Supporting Information

Process Investigations on the One-Pot Synthesis of Rifamycin S Avoiding Chlorinated Solvents

Sebastian A. Löw,[a] Bettina M. Nestl,[a] Martin J. Weissenborn,[a] Ferdinand Zepeck,[b] and Bernhard Hauer*[a]

[a] *Institute of Technical Biochemistry, University of Stuttgart, Allmandring 31, 70569 Stuttgart, Germany. E-mail: bernhard.hauer@itb.uni-stuttgart.de; Fax: +49 711 68563196; Tel: +49 711 68563193*
[b] *Sandoz GmbH, Biocatalysis Lab, Biochemiestraße 10, 6250 Kundl, Austria.*

Contents

Materials and Methods

1.1 Chemicals and enzymes

Solvents, buffer components, APS, H_2O_2 and $FeSO_4$ were obtained from Sigma-Aldrich (Schnelldorf, Germany) and Roth (Karlsruhe, Germany). Rifamycin B, rifamycin O and rifamycin S were kindly provided by Sandoz (Kundl, Austria). Analysis was carried out on a Shimadzu LC/MS-2010 system or on an Agilent 1200 series HPLC system using a Chromolith Performance RP-18 endcapped 100-4.6 HPLC column (Merck KGaA, Darmstadt, Germany). The measurements were performed at room temperature with 10 mM ammonium acetate (pH 6.5)/MeOH in a ratio of 2/3 as mobile phase and a flow rate of 1 mL min^{-1}. The analytes were detected and quantified using mass spectrometry or the DAD area.

1.2 LC/MS and HPLC sample preparation

The organic phase obtained after extraction was evaporated under a nitrogen stream to yield a dry product mixture. 400 µL of MeOH were added and thoroughly shaken to ensure solubilisation. Then 600 µL of ammonium acetate buffer (10 mM, pH 6.5) were added and 1 µL of the sample was analyzed via LC/MS or HPLC. The conversions were determined by LC/MS based on the response in the mass spectrometer.

2.1 Determination of oxidation reaction using Fenton's reagent

20 mg rifamycin B (0.026 mmol) were dissolved in 9.5 mL KPi buffer (10 mM, pH 8) containing 0.5 mL methanol and 0.6 mL H_2O_2 (0.15 % m/v). The reaction was per-formed in presence and absence of 0.5 mL 0.54 mM $FeSO_4$ solution (final concentration 0.027 mM) and was analyzed by LC/MS after 30, 60, 120 and 180 min (see figure S1).

