Published in final edited form as:

Chembiochem. 2014 October 13; 15(15): 2289–2296. doi:10.1002/cbic.201402306.

Synergistic Actions of Tailoring Enzymes in Pradimicin Biosynthesis

Kandy Napan^{[a],†}, Dr. Shuwei Zhang^{[a],†}, Whitney Morgan^[a], Thomas Anderson^[b], Prof. Dr. Jon Y. Takemoto^[b], and Prof. Dr. Jixun Zhan^[a]

Jixun Zhan: jixun.zhan@usu.edu

^[a]Department of Biological Engineering, Utah State University, 4105 Old Main Hill, Logan, UT 84322, Fax: 435-797-1248

^[b]Department of Biology, Utah State University, 5305 Old Main Hill, Logan, UT 84322

Abstract

Three key tailoring enzymes PdmJ, PdmW and PdmN in pradimicin biosynthesis were investigated. PdmW was characterized as the C-6 hydroxylase by structural characterization of the corresponding product 6-hydroxy-G-2A. The efficiencies of the C-5 and C-6 hydroxylations catalyzed respectively by PdmJ and PdmW were low when they were expressed individually with the early biosynthetic enzymes that form G-2A. When these two cytochrome P450 enzymes were co-expressed, a dihydroxylated product 5,6-dihydroxy-G-2A was efficiently produced, indicating that these two enzymes work synergistically in pradimicin biosynthesis. Heterologously expressed PdmN in *Streptomyces coelicolor* CH999 converted G-2A to JX137a by ligating a unit of D-alanine to the carboxyl group. PdmN has relaxed substrate specificity toward both amino acid donors and acceptors. Through combinatorial biosynthesis, a series of new pradimicin analogues were produced.

Keywords

pradimicin; cytochrome P450 hydroxylase; synergistic actions; polyketides; combinatorial biosynthesis

Introduction

The pradimicin family of natural products was first isolated in 1988 from the soil bacterium, *Actinomadura hibisca* P157-2 (ATCC 53557).^[1] Pradimicin A (**1**, Scheme 1) is a major product from this strain. Its structure features a benzo[*a*]naphthacene quinone core, a D-alanine moiety, and a disaccharidemoiety consisting of 4,6-dideoxy-4-methylamino-D-galactose and D-xylose.^[2] *In vitro* and *in vivo* studies indicated that **1** is a promising lead compound for development of antifungal and antiviral drugs.^[3–5] It has shown significant antifungal activity against many clinically important pathogens such as *Candida*,

Correspondence to: Jixun Zhan, jixun.zhan@usu.edu.

[†]These authors contributed equally to this work.

Supporting information for this article is available

Aspergillus, *Microsporum*, *Penicillium*, and *Sporothrix*.^[2, 4] It is also a virus entry inhibitor and possesses selective activities against viruses such as the human immunodeficiency virus (HIV).^[6] Recent studies on the mechanism of action of **1** highlighted its lectin-like property in a Ca²⁺ dependent manner.^[7, 8] The carboxyl group of D-alanine at C-18 and the CDE rings of **1** form the cavity which interacts with the D-mannopyranoside found in the surface envelope of several species of yeasts and in the glycoprotein gp120 of HIV.^[9] This unique mechanism of action could help meet the challenges of multidrug resistant yeast and provide the basis for new therapeutic approaches to stopping the entry of HIV into TCD4+ cells.^[6, 10]

As the only family of natural carbohydrate-binding antibiotics with nonpeptidic skeleton (lectin mimics), pradimicins are a promising group of drug leads. To develop effective anti-infective drugs, BMY-28864 (**2**, Scheme 1) was previously semi-synthesized to have improved water solubility (>40 g L⁻¹ at pH 2) and increased bioactivities than **1**^[11] and its analogue BMS-181184. However, it was found to increase hepatic transaminase level in healthy humans, leading to the discontinuation of the clinical trial of this compound.^[12] New analogues of **1** with improved biological activities and safety profiles are thus needed for new drug development.

1 belongs to a large family of natural products called polyketides. It is assembled via a type II polyketide biosynthetic pathway. Type II polyketide biosynthetic pathways consist of a series of discrete enzymes that work collaboratively to synthesize diverse molecules, such as the broad-spectrum antibiotic oxytetracycline.^[13] Unlike modular type I polyketide synthases (PKSs) and simple type III PKSs, type II PKSs form multi-enzyme complexes to act as an efficient assembly line. For example, ketosynthases (KS) and chain length factor (CLF) form a heterodimer to precisely control the chain elongation. Together with a dissociated acyl carrier protein (ACP), they synthesize a linear poly- β -ketone chain to the desired length, and thus they are called minimal PKS. The nascent polyketide chain is then cyclized and modified by tailoring enzymes to yield final products.

