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rif-orf13 编码的细胞色素 P450 催化利福霉素生物合成过程中 C34a 位的羟化反应

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摘要 利福霉素生物合成途径在经历了二十余年的研究之后，仍然没有得到完全阐明。其中 C34a 甲基的氧化脱除是利福霉素成熟过程中的必需反应步骤，但是催化这一步骤的酶尚未鉴定；推测可能是利福霉素生物合成基因簇编码的某个细胞色素 P450 催化了这一步骤。我们选取利福霉素生物合成基因簇中功能尚未确证的 P450 基因 *rif-orf0*、*4* 和 *13* 在变铅青链霉菌中进行异源表达和底物喂养实验，发现表达了 *rif-orf13* 的链霉菌能够将 16-脱甲基-34a-脱氧利福霉素 W（**1**）转化为 16-脱甲基利福霉素 W（**2**）。将 *rif-orf13* 在大肠杆菌 BL21（DE3）中进行诱导表达，利用纯化的 Orf13 蛋白进行体外酶催化反应，发现 Orf13 能够将底物 **1** 羟化为产物 **2**。结合前人的基因敲除研究，我们认为 *rif-orf13* 是编码 34a-脱氧利福霉素 W 羟化酶的基因，其在胞内的功能可以被另一个负责 C12-C29 双键氧化断裂的 P450 基因 *rif-orf5* 替代。

关键词 细胞色素 P450; 羟化反应; 利福霉素; *rif-orf13*

Cytochrome P450 Encoded by *rif-orf13* Catalyzes 34a-Hydroxylation in Rifamycin Biosynthesis

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Abstract The biosynthetic pathway of rifamycins is still not completely deciphered after decades of study. For example, the gene responsible for the oxidative elimination of C34a is not identified. It was proposed that some cytochrome P450 is related to this essential biosynthetic step in the modification of rifamycin. Here, cytochrome P450 encoding genes *rif-orf0*, *4* and *13* from rifamycin biosynthetic gene cluster were heterologously expressed in *Streptomyces lividans* and fed with 16-demethyl-34a-deoxyrifamycin W (**1**). Compound **1** was completely converted into 16-demethylrifamycin W (**2**) in the strain harboring *rif-orf13*. His_6-tagged Orf13 was prepared from *E. coli* BL21 (DE3) and characterized to be active cytochrome P450. Enzymatic assays demonstrated that compound **1** could be converted into **2** by Orf13 as *in vivo*. Therefore, we concluded that *rif-orf13* is responsible for the hydroxylation on C34a in the biosynthesis of rifamycins. In addition, it's role *in vivo* could be functionally complemented by *rif-orf5*, which encoding another cytochrome P450 enzyme.
Keywords cytochrome P450, hydroxylation, rifamycin, *rif-orf13*

1 Introduction

Rifamycins have been used for more than 50 years in clinic and are still mainstay in the treatment of tuberculosis, leprosy and AIDS-related mycobacterial infections. Biosynthetic study revealed that the backbone of rifamycins is constructed

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by type I polyketide synthase (PKS) using 3-amino-5-hydroxy benzoic acid (AHBA) as starter unit and malonyl-CoA and methylmalonyl-CoA as extending units [1, 2]. The macrolactam backbone further undergo 7, 8-dehydrogenation and 34a-hydroxylation to produce the important intermediate rifamycin W [3]. The latter is oxidized by cytochrome P450 Orf5 on Δ29, 12 olefinic bond [4], followed by 25-O-acetylation catalyzed by Orf20 [5] and 27-O-methylation catalyzed by Orf14 [6], to produce rifamycin S, which is converted into rifamycin SV through reduction on naphtoquinone and finally into end product rifamycin B under the catalysis of transketolase Orf15 and cytochrome P450 enzyme Orf16 [7, 8].

