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FURANOCOUMARIN BIOSYNTHESIS IN *RUTA GRAVEOLENS* CELL CULTURES

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Abstract—The biosynthetic routes to four linear furanocoumarins—psoralen, xanthotoxin, bergapten, and isopimpinellin—co-occurring in *Ruta graveolens* cell cultures have been investigated with six ^{14}C-labelled compounds. Mevalonic acid was only poorly incorporated, in contrast to umbelliferone. In support of previous suggestions, 7-demethylsuberosin and (±)-marmesin were very good precursors of the linear furanocoumarins. 7-*O*-Prenylumbelliferone also was fairly well utilized, but this was probably owing to a prior ether cleavage yielding umbelliferone. Psoralen was well incorporated into bergapten and xanthotoxin, but not into the dimethoxylated isopimpinellin. Differences exist between the organized plant and its cell culture in terms of metabolic products and, by implication, precursor utilization. *S*(+)-Marmesin was obtained in small quantity from an acid-hydrolysable conjugate present in the culture medium. Syntheses of [2-^{14}C]7-demethylsuberosin, [2-^{14}C]osthenol, [2-^{14}C]7-*O*-prenylumbelliferone, [3-^{14}C] (±)-marmesin, and [3-^{14}C]psoralen are described, as well as an improved method for separation of furanocoumarin mixtures by TLC and GLC.

INTRODUCTION

EXPERIMENTS with ^{14}C- and ^{3}H-labelled compounds have clarified the biosynthetic routes to the linear and angular furanocoumarins of various higher plants.[1–7] The initial step in the formation of the furan ring is believed to be an isoprenylation of the benzene ring of umbelliferone (7-hydroxycoumarin) to yield either the 6-alkyl or 8-alkyl derivative (7-demethylsuberosin (DMS) and osthenol, respectively.[5,6] Subsequent cyclization involving the phenolic hydroxyl and the 2′-carbon atom of the isoprenyl side chain (as such, or 'activated' as the corresponding epoxide or diol) gives rise to the α-hydroxydihydrofurano-coumarin structure. In the case of DMS such a cyclization would result in *S*(+)-marmesin[8] or its optical isomer, *R*(−)-marmesin (nodakenetin). Several different routes between marmesin and the simplest linear furanocoumarin, psoralen, may be postulated but little evidence is yet available as to the *in vivo* pathway. The transformation is formally a loss of the elements of isopropanol in one or more stages.

By means of trapping experiments with ^{14}C and direct feedings of G-^{3}H-labelled material,

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[1] FLOSS, H. G. and MOTHES, U. (1966) *Phytochemistry* **5**, 161.
[2] FLOSS, H. G. and PAIKERT, H. (1969) *Phytochemistry* **8**, 589.
[3] STECK, W., EL-DAKHAKHNY, M. and BROWN, S. A. (1969) *Tetrahedron Letters* 4805.
[4] BROWN, S. A. (1970) *Phytochemistry* **9**, 2471.
[5] BROWN, S. A., EL-DAKHAKHANY, M. and STECK, W. (1970) *Can. J. Biochem.* **48**, 863.
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[7] STECK, W. and BROWN, S. A. (1971) *Can. J. Biochem.* **49**, 1213.
[8] HARADA, I., HIROSE, Y. and NAKAZAKI, M. (1968) *Tetrahedron Letters* 5463.

(+)-marmesin has been confirmed as an intermediate in the formation of linear furanocoumarins.[5,7] Detailed reports of direct feedings of the logical earlier precursor, DMS, are not yet to hand (but see Refs. 9 and 10). The present report describes the results of separate time-course feedings of these and four other compounds, all ^{14}C-labelled, to leaf cell cultures of *Ruta graveolens*.

RESULTS AND DISCUSSION

Ruta graveolens L. (garden rue) is well known as a source of furanoquinolines[11] and of linear furanocoumarins such as psoralen, xanthotoxin, and bergapten.[12] In addition, the plant elaborates other compounds with α,α-dimethylallyl substituents at the 3-position of the lactone ring, i.e. rutacultin and rutamarin.[13] Leaf cell cultures of *R. graveolens* can produce rutaretin (8-hydroxymarmesin).[14] In the present work (+)-marmesin has been obtained from an acid-hydrolysable conjugate in the medium while, in confirmation of a previous report,[13] isopimpinellin has also been recovered. This isolation of isopimpinellin, which is not present in organised *Ruta* plants, has meant that non-, mono- and di-oxygenated linear furanocoumarins could all be examined after each feeding experiment, with potential benefits for the overall interpretation of the results. Part of the present work has been a checking by ^{14}C-labelling of data previously obtained from feedings of ^{3}H-labelled compounds to cut *Ruta* shoots.[5]

TABLE 1. INCORPORATION OF [5-^{14}C] (±)-MEVALONIC ACID INTO *Ruta* FURANOCOUMARINS

Metabolic period (days)	Total % inc.	% Incorporation and dilution values			
		Psoralen	Xanthotoxin	Bergapten	Isopimpinellin
1	0·0221	0·0015 (66 000)	0·0014 (93 000)	0·0046 (85 000)	0·0034 (120 000)
4	0·0122	0·0007 (170 000)	0·0012 (170 000)	0·0021 (210 000)	0·0020 (150 000)
7	0·0094	0·0003 (240 000)	0·0006 (470 000)	0·0023 (170 000)	0·0014 (300 000)

Precursor specific activity = 1·18 × 10^{4} μCi/mmol; Dose = 0·146 μM/flask. Dilution values are in parentheses.

