VOL. 55 NO. 4, APR. 2002 THE JOURNAL OF ANTIBIOTICS pp. 396 — 406

New Insights into Rifamycin B Biosynthesis: Isolation of Proansamycin B and 34a-Deoxy-rifamycin W as Early Macrocyclic Intermediates Indicating Two Separated Biosynthetic Pathways

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(Received for publication November 5, 2001)

Proansamycin B, the formerly postulated intermediate of rifamycin B biosynthesis, was isolated from cultures of the *Amycolatopsis mediterranei* mutant F1/24. The structure was determined using UV, IR, NMR and MS techniques. Biotransformation studies demonstrate that proansamycin B is an intermediate of a shunt pathway, a 8-deoxy variant, of rifamycin B biosynthesis leading to 8-deoxy-rifamycin B as the final product.

In addition, 34a-deoxy-rifamycin W, the direct precursor of rifamycin W, could be isolated representing the earliest macrocyclic intermediate obtained so far in the biosynthetic route to rifamycin B. Furthermore, the new rifamycin W-28-desmethyl-28-carboxy and rifamycin W-hemiacetal, intermediates in the transformation sequence of rifamycin W to rifamycin S, were isolated. Application of proton NMR measurements (double resonance and ROESY experiments) on the latter compound indicated that the stereochemistry at the chiral center C-28 is *R*.

The group of ansamycin antibiotics is an example of the enormous diversity in secondary metabolites produced by microorganisms (actinomycetes) and plants (*Maytenus* sp., *Colubrina* sp., *Euphorbia* sp.). This group of secondary metabolites derived their name from the characteristic molecular structure which consists of an aromatic nucleus and a long handle-shaped aliphatic ansa bridge joining two opposite positions of the nucleus (chromophore). Two types of aromatic units can be distinguished: a naphthalenic ring system (*e.g.* rifamycins, streptovaricins, tolypomycins, halomicins, naphthomycin, actamycin and bransarols) or a benzenic ring system (*e.g.* geldanamycin, herbimycins, macbecins, ansatrienins, mycotrienins, and maytansinoids)[1]. The common precursor for this group of metabolites produced by actinomycetes is the unusual starter unit for the type I polyketide synthase, 3-amino-5-hydroxybenzoic acid (AHBA), which is synthesized *via* a variant of the shikimate pathway of aromatic biosynthesis, the recently discovered aminoshikimate pathway[2,3].

The rifamycin biosynthetic gene cluster is the best characterized ansamycin gene cluster up to now[4~6], and a first comparative study on the organization of the gene clusters for the biosynthesis of ansatrienin, naphthomycin and rifamycin was recently published[7]. The biosynthesis of the clinically important rifamycin antibiotics has been extensively studied[8,9] but is still not fully elucidated. In particular, the earliest macrocyclic intermediate of rifamycin biosynthesis, produced by the rifamycin polyketide synthase (PKS) and cyclized by rifamycin amide synthase (RifF)[10], has yet to be characterized.

In our study we describe the isolation and characterization of two early macrocyclic intermediates in the rifamycin biosynthesis in *Amycolatopsis mediterranei*. In addition, we present evidence for a second independent pathway in rifamycin biosynthesis with proansamycin B as

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the first macrocyclic intermediate and 8-deoxy-rifamycin B as the final product.

Materials and Methods

Bacterial Strains and Culture Conditions

The *Amycolatopsis mediterranei* strains used in this work are mutational derivatives of the wild type strain *A. mediterranei* ATCC13685. Mutant strain N/813 is a high rifamycin B producer (Novartis strain collection). F1/24 is a mutant, derived from strain N/813, which is blocked in rifamycin B biosynthesis and produces protorifamycin I and other rifamycin precursors[11]. Strain 3/1 is a mutant of N/813 with disrupted *rifF* gene[10]. Mutant W126 is blocked in the transformation of rifamycin W to rifamycin S[8]. The strains were propagated for 3~4 days in 50 ml liquid medium NL148[12] in 200 ml Erlenmeyer flasks. Cultures for rifamycin production were grown in 40 ml medium FBR42[10] in 200 ml Erlenmeyer flasks (one baffle). All incubations were at 28°C and with shaking at 250 rpm.

