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Engineered Biosynthesis of Novel Polyketides: Analysis of TcmN Function in Tetracenomycin Biosynthesis

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Abstract: Two proteins, encoded by the *tcmJ* and *tcmN* genes, have been previously implicated in early cyclization steps in the biosynthesis of the aromatic polyketide tetracenomycin. In order to elucidate the function of these enzymes and to evaluate their potential for generating novel polyketides, several *tcmJ*- and *tcmN*-containing recombinant polyketide synthase (PKS) gene clusters were constructed and analyzed *in vivo*. These constructs led to the expression of combinations of the TcmJ and TcmN proteins with subunits from the actinorhodin (*act*) and tetracenomycin (*tcm*) PKSs, responsible for the biosynthesis of different polyketide backbones. In addition to three novel polyketides, RM77 (**3**), RM80 (**4**), and RM80b (**5**), which are characterized here, several previously isolated polyketides were produced. While results for TcmJ were inconclusive, comparison of the new molecules to other polyketide structures has allowed us to propose two functions for TcmN. First, consistent with earlier predictions [McDaniel, R.; Ebert-Khosla, S.; Fu, H.; Hopwood, D. A.; Khosla, C. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 11542–11546], the TcmN protein influences the regiospecificity of the intramolecular aldol condensation that forms the first ring. Second, the protein appears to catalyze the aromatization of the second ring, and is deduced to be a *second* ring aromatase. The inability of TcmN to have any affect on C-9 reduced backbones suggests that ketoreduction occurs prior to cyclization of the first ring in reduced polyketides. The analysis of TcmN extends the use of genetically engineered PKSs for novel polyketide design and biosynthesis.

Polyketides are a large family of structurally diverse natural products which possess a wide range of biological activities, including antibiotic and other pharmacological properties. They are synthesized by multifunctional polyketide synthase enzymes (PKSs). PKSs catalyze repeated condensations between acyl thioesters (usually acetyl, propionyl, malonyl, or methylmalonyl) which results in the formation of a β-keto group during each cycle that may undergo none, some, or all of a series of reductive steps. In the case of aromatic PKSs, the β-keto groups are left largely unmodified and the resulting highly reactive polyketide backbone undergoes enzyme-catalyzed regiospecific cyclization.[1,2]

The fact that PKSs are structurally and mechanistically related to each other has prompted interest in their potential use for the rational design and engineering of novel compounds[2] as well as for combinatorial biosynthesis of polyketide libraries.[3,4] Recently, we described the design and construction of a *Streptomyces* host–vector expression system and its application for the engineered biosynthesis of several novel polyketides. Elucidation of the structures and biosynthetic pathways of these molecules has led to significant advances in our understanding of PKS enzyme function and specificity.[3–12]

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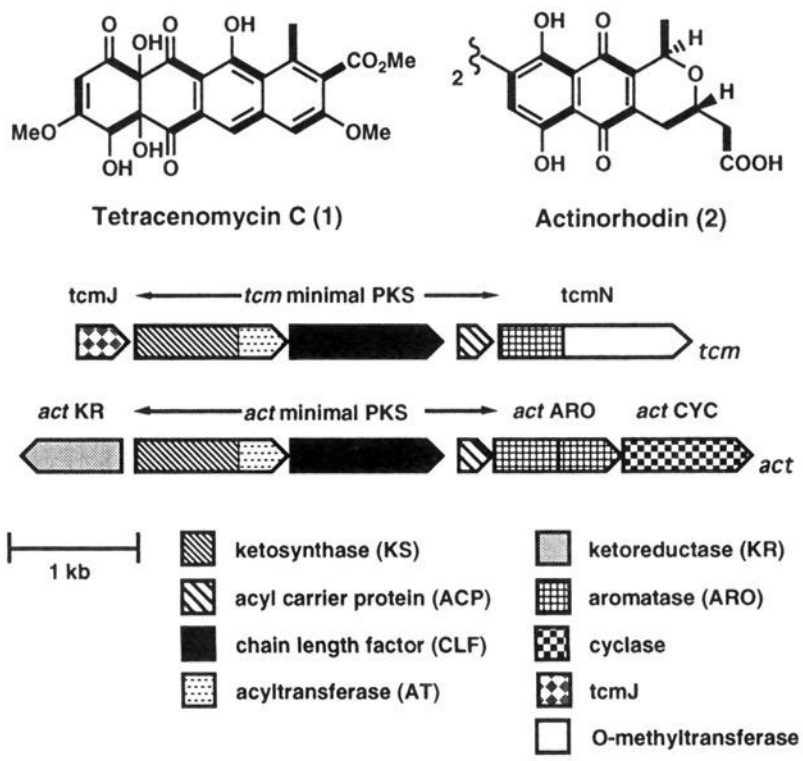

Figure 1. 1. Naturally occuring polyketides tetracenomycin C (**1**) (*tcm*) from *S. glaucescens* and actinorhodin (**2**) (*act*) from *S. coelicolor*, relevant portions of their biosynthetic gene clusters, and deduced protein functions. Each minimal PKS includes a KS/AT, a CLF, and an ACP, which is responsible for biosynthesis of the nascent polyketide backbone. The CLF is involved in dictating carbon chain length. The *act* cluster also contains a KR which reduces a specific carbonyl of the polyketide. The *act* ARO and *act* CYC aromatize the first ring and cyclize the second ring of actinorhodin, respectively. TcmJ and TcmN are analyzed in this work.

