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7-DEMETHYLSUBEROSIN AND OSTHENOL AS INTERMEDIATES IN FURANOCOUMARIN BIOSYNTHESIS

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Key Word Index—*Conium maculatum*; *Heracleum lanatum*; *Ruta graveolens*; *Coronilla glauca*; coumarins, furanocoumarins, biosynthesis, 7-demethylsuberosin, osthenol.

Abstract—^{14}C-Labelled 7-demethylsuberosin (DMS) was a precursor of linear furanocoumarins in *Conium maculatum* and *Heracleum lanatum* (Umbelliferae), and in *Ruta graveolens* (Rutaceae), but not in *Coronilla glauca* (Leguminosae). Trapping experiments with ^{14}C-umbelliferone over 3- to 24-hr periods revealed that DMS is rapidly metabolized, and that in short experiments its specific activity relative to the linear furanocoumarins' is that of an intermediate. The specific activity of umbelliferone exhibited anomalies attributed to pool compartmentation. Analogous but less extensive experiments established that osthenol is an intermediate in angular furanocoumarin biosynthesis, but is much less rapidly metabolized than DMS. No significant incorporation into furanocoumarins of ^{14}C from labelled L-valine, L-leucine, acetate, 2,3-dihydroxyisovaleric acid, senecioic acid, isopentenylpyrophosphate, or mevalonate could be demonstrated. A synthesis of [1-^{14}C]2,3-dihydroxyisovaleric acid is described.

INTRODUCTION

THE FUNCTION of umbelliferone (I, $R_6 = R_8 = H$) as a precursor of the furanocoumarins of *Pimpinella magna* was elucidated in 1964 by Floss and Mothes,[1] and studies from our laboratories have since established that (+)-marmesin (II) and columbianetin (III) are intermediates between umbelliferone and linear and angular furanocoumarins, respectively, in species of the Umbelliferae and Rutaceae.[2–4] These findings suggested[2] that 7-demethylsuberosin (DMS, I, $R_6 = Me_2C{=}CH{-}CH_2{-}$; $R_8 = H$) formed by prenylation of umbelliferone, is on the pathway from umbelliferone to marmesin, and that epoxidation of the side-chain double bond, diol formation, and cyclization could then yield the dihydrofuran ring of marmesin. An analogous sequence of reactions[3] would give the angular series in which osthenol (I, $R_6 = H$; $R_8 = Me_2C{=}CH{-}CH_2{-}$) replaces DMS. Alternative pathways remain possible, especially since formation of DMS and osthenol would appear to signify that mevalonate is implicated although there have been conflicting reports about its role in furanocoumarin biosynthesis.[5–7]

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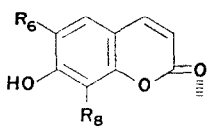
[1] FLOSS, H. G. and MOTHES, U. (1964) *Z. Naturforsch.* **19b**, 770.
[2] BROWN, S. A., EL-DAKHAKHNY, M. and STECK, W. (1970) *Can. J. Biochem.* **48**, 863.
[3] STECK, W. and BROWN, S. A. (1970) *Can. J. Biochem.* **48**, 872.
[4] STECK, W. and BROWN, S. A. (1971) *Can. J. Biochem.* **49**, 1213.
[5] CAPORALE, G., BRECCIO, A. and RODIGHIERO, G. (1964) *Prepn. Bio-Med. Appl. Labeled Mol. Proc. Symp.* p. 103, Venice.
[6] FLOSS, H. G. and MOTHES, U. (1966) *Phytochemistry* **5**, 161.
[7] BROWN, S. A. (1970) *Phytochemistry* **9**, 2471.

Preliminary communications have indeed provided evidence for the participation of DMS in the elaboration of linear furanocoumarins in *Ruta graveolens*, both in organized plants[8] and in cell cultures.[9] Details of the latter work are presented in the preceding paper.[10] In the present paper we report investigations which establish DMS and osthenol as intermediates, as well as precursors, of furanocoumarins in this and other species, and which offer evidence against the involvement of some other possible pathways.



