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Engineered biosynthesis of novel polyketides: Influence of a downstream enzyme on the catalytic specificity of a minimal aromatic polyketide synthase

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ABSTRACT To identify the minimum set of polyketide synthase (PKS) components required for *in vivo* biosynthesis of aromatic polyketides, combinations of genes encoding subunits of three different aromatic PKSs—*act* from *Streptomyces coelicolor* A3(2) (an actinorhodin producer), *fren* from *Streptomyces roseofulvus* (a frenolicin and nanaomycin producer), and *tcm* from *Streptomyces glaucescens* (a tetracenomycin producer)—were expressed in a recently developed *Streptomyces* host–vector system. The "minimal" components (ketosynthase/putative acyltransferase, chain length-determining factor, and acyl carrier protein) were produced with and without a functional polyketide ketoreductase and/or cyclase, and the polyketide products of these recombinant strains were structurally characterized. Several previously identified polyketides were isolated in addition to two previously unidentified polyketides, dehydromutactin and SEK 15b, described here. The results proved that the *act* cyclase is not required for the biosynthesis of several aberrantly cyclized products that have been previously reported. They are also consistent with earlier conclusions that the minimal PKS controls chain length as well as the regiospecificity of the first cyclization and that it can do so in the absence of both a ketoreductase and a cyclase. However, the ability of the minimal *tcm* PKS to synthesize two different singly cyclized intermediates suggests that it is unable to accurately control the course of this reaction by itself. In the presence of a downstream enzyme, the flux through one branch of the cyclization pathway increases relative to the other. We propose that these alternative specificities may be due to the ability of downstream enzymes to associate with the minimal PKS and to selectively inhibit a particular branch of the cyclization pathway.

Polyketides are a large family of structurally diverse natural products with a broad range of biological activities, including antibiotic and pharmacological properties. Polyketide synthases (PKSs) are structurally and mechanistically related to fatty acid synthases (1–4). Both classes are multifunctional enzymes that catalyze repeated decarboxylative condensations between acyl thioesters (usually acetyl, propionyl, malonyl, or methylmalonyl). The main difference between PKSs and fatty acid synthases is that, following each condensation, PKSs introduce enormous structural variability into the product by omitting all, part, or none of the typical fatty acid synthase reductive cycle comprising a ketoreduction, dehydration, and enoylreduction on the β-keto group of the growing polyketide chain.

Within the PKSs, recent molecular genetics and biochemical studies have revealed two different mechanisms for the control of polyketide specificity. In one, exemplified by the PKS for the macrolide antibiotic erythromycin, the synthase provides separate sets of active sites for each condensation and reduction cycle, and product structure is dictated by the number and arrangement of these active sites (5, 6). In the second class, represented by the actinomycete PKSs for aromatic polyketides, this relationship is not apparent because each synthase contains a single set of iteratively used active sites for all condensation and reduction cycles.

Studies on PKS gene clusters, including those based on sequence analysis (7–9), functional complementation *in vivo* (9–16), and enzymological analysis *in vitro* (4), have led to the assignment of specific properties to aromatic PKS gene products. Each PKS consists of a "minimal" set of three protein subunits [a bifunctional ketosynthase/acyltransferase (KS/AT), a chain length-determining protein (CLF), and an acyl carrier protein (ACP)], which is necessary for *in vivo* polyketide biosynthesis. In addition, most aromatic PKSs also contain separate ketoreductase (KR) and cyclase (CYC) enzymes (Fig. 1).

Recently, we developed a potentially general strategy for the biosynthesis of polyketides by the functional expression of recombinant PKSs carrying different combinations of subunits encoded by various gene clusters in a specially constructed expression system (10). Analysis of the structures of these molecules has thus far revealed several key features of the mechanisms by which bacterial aromatic PKSs control product specificities, including carbon-chain length, degree and regiospecificity of ketoreduction, and regiospecificity of cyclization (10–12, 19). A particularly remarkable feature, observed within the set of aromatic polyketides generated by using this genetic approach, is the tremendous diversity in cyclization patterns [compare the structures of the naturally occurring polyketides in Fig. 2 (compounds **1–4**) with those of the genetically engineered polyketides in Fig. 3 (compounds **5–16**)]. Although every recombinant PKS gene cluster in the above studies included the gene for the *act* CYC, it was hypothesized that most of these unusually cyclized molecules were shunt products caused by the inability of the *act* CYC to recognize unnatural polyketide intermediates. To test this hypothesis, we have constructed several deletion mutants lacking the *act* CYC gene. As described here, analysis of the polyketide products in the resulting strains has led to new insights into the role of CYCs in polyketide biosynthesis.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions. *S. coelicolor* CH999 (10) 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]) (21) to generate unmethylated DNA before their use to transform *S. coelicolor*. *E. coli*