Analytical Methods

HPLC

For HPLC analysis of metabolites produced by *A. mediterranei* culture broths were mixed 1 : 1 with butanol and shaked for 4 hours at room temperature (150 rpm). After centrifugation (10 minutes, 5000×*g*) the butanol phase was separated and 10~20 μl were analysed by HPLC (Merck model L-6200) using a 4.6×100 mm Symmetry C18, 3.5 mm column (Waters, Milford, USA) with gradient elution (5 minutes linear gradient of 0~50% acetonitrile; hold for 2 minutes; 5 minutes linear gradient of 50~100% acetonitrile; hold for 3 minutes; 2 minutes linear gradient of 100~0% acetonitrile) using a phosphate (0.55% ortho phosphoric acid, pH adjusted to 4.5 with triethylamine)/acetonitrile buffer system. The region from 200~500 nm was scanned with a diode array detection (DAD)-System L-4500 (Merck, Darmstadt, Germany) and these spectra were compared with rifamycin standards (Table 1).

TLC

To distinguish protorifamycin I and rifamycin W, TLC was used; silica gel plates 'Merck' 60 F_{254}, system CH_2Cl_2 - MeOH - H_2O 70 : 25 : 4 by vol.; Rf values: protorifamycin I 0.56, rifamycin W 0.50.

Isolation of Proansamycin B and Other 8-Deoxy-rifamycin Derivatives

The strain *A. mediterranei* F1/24 was cultivated in 50 ml medium NL148 for 4 days and the culture was used to inoculate 1 liter of FBR42 medium (25 shake flasks). The cultures were supplemented with the adsorber resin XAD-16 (Rohm and Haas) (2.5% w/v) and incubated for 11 days. The cells and adsorber resin were separated by filtration and extracted with 2-propanol (2 liter). After evaporation the extract was taken up in ethyl acetate/water (500 ml/100 ml) and the organic layer was separated and evaporated. The crude residue (1.5 g) was fractionated by medium pressure chromatography on LiChrospher RP18 12 μm (200 g) using a gradient composed of water-triethylammoniumformiate buffer (A) and acetonitrile (B) from 0% B to 60% B. The purity of the fractions was checked by HPLC and TLC (CH_2Cl_2 - MeOH - H_2O 80 : 17.5 : 2.5). The obtained yields were 30 mg proansamycin B (**1**), 143 mg protorifamycin I (**3**), 15 mg 8-deoxy-rifamycin S (**5**), 16 mg 8-deoxy-rifamycin SV (**7**), 7 mg 8-deoxy-rifamycin B (**9**), and 33 mg proansamycin B-M1 (**2**).

Isolation of Rifamycin W Congeners

The mutant W126 was cultivated in 500 ml medium FBR42 for 36 hours and the culture was extracted with 250 ml butanol. Separation of the crude extract (5.8 g) on Sephadex LH20 with MeOH followed by chromatography on LiChrospher RP18 12 μm (200 g) gave 107 mg rifamycin W (**4**), 18 mg rifamycin W-hemiacetal (**12a**) and 6 mg rifamycin W-hemiacetal (**12b**). Preparative TLC (CH_2Cl_2 - MeOH - H_2O 80 : 17.5 : 2) of a side-fraction yielded 4 mg 34a-deoxy-rifamycin W (**11**) in pure form.

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. $^1H/^{13}C$-NMR spectra were recorded in d_6-DMSO on a Bruker Avance DMX-500 spectrometer at 500 MHz with TMS as internal standard. Electrospray mass spectra were measured with a Finnigan SSQ-7000 MS spectrometer.

Biotransformation Studies

Shake flasks (50 ml) with 10 ml of medium NL148 were inoculated from frozen stock cultures of the strains F1/24, 3/1 and W126 and cultivated for 4 days. The strains were transferred into 40 ml FBR42 medium in 200 ml shake flasks (one baffle) and fermented for 6 days. The mycelia

Table 1. UV data and HPLC retention times of various rifamycins.

Substrate	UV maxima [a] nm	HPLC retention time (min.)
Proansamycin B (1)	310, 273, 229	9.25
Protorifamycin I (3)	312, 272, 231	8.62
Rifamycin W (4)	321, 272, 230	8.62
8-Deoxy-rifamycin S (5)	350, 298, 219	13.21
Rifamycin S (6)	410, 317, 219	13.27
Rifamycin SV (8)	445, 314, 223	10.19
8-Deoxy-rifamycin B (9)	416, 302, 219	11.06
Rifamycin B (10)	430, 309, 226	10.01
34a-Deoxy-rifamycin W (11)	321, 272, 231	9.38

[a] aqueous solution at pH 4.5.

were washed twice with 66 mM phosphate buffer (pH 7.0) at 20°C and resuspended in 25 ml of the same buffer. The substrates (proansamycin B (**1**), 8-deoxy-rifamycin S (**5**), 8-deoxy-rifamycin B (**9**), 34a-deoxy-rifamycin W (**11**), rifamycin W (**4**), rifamycin S (**6**)) were dissolved in methanol at a concentration of 6 mg/ml each, with the exception of protorifamycin I (**13**) (10 mg/ml).