The actinorhodin (*act*) PKS from *Streptomyces coelicolor* and the tetracenomycin (*tcm*) PKS from *Streptomyces glaucescens* have been central in our studies on bacterial aromatic polyketide biosynthesis. The entire biosynthetic gene clusters for both of these molecules have been sequenced (Figure 1).[13−19] Actinorhodin (**2**) is derived from an octaketide, whereas tetracenomycin (**1**) is derived from a decaketide. Through the construction and analysis of several hybrid PKS complexes comprised of components from both pathways, it was shown that a specific protein is involved in determining polyketide carbon chain length (Figure 1).[3] The difference in degree of reduction between actinorhodin and tetracenomycin was also exploited to determine the function and regiospecificty of the *act* ketoreductase (KR).[3,9] More recent studies have been aimed at probing the mechanisms by which PKSs control the cyclizations of a nascent polyketide backbone.[6,7]

Table 1. Polyketides Produced by Homologous and Heterologous Combinations of PKS Proteins

plasmid	minimal PKS[a]	KR	TcmJ,N	product	ref
pSEK21	*act*	*act*		mutactin (**9**)	6
pRM70	*act*	*act*	TcmJ	mutactin (**9**)	this work
pRM71	*act*	*act*	TcmN	mutactin (**9**)	this work
pRM72	*act*	*act*	TcmJ,N	mutactin (**9**)	this work
pSEK23	*tcm*	*act*		RM20b (**10**)	6
pRM73	*tcm*	*act*	TcmJ	RM20b (**10**)	this work
pRM74	*tcm*	*act*	TcmN	RM20b (**10**)	this work
pRM75	*tcm*	*act*	TcmJ,N	RM20b (**10**)	this work
pSEK24	*act*			SEK4 (**6**)	6
pRM76	*act*		TcmJ	SEK4 (**6**)	this work
pRM77	*act*		TcmN	RM77 (**3**)	this work
pRM78	*act*		TcmJ,N	RM77 (**3**)	this work
pSEK33	*tcm*			SEK15 (**8**)/SEK15b (**7**)	6
pRM79	*tcm*		TcmJ	SEK15 (**8**)/SEK15b (**7**)	this work
pRM80	*tcm*		TcmN	RM80 (**4**)/RM80b (**5**)	this work
pRM81	*tcm*		TcmJ,N	RM80 (**4**)/RM80b (**5**)	this work

[a] The minimal PKS is comprised of the KS/AT and CLF from the gene cluster indicated, and the *act* ACP (see Figure 1).

Here, we extend our approach to the analysis of two additional PKS enzymes, encoded by *tcmJ* and *tcmN* (Figure 1). Previous studies have speculated on the involvement of these proteins in ring cyclizations during tetracenomycin biosynthesis.[17,18] More recently, it was shown that the *tcm* minimal PKS (ketosynthase/putative acyltransferase (KS/AT), chain length factor (CLF), and acyl carrier protein (ACP)) catalyzes cyclization of the first ring with a relaxed specificity, and that the course of this reaction is influenced by the presence or absence of the *act* aromatase (ARO). It was therefore postulated that a protein such as the *tcmN* gene product, a homolog of the *act* ARO, may control the regiospecificity of the first cyclization in the tetracenomycin pathway.[6]

To evaluate the specific catalytic roles of the proteins, *tcmJ* and *tcmN* were expressed in the presence of additional *act* and *tcm* PKS components in our *Streptomyces* host−vector system. While results for TcmJ were inconclusive, the isolation of three novel polyketides from these genetic constructs has allowed us to assign two distinct catalytic functions to TcmN. While these results are consistent with some of the earlier predictions of the TcmN function, they also provide new insights into the mechanisms of aromatic polyketide cyclization. Furthermore, a new biosynthetic degree of freedom has been identified that can be incorporated in the design and production of novel polyketides.

