STUDIES ON THE BIOSYNTHESIS OF COUMARINS

BY

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ABSTRACT

This thesis describes the investigations on the biosynthesis of coumarins from Thamnosma montana Torr. and Frem plants and tissue cultures.

Part I of this thesis discusses the degradative sequences developed for the furanocoumarins, isoimperatorin (13), allimperatorin methyl ether (7) and isopimpinellin (2) and for the coumarin, umbelliprenin (9). These degradative sequences were developed to gain information as to the distribution of radioactivity in the radioactive compounds made available from the subsequent biosynthetic studies.

In Part II of this thesis, the role of mevalonate (85) in the biosynthesis of the alkyl side chains and the furan ring of furanocoumarins of Thamnosma montana tissue cultures is described. In preliminary studies, it was shown that D,L-phenylalanine-[3-^{14}C] was being efficiently incorporated into three furanocoumarins, isoimperatorin (13), alloimperatorin methyl ether (7) and isopimpinellin (2). Incorporation experiments with various tritium labelled forms of mevalonic acid showed that mevalonic acid-[5-^{3}H] was being incorporated into the alkyl side chain and the 6-position of the furan ring of the furanocoumarins. Similarly, experiments with mevalonic acid-[4-^{3}H] indicated it to be a precursor of the alkyl side chains and the 7-position of the furan ring of the furanocoumarins. Mevalonic acid-[2-^{3}H] was incorporated into the alkyl side chains but was not incorporated into the furan ring. Incorporation studies with mevalonic acid-[5-^{14}C] supported the data already obtained with mevalonic acid-

[5-^{3}H] and revealed that any activity in the methoxy groups of furanocoumarins obtained from the [5-^{3}H]- and [2-^{3}H]-mevalonic acid feeding experiments was either due to a tritium exchange between the tritiated mevalonic acid and the C_1-pool in the tissue culture system or by some other unknown mechanism.

Part III of this thesis describes the role of glycine (124) in the biosynthesis of the coumarins, umbelliprenin (9), alloimperatorin methyl ether (7) and isopimpinellin (2), in Thamnosma montana plants. By specific degradations, it was shown that glycine-[2-^{14}C] was acting as an efficient precursor of the methoxyl groups of alloimperatorin methyl ether (7) and isopimpinellin (2). Glycine-[2-^{14}C] was also shown to incorporate almost exclusively into the farnesyl-ether side chain of umbelliprenin (9) and to a small extent into the C_5-alkyl side chain of allimperatorin methyl ether (7). However, very little activity could be found in the furan ring and the coumarin portion of alloimperatorin methyl ether (7) and isopimpinellin (2). These results suggest that glycine-[2-^{14}C] is acting as an efficient precursor of the C_1 source in Thamnosma montana plants and can also be utilized by the plant system for the biosynthesis of the C_5-alkyl side chain and, in turn, the C_{15}-alkyl-ether side chain of alloimperatorin methyl ether (7) and umbelliprenin (9), respectively.

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ACKNOWLEDGEMENTS

I wish to express my thanks to Dr. J.P. Kutney for his excellent guidance throughout the course of this research. The support provided by his unbounded optimism and unfailing encouragement greatly facilitated the successful completion of this work.

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INTRODUCTION

The world of nature abounds in organic compounds of nearly every conceivable structural class and the study of these constituents represent a fascinating and fruitful area of scientific investigation. The cells of living organisms - plants, fungi, insects, and higher animals - are the sites of intricate and complex synthetic activities that result in the formation of a remarkable array of organic compounds, many of them of great practical importance to mankind. Of increasing interest to organic chemists and biologists alike, is the investigation of biosynthetic pathways and precursors utilized by plants to synthesize these natural products.

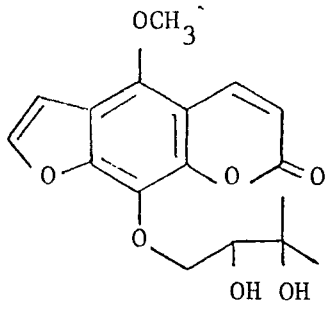
The members of the Rutaceae family (of which the Citrus genus is perhaps the best known) are mostly trees and shrubs, widely distributed in tropical and subtropical habitats, and particularly abundant in Australia and South Africa. This family is well known to contain a large number of benzenoid compounds, coumarins, flavones and some quinoline alkaloids.

Thamnosma montana Torr. and Frem. (Rutaceae), more commonly known as terpentine broom and found in desert mesas and slopes of South Western United States, has over the years attracted the interests of several groups of research workers. It represents a source of coumarins

which exhibit plant-growth-inhibitor properties,[1,2] and its use in folk medicine is also reported.[3] Bennett and Bonner,[1] while studying the toxicity of aqueous extracts of the leaves of desert plants found Thamnosma montana to be the most toxic as judged by the response to young tomato plants. The crude material when exposed to young plants over a period of about seven days caused death of these plants at concentrations of about 1 mg/ml of the administered solution.

These workers isolated three crystalline compounds from Thamnosma montana and identified two of them as byakangelicin (1) and isopimpinellin (2).

The structure of the third and most toxic compound was elucidated by Dreyer[4] and found to be 5-(3'-methyl-2',3'-dihydroxy butanyl)-8-methoxy psoralen (3), hereafter known as alloimperatorin methyl ether diol.



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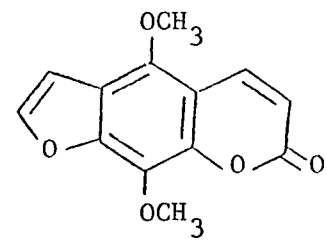

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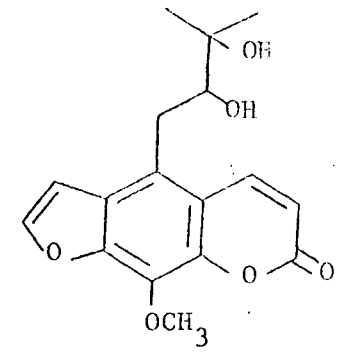

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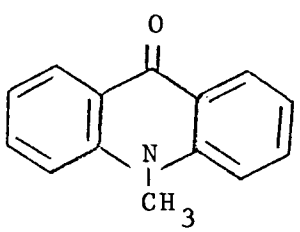
Dreyer developed a better extraction scheme and by chromatography of the acetone extract of Thamnosma montana on alumina was able to isolate not only the three coumarins obtained previously by Bennett and Bonner, but six other compounds as well. These compounds were identified as three known alkaloids; N-methyl acridone (4), skimmianine

(5a) and γ-fagarine (5b); β-sitosterol (6); a known furano coumarin, alloimperatorin methyl ether (7) and an unknown compound, thamnosin. This was the first report of N-methyl acridone (4), the parent member of the acridine alkaloids, occurring as a natural product.

Dreyer proposed a partial structure for thamnosin based on preliminary evidence, which proved to be incorrect. Inaba and Kutney[5,6] working in our laboratories were able to determine the structure of thamnosin as a novel dimeric system (8), a heretofore unknown coumarin.



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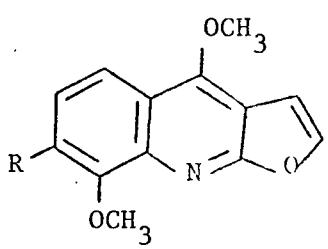
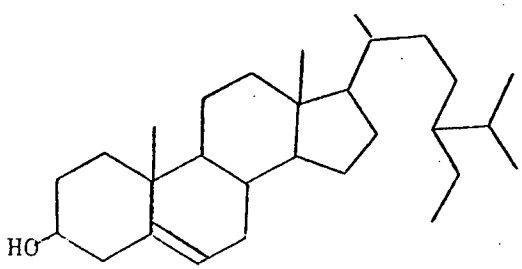


5a, R = OCH_3

5b, R = H



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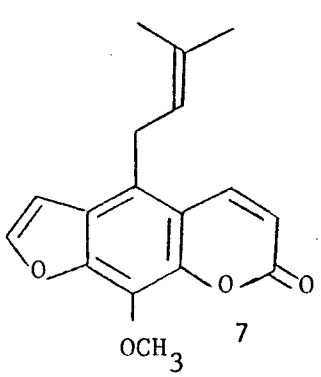

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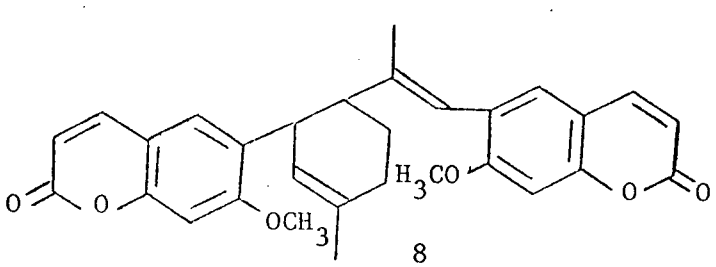

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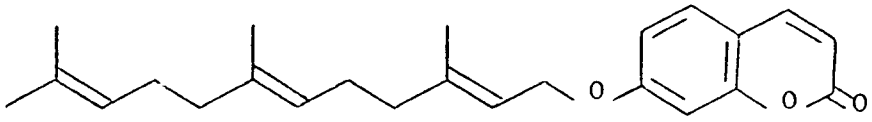
Dreyer could find no trace of the epoxide of alloimperatorin methyl ether,[4] which he had occasion to prepare during the synthesis of alloimperatorin methyl ether diol (3).

The co-occurrence of several types of coumarins in Thamnosma montana, including the interesting dimeric system thamnosin (8), appeared to offer a genuine opportunity to study the biosynthesis of

coumarins. During the course of preliminary investigations for the purpose of biosynthetic studies, it became apparent that Thamnosma montana contained a large number of isolable constituents in addition to those identified by Dreyer.[4] Thus to gain more complete appreciation of the plant system and to determine if these unknown compounds might offer further opportunities for biosynthetic experiments, an exhaustive isolation and structure elucidation study was carried out.

Thamnosma montana shoots and roots, obtained from the region in the Mojave Desert of California, were extracted with acetone and the acetone extract was chromatographed on alumina. By successive chromatography of various fractions, eight new compounds were isolated in addition to the nine already isolated by Dreyer.[4] Six of these compounds were identified to be already known coumarins; umbelliprenin (9); psoralen (10a); bergapten (10b); xanthotoxin (11); phellopterin (12); and isoimperatorin (13). The remaining two compounds were characterized as two novel epoxy coumarins; alloimperatorin methyl ether epoxide (14) and thamnosmin (15).



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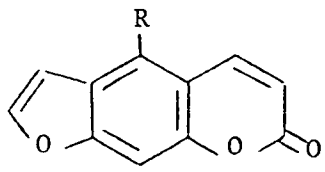
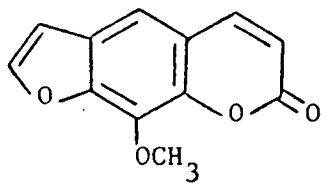


10a, R = H

10b, R = OCH_3



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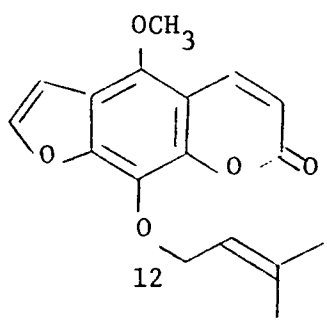

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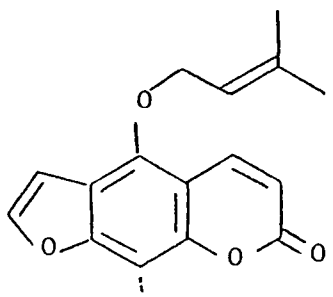

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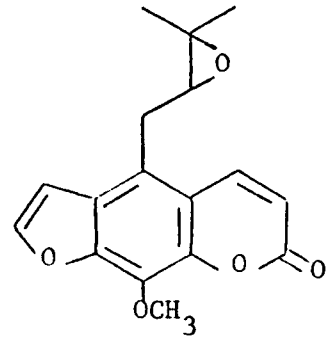

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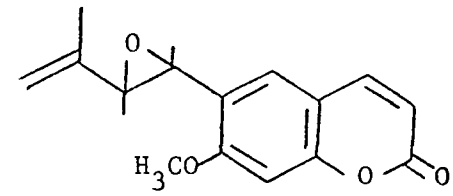

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The detailed study on the isolation and structure elucidation of these new compounds was done in collaboration with Dr. R.N. Young and as this work has already been reported in detail in Dr. Young's Ph.D. thesis,[7] I do not wish to discuss this aspect in any detail. Thus the known chemical constituents of Thamnosma montana can be summarized as in Table 1.

The co-occurrence of thamnosmin (15) and thamnosin (8) within the same plant raises the question of whether they are biogenetically related; since thamnosmin (15) is closely related to the diene (16) which would be produced by a retro Diels-Alder reaction of the co-occurring dimer thamnosin (8). Such a process is actually observed in the mass spectrum of thamnosin (8).[5,6]

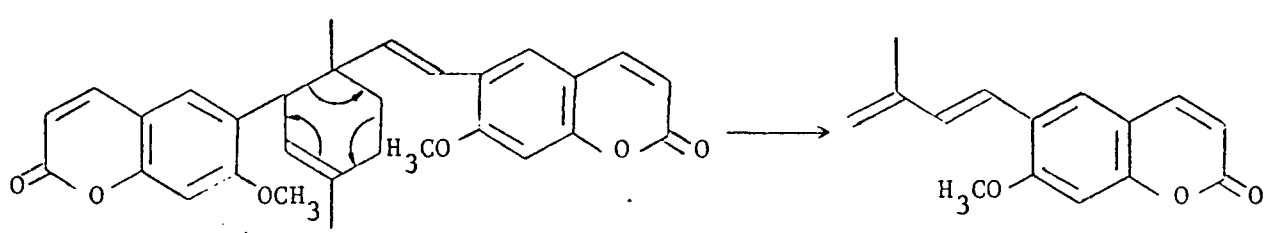

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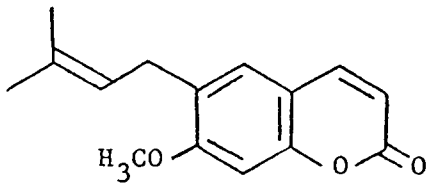
Table 1. Constituents of Thamnosma montana

Name	Formula	m.p.	Reference
β-sitosterol (6)	$C_{29}H_{50}O$	137-9°	4
alloimperatorin methyl ether (7)	$C_{17}H_{16}O_4$	108-10°	4
isopimpinellin (2)	$C_{11}H_{10}O_5$	148-9°	1,4,8
thamnosin (8)	$C_{30}H_{28}O_6$	244-6°	4,5,6
N-methyl acridone (4)	$C_{14}H_{11}ON$	202-3°	4
skimmianine (5a)	$C_{14}H_{13}O_3N$	173-5°	4
γ-fagarine (5b)	$C_{13}H_{11}O_3N$	140-2°	4
byakangelicin (1)	$C_{17}H_{18}O_7$	105-7°	4
alloimperatorin methyl ether diol (3)	$C_{17}H_{18}O_6$	174-6°	4
umbelliprenin (9)	$C_{24}H_{30}O_3$	61-2°	7,8
psoralene (10a)	$C_{11}H_6O_3$	163.5-164.5°	7,8
bergapten (10b)	$C_{12}H_8O_4$	186.5-188.5°	7,8
xanthotoxin (11)	$C_{12}H_8O_4$	146-7°	7,8
phellopterin (12)	$C_{17}H_{16}O_5$	92-4°	7,8
isoimperatorin (13)	$C_{16}H_{14}O_4$	97-8°	7,8
alloimperatorin methyl ether epoxide (14)	$C_{17}H_{16}O_5$	101-3°	7,8
thamnosmin (15)	$C_{14}H_{13}O_3$	101-4°	7,8

Recently Guise et al.[9] and Reyes[10] have reported the isolation of the novel dimeric coumarin thamnosin (8) from two other plant species of Rutoideae subfamily of Rutaceae,[11] namely Zanthoxylum dominianum Merr. and Perry and Ruta pinnata L.fil. Zanthoxylum dominianum also contains two monomeric coumarins which possibly would serve as biogenetic precursors of thamnosin (8); suberosin (17), a known coumarin, and suberenol (18), a new coumarin.



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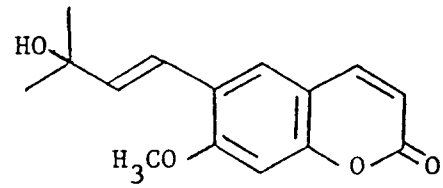

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Biosynthesis of Coumarins

Despite the large number of investigations in recent years into the formation of phenolic compounds in plants, the problem of the origin of the coumarins has attracted relatively little attention. This is perhaps surprising in view of the wide spread distribution and variety of structures associated with this class of compounds.[12] It is notable that with few exceptions, such as coumarin (19) itself, all naturally occurring coumarins have an oxygen atom at the 7-position, i.e. they can be regarded as derivatives of umbelliferone (49), which is one of the most widely distributed compounds of this class.

Before discussing the question of biosynthesis, one important feature of coumarin chemistry which has proved to be relevant to biosynthetic studies should be mentioned.

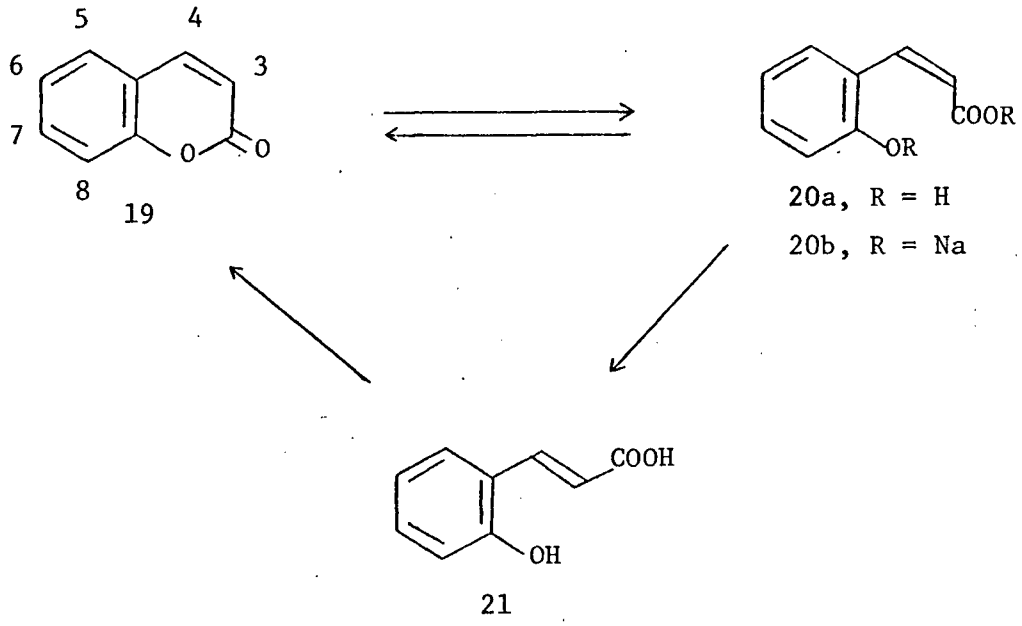
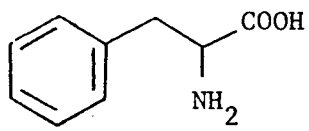


Figure 1. Isomerizations involving coumarin (19)

Coumarin (19) is a lactone of a cis-o-hydroxy cinnamic acid (20a) or coumarinic acid. The lactone ring is easily opened by heating with alkali to form the sodium salt of this coumarinic acid (20b). Upon acidification, the free acid lactonizes spontaneously, but if the salt is treated with mercuric oxide, a cis-trans isomerization takes place to yield stable o-coumaric acid (21). Ultraviolet radiations can catalyze the reverse isomerization to form the coumarin (19).[13]

Before the application of radioactive tracers became possible, attention was focussed on structural regularities within this family of compounds and in this way attempts were made to predict plausible biogenetic pathways. Robinson's[14] theories of biogenesis postulated

that the coumarin system arose by union of C_6 and C_3 units whilst Pavolino, in 1932, suggested pentose sugars as the basis of coumarin biosynthesis, and felt that this would account for the great variety of positions occupied by hydroxyl groups. The C_6-C_3 unit is present in phenylalanine (22) and tyrosine (23), but in earlier speculations these were not considered as direct precursors but rather as related compounds. However in subsequent isotopic labelling studies, both these amino acids (22,23) were in fact found to be the key intermediates.



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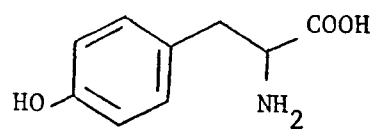

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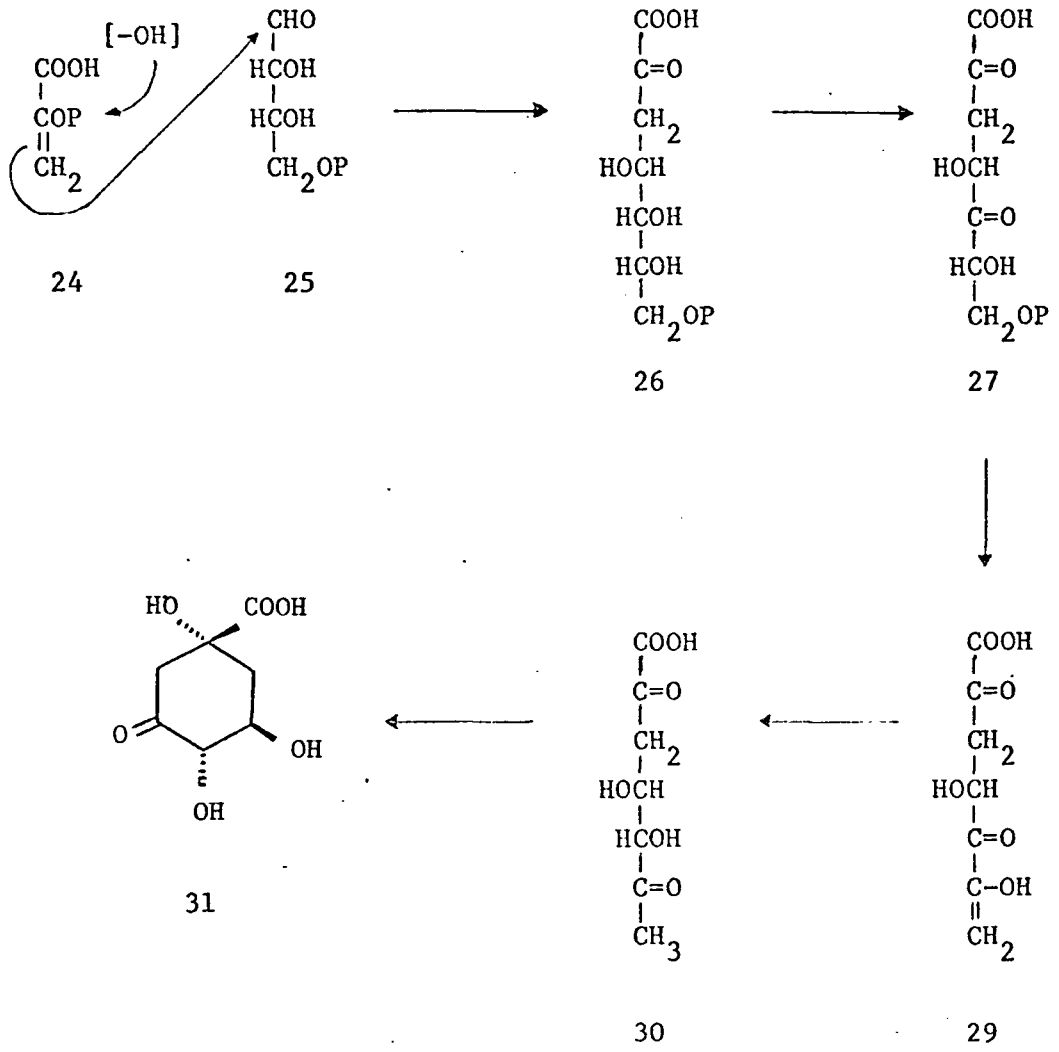
Before discussing the role of these intermediates in the biosynthesis of coumarins, it is instructive to consider their biogenesis.

The Biosynthesis of the C_6-C_3 Unit

Several metabolic pathways for the biosynthesis of aromatic compounds are now known but the phenyl propane unit in many natural products is considered to be biosynthesized from cyclohexane derivatives arising from cyclization of carbohydrates. Davis and coworkers[15-17] established the biogenetic route to phenylalanine (22) (later modified to include chorismic acid) in their work with nutritionally deficient microbial mutants. However, even earlier, when the structures of quinic acid (33a) and shikimic acid (34) were established, their

possible function as intermediates in the biosynthesis of aromatic natural products were suggested by Fischer and Dangschat.[18]

The formation of shikimic acid (34) proceeds from the three- and four-carbon atom precursors, phosphoenolpyruvate (24) and erythrose-4-phosphate (25), through a series of steps as shown in Figure (2).



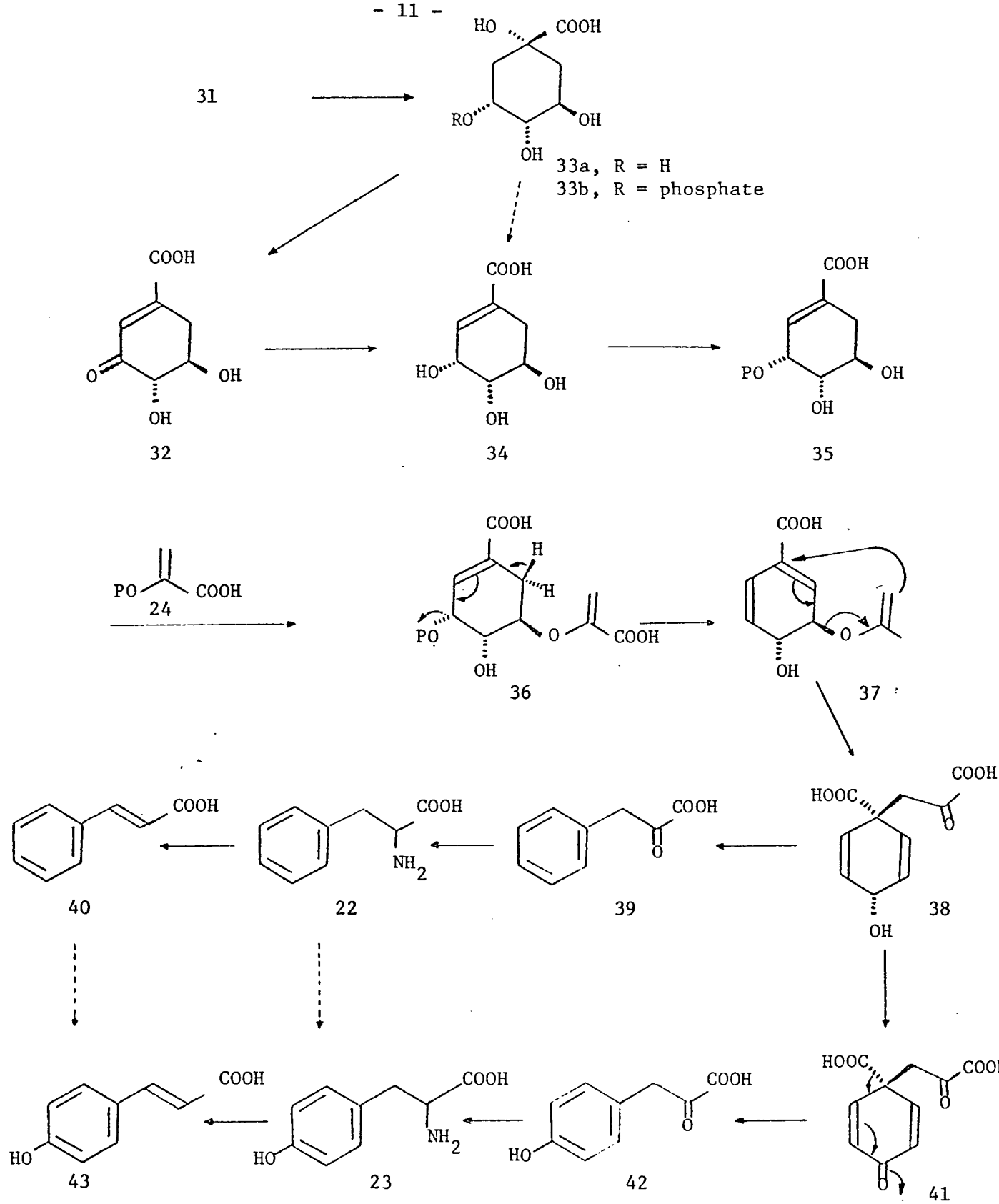


Figure 2. The biogenesis of aromatic compounds via shikimic acid (34).

Further reaction of shikimic acid (34) with phosphoenolpyruvate (24), followed by a stereospecific trans-1,4-elimination of phosphate yields the key intermediate chorismic acid (37)[19] which then rearranges in a reaction reminiscent of the Claisen rearrangement to give prephenic acid (38). Decarboxylation with accompanying expulsion of a hydroxyl group yields phenylpyruvic acid (39). Transamination of phenyl pyruvic acid (39) then leads to phenylalanine (22). The deamination of 22 by phenylalanine ammonia-lysase yields cinnamic acid (40).[20,21] Similarly transamination of p-hydroxyphenyl pyruvic acid (42) yields tyrosine (23) and subsequent deamination leads to p-hydroxy cinnamic acid (43). Tyrosine (23) can also be formed by hydroxylation of phenylalanine (22) and p-hydroxy cinnamic acid (43) can similarly be formed from cinnamic acid (40), but these are minor reactions.

Most of the work in the shikimic acid route to aromatic compounds was done with microorganism (E. coli mutants). However, studies done with plants suggest the pathway is similar if not identical to that elucidated in microorganism[20]. One possible difference may be that plants prefer quinic acid (33a) to shikimic acid (34) as a key intermediate. It is possible that quinic acid (33a) can be dehydrated directly to shikimic acid (34) or that 33a is converted to 5-phospho-shikimic acid (35) via 5-phosphoquinic acid (33b).

Biosynthesis of Coumarin (19)

The first proposal for coumarin biosynthesis from cinnamic acids was offered by Haworth,[22] who postulated para oxidation of a

p-hydroxy cinnamic acid (43) to yield (45) which could then cyclize by a Michael addition of carboxyl group and finally dehydrate to yield 7-hydroxy coumarin (49) (Figure 3).

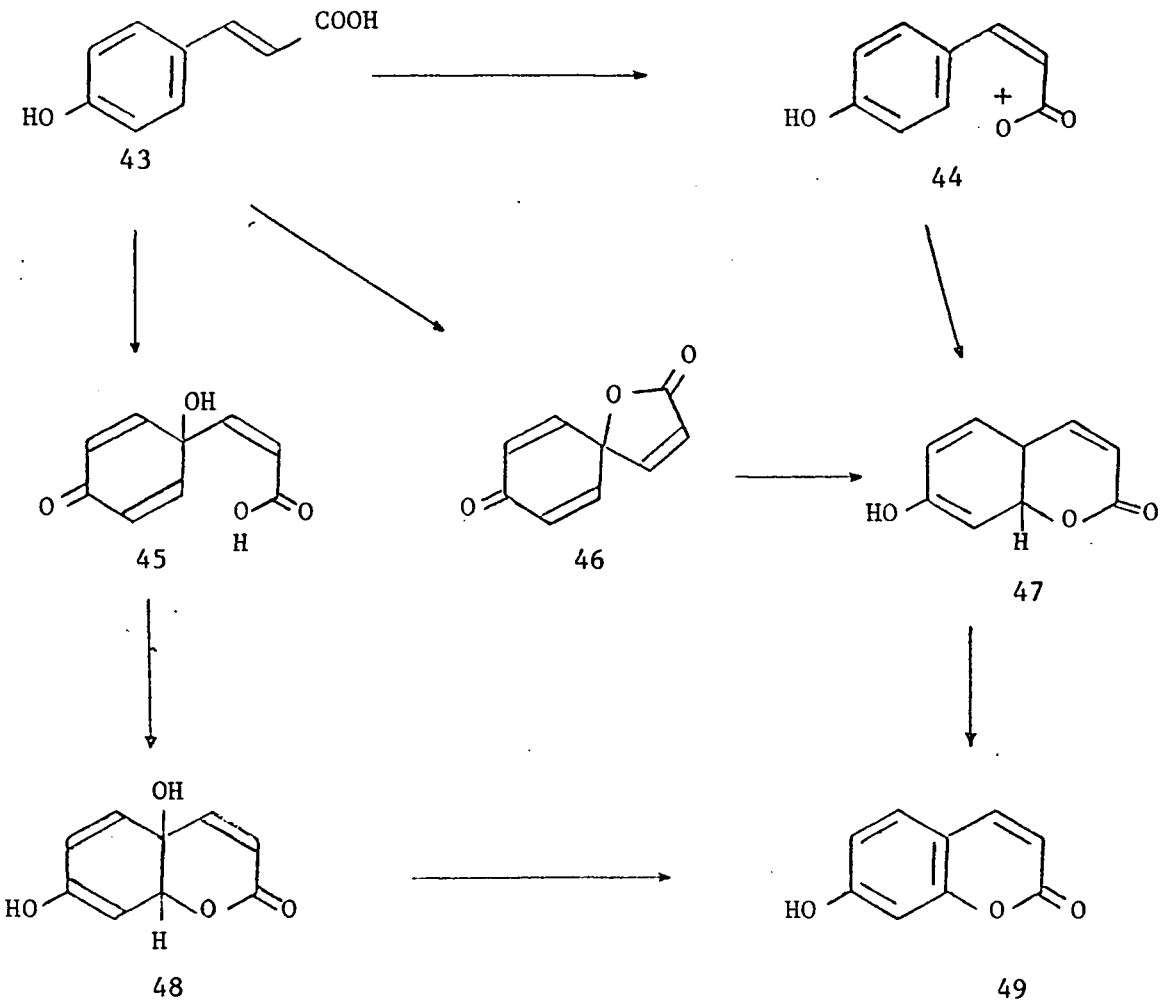


Figure 3. Proposed biosynthetic reactions leading to 7-hydroxy coumarin (49).

