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Review Article

# Biosynthetic Studies on Coumarins

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**Key Word Index:** Biosynthesis; Coumarins; Furocoumarins.

## Abstract

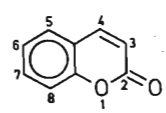
Coumarins, a group of naturally occurring phenylpropanoid lactones exerting a wide range of physiological effects, are elaborated by many plants and in a few microbial species. Early studies on the biosynthetic reactions leading to the simple coumarins in plants demonstrated derivation from the shikimic acid pathway via cinnamic acid and either *ortho*- or *para*-hydroxycinnamic acid. Radiotracer experiments later indicated that the furan ring of the furanocoumarins, found chiefly in the Rutaceae and Umbelliferae, is constructed on the coumarin nucleus following attachment of a mevalonate-derived sidechain to umbelliferone, and cyclization to dihydrofuranocoumarin intermediates. These findings have been substantiated and extended by the isolation and

characterization of several of the enzymes participating in the biosynthetic pathway.

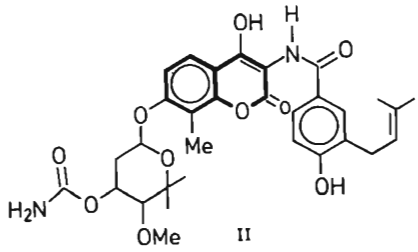
## Introduction

The coumarins are a class of secondary plant and microbial products whose members exhibit a considerable range of physiological effects in animals. The least complex member of the class structurally, known simply as coumarin (I), is toxic to mammals [1], and at the other end of the scale the highly substituted coumarin, novobiocin (II), is a commercial antibiotic. Another microbial coumarin type is the aflatoxins (e. g. III) elaborated by *Aspergillus flavus;* these metabolites are potent hepatotoxins and are among the most intense carcinogens yet discovered [2, 3]. Several coumarins hydroxylated at carbon-4, including the well-known dicoumarol (IV), function as anticoagulants [4, 5]. Dicoumarol results from the action of *Aspergillus* and *Penicillium* spp. on sweet clover (*Melilotus* sp.) hay, the substrate probably being *o*-hydroxycinnamic acid [6]. It is responsible for the very destructive haemorrhagic sweet clover disease of cattle. A synthetic 4-hydroxycoumarin (V) has been sold for many years as an effective rat poison under the name Warfarin.

Of particular interest to the pharmacologist and the phytochemist alike are the linear furanocoumarins, or furo-



I



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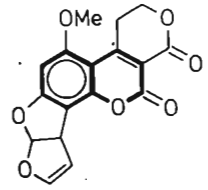

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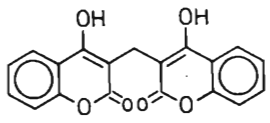

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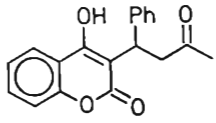
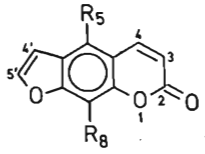


