Polymethylated Flavonols of *Chrysosplenium americanum* I. Identification and Enzymatic Synthesis

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Polymethylated Flavonols, Identification, Enzymatic Synthesis, O-Methyltransferase, *Chrysosplenium americanum*, Saxifragaceae

The major flavonoid constituents of *Chrysosplenium americanum* (Saxifragaceae) were identified by UV-, NMR- and mass spectroscopy and the analysis of their hydrolytic products. They consisted of two 2′-*O*-β-D-glucosides of partially methylated 2′-hydroxyquercetin and four 5′-*O*-β-D-glucosides of partially methylated 6-hydroxyquercetin (quercetagetin).

The O-methyltransferase system of this tissue can catalyse the stepwise O-methylation of quercetin, but not quercetagetin, to its 3,7,4′-triOMe-dervative. It is postulated that the latter compound may serve as a branching-point intermediate in the biosynthesis of the naturally occurring O-methylated flavonols of this tissue.

Introduction

O-Methylated flavonoids are known for their widespread occurrence in the plant kingdom [1]. Those of the genus *Chrysosplenium* (Saxifragaceae) are characterised by a high degree of O-methylation and the occurrence, in several species, of additional hydroxyl and/or methoxyl substituents at positions 6 and/or 2′ [2–4]. Recently, a hepta-OMe-flavone with 2′-substitution has been reported from the Leguminosae [5]. *C. americanum* accumulates an interesting variety of tri-, tetra- and penta-OMe-flavonols which are derived from quercetin and includes 6-OH-quercetin (quercetagetin), 2′-OH-quercetin and 2′,6-diOH-quercetin (2′-OH-quercetagetin).

Our current interest in the O-methylation of flavonoid compounds [6–11] prompted us to study the flavonoid pattern of *C. americanum* in view of using this tissue as source of the O-methyltransferases, as well as other enzymes, involved in the biosynthesis of polymethylated flavonoids.

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Experimental

Plant material

Chrysosplenium americanum Schwein. ex Hooker was collected with its soil from Gatineau National Park or Sutton Junction, Québec and was maintained in the greenhouse under conditions simulating its natural habitat.

Isolation and identification of flavonoids

Extraction: Air-dried shoots were blended in 80% MeOH at 20 °C, filtered and re-extracted twice with the same solvent and three times with absolute MeOH at 20 °C. The combined extracts were evaporated to a syrup at 30 °C, dispersed in 80% MeOH and partitioned against several changes of hexanes to remove lipophilic constituents. The lower phase containing the flavonoids was evaporated to a syrup and taken up in 50% MeOH; to which ethyl acetate was added and the mixture saturated with solid NaCl. The aqueous methanol phase was washed with two changes of ethylacetate and the combined extracts were evaporated to dryness, taken up in a small volume of 50% *i*-PrOH and used for subsequent analyses.

Purification: Flavonoids were purified by repeated chromatography on preparative polyamide DC

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6.6 TLC plates using the following solvent systems: 1. water-2-butanone (85:15); 2. water-*n*-butanol-acetone-dioxane (70:15:10:5); 3. benzene-2-butanone-methanol-water (55:23:20:2); 4. toluene-ethyl formate-ethanol-water (60:20:19:1); 5. toluene-ethyl acetate-ethanol (50:25:25) and 6. toluene-ethyl formate-*n*-butyl acetate-formic acid (25:50:23:2). The flavonol glycosides were purified using solvents 1–4, whereas the aglycones were separated primarily with solvents 4–6. Detection of flavonoids was carried out using UV light (366 nm) and a 0.5% solution of diphenylborinic acid-ethanolamine complex in 75% *i*-PrOH.

Identification: Purified flavonoids were identified by UV, NMR and mass spectroscopy and analysis of their hydrolytic products. Partial and complete acid hydrolysis of glucosides and product identification was carried out as previously described [3]. Instrumental methods (UV and NMR), described by Mabry *et al.* [12], were employed. Mass spectra were obtained using a direct inlet solids probe at 70 eV ionization potential and 100 μA trap current. Under these conditions, the MS were those of the aglycones, free of interfering sugar fragments ions, since the source temperature (ca. 300 °C) was sufficient to cleave the glycosides between the sugar and the aglycone portion.

