Structure-Function Analyses of Cytochrome P450revI Involved in Reveromycin A Biosynthesis and Evaluation of the Biological Activity of Its Substrate, Reveromycin T*

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Shunji Takahashi†§¶∥, Shingo Nagano†∥, Toshihiko Nogawa‡, Naoki Kanoh**§∥, Masakazu Uramoto‡, Makoto Kawatani§∥, Takeshi Shimizu†∥, Takeshi Miyazawa§, Yoshitsugu Shiro, and Hiroyuki Osada*‡¶∥§∥

From the †Chemical Biology Group, RIKEN Center for Sustainable Resource Science, Saitama 351-0198, Japan, the ‡Antibiotics Laboratory, RIKEN, Saitama 351-0198, Japan, the §Department of Chemistry and Biotechnology, Graduate School of Engineering, Tottori University, Tottori 680-8582, Japan, the ¶Biometal Science Laboratory, RIKEN SPring-8 Center, 1-1-1 Kouto, Sayo, Hyogo 679-5148, Japan, and the **Graduate School of Pharmaceutical Sciences, Tohoku University, Aobayama, Sendai 980-8578, Japan

Background: Hydroxylation of natural products is a key step for generating structural diversity.

Results: The revI gene disruptants accumulated reveromycin T, which showed stronger anticancer activity than reveromycin A.

Conclusion: Co-crystal structure analysis, site-directed mutagenesis, and biochemical characterization revealed how P450revI recognizes reveromycin T.

Significance: The co-crystal structure provides insight into the design of regio- and stereo-specific hydroxylation for novel spiroacetal compounds.

Numerous cytochrome P450s are involved in secondary metabolism biosynthesis. The biosynthetic gene cluster for reveromycin A (RM-A), which is a promising lead compound with anti-osteoclastic activity, also includes a P450 gene, revI. To understand the roles of P450revI, we comprehensively characterized the enzyme by genetic, kinetic, and structural studies. The revI gene disruptants (ΔrevI) resulted in accumulation of reveromycin T (RM-T), and revI gene complementation restored RM-A production, indicating that the physiological substrate of P450revI is RM-T. Indeed, the purified P450revI catalyzed the C18-hydroxylation of RM-T more efficiently than the other RM derivatives tested. Moreover, the 1.4 Å resolution co-crystal structure of P450revI with RM-T revealed that the substrate binds the enzyme with a folded compact conformation for C18-hydroxylation. To address the structure-enzyme activity relationship, site-directed mutagenesis was performed in P450revI. R190A and R81A mutations, which abolished salt bridge formation with C1 and C24 carboxyl groups of RM-T, respectively, resulted in significant loss of enzyme activity. The interaction between Arg190 and the C1 carboxyl group of RM-T elucidated why P450revI was unable to catalyze both RM-T 1-methyl ester and RM-T 1-ethyl ester. Moreover, the accumulation of RM-T in ΔrevI mutants enabled us to characterize its biological activity. Our results show that RM-T had stronger anticancer activity and isoleucyl-tRNA synthetase inhibition than RM-A. However, RM-T showed much less anti-osteoclastic activity than RM-A, indicating that hemisuccinate moiety is important for the activity. Structure-based P450revI engineering for novel hydroxylation and subsequent hemisuccinylation will help facilitate the development of RM derivatives with anti-osteoclastic activity.

Streptomycetes produce a wide variety of natural products that are used for medicinal drugs (1) and bioprobes (2) to elucidate biological functions. Reveromycin A (RM-A)3 (3), which is a spiroacetal polyketide compound produced by Streptomyces sp. SN-593, inhibits bone resorption by specifically inducing apoptosis in osteoclasts (4). It has been demonstrated that RM-A inhibited bone metastasis of lung and prostate cancer cells through anti-osteoclastic activity (5–7). We recently reported that RM-A normalized bone metabolism and loss of alveolar bone during continuous tooth movement in osteoprotgerin-deficient (OPG−/−) mice (8). Despite extensive effort to optimize chemical synthesis of RM derivatives to increase biological activity, only limited success has been achieved (9, 10).

Understanding RM-A biosynthetic machinery and utilization of its unique enzymes are promising strategies for the creation of novel RM derivatives. Recently, we identified the RM-A biosynthetic gene cluster, which consists of 21 open reading frames spanning 91 kb (11). Gene disruption and complementation analyses revealed that polyketide synthase (PKS) genes (revA, revB, revC, and revD; Fig. 1A) are responsible for the generation of the polyketide skeleton (RM-A1a). Dihydroxy
ketone synthase (revG) and spiroacetal synthase (revJ) genes that are essential for stereospecific spiroacetal formation have been identified (Fig. 1B).

We also identified the biosynthetic intermediate RM-T, which has been a promising target for the evaluation of biological activity because, in contrast to RM-A, it has a stable 6,6-spiroacetal structure due to the absence of hemisuccinate moiety. However, it was difficult to obtain enough RM-T from the wild-type strain because other biosynthetic reactions also occur.

