Enzymology of Flavonoid Methylation:
Purification and Kinetics of a
Number of Novel O-Methyltransferases

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ABSTRACT

Enzymology Of Flavonoid Methylation: Purification And Kinetics Of a Number Of Novel O-Methyltransferases

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Four novel flavonol-0-methyltransferases (3-, 6-, 7- and 4'-OMTs) from Chrysosplenium americanum and an 8-OMT from Lotus corniculatus were partially purified by precipitation with ammonium sulphate and successive chromatography on gel filtration column, hydroxyapatite, S-adenosyl-L-homocysteine (SAH)-Agarose and chromatofocusing on Polybuffer ion exchanger. They exhibited strict position specificity for positions 3 of quercetin; 7 of 3-methylquercetin; 4' of 3,7-dimethylquercetin and 6 of 3,7,3'-trimethylquercetin and for the 8-OMT, position 8 of 8-hydroxykaempferol. None of these enzymes reacted with phenylpropanoids, flavones, dihydroflavonols or any of their glucosides.

Except for the 7-OMT whose activity was lost during chromatofocusing, the other enzymes had apparent pI values of 4.8, 5.4, 5.77 and 5.8 for 3-, 4', 6- and 8-OMTs respectively. They had similar molecular weights (M= 57,000) and their pH optima varied between 7.5 and 8.8. Unlike the 3- and 4'- enzymes, the 6- and 8-OMTs showed an absolute requirement for Mg²⁺ whose activation was saturable and was inhibited by EDTA.
The steady state kinetic behavior of the 3-, 4', 6- and 8-OMTs suggested that all enzymes studied proceeded by a sequential ordered binding mechanism, whereby both flavonol substrate and the methyl donor S-adenosyl-L-methionine (SAM), had to bind to the enzyme prior to any product release. The three enzymes from Chrysosplenium had SAM and SAH as leading reaction partners and included an abortive EQB complex. The Lotus 8-OMT might also have SAM and SAH as leading reaction partners with an isomerisation of free enzyme or alternatively it could bind kaempferol before SAM, followed by the release of SAH and 8 methoxykaempferol.

The in vitro stepwise 0-methylation of quercetin

3-methylquercetin $\rightarrow$ 3,7-dimethylquercetin $\rightarrow$ 3,7,4'-trimethylquercetin, tends to suggest a coordinated sequence of methyl transfers in Chrysosplenium. Furthermore, these reactions could be controlled by the respective $K_m\text{SAM}/K_i\text{SAH}$ ratio for each enzyme in the sequence, whereas in the case of the 8-OMT of Lotus the regulation of enzyme activity could be controlled solely by the concentration of SAH in the cell.
DEDICATION.

To my parents for their constant encouragement

To Liliana for her love and patience

To Erica and Antonio who are my inspiration
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There is no doubt that without the contributions of several individuals, this thesis would not have been possible.

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A. INTRODUCTION

Flavonoid compounds are widely distributed in the plant kingdom and may be characterized by methyl ether substitution at various positions of the flavonoid ring system. Enzymatic O-methylation, which is catalysed by O-methyltransferase (E.C.2.1.1.6.), will reduce the chemical reactivity of the phenolic hydroxyl groups and render them lipophilic.

The flavonoid O-methyltransferases, so far reported, are known to mediate the transfer of methyl groups from S-adenosyl-L-methionine to the meta or para hydroxyl groups of ring B of flavones/flavonols and isoflavones. More recently, an ortho and A-ring-para directing O-methyltransferase were reported from Glycyrrhiza echinata and tobacco cell cultures, respectively. However, these enzymes catalysed specific, single methylations and did not accept partially methylated substrates for further O-methylation. Recent work in this laboratory demonstrated the stepwise methylation of flavonoid substrates, including quercetin, to various methylated derivatives by partially purified enzyme preparations of Calamondin orange. These enzyme extracts did not lend themselves to further purification and raised the question as to whether sequential methylation of flavonoids is catalysed by one or several distinct enzymes.

Chrysosplenium americanum accumulates several partially methylated flavonol glucosides which have interesting methyl substitutions at positions 3, 6, 7, 2', 4' and/or 5'. Our current interest in the O-methylation of flavonoid compounds prompted us to use this tissue as a source of O-methyltransferases in order to
study the sequential methylation of flavonols, to establish the order of this O-methylation and to isolate and purify these enzymes. In addition, studies with the O-methyltransferase of Lotus corniculatus which catalyses the 8-O-methylation of flavonols, would complement the studies on the Chrysosplenium system which lacks substitution at this position.

Although several flavonoid O-methyltransferases have been isolated from different tissues, very few studies have dealt with the kinetics of these reactions. The only well known kinetic analysis from plant tissues is that of the isoflavone 4'-O-methyltransferase from Cicer arietinum and very recently, there was a report on the vitexin 2''-rhamnoside-7-O-methyltransferase from Avena sativa. These enzymes were postulated to proceed by an ordered bi bi mechanism with S-adenosyl-L-methionine and S-adenosyl-L-homocysteine as leading reaction partners. The highly purified 3-, 6- and 4'-O-methyltransferases from Chrysosplenium as well as the 8-O-methyltransferase from Lotus were submitted to substrate interaction and product inhibition kinetics in order to establish the kinetic mechanisms of these reactions, in relation to the sequential methylation of Chrysosplenium flavonoids versus the final methylation step in Lotus.
B. REVIEW OF LITERATURE

Our knowledge of the biosynthesis of flavonoids has expanded very rapidly in the last decade. In particular, a great deal has been learned about the enzymology and regulation of flavone and flavonol glycoside biosynthesis. Therefore, the aim of this review will be to describe some of the novel enzymes involved in the assembly and modification of the flavonoid ring with special emphasis on how these steps may pertain to the biosynthesis of polymethylated flavonoids.

B.1. Enzymatic synthesis of flavonoids

Grisebach (1) postulated in 1962 that flavonoids were synthesized by the successive condensation of a cinnamoyl-CoA ester with three molecules of malonyl-CoA and the simultaneous liberation of 3 mol of CO₂ (Fig. 1e). This hypothesis, primarily based on feeding experiments with ¹⁴C-labelled acetate and phenylalanine (see ref. 2 for review), has been widely accepted as a result of more recent work at the enzymatic level (see ref. 3 for review).

Much of the enzymology based on the work of the Freiburg group (2, 3) was carried out using parsley cell suspension cultures (Petroselinum hortense). The enzymes of flavonoid biosynthesis shown in Fig. 1 can be classified into two groups. The first (group I) which converts L-phenylalanine to 4-coumaroyl-CoA provides a pool of precursors, not only for flavonoid biosynthesis, but also for most other phenylpropanoid compounds such as lignins (4) and cinnamoyl esters (5). The second (group II) consists of at least 13 enzymes and converts phenylpropanoid precursors into complete acylated flavonol
Figure 1. Common steps in the biosynthesis of flavonoids (3).

(a) Phenylalanine ammonia-lyase (E.C.4.3.1.5.)
(b) Cinnamate-4-hydroxylase (E.C.1.14.13.11)
(c) 4-Coumarate-CoA ligase (E.C.1.12)
(d) Acetyl-CoA carboxylase (E.C.6.4.1.2)
(e) Chalcone synthase
(f) Chalcone isomerase (E.C.5.5.1.6.)
glucosides (3). These enzymes have been extensively reviewed in 1975 (2), 1979 (6) and most recently in 1981 (3).

B.1.1. Chalcone synthase

Chalcone synthase (Fig. 1e), the key enzyme of flavonoid biosynthesis, was previously thought to be a flavanone synthase (7). Recently however, a naringenin chalcone was identified (8) as the immediate product of the reaction. This important enzyme catalyses the sequential condensation of one molecule of 4-coumaroyl-CoA with three molecules of malonyl-CoA (7, 9).

The substrate specificity of the purified enzyme for 4-coumaroyl-CoA, rather than caffeoyl (3,4-dihydroxy-cinnamoyl) or feruloyl (3-methoxy-4-hydroxycinnamoyl)-CoA esters (10), indicates that ring B substitution of flavonoids may be determined at the C₁₅ stage and not at the cinnamoyl level. This was found true for both the enzymes from parsley (10) and *Haplopappus gracilis* (11) cell cultures. The substrate specificity of chalcone synthase also indicates that further hydroxylation of positions other than 5', 7' and 4' as well as subsequent O-methylation, may be catalysed by specific hydroxylases and O-methyltransferases (OMTs).

B.1.2. Hydroxylases

The hydroxyl groups which arise during the assembly of flavonoid rings from their natural precursors are usually located at 5', 7' and 4' positions (Fig. 1). Others (6', 8', 2' and 3' or 5') may be substituted prior to the synthesis of the C₁₅ chalcone, according to the "cinnamic acid starter hypothesis" of Hess (12). However, recent
evidence showed that modifications in the substitution pattern may also take place after the assembly of the basic flavonoid molecule.

There are two alternative strategies for the introduction of a hydroxyl group into a substrate during metabolism. The first involves the addition of a preformed hydroxyl group originating from water to an electron deficient center. The second type, catalyzed by oxygenases (13), occurs with oxygen-deficient lipophilic substrates and involves de novo synthesis of a hydroxyl group due to oxygenation of a substrate with molecular oxygen.

B.1.2.a. Cinnamate-4-hydroxylase (E.C.1.14.13.11) (Fig. 1b)

The second step in the general phenylpropanoid pathway involves the hydroxylation of trans-cinnamic acid to its 4-hydroxy derivative, p-coumaric acid, by the action of cinnamate-4-hydroxylase. This enzyme which was reviewed recently (14), has been established as a member of the cytochrome P-450 dependent oxygenases (15-19). It utilizes 1 mole of NADPH and 1 mole of O₂ for each mole of cinnamic acid as required for a mixed function oxidase (16). Cinnamate-4-hydroxylase seems to be membrane-bound (16, 18-20) and co-migrates with marker enzymes on sucrose density gradients corresponding with the endoplasmic reticulum. Unfortunately, the range of substrate specificity for the microsomal preparations from various plant species that catalyse the 4-hydroxylation of trans-cinnamate has not been extensively investigated (21).
B.1.2.b. Flavonoid-specific hydroxylases

The determination of the substitution pattern of the B-ring of flavonoids remained for a long time an unsolved question in flavonoid biosynthesis (2). Early work using a spinach beet phenolase (22, 23) demonstrated the 3'-hydroxylation of naringenin (5,7,4'-trihydroxyflavanone), dihydrokaempferol and kaempferol (3,5,7,4'-tetrahydroxyflavone). However, this enzyme exhibited broad specificity and accepted p-coumaric and caffeic acids as substrates.

More recently, two laboratories working in collaboration (24), and separately (25, 26) seem to have solved this perplexing question. Extracts from Matthiola incana (24), Antirrhinum majus (snapdragon) flowers (25) and parsely cell cultures (26) were shown to catalyse the 3'-hydroxylation of the B-ring of flavanones and dihydroflavonols. Furthermore, enzymes from flowers of Verbena hybrida (27), not only catalysed the 3'-hydroxylation of flavanones and dihydroflavonols, but also at the 5'-position of these compounds. Enzymes from each source were found in the microsomal fraction, required NADPH as cofactor and could be cytochrome P-450 dependent.
A. majus (25) and parsely cell cultures (26) were also shown to contain a flavanone oxidase which catalysed the oxidation of flavanone to flavone. The enzyme preparation from A. majus which catalysed the conversion of flavanone to flavone and of dihydroflavonol to flavonol (25) was microsomal and required NADPH as cofactor; whereas in parsley (26), this reaction was soluble and was dependent on 2-oxoglutarate, ascorbate and Fe$^{2+}$ for activity. Thus, two distinctly different enzymes, an NADPH-dependent monooxygenase and a 2-oxoglutarate-dependent dioxygenase appear to mediate the oxidation of flavanones and flavonols. Surprisingly, Matthiola incana flowers (24), were shown to contain a soluble flavanone 3-hydroxylase which also required 2-oxoglutarate, Fe$^{2+}$ and ascorbate as cofactors. This preparation catalysed the 3-hydroxylation of naringenin and eriodictyol (5,7,3',4'-tetrahydroxyflavanone) to their respective dihydroflavonols, but did not mediate the oxidation to flavonols.

Further characterisation of these preparations is necessary to establish their specificity for flavonoid compounds in comparison with hydroxycinnamic acids. It would be interesting to compare the specificity of cinnamate-4-hydroxylase in microsomal preparations with that of the flavonoid B-ring-specific hydroxylases (24-27) from these tissues. Such studies may serve to test the hypothesis that multiple forms of plant cytochrome P-450 may confer substrate specificity to the hydroxylase reaction (14, 16).

Nothing is known about the introduction of hydroxyl groups at positions 6 and/or 8 of the flavonoid ring A. Although substitution at these positions was believed to be of less common occurrence
(2), many reports of 6- and 8- substituted flavonoids have appeared recently in the literature (28).

The known example of a flavonoid 8-hydroxylase was reported from the bacterium *Pseudomonas putida* (29). This bacterium was grown on quercetin (3,5,7,3',4'-pentahydroxyflavone) as the sole carbon source, thus inducing the production of enzymes involved in the degradation of this substrate. The first enzyme in the sequence of degradation was shown to be a mixed function oxygenase (29) which introduced a hydroxyl at C8 of ring A. This enzyme had properties typical of a flavoprotein and required NADH as a cofactor in the reaction. This hydroxylation step was necessary for ring cleavage between C8 and the flavonoid C-ring by a dioxygenase, the second enzymatic step in quercetin degradation. It is interesting to speculate whether a similar type of enzyme(s) is responsible for ring A hydroxylation in plants.

B.1.3. Flavonoid-O-methyltransferases (E.C.2.1.1)

Methyl transfer reactions are catalysed by methyltransferases which have been shown to be of widespread occurrence (30-32) in nature. Transmethylation has been demonstrated with DNA, proteins, polysaccharides, tRNA as well as other small molecules in animal systems (30, 31). In plants (32) transmethylation seems to be even more widespread as evidenced by the vast number of secondary products which contain one or more methyl groups. This section of the review will concern itself with the O-methylation of flavonoids and the reader is referred to several excellent reviews on the methylation of other substrates (30-33).
The study of flavonoid OMTs has long been complicated by the fact that plant tissues contain another OMT which transforms caffeic acid (3,4-dihydroxycinnamic) to ferulic acid (3-methoxy-4-hydroxycinnamic). After a hypothetical 5-hydroxylation of the latter to sinapic acid (3,5-dimethoxy-4-hydroxycinnamic) (32), both methoxy acids undergo reduction to their corresponding alcohols before being polymerized to lignin. Caffeic OMT which is found in most plant tissues has been reported to possess broad substrate specificity and has often been compared to the catechol OMT of animal tissues (34-35). Caffeic OMTs which did not accept flavonoids as substrates were recently isolated from bamboo shoots, Japanese black pine, soybean tissue culture and tobacco cell culture (32). The specificity of this enzyme for phenylpropanoids was recognized; however, unlike catechol OMT, it has never been purified to homogeneity (36, 37) or studied kinetically.

Similarly, flavonoid-specific OMTs free of contaminating caffeic OMT have seldom been prepared. Therefore, it was not certain whether methyl groups were introduced into flavonoids at the phenylpropanoid level, at a later stage during flavonoid biosynthesis, or both. It is certain, however, that flavonoid-specific OMTs can only be studied once contaminating caffeic OMT has been eliminated through purification.

B.1.3.a. Substrate specificity

Until recently, our knowledge of O-methylation by SAM-flavonoid-OMTs had been restricted to the formation of monomethyl ether derivatives. These methylations involved the meta-directing 3'-OMT of flavones and flavonols in parsley (38) and soybean cell
cultures (39) and *Tulipa* anthers (40), the *para*-directing OMT which attacked the 4'-position of isoflavones in chick pea cell cultures (41) and the 7-position of quercetin in tobacco cell culture (42, 43) and more recently, the *ortho*-directing OMT of the retrochalcone, licodione in cultured cells of *Glycyrrhiza echinata* (44). On the other hand, 0-methyllations of position 3 of the heterocyclic ring, as well as positions 6 and 8, by specific 0-methyltransferases have not unequivocally been demonstrated. Most of these enzymes (38-43) have recently been reviewed by Poulton (32) and were shown to catalyse single, position-specific methylations on the flavonoid ring. However, none of these enzymes has been shown to accept partially methylated compounds for further 0-methylation. The common occurrence of partially and fully methylated flavonoids (28, 45, 46) raises the question as to whether multiple methylations within one type of flavonoid structure are mediated by one or several distinct enzymes.

Recent work in this laboratory demonstrated the stepwise methylation of a number of flavonoid substrates, including quercetin, to various methylated derivatives by partially purified preparations of Calamondin orange (47, 48). Furthermore, these enzyme preparations accepted partially methylated flavonoid substrates and transformed them to the next higher order of methylation (48), thus indicating the presence of several OMTs. Unfortunately, Calamondin enzyme extracts did not lend themselves to further purification.

More recently, it was demonstrated that the stepwise methylation of phosphatidylethanolamine to phosphatidylcholine in rat liver microsomes was mediated by two distinct N-methyltransferases; the
first of which was rate-limiting and required Mg$^{2+}$ for activity (49). Furthermore, *Ruta graveolens* cell extracts were shown to contain a soluble OMT system which methylated the 5- and 8-hydroxyl groups of linear furanocoumarins (50). One enzyme methylated the 8-hydroxyl groups of xanthotoxol (8-hydroxyfuranocoumarin) and 8-hydroxybergapten (5-methoxy-8-hydroxyfuranocoumarin) and the other methylated the 5-hydroxyl groups of bergapten (5-hydroxyfuranocoumarin) and 5-hydroxyanthotoxin (8-methoxy-5-hydroxyfuranocoumarin) (51). These two examples demonstrate that methylation at different positions of the same molecule may be catalysed by distinct, position-specific enzymes.

**B.1.3.b. Regulation**

The in vitro inhibition of methyltransferases by S-adenosyl-L-homocysteine (SAH) has been extensively studied as a potential regulator of biological methylation. Mudd et al. (52) were the first to show that transmethylation reactions which utilize S-adenosyl-L-methionine (SAM) as methyl donor are sensitive to inhibition by SAH. Since 1963, the list of methyltransferases that are known to be inhibited by SAH has grown considerably. It is now well established that, with the exception of DNA methylation, all transmethylation reactions that utilize SAM as a substrate are inhibited by SAH (32, 53).

Canioni et al. (53), in describing the sensitivity of different methyltransferases to changes in the SAM/SAH ratio, found that these changes did not correlate with either the $K_m$'s for SAM or the $K_i$'s for SAH, but with the ratio $K_m$<sub>SAM</sub>/$K_i$<sub>SAH</sub>. Furthermore, it was
demonstrated that this type of calculation gave a hierarchy of methyltransferases, some of which were very sensitive to $K_{m, \text{SAM}}/K_{i, \text{SAH}}$ and others which were less sensitive to this ratio.

Poulton and Butt (54) were the first to suggest that transmethylation reactions in plants might be controlled by the intracellular SAM/SAH ratio. However, the question of which factors control the intracellular level of SAM and SAH is much more difficult to resolve. These two important metabolites have been measured in plants (55, 56). Their levels, which were similar to those of animal systems (53), were low and within the same orders of magnitude ($\text{SAM}/\text{SAH} = 1.5$, $\text{SAM concentration} = 0.04 \mu\text{moles/g f.w.}$). These observations, as well as the potent inhibition observed with SAH for most methyltransferases (32, 53), demonstrate the requirement for strict regulation of SAH within the cell.

Cleavage of SAH by SAH-hydrolase (E.C.3.3.1.1.) is the only known pathway for SAH metabolism in plants (57). This enzyme has been demonstrated in several plant tissues (56) and has been partially purified from spinach-beet (58) and to homogeneity from yellow-lupin seeds (59). It has been suggested (32, 53) that SAH concentration and thus, the rates of methylation could be regulated by SAH hydrolase. The evidence for this is very convincing in animal systems (53), whereas it has not unequivocally been proven in plants (32).

SAH hydrolase from lupin seeds (59) resembled the enzymes from rat liver (60) and yeast (61) in showing high specificity for both L-homocysteine and adenosine. In addition, all SAH hydrolases studied were shown to strongly favor SAH synthesis (58-61). The equilibrium
could be shifted towards the hydrolysis of SAH if the concentration of products were kept low (58, 60). It has been suggested that the in vivo levels of these products could be decreased via remethylation of homocysteine to methionine by tetrahydro-pteroylglutamate methyltransferase (62, 63) and the conversion of adenosine to ADP by the successive action of adenosine kinase and adenylate kinase (58). However, this hypothesis has not been tested in plants, and there is no clear indication of its contribution to the regulatory mechanisms by which the cell catalyses different methylation reactions.

Whereas several OMTs have been shown to transfer methyl groups from SAM to flavonoids (38-46), inhibition of the reaction by methylated products has not been extensively studied. The determination of inhibition constants for the methylated products, as compared to the $K_m$ for the flavonoid substrate, could be important in assessing the contribution of these terms to the overall rates of methylation observed. For this reason, it is difficult to understand why these types of studies have not been performed (32).

B.1.3.c. Localisation studies: the SAM pathway

The subcellular localisation of the enzymes responsible for the conversion of aspartate to $\alpha$-phosphohomoserine (Fig. 2) has been shown to occur within the chloroplast (64), though it was not known whether these enzymes occurred in other cell fractions. Conclusive evidence for the localisation of these enzymes, including homoserine kinase, cystathionine $\gamma$-synthase and cystathionine $\beta$-lyase (Fig. 2) in the chloroplast was presented by Wallsgrove et al. (65). However, the enzymes responsible for further metabolism of homocysteine to
Figure 2. Localisation of the pathway of S-adenosyl-L methionine biosynthesis (65).

methionine and SAM (Fig. 2) (65) could not be localised within the chloroplast. The inability of plastids to synthesize SAM was also demonstrated when isolated cucumber etioplasts (66), which are dependent on exogenous SAM for the methylation of Mg-protoporphyrin X, could not carry out this reaction in the presence of methionine and ATP.

