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

I. ISOLATION AND CHARACTERIZATION OF A PENTOSE-EXCRETING AUXOTROPHIC MUTANT OF *NOCARDIA MEDITERRANEI* WITH DRASTICALLY REDUCED RIFAMYCIN PRODUCTION

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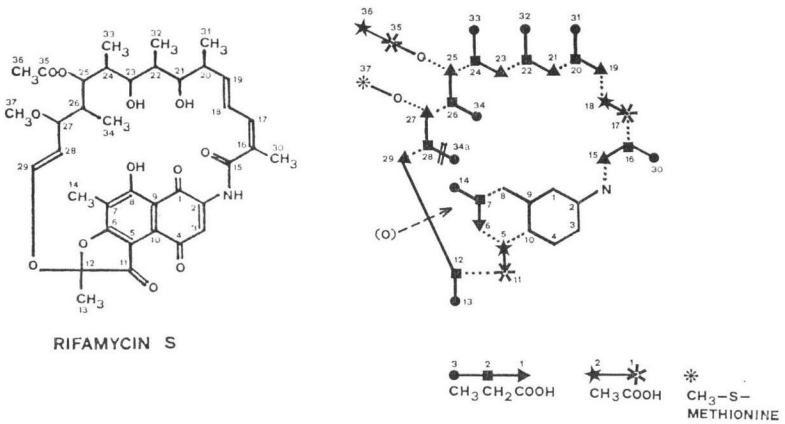
The mutant under study, designated A8, is derived from a *Nocardia mediterranei* strain, N813, which is a high rifamycin B producer. A8 is auxotrophic for aromatic amino acids and produces much less rifamycin B than the parent. A mixture of pentoses with D (−) ribulose as the main product is accumulated in the fermentation broth of this mutant. It was shown to be affected in its transketolase activity as no formation of D-sedoheptulose-7P from pentose-phosphates could be detected *in vitro* using crude extracts.

The only pathway so far known which is derived from D-sedoheptulose-7P is the shikimate pathway leading to aromatic amino acids and vitamins. Biochemical and genetic investigations with mutant A8, which is defective in both the biosynthesis of rifamycins and the biosynthesis of shikimate pathway products, show that the seven-carbon amino unit of the rifamycin-chromophore must be derived from an intermediate of the shikimate pathway.

It has been shown in earlier studies by BRUFANI *et al.*[1], KARLSSON *et al.*[2] and WHITE *et al.*[3] by incorporation of ^{14}C-precursors followed by chemical degradation or by incorporation of ^{13}C-precursors combined with nuclear magnetic resonance (NMR) that the ansa chain of rifamycins and of other ansamycins such as streptovaricins is derived from acetate, propionate and methionine.

Fig. 1. Incorporation of acetate, propionate and methyl from methionine into rifamycin.



The incorporation of acetate (except the O-acetyl group which originates directly from acetyl-CoA) and propionate units into rifamycin takes place *via* malonyl-CoA or methylmalonyl-CoA respectively.

The origin of a seven-carbon amino unit including C(1) to C(4) and C(8) to C(10) of the naphtho-

quinone part of the rifamycins (and other ansamycins) could not be elucidated conclusively by incorporation studies. ^{13}C incorporation studies by KARLSSON *et al.*[2] and WHITE *et al.*[4] led to the hypothesis that this subunit derives from an intermediate of the shikimate pathway. The two other possibilities for the biosynthesis of aromatic nuclei, acetate plus polymalonate or the condensation of a phenolic compound with mevalonate, are excluded by the incorporation pattern of acetate and propionate.

D(1-^{13}C)Glucose labels C(1) and C(10) of rifamycin. C(1) of glucose is known to label C(2) and C(6) of shikimic acid, 3-dehydroquinic acid or 3-dehydroshikimic acid[5,6], which would correspond to C(1) and C(10) of the rifamycin-chromophore. D(1-^{13}C)Glycerate labels C(3) and C(8) of rifamycin. C(1) of glycerate originates from C(3) or C(4) of glucose which are known to label C(4) and C(7) of shikimic acid[5,6] corresponding to C(3) and C(8) of the rifamycin-chromophore.

The incorporation pattern of D(1-^{13}C)glucose and D(1-^{13}C)glycerate would therefore be in accordance with a shikimate-type origin of the seven-carbon amino unit. Identical results have been reported for mitomycins[7] and geldanamycin[8] which both contain a seven-carbon amino unit identical to that in rifamycins. Further incorporation studies were not successful. Benzoate, tryptophan, tyrosine, and phenylalanine (all ^{14}C labelled) are not incorporated into the rifamycin-chromophore. (U-^{14}C) Shikimic acid is not incorporated either; but this is no proof for it not being a precursor of the seven-carbon amino unit of rifamycin as postulated by KARLSSON *et al.*[2] BRUGGISSER[9] and ourselves could show that shikimate is not able to penetrate into the cells of the rifamycin-producing organism *N. mediterranei* within a pH range from 4.0 to 9.0. The addition of DMSO did not improve the uptake of shikimic acid into the cells. Therefore shikimate cannot be excluded as a precursor. Because of the inability of shikimic acid (and probably also of its precursors 3-dehydroshikimic acid and 3-dehydroquinic acid) to penetrate into the cells and because the selection of ^{13}C-precursors available for further incorporation studies is very limited, we chose a genetic approach for our further investigations of the biosynthesis of the rifamycin-chromophore.

