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# BIOSYNTHESIS OF THE BENZOQUINONE, SHANORELLIN, IN *SHANORELLA SPIROTRICHA*

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**Abstract**—The biosynthesis of shanorellin (2,6-dimethyl-3-hydroxymethyl-5-hydroxy-1,4-benzoquinone), an extracellular product of *Shanorella spirotricha* Benjamin (Ascomycetes), has been found to follow the acetate–polymalonate pathway. Studies with carbon-14 indicate that both methyl groups are derived from methionine.

## INTRODUCTION

SOME of the *p*-benzoquinones and related compounds of Basidiomycetes have been shown to be formed by a variety of metabolic routes although those produced by Ascomycetes and Deuteromycetes have been found to be synthesized *via* the acetate-polymalonate pathway.[1–3] For example, coprinin hydroquinone, a product of the basidiomycete, *Lentinus degener*, is derived from tyrosine[4] and helicobasidin from the basidiomycete *Helicobasidium mompa* is synthesized from mevalonate.[5,6] A variant of the acetatepolymalonate pathway is the substitution of propionate for acetate as the starter molecule, e.g. biosynthesis of homoorsellinic acid in *Penicillium baarnense*.[7]

The ascomycete, *Shanorella spirotricha* Benjamin (UBC 240) produces a number of benzoquinones, the major one of which has been identified as 2,6-dimethyl-3-hydroxymethyl-5-hydroxy-1,4-benzoquinone and named shanorellin.[8] In view of the somewhat unusual substitution pattern of this benzoquinone we have investigated its biosynthesis with the use of $^{14}C$-labeled compounds including shikimate, acetate and propionate.

## RESULTS AND DISCUSSION

The production of shanorellin in relation to fungal growth in batch culture is shown in Fig. 1. In the first 8 days of growth there was a rapid increase in dry weight of mycelium, a rise in pH and a rapid utilization of glucose. During this period only traces of phenolic compounds were detected by TLC. Shanorellin appeared in the medium as the mycelial growth rate started to decrease and in about 18 days had reached a maximum. After this it gradually disappeared indicating that it was being metabolized further.

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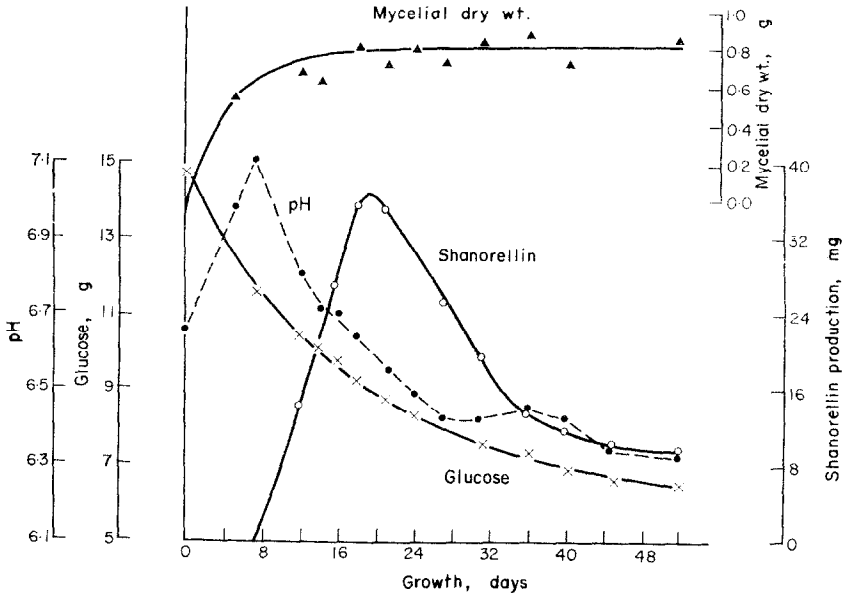


FIG. 1.