Combinatorial biosynthesis is an attractive approach to generating new molecules. In order to rationally engineer the biosynthesis of new pradimicin analogues, it is important to functionally characterize the involved enzymes, understand their catalytic properties, and gain insights into their synergistic actions in the biosynthetic process. The biosynthetic gene cluster of **1** has been previously reported.^[14] Two ABC transporter genes, *pdmR1* and *pdmR2*, were found to act as resistance genes. While overexpression of these genes in *A. hibisca* P157-2 led to elevated production of **1**, their introduction into pradimicin-sensitive microorganisms such as *Streptomyces venezulae* increased their tolerance to **1**.^[15]

In our previous work, we functionally characterized and identified the early steps in the biosynthetic pathway of **1**. The minimal PKS, which consists of PdmA (KS), PdmB (CLF) and PdmC (ACP), assembles the nascent dodecaketide backbone from 12 units of malonyl-CoA. This 24-carbon poly- β -ketone chain is then processed by immediate tailoring enzymes including PdmD, PdmK, PdmL, PdmH and PdmG to give rise to an important benzo[*a*]naphthacene quinone intermediate G-2A (**3**, Scheme 1) with an average yield of 48.8 ± 3.2 mg L⁻¹ in the heterologous host *Streptomyces coelicolor* CH999.^[16] PdmN was

characterized as an amino acid ligase that ligates a molecule of D-alanine to the carboxyl group at C-16.^[17] A new pradimicin analogue JX137a (**4**, Scheme 1) was synthesized at $31.9 \pm 3.1 \text{ mg L}^{-1}$ when PdmN was co-expressed with the enzymes that form **3**. This enzyme can also accept D-serine as the donor to yield another derivative JX137s. PdmJ was characterized both *in vivo* and *in vitro* as a cytochrome P450 (CYP) enzyme that specifically introduces a hydroxyl group at C-5. Hydroxylation of **3** by PdmJ yielded JX134 (**5**, Scheme 1).^[17, 18] This hydroxyl group serves as a site for the subsequent glycosylations. Given the essential roles of the D-alanine and sugar moieties in the biological activities of **1**, it is important to understand the catalytic properties of these tailoring enzymes and how they work together in pradimicin biosynthesis.

In this work, the three key tailoring enzymes, including PdmW, PdmJ and PdmN, were further investigated. PdmW was determined to be the C-6 hydroxylase that acts synergistically with the C-5 hydroxylase, PdmJ. PdmN was discovered to possess broad acceptor substrate specificity, and a series of new pradimicin analogues was generated through combinatorial biosynthesis.

Results and Discussion

Characterization of PdmW as the C-6 hydroxylase

To identify the function of PdmW, we constructed a plasmid pKN92 (Table 1) that consists of *pdmW* and the G-2A-forming genes using an *Escherichia coli*/*Streptomyces* shuttle vector pRM5.^[19] This vector contains a ColE1 replicon for replication in *E. coli* and a SCP2 replicon for *Streptomyces*. For comparison, a similar plasmid pKN35 was constructed containing the same set of genes except that *pdmW* was replaced by *pdmJ* (Table 1). These two plasmids were introduced into *S. coelicolor* CH999 by protoplast transformation and correct transformants were grown on R5 agar supplemented with thiostrepton for product analysis. The products were extracted with ethyl acetate-methanol-acetic acid (89:10:1) and analyzed by LC-MS. As expected, *S. coelicolor* CH999/pKN35 (trace i, Figure 1) produced **3** as the major product and the hydroxylated analogue **5** as a minor metabolite ($1.7 \pm 0.2 \text{ mg L}^{-1}$), which is consistent with our previous discovery.^[17] In contrast, *S. coelicolor* CH999/pKN92 (trace ii, Figure 1) produced a different minor compound **6** ($1.4 \pm 0.4 \text{ mg L}^{-1}$) in addition to **3**. This compound has a UV absorption spectrum similar to that of **3** (Figure S1), indicating that they share a similar structure. ESI-MS revealed that its molecular weight is 448 (Figure S2) and 16 mass units larger than **3**, suggesting that it is a hydroxylated product of **3**. Since **5** and **6** have different retention times, it was proposed that the latter has a hydroxyl group at a position other than C-5. We had previously detected a similar compound produced by *S. coelicolor* CH999/pJX152 possessing the same set of genes, but it was not structurally characterized due to the lack of sufficient amounts of pure compound.^[17] In this work, we scaled up the culture to 6 L, and purified 6.0 mg of **6** for NMR analysis.

The ¹³C NMR spectrum of **6** revealed that it has 24 carbons, confirming that it is a dodecaketide synthesized by the pradimicin biosynthetic enzymes. Compared to **3**, which has two CH₂ groups at C-5 and C-6, respectively, **6** has a single CH₂ group but contains a new oxygenated CH group. This suggested that the hydroxyl group was introduced at C-5 or C-6. Because **5** is already identified as 5-hydroxy-G-2A, **6** was deduced to be a C-6

hydroxylated derivative of **3**. Further 2D NMR analysis of **6** (Figure 2) revealed the ^1H - ^1H COSY correlation of the oxygenated CH signal to 5-CH₂ and its HMBC correlation to C-4a, thus confirming that **6** is 6-hydroxy-G-2A, which was named KN92. The proton and carbon signals were assigned and are presented in Tables 2 and 3, respectively. We then prepared the MTPA esters of **6** to determine the configuration of C-6. As shown in Figure 3, the δ values revealed that C-6 has *R* configuration. Full structural characterization of **6** confirmed that PdmW is the dedicated C-6 hydroxylase in pradimicin biosynthesis. Using the same method, we also determined the absolute configuration at C-5 of **5** to be *R* (Figure 3) which was not determined previously. We also recorded the ^{19}F NMR data of these Mosher esters, which supported the determination of the absolute configurations of **5** and **6** (Figure S3).^[20] Therefore, this work allowed the characterization of PdmW as the C-6 hydroxylase and the *R* configuration at C-5 in **5** and C-6 in **6**, which are consistent with the reported absolute configurations at the same positions in the final product **1**.

