Separation of the S-Adenosylmethionine: 5- and 8-Hydroxyfuranocoumarin O-Methyltransferases of *Ruta graveolens* L. by General Ligand Affinity Chromatography *

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Two S-adenosyl-L-methionine: furanocoumarin O-methyltransferases of *R. graveolens*, acting at the 5- and 8-hydroxyls of the psoralen nucleus, were completely resolved by adsorption on a general affinity ligand, 5-(3-carboxypropanamido) xanthotoxin, followed by specific desorption by bergaptol and xanthotoxol, respectively. The 5-O-methyltransferase was purified 450-fold by this procedure, the 8-O-methyltransferase 112-fold, and both enzyme fractions were electrophoretically homogeneous. No resolution could be achieved of the activity against two 5-hydroxypsoralens or of the activity against two 8-hydroxypsoralens, and conclusive evidence is presented for the existence of only one 5-O-methyltransferase and only one 8-O-methyltransferase acting on linear furanocoumarins.

**Introduction**

O-Methylated phenols are a very common type of natural product, and much attention has been directed toward the study of enzymes mediating the O-methylation reactions necessary for their elaboration in vivo. A number of furanocoumarins bear methoxyl substituents, and in recent studies in this laboratory [1, 2] an O-methyltransferase system from the plant, *Ruta graveolens* L., was reported which catalyses the reactions shown in Scheme I, where R<sub>5</sub> and R<sub>8</sub> are either H or OMe. Unequivocal evidence was presented [2], on the basis of additive activities in the presence of both 5- and 8-hydroxylated linear furanocoumarin substrates, and wide fluctuations in the ratios of the specific activities against the 5- and 8-hydroxy substrates during partial purification, for the existence of at least two discrete enzymes acting at the 5- and 8-positions.

In an earlier paper [3] we reported isolation of the two furanocoumarin O-methyltransferases, uncontaminated by a co-occurring caffeic acid-3-O-methyltransferase, on AH-Sepharose 4B linked to CPAX (5). In the absence of the appropriate co-factor, SAM, these enzymes were shown to have no affinity for this Sepharose-linked xanthotoxin derivative. In the course of these studies [3] it was found that the furanocoumarin O-methyltransferases form strong ternary complexes together with SAM and the phenolic substrate. In the present paper we have demonstrated that this effect can be utilized for complete separation of the 5- from the 8-O-methyltransferase, and also to establish the number of O-methyltransferases involved in the biosynthesis of linear furanocoumarins.

![Scheme I](attachment:image.png)

**Abbreviations:** CPAX, 5-(3-carboxypropanamido) xanthotoxin; SAH, S-adenosyl-L-homocysteine; SAM, S-adenosyl-L-methionine.

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Experimental

The materials used, the procedures for the preliminary purification of the O-methyltransferases, their affinity chromatography with a CPAX ligand in the presence of SAM, activity assays, and the determination of protein were as previously described [3].

Polyacrylamide disc gel electrophoresis

Samples of an aqueous solution of the purified protein extract (100 μg of protein, 0.5 mg/ml) containing furanocoumarin O-methyltransferases, before and after affinity separation, were analysed by 7.5% polyacrylamide disc gel electrophoresis, by the method of Zeldin and Ward [4]. The gels were run at 6 mA/tube for ca. 1 h in 38 mM glycine buffer (titrated to pH 8.2 with concentrated Tris solution). The protein bands were stained with 1% Coomassie Brilliant Blue R-250 (Bio-Rad Laboratories, Richmond, Cal., USA) in water-acetic acid-methanol (45 : 10 : 45) followed by destaining in the same mixture.

Results and Discussion

Resolution of the O-methyltransferases by a general ligand approach

As already reported [3], no binding of either furanocoumarin O-methyltransferase to the CPAX ligand 5 occurs in the absence of SAM, a phenomenon explicable on the basis of induction of the phenolic binding site by prior binding of the methyl donor (SAM) to the enzyme. Because of this, SAM (5 × 10⁻⁵ M) was initially included in the Tris buffer (0.05 M, pH 7.5) employed as irrigant, to effect adsorption of both furanocoumarin O-methyltransferases to the CPAX column.

