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Rational Design and Engineered Biosynthesis of a Novel 18-Carbon Aromatic Polyketide

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Abstract: Heuristics for the rational design of aromatic polyketides were recently proposed and tested via the engineered biosynthesis of two novel products. Here we have applied these rules to a previously untested subclass of aromatic polyketides, the unreduced molecules. A recombinant strain of *Streptomyces coelicolor* expressing the genes for the frenolicin (*fren*) minimal polyketide synthase (PKS) and the TcmN subunit (a putative aromatase/cyclase) from the tetracenomycin PKS was constructed, and its principal product, the nonaketide PK8, was characterized by spectroscopic and isotope labeling methods. The structure of PK8 was exactly as predicted by the design rules. Surprisingly however, no major octaketide product was isolated from this strain. In contrast, a strain expressing the *fren* minimal PKS genes alone produced octaketides to the exclusion of nonaketides. These results differ from earlier reports of both octaketide and nonaketide products from strains containing the *fren* minimal PKS and a regiospecific ketoreductase. We therefore propose a model for bacterial aromatic polyketide biosynthesis in which auxiliary PKS subunits such as ketoreductases, aromatases, and cyclases can modulate the intrinsic specificity of the minimal PKS with respect to *both* the folding pattern *and* the chain length of the final product.

Polyketides are a large family of structurally complex and pharmaceutically important natural products.[1,2] They are synthesized by multifunctional polyketide synthases (PKSs), which catalyze repeated condensations between acyl thioesters (usually acetyl, propionyl, malonyl, or methylmalonyl). Each condensation results in the introduction to a growing carbon chain of a β-keto group that then undergoes some, all, or none of a series of reductive steps. PKSs incorporate enormous structural diversity into their products by varying this reductive cycle and by controlling the overall chain length, choice of primer and extender thioester units, and, particularly in the case of bacterial aromatic polyketides, regiospecific cyclizations of the nascent polyketide chain.[3]

Given the medicinal importance of polyketides, their shared mechanism of biosynthesis, and the high degree of conservation that exists among PKS gene clusters considerable interest has developed in the possibility of generating novel polyketides through the rational design of PKSs. Toward this end, the gene clusters for numerous bacterial PKSs have been cloned and sequenced. This analysis has revealed two major architectural paradigms for the synthases. On one hand, modular PKSs, which are involved in the biosynthesis of natural products such as erythromycin,[4,5] avermectin,[6] and rapamycin,[7] carry a distinct

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active site for each enzyme-catalyzed step in carbon chain assembly and modification. These active sites, encoded as domains within large proteins, are clustered into modules. Each module contains the complement of sites required for one condensation and its associated complete or incomplete reduction cycle. This one-to-one correspondence between protein domains and catalytic steps has been exploited in the case of the erythromycin PKS for the rational design and engineered biosynthesis of several novel polyketides.[5,8−14] In contrast, the PKSs responsible for the biosynthesis of aromatic polyketides such as actinorhodin (*act*),[15] granaticin (*gra*),[16] tetracenomycin (*tcm*),[17] and frenolicin (*fren*)[18] consist of a small number of polypeptides each carrying one (or perhaps occasionally two) active sites. Since aromatic PKSs appear to possess considerably fewer active sites than the total number of enzymatic reactions in the overall catalytic cycle, it has been proposed that some active sites are used iteratively in the biosynthesis of each product molecule. Consequently, an approach to the rational design of novel aromatic polyketides cannot be as conceptually straightforward as, for example, in the case of the erythromycin pathway. Notwithstanding this difficulty, recently a step has been taken in this direction through the rational design and engineered biosynthesis of two novel aromatic polyketides.[19]

Rationally guided biosynthesis of novel aromatic polyketides has two prerequisites. First, a versatile method to assemble and analyze recombinant PKSs is required. For this we designed and constructed a host-vector system, [20] consisting of the *Streptomyces coelicolor* strain CH999, from which the *act* aromatic PKS gene cluster and other *act* genes had been deleted, and the expression plasmid pRM5. Second, one needs a reasonable understanding of the functions and molecular recognition features of individual subunits in aromatic PKSs. Although sequence analysis was useful in suggesting the functions of some aromatic PKS components, it fell short of predicting the specificities of these enzymes and of elucidating the unknown functions of several cyclizing and modifying enzymes that are encoded within aromatic PKS gene clusters.