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Abbreviations: CYC, cyclase; PKS, polyketide synthase; CLF, chain length-determining factor; KS, ketosynthase; AT, acyltransferase; KR, ketoreductase; DMSO, dimethyl sulfoxide.

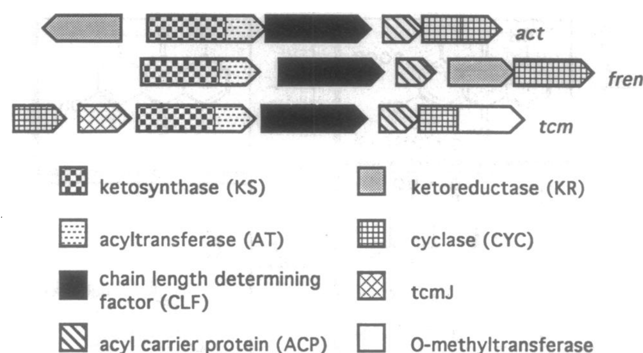

FIG. 1. Aromatic PKS gene clusters. Each PKS includes a KS/AT, a CLF, and an ACP, collectively referred to as the minimal PKS. The *act* and *fren* clusters also contain a KR. The AT presumably transfers the starter unit from CoA to the KS, which catalyzes the condensation between the starter (or growing polyketide) acyl thioester and the extender thioester on the ACP. The KR reduces a specific carbonyl of the actinorhodin polyketide backbone. The gene clusters also encode a CYC involved in cyclization of the nascent polyketide backbone; however, the *tcm* gene cluster is unusual because it encodes at least two CYC genes: a bifunctional CYC/*O*-methyltransferase gene (*tcmN*; located downstream of the *tcm* ACP; ref. 16) and an upstream CYC gene, responsible for the formation of the last ring in tetracenomycin biosynthesis (*tcmI*; ref. 18). In addition, the *tcm* gene cluster also encodes tcmJ, which has been hypothesized to catalyze the second cyclization in tetracenomycin biosynthesis (18).

strains were grown under standard conditions (22). *S. coelicolor* strains were grown on R2YE agar plates (23) rather than in liquid medium because of the apparently more abundant production of metabolites on agar medium.

Manipulation of DNA and Organisms. Standard *in vitro* techniques were used for DNA manipulations (22). *E. coli* was transformed with a Bio-Rad *E. coli* Pulser electroporating apparatus using protocols provided by Bio-Rad. *S. coelicolor* was transformed by the standard procedure (23), and transformants were selected by using a 2-ml overlayer containing 500 μg of thiostrepton per ml of water.

Construction of Plasmids Containing PKS Gene Clusters. Ten plasmids were used (Table 1). The construction of pRM5, pRM34, pRM37, pSEK4, and pSEK15 has been described (10–12). Each plasmid carries the genes encoding