In this and in the succeeding paper[10] we describe the isolation and biochemical characterization of the first two aromatic amino acid deficient mutants of *N. mediterranei* which provide genetic evidence that intermediates of the shikimate pathway must be involved in the biosynthesis of the rifamycin-chromophore.

Materials and Methods

Organisms and Cultivation

Nocardia mediterranei N813 is a high rifamycin B producer from the laboratories of CIBA-GEIGY Ltd. All the auxotrophic strains described in this series of papers are mutants of strain N813.

Liquid minimal medium 150: 90 g glucose, 15 g $(NH_4)_2SO_4$, 2 g K_2HPO_4, 1 g $MgSO_4 \cdot 7H_2O$, 2 g sodiumdiethylbarbiturate, 17 g $CaCO_3$ and 10 ml of a trace element solution in 1 liter of water, pH 6.3 after sterilization for 20 minutes at 120°C.

Trace element solution: 0.33 g $CuSO_4 \cdot 5H_2O$, 1 g $FeSO_4 \cdot 7H_2O$, 5 g $ZnSO_4 \cdot 7H_2O$, 0.4 g $MnSO_4 \cdot 4H_2O$, 0.2 g $CoCl_2 \cdot 6H_2O$, 0.1 g $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ in 1 liter of water.

Liquid complex medium 148: 22 g glucose, 5 g Lab-Lemco beef extract (Oxoid), 5 g peptone C, 5 g yeast extract, 3 g Bacto-Casitone (Difco) and 1.5 g NaCl in 1 liter of water, pH 6.5 after sterilization for 20 minutes at 120°C.

Fermentation: shake flasks, 28°C, 250 rpm, 4~10 days duration.

Isolation of Auxotrophic Mutants

Starting from *N. mediterranei* N813 a number of auxotrophic mutants were selected by UV-irradiation and the replica technique as described by SCHUPP[11].

Reversion of mutants was carried out using the same technique.

Auxanography (Selection for Aromatic Amino Acid Requirement)

Auxanography was carried out by the method of HOLLIDAY[12], which has been slightly modified by SCHUPP[11] using pools of growth factors on agar plates. The mutants showing requirements for aromatic amino acids were auxanographed with the available intermediates of the shikimate pathway.

Supplementation Studies in Liquid Medium

Fermentations with the isolated mutants blocked in the biosynthesis of aromatic amino acids were carried out in liquid minimal medium 150. Aromatic amino acids, intermediates of the shikimate pathway or aromatic vitamins were added as supplements in varying amounts and in all the possible combinations. The dry weight of the mycelium and the production of rifamycin B were determined after 10 days of fermentation.

Photometric Determination of Rifamycin B

Rifamycin B in the fermentation broth was determined by the method described by PASQUALUCCI *et al.*[13] and modified by BRUGGISSER[9].

TLC-Methods

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

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

Solvent system 2: phenol - water (90: 30)

Solvent system 3: propanol - 25% ammonia - water (60: 30 :10)

Spray reagents: periodic acid/benzidine[14,15]: 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.

Ammoniummolybdate-perchloric acid, 2,4-dinitrophenylhydrazine, alkaline silver nitrate, dimedone-phosphoric acid, anthrone, aniline-diphenylamine, naphthoresorcinol-sulfuric acid, carbazole and orcinol-$FeCl_3$ spray reagents were prepared according to STAHL[16]. Orcinol-trichloric acid - isopropanol spray reagent was prepared according to KLEVSTRAND and NORDAL[17].

Colour Reactions for Pentoses

Four different photometric methods were used to determine pentoses in culture filtrates or eluates: The orcinol-method[18] for aldopentoses, ketopentoses and sedoheptulose; the resorcinol-method[19,20] for ketopentoses, sedoheptulose and fructose; the cysteine-carbazole-method[19,20] for ketopentoses, sedoheptulose and fructose; and the phloroglucinol-method[20,21] for aldopentoses (ketopentoses).

Enzyme Tests

The enzyme activities were all tested in crude extracts. A 200-ml shake flask with 40 ml medium 148 was inoculated with mycelium of *N. mediterranei* N813 or A8 (60 mg total dry weight) and fermented for 4 days; 3.5 ml of this culture were transferred into each of ten 500-ml shake flasks with 100 ml medium 148 and fermented for another 5 days. The mycelium was then harvested and washed with 0.066 M phosphate buffer pH 7.0. The mycelium (25~30 g wet) was frozen, passed four times through an X-press at $-45°$~$-50°$C and homogenized in 50~60 ml phosphate buffer. This homogenizate was centrifuged at 27,000$\times g$ for 30 minutes and the clear orange supernatant (crude extract) was frozen in portions of 3 ml and stored at $-30°$C. The crude extracts contained 15~20 mg/ml protein (LOWRY-method).

