Engineered Biosynthesis of Novel Polyketides: Dissection of the Catalytic Specificity of the *act* Ketoreductase

Hong Fu,[1a] Susanne Ebert-Khosla,[1a] David A. Hopwood,[1b] and Chaitan Khosla*,[1a]

Contribution from the Department of Chemical Engineering, Stanford University, Stanford, California 94305-5025, and Department of Genetics, John Innes Institute, John Innes Centre, Norwich NR4 7UH, U.K.

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Abstract: Combinations of genes (*act* and *tcm*, respectively) encoding subunits of polyketide synthases (PKSs) for actinorhodin (**1**) from *Streptomyces coelicolor* A3(2) and tetracenomycin (**2**) from *Streptomyces glaucescens* were functionally expressed in a recently developed *Streptomyces* host–vector system. Recombinant strains lacking any ketoreductase activity associated with the PKS produced novel polyketides, which were structurally characterized using NMR and high-resolution mass spectroscopy in combination with isotopic labeling experiments. As expected, the *act* and *tcm* PKSs produced completely unreduced polyketides with backbones derived from 8 and 10 acetate units, respectively (**9** and **10**). However, the regiospecificities of cyclizations in these two molecules differed from their reduced counterparts, and have not been previously observed in this class of bacterial polyketides. Taken together with earlier findings, our results provide evidence for yet another degree of freedom for the biosynthesis of novel polyketides through combinatorial expression of the *act* ketoreductase with PKSs of varying chain length specificities (including those that are not associated with any ketoreductase in nature). By comparing the structures and deduced backbones of the novel polyketides reported here with those of novel polyketides described earlier, a biosynthetic model is favored in which the regiospecificity of the first cyclization is controlled in part by the chain elongation enzymes themselves and is not influenced by the ketoreductase; however, the cyclase responsible for controlling the regiospecificity of the second cyclization can discriminate between polyketide backbones of different lengths as well as different degrees of reduction.

Polyketides are a large family of structurally diverse natural products possessing a broad range of biological activities, including antibiotic and pharmacological properties. Biosynthetic and molecular genetic studies have demonstrated that polyketide synthases (PKSs) are structurally and mechanistically related to fatty acid synthases (FASs).[2] Both classes of synthases are mutifunctional enzymes that catalyze repeated (decarboxylative) Claisen condensations between acylthioesters (usually acetyl, propionyl, malonyl, or methylmalonyl). After the carbon chain has grown to a length characteristic of each specific product, it is released from the synthase by thiolysis or acyltransfer. Unlike typical FASs, PKSs introduce structural variability into the product by varying the extent of the reductive cycle comprising a ketoreduction, dehydration, and enoylreduction on each β-keto group of the polyketide chain.

An important scientific challenge is to understand the structural basis for the exquisite catalytic specificity of each PKS. Toward this end, we have developed a recombinant system for combinatorially expressing PKS subunits obtained from different aromatic PKS gene clusters (such as the *act* and the *tcm* gene clusters, shown in Figure 1).[3] Structural analysis of the resulting novel molecules reveals key features of the mechanisms by which bacterial aromatic PKSs control the structures of their products.[3,4] The following have already been reported: (i) the chain length is, at least in part, dictated by a specific protein, which was therefore named the chain length determining factor (CLF); (ii) the association of some heterologous ketosynthase/acyltransferase (KS/AT)–CLF pairs gave rise to functional PKSs, but other pairs were nonfunctional; (iii) acyl carrier proteins (ACPs) could be interchanged among different synthases without any effect on product structure; (iv) a specific ketoreductase (*act* KR) reduced polyketide chains of different lengths, probably after the complete polyketide chain had been synthesized; (v) regardless of the chain length, the *act* KR reduced the C-9 carbonyl, counting from the carboxyl end; (vi) in reduced polyketides, the regiospecificity of the first cyclization was constant relative to the position of ketoreduction, regardless of the chain length or the regiospecificity

