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Structure-Based Dissociation of a Type I Polyketide Synthase Module

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SUMMARY

Individual modules of modular polyketide synthases (PKSs) such as 6-deoxyerythronolide B synthase (DEBS) consist of conserved, covalently linked domains separated by unconserved intervening linker sequences. To better understand the protein-protein and enzyme-substrate interactions in modular catalysis, we have exploited recent structural insights to prepare stand-alone domains of selected DEBS modules. When combined in vitro, ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) domains of DEBS module 3 catalyzed methylmalonyl transfer and diketide substrate elongation. When added to a minimal PKS, ketoreductase domains from DEBS modules 1, 2, and 6 showed specificity for the β-ketoacylthioester substrate, but not for either the ACP domain carrying the polyketide substrate or the KS domain that synthesized the substrate. With insights into catalytic efficiency and specificity of PKS modules, our results provide guidelines for constructing optimal hybrid PKS systems.

INTRODUCTION

Modular polyketide synthases (PKSs) are multifunctional enzymes that catalyze the production of polyketide compounds, many of which exhibit antibiotic, antifungal, anticancer, and other important biological activities [1–5]. Each PKS module contains minimally three functional domains—β-ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP)—and a subset of tailoring domains drawn from enzymes such as ketoreductases, dehydratases, enoylreductases, and methyltransferases. Together, the KS, AT, and ACP of a module catalyze decarboxylative condensation between the incoming polyketide chain and a substituted malonyl thioester, while the tailoring domains dictate the final oxidation state of the β carbon (Figure 1). The modular architecture of polyketide synthases has motivated numerous efforts toward combinatorial biosynthesis of novel polyketides [6–11]. Genetic manipulations including insertion, deletion, and substitution of domains or modules have allowed the generation of a variety of polyketide analogs, with varying degrees of success. Nonetheless, limited understanding of domain-domain compatibility, as well as lack of precise information about domain boundaries, has thus far hampered our ability to fully harness the power of biosynthetic engineering.

6-deoxyerythronolide B synthase (DEBS) from *Saccharopolyspora erythraea* is the most extensively studied modular PKS. Organized into three homodimeric polypeptides, DEBS consists of six catalytic modules, each containing a unique set of covalently linked catalytic domains. Together, the six modules of this megasynthase produce 6-deoxyerythronolide B (6-dEB), the macrolide aglycone core of the antibiotic erythromycin (Figure 1) [12, 13]. Because all modules of DEBS are comprised of combinations of covalently fused domains in which individual domains occur in equal molar ratios, we have sought to reconstitute PKS activity by recombining individually expressed domains (Figure 2A) [14, 15]. Prototypical versions of each PKS fragment, whose boundaries were originally defined by limited proteolysis, have now been characterized by high-resolution structural studies [16, 17]. The dissection and subsequent reconstitution of a functional module from its constituent domains not only represent an excellent strategy for defining authentic domain boundaries, it also provides a route to interrogate domain-domain specificity within PKS modules. Here we report the reconstitution of fragmented, fully active PKS modules from isolated recombinant domains (Figures 2B and 2C). A series of assays was developed to monitor domain acylation, polyketide chain elongation, and β-ketoacylthioester reduction. The knowledge gained from this study is expected to strengthen the basis for the rational construction of future generations of hybrid PKSs.

RESULTS

The previously characterized DEBS module 3 was chosen as a target for the development and analysis of a fully dissected PKS module [14, 15, 18]. Because the