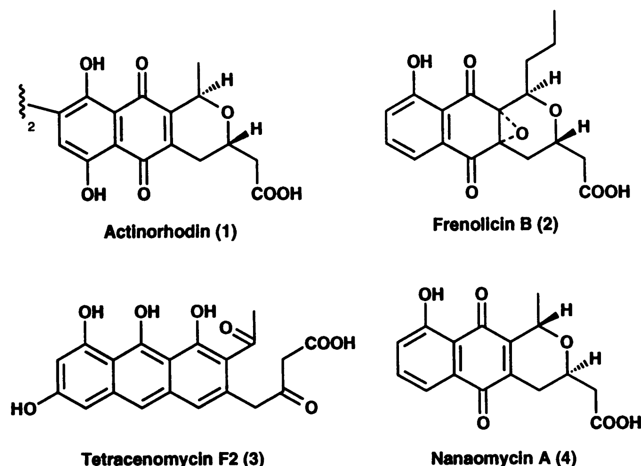

FIG. 2. Naturally occurring polyketides synthesized by PKSs used in this study. The *act* gene cluster, found in *Streptomyces coelicolor* A3(2), produces actinorhodin. The *fren* gene cluster, found in *Streptomyces roseofulvus*, produces frenolicin and nanaomycin. The *tcm* gene cluster, found in *Streptomyces glaucescens*, produces tetracenomycin C; the structure shown here is that of tetracenomycin F2, a biosynthetic intermediate produced by several strains blocked in the tetracenomycin pathway (20).

a KS/AT, CLF, and ACP; in addition, pRM5, pRM34, and pRM37 also carry a gene encoding the *act* KR. All five plasmids possess a copy of the *act*VII (CYC) and *act*IV (putative dehydratase) genes. To generate minimal PKSs, the plasmid pSEK31 was first constructed by replacing a *Pst* I-*Eco*RI fragment in pIJ5639 (17), which includes the *act*VII and *act*IV genes, with a "silent" 0.6-kb *Pst* I-*Eco*RI fragment from a *hyg* cassette (13). The final minimal PKS plasmids, designated pSEK21, pSEK22, pSEK23, pSEK24, and pSEK33, were constructed by replacing the *Xba* I-*Eco*RI fragment in pRM5, pRM34, pRM37, pSEK4, and pSEK15, respectively, with the *Xba* I-*Eco*RI from pSEK31. All plasmids were constructed in *E. coli* before introduction into CH999 by transformation.

Production, Purification, and Characterization of Polyketides. The product profile of each strain was evaluated by methods described earlier (10–12, 19). The structures and isolation methods for mutactin (compound **7** in Fig. 3; ref. 24), RM18 (compound **11**; ref. 11), RM18b (compound **10**; ref. 11), RM20 (compound **12**; ref. 10), RM20b (compound **13**; ref. 19), RM20c (compound **14**; ref. 19), SEK4 (compound **9**; ref. 12), and SEK15 (compound **15**; ref. 12) were reported elsewhere. During the process of purifying mutactin, considerable amounts of a related product [earlier designated dehydromutactin (compound **8**; ref. 17)] were detected. This molecule was also purified via reverse-phase HPLC. The purification of SEK15b (compound **16**) was identical to methods described earlier (11).

Sodium [1,2-$^{13}C_2$]Acetate Feeding Experiments. Confluent lawns of *S. coelicolor* CH999/pSEK33 were grown on eight agar plates, each with approximately 35 ml of R5 medium (23) containing 50 μg of thiostrepton and 0.5 mg of sodium [1,2-$^{13}C_2$]acetate (Sigma) per ml. Isolation of SEK15b (**16**) was carried out as described above. Approximately 0.5 mg of product was obtained. ^{13}C NMR data indicated approximately 2–3% enrichment (estimated by comparing peak areas to the natural abundance ^{13}C peak area).

Mass and NMR Spectroscopy. Field-desorption mass spectroscopy was used for analysis of dehydromutactin (**8**), and fast-atom-bombardment mass spectroscopy was used for analysis for SEK15b (**16**). NMR spectra were recorded on a Varian XL-400. ^{13}C spectra were acquired with continuous broadband proton decoupling. All compounds were dissolved in totally deuterated dimethyl sulfoxide ([*methyl*-$^2H_6$]DMSO; Sigma, 99+ mol % ^{2}H), and spectra were referenced internally to the solvent. Hydroxyl resonances were identified by adding ^2H_2O (Aldrich, 99 mol % ^{2}H) and checking for disappearance of the signal.

Data characterizing dehydromutactin (**8**) are as follows: ^{1}H NMR ([*methyl*-$^2H_6$]DMSO, 400 MHz) δ 11.38 (very broad peak, 2OH), 7.29 (dd, J = 7.73, 8.38 Hz, 1H), 6.98 (d, J = 8.42, 1H), 6.74 (d, J = 7.61 Hz, 1H), 6.69 (s, 1H), 6.19 (d, J = 2.07 Hz), 5.44 (d, J = 2.16 Hz), 2.26 (s, 3H); ^{13}C NMR ([*methyl*-$^2H_6$]DMSO, 100 MHz) δ 170.4, 164.5, 161.3, 155.8, 154.6, 136.9, 135.2, 128.5, 120.4, 115.8, 113.0, 110.8, 108.6, 105.3, 89.8, 20.5. Field-desorption MS (FD-MS) m/e 284. Spectra are consistent with those reported for mutactin (**7**) (24).