Results

Analysis of Recombinant Strains. The series of recombinant gene clusters shown in Table 1 was constructed. Each plasmid contains either *tcmJ*, *tcmN*, or both in addition to the minimal PKS genes responsible for the biosynthesis of 16 (*act*) or 20 (*tcm*) carbon backbones. Half of the plasmids also contain the gene encoding the *act* ketoreductase (KR, *act*III), which catalyzes ketoreduction at the C-9 position of the nascent polyketide backbone.[3,5,9] For analysis, the plasmids were introduced by transformation into *S. coelicolor* CH999, a host in which the entire actinorhodin polyketide cluster has been deleted. The major polyketides produced by the transformed strains were isolated and structurally characterized using a combination of NMR spectroscopy, isotopic labeling, and mass

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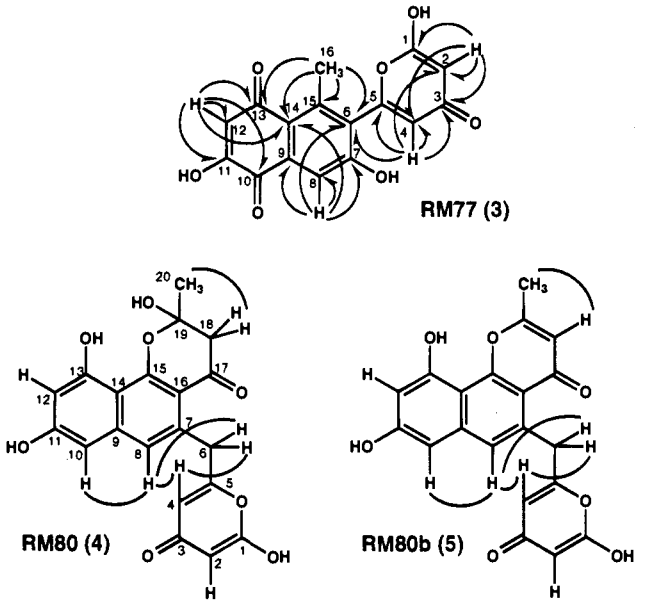

Figure 2. Summary of HMBC analysis of RM77 (**3**) and NOE data from RM80 (**4**) and RM80b (**5**).

Table 2. ^1H and ^{13}C NMR Data from RM77 (**3**)[a]

carbon[b]	^{13}C δ (ppm)	J_{CC} (Hz)	^1H δ (ppm) (m, J_{HH}, area)
1	163.9	79.2	*c*
2	89.7	78.8	5.40 (d, 2.1, 1H)
3	170.0	56.9	
4	104.9	57.3	6.19 (d, 2.1, 1H)
5	158.0	67.7	
6	126.8	66.8	
7	158.9	62.2	*c*
8	111.6	62.5	7.49 (s, 1H)
9	134.8	54.5	
10	181.3	53.5	
11	157.1	70.7	*c*
12	113.6	70.5	6.05 (s, 1H)
13	186.2	55.1	
14	121.4	54.7	
15	141.7	41.5	
16	18.7	42.1	2.49 (s, 3H)

[a] In DMSO-d_6 (400 MHz for ^1H and 100 MHz for ^{13}C). [b] Carbons are labeled according to Figure 2. [c] Hydroxyl resonances at 11.8 (br s, 2OH) and 11.2 (s, 1OH), concentration dependent.

spectroscopy experiments. All of the polyketides isolated have been previously structurally characterized with the exception of the novel polyketides RM77 (**3**), RM80 (**4**), and RM80b (**5**).

Structure Elucidation of RM77 (3). RM77 (**3**) precipitated as a yellow powder from HPLC fractions. Sodium [1,2-$^{13}C_2$]-acetate feeding experiments indicate that RM77 (**3**) is derived from an octaketide (eight acetate units) backbone. The ^{13}C-NMR spectrum contains chemical shifts indicative of a quinone moiety, as well as characteristic peaks associated with 2-hydroxy-4-pyrone rings formed at the carboxyl end of several polyketides.[6-9,20] Unambiguous ^1H- and ^{13}C-NMR chemical shift assignments for RM77 were confirmed via heteronuclear multiple bond correlation (HMBC) studies and ^{13}C coupling constants obtained with an isotopically labeled sample (Figure 2, Table 2). Also, consistent with the proposed structure was the absence of any significant nuclear Overhauser effect (NOE) interaction. High-resolution fast atom bombardment (FAB) mass spectroscopy gave molecular weights of 337.0324 (M + Na^+, 337.0319 expected) and 315 (M + H^+) consistent with the molecular formula $C_{16}H_{10}O_7$.