Recently, we unexpectedly found novel RM-T derivatives (RM-T 1-methyl ester and RM-T 1-ethyl ester) in alcohol-added fermentation broth during feeding experiments (12). This finding prompted us to investigate the biological activity of RM-T derivatives. Although RM-A derivatives that were chemically modified at the C1 carboxyl group had reduced biological activity (9), the cytotoxic activities of the RM-T derivatives exhibited a 2–10-fold reduction of IC50 values against the HL-60 and K562 cell lines (12). Therefore, evaluating the biological activity of RM-T is also of interest.

The presence of only one P450 gene (revI) in the RM-A biosynthetic gene cluster indicates that the P450revI enzyme might be responsible for catalyzing C18-hydroxylation of RM-T. In this study, we disrupted the revI gene to evaluate its biosynthetic intermediate. Additionally, it remains unclear why no hydroxylated or hemisuccinylated metabolites derived from

**FIGURE 1.** Gene organization (A) and RM-A biosynthetic pathway (B). A, the revI gene was organized in revJIH gene sets. B, formation of RM-T from post-PKS biosynthetic precursor RM-A1a into RM-T, C18-hydroxylation of RM-T, and subsequent hemisuccinylation.
Cytochrome P450 Involved in Reveromycin A Biosynthesis

RM-T 1-esters were isolated from the culture broth, even in the presence of functional genes in the RM-A cluster. Kinetic analysis of P450revI is essential to obtain insight into the mechanism underlying this observation.

In this study, we comprehensively characterized RM-T C18-hydroxylase (P450revI) by gene disruption, co-crystal structure analysis, site-directed mutagenesis, and biochemical characterization. Taking advantage of the RM-T-accumulating strain, the biological activities of RM-T were also compared with those of other RM derivatives.

EXPERIMENTAL PROCEDURES

Chemicals—Ampicillin, kanamycin, and chloramphenicol were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Streptomycin, spectinomycin, thiostrepton, ribostamycin, NADPH, spinach ferredoxin (Fd), and spinach Fd-NADP+ reductase were purchased from Sigma-Aldrich. Carumonum was purchased from Takeda Pharmaceutical Co. Ltd. All other chemicals were of analytical grade. Spirofungin A (SF-A) and spirofungin B (SF-B) were chemically synthesized (13). RM-A1a, RM-A1b, RM-A1c, and RM-T were isolated from Streptomyces sp. SN-593, as described previously (3, 11). RM-T 1-methyl ester and RM-T 1-ethyl ester were isolated from alcohol-added fermentation of the wild-type strain (12). C18-hydroxy RM-T (RM-T1) was prepared as described previously, except that NaOH was used instead of LiOH (14).

Analytical Methods—The 1H and 13C NMR spectra were recorded on JEOL ECP-500 spectrometers in CD3OD. Chemical shifts were referenced to the residual solvent signal. UV spectra were measured using a JASCO V-630 BIO spectrophotometer. Optical rotations were recorded with a HORIBA SEPA-300 high-sensitivity polarimeter. The high-resolution mass spectrum was measured using a JEOL JMS-T100LC mass spectrometer. Thin layer chromatography was performed on a Merck 0.25-mm silica gel-precoated plate (60 F 254) with solvent B, acetonitrile. After injection and the hybridization probe are listed in Table 1. To construct a complementation vector, the revI gene was ligated into the BamHI and HindIII sites of pTYM19 (19) with the aphII promoter. The primers used for gene complementation are listed in Table 1. For intergeneric conjugation with Streptomyces sp. SN-593, E. coli GM2929 hsdS::Tn10 (pUB307::Tn7) (15) was used as the plasmid donor strain, as described previously (20).

Extraction and LC-MS Analysis—Wild-type and revI gene disruptants (ΔrevI) were grown in a 500-ml cylindrical flask containing SY medium (70 ml) for 2 days. One milliliter of the preculture was inoculated in a 500-ml cylindrical flask containing RM-A-producing medium (70 ml). Five days after inoculation, 4 ml of culture broth was extracted with an equal volume of acetone and then concentrated to remove acetone. The pH of the aqueous extract was adjusted to 4 by adding acetic acid and then extracted twice with an equal volume of ethyl acetate. The organic layer was concentrated in vacuo and dissolved in 1.2 ml of methanol. An electrospray ionization-MS analysis was performed using a Waters Alliance HPLC system equipped with a mass spectrometer (Q-TRAP, Applied Biosystems). The HPLC conditions were as follows: column, XTerra®MSC 5 μm (2.1 × 150 mm); flow rate, 0.2 ml min⁻¹; solvent A, water containing 0.05% formic acid; solvent B, acetonitrile. After injection of 1 μl of extracted sample into a column equilibrated with 30% solvent B, the column was developed with a linear gradient from 30 to 100% solvent B over the course of 20 min and kept at 100% solvent B for another 20 min. Mass spectra were collected in the electrospray ionization-negative mode.

Plasmid Construction for Heterologous Gene Expression—A PCR fragment of 1,194 bp containing revI was amplified from pCCIFOS-11A02 by PrimeSTAR®HS DNA polymerase in the presence of functional genes in the RM-A cluster. Kinetic analysis of P450revI is essential to obtain insight into the mechanism underlying this observation.
Terrific broth medium containing kanamycin (50 μg/ml) was inoculated into 2 liters of buffer A (50 mM Tris-HCl, pH 7.5, 500 mM NaCl, 10 mM 2-mercaptoethanol, and 20% glycerol) including 0.5 mg of lysozyme ml⁻¹ and 500 units of benzozonase. The cell suspension was sonicated on ice for 10 × 10 s with a 1-min interval after each sonication treatment (TOMY UD-200). Cellular debris was removed by centrifugation (10,000 × g for 30 min) (TOMY SRX-201). The supernatant was applied to the nickel-nitritriacetic acid (Ni-NTA)-agarose column (2 × 4 cm) (Qiagen), equilibrated with buffer A, and washed with 100 ml of buffer A.