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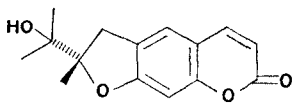

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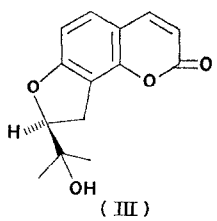

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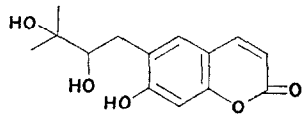

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RESULTS AND DISCUSSION

The possible precursor role of DMS was studied in *Coronilla glauca* (Leguminosae), *Heracleum lanatum*, and *Conium maculatum* (Umbelliferae), and *Ruta graveolens* (Rutaceae), all of which form at least one furanocoumarin. In each experiment a parallel feeding with the known furanocoumarin precursor, umbelliferone, was conducted to ensure that the plants were in a period of active synthesis at the time of the experiment. The results are presented in Tables 1 and 2. In *Conium*, *Heracleum* and *Ruta*, administrations of labelled DMS yielded psoralen of quite high specific activity, and respective dilution values of 8·0, 2·3 and 40, and in agreement with earlier findings the oxygenated psoralens had lower specific activities. But surprisingly, psoralen (both free and bound as a glycoside) recovered from *Coronilla* after labelled DMS had been fed was totally devoid of activity, even though high activity was found in the dihydropyranocoumarin, dihydroxanthyletin. In all three plants a definite incorporation of label was obtained from umbelliferone, but quantitative comparisons with the DMS values are not valid owing to the differing doses of the two given to both *Coronilla* and the umbellifers, and a noticeably poorer translocation of DMS in *Ruta*. An unexplained anomaly in the umbelliferone feeding to *Conium* is the higher specific activity of bergapten in comparison to psoralen, a result at variance with all those obtained earlier.

The data for *Conium*, *Heracleum* and *Ruta* establish a precursor role for DMS in furanocoumarin formation, confirming the cell culture results in the case of *Ruta*.[10] But such a role is contraindicated for *Coronilla*. While a negative result cannot be regarded as conclusive, it is significant that psoralen was being synthesized from umbelliferone in the parallel experiment, and that DMS was readily converted to dihydroxanthyletin. It may be that dihydroxanthyletin synthesis had consumed all the administered DMS before it could be translocated to the site of psoralen formation; this would assume, of course, two distinct synthetic sites of differing accessibility, such as stems and leaves. However, it is also of interest that *C. glauca* seems to be virtually unique among plants elaborating furanocou-

[8] GAMES, D. E. and JAMES, D. H. (1972) *Phytochemistry* **11**, 868.
[9] AUSTIN, D. J. and BROWN, S. A. (1970) Abstracts—*Biochemistry*, 14th Nordiska Kemistmötet, Umeå, Sweden, June 18–22, p. 108.
[10] AUSTIN, D. J. and BROWN, S. A (1973) *Phytochemistry* **12** (7), in press.

marins in having the glycoside of the coumarinic acid as the predominant form of psoralen, the analogy here being rather to the simple coumarins such as herniarin, umbelliferone and coumarin itself.[11] These two anomalies hint at some fundamental differences in the physiological apparatus for synthesis of psoralen or in the biosynthetic pathway. An instance of a different biosynthetic route to the coumarin nucleus has already been reported from another member of the Leguminosae.[12,13]

TABLE 1. INCORPORATION OF DEMETHYLSUBEROSIN (DMS) INTO COUMARINS

Species	Fr wt (g)	Compound administered	Dose (mmol)	Metabolic period (hr)	Coumarins recovered Compound	Activity (μCi/mmol)	Dilution
Coronilla glauca	20	[3-^{14}C]DMS; 1 98 μCi, 80 μCi/mmol	0 025	6†	Psoralen	0	—
	21	[3-^{14}C]DMS; 2 20 μCi, 80 μCi/mmol	0 0275	24†	Psoralen*	0	—
					DMS	32	2 5
					Dihydroxanthyletin	32	2 5
	22	[G-^{3}H]Umbelliferone; 350 μCi, 1 04 × 10^{4} μCi/mmol‡	0 0034		Psoralen	134	77 6
					Psoralen	13 3	780
Conium maculatum	58	[3-^{14}C]DMS; 2 72 μCi, 80 μCi/mmol	0 034	24	Psoralen	8 9	8 0
					Xanthotoxin	2 1	38
	60	[G-^{3}H]Umbelliferone; 200 μCi, 1 04 × 10^{4} μCi/mmol‡	0 0019	72	Psoralen	3 5	3000
					Bergapten	8 6	1200
					Xanthotoxin	2 1	5000
					Isopimpinellin	5 5	1900
					Umbelliferone	25	420
Ruta graveolens	25	[2-^{14}C]DMS; 7 6 μCi, 76 μCi/mmol	0 10	67	Marmesin	65	1 2
					Psoralen	1 9	40
					Bergapten	0 15	510
					Xanthotoxin	0 18	420
	25	[2-^{14}C]Umbelliferone; 10 μCi, 100 μCi/mmol	0 10	67	Marmesin	102	1 0
					Psoralen	4 7	21
					Bergapten	0 70	140
					Xanthotoxin	1 3	77