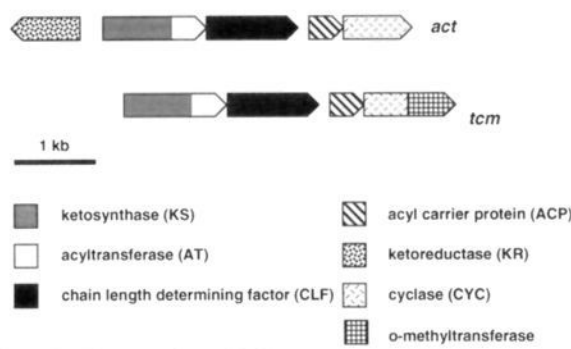

Figure 1. The *act* and *tcm* PKS gene clusters. Each PKS includes a ketosynthase/acyltransferase (KS/AT), a chain length determining factor (CLF), and an acyl carrier protein (ACP); the *act* cluster also contains a ketoreductase (KR). The AT presumably transfers the starter unit from CoA to the KS, which catalyzes the Claisen condensation between the starter (or growing polyketide) acylthioester and the extender thioester on the ACP. The KR reduces a specific carbonyl of the actinorhodin polyketide backbone. The gene clusters also encode a cyclase, involved in cyclization of the nascent polyketide backbone; however, the *tcm* cyclase (tcmN) is unusual, since it is similar to the *act* (and other) cyclases only in the N-terminal half of the polypeptide. Its C-terminal half resembles known O-methyltransferases. All other genetic homology depicted here is conserved throughout most actinomycete aromatic PKSs.

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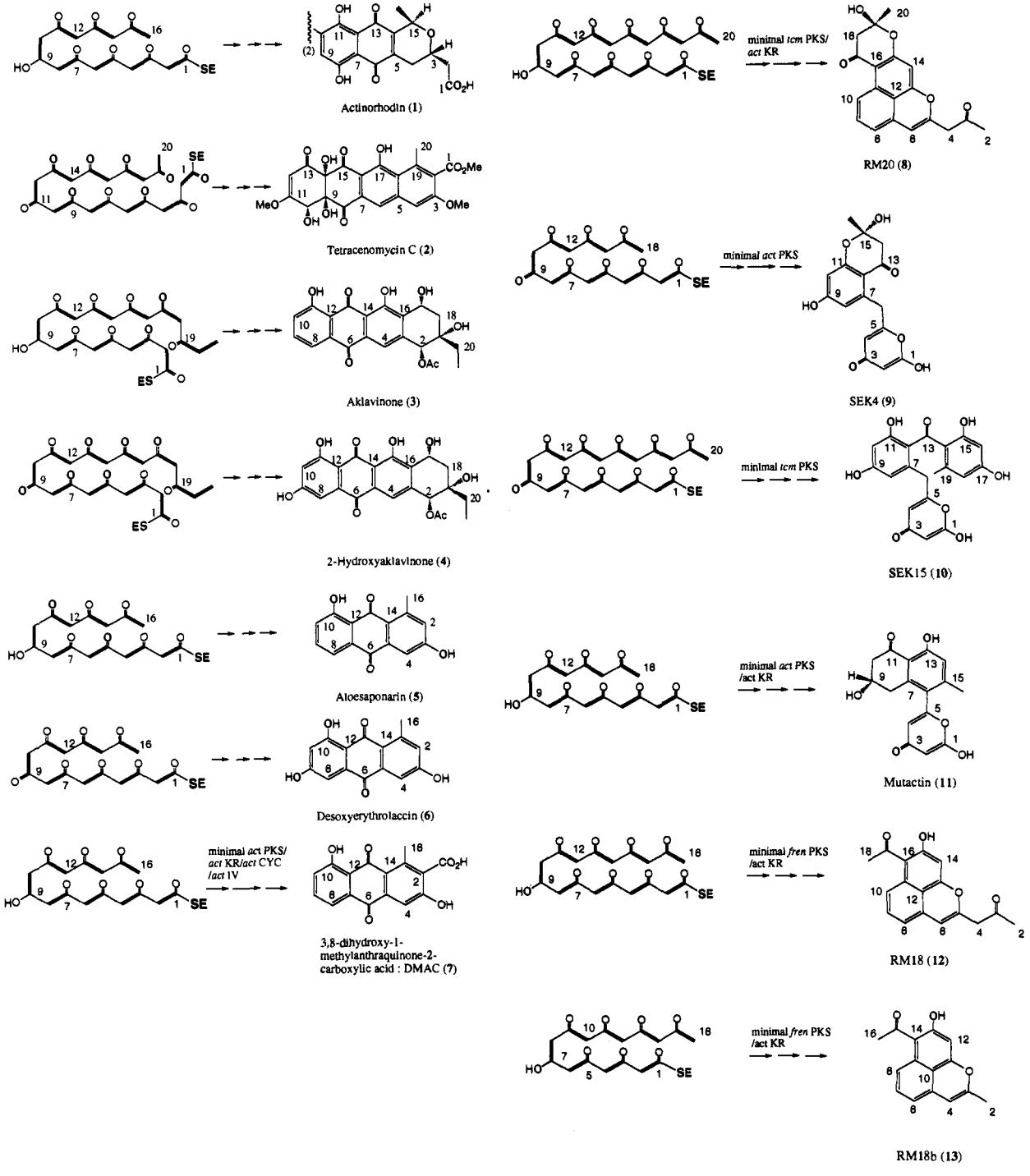

