A GENETIC APPROACH TO THE BIOSYNTHESIS OF THE RIFAMYCIN-CHROMOPHORE IN *NOCARDIA MEDITERRANEI*

II. ISOLATION AND CHARACTERIZATION OF A SHIKIMATE EXCRETING AUXOTROPHIC MUTANT OF *NOCARDIA MEDITERRANEI* WITH NORMAL RIFAMYCIN-PRODUCTION

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The mutant under study, designated A10, is derived from a *Nocardia mediterranei* strain, N813, which is a high rifamycin B producer. A10 is auxotrophic for aromatic amino acids but unlike A8 (see preceding paper)[1] produces the same amount of rifamycin B as the parent. Shikimic acid and 3-dehydroshikimic acid are accumulated in the fermentation broth of this mutant. It was shown to be blocked in one of the enzymes leading from shikimate to chorismate. No formation of shikimate-3-phosphate from shikimate and ATP could be detected *in vitro* using crude extracts of this mutant and of the parent. As mutant A10 is only defective in the biosynthesis of aromatic amino acids and not in the biosynthesis of rifamycins it would appear that the seven-carbon amino unit of the rifamycin-chromophore must be derived from an intermediate of the shikimate pathway not behind shikimate. By referring to the results of the preceding paper[1] it can be seen that the origin of this moiety can definitely be localized between 3-deoxy-D-arabinoheptulosonic acid-7-phosphate and shikimate.

Karlsson *et al.*[2] suggested that 3-dehydroquinate or 3-dehydroshikimate were the most probable precursors for the seven-carbon amino unit of the rifamycin-chromophore because both these intermediates of the shikimate pathway bear carbonyl functions in the correct position to give rise to an amino group in *meta* position to the carboxyl function on transamination (Fig. 1).

All the other aromatic amino compounds deriving from the shikimate pathway at the branch point –chorismate– have amino groups in the *ortho* or *para* position to the carboxyl function (*p*-aminobenzoic acid, anthranilic acid, *p*-aminophenylalanine).

White *et al.*[3] pointed out that if this suggestion is true it should be possible to isolate rifamycins

Fig. 1. Orientation of possible precursors of the seven-carbon amino unit of rifamycin with the carbonyl function in the right position for a direct transamination.

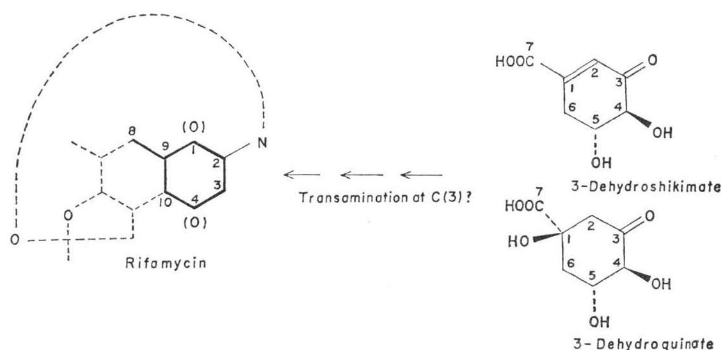
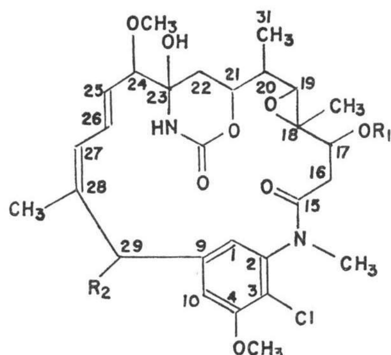
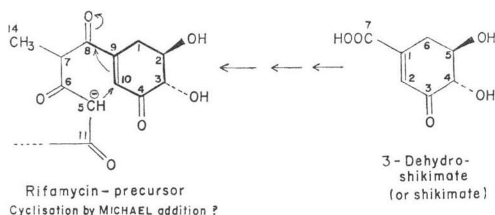


Fig. 2. Some ansamycins of the maytansine group (numbering of the ansa chain and of the aromatic nucleus in analogy to the numbering of rifamycins).



