DOI: 10.1002/cbic.200900082

Investigation of Tailoring Modifications in Pradimicin Biosynthesis

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Pradimicins and benonamicins are pentangular polyphenols[1] that display potent antiviral and antifungal activities.[2–7] Pradimicins bind to the cell-wall mannan of many fungal strains and cause osmotic lysis that leads to cell death.[8,9] Pradimicin A (**1**) is a highly decorated aromatic polyketide (PK) with a benzo[α]-naphthacene aglycone and a number of interesting structural features (Figure 1 A), especially on the D and E rings. Two chiral alcohols are present in the D ring of **1**, and are located at C-5 and C-6. A disaccharide consisting of D-xylopyranose and 4-methylamino-4-deoxy-D-fucopyranose is linked O-glycosidically to the 5-OH group. The two hydroxyls are in opposite stereochemical configurations and are both presumed to be inserted into the nonaromatic ring D by a dedicated P450 hydroxylases during tailoring steps.

On the E ring of **1**, the terminal carboxylic acid is amidated with the nonproteinogenic amino acid D-alanine (Figure 1 A). The D-alanine moiety is a frequently used building block for nonribosomal peptides (NRPs) and NRP/PK hybrids, such as microcystin,[10] daptomycin,[11] and leinamycin.[12] In these biosynthetic pathways, D-alanine can be either converted from an activated L-alanine enantiomer by an epimerization domain in the NRP biosynthetic assembly line, or directly activated by a dedicated adenylation domain. Pradimicin A represents the only known example in which a D-amino acid is appended to the aglycon of an aromatic polyketide. Structure–activity relationships studies have shown that the amide proton in **1** is necessary for the antifungal property.[13] In addition, the D-enantiomer is required for the bioactivity, as the synthetic L-alanine-containing isomer of **1** has no antifungal activity.[14] The more water-soluble semisynthetic derivative BMS-181184, which contains a D-serine substitution at C-16 exhibited broad-spectrum antifungal activity against 167 different fungal strains.[15] The biosynthetic mechanism in which the D-alanine is ligated to the aglycon of **1** has not been elucidated. Because of the importance of this moiety to the biological activity of **1**, characterization of the enzyme involved in the ligation reaction might enable us to biosynthesize new pradimicin analogues.