Grisebach and Ollis[23] suggested the direct oxidative coupling of the para-position with the cinnamoyl carboxyl, as in (46), whereas Kenner et al.[24] have favored initial two-electron oxidation of the carboxyl, as in (44), so as not to have the process dependent on the p-hydroxy function.

However, the first tracer experiment on the biosynthesis of coumarin (19) was done by Brown et al.[25] By feeding various labelled compounds into the perennial grass, Hierochloe odorata, they found that o-coumaric acid (21) and cinnamic acid (40) were utilized most efficiently, while phenylalanine (22) and shikimic acid (34) were less efficiently utilized, with acetate and salicylic acid being incorporated at very low levels. These results indicated that cinnamic acid (40) is being converted to o-coumaric acid (21) by a mechanism found in Melilotus species (sweet clover) to be controlled by a specific gene.[26] The ability to hydroxylate cinnamic acid in the ortho position seems to be a key factor in a plant's ability to synthesize coumarin (19). o-Coumaric acid (21) is next converted to o-coumaryl glucoside (50) and 50 has been shown to be a very good precursor of coumarin (19), having been found in all species containing 19 thus far examined.[13] The glucoside (50) is isomerized to the cis-isomer, coumarinyl glucoside (51) which may cyclize to form coumarin (19).

Haskins and Gorz[27] have shown that, at least in Melilotus, coumarin (19) as such is an artifact, and that what exists in the intact plant cell is actually the coumarinyl glucoside (51). Kosuge and Conn[28] have identified and characterized a β-glucosidase in Melilotus which specifically hydrolyses the cis-glucoside, releasing coumarin (19), when

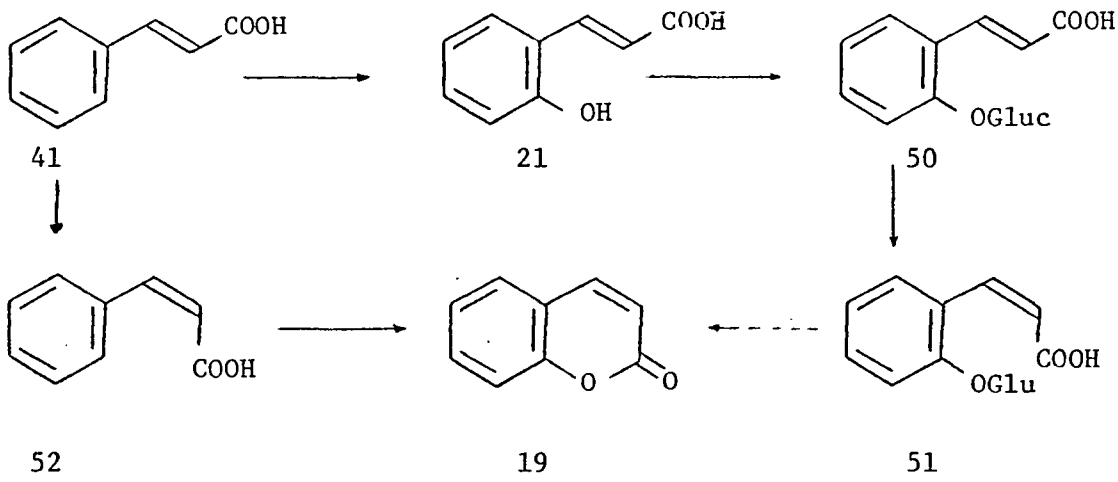


Figure 4. Biosynthesis of coumarin (19).

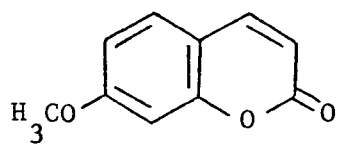
the cells are disrupted. Free coumarin (19), which probably does exist in some plants, may be formed by a route involving ortho-hydroxylation of cis-cinnamic acid (52); the evidence for this has been presented by Stoker and Bellis.[29]

The isomerization of o-coumaryl glucoside (50) to coumarinyl glucoside (51), in sweet clover, has been shown to be photochemically induced.[30,31]

Biosynthesis of 7-Oxygenated Coumarins

The formation of 7-oxygenated coumarins has been the subject of increasing study in recent years. A few of the simple members are

herniarin (53), esculetin (54a) and scopoletin (55). The corresponding cinnamic acid, p-methoxy cinnamic acid (56), caffeic acid (57) and ferulic acid (58) are also naturally occurring, thereby suggesting the existence of a biogenetic relationship between these compounds. A body of evidence supports this hypothesis.[13,32]



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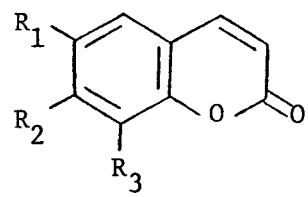
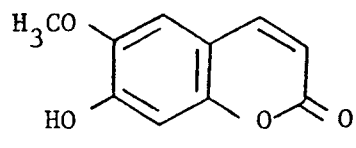


54a, R_1=R_2=OH, R_3=H

54b, R_1=H, R_2=R_3=OH



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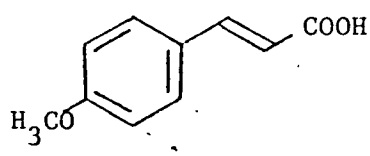

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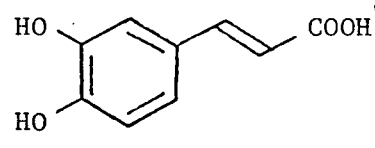

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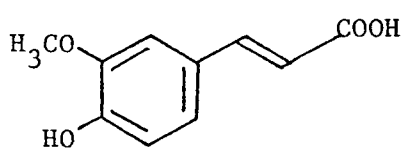

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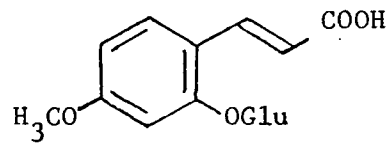
It is noteworthy that the co-occurrence of coumarin (19) and a 7-oxygenated coumarin is a rare phenomenon.[33] However, lavender (Lavandula officinalis) is one of the few species which elaborate both coumarin (19) and a 7-oxygenated coumarin, in this case, 7-methoxy coumarin (53). On this basis, this plant was chosen[34] as a convenient species in which to compare the biosynthesis of coumarin (19) and herniarin (53).

Using lavender plants, Brown[34] studied the biosynthesis of these two coumarins with a number of ^{14}C-labelled compounds. He found that

such precursors as glucose and phenylalanine (22) were incorporated equally well into both, as was cinnamic acid, while oxygenated cinnamic acids showed striking differences. For example, o-coumaric acid (21) and its glucoside (50) were selectively used for the synthesis of coumarin (19) while p-coumaric acid (43) and trans-2-glucosyloxy-4-methoxy cinnamic acid (59) were utilized selectively for herniarin (53) biosynthesis.



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The results of these investigations strongly suggest that the diversion in the pathway occurs at the trans-cinnamic acid stage. ortho-Hydroxylation apparently leads to coumarin (19), and para-hydroxylation to herniarin (53). As with coumarin (19), herniarin (53) is also found in lavender in the bound state (as a glucoside) and, as with 19, intermediary glucosides appear to be important.

Subsequent study has shown that biosynthesis of umbelliferone (49) can proceed by analogous pathways. The formation of umbelliferone (49) has been studied by Brown, Towers and Chen[35] and by Austin and Meyers[36] using Hydrangea macrophylla and the feeding and trapping experiments with ^{14}C-labelled compounds allowed the proposal for umbelliferone biosynthesis as represented in Figure (5).

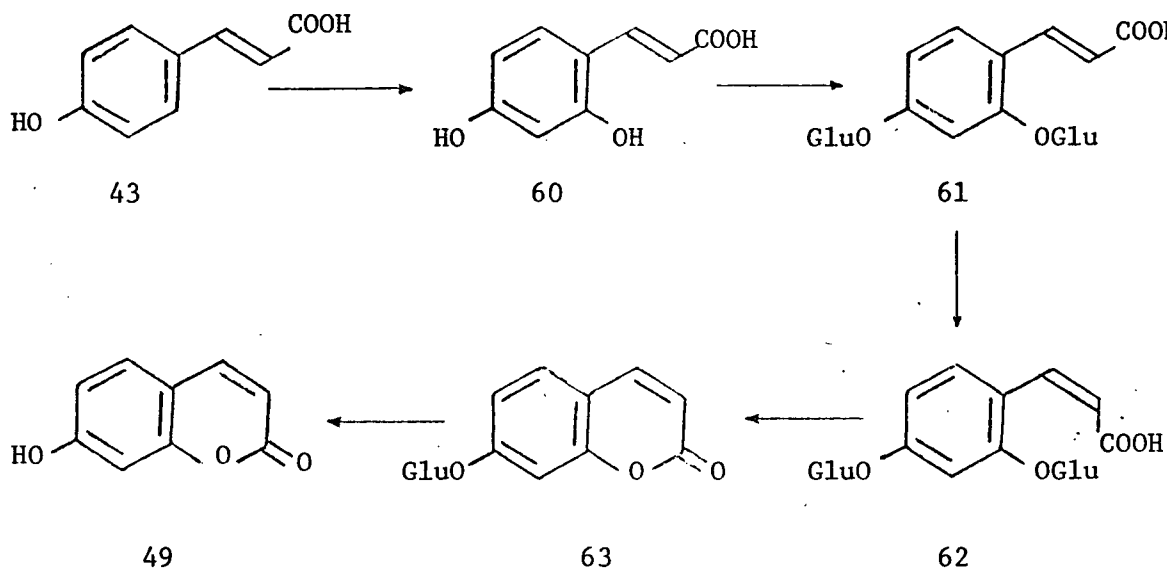


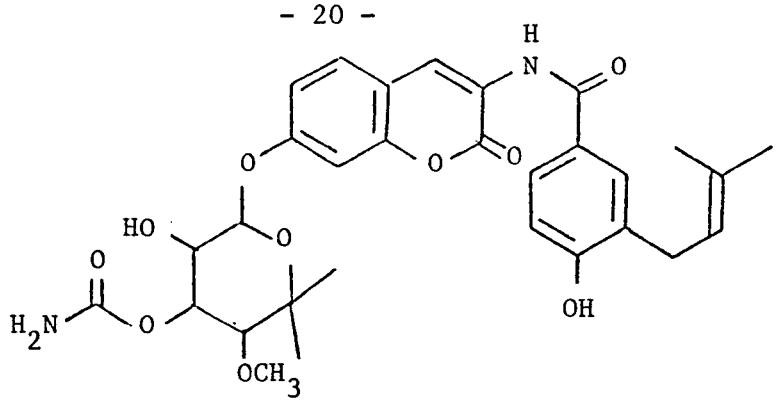
Figure 5. The biosynthesis of umbelliferone (49).[62]

Austin and Meyers[36] have reported that umbelliferone (49) exists as the free coumarin only to a very small extent, if at all, and have identified two bound forms in Hydrangea. The two forms are skimmin (63) and cis-2,4-di-β-D-glucosyloxycinnamic acid (62), the former being predominant. The conversion 63 → 49 may only occur when plant cells are disrupted. Reactions 61 → 62 and 62 → 63 are assumed to be fast as these intermediates cannot be isolated, although the conversion of p-hydroxycinnamic acid (43) to umbellic acid (60) has been detected by trapping experiments.[35]

The final step in the sequence, the trans-cis isomerization, has not been completely clarified. Current evidence suggests that it may be catalyzed in part by ultraviolet radiation[30] and in part by a specific isomerase.[37] However, recently, Edward and Stoker[31] have shown that in lavender, the trans-cis isomerization reaction in herniarin (53) biosynthesis is entirely photochemical with no isomerase involved. They consider that this situation is applicable to all plant coumarin biosynthesis. However, Ourisson and coworkers[38] feel the trans-cis isomerization in scopoletin (55) formation in tobacco tissue cultures is not purely photochemical. Thus there seems to be some doubt about the general validity of Edward and Stoker's statement.

Two other aspects of the formation of coumarins has been studied; one is the origin of the lactone ring, and the other is the stage at which methylation occurs in such coumarins as herniarin (53) and scopoletin (55).

After the studies on coumarin (19), it was assumed that the sequence of events was ortho-hydroxylation, glucosylation, trans-cis inversion, hydrolysis and lactonization; the last two being minor reactions (see Figure 4). However Kenner and coworkers[24,39] have shown that the lactone ring in the coumarin residue of the antibiotic novobiocin (64), formed by Streptomyces niveus, originates in a different way. By the use of ^{14}C and ^{18}O experiments, they showed that the carboxy oxygen atoms of tyrosine (23) served as a source of the heterocyclic coumarin oxygen atom. They postulated an oxidative cyclization of the amino acid to explain their results. Others[23,40]



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have raised the question whether a similar mechanism may not also operate in higher plants, and Scott and Meyers have suggested the involvement of a spiro lactone (46) (see Figure 3) but subsequent work by Austin and Meyers[36] with a ^{14}C-labelled spirolactone did not bear out the theory. The fact that the coumarins in question actually exist in the cell as glucosides of coumarinic acids argues strongly for an o-hydroxylation mechanism, as opposed to oxidative cyclization, in plants.[41]

The stage at which o-methylation occurs remains uncertain. Brown[34] found p-methoxy cinnamic acid (56) a much better precursor of herniarin (53) than was umbellic acid (60), which suggests that methylation occurs prior to ortho-hydroxylation, but trapping experiments failed to detect 56 in Lavandula.[42] Brown[32] feels that this plant can utilize 56 but the main pathway is by way of umbellic acid (60). He proposed that the poor incorporation of 60 could be explained if p-hydroxycinnamic acid (43) proceeded to trans-2-glucosyloxy-4-hydroxycinnamic acid (65) via enzyme substrate complex (X) with ortho-hydroxylation and glucosylation both occurring without

leaving the enzyme surface. The product, trans-2-glucosyloxy-4-hydroxycinnamic acid (65), could then undergo O-methylation to trans-2-glucosyloxy-4-methoxycinnamic acid (59), a known intermediate, followed by isomerization of the double bond to give the bound form of herniarin (53) (see Figure 6).

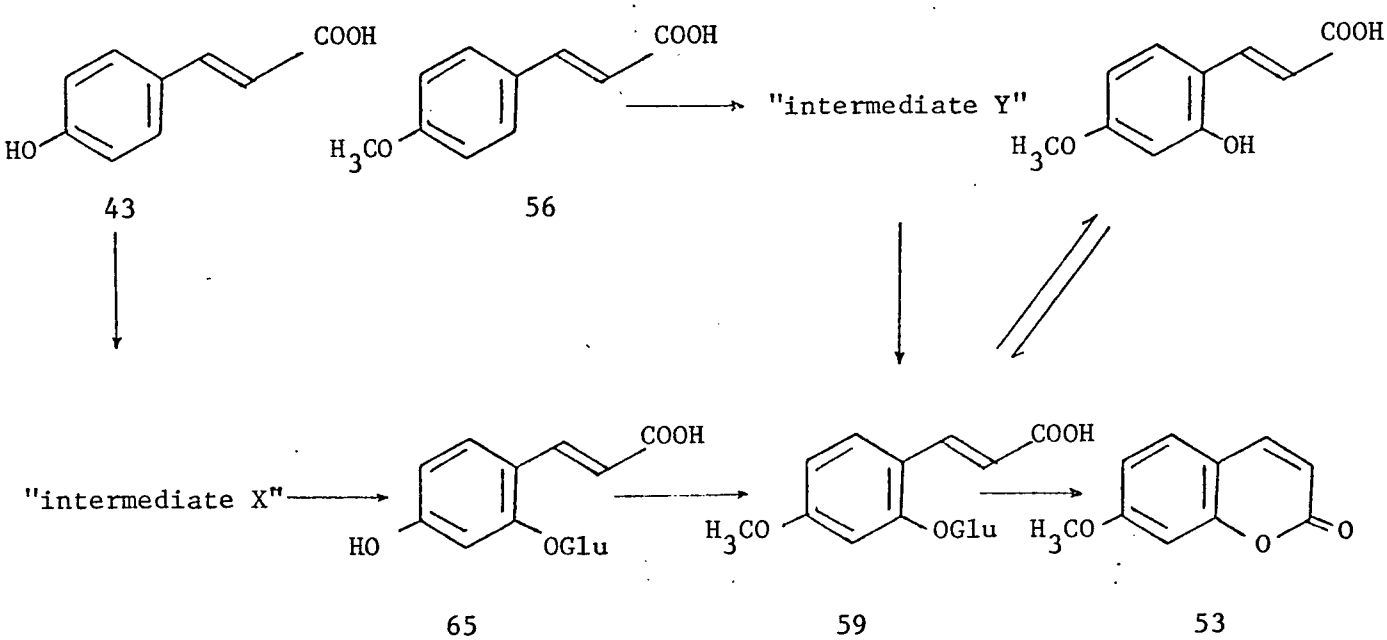


Figure 6. Proposed biosynthesis of herniarin (53) in Lavendula.

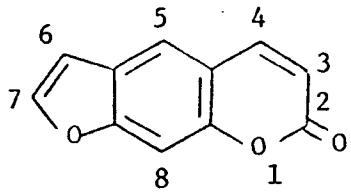
Ourisson[38] however suggests that methylation in scopoletin (55) biosynthesis, in tobacco tissue cultures, occurs prior to ortho-hydroxylation, as supported by incorporation of ferulic acid (58) into 55. However, he failed to find evidence of the o-hydroxylated ferulic acid intermediate and suggests that cyclization may occur via radical coupling without intermediary ortho-hydroxylation.

Recently, a publication has appeared on the biosynthesis of the dihydroxy coumarin daphnin (54b) and its corresponding 8-glucoside.[43] The authors suggest these compounds are produced mainly via p-coumaric acid (43) and not via caffeic acid (57) as has been suggested earlier.[32]

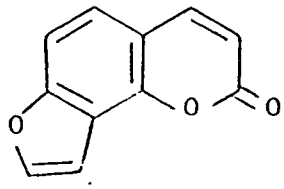
Thus the overall picture of the correct sequence of events is still somewhat confused.

Biosynthesis of Furanocoumarins

The furanocoumarins are fish poisons and insecticides.[44] Plants of the Rutaceae and Umbelliferae families are the principle source of the many naturally occurring members of this group. Several linear and nonlinear structurally isomeric furanocoumarins are theoretically possible, but with few exceptions, derivatives of only two of these isomers have been obtained from natural sources. The two categories are i) the linear system portrayed by psoralen (10a) (6,7-furanocoumarin) and ii) angelicin (66) (7,8-furanocoumarin) representing the nonlinear skeleton.



10a



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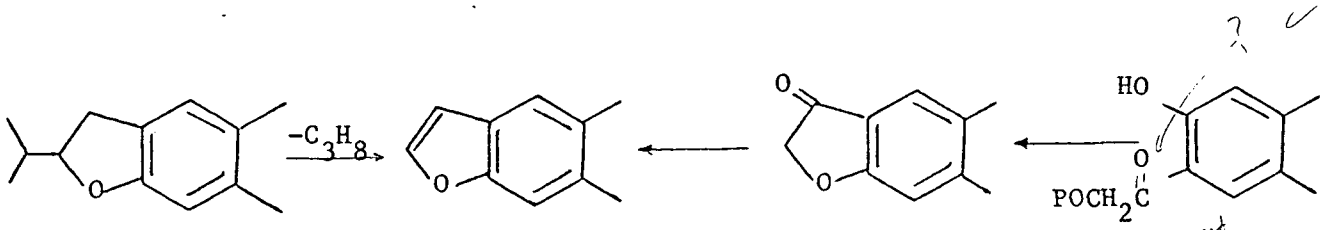
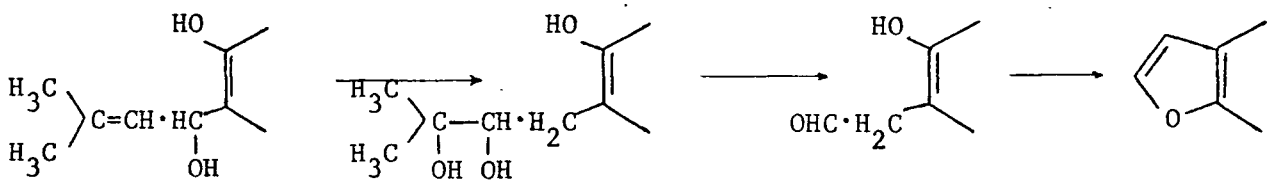
The biogenesis of the two extra carbon atoms of the furan ring (C-6 and C-7) of the furanocoumarins has been a source of controversy over the years. Haworth[45] suggested that theoretically the unsubstituted furan rings of these natural coumarins could be derived by elimination of propane from a hypothetical α-isopropyldihydrofuran (67). Geissman and Hinreiner[46] proposed a two carbon phosphorylated keto alcohol moiety (68), which could cyclize to furan-3-one (69) and subsequently yield a furan ring by reduction and dehydration.



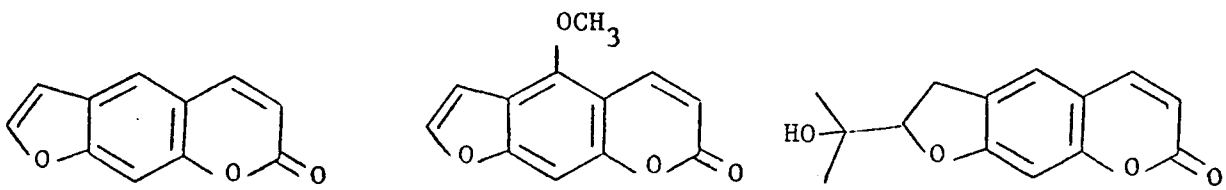
67 70 69 68

A review of natural products within the coumarin and related families reveals a remarkable feature that, frequently, definite isopentane units and unsubstituted furan rings are found incorporated

into a structure of a given natural product. Based on this close association, Seshadri and coworkers[47] suggested structure 71 to be the precursor of the simple furan ring. The transformation of 71 into the glycol (72) and oxidative cleavage of the latter, would result in the loss of three carbons, leaving a residue of two carbon atoms as shown in 73 which on cyclization and dehydration would form unsubstituted furans.



71 72 73

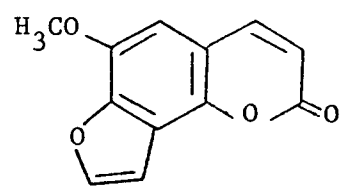


10a 10b 74

The first tracer study on the biosynthesis of furanocoumarins was done by Caporale et al.[48] They reported the incorporation of radioactivity from acetate-[2-^{3}H], tyrosine-[2-^{14}C] (23), [U-^{3}H]-tyrosine, mevalonic acid-[2-^{14}C] (85), and succinic acid-[2,3-^{3}H] into bergapten (10b) and psoralene (10a) utilizing leaves of Ficus carica. However, no degradations were performed to determine the position of the labels. The incorporation of mevalonic acid-[2-^{14}C] into these furanocoumarins is difficult to reconcile with the proposed formation of the

furan ring via a marmesin (74) type intermediate, as C-2 of mevalonic acid should be lost with the degradation of the three extra carbon atoms if Seshedri's hypothesis[47] is followed.

The first direct evidence as to the origin of furanocoumarins in Pimpinella magna was provided by Floss and Mothes.[49] By incorporating radioactively labelled precursors into the plant, they demonstrated that the coumarin nucleus, C_6-C_3 portion, of sphondin (75) is derived from cinnamic acid (40). Umbelliferone (49), a natural constituent of P.magna roots, proved to be a more efficient precursor than coumarin (19) indicating that para-hydroxylation occurs prior to



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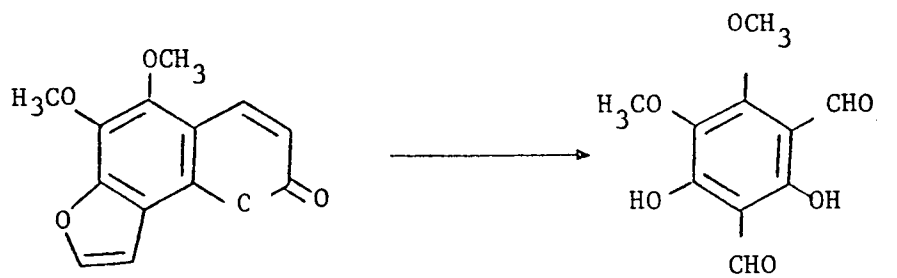
ortho-hydroxylation. By incorporating mevalonic acid-[4-^{14}C] (85) into Pimpinella, they were able to isolate radioactive pimpinellin (76) and its subsequent degradation provided good evidence of specific incorporation of C-4 of mevalonic acid into the 7-position of pimpinellin (76) (Figure 7).[49]

Floss and Mothes[49] also presented evidence as to the general mechanism of furanocoumarin biosynthesis, especially the sequence of the individual steps. On the basis of comparison of the specific activities of the various furanocoumarins after feeding cinnamic acid-[1-^{14}C] and mevalonic acid-[4-^{14}C], they suggested a biogenetic scheme



37.6 dpm/mmole

76

4.0 ± 0.9 dpm/mmole

Figure 7. Degradation of piminellin (76).

which involved isoprenylation occurring after the final oxygenation pattern of the coumarin portion had been established. Since the 7-hydroxy group of umbelliferone (49) moiety must not be methylated, they also suggested that umbelliferone-7-glucoside (63) might be an intermediate and that further hydroxylation and methylations might occur at the glucoside stage.

However in latter work, Floss and Paikert[50] tested this proposed scheme and found evidence which did not support prenylation as a last step in the biosynthetic pathway. They found that umbelliferone-7-glucoside (63) was not a better precursor of the furanocoumarins than umbelliferone (49) itself and that [CH_3-^{3}H]-scopoletin (55) was not incorporated preferentially into any one furanocoumarin as would be expected if isoprenylation were a late step in the biogenetic pathway. It was observed that the labelled methyl group of 55 was being rapidly equilibrated, by demethylation and remethylation, with the C_1 pool of the plant. They proposed an alternative scheme for furanocoumarin biosynthesis, involving

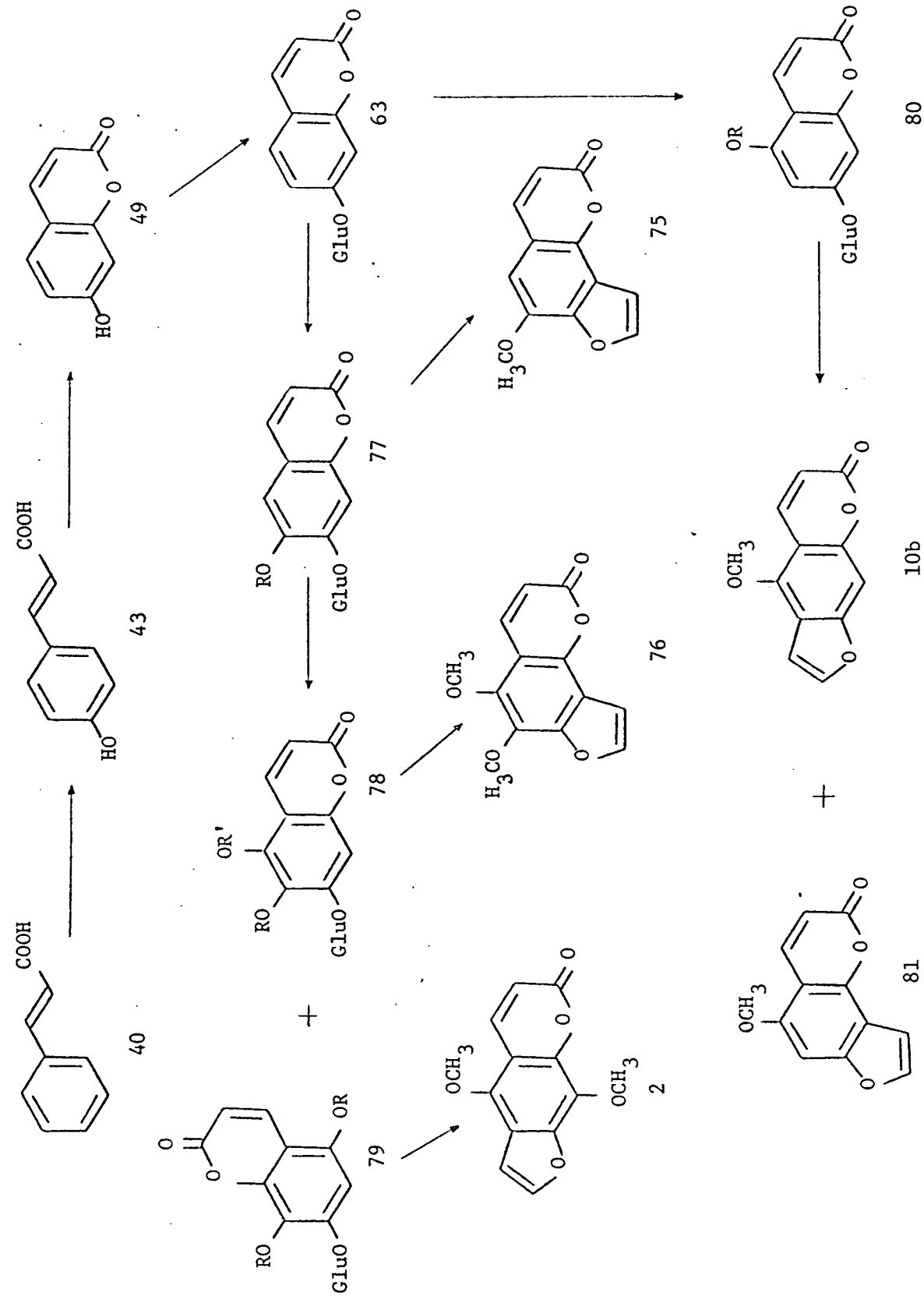


Figure 8. Proposed scheme for furanocoumarin biosynthesis in Pimpinella magna.[49]

isoprenylation of umbelliferone (49) followed by further modification of the resulting 6- and 8-dimethylallyl-umbelliferone (82,83). They feel that such a pathway (Figure 9) would also explain the almost exclusive occurrence, in nature, of 6,7- and 7,8-furanocoumarins. This opinion is supported by Steck and coworkers.[51]

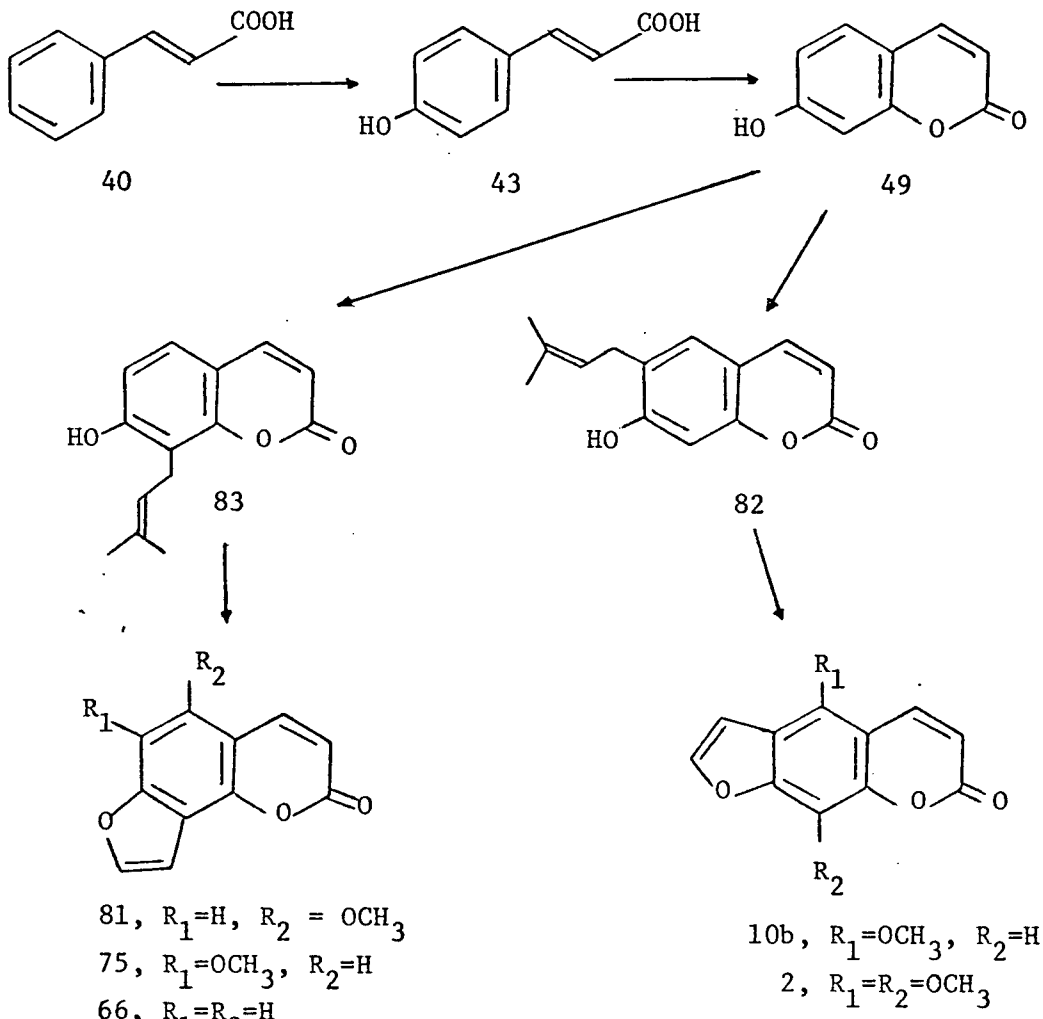
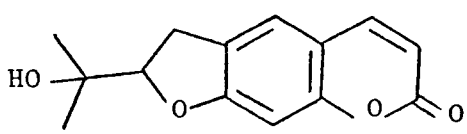


Figure 9. Alternative pathway of furanocoumarin biosynthesis in Pimpinella magna.[50]

Recently Steck, El-Dakhakhny and Brown[51] have demonstrated the intermediacy of mermesin (74) (in Ruta graveolens) in the biosynthesis of psoralen (10a), bergapten (10b) and xanthotoxin (11) and the intermediacy of columbianetin (84) (in Heracleum lanatum) in the biosynthesis of angelicin (66), isobergapten (81) and pimpinellin (76). By feeding umbelliferone-[2-^{14}C] (49) to Ruta graveolens and skimmin-[2-^{14}C] (63) to Heracleum lanatum, they were able to demonstrate the intermediacy of umbelliferone-[2-^{14}C] (49) in mermesin (74) and columbianetin (84). Furthermore, by direct feeding of tritiated marmesin (74) and columbianetin (84), they established their conversion to the appropriate furanocoumarins.