Figure 2. Polyketides (right) and deduced backbones (left) synthesized by PKSs. **1** and **2** are the products of the *act* and *tcm* polyketide pathways in *S. coelicolor* and *S. glaucescens*, respectively. The biosynthetic origins of **3–6** are described elsewhere.[5] **7–10** and **12–13** are products of genetically engineered PKSs. **11** is produced by an *act*VII mutant of *S. coelicolor*. The PKS subunits involved in the biosynthesis of **7–13** are listed.

of subsequent cyclizations; and (vii) a specific cyclase (*act* CYC), responsible for catalyzing the second cyclization reaction in an octaketide, could not cyclize intermediates of longer chain lengths.

Although the *act* KR has been shown to reduce nascent polyketides of different chain lengths,[3-5] the precise role of this subunit is relatively obscure, since the polyketide product of an *act* KR mutant of *S. coelicolor* (derived via classical mutagenesis[6]) has not been structurally characterized. On the basis of an analysis of recombinant PKS gene clusters in *S. galileus*, Bartel et al. concluded that the *act* KR could reduce the C-9 carbonyl of both octaketides and decaketides.[5] As summarized above, this result was subsequently confirmed using an *S. coelicolor*-based expression system.[3] However, while the cyclization patterns of polyketides characterized by Bartel et al. (**3–6**)[5] were not influenced by the presence or absence of a KR, the structure of RM20 (**8**)[3] synthesized by *S. coelicolor* CH999/pRM20 tentatively suggested that the regiospecificity of both the first and the second cyclization reactions could be altered by the *act* KR. Here we investigate the role of the *act* KR by comparing the structures and deduced backbones of the products obtained from two pairs of genetically engineered strains that are identical except for the presence or absence of the KR.

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Table 1. ^1H and ^{13}C NMR Data for SEK4 (**9**) and SEK15 (**10**)[a]

SEK4 (**9**)				SEK15 (**10**)			
C no.[b]	^{13}C δ (ppm)	J_{CC} (Hz)	^1H δ (ppm)	C no.	^{13}C δ (ppm)	J_{CC} (Hz)	^1H δ (ppm)
1	165.4	78.8	11.60 (s, 1OH)	1	164.0	79.1	12.20 (s, 1OH)
2	88.2	79.8	5.19 (d, J = 2.28 Hz, 1H)	2	88.2	79.4	5.08 (d, J = 1.88 Hz, 1H)
3	170.5	55.3		3	172.8	57.9	
4	102.9	60.9	5.66 (d, J = 2.24 Hz, 1H)	4	101.8	53.9	5.66 (d, J = 1.88 Hz, 1H)
5	163.8	51.0		5	163.1	50.4	
6	37.6	50.8	4.07 (d, J = 15.7 Hz, 1H)	6	36.7	50.8	3.50 (s, 2H)
			4.16 (d, J = 16.0 Hz, 1H)	7	135.4	60.7	
7	138.6	60.7		8	109.1	61.7	6.23 (s, 1H)
8	112.9	61.6	6.33 (d, J = 1.6 Hz, 1H)	9	159.8	66.2	
9	161.9	71.9	10.50 (s, 1OH)	10	101.6	66.5	6.20 (s, 1H)
10	100.6	70.9	6.26 (d, J = 1.96 Hz, 1H)	11	157.4	67.3	
11	162.9	60.8		12	121.1	67.6	
12	111.3	61.3		13	200.3	58.1	
13	191.1	39.1		14	117.2	58.6	
14	49.3	39.9	2.54 (d, J = 15.9 Hz, 1H)	15	163.6	68.5	
			2.92 (d, J = 16.0 Hz, 1H)	16	100.6	68.0	6.08 (s, 1H)
15	99.6	46.6	6.90 (s, 1OH)	17	162.2	62.6	
16	27.5	46.8	1.56 (s, 3H)	18	111.0	62.0	6.12 (s, 1H)
				19	141.9	43.3	
				20	21.1	42.7	1.86 (s, 3H)