The results obtained from the time-course feedings are presented in Tables 1–6. The observed percentage incorporation and dilution values are given together with the total incorporation values, but because the complexity of the isolation procedure precluded strictly quantitative recovery, the incorporation values must be regarded only as minimal, in common with most such data. They should, however, be comparable within each feeding experiment. For the racemic precursors, mevalonic acid and marmesin, the percentage

[9] AUSTIN, D. J. and BROWN, S. A. (1970) *Abstracts—Biochemistry 14th Nordiska Kemistmötet, Umeå, Sweden*; 18–20 *June*, p. 108.
[10] GAMES, D. E. and JAMES D. H. (1972) *Phytochemistry* **11**, 868.
[11] SCHNEIDER, G. (1965) *Planta Med.* **13**, 425.
[12] DEAN, F. M. (1963) *Naturally-Occurring Oxygen Ring Compounds*, Butterworths, London.
[13] STECK, W., BAILEY, B. K., SHYLUK, J. P. and GAMBORG, O. L. (1971) *Phytochemistry* **10**, 191.
[14] REINHARD, E., CORDUAN, G. and VOLK, O. H. (1968) *Planta Med.* **16**, 8.

incorporation and dilution values reported here are those actually observed.* Except for mevalonic acid, the molar dose level was held as constant as possible to limit at least one variable.

As anticipated from earlier work,[1,4] the incorporation of mevalonic acid was extremely low, and the corresponding dilution values high (Table 1). This phenomenon has been very frequently observed and alternatives to mevalonate have been considered as precursors for the five-carbon units in certain isoprenoid or prenylated compounds.[4,15,16] Despite this, previous results on the biosynthesis of furanocoumarins[1] and furanoquinolines[17–19] leave no doubt that mevalonate *can be* a precursor of dimethylallyl substituents *ortho* to a 'phenolic' oxygen function and that subsequent cyclization gives rise to dihydrofuran and furan derivatives.

TABLE 2. INCORPORATION OF [2-^{14}C]UMBELLIFERONE INTO *Ruta* FURANOCOUMARINS

Metabolic period (days)		Total % inc.	% Incorporation and dilution values			
			Psoralen	Xanthotoxin	Bergapten	Isopimpinellin
1		3·84	1·51	1·27	0·772	0·283
			(17·1)	(30·3)	(62·9)	(107)
4		2·42	0·528	0·500	1·25	0·144
			(67·3)	(63·5)	(59·4)	(229)
7	Total	1·99	0·322	0·435	0·820	0·411
			(164)	(123)	(86·1)	(93·9)
7	Tissue	1·73	0·212	0·398	0·722	0·400
			(130)	(115)	(83·9)	(92·3)
7	Medium	0·256	0·110	0·037	0·098	0·011
			(228)	(203)	(103)	(150)

Precursor specific activity = 252 μCi/mmol; Dose = 1·92 μM/flask. Dilution values are in parentheses.

The data for (±)-mevalonic acid (I) in Table 1 exhibit two features which, with minor variation, apply to all six feedings to the cell cultures. First, the marked decrease in percentage incorporations (both total and individual) with time indicates that the furanocoumarins, once formed, are subject to further metabolism. *R. graveolens* cell cultures have been shown to metabolize [2-^{14}C]cinnamic acid, phenylalanine, tyrosine, and tryptophan to carbon dioxide.[20] Furanocoumarins may be degraded via initial attack on the lactone or furan rings. Second, the equally marked increase in the dilution values shows that the synthesis of furanocoumarins continues throughout the 7-day metabolic period, in accord with the observed doubling of furanocoumarin weight. Taken together, these features demonstrate a net turnover of radioactive furanocoumarins in the tissue. The coumarin

* The common practice of doubling the incorporation value for a racemic precursor may be suspect, since there is evidence that the relationship between dose on the one hand, and incorporation and dilution values on the other, is not linear. Also, the presence of the unutilized enantiomer could conceivably affect metabolic processes involved in the translocation and conversion of its isomer.

[15] BANTHORPE, D. V., MANN, J. and TURNBULL, K. W. (1970) *J. Chem. Soc.* C, 2689.
[16] BAKKER, H. J., GHISALBERTI, E. L. and JEFFERIES, P. R. (1972) *Phytochemistry* **11**, 2221.
[17] GRUNDON, M. F. and JAMES, K. J. (1971) *Chem. Commun.* 1311.
[18] COLONNA, A. C. and GROS, E. G. (1971) *Phytochemistry* **10**, 1515.
[19] COBET, M. and LUCKNER, M. (1971) *Phytochemistry* **10**, 1031.
[20] ELLIS, B. E. and TOWERS, G. H. N. (1970) *Phytochemistry* **9**, 1457.

