Engineered Biosynthesis of Novel Polyketides: Manipulation and Analysis of an Aromatic Polyketide Synthase with Unproven Catalytic Specificities

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Abstract: Genes for a putative aromatic polyketide synthase (PKS) of unproven catalytic specificity from *Streptomyces roseofulvus*, which produces both frenolicin B (**1**) and nanaomycin A (**2**), were functionally expressed in *Streptomyces coelicolor* A3(2) using a heterologous expression system in combination with components of the actinorhodin (**3**) PKS gene cluster. Spectroscopic characterization and isotopic labeling experiments showed that the primary product of the recombinant strain was a novel tricyclic molecule (**6**), derived from a nonaketide backbone that undergoes a single ketoreduction. In addition, two octaketides (**4** and **7**) were produced by this PKS, suggesting that the chain length determining factor (CLF) of this PKS has a relaxed specificity and providing strong support for the supposition that the cloned genes encoded the PKS for frenolicin and nanaomycin biosynthesis. The regiospecificity of ketoreduction of the putative backbone of one of the octaketides (**7**) argues in favor of a biosynthetic model in which ketoreduction occurs only after the complete polyketide chain has been synthesized. Construction and analysis of hybrid PKSs containing different subunits from the actinorhodin and the above PKS confirmed earlier conclusions regarding the protein determinants of chain length, ketoreduction, and cyclization specificities.

Polyketides are a large, structurally diverse family of natural products possessing a broad range of biological activities, including antibiotic and pharmacological properties. Biosynthetic and molecular genetic studies have clearly demonstrated that polyketide synthases (PKSs) are structurally and mechanistically related to each other and to fatty acid synthases (FASs).[2] The PKSs are multifunctional enzymes that catalyze repeated (decarboxylative) Claisen condensations between acylthioesters (usually acetyl, propionyl, malonyl, or methylmalonyl). Following each condensation, they introduce structural variability into the product by catalyzing all, part, or none of a reductive cycle comprising a ketoreduction, dehydration, and enoylreduction on the β-keto group of the growing polyketide chain. After the carbon chain has grown to a length characteristic of each specific product, it is released from the synthase by thiolysis or acyl transfer.

Recently, we described a generally applicable strategy for the biosynthesis of novel polyketides by the functional expression of recombinant PKSs in a specially constructed expression system.[3] Analysis of the structures of the molecules revealed key features of the mechanisms by which the PKSs for polycyclic aromatic compounds control their catalytic specificities. It was shown that (i) the chain length is, at least in part, dictated by a protein called the chain length determining factor (CLF; Figure 1), (ii) while the association of some heterologous ketosynthase/acyltransferase (KS/AT; Figure 1)–CLF pairs gave rise to functional PKSs, other pairs were nonfunctional, (iii) acyl carrier proteins (ACPs; Figure 1) could be interchanged among different synthases without any effect on product structure, (iv) a given ketoreductase (KR; Figure 1) recognized and reduced polyketide chains of different lengths, (v) regardless of the chain length, this ketoreductase catalyzed a single ketoreduction at the C-9 position, counting from the carboxyl end, (vi) if this ketoreduction was allowed to occur, the regiospecificity of the first cyclization was constant relative to the position of ketoreduction, regardless of the chain length, and (vii) a specific cyclase (Figure 1) responsible for the second cyclization reaction could not cyclize substrates of altered chain lengths.

