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ON THE BIOSYNTHESIS OF FUROCOUMARINS IN *PIMPINELLA MAGNA**

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Abstract—Feeding of cinnamic acid-[COOH-^{14}C] and mevalonic acid-[4-^{14}C] to roots of *Pimpinella magna* and degradation of the labelled furocoumarins show that the coumarin part of the furocoumarin skeleton is formed from cinnamic acid, whereas the two extra-carbons of the furan ring originate from C-4 and C-5 of mevalonic acid. The furan ring thus is likely to be formed by isoprenylation of a coumarin precursor followed by cyclization and loss of a 3-carbon unit. Comparison of the specific activities of the furocoumarins isolated from the two experiments indicates that the hydroxylation pattern is established before isoprenylation occurs. A tentative scheme for furocoumarin formation is discussed. Some evidence is presented that furocoumarins in *Pimpinella magna* are excretion products which are accumulated extracellularly.

RECENTLY, we presented evidence that the coumarin skeleton of furocoumarins is formed from cinnamic acid.[1] The results indicated that para-hydroxylation of the cinnamic acid precursor precedes ortho-hydroxylation, since umbelliferone was a far better precursor than cinnamic acid, whereas coumarin gave only very poor incorporation. This is in agreement with the findings of Brown,[2–4] who showed that the formation of coumarin itself and of 7-oxygenated coumarins involves different pathways, the branch point being the hydroxylation of cinnamic acid. No experimental data are available regarding the origin of the two extra carbon atoms of the furan ring. Although a number of other mechanisms has been put forward,[5,6] the co-occurrence of simple furocoumarins, isopropyl-furocoumarins and isoprenylated coumarins,[7] strongly suggests the possibility that the furan ring originates from carbons 1 and 2 of an isoprene residue or from carbons 5 and 4 of mevalonic acid respectively.[8,8a] We now wish to report results which confirm the incorporation of cinnamic acid and clarify the origin of the two extra carbon atoms of the furan ring. They also allow some conclusions regarding the pathway of furocoumarin formation.

RESULTS

The roots of *Pimpinella magna* used in the present experiments had an unexpectedly high furocoumarin content (6 per cent of the dry weight of the roots, compared to about 0·2 per

* Dedicated to Prof. Dr. K. Mothes on the occasion of his 65th birthday.

[1] H. G. FLOSS and U. MOTHES, *Z. Naturforsch.* **19b**, 770 (1963).
[2] S. A. BROWN, *Science* **137**, 977 (1962).
[3] S. A. BROWN, *Phytochem.* **2**, 137 (1963).
[4] S. A. BROWN, *Lloydia*, **26**, 211 (1963).
[5] E. SPÄTH, *Ber. Deut. Chem. Ges.* **70A**, 83 (1937).
[6] T. A. GEISSMAN and E. HINREINER, *Botan. Rev.* **18**, 229 (1952).
[7] W. KARRER, *Konstitution and Vorkommen der organischen Pflanzenstoffe*. Birkhäuser Verlag, Basel (1958).
[8] R. ANEJA, S. K. MUKERJEE and T. R. SESHADRI, *Tetrahedron* **4**, 256 (1958).
[8a] A. J. BIRCH and H. SMITH, *Chem. Soc. Spec. Publ.* No. 12, 1 (1958).

cent in material used for earlier work). The furocoumarins are found almost exclusively in the root and a simple experiment shows that they are not evenly distributed in the root but are concentrated in a certain structure, the resin canals. On cutting a root, little droplets of a milky liquid appear on the cut surface. Under an u.v. lamp these droplets show an intense brownish-yellow fluorescence, whereas the surrounding tissue is non-fluorescent. Thin-layer chromatography shows this liquid to be extremely rich in furocoumarins. It has the same spectrum of furocoumarins as the ether extract of the whole root.