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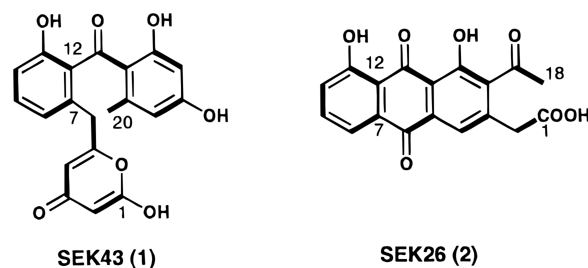

Figure 1. Structures of rationally designed novel polyketides reported earlier.[19] Bold bonds correspond to individual acetate-derived monomers in the polyketide backbones.

Table 1. Polyketides Produced by Combinations of the Frenolicin (*fren*) and the Tetracenomycin (*tcm*) Polyketide Synthase (PKS) Proteins[a]

plasmid	minimal PKS	*tcm*J,N	product
pPK9	fren		SEK4 (**7**), SEK4b (**8**)
pPK7	fren	*tcm*J	SEK4 (**7**), SEK4b (**8**)
pPK8	fren	*tcm*N	PK8 (**5**)
pPK6	fren	*tcm*J,N	PK8 (**5**)

[a] The minimal PKS is comprised of the ketosynthase/putative acyltransferase and chain length factor from the gene cluster indicated and the acyl carrier protein from the actinorhodin (*act*) PKS.

To address these questions the host-vector system was used to express and analyze many recombinant aromatic PKS gene clusters. By relating product structure to PKS subunit composition, it was possible to ascribe functions and specificities to the various subunits. For example, proteins involved in determining chain length, the degree and regiospecificity of ketoreduction, and the regiospecificity of cyclizations and aromatizations were identified. Based on these studies a set of "design rules" for generating aromatic polyketides in a predictive manner was proposed and successfully tested via the engineered biosynthesis of two novel products.[19] Here we extend this idea by generating another polyketide "to order"—the first example of an unreduced compound. In the process new insights have been gained regarding the interplay between chain length, reduction, and cyclization control by aromatic PKSs.

Results

The "design rules" described recently were tested in the context of two *reduced* aromatic polyketides, SEK43 (**1**) and SEK26 (**2**) (Figure 1).[19] The motivation for the experiments described here was to test the applicability of these heuristics for the rational design of novel *unreduced* polyketides. In particular, we predicted that it should be possible to generate 18-carbon polyketides that have undergone a C-9/C-14 cyclization using the *fren* minimal PKS, which includes the ketosynthase/putative acyltransferase, chain length factor, and acyl carrier protein, in combination with the tcmN subunit of the *tcm* PKS whose N-terminal domain encodes a bifunctional aromatase/cyclase. Toward this end a series of recombinant aromatic gene clusters described in Table 1 was constructed and introduced via transformation into *S. coelicolor* CH999. The results from structural and isotope labeling analysis of the polyketides produced by these recombinant strains are presented here.

Properties of a Recombinant PKS Consisting of the *fren* Minimal PKS and tcmN. *S. coelicolor* CH999/pPK8 (Table 1) expresses the genes encoding the *fren* minimal PKS (with the *act* acyl carrier protein substituted for the *fren* acyl carrier protein) and tcmN. Since the *fren* PKS is known to have a relaxed chain length specificity for 16- and 18-carbon

