Direct evidence that the rifamycin polyketide synthase assembles polyketide chains processively

Tin-Wein Yu*, Yuemao Shen*, Yukiko Doi-Katayama†, Li Tang†, Cheonseok Park†, Bradley S. Moore*,
C. Richard Hutchinson†‡§, and Heinz G. Floss*§

*Department of Chemistry, Box 351700, University of Washington, Seattle, WA 98195-1700; and †School of Pharmacy and ‡Department of Bacteriology,
University of Wisconsin, Madison, WI 53706

ABSTRACT The assembly of the polyketide backbone of
rifamycin B on the type I rifamycin polyketide synthase (PKS),
encoded by the *rifA–rifE* genes, is terminated by the product
of the *rifF* gene, an amide synthase that releases the completed
undecaketide as its macrocyclic lactam. Inactivation of *rifF*
gives a rifamycin B nonproducing mutant that still accumu-
lates a series of linear polyketides ranging from the tetra- to
a decaketide, also detected in the wild type, demonstrating
that the PKS operates in a processive manner. Disruptions of
the *rifD* module 8 and *rifE* module 9 and module 10 genes also
result in accumulation of such linear polyketides as a conse-
quence of premature termination of polyketide assembly.
Whereas the tetraketide carries an unmodified aromatic
chromophore, the penta- through decaketides have undergone
oxidative cyclization to the naphthoquinone, suggesting that
this modification occurs during, not after, PKS assembly. The
structure of one of the accumulated compounds together with
^{18}O experiments suggests that this oxidative cyclization pro-
duces an 8-hydroxy-7,8-dihydronaphthoquinone structure
that, after the stage of proansamycin X, is dehydrogenated to
an 8-hydroxynaphthoquinone.

The rifamycins (1), exemplified by rifamycin B (**1**) (2, 3),
isolated in 1959 from *Amycolatopsis mediterranei* (4), are a
group of antibiotics of the ansamycin family (5) with pro-
nounced antibacterial activity. Several chemically modified
rifamycins, such as rifampicin, have been used since the
mid-1960s for the treatment of tuberculosis and other myco-
bacterial infections (6). However, as in the case of many
antibiotics, their effectiveness is threatened by the emergence
of resistant pathogens (7), making the development of mod-
ified rifamycins not subject to these resistance mechanisms a
matter of urgency. Although numerous rifamycin derivatives
have been synthesized from the natural products, the structural
modifications largely were confined to one region of the
molecule, the C-3 position of the naphthoquinone chro-
mophore (6, 8). More deep-seated structural alterations of the
molecule are difficult to accomplish chemically and clearly call
for alternative (biosynthetic) methods of structure modifica-
tion.

The biosynthetic assembly of the rifamycins (1) involves
chain extension of an unusual starter unit, 3-amino-5-
hydroxybenzoic acid (AHBA) (**2**) (ref. 9; ref. 10 and references
therein), by propionate and acetate units on a type I polyketide
synthase (PKS) (Fig. 1). Premature polyketide chain termina-
tion in a mutant strain leads to release of a tetraketide,
P8/1-OG (**3**) (11), from the PKS and its excretion into the
medium, whereas in the wild-type organism, processing con-
tinues to the stage of an undecaketide, which then is released
from the enzyme as a macrocyclic lactam, presumably proan-

samycin X (**4**) (12, 13). The simplest ansamycin isolated from
cultures is protorifamycin I (**5**, shown in Fig. 5), which has been
proposed to be a precursor of the rifamycins (14). The initial
cyclization product then is subject to extensive downstream
processing to give the final biologically active rifamycins.

The rifamycin biosynthetic gene cluster recently has been
cloned from *A. mediterranei* and sequenced (12, 13, 15). On
one side of the cluster of about 90 kb are located about 36 kb
of DNA-containing genes required for AHBA synthesis, genes
involved in export and resistance, regulatory genes, and a
substantial number of downstream-processing genes. The
other side of this cluster consists of 52 kb of DNA encoding a
type I PKS that consists of 10 modules, each with the expected
functional domains, arranged colinear with the sequence of
chain-extension steps that must occur in the biosynthesis of the
antibiotic. These 10 modules are arranged in five genes/
proteins. *RifA* contains the loading domain and the first three
modules, *rifB* contains the next three modules, *rifC* and *rifD*
each contain one module, and *rifE* contains the final two
modules. An interesting feature compared with the well-
characterized PKSs encoding the formation of macrolides,
such as the erythromycin precursor, 6-deoxyerythronolide B
(16), is the absence of a type 1 thioesterase domain (17) at the
end of the last module of the *rif* PKS. Such a thioesterase,
catalyzing the release of the fully assembled polyketide from
the enzyme as the macrocyclic lactone, is characteristic of type
I macrolide PKSs. A type 2 thioesterase (17), similar to one
found additionally in macrolide and nonribosomal peptide
biosynthetic gene clusters (18, 19) is, however, present in the
rif cluster (*rifR*) (12). Immediately after the *rifE* PKS gene is
located a gene, *rifF*, that encodes a protein with pronounced
homology to mammalian arylamine:acetyl-CoA acetyltrans-
ferases (20). Based on the chemical analogy to this reaction, we
have proposed (12) that this gene product catalyzes the release
of the completed polyketide from the PKS by intramolecular
amide formation. Here we describe an unexpected property of
the *rif* PKS, based on the behavior of *rifD*, *rifE*, and *rifF*
mutants, that has widespread implications for modular PKSs in
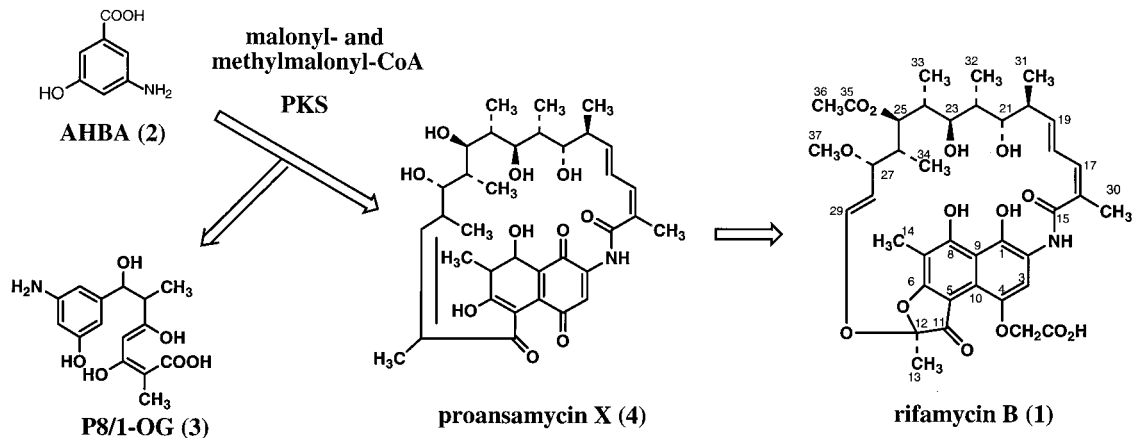general.

