APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Mar. 1982, p. 514–516
0099-2240/82/030514-03$02.00/0
Vol. 43, No. 3

# Biosynthesis of Radiolabeled Verruculogen by *Penicillium simplicissimum*

J. B. DAY AND P. G. MANTLE*

*Department of Biochemistry, Imperial College, London, United Kingdom*

Received 6 August 1981/Accepted 12 November 1981

In surface culture of *Penicillium simplicissimum*, verruculogen was shown to be biosynthesized from the intact carbon skeletons of tryptophan and proline, isoprenoid derivatives of mevalonic acid, and a methyl group donated by methionine. Selected radiolabeled precursors (1 mCi) pulse-fed at the optimum stage of fermentation yielded verruculogen (specific activity, $5.89 \times 10^2$ μCi $mmol^{-1}$) labeled in the prolyl and isoprenyl regions of the molecule and suitable for metabolic studies.

Verruculogen was first isolated from *Penicillium verruculosum* (2), and its structure (Fig. 1) has been determined (1, 6). As the most potent tremorgenic mycotoxin known, its possible role, together with other tremorgens, in animal neurological disorders has been investigated (9). Radiolabeled verruculogen is a desirable tool in these studies, and this paper reports on the investigation of biosynthetic precursors and their optimal use in preparing radiolabeled toxin. A sensitive assay for verruculogen has been developed (4, 5), and this has facilitated the development of toxin production on a large scale with a New Zealand soilborne mould identified as *P. estinogenum* (5). However, in accordance with the authoritative revision of the penicillia (11, 12), this fungus, which was used also in the present studies, will be referred to as *P. simplicissimum*.

## MATERIALS AND METHODS

**Fungal culture.** The medium used throughout this study was Czapek Dox/yeast extract broth (pH 6.8; Difco Laboratories) containing (grams per liter of distilled water): sucrose, 30; $NaNO_3$, 3; $K_2HPO_4$, 1; $MgSO_4 \cdot 7H_2O$, 0.5; KCl, 0.5; $FeSO_4 \cdot 7H_2O$, 0.01; and yeast extract, 5. For the production stage only of shaken-flask cultures, this medium was supplemented with $CaCl_2 \cdot 2H_2O$ (20 g $liter^{-1}$). Media (100 ml each) were dispensed into 500-ml Erlenmeyer flasks and sterilized at 121°C for 30 min. Shaken-flask fermentations were performed as previously described (5). Surface culture flasks were inoculated by scattering spores from potato dextrose agar slant cultures of *P. simplicissimum* onto the surface of the medium. All cultures were incubated at 27°C. When necessary, mycelial dry weight was determined at 50°C.

**Extraction and estimation of verruculogen.** After appropriate incubation (15 to 17 days), mycelia were homogenized in water, freeze-dried, and extracted with chloroform-acetone (1:1, vol/vol). Verruculogen, contaminated with a small amount of fumitremorgin B, was isolated by preparative-layer chromatography on silica gel $GF_{254}$ (Merck & Co., Inc.), with chloroform-acetone (93:7) as the developing solvent. The small amount of fumitremorgin B was regarded as irrelevant in these biosynthetic studies since the carbon skeleton is identical to that of verruculogen. Verruculogen was estimated by fluorimetric assay (4, 5).

**Radioisotopes.** Radioisotopes were obtained from the Radiochemical Centre, Amersham, England, and were injected into the broth of cultures in the following formulations: (i) L-[*methylene*-$^{14}$C]tryptophan (specific activity, 56 mCi $mmol^{-1}$) and L-[*U*-$^{14}$C]proline (specific activity, 285 mCi $mmol^{-1}$), supplied as sterile aqueous solutions containing 2% ethanol; (ii) [*U*-$^{14}$C]acetate (sodium salt; specific activity, 52 mCi $mmol^{-1}$), sterilized as an aqueous solution at 121°C for 15 min; and (iii) L-[*methyl*-$^{14}$C]methionine (specific activity, 65 mCi $mmol^{-1}$) and DL-[2-$^{14}$C]mevalonate (sodium salt; specific activity, 7 mCi $mmol^{-1}$), aqueous solutions sterilized by membrane filtration.

