

STUDIES RELATED TO NATURAL PRODUCTS:

BIOSYNTHESIS OF COUMARINS

BY

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B.Sc. Honours, University of Alberta, 1967

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE  
REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the

DEPARTMENT OF CHEMISTRY

We accept this thesis as conforming to the required  
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THE UNIVERSITY OF BRITISH COLUMBIA

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ABSTRACT

This thesis describes a biosynthetic investigation of coumarins in turpentine-broom, Thamnosma montana Torr. and Frem.

In contrast to simple coumarins, the biosynthetic pathways leading to the furanocoumarins were found to be in a state of confusion as indicated by existing published data. None of the results were internally consistent with any general postulate and there was considerable question concerning the actual meaning of a substantial portion of the experimental data.

Studies as described here were performed in several different areas. In the initial investigations, a detailed study of isolation procedures for the many coumarins present in the plant was necessary. Subsequently, appropriate chemical degradative pathways were developed for umbelliprenin (11), isopimpinellin (2), and alloimperatorin methyl ether (8) to allow isolation of relevant carbon atoms in these coumarins. Finally, incorporation studies with DL-mevalonic-5-<sup>3</sup>H acid were conducted and subsequent degradative reactions were performed on umbelliprenin (11) and alloimperatorin methyl ether (8). The implications of these preliminary experiments in terms of biosynthetic pathways are presented.

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#### ACKNOWLEDGEMENTS

I wish to express my gratitude to Professor James P. Kutney for his encouragement and guidance throughout the course of this research.

I am also extremely grateful to R.N. Young and A.K. Verma for their collaboration and patience with me in this research and for many helpful suggestions.

Special thanks is due also to Miss Diane Johnson for typing the manuscript.

BIOSYNTHESIS OF COUMARINS

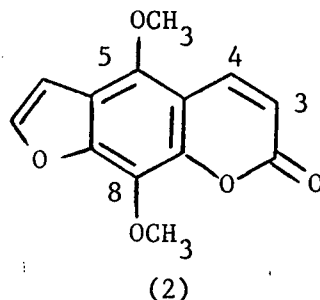
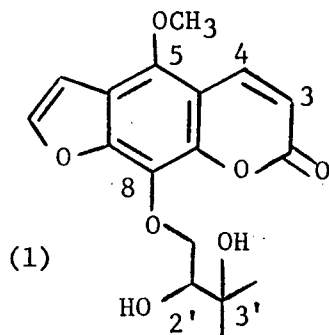


## INTRODUCTION

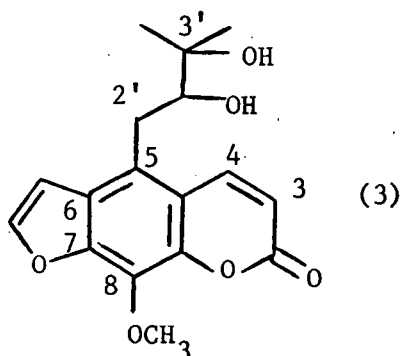
The turpentine-broom, Thamnosma montana Torr. and Frem. (Rutaceae), is found in desert mesas and slopes. These shrubby plants were reported to have plant-growth-inhibitor properties.<sup>1,2</sup> In addition, they were reported to have been used by American Indians in folk medicine.<sup>3</sup>

Bennett and Bonner studied the toxicity of aqueous extracts of leaves of eleven desert plant species and found that Thamnosma montana Torr. and Frem. was the most toxic as judged by the response to tomato.<sup>1</sup> The crude material caused the death of young tomato plants at a concentration of about 1 mg/ml within seven days.

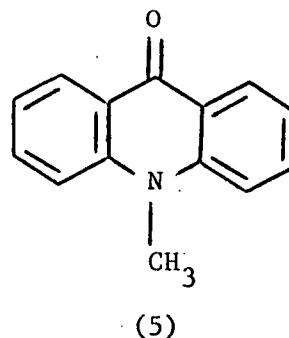
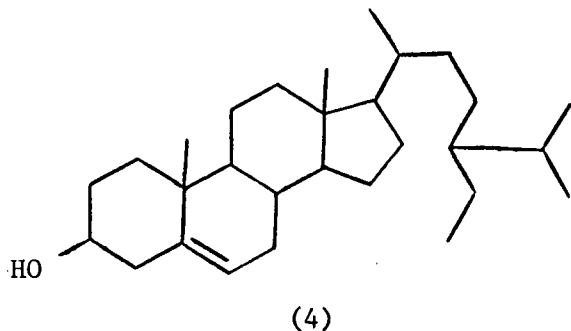
The plants of the Rutaceae family are well known to contain a large number of benzenoid compounds, coumarins, flavones and some quinoline alkaloids. Bennett and Bonner isolated three crystalline compounds from Thamnosma montana and identified two of them as byakangelicin(1) and isopimpinellin (2), respectively.<sup>1</sup>

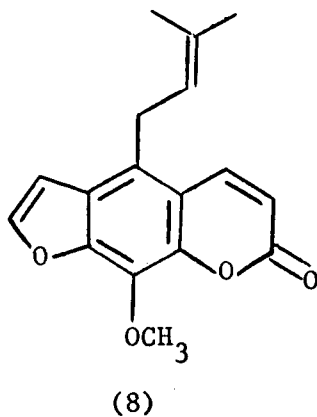
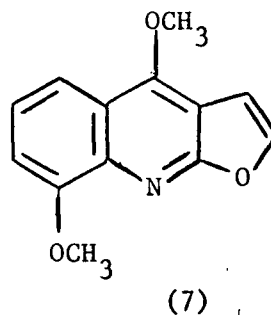
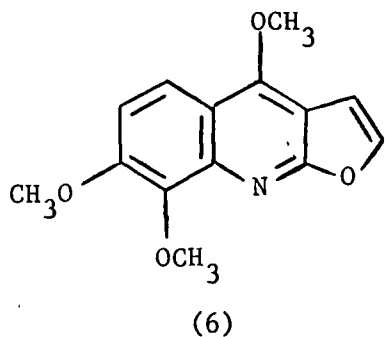


The structure of the third compound was elucidated by Dreyer<sup>4</sup> and found to be alloimperatorin methyl ether diol (3) [5-(3'-methyl-2',3'-dihydroxybutanyl)-8-methoxypsoralen].

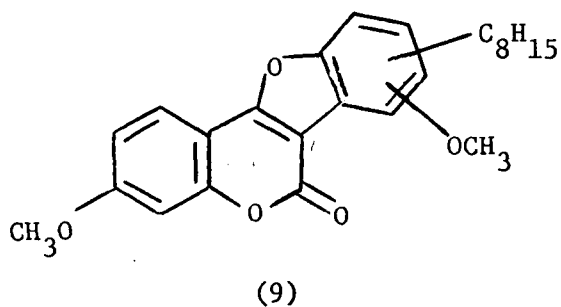


Dreyer developed a better extraction scheme for isolating not only the three compounds obtained by Bennett and Bonner, but was able to separate six other compounds. These six compounds were  $\beta$ -sitosterol (4); three known alkaloids, N-methylacridone (5), skimmianine (6), and  $\gamma$ -fagarine (7); a known furanocoumarin alloimperatorin methyl ether (8); and an unknown compound, thamnosin. This was the first report of N-methylacridone, the parent member of acridone alkaloids,<sup>5</sup> occurring as a natural product.

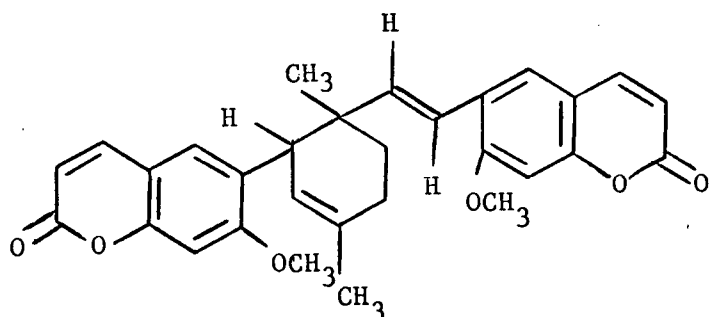




Dreyer's proposal for the structure of thamnosin (9) was the following.<sup>6</sup>

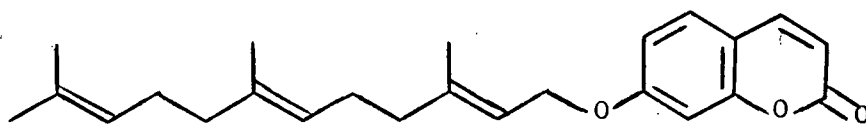


However, recent work in our laboratories by T. Inaba has proved this tentative structure to be incorrect. Furthermore, Inaba<sup>7</sup> as a result of a complete elucidation of the structure of thamnosin, assigned the following structure to the compound.

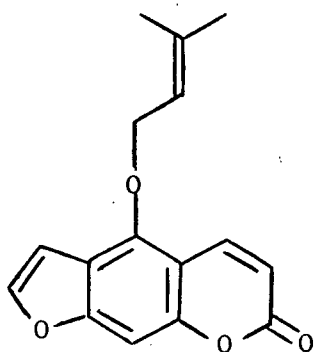


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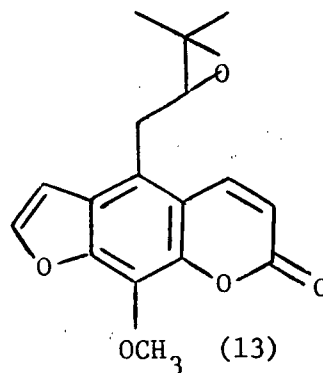
As a result of further recent work in our laboratories on Thamnosma montana, eight other compounds were separated and characterized in addition to the nine previously isolated by the workers mentioned above. These compounds were umbelliprenin (11), isoimperatorin (12), alloimperatorin methyl ether epoxide (13), thamnosmin (14), bergapten (15), xanthotoxin (16), psoralene (17), and phellopterin (18).



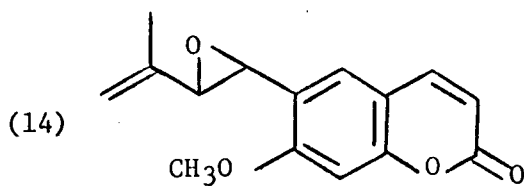
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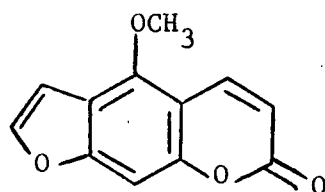
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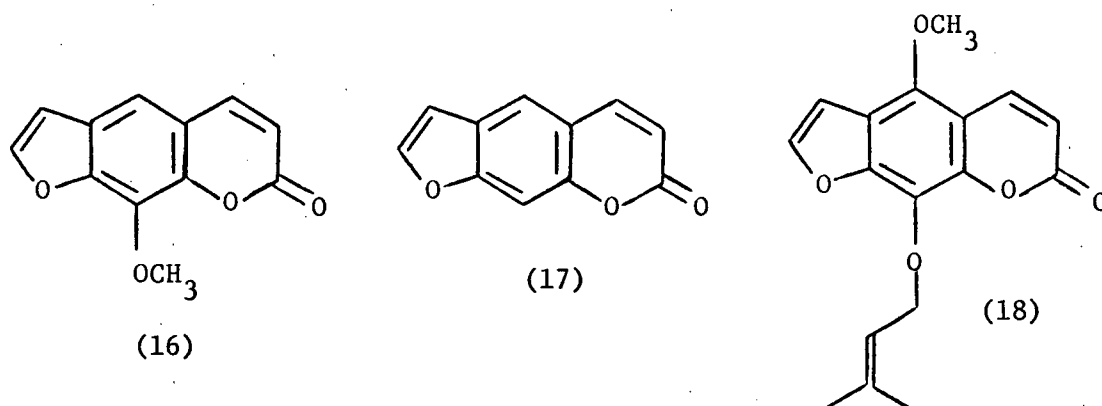
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(14)



(15)



Following the isolation and identification of the numerous compounds in the plant, it was decided to conduct some biosynthetic work on some of the coumarins. This section of the thesis is concerned with some of the biosynthetic work done on some of the coumarins in Thamnosma montana. It is therefore appropriate to discuss the status of biosynthesis in this area.

#### Biosynthesis of Coumarins

The coumarins form a diversified and quite widely distributed class of naturally occurring aromatic compounds. Coumarin itself is the simplest member of this class. Other compounds in this class have substituents of varying complexity, ranging from simple hydroxyl or methoxyl groups to isoprenoid side chains and isoprenoid-derived rings. A striking point about the substituted coumarins is that with only a very few known exceptions, the benzene ring of the coumarin nucleus is oxygenated para to the position of the side chain attachment, that is, at position 7. Figure 1 illustrates some representative naturally occurring coumarins, bearing a variety of different functions

and substitution patterns.

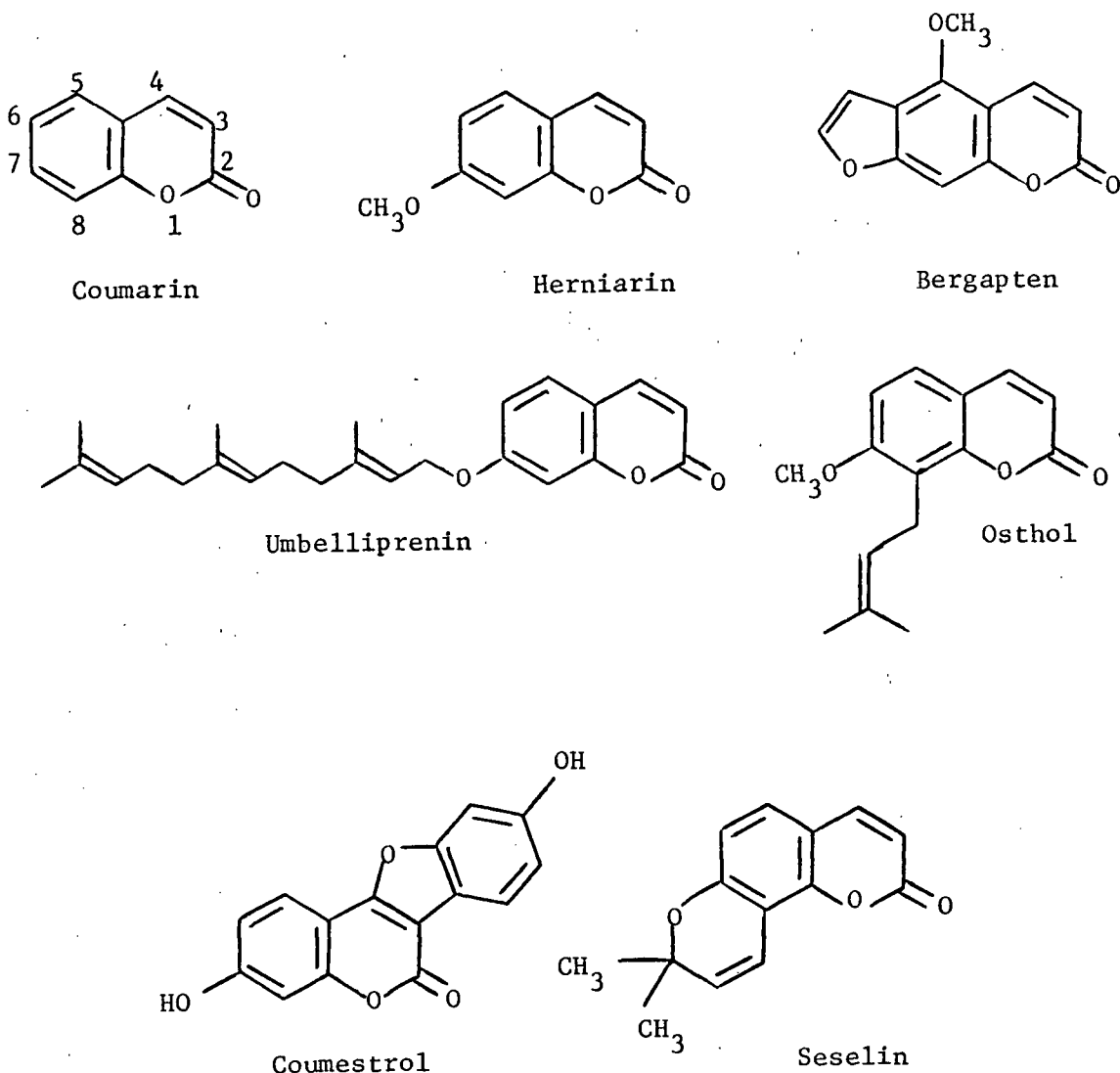
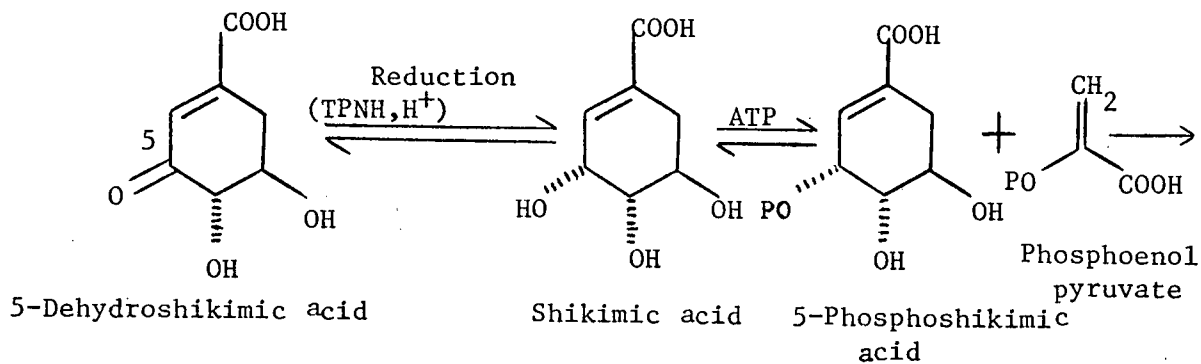
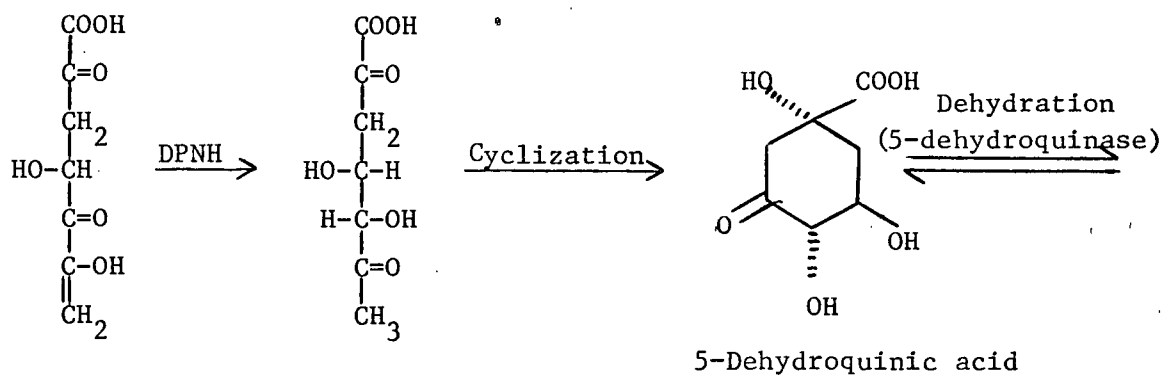
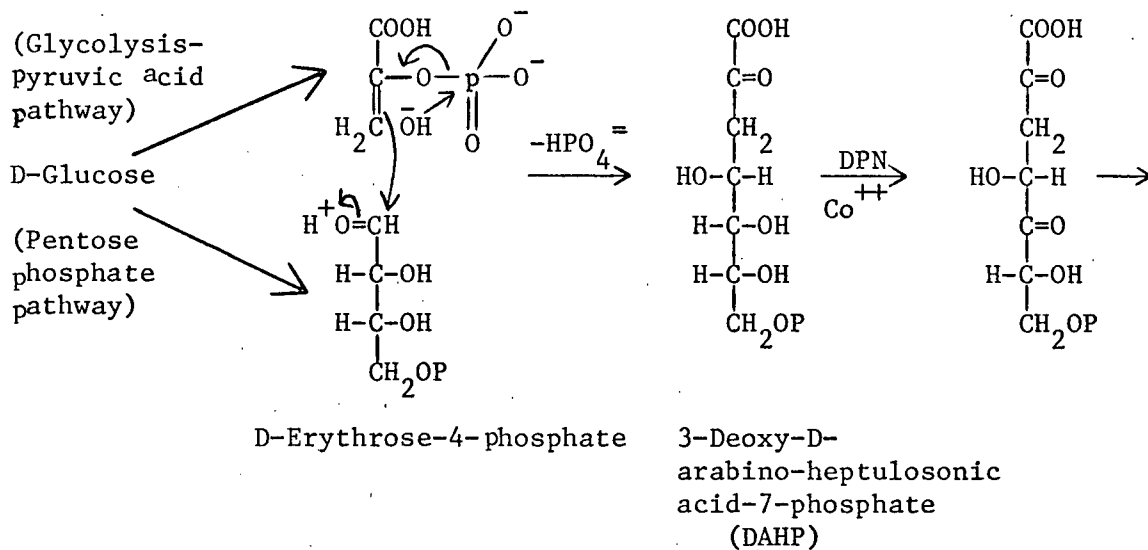


Figure 1. Some representative naturally occurring coumarins.