concentration in the tissue is probably held roughly constant by a balance of synthetic and degradative reactions since, as a rule, these compounds are not released into the medium. Provided that adequate quantities of the required metabolites can still be obtained, short metabolic periods would appear to be greatly preferred for tissue culture feedings, as well exemplified by a recent study of scopoletin biosynthesis.[21]

The experiment reported in Table 2 not only confirmed umbelliferone (II) as an excellent precursor of furanocoumarins, but also provided data of a new type. The tissue gave rise, sporadically before seven days' culture but consistently after nine days, to a cloudy cell suspension in the medium, which on subculturing eventually reproduced the original green nodular tissue. Simultaneously there appeared in the medium four major furanocoumarins. These had acquired about one-eighth of the total incorporation after 7 days (the suspension first appeared on the fifth day) and, in each case, they were appreciably *less* radioactive, in terms of both total and specific activity, than the tissue coumarins. On the assumption that compartmentation exists in the tissue, the radioactive furanocoumarins found in the medium may have been formed from precursor pools diluted to different degrees by endogenous unlabelled material. Alternatively, their observed dilution values may arise by simple dilution of the radioactive coumarins with varying amounts of the four unlabelled compounds released into the medium when the cell suspension formed. The noteworthy decrease in the dilution values for isopimpinellin between the fourth and seventh days suggests that a highly labelled precursor pool became available for its biosynthesis during this period.

TABLE 3. INCORPORATION OF [2-^{14}C]DEMETHYLSUBEROSIN INTO *Ruta* FURANOCOUMARINS

Metabolic period (days)	Total % inc.	% Incorporation and dilution values			
		Psoralen	Xanthotoxin	Bergapten	Isopimpinellin
1	1·77	0·456	0·140	1·05	0·123
		(20·6)	(75·2)	(68·7)	(222)
4	2·18	0·192	0·298	1·39	0·300
		(60·9)	(89·9)	(63·9)	(154)
7	1·42	0·084	0·128	0·903	0·305
		(151)	(191)	(86·7)	(170)

Precursor specific activity = 48·5 μCi/mmol; Dose = 1·79 μM/flask. Dilution values are in parentheses.

The radioactive coumarins, once formed, and the endogenous coumarins are apparently not mutually accessible until the cells have been disrupted. The various possibilities suggest the existence, after radiotracer feedings, of individual labelled compounds distributed among separate metabolic pools, each varying with respect to the total and specific radioactivity present. Since such pools would be expected to have different functions in the overall metabolism, the implication is that compartmentation not only of 'final' metabolites but also of precursors can exist, further increasing the problems of interpretation.

The feeding of 7-demethylsuberosin, DMS (III) provided very good evidence for its status as a furanocoumarin precursor (Table 3). Although the total incorporation values were somewhat lower than for the umbelliferone feedings, the dilution values were closely

[21] FRITIG, B., HIRTH, L. and OURISSON, G. (1970) *Phytochemistry* **9**, 1963.

similar. Examination of the individual incorporation values shows that, for bergapten synthesis, DMS was somewhat more efficient than umbelliferone, while it was less so for the others. The incorporation difference could indicate a recurrence of the frequently encountered permeability problem. A less likely possibility is that the feeding, even on the micromole level, has caused some quantitative alterations in the tissue metabolism. Although it has been assumed in the past that DMS is converted to furanocoumarins via marmesin, it is worth noting that an oxidative cleavage of the side-chain of DMS could yield the aldehyde 6-(2-oxoethyl)umbelliferone, which was used by Seshadri and his associates in their *in vitro* synthesis of psoralen from DMS.[22]

TABLE 4. INCORPORATION OF [2-^{14}C]7-*O*-PRENYLUMBELLIFERONE INTO *Ruta* FURANOCOUMARINS

Metabolic period (days)	Total % inc.	% Incorporation and dilution values			
		Psoralen	Xanthotoxin	Bergapten	Isopimpinellin
1	0·939	0·313	0·220	0·302	0·104
		(83·1)	(154)	(271)	(439)
4	0·784	0·143	0·166	0·316	0·159
		(171)	(204)	(239)	(243)
7	0·555	0·063	0·073	0·340	0·079
		(307)	(270)	(226)	(247)

Precursor specific activity = 14·4 μCi/mmol; Dose = 1·83 μM/flask. Dilution values are in parentheses.

The dilution values for 7-*O*-prenylumbelliferone (IV, Table 4), while higher than for umbelliferone and DMS, were not so high as to rule out its participation. The incorporation observed is most readily explained by some *in vivo* cleavage of the alkenyl oxygen bond to produce [2-^{14}C]umbelliferone, although the possibility of permeability difficulties is not excluded. Another possibility is oxidative cleavage of the isoprenyl side chain to produce the aldehyde 7-(2-oxoethoxy)coumarin, known to give rise to psoralen on base treatment *in vitro*.[23] In this feeding, the constancy of the bergapten dilution values and the rise and subsequent fall in those of xanthotoxin may indicate derivation of isopimpinellin primarily from an 8-oxygenated rather than a 5-oxygenated furanocoumarin.