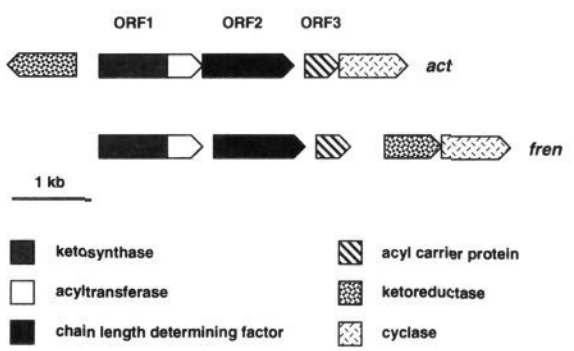

Figure 1. The *act* and *fren* PKS gene clusters. Each PKS includes a ketosynthase/acyltransferase (KS/AT, ORF1), a chain length determining factor (CLF, ORF2), an acyl carrier protein (ACP, ORF3), and a ketoreductase (KR). The AT presumably tranfers the starter unit from CoA to the KS, which catalyzes the Claisen condensation between the starter (or growing polyketide) acylthioester and the extender thioester on the ACP. The KR reduces carbonyls of the polyketide backbone. The gene clusters also code for a cyclase, involved in cyclization of the nascent polyketide backbone. The genetic homology depicted here is conserved throughout most actinomycete aromatic PKSs.

Here, we extend the earlier approach[3] by generating recombinant PKSs incorporating components from a PKS gene set of unproven (though conjectured) specificity, and use the structures of the novel polyketides to confirm the role of the unknown PKS. The genes encoding such PKSs can readily be cloned from actinomycete species by taking advantage of the high degree of

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sequence similarity between members of different PKS subgroups.[4,5] Using this expression system, it should therefore be possible to generate a wide variety of novel polyketide backbone chains by constructing a library of recombinant PKSs with components cloned from different polyketide producers.

Isotope labeling and NMR analysis of the products of the recombinant PKS described here have allowed us to address two key questions that arose in our earlier work[3] and that of others:[6,7] (i) does ketoreduction of polycyclic aromatic polyketide backbones occur in the β-position, or does it occur after the complete carbon backbone is synthesized, and (ii) do cyclases discriminate between polyketide chains of different lengths, or is their inability to catalyze the cyclization of polyketide chains of altered lengths the result of lack of recognition of CLFs having different chain length specificities? We have also identified the first example of a PKS with a relaxed chain length specificity that allows it to produce both octaketide and nonaketide products.

Results

Streptomyces roseofulvus produces both frenolicin B (**1**)[8] and nanaomycin A (**2**).[9] The carbon chain backbones of these aromatic polyketide products have been hypothesized to be derived from nine and eight acetates, respectively,[2e] although *in vivo* acetate labeling studies have not been carried out on this strain. Furthermore, it is presumed that both carbon chains undergo a β-ketoreduction at the C-9 position and that the frenolicin backbone also undergoes a complete cycle of β-ketoreduction, dehydration, and enoylreduction at the C-17 position. A 10-kb DNA fragment (referred to as the *fren* fragment hereafter) was cloned from a genomic library of *S. roseofulvus*[5] using DNA encoding the KS/AT and KR components of the actinorhodin (**3**) (*act*) PKS of *S. coelicolor* A3(2) as a hybridization probe.[4a,10] The *act* PKS catalyzes the biosynthesis of the octaketide precursor of actinorhodin, another aromatic polyketide, whose backbone is derived from eight acetates with a single ketoreduction at the C-9 position (i.e. identical to that of nanaomycin).[11] DNA sequencing of the *fren* fragment[5] revealed the existence of genes with a high degree of identity to those encoding the genes from the *act* cluster involved in carbon chain formation and cyclization; these are *actI*-ORF1, -2, and -3 genes that encode a putative bifunctional ketosynthase/acyltransferase (KS/AT), a chain length determining factor (CLF), and an acyl carrier protein (ACP), respectively; a ketoreductase (KR); and a cyclase (Figure 1).

The host-vector system used for expression and analysis of recombinant PKSs in this work is identical to that reported earlier;[3] the vector was pRM5 and the host was *S. coelicolor* CH999 (a mutant from which the *act* cluster has been precisely deleted).

