

Intermediates in the Penicillic Acid Biosynthesis in *Penicillium cyclopium*

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Feeding experiments using ^{14}C -labelled precursors demonstrate the following sequence of the biological formation of penicillic acid in *Penicillium cyclopium*: orsellinic acid \rightarrow 2-*O*-methylorsellinic acid \rightarrow 1,4-dihydroxy-6-methoxy-2-methylbenzene \rightarrow 6-methoxy-2-methylbenzoquinone(1,4) \rightarrow penicillic acid.

Penicillic acid was isolated already in 1913 by Alsberg and Black.¹ They also established that penicillic acid was toxic to various laboratory animals. The toxicity of penicillic acid has been confirmed by several research groups and its natural occurrence in moldy corn, *e.g.* in blue-eye diseased corn has been demonstrated by Ciegler and Kutzman.²

Penicillic acid is synthesized by a large number of fungi. Mosbach³ has shown that the biological formation of penicillic acid in *Penicillium barnense* proceeds *via* orsellinic acid which in itself is derived from acetate-polymalonate. Later Bentley and Keil⁴ have established in a series of incorporation experiments with *P. cyclopium* the participation of malonate in penicillic acid biosynthesis which is in accordance with its formation from orsellinic acid. Recently Al-Rawi *et al.*⁵ using penicillic acid obtained from tritium labelled acetate in a feeding experiment with *P. cyclopium* confirmed with ^3H NMR spectroscopy the origin of penicillic acid.

It is obvious that there are several metabolic steps with the possible involvement of defined intermediates in the transformation of orsellinic acid into penicillic acid. In order to investigate the details of this reaction sequence the following conceivable intermediates were synthesized

labelled with ^{14}C : 2-*O*- ^{14}C -methylorsellinic acid, 1-*O*- ^{14}C -methylorcinol, 6- ^{14}C -methoxy-2-methylbenzoquinone(1,4), 1,4-dihydroxy-6- ^{14}C -methoxy-2-methylbenzene, 3-hydroxy-6- ^{14}C -methoxy-2-methylbenzoquinone(1,4), 2,5,6-trihydroxy-3- ^{14}C -methoxy-toluene. These ^{14}C -labelled substances were used in feeding experiments with submerged cultures of *P. cyclopium*. In most cases the labelled substrate was added after 74 h growth of the culture when the penicillic acid production just had started. The efficiency of the incorporation of radioactivity into penicillic acid is shown in Table 1.

2-*O*-Methylorsellinic acid is taken up and transformed into penicillic acid by the organism with remarkable efficiency (50 %). The incorporation of the decarboxylated substance, *O*-methylorcinol, is appreciably lower (17 %). It could be explained by differences in abilities to penetrate the cell membrane but it could also reflect the occurrence of separate catabolic pathways both leading to penicillic acid. 6-Methoxy-2-methylbenzoquinone(1,4) as well as the corresponding hydroquinone give rise to penicillic acid, the quinone being significantly more efficient as a precursor than the hydroquinone. Labelled methoxy-methylbenzoquinone could also be isolated from the culture when radioactive 2-*O*-methylorsellinic acid was fed to the organism. The two labelled compounds carrying a further hydroxyl group in 3 position do not function in our experiments as precursors of penicillic acid. These results suggest a sequence of intermediates from orsellinic acid to penicillic acid as described in Fig. 1.

All attempts to demonstrate the presence of

Table 1. Incorporation of radioactive precursors into penicillic acid.

¹⁴ C-Substrate	Added dpm × 10 ⁻³	Penicillic acid dpm × 10 ⁻³	Incor- poration %
2- <i>O</i> -[¹⁴ CH ₃]orsellinic acid	80	40	50
1- <i>O</i> -[¹⁴ CH ₃]orcinol	120	20	17
6- <i>O</i> -[¹⁴ CH ₃]-2-CH ₃ -benzoquinone(1,4)	600	162	27
1,4-DiOH-6- <i>O</i> -[¹⁴ CH ₃]-2-CH ₃ -benzene	400	20	5
3-OH-6- <i>O</i> -[¹⁴ CH ₃]-2-CH ₃ -benzoquinone(1,4)	800	nil	—
2,5,6-TriOH-3- <i>O</i> -[¹⁴ CH ₃]toluene	800	nil	—

a 2-*O*-methylorsellinic acid decarboxylase in the organism have so far failed. It makes the suggested decarboxylation of 2-*O*-methylorsellinic acid coupled with a hydroxylation reaction, with a mechanism resembling the action of salicylate-1-monooxygenase,⁶ very likely. Thus the transformation of *O*-methylorcinol to 6-methoxy-2-methylbenzoquinone(1,4) would not be a part of the main route to penicillic acid.

The natural occurrence of 1,4-dihydroxy-6-methoxy-2-methylbenzene was demonstrated in a short term experiment by feeding the organism with [¹⁴CH₃] methionine. After 40 min of incubation 20 % of the labelling from methionine was found to be incorporated into the hydroquinone derivative.

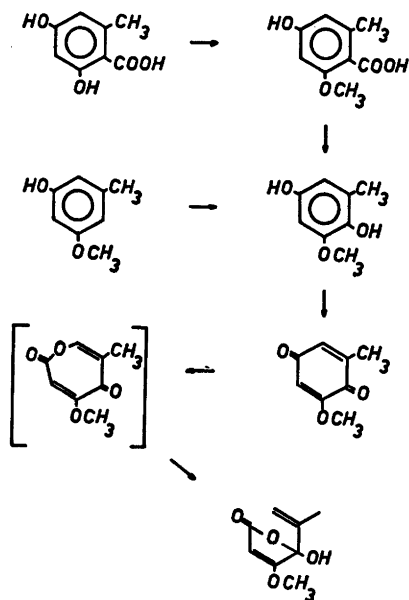


Fig. 1. Metabolic pathway of penicillic acid formation.