Before commencing with the biosynthesis of coumarins, a discussion of the shikimic acid pathway to aromatic compounds (Figure 2) is appropriate.<sup>8</sup>



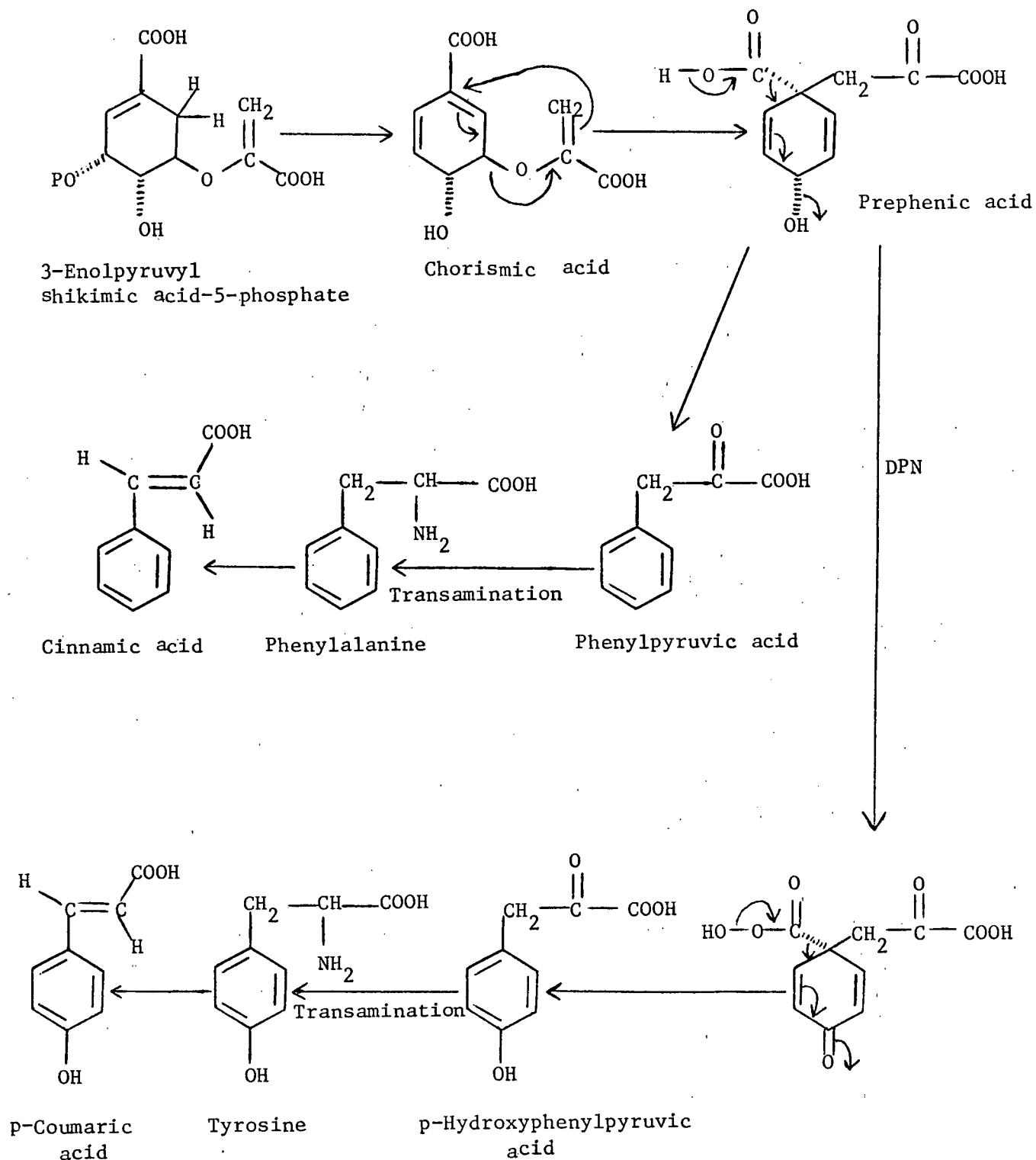


Figure 2. The shikimic acid pathway to aromatic compounds.



Phosphoenolpyruvic acid and D-erythrose-4-phosphate are independent intermediates in the metabolic pathways of D-glucose. The reaction of the two can be formulated as a concerted reaction between two phosphate esters leading to the formation of orthophosphate and 3-deoxy-D-arabino-heptulosonic acid-7-phosphate (DAHP). The D-arabino configuration is favored since, in the assumed cyclization of DAHP to 5-dehydroquinic acid, the hydroxyl groups on C-4 and C-5 of DAHP would correspond to the identical configuration of C-3 and C-4 of 5-dehydroquinic acid.

The enzymic condensation of phosphoenolpyruvic acid and D-erythrose-4-phosphate is formulated as an attack by a nucleophilic group of the enzyme, symbolized here by  $\text{OH}^-$ , on the phosphoenolpyruvic acid. This process is concerted with attack by carbon 3 of the phosphoenolpyruvic acid on the electrophilic carbon atom of the aldehyde and protonation of the carbonyl oxygen atom by an acidic group. The overall result is the release of orthophosphate or of a transient, phosphorylated enzyme and the open chain form of DAHP. In the limited sense that this reaction may be viewed as an attack by a nucleophilic species on an aldehyde, it resembles the chemical aldol condensation and the enzymic aldolase condensation.

In order to convert DAHP to 5-dehydroquinic acid, the hydroxyl group on C-5 of DAHP is first oxidized by diphosphopyridine nucleotide (DPN), to facilitate the elimination of phosphate in the next step. The carbonyl group on C-5 is then reduced by reduced DPN formed in the first reaction to a hydroxyl group having the original configuration. Finally, the resulting 2,6-diketone is cyclized to 5-dehydroquinic acid. All these reactions are catalyzed by one enzyme or enzyme complex and

are probably concerted.

To convert 5-dehydroquinic acid to shikimic acid, the former compound is first dehydrated to 5-dehydroshikimic acid. The enzyme responsible for this was 5-dehydroquinase which was highly specific for this reaction as it was unable to dehydrate the closely related quinic acid to shikimic acid. The reduction of 5-dehydroshikimic acid to shikimic acid by partially purified 5-dehydroshikimate reductase has also been studied. A cofactor requirement for the reduction is triphosphopyridine nucleotide (TPNH).

To convert shikimic acid to prephenic acid, 5-phosphoshikimic acid is first formed by phosphate transfer from adenosine-5-triphosphate (ATP) to shikimic acid. This then reacts with enol pyruvate phosphate to give the 3-enol pyruvate ether of phosphoshikimic acid. The prephenic acid is formed by the attack of nucleophilic enolpyruvate on C-1 of the ring associated with the phosphate leaving group in a trans manner. With external nucleophilic reagents, such reactions are known to require cis stereochemistry. No exact analogies appear to be known for the type of internal rearrangement postulated for the conversion of enol pyruvate to prephenic acid.

Aromatic compounds are formed from prephenic acid by decarboxylation and reductive decarboxylation which yields the immediate precursors of tyrosine and phenylalanine, respectively. The latter two are finally formed by transamination and can undergo conversion to cinnamic acid and p-coumaric acid, respectively. Both the acid catalyzed and the enzymic aromatization of prephenic acid may be visualized as initiated by electrophilic attack on the hydroxyl group. Prephenic acid has also been shown to be converted by enzymes to

p-hydroxyphenylpyruvic acid. Diphosphopyridine nucleotide (DPN) is required for this reaction, suggesting oxidation of C-4 followed by decarboxylation.

#### Biosynthesis of Coumarin:

Evidence from feeding experiments showed that coumarin was synthesized from shikimic acid-derived phenylpropane precursors in preference to acetate condensation.<sup>9,10,11</sup>

Brown, Towers, and Wright<sup>9</sup> studied the biosynthesis of coumarin in Hierochloe odorata and Melilotus officinalis. A number of possible precursors were fed. From this the best precursors of coumarin were found to be o-coumaric acid and cinnamic acid while shikimic acid and L-phenylalanine were found to be slightly less efficient as precursors whereas acetic acid, salicylic acid, o-tyrosine, ferulic acid, and melilotic acid were found to be very poor precursors. This work indicated approximately the same results as Kosuge and Conn's feeding experiments in sweet clover, Melilotus alba<sup>10</sup>. The results obtained by Brown, Towers, and Wright suggested that the shikimic acid-phenylpropanoid acid pathway predominates in coumarin biosynthesis in H. odorata. Furthermore, the relative efficient utilization of cinnamic acid and o-coumaric acid implied that the former undergoes hydroxylation ortho to the side chain during the process.

The effect of varying metabolic periods on the radioactivity of several phenolic constituents of H. odorata including coumarin and structurally related compounds was also studied by Brown, Towers, and Wright<sup>9</sup> after the administration of cinnamic acid-3-<sup>14</sup>C. The radio-

activity of the following compounds isolated from the plant was of particular interest (Figure 3). Changes in specific activity of coumarin

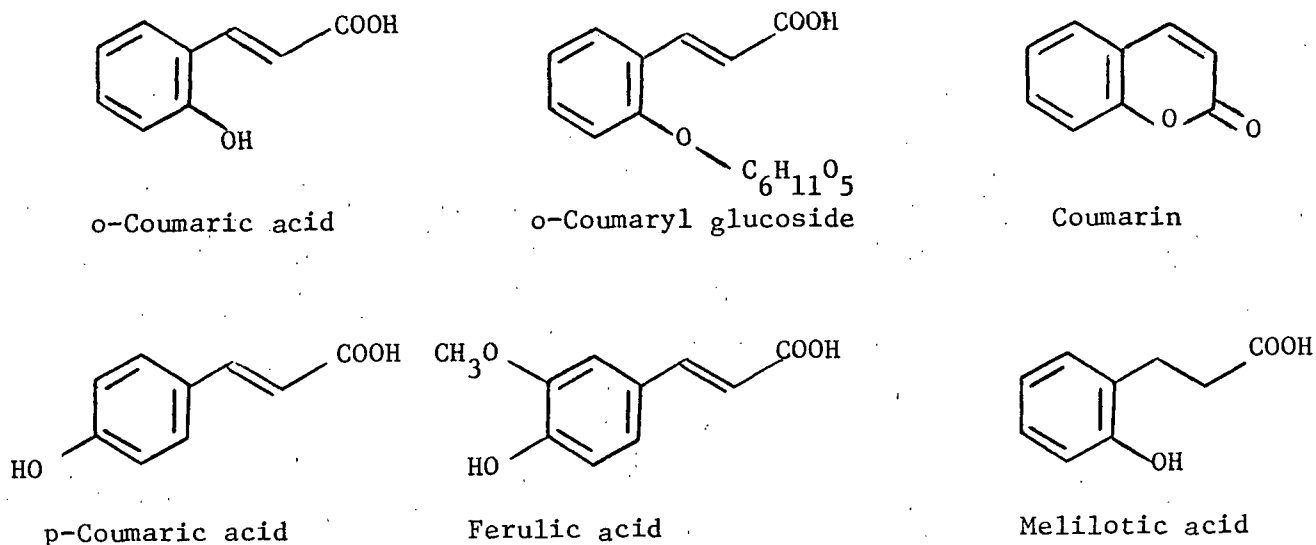


Figure 3. Radioactive compounds isolated from H. odorata.

with time after feeding cinnamic acid-3-<sup>14</sup>C to Hierochloe odorata indicated a definite lag phase persisting until 8 hours from the complete absorption of the precursor. From that time until about 24 hours, there was a linear increase in specific activity. Changes of specific activity of o-coumaric acid, p-coumaric acid, ferulic acid, melilotic acid, and o-coumaryl glucoside with time from the same experiment indicated that all four free acids exhibited a maximum in specific activity at or before 16 hours, and that all but melilotic acid attained a relatively high specific activity in 0 to 4 hours. The slow and slight accumulation of <sup>14</sup>C in melilotic acid again suggested it to be a secondary metabolic product of cinnamic acid.

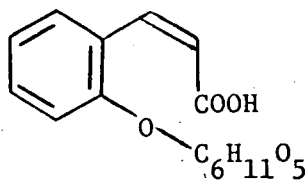
A clearer picture of the distribution of the active carbon was obtained from an examination of the total activity in the various constituents throughout the period of the experiment. From this, it was clear that the largest reservoir of o-hydroxylated phenyl propanoid material throughout the experiment was o-coumaryl glucoside. It was found that the  $^{14}\text{C}$  content of the glucoside was even higher shortly after feeding than at 4 hours, indicating that cinnamic acid administered to H. odorata undergoes rapid ortho hydroxylation and that glucoside formation also occurs rapidly, with a large accumulation of  $^{14}\text{C}$  in o-coumaryl glucoside even while absorption of the precursor is in progress.

In order to determine whether o-coumaryl glucoside is an intermediate in coumarin biosynthesis a sample of radioactive o-coumaryl glucoside and cinnamic acid-3- $^{14}\text{C}$  were fed to H. odorata. The  $^{14}\text{C}$  dilution during conversion of cinnamic acid to coumarin was 315 and that of o-coumaryl glucoside was 435, indicating the two compounds to be comparable in efficiency as precursors of coumarin.

Brown<sup>12</sup> has also shown that p-coumaric acid was seventy times less effective than cinnamic acid as a precursor of coumarin in Hierochloe odorata, while tyrosine was sixty times less effective than phenylalanine. From this it was clear that a preformed phenolic nucleus posed a decided disadvantage in its use as a precursor.

In later studies on the biosynthesis of coumarin in H. odorata, Brown<sup>13</sup> fed  $^{14}\text{CO}_2$  and showed that coumarin exists in plant cells as a glucoside of o-coumarinic acid (19) and can be recovered by emulsin hydrolysis. A study of the incorporation of  $^{14}\text{C}$  from  $\text{CO}_2$  into coumarin

and the aglycone of o-coumaryl glucoside with time was subsequently made. The results indicated that the total activity of coumarin showed



(19)

a slow, almost uninterrupted increase until 17 days after which the total <sup>14</sup>C declined markedly. The maximum activity of the aglycone, however, occurred at about 4 days and was followed by a marked decrease in activity.

In addition to showing the presence of the cis-glucoside of coumarinic acid in H. odorata, the results of Brown also suggested that o-coumaryl glucoside was a metabolic intermediate rather than an end product, since the peak in its total activity is reached earlier than that of coumarin. The demonstration of the presence of coumarinic acid glucoside suggested that glucoside formation was necessary to effect trans-cis inversion which must precede the formation of the coumarin lactone ring. The cis-glucoside would be an intermediate in such a pathway. Also the incorporation of labelled o-coumaryl glucoside to coumarin, supported the belief that o-hydroxylation was an essential feature of coumarin biosynthesis. Furthermore, coumarin like o-coumaryl glucoside was not an end product, but underwent relatively slow metabolism as revealed by the eventual decline in <sup>14</sup>C in the experiments.

Therefore, Brown<sup>14</sup> proposed the following scheme for coumarin biosynthesis in Melilotus and H. odorata (Figure 4).

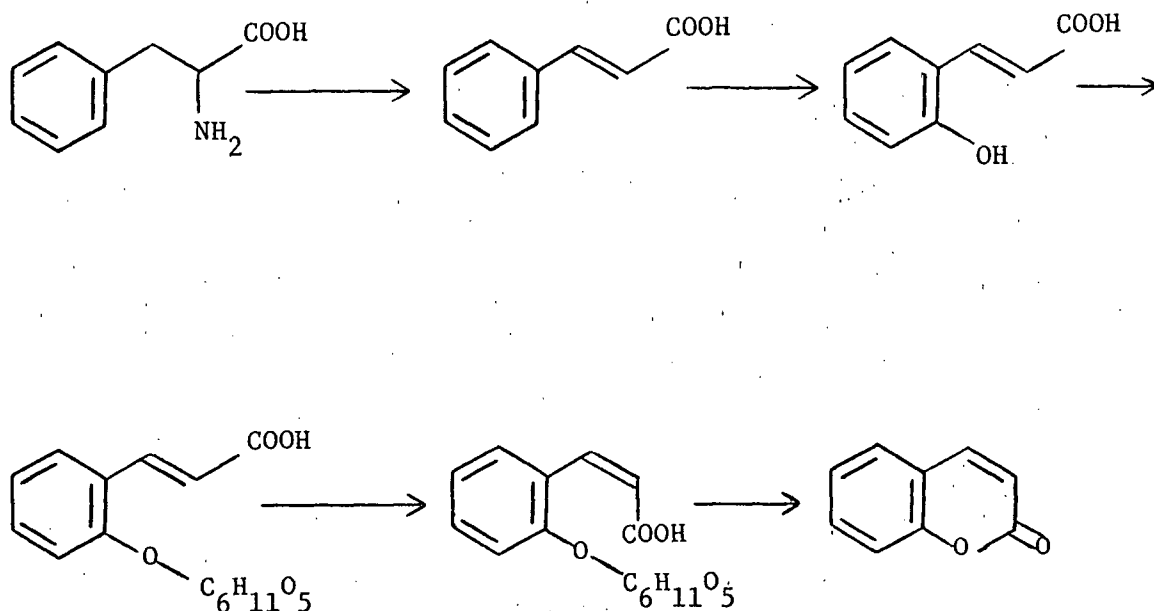
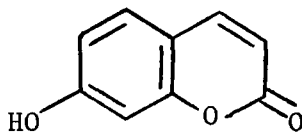


Figure 4. Proposed scheme for coumarin biosynthesis in Melilotus and H. odorata.

Results by Kosuge and Conn<sup>15</sup>, Stoker and Bellis<sup>16</sup>, and Gorz and Haskins<sup>17</sup> also independently showed that the conversion of trans-cinnamic acid to coumarin in Melilotus alba occurred by a similar scheme. Furthermore, this plant was shown to contain a trans-cis isomerase enzyme system<sup>18</sup>.

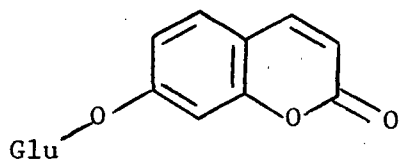
# Biosynthesis of 7-Oxygenated Coumarins:

## A. Umbelliferone (7-hydroxycoumarin)



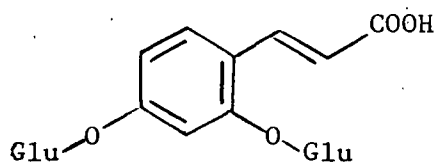
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The formation of umbelliferone (20) has been studied by Brown, Towers, and Chen<sup>19</sup> and by Austin and Meyers<sup>20,21</sup> using Hydrangea macrophylla. The latter workers have reported that umbelliferone exists as the free coumarin only to a very small extent if at all, and have identified two bound forms in Hydrangea (21 and 22). The



7-β-D-Glucosyloxycoumarin or  
skimmin

(21)



cis-2,4-di-β-D-Glucosyloxycinnamic  
acid

(22)

first compound was predominant. Experiments with <sup>14</sup>C-labelled compounds by the two groups have been in substantial accord and led to the following biosynthetic route being proposed for umbelliferone in Hydrangea (Figure 5).



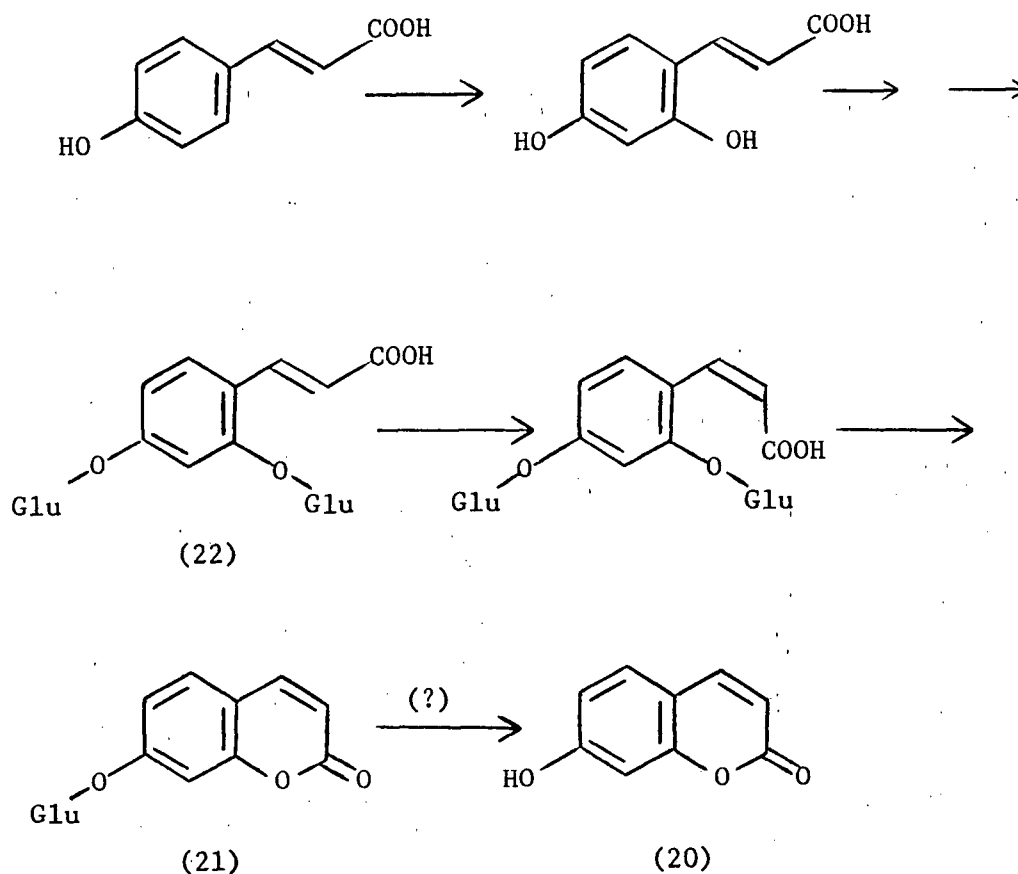
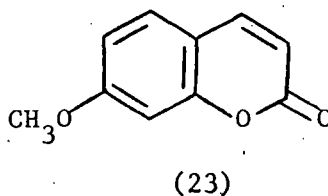


Figure 5. Proposed scheme for biosynthesis of umbelliferone in Hydrangea.

B. Herniarin (7-methoxycoumarin)



Lavender (Lavandula officinalis Chaix) is one of the few species which elaborate both coumarin and a 7-hydroxylated coumarin, in this case herniarin (23). Therefore, it was chosen by Brown<sup>22</sup> as a convenient species in which to compare the biosynthesis of coumarin and a

7-hydroxylated coumarin.