V

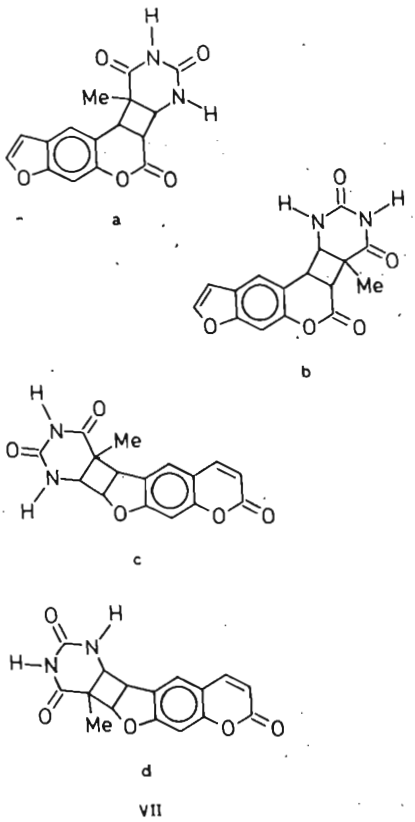


VI

(a) Psoralen: $R_5 = R_8 = H$
(b) Bergapten: $R_5 = OMe$, $R_8 = H$
(c) Xanthotoxin: $R_5 = H$, $R_8 = OMe$
(d) Isopimpinellin: $R_5 = R_8 = OMe$
(e) 5-Hydroxyxanthotoxin: $R_5 = OH$, $R_8 = OMe$
(f) 8-Hydroxybergapten: $R_5 = OMe$, $R_8 = OH$
(g) Bergaptol: $R_5 = OH$, $R_8 = H$
(h) Xanthotoxol: $R_5 = H$, $R_8 = OH$

coumarins. One of these, xanthotoxin (VIc), has been recognized as poisonous to fish for many years, and this property is shared by some others in this class. But more importantly several natural furanocoumarins, and especially psoralen (VIa) and xanthotoxin, are noted for their unique ability to photosensitize the skin to the near ultraviolet, leading to the development of erythema, vesicle formation, and pigmentation of the affected areas [7]. Of all the physiological effects of the coumarins this is the best understood at the molecular level, thanks largely to the work of MUSAJO, RODIGHIERO, DALL'ACQUA and the their collaborators in Italy [8] and PATHAK, FITZPATRICK, and their group in the United States [9]. Studies on the mechanism of their action have shown that linear furanocoumarins can, under the influence of appropriate ultraviolet wavelengths, form cross-linkages between the two strands of the DNA double helix, joining pyrimidine bases, (VIIa–d), and thereby resulting in a partial loss of template activity for RNA synthesis as well as inhibition of DNA replication. The American group have recently taken advantage of the reduced protein synthesis this implies in the epidermal cells to devise an effective treatment for the stubborn and disfiguring skin disease psoriasis, which is characterized by a proliferation of these epidermal cells. Oral doses of xanthotoxin followed by controlled exposure to ultraviolet radiation have shown remarkable success in experimental treatment of this disease [10, 11].

The subject of the present review is the biosynthesis of coumarins, and I wish especially to discuss the linear



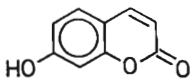

VII

furanocoumarins, which are principally found in species of the two plant families *Umbelliferae* and *Rutaceae*.

## Biosynthetic Routes to the Simple Coumarins

Coumarins, to the biochemist, are lactones of phenylpropanoid acids, a class which derives from the shikimic acid pathway via the protoaromatic amino acids phenylalanine and, in a few plants, tyrosine [12]. The entire carbon skeleton of the simple coumarins, i. e. those having no rings additional to the benzopyrone nucleus, is phenylalanine-derived [13]. In a number of early bio-

synthetic experiments on these compounds in plants it was shown that shikimic acid, phenylalanine, and *trans*-cinnamic acid are common precursors of this benzopyrone nucleus [14–16]. Cinnamic acid, however, represents a branch point in the elaboration of coumarin itself and probably other coumarins lacking 7-oxygenation, on the one hand, and the 7-hydroxycoumarins based on umbelliferone (VIII) on the

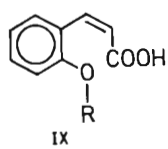


VIII

other [17, 18], the latter group constituting the vast majority of the class. *ortho*-Hydroxylation of *trans*-cinnamic acid leads to coumarin itself (Fig. 1), via a light-catalysed *trans-cis* isomerization, and lactone ring formation which can be formally represented as a dehydration [19, 20]. *para*-Hydroxylation of *trans*-cinnamic acid is a necessary prerequisite for synthesis of the 7-hydroxycoumarins, via *ortho*-hydroxylation and lactonization as before (Fig. 1).

The *ortho*-hydroxylations of cinnamic and *p*-hydroxycinnamic acids are mediated by different enzymes [22, 23], which are apparently seldom found together in the same species, so that coexistence of coumarin and the umbelliferones is an exceptional phenomenon.

