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Synergistic Actions of a Monooxygenase and Cyclases in Aromatic Polyketide Biosynthesis

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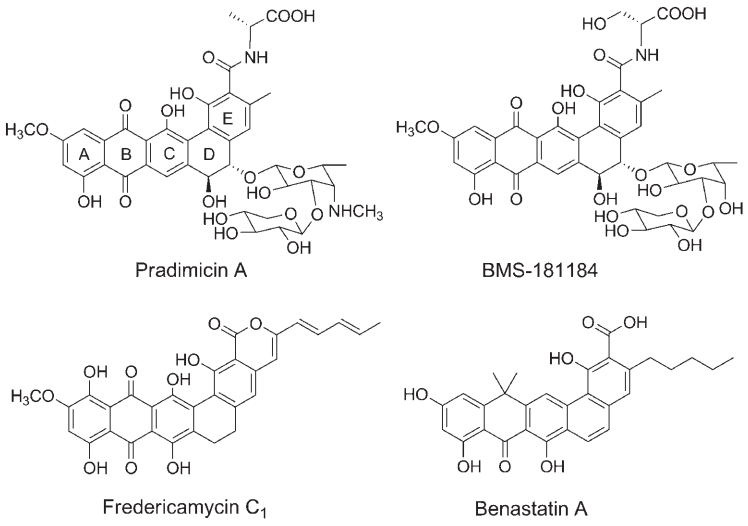
Type II polyketide synthases (PKSs) are enzymes responsible for the biosynthesis of bacterial aromatic polyketides.[1,2] The type II minimum PKS—which consists of the β-ketoacyl synthase (KS_α), chain length factor (CLF or KS_β), and acyl carrier protein (ACP)—synthesizes the nascent polyketide chain by successive Claisen-like decarboxylative condensations. The nascent polyketide chain is then subjected to regioselective reduction and cyclization catalyzed by dedicated ketoreductases (KRs) and cyclases, respectively, to yield an aromatic aglycon. In general, these immediate tailoring enzymes function as independent proteins, a feature that has facilitated the use of heterologous combinations of KRs and cyclases for the biosynthesis of new polyketides.[3]

Pradimicin A (Scheme 1) is an aromatic polyketide biosynthesized by *Actinomadura hibisca* P157–2.[4] It contains a dihydrobenzo[α]naphthacenequinone core, derived from a dodecaketide backbone through five regioselective cyclizations. The carboxyl end of the polyketide at ring E is amidated with D-alanine, while the nonaromatic ring D is substituted with a disaccharide consisting of D-xylopyranose and 4-methylamino-4-deoxy-D-fucopyranose. Together, these structural elements make pradimicin A an effective viral entry inhibitor[5,6] and a broad-spectrum fungicide.[7] Numerous pradimicin analogues, generated by synthetic,[8–10] semisynthetic,[11–13] and strain mutagenesis[14–16] methods, have been reported. As an example, the more water-soluble derivative BMS-181184 (Scheme 1) exhibited antifungal activity against 167 different fungal strains.[17] Because of the complexity of the pradimicin molecule, however, engineered biosynthesis through manipulation of the pradimicin (*pdm*) PKS enzymes remains an attractive approach for further development of pradimicin analogues.

Pradimicin A is a dihydrobenzo[α]naphthacenequinone that belongs to a family of polyketides including rubromycin,[18] griseorhodin,[19] benastatin, and fredericamycin, all of which are derived from biosynthetic intermediates that contain a pentangular aglycon. The recent sequencing of the benastatin[20] and fredericamycin[21] gene clusters, in conjunction with genetic and biochemical experiments, have provided insights into the biosynthesis of this class of polyketides.[22–24] However, the regioselective cyclization steps that yield the pentangular core have remained uncharacterized. The cyclization steps are significantly different from those dictating the formation of anthraquinones,[25] anthracyclines,[26] tetracyclines,[27] and angucyclines.[28] In particular, it is unclear how the cyclases can direct the regioselective intramolecular aldol condensations, while preventing the extended, dodecaketide backbone from spontaneous cyclization.