Radioactivity measurement of labeled toxin samples in acetone was made with scintillant (naphthalene and butyl PBD [2-(4′-*tert*-butylphenyl)-5-(4″-biphenylyl)-1,2,4-oxadiazole] in toluene) counted in a Kontron SL30 liquid scintillation counter. Corrections for quenching and chemiluminescence were made by reference to standard [$^{14}$C]hexadecane (specific activity, $1.13 \times 10^6$ dpm $g^{-1}$).

## RESULTS AND DISCUSSION

Since the option to use either surface or submerged culture was open, the relative efficiencies of the two methods were explored. The typical relationship between biomass production in surface culture and verruculogen accumulation is shown in Fig. 2. Toxin was produced over a period exceeding 2 weeks, eventually comprising over 2% of the mycelial dry weight. Conidiophores were produced after 3 days and, thereafter, the mycelium sporulated profusely.

In submerged culture, described fully elsewhere (5), the process is more rapid, biomass accumulation and sporulation being completed within about 2 days and the verruculogen con-

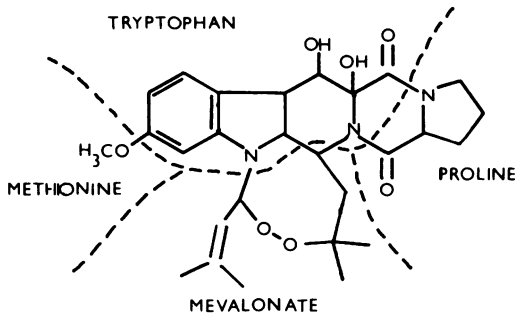


FIG. 1. Structure of verruculogen. The regions of the molecule derived from various precursors are indicated.

TABLE 1. Relative efficiency of incorporation of [$^{14}$C]proline into verruculogen in surface and submerged culture

| [$^{14}$C]proline fed[a] at: | Sp act of verruculogen ($\mu$Ci mmol$^{-1}$) | Incorporation (%) |
|---|---|---|
| Surface culture | | |
| Day 0 | 51.6 | 1.15 |
| Day 2 | 60.5 | 2.38 |
| Submerged culture | | |
| Day 0 | 44.5 | 0.87 |
| Day 2 | 68.6 | 1.18 |

[a] 20 $\mu$Ci (<0.1 $\mu$mol) added to 100-ml culture.

tent of mycelium reaching a maximum, usually not exceeding 1% (wt/wt) in about 1 week. [$^{14}$C]proline (>250 mCi mmol$^{-1}$) was added to shaken or surface culture flasks containing 100 ml of medium (0.2 $\mu$Ci ml$^{-1}$) either at inoculation or 2 days later. The specific activities of verruculogen produced in these ways (Table 1) were of a similar order, but surface culture was shown to be the more efficient process, as indicated by the higher percent incorporation of [$^{14}$C]proline, which in turn reflected the higher overall yield of verruculogen from surface culture.

Subsequent experiments to explore the range of biosynthetic precursors of verruculogen and to optimize the time of addition of radiolabeled precursors were therefore performed in surface culture. Clear evidence of incorporation of all of the putative precursors was obtained (Fig. 3). Mycelia produced similar final verruculogen titers (mean, 2.1% [wt/wt]), and the percent incorporation of radiolabel followed closely the variations in specific activity associated with the stage at which the radiolabeled precursors were administered. For example, 6% of [$^{14}$C]proline fed on day 3 was incorporated into verruculogen, whereas there was only 0.2% incorporation on day 7. Similarly, there was 2.0% incorporation of the methyl group of methionine on day 3 but only 0.1% on day 11. The optimum percent incorporation values are quite favorable, considering the key intermediate roles of all the precursor substances tested. The relative efficiencies

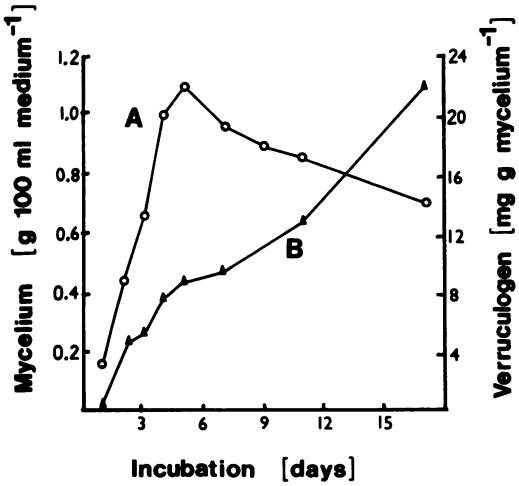


FIG. 2. Typical changes in mycelial dry weight (A) and its verruculogen content (B) during surface culture of *P. simplicissimum*.