Figure 2 shows the suggested localisation of the SAM pathway. All the enzymes necessary for homocysteine biosynthesis are contained in the chloroplast, which is also the site of incorporation of inorganic sulfur into cysteine (56). However, the synthesis of methionine and SAM takes place in the cytoplasm. It is interesting to note that the only enzymes found in animal cells, methionine synthase and methionine adenosyltransferase (Fig. 2), are precisely those not located in the chloroplast (65).

B.1.3.d. Localisation studies: enzymes of the flavonoid pathway.

The enzymes of the phenylpropanoid pathway and flavonoid biosynthesis have been postulated to occur within the plastids (67-68) with the subsequent transport of completed flavonoids to the vacuole (69). On the other hand, the cytosol was shown to be the main site of chalcone synthase, chalcone-flavanone isomerase and UDP-glucose:anthocyanidin 3-O-glicosyltransferase activities in *Hyppeastrum* and *Tulipa* spp. (70). Similarly, the activities of SAM:caffeic acid OMT, flavanone synthase, UDP:flavonoid 3-O-glicosyltransferase and SAM:quercetin OMT could only be detected in the cytoplasm of peas, green beans, spinach and red cabbage (71). These workers concluded that chloroplasts are not involved in flavonoid synthesis.
A recent study by Charriere-Ladreix et al. (72) claimed that the stepwise O-methylation of quercetin and quercetagetin (6-hydroxyquercetin) was catalysed by spinach chloroplast envelope, whereas the chloroplast stroma contained a soluble caffeic OMT. However, spinach contains only trace amounts of methylated flavonoids (73, 74) and low levels of caffeic-OMT and quercetin-OMT (71). Furthermore, the claim that the chloroplast envelope was the site (72) of multiple methylation of flavonoids was based on assays containing 700 μg protein which converted about 4 nmoles of quercetin or 8 nmoles of caffeic acid to their methylated products within 30 min. This represented less than one and two percent of the respective quercetin and caffeic OMT activities, present within the whole tissue (71). These results could easily be attributed to cytoplasmic OMTs contaminating the 'purified' chloroplasts and therefore, could be artifacts of the preparation. Furthermore, these workers (72) failed to give convincing evidence for the identity of the radiolabelled products which were synthesized by this system.

B.2. Enzyme kinetics

Very few studies have dealt with the complete kinetic analysis of enzymes catalysing methyl transfer reactions, most of which were reported from animal systems (75-79). The best characterised of the methyltransferases, catechol-OMT, catalyzed the conversion of epinephrine to metanephrine and paraneaphrine. The kinetic mechanism derived for the catechol-OMT involves random addition of substrates and release of products. Furthermore, the formation of a dead-end
complex between the enzyme, methyl acceptor and methylated product was postulated in order to explain the product inhibition patterns observed (75). A ping pong mechanism for histamine-N-methyltransferase (76) was proposed on the basis that substrate interaction kinetics gave parallel lines in Lineweaver-Burke plots. However, these studies alone cannot exclude a sequential binding mechanism (79). Similarly, two out of five charge-isoenzymes of rabbit adrenal norepinephrine N-methyltransferases (77, 78) gave parallel lines in substrate interaction kinetics. However, these authors postulated an ordered bi bi mechanism with SAM and SAH as leading reaction partners and containing one or more dead-end complexes, based on product inhibition studies (78).

Although plant systems catalyse various types of methylation by specific methyltransferases, no complete kinetic analysis involving initial velocity and product inhibition studies has been reported. Most of the work carried out on plant enzymes demonstrated that SAH was a potent competitive inhibitor versus SAM at physiological concentrations (32).

The isoflavone-4'-O-methyltransferase isolated from Cicer arietinum (41) was postulated to proceed by an ordered bi bi mechanism with SAM and SAH as leading reaction partners. However, product inhibition studies using the methylated isoflavonoid product were not carried out because of poor solubility of this product. Evidence for a sequential binding mechanism was also obtained for SAM:Mg-protoporphyrin methyltransferase isolated from Euglena (80), although the nature of binding was not determined. Indirect evidence for an
ordered binding mechanism was shown by Sharma and Brown (51) for the
5- and 8-hydroxyfuranonconmarin methyltransferases as well as caffeic-
OMT from Ruta graveolens cell cultures. It was found that the latter
enzymes could only bind to xanthotoxin- and ferulic acid-immobilized
Sepharose columns, if SAM or SAH was present in the irrigant buffer.
It was concluded that each of these enzymes had to bind SAM or SAH
before it could bind the phenolic substrate and therefore a
compulsory, ordered kinetic mechanism was proposed (51).

B.3. The stereochemistry of methylation

Stereochemical studies can contribute significantly to our
understanding of the mechanism of enzymatic reactions. Enzymatic
processes, unlike non-biological reactions, are almost always
stereospecific since the enzyme must orient reaction partners within a
rigid conformation in order to achieve high rates of reaction and high
degree of substrate specificity. This fact permits the use of chiral
groups to determine the orientation of transfer and thus, to
distinguish between sequential and ping pong mechanisms (81).

Two laboratories, those of Arigoni (Zurich) and Floss (Ohio
State), have recently initiated studies on the stereochemistry of the
enzymatic transfer of chiral methyl (H,D,T) groups from SAM to various
substrates. Six methyltransferases were studied (82-86) and in each
case, the transfer occurred with inversion of configuration of the
methyl group; thus precluding a ping pong mechanism and methylated-
enzyme intermediate. This suggested a common SN2 mechanism where the
methyl donor and methyl acceptor would have to bind to the enzyme
active site prior to any product release. This is consistent with a sequential mechanism in which a ternary-SAM-methyl acceptor-complex was formed.

Thus, in the case of methyltransferases several enzymes which catalyse methyl group transfer to carbon (84-86), sulphur (86), nitrogen (84) or oxygen (82, 83, 86) nucleophiles do so with the same stereochemistry. This increases the possibility that the steric course of methyl transfer is dictated by its mechanism and is in agreement with the sequential binding of substrates postulated for several methyltransferases through steady state enzyme kinetics (41, 51, 75, 77, 78, 80).

B.4: The regulation of flavonoids in plants

It is apparent from previous reviews (eg 87) that flavonoids may function in higher plants as attractants of agents involved in pollination. Some have speculated that flavonoids act as protective substances against UV light (88) or infection by phytopathogenic organisms (89). It is also well established that flavonoid levels may vary greatly during different stages of plant development (90), thus providing evidence that the plant actively controls the biosynthesis and degradation of these compounds.

Irradiation with ultraviolet light has been shown to induce the enzymes of general phenylpropanoid metabolism (Group I, Fig. 1) and of the flavonoid glycoside pathway (Group II, Fig. 1) in cultures of parsley cells (91). This coordinated induction of enzyme activities was attributed to de novo synthesis of subunits and assembly of these
enzymes (92-94) and not to an activation of preformed inactive proteins. These results have been extended to Phaseolus vulgaris (95) as well as other systems (96) indicating that the phenomena observed in parsley cell cultures may be widespread. It is believed that methyltransferases, which belong to Group II, would also be induced in a similar fashion (3), although this has not been studied in these systems.

The enzymes of Group I could be induced more rapidly when parsley cells were treated with an 'elicitor' from the phytopathogen, Phytophthora megasperma (97). The enzymes of group II were not induced under these conditions. Instead, the accumulation of linear furanocoumarins (phytoalexins) in response to the elicitor treatment (97, 98) suggested the existence of a third group of enzymes which might be involved in an elicitor-specific, light-independent pathway.

Thus, each signal (UV irradiation or treatment with elicitor) appears to trigger the formation of one class of compounds (UV protective flavonoids, or antimicrobial phytoalexins) whose potential function is to protect the plant against possible damage by the inducing agent. The nature of these triggers is not well understood; however, their elucidation should contribute to the understanding of these phenomena, as well as the controls regulating secondary metabolite synthesis in plants.
C. MATERIALS AND METHODS

C.1. The plant material

C.1.1. Chrysosplenium americanum

Chrysosplenium comprises 55 species, that are widely distributed in the northern hemisphere. This genus is characterized by a high degree of flavonoid O-methylation and, in several species, the occurrence of unusual substitution with hydroxyl and/or methoxyl groups at positions 6 and/or 2' (99-101).

*C. americanum*, which occurs in eastern North America, accumulates a variety of tri-, tetra- and penta-O-methylated flavonols which are derived from quercetin and quercetagine (6-hydroxy-quercetin) both with 2'-substitution (102) as shown in Figure 3. These compounds, which are of relatively rare distribution occur as 2'-/5'-monoglucosides, together with smaller amounts of their free aglycones. Conspicuous by their absence, are the early methylated intermediates (mono- and dimethylquercetin and -quercetagine); indicating that these are rapidly transformed into final products in vivo (102).

Our current interest in the O-methylation of flavonoid compounds (42, 43, 47, 48) prompted us to use *C. americanum* as a source of OMTs because of its unusual methyl substitution at positions 3, 6, 7, 2', 4' and/or 5' (Fig. 3). Early work in this laboratory demonstrated that when [2-14C]-cinnamic acid was administered to leaves, the polymethylated flavonol glucosides were labelled within 30 min. (Fig. 3 and Appendix Fig. 1). This demonstrated the presence of the whole pathway and indicated that all the enzymes catalysing these reactions
Figure 3. Structural formulae of the six highly O-methylated flavonol glucosides of *Chrysosplenium americanum* (102).

This tissue contains two 2'-β-D-glucosides of partially methylated 2'-hydroxyquercetin and four 5'-β-D-glucosides of partially methylated 6-hydroxy and 6,2'-dihydroxy-quercetin. Note that since the 2'-position is substituted, then numbering of the o-dihydroxy grouping on ring B becomes A', 5' instead of 3',4' since both 3'– and 5'– positions are identical.
were present in this tissue. The potential of this system was recently demonstrated by the characterization of a glucosyltransferase (103, 104) that catalysed the last step in the biosynthesis of these compounds (Fig. 3).

C.1.2. Lotus corniculatus

Lotus corniculatus, a common legume, is widely distributed in the pastures of temperate regions and has been the subject of many studies with the aim of improving its fodder quality (105). It represents an interesting example of the ontogeny of 8-substituted flavonols in its vegetative and flowering parts. The leaves contain the flavonol aglycones: kaempferol, quercetin and isorhamnetin (3'-methylquercetin), whereas the flowers accumulate their 8-methoxyderivatives: 8-methoxykaempferol, 8-methoxyquercetin and 8-methoxyisorhamnetin (Fig. 4) (106).

Since the methoxyl groups at position 8 and 3' of Lotus flavonols represent meta-methylation of rings A and B, respectively, it was interesting to find out whether both reactions are catalysed by one or two distinct OMTs. Furthermore, the study of the 8-OMT in Lotus complements the work on Chrysosplenium which lacks substitution at the 8-position.

C.1.3. Growth conditions of plant material

C. americanum was collected from Sutton Junction, Province of Quebec, where it thrives in wet bogs, under diffuse light and cool temperature. In order to simulate its natural habitat, the plants
\[
\begin{array}{c|c|c}
R_1 & R_2 & \\
\hline
H & H & \text{Kaempferol} \\
H & OH & \text{Quercetin} \\
OH & OH & \text{Gossypetin} \\
OCH_3 & H & \text{8-methoxykaempferol} \\
H & OCH_3 & \text{isorhamnetin} \\
OCH_3 & OCH_3 & \text{8-methoxyisorhamnetin} \\
\end{array}
\]
Figure 4. Structural formulae of the flavonol derivatives of Lotus corniculatus.
were installed in a compartment of the glass house, equipped with a netting material designed to decrease the light intensity by more than half during the summer months (May-September) and by 25% in the winter months. The temperature ranged between 15-20°C (September-May) and 20-25°C during the summer months. The plants (Fig. 5) were grown in flat plastic containers (25 x 36 cm) that were filled with a potting soil-peat-moss mixture (3:1) and were kept flooded with water at all times.

Both vegetative and reproductive parts of *L. corniculatus* were obtained from glasshouse-raised plants as well as the Agriculture Experimental Farm of Macdonald College, McGill University. The plant material was collected in liquid nitrogen until required for enzyme extraction.

C.2. Chemicals

S-Adenosyl-L-[14CH3] methionine (60 mCi/mmol) was purchased from Amersham (Oakville, Ontario); cold SAM and SAH were obtained from Boehringer Mannheim (Germany). Most commercially unavailable flavonoid compounds were generous gifts from Dr. M. Jay, University of Lyon and Prof. E. Wollenweber, Darmstadt. Other flavonoid substrates were purchased from Roth (Karlsruhe, Germany) and Sarsyntex (Bordeaux, France) and were further purified by recrystallization or preparative TLC. Sephacryl S-200, Sephadex G-100, Polybuffer ion exchanger (PBE-94) and Polybuffer (PB-74) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). Hydroxyapatite (Biogel HT), Bio-Rad protein reagent, Acrylamide (99%) and bis Acrylamide were from
Figure 5. Photograph of *Chrysopteris Americanum*

This tissue was grown under glass house conditions as described in section C.1.3.
Bio-Rad (Richmond, CA). SAH-Agarose was from Bethesda Research Labs (Gaithersburg, MD), sodium dodecyl sulfate from BDH chemicals, commercial Polyamide 6-MN TLC plates and Polyamide powder from Brinkmann Instrument (Mississauga, Ont.). All column chromatographic supports were prepared according to manufacturers' specifications for subsequent use with enzyme purification. Imidazole was recrystallized from ethyl acetate. All other chemicals were of analytical grade.

C.3. Characterization of 3-, 6-, 7- and 4'-OMTs from Chrysosplenium
C.3.1. Preparation of crude extracts

Unless stated otherwise, all purification steps were carried out at 2-4°C. Young shoot tips (ca 90 g) were frozen in liquid N2 and mixed with Polyclar AT (10% w/w), then homogenized in a blender with 500 ml of 0.1 M phosphate buffer, pH 7.6, containing 5 mM EDTA, 10 mM DIECA (diethyl ammonium diethyl-dithiocarbamate) and 14 mM 2-mercaptoethanol. The slurry was filtered through nylon mesh and the filtrate was centrifuged for 15 min at 20,000 g. The supernatant was stirred with Dowex 1 X 2 (10% w/v) which had previously been equilibrated with 0.1 M phosphate, pH 7.6. The filtrate was fractionated with solid (NH4)2SO4 and the protein fraction which precipitated between 30-70% was collected by centrifugation at 20,000 g for 10 min.

C.3.2. Chromatography on Sephacryl S-200

The protein pellet was resuspended in 10.5 ml of 25 mM imidazole-HCl, pH 7.4 containing 10% glycerol, 14 mM 2-mercaptoethanol
and 0.5 M NaCl (buffer B) and 10 ml were chromatographed on a Sephacryl S-200 column pre-equilibrated with buffer B. The column was developed for 20 hours using buffer B at a flow rate of 29 ml/hr and 145 fractions were collected (4 ml/fraction). Enzyme assays were conducted against the five flavonoid substrates shown in Figure 6.

The remaining 0.5 ml was desalted on a Sephadex-G25 column (1.5 x 25 cm) pre-equilibrated with buffer C (buffer B without NaCl) prior to determining the enzyme activity of the partially purified preparation. Preparations could not be assayed before Sephadex-G25 chromatography because of the high background counts obtained in control assays. However after passage on Sephadex-G25, this high background was totally eliminated. It was concluded that DIECA was the cause of this problem (Table 1), since its inclusion in assays with desalted enzyme resulted in the appearance of high background radioactivity.

C.3.3. Fractionation on hydroxyapatite

The OMT activity (40 ml) recovered from the Sephacryl S-200 column was applied onto hydroxyapatite (3 x 3 cm) which had been pre-equilibrated with buffer C. The column was washed with buffer C (25 ml) until the optical density at 280 returned to base level. Enzyme activity was eluted (32 ml/hr) from the hydroxyapatite column using a linear gradient of phosphate (0-100 mM) in buffer C. Seventy fractions were collected (2.8 ml/fraction) and assayed against the five different substrates.
Figure 6. Substrates used to assay for the 3-, 6-, 7- and 4'-OMTs of Chrysosplenium

1. Quercetin (Q) to 3-methyl Q
2. 3-methylquercetin to 3,7-dimethyl Q
3. 3,7-dimethyl Q to 3,7,4'-trimethyl Q
4. 3,7-dimethylquercetagetin to 3,7,4' trimethylquercetagetin or 3,6,7-trimethylquercetagetin
5. 3,7,3'-trimethylquercetagetin to 3,6,7,3'-trimethylquercetagetin
Figure 6A. Structural formulae of other potential substrates for O-methylation by *Chrysosplenium* and *Lotus* enzymes.
Table 1. Effect of DIECA on the assay of Chrysosplenium OMTs

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Enzyme activity (dpm/assay)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>plus 1mM DIECA</td>
</tr>
<tr>
<td>None</td>
<td>7800</td>
</tr>
<tr>
<td>Quercetin-(Q)</td>
<td>6000</td>
</tr>
<tr>
<td>3-Methyl-Q</td>
<td>1600</td>
</tr>
<tr>
<td>3,7-Dimethyl-Q</td>
<td>5300</td>
</tr>
<tr>
<td>Quercetagetin (Qg)</td>
<td>4000</td>
</tr>
<tr>
<td>3,7-Dimethyl-Qg</td>
<td>8400</td>
</tr>
<tr>
<td>3,7,3'-Trimethyl-Qg</td>
<td>6200</td>
</tr>
</tbody>
</table>

\(^a\)Partially purified enzyme preparations (Section C.3.1.) were desalted on Sephadex G-25 in order to remove DIECA that was present in the grinding buffer.  
\(^b\)Standard enzyme assays were performed as described in the Materials & Methods section.
C.3.4. Chromatofocusing on Polybuffer ion exchanger.

Each of the two major OMT peaks eluted from hydroxyapatite were subjected to chromatofocusing on a Polybuffer ion exchanger column (1 x 20 cm) which had been pre-equilibrated with buffer C. The enzyme activity was eluted with Polybuffer-94 which generated a linear, 200 ml gradient of pH 7-4. The eluting buffer consisted of Polybuffer-94:water (1:8) containing 20% glycerol and 10 mM 2-mercaptoethanol and its pH was adjusted to 4 with dilute HCl. A total of 70 fractions (3 ml/fraction) were collected at a rate of 70 ml/hr and assayed for OMT activity against the different substrates.

C.3.5. Removal of contaminating polybuffer

After chromatofocusing on Polybuffer ion exchanger, each purified enzyme (38 to 50 ml) was subsequently applied to hydroxyapatite as described in C.3.3., in order to eliminate contaminating polybuffer and to concentrate each OMT. After enzyme application, the column was washed with buffer C (25 ml) and the enzyme activity was eluted batchwise with 0.2M phosphate buffer pH 8, containing 20% glycerol and 5 mM DTT. Enzyme activity was recovered in a total volume of 6 ml.

C.4. Characterization of Flavonol 8- and 3'-OMTs from Lotus

C.4.1.a. OMT activity in different organs

Leaves, apical meristems or flower buds were obtained from Lotus in order to characterize their OMT activities. One gram of each material was pulverized in liquid nitrogen and the enzymes were extracted using 3 ml of buffer A as described in section C.3.1.
However, these extracts were not submitted to \((\text{NH}_4)_2\text{SO}_4\) fractionation; instead, they were desalted on Sephadex G-25, as in section C.3.2., and were subsequently used for enzyme assays.

C.4.2. Preparation of crude extracts

Flower buds (2 g) were frozen in liquid \(\text{N}_2\), ground to a fine powder with Polyclar AT (20% w/w), then homogenized in buffer A (1:5 w/v). All subsequent procedures were performed as described in section C.3.1.

C.4.3. Chromatography on Sephadex G-100

The protein pellet (section C.3.1.) was resuspended in buffer C and chromatographed on a Sephadex G-100 column which was pre-equilibrated in the same buffer. Enzyme activity was eluted with buffer C at a flow rate of 20 ml/hr and 100, 3-ml fractions were collected. Enzyme assays were performed with 8-hydroxykaempferol and quercetin as substrates for the 8- and 3'-OMTs, respectively.

C.4.4. Chromatofocusing

The enzyme activity from the Sephadex G-100 column (35 ml) was applied on a Polybuffer ion exchanger as was described in section C.3.4. The 8- and 3'-OMTs were freed from contaminating polybuffer and concentrated as described in section C.3.5.

C.5. Purification of 8-OMT from Lotus

Two thousand young flower buds (ca 20 g) were used in order to purify enough 8-OMT for kinetic studies. The protocol followed was
similar to that described in section C.4. up to and including the Sephadex G-100 step.

C.5.1. SAH-Agarose chromatography

The protein fractions from Sephadex G-100 containing high OMT activity were pooled and chromatographed on a SAH-Agarose column (1 x 5 cm) which was pre-equilibrated with buffer C. The column was washed with 100 ml of buffer C and the enzyme activity was eluted in 3 ml fractions using a linear gradient of NaCl (0-1M) in buffer C.

C.5.2. Chromatography on hydroxyapatite

The active fractions from the SAH-Agarose column were pooled and applied to hydroxyapatite as described in section C.3.3.

C.5.3. Chromatofocusing

The enzyme protein from the hydroxyapatite column was subsequently bound to Polybuffer ion exchanger as described in section C.3.4. However, this column was pre-equilibrated in 25 mM histidine, pH 6.4, 10% glycerol and 10 mM-2-mercaptoethanol, instead of buffer C. In addition, the pH of Polybuffer was adjusted to 4.8 instead of 4 and a linear gradient of pH 6-4.8 was generated. Active fractions were pooled and concentrated as in section C.3.5.