It should be noted that a number of the simple coumarins are artifacts of isolation, and do not occur to any very significant extent in the free form in the intact plant cell. Thus coumarin [24], herniarin [25], and umbelliferone [26] have all been shown to occur in the form of glycosides of the corresponding *cis*-*o*-hydroxycinnamic acids (e. g. IX, R = glucosyl). However, glucosidases are also present which gain access to these substrates upon disruption of the cells, and the liberated aglycones (e. g. IX, R = H) lactonize spontaneously to the free coumarins [27]. The pungent odour of coumarin associated with new-mown hay originates in this way.



IX

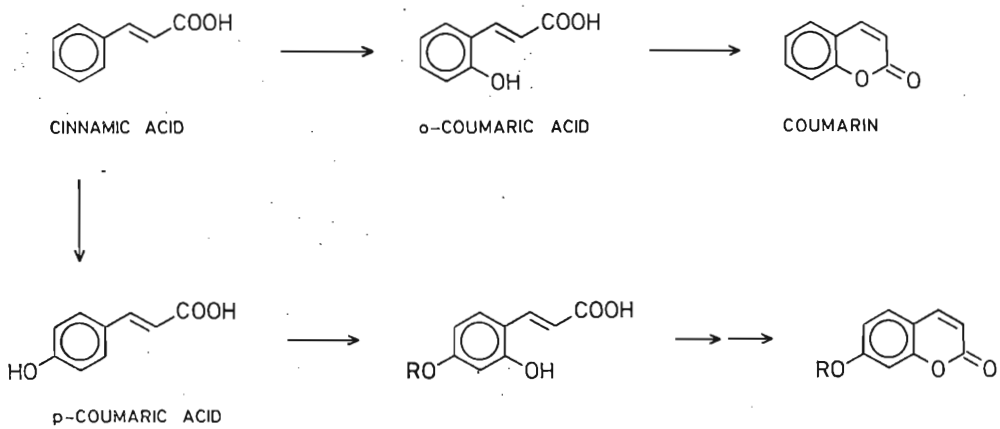



*Fig. 1.* Pathways from *trans*-cinnamic acid to coumarins.

In at least one case in the elaboration of a microbial coumarin – novobiocin (II) by *Streptomyces nivens* – lactone ring closure is not by dehydration but by an oxidative mechanism. KENNER and his co-workers [28, 29] have adduced evidence based on tracer studies with $^{14}C$ and $^{18}O$ that the lactone ring of this compound originates directly from tyrosine, with the ring oxygen deriving from the carboxyl oxygens of this amino acid.

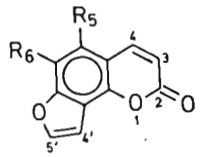
Current evidence, admittedly incomplete, suggests that oxygenation of the benzene ring of *simple* plant coumarins tends to occur befor the benzopyrone nucleus has been elaborated. Thus aesculetin (6, 7-dihydroxycoumarin) is formed from caffeic (3, 4-dihydroxycinnamic) acid [30], and scopoletin (6-methoxy-7-hydroxycoumarin) from ferulic (3-methoxy-4-hydroxycinnamic) acid [31, 32]. But occasional instances exist where a simple coumarin acts as the precursor of another, more highly oxygenated simple coumarin.

## Biosynthetic Routes to the Furanocoumarins

Consideration of possible biosynthetic pathways to the furanocoumarins raises the question of how the fused furan ring is constructed, and the order in which the three rings are formed. The first question has not yet been fully answered, but the second was answered in early studies by FLOSS and MOTHES [33], who showed by $^{14}C$ tracer experiments that umbelliferone (7-hydroxycoumarin) is an efficient precursor of the furanocoumarins of the umbellifer, *Pimpinella magna* L. Coumarin itself was not utilized, an observation consistent with the idea that pathways to coumarin and the oxygenated coumarins diverge at the cinnamic acid hydroxylation stage.

