Deciphering the late steps of rifamycin biosynthesis

Feifei Qi1, Chao Lei2, Fengwei Li1, Xingwang Zhang1, Jin Wang2, Wei Zhang1, Zhen Fan2, Weichao Li2, Gong-Li Tang3, Youli Xiao2,4, Guoping Zhao2,4 & Shengying Li1,4

Rifamycin-derived drugs, including rifampin, rifabutin, rifapentine, and rifaximin, have long been used as first-line therapies for the treatment of tuberculosis and other deadly infections. However, the late steps leading to the biosynthesis of the industrially important rifamycin SV and B remain largely unknown. Here, we characterize a network of reactions underlying the biosynthesis of rifamycin SV, S, L, O, and B. The two-subunit transketolase Rif15 and the cytochrome P450 enzyme Rif16 are found to mediate, respectively, a unique C–O bond formation in rifamycin L and an atypical P450 ester-to-ether transformation from rifamycin L to B. Both reactions showcase interesting chemistries for these two widespread and well-studied enzyme families.
Rifamycins are ansamycin antibiotics that show a wide spectrum of antimicrobial activities against both Gram-positive and Gram-negative bacteria. Their semisynthetic derivatives such as rifampin, rifabutin, rifampentine, and rifaximin have been used for decades in the clinic for the treatment of tuberculosis, leprosy, and AIDS-related mycobacterial infections, and their recognized pharmacological mode-of-action is the specific inhibition of prokaryotic DNA-dependent RNA synthesis.

Floss and co-workers discovered the first rifamycin biosynthetic gene cluster comprised of 34 genes in the bacterium *Amycolatopsis mediterranei* S699. Five type I polyketide synthases (PKSs: RifA-E) encoded by the cluster are responsible for assembling the first macrocyclic intermediate (proansaamycin X) using 3-amino-5-hydroxybenzoic acid as the starter unit, and malonyl-CoA and methylmalonyl-CoA as extender units. Further post-PKS modifications, including the dehydration of C-8 and the hydroxylation of C-34, lead to the intermediate rifamycin W. Subsequently, Rif5 converts the Δ12,29 olefinic bond of rifamycin W into a ketal moiety, followed by Rif20-mediated acetylation of the hydroxyl group at C-25 and Rif14-mediated O-methylation at C-27, producing rifamycin SV (R-SV) (Supplementary Fig. 1). The later biosynthetic steps leading from R-SV to the end product rifamycin B (R-B) (Fig. 1) have not been biochemically characterized.

During fermentation, R-B is the predominant rifamycin product accumulated by wild type *A. mediterranei* and by the first industrial strains. However, as the antibacterial activity of R-B is modest, R-B needs to be transformed back to the more bioactive R-SV before being subjected to the chemical, enzymatic, or biotransformation process that yield multiple highly potent clinical drugs. Consequently, strains that produce high levels of R-SV are now preferred by industry (e.g., the well-studied mutant strain *A. mediterranei* U32). Nonetheless, the strain improvement for R-SV high-producers has not been as successful as that for R-B producing strains, so both kinds of strains are still required by industry.

Recently, comparative analysis of the rifamycin biosynthetic gene clusters of *A. mediterranei* S699 (an R-B producer) and U32 (an R-SV producer), together with corresponding genetic complementation testing, strongly suggested that the cytochrome P450 enzyme Rif16 is involved in the conversion of R-SV to R-B. Furthermore, gene inactivation and complementation experiments showed that Rif16 and the two-subunit transketolase Rif15 (encoded by rif15a and rif15b, two overlapping genes) are essential and sufficient for this transformation. However, the biochemical and chemical mechanisms underlying the unusual ether bond formation between the C-4 phenolic hydroxyl group of R-SV and a glycolic acid moiety leading to R-B remain unknown. Here, by reconstituting the in vitro activity of Rif15 and Rif16, we reveal a biosynthetic network for the inter-conversion of R-SV, R-S, R-L, R-O, and R-B (Fig. 1), finally elucidating the mechanisms for the late reactions of rifamycin biosynthesis.