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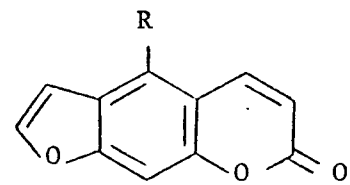
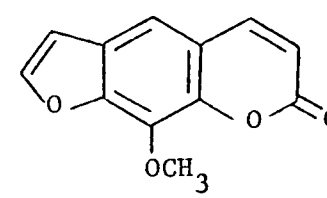


10a, R=H

10b, R=OCH_3



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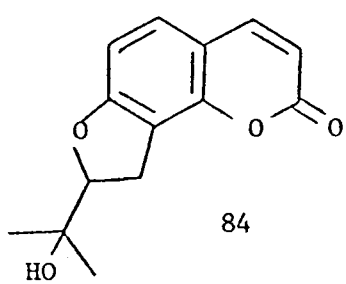

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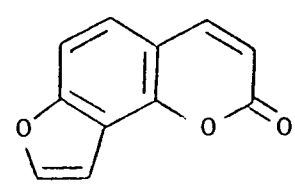

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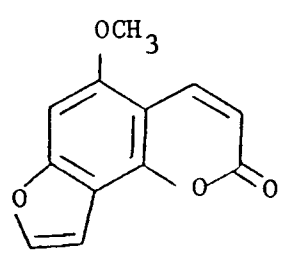

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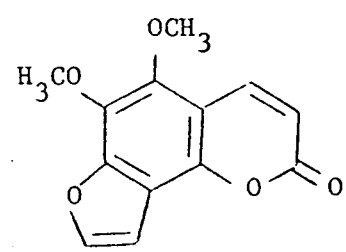

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Little is known as to the actual mechanism of furan ring formation but Brown and Steck[51] envisage the formation of marmesin (74) as depicted in Figure 10.

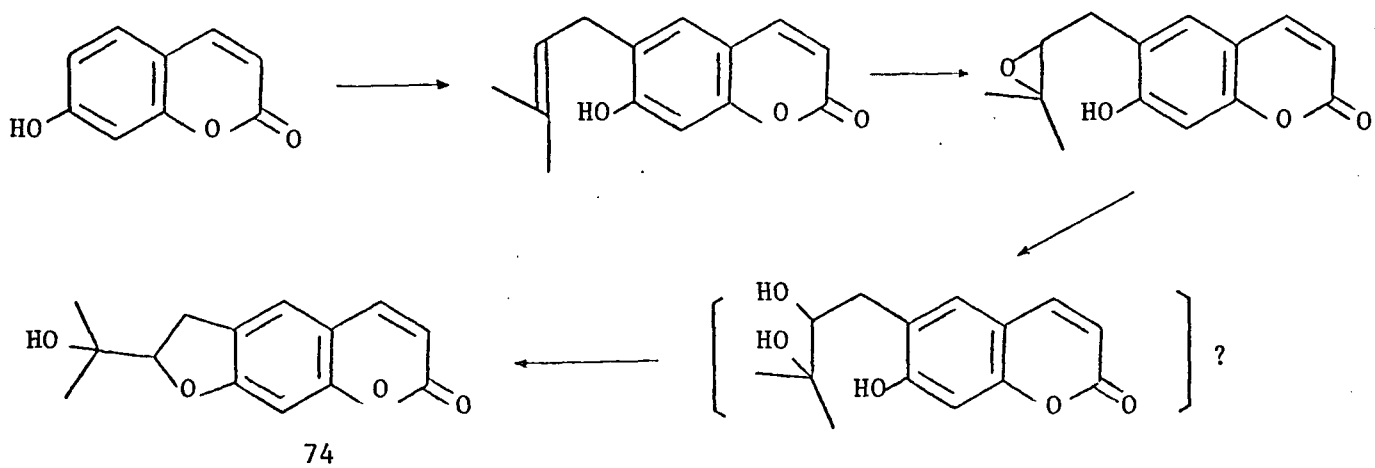
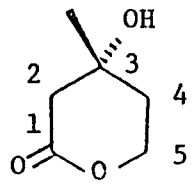
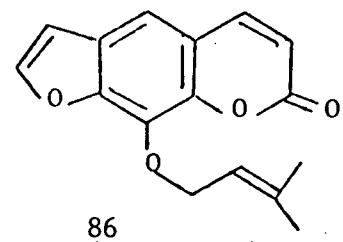


Figure 10. The biosynthetic route to marmesin (74).

Brown[52] has recently attempted to test some of the proposed pathways to furanocoumarins, utilizing parsnips (Pastinaca sativa L.) which contains the furanocoumarins bergapten (10b), imperatorin (86), isopimpinellin (2), and xanthotoxin (11). In his incorporation studies, with 5-methoxy-7-hydroxycoumarin-[2-^{14}C] (87), Brown could only conclude that there is no evidence to indicate that 87 is a precursor of bergapten (10b). From incorporation of mevalonic acid-[2-^{14}C] and -[5-^{14}C] (85), sodium acetate-[1-^{14}C] and -[2-^{14}C] and umbelliferone-[2-^{14}C] (49), Brown showed that mevalonate (85) and acetate were very poorly utilized relative to umbelliferone (49), and

that in general acetate was a better precursor than mevalonate. He reports that mevalonate-[2-^{14}C] was incorporated into all the furanocoumarins to approximately the same extent as the -[5-^{14}C] compound.





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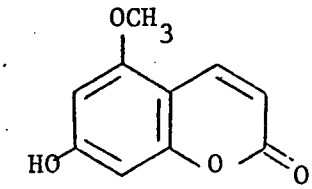
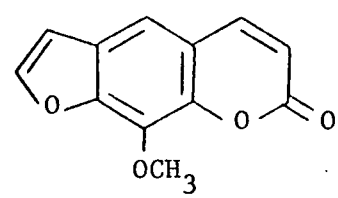


87

These results seem to raise doubts as to the role of mevalonate in the furanocoumarin formation, at least in parsnips. Brown suggests that it may be necessary to consider other possible origins of the 3-methylbutanoid moiety in marmesin (74) and thus also the furan ring carbons in furanocoumarins.

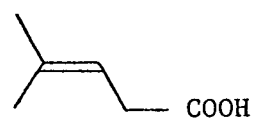
Alkylated Coumarins

Little information is available as to the origin of alkyl side chains often found in natural coumarins. Floss et al.[50] have presented evidence which shows that formate or the S-methyl group of methionine may act as a carbon source for the methoxyl group in xanthotoxin (11).



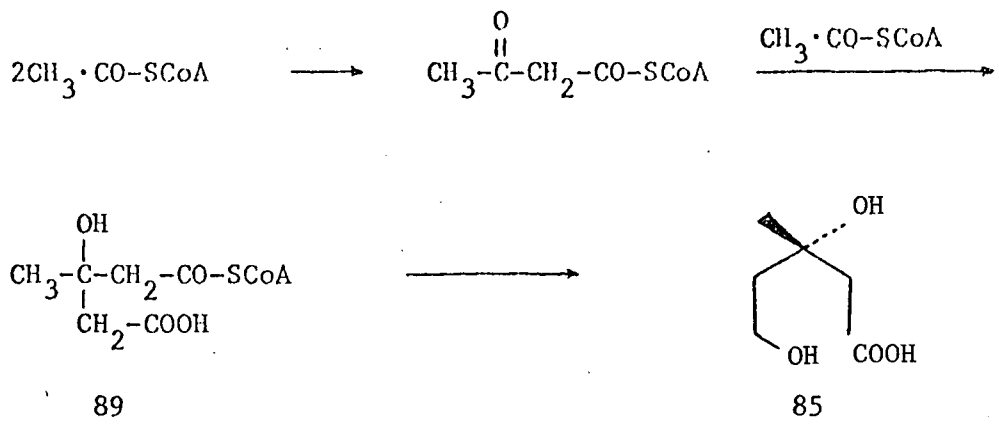
11

Isoprenoid groups are present in many coumarins; they may appear as O- or C-alkyl substituents or involved in ring formation with an adjacent hydroxyl group (see section on furanocoumarins). Multiples of the C_5 unit, such as the geranyl (C_{10}) or farnesyl (C_{15}) side chains are also found. Little definite information is presently known about the origin of these isoprenoid groups. Geissman and Hinreiner[46] have adopted the earlier ideas that the C_5 unit is the result of condensation of C_2 and C_3. Robinson[14] has suggested senecioic acid (β,β-dimethyl-acrylic acid) (88) as the terpene precursor, and the carbonyl group of 88 as the spearhead of the attack on the aromatic nuclei.



88

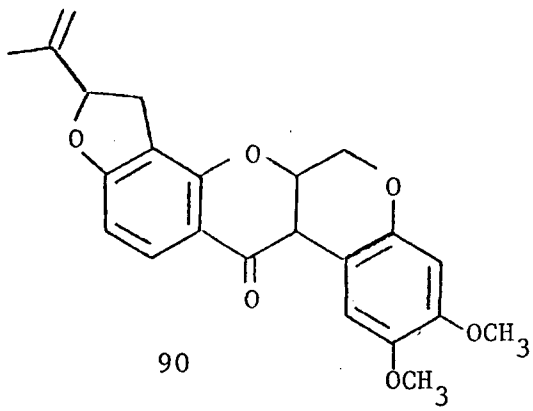
However, more recent work has emphasized the importance of mevalonic acid (85) as theprecursor to these side chains. Mevalonic acid (85) itself is the product resulting from the combination of three units of acetyl coenzyme-A[53] and as shown in Figure (11), its biogenesis is accomplished <u>via</u> Claisen and aldol-like condensations and the reduction of the resultant β-hydroxy-β-methyl glutaryl CoA (89).



89 85

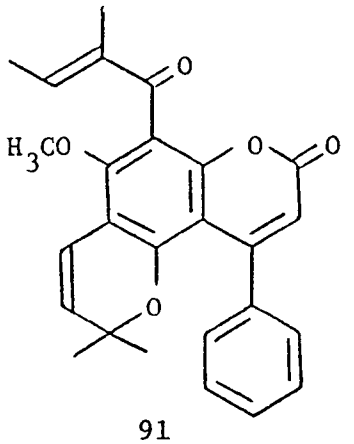
Figure 11. The acetate biogenesis of mevalonic acid (85).

Although no direct evidence is available to substantiate this hypothesis in coumarin biosynthesis, experiments with radioactively labelled mevalonic acid (85) have shown it to be incorporated into the prenyl side chains of some natural phenols and quinones.[54] Recently Hamada and Chabachi[55] have reported that mevalonic acid is utilized in the formation of the isoprene side chain of rotenone (90).



90

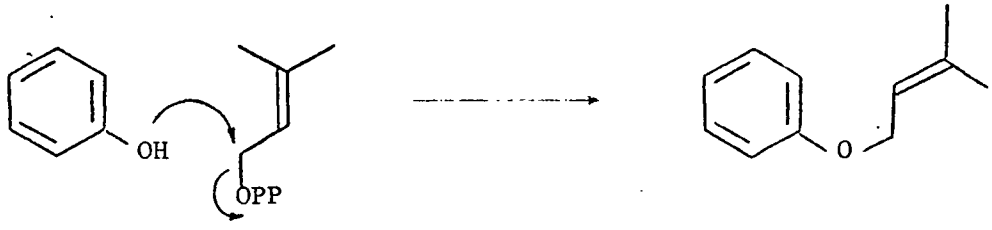
However, Kunesch and Polonsky[56] have shown isoleucine to be the presursor of the tigloyl side chain of the 4-phenyl coumarin calophyllolide (91).



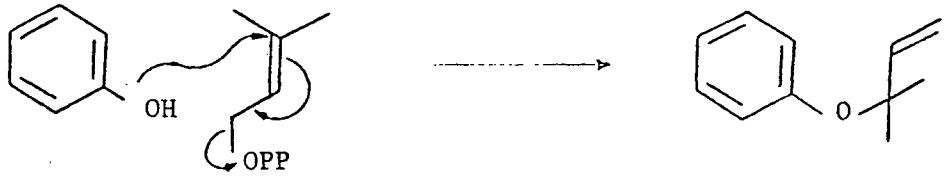
91

C- and O-alkylations of these phenolic compounds are assumed to occur via attack of the phenolic oxygen or of phenol activated ortho or para attack on γ,γ-dimethylallylpyrophosphate. This would give rise to any of the four possible products by the mechanisms indicated in Figure 12.[54]

(a)



(b)



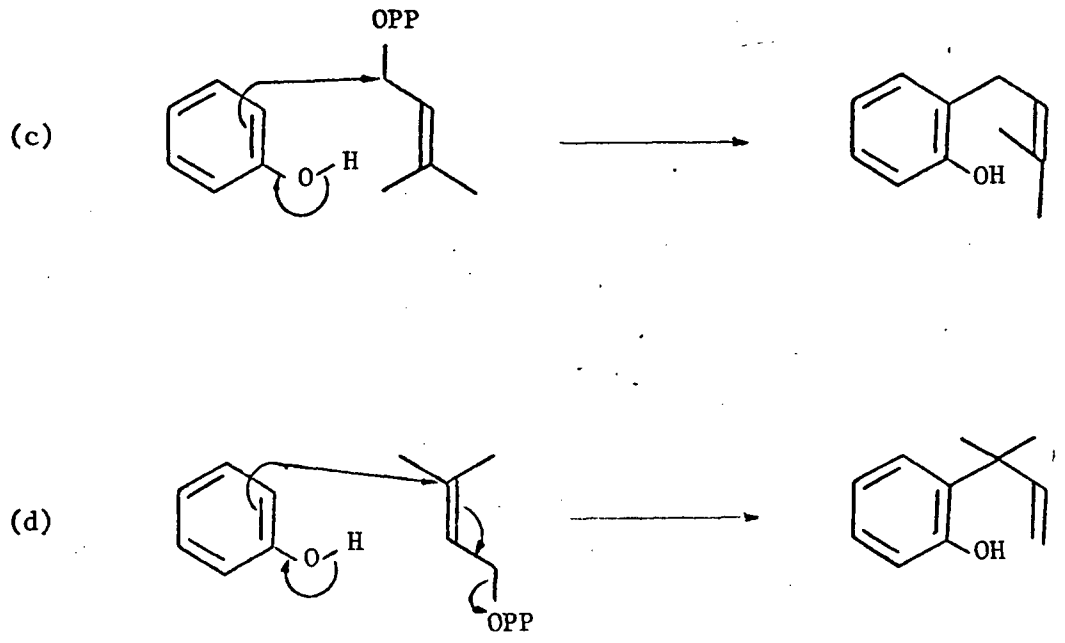


Figure 12. Proposed mechanism for alkylation of phenols.

The two alkylation reactions (a) and (c) leading to C-γ,γ-dimethylallyl derivatives and γ,γ-dimethyl allyl ethers are more likely to occur for obvious steric reasons. However, compounds of all four types have been isolated from natural sources and as expected, the products of the first two processes greatly outnumber the rather rare α,α-dimethylallyl derivatives.

The seemingly general confusion as to the nature of prenylation in coumarin biosynthesis, and the presence in Thamnosma montana of a number of interesting prenylated coumarins and furanocoumarins, led us to consider further experiments which might serve to help clarify some of the many questions that remain unanswered. Such experiments will be discussed in Parts II and III of this thesis.

DISCUSSION (PART I)

Degradations of Coumarins from Thamnosma montana

Before the advent of modern spectroscopy,many compounds were isolated and their structures determined by the use of exhaustive degradative techniques. Coumarins are certainly no exception in this regard. Thus there are a wide variety of methods available in the literature for the degradation of the coumarin system. However, for the purpose of biosynthetic investigations, it is essential that the reactions utilized be applicable to the small quantities of material that would be available from the radioactive incorporation experiments. Keeping this in mind, a detailed study on the degradations of coumarins available from Thamnosma montana was undertaken.

The coumarins umbelliprenin (9), isopimpinellin (2) and alloimperatorin methyl ether (7) were selected for this purpose as they are present in reasonable quantities in Thamnosma montana, are easily isolated and purified and are representative examples of three types of coumarins. However, the later studies on the tissue cultures of Thamnosma montana revealed the absence of umbelliprenin (9), whereas isoimperatorin (13) was present in isolable quantities in the tissue culture extract along with isopimpinellin (2) and alloimperatorin methyl ether (7). Therefore it was decided to study the biosynthesis of

all four coumarins from Thamnosma montana plants and tissue cultures and the degradations utilized in these investigations are discussed below.

To study precursors other than mevalonic acid which could be utilized by plants in the biosynthesis of the isoprenoid side chains in coumarins, umbelliprenin (9), a simple coumarin with a farnesol ether side chain, was considered to be the ideal compound. Since mevalonic acid has been shown to be the precursor of these isoprenoid units in many other compounds (see Introduction), umbelliprenin (9) could also act as an internal standard in any subsequent mevalonic acid feeding experiment. On the other hand, isopimpinellin (2) is a relatively simple furanocoumarin whose chemistry can be correlated with that of other such furanocoumarins as bergapten (10b) and xanthotoxin (11). Thus, although little has been reported on the degradative chemistry of isopimpinellin (2) itself, related compounds, such as bergapten (10b), have been studied in detail. Alloimperatorin methyl ether (7) is also a furanocoumarin but with the added complication of a C_5 alkyl side chain whereas in isoimperatorin (13), a C_5 alkyl-ether side chain is present. Thus isopimpinellin (2), alloimperatorin methyl ether (7) and isoimperatorin (13) offer the possibility of studying the mode of furan ring formation and the origin of isoprenoid side chains in these compounds. With these various objectives in mind, a detailed degradative scheme was prepared for these compounds.

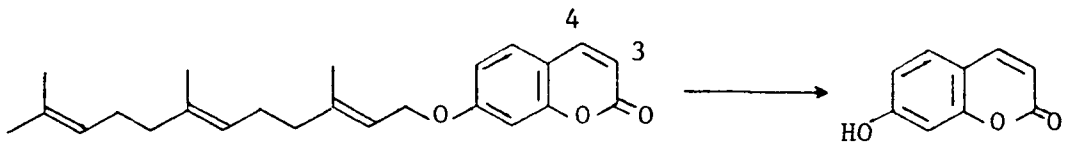
It is necessary to mention at this point that the degradations on umbelliprenin, isopimpinellin and alloimperatorin methyl ether were performed in collaboration with Dr. R.N. Young. A detailed account of

these degradations along with complete characterization of the degradative products can be found in the doctoral thesis of R.N. Young,[7] and consequently for the sake of clarity for further discussions only a brief description of these degradations will be presented here together with full details of any deviation from this sequence. A complete degradation of isoimperatorin (13) was developed more recently and is presented in detail.

Degradations of Umbelliprenin (9)

As mentioned previously, it was our intention to study the biosynthesis of the farnesol ether side chain present in umbelliprenin (9) and therefore it was desirable to determine the amount of radioactivity associated with this side chain in any radioactive incorporation experiment. Kariyone and Matsumo[57] found that the coumarin auraptene (the geranyl ether of umbelliferone (49)) would undergo efficient hydrolysis in hot glacial acetic acid to give umbelliferone (69) and geranyl acetate. However if the hydrolysis was carried out in glacial acetic acid in the presence of a small amount of sulfuric acid, no geranyl acetate could be isolated due to its polymerization. When the hydrolysis of umbelliprenin (9) was carried out in hot glacial acetic acid, umbelliferone (49) was obtained in good yield (75%) but no farnesyl acetate could be isolated. It was observed that under these reaction conditions, the farnesyl acetate being produced was undergoing a series of further reactions. However, since the radioactivity present in umbelliferone (49) derived from the reaction would allow calculation of the radioactivity in the side chain by difference, it was

decided to abandon further attempts to isolate the entire side chain.

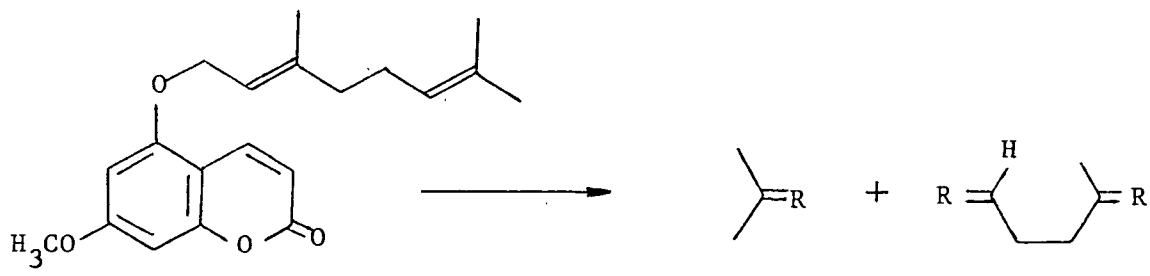


9 49

In order to determine the amount of radioactivity present (if any) in the 2, 3 and 4-positions of umbelliprenin (9), umbelliferone (49) was treated with potassium hydroxide at high temperature. This reaction was previously reported to give resorcinol.[58] However very little resorcinol could be obtained and the major component isolated proved to be 2,4-dihydroxy benzoic acid (48% yield). Thus this degradation allows the determination of radioactivity at the 2- and 3-positions of umbelliprenin (9).

49

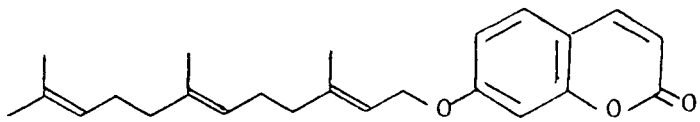
As it was also of interest to determine the distribution of radioactivity in the farnesol ether side chain of umbelliprenin (9), a suitable degradation scheme was devised. Caldwell and Jones[59] have reported the isolation of both acetone and levulinaldehyde (94a) as their 2,4-dinitrophenylhydrazone derivatives, from 7-methoxy-5-geranyl coumarin (92) by ozonolysis and steam distillation of the reaction mixture into a solution of 2,4-dinitrophenylhydrazine (2,4-DNP).



92

93, R=2,4-DNP 94a, R=O
94b, R=2,4-DNP

This procedure when applied in the case of umbelliprenin (9) turned out to be unsatisfactory as the yield of levulinaldehyde-2,4-DNP (94b) was very poor (< 1%). However when the ozonide of 9 was worked up under reductive conditions utilizing catalytic hydrogenation[58] and the resulting reaction mixture treated with a solution of 2,4-DNP reagent in methanolic hydrogen chloride, orange coloured crystals of 94b precipitated (26% yield). In order to isolate the acetone-2,4-DNP produced in the reaction, acetone-free methanol (methanol distilled from iodine and aqueous base) proved to be unsuitable as it was found that the 2,4-DNP reagent would not dissolved satisfactorily in the acetone-free methanolic hydrogen chloride.



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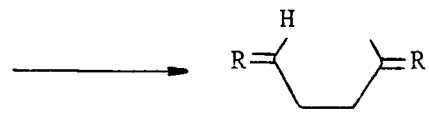


94b, R=2,4-DNP

As mentioned previously, it was desirable to isolate the terminal three carbon system of the farnesol ether side chain of 9 as acetone in an ozonolysis reaction, but the lack of material prevented further evaluation of this reaction.

Degradation of Isopimpinellin (2)

Isopimpinellin (2), as an abundant component of Thamnosma montana shoots, was selected for study of the biosynthesis of the furan ring in furanocoumarins. As mentioned previously, Floss and Mothes[49] have shown specific incorporation of C-4 of mevalonic acid (85) into C-7 of pimpinellin (76). However, the findings of Rodighiero and coworkers[48] and of Brown[52] that the C-2 of mevalonic acid incorporates into simple furanocoumarins as efficiently as C-4 labelled material calls into question the results of Floss and Mothes.[49] Brown has also found acetate to be a much better precursor of furanocoumarins than was mevalonic acid. Therefore, to clarify these uncertainties, it was decided that an extensive degradation procedure to determine the distribution of radioactivity in isopimpinellin (2) was needed. A summary of these degradations is given in Figure 13.

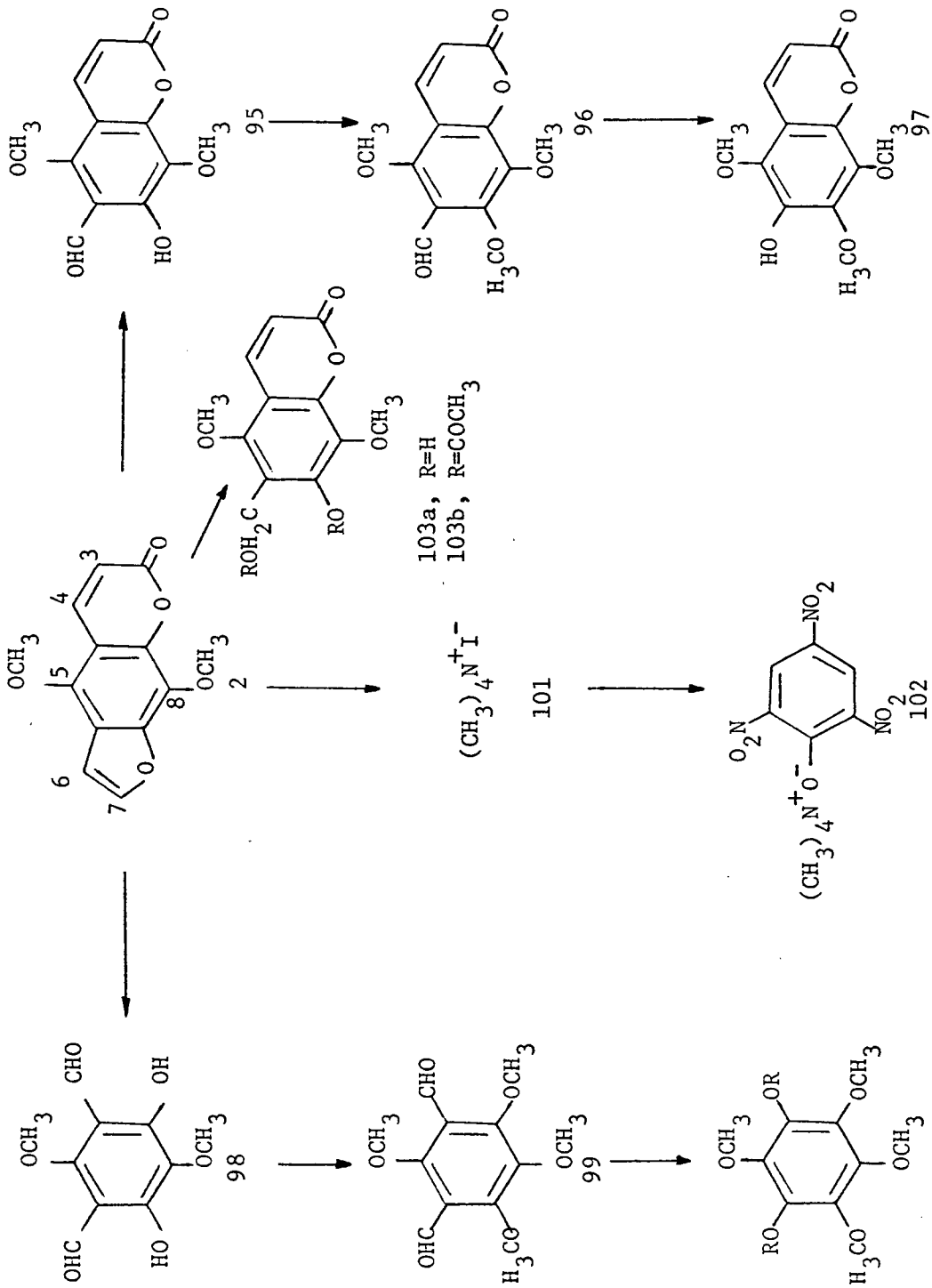


Figure 13. Degradative scheme of isopimpinellin (2).

In order to determine the amount of radioactivity present at the 7-position, isopimpinellin (2) was ozonized under controlled conditions. Previous workers[61] have found that by ozonolysis, furanocoumarins could be converted to the phenolic aldehydes where the furan ring had undergone degradation in preference to the pyrone ring. To accomplish such a selective ozonolysis, acetic acid was saturated with ozone and the ozone concentration determined by titration of the iodine produced when the aliquot of this solution was reacted with aqueous potassium iodide. Thus the ozonolysis of isopimpinellin (2) with a 60-70% molar excess of ozone followed by reduction of the ozonide with zinc dust yielded a product which when chromatographed on a silica gel column followed by fractional crystallization gave pure 6-formyl-7-hydroxy-5,8-dimethoxy coumarin (95), m.p. 214-216° (45% yield). It was characterized on the basis of ir, uv, nmr, mass spectrometry and elemental analysis.