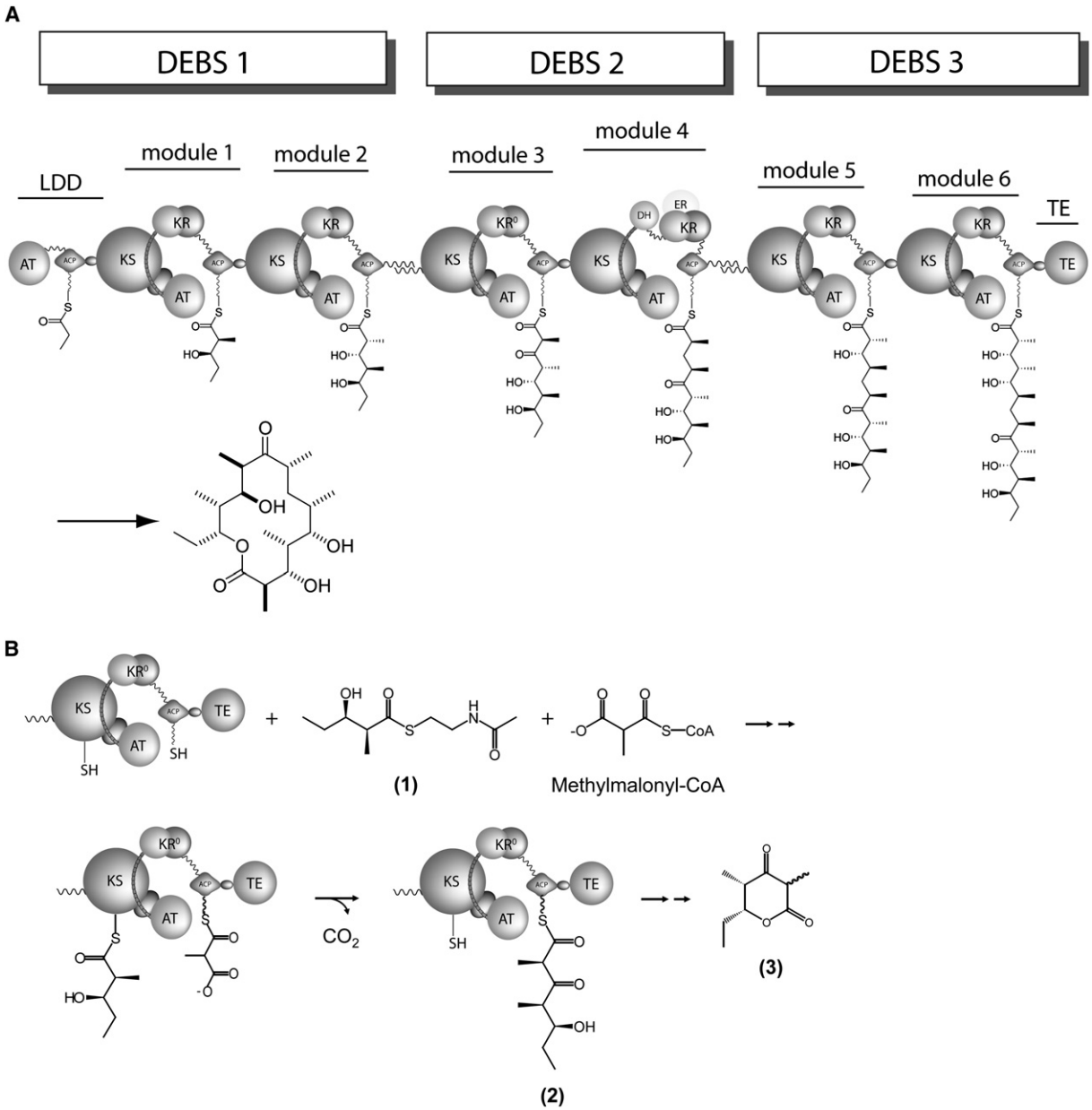

Figure 1. DEBS Organization and Chemistry

(A) Modular organization of 6-deoxyerythronolide B synthase (DEBS). Chain elongation occurs minimally through the combined action of the β-ketosynthase (KS), acyl transferase (AT), and acyl carrier protein (ACP) domains. Optional tailoring domains including ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains control the ultimate oxidation state of the β carbon. Once processed, the polyketide chain is either passed to the KS domain of the downstream module or cyclized and released by the thioesterase (TE) domain at the C terminus of the polyketide synthase. LDD, loading didomain.

(B) Chain elongation cycle catalyzed by DEBS module 3 + TE. AT is acylated with a methylmalonyl extender unit from its CoA derivative, which is then transferred to the phosphopantetheine arm of the ACP domain. KS is primed with a 2-methyl-3-hydroxy pentanoyl unit from diketide **1**. Once both the growing chain and the extender units are covalently loaded onto the module, KS-mediated decarboxylative condensation occurs to form triketide β-ketoacyl-ACP intermediate **2** with the release of carbon dioxide. TE catalyzes the release of the final triketide ketolactone product **3**.

ketoreductase domain from this module is inactive, the ketoreductase domain from DEBS modules 1, 2, and 6 were targeted in this study. As a point of comparison, we used DEBS module 6, whose domain organization is similar that of DEBS module 3.

Expression and Purification of Recombinant KS and AT Domains from DEBS Module 3

Previous attempts to express isolated KS and AT domains derived from modular PKSs have resulted in formation of insoluble proteins. For AT domains, this problem was

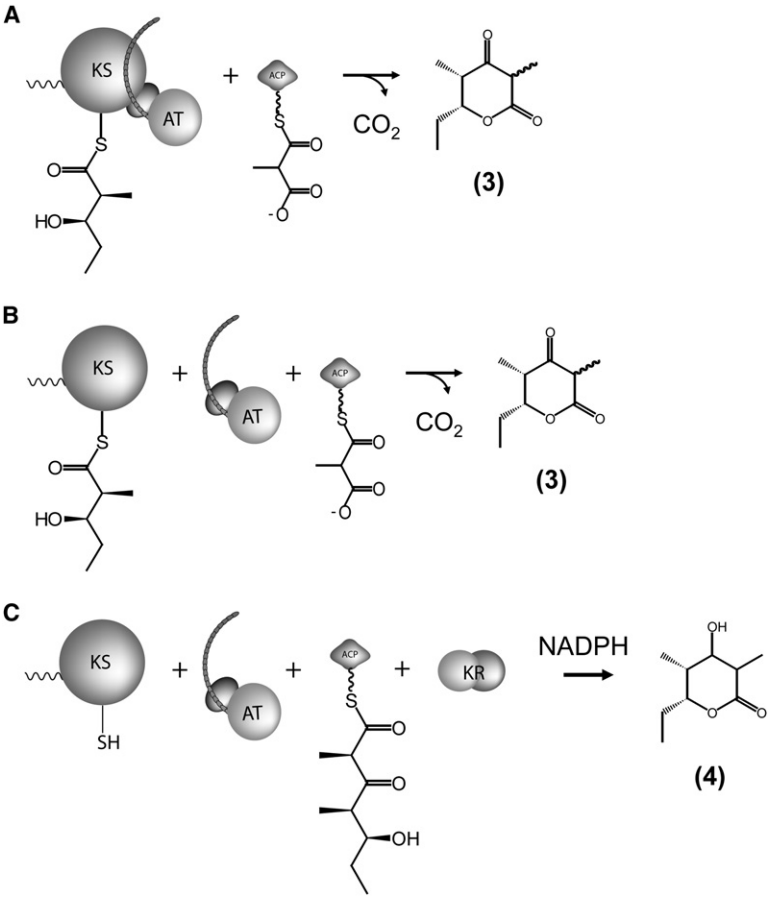

Figure 2. Fragmented Module Systems
(A and B) Chain elongation performed by (A) [KS][AT] didomain and stand-alone ACP, and (B) stand-alone KS, AT, and ACP.
(C) Reduction performed by stand-alone KR to produce product **4**.

originally circumvented by expressing a chimeric AT-ACP didomain construct [19]. Although this hybrid construct facilitated analysis of the properties of individual AT domains, its utility was limited due to the presence of the fused ACP domain.

Based on a careful analysis of two recently solved structures of the [KS3][AT3] and [KS5][AT5] didomains, we have now constructed recombinant KS3 and two versions of recombinant AT3, all three of which have been expressed as soluble proteins. An EEAPERE sequence, which immediately follows the KS3 domain [17], was identified as the junction site between KS3 and the newly recognized KS-to-AT linker. The two distinct AT3 constructs were prepared to examine the role of the C-terminal post-AT linker (30 residues from FALP to LAYR), which wraps around both the AT3 domain and the KS-to-AT linker and interacts with KS3 [17]. The smaller AT protein, designated AT3(0), lacked the C-terminal post-AT linker peptide, while the larger version, designated AT3(3), included the complete post-AT linker region. Both proteins retained the complete KS-to-AT linker at their N termini, which was missing or incomplete in the previously attempted insoluble and/or inactive AT constructs (data not shown). KS3 and AT3(0) were both expressed as soluble proteins at a yield of 10 mg purified protein per liter culture, while AT3(3) was obtained in a yield of 3 mg purified protein per liter culture.

