

STUDY TO THE BIOSYNTHESIS OF  
THE RIFAMYCIN-CHROMOPHORE IN  
*NOCARDIA MEDITERRANEI*

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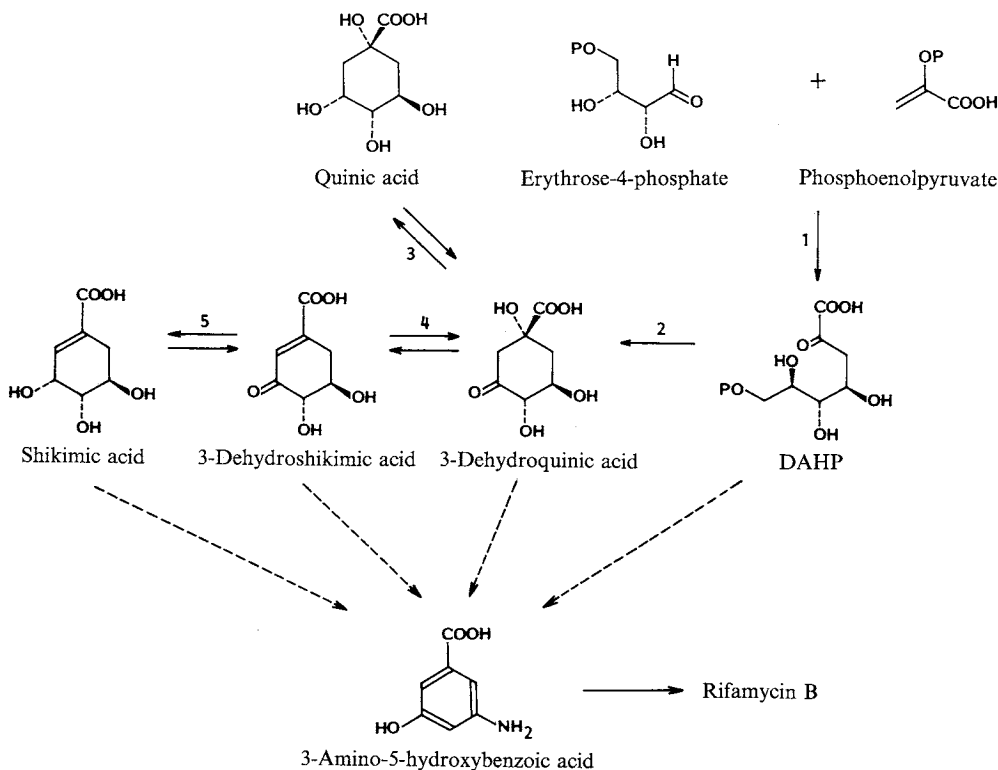
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*Nocardia mediterranei* produces the antibiotic rifamycin B, which belongs to the ansamycin group. The structure of rifamycin consists of the ansa chain and the chromophore. One part of this chromophore, the seven-carbon amino unit, which initiates ansa chain formation in ansamycins, has been identified as 3-amino-5-hydroxybenzoic acid. KIBBY *et al.*<sup>1)</sup> synthesized [carboxy-<sup>14</sup>C]3-amino-5-hydroxybenzoic acid and showed that this labeled compound was incorporated into the ansamycin actamycin. GHISALBA and NÜESCH<sup>2)</sup>, using a

different approach, came to the same result with rifamycin from *N. mediterranei*. By supplementing mutant A8 (transketolase<sup>-</sup>, synthesizes only 10% of the rifamycin B produced by the parent)<sup>3)</sup> with 3-amino-5-hydroxybenzoic acid, they found a strong stimulation of the rifamycin B production, which was comparable to the titer reached with the parent strain N813.

The seven-carbon amino unit itself is derived from an intermediate in the main sequence of the aromatic amino acid biosynthesis. By chemical and biochemical analysis of mutant A8 and mutant A10<sup>4)</sup> (shikimate accumulation, *aro*<sup>-</sup>, rifamycin<sup>+</sup>) it was demonstrated that the branch point is located between 3-deoxy-D-arabinoheptulosonic acid 7-phosphate (DAHP) and shikimic acid. Four pathways for the biosynthesis of 3-amino-5-hydroxybenzoic acid would therefore be possible (Fig. 1). HORNEMANN *et al.*<sup>5)</sup> proposed acyclic DAHP as the starting point for the mitomycins, and more recently CASATI *et al.*<sup>6)</sup> came to the same conclusion for ansatrienin. Mitomycin as well as ansatrienin originate from the same seven-carbon

Fig. 1. Possible pathway for the biosynthesis of the aromatic moiety of the rifamycin chromophore.