[a] ^1H and ^{13}C NMR data were recorded in DMSO-d_6 (400 MHz for ^1H and 100 MHz for ^{13}C). [b] Carbons are labeled according to their number in the polyketide backbone (Figure 2).

Results

To investigate the role of the *act* KR in the biosynthesis of 3,8-dihydroxymethylanthraquinonecarboxylic acid (DMAC; **7**) and RM20 (**8**), we constructed two additional plasmids, pSEK4 and pSEK15. These plasmids were identical to those responsible for DMAC and RM20 biosynthesis, respectively, except for the absence of a functional KR gene (see Experimental Section). pSEK4 and pSEK15 were used to transform CH999, a genetically engineered derivative of *S. coelicolor* A3(2) lacking the entire *act* gene cluster. Both the resulting transformants, CH999/pSEK4 and CH999/pSEK15, produced a single major polyketide product (i.e., in greater than 10-fold abundance over minor products). These products, SEK4 (**9**) and SEK15 (**10**), were structurally characterized using NMR, high-resolution, MS, and sodium [1,2-$^{13}C_2$]acetate feeding experiments.

Structure of SEK4. ^1H and ^{13}C NMR (Table 1) revealed that SEK4 (**9**) had a pyrone moiety, similar to mutactin (**11**), a shunt product from an *act* cyclase mutant of *S. coelicolor*,[8] and a non-aromatic 6-membered ring, similar to RM20 (**8**). The geminal protons on both methylene groups are diastereotopic with a coupling constant of 16 Hz. Sodium [1,2-$^{13}C_2$]acetate feeding experiments confirmed that the carbon chain of SEK4 was derived from 8 acetate units. The coupling constants calculated from the ^{13}C NMR spectrum of the enriched SEK4 sample also facilitated peak assignment. High-resolution fast atom bombardment (FAB) mass spectroscopy gave a molecular weight of 319.0820 (M + H^+), consistent with $C_{16}H_{14}O_7$ (M + H^+ 319.0818). Unequivocal assignments for SEK4 were established with 1D nuclear Overhauser effect (NOE) studies (Figure 3). Deuterium exchange was used to confirm the presence of each hydroxyl in SEK4.

Structure of SEK15. Sodium [1,2-$^{13}C_2$]-acetate feeding experiments showed that SEK15 (**10**) was derived from 10 acetate units. The results of ^1H and ^{13}C NMR studies (Table 1) were consistent with a structure composed of one pyrone moiety and two benzene rings. High resolution FAB mass spectroscopy gave a molecular weight of 385.0920 (M + H^+), consistent with $C_{20}H_{16}O_8$ (M + H^+ 385.0923). Once again, 1D NOE (Figure 3) and deuterium exchange experiments were carried out to confirm the structure proposed here.

Catalytic Control of the First Cyclization. Comparison of the cyclization patterns of DMAC (**7**), RM20 (**8**), SEK4 (**9**), and SEK15 (**10**) provides new insights into how intramolecular cyclizations (aldol condensations) are controlled by this class of bacterial PKSs. It can be seen that, regardless of chain length or degree of reduction, the first cyclization in each of the four nascent polyketide backbones occurs between the C-7 carbonyl and C-12 methylene. Therefore, the possibility that the KR exercises control over the regiospecificity of this reaction can be ruled out. A more likely model is one in which the "minimal" PKS itself defines, at least in part, the specificity of the first cyclization in a manner that is invariant among all naturally occurring bacterial aromatic PKSs studied thus far. (An exception can be made in the case of the *fren* PKS in the frenolicin pathway; see Discussion section). Given such a biosynthetic model, the question of what dictates the unusual regiospecificity of the first cyclization in tetracenomycin C (**2**) still remains unanswered; we speculate on this issue in the Discussion section.