Acylation and Transacylation of Recombinant AT3 Domains

The AT domain of a DEBS module first captures the methylmalonyl-CoA extender unit to generate a covalent methylmalonyl-*O*-AT intermediate, and then transfers the methylmalonyl moiety to the phosphopantetheinyl side chain of the ACP of the same module. To confirm the individual acylation and transacylation activities of recombinant AT3, both with and without the post-AT linker region, AT3(0) and AT3(3) were each incubated with [^{14}C]methylmalonyl-CoA, either alone or in the presence of ACP3. Radio-SDS-PAGE analysis showed that both versions of AT3 were indeed acylated by methylmalonyl-CoA (Figure 3A, lanes 1 and 3) and each could also transfer the methylmalonyl unit to the discrete ACP3 domain (Figure 3A, lanes 2 and 4). Thus, the post-AT linker is not required for either the methylmalonylation or transacylation activity of the AT domain. Furthermore, when either AT3 protein was incubated with [^{14}C]malonyl-CoA, no protein labeling was observed (data not shown), indicating that the isolated, recombinant AT domain retained its intrinsic substrate specificity.

Acylation of the Recombinant KS3 Domain

In polyketide chain elongation by modular polyketide synthases, the KS domain normally receives the growing polyketide chain as an acyl thioester attached to the ACP domain of the upstream module. This ACP can be replaced

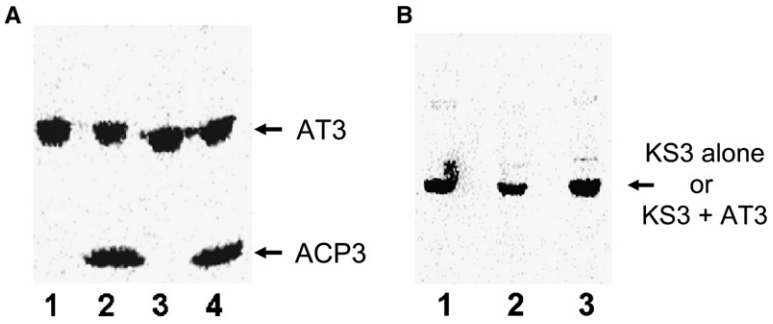

Figure 3. Radio-SDS-PAGE for Protein Acylation

(A) Acylation and transacylation activities of isolated DEBS AT3. Radiolabeled methylmalonyl-CoA with AT3(3) (lane 1), AT3(3) + ACP3 (lane 2), AT3(0) (lane 3), and AT3(0) + ACP3 (lane 4).

(B) Acylation activities of isolated DEBS KS3. Radiolabeled diketide **1** with KS3 (lane 1), KS3 + AT3(3) (lane 2), and KS3 + AT3(0) (lane 3).

by a surrogate *N*-acetylcysteamine thioester analog. To evaluate the self-acylation activity of the discrete KS3 domain, the recombinant KS3 protein was incubated with [^{14}C]**1**, with or without added AT3(3) or AT3(0). Radio-SDS-PAGE showed that the isolated KS3 was indeed acylated by diketide **1** (Figure 3B, lane 1) with comparable occupancy of the active site relative to that of [KS3][AT3]. However, reduced KS acylation was observed at lower ketide substrate concentration (50 μM; data not shown). The presence of recombinant AT3, with or without the post-AT linker, had no observable influence on KS3 acylation (Figure 3B, lanes 2 and 3).

Condensation Activity of the Fragmented KS3 + AT3 + ACP3 System

Once the acylation activities of both KS3 and AT3 were confirmed, we examined the condensation activity of the dissociated system by incubating recombinant KS3 with unlabeled **1**, [^{14}C]methylmalonyl-CoA, *holo*-ACP3, and either AT3(3) or AT3(0). A radio-TLC assay was used to detect the formation of triketide condensation products. Surprisingly, whereas AT3(3) and AT3(0) had performed similarly in self-methylmalonylation, the expected triketide ketolactone **3** was formed only in the presence of AT3(3) in combination with KS3 and ACP3 (Figure 4). This result indicates that the post-AT linker, while not necessary for methylmalonylation of the discrete AT domain, is required for KS-catalyzed β-ketoacyl-ACP synthase activity. Notably, the results summarized in Figure 4 also demonstrate for the first time, to our knowledge, the feasibility of dissecting a type I PKS module into its constituent domains and reconstituting the functions of the complete module from a combination of the individual domains, analogous to the operation of a typical type II PKS as a fully fragmented PKS.

Recombinant Ketoreductases

In addition to the KS, AT, and ACP domains, functional ketoreductases (KRs) are present in five of the six DEBS modules and have been expressed as isolated domains [20]. To construct a fully fragmented system with β-ketoreduction activity, the KR domains from DEBS modules 1, 2, and 6 were each expressed as isolated, soluble proteins, at a yield of 20 mg purified protein per liter culture.

To evaluate the catalytic activity of the individual recombinant KR domains, each KR protein was first tested in combination with both the [KS3][AT3] didomain + ACP3 and the [KS6][AT6] didomain + ACP6 systems. Figure 5 shows the activities of KR1, KR2, and KR6 toward triketide-S-ACP **2** in the module 3 (Figure 5A) and module 6 (Figure 5B) systems. The formation of the reduced β-hydroxy triketide lactone product **4** established that both KR2 and KR6 had comparable activity in both reconstituted PKS systems regardless of the KS-KR combination. That is, KR2 reduced triketide **2** to give triketide lactone **4** exclusively regardless of whether the ACP-bound (2-D-methyl)-triketide **2** was generated by KS3 or KS6. Similarly, KR6 produced a mixture of unreduced triketide lactone **3** and reduced triketide lactone **4** in both systems. The stereochemistry of triketide lactone **4** is currently