Flavonoid A: 5,2′,5′-trihydroxy-3,7,4′-trimethoxyflavone-2′-*O*-*β*-D-glucoside: pale yellow needles from MeOH-water, mp 272–275 °C (uncorrected). NMR per TMS derivative, CCl_4, δ(ppm): 6.81 s (H-3′); 7.02 s (H-6′); 6.35 d, $J_a = 2.5$ Hz (H-6) 6.40 d, $J_a =$ 2.5 Hz (H-8); 4.85 d, $J_a = 7.5$ Hz (H-1″*β*-D-glucopyranosyl); 3.95 s, 3.84 s, 3.71 s ($3 \times OCH_3$); ~ 3.8–3.2 m ($6 \times$ H, glucosyl protons). Total acid hydrolysis: D-glucose (TLC) and aglycone identical to flavonoid G (TLC, UV). MS as aglycone; $C_{18}H_{16}O_8$, MW 360 *m/e*: 360 (100%) M⁺; 359 (18%) (M-1)⁺; 344 (13%) (M-16)⁺; 343 (33%) (M-17)⁺; 331 (10%) (M-29)⁺; 330 (37%) (M-30)⁺; 329 (71%) (M-31)⁺.

Flavonoid B: 5,2′-dihydroxy-3,7,4′,5′-tetramethoxyflavone-2′-*O*-*β*-D-glucoside: amorphous yellowish granules from MeOH-water; NMR per TMS derivative, CCl_4, δ(ppm): 6.80 s (H-3′); 6.96 s (H-6′); 6.36 d, $J_a = 2.5$ Hz (H-6); 6.39 d, $J_a = 2.5$ Hz (H-8); 4.91 d, $J_a = 7.5$ Hz (H-1″*β*-D-glucopyranosyl); 3.93 s, 3.90 s, 3.85 s, 3.70 s ($4 \times OCH_3$); ~ 3.8–3.3 m ($6 \times$ H, glucosyl protons). Total acid hydrolysis: D-glucose (TLC) and aglycone identical to flavonoid H. MS, as aglycone; $C_{19}H_{18}O_8$, MW 374, *m/e*: 374 (100%) M⁺; 373 (1%) (M-1)⁺; 360 (20%) (M-14)⁺; 359 (40%) (M-15)⁺; 357 (19%) (M-17)⁺; 345 (10%) (M-29)⁺; 344 (32%) (M-30)⁺; 343 (70%) (M-31)⁺; 331 (12%) (M-43)⁺.

Flavonoid C: 5,6,5′-trihydroxy-3,7,4′-trimethoxyflavone-5′-*O*-*β*-D-glucoside: amorphous yellow granules from MeOH-water; NMR, per TMS derivative, CCl_4, δ(ppm): 7.78 dd, $J_a = 8.5$, $J_b = 2.5$ Hz (H-2′); 7.75 d, $J_b = 2.5$ Hz (H-6′); 6.81 d, $J_a = 8.5$ Hz (H-3′); 6.40 s (H-8); 4.81 d, $J_a = 7.5$ Hz (H-1″*β*-D-glucopyranosyl); 3.92 s, 3.89 s, 3.85 s ($3 \times OCH_3$); ~ 3.8–3.1 m ($6 \times$ H glucosyl protons). Total acid hydrolysis: D-glucose (TLC) and aglycone identical to flavonoid I (TLC, UV). MS, as aglycone $C_{18}H_{16}O_8$, MW 360, *m/e*: 361 (35%) (M-1)⁺; 360 (100%) M⁺; 359 (28%) (M-1)⁺; 346 (20%) (M-14)⁺; 345 (50%) (M-15)⁺; 342 (9%) (M-18)⁺; 341 (15%) (M-19)⁺; 331 (8%) (M-29)⁺.

Flavonoid D: 5,5′-dihydroxy-3,6,7,4′-tetramethoxyflavone-5′-*O*-*β*-D-glucoside: pale yellow needles from MeOH-water; mp 179–183 °C (uncorrected). NMR, per TMS derivative CCl_4, δ(ppm): 7.72 d, $J_b =$ 2.5 Hz (H-6′); 6.83 d, $J_a = 8.5$ Hz (H-3′); 7.74 dd, $J_a = 8.5$, $J_b = 2.5$ Hz (H-2′); 6.42 s (H-8); 4.86 d, $J_a = 7.5$ Hz (H-1″*β*-D-glucopyranosyl); 3.95 s, 3.88 s, 3.82 s ($4 \times OCH_3$); 3.75–3.2 m ($6 \times$ H, glucosyl protons). Total acid hydrolysis: D-glucose (TLC) and aglycone identical to flavonoid *J* (TLC, UV). MS, as aglycone, $C_{19}H_{18}O_8$, MW 374, *m/e*: 375 (29%) (M+1)⁺; 374 (100%) M⁺; 373 (33%) (M-1)⁺; 360 (16%) (M-14)⁺; 359 (48%) (M-15)⁺; 356 (10%) (M-18)⁺; 355 (18%) (M-19)⁺; 345 (8%) (M-29)⁺.