**Fig. 1** The biosynthetic network of late rifamycin derivatives. R-SV can be oxidized to R-S spontaneously in the presence of dioxygen and divalent metal ions. The transketolase Rif15 is responsible for transferring a C2 keto-containing fragment from a 2-ketose to R-S, giving rise to R-L. The P450 enzyme Rif16 catalyzes the transformation from R-L to R-O in the presence of NADPH, ferredoxin (Fdx), and ferredoxin reductase (FdR). Finally, R-O is non-enzymatically reduced to R-B by NADPH.
Results and Discussion

Initial examination of the enzymatic activity of Rif16. Generally, P450 enzymes catalyze oxidative reactions, and transketolases can, in the presence of the essential cofactor thiamine diphosphate (ThDP), transfer a C-2 keto-containing fragment from a 2-keto (e.g., fructose-6-phosphate (F-6-P), xylulose-5-phosphate (Xu-5-P), ribulose-5-phosphate (Ru-5-P), sedoheptulose-7-phosphate (S-7-P), dihydroxyacetone (DHA), etc.) to the first carbon atom of an aldose (e.g., ribose 5-phosphate, glyceraldehyde 3-phosphate, etc.)\(^{20}\). Based on the previous proposal that R-SV could be a biosynthetic precursor of R-B\(^{21}\), we surmised that Rif16 (CYP105G1\(^{21}\)) may oxidize R-SV to R-S and that R-S (containing a C-4 keto group) may be a substrate of Rif15. To test these enzymatic hypotheses, we sub-cloned rif15a, rif15b, and rif16 (Supplementary Fig. 2) and heterologously expressed these genes in \textit{Escherichia coli} Codon Plus (DE3)-RIPL. We then used Ni-NTA chromatography to purify the N-terminally His\(_{6}\)-tagged Rif15a, Rif15b, and Rif16 to homogeneity (Supplementary Fig. 3). Notably, the N-His\(_{6}\)-tagged Rif15a and the non-tagged Rif15b were able to be co-expressed and co-purified, suggesting a strong interaction between these two Rif15 subunits (Supplementary Fig. 3).

Purified Rif16 appeared to be a functional P450 enzyme, as it had the expected red color and showed a signature peak at 450 nm in its CO-reduced difference spectrum (Supplementary Fig. 4). To reconstitute the in vitro activity of Rif16, we used two surrogate redox partner proteins to shuttle electrons from NADPH to the heme-iron reactive center for P450 catalysis: the ferredoxin \textit{seFdx} (SynPcc7942_1499) and the ferredoxin reductase \textit{seFdr} (SynPcc7942_0978), both of which are from the cyanobacterium strain \textit{Synechococcus elongatus} PCC 7942 and were here expressed heterologously in \textit{E. coli} and purified\(^{22}\). Against our expectations, Rif16 was not able to catalyze the conversion from R-SV to R-S, while R-S was readily reduced to R-SV by addition of NADPH alone (Supplementary Fig. 5). Importantly, the hydroquinone R-SV was spontaneously oxidized to the quinone R-S by ambient O\(_2\), and this transformation was dramatically accelerated by the presence of divalent metal ions (e.g., Cu\(^{2+}\), Mn\(^{2+}\), etc.) (Supplementary Fig. 6), similar to previously reported findings\(^{23}\). However, we cannot exclude the possibility that an oxidase might be responsible for enzymatic oxidation of R-SV into R-S in vivo. Taken together, our results suggest that Rif16, rather than performing a normal bio-oxidation, may catalyze an atypical P450 reaction in rifamycin biosynthesis.

Functional characterization of Rif15. We next evaluated the in vitro activity of Rif15a/Rif15b at a 1:1 ratio (i.e., the reconstituted Rif15 transketolase) in the presence of R-S and F-6-P as the potential C\(_2\) keto acceptor and donor, respectively, with ThDP and MgCl\(_2\) as cofactors. As predicted, Rif15 converted R-S into a different product with higher polarity than R-B, while single subunits (that is, either Rif15a or Rif15b alone) were not able to catalyze the same transformation. Additionally, we found that both ThDP and Mg\(^{2+}\) were required for the catalytic activity of Rif15 (Fig. 2a, trace i–vi). This is unsurprising since the diphosphate moiety of ThDP is bound to the transketolase through a bivalent cation to form the catalytically active apo-enzyme from the \textit{apo}-protein\(^{24,25}\). Protein sequence alignment of multiple transketolases shows that the residues involved in the interactions with ThDP and the metal ion are highly conserved regardless their origins and subunit organization modes (Supplementary Fig. 7).