Recent sequencing of the 39 kb *pdm* gene cluster from *A. hibisca* revealed 28 open reading frames (ORFs).[29] Enzymes that are putatively associated with the biosynthesis of the pradimicin aglycon **5**, including the previously identified minimal PKS and tailoring enzymes, are shown in Table 1.[30] Consistently with the gene clusters reported for other pentangular polyketides, the *pdm* gene cluster contains only three putative cyclases (PdmD, PdmK, and PdmL). Using the *Streptomyces coelicolor* strain CH999[31] as the heterologous host,[32] we had previously confirmed the



Scheme 1. Pradimicins and structurally related aromatic polyketides.

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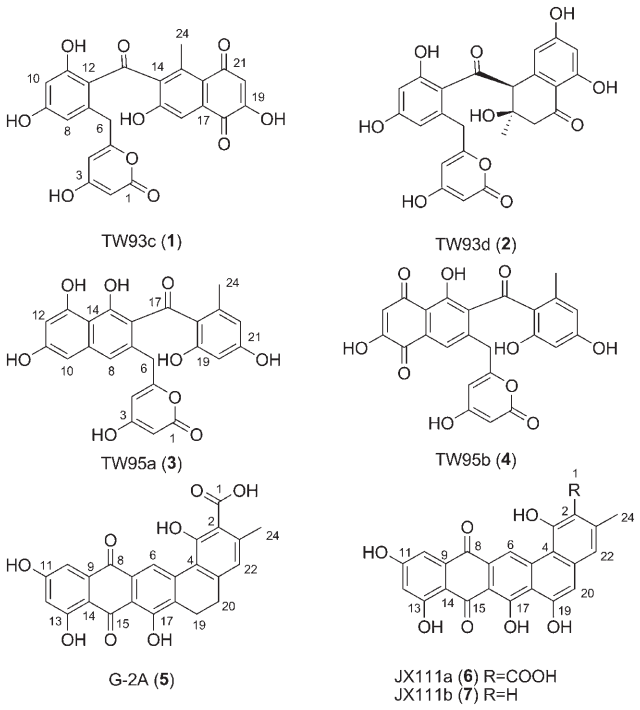
Table 1. Proposed enzymes involved in the biosynthesis of the pentangular aglycon of pradimicin A.

Enzyme	Proposed role	Enzyme	Proposed role
PdmA	β-ketoacyl synthase	PdmG	ketoreductase
PdmB	chain length factor	PdmH	monooxygenase
PdmC	acyl carrier protein	PdmI	monooxygenase
PdmD	cyclase	PdmK	cyclase
PdmE	monooxygenase	PdmL	cyclase

functions of the *pdm* minimal PKS (PdmABC) and PdmD, The *pdm* minimal PKS synthesized a spectrum of compounds in CH999, including the dodecaketides TW93c (**1**) and TW93d (**2**; Scheme 2), first described by Moore and co-workers, who used



Scheme 2.

the *whiE* and *sch* minimal PKSs.[33] Coexpression of PdmD facilitated the cyclization of the A- and B-rings through C9–C14 and C7–C16 intramolecular aldol condensations and afforded predominantly TW95a (**3**) and TW95b (**4**) in high yields. The roles of the remaining enzymes in the synthesis of the pentangular structure, including those that cyclize rings C, D, and E, as well as enzyme candidates that oxidize ring B or reduce ring D, have not been resolved. It is therefore significant to identify the minimum set of enzymes required for the formation of the pradimicin aglycon **5**. Reconstitution of **5** in a robust heterologous host should enable further studies and manipulation of the biosynthesis of this family of aromatic polyketides.

PdmK (115 aa) shows strong sequence homology to cyclases found in angucycline gene clusters, such as SimA4 (54% identity) from the simocyclinone gene cluster,[34] and to cyclases present in spore pigment PKSs, such as WhiE-ORFVII (51%) from *S. coelicolor*. PdmL (147 aa) is predicted to have a cupin 2 barrel fold and is similar to putative cyclases found in several spore pigment PKSs. We first examined the roles of PdmK and PdmL in the biosynthesis of pradimicin A. We constructed pJX70 (Table 2), which contains a multicistronic cassette *pdmABCDGKL*, and transformed it into CH999. The gene *pdmG* encodes an enzyme that is homologous to BenL from the benastatin pathway,[22] which was previously characterized as a C19 KR by Hertweck and coworkers. HPLC analysis of the organic extract of CH999/pJX70 revealed that the predominant