Synergistic actions of two CYP hydroxylases in pradimicin biosynthesis

As described above, PdmJ and PdmW were characterized as CYP hydroxylases that introduce a hydroxyl group to C-5 and C-6 of the pradimicin structure, respectively. However, the yields of **5** and **6** were low, as shown in traces i and ii in Figure 1. The major metabolite in both *S. coelicolor* CH999/pKN35 and *S. coelicolor* CH999/pKN92 was still **3**, the benzo[*a*]naphthacene quinone intermediate formed by the biosynthetic genes *pdmABCDGHLK* in pJX120. Apparently, these CYP enzymes when expressed individually were not efficient in converting **3** into the corresponding monohydroxylated products. To test whether these two CYP hydroxylases work collaboratively, plasmid pKN90 that contains *pdmABCDGHLK* and both *pdmJ* and *pdmW* was constructed. Co-expression of these two hydroxylases in *S. coelicolor* CH999 resulted in the production of **5**, **6**, and a new product **7** ($18.7 \pm 1.7 \text{ mg L}^{-1}$) (trace iii, Fig. 1). This compound has a shorter retention time (9.6 min) than **5** and **6** on a reverse-phase C18 HPLC column, suggesting that it is more polar than these two compounds. The UV absorptions of **7** (Figure S1) indicated that it is an analogue of **1**. ESI-MS spectra of **7** showed the ion peaks including $[\text{M}-\text{H}]^-$ at m/z 462.9 (Figure S2) and $[\text{M}+\text{H}]^+$ at m/z 465.0, indicating that it has a molecular weight of 464. This is 16 mass units larger than **5** and **6**, indicating that **7** is a dihydroxylated product. The ^1H NMR spectrum of **7** exhibited 12 proton signals including three hydroxyl protons, four phenolic protons, two oxygenated CH protons, and a CH₃ group (Table 2).

The ^{13}C NMR spectrum of **7** showed 24 carbons including two carbonyl groups, one carboxyl group, eighteen phenolic carbons, one methyl group and two oxygenated CH groups (Table 3). The NMR data is very similar to those of **3**, **5** and **6**. The major difference is that **7** does not have any CH₂ groups, suggesting that both C-5 and C-6 were hydroxylated. Unlike the monohydroxylated compounds **5** and **6**, the ^1H NMR signals of H-5 and H-6 were doublets at δ 4.50 and δ 5.09, respectively.

The ^{13}C NMR and DEPT spectra showed that the chemical shifts of C-5 and C-6 were δ 71.0 (CH) and δ 62.1 (CH), respectively. The ^1H - ^1H COSY correlation between H-5 and H-6 and the HMBC correlations of H-4 to C-5, H-6 to C-4a at δ 143.3, H-5 to C-6a at δ 132.1 confirmed that C-5 and C-6 were hydroxylated (Figure 2). This was also supported by

the ^1H - ^1H ROESY correlations shown in Figure S4. Thus, **7** was characterized as 5,6-dihydroxy-G-2A, which was named as KN90 according to the name of the producing plasmid pKN90.

We have previously functionally expressed PdmJ in *E. coli* and the engineered strain converted **3** into the C-5 hydroxylated product **5** with low efficiency. The conversion rate was improved when glucose dehydrogenase was co-expressed to recycle the co-factors.^[18] Although both PdmJ and PdmW can catalyze the corresponding hydroxylations of **3**, the efficiencies of both individual reactions were low. However, these two CYP enzymes work collaboratively to efficiently generate a new pradimicin bisynthetic intermediate **7**. A similar synergistic phenomenon with cyclases PdmK and PdmL was found in the formation of the pentangular structure of pradimicins, which require the presence of PdmH, a monooxygenase that oxidizes ring B into the quinone form, to close rings C-E.^[16] It is also known that KS and CLF in type II polyketide biosynthetic pathways form a heterodimer to promote the chain length control. In the case of PdmJ and PdmW, either enzyme can work separately, but it is apparent that both enzymes can work more efficiently when they were co-expressed. Therefore, it is proposed that these two CYPs form a complex to catalyze the dihydroxylation of the substrate. Our previous work on PdmJ revealed substrate inhibition of this enzyme in the *in vitro* reactions.^[18] The synergistic actions of PdmJ and PdmW are likely due to the prevention of substrate inhibition in pradimicin biosynthesis.