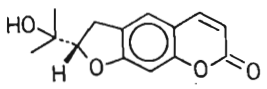
The furan ring is therefore constructed on umbelliferone, the simplest naturally occurring hydroxycoumarin. In the case of the linear furanocoumarins mentioned above the side chain is attached at position-6. In a second subclass, the angular furanocoumarins (X), which have a more restricted distribution, side-chain attachment is at position 8. A clue to the pathway of furan ring construction came from the existence of two substituted dihydrofuranocoumarins, marmesin (XI) in the linear series and columbianetin (XII) in the angular. The isopentane skeleton of the side-chains in those compounds suggested that their origin is in an isoprenoid side-chain attached to carbons 6 or 8 of umbelliferone, respectively, and arising ultimately from acetate via mevalonic acid (XIII).

FLOSS and MOTHES [34] did indeed present evidence, from the feeding of

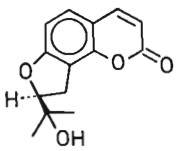



X

(a) Isobergapten: $R_5$ = OMe, $R_6$ = H
(b) Sphondin: $R_5$ = H, $R_6$ = OMe
(c) Pimpinellin: $R_5$ = $R_6$ = OMe

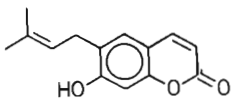



XI

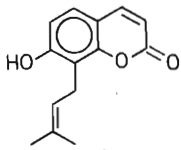


XII

XIII



XIV



XV

$^{14}$C-labelled mevalonic acid to *P. magna*, of a specific incorporation of carbons 4 and 5 of this compound into carbons 5′ and 4′, respectively, of the furan ring of pimpinellin, an angular furanocoumarin, but this precursor was utilized with very low efficiency. However, despite later questions raised about the suggested role of mevalonate as an intermediate in this pathway [35, 36], the studies of KUTNEY's group [37–39] on cell cultures of *Thamnosma montana* TORR. and FRÉM. have amply confirmed this role.

The specific incorporation of $^{14}$C from mevalonate into the furan ring suggests involvement of an isoprenoid unit in the biosynthetic pathway, and its condensation at some stage with the benzopyrone nucleus. Several simple coumarins are known which have isoprenoid side chains [40], but two which are of particular interest in the present context are demethylsuberosin (DMS, XIV) and osthenol (XV), which have dimethylallyl side-chains attached *ortho* to the hydroxyl group of umbelliferone, at positions 6 and 8, respectively. Tracer experiments have demonstrated that DMS is a precursor of the linear furanocoumarins [41–43] and that osthenol is a precursor of the angular furanocoumarins [42]. The use of trapping techniques [42] has shown that DMS, which apparently accumulates in quantity only in *Chloroxylon swietenia* DC., a species of mahogany [40]. is formed from umbelliferone in *Ruta graveolens* L. of the Rutaceae as well. DMS and osthenol are thus presumed to be intermediates in all species forming the respective subclasses of furanocoumarins.

The reaction by which the dimethylallyl side-chain is attached to C-6 of umbelliferone to form DMS, the committed step in linear furanocoumarin biosynthesis, has been the subject of investigation at the enzyme level in the author's laboratory. The participating enzyme, a particulate dimethylallyl (prenyl) transferase, was demonstrated in extracts of *R. graveolens* cell cultures [44], as well as in fresh leaves of the same species. It mediates the reaction shown in Fig. 2. It does not, however, transfer a prenyl group from dimethylallyl pyrophosphate to C-8 of umbelliferone to form osthenol. It is also inactive against pyrophosphates of two or three isoprenoid units – geranyl and farnesyl. DHILLON and BROWN [45] later established that this transferase is a chloroplast enzyme, a point of interest in the light of earlier demonstrations

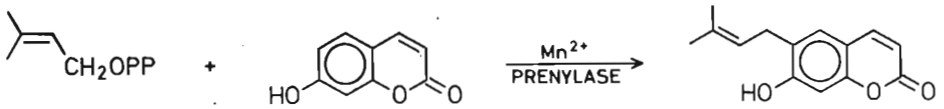



*Fig. 2.* Formation of demethylsuberosin by a prenylase from *Ruta graveolens*.