Flavonoid E: 5,2′,5′-trihydroxy-3,6,7,4′-tetramethoxyflavone-5′-*O*-*β*-D-glucoside: amorphous pale yellow granules from MeOH-water; NMR per TMS derivative, CCl_4, δ(ppm); 6.81 s (H-6′); 6.79 s (H-3′); 6.39 s (H-8); 4.88 d, $J_a = 7.0$ (H-1″*β*-D-glucopyranosyl); 3.92 s, 3.83 s, 3.82 s, 3.57 s ($4 \times OCH_3$); ~ 3.8–3.2 m ($6 \times$ H glucosyl protons). Total acid hydrolysis: D-glucose (TLC) and aglycone identical to flavonoid K (TLC, UV). MS, as aglycone, $C_{19}H_{18}O_9$, MW 390, *m/e*: 391 (45%) (M+1)⁺; 390 (100%) M⁺; 389 (19%) (M-1)⁺; 376 (15%) (M-14)⁺; 375 (52%) (M-15)⁺; 373 (32%) (M-17)⁺; 361 (10%) (M-29)⁺; 360 (38%) (M-30)⁺; 359 (73%) (M-31)⁺; 357 (15%) (M-33)⁺.

Flavonoid F: 5,5′-dihydroxy-3,6,7,2′,4′-pentamethoxyflavone-5′-*O*-*β*-D-glucoside: white needles from MeOH-water; mp. 153–155 °C (uncorrected). NMR, per TMS derivative, CCl_4, δ(ppm): 6.83 s (H-6′); 6.80 s (H-3′); 6.37 s (H-8); 4.85 d, $J_a = 7.0$ Hz (H-1″

β-D-glucopyranosyl); 3.91 s, 3.86–3.84 m, 3.58 (5 × OCH_3); ~ 3.7–3.2 m (6 × H glucosyl protons). Total acid hydrolysis: D-glucose (TLC) and aglycone identical to flavonoid L (TLC, UV). MS, as aglycone, $C_{20}H_{20}O_9$, MW 404, *m/e*: 405 (55%) $(M+1)^+$; 404 (100%) M^+; 403 (21%) $(M-1)^+$; 390 (26%) $(M-14)^+$; 389 (56%) $(M-15)^+$; 387 (18%) $(M-17)^+$; 375 (10) $(M-29)^+$; 374 (32%) $(M-30)^+$; 373 (65%) $(M-31)^+$; 371 (10%) $(M-33)^+$.

Protein extraction and O-methyltransferase assay

Shoot tips were frozen in liquid N_2, ground to fine powder and homogenised with 0.1 M phosphate buffer, pH 7.5 (1:4, w/v) containing 14 mM β-mercaptoethanol, 5 mM EDTA, 10 mM diethylammonium diethyldithiocarbamate and Polyclar AT (2%, w/v). The homogenate was filtered through nylon mesh and the filtrate was centrifuged at 15000 × *g* for 15 min. The supernatant was stirred for 20 min with Dowex 1X2 (2%, w/v), which had previously been equilibrated with the same buffer, then filtered. The filtrate was fractionated with solid $(NH_4)_2SO_4$ and the protein fraction which precipitated between 40–70% salt concentration was collected by centrifugation and desalted on Sephadex G-25 column using 50 mM phosphate buffer, pH 7.5, containing 5 mM, EDTA and 10% glycerol. The standard enzyme assay was the same as that described previously [9], using S-adenosyl-L-[^{14}C-methyl]-methionine as the methyl donor (sp. act. 57.6 mCi/mmol; New England Nuclear, Boston MA.). The O-methylated products from several enzyme assays were pooled and chromatographed together with reference compounds on Polyamid-6 TLC plates (Machery & Nagel, Darmstadt, Ger.), using solvent 6, then autoradiographed.

