Phytochemistry, 1969, Vol. 8, pp. 585 to 588. Pergamon Press. Printed in England.

BIOSYNTHESIS OF FURANOCOUMARINS IN DISEASED CELERY

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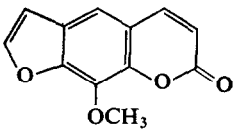
(*Received* 18 *September* 1968)

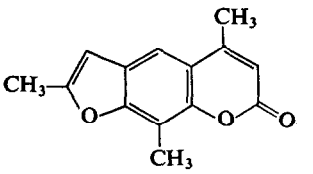
Abstract—Celery infected with the pathogenic fungus *Sclerotinia sclerotiorum* incorporates cinnamic acid-1^{14}C, formate-^{14}C, and methionine-$^{14}CH_3$ into xanthotoxin (8-methoxypsoralen), but not into 4,5′,8-trimethylpsoralen.

INTRODUCTION

UPON infection with the fungus *Sclerotinia sclerotiorum*, celery develops a condition called "pink rot" which has been correlated with the skin-irritating properties of diseased celery. Two phototoxic psoralen derivatives have been isolated from diseased celery tissue and have been characterized as xanthotoxin (8-methoxypsoralen) (I) and 4,5′,8-trimethylpsoralen (II).[1]



(I) Xanthotoxin



(II) 4,5′,8-Trimethylpsoralen

This paper reports the results of some feeding experiments with diseased celery to determine whether the two psoralen derivatives originate from the same biosynthetic precursors as other furanocoumarins found in higher plants. It has been shown that the various furanocoumarins occurring in *Pimpinella magna* (Umbelliferae) originate from cinnamic acid, which provides the coumarin backbone, and from mevalonic acid, which provides the two additional carbon atoms of the furan ring.[2] The *O*-methyl groups are presumed to come from the C_1 pool, e.g. from formate or the methyl group of methionine, although this has not yet been verified experimentally. Xanthotoxin has been found in other higher plants besides diseased celery; it has the same structural pattern as other higher plant furanocoumarins and would thus be expected to be derived from the same biogenetic precursors. 4,5′,8-Trimethylpsoralen, however, has some rather unusual features in that it carries three carbon bound methyl groups. If, as in other furanocoumarins, the ring skeleton of this compound is derived from cinnamate and mevalonate, the three methyl groups, or at least the two attached to the coumarin portion, would be expected to originate from the C_1 pool by *C*-methylation.

[1] L. D. SCHEEL, V. B. PERONE, R. L. LARKIN and R. E. KUPEL, *Biochemistry* **2**, 1127 (1963).
[2] H. G. FLOSS and U. MOTHES, *Z. Naturforsch.* **19b**, 770 (1963); *Phytochem.* **5**, 161 (1966).

RESULTS

In order to examine this question, cinnamic acid-1-^{14}C, methionine-methyl-^{14}C, and formate-^{14}C were fed to celery plants 2–3 days after infection with *Sclerotinia sclerotiorum*. After 1 week the plants were harvested and the furanocoumarins were extracted from the infected parts of the stalks. Xanthotoxin and 4,5′,8-trimethylpsoralen were isolated from the extract by chromatography and were purified to constant specific radioactivity by crystallization with carrier material.

TABLE 1. INCORPORATION OF CINNAMIC ACID-^{14}C, FORMATE-^{14}C AND METHIONINE-CH_3-^{14}C INTO XANTHOTOXIN AND TRIMETHYLPSORALEN BY DISEASED CELERY

		Xanthotoxin isolated			Trimethylpsoralen isolated		
Precursor	Radioactivity fed	Quantity (mg)	Total RA (dpm)	% incorp.	Quantity (mg)	Total RA (dpm)	% incorp.
Cinnamic acid-1-^{14}C	4·45 μc		$2{\cdot}8.10^4$	0·29		160	0·0016*
Formate-^{14}C	15 μc	15·2	$2{\cdot}71.10^4$	0·08	3·3	312	0·0009*
Methionine-CH_3-^{14}C	15 μc	14·3	$1{\cdot}11.10^5$	0·34	3·0	163	0·00047*

* Maximum values, purification to constant specific activity was not possible.

TABLE 2. CRYSTALLIZATION OF XANTHOTOXIN AND TRIMETHYLPSORALEN FROM METHIONINE-(CH_3-^{14}C)

	dpm/mg	dpm total
Xanthotoxin		
Radioactivity after chromatography		$1{\cdot}19.10^5$
52·25 mg carrier added		
1st cryst.	$1{\cdot}63.10^3$	$1{\cdot}08.10^5$
2nd cryst.	$1{\cdot}66.10^3$	$1{\cdot}11.10^5$
Trimethylpsoralen		
Radioactivity after chromatography		$4{\cdot}50.10^4$
51 mg carrier added		
1st cryst.	54	$3{\cdot}07.10^3$
2nd cryst.	9	512
3rd cryst.	3	163