Broad substrate specificity of PdmN

Previously, PdmN was identified as an amino acid ligase that can accept D-alanine or D-serine to give different pradimicin analogues.^[17] The relaxed amino acid substrate specificity of PdmN makes it a useful biocatalyst to generate novel pradimicin analogues. Previous attempts to express PdmN in *E. coli* BL21 (DE3) using the expression vector pET28a failed because an insoluble form of the enzyme was generated^[18] and similar results were obtained with other expression vectors such as pACYC Duet-1 and with different *E. coli* expression conditions. In the current work, PdmN expression in *S. coelicolor* CH999 was attempted since functional expression of PdmN and other biosynthetic enzymes was successful to afford **4**.^[17] Expression plasmid pKN50 was constructed by ligating *pdmN* (1,863 bp) into the pRM5 vector (Table 1). The plasmid was introduced into *S. coelicolor* CH999 and the transformant was used for testing alanylation of different substrates including **3**, **5**, **6** and **7**. To confirm that this strain had functional PdmN, exogenously fed **3** was shown to be converted into the alanylated derivative **4** by cells of *S. coelicolor* CH999/pKN50 (trace i, Figure 4). No product was observed when **3** was incubated with the control strain *S. coelicolor* CH999. To examine the substrate specificity of PdmN toward amino acid acceptors, **5**, **6** and **7** were added to the growing cultures of *S. coelicolor* CH999/pKN50. As shown in Figure 4, incubation of **5** (trace ii) with the PdmN-expressing strain yielded no products, while **6** and **7** (traces iii and iv) were converted into two new products **8** and **9**, respectively. The ESI-MS revealed that the molecular weights of these two products were 519 and 535, indicating that they are the alanylated products of **6** and **7**, respectively. Thus, among the four tested substrates, it can catalyze the alanylation of **3**, **6** and **7** (Figure 4). These results revealed that PdmN has relaxed substrate specificity toward amino acid acceptors, and represents a useful biocatalyst for alanylation.

Combinatorial biosynthesis of new pradimicin analogues

We then made a series of plasmids to generate new pradimicin analogues through combinatorial biosynthesis, which was achieved by combining one, two or all of the three tailoring enzymes (PdmN, PdmJ and PdmW) with the early biosynthetic enzymes (Table 1). All genes are transcribed from a single promoter/regulator *actI/actII-ORF4*. Ligation of *pdmN* into pJX120 yielded pKN93 (Table 1), which was introduced into *S. coelicolor* CH999. *S. coelicolor* CH999/pJX120 that produces **3** as the major product was used as the control (trace i, Figure 5). LC-MS analysis of the culture of *S. coelicolor* CH999/pKN93 in R5 agar showed that **4** was produced in a high yield ($31.9 \pm 3.1 \text{ mg L}^{-1}$), with **3** as the minor product (trace ii, Figure 5). When PdmJ was introduced into the system (pKN89), however, the same products produced by *S. coelicolor* CH999/pKN93 were obtained (trace iii, Figure 5). This result suggested that although both PdmN and PdmJ can use **3** as the substrate for C-16 alanylation and C-5 hydroxylation, respectively, it is apparent that PdmN has higher affinity to the intermediate **3** than PdmJ. **4** was efficiently synthesized from **3** by PdmN. PdmJ cannot further hydroxylate **4** to yield the corresponding alanylated and C-5 hydroxylated product. Accordingly, **5** was not observed due to the low efficiency of the C-5 hydroxylation. This is consistent with the *in vitro* results from our previous work.^[18] In contrast, when pKN102 that harbors *pdmABCDGHLNW* was expressed in *S. coelicolor* CH999, several products including **3**, **4**, **6**, and **8** ($0.3 \pm 0.1 \text{ mg L}^{-1}$) were biosynthesized (trace iv, Figure 5). The UV spectrum of **8** suggested it is a pradimicin analogue (Figure S1). ESI-MS of **8** showed the $[\text{M}-\text{H}]^-$ peak at m/z 518 (Figure S2), indicating that it has a molecular weight of 519, which is 71 mass units larger than that of **6**. This suggested that **8** is the alanylated derivative of **6** and the same as the biotransformation product of **6** by PdmN (trace iii, Figure 4). Although the ^{13}C NMR spectrum was not acquired due to the lack of sufficient amounts of **8**, the presence of the oxygenated H-6 as well as H-17 and CH_3 -19 from the D-alanine moiety was confirmed by the proton signals at δ 5.16 (1H, s), 4.20 (1H, s) and 1.27 (3H, s), respectively, in the ^1H NMR of **8**. Accordingly, the structure of **8** was deduced to be 6-hydroxy-JX137a. Therefore, in addition to **3** that was formed by PdmABCDGHLK, **4** and **6** resulted from the individual tailoring actions catalyzed by PdmN and PdmJ, respectively, and **8** was generated by the actions of both enzymes. However, the overall efficiency is still low and **3** was accumulated as the major metabolite.

Apparently, co-expression of PdmJ or PdmW with PdmN and other early biosynthetic enzymes yield no or small amounts of the expected hydroxylated and alanylated product ($0.3 \pm 0.1 \text{ mg L}^{-1}$ of **8**), suggesting that PdmN cannot efficiently work with either of the two CYP enzymes. Since we have found that PdmJ and PdmW work synergistically to generate the dihydroxylated product **7**, we hypothesized that collaboration of PdmN with both CYP enzymes might be required to generate a new pradimicin analogue. To this end, we co-expressed these three enzymes with the G-2A-forming enzymes by constructing a new expression plasmid pKN87 (Table 1). Expression of this plasmid in *S. coelicolor* CH999 led to the production of a series of products including **3**, **4**, **6**, **7** and **9**, with **9** being the most predominant product ($32.4 \pm 2.2 \text{ mg L}^{-1}$). Compound **9** is identical to the PdmN-catalyzed biotransformation product of **7** in *S. coelicolor* CH999 (trace iv, Figure 4). **9** was isolated from a scaled-up culture as an orange solid. Its molecular weight was found to be 535 according to the ion peaks including $[\text{M}-\text{H}]^-$ at m/z 534.0 (Figure S2) and $[\text{M}+\text{H}]^+$ at m/z