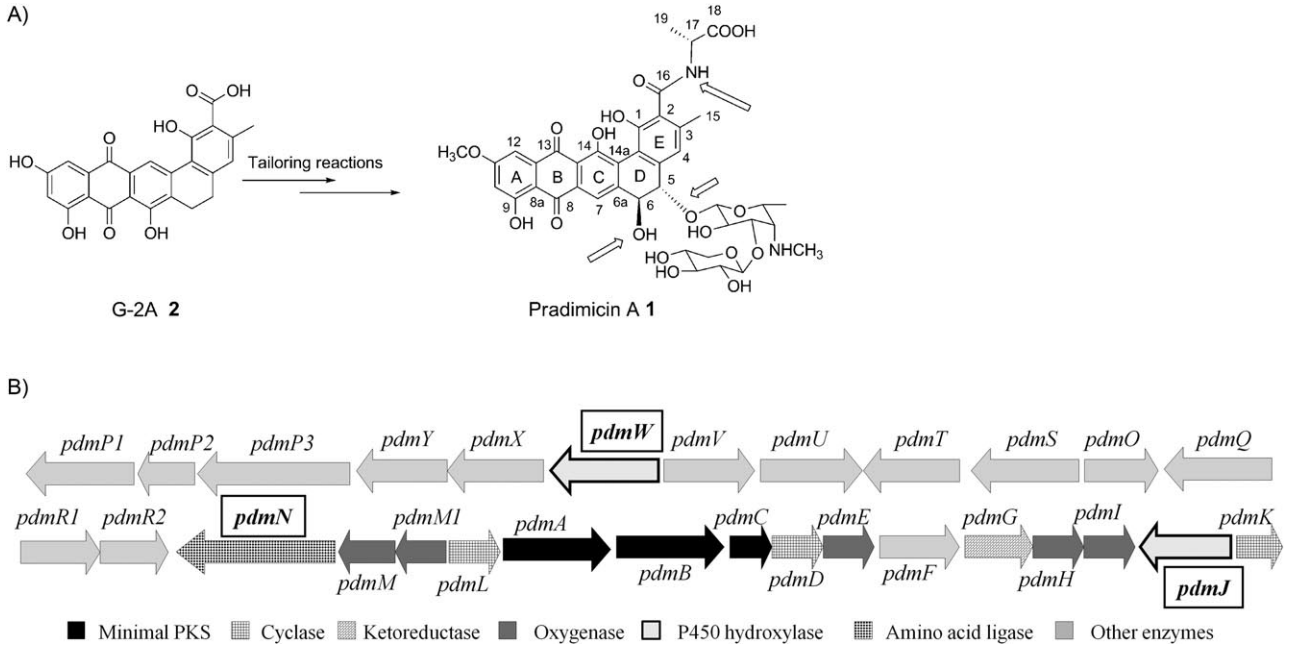

Figure 1. A) Proposed post-PKS tailoring steps involved in converting G-2 A (**2**) to pradimicin (**1**). The three arrows indicate key modifications performed on the aglycon. B) The pradimicin A gene cluster. The enzymes identified in this work are boxed.

Since **1** was first isolated from *Actinomadura hibisca* P157-2 (ATCC 53557),[16] its biosynthesis has been investigated in both

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Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cbic.200900082.

native[17–22] and heterologous[23,24] hosts. The *pdm* biosynthetic gene cluster has been recently sequenced,[17,21] and has provided important genetic information on the biosynthesis of **1**. The dodecaketide carbon backbone of **1** is synthesized by the type II *pdm* minimal polyketide synthase (PKS),[25] which consists of the ketosynthase (KS, PdmA), chain length factor (CLF, PdmB), and acyl carrier protein (ACP, PdmC). The backbone is subsequently converted into the benzo[α]naphthacenequinone aglycon G-2A (**2**) via the actions of immediate tailoring enzymes, which include three cyclases (PdmD, PdmK, PdmL), the ketoreductase PdmG, and the monooxygenase PdmH.[24] Recently, we demonstrated that the concerted actions of PdmK, PdmL, and PdmH are required for the biosynthesis of **2**. We report here that PdmN and PdmJ were identified as D-amino acid ligase and C-5 P450 hydroxylase, respectively, while PdmW was deduced to be the putative C-6 P450 hydroxylase.

Among the open reading frames reported in the *pdm* gene cluster (Figure 1 B), *pdmN* encodes a 620-amino-acid (aa) enzyme that displays significant sequence identity to type II asparagine synthetases, which catalyze the conversion of aspartic acid to asparagine in an ATP dependent fashion. Type II asparagine synthetases contain an N-terminal nucleophile (Ntn) cysteine that hydrolyzes the amine of the nitrogen donor glutamine.[26] PdmN, however, lacks the Ntn cysteine (replaced with serine), and is therefore unlikely to be a glutamine-dependent amidotransferase.[27] Instead, PdmN might act as an amino acid ligase that catalyzes a two-step reaction, in which the first step is activation of the C-16 carboxylic acid through formation of the adenylate, followed by amidation with D-alanine. Despite repeated attempts to express PdmN in *Escherichia coli*, we were not able to obtain any enzymes in soluble form. As an alternative approach, the engineered *Streptomyces coelicolor* CH999–pRM5 host–vector pair was used to reconstitute the activities of PdmN.[28] We have previously shown that CH999 transformed with pJX116, which contains the genes *pdmABCDGHKL*, was sufficient to synthesize **2** in good yield.[24] Although the exact pentangular substrate of PdmN is unknown, we reasoned that **2** might be recognized by PdmN. We introduced *pdmN* into pJX116 to yield pJX137 (Table 1) and transformed the plasmid into CH999. The transformed strain was grown on R5 agar supplemented with thiostrepton (50 mg L^{-1}) for 8 days, extracted, and subjected to HPLC analysis (Figure 2 A). Two major polyketide products, including **2** (35 mg L^{-1}), and a new product JX137a **3** (10 mg L^{-1}) were detected (trace b). HRESI-MS suggested a molecular formula of $C_{27}H_{21}NO_9$ for **3**, which is consistent with that of **2** amidated with alanine (Scheme 1 A). The ^{13}C NMR spectrum of **3** is similar to that of **2**, except for the appearance of three unique signals that are characteristic of alanine (δ_C = 167.5, 48.2 and 16.5 ppm). The doublet proton signal at δ_H = 8.79 ppm with a coupling constant of J_{HH} = 7.0 Hz was assigned to the α-NH, whereas ^1H-^{13}C HMBC correlation of α-NH to C-16 confirmed that the alanine was tethered to C-16 carboxyl group (Scheme 1 B). The remaining 1D and 2D NMR signals (Supporting Information) are consistent with the structure of **3** as shown in Scheme 1 A.

