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The role of erythromycin C-12 hydroxylase, EryK, as a substitute for PikC hydroxylase in pikromycin biosynthesis

Sang Kil Lee,[a] Devi B. Basnet,[b] Cha Yong Choi,[a] Jae Kyung Sohng,[b] Jong Seog Ahn,[c] and Yeo Joon Yoon[d,*]

[a] *School of Chemical Engineering, College of Engineering, Seoul National University, San 56-1, Sinlim-dong, Gwanak-gu, Seoul 151-742, Republic of Korea*
[b] *Institute of Biomolecule Reconstruction, Sunmoon University, Kalsan-ri, Tangjeong-myeon, Asan-si, Chungnam 336-708, Republic of Korea*
[c] *Laboratory of Cellular Signaling Modulator, Korea Research Institute of Bioscience and Biotechnology, 52, Oeun-dong, Yusong-gu, Daejeon 305-333, Republic of Korea*
[d] *Division of Nano Sciences and Department of Chemistry, Ewha Womans University, 11-1, Daehyun-dong, Seodaemun-gu, Seoul 120-750, Republic of Korea*

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Abstract

The substrate flexibility of the erythromycin C-12 hydroxylase from *Saccharopolyspora erythraea*, EryK, was investigated to test its potential for the generation of novel polyketide structures. We have shown that EryK can accept the substrates of PikC from *Streptomyces venezuelae* which is responsible for the hydroxylation of YC-17 and narbomycin. In a *S. venezuelae pikC* deletion mutant, EryK could catalyze the hydroxylation of YC-17 and narbomycin to generate methymycin/neomethymycin and pikromycin, respectively. Molecular modeling of the enzyme-substrate complex suggested the possible interaction of EryK with alternative substrates. The results indicate that EryK is flexible toward some alternative polyketides and can be useful for structural diversification of macrolides by post-polyketide synthase hydroxylation.

Keywords: Cytochrome P450 monooxygenases; Polyketide; EryK; PikC

* Corresponding author. Fax: +82-2-32773419.
E-mail address: joonyoon@ewha.ac.kr (Y.J. Yoon).

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1. Introduction

Polyketides are a large class of natural products that possess diverse structures and pharmacological activities [1]. The biosynthetic pathways of macrolide polyketides involve the formation of macrolactones by the activity of the large multifunctional enzymes, modular polyketide synthases (PKSs), followed by specific post-PKS modifying steps such as oxidation, methylation, acylation, and glycosylation. One of the post-PKS modification reactions, hydroxylation or other oxidative steps catalyzed by cytochrome P450 monooxygenases, are often the key steps leading to structural diversity and biological activity of macrolide antibiotics. Therefore, the identification and investigation of the substrate flexibilities of P450s is significant for the generation of a variety of unnatural polyketides through combinatorial biosynthesis.

