Intermediates of rifamycin polyketide synthase produced by an *Amycolatopsis mediterranei* mutant with inactivated *rifF* gene

Ansgar Stratmann, Christiane Toupet, Wolfgang Schilling, René Traber, Lukas Oberer and Thomas Schupp

Author for correspondence: Thomas Schupp. Tel: +41 61 32 47903. Fax: +41 61 32 43279.
e-mail: thomas.schupp@pharma.novartis.com

Novartis Pharma AG,
Research, Core Technology
Area, CH-4002 Basel,
Switzerland

Rifamycin B biosynthesis in *Amycolatopsis mediterranei* N/813 was inactivated by introducing a small deletion in the *rifF* gene situated directly downstream of the rifamycin polyketide synthase (PKS) gene cluster. The corresponding mutant strain produced a series of linear intermediates of rifamycin B biosynthesis that are most probably generated by obstruction of the normal release of the end product of the rifamycin PKS. This result provides evidence that the *rifF* gene product catalyses the release of the completed linear polyketide from module 10 of the PKS and the intramolecular macrocyclic ring closure by formation of an amide bond, as indicated by sequence similarity of this protein to amide synthases. The chemical structures of the new rifamycin polyketide synthase intermediates released from modules 4 to 10 were determined by spectroscopic methods (UV, IR, NMR and MS) and gave insight into the reaction steps of rifamycin ansa chain biosynthesis and the timing of the formation of the naphthoquinone ring. The intermediates released from modules 6 and 8 were isolated as lactones formed by the terminal carboxyl group; proton NMR double resonance and ROESY (rotated frame nuclear Overhauser enhancement spectroscopy) experiments enabled the deduction of the relative configurations in the linear chain which correspond to the known absolute stereochemistry of rifamycin B.

Keywords: antibiotic biosynthesis, ansamycins, amide synthase, gene replacement, pathway engineering

INTRODUCTION

Rifamycins are clinically important ansamycin antibiotics, composed of a naphthalenic chromophore spanned by a long aliphatic ansa chain. The rifamycins and the semisynthetic drugs derived from them exert their antibiotic activity by specific inhibition of bacterial DNA-dependent RNA polymerase (Wehrli, 1977). At higher concentrations, these antibiotics also inhibit the RNA-dependent DNA polymerase of retroviruses (Szabo *et al.*, 1976). Although rifamycins are primarily used against *Mycobacterium tuberculosis* and *Myco-*

bacterium leprae, causative agents of tuberculosis and leprosy, respectively, they are also active against a variety of other organisms, including bacteria and viruses (Szabo *et al.*, 1976; Oppenheim *et al.*, 1986; Barakett *et al.*, 1993; Bachs *et al.*, 1992). Furthermore, it was shown recently that rifampicin-containing regimens are able to cure staphylococcal implant-related infections (Zimmerli *et al.*, 1998).

The identification and sequencing of the rifamycin polyketide synthase (PKS) gene cluster by our group (Schupp *et al.*, 1998) and by August *et al.* (1998) disclosed five genes, *rifA–rifE*, responsible for the synthesis of the rifamycin polyketide chain. The encoded multifunctional enzymes contain a total of ten PKS modules, which use 3-amino-5-hydroxybenzoic acid (AHBA) as a starter unit to catalyse successive rounds of polyketide

Abbreviations: PKS, polyketide synthase; AHBA, 3-amino-5-hydroxybenzoic acid.

chain elongation to build the polyketide backbone of the rifamycin molecule.

Our interest in further studying rifamycin B biosynthesis led us to undertake the inactivation of the *rifF* gene, situated directly downstream of the PKS genes, and to analyse the effect of this mutation on rifamycin biosynthesis. The *rifF* gene product has been characterized as rifamycin amide synthase by sequence homologies to different arylamine *N*-acetyltransferases (August *et al.*, 1998) and the putative function of the Riff protein in rifamycin biosynthesis was suggested to be a cyclase, catalysing the formation of an intramolecular amide bond between the carboxyl end of the synthesized linear polyketide chain and the amino group of the AHBA starter unit (Chen *et al.*, 1999).

In this paper we describe the inactivation of the *rifF* gene by introducing a 9 bp deletion into the chromosomal *rifF* gene of the rifamycin B high-producing strain *Amycolatopsis mediterranei* N/813 (Ghisalba *et al.*, 1984). We were able to isolate and characterize a series of linear intermediates of rifamycin B biosynthesis. Our results confirm the putative function of Riff postulated from sequence homology, and give new insights into the succession of biosynthetic steps during the rifamycin B biosynthesis.

METHODS

Bacterial strains and culture conditions. *Amycolatopsis mediterranei* N/813 (Ghisalba *et al.*, 1984) a rifamycin high-producing strain (from the Novartis strain collection) and mutants of this strain were propagated in liquid medium NL148 (Schupp & Divers, 1986) or on YMG agar (4 g yeast extract, 10 g malt extract, 4 g glucose and 18 g agar in 1 l water, pH 7.3). Strain N/813 was cultivated in liquid YMG medium for genomic DNA isolation and in SOC medium (Hanahan, 1983) for preparation of electrocompetent cells. Cultures for rifamycin production were grown in 40 ml liquid medium FBR42 [90 g sucrose, 70 g glucose, 32 g soybean flour, 9.5 g CaCO₃, 7.5 g (NH₄)₂SO₄, 1 g MgSO₄·7H₂O, 5 g propylene glycol, 1 g sodium diethylbarbituric acid, 1 ml trace elements solution (1 g Fe₂(SO₄)₃·7H₂O, 0.33 g CuSO₄·5H₂O, 5 g ZnSO₄·7H₂O, 0.4 g MnSO₄·H₂O, 0.2 g CoCl₂·6H₂O and 0.1 g ammonium molybdate tetrahydrate in 1 l water) and 1 ml antifoam B (Sigma) in 1 l water, pH 7.0] in 200 ml shake flasks (one baffle).