Table 1. Plasmids and their resulting polyketide products in *S. coelicolor* CH999

Plasmid	Genes	Products
pJX116	*pdmABCDGHKL*	**2**
pJX137	*pdmABCDGHKLN*	**2**, **3**, **4**[a]
pJX134	*pdmABCDGHKLJ*	**2**, **5**
pJX152	*pdmABCDGHKLW*	**2**, **6**
pJX165	*pdmABCDGHKLJW*	**2**, **5**, **6**

[a] When supplemented with D-serine in R5 agar.

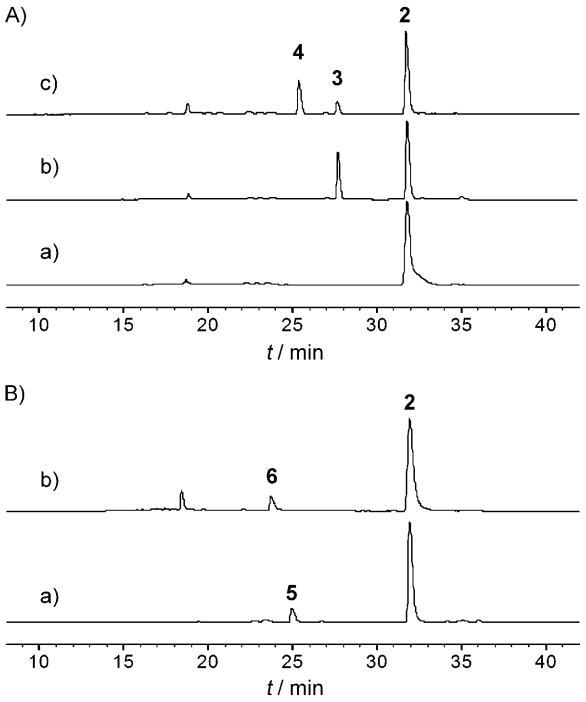
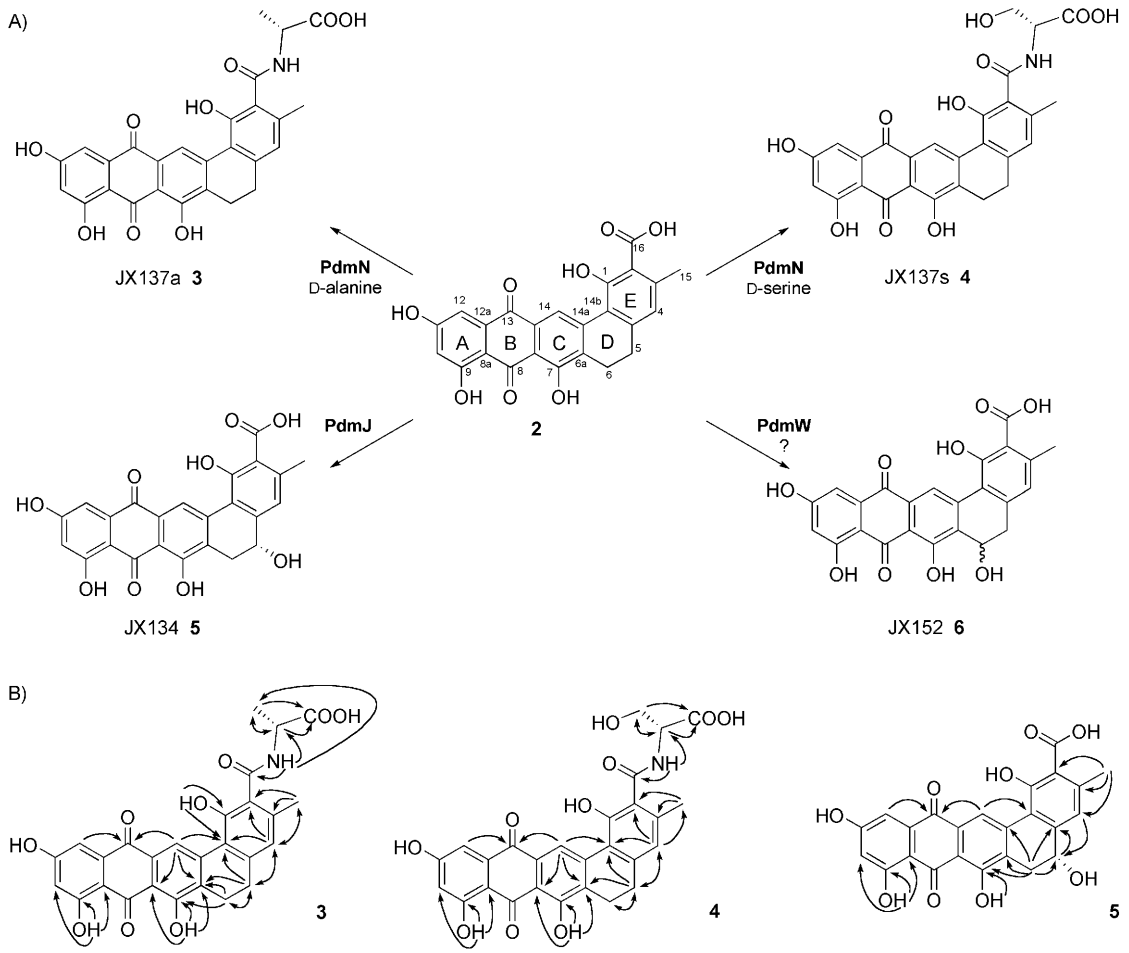