536.1 in the ESI-MS spectra. It showed typical UV absorptions of pradimicins (Figure S1), suggesting that it is a pradimicin analogue. The ^1H and ^{13}C NMR spectra revealed that compared to **7**, **9** has extra proton and carbon signals including one methyl, one carboxyl, one methine and one NH group, which belongs to a D-alanine moiety introduced by PdmN. In the HMBC spectrum, the correlations of NH at δ 8.86 (1H, d, $J = 5.9$) to C-16 at δ 167.5 and C-18 at δ 174.7, as well as H-19 at δ 1.39 (3H, d, $J = 7.2$) to C-18 (Figure 2) confirmed that the amino acid was connected to C-16 to form an amide group. The ^1H - ^1H ROESY correlations also supported the connection (Figure S4). Therefore, **9** was characterized as an alanylated analogue of **7**, which was named KN87. **9** was found to be a major product in the culture of *S. coelicolor* CH999/pKN87 (trace v, Figure 5). It represents an important intermediate in pradimicin biosynthesis that can be further decorated by late tailoring enzymes such as methyltransferases, oxygenases and glycosyltransferases to form the final structure of **1**.

Structural analysis of **8** and **9** confirmed that they are the products from the PdmN-catalyzed alanylation of **6** and **7**, respectively. It is apparent that all the three studied tailoring enzymes (PdmJ, PdmW and PdmN) can use **3** as substrate. As shown in the HPLC traces of *S. coelicolor* CH999/pKN35 (trace i, Figure 1), *S. coelicolor* CH999/pKN92 (trace ii, Figure 1) and *S. coelicolor* CH999/pKN93 (trace ii, Figure 5), PdmN can ligate the D-alanine moiety into **3** to form **4** efficiently, while PdmJ and PdmW are much less efficient in their corresponding hydroxylations. Different combinations of these tailoring enzymes generate various product profiles, which provide important information about substrate specificity of these enzymes. Efficient production of **9** further confirmed that synergistic actions of biosynthetic enzymes are important for the assembly of **1**.

Compounds **3–9** were tested for the antifungal activity against *Candida albicans* ATCC MYA-2876. However, none of these compounds showed inhibition to this fungus at the test concentrations ($0.9\text{--}500\text{ }\mu\text{g mL}^{-1}$). This is consistent with the previous reports on the analogues of the aglycon pradimicinone, which further confirmed that the sugar moieties are essential for the biological activities of pradimicins.^[21, 22]

Conclusion

1 is a promising lead compound for antiviral and antifungal therapeutics development. Combinatorial biosynthesis represents an effective approach to creating diverse analogues for drug development.^[23] In this work, PdmW was characterized as the C-6 hydroxylase and PdmN was found to take different amino acid acceptor substrates. Moreover, this work demonstrated synergistic actions of two tailoring CYP enzymes in pradimicin biosynthesis (Figure 6). Together with our previous discovery on the collaboration of two cyclases and a monooxygenase in the early biosynthetic steps of **1**,^[16] it can be concluded that the pradimicin biosynthetic enzymes have extensive synergistic actions and interactions during the biosynthetic process. Concerted actions of the PKS and three cyclases have also been reported in the resistomycin biosynthetic pathway to form the discoid ring structure.^[24] Thus, we propose that synergistic actions of biosynthetic enzymes are a common approach for type II polyketide biosynthetic pathways that consists of a series of discrete enzymes to achieve the accuracy and overall efficiency of the product assembly lines. Understanding of

the functions of individual enzymes and their synergistic actions will facilitate rational and efficient combinatorial biosynthesis of new molecules for bioactivity screening.

Experimental Section

General

1D and 2D NMR spectra were recorded on a JEOL instrument (300 MHz). ESI-MS were acquired on an Agilent 6130 LC-MS. The extracts were analyzed on an Agilent 1200 HPLC instrument using an Agilent ZORBAX SB-C18 (5 μ m, 4.6 mm $\times$ 150 mm), eluted with a gradient of acetonitrile-water (25–90%) with 0.1% formic acid over 25 min at a flow rate of 1 mL min⁻¹, followed by 100% acetonitrile for 5 min. Compounds were purified on the same HPLC instrument. The yields of the products from three different colonies of the same strain were analyzed by HPLC and determined using the standard curves of the purified compounds.

Bacterial strains and vectors

E. coli XL-1 Blue (Stratagene) was used for routine subcloning. *S. coelicolor* CH999 was used for the expression of *pdm* biosynthetic genes. *A. hibisca* P157-2 was obtained from the American Type Culture Collection (ATCC). The cloning vectors used in this study were pCR-Blunt vector (Life Technologies) and pJET1.2 (Fermentas). The pRM5 *E. coli*/*Streptomyces* shuttle vector was used to construct the expression plasmids.

Growth media and conditions

E. coli strains were routinely grown in Luria-Bertani (LB) medium at 37 °C. For selection of correct transformants, ampicillin (100 μ g mL⁻¹) or kanamycin (50 μ g mL⁻¹) was supplemented. *S. coelicolor* CH999 was used as the heterologous host for expression of the pradimicin biosynthetic enzymes. The expression plasmids were introduced into *S. coelicolor* CH999 through polyethylene glycol (PEG)-mediated protoplast transformation. The transformants were selected on R5 agar supplemented with 50 μ g mL⁻¹ thiostrepton. All the engineered strains of *S. coelicolor* CH999 were cultivated on R5 agar with thiostrepton at 28 °C for the production of the polyketide compounds.