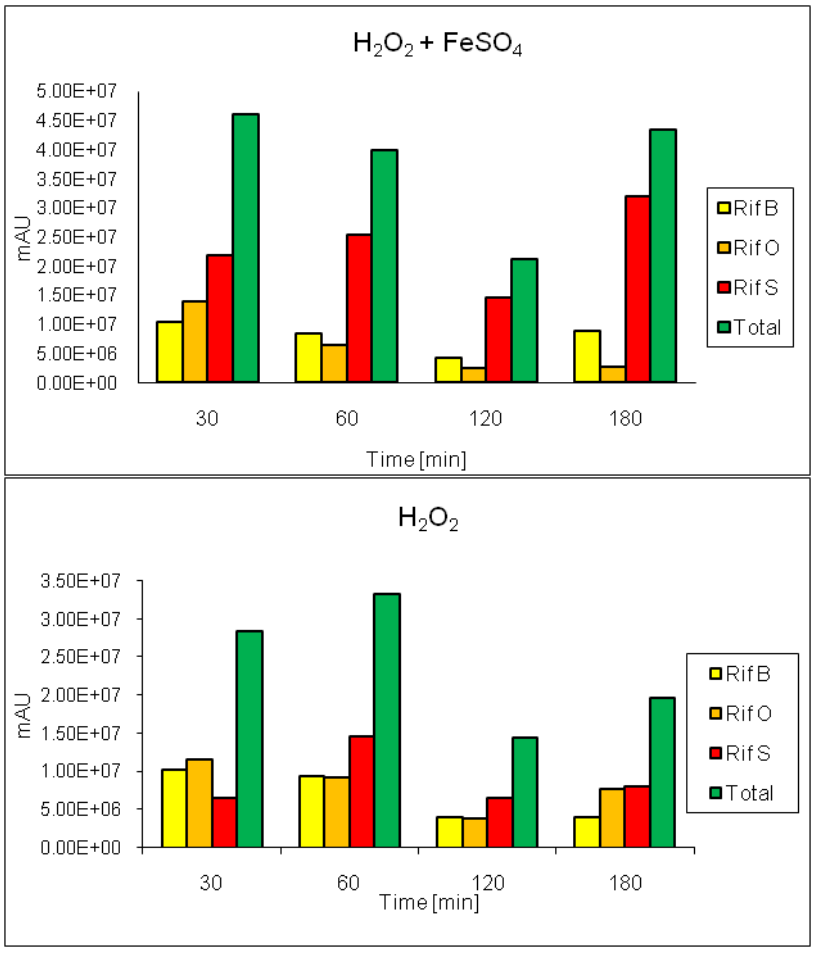

Figure S1: LC/MS Analysis of the conversion of rifamycin B (rif B) to rifamycin O (rif O) and rifamycin S (rif S) with fenton's reagent $FeSO_4$ and hydrogen peroxide (top) and only hydrogen peroxide (bottom). Total shows the sum of all rifamycin compounds.

2.2 Determination of the oxidation reaction using ammonium persulfate (APS)

20 mg rifamycin B (0.026 mmol) were dissolved in 10 mL KPi buffer (10 mM, pH 8) containing 0.5 mL methanol. Then 6 mg ammonium persulfate (APS, 0.026 mmol) were added. The reaction was analyzed after 30, 60, 120 and 180 min.

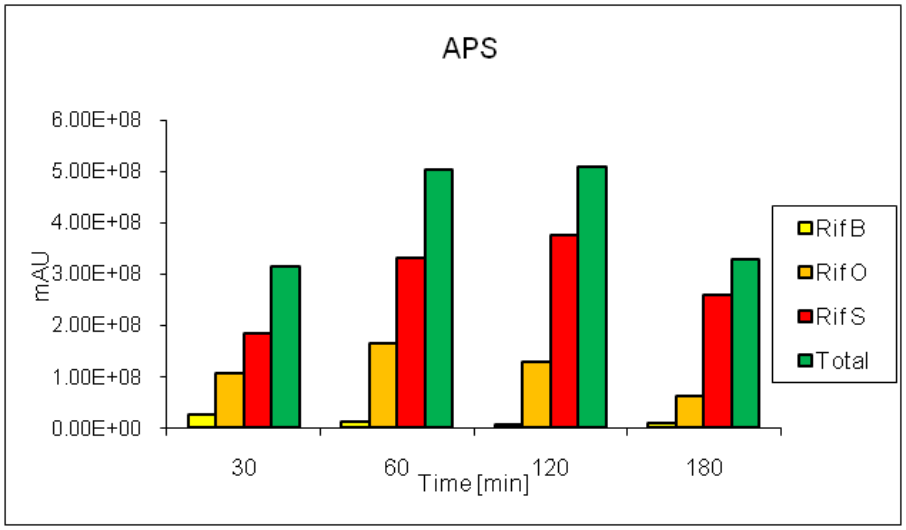

Figure S2: LC/MS Analysis of the conversion of rifamycin B (rif B) to rifamycin O (rif O) and rifamycin S (rif S) with APS. Total shows the sum of all rifamycin compounds.

3 Solvent System

Investigation of solvent systems. To 10 mg rifamycin B (0.013 mmol) 500 µL of the first solvent (acetone, acetonitrile, γ-butyrolactone, dioxane, methanol, THF, DMSO or DMF) were added and after intense mixing 500 µL of the corresponding second solvent (vide supra) were added which led to a final rifamycin B concentration of 13 mM. After repeated mixing the sample was analyzed by eye and rated on a scale from -- (-2, insoluble) to +++ (+3, completely soluble) (see table S1).

Table S1: Matrix of investigated solvent combinations for dissolving rifamycin B.