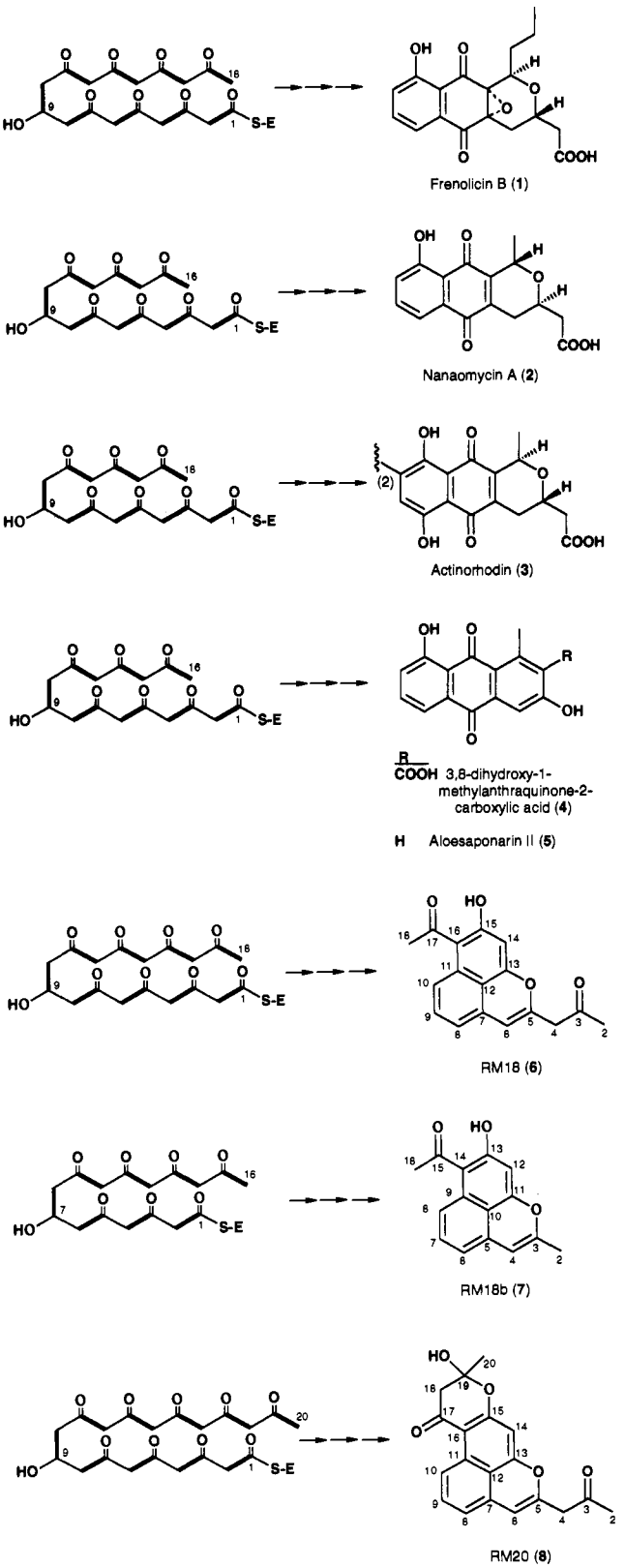

Figure 2. Polyketides (right) and deduced backbones (left) synthesized by PKSs.

The host was transformed with a pRM5 derivative containing the *fren* PKS genes that are presumably responsible for the biosynthesis of a polyketide backbone (i.e. ORFs 1-3) (Table I). In addition, plasmids carrying all possible combinations of *act* and *fren* ORFs 1-3 were also constructed and used to transform CH999 (Table I). The genes encoding the *act* KR and the *act* cyclase were also present on each of these genetic constructs. On the basis of results from similar experiments with *act* and *tcm*

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Table I. Summary of the Major Polyketide Products Isolated from Recombinant Strains with Various Combinations of *Act and Fren* ORFs 1–3 Genes[a]

plasmid	ORF1 (KS/AT)	ORF2 (CLF)	ORF3 (ACP)	major products
pRM5	*act*	*act*	*act*	**4,5**
pRM8	*fren*	*act*	*act*	**4,5**
pRM13	*act*	*fren*	*act*	NP
pRM23	*act*	*act*	*fren*	**4,5**
pRM18	*fren*	*fren*	*act*	**4,5,6,7**
pRM32	*fren*	*act*	*fren*	NP
pRM33	*act*	*fren*	*fren*	NP
pRM34	*fren*	*fren*	*fren*	**4,5,6,7**

[a] NP, no product.