	R_1	R_2
Maytansine	$CO \cdot CHCH_3 \cdot NCH_3 \cdot COCH_3$	H
Maytanprine	$CO \cdot CHCH_3 \cdot NCH_3 \cdot COCH_2CH_3$	H
Maytanbutine	$CO \cdot CHCH_3 \cdot NCH_3 \cdot COCH(CH_3)_2$	H
Maytanacine	$COCH_3$	H
Maytansinol	H	H
Colubrinol	$CO \cdot CHCH_3 \cdot NCH_3 \cdot COCH(CH_3)_2$	OH
Colubrinol-acetate	$CO \cdot CHCH_3 \cdot NCH_3 \cdot COCH(CH_3)_2$	$OCOCH_3$

Fig. 3. Orientation of possible precursors of the seven-carbon amino unit of rifamycin as is indicated by the enrichment factors in ^{13}C incorporation studies.



lacking oxygen at C(1). Such variants are predicted to be biologically inactive. Up to now no such rifamycins have been isolated but the ansamycins of the maytansine group (Fig. 2) lack oxygen at the corresponding position. Unfortunately this proves nothing as maytansinoids are ansamycins isolated from higher plants and not from microorganisms. No biosynthetic data on maytansine are available and therefore it is not known whether the biosynthesis of these compounds is identical with that of rifamycin or geldanamycin.

In a later publication WHITE and MARTINELLI[4] proposed another orientation of the seven-carbon amino unit, placing the double bond of 3-dehydroshikimate and shikimate between C(9) and C(10) of rifamycin with C(1) of rifamycin deriving from C(6) of shikimate. This orientation was proposed analyzing the enrichment factors from incorporation of (1-^{13}C)glucose and (1-^{13}C)glycerate indicating that C(1) of rifamycin derives from the methylene carbon and C(8) of rifamycin derives from the carboxyl group of phosphoenolpyruvate. This orientation of the seven-carbon amino unit would favour a ring closure between C(5) and C(10) by MICHAEL addition, giving rise to the naphtoquinone chromophore of rifamycins in a manner analogous to the menaquinones[5] (Fig. 3).

With this orientation of 3-dehydroshikimate (3-dehydroquinate, shikimate) the carbonyl function is no longer in the right position for a direct transamination. Therefore the introduction of an amino group at C(2) of rifamycin would be more complex than suggested by KARLSSON *et al.*[2].

In this paper we describe the isolation and biochemical characterization of an aro-auxotrophic mutant A10 of *N. mediterranei* providing genetic evidence that the branch point of the biosynthesis of the seven-carbon amino unit of the rifamycin-chromophore cannot be behind the level of shikimate.

Material and Methods

The organisms and the methods of cultivation, isolation of auxotrophic mutants, auxanography, supplementation studies and photometric determination of rifamycin B have been described or cited

in the preceding paper[1].

TLC-Methods

All TLC assays were carried out on pre-coated TLC plates silica gel 60 F-254 (Merck) using 20~30 μl test samples.

Solvent system 1: *n*-butanol - acetic acid - water (80: 20: 20)

Spray reagents: Ammoniummolybdate-perchloric acid, 2,4-dinitrophenylhydrazine, alkaline silver nitrate and *o*-phenylenediamine spray reagents were prepared according to STAHL[6].

Periodic acid/benzidine[7,8]: first spray with 0.1% HIO_4 and after 10 minutes spray again with a solution of 0.37 g benzidine in 20 ml 50% ethanol, 4 ml acetone and 2 ml 0.2 N HCl.

Periodate/aniline[9]: first spray with 0.16 g $NaIO_4$ in 25 ml 1 M acetate buffer pH 4.7 and after 15 minutes spray again with a solution of 3% aniline in ethanol (solutions freshly prepared).

Periodate/thiobarbituric acid[10,11]: first spray with 0.02 M $NaIO_4$, after 15 minutes spray with ethyleneglycol - acetone - 95% H_2SO_4 (50: 50: 0.3) and after 10 minutes spray again with 1.2 g thiobarbituric acid in 8.3 ml 1 N NaOH and 11.7 ml water and develop 5 minutes at 100°C.

3-Dehydroshikimate standard for TLC was prepared as follows[12]: 20 mg 3-dehydroquinic acid are dissolved in 2 ml 0.1 N HCl and boiled for one hour in a water-bath. The resulting solution is used as a TLC standard and contains 3-dehydroquinic acid, 3-dehydroshikimic acid, protocatechuic acid and a fourth unidentified compound (gallic acid?).

