Structural Diversification of Macrolactones by Substrate-Flexible Cytochrome P450 Monooxygenases

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Abstract: The substrate flexibilities of several cytochrome P450 monooxygenases involved in macrolide biosynthesis were investigated to test their potential for the generation of novel macrolides. PikC hydroxylase in the pikromycin producer *Streptomyces venezuelae* accepted oleandomycin as an alternative substrate and introduced a hydroxy group at the C-4 position, which is different from the intrinsic C-12 hydroxylation position in the natural substrate. This is the first report of C-4 hydroxylation activity of cytochrome P450 monooxygenase involved in the biosynthesis of 14-membered macrolides. EryF hydroxylase from the erythromycin biosynthetic pathway of *Saccharopolyspora erythraea* and OleP oxidase from the oleandomycin biosynthetic pathway of *Streptomyces antibioticus* also showed a certain degree of plasticity towards alternative substrates. In particular, EryF and OleP were found to oxidize a 12-membered macrolactone as an alternative substrate. These results demonstrate the potential usefulness of these enzymes to diversify macrolactones by post-PKS oxidations.

Keywords: cytochrome P450 monooxygenase; hydroxylation; macrolide; *Streptomyces*; substrate flexibility

Introduction

Polyketides are structurally diverse natural products that include a range of useful pharmaceuticals such as antibacterial, antifungal, and anticancer compounds. The emerging resistance of pathogenic bacteria has prompted interest in the discovery of new and structurally modified antibiotics.[1]

The biosynthetic pathways of macrolide antibiotics produced by actinomycetes involve the formation of macrolactones by modular type I polyketide synthase (PKS) in the first stage, followed by specific post-PKS modifying steps such as oxidation, methylation, acylation and glycosylation. The structural diversity of PKS products is further increased by these modification reactions. As these modifications are also often critical for biological activity, a current challenge for combinatorial biosynthesis is to develop approaches that lead not only to novel macrolactones but also to ones that provide fully elaborated structures generated by post-PKS tailoring enzymes.

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One of the post-PKS modification reactions, hydroxylation or other oxidative steps catalyzed by polyketide cytochrome P450 monooxygenases, is often the key step leading to structural diversity and biological activity of macrolide antibiotics. Therefore, identification and engineering of the monooxygenases with activities towards a range of alternative substrates would be helpful to generate novel products with potentially greater or altered biological activities.[2] Although the first historical hybrid product that was obtained by genetic engineering involved an oxygenase,[3] studies on combinatorial biosynthetic works involving these enzymes are still rare.[4] Most of the combinatorial biosynthetic studies with polyketides have focused on early biosynthetic steps catalyzed by PKSs. Moreover, most studies in the field of combinatorial biosynthesis involving cytochrome P450 monooxygenases are related to aromatic polyketides produced by type II PKSs. Little work has been reported on the oxidative modification of the macrolides produced by type I PKS through combinatorial biosynthesis.