Figure 2. HPLC traces (460 nm) of extracts from CH999 transformed with different plasmids in Table 1. A) a) CH999/pJX116; b) CH999/pJX137 supplemented with D-alanine; c) CH999/pJX137 supplemented with D-serine; B) a) CH999/pJX134; b) CH999/pJX152. Traces are not drawn to the same scale to facilitate visualization of **5** and **6.**

Biosynthesis of **3** upon coexpression of PdmN in CH999 confirmed that PdmN is indeed the enzyme responsible for transferring the amino acid to the aglycon. To probe the amino acid specificity of PdmN, CH999/pJX137 was grown on R5 agar supplemented with different D- or L-amino acids (0.25 %, *w*/*v*). HPLC analysis of the extracts showed that only the culture that was supplemented with D-serine afforded a new peak **4** in addition to **2** and **3** (Figure 2 A, trace c), whereas feeding other amino acids, including L-serine did not lead to the biosynthesis of new polyketide products in addition to **2**. Compound **4** eluted with an earlier retention time (t_R = 25.8 min) than **3** (t_R = 28.2 min); this is consistent with the likely incorporation of the more polar D-serine. Large-scale culturing and extraction of CH999/pJX137 on R5 medium supplemented with D-serine, followed by purification by using Sephadex LH-20 and prepara-



Scheme 1. A) Compounds produced by CH999, starting from the aglycon **2**; B) ^{1}H,^{13}C HMBC correlations for compounds **3**–**5**.

tive reversed-phase HPLC afforded **4** in a yield of 2.5 mg L^{-1}. The molecular formula of **4** is $C_{27}H_{21}NO_{10}$ and confirms the presence of an extra oxygen atom compared to **3**. The identity of the serine moiety is further confirmed by an oxygenated CH_2 signal of the amino acid moiety at δ_C 60.8 ppm in the ^{13}C NMR spectrum. The structure of **4** was readily established as JX137s (Scheme 1 A) by a full comparison of its NMR spectroscopy data with those of **3**.