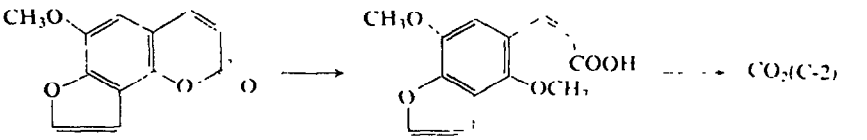
The labelled compounds (Table 1) were fed via capillary tubes into the cut surface of a root. After 14 days the root was separated, dried and extracted with ether. The individual coumarins (Tables 1 and 2) were isolated by chromatography on columns of silica gel, sublimation and crystallisation (see Experimental).

TABLE 1. INCORPORATION OF CINNAMIC ACID-[COOH-^{14}C] AND MEVALONIC ACID-[4-^{14}C] INTO FUROCOUMARINS IN ROOTS OF *Pimpinella magna*

Experiment	Compound fed	Amount and activity fed	Dry root material (g)	Total furocoumarin (g)	Total activity in furocoumarins (dpm* × 10^{-4})	Incorporation (%)
COOH 1	Cinnamic acid-[COOH-^{14}C]	30 μmoles; 8·1 μC; 1·18 × 10^7 dpm	29·5	1·87	30	1·7
2	D,L-mevalonic-acid-[4-^{14}C]	30 μmoles; 9·0 μC; 2·0 × 10^7 dpm	17·3	0·79	9·9	0·5

* Disintegrations per minute,

Experiment 1 (Table 1) confirms our earlier finding that cinnamic acid is incorporated into the furocoumarins. The incorporation obtained in this experiment is 5 times higher than that reported previously.[1] The reason probably is that the amount of precursor per g of plant material in this experiment was lower than previously. In order to establish the direct conversion of cinnamic acid to furocoumarins, a degradation was performed. Spondin from experiment 1 was degraded as shown in Fig. 1.



87·4 dpm/μmole 83·6 dpm/μmole 76·0 dpm/μmole

FIG. 1. DEGRADATION OF SPHONDIN TO SHOW LOCATION OF RADIOACTIVITY FROM CINNAMIC ACID [COOH-^{14}C].

The CO_2 obtained from C-2 of sphondin had about 90 per cent of the specific activity of the starting material. Thus, most of the radioactivity was located at C-2 of the furocoumarin as is to be expected for the direct incorporation of cinnamic acid-[COOH-^{14}C].

Table 1 also shows that mevalonic acid-[4-^{14}C] is incorporated into the furocoumarins. For comparison with cinnamic acid the incorporation value for mevalonic acid should be multiplied by 2, since only one of the optical isomers administered to the plant is biologically active.

To determine the location of the isotope in the furocoumarins isolated in Experiment 2, a degradation was attempted. All the ^{14}C would be expected to be in position 5', if the furan ring is indeed formed from C-4 and C-5 of mevalonic acid. A number of reactions was tried without success. Oxidation with hydrogen peroxide in alkali[9] gave yields of furan-2,3-dicarboxylic acid which were too low for our purposes. Dichromate in acetic acid[10] gave the expected coumarin-aldehyde in the case of bergapten, but did not oxidize pimpinellin, which was the only furocoumarin isolated in a quantity sufficient for degradation. Oxidation with dichromate in acetic acid and sulfuric acid,[11,12] which is supposed to cleave out carbon atom 5', leaving a coumarin-carboxylic acid, did indeed give an acidic material which according to thin-layer chromatography and u.v. spectrum consisted mainly of a coumarin compound. Lack of material, however, prevented further evaluation of this reaction. Ozonization of furocoumarins may either cleave only the furan ring or both the furan and the lactone ring. Brokke and Christensen[13] obtained a coumarin aldehyde from xanthotoxin, but an *m*-phthalaldehyde from 5-bromo-xanthotoxin. Nevertheless, ozonization was chosen as the degradation method for the labelled pimpinellin. The product of the reaction was identified as 2,4-dihydroxy-5,6-dimethoxy-*m*-phthalaldehyde by u.v. and mass-spectra. Thus, both rings had been cleaved (Fig. 2).