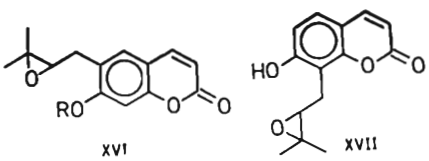
[22, 23] that hydroxylases mediating the committed step in the elaboration of coumarin and the 7-oxygenated coumarins are also chloroplast enzymes. The prenyl transferase was successfully solubilized, and purified over 300-fold by ammonium sulphate fractionation followed by column chromatography on DEAE-cellulose and hydroxylapatite [45].

The next intermediates in the biosynthetic pathways to the linear and angular furanocoumarins, the hydroxyisopropyldihydrofuranocoumarins, were actually the first to be identified. As mentioned above, these are marmesin (XI) in the linear series and columbianetin (XII) in the angular. A glucoside of marmesin accumulates in the umbellifer *Ammi majus* L. [46]. This plant was the subject of tracer studies by BROWN et al. [47], who found after administration of [$^{14}$C] umbelliferone that the specific radioactivity of the aglycone was higher than that of the linear furanocoumarins formed in the same experiment. This indication of an intermediate status for marmesin in the biosynthetic pathway was confirmed by trapping experiments in *R. graveolens*, and in addition tritiated marmesin was efficiently utilized as a linear furanocoumarin precursor in both species. The marmesin used in these species was the (+)-isomer; the enantiomer is inactive as a precursor in *R. graveolens*, *Angelica archangelica* L., and *Heracleum lanatum* MICHX. [48].

Analogous results were obtained in the angular series with columbianetin, in two other umbellifers, *A. angelica* and *H. lanatum*, both of which form angular as well as linear furanocoumarins [49].

The nature of the pathways between DMS and marmesin, and between osthenol and columbianetin, is still not definitely established. It was suggested quite early in the biosynthetic studies that the intermediates in question are coumarins in which the double bonds of the prenyl side chains have been epoxidized to yield structures XVI (R = H) and XVII [47, 49]. A coumarin of this type in the linear series is aculeatin (XVI, R = Me) which occurs in *Toddalia aculeata* PERS. [40]. Its 7-hydroxyl group is methylated, but where such is not the case it is easy to envisage the sort of reaction shown in Fig. 3, involving an attack of the phenol group on the epoxide ring, with a cyclization, and the formation of a substituted dihydrofuranocoumarin. There is no experimental evidence pertaining to this step, and it is still possible that diols such as XVIII, formed by hydrolysis of the analogous epoxides, participate.

Another important lacuna in our knowledge of these pathways is the nature of the mechanism whereby the



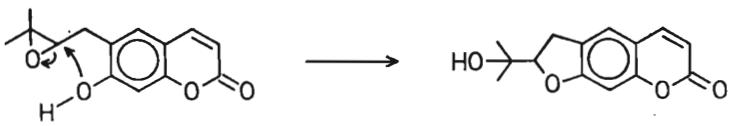
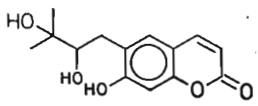




*Fig. 3.* Hypothetical cyclization reaction leading to marmesin formation.



XVIII

isopropyl side-chain is eliminated from marmesin and columbianetin, and the double bond introduced to form the furan ring. The only evidence thus far is negative. In the linear series, despite one early claim to the contrary [50], 4′, 5′-dihydropsoralen (XIX) is almost certainly not an intermediate [51, 52]. KUTNEY and his co-workers [37] deduced from experiments with specifically tritiated mevalonic acid that furan ring formation cannot proceed via any mechanism that involves loss of all the protons from either the 4′ R- or 5′-positions of this intermediate. A plausible mechanism, in which a carbonium ion is generated at C-4′, followed by a 1, 3-elimination, as shown in Fig. 4, has been proposed by Birch and his associates [53], the 3-carbon side-chain being converted to acetone in the process. To date there is no *in vivo* experimental support for this hypothesis.