Results

Identification of flavonoids

A representative two-dimensional TLC separation of the flavonoids identified in *C. americanum* is illustrated in Fig. 1. The major constituents consisted of the monoglucosides (A–F) of each of six variously methylated flavonols, together with small amounts of their free aglycones (G–L), respectively. In addition, trace amounts of the 3-*O*-arabinosides, 3-*O*-monoglucosides, 3-*O*-rutinosides and 3-*O*-diglucosides of both kaempferol (M–P, respectively) and quercetin (Q–T, respectively) were detected along with the free aglycones (kaempferol, U and quercetin, V).

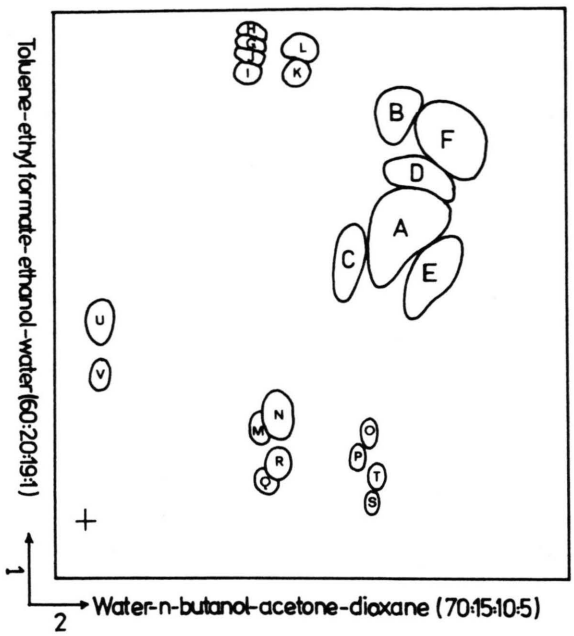
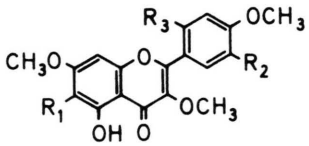


Fig. 1. Two-dimensional TLC of *C. americanum* flavonoids (see Fig. 3. for structrues of major compounds).

The predominant glucosides included two 2′-*O*-β-D-glucosides (A and B) of partially-methylated 2′-hydroxyquercetin, and four 5′-*O*-β-D-glucosides (C–F) of partially-methylated 6-OH-quercetin (quercetagetin). The structures and UV spectral properties of these compounds are given in Table I. From a UV-spectrum diagnostic standpoint, it was found that all the flavonoids with 2′-substitution (A, B, E, F, G, H, K & L) characeristically exhibited a much greater absorptivity at the short wavelength maximum (Bd II) in MeOH than at the long wavelength maximum (Bd I). Ratios of absorptivity (Bd II/I) were always greater then 1.5 for these flavonols, regardless of the presence or absence of substitution at the 6-position. In contrast, 6-*O*-substituted derivatives, lacking additional substitution at 2′ (C, D, I & J) exhibited relatively more intense absorptivity at the long wavelength maximum and the absorptivity ratio Bd II/I were all less than 1.0. Furthermore, the low acidity of the phenolic hydroxyl group at the 2′ position is evident from the spectra of compounds E, G, H and K which exhibited no bathochromic shift of Bd I in