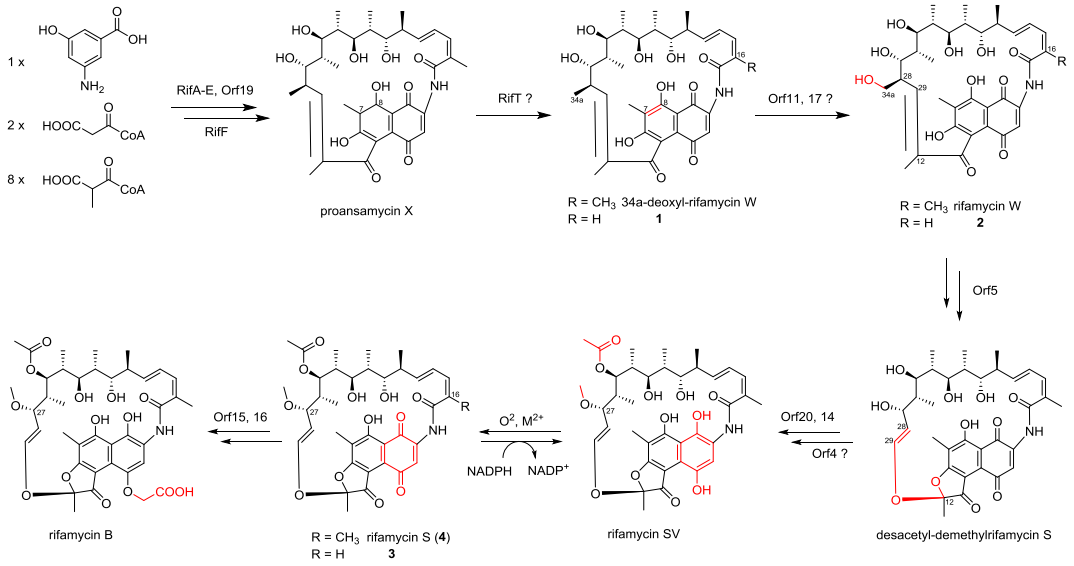


Figure 1 Biosynthetic pathway of rifamycins.

Enzymes labeled with question mark are proposed to catalyze the biosynthetic steps based on functional prediction.

Several steps in the post-PKS modification of rifamycins are still ambiguous after decades of study because of the complicated reaction route and redundant biosynthetic gene cluster (Figure S8). In the maturing of rifamycins, oxidation on C34a followed by oxidative cleavage of C12-C29 double bond is essential step to produce the intermediate harboring ketal moiety on C12, which ensures the proper molecular conformation of rifamycins to effectively bind to RNA polymerase of bacteria via the formation of hydrogen bonds [9]. Cytochrome P450 encoded by *rif-orf5* was proved to participate in the oxidative conversion of Δ29, 12 olefinic bond through *in vivo* experiments [4], and Rif-Orf11 and Orf17 (both are flavin dependent oxidoreductases, see Table S3) were suspected to be responsible for the hydroxylation on C34a [10]. However, no experimental data have been published to prove this hypothesis till now. Yoon *et al* proposed that Orf0 catalyzed the hydroxylation of C34a in proansamycin X[11] based on the accumulation of proansamycin X (Figure 1) in *orf0* deletion mutant, but the same result was not accomplished by Moor *et al* [4] or ourselves (unpublished data). Here, we demonstrate that the cytochrome P450 encoded by *rif-orf13* is responsible for the hydroxylation of 34a-deoxyrifamycin W to produce rifamycin W based on intermediate feeding experiments and *in vitro* enzymatic assays.

2 Results

Recently, we discovered several rifamycin analogs, including compounds **1**, **2** and **3** (Figure 1), from *Micromonospora* sp. TP-A0468[12]. These compounds are structurally identical to the known rifamycins except for the absence of the methyl substitute on C16 (Figure 1). Homologous genes of *orf11* and *17* are missing in the biosynthetic gene cluster of 16-demethylrifamycins (Figure S8), while the 34a-hydroxylated intermediate **2** is produced. We inferred that a cytochrome P450 instead of Orf11/17 probably catalyzed the hydroxylation of C34a.

