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O-METHYLATION OF FLAVONOIDS BY CELL-FREE EXTRACTS OF CALAMONDIN ORANGE

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Key Word Index—*Citrus mitis*; Rutaceae; calamondin orange; *O*-methyltransferase; flavonoids; *ortho*, *meta*, *para* and 3-*O*-methylation.

Abstract—Cell-free extracts of calamondin orange (*Citrus mitis*) catalysed the *O*-methylation of almost all hydroxyls of a number of flavonoids, indicating the existence in citrus tissues of *ortho*, *meta*, *para* and 3-*O*-methyltransferases. The latter, hitherto unreported enzyme, catalysed the formation of 3-*O*-methyl ethers of galangin and quercetin. The stepwise *O*-methylation of a number of compounds, especially quercetin and quercetagetin, tends to suggest a coordinated sequence of *O*-methylations on the surface of a multienzyme complex. The methyl acceptor abilities of the flavonoid substrates used are discussed in relation to their hydroxyl substitution patterns and their negative electron density distribution.

INTRODUCTION

O-Methylated flavonoids are known for their widespread occurrence in the plant kingdom [1]. More than two-thirds of the naturally occurring flavonoids are partially or fully methylated with their methyl distribution frequencies of C8 > C6 > C4′ ≃ C3′ ≃ C7 > C5 for flavones [2] as compared with C6 > C8 > C3 ≃ C7 > C4′ > C3′ > C5 for flavonols [3]. When methylation is effected in the laboratory using Me_2SO_4 in alcoholic Na_2CO_3, the rate of reaction is determined by the acidity of hydroxyl groups and follows the order C7 > C4′ > C3′ > C3 [4].

In contrast with the thoroughly investigated *in vitro* *O*-methylation of ring B of flavonoids [5–10], that of ring A has received very little attention [11–14]. Recently, however, Poulton *et al.* [7] reported the efficient *O*-methylation of texasin (6,7-dihydroxy-4′-methoxyisoflavone) by a purified *O*-methyltransferase (OMT) from soybean cell culture, though the exact position of methylation remains to be determined. Furthermore, the 7-*O*-methylation of quercetin has been recently demonstrated with cell-free extracts of citrus tissues [15] and with a highly purified *para*-directing OMT from tobacco cell culture [9, 10].

Citrus tissues, especially the fruit flavedo, have yielded one of the most complete complements of metabolic intermediates involved in flavonoid biosynthesis [16] and leading to the formation of polymethylated flavones characteristic of the genus [17, 18]. Preliminary work in this laboratory [15] indicated that cell-free extracts of citrus tissues catalysed the stepwise *O*-methylation of quercetin. We report here the results of an investigation of the effect of hydroxyl substitution on the pattern and extent of *in vitro* *O*-methylation of a number of flavonoids in relation to their negative electron density (NED) distribution.

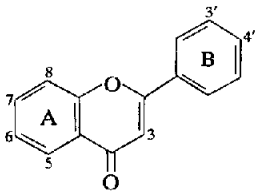
RESULTS

Buffer extracts of seedling root, fruit peel and seed callus tissues of calamondin orange were fractionated with $(NH_4)_2SO_4$ and the protein which precipitated between 30–70% saturation was dissolved in 50 mM Pi buffer (pH 7.5), desalted on Sephadex G-25 columns and was directly used for OMT assays against various flavonoid compounds. The total incorporation of radioactivity from *S*-adenosyl-L-[^{14}Me]-methionine into reaction products was expressed as % of that of quercetin (=100) when used as substrate. Most of the reaction products were identified by cochromatography with reference compounds when available, while others were tentatively identified by comparison of their R_f values with those published [19, 20]. With the solvent systems used (see Methods), it was found that *O*-methylated products have higher R_f values than their hydroxylated substrates; products with OMe groups *meta* to the side chain (6-, 8- or 3′-) run faster than those *O*-methylated at the *Para* position (7- or 4′-), while compounds with 3- or 5-OMe groups have the lowest R_fs as compared with other monomethyl derivatives.