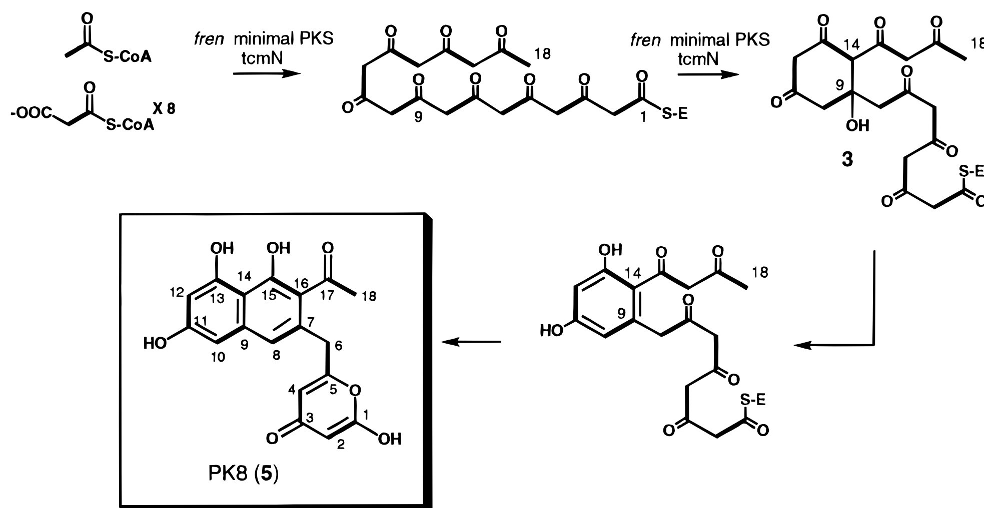

Figure 2. Proposed biosynthetic pathway for PK8, a rationally designed novel polyketide. For details see text.

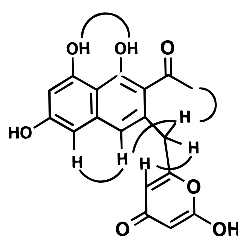

Figure 3. Summary of nuclear Overhauser effect spectroscopic data for PK8.

polyketides,[21] and tcmN is known to channel an unreduced nascent chain toward a C-9/C-14 cyclized intermediate,[22,23] we predicted that a combination of the two components would lead to the biosynthesis of the putative intermediate **3** (Figure 2). It was also anticipated from precedent[19] that the carboxyl end of the full-length chain would undergo lactonization and subsequent dehydration to form a γ-pyrone moiety. Furthermore, the methyl end of the intermediate is long enough to allow a second aldol condensation to occur between C-7 and C-16, as in the case of the decaketide RM80 (**4**) (Figure 4), the primary product of the *tcm* minimal PKS and tcmN.[23] (A similar second aldol condensation is also seen in Tcm F2, a product related in structure to RM80, that is synthesized by the *tcm* minimal PKS and tcmN in *S. glaucescens*[28] and, to a lesser extent, in *S. coelicolor* (unpublished results).) Thus, the structure of the dominant nonaketide product of CH999/pPK8, designated PK8, was expected to be as shown in **5** (Figure 2).

PK8 (**5**) was indeed purified via C_{18} reverse-phase HPLC and characterized by NMR spectroscopy (Table 2), high resolution mass spectroscopy (HRMS), and sodium [1,2-$^{13}C_2$] acetate feeding experiments. Both HRMS (measured M + Cs^+ 474.981, calculated M + Cs^+ 474.9794, consistent with the molecular formula $C_{18}H_{14}O_6$) and ^{13}C-NMR spectroscopy confirmed that the compound consisted of 18 carbon atoms. Chemical shifts indicative of a 2-hydroxy-4-pyrone ring system were detected in the ^{1}H-NMR spectrum; the remaining peaks in the aromatic region of the spectrum were consistent with the bicyclic fused ring structure shown in **5**. Finally, the 203.8 ppm peak in the ^{13}C-NMR spectrum suggested the presence of an aliphatic carbonyl, consistent with a product derived from a C-7/C-16 aldol condensation. Deuterium exchange was used to identify peaks corresponding to the hydroxyl hydrogens. Unambiguous ^{1}H-NMR chemical shift assignments were made by nuclear Overhauser effect spectroscopy (Figure 3), and unambiguous ^{13}C-NMR chemical shift assignments for PK8 were made by measuring ^{13}C coupling constants in an isotopically labeled sample of PK8 derived from sodium [1,2-$^{13}C_2$] acetate feeding experiments (Table 2). Thus, the deduced structure of PK8 was identical to the predicted structure. Furthermore, the structure of PK8 is also consistent with the proposed function of tcmN as a first ring cyclase and a second ring aromatase for unreduced polyketide backbones.[23,28]