Preparation of shikimate-3-phosphate standard for TLC (and for enzyme test): A mixture of 1 ml 0.75 M tris-maleate buffer pH 6.5, 2 ml 0.15 M ATP (neutralized), 2 ml 0.15 M $MgSO_4$, 1 ml 0.1 M KF, 2 ml 0.05 M shikimic acid (neutralized), 1 ml water and 1 ml crude extract of *E. coli* is incubated at 28°C. After 24 hours 90% of the shikimate was transformed into shikimate-3-phosphate. The protein was denatured by boiling for 2 minutes in a water bath and separated by centrifugation. The resulting solution is stable for months if stored at −30°C and can be used directly for TLC or enzyme assays.

Colour Reactions for Shikimic Acid

Two different photometric methods were used to determine shikimic acid in culture filtrates or eluates: The method of YOSHIDA and HASEGAWA[9] with periodate and aniline is specific for shikimic acid. The MILLICAN method[10] with periodate and thiobarbituric acid is not specific for shikimic acid but for polyhydroxy compounds such as sugars and cyclitols. The MILLICAN method was carried out in two ways:

Standard procedure: periodate treatment for 5 minutes at 80°C and thiobarbiturate treatment for 15 minutes at 100°C.

Modified procedure: periodate treatment for 60 minutes at 40°C and thiobarbiturate treatment for 2 minutes at 100°C.

Enzyme Tests

The enzymes were all tested at 28°C in crude extracts of *Nocardia mediterranei* N813 and A10. The crude extracts were prepared as described in the previous paper[1] where the methods for the qualitative assays of pentose shunt enzymes are also mentioned. The enzymes of the phenylalanine/tyrosine branch were tested using the following methods: chorismate mutase, prephenate dehydrogenase and prephenate dehydratase by the methods of COTTON and GIBSON[13], phenylalanine aminotransferase by the method of FUJIOKA *et al.*[14] but using the colour reaction for phenylpyruvate with NaOH[13] and tyrosine aminotransferase by the method of GRANNER and TOMKINS[15] and DIAMONDSTONE[16]. The enzymes 3-deoxy-D-arabinoheptulosonic acid 7-phosphate synthetase (DAHP-synthetase), 3-dehydroquinate synthetase, 3-dehydroquinate dehydratase, 3-dehydroshikimate reductase and shikimate kinase of the basic branch of the shikimate pathway were all assayed with the methods used by GOLLUB *et al.* for *Salmonella*[17]. The DAHP needed for the 3-dehydroquinate synthetase assay was prepared as follows: A mixture of 5 ml 0.005 M erythrose-4-phosphate, 2.5 ml 0.01 M phosphoenolpyruvate, 1.7 ml 0.6 M tris-maleate buffer pH 6.5, 0.4 ml water and 0.4 ml crude extract A10 was incubated at 28°C. The reaction equilibrium is reached after 2 hours and 40% of the theoretical amount of DAHP are formed. The protein is denatured by boiling for 2 minutes in a water bath and separated by centrifugation. The resulting solution is approx. 0.001 M DAHP and is stable for months if stored at −30°C.

Qualitative test for 5-enolpyruvyl shikimate-3-phosphate synthetase (EPSAP-synthetase): 0.1 ml 0.6 M tris-maleate buffer pH 6.5, 0.5 ml 0.01 M shikimate-3-phosphate (preparation see under TLC methods), 0.1 ml 0.065 M phosphoenolpyruvate, 0.1 ml water and 0.2 ml crude extract N813 or A10 were incubated at 28°C. After 7 hours and 24 hours of incubation TLC tests on silica gel plates in solvent system 1 were carried out using periodate/benzidine, 2,4-dinitrophenylhydrazine/NaOH and *o*-phenylenediamine spray reagents. The formation of a new product (Rf 0.37) from shikimate-3-phosphate and phosphoenolpyruvate can be observed.

The quinic dehydrogenase activity was assayed by a modification of the method by MITSUHASHI and DAVIS[18,19]. The addition of KCN proposed in the original method was omitted because CN^- reacts with NAD^+ and the addition product absorbs at the same wave-length as NADH[20].

Sources of Chemicals

Quinic acid, shikimic acid, and Dowex ion-exchangers: Fluka Chemicals

Chorismic acid (barium salt), and prephenic acid (barium salt): Sigma Chemicals

D-Erythrose-4P (dimethylacetal, dicyclohexylammonium salt, monohydrate) and phosphoenol pyruvic acid (sodium salt): Calbiochem Chemicals

3-Dehydroquinic acid was prepared according to the method of GREWE and JESCHKE[21].