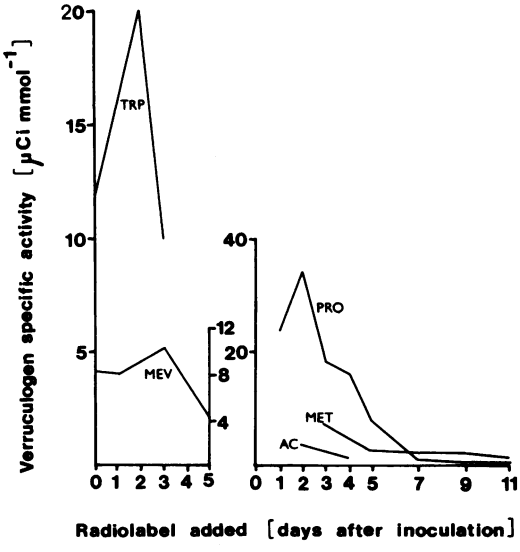


FIG. 3. Specific activities of [$^{14}$C]verruculogen produced in surface cultures of *P. simplicissimum* fed with radiolabel at various times during incubation. L-proline (PRO), L-methionine (MET), and acetate (AC) were added at 0.1 $\mu$Ci ml$^{-1}$. The specific activity scale was adjusted for L-tryptophan (TRP; 0.025 $\mu$Ci ml$^{-1}$) and DL-mevalonate (MEV; 0.05 $\mu$Ci ml$^{-1}$) to account for variations in amounts of radiolabel added and therefore to indicate graphically the relative labeling efficiencies of the putative precursors.

TABLE 2. Effect of increasing the concentration of radiolabel, added early in *P. simplicissimum* fermentation, upon the specific activity of the verruculogen produced

| Concn of [$^{14}$C]proline added ($\mu$Ci ml$^{-1}$)[a] | Sp act of verruculogen ($\mu$Ci mmol$^{-1}$) | |
|---|---|---|
| | Label added at: | |
| | Day 0 | Day 1 |
| 0.1 | 31.0 | 24.2 |
| 1.0 | 447.2 | 208.5 |
| 10.0 | | 1,764.5 |

[a] In this experiment, 10 ml of culture medium was used in a 50-ml conical flask.

of incorporation of biosynthetically acceptable (L-form), radiolabeled precursors, expressed as specific activities adjusted also to take account of the different amounts of $^{14}$C-labeled precursor fed, were calculated to be approximately in the ratio 29:14:11:2:1 for tryptophan, mevalonate, proline, methionine, and acetate, respectively. This confirms the deduction, readily made after considering the structure of verruculogen, that the carbon skeleton is formed from that of one molecule each of tryptophan and proline, two isoprene units derived from mevalonate, and the methyl group donated by *S*-adenosylmethionine. The relatively inefficient incorporation of acetate is in accord with the assumption that there is no polyketide moiety, but that this key metabolic intermediate finds its way into verruculogen primarily via mevalonate but also probably through proline. The optimum time of administration of a single pulse of radiolabeled precursor was about 2 days after inoculation.

The effect of increasing the concentration of radiolabel was explored with [$^{14}$C]proline (Table 2). Within the limits of experimental error, it was concluded that the efficiency of incorporation was constant over the range 0.1 to 10.0 $\mu$Ci ml$^{-1}$, beyond which the cost of producing biosynthetically labeled verruculogen of high specific activity would be unreasonable. Calculations indicated a similar cost effectiveness of radiolabeling the aromatic carbons of verruculogen (via benzene ring-labeled tryptophan) or the two isoprenoid methyl carbons derived from the 2-position of mevalonate. Incorporation of radiolabel of the methylene carbon of tryptophan would have been considerably cheaper and similar in cost to labeling the proline-derived carbons. Since the purpose of the radiolabeling was to obtain a product which, in whole or in part, might be identifiable in spite of possible metabolic transformations in animals or plants, a compromise was reached which involved using labeled proline and mevalonate.