Like coumarin, herniarin also occurred in the bound state as a glucoside. It seemed quite certain that this glucoside was 2-glucosyloxy-4-methoxy-cis-cinnamic acid (cis-GMC) (24). The presence of this compound implied the formation, at some stage, of an ortho-hydroxylated precursor and indicated that the lactone ring of 7-hydroxylated coumarins also can be synthesised via ortho-hydroxylation.

Brown<sup>22</sup> compared in studies on lavender a number of different <sup>14</sup>C labelled compounds as precursors of herniarin, and in some cases coumarin. L-phenylalanine was incorporated with moderate dilution of <sup>14</sup>C into herniarin as well as coumarin. Glucose was used with lower efficiency as a precursor of both coumarins. However, o-coumaric and p-coumaric acids were utilized with a high degree of selectivity. The former was used for the synthesis of coumarin some 150-200 times as efficiently as it was used for herniarin synthesis, and the latter was selectively utilized for herniarin synthesis by a slightly lesser factor. Furthermore, cinnamic acid was found to be a precursor of both coumarins.

These findings showed that herniarin, unlike coumarin, was not synthesized by way of the o-coumaric acid - o-coumaryl glucoside pathway. They confirmed the theory that coumarin and herniarin arise via ortho- and para-hydroxylation, respectively, of a common precursor, probably some form of cinnamic acid.

The low dilution of <sup>14</sup>C in trans-GMC suggested that this compound was the intermediate precursor of cis-GMC. The trans-cis inversion necessary for this step would be analogous to that postulated in the

formation of coumarinyl glucoside. In addition, during the conversion of p-coumaric acid to cis-GMC, an ortho-hydroxylation glucoside formation and an O-methylation of the para-hydroxyl group must occur. Glucoside formation must obviously follow the o-hydroxylation, but the order of the other steps remained in question.

In an attempt to elucidate the above problem Brown compared p-methoxycinnamic acid- $\alpha$ - $^{14}\text{C}$  and 2,4-dihydroxy-trans-cinnamic acid-1- $^{14}\text{C}$  (umbellic acid) with p-coumaric acid as precursors of herniarin. The results clearly demonstrated the very high efficiency with which p-methoxycinnamic acid was converted to herniarin. Umbellic acid and umbelliferone, while moderately well utilized, were both poorer precursors than p-coumaric acid and 25-50 times poorer than p-methoxycinnamic acid. Thus, these experiments revealed the following order of precursor efficiencies for herniarin synthesis: trans-GMC > p-methoxycinnamic acid > p-coumaric acid > umbellic acid  $\geq$  umbelliferone > glucose.

Therefore, the scheme for the herniarin biosynthesis proposed by Brown<sup>22</sup> at the time can be represented by the following scheme (Figure 6).

The stage at which O-methylation occurred in the biosynthesis of herniarin remained uncertain, however. The two possibilities shown below (Figure 7) seemed the most probable, and as has already been noted a comparison of the two labelled intermediates showed that p-methoxycinnamic acid was much the better herniarin precursor.<sup>22</sup>

On the basis of existing evidence, Brown<sup>23</sup> recently proposed the following scheme (Figure 8) for herniarin biosynthesis. Here p-coumaric acid is converted to an intermediate "X", which may be an enzyme-substrate

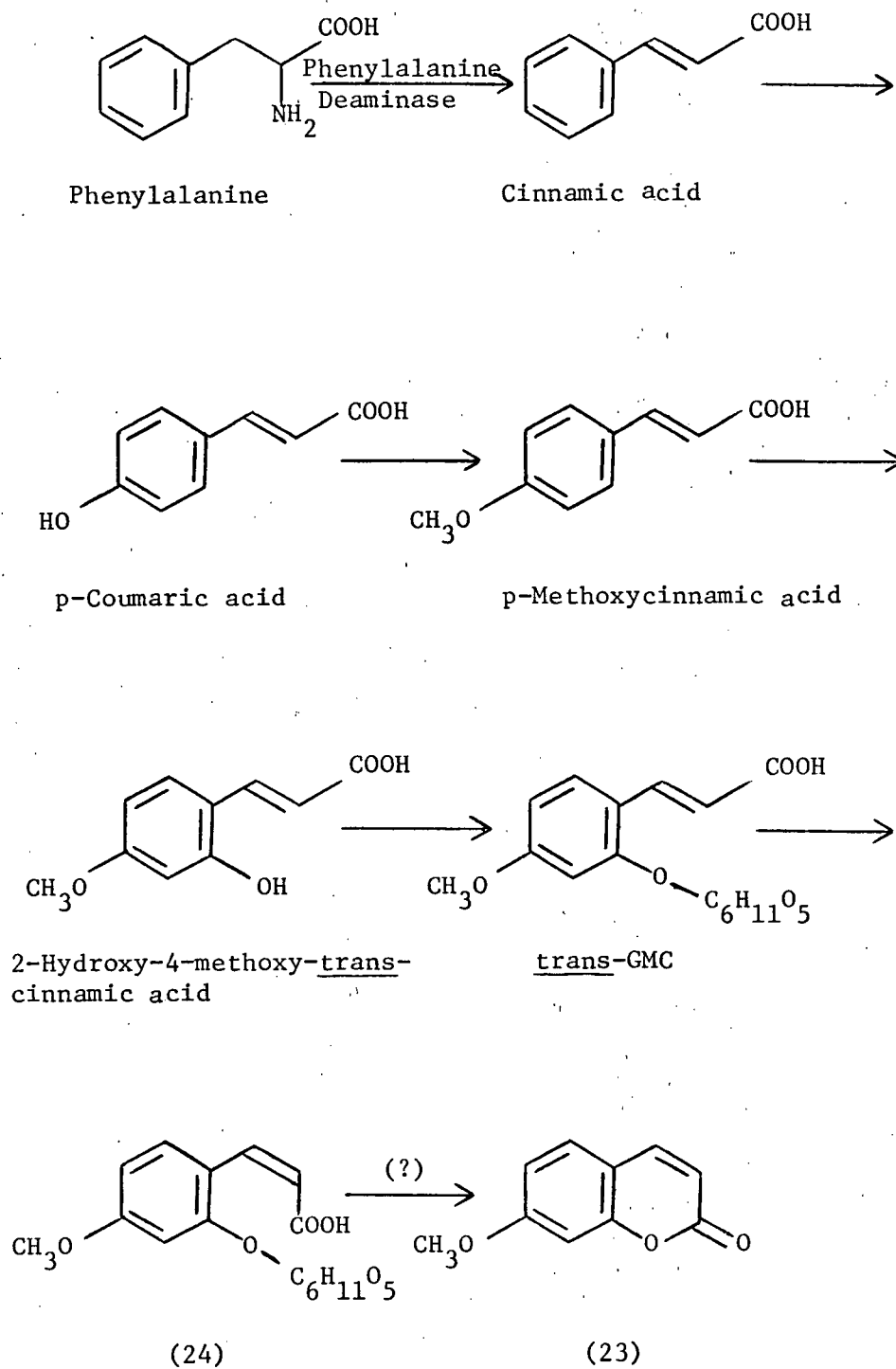


Figure 6. Proposed scheme for herniarin biosynthesis.

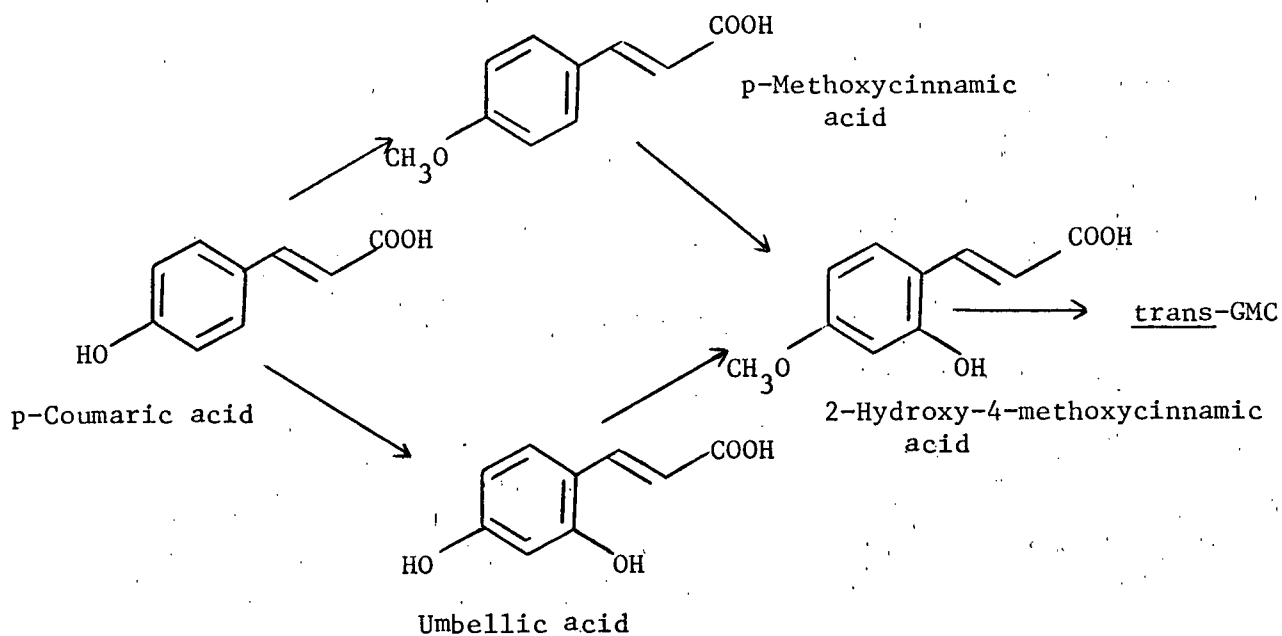


Figure 7. Possible stages of O-methylation in herniarin biosynthesis.

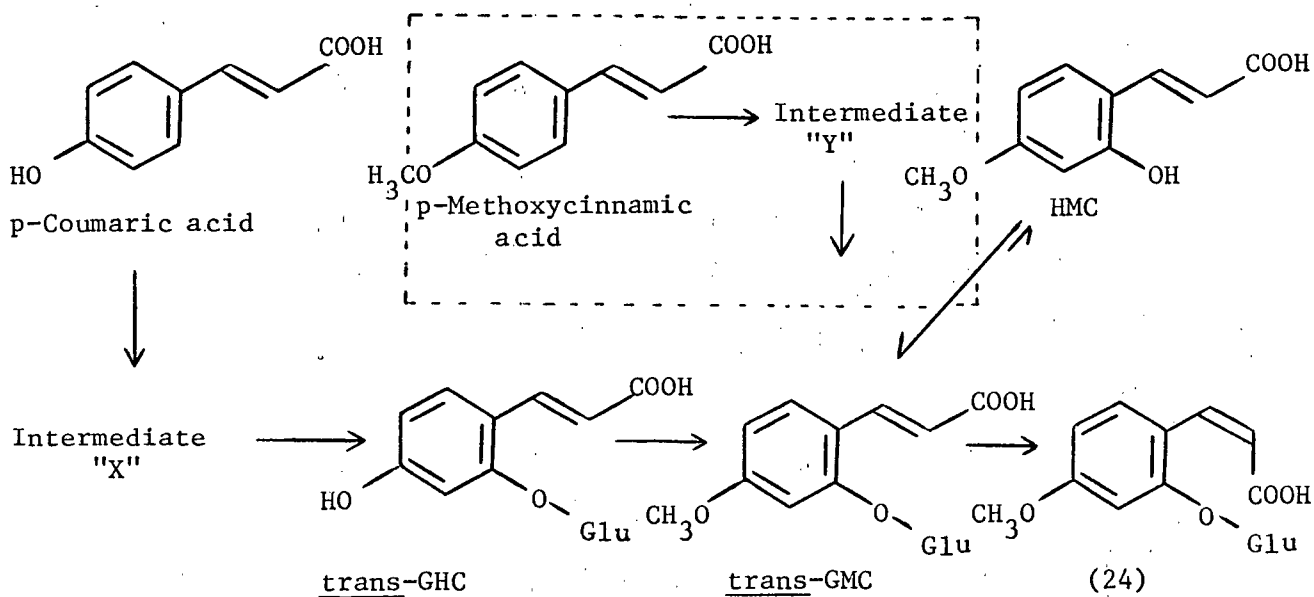
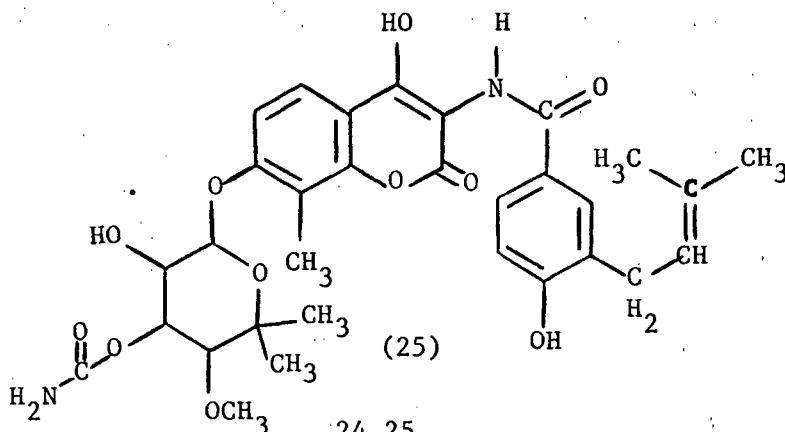


Figure 8. Modified scheme for herniarin biosynthesis.

complex. To overcome the difficulty posed by the poor efficiency of umbellic acid as an intermediate, it was suggested that o-hydroxylation and glucosylation occurred without the substrate leaving the enzyme surface, or whatever other complex may be concerned. The product, trans-2-glucosyloxy-4-hydroxy-cinnamic acid (trans-GHC), could then undergo O-methylation to trans-GMC, a known intermediate. However, trans-GHC, an unknown compound, has not yet been tested as a herniarin precursor.

### C. Novobiocin



Kenner and his co-workers<sup>24,25</sup> have shown that the lactone ring in the coumarin residue of the antibiotic novobiocin (25), formed by a *Streptomyces*, originates in a different way. By the use of <sup>14</sup>C they showed that the coumarin residue was formed from tyrosine, and further work with <sup>18</sup>O yielded good evidence that the ring oxygen originated from the carboxyl oxygens of tyrosine. They postulated an oxidative cyclization of the amino acid to explain their results. Others<sup>26,27</sup> have raised the question whether a similar mechanism may not also operate in higher plants, and Scott, Meyers, and co-workers<sup>27</sup> have suggested the involvement of a spirolactone as shown (Figure 9 ).

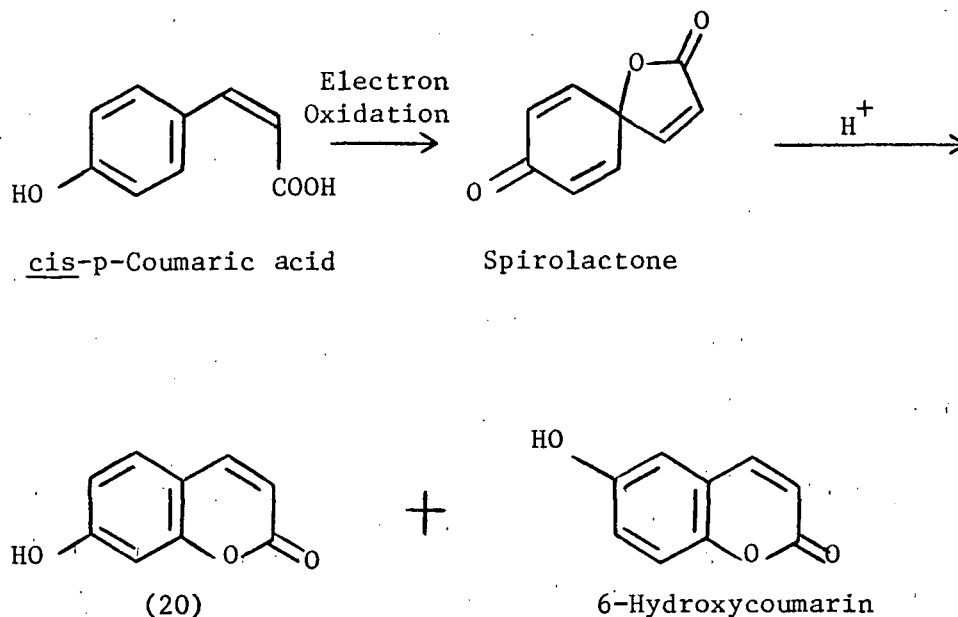
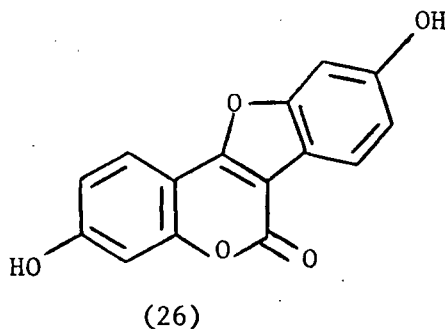


Figure 9. Involvement of a spirolactone in coumarin biosynthesis.

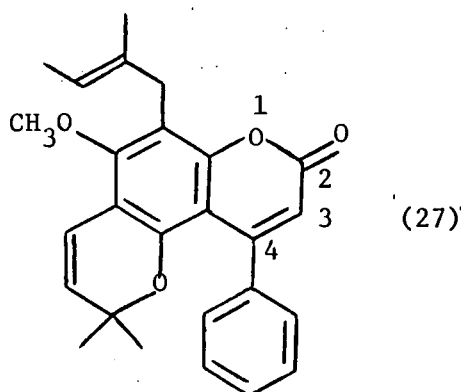
The model reaction shown was demonstrated in vitro,<sup>27</sup> but subsequent in vivo work by Austin and Meyers<sup>20</sup> with a  $^{14}C$ -labelled spirolactone did not bear out the theory. The fact that the coumarins in question actually exist in the cell as glucosides of coumarinic acids argued strongly that at least in plants an o-hydroxylation mechanism, rather than oxidative cyclization is the favored pathway.<sup>28</sup>

#### D. Coumestrol



The investigations of Grisebach and Barz<sup>29,30</sup> on the coumarano-coumarin, coumestrol (26), have shown that this compound is biosynthetically an isoflavone with the benzenoid ring of the coumarin nucleus originating from acetate, and the remaining nine carbons from phenylpropanoid precursors. As in the formation of isoflavones,<sup>26</sup> an aryl-migration was involved, and a chalcone glucoside was apparently an intermediate. These findings demonstrated that identical structures may have entirely different biosynthetic origins, even in species of the same family.

E. Calophyllolide



Recent work by Kunesch and Polonsky<sup>31</sup> showed that the specific incorporation of (-)-phenylalanine-3-<sup>14</sup>C into a 4-phenylcoumarin (neoflavanoid), calophyllolide (27), supported the biogenetic scheme (Figure 10) suggested by Seshadri<sup>32</sup> and Ollis.<sup>33</sup>

Edwards and Stoker presented additional information concerning the isomerization of o-coumaroyl glucoside to coumarinoyl glucoside (19) in the biosynthesis of coumarin<sup>34</sup> and the isomerization of 2-glucosyloxy-4-methoxy-trans-cinnamic acid (trans-GMC) to 2-glucosyloxy-4-methoxy-cis-cinnamic acid (cis-GMC) (24) in the biosynthesis of herniarin.<sup>35</sup>



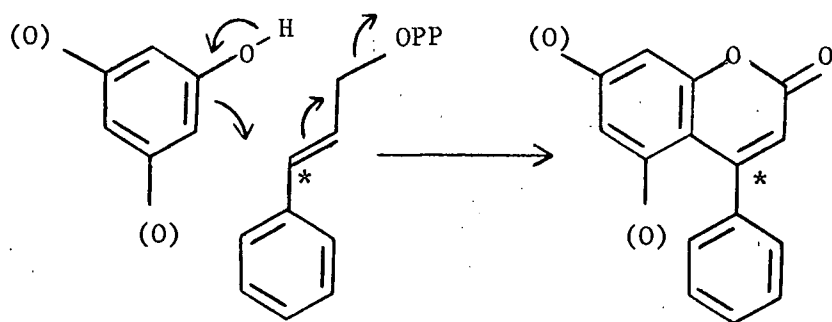
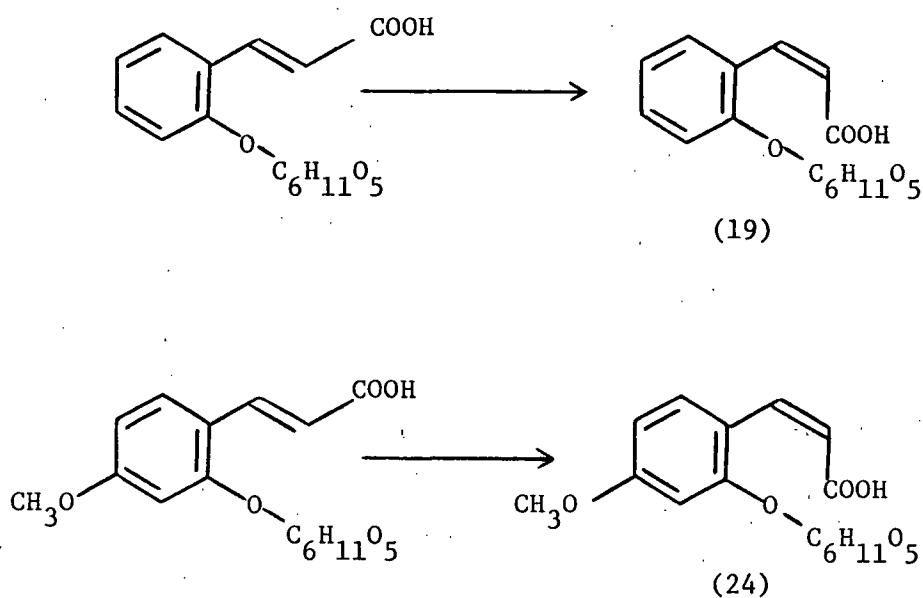


Figure 10. Suggested biosynthetic scheme for calophyllolide.