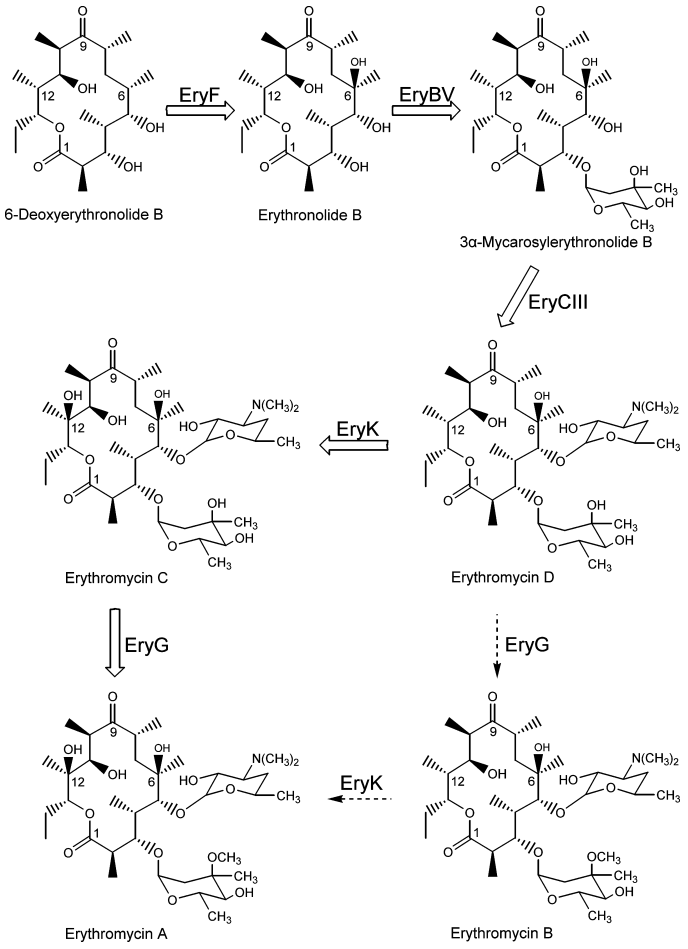

Fig. 1. The function of EryK in erythromycin A biosynthesis. Erythromycin D is the preferred substrate for EryK. The dotted line indicates a shunt biosynthetic pathway.

The erythromycin C-12 hydroxylase from *Saccharopolyspora erythraea*, EryK, is a cytochrome P450 responsible for the stereospecific C-12 hydroxylation of 14-membered precursors, erythromycin D and erythromycin B (Fig. 1) [2,3]. Although EryK can catalyze the hydroxylation of erythromycin B, the preferred pathway for erythromycin A biosynthesis involves oxidation of erythromycin D to erythromycin C, as indicated by a 1200- to 1900-fold kinetic preference for erythromycin D over erythromycin B as a substrate [3]. Erythromycin B is structurally identical to the preferred substrate, erythromycin D, with the exception of the methylation of a distal mycarosyl hydroxyl group. Due to this stringent substrate specificity, little work has been reported on the oxidative modification of alternative substrates by EryK.

The cytochrome P450 monooxygenase in the pikromycin biosynthetic gene cluster from *Streptomyces venezuelae*, designated PikC, can accept 12- and 14-membered ring macrolides as substrates and be active at different positions on the macrolactone system [4,5]. This monooxygenase can catalyze the C-12 hydroxylation of the 14-membered ring macrolide narbomycin **5** to pikromycin **6**, as well as functionalize the C-10 position of the 12-membered ring macrolide YC-17 **1** to yield methymycin **2** or the C-12 position to yield neomethymycin **3**. Functionalization at both positions yields novamethymycin **4** (Fig. 2) [4,6]. This unique substrate flexibility of PikC is useful for the construction of novel macrolides and ketolides [4,5]. Indeed, it has been reported that hybrid macrolides produced by recombinant *S. venezuelae* are further modified by the highly flexible PikC hydoxylase [7]. Moreover, PikC processes a possible substrate of EryK, erythromycin D, as an alternative substrate and catalyzes the hydroxylation at C-12 to yield erythromycin C [8].

We investigated the catalytic ability of EryK toward alternative macrolide substrates in an engineered *S. venezuelae pikC* deletion mutant. Molecular modeling of