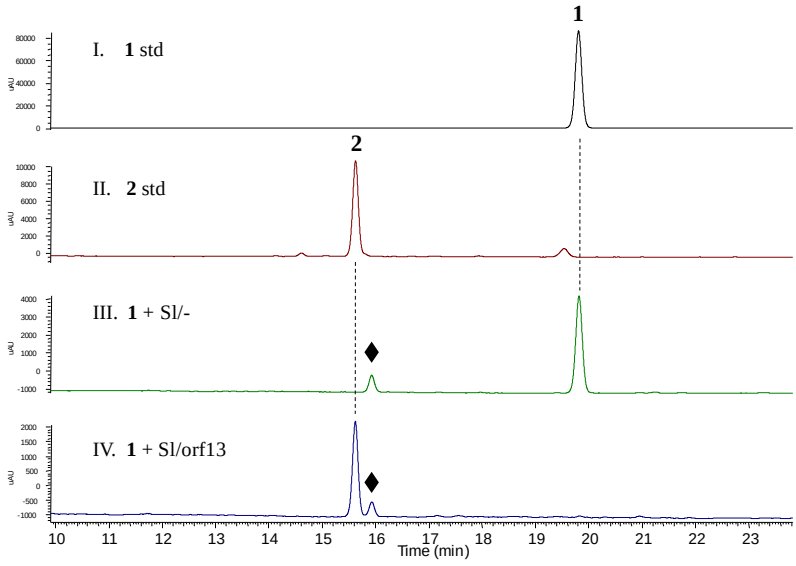


Figure 2 Heterologous expression and feeding experiments of *rif-orf13*.

The cytochrome encoding gene was heterologously expressed in *Streptomyces lividans* 1326 and cultured in ISP-2 medium with purified compound **1** added. Extracts of feeding experiments were analyzed at 423 nm on HPLC. Sl, *Streptomyces lividans* 1326; Sl/-, Sl containing plasmid pSETe, used as negative control. Sl/orf13, Sl containing recombinant plasmid pAT13he. I, standard compound **1**; II, standard compound **2**; III, Sl/- fed with **1** during cultivation; IV, Sl/orf13 fed with **1** during cultivation. ♦, unknown metabolite (*m/z* 362) produced by *Streptomyces lividans* 1326.

Functionally unidentified cytochrome P450 encoding genes, *rif-orf0*, *orf4* and *orf13*, were cloned from another rifamycin producer, *Amycolatopsis* (*A.*) *tolypomycina*, because the original producer of rifamycins, *A. mediterranei* S699, was not available in our laboratory. These genes displayed extremely high similarity to their counterparts from *A. mediterranei* S699 (Table S3). *Rif-orf0, 4* and *13* were cloned into shuttle plasmid pSETe under the control of constitutive promoter *ermE** (Figure S1), and the recombinant plasmids were transferred into *Streptomyces* (*S.*) *lividans* 1326 through conjugation between *S. lividans* and *E. coli* S17-1. Verified conjugants were cultured in ISP-2 medium and added with compound **1**, **2**, **3** or **4** during the cultivation. The cultures were extracted and subject to HPLC and LC-MS analysis with the standard controls of **1**, **2**, **3** or **4**. Recombinant strain containing blank plasmid pSETe was cultured and analyzed in the same way as negative

control.

HPLC analysis showed that most of compound **1** remained in the extract of *S. lividans* 1326 harboring pSETe (Figure 2 III). But in the strain containing *rif-orf13*, compound **1** added into the culture broth was completely disappeared, and a new compound arose in the extract which exhibited identical retention time and mass spectrum with **2** on HPLC and LC-MS analysis (Figure 2 IV). Strains containing *orf0* or *orf4* didn't convert the additive compound **1** into any other detectable product (Figure S3). When compound **2**, **3** or **4** was used as substrate, no product was detected in the culture of the recombinant strain containing *orf13*. These results demonstrate that *rif-orf13* is responsible for the hydroxylation on C34a of compound **1** while *orf0* and *orf4* are irrelevant to this step.