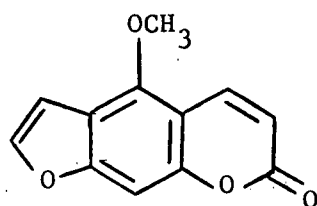


The results of Edwards and Stoker indicated that an enzyme is not involved in the isomerization of o-coumaroyl glucoside to coumarinoyl glucoside in *M. officinalis*, but that the reaction is catalyzed by light. Furthermore, their results also indicated that the isomerization

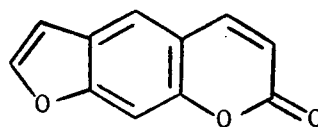
stage in the biosynthesis of herniarin is a photochemical step, no isomerase enzyme being involved. Consequently, this isomerization stage has now been shown to be photochemical both for coumarin itself and for a typical 7-oxygenated coumarin. It is probable, therefore, that the isomerization step in the biosynthesis of all plant coumarins is entirely photochemical.

#### Biosynthesis of Furanocoumarins:

In one of the initial studies on the biosynthesis of furanocoumarins, Caporale and his co-workers<sup>36</sup> reported the incorporation of radioactivity from acetate-2-<sup>3</sup>H, tyrosine-2-<sup>14</sup>C and -U-<sup>3</sup>H, mevalonic acid-2-<sup>14</sup>C, and succinic acid-2,3-<sup>3</sup>H into bergapten (15) and psoralen (17) by leaves of Fiscus carica.



(15)



(17)

Floss and Mothes<sup>37</sup> presented evidence that the coumarin skeleton of furanocoumarins in Pimpinella magna (Umbelliferae) was formed from cinnamic acid and also that para-hydroxylation preceded ortho-hydroxylation, since umbelliferone was a far better precursor than cinnamic acid whereas coumarin gave only poor incorporation, indicating that umbelliferone but not coumarin was an intermediate in the formation of Pimpinella furanocoumarins from cinnamic acid. The co-occurrence of

simple furanocoumarins, isopropylfuranocoumarins and isoprenylated furanocoumarins suggested the possibility that the furan ring originated from carbons 1 and 2 of the isoprene residue or from carbons 4 and 5 of mevalonic acid, respectively.<sup>38</sup> Floss and Mothes<sup>39</sup> reported results which confirmed the incorporation of cinnamic acid and apparently clarified the origin of the two extra carbon atoms of the furan ring, and hence allowed some conclusions to be made regarding the pathway of furanocoumarin formation.

Labelled cinnamic acid-(<sup>14</sup>COOH) and DL-mevalonic acid-4-<sup>14</sup>C were fed to Pimpinella magna and various furanocoumarins present were isolated. In order to establish the direct conversion of cinnamic acid to furanocoumarins, a degradation, using sphondin (28), was performed (Figure 11). The CO<sub>2</sub> obtained from C-2 of sphondin had about

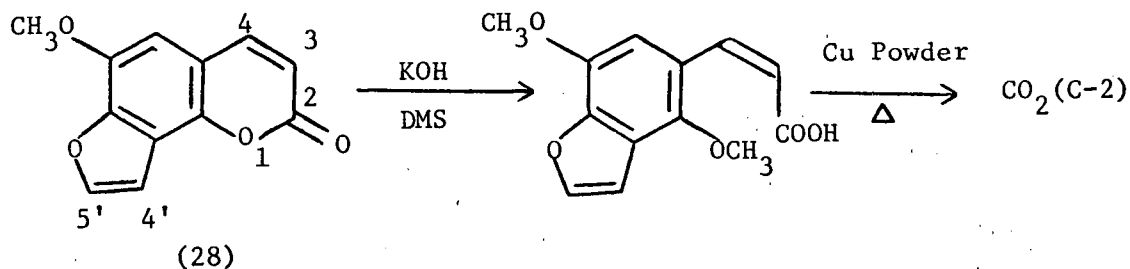


Figure 11. Degradation of sphondin.

90% of the specific activity of the starting material. Thus, most of the activity was located at C-2 of the furanocoumarin, indicating that cinnamic acid was indeed incorporated into the furanocoumarins.

Mevalonic acid-4-<sup>14</sup>C was also incorporated into the furanocoumarins. To determine the location of the isotope in the furanocoumarins isolated,

a degradation was attempted. All the  $^{14}\text{C}$  would be expected to be in position 5', if the furan ring was indeed formed from C-4 and C-5 of mevalonic acid. Labelled pimpinellin (29) was degraded by ozonization (Figure 12) and the product was identified as 2,4-dihydroxy-5,6-dimethoxy-m-phthalaldehyde (30).

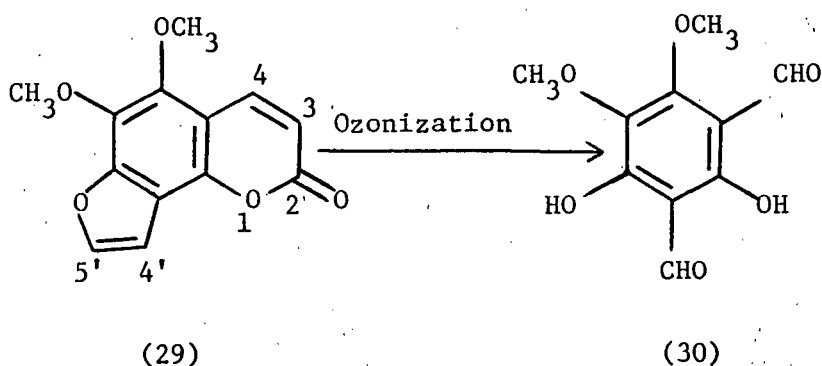


Figure 12. Degradation of pimpinellin.

The ozonization reaction involved removal of carbon atoms 2, 3, and 5'. Since pimpinellin had a constant specific activity and the dialdehyde had only 11% of the original specific activity, most of the  $^{14}\text{C}$  must have been located in C-2, C-3 and/or C-5'. As shown before, the coumarin portion of the molecule, which includes C-2 and C-3, originated from cinnamic acid. A specific incorporation of C-4 of mevalonic acid was highly unlikely. Thus, it confirmed that C-4 of mevalonic acid indeed labelled C-5' furanocoumarins.

Therefore, the results of Floss and Mothes<sup>39</sup> apparently clarified the origin of the carbon skeleton of the furanocoumarins. Thus, the coumarin portion is formed from cinnamic acid, while the furan ring originated from an isoprenoid residue, probably via isoprenylation of

the aromatic ring followed by cyclization and loss of a 3-carbon isopropyl side chain.

The general mechanism of furanocoumarin biosynthesis especially the sequence of the individual steps was also considered by Floss and Mothes.<sup>39</sup> With very few exceptions all furanocoumarins have the furan ring attached to position 6 and 7 or 7 and 8 of the coumarin system, the oxygen being in position 7. This would be explained most easily by the assumption that umbelliferone is the compound which is isoprenylated, and that further hydroxylations and alkylations take place after this step. However, the results of the cinnamic acid and mevalonic acid feeding experiments clearly showed that this was not the case. If isoprenylation of umbelliferone preceded further hydroxylations, it was expected that the relative specific activities of the various furanocoumarins isolated would be the same in the cinnamic acid as in the mevalonic acid experiment, since all the reactions which create the difference between the products would occur after the introduction of the isotope in both cases. However, the relative specific activities of the various furanocoumarins were not the same in the cinnamic acid and mevalonic acid experiments.

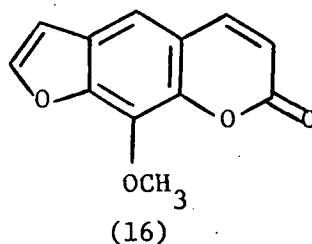
The results pointed to the alternative whereby the hydroxylation and methylation pattern was established first and isoprenylation then occurred as the last step. Since, however, the 7-hydroxy group must be protected from methylation in order to allow furan ring formation, it was likely that umbelliferone was first converted to the glucoside and this compound was the substrate for further hydroxylations and methylations. The sugar could then be removed at a latter stage to allow furan ring formation to take place.

Floss and Mothes<sup>39</sup> also found that the hydroxylation pattern of the

furanocoumarins in Pimpinella could be derived by a combination of three types of reactions:

- (i) a hydroxylation of umbelliferone ortho to the hydroxyl group and para to the lactone oxygen,
- (ii) a hydroxylation in position 5 ("second ortho hydroxylation"),
- (iii) opening of the lactone ring and recyclization in the opposite direction.

An observation in favor of this scheme was that xanthotoxin (16), the only member of all the possible mono- and dimethoxylated furanocoumarins of this type which could not be formed by these reactions, was not found in Pimpinella.



The data also suggested that reaction (i) occurred more readily than reaction (ii), since in the cinnamic acid experiment, sphondin (28) had a much higher specific activity than bergapten (15) and isobergapten (31). As expected bergapten and isobergapten had the same specific activity in the cinnamic acid experiment, whereas the value for pimpinellin (29) and isopimpinellin (2) were lower. Since the value of pimpinellin was lower than that of its isomer it appeared as if it was obligatory for reaction (iii) to occur together with reaction (ii).

The data from the mevalonic acid experiments also indicated that isoprenylation at position 8 was preferred as all angular furanocoumarins had a higher specific activity than the linear ones. This was more pronounced with the pair bergapten and isobergapten, where there was a true competition for the same precursor.

On the basis of the results Floss and Mothes<sup>39</sup> proposed the following scheme for the biosynthesis of furanocoumarins (Figure 13).

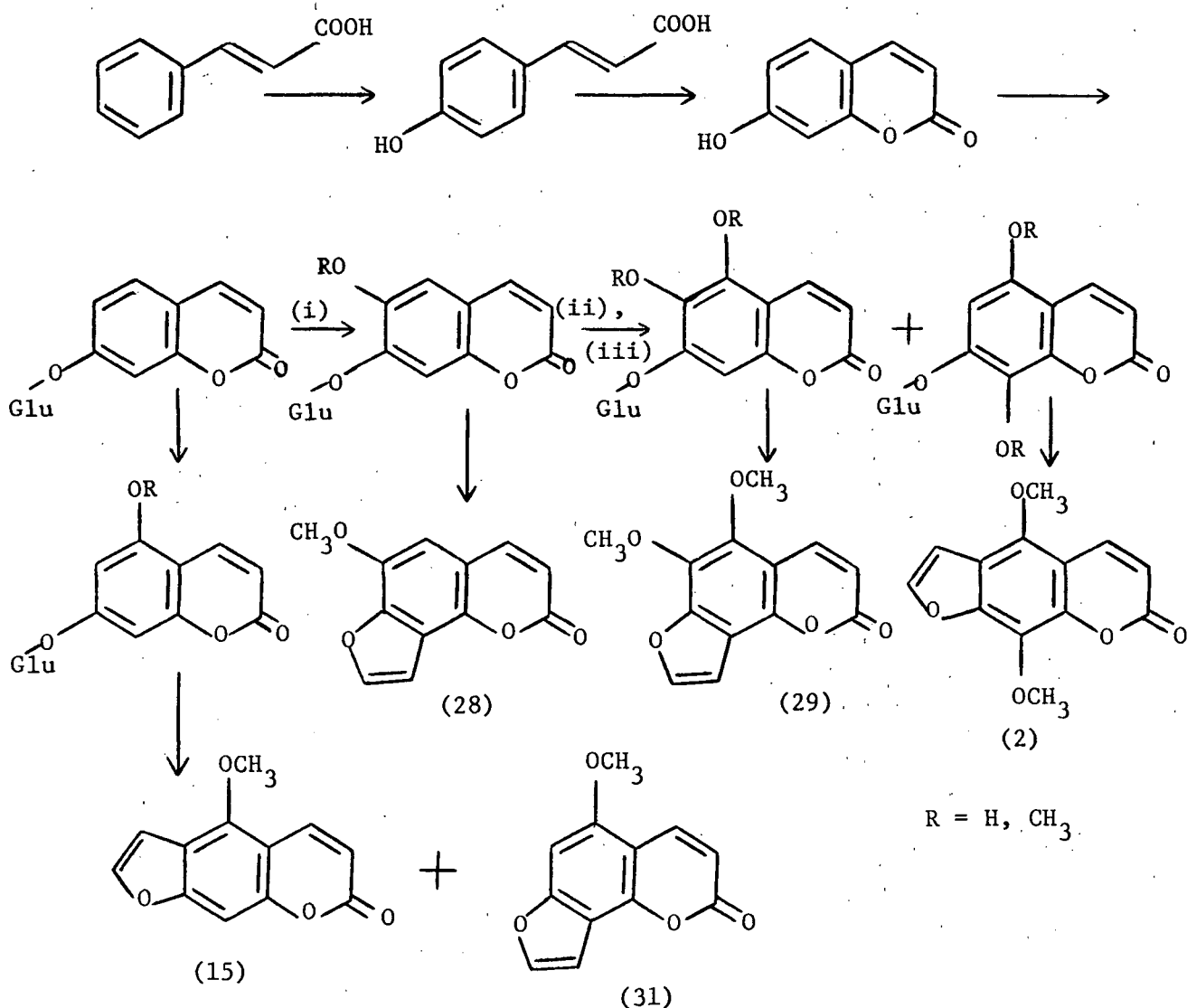


Figure 13. Proposed scheme for the biosynthesis of furanocoumarins.

Recently, Floss and Paikert<sup>40</sup> examined the scheme experimentally. The evidence obtained did not favour the proposed biogenetic scheme and indicated that an alternative pathway, involving isoprenylation at the umbelliferone stage followed by further modification of the benzenoid ring, must be considered.

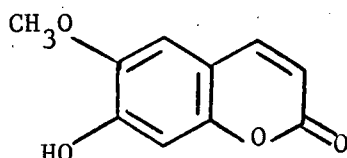
These workers attempted to determine whether the ortho- or the para-hydroxyl group was introduced into the cinnamic acid molecule. The fact that coumarin was not utilized in the biosynthesis did not rule out o-coumaric acid as an intermediate. Both o-coumaric acid and p-coumaric acid with a <sup>14</sup>C label in the carboxyl group were fed to Pimpinella magna. However, the results together with previous work by Brown and his co-workers on the biosynthesis of umbelliferone in Hydrangea macrophylla suggested that o-coumaric acid was not a natural intermediate in the synthesis, but that p-coumaric acid was the likely precursor.

Floss and Paikert<sup>40</sup> also studied the incorporation of umbelliferone and its 7-glucoside into furanocoumarins. It was found that the glucoside was incorporated slightly less efficiently than the aglycone. Furthermore, earlier experiments<sup>41</sup> had shown very rapid glycosylation of added umbelliferone, and thus, they examined the metabolic fate of umbelliferone in P. magna. Umbelliferone-3-<sup>3</sup>H was root fed to the plant. After work up of the plants and subsequent enzymatic hydrolysis of the coumarins to yield the aglycones, it was noted that umbelliferone was the only labelled material, that is, there was no detectable conversion of umbelliferone glucoside to other coumarin glucosides.

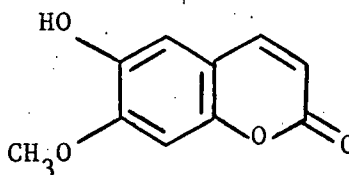
If the hypothetical pathway of furanocoumarin biosynthesis, as



postulated by Floss and Mothes, was correct, it was expected that scopoletin (7-hydroxy-6-methoxycoumarin) (32) would be a precursor of sphondin and pimpinellin, possibly also of isopimpinellin, but not of bergapten and isobergapten. Therefore, scopoletin-methyl-<sup>3</sup>H was administered to the roots of P. magna and, as a reference, its isomer (6-hydroxy-7-methoxycoumarin-methyl-<sup>3</sup>H) (33) was fed in a parallel experiment.



(32)



(33)

It was found that scopoletin-methyl-<sup>3</sup>H was not incorporated preferentially into any one furanocoumarin as would be expected if isoprenylation were a late step in the biogenetic pathway. Furthermore, it labelled the furanocoumarins in about the same manner of magnitude as its isomer 6-hydroxy-7-methoxycoumarin-methyl-<sup>3</sup>H which could not be an intermediate in the formation of the furanocoumarins of Pimpinella. This indicated that the scopoletin molecule was not transformed into furanocoumarins as a unit, but that the incorporation of radioactivity was possibly only due to demethylation and partial reutilization of the labelled C<sub>1</sub> fragment. This was also suggested by the rather low rate of incorporation of scopoletin as compared to umbelliferone. Therefore, it seemed very likely that scopoletin was not a direct precursor of any of the furanocoumarins of P. magna.

Therefore, the results presented by Floss and Paikert<sup>40</sup> did not substantiate the assumption that isoprenylation occurred as a late reaction in furanocoumarin biosynthesis and that the hydroxylations and methylations of the coumarins portion occurred at the glucoside stage. An alternative pathway of furanocoumarin biosynthesis had to be considered, involving isoprenylation of umbelliferone (20) followed by further modification of the resulting 6- and 8-dimethylallylumbelliferone (Figure 14). Such a pathway was attractive from a phytochemical point

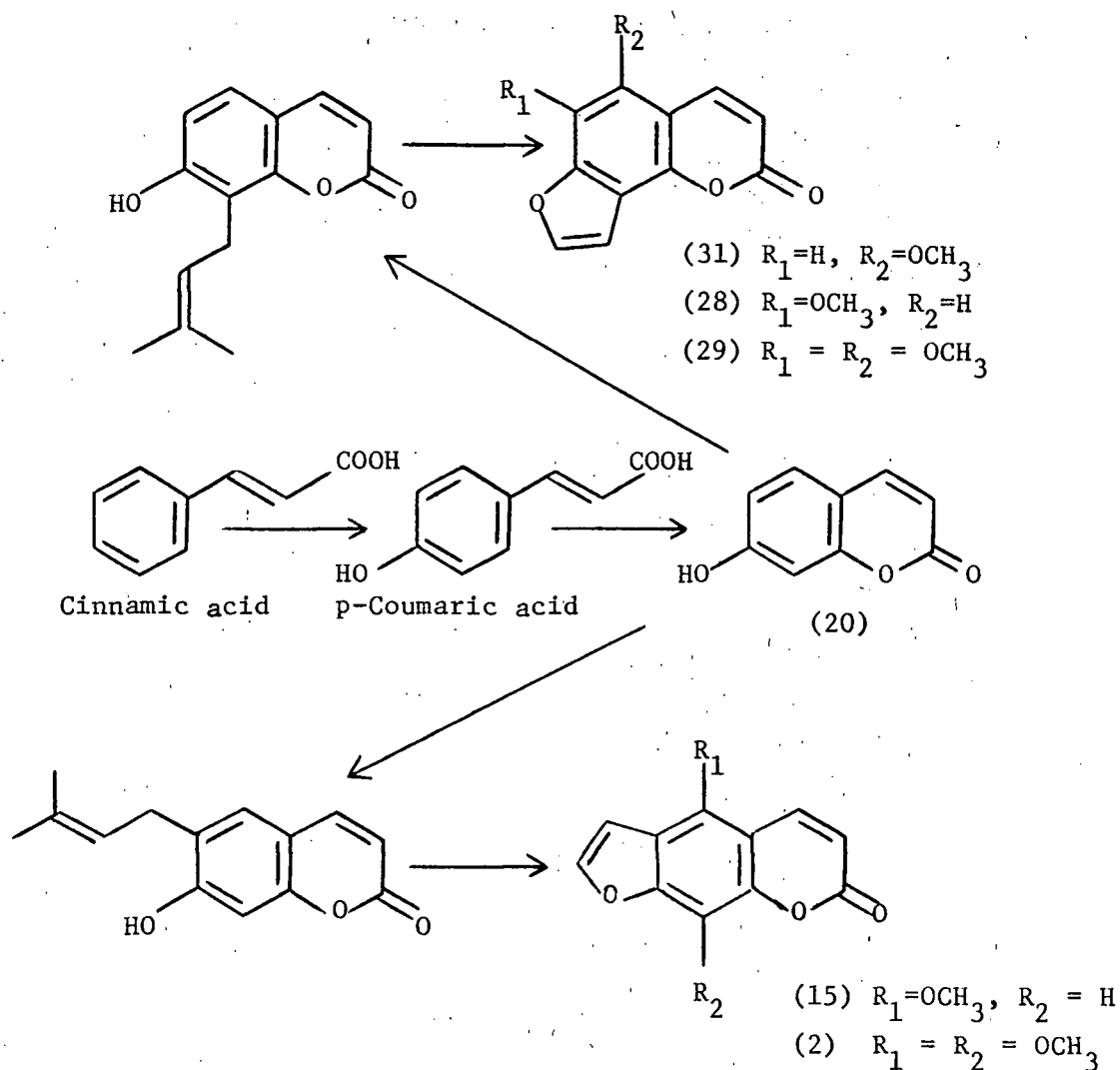
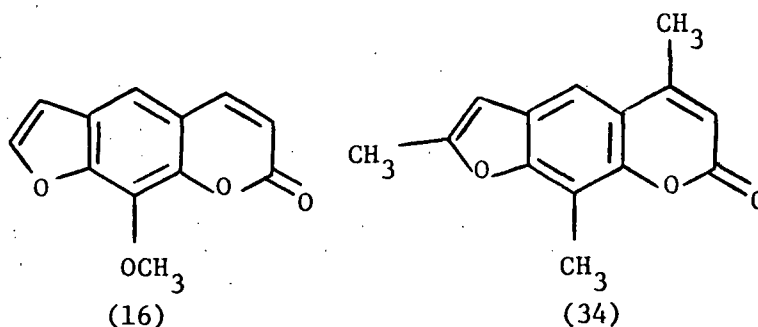


Figure 14. Proposed alternative pathway of furanocoumarin biosynthesis.

of view since it could explain the almost exclusive occurrence, in nature, of 6,7- and 7,8-furanocoumarins.

