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Biosynthesis of Ochratoxin A[1]

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Biosynthesis of ochratoxin A by *Aspergillus ochraceus* Wilh. was investigated by radiolabeling experiments in which phenylalanine-*1*-^{14}C and sodium acetate-*2*-^{14}C were supplied to the fungus in sucrose-yeast extract medium. Results showed that phenylalanine was incorporated unaltered into the phenylalanine moiety of ochratoxin A, whereas the isocoumarin moiety of ochratoxin A was mostly derived via acetate condensation.

Aspergillus ochraceus Wilh. has been isolated from a variety of agricultural commodities (4–7, 19, 24, 27). Symptoms of chronic or acute toxicity have been observed in test animals when *A. ochraceus* was grown in pure culture on corn, wheat, rye, sorghum, rice, buckwheat, soybeans, and peanuts and the molded substrates fed to the test animals (5–7, 17, 27). Shotwell et al. (20) found that ochratoxin A was a natural contaminant of a corn sample. Van Walbeek et al. (28) reported ochratoxin A production by a *Penicillium* species and by *A. ochraceus*.

The toxic agent was first isolated and named ochratoxin by van der Merwe et al. (27). *A. ochraceus* strain K-804, originally isolated from sorghum grain, was grown in bulk on sterilized corn meal, the toxic principle was quantitatively extracted with methanol-chloroform (1:1), and a chemical structure was suggested (27). This structure (Fig. 1, I) was later confirmed by chemical synthesis of ochratoxins A and B (23). Ochratoxin A was shown to be 7-carboxy-5-chloro-8-hydroxy-3,4-dihydro-3-methylisocoumarin, linked through the 7-carboxy-group to L-β-phenylalanine by an amide bond (26).

Microbiological aspects of *A. ochraceus* growth and ochratoxin production were investigated by Ferreira (10), who formulated a synthetic medium suitable for the production of 100 mg of ochratoxin per liter in shaken flasks and 10-liter fermentors. He obtained 50 mg of ochratoxin per liter of medium in a 100-liter pilot plant fermentation. The preferred carbon and nitrogen sources were sucrose (3%) and glutamic acid (1%), respectively. Ferreira (11) further investigated the effect of amino acids on ochratoxin production. Davis et al. (8) developed a semisynthetic medium containing 4% sucrose and 2% yeast extract, in which the fungus produced 29 mg of ochratoxin A per 100 ml of medium in stationary cultures. They also observed that the proportions of ochratoxins A and B could be altered by appropriate variation of the sucrose and yeast extract concentration, thus simplifying the procedure for subsequent purification of ochratoxin A.

From a theoretical consideration of the structure of ochratoxin A, it appears probable that the phenylalanine portion of the molecule is synthesized via the shikimic acid pathway. On the other hand, the isocoumarin portion could arise either via the shikimate pathway as in coumarin synthesis of higher plants, or from acetate units via an isoprenoid type of condensation as in oosponal (18), oospolactone (18), and aflatoxin (1). This research was conducted to determine the pattern of incorporation of the theoretical ^{14}C precursors, phenylalanine and acetate, into ochratoxin A, thereby revealing the pathway of biosynthesis of the metabolite.

MATERIALS AND METHODS

A. ochraceus NRRL 3174, obtained from C. W. Hesseltine, Northern Regional Research Laboratory, U.S. Department of Agriculture, Peoria, Ill., was used throughout this investigation. Cultures were maintained at 28 C on Czapek solution agar with 20% sucrose supplemented with 0.7% Difco yeast extract (8).

Radiolabeled ochratoxin A was prepared by growing *A. ochraceus* in stationary cultures on a 4% sucrose and 2% yeast extract (SYE) medium containing phenylalanine-*1*-^{14}C or sodium acetate-*2*-^{14}C at 25C (8). Ochratoxin A was extracted from the culture medium, purified by preparative thin-layer chromatography (TLC), and hydrolyzed to separate the phenylalanine and isocoumarin moieties. The isocoumarin moiety was selectively degraded to determine the position of the ^{14}C label derived from the

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sodium acetate-*2*-^{14}C. The numbering system used for these compounds is shown in Fig. 2.