Flavonoids with vicinal ring A hydroxyls

Cell-free extracts of citrus tissues catalysed *O*-methylation of eleven flavonoid substrates having vicinal ring A hydroxyls, albeit with different efficiencies (Table 1). Among those lacking ring B substitution, the order of total label incorporation into reaction products was 5,6,7-trihydroxyflavone (baicalein) ≫ 5,7,8-trihydroxyflavone (norwogonin) > 7,8-dihydroxyflavone > 5,6-dihydroxyflavone. Both baicalein and

Table 1. *In vitro* O-methylation of flavonoid compounds by cell-free extracts of calamondin orange*



Flavonoid compound	Source†	R_f (×100)‡	Total methylation§ Root	Total methylation§ Peel	Product identification: OMe-deriv.	Source†	R_f (×100)‡	Rel. activ.‖ (%)	Negative electron density¶
Flavonoids with vicinal ring A hydroxyls									
5,6-Dihydroxy-flavone	MF	66p	42	12	6-	S	81p	62	0.1386
					5-	S	70p	22	0.0865
					5,6-	S	87p	16	
7,8-Dihydroxy-flavone	S	50p	39	20	8-	S	78p	90	0.0369
					7-	S	56p	5	+0.0542
					7,8-	S	89p	5	
6,7-Dihydroxy-4'-methoxyisoflavone (texasin)**	Ga	65s	92	68	6-		80s	77	0.1390
					7-		76s	23	0.1066
6,7,4'-Trihydroxy-isoflavone	P&B	60s	75	30	6-		73s	80	0.0985
					6,4'-		80s	10	0.0235 (4'-)
					7,4'-		76s	10	0.0360 (7-)
5,6,7-Trihydroxy-flavone (baicalein)**	Gb	75s	120	120	6-		80s	40	0.3009
					5,6-/6,7-		92s	60	0.1409 (5-) 0.1114 (7-)
5,7,8-Trihydroxy-flavone (norwogonin)	S	85s	35	70	8-		91s	55	0.2089
					7,8-		95s	45	0.0819 (7-)
5,7,8,4'-Tetra-hydroxyflavone (8-hydroxyapigenin)	S	40p	22	40	8-		63p	60	0.2495
					4'-		49p	40	0.1519
5,7,8-Trihydroxy-4'-methoxyflavone (8-hydroxyacacetin)	S	49p	4	15	8-	S	64p	60	0.2089
					7-	S	59p	40	0.0819
5,6,7,4'-Tetra-hydroxyflavone (scutellarein)	Gc	30s	65	70	4'-		65s	65	0.1395
					6,4'-		71s	35	0.0213 (6-)
3,5,6,7,3',4'-Penta-hydroxyflavone (quercetagetin)	R,Gd	11s	12	19	3,6,7,3'-	Gc	51s	70	0.2969 (3-) 0.1086 (3'-)
					3,7,3'-		49s	30	+0.0821 (6-) +0.0032 (7-)
3,5,7,8,3',4'-Penta-hydroxyflavone (gossypetin)	Gc	11s	24	4	tri-/tetra-		51s	100	

* The standard enzyme assay was used as described in the Experimental.

† Ga-g, gifts (a, W. Hösel; b, J. E. Poulton; c, M. Jay; d, T. Mabry; e, G. Hrazdina; f, L. Jurd; g, B. A. Bohm): K&K, ICN Pharmaceuticals, Plainview, N.Y.; MF, Merck–Frosst Lab., Montreal; P&F, Pflatz & Bauer, Flushings, N.Y.; R, Roth, Karlsruhe, Germany; S, synthesis; Sa, N.A.M. Saleh.

‡ R_f values on: s, Si gel G using C_6H_6–Py–HCOOH (36:18:5 or 86:19:5); p, polyamide DC 6.6 using C_6H_6–petrol–MeCOEt–MeOH (60:26:7:7).