MATERIALS AND METHODS

Materials and General Methods. *A. mediterranei* strain
S699 was obtained from G. C. Lancini (Lepetit S.A., Varese,
Italy); the wild type and mutants (except as described below)
were cultivated as reported previously (ref. 10 and references
therein). [7-^{13}C, ^{18}O$_2$]-AHBA was prepared as described by
Rickards and coworkers (21). Protorifamycin I was a gift from
Novartis. The compounds were fed to the appropriate mutants

FIG. 1.   Biosynthesis of rifamycin B in *A. mediterranei*.

in liquid culture (AHBA) or surface cultures grown on agar plates (protorifamycin I); the cultures then were analyzed for rifamycin B **1** by HPLC and bioassay. In the [7-^{13}C, ^{18}O$_2$]-AHBA feeding, **1** was isolated by HPLC and analyzed by electrospray–MS as described (22). Liquid chromatography–MS (LC-MS) analyses were carried out with a Shimadzu LC-10AD pump connected to a Fisons Quattro II mass spectrometer (Fisons, Loughborough, U.K.).

Gene Inactivations. Gene disruptions were carried out essentially as described for the construction of the *rifK*(−) mutant (ref. 10 and references therein). For the disruption of *rifF*, replacement suicide vector pHGF1087 was created by insertion of a 1.75-kb *Bgl*II fragment carrying the hygromycin resistance gene (*hyg*) from pIJ963 (23) into *Bam*HI-treated pHGF1086, in which the 3.0-kb *Bam*HI–*Bsa*BI and 3.1-kb *Age*I(blunt-ended)–*Xho*I DNA fragments from the *rif* gene cluster (see Fig. 2*A*) were coligated into pBluescript KS(−)/ (*Bam*HI, *Xho*I), generating a 369-bp, in-frame deletion at the

N-terminal end of *rifF* (see Fig. 2*C*). The heat-denatured pHGF1087 was introduced by electroporation into *A. mediterranei* S699, and six transformants resistant to hygromycin were selected for further investigation. Southern blotting of these six transformants (data not shown) demonstrated that they arose by the expected single-crossover integration either upstream or downstream of *rifF*. The four upstream-integrated transformants had lost the ability to produce rifamycin B, whereas the two downstream integrants produced normal rifamycin B titers. One of the downstream-integrated transformants was chosen for maintenance on synthetic agar medium (24) without hygromycin. After propagation through three generations, five colonies showed sensitivity to hygromycin and two of them also showed loss of rifamycin B production. Southern blotting with the *Not*I-digested genomic DNA from these hygromycin-sensitive colonies revealed that all of them had undergone a second crossover event. The three rifamycin B-producing colonies reverted back to wild type, and
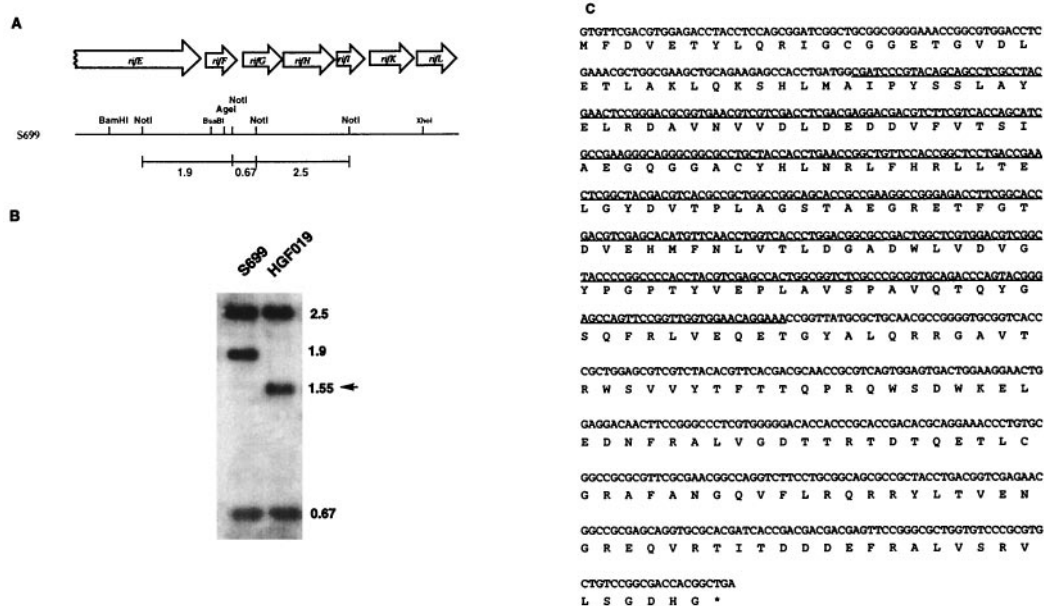