1: DAHP-Synthetase, 2: 3-dehydroquininate synthetase, 3: quinic acid dehydrogenase,  
4: 3-dehydroquininate dehydratase, 5: 3-dehydroshikimate reductase.

amino unit. The amination would then occur in position 4 of DAHP. KARLSSON *et al.*<sup>7)</sup> suggested that 3-dehydroquinic acid and 3-dehydroshikimic acid were the most probable precursors, with amination at position 3.

In this paper we describe the isolation and biochemical characterization of an *aro*<sup>-</sup> auxotrophic mutant B9 and the supplementation of mutant A8 with potential precursors.

The auxotrophic mutant *N. mediterranei* B9 described in this paper was first analyzed by auxanography on agar. The minimal medium was supplemented with the three aromatic amino acids (1%), each alone and in combination and with quinic acid (1%). The results indicate that mutant B9 has a requirement for the aromatic amino acids in combination. Growth was also observed on quinic acid. Supplementation studies in liquid minimal medium 150 (modified)<sup>8)</sup> did not confirm the results from auxanography. A slight growth of mutant B9 was also observed when no supplement was added to the culture. The supplements (the three aromatic amino acids in combination or quinic acid) increased growth.

The production of rifamycin B by strain B9 was slightly reduced in comparison with the parent in liquid complex medium 148<sup>3)</sup> (0.2 g/liter for B9 and 0.35 g/liter for parent N813). In industrial fermentation medium 151b<sup>2)</sup> the rifamycin production of the mutant was higher than with the parent (3.5 g/liter for B9 and 2 g/liter for the parent). Supplementation studies with mutant B9 in this medium with all the three aromatic amino acids in combination (2 g/liter each) or with quinic acid (2 g/liter) yielded an identical rifamycin B titer of 3.5 g/liter.

Each enzyme activity of the early shikimic acid pathway of the mutant B9 and of the parent N813 was tested in a parallel set of experiments and under the same conditions in crude extracts. The results are summarized in Table 1. The only enzyme which showed no activity was the 3-dehydroquinic acid synthetase of mutant B9. The first step was to optimize the 3-dehydroquinic acid synthetase reaction for the parent in order to be able to measure the low enzyme activities anticipated as the mutant actually does grow slowly in unsupplemented liquid minimal medium. The pH-optimum of the 3-dehydroquinic acid synthetase was found to be at 7.2. The enzyme precipitates between 20 and 40% of ammonium sulfate saturation. The reaction rate increases with the temperature, the optimal temperature being 37°C. Within a measured range from 0.7 to 5.5 mg protein (ammonium sulfate

Table 1. Enzyme activities of the early shikimic acid pathway of mutant B9 compared to parent N813.

Enzyme (strain)	Specific activity ( $\mu\text{mol}/\text{mg}\cdot\text{hour}$ )	
	B9	N813
DAHP-Synthetase <sup>8)</sup>	0.77	0.4
3-Dehydroquinic acid synthetase <sup>a</sup>	nm	0.04
3-Dehydroquinic acid dehydratase <sup>9)</sup>	0.06	0.15
3-Dehydroshikimate reductase <sup>9)</sup>	0.25	0.5

<sup>a</sup> Incubation mixture of 1 ml contains: Tris-HCl buffer 20  $\mu\text{mol}$  pH 7.2, KF 10  $\mu\text{mol}$ , CoCl<sub>2</sub> 2  $\mu\text{mol}$ , NAD 0.2  $\mu\text{mol}$ , DAHP 0.12  $\mu\text{mol}$ , enzyme 5 mg.  
nm: Not measurable.

precipitated crude extract) the initial velocity increased linearly from 0.01 to 0.1  $\mu\text{mol}/\text{ml}\cdot\text{hour}$  consumption of DAHP. The kinetic behavior of the 3-dehydroquinic acid synthetase was investigated by measuring the initial velocity of DAHP disappearance at various concentrations of DAHP (from 0.06 to 0.2 mM). Up to a substrate concentration of 0.12 mM the reaction followed regular Michaelis-Menten kinetics. Above this concentration substrate inhibition was observed.