High performance liquid chromatography-high resolution mass spectrometry (HPLC-HRMS) analysis revealed that the m/z value of the product was 754.3069 ([M-H]\(^{-}\), deduced to be [\text{C}_{39}\text{H}_{48}\text{NO}_{14}]\(^{-}\)) (Supplementary Fig. 8), which is consistent with that of R-L or R-B in negative ion mode (calc. 754.3069). Since the retention time of this product was distinct from that of R-B, we suspected that the product here is R-L. Both the 1D (\(^{1}\)H, \(^{13}\)C, DEPT135) and 2D (\(^{1}\)H-\(^{1}\)H COSY, HSQC, HMBC) NMR spectra (Supplementary Figs. 9–13, Supplementary Table 2) of the purified product were acquired, and spectral comparisons of the proton NMR data obtained from the product and the substrate R-S (Supplementary Fig. 9) revealed a new set of CH\(_2\) signals (\(\delta_{1H,13C}\)).
labeled carbons marked by asterisks originate from [1-13C]glucose. enzymatic reaction buffer kinetic constants of Rif15 were determined with this methylene group con product. Further extensive analyses of 2D NMR correlations of Fig. 14). conversion ratios under the same conditions (Supplementary donors for Rif15, with Xu-5-P being optimal in terms of previously proposed2, similar Rif15 reactions were performed the Rif15 reactions to protect R-SV from oxidation (Fig. 2a, trace 4.72, s, 2 H in R-B; δC-39 62.3 vs δC-38 67.8 in R-B) from the product. Further extensive analyses of 2D NMR correlations of this methylene group confirmed it to be R-L. The steady-state kinetic constants of Rif15 were determined with Kcat = 8.8 ± 2.4 μM and kcat = (2.2 ± 0.1) × 10−2 min−1. Moreover, Xu-5-P, Ru-5-P, S-7-P, and DHA were also able to serve as alternative C2 keto donors for Rif15, with Xu-5-P being optimal in terms of conversion ratios under the same conditions (Supplementary Fig. 14).

To examine whether R-SV is also a direct precursor of R-L as previously proposed2, similar Rif15 reactions were performed using R-SV as substrate, and, interestingly, we detected a small amount of R-L as a product (Fig. 2a, trace vii). In light of the spontaneous oxidation from R-SV to R-S by O2 that we had observed in earlier assays (in aqueous solution in the presence of Mg2+, Supplementary Fig. 6), 2 mM ascorbic acid was added to the Rif15 reactions to protect R-SV from oxidation (Fig. 2a, trace viii, ix)26. Upon this addition, we no longer detected R-L as a reaction product (Fig. 2a, trace x), establishing that the previously detected R-L was actually derived from the spontaneously formed R-S.

Collectively, our results from these in vitro assays demonstrate that Rif15 is a two-component transketolase that transforms a ketone (in R-S) into an ester (in R-L), a reaction that has not been found previously in natural product synthesis, to the best of our knowledge. Mechanistically, the deprotonation of ThDP at the thiazolium ring generates a carbanion, which is responsible for cleaving the C-2/C-3 bond in the 2-ketose. The resultant ThDP-bound dihydroxyethyl group then undertakes nucleophilic attack of the C-4 carbonyl carbon of R-S, which is followed by bond rearrangements and re-aromatization, ultimately yielding R-L (Fig. 3a).

Biochemical, structural, and mechanistic characterization of Rif16. Having characterized the R-SV→R-S→R-L transformation, we next sought to resolve the conversion of R-L into R-B. These
two rifamycin derivatives have the same oxidation state, but we still chose to test the activity of Rif16 against R-L, since this P450 enzyme was previously shown to be required for R-B biosynthesis. Indeed, we found that R-L was significantly converted into R-B by Rif16 in the presence of SFdx, SFDR, and NADPH (Fig. 2b, trace xiii, xiv); the structure of the product was confirmed by the identical retention time of the product and the R-B authentic standard, co-elution with R-B in a co-injection experiment (Fig. 2b, trace xv), and the consistently observed m/z value of 754.3069 ([M-H]–, calc. 754.3069) (Supplementary Fig. 15). These results clearly establish that Rif16 is the long-sought R-B synthase of rifamycin biosynthesis.