Quantitative determinations of ochratoxin. Culture filtrates were extracted four times with 100 ml of chloroform in a 500-ml separatory funnel. Extracts were combined, dried by passage through a sodium sulfate column, and evaporated to dryness on a water bath. The residue was then dissolved in 5 ml of chloroform and streaked on Silica Gel TLC sheets (Eastman 6061) for separation of ochratoxin A. The chromatograms were developed in unlined chromatography tanks containing a 2-cm layer of toluene-ethyl acetate-90% formic acid (5:4:1, v/v). They were then air-dried and examined with a shortwave (254 nm) ultraviolet lamp; ochratoxin A was located by comparison with an external standard of authentic ochratoxin A. The ochratoxin A standard solution was prepared in this laboratory and was calibrated against a standard supplied by L. J. Vorster, National Nutrition Research Institute, South African Council for Scientific and Industrial Research, Pretoria.

Ochratoxin A was separated from the TLC sheets and purified as described below. Confirmation of the identity of the extracted compound was ascertained by TLC with authentic ochratoxin A as a control in three different solvent systems, by exposure of fluorescent zones to ammonia fumes, and by the preparation and co-chromatography of methyl esters of extracts and authentic ochratoxin A (9; J. W. Searcy, Ph.D. Thesis, Auburn Univ., Auburn, Ala., 1969).

Experiment 1. Radiolabeled ochratoxin A was prepared by placing 100 μc of phenylalanine-*1*-^{14}C (Calbiochem, Los Angeles, Calif.) in 100 ml of sterile SYE medium. After inoculation with *A. ochraceus* conidia, the culture was incubated at room temperature for 8 days. The culture filtrate was extracted four times with 200 ml of chloroform. Extracts were evaporated to 5 ml and streaked on TLC sheets for development and separation in the toluene-ethyl acetate-90% formic acid solvent system. Ochratoxin A was located by comparison with a standard spotted beside the extract. The areas containing ochratoxin A were cut from the sheets and eluted by the same solvent system running at right angles to the solvent front in a descending manner. The eluates were combined, evaporated to dryness, and redissolved in a known volume of chloroform for measurement of radioactivity in a liquid scintillation spectrometer (Beckman model 1650). Duplicate 100-μliter samples were placed in liquid scintillation (LS) vials and evaporated to dryness before adding the LS cocktail to reduce quenching to a minimum. Radioactivity measurements were corrected to counts per minute (counts/min) by constructing a quenching curve with acetone and sodium acetate-*1,2*-^{14}C, and by counting a standard ^{14}C sample and a reference background sample with each set of counts made. The chloroform solution of ^{14}C-ochratoxin A was then transferred to a 50-ml boiling flask and evaporated to dryness for acid hydrolysis.

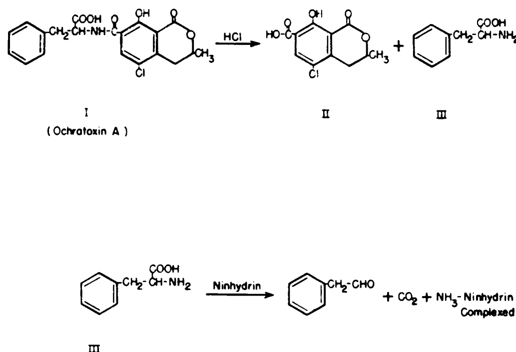

FIG. 1. *Degradation scheme of ochratoxin A radiolabeled with phenylalanine-1-^{14}C.*

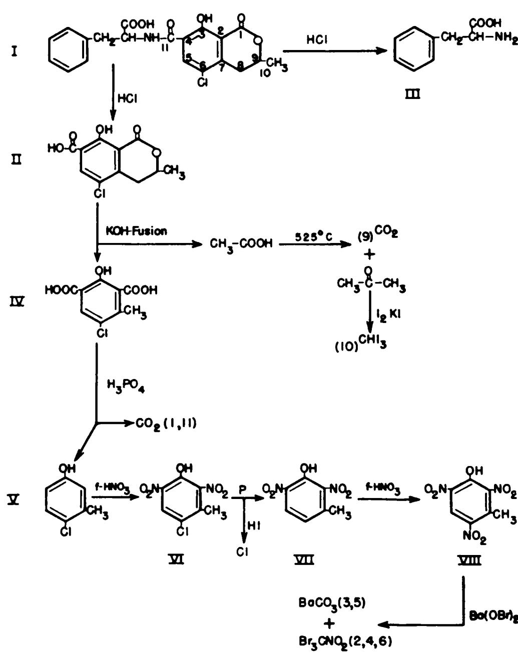

FIG. 2. *Stepwise degradation of the isocoumarin moiety of ochratoxin A radiolabeled with sodium acetate-2-^{14}C.*

