

Meta-Methylation of Flavonol Rings A (8-) and B (3'-) Is Catalysed by Two Distinct O-Methyltransferases in *Lotus corniculatus* *

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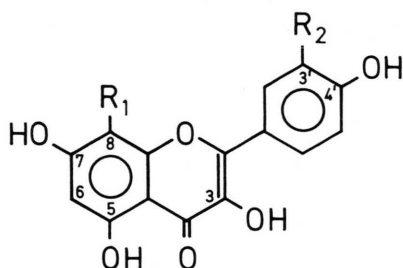
Lotus corniculatus, Leguminosae, O-Methyltransferase, Flavonols, 8-O-Methylation, 3'-O-Methylation

Two O-methyltransferases specific for flavonol rings A and B were isolated from young flower buds of *Lotus corniculatus*. They were partially purified by ammonium sulphate precipitation and successive chromatography on Sephadex G-100 and Polybuffer ion exchanger. One enzyme focused at pI 5.5 and catalysed the O-methylation of position 8 of flavonols with a pH optimum of 8.1. The other enzyme had a pI of 5.1 and preferentially attacked position 3' at an optimum pH of 7.7. The methylated products of both enzymes seem to contribute to the flower colour of *Lotus* and may be used as biochemical markers in genetic studies of this genus.

Introduction

8-Substituted flavonols were thought to be of less common occurrence in plants [1] until recent reports [2] indicated their presence in a number of species belonging to the Compositae [3], Malvaceae [4] and Leguminosae [5] among other families. *Lotus corniculatus*, a common legume, is widely distributed in the pastures of temperate regions and has been the subject of many genetic studies with the aim of improving its fodder quality [6]. It represents an interesting example of the ontogeny of 8-substituted flavonols in its vegetative and flowering parts. Whereas the leaves contain kaempferol, quercetin and isorhamnetin, the flowers accumulate the latter compounds as well as their 8-substituted derivatives: 8-methoxykaempferol, 8-methoxyquercetin and 8-methoxyisorhamnetin (Fig. 1) [5]. Such an interesting substitution pattern may be considered of taxonomic and ecological significance in determining the differences among various species of the genus, as well as the different populations of the same species.

Our recent work on the methylation of flavonoid compounds [7–11] indicates that multiple methylation of flavonols is catalysed by a number of distinct O-methyltransferases with specificity for positions 3, 6, 7 and 4' of the flavonoid ring. Since the methoxyl groups at positions 8 and 3' of *Lotus* flavonols represent *meta* methylation of rings A and B, respectively it was considered of interest, therefore, to find out whether both reactions are catalysed by one or two distinct O-methyltransferases. Furthermore, the substitution pattern of *Lotus* flavonoids allows us to study the O-methyla-



R ₁	R ₂	
H	H	Kaempferol
H	OH	Quercetin
OH	OH	Gossypetin
OMe	H	8-OMe-kaempferol
H	OMe	Isorhamnetin
OMe	OMe	8-OMe-isorhamnetin

Fig. 1. Structural formulae of the flavonol derivatives of *Lotus corniculatus*.

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tion steps at the 8- and 3'-positions which complement our previous knowledge of O-methyltransferases.

We wish to report in this paper the partial purification and some properties of two distinct O-methyltransferases which expressed high specificity for the *meta* positions of ring A(8-) and ring B(3'-) of flavonols.

Materials and Methods

Plant material

Both vegetative and reproductive parts of *Lotus corniculatus* L. were obtained from greenhouse-raised plants as well as the Agricultural Experimental Farm of Macdonald College, courtesy of Dr. B. Coulman, McGill University. The plant material was collected in liquid nitrogen until brought to the laboratory for enzyme extraction.

Chemicals

S-Adenosyl-L-[$^{14}\text{CH}_3$] methionine (56 mCi/mmol) was purchased from New England Nuclear (Boston, MA). All substrates and reference compounds were from our laboratory collection; except 8-hydroxy-7-methylquercetin and 8-hydroxy-3'-methylquercetin which were generous gifts from Professor J. B. Harborne and 8-methoxy-3-methylquercetin from Professor M. Nogradi. Polybuffer ion exchanger (PBE-94) and Polybuffer (PB-74) were obtained from Pharmacia Fine Chemicals. All other chemicals and solvents were of analytical reagent grade.

