TRANSFORMATION OF RIFAMYCIN S INTO RIFAMYCINS B AND L

A REVISION OF THE CURRENT BIOSYNTHETIC HYPOTHESIS

ORESTE GHISALBA, RUTH ROOS, THOMAS SCHUPP and JAKOB NÜESCH

Pharmaceuticals Division and Central Research Laboratories of
CIBA-GEIGY Limited, Basel, Switzerland

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The transformation of rifamycin S into rifamycins B and L was reinvestigated in order to establish more detailed pathways. Our results exclude rifamycin O as a common progenitor in the biosyntheses of rifamycins B and L. Rifamycins B and L are formed from rifamycin S (SV) by different pathways using different C_3-precursors for the biosynthesis of their glycolic acid moieties. A thiamine-dependent enzyme (decarboxylase) seems to be involved in the transformation reaction.

LANCINI, *et al.*[1~3] investigated the origin of the glycolic acid moieties in rifamycins B and L by adding ^{14}C-precursors to growing cultures or washed mycelium of *Nocardia mediterranei* and analyzing the incorporation patterns in the glycolic acid after chemical degradation of the extracted rifamycins B and L. No incorporation into the glycolic acid moiety of rifamycin B was found with C_2-precursors such as (2-^{14}C)-acetate, (1-^{14}C)-glycolate, (2-^{14}C)-glyoxylate, (2-^{14}C)-glycine or (1-^{14}C)-ethanol. On the other hand ^{14}C-labelled carbohydrates were incorporated into the glycolic acid moiety when added to either growing cells or to resting cells in the presence of rifamycin SV. In the latter case about 80% of the rifamycin B radioactivity was located in the glycolic acid moiety. (1-^{14}C)-Glucose, (6-^{14}C)-glucose and (1-^{14}C)-ribose predominantly labelled the hydroxymethyl group (80~90% of the total glycolic acid radioactivity), whereas (2-^{14}C)-glucose, (3,4-^{14}C)-glucose and (1-^{14}C)-glycerol labelled both the hydroxymethyl group (40~60%) and the carboxyl group (40~60%). For the exact data see Table 1 in reference 2.

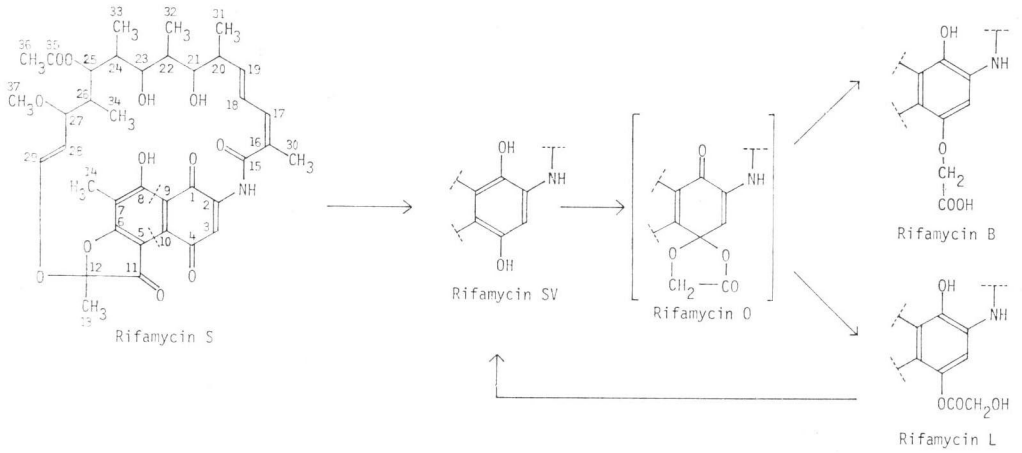
Based on the low degree of randomization observed with (1-^{14}C)- and (6-^{14}C)-glucose and on the non-incorporation of C_2-precursors it was suggested that the glycolic acid moiety derives from a C_3-precursor[1~3]. The incorporation of (1-^{14}C)-ribose indicates the presence of the pentose phosphate cycle. The results with (2-^{14}C)-glucose were explained with labeling the hydroxymethyl group *via* the pentose phosphate cycle and the carboxyl group *via* the glycolytic pathway.