Consequently, 500 $\mu$Ci each of [$^{14}$C]proline and [$^{14}$C]mevalonate were combined, divided among five surface cultures and added on day 2 (2 $\mu$Ci ml of culture$^{-1}$). The mycelium harvested 13 days later yielded verruculogen which was crystallized to a constant specific activity of $5.89 \times 10^2$ $\mu$Ci mmol$^{-1}$ (ca. 1 $\mu$Ci mg$^{-1}$). High-performance liquid chromatography of this product on reversed-phase silica (LiChrosorb-$C_{18}$) with methanol-water (3:1) solvent and UV light (235 nm) detection indicated that the only contaminant was fumitremorgin B (ca. 10%).

The specific activity of verruculogen prepared as described here is, for example, about 500 times greater than that of [$^{14}$C]T-2 toxin (8) and at least 10 times greater than [$^{14}$C]zearalenone (7) produced recently with a view to distribution and metabolism studies in animals. [$^{14}$C]verruculogen of high specific activity, described herein, has therefore facilitated studies of the uptake of this potent tremorgen by plants (3) and its distribution and metabolism in animals (9a; K. P. W. C. Perera, P. G. Mantle, and R. H. C. Penny, Res. Vet. Sci., in press).

ACKNOWLEDGMENT

We thank the Wellcome Trust for support.

LITERATURE CITED

1. **Cole, R. J., and J. W. Kirksey.** 1973. The mycotoxin verruculogen: a 6-O-methylindole. J. Agric. Food Chem. **21**:927–929.
2. **Cole, R. J., J. W. Kirksey, J. H. Moore, B. R. Blankenship, U. L. Diener, and N. D. Davis.** 1972. Tremorgenic toxin from *Penicillium verruculosum*. Appl. Microbiol. **24**:248–256.
3. **Day, J. B., and P. G. Mantle.** 1980. Tremorgenic forage and ryegrass staggers. Vet. Rec. **106**:463–464.
4. **Day, J. B., and P. G. Mantle.** 1981. Analysis and fermentation production of the tremorgenic mycotoxin verruculogen. *In* Mycotoxins and phycotoxins in human and animal health. Pathotox Publishers Inc., Park Forest South, Ill.
5. **Day, J. B., P. G. Mantle, and B. I. Shaw.** 1980. Production of verruculogen by *Penicillium estinogenum* in stirred fermenters. J. Gen. Microbiol. **117**:405–410.
6. **Fayos, J., D. Lokensgard, J. Clardy, R. J. Cole, and J. W. Kirksey.** 1974. Structure of verruculogen, a tremorgen-producing peroxide from *Penicillium verruculosum*. J. Am. Chem. Soc. **96**:6785–6787.
7. **Hagler, W. M., and C. J. Mirocha.** 1980. Biosynthesis of [$^{14}$C]zearalenone from [1-$^{14}$C]acetate by *Fusarium roseum* 'Gibbosum'. Appl. Environ. Microbiol. **39**:668–670.
8. **Hagler, W. M., C. J. Mirocha, and S. V. Pathre.** 1981. Biosynthesis of radiolabeled T-2 toxin by *Fusarium tricinctum*. Appl. Environ. Microbiol. **41**:1049–1051.
9. **Mantle, P. G., and R. H. C. Penny.** 1981. Tremorgenic mycotoxins and neurological disorders—a review. Vet. Annu. **23**:51–62.

9a. **Perera, K. P. W. C., J. B. Day, P. G. Mantle, and L. Rodrigues.** 1982. Metabolism of verrucologen in rats. Appl. Environ. Microbiol. **43**:501–506.

10. **Pitt, J. I.** 1979. *Penicillium crustosum* and *P. simplicissimum*, the correct names for two common species producing tremorgenic mycotoxins. Mycologia **71**:1166–1177.
11. **Pitt, J. I.** 1979. The genus *Penicillium* and its teleomorphic states *Eupenicillium* and *Talaromyces*. Academic Press, Inc., New York.