However, it was observed that when isopimpinellin (2) was ozonized in a similar manner as above but the reduction of the ozonide was carried out with a large amount of zinc and over a longer period of time, no aldehyde (95) could be obtained. The nmr spectrum of the reaction mixture revealed that the desired aldehyde was being reduced to the corresponding alcohol (103a). However, the alcohol (103a) was not isolated but was acetylated with acetic anhydride and pyridine and after preparative layer chromatography of the reaction mixture pure 6-acetoxy methyl-7-acetoxy-5,8-dimethoxy coumarin (103b), m.p. 139-141° was obtained in 66% overall yield. Thus the nmr spectrum (Figure 14) was very similar to that of isopimpinellin (2) but the signals due to the furan protons in the starting material were now absent and instead a

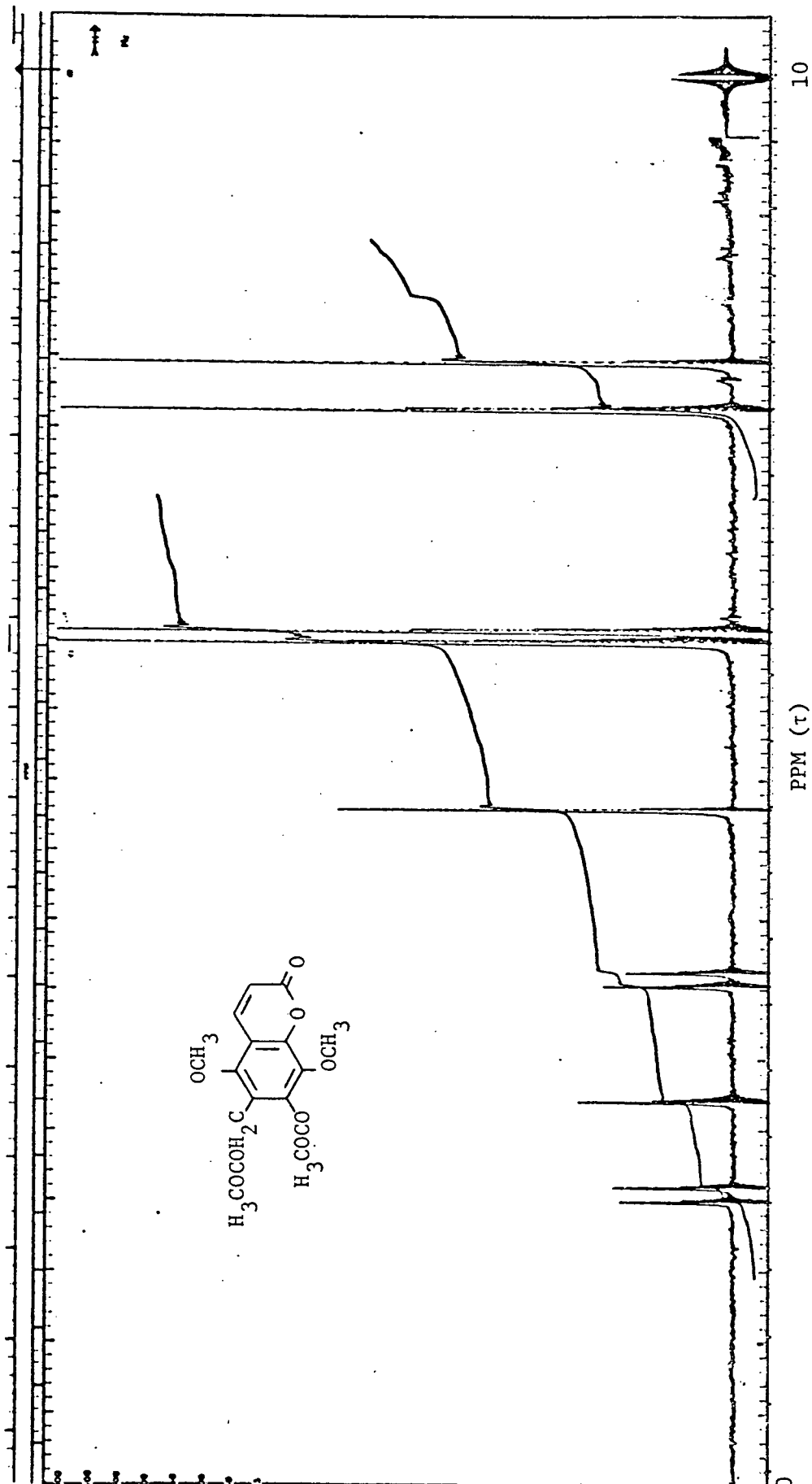


Figure 14. Nmr spectrum of 6-acetoxymethyl-7-acetoxy-5,8-dimethoxycoumarin (103b).

sharp two proton singlet at τ 4.81 was assigned to the benzylic methylene group containing the acetyl function. A sharp three proton singlet at τ 7.65 was assigned to the aromatic acetyl group and another at τ 7.98 was assigned to the aliphatic acetyl group. Elemental analysis and high resolution mass spectrometry supported the assigned structure.

Next it was of interest to determine the proportion of radioactivity in isopimpinellin (2) which resided in the 6-position. The phenolic aldehyde (95) was felt to be the obvious starting material and it was apparent that some kind of oxidative procedure would be necessary for such a degradation. As phenols are generally unstable to oxidative conditions, 95 was methylated with methyl iodide and anhydrous potassium carbonate in acetone to give 6-formyl-5,7,8-trimethoxy coumarin (96), mp 152.5-154° (75% yield). This compound was fully characterized on the basis of analytical and spectral data.

The removal of the formyl group from 96 was achieved by utilizing a modified Dakin reaction.[62] Thus when 96 in glacial acetic acid was treated with a mixture of hydrogen peroxide and sulfuric acid and the residue after work up of the reaction mixture chromatographed on preparative layer chromatography, the major component, after crystallization from 95% ethanol, yielded pure 6-hydroxy-5,7,8-trimethoxycoumarin (97), mp 198.5-199.5° (75% yield).

Next of interest was the determination of the radioactivity in the pyrone portion of isopimpinellin (2). Treatment of furanocoumarins with a large excess of ozone is known to cause degradation of both the furan and the pyrone ring.[63] Therefore, isopimpinellin, in a mixture of

acetic acid and ethyl acetate was ozonized with a large excess of ozone and the resultant ozonide was reduced with zinc dust. The work up of the reaction mixture gave, after crystallization, pure 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98), mp 162-164° in 42% yield. Thus this reaction allowed the determination of radioactivity associated with the 2- and 3-positions of isopimpinellin.

In order to obtain a degradative procedure which would allow the determination of radioactivity associated with the 4-position of isopimpinellin (2), the dialdehyde (98) was choosen as the starting material. It was considered that a Dakin-type of reaction could be utilized for such a purpose on the methylated product of 98. Therefore, 98 was methylated under conditions previously described and the product 1,3-diformyl-2,4,5,6-tetramethoxybenzene (99) mp 49-50°, was obtained in 86% yield. This compound had spectral and analytical data completely consistent with the assigned structure.

The treatment of the methylated dialdehyde (99) in acetic acid with a mixture of hydrogen peroxide and sulfuric acid for 16 hours in cold (i.e. under the conditions described by Schonberg et al.[62]) yielded only a complex mixture of highly coloured products. Thus it was evident that the diphenol (100 a) apparently being formed in the reaction was decomposing under these conditions. In order to minimize the suspected decomposition, the reaction time was reduced to only 15 minutes, less hydrogen peroxide and a nitrogen atmosphere was used and the reaction was then quickly worked up in the cold. The nmr spectrum of the product mixture revealed no dialdehyde protons but the presence of signals due to a formate ester (τ 1.62) and a phenol (τ 4.70). It

was apparent that the reaction had proceeded but the hydrolysis of the intermediate formate esters was incomplete. Due to the apparent instability of the diphenol (100a), it was decided to trap it as the diacetate derivative (100b). However, when the formate ester mixture was treated with aqueous base and acetic anhydride (to trap the resulting diphenolate anion), only a complex mixture of products could be obtained.

Considering the instability of 100a to hydrolysis conditions, a more rigorously controlled method of formate ester hydrolysis was devised. It was expected that a strong nucleophile such as methyllithium could be utilized to effect rapid and complete transformation of the formate ester to the dilithio salt of diphenol (100a). Under strictly anhydrous conditions, the salt would be expected to precipitate from the organic solvent and thus as a solid, perhaps would be less prone to decomposition. Quenching such a reaction mixture with acetic anhydride would then afford the diacetate (100b).

Thus the dialdehyde (99) in acetic acid was treated with a hydrogen peroxide and sulfuric acid mixture at 0°C under a nitrogen atmosphere for 20 minutes and the reaction was worked up quickly in the cold as before. The product mixture was dissolved in anhydrous ether and treated with excess methyllithium. As expected, a precipitate formed and after treatment with acetic anhydride (with some pyridine added to ensure complete acetylation), the work up of this complex mixture yielded a near quantitative yield of 1,3-diacetoxy-2,4,5,6-tetramethoxybenzene (100b) (97% yield from preparative layer chromatography) as a colourless oil which could be induced to crystallize, mp 57-58°. It

was characterized completely on the basis of analytical and spectral data.

Thus by comparison of the molar activity of 100b with that of the dialdehyde (98), the activity associated with the 4- and 6-positions of isopimpinellin (2) could be obtained. Since the radioactivity of the 6-position could be determined from previous degradations, the percentage of radioactivity residing at the 4-position of isopimpinellin (2) is thus determinable.

It was finally of interest to determine the percentage of radioactivity of isopimpinellin (2) which might be associated with the two methoxyl groups. To accomplish this, isopimpinellin (2) was demethylated by refluxing with hydriodic acid[64] and the resulting methyl iodide was swept from the reaction mixture with a stream of nitrogen and trapped as tetramethylammonium iodide (101). After scintillation counting of 101, it was converted to its picrate derivative (102), mp 323-325°C. Elemental analysis was consistent with the molecular formula $C_{10}H_{14}O_7N_4$.

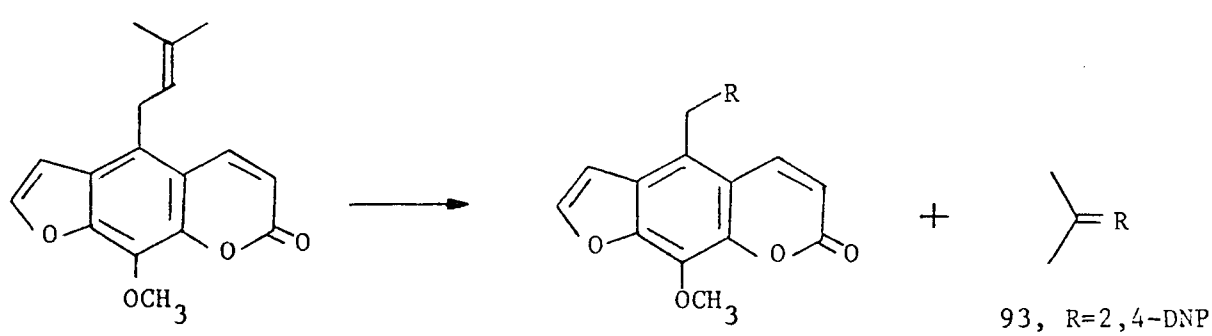
Thus by a series of degradations, isopimpinellin (2) could be degraded to determine the radioactivity associated with all the carbon atoms attached to the benzene portion of the molecule.

Degradations of Alloimperatorin Methyl Ether (7)

Alloimperatorin methyl ether (7) contains a furan ring and a dimethylallyl side chain. Although no direct evidence is available as to the origin of these side chains in furanocoumarins, experiments with similar coumarins have shown them to be mevalonic acid derived (see Introduction). Thus in order to gain some information as to the

specificity of incorporation of such precursors into 7, a series of degradations were devised which would allow the determination of the distribution of radioactivity in alloimperatorin methyl ether (7) in the course of biosynthetic experiments.

Thus to determine the distribution of radioactivity in the prenyl side chain of 7, a cleavage reaction was indicated. It was felt that although 7 has three double bonds which would be reactive to ozone, the partial aromatic character of both the furan and the pyrone rings might allow selective ozonization of the side chain double bond. Thus alloimperatorin methyl ether (7) was treated with 1.5 molar equivalents of ozone in acetic acid and the resultant ozonide was reductively cleaved with zinc dust. The resulting mixture was steam distilled and the 2,4-dinitrophenylhydrazone derivative of acetone (93) was isolated in 43% yield. The non-volatile portion of the reaction mixture was extracted and subsequent preparative layer chromatography allowed the isolation of unreacted 7 (37% yield) and the expected aldehyde (104) in 49% yield. This compound appeared to be unstable to air and therefore it was decided to reduce it to the corresponding alcohol (105). Thus the aldehyde (104) was treated with sodium borohydride and alcohol (105) was isolated in 85% yield, mp 167-169°. The spectra data was consistent with the assigned structure.



7

104, R=CHO
105, R=CH_2OH

93, R=2,4-DNP

This degradation, while giving the desired products, was found to have some serious drawbacks when performed on radioactive 7. The most significant problem was that 7 proved to be very difficult to obtain radiochemically pure. Also, the highly coloured nature of acetone-2,4-DNP (93) made scintillation counting inaccurate when low levels of radioactivity were present.

Thus an alternative scheme for the degradation of alloimperatorin methyl ether (7) was considered. The scheme must incorporate the main objective of determining the distribution of radioactivity in the furan and the pyrone rings of 7. This scheme is summarized in Figure 15.

As has been shown previously, ozone attacks preferentially the side chain double bond and thus it was apparent that if an ozonolysis procedure was to be used to cleave the furan and the pyrone rings, it would be necessary to first modify the side chain double bond to make it resistant to ozonolysis. Alloimperatorin methyl ether diol (3) was considered to be the ideal intermediate as this could be used for the cleavage of the side chain as well as for the degradation of the furan and the pyrone ring. Dreyer[4] had previously shown that alloimperatorin methyl ether (7) could be converted to diol (3) *via* the epoxide (14) in good overall yield. Thus alloimperatorin methyl ether (7) was treated with *m*-chloroperbenzoic acid and the epoxide (14) was isolated in 80% yield. Treatment of the epoxide (14) with 5% oxalic acid gave the desired diol (3) in 70% yield. This compound was identical with authentic alloimperatorin methyl ether diol (3) kindly supplied by Dreyer.

To gain information as to the distribution of radioactivity in the side chain of 3, diol (3) was treated with periodic acid and the acetone

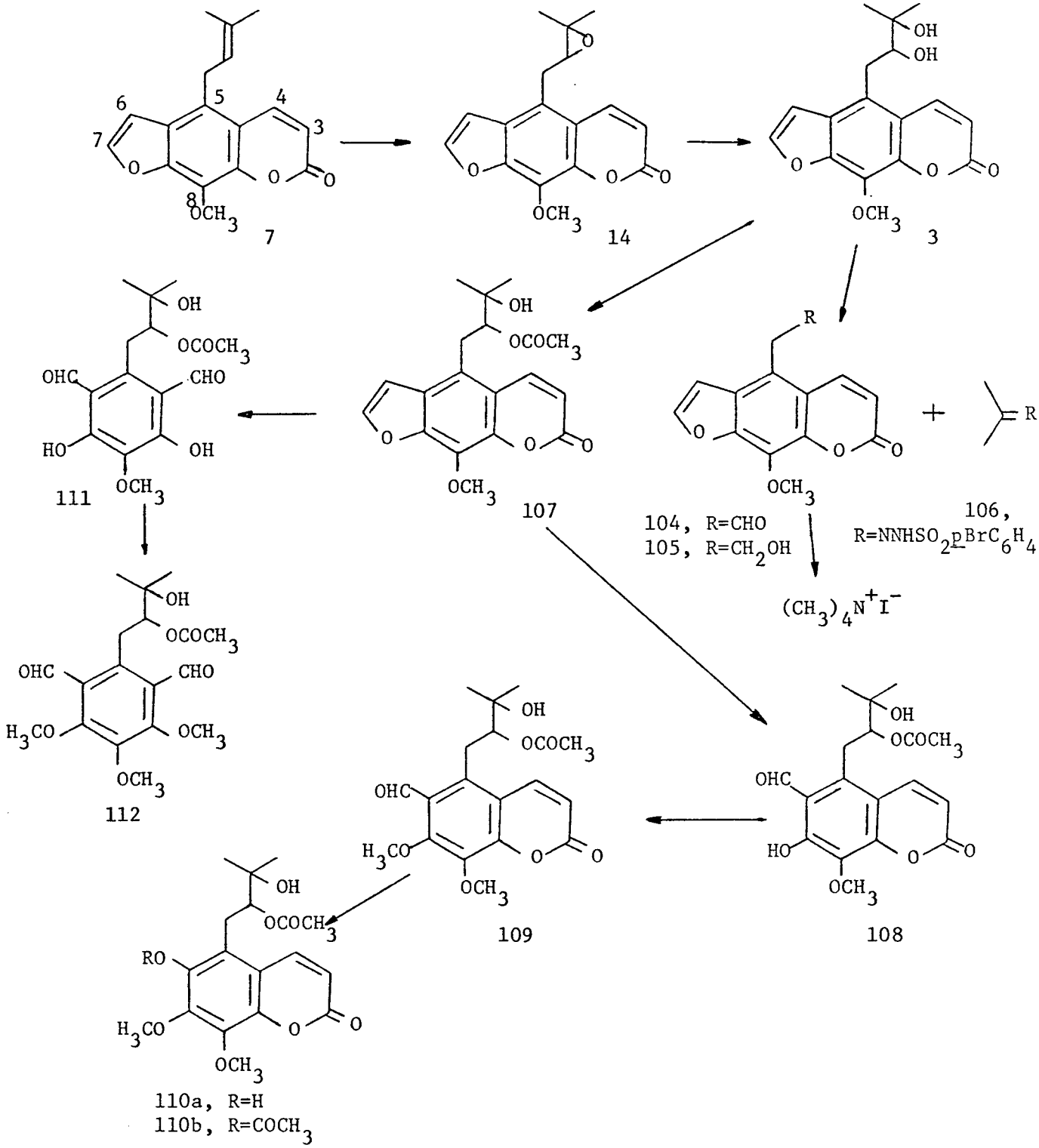


Figure 15. Degradative schemes of alloimperatorin methyl ether (7).

removed from the reaction mixture in the form of the colourless p-bromobenzenesulfonylhydrazone derivative (106). The non-volatile portion gave aldehyde (104) which upon reduction with sodium borohydride gave alcohol (105) in 58% yield.

Next it was of interest to determine the distribution of radioactivity in the furan portion of alloimperatorin methyl ether (7). For this purpose, diol (3) was acetylated with acetic anhydride in pyridine to form a monoacetate derivative[4] (107) in 90% yield. The monoacetate (107) was then treated with a slight excess of ozone and after the reduction of the ozonide with zinc dust, work up of the mixture yielded the desired phenolic aldehyde (108) which could be separated from the starting material (107) by partitioning the mixture between chloroform and aqueous base. The base soluble material, after acidification and extraction, yielded the pure phenolic aldehyde (108) in 35% yield, mp 162-164°. The spectral data was consistent with the assigned structure. Thus this reaction allows the determination of radioactivity in the 7-position of alloimperatorin methyl ether (7).

In order to determine the amount of radioactivity which might reside in the 6-position of 7, the removal of the aldehyde group of 108 in the manner previously found successful in the degradations of isopimpinellin (2), was considered. For this purpose, the phenolic aldehyde (108) was methylated with methyl iodide and potassium carbonate in acetone and the methylated coumarin (109), mp 116-118°, was isolated in 75% yield. This material (109) was treated with a mixture of hydrogen peroxide and sulfuric acid in the cold and preparative layer chromatography of the mixture provided a major band in 90% yield. The nmr spectrum of this

material revealed that the desired phenol (110a) had been obtained. However, this compound could not be induced to crystallize and therefore it was acetylated with acetic anhydride and pyridine to give 110b in 45% overall yield. This material was highly crystalline, mp 143-144°, and had analytical and spectral properties completely consistent with the expected product.

To allow determination of radioactivity associated with the pyrone ring of 7, the diol acetate (107) was ozonized in the manner described by Hegarty and Lahey.[63] The residue, after work up, was crystallized to provide in 48% yield a compound which was fully characterized as the expected product 111, mp 188-190°. This reaction allows the determination of radioactivity associated with the 2- and 3-positions of alloimperatorin methyl ether (7).

To determine the amount of radioactivity associated with the 4-position of 7, a sequence of reactions similar to those performed successfully on isopimpinellin (2) was attempted. The dialdehyde (111) was methylated by standard procedures and the product (112), mp 79.5-80.5°, gave spectral and analytical data consistent with the assigned structure. However, this material when treated under the conditions developed in the degradation of isopimpinellin (2), gave only a complex mixture of coloured products. Thus it was evident that even under highly controlled conditions of the reaction, the resultant diphenol (or perhaps the intermediate diformate ester) was decomposing as fast as it was being formed. Thus attempts to effect this conversion were abandoned.

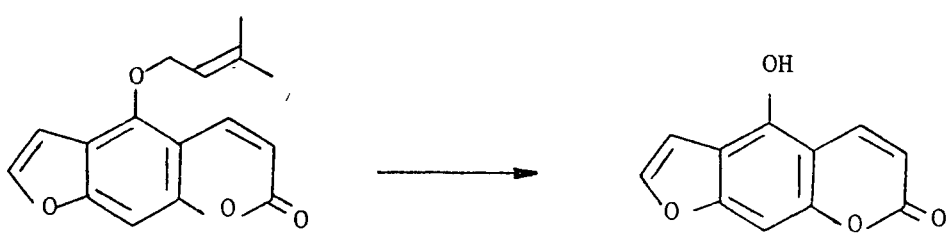
Finally, in order to determine the amount of radioactivity residing in the methoxyl group of alloimperatorin methyl ether (7), alcohol (105),

derived from the cleavage of diol (3), was demethylated with hydriodic acid and the resultant methyl iodide was trapped as tetramethylammonium iodide (101). Conversion to the picrate derivative was carried out as before.

Thus these series of reactions allow the determination of radioactivity associated with most of the carbon atoms of 7. It should also be noted that these degradations are equally applicable to the degradations of alloimperatorin methyl ether epoxide (14).

Degradations of Isoimperatorin (13)

Isoimperatorin (13) contains an alkyl-ether side chain and a furan ring. Thus to study its biosynthesis, it was considered essential to determine the amount of radioactivity associated with the entire side chain and the furan ring. It was felt that an acid hydrolysis of 13 in a manner similar to that of umbelliprenin (9) would allow the determination of radioactivity associated with the C_5-alkyl side chain of isoimperatorin (13). Thus when 13 was refluxed with glacial acetic acid and the residue, after work up, was chromatographed on a preparative layer chromatoplate, bergaptol (113), mp 275°, could be isolated in 85%



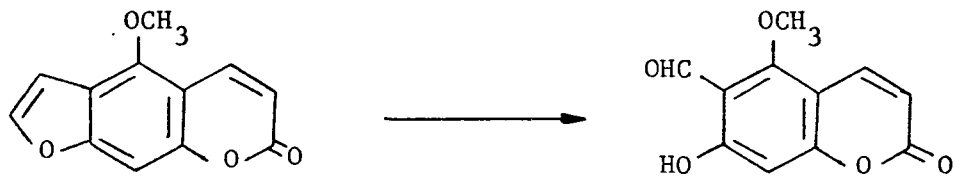
13 113

yield. The nmr spectrum was characteristic of a simple furanocoumarin. The signals at τ 1.72 (doublet of doublets, J = 9.50 Hz and 0.5 Hz) and at τ 3.80 (doublet, J = 9.50 Hz) could be assigned to H(4) and H(3) respectively. Signals attributed to the furan ring were observed at τ 2.49 (doublet, J = 2.50 Hz, H(7)) and at τ 3.01 (multiplet, H(6)). A multiplet at τ 2.82 was assigned to H(8) and a broad signal at τ 7.40 (disappearing on addition of D_2O) was assigned to the phenolic proton. However, recovery during crystallization of 113 was poor and therefore it was methylated with methyl iodide and potassium carbonate in acetone to give bergapten (10b), mp 186-188° (in 90% yield). Its structure was established by analytical and spectral data and comparison with an authentic sample.

113 10b

In order to determine the amount of radioactivity associated with the furan ring of 13, bergapten (10b) was felt to be the ideal starting material. It was expected that by utilizing a controlled ozonolysis procedure bergapten (10b) could be degraded to the corresponding phenolic aldehyde, a reaction previously employed in the case of isopimpinellin (2). Thus bergapten (10b) was treated with a slight excess of ozone in glacial acetic acid and after reduction of the

ozonide with zinc and work up of the mixture yielded a residue which when analyzed by tlc was observed to be a mixture of the starting material and a more polar yellow compound. This reaction mixture was chromatographed on a silica gel column and elution with benzene and benzene-chloroform mixtures afforded the yellow compound. Further purification by means of crystallization gave pure 6-formyl-7-hydroxy-5-methoxy coumarin (114), mp 220-221° (30% yield). This compound was



10b 114

characterized on the basis of the following data. The molecular formula, $C_{11}H_8O_5$, was established by elemental analysis and high resolution mass spectrometry. The uv spectrum of 114 (λ_{max}^{MeOH} 266 and 312 nm) revealed the phenolic nature of the compound, as, on addition of alkali, the spectrum showed a marked bathochromic shift (λ_{max}^{MeOH} (+NaOH) 238, 262, 347, and 394 nm). Acidification of the uv sample reversed the spectrum to its original form (λ_{max}^{MeOH} (+ HCl) 267 and 314 nm). The ir spectrum of 114 indicated that the phenol was strongly hydrogen bonded to the adjacent aldehyde carbonyl group. Thus no hydroxyl absorption was in evidence. The carbonyl region of the spectrum had an absorption at 1742 cm^{-1} and another at 1647 cm^{-1} (aldehyde C=O). An absorption at 1592 cm^{-1} was assigned to the α-pyrone system. The nmr

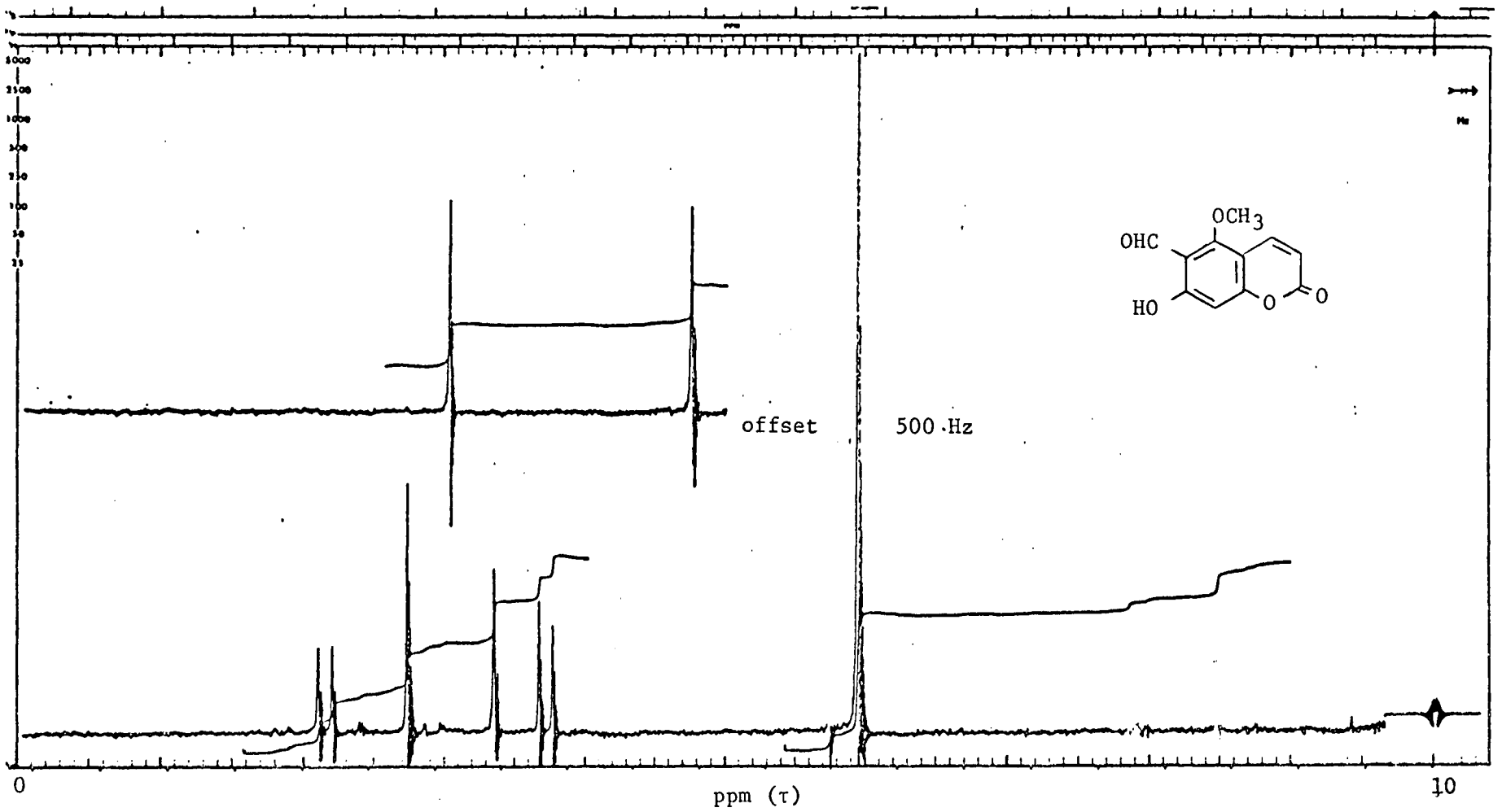


Figure 16. Nmr spectrum of 6-formyl-7-hydroxy-5-methoxycoumarin (114).

spectrum of 114 (Figure 16) fully confirmed the structure of this compound. Thus the spectrum was very similar to that of bergapten (10b) but the signals due to the furan protons in the starting material were now absent and instead low field singlets at τ -0.23 and at τ -1.96 (disappearing on addition of D_2O) were readily assigned to the aldehyde and phenol protons respecitvely. Thus this reaction allowed the determination of radioactivity in the C-7 position of 13.

It was also of interest to determine the amount of radioactivity which resided in the 6-position and in the pyrone ring of isoimperatorin (13). However, the lack of material prevented any further development of these degradations.

EXPERIMENTAL (PART I)

Melting points were determined on a Kofler block and are uncorrected. The ultraviolet (uv) spectra were recorded in methanol solution utilizing a Cary 11 or a Unicam, model SP800 spectrophotometer. The infrared (ir) spectra were recorded on Perkin-Elmer model 21 or 457 spectrometers utilizing a potassium bromide disc. The position of the absorption maxima are quoted in wave numbers (cm^{-1}). Nuclear magnetic resonance (nmr) spectra were recorded in deuterochloroform solution (unless otherwise indicated) at 100 MHz on a Varian HA-100 or a Varian XL-100 instrument and at 60 MHz on a Varian T-60 spectrometer. Chemical shifts are given in the Tiers τ scale with reference to tetramethylsilane as the internal standard. Mass spectra were recorded on an Atlas CH-4 mass spectrometer and high resolution mass spectra were carried out on an AEI-MS 902 instrument. Woelm neutral alumina and silica gel G (acc. to Stahl) containing 1% by weight electronic phosphor were used for analytical and preparative layer chromatography (tlc), unless otherwise noted. Woelm neutral alumina (activity IV - unless otherwise indicated) was used for column chromatography. The tlc plates were activated in an oven at 90° for one hour. For qualitative chromatography, layers of 0.3 mm thickness were used and spots were visualized by viewing under ultraviolet (uv) light. For preparative tlc, large (20 x 20 cm) plates with a thicker

layer (0.5 mm) were used. Developing solvents used were; A anhydrous ether-hexane (1:1) or B, ethyl acetate-chloroform (1:1), unless otherwise noted.

Microanalysis were performed by Mr. P. Borda, Microanalytical Laboratory, University of British Columbia.

Methanol was made acetone-free by treatment with iodine and aqueous sodium hydroxide.[65a] Chloroform was made acetone-free by flushing through a column of Celite impregnated with 2,4-dinitrophenyl hydrazine and the eluent was distilled.[65b]

For detailed experimental procedures on the various degradations discussed above, the reader is referred to the Ph.D. thesis of R.N. Young. Only those procedures developed more recently and not discussed in that thesis are presented below.

Preparation of a Standard Solution of Ozone in Glacial Acetic Acid

Glacial acetic acid was placed in a flask equipped with a bubbler and ozone enriched oxygen was allowed to bubble through the solution for 30 minutes at room temperature, at which time the solution had a definite blue tinge. The bubbler was then removed and the flask was tightly stoppered. Aliquots (20 ml) of this solution were added to a solution of potassium iodide (1 g) in water (20 ml) and the iodine which was liberated was titrated with a standard solution of sodium thiosulfate using starch as an indicator. The sodium thiosulfate solution was standardized against a standard potassium dichromate solution. In a typical experiment, glacial acetic acid was saturated with ozone as described above and two aliquots (20 ml) were removed

and added individually to aqueous solutions of potassium iodide (1 g per flask in two flasks). The iodine liberated was titrated with 0.0125 N sodium thiosulfate solution requiring respectively 17.1 and 16.8 ml to reach the end point. Thus the average of these two values (16.95 ml) required that the ozone concentration at room temperature be 0.106 mmole per 20 ml glacial acetic acid.