C.6. OMT assay

The assay for OMT activity was performed as described previously by Tsang and Ibrahim (42) using S-adenosyl-L-[14CH3]-methionine as methyl donor. The standard assay mixture consisted of 1 n mole of the phenolic substrate (dissolved in 10 µl of dimethylsulfoxide), 0.7
nmole of SAM (containing 25 nCi), 1.4 μmole 2-mercapteothanol and the enzyme protein. The assay was carried out in a final volume of 100 μl containing 0.1 M phosphate buffer, pH 8, and the reaction was started by the addition of enzyme. The reaction mixture was incubated at 30°C for 30 min in a constant temperature water bath and the reaction was terminated by the addition of 20 μl of 6 N HCl.

The O-methylated products were separated from unreacted SAM by extraction with 250 μl of benzene-ethyl acetate (1:1) and shaking in an Eppendorf rotary shaker for 2 min. The organic phase containing the methylated products was separated from the aqueous layer by a 2 min centrifugation using an Eppendorf centrifuge. An aliquot (100 μl) of the organic layer was transferred to plastic scintillation vials (Beckman Bio Vials) together with 2.5 ml scintillation fluid (5 g PPO/l of toluene) and was counted for radioactivity using a Wallac LKB model 1215 Rackbeta Liquid Scintillation Spectrometer which was programmed with a quench correction curve.

C.6.1. Efficiency of extraction of reaction products

In order to estimate the percentage recovery of the methylated flavonoid product after extraction with ethyl acetate-benzene mixture, several assays were performed with the purified 3-, 6-, 8- and 4'-OMTs, and their radioactive products counted. Therefore, 3*-methylquercetin, 3,6*,7,3'-tetramethylquercetigenin, 8*-methoxykaempferol and 3,7,4'-*trimethylquercetin of known radioactivity were placed in separate Eppendorf vials and the organic solvent was allowed to evaporate. DMSO (10 μl, 50% v/v) was added to each vial followed
Table 2. Effect of the extraction procedure on the recovery of flavonoid products

<table>
<thead>
<tr>
<th>Volume (μl)</th>
<th>3-Methylquercetin</th>
<th>3,6,7,3'-Tetramethylquercetagetin</th>
<th>3,7,4'-Trimethylquercetin</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>95</td>
<td>104</td>
<td>92</td>
</tr>
<tr>
<td>100</td>
<td>107</td>
<td>100</td>
<td>99</td>
</tr>
<tr>
<td>150</td>
<td>103</td>
<td>99</td>
<td>76</td>
</tr>
<tr>
<td>200</td>
<td>104</td>
<td>101</td>
<td>100</td>
</tr>
<tr>
<td>250</td>
<td>106</td>
<td>100</td>
<td>98</td>
</tr>
<tr>
<td>300</td>
<td>103</td>
<td>96</td>
<td>99</td>
</tr>
</tbody>
</table>

\(^a\) Fifty μl of the organic layer contained 1250, 1850 and 1750 dpm of labelled 3*-methylquercetin, 3,6*-7,3'-tetramethylquercetagetin and 3,7,4'-trimethylquercetin, respectively (*, \(^14\)CH\(_3\)).
by phosphate buffer (90 μl). Each tube was then processed according to the procedure outlined in section C.6.

Table 2 shows that nearly 100% of the radioactivity was recovered, irrespective of the actual amounts of radioactive product present (marked*) or the position of methylation on the flavonoid ring. Similar results were obtained when 10 μl DMSO-water (50% v/v) containing the respective flavonoid substrate was added to the radioactive product.

C.6.2. Identification of reaction products

The reaction products of each enzyme studied were subjected to similar procedures for identification purposes. Several assays were performed in order to obtain enough radioactive products for autoradiographic analysis. The products were evaporated to dryness and dissolved in 50 μl of methanol, then chromatographed on commercial Polyamide-6 MN TLC plates in different solvent systems (described in figure legends). The identity of methylated products was confirmed by co-chromatography with reference compounds (Table 3), visualisation in UV-light (366 nm) and by autoradiography on X-Ray film.

C.7. Analytical gel electrophoresis

Standard 7.5% polyacrylamide disc gels were prepared according to Davis (107) and were used to establish protein profiles during different steps of purification of OMTs. In addition, some gels were run in the cold room when enzymes were to be extracted for determination of activity profiles.
Table 3. Identification of reaction products of *Chrysosplenium* OMT activity\(^a\)

<table>
<thead>
<tr>
<th>Flavonol substrate</th>
<th>Reaction products(^b)</th>
<th>Methyl substitution</th>
<th>R(_f) values (X100)(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Solvent A</td>
<td>Solvent B</td>
</tr>
<tr>
<td>Quercetin</td>
<td>3-</td>
<td>18</td>
<td>52</td>
</tr>
<tr>
<td>3-Methylquercetin</td>
<td>3,7-</td>
<td>53</td>
<td>75</td>
</tr>
<tr>
<td>3,7-Dimethylquercetin</td>
<td>3,7,4'-</td>
<td>80</td>
<td>75</td>
</tr>
<tr>
<td>3,7-Dimethylquercetagetin</td>
<td>3,6,7-</td>
<td>63</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td>3,7,4'</td>
<td>68</td>
<td>85</td>
</tr>
<tr>
<td>3,7,3'-Trimethylquercetagetin</td>
<td>3,6,7,3'</td>
<td>84</td>
<td>90</td>
</tr>
</tbody>
</table>

\(^a\) Partially purified enzyme preparation (Section C.3.1.) desalted on Sephadex G-25. The standard enzyme assay was used as was described in the Methods section.

\(^b\) The reaction products were identified by co-chromatography with reference compounds, visualization in UV-light and autoradiography.

\(^c\) On Polyamid-6 MN TLC plates in solvents: A, toluene-ethyl formate-butyl acetate-formic acid (25:50:23:2) and B, benzene-methyl ethyl ketone-methanol (4:3:3).
When the latter protocol was used, the gels were sliced with a razor blade into 5 mm segments and each slice was transferred to an Eppendorf tube. Each slice was then extracted with 250 μl of 0.2 M phosphate buffer, containing 10% glycerol and 5 mM DTT. Gel slices were macerated in this buffer using a glass rod until a fine pulp was produced and the gels were stored overnight at 4°C prior to enzyme assay. Enzyme assays were performed as described in section C.2.1, using 100 μl of each extracted fraction.

C.7.1. Sodium dodecyl sulfate PAGE (SDS-PAGE)

SDS-PAGE slab gels (20 cm x 20 cm) were prepared as described by Laemmli (108) and were used to establish protein profiles under denaturing conditions at different stages of OMT purification. SDS gels containing 11% acrylamide were routinely used for this purpose.

C.8. Molecular weight determination

The molecular weights of both crude and purified enzymes were determined by gel filtration on a calibrated column of Sephacryl S-200 (section C.3.2). The column was equilibrated in buffer B and proteins were eluted using this buffer. The column was previously calibrated with a number of standard proteins, and the void volume of the column was determined by elution of a sample of blue dextran (108). OMTs were routinely applied together with a standard protein to ensure the reproducibility of the system. The $K_a b$ values for the proteins used were plotted against their molecular weights on a
logarithmic scale. The $K_{av}$ of each OMT was then calculated using
\[ K_{av} = \frac{V_e - V_0}{V_t - V_0} \]
where $V_t = \text{total volume}$, $V_0 = \text{void volume}$ and $V_e = \text{elution volume}$.

C.9. Localisation of OMT activities on sucrose gradients

The method of Beever$^{+\text{et al.}}$ (110) was used to isolate organelles on sucrose gradients. Five grams of Chrysosplenium leaves were placed in a petri dish containing five ml of grinding buffer consisting of 150 mM tricine, pH 7.6, 10 mM KCl, 1 mM MgCl$_2$, 1 mM EDTA and 13% (w/v) sucrose (Buffer D). The leaves were chopped until the tissue was finely ground then filtered through a nylon mesh (300 micron mesh size) and the filtrate was centrifuged for 5 min. at 500 g to remove cellular debris. The supernatant was applied on top of a linear sucrose gradient in buffer D (without sucrose). This gradient was constructed in 25 ml Polyalomer centrifuge tubes with a 60% (w/w) sucrose cushion (4 ml) followed by 16-60% (w/w) linear sucrose gradient (18 ml) and 2.5 ml supernatant. The gradients were placed in a 3 x 25 ml swing-out rotor (cat. no. 43127-104) and centrifuged in an MSE Superspeed 75 Ultracentrifuge at 60,000 g at 4°C for 2 hr.

After centrifugation, the bottom of each centrifuge tube was taped with masking tape, then punctured with a 15 gauge needle and drops were collected at the open end. Fractions were collected (0.7 ml) and assayed for OMT and glucosyltransferase activities. Relative chlorophyll absorbance was measured at 670 nm. Each fraction was
subsequently extracted with ethyl acetate-benzene (1:1) and the organic phase was evaporated to dryness then chromatographed on Polyamide TLC plates. The solvent system used permitted the separation of chlorophylls from polymethylated flavonol glucosides. The latter could be visualized under UV-light and thus permitted their localization on the sucrose gradient.

C.10. Analysis of kinetic data

Kinetic data were analyzed using the nonlinear regression computer program as described by Duggleby and Dennis (111). All of the substrate interaction data were fitted to both of the following equations:

\[ v = \frac{V_{\text{max}}}{1 + (K_a/[A]) + (K_b/[B]) + (K_{ia}K_b/[A][B])} \] (I)

\[ v = \frac{V_{\text{max}}}{1 + (K_a/[A]) + (K_b/[B])} \] (II)

where A and B are the varied substrates, \( K_a \) and \( K_b \) are the respective limiting Michaelis constants, \( V_{\text{max}} \) is the maximum velocity and \( K_{ia}K_b \) is an interaction term.

All data from inhibition studies were fitted to the equation for non competitive inhibition (III). In addition, the data were fitted to the equation for competitive inhibition (IV) or uncompetitive
inhibition (V) as determined by inspection of the double reciprocal plots.

\[
v = \frac{V'_{\text{max}}}{1 + (K'_a/[A]) + (K'_a[I]/K_{iS}[A]) + ([I]/K_{ii})}
\]

\[
v = \frac{V'_{\text{max}}}{1 + (K'_a/[A]) + (K_a[I]/K_{iS}[A])}
\]

\[
v = \frac{V'_{\text{max}}}{1 + (K'_a/[A]) + [I]/K_{ii}}
\]

In these equations, A is the variable substrate, I is the inhibitor, 
\( K'_a \) and \( V'_{\text{max}} \) are the Michaelis constant and maximum velocity, 
respectively, in the presence of inhibitor, and \( K_{iS} \) and \( K_{ii} \) are the 
slope and intercept inhibition constants, respectively.

The data from the kinetic studies of the 3-, 6-, 8- and 4'-OMTs 
are presented as double reciprocal plots which were fitted by linear 
regression analysis (method of least squares). However, all the data 
(3-, 6-, 8- and 4'-OMTs) were also submitted to the above-mentioned 
non-linear regression analysis and the appropriate model was chosen 
using the variance ratio test. In most cases the model formed by the 
computer programme was identical to that generated by fitting the data 
by the method of least squares. It should also be noted that the 
kinetic constants given in Tables 15 to 20 were those given by the 
computer programme of Duggleby and Dennis (111).
In the method of data analysis described by Duggleby and Dennis (111), replicates of individual rates were not performed since it appeared to be more advantageous to increase the number of data points rather than perform replicates. It was also assumed that the measured velocity, \( v \) was normally distributed around the predicted velocity, \( \bar{v} \) and the variance in initial velocities was constant. Furthermore, the errors in substrate and inhibitor concentrations were assumed to be constant at all concentrations. Since replicates were not performed, no estimate of pure error could be made. However, the residual sum of squares (RSS\(_C\)), which resulted when the data were fitted to equations I and III, gave a measurement of the pure error in the data. When the data were fitted to equations II, IV, and V, the residual sum of squares (RSS\(_r\)) was due both to pure error and lack of fit to the equations. The residual sum of squares (RSS\(_f\)) due to lack of fit to the equation could be estimated from

\[
\text{RSS}_{f} = \text{RSS}_{r} - \text{RSS}_{C}
\]

The significance of the lack of fit to equations II, IV and V could be determined by a variance ratio (F) test

\[
F = \frac{\text{RSS}_{f} (n - p)}{\text{RSS}_{C}}
\]

where \( n \) was the number of assays and \( p \) (the number of parameters in the complete equation) was 4. In all cases, the null hypothesis was used, i.e. equation II, IV or V is chosen if a significantly better
fit is not found with equation I or III i.e. if the residual sum squares due to lack of fit to equation II, IV or V is not significant as compared with the error in the data. Using this analysis, the significance of the $k_{i0}k_{b}$, $[I]/k_{f1}$ and $k_{i}^{'b}[I]/k_{i}^{'b}[A]$ terms in equation I or III was evaluated by comparison of the experimentally determined $F$ value with tables of $F$ values at a probability of 0.99.

C.11. Protein determination

Protein was determined according to Bradford (112) (Fig. 7) using the Bio-Rad protein reagent and bovine serum albumin as a standard protein.

C.12. Definition of enzyme units

Enzyme units are expressed in Katalis as recommended by the International Union of Biochemists (113). One Kat is define as the amount of activity which converts one mole of substrate per second under the assay conditions.
Figure 7. Standard curve for the Bio-Rad protein determination (112).

The standard protein used was bovine serum albumin.
D. RESULTS

D.1. Stabilization of OMTs

Since plant tissues vary in their content of protein-inactivating phenols, no universal procedure for enzyme extraction has gained general acceptance. However, methods designed to remove phenolic compounds during the preparation of tissue extracts are often essential to the isolation of plant enzymes in an active state.

Generally, the removal of polyphenols is achieved by the inclusion of adsorbents such as the widely used Polyclar AT (114) and Dowex 1 X 2 (115) in the homogenization medium. In addition, the presence of the copper chelator, DIECA (116) decreases the formation of protein binding quinones via the copper-dependent polyphenol oxidase.

Preliminary experiments with C. americanum established that in the absence of DIECA, Polyclar AT or Dowex 1 X 2, partially purified preparations were brown in color (indicative of phenol-bound protein) instead of white and showed little or no methylating activity against flavonoid substrates. In addition, loss of enzyme activity of partially purified preparation (C.3.1.) occurred within 24 hr in absence of glycerol. On the other hand, no loss in OMT activity could be observed for several days in the presence of 10% glycerol and storage at 4°C.

D.2. Enzymatic synthesis of methylated flavonoids in Chrysosplenium

Preliminary studies on the OMT system of Chrysosplenium were conducted with partially purified enzyme preparations (C.3.1.).
Figure 8 is a photograph of the autoradiographed reaction products of a Sephadex G-25 OMT preparation that was assayed against a number of flavonoid substrates. Quercetin was readily accepted as substrate and was mainly transformed to 3-methylquercetin, as well as decreasing amounts of 3,7-di- and 3,7,4'-trimethyl derivatives (Fig. 8). Furthermore, partially methylated flavonol intermediates were readily accepted (Table 4) and were transformed to the next higher order of their methyl derivatives (Fig. 8).

D.2.1. Substrate specificity of partially purified preparations

Several flavonoid substrates were tested for their methyl acceptor ability. The results (Table 4) indicate that, unlike quercetagetin, quercetin was the best substrate used and may be considered a possible precursor for the synthesis of polymethylated flavonols in this tissue. When partially methylated quercetagetin derivatives (3,7-dimethyl- and 3,7,3'-trimethyl-) were used as substrates, further methylation resulted in substitution of positions 6 and/or 3' or 4', respectively. This activity was absolutely dependent on Mg$^{2+}$ ions (Fig. 8). However, further O-methylation of 3,6,7-trimethylquercetagetin proceeded poorly and did not require Mg$^{2+}$ ions.

Commercial kaempferol, dihydroquercetin, rhamnetin (7-methylquercetin) and myricetin (5'-hydroxyquercetin) all contained varying amounts of quercetin as contaminant (Appendix Fig. 2). Therefore, these substrates were further purified prior to use for substrate specificity studies (C.3.1.). Upon removal of the quercetin
Figure 8. Photograph of an autoradiograph of the chromatographed reaction products (Solvent A, Table 3) of partially purified (desalted ammonium sulfate pellet) DMT preparation with the indicated substrates. Numbers preceding Q (quercetin) and Qg (quercetagetin) indicate the methylated positions of these substrates. The reaction products were identified by co-chromatography with reference compounds: (1) 3-methyl-Q; (2) 3,7-dimethyl-Q; (3) 3,7,4′-trimethyl-Q; (4) 3,6,7-trimethyl-Qg; (5) 3,7,4′-trimethyl-Qg (6) 3,6,7,4′-tetramethyl-Qg; (7) 3,6,7,3′-tetramethyl-Qg.

Columns a, b, c, e, f and h all contained 10 mM MgCl₂ in the assay mixture (section C.6.), whereas d and g did not contain this divalent cation.
Table 4. Methyl acceptor ability of partially purified OMT preparation

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (EA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin</td>
<td>100</td>
</tr>
<tr>
<td>3,7-Dimethylquercetin</td>
<td>80</td>
</tr>
<tr>
<td>3,7-Dimethylquercerin</td>
<td>68</td>
</tr>
<tr>
<td>3,7,3'-Trimethylquercetin</td>
<td>65</td>
</tr>
<tr>
<td>3-Methylquercetin</td>
<td>32</td>
</tr>
<tr>
<td>3,6,7-Trimethylquercetin</td>
<td>15</td>
</tr>
<tr>
<td>3,7,4'-Trimethylquercetin</td>
<td>9</td>
</tr>
<tr>
<td>Quercetagetin</td>
<td>6</td>
</tr>
<tr>
<td>Dihydroquercetin</td>
<td>3</td>
</tr>
<tr>
<td>Kaempferol</td>
<td>3</td>
</tr>
<tr>
<td>Luteolin</td>
<td>3</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>3</td>
</tr>
</tbody>
</table>

*Partially purified enzyme preparation (Section C.3.1.) desalted on Sephadex G-25 and assayed as described in Section C.6.1.*

*Total methylaing activity of quercetin (control = 100%) amounted to 13,000 dpm/mg protein.*
contaminant, kaempferol and dihydroquercetin were not accepted for methylation by the OMT system of *Chrysosplenium*, whereas rhamnetin and myricetin were poorly accepted (25 and 5% of quercetin activity in Table 4).

Phenylpropanoids such as caffeic or ferulic acids, and flavones such as luteolin or its 7-glucoside were not accepted as substrates (Table 4) indicating the specificity of this enzyme system towards flavonols.

D.3. The OMTs of *C. americanum*

D.3.1. Separation of three distinct flavonol OMTs

Preliminary experiments on the purification of the enzyme system of *C. americanum* were described in sections C.3.1. to C.3.4., except that 20 g of leaves were used in the extraction procedure, Sephadex G-100 was used instead of Sephacryl S-200 and there was no hydroxyapatite step.

Chromatography of the ammonium sulphate pellet on Sephadex G-100 (Fig. 9A) resulted in the recovery of OMT activity against the five different substrates (Fig. 6). All activity was confined to a discrete peak whose molecular weight was larger than ovalbumin and which coincided with the molecular weight of BSA (65,000).

Further purification of the enzyme protein was achieved by chromatofocusing on Polybuffer ion exchanger and resulted in three peaks with OMT activity (Fig. 9B). Activity peak I catalysed the methylation of quercetin and gave one product which was identified as 3-methylquercetin (Fig. 9C). The second peak (II) catalysed further O-methylation of 3,7-dimethylquercetin and 3,7-dimethylquercetatigin
Figure 9. Characterization of the 3-, 6- and 4'-OMTs from C. americanum

A. Sephadex G-100 chromatography (1.5 x 70 cm) of partially purified OMTs from a resuspended 30-70% (NH₄)₂ SO₄ pellet (4 ml). The sample was applied and 2-ml fractions were collected at a rate of 9 ml/hr. OMT activity was assayed using 3,7-dimethylquercetagatin (●) and quercetin as methyl acceptors. The quercetin methylating activity co-eluted with the 6- and 4'-OMTs and was 50% as active as the former activities.

B. Chromatography on Polybuffer ion exchanger (1 x 28 cm) of active fractions (10 ml) from Sephadex G-100, as described in section C.3.4. OMT activity was assayed using quercetin (●) and 3,7-dimethylquercetagatin (●) as substrates.

C. Continued on next page
Figure 9. Continued

C. Photograph of an autoradiogram of the chromatographed reaction products of the 3-, 6- and 4'-OMT peaks. Peak I was only active against quercetin and transformed this substrate into 3-methylquercetin (3-methyl Q) whereas peak II accepted 3,7-dimethylquercetagetin for O-methylation at the 4' position of the A-ring. In addition, Peak III transformed 3,7,3'-trimethylquercetagetin to the 6-substituted tetramethylated derivative. The reaction products were identified by co-chromatography with reference compounds as described in Fig. 8.
to their respective 4'-methyl derivatives. Peak III O-methylated 3,7-dimethylquercetin and 3,7,3'-trimethylquercetin to their respective 3,6,7-tri- and 3,6,7,3'-tetramethyl ethers (Fig. 9B, C). Attempts to detect the 7-OMT activity on chromatofocused fractions (Fig. 9B) were unsuccessful although partially purified enzyme preparations (Fig. 9A) were active against 3-methylquercetin.

D.3.2. Purification of individual OMTs from C. americanum

In order to purify larger amounts of flavonoid OMTs for kinetic studies, the previous procedure (D.3.) was modified as described in section C.3. The elution profiles of enzyme activities from Sephacryl S-200, hydroxyapatite and chromatofocusing on Polybuffer ion exchanger are shown in Figures 10, 11 and 12 respectively.

Sephacryl S-200 chromatography (Fig. 10) resulted in the elution of all OMTs as a single discrete peak similar to that obtained with Sephadex G-100 (Fig. 9A). However, unlike Sephadex G-100, this column permitted the complete removal of chlorophyll-containing protein. Protein fractions with OMT activity were pooled and chromatographed on a hydroxyapatite column as described in section C.3.3.