Floss, Guenther, and Hadwiger<sup>42</sup> recently presented work on the biosynthesis of furanocoumarins in diseased celery. Upon infection with the fungus Sclerotinia sclerotiorum, celery was known to develop a condition called "pink rot" which has been correlated with the skin-irritating properties of diseased celery. Two phototoxic psoralen derivatives have been isolated from diseased celery tissue, xanthotoxin (8-methoxypsoralen) (16) and 4,5',8-trimethylpsoralen (34).

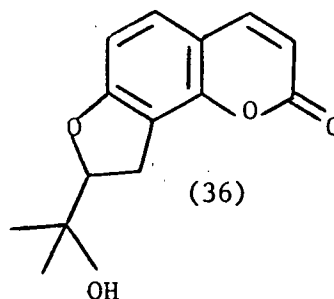
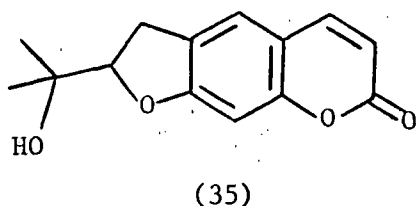


These workers conducted some feeding experiments to determine whether the two psoralen derivatives originated from the same biosynthetic precursors as other furanocoumarins found in higher plants, namely cinnamic acid and mevalonic acid with the O-methyl groups presumably coming from the C<sub>1</sub> pool (formate or the methyl group of methionine). Xanthotoxin has the same structural pattern as other higher plant furanocoumarins and would thus be expected to be derived from the same biogenetic precursors. On the other hand, the psoralen derivative (34) has some rather unusual features in that it carries three carbon bound methyl groups. If, as in other furanocoumarins, the ring skeleton of this compound is derived from cinnamate and mevalonate, the three methyl

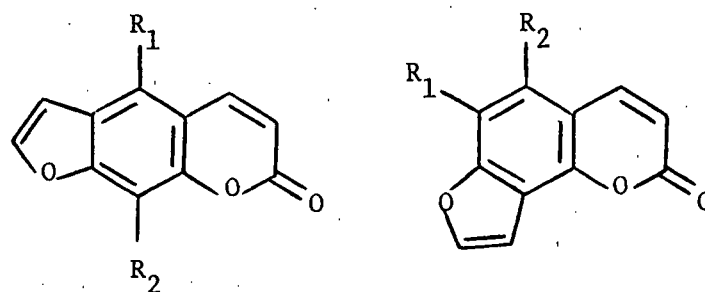
groups, or at least the two attached to the coumarin portion, would be expected to originate from the  $C_1$  pool by C-methylation.

Cinnamic acid-1- $^{14}C$ , methionine-methyl- $^{14}C$ , and formate- $^{14}C$  were fed to celery plants after infection. As expected, all three compounds were incorporated into xanthotoxin, although formate was a considerably less efficient methyl donor than methionine. Interestingly, however, none of the three substrates labelled 4,5',8-trimethylpsoralen to any significant extent. It was conceivable that the two products were possibly synthesized at different sites, that is, xanthotoxin may have been formed by the plant and trimethylpsoralen by the fungal cells. As an alternative explanation of the results of this study, one had, however, to consider the possibility that the two furanocoumarins found in diseased celery were formed by two entirely different biosynthetic pathways.

Steck, El-Dakhakhny and Brown<sup>43</sup> recently presented material strongly indicating the participation of two  $\alpha$ -hydroxyisopropylidihydrofuranocoumarins, marmesin (35) and columbianetin (36) as natural intermediates in the elaboration of linear and angular furanocoumarins, respectively. To date, this was the first time intermediates in the reaction sequence from umbelliferone to furanocoumarins had been identified.



Trapping experiments were conducted in which umbelliferone-2- $^{14}\text{C}$  was administered to shoots of Ruta graveolens together with non-labelled marmesin, and skimmin-2- $^{14}\text{C}$  to Heracleum lanatum together with non-labelled columbianetin. In addition to marmesin, the linear furanocoumarins psoralen (17), bergapten (15), and xanthotoxin (16), were recovered from Ruta. In addition to columbianetin, angelicin (37), isobergapten (31), sphondin (28), and pimpinellin (29) were recovered from Heracleum (Fig. 15).



(17) ( $\text{R}_1 = \text{R}_2 = \text{H}$ )

(15) ( $\text{R}_1 = \text{OCH}_3$ ,  $\text{R}_2 = \text{H}$ )

(16) ( $\text{R}_1 = \text{H}$ ,  $\text{R}_2 = \text{OCH}_3$ )

(37) ( $\text{R}_1 = \text{R}_2 = \text{H}$ )

(31) ( $\text{R}_1 = \text{H}$ ,  $\text{R}_2 = \text{OCH}_3$ )

(28) ( $\text{R}_1 = \text{OCH}_3$ ,  $\text{R}_2 = \text{H}$ )

(29) ( $\text{R}_1 = \text{R}_2 = \text{OCH}_3$ )

Figure 15. Furanocoumarins recovered from Ruta graveolens and Heracleum lanatum.

From the results, it was clear that in each species umbelliferone had been converted to the dihydrofuranocoumarin. Furthermore, the lower specific activities of the furanocoumarins were consistent with their formation from marmesin or columbianetin.

Steck, El-Dakhkhny and Brown<sup>43</sup> also conducted direct feedings of tritiated marmesin and columbianetin to confirm the roles of these compounds in furanocoumarin biosynthesis. In each case there was

good conversion to the analogous unsubstituted furanocoumarin and a lower degree of tritium incorporation into the oxygenated furanocoumarins.

Umbelliferone was known to occur in Ruta graveolens and in Heracleum lanatum. Therefore, it can be regarded as a natural intermediate in furanocoumarin biosynthesis in these species. The formative route to marmesin proposed by Steck, El-Dakhakhny and Brown can be envisaged as the following (Figure 16).

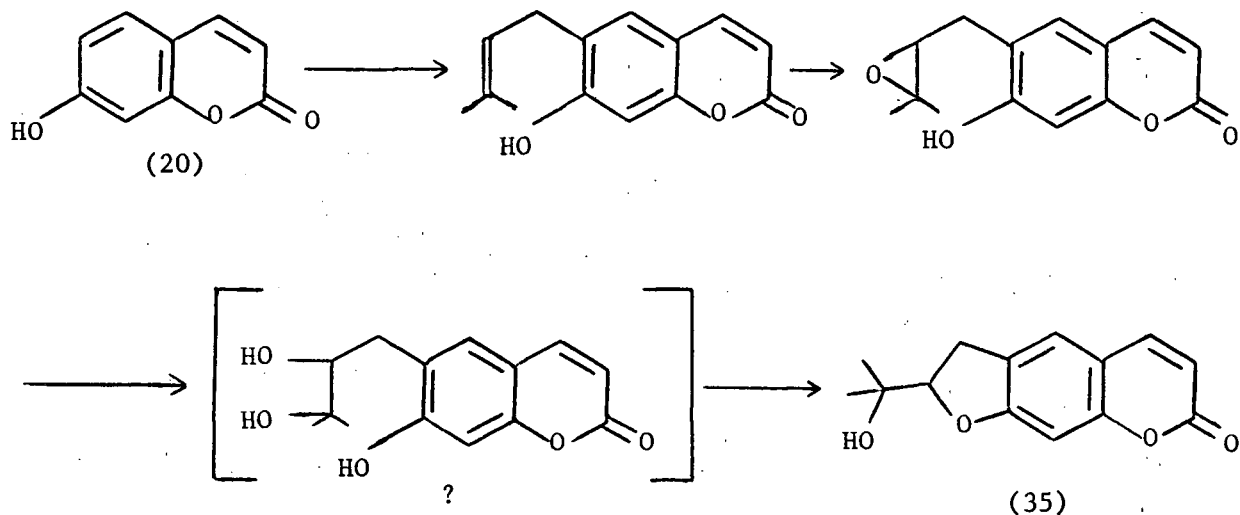


Figure 16. Proposed scheme for the biosynthesis of marmesin.

There was an analogous sequence leading to columbianetin, but there was no firm experimental evidence to support this hypothesis. The nature of the reactions leading on to psoralen and angelicin and ultimately to the substituted furanocoumarins, however, remained unclear.

As the above discussion reveals, the biosynthetic pathways leading

to the furanocoumarins, in contrast to those of simple coumarins are indeed in a state of confusion. None of the results are internally consistent with any general postulate and there was question in our minds about the real meaning of a substantial portion of the experimental data. For this reason we decided to investigate the biosynthesis of several furanocoumarins isolated from Thamnosma montana.

Furthermore, the eventual goal of studying the biosynthesis of the novel dimeric thamnosin system required an initial, careful, investigation of the appropriate monomeric units which would be visualized as the biosynthetic templates for the dimeric natural product.

## DISCUSSION

The discussion within the Introduction section of this thesis has revealed that a considerable amount of work has been done on the biosynthesis of some of the simpler monomeric coumarins. Unfortunately, careful analysis of a large portion of the published data leads one to conclude that much still remains to be done in this area. In many instances the incorporations reported are very low, thereby leading to the isolation of natural products possessing low levels of activity. Furthermore, the eventual characterization and, in particular, sufficient degradation to isolate the relevant carbon atoms is incomplete in a number of instances. It was our feeling that some of the confusions already mentioned in the Introduction are simply due to the incompleteness of the investigations. In order to try and establish more firmly the various postulates in the literature and also to provide an internally consistent picture of the various coumarins within the same plant system, we decided to initiate investigations in turpentine-broom, Thamnosma montana Torr. and Frem. As already mentioned, this plant possesses a wonderful array of monomeric and dimeric coumarins.

In effect these aims required investigations in several different areas. These are the following:



(a) Detailed investigation of suitable isolation procedures to allow isolation and characterization of small quantities of the natural coumarins,

(b) Development of appropriate chemical degradation pathways which allow isolation of relevant carbon atoms in the coumarins,

(c) Incorporation studies with appropriately labelled ( $^{14}\text{C}$  and  $^3\text{H}$ ) precursors.

For this purpose, R.N. Young, A.K. Verma, and myself became involved in separate but complementary areas of the research. I will now discuss those areas with which I was directly involved but wish to emphasize that a great deal of related work which strengthens some of the conclusions made later is available from the efforts of my collaborators.

For the sake of clarity, I propose to discuss my work in the order of research areas (a-c) mentioned above.

Old living plants of the turpentine-broom, Thamnosma montana Torr. and Frem., containing rather thick woody roots, were obtained in the desert region near Morongo Valley, California with the help of Dreyer and his associates at the Fruit and Vegetable Chemistry Laboratory, Pasadena, California. From the whole aerial part of the plant including the crown as well as the roots, it was considered desirable to isolate all the compounds previously found in the plant as well as any new ones which might be present. In addition to the plant material, Dreyer and his associates also provided us with an acetone extract of Thamnosma montana, Torr. and Frem. Both of these were used to develop a suitable isolation procedure.

(a) Isolation of Natural Products from *Thamnosma montana* Torr. and Frem.

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The plants were air dried thoroughly and ground to a coarse powder in a high speed Waring blender. Using a Soxhlet extractor, the pulp was extracted with acetone. The resulting solution was filtered and the clear acetone solution was evaporated to provide a dark heavy tar. The latter was now extracted with chloroform, filtered, and evaporated as before leaving a dark heavy oily residue.

The crude extract was separated into numerous fractions using column chromatography on deactivated alumina. A typical isolation scheme is portrayed in Figure 17 with a typical thin layer silica gel chromatoplate of the isolated fractions illustrated in Figure 18.

For purposes of comparison the appropriate colour characteristics under ultraviolet light of previously characterized compounds found in *Thamnosma montana* Torr. and Frem. are shown on the silica gel chromatoplate in Figure 19.

The compounds of particular interest for our initial biosynthetic work were umbelliprenin, alloimperatorin methyl ether, and isopimpinellin. It was desirable, therefore, to initially accumulate sufficient quantities of the above mentioned compounds along with alloimperatorin methyl ether diol for purposes of subsequent dilution and degradative work in the biosynthetic investigations.

For this purpose the various fractions from the initial column chromatography were combined in an appropriate manner and subjected to additional column chromatography, thin layer chromatography, and finally crystallization to yield the desired products as illustrated in Figure 20.

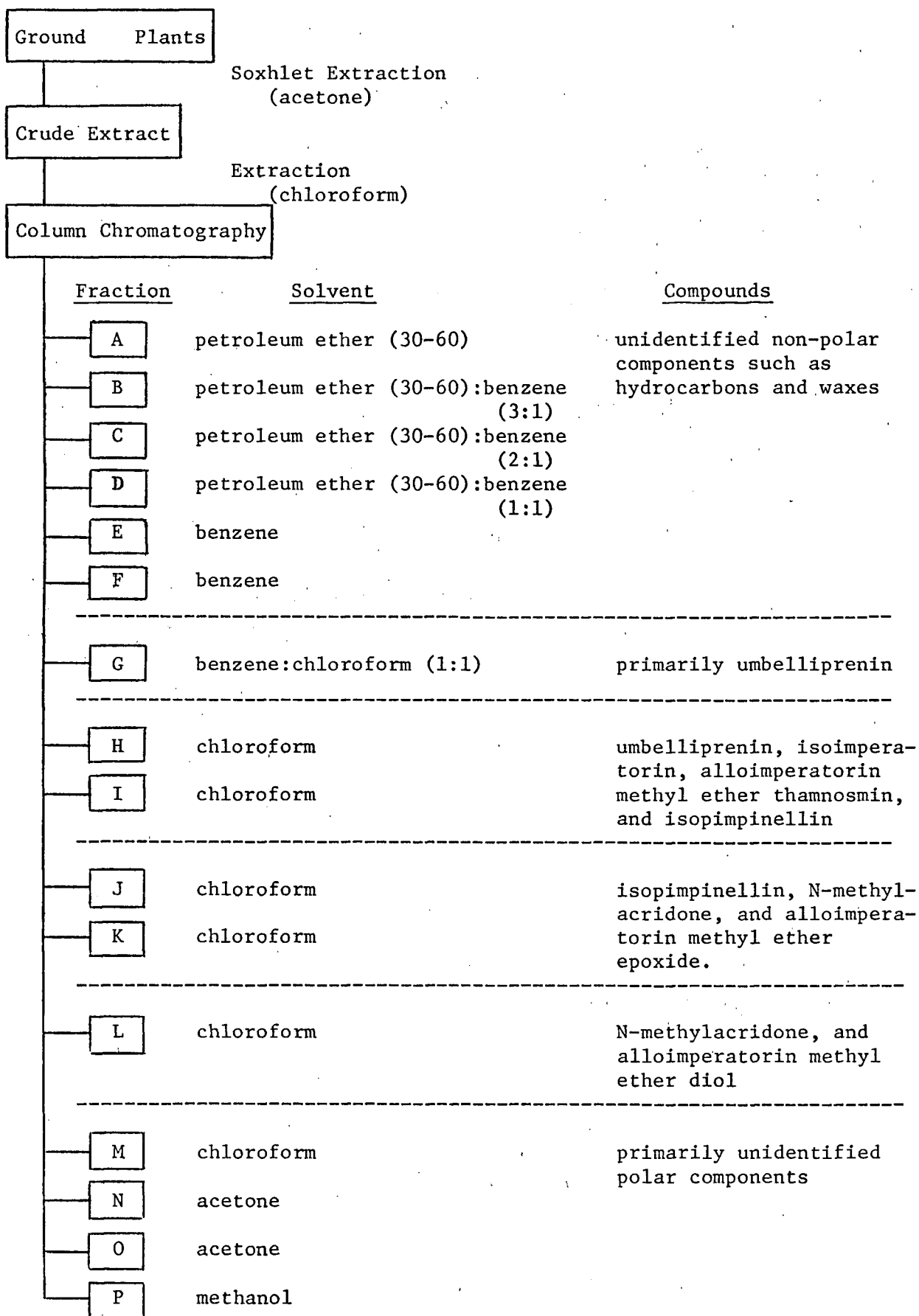


Figure 17. Typical purification sequence of components from Thamnosma montana Torr. and Frem.

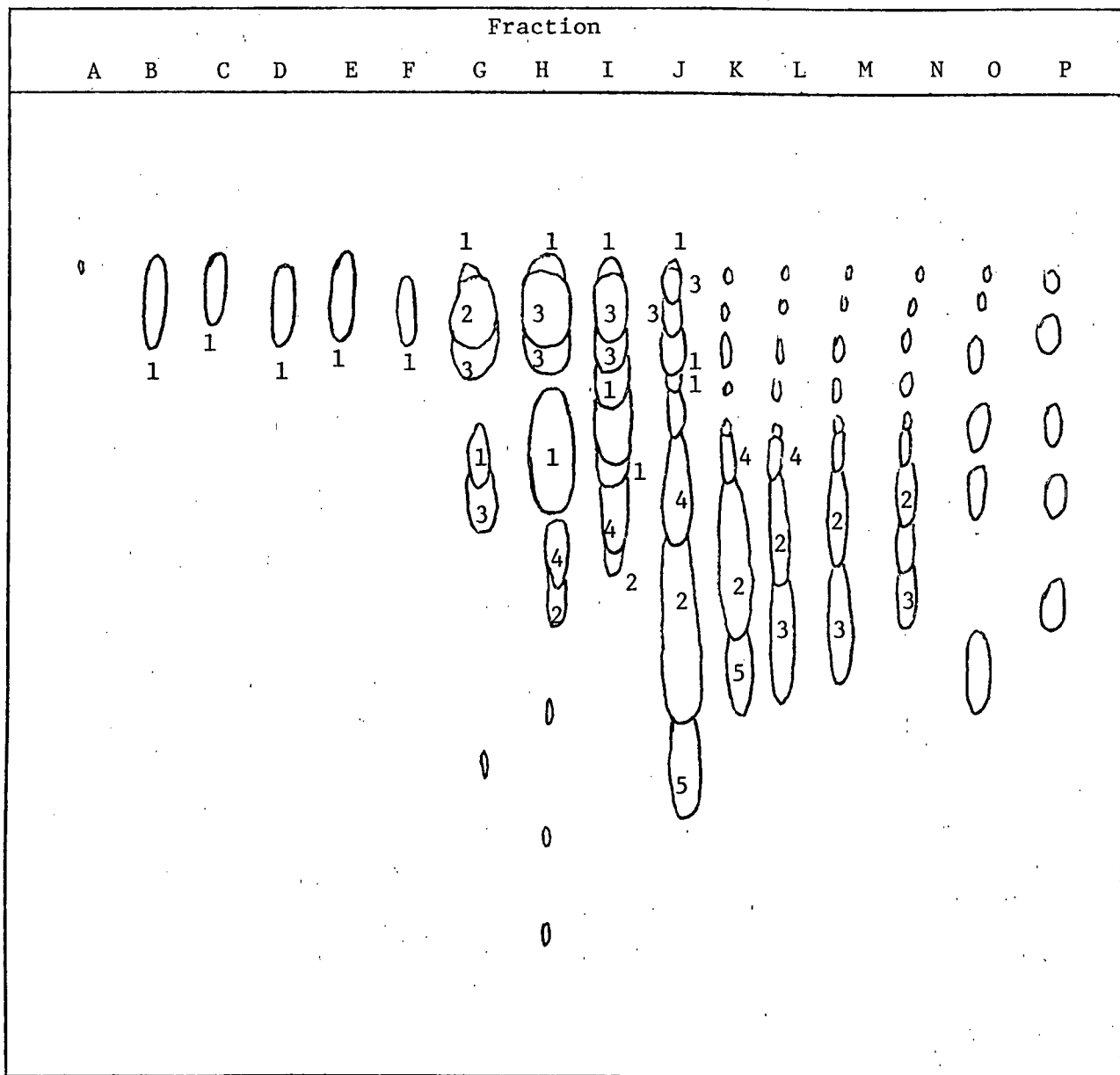


Figure 18. A typical thin layer chromatoplate of isolated fractions obtained from the chloroform extract. Characteristic colours observed on the chromatoplate under ultraviolet light are presented: (1) purple; (2) blue; (3) yellowish-brown; (4) brown; (5) yellowish-green.

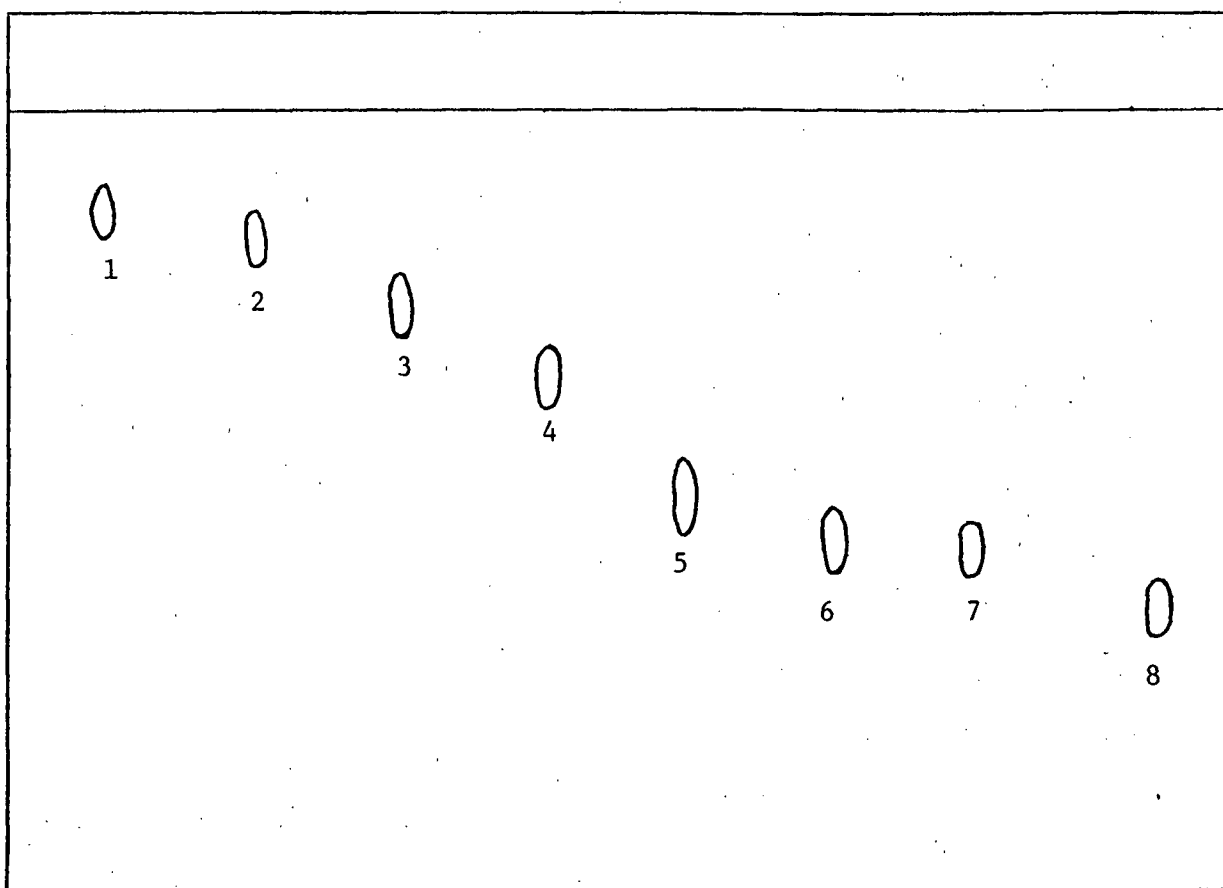


Figure 19. A thin layer chromatoplate of some authentic samples of compounds in Thamnosma montana Torr. and Frem. The legend indicating the names of the compounds and their typical colour under ultraviolet light follows:

- (1) umbelliprenin - blue; (2) isoimperatorin - yellowish-brown;
- (3) alloimperatorin methyl ether - yellowish-brown;
- (4) thamnosmin - purple; (5) isopimpinellin - brown;
- (6) N-methylacridone - blue; (7) alloimperatorin methyl ether epoxide - yellowish-green; (8) alloimperatorin methyl ether diol - yellowish-brown.