Results

Growth and Production of Rifamycin B

The auxotrophic mutant *Nocardia mediterranei* A10 described in this paper was first analyzed by auxanography and found to grow only on a combination of phe, tyr and trp. No growth was observed on double aro-supplements or quinic acid, or 3-dehydroquinic acid or shikimic acid. Supplementation studies in liquid minimal medium 150 showed identical results. An addition of the aromatic vitamins nicotinamide, *p*-hydroxybenzoic acid and *p*-aminobenzoic acid to the supplement of the three aromatic amino acids does slightly inhibit the growth and the production of rifamycin B of mutant A10 in comparison with a supplement of aromatic amino acids alone.

N. mediterranei A10 shows a somewhat lower maximal growth than the parent in liquid complex medium 148 and in liquid minimal medium 150. The production of rifamycin B by strain A10 is almost identical with that of the parent in both media. In the industrial production medium it yields 80%

Table 1. Growth and production of rifamycin B
Comparison of fermentations of strains N813 and A10 in 200 ml shake flasks with 40 ml medium.

Strain	Nutritional requirements	Results of fermentation with two media (inoculum=0.5 g/liter of mycelial dry weight)	
		Liquid minimal medium 150 supplemented with 1‰ phe, 1‰ tyr and 1‰ trp	Liquid complex medium 148
A10	phe, tyr, trp	0.55 g/liter rifamycin B after 10 days 5 g/liter dry weight after 10 days	0.3 g/liter rifamycin B after 8 days 6 g/liter dry weight after 5 days
N813	none	0.65 g/liter rifamycin B after 10 days 6 g/liter dry weight after 10 days	0.35 g/liter rifamycin B after 8 days 7.5 g/liter dry weight after 5 days

In medium 148 the maximal dry weight of the culture is reached after 5 days of fermentation. In medium 150 the dry weight is still increasing after 10 days.

less than the parent. The as yet unknown direct precursor of the seven-carbon amino unit is partially withdrawn from the pool owing to the excretion of shikimic acid. This could be the reason for the much reduced rifamycin B production in industrial production medium.

Identification of Intermediates Accumulated in the Fermentation Broth of Mutant A10

TLC investigations on silica-gel plates in solvent system 1 were carried out with the filtrates of cultures of *N. mediterranei* N813 and A10 grown in liquid complex medium 148. The culture filtrates were concentrated to one tenth of their original volume under reduced pressure at 50°C and both the concentrates and the original culture filtrates were tested by TLC. A spot with Rf 0.55 was detected on the TLC of A10 culture filtrate with ammonium molybdate-perchloric acid and periodic acid/benzidine spray reagents which was not present on the TLC of N813 culture filtrate. The shikimate standard in this system shows the same Rf and the same colour reaction with periodic acid/benzidine (the white spot of shikimate on a dark-blue background becomes characteristically dark-brown after a few minutes). With ammonium molybdate-perchloric acid, 2,4-dinitrophenylhydrazine, alkaline silver nitrate and periodic acid/benzidine spray reagents a second spot with Rf 0.37 is detectable on the TLC of A10 culture filtrate, showing the same properties as the 3-dehydroshikimate standard.

To confirm these findings further tests with spray reagents more specific for shikimic acid were carried out. With periodate/aniline spray reagent (specific for shikimate) shikimate standard and the corresponding spot from A10 produced an intensive red spot on an orange background (the spot fades very soon and becomes brown).

With periodate/thiobarbituric acid spray reagent shikimate standard and the corresponding spot from A10 produced a blue-green spot on a colourless background (the spot fades after a certain time). The spot with Rf 0.37 (3-dehydroshikimate) from A10 is not visible with these two spray reagents because of interference with glucose on the TLC. The spot in the culture filtrate of mutant A10 with Rf 0.55 is clearly identified as shikimic acid with these specific reactions. No shikimate-3-phosphate (Rf 0.15) could be detected in the culture filtrate of A10.

Adsorption studies with several ion-exchange resins confirmed our finding that the accumulation products of mutant A10 must be acidic compounds.