Fractions from hydroxyapatite were assayed against the five different substrates resulting in the separation of two major peaks of activity (Fig. 11). Peak 1 was variably active against all five substrates (Fig. 11B, C) whereas peak 2 was only active against quercetin, 3,7-dimethylquercetin and 3,7-dimethylquercetagetin. This purification step permitted the separation of the 6- and 7-OMTs from the majority of the 3- and 4'-enzymes. Each hydroxyapatite peak was
Figure 10. Sephacryl S-200 (2.5 x 118cm) chromatography of partially purified OMTs.

As described in section C.3.2., OMT activity was assayed using quercetin (○), 3-methylquercetin (△), 3,7-dimethylquercetin (●) 3,7-dimethylquercetagetin (○) and 8,7,3′-trimethylquercetagetin (□) as substrates (Fig. 6).
Figure 11. Hydroxyapatite chromatography of partially purified OMT from Sephadryl S-200.

The procedure was as described in section C.3.3. Note that the high conductivity (▲) was initially due to 0.5 M NaCl present in the sample being applied onto the column. OMTs were assayed using quercetin (▪), 3-methylquercetin (◇), 3,7-dimethylquercetin (■), 3,7-dimethyl-quercetagetin (◇) and 3,7,3'-trimethyl-quercetagetin (◇) as substrates (Fig. 6).
Figure 12. Chromatography on Polybuffer ion exchanger (1 x 28 cm) of peaks 1 and 2 from hydroxyapatite.

A. Chromatofocusing of 6- and 7-OMTs on Polybuffer ion exchanger. Fractions 41-50 from the hydroxyapatite column were applied to and eluted from this column as described in section C.3.4.

B. Chromatofocusing of 3- and 4'-OMTs on Polybuffer ion exchanger. Fractions 51-63 from the hydroxyapatite column were applied to and eluted from this column as described in section C.3.4. OMTs were assayed using quercetin ( ), 3-methylquercetin ( ), 3,7-dimethylquercetin ( ), 3,7-dimethylquercetagetin ( ) and 3,7,3'-trimethylquercetagetin ( ) as substrates.

C. Continued on next page
C. Photograph of an autoradiogram of the chromatographed reaction products of purified 3-, 6- and 4'-OMTs. The 3-OMT activity transformed quercetin (Q) to 3-methyl-Q (3Q); the 7-OMT from hydroxyapatite (Fig. 11, peak 1) accepted 3Q to give 3,7-dimethylquercetin (3,7Q); the 4'-OMT methylated either 3,7Q or 3,7-dimethylquercetagetin (3,7Qg) to their respective trimethylated derivatives (3,7,4'Q and 3,7,4'Qg); the 6-OMT transformed 3,7,3'-trimethylquercetagetin to the 6 methylated derivative (3,6,7,3'-Qg). The reaction products were identified by co-chromatography with reference compounds as described in Fig. 8.
subsequently submitted to chromatofocusing on Polybuffer ion exchanger using Polybuffer PB-74. The 6-OMT was eluted at its apparent isoelectric point (pH 5.77) free from contaminating 3- or 4'-activities (Fig. 12A). The 7-OMT activity was lost during chromatofocusing, possibly due to instability of the enzyme in the purified form, or to the loss of some factor required for its activity. The 3- and 4'-OMTs were further separated on a second Polybuffer ion exchanger and eluted at pH 4.8 and 5.4, respectively (Fig. 12B). This procedure assured that the 6- and 4'-OMTs whose apparent pI's differed only by 0.3 pH unit were not cross-contaminated.

The combined purification steps described above resulted in an increase in specific activity of the three focused enzymes of 85-, 92- and 164-fold for the 3-, 6- and 4'-OMTs, respectively, as compared with those of the crude enzyme preparation (Table 5). The 7-OMT which co-purified with the 6-OMT on hydroxyapatite showed a 6-fold increase in specific activity.

Examination of enzyme preparations after SDS PAGE revealed that the purified enzymes were contaminated with several bands of protein (Appendix I, Fig. 3A). Furthermore, partially purified enzyme preparations (Sephadex G 100, Fig. 9A) were active when extracted from nondenaturing gels (Appendix I, Fig. 3B), whereas purified preparations (after chromatofocusing) were inactivated by this procedure. OMT activity against quercetin, 3,7-dimethylquercetin and 3,7-dimethylquercetagetin could be localized in the same region of the gel, indicating that the 3-, 6- and 4'-OMTs behaved similarly under these electrophoretic conditions.
Table 5. Purification of *Chrysosplenium* O-methyltransferases

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (pkat/mg)</th>
<th>Total activity (pkat)</th>
<th>Purification (-fold)</th>
<th>Recovery %</th>
</tr>
</thead>
<tbody>
<tr>
<td>3'-OMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract b</td>
<td>103</td>
<td>0.62</td>
<td>64</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Amm. sulphate c</td>
<td>80</td>
<td>0.55</td>
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<td>0.9</td>
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<tr>
<td>Sephacryl S-200 20</td>
<td>20</td>
<td>1.5</td>
<td>31</td>
<td>2.4</td>
<td>48</td>
</tr>
<tr>
<td>Hydroxyapatite d</td>
<td>5</td>
<td>5.9</td>
<td>29</td>
<td>9.5</td>
<td>45</td>
</tr>
<tr>
<td>PBE-94 e</td>
<td>0.25</td>
<td>53</td>
<td>13.3</td>
<td>85</td>
<td>21</td>
</tr>
<tr>
<td>6'-OMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract b</td>
<td>103</td>
<td>0.42</td>
<td>50</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Amm. sulphate c</td>
<td>80</td>
<td>0.55</td>
<td>44</td>
<td>1.1</td>
<td>88</td>
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<tr>
<td>Sephacryl S-200 20</td>
<td>20</td>
<td>1.66</td>
<td>33</td>
<td>3.4</td>
<td>66</td>
</tr>
<tr>
<td>Hydroxyapatite d</td>
<td>5</td>
<td>3.1</td>
<td>15.5</td>
<td>6.4</td>
<td>31</td>
</tr>
<tr>
<td>PBE-94 e</td>
<td>0.25</td>
<td>44</td>
<td>11.1</td>
<td>92</td>
<td>22</td>
</tr>
<tr>
<td>4'-OMT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Crude extract b</td>
<td>103</td>
<td>0.84</td>
<td>84</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>Amm. sulphate c</td>
<td>80</td>
<td>0.94</td>
<td>74</td>
<td>1.2</td>
<td>88</td>
</tr>
<tr>
<td>Sephacryl S-200 20</td>
<td>20</td>
<td>1.06</td>
<td>53</td>
<td>1.3</td>
<td>63</td>
</tr>
<tr>
<td>Hydroxyapatite d</td>
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<td>6.1</td>
<td>31</td>
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</tr>
<tr>
<td>PBE-94 e</td>
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<td>120</td>
<td>30</td>
<td>164</td>
<td>38</td>
</tr>
</tbody>
</table>

See following page for footnotes a-g.
Footnotes for Table 5.

a The purification process was performed with 86 g fresh material and the substrates used for the 3-, 6- and 4'-OMTs were quercetin, 3,7,3'-trimethylquercetagenin and 3,7-dimethylquercetin, respectively. The 7-OMT which co-eluted with 6-OMT until the hydroxyapatite step was purified 6-fold.

b After treatment with Dowex 1X2 and desalting on Sephadex G-25.

c 30-70% salt saturation, then desalted on Sephadex G-25.

d Peak 2 from hydroxyapatite column.

e Chromatofocusing column #2.

f Peak 1 from hydroxyapatite column.

g Chromatofocusing column #1.
D.3.3. Substrate specificity of purified OMTs

The 3-OMT, which focused at pH 4.8, exhibited expressed specificity towards the 3-position of flavonols with 3',4'-(or 4',5') hydroxylation pattern such as quercetin, but not quercetagetin (Table 6). Furthermore, the latter did not act as a substrate inhibitor when added at concentrations of up to 80 µM to enzyme assays (Fig. 13). Introduction of a methyl group at the 7-position of quercetin (as in rhamnetin) resulted in a 70% drop in activity, whereas methyl substitution at the 3'-position (as in isorhamnetin) or 4'-position (as in tamarixetin) resulted in complete loss of activity.

Furthermore, the latter compounds were good product inhibitors with inhibition constants of the same order of magnitude as for 3-methylquercetin (Appendix I, Fig. 4). When myricetin was used as a possible substrate, enzyme activity dropped by 90% (Table 6). Such strict position specificity indicates that quercetin is the best substrate for this enzyme and that 3-methylation may be the first step in the sequential methylation of these flavonols.

The 7-OMT, which was studied in the hydroxyapatite fraction, accepted 3-methylquercetin for further methylation at the 7-position (Table 6). 3-Methylquercetagetin was not available as substrate, however, the enzyme was unable to methyleate any of the mono-, di- or trimethyl quercetins tested. The expressed specificity of this enzyme towards the 7-position of quercetin suggests that 7-methylation is the next step in the sequential methyl transfers of Chrysosplenium flavonoids.
Figure 13. Inhibition of 3-OMT by alternate substrate.

1/v versus 1/Q (quercetin) at various fixed concentrations of quercetin (Qg). Each incubation mixture contained 6 g/ml enzyme protein, 10 mM MgCl₂, 10 mM 2-mercaptoethanol and 10% DMSO (in 100 mM phosphate buffer, pH 8) and the reaction was carried out at 30°C for 30 min. Velocities were expressed as μM/s/mg protein.
Table 6. Substrate specificity of Chrysosplenium D-methyltransferases$^a,b$

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)$^c$</th>
<th>3-OMT</th>
<th>6-OMT</th>
<th>7-OMT$^d$</th>
<th>4'-OMT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quercetin (Q)</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Rhamnetin-(7-Methyl-Q)</td>
<td>30</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3-Methyl-Q</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>8-Hydroxykaemferol</td>
<td>0</td>
<td>25</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Quercetalgetin (Qg)</td>
<td>0</td>
<td>5</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>3,7-Dimethyl-Q</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>3,7-Dimethyl-Qg</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>95</td>
<td></td>
</tr>
<tr>
<td>3,7,3'-Trimethyl-Qg</td>
<td>0</td>
<td>40</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Enzyme preparations were used after chromatofocusing (for the 3-, 6- and 4'-OMTs) or after hydroxyapatite (for 7-OMT).

$^b$ The standard enzyme assay was used (Section C.6.1.) using 0.1 - 1.0 µM of the indicated substrates.

$^c$ The methylating activities of these enzymes (100%) amounted to 6500, 14000, 4500 and 9000 dpm/assay for the 3-, 6-, 7- and 4'-OMTs, respectively.

$^d$ The substrate specificity for the 7-OMT was generated considering that this preparation was contaminated with 6-OMT; therefore, the activities against 3,7-dimethyl- and 3,7,3'-trimethylquercetagentin were deducted accordingly.
The two other protein fractions which focused at pH 5.4 and 5.8 accepted partially methylated flavonol intermediates for further O-methylation at the 4'- and 6-positions, respectively. The 4'-OMT converted both 3,7-dimethylquercetin and its 6-hydroxy analog equally well to their respective 4'-methyl derivatives, whereas the 6-OMT accepted 3,7-dimethylquercetin and 3,7,3'-trimethylquercetin producing their respective 6-methyl derivatives at a ratio of 2:1 (Table 6). The fact that the 4'-OMT did not react with trimethyl substrates suggests that 4'-methylation may precede that at position 6; thus establishing the third and fourth steps in the sequential methylation of these flavonols.

None of the four enzymes described here exhibited any activity with phenylpropanoids, flavones, dihydroflavonols or any of their glucosides thus indicating their specificity towards hydroxylated and partially methylated flavonols (Table 6).

D.3.4. Other enzyme properties
D.3.4.a. pH optima

The O-methylating activity of each enzyme was measured against its best substrate in the presence of histidine-HCl, imidazole-HCl, Tris-HCl, and glycine-NaOH, buffers over a pH range of 6.0-9.5. The curves obtained (Fig. 14) show the relative activities of the four OMTs as well as their pH optima which ranged between 7.8 and 9.0. However, there was a gradual shift in optimal activity, from lower to higher pH, with increasing methylation of the substrate used. Therefore, 3- and 7-OMTs which utilised quercetin and 3'-methylquercetin, respectively exhibited their optimal activities at
Figure 14. pH optima of purified OMTs after chromatofocusing, except for the 7-OMT where the partially purified extract from hydroxyapatite (Fig. 11, peak 1) was used. The substrates used were quercetin for the 3-OMT; 3-methylquercetin for the 7-OMT; 3,7-dimethylquercetin for the 4'-OMT; and both 3,7-dimethylquercetin and 3,7,3'-trimethylquercetin for the 6-OMT.
pH 7.8-8.2; whereas those of the 6- and 4'-OMTs, which accepted di- or trimethyl substrates, were between pH 8.8 and 9.0 (Fig. 14).

D.3.4.b. Requirement for co-factors

Of the three purified enzymes studied, the 6-OMT showed absolute requirement for Mg²⁺ (Table 7). The latter could not be replaced by other cations tested. Further investigation of the effect of Mg²⁺ indicated that this activation was saturable (Fig. 15A). Lineweaver-Burke plots (Fig. 15A insert) were linear with 30 μM Mg²⁺ required for half maximal saturation. When the effect of EDTA was investigated (Table 5, Fig. 15B), it was found to inhibit the 6-OMT, whereas it had no effect on the activity of the 3- and 4'-OMTs.

The addition of 14 mM 2-mercaptoethanol in the assay mixture did not increase any of the four enzyme activities, as compared with those of the controls. However, SH-group inhibitors such as PCMB or NEM, were potent inhibitors of enzyme activity (Table 7). The former was particularly effective at 1 mM in inhibiting the three OMTs, whereas the addition of 14 mM 2-mercaptoethanol partially prevented this inhibition.

D.3.4.c. Molecular weight

Partially purified enzyme preparations (C.3.1.) or individual OMTs (C.3.5.) were chromatographed on Sephacryl S-200 which had been calibrated with standard proteins. Both partially purified enzymes and purified OMTs had the same elution volume which corresponded to an approximate molecular weight of 57,000 (Fig. 16).
Table 7. Effect of inorganic ions and other reagents on OMT activity

<table>
<thead>
<tr>
<th>Additions</th>
<th>Concentration (mM)</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>3-OMT</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt; (control)</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>0</td>
<td>95</td>
</tr>
<tr>
<td>Mn&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>1</td>
<td>110</td>
</tr>
<tr>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>K&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1</td>
<td>115</td>
</tr>
<tr>
<td>EDTA</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>EDTA</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>14</td>
<td>105</td>
</tr>
<tr>
<td>PCMB (minus ME)</td>
<td>1</td>
<td>33</td>
</tr>
<tr>
<td>PCMB 14 mM ME</td>
<td>1</td>
<td>90</td>
</tr>
<tr>
<td>N-Ethylmaleimide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>48</td>
</tr>
<tr>
<td>Iodoacetamide&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1</td>
<td>105</td>
</tr>
</tbody>
</table>

<sup>a</sup> The standard enzyme assay was used (Section C.6.1.) using 0.5 μM of quercetin, 3,7-dimethylquercetagetin and 3,7-dimethylquercetin as substrates for the 3-, 6- and 4'-OMTs, respectively.

<sup>b</sup> In absence of 2-mercaptoethanol.
Figure 15. Effect of Mg$^{2+}$ and the metal chelator EDTA on OMT activity.

A. Mg$^{2+}$ saturation curves for the 3- (→) 4'- (←) and 6- (○) OMT activities. A Lineweaver-Burke plot of the latter (inset) gave an activation constant of 30 μM.

B. Effect of EDTA on the 6-OMT activity in the presence of 0.38 (←), 0.76 (→), and 3.8 (→) mM of Mg$^{2+}$Cl$_2$. 
Figure 16. Determination of Molecular Weight of OMTs

Using a calibrated Sephacryl S-200 column

(2.5 x 118 cm)
D.3.4.d. Stability of 3-, 6-, and 4'-OMTs

Except for the partially purified (C.3.1.) enzyme which lost 50% of its activity after 15 days (Fig. 17A), the chromatofocused fractions were quite stable upon storage in 20% glycerol, 5 mM DTT and 0.2M phosphate, pH 8 at -20°C for several months (Fig. 17B).

D.3.5. Localisation studies: OMTs and Glucosyltransferases (GTs) from Chrysosplenium

Preliminary studies on the localisation of the flavonoid specific OMTs and GT were performed as described in section C.9. These experiments demonstrated that all of the OMT activity could be found on top of the gradient indicating their presence in the cytosol (Fig. 18). However, it should be noted that in this system, we cannot differentiate between cytoplasmic and vacuolar constituents. In addition, there seemed to be two GT activities, one cytoplasmic and the other bound to some unidentified membrane fraction. The cytoplasmic activity was present only if a suitable flavonoid substrate was added to the assay mixture, whereas the membrane-bound activity could be detected even without the addition of flavonoid substrate, indicating the glucosylation of some endogenous extractable acceptor. Furthermore, when radioactive products from assays of the respective fractions were submitted to TLC on Polyamide, followed by autoradiography, the cytoplasmic fraction gave products corresponding to the glucosylated flavonoid substrate, whereas the membrane-bound activity produced a single spot which chromatographed with the solvent front (Table 8).
Figure 17. Time course of 3-, 6- and 4'-OMT inactivation

A. Partially purified OMT preparations (section C.3.1.) were stored at 4°C and assayed several times over the course of 5 weeks.

B. Purified 3-, 6- and 4'-OMTs were stored at -20°C and assayed over the course of several months. This represents the time course of inactivation for the 3-OMT, and is representative of the 6- and 4'-OMTs as well.
Figure 18. Fractonation of organelles from *C. americanum* leaf cells.

Sucrose concentrations (---) are expressed on a % w/w basis and (----) is absorbance at 280. The (M) represents the usual location of mitochondria and, in this case, broken chloroplasts as well. The (C) represents intact chloroplasts. Glucosyltransferase activity (---) was assayed using 2'-hydroxy-3,7,4'-trimethylquercetin as substrate. OMTs (---) were assayed with 3,7-dimethylquercetin, a substrate for the 6- and 4'-OMTs; 3,7-dimethylquercetin was 60% as active (specific for the 4' OMT) and quercetin specific for the 3-OMT was 25% as active. No OMT activity could be found beyond 20% w/w sucrose.
Table 8. Identification of reaction products of *Chrysosplenium* O-glucosyltransferases isolated on sucrose gradients.

<table>
<thead>
<tr>
<th>Fraction number&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Substrate</th>
<th>Identification of products&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Relative amount (%)</th>
<th>R&lt;sub&gt;f&lt;/sub&gt;&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1-10)</td>
<td></td>
<td>2'</td>
<td>50</td>
<td>0.55</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5'</td>
<td>50</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td></td>
<td>no products</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(13-18)</td>
<td></td>
<td>unknown</td>
<td>100</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td>unknown</td>
<td>100</td>
<td>0.95</td>
</tr>
</tbody>
</table>

<sup>a</sup> Fractions 1-10 and 13-18 were pooled (Fig. 8) and glucosyltransferase activity was assayed as described by Bajaj (103).

<sup>b</sup> 2'-hydroxy-3,7,4'-trimethylquercetin (#1, Fig. 3) was used as substrate and the products formed were 2'- and 5'-O-glucosylated derivatives.

<sup>c</sup> The reaction products were identified by co-chromatography with reference compounds, visualization in UV light and autoradiography.

<sup>d</sup> On Polyamide 6-MN TLC plates developed in toluene-ethyl formate-ethanol-water (60:20:19:1).
It is clear from these preliminary studies that neither the OMTs nor the GT appeared to be associated with either the chloroplasts or mitochondria of this tissue. However, more detailed studies should be carried out in order to confirm these results as well as to exclude other possible microcompartments (e.g. endoplasmic reticulum).

D.4. The OMTs of *Lotus corniculatus*

D.4.1. Comparative OMT activity of different organs of *Lotus*

The O-methylating activity of partially purified extracts (C.4.1.) of different organs of the plant was compared using three flavonol substrates: quercetin, 8-hydroxyquercetin and 8-hydroxykaempferol and one phenylpropanoid substrate, caffeic acid. The results, shown in Table 9, clearly indicate that the flowering buds exhibited the highest methylating activity towards flavonol substrates and the lowest towards caffeic acid. Both apical buds and mature leaves showed a similar degree of flavonoid O-methylation which was 50-80% lower than that of flowering buds, whereas activity towards caffeic acid was greater in mature leaves than apical buds. Three week-old seedlings, on the other hand, exhibited the lowest degree of flavonoid methylation which amounted to 15% of that of flower buds.

It is interesting to note that the pattern of methylated products formed from each flavonoid substrate was similar for the different plant organs used (Table 10). Both 8-hydroxykaempferol and 8-hydroxyquercetin were methylated either at the 3- or 8- positions at a ratio of 2:1. Quercetin on the other hand, was equally methylated at positions 3 and 3', with trace amount of label found in
Table 9. O-Methylating activity of different organs of *Lotus*\(^a\)

<table>
<thead>
<tr>
<th>Plant organ</th>
<th>8-Hydroxy-kaempferol (DPM/g)</th>
<th>8-Hydroxy-quercetin (DPM/g)</th>
<th>Quercetin (DPM/g)</th>
<th>Caffeic acid (DPM/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Three-wk-old seedlings</td>
<td>2350</td>
<td>2150</td>
<td>2150</td>
<td>1100</td>
</tr>
<tr>
<td>Apical buds</td>
<td>4200</td>
<td>5760</td>
<td>8750</td>
<td>2800</td>
</tr>
<tr>
<td>Mature leaves</td>
<td>4050</td>
<td>6020</td>
<td>6750</td>
<td>4900</td>
</tr>
<tr>
<td>Young flower buds</td>
<td>19750</td>
<td>15600</td>
<td>14290</td>
<td>1000</td>
</tr>
</tbody>
</table>

\(^a\) Buffer extracts of the indicated organs desalted on Sephadex G-25.