Escherichia coli DH5 α (Hanahan, 1983) was used for propagation of plasmids and cloning of DNA. *E. coli* ET12567 (*dam*[−] *dcm*[−] *hsdM*[−]; MacNeil *et al.*, 1992) was used for preparation of plasmid DNA for transfer into *A. mediterranei* by electroporation. *E. coli* was grown in LB medium (Miller, 1972). For plasmid maintenance, tetracycline (25 μ g ml^{−1}), chloramphenicol (50 μ g ml^{−1}) or ampicillin (50 μ g ml^{−1}) were added where appropriate. Cultivation temperatures were 37 °C for *E. coli* and 28 °C for *A. mediterranei*.

Plasmids and construction of new cloning vectors. The *E. coli* cloning vector pUCBM21 (Boehringer Mannheim) was used for the construction of suicide plasmids for gene disruption and gene replacement in *A. mediterranei*. To remove the single *Pst*I site in the multiple-cloning site of pUCBM21, the plasmid was cleaved with *Pst*I, treated with Klenow enzyme

(Boehringer Mannheim) and religated, giving plasmid pBM21*. The *aacC4* cassette (Blondelet-Rouault *et al.*, 1997), which confers apramycin resistance in *E. coli* and *A. mediterranei*, was introduced into the *Hind*III site of pBM21* as a 1.7 kb *Hind*III DNA fragment resulting in plasmid pASN3.

Electroporation of *A. mediterranei* N/813. *A. mediterranei* N/813 cells were harvested after 2 d growth in 40 ml liquid SOC medium and washed three times in 10 % glycerol at room temperature. The cells were resuspended in 4 ml 10 % glycerol containing 40 μ g lysozyme ml^{−1} and incubated for 20 min with gentle shaking (50 r.p.m.). After one washing step with 4 ml 10 % glycerol at 4 °C the cells were resuspended in 1.6 ml ice-cold 10 % glycerol and used immediately for electroporation. Plasmid DNA (0.5–1.0 μ g) was heat denatured (5 min at 95 °C) before it was mixed with 200 μ l cell suspension on ice. The mixture was immediately transferred into a 0.2 cm electrocuvette and a single electric pulse (Gene Pulser II; Pulse Controller Plus; Bio-Rad) was applied (1.5–2.0 kV; 1000 Ω ; 25 μ F). The electroporated mycelium was immediately diluted with 0.8 ml YMG medium, transferred to 10 ml YMG medium and incubated for 14–16 h at 28 °C with vigorous shaking (250 r.p.m.). The whole culture was then plated on a YMG agar plate containing 50 μ g apramycin ml^{−1}.

Genetic procedures. Chromosomal DNA isolation was carried out according to Pospiech & Neumann (1995). DNA fragments were recovered from agarose gels using the Qiaex Kit (Qiagen) and DNA labelling was performed with [³²P]dCTP using a Nick Translation Kit (Boehringer Mannheim). Southern hybridization experiments were performed using Hybond-N+ nylon membranes (Amersham) following the manufacturer's alkali blotting protocol. Genetic procedures involving *E. coli*, and *in vitro* DNA manipulations were performed according to standard protocols (Sambrook *et al.*, 1989).

DNA sequencing. DNA sequencing was by the dideoxynucleotide chain-termination method with an automated laser fluorescence sequencer (Applied Biosystems, ABI PRISM377). Sequencing reactions were done with the standard reverse primer using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Perkin Elmer) according to the supplier's instructions.

HPLC analysis. For HPLC analysis of metabolites, *A. mediterranei* culture broths were mixed 1:1 with butanol and shaken for 4 h at room temperature (150 r.p.m.). After centrifugation (10 min, 5000 g) the butanol phase was separated and 10–20 μ l was analysed by HPLC (Merck model L-6200) using a 4.6 $\times$ 100 mm Symmetry C18, 3.5 mm column (Waters) with gradient elution (5 min linear gradient of 0–50 % acetonitrile, hold for 2 min, 5 min linear gradient of 50–100 % acetonitrile, hold for 3 min, 2 min linear gradient of 100–0 % acetonitrile) using a phosphate (0.55 % orthophosphoric acid, pH adjusted to 4.5 with triethylamine)/acetonitrile buffer system. The region from 200 to 500 nm was scanned with a DAD-System L-4500 (Merck) and the spectra were compared with a rifamycin B standard.