The Baeyer-Villiger type of oxidation in the ring opening reaction in the last step of penicillic acid biosynthesis has been confirmed in a cell free system. The enzymic reaction requires besides oxygen also NADPH indicating a mono-oxygenase system. The reaction is stimulated by the presence of FMN and Fe³⁺.

A report on the isolation of the enzyme and on its properties will be published separately.

EXPERIMENTAL

Culture conditions. *Penicillium cyclopium* NRRL 1888 was grown in 500 ml conical flasks on a shake table (2.5 cm stroke, 240 rpm) at 28 °C. Each flask contained 150 ml of Raulin-Thom medium supplemented with diammonium tartrate as described by Bentley *et al.*⁴

Preparation of radioactive precursors

O-[¹⁴C]Methylorcinol. This substance was synthesized by refluxing 1.0 mmol of anhydrous orcinol with 1.0 mmol of ¹⁴CH₃I and 1.5 mmol K₂CO₃ in anhydrous acetone for 8 h. After acidification with diluted H₂SO₄ the mono-methyl ether of orcinol was separated from the mixture by steam distillation. As some unreacted orcinol followed the methyl ether in the distillation the methyl ether was further purified by fractionation in a counter current distribution apparatus using diethyl ether and 0.5 M Na₂CO₃ respectively as phases. Yield of *O*-[¹⁴C]methylorcinol 29 %, m.p. 133–134 °C.

2-*O*-[¹⁴C]Methylorsellinic acid. This compound was prepared in two different ways.

Method A. Ethylorsellinate synthesized according to Gaucher and Shepherd⁷ was treated with an equivalent amount of ethyl chloroformate in aqueous NaOH. The radioactive isotope was introduced by reacting the obtained carbomethoxy derivative with ¹⁴CH₃I as described above. The 2-*O*-[¹⁴C]methylorsellinic acid was set free by treatment of the ethyl ester of the

carbomethoxy derivative as described by Fischer and Hoesch.⁸ Radioactive yield 41 %, m.p. 175–177 °C (dec.).

Method B. 4-Carbomethoxyrcylaldehyde was methylated by treatment with $^{14}\text{CH}_3\text{I}$ as described above. After oxidation of the aldehyde with aqueous KMnO_4 , the carbomethoxy group was removed from the acid as described in method A. Radioactive yield 27 %, m.p. 175–177 °C (dec.).

6- ^{14}C Methoxy-2-methylbenzoquinone (1,4) and 1,4-dihydroxy-6- ^{14}C methoxy-2-methylbenzene. The phenolic group of 2-nitro-3-hydroxytoluene was methylated with $^{14}\text{CH}_3\text{I}$ as described above. Reduction of the nitro group with hydrogen at atmospheric pressure and Raney-Ni as catalyst yielded quantitatively the amine. Oxidation of the amine with Fremy's salt as described by Teuber and Hasselbach⁹ gave radioactive 6-methoxy-2-methylbenzoquinone(1,4), radioactive yield 52 %, m.p. 155–156 °C. The corresponding hydroquinone was obtained by reduction of the quinone with $\text{Na}_2\text{S}_2\text{O}_4$.

3-Hydroxy-6- ^{14}C methoxy-2-methylbenzoquinone and 2,5,6-trihydroxy-3- ^{14}C methoxytoluene. The benzoquinone derivative was prepared from 6- ^{14}C methoxy-2-methylbenzoquinone(1,4) via the triacetate as described by Anslow and Raistrick.¹⁰ Yield 48 %. 2,4,6-Trihydroxy-3- ^{14}C methoxytoluene was synthesized by reduction of the quinone with $\text{Na}_2\text{S}_2\text{O}_4$.

^{14}C -Incorporation experiments

In the feeding experiments generally 1–4 mg of the synthesized radioactive precursors dissolved in a small volume of ethanol were added to the organism grown for 72 h. After another 24 h of incubation penicillic acid was extracted with ethyl acetate from the acidified culture medium. The ethyl acetate solution was washed with water, dried over anhydrous Na_2SO_4 and evaporated to dryness. After redissolving the residue in a small volume of ethyl acetate penicillic acid crystallized on addition of hot light petroleum.

The radioactive penicillic acid was recrystallized to constant specific radioactivity. When O- ^{14}C methylrcinol and 6- ^{14}C methoxy-2-methylbenzoquinone(1,4) were used as labelled precursors the radioactivity in the penicillic acid was localized to the methoxy group by splitting the molecule with phenylhydrazine and distilling off the formed methanol. The methanol was purified with preparative gas chromatography.

In one experiment a 72 h old culture was exposed for 40 min to 1.25 μCi (0.37 mg) of $^{14}\text{CH}_3$ methionine. The culture filtrate was acidified and extracted with ethyl acetate. The ethyl acetate solution was washed with aqueous NaHCO_3 and water. Thin layer chromatography on silica gel (Kieselgel, G. Merck) of an aliquot of the organic phase demonstrated

the presence of 1,4-dihydroxy-6-methoxy-2-methylbenzene (R_F 0.23). As solvent was used the organic phase of the mixture chloroform-methanol-water-formic acid (250:24:25:1, by vol.)

To the remaining ethyl acetate solution was added after air-oxidation 15 mg of non-labelled 6-methoxy-2-methylbenzoquinone(1,4). The evaporated residue was repeatedly crystallized to constant specific radioactivity from diethyl ether-light petroleum.

Of the added methionine 20 % was incorporated into the hydroquinone derivative.

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