RESULTS AND DISCUSSION

Properties of Minimal Aromatic PKSs. The series of recombinant gene clusters was constructed and functionally expressed in the genetically engineered *S. coelicolor* host CH999 from which the actinorhodin biosynthetic gene cluster had been deleted (Table 1). Since earlier studies had shown that the ACPs from different aromatic PKSs could be exchanged without loss of yield or catalytic specificity (10, 11), the *act* ACP was used in all new constructs described here. The product profile of each recombinant strain is summarized in Table 1. All strains produced abundant quantities (>100 mg/liter) of polyketides. Thus, the minimal PKS is sufficient

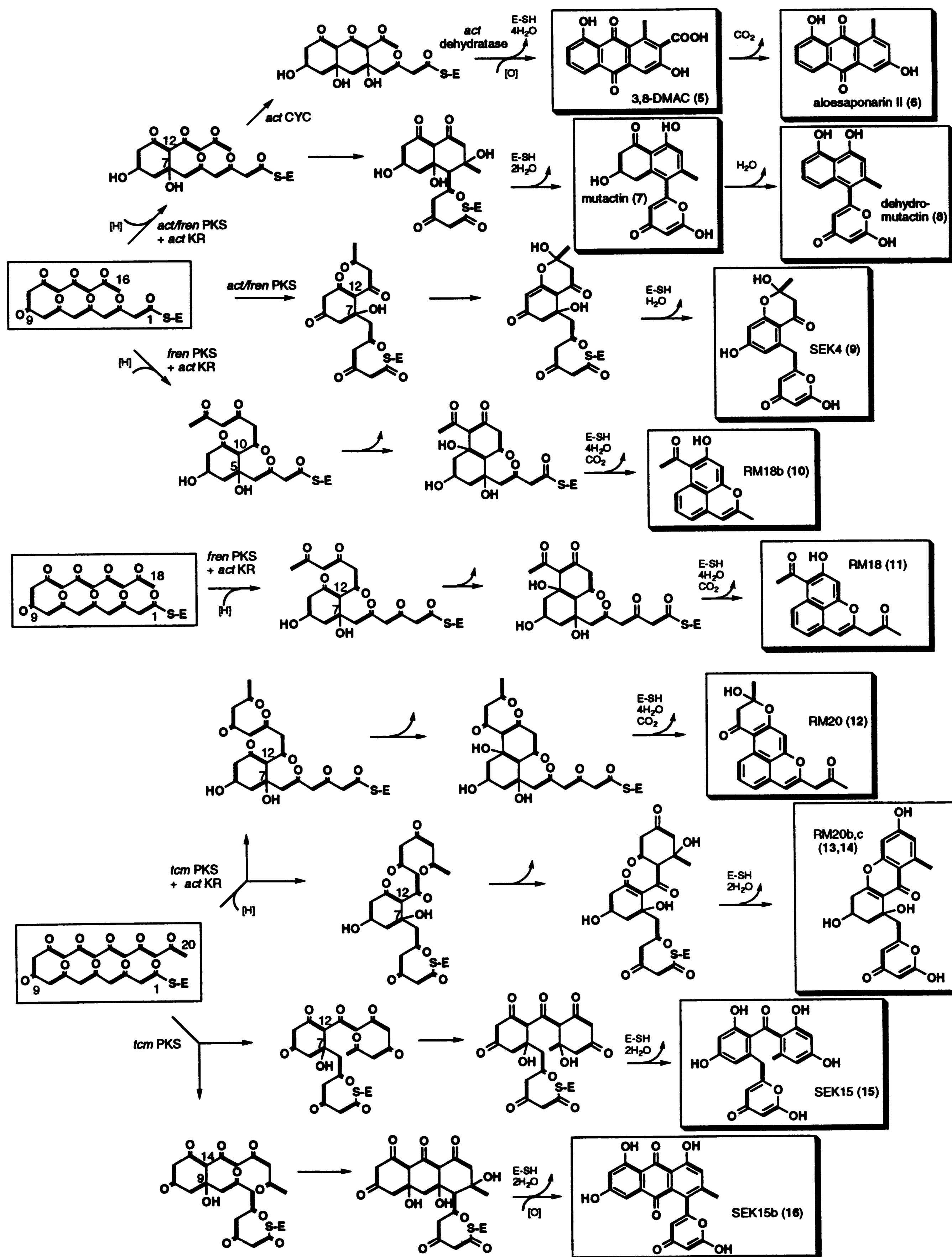

FIG. 3. Structures and proposed biosynthetic pathways of genetically engineered polyketides synthesized by recombinant PKSs. Polyketides produced by genetically engineered PKSs are shown in shadowed boxes. RM20b and RM20c are stereoisomers with opposite configurations at C-7. All of these molecules arise from octaketides, nonaketides, or decaketides (shown in normal boxes). The pathways illustrated here are

Table 1. Polyketides produced by homologous and heterologous combinations of PKS proteins

Plasmid	Minimal PKS			KR	CYC	Major product(s)	Relative yields	Ref.
	KS/AT	CLF	ACP					
pRM5	*act*	*act*	*act*	*act*	*act*	**5, 6**	1:2	10
pSEK21	*act*	*act*	*act*	*act*	–	**7, 8**	3:1	This work
pSEK4	*act*	*act*	*act*	–	*act*	**9**		12
pSEK24	*act*	*act*	*act*	–	–	**9**		This work
pRM34	*fren*	*fren*	*fren*	*act*	*act*	**5, 6, 10, 11**	1:2:2:1	11
pSEK22	*fren*	*fren*	*act*	*act*	–	**7, 8, 10, 11**	4:4:2:1	This work
pRM37	*tcm*	*tcm*	*tcm*	*act*	*act*	**12, 13, 14**	3:7:1	10, 19
pSEK23	*tcm*	*tcm*	*act*	*act*	–	**12, 13, 14**	3:7:1	This work
pSEK15	*tcm*	*tcm*	*tcm*	–	*act*	**15, 16**	16:1	12; This work
pSEK33	*tcm*	*tcm*	*act*	–	–	**15, 16**	1:1	This work