Fig. 1 A shows that when the mixture of furanocoumarin O-methyltransferases, purified by affinity chromatography utilizing CPAX-linked AH-Sepharose 4 B [3], was rechromatographed on a CPAX column in the presence of SAM, the furanocoumarin O-methyltransferases were, in fact, both retarded, no activity against either bergaptol (3, R₈=H) or xanthotoxol (1, R₅=H) being detected in the first two elution volumes. It is thus evident that a specific binding of the 8-O-methyltransferase to the 8-oxygenated ligand, which might have been anticipated on structural grounds, was not achieved in this instance, and that the CPAX ligand can effectively bind the enzymes acting at both the 5- and 8-positions. However, when a second substrate, xanthotoxol (10⁻⁴ M), was added to the irrigant buffer, specific release of the 8-hydroxyfuranocoumarin O-methyltransferase readily occurred. The eluate appearing between 2 and 2.7 elution volumes was active in methylating xanthotoxol under standard assay conditions [2] but was devoid of activity against bergaptol. The other enzyme, 5-hydroxy-
furanocoumarin O-methyltransferase, remained adsorbed on the column as long as SAM was present. The fact that, as shown, discontinuation of the SAM eluted this enzyme within 1.3 elution volumes is consistent with the compulsory-ordered mechanism which we have already established in this laboratory [3]. The fractions in this case were active when assayed against bergaptol, but showed no activity against xanthotoxol.

The reverse approach to specific desorption of the two enzymes was equally effective in achieving separation, as shown in Fig. 1 B. In this experiment the 5-hydroxylating enzyme was released from the column by inclusion of $10^{-4}\text{M}$ bergaptol in the irrigant buffer. The eluate was totally inactive against xanthotoxol, and the 8-hydroxylating enzyme, free of detectable 5-hydroxylating activity, was eluted after omission of SAM from the buffer.

The results of a third approach to the separation, combining the specific desorption features of the other two, are given in Fig. 2, but in this experiment SAH was substituted for SAM in the buffer, as earlier investigations [3] demonstrated it also to be effective in inducing phenolic binding sites on the enzyme. Both enzymes were strongly retarded as long as SAH was present in the irrigant buffer. Addition of bergaptol to the buffer specifically eluted the 5-O-methyltransferase, and finally, application of the xanthotoxol-SAH mixture led to specific elution of the 8-O-methyltransferase.

The effect of xanthotoxol and bergaptol in specifically eluting the 5- and 8-O-methyltransferases, respectively, is entirely consistent with the formation of a ternary complex (SAH- or SAM-enzyme-xanthotoxol or -bergaptol) as postulated earlier [3]. Other examples which could be cited to illustrate the use of ternary complex formation in gaining specificity of elution from a general ligand are certain studies on lactate dehydrogenase, malate dehydrogenase, and alcohol dehydrogenase [5–7].

In such a two-step affinity chromatographic process (i.e., adsorption followed by elution), the importance of the inherent potential of elution for introducing even greater specificity has been increasingly recognized. By using a general ligand (CPAX) with affinity for both the O-methyltransferases one is making greater demands on the specificity of the elution process. Elution from general ligands has been effected by both specific and less specific means. To the latter category belong changes of temperature [8] and pH [9], elution through changes in ionic strength, and elution with hydrophobic agents such as ethylene glycol [10]. Many successful purifications of enzymes from general ligands applying such elution conditions have been reported. However, the view that bioelution is the procedure of choice was borne out in the current work. Nishikawa [11] has laid much stress on the important role of specific desorption in affinity chromatographic separation procedures, especially in the light of well recognized non-specific binding of proteins to various components of the affinity column during the adsorption phase of the process [12, 13]. We have already demonstrated that Ruta O-methyltransferases do not bind to AH-Sepharose 4 B bearing no biospecific ligand [14], and we have recently confirmed that this holds even in the presence of SAM or SAH. The results of the experiments reported here serve to underline the significance of the bioelution procedure in that it...
has permitted a complete resolution of our two enzymes, both of which were retarded by the CPAX ligand.

Polyacrylamide disc gel electrophoresis of the mixture of 5- and 8-O-methyltransferases showed only one protein band, at \( R_f \) 0.57. After separation by affinity chromatography, the two enzymes each yielded a single band \( (R_f \approx 0.57) \), indicating that each was electrophoretically homogeneous. The results suggest that these two enzymes cannot be distinguished by electrophoresis under the experimental conditions used.