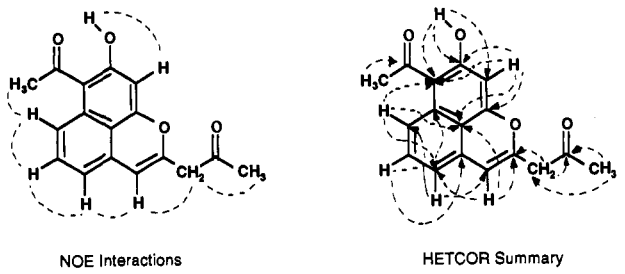

Figure 3. Summary of NOE and HETCOR (HMBC) data from RM18 (**6**).

(tetracenomycin) PKSs,[3] it was expected that the *act* KR would be able to reduce the products of all functional recombinant PKSs, whereas the ability of the *act* cyclase to catalyze the second cyclization would depend upon the chain length of the product of the *fren* PKS.

The results summarized in Table I indicate that most of the transformants expressed functional PKSs, as assayed by their ability to produce aromatic polyketides. Structural analysis of the major products revealed that the producer strains could be grouped into two categories: those that synthesized compound **4** (together with a smaller amount of its decarboxylated side product (**5**)) and those that synthesized a mixture of compounds **4**, **6**, and **7** in a roughly 1:2:2 ratio. (Small amounts of **5** were also found in all strains producing **4**.) Compounds **4** (3,8-dihydroxy-1-methylanthraquinone-2-carboxylic acid)[12] and **5** (aloesaponarin II)[6] had been observed before as natural products and were the metabolites produced by a PKS consisting entirely of *act* subunits.[3] Compounds **6** and **7** (designated RM18 and RM18b, respectively) however are novel structures whose chemical synthesis or isolation as natural products has not been reported previously.

The structures of **6** and **7** were elucidated through a combination of mass spectroscopy, NMR spectroscopy, and isotope labeling experiments. The ^{1}H and ^{13}C spectral assignments are shown in Table II along with ^{13}C–^{13}C coupling constants for **6** obtained through sodium [1,2-$^{13}C_2$]acetate feeding experiments (described below). Unequivocal assignments for compound **6** were established with 1D nuclear Overhauser effect (NOE) and long-range heteronuclear multiple bond correlation (HMBC) studies (Figure 3). Deuterium exchange confirmed the presence of hydroxyls at C-15 of compound **6** and C-13 of compound **7**. Field desorption mass spectrometry (FD-MS) of **6** and **7** revealed molecular weights of 282 and 240, respectively, consistent with $C_{17}H_{14}O_4$-MW (282.2952) and $C_{15}H_{12}O_3$MW (240.2580).

Earlier studies showed that the polyketide backbone of **5**[6] (and by inference, **4**[3]) is derived from iterative condensations of eight acetate residues with a single ketoreduction at C-9 (Figure 2). Nanaomycin (**2**) probably arises from an identical carbon chain backbone (Figure 2). Therefore, it is very likely that nanaomycin is a product of the *fren* PKS genes in *S. roseofulvus*. Regiospecificity of the first cyclization leading to the formation of **4** is constant relative to the position of the ketoreduction,[3] whereas that of the second cyclization is controlled by the *act* cyclase.[7b]