The methods used for measuring the enzymes of the phenylalanine/tyrosine branch and of the basic branch of the shikimate pathway will be listed in the next paper[10]. Of the enzymes of the pentose shunt, ribose-5P-isomerase, ribulose-5P-3-epimerase and transketolase were qualitatively assayed by a set of differential tests with various pentose-phosphates as substrates.

A typical incubation mixture contained:

2.0 ml of a 0.1% solution of pentose-phosphate* (Ba or Na-salt) in 0.1 M tris-HCl buffer pH 7.0 and 250 μl of crude extract from strain N813 or A8.

* or 0.07% of each of two pentose-phosphates

This mixture was incubated at 28°C for 2 hours or more. The formation of sedoheptulose-7P and fructose-6P from pentose-phosphate and the disappearance of pentose-phosphate were observed using the cysteine-carbazole-, the resorcinol- and the phloroglucinol-methods. (The tests were made at 0 hours and after 2 or 4 hours of incubation.) The spectra of these colour reactions were recorded.

Sources of Chemicals

D(−)Ribulose	Calbiochem Chemicals
D-Sedoheptulose monohydrate	Merck, Darmstadt
D-Xylulose-5P, sodium salt D-Ribulose-5P, barium salt D-Sedoheptulose-7P, barium salt	Sigma Chemicals
D-Ribose-5P, barium salt D-Fructose-6P, barium salt Dowex ion-exchangers	Fluka Chemicals

D-Xylulose was prepared according to the method of HOUGH and THEOBALD[25].

Results

Growth and Production of Rifamycin B

The auxotrophic mutant *N. mediterranei* A8 described in this paper was first analyzed by auxanography and found to grow on a combination of phe, tyr and trp or on a combination of phe and tyr alone. Considerable growth was also observed on quinic acid but not on 3-dehydroquinic acid or shikimic acid. No growth was found on pentoses.

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 produced slightly less growth than with the aromatic amino acids alone.

N. mediterranei A8 grows less than the parent N813 in liquid complex medium 148 and the growth rate in liquid minimal medium 150 (supplemented) is also substantially reduced.

The production of rifamycin B by strain A8 is much reduced in comparison with the parent in both media. Even in the industrial production medium the yield is only 5% to 10% of the parent.

Table 1. Growth and production of rifamycin B
Comparison of fermentations of strains N813 and A8 in 200-ml shake flask 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
A8	phe, tyr, trp or phe, tyr (or quinic acid)	<0.05 g/liter rifamycin B after 10 days 3 g/liter dry weight after 10 days	<0.05 g/liter rifamycin B after 8 days 5.5 g/liter dry weight after 5 days
N 813	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.

Identification and Isolation of Intermediates Accumulated in the Fermentation Broth of Mutant A8

TLC investigations on silica gel plates in solvent system 1 were carried out with the filtrates of cultures of *N. mediterranei* N813 and A8 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 0.39 was detected on the TLC of A8 culture filtrate using periodic acid-benzidine, ammoniummolybdate - perchloric acid, alkaline silver nitrate and 2,4-dinitrophenylhydrazine spray reagents which is not present on the TLC of N813 culture filtrate. These reactions indicate a reducing sugar. To confirm this we tried several other sugar specific spray reagents such as orcinol-trichloric acid-isopropanol (specific for heptuloses), dimedone-phosphoric acid and anthrone (specific for ketoses), aniline-diphenylamine and naphtho-resorcinol-sulfuric acid, using a number of sugars as references. D-Ribulose, D-ribose and D-xylose all showed the same Rf value as the spot in question. The colour reactions were those expected from a mixture of aldopentose and ketopentose. No heptulose could be detected.

As a result of adsorption tests with several ion-exchange resins we could conclude that the accumulation products of mutant A8 must be neutral compounds.

In order to optimize the conditions for specific pentose colour reactions in the culture filtrate directly we carried out a series of fermentations in liquid complex medium 148 and found that the formation of excretion products by *N. mediterranei* A8 starts in the late log-phase after 3~4 days of fermentation. A good yield of the accumulation product is reached after 5~7 days.

Specific colour reactions for pentoses were carried out with the unconcentrated culture filtrates of the strains A8 and N813. We obtained the following results with strain A8. The absorption maxima typical for all pentoses at 665 nm and the typical maximum for ribulose at 530 nm but no maximum at 565 nm (typical for sedoheptulose), were found with the orcinol-method using 10~20 μl of culture filtrate. With the resorcinol-method using 100 μl of culture filtrate for the test, a broad maximum at 600 nm typical for ketopentoses but no maxima in the region of 500 nm, 575 nm and 410 nm (all typical for sedoheptulose and fructose) were observed. The cysteine-carbazole-method (20 μl of culture filtrate used for the test) showed the typical maximum for ketopentoses at 536 nm but no maximum at 560 nm typical for sedoheptulose and fructose. The typical maximum for aldopentoses at 555 nm and a second maximum at 610 nm typical for ribulose were detected by means of the phloroglucinol-method (40 μl of culture filtrate used for the test).

None of these maxima typical for pentoses were detected in the corresponding assays with culture filtrate of the parent strain N813.

All these findings show clearly that mutant A8 accumulates a mixture of aldopentoses and ketopentose but no sedoheptulose in the culture broth.