Construction of plasmids

The genes used in this study were amplified by PCR. Specific forward and reverse primers were designed based on the reported sequences to contain compatible restriction enzymes such as XbaI, SpeI, and NheI. The PCR products were then ligated into a cloning vector for DNA sequencing. Each combination of genes was constructed by the subsequent addition of individual genes into a cloning vector and the correct ligation was confirmed by restriction enzyme digestions. Additionally, each group of genes was ligated into a pRM5-derived vector. The gene-cassette (*pdmCGHKLJ*) was excised from pKN31 using XbaI and SpeI and ligated into the pRM5-derived vector pJX111 (*pdmABD*) between the same sites to yield pKN35. The *pdmN* gene was excised from pJX97 using XbaI and SpeI, and ligated into the XbaI-treated pKN35 to yield pKN89. Similarly, the same insert was ligated into XbaI-digested pJX120 to afford pKN93. The *pdmW* gene was excised from pJX151 using XbaI and NheI, and ligated into XbaI-digested pKN93 to yield pKN102. The same insert

was ligated into XbaI-digested pJX120 to yield pKN92. When *pdmW* was ligated into XbaI-digested pKN35, pKN90 was constructed. pKN87 was generated by ligating into pJX111 between XbaI and SpeI. The *pdmN* gene was excised from the plasmid pKN39 using EcoRI and PacI and ligated into pRM5 vector between the same sites to yield the plasmid pKN50. All these plasmids were introduced into *S. coelicolor* CH999 through PEG-mediated protoplast transformation for product analysis or biotransformation.

***In vivo* D-alanylation**

S. coelicolor CH999/pKN50 was cultivated in R5 broth containing 50 $\mu\text{g mL}^{-1}$ thiostrepton at 28°C for 4 days in a rotary shaker. This seed culture (100 μL) was transferred to 10 mL of fresh R5 broth with 50 $\mu\text{g mL}^{-1}$ thiostrepton and cultured for 4 days before the addition of 0.5 mg of **3**, **5**, **6**, or **7** dissolved in pure DMSO. After 10 days of biotransformation, the cultures were centrifuged at 4,000 rpm for 10 min. The pelleted cells were extracted with methanol. This methanol extract was combined with the supernatant and dried *in vacuo*. The samples were subjected to LC-MS analysis.

Extraction and isolation of compounds

The engineered *S. coelicolor* CH999 strains were grown on R5 agar with 50 $\mu\text{g mL}^{-1}$ thiostrepton at 28 °C for 8–10 days. Products were extracted from the cultures using a mixture of ethyl acetate, methanol, and acetic acid (89:10:1). The extracts were then dried under reduced pressure.

The extract of 6 L of *S. coelicolor* CH999/pKN92 was subjected to Diaion HP-20 resin column chromatography, eluted with 0%, 25%, 50%, 75%, and 100% aqueous methanol (v/v) to yield five fractions. HPLC analysis revealed that the 75% fraction contained the target peak. This fraction was further separated by reverse-phase HPLC (Agilent Zorbax SB-C18, 5 μm , 21.2 mm $\times$ 150 mm), eluted with 55% aqueous acetonitrile containing 0.1% formic acid at a flow rate of 1 mL min⁻¹, to yield 6.0 mg of **6**.

The extract of 2 L of *S. coelicolor* CH999/pKN90 was subjected to Diaion HP-20 resin column chromatography as described above. The 50% fraction that contained the target peak was further separated by reverse-phase HPLC (SOPELCO C18, 5 μm , 10 mm $\times$ 150 mm), eluted with a linear gradient of 15–95% aqueous acetonitrile containing 0.1% formic acid at a flow rate of 1 mL min⁻¹ over 16 min, to yield 8.5 mg of **7**.

The extract of 6 L of *S. coelicolor* CH999/pKN102 was subjected to Diaion HP-20 resin column chromatography as described above. The 75% fraction was further separated by reverse-phase HPLC (Agilent Zorbax SB-C18, 5 μm , 21.2 mm $\times$ 150 mm), eluted with 37% aqueous acetonitrile containing 0.1% formic acid at a flow rate of 1 mL min⁻¹, to yield 1.0 mg of **8**. ¹HNMR data of **8** in dimethyl sulfoxide (DMSO)-*d*₆: 89.02 (1H, s, H-14), 7.12 (1H, brs, H-12), 6.61 (1H, s, H-4), 6.45 (1H, brs, H-10), 5.22 (1H, brs, H-6), 4.20 (1H, m, H-17), 2.97 (1H, m, H-5a), 2.76 (1H, m, H-5b), 2.23 (3H, s, H-15), 1.27 (3H, d, H-19, *J* = 7.2 Hz).

The extract of 2 L of *S. coelicolor* CH999/pKN87 was subjected to Diaion HP-20 resin column chromatography as described above. The 50% fraction was found to contain the target peak. This fraction was dissolved in dimethyl sulfoxide (DMSO) after solvent

evaporation, and then subjected to a HW-40F chromatographic column (30 mm × 100 mm) eluted with a gradient of methanol-water to remove the non-pradimicin compounds. The 50% methanol-water elute was further separated by HPLC (ZORBAX SB-C18, 5 µm, 4.6 mm × 150 mm), eluted with 23% aqueous acetonitrile containing 0.1% formic acid at a flow rate of 1 mL min⁻¹, to yield 8.1 mg of **9**.