Radiolabeled ochratoxin A was hydrolyzed by the method of van der Merwe et al. (26). Ochratoxin A was suspended in 25 ml of 6 N HCl and heated under reflux for 30 hr. Chloroform extraction (4 × 100 ml) of the cooled homogenous mixture and evaporation of the solvent from the sodium sulfate-dried organic phase gave 5-chloro-3,4-dihydro-8-hydroxy-3-methyl-isocoumarin-7-carboxylic acid (II) as described by van der Merwe et al. (26). The identity of this compound was confirmed by ultraviolet and infrared spectra and radioautography. The aqueous phase, containing the phenylalanine moiety (III), was adjusted to *p*H 5.85 with NaOH, and a portion was spotted directly on TLC sheets adjacent to a standard solution of phenylalanine. Sheets were developed in *n*-butyl alcohol–acetic acid–water (4:1:1, v/v), dried, sprayed with

ninhydrin spray reagent, and heated at 110 C for 10 min to confirm the presence of phenylalanine.

A portion of the remaining aqueous phase containing the ^{14}C-phenylalanine moiety (4.15×10^5 counts/min) from ochratoxin A was treated with ninhydrin solution reagent in a closed 250-ml, side-arm flask, with the side chamber containing 5 ml of 1 N NaOH. This allowed the determination of the proportion of radioactivity remaining in carbon 1 of the hydrolyzed phenylalanine moiety. The aqueous solution (20 ml, 2.99×10^5 counts/min) was heated in a water bath with the stopper removed from the flask. Ninhydrin solution (40 ml) was added, the stopper was quickly put in place, and the system was allowed to heat for 20 min. The flask was removed from the water bath and allowed to stand for 30 min, while the remaining $^{14}CO_2$ was absorbed in NaOH. This procedure was repeated until no increase in radioactivity in CO_2 was noted.

Experiment 2. Radiolabeled ochratoxin A was prepared by culturing *A. ochraceus* on SYE medium containing 250 μc of sodium acetate-*2-^{14}C* (Calbiochem, Los Angeles, Calif.). Ochratoxin A was extracted and hydrolyzed as in experiment 1. After hydrolysis, the ^{14}C-isocoumarin moiety (II) was identified as in experiment 1 and selectively degraded to determine the radioactivity of individual carbons in the isocoumarin nucleus (see flow chart in Fig. 2.)

The radiolabeled isocoumarin moiety (II) was subjected to KOH fusion to remove carbons 9 and 10 as acetate (18). A chloroform solution of isocoumarin was evaporated to dryness in a three-hole boiling flask. A 4-g amount of KOH and 1 ml of demineralized water were then added. The flask was attached to a reflux condenser; a thermometer was placed in one port through a ground-glass stopper, and the other port was plugged with a cork stopper. The mixture was heated carefully as the temperature increased and was maintained at 208 to 212 C for 10 min. As the mixture cooled, it was dissolved in 10 ml of water which was added slowly through the condenser. After cooling to room temperature, the cork was replaced by a buret, and H_3PO_4 was added to neutralize the KOH. This was done slowly to maintain the temperature below 60 C. A 10-fold excess of H_3PO_4 was then added, and the mixture was steam-distilled to remove the acetate. The distillate was adjusted to *p*H 8 with barium hydroxide and evaporated to dryness on a boiling-water bath. Dried barium acetate was degraded by pyrolysis to CO_2 (carbon 9) and acetone (Fig. 2). The acetone was next converted to iodoform (carbon 10) by the procedure of Calvin et al. (3). Pyrolysis was conducted at 525 C for 10 min in a nitrogen atmosphere flowing though the chamber of a pyrolyzer (model PY-2, Barnes Engineering Co., Stamford, Conn.). Evolved acetone was trapped in 10 ml of water. The aqueous acetone was made strongly basic by addition of 5 ml of saturated NaOH. Potassium tri-iodide was added in portions until the precipitation of iodoform was completed. The radioactivities on the separated iodoform and barium carbonate fractions, respectively, were counted in the liquid scintillation system.

