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Engineered intermodular and intramodular polyketide synthase fusions

Robert McDaniel[1], Camilla M Kao[2], Sue J Hwang[2] and Chaitan Khosla[2,3,4]

Background: Modular polyketide synthases (PKSs) are very large multifunctional enzyme complexes that synthesize a number of medicinally important natural products. The modular arrangement of active sites has made these enzyme systems amenable to combinatorial manipulation for the biosynthesis of novel polyketides. Here, we investigate the involvement of subunit interactions in hybrid and artificially linked PKSs with several series of intermodular and intramodular fusions using the erythromycin (6-deoxyerythronolide B synthase; DEBS) and rapamycin (RAPS) PKSs.

Results: Several two-module and three-module derivatives of DEBS were constructed by fusing module 6 to either module 2 or module 3 at varying junctions. Polyketide production by these intramodular fusions indicated that the core set of active sites remained functional in these hybrid modules, although the ketoreductase domain of module 6 was unable to recognize unnatural triketide and tetraketide substrates. Artificial trimodular PKS subunits were also engineered by covalently linking modules 2 and 3 of DEBS, thereby demonstrating the feasibility of constructing single-chain PKSs. Finally, a series of fusions containing DEBS and RAPS domains in module 2 of an engineered trimodular PKS revealed the structural and functional tolerance for hybrid modules created from distinct PKS gene clusters.

Conclusions: The general success of the intermodular and intramodular fusions described here demonstrates significant structural tolerance among PKS modules and subunits and suggests that substrate specificity, rather than protein–protein interactions, is the primary determinant of molecular recognition features of PKSs. Furthermore, the ability to artificially link modules may considerably simplify the heterologous expression of modular PKSs in higher eukaryotic systems.

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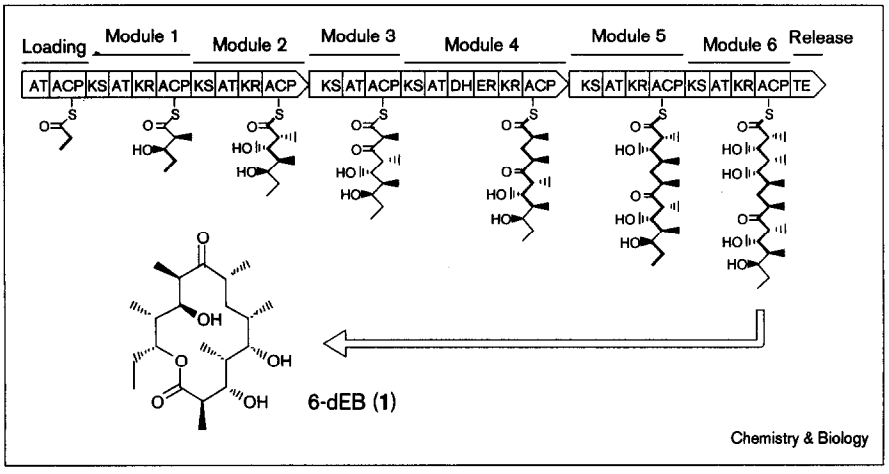
Introduction

Polyketides comprise a large and structurally diverse family of natural products that display a broad range of biological activities. Modular polyketide synthases (PKSs) are multifunctional enzyme complexes that catalyze the biosynthesis of macrocyclic precursors to medicinally important compounds, such as erythromycin [1,2], avermectin [3], FK506 [4], rapamycin [5], and candicidin [6]. A remarkable feature of this class of modular PKSs is the clustering of active sites in 'modules' that each perform a single cycle of condensation and β-ketoreduction in polyketide biosynthesis. Because these catalytic domains exhibit significant homology between modules among all gene clusters, modular PKSs have become attractive targets for genetic manipulation to produce novel polyketides with useful therapeutic activities [7,8].

Using the erythromycin PKS, 6-deoxyerythronolide B synthase (DEBS, Figure 1), as a model system, a variety of mutagenesis strategies have already demonstrated the feasibility of altering the catalytic properties of modular PKSs. These include the inactivation of reductive domains to bypass β-keto processing steps [2,9,10], deletion of modules to manipulate chain length [11–14], substitution of acyltransferase (AT) domains to alter starter [15] and extender unit incorporation [16], and addition of reductive/dehydrative domains to modules which naturally lack these activities [17]. Although these engineered mutants have illustrated the tolerance towards unnatural substrates by the enzymes of modular PKSs, few experiments have systematically addressed the presumed necessity for active-site domains and modules in hybrid systems to fold and associate correctly with upstream and downstream modules.

