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BIOSYNTHETIC STUDIES IN THE COUMARIN SERIES—III[1]

STUDIES IN TISSUE CULTURES OF *THAMNOSMA MONTANA* TORR. AND FREM. THE ROLE OF MEVALONATE†

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Abstract—Feeding experiments with various labeled forms of mevalonic acid were carried out with *Thamnosma montana* tissue cultures. Three furanocoumarins, isopimpinellin (2), alloimperatorin methyl ether (3) and isoimperatorin (4) were isolated and the position of label was determined through specific degradations. Mevalonic acid was shown to be a specific precursor of the furan ring and the alkyl and alkyl-ether side chains of these furanocoumarins.

Plant tissue culture systems have often been used to study many fundamental problems of plant cytology and physiology. Since the early publications by Tuleck[2] and Nickell,[3] there has been considerable interest in the possibility of using plant tissue cultures for secondary product biosynthesis. It has long been known that tobacco root cultures can biosynthesize the alkaloids nicotine[4] and anabasine.[5] Reinhard[6] and Steck[7] have isolated various coumarins and alkaloids from the cell cultures of *Ruta Graveolens* and Ourisson *et al.*[8] have studied the biosynthesis of the coumarin, scopoletin from tobacco tissue cultures. Since tissue cultures can be manipulated to minimize woody or conductive tissue which may be of low metabolic activity, they often contain a much higher percentage of actively metabolizing cells than normal plants do and therefore, frequently higher level of incorporation of precursors of certain metabolites can be achieved in plant tissue cultures than in normally grown plants. Since various workers[9–11] have reported very poor incorporation of mevalonic acid in furanocoumarins in various plant systems, it was decided to study the role of mevalonic acid in the biosynthesis of furanocoumarins of *Thamnosma montana* tissue cultures in the hope that this medium will prove more conducive to efficient utilization of mevalonic acid.

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

Preliminary studies on the constituents of tissue cultures of *Thamnosma montana* revealed the presence of isoimperatorin (4) alloimperatorin methyl ether (3) and isopimpinellin (2) in isolable quantities whereas no umbelliprenin (1) could be detected. However, no attempt was made to identify other constituents of the tissue culture extract. It was considered that the isolation of these substances would offer an opportunity to study the biosynthesis of these three different types of furanocoumarins. As in the preceding publication the numbers assigned to the various compounds discussed here correspond to those given in Part 1 of this series.[11]

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

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

To gain some information as to the biosynthetic

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Table 1. Incorporation of D,L-phenylalanine-[3-^{14}C][a] into coumarins of *Thamnosma montana* tissue cultures

Experiment No.	Feeding time (hr)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation isopimpinellin (2)
1[b]	8	$10{\cdot}93 \times 10^6$	1·20	0·029
2[b]	48	$11{\cdot}30 \times 10^6$	1·38	0·71
3[c]	72	$11{\cdot}00 \times 10^6$	1·30	0·48

[a]Precursor administered in water as sodium salt.
[b]Precursor fed in liquid growth medium.
[c]Precursor applied on the surface of the cultures in solid medium.

interrelationship between these various furanocoumarins of *Thamnosma montana* tissue cultures and to study the role of mevalonate in their biosynthesis, six feeding experiments were set up for different time intervals using D,L-mevalonic acid-[5-^{3}H] as the radioactive precursor. After the desired feeding time, each experiment was worked up and isoimperatorin (4), alloimperatorin methyl ether (3) and isopimpinellin (2) were isolated by the dilution technique (i.e. the tissue culture extract was diluted with non-radioactive coumarins before column chromatography). Isoimperatorin (4) and isopimpinellin (2) were crystallized to constant activity and their radioactivity determined by the scintillation counting method. Alloimperatorin methyl ether (3) was converted to its diol (20) before counting. The results are given in Table 2 and are represented graphically in Fig 1. It should be noted that in each of these experiments, 5-week old tissue cultures were used and the radioactive precursor was mixed with the tissue cultures in sterile distilled water. Only in experiment No. 4 normal liquid growth medium was used instead of distilled water.

It is apparent from these results that the furanocoumarins isolated incorporate D,L-mevalonic acid-[5-^{3}H] at different time intervals. Thus isopimpinellin (2) reaches a maximum incorporation after 2 days and then falls off rapidly reaching a minimum in 7 days. The rise between 7 and 10 days is questionable. Alloimperatorin methyl ether (3) reaches maximum incorporation after 4 days whereas isoimperatorin (4) has a maximum value after 7 days. These results indicate that mevalonic acid-[5-^{3}H] is indeed being incorporated into all three furanocoumarins studied and also the level of incorporation is about five to ten times higher than that achieved in our laboratory[11] with young *Thamnosma montana* plants.

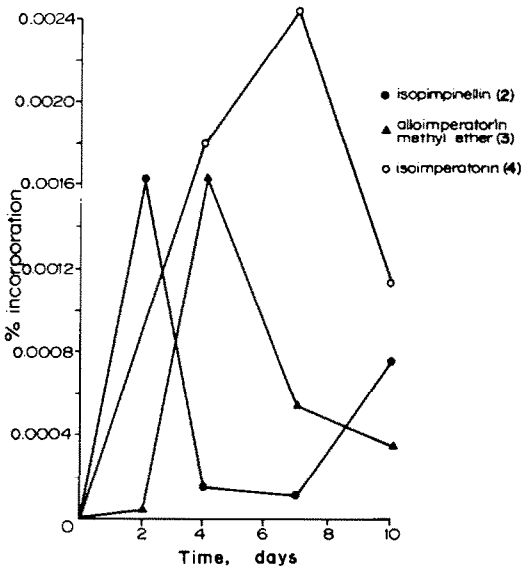


Fig 1. Incorporation of D,L-mevalonic acid-(5-^{3}H) into coumarins of *Thamnosma montana* tissue cultures vs time.