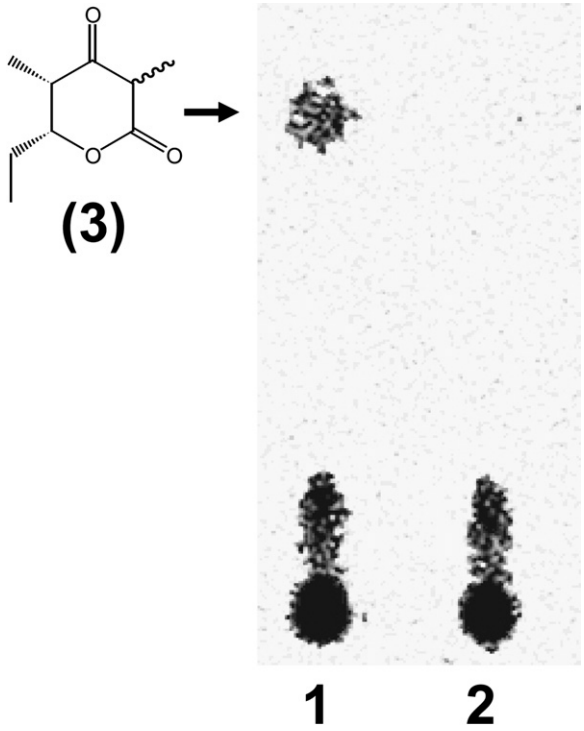

Figure 4. Effect of the Post-AT Linker in Condensation

Radio-TLC/phosphorimaging assay for the formation of triketide ketolactone **3** by DEBS KS3 + AT3(3) + ACP3 (lane 1) and KS3 + AT3(0) + ACP3 (lane 2). This result indicates that the post-AT linker is required for KS-catalyzed β-ketoacyl-ACP synthase activity.

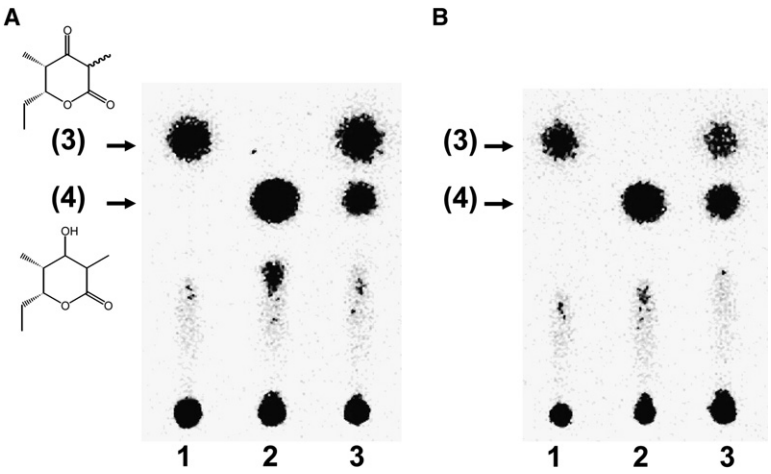

Figure 5. Radio-TLC Assay to Examine the Specificity of KR toward the Partnering KS

DEBS KR1 (lane 1), KR2 (lane 2), and KR6 (lane 3) with [KS3][AT3] + ACP3 (A) or [KS6][AT6] + ACP6 (B). In both module 3 and module 6 systems, KR1 produced no reduced product, KR2 produced the reduced product exclusively, while KR6 produced a mixture of the reduced and unreduced products. Radioactive intensities of the products are tabulated in Table S1.

under investigation. It should be noted that ACP-bound (2-D-methyl)-triketide **2**, the product of condensation of diketide **1** and methylmalonyl-CoA, is the natural substrate for KR2. By contrast, KR1, which normally processes a diketide, did not reduce the acyclic triketide **2** at all. As a control, the activity of recombinant KR1 was independently established using propionyl-SNAc as the primer for KS1 (data not shown).

Because a KR domain normally reduces the condensation product that is tethered to the ACP of the same module, a KR domain might be expected to have an intrinsic specificity toward its cognate ACP domain compared to heterologous ACP domains. To test this hypothesis, KR2 was incubated with [KS3][AT3] and a panel of six recombinant DEBS ACP proteins from modules 1–6 (Figure 6A). By varying the ACP protein partner while holding the [KS][AT] protein and substrates constant, we were able to probe the effect of KR-ACP interaction on the efficiency of reduction, as measured by the effect on the ratio of reduced to unreduced triketide lactones **4** and **3**. The results suggest that KR2 does not discriminate among the six DEBS ACPs, with reduced triketide lactone **4** being the exclusive product in all cases. By contrast, as we have previously reported, the KS3 and KS6 domains each have a marked preference for a distinct subset of ACP partners [15]. For example, KS3 carries out condensation most efficiently in combination with ACP3 or ACP5, relatively well with ACP2 or ACP4, and poorly with ACP1 or ACP6. The inclusion of the recombinant KR domains did not alter the relative k_{cat} profile for generation of triketide, suggesting that KR does not perturb the rate of condensation and that any sensitivity to the identity of the ACP domain has no observable effect on the overall rate of formation of reduced triketide (see Table S2 in the Supplemental Data available with this article online). Analogous results were obtained when KR6 was incubated with [KS6][AT6] and the same panel of six DEBS ACP proteins, with a comparable proportion of reduced and unreduced triketide lactones being formed independently of the origin of the ACP domain (Figure 6B).

Together, the above results suggest that ketoreductase domains have specificity for the structure of their polyketide substrates but are not sensitive to either the ACP domain to which these substrates are anchored or to the KS domain that generates the β-ketoacyl-ACP intermediate. We have further established (e.g., Figure 7) that DEBS modules can be completely dissected into their constituent catalytic protein domains that retain their intrinsic

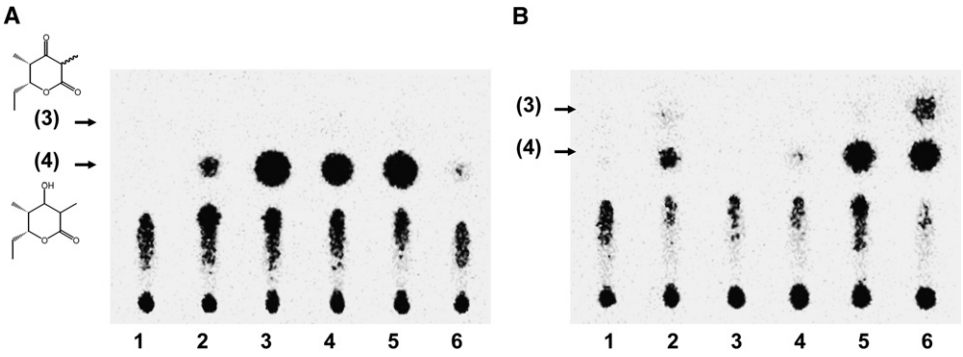

Figure 6. Radio-TLC Assay to Examine the Specificity of KR toward the Partnering ACP

DEBS ACP1–6 (lanes 1–6, respectively) with [KS3][AT3] + KR2 (A) or [KS6][AT6] + KR6 (B). Regardless of the ACP partner, KR2 produced the reduced product almost exclusively, while KR6 produced a mixture of the reduced and unreduced products. Radioactive intensities of the products are tabulated and compared to the k_{cat} profiles in Table S2.