In order to trace the carbon chain backbone of RM18 (**6**), *in vivo* feeding experiments using [1,2-$^{13}C_2$]acetate were performed on CH999/pRM18, followed by NMR analysis of labeled RM18. The ^{13}C coupling data (summarized in Table II) indicate that the polyketide backbone of RM18 is derived from nine acetate residues, followed by a terminal decarboxylation (the C-2 ^{13}C resonance appears as an enhanced singlet), which presumably occurs nonenzymatically (Figure 2). Furthermore, the absence of a hydroxyl group at the C-9 position suggests that a ketoreduction occurs at this carbon. Since such a reduction on an 18-carbon chain would be expected to occur in the putative frenolicin (**1**) backbone, the results suggest that, in addition to synthesizing nanaomycin, the *fren* PKS genes are responsible for the biosynthesis of frenolicin in *S. roseofulvus*. This, to our knowledge, represents the first unambiguous case of a PKS with relaxed chain length specificity. However, unlike the putative backbone of frenolicin, the C-17 carbonyl of RM18 is not reduced. This could either reflect the absence from pRM18 of a specific ketoreductase, dehydratase, and enoylreductase (which might be present in the "*fren*" gene cluster in *S. roseofulvus*), or it could reflect a different origin for carbons 15–18 in frenolicin (for example, butyrate could be used as the starter unit for frenolicin biosynthesis).

Regiospecificity of the first cyclization leading to the formation of RM18 is constant relative to the position of the ketoreduction; however, the second cyclization occurs differently from that in **1** or **4** and is similar to the cyclization pattern observed in RM20 (**8**), a decaketide produced by the *tcm* PKS.[3] Therefore, as in the case of RM20, it could be argued that the *act* cyclase cannot catalyze the second cyclization of the RM18 precursor and that its subsequent cyclizations, which presumably occur nonenzymatically, are dictated by temporal differences in release of different portions of the nascent polyketide chain into an aqueous environment. In view of the ability of CH999/pRM18 (and CH999/pRM34) to produce **4**, one can rule out the possibility that the cyclase cannot associate with the *fren* PKS (KS/AT, CLF, and ACP). A more likely explanation is that the *act* cyclase cannot recognize substrates of altered chain lengths. This would also be consistent with the putative biosynthetic scheme for RM20.[3]

Although isotope labeling analysis on RM18b (**7**) has not been performed, upon the basis of a comparison with the carbon chain precursors of **4** and **6**, it is likely to be derived from eight acetates, followed by a terminal decarboxylation (Figure 2). (The alternative explanation, namely that RM18b results from deacetylation of RM18, is unlikely, particularly in light of the observation that RM20-producing cultures do not contain an equivalent product.) If this is true, then ketoreduction must have occurred at the C-7 position (instead of the more commonly observed C-9 position) in the polyketide chain precursor of RM18b (Figure 2). The simplest explanation for this unusual observation is that the ketoreduction occurs following synthesis of the complete polyketide chain, as opposed to when the target carbonyl is in the β-position (which is how ketoreductions occur in the case of macrolides; for example, see ref 13). Thus, given the observation that the *fren* PKS has a relaxed chain length specificity (see above), the somewhat loose fit of the shorter polyketide within a putative chain length determining pocket would result in occasional "slippage" of the full-length PKS product, giving rise to a stochastic mixture of C-7 and C-9 reduced chains (Figure 4). The same hypothesis has also been proposed earlier upon the basis of the observation that ketoreduction always occurs at a

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Table II. ^1H (400 MHz) and ^{13}C (100 MHz) NMR Data from RM18 (**6**) and RM18b (**7**)