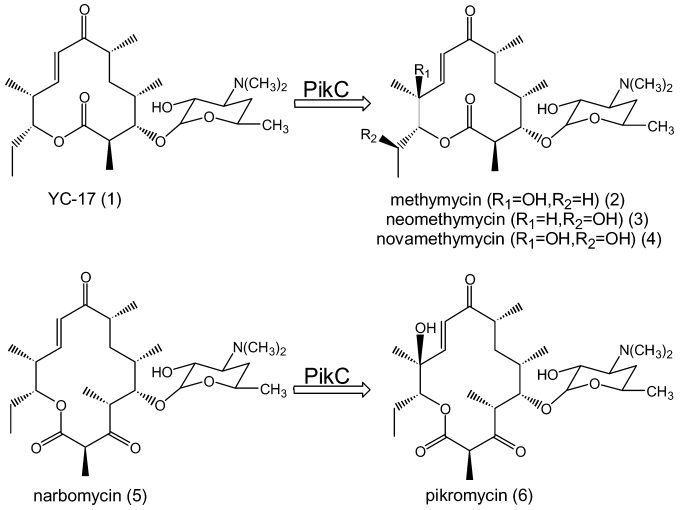

Fig. 2. The structures of the macrolides used as substrates in this study and the function of PikC in pikromycin biosynthesis. PikC can accept 12- and 14-membered ring macrolides as substrates and be active at different positions on the macrolactone system.

the enzyme–substrate complex was performed to determine the possible positioning of alternative substrates within the active site pocket of EryK and potential interactions of them with nearby amino acid residues. Our results demonstrate the interchangeability of EryK with PikC and the potential usefulness of EryK to diversify macrolide structures.

2. Materials and methods

2.1. Bacterial strains and manipulation of DNA and organisms

Escherichia coli DH5α was the host for DNA manipulation, and LB medium was used for *E. coli* propagation. DNA manipulation in *E. coli* and transformation of *E. coli* were performed with standard procedures [9,10]. The routine manipulation of DNA in *E. coli* was performed in LITMUS28 (New England Biolabs). Wild-type *S. venezuelae* ATCC 15439 was used for the production of authentic polyketides. The engineered *S. venezuelae* AX906 mutant strain, in which the gene encoding PikC (*pikC*) was replaced by a kanamycin resistance gene, *aphII*, was a gift from Professor David H. Sherman at the University of Michigan [4]. Protoplast formation and transformation procedure of *S. venezuelae* were performed as described [9]. The polymerase chain reaction (PCR) was performed with *Pfu* polymerase (Stratagene) using conditions recommended by the manufacturer.

2.2. Construction of a plasmid and a mutant for expression of EryK

The plasmid under the control of the *ermE** promoter, pYJ123, was constructed for expression of *eryK* as follows. The expression plasmid was a derivative of pSE34 (derived from pWHM3 [11]), which is a shuttle vector with an *E. coli colE* origin of replication and a high-copy pIJ101 origin [12]. The *eryK* gene was PCR amplified from the genomic DNA of *S. erythraea* using the following oligonucleotide primers (cloning sites in italics); forward 5′-GC*TCTAGA*GGAAACGACCTGCTGCGCG-3′ and reverse 5′-GATGCT*AAGCTT*CTACGCCGACTGCCT-3′. The PCR fragments were isolated, confirmed by DNA sequencing, placed into pSE34 as a *Xba*I–*Hin*dIII fragment and used to transform *S. venezuelae* AX906 to yield *S. venezuelae* YJ010.

2.3. Production and analysis of hydroxylated polyketides

The wild-type *S. venezuelae* strain and mutants were grown on SPA solid medium (1 g yeast extract; 1 g beef extract; 2 g tryptose; 10 g glucose; trace amount of ferrous sulfate; and 15 g agar/L) with an appropriate antibiotic or combination of antibiotics (50 μg/mL kanamycin and 25 μg/mL thiostrepton) for the production and analysis of polyketides. It is known that agar medium production gives more reproducible results than liquid fermentation [7]. After incubation at 30 °C for 3–4 days, the grown culture was sliced and extracted with two volumes of methanol. The extract was washed with water, extracted again with one volume of ethyl acetate, concentrated,

and dissolved with 0.5 mL methanol. A 10 μL aliquot of the solvent extract was analyzed on a reverse-phase C_{18} column (Agilent) with 20–60% acetonitrile in 10 mM ammonium acetate buffer condition using high performance liquid chromatography (HPLC). The flow rate was 1 mL/min, and detection was done with a UV absorbance detector, monitoring peaks at 220 nm. For further characterization, liquid chromatography/mass spectrometry (LC/MS) and mass/mass spectrometry (MS/MS) analyses were performed on a Micromass Quattro LC and on a Finnigan LCQ-DecaXP, respectively, with the electrospray source. The polyketides produced from YJ010 were identified by retention times and mass spectral fragmentation pattern corresponding to those from wild-type *S. venezuelae* in LC/MS and MS/MS, respectively. The relative amount of each compound produced was compared by the peak intensity in the LC/MS.