In an attempt to uncover any structural and association requirements for hybrid PKSs, we constructed a series of PKS mutants with varying intermodular and intramodular fusion boundaries. The biosynthesis of polyketides from these engineered PKSs, which include the fusions between KS and AT (KS: ketosynthase), AT and DH

Figure 1



The erythromycin (6-deoxyerythronolide B synthase; DEBS) polyketide synthase (PKS) which catalyzes the biosynthesis of 6-deoxyerythronolide B (6-dEB, **1**). DEBS consists of three large multifunctional proteins and includes six modules as well as a primer-loading segment and a product-release segment [1,2]. Each module catalyzes one cycle of chain extension and associated β-ketoreduction for the biosynthesis of 6-dEB. All modules include ketosynthase (KS), acyltransferase (AT), and acyl carrier protein (ACP) domains, which are responsible for the assembly of the carbon chain from propionyl and methylmalonyl coenzyme A. The modules also possess reductive segments comprising all, some, or none of the set of ketoreductase (KR), dehydratase (DH), and enoylreductase (ER) domains, which act on the β-carbonyl following each condensation reaction. The loading segment consists of priming AT and ACP domains, whereas a thioesterase (TE) forms the release segment of the PKS. (The relative sizes of the domains are not shown to scale.)

(DH: dehydratase), and between AT and KR (KR: ketoreductase) domains, as well as the covalent linking of modules to generate larger PKS subunits, demonstrates general flexibility in engineering hybrid PKS modules while retaining intact enzyme structure and function. These results have important implications for the engineering of hybrid enzyme systems from two or more different modular PKSs to generate new enzymatic pathways that synthesize novel polyketide products. At the same time, the ability to construct functional single polypeptide chains from multiple PKS subunits offers advantages for engineering these systems in cells where multi-gene expression may be challenging (e.g. in plants and mammals).

Results and discussion

Intramodular DEBS fusions

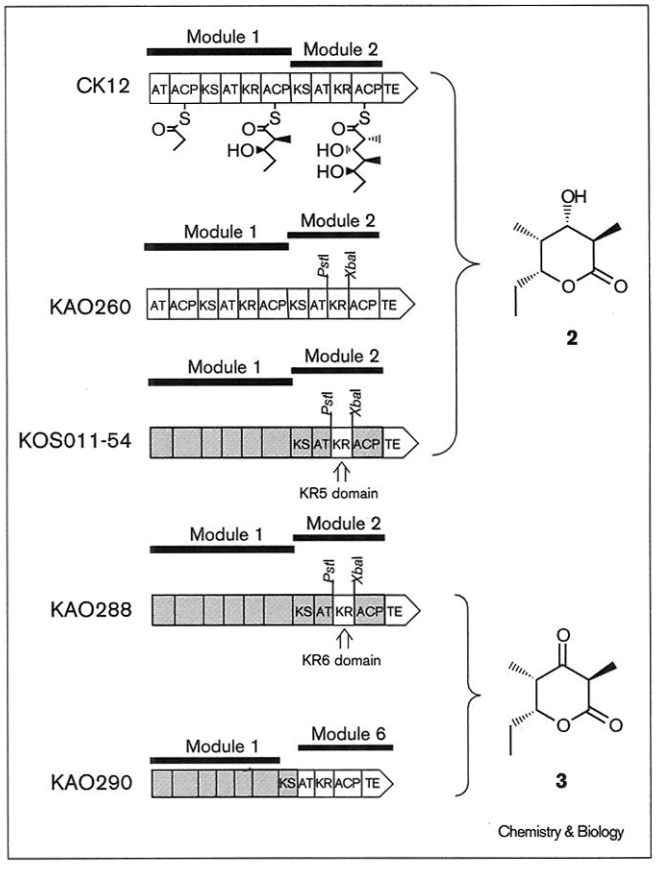
One desirable route to new biosynthetic products involves fusions of modules from two (or more) distinct PKS pathways. The challenge of such fusions is the transfer of an intermediate across a pathway junction; this requires that the active-site domains in a hybrid module remain structurally intact as well as displaying relaxed substrate specificities. In order to explore the structural integrity of different hybrid modules, we constructed a series of fusions between DEBS modules 2, 3 and 6 in which fusion boundaries were varied in the terminal module.

Four derivatives of the two-module DEBS system from *Streptomyces coelicolor* CH999/pCK12 [13] and five derivatives of the three-module DEBS system from *S. coelicolor* CH999/pCK13 [14] were constructed for this study (Figures 2 and 3). Plasmid pKAO260 contains engineered *Pst*I and *Xba*I restriction sites at the conserved boundaries of KR2 (see Figure 2; [18]), which were used to substitute KR2 with the corresponding KR5 and KR6 domains to generate pKOS011-54 and pKAO288, respectively. A two-module fusion between KS2 and AT6 was also constructed in pKAO290, with the beginning of the conserved AT domain [18] as the heterologous junction.