6-Formyl-7-hydroxy-5,8-dimethoxy coumarin (95)

Isopimpinellin (2) (45 mg; 0.183 mmole) was treated with ozone saturated glacial acetic acid (60 ml; 0.30 mmole) and the mixture stirred for one hour at room temperature. Zinc dust (100 mg) was then added and stirring continued for further 10 minutes. The mixture was then filtered and solvent was then evaporated in vacuo. The residue (∿ 70 mg) was dissolved in chloroform-methanol mixture and was chromatographed on silica gel (6 g). The fractions eluted with benzene and benzene-chloroform contained isopimpinellin (2) and a more polar compound (yellow spot; uv and visible). These fractions were combined (33 mg) and crystallized from acetone to yield 6-formyl-7-hydroxy-5,8-dimethoxy coumarin (95) (23 mg; 50% yield), mp 214-216°; ir (KBr) 1758, 1730, 1625, 1592 (α-pyrone), 1640 (aldehyde C=O); uv λ_{max}^{MeOH} (ε) 275 (27,100); uv λ_{max}^{MeOH} (ε) (+NaOH) 238 (19,200), 269 (16,600), 299 (12,900), 360 (14,200); uv λ_{max}^{MeOH} (ε) (+HCl) 208 (29,000), 226 (sh) (15,600), 263 (12,800), 320 (15,600); nmr (100 MHz) in $CDCl_3$, TMS lock, -2.03 (1H, singlet,disappears on addition of D_2O, phenolic O<u>H</u>), -0.23 (1H, singlet, aromatic C<u>H</u>O), 2.17 (1H, doublet, J = 10 Hz, H(4) of coumarin), 3.73 (1H, doublet, J = 10 Hz, H(3) of coumarin), 6.00, 6.02 (6H, two singlets, two aromatic O<u>CH</u>$_3$); mass spectrum m/e 250 (M), 235 (M-15), 221 (M-29),

207 and 179.

Anal. Calcd. for $C_{12}H_{10}O_6$: C, 57.61; H, 4.03. Found: C, 57.38; H, 4.07. High resolution molecular weight determination. Calcd. for $C_{12}H_{10}O_6$: 250.048. Found: 250.046

6-Acetoxy methyl-7-acetoxy-5,8-dimethoxy coumarin (103b)

Isopimpinellin (2) (23.5 mg; 0.095 mmole) was treated with ozone saturated glacial acetic acid (32 ml; 0.160 mmole) and the mixture stirred for 3 hours at room temperature. Zinc dust (400 mg) was added and stirring continued for further one hour. Solution was filtered and solvent was evaporated in vacuo. The residue (30 mg) showed no aldehyde on tlc plate. The nmr of the residue revealed it to be the corresponding alcohol (103a). This (103a) was treated with acetic anhydride and pyridine and solution was stirred for 10 hours. The solvent was evaporated in vacuo and the residue gave almost a single spot on tlc. It was separated on preparative tlc and crystallized from ethyl acetate to yield 6-acetoxy methyl-7-acetoxy-5,8-dimethoxy coumarin (103b) (21 mg; 66% yield), mp 139-141°; ir (KBr) 1780 (aromatic acetate C=O), 1735 (aliphatic acetate C=O), 1598 (α-pyrone); uv λ_{max}^{MeOH} (ε) (23,000), 225 (sh) (15,000), 251 (6, 940), 293 (11,670); nmr (100 MHz) in $CDCl_3$, TMS lock, 2.06 (1H, doublet, J = 10 Hz, H(4) of coumarin), 3.59 (1H, doublet, J = 10 Hz, H(3) of coumarin), 4.81 (2H, singlet, $C\underline{H}_2OCOCH_3$), 6.00, 6.08 (6H, two singlets, two aromatic $OC\underline{H}_3$), 7.65 (3H, singlet, aromatic $OCOC\underline{H}_3$), 7.98 (3H, singlet, aliphatic $CH_2OCOC\underline{H}_3$); mass spectrum m/e 336 (M), 294 (M-42), 251 (M-85), 234 (base peak), 219 and 205.

Anal. Calcd. for $C_{16}H_{16}O_8$: C, 57.14; H, 4.76. Found: C, 56.99; H, 4.88. High resolution molecular weight determination. Calcd. for $C_{16}H_{16}O_8$: 336.084. Found: 336.086.

Acid Catalyzed Hydrolysis of Isoimperatorin (13)

Isoimperatorin (13) (21 mg; 0.078 mmole) was dissolved in glacial acetic acid (5 ml) and the solution was refluxed for 10 hours. The reaction mixture was allowed to cool to room temperature. Water was added and the solution was extracted with chloroform (5 x 20 ml). Chloroform extract was washed with water (20 ml), dried over anhydrous sodium sulfate and solvent was removed under reduced pressure. Residue (23 mg) was crystallized from ethanol (95%) to give bergaptol (113) (11.8 mg, 85% yield), mp 275° (lit.[66] mp 278°); nmr (100 MHz) in $CDCl_3$, TMS lock, 1.72 (1H, doublet of doublets, J = 9.50 Hz and 0.5 Hz, H(4) of furanocoumarin), 2.49 (1H, doublet, J = 2.5 Hz, H(7) of furanocoumarin), 2.82 (1H, multiplet, H(8) of furanocoumarin), 3.01 (1H, multiplet, H(6) of furanocoumarin), 3.80 (1H, doublet, J = 9.50 Hz, H(3) of furanocoumarin), 7.40 (1H, broad singlet, disappearing on addition of D_2O, phenolic hydroxyl group).

Methylation of Bergaptol (113)

Bergaptol (113) (11.8 mg; 0.054 mmole) from previous reaction was dissolved in acetone (1 ml) and anhydrous potassium carbonate (500 mg) and methyl iodide (3 ml) were added. The mixture was refluxed for 15 minutes, cooled to room temperature and stirring continued for further 10 minutes. Water (10 ml) was added, solution was acidified

with concentrated hydrochloric acid and was extracted with chloroform (4 x 20 ml). The chloroform extract was washed with water (20 ml), dried over anhydrous sodium sulfate and the solvent was removed under reduced pressure to yield a residue (18 mg) which was observed to be essentially one component on tlc plate. Preparative tlc from chloroform-ethyl acetate mixture (1:1) gave pure bergapten (10b) (12 mg, 90% yield) which was crystallized from ethyl acetate as colourless plates, mp 186-188° (lit.[67] mp 191°), mixed mp with authentic bergapten (10b) (obtained from Dr. D.L. Dreyer) 186.5-187.5°; ir (KBr) 1726, 1623, 1580 (α-pyrone): uv λ_{max}^{MeOH} (ε) 221 (21,400), 248 (17,800), 257.5 (16,200), 267 (17,500), 309 (14,700); nmr (100 MHz) in $CDCl_3$, TMS lock, 1.91 (1H, doublet of doublets, J = 9.75 Hz and 0.6 Hz, H(4) of furanocoumarin), 2.47 (1H, doublet, J = 2.5 Hz, H(7) of furanocoumarin), 2.92 (1H, multiplet, H(8) of furanocoumarin), 3.05 (1H, doublet of doublets, J = 2.5 Hz and 1.0 Hz, H(6) of furanocoumarin), 3.80 (1H, doublet, J = 9.75 Hz, H(3) of furanocoumarin), 5.80 (3H, singlet, aromatic OCH_3); mass spectrum m/e 216 (M), 201 (M-15), 188 (M-28), 173 and 145.

Anal. Calcd. for $C_{12}H_8O_4$: C, 66.67; H, 3.73. Found: C, 66.57; H, 3.80.

6-Formyl-7-hydroxy-5-methoxy coumarin (114)

Bergapten (10b) (24 mg; 0.111 mmole) was dissolved in glacial acetic acid (4 ml) and 30 ml of ozone saturated glacial acetic acid was added and the mixture was stirred for one hour. Zinc dust (50 mg) was added and stirring continued for another 10 minutes. Solution was filtered and the solvent was evaporated in vacuo. The residue (50 mg)

was dissolved in chloroform-methanol mixture and was chromatographed on silica gel (6 g). Elution with benzene and benzene-chloroform mixture gave the desired phenolic aldehyde (114) (14 mg) which was crystallized from acetone to give pure 6-formyl-7-hydroxy-5-methoxy coumarin (114) (8 mg; 33% yield), mp 220-221° (lit.[68] mp 222-223°); ir (KBr) 1742, 1592 (α-pyrone), 1697 (aldehyde C=O); uv λ_{max}^{MeOH} (ε) 205.5 (5,870), 225 (sh) (2,690), 266 (19,900), 312 (3,180), 340 (sh) (1,345); uv λ_{max}^{MeOH} (ε) (+NaOH) 206 (18,850), 238 (13,700), 262 (8,940), 285 (sh) (4,160), 347 (8,800), 394 (9,800); uv λ_{max}^{MeOH} (ε) (+HCl) 207 (11,370), 222 (sh) (5,740), 267 (15,400), 314 (5,630); nmr (100 MHz) in $CDCl_3$, TMS lock, -1.96 (1H, singlet, disappears on addition of D_2O, phenolic O<u>H</u>), -0.23 (1H, singlet, aromatic C<u>H</u>O), 2.14 (1H, doublet of doublets, J = 9.75 Hz and 0.6 Hz, H(4) of coumarin), 3.35 (1H, multiplet, H(8) of coumarin), 3.72 (1H, doublet, J = 9.75 Hz, H(3) of coumarin), 5.95 (3H, singlet, aromatic O<u>CH</u>$_3$); mass spectrum m/e 220(M), 202(M-18), 191 (M-29), 174, 146 (base peak).

Anal. Calcd. for $C_{11}H_8O_5$: C, 60.00; H, 3.64. Found: C, 60.06; H, 3.71. High resolution molecular weight determination. Calcd. for $C_{11}H_8O_5$: 220.037. Found: 220.036.

DISCUSSION (PART II)

Biosynthetic Studies on Coumarins from Thamnosma montana Tissue Cultures

The Introduction listed many questions on the biosynthesis of coumarins that remain to be answered. Of particular interest to us is the role of mevalonic acid in the formation of the furan ring of furanocoumarins and the origin of the C_5-units found in many coumarins. Floss and Mothes[49] have shown that C-4 of mevalonic acid is incorporated specifically into the 7-position of pimpinellin (76). However reports of Caporale et al.[48] and of Brown[52] that mevalonic acid-[2-^{14}C] incorporates as efficiently as [5-^{14}C]-mevalonic acid into simple furanocoumarins reopens the question as to the true role of mevalonic acid in the biosynthesis of these furanocoumarins. Recent work in our laboratory[7] on young Thamnosma montana plants supports the results of Floss and Mothes[49] but the very low incorporation of mevalonic acid makes the significance of these observations marginal. Thus the main object of this work was to more closely define, through specific degradations, the role of mevalonic acid in the biosynthesis of furanocoumarins of Thamnosma montana tissue cultures.

Plant tissue culture systems have often been used to study many fundamental problems of plant cytology and physiology. Since the early publications by Tuleck[69] and Nickell,[70] there has been considerable interest

in the possibility of using plant tissue cultures for secondary product biosynthesis. It has long been known that tobacco root cultures can biosynthesize the alkaloids nicotine[71] and anabasine.[72] Routien and Nickell[73] have reported plant suspension cultures to produce coumarin and melilotic acid from sweet clover. Similarly, Steck and his group[74] have isolated various coumarins and alkaloids from the cell cultures of Ruta Graveolens and Ourisson et al.[75] have studied the biosynthesis of the coumarin, scopoletin (55) from tobacco tissue cultures. Frequently higher levels of incorporation of precursors of certain metabolites can be achieved in plant tissue cultures than in normally grown plants or in stem-and-leaf or in root cuttings from plants. Since tissue cultures can be manipulated to minimize woody or conductive tissue which may be of low metabolic activity, they often contain a much higher percentage of actively metabolizing cells than normal plants do. Since aggregation of cells in tissue cultures can be divided before they get too large, the distance from the exterior to the inner-most cell is so much less than in the normally grown plant that the problem of transporting precursors to all the cells in the tissue cultures is much easier than for the normal plants.

The initial requirement for preparing tissue cultures from green plants is to eliminate microorganisms from the system which is being set up for the purpose. Microorganisms may be aggressive competitors of green-plant tissue on the nutrient media used and in any event, their biochemical activities might interfere with, or be confused with, those of seed plants being studied. To obtain plant cultures free of microorganisms, the procedure generally followed was to surface-sterilize seeds, germinate them on sterile water-agar in

Petri plates and then use aseptic techniques to transfer seedlings that appear to be free of fungi and bacteria to the appropriate sterile media.

Nutrient requirements in culture media vary with the kind of plant and the purpose for which the culture is prepared. Ourisson and his group[76] have found callus tissue to produce coumarins normal to the plant being cultured and this has been our experience with the callus tissue of Thamnosma montana. Under the circumstances, a general purpose liquid medium (a modified White's nutrient solution) has produced tissue cultures adequate for our purpose. For solid media, 1% agar is added.

Before adopting an experimental design with tissue cultures for biosynthetic studies, a preliminary feasibility study was conducted. This was done by determining the major coumarins of the Thamnosma montana tissue cultures, confirming regular and measurable biosynthesis of these constituents, and finally, postulating biosynthetic relationships between the coumarins of Thamnosma montana tissue cultures.

Preliminary studies on the constituents of tissue cultures of Thamnosma montana revealed the presence of isoimperatorin (13), alloimperatorin methyl ether (7) and isopimpinellin (2) in isolable quantities whereas no umbelliprenin (9) could be detected. Presence of small amounts of thamnosmin (15) was also indicated but no attempt was made to isolate this or to identify other constituents of the tissue culture extract. It was considered that the isolation of isoimperatorin (13), alloimperatorin methyl ether (7) and isopimpinellin (2) would offer an opportunity to study the biosynthesis of these three different types of furanocoumarins.

In order to determine if the biosynthesis of these furanocoumarins was occurring on a regular basis, D,L-phenylalanine-[3-^{14}C] was fed to the 5 week old tissue cultures. Three experiments were set up for different time intervals and in two of these experiment, an Erlenmeyer flask was used containing liquid growth medium, cultures and radioactive precursor and the flask was put on a rotary shaker for incubation. In the third experiment, the cultures were transferred onto Petri plates containing normal growth medium with 1% agar added and the radioactive precursor was applied on the surface of the cultures. After the preselected time period, tissue cultures were freeze-dried and the major constituent, isopimpinellin (2), was isolated in each case and was crystallized to constant radioactivity. The results are given in Table 2.

TABLE 2. Incorporation of D,L-phenylalanine-[3-^{14}C] [a] into coumarins of *Thamnosma montana* tissue cultures.

Experiment no.	Feeding time (hours)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation isopimpinellin (2)
1[b]	8	10.93×10^6	1.20	0.029
2[b]	48	11.30×10^6	1.38	0.71
3[c]	72	11.00×10^6	1.30	0.48

[a] Precursor administered in water as sodium salt.

[b] Precursor fed in liquid growth medium.

[c] Precursor applied on the surface of the cultures in solid medium.

These results indicate that isopimpinellin (2) is being biosynthesized by 5 week old tissue cultures and that the optimum time period for the biosynthesis of isopimpinellin is 2 days.

To gain some information as to the biosynthetic interrelationship between these various furanocoumarins of Thamnosma montana tissue cultures and to study the role of mevalonate in their biosynthesis, six feeding experiments were set up for different time intervals using D,L-mevalonic acid-[5-^{3}H] as the radioactive precursor. After the desired feeding time, each experiment was worked up and isoimperatorin (13), alloimperatorin methyl ether (7) and isopimpinellin (2) were isolated by the dilution technique (i.e. the tissue culture extract was diluted with non-radioactive coumarins before column chromatography). Isoimperatorin (13) and isopimpinellin (2) were crystallized to constant activity and their radioactivity determined by the scintillation counting method. Alloimperatorin methyl ether (7) was converted to its diol (3) before counting. The results are given in Table 3 and are represented graphically in Figure 17. It should be noted that in each of these experiments, 5-week old tissue cultures were used and the radioactive precursor was mixed with the tissue cultures in sterile distilled water. Only in experiment no. 4, normal liquid growth medium was used instead of distilled water.

It is apparent from these results that the furanocoumarins isolated incorporate D,L-mevalonic acid-[5-^{3}H] at different time intervals. Thus isopimpinellin (2) reaches a maximum incorporation after 2 days and then falls off rapidly reaching a minimum in 7 days. The rise between 7 and 10 days is questionable. Alloimperatorin methyl ether (7)

TABLE 3. Incorporation of D,L-mevalonic acid-[5-^{3}H] into tissue cultures of Thamnosma montana.

Experiment no.	Feeding time (days)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation		
				isoimperatorin[a] (13)	alloimperatorin[a] methyl ether (7)	isopimpinellin[a] (2)
4[b]	2	1.11×10^{9}	2.54	--	0.000040	0.0010
5[c]	2	1.11×10^{9}	3.00	--	0.000045	0.00164
6[c]	4	1.11×10^{9}	1.45	0.00182	0.00165	0.00015
7[c]	7	8.0×10^{8}	2.50	0.0025	0.00055	0.000114
8[c]	7	1.11×10^{9}	3.50	0.0021	0.00011	0.00017
9[c]	10	8.0×10^{8}	3.00	0.00113	0.00036	0.00076

[a] All compounds were isolated by dilution technique. Alloimperatorin methyl ether (7) was converted to its corresponding diol (3) before counting.

[b] Precursor mixed with tissue cultures in liquid growth medium.

[c] Precursor mixed with tissue cultures in distilled water.

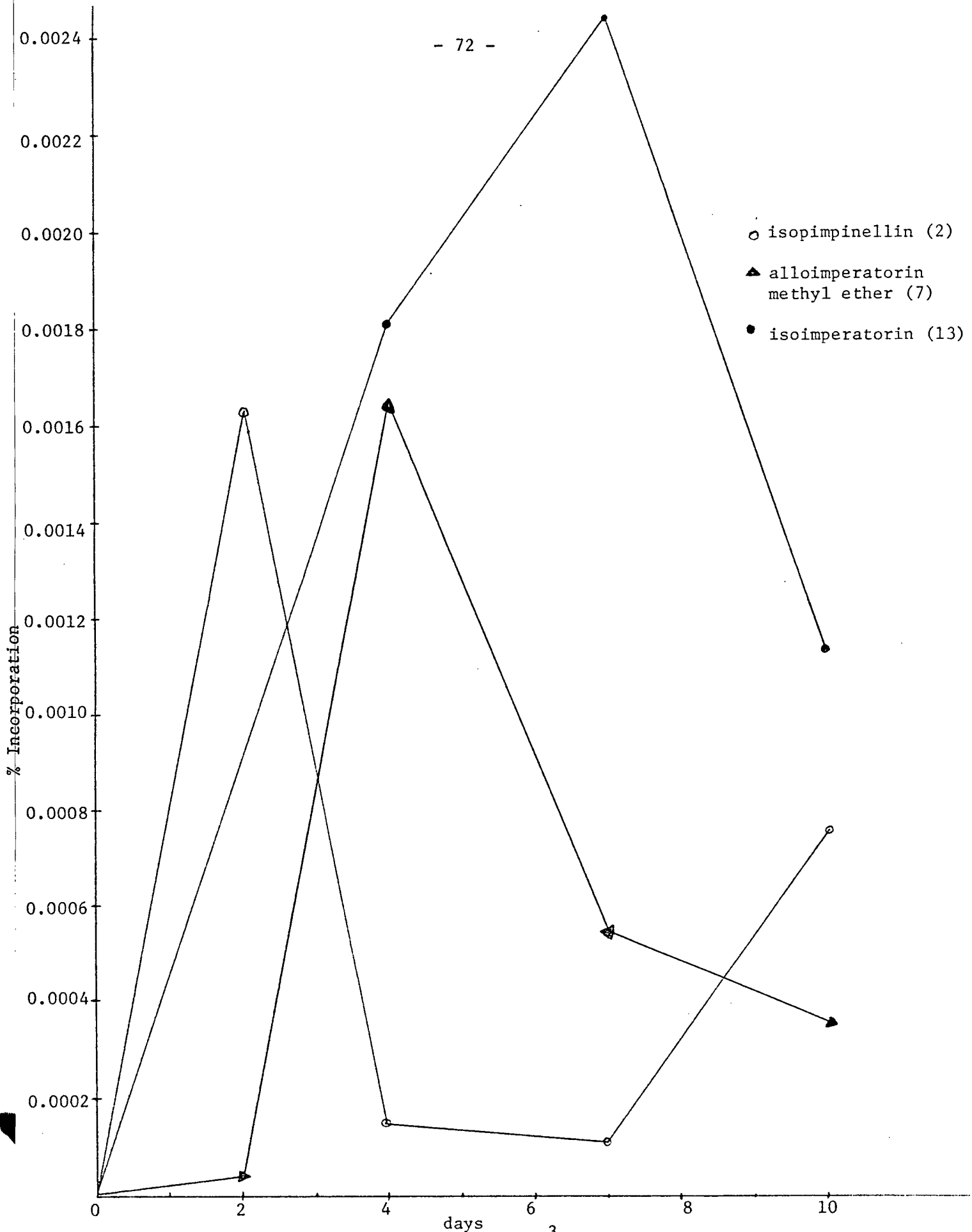


Figure 17. Incorporation of D,L-mevalonic acid-[5-^{3}H] into coumarins of Thamnosma montana tissue cultures versus time.

reaches maximum incorporation after 4 days whereas isoimperatorin (13) has a maximum value after 7 days. These results indicate that mevalonic acid-[5-^{3}H] is indeed being incorporated into all three furanocoumarins studied and also the level of incorporation is about five to ten times higher than that achieved in our laboratory[7] with young Thamnosma montana plants.

To determine the location of radioactivity, isopimpinellin (2) from these various feeding experiments was degraded according to the scheme previously described and as illustrated in Figure 18. The distribution of label determined as a result of these degradations is listed in Table 4.

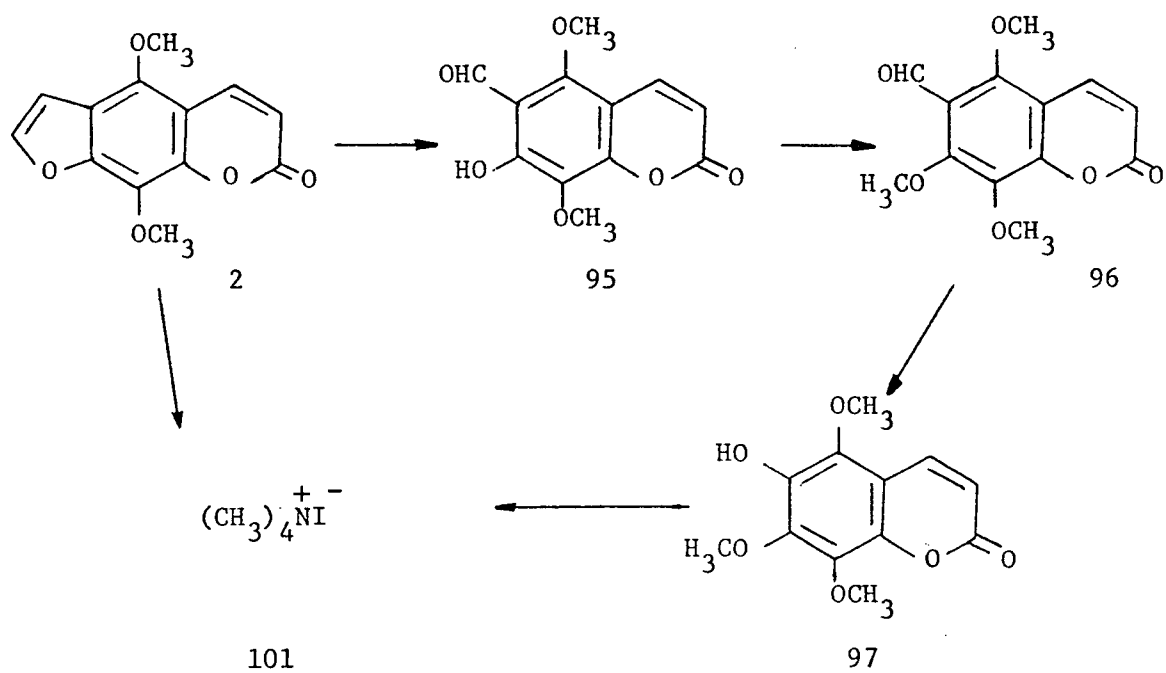


Figure 18. Degradation of radioactive isopimpinellin (2).

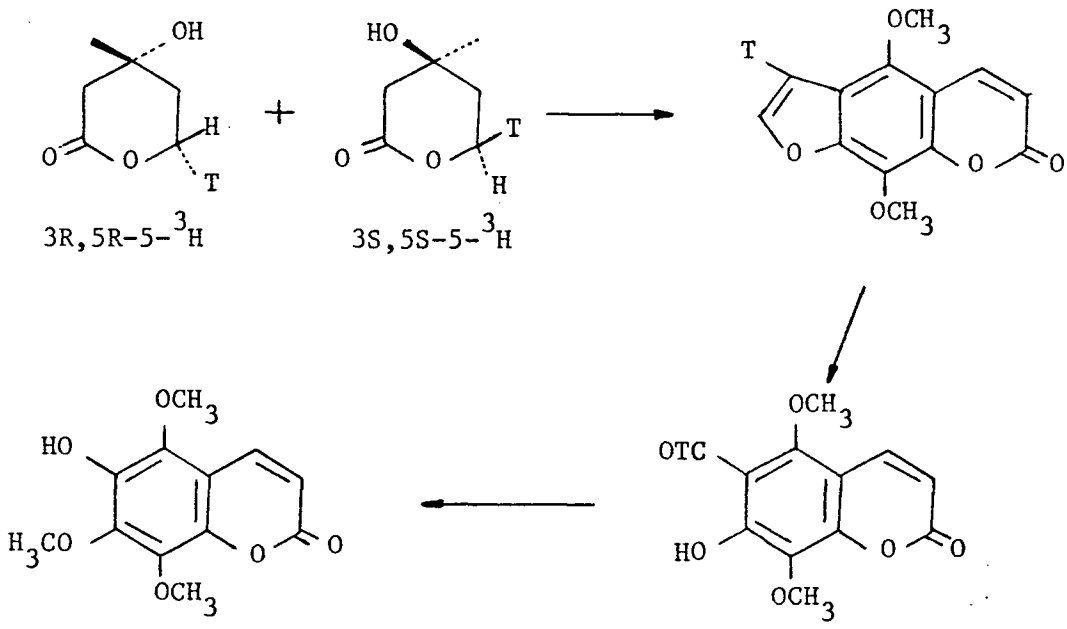
TABLE 4. Distribution of radioactivity in isopimpinellin (2) from D,L-mevalonic acid-[5-^{3}H] incorporation experiments.

Experiment no.	Specific activity of the compounds isolated (dpm/mmole)			
	isopimpinellin[a] (2)	95	97	101
4	2.34×10^4 (100%)	2.25×10^4 (96.5%)	1.025×10^4 (44%)	
5	2.42×10^4 (100%)	2.51×10^4 (103%)	1.067×10^4 (44%)	1.17×10^4 dpm/3 mmole) (48%)
9	1.722×10^4 (100%)	--	--	4.18×10^3 dpm/2 mmole (24.3%)

[a] The total activity in isopimpinellin (2) is set at 100%.

From these results, it is evident that there is no loss of activity in the 7-position of isopimpinellin (2) and that the 6-position contains approximately 56% of the total radioactivity of isopimpinellin (2). This data indicates that mevalonic-[5-^{3}H] is being incorporated specifically into the 6-position of isopimpinellin (2) as would be expected if Seshadri's hypothesis[47] for the furanocoumarin biosynthesis (i.e. the C-4 and C-5 positions of mevalonic acid (85) serve as the precursor of C-7 and C-6 of furanocoumarin) is followed.

Somewhat surprisingly, the remaining radioactivity is found essentially in the methoxyl groups (between 24-44%) of isopimpinellin (2). It is difficult to explain these observed results[48] on the basis of invoked theories about the metabolism of mevalonic acid (85). Mevalonic acid is not considered to be an important source of the "C_1-pool" in plant systems. One possible explanation could be that there is a tritium



exchange between the tritiated mevalonic acid and the biological medium in the system being studied.

To determine the distribution of radioactivity in alloimperatorin methyl ether (7), it was converted to its diol (3). The diol (3) was then acetylated to the monoacetate (107) which was degraded according to the scheme already dscribed and as illustrated in Figure 19. The results are given in Table 5.

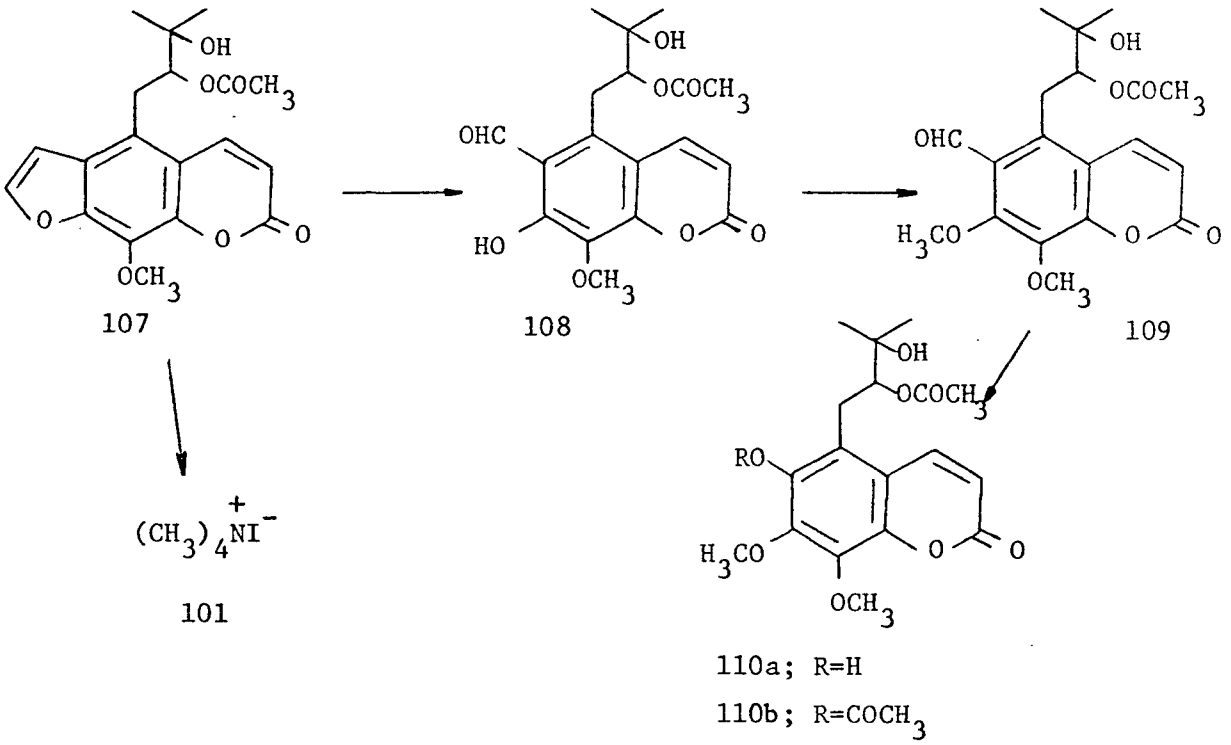


Figure 19. Degradation of radioactive alloimperatorin methyl ether (7).

TABLE 5. Distribution of radioactivity in alloimperatorin methyl ether from D,L-mevalonic acid-[5-^{3}H] incorporation experiments.

Experiment no.	Specific activity of the compounds isolated (dpm/mmole)			
	107[a]	109	110b	101
6	6.63×10^3 (100%)	6.5×10^3 (100%)	4.28×10^3 (68%)	
6	7.12×10^3 (100%)	6.8×10^3 (95.5%)	5.0×10^3 (70%)	
7	1.944×10^4 (100%)	--	--	6.06×10^2 (3%)

[a] The total activity of monoacetate (107) is set at 100%.