Surprisingly, CH999/pPK8 was not found to produce any detectable 16-carbon polyketide. In particular, chromatographic fractions of extracts from this strain were examined for the presence of RM77 (**6**) (the product of the *act* minimal PKS and tcmN[23]) or SEK4 (**7**)/SEK4b (**8**) (the primary products of the *act* minimal PKS alone[24,25]) (Figure 4). Neither of these products were detected. Thus, in contrast to the *act* ketoreductase (KR), which elicits both 16- and 18-carbon polyketides from the *fren* minimal PKS,[19,21,26] coexpression of *tcm*N and the *fren* minimal PKS genes appeared to yield only 18-carbon polyketides. Possible implications of this finding are discussed below.

Properties of the *fren* Minimal PKS: To investigate the chain length specificity of the *fren* minimal PKS alone, strain CH999/pPK9 (Table 1) was constructed and analyzed. The primary products of this strain were SEK4 (**7**) and SEK4b (**8**) (Figure 4), earlier characterized octaketide products of the *act* minimal PKS.[24,25] Remarkably, no nonaketide product was detected from this strain. However, unlike the case of CH999/pPK8, where the expected octaketide product, RM77 (**6**) (Figure 4), was characterized earlier, the structure and physicochemical properties of the possible nonaketide product (if any) from CH999/pPK9 cannot be unambiguously predicted. Thus, our inability to isolate an 18-carbon product could either be due to its inherent instability (at least under the workup conditions used in this study), or it could reflect the genuine absence of such a product from extracts of this strain.

Role of *tcm*J. The *tcm*J gene from the *tcm* gene cluster encodes a protein whose function remains to be elucidated as

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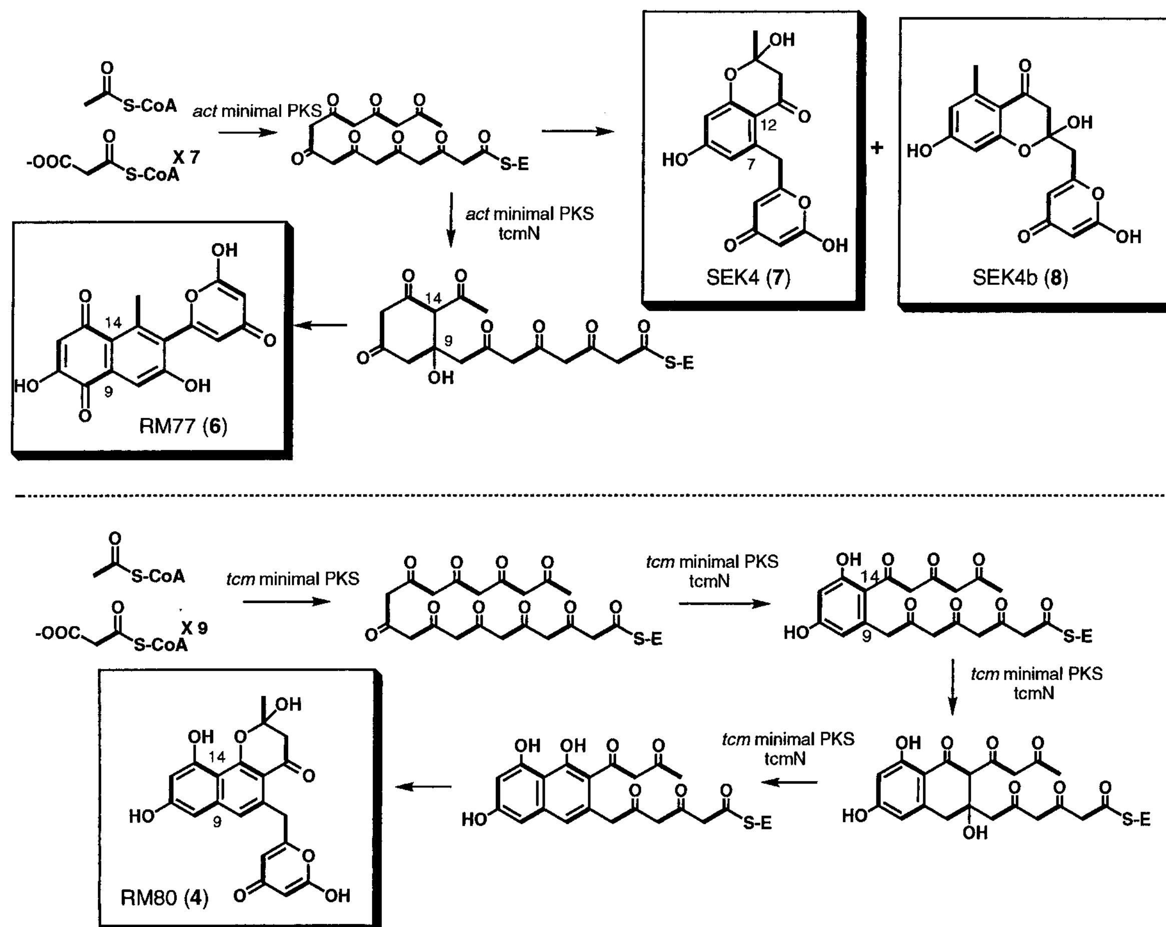