RM18 (**6**)				RM18b (**7**)		
carbon[a]	^{13}C δ (ppm)	(J_{CC} (Hz))	^1H δ (ppm) (m, J_{HH} (Hz), area)	carbon[a]	^{13}C δ (ppm)	^1H δ (ppm) (m, J_{HH} (Hz), area)
2	29.6	NC[b]	2.2 (s, 3H)			
3	203.7	37.7				
4	47.0	36.9	3.6 (s, 2H)	2	18.8	2.1 (s, 3H)
5	149.6	77.2		3	152.3	
6	106.7	77.4	6.2 (s, 1H)	4	104.0	6.1 (s, 1H)
7	129.1	61.9		5	130.0	
8	114.4	62.1	6.7 (d, 7.2, 1H)	6	113.5	6.7 (d, 7.0)
9	130.1	58.9	7.3 (dd, 8.4, 7.4, 1H)	7	130.1	7.3 (dd, 7.1, 8.7, 1H)
10	120.6	59.2	7.6 (d, 8.9, 1H)	8	120.1	7.6 (d, 8.6, 1H)
11	132.7	56.0		9	132.8	
12	116.7	55.7		10	116.6	
13	155.6	74.7		11	155.9	
14	98.4	74.9		12	98.2	
15	158.8	69.6	6.4 (s, 1H)	13	159.1	6.4 (s, 1H)
16	113.6	69.3	11.2 (s, 1OH)	14	113.8	11.2 (s, 1OH)
17	201.7	41.9		15	201.7	
18	32.4	41.7	2.5 (s, 3H)	16	32.4	2.5 (s, 3H)

[a] Carbons are labeled according to their number in the polyketide backbone (Figure 2). [b] NC, not coupled.

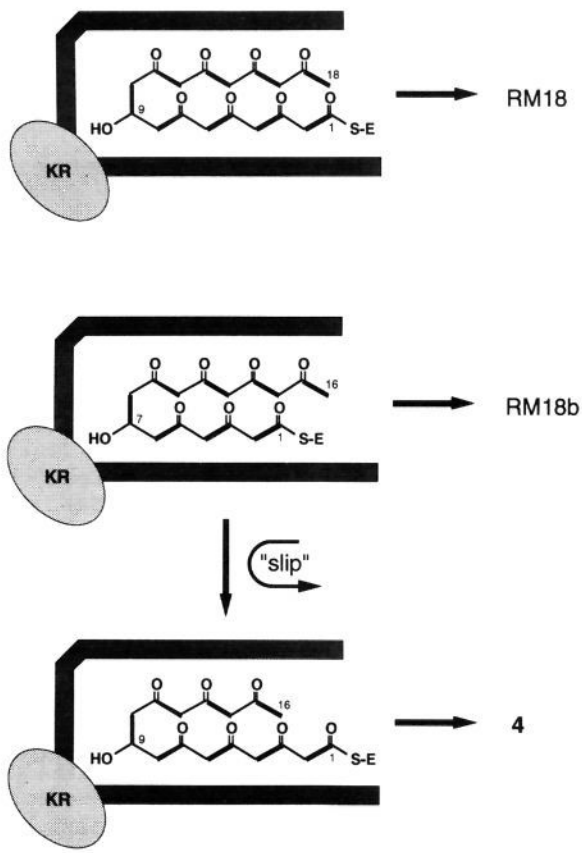

Figure 4. Relaxed specificity of *fren* PKS. This schematic representation illustrates proposed orientations of the different polyketide backbones produced by the recombinant *fren* PKSs and the proposed "slippage" mechanism that explains the aberrent reduction of RM18b (**7**). See text for further details.

constant distance from the end of the chain (i.e. the carboxyl group), as opposed to the beginning of the chain (i.e. the methyl group).[6] With regard to the cyclization of RM18b, the first cyclization is again constant relative to the position of the ketoreduction, while the resulting product, albeit an octaketide, is presumably not recognized by the *act* cyclase.

A comparison of the product profiles of the hybrid synthases reported in Table I with analogous hybrids between *act* and *tcm* PKS components supports the hypothesis that the ORF2 product is the chain length determining factor (CLF).[3] Possible explanations for the hybrid PKSs with ORF1 and ORF2 of different origin that appear to be nonproducing were discussed elsewhere.[3] Two other hybrid PKSs not reported in Table I are also nonproducing. These include a *fren* ORF1/*tcm* ORF2 hybrid and a *tcm* ORF1/*fren* ORF2 hybrid, with the *act* ACP gene present on both constructs.

