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

DANIEL GYGAX, ORESTE GHISALBA,
HANSJÖRG TREICHLER AND JAKOB NÜSCH
Pharmaceuticals Division and Central Research Laboratories of CIBA-GEIGY Limited,
Basel, Switzerland

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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. synthesized [carboxy-14C]3-amino-5-hydroxybenzoic acid and showed that this labeled compound was incorporated into the ansamycin actamycin. Ghisalba and Nüsch, using a different approach, came to the same result with rifamycin from N. mediterranei. By supplementing mutant A8 (transketolase-, synthesizes only 10% of the rifamycin B produced by the parent) 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 (shikimate accumulation, aro^+, rifamycin^+) 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. proposed acyclic DAHP as the starting point for the mitomycins, and more recently Casati et al. 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-dehydroquinate synthetase, 3: quinic acid dehydrogenase, 4: 3-dehydroquinate dehydratase, 5: 3-dehydroshikimate reductase.
amino unit. The amination would then occur in position 4 of DAHP. Karlsson et al. 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 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 (modified) 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 (0.2 g/liter for B9 and 0.35 g/liter for parent N813). In industrial fermentation medium 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-dehydroquinate synthetase of mutant B9. The first step was to optimize the 3-dehydroquinate 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-dehydroquinate 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 precipitated crude extract) the initial velocity increased linearly from 0.01 to 0.1 µmol/ml·hour consumption of DAHP. The kinetic behavior of the 3-dehydroquinate 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.

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 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

<table>
<thead>
<tr>
<th>Enzyme (strain)</th>
<th>Specific activity (µmol/mg·hour)</th>
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<tbody>
<tr>
<td>DAHP-Synthetase</td>
<td>0.77 0.4</td>
</tr>
<tr>
<td>3-Dehydroquinic Synthetase</td>
<td>nm 0.04</td>
</tr>
<tr>
<td>3-Dehydroquinat dehydratase</td>
<td>0.06 0.15</td>
</tr>
<tr>
<td>3-Dehydroshikimate reductase</td>
<td>0.25 0.5</td>
</tr>
</tbody>
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* Incubation mixture of 1 ml contains: Tris-HCl buffer 20 µmol pH 7.2, KF 10 µmol, CoCl₂ 2 µmol, NAD 0.2 µmol, DAHP 0.12 µmol, enzyme 5 mg. nm: Not measurable.
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.

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.

References