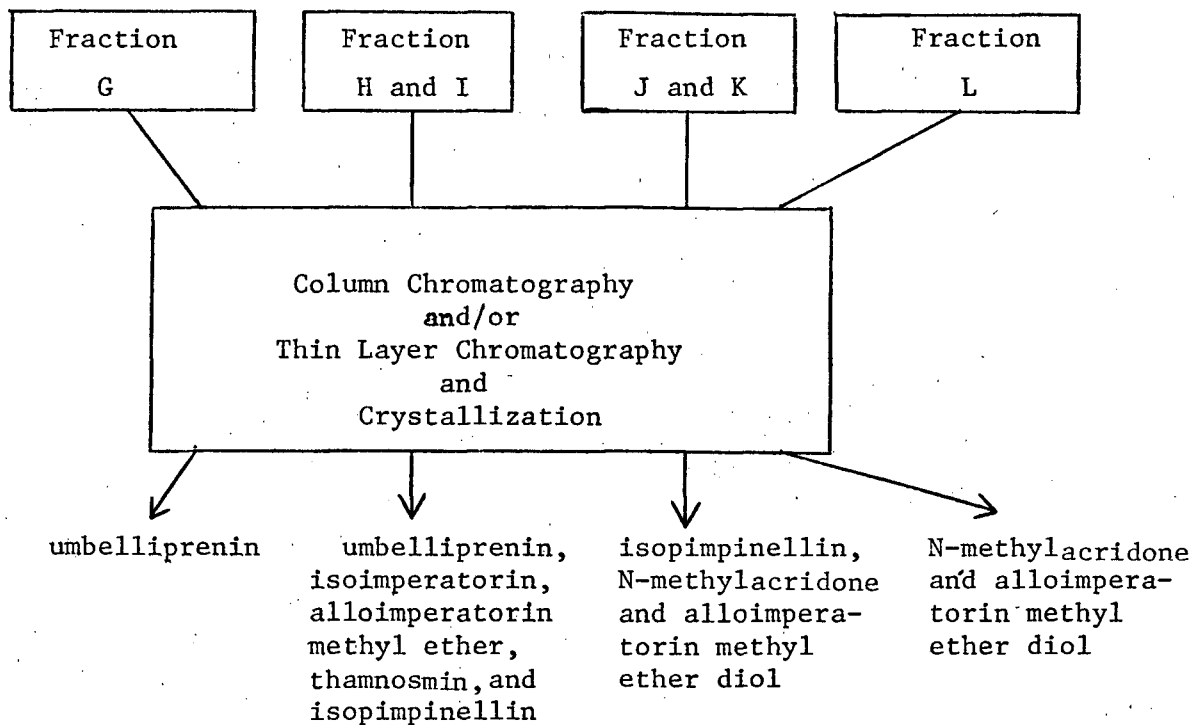


Figure 20. Purification of Fractions G, H. and I, J and K, and L of the initial column chromatography.

(b) Development of Appropriate Chemical Degradation Pathways.

The coumarins of interest primarily for the development of degradative reactions were umbelliprenin (11), isopimpinellin (2), and alloimperatorin methyl ether (8). From these reactions it was thought possible to isolate relevant carbon atoms in the above mentioned coumarins. Before these degradative reactions could be performed on any active material, isolated from the incorporation experiments, it was necessary to perfect them on the inactive compounds previously isolated from the plant. R.N. Young, A.K. Verma and myself, consequently, became involved in the rather extensive research on the chemistry of the appropriate coumarins. For the sake of clarity I will summarize all

of the pertinent reactions which were studied. However, experimental details of only those reactions which were performed by me are recorded in the thesis.

### (i) Degradation of Umbelliprenin

The degradative reaction employed for umbelliprenin involved acidic cleavage of the farnesol side chain to yield umbelliferone (20)<sup>44</sup> (Figure 21). Umbelliprenin (11) was treated with glacial acetic acid to which a catalytic amount of concentrated sulfuric acid had been added.

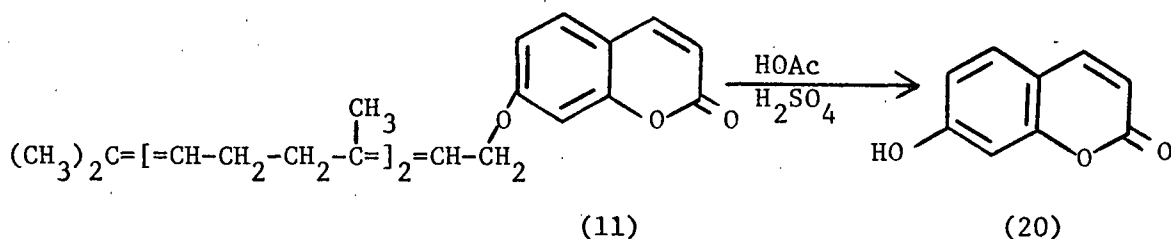


Figure 21. Conversion of umbelliprenin to umbelliferone.

By the above degradative procedure the relevant carbon atoms in the cyclic portion of umbelliprenin could be separated from the isoprenoid side chain in the molecule.

### (ii) Degradation of Isopimpinellin

There were two degradative sequences developed in connection with isopimpinellin (2) (Figure 22). In one sequence isopimpinellin was demethylated by heating it with anhydrous aluminum chloride to yield 5,8-dihydroxypsoralene (38) which in turn was acetylated by treating it with acetic anhydride and pyridine to yield 5,8-diacetylpsoralene (39). This latter substance was more easily handled than the former product. In the other sequence isopimpinellin underwent ozonolysis in

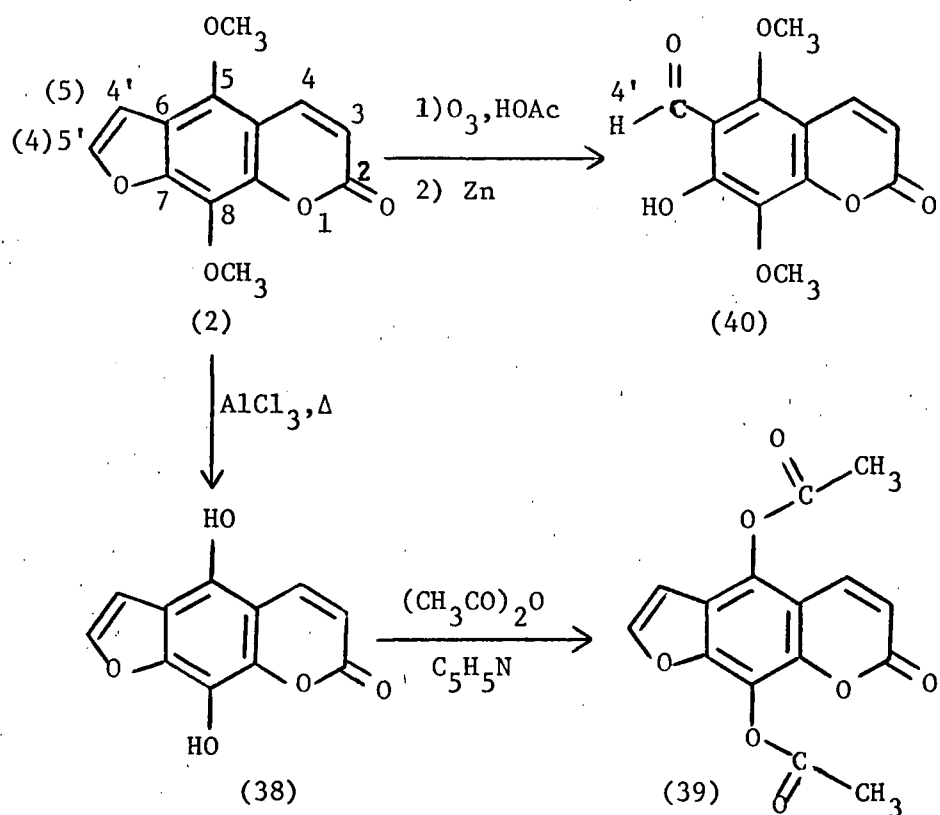
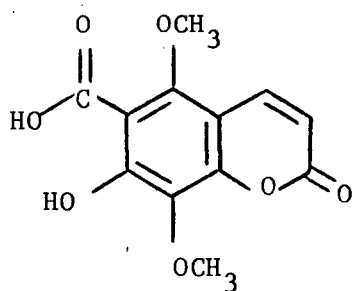


Figure 22. Degradations of isopimpinellin.

glacial acetic acid solution. The resulting ozonide was then reduced by using zinc dust to yield 5,8-dimethoxy-6-formylumbelliferone (40).

Another degradation attempted with isopimpinellin was to convert it to 5,8-dimethoxyumbelliferone-6-carboxylic acid (41). It was felt



(41)



that this compound could in turn be decarboxylated and hence this would provide a technique of isolating the carbon atom attached to position-7 of the coumarin system. In one experiment isopimpinellin was heated with dilute sulfuric acid and potassium dichromate to try and obtain the desired product.<sup>45,46</sup> In the other case isopimpinellin was subjected to ozonolysis in a glacial acetic acid solution, followed by an oxidative work-up using hydrogen peroxide. However, neither procedure produced substantial amounts of the desired carboxylic acid and further attempts to obtain it were abandoned.

(iii) Degradations of Alloimperatorin Methyl Ether

There were two degradative sequences developed in connection with alloimperatorin methyl ether (8) (Figure 23). Alloimperatorin methyl ether in both cases was converted first to alloimperatorin methyl ether diol [5-(3'-methyl-2',3'-dihydroxybutanyl)-8-methoxypsoralen] (3) via the epoxide (13). This latter compound was obtained by first treating the methyl ether with *m*-chloroperbenzoic acid in chloroform at 0°C and then heating the resulting alloimperatorin methyl ether epoxide with dilute oxalic acid.

In one sequence alloimperatorin methyl ether diol was first treated with acetic anhydride in pyridine to yield 5-(3'-methyl-3'-hydroxy-2'-acetoxybutanyl)-8-methoxypsoralene (42) which in turn was ozonized and reduced, respectively, using ozone in glacial acetic acid followed by reduction with zinc dust to yield 5-(3'-methyl-3'-hydroxy-2'-acetoxybutanyl)-6-al-8-methoxyumbelliferone (43).

In the other sequence alloimperatorin methyl ether diol was treated with periodic acid in a mixture of acetone free methanol and

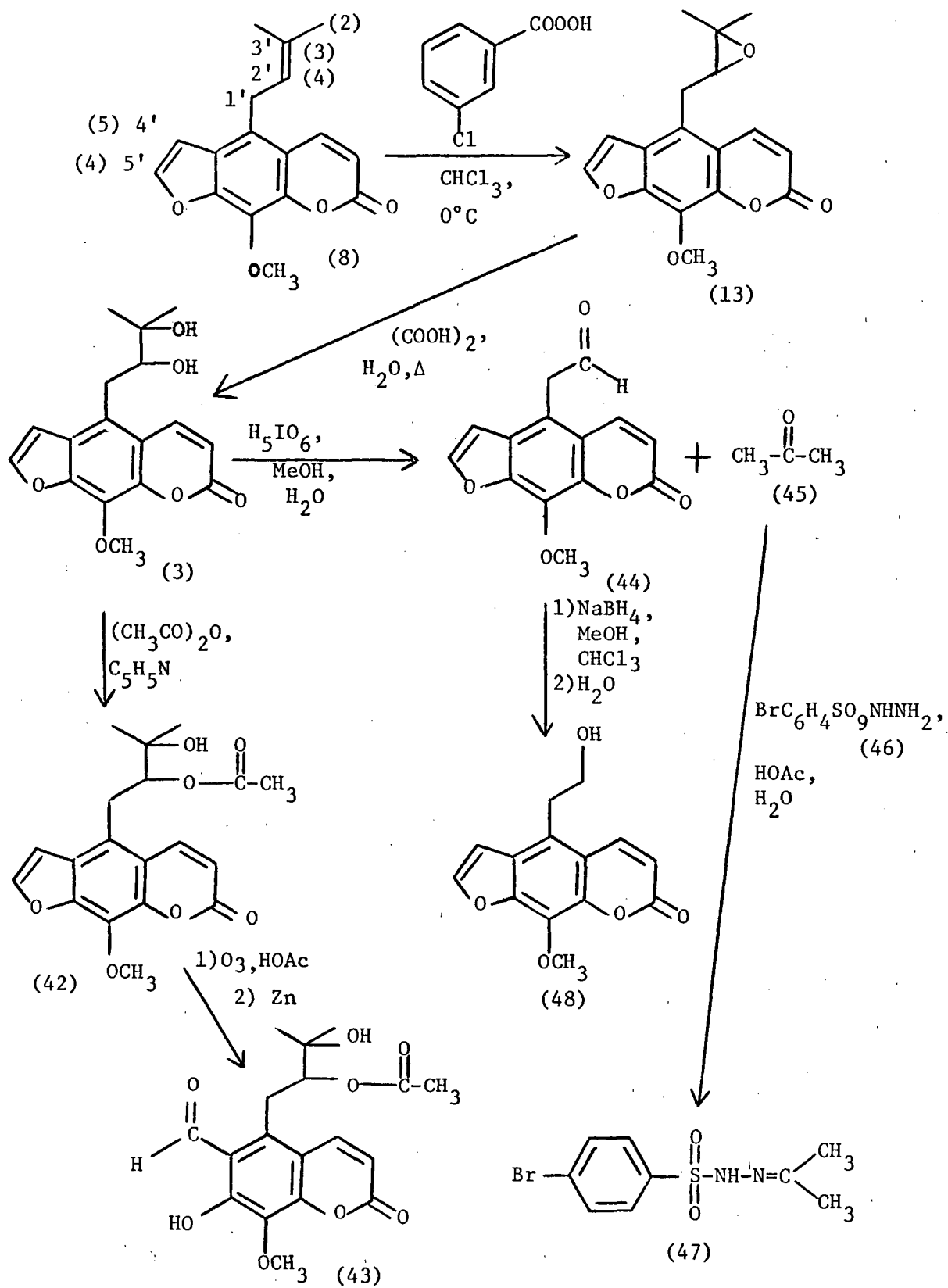
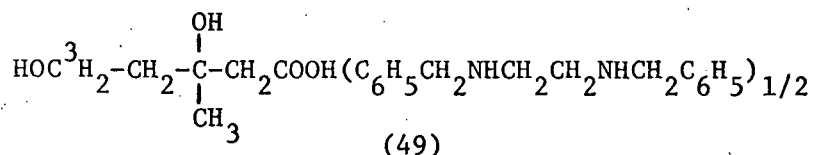


Figure 23. Degradations of alloimperatorin methyl ether.

water to yield the aldehyde, compound (44), and acetone (45). The acetone was distilled into p-bromobenzenesulfonylhydrazide (46), present in a mixture of glacial acetic acid and water, to yield acetone p-bromobenzenesulfonylhydrazone (47).<sup>47</sup> The aldehyde, compound (44), was treated with sodium borohydride in a mixture of methanol and chloroform, followed by hydrolysis with water to yield the alcohol, compound (48).

(c) Biosynthetic Investigations with Radioactive Mevalonic Acid

DL-Mevalonic-5-<sup>3</sup>H acid as its dibenzylethylenediamine salt (49) was utilized in the various experiments discussed in this thesis. The



free acid is unstable and readily lactonizes, therefore, the DBED (dibenzylethylenediamine) salt is normally employed. In preparation for the plant feedings the methanol solution of the radioactive compound was diluted to a known volume (25 ml) and aliquots of this solution were then used in the experiments described.

Similar experiments were conducted by R.N. Young and A.K. Verma using the following possible biosynthetic precursors: glycine-1-<sup>14</sup>C, glycine-2-<sup>14</sup>C, DL-mevalonic acid-2-<sup>14</sup>C, DL-mevalonic acid-4-<sup>14</sup>C, DL-mevalonic acid-2-<sup>3</sup>H, and DL-mevalonic acid-4-<sup>3</sup>H.

In the area of research with which I was directly involved, that is with the biosynthetic investigations of DL-mevalonic-5-<sup>3</sup>H acid as its dibenzylethylenediamine salt (49), Thamnosma montana plants of about 19 to 22 months of age were utilized for the various feedings.

In a typical biosynthetic feeding an aliquot of the previously mentioned solution of the radioactive compound (49) was fed to a small number of the young plants (6 to 8) for a total exposure time of about ten days. The whole plant was used for the feeding experiments conducted, the radioactive compound being incorporated through the roots with the feeding being hydroponic in nature.

After the biosynthetic feeding was completed umbelliprenin, alloimperatorin methyl ether, thamnomin, isopimpinellin, and alloimperatorin methyl ether epoxide were isolated by the procedure discussed under section (a) of the Discussion and summarized briefly in Figures 17 and 20. The activity of the combined residual aqueous solutions in which the plants were fed was determined as was the activity of the acetone and chloroform extracts. Before crystallization was utilized on each of the isolated coumarins, the amount obtained was determined and then diluted with a weighed amount of cold material previously isolated from the plant. Following this procedure, attempts were made to obtain, by crystallization techniques, constant radioactivity counts for each coumarin (Figure 24). Subsequent degradation of these compounds provides an indication of the location of the label, and, hence, allows some conclusions to be drawn concerning the biosynthesis of these compounds.

(i) Degradation of Radioactive Umbelliprenin

After radioactive umbelliprenin (11) had been recrystallized to constant radioactivity (Figure 24), the compound was degraded to umbelliferone (20) as described under Section (b) (i) of the Discussion for inactive umbelliprenin. The umbelliferone obtained was recrystallized to constant radioactivity from ethyl acetate as shown in Figure 25 after partial purification by preparative thin layer chromato-

Figure 24. Recrystallization of various coumarins to constant radioactivity.

Coumarin Isolated	Weight Isolated (mg)	Weight after Dilution (mg)	Recrystallization Number	Weight after Recrystallization (mg)	Radioactive Counts after Recrystallization (dpm/mg)	Counting Time (minutes)
umbelliprenin	24.6	40.8	1	28.6	50,900	40x3(no blank)
			2	24.5	19,610	40x3(no blank)
			3	22.2	31,750	40x3(no blank)
			4	20.6	26,790	40x3
			5	18.7	35,400	40x3
			6	17.7	25,700	40x3
			7	14.2	26,650	40x3
			8	61.1	3,710	100x3
			9	55.8	3,445	100x3
			10	53.5	3,335	100x3
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alloimperatorin methyl ether	18.1	40.1	1	29.3	24,000	40x3(no blank)
			2	24.7	5,770	40x3(no blank)
			3	22.2	1,152	40x3(no blank)
			4	19.1	343	40x3
<hr/>						
thamnosmin	21.3	31.9	1	18.7	21,600	40x3(no blank)
			2	16.1	13,450	40x3(no blank)
			3	11.5	1,542	40x3(no blank)
			4	10.2	1,155	40x3
			5	6.7	368	40x3

Figure 24. (Continued)

Coumarin Isolated	Weight Isolated (mg)	Weight after Dilution (mg)	Recrystallization Number	Weight after Recrystallization (mg)	Radioactive Counts after Recrystallization (dpm/mg)	Counting Time (minutes)
isopimpenellin	32.8	49.5	1	36.3	1,130	40x3(no blank)
			2	30.2	650	40x3(no blank)
			3	28.5	596	40x3(no blank)
			4	27.6	349	40x3
			5	19.3	186	40x3
			6	16.8	175	40x3
			7	15.6	171	40x3
		(Rediluted to 50.4)	8	45.4	37.3	40x3
		9	43.3	23.3	40x3	
		10	38.7	13.4	40x3	
<hr/>						
alloimperatorin methyl ether epoxide	17.8	34.7	1	29.2	1,812	40x3(no blank)
			2	22.4	2,035	40x3(no blank)
			3	19.4	1,405	40x3(no blank)
			4	14.6	266	40x3

graphy.

Recrystallization Number	Radioactive Counts after Recrystallization (dpm/mg)	Counting Time (minutes)
1	122	100x3
2	100	100x3
3	113	100x3

Figure 25. Recrystallization of umbelliferone to constant radio-activity.