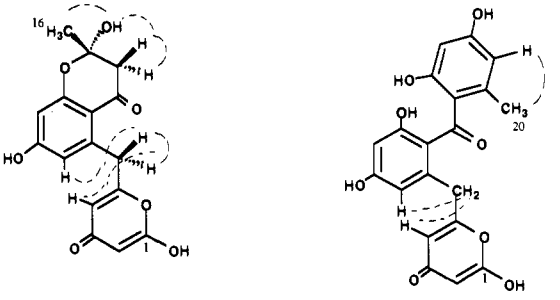

Figure 3. Summary of NOE data from SEK4 (**9**) and SEK15 (**10**).

Catalytic Control of the Second Cyclization. The four polyketides **7–10** also exhibit significant differences with respect to the regiochemistry of their second (and subsequent) cyclizations. It has been argued that the second cyclization (between the C-5 carbonyl and C-14 methylene) in **7** is catalyzed by the *act* cyclase.[3,5] The *act* cyclase, however, does not recognize the singly cyclized precursor of **8**, leading to its unusual cyclization pattern.[3] Likewise, the cyclization patterns of **9** and **10** also argue in favor of a model in which formation of only the first ring of these compounds is enzymatic. In other words, the *act* cyclase is able to discriminate between nascent polyketide substrates that are altered at both the methyl end of the chain and the C-9 position.

Discussion

In order to develop guidelines for the rational design of novel polyketides, it is essential to understand the following: (i) the

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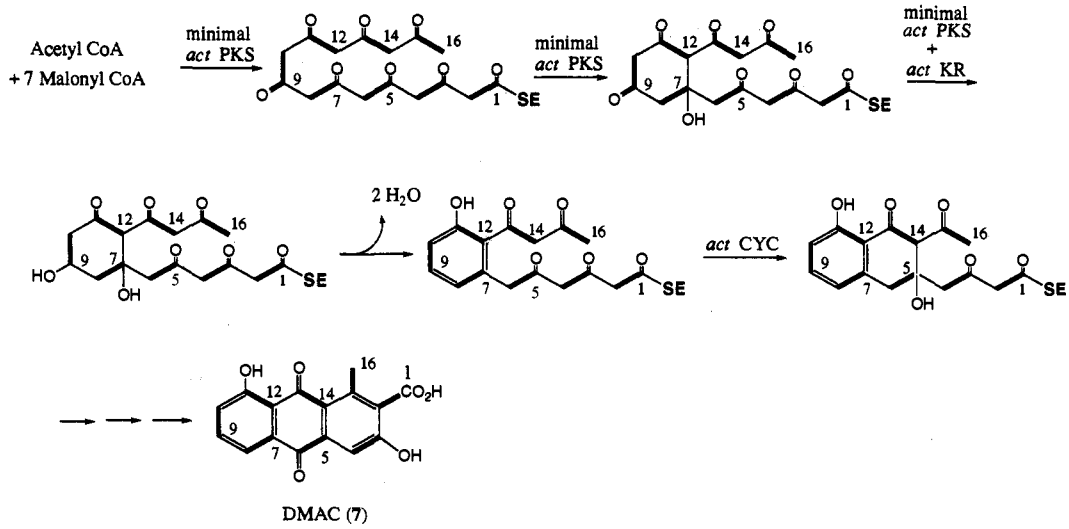

Figure 4. Biosynthesis of DMAC (7). The reactions catalyzed and/or controlled by the minimal *act* PKS (i.e. the KS/AT, CLF, and ACP), the *act* KR, and the *act* CYC in the biosynthesis of DMAC are shown.