The following incubations were carried out (50 ml shake flasks, 24 and 48 hours, 250 rpm, 28°C):

Transformation assay:

5 ml mycelium suspension (F1/24, 3/1, W126) in phosphate buffer, 0.5 ml substrate solution, 1 ml 50% glucose, 3.5 ml 66 mM phosphate buffer (pH 7.0).

Control I:

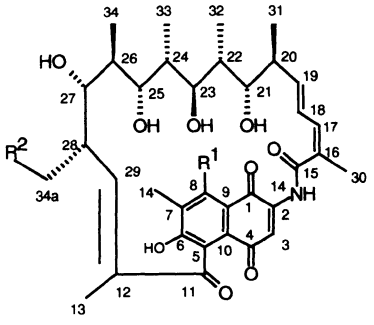
5 ml mycelium suspension (F1/24, 3/1, W126) in phosphate buffer, 4 ml 66 mM phosphate buffer (pH 7.0), 1 ml 50% glucose.

Control II:

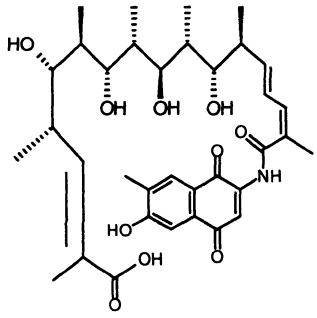
0.5 ml substrate solution, 8.5 ml 66 mM phosphate buffer (pH 7.0), 1 ml 50% glucose.

After 24 and 48 hours of incubation a 1 ml aliquot of each assay was mixed with 1 ml butanol and shaken for 4 hours at room temperature (150 rpm). After centrifugation (10 minutes, 5000×g) the butanol phase was separated and 20 μl were analysed by HPLC. For kinetic studies also shorter incubation times were applied.

Fig. 1. Structures of various rifamycins.