* This psoralen was liberated from psoralen glycoside by treatment with emulsin.
† These replicate experiments yielded identical results for the four samples shown in the last three columns.
‡ Corrected for % loss of tritium calculated from experimentally determined distribution of the label.

Parallel experiments in the angular furanocoumarin series were confined to *Heracleum lanatum*, the only one of our species elaborating an angular series. Although in this case dilution values were much higher, per cent incorporations for angelicin (3·4–6·1) and sphondin (2·5–2·7) leave no doubt that in the angular series osthenol plays an analogous role to DMS (Table 2). The two compounds do not behave completely identically, however; the metabolism of DMS is considerably more rapid than that of osthenol despite the fact that DMS is generally more stable than osthenol *in vitro*. In our feedings to *Heracleum* small amounts of unchanged osthenol were recovered after 24 hr but no DMS remained after the same period. This difference in metabolic rate could be related to the prevalence of 8- rather than 6-prenylated coumarins in nature.

Trapping Experiments

To demonstrate that a known precursor is a natural intermediate in a metabolic pathway one must show its occurrence in the species in question. While both DMS and osthenol

[11] BROWN, S. A. (1966) in *Biosynthesis of Aromatic Compounds* (BILLEK, G., ed.), p. 15, Pergamon Press, Oxford.
[12] GRISEBACH, H. and BARZ, W. (1963) *Z. Naturforsch.* **18b**, 466.
[13] GRISEBACH, H. and BARZ, W. (1964) *Z. Naturforsch.* **19b**, 569.

have been isolated from related umbelliferous and rutaceous plants[14,15] neither has been reported to occur in any of the species of Table 1. In an effort to establish the intermediacy of these two compounds, trapping experiments were undertaken.

TABLE 2. INCORPORATION OF OSTHENOL AND DEMETHYLSUBEROSIN (DMS) INTO FURANOCOUMARINS OF *Heracleum Lanatum**

	Compounds administered	Metabolic period (hr)	% Incorporation into and [sp.act. (μCi/mmol) of] Angular furanocoumarins		
			Angelicin	Isobergapten	Sphondin
1.	[2-^{14}C]Osthenol, 1·3 μCi = 5 μmol	24	6·1% [3 3]	0 3% [0·6]	2·5% [0 4]
2.	[2-^{14}C]Osthenol, 1·6 μCi = 6 μmol	24	3·4% [15·0]	0·2% [0·8]	2·7% [0·4]
3.	[3-^{14}C]DMS, 3·2 μCi = 40 μmol	24	Nil —	Nil —	0 1% [0 1]
4.	[3-^{14}C]DMS, 2·5 μCi = 31 μmol	48	Nil —	Nil —	1% [0·3]

	% Incorporation into and [sp.act (μCi/mmol) of] Linear furanocoumarins				
	Pimpinellin	Psoralen	Bergapten	Xanthotoxin	Isopimpinellin
1.	0·4% [0 4]	Nil —	0·3% [0 1]	0·4% [0·1]	0·1% [0 1]
2.	0·2% [0·8]	Nil —	0·1% [0·1]	0·1% [0·1]	0·2% [0·3]
3.	Nil —	18% [20 0]	(Lost)	2% [1·0]	1% [0 4]
4.	Nil —	11% [34·0]	2% [2 7]	9% [1 8]	1% [1·2]

* Osthenol was administered to 60–70 g leaves; DMS to 45–50 g.