Of the three-module mutants, plasmids pKAO223, pKAO296, and pKAO222 are derivatives of pCK13 generated by fusions between the AT domain of module 3 and the KR domain of module 6 (Figure 3). Fusion junctions occur at the beginning of the conserved KR boundary [18] for pKAO223 and pKAO296 (marked by engineered *Nde*I and *Kpn*I sites, respectively) and at the end of the conserved AT boundary [18] for pKAO222 (marked by an engineered *Pst*I site). Plasmid pKAO301 contains a fusion between the KS domain of module 3 and the AT domain of module 6 at the beginning of the conserved AT boundary [18] (marked by an engineered *Bam*HI site). Finally, plasmid pKAO306 was constructed by linking module 2 with module 6 to give a single 3-module polypeptide in which ACP2 (ACP: acyl carrier protein) and KS3 are joined at the end of the conserved ACP domain [18] (marked by an engineered *Spe*I site).

S. coelicolor CH999/pKAO260 and pKOS011-54 produced the natural triketide lactone **2** (Figure 2) at levels comparable to *S. coelicolor* CH999/pCK12 [13], as determined by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy (see Materials and methods section). This result, which is the second example of a functional KR substitution (after pKOS009-7; see below), indicates that KR5 can recognize and process an unnatural substrate that is considerably shorter than its normal substrate, but which presumably has the 'natural' functionality and stereochemistry at the α, β, and γ positions. In contrast, both

Figure 2



Bimodular DEBS fusions and triketide products. Substitution of the KR2 domain with KR5 (pKOS011-54) leads to the same reduced triketide lactone (**2**) produced by the parent PKS constructs (pCK12 and pKAO260). The KR6 domain, however, is unable to catalyze β-ketoreduction, either as a single-domain substitution (pKAO288) or as a fusion product in module 6 (pKAO290), and results in the formation of the unreduced triketide lactone (**3**). For pKOS011-54, pKAO228, and pKAO290, shaded regions correspond to native sequence and unshaded regions are substituted or fused domains.

CH999/pKAO288 and pKAO290 produced the triketide ketolactone **3**, as determined by ^{1}H and ^{13}C NMR spectroscopy (~20 mg/l; see Materials and methods section; Figure 2). This product, which has been previously characterized [19], is derived from a module 2 intermediate with an unreduced β-carbonyl. Although this result demonstrates that KR6 is nonfunctional in module 2, the pKOS011-54 mutant suggests that this KR6 behavior is most likely to arise from stringent specificity towards unnatural substrates rather than from a perturbed KR6 structure.

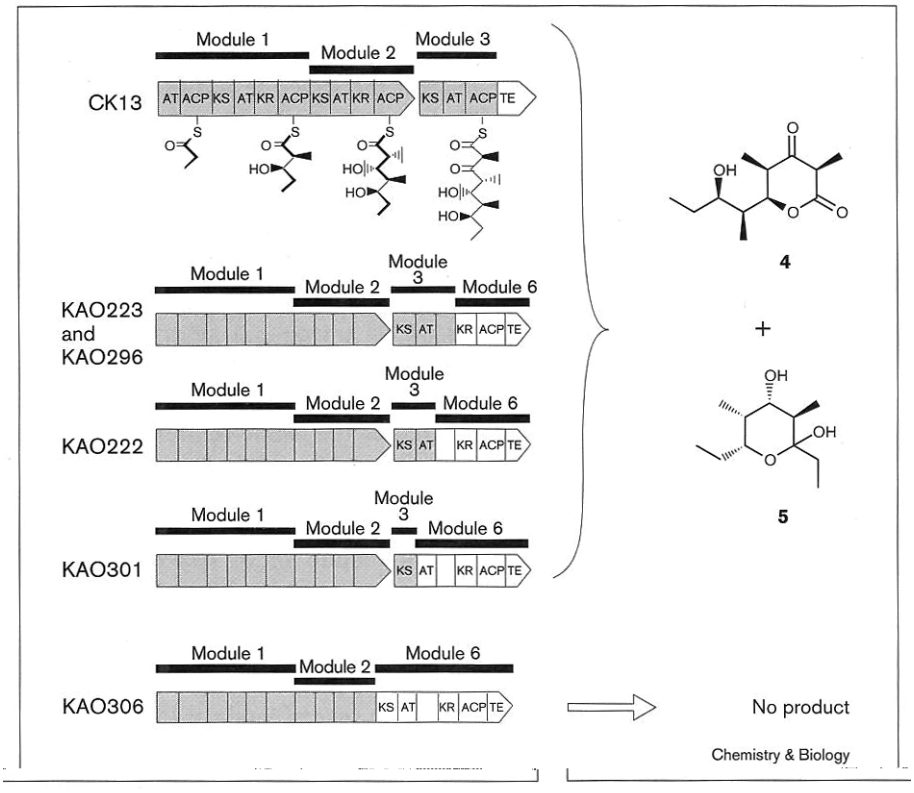
Fusions between modules 3 and 6 were selected because of their substrate resemblance in α-, β-, and γ-carbon functionality and stereochemistry (see Figure 1). The recombinant strains *S. coelicolor* CH999/pKAO223, pKAO296, pKAO222, and pKAO301 (Figure 3) all produced ~10–20 mg/l of the tetraketides **4** and **5**, as determined by ^{1}H NMR spectroscopy (pKAO222 and pKA0301; see Materials and methods section) or thin layer chromatography (TLC; pKA0296; see Materials and methods section). Production of these molecules indicates that the structure and function of the KS, AT and ACP domains remain intact in the hybrid modules, while the KR6 domain appears to be inactive towards the unreduced β-carbonyl of the module 3 intermediate. Although any of the engineered restriction sites may interfere with KR6 function, given the results with the two-module mutants it is likely that KR6 possesses stringent substrate specificity. More significantly, the terminal modules in both pKAO290 and pKAO301 contain a native KS in an otherwise completely heterologous module, suggesting that the correct association with a previous module is dictated by the amino-terminal KS region (i.e. KS2 for the association of module 2 with module 1 and KS3 for the noncovalent assembly of module 3 with module 2). Because trimodule linked systems are functional (see below), the failure of *S. coelicolor* CH999/pKAO306 (Figure 3) to produce any detectable products probably stems from tight KS6 specificity.