sequence of individual reactions within the overall catalytic cycle of multifunctional PKSs, (ii) the structural basis for the specificity of each of these reactions, and (iii) the flexibility existing within a particular PKS for re-engineering any given structural determinant of specificity, without altering the specificities of other reactions in the overall catalytic cycle. The results reported here and elsewhere[3,4] represent an attempt to elucidate the above features for three such reactions that are common to the biosynthesis of many naturally occurring bacterial aromatic polyketides: a C-9 carbonyl reduction, a C-7/C-12 intramolecular aldol condensation (first cyclization), and a C-5/C-14 intramolecular aldol condensation (second cyclization). These three reactions, which are among the first transformations to occur following the biosynthesis of the complete polyketide chain,[4,5] are illustrated in Figure 4 for the case of DMAC (7) biosynthesis.

The relative sequence of the reduction and the first cyclization cannot be unambiguously defined. However, the lack of influence by the KR on the regiospecificity of this cyclization (as observed in compounds **7–10**) suggests that ketoreduction follows the first cyclization and that the regiospecificity of this cyclization is primarily dictated by the "minimal" PKS (KS/AT, CLF, and ACP) itself. In contrast, the specificity of the KR for the C-9 carbonyl is presumably dicated by interactions between the KR and the "minimal" PKS, since relaxation of chain-length specificity in a PKS occurs concomitantly with relaxation of the specificity of ketoreduction, as observed in the structures and deduced backbones of **12** and **13**.[4]

Unlike the ketoreduction and the C-7/C-12 cyclization, the "minimal" PKS does not appear to play a role in controlling the C-5/C-14 cyclization. The regiochemistry of this reaction is primarily controlled by the cyclase. Furthermore, as illustrated by compounds **7–13**, at least the *act* cyclase can discriminate between unnatural substrates that are altered at the methyl end, the carboxyl end, or the C-9 position of the polyketide precursor. It remains to be seen whether other naturally occurring cyclases share these features.

With regard to catalytic flexibility, the degree of reduction of the polyketide backbone can be readily varied, as shown here, but absence of ketoreduction leads to loss of activity of at least some downstream cyclases (such as the *act* cyclase). In contrast, regiospecificity of the first cyclization differs from the C-7/C-12 pattern in only two cases thus far: (i) the *fren* PKS (in the *S. coelicolor* expression system) synthesizes comparable quantities of a C-7/C-12 cyclized octaketide (7) and a C-5/C-10 cyclized octaketide (**13**),[4] and (ii) the tetracenomycin C (**2**) precursor undergoes a C-9/C-14 cyclization (in its natural producer, *S. glaucescens*).[9] The former case is a result of an as yet undefined feature of the PKS, which gives rise to both relaxed chain length and reduction specificities. On the other hand, a plausible explanation for the latter exception is that tcmN, an unusual cyclase of *S. glaucescens* (see Figure 1), which has been postulated to catalyze both the second cyclization and the 11 O-methyltransfer in tetracenomycin C biosynthesis,[9] is also responsible for catalyzing the first cyclization. In order to do so, it must override the intrinsic tendency of the *tcm* PKS to produce a C-7/C-12 cyclized product, as suggested by the structure of SEK15.

Finally, it is noteworthy that the cyclization patterns of SEK4 (**9**) and mutactin (**11**) are different, even though both shunt products have the same chain length and are generated because of the absence of cyclase activity. While the enolic C-11 hydroxyl presumably attacks the C-15 carbonyl to form the hemiketal ring in SEK4, the corresponding reaction does not occur in the case of the mutactin precursor. Instead, mutactin is formed as a result of an aldol condensation between the acidic C-6 methylene and the C-15 carbonyl. This difference in cyclization patterns is presumably due to the inability of the mutactin precursor to enolize at the C-11 carbonyl. In the presence of the *act* cyclase, the same precursor is converted into DMAC (7), which has a phenolic hydroxyl at C-11; however its longer chain counterparts are converted into aberrantly cyclized molecules such as RM18 (**12**) and RM20 (**8**). Perhaps the absence of correctly cyclized products in strains producing RM18 and RM20 is due to the inability of the *act* cyclase (or the *act*IV product) to enolize longer chain intermediates at the C-11 carbonyl.