	Acetone	Acetonitrile	γ-Butyro-lactone	Dioxane	Methanol	THF	DMSO	DMF
Acetone	-2	-2	-1	-1	0	-2	2	2
Acetonitrile	-2	-2	-1	-2	0	-2	2	2
γ-Butyrolactone	1	1	1	-1	1	-1	2	3
Dioxane	-1	-1	1	-1	-1	-1	1	1
Methanol	1	-1	2	1	2	1	1	3
THF	-2	-2	1	-2	1	1	3	3
DMSO	2	2	2	2	2	2	2	3
DMF	2	2	2	2	2	2	2	3

4 Reaction Conditions

4.1 Rifamycin O acetal cleavage to form rifamycin S

4.1.1 Reaction under mild alkaline conditions

To 7.4 mL KPi buffer (100 mM, pH 7.5) 600 µL rifamycin O solution (DMSO, 36 mM) were added to obtain a final concentration of 2.7 mM. The mixture turned orange and was stirred for 60 minutes at 60 °C. The products were extracted with 4 mL of ethyl acetate and analyzed by HPLC.

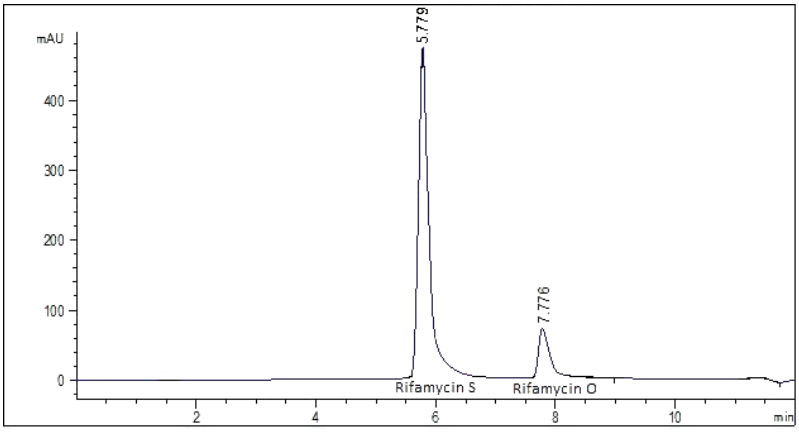

Figure S3: HPLC chromatogram after 1 hour treatment of rifamycin O with alkaline solution

4.1.2 Variation of buffer concentration and pH

10 µL rifamycin O stock solution (DMSO, 100 mM) were mixed with 590 µL KPi buffer (for pH and concentration see Figure S4) to a final concentration of 1.7 mM. The mixtures were stirred at 37 °C for 60 minutes. The reactions were stopped by the addition of 1 mL ethyl acetate and analyzed by HPLC.

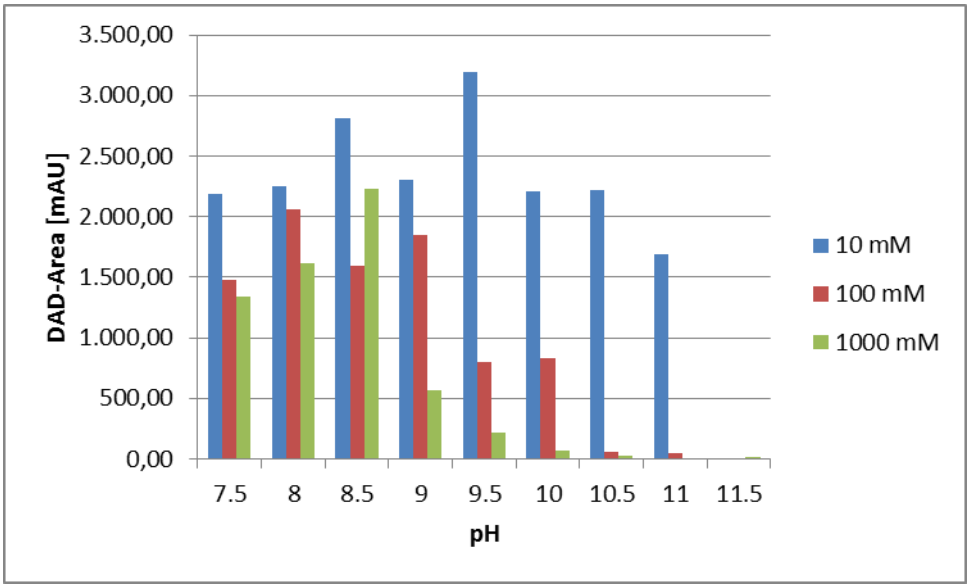

Figure S4: HPLC analysis of the rifamycin O to rifamycin S reaction at different KPi concentrations (10 mM, 100 mM and 1 M) and different pH values (7.5 to 11.5). The bars show the formation of rifamycin S.