Artificially linked PKS subunits

Genetic characterization of several modular PKSs has suggested remarkable flexibility in the lower and higher order structures of these enzymes. Specifically, the number of modules in naturally occurring PKSs varies considerably, as does the number of modules within a polypeptide subunit. For example, DEBS is composed of three two-module polypeptide subunits, while the rapamycin PKS (RAPS) consists of two four-module subunits and one six-module subunit [5]. The spiramycin gene cluster even contains single-module subunits [15]. The regions within a PKS module that dictate proper subunit assembly and chain transfer between noncovalent as well as covalent modules remain undefined, although segments of nonconserved residues at the amino and carboxyl termini of PKS subunits, between covalently joined modules and between certain reductive active sites, may participate in subunit assembly and chain transfer [18].

In the light of the structural variation observed among naturally occurring PKSs, we wished to examine how consecutive modules might be artificially linked and yet retain normal catalytic function. For this purpose, we constructed two derivatives of the well-characterized three-module PKS from *S. coelicolor* CH999/pCK13 [14], in which DEBS modules 2 and 3 were translationally joined to create a single three-module PKS subunit. The first recombinant strain, *S. coelicolor* CH999/pKAO318 (Figure 4), contains a fusion between the stop codon of module 2 and the start codon of module 3. All of the 117 nonconserved amino acids between ACP2 and KS3 are retained, and a diagnostic *Nhe*I restriction site is present at the fusion boundary (Figure 5). In the second linked PKS, expressed by *S. coelicolor* CH999/

Figure 3

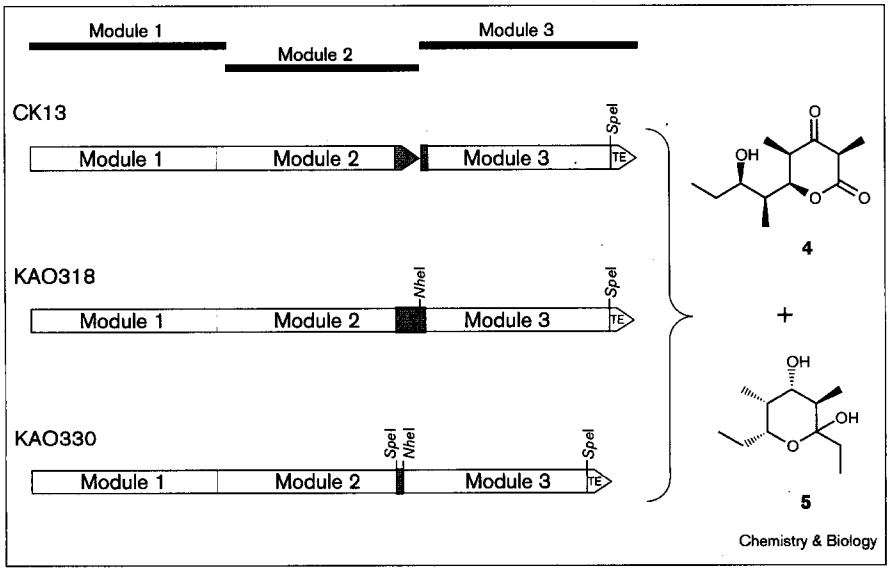


Trimodular DEBS fusions and tetraketide products. Functional fusions give rise to the tetraketide lactone (**4**) and the decarboxylated hemiketal (**5**) [14]. Substitution of the KS6 domain for KS3 leads to an unproductive PKS (pKAO306). Shaded regions correspond to native sequence; unshaded regions are substituted or fused domains.

pKAO330 (Figure 4), the ACP2 and KS3 boundaries are connected by the intermodule segment originally connecting DEBS modules 5 and 6. In this mutant, all 117 natural residues between ACP2 and KS3 are replaced by the foreign 25-residue segment (Figure 5).