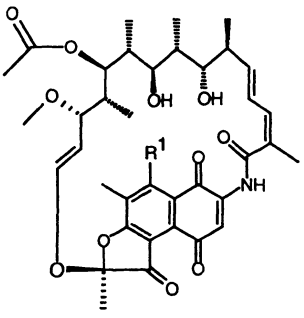
$R^1 = R^2 = H$ Proansamycin B **(1)**

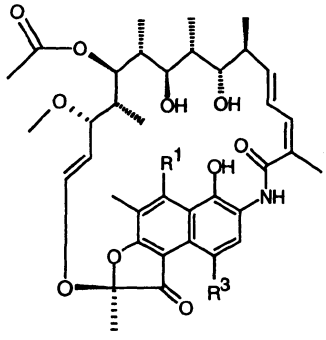


Proansamycin B-M1 **(2)**

$R^1 = H$ $R^2 = OH$ Protorifamycin I **(3)**

$R^1 = R^2 = OH$ Rifamycin W **(4)**



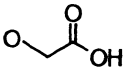


$R^1 = H$ 8-Deoxy-rifamycin S **(5)**

$R^1 = OH$ Rifamycin S **(6)**

$R^1 = H$ $R^3 = OH$ 8-Deoxy-rifamycin SV **(7)**

$R^1 = OH$ Rifamycin SV **(8)**

$R^1 = H$ $R^3 =$  8-Deoxy-rifamycin B **(9)**

$R^1 = OH$ Rifamycin B **(10)**

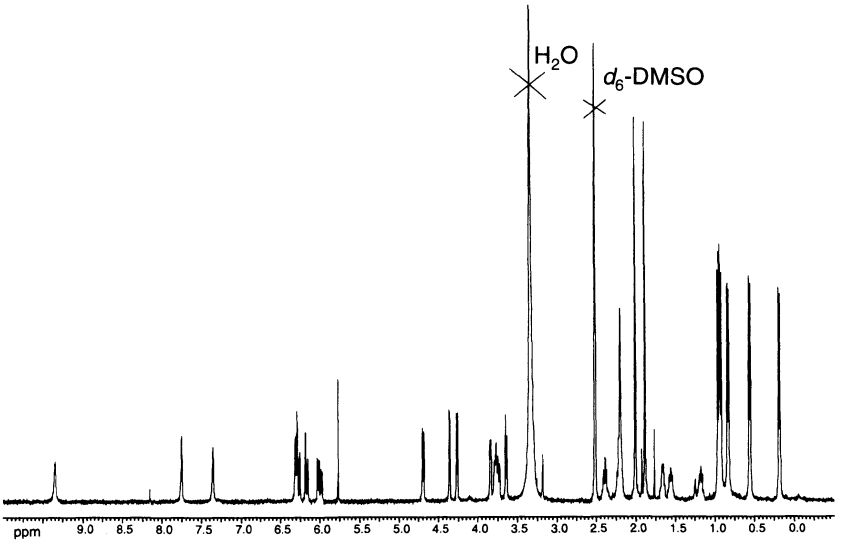
Results

Isolation and Structure Elucidation of Proansamycin B (1) and 8-Deoxy-rifamycin SV (7)

The main product synthesized by *A. mediterranei* F1/24 is protorifamycin I (**3**), an early macrocyclic rifamycin intermediate[11]. In order to isolate further macrocyclic intermediates of rifamycin biosynthesis this strain was cultivated in medium FBR42 (optimized for high rifamycin yield) in the presence of adsorber resin XAD-16. Under these conditions proansamycin B (**1**), hitherto unknown, and 8-deoxy-rifamycin SV (**7**) were isolated as minor components and spectroscopically characterized, besides the already described 8-deoxy-rifamycin S (**5**), 8-deoxy-rifamycin B (**9**), and proansamycin B-M1 (**2**) which were identifiied by comparison of their spectroscopic properties with published data[13].

The UV spectrum of proansamycin B (310 nm, 26'000; 273 nm, 36'000; 229 nm, 54'000) is nearly identical with spectrum of protorifamycin I (see Table 1) indicating the same chromophor; also the IR spectra are very similar. The

Fig. 2. ^{1}H-NMR spectrum of proansamycin B (**1**) in d_6-DMSO.



molecular formula of proansamycin B $C_{35}H_{45}NO_9$ (623) results from the ESI mass spectrum (positive mode: 646 $[M+Na]^+$, negative mode 622 $[M-H]^-$) showing a difference of 16 mass units to protorifamycin I. The structural relationship was deduced by comparison of the corresponding ^{1}H-NMR spectra. In proansamycin B, a new methyl signal appears as doublet at 0.95 ppm for CH_3(34a) (see Table 2), whereas the characteristic signal at 3.2 ppm (dxd) for CH_2(34a) in protorifamycin I is absent in the spectrum of proansamycin B (Fig. 2).

The UV spectrum of 8-deoxy-rifamycin SV (**7**) indicates the hydroquinone chromophor of 8-deoxy-rifamycin B, and the IR spectrum resembles the spectrum of rifamycin SV (**8**). In addition, the molecular mass of 681 deduced from ESI (702 $[M+Na]^+$, 680 $[M-H]^-$) gave evidence for the structure of **7** as 8-deoxy-rifamycin SV.

Isolation and Spectroscopical Properties of 34a-Deoxy-rifamycin W (**11**), Rifamycin W-hemiacetal (**12a/12b**), and Rifamycin W-28-desmethyl-28-carboxy (**13**)

From cultivations of the mutant W126, which contains a block in the transformation of rifamycin W to rifamycin S, several intermediates of the rifamycin W-biosynthesis could be isolated: in a short fermentation run in medium FBR42, the new precursor of rifamycin W still lacking the hydroxy group at C-34a (**11**) is found as minor component, besides rifamycin W as the main product. The structure elucidation of this new compound **11** is based on UV data showing the same features as rifamycin W, thus indicating the 6,8-dihydroxy substituted quinone chromophore. The molecular mass of 639 ($C_{35}H_{45}NO_{10}$) results from ESI spectra and is 16 mass units higher than proansamycin B and 16 mass units lower than rifamycin W. The structural relationship of **11** to rifamycin W is confirmed in the ^{1}H-NMR spectrum by the methyl signal at 0.95 ppm for CH3(34a) and the absence of the HO-substituted methylene group at 3.2 ppm CH_2(34a)-OH characteristic for rifamycin W. In comparison to proansamycin B (**1**), the corresponding member of the 8-deoxy series, the signal at 7.75 ppm for the aromatic proton H-8 is missing in the spectrum of **11**, whereas all other signals show only marginal differences in chemical shift (Table 2).