2.4. Modeling of macrolide conformations and enzyme–substrate complex

The structures of macrolide substrates were modeled using a commercial program Spartan [13]. The molecules were constructed with correct valence according to the general procedure with energy minimized in each step. The conformation of molecules having the lowest energy was subjected to further study. For a molecule in a docking experiment, hydrogens from multivalent atoms were removed without changing the conformation. The coordinates of all atoms were corrected on the basis of the coordinates of 6-deoxyerythronolide B from the crystal structure [14] using the publicly available program SPDBV Viewer [15]. The homology modeling of EryK was carried out by Modeller 6v4 [16]. The best template, EryF, was chosen, which is another cytochrome P450 hydroxylase from the erythromycin biosynthesis gene cluster that hydroxylates 6-DEB at position 6 (Fig. 1). Models were evaluated using different programs (Verify3D, ProsaII and Ival23D) available from the server (http://bioserv.cbs.cnrs.fr/HTMLBIO/frame_valid.html).

For the docking of ligands in the active site of the protein, the AUTODOCK3 set of programs was used [17]. AUTODOCK performs the automated docking of the whole ligand with user-specified dihedral flexibility within a rigid protein binding site. The ligands and macromolecules were prepared for a docking experiment in the usual manner using autodock tools. For ligand preparation, all polar and non-polar hydrogens were added to all atoms in the ligand followed by merging the non-polar hydrogen to ensure that the valences of all atoms were satisfied. The root atom and the planarity of all carbons in the ligand were determined automatically by the program. The number of rotatable bonds was also determined for each ligand. After adding the Gasteiger charges onto the ligand, it was used for the docking experiment. The macromolecule was prepared like the ligand except the polar hydrogens were added followed by addition of Kollman charges on the macromolecule. Since the ligand coordinate is obviously put at the center of the protein active site manually, the grid map was made using the ligand center as the center of grid. For the docking experiment, docking parameters (Simulated Annealing Parameters) were set according to default values with slight modification. All the grids for all atoms present in the ligand (CNHO) were made using autogrid3. Several runs of space search by SA

parameters were carried out to find the best ligand–receptor binding arrangements. A similar experiment was repeated for all ligands.

3. Results

3.1. Hydroxylation of YC-17 and narbomycin by EryK in the S. venezuelae pikC deletion mutant

The potential of EryK to catalyze the hydroxylation of non-natural substrates, both 12- and 14-membered macrolides, was investigated. *S. venezuelae* AX906 mutant strain, which produces only non-hydroxylated polyketides due to the replacement of the gene encoding PikC (*pikC*) by a kanamycin resistance gene, was used as a host for the expression of EryK. Because *S. venezuelae* can produce both 12- and 14-membered macrolides, the catalytic ability of EryK toward **1** and **5** could be tested. The plasmid expressing *eryK*, pYJ123, under the control of the *ermE*∗ promoter [9], was constructed and introduced into *S. venezuelae* AX906, resulting in *S. venezuelae* YJ010.

