Phytochemistry, 1969, Vol. 8, pp. 589 to 596. Pergamon Press. Printed in England.

BIOSYNTHESIS OF FURANOCOUMARINS IN *PIMPINELLA MAGNA* (UMBELLIFERAE)

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(*Received* 5 *October* 1968)

Abstract—The incorporations of *o*-coumaric acid-1-^{14}C, *p*-coumaric acid-1-^{14}C umbelliferone-3-T, umbelliferone-3-T-7-glucoside, scopoletin-CH_3-T and 6-hydroxy-7-methoxycoumarin-CH_3-T into furanocoumarins by *Pimpinella magna* are compared. The results do not provide evidence for a pathway of furanocoumarin biosynthesis proposed earlier, but suggest that an alternate pathway involving isoprenylation of umbelliferone must be considered. Methods for the preparation of the above-labeled compounds are given.

INTRODUCTION

THE BIOSYNTHESIS of furanocoumarins has been studied by two groups. Caporale *et al.*[1] have reported the incorporation of radioactivity from acetate-2-T, tyrosine-2-^{14}C and -U-T, mevalonic acid-2-^{14}C, and succinic acid-2,3-T into bergapten and psoralen by leaves of *Ficus carica*. We have presented evidence for the biosynthetic origin of the furanocoumarins of *Pimpinella magna* roots from cinnamic acid and mevalonic acid, carbon atoms 4 and 5 of the latter providing the two extra carbon atoms of the furan ring.[2] Umbelliferone, a natural constituent of *P. magna* roots,[3] proved to be a very efficient precursor, whereas coumarin gave a much lower incorporation than cinnamic acid,[4] indicating that umbelliferone but not coumarin is an intermediate in the formation of *Pimpinella* furanocoumarins from cinnamic acid. On the basis of a comparison of the specific activities of the various furanocoumarins after feeding cinnamate-1-^{14}C and mevalonate-4-^{14}C, we suggested a biogenetic scheme which involved isoprenylation occurring after the final oxygenation pattern of the coumarin portion had been established. Since the 7-hydroxyl group must not be methylated, we also suggested that umbelliferone-7-glucoside might be an intermediate and that the further hydroxylations and methylations might occur at the glucoside stage.

In the present communication we report our attempts to examine this scheme experimentally. The evidence, although not equivocal, does not favor our proposed biogenetic scheme and indicates that an alternative pathway (Fig. 1), involving isoprenylation at the umbelliferone stage followed by further modification of the benzenoid ring, must be considered.

RESULTS

First, we made a further attempt to determine whether the *ortho*- or the *para*-hydroxyl group is introduced first into the cinnamic acid molecule. The fact that coumarin is not

[1] G. CAPORALE, A. BRECCIA and G. RODIGHIERO, Prepn. Bio-Med. Appl. Labeled Mol., Proc. Symp., p. 103, Venice (1964).
[2] H. G. FLOSS and U. MOTHES, *Phytochem.* **5**, 161 (1966).
[3] A. BAERHEIM-SVENDSEN, in *ZurChemie norwegischer Umbelliferen*, F. Grund Tanum Forlag, Oslo (1954).
[4] H. G. FLOSS and U. MOTHES, *Z. Naturforsch.* **19b**, 770 (1964).

Table 1. Incorporation of potential biosynthetic intermediates into furanocoumarins by *Pimpinella magna*