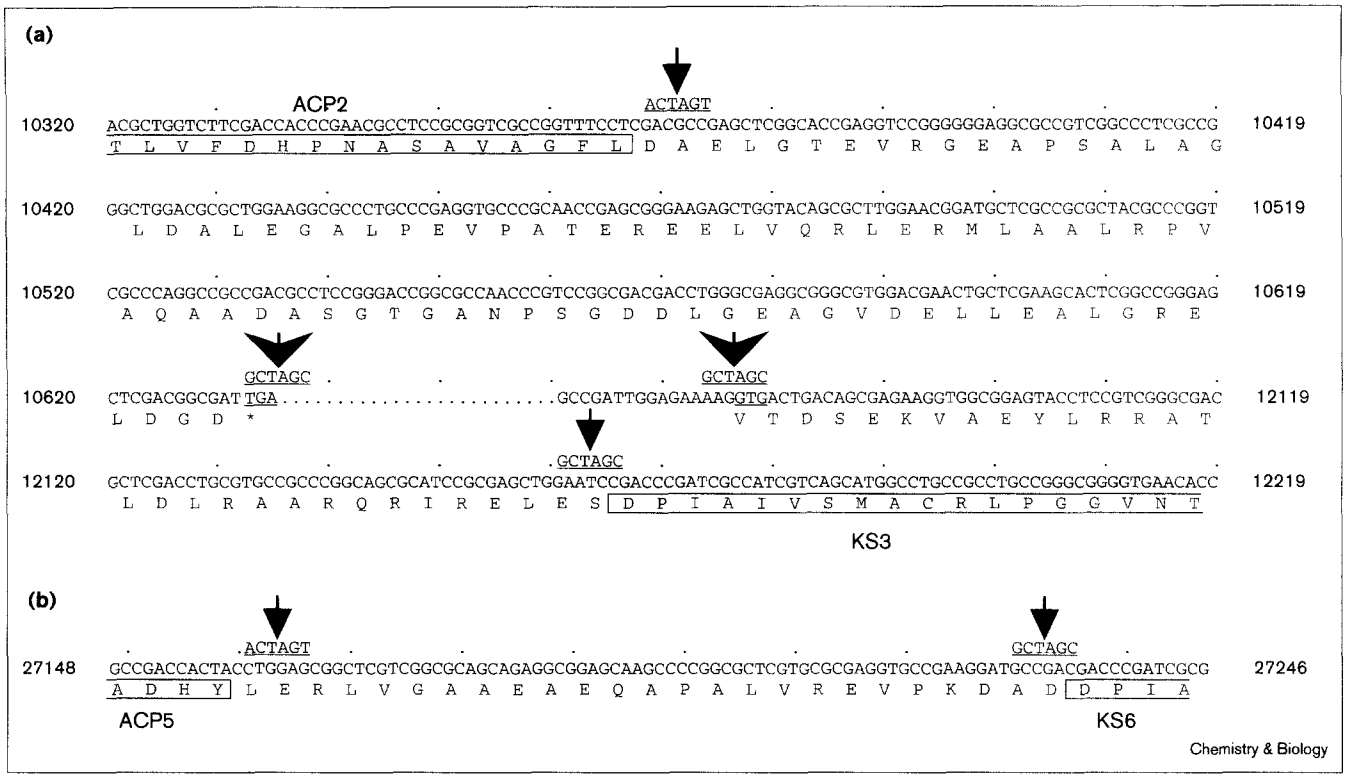
Both *S. coelicolor* CH999/pKAO318 and pKAO330 produced the natural tetraketides **4** and **5** at yields comparable to those of *S. coelicolor* CH999/pCK13 [14], as determined by ^{1}H and ^{13}C NMR spectroscopy. These results show that the amino and carboxyl termini of PKS subunits are not

Figure 4



Intermodular DEBS fusions to create artificially linked modules. In plasmid pCK13, modules 1 and 2 exist as a single open reading frame (ORF) and module 3 as a separate ORF. Translational coupling of modules 2 and 3 in plasmids pKAO318 and pKAO330 results in a functional single trimodular polypeptide that produces the tetraketide lactone (**4**; as in Figure 3) and the decarboxylated hemiketal (**5**; as in Figure 3). Shaded regions correspond to the engineered intermodule domains.

Figure 5



Nucleotide sequence of DEBS regions involved in intermodule fusions. **(a)** The wide arrows indicate the locations of the engineered *Nhe*I sites, which result in the sequence GGCGAT*GCTAGC*ACTGAC (*Nhe*I site italicized) in the linked PKS (pKAO318). The fusion generates a 118-residue linker between the conserved boundaries of ACP2 and KS3 [18]. The sharp arrows denote the engineered *Spe*I and *Nhe*I restriction sites used to join ACP2 and KS3 with the corresponding segment connecting ACP5 and KS6 (pKAO330; see below). The DNA sequences spanning the two boundaries are TTCCTC*ACTAGT*CGGCTC (*Spe*I site italicized) and AAGGAT*GCTAGC*GACCCG (*Nhe*I site italicized). **(b)** The construction generates a 25-residue linker between ACP2 and KS3. One-letter amino acid codes are shown below the DNA sequence.