\(^b\) The standard enzyme assay was used with the indicated substrates as described in Section C.6.1. See Table 10 for identification of reaction products.
Table 10. Identification of the reaction products of *Lotus* OMTs\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Reaction products(^b)</th>
<th>Methyl derivative</th>
<th>Relative amount (%)(^c)</th>
<th>(R_f) value(^d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-Hydroxy-kaempferol</td>
<td>8-</td>
<td>30</td>
<td>0.65</td>
<td></td>
</tr>
<tr>
<td>8-Hydroxy-quercetin</td>
<td>8-</td>
<td>30</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>3-</td>
<td>70</td>
<td>0.30</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3'-</td>
<td>50</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Dimethyl(^e)</td>
<td>45</td>
<td>0.55</td>
<td></td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>3-</td>
<td>90</td>
<td>0.65</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Plant organs were used as source of enzyme as described in Table 9.

\(^b\) Identified by co-chromatography with reference compounds and autoradiography.

\(^c\) As % of total activity shown in Table 9.

\(^d\) On Polyamid-6 using benzene-methyl ethyl ketone-methanol (70:15:15) as solvent system.

\(^e\) 3,3'- or 3,7-Dimethylquercetin.
dimethylated products (Table 10). These results clearly indicate the presence of 8- and 3'-OMT activities in the flower buds of _Lotus_, together with another enzyme activity which attacks position 3 of quercetin, 8-hydroxyquercetin or 8-hydroxykaempferol.

D.4.2. OMT activities in _Lotus_ flower buds

Further characterization of OMT activity was carried out with flower buds at different stages of development (C.4.2) (Table 11). Enzyme activities which catalysed the methylation of positions 3, 8 and 3' of flavonols were assayed with 8-hydroxykaempferol and quercetin whereas caffeic OMT activity was assayed with caffeic acid. The results indicated that 8-OMT activity steadily increased until the third stage of development and then decreased, whereas 3- and/or 3'-OMT activity was already high at stage one and decreased rapidly after stage two (Table 11). On the other hand, caffeic OMT remained uniformly low throughout development, which corresponds to the lack of lignification in flower tissues.

D.4.3. Separation of 8- and 3'-OMTs in _Lotus_ flower buds

OMTs from young flower buds were extracted and purified as described in sections C.4.2.-C.4.4. Chromatography on Sephadex G-100 resulted in a single peak of activity (Fig. 19A) which catalysed the methylation of positions 8, 3 or 3' of different flavonoid substrates. Further purification of the latter by chromatofocusing on Polybuffer ion exchanger resulted in two peaks of OMT activity which focused at pH 5.5 and 5.1 (Fig. 19B).
Table 11. OMT activities of *Lotus* flower buds at different stages of development\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>OMT activity ((\mu)M / mg / min)(^b)</th>
<th>OMT activity ((\mu)M / flower / min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OH-kaempferol</td>
<td>1.88 1.61 2.15 1.73 0.80</td>
<td>0.04 0.08 0.14 0.03 0.01</td>
</tr>
<tr>
<td>Quercetin</td>
<td>1.81 1.18 0.60 0.52 0.36</td>
<td>0.04 0.06 0.04 0.01 0.005</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>0.26 0.14 0.18 0.34 0.38</td>
<td>0.005 0.01 0.01 0.005 0.005</td>
</tr>
</tbody>
</table>

\(^a\) Flower buds at different stages of development were extracted as described in Section C.4.2. without fractionation with ammonium sulphate. Extracts were desalted on Sephadex G-25 and used as the enzyme source.

\(^b\) Enzyme assays were carried out as described in Section C.6.1. Values represent total activity in reaction products.
Figure 19. The OMTs of *Lotus* flower buds

OMT activity was assayed using 8-hydroxykaempferol (−) and quercetin (−) as substrates.

A. Chromatography on Sephadex G-100 (2 x 100 cm) of partially purified OMTs as described in sections C.4.2 - C.4.3.

B. Chromatofocusing on Polybuffer ion exchanger (1 x 28 cm) as described in section C.4.4.

C. Photograph of an autoradiograph of the chromatographed reaction products of the 8- and 3'-OMTs peaks from chromatofocusing. The 8-OMT was assayed against 8-hydroxykaempferol (8-OHK) and gave 8-methoxykaempferol (8-MeK) as a major reaction product. The 3'-OMT was assayed against Quercetin (Q) and 3'-methylquercetin (3-MeQ) and gave 3'-methylquercetin (3'-MeQ) and 3,3'-MeQ as the major reaction products. The reaction products were identified by co-chromatography on Polyamide 6-MN TLC plates with reference compounds, 8-MeK, 3'-MeQ and 3,3'-MeQ. The solvent system used was benzene-methyl ethyl ketone-methanol (70:15:15).
Whereas the Sephadex G-100 fraction catalysed the methylation of a number of flavonoid substrates, the chromatofocused peak 1 exhibited preferential affinity for 8-hydroxyflavonols and gave rise to their 8-methyl derivatives (Table 12). On the other hand, peak 2 methylated 8-hydroxyflavonols as well as luteolin, quercetin and 3-methylquercetin. Examination of the autoradiographed reaction products (Fig. 19C) clearly indicated that peak 1 contained predominantly an 8-OMT activity; whereas peak 2 consisted mainly of 3- and 3'-OMTs.

D.4.4. Separation of OMT activities in Lotus shoots

The OMT profiles of shoot tips were studied using the methods described in sections C.4.2.-C.4.4. A single OMT peak was obtained from Sephadex G-100 (Fig. 20A) similar to that of flower buds (D.4.3.). However, chromatofocusing of active fractions from Sephadex G-100 resolved two peaks of OMT activity at pH 6.1 and 5.2 (Fig. 20B) instead of 5.5 and 5.1 (Fig. 19B). Furthermore, peak 1 (pH 6.1) was very active against quercetin but not 8-hydroxykaempferol, whereas peak 2 (pH 5.2) was more active against 8-methylquercetin than either 8-hydroxykaempferol or quercetin. These results indicated that the flavonoid OMTs present in shoots represent a different group of enzymes from those present in flower buds.

D.4.5. Purification of 8-OMT from Lotus flower buds

The purification procedure described in section C.5.-C.5.3. was carried out in order to purify enough enzyme for kinetic studies. Sephadex G-100 chromatography (Fig. 21A) resulted in a single peak of
Table 12. Substrate specificity of Lotus QMTs at different stages of purification

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)</th>
<th>Sephadex G-100</th>
<th>Peak 1</th>
<th>Peak 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-OH-kaempferol</td>
<td>100</td>
<td>100</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>8-OH-quercetin</td>
<td>80</td>
<td>84</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>Quercetin</td>
<td>45</td>
<td>30</td>
<td>57</td>
<td></td>
</tr>
<tr>
<td>8-MeO-quercetin</td>
<td>10</td>
<td>7</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>Luteolin</td>
<td>32</td>
<td>10</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td>3-Me-quercetin</td>
<td>19</td>
<td>7</td>
<td>52</td>
<td></td>
</tr>
<tr>
<td>7-Me-quercetin</td>
<td>16</td>
<td>6</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Quercetagetin</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*a* Peaks 1 and 2 were recovered after chromatofocusing on Polybuffer ion exchanger (PBE-94).

*b* Control activities (100%) amounted to 17500, 5500 and 2400 dpm/assay for the Sephadex G-100, peak 1 and peak 2, respectively.

*c* The following substrates were assayed and found to be poor methyl acceptors (<5% activity): 3'; 4'; 3',4'; 3,7'; 7,4' and 3,3',4'-methylquercetin, as well as 3,7- and 3,7,3'-methyquercetagetin; kaempferol.
Figure 20. The OMTs of Lotus shoots

OMT activity was assayed using 8-hydroxy-kaempferol (■), quercetin (□) and 3-methylquercetin (△) as substrates.

A. Chromatography on Sephadex G-100 (2 x 100 cm) of partially purified OMTs as described in sections C.4.2. - C.4.3.

B. Chromatofocusing on Polybuffer ion exchanger (1 x 28 cm) as described in section C.4.4.
activity similar to the previous profile (Fig. 18A). Active fractions were applied to SAH-Agarose (Fig. 21B) which resulted in partial separation of quercetin methylating activity from the 8-OMT. The enzyme was concentrated and further purified by chromatography on hydroxyapatite (Fig. 21C) where the activities against quercetin and 8-hydroxykaempferol co-eluted. Final separation of these two activities was achieved by chromatofocusing on Polybuffer ion exchanger (Fig. 21D) where the 8-OMT activity was eluted at pH 5.6. This purification procedure, described in Table 13, resulted in a 1200-fold purification and a 4% yield of the total 8-OMT in the crude enzyme preparation.

D.4.6. Properties of the purified 8-OMT

D.4.6.a. Substrate specificity

The extensively purified 8-OMT was shown to possess strict position specificity for the 8-position of 8-hydroxykaempferol and 8-hydroxyquercetin and did not accept quercetin as substrate (Table 14). Furthermore, TLC of radioactive products on Polyamide (as described in Table 7) followed by autoradiography indicated that 8-methoxykaempferol was the only reaction product formed when 8-hydroxykaempferol was used as substrate. It is interesting to note that quercetagetin, the 6-hydroxy derivative of quercetin was not accepted for methylation at position 6, whereas the corresponding flavone, 6-hydroxyapigenin was a poor methyl acceptor. This is in contrast with the 6 OMT found in Chrysosplenium, where 8-hydroxykaempferol was a good methyl acceptor (Table 6).
Figure 21. Purification of 8-CMT from Lotus flower buds

OMT activity was assayed using 8-hydroxy-
kaempferol (■), and quercetin (□) as
substrates.

A. Chromatography on Sephadex G-100 (2 x 100 cm)
as described in section C.5.

B. Chromatography on SAH-Agarose (1 x 5 cm) as B.described in section C.5.1.

C. Chromatography on hydroxyapatite as described
in section C.5.2.

D. Chromatofocusing on Polybuffer ion exchanger
as described in section C.5.3.
Table 13. Purification of *Lotus* 8-O-methyltransferase

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Specific activity (pkat/mg)</th>
<th>Total activity (pkat)</th>
<th>Purification (-fold)</th>
<th>Recovery (%)</th>
</tr>
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<tbody>
<tr>
<td>Dowex 1X2</td>
<td>98</td>
<td>0.27</td>
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<tr>
<td>Ammonium sulphate</td>
<td>52</td>
<td>0.42</td>
<td>21.85</td>
<td>1.5</td>
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<tr>
<td>Sephadex G-100</td>
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<tr>
<td>SAH-Agarose</td>
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<td>7.48</td>
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<td>28</td>
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<tr>
<td>Hydroxyapatite</td>
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<td>2.32</td>
<td>1230</td>
<td>9</td>
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<tr>
<td>PBE-94</td>
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<td>-</td>
<td>-</td>
<td>1.03</td>
<td>4</td>
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</tbody>
</table>

*The purification procedure was performed with 20 g fresh material and the substrate used with enzyme assays was 8-OM-kaempferol.

30-70% salt saturation, after desalting on Sephadex G-25.

Protein content of the chromatofocused fraction was too low to be measured with the Bio-Rad method.
Table 14. Substrate specificity of purified *Lotus* 8-OMT<sup>a,b</sup>

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity (%)&lt;sup&gt;c&lt;/sup&gt;</th>
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<tr>
<td>8-Hydroxyquercetin</td>
<td>100</td>
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<tr>
<td>8-Hydroxykaempferol</td>
<td>61</td>
</tr>
<tr>
<td>6-Hydroxyapigenin</td>
<td>13</td>
</tr>
<tr>
<td>Luteolin</td>
<td>7</td>
</tr>
<tr>
<td>3,7-Dimethylquercetagetin</td>
<td>6</td>
</tr>
<tr>
<td>8-Methoxyquercetin</td>
<td>5</td>
</tr>
<tr>
<td>3,7-Dimethylquercetin</td>
<td>4</td>
</tr>
<tr>
<td>Quercetin</td>
<td>4</td>
</tr>
<tr>
<td>3-Methylquercetin</td>
<td>4</td>
</tr>
<tr>
<td>3,7,3'-Trimethylquercetagetin</td>
<td>2</td>
</tr>
<tr>
<td>Quercetagetin</td>
<td>2</td>
</tr>
</tbody>
</table>

<sup>a</sup> The enzyme preparation used was that recovered after the chromatofocusing step (Table 13).

<sup>b</sup> The standard enzyme assay was used (Section C.6.1.) with 1.5 μM of the indicated substrates.

<sup>c</sup> The total methylating activity with 8-OH-quercetin (control = 100%) amounted to 17000 dpm/assay.
D.4.6.b. pH optimum

The 8-OMT activity was measured against 8-hydroxykaempferol in the presence of 0.1M phosphate buffer over a pH range of 6.1-8.4. The curve obtained (Fig. 22A) showed an optimum between pH 7-8.4 with maximal activity at pH 7.9.

D.4.6.c. Requirements for cofactors

The activity of the 8-OMT was increased 4-fold upon the addition of MgCl₂ to the reaction mixture (Fig. 22B). However, upon the addition of 1 mM EDTA to the assay mixture, the enzyme activity was completely abolished in the absence of Mg²⁺ ions (Fig. 22B).

D.4.7. Enzyme kinetics of the C. americanum OMTs

Preliminary experiments (Appendix I, Fig. 5) established that the reaction was linear with time at all concentrations of SAM and flavonoid substrate used.

D.4.7.a. Inhibition by flavonoid substrate

When the flavonoid substrate was varied at a constant SAM concentration, Michaelis-Menten kinetics were observed below Km whereas higher flavonoid concentrations resulted in substrate inhibition (Fig. 23). Above 10 uM flavonoid, substrate inhibition was most notable with the 6-OMT whereas the other two enzymes were only slightly inhibited. Such inhibitions may have resulted from the combination of the substrate with the wrong enzyme form and/or from decreased solubility of the flavonoid substrate at higher concentrations. The binding of substrate with the wrong enzyme form
Figure 22. pH optima and Mg$^{2+}$ requirement for 8-OMT of Lotus

A. pH optima of 8-OMT with 200 mM phosphate buffer.

B. Mg$^{2+}$ saturation curve for the 8-OMT in the presence and absence of 1 mM EDTA.
Figure 23. Substrate inhibition by flavonol substrate

1/v versus 1/Flavonol at constant SAM concentrations (76.6 μM, of which 16.6 μM was $[^{14}\text{CH}_3]$-SAM containing 220,000 dpm). Each incubation mixture contained 6 μg/ml of the 3-,4'- or 6-OMTs, 10 mM MgCl$_2$, 14 mM 2-mercaptoethanol, 5% DMSO in 100 mM phosphate buffer pH 8. The amount of $^{14}$CH$_3$ labelled flavonol formed was determined after 30 min. incubation at 30°C. Velocities are expressed as μM/s/mg protein. Q: 3,7-Q; 3,7,3’Qg; and 3,7-Qg represent quercetin, 3,7-dimethylquercetin and 3,7-dimethylquercetin respectively.
usually occurs at high substrate concentrations and/or when the reaction is studied in the non-physiological direction (117).

However, the substrate inhibition observed in Fig. 23 occurred neither at high substrate concentrations nor was it in the non-physiological direction. When SAM was the variable substrate the reaction was saturable without apparent substrate inhibition.

D.4.7.d. Substrate interaction kinetics

Since the 3-, 6- and 4'-OMTs showed Michaelis-Menten kinetics and substrate inhibition was observed with flavonol, therefore, substrate interaction kinetics were performed at flavonol concentrations below 10 μM.

Estimates of the parameters derived from nonlinear regression analysis of the substrate interaction kinetic data are shown in Table 15. The variance ratio test indicated that for the 6-OMT, the $K_{ia}$ $K_b$ term was significant for the interaction between SAM and 3,7,3'-trimethylquercetagetin, i.e. the lines of the double reciprocal plot intersect (Fig. 24C and 25C). For the interaction between SAM and quercetin (3-OMT) (Fig. 24A and 25A) or 3,7-dimethylquercetin (Fig. 24B and 25B) equation one did not give a statistically better fit to the data. However, when the data was fitted by a line intercept computer programme (method of least squares), it was apparent that the reciprocal plots (Fig. 24 A-C and 25 A-C) were converging and not parallel.

In a sequential mechanism, represented by equation I (section C.10.) where $K_{ia}$ is the dissociation constant of enzyme and A, double
Figure 24. Initial velocity - $1/v$ versus $1/\text{Flavonol}$ at various fixed concentrations of SAM for the 3-, 6-, and 4'-OMTs.

A. $1/v$ versus $1/Q$ (quercetin) at various fixed concentrations of SAM. Each incubation mixture contained 6 μg/ml of 3-OMT. Other conditions are as in Fig. 23.

B. $1/v$ versus $1/3,7Q$ (3,7-dimethylquercetin) at various fixed concentrations of SAM. Each incubation mixture contained 6 μg/ml of 4'-OMT. Other conditions were as in Fig. 23.

C. $1/v$ versus $1/3,7,3'Qg$ (3,7,3'-trimethylquercetin) at various fixed concentrations of SAM. Each incubation mixture contained 6 μg/ml of 6-OMT. Other conditions were as in Fig. 23.
Figure 25. Initial velocity - $1/v$ versus $1/SAM$ at various fixed concentrations of SAM for the 3-, 6-, and 4'-OMTs.

A. $1/v$ versus $1/SAM$ at various fixed concentrations of quercetin (Q). The data are the same as those shown in Fig. 24A.

B. $1/v$ versus $1/SAM$ at various fixed concentrations of 3,7-dimethylquercetin (3,7Q). The data are the same as those shown in Fig. 24B.

C. $1/v$ versus $1/SAM$ at various fixed concentrations of 3,7,3'-trimethylquercetagetin (3,7,3'Qg). The data are the same as those shown in Fig. 24C.
reciprocal plots will yield a family of lines which intersect at a point dependent on the ratio of $K_{ia}/K_a$. As $K_{ia}/K_a$ decreases, the intersection point becomes more negative, with minus infinity corresponding to parallel lines (equation II). The possibility was, therefore, considered that the mechanisms of the 3- and 4'-OMTs were sequential and that failure of the computer programme to distinguish between the two equations velocity was due to a small value for $K_{ia}/K_a$ which was not resolved by this programme.

In order to determine the value of the $K_{ia}$ to $K_a$ ratio, $K_{ia}$ was calculated from the kinetic constants given in Table 15. If SAM was assumed to be the first substrate to bind to the enzyme, then the value of $K_{ia}$, the dissociation constant for the Enzyme-SAM complex was 5, 7, and 12 $\mu$M for the 3-, 4'- and 6-OMTs, respectively. On the other hand, the values of $K_a$ were 114, 130 and 50 $\mu$M for the 3-, 4'- and 6-OMTs. Therefore, $K_{ia}$ was 23, 18 and 4 times smaller than $K_a$ for the 3-, 4'- and 6-OMTs, respectively and could explain the tendency of the former two enzymes to give parallel double reciprocal plots.

Another factor which favored a sequential binding mechanism for the three enzymes was the fact that product inhibition patterns (see section D.4.7.c.) did not conform to those expected for a ping pong mechanism. Therefore, the kinetic constants given in Table 15 for the substrate interaction kinetics of the 3- and 4'-OMTs were generated from equation I.

The following equation expresses the initial rate for the three enzymes, assuming that SAM is the first substrate to bind, $K_{ia}$ has a
Table 15. Estimates of parameters of substrate interaction kinetics for Chrysosplenium 3-, 6- and 4'-O-methyltransferases\(^a\)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Parameter value</th>
<th>Kinetic pattern</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
<td>Term</td>
</tr>
<tr>
<td>3-OMT</td>
<td>SAM</td>
<td>Quercetin</td>
<td>(K_a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(K_{1a})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(K_{a}/K_{1a})</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(K_b)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(V)</td>
</tr>
<tr>
<td>6-OMT</td>
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<td>3,7,3'-Tri-methylquercetin</td>
<td>(K_a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(K_{1a})</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td>(V)</td>
</tr>
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<td>4'-OMT</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>(V)</td>
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</tbody>
</table>

See following page for footnote
Footnote for Table 15

The parameter values presented for the 6-OMT were those for the kinetic equation, as chosen by the variance ratio test; whereas the values for the 3- and 4'-OMTs were chosen because the postulated sequential mechanism better fits the product inhibition data.

S.D. is standard deviation
non zero value and the presence of more than one binding site for the flavonol substrate.

\[
V = \frac{V[A][B]}{K_{ia}K_{ib} + K_{b}[A] + K_{a}[B] + [A][B] + [A][B]^2/K_{ib}}
\]

D.4.7.c. Product inhibition kinetics

Previous substrate interaction kinetics established that the 3-, 6- and 4'-OMTs followed a sequential rather than ping pong mechanism. Further information on the order of substrate binding was obtained from product inhibition studies. The rate equations derived for a bi bi sequential mechanism (117, 118) predict that the last product released is a competitive inhibitor with respect to the first substrate since both bind to the same enzyme form, whereas non-competitive inhibition is expected with respect to the other substrate.

The results obtained for the 3-OMT (Fig. 26 A-D) are in agreement with a modified version of the theoretical predictions mentioned above. SAH was a competitive inhibitor with respect to SAM (Fig. 26A) and uncompetitive with respect to quercetin (Fig. 26B). 3-Methylquercetin was a noncompetitive inhibitor with respect to both SAM (Fig. 26C) and quercetin (Fig. 26D). The kinetic patterns obtained (Table 16-18) exclude random addition of substrates and/or release of products, in which case four competitive inhibition patterns would be expected. Three (Fig. 26 A, C, D) of the four (Fig. 26B) kinetic patterns obtained are consistent with an ordered
Figure 26. Product inhibition kinetics of the 3-OMT from *Chrysosplenium americanum*

A. Product inhibition by S-adenosyl-L-homocysteine: $1/v$ versus $1/SAM$ (16.6 μM of [14CH₃]-SAM, 0.1 μCi in addition to varying amounts of unlabelled SAM) at different fixed concentrations of SAH and at a constant concentration of quercetin (4 μM). Each incubation mixture contained 6 μg/ml of the 3-OMT, 10 mM MgCl₂, 14 mM 2-mercaptoethanol, 5% DMSO in 100 mM phosphate buffer, pH 8. The amount of 14CH₃ labelled flavonol formed was determined after 30 min incubation at 30°C. Velocities were expressed as μM/s/mg protein.