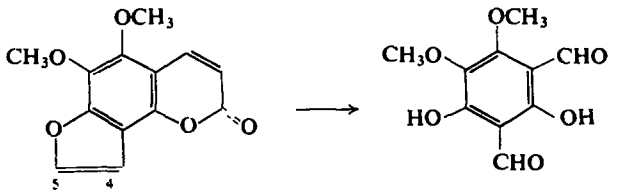


FIG. 2. DEGRADATION OF SPHONDIN TO SHOW LOCATION OF ACTIVITY FROM MEVALONATE-[4-^{14}C].

The reaction involves removal of carbon atoms 2, 3 and 5'. Since the pimpinellin had a constant specific radioactivity and the dialdehyde had only 11 per cent of the original specific activity, most of the ^{14}C of the pimpinellin must be located in C-2, C-3 and/or C-5'. As shown before, the coumarin portion of the molecule, which includes C-2 and C-3, arises from cinnamic acid. A specific incorporation of C-4 of mevalonic acid into C-1 or C-2 of cinnamic acid is highly unlikely. Thus, we conclude that C-4 of mevalonic acid indeed labels C-5' of the furocoumarins.

It seemed possible to obtain some information about the pathway of furocoumarin formation by comparing the specific activities of the different furocoumarins after feeding cinnamic acid and mevalonic acid. We therefore isolated and purified all the single furocoumarins from these two experiments. The results are given in Table 2 in terms of specific activity, relative specific activity (highest spc. act. = 100) and dilution. The dilution values are rather high because of the very large pools of furocoumarins present in the plant.

[9] E. SPÄTH and L. KAHOVEC, *Ber. Deut. Chem. Ges.* **66**, 1146 (1933).
[10] A. SCHÖNBERG, N. BADRAN and N. A. STARKOWSKY, *J. Am. Chem. Soc.* **77**, 1019 (1955).
[11] A. CHATTERJEE and S. S. MITRA, *J. Am. Chem. Soc.* **71**, 606 (1949).
[12] E. A. ABU-MUSTAFA and M. B. E. FAYEZ, *J. Org. Chem.* **26**, 161 (1961).
[13] M. E. BROKKE and B. E. CHRISTENSEN, *J. Org. Chem.* **23**, 589 (1958).

TABLE 2. SPECIFIC ACTIVITIES, RELATIVE SPECIFIC ACTIVITIES AND ^{14}C-DILUTIONS OF FUROCOUMARINS BIOSYNTHESIZED FROM CINNAMIC ACID AND MEVALONIC ACID

Compound	Sp. act. (dpm/μmole)		Rel. sp. act.*		^{14}C dilution	
	Cinnamic acid	Mevalonic acid	Cinnamic acid	Mevalonic acid	Cinnamic acid	Mevalonic acid
Sphondin	87·4	43·6	100	100	6500	15,100
Isobergapten	31·3	26·4	36	60	19,000	25,000
Bergapten	31·8	7·4	36	17	18,700	89,200
Pimpinellin	11·3	37·6	13	86	52,500	17,500
Isopimpinellin	21·9	20·0	25	46	27,100	33,000

* Highest sp. act. = 100.

DISCUSSION

The results clarify the origin of the carbon skeleton of the furocoumarins. Thus, the coumarin portion is formed from cinnamic acid, while the furan ring originates from an isoprenoid residue, probably by isoprenylation of the aromatic ring followed by cyclization and loss of a 3-carbon isopropyl side chain (Fig. 3). Seshadri[8] has previously pointed to the