FIG. 2.   Genetic organization and nucleotide sequence of the rifF gene in *A. mediterranei* S699 and HGF019. (*A*) A restriction map of the region of the *A. mediterranei* S699 chromosome encompassing the C-terminal end of the polyketide synthase (*rifE*) and the whole ORFs of the amide synthase (*rifF*), amino DHQ synthase (*rifG*), amino DAHP synthase (*rifH*), amino quinate/shikimate dehydrogenase (*rifI*), AHBA synthase (*rifK*), and oxidoreductase (*rifL*) genes (12). The three indicated *Not*I DNA fragments, 0.67, 1.9, and 2.5 kb, respectively, were used as a ^{32}P-dCTP-labeled probe for the blot in *B*. (*B*) The autoradiograph of a Southern blot to verify the deletion of a 369-bp DNA fragment of *rifF* in *A. mediterranei* HGF019. A 0.8% agarose gel in which 5 μg of *Not*I-digested genomic DNA from strains S699 and HGF019 had been separated was blotted and hybridized with the three *Not*I DNA fragments shown above. The band corresponding to the deleted DNA fragment, 1.55 kb, of *rifF* in strain HGF019 is indicated by an arrow. (*C*) The nucleotide and deduced amino acid sequences of the *rifF* gene. The deleted 369-bp DNA fragment in strain HGF019 is underlined.

the two rifamycin B nonproducing mutants gave HGF019, in which the endogenous *rifF* was replaced with the inactive, truncated version (Fig. 2*B*).

The *rifE* module 10 disrupted mutant was constructed by cloning a 5.1-kb *Sst*I fragment from *rifE* (Fig. 3*B*) into pUC19 (25) to create pTL139. A 1.8-kb segment containing the hygromycin resistance gene described above was inserted at the unique *Nco*I site of pTL139, which is located inside the KS10 domain of *rifE* module 10, to form pTL175. This plasmid was introduced into the *A. mediterranei* S699 strain, and hygromycin-resistant transformants were selected as described above. Southern analysis of *Sst*I-digested DNA from one of these transformants showed that it had a genotype consistent with the expected single-crossover recombination event diagrammed in Fig. 3*B*.

The *rifD* and *rifE* module 9 disrupted mutants were made by first preparing a PCR product by using the modified splicing by overlap extension–PCR method (26). To delete the entire module 8, the first two PCRs were performed separately. One PCR mixture contained primers (a) 5′-GCGACCAGGTG-GCGGCGCTGC-3′ and (b) 5′-GTCCGTGGCCATT-GAACTCAACTCC CAGAACTC-3′ as primers to obtain a 0.7-kb DNA segment containing the end of module 7 attached via a 12-bp linker to the start of module 9 (underlined). The other PCR mixture used primers (c) 5′-GAGTTGAGT-TCAATGGCCACGGACGAGAAACTC-3′ and (d) 5′-CAGCCGGTGCCGTCCGCGGTC-3′, to result in a product with the 0.5-kb beginning part of module 9 joined via a 12-bp linker to the end of module 7 (underlined). After the first PCR products were purified, a third PCR was carried out with these

products as templates and primers (a) and (b) to assemble a final DNA segment in which all of module 8 had been deleted. From the final PCR product, a 1-kb *Eco*47III–*Xho*I fragment was purified and its sequence was confirmed, by using an Applied Biosystems model 377 sequencer and the Perkin—Elmer Amplitaq Dye-terminator sequencing system. This DNA segment was joined to a 2.1-kb *Eco*47III–*Sst*I fragment from module 7 and a 2.2-kb *Xho*I–*Pst*I fragment from module 9 and inserted into pGEM3zF(+) (Promega) to form pCS101. A 5.3-kb *Bam*HI–*Xba*I segment of pCS101 was transferred into the multiple cloning site of pANT841 (this vector was obtained from W. R. Strohl, Ohio State University, with the *hyg* resistance gene inserted) digested with *Nhe*I and *Bgl*II, to give pCS102 (Fig. 3*A*). Hygromycin-resistant transformants of the S699 strain were obtained as above and analyzed for the position of pCS102 integration by Southern blot hybridization. Two kinds of clones were obtained: in WMH-M101, the single-crossover recombination event had occurred in module 7 to produce a strain with a disrupted *rifD* gene (Fig. 3*A*-1), and in WMH-M102, the single-crossover recombination event had occurred in module 9, thereby disrupting *rifE* module 9 (Fig. 3*A*-2).