To elucidate the catalytic mechanism for this atypical ester-to-ether transformation, the crystal structures of substrate-free Rif16 (PDB ID code: 5YSM, Fig. 4a) and R-L-bound Rif16 (PDB ID code: 5YSW, Fig. 4b) were solved at 1.90 Å and 2.60 Å resolution, respectively. In both of the structures, there was only one typical cytochrome P450 fold existing in an asymmetric unit. The BB’ loop-B’ helix-B’C loop region, which is known to be important for substrate specificity determination, is significantly longer than those of many P450 enzymes that recognize smaller substrates (Supplementary Fig. 16). The missing electron density of this region in both structures suggests the great structural flexibility. Both findings might help explain how Rif16 is able to accommodate its bulky substrate R-L, which represents one of the largest substrates for a P450 enzyme with the substrate-bound crystal structure available. In the absence of substrate, Rif16 adopts an open conformation characterized by retraction of the F and G helices, loss of order in the B’ helix, and missing electron density for the B’C and FG loops (Fig. 4a). A water molecule that is 2.5 Å away from the heme-iron forms the sixth axial ligand of Fe3+ (Supplementary Fig. 17a). Upon binding with R-L, Rif16’s FG loop becomes ordered but the B’ helix and the B’C loop remain disordered (Fig. 4b), thereby adopting a partially open conformation rather than the closed conformation observed in many substrate-bound P450 enzymes (Fig. 4c).

In the substrate-bound structure (Fig. 4d and Supplementary Fig. 17b), R-L forms hydrogen bonds with residues S76, N108, F308, and T407, and additionally interacts with residues 1107, A196, P197, I255, V303, P305, I307, F310, and L406 via hydrophobic interactions (all of these residues are within 5 Å of R-L). Critically, the axial water ligand is displaced and the
hydroxyl group at C-39 of R-L is closest (4.5 Å) to the heme-iron reactive center. These structural features suggest a possible mechanism for R-B production (Fig. 3a): the ferryl-oxo species (Compound I) likely abstracts the hydrogen atom of the C-39 hydroxyl group, leading to formation of a substrate radical and the ferryl-hydroxoro Compound II. The resultant oxygen radical can then directly attack the neighboring arene to form a five-membered ring pendant, and the radical would be delocalized to the aromatic ring. Next, the relocalization of the spirocyclic intermediate could induce the second hydrogen abstraction from the C-1 hydroxyl group by Compound II. This diradical mechanism might result in the formation of R-O. Notably, similar mechanisms—involving two alternative substrate binding poses being responsible for hydrogen abstractions from two distant sites—have been proposed for C–O coupling reactions catalyzed by a number of P450 enzymes. Finally, the pentabasic cyclic compound R-O could be reduced to R-B (rather than R-L) by the NADPH-derived hydride, since the carboxylic acid is a better leaving group than the alcohol.

This R-L→R-B oxidation reaction follows net oxidation-reduction. To dissect this unusual P450 reaction experimentally, we elected to oxidize R-L by taking advantage of the peroxide reduction. To perform a series of 13C-tracer NMR experiments. First, [39–13C]R-L was prepared by mixing [1-13C]glucose, ATP, Mg2+, hexokinase, G-6-P isomerase, Rif15a/Rif15b, ThDP, and Rif16 into a one-pot reaction. We observed that [1-13C]glucose was phosphorylated to [1-13C]G-6-P by hexokinase, which was then converted to [1-13C]F-6-P by G-6-P isomerase (Fig. 3). The Rif15-mediated transfer of the 13C-labeled glycolic acid C2 moiety from [1-13C]-F-6-P to R-S resulted in production of [39,13C]R-L, with an enriched C-39 signal of δC 62.4 (Fig. 3b).

The identity of this product was further confirmed by LC-HRMS analysis indicating an m/z value of 755.3106 ([M–H]−, calc. 755.2924) was observed in a time-course study (Supplementary Fig. 18). These results strongly suggest that R-O is the intermediate that enables the conversion of R-L to R-B.