Isolation of AHBA and rifamycin-biosynthesis intermediates. The culture broth of a 1 l fermentation of mutant 3/1 in FBR42 medium was extracted twice with ethyl acetate (1 l each) and the crude extract was defatted with hexane (3 $\times$ 500 ml). The complex mixture (5.4 g) was submitted to gel filtration on Sephadex LH20 with methanol. Fractions 3 and 4 (1.87 g), containing UV-active compounds, were further separated by reversed-phase chromatography on LiChrospher RP18 12 μ m (200 g) using a gradient composed of water–triethylammoniumformate buffer (A) and acetonitrile (B) from 0–60 % B. Final purification was performed by pre-

Fig. 1. Genomic region of *A. mediterranei* containing the *rifF* gene and scheme for *PstI* deletion in *rifF* and gene replacement. (a) Restriction map of the *rifF* gene, the upstream PKS genes *rifC*-*E* and the downstream *rifG* gene (aminodehydroquinase synthase), cloned in cosmid 3. (b) Subcloned 5.1 kb *KpnI*-*EcoRI* DNA fragment in the suicide plasmid pASN4.1 and the respective chromosomal region of mutant 3/1, with the mutated *rifF* gene introduced by gene replacement through a double crossover (lower map). The short deletion introduced in *rifF* is shown as a black box. The indicated 1.1 kb *BglII*-*SmaI* DNA fragment was used for DNA sequencing to analyse the deletion in the *rifF* gene. The indicated 6.2 kb *PstI* chromosomal DNA fragment of mutant 3/1 was detected by Southern hybridization (Fig. 2). Only one *SmaI* site, relevant for the work, is shown and the *EcoRI** site originates from the cosmid vector pWE15. The thin lines represent cosmid DNA on either side of the inserts.

parative TLC using the systems $\text{CH}_2\text{Cl}_2/\text{MeOH}/\text{H}_2\text{O}$ 80:17.5:2 and 88:11:1 by vol.

The yields were 2.3 g AHBA, 90 mg compound 1, 18 mg 2, 7 mg 4 (lactone), 39 mg 5, 21 mg 6 (lactone), 11 mg 7 and 9 mg 8. From a cultivation on a 5 l scale which was worked up analogously, 44 mg compound 3 was isolated.

Spectroscopic measurements. The UV-absorption spectra were recorded in methanol with a Perkin Elmer Lambda 9 spectrophotometer. The IR spectra were taken in KBr pellets on a FT-IR spectrometer BRUKER IFS 66. $^1\text{H}/^{13}\text{C}$ NMR spectra were recorded in d_6 -DMSO on a Bruker Avance DMX-500 spectrometer at 500 MHz with tetramethylsilane as the internal standard. Electrospray mass spectra were measured with a Finnigan SSQ-7000 MS spectrometer.

RESULTS

Construction of a small deletion in the *rifF* gene of *A. mediterranei*

The *rifF* gene is located directly downstream of the gene cluster for the rifamycin PKS (August *et al.*, 1998). The gene contains one *PstI* site in the 780 bp ORF, 81 bp after the GTG start codon. This site was chosen to introduce a short deletion in *rifF*. For this mutagenesis a 5.1 kb *KpnI*-*EcoRI* fragment containing the *rifF* gene was isolated from the previously described cosmid 3 (Schupp *et al.*, 1998) (Fig. 1) and subcloned into pASN3, a pUCBM21 derivative containing the apramycin resistance gene (*aacC4*) that allows selection in *E. coli* and *A. mediterranei* (see Methods). The resulting plasmid,

Fig. 2. Hybridization pattern of *Pst*I-digested chromosomal DNA from *A. mediterranei* N/813 (lane 1) and the two mutant strains 4/1 (lane 2) and 3/1 (lane 3) with the 5.1 kb *Eco*RI-*Kpn*I DNA fragment (Fig. 1) as the radioactive probe. Fragment size was determined by reference to a 1 kb DNA ladder and *Hind*III-digested lambda DNA (Gibco-BRL).

pASN4, containing the chromosomal *Kpn*I-*Eco*RI fragment of *A. mediterranei*, was used to introduce the deletion in *rifF* by *Pst*I digestion, treatment with Klenow enzyme and self-ligation resulting in plasmid pASN4.1 that has lost the *Pst*I site.

Introduction of the mutated *rifF* gene into *A. mediterranei* by gene replacement

To introduce the mutated *rifF* gene into *A. mediterranei* N/813, electrocompetent cells of this strain were transformed with plasmid pASN4.1 by electroporation. After 8 d growth of the transformed cells on YMG agar plates with apramycin, distinct single colonies became visible. Five of these apramycin-resistant *A. mediterranei* transformants were analysed by Southern hybridization using the *aacC4* gene of pASN4.1 as the radioactive probe. This analysis showed that these transformants contained pASN4.1 integrated into the chromosome by a single crossover through homologous recombination (data not shown). One of these *A. mediterranei* transformants was selected for further analysis and cultivated in the production medium FBR42. HPLC analysis of the extract showed that rifamycin B production is blocked in this strain.

To enable the second crossover, the selected *A. mediterranei* transformant was cultivated four times in

liquid NL148 medium without apramycin (5% inoculation and 3 d cultivation each time). Apramycin-sensitive mutants were then detected by replica plating single colonies, derived from the last NL148 culture, on YMG agar with and without apramycin. From about 1000 colonies tested, two apramycin-sensitive mutants were detected and chosen for further analysis.