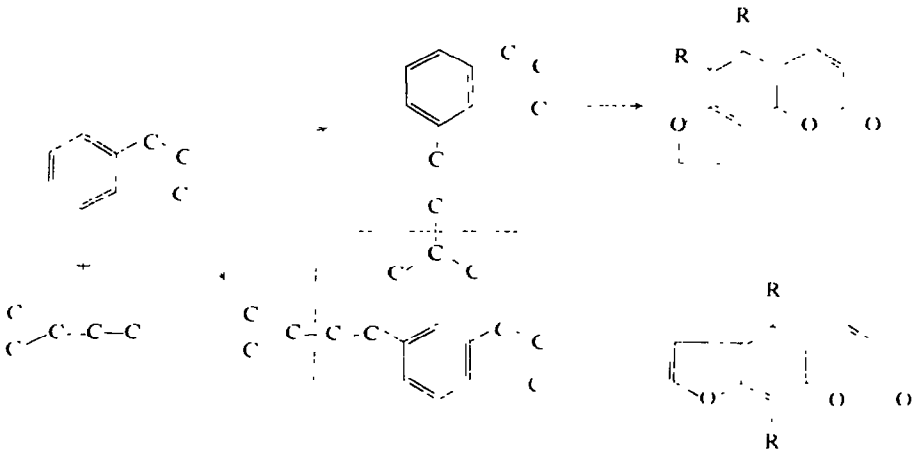


FIG. 3. ORIGIN OF THE CARBON SKELETON OF FUROCOUMARINS.

common occurrence in nature of a furan ring joined to an aromatic ring. Besides the furocoumarins this type of structure is also found in the furochromanes (e.g. khellol), furoflavones (e.g. pinnatin), rotenoids (e.g. elliptone) and in the furoquinoline alkaloids (e.g. dictamnine).[14] The derivation of the furan ring demonstrated here for the furocoumarins is thus very likely a general pathway which is followed in the formation of all the above-mentioned compounds.

It is interesting to consider the general mechanism of furocoumarin biosynthesis, especially the sequence of the individual steps. In general, the formation of the furocoumarins

[14] H. G. BOIT, *Ergebnisse der Alkaloidchemie bis 1960*. Akademie-Verlag, Berlin (1961)

could take place via the ortho-glucosides of the corresponding coumaric acids. By analogy to the biosynthesis of simple coumarins[4] the whole sequence of reactions would then take place at the stage of the corresponding cinnamic acids rather than at the coumarin stage. Such a mechanism would be favoured by the observation of Stoll *et al.*[15] that psoralen in the seeds of *Coronilla glauca* is mainly present as the ortho-glucoside of the corresponding furocoumarinic acid. On the other hand, it is obvious from our observations that in *Pimpinella* the furocoumarins are mainly present as such and not as glucosides.

In view of the good incorporation of umbelliferone into furocoumarins,[1] an alternate pathway involving this compound as a common precursor should be visualized. Umbelliferone itself might be formed via the umbellinic acid ortho glucoside.[16] Evidence for umbelliferone being a precursor of a more hydroxylated coumarin has been obtained by Kindl and Billek[17] in the case of 7-hydroxy-8-methoxycoumarin (hydrangetin[16]).

It is striking that, with a very few exceptions, all the furocoumarins have the furan ring attached to position 6, 7 or 7, 8 of the coumarin system, the oxygen being in position 7. This would be explained most easily by the assumption that umbelliferone is the compound which is isoprenylated, and that the further hydroxylations and alkylations take place after this step. However, the results given in Table 2 clearly show that this is not the case. If, starting from umbelliferone, isoprenylation would precede the further hydroxylations, one would expect the relative specific activities of the various furocoumarins to be the same in the cinnamic acid as in the mevalonic acid experiment, since all the reactions which create the differences between the products would occur *after* the introduction of the isotope in both cases.

The results point to the alternative whereby the hydroxylation and methylation pattern is established first and isoprenylation then occurs as the last step. Since, however, the 7-hydroxyl group must be protected from methylation in order to allow furan ring formation, it is likely that umbelliferone is first converted to the 7-glucoside and this compound is the substrate for further hydroxylations and methylations. The common occurrence of coumarins having a glucosyloxy group in position 7 (e.g. skimmin, cichoriin, scopolin, daphnin), the ready conversion of umbelliferone into a glycoside in *Melilotus officinalis*[18] and the formation of scopoletin from cichoriin in tobacco[19] are good indications for such a pathway. The sugar would be removed at a later stage to allow furan ring formation to take place.