HPLC analysis of the organic extract from *S. venezuelae* YJ010 indicated that the production of **2**, **3**, and **6** was restored (data not shown). The HPLC chromatogram of *S. venezuelae* YJ010 was identical to that of wild-type *S. venezuelae* when the peaks were scanned for the characteristic UV wavelength and retention times. Subsequently, the analysis of YJ010 extract by LC/MS (Fig. 3) and MS/MS (Fig. 4) clearly showed that **2**, **3**, and **6** were produced. The hydroxylated polyketides by EryK were identified by comparison of their LC/MS chromatograms with those corresponding to wild-type *S. venezuelae* and AX906. We detected the parent ions ($M + H^+$) of **2** and **3** at *m*/*z* 470 and **6** at *m*/*z* 526 by LC/MS analysis of YJ010 extract (Fig. 3). Furthermore, MS/MS analyses of the YJ010 extract (Fig. 4) showed the same characteristic fragmentation patterns as compared with that for the wild-type *S. venezuelae* (data not shown). The protonated **2** and **3** at *m*/*z* 470 and **6** at *m*/*z* 526 showed characteristic dehydrated forms at *m*/*z* 452 and at *m*/*z* 508, respectively, and the fragment ion of a desosamine at *m*/*z* 158.

These analyses indicated that EryK could accept both 12- and 14-membered macrolides as substrates. Although the natural substrates of EryK are all 14-membered macrolides, it has now been shown that a 12-membered macrolide can serve as a substrate. This result reconfirms the previously demonstrated unique advantages of the *S. venezuelae* system for combinatorial biosynthetic production of multiple hybrid molecules by a single recombinant strain. However, compound **4** at *m*/*z* 486 was not detected in the YJ010 extract. The absence of **4** suggests that EryK is not flexible enough to catalyze the hydroxylation of the 12-membered macrolide at two different positions simultaneously. It is also possible that **4** could not be detected due to a low yield. In YJ010, the yield of the hydroxylated macrolides by EryK was approximately 100-fold less than those in wild-type *S. venezuelae*. In summary, although the production yields for alternative substrates for EryK were significantly reduced, EryK has a useful activity toward non-natural substrates and can be utilized to diversify the macrolactones by post-PKS hydroxylation.

