nm)

Sulphated conjugate	Source and reference	MeOH	HCl	HCl shift	H_2O	AS	AS shift
Q 3-mono-	CS [13]	350	370	+20	340	340	0
T 3-mono-	CS [13]	340	362	+22			
K 3-mono-	CS [13]	337	360	+23			
R 3-mono-	CS [13]	345	365	+20			
V 3-mono-	CS [13]	340	360	+20			
Q 3,7-di-	Fb [9]	350	365	+15	345	342	−3
I 3,7-di-	Fb [9]	350	365	+15			
T 3,7-di-	CS [12]	345	365	+20			
Q 3,3′-di	CS [13]	340	365	+25	330	338	+8
P 3,3′-di-	Fc [10]	340	365	+25	330	338	+8
R 3,3′-di-	CS [13]	340	365	+25	330	340	+10
T 3,3′-di-	CS [13]	340	365	+25	330	330	0
T 3,7,3′-tri	CS [12]	340	365	+25	333	333	0
Q 3,7,3′-tri	Fb [*]	335	365	+30	333	340	+7
Q 3,4′-di-	Fc [10]	335	365	+30	327	345	+18
R 3,4′-di-	CS [13]	330	367	+37	320	342	+22
Eupal 3,4′-di-	CS [13]	320	355	+35	315	330	+15
Eupatol 3,4′-di-	CS [13]	320	360	+40	315	335	+20
V 3,4′-di-	CS [13]	325	360	+35	320	340	+20
K 3,7,4′-tri	CS [12]	335 s	360	+60	325 s	335	+35
		300			300		
Q 3,7,4′-tri	CS [12]	335	365	+30	325	342	+17
R 3,3′,4′-tri	CS [13]	315	363	+48	310	340	+30
Q 3,7,3′,4′-tetra-	CS [13]	310	363	+53	300	335	+35

AS, aryl-sulphatase; CS, chemical synthesis; Eupal, eupalitin, Eupatol, eupatolitin; Fb, *Flaveria bidentis*; Fc, *F. chloraefolia*; I, isorhamnetin; K, kaempferol; P, patuletin; Q, quercetin; R, rhamnetin; V, veronicafolin.

* Varin, L., unpublished results.

Table 4. Interpretation of the UV spectra of flavone and flavonol sulphates

Reagent	Shift observed for Band I (nm)		Interpretation
	Flavones	Flavonols	
HCl	+8		3′-Sulphate
	+15 to 25		4′-Sulphate
	+35		3′,4′-Disulphate
		+15 to 20	3-Sulphate
		+25	3,3′-Disulphate
		+30 to 40	3,4′-Disulphate
		+48 to 53	3,3′,4′-Trisulphate
Aryl-sulphatase		HCl shift = 15 to 20; No AS shift	3-Monosulphate
		HCl shift > 25; No AS shift	4′-OMe-3,3′-Disulphate
		+7 to 10	4′-OH-3,3′-Disulphate
		+17 to 22	3,4′-Disulphate, with or without free 3′–OH
		+30 to 35	3,3′,4′-Trisulphate

AS, aryl-sulphatase.

Determination of the aryl-sulphatase spectra. The normal spectrum was first recorded in H_2O, and for a second time, 15 min after the addition of 20 μl of aryl-sulphatase preparation to both the reference and assay cuvettes. Complete hydrolysis with aryl-sulphatase was usually achieved in 10 min. In the case of sulphated flavonoid glycosides/uronides, the aryl-sulphatase preparation should be purified from contaminating β-glycosidase or β-glucuronidase activities, both of which can be eliminated by gel filtration.

Acknowledgements—This work was supported in part by operating grants from NSERC and the Fonds FCAR for which we are grateful. We wish to thank Luc Varin for reference samples of quercetin 3,7-disulphate and 3,7,3'-trisulphate.

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