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Biosynthesis of Radiolabeled Phomopsin by *Phomopsis leptostromiformis*

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[^{14}C]phomopsin and [^{36}Cl]phomopsin were synthesized by *Phomopsis leptostromiformis* in liquid cultures containing various labeled compounds. [U-^{14}C]isoleucine, [*U*-^{14}C]phenylalanine, and [*U*-^{14}C]proline were the best precursors in terms of labeling efficiency, whereas [^{36}Cl]hydrochloride was much less efficient. When each of the four precursors was used, a large proportion of recovered label was associated with phomopsin. The specific activities of phomopsin produced with labeled isoleucine, phenylalanine, proline, and hydrochloride were 150, 120, 90, and 17 μCi/mmol, respectively. ^{14}C label from acetate, malate, propionate, sucrose, or tryptophan was neither specifically nor efficiently incorporated into phomopsin.

Phomopsin, a chlorinated hepatotoxic metabolite of *Phomopsis leptostromiformis* (Kühn) Bubák ex Lind, is responsible for the field mycotoxicosis of sheep known as lupinosis. In South Africa, lupinosis has been shown to be due to ingestion of lupin (*Lupinus luteus*, *L. angustifolius*, or *L. albus*) stubbles or hay on which *P. leptostromiformis* grows as a saprophyte (9). Subsequently, the disease in Australia has been shown to be caused by intake of lupins contaminated with *Phomopsis rossiana* (Sacc.) Sacc. et D. Sacc. (10), which was later shown to be synonymous with *P. leptostromiformis* (8).

Phomopsin was originally isolated from cultures of *P. leptostromiformis* grown on moist, autoclaved lupin seeds (1), but more recently, a liquid culture system for laboratory production of phomopsin has been described (4). The chemical structure of phomopsin is not yet known, although analytical and spectral characterizations suggest that the empirical formula is approximately $C_{33}H_{44}N_5O_{13}Cl$ and the molecular weight is 753 (2). More recent studies, including field desorption and electron impact-desorption mass spectrometry studies, suggest that the molecular weight is 770, the value I accept, and show that the molecule is substantially aliphatic, with one aromatic ring as the chromophore (C. C. J. Culvenor, personal communication). The biological effects of phomopsin on animals have been well described (6, 7), but no rapid and specific assay is available for detection of small amounts of phomopsin in field materials.

The purpose of this study was to determine a method for production of radiolabeled phomopsin for use in biochemical and toxicological studies and for possible use in radioimmunoassay and to provide basic biosynthetic data for use in chemical structure determination.

MATERIALS AND METHODS

Media and cultures. *P. leptostromiformis* WA1515 was used throughout. Origin and isolation of the organism, production and storage of pycnidiospores, culture medium, and method of inoculation have been described previously (4). Cultures were grown in 25-ml volumes in cotton-plugged bottles (diameter, 5 cm) for 28 days at 25°C without agitation, after which filtrates from representative bottles were assayed (see below) to confirm phomopsin production. Spent medium was then aseptically removed from duplicate bottles with a syringe and needle, disturbing the mycelia as little as possible, and replaced with 25-ml portions of fresh medium containing 1 μCi of the appropriate labeled compound per ml. Incubation was continued for a further 21 days. The following labeled compounds were used (millicuries per millimole, except as noted): [*U*-^{14}C]acetic acid (40 to 60), [^{36}Cl]hydrochloride (12.5 mCi/g of chlorine), L-[*U*-^{14}C]isoleucine (339), L-[*U*-^{14}C]malic acid (50), L-[*U*-^{14}C]phenylalanine (10), L-[*U*-^{14}]proline (275), [2-^{14}C]sodium propionate (59), [*U*-^{14}C]sucrose (10.1), and DL-[*methylene*-^{14}C]tryptophan (56.5).

The labeled compounds were purchased from Amersham Australia Pty. Ltd. At the end of the incubation period, the medium was decanted and filtered through a layer of cotton wool. Mycelia were washed twice with water, and the washings were combined with the medium filtrate. Mycelia were discarded without extraction since Lanigan et al. found >95% of phomopsin in culture filtrates (4).