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

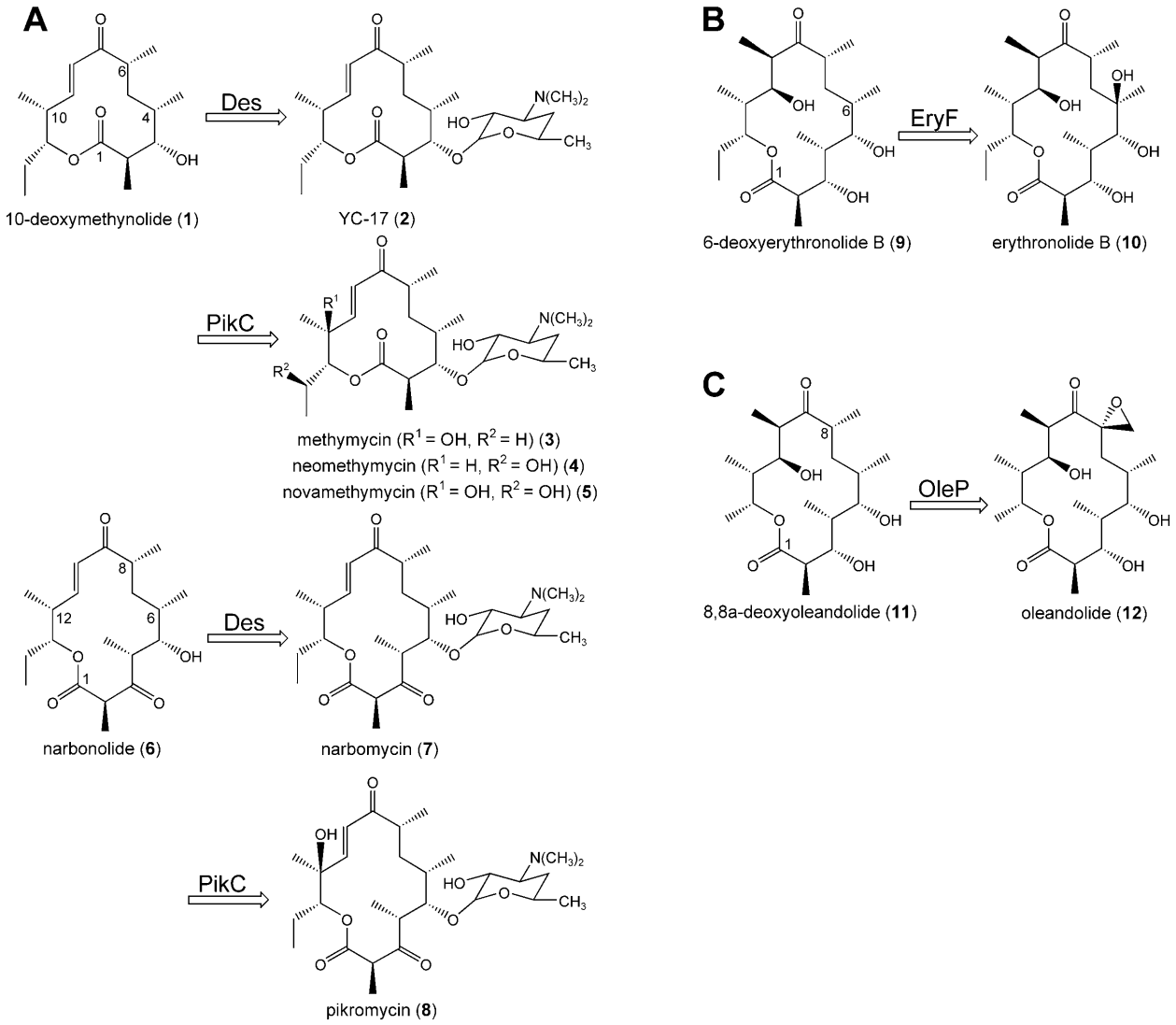

Figure 1. Structures of macrolactone substrates and modified Polyketides by PikC (**A**), EryF (**B**), and OleP (**C**). Des represents all eight enzymes for desosamine biosynthesis and transfer.[13]

The 6-deoxyerythronolide B hydroxylase, EryF, is a cytochrome P450 responsible for the stereospecific C-6 hydroxylation of a 14-membered aglycone, 6-deoxyerythronolide B (**9**), to yield erythronolide B (**10**; Figure 1B).[8] Although changes in the substrate structures resulted in reduced catalytic activity, EryF showed flexibility toward alternative macrolactones.[8c,9] The P450 monooxygenase, OleP, in oleandomycin biosynthesis is involved in the introduction of an epoxy group at C-8/C-8a of 8,8a–deoxyoleandolide (**11**), an aglycone precursor of oleandomycin, yielding oleandolide (**12**; Figure 1C).[10] Recently, some examples were reported indicating that OleP might be tolerant of alternative substrates.[11] Taken together, EryF and OleP are known to be intrinsically flexible towards non-natural substrates to some extent.

In this study, in order to demonstrate the broad substrate flexibility of several cytochrome P450 monooxygenases to novel macrolide biosynthesis, their catalytic abilities toward alternative polyketides were investigated. Our results demonstrate the potential usefulness of these enzymes to diversify macrolactones by post-PKS oxidations.

Results and Discussion

Bioconversion of Oleandomycin by PikC in the *S. venezuelae pikAIV* Deletion Mutant

An engineered *S. venezuelae* HK954 mutant strain, in which the last module of pikromycin PKS (*pikAIV*) was deleted while the gene encoding PikC (*pikC*) remained intact on the chromosome, was previously constructed.[7] In HK954 the biosynthesis of endogenous macrolactone aglycones was blocked, but the *pikC* gene remained functional. Feeding experiments were performed by adding oleandomycin to agar-based cultures of HK954.

A new peak was detected by HPLC analysis from the organic extract of HK954 fed with oleandomycin when compared with the HPLC chromatogram of the extracts from wild-type and HK954 fed with no oleandomycin (data not shown), and the compounds present in this peak were collected. Subsequently, analyses of the collected compounds by LC/MS and MS/MS clearly showed that novel hydroxylated oleandomycin was produced (Figure 2). We detected the hydroxylated compound **14** at $m/z = 704$ (Figure 2C) ahead of oleandomycin (**13**) at $m/z = 688$ (Figure 2B) by LC/MS analysis. This protonated compound gave peaks at $m/z = 560$ for the loss of the oleandrose moiety (*o*) and at $m/z = 403$ for the loss of both oleandrose and desosamine moieties (*d*) (Figure 2A) from the parent ion upon MS/MS spectrometry (Figure 2E). In this MS/MS trace, we detected the peaks at $m/z = 542$ corresponding to the dehydrated compound after the loss of oleandrose, and at $m/z = 385$ and m/z = *367* corresponding to dehydrated compounds after the loss of both sugars. The fragment ion of a desosamine at $m/z = 158$ was not observed under collision activated dissociation according to previous studies.[12] From the MS/MS study of authentic oleandomycin (Figure 2D), all the aforementioned fragmentation patterns were observed. Approximately one percent of the oleandomycin fed to 2 mg/mL was bioconverted to the hydroxylated form, which was compared by the peak intensity in the LC/MS. The expected hydroxylated compound was further purified by preparative HPLC and characterized by ^{1}H NMR. Surprisingly, the catalysis by PikC occurred at the C-4 position of oleandomycin (Figure 2A). The NMR assignments are given in the *Supporting Information*.

To confirm the catalysis of PikC on the substrate, **13** was incubated with PikC purified from an overproducing recombinant strain, *E. coli* BL-21 (DE3)/pYJ106, which was transformed by pikC ligated with pET-28 a–c(+) (Novagen). From the MS/MS analysis, the hydroxylated oleandomycin was detected with the same pattern as the previously described feeding experiment (data not shown). The result of this enzymatic conversion reaction indicated that PikC catalyzed the hydroxylation of **13**.