Determination of the absolute configurations of **5** and **6**

In order to determine the absolute configurations of **5** at C-5 and **6** at C-6, these compounds were reacted with (*S*)- and (*R*)-MTPA chloride separately to afford the corresponding (*R*)- and (*S*)-MTPA esters **5R**, **5S** and **6R**, **6S**. Their ¹H, ¹⁹F and ¹H-¹H COSY NMR spectra were recorded in pyridine-*d*₅ after reacting for 24 hours at room temperature.

Antifungal assays of **3–9**

The antifungal activity of compounds **3–9** was tested against *C. albicans* ATCC MYA-2876. The inoculum was prepared by cultivating a single colony of *C. albicans* in 5mL of potato dextrose broth (PDB) supplemented with 200 µM CaCl₂ at 35°C for 24 hours. The inoculum was diluted with PDB and titrated with a hemocytometer to reach a final inoculum size of 2 × 10⁶ CFU mL⁻¹. Stock solutions of each test compound were prepared in 50% aqueous DMSO with a concentration of 5 mg mL⁻¹. The test concentrations ranged from 0.9 to 500 µg mL⁻¹. Clotrimazole was dissolved in pure DMSO and used as positive control.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by the National Institute of Allergy and Infectious Diseases (R15AI089347). We thank Dr. Chaitan Khosla, Stanford University for the gift of *S. coelicolor* CH999.

References

1. Oki T, Konishi M, Tomatsu K, Tomita K, Saitoh K, Tsunakawa M, Nishio M, Miyaki T, Kawaguchi H. *J Antibiot.* 1988; 41:1701–1704. [PubMed: 3198502]
2. Oki T, Saitoh K, Tomatsu K, Tomita K, Konishi M, Kawaguchi H. *Ann N Y Acad Sci.* 1988; 544:184–187. [PubMed: 3214068]
3. Balzarini J. *Antiviral Chem Chemother.* 2007; 18:1–11.
4. Oki T, Tenmyo O, Hirano M, Tomatsu K, Kamei H. *J Antibiot.* 1990; 43:763–770. [PubMed: 2167304]
5. Walsh TJ, Giri N. *Eur J Clin Microbiol Infect Dis.* 1997; 16:93–97. [PubMed: 9063679]
6. Balzarini J, Van LK, Daelemans D, Hatse S, Bugatti A, Rusnati M, Igarashi Y, Oki T, Schols D. *J Virol.* 2007; 81:362–373. [PubMed: 17050611]
7. Nakagawa Y, Doi T, Takegoshi K, Igarashi Y, Ito Y. *Bioorg Med Chem Lett.* 2012; 22:1040–1043. [PubMed: 22196119]
8. Hoorelbeke B, Kim Y, Oki T, Igarashi Y, Balzarini J. *Curr Top Med Chem.* 2013; 13:1907–1915. [PubMed: 23895096]
9. Nakagawa Y, Doi T, Masuda Y, Takegoshi K, Igarashi Y, Ito Y. *J Am Chem Soc.* 2011; 133:17485–17493. [PubMed: 21942374]

10. van der Meer FJUM, de Haan CAM, Schuurman NMP, Haijema BJ, Verheije MH, Bosch BJ, Balzarini J, Egberink HF. *J Antimicrob Chemother.* 2007; 60:741–749. [PubMed: 17704516]
11. Kakushima M, Masuyoshi S, Hirano M, Shinoda M, Ohta A, Kamei H, Oki T. *Antimicrob Agents Chemother.* 1991; 35:2185–2190. [PubMed: 1803990]
12. Groll AH, Sein T, Petraitis V, Petraitiene R, Callender D, Gonzalez CE, Giri N, Bacher J, Piscitelli S, Walsh TJ. *Antimicrob Agents Chemother.* 1998; 42:2700–2705. [PubMed: 9756780]
13. Zhan J. *Curr Top Med Chem.* 2009; 9:1598–1610.
14. Kim BC, Lee JM, Ahn JS, Kim BS. *J Microbiol Biotechnol.* 2007; 17:830–839. [PubMed: 18051306]
15. Paudel S, Park JW, Lee JH, Yoon YJ, Sohng JK. *Biotechnol Bioproc Eng.* 2012; 17:8–15.
16. Zhan J, Watanabe K, Tang Y. *ChemBioChem.* 2008; 9:1710–1715. [PubMed: 18604835]
17. Zhan J, Qiao K, Tang Y. *ChemBioChem.* 2009; 10:1447–1452. [PubMed: 19431159]
18. Napan KL, Zeng J, Takemoto JY, Zhan J. *Bioorg Med Chem Lett.* 2012; 22:606–609. [PubMed: 22082563]
19. McDaniel R, Ebert-Khosla S, Hopwood DA, Khosla C. *Science.* 1993; 262:1546–1550. [PubMed: 8248802]
20. Seco JM, Quinoa E, Riguera R. *Chem Rev.* 2004; 104:17–117.
21. Aburaki S, Yamashita H, Ohnuma T, Kamachi H, Moriyama T, Masuyoshi S, Kamei H, Konishi M, Oki T. *J Antibiot.* 1993; 46(4):631–640. [PubMed: 8501006]
22. Tsuno T, Yamamoto H, Narita Y, Suzuki K, Hasegawa T, Kakinuma S, Saitoh K, Furumai T, Oki T. *J Antibiot.* 1993; 46(3):420–429. [PubMed: 8478260]
23. Hutchinson CR. *Curr Opin Microbiol.* 1998; 1:319–329. [PubMed: 10066498]
24. Fritzsche K, Ishida K, Hertweck C. *J Am Chem Soc.* 2008; 130:8307–8316. [PubMed: 18533655]

Figure 1.