Figure 4. Proposed biosynthetic pathway for representative unreduced polyketides: above, octaketides; below, decaketides. For details see text.

Table 2. ^{1}H and ^{13}C NMR data from PK8(**5**) in DMSO-d_6[a]

carbon	^{13}C (ppm)	J_{CC} (Hz)	PK8 (**5**) ^{1}H (ppm) (m, J_{HH}(Hz), area)
1	164.2	78.8	hydroxyl
2	88.5	79.6	5.24 (s, 1H)
3	170.7	58.1	
4	100.5	57.7	5.75 (s, 1H)
5	165.5	50.8	
6	37.0	50.2	3.84 (s, 2H)
7	137.8	58.1	
8	119.5	59.8	6.97 (s, 1H)
9	108.0	64.6	
10	101.6	59.2	6.57 (s, 1H)
11	158.1	64.2	hydroxyl
12	101.7	63.0	6.45 (s, 1H)
13	155.1	73.2	hydroxyl
14	119.2	73.2	
15	155.8	64.6	9.97 (s, 1H)
16	131.8	62.6	
17	203.8	41.9	
18	32.4	40.9	2.5 (s, 3H)

[a] Carbons are labeled starting at the carboxyl end. J_{CC}, $^{13}C-^{13}C$ coupling constants obtained from a sample isotopically labeled with [1,2-$^{13}C_2$] acetate.

yet.[22,23,27] In order to see if the presence of tcmJ might influence the activity of the *fren* minimal PKS, plasmids pPK6 and pPK7 were constructed (Table 1) and introduced via transformation into CH999. The products of the resulting strains were purified and spectroscopically analyzed. Both strains were indistinguishable from their counterparts lacking the *tcm*J gene (Table 1). Thus, either tcmJ cannot productively interact with the *fren* minimal PKS, or its properties are undetectable using the combinatorial approach presented here.

Discussion

Recently we proposed a set of structure-function based heuristics for the rational design and engineered biosynthesis of novel bacterial aromatic polyketides.[19] It was argued that these rules could be extended to the design of combinatorial libraries of "unnatural" natural products. The generation of PK8 (**5**) (Figure 2) reported here is consistent with the applicability of these heuristics, specifically for the subclass of unreduced polyketides.