To confirm the D-configurations of the ligated alanine and serine amino acids, the optical rotations of the purified compounds were measured in methanol at 25 °C. The [α] values were found to be +4.4 and +6.9 for **3** and **4**, respectively, which are consistent with those measured for D-alanine (+7.0) and D-serine (+4.0) in methanol under the same conditions. Interestingly, a study on the biosynthesis of **1** by using racemic 1-^{13}C]alanine revealed that the producing strain *A. hibisca* P157-2 can also uptake L-alanine to synthesize the 17-epimer of **1**, albeit with lower efficiency.[20] Although the conversion of **2** to either **3** or **4** was relatively low, our reconstitution studies using CH999/pJX137 confirmed PdmN as the first D-amino acid ligase that uses an aromatic polyketide as the substrate. When additional D-alanine was supplemented to the medium, no improvement in the relative ratio of **3** to **2** was observed; this suggests that the low conversion is not due to limited concentrations of endogenous D-alanine in CH999. We reason the incomplete amidation of **2** might be attributed to the fact that **2** is not the actual biosynthetic substrate of PdmN, thus resulting in an inefficient enzymatic reaction. The PdmN reaction in *A. hibisca* might occur later in the biosynthetic pathway after other tailoring modifications of **2** have taken place. As a result, significant fractions of **2** might have been exported from CH999 before the amidation reaction is completed.

The best known D-amino acid ligases are the D-alanyl-D-amino acid ligase (Ddl) family of enzymes, which are associated with the biosynthesis of bacterial cell wall peptidoglycan assembly.[29] Ddl enzymes catalyze the ATP-dependent ligation of two D-amino acids and do not share any sequence homology with PdmN. Ddl enzymes typically have varied substrate tolerance toward ligating different hydroxyl or amino acids to D-alanine.[30] By using feeding studies, we showed that PdmN has narrow amino acid substrate specificity because it can only ligate D-alanine and D-serine to **2**. This is in good agreement with previous whole-cell semisynthesis studies in which the D-serine analogue of **1** (BMS-181184) was the only C-16 amino acid variant of **1** recovered.[31] We next tested the aglycon substrate specificity of PdmN by inserting the *pdmN* gene into strains that produce other carboxylic acid-containing aromatic polyketides, such as that afforded DMAC or SEK26.[32] No ami-

dated products were recovered during extract analysis; this suggests that PdmN has limited tolerance towards polyketide-derived acceptor substrates.

By using the same heterologous host/vector approach, we next aimed to reconstitute the enzymes that hydroxylate the D-ring in **1**. Hydroxylation of the aliphatic carbons C-5 and C-6 require two cytochrome P450 hydroxylases. Among the oxygenase/hydroxylase enzymes that were identified from bacterial PKS biosynthetic pathways, P450s constitute a relatively small subgroup and are commonly recruited to activate non-reactive aliphatic substituents or macrolide aglycons, such as EryF, EryK,[33] DoxA,[34] and PikC.[35] The *pdm* biosynthetic pathway encodes two P450s, *pdmW* and *pdmJ*, which are possibly involved in the hydroxylation of ring D. PdmW (401 aa) and PdmJ (411 aa) share >45% sequence identity and are both highly homologous to P450s that are found in other actinomycetes.