As all the data described so far indicate that ribulose must be one of the products accumulated by mutant A8 we decided to separate the mixture of pentoses, isolate the compound thought to be ribulose and identify it by chemical and spectroscopical methods. The separation of the pentoses on ion-exchange resins was carried out using a modification of a method described by JONES and WALL[22].

A 200-ml shake flask with 40 ml of liquid complex medium 148 was inoculated with mycelium of *N. mediterranei* A8 (60 mg total dry weight) and fermented for 4 days. Seven ml of this culture were transferred into each of five 500-ml shake flasks with 100 ml of medium 148 and fermented for another 7 days. The mycelium was then filtered off and a total of 450 ml culture filtrate was collected.

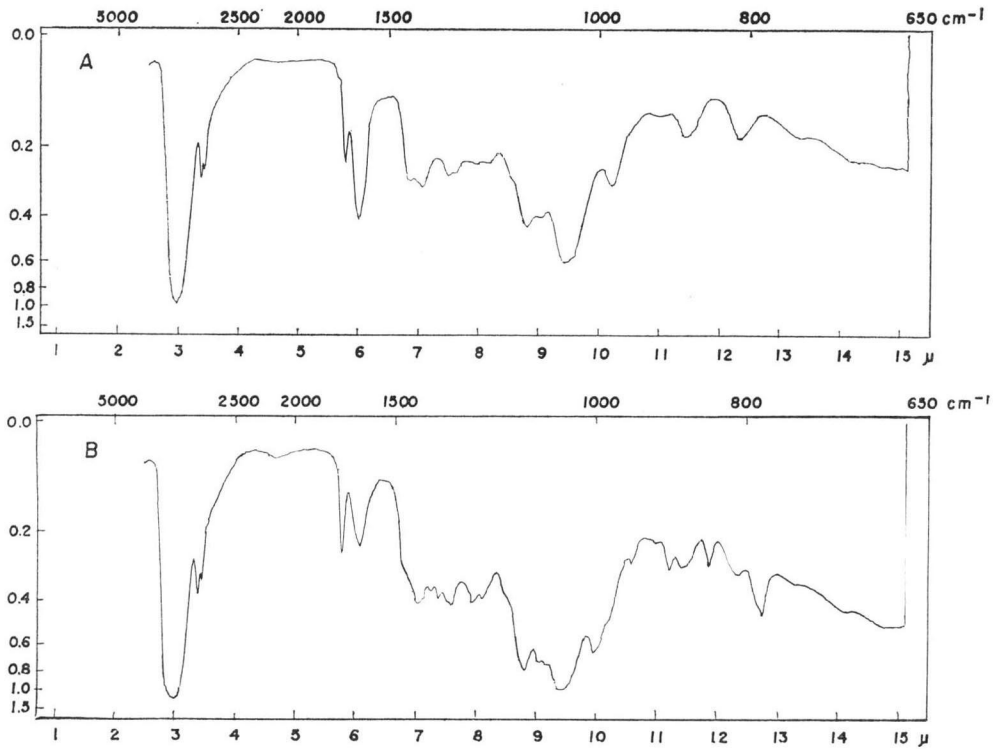
Cation-exchanger Dowex 50W×8 (20~50 mesh, H^+), 150 g, were added to this culture filtrate

and the batch was stirred at room temperature for 30 minutes; 300 g of anion-exchanger Dowex 2×8 (100~200 mesh, Cl^-) were added to the yellow filtrate of this first treatment and the batch was stirred for another 30 minutes. The faint yellow filtrate was neutralized with KOH, treated with 2 g of charcoal for 20 minutes, filtered and concentrated to 10 ml under reduced pressure at 50°C. This concentrate containing only the neutral compounds of the culture filtrate was passed onto a chromatography column (90×2.6 cm) with Dowex 50W×8 (20~50 mesh, Ba^{2+}) at a flow rate of 14 ml per hour and chromatographed with water as the liquid phase at a flow rate of 14 ml per hour. A hundred fractions of 150 drops each (approx. 9 ml) were collected and tested for sugars by TLC on silica gel plates in solvent system 1 using periodic acid/benzidine as the reagent. Fractions 1~5 contained only glucose, fractions 6~43 glucose and pentose, fractions 44~98 only pentose and fractions 99~100 were negative. TLC tests with other spray reagents such as carbazole, orcinol-$FeCl_3$, aniline-diphenylamine and assays with the four specific colour reactions for pentoses showed that fractions 6~43 contained aldopentose (and glucose) and fractions 44~98 contained ketopentose (ribulose).

Fractions 1~30, 31~50, 51~60 and 61~100 were pooled, concentrated to 5 ml each under reduced pressure at 50°C and designated fractions I, II, III and IV. These four fractions were further investigated by TLC on cellulose plates in solvent system 2. The pentoses were detected with carbazole, orcinol-$FeCl_3$ (for developing on cellulose plates, both prepared with HCl instead of H_2SO_4) and periodic acid/benzidine spray reagents. The following results were obtained: fraction I contained glucose (Rf 0.32), arabinose (0.48) and traces of ribulose (0.63); fraction II glucose, arabinose, ribulose and traces of xylulose (0.53); fraction III ribulose and traces of xylulose and fraction IV only ribulose.