It was noted above that oxygenation of the benzene ring of simple coumarins can often occur before the benzopyrone nucleus has been elaborated. The opposite situation seems generally to obtain with furanocoumarins. It is true that CAPORALE's group [54, 55] have reported 8-hydroxymarmesin (rutaretin, XX) to be an intermediate between marmesin and 8-methoxypsoralen (xanthotoxin, 6c) in *R. graveolens*, but this reaction appears to be exceptional, as attempts to demonstrate it in other species have failed [56]. On the other hand, several tracer studies [47, 54, 57, 58] have provided evidence that psoralen (VIa), the unsubstituted linear furanocoumarin, is converted *in vivo* to the mono- and dimethoxypsoralens, and as a few species accumulate the corresponding hydroxypsoralens [40, 59] it was postulated that psoralen is first hydroxylated, and that a transmethylation reaction completes the biosynthetic pathway to furanocoumarins [47].

This hypothesis has now been partially substantiated by experiments in this laboratory at the enzyme level. Extensive work both *in vivo* and with cell-free systems has been done in this laboratory on the transmethylation reactions which constitute the final step in the synthesis of such coumarins as bergapten (VIb), xanthotoxin (VIc), and isopimpinellin (VId) in the linear series, and which are also presumed to be involved in formation of isobergap-

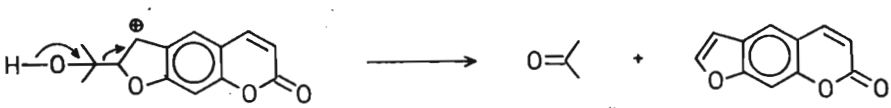
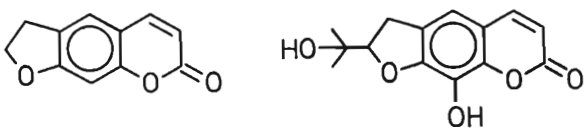


*Fig. 4.* Mechanism proposed by BIRCH et al. [53] for the conversion of marmesin to psoralen.



XIX XX

ten (Xa), sphondin (Xb), and pimpinellin (Xc) in the angular. Isopimpinellin, 5,8-dimethoxypsoralen, appears to arise *in vivo* by the two pathways, depicted in Fig. 5, although the one via xanthotoxin and 5-hydroxyxanthotoxin (VIe) has been shown by tracer experiments to predominate in *R. graveolens* [60].

In cell-free extracts, O-methylation of both 5-hydroxyxanthotoxin and 8-hydroxybergapten (VIf) leads to isopimpinellin, whereas O-methylation of 5-hydroxypsoralen (bergaptol, VIg) yields bergapten, and that of 8-hydroxypsoralen (xanthotoxol, VIh) produces xanthotoxin. All four O-methylations are done in the presence of *S*-adenosyl-L-methionine by a soluble methyltransferase system in cell-free extracts of *R. graveolens* which, in contrast to the enzyme mentioned earlier, does not appear to be confined to the chloroplast [61]. Conditions of stability, molecular weight differences and differing effects of various divalent cations on activity suggested the existence of at least two O-methyltransferases in the system, acting respectively at the 5- and 8-hydroxyls.