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Table 3. ^1H and ^{13}C NMR Data from RM80 (**4**) and RM80b (**5**)[a]

	RM80 (**4**)			RM80b (**5**)		
carbon[b]	^{13}C δ (ppm)	J_{CC} (Hz)	^1H δ (ppm) (m, J_{HH} (Hz), area)	^{13}C δ (ppm)	J_{CC} (Hz)	^1H δ (ppm) (m, J_{HH} (Hz), area)
1	164.2	79.6	*c*	163.8	79.8	*d*
2	88.3	79.6	5.23 (d, 2.1, 1H)	88.0	79.6	5.16 (d, 1.6, 1H)
3	170.7	58.1		170.4	57.9	
4	99.3	58.1	5.52 (d, 1.9, 1H)	98.8	58.3	5.43 (d, 1.2, 1H)
5	166.5	51.2		166.8	51.0	
6	38.4	51.0	4.16 (d, 15.5, 1H) 4.19 (d, 15.2, 1H)	38.1	51.2	4.39 (s, 2H)
7	131.5	63.4		130.4	63.4	
8	123.3	63.0	7.09 (s, 1H)	126.9	62.9	7.38 (s, 1H)
9	139.6	58.8		138.3	58.9	
10	102.1	59.8	6.63 (d, 2.2, 1H)	101.7	59.1	6.68 (d, 2.0, 1H)
11	160.6	64.4	*c*	159.5	63.9	*d*
12	102.8	64.8	6.47 (d, 2.3, 1H)	103.6	64.4	6.65 (d, 2.1, 1H)
13	157.4	62.0	*c*	157.0	66.0	*d*
14	108.0	62.1		107.8	66.3	
15	159.6	66.8		157.0	66.0	
16	110.5	66.7		115.0	65.0	
17	190.9	39.4		177.6	55.9	
18	49.0	39.7	2.74 (d, 15.9, 1H) 3.12 (d, 15.3, 1H)	111.8	56.1	6.19 (s, 1H)
19	104.2	45.7	7.62 (br s, 1OH)	163.8	50.8	
20	27.3	46.0	1.76 (s, 3H)	19.4	50.8	2.40 (s, 3H)

[a] In DMSO-d_6 (400 MHz for ^1H and 100 MHz for ^{13}C). [b] Carbons are labeled according to Figure 2. [c] Hydroxyl resonances at 11.5 (s, 1OH), 10.4 (s, 1OH), and 9.7 (s, 1OH), concentration dependent. [d] Hydroxyl resonances at 11.4 (s, 1OH), 10.3 (s, 1OH), and 10.2 (s, 1OH), concentration dependent.

Structure Elucidation of RM80 (4) and RM80b (5). Both RM80 (**4**) and RM80b (**5**) are pale yellow compounds. In all strains producing these molecules, RM80 (**4**) is presumed to be the major product and spontaneously dehydrates in the presence of water to form RM80b (**5**). Sodium [1,2-$^{13}C_2$]acetate feeding experiments indicate that the molecules are derived from decaketide (10 acetate units) backbones. ^1H- and ^{13}C-NMR spectra for RM80 and RM80b are similar to previously characterized polyketides (Table 3). Specifically, the characteristic chemical shifts associated with carboxy-terminal 2-hydroxy-4-pyrone rings are also present in these molecules. Likewise, the chemical shifts of the pyrone rings formed at the methyl terminus of RM80 (**4**) and RM80b (**5**), respectively, are similar to those of SEK34 and SEK34b (the dehydrated analog of SEK34).[7] NOE studies of both molecules were also consistent with the proposed structures (Figure 2). High-resolution FAB mass spectroscopy of RM80 (**4**) gave molecular weights of 407.0743 (M + Na^+, 407.0762 expected) and 385 (M + H^+) consistent with the molecular formula $C_{20}H_{16}O_8$. Likewise, RM80b (**5**) gave molecular weights of 389.0639 (M + Na^+, 389.0621 expected) and 367 (M + H^+) consistent with the molecular formula $C_{20}H_{14}O_7$.

Roles of TcmJ and TcmN in Aromatic Polyketide Biosynthesis. The product profiles in Table 1 reveal that each strain falls into one of two catagories: those in which TcmJ and TcmN play no biosynthetic role and those which produce a novel polyketide(s), either RM77 (**3**) or RM80 (**4**)/RM80b (**5**). In the former case, the polyketides isolated have been previously characterized from strains containing the identical PKS genes, but without *tcmJ* or *tcmN*. In the latter case, the altered biosynthetic steps leading to formation of a new molecule can be attributed solely to the TcmN protein. That is, no qualitative or significant quantitative differences in product profiles are observed between strains containing only TcmN and those with both TcmJ and TcmN (compare pRM77 with pRM78 and pRM80 with pRM81). Therefore, while no obvious role for TcmJ is apparent from these results, the function of TcmN can be deduced from the structures of RM77 (**3**) and RM80 (**4**)/