Extraction and assay of phomopsin. Phomopsin was extracted from culture filtrates and assayed as described previously (4), except that the polystyrene resin (Amberlite XAD-2) columns were washed with

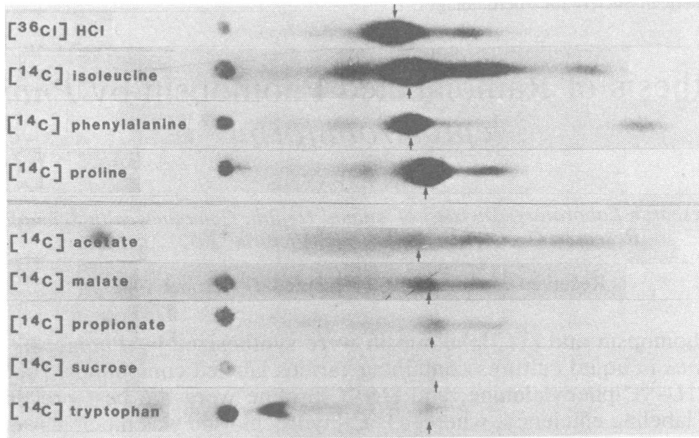

FIG. 1. Autoradiographs of electropherograms for all experiments. Arrows indicate centers of phomopsin spots visible under UV light.

30 ml of methanol-water (40:60 [vol/vol]) before elution of phomopsin with methanol.

Determination of label incorporation. The methanol eluate was evaporated to dryness and redissolved in 200 µl of 50:50 aqueous methanol. Portions (1 and 2 µl) were then subjected to high-voltage paper electrophoresis as previously described (4). The resulting electropherograms were cut into strips to separate each sample, and the strips were examined by autoradiography and liquid scintillation counting. Autoradiographs of the electropherograms of the 2-µl samples were produced by holding strips in contact with X-ray film for 1 or 2 weeks before development by standard methods. The 1-µl papers were cut into portions representing recognizable phomopsin spots visible under UV illumination at 254 nM and similarly sized portions of the interspot regions. Appropriate standards were also spotted onto portions of electrophoresis paper to confirm counting efficiency. The portions of paper were placed into scintillation vials, 3 ml of scintillant (3) was added to each vial, and radioactivity was counted in a Packard 2660 scintillation counter, with an external standard for quench correction. Total recovery of label was determined by counting the activities in 1-µl portions of the concentrated extracts. Labeling specificity was then calculated as the activity of phomopsin expressed as a percentage of total recovered label.

Incorporation of label into phomopsin was also measured by thin-layer chromatography (TLC). Samples (2 µl) of the concentrated extracts in 50:50 aqueous methanol were spotted onto Kieselgel 60 F_{254} plates (E. Merck AG, Darmstadt, West Germany) with concentration zones; the plates were developed with $CHCl_3-CH_3OH-H_2O$ (40:50:5 [vol/vol]) (M. Clarke, personal communication). The plates were examined under UV light and divided into regions as described for the electropherograms. Each region was scraped off the plates and then extracted twice with 1 ml of CH_3OH-H_2O (90:10 [vol/vol]) (L. W. Smith, personal communication). The solvent was allowed to evaporate in a stream of air at room temperature, 3 ml of scintillant was added, the contents of the counting vials were mixed by inversion, and the radioactivity was counted as described above. Counting efficiency was again confirmed with appropriate standards.

RESULTS

All cultures grew readily from initial inoculation and produced 75 to 150 mg of phomopsin per liter within 4 weeks, at which stage the spent medium was replaced with fresh medium containing labeled precursors. Phomopsin again accumulated in all cultures, levels of 75 to 150 mg/liter being reached within the 3-week incubation period. The qualitative and quantitative results of the various incorporations, as determined by electrophoresis, are shown in Fig. 1 to 3 and Table 1. Each autoradiograph (Fig. 1) represents an electropherogram of a constant

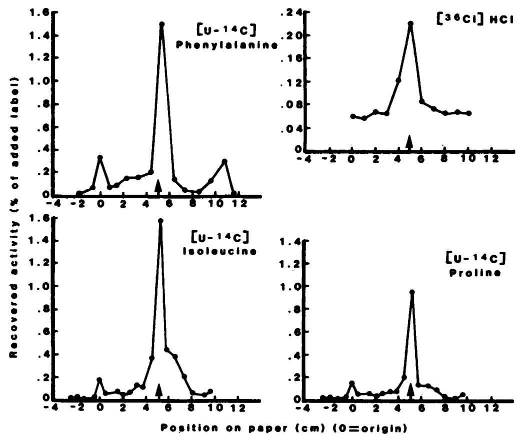

FIG. 2. Quantitative radiolabeling patterns of electropherograms of cultures containing [^{36}Cl]hydrochloride, [^{14}C]isoleucine, [^{14}C]phenylalanine, or [^{14}C]proline. Arrows indicate centers of phomopsin spots visible under UV light.