Enzyme extraction and purification

Unless stated otherwise all purification steps were carried out at 2–4 °C. Frozen tissues were mixed with Polyclar AT (2:1, w/w) and ground to a fine powder, then homogenised with the extraction buffer. The latter consisted of 0.1 M potassium phosphate, pH 7.6 containing 10 mM diethylaminoethyl dithiocarbamate, 5 mM EDTA and 14 mM 2-mercaptoethanol (buffer A). After centrifugation, the supernatant was stirred for 20 min with Dowex 1 × 2 (1:5, w/v) which had previously been equilibrated with the same buffer, then filtered. The filtrate was fractionated with solid ammonium sulphate and the protein pellet which precipitated

between 0–70% salt saturation was dissolved in the minimum amount of 25 mM histidine buffer, pH 7.6 containing 14 mM 2-mercaptoethanol and 10% glycerol (buffer B). The latter was chromatographed on a Sephadex G-100 column and the protein was eluted using the same buffer. The fractions containing O-methyltransferase activity were pooled and applied to a chromatofocusing column which was packed with Polybuffer ion exchanger (PBE-94) and had previously been equilibrated with buffer B. The proteins were eluted at near their isoelectric points using Polybuffer (PB-74) which generated a linear gradient between pH 6 and 4. Two-ml fractions were collected for the assay of enzyme activity.

Enzyme assay and identification of reaction products

The standard assay mixture consisted of 10 µl of 10 µM solution of the flavonoid substrate (in 50% DMSO); 10 µl of S-adenosyl-L-methionine (containing 55000 dpm); 10 µl of 10 mM MgCl_2 and 20–100 µl enzyme protein in a total volume of 130 µl. The assay mixture of chromatofocused fractions contained 50 µl of 0.2 M phosphate buffer, pH 8.0 in order to raise the pH of the enzyme protein above its pI. The enzyme reaction was allowed to proceed for 30 min at 30 °C and was terminated by the addition of 10 µl 6 N HCl. The reaction products were extracted with 400 µl of 1:1 mixture of benzene-ethylacetate (v/v) and an aliquot of the organic phase was counted for radioactivity in a toluene-based liquid scintillation fluid. The remainder was chromatographed on Polyamid-DC6 (MN) TLC plates using benzene-methyl ethyl ketone-methanol (70:15:15) as solvent system, then autoradiographed. Identity of the labelled products was verified by co-chromatography with reference compounds and visualization in UV light (366 nm).

Results

Comparative O-methyltransferase activity of different organs of Lotus

The O-methylating capacity of partially purified extracts (Sephadex G-25 fraction) of different organs of the plant was compared using three flavonol substrates – quercetin, 8-hydroxyquercetin and 8-hydroxykaempferol. The results, shown in Table I, clearly indicate that flowering buds exhibited the highest methylating activity towards the

Table I. O-methylating activity of different organs of *Lotus*^a.

Plant organ	Activity in reaction products ^b		
	8-Hydroxy-kaempferol	8-Hydroxy-quercetin	Quercetin
Three-week-old seedlings	2350	2150	2150
Apical buds	4200	5760	8750
Mature leaves	4050	6020	6750
Young flower buds	19750	15600	14290

^a Protein extracts were fractionated with ammonium sulphate (0–70% saturation) and desalted on Sephadex G-25.

^b The standard enzyme assay was used with the indicated substrates as described in the Methods section. The total activity in reaction products was determined by liquid scintillation as DPM/g fresh weight/min. See Table II for identification of reaction products.

Table II. Identification of the reaction products of *Lotus* O-methyltransferases^a.