4.1.3 Reaction temperature

10 µL of a rifamycin O stock solution (DMSO, 100 mM) were mixed with 590 µL of KPi buffer (pH 7.5 100 mM) to a final concentration of 1.7 mM. The mixtures were stirred at the temperatures and for the times corresponding Figure S5. The reactions were stopped by the addition of 1 mL ethyl acetate and analyzed by HPLC.

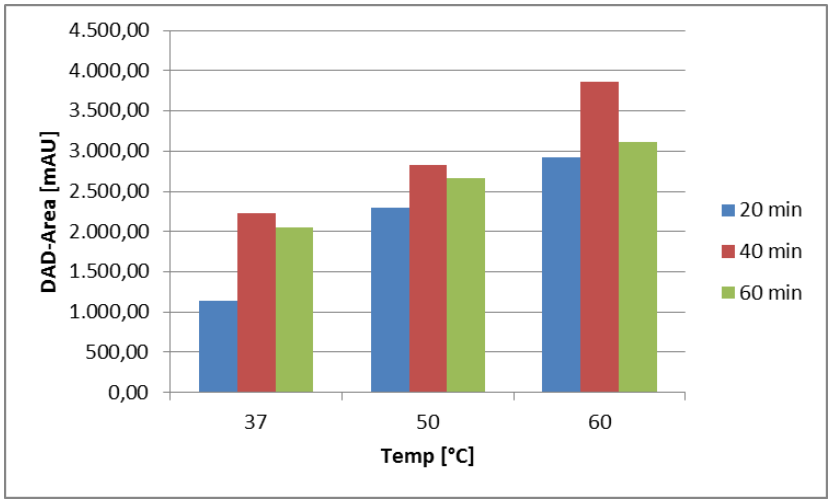

Figure S5: HPLC analysis of the rifamycin O to rifamycin S reaction at different temperatures and reaction times. The bars show the formation of rifamycin S.

4.2 Process options

4.2.1 Minimal methanol content (process 1)

30 mg Rifamycin B (0.039 mmol) were dissolved in different amounts of methanol (see figure S6). The obtained solutions were mixed with 9 mg APS (0.039 mmol) and 5 mL KPi buffer (10 mM, pH 9.5). The mixtures were stirred at 60 °C for 4 h. Then 500 µL of sample were extracted with 500 µL ethyl acetate and analyzed by LC/MS. 5.6 mM (70 % yield) rifamycin S were obtained.

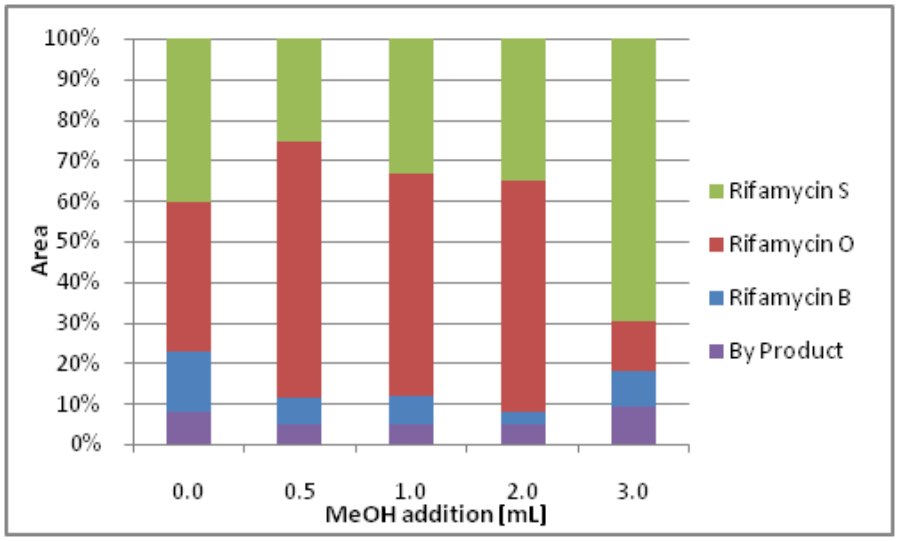

Figure S6: LC/MS analysis of reactions with varying MeOH content. 3.0 mL correspond to 37.5 % MeOH and shows a significant higher rifamycin S amount compared to 2.0 mL due to an improved solubilisation and hence hydrolysis of rifamycin O.