To validate our proposed enzymatic reaction mechanisms, we performed a series of 13C-tracer NMR experiments. First, [39,13C]R-L was prepared by mixing [1-13C]glucose, ATP, Mg2+, hexokinase, G-6-P isomerase, Rif15a/Rif15b, ThDP, and R-S in a one-pot reaction. We observed that [1-13C]glucose was phosphorylated to [1-13C]-G-6-P by hexokinase, which was subsequently transformed into [1-13C]-F-6-P by G-6-P isomerase (Fig. 3). The Rif15-mediated transfer of the 13C-labeled glycolic acid C2 moiety from [1-13C]-F-6-P to R-S resulted in production of [39,13C]R-L, with an enriched C-39 signal of δC 62.4 (Fig. 3b). The identity of this product was further confirmed by LC-HRMS analysis indicating an m/z value of 755.3106 ([M–H]−, calc. 755.3105, Supplementary Fig. 19), which is ~1 Da greater than that of unlabeled R-L [M–H]− = 754.3069]. Next, Rif16, seFdx/ seFdr, and NADPH were added into the above one-pot reaction. As expected, the P450 enzyme converted [39,13C]-R-L into [38,13C]-R-B, confirmed by LC-HRMS with an m/z value of 755.3100 ([M–H]−, calc. 755.3105, Supplementary Fig. 19) and by our observation that the 13C-labeled carbon signal shifted downfield from δC 62.4 to δC 67.9 (Fig. 3b); both analytical results are consistent with the conversion of R-L to R-B via R-O (Fig. 3a).

It was previously reported that the R-SV high-producer A. mediterranei U32 has an R84W single mutation in Rif16. The understanding of Rif16 mechanism allowed us to rationalize this industrially important phenotype. Specifically, the dissociation constant (Ka) of R-L toward Rif16 was determined to be 1.3 ± 0.1 μM (Supplementary Fig. 20), while the purified Rif16R84W mutant (Supplementary Figs. 3 and 21) showed no detectable binding of R-L and lost the ability of catalyzing the transformation from R-L to R-B (Supplementary Fig. 22). Since R84 is located at the B’ loop of Rif16 (Supplementary Fig. 16), which is an important region for P450 substrate recognition35, its replacement by a tryptophan abolishes the productive substrate binding via a mechanism to be elucidated. Furthermore, according to the biosynthetic network shown in Fig. 1, the U32 mutant should accumulate R-L instead of the observed R-SV and R-S10. We reason that the ester R-L might be unstable, which could be hydrolyzed to R-SV either enzymatically or spontaneously (Supplementary Fig. 23).

Our elucidation of the network comprising the last steps of rifamycin biosynthesis revealed a unique C–O bond formation reaction mediated by a transketolase that involves both normal C–C bond formation and unusual bond rearrangements. Notably, transketolases primarily participate in central metabolic pathways such as pentose phosphate pathway and the Calvin cycle, and there have been few reports on transketolases that are involved in natural product biosynthesis. The ether bond formation derived from the concomitant oxidation-reduction reactions and unique C–O bond rearrangements likely represents a highly atypical reaction for a P450 reaction system. The knowledge of slow kinetics and the optimal C2 keto donor of Rif15 could also help direct the future rational strain improvement. Finally, BLAST searches demonstrate that there exist other protein sequences with high similarity to Rif15 and Rif16 (Supplementary Table 3, Supplementary Fig. 24), suggesting that more Rif15-like and Rif16-like functionality could be further identified. Some of these enzymes come from rifamycin producing microorganisms38–41, which may suggest an effective method for discovery of more rifamycin producers by using Rif15 and Rif16 sequences as probes.

Methods

Chemicals. Rifamycin SV and rifamycin O authentic standards were purchased from Sigma Aldrich (USA) and Toronto Research Chemicals (Canada), respectively. Rifamycin S and rifamycin B authentic standards were bought from National Institutes for Food and Drug Control (China).

General DNA manipulation. The E. coli DH5α strain was used for plasmid construction, storage, and isolation. Fast-digest restriction endonucleases (Thermo Fisher Scientific, USA) and T4 DNA ligase (Takara, Japan) were used for construction of vectors. PCR reactions were performed using 1× T MUST plus High-Fidelity Master Mix DNA polymerase (TsingKe Biotech, Beijing, China). Plasmid isolations from E. coli cells were performed using the Plasmid MiniPrep Kit (TsingKe Biotech, Beijing, China). Purification of DNA fragments from agarose gels or PCR reactions was carried out using Gel Extraction Kit (Omega, USA) and Cycle Pure Kit (Omega, USA), respectively. Primers were synthesized by TsingKe (China).