To determine the location of radioactivity, isopimpinellin (2) from these various feeding experiments was degraded according to the scheme

Table 2. Incorporation of D,L-mevalonic acid-[5-^{3}H] into tissue cultures of *Thamnosma montana*

Experiment No.	Feeding time (days)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation		
				Isoimperatorin[a] (4)	Alloimperatorin[a] methyl ether (3)	Isopimpinellin[a] (2)
4[b]	2	$1{\cdot}11 \times 10^9$	2·54	—	0·00004	0·0010
5[c]	2	$1{\cdot}11 \times 10^9$	3·00	—	0·000045	0·0016
6[c]	4	$1{\cdot}11 \times 10^9$	1·45	0·0018	0·00165	0·00015
7[c]	7	$8{\cdot}0 \times 10^8$	2·50	0·0025	0·00055	0·00011
8[c]	7	$1{\cdot}11 \times 10^9$	3·50	0·0021	0·00011	0·00017
9[c]	10	$8{\cdot}0 \times 10^8$	3·00	0·0011	0·00036	0·00076

[a]All compounds were isolated by dilution technique. Alloimperatorin methyl ether (3) was converted to its corresponding diol (20) before counting.
[b]Precursor mixed with tissue cultures in liquid growth medium.
[c]Precursor mixed with tissue cultures in distilled water.

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

Experiment No.	Specific activity of the compounds isolated (dpm/mmol)			
	Isopimpinellin[a] (2)	7	9	14
4	$2{\cdot}34 \times 10^4$ (100%)	$2{\cdot}25 \times 10^4$ (96·5%)	$1{\cdot}025 \times 10^4$ (44%)	
5	$2{\cdot}42 \times 10^4$ (100%)	$2{\cdot}51 \times 10^4$ (103%)	$1{\cdot}067 \times 10^4$ (44%)	$1{\cdot}17 \times 10^4$ (dpm/3 mmole) (48%)
9	$1{\cdot}722 \times 10^4$ (100%)	—	—	$4{\cdot}18 \times 10^3$ (dpm/2 mmole) (24·3%)

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

previously described (see Fig 1 in Part 1).[11] The distribution of label determined as a result of these degradations is listed in Table 3.

From these results, it is evident that there is no loss of activity in the 7-position of isopimpinellin (2) and that the 6-position contains approximately 56% of the total radioactivity of isopimpinellin (2). This data indicates that mevalonic-[5-^{3}H] is being incorporated specifically into the 6-position of isopimpinellin (2) as would be expected if Seshadri's hypothesis[12] for the furanocoumarin biosynthesis (i.e. the C-4 and C-5 positions of mevalonic acid serve as the precursor of C-7 and C-6 of furanocoumarin) is followed.

Somewhat surprisingly, the remaining radioactivity is found essentially in the OMe groups (between 24–44%) of isopimpinellin (2). It is difficult to explain these observed results on the basis of invoked theories about the metabolism of mevalonic acid. Mevalonic acid is not considered to be an important source of the "C_1-pool" in plant systems. One possible explanation could be that there is a tritium exchange between the tritiated mevalonic acid and the biological medium in the system being studied.

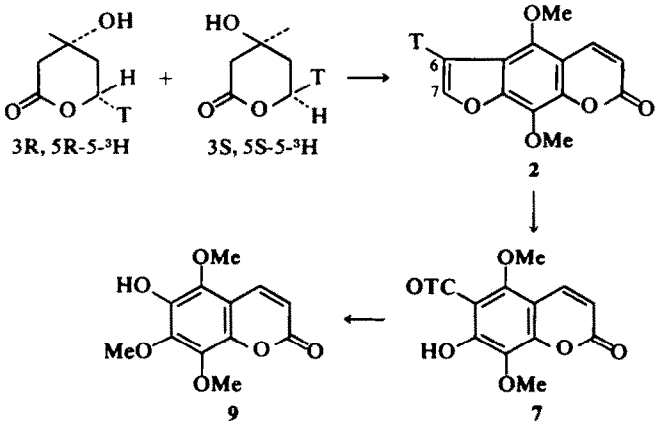
To determine the distribution of radioactivity in alloimperatorin methyl ether (3), it was converted to its diol (20). The diol (20) was then acetylated to the monoacetate (22) which was degraded according to the scheme already described (see Fig 2 in Part 1).[11] The results are given in Table 4.