Nonspecifically bound proteins were washed with 50 ml of buffer B (50 mM Tris-HCl (pH 7.5), 500 mM NaCl, 10 mM 2-mercaptoethanol, and 10% glycerol) containing 5 mM imidazole and 0.2% Tween 20. After washing with 200 ml of buffer B containing 5 mM imidazole, the column was further washed with 50 ml of buffer B containing 40 mM imidazole. His tag fusion protein was eluted with 35 ml of buffer B containing 250 mM imidazole. The purity of P450revI (CYP107E6) was confirmed by SDS-PAGE (Fig. 2A). For the enzyme assay, the purified proteins were dialyzed against 50 mM Tris-HCl (pH 7.5) containing 20% glycerol and frozen at −80 °C.

For x-ray analysis of P450revI, 60 units of thrombin was added to the Ni-NTA column (2 × 7 cm) to remove the His tag fragment. Then the passing fraction (50 ml) was applied to a benzamidine-Sepharose column to remove thrombin. His tag-free P450revI was dialyzed against buffer C (50 mM Tris-HCl (pH 7.5) and 10% glycerol) and applied to a Mono Q™ 5/50 GL column (GE Healthcare) that was previously equilibrated with buffer C at a flow rate of 1 ml min⁻¹. After washing for 5 min, a linear NaCl concentration gradient, from 0 to 500 mM, was established over 55 min. The P450revI fraction was concentrated with Amicon Ultracel-30K (Millipore Corp.), and the buffer was replaced with 10 mM Tris-HCl (pH 7.5). Finally, 10 mg of His tag-free P450revI was concentrated to 43 mg ml⁻¹ for crystal analysis.

To investigate multimeric status, His tag-free P450revI was dialyzed against gel filtration buffer (50 mM Tris-HCl (pH 7.5), 200 mM NaCl, and 10% glycerol) and applied to a Superdex200 column (GE Healthcare), which was previously equilibrated with the same buffer, at a flow rate of 0.8 ml min⁻¹. The column was calibrated with ferritin (440 kDa), aldolase (158 kDa), and cytochrome c (12.4 kDa).
Cytochrome P450 Involved in Reveromycin A Biosynthesis

conalbumin (75 kDa), ovalalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa). The elution was established over 80 min. The P450revI elution peak corresponded to a molecular mass of 42 kDa, suggesting that P450revI was in the monomeric form.

**CO Difference Spectra**—CO difference spectroscopy was performed to quantify and normalize the amount of correctly folded P450 enzyme used in enzyme activity assays. The amount of functional P450 was calculated using the extinction coefficient of 91 mm$^{-1}$ cm$^{-1}$ at 450 nm (Fig. 2B) (21).

**Measurement of P450revI Activity**—Kinetic assays were performed in 1.5-ml tubes with a final reaction volume of 0.2 ml containing 50 mM Tris-HCl (pH 7.5), 1 mM NADPH, 0.1 mg ml$^{-1}$ spinach Fd, 1 unit ml$^{-1}$ spinach Fd-NADP$^+$ reductase, 0.5–15 μM RM-T, and 1 pmol of P450revI (measured by CO difference spectroscopy).

Substrate concentrations of RM-A1c varied from 5 to 30 μM in the presence of 100 pmol of P450revI, and those of SF-A varied from 34 to 224 μM in the presence of 200 pmol of P450revI. After preincubation at 30 °C for 5 min, the reactions were initiated by the addition of NADPH. After incubation for 1 min (RM-T), 0.5 min (RM-A1c), and 10 min (SF-A), the reaction was terminated by the rapid addition of 4 μl of acetic acid. After extraction with ethyl acetate (0.4 ml) of the solution was added to the enzyme.

**Crystallization**—Crystallization of P450revI was performed using sitting drop vapor diffusion. The reservoir solution contained 0.2 m sodium tartrate and 22% polyethylene glycol 3350. The substrate-saturated solution was added to the enzyme solution (0.5 μl) was subjected to LC-MS in the conditions described above. The product peak was calculated from the standard curve, which was obtained from the maximum UV wavelength (238 nm) of the RM-T standard. The enzyme-specific activity (pmol of product formed min$^{-1}$ pmol of enzyme$^{-1}$) was calculated by time-dependent product formation. The kinetic constants were calculated by a nonlinear regression fit to the Michaelis-Menten equation using SigmaPlot11. Kinetic analyses of mutant enzymes were performed in the presence of 100 pmol of R81A, 10 pmol of F91G, 1 pmol of L287F, and 50 pmol of A292V. The concentrations of RM-T varied from 7.5 to 150 μM. After incubation for 0.5 min (F91G and A292V) or 1 min (R81A and L287F), mutant enzyme reactions were terminated and analyzed, as described above.