Molecular cloning. The DNA sequences that encode the two-subunit transketolase Rif15, and the separated subunits Rif15a and Rif15b were amplified from the genomic DNA of Amycolatopsis mediterranei U32 (A. mediterranei U32 was deposited in Institute of Microbiology, Chinese Academy of Sciences designated as CGMCC4.5720) under standard PCR conditions using the primer pairs of rif15-F/rif15-R, rif15a-p52S/rif15a-p52R, and rif15b-p51S/rif15b-p51R, respectively (Supplementary Table 4). The P450 gene rif16 and the mutant gene rif16R84W were amplified from A. mediterranei S699 and U32 (Professor Guoping Zhao’s laboratory collection) gDNA, respectively, using a pair of primers rif16-F and rif16-R (Supplementary Table 4). The rif15a fragment was double digested by BamHI/HindIII and cloned into the expression vector pSJ2 (a derivative of pET21a, which is a gift from Professor Jiachao Zhou at Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences) using standard molecular cloning methods to generate pSJ2-rif15a (Supplementary Fig. 2). The rif15b, rif15b and rif16R84W plasmids were double digested by NdeI/HindIII, NdeI/XhoI, and NdeI/Xhol, respectively, and ligated into the corresponding pre-treated pET28b (Novagen, USA) to afford pET28b-rif15, pET28b-rif15b, pET28b-rif16, and pET28b-rif16R84W (Supplementary Fig. 2). All constructs were confirmed by DNA sequencing (Genezent, China), and transformed into E. coli Codon Plus(DEL3)-RIPI for expression of N-terminal His6-tagged recombinant proteins.

Protein expression and purification. The E. coli Codon Plus(DEL3)-RIPI transformant carrying pSJ2-rif15a was grown at 37 °C overnight in LB media supplemented with ampicillin (50 μg/mL), chloramphenicol (34 μg/mL), and...
streptomycin (50 μg/mL). The transformant carrying pET28b-rf15, pET28b-rf15b, pET28b-rf15 or pET28b-rf15bΔsav was cultured at 37 °C overnight in LB media supplemented with kanamycin (50 μg/mL), chloramphenicol (34 μg/mL) and streptomycin (50 μg/mL). The overnight seed culture was inoculated (1:100) into 1 L LB broth containing 10% glucose, appropriate selective antibiotics and rare salt solution (6.75 mg/L FeCl3, 500 μg/L ZnCl2, 250 μg/L Na2MoO4, 250 μg/L CaCl2, 405 μg/L CuSO4, and 125 μg/L H3BO3), and then cultured at 37 °C until OD600 reached 0.6–1.0 (4 h). Next, 0.1 μM isopropl β-D-thiogalactoside (IPTG, Sigma, USA) was added to induce protein expression, and thiamin (1 mM, Sigma, USA) and δ-aminolevulinic acid (1 mM, Sigma, USA) were supplied to support efficient expression of holop-form P450 enzymes. The cells were grown at 16 °C for 24 h. All recombinant proteins including Rif15, Rif15b, Rif15Δ, Rif16, and Rif16Δsav were purified by Ni-NTA affinity chromatography. Brieﬂy, E. coli cells were collected by centrifugation at 5000 × g for 5 min, the cell pellet was re-suspended with 20 mL lysis buffer (20 mM Tris–HCl, 300 mM NaCl, 10% (w/v) glycerol, and 10 mM imidazole, pH 8.0) and the cells were broken by sonication (5 s on/5 s off) for 30 min on ice. The next, the cellular debris was removed by centrifugation at 12,000 × g for 30 min. To the supernatant 1 mL nickel–titanium acetic acid resin (Qiagen, Germany) was added, and each mixture was incubated for 30 min with gentle shaking at 4 °C. The resin was loaded onto an empty column and washed with wash buffer (20 mM Tris–HCl, 300 mM NaCl, 10% (w/v) glycerol, and 20 mM imidazole, pH 8.0) until no protein could be detected in ﬂow-through. The bound proteins were eluted by 10 mL elution buffer (20 mM Tris–HCl, 300 mM NaCl, 10% (w/v) glycerol, and 250 mM imidazole, pH 8.0). Finally, the eluents were concentrated and buffer-exchanged to reaction buffer (20 mM Tris–HCl, 10% (w/v) glycerol, pH 7.4) via ultraﬁltration using Amicon Ultra-15 centrifugal ﬁlter units (Millipore, Ireland) with a 10-kDa cutoff. All protein puriﬁcation steps were performed at 4 °C. The SDS-PAGE analysis (Supplementary Fig. 3) showed that Rif15, Rif15b, Rif15Δ, Rif16, and Rif16Δsav were unsatisﬁed. Therefore, the collected Rif15 and Rif15Δsav proteins were buffer-exchanged again to 20 mM Tris–HCl buffer (pH 8.0) by ultraﬁltration using Amicon Ultra-15 centrifugal ﬁlter units (Millipore, Ireland) with a 30-kDa cutoff. The buffer-exchanged proteins were further loaded onto a mono Q 5/50 GL column (GE Healthcare, USA) pre-equilibrated with 20 mM Tris–HCl buffer (pH 8.0), and eluted with a gradient volume of 20 CV and an increasing ion strength up to 1 M NaCl at a ﬂow rate of 1 mL/min using AKTA purifier-100 (GE Healthcare, USA). Fractions containing proteins of interest were ﬂash-frozen by liquid nitrogen and stored at −80 °C for later use.