Characterization of two double-crossover mutants

Southern-blot analysis of *Pst*I-digested chromosomal DNA using the 5.1 kb *Kpn*I-*Eco*RI DNA fragment containing *rifF* as the probe clearly demonstrated the elimination of the single *Pst*I site in the *rifF* gene in the chromosome of mutant 3/1. The hybridization pattern of this mutant showed only one band, 6.2 kb in size. In contrast to this, the parental strain N/813 and the other mutant, 4/1, gave two hybridizing *Pst*I fragments, 3.7 kb and 2.5 kb in size (Fig. 2). These hybridization data are in agreement with the restriction pattern expected for replacement of the DNA fragment containing the non-mutated *rifF* gene with the one lacking the internal *Pst*I site in mutant 3/1 and the reconstitution of the parental *rifF* gene in mutant 4/1. This conclusion was confirmed by HPLC analysis of rifamycin B production by the two mutants. Mutant 3/1 does not produce rifamycin B in the production medium FBR42 whereas mutant 4/1 produces about the same amount as the parental strain N/813.

The deletion in the *rifF* gene of mutant 3/1 was analysed by DNA sequencing. To obtain this information, the internal 1.1 kb *Bgl*II-*Sma*I DNA fragment out of the 5.1 kb *Kpn*I-*Eco*RI fragment of pASN4.1 was cloned into the *Bam*HI-*Sma*I sites of pUCBM21 in *E. coli*. The resulting plasmid was named pASN4.2 and the sequence of the cloned DNA fragment was determined. As shown in Fig. 3, mutant 3/1 has an unexpected 9 bp deletion in the *rifF* gene, derived from 3' exonuclease activity of the Klenow enzyme. This mutation results in a loss of three amino acids (Q, K and S) and a change from L to R at position 29 of the RifF protein (Fig. 3).

Isolation and physico-chemical properties of rifamycin biosynthesis intermediates produced by mutant 3/1

To analyse the effect of the truncated RifF protein on rifamycin biosynthesis of mutant 3/1, the strain was cultivated in the vegetative medium NL148 for 3 d and transferred into 1 l FBR42 medium in shake flasks. After 10 d cultivation, an aliquot of the culture broth was extracted with butanol. HPLC analysis of the extract showed that mutant 3/1 does not produce rifamycin B, which is the main rifamycin produced by the parental strain N/813. On the other hand, several new compounds were detected by HPLC that were not found in strain N/813 and showed UV absorption spectra typical for compounds with a naphthoquinone chromophore.

The culture broth of the mutant 3/1 was extracted with

rifF *A. mediterranei* GTG AAC GTG ... 69 bp ... GCG AAG **CTG CAG** AAG AGC CAC CTG ATG GCG ...
M N V A K L Q K S H L M A ...
1 29

rifF mutant 3/1 GTG AAC GTG ... 69 bp ... GCG AAG C GC CAC CTG ATG GCG ...
M N V A K R H L M A ...
1 29

Fig. 3. Comparison of the N-terminal parts of the Riff proteins produced by *A. mediterranei* N/813 and the mutant strain 3/1. The 9 bp deletion inside the *riff* gene and the change from L to R at position 29 inside the mutated Riff protein are indicated.

Fig. 4. Structures of rifamycin biosynthesis intermediates **1** to **8** released from modules 3 to 10.

Table 1. Physico-chemical and chromatographical properties of rifamycin biosynthesis intermediates **1** to **8** released from modules **3** to **10**

ESI-MS, electrospray ionized mass spectroscopy.

(Intermediate) from module	Formula	ESI-MS (<i>m/z</i>)	UV λ max. (MeOH; nm)	TLC <i>R_F</i> [*]	HPLC† (min)
(1) 3	C ₁₅ H ₁₉ NO ₆ (309)	291 (M-H ₂ O)	295, 205	0.33	0.65
(2) 4	C ₁₈ H ₁₇ NO ₆ (343)	299 (M-COOH)	464, 323, 274, 219	0.40	5.02
(3) 5	C ₂₁ H ₂₃ NO ₇ (401)	401	461, 323, 274, 222	0.38	4.91
(4) 6 (lactone)	C ₂₄ H ₂₇ NO ₇ (441)	441	461, 323, 274, 222	0.67	5.31
(5) 7	C ₂₇ H ₃₅ NO ₉ (517)	517	461, 323, 271, 226	0.36	5.25
(6) 8 (lactone)	C ₃₀ H ₃₉ NO ₉ (557)	557	461, 323, 271, 222	0.65	5.50
(7) 9	C ₃₂ H ₄₃ NO ₁₀ (601)	601	461, 321, 271, 215	0.35	5.64
(8) 10	C ₃₅ H ₄₇ NO ₁₀ (641)	641	461, 321, 266, 224	0.53	6.35

^{*} Silica gel plates Merck 60 F₂₅₄, CH₂Cl₂/MeOH/H₂O 70:25:4 by vol.[†] Column HP/HPV Shield RP8 3.5 μ m, 3.9 $\times$ 50 mm (Waters); gradient H₂O (A)–acetonitrile (B) (10–100% B in 9 min).

ethyl acetate and by chromatographical separations the new rifamycin-biosynthesis intermediates **2** to **8** released from modules **4** to **10** (Fig. 4) as well as the starter unit AHBA and the known early intermediate P8/1-OG (Ghisalba *et al.*, 1981) (**1**) were obtained in pure form. P8/1-OG (intermediate **1**) is produced as the main intermediate and was identified by comparison of its spectroscopic properties with published data. The presence of the free acid form **1** was determined by IR spectroscopy (band at 2650 cm⁻¹). The physico-chemical and chromatographic parameters of the new intermediates are shown in Table 1.