Synergistic actions of two CYP hydroxylases in pradimicin biosynthesis. (i) HPLC trace of *S. coelicolor* CH999/pKN35; (ii) HPLC trace of *S. coelicolor* CH999/pKN92; (iii) HPLC trace of *S. coelicolor* CH999/pKN90. These extracts were analyzed at 460 nm.

Figure 2.

Key ^1H - ^1H COSY (—) and HMBC (→) correlations for **6**, **7** and **9**.

Figure 3. Determination of the absolute configurations of **5** and **6** using Mosher's method. δ ($\delta_S - \delta_R$, in ppm) values in pyridine- d_5 are shown.

Figure 4.
HPLC analysis of alanylation of **3** (i), **5** (ii), **6** (iii), and **7** (iv) by *S. coelicolor* CH999/
pKN50 at 460 nm.

Figure 5.

Combinatorial biosynthesis of new pradimicin analogues. (i) HPLC trace of *S. coelicolor* CH999/pJX120; (ii) HPLC trace of *S. coelicolor* CH999/pKN93; (iii) HPLC trace of *S. coelicolor* CH999/pKN89; (iv) HPLC trace of *S. coelicolor* CH999/pKN102; (v) HPLC trace of *S. coelicolor* CH999/pKN87. These extracts were analyzed at 460 nm.

Figure 6.

The bisynthetic pathway of **1**. The steps studied in this work are boxed.

Scheme 1.

Structures of pramidicin A (**1**) and its analogues (**2–9**).

Table 1

Plasmids used in this study and corresponding polyketide products

Plasmid	Genes	Products	Reference
pJX120	<i>pdmABCDGHIKL</i>	3	[16]
pKN35	<i>pdmABCDGHIKLJ</i>	3, 5	This work
pKN92	<i>pdmABCDGHIKLW</i>	3, 6	This work
pKN93	<i>pdmABCDGHIKLN</i>	3, 4	This work
pKN89	<i>pdmABCDGHIKLNJ</i>	3, 4	This work
pKN90	<i>pdmABCDGHIKLJW</i>	3, 5, 6, 7	This work
pKN102	<i>pdmABCDGHIKLNW</i>	3, 4, 6, 8	This work
pKN87	<i>pdmABCDGHIKLJNW</i>	3, 4, 6, 7, 9	This work
pKN50	<i>pdmN</i>	N/A	This work

Table 2

The ^1H NMR data (300MHz) for **6**, **7** and **9** (in DMSO- d_6 , δ in ppm, J in Hz)

Position	6	7	9
4	6.64 (1H, s)	6.89 (1H, s)	6.83 (1H, s)
5	2.83–2.98 (2H, m)	4.50 (1H, d, $J=3.0$)	4.49 (1H, d, $J=3.1$)
6	5.16 (1H, brs)	5.09 (1H, d, $J=2.1$)	5.09 (1H, d, $J=2.8$)
10	6.57 (1H, brs)	6.64 (1H, d, $J=2.4$)	6.64 (1H, d, $J=1.7$)
12	7.12 (1H, brs)	7.19 (1H, d, $J=2.4$)	7.19 (1H, d, $J=2.0$)
14	8.82 (1H, s)	8.93 (1H, s)	8.87 (1H, s)
15	2.47 (3H, s)	2.57 (3H, s)	2.36 (3H, s)
17			4.47 (1H, m)
19			1.39 (3H, d, $J=7.2$)
7-OH	12.47 (1H, s)	12.54 (1H, s)	12.54 (1H, s)
9-OH	12.03 (1H, s)	12.13 (1H, s)	12.14 (1H, s)
11-OH	11.46 (1H, brs)	11.42 (1H, s)	11.62 (1H, brs)
16-NH			8.86 (1H, d, $J=5.9$)

Table 3

The ^{13}C NMR data (75 MHz) for **6**, **7** and **9** (in DMSO- d_6 , δ in ppm)

Position	6	7	9
1	162.3	160.8	153.1
2	113.1	113.8	125.3
3	143.1	144.3	137.8
4	123.4	119.6	124.3
4a	141.9	143.3	140.1[a]
5	37.2	71.0	70.9
6	56.9	62.1	62.4
6a	131.5[a]	132.1	131.4[a]
7	158.5	160.2	160.0
7a	113.2	114.3	113.4
8	189.3	190.1	189.6
8a	109.1	109.1	109.2
9	164.5	164.5	164.5
10	107.9	108.3	107.9
11	165.7	165.9	165.8
12	108.9	109.0	108.9
12a	135.3	135.3	135.4
13	181.4	181.9	181.7
13a	131.3[a]	131.5	131.6[a]
14	119.9	119.6	119.8
14a	140.0	139.2	140.4[a]
14b	117.2	116.9	116.8
15	23.7	23.3	19.4
16	173.9	174.2	167.5
17			48.2
18			174.7
19			16.5

[a] Assignment interchangeable.