To investigate the roles of the two P450s, *pdmJ* or *pdmW* was separately cloned into pJX116 to yield pJX134 or pJX152, respectively (Table 1). HPLC analysis of the extract of CH999/pJX134 showed the biosynthesis of a new, polar compound **5** (t_R = 25.1 min, Figure 2B), albeit with low yield (2 mg L^{-1}), along with **2**. The molecular formula of **5** was predicted to be $C_{24}H_{16}O_9$ by using HRESI-MS; this suggests that an oxygen atom has been introduced into the parent compound **2**. The UV absorption spectrum of **5** is identical to that of **2**, which indicates no modification of the chromophore. A sufficient amount of **5** was purified from a large-scale solid culture for NMR spectroscopic characterization. Both ^{1}H and ^{13}C NMR assignments were consistent with a quinone-containing pentangular compound (Supporting Information). A unique, oxygenated methine signal at δ_C 65.9 ppm in the ^{13}C NMR spectrum suggested that **5** is a D-ring hydroxylated analogue of **2**, which was further supported by the spin system of CH–CH_2 observed in the ^{1}H-^{1}H COSY spectrum. The extra hydroxyl group in compound **5** was determined to be at C-5 based on the ^{1}H-^{13}C HMBC correlations of H-6 to C-7, C-14a and C-4a, as well as H-4 to C-5 (Scheme 1B). The coupling constants between 5-CH and 6-CH_2 are J_{HH} = 10.0 and 4.9 Hz, representing vicinal couplings of 5-H_{ax}-6-H_{ax} and 5-H_{ax}-6-H_{eq}, respectively. These values are in line with the reported NMR spectroscopy data for pradimicin Q, which is a pentangular polyketide isolated from *A. verrucosospora* subsp. *Neohibisca* A10102.[36] The only structural difference between pradimicin Q and JX134 (Scheme 1A) is the lack of 14-OH in JX134. Furthermore, the circular dichroism spectrum of **5** was nearly identical to that reported for pradimicin Q; this indicates that the 5-hydroxyls of both compounds have the same *S* configuration. Taken together, **5** was identified to be JX134, which in turn confirmed the function of PdmJ as the C-5 P450 hydroxylase. This enzyme therefore plays an important role in the biosynthesis of **1** by activating C-5 and inserting a chiral secondary alcohol, which subsequently becomes the site of O-glycosylation by the disaccharide that is essential for the bioactivity of **1**.[21]

Extraction of CH999/pJX152, which coexpresses PdmW, revealed the biosynthesis of a similarly more polar, new product **6** (t_R = 24.6 min, Figure 2B) at lower yield (<1 mg L^{-1}). Compared to **5**, compound **6** displayed the same UV absorption pattern and is predicted to have the same molecular formula by HRESI-MS analysis. However, due to the poor yield of **6**, we were not able to obtain sufficient product for complete NMR spectroscopic analysis. Based on UV, HPLC, and MS analysis, as well as deduction through biosynthetic logic, we conclude the most likely structure for **6** should be the 6-hydroxyl derivative of **2**, or JX152 (Scheme 1A). As a result, we putatively assigned PdmW to be the C-6 P450 hydroxylase. Similar P450-catalyzed hydroxylation of C-6 might take place during the biosynthesis of compounds such as lysolipin[37] and kigamicin.[38] Numerous C-6 hydroxylated shunt products have been observed in the pradimicin[24] and benastatin[1] biosynthetic pathways. In contrast to the P450-catalyzed insertion of the C-6 hydroxyl group in **6**, the phenolic C-6 hydroxyl groups found in the shunt products JX116a,[24] and benastatin H[1] are introduced as a result of removing a regioselective C-6 ketoreductase from the biosynthetic pathways.