Purification data for these two enzymes are given in Table I. A comparison of their specific activities with those of the crude extract revealed a 450-fold purification of the 5-O-methyltransferase and a 113-fold purification of the 8-O-methyltransferase. The much lower value in the latter case doubtless reflects the lesser stability of this enzyme [2].

The separation by affinity chromatography of two discrete O-methyltransferases, each with unique activity against linear furanocoumarin hydroxyls in one position (5- or 8-) of the molecule, serves as final confirmation of the results of the earlier study [2], from which the presence of at least two enzymes was deduced before separation had been achieved.

**Evidence for the existence of only two enzymes**

In a previous paper [2] the question of whether more than two enzymes are present in the *R. graveolens* extracts was addressed, and although no unequivocal conclusion could be reached, the weight of evidence indicated that one enzyme acts on the 8-hydroxyls of both xanthotoxol and 8-hydroxybergapten, and one enzyme on the 5-hydroxyls of both bergaptol and 5-hydroxyxanthotoxin.

Any hypothesis of two discrete enzymes acting on two different 5-hydroxylated substrates, such as bergaptol and 5-hydroxyxanthotoxin \((3, R_8 = \text{OMe})\) would necessarily presuppose a degree of substrate specificity verging on the absolute. It would therefore follow that the addition of xanthotoxol to the irrigant buffer (Fig. 1 A) would selectively desorb only the O-methyltransferase specific for that phenol, and would not desorb any hypothetical O-methyltransferase specific for 8-hydroxybergapten \((1, R_5 = \text{OMe})\). This possibility was tested by (a) assay of the first peak of Fig. 1 A, desorbed by xanthotoxol, against 8-hydroxybergapten, which was found to be converted to its 8-O-methylated product, isopimpinellin \((2, R_8 = \text{OMe})\), by this enzyme, and (b) addition of 8-hydroxybergapten to the irrigant buffer following the desorption of this peak, which resulted in the release of no further enzyme active against that substrate.

An exactly analogous argument could be advanced in the case of hypothetical pairs of 5-O-methyltransferases, and exactly analogous results were obtained: the first peak of Fig. 1 B was active against 5-hydroxyxanthotoxin and no additional enzyme active against this substrate could be released by the subsequent addition of 5-hydroxyxanthotoxin to the buffer.

We regard the above evidence as providing unequivocal proof for the existence in the *Ruta* extract of only two O-methyltransferases acting on linear furanocoumarins: an S-adenosyl-L-methionine:5-hydroxyfuranocoumarin O-methyltransferase converting 1 to 2 (Scheme 1, \( R_5 = \text{H} \) or \( \text{OMe} \)), and an S-adenosyl-L-methionine:5-hydroxyfuranocoumarin O-methyltransferase converting 3 to 4 (\( R_8 = \text{H} \) or \( \text{OMe} \)). There is thus a position specificity directed against the 5- or 8-hydroxyls of the psoralen nucleus.

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**Table I. Purification of *Ruta graveolens* furanocoumarin O-methyltransferases by general ligand affinity chromatography.** After separation by the CPAX-ligand, all the fractions corresponding to the two 5-O-methyltransferase peaks and all those corresponding to the two 8-O-methyltransferase peaks of Fig. 1 were combined and used as the enzyme sources for these measurements. Total activity is expressed as nmol of product formed/h under the standard assay conditions. Purification is calculated on the basis of a specific activity of 0.050 and 0.102 for the crude 5- and 8-O-methyltransferases, respectively.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Volume [mg/ml]</th>
<th>Protein [mg]</th>
<th>Total protein [mg]</th>
<th>Total activity</th>
<th>Specific activity</th>
<th>Purification (-fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-O-methyltransferase</td>
<td>2.0</td>
<td>0.10</td>
<td>0.20</td>
<td>4.50</td>
<td>22.5</td>
<td>450</td>
</tr>
<tr>
<td>8-O-methyltransferase</td>
<td>2.2</td>
<td>0.09</td>
<td>0.20</td>
<td>2.31</td>
<td>11.5</td>
<td>113</td>
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</tbody>
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