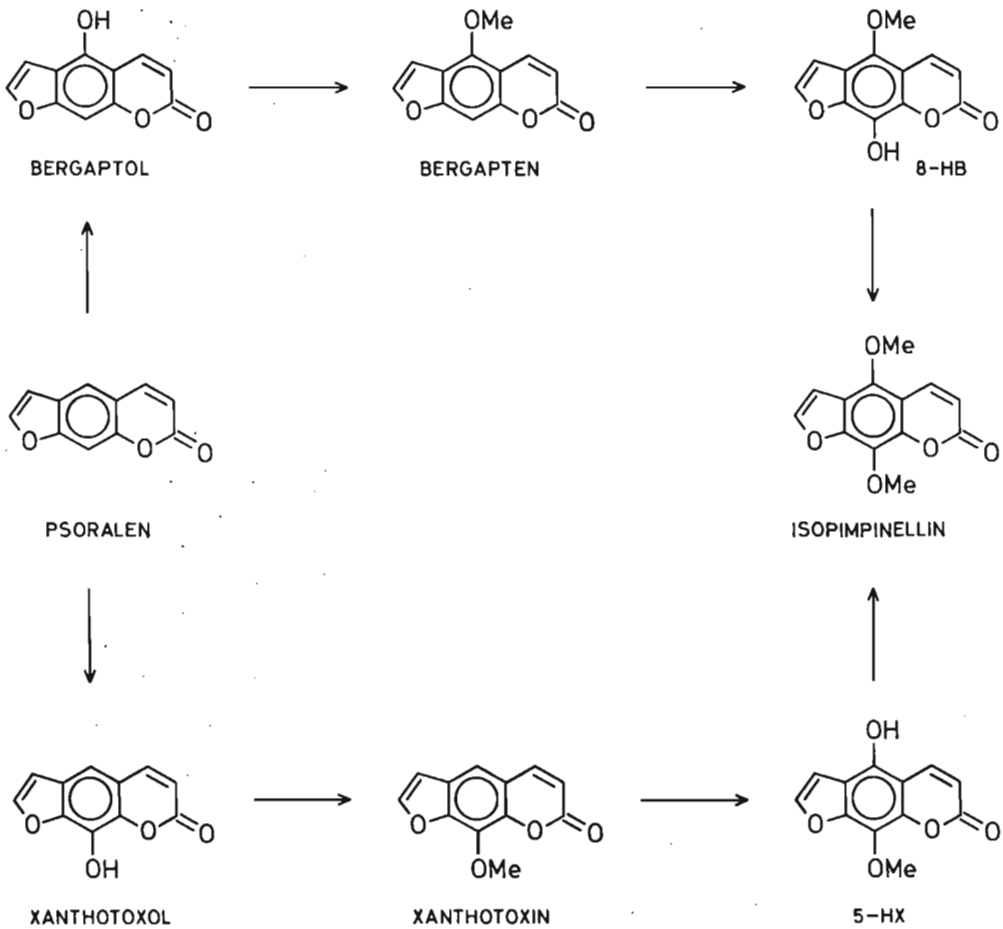



*Fig. 5.* Biosynthetic routes to isopimpinellin in *Ruta*.