Table 2. Plasmids and the corresponding polyketides biosynthesized by CH999 transformed with the plasmid.[a]

	pdm genes							
Plasmids	D	E	G	H	I	K	L	Products
pYT241	–	–	–	–	–	–	–	**1, 2**
pYT259	+	–	–	–	–	–	–	**3, 4**
pJX70	+	–	+	–	–	+	+	**3, 4**
pJX77	+	+	+	–	–	+	+	**3, 4**
pJX114	+	–	+	–	+	+	+	**3, 4**
pJX116	+	–	+	+	–	+	+	**5**
pJX113	+	–	+	+	–	–	+	**3, 4**
pJX115	+	–	+	+	–	+	–	**3, 4**
pJX144	+	–	+	+	–	–	–	**3, 4**
pJX47	–	–	+	–	–	–	–	**1, 2**
pJX51	+	–	+	–	–	–	–	**3, 4**
pJX111	+	–	–	+	–	+	+	**6, 7**

[a] Each plasmid encodes the minimal Pdm PKS (*pdmA*, *pdmB*, and *pdmC*)

products synthesized remained **3** and **4** (Figure 1), the same as those synthesized by CH999/pYT259, which only expresses the minimal PKS and PdmD. This result suggests that the addition of PdmK and PdmL was insufficient to cyclize rings C, D, and E, and additional enzymes may be required.

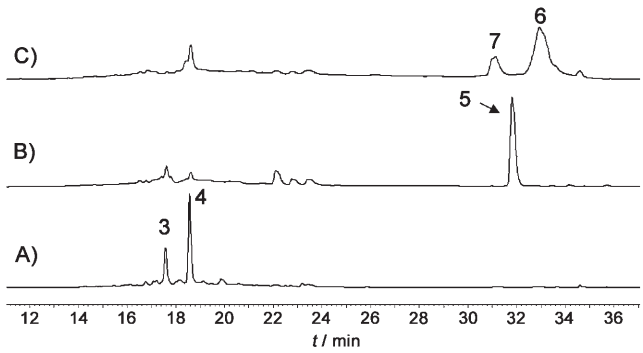


Figure 1. HPLC analysis (430 nm) of extracts of A) CH999/pJX70, B) CH999/pJX116, and C) CH999/pJX111.

Since no other putative cyclases are present in the *pdm* gene cluster, we reasoned that an alternative modification of the polyketide might be a prerequisite step before the cyclization of the C-, D-, and E-rings can take place. A likely such tailoring step is the formation of the quinone moiety in ring B by a monooxygenase. Sequence analysis of the putative oxygenases encoded in the *pdm* gene cluster revealed three possible candidate enzymes: PdmE, PdmH, and PdmI (Table 1). Although all three enzymes were predicted to encode monooxygenases, none showed significant homology to known quinone-forming oxygenases such as DnrG,[35] TcmH,[36] and AknX.[37] To determine the enzyme(s) responsible for ring B hydroxylation, we introduced *pdmE*, *pdmH*, and *pdmI* separately into pJX70 to yield pJX77, pJX116, and pJX114, respectively (Table 2). These plasmids were each transformed into CH999, and the extracts were analyzed by HPLC. Interestingly, while coexpression of PdmE or PdmI produced the same polyketides

3 and **4**, coexpression of PdmH in CH999/pJX116 resulted in the biosynthesis of a new predominant compound **5** (Figure 1) in high yield (50 mg L^{-1}). The emergence of the new compound is accompanied by a corresponding decrease in the levels of **3** and **4** to $<10\%$ of the levels seen in CH999/pJX70. Compound **5** displayed maximum UV absorptions at 243, 300, and 466 nm, suggesting the presence of an extended chromophore. HRESIMS showed that this compound has a molecular formula of $C_{24}H_{16}O_8$, indicating it to be derived from a dodecaketide backbone. The ^{13}C NMR spectrum (Table 3) showed 24

Table 3. ^1H (500 MHz, [D$_6$]DMSO) and ^{13}C (125 MHz, [D$_6$]DMSO) NMR data for compounds **5** and **7**.