Only one incorporation experiment was carried out with rifamycin L[2]. With (1-^{14}C)-glucose 90% of the radioactivity was found in the hydroxymethyl group and 10% in the carboxyl group of the glycolic acid isolated from rifamycin L. From this result it was concluded that the origin of the glycolic acid moiety is the same for both rifamycins B and L. In a transformation assay the conversion of uniformly labelled rifamycin L into rifamycin B was observed but the glycolic acid moiety of rifamycin B was found not to be radioactive. Therefore, rifamycin L was excluded as a precursor of rifamycin B.

The biogenetic hypothesis favored by LANCINI, *et al.*[2,3], is depicted in Fig. 1. It includes rifamycin O as a possible common precursor for both rifamycins B and L.

LANCINI and PARENTI[4] suggested that 3-phosphoglycerate might be a possible precursor for the glycolic acid moiety. This suggestion is based on the finding that (U-^{14}C)-pyruvate does not label the

Fig. 1. Pathway for the transformation of rifamycin S (SV) into the rifamycins B and L as proposed by LANCINI *et al.*[2,8]



glycolic acid moiety of rifamycin B in a transformation assay.

In order to obtain some more detailed information we decided to reinvestigate the rifamycin transformation starting from rifamycin S using different approaches.

Experiments and Results

Transformation Experiments Using Permeabilized Mycelium of *Nocardia mediterranei*

For permeabilizing the cells we used a method applied by FELIX *et al.*[5] for *Cephalosporium acremonium* and adapted it for *Nocardia mediterranei*. Mycelium from 40 ml of a culture of *Nocardia mediterranei* N813[6] in liquid complex medium 148[6] (28°C, 250 rpm, 3 days) was suspended in 12 ml of 0.066 M phosphate buffer pH 7.0. After addition of 12 ml of cold diethyl ether the suspension was mixed by hand agitation for 1 minute and centrifuged at 12,000 × *g* and 0°C. The ether phase was removed and the permeabilized mycelium was resuspended in the aqueous supernatant. To control the efficiency of permeabilization the hexokinase/glucose 6-phosphate dehydrogenase reaction was measured according to the method by SCHLENK and ZYDEK-CWICK[15]. This reaction was shown to be dependent on glucose, ATP and $NADP^+$. It can only be measured with ether permeabilized mycelium and not with untreated intact mycelium of *Nocardia mediterranei*. A specific activity (NADPH formation) of 30~40 μmole/minute/ml ether treated mycelium suspension was found.

Transformation assays were carried out with ether permeabilized mycelium as follows: 10 ml transformation assay mixture containing 2.5 ml of permeabilized cell suspension, rifamycin S (0.5 mM), NADH or NADPH (0.5 mM), $MgCl_2$ (1.5 mM), phosphate buffer pH 7.6 (0.05 M) was incubated at 28°C, 250 rpm. Control experiments were carried out with untreated intact mycelium under the same conditions. After incubation, 3 ml of the reaction mixture was extracted at pH 2 with 3 ml of methylene chloride and the extract evaporated to dryness. The residue was dissolved in 100 μl of chloroform - methanol (4: 1) and chromatographed on silica gel 60 TLC plates (Merck F-254, pretreated with citric acid) using methylene chloride - methanol (95: 5) as solvent system (Rf values: rifamycin S 0.58~0.62, rifamycin SV 0.40~0.41, rifamycin B 0.24~0.28, rifamycin L 0.15~0.17).

In the absence of NADH only traces of rifamycin S are reduced to rifamycin SV by the ether permeabilized cells within 90 minutes but in the presence of NADH about 90% of the added rifamycin S is reduced to SV in the same time. With NADPH instead of NADH the reduction to rifamycin SV is slower but does still occur. After 15 hours rifamycin SV is partially reoxidized. No reaction occurs with NADH/NADPH in the absence of mycelium. Even after 15 hours no traces of rifamycin B or L can be detected in the assay with permeabilized cells. In the control experiment with intact cells the formation of rifamycins SV and L is observed after 3 hours.