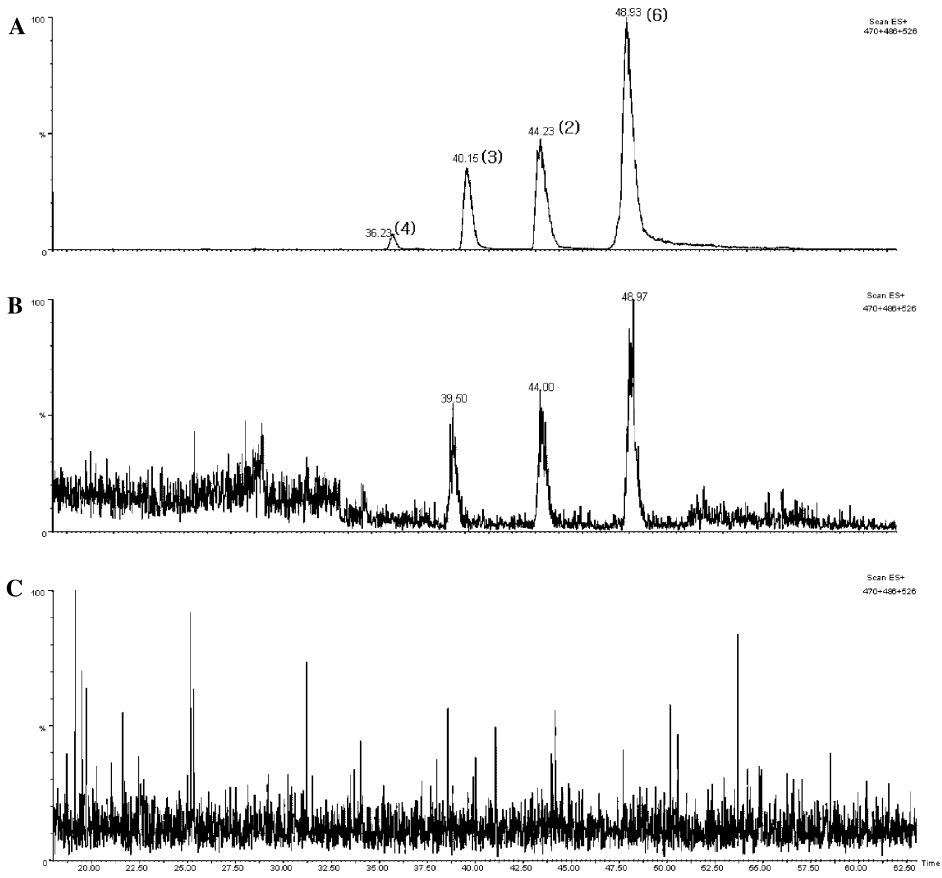

Fig. 3. LC/MS chromatogram of the organic extracts from: (A) wild-type *S. venezuelae*, (B) YJ010, and (C) AX906. LC/MS were selected for *m/z* 470, 486, and 526 corresponding to methymycin/neomethymycin, novamethymycin, and pikromycin, respectively.

3.2. Modeling studies on enzyme-substrate complex

As mentioned above, EryK shows catalytic ability with both 12-membered **1** and 14-membered ring macrolides **5**. The catalytic action on 12-membered ring macrolide may be the result of some flexibility in the active site for the positioning of the smaller substrate [18]. Furthermore, from the three-dimensional models of substrates, the conformations of **1** and **5** in the vicinities of C-10 and C-12, respectively, are nearly identical (Figs. 5A and B).

It is known that the BC loop from most of cytochrome P450s influences the determination of substrate specificity [19]. The length of the BC loop may be directly related to the size of the substrate and selectivity of the enzyme. It has been speculated that the shorter the length of BC, the larger the area is for the substrate binding

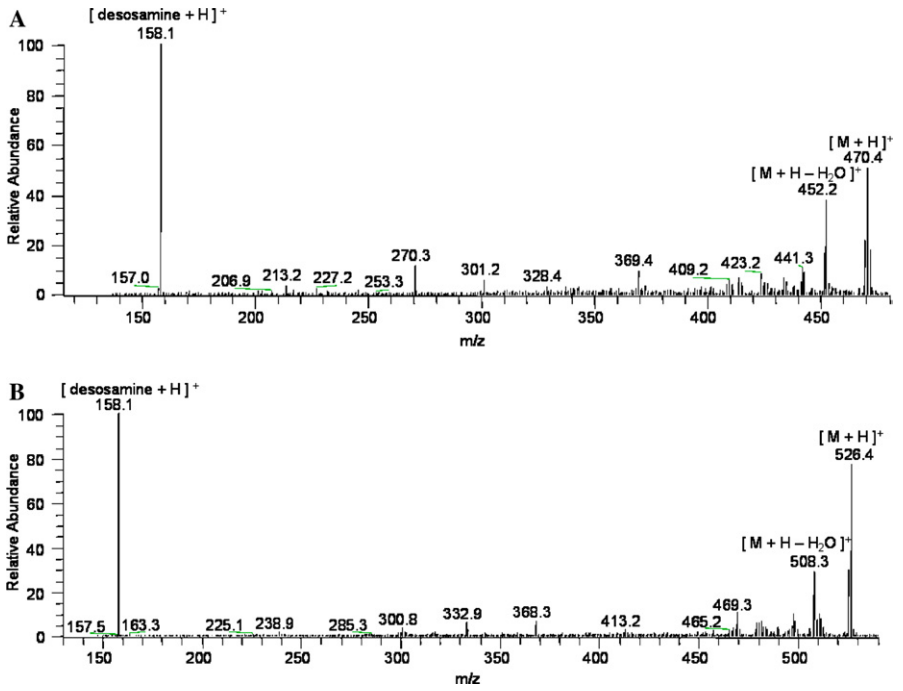

Fig. 4. MS/MS chromatogram of the organic extract of YJ010 showing: (A) methymycin/neomethymycin at *m/z* 470 and (B) pikromycin at *m/z* 526.

such that the active site has more flexibility [20]. The sequence alignment of PikC with other monooxygenases shows that it has a shorter BC loop, which suggests broader substrate specificity [20]. The alignment of EryK with PikC shows that EryK has the similar BC loop length to that of PikC (data not shown), which implies that EryK may be inherently flexible.