Substrate	Reaction products		
	Methyl-derivative ^b	Relative amount [%]	R _F -value ^c
8-Hydroxy-kaempferol	8-Me-	30	0.65
	3-Me-	70	0.50
8-Hydroxy-quercetin	8-Me-	30	0.40
	3-Me-	70	0.30
Quercetin	3-Me-	50	0.40
	3'-Me-	45	0.55
	DiMe- ^d	5	0.70

^a The standard assay was used with partially purified enzyme preparation as described in the Methods section.

^b Identified by co-chromatography with reference compounds.

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

^d 3,3'- or 3,7-Dimethylquercetin.

three substrates. Both apical buds and mature leaves showed similar degree of methylation, which was 50–80% lower than that of flower buds. Three-week-old seedlings, on the other hand, exhibited the lowest degree of methylation which amounted to approximately 15% of that of flower buds.

It is interesting to note that the pattern of methylated products formed from each flavonoid substrate was almost similar for the different plant organs used (Table II). Both 8-hydroxykaempferol and 8-hydroxyquercetin were methylated either at 3- or 8-positions at a ratio of 2:1. Quercetin, on the other hand, was equally methylated at positions 3

and 3', with a trace amount of label found in a dimethylated product which was tentatively identified as 3,3'- or 3,7-dimethylquercetin (Table II). Identification of the methylated products was made possible by the availability of a number of reference compounds, as well as the use of a solvent system which readily separated the monomethyl- and dimethylether derivatives (Table II). These results clearly indicate the presence of 8- and 3'-O-methyltransferase activities in the flower buds of *Lotus*, together with another enzyme activity which attacks position 3 of quercetin, 8-hydroxyquercetin and 8-hydroxykaempferol.

Purification of O-methyltransferase activity from flower buds

The O-methyltransferase activity of young flower bud extracts was purified by successive chromatography of the ammonium sulphate pellet (0–70% saturation) on Sephadex G-100 and chromatofocusing on Polybuffer ion exchanger. The latter step resulted in two peaks with O-methyltransferase activity which focused at pH 5.5 and 5.1 (Fig. 2).

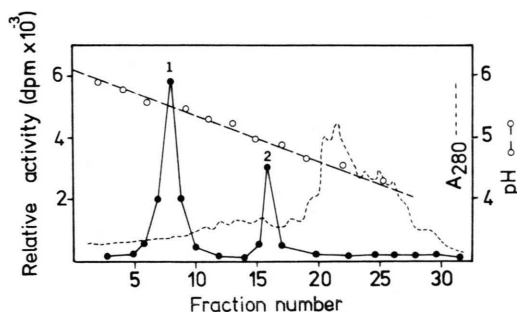


Fig. 2. Elution profile of the O-methyltransferase activities from Polybuffer ion exchanger.

Table III. Characteristic properties of *Lotus* O-methyltransferases^a.

Property	Peak 1	Peak 2
pI value	5.5	5.1
pH optimum	8.1	7.7
Identity	8-OMT ^b	3'-OMT ^b
K _m Substrate (μM) ^c	24	22
Mg ion requirement (mM)	4	4
Inhibition by EDTA (mM)	6	6
Requirement for SH group protectors	absolute	none

^a Fractions were chromatofocused as described in the Methods section.

^b OMT, O-methyltransferase.

^c 8-Hydroxykaempferol and quercetin as substrates for peaks 1 and 2, respectively.

Substrate specificity

Whereas the Sephadex G-100 fraction catalysed the methylation of a number of flavonoid substrates (Table III), the chromatofocused peak 1 (pI 5.5) exhibited preferential affinity for 8-hydroxyflavonols and gave rise to their 8-methyl derivatives (Fig. 3). Peak 2 (pI 5.1), on the other hand, methylated not only 8-hydroxyflavonols but also other compounds, such as luteolin, quercetin, myricetin and isorhamnetin (Table III), presumably attacking their ring B. Examination of the autoradiographed reaction products (Fig. 3) clearly indicates that peak 1 contains predominantly an 8-O-methyltransferase activity; whereas peak 2 consisted mainly of 3'-O-methyltransferase, slightly contaminated with a 3-methylating activity.