Molecular modeling of the active site of PikC was performed to determine the potential interaction of nearby amino acid residues with **13** and to elucidate how PikC can accept an alternative substrate and catalyze the hydroxylation at C-4 (Figure 3). The homology modeling of PikC was performed using the best template, EryF. The residues Ala177 and Phe178 may form hydrophobic interactions with the $N(CH_3)_2$ moiety of the desosamine sugar. And the methyl group of desosamine may have a hydrophobic interaction with Val242. The putative interactions of the CH_2 moiety of the epoxide, the hydroxy group of C-4, the methyl group of C-12, and the methyl group of C-2 with Val290, Lys72, Leu93, and Phe353, respectively, were assigned. The methyl group at C-4 may interact with Ile239. Thr247, which is conserved in most cytochrome P450 s and has been postulated to be involved in a proton shuttle network considered important for delivering the solvent proton required for the activation of oxygen during the catalytic cycle,[13] is found nearby the hydroxylation position at C-4. Although this enzyme-substrate complex modeling study cannot provide direct evidence for the catalysis of PikC, it reveals the possible interaction PikC with **13** and provides potential sites for site-directed mutagenesis.

This is the first example that cytochrome P450 monooxygenase inserts an oxygen atom at the C-4 position in 14-membered ring macrolides. Generation of this novel 4-hydroxy-14-membered macrolide, which has hitherto been unknown in natural biosynthetic pathways, demonstrates the unique substrate- and regio-flexibility of PikC hydroxylase for the creation of structurally diverse

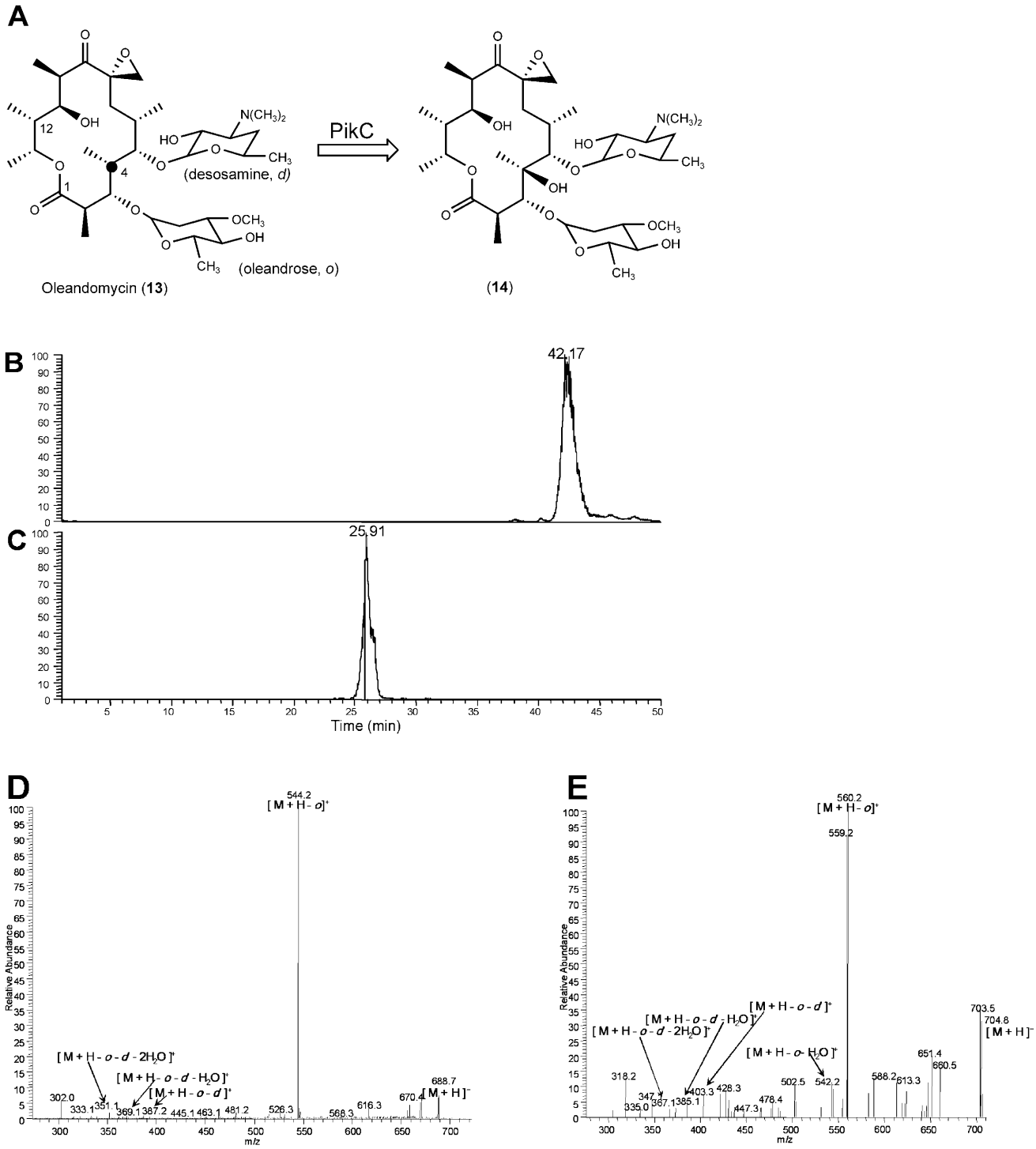

Figure 2. Structures, LC/MS and MS/MS analyses of hydroxylated oleandomycin by PikC. (**A**) Structures of oleandomycin (**13**) and its hydroxylated compound (**14**) by the action of PikC. (**B**) LC/MS selected for $m/z = 688$, corresponding to oleandomycin (**13**). (**C**) LC/MS selected for $m/z = 704$, corresponding to compound **14**. (**D**) MS/MS trace corresponding to oleandomycin (**13**). (**E**) MS/MS trace corresponding to compound **14**.