Precursor	Radioactivity taken up by plant (dpm)	Isolated furanocoumarins			
		Total quantity (μmoles)	Av. spec. radioactivity (dpm/μmoles)	Incorporation (%)	Dilution
o-Coumaric acid-1-^{14}C, 50 μmoles = $2{\cdot}29.10^7$ dpm	$1{\cdot}57.10^7$	187	$6{\cdot}3 \pm 0{\cdot}6.10^1$	0·075	7,300
p-Coumaric acid-1-^{14}C, 120 μmoles = $2{\cdot}96.10^7$ dpm	$2{\cdot}71.10^7$	146	$1{\cdot}08 \pm 0{\cdot}18.10^2$	0·058	2,280
Umbelliferone-3-T, 6·9 μmoles = $2{\cdot}54.10^7$ dpm	$1{\cdot}69.10^7$	102	$1{\cdot}97 \pm 0{\cdot}49.10^3$	1·19	1,880
Umbelliferone-3-T-7-glucoside, 6·9 μmoles = $2{\cdot}56.10^7$ dpm	$1{\cdot}82.10^7$	77	$2{\cdot}07 \pm 0{\cdot}55.10^3$	0·88	1,790
Scopoletin-CH_3-T, 0·79 μmoles = $1{\cdot}08.10^7$ dpm	$9{\cdot}5.10^6$	41	$6{\cdot}3 \pm 4{\cdot}6.10^2$	0·3	21,600
6-Hydroxy-7-methoxycoumarin-CH_3-T, 1·87 μmoles = $2{\cdot}55.10^7$dpm	$2{\cdot}37.10^7$	33	$1{\cdot}44 \pm 0{\cdot}67.10^3$	0·2	9,400

utilized in the biosynthesis does not rule out *o*-coumaric acid as an intermediate. Both *o*-coumaric acid and *p*-coumaric acid were prepared with a ^{14}C label in the carboxyl group and were fed to roots of *Pimpinella magna*. As shown in Table 1, both compounds were about equally efficient precursors of the furanocoumarins. This is reminiscent of the results obtained by Brown *et al.*[5] on the biosynthesis of umbelliferone in *Hydrangea macrophylla*, where also both *o*- and *p*-coumaric acid were incorporated. A trapping experiment, however, showed that only *p*-coumaric acid was formed in the plant,[5] indicating that *o*-coumaric acid is not a natural intermediate in umbelliferone biosynthesis (see also Ref. 6). Although we have not done such a trapping experiment with *Pimpinella*, we assume that the situation in this plant may be similar.

We then compared the incorporation of umbelliferone and its 7-glucoside into furanocoumarins. A useful method for labeling umbelliferone in larger quantities was found to be the decarboxylation of umbelliferone-3-carboxylic acid, which had been equilibrated with tritiated water. This procedure gives rise to umbelliferone tritiated at C-3, a position which

TABLE 2. METABOLISM OF UMBELLIFERONE-3-T IN *Pimpinella magna*

Fed to roots of three 1-year-old plants	$4{\cdot}65.10^7$ dpm (12·6 μmole)
Taken up by plant in 2 days	$4{\cdot}33.10^7$ dpm
Plants extracted with hot ethanol in blendor, water added, extract filtered, concentrated to 15 ml, extracted with 5 × 50 ml of ether	
Ether extracts combined	$3{\cdot}6.10^6$ dpm (8·3%)
Last ether extract	$1{\cdot}2.10^5$ dpm
Aqueous phase	$2{\cdot}98.10^7$ dpm (69%)
Aqueous phase incubated for 3 hr at 37°, pH 8, with β-glucosidase, extracted with 5 × 50 ml ether.	
Ether extracts combined	$2{\cdot}24.10^7$ dpm (52%)
In both ether extracts, all the radioactivity is associated with umbelliferone, except for a small amount, which is present as a polar compound, probably umbelliferone-7-glucoside.	

could be expected to be unaffected during our biosynthetic studies. The labeled umbelliferone was converted into the 7-glucoside by the method of Austin and Meyers.[6] Both compounds were fed to plants in about equal quantities. As shown in Table 1, the glucoside was incorporated slightly less efficiently than the aglycone, but the difference is not very significant. Since earlier experiments with sterile root cultures of sweet clover had shown very rapid glycosylation of added umbelliferone,[7] we examined the metabolic fate of this coumarin in *P. magna*. Umbelliferone was fed through the roots and, after 2 days, the plants were killed and macerated with hot ethanol in order to assure immediate denaturation of glycosidases. The data summarized in Table 2 indicate that very little free umbelliferone was present in the plant (ether extracts before emulsin treatment), and that most of the radioactivity became ether-extractable after treatment of the aqueous phase with β-glucosidase. Chromatography of the aglycones liberated by this enzymatic hydrolysis and scanning of the chromatograms showed that umbelliferone was the only labeled material in this fraction, i.e. there was no detectable conversion of umbelliferone glucoside to other coumarin glucosides.