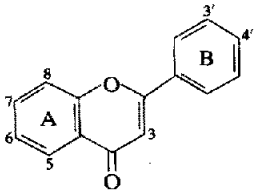
§ % of that of quercetin (=100) which amounted to (cpm/mg protein) 10 500 for root, 27 700 for callus and 14 100 for peel tissues.

‖ Determined after TLC and autoradiography of reaction products as described in the Experimental.

¶ Calculated according to the CNDO/2 computer program [24, 28].

** Total O-methylation of texasin and baicalein by calamondin callus enzyme amounted to 125 and 150% as compared with that of quercetin, respectively.

Table 1.—*Continued*



Flavonoid compound	Source†	R_f (×100)‡	Total methylation§ Root	Total methylation§ Peel	Product identification: OMe-deriv.	Source†	R_f (×100)‡	Rel. activ.‖ (%)	Negative electron density¶
Flavonoids with no vicinal ring A hydroxyls									
3,5,7-Trihydroxy-flavone (galangin)	R	70s	51		3-	Ge	76s	70	0.3273
					7-		73s	10	0.0127
					3,7-		90s	15	
5,7,4′-Trihydroxy-flavone (apigenin)	R	57s	5	10	4′-		69s	90	0.1903
					7,4′-		87s	1	+0.0349
5,7,3′,4′-Tetra-hydroxyflavone (luteolin)	R	31s	150	200	3′-	R	58s	65	0.1217
					4′-		54s	20	0.2176
					7,3′-		66s	10	+0.0242 (7-)
					7,4′-		63s	5	
3,5,7,4′-Tetra-hydroxyflavone (kaempferol)	R	55s	5–10	5–10	3-		60s	40	0.3150
					4′-	R	70s	60	0.1932
3,5,7,3′,4′-Penta-hydroxyflavone (quercetin)††	K&K	30s	100	100	3-	Gf	42s	6	0.3090
					5-	Gg	38s	10	+0.0474
					7-	R	52s	12	0.0162
					3′-	R	57s	15	0.1100
					4′-	Sa	50s	12	0.2080

†† Activities in 7-, 3′- and 4′- were calculated from their respective values in 3,7-, 7,3′- and 7,4′-derivatives (Table 2).

5,6-dihydroxyflavone were *O*-methylated mainly at position 6, norwogonin at positions 7 and 8, whereas 7,8-dihydroxyflavone was predominantly attacked at position 8. Most of these *O*-methylated products are known to occur naturally in plants ([2] and refs. cited therein).

O-Methylation of flavonoid substrates with vicinal ring A hydroxyls and ring B substitution followed the order texasin ≫ 6,7,4′-trihydroxyisoflavone > 5,6,7,4′-tetrahydroxyflavone (scutellarein) > 5,7,8,4′-tetrahydroxyflavone (8-hydroxyapigenin) > 3,5,7,8,3′,4′-pentahydroxyflavone (gossypetin) > 3,5,6,7,3′,4′-pentahydroxyflavone (quercetagetin) > 5,7,8-trihydroxy-4′-methoxyflavone (8-hydroxyacacetin) (Table 1). Both texasin and 6,7,4′-trihydroxyisoflavone were methylated, almost predominantly, at position 6 but not to the exclusion of the 7 position. Scutellarein gave the 4′-OMe and 6,4′-diOMe derivatives, indicating *meta* and *para* *O*-methylation of ring A and ring B hydroxyls, respectively. These methylated products are known to occur naturally in a number of species of the Labiatae and Scrophulariaceae ([2] and refs. cited therein). The *O*-methylation of 8-hydroxyapigenin at positions 8 and 4′ and of 8-hydroxyacacetin at positions 7 and 8 is indicative of both *meta*- and *para*-directing methylations. Quercetagetin and gossypetin were relatively poor methyl acceptors as compared with quercetin (Table 1). The former substrate underwent stepwise methylation and gave two products; the major one cochromatographed with an authentic sample of the 3,6,7,3′-tetramethyl ether, while the minor product had a slightly lower R_f suggesting a tri-OMe derivative. The former product is a natural constituent of a number of *Chrysosplenium* spp. [20–22]. *O*-Methylation of gossypetin gave a tri-/tetra-OMe product whose identity was not further investigated. It is interesting to note that, in contrast with quercetin (see below), *O*-methylation of both quercetagetin or gossypetin failed to produce any mono- or di-OMe intermediates.