The 3-dehydroquinic acid synthetase activity of mutant B9 was compared with 3-dehydroquinic acid synthetase activity of the parent. The consumption of DAHP over 1 hour increased with the parent N813 whereas with mutant B9 no consumption of DAHP could be detected.

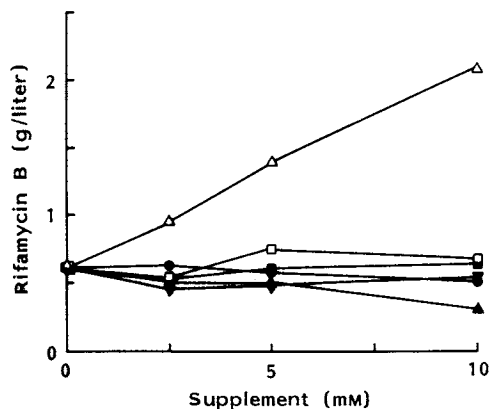
Supplementation experiments were carried out with mutant A8 to confirm the results obtained by the characterization of mutant B9. Mutant A8 which produces only small amounts of rifamycin B is very suitable for supplementation studies.

Each supplement was incubated over 10 days at three different concentrations (2.5, 5 and 10 mM). As it was already demonstrated by GHISALBA and NÜESCH<sup>2)</sup> increasing concentrations of 3-amino-5-hydroxybenzoic acid caused a linear stimulation of rifamycin B production. The amount of rifamycin B produced by strain A8 increased from 0.6 g/liter (unsupplemented) to 2.1 g/liter when supplemented with 10 mM 3-amino-5-hydroxybenzoic acid (Fig. 2).

Shikimic acid, quinic acid and their methyl esters (which are believed to penetrate easier into the cell) showed no stimulation of rifamycin B production. Qualitative analysis of culture filtrates by TLC showed that after the fermentation shikimic acid and shikimic acid methyl ester were still present, but quinic acid and quinic acid methyl ester could not be seen. These results indicate that the penetration

Fig. 2. Stimulation of rifamycin B production in mutant A8.

■ Shikimic acid, ● shikimic acid-methyl ester, ▼ quinic acid, □ quinic acid-methyl ester, ▲ aromatic amino acids, △ 3-amino-5-hydroxybenzoic acid.



of methylated derivatives of shikimic acid and quinic acid into the cell is not facilitated (Fig. 2).

The supplementation with all three aromatic amino acids in combination at 10 mM concentrations caused slight inhibition of rifamycin production (Fig. 2).

The characterization of the mutants A8<sup>3)</sup> and A10<sup>4)</sup> made it possible to limit the choice of potential precursors for the biosynthesis of 3-amino-5-hydroxybenzoic acid and rifamycin B to DAHP, 3-dehydroquinic acid, 3-dehydroshikimic acid and shikimic acid. In this paper two different approaches are described which lead to a more precise identification of the precursor.

Firstly, a mutant B9 was found which can be characterized as follows: Mutant B9 is *aro*<sup>-</sup> (slight growth on minimal medium), 3-dehydroquinase<sup>-</sup> and rifamycin<sup>+</sup>. 3-Dehydroquinase is the second enzyme in the shikimic acid pathway and catalyzes the cyclization of DAHP. Because of the defect in this enzyme DAHP would appear to be the branch point intermediate for the biosynthesis of the aromatic moiety of the rifamycin chromophore.

Secondly, the supplementation study carried out with mutant A8 showed that even quinic acid which is able to penetrate into the cell did not increase the production of rifamycin B. A stimulation of rifamycin B production would have been expected if the branch point is located at a cyclic intermediate.

The results from the two different approaches are in good agreement. Both indicate DAHP as the branch point intermediate. But it should be kept in mind that in both approaches DAHP was only indirectly established as precursor by the exclusion of all the other potential precursors.

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