Attempts to measure shikimate directly in the culture filtrate were not very successful. When using the MILLICAN method with periodate and thiobarbituric acid, glucose and other polyhydroxy-compounds in the culture filtrate interfere with the colour reaction of shikimate (maxima of absorption for glucose 518 nm and for shikimate 534 nm with a shoulder at 500 nm). Using the standard procedure a maximum at 532 nm is observed with both culture filtrates A10 and N813. However, the shoulder at 500 nm is observed only with culture filtrate A10 but not with N813. Using the modified procedure for the development of the colour reaction a maximum at 660 nm occurs with shikimate standard and with A10 culture filtrate but not with N813. The test can be improved if A10 culture filtrate is first treated with Dowex 50W $\times$ 8 (20 ~ 50 mesh, H^+) to remove the cations and then passed over a column with Dowex 2 $\times$ 8 (100 ~ 200 mesh, acetate) to remove the sugars. The test is then carried out with only the acidic compounds of the culture filtrate eluted from the Dowex 2 column with 0.1 N HCl and concentrated under reduced pressure at 50°C. The MILLICAN colour reaction with this concentrated eluate of A10 produced a spectrum in good accordance with the spectrum of authentic shikimic acid: maxima at 535 nm and 452 nm and a strong shoulder at 500 nm in the standard procedure. The control with

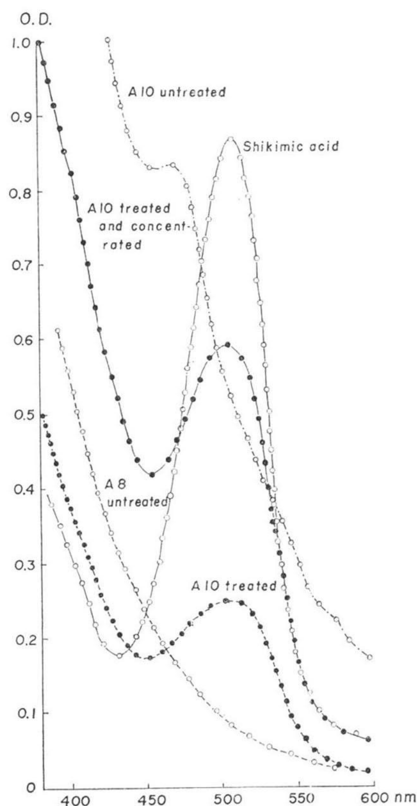
an identical preparation from culture filtrate N813 showed only half of the extinction of A10 at 535 nm and no shoulder at 500 nm.

The concentrated acidic eluate containing the acidic compounds of A10 culture filtrate was neutralized with NaOH and investigated by TLC on silica-gel plates in solvent system 1 using periodic acid/benzidine as the spray reagent. The compounds shikimic acid, 3-dehydroshikimic acid, protocatechuic acid (Rf 0.95) and a compound with Rf 0.87 (gallic acid?) could clearly be identified. They were not present in the control preparation with N813 culture filtrate. Protocatechuic acid and gallic acid (?) are artefacts produced by acidic hydrolysis of 3-dehydroshikimic acid during the concentration step.

A direct measurement of shikimic acid in the culture filtrate is impossible too with the colour reaction of YOSHIDA and HASEGAWA using periodate and aniline which is specific for shikimic acid. In this case not the glucose but the large amount of rifamycin B contained in the A10 culture filtrate interferes with the colour reaction (maxima for shikimic acid 510 nm and for rifamycin B 425 nm). The rifamycin B can be removed from the culture filtrate by treatment with Dowex 50W×8 (20~50 mesh, H^+): 25 ml A10 culture filtrate are stirred at room temperature with 10 g Dowex 50W for 30 minutes. The colour reaction is then carried out directly with the filtrate from this treatment and the characteristic maximum for shikimic acid at 510 nm is observed with mutant A10. Controls with liquid complex medium 148 and with culture filtrate of mutant A8 (containing only very low amounts of rifamycin B) do not show this characteristic peak (Fig. 4).

Fig. 4. Detection of shikimic acid with the periodate/aniline colour reaction.

Tests carried out using 40 μl 0.05 M shikimic acid, 1 ml culture filtrate of mutant A8 untreated, 1 ml culture filtrate of mutant A10 untreated, 1 ml culture filtrate A10 after treatment with Dowex 50 W and 1 ml culture filtrate A10 after treatment with Dowex 50 W, 3× concentrated.