Flavonoids with no vicinal ring A hydroxyls

Whereas chrysin (5,7-dihydroxyflavone) was not methylated by any of the cell-free extracts used, galangin (3,5,7-trihydroxyflavone) was a reasonably good methyl acceptor and gave three products (Table 1); the major one cochromatographed with an authentic sample of 3-OMe-galangin in a number of solvents, thus establishing for the first time the existence in citrus tissues of a 3-*O*-methyltransferase. The fact that an active enzyme preparation from tobacco cell culture catalysed the 3-*O*-methylation of both galangin and quercetin (unpublished results) supports the above finding and provides a good source for the purification of this enzyme. Furthermore, 3-OMe derivatives of galangin and quercetin are known to occur naturally in plants ([3] and refs. cited therein).

Flavones and flavonols with 4′-hydroxylation (apigenin, kaempferol) were very poor methyl acceptors and

Table 2. Stepwise *O*-methylation of quercetin and its intermediate products*

Substrate and source†	Total methylation‡	% Distribution of activity in OMe products									
		3-	5-	7-	3,7-(Gc)	3,3'-/3,4'-	7,3'-/7,4'-(Sa)	3,3',4'-	3,7,3'-/3,7,4'-(Gc)	7,3',4'-	3,7,3',4'-(Gc)
Quercetin	100	6	10	15	18	12	20		12		
3-OMe-quercetin	72				50	25					5
7-OMe-quercetin (rhamnetin)	121				12		40		22		
3'-OMe-quercetin (isorhamnetin)	82					7	12		30		
4'-OMe-quercetin (tamarixetin)	93					12	40		24		
7,4'-Di-OMe-quercetin (Sa) (ombuin)	44						18		65		5
3',4'-Di-OMe-quercetin (MF)	5							35		25	

* The standard assay described in the Experimental was used.

† Source of substrates and products (in brackets) are similar to those listed in Table 1.

‡ % of that of quercetin (=100).

were *O*-methylated mostly at the 4'-position (Table 1). Their 3',4'-dihydroxy analogs (luteolin, quercetin), on the other hand, were readily methylated and gave rise to a number of OMe intermediates. Luteolin was *O*-methylated mainly at the 3'-position with smaller amounts of 4'-OMe and 7,3'- (or 7,4'-) di-OMe ethers formed, all of which are known as natural products [3]. *O*-Methylation of quercetin involved almost all hydroxyls on the flavonoid ring system. The availability of a number of reference compounds made it possible to confirm the presence of 3-, 5-, 7-, 3'-monomethyl 3,7-, 7,3'-dimethyl and 3,7,3'-trimethyl ethers among the reaction products (Tables 1 and 2). The two latter compounds were inseparable on TLC from their 7,4'- and 3,7,4'-isomers, respectively, as were the 7- and 4'-OMe derivatives, rhamnetin and tamarixetin, respectively. Another product was tentatively identified as 3,3'-di-OMe-quercetin after comparison of its R_fs with those published [19]. It should be noted that further *O*-methylation of mono-OMe intermediates into di- or tri-OMe products (see below) may account for the low rates of methylation as compared with their NED values (Table 1).