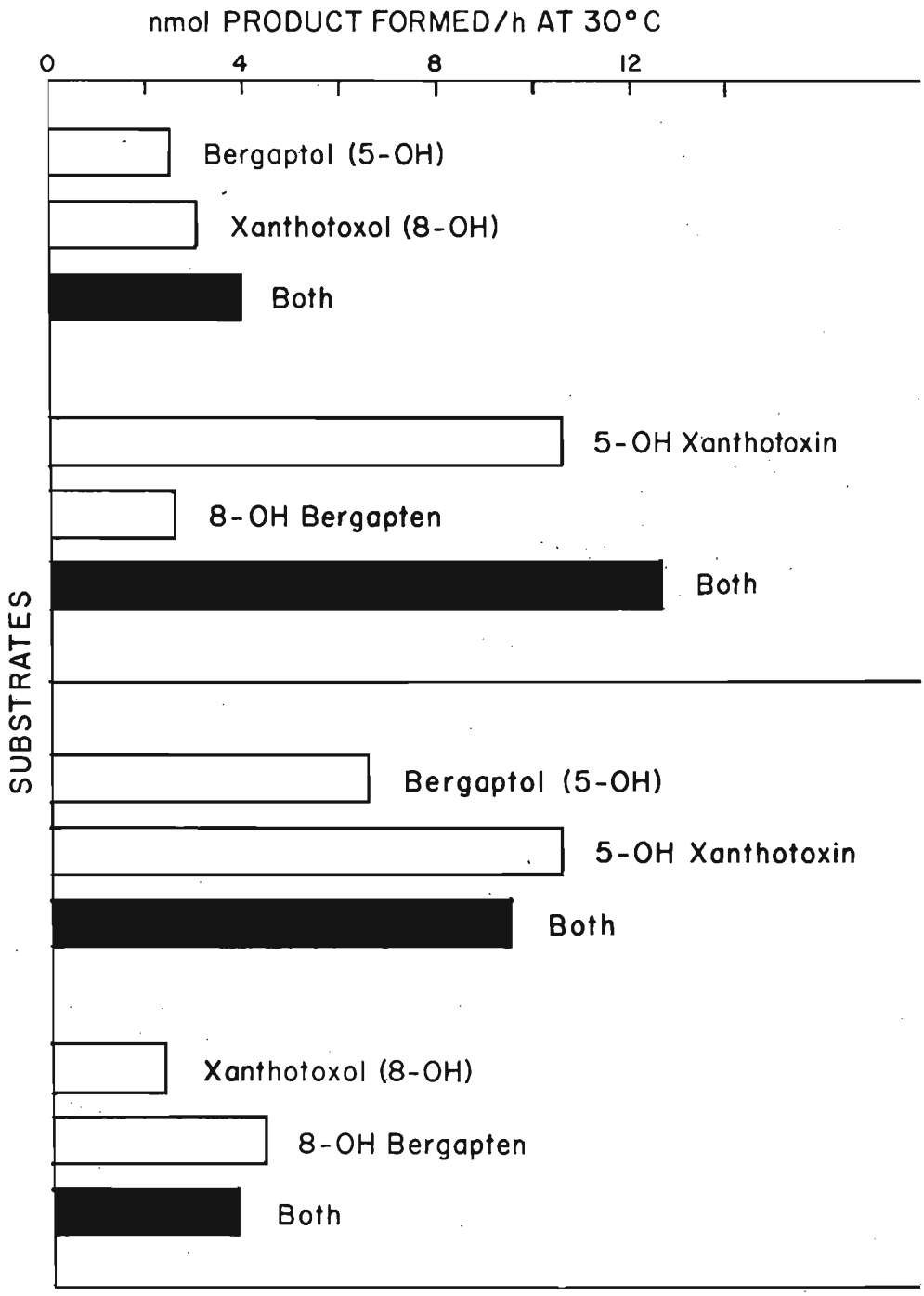



*Fig. 6.* Results of mixed substrate experiments utilizing an extract of *Ruta graveolens* acting on 5- and 8-hydroxylated linear furanocoumarins.

Conclusive evidence for two enzymes came from two further studies. As shown in Fig. 6, additive effects on activity were noted in the simultaneous presence of a 5- and an 8-hydroxyfuranocoumarin, in the two different cases. In contrast, the total activity in the presence of either the two 5-hydroxylated or the two 8-hydroxylated substrates was lower than the higher of the two activities observed when only one of the two substrates was present. Kinetic theory therefore strongly indicated that different enzymes were acting at the 5- and the 8-hydroxyls, but suggested that no more than two enzymes were acting. In a second approach, an affinity system was developed with *S*-adenosyl-L-homocysteine as ligand which selectively retarded O-methyltransferases [62]. In a purification sequence incorporating this step activity ratios against the 5- and 8-hydroxylated substrates fluctuated to a degree explicable only on the basis of discrete enzymes [61]. Both the mixed substrate studies (Fig. 6) and an examination of the divalent cation effects indicate the absence of more than two O-methyltransferases, but this cannot yet be regarded as conclusively established.

It is of interest that the transferase acting at position-5 methylates a hydroxyl *ortho* to the $C_3$ side-chain, the first instance of this pattern observed. Numerous other O-methyltransferases, including the one acting at C-8 of furanocoumarins, methylate *meta* or *para* to the side-chain.

Space has not permitted a consideration of studies done on other coumarins, nor is it possible yet to do more than speculate on the origin of still other classes such as those bearing side-chains on the lactone ring carbons. The existence of these yet unexplored types, together with the still considerable gaps in our knowledge of the biosynthesis of furanocoumarins, serves as a continuing reminder of the scope that remains for biosynthetic research on this remarkable group of secondary plant products.

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