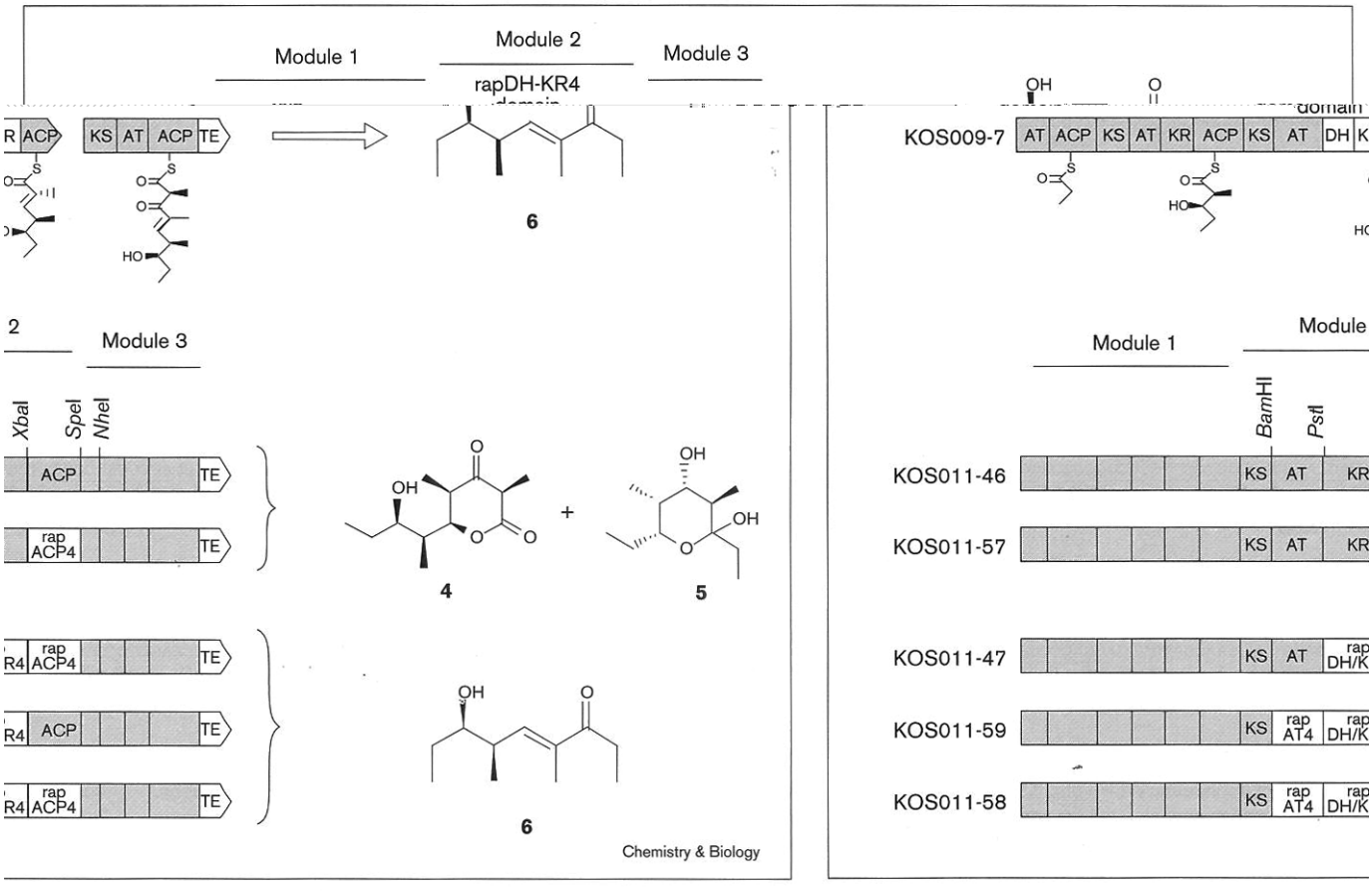
required for chain transfer between covalent modules, and they generally demonstrate that PKS subunits can be covalently linked without apparent loss of activity. The idea that covalently linked subunits can be fully active has immediate implications for the construction of heterologous module series through the artificial joining of modules from different PKS systems.