Properties of 8- and 3'-O-methyltransferases

Both enzyme activities exhibited marked differences in their pI values, pH optima and requirement

for SH group protectors (Table III); thus indicating distinct characteristics for O-methylation at positions 8 and 3'. They showed, however, some similarity with regards to their K_m values for their best substrates and the co-substrate, S-adenosyl-L-methionine. Furthermore, both enzymes exhibited similar requirements for Mg^{2+} and its elimination by EDTA (Table III).

Discussion

This is the first report of an O-methyltransferase which catalysed the methylation of 8-hydroxyflavonols. It was isolated from young flower buds of *Lotus corniculatus* and was partially purified by chromatofocusing on Polybuffer ion exchanger with an apparent pI of 5.5. Another O-methyltransferase (pI 5.1) was isolated from the same tissue and catalysed the methylation of both quercetin and 3-methylquercetin at the 3' position. The substrate specificity of both enzymes clearly indicates that O-methylation of the *meta* positions of ring A (8-position) and ring B (3'-position) is mediated by two distinct enzymes and culminates the predictions [12] with regards to the enzymic synthesis of partially methylated flavonoids. However, it is interesting to note that the 8-O-methyltransferase described here attacked fully hydroxylated flavonols, such as 8-hydroxykaempferol and 8-hydroxyquercetin unlike the 6-O-methyltransferase, another *meta*-directing enzyme from *Chrysosplenium americanum* [9, 11] which only accepted partially methylated flavonols, such as 3,7-dimethyl- or 3,7,3'-trimethyl-6-hydroxyquercetin. Both the 8-O-methyltransferase of *Lotus* and 6-O-methyltransferase of *Chrysosplenium* had similar pI values and required Mg^{2+} ions for activity; though they had different pH optima (8.1 and 8.9, respectively) which reflected the degree of dissociation of the free hydroxyl groups present on their respective substrates. The 3'-O-methyltransferase, on the other hand, resembled the much investigated, *meta*-directing flavonoid O-methyltransferase [13–15], particularly that of tulip anthers [15]. It had similar pI value, pH optimum and required Mg^{2+} for activity.

The physiological role of both O-methyltransferases seems to mediate the methylation of the natural flavonoid substrates found in *Lotus* flowers at both 8- and 3'-positions; namely quercetin, 8-hydroxyquercetin and 8-hydroxykaempferol. It is

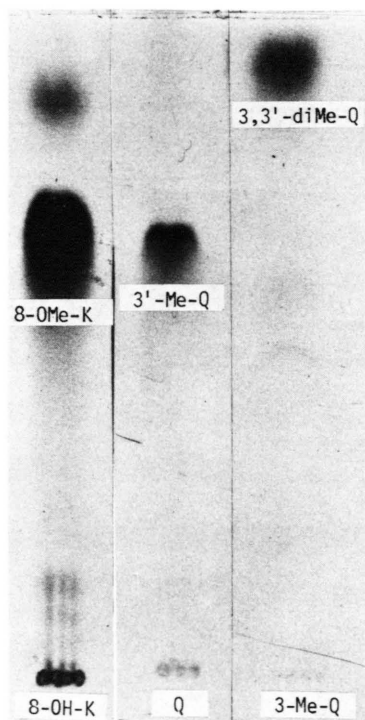


Fig. 3. Photograph of the autoradiographed reaction products of O-methyltransferase activity of peaks 1 and 2 (Fig. 2) against different substrates. Numbers denote the position of hydroxyl or methoxyl groups on kaempferol (K) and quercetin (Q).

remarkable to note that whereas the vegetative parts of *Lotus* contained the only methylated flavonol, isorhamnetin; the flowers accumulated 8-methoxykaempferol, 8-methoxyquercetin and 8-methoxyisorhamnetin as a result of 8-O-methyltransferase activity in the reproductive organs. These three methylated flavonols possess intense yellow colour

and seem to contribute to the colour of flower petals of this species. Furthermore, the presence or absence of an 8-O-methyltransferase may be considered a useful biochemical marker in genetic studies on the biology of populations of this species. Further work on the purification and properties of 8-O-methyltransferase is in progress.

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