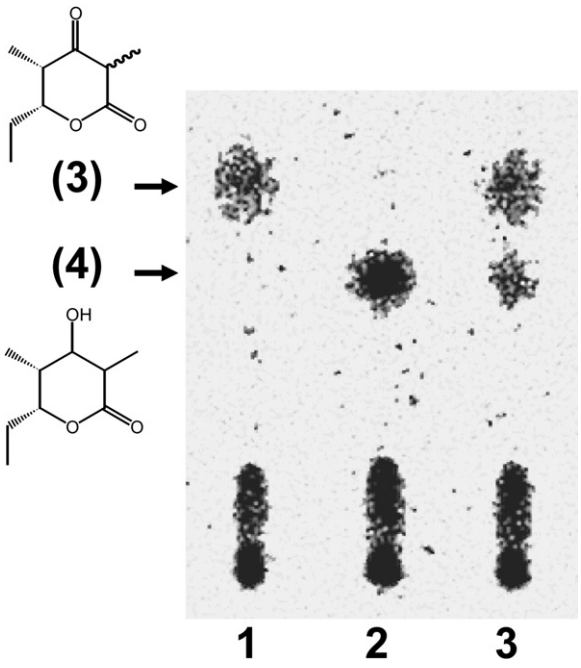

Figure 7. Condensation Performed by the Fully Fragmented Systems

Radio-TLC assay for the formation of triketide lactones **3** and **4** by the fully fragmented systems. DEBS KS3 + AT3(3) with no KR (lane 1), KR2 (lane 2), or KR6 (lane 3). Radioactive intensities of the products are tabulated in Table S3.

function in combination with either cognate or heterologous domain partners.

DISCUSSION

In a type I PKS module, substrates and intermediates are channeled through a defined sequence of domain acylation, chain elongation, polyketide reduction, and polyketide transfer reactions. Although this intramodular organization ensures that complex polyketide biosynthesis takes place with high fidelity, it presents considerable barriers to detailed mechanistic studies of individual reaction steps catalyzed by each PKS domain. By separating the constituent domains of a single PKS module, we can now investigate the contribution of each domain to substrate specificity, catalytic activity, stereocontrol, and interdomain interactions, as well as their combined contribution to overall polyketide biosynthesis. A better understanding of the actual domain boundaries is essential to the rational and efficient engineering of hybrid PKS systems. Previous studies have firmly identified the YRVXW cleavage site between the acyl transferase and ketoreductase domains as well as the RLAGL site between ketoreductase and acyl carrier protein domains [14, 15]. We have now utilized a third domain boundary site to construct discrete, soluble recombinant KS and AT domains. We have also discovered unexpected properties of the interdomain linkers, explored the substrate and protein specificity of KR domains, and reconstituted the activity of a PKS module from isolated recombinant domains.

Acyl Transferase Domain

The AT domain of a given module determines the extender unit, normally methylmalonyl or malonyl, that is incorporated into the final polyketide chain. To introduce noncognate extender units, previous studies have utilized AT domain swaps, but many of the resulting hybrid modules suffer from a significant kinetic penalty, presumably due to the introduction of suboptimal inter- or intradomain junctions or inefficient interdomain interactions [21–26]. To unambiguously establish the proper AT domain boundaries, two versions of the recombinant AT from DEBS module 3 were constructed, based on a detailed analysis of the X-ray crystal structures of the [KS3][AT3] and [KS5][AT5] didomains. One such construct, AT3(3), contained the C-terminal post-AT linker; the other lacked this linker and was designated AT3(0). Both proteins, which retain the ~100 amino acid KS-to-AT linker at their respective N termini, were soluble and active in both self-catalyzed methylmalonylation and AT → ACP transacylation assays. They also retained their characteristic strict intrinsic substrate specificity toward methylmalonyl-CoA. These results, which represent the first successful expression, to our knowledge, of a stand-alone, soluble AT domain, establish that the globular portion of the AT3 domain is structurally stable, catalytically active, and substrate specific.

Ketosynthase Domain and the Effect of the Post-AT Linker

To catalyze polyketide chain elongation, a KS domain first forms a covalent acyl-enzyme intermediate between the growing polyketide chain and the thiol of its active site Cys before catalyzing decarboxylative condensation with the methylmalonyl-ACP or malonyl-ACP cosubstrate. To develop a fully fragmented PKS system, the recombinant KS domain of DEBS module 3 was dissected from the downstream AT domain at the EEAPERE junction site, based on an analysis of the [KS3][AT3] and [KS5][AT5] didomain structures [16, 17]. Both the high level of protein expression and the results of incubation experiments with diketide **1** confirmed the structural stability and intact self-acylation activity of the resulting recombinant KS3 domain. Successful chain elongation in the presence of AT3(3) and ACP3 further confirmed that the recombinant KS3 domain retained condensation activity and demonstrated the feasibility of reconstituting polyketide synthase activity from disconnected PKS domains. The absence of detectable chain elongation activity using AT3(0) as a reaction partner, however, revealed the importance of the post-AT linker in allowing KS-ACP-catalyzed condensation. Models of the docking between the [KS3][AT3] didomain and a discrete ACP protein provide the mechanistic rationale for these observations: when the ACP interacts with the KS domain, it docks in a deep groove formed by the interaction of the KS, AT, and the post-AT linker, thereby implicating both the AT and the post-AT linker in functional KS-ACP

recognition [17]. With a fully fragmented module, preassociation of the KS and AT domains is therefore a likely prerequisite to effective binding of the nucleophilic methylmalonyl-ACP cosubstrate. In fact, we observed a greater than 1000-fold reduction in the observed rate of triketide production by the reconstituted [KS3] + [AT3] system relative to the intact [KS][AT] didomain (data not shown). The structures of the [KS][AT] didomains, along with the results of the biochemical assays, also suggest that the post-AT linker is an intrinsic component of a functional KS domain. This noncontiguity between the primary PKS sequence and three-dimensional domain boundaries presents additional challenges to efficient PKS domain swaps.