4.2.2 Further investigation of methanol content

150 mg Rifamycin B (0.198 mmol) were dissolved in 7, 8 or 9 mL MeOH. After addition of KPi (pH 9.5, 10 mM) to a final volume of 10 mL (19.8 mM final concentration of rifamycin B) and 45 mg APS the reaction was performed at 60 °C for 4 h and analyzed by LC/MS (see figure S7).

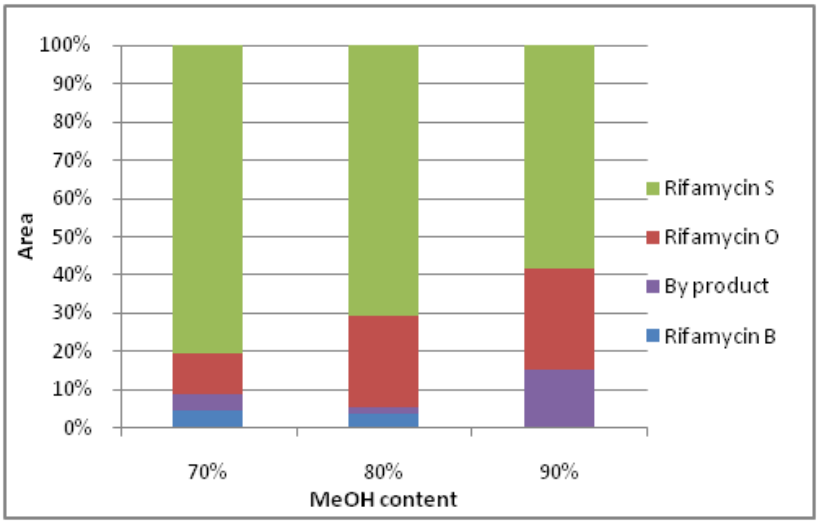

Figure S7: Product distribution at 70, 80 and 90 % *v/v* MeOH. The oxidation improves but hydrolysis decreases with higher MeOH content.

4.2.3 High methanol content (process 2)

40 mg rifamycin B (0.052 mmol) and 12 mg of APS (0.052 mmol) were added to 400 µL MeOH and 4 µL of KPi buffer (10 mM, pH 9.5, 1 % *v/v*). The obtained suspension was heated for 20 min at 60 °C. Then 40 µL of the sample were quenched with 100 µL KPi buffer (pH 9.5, 10 mM) and analyzed by LC/MS. 89.8 mM (68 % yield) rifamycin S were obtained (see figure S8).

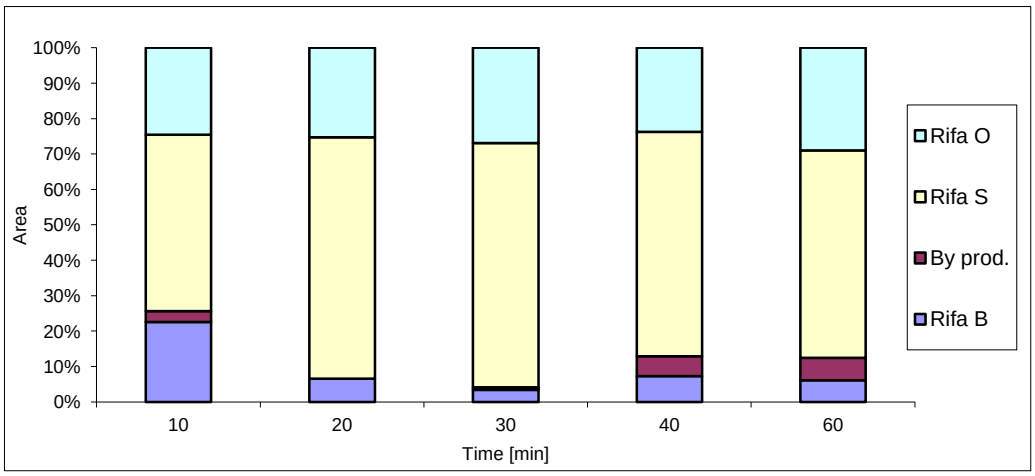

Figure S8: Analysis of the reaction from rifamycin B to rifamycin S in 99 % MeOH at different time points.