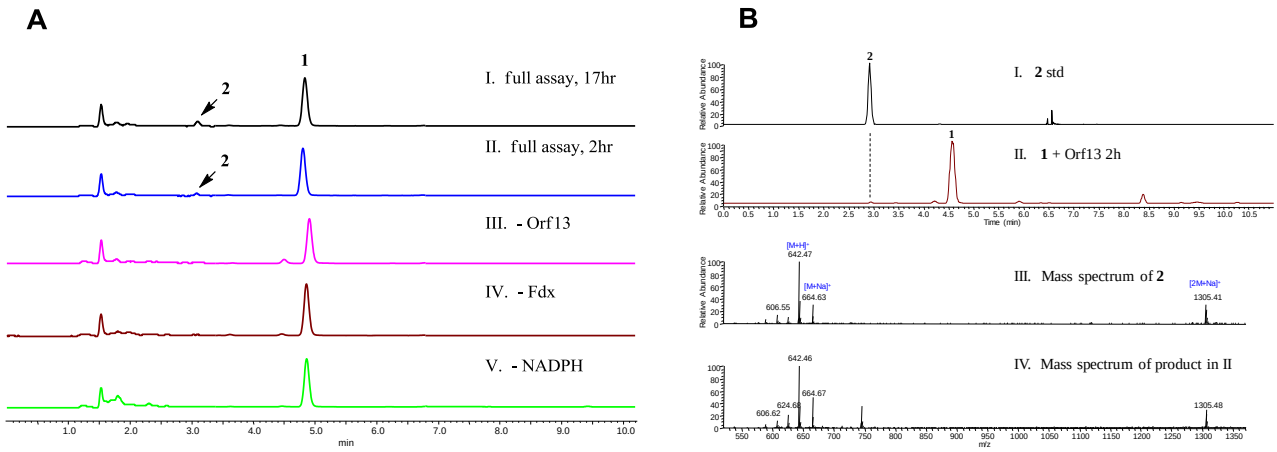


Figure 3 Functional verification of *rif-orf13* through *in vitro* enzymatic assays.

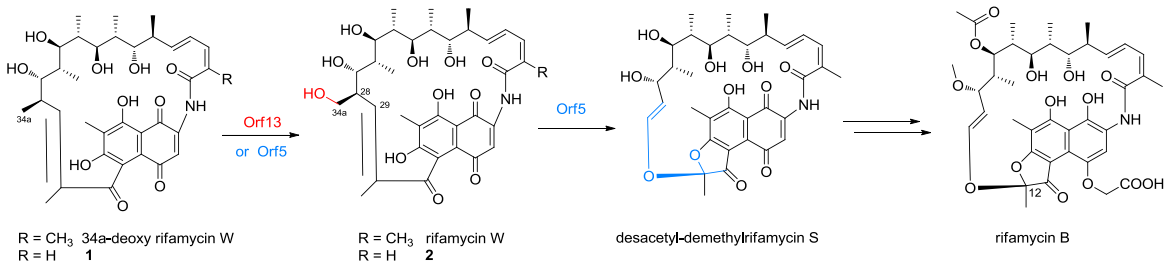
(**A**) HPLC analysis of the enzymatic assays of Orf13. I, complete reaction mixture incubated in 30 ℃ for 17 hours before analysis; II, complete reaction mixture incubated in 30 ℃ for 2 hours before analysis; III, control assay that lack Orf13 in the reaction; IV, control assay that lack ferredoxin in the reaction; V, control assay that lack NADPH in the reaction. (**B**) LC-MS analysis of the product in the enzymatic assay of Orf13.

To further characterize the function of Orf13, we cloned *rif-orf13* into pET28a and expressed the gene in *E. coli* BL21(DE3). Soluble His_6-tagged protein was prepared through induced expression of *rif-orf13* in *E. coli* followed by Nickel affinity chromatography and dialysis. A small aliquot of the characteristic dark red solution of Orf13 was analyzed with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), in which a homogenous band at about 49 kDa (expected 49.1 kDa) was detected (Figure S9), suggesting that Orf13 was successfully prepared. In reduced CO-bound difference spectrum, the absorption peak of Orf13 displayed obvious shift from 420 nm of untreated sample to 450 nm of CO treated sample (Figure S9), confirming that the cytochrome P450 Orf13 was correctly functionalized with active cofactor heme[13].