B. Product inhibition by S-adenosyl-L-homocysteine: $1/v$ versus $1/Q$ (quercetin) at different fixed concentrations of SAH and at constant concentration of SAM (8.3 μM of [14CH₃]-SAM, 0.05 μCi). Other conditions were as in (A).

C & D. Continued on next page
Figure 26. Continued

C. Product inhibition by 3-methylquercetin (3Q): 
1/v versus 1/SAM at different fixed concentrations of 3Q and at constant concentrations of quercetin (4 μM). Other conditions were as in (A), except that DMSO was 10%.

D. Product inhibition by 3-methylquercetin (3Q): 
1/v versus 1/Q (quercetin) at different fixed concentrations of 3Q and at constant concentration of SAM (8.3 μM of [¹⁴CH₃]SAM, 0.05 μCi). Other conditions were as in (A), except that DMSO was 10%.
Table 16. Estimates of parameters of product inhibition kinetics for 3-O-methyltransferase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Parameter value</th>
<th>Kinetic pattern</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>$K'_a$ 25 5.7</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>SAM</td>
<td>SAH</td>
<td>$K'_a$ 44 4.5</td>
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<td></td>
<td>$K_{is}$ 29 5.3</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>$K_{iij}$ 65 9.0</td>
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<td>3-Methylquercetin</td>
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<tr>
<td></td>
<td></td>
<td>$K_{iij}$ 8.4 0.6</td>
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<tr>
<td>Quercetin</td>
<td>SAH</td>
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<td>Noncompetitive</td>
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<tr>
<td></td>
<td></td>
<td>$K_{is}$ 27 2.8</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>$K_{iij}$ 128 36</td>
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<tr>
<td>Quercetin</td>
<td>3-Methylquercetin</td>
<td>$K'_a$ 4.5 0.3</td>
<td>Noncompetitive</td>
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<td></td>
<td></td>
<td>$K_{is}$ 27 2.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{iij}$ 128 36</td>
<td></td>
</tr>
</tbody>
</table>

*a. The parameter values were those chosen for the kinetic equation by a variance ratio test.*
bi bi mechanism where SAM and SAH were leading partners in the reaction.

The results of the product inhibition studies for the 4'-OMT (Fig. 27 A-D) were similar to those of the 3-OMT. When SAM was the variable substrate, SAH (Fig. 27A) was a competitive inhibitor and 3,7,4'-trimethylquercetin (Fig. 27B) a noncompetitive inhibitor. The inhibition by both SAH (Fig. 27C) and 3,7,4'-trimethylquercetin (Fig. 27D) was uncompetitive and noncompetitive, respectively when 3,7-dimethylquercetin was the variable substrate. The presence of SAH at higher concentrations (Fig. 27C inset) enhanced the substrate inhibition observed with 3,7-dimethylquercetin. The kinetic patterns obtained were identical to those for the 3-OMT, indicating that this enzyme also binds substrates and releases products in a given order.

The results obtained for the 6-OMT (Fig. 28 A-D) agree partly with the patterns observed for the 3- and 4'-OMTs. Figure 28A shows that SAH was again a competitive inhibitor when SAM was the variable substrate, whereas 3,6,7,3'-tetramethylquercetagetin (Fig. 28B) was a noncompetitive inhibitor. However, figures 28C and 28D show that both SAH and 3,6,7,3'-tetramethylquercetagetin, respectively, were uncompetitive inhibitors of 3,7,3'-trimethylquercetagetin. However, considering the poor solubility of the tetramethylated product, the results obtained should be interpreted with caution. In contrast with the 4'-OMT, which required higher SAH concentrations to observe substrate inhibition (Fig. 27C inset), the 6-OMT did so at lower concentrations of this inhibitor (Fig. 28D).
Figure 27. Product inhibition kinetics of the 4'-OMT from

Chrysosplenium americanum

A. Product inhibition by S-adenosyl-L-homo-
cysteine: 1/v versus 1/SAM at different fixed
concentrations of SAH and at a constant
concentration of 3,7-dimethylquercetin (3,7Q)
(7 μM). Other conditions were as Fig. 26A
except that 6 μg/ml of the 4'-OMT as used for
assays.

B. Product inhibition by 3,7,4'-trimethylquercetin
(3,7,4'Q): 1/v versus 1/SAM at different
concentrations of 3,7,4'Q and at constant
concentration of 3,7-dimethylquercetin (7 μM)
Other conditions were as in (A) except that the
final DMSO concentration was 10%.

C & D. Continued on next page
C. Product inhibition by S-adenosyl-L-homocysteine: 1/v versus 1/3,7Q (3,7-dimethylquercetin) at different fixed concentrations of SAH and at constant concentration of SAM (8.3 μM of [14CH3]-SAM, 0.05 μCi). Other conditions were as in Fig. 26A. Inset, effect of S-adenosyl-L-homocysteine on substrate inhibition by 3,7Q: 1/v versus 1/3,7Q at different fixed concentrations of SAH and at constant concentration of SAM (8.3 μM of [14CH3]-SAM, 0.05 μCi). Other conditions were as in Fig. 26A.

D. Product inhibition by 3,7,4'-trimethylquercetin (3,7,4'Q): 1/v versus 1/3,7Q (3,7-dimethylquercetin) at different fixed concentrations of 3,7,4'Q and at constant concentrations of SAM (8.3 μM of [14CH3]-SAM, 0.5 μCi). Other conditions were as in Fig. 26A, except that the final DMSO concentrations was 10%.
Figure 28. Product inhibition kinetics of the 6'-OMT from *Chrysosplenium americanum*

A. Product inhibition by S-adenosyl-L-homocysteine: $1/v$ versus $1/$SAM at different fixed concentrations of SAH and at a constant concentration of 3,7,3'-trimethylquercetagetin (3,7,3'Og) (5.8 $\mu$M). Other conditions were as Fig. 26A except that 6 $\mu$g/ml of the 6-OMT was used for assays.

B. Product inhibition of the 6-OMT by 3,6,7,3'-trimethylquercetagetin: (3,6,7,3'Og): $1/v$ versus $1/$SAM at different concentrations of 3,6,7,3'Og and at constant concentration of 3,7,3'-trimethylquercetagetin (5.6 $\mu$M). Other conditions were as in Fig. 28A, except that the final DMSO concentration was 10%.

C & D. Continued on next page
C. Product inhibition of 6-OMT by S-adenosyl-L-homocysteine: 1/ν versus 1/3,7,3′Qg (3,7,3′-trimethylquercetagenin) at different fixed concentrations of SAH and at constant concentration of SAM (8.2 μM of [14CH₃]-SAM, 0.05 μCi). Other conditions were as in Fig. 26A.

D. Product inhibition of 6-OMT by 3,6,7,3′ tetramethylquercetagenin (3,6,7,3′Qg): 1/ν versus 1/3,7,3′Qg (3,7,3′-trimethyl-quercetagenin) at different fixed concentrations of 3,6,7,3′Qg and at constant concentration of SAM (8.3 μM of [14CH₃]-SAM, 0.5 μCi). Other conditions were as in Fig. 26A, except that the final DMSO concentration was 10%.
Table 17. Estimates of parameters of product inhibition kinetics for 6-O-methyltransferase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Product</th>
<th>Parameter value</th>
<th>Kinetic pattern</th>
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</thead>
<tbody>
<tr>
<td>SAM</td>
<td>SAH</td>
<td>$K'_a$ 26</td>
<td>Competitive</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{Is}$ 16</td>
<td></td>
</tr>
<tr>
<td>SAM</td>
<td>3,6,7,3'-Tetra-Me-Qg</td>
<td>$K'_a$ 36</td>
<td>Noncompetitive</td>
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<td></td>
<td></td>
<td>$K_{Is}$ 74</td>
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<td>3,7,3'-Tri-Me-Qg</td>
<td>SAH</td>
<td>n.d.</td>
<td>Uncompetitive</td>
</tr>
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<td>3,6,7,3'-Tetra-Me-Qg</td>
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</tr>
<tr>
<td></td>
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<td>$K_{Ii}$ 167</td>
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*The parameter values were those chosen for the kinetic equation by a variance ratio test.*

*Qg, quercetagetin*

*n.d., not determined*
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<th>Substrate&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> The parameter values were those chosen for the kinetic equation by a variance ratio test.

<sup>b</sup> Q, quercetin
D.5. Enzyme kinetics of Lotus 8-OMT

D.5.1. Saturation with substrate

Preliminary studies on the 8-OMT of Lotus established that saturation with either 8-hydroxykaempferol or SAM gave Michaelis-Menten kinetics (Fig. 29A, B), with no apparent substrate inhibition by either substrate.

D.5.2. Substrate interaction kinetics

Estimates of the parameters derived from non-linear regression analysis of the substrate interaction kinetic data are shown in Table 19. The variance ratio test indicated that the $K_{IA}/K_{IB}$ term was significant for the interaction between SAM and 8-hydroxykaempferol, implying that the lines of the double reciprocal plots intersect (Fig. 30A, B).

The initial velocity results are consistent with a sequential binding mechanism where both substrates must bind prior to any product release, thus excluding a ping pong mechanism. However product inhibition studies are required in order to distinguish between an ordered and a random mechanism.

D.5.3. Product inhibition kinetics

Further information on the order of substrate binding was obtained from product inhibition studies. The results obtained for the 8-OMT (Fig. 31 A-D, Table 20) indicate an ordered binding of substrates and release of products. SAH was a noncompetitive inhibitor with respect to SAM (Fig. 31A) and noncompetitive with respect to 8-hydroxykaempferol (Fig. 31B). 8-Methoxykaempferol was a
Figure 29. Saturation with 8-hydroxykaempferol and SAM as substrate for the 8-OMT of *Lotus corniculatus*.

A. Effect of 8-hydroxykaempferol (8-OHK) concentration on the reaction rate of 8-OMT. The concentration of SAM was 66 μM (containing 10 μM of [1^4 CH_3]-SAM, 0.061 μCi). Inset: double reciprocal plot of 1/v versus 1/8-OHK. Each incubation mixture contained 0.05 μg/ml of 8-OMT, 10 mM MgCl_2, 14 mM 2-mercaptoethanol and 5% DMSO in 100 mM phosphate, pH 8.

B. Effect of SAM concentration on the reaction rate of 8-OMT. (16.6 μM of [1^4 CH_3]-SAM, 0.1 μCi, in addition to varying amounts of unlabelled SAM). The concentration of 8-OHK was 10 μM. Inset: double reciprocal plot of 1/v versus 1/SAM. Other conditions were as in (A).
Figure 30. Substrate interaction kinetics for the 8-OMT of Lotus.

A. Initial velocity. $1/v$ versus $1/8$-hydroxy-kaempferol (8-OHK) at various fixed concentrations of SAM (16.6 $\mu$M of $[^{14}$CH$_3]$-SAM, 0.1 $\mu$Ci), in addition to varying amounts of unlabelled SAM). Each incubation mixture contained 0.05 $\mu$g/ml of 8-OMT, 10 mM MgCl$_2$, 14 mM 2-mercaptoethanol and 5% DMSO in 100 mM phosphate buffer, pH 8. The amount of $^{14}$CH$_3$ labelled flavonol formed was determined after 30 min incubation at 30°C. Velocities are expressed as $\mu$M/s/mg protein.

B. $1/v$ versus $1/8$-OHK fixed concentrations of SAM. The data are the same as those shown in Fig. 29A.
Table 19. Estimates of parameters of substrate interaction kinetics for *Lotus 8-O-methyltransferase*\(^a\)

<table>
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<td></td>
<td>(K_a/K_{ia})</td>
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<tr>
<td></td>
<td>(K_b)</td>
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<td></td>
<td>(V)</td>
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</table>

\(^a\) The parameter values obtained were those for the kinetic equation as chosen by the variance ratio test.
non-competitive inhibitor with respect to SAM (Fig. 31C) and competitive with respect to 8-hydroxykaempferol (Fig. 31D). Therefore, the kinetic patterns obtained (Table 20) exclude random addition of substrates and/or release of products.
Figure 31. Product inhibition kinetics of the 8-OMT from *Lotus corniculatus*.

A. Product inhibition by S-adenosyl-L-homocysteine: $1/v$ versus $1/\text{SAM}$ (16.6 μM of $[^{14}\text{CH}_3]-\text{SAM}$, 0.1 μCi, in addition to varying amounts of unlabelled SAM) at different fixed concentrations of SAH and at a constant concentration of 8-hydroxykaempferol (8-OHK) (0.66 μM). Each incubation mixture contained 0.05 μg/mL of 8-OMT, 10 mM MgCl$_2$, 14 mM 2-mercaptoethanol, 5% DMSO in 100 μM phosphate buffer, pH 8. The amount of $^{14}\text{CH}_3$ labelled flavonol formed was determined after 30 min incubation at 30°C. Velocities are expressed as μM/s/mg protein.

B. Product inhibition by S-adenosyl-L-homocysteine: $1/v$ versus $1/8$-OHK (8-hydroxykaempferol) at different fixed concentrations of SAH and at constant concentrations of SAM (8.3 μM of $[^{14}\text{CH}_3]-\text{SAM}$, 0.05 μCi). Other conditions were as in (A).

C & D. Continued on next page
Figure 31. Continued

C. Product inhibition by 8-methoxykaempferol (8-MeK): $1/v$ versus $1/SAM$ at different fixed concentrations of 8-MeK and at constant concentrations of 8-hydroxykaempferol (0.66 μM). Other conditions were as in (A) except for DMSO which was 10%.

D. Product inhibition of 8-methoxykaempferol (8-MeK): $1/v$ versus $1/8-OHK$ (8-hydroxykaempferol) at different fixed concentrations of 8-MeK and at constant concentrations of SAM (8.3 μM, 0.05 μCi). Other conditions were as in (A) except for DMSO which was 10%.
Table 20. Estimates of parameters of product inhibition kinetics for 8-O-methyltransferase

<table>
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<td></td>
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<td></td>
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<td>$K_{ii}$</td>
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<td>kaempferol</td>
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<td>$K_{is}$</td>
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a The parameter values were those chosen for the kinetic equation by a variance ratio test.
E. DISCUSSION

The results presented here clearly demonstrate the existence in \textit{C. americanum} of four, novel OMTs which exhibited strict position specificity for the 3-, 6-, 7- and 4'-hydroxyl groups of flavonols. In addition, \textit{L. corniculatus} was shown to contain a novel 8-OMT. The fact that none of these enzymes accepted any of the phenylpropanoid compounds tested, or other intermediates of flavonol biosynthesis clearly indicates their expressed specificity for hydroxylated flavonols and their partially methylated derivatives. In view of the differential specificity of the five OMTs for their best substrates, we wish to propose the following systematic names: S-adenosyl-L-methionine:quercetin-3-O-methyltransferase; S-adenosyl-L-methionine:3-methylquercetin 7-O-methyltransferase; S-adenosyl-L-methionine:3,7,3'-trimethyl-quercetagetin 6-O-methyltransferase; S-adenosyl-L-methionine:8-hydroxykaempferol/quercetin 8-O-methyltransferase and S-adenosyl-L-methionine:3,7-dimethylquercetin 4'-O-methyltransferase. Therefore, except for the 5-, 2'- and 5'-positions, the O-methylation of all other hydroxyls on the flavonoid ring system has been demonstrated with these two plant tissues.

\textit{Chrysosplenium} OMTs are quite distinct from the much studied flavonoid enzymes which exhibited a wide range of specificity towards the 3'-position of flavones/flavonols or their glucosides (38-40). The enzymes reported here mediated position-oriented methylations which followed an orderly sequence depicted in Figure 32, thus clearly establishing that O-methylation of flavonoids occurs at the C15 level in \textit{Chrysosplenium} as well as with the \textit{Lotus} 8-OMT. However recent
QUERCETAGETIN (Qg)

QUERCETIN (Q)

3-MeQ

3-MeQg

3,7-MeQg

3,7,4'-MeQg

3,7,4'-MeQ
Figure 32. Proposed pathway for the sequential O-methylation of flavonoids in Chrysosplenium americanum.

Q and Qg are quercetin and quercetagetin respectively, and the numbers preceding Q and Qg indicate their methylated positions. The black arrows demonstrate reactions which have been shown in Chrysosplenium, whereas the white arrows demonstrate potential enzyme reactions which have not been demonstrated.
work with the anthocyanin 3'-OMT of Petunia hybrida (119) and the
vitexin-2'-0-rahmnoside 7-OMT of Avena sativa (120) demonstrated that,
unlike Chrysosplenium and Lotus OMTs, these enzymes accepted only
glycosylated substrates. This suggests that the order of methylation
and glucosylation does not necessarily follow a set pattern, but is
determined by the specific tissue involved.

The fact that feruloyl-CoA (3-methyl-4-hydroxycinnamoyl CoA) was
not converted with any significant efficiency by the chalcone synthase
of Chrysosplenium to the corresponding chalcone or flavanone (D.
Barron, unpublished) suggests that methylation takes place at a later
stage during flavonoid biosynthesis. It is not unexpected, therefore,
that a polyhydroxyflavonol of common occurrence, such as quercetin,
would be the natural methyl acceptor in the multiple sequence of
methyl transfers in Chrysosplenium. This is supported by the fact
that 3-OMT exhibited its highest affinity and strict position
specificity for this substrate. These results are in agreement with
the findings of Brunet and Ibrahim (48) who demonstrated that a number
of 3-hydroxyflavones displayed a high methyl acceptor ability which
correlated with the highest nuclear electron density for the
3-hydroxyl group of flavonols.

Quercetagetin, the 6-hydroxy derivative of quercetin, was neither
accepted for O-methylation nor did it act as a substrate inhibitor.
This raises the question as to how do methylated derivatives of
quercetagetin (Fig. 13) arise in Chrysosplenium. Two possible routes
may be postulated. The first is that quercetin is methylated at
position 3 with subsequent divergence of the pathway into
polymethylated derivatives of quercetin or quercetagetin. Such a hypothesis implies the introduction of a hydroxyl group at position 6 of 3-methylquercetin, or of a later methylated intermediate. The second possibility is that two specific 3-OMTs may be involved, one for quercetin and the other for quercetagetin. However, the latter enzyme has yet to be found in this tissue.

It is interesting to note that neither quercetagetin nor its 3,7-dimethyl- or 3,7,3'-trimethyl derivatives were accepted as substrates by Lotus 8-OMT (Tables 12, 14) whereas the 6-OMT of Chrysosplenium did methylate the 8-position (being meta to the side chain) of 8-hydroxykaempferol. The fact that quercetagetin was not accepted for O-methylation by the 3-, 6- or 8-OMTs, indicates that hydroxylation of position 6 may well take place after quercetin has undergone partial methylation, contrary to earlier proposals (14, 121). Furthermore, the same hypothesis may be true for the hydroxylation at the 2'-position of Chrysosplenium flavonoids (Fig. 3), since any change in the substitution of ring B of quercetin resulted in almost complete loss of activity (Tables 4 & 6). The validity of the latter hypothesis could be tested if a sample of 2'-hydroxyquercetin was available for use as a substrate.

Different OMTs were shown to occur in Lotus flowers (Fig. 19B) as compared with shoot tips (Fig. 20B). 8-OMT activity was found primarily in the flowering stage which coincided with the presence of 8-methoxyquercetin, 8-methoxykaempferol and 8-methoxyisorhamnetin in this tissue (106). Furthermore, the vegetative parts of Lotus contained only the methylated product isorhamnetin (106) and displayed
low 8-OMT activity as compared with the quercetin methylating activities (3- and 3'-OMTs) (Fig. 20B). These results indicate a tissue-specific control of OMT expression in Lotus as a function of morphological differentiation. Therefore, the 8-OMT is expressed during flowering for the production of 8-O-methylated flavonols that are responsible for the intense yellow color of flower petals in this species (106).

The five OMTs reported here had pH optima similar to those reported for other flavonoid-specific enzymes (38-44, 119, 120), whereas their molecular weights were intermediate between those of the flavone/flavonol (38-40) and the isoflavone OMTs (41). The 3-, and 4'-OMTs displayed no Mg$^{2+}$ requirement and were not inhibited by EDTA. Similar results were reported for the isoflavone 4'-OMT of Cicer arietinum (41) and the vitexin-2'-O-rhamnoside 7-OMT of Avena (120).

On the other hand, the 6- and 8-OMTs showed absolute requirement for Mg$^{2+}$ and their activity was inhibited by EDTA. Similar results were reported for the 3'-OMT of parsley cell suspension cultures. (38). Very recently, two distinct N-methyltransferases involved in the stepwise methylation of phosphatidylethanolamine to phosphatidylincholine were isolated from rat liver microsomes. The first enzyme had an absolute requirement for Mg$^{2+}$ ions and was shown to be limiting the rate of synthesis of these phospholipids (49). It is not certain whether the absolute requirement for Mg$^{2+}$ by the 6- and 8-OMTs has any significance in the regulation of O-methylation in Lotus and Chrysosplenium.

Preliminary studies on the subcellular localisation of OMTs and GT in Chrysosplenium (Fig. 18) seem to indicate that none of these
enzymes could be localised within any of the cell organelles. Instead, these reactions seem to take place in the cytosol. Similarly, it was postulated that the enzymes of the latter part of the pathway of flavonol synthesis (70, 71) as well as methionine synthase and methionine adenosyltransferase (65), the enzymes catalysing the synthesis of SAM, were localised in the cytoplasm. These results are in sharp contrast with those of Charrière-Ladreix et al. (72) who claimed that the stepwise methylation of flavonols takes place on the chloroplast membrane. The cytoplasmic localisation of the anthocyanin 3′-OMT from the flowers of *Petunia hybrida* (122) and the demonstration that ultracentrifugation caused non-specific adhesion of this OMT to membranes, confirm that flavonol OMTs are probably not associated with organelles such as mitochondria or chloroplasts.