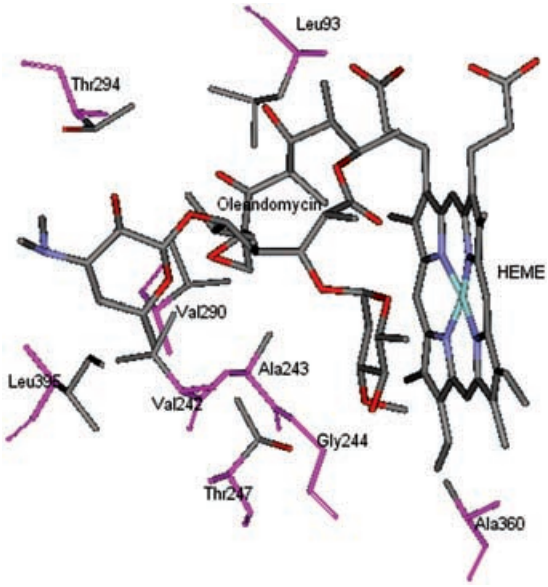

Figure 3. Modeling of oleandomycin in complex with PikC.

macrolides. Moreover, PikC can be an attractive template for engineering novel P450 activities and elucidation of the relationship between structure and substrate specificity for P450 in polyketide biosynthesis.

Analysis of *des* Deletion Strain of *S. venezuelae* as a Host for the Expression of Heterologous Monooxygenases

As the hydroxylation by EryF in erythromycin biosynthesis precedes the attachment of the two sugar moieties, desosamine and mycarose,[9] and the oxidation by OleP can either precede or follow the glycosylation,[11b] an engineered *S. venezuelae* mutant strain which accumulates macrolactone aglycones as substrates for the heterologous oxygenases was required. *S. venezuelae* mutant strain (YJ003[14]) blocked in desosamine biosynthesis by the deletion of the desosamine biosynthetic gene cluster (*des*), located downstream of pikromycin PKS,[15] was previously constructed and used as a host for the expression of EryF and OleP. This system was used in testing catalytic ability of the heterologous oxygenases to modify **1** and **6**. There are two conflicting reports on the sequence of hydroxylation and glycosylation events in methymycin (**3**) and pikromycin (**8**) biosynthesis in *S. venezuelae*. An attempt to detect the oxidation of the aglycones using a cell-free extract of *S. venezuelae* in the presence of appropriate redox cofactors was unsuccessful.[16] This result suggested that the glycosylation of **1** into **2** precedes the hydroxylation by PikC. However, methynolide and neomethynolide, which are the hydroxylated aglycones by PikC before attaching desosamine, were isolated in the mutant strain of *S. venezuelae* in which one of genes, *desI*, involved in desosamine biosynthesis was inactivated, indicating that PikC can catalyze the hydroxylation of non-glycosylated aglycones as well.[17] Analyses by LC/MS and MS/MS from YJ003 extracts have shown that **1** and **6** were accumulated as major metabolites and the oxidation of the aglycones by PikC occurred to yield not only methynolide and neomethynolide but also pikronolide (data not shown) to a detectable extent, suggesting that the pathways for oxidation of 12- and 14-membered macrolactones by PikC are parallel with glycosylation. Despite the hydroxylation of **1** and **6** by endogenous PikC hydroxylase in YJ003, the novel oxidized aglycones should be distinguishable from the endogenously hydroxylated aglycones based on the difference between the relative retention times by LC/MS. Therefore, the genetic background of YJ003 would provide sufficient amounts of **1** and **6** as substrates for heterologous oxygenases and simplify the detection of novel oxidized compounds generated by heterologously expressed oxygenases.

Oxidation of 10-Deoxymethynolide and Narbonolide by EryF and OleP in the *S. venezuelae des* Deletion Mutant

The potential of EryF and OleP to catalyze the oxidation of both 12- and 14-membered macrolactones was investigated. Two plasmids expressing *eryF* and *oleP* (pYJ119 and pYJ118, respectively) under the control of the *ermE** promoter[18] were constructed and introduced into YJ003, respectively. In the organic extracts from both transformants (YJ003/pYJ119 and YJ003/pYJ118), new peaks were detected on LC/MS with predicted molecular weights that have significantly different retention times from those of methynolide/neomethynolide and pikronolide. The compounds present in these new peaks were collected and further characterized by MS/MS (Figures 4 and 5). Surprisingly, analyses of the culture supernatants indicated that EryF and OleP could accept both 12- and 14-membered macrolactones as substrates. Although the natural substrates of EryF and OleP are all 14-membered macrolactones, it was shown that 12-membered macrolactones can also serve as substrates. These results reconfirm the previously demonstrated unique advantages of the *S. venezuelae* system for combinatorial biosynthetic production of multiple hybrid molecules by a single recombinant strain.[7]