Several previous studies revealed that the frenolicin (*fren*) PKS is capable of generating 16- and 18-carbon polyketides in approximately a 1:2 ratio (Figure 5).[19,21,26] Since chain length is controlled by the minimal PKS in the cases of other bacterial aromatic PKSs, it was argued that the *fren* minimal PKS showed relaxed chain length specificity. However, in all recombinants described earlier the gene encoding the actinorhodin (*act*) ketoreductase (KR) was also present. (The corresponding *fren* KR is present in *S. roseofulvus*, the natural producer of both the octaketide nanaomycin and the nonaketide frenolicin.[18,31]) Here we have shown that in the absence of a KR, the *fren* PKS

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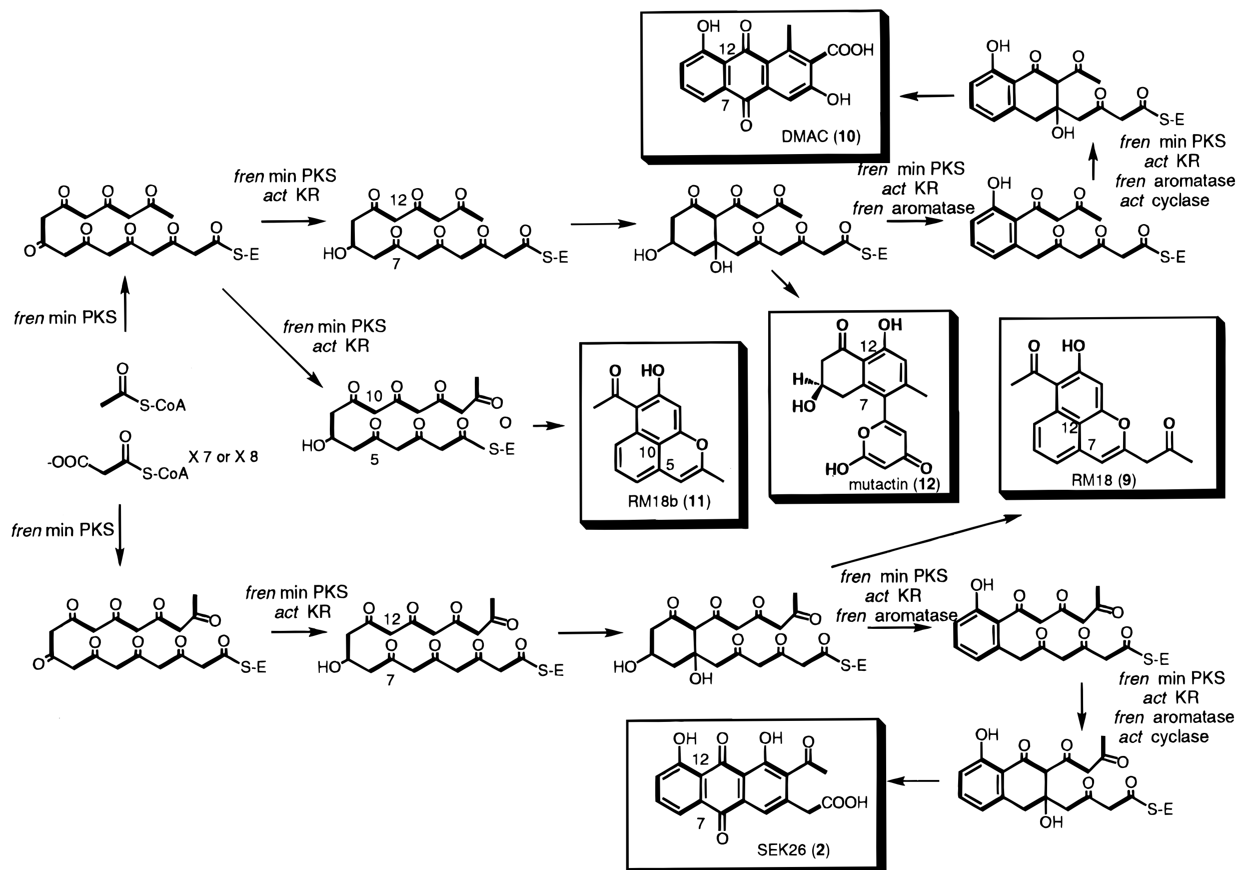