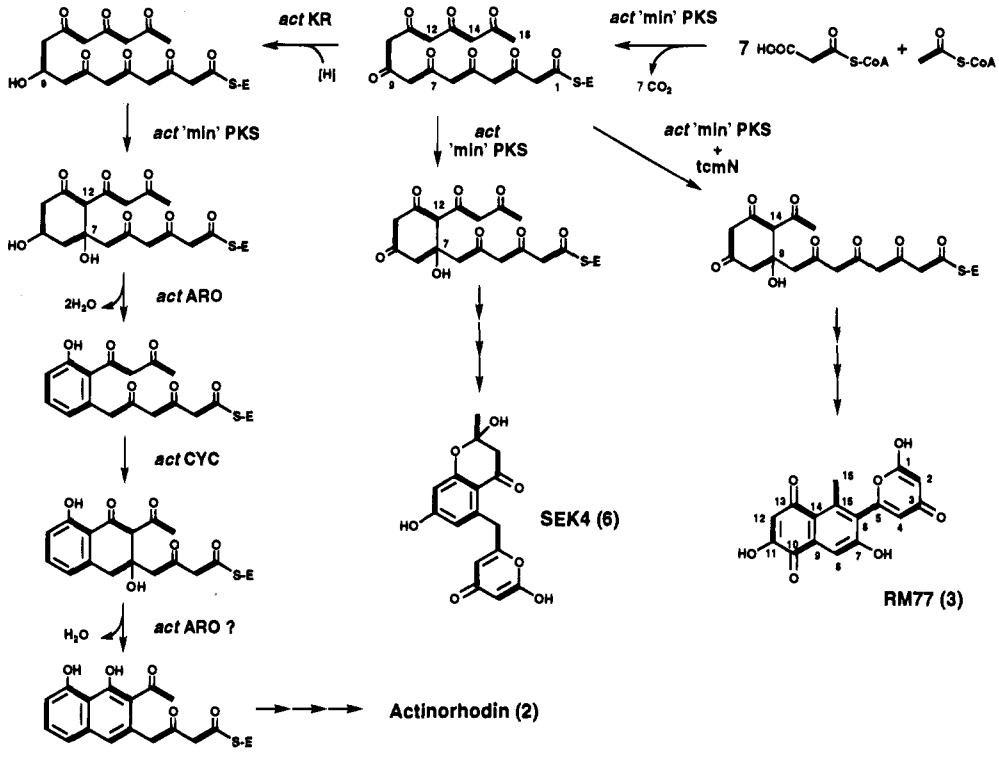

Figure 3. Structures and proposed pathways of octaketide derived polyketides relevant to this study. The pathways shown here and in Figure 4 are hypothetical since no intermediates have been isolated. Therefore, conclusions drawn about the cyclization properties of these molecules are based on structural analysis of final or shunt products. The role of the *act* ARO in the aromatization of the second ring of actinorhodin (**2**) is speculative. See the text for further discussion.

RM80b (**5**). Furthermore, since no effect is observed on biosynthetic pathways in strains containing the *act* KR, ketoreduction is likely to occur prior to any catalytic activity imparted by TcmN (discussed further below).

It should be noted that the expected metabolite Tcm F2 (**11**), isolated from a strain containing the *tcm* minimal PKS and tcmN in a PKS null *S. glaucescens* background,[18,21] was not detected in CH999/pRM80. Whether this is due to differences in host strains or workup conditions is unknown.

TcmN Influences Regiospecificity of First Ring Cyclization. A comparative analysis of the cyclization patterns of these molecules, together with those reported earlier, reveals two functions for TcmN. The first can be illustrated by differences in the proposed pathways for RM77 (**3**; produced by the the *act* minimal PKS + TcmN; pRM77) and SEK4 (**6**; produced by the *act* minimal PKS alone; pSEK24). As shown in Figure 3, it is observed that TcmN influences the regiospecificity of the cyclization of the first ring . In SEK4 (**6**), an intramolecular aldol condensation occurs between the C-7 carbonyl and the C-12 methylene. However, in RM77 (**3**), a similar reaction occurs betweeen the C-9 carbonyl and the C-14 methylene, a shift of one acetate unit in the polyketide backbone. Thus, while earlier results have led us to argue that the course of this reaction is primarily controlled by the minimal PKS,[6,9] RM77 (**3**) clearly illustrates the effect of TcmN on the *act* minimal PKS, which catalyzes molecules with C-7/C-12 cyclizations, exclusively, in the absence of TcmN. The absence of any significant amount of SEK15 (**8**) or other C-7/C-12 cyclized molecules in CH999/pRM80 and CH999/pRM81 also reaffirms our previous hypothesis that regiospecificity of the first aldol condensation can be controlled by enzymes downstream of the minimal PKS.[6,9]

An intriguing feature of RM77 (**3**) is the C-10 oxidation of the first ring. This is the first example of an engineered polyketide in which the first ring contains a quinone moiety. It is unclear, however, whether oxidation occurs as a result of workup conditions or whether a non-PKS oxygenase exists within *S. coelicolor* to catalyze this reaction.