LC/MS (data not shown) and MS/MS analyses of the predicted compounds provided clear information on catalysis of EryF on both **1** and **6** (Figures 4B and 4C). We detected the parent ions ($M+H^+$) of the expected hydroxylated compounds of 12-membered (**15**) and 14-membered (**16**) (Figure 4A) macrolatones at $m/z =$ 313 and 369, respectively. From each MS/MS analysis of 12- and 14-membered macrolactones, two characteristic dehydration products were detected. Prior to the hydroxylation by EryF, there is only one dehydration

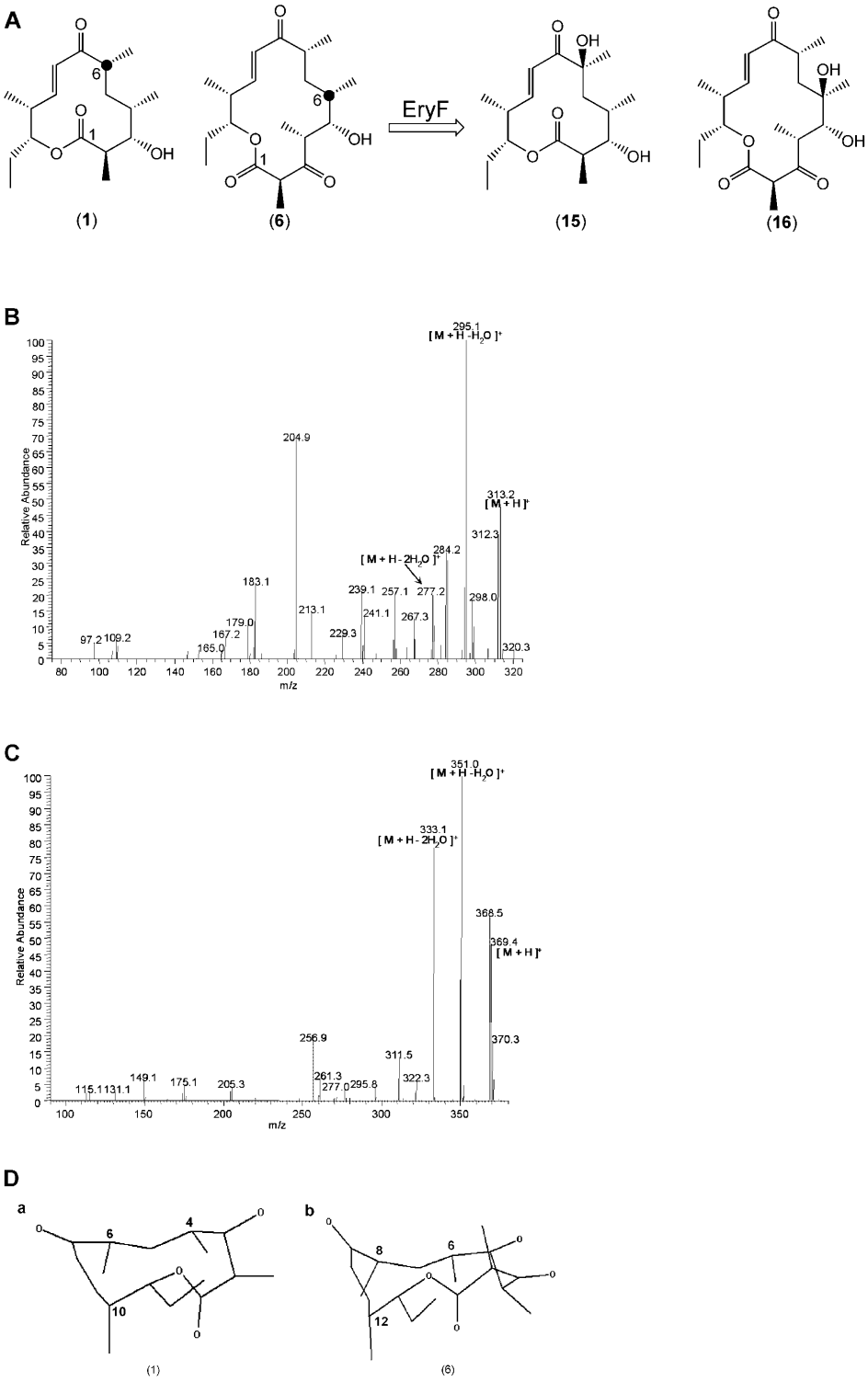

Figure 4. Structures and MS/MS analyses of hydroxylated 10-deoxymethynolide and narbonolide by EryF. (**A**) Structures of 10-deoxymethynolide (**1**) and narbonolide (**6**) and their hydoxylated compounds **15** and **16** by EryF. (**B**) MS/MS trace corresponding to compound **15** produced from *S. venezuelae* YJ003/pYJ119. (**C**) MS/MS trace corresponding to compound **16** produced from *S. venezuelae* YJ003/pYJ 119. (**D**) Structural models of 10-deoxymethynolide **1** (**a**) and narbonolide (**6**) (**b**).

site in both **1** and **6**. MS/MS analyses of **1** and **6** have shown the only one dehydration pattern (data not shown). Therefore, this successive dehydration was due to the hydroxylation of these aglycones by EryF. In the previous work, although it was speculated that **6** was bioconverted to hydroxylated products by EryF,