These results again indicate that there is no loss of activity in the 7-position of alloimperatorin methyl ether (7) and that the 6-position contains between 30-32% of the total activity. The loss of activity in the 6-position is in accord with the proposed hypothesis of furan ring formation in these furanocoumarins. Since alloimperatorin methyl ether also contains a dimethylallyl side chain and a methoxyl group, the remaining activity (68-70%) would be expected in the methoxyl group and/or in the alkyl side chain. However, the demethylation of 107 gave very little activity in the methoxyl group ($\sim$ 3%). Since the coumarin portion of these furanocoumarins has been shown to be cinnamic acid derived,[49] the remaining activity ($\sim$ 65%) in 7 must be present in the alkyl side chain. Thus it is evident that mevalonic acid-[5-^{3}H] is acting as a specific precursor of the 6-position and the alkyl side chains of alloimperatorin methyl ether (7).

To determine the distribution of label in isoimperatorin (13), it was converted to bergapten (10b) as described previously. Thus isoimperatorin (13) (1.728×10^5 dpm/mmole) from experiment 6 was degraded and bergapten (10b) (1.77×10^4 dpm/mmole) was found to contain 10.3% of the total activity of 13. In a similar experiment, isoimperatorin (13) (1.458×10^5 dpm/mmole) from experiment 8 was converted to bergapten (10b) and it was found to have a specific activity of 2.268×10^4 dpm/mmole or 15.5% of the total activity of 13. Therefore, it is evident that 85-90% of the total activity of 13 resides in the alkyl-ether side chain and that only 10-15% of the radioactivity is in the rest of the furanocoumarin molecule. Since isoimperatorin (13) contains a furan ring and as it has already been shown that mevalonic acid-[5-^{3}H]

incorporates into the 6-position of the furanocoumarins, the residual (10-15%) of activity in bergapten (10b) will be expected to reside in the 6-position of isoimperatorin (13). However, due to lack of cold material, this degradation could not be pursued further.

These results show that mevalonic acid-[5-^{3}H] is indeed a specific precursor of the furan ring and the alkyl and alkyl-ether side chains in furanocoumarins.

In order to establish the role of mevalonic acid-[4-^{3}H] in the biosynthesis of furanocoumarins, two feeding experiments were set up for a period of 2 and 4 days and the results are listed in Table 6.

Thus mevalonic acid-[4-^{3}H] is also incorporated into all three furanocoumarins and the level of incorporation is again 5 to 10 times higher than that achieved in young Thamnosma montana plants.[7] Again to determine the location of radioactivity in various furanocoumarins isolated, isopimpinellin (2) (1.96×10^{4} dpm/mmole) from experiment 10 was degraded to 6-acetoxymethyl-7-acetoxy-5,8-dimethoxycoumarin (103b) according to the scheme previously described and 103b was found to be completely inactive. In a similar experiment, isopimpinellin (2) (8.4×10^{3} dpm/mmole) from experiment 11 was selectively ozonized to 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95) which was shown to lack any measurable amount of radioactivity. Thus it is evident that mevalonic acid-[4-^{3}H] is being specifically incorporated into the 7-position of isopimpinellin (2). It is necessary to note that no radioactivity could be found in the methoxyl groups of isopimpinellin (2) as was the case in the mevalonic acid-[5-^{3}H] experiments.

TABLE 6. Incorporation of D,L-mevalonic acid-[4-^{3}H] into the tissue cultures of Thamnosma montana.

Experiment no.	Feeding time (days)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation		
				isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)
10	2	5.50×10^{8}	2.1	0.0057	0.0015	0.001
11	4	5.50×10^{8}	1.7	0.0124	0.00107	0.00117

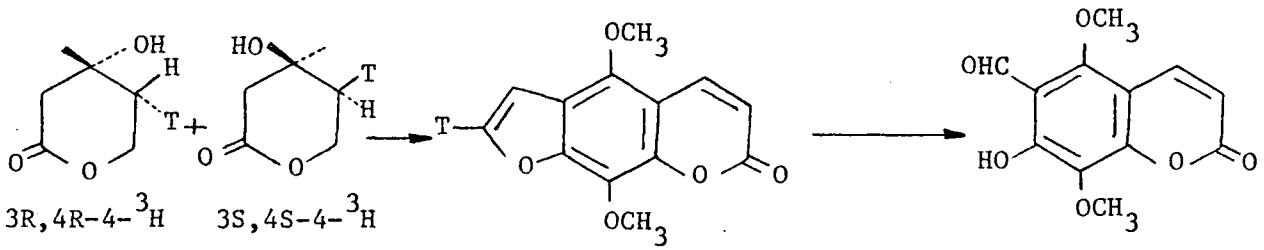
Similarly, alloimperatorin methyl ether diol (3) from experiment 10 was converted to its monoacetate (107). The monoacetate (107) (1.60×10^4 dpm/mmole) was then selectively ozonized to the corresponding phenolic aldehyde, 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108). The phenolic aldehyde (108) (7.11×10^3 dpm/mmole) was found to contain 42% of the radioactivity of 107. In a similar experiment, the monoacetate (107) (1.44×10^4 dpm/mmole) from experiment 11 was converted to the corresponding phenolic aldehyde (108) (5.76×10^3 dpm/mmole) which was shown to have 40% of the original activity of 107.

It is clear from these results that about 58% of the activity of 7 resides in the 7-position as would be expected if mevalonic acid-[4-^{3}H] was acting as a specific precursor of the furan ring. Since there is no activity found in the methoxyl groups of isopimpinellin (2) from experiments 10 and 11 and since the coumarin portion of furanocoumarins has been shown to be cinnamic acid derived,[49] the remaining activity (40-42%) in 7 must reside in the alkyl side chain which would be expected to be mevalonic acid derived.

Location of radioactivity in isoimperatorin (13) (3.46×10^5 dpm/mmole) from experiment 10 was determined by converting 13 to bergapten (10b) and bergapten (8.64×10^4 dpm/mmole) was found to contain 25% of the total activity of 13. Similarly isoimperatorin (13) (5.65×10^5 dpm/mmole) from experiment 11 was degraded to bergapten (10b) and the latter (3.54×10^4 dpm/mmole) was found to contain 6.1% of the activity of isoimperatorin. Thus it is apparent that between 75-94% of the activity in isoimperatorin is located in the C_5-alkyl-ether side chain and only

6-25% of the activity is present in the furanocoumarin molecule. In order to determine the location of radioactivity in the furanocoumarin portion of 13, bergapten (10b) (1.296×10^4 dpm/mmole) from experiments 10 and 11 was combined and ozonized selectively to 6-formyl-7-hydroxy-5-methoxycoumarin (114) and 114 was shown to be completely inactive. Thus all the remaining activity (6-25%) in isoimperatorin (13) resides in the 7-position of 13.

Thus the results of experiments 10 and 11 clearly indicate that mevalonic acid-[4-^{3}H] is acting as a specific precursor of the furan ring and the alkyl side chains of furanocoumarins. These results are in complete agreement with the results of Floss and Mothes[49] that mevalonic acid-[4-^{3}H] is a specific precursor of the 7-position of the furan ring of furanocoumarins.



Finally to determine the role of mevalonic acid-[2-^{3}H] in the biosynthesis of furanocoumarins, two feeding experiments for a time period of 2 and 4 days were set up and three furanocoumarins were isolated. The results are given in Table 7.

Thus mevalonic acid-[2-^{3}H] is incorporated into all three furanocoumarins. However, the level of incorporation is much lower than in the cases of [4-^{3}H]- or [5-^{3}H]-mevalonic acid. Also, the incorporation

TABLE 7. Incorporation of D,L-mevalonic acid-[2-^{3}H] into the tissue cultures of Thamnosma montana.

Experiment no.	Feeding time (days)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation		
				isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)
12	2	1.11×10^{9}	1.70	0.00083	0.00012	0.0002
13	4	1.11×10^{9}	1.69	0.0054	0.00038	0.00036

of mevalonic acid-[2-^{3}H] into isopimpinellin (2), a simple furanocoumarin, is hard to reconcile with the previous results. Therefore to determine the location of radioactivity, isopimpinellin (2) (9.0×10^3 dpm/mmole) from experiment 12 was demethylated and tetramethylammonium iodide (101) (9.05×10^3 dpm/2 mmole) was found to contain all the radioactivity of 2. In a similar experiment, isopimpinellin (2) (4.182×10^4 dpm/mmole) from experiment 13 was demethylated and tetramethylammonium iodide (101) (4.0×10^4 dpm/2 mmole) was found to contain all the radioactivity of isopimpinellin (2).

Thus it is evident that all the radioactivity in isopimpinellin (2) resides in the methoxyl groups and no activity is present in the rest of the furanocoumarin molecule.

To determine the location of radioactivity in alloimperatorin methyl ether (7), alloimperatorin methyl ether diol (3) (6.36×10^3 dpm/mmole) from experiment 12 was cleaved with periodic acid and the alcohol (105) (1.30×10^3 dpm/mmole) was found to contain 20% of the original activity of 3. In a similar experiment, diol (3) (7.63×10^3 dpm/mmole) from experiment 13 was converted to alcohol (105) which was shown to lack any measurable amount of activity. Thus it is evident that between 80-100% of the radioactivity in alloimperatorin methyl ether (7) resides in the terminal three carbon atoms of the dimethylallyl side chain, indicating that mevalonic acid-[2-^{3}H] is acting as a specific precursor of this side chain. Since the furanocoumarin portion of alloimperatorin methyl ether (7) and of isopimpinellin (2) will be expected to be biosynthesized in a similar manner, any residual amount (0-20%) of radioactivity in 7 will be expected to reside in the

methoxyl group of alloimperatorin methyl ether (7) as has been shown to be the case in isopimpinellin (2).

Since isoimperatorin (13) is a simple furanocoumarin with an alkyl-ether side chain and no methoxyl group, all the activity in 13 would be expected to reside in this C_5 side chain. Therefore, isoimperatorin (13) (1.08×10^5 dpm/mmole) from experiment 12 was hydrolyzed and bergapten (10b) (5.85×10^3 dpm/mmole) was shown to have about 5% of the total activity of 13. In a similar experiment, isoimperatorin (8.1×10^5 dpm/mmole) from experiment 13 was degraded to bergapten (10b) (1.728×10^4 dpm/mmole) and this was shown to have about 2.7% of the total radioactivity of 13. Thus it is apparent from these results that between 95-97% of the radioactivity of isoimperatorin (13) resides in the C_5-alkyl-ether side chain indicating that mevalonic acid-[2-^{3}H] is acting as a specific precursor of this side chain.

Finally to determine if mevalonic acid is being degraded to the C_1-pool and is thus being incorporated into the methoxyl groups of furanocoumarins or if there is some sort of tritium exchange between the tritiated mevalonic acid and the C_1-pool in the tissue culture system, mevalonic acid-[5-^{14}C] was fed to the 5-week old tissue cultures over a 2 day period and isopimpinellin (2) was isolated. The results are given in Table 8.

TABLE 8. Incorporation of D,L-mevalonic acid-[5-^{14}C] into tissue cultures of *Thamnosma montana*

Experiment no.	Feeding time (days)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation isopimpinellin (2)
14	2	1.11×10^9	3.33	0.002

To determine the distribution of radioactivity, isopimpinellin (2) (5.00×10^4 dpm/mmole) from experiment 14 was degraded according to the scheme in Figure 18 and phenol (97) (2.75×10^3 dpm/mmole) was shown to contain only 5.5% of the radioactivity of 2. This clearly indicates that about 95% of the radioactivity in isopimpinellin (2)resides in the 6-position. This is in contrast to only 56% of the activity present in the 6-position in the mevalonic acid-[5-^{3}H] feeding experiments. Thus if mevalonic acid was being degraded to the C_1-pool and thus acting as a precursor of the methoxyl groups of isopimpinellin (2), an equivalent amount of activity would be expected in the methoxyl groups of isopimpinellin in both [5-^{3}H] and [5-^{14}C]-mevalonic acid feedings. Since this is not the case and in the mevalonic acid-[5-^{14}C] experiment, about 95% of the radioactivity is present in the 6-position of isopimpinellin (2), it is clearly evident that mevalonic acid is not being degraded to the C_1-pool and thus is not a precursor of the methoxyl groups. Any activity found in the methoxyl groups of furanocoumarins in the [5-^{3}H]- and [2-^{3}H]-mevalonic acid feedings must come from either a tritium exchange between the tritiated mevalonic acid and the C_1-pool in the system or by some other unknown mechanism.

The results of experiments 4-14 are in complete agreement with the results of Floss and Mothes[49] and thus support Seshadri's proposal[47] for furanocoumarin biosynthesis. However, these results are not in agreement with the results of Brown[52] and of Caparole et al.[48] These workers have indicated that the incorporation of mevalonic acid into furanocoumarins was nonspecific. However, our results clearly indicate

that mevalonic acid is acting as a specific precursor of the furan ring and the alkyl groups of furanocoumarins in the tissue cultures of Thamnosma montana. Since in the investigations of Brown[52] and Caparole et al.[48] no degradations to determine the distribution of radioactivity were performed, the significance of their results is questionable.

EXPERIMENTAL (PART II)

For general experimental information see page 59.

Radioactivity was measured with a Nuclear Chicago Mark I or Mark II Liquid Scintillation counter in counts per minute (cpm). The radioactivity of the sample in disintegration per minute (dpm) was subsequently calculated using the counting efficiency which was determined for each sample by the external standard technique[77] utilizing the built-in barium-133 gamma source. The organic scintillator solution used with the counter was made up of the following components: toluene (1 ℓ), 2,5-diphenyloxazole (4 g) and 1,4-bis[2-(5-phenyloxazolyl)]-benzene (0.05 g). In practice, a sample was dissolved in benzene (1ml) or in methanol (1 ml) if the compound was not sufficiently soluble in benzene, in a counting vial. The volume was then made up to 15 ml with the above scintillator solution. In case of water soluble counting samples, an aqueous scintillator solution was utilized made up of the following components: toluene (385 ml), dioxane (385 ml), methanol (230 ml), naphthalene (80 g), 2,5-diphenyloxazole (5 g) and 1,4-bis[2-(5-phenyloxazolyl)] benzene (0.0625 g). In practice, a sample was dissolved in water (as required) and methanol (1 ml) in the counting vial. The solution was made up to 15 ml with the aqueous scintillator solution. For each sample counted, the background was

determined for the counting vial to be used by filling the vial with the appropriate solvent and scintillator solution and counting (3 x 40 min; 3 x 100 min; 2 x 100 min). The difference in the cpm between the background count and the sample count was used for subsequent calculations. Unless otherwise noted, radioactivity was determined by scintillation counting with organic scintillator solution. Deviation from these normal counting procedures will be discussed in the specific instances in which they arise.

Cultivation of Tissue Cultures of Thamnosma montana

All culturing was done at room temperature (24°C). Seeds were rinsed with 70% ethanol, soaked for approximately 2 minutes in 0.1% $HgCl_2$, rinsed with sterile water and were planted on water-agar. Microorganism-free seedlings were cut into 3 or 4 pieces and transferred to the surface of the modified White's agar medium in Erlenmeyer flasks. The seedling pieces were incubated until callus tissue was observed at the cut ends. Then the fragments of callus tissue were transferred to liquid medium, 100 ml per 250 ml Erlenmeyer flask, which was placed on a rotary shaker (about 120 rpm and 30 mm radius). Whenever tissue was transferred to fresh media, the pieces were cut up into smaller pieces.

For nutrient purposes, the following general-purpose medium (a modified White's nutrient solution) was used. For solid medium, 1% agar was added.

Component	mg/ℓ of distilled water
sucrose	20,000
NH_4NO_3	400
KCl	65
KNO_3	80
KH_2PO_4	12.5
$Ca(NO_3)_2 \cdot 4H_2O$	144
$MgSO_4 \cdot 7H_2O$	72
NaFeEDTA	25
H_3BO_3	1.6
$MnSO_4 \cdot 4H_2O$	6.5
$ZnSO_4 \cdot 7H_2O$	2.7
KI	0.75
3IAA	2.0
kinetin	0.2
thiamine HCl	10
nicotinic acid	1.0
pyridoxine HCl	1.0
glycine	2.0
myoinositol	100
casein hydrolysate	1,000

Usually the culturing was done in 250 ml flasks. The amount of tissue cultures in a flask varied according to the size and number of pieces originally transferred to the flask. In a typical experiment, five flasks contained 35 g of wet tissue which when air-dried gave 4.7 g.

When cultures were used for biosynthetic experiments, most of the pieces of wet tissue were 5 mm or less in diameter. The tissue cultures were cultivated by Dr. P. Salisbury of this department.

Constituents of tissue cultures of Thamnosma montana

Five flasks containing tissue cultures (5 week old) were decanted and the solid residue was put on an aspirator for 30 minutes. The residue was washed with water (2 times) and weighed to give 35 g of wet tissue. This was air-dried at room temperature and the dry residue (4.7 g) was extracted with acetone in a Soxhlet extractor. The acetone extract was evaporated to dryness, the residue dissolved in chloroform, filtered and dried over sodium sulfate. The solvent was removed under reduced pressure to give 100 mg of the residue which was pre-adsorbed on 1 g of alumina (activity IV) and was chromatographed on alumina (10 g, neutral activity III). Preparative layer chromatography gave isopimpinellin (2) (1.5 mg), alloimperatorin methyl ether (7) (0.8 mg) and isoimperatorin (13) (1.6 mg). No umbelliprenin (9) was indicated by tlc. Some thamnosmin (15) was present but no attempt was made to isolate this or to identify other components of the tissue culture extract.

In subsequent radioactive experiments, tissue cultures were freeze-dried (unless otherwise noted) and the chloroform soluble extract of the tissue cultures was diluted with cold furanocoumarins before column chromatography. In each experiment, 5 week old tissue cultures were utilized for biosynthetic investigation.

Feeding Experiments 1 and 2

In each of these experiments, two flasks containing tissue cultures were decanted and the solid was transferred to 50 ml of fresh growth medium. D,L-Phenylalanine-[3-^{14}C] (1.2 mg, obtained from New England Nuclear Corp., Boston, Mass.) was dissolved in dilute sodium bicarbonate solution (2 mg in 10 ml of distilled water) and an aliquot was removed, weighed and the radioactivity per unit weight of solution was determined (11.0 x 10^6 dpm/g). Two aliquots were then removed from this solution (∿ 1 g each) and mixed with the tissue cultures in the flasks with fresh growth media, and these flasks were placed on a rotary shaker. When the preselected time period was over, the tissue cultures, including the solutions, were freeze-dried. The dry material was worked up as usual. The chloroform soluble extract was diluted with cold isopimpinellin (2) and chromatographed on neutral alumina (activity IV). Isopimpinellin was isolated by preparative layer chromatography, crystallized and counted to constant radioactivity. The results and experimental details are presented in Table 9.

Feeding Experiment 3

In this experiment, two flasks containing tissue cultures on a solid growth medium were used and D,L-phenylalanine-[3-^{14}C] solution (∿ 1 g) was applied to the surface of the tissue cultures with the help of a pipette. The flasks were left under fluorescent illumination for 72 hours after which the tissue cultures were removed from the solid medium with spatula, freeze-dried and worked up in a usual manner. Isopimpinellin (2) was isolated and the results are given in Table 9.

TABLE 9. Incorporation of D,L-phenylalanine-[3-^{14}C] (sodium salt) into tissue cultures of Thamnosma montana

Experiment no.	Activity fed (dpm)	Specific activity fed (dpm/mmole)	Weight fed (mg)	Dry weight of tissue cultures (g)	Feeding time (hr)	Weight isolated[a] isopimpinellin (2) (mg)	Specific activity isolated isopimpinellin (2) (dpm/mmole)	% Incorporation Isopimpinellin (2)
1	1.093×10^7	1.55×10^{10}	0.12	1.20	8	6.5	1.18×10^5	0.029
2	1.13×10^7	1.55×10^{10}	0.12	1.38	48	7.5	2.62×10^6	0.71
3	1.10×10^7	1.55×10^{10}	0.12	1.30	72	6.5	1.98×10^6	0.48

[a] Isopimpinellin (2) was diluted with inactive material before isolation.

Feeding Experiments 4, 5, 6, 7, 8, and 9

In each of these experiments, three to four flasks containing tissue cultures were used and the tissue cultures transferred into 25 ml of distilled water. Only in experiment 4, 25 ml of nutrient solution was used instead of distilled water. D,L-Mevalonic acid-[5-^{3}H] as dibenzoyl ethylene diamine (DBED) salt (obtained from New England Nuclear Corp.) in methanol was used as precursor and the flasks were put on rotary shaker for a preselected time period. Tissue cultures were freeze-dried and worked up in the usual manner. In each case, chloroform soluble extract was diluted with inactive isopimpinellin (2), alloimperatorin methyl ether (7) and isoimperatorin (13) before chromatography. The experimental details and the results are given in Table 10a and 10b. In each case, alloimperatorin methyl ether (7) was converted to its diol (3) before counting. The specific activity and incorporation values quoted for alloimperatorin methyl ether (7) are calculated from the appropriate information obtained for the diol (3), correcting for the yield in converting 7 to 3. The coumarins were diluted with inactive material as necessary to obtain quantities which could be crystallized to constant radioactivity and to perform degradations.

Degradations of Isopimpinellin (2) from Experiment 4

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95)

Isopimpinellin (2) (45 mg, 2.34 x 10^{4} dpm/mmole) from Experiment 4 was selectively ozonized as described previously[7] and 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95), (19.5 mg) was isolated and shown to have a specific activity of 2.25 x 10^{4} dpm/mmole or 96.5% of the original activity of isopimpinellin (2).

TABLE 10a. Incorporation of D,L-mevalonic acid-[5-^{3}H] (DBED salt) in tissue cultures.

Experiment no.	Activity fed (dpm)	Specific activity fed (dpm/mmole)	Weight fed (mg)	Dry weight of tissue cultures (g)	Feeding time (days)	Weight of compound isolated (mg)		
						isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)
4	1.11×10^{9}	13.86×10^{12}	0.0214	2.54	2	--	5.0	4.5
5	1.11×10^{9}	13.86×10^{12}	0.0214	3.00	2	--	6.2	9
6	1.11×10^{9}	13.86×10^{12}	0.0214	1.45	4	4.9	2.5	7.1
7	8.0×10^{8}	13.86×10^{12}	0.0172	2.50	7	0.8[a]	5.6	6.4
8	1.11×10^{9}	13.86×10^{12}	0.0214	3.50	7	4.0	3.5	7.5
9	8.0×10^{8}	13.86×10^{12}	0.0172	3.00	10	2.9	5.0	6.8

[a] Isoimperatorin (13) from experiment 7 was not diluted with inactive material before chromatography.

TABLE 10b.

Experiment no.	Specific activity isolated (dpm/mmole)			% Incorporation		
	isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)	isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)
4	--	2.52×10^4	6.08×10^5	--	0.000040	0.0010
5	--	2.28×10^4	4.98×10^5	--	0.000045	0.00164
6	1.11×10^6	2.34×10^4	5.78×10^4	0.00182	0.00165	0.00015
7	9.35×10^6	3.49×10^5	4.87×10^4	0.0025	0.00055	0.000114
8	1.57×10^6	9.90×10^4	6.20×10^4	0.0021	0.00011	0.00017
9	1.17×10^6	2.27×10^5	3.22×10^5	0.00113	0.00036	0.00076

Radioactive 6-formyl-5,7,8-trimethoxycoumarin (96)

Pure 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95) and its mother liquor from crystallization were methylated separately and the preparative layer chromatography gave combined 6-formyl-5,7,8-trimethoxycoumarin (6) (23 mg).

Radioactive 6-hydroxy-5,7,8-trimethoxycoumarin (97)

6-Formyl-5,7,8-trimethoxycoumarin (97) (23 mg, 2.25×10^4 dpm/mmole) from the previous reaction was degraded to 6-hydroxy-5,7,8-trimethoxycoumarin (97) (14.5 mg) and 97 was shown to have a specific activity of 1.025×10^4 dpm/mmole or 44% of the original activity of isopimpinellin (2).

Degradations of Isopimpinellin (2) from Experiment 5

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95)

Isopimpinellin (2) (50 mg, 2.42×10^4 dpm/mmole) from Experiment 5 was converted to 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95) (24 mg) and this (95) was shown to have a specific activity of 2.51×10^4 dpm/mmole or all the original activity of isopimpinellin (2).

Radioactive 6-formyl-5,7,8-trimethoxycoumarin (96)

6-Formyl-7-hydroxy-5,8-dimethoxycoumarin (95) from previous reaction was methylated as described previously[7] to give 6-formyl-5,7,8-trimethoxycoumarin (96) (25 mg) by preparative layer chromatography. It was not counted but used as such in the next reaction.

6-Hydroxy-5,7,8-trimethoxycoumarin (97)

Radioactive 6-formyl-5,7,8-trimethoxycoumarin (96) (25 mg) from the above reaction was treated with hydrogen peroxide and sulfuric acid to give 6-hydroxy-5,7,8-trimethoxycoumarin (97) (16.7 mg). This was shown to have a specific activity of 1.067×10^4 dpm/mmole or 44% of the original activity of isopimpinellin (2).

Radioactive tetramethylammonium iodide (101)

Radioactive 6-hydroxy-5,7,8-trimethoxycoumarin (97) (10 mg, 1.067×10^4 dpm/mmole) from a previous reaction was demethylated as described previously[7] and tetramethylammonium iodide (101) (14 mg) was isolated. This was counted by the following method. The salt ($\sim$ 1.5 mg) was dissolved in aqueous sodium thiosulfate solution (0.1 N, 10 drops) and methanol (1 ml) and the solution was made up to 15 ml with aqueous scintillator solution. A blank sample of the same constitution (except that non-radioactive 101 was used) was counted in the same vial to determine background. By this method, 101 gave a specific activity of 1.17×10^4 dpm/mmole or 48% of the original activity of isopimpinellin (2).

Radioactive tetramethylammonium iodide from Experiment 9

Radioactive isopimpinellin (2) (14.5 mg, 1.722×10^4 dpm/mmole) from Experiment 9 was demethylated as described previously and tetramethylammonium iodide (101) (10 mg) was isolated. It was counted by dissolving the salt ($\sim$ 1.5 mg) in sodium thiosulfate solution (0.1 N, 10 drops) and methanol (1 ml) and making the solution up to 15 ml with aqueous

scintillator solution. By this method, 101 gave a specific activity of 4.18×10^3 dpm/2mmole or 24.3% of the original activity of isopimpinellin (2).

Tetramethylammonium iodide (101) (7 mg) from the above reaction was converted to its picrate (102) (6 mg) which was counted as follows: picrate ($\sim$ 2 mg) was dissolved in acetic anhydride (5 drops) and acetic acid (5 drops), zinc dust (10 mg) was added to decolorize the solution. Sodium metabisulfite (100 mg) was added and the mixture was then filtered directly into the counting vial. The original container was washed with methanol (1 ml) and this wash was also filtered into the counting vial. The solution was made up to 15 ml with organic scintillator solution and then counted after standing at least 1 hour in the cold and in the dark. As before, an inactive sample of 102 was counted in the same manner to determine the background prior to counting the radioactive sample. By this method, tetramethylammonium picrate (102) indicated a specific activity of 4.107×10^3 dpm/2mmole or 24% of the original activity of isopimpinellin (2).

Degradations of Alloimperatorin Methyl Ether (7) from Experiment 6

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-8-methoxypsoralen (107)

Radioactive alloimperatorin methyl ether (7) from Experiment 6 was converted to its diol (3). The diol (3) was diluted with non-radioactive material and was acetylated to 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-8-methoxypsoralen (107).

Trial a

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-formyl-7-hydroxy-8-methoxy coumarin (108)

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-8-methoxy-psoralen (107) (42 mg, 6.3×10^3 dpm/mmole) from Experiment 6 was selectively ozonized as previously described[7] and after crystallization, 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-formyl-7-hydroxy-8-methoxy coumarin (108) (30 mg) was isolated. This material (108) was not counted but was used directly in the next reaction.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-formyl-7,8-dimethoxy coumarin (109).

5-(2'-Acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxy coumarin (108) (30 mg) from previous reaction was methylated an 109 (25 mg) was isolated and shown to have a specific activity of 6.5×10^3 dpm/mmole or all the original activity of 107.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-acetoxy-7,8-dimethoxy coumarin (110b)

Radioactive 109 (24.5 mg, 6.5×10^3 dpm/mmole) from the previous reaction was converted to 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-acetoxy-7,8-dimethoxy coumarin (110b) (16 mg) as previously described[7] and 110b was shown to have a specific activity of 4.28×10^3 dpm/mmole or 68% ofthe total activity of 107.

Trial b

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-formyl-7-hydroxy-8-methoxy coumarin (108)

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-8-methoxy coumarin (107) (38 mg, 7.12×10^3 dpm/mmole) was converted to 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-formyl-7-hydroxy-8-methoxy coumarin (108) (25 mg). The product 108 was not counted but used directly for the next reaction.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-formyl-7,8-dimethoxy coumarin (109)

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-formyl-7-hydroxy-8-methoxy coumarin (108) (25 mg) from the previous reaction was methylated to give 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-formyl-7,8-dimethoxycoumarin (109) (23 mg). The product 109 was shown to have a specific activity of 6.8×10^3 dpm/mmole or 95.5% of the original activity of 107.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-acetoxy-7,8-dimethoxy coumarin (110b)

Radioactive 109 (22 mg, 6.8×10^3 dpm/mmole) from the previous reaction was converted to 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-6-acetoxy-7,8-dimethoxy coumarin (110b) (12 mg) and it was shown to have a specific activity of 5.0×10^3 dpm/mmole or 70% of the original activity of 107.

Radioactive tetramethylammonium iodide (101) from Experiment 7

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methyl butyl)-8-methoxy psoralen (107) (19.5 mg, 1.944×10^4 dpm/mmole) was demethylated to give tetramethylammonium iodide (101) (7.3 mg). It was counted as before and was shown to have a specific activity of 6.06×10^2 dpm/mmole or about 3% of the original activity of 107.

Degradations of Isoimperatorin (13) from Experiment 6 and 8

Radioactive bergapten (10b)

Isoimperatorin (13) (24.5 mg, 1.728×10^5 dpm/mmole) from Experiment 6 was converted to bergapten (10b) (10 mg) as previously described and was shown to have a specific activity of 1.771×10^4 dpm/mmole or 10.3% of the original activity of isoimperatorin (13).

Radioactive bergapten (10b)

Radioactive isoimperatorin (13) (21 mg, 1.458×10^5 dpm/mmole) from Experiment 8 was converted to bergapten (10b) (9 mg) and was shown to have a specific activity of 2.268×10^4 dpm/mmole or 15.5% of the original activity of isoimperatorin (13).

Feeding Experiments 10 and 11

In each of these experiments, two flasks containing tissue cultures were used and the tissue cultures were transferred into 25 ml of distilled water. D,L-Mevalonic acid-[4-^{3}H] lactone (obtained from Amersham/Searle Corp.) was converted to its sodium salt by evaporating the benzene solution of the lactone and dissolving the residue in dilute sodium carbonate solution (5 mg in 1 ml of water). This solution was

then added to the tissue cultures in distilled water and the flasks were put on rotary shaker for a preselected time period. Tissue cultures were freeze-dried and worked up as usual. Alloimperatorin methyl ether (7) was converted to its diol (3) before counting. The experimental details and the results are given in Table 11a and 11b.

Degradation of Isopimpinellin (2) from Experiments 10 and 11

Radioactive 6-acetoxymethyl-7-acetoxy-5,8-dimethoxycoumarin (103b)

Isopimpinellin (2) (23.5 mg, 1.96×10^4 dpm/mmole) from Experiment 10 was degraded to 6-acetoxymethyl-7-acetoxy-5,6-dimethoxycoumarin (103b) (21 mg) and was shown to be completely inactive.

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95)

Isopimpinellin (2) (19 mg, 8.4×10^3 dpm/mmole) from Experiment 11 was selectively ozonized as described previously and 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95) (9 mg) was isolated. This was shown to be completely inactive.

Degradations of Alloimperatorin Methyl Ether (7) from Experiments 10 and 11

Radioactive 5-(2'-Acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxy-psoralen (107)

Radioactive alloimperatorin methyl ether diol (3) from Experiments 10 and 11 was diluted with non-radioactive 3 and was converted to 107 separately.

TABLE 11a. Incorporation of D,L-mevalonic acid-[4-^{3}H] (sodium salt) into tissue cultures.

Experiment no.	Activity fed (dpm)	Specific activity fed (dpm/mmole)	Weight fed (mg)	Dry weight of tissue cultures (g)	Feeding time (days)	Weight of compounds isolated (mg)		
						isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)
10	5.55×10^{8}	5.55×10^{11}	0.132	2.1	2	6.9	6.3	9.6
11	5.55×10^{8}	5.55×10^{11}	0.132	1.7	4	6.5	6.6	7.6

TABLE 11b.