Experimental Section

Bacterial Strains, Plasmids, and Culture Conditions. *S. coelicolor* CH999[3] was used as a host for transformation by all plasmids. DNA manipulations were performed in *Escherichia coli* MC1061. Plasmids were passaged through *E. coli* ET12567 (*dam dcm hsdS* Cm^r)[11] to generate unmethylated DNA before their use to transform *S. coelicolor*. *E. coli* strains were grown under standard conditions.[12] *S. coelicolor* strains were grown on R2YE agar plates[13] rather than in liquid media because of the apparently more abundant production of metabolites on agar media.

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Manipulation of DNA and Organisms. Standard *in vitro* techniques were used for DNA manipulations.[12] *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[13] and transformants were selected using 2 mL of a 500 mg/L thiostrepton overlay.

Construction of Plasmids Containing Recombinant PKSs. The construction of the pRM5 derivatives, pSEK4 and pSEK15, was described elsewhere.[3] The relevant biosynthetic genes on pSEK4 are as follows: *act*I ORFs 1–3 (KS/AT, CLF, and ACP, respectively), *act*VII (cyclase), and *act*IV (putative dehydratase). Those present on pSEK15 are as follows: *tcm*KLM (KS/AT, CLF, and ACP, respectively), *act*VII (cyclase), and *act*IV (putative dehydratase).

Production and Purification of SEK4. CH999/pSEK4 was grown on 90 agar plates (~34 mL/plate) at 30 °C for 7 days. The agar was chopped and extracted with ethyl acetate/methanol (4/1) in the presence of 1% acetic acid (3 × 1000 mL). Following removal of the solvent under vacuum, 200 mL of ethyl acetate containing 1% acetic acid was added. The precipitate was filtered and discarded, and the solvent was evaporated to dryness. The product mixture was applied to a Florisil column (Fisher Scientific) and eluted with ethyl acetate containing 3% acetic acid. The first 100-mL fraction was collected and concentrated down to 5 mL. Methanol (1 mL) was added, and the mixture was kept at 4 °C overnight. The precipitate was collected by filtration and washed with ethyl acetate to give 850 mg of pure product. R_f = 0.48 (ethyl acetate with 1% acetic acid). Results from NMR spectroscopy on SEK4 are reported in Table 1. FAB HRMS (NBA), M + H$^+$, calculated *m/e* 319.0818, observed *m/e* 319.0820.

Production and Purification of SEK15. CH999/pSEK15 was grown on 90 agar plates, and the product was extracted in the same manner as SEK4. The mixture was applied to a Florisil column (ethyl acetate with 5% acetic acid), and fractions containing the major product were combined and evaporated to dryness. The product was further purified using preparative C-18 reverse phase HPLC (Beckman) (mobile phase: acetonitrile/water 1/10 to 3/5 gradient in the presence of 1% acetic acid). The yield of SEK15 was 250 mg. R_f = 0.41 (ethyl acetate with 1% acetic acid). Results from NMR spectroscopy on SEK15 are reported in Table 1. FAB HRMS (NBA), M + H$^+$, calculated *m/e* 385.0923, observed *m/e* 385.0920.

[1,2-$^{13}C_2$]Acetate Feeding Experiments. Two 2-L flasks, each containing 400 mL of modified NMP medium,[14] were inoculated with spores of *S. coelicolor* CH999/pSEK4 or CH999/pSEK15. At 72 and 96 h postinoculation, 50 mg of sodium [1,2-$^{13}C_2$]acetate (Aldrich) was added to each flask. After 120 h, the cultures were extracted with two 500-mL volumes of ethyl acetate/1% acetic acid. The organic phase was kept, and purification proceeded as described above. Approximately 5 mg of each product was obtained. ^{13}C NMR data (2048 scans for SEK4 and 2624 scans for SEK15) indicated approximately 0.5–1% enrichment for SEK4 and 1–2% enrichment for SEK15 (estimated by comparing peak areas to the natural abundance ^{13}C peak area).

Mass and NMR Spectroscopy. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer under fast atom bombardment (FAB) conditions. NMR spectra were recorded on a Varian XL-400. ^{13}C spectra were acquired with continuous broadband proton decoupling. For NOE studies, the one-dimensional 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.

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