[5] S. A. BROWN, G. H. N. TOWERS and D. CHEN, *Phytochem.* **3**, 469 (1964).
[6] D. J. AUSTIN and M. B. MEYERS, *Phytochem.* **4**, 245, 255 (1965).
[7] F. WEYGAND, H. SIMON, H. G. FLOSS and U. MOTHES, *Z. Naturforsch.* **15b**, 765 (1960).

If our hypothetical pathway of furanocoumarin biosynthesis were correct, one would expect scopoletin (7-hydroxy-6-methoxycoumarin) to be a precursor of sphondin and pimpinellin, possibly also of isopimpinellin, but not of bergapten and isobergapten. In order to examine this question we tried to prepare labeled scopoletin. Attempts to introduce tritium into the 4-position by decarboxylation of scopoletin-4-carboxylic acid[8] were not very successful, because the thermal decarboxylation led to a complex mixture of products which was difficult to resolve. Scopoletin labeled in the *O*-methyl group was then prepared by reaction of esculetin with diazomethane in the presence of HOT.[9] While the use of excess diazomethane yields quantitatively the dimethyl derivative, at lower molar ratios of diazomethane to esculetin, a mixture of the two monomethyl derivatives together with starting material and some 6,7-dimethoxycoumarin is obtained. This mixture could easily be resolved by TLC. Unfortunately, the 7-hydroxy group is methylated preferentially and scopoletin and its isomer are formed in a molar ratio of about 1:40. Nevertheless, enough labeled scopoletin was obtained by this procedure to carry out a feeding experiment. Labeled 6-hydroxy-7-methoxycoumarin was also prepared independently and in almost quantitative yield by methylation of esculin (6-glucosyloxy-7-hydroxycoumarin) with diazomethane/HOT followed by hydrolysis of the glucoside. Scopoletin-methyl-T was administered to the roots of *P. magna* and, as a reference, its isomer was fed in a parallel experiment. As shown in Table 1, the incorporation of both compounds was of the same order of magnitude and considerably lower than that of umbelliferone. Moreover, there was no preferential labeling of any furanocoumarin, e.g. sphondin, in the scopoletin experiment but rather the specific radioactivities of all furanocoumarins in both experiments were of the same order of magnitude.

DISCUSSION

The results presented do not substantiate the assumption that isoprenylation occurs as a late reaction in furanocoumarin biosynthesis and that the hydroxylations and methylations of the coumarin portion take place at the glucoside stage. Umbelliferone-7-glucoside is not a better precursor of the furanocoumarins than umbelliferone itself and there is no evidence for its conversion into other coumarin glucosides. Scopoletin-methyl-T is not incorporated preferentially into any one furanocoumarin as would be expected if isoprenylation were a late step in the biogenetic pathway. It labels the furanocoumarins in about the same manner as its isomer, 6-hydroxy-7-methoxycoumarin-methyl-T, which cannot be an intermediate in the formation of the furanocoumarins of *Pimpinella*. This indicates that the scopoletin molecule is not transformed into the furanocoumarins as a unit, but that the incorporation of radioactivity is only due to demethylation and partial reutilization of the labeled C_1 fragment. This is also suggested by the rather low rate of incorporation of scopoletin as compared to umbelliferone. One cannot exclude the possibility that scopoletin is a direct precursor of some of the furanocoumarins, but that its labeled methyl group is extremely rapidly equilibrated, by demethylation and remethylation, with the C_1 pool of the plant. In this way the labeling of scopoletin would be immediately reduced to the level of specific radioactivity of other methylated compounds in the cells and this could account for its failure to act like a specific precursor. However, while demethylation and reutilization of the C_1 unit do undoubtedly take place, it seems unlikely that these processes are extremely rapid. Demethylation is an oxidative reaction which involves removal of one of the hydrogens of the methyl

[8] D. G. CROSBY, *J. Org. Chem.* **26**, 1215 (1961).
[9] K. J. VAN DER MERWE, P. S. STEYN and S. H. EGGERS, *Tetrahedron Letters* 3923 (1964).