Molecular modeling of the active site of EryK was performed to determine the potential interaction of nearby amino acid residues with **1** and **5** and to elucidate how EryK can accept alternative substrates (Figs. 5C and D). For substrate **5**, the hydrophobic residues Ala223, Leu224, Leu225, and Leu226 may form hydrophobic interactions with the methyl group at C-6 and C-8 including C-7 of the macrolide ring (Fig. 5C). Similarly, the methyl group at C-12, where hydroxylation occurs, may interact with Ile230 and the ethyl group at C-13 may fit into the hydrophobic pocket which is formed by residues Leu380, Ile378, and Val379. The carbonyl carbon of the ester may interact with Glu276. The sugar part may accommodate towards the BC loop region so that Asp58 could interact with the OH group whereas the methyl groups from the $N(CH_3)_2$ moiety could interact with residues Ile63 and Val62 from BC loop region. Thr231, which is conserved in most cytochrome P450s and has been postulated to be involved in a proton shuttle network considered important for delivering solvent proton required for the activation of oxygen during the catalytic cycle [21], is found nearby the

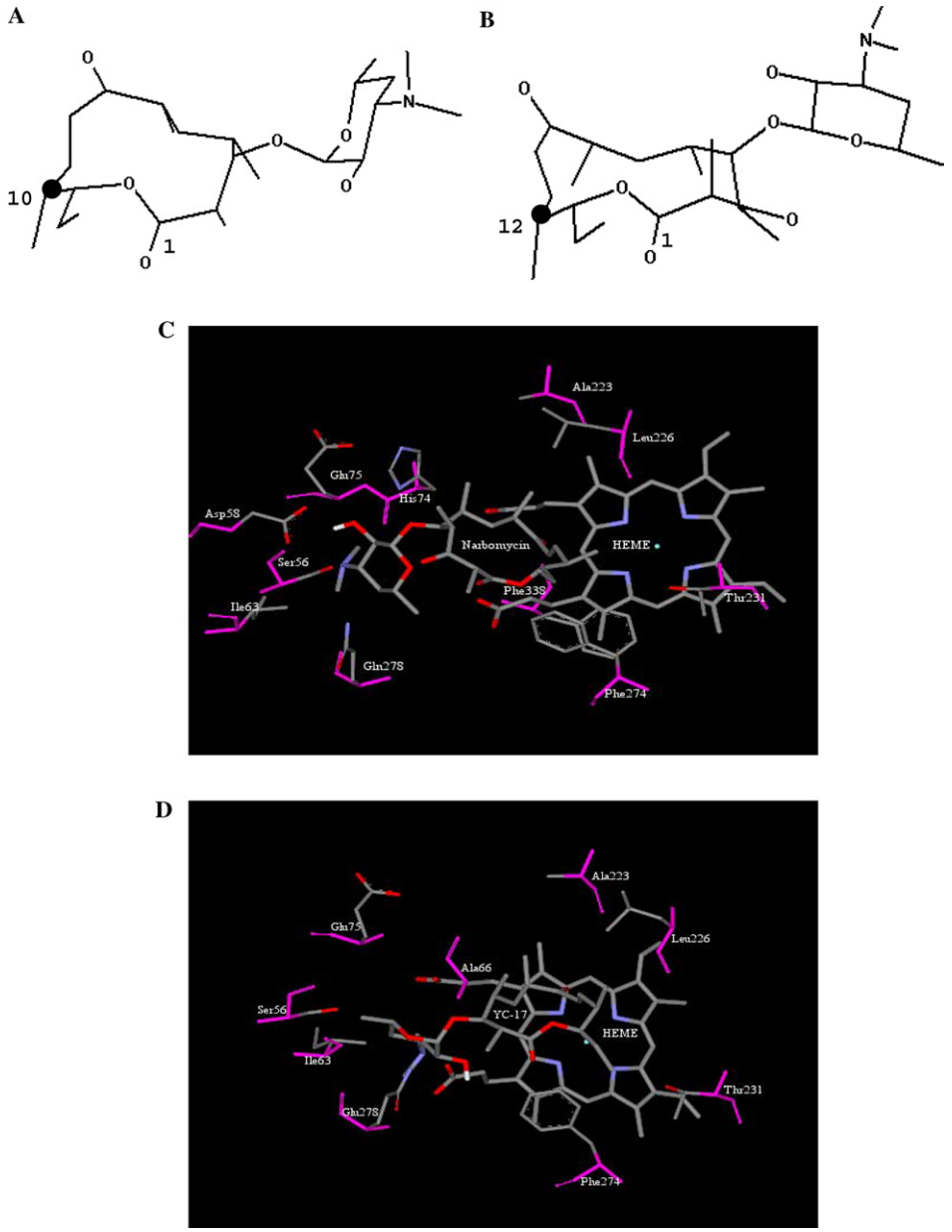

Fig. 5. Three-dimensional structural models of substrates used in this study and complex models with EryK: (A) structural model of YC-17 and (B) that of narbomycin. C-10 of YC-17 and C-12 of narbomycin are marked by black circles. (C) Interaction of narbomycin with the residues at the active site of EryK and (D) interaction of YC-17 with the residues at the active site of EryK. The back bones are shown in pink color and the rest of the residues are shown using the atomic colors (white; carbon bond, red; oxygen, blue; nitrogen, yellow; sulfur, light blue; hydrogen).

hydroxylation position. This analysis indicates that EryK shows broad substrate specificity and thus can accept **1**, a 12-membered ring, as a substrate. The binding of **1** at the active site is similar to that of **5** (Fig. 5D). This analysis would be helpful in revealing potential sites for site-directed mutagenesis in order to engineer novel P450 activities.

4. Discussion