These results again indicate that there is no loss of activity in the 7-position of alloimperatorin methyl ether (3) and that the 6-position contains between 30–32% of the total activity. The loss of

Table 4. Distribution of radioactivity in alloimperatorin methyl ether (3) from D,L-mevalonic acid-[5-^{3}H] incorporation experiments

Experiment No.	Specific activity of the compounds isolated (dpm/mmole)			
	22[a]	24	25b	14
6	$6{\cdot}63 \times 10^3$ (100%)	$6{\cdot}5 \times 10^3$ (100%)	$4{\cdot}28 \times 10^3$ (68%)	
6	$7{\cdot}12 \times 10^3$ (100%)	$6{\cdot}8 \times 10^3$ (95·5%)	$5{\cdot}0 \times 10^3$ (70%)	
7	$1{\cdot}944 \times 10^4$ (100%)	—	—	$6{\cdot}06 \times 10^2$ (3%)

[a]The total activity of monacetate (22) is set at 100%.



activity in the 6-position is in accord with the proposed hypothesis of furan ring formation in these furanocoumarins. Since alloimperatorin methyl ether also contains a dimethylallyl side chain and a OMe group, the remaining activity (68–70%) would be expected in the OMe group and/or in the alkyl side chain. However, the demethylation of **22** gave very little activity in the methoxyl group (~ 3%). Since the coumarin portion of these furanocoumarins has been shown to be cinnamic acid derived,[13] the remaining activity (~ 65%) in 3 must be present in the alkyl side chain. Thus it is evident that mevalonic acid-[5-^{3}H] is acting as a specific precursor of the 6-position and the alkyl side chain of alloimperatorin methyl ether (**3**).

To determine the distribution of label in isoimperatorin (**4**) it was converted to bergapten (**29**) as described previously.[11] Thus isoimperatorin (**4**) ($1{\cdot}728 \times 10^5$ dpm/mmole) from experiment 6 was degraded and bergapten (**29**) ($1{\cdot}77 \times 10^4$ dpm/mmole) was found to contain 10·3% of the total activity of **4**. In a similar experiment, isoimperatorin (**4**) ($1{\cdot}458 \times 10^5$ dpm/mmole) from experiment 8 was converted to bergapten (**29**) and it was found to have a specific activity of $2{\cdot}268 \times 10^4$ dpm/mmole or 15·5% of the total activity of **4**. Therefore, it is evident that 85–90% of the total activity of **4** resides in the alkylether side chain and that only 10–15% of the radioactivity is in the rest of the furanocoumarin molecule. Since isoimperatorin (**4**) contains a furan ring and as it has already been shown that mevalonic acid-[5-^{3}H] incorporates into the 6-position of the furanocoumarins, the residual 10–15% of activity in bergapten (**29**) will be expected to reside in the 6-position of isoimperatorin (**4**). However, due to lack of material, this degradation could not be pursued further.

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

In order to establish the role of mevalonic acid-[4-^{3}H] in the biosynthesis of furanocoumarins, two feeding experiments were set up for a period of 2 and 4 days and the results are listed in Table 5.

Thus mevalonic acid-[4-^{3}H] is also incorporated into all three furanocoumarins and the level of incorporation is again 5 to 10 times higher than that achieved in young *Thamnosma montana* plants.[11] Again, to determine the location of radioactivity in various furanocoumarins isolated, isopimpinellin (**2**) ($1{\cdot}96 \times 10^4$ dpm/mmole) from experiment 10 was degraded to 6-acetoxymethyl-7-acetoxy-5,8-dimethoxycoumarin (**10b**) according to the scheme previously described and 10b was found to be completely inactive. In a similar experiment, isopimpinellin (**2**) ($8{\cdot}4 \times 10^3$ dpm/mmole) from experiment 11 was selectively ozonized to 6-formyl-7-hydroxy-5,7-dimethoxycoumarin (**7**) which was shown to lack any measurable amount of radioactivity. Thus it is evident that mevalonic acid-[4-^{3}H] is being specifically incorporated into the 7-position of isopimpinellin (**2**). It is necessary to note that no radioactivity could be found in the OMe group of isopimpinellin (**2**) as was the case in the mevalonic acid-[5-^{3}H] experiments.

Similarly, alloimperatorin methyl ether diol (**20**) from experiment 10 was converted to its monoacetate (**22**). The monoacetate (**22**) ($1{\cdot}60 \times 10^4$ dpm/mmole) was then selectively ozonized to the corresponding phenolic aldehyde, 5-(2′-acetoxy-3′-hydroxy-3′-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (**23**). The phenolic aldehyde (**23**) ($7{\cdot}11 \times 10^3$ dpm/mmole) was found to contain 42% of the radioactivity of **22**. In a similar experiment, the monoacetate (**22**) ($1{\cdot}44 \times 10^4$ dpm/mmole) from experiment 11 was converted to the corresponding phenolic aldehyde (**23**) ($5{\cdot}76 \times 10^3$ dpm/mmole) which was shown to have 40% of the original activity of **22**.

It is clear from these results that about 58% of the activity of 3 resides in the 7-position as would be expected if mevalonic acid-[4-^{3}H] was acting as a specific precursor of the furan ring. Since there is no activity found in the OMe groups of isopimpinellin (**2**) from experiments 10 and 11 and since the coumarin portion of furanocoumarins has been shown to be cinnamic acid derived,[13] the remaining activity (40–42%) in **3** must reside in the alkyl side chain which would be expected to be mevalonic acid derived.