Table I. Spectral properties of O-methylated flavonols from *C. americanum*



Flavonoid	Structure	MeOH	Band II / Band I	+NaOMe	+$AlCl_3$	+$AlCl_3$/HCl	+NaOAc	+NaOAc/H_3BO_3
A	R_1 = H; R_2 = OH; R_3 = *O*-*β*-D-glu	230[s] 254 299 340	2.67	269 372	270 316 392	270 314 388	256 298[s] 340	255 298[s] 340
B	R_1 = H; R_2 = OCH_3 R_3 = *O* -*β*-D-glu	230 252 299 338	2.60	270 320[s] 366	270 312 390	270 310 387	252 300 338	238[s] 252 299 338
C	R_1 = OH; R_3 = H; R_2 = *O*-*β*-D-glu	234[s] 254[s] 280 346	0.61	290 386	236[s] 268 292 376	236 267 290 372	288 348 386[s]	280 350
D	R_1 = OCH_3; R_3 =H; R_2 = *O*-*β*-D-glu	234[s] 252 272 342	0.89	290 328[s] 386	234[s] 260 284 360 399[s]	234[s] 258 285 362 400[s]	254[s] 272 343	252[s] 272 342
E	R_1=OCH_3; R_3=OH R_2= *O*-*β*-D-glu	230[s] 256 299 334	2.25	269 375	272 326 375	272 325 370	256 302 335	256 304[s] 334
F	R_1 = R_3 = OCH_3; R_2 = *O*-*β*-D-glu	240[s] 254 302[s], 331	1.97	274 378	271 321 370	271 320 366	254 303[s] 332	254 303[s] 330
G	R_1 = H; R_2 = R_3 = OH	228[s] 257 291, 349	1.74	268 380	268 318 394	268 318 392	258 299[s] 352	258 299[s] 350
H	R_1 = H; R_2 = OCH_3; R_3 = OH	230[s] 256 296, 340	2.06	268 370	268 318 388	268 314 384	256 298 340	256 298 340
I	R_1 = R_2 = OH; R_3 = H	234[s] 254[s] 280 348	0.82	290 398	236[s] 268 292 376	236 268 290 372	288 348 388[s]	280 350
J	R_1 = OCH_3; R_2 = OH; R_3 = H	234[s] 256 270 347	0.95	272 390	268 280[s] 378	268 280 376 398	262 270 340	256 270 347
K	R_1 = OCH_3; R_2 = R_3 = OH	228[s] 258 300 344	1.94	270 388	272 328 380	272 326 378	258 302[s] 345	258 302[s] 344
L	R_1 = R_3 = OCH_3; R_2 = OH	230[s] 257 300 338	2.12	270 374	272 322 374	272 322 372	258 302[s] 337	258 302[s] 337

[s] Shoulder or inflection.

the presence of the weak alkali NaOAc. These two spectral features were found helpful in the initial characterisation of polymethylated flavonols in this plant.

Enzymatic synthesis of Chrysosplenium flavonoids

Whereas most of the reaction products were identified by cochromatography with reference compounds, tentative identification of other products was based on comparison of their R_f values with those reported in the literature [3, 4, 6], as well as their polarity in the solvent systems used which reflected the number of hydroxyl and methoxyl substituents present.

In the absence of added substrate the control enzyme assays resulted in trace amounts of two O-methylated products with R_f values indicative of mono- and di-O-methylated flavonoids and visible amount of a penta-OMe-product (Fig. 2). This indicates that the enzyme preparation contained trace amounts of some endogenous substrates which were not removed during partial purification.

Quercetin was readily accepted as substrate (see later, Table II) and was mainly transformed to its 3-OMe-derivative with trace amounts of 3,7-diOMe- and 3,6,7-triOMe-derivatives (Fig. 2). Quercetagetin, on the other hand, was a very poor methyl acceptor (ca. 6% of that of quercetin); nonetheless, it was converted to small amounts of the 3-OMe- and 3,7-diOMe-derivatives. 3-OMe-quercetin was further methylated to a small amount of the 3,7-diOMe-derivative and major product tentatively identified as 3,6,7-triOMe-quercetagetin, suggesting hydroxylation of the substrate or one of its OMe-intermediates at the 6 position and subsequent methylation of the latter (Fig. 2).

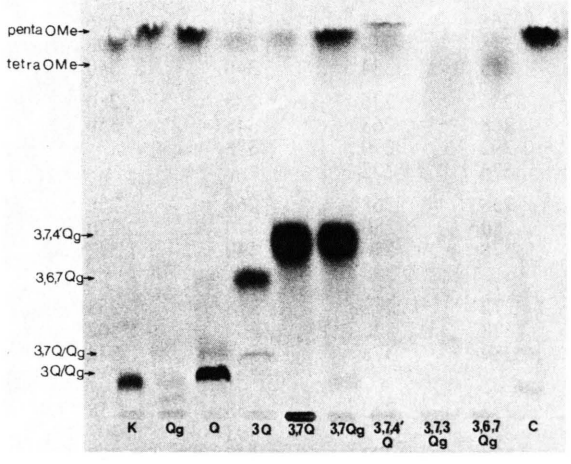


Fig. 2. Autoradiograph of the O-methylation products of various substrates used (see text for experimental details): C, control (no substrate added); K, kaempferol; Q, quercetin; Qg, quercetagetin. Numbers refer to positions of OMe-groups on the flavonol ring.