for polyketide biosynthesis, and the presence or omission of specific KR and/or CYC activities does not appear to affect the overall metabolite flux through the PKS pathway. This is in qualitative, but not quantitative, agreement with recent results of Summers *et al.* (18), who showed that, although the minimal *tcm* PKS (KS/AT, CLF, and ACP) in *S. glaucescens* was sufficient for the biosynthesis of tetracenomycin F2 (3; 20), the polyketide yield from such a strain was drastically reduced compared with strains that also included the *tcmN* gene encoding a bifunctional CYC/O-methyltransferase involved in tetracenomycin biosynthesis (see Fig. 1; ref. 16). Our results also confirm the earlier speculation that the *act* IV gene (which encodes a putative dehydratase) is not necessary for the biosynthesis of any of compounds **7–16** (10–12). While the polyketide product profiles of strains containing most of the new genetic constructs reported here can be rationalized on the basis of results of earlier studies, there are a few unexpected and important findings as follows.

The minimal act *PKS*. The strain containing only the minimal *act* PKS (CH999/pSEK24; Table 1) produced SEK4 (**9**), an unreduced octaketide, which underwent the first cyclization with the same regiospecificity as actinorhodin (**1**) but was unable to cyclize correctly thereafter (see Fig. 3). Likewise, the strain containing a functional KR along with the minimal *act* PKS (CH999/pSEK21; Table 1) produced mutactin (**7**), which had the correct chain length regiospecificities of ketoreduction and first cyclization but an aberrant second (and third) cyclization (Fig. 3). These results are consistent with earlier predictions (10–12) that (*i*) the chain length and regiospecificity of the first cyclization are determined by the minimal PKS, (*ii*) the *act*KR is both necessary and sufficient for reducing the C-9 carbonyl, and (*iii*) the *act* CYC, which influences the regiospecificity of the second intramolecular aldol condensation (cyclization), is required for the biosynthesis of aloesaponarin II (**6**) (14) and its precursor 3,8-dihydroxymethylanthraquinone carboxylic acid (DMAC) (**5**) (10), but not for mutactin (Fig. 3). Furthermore, the absence of any cyclizing enzymes in CH999/pSEK21 and CH999/pSEK24 is consistent with the hypothesis that the difference in cyclization patterns between mutactin (**7**) and SEK4 (**9**) arises from the inability of the C-11 carbonyl in mutactin to enolize, as it must do in SEK4 (12).