	5		**7**	
	^{13}C	^1H (J in Hz)	^{13}C	^1H (J in Hz)
1	173.7			
2	113.1		116.6	6.79 (br s, 1H)
3	161.4		156.7	
4	112.7		112.6	
5	142.8		138.3	
6	119.6	8.81 (s, 1H)	120.1	10.05 (s, 1H)
7	130.2		131.6	
8	181.5		182.8	
9	135.4		135.3	
10	108.8	7.16 (d, J=2.0 Hz, 1H)	106.3	7.12 (d, J=2.0 Hz, 1H)
11	165.5	11.40 (br s, 1H, OH)	163.6	
12	107.8	6.61 (d, J=2.0 Hz, 1H)	108.0	6.53 (d, J=2.0 Hz, 1H)
13	164.4	12.11 (s, 1H, OH)	164.7	13.80 (s, 1H, OH)
14	111.2		111.2	
15	189.5		186.1	
16	113.2		111.5	
17	157.9	12.53 (s, 1H, OH)	158.5	
18	130.2		119.2	
19	19.9	2.83 (m, 2H)	152.2	
20	28.2	2.75 (m, 2H)	109.7	6.89 (s, 1H)
21	144.8		134.6	
22	124.2	6.76 (s, 1H)	117.8	7.00 (br s, 1H)
23	147.6		138.6	
24	23.6	2.52 (s, 3H)	21.2	2.36 (s, 3H)

signals, including those associated with a quinone ($\delta_{C8}=181.5$ and $\delta_{C15}=189.5$ ppm). An additional carbonyl peak ($\delta_{C1}=$ 173.7 ppm) was observed and is indicative of a carboxylic acid. A CH_2–CH_2 spin system ($\delta_{H19}=2.83$ ppm, $\delta_{H20}=2.75$ ppm) was observed in the ^1H–^1H COSY spectrum and was strongly indicative of the presence of a reduced ring D as is found in **5**. All remaining 1D and 2D NMR data were consistent with the structure of **5**, which had previously been isolated from *Frankia* sp. G2 as G–2A.[38,39] The accumulation of **5** by CH999/pJX116 demonstrates that the *pdmABCDHGKL* collection of genes is sufficient for the biosynthesis of the dihydrobenzo[α]naphthacenequinone core of pradimicin A. This result also directly associates PdmH with the formation of the quinone moiety on ring B.

We then constructed plasmid pJX144, which includes *pdmABCDGH,* to test the timing of the quinone formation reaction. We reasoned that in the absence of the cyclases PdmK and PdmL, an oxidized product at C-8 of **3** may be recovered as a result of spontaneous poly-β-ketone cyclization. HPLC analysis of the extract of CH999/pJX144 showed that no new polyketides were synthesized in addition to **3** and **4**, indicating that PdmH cannot catalyze oxidation of ring B in the absence of either PdmK or PdmL. This unexpected finding provides the first evidence for a possible tailoring mechanism in which the monooxygenase and the cyclases work in concert to afford **5**.

To analyze the roles of cyclases and their interactions with PdmH further, *pdmK* or *pdmL* were introduced separately into pJX144 to yield plasmids pJX113 and pJX115, respectively (Table 2). We anticipated that if each cyclase can act independently, we might be able to isolate a shunt product containing a C5–C17 cyclized ring C, which might allow us to assign regiospecificity to the cyclases. Surprisingly, the major metabolites from CH999 transformed with either construct were **3** and **4**, and no new polyketides were produced. Therefore, removal of any one of PdmH, PdmK, or PdmL abolishes the formation of **5**, which suggests that the three enzymes work synergistically to oxidize the B-ring, and to cyclize the C- and D-rings. Formation of the E-ring might either be spontaneous or equally might require the action of either PdmK or PdmL. We propose a model in which the three enzymes form a multienzyme complex that engulfs the cyclized B-ring and uncyclized portions of the polyketide chain. PdmH may be positioned in close proximity to the B-ring to facilitate hydroxylation of C8, while PdmK and PdmL orient the two ends of the polyketide chain to direct the required C5–C17 and C4–C21 aldol condensations. The enzymes cannot act independently without the associated partners, a unique property that gives rise to the product phenotypes listed in Table 2. Neither the minimal PKS, nor the first ring cyclase PdmD is required for the functions of this putative enzyme complex. While multioxygenase complexes have been shown to be involved in angucycline oxidation,[40] this is the first example of multiple cyclases and a monooxygenase working concertedly in the regioselective tailoring of a polyketide backbone.