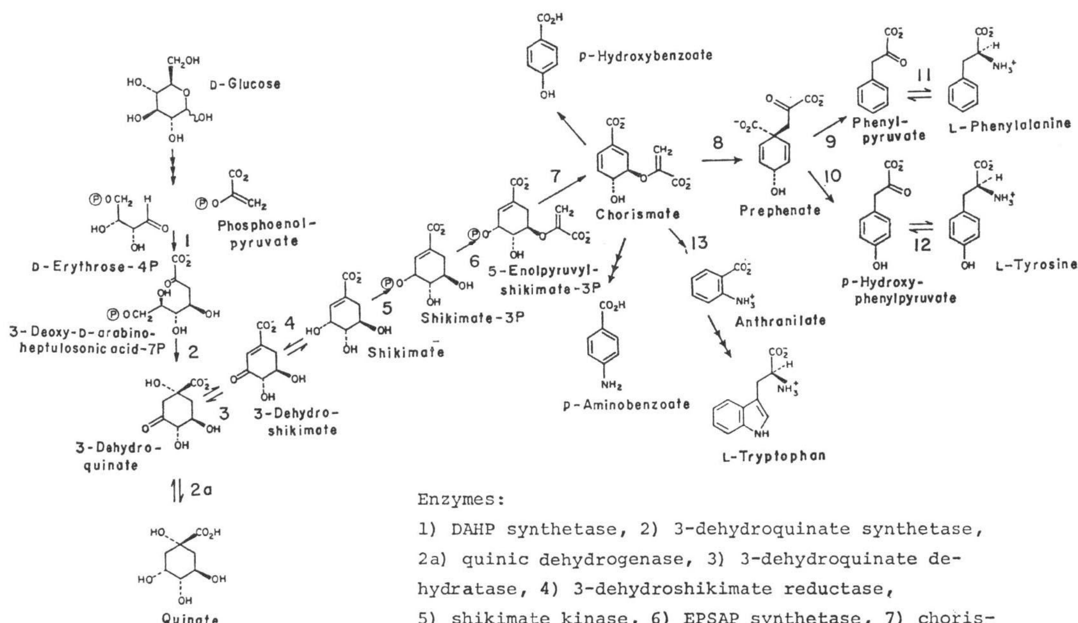


Summing up all our results we can establish that shikimic acid and 3-dehydroshikimic acid but no shikimate-3-phosphate are accumulated by *N. mediterranei* A10. Estimations by TLC showed that shikimic acid is accumulated up to a concentration of 0.1~0.15 g/liter medium 148 after 7 days of fermentation. A similar concentration can be expected for 3-dehydroshikimic acid. Using industrial media the yield of shikimic acid can be increased to 1~2 g/liter.

Enzymatic Studies with *N. mediterranei* A10 (for pathway see Fig. 5)

The enzymes ribose-5P-isomerase, ribulose-5P-3-epimerase and transketolase of the pentose shunt were all detected in mutant A10 and the synthesis of D-sedoheptulose-7-phosphate from pentose-phosphates could also be demonstrated. In all the assays mutant A10 shows the same properties as

Fig. 5. The shikimate pathway.



Enzymes:
1) DAHP synthetase, 2) 3-dehydroquinate synthetase, 2a) quinic dehydrogenase, 3) 3-dehydroquinate dehydratase, 4) 3-dehydroshikimate reductase, 5) shikimate kinase, 6) EPSAP synthetase, 7) chorismate synthetase, 8) chorismate mutase, 9) prephenate dehydratase, 10) prephenate dehydrogenase, 11) L-phenylalanine aminotransferase, 12) L-tyrosine aminotransferase, 13) anthranilate synthetase.

the parent N813. Furthermore the enzymes of the phenylalanine/tyrosine branch leading from chorismic acid to phenylalanine and tyrosine were also found and showed no significant differences to the corresponding activities in the parent N813 (Table 2). The enzymes of the tryptophan branch (except anthranilate synthetase) were not tested because anthranilic acid was found to substitute for tryptophan in the auxanographic studies with mutant A10 and an anthranilate synthetase activity could be detected qualitatively.

All these results, the auxanographic studies (triple requirement for phe, tyr, trp) and the fact that shikimate and 3-dehydroshikimate are accumulated in the culture broth indicate that mutant A10 must be blocked in the basic branch of the shikimate pathway leading from erythrose-4-phosphate and phosphoenolpyruvate to chorismic acid. The enzymes DAHP-synthetase, 3-dehydroquinate synthetase, 3-dehydroquinate dehydratase and 3-dehydroshikimate reductase are all present in mutant A10 and show no significant differences to the corresponding activities in the parent N813 (Table 2). All attempts to measure shikimate kinase failed with both strains N813 (parent) and A10 (even after 24 hours of incubation of crude extract) while in a control assay with a crude extract of *E. coli* under the same conditions shikimate kinase was detectable. Modified assays, such as adding an ATP regenerating system (phosphoenolpyruvate and pyruvate kinase), using ammonium sulfate precipitations of the crude extracts (0~30%, 30~50% and 50~90%), adding sugar phosphates, adding CLELANDS reagent or dialyzing the crude extracts did not lead to any positive results.