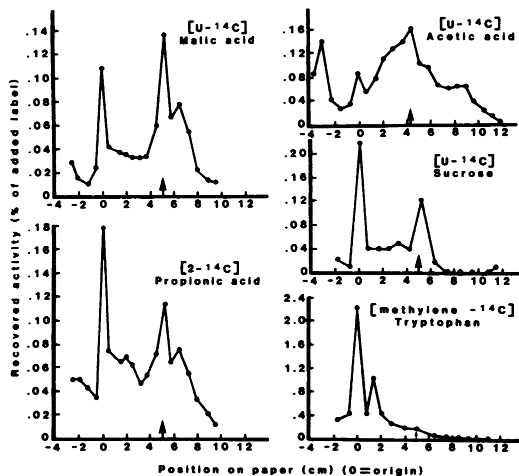

FIG. 3. Quantitative radiolabeling patterns of electropherograms of cultures containing [^{14}C]acetate, [^{14}C]malate, [^{14}C]propionate, [^{14}C]sucrose, or [^{14}C]tryptophan. Arrows indicate centers of phomopsin spots visible under UV light.

proportion (1%) of the extract of each culture fluid. The X-ray film was exposed to electropherograms for 1 week except in the [^{36}Cl]hydrochloride and [^{14}C]acetate experiments, in which the film was exposed for 2 weeks. Addition of [^{14}C]isoleucine, [^{14}C]phenylalanine, and [^{14}C]proline to cultures resulted in reasonably good incorporation of radioactivity into phomopsin, with relatively little other labeled material evident. The best incorporation of label was achieved with [^{14}C]isoleucine and [^{14}C]phenylalanine, which were similar in labeling efficiency and specificity. Incorporation of label from [^{14}C]proline was only about two-thirds the incorporation from labeled phenylalanine and isoleucine, but specificity was slightly greater. Labeling of phomopsin with [^{36}Cl]hydrochloride was fairly specific but much less efficient than labeling with labeled isoleucine, phenylalanine, or proline. The comparatively high intensity of the phomopsin spot in the autoradiograph from this experiment was due to the longer exposure time and the higher energy emissions from ^{36}Cl, compared with exposure time for and emissions from ^{14}C. Addition of [^{14}C]malic acid, [^{14}C]propionic acid, [^{14}C]acetic acid, [^{14}C]tryptophan, and [^{14}C]sucrose led to weak incorporation of label into XAD-2–methanol extracts, the greatest total incorporation being in the [^{14}C]tryptophan cultures. Some incorporation of label into the phomopsin region of the electropherograms was seen, but the majority of the activity was located elsewhere. Consequently, further investigation of labeling specificity by TLC was restricted to experiments with labeled isoleucine, phenylalanine and proline.

TABLE 1. Efficiency and specificity of phomopsin labeling in each experiment, as assessed by electrophoresis and liquid scintillation counting

Label added	Efficiency (%)[a]	Specificity (%)[b]
[^{14}C]acetic acid	0.16	9
[^{36}Cl]hydrochloride	0.22	36
[^{14}C]isoleucine	1.6	28
[^{14}C]malic acid	0.14	12
[^{14}C]phenylalanine	1.5	29
[^{14}C]proline	0.94	34
[^{14}C]propionic acid	0.11	7
[^{14}C]sucrose	0.12	9
[^{14}C]tryptophan	0.19	3

[a] Percentage of added label incorporated into phomopsin.

[b] Activity of phomopsin expressed as a percentage of total label recovered in methanol eluates from XAD-2 columns.