In an assay using permeabilized cells of *Escherichia coli* W71 ETH 2018 instead of *Nocardia mediterranei* N813, surprisingly, the NADH-dependent reduction of rifamycin S to rifamycin SV was also observed within 90 minutes. Thus the reduction to rifamycin SV seems to be an unspecific reaction depending on the reducing conditions of the cells.

Further transformation assays with permeabilized mycelium of *Nocardia mediterranei* N813 were carried out under the same conditions as described above but with the addition of different carbon sources (1~20 mM of glucose, hydroxypyruvate, dihydroxyacetone, 3-phosphohydroxypyruvate, dihydroxyacetone-phosphate, 2-phosphoglycolate, 2-phosphoglycerate, 3-phosphoglycerate, phospho*enol*-pyruvate, glyceraldehyde-3-phosphate, glycerol-1-phosphate, glutamic acid) and cofactors (0.5 mM of NADP, FAD, FMN, thiamine, thiamine pyrophosphate, pyridoxal phosphate, nicotinamide, Mg^{2+}, Fe^{2+}, Fe^{3+}, Cu^{2+}, Mo^{6+}, Zn^{2+}). The carbon sources were tested separately, the cofactors in combination. After incubation and chromatography of the assay mixtures no rifamycin B or L but only rifamycin SV was found irrespective of the carbon sources or cofactors added.

Transformation Experiments Using Cell-free Extracts or Cell Fragments

Transformation experiments were carried out under the same conditions as described above for permeabilized cells. The mycelium of *Nocardia mediterranei* N813 was extracted by X-Press[6] or ultrasonication methods. No formation of rifamycin B or L from rifamycin S was found in the transformation assay (with glycerol and pyruvate as carbon sources) either with crude protein extract or with cell fragments (colony forming units <5% of the total fragments). Negative results were also obtained with protoplasts (protoplast formation with lysozyme).

Transformation Experiments with Intact Mycelium

Specific transformation assays were carried out with washed mycelium (4 mg/ml, dry weight) in tris buffer pH 7.2 (0.05 M) in order to test the biosynthetic hypothesis depicted in Fig. 1.

A mutant strain *Nocardia mediterranei* S/640 produces only rifamycin SV and rifamycin L but no rifamycin B. In the transformation assay rifamycin SV (S) is partially converted into rifamycin L. Surprisingly strain S/640 converts rifamycin O very quickly into rifamycin B and not into rifamycin L as one would expect. Not even traces of rifamycin L are detectable. In a parallel test with strain N813 rifamycin O is also converted to rifamycin B without any traces of rifamycin L. In a test with strain N813 and rifamycin B no formation of rifamycin O or L was observed.

Using the mutant strain *Nocardia mediterranei* P14 (aro$^-$-, P$^-$-mutant derived from strain N813)[7] which is unable to produce rifamycins but still capable of transforming rifamycin S into rifamycin B a sample of (glycolic acid spirolactone-^{14}C)-rifamycin O was synthesized by adding (U-^{14}C)-glycerol to the transformation assay with rifamycin S and oxidizing the resulting (glycolic acid-^{14}C)-rifamycin B with $Na_2S_2O_8$. This preparation of specifically labelled rifamycin O was then tested in a transformation assay with mycelium of strain P14 and after 2 hours of incubation only labelled rifamycin B (25.4 μCi/mmole)

but not even traces of labelled rifamycin L were detected (the radioactivity was measured by liquid scintillation counting of the isolated TLC zones).