Figure 5. Proposed biosynthetic pathway for reduced polyketides derived from the *fren* PKS. For details see text.

appears to produce polyketides of only one chain length, these are either octaketides, when the *fren* minimal PKS acts alone, or a nonaketide when the tcmN protein (a bifunctional protein with cyclase and aromatase properties that exclusively recognizes unreduced polyketide backbones[22,23,28]) is also included. It can therefore be argued that the KR facilitates relaxation in chain-length specificity of the *fren* minimal PKS. In addition, a comparison of the structures of RM18 (**9**), DMAC (**10**), and RM18b (**11**) (Figure 5) had led us to propose earlier that the KR can also influence the regiospecificity of the first cyclization.[21] Likewise, it has been shown that downstream aromatases and cyclases can alter the intrinsic cyclization preferences of the minimal PKS.[22,23,25,26] Now it appears that the auxiliary subunits of bacterial aromatic PKSs (such as ketoreductases, aromatases, and cyclases) can significantly modulate the intrinsic specificity of the minimal PKS with respect to *both the size and the shape* of the final product by influencing the length and folding pattern of the nascent carbon chain. If so, it may be possible to broaden considerably the repertoire of molecular diversity that can be generated from this interesting class of multifunctional enzymes by the application of protein engineering approaches to these subunits.

Experimental Section

Bacterial Strains and Plasmids. The construction of *Streptomyces coelicolor* CH999 has been described elsewhere.[20] Plasmids pPK6-9 (Table 1) are derivatives of pSEK22[29] and pSEK15.[24] pPK9 was generated by ligating the *Pac*I-*EcoR*I fragment of pSEK22 into pSEK15. The construction of pPK6, pPK7, and pPK8 involved ligation of the *Xba*I-*EcoR*I fragments from pIJ5639-tcmJ, pIJ5639-tcmNJ, and pIJ5639-tcmN, respectively,[23] into pPK9. In all cases the *tcm*N and/or *tcm*J genes were cloned downstream of the gene encoding the acyl carrier protein. The plasmids were engineered in *E. coli* strain XL1-Blue or MM294 and were passaged through *E. coli* ET12567 (*dam dcm hsdS* Cm^r)[30] prior to introduction via transformation into *S. coelicolor* CH999.

Polyketide Production and Purification. For polyketide production, the strains were grown on R2YE agar plates.[31] After 6−7 days of growth, the agar was finely chopped and extracted twice with ethyl acetate/1% acetic acid. The organic extract, containing the polyketides, was concentrated by evaporation under vacuum at room temperature. The concentrated extract was flashed through a silica gel (Baker) chromatography column in the same solvent mixture used for extraction. The primary yellow-brown fraction was further purified via high-performance liquid chromatography (HPLC) using a 15−60% acetonitrile/water/1% acetic acid gradient on a preparative reverse phase (C-18) column (Beckman). Absorbance was monitored at 280 nm and 410 nm. The yield of PK8 from CH999/pPK8 and CH999/pPK6 was approximately 50 mg/L, whereas the yields of SEK4 and SEK4b from CH999/pPK7 and CH999/pPK9 were comparable to those reported earlier.[24,25]

NMR, Mass Spectroscopy, and Isotope Labeling Studies. High resolution fast atom bombardment mass spectroscopy, used for analysis of PK8, was performed on a VG ZAB-ZSE spectrometer. ^1H and ^{13}C NMR spectra were recorded on a Varian XL-400 spectrometer. For NOE studies, a one-dimensional difference method was employed. All compounds were dissolved in DMSO-d_6 (99+% D) or DMF-d_7 (99+% D) obtained from Cambridge Isotope Laboratories. Hydroxyl and other acidic proton resonances were identified by adding D_2O (Aldrich, 99% deuterium) and checking for disappearance of signal. Spectra were referenced either internally to the solvent or to TMS. Sodium [1,2-$^{13}C_2$] acetate (99% ^{13}C) labeling experiments were performed as described earlier.[23]

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