Discussion

The isolation of mutant enzymes with relaxed substrate specificities has provided valuable mechanistic insights into enzyme function on many previous occasions.[14] Our deduction that the *fren* PKS catalyzes the biosynthesis of polyketides of two different chain lengths is the first example of a naturally occuring PKS with a relaxed chain length specificity. Such examples, although relatively rare, are suspected to exist in other classes of PKSs as well.[15] Although a better understanding of the molecular basis of this phenomenon must await further analysis of the *fren* PKS, the results presented here prove that ketoreductions of unreduced chains can occur at positions other than C-9 and strongly support an earlier hypothesis that ketoreduction of aromatic polyketides occurs after assembly of the complete polyketide chain.[6] The simplest explanation for the dual specificity of the *fren* PKS is that it has a binding pocket in which the smaller nascent polyketide backbone can only fit loosely, thus leading to chain slipping prior to ketoreduction.

The results reported here also support many of the conclusions drawn from our studies with *act* and *tcm* hybrid PKSs;[3] (i) the ORF2 product (CLF) of aromatic PKSs dictates chain length, at least in part, (ii) the regiochemistry of the first cyclization is constant relative to the position of ketoreduction when it occurs, and (iii) the *act* cyclase does not recognize polyketide chains longer than eight acetates. Furthermore, examination of the structure of compound **7** suggests that, since the cyclase fails to recognize even octaketides with aberrant ketoreductions, substrate specificity must be dictated by the methyl end of the carbon chain.

Finally, the ability to generate novel polyketide structures through genetic engineering of PKSs has been further illustrated by the generation of RM18 (**6**) and RM18b (**7**). Given the tremendously diverse catalytic specificities exhibited by naturally occuring PKSs, heterologous expression of uncharacterized or poorly characterized PKSs could also be exploited as a very useful synthetic and analytical tool. For example, it should be possible to construct a library of randomly cloned ORFs 1 and 2 homologues from a collection of actinomycetes, which would then

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be screened for their ability to catalyze novel aromatic polyketide production as part of hybrid synthases. One could also attempt to correctly cyclize longer polyketides by replacing the *act* cyclase gene with a homologue from PKS gene clusters which produce chains of the correct lengths. It is also possible to alter such polyketide structures by deleting the KR gene.[3] Lastly, a considerable degree of variability exists for non-acetate starter units among certain naturally occurring aromatic PKSs; this might offer another opportunity for obtaining novel polyketides via genetic engineering. Use of an expression system such as that described here, which generates the products of PKSs, without post-PKS modification reactions, may have a particular advantage in producing targets for subsequent synthetic or enzyme-catalyzed derivatization.

Experimental Section

Bacterial Strains, Plasmids, and Culture Conditions. *S. coelicolor* CH999 was used as a host for transformation by all plasmids. The derivation of this strain was described elsewhere.[3] DNA manipulations were performed in *Escherichia coli* MC1061. Plasmids were passaged through *E. coli* ET12567 (*dam dcm hsdS* Cm^r)[16] to generate unmethylated DNA before their use to transform *S. coelicolor*. *E. coli* strains were grown under standard conditions. *S. coelicolor* strains were grown on R2YE agar plates[17] rather than in liquid media because of the apparently more abundant production of metabolites on agar media.

Manipulation of DNA and Organisms. Polymerase chain reaction (PCR) was performed using Taq polymerase (Perkin Elmer Cetus) under conditions recommended by the enzyme manufacturer. Standard *in vitro* techniques were used for DNA manipulations.[18] *E. coli* was transformed with a Bio-Rad *E. Coli* pulsing apparatus using protocols provided by Bio-Rad. *S. coelicolor* was transformed by the standard procedure,[17] and transformants were selected using 2 mL thiostrepton overlay.

Construction of Plasmids Containing Recombinant PKSs. All plasmids are derivatives of pRM5.[3] *Fren* PKS genes were amplified via PCR with 5′ and 3′ restriction sites flanking the genes in accordance with the location of cloning sites on pRM5 (i.e. *PacI–NsiI* for ORF1, *NsiI–XbaI* for ORF2, and *XbaI–PstI* for ORF3). Following subcloning and sequencing, the amplified fragments were cloned in place of the corresponding fragments in pRM5 to generate the plasmids for transformation.