**Growth-inhibiting Activity against Yeast**—Budding yeast (MLC30M strain) was precultured in a medium containing 2% polypeptone, 1% yeast extract, 0.5% meat extract, 0.3% NaCl, and 0.001% SDS at 37 °C. Yeast solution was prepared such that the absorbance at 600 nm was 0.005, and 100 μl of the solution was seeded in a 96-well plate. Each chemical was added to the medium at a final concentration of 0.5% (v/v), prior to incubation at 37 °C for 6 h. A$_{600}$ was measured using a microplate reader.

**Cytotoxic Activity against Osteoclasts**—Bone marrow cells were harvested from thigh and shin bones of 5-week-old male ddY mice (Japan SLC, Inc.) and seeded in a type I collagen-coated plate (IWAKI) in α-minimum Eagle’s medium (Sigma-Aldrich) with 10% fetal bovine serum, 0.5% penicillin/streptomycin solution, 50 ng ml$^{-1}$ human M-CSF (Leukoprol, Kyowa Hakko), and 1 ng ml$^{-1}$ human TGF-β1 (R&D Systems). Bone marrow cells were then maintained at 37 °C in a humidified atmosphere containing 5% CO$_2$ for 3 days. Cells were washed twice with PBS, and cells adhered onto the plate were then used as bone marrow macrophage cells.

Bone marrow macrophage cells were further maintained in a culture medium in which α-minimum Eagle’s medium was added with 10% fetal bovine serum, 0.5% penicillin/streptomycin solution, 50 ng ml$^{-1}$ human M-CSF, and 50 ng ml$^{-1}$ human

**Antibacterial Test**—E. coli (HO141 strain) was precultured in a medium containing 0.5% polypeptone, 0.5% meat extract, 0.3% NaCl, and 0.001% SDS at 37 °C. E. coli solution was prepared such that the absorbance at 600 nm was 0.005, and 100 μl of the solution was seeded in a 96-well plate. Each chemical was added to the medium at a final concentration of 0.5% (v/v), prior to incubation at 37 °C for 6 h. A$_{600}$ was measured using a microplate reader.

**Growth-inhibiting Activity against Animal Cells**—The HL-60, K562, and tsFT210 cell lines were cultured in RPMI 1640 (Invitrogen), supplemented with fetal calf serum (10% for HL-60 and K562) and calf serum (5% for tsFT210) (27), 50 units/ml penicillin, and 50 μg/ml streptomycin at 37 °C for HL-60 and K562 and at 32 °C for tsFT210 in a humidified atmosphere containing 5% CO$_2$.

Each cell line was used to seed a 96-well culture plate (1.5 × 10$^4$ cells/well/100 μl for HL-60 and K562 and 1.6 × 10$^4$ cells/well/100 μl for tsFT210) (IWAKI) and exposed to test compounds for 48 h. Cell growth was measured using Cell Count Reagent SF (Nacalai Tesque), per the manufacturer’s instructions. Briefly, one-tenth volume of the WST-8 solution was added to each well, and the plates were incubated at 37 °C (HL-60 and K562) or 32 °C (tsFT210) for 1 h. Subsequently, cell growth was measured at 450 nm on a microplate reader (PerkinElmer Life Sciences).

**Cytochrome P450 Involved in Reveromycin A Biosynthesis**
soluble RANKL (PeproTech) at 37 °C in a humidified atmosphere containing 5% CO2 for 3 days to differentiate into osteoclasts. Each chemical was added to osteoclasts at 0.5% (v/v), and the culture was maintained at 37 °C in a humidified atmosphere containing 5% CO2 for 24 h. Subsequently, cells were treated with PBS solution containing 3.7% formalin at room temperature for 30 min. After the solution was removed, the cells were further treated with acetic/ethanol solution (1:1, v/v) at room temperature for 1 min, and the solution was removed to dry the cells. Immobilized cells were subjected to a reaction in TRAP solution (50 mM sodium tartrate, 90 mM sodium acetate, 0.01% naphthol AS-MX phosphate (Sigma), 0.05% fast red violet LB salt (Sigma), pH 5.0) at room temperature for 30 min and then washed with distilled water. The number of TRAP-positive multinucleated osteoclasts was counted, and the rate of survival was determined.

Inhibitory Activity against Isoleucyl tRNA Synthetase—To a solution for enzymatic reaction (20 mM imidazole (pH 7.5), 75 mM MgCl2, 0.5 mM DTT, 1 unit ml−1 (GE Healthcare), and 10 μg of protein (HT1080 cell lysate)), each chemical was added at a final concentration of 1% (v/v) to attain a final volume of 100 μl, and the reaction was carried out at 25 °C for 20 min. Subsequently, 1 mg ml−1 BSA solution (400 μl) and 10% TCA solution (500 μl) were then added to terminate the reaction, following which the reaction mixtures were left to stand at 4 °C overnight. A precipitate obtained by centrifugation was transferred onto a GF-C filter (Whatman) and washed three times with 5% TCA solution, and the filter was dried. Two milliliters of Aquasol-2 (PerkinElmer Life Sciences) and the filter were placed in a vial and vigorously stirred. The amount of [3H]isoleucine was measured by a liquid scintillation counter (Beckman) to determine the rate of enzyme activity.