In the linear series two experiments were first conducted by administration of [2-^{14}C]-umbelliferone and unlabelled DMS to *Ruta* shoots with an arbitrary metabolic period of 17 hr. Both gave closely similar and negative results—recovered DMS had a specific activity lower than that of any furanocoumarin and far lower than that of the recovered umbelliferone. The question was then investigated further by the use of metabolic periods of 3, 10, and 24 hr. The specific activities of recovered DMS and three furanocoumarins are plotted against the time of metabolism in Fig. 1. This shows that after 3 hr metabolism following uptake of the solution the specific activity of DMS exceeded that of all three furanocoumarins. But it was clearly falling rapidly at this time, while that of psoralen was

[14] SPÄTH, E. and BRUCK, J. (1937) *Chem. Ber.* **70,** 1023.
[15] KING, F. E., HOUSLEY, J. R. and KING, T. J. (1954) *J. Chem. Soc.* 1392.

rising even more rapidly and those of bergapten and xanthotoxin at slower rates. Except for xanthotoxin a levelling-off is in evidence after 10 hr. Although the shoots used in this experiment were all from the same plant, the necessity of using different samples for the three experiments rules out attempts at more precise quantitation, but the trends are evident, and it is clear that the peak in the specific activity of DMS must occur well under 3 hr after feeding.

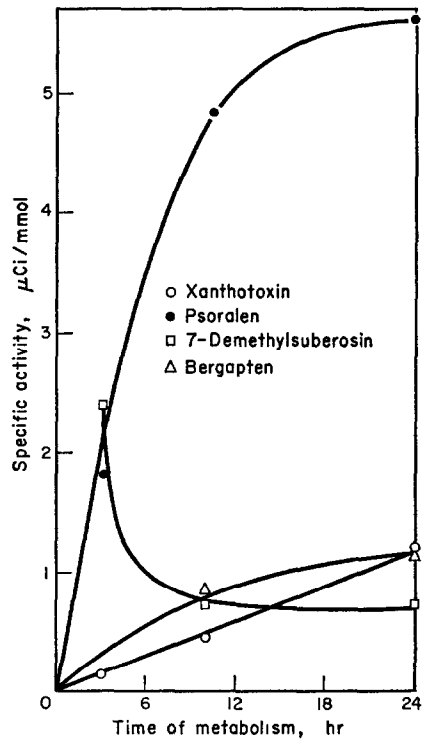

FIG. 1. CHANGES IN SPECIFIC ACTIVITY OF *Ruta* COUMARINS WITH TIME AFTER ADMINISTRATION OF [2-^{14}C]UMBELLIFERONE AND 7-DEMETHYLSUBEROSIN.

The specific activities of the umbelliferone samples recovered in this series of experiments (45 in 3 hr, 69 in 10 hr, and 33 in 24 hr) greatly complicate assessment of the results. The relationship between DMS and umbelliferone that emerges here is clearly not that of an intermediate and its immediate precursor. In an ideal series of specific activity curves covering the sequence $A \rightarrow B \rightarrow C$, where A is an administered precursor, the order of specific activities, $A > B > C$ is observed early in the metabolic period, eventually becoming reversed as the pool of A is flushed free of isotope by non-labelled products of normal metabolism, and as the specific activity of C peaks. In the present case, in which A is umbelliferone, B is DMS, and C is psoralen, the order $A > B > C$ for specific activities is indeed observed after 3 hr, and the shape of the DMS curve indicates that the difference between B and C would have been more pronounced earlier. However, although B has fallen well below C after 10 hr, A remains much higher even after 24 hr. After this period the curves of both psoralen and bergapten appear to be levelling off at values well below that of the recovered umbelliferone.