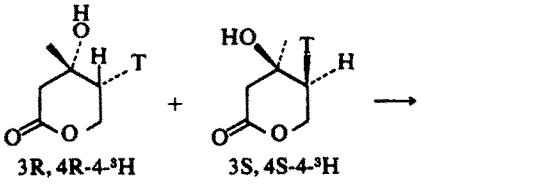
Location of radioactivity in isoimperatorin (**4**) ($3{\cdot}46 \times 10^5$ dpm/mmole) from experiment 10 was determined by converting **4** to bergapten (**29**) and bergapten ($8{\cdot}64 \times 10^4$ dpm/mmole) was found to contain 25% of the total activity of **4**. Similarly isoimperatorin (**4**) ($5{\cdot}65 \times 10^5$ dpm/mole) from experiment 11 was degraded to bergapten (**29**) and the latter ($3{\cdot}54 \times 10^4$ dpm/mmole) was found to contain 6·1% of the activity of isoimperatorin. Thus it is

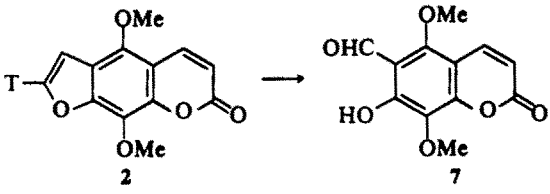
Table 5. Incorporation of D,L-mevalonic acid-[4-^{3}H] into the tissue cultures of *Thamnosma montana*

Experiment No.	Feeding time (days)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation		
				Isoimperatorin (**4**)	Alloimperatorin methyl ether (**3**)	Isopimpinellin (**2**)
10	2	$5{\cdot}50 \times 10^8$	2·1	0·0015	0·0015	0·001
11	4	$5{\cdot}50 \times 10^8$	1·7	0·0124	0·00107	0·00117

apparent that between 75–94% of the activity in isoimperatorin is located in the C_5-alkyl-ether side chain and only 6–25% of the activity is present in the furanocoumarin molecule. In order to determine the location of radioactivity in the furanocoumarin portion of **4**, bergapten (**29**) ($1{\cdot}296 \times 10^4$ dpm/mmole) from experiments 10 and 11 was combined and ozonized selectively to 6-formyl-7-hydroxy-5-methoxycoumarin (**30**) and **30** was shown to be completely inactive. Thus all the remaining activity (6–25%) in isoimperatorin (**4**) resides in the 7-position of **4**.

Thus the results of experiments 10 and 11 clearly indicate that mevalonic acid-[4-^{3}H] is acting as a specific precursor of the furan ring and the alkyl side chains of furanocoumarins. These results are in complete agreement with the results of Floss and Mothes[13] that mevalonic acid-[4-^{3}H] is a specific precursor of the 7-position of the furan ring of furanocoumarins.





Finally to determine the role of mevalonic acid-[2-^{3}H] in the biosynthesis of furanocoumarins, two feeding experiments for a time period of 2 and 4 days were set up and three furanocoumarins were isolated. The results are given in Table 6.

Thus mevalonic acid-[2-^{3}H] is incorporated into all three furanocoumarins. However, the level of incorporation is much lower than in the cases of [4-^{3}H]- or [5-^{3}H]-mevalonic acid. Also, the incorporation of mevalonic acid-[2-^{3}H] into isopimpinellin (**2**), a simple furanocoumarin, is hard to reconcile with the previous results. Therefore to determine the location of radioactivity, isopimpinellin (**2**) ($9{\cdot}0 \times 10^3$ dpm/mmole) from experiment 12 was demethylated and tetramethylammonium iodide (**14**) ($9{\cdot}05 \times 10^3$ dpm/2 mmole) was found to contain all the radioactivity of **2**. In a similar experiment, isopimpinellin (**2**) ($4{\cdot}182 \times 10^4$ dpm/mmole) from experiment 13 was demethylated and tetramethylammonium iodide (**14**) ($4{\cdot}0 \times 10^4$ dpm/2 mmole) was found to contain all the radioactivity of isopimpinellin (**2**).

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

To determine the location of radioactivity in alloimperatorin methyl ether (**3**), alloimperatorin methyl ether diol (**20**) ($6{\cdot}36 \times 10^3$ dpm/mmole) from experiment 12 was cleaved with periodic acid and the alcohol (**17**) ($1{\cdot}30 \times 10^3$ dpm/mmole) was found to contain 20% of the original activity of **20**. In a similar experiment, diol **20** ($7{\cdot}63 \times 10^3$ dpm/mmole) from experiment 13 was converted to alcohol **17** which was shown to lack any measurable amount of activity. Thus it is evident that between 80–100% of the radioactivity in alloimperatorin methyl ether (**3**) resides in the terminal three C atoms of the dimethylallyl side chain, indicating that mevalonic acid-[2-^{3}H] is acting as a specific precursor of this side chain. Since the furanocoumarin portion of alloimperatorin methyl ether (**3**) and of isopimpinellin (**2**) will be expected to be biosynthesized in a similar manner, any residual amount (0–20%) of radioactivity in **3** will be expected to reside in the OMe group of alloimperatorin methyl ether (**3**) as has been shown to be the case in isopimpinellin (**2**).