Ketoreductase Domains

Recent studies on recombinant KR domains have provided valuable insights into both protein structure and reaction stereospecificity [8, 20, 27–31]. Until now, however, protein-protein recognition by individual KS-KR and KR-ACP pairs has been essentially unexplored. We have reported here the lack of discrimination of both DEBS KR2 and KR6 between their cognate and noncognate KS and ACP domains in experiments using discrete [KS][AT] didomains, stand-alone recombinant KR domains, and stand-alone ACP domains. Although it is perhaps not surprising that both KR2 and KR6 are insensitive to the structure of their KS partners (as no direct contact between the KS and KR domains should be required for KR-catalyzed reduction), the lack of specificity for the ACP is somewhat unexpected. These results suggest that the phosphopantetheinyl arm is adequate for substrate recognition by the KR, although it has been independently reported that reduction stereospecificity may be seriously degraded when the ACP thioester is replaced by the commonly used *N*-acetylcysteamine analog [20]. The possibility of combining KR domains with both KS and ACP domains from different modules provides the opportunity for engineering catalytically efficient hybrid PKS modules, as long as the intrinsic substrate specificity of heterologous KR domains can be appropriately taken into consideration. Coupled with the previous studies on AT-ACP and KS-ACP specificities, it appears that the KS-ACP pairing is the most specific of all protein-protein interactions in the PKS assembly line. Armed with this information, it should be possible to avoid severe kinetic penalties in engineered hybrid PKS modules by maximizing constituent KS-ACP interdomain compatibility.

Improperly chosen domain boundaries as well as the intrinsic incompatibility of the component domains are major barriers to rational engineering of PKS modules. By dissecting a PKS module into its constituent domains, we have identified a set of domain junction sites that yield stable and functional domains, have determined domain-domain compatibilities, and have demonstrated the feasibility of reconstituting modular activities from combinations of isolated, recombinant domains. This ''divide and conquer'' approach also provides the toolbox to investigate detailed reaction mechanisms, substrate specificity, and stereocontrol that is not possible using far more complex, intact PKS modules. By adding more functional domains to this biomolecular toolbox, it should be possible to effect practical combinatorial biosynthesis of novel polyketides and polyketide precursors suitable for further synthetic modification.

SIGNIFICANCE

Modular polyketide synthases are well known for their complexity, a feature that prevents detailed mechanistic studies of the individual reaction steps catalyzed by each domain. Here we report the dissection and reconstitution of a fully active PKS module that retains all the intrinsic catalytic activities of its constituent domains. This experimental approach not only confirms that authentic domain boundaries can be used as effective handles for protein engineering, it also allows us to investigate the contribution of each domain to substrate specificity, catalytic activity, reaction kinetics, stereocontrol, and interdomain interactions. With insights provided by fragmented PKS modular systems, we can now avoid the introduction of suboptimal inter- or intradomain junctions and unproductive interdomain interactions, and in so doing, improve the rational design of efficient hybrid PKS systems for biosynthesis of novel polyketides.

EXPERIMENTAL PROCEDURES

Reagents and Chemicals

DL-[2-methyl-^{14}C]methylmalonyl-CoA was from American Radiolabeled Chemicals. [^{14}C](2*S*,3*R*)-2-methyl-3-hydroxypentanoyl-*N*-acetylcysteamine thioester (**1**) was prepared by custom synthesis by Amersham Pharmacia. Unlabeled diketide **1** was prepared by established methods [32–34]. All other chemicals were from Sigma. Thin-layer chromatography (TLC) plates were from J.T. Baker. SDS-PAGE gradient gels (4%–15% acrylamide) were from Bio-Rad. Ni-NTA affinity resin was from QIAGEN. HiTrap-Q anion-exchange column was from Amersham Pharmacia.

Cloning and Expression of KS, AT, and KR Domains from DEBS

The DNA sequence encoding DEBS KS3 was amplified by PCR as an NdeI-EcoRI fragment using primers 5′-AAAAAAACATATGGTGACTG ACAGCGAGAAGG-3′ and 5′-TTTTGAATTCACAGGAGCTTCCTCGAC GATCAC-3′. This NdeI-EcoRI fragment was cloned into the pET21 expression vector to yield plasmid pAYC48, encoding DEBS KS3 with the N-terminal linker and a C-terminal His_6 tag. The DNA sequence encoding DEBS AT3 with the post-AT linker (denoted AT3[3]) was constructed similarly into pET21 with primers 5′-CGAGGAACATATGGAG CGCGAGCACCGGGAGACC-3′ and 5′-CGGAGTCGAATTCACGCG GTAGGCCAGCTCGTCGG-3′ to yield plasmid pAYC23. The DNA sequence encoding DEBS AT3 without the post-AT linker (denoted AT3[0]) was amplified and introduced into pET21 with primers 5′-CGA GGAACATATGGAGCGCGAGCACCGGGAGACC-3′ and 5′-TTTTGA ATTCACGGGCGCGGCGCCGGGCAGAGC-3′ to yield plasmid pAYC47. The DNA sequence encoding DEBS KR1 was amplified and introduced into the pET28 expression vector with primers 5′-AAAAAACATA TGGACGAGGTTTCCGCGCTGCGC-3′ and 5′-TTTTGAATTCACCG CGCCCACCCGCGGTTCGGC-3′ to yield plasmid pAYC59. The DNA sequence encoding DEBS KR2 was amplified and introduced into pET28 with primers 5′-AAAAAACATATGGACGAGCTCGACGGCTG GTTC-3′ and 5′-TTTTGAATTCACCCGGTCGCGCAGGCTCTCCGTC-3′

to yield plasmid pAYC60. The DNA sequence encoding DEBS KR6 was amplified and introduced into pET28 with primers 5′-AAAAA CATATGGCCGACAGCCGCTACCGCGTCG-3′ and 5′-TTTTTTGAATT CACCGTCATCTCCCGCGCCGGG-3′ to yield plasmid pAYC62. All six plasmids were transformed into *Escherichia coli* BL21 for protein expression. Proteins were expressed and purified using the previously described protocol [15].