In addition, rifamycin W-hemiacetal (**12a/12b**) and the hitherto unknown carboxy intermediate rifamycin W-28-desmethyl-28-carboxy (**13**) (Fig. 3) were isolated. The existence of these compounds confirms the normal route for elimination of the methyl group C-34a by oxidation following decarboxylation as proposed[14,15]. The structures of these compounds were elaborated from spectroscopic data. In **13** the carboxy group is supported by the characteristic fragment peak of 624 $[M-COOH]^-$ in the ESI mass spectrum (670 MH^+, 668 $[M-H]^-$; $C_{35}H_{43}NO_{12}$).

Table 2. ^{1}H NMR data for proansamycin B (**1**) and 34a-deoxy-rifamycin W (**11**) (in d_6-DMSO).

Position	Proansamycin B δ (ppm)	34a-Deoxy-rifamycin W δ (ppm)	Position	Proansamycin B δ (ppm)	34a-Deoxy-rifamycin W δ (ppm)
3-H	7.36 (s)	7.13 (br s)	24-H	1.55 (br t)	1.57 (br t)
7-H_3	2.18 (s)	1.80 (s)	25-H	3.75 (m)	3.76 (m)
8-H	7.85 (s)	-	25-OH	3.62 (d)	3.62 (d)
13-H_3	1.88 (s)	1.80 (s)	26-H	1.18 (t)	1.15 (t)
14-NH	9.34 (br s)	9.15 (br s)	27-H	3.83 (d)	3.83 (d)
17-H	6.18 (d)	6.18 (d)	27-OH	4.26 (d)	4.26 (d)
18-H	6.30 (t)	6.32 (t)	28-H	2.28 (t)	2.34 (m)
19-H	6.00 (dd)	6.00 (dd)	29-H	6.28 (d)	6.34 (d)
20-H	2.2 (m)	2.2 (m)	30-H_3	1.99 (s)	2.00 (s)
21-H	3.75 (m)	3.77 (m)	31-H_3	0.84 (d)	0.82 (d)
21-OH	4.36 (d)	4.4 (d)	32-H_3	0.90 (d)	0.91 (d)
22-H	1.65 (br d)	1.66 (br d)	33-H_3	0.55 (d)	0.54 (d)
23-H	3.3-3.4	3.3	34-H_3	0.18 (d)	0.20 (d)
23-H	4.69 (d)	4.64 (d)	34a-H_3	0.91 (d)	0.91 (d)

s, singlet; d, doublet; t, triplet; dd, doublet of doublets; m, multiplet; br, broad.

Absolute Stereochemistry of Rifamycin W (**4**)

For rifamycin B, the absolute stereochemistry of all chiral centers has been determined by X-ray crystallography[16]. As the chirality of the carbon C-28 in the precursor rifamycin W gets lost during the transformation to rifamycin B, its configuration could only be elaborated indirectly by comparison of a rifamycin W-sample prepared by enantioselective total synthesis with the natural product[17]. The hemiacetal (**12a/12b**), an intermediate in the biosynthetic pathway from rifamycin W to rifamycin B[15], could now be separated by chromatography into the two epimeric forms at C-34a. Using the epimer **12a** NMR experiments revealed the relative stereochemistry between the centers C-27 and C-28 as 'trans', deduced from the observed strong NOE's between H-C(25)/H-C(27) and H-C(26)/H-C(28), and measurements of coupling constants (Fig. 4). Thus, the absolute configuration of C-28 is confirmed as *R*.

Evidence for the (*E*)-geometry of the double bond C(12)–C(29) in rifamycin W is based on the strong NOE's in the 1H-NMR spectrum from the methyl group CH_3(13) to the protons of the methylene group CH_2(34a) and the methine proton CH(28); on the other hand, only a weak NOE to the vinylic proton CH(29) is observed.

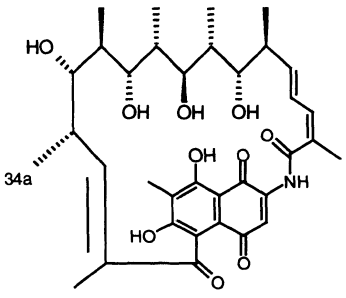
These results elaborated with the genuine natural product are well in accordance with the findings from the enantiospecific total synthesis of rifamycin W[17], which elucidated the configurations of the C-28 position as *R* and the C(12)–C(29) bond to be trans (*E*).