RESULTS

Accumulation of RM-T in the revI Gene Disruptants—Modification of hydroxyl groups by tailoring enzymes is often observed in the biosynthesis of secondary metabolites. We expected that the P450 gene (revI) found in the RM-A gene cluster might catalyze the key hydroxylation step for subsequent hemisuccinylation (Fig. 1B). Therefore, to identify the role of P450revI, we performed revI gene disruption by homologous recombination (Fig. 3, A and B). The metabolite analysis of the ΔrevI mutant strain revealed accumulation of the biosynthetic intermediate RM-T (Fig. 3, C–E). Restoration of RM-A production by the reintroduction of the revI gene into ΔrevI (Fig. 3F) strongly indicated that RM-T is consumed by P450revI.

P450revI Catalyzes the C18 Hydroxylation of RM-T—In order to characterize the function of P450revI, the enzyme was heterologously expressed in E. coli and purified by an Ni-NTA column (Fig. 2A). Proper protein folding was confirmed by CD difference spectroscopy (Fig. 2B). Because of the accumulation of RM-T in the ΔrevI mutant strain, P450revI reaction was examined using RM-T. As expected, P450revI efficiently converted RM-T into RM-T1 (Fig. 4, A and B). The LC-MS analysis clearly demonstrated the P450revI reaction product (m/z 559 [M-H]−). The retention time (12.82 min), UV, and mass spectrum of the reaction product were identical to that of the standard of RM-T1, prepared from RM-A.

To address substrate specificity, we tested the P450revI reaction with RM derivatives obtained from both biosynthesis and chemical synthesis research (11–13). P450revI was unable to catalyze the compounds containing 15S spiroacetal stereochemistry, such as SF-B; acyclic biosynthetic precursor RM-A1a; biosynthetic shunt product RM-A1b, which lost spiroacetal integrity; and the RM-T 1-methyl ester and RM-T 1-ethyl ester, which are modified at the C1 carboxyl group of RM-T. In contrast, the enzyme produced monooxgenated products from SF-A and RM-A1c containing 15S spiroacetal stereochemistry (Fig. 5).

To characterize enzymatic properties, the kinetic parameters were also investigated for RM-T, SF-A, and RM-A1c. We found that P450revI showed the highest catalytic efficiency for RM-T (Table 2). A comparative study using SF-A and SF-B suggested the importance of 15S spiroacetal stereochemistry for substrate recognition by P450revI. Comparison of RM-A1c with RM-A1a and RM-A1b indicates that the integrity of the spiroacetal core structure was required for substrate recognition.

Loss of the catalytic activity for RM-T 1-methyl ester and RM-T 1-ethyl ester strongly indicated the importance of the C1 carboxyl group of RM-T for substrate recognition by P450revI (Fig. 5). Comparison of kcat/Km values between RM-A1c and RM-T suggested an involvement of the C24 carboxyl group in substrate recognition by P450revI. Additionally, comparison of kcat/Km values between SF-A and RM-T suggested that the C18 butyl group attached to the spiroacetal structure has a significant role for substrate recognition by P450revI. Based on gene disruption and biochemical analysis, we concluded that RM-T is a physiological substrate of P450revI.

Co-Crystal Structure of P450revI with Physiological Substrate RM-T—In order to understand the RM-T recognition by P450revI and the substrate structure-reactivity relationship, we determined the crystal structure of P450revI complexed with RM-T. Crystallographic and refinement statistics are summarized in Table 3. P450revI adopts the typical triangular P450 fold with a long I helix, which has two highly conserved residues, Glu244 and Thr245, which are important for oxygen activation (Fig. 6, A and B).

The I helix forms the wall of the large substrate binding cavity, which has a volume of 2,446 Å3. The cavity is covered by a BC and FG loop. A loop region following the K helix also lines the cavity (Fig. 6C). The substrate side chain with the C1 carboxyl group aligns in parallel with the butyl chain at C18 to fit into the cavity, and the substrate thus has a compact conformation (Fig. 7A). The side chain with the C24 carboxyl group is roughly perpendicular to these two substrate side chains. One of the spiroacetal rings is above the heme D ring and is parallel to the heme.

The other spiroacetal ring is almost perpendicular to the heme. Arg390 makes a salt bridge with the C1 carboxyl group at a distance of 2.8 Å. Arg391, which is at the top of the substrate binding cavity, forms a bifurcated hydrogen bond with the C5 hydroxyl and C24 carboxyl groups. The oxygen atom of the spiroacetal ring is hydrogen-bonded with a water molecule (Wat346), which also makes a hydrogen bond with the heme.
7-propionate group. Leu176, Ile240, Leu287, and Val391 come in contact with the hydrophobic moiety of the substrate, which includes C4–C13 atoms.

The phenyl ring of Phe91 is parallel to the butyl side chain of RM-T (Fig. 7B). Although P450revI hydroxylates the C18 atom, and no other hydroxylation product was detected, the nearest carbon atom to the heme iron is C16 (4.5 Å), and C18 is the third nearest one (5.2 Å). Ala292 is located in the loop that adopts two conformations (Gly290–Ala293), and this side chain in conformer A faces the substrate (Fig. 8). Very similar conformational ambiguity was also found at the Thr284–Phe286 region in P450 MycG, which catalyzes sequential hydroxylation and epoxidation in mycinamicin biosynthesis. Carbon atoms that are monooxygenated are relatively distant from the heme iron (9–10 Å), and reorientation of the substrate allows for the required monooxygenation. Such relocation was supported by NMR relaxation experiments in solution, and the attainment of the productive orientation of the substrate is probably coupled with conformational fluctuation of the Thr284–Phe286 (28). Therefore, for P450revI, the flexible loop region, Gly290–Ala293 also probably contributes to translocation of the substrate to allow C18-hydroxylation.