These results suggested that 2-week old cultures would be most suitable for tracer studies. The incorporation of possible $^{14}C$-labeled precursors into shanorellin is shown in Table 1. Shanorellin is obviously derived from the acetate-polymalonate pathway with one or more methyl groups being derived from methionine. Propionate and 2-methylmalonate were not incorporated and the slight activity from phenylalanine-$^{14}C$ and tyrosine-$^{14}C$ may be explained by catabolism of these two amino acids. Although the incorporation of malonate-$^{14}C$ was lower than that of acetate-$^{14}C$ it was significant enough to indicate that it is a

TABLE 1. INCORPORATION OF $^{14}C$-LABELED COMPOUNDS INTO SHANORELLIN BY *Shanorella spirotricha*

| Compound administered | Shanorellin isolated | |
|---|---|---|
| | Amount (mg) | Specific activity ($\mu c/mg$) |
| Acetate-1-$^{14}C$ | 8·26 | 0·036 |
| Acetate-2-$^{14}C$ | 11·93 | 0·050 |
| Malonic-2-$^{14}C$ | 9·10 | 0·019 |
| Shikimic-U-$^{14}C$ | 9·00 | 0·000 |
| Phenylalanine-U-$^{14}C$ | 9·00 | 0·002 |
| Tyrosine-U-$^{14}C$ | 7·40 | 0·005 |
| Propionate-1-$^{14}C$ | 8·70 | 0·000 |
| 2-Methyl-$^{14}C$-malonic | 9·20 | 0·000 |
| Methionine-$^{14}CH_3$ | 8·70 | 0·140 |

Each sample consisted of the contents of three Roux bottles. The specific activity of each of the administered compounds was adjusted to $2\mu c/\mu M/ml$, and $4\mu c$ were added to each bottle of a 14-day-old culture. Incubation time was 2 days.

likely precursor. Formate-$^{14}$C, fed to another batch of *Shanorella* was found to be incorporated to the extent of 6·9%.

When subjected to the Kuhn–Roth oxidation reaction, shanorellin yields 2 moles of acetic acid derived from the methyl carbons and the adjacent ring carbons. The results of Kuhn–Roth degradation of samples of shanorellin isolated from feedings with acetate-1- or -2-$^{14}$C or methionine-$^{14}CH_3$ are shown in Table 2.

TABLE 2. RESULTS OF KUHN–ROTH DEGRADATION OF SHANORELLIN-$^{14}$C OBTAINED ON FEEDING VARIOUS $^{14}$C-LABELED COMPOUNDS

| Activity of isolated shanorellin ($\mu$c) | Activity in recovered acetic acid ($\mu$c) | Recovery* (%) |
|---|---|---|
| A. From acetate-1-$^{14}$C feeding: | | |
| 0·97 | 0·00 | 0·00 |
| 1·13 | 0·00 | 0·00 |
| B. From acetate-2-$^{14}$C feeding: | | |
| 0·23 | 0·11 | 48·7 |
| 0·33 | 0·19 | 57·1 |
| 1·31 | 0·72 | 55·7 |
| C. From methionine-$^{14}CH_3$ feeding: | | |
| 0·18 | 0·16 | 90·2 |
| 0·19 | 0·17 | 86·4 |
| 0·46 | 0·41 | 91·3 |

* $\frac{\text{Specific activity of acetate} \times 2}{\text{Specific activity of shanorellin}} \times 100.$

The results are consistent with the following origin of the carbon skeleton of shanorellin (Fig. 2).

This was further confirmed by the Schmidt degradation of the acetate-$^{14}$C obtained from the Kuhn–Roth degradation. Acetate-$^{14}$C obtained from methionine-labeled shanorellin


FIG. 2.

gave essentially all of the activity in the methyl carbon and that from the acetate-2-$^{14}$C labeled shanorellin, in the carboxyl carbon.

In addition to shanorellin a number of colored compounds as well as compounds giving color reactions of phenols were detected by TLC. Three of the colored compounds were identified as benzoquinones most probably derived from shanorellin; their identification is the subject of a separate paper. Although the other phenols present were not identified, they

were shown to be different from 6-methylsalicylic, orsellinic, 3-methylorsellinic and 3,5-dimethylorsellinic acids and 3,4-dimethyl-5-hydroxyphenol. At no stage of growth could any of these possible precursors be identified in the growth medium. This suggests that methylation of the polyketide precedes aromatization and that a phenolic acid such as 2-hydroxymethyl-3,5-dimethyl-4,6-dihydroxybenzoic acid may be a precursor of shanorellin as suggested in Fig. 3.