Enzymatic assays were carried out using **1** as substrate and Orf13 as catalyst. Since no dedicated ferredoxin and ferre-

doxin reductase was found encoded by rifamycin biosynthetic gene cluster, the partner proteins from *Spinacia oleracea* were used in the *in vitro* assays of Orf13 to deliver electrons from NADPH to the cytochrome P450 [14]. After 2 hours of reaction, a tiny amount of **2** was detected in HPLC analysis (Figure 3A II), which was further verified by LC-MS analysis with standard **2** as control (Figure 3B). Extending the reaction course to 17 hours (Orf13 was thereafter unstable and denatured) led to slight increase of the production of **2** (Figure 3A I), while no product **2** was detected in the reactions minus Orf13 or ferredoxin/reductase or NADPH (Figure 3A III-V). These assays confirmed that Orf13 catalyzed the hydroxylation of **1** to produce **2** with the assist of ferredoxin/ferredoxin reductase and NADPH, despite the extremely low efficiency in the *in vitro* assays.

To enhance the reaction rate and reduce the formation of side products, self-sufficient ferredoxin RhFRED and RhFRED-Fdx [15, 16] were employed to assist Orf13 in the enzymatic hydroxylation of **1**, but no improved efficiency was observed (data not shown).



Scheme 1 Proposed function of Orf13 in the biosynthesis of rifamycins.

3 Discussion

It's quite unexpected to find Orf13 catalyzing the hydroxylation of **1** because former research pointed that the disruption *orf13* in *A. mediterranei* S699 had no effect on the production of rifamycin B [5], and gene inactivation and complementation experiments suggested that *rif-orf0* likely participated in the hydroxylation on C-34a of proansamycin X [11]. Furthermore, there is only one cytochrome P450, Sare_1259 (homologue of Orf5), is involved in the conversion of 34a-deoxy rifamycin W to desacetyl-demethylrifamycin S (Figure 1) in *Salinispora arenicola* CNS-205 [4], thus ruled out a dedicated cytochrome P450 monooxygenase responsible for the hydroxylation of C-34a in the biosynthesis of rifamycins in *S. arenicola* CNS-205. A reasonable proposal is that the function of Orf13 could be undertook by the homologue Orf5 in *S. arenicola* CNS-205, and Orf5 further catalyzes the oxidative elimination of C-34a and cleavage of C12, C29 double bond (Scheme 1). Based on this hypothesis, it's logical that rifamycin W is accumulated in *orf5* inactivated mutant and rifamycin B is normally produced in *orf13* disrupted mutant of *A. mediterranei* S699.

Phylogenetic analysis of cytochrome P450s from different rifamycin biosynthetic gene clusters showed that Orf13 displayed high homology to Orf5 (67% identity, 81% similarity), and they formed a separated clad in the phylogenetic tree (Figure S7). Given the fact that rifamycin W is accumulated in *orf13*-missing strains (*S. arenicola* CNS-205 and *Micromonospora* sp. TP-A0468) [4, 12] but not in *orf13*-harboring strains (*A. mediterranei* and *A. tolypomycina*, see Figure S8) [5, 17], it could be deduced that *orf13* is a reduplicate copy evolved from *orf5* to promote the biosynthetic rate of rifamycins. Our work therefore clarifies the function of *rif-orf13* in the biosynthesis of rifamycin and explains the contradictory results of previous studies.