TABLE 5. INCORPORATION OF [3-^{14}C] (±)-MARMESIN INTO *Ruta* FURANOCOUMARINS

Metabolic period (days)	Total % inc.	% Incorporation and dilution values			
		Psoralen	Xanthotoxin	Bergapten	Isopimpinellin
1	1·91	0·244	0·214	0·337	0·157
		(29·1)	(61·5)	(77·9)	(100)
4	1·37	0·069	0·136	0·366	0·114
		(76·9)	(107)	(89·3)	(113)
7	1·06	0·025	0·121	0·261	0·121
		(320)	(228)	(148)	(171)

Precursor specific activity = 76·6 μCi/mmol; Dose = 1·94 μM/flask. Dilution values are in parentheses.

[22] AREJA, R., MUKERJEE, S. K. and SESHADRI, T. R. (1958) *Tetrahedron* **4**, 256.
[23] MCLEOD, J. K. and WORTH, B. R. (1972) *Tetrahedron Letters* 237.

Table 5 confirms the efficiency of (±)-marmesin (VII) as a furanocoumarin precursor in *Ruta*; the close resemblance to DMS is apparent both in the dilution values and the trend of the incorporations. Although the consistently high incorporation of marmesin, DMS, and umbelliferone into the four linear furanocoumarins strongly supports previously advanced biosynthetic schemes,[3,5] there is still the possibility of hydrative fission of the dioxan ring and oxidative cleavage of the resulting glycol to yield 6-(2-oxoethyl) umbelliferone, as mentioned above. No evidence is currently available that aldehyde intermediates do participate.

TABLE 6. INCORPORATION OF [3-^{14}C]PSORALEN INTO *Ruta* FURANOCOUMARINS

Metabolic period (days)		Total % inc.	% Incorporation and dilution values			
			Psoralen	Xanthotoxin	Bergapten	Isopimpinellin
1		5·59	2·37	0·567	2·59	0·065
			(3·7)	(26·4)	(10·4)	(127)
4		3·29	0·340	0·146	2·79	0·020
			(29·5)	(156)	(16·5)	(1600)
7	Total	3·09	0·128	0·311	2·63	0·021
			(93·4)	(138)	(32·9)	(2300)
7	Tissue	2·49	0·099	0·305	2·07	0·017
			(70·7)	(122)	(27·6)	(2400)
7	Medium	0·600	0·029	0·006	0·561	0·004
			(171)	(1000)	(52·4)	(2000)

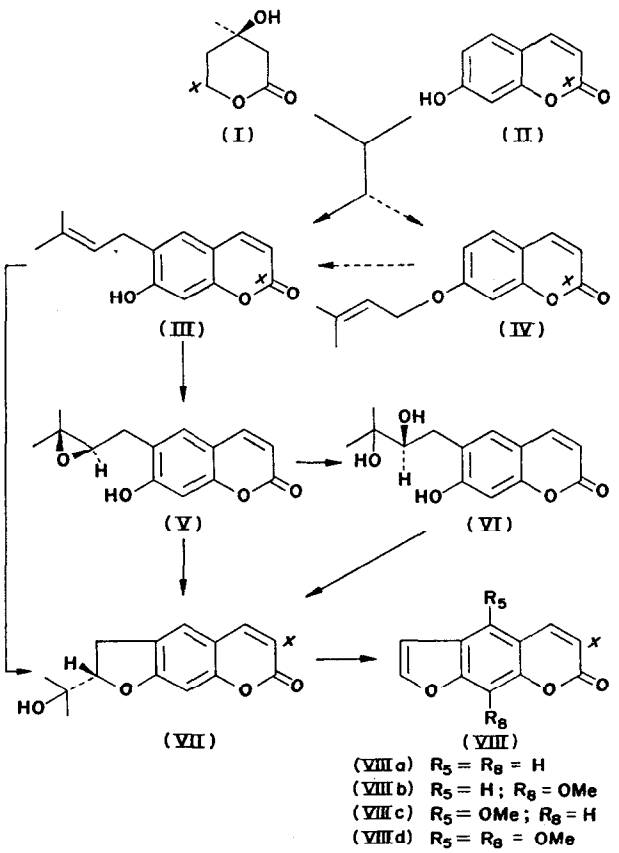
Precursor specific activity = 122 μCi/mmol; Dose = 1·93 μM/flask. Dilution values are in parentheses.

In all feeding experiments, the radioactive psoralen isolated was consistently the least diluted coumarin until late in the metabolic period, when it was superseded by bergapten. Indeed, after 7 days' metabolism with umbelliferone, 7-*O*-prenylumbelliferone, and marmesin, it became the most highly diluted. This behaviour is consistent with psoralen being a precursor of the methoxylated coumarins. This idea, in accord with previous results,[5] is fully supported by the present data. The results of the feedings of ^{14}C-labelled psoralen (VIIIa) to *Ruta* cell cultures (Table 6) show that the simplest linear furanocoumarin is an excellent precursor for the co-occurring methoxylated derivatives, with the exception that isopimpinellin was much less radioactive than expected after 4 and 7 days' metabolism. The discrepancy may be explicable along the lines of selective inhibition by psoralen, but such an approach is highly speculative. Another explanation, based on the theory of Caporale *et al.*,[24] is that xanthotoxin is derived primarily from rutaretin (8-hydroxymarmesin) and, by implication, that its derivation from psoralen is quantitatively less important. If one postulates that isopimpinellin is derived from xanthotoxin, the results of Table 6 can be held to provide some support for this idea, since the xanthotoxin incorporations are also low in relation to those of bergapten. But the data in this and the other tables taken together it make clear that psoralen is also a xanthotoxin precursor, and available evidence is still too meagre to draw any conclusions about the relative importance of these two pathways. With the exception just noted, psoralen is evidently the most efficient precursor among those tested for the biosynthesis of the *Ruta* cell culture furanocoumarins.