The results of these feeding experiments are shown in Table 1. As expected, all three compounds were incorporated into xanthotoxin, although formate is a considerably less efficient methyl donor than methionine. Interestingly, however, none of the three substrates labeled 4,5′,8-trimethylpsoralen to any significant extent. To ensure that sufficient quantities of trimethylpsoralen had been formed, the furanocoumarins isolated in the formate and methionine experiments were characterized and quantitated by their u.v. absorption before the addition of carrier. Even when considering that the plants contained five times more xanthotoxin than trimethylpsoralen, the precursors were used about 100 times more efficiently

in the formation of xanthotoxin than for trimethylpsoralen biosynthesis. Further evidence for the specific incorporation of radioactivity from the methyl group of methionine into xanthotoxin, but not into trimethylpsoralen, is given in Table 2. Whereas essentially all the radioactivity present in the chromatographically isolated xanthotoxin fraction cocrystallized with authentic material, most of the radioactivity of the trimethylpsoralen fraction was eliminated in the crystallization with carrier.

Two feeding experiments with mevalonic acid-4-^{14}C were also carried out, but gave very low incorporations. This is not completely unexpected in view of the difficulties frequently encountered in *in vivo* feedings of mevalonic acid to higher plants. Similar experiments with *Pimpinella* have also resulted in extremely variable incorporations.[2, 3]

DISCUSSION

The results show that radioactivity from the obvious precursors cinnamic acid and methionine-$^{14}CH_3$ is incorporated into xanthotoxin by diseased celery. However, trimethylpsoralen isolated from the same experiments was essentially unlabeled. Negative results of feeding experiments, of course, always have to be interpreted with caution, but at least two frequent sources of error can be ruled out in this case. It is unlikely that trimethylpsoralen was not formed during the time of the experiment, because the compound was present in the extract, the precursors were administered shortly after infection of the plants, and healthy plants are known to be free of trimethylpsoralen.[1] It is equally unlikely that the precursors did not reach the site of synthesis in the plant, since they were incorporated into xanthotoxin. It is, of course, conceivable that the two products are synthesized at different sites, e.g. xanthotoxin may be formed by the plant and trimethylpsoralen by the fungal cells. Because of lack of knowledge about the symbiotic relationship between *Sclerotinia sclerotiorum* and its host, this question can at present not be answered. As an alternative explanation of the results of this study, one has, however, to consider the possibility that the two furanocoumarins found in diseased celery are formed by two entirely different biosynthetic pathways.

METHODS

Cinnamic acid-1-^{14}C and D,L-mevalonic acid-4-^{14}C (administered as the sodium salts) were the same materials used in previous experiments,[2] formate-^{14}C and methionine-methyl-^{14}C were obtained from New England Nuclear, Boston. Radioactivity determinations were carried out with a Packard Tricarb or Beckman LS 100 scintillation counter, using Bray's solution[4] or PPO and POPOP in toluene as scintillation mixtures.

Feeding Experiments

Mature, but not flowering, celery plants grown for 8–9 months in a greenhouse at 70° F were prepared for inoculation by trimming dead yellow leaves and the upper parts of healthy green leaves and by lightly scraping the stalks near the base of the plants. A mycelial pad of *Sclerotinia sclerotiorum*, grown for 4 weeks in a liquid media,[5] was laid in contact with the scraped celery stalks. A moist atmosphere was maintained by covering the plants with plastic bags. After 2–3 days when the infection was initiated, the solutions of the radioactive precursors were injected into the center of the stalk. Each precursor was injected into three plants and these were kept for 7–9 days at room temperature under continuous fluorescent light. The major portions of the roots as well as the green foliage at the end of the stalks were removed, the infected crown and stem material of the three plants from each experiment was combined, homogenized in a Waring blendor and then lyophilized to dryness.

[3] H. G. FLOSS, unpublished.
[4] D. A. BRAY, *Anal. Biochem.* **1**, 279 (1960).
[5] L. H. PURDY, JR. and R. C. GROGAN, *Phytopathol.* **44**, 36 (1954).

Isolation and Purification of Furanocoumarins

The dried plant material was extracted with petroleum ether (cinnamate experiment) or ether (formate and methionine experiments) until no more fluorescent material appeared in the extract. The residue of the extract was chromatographed on a column of silicic acid (Merck) using $CHCl_3$ as the eluent. The fractions containing xanthotoxin and trimethylpsoralen plus xanthotoxin, respectively, were pooled. The latter fractions were further resolved on thin-layer plates (silica gel G, Merck, $CHCl_3$ as solvent) and the trimethylpsoralen and xanthotoxin bands were eluted separately. In the cinnamic acid experiment, the initial column chromatography was omitted and the extract was instead resolved by preparative TLC on silica gel G plates using ether/benzene (1:1) as the solvent. The trimethylpsoralen and the combined xanthotoxin solutions were made up to a defined volume, aliquots were taken for u.v. spectra and radioactivity determinations, and the remainder was taken to dryness. A weighed amount of carrier (30–60 mg) was added and the compounds were recrystallized repeatedly, using alternatingly methanol and benzene as solvents. After each crystallization the specific radioactivity was determined.

Acknowledgements—Supported in part by Public Health Service Grant No. AI 06826-01A1.