Biotransformation of Macrocyclic Rifamycin Precursors

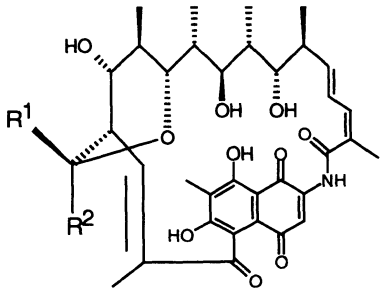
To investigate the biosynthetic interrelation of the different macrocyclic rifamycin intermediates, biotransformation studies were performed with *A. mediterranei* mutant strains. The extracts of the biotransformation reactions were analysed by HPLC-DAD technique. Each compound could be identified by its characteristic UV-spectrum and the unique HPLC retention time (see Table 1) except protorifamycin I and rifamycin W which were distinguishable by TLC (see Materials and Methods).

For these biotransformation studies the blocked mutants F1/24 and W126 were used because of their missing background of rifamycin B and S biosynthesis. In addition strain 3/1 was in particular useful since it is derived directly from strain N/813 (rifamycin B high-producer) by site directed deletion in the rifamycin cyclase gene *rifF*, eliminating all natural macrocyclic rifamycin biosynthesis in this strain[10]. The results are summarized in Table 3.

Fig. 3. Structures of rifamycin 34a-deoxy-rifamycin W (**11**), rifamycin W-hemiacetal (**12a/12b**), and rifamycin W-28-desmethyl-28-carboxy (**13**).

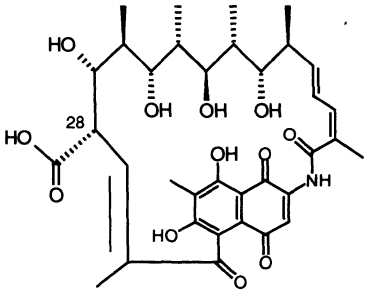


34a-Deoxy-rifamycin W **(11)**



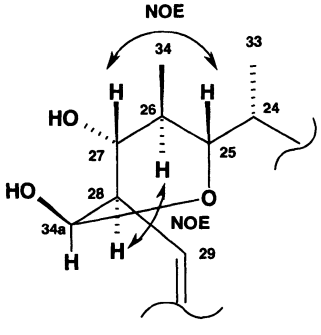
R^1 = OH
R^2 = H
Rifamycin W-hemiacetal **(12a)**

R^1 = H
R^2 = OH
Rifamycin W-hemiacetal **(12b)**



Rifamycin W-
28-desmethyl-28-carboxy
(13)

Fig. 4. Relative stereochemistry in rifamycin W-hemiacetal (**12a**).



Rifamycin W-hemiacetal **(12a)**
(partial structure)

Coupling constants:

$^3J_{H\text{-}27/H\text{-}26}$ = 10.5 Hz
$^3J_{H\text{-}27/H\text{-}28}$ = 10.5 Hz
$^3J_{H\text{-}27/OH}$ = 8.2 Hz
$^3J_{H\text{-}28/H\text{-}34a}$ = 4.5 Hz
$^3J_{H\text{-}25/H\text{-}26}$ = 10.5 Hz

Transformation of 8-Deoxy-intermediates

Important to note is the conversion of proansamycin B, identified and described in this study, to protorifamycin I by all three strains F1/24, 3/1 and W126. On the other hand, protorifamycin I was transformed into 8-deoxy-rifamycin B in low yield only with the strains F1/24 and 3/1, but not with W126. The observed low efficiency may explain why no direct biotransformation of proansamycin B to 8-deoxy-rifamycin B was found. The intermediate 8-deoxy-rifamycin S was converted to 8-deoxy-rifamycin B by all three strains, but a transformation of protorifamycin I, 8-deoxy-rifamycin S or 8-deoxy-rifamycin B to rifamycin W, rifamycin S or rifamycin B, respectively, was not detectable.

Table 3. Biotransformation of rifamycin precursors.

Substrates	Products of the biotransformation		
	Strain F1/24	Strain 3/1	Strain W126
proansamycin B	protorifamycin I	protorifamycin I	protorifamycin I
protorifamycin I	8-deoxy-rifamycin B	8-deoxy-rifamycin B	no transformation
8-deoxy-rifamycin S	8-deoxy-rifamycin B	8-deoxy-rifamycin B	8-deoxy-rifamycin B
8-deoxy-rifamycin B	no transformation	no transformation	no transformation
34a-deoxy-rifamycin W	no transformation	rifamycin W + rifamycin B	rifamycin W
rifamycin W	no transformation	rifamycin B	not tested *)
rifamycin S	rifamycin B	rifamycin B	rifamycin B

*) was not tested since mutant W126 is blocked in the transformation of rifamycin W to rifamycin S[8].