Transformation Experiments under Addition of Specific Inhibitors

As only C_3- but not C_2-precursors seem to be incorporated into the glycolic acid moiety of rifamycin B or L one carbon atom has to be eliminated during the transformation reaction. For this elimination a sequence *via* an activated C_2-aldehyde or *via* a system similar to the pyruvate dehydrogenase complex might serve as a model. Coenzyme A and thiamine pyrophosphate would then be possible cofactors. Therefore the influence of potential inhibitors or antagonists[8,9] for thiamine or pantothenic acid were tested for inhibitory effects. In an exploratory test a suspension of *Nocardia mediterranei* N813 was plated on minimal agar in different concentrations. Then paper discs and stripes were dipped into solutions of 1%, 1‰ or 0.1‰ of the test substances and placed on top of the plates. After two weeks of incubation at 28°C the plates were examined for growth and rifamycin production (orange color due to diffusion into the agar). The following effects were observed (the substances were bought from FLUKA, Merck and SERVA):

— D-Pantothenol, barbituric acid, pyrithiamine, *p*-aminobenzoic acid, 3-acetylpyridine, sodium arsenate and arsenic trioxide caused no inhibition of growth or rifamycin synthesis.

— DL-Pantoyltaurine, salicylic acid, mandelic acid and sodium metaarsenite are effective growth inhibitors.

— Only the rifamycin production but not growth was inhibited by sulfathiazole, oxythiamine chloride-hydrochloride and amobarbital.

The substances showing inhibitory effects in this preevaluation were then tested in the transformation assay with rifamycin S(0.2 mM) and mycelium (4 mg/ml) of *Nocardia mediterranei* N813 or P14 in 0.05 M tris buffer pH 7.2 (14~20 hours, 28°C, 250 rpm). Only oxythiamine chloride-hydrochloride (15 mM) caused a complete inhibition of the transformation into rifamycins B and L with strains N813 and P14 (in the case of N813 traces of rifamycin B were found due to residual activities, this effect disappearing at higher concentrations of the inhibitor). This inhibition is partially suppressed when thiamine (15 mM) is also present in the assay but only when thiamine is added before oxythiamine chloride. Once inhibited by oxythiamine chloride the transformation activity cannot be restored by addition of thiamine or of C_3-precursors (20 mM) such as glycerol, pyruvate, hydroxypyruvate, dihydroxyacetone *etc.* However, if the C_3-precursors are added before the inhibitor only a partial inhibition is observed. Sulfathiazole (15 mM) and amobarbital (15 mM) cause a partial inhibition of the transformation reactions. Thiamine does not counteract the inhibition in these cases. DL-Pantoyltaurine and salicylate do not inhibit under the same conditions.

Incorporation Experiments with ^{14}C-Labelled C_3-Precursors in the Transformation Assay

Washed mycelium (8 ml of a suspension containing 3×10^8 mycelium fragments/ml) of *Nocardia mediterranei* N813 or P14 was added to a transformation assay containing in a final volume of 20 ml tris buffer pH 7.2 (0.04 M), rifamycin S (0.2 mM) and labelled C_3-precursor (20 mM, 40~50 μCi/mmole). The commercial precursors with a specific radioactivity of 25~175 mCi/mmole were mixed with unlabelled precursor to yield the required concentration and radioactivity in the assay. The test mixtures were incubated at 28°C, 250 rpm and after 6 hours (maximum concentration of rifamycin L) and after 20 hours (transformation terminated, practically all rifamycin present in the form of rifamycin B with only traces of rifamycin L) extractions were carried out as described above. The extracts were con-

Table 1. Incorporation of ^{14}C-labelled C_3-precursors into the glycolic acid moiety of the rifamycins B and L in transformation experiments with rifamycin S.

Precursor	% Incorporation into rifamycin B*		% Incorporation into rifamycin L*	
	after 6 hours	after 20 hours	after 6 hours	after 20 hours
(U-^{14}C)-Glycerol	36[c]	36~40[a]	~5[c]	~5[a]
(U-^{14}C)-Pyruvate	4.6[b]	7~10[a]	~67[b]	60~70[b]
(1-^{14}C)-Pyruvate	0.7[b]	3.2[c]	~60[b]	—
(2-^{14}C)-Pyruvate	3.4[b]	6.7[c]	~50[b]	—
(3-^{14}C)-Pyruvate	4.1[b]	11.4[c]	~55[b]	—
(U-^{14}C)-L-Serine	—	1.5[b]	—	<2[b]

* The figures express the percentage of the maximum theoretical incorporation. 100% would then mean that for each mole of rifamycin B or L one mole of labelled precursor is directly incorporated without metabolization (rifamycin B or L in this case shows the same specific radioactivity (40~50 μCi/mmole) as the labelled precursor). With the uniformly labelled compounds the upper limit of incorporation is 67% of this theoretical maximum because one carbon atom is lost during the transformation.