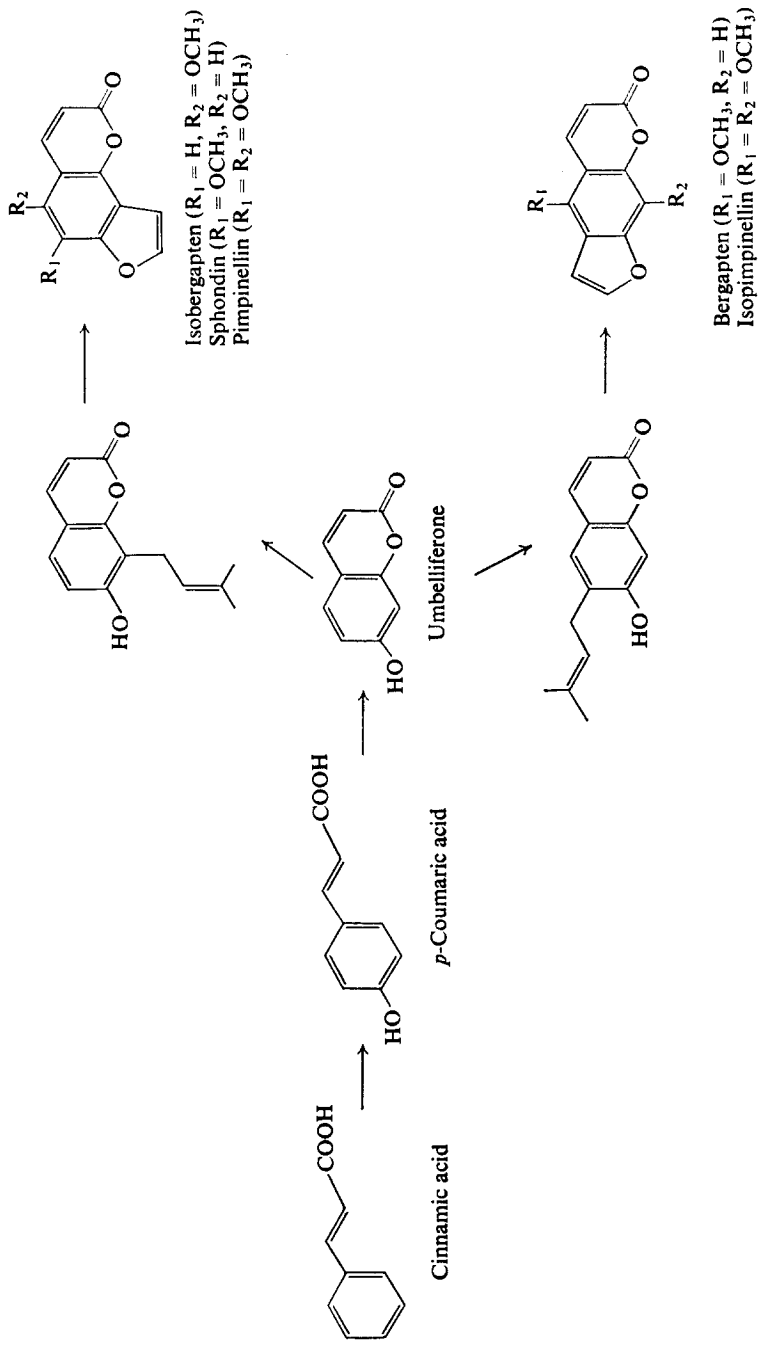

FIG. 1. ALTERNATIVE PATHWAY OF FURANOCOUMARIN BIOSYNTHESIS IN *Pimpinella magna*.