The expression and purification of the [KS][AT] didomains from DEBS module 3 (pAYC02) and module 6 (pAYC10) and the ACP domains from all six DEBS modules have been previously described [15].

Acylation of Recombinant AT3 by [^{14}C]Methylmalonyl-CoA

AT3(3) or AT3(0) (20 μM, in 100 mM phosphate [pH 7.2]) alone or together with *holo*-ACP3 (100 μM) was incubated on ice with 20 μM [^{14}C]methylmalonyl-CoA and 5 mM tris-2-carboxyethyl-phosphine (TCEP) for 10 min. Samples were quenched with SDS-PAGE loading buffer lacking any reducing agents and loaded directly onto an SDS-PAGE gel. The gel was dried using a Bio-Rad gel-drying system and analyzed using a phosphorimager.

Acylation of Recombinant KS3 by [^{14}C]Diketide 1

KS3 (20 μM, in 100 mM phosphate [pH 7.2]) alone or together with AT3(3) or AT3(0) (20 μM) was incubated with 1 mM [^{14}C]**1** and 5 mM TCEP for 90 min at room temperature. Samples were quenched and processed as described above.

Triketide Lactone Formation

The reaction volume was 10 μl for all single time point assays. KS3 + AT3 + ACP3 system (Figure 4): KS3 (10 μM, in 100 mM phosphate [pH 7.2]) was incubated with 10 μM AT3(3) or AT3(0), 10 mM unlabeled diketide **1**, and 5 mM TCEP for 1 hr at room temperature to acylate the KS domain to completion. *Holo*-ACP3 (150 μM) and 150 μM DL-[2-methyl-^{14}C]methylmalonyl-CoA were then added and allowed to react at room temperature for an additional hour. The reaction was quenched by adding 20 μl of 0.5 M potassium hydroxide and heating the mixture for 20 min at 65°C. Hydrochloric acid (10 μl of 1.5 M) was then added, and the mixture was dried in a speedvac for 2 hr. The pellet was resuspended in 10 μl of ethyl acetate and spotted onto a TLC plate. A 60:40 mixture of ethyl acetate:hexane was used for TLC, and the radiolabeled products were then visualized and quantified using a Packard phosphorimager. [KS][AT] + KR + ACP system (Figures 5 and 6): [KS3][AT3] or [KS6][AT6] (10 μM, in 100 mM phosphate [pH 7.2]) was incubated with 5 mM diketide **1** and 5 mM TCEP for 1 hr at room temperature to acylate KS to completion. KR (150 μM), *holo*-ACP (150 μM), 2 mM NADPH, and 150 μM DL-[2-methyl-^{14}C]methylmalonyl-CoA were then added and allowed to react at room temperature for an additional hour. The reactions were quenched and analyzed as described above. KS + AT + KR + ACP system (Figure 7): KS3 (10 μM, in 100 mM phosphate [pH 7.2]) was incubated with 10 μM AT3(3), 10 mM unlabeled compound **1**, and 5 mM TCEP for 1 hr at room temperature to acylate KS to completion. KR (150 μM), *holo*-ACP (150 μM), 2 mM NADPH, and 150 μM DL-[2-methyl-^{14}C]methylmalonyl-CoA were then added and allowed to react at room temperature for an additional hour. The reactions were quenched and analyzed as described above.

Supplemental Data

Supplemental Data include three tables and can be found with this article online at http://www.chembiol.com/cgi/content/full/14/7/784/DC1/.

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REFERENCES

1. Cane, D.E., Walsh, C.T., and Khosla, C. (1998). Harnessing the biosynthetic code: combinations, permutations, and mutations. Science *282*, 63–68.
2. Khosla, C., Gokhale, R.S., Jacobsen, J.R., and Cane, D.E. (1999). Tolerance and specificity of polyketide synthases. Annu. Rev. Biochem. *68*, 219–253.
3. Staunton, J., and Weissman, K.J. (2001). Polyketide biosynthesis: a millennium review. Nat. Prod. Rep. *18*, 380–416.
4. Walsh, C.T. (2002). Combinatorial biosynthesis of antibiotics: challenges and opportunities. ChemBioChem *3*, 125–134.
5. Walsh, C.T. (2004). Polyketide and nonribosomal peptide antibiotics: modularity and versatility. Science *303*, 1805–1810.
6. Donadio, S., and Sosio, M. (2003). Strategies for combinatorial biosynthesis with modular polyketide synthases. Comb. Chem. High Throughput Screen. *6*, 489–500.
7. Weissman, K.J. (2004). Polyketide biosynthesis: understanding and exploiting modularity. Philos. Trans. R. Soc. Lond. B Biol. Sci. *362*, 2671–2690.
8. Weissman, K.J., and Leadlay, P.F. (2005). Combinatorial biosynthesis of reduced polyketides. Nat. Rev. Microbiol. *3*, 925–936.
9. McDaniel, R., Welch, M., and Hutchinson, C.R. (2005). Genetic approaches to polyketide antibiotics. 1. Chem. Rev. *105*, 543–558.
10. Hill, A.M. (2006). The biosynthesis, molecular genetics and enzymology of the polyketide-derived metabolites. Nat. Prod. Rep. *23*, 256–320.
11. Kumar, P., Khosla, C., and Tang, Y. (2004). Manipulation and analysis of polyketide synthases. Methods Enzymol. *388*, 269–293.
12. Cortes, J., Haydock, S.F., Roberts, G.A., Bevitt, D.J., and Leadlay, P.F. (1990). An unusually large multifunctional polypeptide in the erythromycin-producing polyketide synthase of *Saccharopolyspora erythraea*. Nature *348*, 176–178.
13. Donadio, S., Staver, M.J., McAlpine, J.B., Swanson, S.J., and Katz, L. (1991). Modular organization of genes required for complex polyketide biosynthesis. Science *252*, 675–679.
14. Kim, C.Y., Alekseyev, V.Y., Chen, A.Y., Tang, Y., Cane, D.E., and Khosla, C. (2004). Reconstituting modular activity from separated domains of 6-deoxyerythronolide B synthase. Biochemistry *43*, 13892–13898.
15. Chen, A.Y., Schnarr, N.A., Kim, C.Y., Cane, D.E., and Khosla, C. (2006). Extender unit and acyl carrier protein specificity of ketosynthase domains of the 6-deoxyerythronolide B synthase. J. Am. Chem. Soc. *128*, 3067–3074.
16. Tang, Y., Kim, C.Y., Mathews, I.I., Cane, D.E., and Khosla, C. (2006). The 2.7-Å crystal structure of a 194-kDa homodimeric fragment of the 6-deoxyerythronolide B synthase. Proc. Natl. Acad. Sci. USA *103*, 11124–11129.
17. Tang, Y., Kim, C.Y., Chen, A.Y., Cane, D.E., and Khosla, C. (2007). Structural and mechanistic analysis of protein interactions in module 3 of the 6-deoxyerythronolide B synthase. Chem. Biol., in press.
18. Schnarr, N.A., Chen, A.Y., Cane, D.E., and Khosla, C. (2005). Analysis of covalently bound polyketide intermediates on 6-deoxyerythronolide B synthase by tandem proteolysis-mass spectrometry. Biochemistry *44*, 11836–11842.
19. Liou, G.F., Lau, J., Cane, D.E., and Khosla, C. (2003). Quantitative analysis of loading and extender acyltransferases of modular polyketide synthases. Biochemistry *42*, 200–207.