The hydroxylation pattern of the furocoumarins found in *Pimpinella* can be derived by a combination of three types of reactions:

1. A hydroxylation of umbelliferone ortho to the hydroxyl group and para to the lactone oxygen.
2. A hydroxylation in position 5 ("second ortho hydroxylation").
3. Opening of the lactone ring and recyclization in the opposite direction.

An observation in favour of this scheme is that xanthotoxin, the only member of all the possible mono- and dimethoxylated furocoumarins of this type which cannot be formed by these reactions, is not found in *Pimpinella*.[7] The data given in Table 2 would suggest that reaction 1 occurs more readily than reaction 2, since in the cinnamic acid experiment sphondin has a much higher specific activity than bergapten and isobergapten. Alternatively sphondin could be

[15] A. Stoll, A. Pereira and F. Renz, *Helv. Chim. Acta*, **33**, 1637 (1950).
[16] S. A. Brown, G. H. N. Towers and D. Chen, *Phytochem.* **3**, 469 (1964).
[17] H. Kindl and G. Billek, *Monatsh. Chem.* **95**, 1044 (1964).
[18] F. Weygand, H. Simon, H. G. Floss and U. Mothes, *Z. Naturforsch*, **15b**, 765 (1960).
[19] L. J. Dewey and W. Stepka, *Arch. Biochem. Biophys.* **100**, 91 (1963).

formed by a different pathway than the other compounds, involving ferulic acid as an intermediate. As expected, bergapten and isobergapten have the same specific activity in the cinnamic acid experiment, whereas the values for pimpinellin and isopimpinellin are lower. Since the specific activity of pimpinellin is lower than that of its isomer it would appear as if reaction 3 occurs obligatorily together with reaction 2. but the difference may be too small to allow such a conclusion with certainty.

The data from the mevalonic acid experiment are easily explained in terms of two parameters, i.e. the relative rate of isoprenylation at positions 6 and 8 and, to some extent, the availability of substrate for isoprenylation. The angular furocoumarins all have a higher specific activity than the linear ones, indicating a preferential isoprenylation at position 8. This is most pronounced with the pair bergapten-isobergapten, where we have a true competition for the same precursor.

On the basis of these results we would like to propose the biosynthetic scheme given in Fig. 4. It should, however, be emphasized that this scheme at the present time is highly speculative and that further information is needed to check its validity.

Regarding the physiological significance of the furocoumarins in *Pimpinella*, it is interesting to note that these compounds are accumulated extracellularly in the resin canals of the root. Pimpinella roots are known to have a rather low lignin content. The formation of furocoumarins thus may represent an alternative to lignification.

METHODS

Labeled Precursors

Cinnamic acid-[COOH-^{14}C] was prepared from sodium acetate-[COOH-^{14}C] by a Perkin synthesis as modified by Bacharach *et al.*[20] DL-Mevalonic acid-[4-^{14}C] was synthesized according to Cornforth *et al.*[21] The dibenzyl-ethylenediamine salt was fed to the plant.

Feeding Experiments

The plant employed was *Pimpinella magna*, the roots of which according to Baerheim-Svendsen[22] contain the furocoumarins pimpinellin, isopimpinellin, bergapten, isobergapten and sphondin. The solutions of the precursors, 30 μmole (8·1 μc) of cinnamic acid-[COOH-^{14}C] in 0·3 of 0·1 N NaOH and 30 μmole (9·0 μc) of mevalonic acid-[4-^{14}C] salt in 0·3 ml of water, were sucked into a number of melting point capillaries, which were then stuck into the cut surface of a root which had been cut at the lower end. The capillaries were washed several times with small quantities of water and then removed. The roots were wrapped in wet soil and the plants were kept for 14 days in a hood. The roots were then separated from the aerial parts and dried to give 29·5 and 17·3 g dry material in the cinnamic acid and the mevalonic acid experiment respectively. Analysis for radioactivity showed that there was essentially no translocation of precursor to the aerial parts (less than 5 per cent of the administered radioactivity).