(ii) Degradation of Radioactive Isopimpinellin

Radioactive isopimpinellin (2) was not subjected to degradative reactions due to the fact that recrystallization to constant radio-activity was very difficult and that DL-mevalonic-5-<sup>3</sup>H acid was incorporated apparently only to a very limited extent into this furanocoumarin (Figure 24).

(iii) Degradations of Radioactive Alloimperatorin Methyl Ether

Since difficulties were encountered in attempting to recrystallize alloimperatorin methyl ether (8) to constant radioactivity (Figure 24), this furanocoumarin was first partially degraded to alloimperatorin methyl ether diol [5-(3'-methyl-2',3'-dihydroxybutanyl)-8-methoxy-psoralen] (3) as described under section (b) (iii) of the Discussion. It was hoped that this latter compound could be recrystallized to constant radioactivity more easily than alloimperatorin methyl ether (8). After isolation the diol was then recrystallized to constant radioactivity from ethyl acetate as shown in Figure 26 after partial

Recrystallization Number	Weight After Recrystallization (mg )	Radioactive Counts After Recrystallization (dpm/mg)	Counting Time (minutes)
1	37.0	32.8	100x3
2	33.0	18.95	100x3
3	25.4	19.85	100x3
4	22.6	17.30	100x3

Figure 26. Recrystallization of alloimperatorin methyl ether diol to constant radioactivity.

purification by preparative thin layer chromatography.

Alloimperatorin methyl ether diol (3) was subjected to degradation to the aldehyde, compound (44), and acetone (45) which in turn were converted to the alcohol, compound (48), and acetone p-bromobenzenesulfonylhydrazone (47), respectively, as described under section (b) (iii) of the Discussion. Both compounds were partially purified by preparative thin layer chromatography and then recrystallized to constant activity. An insignificant amount of radioactivity was obtained in the acetone derivative. The alcohol, compound (48), however, had significant radioactivity (Figure 27).

Recrystallization Number	Weight After Recrystallization (mg )	Radioactive Counts After Recrystallization (dpm/mg)	Counting Time (minutes)
1	8.9	15.4	100x3
2	7.3	12.6	100x3
3	6.8	12.9	100x3

Figure 27. Recrystallization of the alcohol, compound (48), to constant radioactivity.



### Conclusions

From the degradation of radioactive umbelliprenin (11) to umbelliferone (20) as described under section (c) (i) of the Discussion, it is worthy to note that most of the radioactivity present in umbelliprenin (>95%) was lost. This indicated in turn that most of the original radioactivity present in umbelliprenin must have been present in the farnesyl side chain. Therefore this information would suggest that mevalonic acid is not a direct precursor of the coumarin nucleus or aromatic portion of umbelliprenin in Thamnosma montana Torr. and Frem. However, as expected, the results do indicate that mevalonic acid is the precursor of the farnesyl unit. The well known conversion of mevalonic acid into isopentenyl pyrophosphate and subsequently into  $\gamma,\gamma$ -dimethylallyl pyrophosphate, followed by head-to-tail condensation of three of these 5-carbon units, is obviously the pathway involved.

From the degradation of radioactive alloimperatorin methyl ether (8) to alloimperatorin methyl ether diol (3) and subsequent degradation and conversion to the alcohol, compound (48), and acetone p-bromobenzenesulfonylhydrazone (47), respectively, as described under section (c) (iii) of the Discussion, it is worthy to note that most of the radioactivity present in compound (3) (>70%) was retained in compound (48). On the other hand, only a trace of radioactivity was found in compound (47). This result was in accordance with other experimental data by other research workers and by Verma and Young in our laboratories. That is, in alloimperatorin methyl ether, it was believed that the two carbon atoms (4' and 5') in the furan ring were derived from carbon atoms 4 and 5 of mevalonic acid and that the 5-carbon side chain at

position 5 was derived also from mevalonic acid by condensation with carbon atom 5 of mevalonic acid (Figure 23). Consequently, if DL-mevalonic acid-5-<sup>3</sup>H is incorporated into alloimperatorin methyl ether as described, no appreciable loss in radioactivity should occur in the conversion to compound (48) from compound (3) as the original carbon 5 of mevalonic acid is not lost in such a sequence. The data thus described under Section (c) (iii) supports this theory.

## EXPERIMENTAL

Throughout this work column chromatography was usually performed on Woelm neutral alumina. This adsorbent was deactivated to activity IV by the addition of water as directed by the manufacturers. On occasion silica gel for chromatographic adsorption (B.D.H. reagent) was used for column chromatography. In column chromatography of the crude extract where large quantities of adsorbent were used, the adsorbent used was Shawinigan Aluminum oxide, deactivated by the addition of 6% of a 10% acetic acid solution. Except in large scale column chromatography, the solvents were distilled before use.

For analytical and preparative thin layer chromatography (TLC), Woelm neutral alumina and silica gel G (according to Stahl), containing 2% by weight of General Electric Retma p-1, Type 188-2-7 electronic phosphor, were used. The chromatograms, 0.3 mm in thickness, were air dried and activated in an oven at 100°C for three hours. For preparative thin layer chromatography a thicker layer (0.5 mm) of adsorbent was used. Detection of bands in both cases was possible with ultraviolet light.

Melting points were determined on a Kofler block and are uncorrected.

Radioactivity was measured with a Nuclear-Chicago Mark 1 Model 6860 Liquid Scintillation counter in counts per minute (cpm). The radioactivity of a sample in disintegrations per minute (dpm) was calculated

using the counting efficiency which was determined for each sample by the external standard technique<sup>48</sup> utilizing the built-in barium 133 gamma source. The radioactivity of the sample was determined using an organic scintillator solution made up of the following components: toluene (1 liter); 2,5-diphenyloxazole (4 gm ); and 1,4-bis[2-(5-phenyloxazolyl)]benzene (50 mg ) or an aqueous scintillator solution made up of the following components: toluene (385 ml); dioxane (385 ml); methanol (230 ml); naphthalene (80 gm ); 2,5-diphenyloxazole (5 gm ); and 1,4-bis[2-(5-phenyloxazolyl)]benzene (62.5 mg ). In practice an organic sample was dissolved in benzene (1 ml) in a counting vial or, in the case of a water soluble sample, the sample was dissolved in methanol (1 ml) in a counting vial. Then, in both cases, the volume was made up to 15 mls with the above organic and aqueous scintillator solutions, respectively. For each sample counted the background activity was determined for the counting vial to be used by filling the vial with one of the above scintillator solutions and counting to determine the background in cpm. The counting vial was emptied, refilled with sample to be counted and the scintillator solution, and counted again. The difference in cpm between the background count and the sample count was used for subsequent calculations.

(a) Isolation of Natural Products from *Thamnosma montana* Torr. and Frem.

(i) Extraction of *Thamnosma montana* Torr. and Frem.

The plants for this study were obtained in the desert region near Morongo Valley, California with the help of Dreyer and his associates at the Fruit and Vegetable Chemistry Laboratory, Pasadena California.

An acetone extract of the plant was also obtained from the same source. The plants were air dried and ground to a powder in a Waring blender. The air dried ground powder (393 gm ) was extracted with acetone for 6 1/2 hours in a large glass Soxhlet extractor. Filtration and evaporation of the solvent gave a crude extract (26.3 gm ) as a dark heavy tar. Extraction of the residue with chloroform and subsequent filtration and evaporation gave a dark heavy oily residue (23.8 gm ).

(ii) Column Chromatography of Chloroform Extract

The crude extract (67 gm ) was applied in chloroform to the top of a column of deactivated Shawinigan alumina (2,000 gm , deactivated by the addition of 6% by weight (120 ml ) of 10% acetic acid). Elution of the column was performed with the various solvents as illustrated.

<u>Fraction</u>	<u>Solvent (volume, ml )</u>	<u>wt (gm )</u>	<u>Compounds</u>
A	petroleum ether (2,000)	0.28	unidentified non-polar components such as hydrocarbon or waxes
B	25% benzene in petroleum ether (1,000)	0.02	
C	33 1/3% benzene in petroleum ether (1,000)	0.10	
D	50% benzene in petroleum ether (1,000)	0.37	
E	benzene (1,000)	0.30	
F	benzene (2,000)	0.86	
<hr/>			
G	50% chloroform in benzene (1,750)	1.82	primarily umbelliprenin
<hr/>			
H	chloroform (750)	0.92	umbelliprenin, iso-imperatorin, alloimperatorin methyl ether, thamnomin, and iso-pimpinellin
I	chloroform (1,125)	1.76	

<u>Fraction</u>	<u>Solvent (volume, ml )</u>	<u>wt (gm )</u>	<u>Compounds</u>
J	chloroform (875)	6.42	isopimpinellin, N-methylacridone and alloimperatorin methyl ether epoxide
K	chloroform (500)	6.93	
-----			
L	chloroform (500)	2.45	N-methylacridone and alloimperatorin methyl ether diol
-----			
M	chloroform (1,000)	1.40	primarily unidentified polar components
N	acetone (1,000)	0.77	
O	acetone (1,000)	3.43	
P	methanol (1,000)	0.24	

The numerous fractions obtained from column chromatography were examined under ultraviolet light on a thin layer silica gel G chromatoplate. Authentic samples of umbelliprenin (11), isoimperatorin (12), alloimperatorin methyl ether (8), thamnosmin (14), isopimpinellin (2), N-methylacridone (5), alloimperatorin methyl ether epoxide (13), and alloimperatorin methyl ether diol (3) were examined in a similar manner for purposes of comparison. The chromatograms were developed in an ether-hexane mixture (1:1).

Fractions A to F:

A portion of fractions A to F when examined by TLC showed only the presence of unidentified non-polar components such as hydrocarbons and waxes. Since these fractions had none of the desirable coumarin components, further examination was not conducted.

Fraction G:

A portion of fraction G when examined by TLC showed the presence of at least five compounds with one component being predominant. When compared with authentic samples, this compound proved to be umbelliprenin ( $R_f$  0.88).

Fractions H and I:

A portion of fractions H and I when examined by TLC indicated the presence of at least seven compounds. When compared with authentic samples, umbelliprenin ( $R_f$  0.88), isoimperatorin ( $R_f$  0.85), alloimperatorin methyl ether ( $R_f$  0.75), thamnomin ( $R_f$  0.67), and isopimpinellin ( $R_f$  0.50) were found present.

Fractions J and K:

A portion of fractions J and K when examined by TLC indicated the presence of at least eight compounds. When compared with authentic samples, isopimpinellin ( $R_f$  0.50), N-methylacridone ( $R_f$  0.45), and alloimperatorin methyl ether epoxide ( $R_f$  0.44) were present.

Fraction L:

A portion of fraction L when examined by TLC indicated the presence of at least three compounds. When compared with authentic samples, N-methylacridone ( $R_f$  0.45) and alloimperatorin methyl ether diol ( $R_f$  0.37) were present.

Fractions M to P:

A portion of fractions M to P when examined by TLC showed primarily unidentified polar components and these were not examined further in this study.

The compounds of particular interest, namely, umbelliprenin (11),

alloimperatorin methyl ether (8), isopimpinellin (2), and alloimperatorin methyl ether diol (3) were isolated from the various fractions of the column chromatography by additional chromatographic purification and/or crystallization.

(iii) Isolation of Umbelliprenin from Fraction G

Umbelliprenin (11), was obtained from fraction G (1.82 gm.) primarily by direct crystallization from hexane. Further amounts were obtained by preparative thin layer chromatography on the mother liquors, using thin layer silica gel G chromatoplates developed in an ether-hexane (1:1) mixture. The combined total was then recrystallized several times from hexane to yield a pure white product (0.210 gm.) which was found to be umbelliprenin when compared with an authentic sample previously isolated and characterized in our laboratories by R.N. Young.

(iv) Isolation of Coumarins from Fractions H and I

Fractions H and I (2.68 gm.) were combined and adsorbed on about 10 grams of alumina. This portion was then added to a column of alumina (activity IV, 100 gm.) and eluted with various solvents as illustrated.

<u>Fraction</u>	<u>Solvent (volume, ml.)</u>	<u>Wt (mg.)</u>	<u>Compounds</u>
A	petroleum ether (150)	36	unidentified non-polar components
B	20% benzene in petroleum ether (150)	249	primarily umbelliprenin
C	25% benzene in petroleum ether (150)	564	umbelliprenin, and isoimperatorin
D	33 1/3% benzene in petroleum ether (80)	339	umbelliprenin, isoimperatorin, alloimperatorin methyl ether, and thamnomin



<u>Fraction</u>	<u>Solvent (volume, ml )</u>	<u>Wt (mg )</u>	<u>Compounds</u>
E	50% benzene in petroleum ether (100)	447	isoimperatorin, allo-imperatorin methyl ether, thamnomin, and iso-pimpinellin
F	benzene (80)	315	isoimperatorin, allo-imperatorin methyl ether, thamnomin, and iso-pimpinellin
G	chloroform (140)	315	isoimperatorin, allo-imperatorin methyl ether, thamnomin, and iso-pimpinellin
H	chloroform (45)	27	primarily unidentified polar components
I	chloroform (200)	48	

The fractions from the column chromatography were examined under ultraviolet light on a thin layer silica gel G chromatoplate, developed in an ether-hexane (1:1) mixture. Preparative thin layer chromatography, using similar plates and solvent mixture, was used to obtain the desirable coumarins from fractions B to G, inclusive. Further amounts of umbelliprenin (0.323 gm ) were obtained from fractions B, C, and D by preparative thin layer chromatography, followed by crystallization from hexane. Preparative thin layer chromatography on fractions C to G, inclusive, yielded isoimperatorin (0.258 gm ) which was crystallized from a mixture of hexane and ethyl acetate. Preparative thin layer chromatography on fractions D to G, inclusive, yielded alloimperatorin methyl ether (0.238 gm ) and thamnomin (0.158 gm ) which were both crystallized from a mixture of hexane and ethyl acetate. Preparative

thin layer chromatography on fractions E, F, and G yielded isopimpinellin (0.203 gm ) which was crystallized from a mixture of hexane and ethyl acetate. The coumarins isolated were identified by comparison with authentic samples previously isolated and characterized in our laboratories by R.N. Young.

(v) Isolation of Coumarins from Fractions J and K

Fractions J and K (~10 gm. ) were combined and adsorbed on about 40 gm of alumina. This portion was then added to a column of alumina (Activity IV, 400 gm ) and eluted with various solvents as illustrated.

<u>Fraction</u>	<u>Solvent (volume, ml )</u>	<u>wt (mg )</u>	<u>Compounds</u>
A	33 1/3% benzene in petroleum ether		
B	50% benzene in petroleum ether		
C	33 1/3% petroleum ether in benzene		
D	benzene (300)	288	unidentified non-polar components
E	benzene (200)	577	
F	benzene (125)	519	
G	benzene (300)	2,035	isopimpinellin and N-methylacridone
H	benzene (250)	2,690	primarily N-methylacridone
I	chloroform (250)	211	primarily N-methylacridone, but some alloimperatorin methyl ether epoxide
J	chloroform (1,000)	264	N-methylacridone and unidentified polar components
K	acetone (1,000)	98	

The fractions from the column chromatography were examined under ultraviolet light on a thin layer silica gel G chromatoplate, developed in an ether-hexane (1:1) mixture. Isopimpinellin was crystallized directly out of fraction G and alloimperatorin methyl ether epoxide was crystallized directly out of fraction I, using a hexane-ethyl acetate solvent mixture in both cases. Further amounts of the two compounds were obtained by preparative thin layer chromatography on the mother liquors of the respective crystallizations. The combined total of each compound was then recrystallized from an ethyl acetate-hexane mixture to yield pure isopimpinellin (0.692 gm ) and pure alloimperatorin methyl ether epoxide (0.057 gm ). The coumarins were identified by comparison with authentic samples previously isolated and characterized in our laboratories by R.N. Young.

(vi) Isolation of Alloimperatorin Methyl Ether Diol from Fraction L

Alloimperatorin methyl ether diol (3) was obtained from fraction L (2.45 gm ) primarily by direct crystallization from ethyl acetate. Further amounts were obtained by preparative thin layer chromatography on the mother liquors, using silica gel G chromatoplates developed in an ether-hexane (1:1) mixture. The combined total was then recrystallized from ethyl acetate to yield a pale yellow product (0.772 gm ) which was found to be alloimperatorin methyl ether diol when compared with an authentic sample previously isolated and characterized by D.L. Dreyer.<sup>4</sup>

(b) Development of Appropriate Chemical Degradation Pathways

(i) Degradation of Umbelliprenin (11)

Umbelliprenin (49.8 mg ) was dissolved in glacial acetic acid (1.5 ml ) and concentrated sulfuric acid (4 drops). The reaction mixture was stirred at room temperature for 50 hours. The mixture was treated with sodium hydroxide (5%, 50 ml ) and extracted with ether to remove unreacted material. The aqueous solution was acidified with hydrochloric acid (20%, 50 ml ) and extracted with ether. The ether extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. Preparative thin layer chromatography, using a silica gel G chromatoplate developed in an ether-hexane mixture (3:1), was used to obtain the product which appeared as a bright blue band near the baseline. The compound was crystallized from ethyl acetate to yield 18.6 mg of the desired product. Comparison of the latter by mixed melting point and TLC with a commercially available authentic sample of umbelliferone (20), m.p. 230-231°C, indicated the identity of these compounds.

(ii) Degradations of Isopimpinellin (2)

Degradation of Isopimpinellin (2) to 5,8-Dimethoxy-6-formylumbelliferone (40)

An ozonolysis of isopimpinellin (2) was conducted, using an ozonolysis medium prepared by passing ozone from a Welsbach Ozonator through acetic acid (170 ml ) for 1 1/4 hours. Isopimpinellin (120 mg , 0.5 mmole) was dissolved in a small amount of acetic acid and added to the solution containing ozone (0.91 mmole). The reaction mixture was stirred for 3 3/4 hours followed by reduction with zinc dust (492 mg , 7.6 mmole) for several hours. The solution was filtered, evaporated to small volume

(~25 ml ) and finally taken to dryness in vacuo. The residue (131 mg ) was dissolved in chloroform and subjected to column chromatography, using silica gel (25 gm ) and the various solvents as illustrated.

<u>Fraction</u>	<u>Solvent (volume, ml )</u>	<u>wt (mg )</u>	<u>Compounds</u>
A	benzene (125)	10	unidentified non-polar components
B	33 1/3% chloroform in benzene (125)	1	
C	50% chloroform in benzene (125)	1	
D	33 1/3% benzene in chloroform (125)	2	
<hr/>			
E	chloroform (125)	25	primarily 5,8-dimethoxy-6-formyl-umbelliferone
F	chloroform (125)	34	
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G	chloroform (125)	10	unidentified polar components and possibly some 5,8-dimethoxyumbelliferone-6-carboxylic acid
H	chloroform (125)	12	
I	acetone (125)	15	
J	acetone (125)	17	
K	acetone (125)	5	
L	methanol (250)	30	

Fractions C to K, inclusive, from the column chromatography were examined under ultraviolet light on a thin layer silica gel G chromatoplate, developed in an ethyl acetate-chloroform (2:1) mixture.

Preparative thin layer chromatography on fractions E and F yielded a light yellow product (24.8 mg ). Crystallization from acetone gave an analytical sample, m.p. 214-216°C, which was identified as 5,8-dimethoxy-

6-formylumbelliferone (40) when compared with an authentic sample previously isolated and characterized in our laboratories by R.N. Young. IR (KBr) 1758, 1730 ( $-\text{CO}-\text{O}-\text{C}=\text{C}-$ , aldehyde carbonyl); 1640, 1593 (aromatic ring). UV  $\lambda_{\text{max}}^{\text{MeOH}}$  226  $\text{m}\mu$  (sh,  $\log \epsilon$  4.02), 275  $\text{m}\mu$  ( $\log \epsilon$  4.43);  $\lambda_{\text{max}}^{\text{MeOH}}$  (NaOH added) 238  $\text{m}\mu$  ( $\log \epsilon$  4.26), 269  $\text{m}\mu$  ( $\log \epsilon$  4.20), 299  $\text{m}\mu$  ( $\log \epsilon$  4.08), 360  $\text{m}\mu$  ( $\log \epsilon$  4.16);  $\lambda_{\text{max}}^{\text{MeOH}}$  (HCl added) 226  $\text{m}\mu$  (sh,  $\log \epsilon$  4.17), 263  $\text{m}\mu$  ( $\log \epsilon$  4.08), 320  $\text{m}\mu$  ( $\log \epsilon$  4.16). NMR (100 MHz) -2.03 (1H, singlet, phenolic OH), -0.23 (1H, singlet,  $-\text{CHO}$ ), 2.15 (1H, doublet,  $J = 9.5$  Hz,  $\text{H}-\text{C}_4$  coumarin), 3.72 (1H, doublet,  $J = 9.5$  Hz,  $\text{H}-\text{C}_3$  coumarin), 6.00 (3H, singlet, OMe), and 6.02 (3H, singlet, OMe).

Anal. Calcd. for  $\text{C}_{12}\text{H}_{10}\text{O}_6$ : C, 57.60; H, 4.00. Found: C, 57.38; H, 4.07.

Degradations of Isopimpinellin (2) to 5,8-Dimethoxyumbelliferone-6-carboxylic Acid (41)

Isopimpinellin (56 mg) was reacted with sulfuric acid (3%, 10 ml) and potassium dichromate (169 mg) dissolved in water (2 ml) for 6 hours under reflux. The reaction mixture was extracted with a wide variety of solvents, but no stable product was obtained from these or the residual aqueous reaction mixture.

An ozonolysis of isopimpinellin was attempted, using an ozonolysis medium prepared by passing ozone through acetic acid (140 ml) for 1 hour. Isopimpinellin (120 mg, 0.5 mmole) was dissolved in a small amount of acetic acid and added to the solution containing ozone (0.75 mmole). The reaction mixture was stirred for 4 3/4 hours

followed by oxidation with 30% hydrogen peroxide (0.17 gm , 1.5 mmole) for 1 hour. The solvent was removed in vacuo and the residue (139 mg) dissolved in chloroform and subjected to column chromatography, using silica gel (12 gm) and the various solvents as illustrated.