group.[10] Thus, if the labeled methyl group of scopoletin were to equilibrate very rapidly with the C_1 pool, this would soon result in complete elimination of the tritium from the entire C_1 pool. Since this is obviously not the case, it seems more likely that scopoletin is not a direct precursor of any of the furanocoumarins of *P. magna*. The results therefore indicate that we have to consider an alternative pathway of furanocoumarin biosynthesis, involving isoprenylation of umbelliferone followed by further modification of the resulting 6- and 8-dimethylallylumbelliferone. Such a pathway (Fig. 1) is attractive from a phytochemical point of view since it could explain the almost exclusive occurrence, in nature, of 6,7- and 7,8-furanocoumarins.

METHODS

Labeled Compounds

p-Coumaric acid-1-^{14}C was obtained from *p*-hydroxybenzaldehyde and malonic acid-1,3-^{14}C.[11] *o*-Coumaric acid-1-^{14}C was prepared by emulsin hydrolysis of *o*-coumaric acid-1-^{14}C-β-D-glucoside, which had been obtained by Knoevenagel condensation of helicin and malonic acid-1,3-^{14}C.[12]

Umbelliferone-3-T. 100 mg umbelliferone-3-carboxylic acid[7] were dissolved in 5 ml of isopropanol containing 0·4 ml (200 mc) of tritiated water. The solvent was distilled into a second flask using a closed evacuated system. Another portion of 100 mg of the acid was dissolved in the distillate, the solution was added to the residue of the first distillation and the solvent was again removed. This procedure was repeated until a total of 1 g of umbelliferone-3-carboxylic acid had been tritiated. After drying at 10^{-3} mm Hg, the tritiated acid was transferred into a heavy-wall glass tube, which was sealed under vacuum and heated for 1 hr at 270–280°. The product of the decarboxylation was extracted with 150 ml of boiling methanol in several portions and the solvent was removed in a rotary evaporator. Labile tritium was washed out by dissolving the residue ten times in 20 ml of methanol and evaporating the solvent. The umbelliferone was then sublimed at $5{\cdot}10^{-2}$ mm Hg and 140–150°. Yield 400 mg = 50 per cent, m.p. 227–229°, spec. radioactivity 1·68 mc/mmole.

Umbelliferone-3-T was converted into its 7-β-D-glucoside by the method of Austin and Meyers.[6] Starting from 200 mg of umbelliferone, 160 mg (26·5 per cent) of the glucoside, m.p. 205–210°, were obtained.

Scopoletin-methyl-T and 6-hydroxy-7-methoxycoumarin-methyl-T. A solution of CH_2N_2 in dioxane was prepared by carrying the CH_2N_2 from a dried ether solution (obtained from 1 g of nitrosomethylurea) over into 8 ml of dry dioxane (freshly distilled over $LiAlH_4$) in a stream of dried N_2. The titer of the solution was determined by reaction with a known amount of benzoic acid and titration of the excess acid with 0·1 N NaOH. An aliquot of this solution containing 0·56 mmole of diazomethane was added to 100 mg (0·56 mmole) of esculetin dissolved in 12 ml of dry dioxane and 0·1 ml (50 mc) of tritium water. After standing for 3 hr at room temperature the solvent was removed by lyophilization. 60 mg of the residue were chromatographed on twelve thin-layer plates (silica gel G, Merck, 0·5 mm layers) using ether–benzene (1:1) as the solvent. The bands of scopoletin (R_f 0·28) and 6-hydroxy-7-methoxycoumarin (R_f 0·35) were scraped off, eluted, and each compound was rechromatographed on five plates in the same solvent system. The yield of pure products, as determined by the

[10] S. KIM, L. BENOITON and W. K. PAIK, *J. Biol. Chem* **239**, 3790 (1964); and references therein.
[11] W. BORSCHE, *Ber. Dtsch. Chem. Ges.* **60**, 2112 (1927).
[12] B. HELFERICH and H. LUTZMANN, *Liebigs' Ann. Chem.* **537**, 11 (1939).

u.v. absorption, was 0·25 mg of scopoletin and 10 mg of 6-hydroxy-7-methoxycoumarin, specific radioactivity 6·2 mc/mmole.