Assays to measure the sequences erythrose-4P + phosphoenolpyruvate → anthranilic acid or shikimate → anthranilic acid or shikimate-3-phosphate → anthranilic acid were carried out[22] but in all these cases no formation of anthranilic acid could be detected.

Table 2. Enzyme activities measured in crude extracts of the strains *Nocardia mediterranei* N813 (parent) and A10 (aro⁻ -mutant of N813). Activities in μmole/mg protein·hour

Enzyme	Mutant A10	Parent N813
DAHP-synthetase	0.15	0.1
3-Dehydroquinate synthetase	0.12	0.05
3-Dehydroquinate dehydratase	0.25	0.3
3-Dehydroshikimate reductase	0.45	0.25
Quinic dehydrogenase	0.022	0.016
Chorismate mutase	0.03～0.08	0.01
Prephenate dehydratase	0.02～0.025	0.01
Prephenate dehydrogenase	0.01～0.018	0.005～0.01
L-Phenylalanine aminotransferase	0.03	0.02
L-Tyrosine aminotransferase	0.01～0.015	0.005～0.01

Shikimate kinase was not detectable in both strains and EPSAP synthetase was found to be present in both strains by a qualitative test. Chorismate synthetase was not assayed.

A variation of the protein extraction method without freezing but using a glass bead vibrator showed that the negative result of the shikimate kinase assay was not a question of cold lability of the enzyme and even assays using cells permeabilized by means of an ether treatment showed no positive results. A qualitative test for EPSAP synthetase starting from shikimate-3-phosphate and phosphoenolpyruvate showed that with crude extracts of N813 and A10 a protein dependent reaction requiring shikimate-3-phosphate and phosphoenolpyruvate as substrates takes place. Therefore we concluded that an EPSAP synthetase is present in both strains.

Chorismate synthetase was not tested because no one step assay for this enzyme is known and no EPSAP was available.

Quinic dehydrogenase was detected in all three strains, N813, A10 and A8 but the activities were much less than for the other enzymes of the basic branch of the shikimate pathway (Table 2). However the presence of such an activity in *Nocardia mediterranei* explains the fact that our transketolase⁻-mutant A8 described in the preceding paper[11] shows a moderate growth on a quinic acid supplement.

Reversion of Mutant A10

We tried to revert mutant A10 by UV treatment without success. This non-reversibility might be due to a deletion.

Discussion

Nocardia mediterranei A10 is an auxotrophic mutant needing a triple supplement of the three aromatic amino acids phenylalanine, tyrosine and tryptophan. The mutant accumulates shikimic acid and 3-dehydroshikimic acid in its culture broth. The enzymatic and auxanographic studies showed that mutant A10 is blocked in one of the enzymes leading from shikimic acid to chorismic acid (see Fig. 5). The production of rifamycin B by this mutant was found to be normal in comparison with the parent *N. mediterranei* N813. A qualitative test showed that EPSAP-synthetase, one of the enzymes between shikimic acid and chorismic acid, seems to be present. So we are left with shikimate kinase and chorismate synthetase as possible enzymes for a block in mutant A10.

Shikimate kinase mutants are very rare because many organisms such as *E. coli, Salmonella typhimurium* and *Aerobacter aerogenes* have two isoenzymes for the shikimate kinase activity[23,24]. However

a mutant of *Bacillus subtilis* lacking shikimate kinase activity has been reported[24]. Shikimic acid has been detected in the culture filtrate of polyauxotrophic mutants of *E. coli, S. typhimurium* and *B. subtilis*[25,26,30]. That it is difficult or impossible to detect shikimate kinase activity in crude extracts or density gradient fractions has been reported for several microorganisms such as *Neurospora crassa*[27], *Lactobacillus arabinosus*[28], *B. subtilis*[23,29] and *Streptomyces coelicolor*[23].

Mutants lacking EPSAP synthetase were reported to accumulate shikimate-3-phosphate and shikimate[26,30]. Some mutants of *E. coli* and *S. typhimurium* with shikimate kinase activity accumulated mainly shikimate and only small amounts of shikimate-3-phosphate[28,30]. Finally mutants lacking chorismate synthetase were found to accumulate 5-enolpyruvylshikimate-3-phosphate (EPSAP) and 5-enolpyruvyl shikimate (EPSA) together with shikimate-3-phosphate and shikimate[24,25,30~32]. Only a mutant of *Bacillus subtilis* was found to accumulate exclusively shikimate and no shikimate-3-phosphate[30].