P450revI shows significant structural similarity to CYP105A1 (P450 SU-1), which is isolated from Streptomyces griseolus and can catalyze vitamin D3 hydroxylation. Fig. 9 illustrates the superposed structures of a vitamin D3-bound mutant.

FIGURE 3. Gene disruption of revI and metabolite analysis. A, scheme of revI disruption and restriction map of the wild-type and ΔrevI mutant strains. The bar shows the expected fragment size (in bp) of BamHI digestion. An ∼5-kb DNA fragment was amplified from pIM-ΔrevI and used as a Southern hybridization probe. B, Southern blot analysis of the wild-type (lane 2) and mutant strains (lanes 3–6; individual isolations). Genomic DNA digested with BamHI was resolved on a 0.9% agarose gel and stained by EtBr. The arrows indicate the expected size of the DNA fragments from the wild-type (solid) and mutant (open) strains. C–F, LC-MS analysis of RM standards (C) and RMs from the culture extract of the wild-type strain (D), ΔrevI mutant strain (E), and the ΔrevI mutant strain complemented by pTYM19-Paph-revI (F).
CYP105A1 and RM-T-bound P450revI. CYP105A1 also has a large substrate binding cavity that can accommodate vitamin D₃ (29). The bound substrate makes a salt bridge between Arg¹⁹³ and the 3β-OH of the substrate, and a very similar interaction is found in the P450revI and RM-T complex, where Arg¹⁹⁰ makes a salt bridge with the C₁ carboxyl group (Fig. 7B).

Site-directed Mutagenesis of P450revI—To gain further insight into the mode of substrate recognition by P450revI, site-directed mutagenesis was introduced at the amino acid positions of Arg⁸¹, Phe⁹¹, Arg¹⁹⁰, Leu²⁸⁷, and Ala²⁹², which can interact with RM-T. After purification of mutant P450revIs, CO difference spectra were determined. Like wild-type P450revI, the mutant enzymes showed clear absorption maxima at 450 nm (Fig. 10), which is consistent with that seen for properly folded enzymes that possess the heme cofactor oriented in the correct electron spin state. After estimation of the amount of properly folded enzyme, kinetic properties of the mutant enzymes were characterized. Arg¹⁹⁰, which constructed a salt bridge with the C₁ carboxyl group of RM-T (Fig. 7B), was converted to Ala¹⁹⁰. The abolishment of the salt bridge in the R₁⁹⁰A mutant completely eliminated the RM-T hydroxylase activity (Table 4).

Consistently, wild-type P450revI was unable to catalyze RM-T 1-methyl ester and RM-T 1-ethyl ester (Fig. 5). Similarly,

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kₘ</th>
<th>kₗₐₜ</th>
<th>kₗₐₜ/Kₘ</th>
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<tr>
<td>RM-T</td>
<td>0.89</td>
<td>331 ± 1.7</td>
<td>2.36</td>
</tr>
<tr>
<td>RM-A₁c</td>
<td>11.7</td>
<td>27.6 ± 1.7</td>
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<tr>
<td>SF-A</td>
<td>39.0</td>
<td>8.04 ± 0.15</td>
<td>0.206</td>
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</tbody>
</table>

FIGURE 5. Structure-function analyses of substrates catalyzed by P450revI.
Arg81, which constructed a salt bridge with the C24 carboxyl group of RM-T (Fig. 7B), was converted to Ala81. The elimination of the salt bridge in the R81A mutant revealed 330-fold lower catalytic efficiency than that of the wild-type P450revI (Table 4). Similarly, wild-type P450revI showed 160-fold lower catalytic efficiency for RM-A1c, which is absent from the C24 carboxyl group (Fig. 5). Phe91 in the BC-loop constructed hydrophobic pocket accommodated the butyl residue at C18 of the spiroacetal core structure (Fig. 7B).

The F91G mutant was constructed to evaluate RM-T substrate recognition. As expected, the mutant showed 24-fold lower catalytic efficiency for RM-T than that of the wild-type (Table 4). The recognition of C18 alkyl residue of spiroacetal core structure by wild-type P450revI was also evaluated using SF-A, which has a methyl group instead of a butyl group, which is found in RM-T. Wild-type P450revI showed significantly low catalytic efficiency for SF-A (Table 2 and Fig. 5). Comparative studies support the recognition of C18 butyl residues by Phe91. Moreover, based on co-crystal structure (Figs. 7 and 8), the roles of Leu287 and Ala292, which interact with the substrate, on catalytic efficiency of C18-hydroxylation of RM-T were evaluated. A larger hydrophobic residue was introduced by site-directed mutagenesis to produce L287F and A292V. In this

![FIGURE 6. Overall structure of P450revI (A), close-up view of the substrate binding site (B), and substrate binding pocket of P450revI-RM-T complex (C).](image)

**FIGURE 6.** Overall structure of P450revI (A), close-up view of the substrate binding site (B), and substrate binding pocket of P450revI-RM-T complex (C). Glu244 and Thr344 in the I helix, heme axial ligand Cys353, and the substrate are shown as stick models. BC- and FG-loops and a loop following the K helix are indicated by arrows. Heme and RM-T are shown by gray and green stick models, respectively.