4.2.4 Rifamycin B oxidation in absolute methanol under Argon

40 mg rifamycin B (0.052 mmol) and 12 mg APS (0.052 mmol) were added under argon atmosphere to 400 µL absolute MeOH (dried with CaH_2). The obtained suspension was heated for 20 min at 60 °C. Then 40 µL of the sample were quenched with 100 µL KPi buffer (pH 9.5, 10 mM) and analyzed by LC/MS. (Data not shown)

4.2.5 Rifamycin B to rifamycin S reaction in different solvent systems

40 mg rifamycin B (0.052 mmol) and 12 mg of APS (0.052 mmol) were added to 400 µL - 1000 µL MeOH as well as to 1000 µL acetonitrile, DMSO, ethanol or isopropyl alcohol. The mixtures were shaken for 10 min at 70 °C and analyzed by LC/MS (see figure S9).

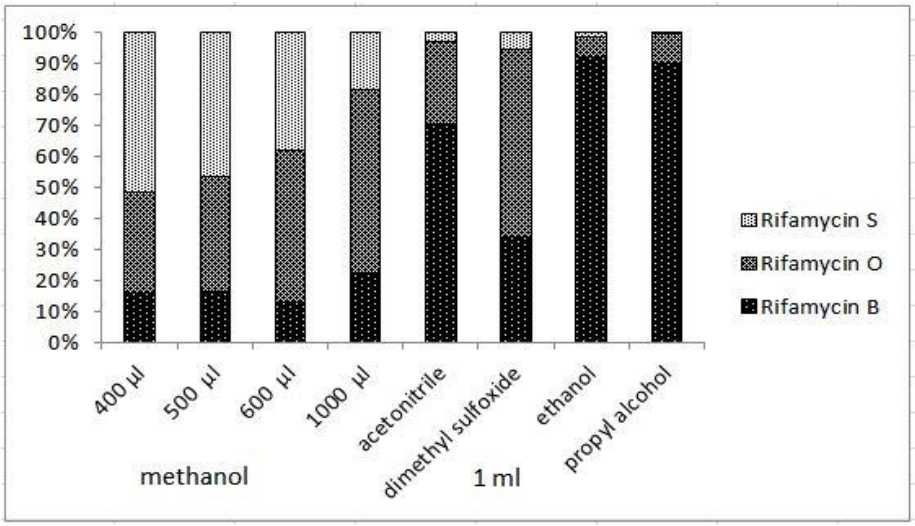

Figure S9: Product distribution of the reaction from rifamycin B to rifamycin S in different solvent systems.

Calculation of the technical process data:
Rifamycin O formation:
10,000 mL with a rifamycin B titre of 2,800 µg/mL equals 28 g Rifamycin B
After **4 h** 30.5 g product with 84 % rifamycin O equals **25.62 g Rifamycin O**.
Yield = (25.62/753) / (28/755) = **91.7 %**

Rifamycin S formation:
642.86 mL with a rifamycin O titre of 70,000 µg/mL equals 45 g Rifamycin O
After **1.8 h** 36 g product with 98 % rifamycin O equals **35.28 g Rifamycin S**.
Yield = (35.28/695) / (45/753) = **85 %**

Combined:
10,000 mL of rifamycin B broth can yield **25.62 g rifamycin O** in 4 h which can yield **20 g of rifamycin S** after **1.8 h** hydrolysis in additional 366 mL solvent.
10.366 l; 20 g product; 5.8 h → **1.9 g L^{-1} and 0.33 g $L^{-1}h^{-1}$ overall yield: 78 %**

Calculation of process 1 data:
3.125 mL with a rifamycin B titre of 2,800 µg/mL equals 8.75 mg Rifamycin B.
Addition of 1.875 mL MeOH to a final volume of **5 mL**
After **4 h** the reaction yielded **70 % rifamycin S**.
((0.00875/755)*0.7)*695 = **5.6 mg rifamycin S**
0.005 l; 0.0056 g product; 4.0 h → **1.12 g L^{-1} and 0.28 g $L^{-1}h^{-1}$ overall yield: 70 %**

Calculation of process 2 data:
0.4 mL with a rifamycin B titre of 100,000 µg/mL equals 40 mg Rifamycin B.
After **0.33 h** the reaction yielded **68 % rifamycin S**.
((0.040/755)*0.68)*695 = **25 mg rifamycin S**
0.0004 l; 0.025 g product; 0.33 h → **62.5 g L^{-1} and 189.4 g $L^{-1}h^{-1}$ overall yield: 68 %**