Elucidation of the structure of rifamycin-biosynthesis intermediates **2** to **8**

Elucidation of the structure of the new rifamycin biosynthesis intermediates **2** to **8** released from modules **4** to **10**, respectively, are based on spectroscopic data (UV, IR, NMR and MS; Table 2); intermediate **7** released from module **9** is used to exemplify the procedure here.

Intermediate 7 from module 9. The UV absorptions at 315, 276 and 203 nm, which are common to the whole series of intermediates **2** to **8** released from modules **4** to **10** and in distinct contrast to the UV spectrum of the known intermediate P8/1-OG (**1**) released by module **3**, clearly indicates a naphthoquinone ring system, as present in rifamycin B. From high resolution ESI-MS (electrospray

ionized mass spectroscopy) (*m/z* 624.27895) the molecular formula C₃₂H₄₃NO₁₀ resulted for intermediate **7**. The IR spectrum shows prominent bands at 1624 (C=O) and 971 cm⁻¹ (C=C *trans*). The substitution pattern of the naphthoquinone ring is supported by the two singlets in the ¹H NMR spectrum (Fig. 5) at 7.57 p.p.m. (8'-H) and 5.38 p.p.m. (3'-H), the methyl signal at 1.98 p.p.m. (11'-H₃) and the broad signal of the amino group (2'-NH₂) at 6.8 p.p.m. The aliphatic chain, substituted with five secondary methyl groups (five doublets in the range of 0.75 to 0.96 p.p.m.), one methyl group bound to a double bond conjugated to carbonyl-1 (singlet at 1.85 p.p.m.) and four methine carrying a hydroxy group was deduced from two-dimensional DQF-COSY (double quantum filtered correlated spectroscopy) NMR measurements (Fig. 6). The *trans* double bond at the carboxy end is revealed by the multiplet (d $\times$ d) at 6.95 p.p.m. (13-H) and the doublet at 5.75 p.p.m. (14-H, *J* = 16 Hz).

Intermediate 4 released from module 6. The UV and IR spectra resemble very closely the corresponding spectra of compound **7**, except the much stronger band at 1720–1730 cm⁻¹ observed in the IR spectrum of **7**. In the proton NMR spectrum, the signal for the methine-5 is shifted downfield to 4.05 p.p.m. (compared to 3.50 p.p.m. in **7**). These spectroscopic data, together with the molecular formula C₂₄H₂₇NO₇ from MS (*m/z* 442 MH⁺), gave evidence for the presence of a 6-membered lactone ring between the terminal carboxy

Table 2. ¹H NMR data for rifamycin biosynthesis intermediates **2** to **8** released from modules 4 to 10 (in d₆-DMSO)

The occurrence of atropic isomers at 25 °C may be due to either tautomeric forms and/or hindered rotation around the 5' 1-carbon bond. The ratio of the two forms differ from 1:1 for intermediates **2** to **6** to 9:1 for intermediates **7** and **8**. When the temperature is increased, the signals of the two forms collapse.

Position	Intermediate						
	2	3	4* (lactone)	5†	6*	7 (lactone)	8‡
2'-NH ₂	7.1 (br)	6.8 (br)	6.61 (br)	6.52 (br)	6.46 (br)	6.8 (br)	6.8 (br)
3'-H	5.58 (s)	5.39 (m)	5.67 (s)	5.69(s)	5.54 (s)	5.38 (s)	5.39 (s)
						5.45 (s)	
8'-H	7.80 (s)	7.55 (m)	7.77 (s)	7.79 (s)	7.67 (s)	7.57 (s)	7.58 (s)
11'-H ₃	2.30 (s)	2.00 (s)	2.31(s)	2.28 (s)	2.15 (s)	1.98 (s)	1.99 (s)
	2.28 (s)	1.97 (s)					
6'-OH				9.3 (br)			
2''-H ₃	1.90 (s)	1.80 (s)	1.91 (s)	1.92 (s)	1.88 (s)	1.85 (s)	1.82 (s)
		1.72 (s)					
3-H	5.95 (d)	6.13 (d)	5.98 (d)	6.09 (d)	6.12 (d)	6.32 (d)	6.28 (d)
	5.90 (d)						
4-H	3.40 (m)	2.52 (m)	2.80 (m)	2.65 (m)	2.65 (m)	2.69 (m)	2.63 (m)
4''-H ₃	1.10 (d)	0.88 (d)	0.91 (d)	0.86 (d)	0.86 (d)	0.87 (d)	0.84 (d)
	1.03 (d)						
5-H	—	3.35 (d)	4.05 (d)	3.65(m)	3.60 (dd)	3.40 (d)	3.42 (br s)
6-H		2.08 (dd)	2.11 (m)	1.74 (m)	1.61 (m)	1.85 (m)	1.84 (m)
6''-H ₃		1.00 (d)	0.82 (d)	0.86 (d)	0.71 (d)	0.75 (d)	0.70 (d)
7-H		—	3.63 (m)	3.58 (m)	3.78 (m)	4.20 (d)	4.15 (d)
8-H			2.22 (dd)	1.57 (m)	1.84 (m)	1.52 (m)	1.52 (m)
8''-H ₃			1.21 (d)	0.66 (d)	0.93 (d)	0.68 (d)	0.67 (d)
9-H			—	3.78 (d)	3.94 (m)	3.50 (d)	3.47 (dd)
10-H				2.63 (m)	2.06 (m)	1.79 (m)	1.80 (m)
10''-H ₃				1.09 (d)	1.00 (d)	0.96 (d)	0.93 (d)
11-H				—	3.56 (d)	3.64 (d)	3.56 (d)
12-H					2.68 (d)	2.36 (dd)	2.28 (m)
12''-H ₃					1.12(d)	0.89 (d)	0.88 (d)
13-H					—	6.95 (dd)	6.00 (dd)
14-H						5.75 (d)	7.01 (dd)
15-H						—	6.46 (d)
16''-H ₃							1.85 (s)

* Shifts at 100 °C.