[24] CAPORALE, G., DALL'ACQUA, F., CAPOZZI, A., MARCIANI, S. and CROCCO, R., (1971) *Z. Naturforsch.* **26b**, 1256.

As in the case of umbelliferone, the feeding of psoralen was marked by the appearance of a cloudy cell suspension in the medium towards the end of the 7-day metabolic period. With the exception of isopimpinellin—here a borderline case—the tissue coumarins were uniformly more radioactive than the medium-derived coumarins, which contained one-fifth of the total radioactivity incorporated. Similar arguments favouring compartmentation within the tissue apply here, with the interesting distinction that psoralen is very much further along the biosynthetic pathway than umbelliferone. Thus, the assumed compartmentation effects still exist even at the stage of the fully elaborated furanocoumarin structures.

Taken overall, the results of the *Ruta* cell culture feedings described here lend strong support to the schemes advanced for linear furanocoumarin biosynthesis.[2–5] Possible routes from umbelliferone to psoralen and its derivatives via DMS and marmesin are shown in Scheme 1; they bear a close analogy to those proposed for the formation of certain furanoquinolines.[17,18] Some aspects of the stereochemistry of these reactions were discussed in earlier publications.[4,5]



SCHEME 1. POSSIBLE ROUTES TO *Ruta* LINEAR FURANOCOUMARINS. '*x*' indicates the ^{14}C-labelling site in the compounds fed.

As previously stated, no evidence is available at present as to the precise mechanism of the loss of the elements of isopropanol which yields psoralen from marmesin. But Caporale and

his associates have recently described experiments on the biosynthesis of psoralen and bergapten in *Ficus carica* and have adduced evidence that the 4',5'-dihydrofuranocoumarins are precursors.[25] It is our belief, nevertheless, that further investigation is required before their role can be regarded as firmly established. The dilution values reported for the ^{3}H-labelled precursors employed by Caporale's group are much higher than those found in the present work and, in fact, their data do not rule out a simple metabolic dehydrogenation of the exogenous dihydrofuranocoumarins rather than their obligatory participation in the pathway. In the latter event the much lower efficiency of incorporation of ^{3}H-umbelliferone into the furanocoumarins which they observed would not be an unexpected result. Their report of a low level of dihydrobergapten-derived activity in psoralen (less than one-sixtieth of the bergapten value) could be an example of the commonly encountered randomization of tritium label. The low activity found in bergapten (one-thirteenth of the psoralen value) after the administration of dihydropsoralen may have arisen similarly, or via 5-methoxylation of ^{3}H-psoralen formed by dehydrogenation, or by both routes together. The presence of small amounts of 4',5'-dihydropsoralen in fig leaves[26] may result from psoralen degradation rather than from an accumulation of a psoralen precursor. Such degradative routes could include the lactone ring attack mentioned above, leading to 3,4-dihydropsoralen formation. An enzyme catalysing the hydrolysis of several dihydrocoumarins to the corresponding *o*-hydroxyphenylpropionic acids[27] (possibly then subject to β-oxidation) has been obtained from higher plant sources.

The isolation of (+)-marmesin from an acid-hydrolysable conjugate present in the *Ruta* cell culture medium supports earlier work[3,5,7] indicating its participation in the pathways shown above. Its very small quantity could be explained by the assumption of its rapid turnover in the tissue itself, from which it is not so far proved isolable. However, the (+)-marmesin recovered could conceivably have been formed by suitable cyclization of either the epoxide or triol during the work-up or the hydrolysis. If this had happened, a chromanol with the decursinol structure[5] would have been expected as a by-product. The marmesin isolated was, however, the only significant oxygenated coumarin derivative found in the 'medium bound' fraction analysed.

Following the preliminary publication of our work,[9] Games and James reported briefly on their studies on the biosynthesis of the coumarins of *Angelica archangelica*.[10] Their work strongly supports the concept that DMS does indeed take part in the pathway to marmesin and the linear furanocoumarins, and together with the present work, leads to the conclusion that the biosynthesis of linear furanocoumarins follows a route such as that shown in Fig. 1.

Beyond psoralen there is no very clear picture of the pathways to the oxygenated psoralens. There are a number of possibilities for hydroxylation followed by alkylation, but the present work permits no firm deductions as to which of these obtain *in vitro*. This question is currently under study.