Experiment no.	Specific activity isolated (dpm/mmole)			% Incorporation		
	isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)	isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)
10	1.24×10^{6}	3.76×10^{5}	1.45×10^{5}	0.0057	0.0015	0.001
11	3.38×10^{6}	2.56×10^{5}	2.1×10^{5}	0.0124	0.00107	0.00117

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108)

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxypsoralen (107) (28 mg, 1.69×10^4 dpm/mmole) from Experiment 10 was ozonized selectively to 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108) (6 mg). This substance (108) was shown to have a specific activity of 7.11×10^3 dpm/mmole or 42% of the original activity of 107.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108)

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxypsoralen (107) (28 mg, 1.44×10^4 dpm/mmole) from Experiment 11 was selectively ozonized as described previously and 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-formyl-7-hydroxy-8-methoxycoumarin (108) (9 mg) was isolated. This was shown to have a specific activity of 5.76×10^3 dpm/mmole or 40% of the original activity of 107.

Degradations of Isoimperatorin (13) from Experiments 10 and 11

Radioactive bergapten (10b)

Isoimperatorin (13) (13 mg, 3.46×10^5 dpm/mmole) from Experiment 10 was converted to bergapten (10b) (9 mg) as described previously and bergapten (10b) was shown to have a specific activity of 8.64×10^4 dpm/mmole or 25% of the original activity of isoimperatorin (13).

Radioactive bergapten (10b)

Radioactive isoimperatorin (13) (11.3 mg, 5.65×10^5 dpm/mmole) from Experiment 11 was converted to bergapten (10b) (7.4 mg) and this compound (10b) was shown to have a specific activity of 3.45×10^4 dpm/mmole or 6.1% of the original activity of isoimperatorin (13).

Radioactive 6-formyl-7-hydroxy-5-methoxycoumarin (114)

Radioactive bergapten (10b) (24 mg, 1.296×10^4 dpm/mmole) from Experiments 10 and 11 was combined and was selectively ozonized to give 6-formyl-7-hydroxy-5-methoxycoumarin (114) (8 mg). This (114) was shown to be completely inactive.

Feeding Experiments 12 and 13

In each experiment, two flasks containing tissue cultures were used and tissue cultures were transferred into 25 ml of distilled water. D,L-Mevalonic acid-[2-^{3}H] lactone (obtained from Amersham/ Searle Corp.) was converted to its sodium salt and mixed with the fresh tissue cultures. The flasks were put on a rotary shaker for preselected time period and tissue cultures were worked up as usual. The alloimperatorin methyl ether (7) was converted to its diol (3) before counting. The experimental details and the results are given in Tables 12a and 12b.

TABLE 12a. Incorporation of D,L-mevalonic acid-[2-^{3}H] (sodium salt) into tissue cultures.

Experiment no.	Activity fed (dpm)	Specific activity fed (dpm/mmole)	Weight fed (mg)	Dry weight of tissue cultures (g)	Feeding time (days)	Weight of compounds isolated (mg)		
						isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)
12	1.11×10^{9}	1.82×10^{11}	0.794	1.70	2	8.6	6.6	3.9
13	1.11×10^{9}	1.92×10^{11}	0.794	1.69	4	4.3	7.1	3.8

TABLE 12b.

Experiment	Specific activity isolated (dpm/mmole)			% Incorporation		
	isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)	isoimperatorin (13)	alloimperatorin methyl ether (7)	isopimpinellin (2)
12	2.89×10^{5}	5.75×10^{4}	1.4×10^{5}	0.00083	0.00012	0.0002
13	3.76×10^{6}	1.69×10^{5}	2.46×10^{5}	0.0054	0.00038	0.00036

Degradation of Isopimpinellin from Experiments 12 and 13

Radioactive tetramethylammonium iodide (101)

Radioactive isopimpinellin (2) (13.5 mg, 9.0×10^3 dpm/mmole) from Experiment 12 was demethylated and tetramethylammonium iodide (101) (12.5 mg) was isolated. This was counted by dissolving 101 (∿ 2 mg) in 10 drops of sodium thiosulfate (0.1 N) solution, 1 ml of methanol was added and the solution was made up to 15 ml with aqueous scintillator solution. By this method, 101 gave a specific activity of 9.05×10^3 dpm/mmole or all the original activity of isopimpinellin (2).

Radioactive tetramethylammonium iodide (101)

Isopimpinellin (16 mg, 4.182×10^4 dpm/mmole) from Experiment 13 was demethylated and tetramethylammonium iodide (101) (18 mg) was isolated. This was converted to tetramethylammonium picrate (102) (10 mg). It was counted by dissolving the picrate (∿ 2 mg) in acetic acid (10 drops) and acetic anhydride (10 drops). Enough zinc dust was added to decolorize the solution. Sodium metabisulfite (100 mg) was added and the solution was filtered into the counting vial. The original container was washed with methanol (1 ml) and the wash was also filtered into the counting vial. The solution was made up to 15 ml with organic scintillator solution and then counted after standing at least 1 hour in the cold and in the dark. By this method, tetramethylammonium picrate (102) was shown to have a specific activity of 4.0×10^3 dpm/2 mmole or all the original activity of isopimpinellin (2).

Degradations of Alloimperatorin Methyl Ether (7) from Experiments 12 and 13

Periodic acid cleavage of radioactive 5-(2',3'-dihydroxy-3'-methylbutyl)-8-methoxypsoralen (3) (alloimperatorin methyl ether diol)

Allimperatorin methyl ether diol (3) (18.5 mg, 6.36×10^3 dpm/mmole) from Experiment 12 was cleaved with periodic acid as described previously and 5-(2'-hydroxy ethyl)-8-methoxypsoralen (105) (5 mg) was isolated and shown to have a specific activity of 1.3×10^3 dpm/mmole or 20.4% of the original activity of 3.

Periodic acid cleavage of radioactive 5-(2',3'-dihydroxy-3'-methylbutyl)-8-methoxypsoralen (3)

Allimperatorin methyl ether diol (3) (36.5 mg, 7.36×10^3 dpm/mmole) from Experiment 13 was cleaved with periodic acid and 5-(2'-hydroxyethyl)-8-methoxypsoralen (105) (9.3 mg) was isolated and was shown to be completely inactive.

Degradations of Isoimperatorin (13) from Experiments 12 and 13

Radioactive bergapten (10b)

Isoimperatorin (13) (12.7 mg, 1.08×10^5 dpm/mmole) from Experiment 12 was converted to bergapten (10b) (7.5 mg) and this was shown to have a specific activity of 5.85×10^3 dpm/mmole or about 5.5% of the original activity of isoimperatorin (13).

Radioactive bergapten (10b)

Isoimperatorin (13) (14 mg, 8.1×10^5 dpm/mmole) from Experiment 13 was degraded to bergapten (10b) (5.6 mg) and this was shown to have a specific activity of 1.728×10^4 dpm/mmole or about 2.7% of the original

activity of isoimperatorin (13).

Feeding Experiment 14

In this experiment, three flasks containing tissue cultures were transferred to 25 ml of distilled water and D,L-mevalonic acid-[5-^{14}C] (obtained from Schwarz/Mann Corp.) was fed as a sodium salt in water. The flask was put on a rotary shaker for 2 days, worked up in the usual manner and only isopimpinellin (2) was isolated and crystallized to constant activity. The experimental details and the results are given in Table 13.

Degradations of Isopimpinellin (2) from Experiment 14

6-Formyl-7-hydroxy-5,8-dimethoxycoumarin (95)

Isopimpinellin (2) (45 mg, 4.92×10^4 dpm/mmole) from Experiment 14 was ozonized selectively and 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95) (22 mg) was isolated. This was shown to have a specific activity of 4.90×10^4 xpm/mmole or all the original activity of isopimpinellin (2).

Radioactive 6-formyl-5,7,8-trimethoxycoumarin (96)

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95) (22 mg) from the previous reaction was methylated as described previously and 6-formyl-5,7,8-trimethoxycoumarin (96) (20 mg) was isolated. This was not counted but used as such for the next reaction.

TABLE 13. Incorporation of D,L-mevalonic acid-[5-^{14}C] (sodium salt) into tissue cultures.

Experiment no.	Activity fed (dpm)	Specific activity fed (dpm/mmole)	Dry weight of tissue cultures (g)	Feeding time (days)	Weight of isopimpinellin (2) (mg)	Specific activity of isopimpinellin (2) (dpm/mmole)	% Incorporation isopimpinellin (2)
14	1.11×10^9	2.62×10^{10}	3.33	2	12	4.55×10^5	0.002

6-Hydroxy-5,7,8-trimethoxycoumarin (97)

Radioactive 6-formyl-5,7,8-trimethoxycoumarin (96) (20 mg) from the previous reaction was converted to 6-hydroxy-5,7,8-trimethoxycoumarin (97) (12 mg) as described previously. This was shown to have a specific activity of 2.75×10^3 dpm/mmole or about 5.5% of the original activity of isopimpinellin (2).

DISCUSSION (PART III)

Biosynthetic Studies on Coumarins from Young Thamnosma montana Plants

As documented in the Introduction, little information is available as to the origin of alkyl side chains often found in many natural coumarins. Although no direct evidence is available, these alkyl groups are often considered to be mevalonate derived. As shown in Figure 11, mevalonic acid (85) itself is the product resulting from the combination of three units of acetyl coenzyme-A.[53] The principal cellular source of acetyl-CoA is the intramitochondrial oxidation of pyruvate (126),[78] which is itself the major product of glucose catabolisis in most cells[78] (Figure 20b). Thus the origin of the C_5 unit of mevalonate can be directly retraced to the carbohydrate products of photosynthesis. This process of carbon dioxide fixation is outlined in Figure 20a. The starred atom illustrates the path of the carbon dioxide carbon atom from its initial fixation by ribulose-1,5-diphosphate (115) through to glucose-6-phosphate (124).[79] In Figure 20b, the glycolytic pathway from glucose-6-phosphate (124) to pyruvate (126) is presented. If the phospho-3-glycerate (118) is directly derived from the carbon dioxide fixation process, then the shortest route to pyruvate (126), and thereby acetyl-CoA is: carbon dioxide → 2-carboxy-3-ketopentitol (117) → phospho-3-glycerate (118) → phospho-2-glycerate

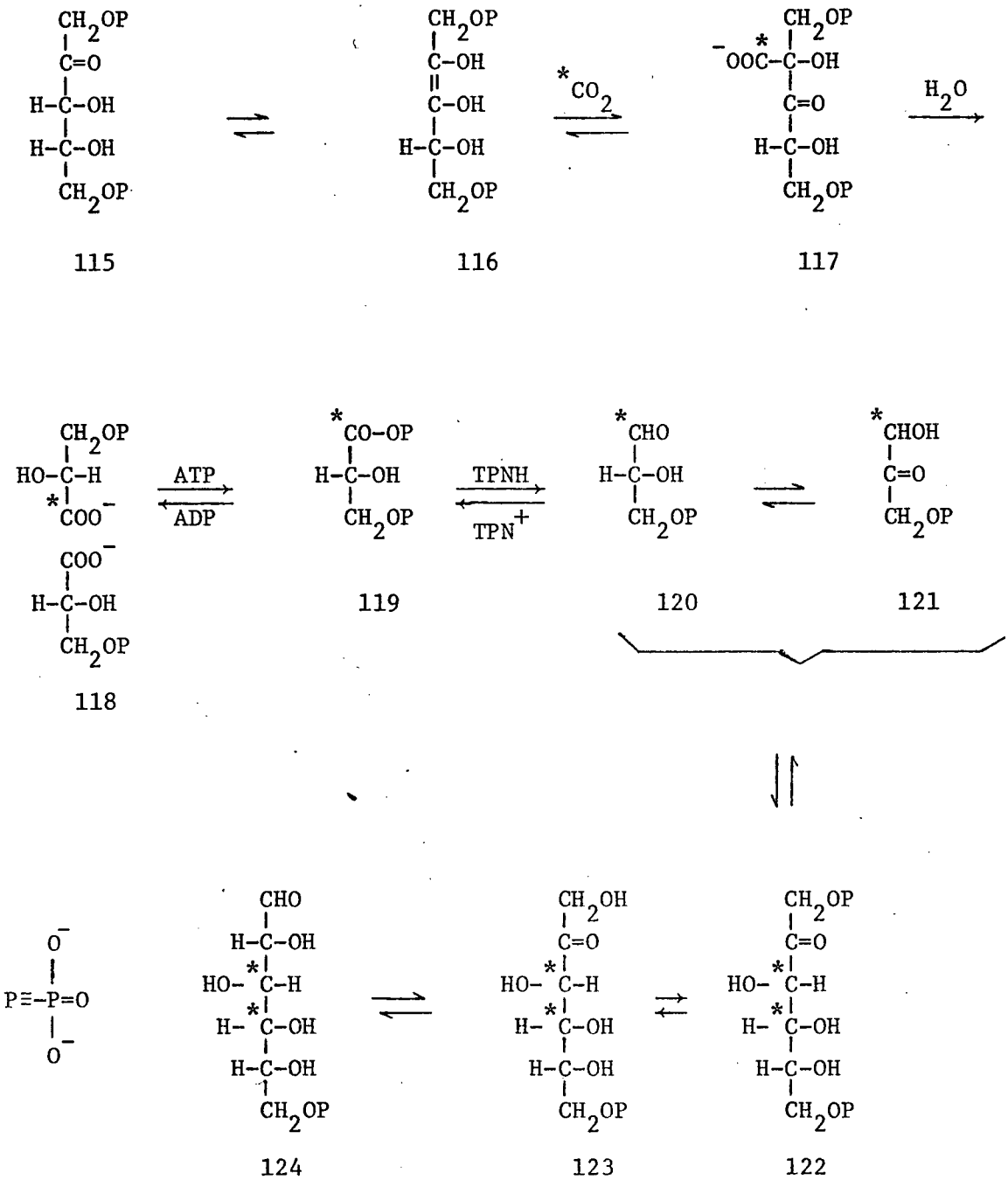


Figure 20a. The photosynthetic fixation of CO_2.

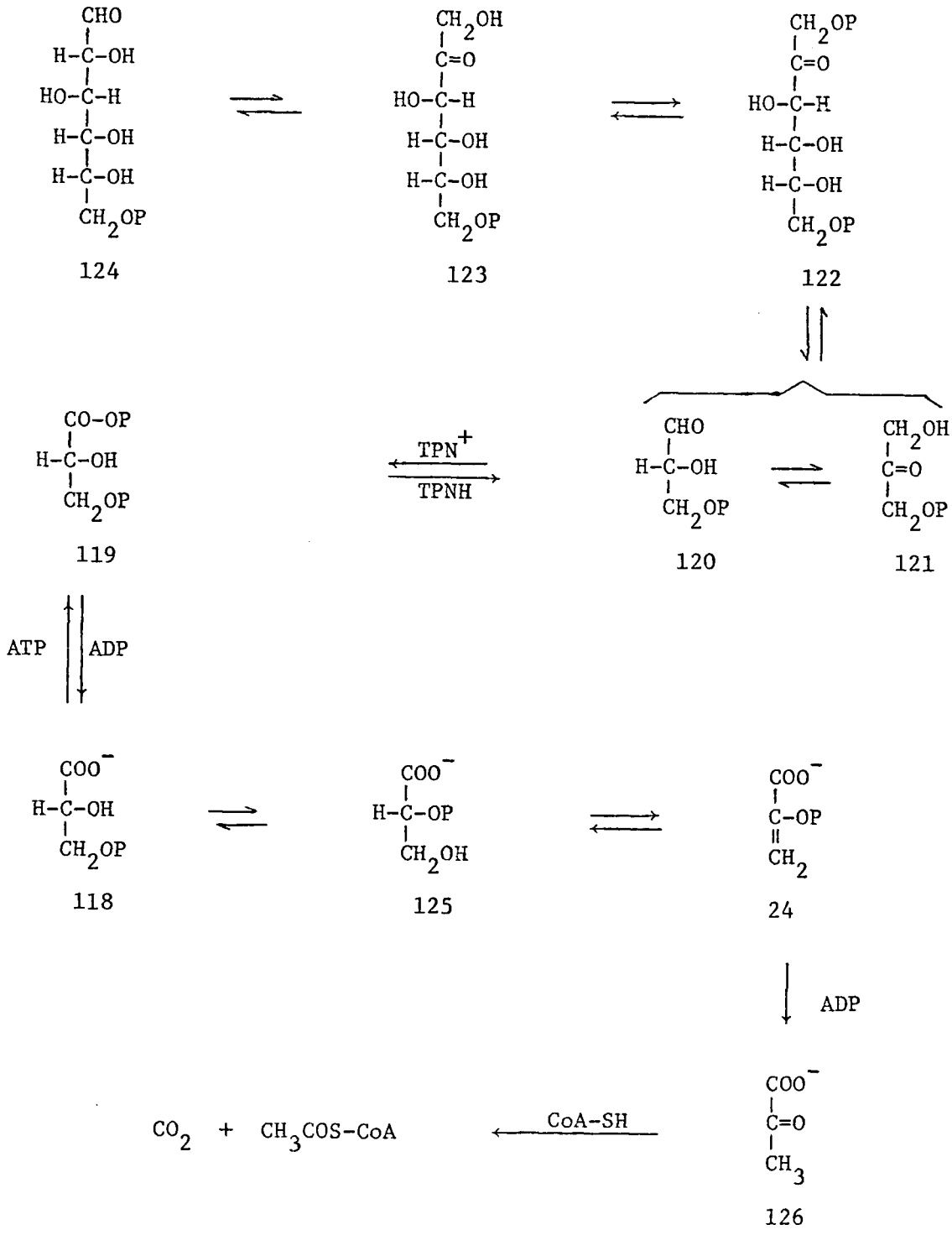


Figure 20b. The glycolytic pathway to pyruvate.

(125) → phosphoenolpyruvate (24) → pyruvate (126) → acetyl-CoA.

The purpose of this precursor delineation is to show that a C_2 unit becomes involved with the biosynthesis of the C_5 unit of mevalonate only at the acetate stage. It was, therefore, of some interest when Shah and Rogers[80] postulated the intermediacy of glycollate (127) via glycine (129) and serine (130) in the biosynthesis of acetyl-CoA. This proposal was based on the work with greening etiolated maize seedlings and involved intra- and extra-chloroplastidic terpenoid synthesis. By means of a wide range of radioisotopic-incorporation studies, radioisotopic dilution studies and experiments with inhibitors of the proposed pathway, Shah and Rogers[80] were able to demonstrate the existence of the pathway, carbon dioxide → glycollate (127) → glyoxylate (128) → glycine (129) → serine (130) → pyruvate (126) → acetyl-CoA, within the chloroplasts (Figure 21). Extrachloroplastidic sterols displayed incorporation patterns consistent with the established sequence, carbon dioxide → glucose (124) → pyruvate (126) → acetyl-CoA → mevalonate (85).

The authors felt that their work offered strong evidence for a number of proposals, specifically the relatively direct synthesis of amino acids from carbon dioxide by-passing the carbohydrate intermediates and the involvement of these amino acids in terpenoid biosynthesis. Both of these proposals are supported in the literature.[81,82]

In an attempt to determine a specifically incorporated (i.e., non-randomized) precursor of mevalonate and the C_9 unit of caphaeline (135), Gear and Garg[83] administered labelled glycollic acid and glycine to three-year-old cephaelis ipecacuanha plants. The [1-^{14}C]-labelled

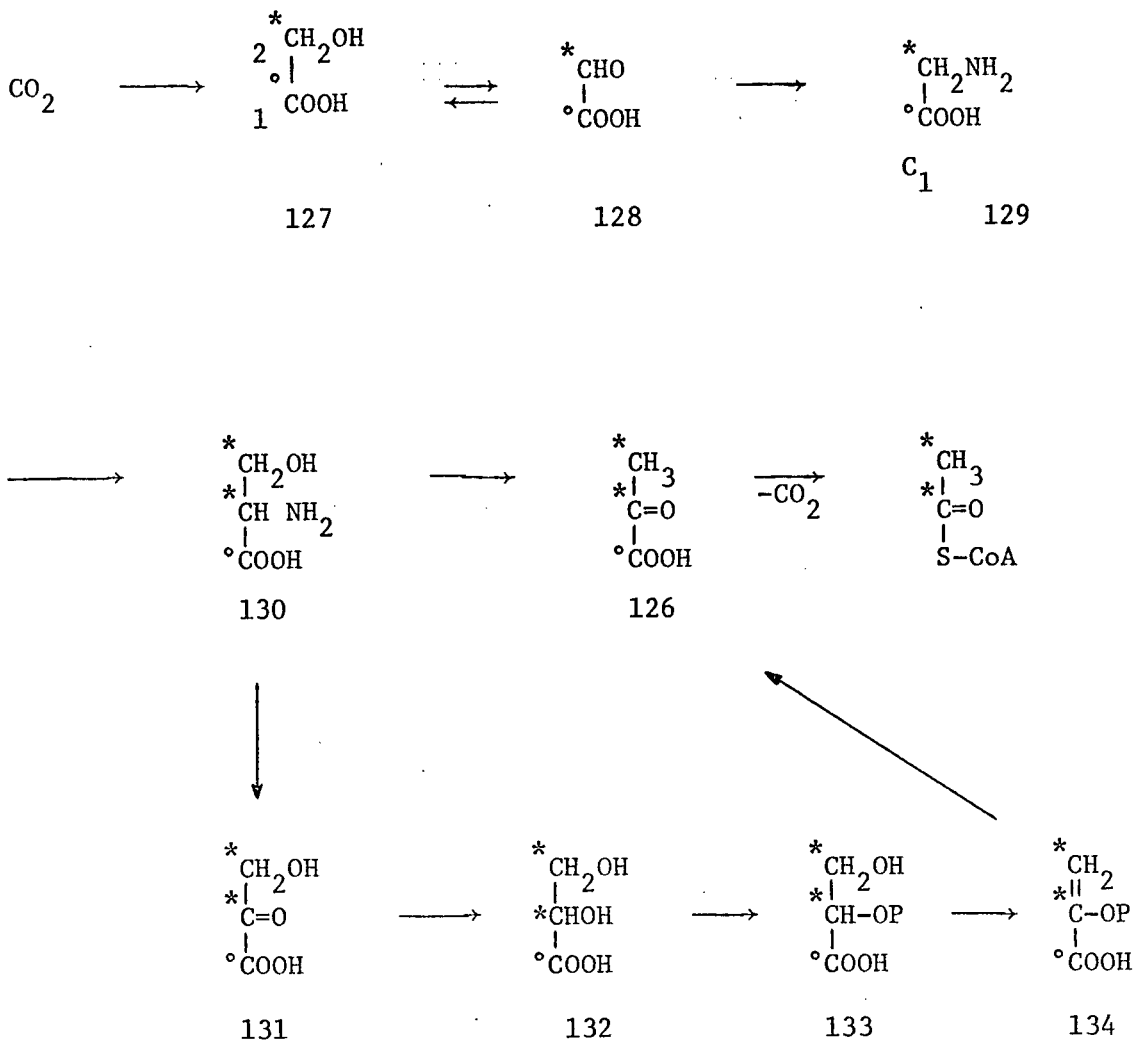
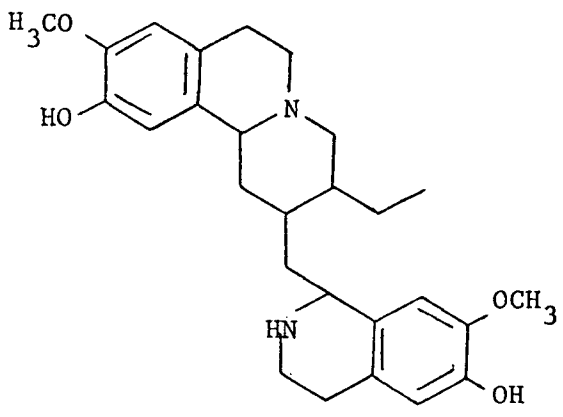
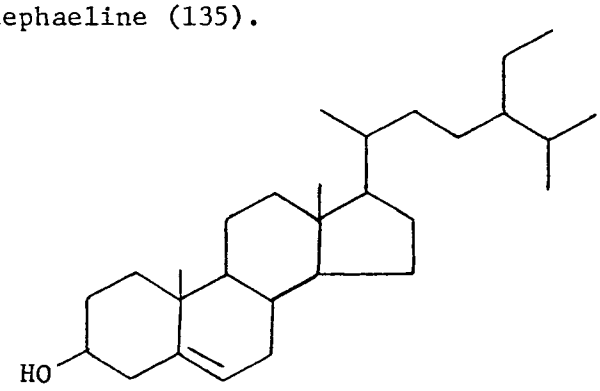


Figure 21. The Shah and Rogers chloroplastidic acetyl-CoA biogenesis.

forms of both these compounds gave either inactive or only slightly active cephaeline (135). This was not unexpected as Shah and Rogers[80] had likewise reported negative incorporation for glyoxylic acid-[1-^{14}C] (128) into β-carotene. The rationale for this finding can be seen in the biogenesis of acetyl-CoA as postulated by these authors (Figure 21). When, however, glycine-[2-^{14}C] (129) and glycollic acid-[2-^{14}C] (127) were fed to the aforementioned plant system, active cephaeline (135) was isolated. It was found that glycollic acid-[2-^{14}C] (127) was being randomized whereas glycine-[2-^{14}C] (129) was acting as a specific precursor of the C_9 unit of cephaeline (135).



135



136

In subsequent publications, glycine-[2-^{14}C] and acetate-[2-^{14}C] were fed to C. acumentata[84] and R. serpentina[85] plants. In the former, cephaeline (135) and the phytosterol, β-sitosterol (136), were isolated and examined. It was found that, whereas acetate was specifically incorporated into the sterol, its activity was randomized whereas glycine-[2-^{14}C] was again reported to be specifically incorporated into the C_9 unit of cephaeline (135) but not at all into

the phytosterol. This result was taken to indicate that glycine and acetate "act as specific and exclusive precursor of different monoterpene moieties, in different compounds, in the same plant."

Since Thamnosma montana contained a large number of alkylated coumarins and the incorporation of mevalonic acid into these coumarins was very poor,[7] it was decided to study precursors other than mevalonic acid which could be utilized by plants in the biosynthesis of these alkyl groups. It was felt that if glycine-[2-^{14}C] can act as a specific precursor of the C_9 unit of cephaeline (135), which has been known to be mevalonate derived, it might also act as a specific precursor of the alkyl side chains found in many natural coumarins. Therefore, it was decided to study the role of glycine in the biosynthesis of these alkyl groups.

Thamnosma montana contains a large array of coumarins (see Introduction) but for the purpose of these biosynthetic investigations, three coumarins, umbelliprenin (9), alloimperatorin methyl ether (7) and ispimpinellin (2) were selected. Since umbelliprenin (9) contains a farnesyl-ether side chain and alloimperatorin methyl ether (7), a C_5-alkyl side chain, it was felt that these two coumarins offer an opportunity to study the role of glycine in the biogenesis of these alkyl groups. Isopimpinellin (2), a simple furanocoumarin with two methoxyl groups, was chosen to evaluate the role of glycine in the biosynthesis of the furan ring and the origin of these methoxyl groups in furanocoumarins.

However, before discussing the studies performed in this regard it is pertinent to discuss some preliminary work done to determine

if the biosynthesis of these coumarins was occurring at a regular and measurable basis. For this purpose young Thamnosma montana plants (about 2 to 3 years old), which had been grown from seeds, were selected and the precursor, D,L-phenylalanine-[3-^{14}C] was fed by the hydroponic method to the roots of these plants. After the preselected feeding time, the plants were mechanically ground to a coarse powder and extracted in a Soxhlet apparatus with acetone. The extract was concentrated and the residue was treated with hot chloroform. The chloroform soluble portion was chromatographed on an alumina column and the compounds were isolated by preparative layer chromatography, crystallized to constant activity and the radioactivity determined by the scintillation counting method. The results are given in Table 14.

TABLE 14. Incorporation of D,L-phenylalanine-[3-^{14}C] into Thamnosma montana.

Experiment no.	Feeding time (days)	Activity[a] fed (dpm)	Weight of the plant (g)	% Incorporation		
				umbelliprenin (9)	allimperatorin methyl ether (7)	isopimpinellin (2)
1	2	13.8×10^6	46.5	--	0.08	0.2

[a] Activity corrected for radioactivity isolated outside the plant.

Thus it is evident that young Thamnosma montana plants were biosynthesizing various coumarins isolated and that D,L-phenylalanine-[3-^{14}C] was being utilized with considerable efficiency. Similar experiments done in our laboratory[7] with young Thamnosma montana

plants indicated that the optimum time period for the biosynthesis of these coumarins was between 7 and 10 days.

Therefore, in the next series of experiments, attention was focussed on the role of glycine in the biosynthesis of these coumarins in Thamnosma montana. In these experiments, glycine-[2-^{14}C] was administered to young Thamnosma montana plants by the hydroponic method and the plants were allowed to grow for a period of 7 days. The plants were worked up in the normal manner and alloimperatorin methyl ether (7) was converted to the diol (3) for counting purposes. The results are presented in Table 15.

TABLE 15. Incorporation of glycine-[2-^{14}C] into Thamnosma montana.

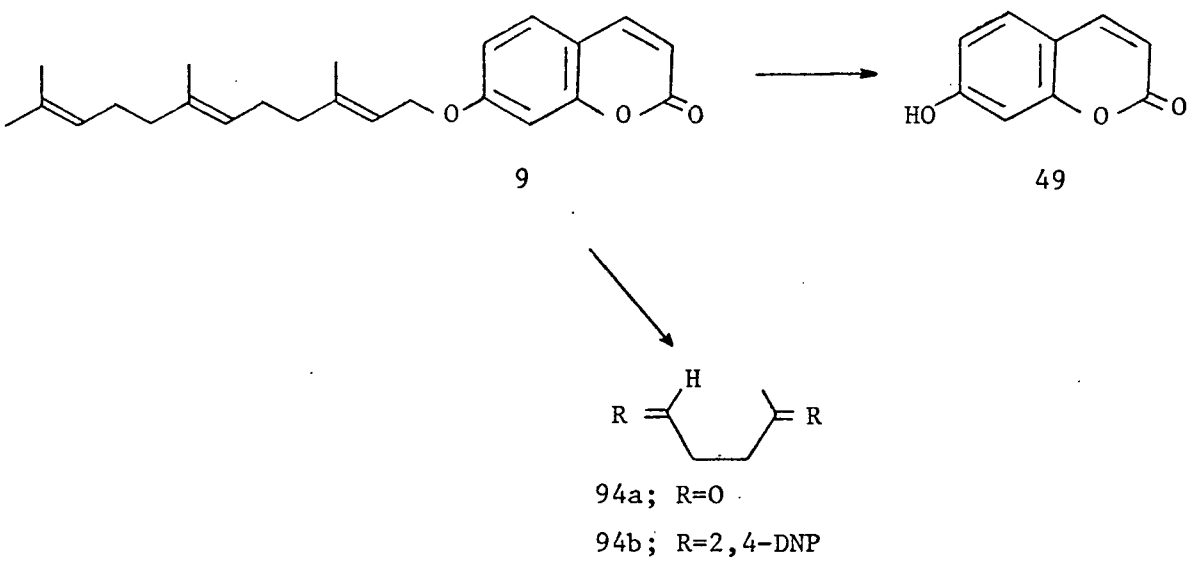
Experiment no.	Activity[a] fed (dpm)	Weight of plant (g)	% Incorporation		
			umbelliprenin (9)	alloimperatorin methyl ether (7)	isopimpinellin (2)
2	8.8×10^{7}	15	0.031	0.073	0.082
3	$2.53c10^{8}$	20	0.019	0.029	0.15
4	5.55×10^{8}	20	0.001	0.01	0.022

[a] Corrected for activity recovered outside the plant.

[b] Counted as alloimperatorin methyl ether diol (3).

Thus the results in Table 15 indicate that glycine-[2-^{14}C] is being incorporated into all three coumarins and the incorporation level is reasonable.

To determine the distribution of radioactivity in umbelliprenin (9), it was degraded according to the scheme previously described and as shown in Figure 22.



9

49

94a; R=O

94b; R=2,4-DNP

Figure 22. Degradation of radioactive umbelliprenin (9).

Thus umbelliprenin (9) (2.23×10^5 dpm/mmole) from experiment 2 was subjected to acid hydrolysis as previously described and umbelliferone (49) was isolated and shown to have a specific activity of 2.43×10^4 dpm/mmole or 10.9% of the original radioactivity of 9. In a similar experiment, umbelliprenin (9) (2.16×10^5 dpm/mmole) from experiment 3 was degraded and umbelliferone (49) (3.24×10^4 dpm/mmole) was shown to have 15% of the original activity of umbelliprenin (9). Thus it is clear that between 85-90% of the radioactivity of 9 resides in the farnesyl side chain and only 10-15% is present in the rest of the coumarin molecule.

