Acta Chemica Scandinavica B 31 (1977) 391—394

Intermediates in the Barnol Biosynthesis in *Penicillium baarnense*

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By using appropriate ^{14}C-labelled phenolic substances as precursors in feeding experiments the following steps in barnol biosynthesis have been established:
acetate + malonate → 2,4-dihydroxy-6-ethyl-5-methylbenzaldehyde → 1,3-dihydroxy-4,6-dimethyl-2-ethylbenzene → barnol
P. baarnense can also reduce orcylaldehyde and 2,4-dihydroxy-5,6-dimethylbenzaldehyde to dimethylresorcinol and trimethylresorcinol, respectively.

Barnol (*9*) is the main secondary product when *Penicillium baarnense* is grown on Raulin-Thom medium whereas orsellinic acid and penicillic acid are the major metabolites when Czapek-Dox medium is used as substrate.

In order to investigate this phenomenon we found it important to know the detailed pathways of the formation of penicillic acid and barnol, respectively. We have recently been able to show how penicillic acid is formed in *Penicillium cyclopium*.[3] Some of the intermediates demonstrated in *P. cyclopium* have been found in *P. baarnense*.[4] One of them, namely 1,4-dihydroxy-2-methoxy-6-methylbenzene was biosynthesized from ^{14}C-labelled acetate and its conversion to penicillic acid in *P. baarnense* was confirmed.

Mosbach and Ljungkrantz [1,2] performed some radioactive feeding experiments that demonstrated the polyketide character of barnol and revealed interesting features of the origin of the three alkyl groups. One of the methyl groups attached to the aromatic nucleus is derived from the C-1 metabolism and is presumably inserted as *S*-adenosylmethionine. The other nuclear methyl group is the result of reduction of a former malonate carboxyl group. Finally, the ethyl group is formed by a *C*-methylation of an original acetate methyl group.

The potentiality of the organism to form orsellinic acid as well as barnol, but on different media, may indicate a biogenetic relationship between the two compounds. Theoretically, orsellinic acid could be a precursor of barnol or alternatively the primary polyketide chain [5] could be identical in the two cases. The former possibility can be tested by feeding ^{14}C-labelled orsellinic acid to the organism. When doing so no radioactivity is incorporated into barnol. Being well aware of the weakness of drawing conclusions of negative incorporation experiments we prefer to point to the successful feeding experiments that will be described for excluding orsellinic acid as a precursor of barnol.

Several other investigations indicate that biological *C*-methylation of an aromatic structure is a rare phenomenon and that the methyl group is preferably introduced at a prearomatic level. 5-Methylorsellinic acid [5] is then a possible precursor of barnol if the nuclear methyl group is substituted onto the open polyketomethylene chain, prior to cyclization and if the ethyl group is subsequently formed by a methylation reaction after cyclization. ^{14}C-Carboxyl labelled 5-methylorsellinic acid was synthesized and fed to the organism but no incorporation was obtained into barnol or any other phenolic compound. However, when ^{14}C-formyl labelled 5-methylorcylaldehyde was used as substrate two radioactive phenolic metabolites were isolated. By recrystallizations to constant specific radioactivity with authentic compounds

Table 1. Incorporation of ^{14}C-labelled precursors.

Radioactive substrate			
Compound	Spec. radioactivity dpm/mmol × 10^{-7}	Product	Incorporation of radioactivity %
Orcylaldehyde (*1*)	12.7	4,5-dimethylresorcinol (*2*)	6.1
2,4-Dihydroxy-5,6-dimethylbenzaldehyde (*3*)	1.3 1.3	4,5,6-trimethylresorcinol (*4*) 4,5,6-trimethylpyrogallol (*7*)	7.8 4.4
2,4-Dihydroxy-5,6-dimethylbenzoic acid (*5*)	4.7	4,5,6-trimethylpyrogallol (*7*)	0
4,5,6-Trimethylresorcinol (*4*)	5.0 5.0 [a]	4,5,6-trimethylpyrogallol (*7*) 4,5,6-trimethylpyrogallol (*7*)	0 10 [b]
2,4-Dihydroxy-6-ethyl-5-methylbenzaldehyde (*8*)	18.4	barnol (*9*)	24.0

[a] Cell-free experiment. [b] Value from radioscanning of TLC.