Table II. O-Methylation of various flavonoid substrates by *Chrysosplenium* O-methyltransferase system [a]

Substrate	Relative activity [b] (% of control)
Quercetin	100
3,7-DiOMe-quercetagetin	80
3,7-DiOMe-quercetin	68
Dihydroquercetin	45
Kaempferol [c]	43
3,6,7-TriOMe-quercetagetin	15
3-OMe-quercetin	12
3,7,3'-TriOMe-quercetagetin	10
3,7,4'-TriOMe-quercetin	9
Quercetagetin	6
Luteolin	3
Caffeic acid	3

[a] The standard enzyme assay was used as described in the Methods section.
[b] Total O-methylating activity of quercetin was taken as control (= 100%) and amounted to 15000 dpm/mg protein/min of reaction. Values were corrected for blanks with no substrate.
[c] Most of activity appeared in 3-OMe-quercetin.

Both 3,7-diOMe-quercetin and 3,7-diOMe-quercetagetin gave rise to the same product, namely 3,7,4'-triOMe-quercetagetin indicating methylation of both substrates at the 4' position. Hydroxylation of the former substrate at position 6 may have occured prior to or after its O-methylation at the 4' position.

When either 3,7,4'-triOMe-quercetin, 3,7,3'- or 3,6,7-triOMe-quercetagetin was supplied as substrate, they were transformed into trace amounts of their tetraOMe-derivatives (Fig. 2) which remain to be identified.

Substrate specificity

Several flavonoid substrates were tested for their methyl acceptor ability. The results (Table II) indicate that, unlike quercetagetin, quercetin was the best substrate used and may be considered the direct precursor for the polymethylated flavonols of this tissue. However, the 3,7-diOMe-derivatives of both quercetin and quercetagetin were readily

methylated as compared with their triOMe-derivatives. The poor methyl acceptor ability of the latter may be due to their slow turn-over as intermediates, or their accumulation as end products. Furthermore, neither caffeic acid, luteolin or its 7-O-glucoside were accepted as substrates indicating the specificity of this enzyme system towards flavonols. Kaempferol, however, gave a small amount of the 3-OMe-derivative but the major product was identified as 3-OMe-quercetin; suggesting hydroxylation of the substrate or its 3-OMe-derivative at the 5′ position (Fig. 2).

Discussion

The major flavonoid constituents of *C. americanum* were two 2′-*O*-*β*-D-glucosides of partially methylated quercetin and four 5′-*O*-*β*-D-glucosides of partially methylated quercetagetin. Whereas the O-methylation pattern of the latter compounds appears similar to that of other members of the Oppositifolia, it differs from that of *C. tetrandrum* in the absence of mono- and diOMe derivatives as well as the 2′ substitution [3, 4 & refs. cited therein]. 2′-Hydroxylation turns out to be of limited occurrence [1, 5, 13] and may result from the electrophilic attack at the 2′-position that is favored by H-bonding between the 5-OH and 4′-OMe [14, 15].

The results of enzymatic synthesis clearly indicate that the O-methyltransferase system of this tissue can catalyse the methylation of a number of hydroxyl groups on the flavonol ring system. This enzyme system is specific for flavonols since neither caffeic acid nor luteolin were accepted as substrates. Unlike quercetagetin, quercetin was a very good methyl acceptor and it seems reasonable to suggest that the latter is stepwise O-methylated to its 3,7,4′-triOMe-derivative which may serve as a branching