It must be pointed out that the catalytic efficiency of Rif-Orf13 *in vivo* and *in vitro* were dramatically different from each other. In the complex of cytochrome P450, ferredoxin and ferredoxin reductase, cytochrome P450 determines the substrate specificity and the reaction mode while the partner proteins account for the reaction rate [14]. Generally, only a few partner proteins are encoded in a genome, while plenty of P450 enzymes maybe exist. We failed to identify the authentic ferredoxin/ferredoxin reductase of Orf13 but used heterologous partners from *Spinacia oleracea* to ensure the supplying of electrons, which probably resulted in the low efficiency of hydroxylation in the enzymatic assays. However, compound **1**, the proposed substrate of Rif-Orf13, was totally converted into the product *in vivo*. It could be proposed that ferredoxin and ferredoxin reductase possessing better compatibility with Rif-Orf13 were harbored in *Streptomyces lividans*. Meanwhile, higher stability of Rif-Orf13 and smooth supply of NADPH was probably more available in the intracellular environment of *Streptomyces lividans*, and thus higher catalytic efficiency was acquired *in vivo*.

4 Experimental section

Genomic sequence of *A. tolypomycina* DSM 44544 was downloaded from genbank (Accession No. FNSO00000000.1), and the homologues of *rif-orf0*, *4* and *13* were identified as gene SAMN04489727_0263, 0292 and 0305, respectively (Table S3), using Basic Local Alignment Search Tool (BLAST, https://blast.ncbi.nlm.nih.gov/Blast.cgi). To heterologously express these genes, we constructed pSET152 derivate plasmid pSETe which contains constitutive promoter *ermE** in the multiple cloning site (Figure S1). Target genes were amplified from genomic DNA of *A. tolypomycina* through polymerase chain reaction (PCR) with specific primers AT-orf0-F/AT-orf0-R, AT-orf4-F/AT-orf4-R or AT-orf13-F/AT-orf13-R, and then digested and cloned into NdeI/XbaI site of pSETe to give recombinant plasmids pAT0he, pAT4he and pAT13he. The recombinant plasmids were verified by DNA sequencing and transferred into *E. coli* S17-1 through transformation, and finally into *S. lividans* 1326 via conjugation between *E. coli* S17-1 and *S. lividans*. Conjugants harboring pAT0he, pAT4he or pAT13he were identified by the resistance to apramycin. Parent plasmid pSETe was trans-

ferred into *S. lividans* 1326 to make blank control (Sl/-) in the following substrate feeding experiments.

Recombinant strains Sl/orf0, Sl/orf4, Sl/orf13 and negative control Sl/- were cultured in 50 mL ISP-2 broth containing 50 μg/mL apramycin in 250 ml flask at 30 ℃ and 220 round per minute. After 2 days of cultivation, the broths were added with 1 mg compound **1**, **2**, **3** or **4** (in 100 μL methanol), respectively, and then continued to cultivate for 24 hours under the same condition. Next, the cultures were individually extracted with 50 mL ethyl acetate twice. The organic extracts were combined and evaporated to dry to yield yellow pastes, which were dissolved in 1 mL methanol and subject to HPLC and LC-MS analysis.

HPLC analysis was carried out on Agilent Technology 1260 infinity with Dickma Diamonsil C18 column (5μm, 4.6 x 250 mm), using H_2O added with 0.1% formic acid (solvent A) and CH_3CN added with 0.1% formic acid (solvent B) as mobile phase. The samples were washed with a flow of 1 mL/min under the gradient elution program as follow: 0 min, 24% solvent B, 24 min, 60% solvent B, 28 min, 80% solvent B, 30 min, 95% solvent B, 33 min, 95% solvent B and 35 min, 24% solvent B. LC-MS analysis of *in vitro* assays was carried out on Thermo Scientific LTQ XL Mass spectrometer with Zorbax Eclipse XDB-C18 column (5μm, 4.6 x 150 mm) and the same mobile phase and flow rate as above but using gradient elution program as follow: 0 min, 40% solvent B, 4.5 min, 50% solvent B, 5 min, 70% solvent B, 9 min, 95% solvent B, 10 min, 40% solvent B and 11 min, 40% solvent B.