Since isoimperatorin (**4**) is a simple furanocoumarin with an alkyl-ether side chain and no OMe group, all the activity in **4** would be expected to reside in this C_5 side chain. Therefore, isoimperatorin (**4**) ($1{\cdot}08 \times 10^5$ dpm/mmole) from experiment 12 was hydrolyzed and bergapten (**29**) ($5{\cdot}58 \times 10^3$ dpm/mmole) was shown to have about 5% of the total activity of **4**. In a similar experiment, isoimperatorin ($8{\cdot}1 \times 10^5$ dpm/mmole) from experiment 13 was degraded to bergapten (**29**) ($1{\cdot}728 \times 10^4$ dpm/mmole) and this was shown to have about 2·7% of the total radioactivity of **4**. Thus it is apparent from these results that between 95–97% of the radioactivity of isoimperatorin (**4**) resides in

Table 6. Incorporation of D,L-mevalonic acid-[2-^{3}H] into the tissue cultures of *Thamnosma montana*

Experiment No.	Feeding time (days)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation		
				Isoimperatorin (4)	Alloimperatorin methyl ether (3)	Isopimpinellin (2)
12	2	$1{\cdot}11 \times 10^9$	1·70	0·0008	0·0001	0·0002
13	4	$1{\cdot}11 \times 10^9$	1·69	0·0054	0·0004	0·0004

the C_5-alkyl-ether side chain indicating that mevalonic acid-[2-^{3}H] is acting as a specific precursor of this side chain.

Finally to determine if mevalonic acid is being degraded to the C_1-pool and is thus being incorporated into the OMe groups of furanocoumarins or if there is some sort of tritium exchange between the tritiated mevalonic acid and the C_1-pool in the tissue culture system, mevalonic acid-[5-^{14}C] was fed to the 5-week old tissue cultures over a 2 day period and isopimpinellin (**2**) was isolated. The results are given in Table 7.

Table 7. Incorporation of D,L-mevalonic acid-[5-^{14}C] into tissue cultures of *Thamnosma montana*

Experiment no.	Feeding time (days)	Activity fed (dpm)	Dry weight of tissue cultures (g)	% Incorporation isopimpinellin (**2**)
14	2	$1{\cdot}11 \times 10^9$	3·33	0·002

To determine the distribution of radioactivity, isopimpinellin (**2**) ($5{\cdot}00 \times 10^4$ dpm/mmole) from experiment 14 was degraded according to the previous scheme and phenol (**9**) ($2{\cdot}75 \times 10^3$ dpm/mmole) was shown to contain only 5·5% of the radioactivity of **2**. This clearly indicates that about 95% of the radioactivity in isopimpinellin (**2**) resides in the 6-position. This is in contrast to only 56% of the activity present in the 6-position in the mevalonic acid-[5-^{3}H] feeding experiments. Thus if mevalonic acid was being degraded to the C_1-pool and thus acting as a precursor of the OMe groups of isopimpinellin (**2**) an equivalent amount of activity would be expected in the OMe groups of isopimpinellin in both [5-^{3}H] and [5-^{14}C]-mevalonic acid feedings. Since this is not the case and in the mevalonic acid-[5-^{14}C] experiment, about 95% of the radioactivity is present in the 6-position of isopimpinellin (**2**), it is clearly evident that mevalonic acid is not being degraded to the C_1-pool and thus is not a precursor of the OMe groups. Any activity found in the OMe groups of furanocoumarins in the [5-^{3}H]- and [2-^{3}H]-mevalonic acid feedings must come from either a tritium exchange between the tritiated mevalonic acid and the C_1-pool in the system or by some other unknown mechanism.

The results of experiments 4–14 are in complete agreement with the results of Floss and Mothes[13] and thus support Seshadri's proposal[12] for furanocoumarin biosynthesis. However, these results are not in agreement with the results of Brown[9] and of Caporale *et al.*[14] These workers have indicated that the incorporation of mevalonic acid into furanocoumarins was nonspecific. However, our results clearly indicate that mevalonic acid is acting as a specific precursor of the furan ring and the alkyl groups of furanocoumarins in the tissue cultures of *Thamnosma montana*. Since in the investigations of Brown[9] and Caporale *et al.*[14] no degradations to determine the distribution of radioactivity were performed, the significance of their results is questionable.

Finally, it should be noted that the above results, which reveal that tritium from the 4R- and the 5-positions of mevalonate appears at C-7 and C-6 respectively of isopimpinellin, provide some obvious mechanistic implications. Any mechanism of furan ring formation and removal of the isopropyl side chain which would involve loss of all H atoms from either of these positions is clearly eliminated from consideration.

EXPERIMENTAL

For detailed description of experimental procedure employed in the various degradations, see accompanying publication.

Cultivation of tissue cultures of Thamnosma montana. All culturing was done at room temp (24°). Seeds were rinsed with 70% EtOH, soaked for approximately 2 min in 0·1% $HgCl_2$, rinsed with sterile water and were planted on water-agar. Microorganism-free seedlings were cut into 3 or 4 pieces and transferred to the surface of the modified White's agar medium in Erlenmeyer flasks. The seedling pieces were incubated until callus tissue was observed at the cut ends. Then the fragments of callus tissue were transferred to liquid medium, 100 ml per 250 ml Erlenmeyer flask, which was placed on a rotary shaker (about 120 rpm and 30 mm radius). Whenever tissue was transferred to fresh media, the pieces were cut up into smaller pieces.