Protein concentration determination. For Rif15 and Rif15Δsav, the UV-visible spectra were recorded on a DU 800 spectrophotometer (Beckman Coulter, USA). The CO-bound reduced difference spectrum was employed to determine the functional concentration of P450 enzymes using the extinction coefficient (ε280nm–400nm) of 91,000 M−1·cm−1. Brieﬂy, CO was slowly bubbled through the Na2S2O4 reduced P450 enzyme solution using a Pasteur pipette in a fume hood. The spectra of ferric, CO-bound, and CO-bound reduced forms of the P450 enzyme were recorded between 250 and 550 nm for generation of the CO-bound reduced difference spectrum. The protein concentrations of other proteins were determined using the Bradford assay with bovine serum albumin as standard as previously described.

Spectral substrate binding assays. Spectral substrate binding assays were carried out on a UV-visible spectrophotometer 50 Bio (Cary, USA) at room temperature by titrating 100 μM rifamycin L, DMSO solution (blank DMSO for the reference group), and 0.5 mL Rif16 or Rif16Δsav solution in 1 μL dimethyl sulfoxide with samples from 10 mL of the substrate concentrations ranging from 0.1 to 1.2 μM. The series of Type I difference spectra were used to deduce ΔA (Apeak–390nm–Ashoulder–420nm). Then, the ΔA data versus substrate concentrations were ﬁt to Michaelis–Menten equation to calculate the dissociation constant Ks.

In vitro enzymatic assays of Rif15 and Rif16. The Rif15 reaction mixture contained 10 μM Rif15a, 10 μM Rif15b, 200 μM R-S (or other rifamycin derivatives, Merck, USA) and 0.5 mL THDP (Sigma, USA), 2.5 mM MgCl2, and 2 mM P–6–P (18K Scientiﬁc China) or Xu–5–P, Ru–5–P, S–7–P, DHA from Sigma, in 100 μL of reaction buffer (20 mM Tris–HCl, 10% (w/v) glycerol, pH 7.4). The Rif15 reaction assay was carried out in 100 μL of the same reaction buffer containing 2 μM Rif15, 200 μM R-L, 20 μM seFdx plus 10 μM seADP, and 1 mM TDP (Solarbio, China) or 2 μM Rif16, 200 μM R-B, and 20 μM H2O2. The Rif15 and Rif16 reactions were incubated at 28 °C for 4 h and 1 h (unless otherwise speciﬁed), respectively, and quenched by mixing with the same volume of methanol. After high-speed centrifugation (20,000 × g) for 15 min, the supernatants were analyzed on an Agilent 1260 infinity HPLC system (Agilent Technologies, USA) equipped with an ultraviolet detector. Compounds were separated by SB–C18 reverse-phase column (Thermo, 5 μm, 46 mm, USA) in a gradient system consisting of dH2O+0.1% triﬂuoroacetic acid as solvent A and acetonitrile as solvent B. The program of solvent gradient is as follow: 40% B for 5 min, 40–80% B over 15 min, and 80–100% B over 5 min at a ﬂow rate of 1 mL/min. The wavelength of detection was 425 nm for all rifamycin derivatives except for R-L, R-B, and R-fdx. Data availability. Data that support the ﬁndings of this study have been deposited in Protein Data Bank and the PDB ID codes 5YSM and 5YSW for substrate-free Rif16 and R-L-bound Rif16, respectively. Figures were prepared using PYMOL (http://www.pymol.org).

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Author contributions
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