† Shifts at 120 °C.

‡ Shifts at 80 °C.

group and the hydroxy group at C-5. The analogous situation is found in intermediate **6** released from module 8, which contains a lactone ring built by the hydroxy function at C-9.

Intermediate 8 released from module 10. In NMR ROESY (rotated frame nuclear Overhauser enhancement spectroscopy) experiments, a strong nuclear Overhauser enhancement (NOE) is measured between the protons of the methyl group at C-16 and the olefinic proton at C-14, thus indicating the geometry of the terminal carbon double bond as *trans*.

Determination of the relative stereochemistry

As intermediates **4** and **6** released from modules 6 and 8 contain a 6-membered lactone ring, the relative stereochemistry for the two carbon sequences C-5, C-6, C-7, C-8 and C-9, C-10, C-11, C-12, respectively, could be deduced from measurements of coupling constants in proton NMR spectra and from NOE experiments (Fig. 7). The overlapping carbon sequence C-7, C-8, C-9, C-10 is covered by the lactone form of the intermediate released from module 7, which was found in addition to the corresponding linear intermediate **5**. The results,

Fig. 5. ^1H NMR spectrum of rifamycin biosynthesis intermediate **7** released from module 9 (in d_6 -DMSO).

therefore, revealed the complete relative configurations for the methine carbons of the side chain (except for C-4 which is achiral in the final rifamycins); the determined relative stereochemistry corresponds to the known absolute chirality of the ansa ring in the rifamycins (Brufani *et al.*, 1964).

DISCUSSION

In this work we have described the inactivation of the *rifF* gene in the rifamycin B biosynthesis gene cluster and the production of a series of linear rifamycin B bio-

synthesis intermediates by the corresponding mutant strain 3/1. Interestingly, all intermediates **1** to **8** released from modules 3 to 10 were obtained, whereas the intermediates produced by modules 1 and 2 have not been detected so far. The absence of these compounds in the culture broth of mutant strain 3/1 may result from an efficient conversion of these intermediates to P8/1-OG (**1**), which was found as the predominant compound.

The RifF protein shows a high degree of similarity to different arylamine *N*-acetyltransferases, e.g. of *Mycobacterium*

Fig. 6. DQF-COSY (double quantum filtered correlated spectroscopy) ^1H NMR spectrum of rifamycin biosynthesis intermediate **7** released from module 9 (in d_6 -DMSO).

Coupling constants:

H atoms	<i>J</i> (Hz)
5 - 6	2.0
6 - 7	4.3
7 - 8	10.9

H atoms	<i>J</i> (Hz)
7 - 8	3.0
8 - 9	7.9
9 - 10	6.1

H atoms	<i>J</i> (Hz)
9 - 10	8.0
10 - 11	2.8
11 - 12	3.3

Fig. 7. Relative stereochemistry in the corresponding lactones of intermediates 4, 5 and 6 released from modules 6, 7 and 8. The arrows on the chemical structures represent strong nuclear Overhauser enhancement (NOE).

																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																					</
--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	--	----

Fig. 8. Alignment of the Riff protein and the inactivated Riff protein produced by the mutant strain 3/1 with arylamine *N*-acetyltransferases from different organisms. GenBank accession numbers are shown in parentheses. *S. typhimurium*, *Salmonella typhimurium* (Q00267); *E. coli* (P77567); *M. smegmatis*, *Mycobacterium smegmatis* (O86309); *H. sapiens*, *Homo sapiens* (O15159). The altered or lost amino acids in the mutated Riff protein are underlined. Only the N-terminal regions of the proteins are shown. Identical(*) or similar (°) amino acids are marked above the sequences.

bacterium smegmatis (36.5% identity over 178 aa), of *E. coli* (28% identity over 230 aa). These sequence similarities together with our results demonstrate the function of the *rifF* gene product as a cyclase (rifamycin amide synthase) performing the intramolecular ring closure by amide bond formation between the carboxyl group at one end and the amino group at the opposite end of the mature linear polyketide molecule synthesized by the rifamycin PKS. The role of the Riff protein in rifamycin B biosynthesis is therefore the cyclization and simultaneous release of the polyketide chain from the acyl carrier protein of module 10 of the rifamycin PKS.