An important consequence that follows the above assignment of the TcmN function is the temporal relationship between ketoreduction and cyclization of the first ring. So far, in all naturally occurring and recombinant polyketides undergoing a C-9 ketoreduction, initial cyclization occurs between carbons 7 and 12.[3,5,6] Therefore, the inability of strains expressing *tcmN* to produce significant quantities of a polyketide with a C-9/C-14 cyclization in the presence of the *act* KR (pRM71, pRM72, pRM74, pRM75; Table 1) demonstrates that ketoreduction is likely to occur prior to formation of the first ring (Figure 3).

TcmN Is a Second Ring Aromatase. The second function of TcmN is apparent from comparison between the proposed cyclization pathways of RM80 (**4**; produced by the *tcm* minimal PKS + TcmN; pRM80) and SEK15b (**7**; produced by the *tcm* minimal PKS alone; pSEK33). Production of these two molecules is mutually exclusive in these strains. As seen in Figure 4, the regiospecificities of the first and second intramolecular aldol condensations in both molecules are identical. However, in SEK15b (**7**) the third ring forms via an aldol condensation between C-6 and C-19, whereas in RM80 (**4**) it forms via hemiketalization between C-15 and C-19. The difference in these two cyclization pathways can be attributed to enolization of the C-15 carbonyl in RM80 (**4**), but not in SEK15b (**7**). This is reminiscent of the related polyketides SEK34 and mutactin, shunt products from the early stages of actinorhodin biosynthesis[7] which led to the hypothesis that the *act* aromatase (ARO) catalyzes the enolization of the C-11 carbonyl. Therefore, it is not surprising that TcmN, a homolog of the *act* ARO, should catalyze the same reaction. However, the specificities of the two proteins differ. Whereas the *act* ARO acts on the first ring, TcmN appears to act on the second ring.

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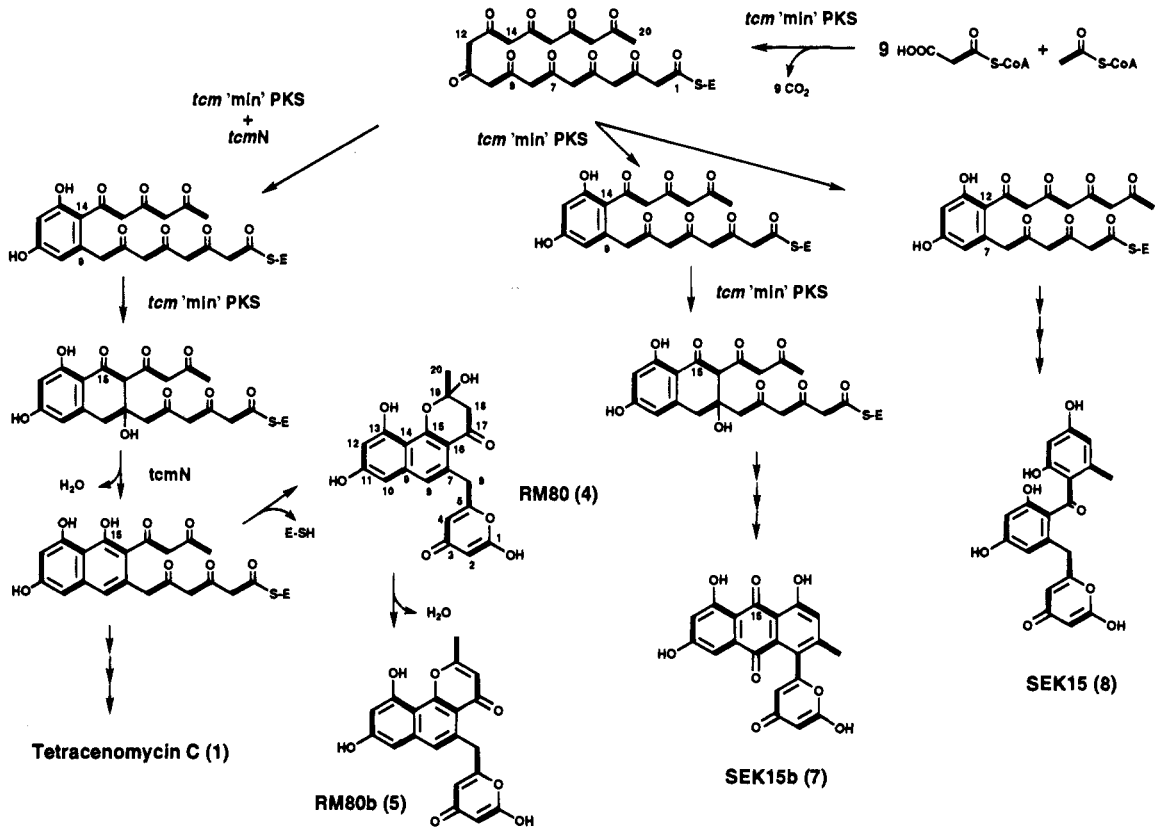

Figure 4. Structures and proposed pathways of decaketide derived polyketides relevant to this study. In the absence of TcmN, the *tcm* minimal PKS catalyzes the cyclization of the first ring with two different regiospecificites, leading to the formation of SEK15 (**8**) and SEK15b (**7**). See the text for further discussion.