Production and Purification of Polyketides. For initial screening, all strains were grown at 30 °C as confluent lawns on 10–20 plates each containing approximately 30 mL of agar medium for 6–8 days. Additional plates were made as needed to obtain sufficient material for complete characterization. CH999 was a negative control when screening for potential polyketides. The agar was finely chopped and extracted with ethyl acetate/1% acetic acid. The concentrated extract was then flashed through a silica gel (Baker 40 μm) chromatography column in ethyl acetate/1% acetic acid. The primary yellow fraction was further purified via high-performance liquid chromatography (HPLC) using a 20–60% acetonitrile/water/1% acetic acid gradient on a preparative reverse-phase (C–18) column (Beckman). Absorbance was monitored at 280 and 410 nm. The compounds reported in this paper are the major polyketide products obtained with the above purification procedure. Several other minor compounds were present but no effort was made to characterize them. In general, the yield of purified product from these strains was approximately 10 mg/L for compounds **6** and **7** and 5 mg/L for compounds **4** and **5**.

[1,2-$^{13}C_2$]Acetate Feeding Experiments. Two 2-L flasks each containing 400 mL of modified NMP medium[19] were inoculated with spores of *S. coelicolor* CH999/pRM18 and incubated in a shaker at 30 °C and 300 rpm. To each flask was added 50 mg of sodium [1,2-$^{13}C_2$]acetate (Aldrich) at 72 and 96 h. After 120 h, the cultures were pooled and extracted with two 500-mL volumes of ethyl acetate/1% acetic acid. The organic phase was kept, and purification proceeded as described above. Approximately 2 mg of RM18 was obtained in this experiment. ^{13}C NMR data (16 512 scans) indicate approximately a 2–3% enrichment (estimated by comparing peak areas to the natural abundance ^{13}C peak area).

NMR Spectroscopy. All spectra were recorded on a Varian XL-400 except for HMBC analysis of RM18 (**6**), which was performed on a Nicolet NT-360. ^{13}C spectra were acquired with continuous broadband proton decoupling. For NOE studies of RM18 (**6**), the 1D difference method was employed. All compounds were dissolved in DMSO-d_6 (Sigma, 99+ atom % D), and spectra were referenced internally to the solvent. Hydroxyl resonances were identified by adding D_2O (Aldrich, 99 atom % D) and checking for disappearance of signal.

3,8-Dihydroxy-1-methylanthraquinone-2-carboxylic Acid (4): ^1H NMR (DMSO-d_6 400 MHz) δ 12.86 (s, 1OH), 7.73 (dd, J = 8.1, 7.6, Hz, 1H), 7.64 (dd, J = 7.6, 1.2 Hz, 1H), 7.61 (s, 1H), 7.34 (dd, J = 8.3, 1.2 Hz, 1H), 2.69 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 189.4, 182.2, 168.5, 161.4, 158.9, 140.8, 136.3, 136.0, 132.5, 131.4, 124.4, 122.5, 118.3, 116.8, 112.3, 19.9; FD-MS m/e 298.

Aloesaponarin II (5): ^1H NMR (DMSO-d_6, 400 MHz) δ 13.00 (s, 1OH), 7.72 (t, J = 8.0 Hz, 1H), 7.63 (dd, J = 7.5, 1.2 Hz, 1H), 7.47 (d, J = 2.6 Hz, 1H), 7.34 (dd, J = 8.3, 1.2 Hz, 1H), 7.06 (d, J = 2.6 Hz, 1H), 2.72 (s, 3H); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 189.1, 182.5, 163.4, 161.5, 145.4, 137.0, 135.9, 132.7, 124.9, 124.3, 123.0, 118.2, 116.6, 112.5, 23.7; FD-MS m/e 282.

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