EXPERIMENTAL

Cell cultures. Sterile leaf cell cultures of *R. graveolens* were maintained on 100-ml portions of medium in 500-ml flasks on a rotary shaker (240 rpm) under fluorescent lighting. B5 medium as finally adopted by Gamborg *et al.*[28] was employed, with the addition of 0·2% N-Z Amine, Type A. Under these conditions the

[25] CAPORALE, G., DALL'ACQUA, F., MARCIANI, S. and CAPOZZI, A. (1970) *Z. Naturforsch.* **25b**, 700.
[26] DALL'ACQUA, F., MARCIANI, S., and CHIARELOTTO, G. (1968) *Atti Ist. Veneto Sci. Lettre Arti. Cl. Sci. Mat. Natur.* **126**, 103.
[27] CONN, E. E. (1964) in *Biochemistry of Phenolic Compounds* (HARBORNE, J. B. ed.), pp. 427–8, Academic Press, New York.
[28] GAMBORG, O. L., MILLER, R. and OJIMA, K. (1968) *Exp. Cell Res.* **50**, 151.

fresh weight of the rough green nodular tissues increased from *ca.* 3·5 g/flask to *ca.* 7 g/flask in 7 days, after which transfers were made. This doubling of fr. wt corresponded to an average increase in furanocoumarin wt from 0·36 to 0·72 mg/flask over the same period (see Ref. 13). Each flask of 7-day culture yielded *ca.* 600 mg of dry tissue after the extraction procedure described below.

Feeding and isolation. Labelled compounds were fed as the sodium salts to 16-flask batches of freshly inoculated *Ruta* cultures by injection of portions of a stock solution through a microporous filter (Millipore GSWP, 0·22 mμ). Later analyses of the residual stock solutions showed no alteration of these compounds. 5 flasks were harvested after 1 and 4 days, and 6 flasks after 7 days' metabolism. After the required metabolic period the tissue was filtered off and treated 10–15 min with boiling EtOH. After cooling, the solvent was diluted to *ca.* 80% EtOH with H_2O and heated to boiling. The partially extracted tissue was then homogenized in this hot solvent, and the mixture refluxed 1 hr to complete the extraction. The procedure previously described[6] was then followed to yield an Et_2O extract and an aq. residue. The former contained the 'tissue free' fraction. The latter was made 2 N with conc. HCl, heated on the steam bath 1 hr, and continuously Et_2O-extracted as above to give the 'tissue bound' fraction. The combined medium from each set of flasks was filtered through Celite analytical filter aid and concentrated to a suitable vol. *in vacuo*. It was then continuously Et_2O-extracted as above to yield the 'medium free' fraction. The aq. residue from the extraction was acidified, heated, and extracted as above to give the 'medium bound' fraction.

Non-phenolic lactones. The individual fractions were rapidly sublimed at 190–200° (0·02 torr) to remove polymeric materials. Each sublimate was dissolved in 5 ml of methanolic KOH (10%, w/v) and allowed to stand overnight at room temp. The solution was diluted with 40 ml H_2O, extracted with three 40-ml portions Et_2O to remove neutral and basic material, and the Et_2O extracts were back-extracted with 20 ml H_2O. The combined aqueous phases were acidified with 2 ml conc. HCl and extracted with four 40-ml portions of Et_2O. The combined Et_2O solutions were extracted with three 40-ml portions of aq. KOH (0·5% w/v) to remove acids and phenols, and the aq. extracts back-extracted with two 40-ml portions of Et_2O. The combined Et_2O phases were washed with two 50-ml portions of H_2O, dried, and the solvent was evaporated to yield the required non-phenolic lactone fraction. UV spectral analyses of the concentrates revealed furanocoumarins only in the 'tissue free' and in two of the 'medium free' fractions.

Furanocoumarins. The non-phenolic lactone fraction was applied as a band on a 0·5-mm Silica gel G plate, 20 × 20 cm, and developed with $CHCl_3$, effecting excellent separation of 2 main UV-fluorescing bands containing psoralen + bergapten and xanthotoxin + isopimpinellin, respectively. Together they amounted to over 90% of the fraction weight. The bands were each excised, eluted with hot acetone, and the extracts filtered through Celite before removal of the solvent to yield >95% recovery of the coumarins. The 4 furanocoumarins were isolated from the mixtures by preparative GLC with helium carrier gas. A column of 3% XE 60 on 80–100 mesh Gas-Chrom Q was contained in a 3-m length of commercial ¼″ (6·3 mm) copper tubing. Oven temps. of 220° and 240°, respectively, were used for the 2 mixtures described above. The eluted coumarins were collected in thin-walled glass capillaries of 1·5-mm bore inserted in an aluminium block at the exit, heated to 280°. They were identical in all respects (UV spectrum, TLC, GLC retention time, fluorescence under UV, m.p. and m.m.p.) with authentic samples. The wt of each compound was estimated by UV spectrometry in MeOH and its radioactivity measured in Omnifluor*-toluene solution by scintillation spectrometry.