[a] Two different assays with strains N813 and P14.

[b] Assay with strain N813.

[c] Assay with strain P14.

centrated and chromatographed as already described. The TLC plates were first scanned on a TLC radioactivity scanner (Berthold LB 2723) and then the TLC zones of rifamycins B and L were isolated, extracted with methanol and the radioactivity was measured by liquid scintillation counting in Instagel.

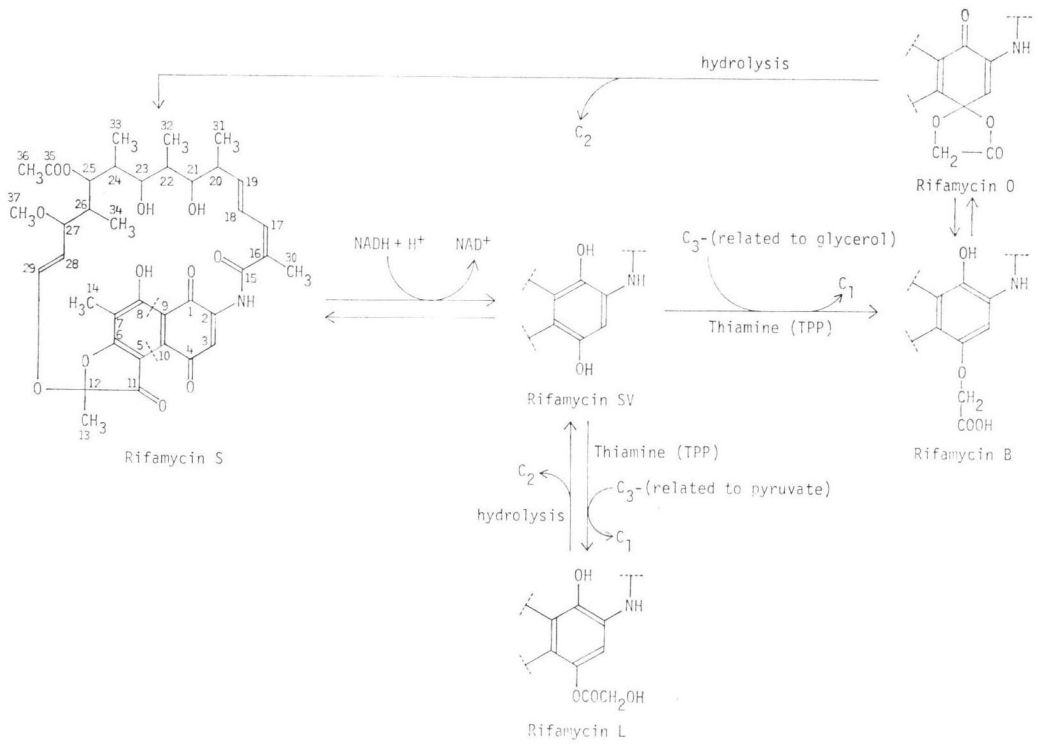
The rifamycins B and SV concentrations were determined with the photometric test described by PASQUALUCCI, *et al.*[10] and modified by BRUGGISSER[11]. In the assays with strain N813 the *de novo* synthesis of rifamycin B (determined in a control experiment without rifamycin S) is subtracted. With strain P14 no *de novo* synthesis occurs. The rifamycins B, SV and L concentrations were also estimated in the TLC test by comparing the assay with different standard concentrations of the corresponding rifamycins. A standard of rifamycin L was prepared from rifamycin S using the mutant strain S/640 (see above) and purified by repeated preparative TLC on silica gel. The spectroscopic properties (UV, IR-spectrum) were in good agreement with reference 2. Alkaline hydrolysis of the product yielded rifamycin S.