Fraction IV was evaporated under reduced pressure to a syrup and a very small amount of solids was precipitated with absolute ethanol and filtered off. The alcoholic solution was again evaporated and 200 mg of a yellow syrup were obtained. This syrup showed a specific rotation in water of $[\alpha]_D^{20}$

Fig. 2. IR-spectra (syrup) of A) isolate from mutant A8 (fraction IV) and B) standard D (−) ribulose. In spectrum B) the peaks at 7.95, 8.1, 10.0, 10.6, 11.2, 11.85, 12.75 and 14.75 microns are due to impurities of arabinose in the authentic sample of D (−) ribulose.



$-20\pm1°$ (recognized value for D(−)ribulose $[\alpha]_D^{20}-17.2°$[23]). Another 110 mg of yellow syrup were obtained from fraction III. The IR-spectra of the syrup obtained from fraction IV and of a commercial product of D(−)ribulose (containing approx. 15% of arabinose) were nearly identical but the resolution of the spectra was not very good (see Fig. 2). For this reason we decided to prepare derivatives of the isolate from fraction IV and of the commercial sample. The *o*-nitrophenylhydrazones were prepared by the method described by GLATTHAAR and REICHSTEIN[24] starting from 100 mg of syrup and 100 mg of commercial product.

After one recrystallization from absolute ethanol 65 mg of orange needles were recovered. Melting points, specific rotations and elemental analysis were identical for both preparations (see Table 2) and were in good agreement with the literature values[23,24].

The IR- and NMR-spectra of the *o*-nitrophenylhydrazone of the isolate from fraction IV are identical with the corresponding spectra of authentic D(−)ribulose-*o*-nitrophenylhydrazone (see Fig. 3 and Fig. 4).

Thus we could establish that D(−)ribulose is the major product of the mixture of pentoses accumulated by *N. mediterranei* A8. With the orcinol- and the cysteine-carbazole-methods it was estimated that D(−)ribulose is accumulated to a concentration of 0.8 g/liter in medium 148 after 8 days of fermentation. The total concentration of all pentoses was estimated to be about 1.2g/liter. Using industrial production media the yield of D(−)ribulose can reach 6 g/liter.

Enzymatic Studies with *N. mediterranei* A8 (for pathway see Fig. 7)

The enzymes of the phenylalanine - tyrosine branch and of the basic branch of the shikimate pathway between erythrose-4P and shikimate were all present in the crude extract of A8. The following

Fig. 3. IR-spectra (Nujol mull) of A) *o*-nitrophenylhydrazone of isolate from mutant A8 (fraction IV) and B) *o*-nitrophenylhydrazone of standard D (−) ribulose.

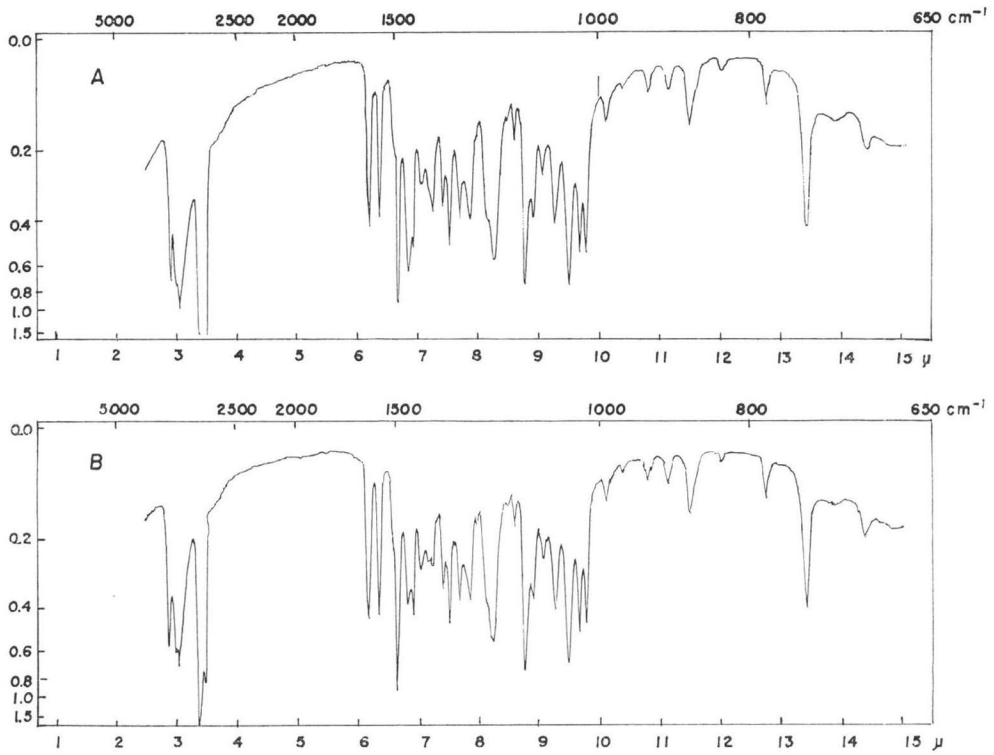
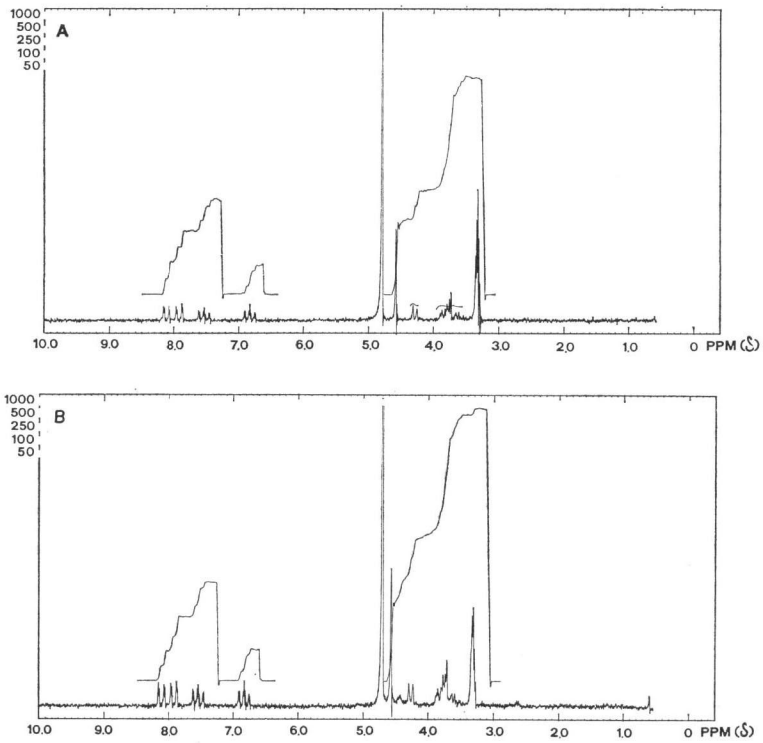