For nutrient purposes, a general-purpose medium (a modified White's nutrient soln)[15] was used. For solid medium, 1% agar was added. When cultures were used for biosynthetic experiments, most of the pieces of wet tissue were 5 mm or less in diameter. The tissue cultures were cultivated by Dr. P. Salisbury of this department.

Constituents of tissue cultures of Thamnosma montana. Five flasks containing tissue cultures (5 week old) were decanted and the solid residue was put on an aspirator for 30 min. The residue was washed with water (2 times) and weighed to give 35 g of wet tissue. This was air-dried at room temp and the dry residue (4·7 g) was extracted with acetone in a Soxhlet extractor. The acetone extract was evaporated to dryness, the residue dissolved in chloroform, filtered and dried over Na_2SO_4. The solvent was removed under reduced pressure to give 100 mg of the residue which was pre-adsorbed on 1 g of alumina (activity IV) and was chromatographed on alumina (10 g, neutral, activity IV). Preparative layer chromatography gave **2** (1·5 mg), **3** (0·8 mg) and **4** (1·6 mg). No **1** was indicated by TLC.

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

Labeled compounds. D,L-Phenylalanine-[3-^{14}C] (obtained from New England Nuclear Corp., Boston, Mass.)

was used as a Na salt soln. D,L-mevalonic acid-[5-^{3}H] as dibenzoyl ethylene diamine (DBED) salt (obtained from New England Nuclear Corp.) in MeOH was used as such. D,L-mevalonic acid-[4-^{3}H] lactone (obtained from Amersham/Searle Corp.) was used as a sodium salt by evaporating the benzene soln of the lactone and dissolving the residue in dilute Na_2CO_3aq. D,L-Mevalonic acid-[2-^{2}H] lactone was also used as Na salt. D,L-Mevalonic acid-[5-^{14}C] (obtained from Schwarz/Mann Corp.) was fed as a sodium salt in water.

Feeding experiments. In each experiment (unless otherwise noted), tissue cultures were transferred to 30 ml of sterilized distilled water in an Erlenmeyer flask and the precursor was mixed with the tissue culture. The flasks were left on a rotary shaker for a preselected time period after which tissue cultures were freeze-dried and worked up as before. The compounds were isolated by dilution technique and the radioactivity was determined by scintillation counting method.

Degradation of isopimpinellin (**2**) *from experiment* 4

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (**7**). Isopimpinellin **2** (45 mg, $2{\cdot}34\times10^4$ dpm/mmole) from experiment 4 was selectively ozonized as described previously[11] and **7** (19·5 mg) was isolated and shown to have a specific activity of $2{\cdot}25\times10^4$ dpm/mmole or 96·5% of the original activity of **2**.

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

Radioactive 6-hydroxy-5,7,8-trimethoxycoumarin (**9**). Compound **8** (23 mg, $2{\cdot}25\times10^4$ dpm/mmole) from the previous reaction was degraded to **9** (14·5 mg) and **9** was shown to have a specific activity of $1{\cdot}025\times10^4$ dpm/mmole or 44% of the original activity of **2**.

Degradations of isopimpinellin (**2**) *from experiment* 5

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (**7**). Isopimpinellin **2** (50 mg, $2{\cdot}42\times10^4$ dpm/mmole) from experiment 5 was converted to **7** (24 mg) and this **7** was shown to have a specific activity of $2{\cdot}51\times10^4$ dpm/mmole or all the original activity of **2**.

Radioactive 6-formyl-5,7,8-trimethoxycoumarin (**8**). Compound **7** from previous reaction was methylated as described previously to give **8** (25 mg) by preparative layer chromatography. It was not counted but used as such in the next reaction.

6-Hydroxy-5,7,8-trimethoxycoumarin (**9**). Radioactive **8** (25 mg) from the above reaction was treated with H_2O_2 and H_2SO_4 to give **9** (16·7 mg). This was shown to have a specific activity of $1{\cdot}067\times10^4$ dpm/mmole or 44% of the original activity of **2**.

Radioactive tetramethylammonium iodide (**14**). Radioactive (**9**) (10 mg, $1{\cdot}067\times10^4$ dpm/mmole) from a previous reaction was demethylated as described previously[11] and **14** (14 mg) was isolated. This was counted by the following method. The salt (~ 1·5 mg) was dissolved in 0·1 N $Na_2S_2O_3$aq (10 drops) and MeOH (1 ml) and the soln was made up to 15 ml with aqueous scintillator soln. A blank sample of the same constitution (except that nonradioactive **14** was used) was counted in the same vial to determine background. By this method, **14** gave a specific activity of $1{\cdot}17\times10^4$ dpm/3 mmole or 48% of the original activity of **2**.

Radioactive tetramethylammonium iodide from experiment 9. Radioactive **2** (14·5 mg, $1{\cdot}722\times10^4$ dpm/mmole) from experiment 9 was demethylated as described previously and **14** (10 mg) was isolated. It was counted by dissolving the salt (~ 1·5 mg) in 0·1 N $Na_2S_2O_3$aq (10 drops) and MeOH (1 ml) and making the soln up to 15 ml with aqueous scintillator soln. By this method, **14** gave a specific activity of $4{\cdot}18\times10^3$ dpm/2 mmole or 24·3% of the original activity of **2**.