Isolation and Purification of the Furocoumarins

The dry root material was powdered in a small blender and extracted continuously with ether for three days. The ether extract was washed with Na_2CO_3 solution to remove unreacted

[20] G. BACHARACH and F. BROGAN, *J. Am. Chem. Soc.* **50**, 3333 (1928).
[21] J. W. CORNFORTH, R. M. CORNFORTH, A. PETTER, M. G. HORNING and G. POPJAK, *Tetrahedron* **5**, 311 (1959).
[22] A. BAERHEIM-SVENDSEN, *Zur Chemie Norwegischer Umbelliferen*. J. Grund Tanum Forlarg, Oslo, 1954.

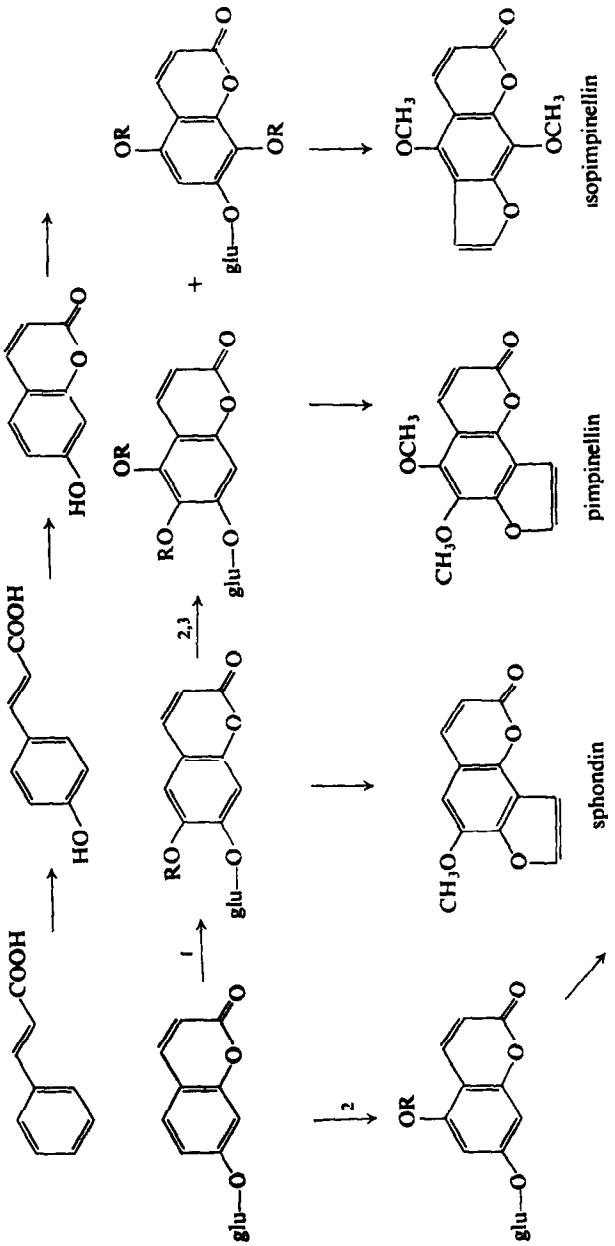


FIG. 4. TENTATIVE SCHEME OF FUROCOUMARIN BIOSYNTHESIS IN *Pimpinella magna*.

precursory material. It was then dried, concentrated to a small volume and chromatographed on a column of silica gel (Merck, activated for 30 min at 110°) of 4 cm diameter and 30 cm length with ether: benzene 1:1. Fractions of 5 ml were collected and the elution was followed by TLC on silica gel G in the same solvent. The fractions were combined to obtain three samples. The first consisted of pimpinellin and isobergapten, the second contained a mixture of all five furocoumarins and the last one consisted of sphondin. Pimpinellin and isobergapten were separated by treating the dry residue from the first sample with a small portion of ether, which only dissolved pimpinellin. Isobergapten was then purified by sublimation and pimpinellin by recrystallization from ethanol and sublimation. Sphondin was isolated from the last sample by recrystallization from ethanol and sublimation.