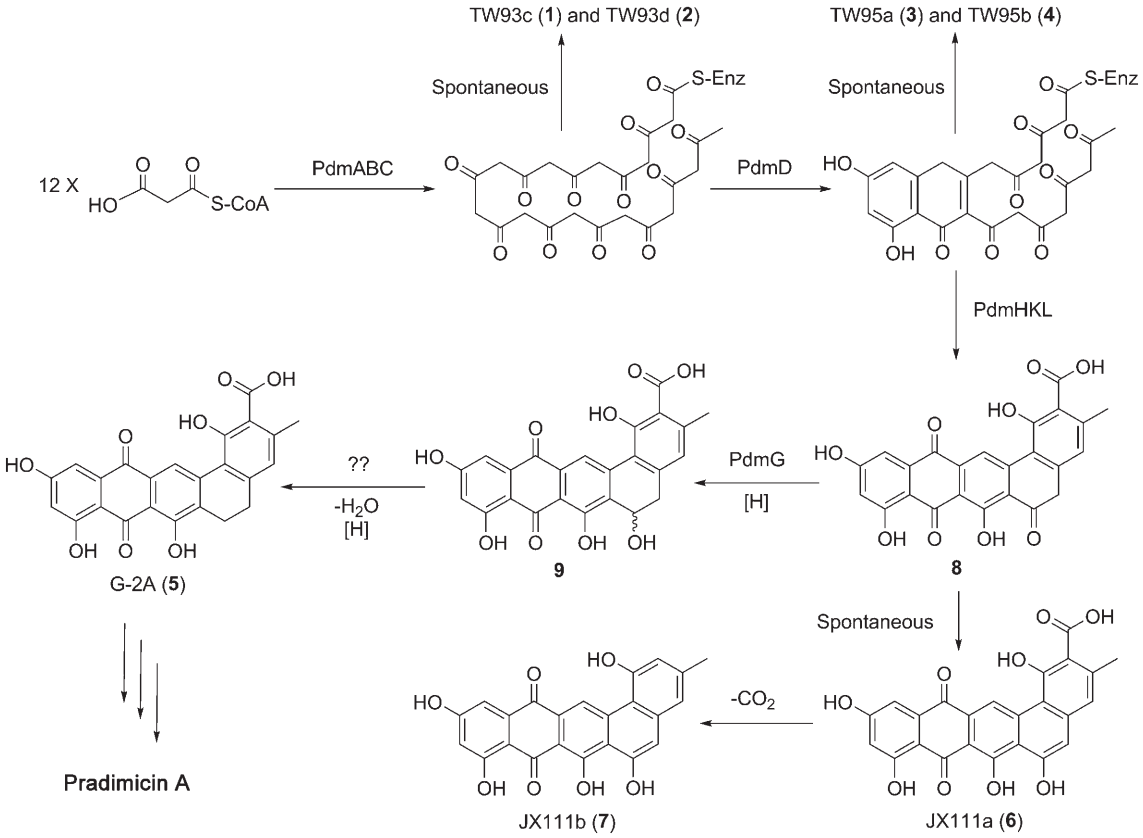
This model can be applied to interpret a phenomenon reported by Moore and co-workers involving *whiE*-ORFII and *whiE*-ORFVII, two uncharacterized cyclases in the *whiE* spore pigment PKS. Individual expression of either cyclase with the *whi*E minimal PKS, along with the first- and second-ring cyclase *whiE*-ORFVI in CH999, produced **3** and **4**. In contrast, **3** and **4** were no longer present and new compounds were produced when the minimal PKS and all three cyclases were coexpressed at the same time.[33] Although these new products were not characterized, the alteration in product profile is consistent with the results reported here. Therefore, the synergistic actions of cyclases and other tailoring enzymes may be a general mechanism associated with dodecaketide synthases. Why is the multienzyme complex needed for the longer polyketide chain? We reason that sequential binding and tailoring of the long poly-β-ketone by individual enzymes may not be fast enough to prevent spontaneous cyclization of the reactive backbone. While an individual tailoring enzyme can only bind to a portion of the extended polyketide chain, a multienzyme complex can effectively span the entire length of the uncyclized polyketide to lock the backbone in place and to suppress aberrant cyclizations. Tailoring of the shorter octaketide

and decaketides requires fewer steps and is therefore less prone to spontaneous cyclization. As a result, separate actions of cyclases are sufficient to yield the correctly cyclized three- and four-ring aromatic structures.