Table 2. Constants of *o*-nitrophenylhydrazones prepared from standard D (−) ribulose and from an accumulation product of *N. mediterranei* A8.

Preparation	Melting point after one recrystallization from ethanol	$[\alpha]_D^{20}$ in methanol	Elemental analysis calculated for $C_{11}H_{15}O_6N_3$ (285.26)
Standard D (−) ribulose-*o*-nitrophenylhydrazone	160~161°C	−50±1°	C, 46.26 H, 5.24 found N, 15.05
o-Nitrophenylhydrazone of isolate from fraction IV	160~161°C mixed melting point 160~161°C	−52±1°	C, 46.38 H, 5.21 found N, 15.07
Reference values[23,24]	166~167°C[23] 168~169.5°C[24] after several recrystallizations	−47°[23] −48.3±3°[24]	C, 46.32 H, 5.30 calculated N, 14.73 and ref. 24)

Fig. 4. 100 Mc NMR-spectra (CD_3OD) of A) *o*-nitrophenylhydrazone of isolate from mutant A8 (fraction IV) and B) *o*-nitrophenylhydrazone of standard D (−) ribulose.



tests were carried out to detect the enzymes of the pentose shunt:

(1) Incubation of ribose-5P (R5P) with crude extracts N813 or A8. After 2 hours of incubation the cysteine-carbazole test showed the typical maximum for the ketopentose-phosphates ribulose-5P (Ru5P) and xylulose-5P (Xu5P) at 536 nm with crude extract of A8 and a maximum at 555 nm with crude extract of N813 [sedoheptulose-7P (Su7P) max. 555 nm and fructose-6P (F6P) max. 560 nm] (see Fig. 5).

Fig. 5. Cysteine-carbazole test with 0.5 ml incubation mixture containing 2.0 ml 0.1% solution of ribose-5P (barium salt) in 0.1 M tris-HCl buffer pH 7.0 and 0.25 ml of crude extract from strain N813 or A8 after 2 hours of incubation at 28°C.

Control: incubation of R5P solution without crude extract.

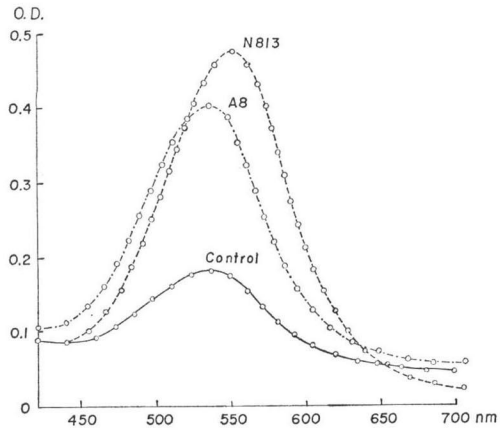
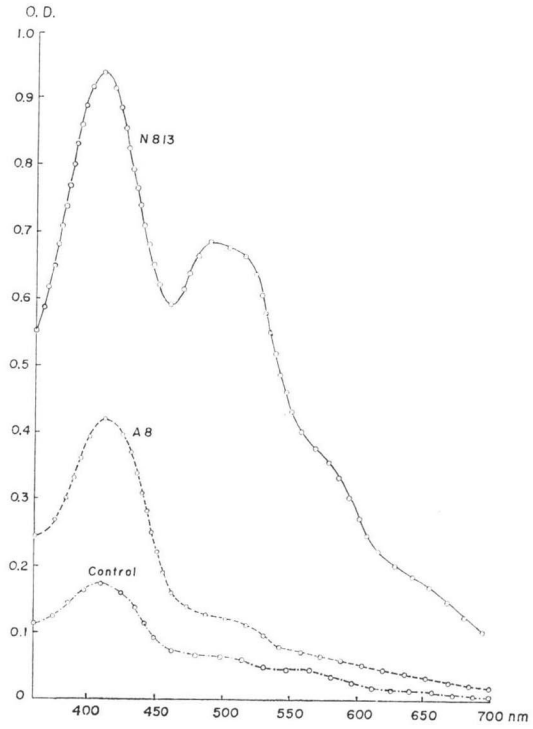