Compartmentation of metabolic pools of umbelliferone could conceivably explain the above results; this phenomenon is well documented in a number of other cases.[16] In the present instance compartmentation could involve umbelliferone equilibrium with, or the intermediacy of its glucoside skimmin. In fact, skimmin is known to be a good furanocoumarin precursor,[3] and is readily translocated from petiole cut ends to leaf blades, where subsequent metabolism takes place. The first step in this metabolism must be cleavage of the glucoside back to umbelliferone, now compartmented and probably prenylated at once. On this basis there would be three umbelliferone pools (exogenous, glucoside and compartmented) of differing specific activities. The data of Table 4 provide support for this. Within 5 hr of entering the plant's vascular system, 83% of umbelliferone administered to *Conium maculatum* was converted to skimmin. The corresponding values of 55% for *A. archangelica*, 50% for *H. lanatum* and 37% for *Ruta graveolens* show that rapid glucoside formation occurs in all these furanocoumarin producers. Even after only 1 hr in the plant tissues about 10% of the substrate was converted to the glucoside. Thus these plants are able rapidly to transform exogenous umbelliferone to skimmin, to translocate this skimmin readily to the leaf blades, and to incorporate the aromatic portion of the glucoside into furanocoumarins. In contrast, umbelliferone itself is translocated to the leaves with difficulty, and in nature possibly not at all, skimmin being the predominant detectable form of umbelliferone.

If the shapes of the curves in Fig. 1 are taken at their face value, the picture without umbelliferone has some indicative features. Over the time period studied the specific activity of DMS decreases, that of psoralen increases and suggests a peak at or before 24 hr, while those of the substituted psoralens rise slowly. Structural considerations imply the existence of several intermediates (one of them is definitely (+)-marmesin[4]) between DMS and psoralen, and if this is so it is easy to construct a hypothetical series of curves consistent with the idea that DMS, already shown (Tables 1 and 2) to be a precursor of psoralen, is an intermediate in its formation as well. Granted the availability of the presumed intermediates between DMS and psoralen, testing them should present no particular difficulties, since such an experiment has already been carried out with (+)-marmesin.[2] The study of their specific activities seems scarcely possible with present techniques because the metabolic period becomes small in comparison to the period needed for solution uptake. This factor must distort the shapes of the curves in Fig. 1, especially the earlier parts, to an unknown extent.

Trapping experiments with osthenol were neither so elaborate nor so intensively pursued. Because residual osthenol recovered from feedings to *Heracleum* was found to have specific activity much below that of the administered material one may presume at once a dilution due to endogenous material. In separate experiments in which [2-^{14}C]umbelliferone glucoside and osthenol were fed concurrently for 32 hr metabolism to *H. lanatum* and to *Angelica archangelica* (an umbellifer from which osthenol has been isolated[14]) radioactive osthenol was recovered with specific activity higher than that of other isolated angular furanocoumarins (Table 3). The presence of intermediates between osthenol and angelicin is certain: columbianetin is known[3] to be one such compound, analogous to marmesin in the linear pathway.

Investigation of Alternative Pathways

Although the evidence which has been presented here and elsewhere establishes DMS and

[16] OAKS, A. and BIDWELL, R. G. S. (1970) *Ann. Rev. Plant Physiol.* **21**, 43.

osthenol as intermediates in the respective formation of linear and angular furanocoumarins, it does not rule out the existence of other biosynthetic routes. The evidence relating to the possible role of mevalonate as the source of the prenyl unit assumed to attack the 6- and 8-positions of umbelliferone has been conflicting. For these reasons we felt it desirable to investigate other possible precursors which could participate in alternative pathways to furanocoumarins. These experiments have been largely confined to the linear series.

TABLE 3. TRAPPING EXPERIMENTS WITH *H. lanatum* AND *A. archangelica**

Plant fed	Specific activities of recovered compounds, μCi/mmol					
	Umbelliferone	Osthenol	Angelicin	Spondin	Xanthotoxin	Isopimpinellin
Angelica archangelica	10·0	2·0	0·9	—	0·9	—
Heracleum lanatum	14·0	2·2	—	1·3	3·2	2·7

* 0·5 μCi[2-^{14}C]umbelliferone glucoside (180 μCi/mmol) and 2–3 mg non-labelled osthenol were administered to 9–10 g fresh leaves for 32 hr metabolism.