Carbons 1 and 11 were removed as CO_2 from 3-carboxy-5-chloro-6-methyl salicylic acid (IV) by refluxing for 30 min in 25 ml of 85% H_3PO_4 by the method of Nitta et al. (18). The top of the condenser was attached to a barium hydroxide trapping solution. A stream of CO_2-free nitrogen was flushed through the system to force evolved CO_2 into the barium hydroxide. Carbon dioxide was evolved and reabsorbed in fresh barium hydroxide by acidification before it was isolated by filtration and the radioactivity was measured. The phosphoric acid solution was diluted to 100 ml and extracted with 4×200 ml of ethyl acetate which was then evaporated to near dryness in a water bath at reduced pressure. The remaining solution was transferred to a 50-ml flask and evaporated to dryness for nitration.

Methylpicric acid for the final degradation reaction was prepared by dechlorinating 2-chloro-5-hydroxytoluene (V). Dechlorination was facilitated by nitration of the ring positions ortho to the hydroxyl group. A 1-ml amount of fuming nitric acid was added to compound V in a 100-ml, round-bottom boiling flask and then was evaporated to dryness on a water bath (12). In the same flask, now containing 2,4-dinitro-3-hydroxy-6-chloro toluene (VI), we placed 2.8 mg of red phosphorus and 20 ml of hydriodic acid (22). The mixture was refluxed for 14 hr, cooled, and filtered through a sintered-glass funnel to remove the phosphorus. The flask and the solid were washed with two 20-ml portions of chloroform. The clear filtrate was transferred to a flash-evaporator flask and concentrated to near dryness by heating in a water bath at reduced pressure. The residue was washed into a beaker with a small portion of hot chloroform. The chloroform solution containing 2,4-dinitro-3-hydroxytoluene (VII) was next evaporated to dryness. The third nitro group was introduced para to the hydroxy group by twice repeating the procedure previously described for nitration of compound V. This procedure yielded methyl picric acid (VIII), which consisted of carbons 2, 3, 4, 5, 6, 7, and 8 of the isocoumarin moiety of ochratoxin A.

The final reaction of the stepwise degradation of the isocoumarin moiety of ^{14}C-labeled ochratoxin A was accomplished by using barium hypobromite to convert methyl picric acid to barium carbonate and nitrobromomethane (bromopicrin; 12). Barium hypobromite was prepared by mixing 100 mg of barium hydroxide octahydrate and 0.38 ml of bromine (99.5% pure) in 60 ml of water. A 30-ml amount of barium hypobromite at 0 C was added to the methyl picric acid, brought to room temperature, and allowed to stand for 30 min. Carbons 3 and 5 of the original isocoumarin were removed as barium carbonate, which was then converted to carbon dioxide. The liquid containing barium carbonate was decanted, and the residual bromopicrin was washed with 3 small volumes of water. Both liquid and washings were placed in a side-arm flask the side chamber of which contained 5 ml of 1 N NaOH. The carbonate was then recycled by acidification into NaOH, and the radioactivity was measured by liquid scintillation. Bromopicrin (carbons 2, 4, and 6) was dissolved in ethyl ether for measurement of radioactivity by liquid scintillation. The presence of bromopicrin was confirmed

by comparing ultraviolet and infrared spectra of the radioactive material with authentic bromopicrin prepared by hypobromite degradation of picric acid (12).

Safety precautions. Because of the explosive nature of derivatives of picric acid, the reactions involving these compounds were conducted behind safety glass of a closed chemical fume hood. Reaction vessels were placed in an ice bath during reactions that liberated heat and then were allowed to come slowly to room temperature.

RESULTS

Radioactivity from phenylalanine-*1*-^{14}C was readily incorporated into ochratoxin A by *A. ochraceus* (Table 1). After 8 days of incubation on SYE medium containing 100 μc (2.22×10^8 counts/min) of phenylalanine-*1*-^{14}C, chloroform extraction, and TLC purification, radiolabeled ochratoxin A with 4.55×10^5 dpm was obtained (0.2% incorporation into ochratoxin A). Hydrolysis of the ^{14}C-ochratoxin A with 6 N HCl and separation of the phenylalanine and isocoumarin moieties revealed that 91% (4.15×10^5 counts/min) of the incorporated phenylalanine was in the pyenylalanine moiety of ochratoxin A (Table 1). Only 0.95% (4.3×10^3 counts/min) of the incorporated radioactivity was present in the isocoumarin moiety of ochratoxin A. The remaining 8% of the radioactivity was lost, presumably through incomplete extraction of the hydrolysis products.