Isolation of Products from Mutants. *rifF*(−). After 7 days of fermentation at 28°C as described (ref. 10 and references therein), the broth together with mycelia were frozen at −80°C and dried by lyophilization. The residue from 4 liters of fermentation was extracted with 500 ml of 80% aqueous methanol containing 1% formic acid. Solvent was removed under vacuum, and the residue was dissolved in 100 ml of 1% aqueous formic acid and extracted twice with 100 ml of *n*-butanol. After removal of the solvent, the residue from this butanol extract was partitioned between 200 ml each of ethyl acetate and 1% formic acid and the aqueous layer was extracted once more with 200 ml of ethyl acetate. The combined organic extract then was subjected to flash chromatography on C_{18} reversed-phase silica gel (5 μ; YMC, Kyoto), eluting with a gradient of 9–26% of CH_3CN in 1% aqueous formic acid. The individual compounds were purified further by HPLC on a semipreparative C_{18} column (ultrasphere, 5 μ; Alltech Associates) with UV detection at 274 and 322 nm and acetonitrile/water/formic acid as the liquid phase. The approximate isolated yields of products were 4 mg of SY4a, 1 mg of SY4b, 0.75 mg of SY5, 0.6 mg of SY6, 0.75 mg of SY7, 0.75 mg of SY8, 0.5 mg of SY9, and 0.25 mg of SY10 from 1 liter of culture.

RifE(−). The mycelium of the *rifE* mutant, which was stocked in 20% glycerol at −80°C, was transferred into three 250-ml baffled flasks, each containing 50 ml of seed medium (4 g of yeast extract, 10 g of malt extract, and 4 g of glucose in 1 liter of water), hygromycin (250 μg/ml), and 4 g glass beads and incubated for 3 days on a shaker (250 rpm, 30°C). The seed culture then was inoculated into three 2-liter baffled flasks, each containing 500 ml of medium (5 g of meat extract, 5 g of peptone, 5 g of yeast extract, 2.5 g of enzyme hydrolysate of casein, 20 g of glucose, and 1.5 g of NaCl in 1 liter of water), and fermented for 7 days. The cultures were combined and adjusted to pH 4.5 with 15% aqueous trichloroacetic acid. The mycelium was removed by centrifugation, and the broth was extracted with ethyl acetate (two times with same volume as the broth). The ethyl acetate-soluble fraction was evaporated under reduced pressure (<30°C), and the resulting residue was passed through a Sep-Pak C_{18} cartridge (Waters), eluting with 40% CH_3CN. Acetic anhydride (1.5 ml) and pyridine (3 ml) were added to the material (300 mg), and the mixture was stirred for 20 h at room temperature. The solution was evaporated and the residue was separated by silica gel column chromatography (1.0 × 30 cm; Aldrich), eluting with toluene/MeOH from 95:5 to 1:1 (vol/vol). The material eluting from 95:5 to 90:10 toluene/MeOH (233 mg) was subjected to silica gel column chromatography, eluting with hexane/ethyl acetate
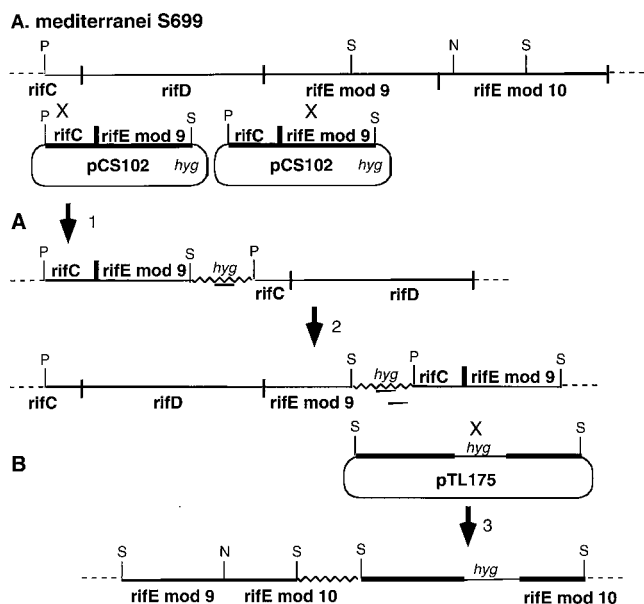

Fig. 3. Construction of *rifD* and *rifE* mutants. (*A*) The *rifD* mutant resulted from crossover 1 to produce a hygromycin-resistant strain in which the entire *rifD* gene has been deleted, which, in effect, produces a strain without the RifD and RifE subunits of the PKS, because transcription does not reinitiate downstream of *rifC*. The *rifE* module 9 mutant resulted from crossover 2 to produce a hygromycin-resistant strain in which module 9 for the *rifE* gene has been disrupted, which results in a strain without the RifE subunit of the PKS because transcription does not reinitiate downstream of *rifD*. (*B*) The *rifE* module 10 mutant resulted from crossover 3 to produce a hygromycin-resistant strain in which module 10 of the *rifE* gene has been disrupted, producing a strain with a truncated RifE protein. Thicker black lines represent the genomic DNA and the boundaries of the *rif* PKS genes. The thick, black line in pCS102 between *rifC* and *rifE* module 9 shows the place at which these two genes were fused. The jagged line presents the vector DNA. P, *Pst*I; S, *Sst*I; N, *Nco*I; Hyg, hygromycin resistance gene.

from 90:10 to 10:90, and a 73-mg portion of the material recovered from 50:50 to 40:60 hexane-ethyl acetate was passed through a Sep-Pak C_{18} cartridge. The fraction eluting with 35% CH_3CN finally was purified by RP-HPLC [Nova-Pak C_{18}; Waters; 10×300 mm; flow rate, 2.5 ml/min; detection by UV at 320 nm; eluant CH_3CN plus 0.05% trifluoroacetic acid/H_2O (35:65, vol/vol)] to yield SY6a (t_{ret}, 25.5 min; 31.6 mg) and SY5a (t_{ret}, 12.6 min; 23.6 mg).