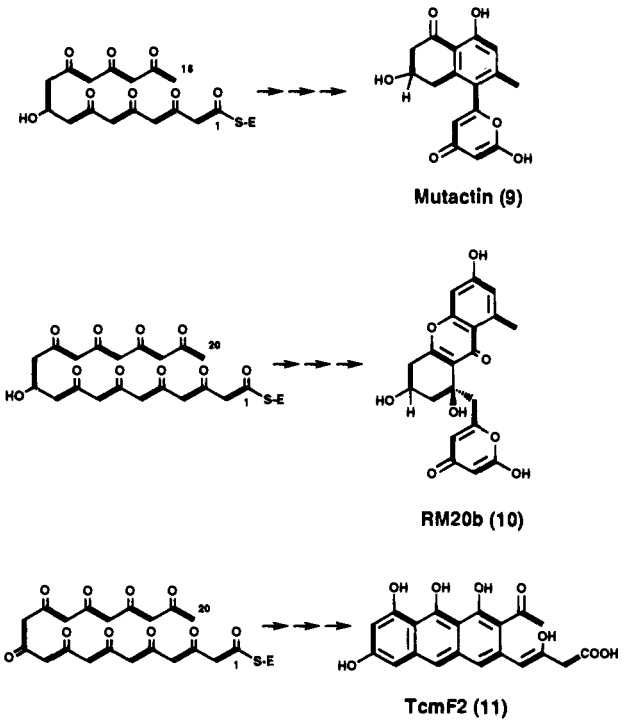

Figure 5. Structures and deduced backbones of mutactin (**9**), RM20b (**10**), and TcmF2 (**11**).

Discussion

The *tcmN* gene product is a bifunctional protein in which the C-terminal portion has been implicated in post-polyketide *O*-methylation of a tetracenomycin C precursor.[17] The N-terminal domain, however, is homologous with the N-terminal domains of a family of aromatases present in several PKS gene clusters, including those involved in the biosynthesis of actinorhodin,[13] granaticin (*gra*),[22] frenolicin (*fren*),[23] and griseusin (*gris*).[24] Although sequence analysis has suggested little about the function of this class of enzymes, recently it was proposed that the *act* protein functions as an aromatase.[7] However, the existence of *tcmN* in the tetracenomycin cluster, which lacks a ketoreductase, was inconsistent with our observation that the first ring of unreduced intermediates can aromatize in the absence of an aromatase.[6]

Results presented here provide further insight into the mechanisms by which PKSs process reduced versus unreduced polyketide chains. It has been noted that aromatases present in KR containing PKS gene clusters (*act, gra, fren,* and *gris*) share an internal "duplication" motif in which the N- and C-terminal halves of the protein are homologous.[23] Aromatases from non-KR containing clusters such as *tcm*[17] and the *S. coelicolor* spore pigment (*whiE*),[25] on the other hand, have only a single region of homology. In light of the newly proposed TcmN function, it may be possible that the N-terminal domains of aromatases from "reduced" systems also encode *second* ring aromatases that function after the second ring cyclase, and the C-terminal domains encode the *first* ring aromatase, a reaction that occurs spontaneously in completely "unreduced" polyketides (see the actinorhodin pathway in Figure 3). This is consistent with the fact that the N-terminal domains of these proteins show

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greater similarity to TcmN than to their respective C-terminal domains.[23]

At this point, it is not known which domain of TcmN is responsible for control of the first ring regiospecificity. However, experiments in which TcmF2 was isolated from strains containing only the N-terminal domain of TcmN,[17,18] in addition to recent studies with the *whiE* aromatase (which lacks the C-terminal domain of TcmN) (T. W. Yu, D. A. Hopwood, R. McDaniel, and C. Khosla, manuscript in preparation) support the hypothesis that the N-terminal domain plays this role. Furthermore, it appears that the two-carbon shift in regiospecificity is determined by counting from the carboxyl end of the polyketide chain, a property that is also shared by the *act* KR.[3,5]

TcmN provides an additional tool for the design and biosynthesis of novel polyketides through the genetic manipulation of PKSs. RM77 (**3**) represents the first example of a 16-carbon polyketide with an engineered first cyclization different from that of the expected "natural" one. Therefore, it is likely that other heterologous PKS complexes containing TcmN (or homologs) along with various minimal PKSs will produce polyketides of different chain length with the alternative first cyclization. However, it appears that this biosynthetic degree of freedom may be limited to only unreduced molecules.