Feeding Experiments and Isolation of Furanocoumarins

The roots of medium-size, 2–4-year-old field-grown *Pimpinella magna* plants were carefully freed from soil and were placed one each in large test-tubes (20 mm dia.). The test tubes were filled with quartz sand, the solutions of the precursors were pipetted into the tubes and water was added to cover the roots (total liquid volume 10–12 ml). The test tubes were sealed with parafilm and the plants were kept in continuous light for 7 days (5 days in the scopoletin and 6-hydroxy-7-methoxycoumarin experiments). The water was replaced at intervals as it was taken up. At the end of the experiment the roots and the test tubes were rinsed and the combined washings were counted to determine the residual radioactivity. The aerial parts of the plants were discarded. The roots were sliced, dried for 15 hr at 40° and then extracted with ether for 3 days in a soxhlet. The ether extracts (40–50 ml) were washed three times with 20 ml of 0·1 N NaOH, followed by washing once with water and drying. The extracts were made up to 100 ml and 0·1 ml aliquots were assayed for total furanocoumarin content (λ_{max} 300–312 nm, ϵ $1{\cdot}2.10^4$) and total radioactivity. 75 ml of the extracts were chromatographed on a column of silicic acid (Merck) (1·5 × 30 cm) using benzene/ether (1:1) as the eluent. Fractions containing isobergapten plus pimpinellin, bergapten plus isopimpinellin, and sphondin were collected and were further resolved by TLC in the same system. In addition, the furanocoumarins from the scopoletin and 6-hydroxy-7-methoxycoumarin experiments were subjected to another rechromatography on silica gel G using chloroform as the solvent. Aliquots of the single pure compounds eluted from the thin-layer plates were assayed for quantity by the u.v. method using calibration curves obtained with reference material, and for radioactivity. From the specific radioactivities of the single compounds the average specific radioactivity of the furanocoumarins and the percentage incorporation was calculated.

Metabolism of Umbelliferone

Three 1-yr-old pot-grown plants of *P. magna* were fed umbelliferone-3-T in the same way as described above. After 49 hr the plants were collected and the residual activity was determined. The whole plants were homogenized with 200 ml of hot ethanol in a blendor, 40 ml of water were added and, after filtration, the extract was concentrated to 15 ml. This solution was treated as indicated in Table 2. Each ether extract was counted for radioactivity, aliquots of both combined ether extracts were chromatographed on thin-layer plates and the plates were scanned for radioactivity. The limit of detection of radioactivity in a single compound by this technique was $\sim 4{\cdot}10^5$ dpm total in the ether extract before emulsin treatment and $\sim 1{\cdot}10^5$ dpm total in the extract obtained after hydrolysis.

Radioactivity determinations were carried out by gas phase proportional counting following dry combustion[13] or by liquid scintillation counting in a Beckman LS 100 instrument using Bray's solution[14] and internal standards. Radioactivity on chromatograms was detected with a Packard Model 7201 radiochromatogram scanner.

R_x values (bergapten = 1·0) of coumarins on silica gel G (Merck) in solvent systems (A)

[13] H. SIMON and F. BERTHOLD, *Atomwirtschaft* **7**, 498 (1962).
[14] D. A. BRAY, *Anal. Biochem.* **1**, 279 (1960).

ether/benzene (1:1) and (B) chloroform were: scopoletin (A) 0·38; 6-hydroxy-7-methoxy-coumarin (A) 0·47; umbelliferone (A) 0·61; isobergapten (A) 1·12, (B) 1·67; pimpinellin (A) 1·08, (B) 0·86; isopimpinellin (A) 0·97, (B) 0·43; sphondin (A) 0·92, (B) 0·90.

Acknowledgements—This work was supported in part by the Deutsche Forschungsgemeinschaft. We are indebted to Mr. W. Lösel, Technische Hochschule, Munich, for the preparation of the labeled *o*-coumaric acid glucoside.