Table 2 shows that in all cases the results of feeding these precursors, labelled with ^{14}C or ^{3}H, were negative, but the rationale behind the choice of these compounds should be outlined briefly. Leucine and one of its recognized precursors, 2,3-dihydroxyisovaleric acid (DHIA) were possible sources of the 3-methylbutyl skeleton that would be independent of mevalonate; the isovaleryl sidechain of mammeisin of *Mesua ferrea* derives from leucine,[17]

TABLE 4. INITIAL METABOLISM OF UMBELLIFERONE IN FURANOCOUMARIN-BEARING PLANTS*

Plant used	Metabolic period (hr)	Compounds recovered, μCi		
		Umbelliferone	Skimmin	"C_5 products"†
Angelica archangelica	1	45	5	Nil
0·5 g	5	22	27	1
	24	1	36	7
Heracleum lanatum	5	17	25	5
1·0 g	24	Nil	39	10
Conium maculatum	5	8	41	1
0·5 g	24	1	47	1
Ruta graveolens	1	44	5	1
0·5 g	5	18	27	4
	24	2	36	8

* 50 μCi[^{3}H]Umbelliferone, 105 mCi/mmol, administered.
† Includes furanocoumarins, marmesin, DMS and (in the case of *Heracleum*) angular analogs.

and the condensation of DHIA (or a derivative) with umbelliferone, followed by reduction of the presumed carbonyl adjacent to the ring, would yield IV, a possible immediate precursor of (+)-marmesin, without either mevalonate or DMS participating. Senecioic acid was another conceivable source of 3-methylbutyl skeleton. Isopentenyl pyrophosphate would be expected to be the immediate precursor of the side-chain in a mevalonate pathway;

[17] KUNESCH, G., HILDESHEIM, R. and POLONSKY, J. (1969) *Compt. Rend.* **268D**, 2143.

TABLE 5. TESTING OF VARIOUS POSSIBLE

Species	Fresh wt. (g)	Compound administered: Name	Dose (mmol)
R. graveolens	28	L-Leucine; 12·9 μCi, 129 μCi/mmol	0·10
	29	[1-^{14}C]Sodium acetate; 9·7 μCi, 97·3 μCi/mmol	0·10
	30	[2-^{14}C]Umbelliferone; 10·1 μCi, 101 μCi/mmol	0·10
R. graveolens	28	[1-^{14}C]DHIA; 11·7 μCi, 128 μCi/mmol	0·092
	29	[G-^{3}H]Umbelliferone; 9·44 mCi, 106 mCi/mmol	$8{\cdot}9 \times 10^{-3}$
C. glauca	65	[U-^{14}C]L-Valine; 4·5 μCi, 110 μCi/mmol	0·041
	55	[2-^{14}C]Senecioic acid; 12·2 μCi, 56 μCi/mmol	0·22
H. lanatum	76	[1-^{14}C]Isopentenyl pyrophosphate; 28·3 μCi, 5350 μCi/mmol + 10 mg (+)-marmesin	$5{\cdot}3 \times 10^{-3}$
	47	[5-^{3}H]Mevalonic acid DBED salt; 68 8 μCi, 66 000 μCi/mmol	$1{\cdot}04 \times 10^{-3}$

the negative result here was not unexpected because of well-recognized permeability problems with phosphorylated metabolites, and certainly does not rule out its possible involvement. Interestingly, and in apparent contradiction to the findings of Floss and Mothes with *Pimpinella*, zero incorporation of [5-^{3}H]mevalonic acid (as the DBED salt) was observed in *Heracleum*. We feel that the role of mevalonate in furanocoumarin biosynthesis must remain in question, and as Banthorpe *et al.*[18] have pointed out in their discussion of monoterpene biosynthesis, we are compelled to recognize it at present only because no evidence whatever exists for an alternative pathway.

EXPERIMENTAL

Cultivation and feeding of plants. H. lanatum seedlings were transplanted from their natural habitat and grown hydroponically in a greenhouse. *Conium maculatum* and *Coronilla glauca* were grown hydroponically from seed, as were the *R. graveolens* plants used in the second experiment in Table 5. Other plants of this last species were grown in soil, either in a greenhouse or outdoors. For feeding experiments with *Conium*, *Coronilla*, and *Ruta*, cut shoots consisting of both stems and leaves were employed; in the case of *Coronilla* young growth not yet extensively lignified was used *Heracleum* leaves were taken and the petioles cut down to *ca.* 2 cm before immersion of the cut ends in the solutions to be fed Labelled compounds were administered in small vols. of aqueous solutions, which were completely absorbed within a few hr. To facilitate solution of DMS, osthenol, senecioic acid, and the larger doses of umbelliferone, NaOH was added in minimal quantities $NaHCO_3$ was used to dissolve DHIA.