<u>Fraction</u>	<u>Solvent (volume, ml )</u>	<u>wt (mg )</u>	<u>Compounds</u>
A	benzene (100)	13	unidentified non-polar components
B	33 1/3% chloroform in benzene (50)	3	
C	50% chloroform in benzene (50)	3	
D	33 1/3% benzene in chloroform (50)	2	
E	chloroform (15)	2	
<hr/>			
F	chloroform (25)	29	primarily 5,8-dimethoxy-6-formyl-umbelliferone
<hr/>			
G	chloroform (25)	15	5,8-dimethoxy-6-formylumbelliferone and possibly some 5,8-dimethoxy-umbelliferone-6-carboxylic acid
H	chloroform (50)	21	
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I	50% acetone in chloroform (25)	21	possibly 5,8-dimethoxy-umbelliferone-6-carboxylic acid
<hr/>			
J	acetone (125)	30	unidentified polar components
K	acetone (125)	13	
L	methanol (250)	31	

The fractions from the column chromatography were examined under ultraviolet light on a thin layer silica gel G chromatoplate, developed in an ethyl acetate-chloroform (1:1) mixture. Preparative thin layer chromatography on fractions F, G, and H yielded some of the aldehyde (27.6 mg), 5,8-dimethoxy-6-formylumbelliferone (40). However, no significant amounts of the carboxylic acid, 5,8-dimethoxyumbelliferone-6-carboxylic acid (41) was obtained either by preparative TLC on fractions G and H followed by sublimation or by direct sublimation of fraction I.

(iii) Degradations of Alloimperatorin Methyl Ether (8)

Conversion of Alloimperatorin Methyl Ether (8) to Alloimperatorin Methyl Ether Diol (3):

Alloimperatorin methyl ether (67.8 mg, 0.239 mmole) and m-chloroperbenzoic acid (48.8 mg, 0.284 mmole) were dissolved in separate portions (6 1/2 ml) of chloroform. The solutions were cooled to 0°C, mixed, and stirred for 11 hours at ice bath temperature. The reaction mixture was washed with 5% sodium bicarbonate and water, respectively. Evaporation to dryness and crystallization from an ethyl acetate-hexane mixture yielded a pale yellow solid identified as alloimperatorin methyl ether epoxide (13), m.p. 103-104°C, when compared with an authentic sample previously isolated and characterized in our laboratories by R.N. Young. IR (KBr) 1717 (C=O), 1588 (C=C). UV  $\lambda_{\text{max}}^{\text{MeOH}}$  221 m $\mu$  (log  $\epsilon$  4.40), 244 m $\mu$  (sh, log  $\epsilon$  4.30), 251.5 m $\mu$  (log  $\epsilon$  4.36), 266 m $\mu$  (log  $\epsilon$  4.31), 305 m $\mu$  (log  $\epsilon$  4.11). NMR (100 MHz) in CDCl<sub>3</sub>, TMS lock, 1.82 (1H, doublet, J = 10 Hz, H-C<sub>4</sub> furanocoumarin), 2.33 (1H, doublet, J = 2 Hz, H-C<sub>7</sub> furanocoumarin), 3.11 (1H, doublet, J = 2 Hz, H-C<sub>6</sub> furano-



coumarin), 3.64 (1H, doublet,  $J = 10$  Hz, H-C<sub>3</sub> furanocoumarin), 5.78 (3H, singlet, OMe), 6.5-7.2 (3H; ABC multiplet;  $J_{AB} = 14.5$  Hz,  $J_{AC} = 3$  Hz,  $J_{BC} = 7.5$  Hz; benzylic methylene and epoxide protons), 8.51 and 8.70 (6H, two singlets,  $\text{>C} \begin{array}{c} \text{O} \\ \diagup \quad \diagdown \\ \text{C} \quad \text{C} \end{array} \text{C(CH}_3)_2$ ). Mass spectrum (m/e) 300(M), 285 (M-15), 271(M-29), 257(M-43), 229(M-71), 201, 199, 186, 171, and 158.

Anal. Calcd. for C<sub>17</sub>H<sub>16</sub>O<sub>5</sub>: C, 67.99; H, 5.37. Found: C, 67.91; H, 5.98.

Alloimperatorin methyl ether epoxide was treated with oxalic acid (5%, 25 ml ) for 2 hours under reflux. The reaction mixture was extracted with chloroform which was washed with 5% sodium carbonate and water, respectively. The chloroform solution was dried with anhydrous magnesium sulfate and evaporated to dryness to yield a pale yellow solid (75.5 mg ). Preparative thin layer chromatography, using silica gel G chromatoplates developed in a chloroform-ethyl acetate (1:1) mixture yielded a pale yellow solid (65.8 mg ). Crystallization from ethyl acetate gave an analytical sample, m.p. 174-176°C, identified as alloimperatorin methyl ether diol (40.7 mg ) when compared with characterization data previously acquired for the compound by D.L. Dreyer.<sup>4</sup>

$[\alpha]_D^{23} -30.6^\circ$  (95% EtOH). IR (nujol) 3410 (hydroxyl), 1713 (C=O), 1592 (C=C). UV  $\lambda_{\text{max}}^{\text{EtOH}}$  220 m $\mu$  (log  $\epsilon$  4.46),  $\sim$ 244 m $\mu$  (log  $\epsilon$  4.19), 251 m $\mu$  (log  $\epsilon$  4.23), 266 m $\mu$  (log  $\epsilon$  4.19), 309 m $\mu$  (log  $\epsilon$  4.06). NMR (60 MHz) in CDCl<sub>3</sub>-deuteriodimethylsulfoxide, TMS lock, 1.75 (1H, doublet,  $J = 10$  Hz, H-C<sub>4</sub> furanocoumarin), 2.25 (1H, doublet,  $J = 2$  Hz, H-C<sub>7</sub> furanocoumarin), 3.00 (1H, doublet,  $J = 2$  Hz, H-C<sub>6</sub> furanocoumarin), 3.73 (1H, doublet,  $J = 10$  Hz, H-C<sub>3</sub> furanocoumarin), 5.83 (3H, singlet, OMe), 6.53 (1H, triplet,  $J = 7$  Hz, H-C<sub>2</sub>, furanocoumarin), 6.87 (2H, doublet,  $J = 7$  Hz, benzylic protons),

8.70 (6H, singlet, C-methyls).

Degradation of Alloimperatorin Methyl Ether Diol (3):

Acetone free chloroform was prepared by passing chloroform through a celite column impregnated with 2,4-dinitrophenylhydrazine and distilling the eluant. Acetone free methanol was prepared by the addition of iodine (25 gm ) to methanol (1,000 ml ), followed by addition of the mixture to sodium hydroxide (500 ml , 1 N) with constant stirring. Water (150 ml ) was added to precipitate iodoform. The mixture was filtered, refluxed to remove the iodoform odours, and finally distilled.

Alloimperatorin methyl ether diol (21.3 mg , 0.0671 mmole) was dissolved in acetone free methanol (3 ml ) and periodic acid (47.8 mg 0.215mmole) was dissolved in water (3 ml ). The solutions were mixed and stirred for ~24 hours. Nitrogen was flushed slowly through the reaction mixture, which was maintained at ~ 50°C, in order to distill over any acetone formed during the course of the reaction. The acetone was collected as a derivative (47) by distillation into p-bromobenzenesulfonhydrazide (150 mg ), dissolved in a mixture of water (5 ml ) and glacial acetic acid (5 ml ). Prior to this distillation a blank was distilled into the reagent in a similar manner. Both the blank and the reaction mixture were extracted with acetone free chloroform. The extracts were washed with water, dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. Preparative thin layer chromatography, using Woelm neutral alumina chromatoplates developed in a

chloroform-methanol (20:1) mixture was used to isolate the derivative, acetone p-bromobenzenesulfonylhydrazone ( $R_f$  0.62), separate from the reagent, p-bromobenzenesulfonylhydrazide ( $R_f$  0.76). The blank was discarded as no significant amount of derivative was apparent when the chromatoplate was analyzed under ultraviolet light. Crystallization from chloroform yielded a white analytical sample (10.4 mg), m.p. 146-148°C, identified as the derivative, acetone p-bromobenzenesulfonylhydrazone (47) when compared with an authentic sample previously isolated and characterized in our laboratories by R.N. Young. IR (KBr) 3220 ( $>N-H$ ); 1343, 1180 ( $>SO_2$ ). UV  $\lambda_{max}^{MeOH}$  235 m $\mu$  (log  $\epsilon$  4.15). NMR (100 MHz) in  $CDCl_3$ , TMS lock, 2.1-2.6 (4H,  $A_2B_2$  multiplet, aromatic protons), 3.24 (1H, broad singlet,  $>N-H$ ), 8.12 and 8.24 (6H, two singlets,  $-N=C-(CH_3)_2$ ). Mass spectrum (m/e) 292 and 290 (M).

Anal. Calcd. for  $C_9H_{11}N_2O_2SBr$ : C, 37.18; H, 3.81; N, 9.62. Found: C, 36.86; H, 3.88; N, 9.40.

The aqueous non-volatile portion of the reaction mixture was extracted with chloroform. The extract was washed with water, dried with anhydrous sodium sulfate, and evaporated to dryness to yield an aldehyde, compound (44), as a crude residue, identified by TLC comparison to a sample previously isolated and partially characterized in our laboratories by R.N. Young. NMR (100 MHz) in  $CDCl_3$ , TMS lock, 0.19 (1H, singlet,  $-CHO$ ), 1.83 (1H, doublet,  $J = 10$  Hz,  $H-C_4$  furanocoumarin), 2.02 (1H, doublet,  $J = 2$  Hz,  $H-C_7$  furanocoumarin), 2.87 (1H, doublet,  $J = 2$  Hz,  $H-C_6$  furanocoumarin), 3.65 (1H, doublet,  $J = 10$  Hz,  $H-C_3$  furanocoumarin), 5.62 (2H, singlet, benzylic protons), 5.82 (3H, singlet, OMe). Mass spectrum (m/e) 258(M), 229(M-29), 214(M-44), 201, 186, and 158.

The residue was dissolved in a mixture of chloroform and methanol (1:1) and cooled to 0°C. Sodium borohydride (41.6 mg) was dissolved in methanol (~1 ml) and added to the solution of the aldehyde. The reduction was conducted for 1 hour with stirring followed by hydrolysis with water (~10 ml) for 1 hour with stirring. The reaction mixture was extracted with chloroform. The extract was washed with water, dried with anhydrous sodium sulfate, filtered, and evaporated to dryness to yield the crude product. Preparative thin layer chromatography, using silica gel G chromatoplates developed in an ethyl acetate-chloroform (2:1) mixture yielded the desired product. Crystallization from ethyl acetate gave a pale yellow analytical sample (5.6 mg), m.p. 167-167.5°C, identified as the alcohol, compound (48), when compared with an authentic sample previously isolated and characterized in our laboratories by R.N. Young. IR (KBr) 3450 (OH), 1705-1690 (C=O), 1585 (C=C). UV  $\lambda_{\text{max}}^{\text{MeOH}}$  220 m $\mu$  (log  $\epsilon$  4.39), 251 m $\mu$  (log  $\epsilon$  4.32), 265 m $\mu$  (log  $\epsilon$  4.24), 306 m $\mu$  (log  $\epsilon$  4.11). NMR (100 MHz) in CDCl<sub>3</sub>-D<sub>2</sub>O, TMS lock, 1.92 (1H, doublet, J = 10 Hz, H-C<sub>4</sub> furanocoumarin), 2.32 (1H, doublet, J = 2 Hz, H-C<sub>7</sub> furanocoumarin), 3.12 (1H, doublet, J = 2 Hz, H-C<sub>6</sub> furanocoumarin), 3.66 (1H, doublet, J = 10 Hz, H-C<sub>3</sub> furanocoumarin), 5.78 (3H, singlet, OMe), 6.08 (2H, triplet, J = 6 Hz, -CH<sub>2</sub>OH), 6.74 (3H, triplet, J = 6 Hz, benzylic protons). Mass spectrum (m/e) 260(M), 229(M-31), 214(M-46), 201, 186, and 158.

Anal. Calcd. for C<sub>14</sub>H<sub>12</sub>O<sub>5</sub>: C, 64.62; H, 4.62. Found: C, 64.66; H, 4.61. Mol. Wt. Calcd. for C<sub>14</sub>H<sub>12</sub>O<sub>5</sub>: 260.068. Found (high resolution mass spectrometry): 260.070.

(c) Biosynthetic Investigations with Radioactive Mevalonic Acid

DL-Mevalonic-5-<sup>3</sup>H acid as its dibenzylethylenediamine salt (49) was obtained from the New England Nuclear Corporation for the purpose of conducting the biosynthetic investigation. The radioactivity of the DL-mevalonic-5-<sup>3</sup>H acid (DBED salt) was 1.0 millicuries ( $2.2 \times 10^9$  dpm) and the specific activity was 221 millicuries per millimole. The radioactive compound (1.21 mg) was obtained dissolved in methanol (1.0 ml). In preparation for the biosynthetic feeding the methanol solution of the radioactive compound was diluted with distilled water to a known volume (25 ml) in a volumetric flask.

(i) Feeding of DL-Mevalonic-5-<sup>3</sup>H Acid (DBED Salt)

In a typical biosynthetic feeding an aliquot ( $6.4060 \text{ gm}, 6.4060/25 \times 2.2 \times 10^9 \text{ dpm} = 5.64 \times 10^8 \text{ dpm}$ ) of the solution of the radioactive compound was fed to six young Thamnosma montana plants (~19 months old) for a total exposure time of about 10 days. The aliquot was divided up approximately into three equal portions, each portion to be fed to two plants contained in a test tube. The whole plant was used for the feeding which was hydroponic in nature with the radioactive compound being incorporated through the roots. After the plants had taken up the initial radioactive solution, the test tubes were maintained full with distilled water to a level just near the top of the roots.

(ii) Isolation of Coumarins from the Biosynthetic Feeding

The six plants from the biosynthetic feeding were ground to a powder in a Waring blender. The ground powder (~11 gm) was extracted with acetone (750 ml) for 9 3/4 hours in a Soxhlet extractor. Filtration

and evaporation of the solvent yielded a dark residue (779.4 mg ).

Extraction of the residue with chloroform and subsequent filtration and evaporation yielded a further dark residue (549.3 mg ). The total radioactivity of the combined residual aqueous solutions in which the plants were fed was determined ( $7.41 \times 10^6$  dpm) as was the total radioactivity of the acetone extract ( $23.2 \times 10^7$  dpm) and the chloroform extract ( $14.6 \times 10^7$  dpm). From the total radioactivity recovered from outside the plants, the corrected radioactivity fed was established ( $5.64 \times 10^8$  dpm -  $0.0741 \times 10^8$  dpm =  $5.57 \times 10^8$  dpm).

The crude extract (549.3 mg ) was adsorbed on about 3 grams of alumina. This portion was then added to a column of alumina (Activity IV, 30 gm ) and eluted with the various solvents as illustrated.

<u>Fraction</u>	<u>Solvent (volume, ml )</u>	<u>Wt (mg )</u>	<u>Compounds</u>
A	petroleum ether (125)	63	unidentified non-polar compounds such as hydrocarbons and waxes
B	petroleum ether (125)	11	
C	25% benzene in petroleum ether (125)	24	
D	33% benzene in petroleum ether (125)	56	umbelliprenin, allo-imperatorin methyl ether, and thamnomin
E	50% benzene in petroleum ether (125)	26	
F	benzene (75)	16	umbelliprenin, allo-imperatorin methyl ether, thamnomin, and isopimpinellin
G	benzene (50)	15	isopimpinellin and N-methylacridone
H	benzene (50)	9	

<u>Fraction</u>	<u>Solvent (volume, ml )</u>	<u>Wt (mg )</u>	<u>Compounds</u>
I	benzene (125)	28	chlorophylls, isopimpinellin, alloimperatorin methyl ether epoxide, and N-methylacridone
J	chloroform (125)	16	primarily unidentified polar components
K	chloroform (125)	19	
L	chloroform (125)	11	

The fractions from the column chromatography were examined under ultraviolet light on a thin layer silica gel G chromatoplate, developed in an ether-hexane (1:1) mixture. Authentic samples of umbelliprenin (11), isoimperatorin (12), alloimperatorin methyl ether (8), thamnomin (14), isopimpinellin (2), N-methylacridone (5), alloimperatorin methyl ether epoxide (13), and alloimperatorin methyl ether diol (3) were examined in a similar manner for purposes of comparison. Preparative thin layer chromatography was used to obtain umbelliprenin (24.6 mg ), alloimperatorin methyl ether (18.1 mg), thamnomin (21.3 mg), isopimpinellin (32.8 mg), and alloimperatorin methyl ether epoxide (17.8 mg), identified by comparison to authentic samples previously isolated and characterized in our laboratories.

In preparation for degradative work to be performed on some of the coumarins isolated, each coumarin was diluted with a weighed amount of cold material previously isolated from the plant. Attempts were made to obtain by crystallization techniques, constant radioactivity counts for each coumarin as illustrated.

Coumarin Isolated	Weight Isolated (mg)	Weight After Dilution (mg)	Recrystallizing Solvent	Weight After Final Recrystallization (mg)	Radioactive Counts After Final Recrystallization (dpm/mg)	Radioactive Status of Coumarin
umbelliprenin	24.6	40.8 (first dilution) 64.2 (second dilution)	hexane	53.5	3,335	constant (incorporation 0.032%)
alloimperatorin methyl ether	18.1	40.1	hexane and ethyl acetate	19.1	343	not constant
thamnosmin	21.3	31.9	hexane and ethyl acetate	6.7	368	not constant
isopimpinellin	32.8	49.5 (first dilution) 50.4 (second dilution)	hexane and ethyl acetate	38.7	13.4	not constant
alloimperatorin methyl ether epoxide	17.8	34.7	hexane and ethyl acetate	14.6	266	not constant



(iii) Degradation of Radioactive Umbelliprenin

Radioactive umbelliprenin (53.5 mg ,3.335 dpm/mg) was dissolved in glacial acetic acid (2 ml )and concentrated sulfuric acid (5 drops). The reaction mixture was treated with sodium hydroxide (5%, 50 ml )and extracted with ether. The aqueous solution was acidified with hydrochloric acid (20%, 50 ml )and extracted with ether. The ether extract was dried over anhydrous sodium sulfate, filtered, and evaporated to dryness. Preparative thin layer chromatography, using a silica gel G chromatoplate developed in an ether-hexane (3:1) mixture was used to obtain the product (21.5 mg ),identified as umbelliferone (20) by comparison with a commercially available authentic sample. Umbelliferone was recrystallized several times from ethyl acetate to constant radioactivity (14.0 mg ,122 dpm/mg; 12.2 mg ,100 dpm/mg; 10.4 mg ,113 dpm/mg).

(iv) Degradations of Radioactive Alloimperatorin Methyl Ether

Since radioactive alloimperatorin methyl ether (19.1 mg , 343 dpm/mg) was not recrystallized to constant radioactivity efficiently, it was diluted again (to 68.6 mg.)with cold material and converted to alloimperatorin methyl ether diol which was recrystallized to constant radioactivity.

Conversion of Radioactive Alloimperatorin Methyl Ether (8) to Radioactive Alloimperatorin Methyl Ether Diol (3):

Radioactive alloimperatorin methyl ether (68.8 mg ,0.243 mmole) and m-chloroperbenzoic acid (47.6 mg ,0.278mmole)were dissolved in separate portions (6 1/2 ml )of chloroform. The solutions were cooled to 0°C,

mixed, and stirred for 12 1/2 hours at ice bath temperature. The reaction mixture was washed with 1% sodium bicarbonate and water, respectively. The chloroform extract was dried with anhydrous sodium sulfate, filtered, and evaporated to dryness to yield crude radioactive alloimperatorin methyl ether epoxide (13), identified by TLC comparison to an authentic sample.

Radioactive alloimperatorin methyl ether epoxide was treated with oxalic acid (5%, 26 ml )for 3 hours under reflux. The reaction mixture was extracted with chloroform. The chloroform extract was washed with 5% sodium carbonate and water, respectively, followed by drying with anhydrous sodium sulfate and evaporation to dryness to yield the crude product (62.8 mg ). Preparative thin layer chromatography, using silica gel G chromatoplates developed in a chloroform-ethyl acetate (1:1) mixture yielded a pale yellow solid (46.1 mg ), identified as alloimperatorin methyl ether diol (3) by TLC comparison to an authentic sample. Alloimperatorin methyl ether diol was recrystallized several times from ethyl acetate to constant radioactivity (37.0 mg , 32.8 dpm/mg; 33.0 mg , 18.95 dpm/mg; 25.4 mg , 19.85 dpm/mg; 22.6 mg , 17.30 dpm/mg). This represented an incorporation of 0.00007% into the original alloimperatorin methyl ether.

Degradation of Radioactive Alloimperatorin Methyl Ether Diol (3):

Radioactive alloimperatorin methyl ether diol (22.3 mg , 0.0704 mm) was dissolved in acetone free methanol (3 ml )and periodic acid (47.4 mg , 0.213mmole)was dissolved in water (3 ml ). The solutions were mixed and stirred for ~24 hours. Acetone, formed during the course of the reaction was isolated as a derivative (47) in a similar manner as it

was during the reaction previously conducted on cold material. Crystallization from chloroform yielded the purified derivative (8.0 mg ), identified as acetone p-bromobenzenesulfonhydrazone (47) by TLC comparison to an authentic sample. The derivative when counted, however, displayed insignificant radioactivity.

The aqueous non-volatile portion of the reaction mixture was worked up as in the reaction previously conducted on cold material to yield crude aldehyde, compound (44), identified by TLC comparison with an authentic sample. Reduction with sodium borohydride (42.5 mg ) and subsequent hydrolysis and workup as before yielded crude alcohol (9.1 mg), compound (48), identified by TLC comparison with an authentic sample. This degradation product was recrystallized several times from ethylacetate to constant radioactivity (8.9 mg ,15.4 dpm/mg; 7.3 mg , 12.6 dpm/mg; 6.8 mg ,12.9 dpm/mg).

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