A cytoplasmic localisation of OMTs and GT raises the question as to the assembly and accumulation of the final products in *C. americanum*. Many of the partially methylated intermediates and end products of this pathway are mostly lipophilic and thus, sparingly soluble in the aqueous environment of the cytoplasm. Therefore, both intermediates and end-products could be compartmented within a loose protein aggregate where these intermediates could be channeled rapidly through the biosynthetic sequence (123), without coming in contact with the cytoplasmic pool. Furthermore, it may be suggested that the enzymes of flavonol synthesis are associated with a membrane system where the final hydrophobic products could be sequestered and/or shuttled towards the site of accumulation. The fact that polymethylated flavonol glucosides of *Chrysosplenium* were found on top
of sucrose gradients (Fig. 18), which may contain cytoplasmic as well as vacuolar contents, seems to indicate that the plant vacuole could be the site of accumulation of these compounds. This hypothesis is supported by the fact that a number of secondary products of plant metabolism accumulate in the central vacuole of the plant cell (122, 124, 125). However, a more rigorous approach is required to provide conclusive evidence for the localisation of these enzymes and their methylated end-products in this tissue.

The purification procedures utilized for the OMTs of both Chrysosplenium and Lotus were successful in separating a number of flavonoid-specific OMTs. The isolation of these OMTs would not have been achieved without the use of chromatofocusing on Polybuffer ion exchanger (Fig. 14 & 25C). Furthermore, the availability of specific flavonoid substrates made it possible to purify these enzymes and study their substrate specificity. These facts may explain why such enzymes could not be studied previously.

It should be noted that purified Chrysosplenium OMT preparations were extensively contaminated with other proteins (Appendix 1, Fig. 3A). This problem is related to the fact that, unlike the enzymes of primary metabolism, those of secondary metabolism are low in abundance. Furthermore, the amount of protein found in plants is extremely low as compared with that in animal tissues. Gulliver and Tipton (126) isolated 55 mg of pure catechol OMT (with a 25% yield) from 1 Kg of porcine liver containing 700 g of protein. Assuming that the abundance of Chrysosplenium OMTs was similar to that of the porcine liver, then 72 Kg of plant material would be required to
isolate similar amounts of flavonoid OMTs from *Chrysoplehium*. This comparison illustrates one of the many difficulties involved in obtaining purified enzymes from plant materials.

The 3-, 6-, and 4'-OMTs of *C. americanum* exhibited similar steady state kinetic characteristics. One noticeable difference with respect to substrate inhibition was observed, namely that the 6-OMT was severely inhibited by substrate concentrations close to \( K_m \) whereas the other two enzymes, though inhibited, were less affected by their respective flavonoid substrates. In view of the substrate inhibition observed with these three enzymes, substrate interaction kinetics were carried out at flavonoid substrate concentrations below \( K_m \).

Substrate interaction kinetics were performed with the 3-, 4'- and 6-OMTs. For the 6-OMT, both the computer programme (Table 15) and the plotting of data by the method of least squares (Fig. 24C) indicated converging Lineweaver-Burke plots, suggesting a sequential reaction mechanism. For the 3- and 4'-OMTs, the computer programme indicated that the equation for a sequential mechanism (equation I) did not give a significantly better fit than the equation for a ping pong mechanism (equation II) (Appendix II, Table VI), whereas the data plotted by the method of least squares gave clearly converging lines (Fig. 24A, B). Therefore, the data suggest either a ping pong mechanism giving a series of parallel lines or a sequential mechanism giving a series of quasi-parallel lines (79). As stated in the results, the degree of convergence will depend on the value of \( K_{iA} \), the dissociation constants for the first substrate to bind. Assuming
that SAM was the first substrate to bind, the apparent dissociation constants (K_{ia}) for the enzyme-SAM complex were calculated from the values of K_{ia} K_{b} and K_{b} given in Table 15. It is remarkable that the values so obtained were similar for each OMT, and might be identical given that K_{ia} was derived from kinetic constants with standard deviations close to 20%. Assuming an average K_{ia} value of 8 \mu M for all three OMTs (as calculated from Table 15), this value would be 14, 16 and 6 times smaller than K_{a} for the 3-, 4'- and 6-OMTs, respectively. The fact that the K_{ia} values were small relative to K_{a} may explain why the computer programme could not better fit the data for the 3- and 4'-OMTs to equation I than II.

Since the substrate interaction kinetics provided equivocal results for two of the three Chrysosplenium OMTs, more information was obtained from product inhibition studies. In a ping pong mechanism, SAM must bind first and SAH be released prior to binding of flavonol substrate. Therefore, a methylated enzyme intermediate is produced in which case SAM and SAH will bind to different forms of the enzyme that are reversibly connected. Slope and intercept effects would be expected resulting in noncompetitive inhibition between SAM and SAH. However, the inhibitions observed for the 3-, 6- and 4'-OMTs were competitive (Fig. 26A, 27A & 28A). Similarly, the inhibition by the flavonol product with SAM as variable substrate should be competitive for a ping pong mechanism, whereas the inhibition observed for the three enzymes was noncompetitive (Fig. 26C, 27B & 28B). Furthermore, the fact that three out of four inhibition patterns could not be fitted to those expected for a ping pong mechanism suggests that the
3-, 6- and 4'-OMTs followed a sequential binding mechanism where both substrates had to bind prior to any product release.

Assuming that the mechanism for the 3-, 6- and 4'-OMTs of C. americanum is sequential, the binding of substrates and the release of products could be random or ordered. In a random mechanism four competitive product inhibition patterns would be expected (79); except when dead-end inhibition occurs, alternative patterns are possible. Coward et al. (34), studying rat liver catechol-OMT, proposed that this reaction proceeds by random binding of substrates and products with the formation of a dead-end complex between SAM and the methylated products, metanephrine or paraneprine. This mechanism was proposed because these two products were noncompetitive inhibitors against SAM as the variable substrate, rather than giving the expected competitive inhibition. However, SAH was a competitive inhibitor against either SAM or epinephrine (methyl acceptor) as the variable substrate and suggested that this inhibitor was competing with both substrates for the same enzyme form. The results obtained from our studies with the 3-, 6- and 4'-OMTs indicated that competitive inhibition was observed only between SAM and SAH (Fig. 26A, 27A and 28A), whereas uncompetitive inhibition was observed between the flavonoid substrate and SAH (Fig. 26B, 27C & 28C). Therefore, most product inhibition patterns obtained for Chrysosplenium OMTs could not be fitted to those expected for a random mechanism.

In an ordered mechanism one competitive and three noncompetitive product inhibition patterns would be expected (79). In the case of the 3- and 4'-OMTs of Chrysosplenium, three out of four kinetic
patterns obtained fit this mechanism whereas in the case of the 6-OMT, two out of four patterns are consistent with ordered binding of substrates and release of products. The 'simplest' mechanism that fits the kinetic data for substrate interaction and product inhibition of *C. americanum* OMTs is that shown in Figure 33. In this reaction sequence, A represents SAM; B, the flavonoid substrate; P, the flavonoid product and Q, SAH. All three enzymes exhibited the common kinetic behavior that SAH was a competitive inhibitor of SAM. This observation is consistent with the hypothesis that SAM and SAH are leading reaction partners in the catalytic cycle, with the flavonoid substrate or product binding only when the SAM-SA H site is occupied. Similarly, the other data would fit this reaction mechanism if the effect of substrate inhibition was taken into account.

At high concentrations, substrates will often act as dead-end inhibitors particularly when the reaction is being studied in the non-physiological direction (117, 118). We found that each of these enzymes was inhibited by its respective flavonoid substrate at concentrations near *K_m* values and occurring in the physiological direction (Fig. 23). Furthermore, inhibition by the flavonoid substrate at concentrations below *K_m* was observed when SAH was added to enzyme assays (Fig. 27C inset and Fig. 28C). Thus, in two cases (6- and 4'-OMTs) the abortive complex EQB could be distinguished kinetically and might explain why SAH was an uncompetitive inhibitor when flavonoid was the variable substrate (Fig. 26B, 27C & 28C).

Uncompetitive substrate inhibition is characteristic of ordered systems and occurs when B reacts in dead-end fashion with EQ (Fig. 33) (79, page 824).
Figure 33. Proposed kinetic mechanism for the 3-, 6- and 4'-OMTs of *Chrysosplenium americanum*: A, S-adenosyl-L-methionine; B, flavonol substrate; P, methylated flavonol product; Q, S-adenosyl-L-homocysteine.
A dead-end inhibitor will affect the slope of the double reciprocal plot if (a) it and the varied substrate combine with the same enzyme form or (b) it combines before the varied substrate with an enzyme form that is reversibly connected to the form combining with the varied substrate (79). A dead-end inhibitor affects the intercept of a reciprocal plot if it and the varied substrate combine with different enzyme forms (79). In the case of Chrysosplenium OMTs, the flavonol (B, Fig. 33) would act both as a substrate and as a dead-end inhibitor; the substrate combines with E-SAM (EA) and the dead-end inhibitor combines with E-SAH (EQ). Furthermore, the reversible connection between these two enzyme forms is broken since there is a product release step (P) and free E is removed to form E-SAH-Flavonol. Therefore, inhibition by SAH against flavonol as variable substrate gives only the intercept effects seen in Figures 26B, 27C and 28C. All other inhibitions were noncompetitive (Figures 26C, D; 27B, D; 28B) except for the 6-OMT where the flavonol product was uncompetitive with the flavonol substrate (28D). The latter inhibition is difficult to explain in terms of an ordered binding mechanism, since an abortive EAP or EP complex would give noncompetitive inhibition patterns. However, as stated before, the problems encountered with the solubility of the tetramethylated product may limit the value of the latter results (Fig. 28D). Given the overall similarity in inhibition patterns for the three enzymes, it is reasonable to suggest that they follow the same reaction sequence (Fig. 33).
Similar substrate interaction and product inhibition kinetics were observed for the five charge isozymes of rabbit adrenal glands (76, 77) which catalyze the N-methylation of norepinephrine. Several isozymes gave quasi-parallel double reciprocal plots when the interaction of substrates was investigated, and in the case of isozyme 3, SAH was an uncompetitive inhibitor of norepinephrine. The only characteristic that was common to all isozymes was the competitive inhibition observed between SAM and SAH. Therefore, a model similar to that shown in Figure 33 was proposed which also included a number of abortive complexes.

Substrate inhibition which does not normally occur at physiological concentrations may be of interest in that it may set upper limits to the \textit{in vivo} levels of these flavonoid substrates. For example, the different sensitivities of muscle and heart isozymes of lactate dehydrogenase to substrate inhibition by pyruvate reflect the different pyruvate levels in these two organs (127, 128). Similarly, the five norepinephrine N-methyltransferase isozymes of rabbit adrenal glands had different sensitivities to substrate inhibition and were postulated to appear at various stages of growth of the animal depending on the requirements for epinephrine (76, 77). The fact that \textit{Chrysosplenium} OMTs were inhibited by concentrations above 10 \( \mu \text{M} \) of the flavonoid substrate indicates that their \textit{in vivo} levels may be substantially lower than this value. Furthermore, the low levels of SAH effective in enhancing inhibition of flavonoid substrate might be physiologically significant. Alternatively, these data may indicate a very tight control of the levels of all substrates and products which would serve to regulate the methylation sequence in this plant.
The kinetic parameters (Table 15) showed that the three enzymes displayed similar affinities for their respective flavonoid substrates as indicated by their apparent $K_d$ values. However, the affinity for SAM ($K_d$) was similar for the 3- and 4'-OMTs whereas it was at least 2 times greater for the 6-OMT. The numerical value of $K_m$ is of interest (79, page 34) since this may establish a measure for the intracellular level of the substrate. For example, if the intracellular concentration of SAM was much smaller than $K_m$, then the reaction rate would be very sensitive to changes in total SAM concentration, but most of the catalytic potential of the enzyme would be wasted since $v$ would be much smaller than $V_{\text{max}}$. Previous studies with plant tissues (55, 56) have established the levels of SAM at 16 $\mu$M whereas in animal tissues (53) this level was 40 $\mu$M. Assuming that *Chrysosplenium* contained levels of SAM corresponding to 16 $\mu$M, that $V_{\text{max}}$ was similar for all three enzymes and that the levels of other substrates were saturating, then the in vitro velocity for the 3-, 4' and 6-OMTs would be 12, 11 and 24% of $V_{\text{max}}$, respectively. These calculations reflect the higher affinity of the 6-OMT for SAM and suggest that these enzymes would only be operating at a fraction of their maximal efficiencies under such conditions while being very sensitive to changes in SAM concentration. Such predictions, however, may not take into account other known or unknown variables which may affect the rates of these enzyme-catalysed reactions. It would be of interest to determine the actual concentrations of substrates and products which occur in *Chrysosplenium* in order to better evaluate the former predictions.
Whereas the 3-, 6- and 4'-OMTs of *Chrysosplenium* showed similar types of product inhibition, there were marked differences in the magnitude of their apparent inhibition constants (Table 16-18). These observations could provide further support for the view that the reactants, both substrates and intermediate products, of the OMT sequence play an important role in the regulation of each enzyme and thus, the whole pathway.

Poulton *et al.* (54) suggested that transmethylation reactions in plants might be controlled by the intracellular SAM/SAH ratio and Cantoni (53) found that some methyltransferases were more sensitive to this ratio than others. It can be seen in Table 21 that when the $K_m$ for SAM is high relative to the apparent $K_i$ for SAH, as demonstrated for the 3- and 4'-OMTs of *Chrysosplenium*, the percent relative activity decreases. However, when the $K_m$ for SAM approaches the apparent $K_i$ for SAH (6-OMT) the percent relative activity increases. Therefore, the 3- and 4'-OMTs were more sensitive to inhibition than the 6-OMT. These characteristics suggest that OMTs earlier in the sequence of O-methylation may regulate the rate of O-methylation in *Chrysosplenium*. It is interesting to note that the isoflavone 4'-OMT from *Cicer* (41); the two anthocyanin 3'/5'-OMTs from *Petunia* (129) and the caffeic OMT from soyabean (130), which catalyse final methylation steps, showed a behavior similar to that of the 6-OMT rather than the 3- or 4'-OMTs (Table 21). Whether these findings will correlate with the behavior of these enzymes *in vivo* remains to be established. However, we believe that the *Chrysosplenium* system demonstrates how the SAM/SAH ratio could serve to regulate the overall pathway of methylation in this tissue.
Table 21. Effect of SAM/SAH ratio on O-methyltransferase activity

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Kinetic pattern</th>
<th>( K_m ) SAM (( \mu M ))</th>
<th>( K_m/K_i )</th>
<th>% Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-OMT</td>
<td><em>C. americanum</em></td>
<td>C</td>
<td>114</td>
<td>4.5</td>
<td>25.3</td>
</tr>
<tr>
<td>4'-OMT</td>
<td><em>C. americanum</em></td>
<td>C</td>
<td>130</td>
<td>4.4</td>
<td>29.5</td>
</tr>
<tr>
<td>6-OMT</td>
<td><em>C. americanum</em></td>
<td>C</td>
<td>51</td>
<td>16</td>
<td>3.2</td>
</tr>
<tr>
<td>4'-OMT</td>
<td><em>Cicer arietinum</em></td>
<td>C</td>
<td>160</td>
<td>30</td>
<td>5.3</td>
</tr>
<tr>
<td>3'/5'-OMT</td>
<td><em>Petunia hybrida</em></td>
<td>C</td>
<td>50</td>
<td>70</td>
<td>0.7</td>
</tr>
<tr>
<td>3'/5'-OMT</td>
<td><em>Petunia hybrida</em></td>
<td>C</td>
<td>95</td>
<td>70</td>
<td>1.35</td>
</tr>
<tr>
<td>Caffeic OMT</td>
<td><em>Glycine max</em></td>
<td>C</td>
<td>15</td>
<td>6.9</td>
<td>2.2</td>
</tr>
<tr>
<td>8-OMT</td>
<td><em>L. corniculatus</em></td>
<td>NC</td>
<td>53</td>
<td>4</td>
<td>13.2</td>
</tr>
<tr>
<td>7-OMT</td>
<td><em>Avena sativa</em></td>
<td>NC</td>
<td>1.6</td>
<td>2.5</td>
<td>0.64</td>
</tr>
</tbody>
</table>

\( a \) SAM 42.4 \( \mu M \), SAH 10.6 \( \mu M \) (55)

\( b \) SAM 16 \( \mu M \), SAH 10.6 \( \mu M \) (55)

\( c \) The enzymes from *C. americanum* were: quercetin 3-OMT, 3,7-dimethylquercetin 4'-OMT, 3,7,3'-trimethylquercetin 6-OMT; *C. arietinum*, 3s-biflavone 4'-OMT (41); *P. hybrida*, anthocyanin 3'/5'-OMT (129).

See following page for footnotes b-g
Footnotes to Table 21 (contd.)

contd. *G. max*, caffeic acid 3-OMT (130); *L. corniculatus*, 8-hydroxy-
kaempferol/8-hydroxyquercetin 8-OMT; *A. sativa*, vitexin-2"-O-rhamnoside
7-OMT (120).

C, competitive; NC, noncompetitive

e When the kinetic pattern for product inhibition was competitive the
following equation was used to determine % relative activity:

\[
\text{Inhibition} = \frac{[I]}{[I] + K_i[I]} \times 100
\]

\[
[I] + K_i[I]
\]


f Assuming that SAM and SAH are leading reaction partners (Fig. 34b).

g When the kinetic pattern for product inhibition was noncompetitive,
the following equation was used to determine the percentage
relative activity:

\[
\text{Inhibition} = \frac{[I]}{K_i + [I]}
\]

In both e and g the % relative activity was obtained from 100% minus
% inhibition.

Also, the effects of other products has not been considered in this
determination.
Our studies with *Chrysosplenium* OMTs indicate that flavonoid products were good inhibitors of the reaction (Table 16-18). In general, flavonoid product concentrations in the same range of $K_m$ for flavonol were required to inhibit the reaction (Fig. 26D, 27D & 28D). It is interesting to note that the Michaelis constant for the glucosylation of the flavonoid substrate (104), the last step in the biosynthesis of polymethylated flavonols, was in the same concentration range (10 $\mu$M) as those found for the OMTs studied here. On the other hand, the apparent inhibition constant for the glucosylated product was in the mM range (104) indicating that glucosylation was not inhibited by high concentrations of products formed. The fact that mono- and dimethylated flavonol intermediates do not accumulate *in vivo* (102), suggest that only catalytic amounts of each intermediate would be synthesized for its utilization by the next enzyme in the biosynthetic sequence, until final products are formed. Therefore, inhibition by flavonol product may not be important in regulation of methylation if methylated intermediates do not accumulate.

The product inhibition patterns which were observed for the 8-OMT from *L. corniculatus* could be expected for a steady-state ordered bi bi mechanism with 8-hydroxykaempferol binding before SAM, followed by the release of SAH and 8-methoxykaempferol (Fig. 34a). An alternative mechanism which may also fit the data is the mono-iso Theorell-Chance mechanism with the inverse binding sequence proposed above and an isomerization of the free enzyme (92) (Fig. 34b). The latter hypothesis is attractive in that SAM and SAH would be leading reaction
Figure 34. Proposed kinetic mechanisms for the 8-DMT of *Lotus corniculatus*

A. A, 8-hydroxykaempferol; B, S-adenosyl-L-methionine; P, S-adenosyl-L-homocysteine; Q, 8-methoxykaempferol.

B. Order of substrate binding and release of products is the inverse of (A).
partners, as was found in most other methyltransferase systems studied (41, 51, 77, 78). However, a distinction between both mechanisms can only be obtained by binding studies.

Very recently, a vitexin 2'-O-rhamnoside 7-OMT from *Avena sativa* (oat) was purified and characterized kinetically (120). The kinetic patterns generated for substrate interaction and product inhibition were identical to those of the *Lotus* 8-OMT. The authors favored the mechanism shown in Figure 34b, based on the efficient binding of the *Avena* enzyme to SAH-Sepharose. Unlike the 7-OMT from oat which was eluted from SAH-Sepharose only in the presence of SAM, the *Lotus* 8-OMT did not bind as tightly to SAH-Agarose (Fig. 218) and was eluted from this column with 0.1M NaCl gradient. Furthermore, both enzymes were inhibited noncompetitively by SAH when SAM was the variable substrate and competitively by the flavonoid product when the flavonoid was the variable substrate. Therefore, these two enzymes differ from those of *Chrysosplenium* as well as other OMTs (Table 21) since the inhibition between SAM and SAH for the latter was competitive.

It is interesting to note that the degree of inhibition caused by a competitive inhibitor depends on the concentration of the substrate, that of the inhibitor, the $K_m$ and the $K_i$. However, in the case of a noncompetitive inhibitor the degree of inhibition depends only upon the concentration of inhibitor and the $K_i$. Therefore, in the latter case, when the concentration of inhibitor is equal to $K_i$, 50% inhibition will be observed at all substrate concentrations. Thus, the 7-OMT of *Avena* and the 8-OMT of *Lotus* require that the level of SAH be kept very low for the continuation of these reactions (Table
On the other hand, the 3-, 6- and 4′-OMTs of Chrysosplenium were variably active at SAH concentrations similar in magnitude to those of SAM. These examples illustrate how the mechanism of the reaction could determine the requirements for regulation of different methyltransferases. Therefore, the regulation of methyltransferase activity may not always be controlled by the intracellular SAM/SAH ratio, but by the concentration of SAH only.

The kinetic data obtained for the four OMTs reported here cannot, however, exclude other uncommon reaction mechanisms. For example, a covalent enzyme-methylated intermediate cannot be totally excluded. If the methyl group from SAM was transferred to the enzyme, but SAH could not dissociate before the binding of flavonoid substrate, sequential kinetics are obtained (131).