Isolation and analysis of coumarins. After the desired metabolic period the plant material in most cases was cut in small pieces into boiling ethanol. Isolation of coumarins was essentially by one or other of the procedures published earlier.[2,3] In some cases 75% MeOH was used for partitioning with hexane, and

[18] BANTHORPE, D. V., CHARLWOOD, B. V. and FRANCIS, M. J. O. (1972) *Chem. Rev.* **72**, 115.

PRECURSORS OF THE FURAN RING

Metabolic period (hr)	Results	Sp. act. (μCi/mmol)	Dilution value
72	Psoralen	0·084	(1500)
	Bergapten	0·199	(650)
	Xanthotoxin	0·081	(1600)
72	Psoralen	0·083	(1200)
	Bergapten	0·058	(1700)
	Xanthotoxin	0·047	(2100)
72	Psoralen	5·90	(17·2)
	Bergapten	3·40	(29·7)
	Xanthotoxin	1·59	(63·6)
72	Psoralen	$9{\cdot}6 \times 10^{-3}$	(13000)
	Bergapten	$4{\cdot}4 \times 10^{-3}$	(29000)
	Xanthotoxin	$4{\cdot}5 \times 10^{-3}$	(28000)
72	Psoralen	915	(85·8)
	Bergapten	110	(502)
	Xanthotoxin	56	(806)
24	(No label in free or bound psoralen)		
24	(No label in free or bound psoralen)		
24	(No label in furanocoumarins, trace of activity in marmesin)		
24	(Non-radioactive chloroform extract, not further examined)		

CH_2Cl_2 substituted for Et_2O in extraction of the aq. residue obtained after removal of the MeOH. The procedure for *C. glauca*, which differed somewhat, was as follows. The plant material was cut into boiling *n*-PrOH, and after removal of the solvent the residue was partitioned between 75% MeOH and hexane. MeOH was removed from the lower phase by concentration *in vacuo*, and the residual solution, after being diluted with H_2O, was continuously extracted with CH_2Cl_2. GLC of the extracted material yielded free psoralen. The aq. residue was then treated with emulsin, and the extraction and GLC were repeated to obtain the psoralen originally bound in the form of the coumarinic acid glycoside. GLC of neutral coumarins was done with two systems. One was the SE30 on Chromosorb W previously described,[19] with column temps. of 200° or 205°, and helium flow rates of 60 or 100 ml/min. The other was a 3 m column of nitrile silicone gum XE 60 (3% on Gas-Chrom Q, 80–100 mesh) in commercial 0·8 cm glass or stainless steel tubing, operated with a helium flow rate of 100 ml/min at 220°. These procedures normally yielded fractions showing only one fluorescent spot on TLC and giving UV curves identical to those from authentic samples. In a few cases where there was still evidence of impurity, TLC on silica gel G in $CHCl_3$–MeOH (19:1), Et_2O–hexane (1:1) or both successively, gave a satisfactory product. Free phenolic coumarins obtained from column chromatography on silica were purified by these TLC procedures only, since they cannot be separated by GLC. The quantity of coumarin in each case was calculated from its measured UV absorption, and the sample analysed for ^{14}C by scintillation spectrometry.

Labelled compounds. [U-^{14}C]Valine, [1-^{14}C]isopentenyl pyrophosphate, [5-^{3}H]mevalonic acid DBED salt, [2-^{14}C]leucine, and [1-^{14}C]sodium acetate were obtained from commercial souces Previous publications have described the preparation of [G-^{3}H]umbelliferone,[2] [3-^{14}C]DMS,[20] [2-^{14}C]DMS,[10] and [2-^{14}C]-osthenol.[10] [2-^{14}C]Senecioic acid was prepared in 15–25% yield by the method of Massot.[21] Acetone (1·0 ml), [2-^{14}C]malonic acid (0·3 g, 250 μCi), and Ac_2O (0·3 g) were refluxed 72 hr, and excess acetone was then evaporated in an air stream. The dark residue was treated 15 min with 8 ml of boiling H_2O, the solution cooled, diluted with 8 ml of 6 N HCl, saturated with NaCl, and extracted with Et_2O (3 × 10 ml). Evaporation of the Et_2O left a syrup which was chromatographed on silicic acid, elution being first with hexane,