Interestingly, relatively large quantities (200 mg per liter of culture) of a dehydrated analog of mutactin were also produced by the strain expressing the *act* minimal PKS and the *act* KR genes (CH999/pSEK21; Table 1). Based on mass spectrometric analysis of small quantities of this molecule isolated from another mutactin producer, this molecule had earlier been called dehydromutactin (**8**) (17). Dehydromutactin is presumably formed via a nonenzymatic dehydration of mutactin (Fig. 3).

The minimal fren *PKS*. The strain containing the minimal *fren* PKS with the *act* KR (CH999/pSEK22; Table 1) produced four polyketides: RM18 (**11**) (11), RM18b (**10**) (11), mutactin (**7**), and dehydromutactin (**8**). This is consistent with the observed relaxed chain-length specificity of the *fren* PKS, producing both 16- and 18-carbon polyketide chains, and verifies an earlier assumption that the *act* CYC is not required for the biosynthesis of RM18 and RM18b.

The minimal tcm *PKS*. The *act* CYC is also not required for the biosynthesis of the decaketides RM20 (**12**) (10), RM20b (**13**) (19), and RM20c (**14**) (19), as demonstrated by the product profile of the strain expressing the minimal *tcm* PKS and *act* KR genes (CH999/pSEK23; Table 1). Unexpectedly, however, the strain containing the minimal *tcm* PKS alone (CH999/pSEK33; Table 1) produced two decaketides, SEK15 (**15**) (12) and SEK15b (**16**) in approximately equal amounts. SEK15 was earlier isolated in CH999/pSEK15, which is identical to CH999/pSEK33 except for the presence of the *act* CYC (Table 1). SEK15b (**16**) is an unusual compound whose structure and deduced backbone are described below. Upon further examination, CH999/pSEK15 was also found to produce this new polyketide, although in significantly smaller quantities compared with SEK15 (Table 1).

Structure and Biosynthesis of SEK15b (16). ^{1}H and ^{13}C NMR (Table 2) suggested that SEK15b consisted of an unreduced anthraquinone moiety and a pyrone moiety. Sodium [1,2-$^{13}C_2$]acetate feeding experiments confirmed that the carbon chain of SEK15b was derived from 10 acetate units. The coupling constants calculated from the ^{13}C NMR spectrum of the enriched SEK15b sample also facilitated peak assignment. Fast-atom-bombardment mass spectroscopy gave a molecular weight of 381 ($M + H^+$), consistent with $C_{20}H_{12}O_8$. Deuterium exchange was used to confirm the presence of each hydroxyl group in SEK15b.

The cyclization pattern of SEK15b has two interesting features, which provide important clues regarding the enzymatic control of this class of reactions in aromatic polyketides. First, the regiospecificity of the initial cyclization in SEK15b differs from that observed in SEK15 (Fig. 3) but is the same as that observed in tetracenomycin F2, the natural product of the *tcm* PKS in *S. glaucescens*. The production of

FIG. 3. (*Continued*). hypothetical, since no intermediates have been isolated. In each pathway, after biosynthesis of the full-length polyketide chain, the nascent polyketide undergoes an initial cyclization, whose regiospecificity may be influenced by certain enzymes (see text), and ketoreduction (if *act* KR is present). This is followed by a second cyclization reaction, whose regiospecificity is influenced by the *act* CYC. The *act* CYC can discriminate between carbon chains of different lengths as well as degrees and regiospecificities of reduction (10–12). In the absence of CYC activity, the methyl and carboxyl ends of the polyketide appear to cyclize independently (the only exception is in the case of the mutation precursor, whose methyl end is relatively inert). In cases where abberant cyclizations are observed, it is proposed that the methyl end of the singly cyclized polyketide intermediate undergoes cyclization, while the carboxyl end remains bound to the enzyme. DMAC, 3,8-dihydroxymethylanthraquinone carboxylic acid.