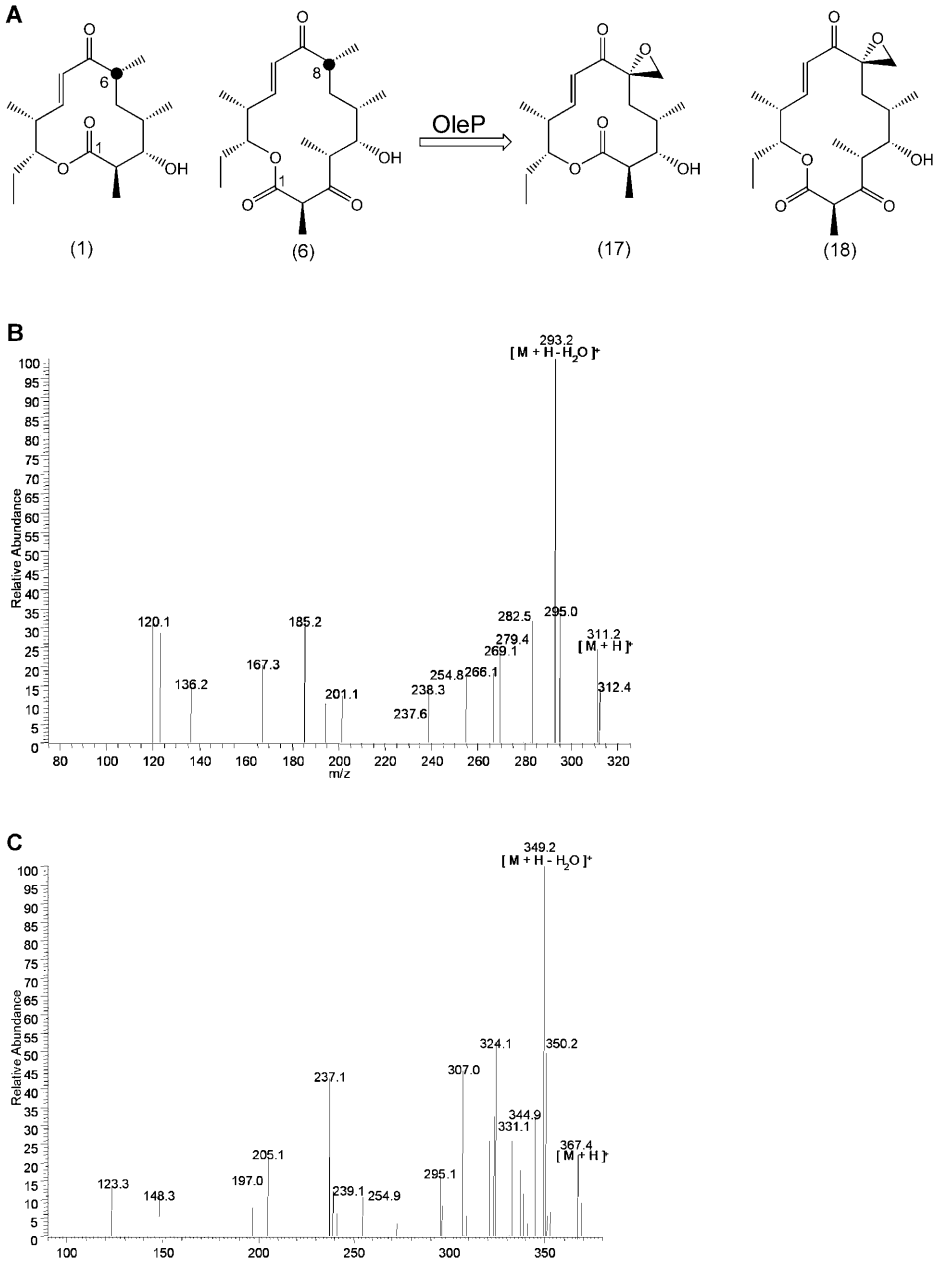

Figure 5. Structures and MS/MS analyses of oxygenated 10-deoxymethynolide and narbonolide by OleP. (**A**) Structures of 10-deoxymethynolide (**1**) and narbonolides (**6**) and their expected oxygenated compounds **17** and **18** by OleP. (**B**) MS/MS trace corresponding to compound **17** produced from *S. venezuelae* YJ003/pYJ118. (**C**) MS/MS trace corresponding to compound **18** produced from *S. venezuelae* YJ003/pYJ118.

the identity of this compound was not determined.[9] Our work has provided supportable results that **6** could be accepted as a substrate by EryF. More interestingly, the 12-membered macrolactone **1** was shown to be converted to the hydroxylated macrolactone by EryF. The ability of catalysis on a 12-membered ring macrolactone may be the result of some looseness of the active site in the positioning of the smaller macrolactone as compared to the larger one.[4] The hydroxylated compounds by EryF were partially purified and characterized by ^{1}H NMR. Compound **6** was found to be hydroxylated at the C-6 position to yield **16**. Although the conformation at C-4 of **1** is very similar to that at C-6 of **6** in the three-dimensional models of **1** and **6** (Figure 4D), compound **1** was hydroxylated at C-6 to yield **15**, not at the C-4 position. This result with the case of PikC indicates that the catalytic position of P450 s in the alternative substrates is possibly diverse. The NMR assignments are given in the *Supporting Information*.

Analyses of the culture extracts using LC/MS (data not shown) and MS/MS revealed that both **1** and **6** were accepted as substrates by OleP (Figures 5B and

Table 1. Natural and unnatural substrates of the enzymes and novel hydroxylated compounds in this study.