Few cytochrome P450 mono-oxygenases have been studied in the context of bacterial aromatic polyketide biosynthesis.[39] The biosynthesis of **5** and **6** (although the structure has not been confirmed) reveals the role of the two P450 enzymes in the *pdm* biosynthetic pathway. Whereas PdmJ has been unequivocally assigned as the C-5 hydroxylase, the assignment of PdmW as the C-6 hydroxylase is putatively based on UV and MS data only. Reconstitution of either P450s has resulted in very small conversion (<5%) of **2** to **5** or **6** in CH999. The main obstacle for P450 reconstitution in a heterologous host has always been the lack of an efficient P450 reductase partner, which is required to reconstitute P450 activity together with a ferredoxin-like protein. The endogenous reductase partners of most P450s associated with polyketide biosynthesis are not present in the gene cluster. Similarly, no P450 reductase partners were found in the *pdm* gene cluster. Hence, the reconstituted P450s in CH999 are required to partner with *S. coelicolor* P450 reductases. Although *S. coelicolor* hosts an impressive number of P450 reductases,[40] the heterologous interactions are most likely highly inefficient, and result in poor enzymatic activity and low yield of the hydroxylated products. Recently, naturally occurring[41] and bioengineered[42] self-sufficient P450 systems with covalently fused reductase partners have been reported to have much higher catalytic efficiency. These strategies can be similarly adopted here to improve the efficiency of PdmJ and PdmW. Furthermore, analogous to the explanation provided for the lower conversion observed for PdmN, the low yield of **5** and **6** might be due to **2** not being the natural or preferred substrate of PdmJ and PdmW. In fact, **2** can at best be the natural substrate of one of these P450 enzymes. When we coexpressed PdmN with either of the P450 enzymes, we did not see compounds that are modified with both D-amino acid amidation and D-ring hydroxylation. Similarly, when both P450 enzymes were coexpressed, we were not able to detect the 5,6-dihydroxylated analogue of **2**; this is likely due to the low efficiencies of the individual hydroxylation reactions.

In summary, we have investigated three important tailoring steps in the *pdm* biosynthetic pathway starting from the pen-

tangular aglycon **2**. Both PdmN and PdmJ have been shown to catalyze highly stereospecific and regiospecific reactions en route to the complete assembly of **1.** Whereas the continued reconstitution of the pradimicin biosynthetic pathway might be complicated by the low efficiencies of the enzymes in the heterologous host, in particular the P450 enzymes, our work has revealed new enzymes that may be used in the semisynthesis of pradimicin analogues, as well as combinatorial biosynthesis of other aromatic polyketides.

Experimental Section

General: 1D and 2D NMR spectra were recorded in [D_6]DMSO on a Bruker DRX-500 instrument (500 MHz for ^{1}H NMR and 125 MHz for ^{13}C NMR). The chemical shift values (δ) are given in parts per million (ppm), and the coupling constants are in Hz. High resolution ESI-MS were measured on an IonSpec Ultima 7T FTICR instrument. IR spectra were measured on an Avatar 370 FT-IR instrument (Thermo Nicolet). Optical rotations were measured in MeOH on an Autopol® IV automatic polarimeter. Analyses and separations of extracts were performed on a Beckman–Coulter HPLC instrument.

Strains and culture conditions: *Escherichia coli* TOPO 10 (Invitrogen) and XL-1 Blue (Stratagene) strains were used for cloning and plasmid manipulations. *S. coelicolor* CH999 was used as the heterologous host for expression for the pRM5 derived plasmids and engineered biosynthesis of pradimicin compounds. Protoplast preparation and PEG-mediated transformation were performed as described previously.[43] The transformants were grown on R5 agar supplemented with thiostrepton (50 mg L^{-1}) at 28 °C for 8 days.

Construction of plasmids: PCR was performed by using Platinum Pfx DNA polymerase (Invitrogen). The primers were designed based on the gene sequence information from the gene cluster to amplify the individual genes (Table S1). Restriction sites such as XbaI and NheI were introduced into the primers for subcloning. All genes were amplified through PCR with the genomic DNA of *A. hibisca* P157-2 as the template. The PCR products were cloned into pCR-Blunt vector (Invitrogen) for subcloning and plasmids are constructed by the sequential addition of each gene of interest by using T4 DNA ligase (Invitrogen). The recombined gene cassettes are finally ligated into pRM5 shuttle vector before transformed into *S. coelicolor* CH999.

Isolation of compound 3: Plasmid pJX137 was transformed into *S. coelicolor* CH999 and the transformant was grown on R5 agar with thiostrepton (50 mg L^{-1}). After five days, a single colony was transferred to a new R5 plate and cultured for additional eight days. The solid culture was minced and extracted with EtOAc/MeOH/AcOH (89:10:1). The extract was dried with anhydrous Na_2SO_4 and evaporated to dryness. The residue was then dissolved in DMSO and analyzed on HPLC by using a Varian C_{18} reversed-phase column (5μ, 250×4.6 mm) with gradient elution from 5 to 95% MeCN/H_2O (0.1% TFA) over 30 min at a flow rate of 1 mL min^{-1}. To purify the compound, the extract was separated on a Sephadex LH-20 column, eluted with MeOH. Fractions containing **3** were pooled and further separated on an Alltima semi-preparative C_{18} reversed-phase column (5μ, 250×10 mm) with isocratic elution of 75% ACN-H_2O (0.1% TFA) at a flow rate of 4 mL min^{-1}, yielding pure yellow compound **3** (t_R = 8.0 min).