Fig. 6. Resorcinol test with 1.0 ml incubation mixture containing 2.0 ml 0.1% solution of ribose-5P (barium salt) in 0.1 M tris-HCl buffer pH 7.0 and 0.25 ml of crude extract from strain N813 or A8 after 4 hours of incubation at 28°C.

Control: incubation of R5P solution without crude extract.



After 4 hours of incubation the resorcinol test showed clearly that Su7P and F6P are present in the crude extract of N813 but not in the crude extract of A8 (maxima at 410 nm, 488 nm and 512 nm) (see Fig. 6). A dramatic decrease of R5P (555 nm) is observed after 4 hours of incubation by means of the phloroglucinol test in crude extract N813 and to a smaller extent also with crude extract A8.

From this assay it can be seen that strain N813 converts R5P into Su7P and F6P but strain A8 only converts it to Ru5P (and Xu5P). The mutant strain A8 is thus unable to synthesize Su7P from R5P. From these findings it appears that one of the enzymes ribose-5P-isomerase, ribulose-5P-3-epimerase or transketolase must be blocked.

(2) Incubation of ribulose-5P with crude extracts N813 or A8. The cysteine-carbazole test shows a shift of the maximum from 536 nm to 550 nm in crude extract of N813 after 2 hours of incubation but not in crude extract of A8. The formation of Su7P and F6P can be observed in N813 by means of the resorcinol test but not in A8. An increase of aldopentose-phosphate (formation of R5P from Ru5P) is observed in both crude extracts in the phloroglucinol test.

This assay shows that ribose-5P-isomerase is not the blocked enzyme.

(3) Incubation of ribose-5P and ribulose-5P with crude extracts N813 or A8.

In the cysteine-carbazole test after 2 hours of incubation the maximum shifts from 536 nm to 555 nm with crude extract of N813 but not with A8. In the resorcinol test the formation of Su7P and F6P is observed in N813 but not in A8. Strain A8 is thus unable to synthesize Su7P from a mixture of R5P and Ru5P.

Ribose-5P-isomerase is therefore definitely excluded and the possibilities of blocks at ribulose-5P-3-epimerase or transketolase remain.

(4) Incubation of xylulose-5P with crude extracts N813 or A8. Again strain A8 cannot synthesize Su7P from Xu5P.

(5) Incubation of ribose-5P and xylulose-5P with crude extracts N813 or A8.
A shift from 536 nm to 552 nm is observed with the cysteine-carbazole test in N813 but not in A8 and with the resorcinol test the formation of Su7P and F6P is only observed in crude extract of N813.
This assay excludes both ribose-5P-isomerase and ribulose-5P-3-epimerase as possible blocks. The blocked enzyme in *N. mediterranei* A8 must therefore be the transketolase.

(6) Because transketolase not only catalyzes the reaction R5P + Xu5P $\leftrightharpoons$ Su7P + GAP (glyceraldehyde-3P) but also the reaction Xu5P + E4P $\leftrightharpoons$ F6P + GAP, crude extracts were also incubated with a mixture of xylulose-5P and erythrose-4P (E4P). The formation of F6P using the cysteine-carbazole and resorcinol tests was only found with N813. This again shows that the blocked enzyme in *N. mediterranei* A8 must be transketolase.

To confirm the fact that mutant A8 is unable to synthesize Su7P we analyzed crude extracts of N813 or A8 incubated for 4 hours in a 1% solution of ribose-5P in 0.1 M tris-HCl pH 7.0. The solutions were tested after incubation by TLC on silica gel plates in solvent systems 2 and 3 with Ru5P, Xu5P, Su7P, R5P and F6P as reference substances. Using orcinol - trichloric acid - isopropanol and carbazole as spray reagents, Su7P, F6P, Ru5P and Xu5P were detected in the assay using crude extract N813 but only Ru5P and Xu5P without Su7P and F6P using crude extract A8.

Concentrated HCl, 150 μl, was added to 0.65 ml of the solutions after incubation and boiled for 2 hours in a water bath to hydrolyze the phosphate esters. The solutions were then neutralized with NaOH and tested by TLC on silica gel plates in solvent system 1 with sedoheptulose and fructose (both untreated and acid treated) as reference substances. The decomposition products of sedoheptulose and fructose were detected with orcinol-trichloric acid-isopropanol and carbazole spray reagents in crude extract of N813 but not with A8.