	Natural substrate	Unnatural substrate in this study	Novel hydroxylated compound in this study
PikC	YC-17 (**2**), narbomycin (**7**)	oleandomycin (**13**)	(**14**)
EryF	6-deoxyerythronolide B (**9**)	10-deoxymethynolide (**1**), narbonolide (**6**)	(**15**), (**16**)
OleP	8,8a–deoxyoleandolide (**11**)	10-deoxymethynolide (**1**), narbonolide (**6**)	(**17**), (**18**)

5C). The parent ions ($M+H^+$) of the epoxidated 10-deoxymethynolide derivative **17** and narbonolide derivative **18** (Figure 5A) at $m/z=311$ and $m/z=367$, respectively, were detected and the characteristic dehydration patterns were observed. Further MS/MS analysis of novel compounds in our experiment indicated the presence of the 12- and 14-membered dihydroxy derivatives as well (data not shown). The presence of the diol in our experiment could be explained by the formation of the epoxide and subsequent hydrolysis by endogenous enzyme or spontaneously. It was speculated that oxidation by OleP occurred only after attachment of the neutral sugar.[19] However, recent studies have shown that OleP can also catalyze the epoxidation of several non-natural substrates prior to glycosylation,[11] which revealed that the catalytic pathways of OleP are parallel. Our results gave further evidence that OleP can catalyze the oxidation of non-glycosylated macrolactones. From the comparison by the peak intensity in the LC/MS, the conversion yields of novel compounds by EryF and OleP were approximately twenty-fold and one hundred-fold, respectively, less than those of methynolide, neomethynolide, and pikronolide by PikC in YJ003. Although the regio- and stereochemistry of the introduced epoxide group were not determined due to their low conversion yield, the modified structure of **6** by OleP in Figure 5A was deduced from its intrinsic catalytic specificity. Previous work has also shown that **9** was bioconverted to the 8,8a–epoxy- or 8,8a–dihydroxy derivative by OleP.[11a] However, as shown in the case of PikC and EryF, it is possible that the oxidation in **1** might occur at an unexpected position.

Conclusions

It was demonstrated that structurally novel macrolides can be generated by several substrate-flexible cytochrome P450 monooxygenses. The cytochrome P450 monooxygenases, PikC, EryF, and OleP expressed in the engineered mutants of *S. venezuelae* resulted in the production of structurally novel macrolactones modified by hydroxylation or oxidation. Table 1 summarizes the results of this study. Interestingly, PikC modifies an alternative substrate at a different position from its natural substrate, and EryF and OleP can accept smaller macrolactones than their natural substrates as alternative substrates. Furthermore, modeling of the PikC-substrate complex has been conducted to elucidate the potential interaction of amino acid residues with the substrate. In the past years, most studies on the generation of structurally novel macrolides were related to the modification of modular PKS and there have been only a few reports on the production of novel elaborated macrolides by cytochrome P450 monooxygenases. However, since the identification of the cytochrome P450 monooxygenases with catalytic activities towards alternative macrolactones would be very useful for the generation of a variety of non-natural polyketides,[2] extensive efforts should focus on investigating and improving the substrate flexibility of these enzymes. Although the P450 monooxygenases described above possess noticeable substrate flexibility towards alternative substrates, the catalytic efficiencies of P450 s towards alternative substrates are significantly lower as compared with their intrinsic productivities. Therefore, it is essential to develop P450 monooxygenases with improved catalytic activity and potentially broad substrate specificity through genetic approaches such as gene shuffling[20] and mutagenic PCR.[21] Also, substrate-enzyme complex modeling through protein crystallization[22] would provide precise information in the engineering the monooxygenases. Particularly, such studies combined with hybrid modular PKS systems would also support their use in searching for new therapeutic polyketide macrolides.

Experimental Section

Bacterial Strains and Growth Conditions

S. venezuelae ATCC 15439 was used for the construction of *pikAIV*[7] and the *des* deletion mutant.[14] *S. venezuelae* transformants were selected on R2YE[18] agar plates by overlaying with appropriate antibiotics, apramycin (0.5 mg/mL), kanamycin (1 mg/mL), or thiostrepton (0.5 mg/mL). For propagation of *S. venezuelae*, SGGP[23] liquid medium was used. *Escherichia coli* DH5α was the host for DNA manipulation, and LB medium was used for *E. coli* propagation.

Manipulation of DNA and Organisms

DNA manipulation in *E. coli* and transformation of *E. coli* were performed with standard procedures.[24] The routine manipulation of DNA in *E. coli* was performed in LITMUS28 (New England Biolabs). Protoplast formation and transformation procedures of *S. venezuelae* were performed as described.[18] The polymerase chain reaction (PCR) was performed

with *Pfu* polymerase (Stratagene) using conditions recommended by the manufacturer.

Construction of Plasmids for Cytochrome P450 Monooxygenases Expression

Two plasmids under the control of the *ermE** promoter were constructed for expression of *eryF* and *oleP* (pYJ119 and pYJ118, respectively) in YJ003 as follows. The expression plasmid were derivatives of pSE34 (derived from pWHM3[25]), which is a shuttle vector with an *E. coli colE* origin of replication and a high-copy pIJ101 origin.[26] The *eryF* gene was PCR amplified from the genomic DNA of *S. erythraea* using the following oligonucleotide primers; forward carrying *Bam*HI site (underlined) 5′-GGGGATCCTCGCTGGCGGCGGAAAGCT-3′ and reverse carrying *Hind*III site (underlined) 5′-GCAGCAAGCTTCTCATCCGTCGAGCCGCAC-3′. For expression of *oleP*, the *oleP* gene was PCR amplified from the genomic DNA of *S. antibioticus* using the following oligonucleotide primers; forward carrying *Bam*HI site (underlined) 5′-GCGGATCCGGAACCACCTCCAGCAAAG-3′ and reverse carrying *Hind*III site (underlined) 5′-GGCAAGCTTTCACCAGGAGACGATCTG-3′. The PCR fragments were isolated, ligated with the pSE34 and used to transform YJ003.