Transformation of 8-Hydroxy-intermediates

Rifamycin W is transformed to rifamycin B by the strain 3/1, but not by the strain F1/24. The analogous situation holds true for 34a-deoxy-rifamycin W which is very efficiently converted to rifamycin W by the strain 3/1 and, subsequently, to rifamycin B, whereas with the strain F1/24 no transformation occurred. 34a-Deoxy-rifamycin W is also easily transformed to rifamycin W as final product by the blocked mutant W126. The well documented transformation of rifamycin S to rifamycin B[9,18] was found with all three strains. Of the 8-hydroxy-intermediates none was transformed to a corresponding 8-deoxy-rifamycin.

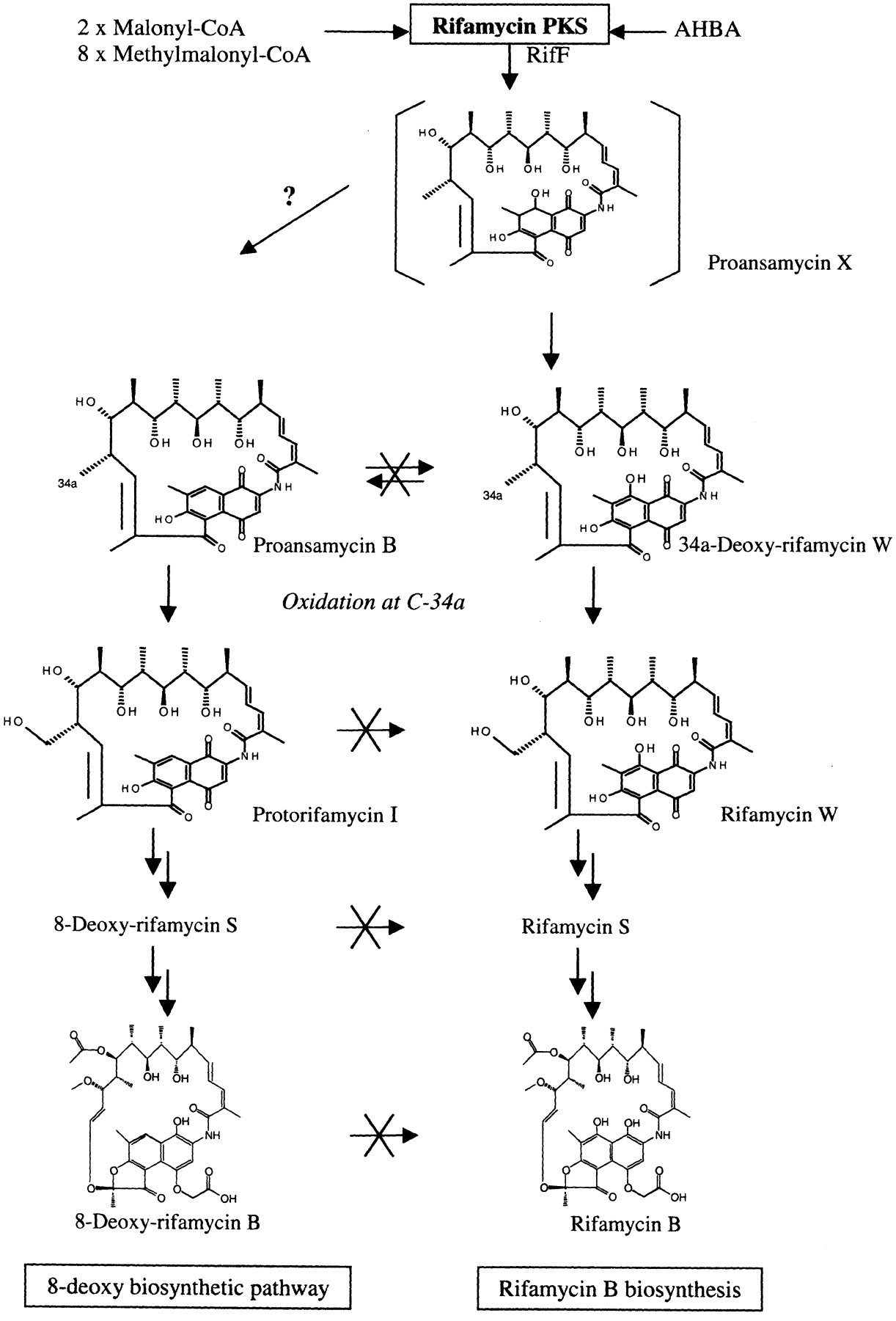
Discussion

In the present study we were able to isolate and structurally characterize two early macrocyclic rifamycin precursors, namely proansamycin B (**1**) and 34a-deoxy-rifamycin W (**11**), from fermentations of mutant strains of *A. mediterranei* in the high production medium FBR42. Proansamycin B, produced by mutant F1/24, had been postulated as early macrocyclic intermediate of rifamycin B biosynthesis[11], but was not detected and isolated so far. The closely related 34a-deoxy-rifamycin W has now been isolated from the rifamycin W-producing mutant W126 in a short fermentation run. These two compounds are the earliest macrocyclic rifamycin precursors described so far.

In transformation experiments using *A. mediterranei* mutants proansamycin B (**1**) was converted to protorifamycin I (**3**), but a subsequent transformation to rifamycin B is not observed. However, protorifamycin I is converted to 8-deoxy-rifamycin B in a separate assay, showing that a hydroxylation in the 8-position of the aromatic nucleus does not take place. These results indicate an independent biosynthesis pathway in the strain F1/24, an 8-deoxy variant, starting with proansamycin B as the first macrocyclic product and 8-deoxy-rifamycin B as the final product, with no connection between this 8-deoxy pathway and the "normal" (8-hydroxy) biosynthesis of rifamycin B (Fig. 5). These findings, supported by the failure of the transformation of 8-deoxy-rifamycin S or 8-deoxy-rifamycin B to rifamycin B, are in distinct contrast to the hitherto postulated biosynthetic scheme for rifamycin B *via* proansamycin B and protorifamycin I as early intermediates[10,11].