The iodide **14** (7 mg) from the above reaction was converted to its picrate **15** (6 mg) which was counted as follows: Picrate (~ 2 mg) was dissolved in Ac_2O (5 drops) and AcOH (5 drops), Zn dust (10 mg) was added to decolorize the soln. Sodium metabisulfite (100 mg) was added and the mixture was then filtered directly into the counting vial. The original container was washed with MeOH (1 ml) and this wash was also filtered into the counting vial. The soln was made up to 15 ml with organic scintillator soln and then counted after standing at least 1 hr in the cold and in the dark. As before, an inactive sample of **15** was counted in the same manner to determine the background prior to counting the radioactive sample. By this method, **15** indicated a specific activity of $4{\cdot}107\times10^3$ dpm/2 mmole or 24% of the original activity of **2**.

Degradations of alloimperatorin methyl ether (**3**) *from experiment* 6

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxypsoralen (**22**). Radioactive **3** from experiment 6 was converted to **20**. The diol **20** was diluted with nonradioactive material and was acetylated to **22**.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (**23**) – *trial a.* **22** (42 mg, $6{\cdot}3\times10^3$ dpm/mmole) from experiment 6 was selectively ozonized as previously described[11] and after crystallization, **23** (30 mg) was isolated. This material (**23**) was not counted but was used directly in the next reaction.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7,8-dimethoxycoumarin (**24**). Compound **23** (30 mg) from previous reaction was methylated and **24** (25 mg) was isolated and shown to have a specific activity of $6{\cdot}5\times10^3$ dpm/mmole or all the original activity of **22**.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-acetoxy-7,8-dimethoxycoumarin (**25b**). Radioactive **24** (24·5 mg, $6{\cdot}5\times10^3$ dpm/mole) from the previous reaction was converted to **25b** (16 mg) as previously described and **25b** was shown to have a specific activity of $4{\cdot}28\times10^3$ dpm/mmole or 68% of the total activity of **22**.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (**23**)–*trial b.* Radioactive **22** (38 mg, $7{\cdot}12\times10^3$ dpm/mmole) was converted to **23** (25 mg). The product **23** was not counted but used directly for the next reaction.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7,8-dimethoxycoumarin (**24**). Radioactive **23** (25 mg) from the previous reaction was methylated to give **24** (23 mg). The product **24** was shown to have a specific activity of $6{\cdot}8\times10^3$ dpm/mmole or 95·5% of the original activity of **22**.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-acetoxy-7,8-dimethoxycoumarin (**25b**). Radioactive **24** (22 mg, $6{\cdot}8\times10^3$ dpm/mmole) from the previous reaction was converted to **25b** (12 mg) and it was shown to have a specific activity of $5{\cdot}0\times10^3$ dpm/mmole or 70% of the original activity of **22**.

Radioactive tetramethylammonium iodide (**14**) *from experiment* 7. Radioactive **22** (19·5 mg, $1{\cdot}94\times10^4$ dpm/mmole) was demethylated to give **14** (7·3 mg). It was

counted as before and was shown to have a specific activity of $6{\cdot}06 \times 10^3$ dpm/mmole or about 3% of the original activity of **22**.

Degradations of isoimperatorin (**4**) *from experiments* 6 *and* 8

Radioactive bergapten (**29**). Isoimperatorin **4** (24·5 mg, $1{\cdot}728 \times 10^5$ dpm/mmole) from experiment 6 was converted to **29** (10 mg) as previously described and was shown to have a specific activity of $1{\cdot}771 \times 10^4$ dpm/mmole or 10·3% of the original activity of **4**.

Radioactive bergapten (**29**). Radioactive **4** (21 mg, $1{\cdot}458 \times 10^5$ dpm/mmole) from experiment 8 was converted to **29** (9 mg) and was shown to have a specific activity of $2{\cdot}268 \times 10^4$ dpm/mmole or 15·5% of the original activity of **4**.

Degradation of isopimpinellin (**2**) *from experiments* 10 *and* 11

Radioactive 6-acetoxymethyl-7-acetoxy-5,8-dimethoxycoumarin (**10b**). Isopimpinellin **2** (23·5 mg, $1{\cdot}96 \times 10^4$ dpm/mmole) from experiment 10 was degraded to **10b** (21 mg) and was shown to be completely inactive.

Radioactive 6-formyl-7-hydroxy-5,8-dimethoxycoumarin (**7**). Isopimpinellin **2** (19 mg, $8{\cdot}4 \times 10^3$ dpm/mmole) from experiment 11 was selectively ozonized as described previously and **7** (9 mg) was isolated. This was shown to be completely inactive.

Degradations of alloimperatorin methyl ether (**3**) *from experiments* 10 *and* 11

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-8-methoxypsoralen (**22**). Radioactive **20** from experiments 10 and 11 was diluted with non-radioactive **20** and was converted to **22** separately.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (**23**). Radioactive **22** (28 mg, $1{\cdot}69 \times 10^4$ dpm/mmole) from experiment 10 was ozonized selectively to **23** (6 mg). This substance **23** was shown to have a specific activity of $7{\cdot}11 \times 10^3$ dpm/mmole or 42% of the original activity of **22**.

Radioactive 5-(2'-acetoxy-3'-hydroxy-3'-methylbutyl)-6-formyl-7-hydroxy-8-methoxycoumarin (**23**). Radioactive **22** (28 mg, $1{\cdot}44 \times 10^4$ dpm/mmole) from experiment 11 was selectively ozonized as described previously and **23** (9 mg) was isolated. This was shown to have a specific activity of $5{\cdot}76 \times 10^3$ dpm/mmole or 40% of the original activity of **22**.