When the partially methylated intermediates of quercetin were used as substrates, they were further *O*-methylated to a higher order of methyl substitution (Table 2). Their order of efficiency as methyl acceptors was 7->4'->3'->3->7,4'->3',4'-OMe quercetin; indicating that monomethyl ethers were better acceptors than the dimethyl ethers. It is interesting that further *O*-methylation of quercetin intermediate products favored the positions with high nucleophilicity and NED of the flavonoid ring system (Tables 1 and 2). These results clearly demonstrate the stepwise *O*-methylation of quercetin into products most of which are known to occur naturally in plants [3].

DISCUSSION

Cell-free extracts of calamondin orange tissues catalysed the *in vitro* *O*-methylation of ring A hydroxyls of a number of flavonoids, but not to the exclusion of those of ring B. They also catalysed the 3-*O*-methylation of both galangin and quercetin, thus indicating the existence in calamondin tissues of at least three OMTs which attack the *meta* (6-, 8-, 3'-), *para* (7-, 4'-) and 3-positions of the flavonoid ring system. The latter, hitherto unreported, enzyme is of particular interest since it methylated the 3-OH group that is known for its fairly high acidity [4, 23] and NED [24]. We have also demonstrated the 5-*O*-methylation of both 5,6-dihydroxyflavone and quercetin, which seems to be catalysed by an *ortho*-directing OMT similar to that reported for the *O*-methylation of linear furanocoumarins in *Ruta graveolens* [25]. However, it is not clear whether the *meta*- and *para*-directing OMTs of calamondin are specific for either, or common to both rings A and B of flavonoids. This must await the purification of both enzymes from a suitable source and the study of their position specificity using appropriate substrates and products. Very recently, we presented evidence for the existence of two distinct enzymes which catalysed the *meta* *O*-methylation of caffeic acid and the 7-*O*-methylation of quercetin [9, 10]. Unfortunately, calamondin tissues were not suitable for the purification of both enzymes and attempts to use affinity chromatography ligands were unsuccessful (Matern, U., personal communication).

The stepwise *O*-methylation of a number of flavonoids to their di-, tri-, or tetra-OMe derivatives is remarkable and suggests a coordinated sequence of *O*-methylation steps that may occur on the surface of a multi-OMt complex [26, 27]. Depending on the pool

size of substrates and intermediate products, the latter may either accumulate (as was shown with quercetin) or may be transformed directly to a higher order of methyl substitution (as with quercetagetin and gossypetin). Whereas this model may explain the biosynthesis of polymethylated flavones known to occur in *Citrus* [17, 18] and *Chrysosplenium* [20–22], it does not exclude the possibility of the existence of a single enzyme with broad specificity or one with different active sites.

Whereas no precise order of *in vitro* O-methylation could be established due to the limited number of flavonoids available and the frequent occurrence of stepwise methylation, there appears, however, some interesting relationship between the hydroxyl substitution pattern of the substrates tested, their methyl acceptor ability and the NED distribution (Table 1) as determined by the complete neglect of differential overlap (CNDO/2) method [28]. Flavonoids with vicinal hydroxyl groups at positions 5-, 6-, 7- or 8- and lacking ring B substitution exhibited the highest O-methylation and NED for positions 6 and 8. The overall pattern appeared to follow the order $6 \simeq 8 > 7 > 5$. Substitution of ring B seems to affect the nucleophilicity of ring A hydroxyls and hence their reactivity towards O-methylation. Hydroxylation at 4'-position may result in a *para* quinoid, rigid structure and concomitant resonance of the flavonoid ring system that may limit O-methylation as in apigenin and kaempferol (Table 1), except where vicinal hydroxyl groups exist at positions 6 or 8. Hydroxylation at 3',4'- as in luteolin or quercetin, on the other hand, results in an *ortho* quinoid structure which seems to enhance O-methylation at both positions. Furthermore, 3-hydroxylation as in galangin and quercetin appears to have a stabilizing effect on the flavonoid ring system [23] in that it decreases the high positive charges of positions 2 and 4 [29] thus enhancing the reactivity of both ring A and ring B hydroxyls. These observations compare well with the rates of O-methylation and NED distribution of most flavonoids tested (Table 1). However, the poor rate of O-methylation of the hexahydroxyflavonoids, quercetagetin and gossypetin, is difficult to explain. The lack of formation of mono- and di-OMe derivatives among the reaction products makes it hard to decide whether the correlation observed with other flavonoids exists in this case. Factors such as substrate solubility, K_m values, product inhibition or competition among different OMTs for either the substrate or its O-methylated intermediates may affect the rate of O-methylation of flavonoids with such a high order of hydroxylation.