(U-^{14}C)-Glycerol, (U-^{14}C)-pyruvate, (1-^{14}C)-pyruvate, (2-^{14}C)-pyruvate, (3-^{14}C)-pyruvate and (U-^{14}C)-L-serine were tested for incorporation into the glycolic acid moieties of rifamycins B and L. The results calculated from the determined rifamycin concentrations and radioactivities expressed in percent of the maximum theoretical incorporation (direct incorporation without further metabolization) are listed in Table 1. Surprisingly glycerol incorporates very well into rifamycin B but badly into rifamycin L, whereas pyruvate incorporates well into rifamycin L and badly into rifamycin B.

Discussion

Our transformation experiments with permeabilized cells of *Nocardia mediterranei* and *E. coli* demonstrate that the reduction of rifamycin S to rifamycin SV is NADH-dependent but not specific for the rifamycin biosynthetic pathway. The reduction is probably due to the reducing conditions of the cells. All the transformation$^-$-mutants of *Nocardia mediterranei* (six strains) isolated in our laboratories, as well as a mutant described by LANCINI and HENGELLER[12], excrete predominantly rifamycin SV and only traces of rifamycin S. The transformation systems for the biosynthetic steps leading from

Fig. 2. Revised biosynthetic model for the transformation of rifamycin S into the rifamycins B and L.



rifamycin SV to rifamycin B or L do not work in the assay using permeabilized mycelium of *Nocardia mediterranei*, crude protein extracts, cell fragments or protoplasts. This indicates that the transformation system might be a membrane associated labile multienzyme complex which only operates in intact cells.

According to the hypothesis in Fig. 1 one would expect that our mutant strain S/640 (producing rifamycins SV and L) is blocked in a biosynthetic step between rifamycin O and rifamycin B and would therefore only transform rifamycin O into rifamycin L. However, the strain was shown to convert rifamycin O very quickly into rifamycin B and not into rifamycin L (the same result is found for rifamycin O and mycelium of the strain N813 or P14). This contradicts the hypothesis in Fig. 1 and rules out rifamycin O as a precursor for both rifamycins B and L.

For this reason we propose the pathway depicted in Fig. 2. In this modified pathway rifamycin O is interpreted as an oxidation product of rifamycin B which can not be detected in the fermentation broth due to the reducing conditions in *Nocardia mediterranei*.

The thiamine antagonist oxythiamine chloride-hydrochloride completely inhibits both the transformation of rifamycin S into rifamycin B and its transformation into rifamycin L. The fact that this inhibition is partially counteracted by thiamine (or by C_3-precursors) but only when oxythiamine chloride is added after thiamine (or the C_3-precursors) indicates that oxythiamine chloride irreversibly binds to a thiamine- or thiamine pyrophosphate (TPP)-dependent transformation enzyme. Another argument for the participation of thiamine or TPP in the transformation reactions is provided by an auxotrophic strain *Nocardia mediterranei* T137 (isolated by R. ROOS and T. SCHUPP). This strain requires thiamine for growth and in the transformation assay the rifamycins B and L are synthesized from rifamycin S only when thiamine is added. TPP is a well-known cofactor for decarboxylating enzymes.

Based on the hypothesis in Fig. 1 one would expect to find identical levels of radioactivity incorporated for both rifamycins B and L in transformation experiments with labelled C_3-precursors. However, this is not the case either with (U-^{14}C)-glycerol or with the different labelled pyruvates.

This again rules out rifamycin O as a common progenitor and supports the model in Fig. 2. The fact that glycerol predominantly labels the glycolic acid moiety of rifamycin B and pyruvate the glycolic acid moiety of rifamycin L, indicates that two completely different biosynthetic pathways using different C_3-precursors for the biosynthesis of the glycolate ether rifamycin B and for the glycolate ester rifamycin L must be operating. Considering the percentage of incorporation (Table 1) one would expect a precursor related to glycerol for the glycolic acid moiety in rifamycin B and a (symmetric) precursor related to pyruvate for rifamycin L.

In contrast to rifamycin S, 3-hydroxyrifamycin S[13] and 8-deoxy-3-hydroxyrifamycin S[14] are not transformed into the corresponding 3-hydroxyrifamycins of the B- or L-type by the mycelium of *Nocardia mediterranei*.

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