JX137a (**3**) was obtained as a yellow powder. $[\alpha]_D$ = +4.4 (c = MeOH, 0.045); IR (KBr): ν_{max} = 3444, 2844, 2344, 1650, 1624, 1597, 1362, 1264, 1199 cm^{-1}; HRESI-MS: *m/z* calcd for $C_{27}H_{20}O_9N$: 502.1138; found: 502.1136 $[M-H]^-$; for the ^{1}H and ^{13}C NMR data see Table S2 in the Supporting Information.

Isolation of compound 4: Transformant CH999/pJX137 was grown on a R5 agar plate that was supplemented with thiostrepton and D-serine (0.25%, *w/v*). The culturing conditions and separation procedures were same as those for compound **3** except that the retention times on the analytical column and semi-preparative column for **4** were 25.8 and 5.8 min, respectively.

JX137s (**4**) was obtained as a yellow powder. $[\alpha]_D$ = +6.9 (c = MeOH, 0.029); IR (KBr) ν_{max} = 3412, 2932, 2839, 1722, 1621, 1597, 1475, 1398, 1257, 1165, 1097 cm^{-1}; HRESI-MS: *m/z* calcd for $C_{27}H_{20}O_{10}N$: 518.1093; found: 518.1108 $[M-H]^-$; for the ^{1}H and ^{13}C NMR data see Table S3 in the Supporting Information.

Isolation of compound 5: Transformant CH999/pJX134 was grown on R5 agar plates that were supplemented with thiostrepton (50 mg L^{-1}) and under the same conditions as described for compounds **3** and **4**. The extraction and separation procedures are same as those for **3** and **4**. The retention time for **5** is 6.5 min on the Alltima semipreparative C_{18} reversed-phase column.

JX134 (**5**) was obtained as a orange powder. $[\alpha]_D$ −2.99 (c = 0.17, MeOH); IR (powder) ν_{max} = 3372, 2845, 2475, 1758, 1589, 1464, 1403, 1291, 1262, 1147, 989, 942, 776 cm^{-1}; UV (MeOH): λ_{max} (logε) = 230 (4.41), 298 (4.23), 459 nm (3.93); CD $[\theta]_{209}$ +73 899, $[\theta]_{224}$ −28 345, $[\theta]_{241}$ +35 431, $[\theta]_{259}$ −89 084, $[\theta]_{266}$ −95 158, $[\theta]_{303}$ +41 505, $[\theta]_{337}$ +24 296; ESI-MS *m/z*: 447 $[M-H]^-$; HRESI-MS; *m/z* calcd for $C_{24}H_{15}O_9$: 447.0722; 447.0715 $[M-H]^-$; for the ^{1}H and ^{13}C NMR data see Table S4 in the Supporting Information.

JX152 (**6**): UV (MeOH) λ_{max} (logε) = 230 (4.41), 298 (4.23), 459 nm (3.93); ESI-MS *m/z*: 447 $[M-H]^-$; HRESI-MS: *m/z* calcd for $C_{24}H_{15}O_9$: 447.0722; found: 447.0731 $[M-H]^-$.

Acknowledgements

This work is supported by funds from the University-wide AIDS Research Program of the University of California, grant number ID06-LA-193 and a National Science Foundation CAREER award CBET 0545860. We thank Prof. Michael Jung for access to optical rotation measurements, and Wenjun Zhang for careful reading of the manuscript.

Keywords: amino acids · biosynthesis · metabolism · P450 hydroxylase · polyketides

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Received: February 14, 2009
Published online on May 8, 2009