The constitutional structures of the isolated compounds were determined by high-resolution fast atom bombardment MS and by one-dimensional (^{1}H, ^{13}C, difference nuclear Overhauser effect) and two-dimensional (heteronuclear multiple bond correlation) NMR spectroscopy. Rotation measurements showed them to be optically active, but their stereochemistries, assumed to correspond to those of the natural rifamycins, were not determined independently. The structure elucidation of these compounds will be reported in a forthcoming paper.

RESULTS

Disruption of *rifF*. To probe the function of the proposed off-loading enzyme RifF, we constructed a mutant of *A. mediterranei* S699 in which part of the *rifF* gene had been deleted from the genome (Fig. 2). The mutant construction provided evidence that there is no endogenous promoter located on the 2.9-kb C-terminal end of *rifE*. Taken together with the deduced translational coupling between *rifE* and *rifF*, this shows that the PKS products (RifA–E), or at least the one from *rifE*, and the product from *rifF* should share the same transcription unit. It also revealed that there might be a promoter located downstream of *rifF* that directly controls the downstream genes such as *rifG*, *rifH*, and *rifI*, which are thought to be involved in the formation of the starter unit AHBA (12). The *rifF*(−) mutant no longer synthesizes rifamycin B, the main product of this fermentation, but HPLC analysis of the organic extract revealed a very complex product pattern. Isolated and fully characterized by HR-MS and one- and two-dimensional NMR was a series of linear polyketides, from the pentaketide SY5 to the decaketide SY10, all carrying a naphthoquinone chromophore without a hydroxyl group at C-8 (Fig. 4). The heptaketide and the nonaketide were isolated

as the carboxyl-terminal lactones, but LC-MS also indicated the presence of the corresponding open-chain acids. The corresponding undecaketide, if present at all, is not a prominent metabolite as judged from LC-MS evidence. Also isolated and structurally characterized were *N*-formyl and *N*-acetyl derivatives of the tetraketide, SY4a and SY4b, lactone analogs of the previously reported compound **3** (11), carrying a 3-amino-5-hydroxybenzene chromophore, and a hydroxyl group at the position corresponding to C-8 in the rifamycins (Fig. 4). LC-MS evidence additionally suggests the presence of the corresponding free amino compound P8/1-OG lactone. The tetra- and pentaketides are the most prevalent compounds; the amounts of metabolite obtained decrease with increasing chain length, but the di- and triketides, if present at all, also are formed only in trace amounts. The sum of the accumulated metabolites adds up to approximately 10–20% of the amount of rifamycin B formed in a wild-type fermentation. Original indications based on feeding experiments with ^{13}C-AHBA and NMR analysis were that these compounds are not formed by the wild-type organism. However, subsequent LC-MS analysis with selective ion monitoring and comparison with the authentic samples revealed that the same compounds also are present in extracts of wild-type *A. mediterranei* S699.

Disruption of PKS Genes. Disruption of the *rifD* module 8 and *rifE* modules 9 and 10 genes, as shown in Fig. 3, also led to the production of linear polyketides. Acetylation of the crude material obtained from the *rifE* module 10 mutant before further purification resulted in isolation of SY6a, the diacetate of the 8-hydroxy-7,8-dihydronaphthoquinone analog of hexaketide SY6, plus SY5a, the monoacetate of the enol of pentaketide SY5 (Fig. 4). The structure of SY6a was fully consistent with its FAB-MS and one- and two-dimensional NMR data. Moreover, tetraketide **3** to decaketide SY10 were detected by HPLC analysis of the *rifE* module 10 mutant (which cannot make the undecaketide), whereas the *rifD* mutant produced the tetraketide-to-octaketide products (heptaketide SY7 and octaketide SY8 were the main products); the *rifE* module 9 mutant accumulated a similar set of compounds (octaketide and nonaketide were the main products). Peaks with a UV spectrum characteristic of a dihydronaphthoquinone ring, as in SY6a, were seen in these extracts as well, but not in the *rifF*(−) extract.