In view of the fact that our mutant *N. mediterranei* A10 accumulates only shikimate and 3-dehydroshikimate but not even traces of shikimate-3-phosphate or other intermediates behind shikimate and that EPSAP synthetase seems to be present, it seems very likely that the missing enzyme is shikimate kinase. This would be in good accordance with the data from other microorganisms we have mentioned above.

Another explanation would be that two different pathways leading from shikimate to EPSAP exist. In several higher plants (as in the microorganisms mentioned above) the presence of shikimate kinase has not been shown conclusively yet[33]. For some plants BERLYN *et al.*[34] reported that neither shikimate kinase nor EPSAP synthetase could be detected. In these cases several alternative pathways have been proposed.

NEISH[35] suggested that it is possible in plants to convert quinic acid into shikimate-3-phosphate *via* quinate-3-phosphate thus not using shikimate as a precursor for shikimate-3-phosphate. YOSHIDA[36] proposed the possibility that shikimate is first converted to EPSA and then phosphorylated to produce EPSAP. If we assume that such alternative pathways can also occur in microorganisms the fact that shikimate kinase is not detectable in *N. mediterranei* and other microorganisms could easily be explained. YOSHIDA's[36] theory is supported by the fact that a non-growing cellular suspension of an EPSA accumulating strain of *E. coli* incubated with shikimate in a nitrogen-free medium could be shown to convert shikimate to EPSA without the formation of shikimate-3-phosphate as reported by DAVIS and MINGIOLI[30].

If we postulate the two alternative pathways shikimate → shikimate-3-phosphate → EPSAP and shikimate → EPSA → EPSAP, organisms with one or the other or both pathways operating should exist. We would then have a similar situation as has been found for the biosynthesis of L-tyrosine from prephenate in microorganisms[37]. Also in this case two alternative pathways with an inverted reaction sequence between prephenate and L-tyrosine were found and some organisms can synthesize L-tyrosine by both, other organisms only by one or the other of these pathways.

This is a problem to be further investigated. However the answer to this question is not necessary for our aim to explain the biosynthesis of the seven-carbon amino unit of the rifamycin-chromophore. We know that the production of rifamycin B is not affected in our mutant A10 and that therefore shikimate is the last possible precursor for the seven-carbon amino unit.

Referring to the results of the preceding paper[1] we can localize the origin of this moiety between D-sedoheptulose-7-phosphate (Su7P) and shikimate. A direct cyclization of Su7P to yield a precursor of the seven-carbon amino unit can definitely be excluded for the following reason: As mentioned in the preceding paper KARLSSON *et al.*[2] and WHITE *et al.*[4] have shown that D(1-^{13}C)glucose enriches C(1) and C(10) of rifamycin which would correspond to C(2) and C(6) of 3-dehydroquinate, 3-dehydroshikimate or shikimate. Assuming a direct cyclization of Su7P C(3) and C(7) of Su7P would correspond to C(1) and C(10) of rifamycin. It is known that C(1) and C(7) of Su7P but not C(3) originate from C(1) of glucose[38]. Su7P is therefore excluded as a precursor of the seven-carbon amino unit. During the biosynthesis of 3-deoxy-D-arabinoheptulosonic acid-7-phosphate (DAHP) from Su7P an inversion of the triose fragment C(1) to C(3) of Su7P takes place and C(3) of DAHP corresponds to C(1) of Su7P originating from C(1) of glucose. DAHP is the first intermediate of the shikimate pathway

showing the incorporation pattern needed for a precursor of the seven-carbon amino unit.

The selection of possible precursors is now reduced to only four intermediates of the shikimate pathway, namely DAHP, 3-dehydroquinate, 3-dehydroshikimate and shikimate. DAHP cannot be definitely excluded because it is not known if the 3-dehydroquinate synthetase reaction is the only way for a cyclization of DAHP.

Our further investigations will deal with the isolation and analysis of aro$^-$-mutants of *N. mediterranei* N813 blocked either in DAHP synthetase, 3-dehydroquinate synthetase, 3-dehydroquinate dehydratase or shikimate reductase in order to find the branch point of the shikimate pathway and the biosynthesis of rifamycin.

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