PdmG is the only putative KR found in the pradimicin biosynthetic pathway, and it is proposed that it catalyzes the reduction of C19 during the biosynthesis of **5**. The timing of the reduction is unclear, with one proposal suggesting that PdmG might function on the uncyclized polyketide in a fashion similar to that of the well-studied *act* KR.[2] We first confirmed the function of PdmG by constructing pJX111, which harbors the genes *pdmABCDHKL*. Two major products—JX111a (**6**) and JX111b (**7**)—were observed in the extract of CH999/pJX111, while no trace of **5** could be detected. HPLC-ESIMS showed that the molecular weights of these two compounds were 446 and 402, respectively. Compound **6** gradually converted into **7** during the extraction process, suggesting that **7** is probably the decarboxylated product of **6**. Compound **7** was then purified in a yield of 33 mg L^{-1} and was structurally characterized as shown in Scheme 2. HRESIMS predicted a molecular formula of $C_{23}H_{14}O_7$, while the ^{13}C NMR spectrum contained 23 unique signals, both of which agree with those of a dodecaketide compound with the loss of CO_2. With the exception of the methyl group on the acetyl starter unit ($\delta_{H19} = 2.83$ ppm), all the proton signals in the ^1H NMR spectrum of **7** were at low field, consistent with those of a highly conjugated polyphenol. The phenol group in ring D was positioned at C19 on the basis of ^1H,^{13}C HMBC correlations. Through its 1D and 2D spectra, compound **7** was identified as a pentahydroxy-methylbenzo[α]tetracenedione. By inference, the structure of **6** was established as the corresponding carboxylic acid as shown in Scheme 2. The biosynthesis of **6** and **7** unambiguously confirmed the function of PdmG as a C19 KR, analogously with the assigned role of BenL during benastatin biosynthesis.[22]

We further tested the timing of the PdmG-catalyzed reduction reaction. *pdmG* was inserted into pYT241 and pYT259 to yield pJX47 and pJX51, respectively (Table 2). CH999/pJX47 continued to produce **1** and **2**, indicating that PdmG does not function on the nascent polyketide chain. Similarly, CH999/pJX51 and CH999/pYT259 produced the same products **3** and **4** (Table 2). Taken together, these in vivo results suggest that PdmG most likely acts on the C19 ketone after the pentangular core has been established by the PdmH-PdmK-PdmL trio (Scheme 3). Although we were able to obtain soluble PdmG by using *E. coli* as an expression host, definitive confirmation of the reduction reaction was hampered by the inaccessibility of the C19 ketone-containing compound **8**, which rapidly isomerizes into the more stable phenol **6**.

Our results provide significant new insight into the biosynthesis of pentangular aromatic polyketides. The early steps leading to the biosynthesis of the dihydrobenzo[α]naphthacenequinone core of pradimicin A are shown in Scheme 3. The minimum set of enzymes required to afford the benzo[α]naphthacenequinone **8** includes PdmABCDHKL. After biosynthesis



Scheme 3. Early steps of pradimicin A biosynthesis.

of the dodecaketide backbone by the minimal PKS, the C9–C14 and C7–C16 regioselectivities of the first two rings are determined by PdmD. Whereas PdmD can function as a standalone enzyme, the subsequent tailoring reactions require concerted action of the monooxygenase PdmH and the two remaining cyclases PdmK and PdmL. Deletion of any one of the three enzymes leads to the shunt products **3** and **4**. The synergistic actions of multiple enzymes during the synthesis of **8** may be a representative example of nature's strategy to suppress undesirable, spontaneous cyclizations of extended polyketide backbones, especially those requiring extensive tailoring steps.