Mutant strain 3/1 produced a series of linear intermediates of the rifamycin B biosynthesis and not, as expected, only the full-length linear intermediate produced by module 10. To explain this result, the activity of a second enzyme is needed that is able to catalyse the release of the linear intermediates from the acyl carrier proteins of modules 3 to 10. The thioesterase encoded by *orf12* (August *et al.*, 1998), almost 30 kb downstream of *rifF*, may be the protein having this function. In addition, the obstruction of the normal release of the full-length acyl chain from the rifamycin PKS probably leads to an accumulation of linear intermediates docked at the acyl

Fig. 9. Function of RifF and pathway for the biosynthesis of rifamycin B starting from the completed linear polyketide (8) released from module 10.

carrier proteins of the different modules, which are then released from the PKS by the thioesterase, thereby preventing blockage of the enzyme complex. Such an editing activity was recently discussed for the thioesterase TyLO in the tylosin biosynthesis (Butler *et al.*, 1999).

Noncyclized polyketide intermediates of 16-membered macrolides were also isolated from the culture broth of

the mycinamicin producer '*Micromonospora griseorubida*' sp. nov. (tetraketide and pentaketide) and the corresponding triketide in a nonproducing mutant (Kinoshita *et al.*, 1988). Tetra- and pentaketides were observed in nonproducing mutants of the tylosin producer *Streptomyces fradiae* (Huber *et al.*, 1990). These observations, together with our results, suggest that the formation of noncyclized polyketide intermediates is perhaps a general phenomenon during either the normal or impaired catalysis by type I PKS.

The introduction of the 9 bp deletion inside the *rifF* gene results in the loss of three amino acids (Q, K and S) and the exchange of L to R at position 29 (Fig. 8). These alterations in the RifF protein caused the inactivation of the enzyme because the cyclization reaction at the end of the biosynthesis of the polyketide backbone is no longer performed. The exact function of the altered or lost amino acids inside the RifF protein is not clear at the moment, but one possible candidate for an essential function for the cyclization reaction is the conserved L at position 29 (Fig. 8).

Furthermore we have shown that the linear intermediates released by module 4 and the following modules already have the naphthoquinone chromophore, whereas the linear intermediate released by module 3 still has the benzenoid structure derived from the AHBA starter unit. A proposal for the mechanism of the naphthoquinone unit formation was made by Tang *et al.* (1998); however, the timing of these reactions was not clear. The naphthoquinone structure of the intermediates derived from modules 4–10 indicates that the formation of the naphthoquinone ring takes place at the transition from module 3 to module 4. With the results obtained it is not clear, however, if the reactions for the naphthoquinone ring formation take place with the respective intermediates bound to the rifamycin PKS or after their release from the enzyme. It seems that the transition from module 3 to module 4 is a critical step during the rifamycin B synthesis. This is strengthened by results from Hunziker *et al.* (1998), who showed that feeding of altered starter units, with a missing amino group at C-3 or an additional missing hydroxyl group at C-5, to a mutant in AHBA synthesis results in a termination of the rifamycin synthesis after module 3. In those feeding experiments, the corresponding derivatives of the well-known tetraketide shunt product P8/1-OG were detected (Hunziker *et al.*, 1998).

Different rifamycin molecules have been postulated as the earliest macrocyclic intermediates of rifamycin biosynthesis: proansamycin A or B deduced from the early intermediate protorifamycin I and structure comparison with streptovaricin precursors (Ghisalba *et al.*, 1978, 1979) and proansamycin X, based on hypothetical intermediates of the rifamycin PKS gene cluster (August *et al.*, 1998; Tang *et al.*, 1998). The structure of the observed module 10 intermediate 8 and the deduced function of RifF, performing the ring closure of the linear endproduct 8 of the PKS, now provides strong evidence that proansamycin B is the direct macrocyclic

product of the rifamycin PKS (Fig. 9). The presence of the *trans-cis* sequence for the conjugated olefinic system in rifamycin B (and its precursor proansamycin B) can be explained by migration of the (13,14)*trans*-(15,16)*trans* double bonds via enolysation and subsequent isomerization of the geometry. From the hypothetical proansamycin B, a simple one-step transformation, oxidation at C-4'', would lead to protorifamycin I, the earliest macrocyclic precursor of the rifamycins isolated so far (Ghisalba *et al.*, 1978).

The inactivation of the *rifF* gene in *A. mediterranei* S699 has also been carried out independently by Yu *et al.* (1999), whose report appeared while our paper was being reviewed.

ACKNOWLEDGEMENTS

The authors thank A. Pospiech (Novartis Services, Bioanalytik) for DNA sequencing and Ch. Quiquerez for the measurements of the MS spectra.