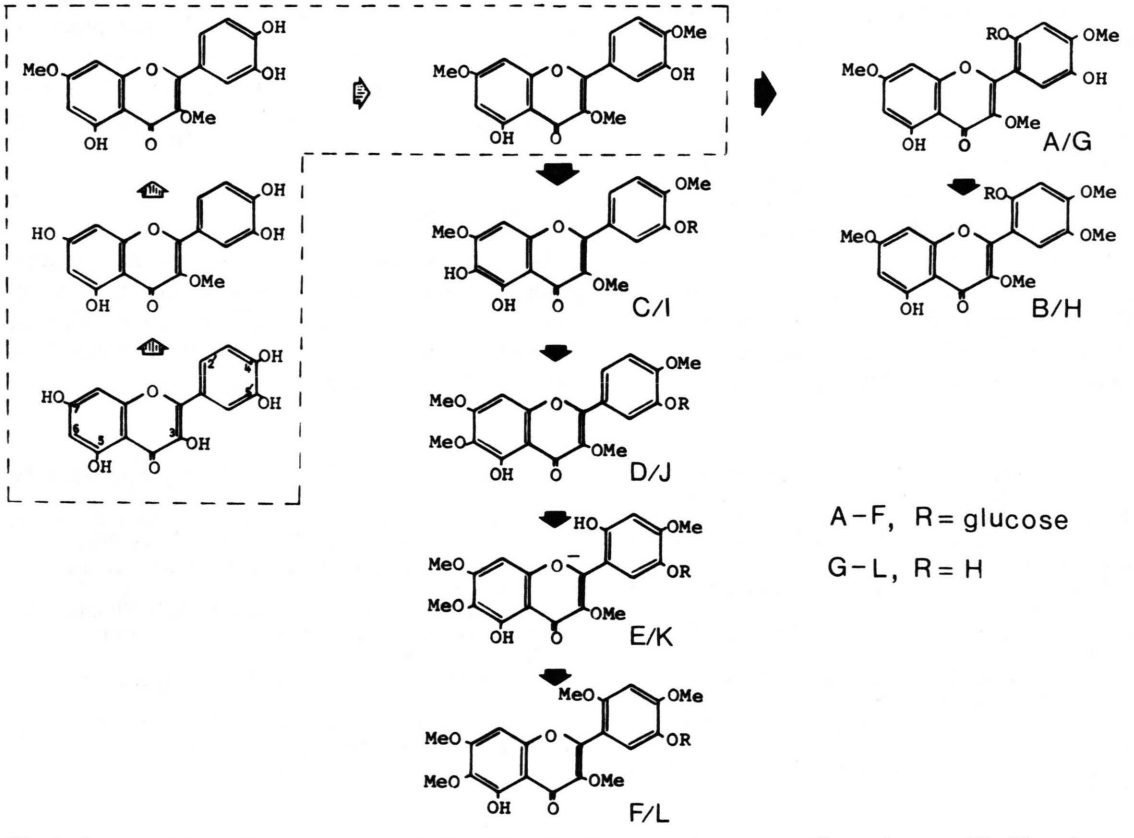


Fig. 3. Structural formulae of the flavonol glucosides (A–F) and their corresponding aglycones (G–L) and a proposed pathway for their enzymic synthesis in *C. americanum* (compounds in dotted box are intermediates with rapid turn over).

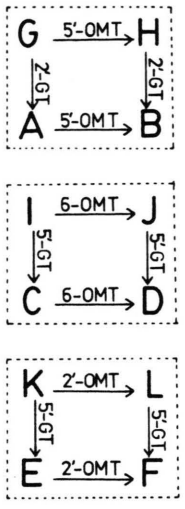


Fig. 4. Three "grids" showing the proposed methylation and glucosylation of the structurally related pairs of *C. americanum* flavonols.

point intermediate in the formation of *C. americanum* flavonols (Fig. 3). Further hydroxylation of this intermediate at the 2′ position would give rise to the aglycones G and H and their glucosides A and B. On the hand, hydroxylation at the 6-position would result in the formation of the aglycones I–L and their corresponding glucosides C–F (Fig. 3). 3-OMe-, 3,7-diOMe- and 3,7,4′-triOMe-quercetin would be considered as intermediates with rapid turn over rates and therefore do not accumulate in the intact tissue. These successive O-methylations seem to be catalysed by a number of highly coordinated O-methyltransferases; since they can accept partially methylated intermediates for further methylation (Fig. 2). This system is similar to that of Calamondin orange [6, 9] except that the latter can methylate quercetin its 3-, 7- and 3′-OMe-derivatives as well as the di-, tri- and tetraOMe-quercetin.

It is not clear which of the O-methylated intermediates of quercetin (Fig. 3) is further hydroxylated at the 6- and/or 2′ positions for the biosynthesis of the polymethylated flavonols I–L. This must await the isolation and purification of these hydroxylases and the study of their substrate specificity.

Furthermore, it is not known at this point whether methylation of flavonols proceeds through the aglycones or their respective glucosides. It appears that the six naturally occurring flavonol glucosides of this tissue fall into three "grids" where each pair of structurally related compounds may be O-methylated along the horizontal direction or O-glucosylated along the vertical one (Fig. 4). The O-methyltransferases which catalyse these reactions seem to be specific to positions 6, 2′ and 5′ due to the inherent differences in their acidity and their nuclear electron density [9]. Likewise, the two glucosylation reactions involving the 2′ and 5′ hydroxyl groups may be catalysed by two distinct glucosyltransferases following the same reasoning given above. Purification of the O-methyltransferases of this tissue is in progress and will be reported elsewhere.

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