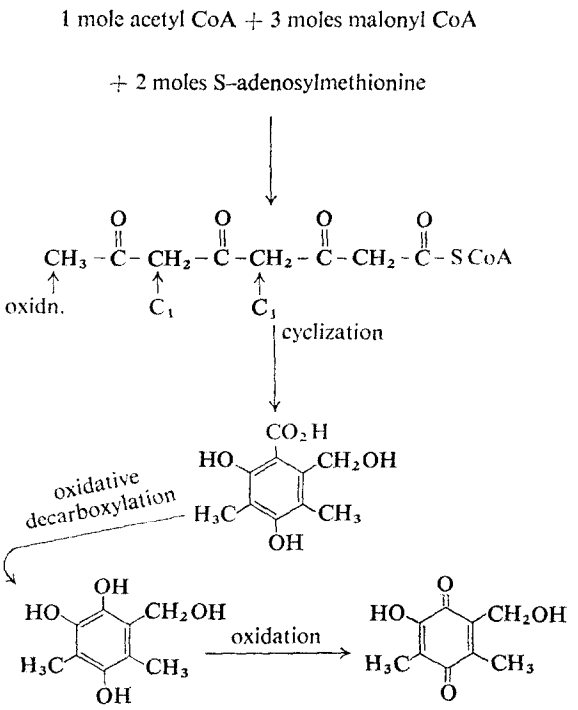


FIG. 3. SUGGESTED SCHEME FOR THE BIOSYNTHESIS OF SHANORELLIN.

## EXPERIMENTAL

*Administration of Radioactive Compounds*

*Shanorella* was grown in Roux bottles under the same culture conditions as described previously.[7] In tracer experiments each sample consisted of the contents of three Roux bottles. All radioactive solutions were autoclaved before being used, except for malonic acid which was sterilized by filtration through Millipore filters (0·22 $\mu$ pore size).

*Isolation of Shanorellin-$^{14}C$*

The medium was separated from the mycelial mat by filtration through three layers of cheesecloth. The filtrate was acidified to pH 2 with dilute HCl and extracted with EtOAc. The EtOAc extract was evaporated *in vacuo* and the residue obtained dissolved in 1 ml ethanol. An aliquot was used for TLC. The remainder was taken to dryness and the residue dissolved in $CHCl_3$. This solution was passed on to a column (27 cm × 2·5 cm) prepared by mixing three parts of silicic acid to one part of Hyflo Super Cel. Analytical grade $CHCl_3$ was used as eluant. The shanorellin band, which appeared as the third colored one on the column, was collected, evaporated to dryness and sublimed at 100°. Shanorellin that was used for degradation studies was further purified by preparative TLC developed first with $CHCl_3$–HOAc (500:37·5, v/v) and then with Cyclohexane–EtOAc–HOAc (20:10:1, by vol.). The compound was re-sublimed and the sublimate crystallized from benzene–petrol to constant specific activity. Solutions of shanorellin in $CHCl_3$ were found to obey the Beer–Lambert Law at concentrations up to 0·9 × $10^{-2}$ mg/ml when measured at 272 nm.

*Radioactivity Measurements*

All $^{14}C$-labeled compounds were counted with a Nuclear Chicago Liquid Scintillation System 724 and 725 in 15 ml of scintillator (PPO 6 g/l., POPOP 0·1 g/l. toluene 250 ml, ethanol 150 ml). All readings were corrected using efficiency curves calibrated from standard quenching solutions by the channel ratio method.

*Degradation of Shanorellin-$^{14}C$*

Purified Shanorellin-$^{14}C$ was first degraded by the Kuhn-Roth method as described by Eisenbraun, McElvain and Aycock.[9] The acetate obtained was subsequently degraded by the Schmidt reaction as described by Phares,[10] the evolved $CO_2$ being trapped in aq. 80% 2-phenylethylamine instead of 0·5N NaOH as described by Phares. The Schmidt degradation of commercially obtained acetate-2-$^{14}C$ gave over 95% of the activity in the methyl carbon (activity remaining in the flask) and none in the carboxyl carbon (activity in the trapping solution). With commercial samples of acetate-1-$^{14}C$, however a range of 50–80% of the total activity was isolated in the trapping solution and less than 5% the total activity from the flask. With the acetic acid obtained from acetic-1-$^{14}C$ labeled shanorellin by the Kuhn–Roth method no more than 80% of the activity was ever recovered in the trapping solution, 2–5% of the activity remaining in the flask. These yields could not be improved in spite of various minor modifications of the method.

*Determination of Mycelial Dry Weight*, pH *and Glucose Concentration*

Mycelial mats were dried overnight on petri dishes in an oven at 90° and weighed. The pH of the medium was measured with a Leeds & Northrup pH meter. For glucose determination, the medium after filtration was diluted with distilled water to 500 ml and 10 ml was removed for determination of glucose concentration by the method of Nelson.[11] The remaining solution was acidified and extracted with EtOAc for isolation of shanorellin.

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