Origin of C-8 Oxygen in Rifamycins. The isolation of a series of polyketides mostly containing a naphthoquinone chromophore without the 8-hydroxy group raises a question about the pathway of rifamycin formation that is unsettled in the literature. Clearly, the structure of the chromophore initially generated on the enzyme, and presumably present in the first cyclic product released, proposed to be **4**, must be that of an 8-hydroxylated 7,8-dihydronaphthoquinone. This follows from the structure of the first PKS module, which has a functional ketoreductase domain but an inactive dehydratase domain and is supported by the structures of **3**, SY4a, SY4b, and, particularly, SY6a. The question, however, is whether this 8-hydroxy-7,8-dihydronaphthoquinone is converted to the 8-hydroxynaphthoquinone chromophore of the rifamycins by (*i*) dehydration followed by hydroxylation at C-8 or (*ii*) by a direct dehydrogenation to introduce the 7,8-double bond (Fig. 5). The first pathway has been proposed by Ghisalba *et al.* (14) based on the structure of **5** and their finding of its partial conversion into rifamycins. On the other hand, Rickards and coworkers (21) fed AHBA carrying ^{18}O in the carboxyl group and reported incorporation of ^{18}O into the 8-hydroxy group of rifamycins, implying that route *ii* must be operating. Because the two reports are in conflict, we reexamined both of these experiments. Feeding of **5** to a mutant, HGF010 (unpublished data), of *A. mediterranei* carrying a deletion from the *rif* gene cluster covering part of the last PKS module through *rifI*, i.e., a mutant that can neither synthesize AHBA nor assemble a complete rifamycin polyketide, but that contains all the down-
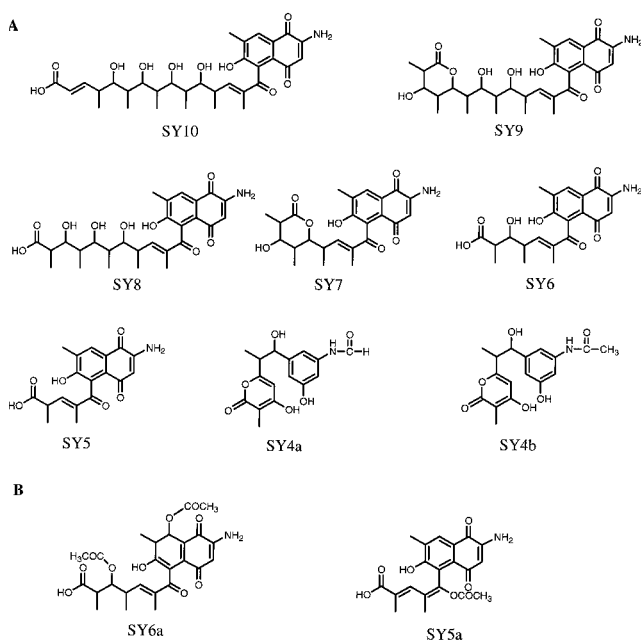

FIG. 4.   Structures of compounds isolated and characterized from the *rifF*(−) mutant (*A*) and the *rifE* disrupted mutant (*B*).
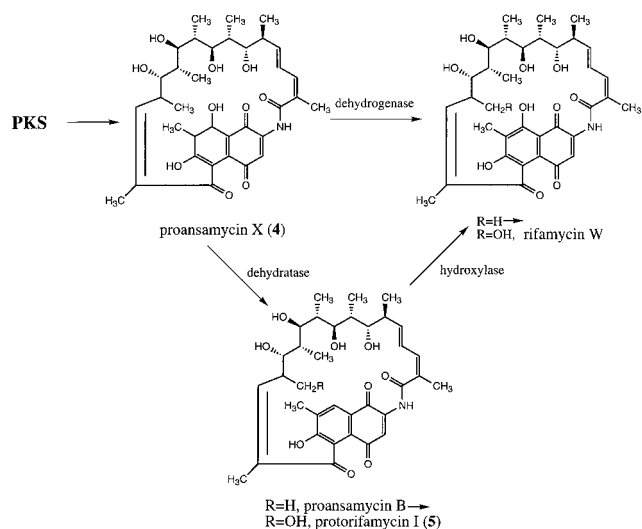
FIG. 5.   Two possible routes from proansamycin X (**4**) to rifamycin W, the precursor of rifamycin B (**1**).

stream-processing genes, resulted in no detectable formation of rifamycin B (although small amounts of an unidentified yellow pigment were produced). Although one cannot rule out the possibility that the precursor does not penetrate the cell membrane, this result provides no support for the proposed precursor role of **5** in rifamycin biosynthesis. Conversely, [7-^{13}C,^{18}O$_2$]AHBA (85% ^{18}O, 89% ^{13}C) was fed to a *rifK*(−) mutant that depended on external AHBA for **1** synthesis. The resulting rifamycin B from this mutant contained 82% ^{18}O in addition to the ^{13}C, as demonstrated by electrospray ionization–MS, indicating that the C-8 oxygen is derived from the carboxyl group of AHBA. These results thus strongly support a pathway of rifamycin biosynthesis involving direct dehydrogenation of **4**. Hence, both **5** and the naphthoquinone compounds SY5 through SY10 isolated in this work are either shunt metabolites or artifacts of the work-up process arising from the true intermediate structures carrying an 8-hydroxy-7,8-dihydronaphthoquinone chromophore, as in SY6a.

DISCUSSION

Because inactivation of *rifF* abolished the synthesis of **1** but not that of the open-chain polyketides, the results of this study support the proposed role of RifF as the chain-termination enzyme in rifamycin B **1** biosynthesis. This represents another mode of release of an assembled polyketide chain from a type I PKS, an alternative to the well-known chain-termination catalyzed by thioesterase domains, as exemplified by the erythromycin PKS system. The data also provide very clear evidence that the *rif* PKS assembles polyketide chains in a processive manner as illustrated in Fig. 6. It seems plausible, and is consistent with the data, that multiple polyketide chains are synthesized simultaneously on the same enzyme molecule, but an alternative model in which ketides of different chain length are released from different protein molecules cannot be ruled out. Evidence for the processive nature of polyketide assembly on type I PKSs comes primarily from feeding experiments demonstrating the intact incorporation of partially assembled precursor oligoketides, which first were carried out in the erythromycin and tylosin systems (27, 28). Only a few studies have provided evidence for the accumulation of such intermediates during either the normal or impaired function of a type I PKS. Working on the mycinamicin producer, *Micromonospora griseorubida,* Kinoshita *et al.* (29) isolated from a nonproducing mutant a triketide containing the starter and first two chain-extension units and, from the wild type, the corresponding tetraketide and the decarboxylation product of the pentaketide. Tetra- and decarboxylated pentaketides were isolated from nonproducing mutants of the tylosin producer *Streptomyces fradiae* (30). However, noncyclized polyketide intermediates have not been observed during extensive work with 6-deoxyerythronolide B synthase (31) *in vivo* and *in vitro* (R. McDaniel and C. Khosla, personal communication). The present work, showing the accumulation of a whole series of ketides of different chain length, represents perhaps the most direct support for the widely accepted notion that type I PKSs assemble polyketide chains processively.