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[20] Steck, W. (1971) *Can. J. Chem.* **49**, 2297.
[21] Massot, H. (1894) *Ber.* **27B**, 1225.

then an equal mixture of hexane and Et_2O. The labelled senecioic acid emerged from the column as the only major radioactive product, and slowly crystallized. The crystalline product (27 mg) was diluted with carrier (*ca.* 75 mg) and recrystallized from warm H_2O to yield needles of [2-^{14}C]senecioic acid, m.p. 67–68°. The procedure involving acetone–malonate condensation described by Dutt[22] gave zero yield in our hands, as did an attempted condensation of acetone and cyanoacetate. A procedure[23] based on the reaction of $^{14}CO_2$ with methylallylmagnesium chloride, a reagent which proved to be Et_2O-insoluble, gave poor yields.

[1-^{14}C]2,3-Dihydroxyisovaleric acid was synthesized via the cyanohydrin. 2-Bromoisobutyraldehyde was prepared by bromination of isobutyraldehyde diethylacetal[24] and acid hydrolysis of the product.[25] 1 g of 2-bromoisobutyraldehyde was added to a suspension of 500 mg $CaCO_3$ in 7 ml H_2O in a 25-ml flask equipped with an air condenser. The mixture was stirred vigorously and heated under reflux while evolved CO_2 was bubbled through H_2O until no further gas evolution was observed (0·5 hr). After filtration, the aq solution was saturated with NaCl and the product recovered by continuous Et_2O extraction for 3·5 hr. Removal of the Et_2O yielded 0·25–0·3 g of crude 2-hydroxyisobutyraldehyde. This product (0·26 g) was dissolved in 1 ml H_2O and a slight excess (300 mg) of solid sodium bisulphite dissolved in the solution. The solution was chilled in an ice bath and a solution of 0·93 mg of $Na^{14}CN$ (1·0 mCi, 52 8 mCi/mmol) in 0·5 ml H_2O stirred in. After 1 hr at room temp. the solution was again cooled in ice and 139 mg of carrier NaCN, as a near-saturated solution, was added slowly with stirring The solution was then allowed to stand overnight at room temp. Saturation of the reaction solution with NaCl and continuous Et_2O extraction for 4 hr yielded 286 mg (82%) of crude [1-^{14}C]2,3-dihydroxyisovaleronitrile (0·968 mCi, radiochemical yield 97%) whose identity was confirmed by comparison of its IR spectrum with one from a sample prepared by a different route[26]

To this compound dissolved in 6·4 ml H_2O was added 440 mg Na_2CO_3. H_2O, and the solution was heated under an air condenser in a test tube in a 60° oil bath, with slow aeration,[27] 36 hr. It was then continuously Et_2O-extracted 3 hr and the aq. residue stirred with enough Amberlite IR-120H resin to decompose the carbonate and give a distinctly acid reaction (*ca.* pH 4). The combined filtrate and washings from this treatment were concentrated *in vacuo* to a small vol. and the residue was adsorbed from acetone solution on 1 g of silica gel H This was packed on top of a 3- × 9-cm column of silica gel H slurried in $CHCl_3$–MeOH (17:3), and developed with that solvent mixture. A band appearing between 140 and 180 ml, and giving a positive test with the 1,2-diol reagent of Lemieux and Bauer,[28] was recovered by evaporation as a colourless syrup which rapidly crystallized on seeding with crystals from an earlier synthesis. Yield was 40 6 mg, 12% based on the nitrile. Carrier (61 mg) was added and the product recrystallized from EtOAc to yield 92 mg of [1-^{14}C]2,3-dihydroxyisovaleric acid, m p. 123–124·5°,* 128 μCi/mmol. The MS showed the molecular ion at the expected 134. The IR spectrum matched a published spectrum,[30] although showing better resolution.

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* Kogl *et al.*,[29] apparently the only others to have crystallized this compound, reported m.p. 94°. The discrepancy may be due to hydrate formation in our compound, a possibility supported by its NMR spectrum

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