these phenols were identified as 4,5,6-trimethylresorcinol (*4*) and 4,5,6-trimethylpyrogallol (*7*), respectively. In the same way ^{14}C-formyl labelled orcylaldehyde gave rise to labelled 4,5-dimethylresorcinol (*2*). The results indicate the following. The methionine derived nuclear methyl group is introduced into the structure before the polyketide chain is ring-closed. The aldehyde group is reduced to a methyl group. The reduction of the original carboxyl group may thus pass the aldehyde level. The failure of metabolizing exogenous 5-methylorsellinic acid to trimethyl resorcinol or trimethylpyrogallol may depend on the inability of the organism to activate the aromatic carboxyl group with the possible formation of a thiol ester which may be the site for the initial reduction. The methylation of the acetate methyl group with the formation of the ethyl group of barnol apparently also takes place prior to cyclization of the open polyketide chain, as judged from the formation of the lower homolog and not barnol when 2,4-dihydroxy-5,6-dimethylbenzaldehyde (*3*) is given to the organism. Furthermore, when the organism is fed with ^{14}C-formyl labelled 2,4-dihydroxy-6-ethyl-5-methylbenzaldehyde (*6*) an appreciable conversion of the aldehyde into barnol is observed. Finally, the experiments indicate that in the formations of barnol and trimethylpyrogallol the methylation reactions and the reduction of the carboxyl group precede the hydroxylation of the aromatic structure. In an attempt to show the hydroxylation in a single step reaction ^{14}C-labelled 4,5,6-trimethylresorcinol was fed to the organism but no radioactive 4,5,6-trimethylpyrogallol could be isolated. To avoid permeability problems a corresponding experiment was performed with a homogenate of the cells whereby a significant formation of the pyrogallol derivative was obtained giving support to the suggestion that hydroxylation is the last step in the formation of barnol. The results from the various feeding experiments are shown in Table 1. Fig. 1 summarizes the reactions performed by the organism and Fig. 2 proposes the metabolic sequence of barnol formation as supported by the experimental evidences.

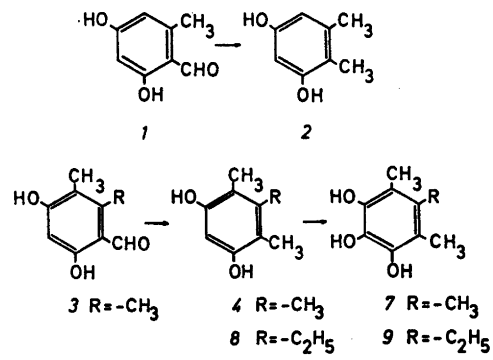

Fig. 1. Chemical transformations performed by *P. baarnense.*

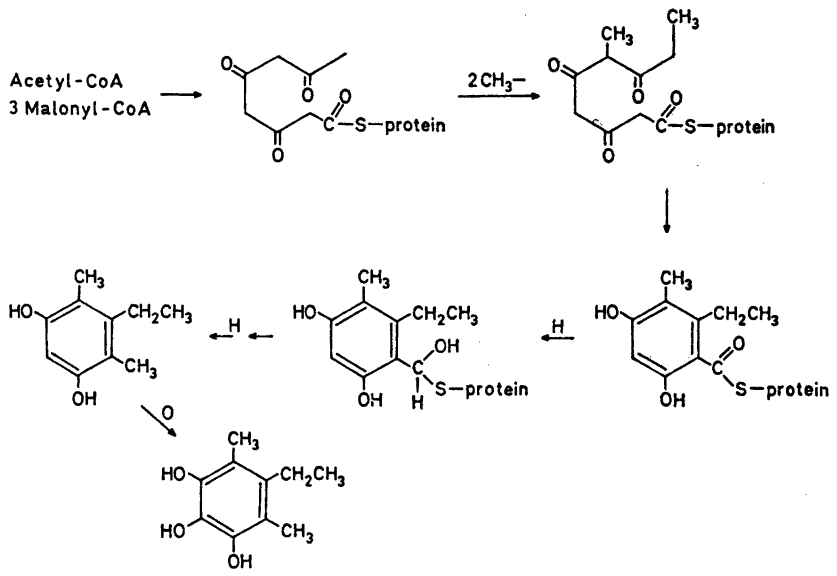

Fig. 2. Pathway suggested for the biosynthesis of barnol.

EXPERIMENTAL

Culture conditions. Penicillium baarnense v. Beyma, CBS 315.59 was grown in 500 ml conical flasks on a rotary shaker (2.5 cm stroke, 240 rpm) at 28 °C. Each flask contained 150 ml of Raulin-Thom medium with the following composition: $(NH_4)_2HPO_4$, 0.6 g; $(NH_4)_2SO_4$, 0.25 g; K_2CO_3, 0.6; $MgCO_3$, 0.4 g; $FeSO_4 . 7H_2O$, 0.07 g; $ZnSO_4 . 7H_2O$, 0.07 g; tartaric acid, 4.0 g; ammonium tartrate, 4.0 g; glucose, 75 g; yeast extract, 1.5 g; distilled water, 1500 ml.