Production and Analysis of Novel Polyketide Analogues

HK954 was cultured on SPA solid medium (1 g of yeast extract; 1 g of beef extract; 2 g of tryptose; 10 g of glucose; trace amount of ferrous sulfate; 15 g of agar/L) at 30 °C for 3–4 days under kanamycin selection. It is known that agar medium production gives more reproducible results than liquid fermentation.[7] The sporulating HK954 was fed with oleandomycin (Sigma) to 2 mg/mL and incubated at 30 °C for 5 days. The grown cultures were sliced and extracted with two volumes of methanol. The extracts were washed with water, extracted again with one volume of ethyl acetate, and concentrated with methanol. 10 μL aliquots of the solvent extracts were analyzed on a reverse-phase C_{18} column (Agilent) with 20–60% acetonitrile in 10 mM ammonium acetate buffer condition over 60 min using high performance liquid chromatography (HPLC) analysis. The flow rate was 1 mL/min, and detection was done with a UV absorbance detector, monitoring peaks at 210 nm for the hydroxylated oleandomycin sample and at 220 nm for oxygenated samples by EryF and OleP. New peaks with absorbance spectra different to that of HK954 fed with no oleandomycin were collected. To obtain further structural information, liquid chromatography/mass spectrometry (LC/MS) and mass/mass spectrometry (MS/MS) analyses were performed on a Finnigan LCQ-DecaXP with the electrospray source. The novel polyketides were identified by mass spectral fragmentation patterns corresponding either to the predicted products or to known standards.

YJ003 harboring the appropriate expression vectors were grown on SPA solid medium containing kanamycin (50 μg/mL) and thiostrepton (25 μg/mL) for the production and analysis of macrolactones. After incubation at 30 °C for 3–4 days, the cultures of these transfomants were extracted and analyzed as described earlier.

The crude extracts were purified for ^{1}H NMR analysis on a Capcell pak C_{18} column (10 × 250 mm, Shiseido) by preparative reverse phase HPLC using a gradient from 20 to 50% acetonitrile in aqueous solvent consisting of 20% acetonitrile and 5 mM ammonium acetate buffer at pH 8.0 over 30 min, then 100% acetonitrile for 20 min with a 2 mL/min flow rate. The partially purified compounds were subjected to analysis by ^{1}H NMR and COSY.

Purification of PikC and Enzymatic Conversion

The *pikC* gene was PCR amplified from the genomic DNA of *S. venezuelae* using the following oligonucleotide primers; forward carrying *Nde*I site (underlined) 5′-GGAGTTCCATATGCGCCGTACCCAGCAG-3′ and reverse carrying *Hind*III site (underlined) 5′-CGTAAGCTTGACGTGCGGGTTCAACC-3′. The PCR fragments were isolated and ligated with the pET-28a–c(+) (Novagen) resulting in pYJ106. This plasmid was used to transform *E.coli* BL-21 (DE3). The purification of PikC was performed with Ni-NTA agarose (QIAGEN) using conditions recommended by the manufacturer.

The reaction mixture for enzymatic conversion consisted of 2 μM PikC, 4 μM spinach ferredoxin (Sigma), 0.1 unit of ferredoxin-NADP$^+$ reductase (Sigma), 1 mM NADPH (Sigma), and 1 mM oleandomycin (Sigma) in a total volume of 1 mL of 100 mM potassium phosphate buffer (pH 7.2). The reaction proceeded at 37 °C for 1 h and terminated by extraction with ethyl acetate two times.

Modeling of Macrolide Conformations and Enzyme-Substrate Complex

The conformations of substrates were modeled using the commercial program Spartan.[27] The molecules were constructed with the correct valencies 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, the hydrogen atoms from multivalent atoms have been 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[28] using the publicly available program SPDBV Viewer.[29]

The homology modeling of PikC has been carried out by Modeller 6v2.[30] The best template, EryF, has been chosen, which has 46% identity with PikC. Models were evaluated using different programs (Verify3D, ProsaII and Ival23D) from the server (http://bioserv.cbs.cnrs.fr/HTML BIO/frame valid.html).

For the docking of ligands in the active site of the protein, the AUTODOCK3[31] set of programs have been used. 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 according to the usual procedure 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 automati-

cally by the program. The number of rotatable bonds was also determined for each ligand. After adding the Gasteiger charges on each ligand, it was used for the docking experiment. On the other hand, the macromolecule has been prepared similar to the ligand except that only the polar hydrogen atoms were added followed by addition of Kollman charges on the macromolecule. Since the ligand coordinate was obviously put at the center of the protein active site manually, the grid map was made using the ligand center as the center of the grid. For a docking experiment, docking parameters (Simulated Annealing Parameters) have been set according to default values with slight modification. All the grids for all atoms present in the ligand (CNHO) have been 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.

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