![FIGURE 7. Substrate conformation and P450revI-substrate interactions.](image)

**FIGURE 7.** Substrate conformation and P450revI-substrate interactions. Simulated annealing-composite omit maps for the substrate (A) as well as side chains and a water molecule that interact with the substrate (B) are shown as mesh at 1.0sigma level. C, amino acid side chains, which make hydrogen bonding or hydrophobic interactions with the substrate.
experiment, we expected a different regio-specific hydroxylation product from the mutants. However, the mutants dominantly produced RM-T1. Additionally, the mutants showed 23 and 34 times lower catalytic efficiency than that of the wild-type P450revI, primarily because of the higher $K_m$ values of these mutants (Table 4). These results suggest that P450revI does not have extra room to facilitate relocation of the substrate and that the substrate binding site of this enzyme is highly optimized for C18-hydroxylation of RM-T.

**Biological Activity of RM-T—**
RM-T accumulation in the Δrevl mutant strain (Fig. 3) enabled us to evaluate its biological activity (Table 5). RM-T efficiently inhibited the activity of isoleucyl tRNA synthetase, which is the molecular target of RM-A (30). Consistently, RM-T showed stronger cytotoxic activity against cancer cell lines than any other RM derivatives tested. RM-T also demonstrated growth-inhibitory activity against yeast cells, suggesting its application as an antifungal agent. On the other hand, RM-T showed much less anti-osteoclastic activity than RM-A, suggesting that the hemisuccinate moiety in addition to the C1 and C24 carboxyl groups was essential to exhibit the strong activity (10).

**DISCUSSION**

In natural product biosynthesis, introduction of a hydroxyl group by P450 is a key step for subsequent modifications, such as glycosylation, acetylation, and methylation. Understanding a

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_m$</th>
<th>$k_{cat}$</th>
<th>$k_{cat}/K_m$</th>
<th>Relative activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>0.89 ± 0.05</td>
<td>331 ± 5</td>
<td>372</td>
<td>100</td>
</tr>
<tr>
<td>L287F</td>
<td>5.58 ± 0.82</td>
<td>89.5 ± 3.5</td>
<td>16.0</td>
<td>4.3</td>
</tr>
<tr>
<td>F91G</td>
<td>12.5 ± 2.0</td>
<td>197 ± 11</td>
<td>15.8</td>
<td>4.2</td>
</tr>
<tr>
<td>A292V</td>
<td>13.1 ± 1.1</td>
<td>143 ± 3</td>
<td>10.9</td>
<td>2.9</td>
</tr>
<tr>
<td>R81A</td>
<td>75.2 ± 9.4</td>
<td>84.4 ± 5.2</td>
<td>1.12</td>
<td>0.3</td>
</tr>
<tr>
<td>R190A</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^a$ Data are from Table 2.

$^b$ NA, no activity.
variety of substrate recognitions and reactions by P450s will expand the chemical diversity for drug leads (31). Here, we comprehensively characterized P450revI involved in RM-A biosynthesis. We demonstrated that the C18-hydroxylation of RM-T by P450revI is essential for subsequent hemisuccinate formation (Figs. 3 and 4). The 1.4 Å resolution co-crystal structure of P450revI with RM-T demonstrated the mode of substrate recognition (Figs. 6–8). The biosynthesis of RM-A is unique because C18-hydroxylation to afford the tertiary alcohol was followed by ester formation, which chemically required high pressure (1.5 gigapascals) (32, 33).

Structure-function analyses demonstrated that P450revI efficiently catalyzed substrates with 15\(^\text{S}\)spiroacetal structure whose stereochemistry was produced by RevG and RevJ in the RM-A biosynthetic pathway (11). Moreover, the interaction between Arg\(^{190}\) of P450revI and the C1 carboxyl group of RM-T (Fig. 7B) clearly explained the reason why alcohol-added fermentation resulted in the accumulation of RM-T 1-methyl ester and RM-T 1-ethyl ester, the latter of which does not contain hemisuccinylated moieties. Additionally, this finding led us to speculate that RM-T 1-methyl and 1-ethyl ester intermediates might be released as a post-PKS biosynthetic precursor when either methanol or ethanol was added to the culture medium.

Interestingly, RM-T showed stronger anticancer and isoleucyl tRNA synthetase inhibition activity than RM-A (Table 5). Because the presence of hemisuccinate moiety at the C18 position causes the conversion of bioactive 6,6-spiroacetal (RM-A) into inactive 5,6-spiroacetal structure (RM-B) (Fig. 1B) (11), the stability of 6,6-spiroacetal structure of RM-T might be one of the reasons for the strong biological activities observed. Unfortunately, RM-T showed weak anti-osteoclastic activity, suggesting that hemisuccinate moiety is important for efficient incorporation into osteoclasts in an acidic environment (4).