The steric course of the one-carbon transfer to nucleophilic O, N, S or C atoms of different acceptor molecules has been investigated for several SAM-dependent methyltransferases. All transfer reactions examined so far resulted in an inversion of configuration of the transferred methyl group. Therefore, a direct transfer of the methyl group from SAM to the acceptor substrate via an SN2-type mechanism appears to be involved (81-86). Based on the results from binding studies with different affinity gels, the ordered mechanism was also postulated for three OMTs from cell cultures of Ruta graveolens; two different furanocoumarin OMTs and a caffeic OMT, respectively (51).

It is tempting to postulate that the synthesis of polymethylated flavonoids in Chrysosplenium may occur on the surface of a protein aggregate (123). It is evident from our work that if such an
aggregate exists in vivo, the various components appear to be loosely associated, unlike the membrane-bound pathway of cyanogenic glucoside biosynthesis (132) or the multifunctional protein of the arom complex (133). One of the problems associated with the latter systems (132, 133) was the difficulty in studying the individual enzyme reactions involved. In contrast, we have not been able to isolate a protein aggregate catalysing the sequence of methylation in this tissue. Whereas the results reported here do not establish the existence of an OMT aggregate in Chrysosplenium, however, the following lines of evidence tend to support this concept: (a) incorporation of 14C-cinnamate into end-products after 5-10 min pulse, without labelling of low methylated intermediates, (b) sequential multistep methylation of quercetin at positions 3, 7 and 4' by cell free extracts, (c) absence of mono- and dimethylated intermediates among the products which accumulate in vivo, (d) similarity of the kinetic mechanism of the enzymes studied, (e) regulation of all enzymes studied by a very specific range of substrate and product concentrations as shown by kinetic constants, (f) the presence of inhibition by flavonoid substrate in the three OMTs studied. This indirect evidence, however, does not unequivocally demonstrate the existence of a protein aggregate, and presents a major challenge to be investigated in future research on the OMT system of Chrysosplenium.
F. REFERENCES


G. APPENDIX

G.1. Appendix I: Miscellaneous figures.

G.2. Appendix II: Statistical Tables.

G.3. Appendix III: Perspectives for future work.
Appendix I.

Figure 1. Incorporation of $^{14}$C-cinnamic acid into methylated flavonol glucosides of Chrysosplenium.

The labelled products are numbered as in figure 3. It should be noted that except for number 2, all the flavonols which accumulate in this tissue were readily labelled. The other major spots of radioactivity do not correspond to flavonoids and remain to be identified.

The reaction products were chromatographed on Polyamide 6-MN TLC plates in two directions:

1. Toluene-ethyl formate-ethanol-water (60:20:19:1)
2. Water-n-butanol-acetone-dioxane (70:15:10:5)

(102) followed by autoradiography.
Appendix 1.

Figure 2. Effect of impure substrates in the study of substrate specificity for the 3-QMT.

Myricetin (M) (5'-hydroxyquercetin) and rhamnetin (R) (7-methylquercetin) both contained quercetin (Q) impurities which accounts for the major incorporation of radioactivity into 3-methylquercetin. Products were chromatographed with reference compounds on Polyamide 6-MN TLC plates in solvent A (Table 3) followed by autoradiography.
Appendix I.

Figure 3A. Photograph of Coomasie Blue stained proteins submitted to SDS-PAGE as described in section C.3.7.1.

Lane 1 is desalted (NH₄)₂SO₄ pellet (120 µg); lane 2 is after Sephacryl S-200 (24 µg) (Fig. 10); lane 3 is hydroxyapatite peak 1 (30 µg) (Fig. 11); lane 4 is hydroxyapatite peak 2 (20 µg) (Fig. 11); lane 5 is protein standards: bovine serum albumin(a) (MW, 66000), ovalbumin(b) (MW, 45000), pepsin(c) (MW, 34700), trypsinogen(d) (MW, 24000), α-lactoglobulin(e) (MW, 84000) and lysozyme(f) (MW, 14300); lane 6 is 6-OMT from Polybuffer ion exchanger (28 µg) (Fig. 12A); lanes 7 and 8 are 4'- and 3-OMTs, respectively, from Polybuffer ion exchanger (10 µg) (Fig. 12B).

Figure 3B. Activity profile of OMTs isolated from native polyacrylamide gels (section C.7.).

OMT activity was assayed using quercetin (←), 3,7-dimethylquercetin (←) and 3,7-dimethylquercetagetin (←) for the 3-, 4'-, and 6-OMTs, respectively.
Appendix I.

Figure 4. Product inhibition by 20 μM 3-methylquercetin (—), 3'-methylquercetin (—■—) or 4'-methylquercetin (—○—) of 3-OMT activity. (—△—) is the control without flavonol product inhibitor. 1/v versus 1/Quercetin with assay conditions as described in figure 26D.
Appendix 1.

Figure 5. Linearity with time for the 3-, 6- and 4'-OMT assays.

In A, C and E the concentration of SAM was constant at 8.3 μM (0.05 μCi); whereas for A the concentration of quercetin was 0.5, 1, 3, and 10 μM; for C the concentration of 3,7-dimethylquercetin was 0.6 and 6 μM; for E the concentration of 3,7,3'-trimethylquercetagetin was 0.6 and 6 μM.

In B, D and F the concentration of SAM was constant at 116.6 μM (0.1 μCi), whereas the concentrations of flavonol substrates were identical to A, C and E, respectively.
G.2. Appendix II: Statistical Tables.
Table I. Substrate interaction kinetics; estimation of kinetic parameters for the 3-, 6-, 8- and 4'-OMTs.

<table>
<thead>
<tr>
<th>Varied Substrates</th>
<th>Parameter</th>
<th>( \text{Value (standard deviation)} ) (( \mu \text{M} ))</th>
<th>( \text{Complete Model} )</th>
<th>( \text{Reduced Model} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( K_a )</td>
<td></td>
<td>114 (33)</td>
<td>195 (52)</td>
<td></td>
</tr>
<tr>
<td>( K_b )</td>
<td></td>
<td>12 (3.3)</td>
<td>19 (5.3)</td>
<td></td>
</tr>
<tr>
<td>( K_{ia}K_b )</td>
<td></td>
<td>85 (34)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>( V )</td>
<td></td>
<td>24 (4.6)</td>
<td>34 (8)</td>
<td></td>
</tr>
<tr>
<td>SAM Q(^a)</td>
<td></td>
<td>51 (27)</td>
<td>411 (478)</td>
<td></td>
</tr>
<tr>
<td>( K_a )</td>
<td></td>
<td>18 (6)</td>
<td>87 (103)</td>
<td></td>
</tr>
<tr>
<td>( K_{ia} )</td>
<td></td>
<td>214 (55)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>( V )</td>
<td></td>
<td>20 (5.5)</td>
<td>82 (93)</td>
<td></td>
</tr>
<tr>
<td>SAM 3,7,3'Qg</td>
<td></td>
<td>130 (25)</td>
<td>176 (29)</td>
<td></td>
</tr>
<tr>
<td>( K_a )</td>
<td></td>
<td>15 (2.6)</td>
<td>19 (3.2)</td>
<td></td>
</tr>
<tr>
<td>( K_{ia} )</td>
<td></td>
<td>73 (33)</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>( V )</td>
<td></td>
<td>29 (3.9)</td>
<td>36 (5)</td>
<td></td>
</tr>
<tr>
<td>SAM 3,7Q'</td>
<td></td>
<td>53 (10)</td>
<td>142 (41)</td>
<td></td>
</tr>
<tr>
<td>( K_a )</td>
<td></td>
<td>1.33 (0.27)</td>
<td>3.68 (1.1)</td>
<td></td>
</tr>
<tr>
<td>( K_{ia} )</td>
<td></td>
<td>45 (6.6)</td>
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<td>--</td>
</tr>
<tr>
<td>( V )</td>
<td></td>
<td>3.6 (0.4)</td>
<td>6.3 (1.5)</td>
<td></td>
</tr>
</tbody>
</table>

\(^{a}\) Q, 3,7,3'Qg, 3,7Q and 8-OHK r\'epresent quercetin, 3,7,3'-trimethyl- tagetin, 3,7-dimethylquercetin and 8-hydroxykaempferol, respectively, and were used for the 3-, 6-, 4'- and 8-OMTs, as the respective flavonoid substrate.
Table II. Product inhibition kinetics; estimation of kinetic parameters for the 3-OMT.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Parameter</th>
<th>Value (standard deviation) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>I Term</td>
<td>Complete Model</td>
<td>Reduced Model (Eq. IV or V Competitive(C) or uncompetitive(U))</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Equation III: non-competitive)</td>
<td></td>
</tr>
<tr>
<td>Q&lt;sup&gt;a&lt;/sup&gt;</td>
<td>SAH</td>
<td>$K_b$</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{is}$</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>--</td>
</tr>
<tr>
<td>Q</td>
<td>3Q</td>
<td>$K_b$</td>
<td>4.5 (0.31) (C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{is}$</td>
<td>27 (2.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>128 (36)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>2.5 (0.09)</td>
</tr>
<tr>
<td>SAM</td>
<td>3Q</td>
<td>$K_a$</td>
<td>44 (4.5)</td>
</tr>
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<td></td>
<td></td>
<td>$K_{is}$</td>
<td>29 (5.3)</td>
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<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>65 (9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>5.5 (0.18)</td>
</tr>
<tr>
<td>SAM</td>
<td>SAH</td>
<td>$K_a$</td>
<td>--&lt;sup&gt;b&lt;/sup&gt;</td>
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</tr>
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<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>--</td>
</tr>
</tbody>
</table>

<sup>a</sup> Q and 3Q represent quercetin and 3-methylquercetin.

<sup>b</sup> The data could not be fitted to this equation.
Table III. Product inhibition kinetics; estimation of kinetic parameters for the 6-OMT.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Parameter</th>
<th>Value (standard deviation) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td>Complete Model</td>
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<td>A</td>
<td>I</td>
<td>Term</td>
<td>(Equation III: non-competitive)</td>
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<tr>
<td>SAM</td>
<td>3,6,7,3'Qg</td>
<td>(K_a)</td>
<td>36 (2.2) (U)</td>
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<td></td>
<td></td>
<td>(K_i_s)</td>
<td>74 (13.5)</td>
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<td></td>
<td></td>
<td>(K_i_i)</td>
<td>81 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>5 (0.09)</td>
</tr>
<tr>
<td>SAM</td>
<td>SAH</td>
<td>(K_a)</td>
<td>-- (b)</td>
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</tr>
<tr>
<td>3,7,3'Qg</td>
<td>3,6,7,3'Qg</td>
<td>(K_b)</td>
<td>9.4 (0.7) (U)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(K_i_s)</td>
<td>1413 (820)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(K_i_i)</td>
<td>204 (31)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>3.1 (0.14)</td>
</tr>
<tr>
<td>3,3,3'Qg</td>
<td>SAH</td>
<td>-- (c)</td>
<td>--</td>
</tr>
</tbody>
</table>

\[\text{a} \] 3,6,7,3'Qg and 3,7,3'Qg represent 3,6,7,3'-tetramethylated and 3,7,3'-trimethylated quercetin derivatives.

\[\text{b} \] The data could not be fitted to this equation.

\[\text{c} \] The data did not give linear double reciprocal plots and could not be fitted to any of the equations.
Table IV. Product inhibition kinetics; estimation of kinetic parameters for the 4'-OMT.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Parameter</th>
<th>Value (standard deviation) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Complete Model (Eq. III: non-competitive)</td>
</tr>
<tr>
<td>A</td>
<td>I Term</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SAM</td>
<td>3,7,4'Q</td>
<td>$K_a$</td>
<td>27 (1.2) (C) 25 (2.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{is}$</td>
<td>23 (0.8) 16 (1.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>140 (32) -- --</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>5.5 (0.14) 5.3 (0.15)</td>
</tr>
<tr>
<td>SAM</td>
<td>SAH</td>
<td>$K_a$</td>
<td>-- (b) (C) 21 (2.8)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{is}$</td>
<td>-- -- 4.4 (0.44)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>-- -- --</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>-- -- 5.3 (0.21)</td>
</tr>
<tr>
<td>3,7Q</td>
<td>3,7,4'Q</td>
<td>$K_a$</td>
<td>1.4 (0.08) (C) 1.3 (0.14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{is}$</td>
<td>3 (0.33) 1.5 (0.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>10 (1.4) -- --</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>4 (0.09) 4 (0.16)</td>
</tr>
<tr>
<td>3,7Q</td>
<td>SAH</td>
<td>$K_a$</td>
<td>-- (b) (U) 1.1 (0.24)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{is}$</td>
<td>-- -- --</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>-- -- 10 (1.9)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>-- -- 1.4 (0.13)</td>
</tr>
</tbody>
</table>

a 3,7,4'Q and 3,7Q represent 3,7,4'-trimethyl and 3,7-dimethylated quercetin.
b The data could not be fitted to this equation.
Table V. Product inhibition kinetics; estimation of kinetic parameters for the 8-OMT.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Inhibitor</th>
<th>Parameter</th>
<th>Value (standard deviation) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Complete Model</td>
<td>Reduced Model</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Equation III: non-</td>
<td>(Eq. IV or V Competitive (C)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>competitive)</td>
<td>or uncompetitive (U)</td>
</tr>
<tr>
<td>A</td>
<td>I</td>
<td>Term</td>
<td></td>
</tr>
<tr>
<td>SAM</td>
<td>8MeK</td>
<td>$K_a$</td>
<td>14 (3)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_is$</td>
<td>14 (6)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>28 (7.5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>0.47 (0.03)</td>
</tr>
<tr>
<td>SAM</td>
<td>SAH</td>
<td>$K_a$</td>
<td>20 (2.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_is$</td>
<td>4 (1.1)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>15 (6.2)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>0.72 (0.03)</td>
</tr>
<tr>
<td>8OHK</td>
<td>8MeK</td>
<td>$K_a$</td>
<td>1.34 (0.15)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_is$</td>
<td>6.2 (0.96)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>215 (518)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>1 (0.05)</td>
</tr>
<tr>
<td>8OHK +</td>
<td>SAH</td>
<td>$K_a$</td>
<td>1.7 (0.14)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_is$</td>
<td>3.8 (0.82)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{ii}$</td>
<td>2.7 (0.42)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V$</td>
<td>0.84 (0.04)</td>
</tr>
</tbody>
</table>

a 8MeK and 8OHK represent 8-methoxykaempferol and 8-hydroxykaempferol.
Table VI. Variance ratio test. Evaluation of the $K_{ia} K_{ib}$ and $K_{ii}$ terms in the complete model for the 3-, 6-, 8- and 4'-OMTs.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Experiment</th>
<th>Residual Sum of Squares</th>
<th>Complete Model</th>
<th>Reduced Model</th>
<th>Calculated</th>
<th>(Degrees of freedom)</th>
<th>Tables (α=99)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-OMT</td>
<td>SAM/Q$^a$</td>
<td>0.4207</td>
<td>0.5237</td>
<td>4.76</td>
<td>(1;20)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAM/SAH</td>
<td>--</td>
<td>0.8007</td>
<td>--</td>
<td>(1;16)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAM/3Q</td>
<td>0.2334</td>
<td>1.0053</td>
<td>53</td>
<td>(1;16)</td>
<td>8.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q/SAH</td>
<td>--</td>
<td>0.0362</td>
<td>--</td>
<td>(1;16)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Q/3Q</td>
<td>0.0144</td>
<td>0.2765</td>
<td>14.8</td>
<td>(1;16)</td>
<td>8.53</td>
<td></td>
</tr>
<tr>
<td>6-OHT</td>
<td>SAM/3,7,3'Qg</td>
<td>0.2432</td>
<td>0.4005</td>
<td>13.3</td>
<td>(1;20)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAM/SAH</td>
<td>--</td>
<td>0.0505</td>
<td>--</td>
<td>(1;16)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAM/3,6,7,3'Qg</td>
<td>0.0623</td>
<td>0.2262</td>
<td>58</td>
<td>(1;16)</td>
<td>8.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,7,3'Qg/SAH</td>
<td>--</td>
<td>0.0505</td>
<td>--</td>
<td>(1;16)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td>4'-OMT</td>
<td>SAM/37Q</td>
<td>0.4471</td>
<td>0.5392</td>
<td>4</td>
<td>(1;20)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAM/SAH</td>
<td>--</td>
<td>0.4943</td>
<td>--</td>
<td>(1;26)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAM/3,7,4'Q</td>
<td>0.0421</td>
<td>0.2352</td>
<td>92</td>
<td>(1;20)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,7Q/SAH</td>
<td>--</td>
<td>0.2013</td>
<td>--</td>
<td>(1;20)</td>
<td>--</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3,7Q/3,7,4'Q</td>
<td>0.0574</td>
<td>0.2110</td>
<td>53</td>
<td>(1;20)</td>
<td>8.1</td>
<td></td>
</tr>
<tr>
<td>8-OMT</td>
<td>SAM/80HK</td>
<td>0.0574</td>
<td>0.1706</td>
<td>39.3</td>
<td>(1;21)</td>
<td>8.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAM/SAH</td>
<td>0.0045</td>
<td>0.0091</td>
<td>17</td>
<td>(1;16)</td>
<td>8.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SAM/8MeK</td>
<td>0.0041</td>
<td>0.0111</td>
<td>17</td>
<td>(1;12)</td>
<td>9.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80HK/SAH</td>
<td>0.0034</td>
<td>0.0139</td>
<td>64</td>
<td>(1;21)</td>
<td>8.01</td>
<td></td>
</tr>
<tr>
<td></td>
<td>80HK/8MeK</td>
<td>0.0069</td>
<td>0.0069</td>
<td>0.16</td>
<td>(1;16)</td>
<td>8.53</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Tables I to V have the abbreviations for the substrates and products.
G.3. Appendix III: Perspectives for future work.
III. Perspective for future work.

The OMT system of *C. americanum* has proven to be ideal in the study of closely related enzymes involved in the sequential O-methylation of flavonoids. Therefore, this tissue unlike that of spinach (72) should prove to be ideal for studies on the in situ localisation of these enzymes. Apart from the localisation studies described earlier in this thesis, a more direct approach would involve the localisation of OMT antigens at the electron microscope level using specific OMT antibodies. The methodology for the latter technique is well established in plant systems (134), given that the antibody used is prepared from pure antigen.

The procedures reported in this thesis involved the partial purification and isolation of separate OMTs from *Chrysosplenium*. Affinity chromatography with SAH-Agarose could be useful in further purifying these enzymes. Preliminary work by this author demonstrated that the 3-, 6-, 7- and 4'-OMTs of *Chrysosplenium* did bind to SAH-Agarose and were not eluted with SAM (1 mM) Appendix, Fig. 6). However it was found that the 3-, 6-, 7- and 4'-OMTs could be partially separated from each other by a 0-100 mM NaCl gradient. This demonstrates the potential use of affinity chromatography on SAH-Agarose in the purification of individual OMTs. Hopefully, the refinement of this technique may allow the preparation of pure OMTs for antibody production.

Another potential use of *Chrysosplenium* could involve studies on the in vivo regulation of O-methylation in this tissue. It has been shown that the levels of SAM can be modified in plant tissues by
Appendix III.

Figure 6. Chromatography on SAH Agarose (1 x 4 cm) of active fractions from Sephadex G-100 (as described in figure 9).

OMT activity was assayed using quercetin (---), 3-methylquercetin (→), 3,7-dimethylquercetin (←) and 3,7,3'-trimethylquercetagetin (△) as substrates for the 3-, 7-, 4'- and 6-OMTs, respectively.
simply placing the latter in a solution containing methionine. It was shown that addition of methionine to the growth medium of *Lemma paucicostata* (56) increased the steady state concentration of SAM between 10- to 20-fold. Similarly, turnip discs incubated with methionine accumulated SAM to approximately 200 μM (135). It would be interesting, therefore, to raise the levels of SAM in *Chrysosplenium* before the administration of [2-14C]-cinnamic acid and determine if the rate of partially methylated flavonoid synthesis could be enhanced (Appendix, Fig. 1). This may provide indirect evidence for the effect of SAM/SAH ratio on the biosynthesis of such compounds.

Photoaffinity labelling can be useful in the further characterization of *Chrysosplenium* OMTs as demonstrated with the catechol OMT of porcine liver (136). It was shown that in the absence of UV light, the 8-Azido analog of S-adenosyl-L-[35S]-methionine could serve as a methyl donor for porcine catechol-OMT, whereas photolysis of this analog in the presence of the enzyme resulted in covalent binding. The specificity of the incorporation indicated that it occurred at the SAM binding site on the catechol-OMT.

Preliminary work by this author demonstrated that in the absence of light 8-Azido-S-adenosyl-L-[C3H3]-methionine (80 Ci/mmol, New England Nuclear) also served as a methyl donor with the 3-, 6- and 4'-OMTs of *Chrysosplenium*. The rate of methylation when 8-Azido-SAM was the methyl donor was similar for the 3- and 6-OMTs whereas the rate of incorporation with the 4'-OMT was very low. However, the rate of incorporation from [14CH3]-SAM was highest with the 4'-OMT followed by the 3-OMT and the 6-OMT. This demonstrates different efficiency
of incorporation of this analog for each of the Chrysosplenium OMTs as well as a possible tool for further characterization of these enzymes. The fact that this analog acted as a methyl donor with the three OMTs, indicates that these enzymes could also be subjected to photoaffinity labelling for covalent binding to their respective active sites. Radioactively labelled enzymes could be subjected to SDS-PAGE followed by fluorography and identification of the 3-, 6- or 4'-OMTs. The latter could be submitted to proteolytic digestion in order to isolate the polypeptide fragment containing the active site and subsequent amino acid analysis. The 'finger prints' of each fragment could be compared and perhaps determine which differences may confer specificity to these enzymes.

These are only a few of the potential studies which could be carried out with the OMT system of Chrysosplenium. However, these examples serve to illustrate the fundamentally interesting biochemistry which can be carried out with the advent of technological innovations and the knowledge that plants like Chrysosplenium do exist.