DEBS/RAPS hybrid module fusions

To systematically examine various module hybrids generated from two distinct PKS systems, we constructed a series of module 2 fusions using DEBS and RAPS. We based these experiments on the recently described plasmid pKOS009-7 (Figure 6), a three-module derivative of pCK13 in which KR2 is substituted by the rapDH/KR4 domain [17]. *S. coelicolor* CH999/pKOS009-7 produces compound **6** (Figure 6), a tetraketide product derived from a module 2 intermediate with a *trans* double bond introduced by the rapDH/KR4 activity. Because the chemical structure of rapamycin suggests that the rapAT4 domain encodes methylmalonyl transferase activity [5], we reasoned that, in the pCK13 scaffold, a hybrid module 2 containing the active sites of DEBS module 2 and RAPS module 4 could generate only two different intermediates — the wild-type β-hydroxyl triketide or the pKOS009-7 β-enoyl triketide. Because CH999/pKOS009-7 demonstrated that the RAPS DH/KR4 domains and DEBS module 3 can process the unnatural as well as the natural triketide substrates, we could attribute any defects in polyketide catalysis to incorrect protein folding or association in the module fusions, thereby decoupling structural issues from substrate-specificity requirements.

A series of four hybrid fusions containing domains from DEBS module 2 and RAPS module 4 were designed. To initially bypass potential subunit association problems, these hybrids were constructed in the linked three-module scaffold using the plasmid pKOS011-46 (Figure 6), a derivative of pKAO330 (Figure 4) with engineered restriction sites between the module 2 active sites. Substitutions in module 2 included rapACP4 (pKOS011-57), rapDH/KR4+ ACP4 (pKOS011-47), rapAT4+DH/KR4 (pKOS011-59),

Figure 6



Engineered DEBS/RAPS hybrid fusions. Hybrids that lack a dehydratase produce **4** and **5**; those with dehydratase activity produce **6** (KOS009-7a). All active sites in the hybrid fusions remained fully functional. Unshaded regions (except for the TE) refer to rapamycin module 4 domains.

and rapAT4+DH/KR4+ACP4 (pKOS011-58) (Figure 6). The resulting plasmids were introduced into *S. coelicolor* CH999 and screened for polyketide production.

Both *S. coelicolor* CH999/pKOS011-46 and pKOS011-57 produced the expected compounds **4** and **5** by TLC analysis. The ability of rapACP4 to complement ACP2 in pKOS011-57 with little or no effect on polyketide production is consistent with a previously described fusion between DEBS KR5 and ACP6 domains [13]. The recombinant strains *S. coelicolor* CH999/pKOS011-47, pKOS011-58, and pKOS011-59 all produced the anticipated tetraketide **6**, as determined by ^{1}H NMR spectroscopy, showing that all the catalytic domains in these fusions retain their expected activity. Although the yields for these strains were ~10–20% of those originally reported for pKOS009-7 [17], no difference in production levels was observed between the newly engineered constructs. Note that the intact PKS function in CH999/pKOS011-58, whose module 2 consists primarily (68%) of RAPS amino acids, is consistent with the results of pKAO290 and pKAO301, and further supports the notion that the KS region participates in the productive association of adjacent modules.

Significance

Modular polyketide synthases (PKSs) are multifunctional enzyme complexes that synthesize a number of medicinally important natural products. With their modular arrangement of active sites, the enzymes possess tremendous potential for the generation of novel compound libraries. In this work, we have generated a series of hybrid modular PKSs by engineering intermodular and intramodular fusions between PKS subunits, thereby extending the range of structural manipulations of these multifunctional enzymes from the previous active-site substitution and inactivation experiments. We have shown that the PKS structure and the active-site specificity are flexible enough to allow fusions between modules from the same or different PKSs, and separate modules can also be linked to generate enlarged PKS subunits. Although certain active sites appear to possess stringent specificities towards unnatural substrates, most active sites tested in this work suggest that significant

tolerance towards unnatural substrates exists in these enzyme systems. Furthermore, because hybrid modules containing only a native ketosynthase domain productively interact with neighboring modules in a heterologous PKS system, some determinants of modular assembly appear to reside in the amino termini of modules.

Should the facile construction of intermodular and intramodular fusions prove to be a general feature of modular PKSs, the task of generating hybrid pathways from different PKS systems may be restricted more by substrate stringency of active sites than by the structural limitations imposed by hybrid modules. Indeed, further understanding of structure–function relationships underlying PKS molecular recognition will be required to explain and overcome the observed specificity differ-

artificially link modules should, however, minimize subunit dissociation problems in these engineered systems; this has immediate implications for the construction of heterologous module series. Furthermore, single-chain PKSs would be more desirable for expression in higher eukaryotic organisms such as plants or mammalian cell lines. These approaches, in conjunction with other emerging technologies in PKS pathway manipulation, should provide access to a broad range of polyketide structures and pharmacophores that are not currently accessible by synthetic methods and may not even be present in nature.

Materials and methods

Bacterial strains and culture conditions

S. coelicolor CH999 [20] was used as the host for production of polyketides from the engineered plasmids. DNA manipulations were performed in *Escherichia coli* XL1 Blue (Stratagene) using standard culture conditions [21]. *S. coelicolor* strains were grown on R2YE agar plates [22].

Manipulation of DNA and organisms

Manipulation and transformation of DNA in *E. coli* was performed by standard procedures [21]. Polymerase chain reaction (PCR) was performed with Pfu polymerase (Stratagene) under conditions recommended by the manufacturer. *S. coelicolor* protoplasts were transformed by the standard procedure [22] and transformants were selected using 2 ml of a 500 μg/ml thiostrepton (Sigma) overlay.