Reduction of **8** by PdmG led to the formation of **9**. Surprisingly, neither **9** nor the C19–C20 dehydrated version of **9**, which can form spontaneously, were found in the extracts of CH999/pJX116. Instead, the completely reduced **5** was isolated as a predominant product. The nonaromatic ring D was observed among other pentangular polyketides, including fredericamycin C1 and several benastatins.[22] It remains unclear how the final reduction step that give rise to the C19–C20 single bond, which may involve endogenous *Streptomyces* enoylreductases, is achieved. The reconstitution of **5** establishes a versatile platform for further biochemical analysis of enzymes that give rise to the unique structural features of pradimicin A, including those involved in ligation of the carboxyl end with D-alanine, and hydroxylation of C19 and C20 on ring D.

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). Low-resolution ESIMS were obtained on a Finnigan LCQ Deca XP quadrupole ion trap mass spectrometer. High-resolution ESIMS were measured on an IonSpec Ultima 7T FTICR instrument. Analyses and separations of extracts were performed on a Beckman–Coulter HPLC instrument.

Strains and culture conditions: *S. coelicolor* CH999 was used as the host for heterologous expression for all pRM5-derived plasmids. Protoplast preparation and PEG-mediated transformation were performed as described previously.[41] The transformants were grown on R5 agar supplemented with thiostrepton at 50 μg mL^{-1} at 28 °C for eight days. *E. coli* Topo 10 (Invitrogen) and XL-1 Blue (Stratagene) strains were used for subcloning and plasmid manipulations. Growth of *E. coli* was on Luria–Bertani (LB) agar and in LB liquid medium with kanamycin or ampicillin at 37 °C.

Construction of plasmids: PCR was performed with Platinum *Pfx* DNA polymerase (Invitrogen). 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 all the recombined gene cassettes were ligated into pRM5 shuttle vector before transformation into CH999. T4 DNA ligase (Invitrogen) was used for ligation of fragments. The different combinations of genes were ligated as a polycistronic cassette into the shuttle vector. Each plasmid is constructed by the sequential addition of each gene of interest. We did not delete any genes of a polycistronic cassette in the shuttle vector to yield new constructs with fewer genes.

Extraction and isolation: HPLC analyses were carried out on a Varian C-18 reversed-phase column (5 μm, 250×4.6 mm) with gradient elution from 5% to 95% ACN/H_2O (0.1% TFA) over 30 min at a flow rate of 1 mL min^{-1}. All large-scale culturing experiments were performed in R5 agar (3 L) over eight days. The following separation procedure was used to isolate the pure compounds. The cultures (3 L) were homogenized and extracted three times with EtOAc/MeOH/AcOH (89:10:1, 3 L). The extracts were dried with anhydrous Na_2SO_4 and evaporated to dryness. These extracts were first placed on a Sephadex LH–20 column and eluted with MeOH/AcOH (9:1). The fractions containing the target compounds were then further separated on an XTerra preparative MS C18 column (5 μm, 50×19 mm) with isocratic elution (ACN/H_2O, 75%, 0.1% TFA) at a flow rate of 4 mL min^{-1}, yielding pure compounds **5** (t_R = 6.8 min) and **7** (t_R = 2.4 min).

G-2A (5): Orange powder; ^{1}H and ^{13}C NMR see Table 3; IR (powder): $\tilde{\nu}$ = 3392, 2917, 2352, 1683, 1622, 1410, 1338, 1252, 1201, 1132, 801, 722 cm^{-1}; UV (MeOH): λ_{max} (log ε) = 243 (4.20), 300 (4.19), 466 nm (3.91 mol^{-1} dm^3 cm^{-1}); ESIMS: *m/z* 431 [*M*−H]$^-$; HRESIMS: *m/z* 431.0772 [*M*−H]$^-$ (calcd for $C_{24}H_{15}O_8$: 431.0767).

JX111b (7): Red powder; ^{1}H and ^{13}C NMR see Table 3; IR (powder): $\tilde{\nu}$ = 3386, 2921, 1602, 1563, 1345, 1278, 1172, 1021, 839, 749 cm^{-1}; UV (MeOH): λ_{max} (log ε) = 226 (4.16), 266 (4.04), 300 (3.91), 336 (3.71), 493 nm (3.55 mol^{-1} dm^3 cm^{-1}); ESIMS: *m/z* 401 [*M*−H]$^-$; HRESIMS: *m/z* 401.0668 [*M*−H]$^-$ (calcd for $C_{23}H_{13}O_7$: 401.0661).

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Keywords: cyclases · engineered biosynthesis · monooxygenases · multienzyme complexes · polyketides

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