To characterize the *in vitro* function of Orf13, *rif-orf13* was amplified from genomic DNA of *A. tolypomycina* with specific primers AT-13e-F / AT-13e-R, and then cloned into the NdeI/HindIII site of pET28a to give recombinant plasmid pAT13p (Figure S2). This plasmid was verified by DNA sequencing and finally transferred into heterologous expression host *E. coli* BL21 (DE3).

E. coli BL21 (DE3)/pAT13p was cultivated in 700 mL LB broth in 2.5 L flask with 50μg/mL kanamycin added at 37 ℃ and 250 round per minute. The temperature was adjusted to 16 ℃ when the optical density at 600 nm (OD_{600}) arrived at 0.5, and 30 minutes later final concentration of 100 μM IPTG, 50 mg/L ALA and 200 mg/L NH_4FeSO_4 were added into the culture broth to induce the production of Orf13. The induced culture was precipitated after 20 hours to harvest cells. The sediment was washed with and then suspended in 40 mL phosphate buffer (50 mM NaH_2PO_4, 500 mM NaCl and 10 mM imidazole, pH 8.0). The suspension was subject to digestion with 1 mg/mL lysozyme for one hour and then ultrasonication in ice-bath condition to thoroughly smash the cells. After centrifugation, the lysate supernatant was mixed with nickel affinity beads to adsorb target his-tagged Orf13, then filtered to gather beads and washed with phosphate buffer containing imidazole gradient from 50 mM to 500 mM. The eluents containing pure target protein were combined and dialyzed against tris-HCl buffer (50mM tris-HCl, 50 mM NaCl, 10% glycerol, pH 8.0) to yield Orf13 product.

Purified Orf13 was subject to reduced CO-bound difference spectrum measurement to check whether the cofactor heme was properly loaded in the active center. The protein solution was bubbled with CO for 1 minute in the presence of 1 mM sodium hyposulfite and the absorption spectra of treated and untreated protein were measured with Thermo Scientific NanoDrop 2000c spectrophotometer.

The enzymatic activity of Orf13 was tested in 50μL reaction containing 50mM sodium phosphate buffer (pH 6.5), 1 mM **1**, 3 mM NADPH, 50 μM Orf13 and 10 μM ferredoxins and ferredoxins reductases at 30 ℃ for 2 to 17 hours. The reaction was quenched by adding 100μL methanol and vortex. The product was analyzed on HPLC and LC-MS after removing the denatured protein by centrifugation.

Supporting Information Strains, plasmids and primers used in this study, more feeding experiments, construction of recombinant plasmids and bioinformatic analysis of genes are available free of charge via the Internet at http://sioc-journal.cn/.

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Cytochrome P450 Encoded by rif-orf13 Catalyzes 34a-Hydroxylation in Rifamycin Biosynthesis

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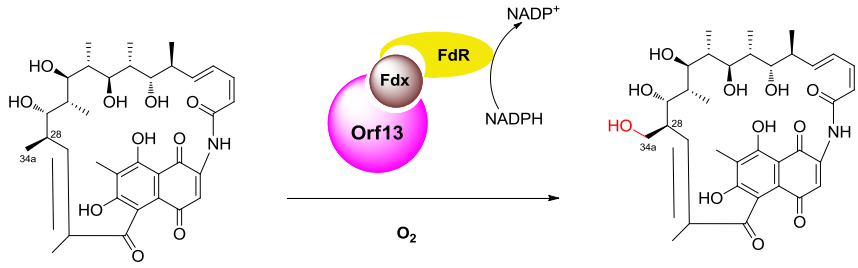

Cytochrome P450 encoded by *rif-orf13* can convert 16-demethyl-34a-deoxyrifmycin W into 16-demethyl-rifamycin W when heterologously expressed in *Streptomyces lividans*. The same conversion was observed in the *in vitro* enzymatic assay using Orf13 as catalyst at the presence of ferredoxin, ferredoxin reductase and NADPH. Therefore, *rif-orf13* is responsible for the hydroxylation of C34a in the biosynthesis of rifamycins.