Radioactivity from sodium acetate-*2*-^{14}C was incorporated into ochratoxin A by *A. ochraceus* (Table 2). After 8 days of incubation on SYE medium containing 250 μc (5.55×10^8 counts/min) of sodium acetate-*2*-^{14}C, chloroform extraction, and TLC purification, radiolabeled ^{14}C-

TABLE 1. *Distribution of radioactivity in ochratoxin A produced by A. ochraceus from phenylalanine-1-^{14}C*[a]

Material degraded	Degradation products	Total radioactivity	Radioactivity as per cent of material degraded
Ochratoxin A (I)[b]		$4.55 \times 10^5 \pm 2\%$	100.0
	Isocoumarin (II)	$0.04 \times 10^5 \pm 5\%$	0.9
	Phenylalanine (III)	$4.15 \times 10^5 \pm 2\%$	91.0
Phenylalanine		$4.15 \times 10^5 \pm 2\%$	100.0
	CO_2 (carboxyl group)	$2.56 \times 10^5 \pm 2\%$	61.2

[a] Incubation was for 8 days in SYE medium containing 100 μc of phenylalanine-*1*-^{14}C.
[b] Numbers refer to structures in Fig. 1.

TABLE 2. *Distribution of radioactivity in ochratoxin A produced by A. ochraceus from sodium acetate-2-^{14}C*[a]

Material degraded	Degradation products	Total radioactivity	Radioactivity as per cent of material degraded
		counts/min	
Ochratoxin A (I)[b]		$769.0 \times 10^5 \pm 2\%$	100.0
	Isocoumarin (II)	$478.0 \times 10^5 \pm 2\%$	62.0
	Phenylalanine (III)	$127.0 \times 10^5 \pm 2\%$	16.5
Isocoumarin (II)		$478.0 \times 10^5 \pm 2\%$	100.0
	Acetate (C9, 10)[c]	$0.82 \times 10^5 \pm 2\%$	0.17
Acetate (C9, 10)		$0.82 \times 10^5 \pm 5\%$	100.0
	$BaCO_3$ (C9)	$0.33 \times 10^5 \pm 5\%$	40.2
	CHI_3 (C10)	$0.30 \times 10^5 \pm 5\%$	36.6
3-Carboxy-5-chloro-6-methyl salicylic acid (IV)		$450.0 \times 10^5 \pm 2\%$	100.0
	2 CO_2 (C1, 11)	$13.5 \times 10^5 \pm 5\%$	3.0
Methyl picric acid (VIII)		$145.5 \times 10^5 \pm 2\%$	100.0
	$BaCO_3$ (C3, 5)	$1.0 \times 10^5 \pm 5\%$	0.69
	Br_3CNO_2 (C2, 4, 6)	$119.0 \times 10^5 \pm 3\%$	81.8

[a] Incubation was for 8 days in SYE medium with 250 μc of sodium acetate-*2*-^{14}C.
[b] Numbers refer to structures in Fig. 2.
[c] Carbon numbers refer to numbering scheme of ochratoxin A in Fig. 2.

ochratoxin A with 7.69 × 10^5 counts/min was obtained (1.39% incorporation into ochratoxin A). Acid hydrolysis of ^{14}C-ochratoxin A and separation of the isocoumarin and phenylalanine moieties showed that 62% (4.78 × 10^5 counts/min) of the radioactivity incorporated from acetate appeared in the isocoumarin moiety, with only 16.5% (1.27 × 10^5 counts/min) being present in the phenylalanine moiety of ochratoxin A.

After acid hydrolysis of the acetate-labeled ochratoxin A, the isocoumarin moiety was isolated and degraded stepwise to determine the radioactivity of various individual carbons in the isocoumarin nucleus. The degradation scheme is illustrated in Fig. 2, and the results are shown in Table 2. The barium acetate that was recovered after KOH fusion of isocoumarin yielded only 0.17% (820 counts/min) of the radioactivity in the parent compound. The radioactivity was found to be distributed equally in the acetate between carbons 9 and 10 of isocoumarin (Table 2).

Decarboxylation of 3-carboxy-5-chloro-6-methyl salicylic acid (IV), by refluxing for 30 min in 85% H_3PO_4, produced $^{14}CO_2$ (carbons 1 and 11) with 3% (1.35 × 10^4 counts/min) of the radioactivity originally present in IV.