EXPERIMENTAL

Flavonoids. Most substrates were available from commercial sources, the remaining compounds were either generous gifts from various laboratories (mentioned in Table 1) or were synthesized. 7,8-Dihydroxyflavone was synthesized by the method of Baker [30] as modified by ref. [31]. Norwogonin and 8-hydroxyacacetin were prepared by persulphate oxidation of chrysin [32] and acacetin [33], respectively. 8-Hydroxyapigenin was obtained by demethylation of 8-hydroxyacacetin. Synthetic compounds were recrystallized from appropriate solvents and their mps were compared with those published. Partial O-methylation of flavonoids was carried out using diazomethane in dry Py [34]. The purity and identity of the methylated products were verified by the standard chromatographic and spectral techniques [35] and by comparison of their R_fs with those published [21, 35].

Plant material. Calamondin orange (*Citrus mitis*) plants were obtained from a commercial source and their growth was maintained under greenhouse conditions. The tissues used for enzyme extraction were (a) roots from 4–6-week-old seedlings, (b) immature fruit peel, and (c) callus tissue that was initiated from calamondin seed, and its growth was maintained on MS-medium [36] supplemented with 3% sucrose and 0.5 ppm 2,4-D.

Preparation of cell-free extracts. Fresh tissue was homogenized with 3 vols. of 0.2 M Pi buffer (pH 7.5) containing 5 mM EDTA and 0.1% (w/w) Polyclar AT then filtered. The filtrate was centrifuged at 20 000g for 20 min. The supernatant was fractionated with solid $(NH_4)_2SO_4$ while maintaining the pH at 7.5 and the protein which precipitated between 30 and 70% satn was collected by centrifugation. The latter was suspended in the minimal amount of 50 mM Pi buffer (pH 7.5) and desalted on Sephadex G-25 using the same buffer. The eluate was used directly for OMT assays and its protein content was determined by the Lowry method [37].

OMT assay and product identification. The standard assay mixture consisted of 50–100 nmol substrate (in 10 μl DMSO), 7 nmol S-adenosyl-L-^{14}Me-methionine (containing 0.02 μCi), 1.4 μmol β-mercaptoethanol and 50–100 μg enzyme protein (in Pi buffer, pH 7.5) in a total vol. of 150 μl. The reaction proceeded for 30 min at 35° and was stopped by the addition of 20 μl of 6 N HCl. The methylated products were extracted with 2×0.7 ml of C_6H_6–EtOAc (1:1) and measured aliquots (200 μl) of the organic layer were used for determination of total activity by liquid scintillation counting. The remaining fraction was chromatographed together with reference compounds on Si gel G (Eastman Kodak) using C_6H_6–Py–HCOOH (36:18:5 or 86:19:5) or polyamide DC6.6 (Macherey Nagel) using C_6H_6–petrol–MeCOEt–MeOH (60:26:7:7). Both solvents were also used for 2D TLC. When reference compounds were not available, other solvent systems were used for comparison of their R_fs with those published [19–21]. Reaction products were located on TLC plates by autoradiography; individual compounds were scraped off and mixed with Cab-O-Sil for liquid scintillation counting.

CNDO/2 program. Structures of flavonoids were generated using the Merck Molecular Modeling System [24] and the standard bond lengths and angles. The normal CNDO/2 parameters program [28] was used for calculation of electron densities at the different positions of the flavonoid ring system.

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