Proansamycin X has been proposed as the first macrocyclic intermediate of rifamycin B biosynthesis, based on hypothetical products of the rifamycin PKS gene cluster and on incorporation of [carboxyl-^{18}O]AHBA into rifamycin B with retention of ^{18}O at the C-8 position of the naphthoquinone ring[6,19]. Inactivation of the *rifF* gene

Fig. 5. Rifamycin biosynthesis pathways.



(rifamycin amide synthase), the enzyme responsible for the intramolecular ring closure and release of the polyketide chain from the PKS, did not provide final evidence for proansamycin X as the first *post* PKS product[10,20]. Also all attempts in our study to detect and isolate the putative intermediate proansamycin X failed. On the other hand, the intermediate 34a-deoxy-rifamycin W which we have isolated as a minor product of the mutant W126 of *A. mediterranei*, represents the earliest macrocyclic intermediate in rifamycin B biosynthesis found so far. It is distinguished from the postulated proansamycin X by two hydrogen atoms and contains a substituted naphthoquinone instead of the corresponding 7,8-dihydronaphthoquinone system. 34a-Deoxy-rifamycin W could be successfully transformed to rifamycin W, thus it is a true intermediate in the pathway to rifamycin B, whereas proansamycin B or protorifamycin I are not formed out of 34-deoxy-rifamycin W. Therefore, branching into the two parallel biosynthetic routes which lead to 8-deoxy-rifamycin B and rifamycin B, respectively, must occur before the formation of 34a-deoxy-rifamycin W, but at which exact stage remains unclear. These results are well in accordance with the ^{18}O incorporation experiments demonstrating retention of the label in the 8-hydroxy group[6,19]. The 8-deoxy biosynthesis pathway seems to be much less efficient than the normal rifamycin B pathway, because the respective intermediates of it were only detectable in fermentations of the mutant F1/24 which has an early block in the normal 8-hydroxy pathway. This shunt pathway is also present in the other two mutants, 3/1 and W126, as demonstrated by the biotransformation studies, and its low efficiency is reflected by the slower turnover of the analysed 8-deoxy-intermediates relative to the 8-hydroxy-analogues of the normal pathway of rifamycin B biosynthesis.

Acknowledgements

The authors thank the late CH. QUIQUEREZ (Novartis Pharma AG) for the measurements of the mass spectra and F. PETERSEN (Head of Natural Products, Novartis Pharma AG) for supporting this work.

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