Because the ketides isolated from the *rifF*(−) mutant also are found in the wild-type extract, although their presence is masked by large amounts of rifamycin, their formation is not the result of the mutation to eliminate the off-loading mechanism. It is noteworthy that all of the isolated ketides, with the
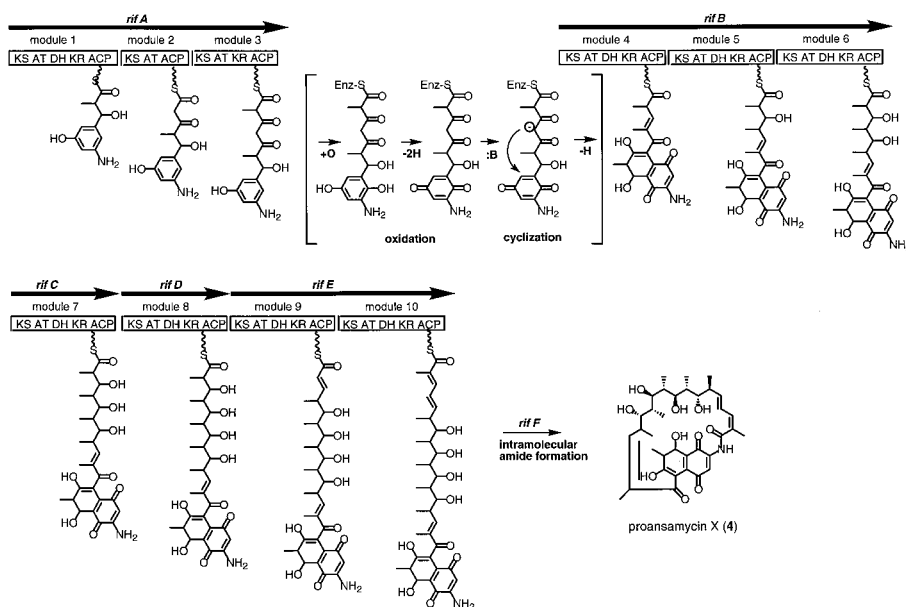

FIG. 6.   Proposed mechanism of polyketide assembly, dihydronaphthoquinone ring closure, and macrolactam ring closure on the type I rifamycin polyketide synthase of *A. mediterranei*.

exception of SY6a, which comes from a PKS-truncated mutant, have undergone a structural modification in the aromatic moiety, either a dehydration from the 8-hydroxy-7,8-dihydronaphthaline to the naphthalene ring structure in SY5-SY10 or an acylation of the nitrogen in SY4a and SY4b. If these modifications occurred on the enzyme-bound ketides, either spontaneously or by the aberrant action of an enzyme, they may prevent the further processing of the modified ketide, with consequent inactivation of the PKS. The release of these products then may reflect the operation of a repair mechanism, which removes the aberrant product from the enzyme. Such a repair process may be an inherent function of the PKS itself, or it may be catalyzed by an auxiliary enzyme, such as the type 2 thioesterase encoded by *rifR* (12), as has been suggested for this type of enzyme in macrolide (19) and nonribosomal peptide biosynthesis (18). Alternatively, however, the structural modifications may be introduced only after release of the incomplete polyketides from the PKS, and the accumulated ketides simply may represent normal shunt metabolites of this fermentation.

Our data indicate that the ring closure from the benzenoid to the naphthoquinoid system may not be a postsynthetic modification of the polyketide produced by the *rifA–F* genes, but could occur either during its assembly or before the ansamacrolide ring is closed by the RifF enzyme, to form **4** (Fig. 6). Mechanistically, this transformation presumably requires enzymatic oxidation to the benzoquinone followed by Michael addition of a C-5 carbanion, which may or may not be enzyme-controlled, driven by reoxidation of the resulting naphthohydroquinone to the quinone (Fig. 6). This modification most likely occurs between the tetraketide and pentaketide stage, coincident with the handover of the substrate from the last module of RifA to the first module of RifB. The absence of uncyclized pentaketide among the products isolated from the *rifF* mutant implies that oxidative cyclization is less likely to occur after formation of the pentaketide by the first module of RifB. The fact that **3** does not seem to be a free intermediate in rifamycin biosynthesis (C. Khosla, personal communication) suggests that the cyclization may occur on a PKS-bound tetraketide chain. (Cyclization after release would require that the enzymes involved have an unusually broad substrate specificity, encompassing the pentaketide-to-undecaketide substrates but ignoring the tetraketide.) Thus, the enzyme(s) catalyzing it either must be part of the PKS or must dock to the PKS, and module 4 of RifB may only chain-extend a (dihydro)naphthoquinoid, i.e., cyclized tetraketide. Downstream recognition of modifications in the starter unit had been demonstrated earlier by the observation that 3-hydroxy- and 3,5-dihydroxybenzoic acid were used as starter units but resulted only in the formation of the desamino or 3-hydroxy analogs of **3**, respectively (32). The cumulative evidence points to module 4 as the site of this discrimination. This obviously has implications for future efforts to produce modified rifamycins by genetic engineering of the biosynthetic process.