REFERENCES

- August, P. R., Tang, L., Yoon, Y. J. & 9 other authors (1998). Biosynthesis of the ansamycin antibiotic rifamycin: deductions from the molecular analysis of the *rif* biosynthetic gene cluster of *Amycolatopsis mediterranei* S699. *Chem Biol* **5**, 69–79.
- Bachs, L., Pares, A., Elena, M., Piera, C. & Rodes, J. (1992). Effects of long-term rifampicin administration in primary biliary cirrhosis. *Gastroenterology* **102**, 2077–2080.
- Barakett, V., Lesage, D., Delisle, F., Vergez, P. & Petit, J. C. (1993). Killing kinetics of vancomycin and rifamycin tested alone and in combination against penicillin-resistant *Streptococcus pneumoniae*. *Eur J Clin Microbiol Infect Dis* **12**, 69–71.
- Blondelet-Rouault, M.-H., Weiser, J., Lebrihi, A., Branny, P. & Pernodet, J.-L. (1997). Antibiotic resistance gene cassettes derived from the Ω interposon for use in *E. coli* and *Streptomyces*. *Gene* **190**, 315–317.
- Brufani, M., Fedeli, W., Giacomello, G. & Vaciago, A. (1964). The X-ray analysis of the structure of rifamycin B. *Experientia* **20**, 339–342.
- Butler, A. R., Bate, N. & Cundliffe, E. (1999). Impact of thioesterase activity on tylosin biosynthesis in *Streptomyces fradiae*. *Chem Biol* **6**, 287–292.
- Chen, S., von Bamberg, D., Hale, V., Breuer, M., Hardt, B., Müller, R., Floss, H. G., Reynolds, K. A. & Leistner, E. (1999). Biosynthesis of ansatrienin (mycotrienin) and naphthomycin: identification and analysis of two separate biosynthetic gene clusters in *Streptomyces collinus* Tü 1892. *Eur J Biochem* **261**, 98–107.
- Ghisalba, O., Traxler, P. & Nüesch, J. (1978). Early intermediates in the biosynthesis of ansamycins. I. Isolation and identification of protorifamycin I. *J Antibiot* **31**, 1124–1131.
- Ghisalba, O., Traxler, P., Fuhrer, H. & Richter, W. J. (1979). Early intermediates in the biosynthesis of ansamycins. II. Isolation and identification of proansamycins B-M1 and protorifamycin I-M1. *J Antibiot* **32**, 1267–1272.
- Ghisalba, O., Fuhrer, H., Richter, W. J. & Moss, S. (1981). A genetic approach to the biosynthesis of the rifamycin chromophore in *Nocardia mediterranei*. III. Isolation and identification of an early aromatic ansamycin precursor containing the seven-carbon amino starter-unit and three initial acetate/propionate units of the ansa chain. *J Antibiot* **34**, 58–63.
- Ghisalba, O., Auden, J. A. L., Schupp, T. & Nüesch, J. (1984). The rifamycins: properties, biosynthesis, and fermentation. In *Bio-technology of Industrial Antibiotics*, pp. 281–327. Edited by E. J. Vandamme. New York & Basel: Marcel Dekker.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Huber, M. L. B., Paschal, J. W., Leeds, J. P., Kirst, H. A., Wind, J. A., Miller, F. D. & Turner, J. R. (1990). Branched-chain fatty acids produced by mutants of *Streptomyces fradiae*, putative precursors of the lactone ring of tylosin. *Antimicrob Agents Chemother* **34**, 1535–1541.
- Hunziker, D., Yu, T.-W., Hutchinson, C. R., Floss, H. G. & Khosla, C. (1998). Primer unit specificity in rifamycin biosynthesis principally resides in the later stages of the biosynthetic pathway. *J Am Chem Soc* **120**, 1092–1093.
- Kinoshita, K., Takenaka, S. & Hayashi, M. (1988). Isolation of proposed intermediates in the biosynthesis of mycinamicins. *J Chem Soc Chem Commun* **1988**, 943–945.
- MacNeil, D. J., Gewain, K. M., Ruby, C. L., Dezeny, G., Gibbons, P. H. & MacNeil, T. (1992). Analysis of *Streptomyces avermitilis* genes required for avermectin biosynthesis utilizing a novel integration vector. *Gene* **111**, 61–68.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Oppenheim, B., Koornhof, H. J. & Austrian, R. (1986). Antibiotic-resistant pneumococcal disease in children at Baragwanath hospital, Johannesburg. *Pediatr Infect Dis* **5**, 520–524.
- Pospiech, A. & Neumann, B. (1995). A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends Genet* **11**, 217–218.
- Sambrook, J., Fritsch, E. F. & Manniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schupp, T. & Divers, M. (1986). Protoplast preparation and regeneration in *Nocardia mediterranei*. *FEMS Microbiol Lett* **36**, 159–162.
- Schupp, T., Toupet, C., Engel, N. & Goff, S. (1998). Cloning and sequence analysis of the putative rifamycin polyketide synthase gene cluster from *Amycolatopsis mediterranei*. *FEMS Microbiol Lett* **159**, 201–207.
- Szabo, C., Bissel, M. J. & Calvin, M. (1976). Inhibition of infectious rous sarcoma virus production by a rifamycin derivative. *J Virol* **18**, 445–453.
- Tang, L., Yoon, Y. J., Choi, C.-Y. & Hutchinson, C. R. (1998). Characterization of the enzymatic domains in the modular polyketide synthase involved in rifamycin B biosynthesis by *Amycolatopsis mediterranei*. *Gene* **216**, 255–265.
- Wehrli, W. (1977). Ansamycins: chemistry, biosynthesis and biological activity. *Topics Curr Chem* **72**, 21–49.
- Yu, T.-W., Shen, Y., Doi-Katayama, Y., Tang, L., Park, C., Moore, B. S., Hutchinson, C. R. & Floss, H. G. (1999). Direct evidence that the rifamycin polyketide synthase assembles polyketide chains processively. *Proc Natl Acad Sci USA* **96**, 9051–9056.
- Zimmerli, W., Widmer, A. F., Blatter, M., Frei, R. & Ochsner, P. E. (1998). Role of rifampin for treatment of orthopedic implant-related staphylococcal infections: a randomized controlled trial. *J Am Med Assoc* **279**, 1537–1541.

Received 9 August 1999; accepted 3 September 1999.