The 2-chloro-5-hydroxytoluene (V) that resulted from decarboxylation of IV was next dechlorinated and trinitrated to produce methyl picric acid (VIII). The picric acid was then subjected to barium hypobromite degradation to yield barium carbonate (carbons 3 and 5) and bromopicrin (carbons 2, 4, and 6). The barium carbonate was found to contain only 0.69% (1.03 × 10^3 counts/min) of the methyl picrate activity, whereas the bromopicrin contained 81.8% (1.19 × 10^5 counts/min).

DISCUSSION

A strictly theoretical consideration of the structure of the ochratoxin A molecule suggests that both an aromatic and an aliphatic pathway could be involved in its biosynthesis. The presence of phenylalanine in the molecule strongly implies that the shikimic acid pathway is responsible for the biosynthesis of that portion of ochratoxin A. The isocoumarin moiety could possibly be synthesized either via shikimate, as in coumarin synthesis in higher plants (16), or from acetate units via acetate condensation, as in the formation of oosponol and oospolactone by *Oospora astringenes* (18) and of several other similar fungal metabolites (1, 16).

Results of experiment 1 show that *A. ochraceus* readily incorporated phenylalanine-*1-^{14}C* into ochratoxin A. Of the 100 μc of radioactivity supplied in the medium, 0.2% was recovered in ^{14}C-ochratoxin A. Ninety-one per cent of this activity was contained in the phenylalanine moiety, with 61.2% of the ^{14}C-phenylalanine radioactivity being in the carboxyl group. The isocoumarin nucleus was not comparably labeled from phenylalanine-*1-^{14}C*. The fungus did degrade phenylalanine to some extent and appeared to incorporate some radioactivity randomly into both the phenylalanine ring and the isocoumarin ring. However, the isocoumarin moiety contained only 0.9% of the radioactivity present in the ochratoxin. *A. ochraceus* can degrade phenylalanine to some extent, since uniformly labeled ^{14}C-phenylalanine, supplied to the fungus, was catabolized by 38% to $^{14}CO_2$ (*unpublished data*).

Results of experiment 2 show that *A. ochraceus* incorporated sodium acetate-*2-^{14}C* into ochratoxin A. The fungus utilized 1.39% of the 250 μc of radiolabeled acetate supplied in the medium in the synthesis of ochratoxin A, with 62% being incorporated into the isocoumarin moiety and 16.5% being incorporated into phenylalanine. The 62% incorporation into isocoumarin strongly supports the acetate pathway hypothesis. The incorporation of acetate into phenylalanine may have resulted from the metabolism of acetate through the citric acid cycle or the glyoxylate cycle to malate or oxalacetate with subsequent decarboxylation to phosphoenolpyruvate. These intermediates could then have been incorporated into phenylalanine via the shikimic acid pathway. Such pathways are known to occur in microorganisms (13, 21, 29).

The stepwise degradation of the acetate-labeled isocoumarin moiety revealed that most of the incorporated radioactivity was contained in ring carbons 2, 4, and 6, with little or no radioactivity in carbons 1, 3, 5, 9, 10, or 11. This pattern of labeling is consistent with the hypothesis that the major portion of the isocoumarin moiety of ochratoxin A is synthesized via acetate condensation. The absence of any significant amount of radioactivity in carbon 10 suggests that this methyl group is not derived from acetate. This absence of radioactivity raises the possibility that the carbons of the lactone ring of the isocoumarin arose from a phenylpropanoid precursor in a manner similar to that of several carbon atoms in the isocoumarin portion of hydrangenol from the plant *Hydrangea macrophylla* (14, 15).

The small but significant amount of radioactivity found in isocoumarin carbons 1 and 11 suggests that the lactone carbonyl group results from a head-to-head condensation of acetate, with subsequent decarboxylation and oxidation of the remaining methyl group to a carboxy group with subsequent closure of the ring. This oxidation of

a methyl group would then be similar to the situation reported by Gatenbeck (12) for the biosynthesis of 3-hydroxy-phthalic acid. If this is true, the carboxyl group attached to carbon 4 probably arose from the one-carbon pool of the organism, as demonstrated by Birch et al. for a corresponding carboxyl group in citrinin from *A. candidus* (2).

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