Preparation of radioactive precursors

Orcylaldehyde (1). The aldehyde was prepared as described by Adams and Levine.[5] M.p. 184 °C (lit.[5] 178–180 °C), yield 85 %. Orcylaldehyde-^{14}C-formyl (12.7×10^7 dpm/mmol) was obtained by adding $Na^{14}CN$ (0.5 mCi) to the reacting $Zn(CN)_2$.

4,5-Dimethylresorcinol (2). Clemmensen reduction of *1* yielded *2* which was purified by recrystallization from water, m.p. 137–138 °C (lit.[6] 137–138 °C), yield 90 %.

2,4-Dihydroxy-5,6-dimethylbenzaldehyde (3). A Gattermann reaction on *2* gave the formyl derivative. The ^{14}C-labelled product was obtained by adding $Na^{14}CN$ (0.5 mCi) to the reaction mixture, m.p. 199–201 °C (lit.[7] 196–197 °C), yield 90 %, specific radioactivity 1.3×10^7 dpm/mmol.

4,5,6-Trimethylresorcinol (4). Compound *3* was reduced in a Clemmensen reaction. The product was extracted into ether, washed with aqueous $NaHCO_3$, water, and finally filtered through Florisil. Yield 85 %, m.p. 165–166 °C (from cyclohexane) (lit.[8] 163–165 °C). The ^{14}C-labelled substance was prepared in a similar manner.

2,4-Dihydroxy-5,6-dimethylbenzoic acid (^{14}COOH) *(5).* Acetylation of *3* (yield 90 %) and subsequent $KMnO_4$-oxidation (yield 65 %) gave the acetylated *5* m.p. 138–141 °C. Hydrolysis of the acetylated compound in 10 % aqueous KOH gave *5*.

2,6-Dihydroxy-3,4,5-trimethylbenzaldehyde (6). A Gattermann reaction was performed with $Zn(CN)_2$ and *4*. Compound *6* was purified by filtration of an ether solution through Florisil and subsequent sublimation at 130 °C/0.1 mmHg, m.p. 138–140 °C (lit.[8] 136 °C), yield 54 %.

4,5,6-Trimethylpyrogallol. (7). Compound *7* was obtained in a Dakin oxidation of *6* by adding 125 ml 3 % H_2O_2 for one hour to 180 mg (1 mmol) *6*, 0.36 ml (1 mmol) $[(CH_3)_4N]OH$ and 2 ml 50 % aqueous ethanol. The temperature was kept at 40–50 °C and the reaction performed under nitrogen. After 3 h 15 ml saturated aqueous $(NH_4)_2SO_4$ was added and *7* repeatedly extracted with ether. The extract was washed in order with aqueous $NaHCO_3$, $Na_2S_2O_4$, and water. The solid residue remaining after evaporation of the solvent was sublimed at 140 °C/1 mmHg and recrystallized from CCl_4. Yield 67 %, m.p. 166–169 °C (lit.[6] 165–166 °C). Anal. $C_9H_{12}O_3$: C, H.

2,4-Dihydroxy-6-ethyl-5-methylbenzaldehyde (8). 14.2 g 3,5-dimethoxyacetophenone was re-

duced in a Clemmensen reduction using a two phase solvent system (benzene-water). The product, a colorless oil (10.6 g, yield 81 %), was demethylated by refluxing with HI and red P in glacial acetic acid. The obtained 5-ethylresorcinol was formylated in a Gattermann reaction. The aldehyde was sublimed at 100 °C/0.1 mmHg and recrystallized from benzene, m.p. 99 – 100 °C (lit.[9] 99 – 100 °C), yield 98 %. 5-Ethyl-4-methylresorcinol was prepared in quantitative yield by a Clemmensen reduction of the aldehyde. ^{14}C-labelled *8* was synthesized as described for *1*. Purification of *8* was made by sublimation at 150 °C/0.1 mmHg and recrystallization from benzene, m.p. 158 – 159 °C (lit.[6] 157 – 158 °C), specific radioactivity 18.4×10^7 dpm/mmol.

^{14}C-Incorporation experiments

In the feeding experiments generally 2 – 9 mg of the synthesized radioactive precursors dissolved in a small volume of water were added to the organism grown for 3 to 4 days. After another day of incubation the phenolic products were extracted with ether from the acidified culture filtrate. The metabolic conversion of the added precursors and the nature of the formed radioactive phenolic compounds were checked by analyzing a small portion of the ether extract by TLC (benzene:dioxane:acetic acid (95:25:4), silica gel). After washing the ether solution with aqueous $NaHCO_3$ and water it was evaporated to dryness. The residue was usually sublimed before recrystallization to constant specific radioactivity with non-labelled carrier substance.

Acknowledgement. This work was supported by grants from the Swedish Natural Science Research Council.

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Received November 30, 1976.