1. Lancini, G. & Cavalleri, B. (1997) in *Biotechnology of Antibiotics*, ed. Strohl, W. R. (Dekker, New York), 2nd Ed., pp. 521–549.
2. Brufani, M., Fedeli, W., Giacomello, G. & Vaciago, A. (1964) *Experientia* **20,** 339–342.
3. Oppolzer, W. & Prelog, V. (1973) *Helv. Chim. Acta* **56,** 2287–2314.
4. Sensi, P., Margalith, P. & Timbal, M. T. (1959) *Farmaco Ed. Sci.* **14,** 146–147.
5. Rinehart, K. L., Jr. & Shields, L. S. (1976) *Fortsch. Chem. Org. Naturst.* **33,** 231–307.
6. Sepkowitz, K. A., Rafalli, J., Riley, L., Kiehn, T. E. & Armstrong, D. (1995) *Clin. Microbiol. Rev.* **8,** 180–199.
7. Service, R. F. (1995) *Science* **270,** 724–727.
8. Lancini, G. C. & Zanichelli, W. (1977) in *Structure-Activity Relationships Among the Semisynthetic Antibiotics*, ed. Perlman, D. (Academic, New York), pp. 537–600.
9. Ghisalba, O. (1985) *Chimia* **39,** 79–88.
10. Kim, C.-G., Yu, T.-W., Fryhle, C. B., Handa, S. & Floss, H. G. (1998) *J. Biol. Chem.* **273,** 6030–6040.
11. Ghisalba, O., Fuhrer, H., Richter, W. & Moss, S. (1981) *J. Antibiot.* **34,** 58–63.
12. August, P. R., Tang, L., Yoon, Y. J., Ning, S., Müller, R., Yu, T.-W., Taylor, M., Hoffmann, D., Kim, C.-G., Zhang, X., *et al.* (1998) *Chem. Biol.* **5,** 69–79.
13. Tang, L., Yoon, Y. J., Choi, C. Y. & Hutchinson, C. R. (1998) *Gene* **216,** 255–265.
14. Ghisalba, O., Traxler, P. & Nüesch, J. (1978) *J. Antibiot.* **31,** 1124–1131.
15. Schupp, T., Toupet, C., Engel, N. & Goff, S. (1998) *FEMS Microbiol. Lett.* **159,** 201–207.
16. Donadio, S., Staver, M. J., McAlpine, J. B., Swanson, S. J. & Katz, L. (1991) *Science* **252,** 675–679.
17. Magnuson, S., Rock, C. O. & Cronin, J. E., Jr. (1993) *Microbiol. Revs.* **57,** 522–542.
18. Schneider, A. & Marahiel, M. A. (1998) *Arch. Microbiol.* **169,** 404–410.
19. Xue, X., Zhao, L., Liu, H.-w. & Sherman, D. H. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 12111–12116.
20. Ohsako, S., Ohtomi, M., Sakamoto, Y., Uyemura, K. & Deguchi, T. (1988) *J. Biol. Chem.* **263,** 7534–7538.
21. Anderson, M. G., Monypenny, D. & Rickards, R. W. (1989) *J. Chem. Soc. Chem. Commun.,* 311–313.
22. Kim, C.-G., Kirschning, A., Bergon, P., Zhou, P., Su, E., Sauerbrei, B., Ning, S., Ahn, Y., Breuer, M., Leistner, E. *et al.* (1996) *J. Am. Chem. Soc.* **118,** 7486–7491.
23. Lydiate, D. J., Malpartida, F. & Hopwood, D. A. (1985) *Gene* **35,** 223–235.
24. Lancini, G. (1986) in *Biotechnology*, eds. Rehm, H.-J. & Reed, G. (VCH, New York), Vol. 4, pp. 431–469.
25. Yanish-Perron, C., Vieira, J. & Messing, J. (1985) *Gene* **33,** 103–109.
26. Horton, R. M., Hunt, H. D., Ho, S. N., Pullen, J. K. & Pease, L. R. (1989) *Gene* **77,** 61–68.
27. Yue, S., Duncan, J. S., Yamamoto, Y. & Hutchinson, C. R. (1987) *J. Am. Chem. Soc.* **109,** 1253–1255.
28. Cane, D. E. & Yang, C. C. (1987) *J. Am. Chem. Soc.* **109,** 1255–1257.
29. Kinoshita, K., Takenaka, S. & Hayashi, M. (1988) *J. Chem. Soc. Chem. Commun.,* 943–945.
30. Huber, M. L. B., Paschal, J. W., Leeds, J. P., Kirst, H. A., Wind, J. A., Miller, F. D. & Turner, J. R. (1990) *Antimicrob. Agents Chemother.* **34,** 1535–1541.
31. Cane, D. E., Walsh, C. T. & Khosla, C. (1998) *Science* **282,** 63–68.
32. Hunziker, D., Yu, T.-W., Hutchinson, C. R., Floss, H. G. & Khosla, C. (1998) *J. Am. Chem. Soc.* **120,** 1092–1093.