It was also of interest to gain information as to the distribution of radioactivity present in the farnesyl side chain of umbelliprenin (9). To this end, umbelliprenin (9) (2.23×10^4 dpm/mmole) from experiment 3 was ozonized as previously described and levulinaldehyde bis-2,4-dinitrophenylhydrazone (94b) was isolated. This compound was shown to have a specific activity of 4.55×10^3 dpm/mmole or 20.4% of the original activity of 9. As two molar equivalents of levulinaldehyde (94a) should be produced in this reaction, this result indicates that only 41% of the radioactivity in the side chain resides in the internal ten carbon portion. Whether this represents an unequal labeling of the farnesol or reflects some error in the counting method is difficult to determine at this time as the lack of nonactive umbelliprenin (9) precludes further experimentation.

To determine the distribution of radioactivity, isopimpinellin (2) from experiment 2, 3 and 4 was degraded according to the scheme previously described and as depicted in Figure 23. The results are presented in Table 16.

From these results it is evident that more than 90% of the activity in isopimpinellin (2) resides in the two methoxyl groups and very little (less than 10%) activity is present in the rest of the molecule. The incorporation of glycine into the methoxyl groups of isopimpinellin (2) is explicable in terms of the degradation of glycine to a C_1-unit, a process observed previously. Byerrum and coworkers[86] showed that in plant systems, the C-2 of glycine could function as a C_1-unit as efficiently as the methyl groups of methionine and choline, and ten times as efficiently as formate whereas the carboxyl carbon of

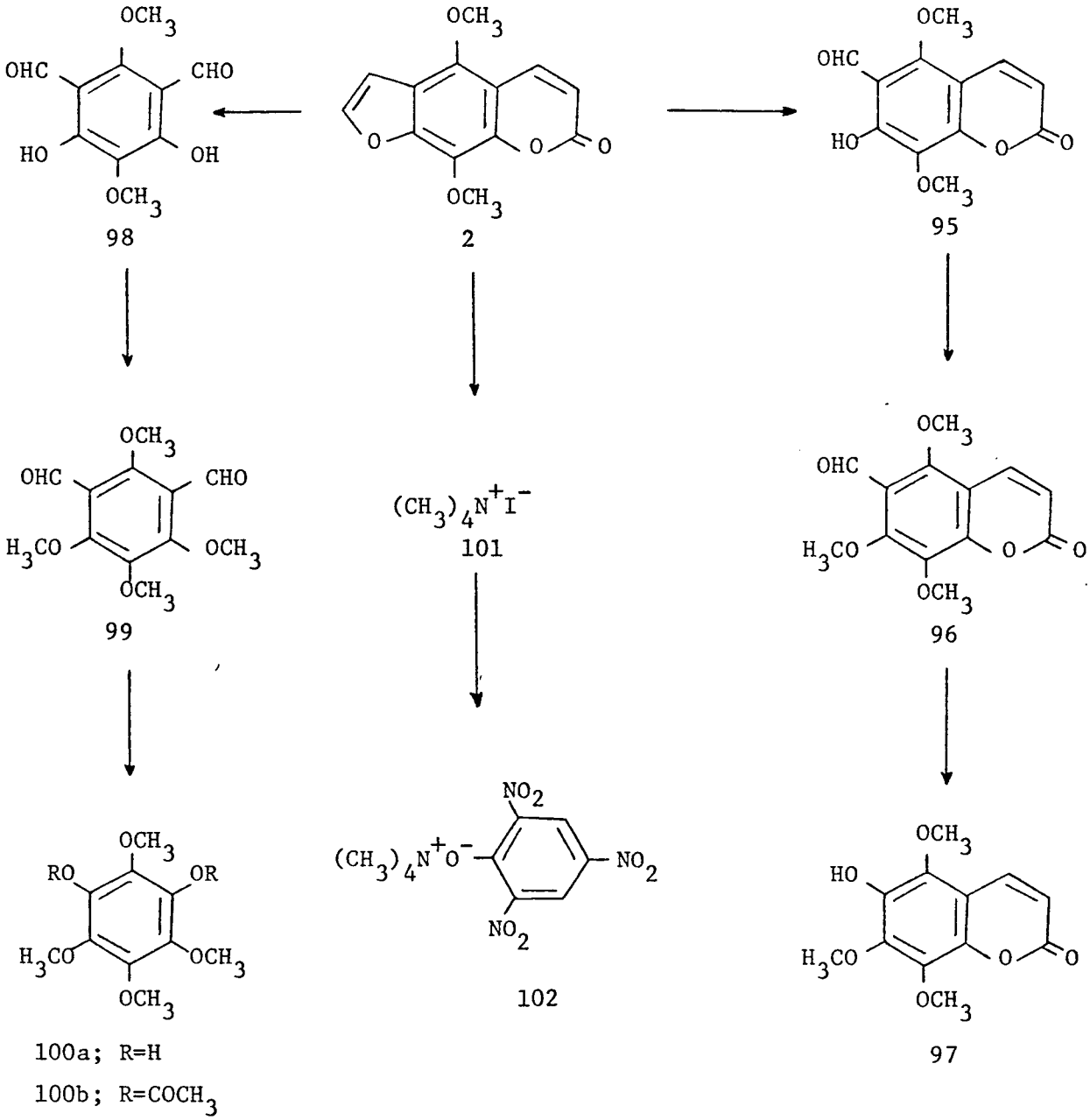


Figure 23. Degradation of radioactive isopimpinellin (2).

TABLE 16. Degradation of radioactive isopimpinellin (2) (Experiments 2, 3 and 4).

Compound	Specific activity (% total activity in isopimpinellin)[a]		
	Experiment 2	Experiment 3	Experiment 4
isopimpinellin (2)	3.208×10^5 (100%) dpm/mmole	2.80×10^4 (100%) dpm/mmole	1.00×10^5 (100%) dpm/mmole
6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95)	3.12×10^5 (97.5%)	2.57×10^4 (91.8%)	8.2×10^4 (82%)
6-formyl-5,7,8-trimethoxy-coumarin (96)	--	--	--
6-hydroxy-5,7,8-trimethoxy-coumarin (97)	2.91×10^5 (90.5%)	--	--
1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98)	3.03×10^5 (94.5%)	2.57×10^4 (91.8%)	9.27×10^4 (92.7%)
1,3-diformyl-2,4,5,6-tetra-methoxybenzene (99)	3.06×10^5 (95.5%)	--	--
1,3-diacetoxy-2,4,5,6-tetra-methoxybenzene (100b)	3.08×10^5 (96%)	--	9.17×10^4 (91.7%)
tetramethylammonium iodide (101)	$2 \times 1.44 \times 10^5$ (89.8%)	$2 \times 1.44 \times 10^5$ (100%)	$2 \times 4.75 \times 10^4$ (95%)
tetramethylammonium picrate (102)	$2 \times 1.38 \times 10^5$ (86%)	$2 \times 1.26 \times 10^5$ (90.5%)	--

[a] The total activity in isopimpinellin is set at 100%.

glycine showed no such activity. Our studies are consistent with these findings.

To determine the distribution of radioactivity in alloimperatorin methyl ether (7) from experiment 2, 3, and 4, it was degraded according to the scheme already described and as depicted in Figure 24. The results are given in Table 17.

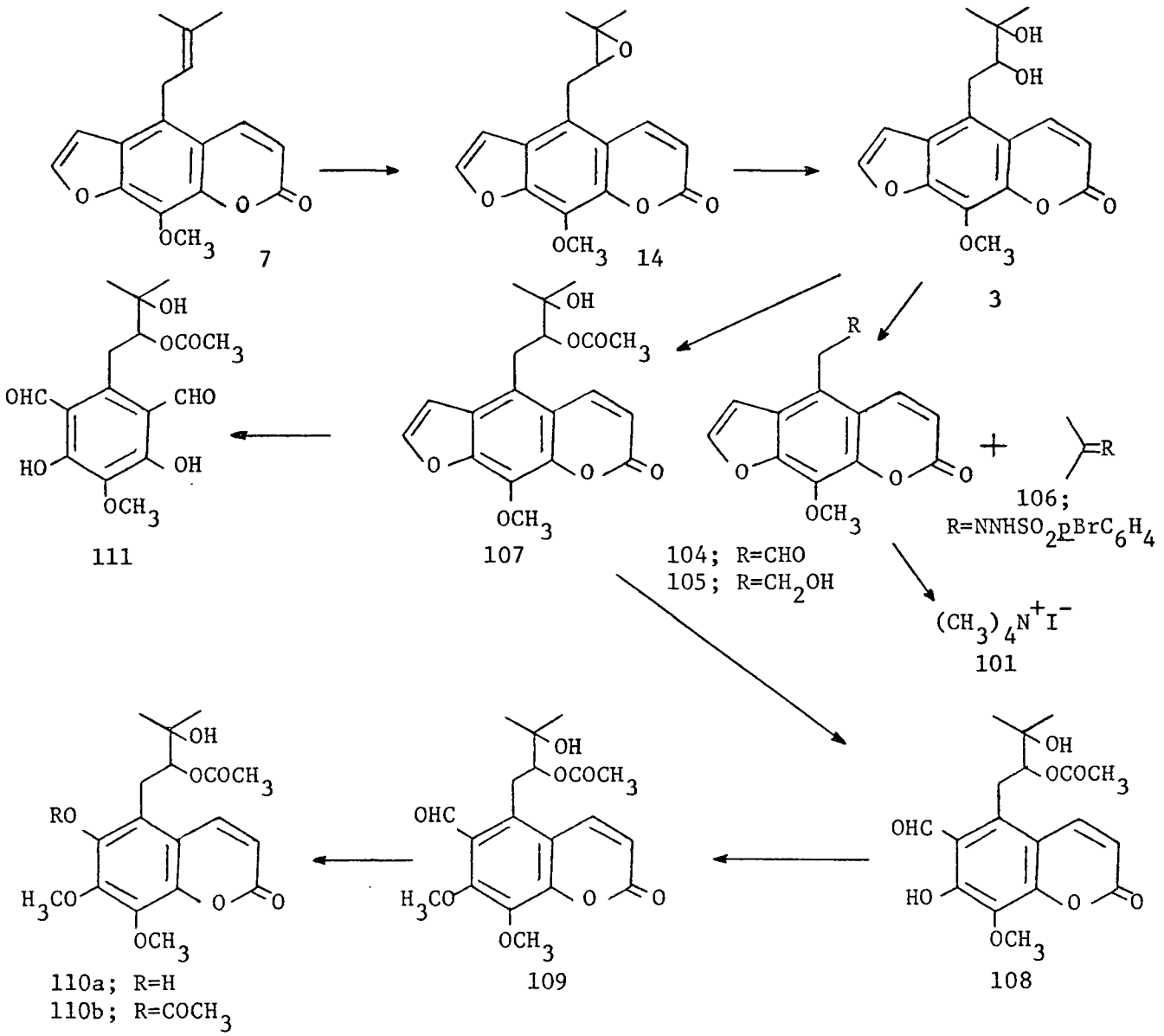


Figure 24. Degradations of radioactive alloimperatorin methyl ether (7).

TABLE 17. Degradations of radioactive alloimperatorin methyl ether (7).

Compound	Specific activity (% of alloimperatorin methyl ether)			
	Experiment 2	Experiment 3	Experiment 4	
			Trial a	Trial b
alloimperatorin methyl ether diol (3)	3.29×10^4 (100%) dpm/mmole	1.10×10^4 (100%) dpm/mmole	1.22×10^4 (100%) dpm/mmole	
5-(2'-hydroxyethyl)-8-methoxy-psoralen (105)	--	1.00×10^4 (91.5%)	1.11×10^4 (92%)	
acetone p-bromobenzenesulfonyl-hydrazone (106)	2.19×10^3 (6.7%)	5.72×10^2 (5.2%)	--	
tetramethylammonium iodide (101)	--	7.7×10^3 (70%)	1.12×10^4 (92%)	
tetramethylammonium picrate (102)	--	--	1.18×10^4 (97.5%)	
5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxypsoralen (107)	3.29×10^4 (100%)	1.10×10^4 (100%)	1.22×10^4 (100%)	4.85×10^4 (100%)
5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108)	2.91×10^4 (88.5%)	1.10×10^4 (100%)	1.18×10^4 (96.6%)	4.75×10^4 (97.5%)
5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-acetoxy-7,8-dimethoxycoumarin (110b)	--	1.06×10^4 (96.5%)	--	
1-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-2,6-diformyl-3,5-dihydroxy-4-methoxybenzene (111)	--	1.04×10^4 (94.5%)	--	

These results indicate that glycine-[2-^{14}C] is being incorporated into the dimethylallyl side chain of alloimperatorin methyl ether (7). Also it is clear from these results that most of the activity in 7 is present in the methoxyl groups. It is significant to note that very little activity could be found in the furanocoumarin portion of the molecule indicating that glycine-[2-^{14}C] is acting as a specific precursor of the C_1-pool and the C_5-alkyl side chain. This result is in contrast to the experiments with acetate-[2-^{14}C] done in our laboratory[7] with young Thamnosma montana plants. In these experiments, it was found that acetate-[2-^{14}C] was being incorporated into the furanocoumarin portion of the molecule as well as in the methoxyl groups and the alkyl side chain indicating a randomization of the activity.

EXPERIMENTAL (PART III)

For general experimental information, see page 59 and 87.

Thamnosma montana plants used in this study were collected in the summer as seeds and mature plants from the north-facing slopes of small hills in the vicinity of Joshua Tree National Monument, in the Mojave Desert area of Southern California. Some seeds could be propagated by Dr. P. Salisbury of our Department. Small Thamnosma montana plants (2-3 years old) were obtained from Molecular Biochemical Corporation, Tempe, Arizona.

Isolation of the Constituents of Young Thamnosma montana Plants

Whole Thamnosma montana plants (wet weight 15 g) which had been grown from seeds (∿ 16 months old) were ground in a Waring blender to a coarse powder and extracted with acetone (300 ml) in a Soxhlet extractor for 3 hours. The acetone extract was reduced to dryness under reduced pressure to yield a residue (0.6 g) which was treated with hot chloroform (100 ml). The chloroform soluble portion was filtered and the solvent was removed under reduced pressure to yield a residue (0.28 g) which was preadsorbed on alumina (neutral, 3 g, activity IV). The preadsorbed material was placed on top of a column of alumina (neutral, 27 g, activity IV) which had been made up in petroleum ether and elution was begun immediately. The fractions

eluted with petroleum ether and petroleum ether-benzene contained waxy material and were not examined further. Also eluted with petroleum ether-benzene was a fraction (20 mg) noted as a purple (uv) band on the column. Preparative layer chromatography on this fraction allowed the isolation of umbelliprenin (9) (10 mg), mp 62-63°. Also noted in this fraction was a yellow (uv) band corresponding to isoimperatorin (13), but this material was not isolated. Later fractions eluted with petroleum ether-benzene and benzene were noted (by tlc) to contain alloimperatorin methyl ether (7), thamnosmin (15) and phellopterin (12). Preparative layer chromatography on these combined (25 mg) allowed isolation of alloimperatorin methyl ether (7) (8 mg), mp 108-110°. The fractions eluted with benzene gave isopimpinellin (2) (9.4 mg) by preparative layer chromatography, mp 150-151°. Examination of other fractions by tlc suggested the presence of alloimperatorin methyl ether epoxide (14), bergapten (10b), xanthotoxin (11) and possibly psoralen (10a) but the quantity of these materials was small.

Feeding Experiment 1

In this experiment, D,L-phenylalanine-[3-^{14}C] (obtained from New England Nuclear Corp., Boston, Mass.) was administered to whole young Thamnosma montana plants (∿ 16 months old) by hydroponic methods. The precursor (2.22 x10^9 dpm, 37.4 mg) was dissolved in distilled water (25 ml) and an aliquot was removed, weighed and the radioactivity per unit weight of solution determined (9.11 x 10^6 dpm/g). Young plants were carefully uprooted so as to cause minimum damage to the fine rootlets and the roots were placed in the test tube containing precursor

in solution. Several plants (5 to 10) were used and the precursor solution was distributed equally among the test tubes to be used. After the plants had absorbed the precursor solution, the original container of the precursor solution was washed with distilled water and the plants were allowed to absorb the washings. The plants were left to grow under continuous fluorescent illumination. After the preselected time, the plants were worked up as previously described. The experimental details are given in Table 18. As necessary, the compounds isolated were diluted with the corresponding inactive compound to allow the necessary crystallizations to be performed.

TABLE 18. Incorporation of D,L-phenylalanine-[3-^{14}C] into young *Thamnosma montana* plants

Experiment no.	Feeding time (days)	Activity[a] fed (dpm)	Plant weight (g)	Specific activity [% incorporation]		
				umbelliprenin (9)	alloimperatorin methyl ether (7)	isopiminellin (2)
1	2	13.8x 10^6	46.5	--	4.629×10^5 [0.08] dpm/mmole	1.279×10^5 [0.2] dpm/mmole

[a] Corrected for activity isolated outside the plant.

Feeding Experiment 2, 3, and 4

In these experiments, glycine-[2-^{14}C] (obtained from New England, Nuclear Corp., Boston, Mass.) was administered to young whole plants (18-24 months old) by the hydroponic technique (feeding directly into the roots). The precursor, obtained as 0.1 N HCl solution, was used

TABLE 19a. Incorporation of glycine-[2-^{14}C] into Thamnosma montana

Experiment No.	Activity fed (dpm)	Specific activity fed (dpm/mmole)	Weight fed (mg)	Wet weight of plant (g)	Weight of compounds isolated (mg)		
					umbelliprenin (9)	alloimperatorin methyl ether (7)	isopimpinellin (2)
2	8.8×10^{7}	5.9×10^{10}	0.116	15	10	16	9.4
3	2.53×10^{8}	5.9×10^{10}	0.350	20	23.5	13.8	17.3
4	5.55×10^{8}	5.9×10^{10}	0.705	20	7.5	12.0	20

TABLE 19b.

Experiment no.	Specific activity isolated (dpm/mmole)			% Incorporation		
	umbelliprenin (9)	alloimperatorin methyl ether (7)	isopiminellin (2)	umbelliprenin (9)	alloimperatorin methyl ether (7)	isopimpinellin (2)
2	9.99×10^{5}	11.4×10^{5}	18.9×10^{5}	0.031	0.073	0.082
3	4.13×10^{5}	15.1×10^{5}	5.4×10^{6}	0.019	0.029	0.15
4	2.72×10^{5}	13.1×10^{5}	15.0×10^{5}	0.001	0.01	0.022

as such and the plants were allowed to grow for 7 days. After the solution was absorbed, the original vial containing the precursor was rinsed with distilled water (2 ml) and these washes were allowed to be absorbed by the plants. The procedure was continued throughout the course of the feedings. In each case, the plants were worked up as before and umbelliprenin (9), alloimperatorin methyl ether (7) and isopimpinellin (2) were isolated. In each case, alloimperatorin methyl ether (7) was converted to diol (3). The values quoted for 7 are based on the appropriate values obtained from 3, corrected for the yield of converting 7 to 3. Where necessary, dilutions were performed to provide sufficient samples for purification and degradations. The experimental details are presented in Table 19a and 19b.

Degradations of Umbelliprenin (9) from Experiments 2 and 3

Acid-catalyzed hydrolysis of umbelliprenin (9)

Umbelliprenin (9) (40 mg, 2.23×10^5 dpm/mmole) from Experiment 2 was hydrolyzed with acetic acid as described previously[7] and umbelliferone (49) (12 mg) was isolated and shown to have a specific activity of 2.43×10^4 dpm/mmole or 10.9% of the original activity of umbelliprenin (9).

Acid-catalyzed hydrolysis of umbelliprenin (9)

Radioactive umbelliprenin (9) (50 mg, 2.16×10^5 dpm/mmole) from Experiment 3 was degraded as described previously[7] and umbelliferone (49) (14 mg) was isolated and shown to have a specific activity of 3.24×10^4 dpm/mmole or 15% of the total activity of 9.

Ozonolysis of radioactive umbelliprenin (9)

Umbelliprenin (9) (25 mg, 2.23×10^4 dpm/mmole) from Experiment 3 was ozonized under optimum conditions as described previously[7] and levulinaldehyde bis-2,4-dinitrophenylhydrazone (94b) (20 mg) was isolated after crystallization. This material (94b) was counted in the following manner. The derivative (94b) (∿ 2 mg) was dissolved in the counting vial in a mixture of glacial acetic acid (10 drops), acetic anhydride (5 drops) and dimethylformamide (20 drops). The mixture was then heated to complete dissolution and zinc dust (∿ 50 mg) was added to decolourize the solution. Sodium metabisbulfite (100 mg) was added, then benzene (∿ 0.5 ml) and the solution was made up to 15 ml with organic scintillator solution. After standing in the cold and dark for 1 hour, the sample was counted. Due to the unorthodox counting solution employed, counting efficiency was determined by adding an accurately weighed sample of ^{14}C-hexadecane standard to the already counting sample and it was counted again. The ratio of the expected dpm to found cpm for hexadecane determined the counting efficiency (∿ 64%). In each case a blank sample containing an equal amount of inactive levulinaldehyde bis-2,4-DNP (94b) was counted first to determine the accurate background. In this manner, the radioactive levulinaldehyde bis-2,4-DNP (94b) was shown to have a specific activity of 4.55×10^3 dpm/mmole or 20.4% of the total activity of umbelliprenin (9).

Degradations of Isopimpinellin (2) from Experiment 2

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95)

Radioactive isopimpinellin (2) (50 mg, 3.208×10^5 dpm/mmole) was selectively ozonized as previously described[7] and 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95) (20 mg) was isolated. It was shown to have a specific activity of 3.12×10^5 dpm/mmole or 97.5% of the total activity of isopimpinellin (2).

Radioactive 6-formyl-5,7,8-trimethoxycoumarin (96)

6-Formyl-7-hydroxy-5,8-dimethoxycoumarin (95) (20 mg, 3.12×10^5 dpm/mmole) from the above reaction was methylated and 6-formyl-5,7,8-trimethoxycoumarin (96) (18 mg) was isolated by preparative layer chromatography. It was not counted but was used as such in the next reaction.

Radioactive 6-hydroxy-5,7,8-trimethoxycoumarin (97)

Radioactive 6-formyl-5,7,8-trimethoxycoumarin (96) (18 mg) from the previous reaction was treated with hydrogen peroxide and sulfuric acid and 6-hydroxy-5,7,8-trimethoxycoumarin (97) (10 mg) was isolated and shown to have a specific activity of 2.99×10^5 dpm/mmole or 90.5% of the total activity of isopimpinellin (2).

Radioactive 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98)

Isopimpinellin (2) (60 mg, 3.208×10^5 dpm/mmole) was ozonized as described previously[7] and crystalline 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98) (23 mg) was isolated and shown to have a specific

activity of 3.03×10^5 dpm/mmole or 94.5% of the total activity of isopimpinellin (2).

Radioactive 1,3-diformyl-2,4,5,6-tetramethoxybenzene (99)

Radioactive 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98) (23 mg, 3.03×10^5 dpm/mmole) from the above reaction was methylated and 1,3-diformyl-2,4,5,6-tetramethoxybenzene (99) (19 mg) was isolated. This was shown to have a specific activity of 3.06×10^5 dpm/mmole or 95.5% of the total activity of isopimpinellin (2).

Radioactive 1,3-diacetoxy-2,4,5,6-tetramethoxybenzene (100b)

1,3-Diformyl-2,4,5,6-tetramethoxybenzene (99) (19 mg, 3.06×10^5 dpm/mmole) from the previous reaction was degraded as described previously[7] and 1,3-diacetoxy-2,4,5,6-tetramethoxybenzene (100b) (12 mg) was isolated and shown to have a specific activity of 3.08×10^5 dpm/mmole or 96% of the total activity of isopimpinellin.

Radioactive tetramethylammonium iodide (101)

Radioactive isopimpinellin (2) (40 mg, 3.208×10^5 dpm/mmole) was demethylated with hydriodic acid and tetramethylammonium iodide (101) (48 mg) was isolated. It was counted as previously described (page 97) and was shown to have a specific activity of $2 \times 1.44 \times 10^5$ dpm/mmole or 89.8% of the total activity of isopimpinellin. Tetramethylammonium iodide (101) was converted to tetramethylammonium picrate (102) and this was counted as previously described (page 107) and was shown to have a specific activity of $2 \times 1.38 \times 10^5$ dpm/mmole or 86% of the total activity of isopimpinellin (2).

Degradations of Isopiminellin (2) from Experiment 3

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95)

Isopimpinellin (2) (50 mg, 2.80×10^4 dpm/mmole) from Experiment 3 was ozonized and 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95) (20 mg) was isolated and shown to have a specific activity of 2.57×10^4 dpm/mmole or 91.8% of the total activity of isopimpinellin (2).

Radioactive 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98)

Isopimpinellin (2) (43 mg, 2.80×10^4 dpm/mmole) was ozonized as described previously[7] and 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98) (8.5 mg) was isolated and shown to have a specific activity of 2.57×10^4 dpm/mmole or 91.8% of the total activity of isopimpinellin (2).

Radioactive tetramethylammonium iodide (101)

Isopimpinellin (2) (23 mg, 2.80×10^4 dpm/mmole) was demethylated and tetramethylammonium iodide (101) (25 mg) was isolated and shown to have a specific activity of $2 \times 1.44 \times 10^4$ dpm/mmole or all the original activity of isopimpinellin (2). Tetramethylammonium iodide (101) was converted to tetramethylammonium picrate (102) and this was shown to have a specific activity of $2 \times 1.26 \times 10^4$ dpm/mmole or 90% of the activity of isopimpinellin.

Degradations of Isopimpinellin (2) from Experiment 4

6-Formyl-7-hydroxy-5,8-dimethoxycoumarin (95)

Radioactive isopimpinellin (35 mg, 1.00×10^5 dpm/mmole) was selectively ozonized and 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (95)

(15 mg) was isolated and was shown to have a specific activity of 8.2×10^4 dpm/mmole or 82% of the total activity of isopimpinellin (2).

Radioactive 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98)

Isopimpinellin (55 mg, 1.00×10^5 dpm/mg) was ozonized as described previously[7] and 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98) (19.5 mg) was isolated and shown to have a specific activity of 9.27×10^4 dpm/mmole or 92.7% of the total activity of isopimpinellin (2).

Radioactive 1,3-diformyl-2,4,5,6-tetramethoxybenzene (99)

Radioactive 1,3-diformyl-4,6-dihydroxy-2,5-dimethoxybenzene (98) (19.5 mg, 9.27×10^4 dpm/mmole) from the previous reaction was methylated and 1,3-diformyl-2,4,5,6-tetramethoxybenzene (99) (16 mg) was isolated. It was not counted but was used as such in the next reaction.

Radioactive 1,3-diacetoxy-2,4,5,6-tetramethoxybenzene (100b)

1,3-Diformyl-2,4,5,6-tetramethoxybenzene (99) (16 mg) from the previous reaction was degraded to 1,3-diacetoxy-2,4,5,6-tetramethoxybenzene (100b) (10 mg). This was shown to have a specific activity of 9.17×10^4 dpm/mmole or 91.7% of the total activity of 2.

Radioactive tetramethylammonium iodide (101)

Isopimpinellin (2) (25 mg, 1.00×10^5 dpm/mmole) from Experiment 4 was demethylated and tetramethylammonium iodide (101) (28 mg) was isolated and shown to have a specific activity of $2 \times 4.75 \times 10^4$ dpm/mmole or 95% of the total activity of isopimpinellin.

Degradations of Alloimperatorin Methyl Ether (7) from Experiment 2

Periodic acid cleavage of 5-(2',3'-dihydroxy-3'-methylbutyl)-8-methoxypsoralen (3) (alloimperatorin methyl ether diol)

Radioactive alloimperatorin methyl ether diol (3) (67.5 mg, 3.29×10^4 dpm/mmole) from Experiment 2 was cleaved with periodic acid and acetone p-bromobenzenesulfonylhydrazone (106) (41 mg) was isolated and shown to have a specific activity of 2.19×10^3 dpm/mmole or 6.7% of the total activity of 3.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxypsoralen (107)

Alloimperatorin methyl ether diol (3) from Experiment 2 was acetylated to 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxypsoralen (107).

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108)

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methylpsoralen (107) (56 mg, 3.29×10^4 dpm/mmole) was selectively ozonized to 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methyoxycoumarin (108) (36 mg). This was not counted but was used as such in the next reaction.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7,8-dimethoxycoumarin (109)

5-(2'-Acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108) (36 mg) from the previous reaction was methylated

to give 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7,8-dimethoxycoumarin (109) (15 mg). This was shown to have a specific activity of 2.91×10^4 dpm/mmole or 88.5% of the total activity of 107.

Degradations of Alloimperatorin Methyl Ether (7) from Experiment 3

Periodic acid cleavage of alloimperatorin methyl ether diol (3)

Radioactive alloimperatorin methyl ether diol (3) (55 mg, 1.10×10^4 dpm/mmole was cleaved with periodic acid as previously described[7] and 5-(2'-hydroxyethyl)-8-methoxypsoralen (105) (25.8 mg) was isolated and was shown to have a specific activity of 1.00×10^4 dpm/mmole or 91.5% of the total activity of 3. Also isolated was acetone p-bromobenzenesulfonylhydrazone (106) (11 mg). This was shown to have a specific activity of 5.72×10^2 dpm/mmole or 5.2% of the total activity of 3.

Demethylation of radioactive 5-(2'-hydroxyethyl)-8-methoxy psoralen (105)

Radioactive 5-(2'-hydroxyethyl)-8-methoxypsoralen (105) (23 mg, 1.00×10^4 dpm/mmole) from the previous reaction was demethylated and tetramethylammonium iodide (101) (10 mg) was isolated and was shown to have a specific activity of 7.70×10^3 dpm/mmole or 70% of the total activity of 3.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxy-psoralen (107)

Radioactive alloimperatorin methyl ether diol (3) from Experiment 3 was acetylated to 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-

methoxypsoralen (107).

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108)

Radioactive 107 (67 mg, 1.10×10^4 dpm/mmole) was selectively ozonized and 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108) (32 mg) was isolated. This was shown to have a specific activity of 1.10×10^4 dpm/mmole or all the activity of 107.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7,8-dimethoxycoumarin (109)

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108) (32 mg, 1.10×10^4 dpm/mmole) from the previous reaction was methylated and 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7,8-dimethoxycoumarin (109) (18.5 mg) was isolated. This was not counted but was used as such for the next reaction.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-acetoxy-7,8-dimethoxycoumarin (110b)

Radioactive 109 (18.5 mg) from the above reaction was degraded and 110b (8.3 mg) was isolated and shown to have a specific activity of 1.06×10^4 dpm/mmole or 96.4% of the total activity of 107.

Radioactive 1-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-2,6-diformyl-3,5-dihydroxy-4-methoxybenzene (111)

Radioactive 107 (50 mg, 1.10×10^4 dpm/mmole) from Experiment 3 was ozonized as described previously[7] and 111 (15 mg) was isolated. This was shown to have a specific activity of 1.04×10^4 dpm/mmole or 99.5% ofthe total activity of 107.

Degradations of Alloimperatorin Methyl Ether (7) from Experiment 4

Periodic acid cleavage of alloimperatorin methyl ether diol (3)

Radioactive alloimperatorin methyl ether diol (3) (50 mg, 1.22×10^4 dpm/mmole) from Experiment 4 was cleaved with periodic acid and 5-(2'-hydroxyethyl)-8-methoxypsoralen (105) (32 mg) was isolated and shown to have a specific activity of 1.11×10^4 dpm/mmole or 92% of the total activity of 3.

Demethylation of radioactive 5-(2'-hydroxyethyl)-8-methoxypsoralen (105)

Radioactive 5-(2'-hydroxyethyl)-8-methoxypsoralen (105) (20 mg, 1.11×10^4 dpm/mmole) from the previous reaction was demethylated and tetramethylammonium iodide (101) (12 mg) was isolated and was shown to have a specific activity of 1.12×10^4 dpm/mmole or 92% of the original activity of 3. Tetramethylammonium iodide (101) was converted to tetramethylammonium picrate (102) which was shown to have a specific activity of 1.18×10^4 dpm/mmole or 96.6% of the total activity of 3.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108). Trial a

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxypsoralen (107) (60 mg, 1.22×10^4 dpm/mmole) was selectively ozonized and 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108) (25 mg) was isolated and was shown to have a specific activity of 1.18×10^4 dpm/mmole or 96.6% of the total activity of 107.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108). Trial b

Radioactive 107 (60 mg, 4.85×10^4 dpm/mmole) from Experiment 4 was selectively ozonized and 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (108) (15 mg) was isolated and shown to have a specific activity of 4.73×10^4 dpm/mmole or 97.5% of the total activity of 107.

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