Thus it could be demonstrated by various methods that *N. mediterranei* A8 is blocked in transketolase activity and unable to synthesize sedoheptulose-7P, the essential precursor for the shikimate pathway.

Reversion of Mutant A8

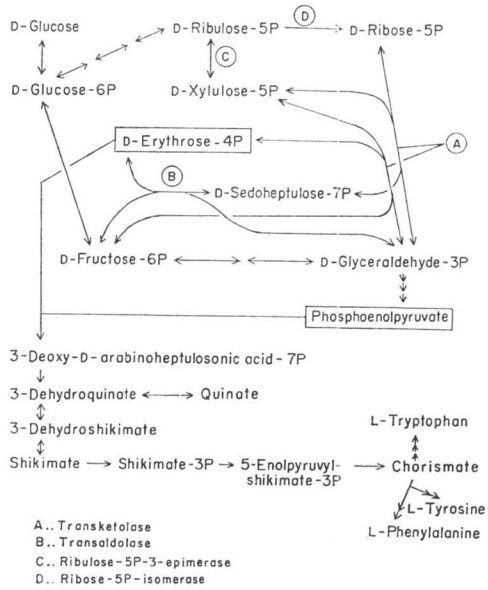
Three revertants of *N. mediterranei* A8 were isolated after UV treatment. The revertants showed normal production of rifamycin B and no accumulation of pentoses in the culture filtrate. This indicated that the mutation in *N. mediterranei* A8 must be a single point mutation. It also proved that there is a direct correlation between the functioning of the transketolase and the production of rifamycin B. Furthermore one step reversion of the mutant excludes the possibility of a double mutation having taken place.

Discussion

Nocardia mediterranei A8 is an auxotrophic mutant requiring aromatic amino acids but not pentoses for growth. Although it is phenotypically an aro$^-$-mutant, no block in the enzymes of the shikimate pathway was found but only a block in the transketolase activity (see Fig. 7). A mixture of pentoses

with D(−)ribulose as the major product and arabinose, xylose and xylulose as minor products was found to be accumulated by mutant A8 in the culture broth. The mutant must be somewhat leaky because growth could be observed on an incomplete supplement of aromatic amino acids containing only phenylalanine and tyrosine but no tryptophan. A very small amount of shikimate pathway precursors must therefore be synthesized which can produce tryptophan and a trace of rifamycin B. The mutation was shown by reversion to be a single point one.

Fig. 7. Biosynthetic pathways: pentose shunt, glycolysis and shikimate pathway.



Similar findings have been reported by JOSEPHSON and FRAENKEL[26,27] with transketolase$^-$-mutants of *E. coli*. These mutants were found to be phenotypically aro$^-$-mutants but were somewhat leaky for the biosynthesis of aromatic compounds of the shikimate pathway. The mutants did not require exogenous pentose for growth. No accumulation of pentoses in the culture broth was observed. The authors explained the reason for the leakiness only being apparent in the aromatic requirement but not in pentose induced growth by the fact that the requirement of transketolase activity for aromatic biosynthesis is much less than for pentose degradation, for which it is a major catabolic enzyme. The same properties were observed in transketolase$^-$-mutants of *Salmonella typhimurium*[28] and a *Bacillus* species[29,30]. The transketolase$^-$-mutant of the *Bacillus* species was found to excrete D-ribose. Incubation of a crude extract of this mutant with ribose-5P gave the same results in the cysteine-carbazole test as we describe in this paper for *N. mediterranei* A8. A ribulose-5P-3-epimerase$^-$-mutant of this *Bacillus* sp. was found to accumulate ribose and ribulose.

An accumulation of D-ribulose in the culture broth has been observed with *Agrobacterium tumefaciens*[23], *Brevibacterium fuscum*[31], *Brevibacterium* and *Corynebacterium*[32], but all these organisms were isolated from natural sources and no enzymatic studies are published. A thiamine requiring species of *Corynebacterium* has also been found to accumulate D-ribulose in a thiamine deficient medium[33,34]. The ribulose production in this case is related to the thiamine requirement of this organism. Thiamine deficiency has been found to cause a reduction of transketolase activity[34].

The fact that production of rifamycin B in *N. mediterranei* N813 is dependent on the formation of D-sedoheptulose-7P and the presence of transketolase activity shows clearly that the seven-carbon amino unit of the rifamycin-chromophore must be derived from an intermediate of the shikimate pathway. No other pathway for synthesizing D-sedoheptulose-7P except the transketolase reaction is known so far. Likewise D-erythrose-4P is only known to be synthesized from Su7P by means of the transaldolase reaction or from fructose-6P and glyceraldehyde-3P by the transketolase reaction. Thus a mutant lacking transketolase activity is not able to synthesize both D-erythrose-4P and D-sedoheptulose-7P. This biochemical evidence is fully supported by the fact that the mutation is reversible. No other pathway except the shikimate one is known which starts from D-sedoheptulose-7P or D-erythrose-4P and leads to aromatic compounds. Therefore the seven-carbon amino unit can only be derived from an intermediate of the shikimate pathway behind D-sedoheptulose-7P.

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