Table 2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data from SEK15b (**16**)

Carbon*	^{13}C, ppm	J_{cc}, Hz	^{1}H, ppm (m, area)
1	164.5	79.3	11.98 (s, 1OH)
2	89.4	78.7	5.42 (s, 1H)
3	170.6	57.5	
4	105.3	57.8	6.23 (s, 1H)
5	157.8	67.5	
6	127.6	67.5	
7	136.9	52.1	
8	182.1	52.2	
9	134.1	63.5	
10	107.1	64.6	7.04 (s, 1H)
11	164.2	66.5	11.54 (s, 1OH)
12	108.4	67.5	6.57 (s, 1H)
13	164.0	61.9	13.10 (s, 1OH)
14	110.3	61.6	
15	187.8	56.5	
16	122.2	57.6	
17	160.6	62.2	11.11 (s, 1OH)
18	112.4	62.9	7.62 (s, 1H)
19	143.8	42.2	
20	20.0	42.0	2.59 (s, 3H)

*Carbons are labeled according to their number in the polyketide backbone (Fig. 3). J_{cc}, carbon–carbon coupling constant.

approximately equal quantities of the two cyclized forms (**15** and **16**) by CH999/pSEK33 suggests that the *tcm* PKS has a relaxed specificity for the regiochemistry of the first cyclization. However, in the presence of the *act* CYC, the "unnatural" C7–C12 cyclization pathway is significantly favored over the "natural" C9–C14 cyclization pathway (compare the relative quantities of SEK15 and SEK15b produced by CH999/pSEK33 and CH999/pSEK15 in Table 1). In other words, the *act* CYC in CH999/pSEK15 affects the specificity of the first aldol condensation, even though it does not play a role in catalyzing the appropriate second condensation reaction. In contrast, the expression of *tcmN* (encoding a bifunctional CYC/*O*-methyltransferase; see Fig. 1) in *S. glaucescens* leads to the biosynthesis of dominant quantities of tetracenomycin F2 (16), which contains the "natural" C9–C14 cyclized product. Thus, enzymes acting after initial cyclization may alter the catalytic specificity of the minimal PKS. While the existence of editing mechanisms cannot be ruled out, a more likely explanation involves the ability of downstream enzymes, such as CYCs (bifunctional CYC/*O*-methyltransferases), to selectively inhibit the occurrence of one type of initial cyclization, thereby increasing the metabolic flux through the other branch of the cyclization pathway. This could be easily rationalized by a model in which downstream enzymes associate with the minimal PKS to form a complex that either constrains the catalytic specificity of the minimal PKS or allows the enzymes to interact with the nascent polyketide substrate.

Another observation concerning the specificity of the first cyclization is that only "unnatural" C7–C12 cyclizations are observed in reduced molecules produced by the minimal *tcm* PKS. Since it is not known specifically when ketoreduction occurs in biosynthesis, this preferred cyclization may represent another example in which specificity is influenced by an associated downstream enzyme. Alternatively, if ketoreduction occurs before the first cyclization, the minimal *tcm* PKS does not have a relaxed specificity for reduced substrates.

Finally, it should be noted that the regiospecificity of the second cyclization of SEK15b is identical to that observed in tetracenomycin F2. This is so, despite the fact that CH999/pSEK33 contains neither the *act* CYC (25) nor any of the *tcm* CYCs (16, 18) and suggests that the minimal *tcm* PKS is capable of controlling the regiochemical course of this reaction even in the absence of additional CYCs. However, it can be contrasted to the absence of detectable levels of products with regiochemically correct second aldol condensations in other strains lacking CYC activities (Table 1). Further studies along the above lines using CYCs from different PKS gene clusters will prove invaluable in helping to clarify the functions and specificities of this class of enzymes.

Note added in proof. More recent results indicate that the *act*IV gene product catalyzes the second cyclization in the biosynthesis of compound **6**, whereas the *act*VII gene product plays a role in the formation of the first ring (26).

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