Degradations of isoimperatorin (**4**) *from experiments* 10 *and* 11

Radioactive bergapten (**29**). Isoimperatorin **4** (13 mg, $3{\cdot}46 \times 10^5$ dpm/mmole) from experiment 10 was converted to **29** (9 mg) as described previously and **29** was shown to have a specific activity of $8{\cdot}64 \times 10^4$ dpm/mmole or 25% of the original activity of **4**.

Radioactive bergapten (**29**). Radioactive **4** (11·3 mg, $5{\cdot}65 \times 10^5$ dpm/mmole) from experiment 11 was converted to **29** (7·4 mg) and this compound **29** was shown to have a specific activity of $3{\cdot}45 \times 10^4$ dpm/mmole or 6·1% of the original activity of **4**.

Radioactive 6-formyl-7-hydroxy-5-methoxycoumarin (**30**). Radioactive **29** (24 mg, $1{\cdot}296 \times 10^4$ dpm/mmole) from experiments 10 and 11 was combined and was selectively ozonized to give **30** (8 mg). This **30** was shown to be completely inactive.

Degradation of isopimpinellin from experiments 12 *and* 13

Radioactive tetramethylammonium iodide (**14**). Radioactive **2** (13·5 mg, $9{\cdot}0 \times 10^3$ dpm/mmole) from experiment 12 was demethylated and **14** (12·5 mg) was isolated. This was counted by dissolving **14** (~ 2 mg) in 10 drops of 0·1 N $Na_2S_2O_3$, 1 ml of MeOH was added and the soln was made up to 15 ml with aqueous scintillator soln. By this method, **14** gave a specific activity of $9{\cdot}05 \times 10^3$ dpm/mmole or all the original activity of **2**.

Radioactive tetramethylammonium iodide (**14**). Isopimpinellin (16 mg, $4{\cdot}182 \times 10^4$ dpm/mmole) from experiment 13 was demethylated and the iodide **14** (18 mg) was isolated. This was converted to the picrate **15** (10 mg). It was counted by dissolving the picrate (~ 2 mg) in AcOH (10 drops) and Ac_2O (10 drops). Enough Zn dust was added to decolorize the soln. Sodium metabisulfite (100 mg) was added and the soln was filtered into the counting vial. The original container was washed with MeOH (1 ml) and the wash was also filtered into the counting vial. The soln was made up to 15 ml with organic scintillator soln and then counted after standing at least 1 hr in the cold and in the dark. By this method, the picrate **15** was shown to have a specific activity of $4{\cdot}0 \times 10^3$ dpm/2 mmole or all the original activity of **2**.

Degradations of alloimperatorin methyl ether (**3**) *from experiments* 12 *and* 13

Periodic acid cleavage of radioactive 5-(2',3'-dihydroxy-3'-methylbutyl)-8-methoxypsoralen (**20**) (*alloimperatorin methyl ether diol*). Compound **20** (18·5 mg, $6{\cdot}36 \times 10^3$ dpm/mmole) from experiment 12 was cleaved with periodic acid as previously described and **17** (5 mg) was isolated and shown to have a specific activity of $1{\cdot}3 \times 10^3$ dpm/mmole or 20·4% of the original activity of **20**.

Periodic acid cleavage of radioactive 5-(2',3'-dihydroxy-3'-methylbutyl)-8-methoxypsoralen (**20**). Diol **20** (36·5 mg, $7{\cdot}36 \times 10^3$ dpm/mmole) from experiment 13 was cleaved with periodic acid and **17** (9·3 mg) was isolated and was shown to be completely inactive.

Degradations of isoimperatorin (**4**) *from experiments* 12 *and* 13

Radioactive bergapten (**29**). Isoimperatorin **4** (12·7 mg, $1{\cdot}08 \times 10^5$ dpm/mmole) from experiment 12 was converted to **29** (7·5 mg) and this was shown to have a specific activity of $5{\cdot}85 \times 10^3$ dpm/mmole or about 5·5% of the original activity of **4**.

Radioactive bergapten (**29**). Isoimperatorin **4** (14 mg, $8{\cdot}1 \times 10^5$ dpm/mmole) from experiment 13 was degraded to **29** (5·6 mg) and this was shown to have a specific activity of $1{\cdot}728 \times 10^4$ dpm/mmole or 2·7% of the original activity of **4**.

Degradations of isopimpinellin (**2**) *from experiment* 14

6-Formyl-7-hydroxy-5,8-dimethoxycoumarin (**7**). Isopimpinellin **2** (45 mg, $4{\cdot}92 \times 10^4$ dpm/mmole) from experiment 14 was ozonized selectively and **7** (22 mg) was isolated. This was shown to have a specific activity of $4{\cdot}90 \times 10^4$ dpm/mmole or all the original activity of **2**.

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

6-Hydroxy-5,7,8-trimethoxycoumarin (**9**). Radioactive **8** (20 mg) from the previous reaction was converted to

9 (12 mg) as described previously. This was shown to have a specific activity of $2{\cdot}75 \times 10^3$ dpm/mmole or about 5·5% of the original activity of 2.

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