20. Siskos, A.P., Baerga-Ortiz, A., Bali, S., Stein, V., Mamdani, H., Spiteller, D., Popovic, B., Spencer, J.B., Staunton, J., Weissman, K.J., and Leadlay, P.F. (2005). Molecular basis of Celmer's rules: stereochemistry of catalysis by isolated ketoreductase domains from modular polyketide synthases. Chem. Biol. *12*, 1145–1153.

21. Liu, L., Thamchaipenet, A., Fu, H., Betlach, M., and Ashley, G. (1997). Biosynthesis of 2-nor-6-deoxyerythronolide B by rationally designed domain substitution. J. Am. Chem. Soc. *119*, 10553–10554.

22. Stassi, D.L., Kakavas, S.J., Reynolds, K.A., Gunawardana, G., Swanson, S., Zeidner, D., Jackson, M., Liu, H., Buko, A., and Katz, L. (1998). Ethyl-substituted erythromycin derivatives produced by directed metabolic engineering. Proc. Natl. Acad. Sci. USA *95*, 7305–7309.

23. McDaniel, R., Thamchaipenet, A., Gustafsson, C., Fu, H., Betlach, M., and Ashley, G. (1999). Multiple genetic modifications of the erythromycin polyketide synthase to produce a library of novel "unnatural" natural products. Proc. Natl. Acad. Sci. USA *96*, 1846–1851.

24. Kato, Y., Bai, L., Xue, Q., Revill, W.P., Yu, T.-W., and Floss, H.G. (2002). Functional expression of genes involved in the biosynthesis of the novel polyketide chain extension unit, methoxymalonyl-acyl carrier protein, and engineered biosynthesis of 2-desmethyl-2-methoxy-6-deoxyerythronolide B. J. Am. Chem. Soc. *124*, 5268–5269.

25. Hans, M., Hornung, A., Dziarnowski, A., Cane, D.E., and Khosla, C. (2003). Mechanistic analysis of acyl transferase domain exchange in polyketide synthase modules. J. Am. Chem. Soc. *125*, 5366–5374.

26. Oliynyk, M., Brown, M.J., Cortes, J., Staunton, J., and Leadlay, P.F. (1996). A hybrid modular polyketide synthase obtained by domain swapping. Chem. Biol. *3*, 833–839.

27. Zhu, D.M., Mukherjee, C., Rozzell, J.D., Kambourakis, S., and Hua, L. (2006). A recombinant ketoreductase tool-box. Assessing the substrate selectivity and stereoselectivity toward the reduction of β-ketoesters. Tetrahedron *62*, 901–905.

28. Baerga-Ortiz, A., Popovic, B., Siskos, A.P., O'Hare, H.M., Spiteller, D., Williams, M.G., Campillo, N., Spencer, J.B., and Leadlay, P.F. (2006). Directed mutagenesis alters the stereochemistry of catalysis by isolated ketoreductase domains from the erythromycin polyketide synthase. Chem. Biol. *13*, 277–285.

29. O'Hare, H.M., Baerga-Ortiz, A., Popovic, B., Spencer, J.B., and Leadlay, P.F. (2006). High-throughput mutagenesis to evaluate models of stereochemical control in ketoreductase domains from the erythromycin polyketide synthase. Chem. Biol. *13*, 287–296.

30. Keatinge-Clay, A.T., and Stroud, R.M. (2006). The structure of a ketoreductase determines the organization of the β-carbon processing enzymes of modular polyketide synthases. Structure *14*, 737–748.

31. Bali, S., O'Hare, H.M., and Weissman, K.J. (2006). Broad substrate specificity of ketoreductases derived from modular polyketide synthases. ChemBioChem *7*, 478–484.

32. Cane, D.E., Kudo, F., Kinoshita, K., and Khosla, C. (2002). Precursor-directed biosynthesis: biochemical basis of the remarkable selectivity of the erythromycin polyketide synthase toward unsaturated triketides. Chem. Biol. *9*, 131–142.

33. Jacobsen, J.R., Hutchinson, C.R., Cane, D.E., and Khosla, C. (1997). Precursor-directed biosynthesis of erythromycin analogs by an engineered polyketide synthase. Science *277*, 367–369.

34. Harris, R.C., Cutter, A.L., Weissman, K.J., Hanefeld, U., Timoney, M.C., and Staunton, J. (1998). Enantiospecific synthesis of analogues of the diketide intermediate of the erythromycin polyketide synthase (PKS). J. Chem. Res. *283*, 1230–1247.