Finally, our failure to determine the function of TcmJ suggests that it probably plays a nonessential role in the early steps of tetracenomycin biosynthesis. On the basis of the observation that the presence of TcmJ dramatically increases yields of TcmF2 (**11**) in *S. glaucescens*, Summers *et al.*[18] have suggested that the protein catalyzes formation of the second ring, a reaction requiring an unrelated enzyme (the *act* cyclase (CYC)) in actinorhodin biosynthesis.[7] However, our results reported here and elsewhere[6] indicate that the *tcm* minimal PKS is capable of catalyzing formation of the second ring prior to aromatization. Therefore, further studies are necessary to determine the role of TcmJ.

Experimental Section

Bacterial Strains and Culture Conditions. *S. coelicolor* CH999[3] was used as a host for transformation by all plasmids. DNA manipulations were performed in *Escherichia coli* XL1 Blue. Plasmids were passed through *E. coli* ET12567 (*dam dcm hsdS* Cm^r)[26] to generate unmethylated DNA before their use to transform *S. coelicolor*. *E. coli* strains were grown under standard conditions.[27] *S. coelicolor* strains were grown on R2YE agar plates[28] rather than in liquid media because of the apparently more abundant production of metabolites on agar media.

Manipulation of DNA and Organisms. The polymerase chain reaction (PCR) was performed using Pfu polymerase (Stratagene) under conditions recommended by the manufacturer. Standard *in vitro* techniques were used for DNA manipulations.[27] *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,[28] and transformants were selected using 2 mL of a 500 μg/mL thiostrepton (Sigma) overlay.

Construction of Plasmids Used for Transformation. The construction and analysis of pSEK21, pSEK23, pSEK24, and pSEK33 have been described elsewhere.[6] Each of these plasmids contains genes encoding the KS/AT and CLF from *act* or *tcm* and the *act* ACP. Plasmids pSEK21 and pSEK23 also contain the gene encoding the *act* KR. The *tcmJ* and *tcmN* genes were amplified via PCR with flanking restriction sites. *tcmN* was amplified with a 3′ *Pst*I site and nested 5′ *Sph*I and *Eco*RI sites. *tcmJ* was amplified with nested 3′ *Pst*I and *Sph*I sites and a 5′ *Eco*RI site. The genes were subcloned independently and together, joined at the *Sph*I site, as *Pst*I/*Eco*RI cassettes into pIJ5639.[29] The final plasmids were constructed by moving *Xba*I/*Eco*RI fragments from each of the three subclones into the corresponding sites of pSEK21, pSEK23, pSEK24, and pSEK33. All constructs carry the *act* ACP which has been shown not to affect product specificity.[3,5,6]

Production and Purification of Polyketides. Analysis of the transformed strains was performed as described for similar experiments.[5,7] The isolation and characterization of mutactin (**9**),[6] RM20b (**10**),[8] SEK4 (**6**),[9] SEK15 (**7**),[9] and SEK15b (**8**)[6] have been described elsewhere. The workup of RM77 (**3**), RM80 (**4**), and RM80b (**5**) was identical to methods described earlier.[5,7] Each compound was identified as a major polyketide product from preparative HPLC (Beckman C-18 reversed-phase column) fractions using a 15−60% acetonitrile/water/1% acetic acid gradient over 60 min. Compounds were analyzed via NMR spectroscopy. For structural characterization, RM77 (**3**) was further purified (>95%) via HPLC under isocratic (15:85 acetonitrile/water) conditions. Yields were approximately 20 mg/L for RM77 (**3**) and 100 mg/L of RM80 (**4**) and RM80b (**5**) combined (ratios of these two molecules varied, as **4** is converted into **5** during workup).

Sodium [1,2-$^{13}C_2$]Acetate Feeding Experiments. Sixteen confluent lawns each of *S. coelicolor* CH999/pRM78 and CH999/pRM81 were grown on agar plates as described above. Each plate contained approximately 35 mL of R5 media[28] with 50 mg/mL thiostrepton (Sigma) and 0.5 g/L sodium [1,2-$^{13}C_2$]acetate (99% ^{13}C, Cambridge Isotope Laboratories). Isolation of RM77 (**3**), RM80 (**4**), and RM80b (**5**) was carried out as described above with similar yields. ^{13}C-NMR data indicated approximately 0.5−1% enrichment (estimated by comparing peak areas to the natural abundance ^{13}C peak area).

Mass and NMR Spectroscopy. High-resolution fast atom bombardment (FAB) mass spectroscopy (VG ZAB-ZSE spectrometer) was used for analysis of compounds. NMR spectra were recorded on a Varian XL-400. ^{13}C spectra were acquired with continuous broadband proton decoupling. HMBC analysis was performed on a JEOL A500 spectrometer using a pulsed field gradient. For NOE studies, the one-dimensional difference method was employed. All compounds were dissolved in DMSO-d_6 (99+% D, Cambridge Isotope Laboratories) and spectra were referenced internally to the solvent.

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