Figure 4 shows the measured activities (in counts per minute) of the various bands removed from thin-layer chromatograms of 1% of the methanol extract concentrates used in each experiment. The results for all three experiments closely parallel those obtained by electrophoresis. With phenylalanine and isoleucine, a smaller peak of activity, which coincided with a region of UV light absorption on the plates, was seen at a higher R_f than that shown by phomopsin. The identity of this material is unknown.

Table 1 summarizes specificities and efficiencies of incorporation of label into phomopsin, as determined by electrophoretic separations. Specificity calculations were based on the total amount of phomopsin produced, as measured by high-voltage electrophoresis, and the measured radioactivity of the phomopsin spots. Only in

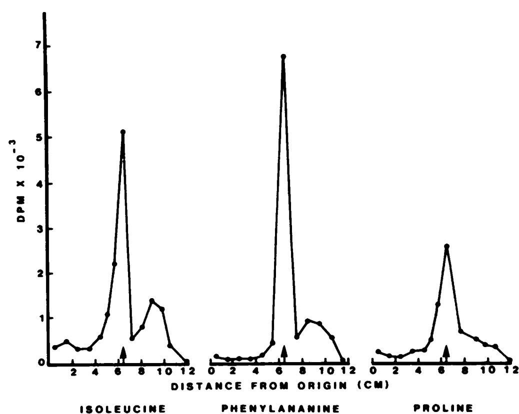

FIG. 4. Quantitative radiolabeling patterns of cultures containing [^{14}C]isoleucine, [^{14}C]phenylalanine, or [^{14}C]proline, as determined by TLC separations. Arrows indicate centers of phomopsin spots visible under UV light.

TABLE 2. Specific activity of phomopsin in each experiment, as assessed by electrophoresis and liquid scintillation counting

Label added	Sp act	
	μCi/mmol	dpm/μg
[14C]acetic acid	15.4	44
[36Cl]hydrochloride	16.9	48
[14C]isoleucine	152.5	440
[14C]malic acid	13.1	38
[14C]phenylalanine	115.5	330
[14C]proline	90.1	260
[14C]propionic acid	11.6	32
[14C]sucrose	9.2	26
[14C]tryptophan	14.6	42

the cases of isoleucine, proline, and phenylalanine was specificity of phomopsin labeling further checked by TLC.

DISCUSSION

The efficiency of incorporation of radioactivity from isoleucine, phenylalanine, and proline into phomopsin, although not great, was quite significant, especially in view of the relatively slow rate of phomopsin accumulation and the possible metabolic fates of the amino acids. Furthermore, the very poor incorporations achieved with other metabolically useful compounds indicate that isoleucine, phenylalanine, and proline are incorporated into phomopsin substantially intact, rather than through metabolism to smaller fragments. Also important in terms of biosynthesis is that the chlorine in phomopsin may be derived from inorganic chloride, rather than from an obligatory chlorine-containing organic precursor. This is similar to synthesis of griseofulvin by *Penicillium griseofulvum* and *P. nigricans*, which have been shown to incorporate bromine (and, by inference, chlorine) from inorganic sources (5).

Although little definitive information on the chemical structure of phomopsin has been published, the production of oxaloacetic acid upon mild hydrolysis has been reported (2). An attempt to label the part of the phomopsin molecule which yields oxaloacetic acid by addition of labeled malic acid to cultures produced disappointing results, suggesting that oxaloacetic acid is not a direct precursor of phomopsin but is generated upon hydrolysis by some other chemical moiety. Owing to rather poor label incorporation, no further attempt was made to characterize the phomopsin produced in this experiment.

The results of the above experiments suggest that synthesis of phomopsin by *P. leptostromiformis* consists, at least in part, of assembly of a relatively small number of precursors such as amino acids or peptides, rather than sequential addition of a larger number of small units such as acetate or propionate. This conclusion is consistent with the findings of Lanigan et al., who have shown that a complex undefined mixture containing amino acids and peptides is necessary for phomopsin production (4).

The specific activities of labeled phomopsin produced in the isoleucine, phenylalanine, and proline experiments (Table 2) were great enough to be useful in metabolism studies or radioimmunoassay. However, higher-specific-activity phomopsin could be readily produced by increasing the amount of label added or by using more than one precursor in cultures, since it is unlikely that label from the three compounds would be incorporated into the same part of the phomopsin molecule.

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