This study shows that the hydroxylation of alternative macrolactones could be catalyzed by the heterologously expressed erythromycin C-12 hydroxylase, EryK. The natural substrates of PikC, **1** and **5**, could be hydroxylated by EryK to yield **2**, **3**, and **6**. This result demonstrates that EryK can catalyze the hydroxylation of a 12-membered ring macrolide as well as 14-membered ring macrolides. Previous to our work, EryK was known to have stringent substrate specificity [3]. However, our results show a certain degree of substrate flexibility for EryK. Furthermore, modeling of the enzyme–substrate complex has elucidated the potential interaction of amino acid residues with substrate.

Over the past years, most studies of the generation of structurally novel macrolides focused on the modification of modular PKS. There have been only a few reports on the production of novel macrolides by the cytochrome P450 monooxygenases [8,22,23]. However, since the identification of the cytochrome P450 monooxygenases with catalytic activities toward alternative polyketides would be very useful for the generation of a variety of unnatural polyketides, extensive efforts should be directed towards the investigation and improvement of the substrate flexibilities of these enzymes. It would also be essential to develop P450 monooxygenases with improved catalytic activity and potentially broad substrate specificity through genetic approaches such as gene shuffling [24] and site-directed mutagenesis. Such studies combined with hybrid modular PKS systems would be useful in developing new therapeutic polyketide macrolides.

The presence of a hydroxyl group at the C-12 position is necessary for ketolide intermediates. Ketolides are derived from 14-membered ring macrolides which possess remarkable in vitro antibacterial activity against macrolide-resistant pathogens [25,26]. It is expected that the substrate-flexibility of EryK investigated in this study would be utilized in the biosynthesis of C-12 hydroxylated ketolide intermediates, as well as in the construction of novel macrolides.

Acknowledgments

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