To minimize the conversion of 5,6-spiroacetal formation, mutagenesis in P450revI for novel regio- and stereo-specific hydroxylation in 6,6-spiroacetal structure and subsequent hemisuccinylation might be an approach to enhance the stability of RM derivatives that retain strong anti-osteoclast activity. Therefore, future research should focus on the engineering of P450revI to catalyze novel regio- and stereo-specific hydroxylation.

Reintroduction of the engineered P450revI into the \(\Delta\text{revI}\) mutant is promising for the production of novel RM derivatives. Based on co-crystal structure (Fig. 7B), C16 of RM-T was located closer to the heme-iron reaction center (4.5 Å) than C18 (5.2 Å). Therefore, changing the hydroxylation position from C18 to C16 will be the first step. The co-crystal structure

---

### TABLE 5

**Biological activity of RM derivatives**

The assay conditions are described under “Experimental Procedures.”

<table>
<thead>
<tr>
<th>Compd.</th>
<th>Structure</th>
<th>HL60(^{a})</th>
<th>K562(^{a})</th>
<th>tsFT210(^{a})</th>
<th>E. colia</th>
<th>Yeast(^{a})</th>
<th>OC(^{a})</th>
<th>IRSb</th>
</tr>
</thead>
<tbody>
<tr>
<td>RM-A</td>
<td><img src="image" alt="RM-A structure" /></td>
<td>2.7(^{c})</td>
<td>4.9(^{c})</td>
<td>8.5(^{c})</td>
<td>&gt;50(^{c})</td>
<td>4.3</td>
<td>0.06</td>
<td>6.2 (1.3(^{d})) 2.95(^{e})</td>
</tr>
<tr>
<td>RM-T</td>
<td><img src="image" alt="RM-T structure" /></td>
<td>0.2</td>
<td>0.7</td>
<td>0.4</td>
<td>&gt;50</td>
<td>0.05</td>
<td>6.2</td>
<td>0.2</td>
</tr>
<tr>
<td>RM-T1-methyl ester</td>
<td><img src="image" alt="RM-T1-methyl ester structure" /></td>
<td>0.9(^{c})</td>
<td>2.5(^{c})</td>
<td>8.2(^{c})</td>
<td>&gt;50(^{c})</td>
<td>&gt;50</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>RM-T1-ethyl ester</td>
<td><img src="image" alt="RM-T1-ethyl ester structure" /></td>
<td>0.2(^{c})</td>
<td>0.7(^{c})</td>
<td>11.4(^{c})</td>
<td>&gt;50(^{c})</td>
<td>&gt;50</td>
<td>6.0</td>
<td>200</td>
</tr>
<tr>
<td>SF-A</td>
<td><img src="image" alt="SF-A structure" /></td>
<td>1.2</td>
<td>3.4</td>
<td>&gt;30</td>
<td>&gt;50</td>
<td>0.3</td>
<td>NT</td>
<td>565(^{c})</td>
</tr>
<tr>
<td>SF-B</td>
<td><img src="image" alt="SF-B structure" /></td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>&gt;30</td>
<td>&gt;50</td>
<td>4.0</td>
<td>NT</td>
<td>&gt;1000(^{c})</td>
</tr>
</tbody>
</table>

\(^{a}\) IC\(_{50}\) (\(\mu\)g ml\(^{-1}\)).

\(^{b}\) IC\(_{50}\) (ng ml\(^{-1}\)) for IRS.

\(^{c}\) Cytotoxicity data reported (12).

\(^{d}\) IRS activity reported by Shimizu et al. (9).

\(^{e}\) IRS activity reported by Shimizu et al. (10).

\(^{f}\) NT, not tested.
of P450revl-RM-T provided insight into the rational design of novel hydroxylation products. Phe⁹¹, Leu²⁸⁷, and flexible loop Gly²⁹⁰–Ala²⁹³ found in P450revl (Figs. 7B and 8) will be the target amino acid residues for research on combinatorial mutagenesis.

Moreover, because of the biological activity of SF-A, which is structurally similar to RM-A (Table 5) (10, 34), 35 creating its biosynthetic gene cluster has been found in the genome sequence of Streptomyces violaceusniger Tü4113 (NC_015957), which is a strain that produces both SF-A and SF-B (34). Consistent with the structure of SF-A (Fig. 5), cytochrome P450 and possible modifier genes were not found in the putative cluster.

For the creation of novel hemisuccinylated compounds, one approach is the introduction of modification genes (revl, revK, revL, and revM) found in the RM-A gene cluster into S. violaceusniger Tü4113. The other approach is to perform domain exchange of butylmalonyl-CoA-specific acyltransferase (AT4) from RM-PKS into methylmalonyl-CoA-specific AT from the putative SF-A gene cluster. In the latter combinatorial biosynthesis approach, the utilization of the Δrevl strain and reintroduction of P450revl mutants catalyzing regio- and stereo-specific hydroxylation will further expand chemical diversity for drug leads.

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Structure-Function Analyses of Cytochrome P450revI Involved in Reveromycin A Biosynthesis and Evaluation of the Biological Activity of Its Substrate, Reveromycin T

Shunji Takahashi, Shingo Nagano, Toshihiko Nogawa, Naoki Kanoh, Masakazu Uramoto, Makoto Kawatani, Takeshi Shimizu, Takeshi Miyazawa, Yoshitsugu Shiro and Hiroyuki Osada


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