In order to isolate isopimpinellin and bergapten, the second sample of the column was subjected to the same separation again. The fractions containing mainly isopimpinellin and bergapten were combined and subjected to a third chromatography on a column of aluminum oxide, which was eluted first with benzene followed by benzene containing 1% of methanol.[22] Isopimpinellin and bergapten were purified from the corresponding fractions by sublimation. The identity of the isolated compounds was confirmed by chromatographic and mixed melting point comparison with authentic samples.

When possible, the radiochemical purity was checked by either charcoal treatment and recrystallization from ethanol, using a large quantity of charcoal, or by the following procedure: The furocoumarin was dissolved in methanolic KOH with slight warming, water was added and the solution extracted several times with ether. The ether extract was discarded and the aqueous phase was acidified with HCl and heated on the steam-bath for a few minutes. It was then extracted with ether and the ether in turn washed with sodium bicarbonate solution and evaporated to dryness. The residue was crystallized from ethanol.

The isolated compounds had the following specific radioactivities. Cinnamic acid experiment: isobergapten 31·3 dpm (disintegrations per min)/μmole; pimpinellin 11·3 dpm/μmole; unchanged by purification via the acid; bergapten 31·8 dpm/μmole; isopimpinellin 21·9 dpm/μmole; unchanged by recrystallization; sphondin, 87·4 dpm/μmole; unchanged by conversion to sphondinic acid methyl ether. Mevalonic acid experiment: isobergapten 26·4 dpm/μmole; pimpinellin, 37·6 dpm/μmole; unchanged by purification via the acid; bergapten, 7·4 dpm/μmole, unchanged by recrystallization; isopimpinellin 20·0 dpm/μmole, unchanged by recrystallization; sphondin, 43·6 dpm/μmole, unchanged by purification via the acid.

Degradation of Sphondin

Fifty mg of sphondin were dissolved in 2·5 ml of acetone and refluxed with 0·5 ml of distilled dimethyl sulfate and 2·5 ml of 20% KOH. After 30 min the same quantities of dimethylsulfate and KOH were added again and refluxing was continued for another 2 hr. Water was added and the acetone was evaporated. After two washings with ether, the aqueous phase was acidified and the methyl ether (40 mg) of sphondinic acid was collected by filtration. A 35-mg aliquot of this acid were decarboxylated by refluxing with 100 mg of copper powder in 3 ml of quinoline and the CO_2 was condensed in a spiral trap cooled with liquid oxygen. Its quantity was determined manometrically and its radioactivity by gas-phase counting.[23]

Degradation of Pimpinellin

Twenty mg of pimpinellin dissolved in 3 ml of dry methylene chloride were ozonized for 1·5 min (approximately 1 mmole O_3/min). The solution was added to 20 mg of zinc dust in

[23] H. SIMON, H. DANIEL and J. F. KLEBE, *Angew. Chem.* **71**, 303 (1959). H. SIMON and F. BERTHOLD, *Atomwirtschaft* 7, 498 (1962).

1·5 ml of 30% aq. acetic acid and kept at room temperature for 1·5 hr with occasional shaking. The methylene chloride was then removed on a steam-bath and the solution was filtered and cooled in the refrigerator overnight. The product (2·7 mg) was collected by filtration. It had a melting point of 124°*[24] and gave a single spot on thin-layer chromatography in several solvents. The u.v. spectrum showed maxima at 224, 252, and 303 mμ, the mass-spectrum gave a molecular weight of 226. With 2,4-dinitrophenylhydrazine, a precipitate was obtained which consisted of two components, the major one being insoluble in ethanol and having a m.p. over 295° and the minor component being ethanol-soluble.

Determination of Radioactivity

Radioactivities were determined by gas-phase counting in a proportional counting tube according to the method of Simon[23] or by liquid scintillation counting in a Tri-carb scintillation counter using internal standards.

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* Hegarty and Cahey[24] reported m.p. 114–15° for this compound. However, they used a different solvent for crystallization of their compound.

[24] M. P. Hegarty and F. N. Cahey, *Australian J. Chem.* **9**, 120 (1956).