PCR amplification of active-site domains used for substitutions

The active site domains used for substitution in plasmids below were PCR amplified from plasmids or cosmids containing DEBS (pCK7) [23] or RAPS [17] DNA. The following oligonucleotides containing appropriate flanking restriction sites (shown in italics) for substitution were used: eryKR5, forward 5′-TTT*CTGCAG*ATCCCCACCGGCGGGCGCGCA-3′ and reverse 5′-TTT*TCTAGA*GCCCTCCTCGCTCTGCCGGGC-3′; eryKR6, forward 5′-TTT*CTGCAG*CCGGAGGTGTCCGACCAGCTC-3′ and reverse 5′-TTT*TCTAGA*GGCCCCCACCGCCGGCGTGACGAGCTCGTC-3′; rapAT4, forward 5′-TTT*GGATCC*GTGTTTGTTTTCCCGGGGCAG-3′ and reverse 5′-TTT*CTGCAG*CCAGTACCGCTGGTGTTGGAA-3′; rapDH/KR4 [17], forward 5′-TT*CTGCAG*AGCGTGGACCGGGCGGCT and reverse 5′-TTT*TCTAGA*GTCACCGGTAGAGGCGGCCCT-3′; rapACP4, forward 5′-TTT*TCTAGA*GTGCAGTGGTTGGCAGCTTTG-3′ and reverse 5′-TTT*ACTAGT*CAGCCCGGCGGCCAGGGCCGT. Restriction sites: *CTGCAG*, *PstI*; *TCTAGA*, *XbaI*; *GGATCC*, *BamHI*; *ACTAGT*, *SpeI*.

Construction of DEBS KR substitution plasmids

Plasmid pKAO260 is a derivative of pCK12 [13] in which *PstI* and *XbaI* restriction sites were engineered identical those previously described [10]. Plasmids pKOS011-54 and pKAO288 were obtained by replacing the KR2 domain (*PstI*/*XbaI* fragment) of pKAO260 with the eryKR5 and eryKR6 cassettes, respectively.

Construction of plasmids containing intramodular fusions and artificially linked DEBS genes

Plasmids pKAO290, pKAO223, pKAO296, pKAO222, pKAO301, pKAO306, pKAO318, and pKAO330 were all constructed as *in vivo* deletions of pCK7 [23] using a homologous *in vivo* recombination method previously described [23]. To create the fusion junctions PCR fragments ~1 kb in length and containing the appropriate engineered restriction site at the boundary were generated. The two corresponding fragments from each side of the junction were ligated together in the vector pMAK705 [24] used for homologous recombination. DNA sequences of the resulting junctions are as follows (engineered restriction sites italicized): pKAO290, eryKS2..GCCTCCGACGGTG-*GATCC*GTTTTCGTCTTC..eryAT6; pKAO223, eryAT3..CGCGCAA-CGCGGAGCTG*GGTACC*GGCGGGCACGGCG..eryKR6; pKAO222, eryAT3..AAGCGCTACTGG*CTGCAG*CCGGAGGTGTCC..eryKR6; pKAO301, eryKS3..GTGGACGGGCGC*GGATCC*GTTTTCGTCTTC-..eryAT6; pKAO306, eryACP2..GCCGGTTTCCTC*ACTAGT*GACCCGATCGCG..eryKS6. *GGATCC*, *BamHI*; *CATATG*, *NdeI*; *GGTACC*, *KpnI*, *CTGCAG*, *PstI*; *ACTAGT*, *SpeI*. The engineered boundaries for plasmids pKAO318 and pKAO330 are shown in Figure 5.

Construction of DEBS/RAPS hybrid plasmids

Plasmid pKOS011-33 is a derivative of pKAO330 (see above) with engineered *BamHI*, *PstI*, and *XbaI* restriction sites. The location of the *PstI* and *XbaI* sites are identical to those previously described [10]. The *BamHI* site resulted in the following altered DNA sequence between the DEBS KS2 and AT2 domains: 5′-GCCTCCGACGGT*GGATCC*-GTGTTCGTCTTC-3′ (*BamHI* site italicized). The rapAT4, rapDH/KR4, and rapACP4 domains were inserted into corresponding sites in pKOS011-33 to generate pKOS011-46, pKOS011-47, pKOS011-57, pKOS011-58, and pKOS011-59 (see Figure 6).

Purification and characterization of polyketides

Each strain was grown on a total of 200–500 ml of R2YE agar on petri plates. The purification and characterization of compounds **2** [11], **3** [10], **4** [14], **5** [14], and **6** [17] proceeded as previously described. TLC analysis was performed with the previously purified and characterized compounds as controls. ^{1}H and ^{13}C NMR spectra, recorded at 400 MHz, were identical to the reported data.

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