(+)-*Marmesin.* The combined medium from 90 flasks of 7-day *Ruta* culture was treated as above to provide 'medium free' and, after acid hydrolysis, 'medium bound' fractions, which were then rapidly sublimed. PC revealed a marmesin-like compound in the 'bound' fraction only. This was isolated and purified by repeated preparative TLC on 0·5-mm Silica gel G plates, 10 × 20 cm in MeOH–$CHCl_3$ (1:9). The pure compound (*ca.* 0·1 mg) was indistinguishable from authentic (+)-marmesin by UV spectrum, R_fs on PCs and TLCs, or GLC retention times. Co-chromatography in all the systems confirmed that the unknown is marmesin. Although the small amount of the isolated compound available precluded accurate measurement of the optical rotation, this was in the same sense as that of authentic *S*(+)-marmesin. The present finding suggests the existence in the culture medium of an acid-hydrolysable conjugate of *S*(+)-marmesin, possibly the known β-D-glucoside, marmesinin.[29]

Radioactive compounds. [5-^{14}C]*Mevalonolactone* (I). This was obtained from Schwarz/Mann. Radiochromatogram scanning indicated a purity of >98%. [2-^{14}C]*Umbelliferone* (II). This was prepared as previously described[30] from 2,4-dihydroxybenzaldehyde and [3-^{14}C]*cyanoacetate*. [2-^{14}C]*7-Demethylsuberosin* (III), [2-^{14}C]*osthenol, and* [2-^{14}C]-7-O-*prenylumbelliferone* (IV). After repeated failure of attempts to prepare DMS directly from 2,4-dihydroxy-5-prenylbenzaldehyde, the following low-yield procedure was adopted. The direct synthesis has since been achieved.[31] [2-^{14}C]Umbelliferone (120 mg, 276 μCi/mmol) was dissolved in dry purified dioxan (11 ml), and black silver oxide (190 mg) added in portions to the cooled solution. The suspension was shielded from light and continuously stirred. Redistilled 3-methyl-2-butenyl

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[30] BROWN, S. A. (1963) *Phytochemistry* **2**, 137.
[31] STECK, W. (1971) *Can. J. Chem.* **49**, 2297.

bromide[32] (256 mg) was added dropwise and the flask was well stoppered. After 3 hr further portions of silver oxide (120 mg) and 3-methyl-2-butenyl bromide (128 mg) were added as above. After 52 hr stirring at room temp. an excess of the bromide was still present, and a third portion of silver oxide (120 mg) was added to the yellow-gray suspension.

The reaction was monitored by TLC and stopped after 5 days. The suspension was extracted with boiling acetone and the extract diluted with H_2O (20 ml). The mixture was then repeatedly distilled azeotropically with benzene, yielding the reaction product as a pale yellow gum which, on radio-TLC scanning, was shown to contain umbelliferone, DMS, osthenol, and 7-*O*-prenylumbelliferone as the major radioactive components. The gum was dissolved in ether (100 ml) and extracted with seven 40-ml portions 0·5% aq. KOH to provide 'neutral' and 'phenolic' fractions. Surprisingly, although the 'phenolic' fraction contained almost all the unreacted umbelliferone, it contained none of the expected DMS and osthenol, both of which appeared in the 'neutral' fraction. The latter fraction was applied to four 0·5-mm Silica gel G plates, 20 × 20 cm, and developed once with $CHCl_3$, then twice with 2% MeOH in $CHCl_3$. This gave a clear-cut separation of the 4 compounds present; R_fs were: umbelliferone 0·39, DMS 0·54, osthenol 0·67, 7-*O*-prenylumbelliferone 0·90. The 4 bands were excised separately and eluted with Et_2O–acetone mixtures. TLC and radio-TLC scanning demonstrated the high radiochemical purity of the first three, while the last contained a small proportion of a less polar impurity. In order to obtain pure radioactive DMS, a known amount (17·8 mg) of the unlabelled compound was added to the appropriate eluate and the mixture rapidly sublimed at 195–200° (0·02 torr), yielding a cloudy yellow gum (39·8 mg). This was crystallised from benzene to give [2-^{14}C]DMS (168· mg) as colourless needles, m.p. 128–31° (lit. m.p. 130–2°),[33] identical in all respects to authentic material (UV, TLC, undepressed m.p.) and pure as judged from radio-TLC scanning. A second crop (3·3 mg) was obtained from the yellow mother liquors. The specific activity of the pure compound was 48·5 μCi/mmol, consistent with a yield of 11·7 mg (6·8% of theoretical). The osthenol fraction was twice sublimed as for DMS to yield 11·6 mg of a pale yellow gum which slowly crystallized on standing. TLC and radio-TLC scanning showed a trace of a less polar impurity, not 7-*O*-prenylumbelliferone. The yield was 6·8% of theoretical. The 7-*O*-prenylumbelliferone fraction was rechromatographed on a 2-mm Silica gel G plate, 20 × 20 cm, developed partially with 2% MeOH in $CHCl_3$ and fully with $CHCl_3$. The required component, which was well separated from less polar impurities, was eluted from the excised band with acetone, and the solute sublimed to yield the pure compound (60·9 mg, 35·8% of theoretical) as an almost colourless gum. This was combined with another sample, also 2-^{14}C-labelled, obtained in 88% yield by Steck's method,[34] and the combined lot was sublimed and then recrystallized from MeOH. The compound melted at 77–8° (lit. m.p. 76–7°)[34] and was shown to be pure and identical with authentic material by UV, TLC, and radio-TLC scanning. The final specific activity was 14·4 μCi/mmol. The appropriate eluates from the 'neutral' and 'phenolic' fractions were combined and 2× sublimed as above for recovery of unreacted umbelliferone (47·1 mg, 39·2% of starting material).

[3-^{14}C](±)-*Marmesin* (VII). 5-Prenylresorcylaldehyde (1 g) was dissolved in $CHCl_3$ (13·4 ml) and treated with a solution of *m*-chloroperbenzoic acid (1 g, 85% grade) in $CHCl_3$ (13·4 ml) and the mixture allowed to stand overnight at room temp. (The product formed is strongly dependent on the nature of the solvent.[31]) The pale yellow solution was extracted with 5% aq. $NaHCO_3$, washed with H_2O, and dried. After removal of the solvent the crystalline product was rapidly sublimed at 190° (0·02 torr) to yield the cyclized racemic salicylaldehyde derivative (963 mg, 90·0%). This product was recrystallized from EtOH–H_2O to yield a first crop (452 mg) of the required product, m.p. 117·5–8·5° (lit. m.p. 117–8°).[35] A second crop (258 mg, m.p. 116·5–7·5°) was recovered from the mother liquors. The combined yield was 71% of theoretical. The salicylaldehyde derivative (169 mg) was dissolved in EtOH (2 ml) containing diethyl [2-^{14}C]malonate (144 mg) and 0·02 ml piperidine was added. The yellow solution was refluxed 20 min and cooled, yielding a mass of yellow needles. This was subjected repeatedly to azeotropic distillation with C_6H_6–acetone, and the yellow crystalline ethyl [3-^{14}C] (±)-marmesin-3-carboxylate dried under vacuum at 110° for 3 hr. It melted at 220–1°, and the yield was 219·4 mg (90·4% of theoretical). It was shown to be pure by TLC and radio-TLC scanning. The (±)-marmesin ester (219 mg) was dissolved in 3·5 ml HOAc and 1 ml conc. HCl. The solution was refluxed 2 hr and then poured on crushed ice to yield a gray–green powder. The powder was sublimed at 220–5° (0·02 torr) to give the required (±)- marmesin-3-carboxylic acid (187 mg, 94% of theoretical) as a yellow powder, m.p. 219–22° (lit. m.p. 223–4°).[35] The (±)-marmesin-3-carboxylic acid (187 mg) was refluxed 10 min with 204 mg copper powder in 4 ml quinoline. The cooled solution was diluted with EtOAc and Et_2O, and decanted from the copper. The solution was washed with dil. HCl and H_2O, then dried. The solvent was removed on the steam bath and the oily product rapidly sublimed at 180–90° (0·02 torr) to yield a yellow crystalline product (129·2 mg, 75·4%), predominantly (±)-marmesin. This was dissolved in 5 ml of $CHCl_3$–light petrol. (1:1) and cooled below 0°. For feeding purposes, sufficient [3-^{14}C] (±)-marmesin was obtained in the first crop (19·3 mg, 11·4%) of colourless powdery crystals, m.p. 150–2° (lit. m.p. 153–4°).[35] The com-

[32] CLAISEN, L., KREMERS, F., ROTH, F. and TIETZE, E. (1922) *J. Prakt. Chem.* **105,** 65.
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[34] STECK, W. and BAILEY, B. K. (1969) *Can. J. Chem.* **47,** 3577.
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pound was identical with authentic (+)-marmesin in its UV spectrum, TLC, and GLC retention time, and was shown to be pure by radio-TLC scanning. The specific activity was 76·6 μCi/mmol. Subsequent crops were increasingly contaminated with a less polar impurity, probably 2'-isopropyl-psoralen formed by dehydration during the decarboxylation step, and the possibility of purification by other techniques was not pursued.

[3-^{14}C]*Psoralen* (VIIIa). 5-Formyl-6-hydroxybenzofuran (108 mg) was dissolved in 2 ml EtOH, and diethyl [2-^{14}C]malonate (127 mg) added. Addition of 0·03 ml of piperidine resulted in an intense orange-red coloration. The solution was refluxed 15 min and then cooled, yielding a mass of orange needles. The material was repeatedly dissolved in EtOH and the solvent removed under vacuum at 80° to give a quantitative yield (172 mg) of ethyl [3-^{14}C]psoralen-3-carboxylate as orange needles, m.p. 150–2° (lit. m.p. 153–4°).[36] The psoralen ester derivative (171 mg) was dissolved in 3·5 ml HOAc containing 1 ml conc. HCl and refluxed 5 hr, with addition of a further 1 ml HCl after 2·5 hr. The resulting yellow suspension was poured on crushed ice and the slightly impure [3-^{14}C]psoralen-3- carboxylic acid (143·1 mg, 93% of theoretical) recovered as a yellow powder, m.p. 255–8° (lit. m.p. 264–5·5°).[36] The psoralen-3-carboxylic acid (143 mg) was heated in a sealed evacuated tube 1 hr at 265–70°. The resulting mixture of brown tar and crystalline colourless sublimate was removed from the tube with hot MeOH and sublimed at 180° (0·02 torr) to give [3-^{14}C]psoralen (42·1 mg, 34% of theoretical), m.p. 160–1° (lit. m.p. 160–2°),[36] undepressed by authentic material, with which it was identical as judged by UV spectrum, TLC, and GLC retention time. The compound contained no radioactive impurities on radio-TLC scanning, and its specific activity was 122 μCi/mmol.

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